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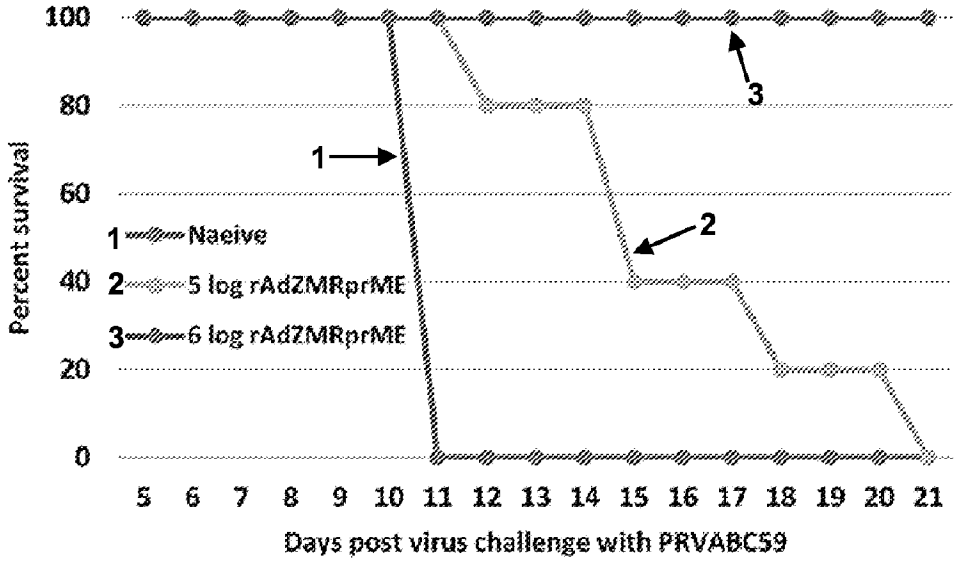
(54) Titre : ACIDES NUCLEIQUES CODANT POUR DES PARTICULES DU TYPE DU VIRUS ZIKA ET LEUR UTILISATION DANS DES VACCINS CONTRE LE VIRUS ZIKA ET DES DOSAGES DE DIAGNOSTIC

(54) Title: NUCLEIC ACIDS ENCODING ZIKA VIRUS-LIKE PARTICLES AND THEIR USE IN ZIKA VIRUS VACCINES AND DIAGNOSTIC ASSAYS

FIG. 2C



(57) Abrégé/Abstract:
Transcriptional units encoding Zika virus (ZIKV) premembrane (prM) and envelope (E) proteins, which upon translation form Zika virus-like particles (VLPs), are described. Use of the transcriptional units and VLPs in three different ZIKV vaccine platforms is described. Immunoassay- based detection methods using ZIKV VLPs are described for the diagnosis of ZIKV infection.

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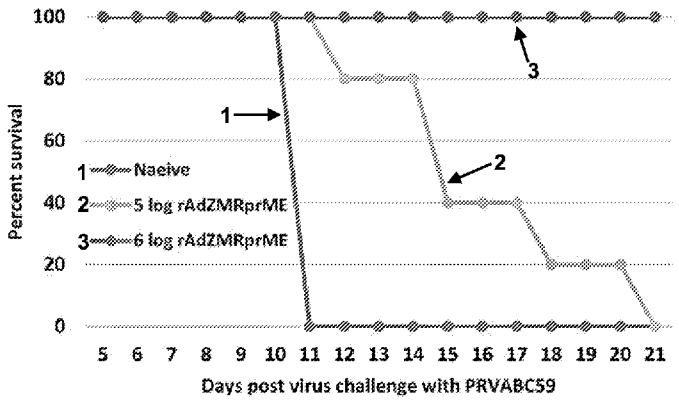
(54) Title: NUCLEIC ACIDS ENCODING ZIKA VIRUS-LIKE PARTICLES AND THEIR USE IN ZIKA VIRUS VACCINES AND DIAGNOSTIC ASSAYS

FIG. 2C



(57) Abstract: Transcriptional units encoding Zika virus (ZIKV) premembrane (prM) and envelope (E) proteins, which upon translation form Zika virus-like particles (VLPs), are described. Use of the transcriptional units and VLPs in three different ZIKV vaccine platforms is described. Immunoassay- based detection methods using ZIKV VLPs are described for the diagnosis of ZIKV infection.

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NUCLEIC ACIDS ENCODING ZIKA VIRUS-LIKE PARTICLES AND THEIR USE IN ZIKA VIRUS VACCINES AND DIAGNOSTIC ASSAYS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 62/349,537, filed June 13, 2016, which is herein incorporated by reference in its entirety.

FIELD

This disclosure concerns Zika virus (ZIKV) transcriptional units encoding ZIKV premembrane (prM) and envelope (E) proteins (prME) and their use in ZIKV vaccine platforms and ZIKV diagnostic assays.

BACKGROUND

The twentieth and twenty-first centuries have demonstrated the benefits and risks of living in a globalized world. A microcosm of those risks is the repeat introduction and expansion of vector-borne viruses within the *Flavivirus* genus (such as dengue virus, West Nile virus, and Zika virus) across the world and their emergence as global public health concerns (Musso and Gubler, *Clin Microbiol Rev* 29, 487-524, 2016). The explosive expansion of an Asian genotype of Zika virus (ZIKV) across the Pacific Islands in 2013-2014, which by May of 2015 emerged in Brazil, underscores this reality (Haddow *et al.*, *PLoS Negl Trop Dis* 6, e1477, 2012; Duffy *et al., N Engl J Med* 360, 2536-2543, 2009; Nishiura *et al.*, *Int J Infect Dis* 45, 95-97, 2016). Since then, the Centers for Disease Control and Prevention (CDC) has established a causal link between prenatal exposure to ZIKV and an increased risk for congenital birth abnormalities, including the much publicized increased incidence of neonatal microcephaly (Driggers *et al.*, *N Engl J Med* Epub March 30, 2016; Petersen *et al.*, *MMWR Morb Mortal Wkly* Rep 65, 30-33, 2016; Karwowski *et al.*, *Pediatrics* Epub March 23, 2016; Petersen *et al.*, *N Engl J Med* 374, 1552-1563, 2016). Additionally, there is mounting evidence of a link between ZIKV exposure and Guillian-Barré syndrome (Cao-Lormeau *et al.*, *Lancet* 387, 1531-1539, 2016), encephalitis (Carteaux *et al.*, *N Engl J Med* 374, 1595-1596, 2016), and myelopathy (Mecharles *et al.*, *Lancet* 387, 1481, 2016) in adults. Because of the global risks, particularly the risk posed to the populations of the Americas, the World Health Organization (WHO) has declared the epidemics as a Public Health Emergency of International Concern, and launched a global Strategic Response

Framework and Joint Operations Plan in order to mitigate the spread and impact of the virus (Maurice, *Lancet* 387, 1147, 2016). However, with a very short window of viremic phase in humans, Zika virus provides a unique challenge to using ZIKV-specific nucleic acid based diagnostic procedures (Bingham *et al.*, *MMWR Morb Mortal Wkly Rep* 65, 475-478, 2016), and control measures focus primarily on vector control. Thus, in order to comprehensively address the threat of ZIKV, an improved serodiagnostic assay must be developed and an effective vaccine must be made available.

ZIKV contains a single, positive sense viral RNA of 10.7 kb in-length that translates into a single poly-protein, which is subsequently cleaved into three structural proteins (capsid, premembrane/membrane, envelope; C, prM/M, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Kuno and Chang, *Arch Virol* 152, 687-696, 2007). It has been previously demonstrated with other flaviviruses that expression of prM and E glycoproteins alone can self-assemble and be secreted as immunogenic virus-like particles (VLPs) (Chang *et al.*, *J Virol* 74, 4244-4252, 2000; Davis *et al.*, *J Virol* 75, 4040-4047, 2001; Chang *et al.*, *Virology* 306, 170-180, 2003; Konishi *et al.*, *J Virol* 72, 4925-4930, 1998; Konishi *et al.*, *Vaccine* 21, 3713-3720, 2003).

SUMMARY

Disclosed herein are transcriptional units encoding ZIKV prM and E proteins, which upon translation, form ZIKA VLPs. The disclosed transcriptional units and VLPs are used in a variety of ZIKV vaccine platforms, as well as in detection methods for the diagnosis of ZIKV infection.

Provided herein are isolated nucleic acid molecules including a transcriptional unit. The transcriptional unit includes a sequence encoding a modified Japanese encephalitis virus (JEV) signal sequence and a ZIKV prM and E protein (prME) coding sequence. In some embodiments, the nucleic acid molecules further include a promoter operably linked to the prME coding sequence; a transcription termination sequence; and/or a translation initiation sequence. In some examples, the prME coding sequence is codon-optimized for expression in human cells.

Further provided herein are vectors that include the disclosed nucleic acid molecules. In some embodiments, the vector is an adenovirus vector. Recombinant adenoviruses that include a nucleic acid molecule disclosed herein are also provided. The recombinant adenoviruses express ZIKV VLPs. Also provided are isolated cells that include a nucleic acid or vector disclosed herein.

Further provided herein are VLPs encoded by the nucleic acid molecules and vectors disclosed herein. In some embodiments, the VLPs include at least one amino acid substitution that reduces flavivirus cross-reactive immune responses.

Compositions, such as immunogenic compositions, that include the nucleic acid molecules, vectors, recombinant adenoviruses or VLPs disclosed herein are also provided by the present disclosure. Further provided herein are methods of eliciting an immune response against Zika virus in a subject by administering a disclosed nucleic acid molecule, vector, recombinant adenovirus, VLP or composition.

Also provided herein are methods of detecting ZIKV-specific antibodies in a biological sample. In some embodiments, the method includes contacting the sample with a ZIKV VLP disclosed herein under conditions sufficient to form VLP-antibody complexes if ZIKV antibodies are present in the sample; and detecting the VLP-antibody complexes in the sample. In other embodiments, the method includes providing a secondary antibody bound to a solid support; contacting the secondary antibody-bound solid support with the biological sample under conditions sufficient to allow binding of the secondary antibody to any ZIKV-specific antibodies present in the biological sample, thereby forming antibody-antibody complexes; contacting the antibody-antibody complexes with a ZIKV VLP disclosed herein under conditions sufficient for the VLP to bind the ZIKV-specific antibodies, thereby forming immune complexes; and detecting the presence of the immune complexes. In yet other embodiments, the method includes providing a ZIKV-specific antibody bound to a solid support; contacting the antibody-bound solid support with a ZIKV VLP disclosed herein under conditions sufficient for the VLP to bind the ZIKV-specific antibody to form antibody-VLP complexes; contacting the antibody-VLP complexes with the biological sample to allow binding of any ZIKV-specific antibodies present in the sample to the VLP, thereby forming immune complexes; contacting the immune complexes with a secondary antibody; and detecting binding of the secondary antibody to the immune complexes.

The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1D: Characterization of virus-like particle (VLP) expressed prM and E proteins of ZIKV MR766 strain. (FIG. 1A) Schematic representation of plasmid vector pEZMRprME1-8. This plasmid includes the cytomegalovirus (CMV) promoter/enhancer element, the modified Japanese encephalitis virus (JEV) signal sequence (SS), bovine growth hormone (BGH) poly(A) signal and

transcription termination sequence [BGH(A)n], kanamycin resistance gene (KanR), and pUC origin (ori) for selection and maintenance in *E. coli*. (FIG. 1B) Immunofluorescence analysis of prM and E protein expression in COS-1 cells transfected with the plasmids pEZMRprME1-8, pEBZHu8 (expressing human codon-optimized, synthetic prME gene of the BPH2015 strain) and pEBZHu2-3 (pr1Ala deletion clone derived from pEBZHu8). After fixation, prM/M and/or E proteins were detected with anti-ZIKV mouse hyper-immune ascetic fluid (MHIAF) or flavivirus group-cross reactive murine monoclonal antibody 4G2 (MAb 4G2), followed by incubation with goat anti-mouse IgG-FITC and Evan's blue to counterstain the cells. Fluorescence in cells indicated positive intracellular expression of prM and/or E proteins. (FIG. 1C) Detection and quantification of secreted MR766-VLPs in culture supernatants harvested from transiently transformed COS-1 cells by antigen (Ag)-capture ELISA using 4G2 and a ZIKV-specific human polyclonal serum (αZHS) as the detector antibodies. Culture supernatants were harvested on day 5 (VLP1) and day 10 (VLP2, second harvest) and concentrated 40-fold. Data points are presented as means of two independent assays. (FIG. 1D) Characterization of ZIKV VLPs and virions by Western blot analysis. The forth and eight lanes contain pre-stained protein standards. Bands corresponding to E and prM proteins are labeled on the left side of the panel. Reactivity of pelleted VLPs expressed by pEZMRprME1-8 (1-8), pEBZHu2-3 (2-3) and purified MR766 virion particles (V) with αZHS and 4G2 was tested. E and prM bands were detected by αZHS, while only E bands were detected by 4G2 in purified virions, 1-8 and 2-3 VLPs. Capsid and processed pr and M proteins were not detected by this αZHS.

FIGS. 2A-2D: Characterization of AG129 mouse as the disease and vaccine efficacy model. Cumulative survival rate of AG129 mice challenged with various doses of MR766 (FIG. 2A) and PRVABC59 (FIG. 2B) virus. *In vivo* protective efficacy of a ZIKV vaccine is dose-dependent (FIG. 2C). The protective efficacy of a non-infectious recombinant adenovirus-vector ZIKV vaccine candidate (rAdMR1-8) was determined by challenging four weeks post-vaccinated immunized AG129 mice with 10^3 focus forming units (ffu) of PRVABC59 virus. Mice that had received 10^6, but not 10^5 transduction units (TU) of candidate vaccine were fully protective from viral challenge. ZIKV-specific reactivity of pre-challenge and post challenge serum specimens immunized with 10^6 TU of vaccine was characterized by Western blot analysis (FIG. 2D). E and prM bands were detected by pre-and post-challenge pooled serum in purified virions, 1-8 and 2-3 VLPs. Mature M protein was only detected by post-challenge serum in purified virions. 4WPC = 4 weeks post-challenge; 4WPV = 4 weeks post-vaccination.

FIGS. 3A-3D: ZIKV-specific neutralizing (Nt) antibody against MR766 and PRVABC59 virus in AG129 and ICR mice immunized with 10^6 TU of rAdMR1-8 vaccine. Only AG129 mice were challenged with 10^3 ffu of PRVABC59 virus at four weeks post vaccination. For FIGS. 3A and 3B, bars are from left to right: AG1, AG2, AG3, AG4 and AG5. For FIGS. 3C and 3D, bars are from left to right: ICR1, ICR2, ICR3, ICR4 and ICR5.

FIGS. 4A-4D: Generation of a non-infectious recombinant adenovirus vaccine (rAdMR1-8) expressing ZIKV virus-like particles. (FIG. 4A) The transcription unit expressing prM and E protein was transferred to pAdMR1-8 plasmid by homologous recombination. (FIG. 4B) Schematic representation of the procedure to generate rAdMR1-8 in 293A cells. (FIG. 4C) rAdMR1-8 is used to transduce Vero cells and its titer is measured by an antigen focus assay. (FIG. 4D) Cells expressing ZIKV VLP are counted to determine the transduction unit titer of rAdMR1-8.

FIGS. 5A-5B: Total IgG titers in AG129 (FIG. 5A) and ICR (FIG. 5B) mice immunized with 10^6 transduction units of rAdMR1-8 vaccine. Only AG129 mice were challenged with PRC59 virus at four weeks post-vaccination. IgG reactivity was determined using MR766 (1-8 VLP) and BPH2015 (2-3 VLP) antigens. For FIG. 5A, bars are from left to right: AG1, AG2, AG3, AG4 and AG5. For FIG. 5B, bars are from left to right: ICR1, ICR2, ICR3, ICR4 and ICR5.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file, created on May 31, 2017, 71.7 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NO: 1 is the nucleotide sequence of plasmid pEZMRprME1-8 having the following features:

Nucleotides 517-999 – CMV promoter

Nucleotides 1105-1117 – Kozak consensus sequence

Nucleotides 1114-1185 – coding sequence for modified JEV signal sequence

Nucleotides 1186-3204 – prME coding sequence

Nucleotides 3279-3479 – BGH) poly(A) signal and transcription termination sequence.

SEQ ID NO: 2 is the nucleotide sequence of plasmid pEBZHu8, having the following features:

Nucleotides 517-999 – CMV promoter

Nucleotides 1105-1117 – Kozak consensus sequence

Nucleotides 1114-1185 – coding sequence for modified JEV signal sequence

Nucleotides 1186-3213 – prME coding sequence

Nucleotides 3288-3488 – BGH poly(A) signal and transcription termination sequence.

SEQ ID NO: 3 is the nucleotide sequence of plasmid pEBZHu2-3, having the following features:

Nucleotides 517-999 – CMV promoter

Nucleotides 1105-1117 – Kozak consensus sequence

Nucleotides 1114-1185 – coding sequence for modified JEV signal sequence

Nucleotides 1186-3210 – prME coding sequence

Nucleotides 3285-3485 – BGH) poly(A) signal and transcription termination sequence.

SEQ ID NO: 4 is the amino acid sequence of a modified JEV signal sequence.

SEQ ID NO: 5 is the amino acid sequence of prME expressed by plasmid pEZMRprME1-8, having the following features:

Residues 1-93 – pr sequence

Residues 94-168 – M protein

Residues 169-672 – E protein.

SEQ ID NO: 6 is the amino acid sequence of prME expressed by plasmid pEBZHu8, having the following features:

Residues 1-94 pr sequence

Residues 95-169 – M protein

Residues 170-675 – E protein.

SEQ ID NO: 7 is the amino acid sequence of prME expressed by plasmid pEBZHu2-3, having the following features:

Residues 1-93 pr sequence

Residues 94-168 – M protein

Residues 169-674 – E protein.

SEQ ID NO: 8 is the nucleotide sequence of a Kozak consensus sequence.

SEQ ID NOs: 9-19 are amino acid sequences containing furin and signalase cleavage sites (see Table 1).

SEQ ID NO: 20 is the nucleotide sequence of plasmid pEZMRprME KD having the following features:

Nucleotides 517-999 – CMV promoter

Nucleotides 1105-1117 – Kozak consensus sequence

Nucleotides 1114-1185 – coding sequence for modified JEV signal sequence

Nucleotides 1186-3204 – prME coding sequence with modifications at E106/107

Nucleotides 3279-3479 – BGH) poly(A) signal and transcription termination sequence.

SEQ ID NO: 21 is the amino acid sequence of prME expressed by plasmid pEZMRprME KD, having the following features:

Residues 1-93 – pr sequence

Residues 94-168 – M protein

Residues 169-672 – E protein with K/K at residues 274/275.

SEQ ID NO: 22 is the nucleotide sequence of plasmid pEBZHu2-3 KD, having the following features:

Nucleotides 517-999 – CMV promoter

Nucleotides 1105-1117 – Kozak consensus sequence

Nucleotides 1114-1185 – coding sequence for modified JEV signal sequence

Nucleotides 1186-3210 – prME coding sequence with modifications at E106/107

Nucleotides 3285-3485 – BGH) poly(A) signal and transcription termination sequence.

SEQ ID NO: 23 is the amino acid sequence of prME expressed by plasmid pEBZHu2-3 KD, having the following features:

Residues 1-93 pr sequence

Residues 94-168 – M protein

Residues 169-674 – E protein with K/D at residues 274/275.

SEQ ID NOs: 24-29 are primer sequences.

DETAILED DESCRIPTION

I. Abbreviations

Ad	adenovirus
Ag	antigen
BGH	bovine growth hormone
CMV	cytomegalovirus

E	envelope (protein)
ELISA	enzyme-linked immunosorbent assay
IFA	immunofluorescent antibody assay or immunofluorescence assay
i.m.	intramuscularly
i.p.	intraperitoneally
ffu	focus forming unit
FRμNT	focus-reduction micro-neutralization test
GAC-ELISA	IgG antibody-captured ELISA
GBS	Guillan-Barré syndrome
JESS	Japanese encephalitis signal sequence
JEV	Japanese encephalitis virus
M	membrane (protein)
MAb	monoclonal antibody
MHIAF	mouse hyper-immune ascetic fluid
NS	non-structural (protein)
Nt	neutralizing
OD	optical density
PC	post challenge
pfu	plaque forming unit
prM	premembrane (protein)
PV	post vaccination
RT-PCR	reverse transcriptase polymerase chain reaction
SS	signal sequence
TU	transduction unit
VLP	virus-like particle
WHO	World Health Organization
ZIKV	Zika virus

II. Terms and Methods

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of*

Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

Adenovirus (Ad): A non-enveloped virus with a liner, double-stranded DNA genome and an icosahedral capsid. There are at least 68 known serotypes of human adenovirus, which are divided into seven species (species A, B, C, D, E, F and G). Different serotypes of adenovirus are associated with different types of disease, with some serotypes causing respiratory disease (primarily species B and C), conjunctivitis (species B and D) and/or gastroenteritis (species F and G). Modified adenoviruses are often used for delivery of exogenous DNA, such as for vaccination or gene therapy.

Adjuvant: A substance or vehicle that non-specifically enhances the immune response to an antigen. Adjuvants can include a suspension of minerals (alum, aluminum hydroxide, or phosphate) on which antigen is adsorbed; or water-in-oil emulsion in which antigen solution is emulsified in mineral oil (for example, Freund's incomplete adjuvant), sometimes with the inclusion of killed mycobacteria (Freund's complete adjuvant) to further enhance antigenicity. Immunostimulatory oligonucleotides (such as those including a CpG motif) can also be used as adjuvants (for example, see U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; 6,339,068; 6,406,705; and 6,429,199). Adjuvants also include biological molecules, such as costimulatory molecules. Exemplary biological adjuvants include IL-2, RANTES, GM-CSF, TNF-α, IFN-γ, G-CSF, LFA-3, CD72, B7-1, B7-2, OX-40L and 41 BBL.

Administer: As used herein, administering a composition (*e.g.* an immunogenic composition) to a subject means to give, apply or bring the composition into contact with the subject. Administration can be accomplished by any of a number of routes, such as, for example, topical, oral, subcutaneous, intramuscular, intraperitoneal, intravenous, intrathecal and intramuscular.

Animal: Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects, for example, humans, non-human primates, dogs, cats, horses, and cows.

Antibody: An immunoglobulin molecule produced by B lymphoid cells with a specific amino acid sequence. Antibodies are evoked in humans or other animals by a specific antigen (immunogen). Antibodies are characterized by reacting specifically with the antigen in some demonstrable way,

antibody and antigen each being defined in terms of the other. "Eliciting an antibody response" refers to the ability of an antigen or other molecule to induce the production of antibodies.

Antigen: A compound, composition, or substance that can stimulate the production of antibodies or a T-cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous immunogens.

Biological sample: A sample obtained from a subject (such as a human or veterinary subject). Biological samples, include, for example, fluid, cell and/or tissue samples. In some embodiments herein, the biological sample is a fluid sample. Fluid sample include, but are not limited to, serum, blood, plasma, urine, feces, saliva, cerebral spinal fluid (CSF) and bronchoalveolar lavage (BAL) fluid.

Capsid protein (C protein): One of three flavivirus structural proteins that forms the flavivirus particle. The C protein is a dimeric, alpha-helical protein with an unstructured N-terminus. In flavivirus particles, the C protein is found internal to the lipid bilayer and directly contacts the flavivirus genomic RNA.

Codon-optimized: A "codon-optimized" nucleic acid refers to a nucleic acid sequence that has been altered such that the codons are optimal for expression in a particular system (such as a particular species or group of species). For example, a nucleic acid sequence can be optimized for expression in mammals, or more specifically, humans. Codon optimization does not alter the amino acid sequence of the encoded protein.

Contacting: Placement in direct physical association; includes both in solid and liquid form. "Contacting" is often used interchangeably with "exposed." In some cases, "contacting" includes transfecting, such as transfecting a nucleic acid molecule into a cell. In other examples, "contacting" refers to incubating a molecule (such as an antibody) with a biological sample.

Control: A reference standard, for example a positive control or negative control. A positive control is known to provide a positive test result. A negative control is known to provide a negative test result. However, the reference standard can be a theoretical or computed result, for example a result obtained in a population.

Detectable label: A detectable compound or composition that is conjugated directly or indirectly to another molecule, such as an antibody, protein or microparticle, to facilitate detection of that molecule. Specific, non-limiting examples of labels include fluorescent tags, enzymatic linkages, and radioactive isotopes. In one example, a "labeled antibody" refers to incorporation of another molecule in the antibody. For example, the label is a detectable marker, such as the incorporation of a

radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (for example, streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes or radionucleotides (such as ^{35}S, ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{19}F, ^{99m}Tc, ^{131}I, ^{3}H, ^{14}C, ^{15}N, ^{90}Y, ^{99}Tc, ^{111}In and ^{125}I), fluorescent labels (such as fluorescein isothiocyanate (FITC), rhodamine, lanthanide phosphors), enzymatic labels (such as horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase), chemiluminescent markers, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (such as a leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags), or magnetic agents, such as gadolinium chelates. In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

Envelope (E) glycoprotein: A flavivirus (including Zika virus) structural protein that mediates binding of flavivirus virions to cellular receptors on host cells. The flavivirus E protein is required for membrane fusion, and is the primary antigen inducing protective immunity to flavivirus infection. Flavivirus E protein affects host range, tissue tropism and viral virulence. The flavivirus E protein contains three structural and functional domains, DI-DIII. In mature virus particles the E protein forms head to tail homodimers lying flat and forming a dense lattice on the viral surface. As used herein, "positions 106 and 107" or "residues 106 and 107" of the ZIKV E protein refer to the amino acids corresponding to residues 274 and 275 of the prME amino acid sequences set forth herein as SEQ ID NO: 21 and SEQ ID NO: 23.

Fluorophore: A chemical compound, which when excited by exposure to a particular wavelength of light, emits light (*i.e.*, fluoresces), for example at a different wavelength.

Examples of fluorophores that may be used in the compositions and methods disclosed herein are provided in U.S. Patent No. 5,866,366 to Nazarenko *et al.*: 4-acetamido-4'-isothiocyanatostilbene-2,2'disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), 4-amino-N-[3-vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)maleimide, anthranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumarin 151); cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5', 5"-dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin;

diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino]naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC (XRITC); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; R-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron .RTM. Brilliant Red 3B-A); rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives.

Other suitable fluorophores include thiol-reactive europium chelates which emit at approximately 617 nm (Heyduk and Heyduk, *Analyt. Biochem.* 248:216-27, 1997; *J. Biol. Chem.* 274:3315-22, 1999).

Other suitable fluorophores include GFP, Lissamine™, diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Patent No. 5,800,996 to Lee *et al.*) and derivatives thereof. Other fluorophores known to those skilled in the art may also be used.

Heterologous: A heterologous protein or polypeptide refers to a protein or polypeptide derived from a different source or species.

Immune complex: A protein complex that comprises an antibody bound to an antigen. In the context of the present disclosure, the term "**immune complex**" is used to indicate a protein complex that includes an antigen (such as a VLP) bound to at least one antibody. In some cases, the immune complex includes an antigen (such as a VLP) bound to two separate antigen-specific antibodies (each binding a different epitope of the antigen), or includes an antigen (such as a VLP) bound to an antigen-specific antibody, which is further bound to a secondary antibody. The term "**antibody-antigen**

complex" or "**antibody-VLP complex**" is used to refer to an antigen (or VLP) bound to one antibody. Furthermore, the term "**antibody-antibody complex**" is used to refer to an antibody bound to a different antibody (such as an antigen-specific antibody bound to a secondary antibody).

Immune response: A response of a cell of the immune system, such as a B-cell, T-cell, macrophage or polymorphonucleocyte, to a stimulus such as an antigenic polypeptide or vaccine. An immune response can include any cell of the body involved in a host defense response, including for example, an epithelial cell that secretes an interferon or a cytokine. An immune response includes, but is not limited to, an innate immune response or inflammation. As used herein, a protective immune response refers to an immune response that protects a subject from infection (prevents infection or prevents the development of disease associated with infection). Methods of measuring immune responses are well known in the art and include, for example, measuring proliferation and/or activity of lymphocytes (such as B or T cells), secretion of cytokines or chemokines, inflammation, antibody production and the like.

Immunize: To render a subject protected from an infectious disease, such as by vaccination.

Isolated: An "isolated" or "purified" biological component (such as a nucleic acid, peptide, protein, protein complex, or virus-like particle) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, that is, other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins that have been "isolated" or "purified" thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell, as well as chemically synthesized nucleic acids or proteins. The term "isolated" or "purified" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, an isolated biological component is one in which the biological component is more enriched than the biological component is in its natural environment within a cell, or other production vessel. Preferably, a preparation is purified such that the biological component represents at least 50%, such as at least 70%, at least 90%, at least 95%, or greater, of the total biological component content of the preparation.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

Pharmaceutically acceptable carrier: The pharmaceutically acceptable carriers (vehicles) useful in this disclosure are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of one or more therapeutic compositions, such as one or more Zika virus vaccines, and additional pharmaceutical agents.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Polypeptide: A polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used. The terms "polypeptide" or "protein" as used herein are intended to encompass any amino acid sequence and include modified sequences such as glycoproteins. The term "polypeptide" is specifically intended to cover naturally occurring proteins, as well as those which are recombinantly or synthetically produced. The term "residue" or "amino acid residue" includes reference to an amino acid that is incorporated into a protein, polypeptide, or peptide.

A conservative substitution in a polypeptide is substitution of one amino acid residue in a protein sequence for a different amino acid residue having similar biochemical properties. Typically, conservative substitutions have little to no impact on the activity of a resulting polypeptide. For example, a flavivirus protein including one or more conservative substitutions (for example no more than 2, 5, 10, 20, 30, 40, or 50 substitutions) retains the structure and function of the wild-type protein. A polypeptide can be produced to contain one or more conservative substitutions by manipulating the nucleotide sequence that encodes that polypeptide using, for example, standard procedures such as site-directed mutagenesis or PCR. In one example, such variants can be readily selected by testing antibody cross-reactivity or its ability to induce an immune response. Examples of conservative substitutions are shown below.

Original Residue	Conservative Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
His	Asn; Gln
Ile	Leu, Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Conservative substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

The substitutions which in general are expected to produce the greatest changes in protein properties will be non-conservative, for instance changes in which (a) a hydrophilic residue, for example, seryl or threonyl, is substituted for (or by) a hydrophobic residue, for example, leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, for example, lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, for example, glutamyl or aspartyl; or (d) a residue having a bulky side chain, for example, phenylalanine, is substituted for (or by) one not having a side chain, for example, glycine.

Premembrane (prM) protein: A flavivirus structural protein. The prM protein is an approximately 25 kDa protein that is the intracellular precursor for the membrane (M) protein. prM is believed to stabilize the E protein during transport of the immature virion to the cell surface. When the virus exits the infected cell, the prM protein is cleaved to the mature M protein, which is part of the viral envelope (Reviewed in Lindenbach and Rice, In: *Fields Virology*, Knipe and Howley, eds., Lippincott, Williams, and Wilkins, 991-1041, 2001).

Preventing, treating or ameliorating a disease: "Preventing" a disease refers to inhibiting the full development of a disease. "Treating" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. "Ameliorating" refers to the reduction in the number or severity of one or more signs or symptoms of a disease.

Promoter: A promoter is an array of nucleic acid control sequences which direct transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences near the start site of transcription. A promoter also optionally includes distal enhancer or repressor elements. A "constitutive promoter" is a promoter that is continuously active and is not subject to regulation by external signals or molecules. In contrast, the activity of an "inducible promoter" is regulated by an external signal or molecule (for example, a transcription factor). In some embodiments herein, the promoter is a cytomegalovirus (CMV) promoter, such as the CMV E1A promoter.

Recombinant: A recombinant nucleic acid, protein or virus is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acids, for example, by genetic engineering techniques. The term recombinant includes nucleic acids, proteins and viruses that have been altered solely by addition, substitution, or deletion of a portion of a natural nucleic acid molecule, protein or virus.

Secondary antibody: An antibody that specifically recognizes the Fc region of a particular isotype of antibody (for example specifically recognizes human IgG or human IgM). Secondary antibodies for use with the methods disclosed herein include, but are not limited to, anti-human IgG and anti-human IgM. In some embodiments herein, the secondary antibody is conjugated to a detectable label, such as a fluorophore, enzyme or radioisotope, to facilitate detection of antibodies and/or immune complexes to which the secondary antibody is bound.

Sequence identity/similarity: The identity/similarity between two or more nucleic acid sequences, or two or more amino acid sequences, is expressed in terms of the identity or similarity

between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences are. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the percentage, the more similar the sequences are. Homologs or orthologs of nucleic acid or amino acid sequences possess a relatively high degree of sequence identity/similarity when aligned using standard methods.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988; Higgins & Sharp, *Gene*, 73:237-44, 1988; Higgins & Sharp, *CABIOS* 5:151-3, 1989; Corpet *et al.*, *Nuc. Acids Res.* 16:10881-90, 1988; Huang *et al. Computer Appls. in the Biosciences* 8, 155-65, 1992; and Pearson *et al.*, *Meth. Mol. Bio.* 24:307-31, 1994. Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site.

BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. If the two compared sequences share homology, then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

Serum: The fluid portion of the blood that separates out from clotted blood. Serum contains many proteins, including antibodies, but does not contain clotting factors.

Signal sequence: A short amino acid sequence found at the N-terminus of most newly synthesized proteins that are targeted to the secretory pathway. In some embodiments herein, the signal sequence is a JEV signal sequence, such as the JEV signal sequence present at the N-terminus of the prM protein. In particular examples, the signal sequence is a modified JEV prM signal sequence having the amino acid sequence of SEQ ID NO: 4.

Subject: Living multi-cellular vertebrate organisms, a category that includes both human and non-human mammals (such as mice, rats, rabbits, sheep, horses, cows, and non-human primates).

Therapeutically effective amount: A quantity of a specified agent (such as an immunogenic composition) sufficient to achieve a desired effect in a subject being treated with that agent. For example, this may be the amount of a virus vaccine useful for eliciting an immune response in a subject and/or for preventing infection by the virus. In the context of the present disclosure, a therapeutically effective amount of a Zika virus vaccine, for example, is an amount sufficient to increase resistance to, prevent, ameliorate, and/or treat infection caused by Zika virus in a subject without causing a substantial cytotoxic effect in the subject. The effective amount of a Zika virus vaccine (or Zika virus immunogenic composition) useful for increasing resistance to, preventing, ameliorating, and/or treating infection in a subject will be dependent on, for example, the subject being treated, the manner of administration of the therapeutic composition and other factors.

Transcriptional unit: A nucleic acid that codes for a single RNA molecule and includes the sequences necessary for transcription of the encoded RNA.

Transcription termination sequence: A nucleic acid sequence that marks the end of a gene or operon during transcription. A transcription termination sequence mediates termination of transcription by providing signals in the newly synthesized RNA that trigger processes that release the mRNA from the transcriptional complex. In some embodiments herein, the transcription termination sequence is a BGH transcription termination sequences, such as the sequence set forth as nucleotides 3279-3479 of SEQ ID NO: 1.

Translation initiation sequence: A nucleic acid sequence that mediates the initiation of translation of an RNA. In some embodiments herein, the translation initiation sequence is a Kozak consensus sequence comprising SEQ ID NO: 8.

Vaccine: A preparation of immunogenic material capable of stimulating an immune response, administered for the prevention, amelioration, or treatment of infectious or other types of disease. The immunogenic material may include attenuated or killed microorganisms (such as bacteria or viruses), or antigenic proteins (including VLPs), peptides or DNA derived from them. An attenuated vaccine is a virulent organism that has been modified to produce a less virulent form, but nevertheless retains the ability to elicit antibodies and cell-mediated immunity against the virulent form. A killed vaccine is a previously virulent microorganism that has been killed with chemicals or heat, but elicits antibodies against the virulent microorganism. Vaccines may elicit both prophylactic (preventative) and therapeutic responses. Methods of administration vary according to the vaccine, but may include inoculation, ingestion, inhalation or other forms of administration. Vaccines may be administered with an adjuvant to boost the immune response.

Vector: A vector is a nucleic acid molecule allowing insertion of foreign nucleic acid without disrupting the ability of the vector to replicate and/or integrate in a host cell. A vector can include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. An insertional vector is capable of inserting itself into a host nucleic acid. A vector can also include one or more selectable marker genes and other genetic elements. An expression vector is a vector that contains the necessary regulatory sequences to allow transcription and translation of inserted gene or genes.

Virus-like particle (VLP): Virus particles made up of one of more viral structural proteins, but lacking the viral genome. Because VLPs lack a viral genome, they are non-infectious. In some embodiments, the VLPs are flavivirus VLPs, such as Zika virus VLPs. In particular examples, flavivirus VLPs include two flavivirus structural proteins – prM/M and E.

Zika virus (ZIKV): A member of the virus family *Flaviviridae* and the genus *Flavivirus*. Other members of this genus include dengue virus, yellow fever virus, Japanese encephalitis virus (JEV), West Nile virus and Spondweni virus. ZIKV is spread by the daytime-active mosquitoes *Aedes aegypti* and *A. albopictus*. This virus was first isolated from a *Rhesus* macaque from the Zika Forest of Uganda in 1947. Since the 1950s, ZIKV has been known to occur within a narrow equatorial belt from Africa to Asia. The virus spread eastward across the Pacific Ocean in 2013-2014, resulting in ZIKV outbreaks in Oceania to French Polynesia, New Caledonia, the Cook Islands, and Easter Island. In 2015, ZIKV spread to Mexico, Central America, the Caribbean and South America, where ZIKV has reached pandemic levels. Infection by ZIKV generally causes either no symptoms are mild symptoms, including mild headache, maculopapular rash, fever, malaise, conjunctivitis and joint pain. ZIKV causes symptoms in about 20% of infected individuals, and no deaths from the virus have yet been reported. However, ZIKV infection has been linked to the birth of microcephalic infants following maternal infection, as well an increase in cases of GBS. Reports have also indicated that ZIKV has the potential for human blood-borne and sexual transmission. ZIKV has also been found in human saliva and breastmilk. There are currently no available medical countermeasures for the treatment or prevention of Zika virus infection (Malone *et al.*, *PLoS Negl Trop Dis* 10(3):e0004530, 2016).

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. "Comprising A or B" means including A, or B, or A and B. It is further to be understood

that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

III. Introduction

To address the public health emergency that has arisen from the rapid spread of ZIKV, the present disclosure provides compositions for use as ZIKV vaccines, as well as reagents and methods for detection of ZIKV infection in susceptible individuals. In particular, disclosed herein are transcriptional units that encode ZIKV prM and E proteins (prME), which upon expression, form virus-like particles (VLPs). In specific embodiments, the transcriptional units encode a modified Japanese encephalitis virus (JEV) prM signal sequence to improve protein translocation and VLP secretion. In some examples, the transcriptional units also include a CMV promoter/enhancer element to improve mRNA synthesis, a Kozak translation initiation sequence to enhance translation, and a bovine growth hormone (BGH) poly(A) signal and transcription termination sequence. Three prME expression plasmids derived from three different ZIKV strains (MR766, P6-740 and BPH2015) were generated. MR766 is the prototype African genotype virus; P6-740 is the prototype Asian genotype virus; and BPH2015 is the current circulating Asian genotype virus. Also disclosed are two mutant constructs based on MR766 and BHP2015 that express VLPs having amino acid substitutions at positions 106 and 107 of the E protein.

The ZIKV transcriptional units were used in the development of three different vaccine platforms – a plasmid DNA vaccine that includes the transcriptional unit; a recombinant adenovirus (rAd) harboring the transcriptional unit (and that expresses ZIKV VLPs upon transduction of a cell); and VLPs isolated from cells expressing the transcriptional unit.

Though previous flavivirus vaccine work has focused on using a plasmid DNA based vaccine, there is evidence to suggest that a non-replicating vector-based protein nanoparticle (Ledgerwood *et al.*, *Vaccine* 29, 304-313, 2010; Smaill *et al.*, *Sci Transl Med* 5(205):205ra134, 2013; Zhu *et al.*, *Lancet* 385, 2272-2279, 2015) would be an efficient platform to deliver a transcription, translation and protein processing optimized vaccine component, thereby producing a ZIKV vaccine capable of

eliciting a strong immune response. Thus, in one aspect, disclosed herein is the construction of a ZIKV prME transcriptional unit and insertion of the optimized transcriptional unit into a non-infectious rAd serotype 5 vector. The rAd ZIKV vaccine was tested for efficacy as a single-dose vaccine and shown to provide protective immunity in a mouse challenge model.

In addition, methods of using ZIKV VLPs encoded by the transcriptional units to develop immunoassays, such as antibody capture ELISAs, to enable detection of anti-ZIKV antibodies from patient samples is also described.

IV. Overview of Several Embodiments

Disclosed herein are transcriptional units encoding ZIKV prM and E proteins, which upon translation, form ZIKA VLPs. The disclosed transcriptional units and VLPs are suitable for use with a variety of ZIKV vaccine platforms, as well as in multiple different detection methods for the diagnosis of ZIKV infection.

Provided herein are isolated nucleic acid molecules that include a ZIKV transcriptional unit. In some embodiments, the transcriptional units include a sequence encoding a modified Japanese encephalitis virus (JEV) signal sequence, and include a ZIKV prME coding sequence. In some examples, the modified JEV signal sequence comprises SEQ ID NO: 4, or comprises no more than 5, no more than 4, no more than 3, no more than 2 or no more than 1 substitution(s) relative to SEQ ID NO: 4.

In some embodiments, the transcriptional unit further includes a promoter operably linked to the prME coding sequence. In some examples, the promoter is a CMV promoter, such as the CMV E1A promoter. In specific examples, the promoter sequence is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to nucleotides 517-999 of SEQ ID NO: 1. In one non-limiting example, the promoter sequence comprises or consist of nucleotides 517-999 of SEQ ID NO: 1.

In some embodiments, the transcriptional unit further includes a transcription termination sequence. In some examples, the transcription termination sequence comprises a bovine growth hormone (BGH) transcription termination sequence. In specific examples, the transcription termination sequence is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to nucleotides 3279-3479 of SEQ ID NO: 1. In one non-limiting example, the transcription termination sequence comprises or consists of nucleotides 3279-3479 of SEQ ID NO: 1.

In some embodiments, the transcriptional unit further includes a translation initiation sequence. In some examples, the translation initiation sequence is a Kozak consensus sequences, such as the sequence GCCGCCGCCATGG (SEQ ID NO: 8).

In some embodiments, the ZIKV is an African genotype strain, such as MR-766. In other embodiments, the ZIKV is an Asian genotype strain, such as SPH2015, P6-740, or FSS 13025.

In some embodiments, the prME coding sequence is codon-optimized for expression in human cells.

In some embodiments, the ZIKV prME coding sequence is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to nucleotides 1186-3204 of SEQ ID NO: 1, nucleotides 1186-3213 of SEQ ID NO: 2, nucleotides 1186-3210 of SEQ ID NO: 3, nucleotides 1186-3204 of SEQ ID NO: 20 or nucleotides 1186-3210 of SEQ ID NO: 22. In some examples, the ZIKV prME coding sequence comprises or consists of nucleotides 1186-3204 of SEQ ID NO: 1, nucleotides 1186-3213 of SEQ ID NO: 2, nucleotides 1186-3210 of SEQ ID NO: 3, nucleotides 1186-3204 of SEQ ID NO: 20 or nucleotides 1186-3210 of SEQ ID NO: 22.

Also provided herein is a vector that includes a nucleic acid molecule (a transcriptional unit) disclosed herein. In some embodiments, the vector is a plasmid vector. In other embodiments, the vector is an adenovirus vector. In some examples, the vector is a replication-incompetent adenovirus vector.

Further provided are isolated cells that contain a nucleic acid molecule (transcriptional unit) or vector disclosed herein.

Recombinant adenoviruses that include a nucleic acid molecule disclosed herein are also provided. By harboring the transcriptional unit, the recombinant adenoviruses express ZIKV VLPs upon transduction of a host cell.

Also provided herein are VLPs encoded by a nucleic acid molecule (or vector) disclosed herein. In some embodiments, the E protein of the VLP includes at least one amino acid substitution that reduces cross-reactivity. In some examples, the at least one amino acid substitution is at position 106 and/or position 107 of the E protein (corresponding to residues 274 and 275 of the prME sequences set forth herein as SEQ ID NO: 21 and SEQ ID NO: 23). In specific examples, the E protein of the VLP has a lysine at position 106 and an aspartic acid at position 107; an arginine at position 106 and an aspartic acid at position 107; an arginine at position 106 and a histidine at position 107; a glutamic acid at position 106 and an aspartic acid at position 107; or a glutamic acid at position

106 and an arginine at position 107. In particular non-limiting examples, the prME amino acid sequence of the VLP comprises SEQ ID NO: 21 or SEQ ID NO: 23.

Compositions, such as immunogenic compositions, that include a nucleic acid molecule, vector, recombinant adenovirus or VLP disclosed herein, and a pharmaceutically acceptable carrier, are further provided herein.

Also provided herein are methods of eliciting an immune response against ZIKV in a subject by administering to the subject a nucleic acid molecule, vector, recombinant adenovirus, VLP or composition disclosed herein. In some embodiments, the subject is a human. The immune response may include, for example, induction of ZIKV-specific antibodies (such as IgM and/or IgG antibodies) or induction of a virus-specific T cell response. In some examples, the immune response is a protective immune response.

Further provided is a method of immunizing a subject against ZIKV by administering to the subject a nucleic acid molecule, vector, recombinant adenovirus, VLP or composition disclosed herein. In some embodiments, the subject is a human.

Also provided herein are methods of detecting ZIKV-specific antibodies in a biological sample. In some embodiments, the method includes contacting the sample with a ZIKV VLP disclosed herein under conditions sufficient to form VLP-antibody complexes if ZIKV antibodies are present in the sample; and detecting the VLP-antibody complexes in the sample. In some examples, detecting the VLP-antibody complexes includes contacting the VLP-antibody complexes with an antibody that specifically binds the VLP and comprises a detectable label. In other examples, detecting the VLP-antibody complexes comprises contacting the VLP-antibody complexes with a secondary antibody comprising a detectable label. In specific examples, the secondary antibody is an anti-IgM antibody or an anti-IgG, such as anti-human IgM antibody or an anti-human IgG antibody.

In other embodiments, the method includes providing a secondary antibody bound to a solid support; contacting the secondary antibody-bound solid support with the biological sample under conditions sufficient to allow binding of the secondary antibody to any ZIKV-specific antibodies present in the biological sample, thereby forming antibody-antibody complexes; contacting the antibody-antibody complexes with a ZIKV VLP disclosed herein under conditions sufficient for the VLP to bind the ZIKV-specific antibodies, thereby forming immune complexes; and detecting the presence of the immune complexes. In some examples, detecting the presence of the immune complexes includes contacting the immune complexes with an antibody that specifically binds the VLP and comprises a detectable label. In some examples, the secondary antibody is an anti-IgM

antibody, such as anti-human IgM antibody. In other examples, the secondary antibody is an anti-IgG antibody, such as anti-human IgG antibody.

In yet other embodiments, the method includes providing a ZIKV-specific antibody bound to a solid support; contacting the antibody-bound solid support with a ZIKV VLP disclosed herein under conditions sufficient for the VLP to bind the ZIKV-specific antibody to form antibody-VLP complexes; contacting the antibody-VLP complexes with the biological sample to allow binding of any ZIKV-specific antibodies present in the sample to the VLP, thereby forming immune complexes; contacting the immune complexes with a secondary antibody; and detecting binding of the secondary antibody to the immune complexes. In some examples, the secondary antibody is an anti-IgM antibody, such as anti-human IgM antibody. In other examples, the secondary antibody is an anti-IgG antibody, such as anti-human IgG antibody.

In some embodiments of the methods of detecting ZIKV-specific antibodies, the biological sample is a biological fluid sample. In some examples, the biological fluid sample comprises serum, blood or plasma. In particular examples, the biological sample comprises serum.

V. Immunogenic Compositions and Administration Thereof

Immunogenic compositions that include a nucleic acid (such as a vector) comprising a ZIKV transcriptional unit encoding prME, a rAd comprising the transcriptional unit, or VLPs encoded by the transcriptional unit, can be administered to a subject to induce a ZIKV-specific immune response in a subject. The immunogenic compositions can be used prophylactically to prevent ZIKV infection, or therapeutically to promote a ZIKV immune response. The provided nucleic acid molecules, vectors, recombinant adenoviruses and VLPs are combined with a pharmaceutically acceptable carrier or vehicle for administration as a composition to human or animal subjects.

In embodiments in which a nucleic acid encoding prME is administered (either as part of a plasmid DNA or encoded by a recombinant adenovirus), the composition administered to a subject directs the synthesis of a ZIKV prME as described herein, and a cell within the body of the subject, after incorporating the nucleic acid within it, secretes ZIKV VLPs. VLPs then serve as an *in vivo* immunostimulatory composition, stimulating the immune system of the subject to generate protective immunological responses against ZIKV.

The immunogenic formulations may be conveniently presented in unit dosage form and prepared using conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the

formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers. Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets commonly used by one of ordinary skill in the art.

In certain embodiments, unit dosage formulations are those containing a dose or unit, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients particularly mentioned above, formulations encompassed herein may include other agents commonly used by one of ordinary skill in the art.

The compositions provided herein, including those for use as immune stimulatory compositions, may be administered through different routes, such as oral, including buccal and sublingual, rectal, parenteral, aerosol, nasal, intramuscular, subcutaneous, intradermal, and topical. They may be administered in different forms, including but not limited to solutions, emulsions and suspensions, microspheres, particles, microparticles, nanoparticles, and liposomes.

The volume of administration will vary depending on the route of administration. By way of example, intramuscular injections may range from about 0.1 ml to about 1.0 ml. Those of ordinary skill in the art will know appropriate volumes for different routes of administration.

Immune stimulatory compounds (for example, vaccines) can be administered by directly injecting nucleic acid molecules encoding polypeptide antigens (broadly described in Janeway & Travers, *Immunobiology: The Immune System In Health and Disease,* page 13.25, Garland Publishing, Inc., New York, 1997; and McDonnell & Askari, *N. Engl. J. Med.* 334:42-45, 1996), including virus-like particles. Vectors that include nucleic acid molecules described herein, or that include a nucleic acid sequence encoding ZIKV prME may be utilized in such DNA vaccination methods.

Thus, the term "immune stimulatory composition" or "immunogenic composition" as used herein also includes nucleic acid vaccines in which a nucleic acid molecule encoding a ZIKV prME is administered to a subject in a pharmaceutical composition. For genetic immunization, suitable delivery methods known to those skilled in the art include direct injection of plasmid DNA into

muscles (Wolff *et al., Hum. Mol. Genet.* 1:363, 1992), delivery of DNA complexed with specific protein carriers (Wu *et al., J. Biol. Chem.* 264:16985, 1989), co-precipitation of DNA with calcium phosphate (Benvenisty and Reshef, *Proc. Natl. Acad. Sci.* 83:9551, 1986), encapsulation of DNA in liposomes (Kaneda *et al., Science* 243:375, 1989), particle bombardment (Tang *et al., Nature* 356:152, 1992; Eisenbraun *et al., DNA Cell Biol.* 12:791, 1993), and *in vivo* infection using cloned retroviral vectors (Seeger *et al., Proc. Natl. Acad. Sci.* 81:5849, 1984). Similarly, nucleic acid vaccine preparations can be administered via viral carrier.

The amount of antigen in each dose of an immunogenic composition is selected as an amount that induces an immunostimulatory or immunoprotective response without significant, adverse side effects. Such amount will vary depending upon several factors. Initial injections may range from about 1 μg to about 1 mg, with some embodiments having a range of about 10 μg to about 800 μg, and still other embodiments a range of from about 25 μg to about 500 μg. Following an initial administration of the immune stimulatory composition, subjects may receive one or several booster administrations, adequately spaced. Booster administrations may range from about 1 μg to about 1 mg, with other embodiments having a range of about 10 μg to about 750 μg, and still others a range of about 50 μg to about 500 μg. Periodic boosters at intervals of 1-5 years, for instance three years, may be desirable to maintain the desired levels of protective immunity.

The pharmaceutical or immunogenic compositions may be administered in combination with other therapeutic treatments. For example, the compositions provided herein can be administered with an adjuvant, such as Freund incomplete adjuvant or Freund's complete adjuvant.

Optionally, one or more cytokines, such as IL-2, IL-6, IL-12, RANTES, GM-CSF, TNF-α, or IFN-γ, one or more growth factors, such as GM-CSF or G-CSF; one or more molecules such as OX-40L or 41 BBL, or combinations of these molecules, can be used as biological adjuvants (see, for example, Salgaller *et al.*, 1998, *J. Surg. Oncol.* 68(2):122-38; Lotze *et al.*, 2000, *Cancer J. Sci. Am.* 6(Suppl 1):S61-6; Cao *et al.,* 1998, *Stem Cells* 16(Suppl 1):251-60; Kuiper *et al.,* 2000, *Adv. Exp. Med. Biol.* 465:381-90). These molecules can be administered systemically (or locally) to the host.

VI. Detection of Zika Virus Antibodies in Patient Samples

Serological methods of detecting ZIKV-specific antibodies in a biological sample, such as a serum sample, are disclosed herein. These methods use the ZIKV VLPs disclosed herein. Detection assays based on binding of an antigen to an antibody are well known in the art and include, for example, ELISA, microsphere immunoassay (MIA), immunofluorescence assay (IFA), Western blot,

fluorescence activated cell sorting (FACS), radioimmunoassay (RIA), immunohistochemistry (IHC) and plaque reduction neutralization test (PRNT). As is well known to one of skill in the art, in some cases the detection assay further includes the step of contacting an antigen-antibody complex with a detection reagent, such as a labeled secondary antibody (*e.g.*, an anti-isotype antibody, such as an anti-IgG antibody), or in the case of a sandwich ELISA, a second antibody that recognizes the same antigen as the first antibody and is labeled for detection. Secondary antibodies can also be conjugated to magnetic beads to allow for magnetic sorting. In other cases, the primary antibody is directly labeled. Directly labeled antibodies can be used for a variety of detection assays, such as FACS. The ZIKV VLPs disclosed herein can be used with a variety of immune-based detection assays for the diagnosis of ZIKV infection. Several exemplary immune-based detection assays are described below.

A. IgM or IgG Antibody Capture ELISAs

The immune response following a flavivirus infection includes the production of IgM and IgG antibodies, which are primarily directed against the flavivirus E protein. IgM antibody capture (MAC) or IgG antibody capture (GAC) ELISAs are commonly used to detect the level of IgM or IgG (respectively) in serum samples of patients suspected of having a flavivirus infection. In these assays, anti-human IgM or anti-human IgG serves as a capture antibody and is coated onto an appropriate assay plate, such as a multi-well plate. After blocking of the plate, such as with nonfat dry milk, diluted human sera are reacted with the anti-human IgM or IgG. In the context of the present disclosure, purified ZIKV VLPs, which serve as the antigen, are added to the plates. A ZIKV antigen-specific antibody conjugated to detectable label (for example, an enzyme or fluorophore) is then reacted with the immobilized VLPs. The detectable label is then measured to detect the presence of ZIKV-specific antibodies that were present in the serum sample. Serial dilutions of positive sera can be evaluated. The maximum dilution that exhibits positive signal is the titer for the serum. The titer of the MAC-ELISA or GAC-ELISA can be compared with the titers of other tests, such as hemagglutination inhibition tests (HIT) or PRNT. Serum samples can also be tested on control antigen in addition to viral antigen, to reduce the number of false-positive results due to non-specific binding of the serum or other factors (U.S. Patent Application Publication No. 2006/0115896).

B. Microsphere Immunoassay (MIA)

Microsphere immunoassays are becoming increasingly popular for laboratory diagnosis of many diseases (Earley *et al.*, *Cytometry* 50:239-242, 2002; Kellar *et al.*, *Cytometry* 45:27-36, 2001). The technology involves the detection and analysis of a reaction (such as an antibody or other ligand) attached to microspheres or beads. The detecting instrument is a simplified flow cytometer, and lasers

simultaneously identify the microsphere sets and measure the fluorescence associated with the reaction. The speed at which these tests can be performed and the ability to multiplex make this methodology particularly useful.

A MIA can be used to detect the presence of Zika virus-specific antibodies in a sample. In some embodiments, microsphere beads are coated with a ZIKV-specific antibody and contacted with a ZIKV VLP (as disclosed herein) such that the ZIKV VLPs bind to the microsphere-bound Zika virus-specific antibodies. The microsphere immune complexes are mixed with a serum sample such that antibodies in the sample that are specifically reactive with the ZIKV VLPs bind the VLPs bound (indirectly) to the microsphere. The bead-bound immune complexes are allowed to react with fluorescent-dye labeled anti-species antibody (such as PE-labeled anti-human IgM or anti-human IgG), and are measured using a microsphere reader (such as a Luminex instrument). In an alternative embodiment, microsphere beads are coated directly with the ZIKV VLPs and VLP-bound microspheres are contacted with the serum samples.

C. Indirect ELISA

Indirect ELISAs to detect the presence of virus-specific antibodies are typically carried out by coating a microtiter plate with an antigen-specific antibody (such as a ZIKV-specific antibody), blocking the plates to prevent non-specific binding to the plate surface, and adding virus antigen (such as a ZIKV VLP) to allow binding of the antigen to the virus-specific antibody. After several washes, diluted human sera is added to allow binding of any antibodies present in the sample to the immobilized viral antigens (*e.g.* the VLP antigens). IgM or IgG antibodies that were present in the sample are then detected using a labelled secondary antibody, such as anti-human IgG or anti-human IgM conjugated to a detectable label (such as an enzyme or fluorophore). The presence of ZIKV-specific antibody is detected by measuring the detectable label (for example, by measuring fluorescence, optical density or colorimetric absorbance).

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the disclosure to the particular features or embodiments described.

EXAMPLES

Example 1: Materials and Methods

This example describes the materials and experimental procedures used for the studies described in Example 2.

Cells and viruses

COS-1, Vero E6, 293A and C6/36 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO®, Grand Island, NY, USA) with 10% heat-inactivated fetal bovine serum (FBS, Hyclone®, Logan, UT, USA). All media were supplemented with 2 mM L-glutamine, 110 mg/L sodium pyruvate, 0.1 mM non-essential amino acids, 20 mL/L 7.5% $NaHCO_3$, 100 U/mL penicillin, and 100 µg/mL streptomycin. All cells were maintained at 37°C with 5% CO_2 except for C6/36 cells, which were maintained at 28°C without CO_2.

The strains of Zika virus used in this study included viruses belonging to the African genotype (Monkey/Uganda/1947/MR766) and Asian genotype (Mosq/Malaysia/1966/P6-740; Hu/Cambodia/2010/FSS 13025; and Hu/Puerto Rico/2015/PRVABC59). All viruses were obtained from the collection of Division of Vector-borne Diseases (DVBD), CDC, Fort Collins, CO. Virus stocks were propagated by infecting Vero E6 cells in DMEM with 2% FBS for 5-7 days. Culture supernatants were harvested, clarified by centrifugation, and stored in aliquots at -80°C.

Virus titer determination

Virus quantification was determined by antigen focus forming test in Vero cells. Briefly, 2.475 × 10^4 Vero cells/well were seeded into flat-bottom 96-well Costar® cell culture plates (Corning Inc., Corning, NY) and incubated 16 hours overnight at 37°C with 5% CO_2. Viruses to be quantified were diluted at 10-fold serial dilution in BA1 medium (5% BSA in 1x DMEM) and 25 µL of the serial diluted viruses were inoculated in duplicate into plates containing a Vero cell monolayer. Plates were incubated for 1 hour at 37°C with 5% CO_2 and rocked every 10 minutes to allow infection. Overlay medium containing 1% methylcellulose (Sigma-Aldrich Inc., St. Louis, MO) in DMEM with 2% FBS was added and plates were incubated at 37°C with 5% CO_2. Forty hours later, plates were washed, fixed with 75% acetone in PBS and air-dried. Immunostaining was performed by adding anti-ZIKV MHIAF at 1:2,000 in PBS and incubating for 60 minutes at 37°C, washing and adding goat anti-mouse IgG-HRP (Jackson labs, West Grove, PA) at 1:100 in 5% skim milk in PBS and incubating for 45 minutes at 37°C. Infection foci were developed using peroxidase substrate kit Vector® VIP SK-4600

(Vector Laboratories, Inc., Burlingame, CA, USA) following the manufacturer's instructions. Viral antigen foci were counted using AID Reader system (Advance Instrument Device, Strassberg, Germany).

Antibodies

Flavivirus group cross-reactive murine monoclonal antibodies (MAbs, 4G2 recognizing viruses of the four major pathogenic flavivirus serocomplexes) and anti-ZIKV mouse hyper-immune ascetic fluid (MHIAF) were obtained from DVBD, CDC, Fort Collins, CO. Anti-ZIKV VLP rabbit polyclonal serum was obtained by intramuscular (i.m.) immunization of a non-infectious recombinant adenovirus serotype 5-vectored, MR766 VLP-expressed vaccine candidate (rAdMR1-8; detail in next section). The antibodies were used in the indirect immunofluorescent antibody assay (IFA) and enzyme-linked immunosorbent assays (ELISA) as described below.

Construction of plasmids

To construct the ZIKV prM and E expressed plasmids, genomic RNA was extracted from 150 µL of Vero cell culture medium infected with MR766, P6-740 and FSS 13025 strains using the QIAmp Viral RNA Kit (Qiagen, Santa Clarita, CA). Extracted RNA was eluted in 80 µL of DEPC-treated water (Sigma-Aldrich Inc., St. Louis, MO) and used as template in reverse transcription-PCR (RT-PCR) for the amplification of prM and E genes. AfeI, TGA (stop codon) and NotI restriction enzyme sites were incorporated at the 5'- and 3'-termini of the cDNA amplicons, respectively. cDNA amplicons were digested with AfeI and NotI enzymes and inserted into the AfeI and NotI cutting sites of pED1i vector plasmid to obtain the plasmids pEZMRprME1-8 and pEZP6 3-2. pED1i expressed prME of dengue virus serotype 1 was used as the vector because of the available CMV promoter, Kozak consensus sequence (GCCGCCGCCATGG; SEQ ID NO: 8), a modified Japanese encephalitis signal sequence (JESS), restriction enzyme sites (AfeI and NotI) and BGH poly-A to replace ZIKV prME (FIG. 1A).

Amino acid sequence of prM and E protein of BPH2015 (Brazil/human/2015/BPH2015) was retrieved from GenBank (accession number: KU321639.1) and used as a template to design human codon optimized coding sequence (BZHuprME) that was synthesized commercially (Thermofisher) and inserted between AfeI and NotI sites of pED1i to generate pEBZHu8. A pr1-Ala deletion clone (deletion of the alanine residue at position 1 of prM), pEBZHu2-3, derived from BZHu8 was constructed by a site directed mutagenesis kit (Q5® Site-Directed Mutagenesis Kit, New England

BioLabs, Ipswich, MA). pAdPL/DEST (Invitrogen, Carlsbad, CA) gateway plasmid was used to receive the optimum transcription unit containing ZIKV prME transcriptional unit to generate pAdMR1-8, pAdBZHu8 and pAdBZHu2-3. PRVABC59 (accession number: KU501215.1) virus was used as the challenge virus in the mouse studies. Only one amino acid substitution (E23 of Ile-Val) at the prME region was identified between BPH2015 and PRVABC59 (accession number: KU501215.1) viruses.

Automated DNA sequencing was performed on an ABI Prism 3730 sequencer (Applied Biosystems, Foster City, CA, USA) and recombinant plasmids with correct prM and E sequences were identified using Lasergene® software (DNASTAR, Madison, WI). Plasmids were purified from DH5α *E. coli* cells using QIAGEN Plasmid Maxi Kit™ (Qiagene, Valencia, CA) and reconstituted in DEPC-treated water.

Generation of non-infectious recombinant expressing prME containing ZIKV VLPs

293A cells at 85% confluency were transduced with pAdMR1-8 and pAdBZHu2-3 using calcium phosphate precipitation protocol (Invitrogen) to generate rAd5ZMR1-8 (referred to herein as "rAdMR1-8") and rAd5BZHu2-3 (referred to herein as "rAdBZHu2-3") recombinants. rAdMR1-8 and rAdBZHu2-3 were titrated using antigen focus forming test in Vero cells similar to the protocol used to determine the infectivity of ZIKV.

Antigen production, secretion level characterization and immunofluorescence assay (IFA)

To produce VLP antigens, COS-1 cells at a density of 1.5×10^7 cells/mL were electroporated with 30 μg of ZIKV plasmids following the described protocol (Chang *et al.*, *J Virol* 74, 4244-4252, 2000). After electroporation, cells from two separate electroporations were combined and seeded into a 150-cm^2 culture flasks containing 50 mL growth medium. Portions of an electroporated cell suspension were seeded into a Costa 96-well black clear plate (Corning, Corning, NY), 100 μL/well. At 24 to 48 hours after electroporation, cells in the 96-well plate were fixed with 3:1 acetone in PBS at room temperature for 10 minutes, air dried, stored in a Ziploc bag and kept at 4°C until processing. The remaining cells were allowed to recover overnight at 37°C. The growth medium was replaced the next day with a maintenance medium containing 2-3% FBS and cells were continuously incubated at 28°C with 5% CO_2 for VLP secretion. Tissue-culture media were harvested twice in 5-day intervals after transfection and clarified by centrifugation at 10,000 rpm for 30 minutes at 4°C and concentrated

40-fold using T19 rotor (Beckman Coulter, Indianapolis IN) and re-suspended in TNE buffer (50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 0.1 mM EDTA).

Antigen-capture ELISA as previously described (Chang *et al.*, *J Virol* 74, 4244-4252, 2000) was performed to detect and quantify the secretion level of VLP antigens harvested from COS-1 cells transfected with ZIKV plasmids. Briefly, flat-bottom 96-well Immulon 2HB™ plates (Thermo Scientifics, Rochester, NY) were coated with 50 µL of polyclonal rabbit anti-ZIKV VLP hyper-immune serum at 1:8,000 in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), incubated overnight at 4°C, and wells were blocked with 300 µL of blocking buffer (5% skim milk, 0.5% Tween-20 in PBS) for 1 hour at 37°C. Harvested culture media and normal COS-1 culture fluid were titrated two-fold in PBS with 0.05% Tween-20 (wash buffer) and 50 µL were added to wells in duplicate or triplicate, incubated for 2 hours at 37°C, and washed 5 times with 300 µL of wash buffer (BioTek ELx405, Winooski, VT). Captured antigens were detected by adding 50 µL of anti-ZIKV MHIAF (1:2000) or a human serum recovered from primary ZIKV infection (1:1000; ZIKV Nt_{90} = 946.5; dengue serotype 2 Nt_{90} <20) in wash buffer, incubated for 1 hour at 37°C, and washed 5 times. Fifty microliters of HRP-conjugated goat anti-mouse IgG or goat anti-human IgG (Jackson ImmunoResearch, Westgrove, PA, USA) at 1:8,000 in blocking buffer were added to wells and incubated for 1 hour at 37°C to detect antigen-bound mouse or human IgG, respectively. Subsequently, plates were washed 10 times. Bound conjugate was detected with 100 µL of 3,3',5,5'-tetramethylbenzidine substrate (Enhanced K-Blue® TMB, NEOGEN® Corp., Lexington, KY, USA), incubated at room temperature for 10 minutes, and stopped with 50 µL of 2N H_2SO_4. Reactions were measured at A_{450} using BioTek Synergy HTX™ microplate reader (BioTek). Endpoint antigen secretion titers from two or three independent experiments were determined, as deduced from twice the average optical density (OD) of negative control antigen (P/N=2), after curve-fitting using a sigmoidal dose-response equation in GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

For IFA, ZIKV MHIAF and 4G2 were diluted 1:200 in PBS and 50 µL/well of each were used to stain acetone fixed cells in a 96-well plate at 37°C for 1 hour in a humidified Ziploc bag, then washed five time with 300 µL of PBS. Fifty µL of a goat anti-mouse-FITC conjugated IgG (Jackson ImmunoResearch, Westgrove, PA, USA) at 1:6,000 in blocking buffer were added to wells and incubated at 37°C for one hour in a humidified Ziploc bag to detect cell-bound mouse IgG, washed four times with 300 µL of PBS, incubated with 300 µL of 0.0005% Evan's blue in PBS at room temperature for 5 minutes and washed two additional times in PBS. Fifty µL of mounting medium (4% of DABCO; 1,4-Diazabicyclo-(2,2,2) Octoane dissolved in 80% glycerol-20% PBS) were added

to wells and cells visualized using 20X objective and recorded using a fluorescent microscope (AXiovert 200M, Zeiss, Thornwood, NY).

Mouse experiment

To establish immunogenicity and vaccine efficacy models, the ICR (outbreed mice, Harlan Sprague Dawley, Madison, WI) and AG129 mice (α, β and γ interferon receptor-deficient mice, in-house colony) were used at between 4 to 8 weeks old. Five groups of five female ICR mice per group or AG129 mice (3 male and 2 females or 2 males and 3 females) at age between 4 to 8 weeks old were injected intraperitoneally (i.p.) with 10^7, 10^6, 10^5, 10^4 or 10^3 pfu/100 µL (diluted in PBS) of MR766 viruses, respectively. Seven groups of five female ICR mice or AG129 mice at age between 4 to 8-week old (3 male and 2 females or 2 males and 3 females) were injected i.p with 10^7, 10^6, 10^5, 10^4 10^3, 10^2 or 10^0 pfu/100 µL (diluted in PBS) of PRVABC59 viruses, respectively. Experimental mice were observed daily and percent survival in each group was recorded for 21 days. All virus challenged ICR mice, regardless of viral strain and dosage used, survived challenge with no observable morbidity for 21 days. All virus challenged AG129 mice, regardless its sex, viral strain and dosage used, showed 100 percent mortality between day-6 and day-21. Thus, we chose ICR and AG129 mice to evaluate immunogenicity and vaccine efficacy, respectively.

Groups of 4 to 8-week-old female ICR mice, 5 mice per group, were injected intramuscularly with rAdMR1-8 at week-0 at a dose of 10^5 or 10^6 transduction units (TU)/100 µL (diluted in PBS) divided between the right and left quadriceps muscle. Similarly, groups of 4 to 8-week-old (2 males and 3 females or 3 males and 2 females) AG129 mice were i.m. injected at week-0 at a dose of 10^5 or 10^6 transduction units (TU)/100 µL in PBS. ICR Mice were bled from the tail vein at day 7 and every 4 weeks post vaccination. Serum specimens from individual mice were stored separately at 4°C to determine the total IgG and neutralization antibody by IgG antibody-captured ELISA (GAC-ELISA) and antigen focus-reduction micro-neutralization test (FRµNT), respectively. Two groups of vaccinated and one group of age-matched naïve AG129 mice were challenged by i.p. with 1,000 ffu of PRVABC56 in 100 µL of PBS at 4 weeks post-vaccination (PV) to determine the protective efficacy of the vaccine. Prior to virus challenge, at day 7, 4 weeks PV and 4 weeks post viral challenge (PC) of survival mice, serum specimens were collected from tail vein and stored at 4°C to determine the total IgG and neutralization antibody. Percent survival in mice was observed two to four times daily up to 21 days. ZIKV-specific total IgG antibodies by ELISA and FRµNT were measured as described in the following section.

ELISA

Mouse serum specimens were assayed for the presence of ZIKV-specific total IgG with the same Ag-capture ELISA protocol described above with minor modifications. MR766 and BHP2015-VLP antigens were standardized by Ag-capture ELISA at a single concentration producing an OD of 1.0, within the region of antigen excess near the upper asymptote of the sigmoidal antigen dilution curve, and were used to determine total IgG titer after appropriate dilutions. Individual serum specimens, initially diluted at 1:1,000, were titrated two-fold and added to wells in duplicate and incubated for 1 hour at 37°C. Pre-vaccination mouse sera were included as negative controls. Incubations with conjugate and substrate were carried out according to the standard Ag-capture ELISA as above. OD_{450} values were modeled as non-linear functions of the log10 serum dilutions using a sigmoidal dose-response (variable slope) equation and endpoint antibody titers were determined at the dilutions where the OD value was twice the average OD of negative control. Each serum specimen was tested in two or three independent experiments.

Virus neutralization

To measure the neutralizing ability of the immune mice serum specimen against MR766 and PRVABC56 representing prototype African genotype and a current circulating Asian genotype strains, an antigen focus-reduction micro-neutralization test (FRμNT) was utilized as previously described (Crill *et al.*, *Front Immunol* 3, 334, 2012; Galula *et al.*, *J Virol* 88, 10813-10830, 2014). Briefly, 2.475×10^4 Vero cells/well were seeded into flat-bottom 96-well Costar® cell culture plates (Corning Inc., Corning, NY, USA) and incubated for 16 hours overnight at 37°C with 5% CO_2. Serum specimen were initially diluted at 1:10, heat-inactivated for 30 minutes at 56°C, titrated two-fold to a 40 μL volume, and 320 pfu/40 μL of MR766 or PRVABC56 (8% normal human serum in DMEM) was added to each dilution. The mixtures were then incubated for 1 hour at 37°C. After incubation, 25 μL of the immune complexes were inoculated in duplicate into plates containing a Vero cell monolayer. Plates were incubated for 1 hour at 37°C with 5% CO_2 and rocked every 10 minutes to allow infection. Overlay medium containing 1% methylcellulose (Sigma-Aldrich Inc., St. Louis, MO, USA) in DMEM with 2% FBS was added and plates were incubated at 37°C with 5% CO_2. Forty hours later, plates were washed, fixed with 75% acetone in PBS and air-dried. Immunostaining was performed by adding anti-ZIKV MHIAF at 1:1,000 in PBS and incubated for 60 minutes at 37°C, washing and adding goat anti-mouse IgG-HRP at 1:200 in 5% skim milk in PBS and incubated for 30 minutes at 37°C.

Infection foci were visualized using peroxidase substrate kit Vector® VIP SK-4600 (Vector Laboratories, Inc., Burlingame, CA) following the manufacturer's instructions. FRμNT titers were calculated for each virus relative to a virus only control back-titration. Titers of exact 90%, 75% or 50% reduction of infection foci ($FR\mu NT_{90}$, $FR\mu NT_{75}$ and $FR\mu NT_{50}$ titer) were modeled using a sigmoidal dose-response (variable slope) formula. All values were taken from the average of two independent experiments. Viral antigen foci were counted using AID Reader system (Advance Instrument Device, Strassberg, Germany).

Example 2: A recombinant ZIKV vaccine that prevents ZIKV infection and mortality in an animal model

This example describes an adenovirus-vectored ZIKV vaccine that is capable of eliciting protective immunity and prevents ZIKV infection as early as seven days post-immunization.

VLPs of several non-ZIKV flaviviruses have been previously generated (Chang *et al.*, *J Virol* 74, 4244-4252, 2000; Davis *et al.*, *J Virol* 75, 4040-4047, 2001; Hunt *et al.*, *J Virol* Methods 97, 133-149, 2001). The present study includes the construction of three prME expression plasmids derived from three ZIKV strains (MR766, P6-740 and BPH2015). MR766 (the prototype African genotype virus; AY632535) and P6-740 (the prototype Asian genotype virus; HQ234499) prME coding sequences were directly amplified from viral RNA. Human codon optimized prME sequences were designed and synthesized commercially to express the BPH2015 (current circulating Asian genotype virus; KU321639) prME coding region. Sequence verified plasmid clones pEZMRprME1-8 (FIG. 1A), pEZP6 3-2 and pEBZHu8 containing MR766, P6-740 and human codon optimized BPH2015 prME gene insert, respectively, were electroporated into COS-1 cells. Plasmid-transformed COS-1 cells and culture media were harvested at 24 hours and twice every 5 days after electroporation, respectively, to determine antigen expression by indirect fluorescent antibody assay (IFA), and the level of VLP secretion by antigen-capture ELISA (AG-ELISA) and Western blot (FIGS. 1B-1D). All transcription units have the identical regulatory elements for transcriptional (CMV promoter and BGH(A)n), translational (Kozak consensus sequence; GCCGCCGCCATGG, SEQ ID NO: 8) and protein processing (modified Japanese encephalitis virus signal sequence) with a similar signalase cleavage site potential predicted by the Signal IP 4.1 program (Table 1). The end-point titer of VLPs secreted from COS-1 cells were 274.8, 4.0 and 58.80 from pEZMRprME1-8, pEZP6 3-2 and pEBZHu8, respectively. The pEZP6 3-2 clone secreted the fewest VLPs. The pEBZHu8 clone secreted 4-fold less VLPs than the pEZMRprME1-8 clone. A pr1-A deletion clone derived from

pEBZHu8, pEBZHu2-3, exhibited 3-fold increased VLP secretion to the end-point titer of 194.6 (Table 1), compared to the pEBZHu8 clone transformed COs-1 cells. Thus, the studies disclosed herein focused on the pEZMRprME1-8 and pEBZHu2-3 constructs. pEZMRprME1-8, pEBZHu8 and pEBZHu2-3 transformed COS1 cells were IFA positive (FIG. 1B) using Zika virus recovered convalescent human serum (αZHS, neutralization (Nt) antibody titer=45,960 against MR766 and Nt=19.4 against dengue virus serotype 2 16681) and MAb 4G2. Using a rabbit polyclone anti-ZIKV antibody as the capture antibody to capture VLPs (1:40 concentrated culture media harvested every 5 days from two independent pEZMRprME1-8 plasmids transformed cells) and 4G2 or αZHS as a detector in the Ag-ELISA, 4G2 and αZHS detected both concentrated VLPs equally well (FIG. 1C). By Western blot, 4G2 detected only E protein (predicted MW of 54.6 kd) from purified MR 766 virus, pEZMRprME1-8 and pEBZHu2-3 VLPs. In addition to E, αZHS detected the un-processed prM protein (predicted MW of 19.0 kd).

Table 1. Signal sequence cleavage potential predicted by Signal IP 4.1 program

		Signal IP 4.1 prediction			
Gene	**Character**	**Predicted furin and signalase cleavage site ↓**	**SEQ ID NO:**	**Cleavage potential D**	**Plasmid clone (AG-ELISA)**
MR766 furin	Furin cleavage motif + ZIKV SS	**RKEKKRR**↓GADTSIGIVGLLLTTAMA↓AEITRRGSAYYMYLDRSD	9	0.49	(N.D.)
MR766 after furin	ZIKV SS	GADTSIGIVGLLLTTAMA↓AEITRRGSAYYMYLDRSD	10	0.481	(N.D.)
P6-740 after furin	ZIKV SS	GADTSIGIVGLLLTTAMA↓AEVTRRGSAYYMYLDRND	11	0.485	pEZP6-740 (4.00)
BPH2015 furin	Furin cleavage motif + ZIKV SS	**RKEKKRR**↓GADTSVGIVGLLLTTAMA↓AEVTRRGSAYYMYLDRND	12	0.518	(N.D.)
BPH2015 after furin	ZIKV SS	GADTSVGIVGLLLTTAMA↓AEVTRRGSAYYMYLDRND	13	0.493	(N.D.)
JESSMR766	Modified JE SS	MGKRSAGSIMWLASLAVVIAGTSA↓AEITRRGSAYYMYLDRSD	14	0.797	pEZMRprME1-8 (274.90)
JESSBPH2015	Modified JE SS	MGKRSAGSIMWLASLAVVIAGTSA↓AEVTRRGSAYYMYLDRND	15	0.805	(N.D.)
JESSd1ABPH2015	Delete A at pr1	MGKRSAGSIMWLASLAVVIAGTSA↓EVTRRGSAYYMYLDRND	16	0.747	(N.D.)
JESSd3VBPH2015	Delete V at pr3	MGKRSAGSIMWLASLAVVIAGTSA↓AETRRGSAYYMYLDRND	17	0.756	(N.D.)
JESS+V	V insertion	MGKRSAGSIMWLASLAVVIAGTSA↓AVEVTRRGSAYYMYLDRND	18	0.774	pEBZHu8 (58.80)
JESS-A+V	A deletion and V insertion	MGKRSAGSIMWLASLAVVIAGTSA↓VEVTRRGSAYYMYLDRND	19	0.792	pEBZHu2-3 (194.60)

JESS: Modified JEV signal sequence (underlined characters) derived from the carboxy terminal of C protein

AG-ELISA titer: P/N=2 (P: OD450 of VLP; N: OD450 of COS-1 cell culture media)

N.D: not done

Immunogenicity of DNA vaccine candidates expressing flavivirus VLPs is directly correlated with the level of VLP expression (Chang *et al.*, *Virology* 306, 170-180, 2003; Galula *et al.*, *J Virol* 88, 10813-10830, 2014). The transcriptional units from the pEZMRprME1-8 and pEBZHu2-3 plasmids were transferred to pAd/PL-DEST plasmid to generate pAdMR1-8 (FIG. 4A) and pAdBZHu2-3, respectively. pAdMR1-8 and pAdBZHu 2-3 were used to rescue non-infectious recombinant adenovirus serotype 5 particles by transducing 293A cells (Invitrogen). rAdMR1-8 and rAdBZHu2-3 were amplified twice in 293A cells and titrated in Vero cells to determine their transduction unit (TU, FIGS. 4C-4D).

In an initial study, two groups (5 mice per group) each of ICR and AG129 mice were immunized by intramuscular injection with 10^6 and 10^5 TU/100 μL of rAdMR1-8. Serum specimens from vaccinated mice were collected at day 8 and 4 weeks post vaccination (PV), and ZIKV specific antibodies were measured by an antigen-capture IgG ELISA (IgG-ELISA) using MR1-8 and BZHu2-3 VLPs, respectively. In the 10^5 TU groups, IgG antibodies were only detected using MR1-8 VLPs in serum specimens of AG129 mice collected at 4 weeks PV (Avg=2382.6), but none from ICR mice using either one of the VLP antigens. 10^6 TU of rAdMR1-8 are highly immunogenic in vaccinated ICR and AG129 mice. In ICR mice, the average IgG titer increased 18- and 11-fold (MR1-8 and BZHu2-3 VLP antigens, respectively, FIG. 5) between day 8 and 4 weeks PV. Compared to the ICR mouse group, AG129 mice had 3.6- and 1.7-fold (MR1-8 and BZHu2-3 VLPs, respectively) higher average IgG titer than ICR mice on day 8 PV serum specimens. But this trend is reversed in week 4 PV serum collections. On average, AG129 mouse serum IgG titers only reached 27% and 20% of ICR serum titers at week 4 PV collections.

AG129 mice have been used to examine the pathogenesis/virulence of various Zika viruses (Aliota *et al.*, *PLoS Negl Trop Dis* 10, e0004682, 2016; S. L. Rossi *et al.*, *Am J Trop Med Hyg* Epub March 28, 2016). Groups of ICR and AG129 mice (5 per group) were infected i.p. with MR766 (10^7, 10^6, 10^5, 10^4, 10^3 per 100 μL in PBS) or PRVABC59 (PR59; 10^6, 10^5, 10^4, 10^3, 10^2, 10^0 per 100 μL in PBS). Mice were observed 2 to 4 times daily and mortality was recorded. All ICR female and male mice, regardless of infected virus strains and dosages, showed no sign of illness. All AG129 female and male mice, regardless of infected virus strains and dosages, were 100% circumvented by infection. Cumulative survival rates over time in AG129 mouse groups are shown in FIGS. 2A and 2B for MR766 and PR59 virus, respectively. In AG129 mice, MR766 i.p. challenge led to weight loss and disease characterized by ruffled fur, hunched back and hind-leg paralysis on day 5 to day 6 post

challenge (PC). The disease progression developed rapidly; mice had to be euthanized within 12 to 24 hours after the first observable disease symptom (FIG. 2A). However, in PR56 challenged groups, the development of disease symptoms, such as weight loss, ruffed fur and hunched back, was delayed between 3 to 10 days relative to MR766 challenge, but no signs of neurological involvement, such as hind-leg paralysis, were observed (FIG. 2B). One mouse each from the 1 and 100 pfu groups did not show signs of morbidity until day 19 PC. One male and one female each were euthanized on day 21.

AG129 mice have been a useful animal model for estimating the protective efficacy of dengue vaccine candidates (Crill *et al.*, *Front Immunol* 3, 334, 2012). One dose of rAdMR1-8 vaccinated AG129 mice did elicit ZIKV-specific IgG antibodies recognizing MR766 or PR59 antigens (FIG. 5A). Therefore, both 10^5 and 10^6 TU immunized mice groups were i.p. challenged with 1,000 ffu of PR59 virus, a virus isolated in 2015 in Puerto Rico that is highly similar to viruses circuiting in the on-going epidemics in Latin Americas (Lanciotti *et al.*, *Emerg Infect Dis* 22, 933-935, 2016). 10^5 TU immunized mice did not survive virus challenge; none of the mice had a measurable neutralizing (Nt) antibody prior to virus challenge (Table 2). However, the average survival time in the vaccinated mice (AST=16.2 days) was statistically longer than naïve challenge control mice (AST=11 days; FIG. 2C). In the 10^6 TU immunized group, 100% of mice survived the virus challenge with no observable morbidity during the 21 day observation period (FIG. 2C). Nt antibody titers from immunized AG129 mice were determined for serum specimens collected at day 8 (Table 2), 4 weeks PV (FIG. 3A) and 4 weeks PC (FIG. 3B) against MR766 virus (homologous neutralization) and PR56 virus (heterologous neutralization). In the 10^6 TU immunized group, the average $FR\mu NT_{50}$ titers at day 8 and 4 weeks PV serum specimens were 385 and 55, and 1529.1 and 97.2, against MR766 and PR59 virus, respectively (Table 2, and FIG. 3A). Although Nt titers were significantly lower against PR59 than MR766, they were sufficient to prevent lethal heterologous PR59 viral challenge with no observable morbidity. $FR\mu NT_{50}$ from week 4 PC serum specimens increased slightly from the average of 97.2 to 153.6 against challenge viral titers, but the titers decreased from 1529.1 to 1368.1 against MR766 virus (Table 2, FIG. 3B). Pooled serum collected 4 weeks PV and 4 weeks PC were used in the Western blot (FIG. 3D). Both sera detected E protein (predicted MW of 54.6 kd) and un-processed prM protein (predicted MW of 19.0 kd) from purified MR 766 virus, MR 1-8 and BZHu 2-3 VLPs. However, 4-week PC serum detected the presence of M protein (predicted MW of 8.5 kd) in purified virions, but not in MR 1-8 and BZHu 2-3 VLPs.

Table 2. Neutralizing antibodies of post-vaccination (PV) and post-viral (PC) challenged AG129 mouse serum specimens collected post viral challenge

		8-day PV-FRμNT					
		ZIKV MR766			ZIKV PR59		
Mouse	**Dose**	**90**	**75**	**50**	**90**	**75**	**50**
AG1	1E+6TU	139	271	524	<20	24	46
AG2		24	55	222	<20	23	58
AG3		72	159	269	<20	10	55
AG4		80	187	439	<20	10	54
AG5		69	180	471	<20	27	62
	Average	**77**	**170**	**385**		**15**	**55**
AG1	1E+E5	<20	<20	<20	<20	<20	<20
AG2		<20	<20	<20	<20	<20	<20
AG3		<20	<20	<20	<20	<20	<20
AG4		<20	<20	<20	<20	<20	<20
AG5		<20	<20	<20	<20	<20	<20

		4-week PV-FRμNT					
		ZIKV MR766			ZIKV PR59		
Mouse	**Dose**	**90**	**75**	**50**	**90**	**75**	**50**
AG1	1E+6TU	59	183	552	<20	26	54
AG2		142	258	5120	<20	30	82
AG3		36	130	411	<20	28	112
AG4		91	259	778	<20	32	122
AG5		79	237	785	<20	33	116
	Average	**81**	**213**	**1529**		**30**	**97**
AG1	1E+E5	<20	<20	<20	<20	<20	<20
AG2		<20	<20	<20	<20	<20	<20
AG3		<20	<20	<20	<20	<20	<20
AG4		<20	<20	<20	<20	<20	<20
AG5		<20	<20	<20	<20	<20	<20

		4-week PC-FRµNT					
		ZIKV MR766			ZIKV PR59		
Mouse	**Dose**	**90**	**75**	**50**	**90**	**75**	**50**
AG1	1E+6TU	21	83	452	10	24	59
AG2		21	105	575	10	31	81
AG3		170	356	5120	74	162	442
AG4		10	65	203	10	10	54
AG5		71	170	491	36	66	133
	Average	**59**	**156**	**1368**	**55**	**71**	**154**

		8-day PV-FRµNT					
		ZIKV MR766			ZIKV PR59		
Mouse	**Dose**	**90**	**75**	**50**	**90**	**75**	**50**
ICR1	1E+6TU	43	79	177	<20	28	84
ICR2		59	97	141	<20	10	79
ICR3		413	654	1059	<20	10	176
ICR4		228	310	397	<20	38	161
ICR5		101	221	513	<20	48	193
	Average	**169**	**272**	**457**		**23**	**139**
ICR1	1E+E5	<20	<20	<20	<20	<20	<20
ICR2		<20	<20	<20	<20	<20	<20
ICR3		<20	<20	<20	<20	<20	<20
ICR4		<20	<20	<20	<20	<20	<20
ICR5		<20	<20	<20	<20	<20	<20

		4-week PV-FRµNT					
		ZIKV MR766			ZIKV PR59		
Mouse	**Dose**	**90**	**75**	**50**	**90**	**75**	**50**
ICR1	1E+6TU	733	1161	5120	35	69	131

		4-week PV-FRμNT					
		ZIKV MR766			ZIKV PR59		
Mouse	**Dose**	**90**	**75**	**50**	**90**	**75**	**50**
ICR2		471	1101	2378	10	57	126
ICR3		404	1395	5120	42	103	259
ICR4		846	1860	5120	44	126	401
ICR5		915	5120	5120	73	171	436
	Average	**674**	**2127**	**4572**	**41**	**105**	**271**
ICR1	1E+E5	<20	<20	23	<20	<20	<20
ICR2		<20	<20	32	<20	<20	<20
ICR3		<20	<20	37	<20	<20	<20
ICR4		<20	<20	22	<20	<20	<20
ICR5		<20	<20	28	<20	<20	<20

		8-week PV-FRμNT					
		ZIKV MR766			ZIKV PR59		
Mouse	**Dose**	**90**	**75**	**50**	**90**	**75**	**50**
ICR1	1E+6TU	1184	2314	5120	415	699	2306
ICR2		1001	1916	5120	235	574	1986
ICR3		478	994	5120	224	417	917
ICR4		1262	5120	5120	302	634	1486
ICR5		1423	5120	5120	551	1006	2529
	Average	**1070**	**3093**	**5120**	**345**	**666**	**1845**
ICR1	1E+E5	<20	<20	<20	<20	<20	<20
ICR2		<20	<20	<20	<20	<20	<20
ICR3		<20	<20	<20	<20	<20	<20
ICR4		<20	<20	<20	<20	<20	<20
ICR5		<20	<20	<20	<20	<20	<20

		12-week PV-FRμNT					
		ZIKV MR766			ZIKV PR59		
Mouse	**Dose**	**90**	**75**	**50**	**90**	**75**	**50**
ICR1	1E+6TU	1462	2730	4973	536	1100	2278
ICR2		1288	1866	>5120	448	813	1447
ICR3		2221	2372	2602	341	837	1908
ICR4		2458	2754	>5120	408	1066	2435
ICR5		1753	3249	>5120	679	1628	3508
	Average	**1836**	**2594**	**4587**	**482**	**1089**	**2315**

Since both male and female mice are similarly susceptible to MR766 and PR59 infection and MR766 is more virulent than PR59 virus, it was investigated whether the protective efficacy would be different among them. Two groups of AG129 mice (6 males plus 6 females per group) were immunized with a single 10^6 TU of rAdMR1-8 vaccine candidate. Two age- and sex-matched naïve control and two vaccinated groups were challenged on the same day with 200 and 840 ffu/100 μL of MR766 and PR59 virus, determined precisely by titrating the duplicate of both challenge viruses, respectively. Serum specimens were collected from naïve and vaccinated mice on day 2, 3, 5, 6, 7 and 9 PC for the viremic study. Vaccinated mice were virus isolation negative throughout 9 collection days for both challenge groups (Table 3). MR766 challenged morbid-bound naïve mice were euthanized on day 6 PC. The viremic titers in this mouse group ranged from 3.17×10^7 to 8.53×10^6 ffu/mL throughout collection period. PR59 challenged naïve mice had no signs of illness for the first 9-day PC and virus was detected between day-2 and day-6 but not on day-7 and day-9 collection. The average viremic titers ranged from 1.62×10^5 to 4.8×10^4, significantly lower titer than MR766 challenged mice (Table 3).

Table 3. Post-challenge viremic viral titers determined from subset of mice

	Day post challenge viremic titer (ffu/mL)					
Treatment	2	3	5	6	7	9
Naïve/MR766	6.40×10^3	3.07×10^7	3.07×10^7	1.74×10^7	N/A	N/A
	6.40×10^5	3.07×10^7	1.13×10^7	8.19×10^6	N/A	N/A
	3.20×10^3	3.07×10^7	3.17×10^7	N/A	N/A	N/A

	Day post challenge viremic titer (ffu/mL)					
Treatment	2	3	5	6	7	9
Average	1.62×10^5	2.30×10^7	1.84×10^7	8.53×10^6		
Naïve/PR59	1.28×10^5	0.00	3.84×10^4	8.32×10^4	-	-
	1.41×10^5	4.80×10^3	8.32×10^4	4.48×10^4	-	-
	2.18×10^5	1.02×10^6	2.24×10^4	1.06×10^5	-	-
Average	1.62×10^5	3.43×10^5	4.80×10^4	7.79×10^4		
rAdMR1-8/MR766	-	-	-	-	-	-
	-	-	-	-	-	-
	-	-	-	-	-	-
rAdMR1-8/PR59	-	-	-	-	-	-
	-	-	-	-	-	-
	-	-	-	-	-	-

Naïve/MR766 challenged mice were euthanized on day-6 post-challenge.

N/A = not available

- = virus undetected

Example 3: Generation and characterization of ZIKV VLPs with mutations at positions 106 and 107 of the E protein

Previous studies of dengue virus identified immunodominant cross-reactive epitopes within the E glycoprotein that are associated with immune enhancement. Mutation of particular residues of the E protein, including positions 106 and 107, led to a reduction in cross-reactivity amongst dengue virus serotypes (WO 2013/059493), which is an important safety feature for a flavivirus vaccine. The studies described in this example introduce this safety feature into the ZIKV VLP constructs.

Using pEBZHu2-3 as a template and the mutagenesis primers listed in Table 4, five different mutant constructs were generated that contain mutations in the codons for E protein residues 106 and 107, resulting in substitution of the native glycine and leucine (GL) residues at E106/107 with lysine and aspartic acid (KD); arginine and aspartic acid (RD); arginine and histidine (RH); glutamic acid and aspartic acid (ED); or glutamic acid and arginine (ER). VLP secretion of each mutant was tested as described in Example 1. The results demonstrated that the KD mutant exhibited the highest levels of

VLP secretion (Table 4). Therefore, the same mutations were introduced into the pEZMRprME 1-8 construct.

Table 4. Primer sequences to derive E 106/107 mutants and ranking of mutated VLPs secretion

pEBZHu2-3 as the template		**SEQ ID NO**	**Ranking**
GL106/107KD	CAATGGCTGCaaggacTTTGGCAAGGGCAGCC	24	1
GL106/107RD	CAATGGCTGCcgagacTTTGGCAAGGGCAGCCTCG	25	2
GL106/107RH	CAATGGCTGCcgacatTTTGGCAAGGGCAGCC	26	3
GL106/107ED	CAATGGCTGCgaagatTTTGGCAAGGGCAG	27	4
GL106/107ER	CAATGGCTGCgaacgaTTTGGCAAGGGCAGC	28	5

pEZMRprME 1-8 as the template			
GL106/107KD	GAAACGGTTGTaaggaTTTTGGCAAAGGGAG	29	n.a.

The wild-type and mutant ZIKV VLPs were tested for cross-reactivity with a panel of flavivirus E protein-specific murine monoclonal antibodies. COS-1 cells (2 x 10^7/ ml) were electroporated with 30 µg of pEZMR766 prME 1-8 (wt), pEZMR KD, pEBzHu 2-3 (wt) and pEBzHu KD. Tissue culture supernatants were harvested and clarified at 10,000 rpm for 30 minutes. The clarified supernatants were used to compare cross-reactivities of the ZIKV VLPs against a panel of E-specific murine monoclonal antibodies (MAbs). As shown in Table 5, the E106/107 KD mutants drastically reduced 5 group cross-reactive MAbs, but did not alter ZIKV virus-specific MAbs.

Table 5. E106/107 mutations of ZIKV VLPs influence monoclonal antibodies

Antibodies	**MHIAF**	**2H2**	**4G2**	**6B6C-1**	**4A1B-9**	**23-1**
Source of immunogen	Zika MR766	DENV-2	DENV-2	SLEV	MVEV	WNV
Antigenic group		DENV SC	Group	Group	Group	Group
Antigen specificity		prM	E	E	E	E
	Antibody end-point titers					
MR766wt VLPs	>2,187,000	<1,000	>2,187,000	>2,187,000	81,000	>2,187,000
MR766KD VLPs	243,000	<1,000	<1,000	27,000	<1,000	<1,000
Fold changes	>9	NA	>2,187	>81	>81	>2,187

Antibodies	MHIAF	2H2	4G2	6B6C-1	4A1B-9	23-1
BzHuwt VLPs	243,000	<1,000	729,000	729,000	81,000	>2,187,000
BzHuKD VLPs	243,000	<1,000	<1,000	9,000	<1,000	<1,000
Fold changes	0	NA	>729	81	>81	>2,187

Antibodies	23-2	1B7	D35C9-1	6B4A-10	9D12	1A1D-2
Source of immunogen	JEV	DENV-3	DENV-4	JEV	DENV-1	DENV-2
Antigenic group	Group	DENV SC	DENV SC	JEV SC	DENV-1, -2 ,-4	DENV-1, -2, -3
Antigen specificity	E	E	E	E	E	E

MR766wt VLPs	>2,187,000	<1,000	<1,000	9,000	<1,000	<1,000
MR766KD VLPs	3,000	<1,000	<1,000	27,000	<1,000	<1,000
Fold changes	>729	NA	NA	3	NA	NA
BzHuwt VLPs	729,000	<1,000	<1,000	3,000	<1,000	<1,000
BzHuKD VLPs	9,000	<1,000	<1,000	9,000	<1,000	<1,000
Fold changes	81	NA	NA	3	NA	NA

Antibodies	14H5	T5-1	3H5	D6-8A1	INB9164	INB9165
Source of immunogen	JEV	JEV	DENV-2	DENV-3	ZIKV	ZIKV
Antigenic group	JEV, DENV	JEV, DENV-2	DENV-2	DENV-3	ZIKV	ZIKV
Antigen specificity	E	E	E	E	E	E

MR766wt VLPs	<1,000	9,000	<1,000	<1,000	2,187,000	2,187,000
MR766KD VLPs	<1,000	9,000	<1,000	<1,000	729,000	729,000
Fold changes	NA	0	NA	NA	3	3
BzHuwt VLPs	<1,000	9,000	<1,000	<1,000	729,000	729,000
BzHuKD VLPs	<1,000	3,000	<1,000	<1,000	729,000	729,000
Fold changes	NA	3	NA	NA	0	0

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

CLAIMS

1. An isolated nucleic acid molecule comprising a transcriptional unit, wherein the transcriptional unit comprises:

a sequence encoding a modified Japanese encephalitis virus (JEV) signal sequence comprising SEQ ID NO: 4; and

a Zika virus (ZIKV) premembrane (prM) and E protein (prME) coding sequence.

2. The isolated nucleic acid molecule of claim 1, wherein the transcriptional unit further comprises a promoter operably linked to the prME coding sequence.

3. The isolated nucleic acid molecule of claim 2, wherein the promoter comprises the cytomegalovirus (CMV) E1A promoter.

4. The isolated nucleic acid molecule of any one of claims 1-3, wherein the transcriptional unit further comprises a transcription termination sequence.

5. The isolated nucleic acid molecule of claim 4, wherein the transcription termination sequence comprises a bovine growth hormone (BGH) transcription termination sequence.

6. The isolated nucleic acid molecule of any one of claims 1-5, wherein the transcriptional unit further comprises a translation initiation sequence.

7. The isolated nucleic acid molecule of claim 6, wherein the translation initiation sequence comprises GCCGCCGCCATGG (SEQ ID NO: 8).

8. The isolated nucleic acid molecule of any one of claims 1-7, wherein the ZIKV is a strain selected from MR-766, SPH2015, P6-740, and FSS 13025.

9. The isolated nucleic acid molecule of any one of claims 1-8, wherein the prME coding sequence is codon-optimized for expression in human cells.

10. The isolated nucleic acid molecule of any one of claims 1-9, wherein the ZIKV prME coding sequence is at least 95% identical to nucleotides 1186-3204 of SEQ ID NO: 1, nucleotides 1186-3213 of SEQ ID NO: 2, nucleotides 1186-3210 of SEQ ID NO: 3, nucleotides 1186-3204 of SEQ ID NO: 20 or nucleotides 1186-3210 of SEQ ID NO: 22.

11. The isolated nucleic acid molecule of claim 10, wherein the ZIKV prME coding sequence comprises or consists of nucleotides 1186-3204 of SEQ ID NO: 1, nucleotides 1186-3213 of SEQ ID NO: 2, nucleotides 1186-3210 of SEQ ID NO: 3, nucleotides 1186-3204 of SEQ ID NO: 20 or nucleotides 1186-3210 of SEQ ID NO: 22.

12. A vector comprising the nucleic acid molecule of any one of claims 1-11.

13. The vector of claim 12, which is an adenovirus vector.

14. An isolated cell comprising the vector of claim 12 or claim 13.

15. A recombinant adenovirus comprising the nucleic acid molecule of any one of claims 1-11.

16. A virus-like particle (VLP) encoded by the nucleic acid molecule of any one of claims 1-11.

17. The VLP of claim 16, wherein the E protein comprises an amino acid substitution at position 106, position 107, or both, relative to a wild-type ZIKV E protein.

18. The VLP of claim 17, wherein the E protein comprises a lysine at position 106 and an aspartic acid at position 107 of the E protein.

19. The VLP of claim 17 or claim 18, comprising the prME amino acid sequence set forth as SEQ ID NO: 21 or SEQ ID NO: 23.

20. A composition comprising the nucleic acid molecule of any one of claims 1-11, the vector of claim 12 or claim 13, the recombinant adenovirus of claim 15, or the VLP of any one of claims 16-19, and a pharmaceutically acceptable carrier.

21. A method of eliciting an immune response against Zika virus in a subject, comprising administering to the subject the composition of claim 20.

22. A method of detecting Zika virus (ZIKV)-specific antibodies in a biological sample, comprising:

contacting the sample with the VLP of any one of claims 16-19 under conditions sufficient to form VLP-antibody complexes if ZIKV antibodies are present in the sample; and

detecting the VLP-antibody complexes in the sample, thereby detecting ZIKV antibodies in the sample.

23. The method of claim 22, wherein detecting the VLP-antibody complexes comprises contacting the VLP-antibody complexes with an antibody that specifically binds the VLP and comprises a detectable label.

24. The method of claim 22, wherein detecting the VLP-antibody complexes comprises contacting the VLP-antibody complexes with a secondary antibody comprising a detectable label.

25. A method of detecting ZIKV-specific antibodies in a biological sample, comprising:

providing a secondary antibody bound to a solid support;

contacting the secondary antibody-bound solid support with the biological sample under conditions sufficient to allow binding of the secondary antibody to any ZIKV-specific antibodies present in the biological sample, thereby forming antibody-antibody complexes;

contacting the antibody-antibody complexes with the VLP of any one of claims 16-19 under conditions sufficient for the VLP to bind the ZIKV-specific antibodies, thereby forming immune complexes; and

detecting the presence of the immune complexes, thereby detecting ZIKV-specific antibodies in the biological sample.

26. The method of claim 25, wherein detecting the presence of the immune complexes comprises contacting the immune complexes with an antibody that specifically binds the VLP and comprises a detectable label.

27. A method of detecting ZIKV-specific antibodies in a biological sample, comprising:

providing a ZIKV-specific antibody bound to a solid support;

contacting the antibody-bound solid support with the VLP of any one of claims 16-19 under conditions sufficient for the VLP to bind the ZIKV-specific antibody to form antibody-VLP complexes;

contacting the antibody-VLP complexes with the biological sample to allow binding of any ZIKV-specific antibodies present in the sample to the VLP, thereby forming immune complexes;

contacting the immune complexes with a secondary antibody; and

detecting binding of the secondary antibody to the immune complexes, thereby detecting ZIKV-specific antibodies present in the biological sample.

28. The method of any one of claims 24-27, wherein the secondary antibody comprises an anti-IgM antibody.

29. The method of any one of claims 24-27, wherein the secondary antibody comprises an anti-IgG antibody.

30. The method of any one of claims 22-29, wherein the biological sample comprises serum.

FIG. 1A

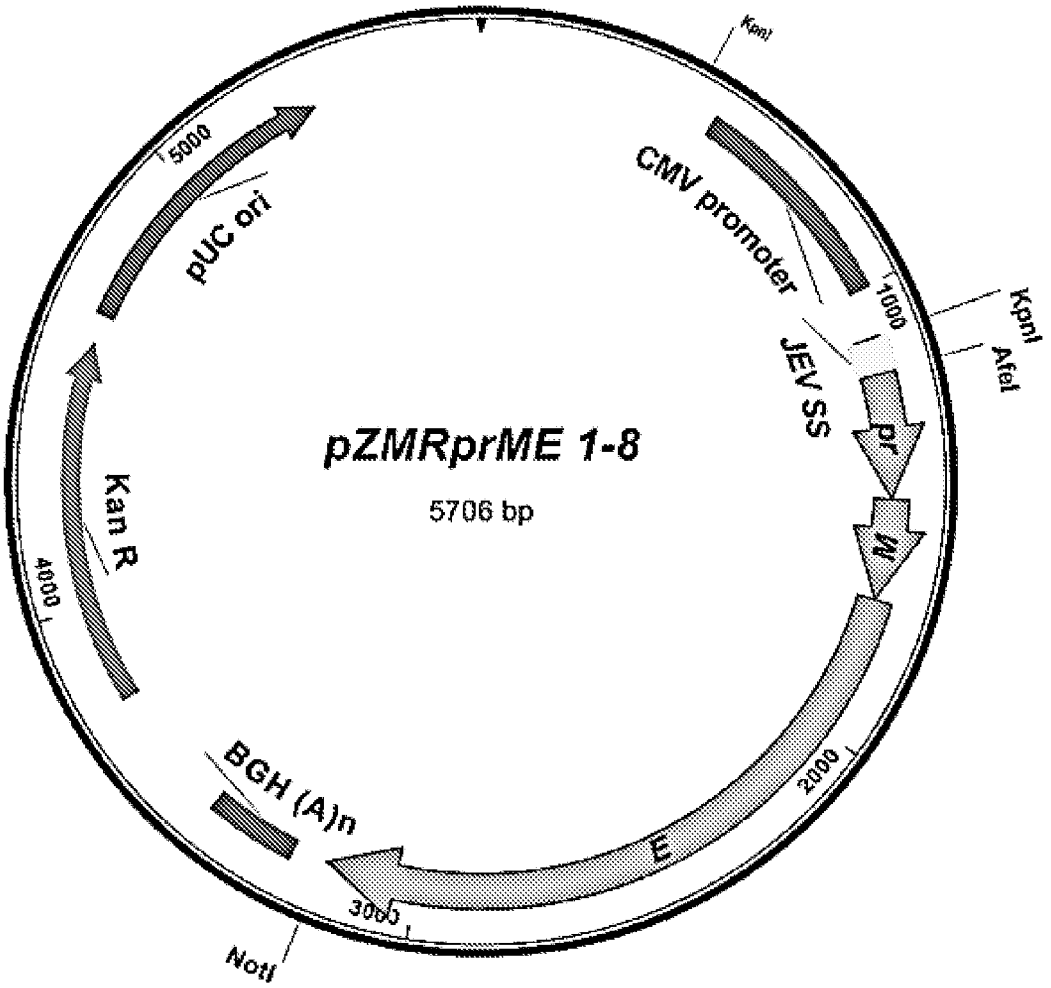

KpnI
5000
pUC ori
CMV promoter
1000
KpnI
AfeI
JEV SS
pr
M
pZMRprME 1-8
5706 bp
Kan R
4000
2000
BGH (A)n
E
3066
NotI

FIG. 1B

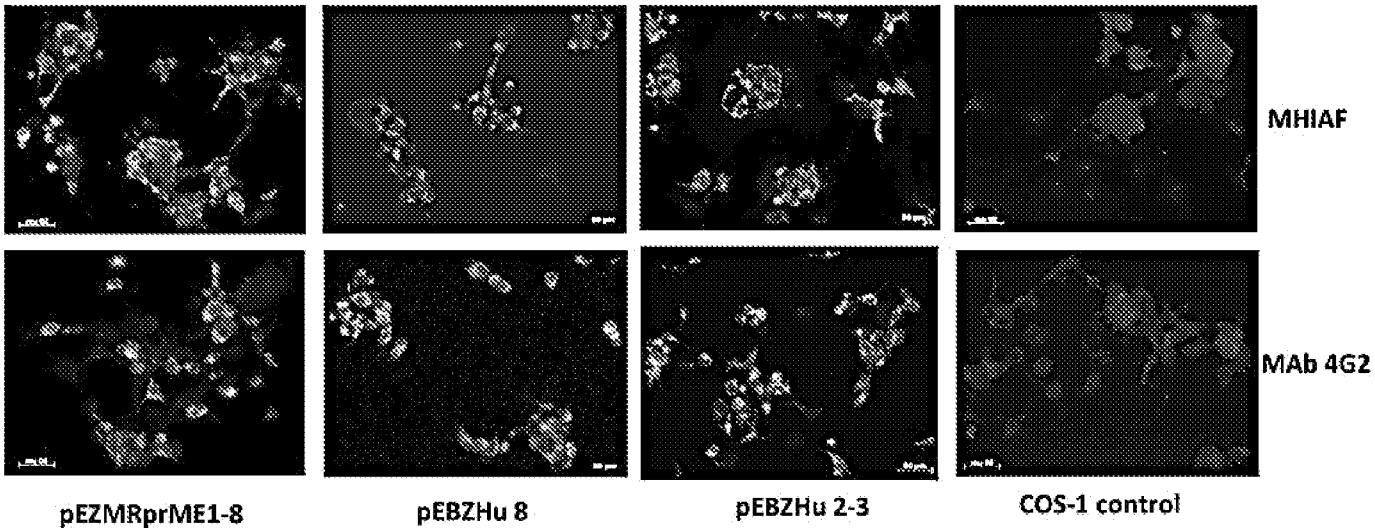

MHIAF
MAb 4G2
pEZMRprME1-8
pEBZHu 8
pEBZHu 2-3
COS-1 control

FIG. 1C

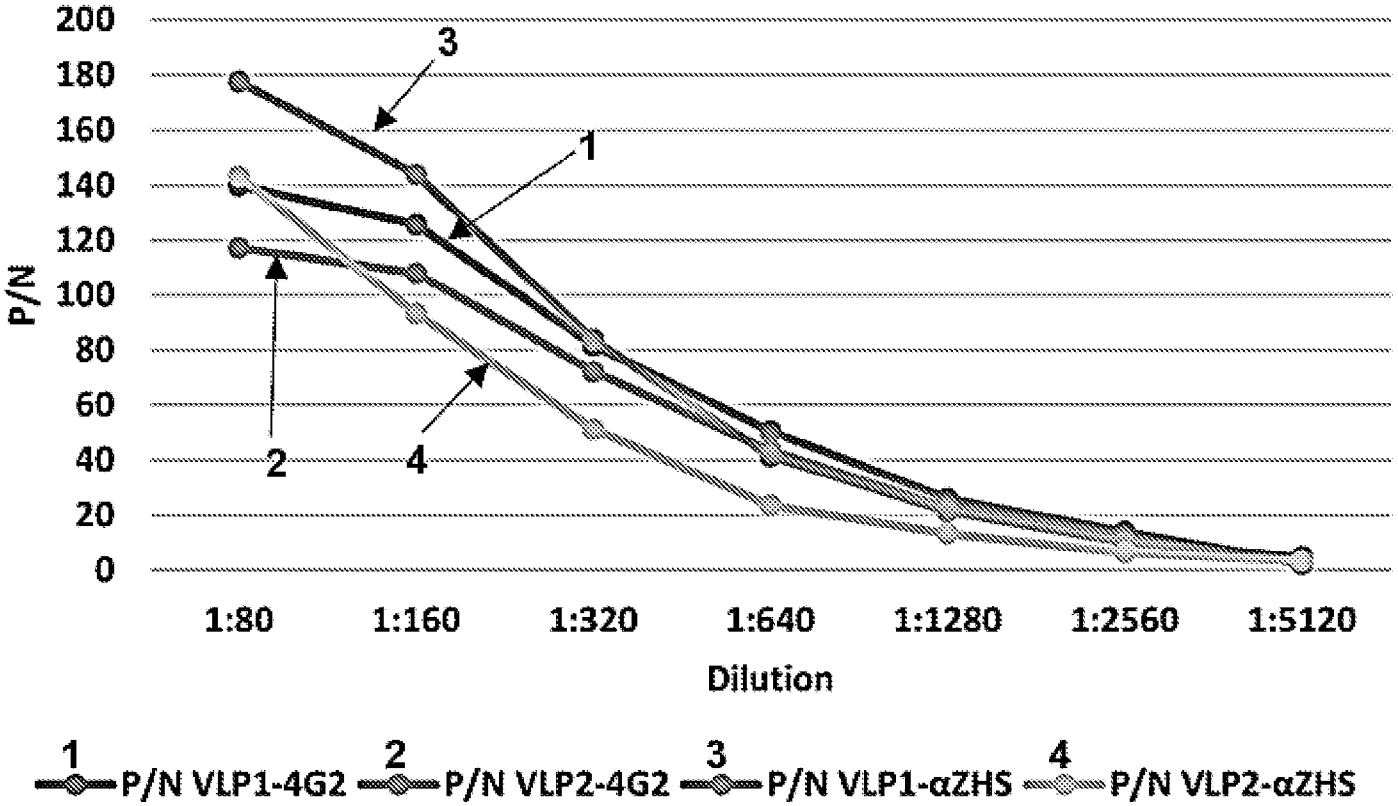

200
180
160
140
120
100
80
60
40
20
0
P/N
1:80
1:160
1:320
1:640
1:1280
1:2560
1:5120
Dilution
1
2
3
4
1 P/N VLP1-4G2
2 P/N VLP2-4G2
3 P/N VLP1-αZHS
4 P/N VLP2-αZHS

FIG. 1D

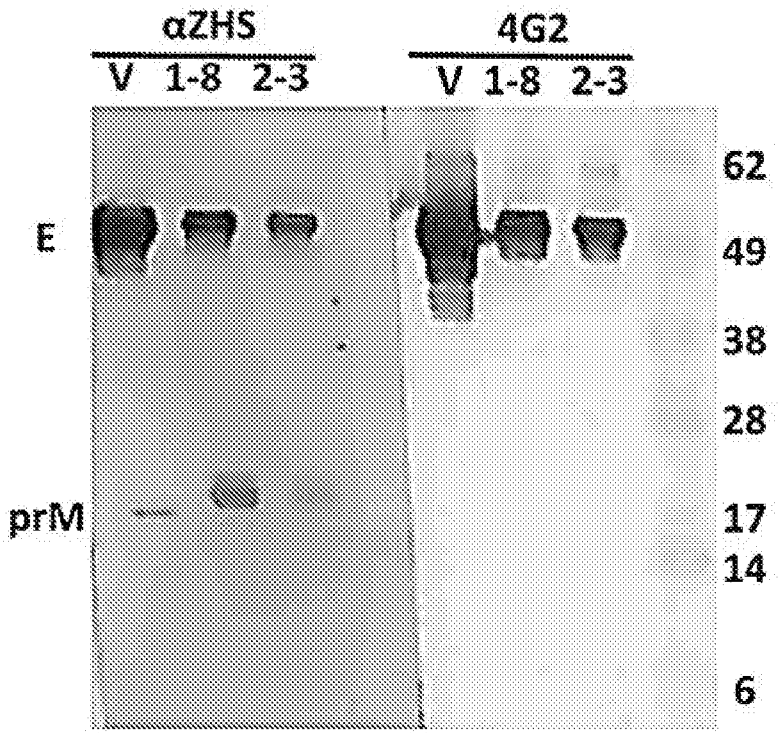

αZHS
V 1-8 2-3
4G2
V 1-8 2-3
E
prM
62
49
38
28
17
14
6

FIG. 2A

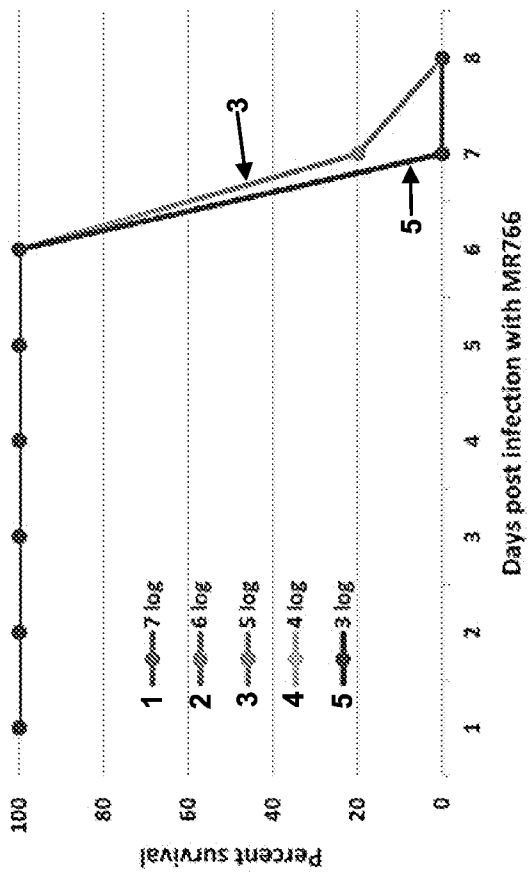


FIG. 2B

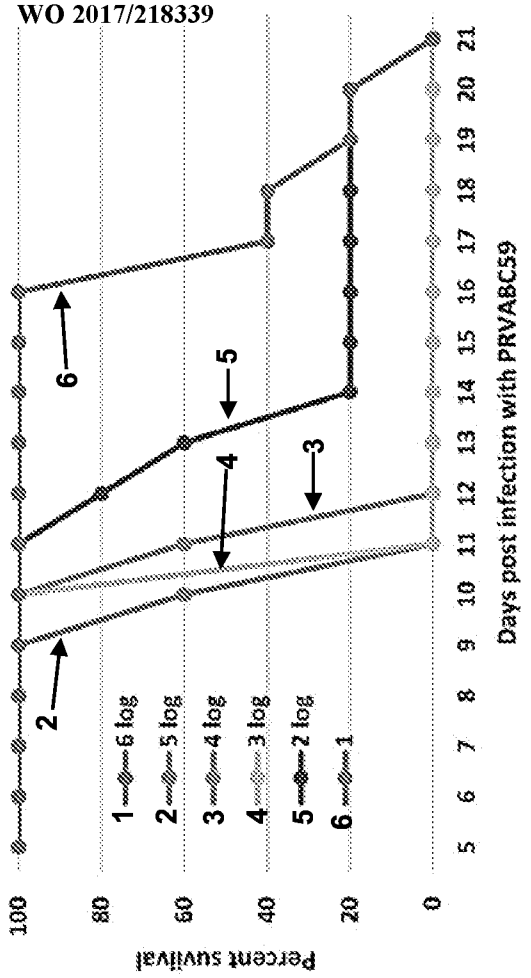


FIG. 2C

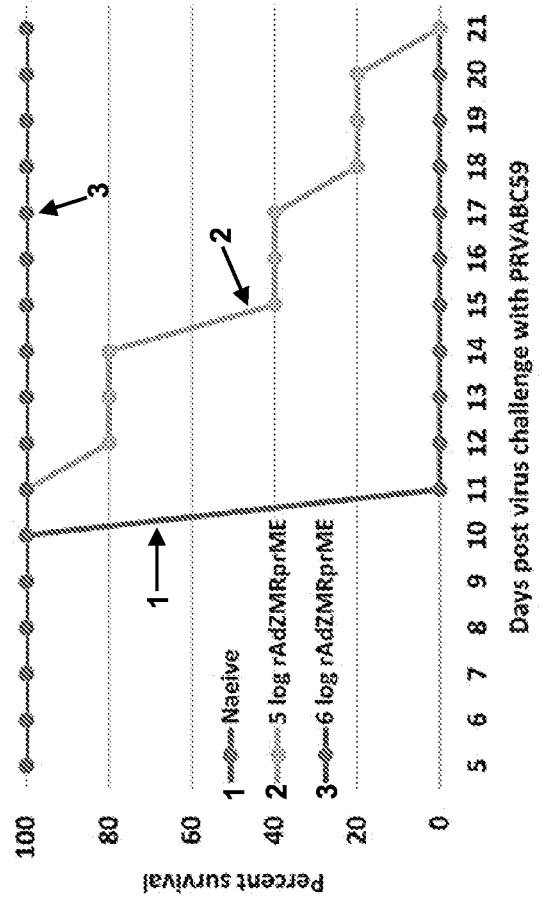


FIG. 2D

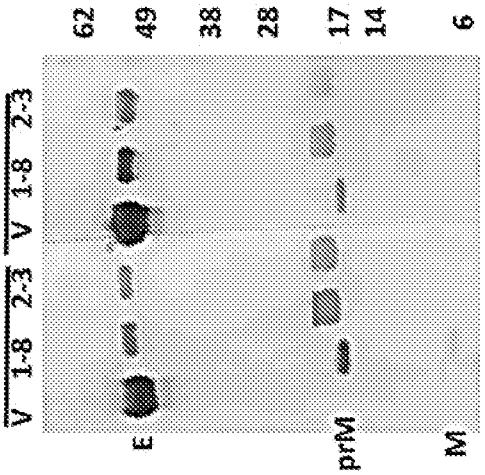


FIG. 3A

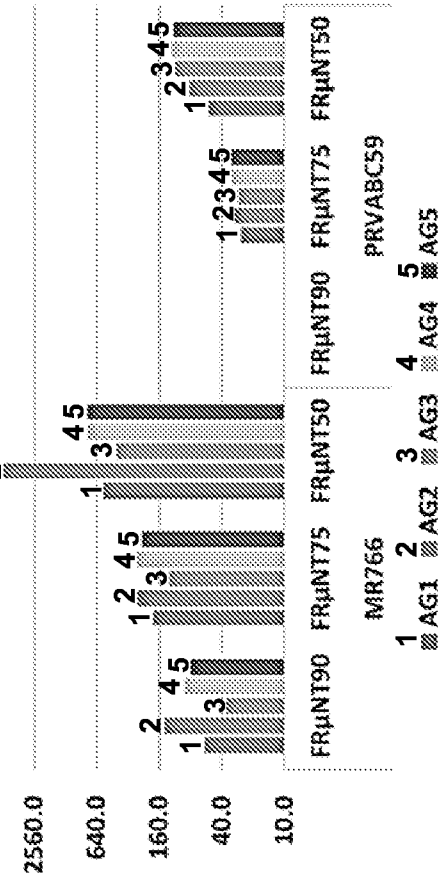


FIG. 3B

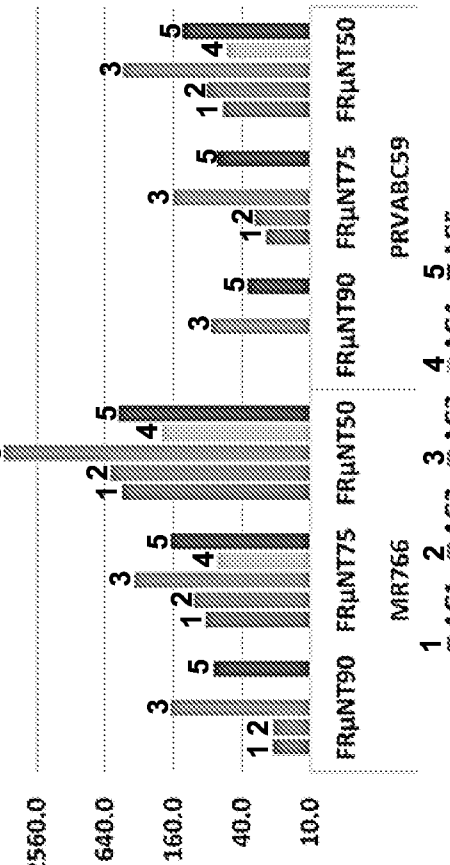
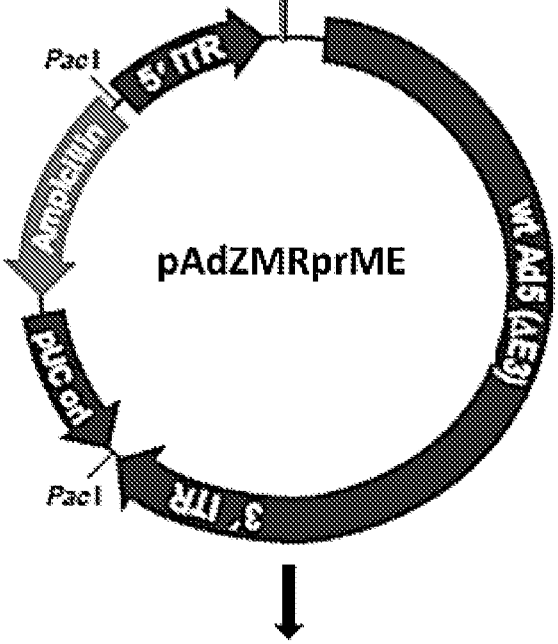


FIG. 3C

FIG. 3D

FIG. 4A



rAdMR1-8

FIG. 4B

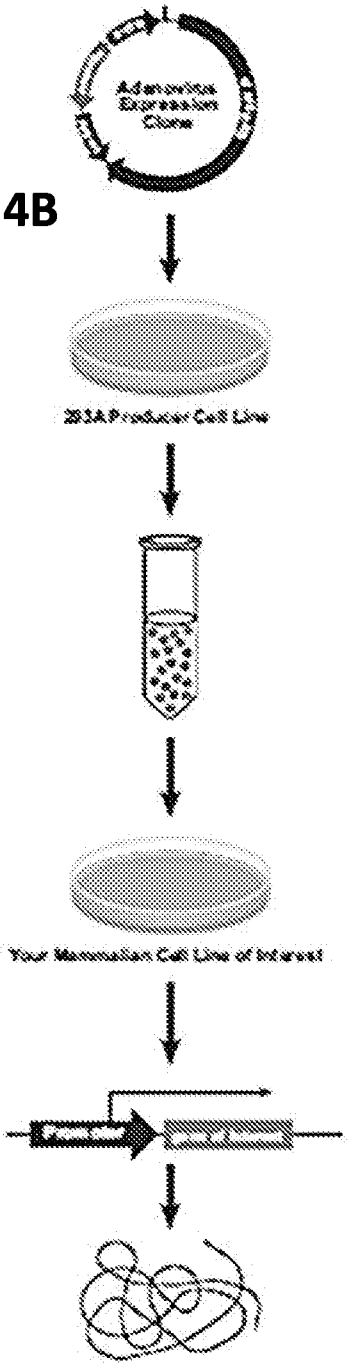
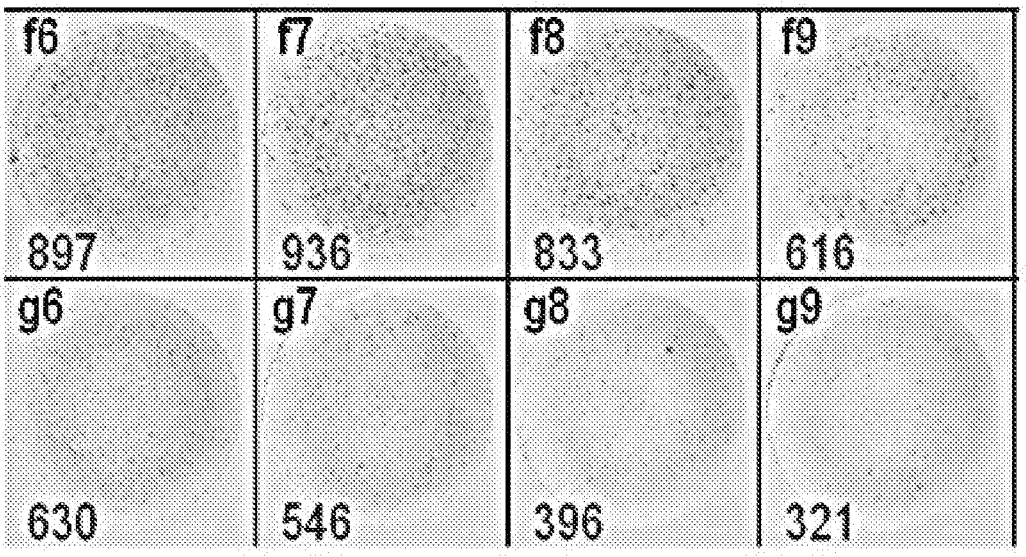


FIG. 4C



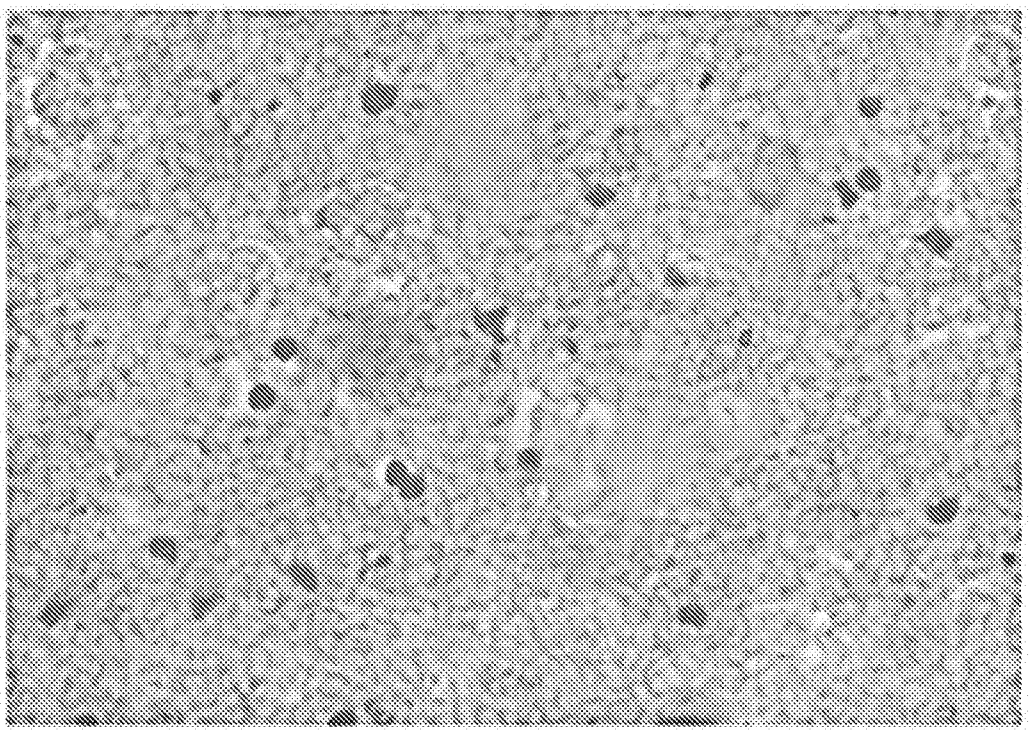


FIG. 4D

FIG. 5B

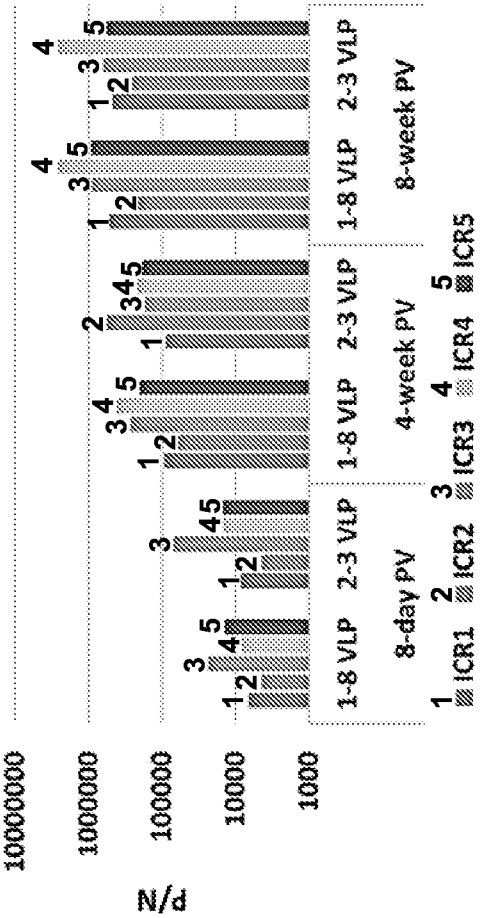

End-point total IgG titer
10000000
1000000
100000
10000
1000
N/d
1-8 VLP
2-3 VLP
8-day PV
1-8 VLP
2-3 VLP
4-week PV
1-8 VLP
2-3 VLP
8-week PV
ICR1
ICR2
ICR3
ICR4
ICR5

FIG. 5A

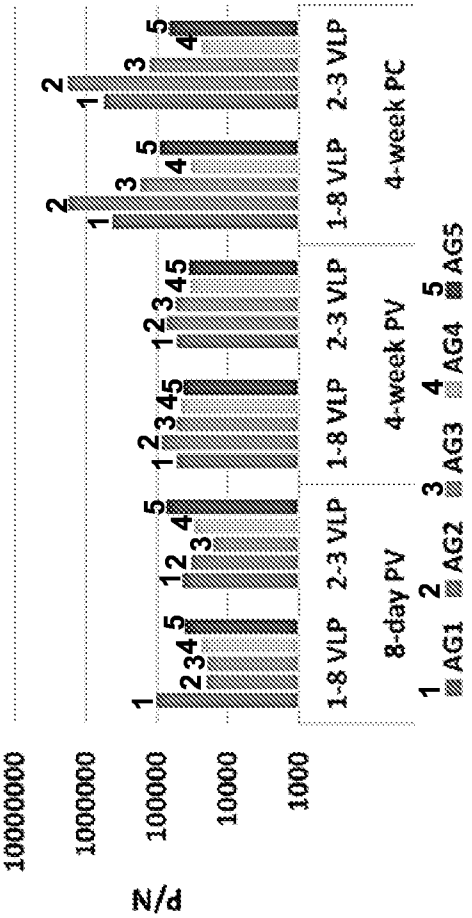
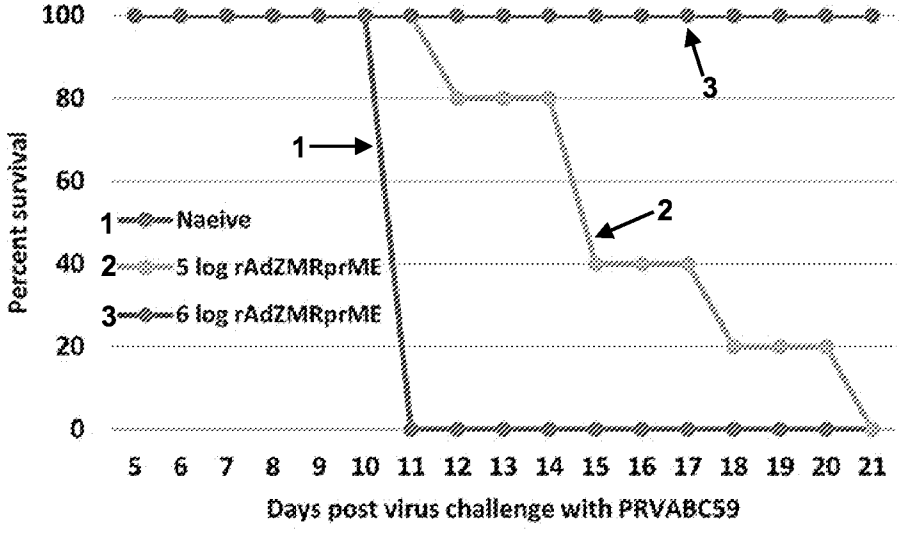

End-point total IgG titer
10000000
1000000
100000
10000
1000
N/d
1-8 VLP
2-3 VLP
8-day PV
1-8 VLP
2-3 VLP
4-week PV
1-8 VLP
2-3 VLP
4-week PC
AG1
AG2
AG3
AG4
AG5

FIG. 2C


100
80
60
40
20
0
Percent survival
1
2
3
1 Naeive
2 5 log rAdZMRprME
3 6 log rAdZMRprME
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days post virus challenge with PRVABC59