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(54) Title: ATTENUATED ZIKA VIRUS CONSTRUCTS AND USES THEREOF

(57) Abstract: Novel attenuating deletions of Zika virus E2 polypeptides are provided as are attenuated viruses comprising the deletions. Also provided are immunogenic compositions that comprise the attenuated viruses and methods of producing such viruses in cells (such as insect cells). Viruses of the embodiments can be used for immunization of animals to provide protection from the pathogenic effects of Zika virus infection.



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DESCRIPTION

ATTENUATED ZIKA VIRUS CONSTRUCTS AND USES THEREOF

[0001] The present application claims the priority benefit of United States provisional application number 62/314,193, filed March 28, 2016, the entire contents of which are
5 incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0002] The present invention relates generally to the fields of molecular biology, virology and disease control. More particularly, it concerns attenuated Zika virus constructs
10 for use in preparing vaccines.

2. Description of Related Art

[0003] Arthropod vectored viruses (Arboviruses) are viral agents which are transmitted in nature by blood sucking insects. Arboviruses include members of the Alpha-, Flavi- and Bunyaviridae. The family of flaviviruses includes approximately 60 enveloped,
15 positive strand RNA viruses, most of which are transmitted by an insect vector. Many members of this family cause significant public health problems in different regions of the world (Monath, 1986). The genome of all flaviviruses sequenced thus far has the same gene order: 5'-C-preM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' in which the first three genes code for the structural proteins the capsid (C), the pre-membrane protein (preM) and
20 the envelope protein (E).

[0004] By their very nature, flaviviruses, like other Arboviruses, must be able to replicate in the tissues of both the invertebrate insect and the mammalian host (Brown and Condreay, 1986, Bowers *et al.*, 1995). Differences in the genetic and biochemical environment of these two host cell systems provide a basis for the production of host range
25 mutant viruses which can replicate well in one host but not the other.

[0005] Zika virus is a positive-sense RNA virus belonging to the Flavivirus genus of the family Flaviviridae. Zika virus is widely distributed throughout the tropical and semitropical regions of the world and is transmitted to humans by mosquito vectors. The virus poses a significant health risk, especially in the case of pregnant women and their
30 unborn children since infection with the virus results in a significant risk for devastating birth

defects. To date, however, there remains no effective vaccine to help prevent Zika virus infection and spread.

SUMMARY OF THE INVENTION

[0006] Embodiments of the present disclosure provide methods and compositions
5 concerning recombinant Zika virus polypeptides. In a first embodiment, there is provided a recombinant polypeptide wherein the polypeptide comprises an amino acid sequence at least 90% identical to the Zika virus envelope protein of SEQ ID NO: 1, wherein the transmembrane domain (TMD) comprises the amino acid sequence SWFSQILIVWLG (SEQ ID NO: 5), SWFSQILIGWLG (SEQ ID NO: 8) or SWFSQILIWLG (SEQ ID NO: 11). In
10 particular aspects, the TMD comprises the amino acid sequence SWFSQILIVWLG (SEQ ID NO: 5). In some aspects, the polypeptide is at least 91%, 92%, 93%, 94%, 95% or 96% identical to the Zika virus envelope protein of SEQ ID NO: 1.

[0007] In certain aspects, the polypeptide comprises a deletion of 4 amino acids in the TMD. In some aspects, the polypeptide comprises a deletion of the amino acids
15 corresponding to amino acid positions 465-468 of SEQ ID NO: 1. For example, the polypeptide comprises SEQ ID NO: 3. In other aspects, the polypeptide comprises a deletion of the amino acids corresponding to amino acid positions 466-469 of SEQ ID NO: 1. For example, the polypeptide comprises SEQ ID NO: 6.

[0008] In some aspects, the polypeptide comprises a deletion of 5 amino acids in the
20 TMD. In certain aspects, the polypeptide comprises a deletion of the amino acids corresponding to amino acid positions 465-469 of SEQ ID NO: 1. For example, the polypeptide comprises SEQ ID NO: 9.

[0009] In another embodiment, there is provided a polynucleotide molecule encoding a polypeptide of the embodiments. In some aspects, the polynucleotide comprises a sequence
25 at least 90% identical to SEQ ID NO: 2. In particular aspects, the polynucleotide comprises a sequence of SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 10. In one specific aspects, the polynucleotide comprises a sequence of SEQ ID NO: 4.

[0010] In yet another embodiment, there is provided a host cell comprising the polynucleotide of the embodiments. In some aspects, the cell is an insect cell. For example,
30 the cell is a Sf9 cell.

[0011] In further embodiments, there is provided a recombinant virus particle comprising a polypeptide or a polynucleotide of the embodiments. In some aspects, the recombinant virus particle is further defined as a live attenuated Zika virus. In certain aspects, the recombinant virus particle further comprises a genome encoding at least one additional
5 attenuating mutation. In some aspects, the virus is adapted for growth in insect cells. For example, a live attenuated Zika virus of the embodiments may replicate at least 2, 3, 4, 5, 10, 20, 50, 100, 500 or 1,000 times more efficiently in insect cells than in mammalian cells. Preferably, a live attenuated Zika virus of the embodiments replicates at least 10 times more efficiently in insect cells than in mammalian cells.

10 [0012] An even further embodiment provides an immunogenic composition comprising a recombinant virus of the embodiments in a pharmaceutically acceptable carrier. In some aspects, the immunogenic composition further comprises an adjuvant, a preservative or a stabilizer.

[0013] Another embodiment provides a method of producing an immune response in
15 a subject comprising administering an immunogenic composition of the embodiments to the subject. In some aspects, the subject is human. In some aspects, the composition is administered by injection. In particular aspects, the composition is administered by an intramuscular or subcutaneous injection. In some aspects, the method is further defined as a method for preventing the symptoms of a Zika virus infection in a subject. In some aspects,
20 the subject is at risk of a Zika virus infection.

[0014] In yet another embodiment, there is provided a composition for use in preventing the symptoms of a Zika virus infection, said composition comprising a recombinant virus of the embodiments in a pharmaceutically acceptable carrier.

[0015] As used herein, “essentially free,” in terms of a specified component, is used
25 herein to mean that none of the specified component has been purposefully formulated into a composition and/or is present only as a contaminant or in trace amounts. The total amount of the specified component resulting from any unintended contamination of a composition is therefore well below 0.01%. Most preferred is a composition in which no amount of the specified component can be detected with standard analytical methods.

[0016] As used herein the specification, “a” or “an” may mean one or more. As used herein in the claim(s), when used in conjunction with the word “comprising,” the words “a” or “an” may mean one or more than one.

5 [0017] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.” As used herein “another” may mean at least a second or more.

10 [0018] Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0019] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications
15 within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better
20 understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0021] **FIG. 1:** Percentage seroconversion of WT MR766 infected mice compared to ΔGTLL vaccinated mice. The mice were vaccinated on day one and challenged with wild type virus on day 76. Mock infected mice were also challenged on day 76. Seroconversion is
25 defined as NAb titers ≥ 20 which is also the detection level of the assay.

[0022] **FIG. 2:** RNA copies measured by RT-qPCR (LOD = 10 copies) in the ΔGTLL vaccinated and the mock vaccinated mice. The RT-qPCR shows that virus replication was suppressed in the ΔGTLL vaccinated group compared to the mock vaccinated group on all days after challenge.

[0023] **FIG. 3:** Qualitative evaluation of amount of WT RNA replication post challenge. Challenge virus load was reduced to non-detectable levels by day 2 post challenge in vaccinated mice.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

5 [0024] The Zika virus is a pathogen that is known to circulate in Africa, the Americas, Asia and the Pacific and has recently established itself in Latin America. Zika virus is transmitted to people through the bite of an infected mosquito from the *Aedes* genus, mainly *Aedes aegypti* in tropical regions. This is the same mosquito that transmits dengue, chikungunya and yellow fever. Zika virus disease outbreaks were reported for the first time
10 from the Pacific in 2007 and 2013 (Yap and French Polynesia, respectively), and in 2015 from the Americas (Brazil and Colombia) and Africa (Cape Verde). In addition, more than 13 countries in the Americas have reported sporadic Zika virus infections indicating rapid geographic expansion of Zika virus. The virus poses a significant health risk, especially in the case of pregnant women and their unborn children since infection with the virus results in a
15 significant risk for devastating birth defects. However, there is currently no specific treatment or vaccine available.

 [0025] Thus, the present invention overcomes challenges associated with current technologies by providing Host range mutants of Zika virus that render the virus highly attenuated in mammalian hosts. In certain aspects of the invention, there is provided an
20 engineered nucleic acid comprising a sequence encoding a modified Zika virus protein comprising a transmembrane domain mutation, wherein the mutation inhibits the production or infectivity of the mutant Zika virus. In particular, a deletion of a portion of the transmembrane domain results in the attenuated mutant. Moreover, though highly attenuated in mammalian cells, the viruses can be grown to near wild type titers in insect cells, thereby
25 allowing for efficient production of vaccine strains. The mutant viruses described here provide ideal vaccine candidates. First, they are highly attenuated as demonstrated by their reduced replication efficiency in mammalian cells and the lack of persistence and symptoms of infection upon introduction into test animals. Second because of the large deletions that are used, the chance of reversion to wild type has been minimized. Thus, the present invention
30 provides highly attenuated, non-reactogenic, and efficacious strains of Zika virus which can be further developed for use in human vaccines.

I. Reference to the Sequence Listing

[0026] The following sequences are provided in the sequence listing and may be used in accordance with certain aspects of the embodiments.

SEQ ID NO: 1 - amino acid sequence for WT Zika virus polypeptide strain MR766 (Genbank # AY632535.2, incorporated herein by reference)

SEQ ID NO: 2 - polynucleotide sequence encoding SEQ ID NO:1

SEQ ID NO: 3 - amino acid sequence for Zika virus mutant 1 “E-TM-1” (Δ GTLL)

SEQ ID NO: 4 – polynucleotide sequence encoding SEQ ID NO:3

SEQ ID NO: 5 - Zika virus mutant 1, TM domain

SEQ ID NO: 6 - amino acid sequence for Zika mutant 2 “E-TM-2”

SEQ ID NO: 7 – polynucleotide sequence encoding SEQ ID NO: 6

SEQ ID NO: 8 - Zika virus mutant 2, TM domain (Δ TLLV)

SEQ ID NO: 9 - amino acid sequence for Zika mutant 3 “E-TM-3”

SEQ ID NO: 10 – polynucleotide sequence encoding SEQ ID NO: 9

SEQ ID NO: 11 - Zika virus mutant 3, TM domain (Δ GTLLV)

II. Zika Virus Mutants

[0027] The recombinant polypeptides and viruses of certain aspects of the embodiments are based on deletion mutations in the transmembrane domains of membrane glycoproteins of Zika virus, in particular the Zika virus TMD. Like other viruses, the E membrane glycoprotein has a hydrophobic membrane-spanning domain which anchors the protein in the membrane bilayer (Rice *et al.*, 1982). The membrane-spanning domain needs to be long enough to reach from one side of the bilayer to the other in order to hold or anchor the proteins in the membrane. Unlike mammalian cell membranes, the membranes of insect cells contain no cholesterol (Clayton 1964; Mitsuhashi *et al.*, 1983). Because insects have no cholesterol in their membranes, the insect-generated viral membrane will be thinner in cross section than the viral membranes generated from mammals. Consequently, the membrane-spanning domains of proteins integrated into insect membranes do not need to be as long as those integrated into the membranes of mammals. Accordingly, certain aspects of the present

inventions provide Zika virus polypeptides with a 3-5 amino acid deletion in their TMD result in viruses that can replicate efficiently in insect cells but show reduced replication in mammalian cells that comprise thicker membranes. Further methods of modifying the glycoprotein transmembrane domain are provided for instance in U.S. Patent 6,306,401; 5 6,589,533; 7,128,915 and 7,335,363, each incorporated herein by reference.

[0028] In certain embodiments, recombinant viruses or polypeptides according to the current embodiments may comprise two or more host range mutations or additionally comprise other mutations such as attenuating mutations, mutations to increase immunogenicity or viral stability or any mutations that may be used for vaccine production 10 and that are current known in the art.

[0029] In additional aspects, recombinant polynucleotide, polypeptides or viruses of the embodiments can comprise additional deletions, substitutions or insertions (or amino acids or nucleic acids). For example, sequences from other Zika virus strains can be incorporated into the recombinant molecules of the embodiments. Thus, in some aspects, 15 amino acid or nucleic acid changes can be made in molecules by substituting the position for a corresponding position from another strain of virus. Similarly, in the case of amino acid substitution, changes can be made with amino acids having a similar hydrophilicity. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte & Doolittle, 1982). It is accepted that the 20 relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 $\pm$ 1); glutamate (+3.0 $\pm$ 1); serine 25 (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 $\pm$ 1); alanine (0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (2.3); phenylalanine (-2.5); tryptophan (-3.4). These values can be used as a guide and thus substitution of amino acids whose hydrophilicity values are within $\pm$ 2 are preferred, those that are within $\pm$ 1 are particularly preferred, and those within $\pm$ 0.5 are 30 even more particularly preferred. Thus, any of the E polypeptides described herein may be modified by the substitution of an amino acid, for different, but homologous amino acid with

a similar hydrophilicity value. Amino acids with hydrophilicities within +/- 1.0, or +/- 0.5 points are considered homologous.

[0030] The recombinant polynucleotide, polypeptides or viruses of the embodiments of the present invention are based on deletion mutations in the transmembrane domain of the membrane glycoprotein E of Zika virus. In certain embodiments, the mutation of Zika virus may comprise a deletion at amino acids 465 to 468 (*i.e.*, deletion of G465, T466, L667 and L668), at amino acids 465 to 469 (*i.e.* deletion of G465, T466, L667, L668, and V469) or at amino acids 466 to 469 (*i.e.*, T466, L667, L668 and V469). Alternatively, the Zika virus mutation may comprise a deletion at amino acids 467 to 470, amino acids 458 to 461, amino acids 460 to 463, amino acids 457 to 460, amino acids 460 to 464, or amino acids 459 to 461.

III. Viral Vaccines

[0031] Certain aspects of the present invention are drawn to a method of producing an immunogenic composition or viral vaccine from genetically engineered membrane-enveloped Zika virus for vaccination of mammals, comprising the steps of introducing the engineered virus into insect cells and allowing the virus to replicate in the insect cells to produce a viral vaccine.

[0032] Certain aspects of the embodiments concern host-range mutant viruses. It is contemplated in certain aspects of the invention that one, two, three, four or more of these types of mutations can be combined, for example, to formulate a tetravalent vaccine. Furthermore, certain aspects of the present invention provide a method of producing a viral vaccine against a disease spread by a wild mosquito population to a mammal, comprising the steps of genetically engineering a mutation of one or more amino acids in a Zika virus protein such as the TMD to produce an engineered virus, wherein the transmembrane protein is able to span the membrane envelope when the virus replicates in mosquito cells, but is unable to efficiently span the membrane envelope when the virus replicates in mammalian cells, and wherein the virus remains capable of replicating in mosquito cells; introducing the engineered virus into a wild mosquito population; and allowing the virus to replicate in cells of the wild mosquito population to produce a population of mosquitoes which excludes the wild-type pathogenic virus and harbors the vaccine strain of the virus such that a mosquito bite delivers the vaccine to a mammal that is bitten.

[0033] In addition, certain aspects of the present invention provide a method of vaccinating an individual in need of such treatment, comprising the steps of introducing the viral vaccine of the present invention into the individual and allowing the vaccine to produce viral proteins for immune surveillance and to stimulate the immune system for antibody production in the individual.

A. Vaccine Preparations

[0034] In any case, a vaccine component (*e.g.*, an antigenic peptide, polypeptide, nucleic acid encoding a proteinaceous composition, or virus particle) may be isolated and/or purified from the chemical synthesis reagents, cell, or cellular components. A vaccine component may be cultured in a population of cells, such as a cell line. Any suitable cell population or cell line may be used. For example, a vaccine component (*e.g.*, a polypeptide, a nucleic acid encoding a polypeptide, or a virus particle) may be cultured in insect cells. Suitable insect cells include, but are not limited to, Sf9 cells, other Sf series cells, Drosophila S1 cells, other Drosophila cell lines, or TN368 cells. It is anticipated that any cultured insect cells may be used to grow the vaccine components or viruses disclosed herein.

[0035] The C6/36 cell line (derived from *Aedes albopictus*) is made up of mosquito cells and is frequently used to study arboviruses. C6/36 cells can be transfected with a vaccine component, such as a polypeptide or a nucleic acid encoding a polypeptide. The production of viruses can be visualized and monitored using a focus assay during vaccine development.

[0036] The Sf9 cell line (derived from *Spodoptera frugiperda*) is commonly used to express recombinant proteins and can be infected by viruses, including arboviruses. For example, Sf9 cells can be infected by viruses including recombinant baculovirus and St. Louis encephalitis, Yellow fever, DEN-1, DEN-2, Gumbo limbo, Eastern equine encephalomyelitis, herpes simplex virus type 1, and vesicular stomatitis viruses (Zhang *et al.*, 1994). Yellow fever, DEN-1-4 viruses can replicate in Sf9 cells (Zhang *et al.*, 1994) such that Sf9 cells can be used to culture and produce such viruses. Likewise, Sf9 cells can be used for production of the recombinant Zika virus of the embodiments.

[0037] In a method of producing a vaccine component, purification is accomplished by any appropriate technique that is described herein or well known to those of skill in the art (*e.g.*, Sambrook *et al.*, 1987). Although preferred for use in certain embodiments, there is no

general requirement that an antigenic composition of the present invention or other vaccine component always be provided in their most purified state. Indeed, it is contemplated that a less substantially purified vaccine component, which is nonetheless enriched in the desired compound, relative to the natural state, will have utility in certain embodiments, such as, for example, total recovery of protein product, or in maintaining the activity of an expressed protein. However, it is contemplated that inactive products also have utility in certain embodiments, such as, *e.g.*, in determining antigenicity via antibody generation.

[0038] Certain aspects of the present invention also provide purified, and in preferred embodiments, substantially purified vaccines or vaccine components. The term “purified vaccine component” as used herein, is intended to refer to at least one vaccine component (*e.g.*, a proteinaceous composition, isolatable from cells), wherein the component is purified to any degree relative to its naturally obtainable state, *e.g.*, relative to its purity within a cellular extract or reagents of chemical synthesis. In certain aspects wherein the vaccine component is a proteinaceous composition, a purified vaccine component also refers to a wild-type or mutant protein, polypeptide, or peptide free from the environment in which it naturally occurs.

[0039] Where the term “substantially purified” is used, this will refer to a composition in which the specific compound (*e.g.*, a protein, polypeptide, or peptide) forms the major component of the composition, such as constituting about 50% of the compounds in the composition or more. In preferred embodiments, a substantially purified vaccine component will constitute more than about 60%, about 70%, about 80%, about 90%, about 95%, about 99% or even more of the compounds in the composition.

[0040] In certain embodiments, a vaccine component may be purified to homogeneity. As applied to the present invention, “purified to homogeneity,” means that the vaccine component has a level of purity where the compound is substantially free from other chemicals, biomolecules or cells. For example, a purified peptide, polypeptide or protein will often be sufficiently free of other protein components so that degradative sequencing may be performed successfully. Various methods for quantifying the degree of purification of a vaccine component will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific protein activity of a fraction (*e.g.*, antigenicity), or assessing the number of polypeptides within a fraction by gel electrophoresis.

[0041] It is contemplated that an antigenic composition of the invention may be combined with one or more additional components to form a more effective vaccine. Non-limiting examples of additional components include, for example, one or more additional antigens, immunomodulators or adjuvants to stimulate an immune response to an antigenic composition of the present invention and/or the additional component(s). For example, it is contemplated that immunomodulators can be included in the vaccine to augment a cell or a patient's (*e.g.*, an animal's) response. Immunomodulators can be included as purified proteins, nucleic acids encoding immunomodulators, and/or cells that express immunomodulators in the vaccine composition.

[0042] Immunization protocols have used adjuvants to stimulate responses for many years, and as such adjuvants are well known to one of ordinary skill in the art. Some adjuvants affect the way in which antigens are presented. For example, the immune response is increased when protein antigens are precipitated by alum. Emulsification of antigens also prolongs the duration of antigen presentation.

[0043] Optionally, adjuvants that are known to those skilled in the art can be used in the administration of the viruses of the invention. Adjuvants that can be used to enhance the immunogenicity of the viruses include, for example, liposomal formulations, synthetic adjuvants, such as (*e.g.*, QS21), muramyl dipeptide, monophosphoryl lipid A, or polyphosphazine. Although these adjuvants are typically used to enhance immune responses to inactivated vaccines, they can also be used with live vaccines. In the case of a virus delivered via a mucosal route (for example, orally) mucosal adjuvants such as the heat-labile toxin of *E. coli* (LT) or mutant derivations of LT can be used as adjuvants. In addition, genes encoding cytokines that have adjuvant activities can be inserted into the viruses. Thus, genes encoding cytokines, such as GM-CSF, IL-2, IL-12, IL-13, or IL-5, can be inserted together with foreign antigen genes to produce a vaccine that results in enhanced immune responses, or to modulate immunity directed more specifically towards cellular, humoral, or mucosal responses.

[0044] An immunologic composition of the present invention may be mixed with one or more additional components (*e.g.*, excipients, salts, etc.) that are pharmaceutically acceptable and compatible with at least one active ingredient (*e.g.*, antigen). Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol and combinations thereof.

[0045] An immunologic composition of the present invention may be formulated into the vaccine as a neutral or salt form. A pharmaceutically acceptable salt, includes the acid addition salts (formed with the free amino groups of the peptide) and those that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acid, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. A salt formed with a free carboxyl group also may be derived from an inorganic base such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxide, and such organic bases as isopropylamine, trimethylamine, 2 ethylamino ethanol, histidine, procaine, and combinations thereof.

[0046] In addition, if desired, an immunologic composition may comprise minor amounts of one or more auxiliary substances such as for example wetting or emulsifying agents, pH buffering agents, etc. that enhance the effectiveness of the antigenic composition or vaccine.

B. Vaccine Administration

[0047] Viruses of the embodiments can be administered as primary prophylactic agents in adults or children at risk of infection, or can be used as secondary agents for treating infected patients. Examples of patients who can be treated using the Zika virus-related vaccines and methods of the invention include (i) children in areas in which Zika virus is endemic, such as Latin America, (ii) foreign travelers, (iii) military personnel, and (iv) patients in areas of a Zika virus epidemic. Moreover, inhabitants of regions where the disease has been observed to be expanding (*e.g.*, Brazil), or regions where it may be observed to expand in the future (*e.g.*, regions infested with *Aedes aegypti* or *Aedes albopictus*), can be treated according to the invention.

[0048] Formulation of viruses of the invention can be carried out using methods that are standard in the art. Numerous pharmaceutically acceptable solutions for use in vaccine preparation are well known and can readily be adapted for use in the present invention by those of skill in this art (*see, e.g.*, Remington's Pharmaceutical Sciences, 18th Ed., 1990). In two specific examples, the viruses are formulated in Minimum Essential Medium Earle's Salt (MEME) containing 7.5% lactose and 2.5% human serum albumin or MEME containing 10% sorbitol. However, the viruses can simply be diluted in a physiologically acceptable solution, such as sterile saline or sterile buffered saline. In another example, the viruses can be administered and formulated, for example, in the same manner as the yellow fever 17D vaccine, *e.g.*, as a clarified suspension of infected chicken embryo tissue, or a fluid harvested

from cell cultures infected with the chimeric yellow fever virus. Preferably, virus can be prepared or administered in FDA-approved insect Sf9 cells.

[0049] The immunogenic compositions of the embodiments can be administered using methods that are well known in the art, and appropriate amounts of the vaccines administered can readily be determined by those of skill in the art. For example, the viruses of the invention can be formulated as sterile aqueous solutions containing between 10^2 and 10^7 infectious units (*e.g.*, plaque-forming units or tissue culture infectious doses) in a dose volume of 0.1 to 1.0 ml, to be administered by, for example, intramuscular, subcutaneous, or intradermal routes. Further, the immunogenic compositions of the embodiments can be administered in a single dose or, optionally, administration can involve the use of a priming dose followed by a booster dose that is administered, *e.g.*, 2–6 months later, as determined to be appropriate by those of skill in the art.

[0050] The manner of administration of an immunogenic composition of the embodiments may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. For example, a vaccine may be conventionally administered intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intratumorally, intramuscularly, intraperitoneally, subcutaneously, intravesicularly, mucosally, intrapericardially, orally, rectally, nasally, topically, in eye drops, locally, using aerosol, injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter, via a lavage, in creams, in lipid compositions (*e.g.*, liposomes), or by other methods or any combination of the foregoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed., 1990, incorporated herein by reference).

[0051] A vaccination schedule and dosages may be varied on a patient-by-patient basis, taking into account, for example, factors such as the weight and age of the patient, the type of disease being treated, the severity of the disease condition, previous or concurrent therapeutic interventions, the manner of administration and the like, which can be readily determined by one of ordinary skill in the art.

[0052] An immunogenic composition of the embodiments is administered in a manner compatible with the dosage formulation, and in such amount as will be

therapeutically effective and immunogenic. For example, the intramuscular route may be preferred in the case of toxins with short half lives in vivo. The quantity to be administered depends on the subject to be treated, including, *e.g.*, the capacity of the individual's immune system to synthesize antibodies, and the degree of protection desired. The dosage of the vaccine will depend on the route of administration and will vary according to the size of the host. Precise amounts of an active ingredient required to be administered depend on the judgment of the practitioner. In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of an active compound. In other embodiments, an active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. However, a suitable dosage range may be, for example, of the order of several hundred micrograms active ingredient per vaccination. In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per vaccination, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, etc., can be administered, based on the numbers described above. A suitable regime for initial administration and booster administrations (*e.g.*, inoculations) are also variable, but are typified by an initial administration followed by subsequent inoculation(s) or other administration(s).

[0053] In many instances, it will be desirable to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be at from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1.5 years, usually three years, will be desirable to maintain protective levels of the antibodies.

[0054] The course of the immunization may be followed by assays for antibodies for the supernatant antigens. The assays may be performed by labeling with conventional labels, such as radionuclides, enzymes, fluorescents, and the like. These techniques are well known and may be found in a wide variety of patents, such as U.S. Pat. Nos. 3,791,932; 4,174,384
5 and 3,949,064, as illustrative of these types of assays. Other immune assays can be performed—and assays of protection from challenge with the Zika virus—can be performed following immunization.

[0055] Certain aspects of the present invention include a method of enhancing the immune response in a subject comprising the steps of contacting one or more lymphocytes
10 with a Zika virus immunogenic composition, wherein the antigen comprises as part of its sequence a nucleic acid or amino acid sequence encoding mutant E2 protein, according to the invention, or an immunologically functional equivalent thereof. In certain embodiments the one or more lymphocytes is comprised in an animal, such as a human. In other embodiments, the lymphocyte(s) may be isolated from an animal or from a tissue (*e.g.*, blood) of the animal.
15 In certain preferred embodiments, the lymphocyte(s) are peripheral blood lymphocyte(s). In certain embodiments, the one or more lymphocytes comprise a T-lymphocyte or a B-lymphocyte. In a particularly preferred facet, the T-lymphocyte is a cytotoxic T-lymphocyte.

[0056] The enhanced immune response may be an active or a passive immune response. Alternatively, the response may be part of an adoptive immunotherapy approach in
20 which lymphocyte(s) are obtained from an animal (*e.g.*, a patient), then pulsed with a composition comprising an antigenic composition. In a preferred embodiment, the lymphocyte(s) may be administered to the same or different animal (*e.g.*, same or different donors).

C. Pharmaceutical Compositions

[0057] It is contemplated that pharmaceutical compositions may be prepared using
25 the novel mutated viruses of certain aspects of the present invention. In such a case, the pharmaceutical composition comprises the novel virus and a pharmaceutically acceptable carrier. A person having ordinary skill in this art readily would be able to determine, without undue experimentation, the appropriate dosages and routes of administration of this viral
30 vaccination compound. When used *in vivo* for therapy, the vaccine of certain aspects of the present invention is administered to the patient or an animal in therapeutically effective amounts, *i.e.*, amounts that immunize the individual being treated from the disease associated

with the particular virus. It may be administered parenterally, preferably intravenously or subcutaneously, but other routes of administration could be used as appropriate. The amount of vaccine administered may be in the range of about 10^3 to about 10^6 pfu/kg of subject weight. The schedule will be continued to optimize effectiveness while balancing negative effects of treatment (*see* Remington's Pharmaceutical Science, 18th Ed., (1990); Klaassen In: Goodman and Gilman's: The Pharmacological Basis of Therapeutics, 8th Ed. (1990); which are incorporated herein by reference). For parenteral administration, the vaccine may be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are preferably non-toxic and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin.

IV. Examples

[0058] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 – Host Range Mutants of Zika Virus

Host Range mutant design

[0059] A series of 3 Zika Δ GTLL, Δ TLLV and Δ GTLLV mutants will be made, deleting the sequences shown in Table 1. Virus titers of the Zika virus mutants were determined after growth in both C6/36 and Vero cells and with titration on C6/36 cells.

[0060] **Table 1:** Transmembrane domain sequences of Zika virus WT and mutants are shown. Three transmembrane deletions (each deletion of 4 or 5 amino acids) of Zika virus were produced *in vitro* and studied. The underlined portions of sequence represent the segments of the TMD which will be deleted.

Virus		E2 TMD Sequence
Zika virus	MR766	457SWFSQILIGTLLVWLG ₄₇₂
Zika virus	E-TM-1	457SWFSQILIGTLLVWLG ₄₇₂
Zika virus	E-TM-2	457SWFSQILIGTLLVWLG ₄₇₂
Zika virus	E-TM-3	457SWFSQILIGTLLVWLG ₄₇₂

Biosafety

[0061] All studies involving viable Zika virus were performed in certified BSL-3 laboratories in biosafety cabinets using biosafety protocols approved by the Institutional Biosafety Committee of North Carolina State University. Animal husbandry and mouse experiments were performed in accordance with all North Carolina State University Institutional Animal Care and Use Committee guidelines.

Construction of Zika Virus TM deletion mutants.

[0062] A full-length cDNA clone of Zika virus strain MR766 (Genbank # AY632535.2, incorporated herein by reference (SEQ ID NO: 1) was obtained by *de novo* chemical synthesis and cloned into the pCCI vector with modifications including substitution of unique restriction sites at the virus 5' end to obtain Zika mutant 1 ΔGTLL, Zika mutant 2 ΔTLLV and Zika mutant 3 ΔGTLLV (Table 1). PCR screen and restriction enzyme analysis were used to identify correct mutations. Growth of all Zika clones in EPI300 cells was in LB containing 12.5 μg/mL chloramphenicol at 28 to 30°C for approximately 24 to 48 hours. Zika plasmid DNA was recovered using the Wizard® Plus Minipreps (Promega, Madison, WI). All Zika deletion mutant clones were confirmed by sequence analysis. Transcripts were generated for each Zika virus mutant clone using the RiboMAX™ Large Scale RNA Product Systems for SP6 RNA Polymerase (Promega) following manufacturer's instructions, with the addition of RNA cap analog 7mg (ppp)G (NEB # S1404S). The RNA transcripts were transfected into Sf9, Vero and C6/36 cells.

Cell and Virus Culture

[0063] C6/36 cells (*Aedes albopictus*, American Type Culture Collection [ATCC] # CRL-1660, Manassas, VA) were maintained in minimal essential medium (MEM) containing

Earl's salts supplemented with 10% fetal bovine serum (FBS), 5% tryptose phosphate broth (TPB) and 2 mM L-glutamine. Vero cells (African Green monkey kidney, ATCC #CCL-81) were maintained in 1X MEM supplemented with 10% FBS, 5% TPB, 2 mM glutamine, 10 mM Hepes pH 7.4 and 1X MEM nonessential amino acids (NEAA) (1:100 dilution of NEAA from Gibco #11140, Carlsbad, CA). SF9 cells *Spodoptera frugiperda* (ATCC #CRL 1711) were maintained in ESF 921 (Expression Systems).

[0064] C6/36 and Vero cells were transfected by electroporation with WT Zika virus and Zika virus Δ GTLL (encoding a transmembrane domain of SEQ ID NO: 5), Δ TLLV (encoding a transmembrane domain of SEQ ID NO: 8) and Δ GTLLV (encoding a transmembrane domain of SEQ ID NO: 11) mutant RNAs as follows: cells were pelleted and washed in RNase free electroporation buffer (PBS-D for Vero and MOPS for C6/36) and resuspended in their respective buffers at a concentration of 1×10^7 to 5×10^7 cells/ml. RNA transcripts will then be added to 400 μ l of cells and electroporated at 1.0 KV, 50 μ F and ∞ resistance using the BioRad Gene Pulsar II (Bio-Rad Laboratories, Hercules, CA). The transfected cells were then be plated in 24 well plates with 1.0 ml of the media and incubated at 37°C for Vero cells and 28°C for C6/36 cells for 1 hour with slow rocking. The media was removed and the plates overlayed with 10 ml of 1X Vero media or 1X C6/36 media and incubated for 7 days. The supernatant from the plates was harvested and quick frozen for titer analysis by focus or plaque assay.

[0065] Focus assay. The focus assay may be developed as a colorimetric or fluorescent assay using antibodies labeled with either HRPO (color substrate) or Alexa Fluor fluorescent dye. For the color assay, plates with transfected or infected cells were washed twice with 1X PBS and fixed with 80% methanol for 15 minutes at room temperature, followed by incubation with antibody dilution buffer (5% skim milk in 1X PBS-D) for 10 minutes. Primary antibody (α -DV NS1 glycoprotein, Abcam #ab41623, Cambridge, MA) was added at a dilution of 1:400 in antibody (Ab) dilution buffer and incubated for 1 hour at 37°C with slow rocking. The wells were then be washed twice with 1X PBS followed by the addition of secondary antibody conjugated with horse radish peroxidase (HRP) (Sigma # 8924, St. Louis, MO) at a dilution of 1:500 in Ab dilution buffer. Wells were washed again twice with 1X PBS. Foci were visualized by the addition of 150 μ l TrueBlue™ peroxidase substrate (KPL# 50-78-02, Gaithersburg, MD) to each well and developing for about 15 minutes. Foci were counted and titer determined in focus forming units/ml (ffu/mL) of virus.

For the fluorescent assay, the protocol is similar to the color assay with the following exceptions: Cells were fixed for 20 minutes at room temperature in 100% methanol. A second 10 minute incubation was performed with 1 X PBS plus 0.05% Tween, followed by 2 washes with 1 X PBS plus 0.2% BSA. Antibody was diluted in 1 X PBS + 0.2% BSA. The washes between the primary and secondary antibodies were performed in 1 X PBS + 0.2% BSA. The secondary antibody, Alexa fluor® 488 F(ab')₂ fragment of goat anti-mouse IgG (Invitrogen # A-11017, Carlsbad, CA), incubation was conducted for 45 minutes in darkness. After the final wash, 50 µl of water were added to each well for visualization of the fluorescent foci.

[0066] Plaque assay. Titration of virus produced was done using C6/36 cells as indicator cell monolayers. Modification of the standard plaque assay was necessary to accommodate the specific cell line. Virus stocks were thawed slowly on ice, and serial virus dilutions were made, on ice, into cold phosphate-buffered saline (PBS) deficient in MgCl and CaCl (PBS-D) containing 1% FBS. The 1% agarose (Sigma, St. Louis, Mo.) overlay was as described (Hernandez *et al.*, 2010).

[0067] Infection and purification of selected mutants. The WT and Zika mutants were grown in the *Aedes albopictus* mosquito-derived C6/36 cell line. Cells will be split one day prior to infection at a ratio of 1:3. Subconfluent monolayers of C6/36 cells were infected at an MOI of ~0.03 pfu/cell. Virus was diluted in C6/36 media and each 75 cm³ flask infected with 1.0 ml of diluted virus for 1 hour at room temperature with slow rocking. After the initial infection, 4.0 ml of fresh media were added to each flask. Flasks were then be incubated for 7 days at 28°C. Virus was harvested by centrifugation of the supernatant at 4000 rpm for 10 min. Purification and concentration of WT and mutant Zika virus was achieved using isopycnic ultracentrifugation with iodixanol (Optiprep) gradients (Sigma, St. Louis, MO). Virus was spun to equilibrium in gradients of 12% to 35% iodixanol and isolated.

[0068] *Spodoptera frugiperda* (Sf9) cells were cultured at 28°C in ESF 921 (Expression Systems) serum free medium. Suspension cultures were seeded at a density of 3x10⁵ cells per mL, and allowed to grow to a density of 2x10⁶ cells/mL. 24 hours prior to infection, adherent flasks were seeded with cells from suspension cultures and incubated at 28°C. Subconfluent adherent Sf9 cells were infected with a multiplicity of infection (MOI) of >1 plaque forming units (pfu)/cell of Zika virus or Zika virus mutants ΔGTLL, ΔTLLV and ΔGTLLV, for 1 hr. with rocking and inoculum were removed and replaced with fresh ESF

921 medium. Supernatants were harvested after 7 days of incubation at 28°C. Virus was titrated via plaque assay on C6/36 cells.

Mouse Studies

[0069] Upon advice from the NIAID, BALB/c mice were used as a model system for this virus. All Zika LAV vaccine candidates were grown in Sf-9 cells, purified in potassium tartrate, measured by ELISA and titered by plaque assay. Experimental design: 3 vaccine groups + 2 control groups, 8 mice/group and were inoculated subcutaneously. The 5 groups included an inoculation with; WT MR766 10e4 total pfu (group1), ΔGTLL 10e3 total pfu (group 2), ΔGTLLV (group 3) ΔTLLV 10e3 total pfu (group 4) Zika virus HR vaccine strains and a mock immunization with saline. A pre-bleed was done on all mice on the day previous to inoculation. A total of forty male and female BALB/c mice were inoculated on day zero (D0) with 10e4 PFU of either wild type Zika virus MR766 (Group 1), or 10e3 of the candidate vaccines (Groups 2-4). Serum samples were collected at predetermined time points; day (D) -1, D14, D28, and D42, Vaccine candidates not showing significant immune response (≥ 40 PRNT 50 titer), ΔGTLLV (group 3) ΔTLLV 10e3 total pfu (group 4) were dropped from the study. Mice vaccinated with the remaining HR candidate, ΔGTLL and the WT MR766 were further bled on day 42. The remaining groups, 1,2 and 5 were challenged with a dose of 10e4 total MR766 pfu per mouse intravenously (i.v.) on D76 of the study. Mice were again sampled on Days 77-80 (for PCR analysis of challenge viremia), and D89 for a final PRNT50 titer. To check the virus replication (viremia), blood samples of all mice groups (1,2 and 5) were collected during days 77-80 post challenge and their sera tested for Zika genome.

Viremia from Mice

[0070] Due to the selective nature of the Zika virus strain for growth in mosquitoes, and the attenuation of the vaccine strains in mammalian cells, vaccine titers were quantified by plaque assay in C6/36 as described (Hernandez *et al.* 2010). Viremias resulting from the challenge virus Zika virus MR766 were quantified by RT,qPCR. The limit of detection (LOD) for these assays is 10 RNA genome equivalents..

[0071] Extracted RNA was then analyzed via RT-qPCR (reverse transcription-quantitative polymerase chain reaction) using the following primer pairs; Sense primer: ZIKA VIRUS F (5'-CCTTCAAATCACTGTTTGG -3'; SEQ ID NO:12) Anti-sense primer:

ZIKA VIRUS R (5'-GTGGAGAGGAAGATCATC -3'; SEQ ID NO:13) which recognize the Zika virus strain. The infectious ZIKA virus was used as a positive control, and extracted RNA was used as a negative control. RT-PCR has a sensitivity of detection for Zika virus of about 10 pfu.

5 *Plaque Reduction Neutralization Test*

[0072] Neutralizing antibody (NAb) titers are determined by plaque reduction neutralization test (PRNT) in Vero cells (Briggs et al 2014). Mice sera are heat inactivated at 56°C for 20 minutes prior to being serially diluted in duplicate 1 to 2, starting with a 1 to 20 dilution. After diluting the sera, approximately 20 pfu of WT Zika virus are added to each dilution, allowed to incubate at RT for 15 minutes, and then plated on WHO Vero cells and allowed to produce plaques for 4 days at 37°C. NAb titers (PRNT50) are determined based upon the highest serial dilutions where 50% of the pfu added is observed, and results are expressed as the geometric mean of titers from the each mouse per group per day. Mouse group size may vary.

15 **Example 2 – Results of Mouse Studies**

[0073] As described in Example 1, Zika virus vaccine candidates were produced in the reference strain MR766 using the dengue virus and West Nile virus host range virus vaccine candidates previously tested as templates. Vaccines were grown in insect Sf9 cells because these strains are host adapted to insect cells with limited growth in mammalian cells. Three deletion mutants of ZIKV MR766 were made in the TMD 1 region and included deletions of the amino acids GTLL, GTLLV, and TLLV (Table 1).

[0074] These three vaccine candidates were tested for immunogenicity in BALB/c mice. The presence of neutralizing Ab was tested by PRNT50 on days 14, 28, and 42 after vaccination. Mutants Δ GTLLV and Δ TLLV did not confer significant neutralizing antibody (NAb) production on days 14 or 28 and were not tested further. It was of interest to test the quality of the mouse NAb response to the Δ GTLL vaccine candidate by evaluating the response to a wild-type virus challenge after clearance of the infecting virus (day 14) and a return of the Ab response to baseline (day 76) had occurred. The Δ GTLL, MR766 and mock control groups were thus challenged with wild-type virus using the more invasive IV route to measure the anamnestic response of the Δ GTLL vaccinated group. Viremia post challenge

was also measured for four days after inoculation to determine replication of the virus in the three groups.

5 **[0075]** BALB/c mice, 8 per group were immunized subcutaneously with Zika virus (ZIKV) WT MR766, vaccine strains Δ GTLL, Δ GTLLV, Δ TLLV, or mock immunized on day 0 of the trial. Group 1 was immunized with 10e4 total pfu Zika WT MR766; Group 2 was immunized with 10e3 total pfu Zika Δ GTLL vaccine strain; Group 3 was immunized with 10e3 total pfu Zika Δ GTLLV vaccine strain; Group 4 was immunized with 10e3 total pfu Zika Δ TLLV vaccine strain; and Group 5 was mock inoculated with saline buffer.

10 **[0076]** Groups 1, 2, and 5 were challenged on day 76 post vaccination with 1e4 total ZIKV MR766 pfu. Blood samples were drawn from all mice on days 0, 14, and 28 to test for neutralizing Ab (NAb). Results are shown in Table 2. PRNT₅₀ values are the dilutions of the mouse sera capable of neutralizing 50% of the virus particles added and are the standard metric of NAb production. The Δ GTLL vaccine, MR766 and mock vaccinated groups, were also tested on day 42 to determine the stability of the immune response.

15 **[0077]** On day 76 of the study, all groups were challenged with 10e4 total pfu of Zika WT MR766. This was done to evaluate the anamnestic response of the mice to the vaccine and to measure replication of the challenge virus. Thus, blood was drawn from mice on four days post challenge, days 77, 78, 79, and 80. The trial was stopped on day 89 with a terminal bleed.

20 **[0078]** Blood draws on consecutive days cannot be done on mice so this part of the trial was conducted in two groups. Four of eight mice were bled on days 1 and 3 and the remaining four mice were bled on days 2 and 4, allowing for a mouse to mouse comparison for all days tested.

25 **[0079] Table 2.** PRNT₅₀ titers of ZIKV MR766, Δ GTLL and mock vaccinated BALB/c mice post vaccination and post challenge.

PRNT₅₀						 Challenge day 76
MR766						
Days of serum sampling						
Animal ID	-1	14	28	42	80	89
10829	<20	2560	640	1280	1280	2560
10842	<20	160	160	1280	640	2560
10822	<20	40	80	40	1280	5120
10844	<20	320	1280	640	640	2560
10852	<20	160	160	320	320	640
10869	<20	640	320	640	2560	5120
10870	<20	640	1280	2560	2560	2560
10851	<20	640	640	1280	1280	5120
GMT:	<20	349	380	640	1076	2791
Seroconversion:	0/8	8/8	8/8	8/8	8/8	8/8
Seroconversion (%):		100.0%	100.0%	100.0%	100.0%	100.0%
ΔGTLL						
10837	<20	<20	<20	<20	1280	320
10824	<20	20	40	20	1280	1280
10830	<20	20	40	20	1280	1280
10833	<20	20	40	20	1280	1280
10850	<20	<20	40	80	2560	1280
10857	<20	20	<20	<20	640	160
10853	<20	20	80	80	160	160
10845	<20	<20	80	20	5120	5120
GMT:	<20	20.0	51	32	1174	761
Seroconversion:	0/8	5/8	6/8	6/8	8/8	8/8
Seroconversion (%):		63%	75%	75%	100.0%	100.0%
Mock						
10825	<20	<20	<20	<20	NA	2560
10819	<20	<20	<20	<20	NA	160
10831	<20	<20	<20	<20	NA	40
10823	<20	<20	<20	<20	NA	320
10856	<20	<20	<20	<20	NA	160
10849	<20	<20	<20	<20	NA	640
10858	<20	<20	<20	<20	NA	640
10864	<20	<20	<20	<20	2560	1280
GMT:	<20	<20	<20	<20		
Seropositive:	0	0	0	0	NA	8/8
Seropositive (%):						100.0%
Seroconversion:	NA	NA	NA		PC	PC

NA: not available; PC: post challenge

[0080] The kinetics of antibody synthesis in the WT and vaccinated mice can be visualized graphically in FIG. 1. From these data, it was concluded that the ΔGTLL vaccinated mice had responded to the vaccination and produced antibodies at stable levels during the period between days 28 and 42. Compared to the WT infected group, the vaccinated mice generated protective NAb that remained stable and consistent throughout the

trial until challenge with the WT on day 76. In addition, after the challenge a strong and rapid anti-ZIKV NAb response was observed in the WT and vaccinated mice. This response was well above the 4 fold increase that is indicative of an anamnestic response. This response remained relatively high for all mice throughout the remaining ~ 2 weeks post challenge and
5 was seen in all mice including the ones that did not have a titer of ≥ 20 . This finding indicated that all mice were primed after immunization with Δ GTLL.

[0081] To measure the levels of virus in the mouse serum post challenge, mice were bled on days 77, 78, 79 and 80 post vaccination and ZIKV RNA levels determined by RT-PCR. The PCR data shown in FIGS. 2 and 3 confirmed that the NAb generated by the
10 vaccinated group was able to strongly suppress virus replication (*i.e.*, viremia) in the mice, when compared to non-vaccinated group. Thus, vaccination with the Zika virus host range mutant Δ GTLL resulted in strong immunogenicity to wild type Zika virus challenge.

* * *

[0082] All of the methods disclosed and claimed herein can be made and executed
15 without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain
20 agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Bell *et al.* Zika virus infection of the central nervous system of mice. *Archiv für die gesamte Viursforschung*, 35, 183-193, 1971.

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WHAT IS CLAIMED IS:

1. A recombinant polypeptide wherein the polypeptide comprises an amino acid sequence at least 90% identical to the Zika virus envelope protein of SEQ ID NO:1, wherein the transmembrane domain (TMD) comprises the amino acid sequence SWFSQILIVWLG (SEQ ID NO: 5), SWFSQILIGWLG (SEQ ID NO: 8) or SWFSQILIWLG (SEQ ID NO:11).
2. The polypeptide of claim 1, wherein the TMD comprises the amino acid sequence SWFSQILIVWLG (SEQ ID NO: 5).
3. The polypeptide of claim 1, wherein the polypeptide is at least 91%, 92%, 93%, 94%, 95% or 96% identical to the Zika virus envelope protein of SEQ ID NO:1.
4. The polypeptide of claim 3, wherein the polypeptide comprises a deletion of 4 amino acids in the TMD.
5. The polypeptide of claim 4, wherein the polypeptide comprises a deletion of the amino acids corresponding to amino acid positions 465-468 of SEQ ID NO:1.
6. The polypeptide of claim 4, wherein the polypeptide comprises a deletion of the amino acids corresponding to amino acid positions 466-469 of SEQ ID NO:1.
7. The polypeptide of claim 1, wherein the polypeptide comprises SEQ ID NO:3.
8. The polypeptide of claim 1, wherein the polypeptide comprises SEQ ID NO:6.
9. The polypeptide of claim 3, wherein the polypeptide comprises a deletion of 5 amino acids in the TMD.
10. The polypeptide of claim 9, wherein the polypeptide comprises a deletion of the amino acids corresponding to amino acid positions 465-469 of SEQ ID NO:1.
11. The polypeptide of claim 1, wherein the polypeptide comprises SEQ ID NO:9.
12. A polynucleotide molecule encoding a polypeptide of anyone of claims 1-11.
13. The polynucleotide of claim 12, comprising a sequence at least 90% identical to SEQ ID NO:2.

14. The polynucleotide of claim 13, comprising a sequence of SEQ ID NO:4, SEQ ID NO:7, or SEQ ID NO:10.
15. The polynucleotide of claim 13, comprising a sequence of SEQ ID NO:4.
16. A host cell comprising the polynucleotide of claim 12.
17. The cell of claim 16, wherein the cell is an insect cell.
18. The cell of claim 17, wherein the cell is a SF9 cell.
19. A recombinant virus particle comprising a polypeptide of anyone of claims 1-11 or the polynucleotide of claim 12.
20. The recombinant virus of claim 19, further defined as a live attenuated Zika virus.
21. The recombinant virus of claim 20, further comprising a genome encoding at least one additional attenuating mutation.
22. The recombinant virus of claim 20, wherein the virus is adapted for growth insect cells.
23. An immunogenic composition comprising a recombinant virus of claim 20 in a pharmaceutically acceptable carrier.
24. The immunogenic composition of claim 23, further comprising an adjuvant, a preservative or a stabilizer.
25. A method of producing an immune response in a subject comprising administering an immunogenic composition of claim 23 to the subject.
26. The method of claim 25, wherein the subject is human.
27. The method of claim 26, wherein the composition is administered by injection.
28. The method of claim 27, wherein the composition is administered by an intramuscular or subcutaneous injection.
29. The method of claim 25, further defined as a method for preventing the symptoms of a Zika virus infection in a subject.

30. The method of claim 25, wherein the subject is at risk of a Zika virus infection.
31. A composition for use in preventing the symptoms of a Zika virus infection, said composition comprising a recombinant virus of claim 20 in a pharmaceutically acceptable carrier.

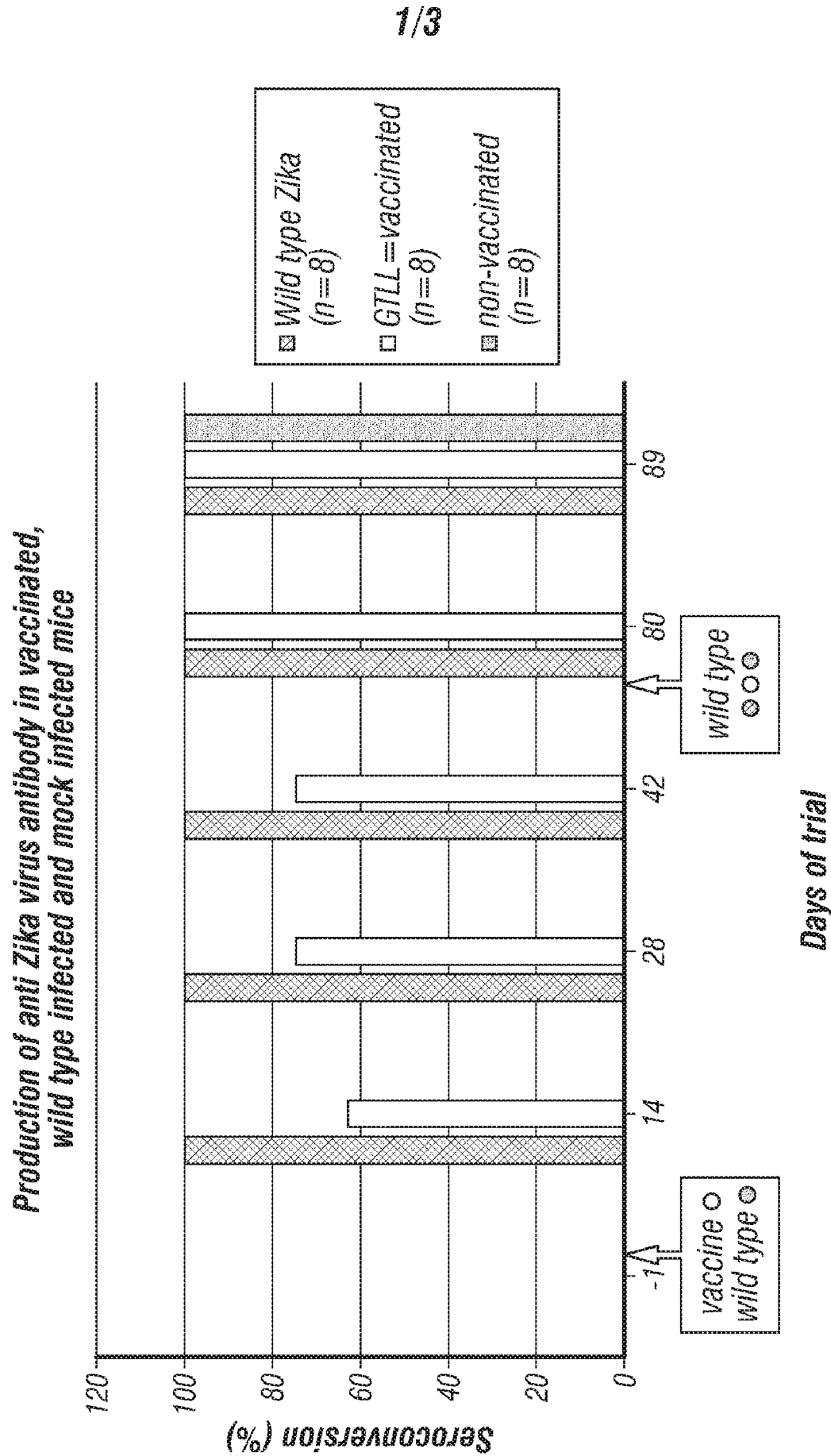


FIG. 1

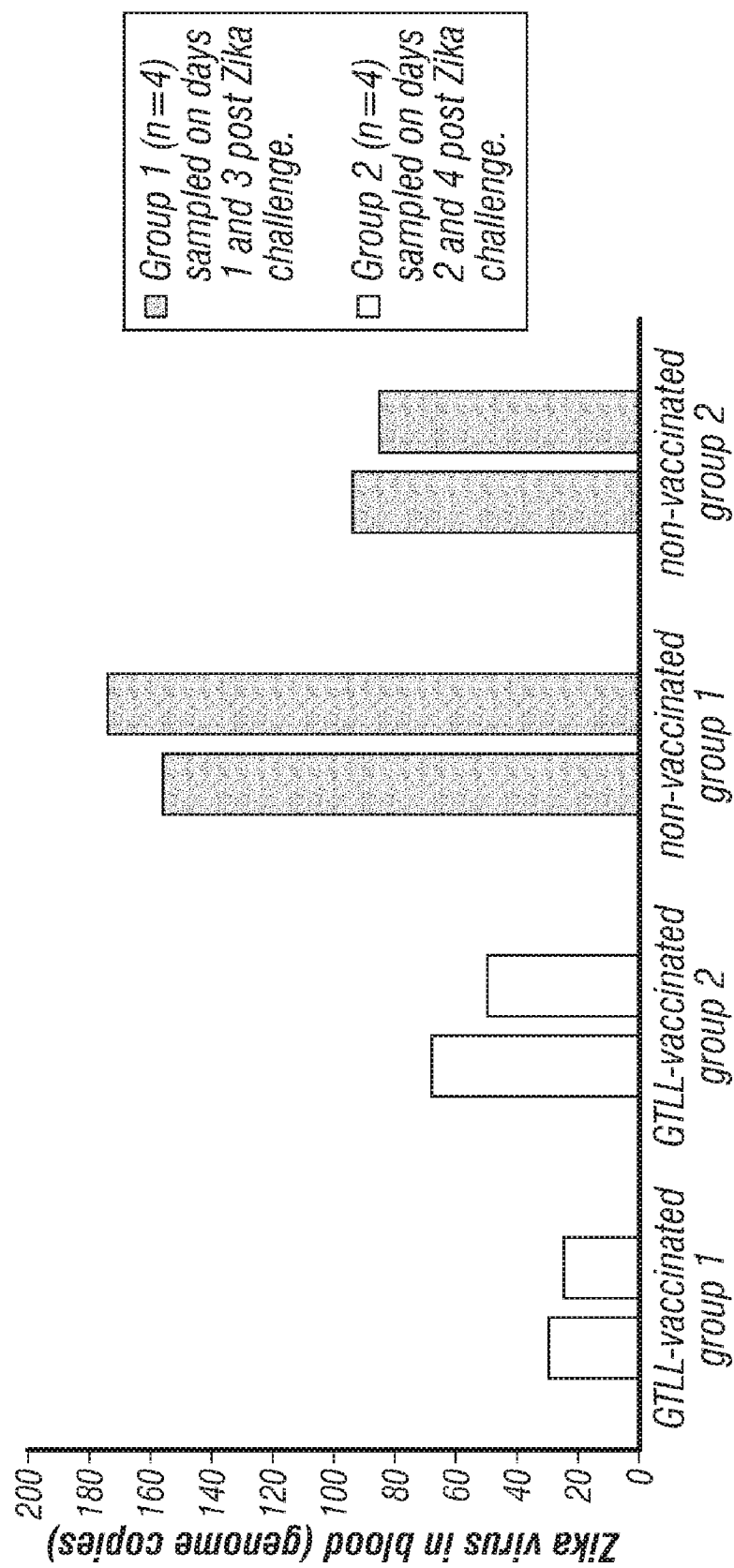


FIG. 2

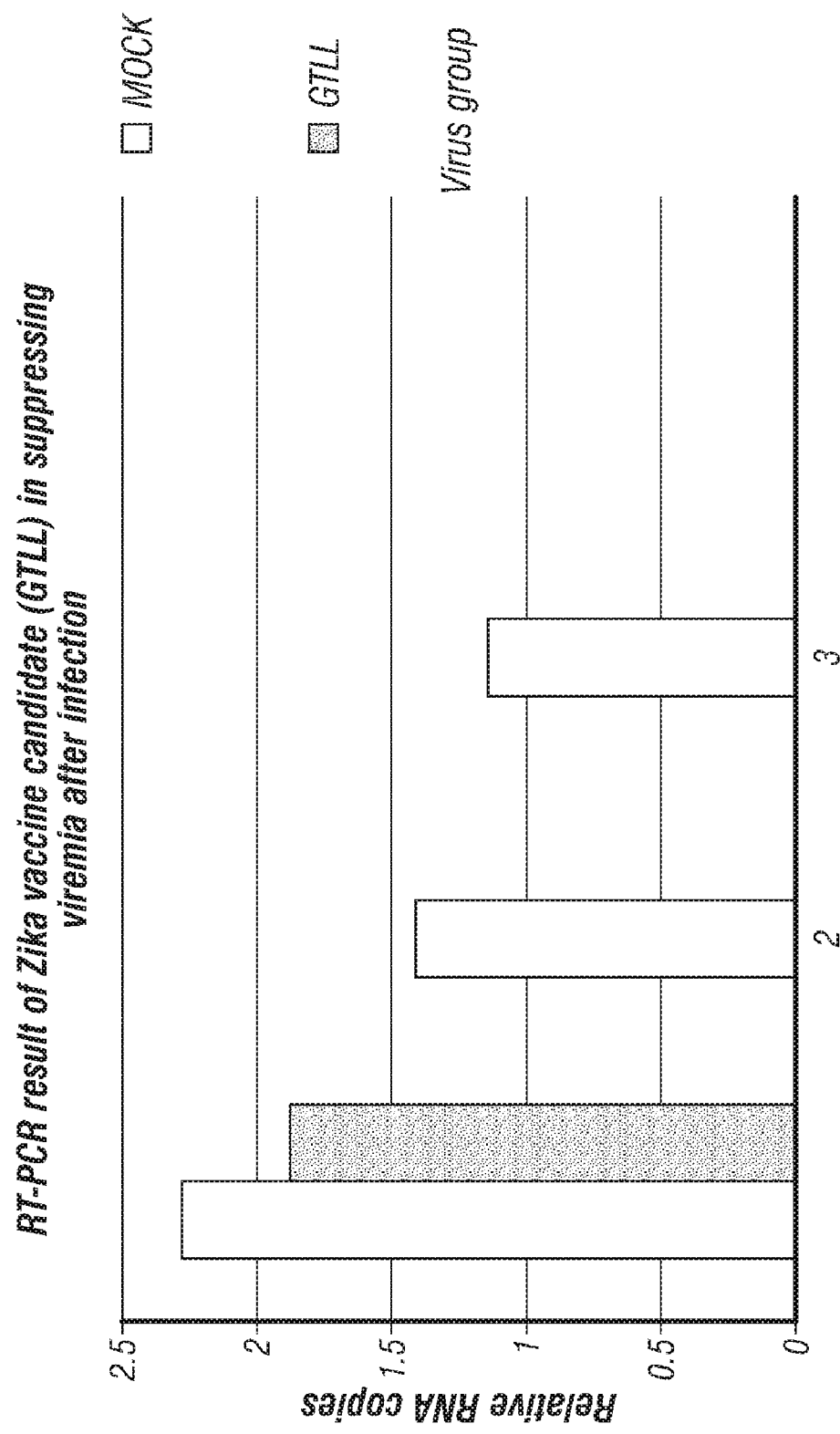


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/24483

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 14/18, 14/08; C12N 7/01; A61K 39/12; A61P 31/14 (2017.01)

CPC - A61K 39/12, 2039/5254; C07K 14/1825; C12N 2770/24111, 2770/24162

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2012/0003255 A1 (BROWN et al.) 5 January 2012 (05.01.2012). Especially para [0012], [0013], pg 2 table 1, SEQ ID NO: 14	1-5, 7, (12-18)/(1-5,7)
A	GenBank Accession No. AY632535.2. Zika virus strain MR 766, complete genome [online] 23 November 2010 [retrieved 5 June 2017] Available on the internet: <URL: https://www.ncbi.nlm.nih.gov/nuccore/AY632535 >. pg 1-2.	1-5, 7, (12-18)/(1-5,7)

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

8 June 2017

Date of mailing of the international search report

25 AUG 2017

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/24483

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

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

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

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

3. ☒ Claims Nos.: 19-31
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
-----Go to Extra Sheet for continuation-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-5, 7, (12-18)/(1-5,7) limited to SEQ ID NOs: 3, 4 and 5

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/24483

----continuation of Box III (Lack of Unity of Invention)-----

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-18, drawn to a recombinant Zika virus envelope protein (wild type, SEQ ID NO: 1) mutant. The Zika virus envelope protein mutant will be searched to the extent to encompass "E-TM-1" (.delta.GTLL) comprising SEQ ID NOs: 3, 4 and 5 [note: see instant application para [0026]]. It is believed that claims 1-5, 7, (12-18)/(1-5,7) read on this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NOs: 3, 4 and 5. Additional Zika virus envelope protein mutant(s) will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional elected Zika virus envelope protein mutant(s). Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be: a Zika virus envelope protein mutant encompasses "E-TM-2" (.delta.TLLV) comprising SEQ ID NOs: 6, 7, 8 (claims 1, 3, 4, 6, 8, (12-18)/(1,3,4,6,8)).

The inventions listed as Group I+ inventions do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Among the inventions listed as Groups I+ is the specific Zika virus envelope protein mutants, recited therein. Each of the inventions of Group I+ requires an unique Zika virus envelope protein mutant, not required by the other inventions.

Common Technical Feature:

Group I+ share the common technical feature of a recombinant Zika virus envelope protein (wild type, SEQ ID NO: 1) mutant, a polynucleotide encoding the same, and a host cell comprising the same.

However, said common technical features do not represent a contribution over the prior art, and are obvious over US 2012/0003255 A1 to BROWN et al. (hereinafter "Brown"), in view of GenBank Accession No. AY632535.2, "Zika virus strain MR 766, complete genome" (hereinafter "GenBank AY632535.2") [published 23 November 2010 and available on the internet: <URL: <https://www.ncbi.nlm.nih.gov/nuccore/AY632535> >].

As to claim 1, Brown teaches Zika virus envelope protein encoded by GenBank AY632535 and the envelope transmembrane domain is indicated (pg 2 Table 1; GenBank Accession AY632535 and transmembrane amino acid sequence 457-SWFSQILIGTLLVWLG-472) and making a mutation in the transmembrane domain (para [0012]; "the present invention, the mutation will preferably reside in a transmembrane protein of a flavivirus, for example, the envelope (E) protein of a flavivirus. The mutation may be in a transmembrane domain of the E protein"; para [0013]- "the mutation may comprise a deletion of at least two, three, four, five or more amino acids comprising the central amino acids").

As the common technical features were known in the art at the time of the invention, they cannot be considered a common special technical feature that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Group I+ inventions lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature

Note concerning item 4: Claims 19-31 are multiple dependent claims and are not drafted according to the second and third sentences of PCT Rule C.4(a).

Note concerning claims 5, 6 and 10. The deleted amino acid positions referred in claims 5, 6 and 10 are incorrect. For the purpose of International Search and Opinion, claims 5, 6 and 10 are construed as follow:

Claim 5 ".....wherein the polypeptide comprises a deletion of the amino acids corresponding to amino acid positions 755-758 of SEQ ID NO: 1.

Claim 6 ".....wherein the polypeptide comprises a deletion of the amino acids corresponding to amino acid positions 756-759 of SEQ ID NO: 1.

Claim 10 ".....wherein the polypeptide comprises a deletion of the amino acids corresponding to amino acid positions 755-759 of SEQ ID NO: 1.