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(54) Title: FLAVIVIRUS AND ALPHAVIRUS VIRUS-LIKE PARTICLES (VLPs)

Fig. 1

(57) Abstract: Described herein are flavivirus virus-like particles (VLPs) that display on their surfaces antigenic flavivirus proteins. Also described are methods of making and using these VLPs.
FLAVIVIRUS AND ALPHAVIRUS VIRUS-LIKE PARTICLES (VLPS)

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from U.S. Provisional Application No. 62/184,738, filed June 25, 2015 and U.S. Provisional Application No. 62/292,936, filed February 9, 2016, the disclosures of which are incorporated herein by reference.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0002] This work was supported in part by a Qualifying Therapeutic Discovery Project Grant from Health and Human Service (HHS) for the Development of a Multivalent Dengue Virus-Like Particle (VLP) Vaccine to TechnoVax, Inc. The U.S. Government may have certain rights in this invention.

TECHNICAL FIELD

[0003] The present invention relates to compositions comprising flavivirus (e.g., dengue or Zika) and/or alphavirus (e.g., chikungunya) virus-like particles (VLPs) and to methods of making and using these VLPs, including the creation and production of virus-like particle (VLP) based vaccines (e.g., for dengue, Zika, and/or chikungunya) as well as its use for diagnostic and therapeutic indications. In particular, the present disclosure includes strategies and methods used for the development of novel monovalent or multivalent vaccines that are able to protect humans against infection with one or more clades or antigenic variants of the flavivirus (dengue, Zika) and/or alphavirus viruses. Also described herein are VLP production methods that produce VLPs that display certain antigenic configurations. These VLPs feature conformational epitopes relevant for the generation of an enhanced neutralizing immune response to the virus. Single particle monovalent, bivalent, or multivalent (e.g., tetravalent, for example for the 4 dengue serotypes) VLPs are assembled and used to formulate vaccine compositions, which allows for immunization and subsequent protection against one or more clades or antigenically distinct virus (e.g. Asian clade, South America clade, etc. for Zika; 1, 2, 3, or 4 for dengue serotypes). Furthermore, VLPs are also used for the diagnosis of infection or for therapeutic indications. VLP vaccines can be produced in suspension culture of eukaryotic cells and released into the culture medium. After purification, concentration, and formulation the vaccine can be administered by any suitable route,
for example, via either mucosal or parenteral routes, and induce an immune response able to protect against any or all of the Zika, dengue, chikungunya virus clades, antigenic variants or serotypes. VLPS comprising combinations of Zika, dengue and/or chickungunya and methods of providing immune responses to additional viruses are also provided.

BACKGROUND

Flaviviruses such as dengue and Zika and alphaviruses such as chikungunya are the causative agents of infections in humans and birth defects when pregnant women are infected with Zika. Zika fever disease results from an infection with Zika virus (ZIKV), which is transmitted to human by the bite of an infected Aedes mosquito (A. aegypti, A. albopictus and polynesiensis). Zika virus was isolated for the first time in the Zika Forest in Uganda from a Rhesus monkey in 1947 and later from humans in 1952. ZIKV belongs to the flavivirus genus within the Flaviviridae family. Members of this family possess a single stranded positive sense RNA genome (~10,794 nucleotides long) that encodes only one open reading frame (ORF) translated into a single polyprotein which is cleaved by both cellular and virus-encoded proteases into three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5.) that enables virus replication. Zika virus protein processing and maturation appears to be similar to that of other members of the family and it occurs through the secretory pathway beginning with the self-cleavage of NS3 protease with its cofactor NS2B. Then, the NS2B/NS3 complex cleaves the cytoplasmic tail of the C protein and host cell signalases and proteases perform other cleavages within the polyprotein.

During replication and virus morphogenesis, which occurs in closed association with intracellular membranes, nascent virions are assembled and transported through the secretory pathway and released at the cell surface. Enveloped virions are composed of a cell-derived lipid bilayer encapsulating the C-protein wrapped viral RNA genome and studded with multiple copies of the proteins E and M. During maturation within the secretory pathway (trans-Golgi network) the precursor prM protein is cleaved by the host cell’s furin protease to produce the small M protein and the fragment pr, which is released upon virus egress from the cell. The surface of the virus displays E protein (dimers arranged in head to tail herringbone arrays) as the major antigenic determinant of the virus and mediates receptor binding
and fusion during virus entry into cells. Structural studies of an analogous protein of
the genus flavivirus reveals three domains, DI, DII and DIII followed by two helices
and two transmembrane domains, which anchor this protein to the surface of the
virion particle (Crill WD, Chang G-JJ, 2004, Localization and Characterization of
13986). Therefore, this protein is a major target of the host immune response and a
suitable candidate for vaccine development and diagnostic applications.

[0006] ZIKV has been transmitted in Africa for many years through a sylvatic
cycle between the mosquito vectors and nonhuman primates, with occasional human
infections. In recent years, however, epidemics of Zika have resulted from cycles of
transmission between vectors and humans spreading the disease beyond the African
continent into the French Polynesia and other Pacific regions. Since 2015 a dramatic
spread of ZIKV that started in Brazil is taking place in South America and the
Caribbean Islands and some sporadic cases of travelers have been identified in the
USA and Europe. Although Zika fever appears to cause a mild illness in 1 of 5
people infected, contracting the virus during pregnancy has been associated with birth
defects, primarily microcephaly (defective brain development). Furthermore, an
increase of cases of Guillain-Barre syndrome has been observed following ZIKV
infection. The seriousness of these disorders imposes a tremendous burden on public
health and human life. In addition to vectors transmission, ZIKV can also be
transmitted by sexual contact, making disease control more difficult.

[0007] It has been observed with other flaviviruses that in addition to mature
and immature particles (virions that carry uncleaved pr peptide) produced by
flavivirus-infected cells, small non-infectious particles composed of M and E are also
assembled and released. Furthermore, it was shown that recombinant expression of
proteins prM and E from tick-borne encephalitis (TBE) virus was sufficient to drive
assembly and budding of this type of particle. Other flavivirus sub-viral particles
assembled with prM and E proteins have also been produced in the yeast Pichia
pastoris expression system as well as in mammalian cells. Vaccine compositions
containing these sub-viral particles have been shown to induce neutralizing antibodies
and specific cytotoxic T lymphocyte responses in mice.

[0008] Dengue fever results from infection with dengue virus, which is
transmitted to humans by the bite of infected Aedes mosquitoes (A. aegypti, A.
*albopictus* and *A. polynesiensis*). This mosquito-borne illness is responsible for 100 million cases of dengue each year worldwide. The World Health Organization (WHO) estimates that two-thirds of the world human population is at risk of contracting dengue infection. Furthermore, the relentless spread of the mosquito vectors in recent years continues to expand the illness to new regions of the world. Four distinct virus serotypes (DENV1-4) can be transmitted by infected Aedes mosquitoes causing an infection characterized by fever, headache, myalgia, arthralgia and, depending on the severity of the infection, may progress to Dengue hemorrhagic fever/Dengue shock syndrome (DHF/DSS) (WHO (2014) Dengue and severe Dengue Fact Sheet N° 117. Available at: http://www.who.int/mediacentre/factsheets/fs117/en/ [Accessed September 4, 2014]). These life-threatening outcomes are more common following subsequent infections with a dengue virus of a different serotype. The presence of a low concentration of poorly neutralizing antibodies produced after the primary infection, appears to heighten infection of Fc-receptor bearing cells, increasing virus replication and clinical signs by the mechanism of antibody-dependent enhancement (ADE) of disease. This complex interaction between the host-immunity and dengue viruses has hindered the development of a safe and effective dengue vaccine, which has to elicit a robust and balanced neutralizing antibody response against each one of the four virus serotypes in order to avoid potential induction of ADE.

Dengue virus is an enveloped positive sense single-strand RNA virus, which belongs to the flaviviridae family within the Flavivirus genus. The dengue RNA genome (~10.7-Kb) encodes only one open reading frame (ORF) and translates into a single polyprotein cleaved by both cellular and virus-encoded proteases into three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5.) that enables virus replication (Lindenbach BD, Rice CM, 2003, Molecular biology of flaviviruses. Adv Virus Res 59:23–61). Protein processing and maturation occur through the secretory pathway and begins with the self-cleavage of NS3 protease with its cofactor NS2b. Then, the NS2B(NS3 complex cleaves the cytoplasmic tail of the C protein and host cell signalases and proteases perform other cleavages within the polyprotein.

During replication and virus morphogenesis, which occurs in closely association with intracellular membranes, nascent virions are assembled and transported through the secretory pathway and released at the cell surface. Enveloped
virions are composed of a cell-derived lipid bilayer encapsulating the C-protein wrapped viral RNA genome and studded with multiple copies of the proteins E and M. During maturation within the secretary pathway (trans-Golgi network) the precursor prM protein is cleaved by the host furin protease to produce the small M protein and the fragment pr, which is released upon virus egress from the cell. The surface of the virus displays E protein (dimers ordered in head to tail herringbone arrays) as the major antigenic determinant of the virus and mediates receptor binding and fusion during virus entry. This protein has three domains, DI, DII and DIII followed by two helices and two transmembrane domains, which anchor this protein to the surface of the virion particle (Crill WD, Chang G-JJ, 2004, Localization and Characterization of Flavivirus Envelope Glycoprotein Cross-Reactive Epitopes. J Virol 78(24):13975–13986). Therefore, this protein is a major target for vaccine development. It has been observed that in addition to mature and immature particles (virions that carry uncleaved pr peptide) produced by dengue-infected cells; small non-infectious particles composed of M and E are also assembled and released. Furthermore, it was shown that recombinant expression of proteins prM and E from tick-borne encephalitis (TBE) virus was sufficient to drive assembly and budding of this type of particle. Dengue sub-viral particles assembled with prM and E proteins have also been produced in the yeast Pichia pastoris expression system as well as in mammalian cells. Vaccine compositions containing these sub-viral particles have been shown to induce neutralizing antibodies and specific CTL responses in mice. 

[0011] At this time there is no vaccine or specific treatment to control, combat or prevent ZIKV infection. For their parts, dengue vaccines are being developed using conventional strategies such as chimeric, live-attenuated, and inactivated viruses or DNA and some of these vaccine are far advanced in their development. However, due to a prolong immunization regimen, as well as unbalanced and insufficient immunity against some serotypes raises concern as to whether these vaccines are safe and efficacious. Therefore, new technologies are needed to develop safer and more effective dengue vaccines. The prevention of infection by vaccination represents a critical unmet need to control the spread and the effects of these diseases globally. Here, we described the formation of virus-like particles as a strategy for flavivirus (Zika and dengue) and/or alphavirus (chickungunya) vaccine development and formulations for an specific virus as example dengue containing four serotypes or
Zika containing one or more antigenic variants as well as combination as dengue, Zika and chikungunya and alternative dual compositions e.g. dengue/Zika or dengue/Chikungunya or Zika/Chikungunya. Furthermore, the VLPs as described herein can be used for diagnostic as well as therapeutic applications.

SUMMARY

[0012] Described herein are virus-like particles (VLPs) comprising at least one antigenic flavivirus or alphavirus protein (Zika, dengue or chikungunya). Also described are compositions comprising these VLPs, as well as methods for making and using these VLPs. The VLPs described herein are devoid of viral genetic material and therefore unable to replicate or cause infection; however given their morphological, biochemical and antigenic similarities to wild type virions, VLPs are highly immunogenic and able to elicit robust protective immune responses. Unlike virion inactivated based vaccines, VLPs are not infectious eliminating the need for chemical treatment, thus maintaining the native conformation of structural components and antigenic epitopes.

[0013] Thus, the invention describes a novel approach for Zika, dengue and/or chikungunya virus-like particle (VLP) development. In particular, we describe the creation, development and production of VLP vaccines for Zika, dengue as well as chikungunya that will trigger, upon human immunization, a strong and balanced immune response characterized by the induction of high level of neutralizing antibodies. In certain embodiments, the VLP triggers a high level of neutralizing antibodies against the four-dengue virus serotypes and/or multiple Zika clades concurrently. In other embodiments, the VLP vaccine as a virus specific composition triggers a high level of neutralizing antibodies against either the four-Dengue serotypes, or against a single or multiple Zika clades. In another embodiment, a combination vaccine elicits a high level of neutralizing response against the four-dengue serotypes, Zika clades and chikungunya.

[0014] Based on flavivirus subviral particles studies as well as on our own experience in virus-like particle assembly, we have designed a new and more effective strategy for the formation and release of virus-like particles (e.g., dengue and/or Zika). We have found that the co-expression of flavivirus (dengue, Zika) virus structural proteins capsid (C), preMembrane (prM), envelope (E) together with the
non-structural protein NS2B/NS3 drives the assembly and release of virus-like particles. The presence of the complex NS2B/NS3 contributes not only to the processing of the polyprotein CprME by its protease functions but also to the particles assembly and release. We also produce VLPs displaying E protein with different reactivites as demonstrated with a monoclonal antibody that recognizes a conformation epitope of the E protein that is shared by other flaviviruses. These different E protein conformations seem to be highly relevant for the elicitation of potent neutralizing antibody in humans.

[0015] In one aspect, described herein is a flavivirus (e.g., dengue, Zika, yellow fever, Japanese encephalitis, tick-borne encephalitis, hepatitis C and/or West Nile virus) virus-like particle (VLP) comprising at least one flavivirus structural protein and at least one non-structural flavivirus protein. In certain embodiments, the VLP comprises all of the CPrME proteins. Any CPrME proteins can be employed, including wild-type or mutated (e.g., codon optimized) sequences from any flavivirus species and serotype (dengue 1, 2, 3, 4, Zika, etc.). In an exemplary embodiment, the wild-type nucleotide sequence of CprME of dengue-2 is shown in SEQ ID NO:1, and the amino acid sequence is described in SEQ ID NO:2. Other wild-type CprME sequences are known in the art and may be readily aligned with any of the exemplary Zika or dengue (e.g., dengue-2) sequences disclosed herein. In certain embodiments, the CprME sequence comprises a sequence with one or more mutations (substitutions, additions and/or deletions) as compared to wild-type and/or a codon optimized sequences. See, e.g., SEQ ID NO:3 (mutated) and SEQ ID NO:4 (codon optimized with mutations), which both include the amino acid sequences shown in SEQ ID NO:5. In other embodiments, the VLP consists of less than all of the CPrME proteins (e.g., CPrME, PrME, CME, CPrE or ME), as compared to the full length wild-type or mutated sequences. Similar mutations can be made in any flavivirus CprME protein following the teachings described herein. In any of the assembly of VLPs as described herein the non-structural proteins may comprise NS2B and/or NS3 proteins derived from any flavivirus species or serotype (e.g. an example of wild type nucleotide sequence of NS2B/NS3 is shown in SEQ ID NO:6 and amino acid sequence described in SEQ ID NO:7) or modified (e.g., truncated, mutated and/or codon optimized) proteins (e.g. example of modified NS2B/NS3 nucleotide sequence is shown in SEQ ID NO:8 and amino acid sequence shown in SEQ ID NO:9). NS2B
or NS3 may also be used as single proteins the nucleotide sequences (e.g., fragments derived from any flavivirus NS2B/NS3 protein. Exemplary, non-limiting sequences are shown in SEQ ID NO: 10 (NS2B nucleotide sequence), SEQ ID NO:11 (NS2B amino acid sequence), SEQ ID NO:12 (NS3 nucleotide sequence) and SEQ ID NO: 13 (NS3 amino acid). The VLP may be monovalent, bivalent or multiple valent and display on its surface one or more antigenic flavivirus proteins (1, 2, 3, 4 or more proteins): from a single flavivirus, from one or more serotypes (or clades or isolated) of a single flavivirus (e.g., a bivalent or multivalent; from multiple flaviviruses (e.g., dengue and/or Zika and/or alphaviruses), as well as combinations thereof.

[0016] Also provided is an immunogenic composition comprising at least one VLP as described herein. In certain embodiments, the immunogenic compositions further comprise an adjuvant. Thus, described herein are flavivirus (e.g., dengue, Zika) virus-like particles (VLP)-also known as subviral particles, recombinant subviral particles, biological nanoparticles, nanoparticles, etc.-utilizing structural (CPM-E) and non-structural (NS2B/NS3) viral proteins. These VLPs are designed as vaccine or immunogens for protecting against infection with any one of the four-dengue virus serotypes and/or any of the known Zika clades/isolates. In certain embodiments, the VLP also comprises alphavirus antigenic proteins (e.g., chickungunya).

[0017] Also provided are DNA constructs comprising sequences encoding flavivirus viral proteins (structural and non-structural) used to assemble the VLP of any of claims 1 to 8. The constructs may further comprise one or more sequences encoding one or more linkers between one or more of the sequences encoding the structural and non-structural proteins (e.g., a linker comprising amino acids corresponding to amino acids 1 to 8 or 9 or 10 of any flavivirus NS1 protein, numbered relative to any flavivirus NS1 protein, for example as shown in SEQ ID NO:15 (amino acid) (SEQ ID NO:14 shows the nucleotide sequence encoding these residues); a linker comprising amino acids corresponding to 186 or 187 or 188 or 189 to amino acids corresponding to 218 or 225 of any flavivirus NS2A protein, numbered relative to any wild-type protein (e.g. as shown in SEQ ID NO:17); a linker comprising amino acid 1 to 8 or 9 or 10 of NS1, amino acids 1 to 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 of NS2A, amino acids 186 or 187 or 188 or 189 to 218 or 225 of NS2A; a linker comprising amino acids 1 to 8 or 9 or 10 of NS1 and the
second transmembrane domain of NS2B (e.g. nucleotide sequence amino acid sequence SEQ ID NO:11); a linker comprising amino acid 1 to 8 or 9 or 10 of NS1 and the first transmembrane domain of NS2A (e.g., amino acids encoded by nucleotides 51 to 100 of nucleotide sequence, SEQ ID NO: 16 and amino acid sequence SEQ ID NO: 17); and a linker comprising amino acid 1 to 8 or 9 or 10 of NS1 and the C terminal portion of NS2B comprising the second transmembrane domain to the end of the protein. The DNA constructs may comprise the flavivirus protein-encoding sequences in any order (e.g., a full length NS2B (e.g. SEQ ID NO:10); and a full NS3 (e.g. SEQ ID NO: 18) or modified truncated NS2B/NS3 (SEQ ID NO: 8) or wild-type NS2B/NS3 (e.g. SEQ ID NO: 6) operably linked directly to the structural proteins CprME (e.g. SEQ ID NO: 4) in any order). In certain embodiments, the furin protease cleavage site between pr and M protein of the constructs describes herein (e.g., SEQ ID NO:5) is modified by substituting amino acids residues at position P3 with hydrophobic one and/or wherein the NS3 protease active site is modified (e.g. as shown in NS3 alone SEQ ID NO: 13 and NS2B/NS3 SEQ ID NO: 9, which correspond to nucleotide sequences SEQ ID NO: 12 and SEQ ID NO: 8) in such that its enzymatic activity is enhanced. In other embodiments, sequence(s) encoding the E protein (e.g. SEQ ID NO: 47) is (are) modified to enhance VLP assemble and release (e.g., amphipathic helix 1 in the stem domain of the E protein is modified to enhance the hydrophobic properties of one side of the helix; one, two, three or more amino acids in the hydrophobic side of the helix are substituted, for example, at positions corresponding to 398, 401 and / or 412 (e.g., numbered relative to SEQ ID NO:47), including but not limited to I398L, I398M, I398V, I398A or M401A, M401L, M401V, M401I, or M412A, M412L, M412V, M412I, and/or helix 1 and/or helix 2 of the E protein (e.g. as example I398L, M401A, and M412L of SEQ ID NO: 19) are exchanged with the helix sequences of other flaviviruses or analogous motif from other viruses and cellular sources).

[0018] In another aspect, methods of generating (assembling) the VLPs described herein are provided. In certain embodiments, such methods and strategies involve mutations, deletions, insertions, gene organization and/or expression conditions to enhance particle morphogenesis and egress from producing cells. Also delineated are strategies for the assembly of VLPs displaying on its surface the E protein of a single serotype (monovalent), or the E protein of two distinct serotypes
(bivalent), or the E protein of three distinct serotypes (trivalent) or the E protein of each one of the four-dengue virus serotypes (tetravalent) or again multiple Zika clades. In certain embodiments, combining VLPs with alternative antigenic composition allows for the formulation of a tetravalent vaccine. Furthermore, production of VLP can be attained in suspension cultures of transfected eukaryotic cells following the expression of the selected structural and non-structural genes. Transient or stable transfection methods can be used to introduce into cells the plasmids that direct proteins expression. VLPs are released from the producing cells into the culture medium from where they are collected and purified by different methods such as gradient centrifugation, filtration and chromatography or combination thereof. Thus, also provided is a method of producing a VLP, the method comprising introducing into a host cell (e.g., a eukaryotic cells such as a mammalian, yeast, insect, plant, amphibian and avian cells) one or more DNA constructs as described herein under conditions such that the cells produces the VLP.

In certain embodiments, host cell(s) are cultured at temperatures ranging from 25°C to 33°C (e.g., 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C or 33°C). Also provided are VLPs generated by the methods as described herein as well as a method of generating an immune response to one or more flaviviruses in a subject (e.g., human), the method comprising administering (e.g., mucosally, intradermally, subcutaneously, intramuscularly, or orally) to the subject an effective amount of the VLPs and/or immunogenic compositions as described herein. The methods described herein can result in an immune response that treats and/or prevents (vaccinates) the subject against multiple serotypes or clades of one or more flaviviruses.

[0019] In yet another aspect, described herein is the formation of VLPs containing E proteins of different structural conformations resulting from the production at different temperatures (e.g., 37°C or 31°C). These VLPs show differential reactivity with a specific monoclonal antibody that recognizes the E protein, reflecting their conformational differences. In addition, VLPs produced at 31°C elicited stronger titers of neutralizing antibodies than those induced by VLP produced at 37°C when administered as vaccine to small animal models. A tetravalent VLP based vaccine can be formulated with single tetravalent VLPs (one particle carrying E antigens of all serotypes/clades) or by blending VLP of alternative antigenic compositions. The utility of the VLPs as described herein may include, but
it is not limited to, vaccine and immunological use, as adjuvant, and/or immune-modulators, delivery vehicle for heterologous proteins or small molecules and RNA molecules as well as prophylactic and therapeutic applications.

[0020] In another aspect of the invention described provides dengue and/or Zika virus-like particles (VLP) (also known as subviral particles), recombinant subviral particles, biological nanoparticles, nanoparticles, etc.-utilizing structural prM-E or C-prM-E and non-structural (NS2B/NS3) viral proteins. These VLPs are designed as vaccine or immunogens for protecting against infection with any one of the dengue and/or Zika virus clades, antigenic variants or serotypes. Furthermore, the flavivirus (e.g., dengue/Zika) vaccine could be combined with one or all of other VLP based vaccine such as dengue, yellow fever, West Nile, or chikungunya, which are viral diseases transmitted by mosquito vectors.

[0021] In addition, methods for generating (assembling) the VLPs (e.g., dengue and/or Zika) described herein are provided. In certain embodiments, such methods and strategies involve mutations, deletions, insertions, gene organization arrangements and the conditions under which the VLPs are produced recombinantly to better retain particle resemblance to the virus and enhance egress from the producing cells. Also delineated are strategies for the assembly of VLPs displaying on their surface the E protein of a single clade/antigenic variant or the E protein of two distinct clades/antigenic variants (bivalent), or the E protein of distinct clades/antigenic variants (multivalent). In certain embodiments, combining VLPs with alternative antigenic composition allows for the formulation of a multivalent vaccine. Furthermore, production of VLPs can be attained in suspension cultures of eukaryotic cells following the expression of the selected structural proteins prME or CprME alone or structural and non-structural proteins NS2B/NS3 combined. Transient or stable transfection methods can be used to introduce into cells the plasmids that direct proteins expression. VLPs are released from the producing cells into the culture medium from where they are collected and purified by different methods such as gradient centrifugation, filtration and chromatography or combination thereof.

[0022] In yet another aspect, described herein is the formation of VLPs containing E proteins of different structural conformation resulting from the production of the VLPs at different temperatures, one set ranging from 27°C to 33°C (e.g. 31°C) and a second set ranging from 34°C to 41°C (e.g. 37°C). These VLPs
show differential reactivity with a specific monoclonal antibody that recognizes shared epitopes of E protein amongst other flaviviruses and reflects their conformational differences when produced at distinct temperatures. In addition, VLPs produced at 31°C elicits stronger titers of neutralizing antibodies than those induced by VLP produced at 37°C when administered as vaccine to small animals. Single or multivalent antigenic VLP based vaccine (Zika, dengue and/or chickunguna) can be formulated (one particle carrying E antigens of several clades or by blending VLP of alternative antigenic compositions). The utility of the these VLPs may include, but it is not limited to, vaccine and immunological use, as adjuvant, and / or immune-modulators, delivery vehicle for heterologous proteins or small molecules as well as prophylactic, therapeutic and diagnostic applications.

[0023] Also provided are VLPs produced by any of the methods described herein.

[0024] In a still further aspect, provided herein is a method of generating an immune response to a flavivirus and/or alphavirus in a subject, the method comprising administering to the subject (e.g., human) an effective amount of a VLP and/or immunogenic composition as described herein to the subject. In certain embodiments, the composition is administered mucosally, intradermally, subcutaneously, intramuscularly, or orally. In certain embodiments, the methods generate an immune response to multiple strains or subtypes of flaviviruses, thereby providing a “universal” vaccine that protects the subject against infection from various flaviviruses and/or over time (more than one season).

[0025] Any of the methods may involve multiple administrations (e.g., a multiple dose schedule).

[0026] In another aspect, a packaging cell line is provided for producing VLPs as described herein. The cell line may be stably transfected with one or more polynucleotides encoding structural proteins and upon introduction and expression of the one or more flavivirus protein-encoding sequences not stably transfected into the cell, the VLP is produced by the cell. The packaging cell may be an insect, plant, mammalian, bacterial or fungal cell. In certain embodiments, the packaging cell is a mammalian (e.g., human) cell line.

[0027] Thus, the invention includes but is not limited to the following embodiments:
1. A flavivirus virus-like particle (VLP) comprising the proteins CPrME that are assembled following the co-expression of structural and non-structural proteins, wherein said flavivirus is dengue and/or Zika.

2. A flavivirus virus-like particle (VLP) comprising of the structural proteins CPrME that are assembled following the expression of the same structural proteins, wherein said flavivirus is dengue and/or Zika.

3. A flavivirus virus-like particle comprising less than the structural protein CPrME such as PrME or CME or CPrE or ME that are assembled following their expression or co-expressed with the non-structural proteins, wherein said flavivirus is dengue and/or Zika.

4. A virus-like particle of 1, 2 and/or 3, wherein the structural proteins are produced from separate transcription units.

5. A virus-like particle (VLP) of 1, 2, 3 and/or 4 where the non-structural proteins comprise the full length or truncated form of NS3 co-expressed with the full length or truncated forms of NS2B.

6. A DNA construct comprising sequences encoding dengue and/or Zika viral proteins used to assemble VLPS, wherein the structural and non-structural viral proteins are operably linked to form a single segment with a defined order optionally comprising a linker such as a sequence of different portions of the NS1, NS2A and/or NS2B proteins.

7. The DNA construct of 6, wherein the linker comprises amino acids corresponding to amino acids 1 to 8 or 9 or 10 of NS1 connected to a portion of NS2A comprising of amino acids corresponding to 186 or 187 or 188 or 189 to amino acids corresponding to 218 of NS2A.

8. The DNA construct of 6, wherein the linker comprises amino acid 1 to 8 or 9 or 10 of NS1 are connected to a first portion of NS2A comprising amino acid 1 to 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 connected to a second portion of NS2A comprising of amino acid 186 or 187 or 188 or 189 to 218 of NS2A.

9. The DNA construct of 6, wherein the linker comprises amino acid 1 to 8 or 9 or 10 of NS1 connected to the second transmembrane domain of NS2B.

10. The DNA construct of 6, wherein the linker comprises amino acid 1 to 8 or 9 or 10 of NS1 connected to the first transmembrane domain of NS2A.
11. The DNA construct of 6, wherein the linker comprises amino acid 1 to 8 or 9 or 10 of NS1 connected to the C terminal portion of NS2B comprising the second transmembrane domain to the end of the protein.

12. The DNA construct of any of 6 through 11, wherein the order of the structural and non-structural segments is inverted and the non-structural segment is operably linked to the structural segment with or without a connecting linker.

13. The DNA construct of 12, wherein the non-structural protein optionally includes a full length NS2B and a full NS3 genetically linked directly to the structural proteins CprME.

14. The DNA construct of 12 and 13, wherein the non-structural proteins comprise a truncated variant of NS3 in which the helicase domain is deleted but the protease domain and its recognizable self-cleavage site located at the carboxyl terminal of the protein is preserved. The self-cleavage site serves as one example of a linker that may be used to connect the non-structural and structural proteins.

15. A DNA construct comprising sequences encoding dengue and/or Zika viral proteins are used to assemble VLPs wherein the furin protease cleavage site between pr and M protein is modified by substituting amino acids residues at position P3 with hydrophobic amino acids.

16. A DNA construct comprising sequences encoding dengue and/or Zika viral proteins are used to assemble VLPs wherein the helices of the E protein are modified to enhance VLP assemble and release.

17. The DNA construct of 16, where the amphipathic helix 1 in the stem domain of the E protein is modified to enhance the hydrophobic properties of one side of the helix.

18. The DNA construct of 16 and/or 17 where one, two, three or more amino acids in the hydrophobic side of the helix are substituted, for example at positions corresponding to 398, 401 and / or 412, including but not limited to I398L, I398M, I398V, I398A or M401A, M401L, M401V, M401I, or M412A, M412L, M412V, M412I.

19. A DNA construct of 16, wherein the helix 1 and/or helix 2 are exchanged with the helix sequences of other flaviviruses or analogous motif from other viruses and cellular sources.
20. A DNA construct comprising sequences encoding flavivirus (Zika, dengue, yellow fever, Japanese encephalitis, West Nile virus etc.) viral proteins used to assemble VLPs wherein the NS3 protease active site is modified in order to enhance its enzymatic activity. Such modification may include but are not limited to the substitution of the amino acid corresponding to leucine at position 115 to a preferred amino acid with a shorter side chain such as alanine.

21. A method of increasing the amount of mature particles produced by a cell, the method comprising enhancing cleavage between pr and M by the furin protease wherein said protease is furnished to the culture media of VLP producing cells, or co expressed with the VLP producing genes or produced constitutively in stably transfected cells used for VLP production.

22. A method of producing VLPs comprising selected gene products (e.g., dengue and/or Zika proteins), the method comprising transiently transfecting a eukaryotic cell with one or more plasmids comprising sequences encoding the selected gene products such that the VLPs are produced by the eukaryotic cell.

23. A method of producing VLPs comprising selected gene products, the method comprising stably integrating with one or more sequences encoding the selected gene products into the genome of a eukaryotic cell such that the VLPs are produced by the eukaryotic cell.

24. The method of 22 and 23, wherein said eukaryotic cell is selected from the group consisting of mammalian, yeast, insect, plant, amphibian and avian cells.

25. A method of producing VLPs with selected gene products and distinct structural conformation of the E, the method comprising transiently or stably transfecting a eukaryotic cell one or more sequences encoding the selected gene products and culturing cells at temperatures ranging from 25°C to 33°C, in which the optimal temperature is 31°C such that the VLPs are produced by the eukaryotic cell.

26. The method of 25, wherein said VLP elicits higher neutralizing antibodies titers in humans or animals and are more protective against one or all dengue virus serotypes than those induced by VLPs produced at higher temperature (e.g. 37°C) when administered to said humans or animals.

27. The method of any of 22 to 26, wherein the VLP is a single bivalent VLP that displays on its surface the E antigen of two dengue virus serotypes (e.g. 1 and 2 or 1 and 3 or 1 and 4 or combination thereof) or a single tetravalent VLP wherein said
VLP displays on its surface the E antigen of all four-dengue virus serotype (e.g. 1, 2, 3 and 4) or a single bivalent VLP that displays on its surface the E antigen of two Zika virus clades or a single multivalent VLP wherein said VLP displays on its surface the E antigen of multiple antigenic variants / clades of the Zika virus.

28. A VLP generated by the method of any of 22 to 27.

29. A single bivalent VLP that displays on its surface the E antigen of two dengue virus serotypes (e.g. 1 and 2 or 1 and 3 or 1 and 4 or combination thereof).

30. A single tetravalent VLP wherein said VLP displays on its surface the E antigen of all four-dengue virus serotype (e.g. 1, 2, 3 and 4).

31. A monovalent vaccine comprising at least one of the four-dengue serotypes monovalent.

32. A tetravalent vaccine composed of four monovalent VLPs or two bivalent VLPs wherein said induces strong and balance immune response to all dengue virus serotypes.

33. A single bivalent VLP that displays on its surface the E antigen of two or more flavivirus (Zika, dengue and/or yellow fever, Japanese encephalitis, West Nile virus etc.) virus clades/ antigenic variants or combinations thereof.

34. A single multivalent VLP wherein said VLP displays on its surface the E antigen of multiple flavivirus (Zika, dengue and/or yellow fever, Japanese encephalitis, West Nile virus etc.) virus clades / antigenic variants or serotypes.

35. A monovalent VLP vaccine comprising at least one of the clades of flavivirus virus (monovalent).

36. A multivalent vaccine composed of various monovalent VLPs or two bivalent VLPs wherein said induces strong and balance immune response to (i) one or all virus clades / antigenic variants or serotypes of a flavivirus (e.g., Zika) and/or (ii) one or more virus clades / antigenic variants or serotypes of at least one other flavivirus (e.g., dengue and/or yellow fever, Japanese encephalitis, West Nile virus) or Alphavirus chikungunya.

37. The VLP or vaccines of any of 31 to 35, wherein the vaccine also comprises adjuvant.

38. Flavivirus vaccine compositions where the Zika vaccine is blended in bivalent, trivalent, tetravalent or pentavalent formulations and combination thereof.
with VLPs derived from dengue virus, yellow fever virus, West Nile virus, Japanese encephalitis virus or Alphavirus chikungunya.

39. The VLP or vaccines of any of 29 to 38, wherein the vaccine comprises an adjuvant. In another aspect, described herein is a host cell comprising any of the VLPs as described above. In certain embodiments, the host cell permits assembly and release of a VLP as described herein from one or more vectors encoding the polypeptides of the VLP. In certain embodiments, the eukaryotic cell is selected from the group consisting of a yeast cell, an insect cell, an amphibian cell, an avian cell, a plant cell or a mammalian cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] Figure 1 is a schematic depicting the structural and non-structural genes selected for virus-like particle assembly and their different configurations.

[0029] Figures 2A through 2C are schematics depicting exemplary arrangements for constructs comprising the genes encoding the structural and non-structural protein utilized for the assembly of virus-like particles.

[0030] Figures 3A through 3E show exemplary antigenic composition of single VLPs and options for formulating multivalent (e.g., tetravalent) vaccines.

[0031] Figures 4A and 4B are schematics depicting dengue virus genome and VLP assembly strategy. Figure 4A is a schematic showing the dengue virus genome, which contains a single open reading frame (ORF) that expresses a polyprotein comprised of both structural and non-structural proteins, which arise via several proteolytic cleavages (top panel). In early stages, the complex viral protease NS3 with its cofactor NS2B (\(\perp\)) self-cleaves before cleaving the capsid protein. A host cell signalase is also involved in the maturation of the polyprotein (\(\circ\)). In a final step, the furin protease (\(\nabla\)) cleaves the pr portion from the M protein to uncover E protein fusion peptide. Figure 4B depicts the VLP assembly strategy relies on the native properties of the structural genes although key point mutations were introduced to improve protein processing and particle assembly. The furin cleavage site (\(\nabla\)) was mutated at E88A to enhance cleavage at this location. The E protein was mutated as followed I398A, M401A, and M412L to improve the amphipathic properties of the helical domain 1 and enhance trafficking and secretion of E. The viral NS3 protein
was truncated maintaining only its N-terminal protease domain that was mutated at L115A to enhance its catalytic activity. The protease domain is kept as a single transcription unit together with its cofactor NS2B.

**Figures 5A through 5D** show dengue VLPs were produced in Exp293™ cells by co-expression of CprME-NS2B/NS3 or CprME alone. Analysis of transfected cells lysates show that capsid protein cleavage was efficient when CprME protease was co-expressed with CprME. The distinct cell lysates (20μg of total protein per lane) were tested by Western blot using specific antibodies: anti-E (shown in Figure 5A), anti-pr (shown in Figure 5B), anti-C (shown in Figure 5C) and anti-NS2B (shown in Figure 5D) antibodies.

**Figure 6** shows dengue VLP secretion is enhanced by co-expression of CprME and the viral protease complex NS2B/NS3. Exp293™ cells were transfected with CprME alone or CprME together with NS2B/NS3. Cell supernatant from mock transfected or CprME or CprME-NS2B/NS3 transfected cells were purified by ultracentrifugation. Purified VLPs were analyzed via Western blot using 20μg of total protein per lane. Purified DENV-2 virus was used as control (20μg). The Western blot membranes were probed with (top panel) an anti-E antibody and (bottom panel) an anti-prM antibody.

**Figures 7A through 7C** show analysis of the effect of temperature on the reactivity of the envelope (E) protein display on the surface of the dengue VLP with monoclonal antibodies recognizing conformational epitopes. Samples of gradient purified VLP fractions were applied to nitrocellulose membranes and probed by dot blot with the following antibodies: Figure 7A shows anti-E polyclonal antibody; Figure 7B shows 4G2 MAb; and Figure 7C shows 3H5 MAb. VLPs produced at a lower temperature (31°C) demonstrate better protein folding of conformational epitopes as shown by reactivity with MAbs 4G2 and 3H3, which also react with DENV-2 virus control.

**Figures 8A and 8B** show electron microscopy study of gradient purified DENV-2 VLP (bar represents 100nm). Figure 8A shows negative staining with 2% Uranyl acetate. Arrows point to the dengue VLPs. Figure 8B shows two particles observed after Immuno-gold staining with 3H5 monoclonal antibody.

**Figure 9** is a graph showing results of ELISA assays at the indicated conditions. Analysis of anti-DENV specific antibodies (total IgG) in mouse serum (n=
4) following immunization with dengue VLP vaccine (TVX-31°C and TVX37°C) and inactivated dengue-2 virus control (DENV-2) via chemiluminescent ELISA. Pre-immune sera (Pre I.) show statistical difference (*) (P < 0.05) with the immunized groups.

[0037] Figure 10 is a table showing neutralization power of vaccinated mice sera is presented as the reciprocal of serum dilution for which 50% of the virus is neutralized (PRNT50). PRNT50 is calculated using PROBIT method (Finney, 1952).

[0038] Figures 11A through 11F shows electron microscopy studies of Zika VLPs. Zika virus-like particles (zVLPs) were purified by ultracentrifugation through a potassium tartrate (10%-35%) /glycerol (7%-28%) linear gradient and examined by electron microscopy. Figures 11A, 11B and 11C show images of negatively stained zVLPs, which have round shape of ~ 60nm diameter. Figures 11D, 11E and 11F show images of immuno-gold labeled zVLP using an anti-E specific MAb as primary and a gold labeled (10nm beads) secondary Ab. Black dots (beads) demonstrates the presence of the E protein on the surface of the particles.

[0039] Figure 12 shows dot blot evaluation of gradient purification profile of Zika virus-like particles (zVLPs) and Zika virus. Aliquots (3 ul) from each fraction of gradient purified VLPs and ZIKV virus were tested by dot blot with an anti-E specific MAb. VLPs were detected in fractions 15 to 20 whereas the ZIKV virus was detected in fractions 13 to 15.

[0040] Figure 13 shows Western blot examination of gradient purified VLPs and Zika virus. The Western blot membrane was probed with an anti-E specific antibody and each lane correspond to the following: (1) Dengue control, (2) mock, (3) Zika virus (ZIKV) fractions 6-9, (4) Zika VLPs fractions 10-12, and (5) Zika VLPs fractions 7-8. The anti-E antibody detected the E protein of VLPs (lanes 4 and 5) and ZIKV (lane 3) as well as dengue control (lane 1).

DETAILED DESCRIPTION

[0041] The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Remington's Pharmaceutical Sciences, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); Methods In Enzymology (S. Colowick and N. Kaplan, eds., Academic Press, Inc.); and Handbook

All publications, patents and patent applications cited herein are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise. Thus, for example, reference to "a VLP" includes a mixture of two or more such VLPs.

Definitions

As used herein, the terms “sub-viral particle” "virus-like particle", "recombinant subviral particles” or "VLP" refer to a nonreplicating, viral shell. VLPs are generally composed of one or more viral proteins, such as, but not limited to those proteins referred to as capsid, coat, shell, surface and/or envelope proteins, or particle-forming polypeptides derived from these proteins. VLPs can form spontaneously upon recombinant expression of the protein in an appropriate expression system. Methods for producing particular VLPs are known in the art and discussed more fully below. The presence of VLPs following recombinant expression of viral proteins can be detected using conventional techniques known in the art, such as by electron microscopy, biophysical and immunological characterizations, and the like. See, e.g., Baker et al., Biophys. J. (1991) 60:1445-1456; Hagensee et al., J. Virol. (1994) 68:4503-4505. For example, VLPs can be isolated by density gradient centrifugation and/or identified by characteristic density banding. Alternatively, cryoelectron microscopy can be performed on vitrified aqueous samples of the VLP preparation in question, and images recorded under appropriate exposure conditions. Additional methods of VLP purification include but are not limited to chromatographic
techniques such as affinity, ion exchange, size exclusion, and reverse phase procedures.

[0045] By "particle-forming polypeptide" derived from a particular viral protein is meant a full-length or near full-length viral protein, as well as a fragment thereof, or a viral protein with internal deletions, which has the ability to form VLPs under conditions that favor VLP formation. Accordingly, the polypeptide may comprise the full-length sequence, fragments, truncated and partial sequences, as well as analogs and precursor forms of the reference molecule. The term therefore intends deletions, additions and substitutions to the sequence, so long as the polypeptide retains the ability to form a VLP. Thus, the term includes natural variations of the specified polypeptide since variations in coat proteins often occur between viral isolates. The term also includes deletions, additions and substitutions that do not naturally occur in the reference protein, so long as the protein retains the ability to form a VLP. Preferred substitutions are those which are conservative in nature, i.e., those substitutions that take place within a family of amino acids that are related in their side chains. Specifically, amino acids are generally divided into four families: (1) acidic—aspartate and glutamate; (2) basic—lysine, arginine, histidine; (3) non-polar—alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar—glycine, asparagine, glutamine, cysteine, serine threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids.

[0046] An "antigen" refers to a molecule containing one or more epitopes (either linear, conformational or both) that will stimulate a host's immune-system to make a humoral and/or cellular antigen-specific response. The term is used interchangeably with the term "immunogen." Normally, a B-cell epitope will include at least about 5 amino acids but can be as small as 3–4 amino acids. A T-cell epitope, such as a CTL epitope, will include at least about 7–9 amino acids, and a helper T-cell epitope at least about 12-20 amino acids. Normally, an epitope will include between about 7 and 15 amino acids, such as, 9, 10, 12 or 15 amino acids. The term includes polypeptides which include modifications, such as deletions, additions and substitutions (generally conservative in nature) as compared to a native sequence, so long as the protein maintains the ability to elicit an immunological response, as defined herein. These modifications may be deliberate, as through site-directed
mutagenesis, or may be accidental, such as through mutations of hosts which produce the antigens.

[0047] An "immunological response" to an antigen or composition is the development in a subject of a humoral and/or a cellular immune response to an antigen present in the composition of interest. For purposes of the present disclosure, a "humoral immune response" refers to an immune response mediated by antibody molecules, while a "cellular immune response" is one mediated by T-lymphocytes and/or other white blood cells. One important aspect of cellular immunity involves an antigen-specific response by cytolytic T-cells ("CTL"s). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A "cellular immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells. Hence, an immunological response may include one or more of the following effects: the production of antibodies by B-cells; and/or the activation of suppressor T-cells and/or γΔ T-cells directed specifically to an antigen or antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection to an immunized host. Such responses can be determined using standard immunoassays and neutralization assays, well known in the art.

[0048] An "immunogenic composition" is a composition that comprises an antigenic molecule where administration of the composition to a subject results in the development in the subject of a humoral and/or a cellular immune response to the antigenic molecule of interest.

[0049] "Substantially purified" general refers to isolation of a substance (compound, polynucleotide, protein, polypeptide, polypeptide composition) such that the substance comprises the majority percent of the sample in which it resides.
Typically in a sample a substantially purified component comprises 50%, preferably 80%-85%, more preferably 90-95% of the sample. Techniques for purifying polynucleotides and polypeptides of interest are well-known in the art and include, for example, ion-exchange chromatography, affinity chromatography and sedimentation according to density.

[0050] A "coding sequence" or a sequence which "encodes" a selected polypeptide, is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide in vivo when placed under the control of appropriate regulatory sequences (or "control elements"). The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from viral, prokaryotic or eukaryotic mRNA, genomic DNA sequences from viral or prokaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence may be located 3' to the coding sequence.

[0051] Typical "control elements", include, but are not limited to, transcription promoters, transcription enhancer elements, transcription termination signals, polyadenylation sequences (located 3' to the translation stop codon), sequences for optimization of initiation of translation (located 5' to the coding sequence), and translation termination sequences, and/or sequence elements controlling an open chromatin structure see e.g., McCaughan et al. (1995) PNAS USA 92:5431-5435; Kochetov et al (1998) FEBS Letts. 440:351-355.

[0052] A "nucleic acid" molecule can include, but is not limited to, prokaryotic sequences, eukaryotic mRNA, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. The term also captures sequences that include any of the known base analogs of DNA and RNA.

[0053] "Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, a given promoter operably linked to a coding sequence is capable of effecting the expression of the coding sequence when active. The promoter need not be contiguous with the coding sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present
between the promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

[0054] "Recombinant" as used herein to describe a nucleic acid molecule means a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of the polynucleotide with which it is associated in nature; and/or (2) is linked to a polynucleotide other than that to which it is linked in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide. "Recombinant host cells," "host cells," "cells," "cell lines," "cell cultures," and other such terms denoting prokaryotic microorganisms or eukaryotic cell lines cultured as unicellular entities, are used interchangeably, and refer to cells which can be, or have been, used as recipients for recombinant vectors or other transfer DNA, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement to the original parent, due to accidental or deliberate mutation. Progeny of the parental cell which are sufficiently similar to the parent to be characterized by the relevant property, such as the presence of a nucleotide sequence encoding a desired peptide, are included in the progeny intended by this definition, and are covered by the above terms.

[0055] Techniques for determining amino acid sequence "similarity" are well known in the art. In general, "similarity" means the exact amino acid to amino acid comparison of two or more polypeptides at the appropriate place, where amino acids are identical or possess similar chemical and/or physical properties such as charge or hydrophobicity. A so-termed "percent similarity" then can be determined between the compared polypeptide sequences. Techniques for determining nucleic acid and amino acid sequence identity also are well known in the art and include determining the nucleotide sequence of the mRNA for that gene (usually via a cDNA intermediate) and determining the amino acid sequence encoded thereby, and comparing this to a second amino acid sequence. In general, "identity" refers to an exact nucleotide to nucleotide or amino acid to amino acid correspondence of two polynucleotides or polypeptide sequences, respectively.
Two or more polynucleotide sequences can be compared by determining their "percent identity." Two or more amino acid sequences likewise can be compared by determining their "percent identity." The percent identity of two sequences, whether nucleic acid or peptide sequences, is generally described as the number of exact matches between two aligned sequences divided by the length of the shorter sequence and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be extended to use with peptide sequences using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, Nucl. Acids Res. 14(6):6745-6763 (1986). Suitable programs for calculating the percent identity or similarity between sequences are generally known in the art.

A "vector" is capable of transferring gene sequences to target cells (e.g., bacterial plasmid vectors, viral vectors, non-viral vectors, particulate carriers, and liposomes). Typically, "vector construct," "expression vector," and "gene transfer vector," mean any nucleic acid construct capable of directing the expression of one or more sequences of interest in a host cell. Thus, the term includes cloning and expression vehicles, as well as viral vectors. The term is used interchangeable with the terms "nucleic acid expression vector" and "expression cassette."

By "subject" is meant any member of the subphylum chordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. The system described above is intended for use in any of the above vertebrate species, since the immune systems of all of these vertebrates operate similarly.

By "pharmacologically acceptable" or "pharmacologically acceptable" is meant a material which is not biologically or otherwise undesirable, i.e., the material
may be administered to an individual in a formulation or composition without causing any unacceptable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

[0060] As used herein, "treatment" refers to any of (i) the prevention of infection or reinfection, as in a traditional vaccine, (ii) the reduction or elimination of symptoms, and (iii) the substantial or complete elimination of the pathogen in question. Treatment may be effected prophylactically (prior to infection) or therapeutically (following infection).

[0061] As used herein the term "adjuvant" refers to a compound that, when used in combination with a specific immunogen (e.g. a VLP) in a formulation, will augment or otherwise alter or modify the resultant immune response. Modification of the immune response includes intensification or broadening the specificity of either or both antibody and cellular immune responses. Modification of the immune response can also mean decreasing or suppressing certain antigen-specific immune responses.

[0062] As used herein an "effective dose" generally refers to that amount of VLPs of the invention sufficient to induce immunity, to prevent and/or ameliorate an infection or to reduce at least one symptom of an infection and/or to enhance the efficacy of another dose of a VLP. An effective dose may refer to the amount of VLPs sufficient to delay or minimize the onset of an infection. An effective dose may also refer to the amount of VLPs that provides a therapeutic benefit in the treatment or management of an infection. Further, an effective dose is the amount with respect to VLPs of the invention alone, or in combination with other therapies, that provides a therapeutic benefit in the treatment or management of an infection. An effective dose may also be the amount sufficient to enhance a subject's (e.g., a human's) own immune response against a subsequent exposure to an infectious agent. Levels of immunity can be monitored, e.g., by measuring amounts of neutralizing secretory and/or serum antibodies, e.g., by plaque neutralization, complement fixation, enzyme-linked immunosorbent, or microneutralization assay. In the case of a vaccine, an "effective dose" is one that prevents disease and/or reduces the severity of symptoms.

[0063] As used herein, the term "effective amount" refers to an amount of VLPs necessary or sufficient to realize a desired biologic effect. An effective amount of the composition would be the amount that achieves a selected result, and such an amount could be determined as a matter of routine experimentation by a person

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skilled in the art. For example, an effective amount for preventing, treating and/or ameliorating an infection could be that amount necessary to cause activation of the immune system, resulting in the development of an antigen specific immune response upon exposure to VLPs of the invention. The term is also synonymous with "sufficient amount."

[0064] As used herein, the term "multivalent" refers to VLPs which have multiple antigenic proteins against multiple types or strains of infectious agents.

[0065] As used herein the term "immune stimulator" refers to a compound that enhances an immune response via the body's own chemical messengers (cytokines). These molecules comprise various cytokines, lymphokines and chemokines with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interferons, interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (e.g., granulocyte-macrophage (GM)-colony stimulating factor (CSF)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The immune stimulator molecules can be administered in the same formulation as VLPs of the invention, or can be administered separately. Either the protein or an expression vector encoding the protein can be administered to produce an immunostimulatory effect.

[0066] As used herein the term "protective immune response" or "protective response" refers to an immune response mediated by antibodies against an infectious agent, which is exhibited by a vertebrate (e.g., a human), that prevents or ameliorates an infection or reduces at least one symptom thereof. VLPs of the invention can stimulate the production of antibodies that, for example, neutralize infectious agents, blocks infectious agents from entering cells, blocks replication of said infectious agents, and/or protect host cells from infection and destruction. The term can also refer to an immune response that is mediated by T-lymphocytes and/or other white blood cells against an infectious agent, exhibited by a vertebrate (e.g., a human), that prevents or ameliorates flavivirus infection or reduces at least one symptom thereof.

[0067] As use herein, the term "antigenic formulation" or "antigenic composition" refers to a preparation which, when administered to a vertebrate, e.g. a mammal, will induce an immune response.

[0068] As used herein, the term "vaccine" refers to a formulation which contains VLPs of the present invention, which is in a form that is capable of being
administered to a vertebrate and which induces a protective immune response sufficient to induce immunity to prevent and/or ameliorate an infection and/or to reduce at least one symptom of an infection and/or to enhance the efficacy of another dose of VLPs. Typically, the vaccine comprises a conventional saline or buffered aqueous solution medium in which the composition of the present invention is suspended or dissolved. In this form, the composition of the present invention can be used conveniently to prevent, ameliorate, or otherwise treat an infection. Upon introduction into a host, the vaccine is able to provoke an immune response including, but not limited to, the production of antibodies and/or cytokines and/or the activation of cytotoxic T cells, antigen presenting cells, helper T cells, dendritic cells and/or other cellular responses.

**General Overview**

[0069] This invention describes the formation of biological particles (e.g., VLPs) that mimic the structure in size, morphology and biochemical composition of native Zika viruses and other flaviviruses; however, they are devoid of a fully competent viral genome and therefore unable to cause infection or disease. The lack of viral genome and lack of infectivity of the flavivirus (Zika) VLPs eliminate the need of chemical inactivation better preserving therefore their structures, protein conformations and antigenic properties enhancing immunogenicity and potency as vaccine. These biological mimics are identified as virus-like particles (VLPs). VLPs are assembled using genetic information comprising segments of the virus genome encoding selected proteins that may include but not limited to structural and non-structural protein. As shown in Figure 1, the viral sequences in DNA form can be organized in a single transcription unit (segment) that expresses a single polypeptide or in separate transcription units (segments) each one expressing a single protein.

**Virus-Like Particles**

[0070] The present disclosure relates to flavivirus VLPs, which VLPs carry on their surfaces one or more modified antigenic flavivirus proteins. This VLP, alone or in combination with one or more additional VLPs and/or adjuvants, stimulates an immune response that protects against flavivirus infection.
In one embodiment of the invention, the structural proteins of interest comprise CprME, which after expression leads to the formation of VLPs. In order to enhance assembly and release of these VLPs from the producing cells, non-structural proteins may be co-expressed with the structural proteins (e.g. NS2B/NS3).

Exemplary wild-type and mutant CprME nucleotide and amino acid sequences are shown in the “Sequences” Section below (e.g. Zika CprME wild type nucleotide sequence, SEQ ID NO: 20 and codon optimized SEQ ID NO: 21 and the amino acid sequence is shown in SEQ ID NO: 22). Exemplary of Zika NS2B/NS3 full-length nucleotide sequence (e.g. SEQ ID NO: 23) and amino acid sequence (e.g. SEQ ID NO: 24). Also, examples of truncated and codon optimized nucleotide sequence of Zika NS2B/NS3 is shown in SEQ ID NO: 25 and its corresponding amino acid sequence is described in SEQ ID NO: 26. It will be apparent proteins from any Zika serotype or strain can be used in the compositions described herein, for example by alignment with the exemplary Zika sequences disclosed herein.

In any of the VLPs and methods described herein, the non-structural proteins may comprise either a full-length NS3 segment or a truncated version of the segment containing the protease domain, which is localized at the amino terminal of the polypeptide (e.g. truncated version of NS3 comprising aa 1 to aa 181). Exemplary sequences are shown below (e.g. full length amino acid sequence of dengue NS3, SEQ ID NO: 27, and truncated aa 1 to aa 181 and mutated, SEQ ID NO: 13).

In any of the VLPs or methods described herein, the non-structural protein segment comprising the full length or truncated form of NS3 is co-expressed with the full length or truncated forms of NS2B. Exemplary wild-type and mutant NS3 and/or NS2B nucleotide and amino acid sequences are shown in the “Sequences” section below. (e.g. an example of wild type nucleotide sequence of dengue NS2B/NS3 is shown in SEQ ID NO: 6 and amino acid sequence described in SEQ ID NO: 7). Modified dengue NS2B/NS3 (e.g., truncated, mutated and codon optimized) nucleotide sequence is shown in SEQ ID NO: 8 and amino acid sequence shown in SEQ ID NO: 9. When NS2B or NS3 are used as single proteins the nucleotide sequence is derived from SEQ ID NO: 8 resulting in NS2B nucleotide sequence (e.g. SEQ ID NO: 10) and amino acid sequence (SEQ ID NO: 11) and NS3 nucleotide sequence (e.g. SEQ ID NO: 12) and amino acid (e.g. SEQ ID NO: 13). In certain embodiments, the structural and non-structural proteins may be genetically linked, (as
shown in Figure 2). This is a non-limiting example of a single segment with a defined order using a linker that may encode. Additional exemplary sequences that may be used include but are not limited to a sequence of different portions of the NS1, NS2A and / or NS2B proteins as follows:

[0074] Linker 1: amino acids (aa) 1 to 8 or 9 or 10 of NS1 (e.g. nucleotide sequence SEQ ID NO: 14 and amino acid sequence SEQ ID NO: 15) connected to a portion of NS2A comprising of aa186 or 187 or 188 or 189 to aa 218 of NS2A (e.g. nucleotide sequence SEQ ID NO: 16 and amino acid sequence SEQ ID NO: 17).

[0075] Linker 2: aa 1 to 8 or 9 or 10 of NS1 (SEQ ID NO: 14) connected to a first portion of NS2A comprising aa 1 to 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 connected to a second portion of NS2A comprising of aa186 or 187 or 188 or 189 to 218 of NS2A (SEQ ID NO: 16).

[0076] Linker 3: aa 1 to 8 or 9 or 10 of NS1 (SEQ ID NO: 14) connected to the second transmembrane domain of NS2B (e.g. nucleotide sequence SEQ ID NO: 10 and amino acid sequence SEQ ID NO: 11)

[0077] Linker 4: aa 1 to 8 or 9 or 10 of NS1 (SEQ ID NO: 14) connected to the first transmembrane domain of NS2A (Amino acid 51 to 100 of nucleotide sequence, SEQ ID NO: 16 and amino acid sequence SEQ ID NO: 17)

[0078] Linker 5: aa 1 to 8 or 9 or 10 of NS1 (SEQ ID NO: 14) connected to the C terminal portion of NS2B comprising the second transmembrane domain to the end of the protein (e.g. nucleotide sequence SEQ ID NO: 10 and amino acid sequence SEQ ID NO: 11).

[0079] In still further embodiments, the positional order of the structural and non-structural segments may be inverted (with respect to each other) where the non-structural segment is genetically linked to the structural segment with or without a connecting linker. For example, the non-structural protein may include a full length NS2B and a full NS3 genetically linked directly to the structural proteins CprME. Alternatively, the non-structural proteins may comprise a truncated variant of NS3 in which the helicase domain is deleted but the protease domain and its recognizable self-cleavage site located at the carboxyl terminal of the protein are preserved. The self-cleavage site serves as one example of a linker that may be used to connect the non-structural and structural proteins.
In still further embodiments, the VLPs and methods described herein may include changes in the sequence of the structural and non-structural proteins (e.g., modifications to the nucleotide sequence which result in amino acid modifications), which can be used to enhance the formation and release of the VLP from the producing cells.

For example, the furin protease cleavage site between pr and M protein may be modified to enhance furin activity. The recognition consensus sequence is defined as R-Xaa-L/R-R where amino acids immediately prior to the cleavage site specifies positions P1 (R), P2 L/R, P3 (Xaa) and P4 (R). The naturally occurring acid residue in the cleavage site at position P3 can be substituted, but not limited to, by a hydrophobic residue. Exemplary sequences are shown below in “Sequences” section (e.g SEQ ID NO: 4)

In other embodiments, one or more helices of the E protein may be modified to enhance VLP assemble and release (Purdy DE, Chang G-JJ, 2005, Virology 333(2): 239–250).

For example, the amphipathic helix 1 in the stem domain of the E protein may be modified to enhance the hydrophobic properties of one side of helix.

In one exemplary embodiments of this modification: one, two, three or more amino acids in the hydrophobic side of the helix are substituted. These substitutions may occur at positions 398, 401 and / or 412, including but not limited to I398L, I398M, I398V, I398A or M401A, M401L, M401V, M401I, or M412A, M412L, M412V, M412I.

In other embodiments, the helix 1 and / or helix 2 are exchanged with the helix sequences derived from other flaviviruses or other viruses and sources.

In other embodiments, the NS3 protease active site may be modified in order to enhance its enzymatic activity. Non-limiting examples of such modifications include the substitution of amino acid leucine at position 115 (e.g., with an amino acid with a shorter side chain such as glycine or alanine).

In still further embodiments, to boost the protease cleavage between pr and M by furin and therefore increase the amount of mature particles, additional furin
enzymes may be furnished throughout the VLP production using any of the methods described below:

[0088] One version of this example may include the addition of recombinant furin protein to the VLPs producing cells culture medium.

[0089] A second version of this example: an expression plasmid carrying the furin gene as a single transcription unit (segment) may be co-transfected with one or more plasmids expressing the structural and non-structural genes. Another method may include an expression plasmid carrying a single transcription unit (segment) where the furin gene is genetically linked to either structural or non-structural protein or both.

[0090] A third version of this example: a cell line stably transfected and selected for its constitutive expression of furin, may be used in VLP production. Alternatively, the furin gene is stably transfected and / or inducible in a cell line already modified to constitutively produce VLPs.

VLP Production

[0091] The production of VLPs as described herein may be achieved by any suitable method, including but not limited to transient and / or stable expression of the structural and/or non-structural genes in a suspension culture of eukaryotic cells, typically requiring a period of continued cell culture after which the VLPs are harvested from the culture medium. The VLPs produced as described herein are conveniently prepared using standard recombinant techniques. Polynucleotides encoding the VLP-forming protein(s) are introduced into a host cell and, when the proteins are expressed in the cell, they assemble into VLPs.

[0092] Polynucleotide sequences coding for molecules (proteins) that form and/or are incorporated into the VLPs can be obtained using recombinant methods, such as by screening cDNA and genomic libraries from cells expressing the gene, or by deriving the gene from a vector known to include the same. For example, plasmids which contain sequences that encode naturally occurring or altered cellular products may be obtained from a depository such as the A.T.C.C., or from commercial sources. Plasmids containing the nucleotide sequences of interest can be digested with appropriate restriction enzymes, and DNA fragments containing the nucleotide
sequences can be inserted into a gene transfer vector using standard molecular biology techniques.

[0093] Alternatively, cDNA sequences may be obtained from cells which express or contain the sequences, using standard techniques, such as phenol extraction and PCR of cDNA or genomic DNA. See, e.g., Sambrook et al., supra, for a description of techniques used to obtain and isolate DNA. Briefly, mRNA from a cell which expresses the gene of interest can be reverse transcribed with reverse transcriptase using oligo-dT or random primers. The single stranded cDNA may then be amplified by PCR (see U.S. Pat. Nos. 4,683,202, 4,683,195 and 4,800,159, see also PCR Technology: Principles and Applications for DNA Amplification, Erlich (ed.), Stockton Press, 1989)) using oligonucleotide primers complementary-to sequences on either side of desired sequences.

[0094] The nucleotide sequence of interest can also be produced synthetically, rather than cloned, using a DNA synthesizer (e.g., an Applied Biosystems Model 392 DNA Synthesizer, available from ABI, Foster City, Calif.). The nucleotide sequence can be designed with the appropriate codons for the expression product desired. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) Nature 292:756; Nambair et al. (1984) Science 223:1299; Jay et al. (1984) J. Biol. Chem. 259:6311.

[0095] Preferably, the sequences employed to form flavivirus VLPs exhibit between about 60% to 80% (or any value therebetween including 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78% and 79%) sequence identity to a naturally occurring flavivirus nucleotide sequence and more preferably the sequences exhibit between about 80% and 100% (or any value therebetween including 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99%) sequence identity to a naturally occurring polynucleotide sequence.

[0096] Any of the sequences described herein may further include additional sequences. For example, to further to enhance vaccine potency, hybrid molecules are expressed and incorporated into the sub-viral structure. These hybrid molecules are generated by linking, at the DNA level, the sequences coding for the protein genes with sequences coding for an adjuvant or immuno-regulatory moiety. During sub-
viral structure formation, these hybrid proteins are incorporated into or onto the particle. The incorporation of one or more polypeptide immunomodulatory polypeptides (e.g., adjuvants describe in detail below) into the sequences described herein into the VLP may enhance potency and therefore reduces the amount of antigen required for stimulating a protective immune response. Alternatively, as described below, one or more additional molecules (polypeptide or small molecules) may be included in the VLP-containing compositions after production of the VLP from the sequences described herein.

These sub-viral structures do not contain infectious viral nucleic acids and they are not infectious eliminating the need for chemical inactivation. Absence of chemical treatment preserves native epitopes and protein conformations enhancing the immunogenic characteristics of the vaccine.

The sequences described herein can be operably linked to each other in any combination. For example, one or more sequences may be expressed from the same promoter and/or from different promoters. As described below, sequences may be included on one or more vectors. Non-limiting examples of vectors that can be used to express sequences that assemble into VLPs as described herein include viral-based vectors (e.g., retrovirus, adenovirus, adeno-associated virus, lentivirus), baculovirus vectors (see, Examples), plasmid vectors, non-viral vectors, mammalian vectors, mammalian artificial chromosomes (e.g., liposomes, particulate carriers, etc.) and combinations thereof. The expression vector(s) typically contain(s) coding sequences and expression control elements which allow expression of the coding regions in a suitable host. Enhancer elements may also be used herein to increase expression levels of the mammalian constructs.

Vaccine formulation is accomplished according to standard procedures, for example as shown in Figure 3.

Monovalent

Different exemplary strategies are described to assemble monovalent VLPs including:

1. Use homologous clades / antigenic variants for both structural and non-structural proteins
2. Use heterologous clades between the structural proteins and non-structural proteins with the exception of the viral cleavage site within the sequence of the C protein that matches the clade/antigenic variant of the non-structural proteins.

3. Use heterologous clades between the structural proteins and non-structural proteins with the exception of the cytoplasmic domain including the viral protease cleavage site of the C protein, which matches the serotype of the non-structural proteins.

**Bivalent**

Two exemplary strategies to generate a bivalent vaccine:

1. Blending two monovalent VLP in a single formulation.
   1a. Use homologous clades/antigenic variants for both structural and non-structural proteins.
   1b. Use heterologous clades/antigenic variants between the structural proteins and non-structural proteins with the exception of the viral cleavage site within the sequence of the C protein that matches the serotype of the non-structural proteins.
   1c. Use heterologous clades/antigenic variants between the structural proteins and non-structural proteins with the exception of the cytoplasmic domain including the viral protease cleavage site of the C protein, which matches the serotype of the non-structural proteins (e.g. SEQ ID NO: 35 and SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 39 and SEQ ID NO: 42, SEQ ID NO: 43).

2. Assembly of a single bivalent particle VLP. Alternative exemplary approach may be used to build these structures:
   2a. Co-expression of two heterologous set of both structural and non-structural proteins.
   2b. Co-expression of two heterologous structural proteins containing the same viral cleavage site within the sequence of the C protein together with non-structural proteins that recognized this viral cleavage site.
   2c. Co-expression of two heterologous structural proteins that share the analogous cytoplasmic domain sequence including the viral protease cleavage
site of the C protein together with non-structural proteins that recognize this viral cleavage site

Multivalent

[0102] Several exemplary approaches may be used to create multivalent vaccine formulations:

1. Blending of several single monovalent VLPs as described in the monovalent sections 1, 2 and 3

2. Blending of two single particle bivalents in a sole combination. Assembly of the single particle bivalent is as described in section 2a, 2b and 2c.

3. Assembly of single multivalent particle: To assemble this particle
   3a. Co-expression of several heterologous sets of both structural and non-structural proteins

   3b. Co-expression of several heterologous structural proteins containing the same viral cleavage site within the sequence of the C protein together with non-structural proteins that recognize this viral cleavage site

   3c. Co-expression of several heterologous structural proteins that share the analogous cytoplasmic domain sequence including the viral protease cleavage site of the C protein together with non-structural proteins that recognize this viral cleavage site.

[0103] Furthermore, combination vaccine can be created by blending in a single formulation monovalent, bivalent or multivalent compositions of a disease-causing virus (e.g. dengue) with composition of another disease-causing virus such as Zika or chikungunya.

[0104] The utility of the VLPs include, but it is not limited to, the generation of immunogenic (vaccine) compositions that when administered to humans are able to treat and/or prevent flavivirus (dengue, Zika) infection, including treatment and/or prevention of infection with one and / or more flavivirus virus clades / antigenic variants or serotypes. Flavivirus VLPs may be used in combination with other flavivirus VLPs (e.g., Zika with dengue, and / or Alpha chikungunya, etc.), including VLPs that include one or more antigenic determinants from two or more flaviviruses and combinations of VLPs that include one or more antigenic determinants from one, two or more flaviviruses with VLPs that includes one or more antigenic determinants from one, two or more flaviviruses (e.g., monovalent, bivalent or multivalent Zika
VLP in a pharmaceutical composition with monovalent, bivalent or multivalent Zika, dengue and/or chikungunya VLP). Other utility of the Zika VLP relates to diagnostic and therapeutic applications.

[0105] Suitable host cells for producing VLPs as described herein include, but are not limited to, bacterial, mammalian, baculovirus/insect, yeast, plant and *Xenopus* cells. For example, a number of mammalian cell lines are known in the art and include primary cells as well as immortalized cell lines available from the American Type Culture Collection (A.T.C.C.), such as, but not limited to, MDCK, BHK, VERO, MRC-5, WI-38, HT1080, 293, 293T, RD, COS-7, CHO, Jurkat, HUT, SUPT, C8166, MOLT4/clone8, MT-2, MT-4, H9, PM1, CEM, myeloma cells (e.g., SB20 cells) and CEMX174 (such cell lines are available, for example, from the A.T.C.C.).

[0106] The immunogenicity of VLP vaccines may be affected by the structural conformation of the E protein displayed on the particles’ surface. Changing the temperature of the fermentation process may alter this conformation. In one embodiment, the VLPs are produced at lower temperature (31°C, plus or minus 3 degrees centigrade) than the standard temperature of fermentation of 37°C. VLPs produced at the lower temperature when administered as vaccine may induce higher neutralizing antibody titers than those produced at 37°C.

[0107] The VLPs as described herein may be purified following production. Non-limiting examples of suitable purification (isolation) from the cell culture medium procedures include using centrifugation and/or gradient centrifugation under suitable conditions. Other methods of purification may include sequential steps of filtration and/or chromatography procedures including ion exchange, affinity, size exclusion and/or hydrophobic interaction chemistries.

[0108] Cell lines expressing one or more of the sequences described above can readily be generated given the disclosure provided herein by stably integrating one or more expression vector constructs encoding the proteins of the VLP. The promoter regulating expression of the stably integrated flavivirus sequences (s) may be constitutive or inducible. Thus, a cell line can be generated in which one or more structural proteins are stably integrated such that, upon introduction of the sequences described herein (e.g., hybrid proteins) into a host cell and expression of the proteins encoded by the polynucleotides, non-replicating viral particles that present antigenic glycoproteins are formed.
In certain embodiments, a mammalian cell line that stably expressed two or more antigenically distinct flavivirus proteins is generated. Sequences encoding structural and/or non-structural proteins can be introduced into such a cell line to produce VLPs as described herein. Alternatively, a cell line that stably produces structural proteins can be generated and sequences encoding the antigenic flavivirus protein(s) from the selected strain(s)/serotype(s)/clade(s) introduced into the cell line, resulting in production of VLPs presenting the desired antigenic glycoproteins.

The parent cell line from which an VLP-producer cell line is derived can be selected from any cell described above, including for example, mammalian, insect, yeast, bacterial cell lines. In a preferred embodiment, the cell line is a mammalian cell line (e.g., 293, RD, COS-7, CHO, BHK, MDCK, MDBK, MRC-5, VERO, HT1080, and myeloma cells). Production of VLPs using mammalian cells provides (i) VLP formation; (ii) correct post translation modifications (glycosylation, palmitylation) and budding; (iii) absence of non-mammalian cell contaminants and (iv) ease of purification.

In addition to creating cell lines, flavivirus-encoding sequences may also be transiently expressed in host cells. Suitable recombinant expression host cell systems include, but are not limited to, bacterial, mammalian, baculovirus/insect, vaccinia, Semliki Forest virus (SFV), Alphaviruses (such as, Sindbis, Venezuelan Equine Encephalitis (VEE)), Retrovirus vectors (lentivirus), mammalian, yeast and Xenopus expression systems, well known in the art. Particularly preferred expression systems are mammalian cell lines, vaccinia, Sindbis, insect and yeast systems.


When expression vectors containing the altered genes that code for the proteins required for sub-viral structure vaccine formation are introduced into host cell(s) and subsequently expressed at the necessary level, the sub-viral structure vaccine assembles and is then released from the cell surface into the culture media (Fig. 7).

Depending on the expression system and host selected, the VLPs are produced by growing host cells transformed by an expression vector under conditions whereby the particle-forming polypeptide(s) is (are) expressed and VLPs can be formed. The selection of the appropriate growth conditions is within the skill of the art. If the VLPs are formed and retained intracellularly, the cells are then disrupted, using chemical, physical or mechanical means, which lyse the cells yet keep the VLPs substantially intact. Such methods are known to those of skill in the art and are
described in, e.g., Protein Purification Applications: A Practical Approach, (E. L. V. Harris and S. Angal, Eds., 1990). Alternatively, VLPs may be secreted and harvested from the surrounding culture media.

The particles are then isolated (or substantially purified) using methods that preserve the integrity thereof, such as, by density gradient centrifugation, e.g., sucrose, potassium tartrate or Iodixanol gradients, PEG-precipitation, pelleting, and the like (see, e.g., Kirnbauer et al. J. Virol. (1993) 67:6929-6936), as well as standard purification techniques including, e.g., ion exchange and gel filtration chromatography, tangential filtration, etc.

Compositions

VLPs produced as described herein can be used to elicit an immune response when administered to a subject. As discussed above, the VLPs can comprise a variety of antigens (e.g., one or more modified flavivirus antigens from one or more flaviviruses and/or one or more strains, serotypes, clades or isolates of a particular flavivirus). Purified VLPs can be administered to a vertebrate subject, usually in the form of vaccine compositions. Combination vaccines may also be used, where such vaccines contain, for example, other proteins derived from other flaviviruses or other organisms and/or gene delivery vaccines encoding such antigens.

VLP immune-stimulating (or vaccine) compositions can include various excipients, adjuvants, carriers, auxiliary substances, modulating agents, and the like. The immune stimulating compositions will include an amount of the VLP/antigen sufficient to mount an immunological response. An appropriate effective amount can be determined by one of skill in the art. Such an amount will fall in a relatively broad range that can be determined through routine trials and will generally be an amount on the order of about 0.1 µg to about 10 (or more) mg, more preferably about 1 µg to about 300 µg, of VLP/antigen.

Sub-viral structure vaccines are purified from the cell culture media and formulated with the appropriate buffers and additives, such as a) preservatives or antibiotics; b) stabilizers, including proteins or organic compounds; c) adjuvants or immuno-modulators for enhancing potency and modulating immune responses (humoral and cellular) to the vaccine; or d) molecules that enhance presentation of vaccine antigens to specific cell of the immune system. This vaccine can be
prepared in a freeze-dried (lyophilized) form in order to provide for appropriate storage and maximize the shelf-life of the preparation. This will allow for stock piling of vaccine for prolonged periods of time maintaining immunogenicity, potency and efficacy.

[0119] A carrier is optionally present in the compositions described herein. Typically, a carrier is a molecule that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Examples of particulate carriers include those derived from polymethyl methacrylate polymers, as well as microparticles derived from poly(lactides) and poly(lactide-co-glycolides), known as PLG. See, e.g., Jeffery et al., Pharm. Res. (1993) 10:362-368; McGee J P, et al., J Microencapsul. 14(2):197-210, 1997; O'Hagan D T, et al., Vaccine 11(2):149-54, 1993. Such carriers are well known to those of ordinary skill in the art.

[0120] Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Exemplary adjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (International Publication No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, Mass.), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphoryl lipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL+CWS (Detoxu); (3) saponin adjuvants, such as Stimulon™. (Cambridge Bioscience, Worcester,
Mass.) may be used or particle generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (5) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), beta chemokines (MIP, 1-alpha, 1-beta Rantes, etc.); (6) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an E. coli heat-labile toxin (LT), particularly LT-K63 (where lysine is substituted for the wild-type amino acid at position 63) LT-R72 (where arginine is substituted for the wild-type amino acid at position 72), CT-S109 (where serine is substituted for the wild-type amino acid at position 109), and PT-K9/G129 (where lysine is substituted for the wild-type amino acid at position 9 and glycine substituted at position 129) (see, e.g., International Publication Nos. WO93/13202 and WO92/19265); and (7) other substances that act as immunostimulating agents to enhance the efficacy of the composition.

![0121] Muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetyluramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.


(1989) *Can. Immunol. Immunother.* 30:34-42, and U.S. Pat. Nos. 4,762,791 and 4,727,138; G-CSF (U.S. Pat. Nos. 4,999,291 and 4,810,643); GM-CSF (International Publication No. WO 85/04188); tumor necrosis factors (TNFs) (Jayaraman et al. (1990)*J. Immunology* 144:942-951); CD3 (Krissanen et al. (1987) Immunogenetics 26:258-266); ICAM-1 (Altman et al. (1989)*Nature* 338:512-514, Simmons et al. (1988)*Nature* 331:624-627); ICAM-2, LFA-1, LFA-3 (Wallner et al. (1987)*J. Exp. Med.* 166:923-932); MHC class I molecules, MHC class II molecules, B7.1-β2-microglobulin (Parnes et al. (1981)*Proc. Natl. Acad. Sci. USA* 78:2253-2257); chaperones such as calnexin; and MHC-linked transporter proteins or analogs thereof (Powis et al. (1991)*Nature* 354:528-531). Immunomodulatory factors may also be agonists, antagonists, or ligands for these molecules. For example, soluble forms of receptors can often behave as antagonists for these types of factors, as can mutated forms of the factors themselves.

Nucleic acid molecules that encode the above-described substances, as well as other nucleic acid molecules that are advantageous for use within the present invention, may be readily obtained from a variety of sources, including, for example, depositories such as the American Type Culture Collection, or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Representative examples include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), A.T.C.C. Deposit No. 39656 (which contains sequences encoding TNF), A.T.C.C. Deposit No. 20663 (which contains sequences encoding alpha-interferon), A.T.C.C. Deposit Nos. 31902, 31902 and 39517 (which contain sequences encoding beta-interferon), A.T.C.C. Deposit No. 67024 (which contains a sequence which encodes Interleukin-1b), A.T.C.C. Deposit Nos. 39405, 39452, 39516, 39626 and 39673 (which contain sequences encoding Interleukin-2), A.T.C.C. Deposit Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), A.T.C.C. Deposit No. 57592 (which contains sequences encoding Interleukin-4), A.T.C.C. Deposit Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and A.T.C.C. Deposit No. 67153 (which contains sequences encoding Interleukin-6).

Plasmids encoding one or more of the above-identified polypeptides can be digested with appropriate restriction enzymes, and DNA fragments containing
the particular gene of interest can be inserted into a gene transfer vector (e.g., expression vector as described above) using standard molecular biology techniques. (See, e.g., Sambrook et al., supra, or Ausubel et al. (eds) Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience).

Administration

[0125] The VLPs and compositions comprising these VLPs can be administered to a subject by any mode of delivery, including, for example, by parenteral injection (e.g. subcutaneously, intraperitoneally, intravenously, intramuscularly, or to the interstitial space of a tissue), or by rectal, oral (e.g. tablet, spray), vaginal, topical, transdermal (e.g. see WO99/27961) or transcutaneous (e.g. see WO02/074244 and WO02/064162), intranasal (e.g. see WO03/028760), ocular, aural, pulmonary or other mucosal administration and / or inhalation of powder compositions. Multiple doses can be administered by the same or different routes. In a preferred embodiment, the doses can be intranasally administered.

[0126] The VLPs (and VLP-containing compositions) can be administered prior to, concurrent with, or subsequent to delivery of other vaccines. Also, the site of VLP administration may be the same or different as other vaccine compositions that are being administered.

[0127] Dosage treatment with the VLP composition may be a single dose schedule or a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals, chosen to maintain and/or reinforce the immune response, for example at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the potency of the modality, the vaccine delivery employed, the need of the subject and be dependent on the judgment of the practitioner.

[0128] All patents, patent applications and publications mentioned herein are hereby incorporated by reference in their entireties.

[0129] Although disclosure has been provided in some detail by way of illustration and example for the purposes of clarity and understanding, it will be apparent to those of skill in the art that various changes and modifications can be
practiced without departing from the spirit or scope of the disclosure. Accordingly, the foregoing disclosure and following examples should not be construed as limiting. Thus, it will be apparent that while exemplary results are presented with respect to dengue virus, the teachings herein are equally applicable to any flavivirus (e.g. Zika, yellow fever, Japanese encephalitis, hepatitis C, West Nile, tick-borne encephalitis) or alphavirus (e.g. chikungunya).

EXAMPLES

Example 1: Dengue VLP production

[0130] A dengue vaccine capable of rapidly eliciting a robust and balanced immunity against the four virus serotypes after only a few immunizations is greatly needed. We describe a new strategy to develop dengue vaccines based on the assembly of virus-like particles (VLPs) utilizing the structural proteins CprME together with a modified complex of the NS2B/NS3 protease, which enhances particle formation and yield. These VLPs are produced in mammalian cells and resemble native dengue virus as demonstrated by negative staining and immunogold labeling electron microscopy (EM). Realizing that in the mosquito that the virus replicates at a lower temperature than in humans, we found that VLPs produced at a lower temperature (31°C) were recognized by conformational monoclonal antibodies (MAbs) 4G2 and 3H5, whereas VLPs produced at a higher temperature (37°C) were not recognized by either MAbs. To evaluate the significance of these conformational discrepancies in vaccine performance, we tested the immunogenicity of VLP vaccines produced at 31°C or 37°C in alternative formulations. Mice immunized with the VLP vaccine produced at 31°C (TVXDO-31°C) elicited higher titer of neutralizing antibodies as compared to those elicited by equivalent doses of the vaccine produced at 37°C (TVXDO-37°C) as well as by inactivated dengue virus vaccine or the titer seen with a human anti-dengue-2 convalescence serum used as a reference. Our results demonstrate that the conformation of the E protein displayed on the VLP vaccine plays a critical role in the induction of highly neutralizing antibodies. These findings will guide development of a tetravalent vaccine capable of eliciting a robust and balanced neutralizing response against four dengue serotypes regardless of background immunity.
Dengue is a mosquito-borne viral disease that affects humans of all ages in tropical and subtropical regions around the world. Due to global expansion of the mosquito vectors (primarily A. aegypti, and A. albopictus) the virus has spread to more than 120 countries, infecting approximately 390 million people annually (1). This dramatic spread of the vector poses a threat to almost half of the world’s population with disease outbreaks imposing a hefty public health and economic burden upon all affected areas. Four distinct virus serotypes (DENV1-4) can be transmitted by the bite of an infected Aedes Sp. mosquito causing an infection characterized by fever, headache, myalgia, arthralgia and, depending on the severity of the infection, may progress to Dengue hemorrhagic fever/Dengue shock syndrome (DHF/DSS) (2). Primary infection generates long-term protection against the homologous serotype, but a short-lived defense against heterologous serotypes. A secondary infection with a different serotype can trigger an antibody-dependent enhancement (ADE) of disease that may result in potentially fatal DHF and DSS (3). Currently, there are no specific interventions to treat dengue infections and prophylactic vaccines are the best hope to control the disease. The need to elicit a robust and balanced neutralizing response against the four-dengue serotypes, in order to prevent ADE, has hindered the development of a safe and effective dengue vaccines.

Dengue virus is a positive sense single-stranded RNA virus that belongs to the Flavivirus genus within the Flaviviridae family. There are four distinct identified virus serotypes: DENV-1, DENV-2, DENV-3 and DENV-4. These serotypes appeared independently during endemic cycles of transmission between humans and arthropod vectors (4). The dengue viral genome has a single open reading frame (ORF) encoding a polyprotein that is cleaved co- and post-translationally by cellular and viral proteases into three structural proteins: capsid (C), the pre-membrane (prM) and the envelope (E) as well as seven non-structural proteins: NS1, NS2A/B, NS3, NS4A/B and NS5 (5). Virus replication and morphogenesis takes place on virus-induced remodeled endoplasmic reticulum (ER) membranes leading to the assembly and budding of complete but immature particles into the ER system. (6). The spiky immature particles undergo an additional furin protease cleavage of prM during trafficking through the ER and the trans-Golgi network (tGN) (7) (Figure 1A)
that together, with a rearrangement of the E protein, turn the virions into smooth mature particles that are released from infected cells.

[0133] The surface E protein is the main antigenic determinant of the virus and the target of the immune system. Immunity directed toward the E protein is primarily mediated by neutralizing antibody which, when present, confers protection against dengue (8).

[0134] After decades of ceaseless effort, recently a dengue vaccine has reached commercialization stage. This live-attenuated chimeric vaccine is based on the yellow fever virus 17D vaccine strain, which provides the backbone to carry the DENV prM-E structural proteins of each serotype as chimeric viruses. Results of clinical phase III trials in Asia and South America show better results than did the clinical phase II trials, but both present serotype-dependent differences in vaccine efficacy and a lower efficacy in young children. In particular, the response against DENV-2 is suboptimal and after three immunization doses, the vaccination efficacy is only approximately 35% to 50% (9-11). Other vaccine candidates currently in clinical trials include live-attenuated virus vaccines, purified inactivated vaccine, a DNA vaccine, and a subunit vaccine (12, 13). The later vaccine formulation is comprised of four recombinant truncated E proteins and is currently the only recombinant vaccine candidate in clinical trials.

[0135] An alternative strategy for vaccine development involves the generation of viral-like particle (VLP) vaccine. These recombinant particles are self-assembling complex structures morphologically similar to wild-type virus but are devoid of viral genetic material and are thereby unable to replicate or cause infection. Flavivirus subviral particles or VLPs were initially detected in the supernatant of flavivirus-infected cells (14) and subsequent studies have shown that the sole expression of recombinant prM and E were sufficient to drive the assembly and budding of subviral particles (15-17). These structures provide an attractive strategy for vaccine development because the particulate nature of recombinant VLPs composed of native proteins enhances antigen recognition, presentation and immune stimulation (18-20). Furthermore, the system allows for surface protein engineering to optimize antigen configuration seeking enhancement of the immune response. The proven efficacy and safety of licensed human VLP based vaccines for HBV, HPV and
HEV (21-24) provide strong evidence of the value of this approach for vaccine development.

[0136] Here, we present data on the assembly of dengue virus-like particles utilizing a unique set of viral structural and non structural (NS) proteins and production conditions in which suspension cultures of mammalian cells render particles with distinct immunological properties which, as vaccines, elicit the production of highly effective neutralizing antibodies. This study describes a new strategy to efficiently develop a VLP based dengue vaccine that is assembled with native dengue protein and manufactured in a mammalian cell suspension culture system suitable for scale up manufacturing.

A. MATERIALS & METHODS

Genes and plasmids construct

[0137] The structural and non structural genes of DENV-2 were chemically synthesized by GeneArt (Life Technology) according to a specifically designed and codon-optimized sequence. DNA fragments were subcloned into the plasmid vector pcDNA3.4 (Life Technologies) utilizing the NheI/NotI restriction enzyme sites. Specific mutations were introduced in the prM gene G88A, and in the E gene, I398L, M401A, M412L, as described by Purdy and Chang (25).

[0138] The non-structural genes of NS2B and viral protease NS3 were synthesized as a single codon optimized unit and subcloned into plasmid vector pcDNA3.4 via Xhol/EcoRV. The mutation L115A was introduced into the synthesized NS3 gene.

[0139] Plasmids were amplified in MAX Efficiency® Stbl2™ Competent E. Coli Cells (Life Technologies 10268-019) and purified from the bacteria utilizing an EndoFree Plasmid Maxi Kit (Qiagen).

Virus, cells, and antibodies

[0140] Cultures of Vero cells (ATCC® CCL-81™) were maintained in VP-SFM media (Life Technologies 11681-020) supplemented with 2mM L-Glutamine (Life Technologies 25030-081), 2mM GlutaMAX™ Supplement (Life Technologies
35050-061), 1X Non-essential amino acids solution (Life Technologies 11140-050), 1X ITSE (Invitria 777ITS032) and 500ng/ml rhEGF (Life Technologies PHG0314).

Dengue virus: DENV-2 Th-36 (ATCC® VR-1810™) was amplified in Vero cells following virus inoculation at a low multiplicity of infection (MOI: 0.01). Expi293TM (Life Technologies) cells were expanded in Expi293 medium (Gibco A1435101) and transfected using ExpiFectamine following the manufacturer’s instructions (Life Technologies, A14635). Monoclonal antibody 4G2 is an in-house protein G purified from hybridoma D1-4G2-4-15 culture supernatant (ATCC HB-112). Mouse monoclonal antibody 3H5 was acquired through BEI Resources (NR-2556). Rabbit polyclonal antibodies, anti-C (GTX124247), anti-E (GTX127277), anti-prM (GTX128093) and anti-NS2B (GTX124246) were purchased from GeneTex, CA.

These secondary antibodies anti-mouse and anti-rabbit were both purchased from Pierce Thermo Fisher, MA (#31430 and #31460 respectively).

VLP production and purification

Expi293TM cells were transfected with a 1:2 ratio of pcDNA3.4-NS2b/NS3 and pcDNA3.4-CprME at 37°C and transferred after 4h post-transfection to incubators set at either 37°C or 31°C. Transfected cells were harvested 72hs post-transfection and clarified via two successive centrifugations. The first clarification was performed at 400xg for 10min at 4°C followed by a second clarification at 10,000 x g for 10min at 4°C. Clarified supernatant fluid was concentrated by ultracentrifugation for 2h at 140,000 x g at 4°C. The pellet was resuspended with 1X PBSCaMg pH 7.2 (1X phosphate buffered saline supplemented with 1mM MgCl2; and 1mM CaCl2). VLPs were further purified by ultracentrifugation through a 20-60% step sucrose gradient in TN buffer (50mM Tris-HCl pH 7.2; 150mM NaCl) for 4h at 180,000xg at 4°C using an SW40Ti rotor (Beckman Coulter, CA). The protein content of the collected fractions was analyzed by dot blot using a dengue specific antibody. Selected fractions were combined, dialyzed overnight versus 1X PBS and concentrated by ultracentrifugation for 2h at 140,000 x g at 4°C. The pellet was then suspended in 80µl of 1XPBSCaMg and loaded onto a second sucrose gradient (20%-60%) in TN buffer. Fractions were collected, analyzed and processed in the same fashion as in the first linear gradient above.
Dot blot and Western blot assays

The cell protein content was analyzed after clarification of transfected Expi293TM cells. The cell pellet was collected and cells were lysed with RIPA buffer (PI-89901, Pierce Thermo Fisher, MA). For Western blotting, cell lysates and concentrated culture supernatants were loaded onto a 10-20% Tris-glycine SDS-PAGE gel (EC61352BOX, Life Technologies, CA). After electrophoresis separation, proteins were electro-transferred from the gel onto a 0.45µm nitrocellulose membrane (Life Technologies LC2001). For dot blot, 3µl of sample was applied on top of a 0.45µm nitrocellulose membrane and allowed to dry for 5 min. The nitrocellulose membranes were then treated for 1h at room temperature with blocking solution (5% non-fat milk 1X TBS 0.1% Tween-20) followed by overnight incubation at room temperature in primary antibody diluted in blocking solution. Membranes were washed three times for 5min with 1X TBS 0.1% Tween-20 and then incubated for 1.5h in secondary antibody diluted in blocking solution. Finally, membranes were washed three times with 1XTBS-0.1% Tween-20 and developed with ECL system (WP20005, Life Technologies, CA).

Negative staining and immuno-gold labeling electron microscopy

Gradient purified VLP samples were blotted onto 200-mesh carbon coated grid (EMS CF200-Cu) for 5min. The grids were then washed and stained with 2% uranyl acetate (EMS 22400-2). Examination of VLPs by immunogold labeling EM was performed as follows: sample coated carbon grids were blocked with 3% BSA in 0.1M Sodium cacodylate buffer for 5min, followed by incubation for 20min in primary monoclonal antibody 3H5 diluted in PBS (1:100 dilution). Grids were then washed three times with 0.1M sodium cacodylate buffer and then incubated for 20min with secondary goat anti-mouse antibody (1:30 dilution). After a final series of three washes with 0.1M Sodium Cacodylate buffer, grids were stained with 2% uranyl acetate solution and examined with a JEOL-1400 electron microscope at the Rockefeller University Imaging Center.

Mouse immunogenicity study
Ten groups of 4-week old BALB/c mice were inoculated twice (day 0 and day 24) via the intramuscular (IM) route with VLP vaccines (TVXDO-31°C or TVXDO37°C). Groups of mice received doses of either 1µg or 5µg of total E protein content and formulated alone or admixed in a 1:1 volume with a squalene-based oil-in-water nano-emulsion AddaVax (InvivoGen, CA). Control groups were immunized with formalin inactivated (0.05%) DENV-2 Th-36 virus at the dose of either 1µg or 5µg of total E protein content. Serum samples for immunogenicity evaluation were collected at day 39.

ELISA

ELISA assays were performed in 96-well plates coated with 100µl/well of formaldehyde inactivated DENV-2 virus (1µg/ml of E protein content) and incubated overnight at 4°C. The plates were washed three times with PBST buffer (phosphate buffered saline plus 0.05% Tween-20) and then blocked with 100µl of blocking buffer (PBST plus 5% non-fat milk) for one hour at room temperature. Mouse sera were diluted following a 4-fold serial dilution in blocking buffer starting at 1:10; 50µl of the diluted sera were incubated for 2h at room temperature. After a set of 6 washes with PBST, plates were incubated for 2h with 50µl of HRP-conjugated goat anti-mouse antibody diluted 1:2,000 in blocking buffer. Subsequently, plates were washed (6X) and developed by adding 50µl/well of ECL reagent. Light emission was measured at 425 nm using a plate reader (Synergy, H1; BioTek, VT).

Plaque reduction neutralization test (PRNT)

The plaque reduction neutralization test was carried out in Vero cells using dengue virus serotype 2 (DENV-2) and sera from immunized mice according to the method described for the evaluation of vaccine efficacy (26, 27). Briefly, DENV-2 was amplified and titrated in Vero cells using the same plaque visualization procedure described below. Vero cells were seeded in 24 well plates at a density of 5x104 cells per well 24h prior to the initiation of the test. Control and tests sera were heat inactivated at 56°C for 30 min. and then two-fold serially diluted in cell culture media supplemented with penicillin and streptomycin. An equal amount of diluted virus to form ~60 plaques per well was added to each serum dilution. The sera-virus mixture was incubated for 1h at 37°C in a 5% CO2 environment. Subsequently, each dilution
was applied in duplicate wells of an 85% confluent monolayer of Vero cells and incubated for 1h at 37°C in a 5% CO2 incubator.

[0149] Thereafter, the inoculum was removed and a 3ml overlay of 2% carboxymethyl cellulose (CMC) in culture medium was added to each well. Plates were then incubated for six days at which time the CMC overlay was removed, cells washed 1X with PBST (phosphate buffer saline plus 0.05% Tween 20) and fixed with cold 80% acetone for 10 min at room temperature (RT). Subsequently, plates were washed 1X with PBST and then incubated with blocking buffer (2.5% non-fat milk in PBS plus 0.5% of Triton X-100) for 1h in a 37°C incubator. After one wash, the primary antibody (MAb 4G2) diluted 1/200 in blocking buffer was applied for 2h at RT, followed by two PBST washes and incubation for 1h at RT with a goat anti-mouse AP conjugated secondary antibody (1/2000) in blocking buffer. Finally, plates were washed twice with PBST and one time with alkaline phosphate buffer (APB: 100mM Tris-HCl pH9.0, 150mMNaCl, 1mM MgCl2). Viral plaques were detected by adding 100µl per well of the alkaline phosphate (AP) substrate nitro blue tetrazolium chloride (NTB) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) prepared and used as follows: 33µl of NTB (50mg/ml in 70% dimethylformamide) was added to 5ml of APB, mixed well and then added 16.5ul of BCIP (50mg/ml in 100% dimethylformamide) and the mixture was used within 1h. Plaques were counted and PRNT50s were determined using the PROBIT method (28). The neutralization power calculated is expressed as the reciprocal of the highest serum dilution that neutralizes 50% of the virus. Human pre-immune serum control and human anti-DENV-2 convalescent serum references were obtained from National Institute for Biological Standards and Control (NIBSC), UK.

B. RESULTS

[0150] Processing of the structural and non-structural polyproteins and formation of dengue virus-like particles (VLPs)

[0151] To assemble and release dengue virus-like particles (VLPs), we co-transfected into suspension cultures of Expi293TM human cells two DNA plasmids, one expressing the structural protein CprME and the other the non-structural proteins NS2B/NS3 (Fig. 4B). Expression of this protein combination resulted in the
processing of the structural proteins CprME in an analogous fashion as occurs within the whole viral polyprotein (Fig. 4A) and therefore drove the formation and release of dengue VLPs.

The genes encoding CprME (plasmid TVXDO2) and NS2B/NS3 (plasmid TVXDO3) were de novo-synthesized and codon optimized to enhance protein expression in the HEK293 derived human cell line. Both the structural and non-structural protein coding sequences were modified by truncation and substitution mutations to facilitate polyprotein processing and enhance assembly and release of the VLPs. We introduced mutations at positions I398A, M104A and M412L within the amphipathic domain-1 of the E protein to overcome restrictions in the processing and maturation of the dengue polyprotein and thereby enhance VLP yield. (e.g. retention signal in the E protein) (25). We also mutated E88A in the furin recognition sequence of prM protein to enhance furin cleavage at this location. Furthermore, the viral NS3 protease/helicase was truncated retaining only the N-terminal protease domain, which contains the L115A mutation to enhance its catalytic activity. The remaining protease cleavage sites within the CprME polypeptide, however, were preserved in order to maintain a processing pattern analogous to the one occurring in dengue virus infected cells (Fig. 4B).

Western blot analysis of transfected Expi293TM cell lysates showed not only expression of both structural and non-structural proteins but also complete processing of the structural polyprotein. Indeed, the capsid (12Kda), the prM predicted size (23/8Kda) and the E protein (64KDa) demonstrated the appropriate molecular size, as would be expected of a fully processed polyprotein (Fig. 5). The molecular weight of the NS2B protein is similar to the one detected in the DENV-2 cell lysate, suggesting that the self-cleavage between NS2B/NS3Pro occurs efficiently at the corresponding cleavage site (Fig. 2D). The NS3 portion of NS2B/NS3 could not be detected due to the lack of a suitable specific antibody. The efficient self-cleavage of NS2B/NS3Pro and the cleavage of the capsid protein confirm that the amino acids 1 to 183 are sufficient for proper protease activity as described by Li et. al. (29). Our construct contains the substitution mutation L115A in the NS3Pro, which does not appear to interfere with proper protease activity.

To determine whether the NS2B/NS3pro have an effect on VLP production, we transfected Expi293TM cells with the TVXDO2 (CprME) plasmid
alone or TVXDO2 together with TVXDO3 (NS2B/NS3pro) plasmid. The culture supernatants were clarified twice and VLPs concentrated by ultracentrifugation. Western blot analysis of concentrated supernatant of TVXDO2 alone showed that a small amount of prM and E proteins were secreted into the culture medium (Fig. 6). Release of the structural proteins, however, was enhanced when the plasmid TVXDO2 expressing the structural genes (CprME) was co-transfected with the plasmid TVXDO3 that expresses the modified non-structural proteins NS2/NS3pro. There was an increase in the amount of secreted E and prM when NS2B/NS3pro was co-expressed with CprME (Fig. 6). Furthermore, these results revealed an interdependence of cleavage between the C-anchored to C and the C anchored to prM, which is in agreement with findings in studies previously reported (30, 31). Thus, this protein combination offers an effective approach for VLP production.

**Dot blot analysis of VLPs produced at different temperatures**

[0155] Dengue virus is able to replicate in both mosquitoes and humans, which illustrates the broad temperature range (ambient versus 37°C respectively) over which the virus can complete its replication cycle. Several studies have shown that higher VLP yields can be obtained when the producing mammalian cell culture is incubated at a lower temperature underlying the significance of a slower assembly process (32, 33). Based on this information, we chose to evaluate the effect of different temperatures on particle formation and surface protein structure by dot blot using specific MAbs recognizing conformation epitopes of the E protein. VLPs were produced at two distinct temperatures 31°C and 37°C, purified through a linear sucrose gradient (15%-50%) and their sedimentation profile analyzed by dot blot using an anti-E polyclonal antibody. This comparative examination showed that the migration profile of the VLPs produced at both temperatures was similar with the strongest reactivity in fractions 10 to 13, indicating that the differing temperatures did not affect the migration profile of the assembled VLPs (Fig. 7).

[0156] To examine the structural conformation of the E protein, we utilized the dot blot method that allows for the study of dengue VLP in its native conformation. The monoclonal antibodies 4G2 and 3H5 that recognize structural epitopes on the E protein were used to search for conformational differences between VLPs produced at 31°C (TVXDO-31°C) and 37°C (TVXDO-37°C). The MAb 4G2 recognizes a structural epitope in the domain DII of E at the fusion loop comprised of
amino acids G104, G106, L107 and W231. The fusion loop of the E protein plays a key role in membrane fusion and therefore in dengue virus cell entry and replication.

Purified VLPs produced at 31°C (fractions 10-13) demonstrated the strongest reactivity with the MAb 4G2, whereas VLPs produced at 37°C failed to be recognized by this MAb (Fig. 7). It was evident, therefore, that the recombinant E protein displayed on the VLPs produced at either 31°C or 37°C was recognized by the anti-E polyclonal antibody but that only the VLPs produced at 31°C exhibited the epitope recognized by 4G2 MAb, suggesting a conformational difference between these E proteins.

To further examine the structural features of the VLP E protein, we used a second MAb (3H5) that recognizes a conformation epitope in the domain DIII of the E protein, comprised of amino acid residues K305, P384 which are essential for binding to cellular receptors (34, 35). Dot blot analysis of purified VLPs with the 3H5 MAb showed strong reactivity with TVXDO-31°C but failed to recognize TVXDO-37°C (Fig. 7). Thus, equivalent fractions of the two VLP preparations reacted strongly with the anti-E polyclonal antibody, however only the VLPs prepared at 31°C (TVXDO-31°C) were recognized by both 3H5 and 4G2 (Fig. 7). TVXDO-31°C shows closer immunological characteristics to the wild type virus than does the TVXDO-37°C VLP. All three tested antibodies recognized DENV-2 control. These results support the conclusion that the E protein display on VLPs produced at a lower temperature adopt structures that better exhibit conformational epitopes, which are critical for the induction of neutralizing antibodies.

Electron microscopy (EM) examination of DEN-2 VLPs

In view of the conformational differences due to incubation at either 31°C or 37°C, we carried out a closer examination of purified VLPs by negative staining and electron microscopy to further evaluate the morphology, shape, size and surface composition of the secreted virus-like particles. EM examination of TVXDO-31°C VLPs (Fig. 8A) showed that they are spherical in shape and with an approximately 50nm diameter, resembling structural characteristics of mature dengue virus as has been previously shown (36). Immuno-gold labeling EM of dengue-2 VLPs using the conformational-epitope recognized by MAb 3H5 showed surface reactivity and labeling only of TVXDO-31°C VLP, confirming the assembly and
release of particles composed of prM/E that exhibited the conformational epitopes recognized by these MAbs (Fig. 8B). In contrast, the TVXDO-37°C VLPs did not react with either 4G2 or 3H5.

5 Immunogenicity evaluation of VLPs in mice by ELISA
[0160] The recombinant DENV-2 VLPs showed immunological and structural similarities with DENV-2 virus when produced at 31°C but not at 37°C. To ascertain whether these distinctions play a significant role in the immunogenicity of the VLPs and therefore in their effectiveness as vaccine candidates, we performed

immunization studies in BALB/c mice. Groups of mice (n=4) were immunized twice, two weeks apart, via the intramuscular route with either TVXDO-31°C or TVXDO-37°C at the dose of 1μg or 5μg of total E protein content and formulated with or without adjuvant. Two weeks after the booster immunization, we collected serum samples from vaccine and control mice and assessed the antibody response by measuring total IgG levels by ELISA and neutralizing antibody titers by plaque reduction neutralization assay. Mice immunized with 5μg of TVXDO-31°C VLP vaccine with or without adjuvant demonstrated the highest level of IgG production as compared to the equivalent dose and formulations of TVXDO-37°C vaccine or the inactivated DENV-2 virus control (Fig. 6). Similarly, the TVXDO-31°C VLP vaccine administered at the dose of 1μg elicited production of higher IgG levels than the TVXDO-37°C VLP or inactivated virus vaccine. Although the TVXDO-31°C VLP vaccine induced the highest IgG levels, in a dose response manner, it was only the 1μg adjuvanted dose that showed a statistically significant difference with the TVXDO-37°C VLP vaccine (Fig. 9).

25 Immunogenicity assessment by plaque reduction neutralization test (PRNT)
[0161] Elicitation of high titers of neutralizing antibody is paramount for dengue protection and therefore we measured the levels of neutralizing antibodies elicited by these vaccines via PRNT. The assay was performed according to the World Health Organization (WHO) guidelines and protocols (26, 27). The PRNT50 of reciprocal dilutions was calculated using the PROBIT methods and compared with pre-immune mouse serum and reference controls (Fig 10). Both adjuvanted vaccines, at the dose of either 1 μg or 5 μg elicited higher neutralizing titers than the non-
adjuvanted preparations. The 1 µg dose of TVXDO-37°C vaccine, with and without adjuvant induced lower neutralizing antibody titers (PRNT50s: <25 and 57) than an equivalent dose of the inactivated dengue virus control (Fig. 10). However, the adjuvanted 5µg dose of the TVXDO-37°C elicited neutralizing titers (PRNT50: 382) that were greater than the one induced by the 5 µg (PRNT50: 201) and 1 µg (PRNT50: 158) doses of the inactivated dengue virus control.

On the other hand, the non-adjuvanted TVXDO-31°C vaccine at the dose of 1 µg and 5 µg stimulated neutralizing antibody in the PRNT50 ranging from 99 to 196. However, when this vaccine was admixed with adjuvant the potency of the neutralizing response increased 3.7-fold to a PRNT50 of 371 for the 1µg dose and greater than 5-fold to a PRNT50 of 1067 for the 5 µg dose. The high neutralizing potency stimulated by the adjuvanted 5 µg dose of the TVXDO-31°C is almost 3-fold greater than the equivalent dose of the TVXDO-37°C as well as 5.3 fold greater than the inactivated dengue virus control. Furthermore, to better judge the neutralization power elicited by the different vaccine doses, formulations and controls, we performed PRNT50 assays with a standard of human pre-immune and human convalescence DENV-2 sera obtained from the National Institute for Biological Standard and Control, UK (NIBSC). The PRNT50 of human seronegative control was similar to mouse pre-immune sera whereas the neutralizing power of the human convalescent DENV-2 serum (PRNT50: 297), was slightly higher than the high dose (5µg) of inactivated dengue virus control (PRNT50: 201). On the other hand, the adjuvanted TVXDO-31°C VLP vaccine at the dose of 1µg and 5µg elicited neutralizing antibody responses that were 1.2 fold and 3.6 fold higher than the human convalescence sera control, PRNT50: 371 versus 297 and PRNT50: 1067 versus 297 respectively. Although the TVXDO-37°C adjuvanted 5µg dose vaccine performed better than the inactivated DENV-2 and the anti-DENV-2 human serum standard it did not reach the neutralizing power induced by the TVXDO-31°C vaccine (PRNT: 382 versus PRNT: 1067). These results clearly show that the VLP vaccine produced at lower temperature, TVXDO-31°C, and formulated with adjuvant elicits the strongest anti-DENV-2 neutralizing antibody response, which potency correlated with the amount of antigen and inclusion of adjuvant.

Dengue is an expanding disease due to the increase numbers and geographic spread of the Aedes sp. mosquito population that disseminates the virus.
The only vaccine approved for DENV is a live attenuated virus that requires a year of multiple immunizations to reach, in many cases, an unbalanced immunity against the four-dengue serotypes (Dengvaxia, Sanofi Pasteur’s) (10, 11). Importantly, vaccine efficacy against the most disseminated DENV-2 serotype is only approximately 35% to 50%. Thus, new approaches for developing highly effective and safe dengue vaccines are needed. Here we report a novel strategy that utilizes a new set of structural and non-structural dengue proteins to assemble virus-like particles (VLPs) for dengue vaccine development. Most dengue VLPs have been assembled using the sole expression of prM and E proteins (37) and these attempts have revealed limitations in protein processing and domain restrictions, such as the presence of E retention sequences, which reduce assembly efficacy and yield. Our approach utilizes the complete set of structural proteins (CprME) expressed as a single polypeptide together with a modified NS2B/NS3 protease complex. In order to optimize authentic protein processing, trafficking and particle assembly, we introduced specific mutations, substitutions and a deletion within the structural and non-structural proteins. We mutated the furin recognition site (E88A) in prM protein to enhance cleavage and protein processing as well as introduced changes in the helical domain 1 of the E protein (I398A, M401A, and M412L) to improve its amphipathic properties and thus enhance trafficking and secretion of E. Lack of furin cleavage and retention of the E protein by its helical domains may impede protein transport and the assembly of mature particles. Furthermore, the C-terminal of the NS3 protein was truncated retaining only its N-terminal protease domain that contains a mutation (L115A) to enhance its catalytic activity. The NS3 protease domain was genetically linked to its cofactor NS2B and expressed as a single polypeptide in order to maintain enzymatic activity. We selected this approach rather than replacing C with a heterologous signal peptide for protein translocation into the ER, as has been reported (38, 39) because our optimized CPrME construct spurred significant expression of the structural polyprotein, which was effectively processed by the co-expressed viral protease (NS2B/NS3) and cell host proteases. Co-expression of these proteins in suspension culture of mammalian cells resulted in the assembly and release of dengue virus-like particles (VLPs) into the culture media. Their structures resemble native dengue virus in shape, size and surface antigenic composition as demonstrated by negative staining and immunogold labeling electron microscopy with VLPs produced at 31°C.
[0164] Because of the temperature range of dengue virus replication (ambient in the mosquito vector and 37°C in the human host) and the observation that lower temperature increased the yield of dengue replicons (32, 33), we investigated whether the VLP production temperature enhances yield. This study revealed that lower temperature (31°C) not only improves VLP yield but also has a significant effect on the antigenic properties of the E protein displayed on the particle surface. VLPs produced at 31°C were strongly recognized by two distinct MAbs (4G2 and 3H5) that bind the conformational epitopes in domain II (4G2) and domain III (3H5) of the E protein. In contrast, VLPs produced at 37°C were recognized by neither 4G2 nor 3H5, revealing that the targeted epitopes were not properly folded in two different domains of the E protein. This finding suggests that production temperature has a significant effect on the folding and conformation of the E protein exhibited on the surface of the VLPs. Several studies have demonstrated the effect of different temperatures on dengue virus structures. Detailed cryo-electron microscopy analysis of dengue virus showed that mature virus produced at 28°C is smooth, however when incubated at temperatures higher than 33°C it appears bumpy, with an increase in diameter and heterogeneity (40, 41). Although it has been reported that MAbs react differently with dengue virus of distinct conformations (42, 43), the role of dissimilar conformations of E in the elicitation of neutralizing antibodies has not been completely elucidated.

[0165] In our vaccine studies, we have shown that VLPs produced at 31°C elicit higher neutralizing antibody titers than those produced at 37°C. This finding indicates that the TVXDO-31°C vaccine exhibits a better conformation of E as was initially recognized with the MAbs 4G2 and 3H5. This structure appears to displays a larger repertoire of neutralizing sites, which is evident in the stronger neutralizing response stimulated by this vaccine. Furthermore, the higher neutralizing activity stimulated by the TVXDO-31°C VLP vaccine cannot be merely attributed to the sites recognized by 4G2 and 3H5 because these antibodies are not highly neutralizing, suggesting that additional epitopes capable of stimulating neutralizing antibodies are also exposed. Neutralizing titers were markedly enhanced with the addition of an adjuvant and the improvement was significantly higher with both doses (1µg and 5µg) of TVXDO-31°C (PRNTs: 371 and 1067) as compared to equivalent doses of the TVXDO-37°C vaccine (PRNTs: 57 and 382). To provide context to these PRNT
results, we tested a human anti-DENV-2 convalescent serum (NIBSC/WHO) and found that it had lower neutralizing titers than that obtained with either dose of the adjuvanted TVXDO-31°C vaccine or even higher dose of the TVXDO-37°C vaccine. The difference in neutralizing power between the two VLP vaccines (TVXDO-31°C and TVXDO-37°C) at either dose with and without adjuvant illustrates intrinsic distinctions in the antigenic conformation of the E protein of the VLPs with better display, arrangement or frequency of epitopes in the TVXDO-31°C vaccine. It is even possible that these conformational attributes arise after inoculation as the consequence of molecular breathing at the higher temperature of the recipient host (37°C). In addition, the display of quaternary epitopes that result from adjacent domains of two surface proteins may further explain why the VLPs produced at 31°C elicit higher neutralizing titers, since molecular breathing may also impact the quaternary structure of the VLP. Reactivity with MAb 4G2 suggests better E protein folding, which correlates with quaternary epitope display as has been reported (42, 44-46). These results highlight how conformational differences of the major dengue surface antigens affect the elicitation of neutralizing antibodies. Given the significance of the level of neutralizing antibody against dengue needed to achieve protection, these findings may be of great significance in dengue vaccine design and development. While other dengue VLP vaccine studies have reported efficacy, some of these preclinical tests used significantly higher doses than the one used with our VLP vaccine formulations (100μg versus 5μg) (38) and the assays used to assess efficacy were not comparable to our PRNTs. Thus, it seems impractical to compare the effectiveness of our TVXDO-31°C or TVXDO-37°C with other vaccine constructs. The convalescent human DENV-2 serum standard may provide the best benchmark for comparison.  

[0166] In summary, this work not only describes a new strategy for VLP based dengue vaccine development but also shows the effect of temperature on the VLP E protein conformation and how these structural dissimilarities dictate the neutralizing antibody response, which is higher with VLP vaccines produced at a lower temperature. These findings hold great promise for the development of a VLP-based dengue vaccine.  

Example 2: Zika VLP Production
Emerging viral infections are those that either newly infect the human population such as Zika or rapidly disseminate increasing the geographical range of infection and the number of cases of disease, as is the case with dengue. One of the most effective countermeasures to fight these arthropod transmitted viral infections is prophylactic vaccination. We have developed a flavivirus VLP vaccine platform technology to generate vaccines against these pathogens and using it to produce a Zika vaccine.

Zika fever disease results from an infection with Zika virus (ZIKV), which is transmitted to humans by the bite of an infected Aedes mosquito (A. aegypti, A. albopictus and polynesiensis). Zika virus was isolated for the first time from a Rhesus monkey in the Zika Forest in Uganda in 1947 and later from humans in 1952 [2].

ZIKV has been transmitted in Africa for many years through a sylvatic cycle between mosquito vectors and nonhuman primates, with occasional human infections [3]. In recent years, however, epidemics of Zika have resulted from cycles of transmission between vectors and humans resulting in the spread of disease beyond the African continent into French Polynesia and other Pacific regions [4-6]. Since 2015 a dramatic spread of ZIKV that began in Brazil is taking place in South America and the Caribbean Islands with the occurrence of sporadic cases in travelers identified in the USA and Europe [7].

Zika infection appears to cause a mild illness in most people infected, however, contracting the virus during pregnancy is associated with birth defects, primarily microcephaly (defective brain development). Furthermore, an increase in cases of Guillain-Barre syndrome has been observed following ZIKV infection. The seriousness of these disorders imposes a tremendous burden on public health. In addition to vector transmission, ZIKV is also likely transmitted via sexual contact [8], this fact taken together with its often asymptomatic nature makes disease control more difficult.

Zika virus is a member of the flavivirus genus within the Flaviviridae family. This is a large group of enveloped viruses including dengue, yellow fever, West Nile, Japanese encephalitis and others that possess a single stranded RNA genome of positive polarity, which serves as mRNA upon the infection of susceptible cells. The ZIKV RNA genome encodes only one open reading frame (ORF,
and translates into a single polyprotein that is co- and post-translationally cleaved by cellular and virus-encoded proteases into three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5.) that enable virus replication [9, 10]. Flaviviruses replication and morphogenesis occurs closely associated with intracellular membranes and nascent virions are assembled and transported through the secretory pathway and released at the cell surface. Enveloped virions are composed of a cell-derived lipid bilayer encapsulating the C-protein wrapped viral RNA genome and studded with multiple copies of the proteins E and M. During maturation within the secretory pathway the precursor prM protein is cleaved by the host cell furin protease to produce the small M protein and the fragment pr, which is released upon virus egress from the cell. The surface displays E protein as the major antigenic determinant of the virus and mediates receptor binding and fusion during virus entry. Therefore, this protein is a major target for vaccine development [11].

[0172] At this time there is no vaccine or specific treatment to control, combat or prevent ZIKV infection. The prevention of infection by vaccination represents a critical unmet need to control the spread and the effects of the disease globally. Development of a ZIKV vaccine is highly significant given the public health concerns raised by the dramatic spread of the disease, its possible effects and unclear epidemiology (birth defects, Guillain-Barre syndrome and sexual transmission).

[0173] We implemented our flavivivirus virus-like particle (VLP) vaccine platform technology to create immunoprotective countermeasures against Zika. VLP vaccines are produced in cell-based systems as structural and biochemical mimics of wild-type viruses, however, VLPs lack viral genetic material and are unable to replicate or cause infection. Therefore, vaccine inactivation is not required, better maintaining their antigenic epitopes and enhancing immunogenicity. The strategy to create flavivirus VLPs, and as an example Zika, is based on the simultaneous expression in mammalian cells of the structural proteins CprME together with a modified complex of the non-structural protein NS2B/NS3 protease to maximize assembly and production.

[0174] These polypeptides suffice for the efficient self-assembly and release of particles into culture media. Since only sequence information of viral genes is needed and because of the flexibility, speed and safety of the technology, vaccines
against emerging viruses such as Zika can be generated rapidly and without risk of disseminating infectious material [12-14]. Furthermore, VLP vaccines can be manufactured via transient transfection of mammalian cells with an expressing plasmid or by using engineered and selected high producer stably transfected cells. This later approach will allow not only for the continuous vaccine production to the desired scale but also for the storage of specific vaccine producing cell lines that can be activated at the time, location and levels required. In addition, this technology allows for the generation of combination vaccines via either blending distinct VLPs in a single formulation or by assembling chimeric VLPs following the co-expressing of E proteins from different pathogens or serotypes.

Results

[0175] Transient transfection of the structural protein CprME and non-structural protease complex NS2B/NS3 lead to secretion of particles. Purification of ZIKA VLPs by ultracentrifugation through a potassium tartrate (10-35%) / glycerol (7%-30%) linear demonstrates a similar migration pattern as the ZIKV when gradient fractions were probed with an anti-E specific MAb, as shown in Figure 12. Furthermore, examination of E positive gradient fractions of negative staining electron microscopy (EM) showed the presence of spherical particles (~60nm diameter) that closely resembled the structure of the wild type ZIKV. (Figures 11A, 11B, 11C). To assess whether the E protein was present on the surface of the VLPs, we evaluated the same ZIKA VLP fraction by immune-gold labeling EM using an anti-E MAb as primary and an anti-mouse gold bead conjugated secondary antibodies, respectively.

[0176] This study demonstrated that indeed the E protein was detected on the VLPs as shown by the presence of beads on the particles surface (Figures 11, 11D, 11E, and 11F). In addition, Western blot analysis of ZIKA VLPs and wild type Zika virus revealed the presence of a correct size E protein in both VLP and virus samples when probed with anti-E antibody, Figure 13. These data demonstrates that transfected cells release VLPs, which resemble native Zika virus and display the E protein a major antigenic target for the elicitation of neutralizing antibodies.
The immunogenic attributes of alternative ZIKA VLP vaccine formulations are assessed in mice to evaluate the quality and magnitude of ZIKA virus specific neutralizing antibody response.

Based on the high neutralizing antibody titers elicited by a monovalent dengue-2 VLP vaccine, assessed by plaque reduction neutralization assay (PRNT) (Figure 10), a ZIKA VLP vaccine should also stimulate a robust virus neutralizing antibody response. Vaccine for flaviviruses based on the VLP platform technology offers a safe, effective and scalable system and warrants further development, particularly for the Zika virus problem.

REFERENCES


SEQUENCES

Exemplary sequences are shown below. It will be apparent that the same proteins can be used from any flavivirus, for example by aligning the sequences to those disclosed herein. The particular amino acid residues identified herein are numbered relative to the indicated sequences but their relative numbering in other flaviviruses can be readily determined, for example by alignment.

Dengue virus 2 Strain 168861 (GenBank U87411.1)

1. DEN-2 CprME wild-type nucleotide sequence (SEQ ID NO:1)
ATGAATAACCAACGGAAAAAGGCAGAAAACACGCCTTTTAAATATGCTGA
AACCGGAGAGAAAACCGGTGCTGACTGTGCAACAGCTGCAAAAGAGATT
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GTTTGGAGAACAACAAATGGAGGGGCGGCAAGAAGATGCCCCATTTAGGTGAC  
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GGCTCTCCACAAACATTGTGCGACATCTATGAGCTGCAGTGGGT  
TTCATGGACATATGAAAAATTCTCATAGGAGATCTTACGATAGGAA  
TAGAATTCACGCACACTCCTACTGTCGTGGACACTATGATTGTTGGAATTTG  
TAGAATGCTTATGGGACGTATGGGACGCCC

2. DEN-2 CprME wild-type amino acid sequence (SEQ ID NO:2)

MNNQRKKAKTNPFNMLKR ERNRVSTVQQLTKRFSGLMLQGRPLKLFLMAL  
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IIMLLPFTVMAFHLRTTNGEPHMIVSRQEGKSSLLKLDFTAGVNMCTLMAMDLG  
ELCEDITYKCPNRQEPEDIDCCNWSTTWYGTCTTGMHEERKEKRSVA  
LPVPHVMGLERTETWMSSEGAWKVQRRIETWILRPFTMMAAILAYTIG  
TTHFORALISLEFTAVTPSTEMGRCMSGNRDFVEGGSWVDIVLEHSGCVT  
TMAKNPKTLSFELIKTEAKQPATLKRUCIEAKLTNTTPERSRCPQTQEPSSLNEE  
QDKRFFCKHSMDVDRGWNGCFLGKFKGGIGVTACMRCKNNMGEKVKVQVQPEN  
LEYTIVTPHSGEHAVGNDTGKHGKEIKTPQSSITAEILTYGTVMTESCPR  
TGLDFNEMVLQOMENKAWLVHRQWFLDLPLPWLPGATDQGNSWQKETLKV  
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DLKQLKGMSSMCTGKFKVVKWEIATQHGTIVIRVQYEGDGSPCKIPFIMDL  
EKHRVLGRILTVNVPIEKEDFPGDSYIIIGVPEQQLNWFKGGSS  
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3. DEN-2 CprME wild-type nucleotide sequence with Mutations (SEQ ID NO:3)

ATGAAATAACCAACGGAAGAAAAAGCGCAGAAACACGCCTTTCAATATGCTGA
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CATCTGCAGGAATGTCATATTCATGTGAGATCTCAGACCTGGAGGTCGCCC
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4. DEN-2 CprME codon optimized nucleotide sequence with Mutations

(SEQ ID NO:4)

ATGAACAACCAGCGCAAGAGCCAAAAACACCTCCGTCAATTATGCTCAA
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GAGAGAGCGCAATCGGGTTTCTACCGTGACAGCGACGCTGACGAAGAGATTTCT
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GGCGTGAGGTGGCAGAACCTG

5  5. DEN-2 CPrME amino acid sequence with mutations (SEQ ID NO: 5)
MNQRKKAKNTPFNLKRNVRNVTSTVQQLTJRSLGLMLGGRPLKFLMAL
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IIMLPTVMAFHLTTTRENPEHPHMVRSSQEGKKSFLKFTEDGVMCTLMADMGL
ELCEDDTITYCPLLRQNEPEWDICWCNSTSTWTVYGTCTTCTMGHERAKRVS
LVPVHVGMLERTRTETWMSSEGAWKHVQRHETWILLRHPFGTMMAAILAYTIG
TTHFQRALIFILTTAVTPSMTMRICGMSNRFDEFGVSGSWVDLHEHSCVT
TMAMNKPTDLFEIKTEAKQPATLRLKCYEIAKLTNTTESRCPTOEPSLNEE
QDKKRFVCKHSMDRGWRNGCGLFKGIIIVTCAMFRCNNMEKGQVQPEN
LEYTVITPHSGEHHAVGNDTGHKHEIKITPQSSITAEALTYGTVTMECSPR
TGLDNSYMVLQENKAWLHRQWFLDLPLVWPLGADTQGNSWQIKETLTV
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DKLQLQKGMYSDCTGKFQVKEIQHTQGTVRQVGRDGPLCIPFEIMDL
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LGQAFETTRMRGAKRLAIGDTAWDFQSLGGVFTSIGKALHQQVFGAIYGAFAFS
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25  6. DEN-2 NS2B3NS wild-type nucleotide sequence (SEQ ID NO:6)
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GGACGTCAGAAAGACACATATATCATAATAGGAGGTGGCAGCAAGTTAGAA
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7. DEN-2 NS2B/NS3 wild-type amino acid sequence (SEQ ID NO:7)
MSWPLNEAIMAVGMVSLASSLKDIPMTGPLVAGGLTVCTYVLTGRSADL
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ALKEFKEFAAGR

8. DEN-2 NS2B/NS3Pro codon optimization nucleotide sequence with mutations (SEQ ID NO:8)
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ATGAGTTGGCCTCTGTAACAGAGGCAATAATGGCGGTTGGGATGGTGACAT
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AGATGTGAAAGGAAAGGAGAGAGGAGTCAGTCCTGCTGAAAGCTGGAAG
GGGAATGGAAGGAGGAGAGGAGTTCAGTCCTGCTGAAACCCAG
GTAAGAATCCCCCGCGCGGTCGACCAAGAACCCGCGGCGGCTTCAAGACTAAT
GCCGGAAACCATCGAGACCTGTCTCTTGGATTTACGCAACCGACCTCCCG
GTTTCATCATCGACAAAAAGGGCAATGCTGGCTGCTATGGGAATGG
GGGTGGTGACGCAGCTCAGGAGCCTATGTTTCTGCAATAGCTCAGACCGAG
AAGTCAATCGAAGATAACCCCGGAAATCGAAGATGTAG

9. DEN-2 NS2B/NS3Pro amino acid sequence with mutations (SEQ ID NO:9)
MSWPLNEAIMAVGVMVISLASSLLKNDIPMTGPLVAGGLLTVCYVLTGRSADL
ELERAAADVWEDQAEISGSPILSITISEDGSMIKNEEEEQTLTILIRTGLLVIS
GLFPVSPITAAAAYLWEVKKQRAVGVLWDVPSSPPMKGAELLEDGAYRIKQK
GILGYSQIGAGVYKEGFFHMWHVTRGAVLMHKGKRPSWADVKKDLISY
GGGKWLEGEWKEEVEQVLALEPKKNPRAVQTPQAFKTNAGTIGAVSLDF
SPGTSGSPIIDKKGKVGYGNGYVGVRSGAYVSAIAQTEKSIENPEIEDD

10. DEN-2 NS2B codon optimized nucleotide sequence (SEQ ID NO:10)
ATGAGTTTGCCCTGTAACGACGGAATATAATGCGGTGGGATGTTGACAGAT
ACTTGCAATACGCGCTGCTGAAAGGACACATCCCTATGACTGCTTCCTGGT
GGCGGCGCGCCCTGCTGACGCTCTGGTATATTGCTGACGCCGACGTCCGACAG
ACTTTGGAAGTGGGAAAGGCTGCCGACGTAAGTGGGAGCGACAGCAGCA
AATTTCCAGGAAAGACCTCCATCTGAGTATCACAATTTCCGAGCAGGTT
CAATGTCCATCAAGAATGAAAGGAGAGACAGACTGACCATACTGATT
CGACCCGCGCTGTTATATGAGCTGGCTTCCTCTGTATCTATCCCTATCA
CTGCCGCCGCCCTTGGTATCTCTCTGGGAAGTAAAGAACAGCGGCGAGCGTAC
CTCTGGGATGTGCGCTTCCCCCACAATGCGAACAAAGGCGGAATGGGACAGAG
CGGTGCAATACGCATTAAAGCATAACCTCGGATACAGCAGATTG
GAGCCCGGGTGTATAAAAGAAGGAACTTTACATACATAGTGGCAGCTGACT
AGAGGGGCGGTCTTATGACAAAGGTTAATAAGGACATCCATTGAGCAGACAG
AGATGTGAAAGGAAAGGAGAGGAGTTCAGTCCTGCTGAAACCCAG
GTAAGAATCCCCCGCGCGGTCGACCAAGAACCCGCGGCGGCTTCAAGACTAAT
GCCGGAAACCATCGAGACCTGTCTCTTGGATTTACGCAACCGACCTCCCG
GTTTCATCATCGACAAAAAGGGCAATGCTGGCTGCTATGGGAATGGGAATGG
GGGTGGTGACGCAGCTCAGGAGCCTATGTTTCTGCAATAGCTCAGACCGAG
AAGTCAATCGAAGATAACCCCGGAAATCGAAGATGTAG

11. DEN-2 NS2B amino acid sequence (SEQ ID NO:11)
MSWPLNEAIMAVGVMVISLASSLLKNDIPMTGPLVAGGLLTVCYVLTGRSADL
ELERAAADVWEDQAEISGSPILSITISEDGSMIKNEEEEQTLTILIRTGLLVIS
GLFPVSPITAAAAYLWEVKKQRAVGVLWDVPSSPPMKGAELLEDGAYRIKQK
GILGYSQIGAGVYKEGFFHMWHVTRGAVLMHKGKRPSWADVKKDLISY
GGGKWLEGEWKEEVEQVLALEPKKNPRAVQTPQAFKTNAGTIGAVSLDF
SPGTSGSPIIDKKGKVGYGNGYVGVRSGAYVSAIAQTEKSIENPEIEDD

12. DEN-2 NS3Pro codon optimized nucleotide sequence with mutation (SEQ ID NO:12)
GCAGGCCGTACTCTGGGATGTCCTTTCCCCCCACCTATGGGAAGGGGGA
ACTGGAGGACGGTGCTACTCATTGCAAAACGGCAGCTCGAGATACA
GCGGACGTCGAGCCGGGGTCTATTTCAATACAGGTGATGAACTGAG
CACGTGACGGAGGGCCGGGGTCTATTTCAATCGAAGGGATGAAACC
ATCCGGCGAGATGTAAGAACACGTGAGTCTCAGTCCCTTGGCCCT
GGAACCCAGGTAAAGAATCCCGCCGGAGTACAGCAAGGGCAGCTCTC
AGACTAATGCGGGAACCATCGGAGCTGCTTCTCTTGAGATTCAGCCAG
ACCTCGGTTCTCCTACATGCAAAAAAGGGCAAGTGTGGGGTCTCTA
TGAGGAATGGGTTGTTGACGCGCTAGGACCTATATTTCTCTGCTAGAT
AGGCCAGAGTGAATCCGAGATGAACTGAG

13. DEN-2 NS3Pro amino acid sequence with mutation (SEQ ID NO:13)
AGVLWDPSPPPMPMKAELEDGAYRIKQKGILGYSQIAGAVYKEGTFTHWMVH
VTRGAVLHMKGRKIEPSWADVKKDLISYGGGWKLGEWKEEGVQVLAE
PGKNPRAVQTPKPGAFKTNACTGAVSLDFSPGTSGPIIDKKGKVVGLYNGV
VTRSGAYVSAIAQTEKSIEDNPEIUED

14. DEN-2 nucleotide sequence (SEQ ID NO:4)
GATAGTGCTGCACTTACCTGAAATAGGCTGGCAG
TGGGAAAATTTCGAGTGGTGCTGCACTTACCTGAAATAGGCTGGCAG
TCCAAACGAGACTCGCTACGTACACATTACATGGAATAGGCTGGCAG
GAAAGGCGAGATGGTATGGGAATAGGCTGGCAG
TGGGAAAATTTCGAGTGGTGCTGCACTTACCTGAAATAGGCTGGCAG
TCCAAACGAGACTCGCTACGTACACATTACATGGAATAGGCTGGCAG

15. DEN-2 NS1 wild-type amino acid sequence (SEQ ID NO:15)
DSGCVVSWKNEALCGCGSFIFDDNVHTWEYKFQFPEPSKLASAIQKAHEE
GICGIRSVTRLENLMSWQITPELENHILSENVKLTIMTGDIKIMGQAGKRSLRP
16. DEN-2 NS2A wild-type nucleotide sequence (SEQ ID NO:16)
GACATGGCGAGGGTGCAGAATTTTCATAGGACTTGGGAATGGCATT
GTTTCCCTAGGAAATGCCTAGGACCCGAGATGAAGGGAAACATGGCAATAC
TACCTAGTGCAGTTTCTTTTGACATCTTGTACACAGGGAAACATGGCTTT
GAGACTCTGGGAAAGATGGTAGATGGTAGATGGACTGGAAGTGGCACCTATGAC
GATAGGTATGCGGCGTACTATCTTTGCACTAGCAGCTTTAAGAGTCA
CCAACCTTTTGCAGCTGACACTCTCTTGAGAAGAGCTGACCTCCAGGAATT
GATGATGACACTATAGGAATTGTACCTCTCTCCAGAGCCATACAG
AGACACATTCTTTGAGTGCTAGCTGATAGCTGTCTCTACA
AAATGGTGAGAAATAAGTGGAAAGATCAATTGCGACTATCATGCT
ATCTTTGCGCTCACCAGCAGTGATATTACAAACAGCAGTAGAAAGTGA
TTGCAAAATATTGGGGCTGAGTTGCCTCCCCACGTGCTTAAATCTATT
ACACGAAAAACAGATTGGATACATTAGCTAGCTGGACATCAAGAGTCTCA
ATCCACAGCTATTTTTCTAAACACCCTCTCAAGAACCAGCAAGAAAGG

17. DEN-2 NS2A wild-type amino acid sequence (SEQ ID NO:17)
GHGQVDFNSFLVGMAFLLEELRTRGVGTKHAILLIVAVSVFVLTGNSFRD
LGRVVMVGATMDDGMGVYLLALLAFAKVRPFAAGLLRLKTSKELM
MTTIGIVLSQSTPPETILELTDALALGMMLKLMVRNMEYQLAVTIMAICV
PNAVILQNAWKVSTLAVVSVSPLLTTSSQKTDWPLALTIGGLNPTAIFLT
TLSRTSKKR

18. DEN-2 NS3 Full length nucleotide sequence (SEQ ID NO:18)
GCCGAGATATTGTGGGATGTCTCTCCACCCCCACCCATGGGAAAGCTGA
ACTGGAAAGATGGGACCTATAGAATTAAAGCAAAAAAGGATTTCTTGAGATATT
CCGAGATCGGACCCGGGATTTACAAAGAAAGGAACTTCCATCAAATGTGG
CATGTCACACGTGGCTGCTTAAATAGCAAAAAAGGAAAGGATTTGCAACC
ATCATGGGCAGCAGTCAAGAAGACCTAATATCATATGAGAGGAGGTGAG
AAGTTGAAAGGAAATGGGAAAGGAAAGGAAAGAAGTCCAGGTATTGCGAC
TGGAGCCTGGGAAAAATTCCACAGACCCTGCTACAACCAGAACCTGTGGTCTTC
AAAACCAACGGGCAACAAATAAGGTGCTGTATCTCGGACTTCTCTCCTGG
AACGTCAAGATCTCACAATATGCAAAAAAGGAAAGGTGCGTTCTCT
ATGGTAATGGTGGTGTGTAACAGGAGTGGGAGCATATGTGAGTCTATAGCC
CACGACTGAAAAAGCAACTGGAAGACACCCCCGAGATCGAAGATGGAATTTT
CCGAAAGAGAAGAGCTACATAGGACCTCCACCCAGGAGGAGGAAAG
AGCGAGAGGATACCCTCCGCGCACTAGCAAGATCATAAAACCGGGGGTTT
GAGAACATATACTTTGGCCTCCACTAGGTGTCGGCAGTGAATGGAGG
AAGCCCTTAGAGGCTTTCCACCAAAGATACCCAGACCCCCACCATCAGAGCT
GAGCAGAGGAGGAGAATTGTGTGACCTAAATGTGTCTGATGCACATTTAC
CATGAGGGTGCTATACCACTGGAGTGGCCAAACTACAACTCTAGGATTATCA
TGAGCGAAGCCCATTTTACAGCCAGCAAGATATAGCAGCTAGAGGATAC
ATCTCAAGTCAGTGAGAGGTGGTGCAGGAGCGCAGCTGGGATTTTATGCAACGC
CACTCCCCCGGAGACCCATTTTTCCTACGAGAACCAATCGA
TAGATGAAAGAAAGAGAATACCTCTGAAAGCTTGAGATTCGGGACATGAA
TGGGTCAGGATTATAAGGGAAGACGTTTTGGTCGATGAAAATGAA
AGCAGGAAATGAGATAGCAGCTTGAGGAGGAAATAGGAAAGAGATG
ATAACAATGCTAGGAAGCCCTTGGAGTCTGAGTCAAGCTAGAAC
CAATGATTTGGGACTTCGTGTGTTACAACTGTGACATTCAGAAGATGGTGCCCA
ATTCCAAAGCGTGAAGGAGTTGATAGCACCCAGACGTGTGAAAGCCATGTC
ATACTAAACAGATGTTGAAAGACGCGGGTGATTCTTGGCGAGACATATGCCAG
GACCACACTCTAGTGCAGCAAAAGAAAGAGAGGGAGATAGGAAGAAATCCA
AAAAATGGAAGATAGGGCATGCTCATATACATATCTGGGGAACCTGGGAAATG
ATGAAAGACTGTGCAACACTGGAAAGAAGCTAATATGCTCCTAGATACATC
AACAGCAGGAAGAATCTCTAGTGCGAAGAGCCTGGTGA
GGTGTAGTGGCATTGTGGCAGAATACCGCTTGAGAGGAGGAAGCAAGGAA
ACCTTTTTGAGACACTTAAATGAGAAGAGGAGACCTACAGCTGTTGGCCCTA
CAGAGTGCCAGTGAACGCCATCAACTACGCCAGAAGGTTGGTGTCTGG
ATGGAGTCGAAGAAACACCAAATCTTCTAGAAGAAAAAGTTGGAGATG
CTGGACAAAAAGAAAGGGAAGAGAATAGGAACAGGATTTGCGGAGAT
GCTAGGATCTATTTTCTGACCCACTGGCGCTAAAAGAATTTAAGGAAATTTGCG
AGCCGGAGGAAG

19. DEN-2 E protein amino acid sequence with mutations (SEQ ID NO:19)
MERGMSGNRDFVEGYGVSGLSVDFLHEGSCVTMMIKNKPTLDPEKLITEAKQ
PATLRLKYCEAKTLNTTTESRCPTQGEPSLNEEQDKRFVCKHSMVDRGWNG
CLGFLGKGGIVTCAMFRCKKNMEQKVQPENLEYITIVTPSHGEEHAVGN
DTHKKGHEIKITPOSSITEAEILTGTVTMESPRTRGLDFNEMVLLQMEMKAL
VARQWFLDLPLPWLPAGATQGNSWIKETLVTFKKHPKQDVVVLGGSQEGA
MHTALTGATEIQMSSGNLNFTHKLKRLMMDLQKSLKGSYMSTMCTGKFVVK
EIAETQHTGHTIRVQRQPSKCIPEFIDLEKRHLGRLITVNPITEKDSVP
NIEAEPPFSGSYSIIIGVEPQQLKLNWFKKGGSSLQFETTMRGAKRILLAILGD
TAWDFGSLGGVFTISIGKAIHQVHFQGAYGAAFSGVSWTMKILGVITIWGMNSRS
TSLSVTLLVGLTVLYLGMVQA

20. ZIKV CprM wild-type nucleotide sequence (SEQ ID NO:20)
ATGAAAAACCAAAAGAAATCCGGGAGGATTCCGGATTTGCAATATGCT
AAAAACGCGGAGTACCCGGCACTGGACCCCTTTAGGGGCTGAGAGCTGC
CAGCCGGACTTCTTGCTTTGTTGCTGATGGGCTACATCGGCTATGGCTGTG
AGCCTTTTGTGAGATTAAGGCAATCAAGCCATACGGGCTGGTCTATCAATA
GATGGGGTTCTAGGGGAAAAAGAGGCTATGGAAATAATAAGAAAGT
CAAGAAAGATCTGCTGCCCATTGTGAGAATATCTGCAAGAAGAG
AAGAAGAGACAGGGCGCAAGATACTAGTGTGCAATTGTGGGCTCTGTGCT
GACCACAGCTATGGCAAGCAAGAGTATGTCGATGATGTCGATATATA
TGTCATTGGACAAAGAGCGCTGAGGAGCCATATTTTCAACCCACAA
TTGGGAGTAATGAATGTTTTATATACAGTATGACTTGAGCTTGGGACAG
TAGTGATGCCACCCTAGTCTATAAGCCTATGCTGATTGAGGGGTTGGAAC

77
CAGATGACGTGATTGTGTTGTGACACACACGACGTCACAATGGTTGTGTGAC
GGAACCTGCCCATTCAAAAAAGATGGAAGCAGGAGATCTAGAAGAGCTG
TGACGTCCCCTCCATCCATCAGGCTAGCTGAAGATGACGCTGAAACC
TGTTGGAATACAGAAGGATATAACAGACATGTTTGAGCTGATGACATG
5
GATAATCCAGAACAAGGCTCTGTCGTAAAGCAGATCGCTACTGTTCCTGGC
TTGGGAGCTCAACACAGGCACAAAAAGCTCATTACCTGTGACTGACTGCT
TGATTTGGCCAGCATACGCAGCTACAGTGTGACATGGATGCACATAGGAC
TTTGTTGAAGGTATGTTAGGTGGAGACCTTGTTGTTGAGTGTTCTTGGAAACAT
GGAGGTTTGTCTACCGTAAATGCGACAGACACAAACGACCTGTGACATAG
10
GCTGGTTAACACACACAGTCAAGCAACATGGGCGGAGGATAGATCCTACTGCT
ATGAGGCACTCATATCGACATGGCTCGGCAGACGGCCTGCCCACACACAA
GGTGAAGCTACCTTGAACAGCATACAGACACTCTTACATGTCGTCAAAAG
AAGCTTAAAGGGAAGAGACGGTGGAGAAGCTAGAGCACTTTTGGCACAAG
GGAGAGCTTGTGTGCTACGACGCTTTCTATAGTTTTGACGTGCTAATACAA
15
AAGAGCATCCACGCGAGAGAACTCGGAGTACCCGATAATGCTGTCAGTCA
TGCGTCCCCAGCACGATGCTGATGTAGAATAGACACAGACATGAAACTG
ATGAGAATAAGAGCAAGTTTAGTGAATAAGCAGCCCAATCCACCAAGAGCGCA
AGCCACCTACGTTGAAGCTAGAGCAAAGAGCTAGTTTGAGGAGTGCT
20
GATTGGTTCACAAGGAGTTGAGGCTTCCACAGCAGACATTCCTACTTATCCGCTG
GGGCAAGACCCGGAACCTTGAGAAGGACACATGGGAAACACAAAAAGACGTGATGA
GTTCAAGGACGCACATGCAAGGCAAAAGGCAAACACTGTGCTGTTCTCTGACTG
25
AAGAAGGACGAGCAGCTTCCAGCAGCAAGCCCATGCTGAGTGATGACGCTGCA
AGTGCTGCAAAAGGGAAGGAGCTGTCCTCTGCTGGCCACTTTGAATGTGCCTGGA
CTAACCCTGTAATCAGAAGACTGAGAATCTAAGATGATGCTGCTGAA
30
CTTGGATCCACATTGTTGGAAGAGACTTCTACATGGTACAGTGGGAGAA
GAAAGATCACCACCTGTCACAGGAGTTGCGAGACACCATTGGAAGAAGCAG
TTTGTAAAGACACTGTGAGAGCTGCAAGGAATATGAGCAGCTGCTGGAGACAC
AGCCCTGGACATTTTGAGATTTGGAAGGCGCTCCTCAACACTCATTGGCCAAGG
GCACTCCATTAAATTTTTGGAAGCAGCATTTCAAAAATCATTTGTTGAGGAAATGT
35
CCTGGTTTCACAAATTTTATCTGGAACAGTTTGCTAGTGTTGCTTGCTGA
ACACAAAAATGGGATCTATATTTCCCTTATTTGCTGTTGGCCTTAGGGGAGATGTG
TGATCTTCTTATCCACAGCTGCTGTCTGCT

21. ZIKV CprME codon optimized nucleotide sequence (SEQ ID NO:21)
ATGAAGAAACCAAAAGAAGGATTCGCGCGGTCCGAGATCGAATGCT
GAAGAGAGCGCTGCGACAGATCGACCCCTCTGCGGAGACATGAAAGCATG
CCTGCAGACTGCTGCTGTTGGCACGCGCTATTAGAATGGTGTGCTGCGCAT
CCTGCGTTTCTGCGGTCTCAACGCGGATCAAGCCCTCTTGGGCTGTACAA
45
CAGATGGCGCGACAGGCAAGAAAGAAGGCGCATGGAATCATCAAGAA
TTCAAGAAAGGACCTGCGCGCCCATGCTGCGGAATCATCAAGCAGCGCC
GAAAGAGCGCAGAGGGCGCGGATACCTCCTGCGGGATTTGGGGCTGTGCG
TGACAACAGCCATGGCGCGCGAAGTGACAGCAAGAGGCAGCGCTACTA
CATGTACCTGGACCGGAATGAGCGCGGCAGCGCAGCTACGCTTCCACACCA
CCCTGGGCGATGAAACAGTGACTCATCCAGATCATTGGACTGGCCACATG
TGCGACCGCCCAAGATAGCTAGCTAGCTGCAAGGCACTGAGAGACGGCTGGA
ACCCGAGATGTTGAGTTGACTGCTGGTGCAACACCACCAGCCCTGGTGTG
ACGGCACACTTCCTGGAACCCCGCTGGTCAATTCGCGTCGGCTGA
GGCGAAGGTGCAACGACCAGCCAGAAGATGATCTACCTGGTACATGC
CTGCTGATGCCTGACCCTGCAAGACTGCGGCTGTCCAGCTGGGA
GACTTGCTGGAAGGAGCTAGAGCGGCGACATGGGACTGTGCTGTGG
ACACGCGCGCTGTTGACCCGATGGCCAGTAAACCCACCAGTGGCA
TCGAGCTGCTGCAACTCCCGTCAAAATCGCAGGGCAGTGGAGGCT
TGTCAGACGAGCCGACTCACGACAGCAGCAGATGCCCTACAC
ACAGGGCGCAGGCTCATTACGGCAGAAGATGCTGCAGCTGAGCG
CGCAAGAGACATCCAGCCCAGAATACCGGATCATGCTGAGCG
TGACAGCAGCAGACTCCCGATGATGTAAGACTGCAGCAGAGCAG
ACAGACGAGAACGGGCAAGTGGGAAATCACCCTTAACAGCCTAGAG
CCGAGGCCACACTGGGCGGCCTTGGATCTCTGGGACTGGCACTGCCGAGCCC
CGGACGCGCTGTTGCACTCCCGTCAACGGCAGATGCAGCTGAGCG
ATGGCGCGCTGTTGCACTCCCGTCAACGGCAGATGCAGCTGAGCG
CTGAGATGGAAGCTGAGCCTAGCATCATCTGTAGAGCTGAGCAG
GCTAGATGGGAAGCTGAGCCTAGCATCATCTGTAGAGCTGAGCAG
CCAGCGCTTCCGAAGGGAGGCTGTGCTACGGCTGCTGCAGCTGAGCG
AGGGCTTCCGAGCTCATAGGGGAGGCGACAGAATGCGCGTCGGG
CGATACAGCCTGGAATTCTGTGGCTGCTGCAGCGCTGCTGCAGCTGAGCG
GCAAGGGAATAACCCAGATCTCTGGGAGCCGCCTTTAAGAGCCTGTTCCGC
GGCATGGTCTCTGTGGACCCCTGAGCTGAGCTGAGCG
CGGAGTCGCTGATGCTGAGCGCTGCTGCAGCTGAGCG
22. ZIKV CprME amino acid sequence (SEQ ID NO:22)
MKNPKKSSGGFRIVNMLRGRVARVSPFGGLKLRLPAGLLLHGHGPIRMVISLAIL
AFLRFTAIPSLGLNIRWSGVKKEAMIEIIKFKDDALMLRINARKKRRG
ADTSVGIYLLTTTAMAAEVTRRGASAYMYLLDNRDAGEIASTPLTGLMNKC
YIQMDLGHMCATMSYECPMLDEGVEPDVDWCNTSTSWVYTGCHHK
KGEARRSRRAVLTSPHSTRKLQTRSQTWLESREYTKHLIRVENWIRPFGAL
AAAAIAWLLGSGSQKVIYIVLMMIAPAYSRCIGVSNRDFVEGMSGGTWVD
VVLHGCGCVTVMAQDKPTVDIELVTTVSNMAEYRSCYEASIDMSADSRC
23. ZIKV NS2B/NS3 Full length nucleotide sequence (SEQ ID NO:23)
AGCTGGCCTCCCTAGCCAGATCATCAGCTACGTTGGGCTGATATAGCGCAAT
GGCTGGACGGGTTCGCAAGGCGATAGATAGATGGTGAC
CGGTGCTCGTCTCATAATTTGCAGTTACGTGGTCCTAGAGAAAGATGTGGAC
ATGTACATTGAACGGATGGAGCATTACAGCATAGAGGAAAAGTGGGAAAG
TCACCTGGAACAGTCCCCGACTGGTAGTGCCAGATTAGAGAGTTGGAT
TTCTCCTGTGTTAGACGGCTGTTTCTCCTACATGGGACTGCA
GGTGTTCTGATGACCATCTGTGGCATGAACCAATAGGATACCTACCTTTG
AGCTGGAGCGTGTAGCTATACGTGAAGACTGAGAAAAGGAGATTGGGCTC
TATGGGAGTGTGCTGCTGCTCCAAAGGAATGAAAAAGGAGACCCAGAT
TGAGCTATAGATAGAATGGCTGAGCGTCTGCTTCACACACAAAGTTG
GAGTGGAGTTAGTGCAAGGGGCTTCTTCCTACATGGGACTGCA
AAGAGATCGCCGCTGAAGAAGCGGTGAAAGGAGACTGATGCCATACGG
25 GAGATGTCACAGCAGAGTCTGTCTGAGATGGCAAGTGAGCTGAC
GCCCGCTTGAGGGGCAACACCGAGGAGCTGGGCTCCTGCCCCGG
AGAGAGAGCGAAGACATTGAACACATGCTGCTGCTGCTGAGAAATGGAGGAAG
GATGGCCACATTAGGCGGTTCGGCTGGATACCCAGGAACTCCAGGG
ATCCTCAATCTCAGAAACGGATGTGGAAGATGAGACTTTATGTGCAATG
30 GGTCGTGATCAAAAATGAGGATTGTGTGTGCGATCCACAAAGGGAG
AGGGAGAAGAGACCTCCTTTAGTGTCTCTAGCCCTAGTGGTAGAA
GAAGCATCATACCTTTCTGATCCTTTGGAGCTGTTGGAAACCAGGA
GAGTTCTCTCCTGAAAATGCTTGAGGAAACCCAAACAGCTCCGAT
GTATCTTAGCTGCCAACAGGGGTTCGGCTGGCTGCTGAATGGAGGAAGCC
35 TAGAGGGGCTTCGTGGTATATAAGCATAGCAACAGCATGTCAATCCACCT
CTGGAGAAGAATCGCTGACTTAAATGTGCGATCCACCTCACTTCATCATG
TCACTACAGCAATCCAGTGTCTCCAAACTATAACTGTATATGGTAGA
GCCCACCTCAGATCCCTCTAGTATAGCAGACAGAGGGTAGACATTTCAAC
AAGGGTGAGATGAGGGCAGGCGGCTGCTCCTCTAGCCAGCCCGACCCAC
40 CAGGAACCCGTGACGATTCCGGACTCCTACCTACCAATATGGACACC
GAAATGTGAAAGTGGCAAGAGAGCTGACAGCTGGCTTATTTGGGTGAC
GCAATTCATTGCTGAAACAGATGTTGTGTCCAAAGCGTGAAGAACCGCA
ATGAGATCAGCAGCTGCTGCAACAGGCTGGAAGACGATGCTACAGCT
AGACAAAAGACTTTTGGAGACAGACAGTGCCACAAACAAACATCCAGAGT
45 GGCACTTTGGTCTGAGAAGCTGCATTTGCTAGATGGGCGCAACTTAAAA
GCTGACGGCTGTCATAGATGGCAGAGATGGCAAAAGCCGGCTGACTTTGA
TGCGGAGAAGACATCCTGTGCGTGGACCAGTGGCCTGAGCATAGCTGGC
ACGCTGCCCCAGAGGGGGGCGCATAGGCGAACATCCACAAACACCTGGGA
TAGATATCTGTATGAGGTGGTGGCTGGAGACTGACGCAAGACCATCGAC
ACTGGCTTTGAAGCAAGAATGCTCTTGGCAAAATTTTACCTCCAAGATGTCGCTCATAGCCTCCTATGACCTGAAGGGCCGACAAAGATGACGCCCATTTG
GGGAGAGGTCAAGCTTAGGCAGAGGAGCAAAGGAGACCTTTTGGGAACTC
ATGAAAGAGAGAGATCTCCTTCTGATGTTGCTGGCCATCATATGGCC
GGAAATACACTACAGATAAGATGTTGCTTTGATGCGACGACAAAAC
CACCATATGGAAAGACAGTGGTGGCCGCGAGAGGTGTTGGGACGAGACACCGA
GAGAAAGAAGGAGTTCAACAGGAGTGGATGGGCAGAGTGTGGCCAGATGTCG
ATCATGCGCCTCTGAAGTCTCATTACAGGAGATTTGCGGACTAGAAG

24. ZIK NS2B/NS3 full length amino acid sequence (SEQ ID NO:24)
MSWPPSEVLTAAGVGLAAGAGFAKADIEMAPMAAVGLLIVSVYVSQKSVD
MYIERAGITWEKDAEVTNPSRLDVALDESDGFSLVDEDDPGMPREILKVL
MTICGMMPIAPFAGAWYVVYVTGKRSALGALLDWPVAPKEVKGETTTGVYR
VMTRRLGSLSTQVGVMQEGVFHMTMHWVTKGALSRSREGRLDPYGDVK
QDLVSYCPWKLDDAAWGDHSEVQLAVPPGERARIIQTLPGIFKTGDGIG
VALDYAPGTSGSPILKCRGVLGNYGVNYSVAXGTGRRTEPVECT
FEPMLKMKNLTVLHDHPGAKTRVRLEIVREAILKRTLVRVTPLTVVAEE
MEELAGVLGPLMVTATTAMVNTSHGTEIVDLIMCHAFSTSRLQPVRNPYNLYI
MDEAHAFTDPSSIAARLYSTRVEMGEGAAAIIFMATATTPGTPDAPFDSNPIMTDM
EVEPVRAWSSGFVVDTHSGKTVWVFPSVNRNEIAACLTKAGKVRVQLS
RKTTFETEFQTKHDFEVDFTVIDESEGMAFKNARKIDVRSRCLKPVLIDGER
VLAGPMVTHASKAQHRGRJIRNPNKPDQELYLGCGCAGTEDHHAWLE
RMMLDNYIQLDGLIASLYPREADKVAAIEGEFKLRERTEQRKTFRVLMKRGDLP
VWLAYQVAVASAGITYTDWRFDFGTNNTIMEDSVPAEVWTHGKEKVRVLKPR
WMDARVCSDHAALKSFKEAAAGKR

25. ZIK NS2B/NS3Pro codon optimized nucleotide sequence (SEQ ID NO:25)
ATGTCTTGGCGCTTCCAATCTGAGTGCTAGCCCGCTTGGGACTGATTTTTGTC
CTGCTGTTCCGAGATCCACGTAGATGGCCGGACATCTATGCG
CGCGCTGGGCTGCTGCCCACTCTGAGATGTGGCCGGACCTATGCG
GACTTCTCCCTGTTGGAAGATGACGGCCCTTCCATGCGGAGATCATCTGCT
GAAGTTTGTGCTGATGACCATCTGCGGAAGTGAACCCTATCGGCAATCCCTT
TCGCCGGCTGGCGCTGCAGATCGTGGAAGAGCCAGACCCGGAGAGCGGAGAGCC
GCCCTTGGGAGCTGCCACCACCGCCCTAAGAAGATGAAAGGCGGACAGAAC
CCAGAGGCGCTGTGAGATGAGTGGACAGACGCGCTGGGACAGCACA
AGTCGAGGAGTTGATGCAAGGAAGGGGTCTCTCCACCAACCATCTGTC
CGACCAAGGCAGCGCCTGAGATCTGCGAAGGCGACAGACTCTAC
TGCGGCGAGTGATACAGAAGCAGACTCTGCTCCTACTGCCGGCCTCTGGAACAT
GGATCCCGTGATGCGATGCGACAGCGAATGGCAGCTGCGCTGCTGCTGCTGCTGCTGCTGCTGCTG
CCGGCCAGAGGCGCAAGAAATATCAGACACCCGGCCATCCTAAAGAC
AAGGATTGCGCACTCCGGCGGCGGTGGCGCATTACCTGCACGAGAC
TCGGAGGCCCAGCTCCTGTGAAGTGGCGACAGTGTCGTGCTGACG
ACCCCGCGGCGGACACAGCAGCGACAGCGAGACTGCTGCGCTGCTGCTGCTGCTGCTGCTGCTGCTG
AGACCGAGGAGGACAGACCCGGCTGGAAGTAGCTCTCGAGTGA
26. ZIKV NS2B/NS3Pro amino acid sequence (SEQ ID NO:26)
MSWPPSEVLTAVGLCAAGGFAKADIEMAGPMMAVGLLLYVSVVSGSVVD
MYIERAGDITWEKDAEVTGNSPRDLVALDESGDFSILVEDGGPPMREILKVVL
MTICGMNPILAFPAAGAWYYVYVKTGKRSGALWDVPAPEVKKGETTDDGVYR
VMTRRLGLGSTQVGVGVMQEGVFHTMWHVTKGSALRSGEGRLDPYWGDVK
QDLVSYCGPWKLDAAWDGHEVQLAVPPGERARNIQTLPGIFKTGDIGA
VALDYPAGTSGPSILDKCGVRVGLYNGNYVKKNSYVSATQQRREETPVEC
FE

27. DEN-3 NS3 wild-type amino acid sequence (SEQ ID NO:27)
AGVLVWDPVSPPPMGKAELTEDGAYRIKQKGLGYSIQAGAVYKEGGTFHMTWH
VRTGAVLMHKKGRIECSWADVLKDSLISYGWGLKEGSEWKEEGVEQQVLALE
PGKNPRAVQTKPGLFNTAGTAVSLDFSPGTSGPSIIKKGGKVVGILYGNV
VRTSGAVSIAHTQETKSEIDNPNEIEDDFRKKRLTMALHPGAKTFRKPLAIV
REAIKRGLRLTILAPTRVVAEMEEALRGLPIRYQTPAIRAEHTGREIVDLMCH
ATFTMRLSSPVRVPNYLIMALMDEAHFTDPASAAGRYSTRVMEGAAAGFMKAT
ATPPGSRDPFPQNSNPIIDEEREIPERSWSNWEHWTDFKGTWVNVPSIKAGN
DIAACLRRKNGKVIQLSRTDFSEYVKTRTNDWDFVTTIDISEMGANFKAE
VIDPRRCMKPVILTDGEVILAGMPVPVTHSSAAQQRGRGPNKENDQYIYM
MGEPELENDCAHKEAKMLDDNINTPEGIIPSMFEPEREKVDADGEYRLR
GEARKTFVLMMRGRDLPVWLYRAYVAEAGNYADRWRCDGVYNKNNIQLEEN
VEVEIWTKEERKKLKRPLWALADRIYSDPLALKEFKEAAGR

28. DEN-2 NS3 Helicase portion wild type nucleotide sequence (SEQ ID NO:28)
ATTGTTCCGGAAACAGAAAGACTGACCATCATGGGCTCCCACCCGGAGGCGG
AAAGACGAAAGAGATACCTTCCGGGCTCAGATGAAACATAAAACCG
GGTTTGAGAACATTATCTCTGGCCTCCTCAACTGAGTGGTGACCAGTAGAAAT
GGAGGAGAAGCTCACTTCTTCTTTCAATAGACCAGACCCCATCCAACCAACCA
GAGCTGAGCAGACACCGGCGGGAGATTTGGGACCTATATGTCATGCCACA
TTACCTAGTACGCTGCTATACACAGTACGCTGCAATACCACTACCTGATT
ATCATGGACGAGGCCTACATCGAGGCTAGCAAGCTACAGCAGCTAGG
AGACATCTCTGACTGACTGGGAGATGGGTGGGACAGTGGGATTTTTATAG
CAAGCCACTCTCCCGGAGCAAGCACAGACCCATTTCCTCAGACCCCATACCA
ATCATAGAGAGACAGAAATCATCCTGAAAGCTCGTGGGAACTCCGGACA
TGAATGGGTAAAGGGAAGACTGTTTTGCTGCTACCAGATT
AAAAGCAGAAAGATATAGCAGGCTTGGCAGAAGAAATGGAAGAAAA
GTGATACAAACTCGAGTGAAGACCTTTGATTTCTGATATGTAGACTAG
AAACCATTTGGGGCTTCCGTTGTTACAACTGACATTTCAGAATGTTGGT
CCAACTCCAGGCTAGGAGGGTTATAGACCCAGACGCTGATGAAACCA
GTCATACAAACAGATGTTGAAAGCGGCTTGGATTTCTGAGACCTAGGAA
AGTTGACCACCTCAGTAGTCAGCAAGCAAGAAAGAGGAGAATAGGAGAAAT
CCAAAAAGAGAAAAAGGGAAGACCTTCAACTCATACTTACAGCAAGCA
CTTCAAAAGAGAAAGAGGAGAAGAAAGAGAAGAGGAGAAGAAAGAGGAGA

82
29. DEN-2 NS3 Helicase wild type amino acid sequence (SEQ ID NO:29)
IFRKRLRTIDMLHPGAGKTKRYLPAILVREIAKRGLRLVILIAPPTRVVAEMEEAL
RGLPIRYQTPAIRAHTGREIVDLMCHATFTMRLSPVRVPNYLIMDEAHFT
DPASIAARGYISTRVEMGAAAGIFMTATPPGRDPFPQSNAPIDEREIPERSW
NSGHWETFDFKVFWFVPSIKAGNDIAACLRLNKKQVIQLSRKTFDSEYVK
TRNTDWFVFVTTIDSEGMANFKAERVIDPRRCMKPVTLDGEEVILAGPMP
VTHSSAAQRRGRGHRPNKNENDQYIYMGEPLENDEDCAHWKEAKMILLDNIN
TGPIEIPERMERKVDAYDINGCREKVTDFVNLGDRDLPLYFVYRYVA
AEGINYADRRWCFDGKVNQILENEVENVIEWTKEGERKLKPRWLARIYSD
PLALKEFKEAAGR

30. DEN-2 NS2B/NS3Pro wild-type nucleotide sequence with Mutations
(SEQ ID NO:30)
ATGAGCTGGCCATTAATAGAGGCTCATCATGCGAGTCGGAGTGTAGCAT
TTAGCCAGTTTCTCTTCTCTAAAAATGATATATTCATGACAGAGGACATTATGT
GGCTGGAGGCTTTCTCTACGTGTGCTACGTGCTACGTGCTACGTGAAGCTGCCG
ATTGGAACTGGAAGAGGACGACGTGTAACATGGAAGATGGTA
GATATGCAGGGAGAGCCATCCTGGAATACATCAATACGAAAGATGG
GATGAGCGGGATGGTTTACAAAAAGAGGAACATTCATAAATGTCGGAGCTGCA
ACGCTGCGCTTTAATGCTAAGAAAGGAAGGATGGAATCCCGTGAG
GGGACCGTCAGAAAGACCTATACATGAGAGGTGAGCTGGAAGT"
32. DEN-2 Capsid (C) amino acid sequence (SEQ ID NO: 32)
MNQRKKKAKTNPKFNLKRRNRVSTVQQLTRKSFLGMLQGRPPLKLMAL
VAFRFLFIPPTAGILKRWGTIIKSKAINVLRGRKRIEGRMLNILNRARSAGM
IIIMLIPTVMA

33. DEN-1 prME wild type nucleotide sequence (SEQ ID NO:33)
TTCCATCTGACCAACCGAGGGGAGAGGCCACATGATATGTTAGCAAGCA
GAAAGAAGAAATCACTTTTTGTTTAAGACCTTCGAGTGCACATGT
GCACCCCTATTGCAATGGATTTTGAGAGTATTTGAGCACAATAGCC
TACAAATGCCCCCGGATCACGTAGAACGAGAACCAGATGACGTTGACTGTG
GTGCAATGCCCAGCAGACATGTTGACCTATGGAACATGTTCCACTAAACG
GTGACACCCGAGAGACAAACGTGCTGACCTGACACACAGAGTCGAGGG
CTTGGTCTAGAAACAAGAACGCAAACGTTGAGATGCTCTCTGAGAGCGCTTG
GAAACAAATACAAAACTTGGAGACCTTGCGTCTGAGACACCAAGAGTTTC
ACGCACAGAATACCGAGAGGCAATATGGAAGAAAAATTCTGAAATGAAA
ACTTAAAAATATCTGACGAGACATGCACACTGACAGCGGAGACAGGAC
GGACATGGAGTTGTGGGCTATTCCGGAAAAGTAGACCTTTATAACACGGTGCT
AAGTTAAATGTTGCTGACAAAACACTGCGAAGGAGAAGTGAATGTCACATGAAA
ACTTAAATATTTCAATGTCAGATAGTCACCAGTACACATCGGAGAGCCAGCACAA
GTTGGAATATGAGACCAGAATGACCACTACCCGATGACACACATACCCGAC
ATGCTCCAGCTCGGAAAATACAGCTGACAGACTACCGAGACTCCTACATTTG
ATGTCTACCTGAGACACGACTTTTAAATGGAATGTGTGTTGACAA
ATGGAAAAAAATCATGTCGTCGCCACAAAAAATGGTTTTCTAGACTTACC
ACTGCTTCGAGCTCGGAGGCTTCAATCACCACAGAACAGACTTTGGAATAGAC
AAGACTTGGCGTGTCACATTTAGAACAGCTCATGCACAAAAAGCAAGAAATGA
GTCGCTAATGGCAATCAGAAGAGACATGACACCTGCGTTGACAGAGAC
GACGAGAACCTAACACGTCGGGAGACACAAATTTTGCGACCAACTCTGA
AATGCGAATCCAAATGTTAACTGACCTTTTTAAAGGATGTCATATGTA
ATGTGGACAGGGGTATTTGCAAGAAGGAGTGGTTGCTGACAGAGAC
TGAAGGCGAGCCACACACCCATTTTGTTGAGAGCTACATTGTTGAGAGGAGGTG
AATGTTTTTAAACTAAGCTGTTTGCAAGAAGGAGATGTTGCTGACAGAGA
AATGGTTGAAAGCACAATCGCCCGTGAGACAAGAGGAGGATGACACATTCCGAG
ACACTGTACAGGGCTGTTCTAGATAGGGGTGTTGACAGAGTTGGA
TTCAGATTACACAGCCTACCCATAGTCTACGACAAAGAAAAACAGTCAACAT
TGAAGGCGGACGACACACCCATTTTGTTGAGAGCTACATTGTTGAGAGGAGGTG
AATGTTTTTAAACTAAGCTGTTTGCAAGAAGGAGATGTTGCTGACAGAGA
AATGGTTGAAAGCACAATCGCCCGTGAGACAAGAGGAGGATGACACATTCCGAG
ACACTGTACAGGGCTGTTCTAGATAGGGGTGTTGACAGAGTTGGA

34. DEN-1 prME wild type amino acid sequence (SEQ ID NO:34)
35. TVXDO21 (Capsid of DENV-2 and prME of DENV-1) Codon Optimized nucleotide sequences with mutations (SEQ ID NO:35)

ATGAAACACAGCGCAAGAAGCCAAAACAACCTCGTATCATGCTCAA
GAGAGAGCCAAATCTGGTCTTACTGTTACAGCACTGCGAAGAGATCTCT
CCCTGGCCCATGCTCAAGTGTCGGCGACCACTGAGTCTAGTGGCCCTT
GTTGCTATTTTCTTTCTTATATTTCACCTCCACCTCTGCTGGAAATCTGAAG
CGGTGGGGGACACCATAAAAAAAGTTCAAGGCTATTTAAATGTGCTGCAAGGGTT
CAGGAAGAGATGCTGGGAGTGTGAACTGACAATCTACTGAGGACAGGCTG
CCGCTGGCCATGATAATCATGTGTAACCACTGATGACTTTTACG
CCACTAGGGGGCAGTGAGACACCACATATGATGTAGTTAGTTAAACAGGAAAGGG
TAAAGATGCTCTTAAAAACAGCCCGGAGTAAATATGTCACACTGATGAGCCATGGAG
AGCCATGGAATTTGCGAGCTTTTGCGAGGATACCATGACATAAATGCC
CCCGGATACAGAGAGACAGACAGAGGACAGTTGCTCGTGGCAGAGCCACG
ACCGAGACTTTGGGTTACATACGGACTTCGACGCAAACGGGAAGACATAG
ACGCAGCAGAGATCTTCTCGCCTGGCACATGAGTGAGG
AGACAAAGACAGAACCACTGGAATGAGTGTGAGAAGGCGCTTGGAAAACAGAT
CCAAAGGTTGGAAGATTTCGGCTCCTGCGACACCTCGGGTCAAGTGATCG
CATTGTTTCTGGCCATGCAATAGGAACCTTCTATACACAGAAAGGCGATT
TCTTACATCTCGTCTGATGTTGTTACACCTCTAATGGCCCATAGTGTCGTCG
GTATCGAAACAGAGATTTCTGGAAGGCTGAGCGGCTTGGACCTGGGTT
GATGTCGTCCTCCTGCAACCCGAGTATCTGTGTCAGCAGCATTAGGGCCAAAAGATAA
GCCTACCTCGATATTGTCCTGGGAAGAGCAGGTTACTAACCCTCGCTGT
GCTCGCAAACCTGTAATGGGCAAAAGATTTCTACACACAAACCCGACA
GTAGAATGTCGGGCAAGAGCAAGCCAGTCGGTGAAGAGCAAGGACAC
CAACATTTGTAGTGAAGAAGGACCTCTTCGTCGATCGGCGATGGGGAACGGGT
GCAGACTTTCCTGGAAGAGATCCTGTAGATCTGCAACAATATCGC
GTCGACTAAACATTGAAGGAAACATTTGCTAAGCAGAAAATTGGAAATTTTGAGTACT
TGTTATCTGGTTACGGGTACAGGACATACAACAGGTTGGGAAAGGAGA
CCACCGGAACACGCGACTTCAAGCCGAACAGTAACTGCAACGCTCAGTCC
GAAATACAACCTACCCGACTATGCGGCCTCTTACACTGGAAGTTCCACACG
CAGTGGCAGACTTCCAACGAAATTTGGCTTCCTGCCAAATGGAAGAAAGAA
GTCGTCATTTGTAACAGAATGGTTTCTGGACACCTCGCGCTCCCCATGGAAGC
AGTGGCCGAGATCTAGCCAGAGAACCTGGAACGGAGAAGGCTTCTGAGT
AACATTTCAAGACAGCAGACGCCCTAAAAGAAGAGGAGATGGTGTCGTTTTGAGAT
CCAAAGAGGCTGCAATGCAACACCTCAGCTACAGTAGTGCAACCGAGATCCAG
ACCTCCGGGACTACATTACATCTTTTGCGAGGCCACTCTAAATGCAGACTGGAA
ATGGGATAAATTACATCCAGGATGCTAATGCTATGTACGTACGGG
CTTTAAAACTTGAAGAGGTGCCGCTGAACACACAGCAAGAATCTTTG
GTTCGACAGCAATACGAAAAGGTGACGTACGAGTGGCTCCCTTGA
CTCTCAGAGCAAAAGCTGTATTACTCAAGATGAGGTAGCTGATCCGCTAA
ATCCAAATTGAACGCAGAAACCCGTAATGTGAGGCGAGACC
CCCTTCCGTTGAATCTTATATTGTATTTGAGGACAGAACG
ACTACAGTTGGTCACAGAGGAGATCTCCTCCTGGAAAAAGCATTGGAAGCTA
CGGCTCGGGGACGCGCGGTGGCTGCTATCCCTGGGAGCAGCCTGGGA
TTTGGGAAGCATTTGCGTGCGTCTTTTACATCCTGGGAAGAGTTGATACCA
AACTTCTTCGGGACCACGCTACCTTTACATCTTGACATTCACTCTCGT
GAAGATCAGGAATTTGCGATACCTGAGCTGTGCTTGCTAGTGAATCCGTC
TACTCCTTTTCAGTATGACTTGCCATTGCTGGTGCTGGGAGATGTCAGTACTCTCTACCT
CGGCTGTAGTGGCTCAGCGGTACCTC

36. TVXDO21 amino acid sequence [SEQ ID NO: 36]

MNNQRKAKKPPNMLRERNVSTVQLTKRFSLGMLQGRGPLKLFMAL
VAFLRFLTITPAAGILKSKAKINVLRFGRKEIGRMLNRRRRSA
IIMLIPVMAFHTTTRGEPHMIVSKQERGKSSLKFKTSAGYVNMCTIAMDLE
LCEDMTYKCPRTETEPPDVDDCWNATETWVTGYTCSQTGEHRIRKSV
LAPHVGLGLETTRKTWWSMAESGKQKIVKQVETWLPFHGLGTFVIALFHAI
SITQKGIIFILLMLVTSPMAMRCVGIIGNRFDFVEGLSGATWDDVVLHEHSCVTT
MAKDKPTLDELLKTECTNPAVRLTCLCEKAIQQNNTDDSCRPQGEATLVEEQ
DTNFVCRRFTFVDRGWNCGCLFGKSLTICAKFKCVKLEIGIQVYNLYK

25
VIVVTHTGDQHQVNGNETTEHTATITTPAQPTSEIQTDYGALTLDSPRTL
DFNEMVLLTMKMKWKQWFLIDLPLWTSGASTQETWNQDLDLTFTK
AHAKKQEVVVLGSQEGAMHTALTGATEIQTSGTTIFAGHLCRLKMDDKLT
LKGMSYVMCTGSFKLEKEVGAETQHGTBLVQVKYEGTDPACKPFSQDEKGV
TQNGRLITANPVTDMKPKYVIEAEPPFGESYIVVAGEKLHFWFKKGSSLG

30 KAFEATARGARRALIIGDFTFSWGGFTSVKGKLIQHIFGTAYGVLFSGVS
WTMKIGIILLTWLGLNSRSTSLSMTCIAVMGMTYLGLVMVQA

Dengue virus 3 Strain CH53489 Genbank DQ863638.1

37. DEN-3 prME wild-type nucleotide sequence [SEQ ID NO:37]
TTCACCTCAAATCCTACGAGATGGAGAGCCGCATGATTGAGGGAAAGAA
TGAAAGAGAGCGCCATGATGAGAAGAGATGGATGGAGCGAGA
TACAAATGGCCCAACATTGCCAGAAGACGTAGTGGAGCGGCATGACTCTGCT
GTGAACACTCCCATTGAAAAAGAGAGATGATGGAACTGAGCATTAGAGTACGCTG
GAGGCACAGACGGCGCAAAAGAGATGATGCCATTGAGCTGAGCGGGCAGCTT
GAGAACATGCAGAAGACGACTAGAGCAGCCATGACCTAAGG
ACCATACTAGCTATTCTTGCCATTACATAGGCACGTCTCTTGACCACAG
AAAGGTTATTTTTATATCATATGACTGCTACATCCTACCATGCGGAAATG
AGATGCCGGAGATGAGAGAAAAACAGAGATTTGGGAGGCTGCTCAGGGAC
TACGTGGGCGACTGCTGAGCGAGCCTGAGGGTGGTGGTTGATGACACATGG
CTAAGAAACAGCCACCGCTGACATAGACGTCCATGACAGAGGGACGACC

50
DEN-3 prME wild type amino acid sequence (SEQ ID NO:48)

MIVGKNERGKSLLFTASINGMCTLIMADLMGEMDFTVYKCPHIAEVPEDI
DCWNCNLSTSWTNTYGTNCQAGHEHRDKRSVALAPHVGMLDTQRTQTWMSA
EGAWRQVEKVEWTWARHPGFTILAFLHAYGTSQKVVKVVIPLLTVPSMA
MRCVGVRNDRFVEGLSGATWVDVLEHGGCVCITMAKNKPTLDEIKQTEA
QQLAALRLKLCGIEKTNITTTSRCPQTEAILPEEQDQNYVCVKHYVDRGWG
NCGLFKGSLVTCAKFCQLESIEKVQVENLKYTVIIVTHTDQHQVGNET
3QGV TalFqPqASTEVJYSPARENTTLGFNEMILLTMKNAWMVHV
RQWFFDLPLWTSGATETPWTNRKELLVTTFKNAHAKKQEVQVLGSQEGAM
HTALTGATIEIONSGTSFAFLHKLRCMLDKELEKMGSMYAMNFTVLKKEV
SETQHGTILKVEYGKDAPCKIPSTEDQGKAHNRLITANPVTKEKPV
NIEAAPFPEGSNIVIGIDKLAINWYKKSSGIGMFEATARGARMAILGDTA
WDFGSVGVGLNLSKGMVHQIIFGSAYTALFSGVSWIMKIGIVLTTWIGLNSK
NTSMSFSCIAIGIIYTRLGAVVQA

38. TVXDO23 (Capsid of DENV-2 and prME of DENV-3) Codon Optimized nucleotide sequence with mutations (SEQ ID NO:38)

ATGAAACACCAGCGCAAGAAGGCCAACACACTCCGGTCAATATGCTCAA
GAGAGAGCCGCAATTGGTTTCTACGGTACAGCAGCTGAAGAGAAGATCC
CCCTGGGCTGGTCGAAGCTCAGGGGCTGGCAAGTGTCGAAGCTGACC
GTTCATGTTTATGTAATATATGGTTTATGCTATTATATATATATATATAT
CAGGAAAGAGATTTGGGCGGATGGCTGAACATCCTTAATAGACGCAGACGGT
DEN-4 prME wild type nucleotide sequence (SEQ ID NO:40)

TTCCACTTATGCAAGACGCGCAACCCCCTCAGTATAGTGACGGAAACA
CGAAAGGGAGCACCTTTTCTGTTATGACAGCAACGAGAAGTCAACAGAT
GCACTCATTCCACATGGGTACGTCATGGAACGTGACCCAGAGTAGGT
GGGAAACACGAGACGAGGAGAAGCGCTACTGAGCTGACCCACCACACAGG
GATTAACTGGAGGCAACCCAGAGGAGGATCCATTATGGAACACGAGAG
TTCCAGATGCGGAGGATGAGGGAAATAGAGATTTTGGGAAGGAGTCTACAGGA
GCAAATGATGTTGCGGTTATGGAACACGAGAGGATGTTGACATACACC
GCAAGGGAACAGCCGACGATCTACATTGCGAAAGAGAGACGTTGGTAGAACAGAAG
TGAGGTAAAGCAGCTGCTGAGGCCAAATACCTGCTAGCAAGAACCTGGAG
ACCTTCAATACAGTGTGTTGAGACATCTATGGAGTTGGACACCCAGCA
GGTGGAAACACGACGTCCCAATCTGGAAGTAAACACCACAATACCCCCAG
GTCACCCATCGGTAGAAGGTAAACTACCAATTAGGGAAGCTAACCCTCAG
ATTCGGAACCCGAGCTCCGAATCTTACAAAAGATATAGGTGACGGAAACCTGGA
ATGAGGAAACACACAGCTCCTGGTACAAACAAATGGTTCTTATGACATCTAC
CAGTGAAAAGTTGCAGTAAAAATGGGAGTTCAAGAAATGGCCCCAGACCA
GATGTGCTCAGGAAAGTTTTCATCTGACAAGGAAATGTCGAAACACGCAG
ACGAGGAGACACGTGGTAGGCTACAGTGAAGGCATGGCCTCATG
CAGAAGGGAAATGAAGACGAGGATGTTTCATCCACAGGACGAACTCTCAG
GACGCAACTCTGCAACATGGAGCTTGAACAGCCACATCGTATGGAACGAG
AGAGAGAATGTTGACGTTTCAAAAGTACTCTCATGCCAAGACAGAGATGT
ACAGTGCTAGGATCCCAAGAGAGCACTCCACACTCCGATGGGACGCTAAG
TCACGAGGATGATTCTGTGACGGGAAACACACAGCTTGTGCAAGAACACCCAG
ACGACAGTCATTGCACACTCTATGGTTAGGAAGGAAGTTCTACTCGG
GAGATGTTTGTGACCCATTTAGAGGTGCAGAAAAAGAATGGGACTTGGGAA
GCACTCATATGCTTCATATCTCCTTCTGGGGAGCTCACATAGTGAGGCGCTAG
41. DEN-4 prME wild type amino acid sequence (SEQ ID NO:41)
MIVAKHERGRPLLFTTGENRCLIAMDVEMCSTDVTYKCPPLNVTEPEDI
DCWCNSTSTWVTYGTQSGSRRKESVAPHSGMLGTRTETMWSSEG
AWKHAAQREVSWILRNPGLFALLAGFMYMIGQTIQRVTFFVLMMLVAPSYG
MRCVGVNRFVGEVSGGTWTVDVLVLEHHGVCCTTTAAGKPTLDFELIKTTA
KEVALLRTRYEIASNITATTARCTPQGEYLPKEEKQDQQYICRRDVDRGWGN
GCCLFGBKGGVTCAKFSGCNGITGLNVQVENLTYVTVTVHNGDAHAVGNS
TSNHGVTITTPRPSVEVKLPDYLGETLDCSPRSGDIFNEMLIKMKGKTWL
VHKNFWLDFLPWTAGADTSEHVWNYKERMVTFKVPHAKRQDVVTVLGSQE
GAMHSALTGATEVDSGDGHNFAKLMCKVREMKLRKMGYSCGKFSI
15 DKEMAETQHGTTVKVKYEGTGAPCKPIEIKDMMKENKVVGVRIISIPFAENT
NSITNIELEERPFGDSYIVIGADSALTLLHWFRGKSSKGFMESTYRGARKMAIL
GETAWDFGSVGGILFTSLGKAVHVQFGSVYTTMFGGVSWMIRILIGILVWIG
TNSRNTSMASCIAVGGITFLGFTVQA

42. TVXDO24 (Capsid of DENV-2 and prME of DENV-4) Codon Optimized
nucleotide sequence with mutations (SEQ ID NO:42)
ATGAAACACCAAGCGCAAAGACACCTCCCCCCATATGCTCAA
GAGAGAGCGAATCTGGGTTTCTACGCTACAGCAGCAGCGAAGGATTCTT
25 CCCTGGGATCTGTCGAAAGTGCGCGACACTAGAAGCTGTCATAGCTGCC
GTTGACATTTTCTAAGTTTATATCTACTTCCTCAAAACTTCGCAAGGACCGGG
CGCTGGGGGACCAATCAAAAATCTCCACATTTTCTAGGATGATACTCA
30 CGAAAAAGGAGATTGCGGCGGATGTAACACTCTCTCTTTTATTATAGGCTTAC
ACCTCACTCAGGGCTACTCATGAACTTGCAACAAAACCGGGAGAGAAAGAG
35 AAGAGCGAACCCGGGAGCTGGCTTCGGCACCACCTCCAGGAAATTTG
AAACTAGAACAAGAAACTTGGAAGATGAGAGGAGAGCGAAGCTTGGAACATGC
CCAAACGCGTGAAAGCCTGGAATTCTGGAACCGCACTCGCTGTGT
40 CCCTGTGTGTAATCGGATGGGGATTTGTGGAAAGCGGCAGGCGGCACCTCGGT
GGACACTCGTGCTGGAAGCATGGAAGGTGCGTTAACAACCATTGGCCAAAGGAA
AACCTAATCTTGAATTTGGAAGATGAGAGGAGAGCGAAGCTTGGAACATGC
45 CGTGGGTAGTAAACATCGTTTTTGGAGAAAGCGCAGGCGGCACCTCGGT
TCAGAAAAATACGCGGAACCTCCTGCTGCAGGTGGAACCTGGAATAC
ACACCCGTGGCACCACCTCAGGGAAACACCTATTGAGGAGAACGAAGATAC
50 ACAGGTGTAAGCTGCTGATTATGGGGAGCTCACCCTTTGATTCGAGCCAG
AACTGGGCAATGACTTAAAGGAGATGATACGAGATAGAAAGGAAA
CCGTTGCTGTTGTCATATAAACGCTGTCGCTCCGCCTTCATCGG
ACAGCAGCCGACACCTCCGGAGTTCTACTTAAATACAAAGAGAA
ATCGTACGCACTGCACTCCGCAATCCGAGGAGGATGTCATCAGTG
ACTGGAGTTGAATATAGAAAGGACTGGAAGCTCCACTGTAAAT
CCCGAGGATGGAATACCATGTTCCGGGCACTCTGAAAAGTGAAG
ATGGAGGAAACTGCAATCAGAATGATGTTATACATAGTCAGGT
GTTTCTTATAGCAAGGAAATATGCGAGAAAACCACAAGTATGTA
ACTGACTCTGAGTGAGTGAAATGCAAGTCGAGTTGAGTTGAAT
ATCGGAGGACCTGTCGAGATTTAAGGGAAGAAATGCGAGAAAAC
CCGAGAACTGCTACTCATAACTACACTTACACAAATAGAGAATC
CTTGTCGTTGATTTCTCATTACTAATCGAGCCGCGGCTTACGGAC
ACTTACTGCACTCCGCAAAAGAGGAAATGCTACTCAGGGAAG
ATTCGGATTTTACGAAATAGGGAATAGTAAATCAGAGTACGTG
GTTTCTCTATGCAATCTTTAGGTAATTTTACGTTATAGGAGGA
AGTGCAGAGGAGGAGTTTGTGAGAGAGGAGGAGGAGGAGGAGG
AGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
AGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
AGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
AGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
AGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
AGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
43. TVXDO24 amino acid sequence (SEQ ID NO:43)
MNNQKRKAKNTTPFNMKRRNRRVSTQVPQLTKRFSLGMLQGRPLKFLMAL
VAFLRTFDTPTAGILKRWGTEIKSRAINLVRGFRKEIRMLNLNRRRASGM
25 JIMILPTVMAFHSRLDDGPLEMYAKHERGPRPLLKTTEGNEGRTLIAMV
CEDTVKCPVLNEPVEDCWCNSNSTWVTYGTQSERRKRSVALAPHS
MGLERTRTETWMSSEGAWKHAQRVESWLRNPFGFALLAGFMAYMIGQT
GIQRTVFVLMLVAPSYSRCVGVGNRFVEGVSHTWLDVLEHGGCV
TTMAAQKGBKTDLEIFIKTTAKEVALLRTYCEIAISINTTATRCPTQ
QDQYYICRDRDVGRWNGGCGLFGKGVVTACFKSCSRIKTNQVLQVENL
30 YTVVTVTHNGDAHAVGNSNHGVTTITTPSVEVKLPDEVGLTDCEPR
SGIDFNEMLMKMKGTZTWVKQWFDLPLPTWAGADEVSEWHWNYKMRV
TFKVPHPBEKQDVTLVSGEQAMHSALTGAETVSDGDNHMFAHLKKCVR
MEKLRIKMSYTMCSKFSIDKEMAEOTQHGTVCVKYEGTGAPCPIKEK
35 MNKEKYVRGRSISIPFAENTNSITNIELEPPFDSYIVIGAGDIALTWHF
SGLKGAFESTYRGAKLAILGETAWDFGSVGGLFTLSLGKAVHQMFSVTV
TTMGGGVSWMIILGFLVWIGTNSRNTSMAVIDGAVGGITLFLGFTVQA

40 ZIKA

44. ZIKV NS2B/NS3Pro wild-type nucleotide sequence (SEQ ID NO:44)
AGCTGGCCCAACTAGCGAAAGTACACTCACAGCTGTGGCGTATAGTCGCTATT
GGCTGAGGGTCTGACCAACAGATAGAAAAATGCTGGGGCTCCATCGGCG
45 CGGTCCGTTCTGCTAATTGTACGTTAGCTGCTACAGGAAAGATGGCTGGAC
ATGTACATTGAAAGAGCAGGTGACTACATCGGAAAGAGATGGCAGGAAG
TCACCTGGAACAGTCCCAGGGTCGCTAGTGCGCTGATAGATGATGGTGAT
TTCCCTCGTGTGGAAGACGACGTCGCCCCATCGAGAGACGCTACCTCAGA
GTTGGTCCTGATGACCATCTGTGACATGAAACATAGCATACCCCTTG
50 AGCTGGAGAGCTGGTACGTATACGCTGAAAGAGAGGAGTGTTGGCTC
TATGGGATGTGCCTGCTCCCAAGGAAGTAAAGAGGGGAGACCACAGA
TGGAGTGTCAGAGTAAAGAATGACTGCTGAGACTGCTAGTTCAACAAACAGTTG
GAGTGGGAGTCTATGCAAGAGGGGCTTTTACACTATGTTGCCAGTCACA
AAAGGATCCGCCTGAGAAGAAGCGTGAGAAGGAGACTTGATCCATACTGGG
5
GAGATGTCAGTCCGCTTCGTCTCATCTGTGGTCCATGGAGCTAGAT
GCCGCTTGGAGCCGACAGCAGGAGGTCAGCTTTGGGGGTCCTGCCCCGGG
AGAGAGAGCGAGGAAACTCCACTGACTGCTGCTGCAAATATTAGAAAG
GATGAGGACATTTGAGCCTGCTGAGTATCCACCAGAAGGCAAGCAGGACC
10
GGTCTGGATCAAAAAGTTGGAGATTGTTAGTGCCCATCACCCTAAGGGAGG
AGGGAGAAGAGACTCTCTGGATGCTGCTCCAG

45. ZIKV NS3 Helicase wild type nucleotide sequence (SEQ ID NO:45)

CCTTCGATGCTGAAGAAAGAAGCAGCTAACTGCTCTAGACTTGCATCTCTGG
AGCTGGGAAACCAAGGAAGGAGCTTCTCTCTGAAATACTCGTGAACCCATAAA
AAACAAAGAATCCCTGCTAGTCTGATCTTAGCTCCAACCAAGGTTGTGCCTGCTG
AAATGGAGGAAGCCCCTAGGAGGGCTCTCAAGTGCTATATAGCAACAGCA
GTCAATGTTCACCCACTCCTGCAAGAAGATCTGCAGTTAAGTGGCATTG
20
CACCCTACCTCCACTAGCTACGCACATACACAGAATCCCTAGGAAAACACGTTTG
GGAGGTAGGAGGAGACATGCGAGCTCTGGTACAAAAGGCTGGAA
AACGAGAGCTACATTCCACCAGAGCCGACAAAGAAGTCTCTTGAGCACAGACTTCCAGAAA
ACAAAACATCAAGGATGGGACCTTTTGTCGTCACAACGTGACATTTGACAGAT
GGGCGCAACTTTAAATGACGCGTTCTGTCAGATAGGAGAGAAGACCTACCA
25
AGCCTGTTGCTAGGCGAGACTACGTAGCGAGCTGAGTCTGCAAGAAGGCTGGAA
AACGAGAGCTACATTCCACCAGAGCCGACAAAGAAGTCTCTTGAGCACAGACTTCCAGAAA
ACAAAACATCAAGGATGGGACCTTTTGTCGTCACAACGTGACATTTGACAGAT
GGGCGCAACTTTAAATGACGCGTTCTGTCAGATAGGAGAGAAGACCTACCA
30
AGCCTGTTGCTAGGCGAGACTACGTAGCGAGCTGAGTCTGCAAGAAGGCTGGAA
AACGAGAGCTACATTCCACCAGAGCCGACAAAGAAGTCTCTTGAGCACAGACTTCCAGAAA
ACAAAACATCAAGGATGGGACCTTTTGTCGTCACAACGTGACATTTGACAGAT
GGGCGCAACTTTAAATGACGCGTTCTGTCAGATAGGAGAGAAGACCTACCA
35
AGTACGGGATGCGCTTCAGTTGGAGGAGGAGACATGCGAGCTCTGGTACAAAAGGCTGGAA
AACGAGAGCTACATTCCACCAGAGCCGACAAAGAAGTCTCTTGAGCACAGACTTCCAGAAA
ACAAAACATCAAGGATGGGACCTTTTGTCGTCACAACGTGACATTTGACAGAT
GGGCGCAACTTTAAATGACGCGTTCTGTCAGATAGGAGAGAAGACCTACCA
40
CCAGAGGTGGGTTCTAGACATGCGGCTCTGAGAAGCTTACCAAGGTGGTACAAAAGGCTGGAA
AACGAGAGCTACATTCCACCAGAGCCGACAAAGAAGTCTCTTGAGCACAGACTTCCAGAAA
ACAAAACATCAAGGATGGGACCTTTTGTCGTCACAACGTGACATTTGACAGAT
GGGCGCAACTTTAAATGACGCGTTCTGTCAGATAGGAGAGAAGACCTACCA
45

46. ZIKV NS3 Helicase wild type amino acid sequence (SEQ ID NO:46)

PSMLKKKQLTVLDLPAGKTRPRVPEIVREAIKTRLTVILAPTRVVAAME
45
EALRGFLPVYMTATVNSHGTEIVDLCHAPTSSRLQPPrVPNLYIMD
EAHFTDPSYRISTVMEGAAIAIFMTATPPGTRFAPDSNPSMIDTEVE
VPERAWSGFDWVDHSGKTVWFVPSVRNGNEIAACLTKAGKRVQLSRKT
ETEFQKTQHVEWDFVTVTDIEMGANFKAQDRVSDRCLPVLIDGERVILAG
PMPVTHASAAQRGGRNPKPGEFELFVGCGCAEETDHHWLEARM
50
DNIYLQDGILASLYRPEADKVAIEEGFKLRTEQRFKTFVEMKRGDLPVWLA
YQVASAGITYTDRRWCDFGTNNNTIMEDSVPAEVWTRHGEKRVLKPRWMD
ARVCSDAALKSFKFEAGKR

47. DEN-2 E protein wild-type amino acid sequence (SEQ ID NO:47)
MRICIGMSNRDFVEGVSQGSGSVIVLHEHGSCVTTMAKNKPTLDFELIKTEAKQ
PATLRKVCIEAKLTNTTTESRCPQGEPSLNNEEQDKRFVCKHSMDVDRGWGN
CGLFKGGGVTCAMFRCKNNMEGKVQPNLEYTIVTPHSGEEHAVGNDTG
KRGKEIKTPQSSITEAELTTYGTVTMECSPTGLDFNEMVLLQMNKAWLV
HRQWFLDLPWLPGADTOQSNWIQKETLVTFKNPHAKKQDQVVLQSGQEGA
MHATALGTAEIQMSGNNLFTGHKCRRLDMLQLAKGMSYSCTGKFKVKV
EIAETQHGTIVRVQEGDSPCKIPFEIMDLEKRHLGRLITVNPIVTEKDSPV
NIEAEPPFGDSYIIIGVEPQLKLNWFKKGSSIQMFETTMGRAKRMALGDT
AWDFGLGGVFTSIQKLHAAVFGAIAFYGAAFSVWSLMNTLIGVIITWIGMNSR
STSLSVTLVSVTLVLYLGVMVQA
CLAIMS

What is claimed is:

1. A virus-like particle (VLP) comprising at least one flavivirus structural protein; and at least one non-structural flavivirus protein.

2. The VLP of claim 1, wherein the structural proteins comprise one or more of CPrME.

3. The VLP of claim 1 or claim 2, wherein the structural proteins consist of CPrME, PrME, CME, CPrE or ME.

4. The VLP of any of claims 1 to 3, wherein the non-structural proteins comprise NS2B and/or NS3 proteins.

5. The VLP of any of claims 1 to 4, wherein the NS2B and/or NS3 proteins are truncated as compared to wild-type.

6. The VLP of any of claims 1 to 4, wherein the flavivirus is Dengue, Zika, yellow fever, Japanese encephalitis and/or West Nile virus.

7. The VLP of any of claims 1 to 6, wherein the VLP is a single bivalent VLP that displays on its surface the E antigen of two flavivirus virus serotypes or clades or a multivalent VLP that displays on its surface the E antigen of multiple flavivirus serotypes or clades.

8. The VLP of claim 7, wherein the VLP comprises E antigens from at least two different flaviviruses.

9. A DNA construct comprising sequences encoding flavivirus viral proteins used to assemble the VLP of any of claims 1 to 8, the DNA construct comprising sequences encoding the structural and non-structural proteins.
10. The DNA construct of claim 9, further comprising one or more sequences encoding a linker between one or more of the sequences encoding the structural and non-structural proteins.

11. The DNA construct of claim 10, wherein the linker comprises amino acids corresponding to amino acids 1 to 8 or 9 or 10 of NS1 and amino acids corresponding to 186 or 187 or 188 or 189 to amino acids corresponding to 218 of NS2A; amino acid 1 to 8 or 9 or 10 of NS1, amino acids 1 to 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 of NS2A, amino acids 186 or 187 or 188 or 189 to 218 of NS2A; amino acids 190 or 191 or 192 or 193 to amino acids corresponding to 225 of NS2A; amino acids 1 to 8 or 9 or 10 of NS1 and the second transmembrane domain of NS2B; amino acid 1 to 8 or 9 or 10 of NS1 and the first transmembrane domain of NS2A; and amino acid 1 to 8 or 9 or 10 of NS1 and the C terminal portion of NS2B comprising the second transmembrane domain to the end of the protein.

12. The DNA construct any of claims 9 to 11, wherein the non-structural protein optionally includes a full length NS2B and a full NS3 operably linked directly to the structural proteins CprME.

13. The DNA construct any of claims 9 to 11, wherein the NS3 protease active site is modified such that its enzymatic activity is enhanced.

14. The DNA construct of any of claims 9 to 11, wherein the furin protease cleavage site between pr and M protein is modified by substituting amino acids residues at position P3 with hydrophobic one such that furin cleavage is enhanced.

15. The DNA construct of any of claims 9 to 11, wherein the sequence encoding the E protein is modified to enhance VLP assemble and release.
16. A method of producing a VLP, the method comprising introducing into a host cell one or more DNA constructs according to any of claims 9 to 14 under conditions such that the cell produces the VLP.

17. The method of claim 16, wherein the host cell is a eukaryotic cell selected from the group consisting of mammalian, yeast, insect, plant, amphibian and avian cells.

18. The method of claim 16 or claim 17, wherein the cells are cultured at temperatures ranging from 25°C to 37°C.

19. A VLP generated by the method of any of claims 16 to 18.

20. An immunogenic composition comprising at least one VLP according to any of claims 1 to 8 or 19.

21. The immunogenic composition of claim 20, further comprising an adjuvant.

22. The immunogenic composition of claim 20 or claim 21, wherein the composition comprises at least two VLPs comprising different flavivirus E proteins.

23. A method of generating an immune response to one or more flaviviruses in a subject, the method comprising administering to the subject an effective amount of the immunogenic composition according to any of claims 20 to 22.

24. The method of claim 23, wherein the composition is administered mucosally, intradermally, subcutaneously, intramuscularly, or orally.

25. The method of claim 23 or claim 24, wherein the immune response vaccinates the subject against multiple serotypes or clades of one or more flaviviruses.
26. The method of any of claims 23 to 25, wherein the subject is a human.
Figure 4A  Dengue Polyprotein

Figure 4B  TVXDO Strategy
<table>
<thead>
<tr>
<th></th>
<th>Fig 7A. Polyclonal anti-E antibody.</th>
<th>Fig 7B. 4G2</th>
<th>Fig 7C. 3H5</th>
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<td>TVXDO-31°C</td>
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<td></td>
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<tr>
<td>Controls</td>
<td>+ –</td>
<td>+ –</td>
<td>+ –</td>
</tr>
<tr>
<td>Neutering Titer Elicited by DENV-2 VLP Vaccine and Inactivated Virus</td>
<td>Adjuvant PRNT&lt;sub&gt;50&lt;/sub&gt;</td>
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<td></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td>--------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Immune 1</td>
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<td></td>
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</tr>
<tr>
<td>Group 2 1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Group 3 2</td>
<td>5</td>
<td></td>
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</tr>
<tr>
<td>Group 4 3</td>
<td>5</td>
<td></td>
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</tr>
<tr>
<td>Group 5 4</td>
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<td></td>
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</tr>
<tr>
<td>Group 6 5</td>
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<td></td>
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</tr>
<tr>
<td>Group 7 6</td>
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</tr>
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<td>Group 8 7</td>
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</tr>
<tr>
<td>Group 9 8</td>
<td>5</td>
<td></td>
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</tr>
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<tr>
<td>Group 11 NIBSC Human anti-DENV-2 Ser Control</td>
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</table>

Dose (µg):
- Pre-Immune: 1
- Group 2: 5
- Group 3: 5
- Group 4: 5
- Group 5: 5
- Group 6: 5
- Group 7: 5
- Group 8: 5
- Group 9: 5
- Group 10: 5
- NIBSC Human Pre-Immune Serum Control: 1
- NIBSC Human anti-DENV-2 Serum Control: 1

PRNT<sub>50</sub>:
- Pre-Immune: <25
- Group 2: 37
- Group 3: <25
- Group 4: 382
- Group 5: 99
- Group 6: 371
- Group 7: 196
- Group 8: 1067
- Group 9: 158
- Group 10: 201
- NIBSC Human Pre-Immune Serum Control: <25
- NIBSC Human anti-DENV-2 Serum Control: 287
Figure 12

Dot Blot Analysis of Fractions of Gradient Purified VLPs and ZIKV Virus
Figure 13
Western Blot Analysis of Purified VLPS and Zika Virus Controls ZIKV VLPS 1 2 3 4 5
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

<table>
<thead>
<tr>
<th>IPC(B)</th>
<th>A61K 39/12; A61K 39/145; A61K 47/22 (2016.01)</th>
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<tr>
<td>CPC</td>
<td>A61K 39/07; A61K 2039/544; A61K 2039/5555 (2016.11)</td>
</tr>
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</table>

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)

- IPC: A61K 39/12; A61K 39/145; A61K 47/22
- CPC: A61K 39/07; A61K 2039/544; A61K 2039/5555

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

USPC - 128/203.15; 424/199.1; 424/210.1 (keyword delimited)

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

- Patbase, Google Patents, PubMed, Google
- Search terms used: VLP flavivirus CMV Cp/E Cp/ME mutant capsid vaccine

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

**Date of the actual completion of the international search**

01 November 2016

**Date of mailing of the international search report**

29 Nov 2016

**Name and mailing address of the ISA/US**

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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**Authorized officer**
Blaine R. Copenehave
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)
### 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.: 4-26 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

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

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

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

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

### Remark on Protest

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

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

□ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)