MUTATIONS THAT STABILIZE AND ATTENUATE DENGUE VIRUS GENOME

FIELD OF THE INVENTION

[0001] The invention is in the field of immunology and virology. The disclosure relates to flaviviruses, and immunogenic compositions of flaviviruses, for use in these fields. The disclosure also relates to methods of eliciting an immune response against flavivirus infection. The disclosure further relates to methods of preventing, ameliorating, or treating a disease caused by flaviviruses. In particular, the disclosure relates to vaccines against dengue virus infection.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. Said XML copy, created on 22 July 2022, is named 75899SG1_SL.xml and is 373,336 bytes in size.

BACKGROUND OF THE INVENTION

[0003] Flaviviruses are enveloped and positive-sense single-stranded RNA viruses of the genus *Flavivirus*, which is classified within the family *Flaviviridae*. Flavivirus infection causes a wide spectrum of diseases in human and animals.

[0004] One example of flaviviruses is dengue virus. Dengue, caused by dengue virus infection, is an *Aedes* mosquito-transmitted disease that is prevalent throughout the tropics and is now encroaching in the subtropics. The acute illness caused by dengue afflicts an estimated 100 million people each year, some with life-threatening severe dengue due to the presence of four antigenically distinct serotypes of dengue viruses (DENV1, DENV2, DENV3 and DENV4). Each of the four DENVs has a ~10.7kb positive-sense, single stranded RNA genome that is encapsidated and enveloped. The genome encodes a single open reading frame with 3 structural (capsid (C), precursor membrane (prM), envelope (E)) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) flanked by the 5'- and 3'-untranslated regions (UTR). With such a small genome, dengue virus necessarily relies on key interactions with host factors for successful infection and transmission. Prevention of

infection with any of the four serotypes of dengue viruses will require an effective vaccine that generates both humoral and cellular immunity.

[0005] There is currently no licensed antiviral drug to treat flavivirus infection, such as dengue infection, and case management relies entirely on supportive care. While there are licensed vaccines, they are associated with certain problems and/or disadvantages. In particular, the only licensed dengue vaccine, Dengvaxia™ shows limitations in the efficacy whereby naïve individuals are more prone to severe dengue upon infection after vaccination, as a consequence of incomplete protection against dengue virus.

[0006] As such, there is a need to provide alternative vaccines against such flavivirus infections, and methods for eliciting an immune response against the flavivirus infections. Specifically, vaccines with increased attenuation of virulence, enhanced stability of genetic mutation, complete and balanced immunity against each serotype are desired.

SUMMARY

[0007] In one aspect, there is provided an attenuated flavivirus comprising at least one mutation in its genome sequence encoding an NS2B protein, wherein the at least one mutation replaces an amino acid at position 114 or an amino acid at a position equivalent to amino acid position 114 of SEQ ID NO: 55, and wherein the attenuated flavivirus has an increased immunogenicity, an increased rate of replication and decreased plaque size compared to a wild type flavivirus.

[0008] In another aspect, there is provided an immunogenic composition comprising one or more flaviviruses as described herein.

[0009] In another aspect, there is provided a method of eliciting an immune response against one or more flaviviruses in a subject comprising administering an effective amount of the immunogenic composition as described herein to the subject.

[0010] In yet another aspect, there is provided a method of preventing, ameliorating, or treating a disease caused by one or more flaviviruses in a subject comprising administering an effective amount of the immunogenic composition as described herein to the subject.

DEFINITIONS

[0011] The following words and terms used herein shall have the meaning indicated:

[0012] The term “virus” refers broadly to an infectious agent that replicates within the cells of other organisms. Viruses may be classified based on their nucleic acid (RNA or

DNA), whether the nucleic acid is single stranded or double stranded, whether reverse transcriptase is utilized, and if their nucleic acid is single stranded RNA, whether it is sense (+) or antisense (-). Viruses can be classified by family, genus, species, and serotype.

[0013] As used herein, the term "attenuated" virus refers to a virus which is infectious but less virulent, as compared to the wild type virus. The term "live attenuated" virus refers to a virus which is viable, infectious but less virulent as compared to the wild type virus. In some examples, the two terms "attenuated" and "live attenuated" may be used interchangeably. Attenuated virulence may be reflected in various phenotypes or features. As used herein, examples of attenuating features may include, but are not limited to one or more of increased interferon response, higher sensitivity of interferon response, and smaller plaque size. Additionally, the attenuating features may be accompanied by increased replication rate.

[0014] As used herein, the term "wild type" refers to a flavivirus strain that is used as a backbone to derive a mutant virus. For example, a wild type DENV1 strain may be D1/SG/05K2402DK1/2005 having the amino acid sequence as set forth in SEQ ID NO: 1, and a wild type DENV2 strain may be D2/SG/05K3295DK1/2005 having the amino acid sequence as set forth in SEQ ID NO: 2. Within a serotype, there can be many wild type strains. For example, the wild type DENV1 strains may include, but are not limited to, D1/SG/05K2402DK1/2005 (SEQ ID NO: 1), DF01-HUB01021093 (SEQ ID NO: 17), and DENV-1/China/YN/GM1502 (2015) (SEQ ID NO: 18).

[0015] "Dengue virus" refers to a small, enveloped, positive-stranded RNA virus that belongs to the Flavivirus genus of the *Flaviviridae* family. There are four dengue virus serotypes: dengue-1 (DENV-1 or D1), dengue-2 (DENV-2 or D2), dengue-3 (DENV-3 or D3), and dengue-4 (DENV-4 or D4). Each one of these serotypes forms a genetically related but antigenically distinct subgroup.

[0016] The term "clinical isolate" in the context of a virus refers to a virus that is isolated from an infected host, such as a dengue patient. For instance, D1/SG/05K2402DK1/2005 is a clinical isolate of DENV1 virus obtained from infected subjects during a major dengue epidemic that took place in Singapore in 2005. Clinical isolates are also known as circulating strains. They may have similar or have different genotypic and phenotypic characteristics compared to a laboratory-adapted strain. A "laboratory-adapted strain" of a virus refers to a virus that has been cultivated in the laboratory. A laboratory-adapted virus strain may originate from a clinical isolate. A laboratory-adapted strain may have characteristics which

are different from clinical isolates or characteristics which are different from the original clinical isolate from which it originated. Such characteristics may be genotypic or phenotypic characteristics. A laboratory-adapted strain may acquire one or more mutations within the genome during laboratory culture and may comprise accumulated mutations within the genome. One example of such laboratory-adapted strain is DENV-2 strain new guinea C. The one or more mutations that a laboratory-adapted strain acquires is random and varies between different laboratory-adapted strains as well as after different lengths of time in culture. The accumulated mutations may have an unknown impact on the phenotype of the virus, such as its clinical fitness. Accordingly, it will generally be understood that clinical development of laboratory-adapted strains into live attenuated vaccines may be challenging and the use of such laboratory-adapted strains to generate live attenuated vaccines is not ideal.

[0017] The term "serotype" refers to a distinct variation within a species of virus. These viruses are classified together based on their surface antigens, allowing the epidemiologic classification of organisms to the subspecies level. For example, the species *Dengue virus* comprises four serotypes, namely, DENV1, DENV2, DENV3 and DENV4. It would be understood that the serotypes are immunologically distinguishable from each other but genetically related.

[0018] The term “virus strain” refers to a genetic variant or subtype of a virus species or serotype. It will be generally understood that strains within a serotype share high similarity in their genetic makeup and structure.

[0019] The term “immune response” refers to a reaction which occurs within an organism for the purpose of defending against foreign substances or invaders. One example of the foreign substances is a virus. The immune response comprises innate and adaptive immune responses. The innate immunity refers to the non-specific defense mechanism that is activated immediately or shortly after exposure to an antigen. Mechanisms involved in the innate immune response may include physical barriers such as epithelial surfaces, activation of inflammatory responses, interferon response, phagocytosis, complement activation, activation of Toll-like receptors and secretion of cytokines such as interferons. The adaptive immune responses comprise humoral and cellular immune responses. It would generally be understood that humoral and cellular immune responses respond to B- and T-cell immune responses, respectively.

[0020] As used herein, the term “interferon response” refers to proteins or cytokines that are secreted by cells in response to an infection. An interferon response can be triggered in response to viral or bacterial infections. Interferons are a subtype of these secreted proteins or cytokines and involved in stimulating the immune response and have immunoregulatory and anti-inflammatory functions. The secreted proteins or cytokines influence cell growth and differentiation, modulate the immune response and inhibit the replication of viruses. When infection by a microorganism (e.g. virus) results in an increased interferon response in a host, the proteins or cytokines are secreted at a higher concentration, at a faster rate and/or over a longer period of time compared to infection by a microorganism that does not result in an increased interferon response. Similarly, when infection by a microorganism (e.g. virus) results in a decreased interferon response in a host, the proteins or cytokines are secreted at a lower concentration, at a slower rate and/or over a shorter period of time compared to infection by a microorganism that does not result in a decreased interferon response

[0021] The term “sensitivity to interferon response” refers to the susceptibility of a virus to the effects of interferons. When a microorganism (e.g., a virus) has high sensitivity to interferon response, the viability and/or proliferation of the microorganism is highly inhibited by interferons. As such, in the presence of interferons, the spread or dissemination of the microorganism is more effectively restricted, as compared to a microorganism with low sensitivity to interferon response.

[0022] The growth or replication rate of virus may be determined by measuring the levels of a specific nucleic acid in a host cell that has been infected. For example, the growth rate of a dengue virus may be measured using the specific viral DENV E protein or nucleic acid. Growth or replication rate may also be measured at predetermined time intervals, for example, at 6-hour, 12-hour, 24-hour and 36-hour intervals. In one embodiment, measurement of growth or replication rate may be determined at 24-hour intervals.

[0023] It will be understood that prior to measuring the growth rate of a virus, the host cell may be infected for a preselected period of time. For example, the host cell may be infected for 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 24 hours or 36 hours prior to the levels of a specific viral nucleic acid in a host cell being measured. In one embodiment, the host cell is infected for 2 hours prior to measurement. In a preferred embodiment, the host cell is infected for 1 hour prior to measurement. For example, the virus may be added to the host cell for 1 hour and then removed, and media solution may be then supplemented.

Measurement of parameters, such as viral titres and immune gene expression, may be taken at a later time point. For example, the measurement may be taken 6 hours, 12 hours, 18 hours, 24 hours, 30 hours, or 36 hours post-infection. In one embodiment, the measurement may be taken 24 hours post-infection. In another embodiment, the measurement may be taken 48 hours, or 72 hours post-infection.

[0024] The term “plaque size” as used herein refers to the sizes of plaques in a plaque assay. Plaque assays are generally understood in the art to be used for purifying or isolating a clonal population of virus or to determine viral titer. Plaque sizes may be compared relative to another plaque within the same assay or within the same plate, or relative to another plaque in a separate assay or on a separate plate. A plaque of interest may then be selected by picking the plaque from the assay or plate for subsequent applications.

[0025] It will be generally understood that the term “immunogenicity” of a virus refers to the propensity of a virus to trigger the immune response of a host cell or organism. Immunogenicity may be measured by the expression or upregulation of the expression of markers associated with the immune response. Examples of immunogenic markers include markers associated with interferon response, inflammatory response, phagocytosis, complement activation, cytokines secretion and B- and T-cell immune responses.

[0026] The term “pathogenicity” refers to the ability of a microorganism to cause disease in a host.

[0027] The term “virulence” refers to the degree of pathogenicity caused by a microorganism (e.g., bacteria, fungi, or viruses) to a host. The extent of the virulence is usually correlated with the ability of the organism to multiply within the host.

[0028] A “host” refers to a cell or organism or subject that is to be, or is, infected with a virus of interest.

[0029] The term “genome sequence” refers to the complete genetic information (either DNA or RNA) of an organism, typically expressed in the number of basepairs. As used herein, the genome sequence refers to the nucleic acid sequence encoding the complete genetic information of a virus. In some examples, the term “genome sequence” is used interchangeably with the term “nucleic acid sequence”.

[0030] The term “nucleic acid sequence” means any sequence of single or double-stranded RNA or DNA molecule. For example, a nucleic acid sequence may refer to an mRNA, cDNA, genomic DNA, or genomic RNA sequence.

[0031] The term “reference sequence” refers to an amino acid or nucleic acid sequence with known sequence. A reference sequence may be compared against a sequence of interest to determine a specific position of an amino acid or nucleotide in the sequence of interest or a sequence identity between the reference sequence and the sequence of interest. For example, a reference sequence may be a consensus sequence.

[0032] The term “consensus sequence” refers to a sequence that represents aligned or related sequences. The consensus sequence may be a nucleic acid sequence or an amino acid sequence. A person skilled in the art would understand that the consensus sequence can be generated by aligning multiple related sequences using sequences alignment software/programs. For an amino acid consensus sequence, the sequence can be defined by the conserved amino acid residue at each position. Non-conserved amino acid residue may be denoted as Xaa or X, i.e., any amino acid. A consensus sequence may be used to determine a specific position of an amino acid in a sequence of interest by aligning the sequence of interest against the consensus sequence. As used herein, the term “consensus sequence” may be used interchangeably with “reference sequence” or “representative sequence”.

[0033] The term “replacement”, when used in reference to the amino acid sequence, refers to the change of an amino acid at a specific position in an amino acid sequence, which is caused by a mutation in the genomic or nucleic acid sequence encoding the amino acid sequence. The term “replacement” may be used interchangeably with “substitution”.

[0034] The term “equivalent”, when used in reference to the position of an amino acid in an amino acid sequence, refers to a position of the amino acid in the sequence of a given amino acid sequence, which corresponds in position (in either primary or tertiary structure) to a position in a reference amino acid sequence. Such equivalent positions in a particular sequence can be determined using methods known in the art, for example based on sequence alignment against the reference sequence or by comparing experimentally revealed or predicted 3D-structures of corresponding proteins. For example, amino acid position 114 of the non-structural protein NS2B of DENV2 strain D2/SG/05K3295DK1/2005 is equivalent to amino acid position 114 of the non-structural protein NS2B of DENV2 strain D2/16681, or amino acid position 115 of the non-structural protein NS2B of Japanese encephalitis virus strain JEV/Nakamaya.

[0035] As used herein, the term “variant” includes a reference to substantially similar sequences. The variant may refer to a variant of the genome or amino acid sequence. These

sequence variants may have at least about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity to a reference sequence, or to a section within the reference sequence. It would be understood that a higher sequence identity indicates a higher similarity in sequence and a closer relationship between the organism comprising the variant sequence and the organism comprising the reference sequence. A virus comprising a variant sequence with higher sequence identity will be more closely related to the virus comprising the reference sequence, compared to a virus comprising a variant sequence with lower sequence identity. Thus, it would be understood that a virus comprising a variant sequence refers to a virus that is related to the virus comprising the reference sequence. For example, two strains under the same serotype may share at least about 60%, at least about 65%, or at least about 70% sequence similarity. In one embodiment, two strains under the same serotype may share at least about 65% sequence similarity.

[0036] The term “vaccine” refers to a biological preparation that provides acquired immunity to a particular disease. A vaccine comprises an agent that stimulates the immune response to give rise to acquired immunity. The agent in a vaccine may include but is not limited to one or more of an inactivated pathogen, an attenuated pathogen, an inactivated toxin (toxoid), a protein subunit, a conjugate of an antigen and a carrier, or a polynucleotide.

[0037] The term “live attenuated vaccine” refers to a vaccine that contains pathogens, including viruses, that are viable but have reduced virulence. For example, the live attenuated vaccine may contain live attenuated flaviviruses. Live attenuated vaccines are typically more effective than inactivated vaccines and have been successful in preventing many viral diseases, such as smallpox, chickenpox, measles, mumps, and rubella.

[0038] The term “immunogenic composition” as used herein refers to a composition which is capable of stimulating immune responses in a subject. In this way, immune protection may be provided against an antigen not recognized as a self-antigen by the immune system.

[0039] The term “administering” and variations of that term including “administer” and “administration”, includes contacting, applying, delivering or providing a composition of the disclosure to subject by any appropriate means.

[0040] The term “subject” refers to a human or non-human mammal. Examples of such mammals include but are not limited to a primate, a mouse, a rat, a guinea pig, a rabbit, and a dog. In a preferred example, the subject is a human. The subject may be at risk of virus infection or desired to be treated using the immunogenic compositions and methods described herein.

[0041] As used herein, the term “about”, in the context of, but not limited to, sequence identity, amount of immunogenic composition or vaccine to be administered, typically means +/- 5% of the stated value, more typically +/- 4% of the stated value, more typically +/- 3% of the stated value, more typically, +/- 2% of the stated value, even more typically +/- 1% of the stated value, and even more typically +/- 0.5% of the stated value.

[0042] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0043] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0044] Other embodiments are within the following claims and non- limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

BRIEF DESCRIPTION OF THE DRAWINGS

[0045] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0046] **Figure 1.** Alignment of NS2B and NS1 amino acid sequence of DENV1-4 with arrow indicating mutation of **(A)** NS2B at position 114 from isoleucine (I) to threonine (114T) and **(B)** NS1 at position 53 from glycine (G) to aspartic acid (53D) and.

[0047] **Figure 2. (A)** Plaque morphology of DENV1 wild type (WT), prM-29V, NS1-53D, NS2B-114T, NS1+NS2B, prM+NS1 and prM+NS2B mutant viruses. Mutant viruses show smaller plaque size compared to WT virus. NS2B-I114T mutation when coupled with prM-29V is stable up to 2 passages Vero cells. **(B)** (Top) Increased viral replication and IFNβ expression levels induced by DENV1-NS1 and DENV1-NS2B viruses in Huh7 cells 48 and 72 hours after infection. (Bottom) Increased IFNβ and MX1 expression levels induced by D1-prM+NS2B in Huh7 cells despite similar viral gene expression to DENV1 WT virus.

[0048] **Figure 3. (A)** Plaque morphology of DENV2 wild type (WT), NS1-53D, NS2B-114T and NS1+NS2B mutant viruses. DENV2-NS2B-114T and NS1+NS2B shows smaller plaque size compared to WT virus. NS1-53D mutation is unstable in Vero cells. NS2B-114T and NS1+NS2B is stable up to 4 passages. **(B)** Increased viral replication and IFNβ expression levels induced by WT and mutant DENV2 viruses in Huh7 (24 and 48 hours after infection) and monocyte derived dendritic cells (moDCs, 24 hours after infection).

[0049] **Figure 4. (A)** Plaque morphology of DENV3 wild type (WT), prM-29V, NS1-53D, NS2B-114T, NS1+NS2B and prM+NS2B mutant viruses. All DENV3 mutant virus shows smaller plaque size compared to WT virus. NS1-53D and NS2B-114T mutations are unstable. However, when engineered together, they become stable up to 4 passages. **(B)** Increased viral replication and IFNβ expression levels induced by WT and mutant DENV3 viruses in Huh7 and monocyte-derived dendritic cells (moDCs) 48 hours after infection.

[0050] **Figure 5. (A)** Plaque morphology of DENV4 wild type (WT), NS1-53D, NS2B-114T and NS1+NS2B mutant viruses. DENV4- NS1-53D, NS2B-114T and NS1+NS2B shows smaller plaque size compared to WT virus. Mutant viruses are stable up to 2 passages. **(B)** Increased viral replication and IFNβ expression levels induced by WT and mutant

DENV4 viruses in Huh7 (24 and 48 hours after infection) and monocyte derived dendritic cells (moDCs, 24 hours after infection).

[0051] **Figure 6. (A)** Flow cytometry gating of uninfected, NS3-negative (negative) and NS3-positive (positive) Huh-7 cells infected with DENV2-NS2B-114T at 72 hours post-infection. 10,000 cells from each gate were isolated for RNA extraction. DENV NS3-positive (active DENV replication in cells) and NS3-negative (uninfected cells in the same culture) populations were sorted for RNA extraction. **(B)** mRNA levels of DENV genome copies, endoplasmic reticulum (ER) stress marker GADD34, CHOP, immune genes such as IFNβ, MX1, IFITM1, IFITM2 and STAT1 relative to housekeeping gene, GAPDH were determined.

[0052] **Figure 7. (A)** Plaque sizes of DENV2-WT and DENV2-NS2B-114T after siRNA knockdown of IRF3 in BHK-21 cells. Cropped image shows western blot analysis of cells after 48 hour of siRNA transfection to determine IRF3 knockdown efficiency. **(B)** Percent inhibition of virus replication as determined by qPCR of viral RNA on Huh7 culture supernatant after 48 hours of infection with co-treatment of recombinant IFNβ at different concentrations.

[0053] **Figure 8.** Plaque morphology of DENV3-WT, D3-NS1+NS2B and DENV3-NS2B-114T after siRNA knockdown of IRF3 in BHK-21 cells. Upon siIRF3 knockdown, D3-NS1+NS2B showed smaller and increased number of plaques while D3-NS2B-114T showed increased number of plaques. Cropped image shows western blot analysis of cells after 48 hour of siRNA transfection to determine IRF3 knockdown efficiency.

[0054] **Figure 9.** Amino acid alignment of NS1 protein from dengue, Japanese encephalitis and yellow fever virus, all of which belongs to the flavivirus family. The position equivalent to position 53 of the amino acid sequence of NS1 protein as set forth in SEQ ID NO: 13, 14, 15, or 16 is indicated with arrow.

[0055] **Figure 10.** Amino acid alignment of NS2B protein from dengue, Japanese encephalitis and yellow fever virus, all of which belongs to the flavivirus family. The position equivalent to position 114 of the amino acid sequence of NS2B protein as set forth in SEQ ID NO: 5, 6, 7, or 8 is indicated with arrow.

[0056] **Figure 11.** Amino acid alignment of prM protein from dengue, Japanese encephalitis and yellow fever virus, all of which belongs to the flavivirus family. The position

equivalent to position 29 of the amino acid sequence of prM protein as set forth in SEQ ID NO: 9, 10, 11, or 12 is indicated with arrow.

[0057] **Figure 12.** (A) Viremia levels in AG129 mice (mice deficient in IFN α/β/γ receptor signaling) infected with 10^7 pfu/mouse DENV2 or DENV2 NS2B-114T viruses over a period of 8 days. (B) Viremia levels in BALB/C mice treated given anti-IFNAR antibody 3 days prior to infection with 10^7 pfu/mouse of DENV2 or DENV2 NS2B-114T viruses.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0058] In one aspect, the present invention refers to an attenuated flavivirus comprising at least one mutation in its genome sequence encoding an NS2B protein, wherein the at least one mutation replaces an amino acid at position 114 or an amino acid at a position equivalent to amino acid position 114 of a reference sequence as set forth in SEQ ID NO: 55, and wherein the attenuated flavivirus has an increased immunogenicity, an increased rate of replication and decreased plaque size compared to a wild type flavivirus.

[0059] A flavivirus is a genus of positive-strand RNA viruses in the family of *Flaviviridae*. Examples of flaviviruses include, but are not limited to, Zika viruses (ZIKVs), Japanese encephalitis viruses (JEVs), yellow fever viruses (YFVs), West Nile viruses (WNVs), tick-borne encephalitis viruses (TBEVs), and Dengue viruses (DENVs). In a preferred example, the flavivirus is a dengue virus.

[0060] Within the species of Dengue virus, there are four genetically similar but antigenically distinct serotypes, namely, dengue virus serotype 1 (DENV1), dengue virus serotype 2 (DENV2), dengue virus serotype 3 (DENV3), and dengue virus serotype 4 (DENV4). In one example, the flavivirus is a DENV1 virus. In another example, the flavivirus is a DENV2 virus. In another example, the flavivirus is a DENV3 virus. In yet another example, the flavivirus is a DENV4 virus.

[0061] It is understood that there are many different specific strains of dengue viruses within each serotype. A DENV1 virus may be, but not limited to, D1/SG/05K2402DK1/2005, DF01-HUB01021093, DENV-1/China/YN/GM1502 (2015), D1/05K872DK1, or D1/WestPac. In one example, the DENV1 is D1/SG/05K2402DK1/2005. A DENV2 virus may be, but not limited to, D2/SG/05K3295DK1/2005, DENV-2/PR/28DN/1994, DENV-2/China/YN/15DGR8(2015), D2/16681 or D2/Tonga. In one example, the DENV2 virus is D2/SG/05K3295DK1/2005. A DENV3 virus may be, but not limited to,

D3/SG/05K863DK1/2005, 98TW434, 18XN10607, D3/C0360, or D3/Sleman. In one example, the DENV3 virus is D3/SG/05K863DK1/2005. A DENV4 virus may be, but not limited to, D4/SG/06K2270DK1/2005, DENV-4/China/YN/15DGR394 (2015), DENV-4/China/YN/15DGR32 (2015), D4/Dominica, or D4/VE_61013. In one example, the DENV4 is D4/SG/06K2270DK1/2005. As disclosed herein, the DENV1 virus may be any specific strain belonging to the DENV1 serotype, the DENV2 virus may be any specific strain belonging to the DENV2 serotype, the DENV3 virus may be any specific strain belonging to the DENV3 serotype, and the DENV4 virus may be any specific strain belonging to the DENV4 serotype. Examples of dengue virus strains may also be found from databases, such as the NCBI Dengue Virus Database.

[0062] In one embodiment, the virus may be a clinical isolate. A clinical isolate virus may be isolated from a host biological sample, which may include but is not limited to blood, blood plasma, serum, buccal smear, amniotic fluid, prenatal tissue, sweat, nasal swab, urine, organs, tissues, fractions, and cells isolated from mammals including humans. Clinical isolates of a virus may also be isolated from sections of the host biological sample including tissues (for example, sectional portions of an organ or tissue). Clinical isolates of a virus may also be isolated from extracts from a biological sample, for example, an antigen from a biological fluid (for example, blood or urine). In a preferred embodiment, a clinical isolate is a virus strain isolated from blood or serum. For example, a clinical isolate of a DENV1 virus may be D1/SG/05K2402DK1/2005, DF01-HUB01021093, DENV-1/China/YN/GM1502 (2015), D1/05K872DK1, or D1/WestPac; a clinical isolate of a DENV2 virus may be D2/SG/05K3295DK1/2005, DENV-2/PR/28DN/1994, DENV-2/China/YN/15DGR8(2015), D2/16681 or D2/Tonga; a clinical isolate of a DENV3 virus may be D3/SG/05K863DK1/2005, 98TW434, 18XN10607, D3/C0360, or D3/Sleman; and a clinical isolate of a DENV4 virus may be D4/SG/06K2270DK1/2005, DENV-4/China/YN/15DGR394 (2015), DENV-4/China/YN/15DGR32 (2015), D4/Dominica, or D4/VE_61013.

[0063] A flavivirus has a positive-sense, single-stranded RNA genome encoding 3 structural proteins (capsid (C), precursor membrane (prM), envelope (E)) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The original genome of a flavivirus may be mutated to produce a flavivirus with attenuated virulence. The mutation may be introduced by common mutagenesis methods known in the art. Examples of

mutagenesis methods may include, but are not limited to, Kunkel's method, Cassette mutagenesis, site-directed mutagenesis, whole plasmid mutagenesis, and CRISPR. In one example, the mutation is introduced by site-directed mutagenesis.

[0064] In order to introduce a mutation into a flavivirus, the flavivirus may be first isolated from a clinical isolate obtained from a subject who is currently infected or was previously infected with the virus. Clinical isolates include, but are not limited to, blood, plasma, serum, buccal smear, amniotic fluid, prenatal tissue, sweat, nasal swab, urine, organs, tissues, fractions, and cells isolated from mammals including humans. Clinical isolates may also include sections of the biological sample including tissues (for example, sectional portions of an organ or tissue). Clinical isolates may further include extracts from a biological sample, for example, an antigen from a biological fluid (for example, blood or urine). In a preferred embodiment, a clinical isolate is a virus strain isolated from blood or serum.

[0065] As disclosed herein, the flavivirus comprises at least one mutation in the genome encoding the non-structural protein NS2B. The at least one mutation results in replacement of an amino acid at position 114 or an amino acid at a position equivalent to position 114 of the reference sequence as set forth in SEQ ID NO: 55. SEQ ID NO: 55 is a representative amino acid sequence of the NS2B protein in flaviviruses. The representative amino acid sequence is also known as the consensus sequence, which is generated by aligning the amino acid sequences of the NS2B protein of flaviviruses (Figure 10). A person skilled in the art would understand position 114 of NS2B or an equivalent position thereof in a given sequence may be determined by aligning the sequence with the consensus sequence.

[0066] In one embodiment, the at least one mutation replaces an amino acid at position 114 or an amino acid at a position equivalent to amino acid position 114 of the amino acid sequence as set forth in SEQ ID NO: 5, 6, 7, or 8.

[0067] SEQ ID NOs: 5, 6, 7, and 8 correspond to the amino acid sequence of NS2B protein of wild type DENV1 strain D1/SG/05K2402DK1/2005, DENV2 strain D2/SG/05K3295DK1/2005, DENV3 strain D3/SG/05K863DK1/2005, and DENV4 strain D4/SG/06K2270DK1/2005, respectively.

[0068] A position equivalent to position 114 of NS2B amino acid sequence as set forth in SEQ ID NO: 55, 5, 6, 7, or 8 refers to the position in an amino acid sequence which corresponds in position in primary protein structure to position 114 of the sequence of SEQ ID NO: 55, 5, 6, 7, or 8. In a preferred example, the equivalent position refers to the position

in the primary protein structure. Such positions in the primary protein structure may be determined according to sequence alignment against SEQ ID NO: 5, 6, 7, or 8 (Figure 10). Examples of positions equivalent to position 114 of the NS2B amino acid sequence as set forth in SEQ ID NO: 55, 5, 6, 7, or 8 may include, but are not limited to, position 114 of the NS2B amino acid sequence of DENV1 strain D1/05K872DK1 or D1/WestPac, position 114 of the NS2B amino acid sequence of DENV2 strain D2/16681 or D2/Tonga, position 114 of the NS2B amino acid sequence of DENV3 strain D3/C0360 or D3/Sleman; position 114 of the NS2B amino acid sequence of DENV4 strain D4/Dominica or D4/VE_61013, position 114 of the NS2B amino acid sequence of YFV strain asibi, and position 115 of the NS2B amino acid sequence of JEV strain JEV/Nakamaya.

[0069] The said mutation in the NS2B protein increases immunogenicity of the flavivirus. Immunogenicity may be determined by an interferon response in a host. In one example, the said mutation in the NS2B protein induces an increased interferon response in a host. When a host is infected with the flavivirus comprising the said NS2B mutation, the infected cells in the host release an increased amount of interferons, as compared to when infected with the wild type flavivirus. The increased amount of interferons causes neighboring cells to heighten their anti-viral defenses, and thereby more effectively restrict the viral dissemination in the host and attenuate the virulence of the flavivirus. Furthermore, the flavivirus comprising the said NS2B mutation displays an increased sensitivity to the interferon response, as compared to the wild type flavivirus. A wild type flavivirus refers to a flavivirus that is used as a backbone to derive a mutant virus. Compared to the wild type flavivirus, a mutated flavivirus with an increased sensitivity to interferon response will be more susceptible to the inhibitory effects of interferons. It would be appreciated that in addition to the increased interferon response induced in the host, the increased sensitivity of the flavivirus to interferons further attenuates the virulence of the flavivirus.

[0070] To test the attenuated virulence, a host is required to be infected by the flavivirus. The host may be a subject or a cell. In one example, the host is a human or a non-human mammal subject. In another example, the host is an immortalized cell line or a primary cell culture. The cell may be an adherent cell culture or a suspension cell culture. The sources of cell may include, but are not limited to, a human, a bovine, a canine, a murine, a rat, a fish, an insect, a rabbit or monkey. Suitable examples of cells that may be used include, but are not limited to, the human hepatoma cell line HuH-7, the human embryonic kidney cell line

HEK293T, human embryonic diploid cells (e.g. human lung fibroblast cells WI-38 and MRC5), C6/36 mosquito cell line, Vero cells, baby hamster kidney fibroblast cells BHK-21, MDCK cells, primary green monkey kidney cells, monocyte derived dendritic cells (moDCs). In a preferred example, the host cell is Huh-7 cells. In another preferred example, the host cell is Vero cells. In another preferred example, the host cell is BHK-21 cells. In a more preferred example, the host cell is moDCs. It is understood that moDCs may be obtained from the whole blood of a healthy donor and the moDCs closely resemble the biological activities or processes *in vivo* during the flavivirus infection. It is generally agreed in the field that cell models, especially the primary cells, are the gold-standard to test dengue virus fitness as an attenuated virus strain.

[0071] In response to the flavivirus infection, a host releases interferons, such as type I, II, and III interferons. In one example, the interferons are type I interferons. Examples of type I interferons may include, but are not limited to, IFN-α (alpha), IFN-β (beta), IFN-κ (kappa), IFN-δ (delta), IFN-ε (epsilon), IFN-τ (tau), IFN-ω (omega), and IFN-ζ (zeta). In a preferred example, the IFN is IFN-β (beta). In another example, the interferons are type II interferons. In yet another example, the interferons are type III interferons. Type I and type III interferons may induce strong antiviral state in responsive cells by regulating expression of several hundred genes known as IFN-stimulating genes (ISGs). Examples of ISGs may include, but are not limited to, MX1, IFITM1, and IFITM2. Type I interferons may be produced by fibroblast and monocytes. Type III interferons may be found on mucosal surfaces. Type II interferons may be immune interferons released by T cells. It is understood that the interferon response in the host may be determined by measuring the mRNA and/or protein levels of the interferons using the common methods known in the field. For example, the levels of interferons are measured by quantitative Real-Time PCR (qPCR), ELISA, western blot, or immunostaining. It is also understood that the sensitivity of the flavivirus to interferon response can be determined by measuring the viral count in the presence of interferons. The viral count may be measured by routine methods known in the art. Methods for quantifying the viral RNA include but are not limited to qPCR.

[0072] Advantageously, the flavivirus comprising the said NS2B mutation may be associated with other attenuating features, such as a smaller plaque size *in vitro*, a faster virus clearance, and an increased survival rate of the infected host, relative to the wild type

flavivirus. Additionally, the attenuating features are accompanied by an increased growth or replication rate.

[0073] In one example, the flavivirus comprising the said NS2B mutation is also associated with a smaller plaque size in a plaque assay, as compared to a wild type flavivirus. Plaque assays are generally known to be the standard method used to determine virus titer or concentration. A reduced plaque size may be indicative of reduced virulence or may be indicative of a slow growing virus. Plaque sizes may be compared relative to another plaque within the same assay or within the same plate, or relative to another plaque in a separate assay or on a separate plate. In a preferred example, the mutated flavivirus is associated with a smaller plaque size relative to the wild type flavivirus in a host cell that has been infected with. In another example, the mutated flavivirus is associated with a smaller plaque size relative to another plaque within the same assay or plate. In some examples, focus forming assay may be carried out. It is understood that the focus forming assay is a variation of the plaque assay. Instead of detecting the plaque formation after virus-induced cell lysis, the focus forming assay detects infected host cells and infectious virus particles before a plaque is formed.

[0074] In another example, the flavivirus comprising the said NS2B mutation has an increased replication rate, as compared to the wild type flavivirus. The replication rate may be determined by the levels of the flavivirus in the infected host, which may in turn be quantified by the level of specific proteins or nucleic acids of the flavivirus. In one example, the replication rate is determined by quantifying the viral RNA using qPCR. Increased replication allows faster and enhanced immune response which leads to activation of antiviral state in neighbouring cells making them refractory to infection. While accelerating the replication, the mutated flavivirus concurrently induces an increased IFN response in the host and the mutated flavivirus responds to the interferon with an increased sensitivity. The increased IFN response and the increased sensitivity to the interferon effectively restrict the viral dissemination to neighboring uninfected cells, thus leading to attenuated virulence.

[0075] In another example, the flavivirus comprising the said NS2B protein is associated with a smaller plaque size and has increased replication rate, as compared to the wild type flavivirus.

[0076] In yet another example, the flavivirus comprising the said NS2B protein is associated with an increased interferon response, an increased replication rate and smaller plaque size, as compared to the wild type flavivirus.

[0077] In addition to the mutations in NS2B protein, the flavivirus may comprise one or more mutations in the genome sequence, wherein the one or more mutations occur in the nucleic acid sequence encoding one or more of proteins selected from the group consisting of capsid (C), precursor membrane (prM), envelope (E), NS1, NS2A, NS3, NS4A, NS4B, and NS5 proteins. The additional mutations may occur at one or more positions within a consensus nucleic acid sequence.

[0078] In a preferred example, the flavivirus further comprises at least one mutation in the genome sequence encoding a precursor membrane (prM) protein, or at least one mutation in the genome sequence encoding a NS1 protein, or at least one mutation in the genome sequence encoding a prM protein and at least one mutation in the genome sequence encoding a NS1 protein; wherein the at least one mutation in the nucleic acid sequence encoding the prM protein replaces an amino acid at position 29 or an amino acid at a position equivalent to amino acid position 29 of the reference sequence as set forth in SEQ ID NO: 56, and the at least one mutation in the genome sequence encoding the NS1 protein replaces an amino acid at position 53 or an amino acid at a position equivalent to amino acid position 53 of a reference sequence as set forth in SEQ ID NO: 57. SEQ ID NO: 56 is a representative amino acid sequence of the prM protein in flaviviruses. The representative amino acid sequence is also known as the consensus sequence, which is generated by aligning the amino acid sequences of the prM protein of flaviviruses (Figure 11). A person skilled in the art would understand position 29 of prM or an equivalent position thereof in a given sequence may be determined by aligning the sequence with the consensus sequence. Similarly, SEQ ID NO: 57 is a representative amino acid sequence of the NS1 protein in flaviviruses. The representative amino acid sequence is also known as the consensus sequence, which is generated by aligning the amino acid sequences of the NS1 protein of flaviviruses (Figure 9). A person skilled in the art would understand position 53 of NS1 or an equivalent position thereof in a given sequence may be determined by aligning the sequence with the consensus sequence.

[0079] In another embodiment, the at least one mutation replaces an amino acid at position 29 or an amino acid at a position equivalent to amino acid position 29 of the amino

acid sequence as set forth in SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12.

[0080] In another embodiment, the at least one mutation replaces an amino acid at position 53 or an amino acid at a position equivalent to amino acid position 53 of the amino acid sequence as set forth in SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, and SEQ ID NO: 16.

[0081] In one example, the further mutations comprise at least one mutation in a nucleic acid sequence encoding a prM protein. The resulting flavivirus comprises at least one mutation in a nucleic acid sequence encoding a NS2B protein and at least one mutation in a nucleic acid sequence encoding a prM protein, wherein the NS2B mutation results in replacement of an amino acid at position 114 or an amino acid at a position equivalent to position 114 of the amino acid sequence as set forth in SEQ ID NO: 55, 5, 6, 7, or 8, and the prM mutation results in replacement of an amino acid at position 29 or an amino acid at a position equivalent to amino acid position 29 of the amino acid sequence as set forth in SEQ ID NO: 56, 9, 10, 11, or 12.

[0082] In another example, the further mutations comprise at least one mutation in a nucleic acid sequence encoding a NS1 protein. The resulting flavivirus comprises at least one mutation in a nucleic acid sequence encoding a NS2B protein and at least one mutation in a nucleic acid sequence encoding a NS1 protein, wherein the NS2B mutation results in replacement of an amino acid at position 114 or an amino acid at a position equivalent to position 114 of the amino acid sequence as set forth in SEQ ID NO: 55, 5, 6, 7, or 8, and the NS1 mutation results in replacement of an amino acid at position 53 or an amino acid at a position equivalent to amino acid position 53 of the amino acid sequence as set forth in SEQ ID NO: 57, 13, 14, 15, or 16.

[0083] In yet another example, the further mutations comprise at least one mutation in a nucleic acid sequence encoding a prM protein and at least one mutation in a nucleic acid sequence encoding a NS1 protein. The resulting flavivirus comprises at least one mutation in a nucleic acid sequence encoding a NS2B protein, at least one mutation in a nucleic acid sequence encoding a prM protein, and at least one mutation in a nucleic acid sequence encoding a NS1 protein, wherein the NS2B mutation results in replacement of an amino acid at position 114 or an amino acid at a position equivalent to position 114 of the amino acid sequence as set forth in SEQ ID NO: 55, 5, 6, 7, or 8, the prM mutation results in replacement

of an amino acid at position 29 or an amino acid at a position equivalent to amino acid position 29 of the amino acid sequence as set forth in SEQ ID NO: 56, 9, 10, 11, or 12, and the NS1 mutation results in replacement of an amino acid at position 53 or an amino acid at a position equivalent to amino acid position 53 of the amino acid sequence as set forth in SEQ ID NO: 57, 13, 14, 15, or 16.

[0084] SEQ ID NOs: 9, 10, 11, and 12 correspond to the amino acid sequence of prM protein of wild type DENV1 strain D1/SG/05K2402DK1/2005, DENV2 strain D2/SG/05K3295DK1/2005, DENV3 strain D3/SG/05K863DK1/2005, and DENV4 strain D4/SG/06K2270DK1/2005, respectively. SEQ ID NOs: 13, 14, 15, and 16 correspond to the amino acid sequence of NS1 protein of wild type DENV1 strain D1/SG/05K2402DK1/2005, DENV2 strain D2/SG/05K3295DK1/2005, DENV3 strain D3/SG/05K863DK1/2005, and DENV4 strain D4/SG/06K2270DK1/2005, respectively.

[0085] It would also be understood that the position "equivalent" to position 29 of the prM amino acid sequence as set forth in SEQ ID NO: 56, 9, 10, 11, or 12 refers to the position in an amino acid sequence which corresponds in position in either primary or tertiary protein structure to position 29 of the amino acid sequence of SEQ ID NO: 56, 9, 10, 11, or 12. In a preferred example, the equivalent position refers to the position in the primary protein structure. Such positions in the primary protein structure may be determined according to sequence alignment against a reference amino acid sequence selected from SEQ ID NO: 56, 9, 10, 11, or 12 (Figure 9). Examples of positions equivalent to position 29 of the amino acid sequence as set forth in SEQ ID NO: 56, 9, 10, 11, or 12 may include, but are not limited to, position 29 of the prM amino acid sequence of DENV1 strain D1/05K872DK1 or D1/WestPac, DENV2 strain D2/16681 or D2/Tonga, DENV3 strain D3/C0360 or D3/Sleman, DENV4 strain D4/Dominica or D4/VE_61013, and JEV strain JEV/Nakamaya, and position 25 of the prM amino acid sequence of Yellow fever virus strain asibi. Similarly, the position "equivalent" to position 53 of the NS1 amino acid sequence as set forth in SEQ ID NO: 57, 13, 14, 15, or 16 refers to the position in an amino acid sequence which corresponds in position in either primary or tertiary protein structure to position 53 of the sequence of SEQ ID NO: 57, 13, 14, 15, or 16. In a preferred example, the equivalent position refers to the position in the primary protein structure. Such positions in the primary protein structure may be determined according to sequence alignment against a reference amino acid sequence selected from SEQ ID NO: 13, 14, 15, or 16 (Figure 11). Examples of

positions equivalent to position 53 of the NS1 amino acid sequence as set forth in SEQ ID NO: 57, 13, 14, 15, or 16 may include, but are not limited to, position 53 of the NS1 amino acid sequence of DENV2 strain D2/16681 or D2/Tonga, DENV1 strain D1/05K872DK1 or D1/WestPac, DENV3 strain D3/C0360 or D3/Sleman, DENV4 strain D4/Dominica or D4/VE_61013, JEV strain JEV/Nakamaya, or YFV strain asibi.

[0086] The said further mutations in prM and/or NS1 proteins may work *in sync* with the mutation in the said NS2B protein to further attenuate the flavivirus. The further attenuation of the flavivirus may stabilize the viral genome and prevent the mutated genome from reverting back to the wild type sequence that is associated with higher virulence. The flavivirus comprising said further mutations may be characterized with one or more attenuating features selected from a smaller plaque size *in vitro*, an increased rate of virus replication, and an increased survival rate of the infected host, relative to a wild type flavivirus. Additionally, the one or more attenuating features are accompanied by an increased growth or replication rate. Increased replication allows faster and enhanced immune response which leads to activation of antiviral state in neighbouring cells making them refractory to infection. Nonetheless, the spread of the virus to the neighbouring cells is restricted due to the increased susceptibility of the virus to interferon response.

[0087] In one example, the flavivirus with the said further mutations is characterized *in vitro* by a smaller plaque size, as compared to the wild type flavivirus. In another example, the flavivirus with the said further mutations is characterized by an increased interferon response. In another example, the flavivirus with the said further mutation is characterized *in vitro* by smaller plaque size and increased interferon response. In yet another example, the flavivirus with further mutation has increased replication rate. It is understood that while the flavivirus has accelerated replication, the infection dissemination will be restricted by the increased IFN response in the host and increased sensitivity of the flavivirus to the interferon response, thereby leading to the attenuation in the virulence of the virus.

[0088] Another important consideration for an attenuated flavivirus is the stability of the flavivirus genome that harbours the attenuating mutations. In one example, the mutation in NS2B protein is stable on its own. For example, NS2B mutation is stable in DENV2 and DENV4. In another example, the combination of mutation in NS2B protein with the further mutation in prM and/ or NS1 proteins stabilizes the genome of the flavivirus. For example, NS2B mutation in DENV1 and DENV3 is stabilized when prM and/or NS1 mutations are

introduced. The increased genomic stability would prevent the mutated genome from reverting back to the wild type sequence that is associated with higher virulence. The genome may remain stable after 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 passages *in vitro*. In one example, the genome remains stable after 2 passages *in vitro*. In another example, the genome remains stable after more than 2 passages, for example, 3, or 4 passages. It is understood that the genome stability may be tested using common methods known in the art. For example, the genome stability can be tested by genotyping the virus progeny *in vitro* in host cells. Examples of genotyping methods include but are not limited to sequencing, INNO-LiPA, restriction fragment polymorphism (RFLP), multiplex PCR, serotyping, oligonucleotide microarray chips, reverse dot blot, restriction fragment mass polymorphism (RFMP), invader assay, and real-time PCR. The sequencing methods may be Sanger sequencing or Next-Generation Sequencing. Examples of Next-Generation Sequencing include but are not limited to whole genome sequencing, transcriptome sequencing, epigenome sequencing. The Next-Generation Sequencing may be carried out using various platforms, such as Deep Sequencing.

[0089] As disclosed herein, the mutations may result in replacement of amino acids at the said positions. The amino acid may be replaced with a neutral, hydrophobic, or hydrophilic amino acid.

[0090] In one example, the amino acid at position 114 or an equivalent position thereof in the NS2B protein may be replaced with a neutral amino acid, such as serine (Ser, S), threonine (Thr or T), tyrosine (Tyr or Y), glycine (Gly or G), histidine (His or H), and proline (Pro or P). In a preferred example, the amino acid at position 114 or an equivalent position thereof is replaced with threonine (Thr or T). In a more preferred example, the amino acid replacement is from isoleucine (Ile or I) to threonine (Thr or T).

[0091] In another example, the amino acid at position 29 or an equivalent position thereof is replaced with a hydrophobic amino acid, such as alanine (Ala or A), valine (Val or V), leucine (Leu or L), isoleucine (Ile or I), cysteine (Cys or C), phenylalanine (Phe or F), methionine (Met or M), and tryptophan (Trp or W). In a preferred example, the amino acid at position 29 of prM amino acid sequence or an equivalent position thereof is replaced with valine (Val or V). In a more preferred example, the amino acid replacement is from asparagine (Asn or N) to valine (Val or V). In another more preferred example, the amino acid replacement is from alanine (Ala or A) to valine (Val or V). In another more preferred

example, the amino acid replacement is from serine (Ser or S) to valine (Val or V). In yet another more preferred example, the amino acid replacement is from glutamic acid (Glu or E) to valine (Val or V). In yet another preferred example, the amino acid replacement is from aspartic acid (Asp or D) to valine (Val or V).

[0092] In yet another example, the amino acid at position 53 of NS1 amino acid sequence or an equivalent position thereof is replaced with a hydrophilic amino acid, such as asparagine (Asn or N), aspartic acid (Asp or D), glutamine (Gln or Q), glutamic acid (Glu or E), lysine (Lys or K), and arginine (Arg or R). In a preferred example, the amino acid at position 53 of NS1 amino acid sequence or an equivalent position thereof is replaced with aspartic acid (Asp or D). In one example, the amino acid replacement is from glycine (Gly or G) to aspartic acid (Asp or D).

[0093] It would be understood that a specific virus strain can be defined by its amino acid sequence or genome sequence.

[0094] In a preferred example, the flavivirus is a dengue virus of DENV1 serotype. In one example, the flavivirus is a DENV1 strain comprising an amino acid sequence as set forth in SEQ ID NO: 23, 24, 25, or 26. In another example, the flavivirus is a DENV1 strain comprising a variant amino acid sequence of the sequence as set forth in SEQ ID NO: 23, 24, 25, or 26. In one example, the flavivirus is a DENV1 strain comprising a genome sequence as set forth in SEQ ID NO: 39, 40, 41, or 42. In another example, the flavivirus is a DENV1 strain comprising a variant genome sequence of the sequence as set forth in SEQ ID NO: 39, 40, 41, or 42.

[0095] In another preferred example, the flavivirus is a dengue virus of DENV2 serotype. In one example, the flavivirus is a DENV2 strain comprising an amino acid sequence as set forth in SEQ ID NO: 27, 28, 29, or 30. In another example, the flavivirus is a DENV2 strain comprising a variant amino acid sequence of the sequence as set forth in SEQ ID NO: 27, 28, 29, or 30. In one example, the flavivirus is a DENV2 strain comprising a genome sequence as set forth in SEQ ID NO: 43, 44, 45, or 46. In another example, the flavivirus is a DENV2 strain comprising a variant genome sequence of the sequence as set forth in SEQ ID NO: 43, 44, 45, or 46.

[0096] In another preferred example, the flavivirus is a dengue virus of DENV3 serotype. In one example, the flavivirus is a DENV3 strain comprising an amino acid sequence as set forth in SEQ ID NO: 31, 32, 33, or 34. In another example, the flavivirus is a DENV3 strain

comprising a variant amino acid sequence of the sequence as set forth in SEQ ID NO: 31, 32, 33, or 34. In one example, the flavivirus is a DENV3 strain comprising a genome sequence as set forth in SEQ ID NO: 47, 48, 49, or 50. In another example, the flavivirus is a DENV3 strain comprising a variant genome sequence of the sequence as set forth in SEQ ID NO: 47, 48, 49, or 50.

[0097] In yet another preferred example, the flavivirus is a dengue virus of DENV4 serotype. In one example, the flavivirus is a DENV4 strain comprising an amino acid sequence as set forth in SEQ ID NO: 35, 36, 37, or 38. In another example, the flavivirus is a DENV4 strain comprising a variant amino acid sequence of the sequence as set forth in SEQ ID NO: 35, 36, 37, or 38. In one example, the flavivirus is a DENV4 strain comprising a genome sequence as set forth in SEQ ID NO: 51, 52, 53, or 54. In another example, the flavivirus is a DENV4 strain comprising a variant genome sequence of the sequence as set forth in SEQ ID NO: 51, 52, 53, or 54.

[0098] The variant sequence may have at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to a reference sequence, or to a section within the reference sequence. In one example, the variant sequence may have at least about 60%, at least about 65%, or at least about 70% sequence identity to a reference sequence. In a preferred example, the variant sequence may have at least about 65% sequence identity to a reference sequence. In another preferred example, the variant sequence may have about 65% sequence identity to a reference sequence. The reference sequence may be an amino acid sequence or a genome sequence. The reference amino acid sequence may be any one of SEQ ID NO: 23-38. The reference genome sequence may be any one of SEQ ID NO: 39-54. The section within the reference sequence may be the amino acid or genome sequence corresponding to NS2B, prM, and/or NS1 protein. It would be understood that a dengue virus comprising a variant sequence of a reference sequence as defined herein may refer to a specific virus strain within the serotype of DENV1, DENV2, DENV3, or DENV4 harboring the mutations of interest as described herein.

[0099] In some other examples, the flavivirus may be a non-dengue virus. In one example, the non-dengue virus comprises a variant amino acid sequence of the amino acid sequence as

set forth in SEQ ID NO: 23-38. In another example, the non-dengue virus comprises a variant genome sequence of the sequence as set forth in SEQ ID NO: 39-54.

[00100] The sequence variant may have at least about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% sequence identity to a reference sequence, or to a section within the reference sequence. The reference amino acid sequence may be any one of SEQ ID NO: 23-38. The reference genome sequence may be any one of SEQ ID NO: 39-54. The section within the reference sequence may be the amino acid or genome sequence corresponding to NS2B, prM, and/or NS1 protein. It would also be understood that a non-dengue virus comprising a variant sequence of a reference sequences as described herein may refer to a specific non-dengue virus within the genus of *Flavivirus* harboring the mutations of interest as described herein.

[00101] Additionally, there may be more than one mutation in the nucleic acid sequence encoding the NS2B protein, the prM protein, or the NS1 protein. In one example, there are 1, 2, 3, 4, 5, or 6 additional mutations in the nucleic acid sequence encoding the NS2B protein. In another example, there are 1, 2, 3, 4, 5, or 6 additional mutations in the nucleic acid sequence encoding the prM protein. In yet another example, there are 1, 2, 3, 4, 5, or 6 additional mutations in the nucleic acid sequence encoding the NS1 protein. These additional mutations may or may not cause replacement of amino acids. These additional mutations may or may not contribute to the attenuation of virulence.

[00102] As disclosed herein, the flavivirus comprises the whole genome encoding for various structural and non-structural proteins from a single virus strain. It would be appreciated that the flavivirus as described herein is a non-chimeric virus. When infected with a non-chimeric virus, humoral and cellular immune responses induced in the host will be directed to the entire virus of the same strain, i.e., the entire virus includes both structural proteins (capsid (C), precursor membrane (prM), envelope (E)) and non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). Therefore, a non-chimeric virus confers complete protection against the virus. In contrast, a chimeric virus strain that comprises structural/non-structural proteins from different viruses, such as those used in the conventional dengue vaccines, has incomplete protection against the virus strain and thus limits the efficacy whereby naïve individuals are more prone to sever diseases upon infection

after vaccination. Thus, it would be appreciated that a non-chimeric virus is superior to the chimeric virus at least from this perspective.

[00103] In a preferred example, the flavivirus is a live attenuated virus. It would be understood a live attenuated virus refers to a virus which is viable but less virulent as compared to the wild type virus. A live attenuated virus is a suitable virus strain to be used in live attenuated vaccines.

[00104] In another aspect, the present invention refers to an immunogenic composition comprising one or more flaviviruses as described herein. In a preferred example, the composition is formulated as a vaccine composition.

[00105] The immunogenic composition may comprise one or more flaviviruses from any species within the genus of *Flavivirus*. Examples of species within the genus of *Flavivirus* may include, but are not listed to, Zika viruses (ZIKVs), Japanese encephalitis viruses (JEVs), yellow fever viruses (YFVs), West Nile viruses (WNVs), tick-borne encephalitis viruses (TBEVs), and Dengue viruses (DENVs). The one or more flaviviruses may be selected from the same or different species.

[00106] In a preferred example, the immunogenic composition comprises one or more dengue viruses. The one or more dengue viruses may be selected from any serotype selected from DENV1, DENV2, DENV3, DENV4, or combinations thereof.

[00107] In one example, the immunogenic composition comprises the flavivirus of one dengue virus serotype. For example, the one dengue virus serotype may be DENV1, DENV2, DENV3, or DENV4. It would be understood that such immunogenic composition may be formulated as a monovalent vaccine against the one dengue virus serotype.

[00108] In another example, the immunogenic composition comprises the flavivirus of two dengue virus serotypes. For example, the two dengue virus serotypes may be selected from the group consisting of DENV1 and DENV2, DENV1 and DENV3, DENV1 and DENV4, DENV2 and DENV3, DENV2 and DENV4, and DENV3 and DENV4. It would be understood that such immunogenic composition may be formulated as a bivalent vaccine against the two dengue virus serotypes.

[00109] In another example, the immunogenic composition comprises the flavivirus of three dengue virus serotypes. For example, the three dengue virus serotypes may be DENV1, DENV2 and DENV3, DENV1, DENV2 and DENV4, DENV1, DENV3 and DENV4, or

DENV2, DENV3 and DENV4. It would be understood that such immunogenic composition may be formulated as a trivalent vaccine against the three dengue virus serotypes.

[00110] In a preferred example, the immunogenic composition comprises the flavivirus of all four dengue virus serotypes DENV1, DENV2, DENV3 and DENV4. It would be understood that such immunogenic composition may be formulated as a tetravalent vaccine against all four dengue virus serotypes.

[00111] When immunogenic composition comprises the flavivirus of different dengue virus serotypes, the flavivirus of different dengue virus serotypes may comprise the same or different mutations selected from the mutations of interest as described herein.

[00112] In one preferred example, the flavivirus of different dengue virus serotypes comprises the same mutations selected from the group consisting of mutations in NS2B protein, mutations in NS2B and prM proteins, mutations in NS2B and NS1 proteins, and mutations in NS2B, prM and NS1 proteins. In one example, the flavivirus of each dengue virus serotype comprises the same mutation in NS2B protein, wherein the NS2B mutation results in replacement of an amino acid at position 114 or an amino acid at a position equivalent to position 114 of the NS2B amino acid sequence as set forth in SEQ ID NO: 55, 5, 6, 7, or 8. In another example, the flavivirus of each dengue virus serotype comprises the same mutation in NS2B and prM proteins, wherein the NS2B mutation results in replacement of an amino acid at position 114 or an amino acid at a position equivalent to position 114 of the NS2B amino acid sequence as set forth in SEQ ID NO: 55, 5, 6, 7, or 8, and the prM mutation results in replacement of an amino acid at position 29 or an amino acid at a position equivalent to amino acid position 29 of the prM amino acid sequence as set forth in SEQ ID NO: 56, 9, 10, 11, or 12. In another example, the flavivirus of each dengue virus serotype comprises the same mutation in NS2B and NS1 proteins, wherein the NS2B mutation results in replacement of an amino acid at position 114 or an amino acid at a position equivalent to position 114 of the NS2B amino acid sequence as set forth in SEQ ID NO: 55, 5, 6, 7, or 8, and the NS1 mutation results in replacement of an amino acid at position 53 or an amino acid at a position equivalent to amino acid position 53 of the NS1 amino acid sequence as set forth in SEQ ID NO: 57, 13, 14, 15, or 16. In yet another example, the flavivirus of each dengue virus serotype comprises the same mutation in NS2B, prM, and NS1 proteins, wherein the NS2B mutation results in replacement of an amino acid at position 114 or an amino acid at a position equivalent to position 114 of the NS2B amino acid sequence as set forth in SEQ ID

NO: 55, 5, 6, 7, or 8, the prM mutation results in replacement of an amino acid at position 29 or an amino acid at a position equivalent to amino acid position 29 of the prM amino acid sequence as set forth in SEQ ID NO: 56, 9, 10, 11, or 12, and the NS1 mutation results in replacement of an amino acid at position 53 or an amino acid at a position equivalent to amino acid position 53 of the NS1 amino acid sequence as set forth in SEQ ID NO: 57, 13, 14, 15, or 16.

[00113] In some examples, when the flavivirus of each dengue virus serotype comprises the same mutations, viruses of different serotypes may share the same mechanism of action for immune response activation, leading to an equal and balanced immune response against each dengue virus serotype, i.e., there is no single serotype that outcompetes the others. It is understood that a balanced immune response against different dengue virus serotype is particularly desirable for a tetravalent vaccine.

[00114] In a preferred example, the immunogenic composition comprises the flavivirus of all four dengue virus serotypes, each of the serotypes comprises the same mutation, and said immunogenic composition induces a balanced immune response against each of the four dengue virus serotypes.

[00115] In another preferred example, the vaccine is a live attenuated vaccine. Advantageously, such vaccine may induce an increased IFN response in a host and the flavivirus in the vaccine may respond to the IFN response with higher sensitivity. A host is a subject that is to be, or is, administered with the immunogenic composition.

[00116] In general, suitable compositions may be prepared according to methods which are known to those of ordinary skill in the art and accordingly may further include a preservative, a stabilizer, a pharmaceutically acceptable carrier, or combinations thereof.

[00117] Generally, an effective dosage to achieve the desired immunogenic response is expected to be in the range of about 0.0001mg to about 1000mg per kg body weight per 24 hours; typically, about 0.001mg to about 750mg per kg body weight per 24 hours; about 0.01mg to about 500mg per kg body weight per 24 hours; about 0.1mg to about 500mg per kg body weight per 24 hours; about 0.1mg to about 250mg per kg body weight per 24 hours; about 1.0mg to about 250mg per kg body weight per 24 hours. More typically, an effective dose range is expected to be in the range about 1.0mg to about 200mg per kg body weight per 24 hours; about 1.0mg to about 100mg per kg body weight per 24 hours; about 1.0mg to about 50mg per kg body weight per 24 hours; about 1.0mg to about 25mg per kg body weight

per 24 hours; about 5.0mg to about 50mg per kg body weight per 24 hours; about 5.0mg to about 20mg per kg body weight per 24 hours; about 5.0mg to about 15mg per kg body weight per 24 hours.

[00118] In another example, the amount of vaccine administered to elicit the desired immunogenic response is quantified based on the number of viruses. The number of viruses can be determined using methods known in the art, such as, but not limited to plaque assay, focus forming assay and endpoint dilution assay. The number of viruses to achieve the desired immunogenic response is expected to be in the range of about 10 to 10 million plaque forming units (PFU).

[00119] For example, an effective dosage may be measured by PFU in logarithmic-sclae (base 10). In some examples, the effective dosage to achieve the desired immunogenic response may be between about 2.0 and about 10.0 $\log_{10}$ PFU. The effective dosage may be about 2.0, about 2.5, about 3.0, about 3.5, about 4.0, about 4.5, about 5.0, about 5.5, about 6.0, about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, about 9.0, about 9.5, or about 10 $\log_{10}$ PFU. In a preferred example, the effective dosage may be about 3.0, about 3.1, about 3.2, about 3.3, about 3.4, about 3.5, about 3.6, about 3.7, about 3.8, about 3.9, about 4.0, about 4.1, about 4.2, about 4.3, about 4.4, about 4.5, about 4.6, about 4.7, about 4.8, about 4.9, about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, or about 6.0 $\log_{10}$ PFU.

[00120] In another aspect, there is provided a method of eliciting an immune response against one or more flaviviruses in a subject comprising administering an effective amount of the immunogenic composition as described herein to the subject.

[00121] There is also provided use of the immunogenic composition as described herein in the manufacture of a medicament for eliciting an immune response against one or more flaviviruses in a subject, wherein an effective amount of the immunogenic composition is to be administered to the subject.

[00122] There is further provided the immunogenic composition as described herein for use in eliciting an immune response against one or more flaviviruses in a subject, wherein an effective amount of the immunogenic composition is to be administered to the subject.

[00123] When a subject is administered with the immunogenic composition comprising the flavivirus as described herein, the subject will develop immune responses against the flavivirus, and the immune responses may include innate and/or adaptive immune responses.

The adaptive immune response may include humoral and/or cellular immune responses. Such immune responses protect the subject from infection with the flavivirus. In one example, the immune responses provide protection against one or more flaviviruses within the genus of *Flavivirus*. In another example, the immune responses provide protection against one or more dengue viruses.

[00124] The immunogenic composition to be administered may comprise one or more flaviviruses selected from any flavivirus within the genus of *Flavivirus*, such as Zika viruses (ZIKVs), Japanese encephalitis viruses (JEVs), yellow fever viruses (YFVs), West Nile viruses (WNVs), tick-borne encephalitis viruses (TBEVs), and Dengue viruses (DENVs). In a preferred example, the immunogenic composition comprises one or more dengue viruses.

[00125] In a preferred example, the one or more flaviviruses are one or more dengue virus serotypes selected from the group consisting of DENV1, DENV2, DENV3, DENV4, and combinations thereof. In one example, the immunogenic composition is formulated as a monovalent vaccine comprising the dengue virus of one serotype which confers protection against the one dengue virus serotype. In another example, the immunogenic composition is formulated as a bivalent vaccine comprising the dengue virus of two serotypes which confers protection against the two dengue virus serotypes. In another example, the immunogenic composition is formulated as a trivalent vaccine comprising the dengue virus of three serotypes which confers protection against the three dengue virus serotypes. In yet another example, the immunogenic composition is formulated as a tetravalent vaccine comprising the dengue virus of all four serotypes which confers protection against all four dengue virus serotypes.

[00126] The subject to be administered with the immunogenic composition may be a human or a non-human mammal. Examples of non-human mammals include but are not limited to non-human primates, livestock animals (e.g. sheep, cows, horses, donkeys, pigs), laboratory test animals (e.g. rabbits, mice, rats, guinea pigs, hamsters), companion animals (e.g. cats, dogs) and captive wild animals (e.g. foxes, deer, dingoes). In one example, the subject is a mouse. The human subjects can be either adults or children. In one example, the subject is a human at risk of dengue virus infection, such as subjects living in areas (or in close proximity to areas) with a Dengue outbreak. In one example, the subject has prior history of dengue viral infection. In another example, the subject does not have prior history of dengue viral infection.

[00127] The immunogenic composition may be administered to the subject by a route selected from the group consisting of intramuscular, intradermal, subcutaneous, intravenous, oral, and intranasal administration. Thus, the immunogenic compositions may be prepared in a form suitable for parenteral administration (that is, subcutaneous, intramuscular or intravenous injection), in the form of a formulation suitable for oral ingestion (such as capsules, tablets, caplets, elixirs, for example), or in an aerosol form suitable for administration by inhalation (such as by intranasal inhalation or oral inhalation). In a preferred example, the route of administration is intramuscular or subcutaneous injection.

[00128] In a fourth aspect, there is provided a method of preventing, ameliorating, or treating a disease caused by one or more flaviviruses in a subject comprising administering an effective amount of the immunogenic composition as described herein to the subject.

[00129] There is also provided use of the immunogenic composition as described herein in the manufacture of a medicament for preventing, ameliorating, or treating a disease caused by one or more flaviviruses in a subject, wherein an effective amount of the immunogenic composition is to be administered to the subject.

[00130] There is also provided the immunogenic composition as described herein for use in preventing, ameliorating, or treating a disease caused by one or more flaviviruses in a subject, wherein an effective amount of the immunogenic composition is to be administered to the subject.

[00131] In a preferred example, the one or more flaviviruses are one or more dengue virus serotypes selected from the group consisting of DENV1, DENV2, DENV3, DENV4, and combinations thereof.

[00132] The disclosure also provides a polynucleotide encoding any one of the flaviviruses as described herein. In one example, the polynucleotide encodes the complete flavivirus. In another example, the polynucleotide encodes one or more proteins of the flavivirus. The polynucleotide may be DNA or RNA.

[00133] The disclosure further provides a polypeptide encoded by any one of the polynucleotides as described herein. In one example, the polypeptide includes one or more proteins of the flavivirus. In another example, the polypeptide includes all proteins of the flavivirus.

[00134] In addition, the disclosure provides an immunogenic composition comprising the polynucleotide or polypeptide as described herein. It would be understood that such

immunogenic composition may be used in a method to elicit an immune response against one or more flaviviruses as well as to prevent, ameliorate, or treat a disease caused by one or more flaviviruses in a subject in need thereof. In a preferred example, the immunogenic composition is a vaccine.

[00135] It would be generally understood that the vaccine as disclosed herein may be administered to a subject in need thereof either in a single dose or in multiple doses. In one example, the vaccine may be administered in a single dose. In another example, the vaccine may be administered in two or more doses. The vaccine may be administered alone or in combination with a buffer. An example of a suitable buffer is phosphate buffered saline. The vaccine may be in lyophilized or aqueous form, with or without stabilizers in the final formulation. It will generally be understood by one of skill in the art that a lyophilized vaccine must be reconstituted in a suitable medium prior to administration to a subject.

EXPERIMENTAL SECTION

[00136] Non-limiting examples of the invention and comparative examples will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

[00137] ***Materials and Methods***

[00138] General materials and methods used in the study are provided below.

[00139] *Viruses*

[00140] DENV1-4 are clinical isolates obtained from patients from the Early DENgue (EDEN) study that was done in Singapore, including DENV1 (Dengue virus type 1 strain D1/SG/05K2402DK1/2005, GenBank: EU081230.1), DENV2 (Dengue virus type 2 strain, D2/SG/05K3295DK1/2005, GenBank: EU081177.1), DENV3 (Dengue virus type 3 strain D3/SG/05K863DK1/2005, GenBank: EU081190.1), DENV4 (Dengue virus type 4 strain, D4/SG/06K2270DK1/2005, GenBank: GQ398256).

[00141] *Infectious clone generation*

[00142] Genomic RNA of DENV1-4 were extracted using QIAamp Synthesis Kir (Qiagen) and cDNA synthesized using Superscript III First-Strand Synthesis Kit (Invitrogen). Six PCR products from each DENV were generated by 6 sets of primer pairs that are routinely used in the laboratory for Gibson assembly using Q5® High-Fidelity DNA polymerase (New

England BioLabs). The PCR products were gel purified for TA cloning into pGEMT® vector (Promega) to generate individual plasmids for each fragment. Site-directed mutagenesis on relevant plasmids was performed to generate specific mutations using NEB Q5 Site-Directed Mutagenesis Kit (New England Biolabs) and assembled by Gibson assembly to obtain infectious clone. The nucleotide (nt) and amino acid (aa) positions for mutations in prM, NS1, and NS2B proteins for all 4 DENV serotypes are listed in Table 1. Full amino acid and nucleic acid sequences are listed in Table 2. Infectious clone is transfected into HEK293T cells using Lipofectamine 2000 as per manufacturer's instructions. Viruses produced from these cells were harvested 48 hours after transfection. Virus progeny was amplified in Vero or C6/36 cells up to 5 passages to generate working stocks.

[00143] **Table 1**: Nucleotide (nt) and amino acid (aa) positions for mutations in NS1 and NS2B proteins for all 4 DENV serotypes.

	Gene	nt position	aa position	WT (aa)	mutant (aa)
DENV1	prM	521 – 523	29	GCA (Ala)	GTA (Val)
	NS1	2576 – 2578	53	GGT (Gly)	GAC (Asp)
	NS2B	4469 – 4471	114	ATA (Ile)	ACA (Thr)
DENV2	prM	523 – 525	29	AAC (Asn)	GTC (Val)
	NS1	2578 – 2580	53	GGC (Gly)	GAC (Asp)
	NS2B	4471 – 4473	114	ATA (Ile)	ACA (Thr)
DENV3	prM	521 – 523	29	TCT (Ser)	GTG (Val)
	NS1	2570 – 2572	53	GGA (Gly)	GAC (Asp)
	NS2B	4463 – 4465	114	ATA (Ile)	ACA (Thr)
DENV4	prM	526 – 528	29	GAA (Glu)	GTA (Val)
	NS1	2581 – 2583	53	GGG (Gly)	GAC (Asp)
	NS2B	4474 – 4476	114	ATT (Ile)	ACT (Thr)

[00144] Table 2: List of amino acid and nucleic acid sequences of the disclosure.

Wild type amino acid sequences	
SEQ ID NO: 1	complete amino acid sequence of wild type Dengue virus type 1 strain D1/SG/05K2402DK1/2005
SEQ ID NO: 2	complete amino acid sequence of wild type Dengue virus type 2 strain D2/SG/05K3295DK1/2005

SEQ ID NO: 3	complete amino acid sequence of wild type Dengue virus type 3 strain D3/SG/05K863DK1/2005
SEQ ID NO: 4	complete amino acid sequence of wild type Dengue virus type 4 strain D4/SG/06K2270DK1/2005
SEQ ID NO: 5	amino acid sequence of NS2B protein of wild type Dengue virus type 1 strain D1/SG/05K2402DK1/2005
SEQ ID NO: 6	amino acid sequence of NS2B protein of wild type Dengue virus type 2 strain D2/SG/05K3295DK1/2005
SEQ ID NO: 7	amino acid sequence of NS2B protein of wild type Dengue virus type 3 strain D3/SG/05K863DK1/2005
SEQ ID NO: 8	amino acid sequence of NS2B protein of wild type Dengue virus type 4 strain D4/SG/06K2270DK1/2005
SEQ ID NO: 9	amino acid sequence of prM protein of wild type Dengue virus type 1 strain D1/SG/05K2402DK1/2005
SEQ ID NO: 10	amino acid sequence of prM protein of wild type Dengue virus type 2 strain D2/SG/05K3295DK1/2005
SEQ ID NO: 11	amino acid sequence of prM protein of wild type Dengue virus type 3 strain D3/SG/05K863DK1/2005
SEQ ID NO: 12	amino acid sequence of prM protein of wild type Dengue virus type 4 strain D4/SG/06K2270DK1/2005
SEQ ID NO: 13	amino acid sequence of NS1 protein of wild type Dengue virus type 1 strain D1/SG/05K2402DK1/2005
SEQ ID NO: 14	amino acid sequence of NS1 protein of wild type Dengue virus type 2 strain D2/SG/05K3295DK1/2005
SEQ ID NO: 15	amino acid sequence of NS1 protein of wild type Dengue virus type 3 strain D3/SG/05K863DK1/2005
SEQ ID NO: 16	amino acid sequence of NS1 protein of wild type Dengue virus type 4 strain D4/SG/06K2270DK1/2005
SEQ ID NO: 17	complete amino acid sequence of wild type Dengue virus type 1 strain DF01-HUB01021093
SEQ ID NO: 18	complete amino acid sequence of wild type Dengue virus type 1

	strain DENV-1/China/YN/GM1502
SEQ ID NO: 58	amino acid sequence of NS2B protein of wild type Dengue virus type 1 strain D1/05K872DK1
SEQ ID NO: 59	amino acid sequence of NS2B protein of wild type Dengue virus type 1 strain D1/WestPac
SEQ ID NO: 60	amino acid sequence of NS2B protein of wild type Dengue virus type 2 strain D2/16681
SEQ ID NO: 61	amino acid sequence of NS2B protein of wild type Dengue virus type 2 strain D2/Tonga
SEQ ID NO: 62	amino acid sequence of NS2B protein of wild type Dengue virus type 3 strain D2/C0360
SEQ ID NO: 63	amino acid sequence of NS2B protein of wild type Dengue virus type 3 strain D2/Sleman
SEQ ID NO: 64	amino acid sequence of NS2B protein of wild type Dengue virus type 4 strain D4/Dominca
SEQ ID NO: 65	amino acid sequence of NS2B protein of wild type Dengue virus type 4 strain D4/VE_61013
SEQ ID NO: 66	amino acid sequence of NS2B protein of wild type Japanese encephalitis strain virus JE/Nakayama
SEQ ID NO: 67	amino acid sequence of NS2B protein of wild type Yellow Fever virus strain YF/Asibi
SEQ ID NO: 68	amino acid sequence of prM protein of wild type Dengue virus type 1 strain D1/05K872DK1
SEQ ID NO: 69	amino acid sequence of prM protein of wild type Dengue virus type 1 strain D1/WestPac
SEQ ID NO: 70	amino acid sequence of prM protein of wild type Dengue virus type 2 strain D2/16681
SEQ ID NO: 71	amino acid sequence of prM protein of wild type Dengue virus type 2 strain D2/Tonga
SEQ ID NO: 72	amino acid sequence of prM protein of wild type Dengue virus type 3 strain D3/C0360

SEQ ID NO: 73	amino acid sequence of prM protein of wild type Dengue virus type 3 strain D3/Sleman
SEQ ID NO: 74	amino acid sequence of prM protein of wild type Dengue virus type 4 strain D4/Dominica
SEQ ID NO: 75	amino acid sequence of prM protein of wild type Dengue virus type 4 strain D4/VE_61013
SEQ ID NO: 76	amino acid sequence of prM protein of wild type Japanese encephalitis virus strain JE/Nakayama
SEQ ID NO: 77	amino acid sequence of prM protein of wild type Yellow Fever virus strain YF/Asibi
SEQ ID NO: 78	amino acid sequence of NS1 protein of wild type Dengue virus type 1 strain D1/05K872DK1
SEQ ID NO: 79	amino acid sequence of NS1 protein of wild type Dengue virus type 1 strain D1/WestPac
SEQ ID NO: 80	amino acid sequence of NS1 protein of wild type Dengue virus type 2 strain D2/16681
SEQ ID NO: 81	amino acid sequence of NS1 protein of wild type Dengue virus type 2 strain D2/Tonga
SEQ ID NO: 82	amino acid sequence of NS1 protein of wild type Dengue virus type 3 strain D3/C0360
SEQ ID NO: 83	amino acid sequence of NS1 protein of wild type Dengue virus type 3 strain D3/Sleman
SEQ ID NO: 84	amino acid sequence of NS1 protein of wild type Dengue virus type 4 strain D4/Dominica
SEQ ID NO: 85	amino acid sequence of NS1 protein of wild type Dengue virus type 4 strain D4/VE_61013
SEQ ID NO: 86	amino acid sequence of NS1 protein of wild type Japanese encephalitis virus strain JEV/Nakayama
SEQ ID NO: 87	amino acid sequence of NS1 protein of wild type Yellow Fever virus strain YF/Asibi
Wild type nucleic acid sequences	

SEQ ID NO: 19	complete nucleotide sequence of wild type Dengue virus type 1 strain D1/SG/05K2402DK1/2005
SEQ ID NO: 20	complete nucleotide sequence of wild type Dengue virus type 2 strain D2/SG/05K3295DK1/2005
SEQ ID NO: 21	complete nucleotide sequence of wild type Dengue virus type 3 strain D3/SG/05K863DK1/2005
SEQ ID NO: 22	complete nucleotide sequence of wild type Dengue virus type 4 strain D4/SG/06K2270DK1/2005
Mutant amino acid sequences	
SEQ ID NO: 23	complete amino acid sequence of Dengue virus type 1 strain D1/SG/05K2402DK1/2005 with mutation at position 114 from Ile to Thr and mutation at position 29 from Ala to Val, corresponding to mutant DENV1-NS2B+prM
SEQ ID NO: 24	complete amino acid sequence of Dengue virus type 1 strain D1/SG/05K2402DK1/2005 with mutation at position 114 from Ile to Thr and mutation at position 53 from Gly to Asp, corresponding to mutant DENV1-NS2B+NS1
SEQ ID NO: 25	complete amino acid sequence of Dengue virus type 1 strain D1/SG/05K2402DK1/2005 with mutation at position 114 from Ile to Thr, mutation at position 53 from Gly to Asp, and mutation at position 29 from Ala to Val, corresponding to mutant DENV1-NS2B+NS1+prM
SEQ ID NO: 26	complete amino acid sequence of Dengue virus type 1 strain D1/SG/05K2402DK1/2005 with mutation at position 114 from Ile to Thr, corresponding to mutant DENV1-NS2B
SEQ ID NO: 27	complete amino acid sequence of Dengue virus type 2 strain D2/SG/05K3295DK1/2005 with mutation at position 114 from Ile to Thr and mutation at position 29 from Asn to Val, corresponding to mutant DENV2-NS2B+prM
SEQ ID NO: 28	complete amino acid sequence of Dengue virus type 2 strain D2/SG/05K3295DK1/2005 with mutation at position 114 from Ile to Thr and mutation at position 53 from Gly to Asp, corresponding to

	mutant DENV2-NS2B+NS1
SEQ ID NO: 29	complete amino acid sequence of Dengue virus type 2 strain D2/SG/05K3295DK1/2005 with mutation at position 114 from Ile to Thr, mutation at position 53 from Gly to Asp, and mutation at position 29 from Asn to Val, corresponding to mutant DENV2-NS2B+NS1+prM
SEQ ID NO: 30	complete amino acid sequence of Dengue virus type 2 strain D2/SG/05K3295DK1/2005 with mutation at position 114 from Ile to Thr, corresponding to mutant DENV2-NS2B
SEQ ID NO: 31	complete amino acid sequence of Dengue virus type 3 strain D3/SG/05K863DK1/2005 with mutation at position 114 from Ile to Thr and mutation at position 29 from Ser to Val, corresponding to mutant DENV3-NS2B+prM
SEQ ID NO: 32	complete amino acid sequence of Dengue virus type 3 strain D3/SG/05K863DK1/2005 with mutation at position 114 from Ile to Thr and mutation at position 53 from Gly to Asp, corresponding to mutant DENV3-NS2B+NS1
SEQ ID NO: 33	complete amino acid sequence of Dengue virus type 3 strain D3/SG/05K863DK1/2005 with mutation at position 114 from Ile to Thr, mutation at position 53 from Gly to Asp, and mutation at position 29 from Ser to Val, corresponding to mutant DENV3-NS2B+NS1+prM
SEQ ID NO: 34	complete amino acid sequence of Dengue virus type 3 strain D3/SG/05K863DK1/2005 with mutation at position 114 from Ile to Thr, corresponding to mutant DENV3-NS2B
SEQ ID NO: 35	complete amino acid sequence of Dengue virus type 4 strain D4/SG/06K2270DK1/2005 with mutation at position 114 from Ile to Thr and mutation at position 29 from Glu to Val, corresponding to mutant DENV4-NS2B+prM
SEQ ID NO: 36	complete amino acid sequence of Dengue virus type 4 strain D4/SG/06K2270DK1/2005 with mutation at position 114 from Ile to Thr and mutation at position 53 from Gly to Asp, corresponding to

	mutant DENV4-NS2B+NS1
SEQ ID NO: 37	complete amino acid sequence of Dengue virus type 4 strain D4/SG/06K2270DK1/2005 with mutation at position 114 from Ile to Thr, mutation at position 53 from Gly to Asp, and mutation at position 29 from Glu to Val, corresponding to mutant DENV4-NS2B+NS1+prM
SEQ ID NO: 38	complete amino acid sequence of Dengue virus type 4 strain D4/SG/06K2270DK1/2005 with mutation at position 114 from Ile to Thr, corresponding to mutant DENV4-NS2B
Mutant nucleic acid sequences	
SEQ ID NO: 39	complete nucleotide sequence of Dengue virus type 1 strain D1/SG/05K2402DK1/2005 with mutation at positions 4469-4471 from ATA to ACA and mutation at positions 521-523 from GCA to GTA, corresponding to mutant DENV1-NS2B+prM
SEQ ID NO: 40	complete nucleotide sequence of Dengue virus type 1 strain D1/SG/05K2402DK1/2005 with mutation at positions 4469-4471 from ATA to ACA and mutation at positions 2576-2578 from GGT to GAC, corresponding to mutant DENV1-NS2B+NS1
SEQ ID NO: 41	complete nucleotide sequence of Dengue virus type 1 strain D1/SG/05K2402DK1/2005 with mutation at positions 4469-4471 from ATA to ACA, mutation at positions 2576-2578 from GGT to GAC, and mutation at positions 521-523 from GCA to GTA, corresponding to mutant DENV1-NS2B+NS1+prM
SEQ ID NO: 42	complete nucleotide sequence of Dengue virus type 1 strain D1/SG/05K2402DK1/2005 with mutation at positions 4469-4471 from ATA to ACA, corresponding to mutant DENV1-NS2B
SEQ ID NO: 43	complete nucleotide sequence of Dengue virus type 2 strain D2/SG/05K3295DK1/2005 with mutation at positions 4471-4473 from ATA to ACA and mutation at positions 523-525 from AAC to GTC, corresponding to mutant DENV2-NS2B+prM
SEQ ID NO: 44	complete nucleotide sequence of Dengue virus type 2 strain D2/SG/05K3295DK1/2005 with mutation at positions 4471-4473

	from ATA to ACA and mutation at positions 2578-2580 from GGC to GAC, corresponding to mutant DENV2-NS2B+NS1
SEQ ID NO: 45	complete nucleotide sequence of Dengue virus type 2 strain D2/SG/05K3295DK1/2005 with mutation at positions 4471-4473 from ATA to ACA, mutation at positions 523-525 from AAC to GTC and mutation at positions 2578-2580 from GGC to GAC, corresponding to mutant DENV2-NS2B+NS1+prM
SEQ ID NO: 46	complete nucleotide sequence of Dengue virus type 2 strain D2/SG/05K3295DK1/2005 with mutation at positions 4471-4473 from ATA to ACA, corresponding to mutant DENV2-NS2B
SEQ ID NO: 47	complete nucleotide sequence of Dengue virus type 3 strain D3/SG/05K863DK1/2005 with mutation at positions 4463-4465 from ATA to ACA and mutation at positions 521-523 from TCT to GTG, corresponding to mutant DENV3-NS2B+prM
SEQ ID NO: 48	complete nucleotide sequence of Dengue virus type 3 strain D3/SG/05K863DK1/2005 with mutation at positions 4463-4465 from ATA to ACA and mutation at positions 2570-2572 from GGA to GAC, corresponding to mutant DENV3-NS2B+NS1
SEQ ID NO: 49	complete nucleotide sequence of Dengue virus type 3 strain D3/SG/05K863DK1/2005 with mutation at positions 4463-4465 from ATA to ACA, mutation at positions 521-523 from TCT to GTG, and mutation at positions 2570-2572 from GGA to GAC, corresponding to mutant DENV3-NS2B+NS1+prM
SEQ ID NO: 50	complete nucleotide sequence of Dengue virus type 3 strain D3/SG/05K863DK1/2005 with mutation at positions 4463-4465 from ATA to ACA, corresponding to mutant DENV3-NS2B
SEQ ID NO: 51	complete nucleotide sequence of Dengue virus type 4 strain D4/SG/06K2270DK1/2005 with mutation at positions 4474-4476 from ATT to ACT and mutation at positions 526-528 from GAA to GTA, corresponding to mutant DENV4-NS2B+prM
SEQ ID NO: 52	complete nucleotide sequence of Dengue virus type 4 strain D4/SG/06K2270DK1/2005 with mutation at positions 4474-4476

	from ATT to ACT and mutation at positions 2581-2583 from GGG to GAC, corresponding to mutant DENV4-NS2B+NS1
SEQ ID NO: 53	complete nucleotide sequence of Dengue virus type 4 strain D4/SG/06K2270DK1/2005 with mutation at positions 4474-4476 from ATT to ACT and mutation at positions 2581-2583 from GGG to GAC, and mutation at positions 526-528 from GAA to GTA, corresponding to mutant DENV4-NS2B+NS1+prM
SEQ ID NO: 54	complete nucleotide sequence of Dengue virus type 4 strain D4/SG/06K2270DK1/2005 with mutation at positions 4474-4476 from ATT to ACT, corresponding to mutant DENV4-NS2B
Reference amino acid sequences for "equivalent" positions (Consensus sequences)	
SEQ ID NO: 55	Representative/Consensus amino acid sequence for NS2B as a comparative sequence relative to position 114
SEQ ID NO: 56	Representative/Consensus amino acid sequence for prM as a comparative sequence relative to position 29
SEQ ID NO: 57	Representative/Consensus amino acid sequence for NS1 as a comparative sequence relative to position 53

[00145] *Virus Infection and Quantification of Viral and Immune Genes by Quantitative PCR (qPCR)*

[00146] Huh-7 cells were seeded at 1 x 10^5 cells per well in 24-well tissue culture plate 1 day prior to infection. Cells were infected with DENV at multiplicity of infection (MOI) of 1 for 1 h before replacement with maintenance media. At various times post-infection, cells were washed once in PBS before lysis in RLT buffer from RNeasy Mini Kit (Qiagen) for RNA extraction. cDNA was synthesised using qScript cDNA Synthesis Kit (Quantabio) according to manufacturer's protocols. Quantitative real-time PCR was performed using LightCycler 480 SYBR Green I (Roche).

[00147] *Plaque Assay*

[00148] Plaque assay was performed on BHK-21 as previously described. Briefly, serial dilutions (10-fold) of virus were added to BHK-21 cells in 24-well plates and incubated for 1 h at 37°C. Media was aspirated and replaced with 0.9% methyl-cellulose in maintenance media. 5 days later, cells were fixed with 20% formalin and stained with 1% crystal violet.

[00149] *siRNA knockdown of IRF3*

[00150] BHK-21 cells in 24-well tissue culture plates were transfected with either control small-interfering RNA (siCtrl) or siRNA targeting IRF3 (sense: GGAACAAUGGGAGUUCGAAdTdT and antisense: UUCGAACUCCCAUUGUUCCdTdT) (SABio) using Lipofectamine RNAiMax reagent (Invitrogen) as per manufacturer's protocols. 48 h post-transfection, plaque assay was performed as described above. Plate was scanned using ImmunoSpot Analyzer (Cellular Technology Ltd.) and smart counting was performed with BioSpot 5.0 software. Transfection efficiency was determined by Western blot using 1:1000 anti-IRF3 (Cell Signaling Technology, #4302S) and 1:1000 anti-β-actin (Cell Signaling Technology, #3700) antibodies.

[00151] *Monocyte derived dendritic cells*

[00152] Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of a healthy donor. CD14+ monocytes were obtained from PBMCs using CD14 microbeads (Miltenyi Biotec) according to manufacturer's protocol. Differentiation of CD14 cells into dendritic cells (moDCs) were done in 6-well plates using RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 ng/ml IL-4 (eBioScience) and 50 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF, eBioScience) for 6 days with media change on the third day. moDCs were seeded at 2 x 10^4 cells per well in 96-well tissue culture plate and infected with DENV2 at MOI 5 with media change 6 h post-infection. At each time point post-infection, supernatant was collected and frozen at -80°C until plaque assay. Cells were washed once in PBS before lysis in RLT buffer from RNeasy Mini Kit (Qiagen) for RNA extraction.

[00153] *Interferon treatment*

[00154] Huh-7 cells were seeded at 1 x 10^5 cells per well in 24-well tissue culture plate 1 day prior to infection. Cells were infected at multiplicity of infection (MOI) of 1 with or without indicated concentrations of IFN (R&D Systems). Supernatant was collected 48 h post-infection and RNA extracted using QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer's protocols. Viral RNA was quantified using qPCR. Percent inhibition from IFN treatment was quantified relative to infection without IFN treatment.

[00155] *Flow cytometry*

[00156] Huh-7 cells were infected with D2C virus at MOI 1. 72 hours later, cells were harvested and fixed in 80% methanol for 20 m at -20ºC. Cells were washed 3 times with 0.04% BSA in PBS followed by 1:1000 mouse anti-NS3 antibody (Genetex) for 1 h at 4ºC.

Cells were washed 3 times with 0.04% BSA in PBS followed by 1:400 anti-mouse Alexa488 for 30 m at 4°C. Cells were washed and resuspended in FACs buffer before sorting using FACSAria cell sorter (BD Biosciences).

[00157] *Animal studies*

[00158] All animal studies were performed in accordance to protocols approved by the Institutional Animal Care and Use Committee at Singapore Health Services, Singapore (ref no.: 2016/SHS/1197). BALB/c and AG129 mice deficient in IFNα/β/γ receptor were housed in a BSL-2 animal facility in Duke-NUS Medical School. Eight-week-old mice were used in the experiment. Mice were injected intraperitoneally (i.p.) with viruses diluted in PBS to stated doses (10^6 or 10^7 pfu in 200 µl). Daily weight measurements were obtained and submandibular bleed was performed to obtain serum samples. Serum viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer's instructions.

[00159] **Example 1**

[00160] *Investigation of NS2B mutation*

[00161] To identify a DENV mutant that induces robust IFN expression shortly following infection, we developed a method using Huh7 cell line stably transfected with EGFP under the control of the IFNβ promoter (Huh7-IFNβ-EGFP). DENV2 (strain D2/SG/05K3295DK1/2005), which was isolated from the blood of a dengue patient (Low at al 2006), was propagated in the presence of a mutagen, 5-fluorouracil (5-FU) and the culture harvest was then inoculated onto Huh7-IFNβ-EGFP cells. Cell sorting was then used to obtain a pool of Huh7 cells with strong EGFP expression. Using full viral genome sequencing and reverse genetics, we were able to rescue a homologous population of virus that contained only a single nucleotide change, T4472C that produced small plaque size. This nucleotide change corresponded to a isoleucine (I) to threonine (T) amino acid (aa) substitution at position 114 of the NS2B protein (NS2B-I114T).

[00162] Previous work performed by the laboratory defined the determinents of viral attenuation using DENV2 PDK53 strain. PDK53 is a candidate live-attenuated dengue vaccine strain that has completed phase 3 clinical trials. It was found that a single amino acid substitution in the NS1 protein restricted dissemination of infection in both mosquite and

mammalian cells. It was also found that prM mutation alone produced virus with smaller plaque size.

[00163] Knowing that prM, NS1 and NS2B mutation produced smaller plaque size which is one of the criteria for attenuation, we further characterized these mutations on its own or in combination to determine viral attenuation and genomic stability of such mutants.

[00164] DENV1, DENV2, DENV3 and DENV4 with the NS2B-114T mutation showed higher IFNβ response either in Huh7 or monocyte derived dendritic cells (moDCs) (Figures 2 – 5).

[00165] Furthermore, DENV2-NS2B-114T was highly susceptible to IFNβ as observed in Figure 7 (bottom) where the virus was inhibited by recombinant IFNβ as opposed to DENV2-WT virus. In addition, knockdown of IRF3, an IFN regulatory factor, showed increase in DENV2 and DENV3 plaque size thus confirming that IFN restriction is key in preventing virus spread (Figure 7, top and Figure 8). This is further emphasized when IFN stimulatory genes (ISGs) such as MX1, IFITM1, IFITM2 and STAT1 were increased in uninfected cells adjacent to infected cells within the same population of DENV2 NS2B-114T infection suggesting activation of anti-viral state in neighbouring cells (Figure 6).

[00166] Increase in IFN susceptibility was also observed in vivo. In an IFN-deficient mouse model (AG129), mice were unable to clear DENV2-NS2B-114T virus in the blood (Figure 12). However, if we treat wild type mice (BALB/c) with anti-IFNAR antibody which dampens the immune response initially for virus establishment, we observed that although DENV2-NS2B-114T replicated more rapidly compared to WT virus in the first 3 days of infection, viremia cleared by day 6 post-infection upon clearance of anti-IFNAR antibody (half-life of 5 days) and IFN response was intact again (Figure 12). This shows that NS2B-114T mutation is responsible for the increase in IFN response upon infection that prevents the spread and dissemination of DENV.

[00167] **Example 2**

[00168] *Investigation of NS2B mutation in combination with prM and/or NS1 mutations*

[00169] Another important feature for LAV strains is the stability of the DENV genome that harbours the mutation. Individually, NS1-53D and NS2B-114T mutations showed attenuated features, however, there were genetically unstable as when engineered onto different serotype backbones, they reverted back to wild type amino acid after passaging in

cell lines. When engineered together, NS1-53D with NS2B-114T stabilised on DENV2 and DENV3 serotypes (Figures 2 – 4).

[00170] This invention with either 2 or 3 of the identified mutations in each of the DENV serotype is sufficient to attenuate and stabilize the virus genome. As all four serotypes will harbour the same mutations, the mechanism of action for immune response activation would be the same. It is expected that with all four intact DENV genome, the tetravalent formulation will induce an equal and balanced immune response.

[00171] **Equivalents**

The foregoing examples are presented for the purpose of illustrating the invention and should not be construed as imposing any limitation on the scope of the invention. It will readily be apparent that numerous modifications and alterations may be made to the specific embodiments of the invention described above and illustrated in the examples without departing from the principles underlying the invention. All such modifications and alterations are intended to be embraced by this application.

Claims

1. An attenuated flavivirus comprising at least one mutation in its genome sequence encoding an NS2B protein, wherein the at least one mutation replaces an amino acid at position 114 or an amino acid at a position equivalent to amino acid position 114 of SEQ ID NO: 55, and

wherein the attenuated flavivirus has an increased immunogenicity, an increased rate of replication and decreased plaque size compared to a wild type flavivirus.

2. The flavivirus of claim 1, wherein the flavivirus further comprises

at least one mutation in its genome sequence encoding a precursor membrane (prM) protein, or

at least one mutation in its genome sequence encoding a NS1 protein, or

at least one mutation in its genome sequence encoding a prM protein and at least one mutation in its genome sequence encoding a NS1 protein;

wherein

the at least one mutation in the genome sequence encoding the prM protein replaces an amino acid at position 29 or an amino acid at a position equivalent to amino acid position 29 of as set SEQ ID NO: 56, and

the at least one mutation in the genome sequence encoding the NS1 protein replaces an amino acid at position 53 or an amino acid at a position equivalent to amino acid position 53 of SEQ ID NO: 57.

3. The flavivirus of claim 1 or 2, wherein the flavivirus comprises at least one mutation in the genome sequence encoding a NS2B protein and at least one mutation in the genome sequence encoding a prM protein.

4. The flavivirus of claim 1 or 2, wherein the flavivirus comprises at least one mutation in the genome sequence encoding a NS2B protein and at least one mutation in the genome sequence encoding a NS1 protein.

5. The flavivirus of claim 1 or 2, wherein the flavivirus comprises at least one mutation in the genome sequence encoding a NS2B protein, at least one mutation in the genome

sequence encoding a prM protein, and at least one mutation in the genome sequence encoding a NS1 protein.

6. The flavivirus of any one of claims 1-5, wherein the flavivirus is a dengue virus.

7. The flavivirus of claim 6, wherein the dengue virus is selected from the group consisting of dengue virus type 1 (DENV1), dengue virus type 2 (DENV2), dengue virus type 3 (DENV3), and dengue virus type 4 (DENV4) serotypes.

8. The flavivirus of any one of the preceding claims, wherein the mutation in the genome sequence encoding the NS2B protein is a replacement of the amino acid at the position equivalent to amino acid position 114 with threonine (Thr).

9. The flavivirus of any one of the preceding claims, wherein the mutation in the genome sequence encoding the prM protein is a replacement of the amino acid at the position equivalent to amino acid position 29 with valine (Val).

10. The flavivirus of any one of the preceding claims, wherein the mutation in the genome sequence encoding the NS1 protein is a replacement of the amino acid at the position equivalent to amino acid position 53 with aspartic acid (Asp).

11. The flavivirus of any one of the preceding claims, wherein the flavivirus is a DENV1 serotype comprising a genome sequence as set forth in SEQ ID NO: 39, 40, 41, or 42, or a variant thereof.

12. The flavivirus of any one of the preceding claims, wherein the flavivirus is a DENV1 serotype comprising an amino acid sequence as set forth in SEQ ID NO: 23, 24, 25, or 26, or a variant thereof.

13. The flavivirus of any one of the preceding claims, wherein the flavivirus is a DENV2 serotype comprising a genome sequence as set forth in SEQ ID NO: 43, 44, 45, or 46, or a variant thereof.

14. The flavivirus of any one of the preceding claims, wherein the flavivirus is a DENV2 serotype comprising an amino acid sequence as set forth in SEQ ID NO: 27, 28, 29, or 30, or a variant thereof.

15. The flavivirus of any one of the preceding claims, wherein the flavivirus is a DENV3 serotype comprising a genome sequence as set forth in SEQ ID NO: 47, 48, 49, or 50, or a variant thereof.

16. The flavivirus of any one of the preceding claims, wherein the flavivirus is a DENV3 serotype comprising an amino acid sequence as set forth in SEQ ID NO: 31, 32, 33, or 34, or a variant thereof.

17. The flavivirus of any one of the preceding claims, wherein the flavivirus is a DENV4 serotype comprising a genome sequence as set forth in SEQ ID NO: 51, 52, 53, or 54, or a variant thereof.

18. The flavivirus of any one of the preceding claims, wherein the flavivirus is a DENV4 serotype comprising a genome sequence as set forth in SEQ ID NO: 35, 36, 37, or 38, or a variant thereof.

19. The flavivirus of any one of the preceding claims, wherein the flavivirus is a live attenuated virus.

20. The flavivirus of any one of the preceding claims, wherein the flavivirus is characterized *in vitro* by smaller plaque size, increased interferon response, or a combination thereof, as compared to the wild type flavivirus.

21. The flavivirus of any one of the preceding claims, wherein the one or more mutations in the genome remain stable after at least 2 passages *in vitro*.

22. The flavivirus of any one of the preceding claims, wherein the flavivirus has increased replication rate, as compared to the wild type flavivirus.

23. An immunogenic composition comprising one or more flaviviruses of any one of the preceding claims.

24. The immunogenic composition of claim 23, wherein the composition is a vaccine.

25. The immunogenic composition of claim 24, wherein the composition comprises the flavivirus of one or two or three or four dengue virus serotypes.

26. The immunogenic composition of claim 24, wherein the composition comprises the flavivirus of all four dengue virus serotypes DENV1, DENV2, DENV3 and DENV4.

27. The immunogenic composition of claim 26, wherein the flavivirus of each dengue virus serotype comprises the same mutations.

28. The immunogenic composition of claim 27, wherein the composition induces a balanced immune response against each dengue virus serotype.

29. The immunogenic composition of any one of claims 24-28, wherein the vaccine is a live attenuated vaccine.

30. The immunogenic composition of any one of claims 24-29, wherein the vaccine induces an increased interferon response in a host.

31. The immunogenic composition of any one of claims 24-30, further comprising a preservative, a stabilizer, a pharmaceutical acceptable carrier, or combinations thereof.

32. A method of eliciting an immune response against one or more flaviviruses in a subject comprising administering an effective amount of the immunogenic composition of any one of claims 24-31 to the subject.

33. The method of claim 32, wherein the one or more flaviviruses are one or more dengue virus serotypes.

34. The method of claim 32 or 33, wherein the subject is a human or non-human mammal.

35. The method of claim 34, wherein the subject is a mouse.

36. The method of claim 34, wherein the subject is a human at risk of dengue virus infection.

37. The method of claim 36, wherein the subject has prior history of dengue viral infection.

38. The method of claim 36, wherein the subject does not have prior history of dengue viral infection.

39. The method of any one of claims 32-38, wherein the vaccine composition is to be administered by a route selected from the group consisting of intramuscular, intradermal, subcutaneous, intravenous, oral, and intranasal administration.

40. A method of preventing, ameliorating, or treating a disease caused by one or more flaviviruses in a subject comprising administering an effective amount of the immunogenic composition of any one of claims 24-31 to the subject.

41. The method of claim 40, wherein the flaviviruses are one or more dengue virus serotypes.