ATTENUATED FLAVIVIRUSES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application includes a claim of priority under 35 U.S.C. §119(e) to U.S. provisional patent application No. 62/640,355 filed March 8, 2018, the entirety of which is hereby incorporated by reference.

FIELD OF THE INVENTION

[0002] This invention provides highly attenuated flaviviruses and particularly, Zika viruses and vaccines. The attenuated viruses and vaccines proliferate well and have high safety factors. The attenuated viruses providing protective immunity from challenge by virus of the same lineage, as well as cross protection against heterologous viruses.

BACKGROUND OF THE INVENTION

[0003] Zika virus (ZIKV) is a single-stranded positive-sense RNA virus belonging to the *Flaviviridae* family, in the same genus (Flavivirus) that includes dengue virus (DENV), West Nile virus (WNV), and yellow fever virus (YFV). It is closely related phylogenetically to DENV and shares a common mosquito vector, *Aedes aegypti*, with both DENV and Chikungunya virus (an alphavirus). Although first isolated from a sentinel rhesus macaque in Uganda in 1947, ZIKV has remained fairly obscure until a few years ago. Despite sporadic evidence of human infection in Africa (3) and Indonesia (4) ZIKV was not associated with any major outbreaks or epidemics until it first emerged in the island of Yap in the South Pacific where it then jumped from island to island, spreading throughout Polynesia and to Southeast Asia. In 2014 and 2015, ZIKV spread to the Americas. Recently, there is active transmission of ZIKV throughout Central and South America as well as on the African islands of Cape Verde and several U.S. territories, including U.S. Virgin Islands, Puerto Rico, and American Samoa. Infection with ZIKV is usually mild with a rash, muscle aches, and conjunctivitis being the most widely reported symptoms. Of public health concern, however, is the association of ZIKV infection with neurological sequelae (Guillain-Barre Syndrome) observed in French Polynesia and microcephaly cases in Brazil. In a preliminary case-control study, up to 30% of fetuses in infected mothers displayed microcephaly, some of which were fatal. Unusual for mosquito-transmitted viruses, ZIKV is capable of vertical (mother to fetus) transmission and possible horizontal (sexual) transmission. The WHO raised a state of alarm as ZIKV continues to spread "explosively" throughout the Americas. There is special concern for tourists at risk for ZIKV infection, with an added concern of importation and spread of the epidemic to places like the southern U.S. The vector for ZIKV, *Aedes aegypti*, is found throughout the southern U.S. *Aedes albopictus*, the Asian tiger

mosquito, is also competent for transmitting ZIKV and has been implicated as a vector for transmission in Singapore. If ZIKV enters *A. albopictus* populations in the U.S., it will have a vastly expanded range extending into the American Midwest and Northeast.

[0004] Accordingly, there remains a need in the art to develop a vaccine to prevent or reduce infection.

[0005] Flavivirus vaccine development is compounded by the phenomenon of Antibody-Dependent Enhancement (ADE). ADE occurs when prior infection with one flavivirus predisposes an individual to an enhanced severity of disease upon re-infection with a different serotype. During ADE, antibodies against the first virus bind, but do not neutralize the second virus, instead increasing its infectivity. ZIKV is prevalent in DENV endemic countries, thus **any** vaccine strategy must consider the impact on a population with established dengue immunity. There is *in vitro* evidence for cross-flavivirus enhancement of ZIKV infection by dengue immune serum. It is therefore important that an inactivated anti-ZIKV vaccine does not predispose the vaccine recipient to ADE from a subsequent DENV infection. Conversely, it is worth noting that people with underlying DENV immunity could experience increased adverse events from a live ZIKV vaccine, since their DENV immunity could enhance infectivity of the ZIKV vaccine strain, leading to increased adverse events. Both of these are plausible scenarios that needs to be considered when developing a ZIKV vaccine, whether live, inactivated or antigen/VLP/backbone carrier-based. Because of the high likelihood of enhancing flavivirus infection due to ADE, all vaccine development strategies need to consider how any given ZIKV vaccine might interact with existing antibodies to a flavivirus or a subsequent flavivirus infection.

[0006] Zika virus is a virus belonging to the Spondweni serogroup of the mosquito-borne Flaviviruses in the family Flaviviridae. Zika viruses are enveloped viruses with an icosahedral virion comprised of C (Core), M(Membrane), and E (Envelope) glycoproteins that is 40-65 nanometers in diameter. The Zika virus genome is a single positive-strand RNA molecule of 10,000-11,000 bases in length encoding structural (C, prM, E) and nonstructural (NS1, NS2, NS3, NS4, and NS5) proteins. Zika viruses transcribe and replicate their genome in the cell cytoplasm, with the genome translated into a single polypeptide that is cleaved and processed by both host and viral proteins. Zika virus is an emerging agent of international concern in the tropics that has been divided in African and Asian lineages, although both lineages are fairly homologous and no individual serotypes have yet to be identified.

SUMMARY OF THE INVENTION

[0007] The following embodiments and aspects thereof are described and illustrated in conjunction with compositions and methods which are meant to be exemplary and illustrative, not limiting in scope.

[0007] It is described herein that recoded Zika viruses made by modification of the E and NS3 regions by large numbers of synonymous nucleotide mutations are highly effective in providing protective immunity against lethal wild type challenge and cross protection against different lineages. Further, the viruses have exceptional safety profiles.

[0008] Accordingly, the invention provides an attenuated Zika virus in which expression of viral proteins is reduced through codon-pair deoptimization of the E and NS3 coding regions. In certain embodiments, E and NS3 are the only virus protein coding regions targeted. In other embodiments of the invention, the expression of one or more other virus protein encoding regions may also be reduced, such as, for example, C, prM, NS1, NS2, NS4, or NS5. In certain embodiments, when another Zika virus protein encoding region other than E and NS3 is deoptimized, the reduction is small compared to the reduction of E and NS3. According to the invention, reduction in expression of virus proteins of the invention is accomplished by changes in protein encoding sequence, for example by lowering the codon pair bias of the protein-encoding sequence, substituting rare codons, modifying G+C content, modifying CG and/or TA (or UA) dinucleotide content, or combinations. Reduced expression can also be accomplished by modifications to the regulatory sequences of the proteins.

[0009] In one such embodiment, reducing the codon-pair bias can comprise identifying a codon pair in the parent protein-encoding sequence having a codon-pair score that can be reduced, and reducing the codon-pair bias by substituting the codon pair with a codon pair that has a lower codon-pair score. In another such embodiment, reducing the codon-pair bias can comprise rearranging the codons of a parent protein-encoding sequence. In certain embodiments, the E protein-encoding sequence and the NS3 protein-encoding sequence individually have a codon pair bias less than −0.1, or less than −0.2, or less than −0.3, or less than −0.4. Codon pair bias of a protein-encoding sequence (i.e., an open reading frame) is calculated as described in Coleman et al., 2000 and herein.

[0010] In an embodiment of the invention, expression of one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence is reduced by replacing one or more codons with synonymous codons that are less frequent in the host.

[0011] The invention further provides a Zika vaccine composition for inducing a protective immune response in a subject, wherein the vaccine composition comprises virus in which viral translation is reduced while maintaining antigenic identity with wt virus.

[0012] The invention also provides a method of eliciting a protective immune response in a subject comprising administering to the subject a prophylactically or therapeutically effective dose of a vaccine composition comprising an attenuated Zika virus, wherein expression of viral proteins is reduced. In an embodiment of the invention, an immune response is elicited that is effective against Zika virus of the same lineage as the attenuated virus of the vaccine. In another embodiment, an immune response is elicited that is effective against a heterologous Zika virus.

[0013] The invention also provides a method of making an attenuated Zika virus genome comprising a) obtaining the genomic nucleotide b) recoding the envelope-encoding nucleotide sequence to reduce expression and recoding the nonstructural protein 3-encoding nucleotide sequence to reduce expression, and substituting the recoded nucleotide sequences into a Zika virus genome to make an attenuated Zika virus genome. In certain embodiments, only the E and NS3 regions are targeted. In some embodiments, expression of another virus protein encoding region is also reduced.

[0014] The invention also provides a method of constructing template Zika virus DNA sequences for transcription of infectious viral RNA genomes by T7 polymerase using overlapping PCR. All Zika genomes were divided into three fragments starting from 5' end (fragment 1: nt1-3596; fragment 2: nt3030-6959, and fragment 3: nt: nt6851-end) and chemically/biochemically synthesized. Instead of constructing an infectious cDNA clone, a novel overlap extension PCR strategy (or: long PCR-based fusion strategy) was used to obtain full-length Zika genome (or: the syn-wt and min Zika genomes simultaneously).

[0015] Various embodiments of the invention provide for a modified Zika virus in which expression of viral proteins is reduced compared to a parent virus, wherein the reduction in expression is the result of recoding the prM, or envelope (E) region, or the nonstructural protein 3 (NS3) region or both the E and NS3 regions.

[0016] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence can be recoded by reducing the codon pair bias or codon usage bias of the protein-encoding sequence. In various embodiments, reducing the codon-pair bias can comprise identifying a codon pair in the parent protein-encoding sequence having a codon-pair score that can be reduced, and reducing the codon-pair bias by substituting the codon pair with a codon pair that has a lower codon-pair score. In various embodiments, reducing the codon-pair bias can comprise rearranging the codons of a parent protein-encoding sequence.

[0017] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence can be recoded by increasing the number of CpG or UpA di-nucleotides compared to a parent virus.

[0018] In various embodiments, each of the recoded prM/E protein-encoding sequence and the recoded NS3 protein-encoding sequence can have a codon pair bias less than, -0.05, −0.1, or less than −0.2, or less than −0.3, or less than −0.4.

[0019] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence can be recoded by replacing one or more codons with synonymous codons that are less frequent in the host.

[0020] Various embodiments of the invention provide for a Zika vaccine composition for inducing a protective immune response in a subject, comprising a modified virus of the present application as described herein.

[0021] Various embodiments of the invention provide for a method of eliciting a protective immune response in a subject, comprising: administering to the subject a prophylactically or therapeutically effective dose of a vaccine composition comprising a modified virus of the present application as described herein.

[0022] In various embodiments, the method can further comprise administering to the subject at least one adjuvant.

[0023] In various embodiments, the immune response is cross-protective against a heterologous Zika virus.

[0024] Various embodiments of the invention provide a method of making a modified Zika virus genome comprising: obtaining the nucleotide sequence encoding the envelope protein of a Zika virus and the nucleotide sequence encoding the nonstructural 3 proteins of a Zika virus; recoding the envelope encoding nucleotide sequence to reduce protein expression and recoding the nonstructural protein 3-encoding nucleotide sequence to reduce protein expression, and substituting a nucleic acid having the recoded envelope-encoding nucleotide sequence and a nucleic acid having the recoded nonstructural protein 3-encoding nucleotide sequence into a parent Zika virus genome to make a modified Zika virus genome; whereby expression of the recoded envelope-encoding nucleotide sequence and expression of the recoded nonstructural protein 3-encoding nucleotide sequence is reduced compared to the parent virus.

[0025] Various embodiments of the invention provide a modified Flavivirus virus in which expression of viral proteins is reduced compared to a parent virus, wherein the reduction in expression is the result of recoding the prM, or envelope (E) region, or the nonstructural protein 3 (NS3) region or both the E and NS3 regions.

[0026] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence can be recoded by reducing the codon pair bias or codon usage bias of the protein-encoding sequence.

[0027] In various embodiments, reducing the codon-pair bias can comprise identifying a codon pair in the parent protein-encoding sequence having a codon-pair score that can be reduced, and reducing the codon-pair bias by substituting the codon pair with a codon pair that has a lower codon-pair score.

[0028] In various embodiments, reducing the codon-pair bias can comprise rearranging the codons of a parent protein-encoding sequence.

[0029] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence can be recoded by increasing the number of CpG or UpA di nucleotides compared to a parent virus.

[0030] In various embodiments, each of the recoded prM/E protein-encoding sequence and the recoded NS3 protein-encoding sequence can have a codon pair bias less than, -0.05, −0.1, or less than −0.2, or less than −0.3, or less than −0.4.

[0031] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence can be recoded by replacing one or more codons with synonymous codons that are less frequent in the viral host.

[0032] In various embodiments, the parent virus can be a Flavivirus selected from the group consisting of dengue fever virus, West Nile virus, yellow fever virus, Japanese encephalitis virus, Spondweni virus, Saint Louis encephalitis virus, and Powassan virus. In various embodiments, the parent virus can be a natural isolate. In various embodiments, the parent virus can be a mutant of a natural isolate.

[0033] Various embodiments of the invention provide for a Flavivirus vaccine composition for inducing a protective immune response in a subject, which comprises a modified virus of the present invention as described herein.

[0034] Various embodiments of the invention provide for a method of eliciting a protective immune response in a subject comprising administering to the subject a prophylactically or therapeutically effective dose of a vaccine composition comprising a modified virus of the present invention as described herein. In various embodiments, the method can further comprise administering to the subject at least one adjuvant.

[0035] In various embodiments, the immune response can be cross-protective against a heterologous Flavivirus virus.

[0036] Various embodiments of the present invention provide for a method of making a modified Flavivirus virus genome comprising: obtaining the nucleotide sequence encoding the envelope protein of a Flavivirus virus and the nucleotide sequence encoding the nonstructural 3 proteins of a Flavivirus virus; recoding the envelope encoding nucleotide sequence to reduce protein expression and recoding the nonstructural protein 3-encoding nucleotide sequence to reduce protein expression, and

substituting a nucleic acid having the recoded envelope-encoding nucleotide sequence and a nucleic acid having the recoded nonstructural protein 3-encoding nucleotide sequence into a parent Flavivirus virus genome to make a modified Flavivirus virus genome; whereby expression of the recoded envelope-encoding nucleotide sequence and expression of the recoded nonstructural protein 3-encoding nucleotide sequence is reduced compared to the parent virus.

[0037] Various embodiments of the present invention provide for a method of eliciting an immune response in a subject in need thereof, comprising: administering a prime dose of an attenuated Flavivirus produced by a method other than codon-pair deoptimization or a modified Flavivirus in which expression of viral proteins is reduced compared to a parent virus, wherein the reduction in expression is the result of recoding the prM, or envelope (E) region, or the nonstructural protein 3 (NS3) region or both the E and NS3 regions; and administering one or more boost dose of the attenuated Flavivirus by methods other than codon-pair deoptimization or the modified Flavivirus to the subject in need thereof, wherein at least the prime dose or the one or more boost dose is the modified virus.

[0038] In various embodiments, a first of the one or more boost dose can be administered about 2 weeks after the prime dose.

[0039] In various embodiments, the Flavivirus can be a Zika virus.

[0040] In various embodiments, the Flavivirus can be selected from the group consisting of dengue fever virus, West Nile virus, yellow fever virus, Japanese encephalitis virus, Spondweni virus, Saint Louis encephalitis virus, and Powassan virus.

[0041] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence can be recoded by reducing the codon pair bias or codon usage bias of the protein-encoding sequence.

[0042] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence can be recoded by increasing the number of CpG or UpA di nucleotides compared to a parent virus.

[0043] In various embodiments, reducing the codon-pair bias can comprise identifying a codon pair in the parent protein-encoding sequence having a codon-pair score that can be reduced, and reducing the codon-pair bias by substituting the codon pair with a codon pair that has a lower codon-pair score.

[0044] In various embodiments, reducing the codon-pair bias can comprise rearranging the codons of a parent protein-encoding sequence.

[0045] In various embodiments, each of the recoded prM/E protein-encoding sequence and the recoded NS3 protein-encoding sequence can have a codon pair bias less than, -0.05, −0.1, or less than −0.2, or less than −0.3, or less than −0.4.

[0046] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence can be recoded by replacing one or more codons with synonymous codons that are less frequent in the viral host.

[0047] [0009] Other features and advantages of the invention will become apparent from the following detailed description, taken in conjunction with the accompanying drawings, which illustrate, by way of example, various features of embodiments of the invention

BRIEF DESCRIPTION OF THE FIGURES

[0048] FIG. 1A-1B depicts Rapid Construction of six SAVE-deoptimized, live-attenuated Zika vaccine candidate in less than 1 month with growth in Vero cells under animal component-free conditions. (1A) Codon pair bias of the Zika prM/E and NS3 genes and their SAVE-deoptimized counterparts in relation to the human ORFeome. Codon-Pair Bias (CPB) is expressed as the average codon pair score of a given gene's open reading frame (ORF). Positive and negative CPB value signifies the predominance of statistically over- or under-represented codon-pairs, respectively in an ORF. Red circles indicate the CPB of each of the 14,795 human ORFs, representing the majority of the known, annotated human genes at the time of our analysis (ORFeome). The CPB of wild-type prM/E and NS3 genes fall within the normal range of human host cell's genes. Following codon pair-'deoptimization' via SAVE, the resulting deoptimized prM/E and NS3 gene segments were now encoded predominately by under-represented human codon-pairs as evident by their extremely negative CPB, and are drastically different from any human gene. (1B) cDNA genomes of wild-type and synthetically 'de-optimized' chimeric Zika vaccine variants. The SAVE-deoptimized synthetic prM/E and NS3 from Figure 1A were synthesized *de novo* and using overlapping PCR subcloned individually into the WT PR15 or MR766 genomes - yielding six independent cDNA genomes each containing a synthetically 'de-optimized' fragment(s). We constructed infectious cDNA genomes for wt PR15 and MR766 in 7 days and then recovered fully infectious, replicating virus for the six deoptimized ZIKV vaccine candidates in 27 days via transfection of RNA into Vero or BHK cells.

[0049] FIG. 2 shows a diagram of subcloning strategies for decreasing attenuation or increasing immunogenicity by reducing the length of deoptimized sequence in the prM + E encoding region. The second generation of Zika vaccine candidates leverages the flexibility of the SAVE platform to reduce the deoptimized region from 2014 bp (E-min) to 997 bp (W-E-Min) or further to 664 bp (W-W-E-Min) while keeping the amino acid sequence 100% identical.

[0050] FIG. 3 depicts growth of Zika vaccine candidates in Vero cells under animal component-free conditions. The 6 chimera Zika vaccine strains were used to infect Vero cells under animal-component free conditions at a MOI of 0.1. Of the six strains, four grew to detectible titers in Vero cells under these conditions. MR766-Emin grew to a peak titer of $5x10^7$ PFU/ml after five days and

PR15-E*hMin* to a peak titer of $5x10^6$ PFU/ml after 5 days in Vero cells grown in animal-free conditions.

[0051] FIG. 4 depicts neuro-attenuation of MR766-EMin in human neuronal cells. The human neuronal cell lines HTB-14 and HTB-15 which have been used previously to characterize wt Zika virus cell tropism in the developing human brain were infected at a MOI of 0.1 and 4 days post infection, replicating virus was titrated on BHK cells, with wt MR766 growing to high titers (5×10^7 PFU/mL for HTB-14 and 8×10^5 PFU/mL for HTB-15. The lead candidate, MR766 E-Min, was approximately 10,000-fold attenuated in both of these cell lines, indicating a high level of neuro-attenuation in human cells.

[0052] FIG. 5 depicts reduction of protein and RNA expression in ZIKV infected cells. Western blot was used to measure envelope glycoprotein expression in Vero cells infected by E-Min (min) and wildtype (WT) variants of ZIKV strains PRVABC59 and MR766 after being incubated for 24 hours at 33°C. In both strains of ZIKV, envelope glycoprotein expression was greatly reduced for the E-Min variants.

[0053] FIG. 6 depicts virus phenotypes in infected mice. Attenuation of SAVE ZIKV vaccine candidates in AG129 mice. AG129 mice were injected with either: i) synthetically derived wild-type virus MR766 and PR15 virus at a dose of 10^2 (positive control); ii) either a dose of 10^4 or 10^2 PFU of the vaccine candidates PR15 E-Min or MR766 E-Min and NS3-Min; iii) a single dose of 10^4 of the vaccine candidate PR15 NS3+E-Min; all delivered in 100 µl subcutaneously. Injected mice were examined for mortality and morbidity (weight loss). All mice inoculated with 10^2 wt virus and 80% of mice inoculated with 10^4 MR766-NS3-Min succumbed to infection. All other mice survived including all mice vaccinated with 10^4 PFU of the lead candidate MR 766 E-Min and none exhibited any weight loss.

[0054] FIG. 7 depicts SAVE-attenuated ZIKV vaccine candidates induce high levels of neutralizing antibodies and protect from lethal challenge in AG129 mice. A) Anti-PR15 Zika antibodies after a single dose. Serum from vaccinated animals was harvested on Day 28 and antibodies were quantified against ZIKV strain Puerto Rico 2015 PRVABC-59 via $PRNT_{50}$ assay on Vero cells. Mice vaccinated with both PR15 E-Min and MR766 E-Min at the 10^4 dose developed high levels of neutralizing antibodies (2.8 $\log_{10}$ PRNT50).

[0055] FIG. 8 depicts survival post-lethal ZIKV challenge. AG129 Mice were vaccinated on day 28 and boosted on Day 49. Mice were then challenged with a lethal dose ($10^{2.3}$ $CCID_{50}$/animal) of Wt PR15 PRVABC59 ZIKV virus in 0.1 mL delivered s.c. Vaccination was successful in preventing or reducing mortality and weight loss (not shown) compared to unvaccinated controls. Lead candidate MR 766 E-Min, PR-15-EMin, and MR766 NS3-Min vaccinated mice were all protected at a dose of 10^4 PFU.

[0056] FIG. 9 depicts immunogenicity of E-W/Min vaccination in Cynomolgus macaques. Zika virus seronegative macaques were vaccinated with either 10^5 or 10^7 plaque-forming units (PFU) or E-W/Min delivered subcutaneously in a volume of 0.5 ml. Mock vaccinated animals were injected with 0.5 mL of vaccine diluent. Macaques were initially vaccinated on day 0, and then boosted on day 28. Serum samples were collected on days 0, 14, 21, 28, and 50 and tested for neutralizing activity against wildtype ZIKV strain MR766 using a focus-reduction neutralization 50% ($FRNT_{50}$) assay in Vero cells. Vaccination with 10^7 PFU E-W/Min was found to be superior to the NIH inactivated ZIKV vaccine candidate after the first vaccination and comparable post-boost. The lower 10^5 PFU dose was also effective in triggering neutralizing antibodies. All animals vaccinated with E-W/Min seroconverted by day 14 post-vaccination.

[0057] FIG. 10 depicts immunogenicity of E-W/W/Min vaccination in Cynomolgus macaques. Zika virus seronegative macaques were vaccinated with either 10^5 or 10^7 plaque-forming units (PFU) or E-W/W/Min delivered subcutaneously in a volume of 0.5 mL. Mock vaccinated animals were injected with 0.5 mL of vaccine diluent. Macaques were initially vaccinated on day 0, and then boosted on day 28. Serum samples were collected on days 0, 14, 21, 28, and 50 and tested for neutralizing activity against wildtype ZIKV strain MR766 using a focus-reduction neutralization 50% ($FRNT_{50}$) assay in Vero cells. Vaccination with 10^7 or 10^5 PFU E-W/W/Min was found to be superior to the NIH inactivated ZIKV vaccine candidate after the first vaccination and the two doses triggered comparable levels of neutralizing antibodies. All animals vaccinated with E-W/Min seroconverted by day 14 post-vaccination. No increase in $FRNT_{50}$ titer was observed post-boost on day 28, indicating sterilizing immunity that prevented secondary infection from triggering an anamnestic response. This further indicates that a single, relatively low dose, of W-W-E-Min may be sufficient for triggering high levels ($\geq$1,028 $FRNT_{50}$).

DETAILED DESCRIPTION

[0058] The present invention relates to the production of attenuated Zika viruses that can be used to protect against viral infection and disease. A basic premise in vaccination is adequate delivery of protective antigens to vaccine recipients assuming that a very high dose ("Peptide or Virus-Like Particle") or a dose corresponding to live viral infection ("ChimeriVax") of these traditionally dominant antigenic polypeptides alone are sufficient for adequate vaccine efficacy. Those expectations aside, the present invention benefits from a contrary approach. The invention provides attenuated Zika viruses in which expression of viral proteins is reduced, which have excellent growth properties useful to vaccine production, yet possess an extraordinary safety profile and enhanced protective characteristics. The attenuated viruses proliferate nearly as well as wild type virus, have highly attenuated phenotypes, as revealed by LD_{50} values, are unusually effective in providing protective

immunity against challenge by Zika virus of the same strain, and also provide protective immunity against challenge by Zika virus of other strains.

[0059] In certain embodiments of the invention, the attenuated Zika viruses of the invention comprise a recoded pre-membrane/Envelope (E) encoding region, a recoded nonstructural protein 3 (NS3) encoding region, or both E and NS3 encoding regions. That the C, NS1, NS2, NS4, or NS5 protein encoding regions are not recoded does not exclude mutations and other variations in those sequences, but only means that any mutations or variations made in those sequences have little or no effect on attenuation. Little or no effect on attenuation includes one or both of the following: 1) The mutations or variations in the That the C, NS1, NS2, NS4, or NS5 encoding regions do not reduce viral replication or viral infectivity more than 20% when the variant C, NS1, NS2, NS4, or NS5 encoding region is the only variant in a test Zika virus; 2) Mutations or variations in any of the C, NS1, NS2, NS4, or NS5 encoding regions represent fewer than 10% of the nucleotides in that coding sequence.

[0060] The viruses of the invention are highly attenuated. In embodiments of the invention, compared to wild type, the viruses are at least 5,000 fold attenuated, or at least 10,000 fold attenuated, or at least 20,000 fold attenuated, or at least 33,000 fold attenuated, or at least 50,000 fold attenuated, of at least 100,000 fold attenuated in the AG129 mouse model compared to a wild type virus having proteins of the same sequence but encoded by a different nucleotide sequence.

[0061] The attenuated viruses are also highly protective against wild type virus of the same strain. In embodiments of the invention, the protective dose (PD_{50}) of the viruses is less than 1,000 PFU, or less than 100 PFU, or less than 50 PFU, or less than 20 PFU, or less than 10 PFU, or less than 5 PFU, when measured by a mouse model, such as exemplified herein.

[0062] The attenuated viruses of the invention also exhibit a large margin of safety (i.e., the difference between LD_{50} and PD_{50}), thus have high safety factors, defined herein as the ratio of LD_{50}/PD_{50}. In certain embodiments of the invention, the safety factor is at least 10^2, or at least 10^3, or at least 10^4, or at least 10^5, or at least 2×10^5, or at least 5×10^5, or at least 10^6, or at least 2×10^6, or at least 5×10^6. In certain embodiments, the safety factor is from 10^2 to 10^3, or from 10^3 to 10^4, or from 10^4 to 10^5, or from 10^5 to 10^6.

[0063] The attenuated viruses of the invention are also highly protective against heterologous strains of the Zika virus. In certain embodiments of the invention, the protective dose (PD_{50}) of an attenuated virus of the invention is less than 1000 PFU, or less than 500 PFU, or less than 200 PFU, or less than 100 PFU, when measured by a mouse model, such as exemplified herein.

[0064] The recoding of E and NS3 protein encoding sequences of the attenuated viruses of the invention can have been made utilizing any algorithm or procedure known in the art or newly devised for recoding a protein encoding sequence. According to the invention, nucleotide substitutions are

engineered in multiple locations in the E and NS3 coding sequences, wherein the substitutions introduce a plurality of synonymous codons into the genome. In certain embodiments, the synonymous codon substitutions alter codon bias, codon pair bias, the density of infrequent codons or infrequently occurring codon pairs, RNA secondary structure, CG and/or TA (or UA) dinucleotide content, C+G content, translation frameshift sites, translation pause sites, the presence or absence of microRNA recognition sequences or any combination thereof, in the genome. The codon substitutions may be engineered in multiple locations distributed throughout the E and NS3 coding sequences, or in the multiple locations restricted to a portion of the E and NS3 coding sequences. Because of the large number of defects (i.e., nucleotide substitutions) involved, the invention provides a means of producing stably attenuated viruses and live vaccines.

[0065] As discussed further below, in some embodiments, a virus coding sequence is recoded by substituting one or more codon with synonymous codons used less frequently in the Zika host (e.g., humans, mosquitoes). In some embodiments, a virus coding sequence is recoded by substituting one or more codons with synonymous codons used less frequently in the Zika virus. In certain embodiments, the number of codons substituted with synonymous codons is at least 5. In some embodiments, at least 10, or at least 20 codons are substituted with synonymous codons.

[0066] In some embodiments, virus codon pairs are recoded to reduce (i.e., lower the value of) codon-pair bias. In certain embodiments, codon-pair bias is reduced by identifying a codon pair in an E or NS3 coding sequence having a codon-pair score that can be reduced and reducing the codon-pair bias by substituting the codon pair with a codon pair that has a lower codon-pair score. In some embodiments, this substitution of codon pairs takes the form of rearranging existing codons of a sequence. In some such embodiments, a subset of codon pairs is substituted by rearranging a subset of synonymous codons. In other embodiments, codon pairs are substituted by maximizing the number of rearranged synonymous codons. It is noted that while rearrangement of codons leads to codon-pair bias that is reduced (made more negative) for the virus coding sequence overall, and the rearrangement results in a decreased CPS at many locations, there may be accompanying CPS increases at other locations, but on average, the codon pair scores, and thus the CPB of the modified sequence, is reduced. In some embodiments, recoding of codons or codon-pairs can take into account altering the G+C content of the E and NS3 coding sequences. In some embodiments, recoding of codons or codon-pairs can take into account altering the frequency of CG and/or TA dinucleotides in the E and NS3 coding sequences.

[0067] In certain embodiments, the recoded E protein-encoding sequence has a codon pair bias less than −0.1, or less than −0.2, or less than −0.3, or less than −0.4. In certain embodiments, the recoded (i.e., reduced-expression) NS3 protein-encoding sequence has a codon pair bias less than −0.1, or less than −0.2, or less than −0.3, or less than −0.4. In certain embodiments, the codon pair bias of

the recoded HA protein encoding sequence is reduced by at least 0.1, or at least 0.2, or at least 0.3, or at least 0.4, compared to the parent E protein encoding sequence from which it is derived. In certain embodiments, the codon pair bias of the recoded NS3 protein encoding sequence is reduced by at least 0.1, or at least 0.2, or at least 0.3, or at least 0.4, compared to the parent NS3 protein encoding sequence from which it is derived. In certain embodiments, rearrangement of synonymous codons of the E protein-encoding sequence provides a codon-pair bias reduction of at least 0.1, or at least 0.2, or at least 0.3, or at least 0.4, parent E protein encoding sequence from which it is derived. In certain embodiments, rearrangement of synonymous codons of the NS3 protein-encoding sequence provides a codon-pair bias reduction of at least 0.1, or at least 0.2, or at least 0.3, or at least 0.4, parent NS3 protein encoding sequence from which it is derived.

[0068] Usually, these substitutions and alterations are made and reduce expression of the encoded virus proteins without altering the amino acid sequence of the encoded protein. In certain embodiments, the invention also includes alterations in the E and/or NS3 coding sequences that result in substitution of non-synonymous codons and amino acid substitutions in the encoded protein, which may or may not be conservative.

[0069] Most amino acids are encoded by more than one codon. See the genetic code in Table 1. For instance, alanine is encoded by GCU, GCC, GCA, and GCG. Three amino acids (Leu, Ser, and Arg) are encoded by six different codons, while only Trp and Met have unique codons. "Synonymous" codons are codons that encode the same amino acid. Thus, for example, CUU, CUC, CUA, CUG, UUA, and UUG are synonymous codons that code for Leu. Synonymous codons are not used with equal frequency. In general, the most frequently used codons in a particular organism are those for which the cognate tRNA is abundant, and the use of these codons enhances the rate and/or accuracy of protein translation. Conversely, tRNAs for the rarely used codons are found at relatively low levels, and the use of rare codons is thought to reduce translation rate and/or accuracy.

Table 1. Genetic Code

U	C	A	G		
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	STOP	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U

	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

[a]The first nucleotide in each codon encoding a particular amino acid is shown in the left-most column; the second nucleotide is shown in the top row; and the third nucleotide is shown in the right-most column.

Codon Bias

[0070] As used herein, a "rare" codon is one of at least two synonymous codons encoding a particular amino acid that is present in an mRNA at a significantly lower frequency than the most frequently used codon for that amino acid. Thus, the rare codon may be present at about a 2-fold lower frequency than the most frequently used codon. Preferably, the rare codon is present at least a 3-fold, more preferably at least a 5-fold, lower frequency than the most frequently used codon for the amino acid. Conversely, a "frequent" codon is one of at least two synonymous codons encoding a particular amino acid that is present in an mRNA at a significantly higher frequency than the least frequently used codon for that amino acid. The frequent codon may be present at about a 2-fold, preferably at least a 3-fold, more preferably at least a 5-fold, higher frequency than the least frequently used codon for the amino acid. For example, human genes use the leucine codon CTG 40% of the time, but use the synonymous CTA only 7% of the time (see Table 2). Thus, CTG is a frequent codon, whereas CTA is a rare codon. Roughly consistent with these frequencies of usage, there are 6 copies in the genome for the gene for the tRNA recognizing CTG, whereas there are only 2 copies of the gene for the tRNA recognizing CTA. Similarly, human genes use the frequent codons TCT and TCC for serine 18% and 22% of the time, respectively, but the rare codon TCG only 5% of the time. TCT and TCC are read, via wobble, by the same tRNA, which has 10 copies of its gene in the genome, while TCG is read by a tRNA with only 4 copies. It is well known that those mRNAs that are very actively translated are strongly biased to use only the most frequent codons. This includes genes for ribosomal proteins and glycolytic enzymes. On the other hand, mRNAs for relatively non-abundant proteins may use the rare codons.

Table 2. Codon usage in Homo sapiens (source: www.kazusa.or.jp/codon/)

Amino Acid	Codon	Number	/1000	Fraction
Gly	GGG	636457.00	16.45	0.25
Gly	GGA	637120.00	16.47	0.25
Gly	GGT	416131.00	10.76	0.16
Gly	GGC	862557.00	22.29	0.34
Glu	GAG	1532589.00	39.61	0.58
Glu	GAA	1116000.00	28.84	0.42
Asp	GAT	842504.00	21.78	0.46
Asp	GAC	973377.00	25.16	0.54

Val	GTG	1091853.00	28.22	0.46
Val	GTA	273515.00	7.07	0.12
Val	GTT	426252.00	11.02	0.18
Val	GTC	562086.00	14.53	0.24
Ala	GCG	286975.00	7.42	0.11
Ala	GCA	614754.00	15.89	0.23
Ala	GCT	715079.00	18.48	0.27
Ala	GCC	1079491.00	27.90	0.40
Arg	AGG	461676.00	11.93	0.21
Arg	AGA	466435.00	12.06	0.21
Ser	AGT	469641.00	12.14	0.15
Ser	AGC	753597.00	19.48	0.24
Lys	AAG	1236148.00	31.95	0.57
Lys	AAA	940312.00	24.30	0.43
Asn	AAT	653566.00	16.89	0.47
Asn	AAC	739007.00	19.10	0.53
Met	ATG	853648.00	22.06	1.00
Ile	ATA	288118.00	7.45	0.17
Ile	ATT	615699.00	15.91	0.36
Ile	ATC	808306.00	20.89	0.47
Thr	ACG	234532.00	6.06	0.11
Thr	ACA	580580.00	15.01	0.28
Thr	ACT	506277.00	13.09	0.25
Thr	ACC	732313.00	18.93	0.36
Trp	TGG	510256.00	13.19	1.00
End	TGA	59528.00	1.54	0.47
Cys	TGT	407020.00	10.52	0.45
Cys	TGC	487907.00	12.61	0.55
End	TAG	30104.00	0.78	0.24
End	TAA	38222.00	0.99	0.30
Tyr	TAT	470083.00	12.15	0.44
Tyr	TAC	592163.00	15.30	0.56
Leu	TTG	498920.00	12.89	0.13
Leu	TTA	294684.00	7.62	0.08
Phe	TTT	676381.00	17.48	0.46
Phe	TTC	789374.00	20.40	0.54
Ser	TCG	171428.00	4.43	0.05
Ser	TCA	471469.00	12.19	0.15
Ser	TCT	585967.00	15.14	0.19
Ser	TCC	684663.00	17.70	0.22
Arg	CGG	443753.00	11.47	0.20
Arg	CGA	239573.00	6.19	0.11
Arg	CGT	176691.00	4.57	0.08
Arg	CGC	405748.00	10.49	0.18
Gln	CAG	1323614.00	34.21	0.74
Gln	CAA	473648.00	12.24	0.26
His	CAT	419726.00	10.85	0.42
His	CAC	583620.00	15.08	0.58
Leu	CTG	1539118.00	39.78	0.40
Leu	CTA	276799.00	7.15	0.07

Leu	CTT	508151.00	13.13	0.13
Leu	CTC	759527.00	19.63	0.20
Pro	CCG	268884.00	6.95	0.11
Pro	CCA	653281.00	16.88	0.28
Pro	CCT	676401.00	17.48	0.29
Pro	CCC	767793.00	19.84	0.32

[0071] The propensity for highly expressed genes to use frequent codons is called "codon bias." A gene for a ribosomal protein might use only the 20 to 25 most frequent of the 61 codons, and have a high codon bias (a codon bias close to 1), while a poorly expressed gene might use all 61 codons, and have little or no codon bias (a codon bias close to 0). It is thought that the frequently used codons are codons where larger amounts of the cognate tRNA are expressed, and that use of these codons allows translation to proceed more rapidly, or more accurately, or both. The PV capsid protein, for example, is very actively translated, and has a high codon bias.

Codon Pair Bias

[0072] In addition, a given organism has a preference for the nearest codon neighbor of a given codon A, referred to a bias in codon pair utilization. A change of codon pair bias, without changing the existing codons, can influence the rate of protein synthesis and production of a protein.

[0073] Codon pair bias may be illustrated by considering the amino acid pair Ala-Glu, which can be encoded by 8 different codon pairs. If no factors other than the frequency of each individual codon (as shown in Table 2) are responsible for the frequency of the codon pair, the expected frequency of each of the 8 encodings can be calculated by multiplying the frequencies of the two relevant codons. For example, by this calculation the codon pair GCA-GAA would be expected to occur at a frequency of 0.097 out of all Ala-Glu coding pairs (0.23×0.42; based on the frequencies in Table 2). In order to relate the expected (hypothetical) frequency of each codon pair to the actually observed frequency in the human genome the Consensus CDS (CCDS) database of consistently annotated human coding regions, containing a total of 14,795 human genes, was used. This set of genes is the most comprehensive representation of human coding sequences. Using this set of genes, the frequencies of codon usage were re-calculated by dividing the number of occurrences of a codon by the number of all synonymous codons coding for the same amino acid. As expected, the frequencies correlated closely with previously published ones such as the ones given in Table 2. Slight frequency variations are possibly due to an oversampling effect in the data provided by the codon usage database at Kazusa DNA Research Institute (http://www.kazusa.or.jp/codon/codon.html) where 84949 human coding sequences were included in the calculation (far more than the actual number of human genes). The codon frequencies thus calculated were then used to calculate the expected codon-pair frequencies by first multiplying the frequencies of the two relevant codons with each other (see Table 3 expected frequency), and then multiplying this result with the observed frequency (in the entire CCDS data set)

with which the amino acid pair encoded by the codon pair in question occurs. In the example of codon pair GCA-GAA, this second calculation gives an expected frequency of 0.098 (compared to 0.097 in the first calculation using the Kazusa dataset). Finally, the actual codon pair frequencies as observed in a set of 14,795 human genes was determined by counting the total number of occurrences of each codon pair in the set and dividing it by the number of all synonymous coding pairs in the set coding for the same amino acid pair (Table 3; observed frequency). Frequency and observed/expected values for the complete set of 3721 (61^2) codon pairs, based on the set of 14,795 human genes, are provided herewith as Table 3.

Table 3. Codon Pair Scores Exemplified by the Amino Pair Ala-Glu

amino acid pair	codon pair	expected frequency	observed frequency	obs/exp ratio
AE	GCAGAA	0.098	0.163	1.65
AE	GCAGAG	0.132	0.198	1.51
AE	GCCGAA	0.171	0.031	0.18
AE	GCCGAG	0.229	0.142	0.62
AE	GCGGAA	0.046	0.027	0.57
AE	GCGGAG	0.062	0.089	1.44
AE	GCTGAA	0.112	0.145	1.29
AE	GCTGAG	0.150	0.206	1.37
Total		1.000	1.000	

[0074] If the ratio of observed frequency/expected frequency of the codon pair is greater than one the codon pair is said to be overrepresented. If the ratio is smaller than one, it is said to be underrepresented. In the example, the codon pair GCA-GAA is overrepresented 1.65 fold while the coding pair GCC-GAA is more than 5-fold underrepresented.

[0075] Many other codon pairs show very strong bias; some pairs are under-represented, while other pairs are over-represented. For instance, the codon pairs GCCGAA (AlaGlu) and GATCTG (AspLeu) are three- to six-fold under-represented (the preferred pairs being GCAGAG and GACCTG, respectively), while the codon pairs GCCAAG (AlaLys) and AATGAA (AsnGlu) are about two-fold over-represented. It is noteworthy that codon pair bias has nothing to do with the frequency of pairs of amino acids, nor with the frequency of individual codons. For instance, the under-represented pair GATCTG (AspLeu) happens to use the most frequent Leu codon, (CTG).

[0076] As discussed more fully below, codon pair bias takes into account the score for each codon pair in a coding sequence averaged over the entire length of the coding sequence. According to the invention, codon pair bias is determined by

[0077] $$\mathrm{CPB} = \sum_{i=1}^{k} \frac{CPSi}{K-1}$$

[0078] Accordingly, similar codon pair bias for a coding sequence can be obtained, for example, by minimized codon pair scores over a subsequence or moderately diminished codon pair scores over the full length of the coding sequence.

Calculation of Codon Pair Bias

[0079] Every individual codon pair of the possible 3721 non-"STOP" containing codon pairs (e.g., GTT-GCT) carries an assigned "codon pair score," or "CPS" that is specific for a given "training set" of genes. The CPS of a given codon pair is defined as the log ratio of the observed number of occurrences over the number that would have been expected in this set of genes (in this example the human genome). Determining the actual number of occurrences of a particular codon pair (or in other words the likelihood of a particular amino acid pair being encoded by a particular codon pair) is simply a matter of counting the actual number of occurrences of a codon pair in a particular set of coding sequences. Determining the expected number, however, requires additional calculations. The expected number is calculated so as to be independent of both amino acid frequency and codon bias similarly to Gutman and Hatfield. That is, the expected frequency is calculated based on the relative proportion of the number of times an amino acid is encoded by a specific codon. A positive CPS value signifies that the given codon pair is statistically over-represented, and a negative CPS indicates the pair is statistically under-represented in the human genome.

[0080] To perform these calculations within the human context, the most recent Consensus CDS (CCDS) database of consistently annotated human coding regions, containing a total of 14,795 genes, was used. This data set provided codon and codon pair, and thus amino acid and amino-acid pair frequencies on a genomic scale.

[0081] The paradigm of Federov et al. (2002), was used to further enhanced the approach of Gutman and Hatfield (1989). This allowed calculation of the expected frequency of a given codon pair independent of codon frequency and non-random associations of neighboring codons encoding a particular amino acid pair. The detailed equations used to calculate CPB are disclosed in WO 2008/121992 and WO 2011/044561, which are incorporated by reference.

$$S(P_{ij}) = \ln\left(\frac{N_O(P_{ij})}{N_E(P_{ij})}\right) = \ln\left(\frac{N_O(P_{ij})}{F(C_i)F(C_j)N_O(X_{ij})}\right)$$

[0082] In the calculation, P_{ij} is a codon pair occurring with a frequency of $N_O(P_{ij})$ in its synonymous group. C_i and C_j are the two codons comprising P_{ij}, occurring with frequencies $F(C_i)$ and $F(C_j)$ in their synonymous groups respectively. More explicitly, $F(C_i)$ is the frequency that corresponding amino acid X_i is coded by codon C_i throughout all coding regions and $F(C_i)=N_O(C_j)/N_O(X_i)$, where $N_O(C_i)$ and $N_O(X_i)$ are the observed number of occurrences of codon C_i and amino acid X_i respectively. $F(C_j)$ is calculated accordingly. Further, $N_O(X_{ij})$ is the number of occurrences of amino acid pair X_{ij} throughout all coding regions. The codon pair bias score $S(P_{ij})$ of

P_{ij} was calculated as the log-odds ratio of the observed frequency $N_o(P_{ij})$ over the expected number of occurrences of $N_e(P_{ij})$.

[0083] Using the formula above, it was then determined whether individual codon pairs in individual coding sequences are over- or under-represented when compared to the corresponding genomic $N_e(P_{ij})$ values that were calculated by using the entire human CCDS data set. This calculation resulted in positive $S(P_{ij})$ score values for over-represented and negative values for under-represented codon pairs in the human coding regions.

[0084] The "combined" codon pair bias of an individual coding sequence was calculated by averaging all codon pair scores according to the following formula:

$$S(P_{ij}) = \sum_{l=1}^{k} \frac{S(P_{ij})l}{k-1}$$

[0085] The codon pair bias of an entire coding region is thus calculated by adding all of the individual codon pair scores comprising the region and dividing this sum by the length of the coding sequence.

Calculation of Codon Pair Bias, Implementation of Algorithm to Alter Codon-Pair Bias.

[0086] An algorithm was developed to quantify codon pair bias. Every possible individual codon pair was given a "codon pair score", or "CPS". CPS is defined as the natural log of the ratio of the observed over the expected number of occurrences of each codon pair over all human coding regions, where humans represent the host species of the instant vaccine virus to be recoded.

$$CPS = \ln\left(\frac{F(AB)_O}{\frac{F(A) \times F(B)}{F(X) \times F(Y)} \times F(XY)}\right)$$

[0087] Although the calculation of the observed occurrences of a particular codon pair is straightforward (the actual count within the gene set), the expected number of occurrences of a codon pair requires additional calculation. We calculate this expected number to be independent both of amino acid frequency and of codon bias, similar to Gutman and Hatfield. That is, the expected frequency is calculated based on the relative proportion of the number of times an amino acid is encoded by a specific codon. A positive CPS value signifies that the given codon pair is statistically over-represented, and a negative CPS indicates the pair is statistically under-represented in the human genome

[0088] Using these calculated CPSs, any coding region can then be rated as using over- or under-represented codon pairs by taking the average of the codon pair scores, thus giving a Codon Pair Bias (CPB) for the entire gene.

$$CPB = \sum_{i=1}^{k} \frac{CPSi}{k-1}$$

[0089] The CPB has been calculated for all annotated human genes using the equations shown and plotted (FIG. 1). Each point in the graph corresponds to the CPB of a single human gene. The peak of the distribution has a positive codon pair bias of 0.07, which is the mean score for all annotated human genes. Also, there are very few genes with a negative codon pair bias. Equations established to define and calculate CPB were then used to manipulate this bias.

[0090] Algorithm for Reducing Codon-Pair Bias.

[0091] Recoding of protein-encoding sequences may be performed with or without the aid of a computer, using, for example, a gradient descent, or simulated annealing, or other minimization routine. An example of the procedure that rearranges codons present in a starting sequence can be represented by the following steps:

(1) Obtain wildtype viral genome sequence.

(2) Select protein coding sequences to target for attenuated design.

(3) Lock down known or conjectured DNA segments with non-coding functions.

(4) Select desired codon distribution for remaining amino acids in redesigned proteins.

(5) Perform random shuffle of at least two synonymous unlocked codon positions and calculate codon-pair score.

(6) Further reduce (or increase) codon-pair score optionally employing a simulated annealing procedure.

(7) Inspect resulting design for excessive secondary structure and unwanted restriction site:

- if yes-> go to step (5) or correct the design by replacing problematic regions with wildtype sequences and go to step (8).

(8) Synthesize DNA sequence corresponding to virus design.

(9) Create viral construct and assess viral phenotype:

- if too attenuated, prepare subclone construct and go to 9;
- if insufficiently attenuated, go to 2.

[0092] Attenuation of viruses by reducing codon pair bias is disclosed in WO 2008/121992 and WO 2011/044561, which are incorporated by reference.

[0093] Methods of obtaining full-length Flavivirus or Zika genome sequence or codon pair deoptimized sequences embedded in a wild-type Flavivirus or Zika genome sequence can include for example, constructing an infectious cDNA clone, using an overlap extension PCR strategy, or long PCR-based fusion strategy.

Modified Flavivirus

[0094] Various embodiments of the invention provide for a modified Flavivirus virus in which expression of viral proteins is reduced compared to a parent virus. The reduction in expression is the result of recoding the prM, or envelope (E) region, or the nonstructural protein 3 (NS3) region or both the E and NS3 regions.

[0095] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence are recoded by reducing the codon pair bias or codon usage bias of the protein-encoding sequence. In various embodiments, reducing the codon-pair bias comprises identifying a codon pair in the parent protein-encoding sequence having a codon-pair score that can be reduced, and reducing the codon-pair bias by substituting the codon pair with a codon pair that has a lower codon-pair score. In other embodiments embodiments, reducing the codon-pair bias comprises rearranging the codons of a parent protein-encoding sequence.

[0096] In various embodiments, each of the recoded prM/E protein-encoding sequence and the recoded NS3 protein-encoding sequence have a codon pair bias less than, -0.05, −0.1, or less than −0.2, or less than −0.3, or less than −0.4.

[0097] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence are recoded by increasing the number of CpG or UpA di nucleotides compared to a parent virus.

[0098] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence are recoded by replacing one or more codons with synonymous codons that are less frequent in the viral host.

[0099] In various embodiments, the parent virus is a Flavivirus selected from the group consisting of dengue fever virus, West Nile virus, yellow fever virus, Japanese encephalitis virus, Spondweni virus, Saint Louis encephalitis virus, and Powassan virus. In various embodiments, the parent virus is a natural isolate. In various embodiments, the parent virus a mutant of a natural isolate.

Modified Zika Viruses

[0100] Various embodiments of the present invention provide for modified Zika viruses as the modified Flavivirus.

[0101] In various embodiments, a modified Zika virus is provided in which expression of viral proteins is reduced compared to a parent virus, wherein the reduction in expression is the result of recoding the prM, or envelope (E) region, or the nonstructural protein 3 (NS3) region or both the E and NS3 regions.

[0102] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence are recoded by reducing the codon pair bias or codon usage bias of the protein-encoding sequence. In various embodiments, reducing the codon-pair bias comprises identifying a codon pair in the parent protein-encoding sequence having a codon-pair score that can

be reduced, and reducing the codon-pair bias by substituting the codon pair with a codon pair that has a lower codon-pair score. In various embodiments, reducing the codon-pair bias comprises rearranging the codons of a parent protein-encoding sequence.

[0103] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence are recoded by increasing the number of CpG or UpA di-nucleotides compared to a parent virus. In various embodiments, each of the recoded prM/E protein-encoding sequence and the recoded NS3 protein-encoding sequence have a codon pair bias less than, -0.05, −0.1, or less than −0.2, or less than −0.3, or less than −0.4. In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence are recoded by replacing one or more codons with synonymous codons that are less frequent in the host.

[0104] Various embodiments of the present invention provide for a method of making a modified Flavivirus virus genome. The method comprises obtaining the nucleotide sequence encoding the envelope protein of a Flavivirus virus and the nucleotide sequence encoding the nonstructural 3 proteins of a Flavivirus virus; recoding the envelope encoding nucleotide sequence to reduce protein expression and recoding the nonstructural protein 3-encoding nucleotide sequence to reduce protein expression, and substituting a nucleic acid having the recoded envelope-encoding nucleotide sequence and a nucleic acid having the recoded nonstructural protein 3-encoding nucleotide sequence into a parent Flavivirus virus genome to make a modified Flavivirus virus genome; whereby expression of the recoded envelope-encoding nucleotide sequence and expression of the recoded nonstructural protein 3-encoding nucleotide sequence is reduced compared to the parent virus.

[0105] Various embodiments of the present invention provide for a method of making a modified Zika virus genome comprising: obtaining the nucleotide sequence encoding the envelope protein of a Zika virus and the nucleotide sequence encoding the nonstructural 3 proteins of a Zika virus; recoding the envelope encoding nucleotide sequence to reduce protein expression and recoding the nonstructural protein 3-encoding nucleotide sequence to reduce protein expression, and substituting a nucleic acid having the recoded envelope-encoding nucleotide sequence and a nucleic acid having the recoded nonstructural protein 3-encoding nucleotide sequence into a parent Zika virus genome to make a modified Zika virus genome; whereby expression of the recoded envelope-encoding nucleotide sequence and expression of the recoded nonstructural protein 3-encoding nucleotide sequence is reduced compared to the parent virus.

[0106] According to various embodiments the invention, viral attenuation is accomplished by reducing expression viral proteins through codon pair deoptimization of E and NS3 coding sequences. One way to reduce expression of the coding sequences is by a reduction in codon pair bias, but other methods can also be used, alone or in combination. While codon bias may be changed, adjusting codon pair bias is particularly advantageous. For example, attenuating a virus through codon bias generally

requires elimination of common codons, and so the complexity of the nucleotide sequence is reduced. In contrast, codon pair bias reduction or minimization can be accomplished while maintaining far greater sequence diversity, and consequently greater control over nucleic acid secondary structure, annealing temperature, and other physical and biochemical properties.

[0107] Codon pair bias of a protein-encoding sequence (i.e., an open reading frame) is calculated as set forth above and described in Coleman et al., 2008.

[0108] Viral attenuation and induction or protective immune responses can be confirmed in ways that are well known to one of ordinary skill in the art, including but not limited to, the methods and assays disclosed herein. Non-limiting examples include plaque assays, growth measurements, reduced lethality in test animals, and protection against subsequent infection with a wild type virus.

[0109] In preferred embodiments, the invention provides viruses that are highly attenuated, and induce immunity against a plurality of Zika types and/or subtypes. Such Zika virus varieties include viruses in the so-called African and Asian lineages. Examples of attenuated Zika protein coding sequences are provided below.

Table 4. Reduced-Expression Zika Virus Genes

	WT Coding Sequence	Recoded Coding Sequence
Gene	SEQ ID NO:	SEQ ID NO:
PR15 Syn WT	1	
PR15 E-Min		2
PR15 NS3-Min		3
PR15 E-W/min		4
PR15 E-W/W/min		5
MR766 Syn WT	6	
MR766 E-Min		7
MR766 E-W/Min		8
MR766 E-W/W/min		9
MR766 NS3-Min		10
MR766 NS3-W/Min		11
MR766 NS3-W/W/Min		12

Vaccine Compositions

[0110] Various embodiments provide for a Flavivirus vaccine composition for inducing a protective immune response in a subject, which comprises the modified Flavivirus of the present invention as described herein.

[0111] Various embodiments of the present invention provide for a Zika vaccine composition for inducing a protective immune response in a subject, comprising the modified Zika virus of the present invention. Non-limiting examples of modified Zika viruses are provided:

[0112] SEQ ID NO:1 – PR15 Syn WT

Agttgttgatctgtgtgaatcagactgcgacagttcgagtttgaagcgaaagctagcaacagtatcaacaggttttattttggatttggaaacgagag
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[0116] SEQ ID NO:5 – PR15 E-W/W/Min

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CGGCCGGTGTGGGGAAATCCATGGTTTCT

[0124] The present invention provides a vaccine composition for inducing an immune response in a subject comprising any of the attenuated viruses described herein and a pharmaceutically acceptable carrier. In various embodiments, the immune response is a protective immune response.

[0125] It should be understood that an attenuated virus of the invention, where used to elicit an immune response in a subject (or protective immune response) or to prevent a subject from or reduce the likelihood of becoming afflicted with a virus-associated disease, is administered to the subject in the form of a composition additionally comprising a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, one or more of 0.01-0.1M and preferably 0.05M phosphate buffer, phosphate-buffered saline (PBS), or 0.9% saline. Such carriers also include aqueous or non-aqueous solutions, suspensions, and emulsions. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and buffered media. Examples of non-aqueous solvents are propylene glycol,

polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Solid compositions may comprise nontoxic solid carriers such as, for example, glucose, sucrose, mannitol, sorbitol, lactose, starch, magnesium stearate, cellulose or cellulose derivatives, sodium carbonate and magnesium carbonate. For administration in an aerosol, such as for pulmonary and/or intranasal delivery, an agent or composition is preferably formulated with a nontoxic surfactant, for example, esters or partial esters of C6 to C22 fatty acids or natural glycerides, and a propellant. Additional carriers such as lecithin may be included to facilitate intranasal delivery. Pharmaceutically acceptable carriers can further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives and other additives, such as, for example, antimicrobials, antioxidants and chelating agents, which enhance the shelf life and/or effectiveness of the active ingredients. The instant compositions can, as is well known in the art, be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to a subject.

[0126] In various embodiments of the instant vaccine composition, the attenuated virus (i) does not substantially alter the synthesis and processing of viral proteins in an infected cell; (ii) produces similar amounts of virions per infected cell as wt virus; and/or (iii) exhibits substantially lower virion-specific infectivity than wt virus. In further embodiments, the attenuated virus induces a substantially similar immune response in a host animal as the corresponding wt virus.

[0127] This invention also provides a modified host cell line specially isolated or engineered to be permissive for an attenuated virus that is inviable in a wild type host cell. Since the attenuated virus cannot grow in normal (wild type) host cells, it is absolutely dependent on the specific helper cell line for growth. This provides a very high level of safety for the generation of virus for vaccine production. Various embodiments of the instant modified cell line permit the growth of an attenuated virus, wherein the genome of said cell line has been altered to increase the number of genes encoding rare tRNAs.

Methods of eliciting an immune response

[0128] Various embodiments provide for a method of eliciting an immune response in a subject comprising administering to the subject a prophylactically or therapeutically effective dose of a vaccine composition comprising a modified Flavivirus of the present invention. Particular embodiments provide for a method of eliciting a protective immune response in a subject comprising administering to the subject a prophylactically or therapeutically effective dose of a vaccine

composition comprising a modified Flavivirus of the present invention. In various embodiments, the immune response is cross-protective against a heterologous Flavivirus virus.

[0129] In various embodiments, the method further comprises administering to the subject at least one adjuvant. Non-limiting examples of adjuvants are discussed herein. Particular embodiments provide for a method of eliciting an immune response in a subject, comprising: administering to the subject a prophylactically or therapeutically effective dose of a vaccine composition comprising a modified Zika virus the present invention.

[0130] Various embodiments provide for a method of eliciting a protective immune response in a subject, comprising: administering to the subject a prophylactically or therapeutically effective dose of a vaccine composition comprising a modified Zika virus the present invention.

[0131] In various embodiments, the method further comprises administering to the subject at least one adjuvant. Non-limiting examples of adjuvants are described herein.

[0132] In various embodiments, the immune response is cross-protective against a heterologous Zika virus.

[0133] Various embodiments of the present invention provide for a method of eliciting an immune response in a subject in need thereof, comprising: administering a prime dose of an attenuated Flavivirus produced by a method other than codon-pair deoptimization or a modified Flavivirus in which expression of viral proteins is reduced compared to a parent virus, wherein the reduction in expression is the result of recoding the prM, or envelope (E) region, or the nonstructural protein 3 (NS3) region or both the E and NS3 regions; and administering one or more boost dose of the attenuated Flavivirus by methods other than codon-pair deoptimization or the modified Flavivirus to the subject in need thereof, wherein at least the prime dose or the one or more boost dose is the modified virus. In various embodiments, a first of the one or more boost dose is administered about 2 weeks after the prime dose.

[0134] In various embodiments, the Flavivirus is a Zika virus. In other embodiments, the Flavivirus is selected from the group consisting of dengue fever virus, West Nile virus, yellow fever virus, Japanese encephalitis virus, Spondweni virus, Saint Louis encephalitis virus, and Powassan virus.

[0135] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence are recoded by reducing the codon pair bias or codon usage bias of the protein-encoding sequence. In various embodiments, reducing the codon-pair bias comprises identifying a codon pair in the parent protein-encoding sequence having a codon-pair score that can be reduced, and reducing the codon-pair bias by substituting the codon pair with a codon pair that has a lower codon-pair score.

[0136] In various embodiments, reducing the codon-pair bias comprises rearranging the codons of a parent protein-encoding sequence. In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence are recoded by increasing the number of CpG or UpA di nucleotides compared to a parent virus. In various embodiments, each of the recoded prM/E protein-encoding sequence and the recoded NS3 protein-encoding sequence have a codon pair bias less than, -0.05, −0.1, or less than −0.2, or less than −0.3, or less than −0.4. In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence are recoded by replacing one or more codons with synonymous codons that are less frequent in the viral host.

[0137] In addition, the present invention provides a method for eliciting a protective immune response in a subject comprising administering to the subject a prophylactically or therapeutically effective dose of any of the vaccine compositions described herein. This invention also provides a method for preventing a subject from becoming afflicted with a virus-associated disease comprising administering to the subject a prophylactically effective dose of any of the instant vaccine compositions. In embodiments of the above methods, the subject has been exposed to a pathogenic virus. “Exposed” to a pathogenic virus means contact with the virus such that infection could result.

[0138] The invention further provides a method for delaying the onset, or slowing the rate of progression, of a virus-associated disease in a virus-infected subject comprising administering to the subject a therapeutically effective dose of any of the instant vaccine compositions.

[0139] As used herein, “administering” means delivering using any of the various methods and delivery systems known to those skilled in the art. Administering can be performed, for example, intranasally, intraperitoneally, intracerebrally, intravenously, orally, transmucosally, subcutaneously, transdermally, intradermally, intramuscularly, topically, parenterally, via implant, intrathecally, intralymphatically, intralesionally, pericardially, or epidurally. An agent or composition may also be administered in an aerosol, such as for pulmonary and/or intranasal delivery. Administering may be performed, for example, once, a plurality of times, and/or over one or more extended periods.

[0140] Eliciting a protective immune response in a subject can be accomplished, for example, by administering a primary dose of a vaccine to a subject, followed after a suitable period of time by one or more subsequent administrations of the vaccine. A suitable period of time between administrations of the vaccine may readily be determined by one skilled in the art, and is usually on the order of several weeks to months. The present invention is not limited, however, to any particular method, route or frequency of administration.

[0141] In various embodiments, the present invention provides for a method of eliciting an immune response in a subject in need thereof, comprising: administering a prime dose of an attenuated Flavivirus produced by a method other than codon-pair deoptimization or a modified Flavivirus in which expression of viral proteins is reduced compared to a parent virus, wherein the reduction in

expression is the result of recoding the prM, or envelope (E) region, or the nonstructural protein 3 (NS3) region or both the E and NS3 regions; and administering one or more boost dose of the attenuated Flavivirus by methods other than codon-pair deoptimization or the modified Flavivirus to the subject in need thereof, wherein at least the prime dose or the one or more boost dose is the modified virus.

[0142] In various embodiments, the one or more boost dose is administered about 2 weeks after a prime dose. In various embodiments, 2, 3, 4, or 5 boost doses are administered. In various embodiments, the intervals between the boost doses can be 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 weeks. In additional embodiments, the intervals between the boost doses can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 months. As a non-limiting example, the prime dose can be administered, about two weeks thereafter a first boost dose can be administered, about one month after the first boost dose, a second boost dose can be administered, about 6 months after the second boost dose, a third boost dose can be administered. As another non-limiting example, the prime dose can be administered, about two weeks thereafter a first boost dose can be administered, about six months after the first boost dose, a second boost dose can be administered, about 12 months after the second boost dose, a third boost dose can be administered. In further embodiments, additional boost dosages can be periodically administered; for example, every 5 years, every 10 years, etc.

[0143] In various embodiments, the Flavivirus is a Zika virus. In various embodiments, the Flavivirus is selected from the group consisting of dengue fever virus, West Nile virus, yellow fever virus, Japanese encephalitis virus, Spondweni virus, Saint Louis encephalitis virus, and Powassan virus.

[0144] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence are recoded by lowering the codon pair bias or codon usage bias of the protein-encoding sequence. In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence are recoded by increasing the number of CpG or UpA di nucleotides compared to a parent virus.

[0145] In various embodiments, reducing the codon-pair bias comprises identifying a codon pair in the parent protein-encoding sequence having a codon-pair score that can be reduced, and reducing the codon-pair bias by substituting the codon pair with a codon pair that has a lower codon-pair score.

[0146] In various embodiments, reducing the codon-pair bias comprises rearranging the codons of a parent protein-encoding sequence. In various embodiments it includes the increase of the CpG dinucleotide in the modified virus. In various embodiments it includes the increase of the UpA dinucleotide in the modified virus.

[0147] In various embodiments, each of the recoded prM/E protein-encoding sequence and the recoded NS3 protein-encoding sequence have a codon pair bias less than, -0.05, −0.1, or less than −0.2, or less than −0.3, or less than −0.4.

[0148] In various embodiments, one or both of the E protein-encoding sequence and the NS3 protein-encoding sequence are recoded by replacing one or more codons with synonymous codons that are less frequent in the viral host.

[0149] In various embodiments, the prime dose is administered subcutaneously, intramuscularly, intradermally, or intranasally. In various embodiments, the one or more boost dose is administered intratumorally, intravenously, or intrathecally.

[0150] The timing between the prime and boost dosages can vary, for example, depending on the stage of infection or disease (e.g., non-infected, infected, number of days post infection), and the patient's health. In various embodiments, the one or more boost dose is administered about 2 weeks after the prime dose. That is, the prime dose is administered and about two weeks thereafter, a boost dose is administered.

[0151] In various embodiments, the dosage amount can vary between the prime and boost dosages. As a non-limiting example, the prime dose can contain fewer copies of the virus compared to the boost dose.

[0152] In other embodiments, the type of attenuated virus produced by a method other than codon-pair deoptimization or modified virus of the present invention can vary between the prime and boost dosages. In one non-limiting example, a modified virus of the present invention can be used in the prime dose and an attenuated virus (produced by a method other than codon-pair deoptimization) of the same or different family, genus, species, group or order can be used in the boost dose.

[0153] In other embodiments, the route of administration can vary between the prime and the boost dose. In a non-limiting example, the prime dose can be administered subcutaneously, and the boost dose can be administered via injection into the tumor; for tumors that are in accessible, or are difficult to access, the boost dose can be administered intravenously.

[0154] A "subject" means any animal or artificially modified animal. Animals include, but are not limited to, humans, non-human primates, cows, horses, sheep, pigs, dogs, cats, rabbits, ferrets, rodents such as mice, rats and guinea pigs, and birds. Artificially modified animals include, but are not limited to, SCID mice with human immune systems, and CD155tg transgenic mice expressing the human poliovirus receptor CD155. In a preferred embodiment, the subject is a human. Preferred embodiments of birds are domesticated poultry species, including, but not limited to, chickens, turkeys, ducks, and geese.

[0155] A "prophylactically effective dose" is any amount of a vaccine that, when administered to a subject prone to viral infection or prone to affliction with a virus-associated disorder, induces in the

subject an immune response that protects the subject from becoming infected by the virus or afflicted with the disorder. "Protecting" the subject means either reducing the likelihood of the subject's becoming infected with the virus, or lessening the likelihood of the disorder's onset in the subject, by at least two-fold, preferably at least ten-fold. For example, if a subject has a 1% chance of becoming infected with a virus, a two-fold reduction in the likelihood of the subject becoming infected with the virus would result in the subject having a 0.5% chance of becoming infected with the virus. Most preferably, a "prophylactically effective dose" induces in the subject an immune response that completely prevents the subject from becoming infected by the virus or prevents the onset of the disorder in the subject entirely.

[0156] As used herein, a "therapeutically effective dose" is any amount of a vaccine that, when administered to a subject afflicted with a disorder against which the vaccine is effective, induces in the subject an immune response that causes the subject to experience a reduction, remission or regression of the disorder and/or its symptoms. In preferred embodiments, recurrence of the disorder and/or its symptoms is prevented. In other preferred embodiments, the subject is cured of the disorder and/or its symptoms.

[0157] Certain embodiments of any of the instant immunization and therapeutic methods further comprise administering to the subject at least one adjuvant. An "adjuvant" shall mean any agent suitable for enhancing the immunogenicity of an antigen and boosting an immune response in a subject. Numerous adjuvants, including particulate adjuvants, suitable for use with both protein- and nucleic acid-based vaccines, and methods of combining adjuvants with antigens, are well known to those skilled in the art. Suitable adjuvants for nucleic acid based vaccines include, but are not limited to, Quil A, imiquimod, resiquimod, and interleukin-12 delivered in purified protein or nucleic acid form. Adjuvants suitable for use with protein immunization include, but are not limited to, alum, Freund's incomplete adjuvant (FIA), saponin, Quil A, and QS-21.

[0158] The invention also provides a kit for immunization of a subject with an attenuated virus of the invention. The kit comprises the attenuated virus, a pharmaceutically acceptable carrier, an applicator, and an instructional material for the use thereof. In further embodiments, the attenuated virus may be one or more dengue virus, one or more Japanese encephalitis virus, one or more West Nile virus, one or more yellow fever virus, one or more Zika virus, etc. More than one virus may be preferred where it is desirable to immunize a host against a number of different isolates of a particular virus. The invention includes other embodiments of kits that are known to those skilled in the art. The instructions can provide any information that is useful for directing the administration of the attenuated viruses.

[0159] Throughout this application, various publications, reference texts, textbooks, technical manuals, patents, and patent applications have been referred to. The teachings and disclosures of these

publications, patents, patent applications and other documents in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which the present invention pertains. However, the citation of a reference herein should not be construed as an acknowledgement that such reference is prior art to the present invention.

[0160] It is to be understood and expected that variations in the principles of invention herein disclosed can be made by one skilled in the art and it is intended that such modifications are to be included within the scope of the present invention. The following Examples further illustrate the invention, but should not be construed to limit the scope of the invention in any way. Detailed descriptions of conventional methods, such as those employed in the construction of recombinant plasmids, transfection of host cells with viral constructs, polymerase chain reaction (PCR), and immunological techniques can be obtained from numerous publications, including Sambrook et al. (1989) and Coligan et al. (1994). All references mentioned herein are incorporated in their entirety by reference into this application. The contents of WO 2008/121992 and WO 2011/044561 are incorporated by reference.

EXAMPLES

Example 1

Construction and Characterization of E, NS3 and E-NS3 Codon Pair-Bias Reduced Zika Virus in Tissue Culture

[0161] To achieve attenuation of Zika virus strains PRVABC59 and MR766, codon pair bias of the prM/E and NS3 genes was reduced (introducing underrepresented codon pairs) in viral genes according to computer algorithms and chemical synthesis in order to reduce the expression level of the viral genes (Figure 1).

Table 6. CpG and UpA increase

Virus	CpG (%)	UpA (%)
PR15 SYN WT	24 (2.39%)	26 (2.59%)
PR15 E-W/W/Min	59 (5.88%)	52 (5.18%)

Example 2

Leveraging SAVE platform flexibility to create second-generation Zika virus vaccine candidates

[0162] To fine-tune attenuation and immunogenicity, a second generation of Zika virus vaccine candidates were constructed using the SAVE platform (Figure 2). Based on the E-Min design (Figure 1), the new vaccine candidates contained a smaller proportion of deoptimized sequence lowered from

2014 base-pairs (E-Min) to either 997 base-pairs (E-W/Min) or 664 base-pairs (E-W/W/Min) with the remainder restored to wild-type sequence.

[0163] The sequences of the three candidates MR766 E-Min (SEQ ID NO: 7), W-E-Min (SEQ ID NO: 8), and E-W/W/Min (SEQ ID NO: 9) are provided herein as examples.

Example 3

Characterization of Reduced Codon-Pair Bias Variants' Attenuation in Vero cells

[0164] MR766-EMin replicated with similar kinetics compared to wildtype MR766 in Vero cells, reaching a titer of $\geq 2 \times 10^7$ PFU/mL. PR15 E-Min was more attenuated, reaching a titer of $\sim 2 \times 10^6$ PFU/mL in Vero cells. The NS3-Min variants were more highly attenuated; with maximum titers of 1×10^3 PFU/mL and 5×10^5 PFU/mL for MR766 NS3-Min and PR15 NS3-Min respectively (Figure 3).

Example 4

Neuroattenuation of Reduced Codon-Pair Bias Variants' in Human Neuronal Cell Lines

[0165] Unlike the older ZIKV strains such as MR766, the current strains of ZIKV cause neurological disease such as microcephaly and Guillain-Barre syndrome. Thus, based on our pre, pre-IND meeting with the FDA, any live-attenuated Zika vaccine would need to demonstrate neuro-attenuation in human neuronal cells *in vitro* and neuro-attenuation in nonhuman primates *in vivo* (proposed Phase II work). To begin the pre-clinical development of our lead candidate, we infected two well-characterized human neuronal cell lines HTB-14 (also known as U-87) and HTB-15 (also known as U-118), which have been used previously to characterize Zika virus cell tropism in the developing human brain and to test potential anti-Zika inhibitors. We infected human neuronal cell lines HTB-14 and HTB-15 and quantified peak titers after 4 days. We observed that in human HTB-14 cells, MR766 E-Min was nearly 4 Log_{10}s attenuated and in human HTB-15 cells, growth was either undetectable in two independent experiments or at the limit of detection in one experiment (100 PFU/ml) also representing a nearly 4 Log_{10} level of neuro attenuation *in vitro* (Figure 4)

Example 5

Levels of Protein are Reduced in PRVABC59 or MR766 derived E-Min Infected Cells.

[0166] Western Blot of whole cell lysates taken from ZIKV infected cells were used to compare levels of protein expression between different PRVABC59 and MR766 variants. For virus infection, Vero cells were grown in the OptiPRO medium at 37°C till 90% confluent. Zika viruses, including synthetic wildtype and de-optimized (E-Min) MR766 and PRVABC59, were diluted to an MOI of 0.5 and were added to the cells. The cells were rocked for 15min at R.T., then incubated at 33°C for 2hrs.

Next, the inocula were removed, and the infected cells were continued to culture in OptiPRO medium at 33C for 24hrs.

[0167] For whole cell lysate preparation after 24hr incubation, cells were briefly rinsed with cold PBS, then lysed on ice with RIPA buffer (150mM NaCl, 5mM b-mercaptoethanol, 1% NP-40, 0.1% sodium dodecyl sulfate, 50mM Tris-HCl, pH8.0). Whole cell lysates were collected, directly mixed with 6x Laemmli buffer with b-mercaptoethanol, boiled and aliquoted for storage.

[0168] For Western blot, an equal volume of WCL from each sample was fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% bovine calf serum (BCS) in PBS for 1hr at R.T., then incubated with a mouse monoclonal antibody against dengue type-2 envelope(E) protein 4G2 overnight at 4C, washed three times with PBS-Tween, subsequently incubated with HRP conjugated anti-mouse secondary antibody in 5% BCS for 1hr at R.T. The membrane was washed three times, and proteins were visualized using Pierce 1-Step Ultra TMB Blotting solution.

[0169] Levels of the envelope glycoprotein were found to be reduced in E-Min variants of PRVABC59 and MR766 strains of ZIKV compared to wildtype (Figure 5).

Example 6

Attenuation in AG129 Mice

[0170] As described above, the AG129 adult mouse model is gaining acceptance for studies of flavivirus vaccines and therapeutics. We used AG129 mice to test: **1)** each synthetically derived wild-type virus MR766 and PR15 virus at a dose of 10^2 (positive control); **2)** two doses (10^4 and 10^2 PFU) of the vaccine candidates PR15 E-Min or MR766 E-Min and NS3-Min; and **3)** a single dose of 10^4 of the vaccine candidates PR15 NS3/E-Min. In this study we examined attenuation, efficacy, and immunogenicity. Animals were randomly assigned to groups of 5 animals. Groups were infected with various attenuated and synthetic wild-type viruses. Two rounds of vaccination were performed on Day 0 and Day 28 to vaccinate mice. To measure attenuation, survival and weight was measured post-vaccination (Figure 6).

[0171] Survival, weight, and clinical sign data were collected daily throughout the course of the experiment. SAVE deoptimized PRVABC59and MR766 strains were highly attenuated compared to synthetic wild-type viruses, with only the MR766 NS3-Min at a dose of 10^4 inducing death in 40% of mice. Thus, all other strains were at least 500-fold attenuated. Mice infected with 10^2 PFU of synthetic wild-type PR15 or MR766 ZIKV experienced a dramatic weight loss just prior to death, similar to the pattern observed with "natural" wild-type ZIKV. Mice infected with 10^4 PFU of the candidate MR766 E-Min did not experience significant weight loss or mortality (Figure 6).

[0172] The growth phenotype and pathogenesis of the PRVABC59 E-Min and E+NS3-Min variant as well as the MR766 E-Min and NS3-Min was examined in an animal model. Groups of five AG129 mice received each virus at doses of 10^2 or 10^4 PFU subcutaneously, and body weight and survival of the animals was monitored continuously for 28 days p.i. Morbidity and mortality (weight loss, reduced activity, death) was monitored. The Lethal Dose 50 (LD_{50}) of the wildtype virus and the vaccine candidates was calculated by the method of Reed and Muench (Reed, L. J.; Muench, H., 1938, The American Journal of Hygiene 27: 493-497). Remarkably, the E-Min variants for PRVABC59 and MR766 and the PRVABC59 E+NS3-Min virus did not induce apparent disease after a dose up to 10^4 PFU with no mortality and minimal weight loss. Therefore, the theoretical LD50 of the E-Min variants was calculated to be equal or greater than 3.16×10^4 PFU, which exceeds that of wt PRVABC59 or MR766 by a factor of at least 1,000 (Table 5). The LD50 of the MR766 NS3-Min virus was calculated to be ≤42.

Table 5. LD_{50} and PD_{50} of Attenuated Virus

Virus	LD50	PD50
WT MR766	$<3.16x10^1$	NA
MR766 E-Min	$>3.16\times10^4$	$6.81x10^1$
MR766 NS3-Min	$<4.20\times10^1$	$<6.81x10^3$
WT PRVABC59	$<3.16x10^1$	NA
PRVABC59 E-Min	3.16×10^4	$1.47x10^2$
PRVABC59 E+NS3-Min	3.16×10^4	$<6.81x10^3$

Example 7

Immunogenicity in AG129 Mice

[0173] Vaccine candidates should be capable of providing, at low dose, long-term protection from challenge with a lethal dose of wt virus equal to $10^{2.3}$ cell-culture infectious dose ($CCID_{50}$) per animal. Although the E-Min variants were highly attenuated in cell culture and in AG129 mice, they were successful in preventing mortality in AG129 mice after challenge with as little as 68 PFU (MR766 E-Min) or 147 PFU (PRVABC59 E-Min). We tested vaccine efficacy in the survivors of each mouse from the attenuation study. First, serum was harvested from survivors via superficial temporal vein on Day 28 and animals were boosted with the same vaccine dose a second time on Day 28. Serum was also collected on day 49 (21 days post-boost). Neutralizing antibodies from these animals were quantified using a 50% plaque reduction neutralization titer ($PRNT_{50}$) assay of serum harvested at 14, 28, and 49 days post vaccination. One half serial dilutions, starting at a 1/10 dilution, of test sera were made. Dilutions were then mixed 1:1 with $10^{2.4}$ PFU of ZIKV strain PRVABC59 (PR15). The virus-serum mixture was then added to individual wells of a 12-well tissue culture plate with Vero 76 cells.

The reciprocal of the dilution of test serum that resulted in ≥50% reduction in average plaques from virus control was recorded as the $PRNT_{50}$ value. On day 28 and after only a single vaccination, MR766 E-Min was *able to generate a robust antibody response to the current PR15 strain in all animals* (Figure 7). Similarly, PR15 E-Min also provided a robust antibody response after only a single vaccination.

Example 8

Protective Efficacy in AG129 Mice

[0174] On Day 49 after initial vaccination, each group was challenged with a lethal dose ($10^{2.3}$ $CCID_{50}$/animal) of PR15 WT virus in 0.1 mL delivered s.c. Challenge virus was provided to Utah State via BEI Resources (Manassas, VA). Mice were observed at least twice daily for mortality, and weights were taken every other day from 7-21 days post challenge to track weight change. Codagenix's SAVE vaccine candidates were successful in preventing or reducing mortality and morbidity (weight loss, not shown) in challenged AG129 mice. MR766 E-Min and PR15 E-Min at a dose of 10^4 could both protect all mice and induce a robust antibody response, whereas other vaccine variants (e.g. PR15 E+NS3-Min) could only provide partial protection (Figure 8). The protective-dose 50% (PD_{50}) for MR766 E-Min was $<10^2$ PFU, at least 1000-fold less than MR766 E-Min's LD_{50}, indicating at least a 3-orders of magnitude safety margin.

Example 9

Efficacy in Non-Human Primates

[0175] Cynomolgus macaques (CM) are commonly used as a model for evaluating flavivirus infection including Yellow Fever, Dengue, West Nile and ZIKV. ZIKV isolates have also been recovered from naturally infected Cynomolgus macaques. Previous work suggests that while experimental ZIKV infection of CM is not likely to produce clinical disease, ZIKV can replicate and be found in blood, urine and other tissues. In a non-GLP study conducted by Southern Research, we evaluated the efficacy of live attenuated Zika virus (ZIKV) vaccine candidates in naïve CM. A total of fifteen (15) (10 male and 5 female) ZIKV seronegative CMs were randomized into five (5) treatment groups. CMs in Groups 1-4 were vaccinated using a prime-boost regimen (Days 0 and 28) by subcutaneous (SC) injection with attenuated viruses (MR776 E-W/Min or MR776 E-W/W/Min) at doses of 10^7 or 10^5 PFU in a volume of 500 µL. CMs assigned to the mock control group (Group 5) received PBS via SC injection. The presence of vaccine induced anti-ZIKV neutralizing antibody (Nab) was assessed on Day 14, 21, 28, 50 and 61 by focus reduction neutralization test (FRNT). FRNT values were 2-4 times higher for both MR766 E-W/Min (Figure 9) and MR766 E-W/W/Min (Figure 10) vaccinated macaques compared to the NIH Inactivated vaccine after a single dose. FRNT values

in E-W/W/Min vaccinated macaques did not increase after the boost on day 28, indicating that the vaccines induced sterilizing immunity that prevented further infection by the boosting virus. All animals vaccinated with E-W/Min or E-W/W/Min seroconverted by day 14 post-vaccination. This data indicates that a single, relatively low dose, of E-W/W/Min may be sufficient for triggering high levels ($\geq$1,028 $FRNT_{50}$).

[0176] Various embodiments of the invention are described above in the Detailed Description. While these descriptions directly describe the above embodiments, it is understood that those skilled in the art may conceive modifications and/or variations to the specific embodiments shown and described herein. Any such modifications or variations that fall within the purview of this description are intended to be included therein as well. Unless specifically noted, it is the intention of the inventors that the words and phrases in the specification and claims be given the ordinary and accustomed meanings to those of ordinary skill in the applicable art(s).

[0177] The foregoing description of various embodiments of the invention known to the applicant at this time of filing the application has been presented and is intended for the purposes of illustration and description. The present description is not intended to be exhaustive nor limit the invention to the precise form disclosed and many modifications and variations are possible in the light of the above teachings. The embodiments described serve to explain the principles of the invention and its practical application and to enable others skilled in the art to utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. Therefore, it is intended that the invention not be limited to the particular embodiments disclosed for carrying out the invention.

[0178] While particular embodiments of the present invention have been shown and described, it will be obvious to those skilled in the art that, based upon the teachings herein, changes and modifications may be made without departing from this invention and its broader aspects and, therefore, the appended claims are to encompass within their scope all such changes and modifications as are within the true spirit and scope of this invention. It will be understood by those within the art that, in general, terms used herein are generally intended as "open" terms (*e.g.*, the term "including" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to," etc.).

[0179] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are useful to an embodiment, yet open to the inclusion of unspecified elements, whether useful or not. It will be understood by those within the art that, in general, terms used herein are generally intended as "open" terms (e.g., the term "including" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to," etc.). Although the open-ended term "comprising," as a synonym of terms such as including, containing, or

having, is used herein to describe and claim the invention, the present invention, or embodiments thereof, may alternatively be described using alternative terms such as “consisting of” or “consisting essentially of.”