IMPROVED METHOD FOR PRODUCING VIRUS LIKE PARTICLES

FIELD OF THE INVENTION

The present invention relates to a platform technology to produce virus-like particles (VLPs). More particularly, the present invention relates to expression cassettes, for producing high yields of immunogenic flavivirus virus-like-particles (VLPs) for use in vaccines, vaccines comprising the VLPs and methods of prophylaxis or treatment of virus infection.

BACKGROUND

Dengue virus (DENV) and Zika virus (ZIKV) are enveloped, single-stranded positive-sense RNA viruses in the family *Flaviviridae* that cause significant morbidity and mortality in humans. Both viruses are transmitted by mosquitoes and DENV is endemic in most of the world's tropical and sub-tropical regions, whereas ZIKV has been endemic in Asia and Africa historically but is currently expanding its geographical range. Dengue virus (DENV) is a major public health problem worldwide with 50-100 million new infections each year [Pang T, Cardosa MJ, Guzman MG, *Immunol Cell Biol* 85(1): 43-5 (2007)]. DENV is considered to be the most important arbo-viral (vector-borne) disease in the world and there are four closely related, yet antigenically distinct, serotypes, all of which can cause severe disease in humans. The majority of DENV-infected individuals present with a febrile illness that resolves after several days [Halstead SB, *Yale J Biol Med* 42(5): 350-62 (1970)]. Approximately 15 to 60% of patients suffer from classic dengue fever (DF) which is characterized by headaches, nausea, high fever and retro-orbital and bone pain. Leukopenia, thrombocytopenia and elevation in serum transaminases are common in symptomatic DF patients [Pang T, Cardosa MJ, Guzman MG, *Immunol Cell Biol* 85(1): 43-5 (2007)]. A small percentage of patients also develop dengue hemorrhagic fever (DHF), a severe disease that can lead to dengue shock syndrome (DSS) and death. Fever, thrombocytopenia, hemorrhagic manifestations and evidence of increased vascular permeability and plasma leakage are common in DHF and DSS patients and viral loads are generally 10 to 100 fold higher as compared to DF patients [Pang T, Cardosa MJ, Guzman MG, *Immunol Cell Biol* 85(1): 43-5 (2007)]. In total, there are more than 500,000 cases of DHF/DSS each year and depending on the country and clinician experience managing DHF/DSS, case fatality ranges but can be as high as 20% [Pang T, Cardosa MJ, Guzman MG, *Immunol Cell Biol* 85(1): 43-5 (2007)]. DENV is transmitted mainly by *Aedes* mosquito species and although infection by one serotype can induce long-lasting immunity, no long-term cross protection to other (heterotypic) DENV serotypes is conferred. In fact,

subsequent infection by a heterotypic serotype can result in immune-mediated enhancement of disease which significantly increases the risk of developing DHF [Pang T, Cardosa MJ, Guzman MG, *Immunol Cell Biol* 85(1): 43-5 (2007)]. Globalization and modern transportation have facilitated the spread of DENV and consequently all four DENV serotypes are now found in most endemic regions increasing the risk of sequential serotype infections and the potential for severe disease [Gubler DJ, *Ann N Y Acad Sci*. 951:13-24 (2001)]. The worldwide public health burden of DENV, DHF and DSS has led to classification of DENV as a category A priority pathogen by NIAID and currently, the only available treatment option for DENV is supportive care.

Zika virus (ZIKV) is another mosquito-borne flavivirus that has been shown to cause a febrile illness in humans that can resemble dengue fever with clinical symptoms including fever, headache, myalgia and rash. Since discovery of the virus in 1947 until recently, ZIKV appeared sporadically in Africa and Asia and a high seroprevalence of ZIKV antibodies in humans throughout Africa and Asia has been demonstrated [Haddow AD, et al., *PLoS Negl Trop Dis* 6(2): e1477 (2012)]. However, in 2007 the first large ZIKV epidemic occurred in Micronesia which was also the first time ZIKV was detected outside of Africa and Asia [Song, B.-H., et al., *J. Neuroimmunol*. (2017), http://dx.doi.org/10.1016/j.jneuroim.2017.03.001]. Phylogenetic analysis of the isolate as well as another recent ZIKV isolate from a pediatric case in Cambodia demonstrated both recent strains were closely related to the Asian lineage of ZIKV and suggested the geographical range of the Asian ZIKV lineage was expanding [Haddow AD, et al. *PLoS Negl Trop Dis* 6(2): e1477 (2012)]. In 2013 the largest outbreak of ZIKV ever described was reported in French Polynesia, and for the first time clinical symptoms included severe autoimmune and neurological complications, including Guillain-Barré Syndrome and fetal abnormalities in pregnant women [Song, B.-H., et al., *J. Neuroimmunol*. (2017), http://dx.doi.org/10.1016/j.jneuroim.2017.03.001]. The French Polynesian outbreak demonstrated for the first time the severe pathogenic potential of ZIKV. Further, phylogenetic analysis revealed the virus was most closely related to ZIKV isolates from Micronesia and Cambodia again demonstrating the expanding range of Asian lineage isolates with pathogenic potential [Song, B.-H., et al., *J. Neuroimmunol*. (2017), http://dx.doi.org/10.1016/j.jneuroim.2017.03.001]. In 2013 and 2014, ZIKV appeared in New Caledonia, the Cook Islands, and Easter Island [Song, B.-H., et al., *J. Neuroimmunol*. (2017), http://dx.doi.org/10.1016/j.jneuroim.2017.03.001] and an isolate from Easter Island was found to be most closely related to French Polynesian ZIKV [Song, B.-H., et al., *J. Neuroimmunol*. (2017), http://dx.doi.org/10.1016/j.jneuroim.2017.03.001]. In May of the following year, the first ZIKV outbreak was reported in Northeastern Brazil and again the

virus was determined to be most closely related to ZIKV from French Polynesia and the Cook islands [Song, B.-H., et al., *J. Neuroimmunol.* (2017), http://dx.doi.org/10.1016/j.jneuroim.2017.03.001]. Unfortunately, by October 2015, autochthonous transmission of ZIKV had been demonstrated in 14 states in Brazil and in Columbia. Over the next 6 months, ZIKV spread rapidly across South America and the Caribbean with significant severe disease, including Guillain-Barré Syndrome, acute myelitis and severe birth defects, including over 4700 suspected cases of microcephaly [Song, B.-H., et al., *J. Neuroimmunol.* (2017), http://dx.doi.org/10.1016/j.jneuroim.2017.03.001]. Further, infectious ZIKV has been isolated from breast milk and semen and sexual transmission has been documented in numerous incidences [Song, B.-H., et al., *J. Neuroimmunol.* (2017), http://dx.doi.org/10.1016/j.jneuroim.2017.03.001]. Most recently, ZIKV has been associated with a rare neurological disorder in adults called acute disseminated encephalomyelitis (ADEM) which is characterized by autoimmune-mediated damage of the myelin in the brain and spinal cord. In February 2016 the World Health Organization declared a global health emergency of international concern related to ZIKV, and currently, the only available treatment option for ZIKV is supportive care.

As ZIKV exploded across the Americas in 2016, another mosquito-borne flavivirus, Yellow fever virus (YFV), was causing an epidemic in Africa [Bagcchi S. *The Lancet Infectious Diseases*, Volume 17, Issue 3, 269-270, (2017)]. Like DENV and ZIKV, YFV is found in tropical regions of Africa and the Americas and it can produce devastating outbreaks of disease. Most YFV infections are asymptomatic; however, some patients develop an acute illness characterized by one or two phases of disease. In the first phase symptoms include muscular pain, headache, chills, anorexia, nausea and/or vomiting, often with bradycardia. Patients who progress to the second phase (~15%) present with resurgence of fever, development of jaundice, abdominal pain, vomiting and haemorrhagic manifestations and approximately 50% of these patients die 10–14 days after the onset of disease. Mass vaccination is used to prevent and control YFV in the event of an outbreak. In response to the African outbreak in 2016, a mass YFV vaccination campaign was initiated and by September 2016 the outbreak had been contained [Bagcchi S. *The Lancet Infectious Diseases*, Volume 17, Issue 3, 269-270, (2017)]. In total, 961 cases of YFV and 137 deaths occurred. Importantly, although the YFV vaccine was effective, the global emergency YFV vaccine stockpile reserved for epidemic response was not enough to complete the African YFV vaccination campaign. Additional doses of vaccine had to be sourced from other nations and diluted prior to use to stretch supplies [Joseph T Wu, et al., *The Lancet*, Volume 388, Issue 10062, 10–16, Pages 2904-2911, 2016]. Beyond exhausting the world's

emergency YFV vaccine stockpile, the epidemic revealed significant weaknesses in the emergency YFV vaccine supply pipeline. Just six months after the African YFV epidemic, a significant increase in YFV was reported in South America and to date, 234 cases and 80 deaths have been reported [Paules CI, Fauci AS. *N Engl J Med.* 2017 Mar 8. doi: 10.1056/NEJMp1702172 (Epub ahead of print)]. The current lack of a global YFV vaccine stockpile and the long time needed to produce additional YFV vaccine is concerning given the ongoing YFV outbreak in Brazil.

In addition to YFV, hepatitis C virus (HCV) can cause a YFV-like disease and differential diagnosis of YFV and HCV based on acute symptoms can be difficult in endemic regions [Makiala-Mandanda S, et al., *J Clin Microbiol*. 2017 Feb 15. pii: JCM.01847-16. doi: 10.1128/JCM.01847-16. (Epub ahead of print)]. Like YFV, HCV is a flavivirus and acute symptoms including fever, fatigue, decreased appetite, nausea, vomiting, abdominal pain, joint pain and jaundice. However, unlike YFV, HCV occurs worldwide and can cause chronic infection with a risk of cirrhosis of the liver within 20 years (15–30% of chronic patients). Globally, it is estimated that ~150 million people are chronically infected with HCV and that 25% of all liver cancer worldwide is caused by HCV [Thursz M, Fontanet A. *Nat Rev Gastroenterol Hepatol.* 2014; 11(1): 28-35. doi: 10.1038/nrgastro.2013.179. Epub 2013 Oct 1]. Significant progress has been made in developing effective HCV therapeutics [Hull MW, Yoshida EM, Montaner JS. *Curr Infect Dis Rep*. 2016; 18(7): 22. doi: 10.1007/s11908-016-0527-8]; however, limited access to treatment largely due to cost has hampered efforts to reduce HCV burden globally. Further, there are six genotypes of HCV that demonstrate different sensitivities to therapeutic drugs and drug resistance HCV variants have also been documented [Jacobson IM. *Gastroenterol Hepatol (N Y)*. 2016; 12(10 Suppl 4): 1-11]. The lack of robust and reliable cell culture systems and lack of HCV animal models have slowed HCV drug development and significantly hampered vaccine research. Coinfection of HCV and DENV has also been demonstrated which may contribute to an increased susceptibility to hepatic damage in coinfected patients, complicating the symptoms of either DENV or HCV infection [Machain-Williams, Biomed Res Int. 2014; 321286. Doi: 10.1155/2014/321286].

DENV vaccine design and development have proven to be difficult due to antigenic differences between serotypes and enhancement of disease upon reinfection by a different serotype. Numerous monovalent and multivalent DENV vaccine candidates are currently in development including live attenuated DENV derived by passage in cell culture, engineering mutations in the 3' untranslated region or creating chimeric YFV expressing DENV prM or E, purified inactivated virus, purified recombinant DENV prM and E proteins and DNA molecules encoding DENV precursor membrane protein (prM) and E [Whitehead SS, et al.,

Nature reviews Microbiology 5(7): 518-28 (2007)]. All of these vaccine candidates have been evaluated for immunogenicity in rhesus macaques in monovalent or multivalent combinations and each has been shown to elicit high levels of neutralizing antibody and prevent viremia in vaccinated and then challenged animals [Whitehead SS, et al., *Nature reviews Microbiology* 5(7): 518-28 (2007)]. Based on these data, many of the monovalent, divalent and tetravalent vaccine candidates have transitioned to phase I and II safety trials. Unfortunately, the only tetravalent DENV vaccine candidate being evaluated in large efficacy trials in humans has returned disappointing results as DENV-mediated disease was exacerbated in vaccinated children under the age of 9 and no efficacy was demonstrated against DENV-2, the most common serotype in the region where the clinical trial was conducted [Halstead SB, *Lancet* 380(9853): 1535-6 (2012)]. Regardless, three countries, have approved the vaccine for use even though the efficacy against DENV-2 was low; however, vaccine use is restricted to individuals 9 and older. Interestingly, low vaccine efficacy did not correlate with the levels of neutralizing antibody, demonstrating that better models and more effective DENV vaccine candidates are needed.

In view of the above vaccine deficiencies, it is desirable to provide methods and constructs for more efficient production of recombinant secreted antigenic flavivirus proteins.

SUMMARY OF INVENTION

Accordingly, we have developed a novel virus-like-particle (VLP) expression cassette that features bicistronic transcription, simultaneous translation and synchronized intracellular trafficking of the flavivirus structural proteins and a host protease. Our underlying hypothesis is that the novel flavivirus VLP expression cassettes can be used to generate highly native flavivirus VLPs in sufficient yields to enable vaccine development. Results obtained using our novel DENV and ZIKV VLP expression cassettes support these claims.

According to a first aspect of the invention, there is provided an expression cassette comprising;

i. a flavivirus structural gene, and

ii. a furin gene.

The presence of the furin gene advantageously produces furin protein within the same cell as the virus proteins, which provides enhanced processing of the recombinant flavivirus proteins which increases yield of secreted mature virus-like particles containing

neutralizing epitopes for vaccine use. Advantageously, co-expression of two genes may be achieved by inserting an element between the genes that allows bicistronic expression.

In a preferred embodiment the expression cassette comprises;

i. a flavivirus structural gene,

ii. a furin gene, and

iii. a bicistronic expression element positioned between the flavivirus structural gene and the furin gene.

A suitable bicistronic expression element is an internal ribosome entry site (IRES). It would be understood that the IRES in the cassette between the virus protein and furin protein may be replaced by other elements suitable for multicistronic expression. For example, the IRES could potentially be replaced with DNA encoding the 2A peptide from the foot-and-mouth disease virus (FDMV) (F2A) for bicistronic or polycistronic expression [Chan HY, et al., *PLoS ONE* 6.12 (2011)].

In another preferred embodiment the expression cassette comprises;

i. a flavivirus structural gene,

ii. a furin gene, and

iii. IRES positioned between the flavivirus structural gene and the furin gene.

In a preferred embodiment, the flavivirus structural gene comprises a partial capsid protein (delC), and either a complete membrane precursor (prM) protein coding sequence and a complete envelope (E) protein coding sequence or a complete membrane protein (p7) coding sequence and complete E protein coding sequences (E1 and E2).

The partial capsid protein (delC) is preferably one that functions to anchor the flavivirus structural protein in the cellular endoplasmic reticulum (ER) membrane

In another preferred embodiment, the furin gene comprises a furin signal peptide (fsp) and a furin proprotein coding sequence. Preferably the furin coding sequence is full length.

In another preferred embodiment, the expression cassette further comprises a partial capsid protein (delC) fused in frame to the 5' end of the furin signal peptide.

The partial capsid protein (delC) is preferably one that functions to anchor the flavivirus structural protein and/or the furin proprotein in the cellular ER membrane. More preferably, when the same delC protein is used for the flavivirus structural protein and the furin proprotein, it co-localizes both proteins to the same site in the ER membrane and provide a temporal balance that is optimal for flavivirus processing. Co-localisation has the advantage of increasing the efficiency of the furin protein processing of the flavivirus structural proteins and increases the yield of mature secreted structural proteins and VLPs.

In a preferred embodiment the delC protein spans the cellular ER membrane and does not contain the first 108 nucleotides of the capsid protein coding sequence. An example is the del108C ZIKA, DENV, YFV and HCV sequences used in the Examples.

In a preferred embodiment, expression cassettes comprising p7 are fused in frame to the 5' end of a partial nonstructural 2 protein (NS2del).

The partial NS2 (NS2del) is preferably one that contains a furin cleavage site that regulates cleavage of flavivirus structural proteins. Inclusion of delNS2 has the advantage of increasing the efficiency of the furin protein processing of the flavivirus structural proteins.

In a preferred embodiment the NS2del protein spans a putative furin cleavage site and contains the first 267 nucleotides of the NS2 coding sequence. An example is the NS2del384 HCV sequence used in the Examples.

In another preferred embodiment, the expression cassette further comprises a promoter to drive transcription of the expression cassette.

The promoter initiates the transcription and is therefore the point of control for the expression of the cloned genes in the expression cassette. The promoters used in expression vectors are normally inducible, meaning that protein synthesis is only initiated when required by the introduction of an inducer such as IPTG. Gene expression however may also be constitutive. Several types of promoters could be selected from to drive expression of the genes in the expression cassette. For example, constitutive promoters may include the simian virus 40 early promoter (SV40), cytomegalovirus immediate-early promoter (CMV), human Ubiquitin C promoter (UBC), human elongation factor 1α promoter (EF1A), mouse phosphoglycerate kinase 1 promoter (PGK), and chicken β-Actin promoter coupled with CMV early enhancer (CAGG), and copia transposon promoter (COPIA) and actin 5C promoter (ACT5C) for *Drosophila* systems). For expression in insect cells, the baculovirus *Autographa californica multiple nuclear polyhedrosis virus* (AcMNPV) Polyhedrin promoter may be used, as disclosed in the Examples herein. It would be understood by the

person skilled in the art that the choice of promoter may depend on the type of cell intended to express the viral proteins and host protease and the level of expression desired.

In a preferred embodiment, the expression cassette comprises a baculovirus *Autographa californica multiple nuclear polyhedrosis virus* (AcMNPV) Polyhedrin promoter to drive transcription of the expression cassette.

In another preferred embodiment, the expression cassette further comprises a polyA signal sequence positioned at the 3' end of the furin gene.

In a preferred embodiment, the polyA signal sequence is a SV40 late polyA signal sequence.

In another preferred embodiment, the furin gene is selected from the group comprising human, non-human mammal or insect furin gene. In a preferred embodiment, the furin gene is human.

An example of two expression cassettes according to the invention is shown in Figure 1.

It would be understood by a skilled person in the art that the expression cassette could be designed to express virus proteins in various types of cells in vitro. In another preferred embodiment, the flavivirus structural gene and the furin gene are codon-optimised for expression in insect cells.

In a preferred embodiment the expression cassette according to the invention has the structure shown in Figures 2A-E with positions of elements described in Figures 2F-J, respectively. More preferably, the cassette represented in Figure 2A has a nucleotide sequence comprising SEQ ID NO: 1; the cassette represented in Figure 2B has a nucleotide sequence comprising SEQ ID NO: 9; the cassette represented in Figure 2C has a nucleotide sequence comprising SEQ ID NO: 8; the cassette represented in Figure 2D has a nucleotide sequence comprising SEQ ID NO: 11 and the cassette represented in Figure 2E has a nucleotide sequence comprising SEQ ID NO: 12.

Another example of two expression cassettes according to the invention is shown in Figure 3. In preferred embodiments the expression cassette has the structure represented in Figure 4A, 4B, 4C or 4D with positions of elements described in Figures 4E-4H, respectively, having, respectively, a nucleotide sequence comprising SEQ ID Nos: 2, 10, 13 and 14.

In another preferred embodiment, the expression cassette further comprises a promoter, a flavivirus NS1 gene and a polyA signal sequence on the complementary DNA strand upstream of the flavivirus structural gene-IRES- furin proprotein sequences.

In a preferred embodiment, the promoter for the flavivirus NS1 gene is baculovirus AcMNPV p10 promoter.

In a preferred embodiment, the polyA signal sequence for the flavivirus NS1 gene is *Herpes Simplex virus* thymidine kinase (HSV tk) polyA signal sequence.

Preferred examples of an expression cassette according to the invention are shown in Figures 9 and 11. In a preferred embodiment, the expression cassette may have the structure shown in Figures 10A, 10B or 10C with positions of elements described in Figures 10D-10F, respectively. More preferably, the cassette of Figure 10A has a nucleotide sequence represented by SEQ ID NO: 3; the cassette of Figure 10B has a nucleotide sequence represented by SEQ ID NO: 6 and the cassette of Figure 10C has a nucleotide sequence represented by SEQ ID NO: 5.

Another example of an expression cassette according to the invention is shown in Figure 12A-B with positions of elements described in Figures 12C-D, respectively. More preferably, the cassette of Figure 12A has a nucleotide sequence represented by SEQ ID NO: 4 and the cassette of Figure 12B has a nucleotide sequence represented by SEQ ID NO: 7.

In another preferred embodiment, the flavivirus is selected from at least one of the group comprising Dengue virus, Zika virus, Yellow fever virus, West Nile virus and Japanese encephalitis virus and serotypes thereof.

In another preferred embodiment, the flavivirus is Hepatitis C virus.

In a preferred embodiment, the flavivirus is selected from at least one of the group comprising Dengue virus serotype 1, Dengue virus serotype 2, Dengue virus serotype 3, Dengue virus serotype 4 and Zika virus.

In a preferred embodiment, the Dengue virus serotype is DENV2.

In another preferred embodiment, the cassette is homologous or heterologous with respect to the partial capsid protein delC.

In a preferred embodiment, the cassette is heterologous and comprises a Dengue virus delC and/or a Zika virus delC.

Preferred embodiments of the expression cassettes of the invention are shown in the Figures and Sequence Listing.

According to another aspect of the invention there is provided the use of an expression cassette according to any aspect of the invention described herein for the recombinant production of secreted virus proteins.

According to a preferred embodiment there is provided the use of an expression cassette according to any aspect of the invention described herein for the recombinant production of virus-like particles (VLPs).

According to another aspect of the invention there is provided a method for the production of recombinant secreted flavivirus structural proteins and/or VLPs comprising the steps: cultivating a eukaryotic cell that has been transfected with a plasmid containing an expression cassette as defined according to any aspect of the invention, and recovering the recombinant secreted virus structural proteins and/or VLPs from the cell or the cultivation medium.

In a preferred embodiment, the eukaryotic cell is a mammalian cell. More preferably the mammalian cell is Chinese hamster ovary of human kidney.

In a preferred embodiment the method of production comprises the steps: cultivating a eukaryotic cell that has been infected with a recombinant baculovirus expressing the novel VLP expression cassette as defined herein, and recovering recombinant secreted flavivirus structural proteins and/or VLPs from the cell or the cultivation medium.

In a preferred embodiment, the eukaryotic cell is an insect cell. Preferably the insect cell is Sf9 from *Spodoptera frugiperda*.

It would be understood by the person skilled in the art that the choice of cell type and cell-specific promoter may depend on the type of cell intended to express the secreted viral proteins and host protease and the level of expression desired.

According to another aspect of the invention there is provided at least one isolated recombinant secreted flavivirus structural protein and/or VLP produced by the method of the invention herein defined.

According to another aspect of the invention there is provided a vaccine comprising at least one recombinant secreted flavivirus structural protein and/or VLP produced by the method of the invention herein defined.

In a preferred embodiment, the at least one recombinant secreted flavivirus structural protein and/or VLP comprises neutralizing epitopes from Dengue virus serotypes 1, 2, 3 and/or 4.

In a preferred embodiment, the at least one recombinant secreted flavivirus structural protein and/or VLP comprises Zika virus neutralizing epitopes.

In a preferred embodiment, at least one recombinant secreted flavivirus structural protein and/or VLP comprises Yellow fever virus neutralizing epitopes.

In a preferred embodiment, at least one recombinant secreted flavivirus structural protein and/or VLP comprises Hepatitis C virus neutralizing epitopes.

According to another aspect of the invention there is provided a method of treatment or prophylaxis comprising administering to a subject in need of such treatment or prophylaxis an efficacious amount of vaccine according to any aspect of the invention.

According to another aspect of the invention there is provided the use of at least one isolated recombinant secreted flavivirus structural protein and/or VLP as herein defined or a vaccine as herein defined for the manufacture of a medicament for the treatment or prophylaxis of a flavivirus infection. Preferably, the flavivirus infection is selected from at least one of the group comprising Dengue virus infection and Zika virus infection.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1D show general flavivirus structural gene bicistronic human furin proprotein expression cassettes. The flavivirus structural gene includes a partial flavivirus capsid protein coding sequence (delC) and the complete membrane precursor (prM) and envelope (E) protein coding sequences (Fig. 1A, Fig. 1C) or complete membrane (p7) and E (E1 and E2) protein coding sequences (Fig. 1B, Fig. 1D). For cassettes containing p7, a partial 5'-NS2 fragment has been fused in frame downstream (*). An Internal Ribosome Entry Site (IRES) sequence is located downstream of the flavivirus structural gene and upstream of a novel fusion protein sequence that encodes flavivirus delC fused in frame to the human furin signal peptide (hfsp) and the entire coding sequence of the human furin proprotein gene. Transcription of the bicistronic expression cassette is dependent on the baculovirus *Autographa californica multiple nuclear polyhedrosis virus* (AcMNPV) Polyhedrin promoter and mRNA is stabilized by the *Simian virus 40* (SV40) late polyA signal.

Figures 2A-2J show specific flavivirus structural gene bicistronic human furin proprotein expression cassettes. The flavivirus structural gene includes a partial dengue 2

(DENV2, Figs. 2A and 2F) or Zika virus (ZIKV, Figs. 2B-C and 2G-H), Yellow fever virus (YFV, Figs. 2D and 2I) or Hepatitis C virus (HCV, Figs. 2E and 2J) capsid protein coding sequence (del108CDENV2, del108CZIKV, del108CYFV or del108CHCV) and the complete DENV2, ZIKV, YFV membrane precursor (prM) and envelope (E) protein coding sequences or the complete HCV membrane (p7) and envelope coding sequences (HCV E1 and HCV E2). For all, del108C does not contain the first 108 nucleotides of the capsid protein coding sequence. An Internal Ribosome Entry Site (IRES) sequence is located downstream of the DENV2, ZIKV, YFV or HCV structural gene and upstream of a novel fusion protein sequence that encodes DENV2, ZIKV, YFV or HCV del108C fused in frame to the human furin signal peptide (hfsp) and the entire coding sequence of the human furin proprotein gene. Transcription of the bicistronic expression cassette is dependent on the baculovirus AcMNPV Polyhedrin promoter and mRNA is stabilized by the SV40 late polyA signal. The overall size of the expression cassette in nucleotides is indicated by the number after the SV40 late polyA signal box.

Figures 3A-3D show general flavivirus structural gene bicistronic human furin proprotein expression cassettes without del108C upstream of the human furin proprotein. The flavivirus structural gene includes a partial flavivirus capsid protein coding sequence (delC) and either the complete membrane precursor (prM) and envelope (E) protein coding sequences (Figs. 3A and 3C) or complete membrane (p7) and E (E1 and E2) protein coding sequences (Figs. 3B and 3D). For cassettes containing p7, a partial 5'-NS2 fragment has been fused in frame downstream (*). An Internal Ribosome Entry Site (IRES) sequence is located downstream of the flavivirus structural gene and upstream of the human furin signal peptide (hfsp) and the entire coding sequence of the human furin proprotein gene. Transcription of the bicistronic expression cassette is dependent on the baculovirus AcMNPV Polyhedrin promoter and mRNA is stabilized by the SV40 late polyA signal.

Figures 4A-4H show specific flavivirus structural gene bicistronic human furin proprotein expression cassette without del108C upstream of the human furin proprotein. The flavivirus structural gene includes a partial DENV2 (Figs. 4A and 4E), ZIKV (Figs. 4B and 4F), YFV (Figs. 4C and 4G) or HCV (Figs. 4D and 4H) capsid protein coding sequence (del108CDENV2, del108CZIKV, del108CYFV or del108CHCV) and the complete DENV2, ZIKV, YFV membrane precursor (prM) and envelope (E) protein coding sequences or the complete HCV membrane (p7) and envelope coding sequences (HCV E1 and HCV E2). For all, del108C does not contain the first 108 nucleotides of the capsid protein coding sequence. An Internal Ribosome Entry Site (IRES) sequence is located downstream of the DENV2, ZIKV, YFV or HCV structural gene and upstream of the human furin signal peptide

(hfsp) and the entire coding sequence of the human furin proprotein gene. Transcription of the bicistronic expression cassette is dependent on the baculovirus AcMNPV Polyhedrin promoter and mRNA is stabilized by the SV40 late polyA signal. The overall size of the expression cassette in nucleotides is indicated by the number after the SV40 late polyA signal box.

Figures 5A-5C show codon Optimization of DENV2 del108C-prM-E for expression in insect cells. The Codon Adaptation Index (CAI, Fig. 5A) and Frequency of Optimal Codons (FOP, Fig. 5B) are shown. In addition, GC Content Adjustment (Fig. 5C) is also shown.

Figures 6A-6C show codon Optimization of ZIKV del108C-prM-E for expression in insect cells. The Codon Adaptation Index (CAI, Fig. 6A) and Frequency of Optimal Codons (FOP, Fig. 6B) are shown. In addition, GC Content Adjustment (Fig. 6C) is also shown.

Figures 7A-7C show codon Optimization of HCV del108C-E1-E2-p7-delNS2 for expression in insect cells. The Codon Adaptation Index (CAI, Fig. 7A) and Frequency of Optimal Codons (FOP, Fig. 7B) are shown. In addition, GC Content Adjustment (Fig. 7C) is also shown.

Figures 8A-8C show codon Optimization of del108CDENV2-hfsp-human furin proprotein for expression in insect cells. The Codon Adaptation Index (CAI, Fig. 8A) and Frequency of Optimal Codons (FOP, Fig. 8B) are shown. In addition, GC Content Adjustment (Fig. 8C) is also shown.

Figure 9 shows general flavivirus structural gene bicistronic human furin proprotein dual promoter nonstructural protein 1 (NS1) expression cassette. The flavivirus structural gene includes a partial flavivirus capsid protein coding sequence (delC) and the complete membrane precursor (prM) and envelope (E) protein coding sequences. An Internal Ribosome Entry Site (IRES) sequence is located downstream of the flavivirus structural gene and upstream of a novel fusion protein sequence that encodes flavivirus delC fused in frame to the human furin signal peptide (hfsp) and the entire coding sequence of the human furin proprotein gene. Transcription of the bicistronic mRNA is dependent on the baculovirus AcMNPV Polyhedrin promoter and mRNA is stabilized by the SV40 late polyA signal. Transcription of the flavivirus nonstructural protein 1 (NS1) coding sequence is dependent on the baculovirus AcMNPV p10 promoter and both p10 and NS1 are encoded on the complement DNA strand upstream of the bicistronic flavivirus structural gene-IRES-human furin proprotein sequences. The NS1 mRNA is stabilized by the *Herpes Simplex virus* thymidine kinase (HSV tk) polyA signal.

Figures 10A-10F show specific flavivirus structural gene bicistronic human furin proprotein dual promoter nonstructural protein 1 (NS1) expression cassettes. The flavivirus structural gene includes a partial DENV2 (Figs. 10A and 10D) or ZIKV (Figs. 10B, 10C, 10E and 10F) capsid protein coding sequence (del108CDENV2 or del108CZIKV) and the complete DENV2 or ZIKV membrane precursor (prM) and envelope (E) protein coding sequences. For DENV2 and ZIKV, del108C does not contain the first 108 nucleotides of the capsid protein coding sequence. An Internal Ribosome Entry Site (IRES) sequence is located downstream of the DENV2 or ZIKV structural gene and upstream of a novel fusion protein sequence that encodes del108CDENV2 (Figs. 10A, 10C, 10D and 10F) or del108CZIKV (Figs. 10B and 10E) fused in frame to the human furin signal peptide (hfsp) and the entire coding sequence of the human furin proprotein gene. Transcription of the bicistronic mRNA is dependent on the baculovirus AcMNPV Polyhedrin promoter and mRNA is stabilized by the SV40 late polyA signal. Transcription of the DENV2 or ZIKV nonstructural protein 1 (NS1) coding sequence is dependent on the baculovirus AcMNPV p10 promoter and both p10 and NS1 are encoded on the complement DNA strand upstream of the bicistronic flavivirus structural gene-IRES-human furin proprotein sequences. The DENV2 or ZIKV NS1 mRNA is stabilized by the HSV tk polyA signal. The overall size of the dual promoter expression cassette in nucleotides is indicated by the number after the SV40 late polyA signal box.

Figure 11 shows a general flavivirus structural gene bicistronic human furin proprotein-dual promoter nonstructural protein 1 (NS1) expression cassette without del108C upstream of the human furin proprotein. The flavivirus structural gene includes a partial flavivirus capsid protein coding sequence (delC) and the complete membrane precursor (prM) and envelope (E) protein coding sequences. An Internal Ribosome Entry Site (IRES) sequence is located downstream of the flavivirus structural gene and upstream of the human furin signal peptide (hfsp) and the entire coding sequence of the human furin proprotein gene. Transcription of the bicistronic mRNA is dependent on the baculovirus AcMNPV Polyhedrin promoter and mRNA is stabilized by the SV40 late polyA signal. Transcription of the flavivirus nonstructural protein 1 (NS1) coding sequence is dependent on the baculovirus AcMNPV p10 promoter and both p10 and NS1 are encoded on the complement DNA strand upstream of the bicistronic flavivirus structural gene-IRES-human furin proprotein sequences. The NS1 mRNA is stabilized by the HSV tk polyA signal.

Figures 12A-12D show specific flavivirus structural gene bicistronic human furin proprotein dual promoter nonstructural protein 1 (NS1) expression cassettes without del108C upstream of the human furin proprotein. The flavivirus structural gene includes a

partial DENV2 (Figs. 12A and 12C) or ZIKV (Figs. 12B and 12D) capsid protein coding sequence (del108CDENV2 or del108CZIKV) and the complete DENV2 or ZIKV membrane precursor (prM) and envelope (E) protein coding sequences. For DENV2 and ZIKV, del108C does not contain the first 108 nucleotides of the capsid protein coding sequence. An Internal Ribosome Entry Site (IRES) sequence is located downstream of the DENV2 or ZIKV structural gene and upstream of the human furin signal peptide (hfsp) and the entire coding sequence of the human furin proprotein gene. Transcription of the bicistronic mRNA is dependent on the baculovirus AcMNPV Polyhedrin promoter and mRNA is stabilized by the SV40 late polyA signal. Transcription of the DENV2 or ZIKV nonstructural protein 1 (NS1) coding sequence is dependent on the baculovirus AcMNPV p10 promoter and both p10 and NS1 are encoded on the complement DNA strand upstream of the bicistronic flavivirus structural gene-IRES-human furin proprotein sequences. The DENV2 or ZIKV NS1 mRNA is stabilized by the HSV tk polyA signal. The overall size of the dual promoter expression cassette in nucleotides is indicated by the number after the SV40 late polyA signal box.

Figures 13A-13C show codon Optimization of DENV2 NS1 for expression in insect cells. The Codon Adaptation Index (CAI, Fig. 13A) and Frequency of Optimal Codons (FOP, Fig. 13B) are shown. In addition, GC Content Adjustment (Fig. 13C is also shown.

Figures 14A-14B show expression of secreted DENV2 E using a recombinant baculovirus containing the DENV2 structural gene and del108CDENV2-human furin proprotein bicistronic expression cassette. Insect Sf9 cells were infected with recombinant baculovirus containing the DENV2 structural gene and del108CDENV2-human furin proprotein bicistronic expression cassette. Insect cell culture supernatants and cell pellets were harvested as described in the methods and all materials were analyzed using non-reducing SDS-PAGE and Western blot. Nitrocellulose membranes were cut into strips for Western blotting and each strip was probed with either human sera (Fig. 14A) or DENV-specific mouse monoclonal antibodies (Fig. 14B) as indicated. Abbreviations: -= negative human serum; PNR=pooled negative DENV reference sera; PCS=pooled convalescent DENV sera; HPR=pooled high positive DENV reference sera; DN=DENV NS1-specific monoclonal antibody; DE=DENV envelope (E)-specific monoclonal antibody; FE=flavivirus envelope (E)-specific monoclonal antibody.

Figures 15A-15B show characterization of secreted DENV2 E expressed using a recombinant baculovirus containing the DENV2 structural gene and del108CDENV2-human furin proprotein bicistronic expression cassette. Insect Sf9 cells were infected with

recombinant baculovirus containing the DENV2 structural gene and del108CDENV2-human furin proprotein bicistronic expression cassette. Cell culture supernatants and cell pellets were harvested as described in the methods and, following ultrafiltration (membrane cutoff size of 750 kDa), permeate and retentate were collected. All materials were analyzed using SDS-PAGE under non-reducing (Fig. 15A) or reducing (Fig. 15B) conditions and Western blot. Western blots were probed with the flavivirus envelope (E)-specific monoclonal antibody FE1. Abbreviations: FGUS=negative baculovirus control; LYS=cell lysate; VS=viral supernatant; VS (1)=freeze-thawed viral supernatant; VS (1) SF=sterile filtered viral supernatant; VS (1) UF-P1= sterile filtered viral supernatant ultrafiltration permeate; VS (1) UF-R1=sterile filtered viral supernatant ultrafiltration retentate.

Figures 16A-16B show further characterization of secreted DENV2 E expressed using a recombinant baculovirus containing the DENV2 structural gene and del108CDENV2-human furin proprotein bicistronic expression cassette. Insect Sf9 cells were infected with recombinant baculovirus containing the DENV2 structural gene amd del108CDENV2-human furin proprotein bicistronic expression cassette. Cell culture supernatants and cell pellets were harvested as described in the methods. Following ultrafiltration (membrane cutoff size of 750 kDa), permeate and retentate were collected. All materials were analyzed using SDS-PAGE under non-reducing (Fig. 16A) or reducing (Fig. 16B) conditions and Western blot. Western blots were probed with pooled high positive DENV reference sera. Abbreviations: FGUS=negative baculovirus control; LYS=cell lysate; VS=viral supernatant; VS (1)=freeze-thawed viral supernatant; VS (1) SF=sterile filtered viral supernatant; VS (1) UF-P1= sterile filtered viral supernatant ultrafiltration permeate; VS (1) UF-R1=sterile filtered viral supernatant ultrafiltration retentate.

Figures 17A-17B show additional characterization of secreted DENV2 E expressed using a recombinant baculovirus containing the DENV2 structural gene and del108CDENV2-human furin proprotein bicistronic expression cassette. Insect Sf9 cells were infected with recombinant baculovirus containing the DENV2 structural gene and del108CDENV2-human furin proprotein expression cassette. Cell culture supernatants and cell pellets were harvested as described in the methods. Following ultrafiltration (membrane cutoff sizes of 100 or 300 kDa), permeate and retentate were collected. All materials were analyzed using SDS-PAGE under non-reducing (Fig. 17A) or reducing (Fig. 17B) conditions and Western blot. Western blots were probed with pooled high positive DENV reference sera. Abbreviations: FGUS=negative baculovirus control; LYS=cell lysate; VS=viral supernatant; VS (1)=freeze-thawed viral supernatant; VS (1) SF=sterile filtered viral supernatant; VS (1)

UF-P1= sterile filtered viral supernatant ultrafiltration permeate; VS (1) UF-R1=sterile filtered viral supernatant ultrafiltration retentate.

Figures 18A-18B show characterization of secreted ZIKV E expressed using a recombinant baculovirus containing the ZIKV structural gene and del108CDENV2-human furin proprotein bicistronic expression cassette. Insect Sf9 cells were infected with recombinant baculovirus containing the ZIKV structural gene and del108CDENV2-human furin proprotein bicistronic expression cassette. Cell culture supernatants and cell pellets were harvested as described in the methods.and all materials were analyzed using SDS-PAGE under non-reducing (Fig. 18A) or reducing (Fig. 18B) conditions and Western blot. Western blots were probed with the flavivirus envelope (E)-specific monoclonal antibody FE5. Abbreviations: FGUS=negative baculovirus control; LYS=cell lysate; VS=viral supernatant. Results from two recombinant baculoviruses derived from two independent clones (3 and 6) are shown.

Figures 19A-19B show further characterization of secreted ZIKV E expressed using a recombinant baculovirus containing the ZIKV structural gene and del108CDENV2-human furin proprotein bicistronic expression cassette. Insect Sf9 cells were infected with recombinant baculovirus containing the ZIKV structural gene and del108CDENV2-human furin proprotein bicistronic expression cassette. Cell culture supernatants and cell pellets were harvested as described in the methods, and all materials were analyzed using SDS-PAGE under non-reducing (Fig. 19A) or reducing (Fig. 19B) conditions and Western blot. Western blots were probed with pooled high positive DENV reference sera. Abbreviations: FGUS=negative baculovirus control; LYS=cell lysate; VS=viral supernatant. Results from two recombinant baculoviruses derived from two independent clones (3 and 6) are shown.

Figures 20A-20B show additional characterization of secreted DENV2 or ZIKV E expressed using a recombinant baculovirus containing the DENV2 or ZIKV structural gene bicistronic human furin proprotein expression cassette. Insect Sf9 cells were infected with recombinant baculovirus containing the DENV2 (A, left panels) or ZIKV (B, right panels) structural gene and del108C-DENV2-human furin proprotein expression cassette. Cell culture supernatants and cell pellets were harvested as described in the methods. Following ultrafiltration and diafiltration (membrane cutoff sizes of 300 or 100 kDa), retentate was collected and imaged using electron microscopy. Electron micrographs of two different grids showing DENV2 VLPs (A; left) or two different grids showing ZIKV VLPs (B; right). Magnification was 52000X scale bars are shown. Baculovirus particles (rod shaped ~45nm

in diameter and ~250 nm in length) as well as enveloped virus-like-particles similar in size to DENV (~40-50 nm, black arrows) were present in the material imaged.

Figures 21A-21B show additional characterization of secreted ZIKV E expressed using three different ZIKV structural gene and human furin proprotein bicistronic expression cassettes: bacIRA3 (ZIKV del108C-prM-E and del108CDENV2-human furin proprotein), bacIRA4 (ZIKV del108C-prM-E and human furin proprotein) and bacIRA5 (ZIKV del108C-prM-E and del108CZIKV-human furin proprotein). Insect Sf9 cells were infected with recombinant baculovirus bacIRA3, bacIRA4 or bacIRA5. Cell culture supernatants and cell pellets were harvested as described in the methods. Following ultrafiltration and diafiltration (membrane cutoff size of 300 kDa), retentate was collected. Cell lysates and retentates were analyzed using SDS-PAGE and Western blot. Western blots were probed with ZIKV patient serum (Fig. 21A) or mouse monoclonal antibody FE5 (Fig. 21B); for both blots 1: baculovirus control; 3: bacIRA3; 4: bacIRA4; 5: bacIRA5.

Figure 22A-22B shows binding of neutralizing monoclonal antibodies to secreted ZIKV or DENV2 E present in retentates generated using 3 different ZIKV VLP expression cassettes (Fig. 22A) or a single DENV2 VLP expression cassette (Fig. 22B). The specific VLP expression cassette used to make the retentate and the membrane cutoff size used for ultrafiltration and diafiltration are shown in the legend. ELISA plates were coated with human anti-IgG, blocked, and incubated with ZIKV or DENV patient serum. Retentate, infectious ZIKV, DENV2 or a nonspecific insect antigen (C636 and C6362) was added to appropriate wells. Subsequently, 8 cross-reactive mouse monoclonal antibodies (FE1-FE8; shown on the x-axis) were added followed by rabbit anti-mouse-HRP. Plates were developed using TMB using standard protocols and the optical density (O.D.) at 450 nm was measured (y-axis). Of the mouse monoclonal antibodies used, all neutralize both ZIKV and DENV2 except FE2, which does not neutralize ZIKV or DENV2. All 7 ZIKV-neutralizing antibodies bound ZIKV and DENV2 secreted E except FE4, which did not bind well to ZIKV or DENV2 secreted E captured from retentates.

Figures 23A-23B show the results of sucrose gradient sedimentation experiments. Retentates (described in Figure 22) were centrifuged at 21,000xg. Supernatants were removed and pellets were resuspended in NTE buffer and overlaid onto discontinuous sucrose gradients (15-60%). Gradients were centrifuged at 17,000 rpm for 18 hours and 2 ml fractions were collected from each gradient. Fractions were analyzed using SDS-PAGE and Western blot and blots were probed with ZIKV or DENV patient serum. Sucrose gradient fractions from bacIRA5 retentate (500 kDA) and bacIRA1 retentate (500 kDA) are shown as

representative examples (Fig. 23A). For each gradient, fractions containing ZIKV or DENV2 VLPs were pooled, diluted, buffer exchanged and concentrated. For each, retentate starting material, diluted fractions containing ZIKV or DENV2 VLPs, concentrator flow through and purified ZIKV or DENV2 VLPS were analyzed using SDS-PAGE and Western blot and blots were probed with ZIKV or DENV patient serum (Fig. 23B). The specific ZIKV or DENV2 VLP expression cassette used to make the retentate and the membrane used for ultrafiltration and diafiltration are shown above the blots. Samples were loaded in the following order: retentate starting material, diluted fractions containing ZIKV or DENV2 VLPs, concentrator flow through and purified ZIKV or DENV2 VLPs. Purified ZIKV or DENV2 VLPs are further indicated by the arrows.

Figure 24 shows binding of neutralizing monoclonal antibodies to purified ZIKV or DENV2 VLPs. Plates were coated with human anti-IgG, blocked and incubated with ZIKV or DENV patient serum (1:1000). Purified ZIKV VLPs, infectious ZIKV or nonspecific insect cell antigen (C636), purified DENV2 VLPs, DENV2 or nonspecific insect cell antigen (C6362) was added to appropriate wells. Subsequently, 8 cross-reactive mouse monoclonal antibodies (FE1-FE8; shown on the x-axis) were added followed by rabbit anti-mouse-HRP (1:8000). Plates were developed using TMB using standard protocols and the optical density (O.D.) at 450 nm was measured (y-axis). Of the mouse monoclonal antibodies used, all neutralize both ZIKV and DENV2 except FE2, which does not neutralize ZIKV or DENV2. All 7 ZIKV-neutralizing antibodies bound ZIKV and DENV2 secreted E except FE4, which did not bind well to purified ZIKV VLPs.

Figure 25 shows total ZIKV-specific immunoglobulins (Ig) in mice immunized with purified ZIKV VLPs. Plates were coated with human anti-IgG. After blocking, ZIKV patient serum (1:1000) was added followed by infectious ZIKV or nonspecific insect cell antigen (C636). Subsequently, serum samples from ZIKV VLP-immunized mice were added to followed by rabbit anti-mouse-HRP (1:8000). Plates were developed using TMB using standard protocols and the optical density (O.D.) at 450 nm was measured (y-axis). Serum dilution is shown on the x-axis and each line represents one animal. Group number and ZIKV VLP dose are shown above the graphs and sample day is shown to the left of the graphs. Serum samples from day 0 for each group are shown in gray on day 21 and day 42 graphs.

Figure 26 shows survival of mice immunized with purified ZIKV VLPs and then challenged with a lethal dose of ZIKV. As detailed in the methods, all ZIKV VLP-immunized mice with challenged with a lethal dose of ZIKV. Animals were monitored for 5 weeks post-

challenge and survival percentage on relevant days is shown for each group. All animals in the adjuvant alone group did not survive ZIKV challenge; whereas 50% of animals in Group 1 (1 µg) and 80% of animals in Group 2 (2.5 µg) survived ZIKV challenge.

Figure 27 shows nucleotide sequence of DENV2 del108C-prM-E & del108CDENV2-human furin proprotein bicistronic cassette shown in Figure 2A; SEQ ID NO: 1.

Figure 28 shows nucleotide sequence of DENV2 del108C-prM-E & human furin proprotein bicistronic cassette shown in Figure 4A; SEQ ID NO: 2.

Figure 29 shows nucleotide sequence of Dual Promoter-DENV2 del108C-prM-E & del108CDENV2-human furin proprotein bicistronic cassette + DENV2 NS1 shown in Figure 10A; SEQ ID NO: 3.

Figure 30 shows nucleotide sequence of Dual Promoter-DENV2 del108C-prM-E & human furin proprotein bicistronic cassette + DENV2 NS1 shown in Figure 11A; SEQ ID NO: 4.

Figure 31 shows nucleotide sequence of Dual Promoter-Heterologous ZIKV del108C-prM-E & del108CDENV2-human furin proprotein bicistronic cassette + ZIKV NS1 shown in Figure 10C; SEQ ID NO: 5.

Figure 32 shows nucleotide sequence of Dual Promoter-Homologous ZIKV del108C-prM-E & del108CZIKV-human furin proprotein bicistronic cassette + ZIKV NS1 shown in Figure 10B; SEQ ID NO: 6.

Figure 33 shows nucleotide sequence of Dual Promoter-ZIKV del108C-prM-E & human furin proprotein bicistronic cassette + ZIKV NS1 shown in Figure 11B; SEQ ID NO: 7.

Figure 34 shows nucleotide sequence of Heterologous ZIKV del108C-prM-E & del108CDENV2-human furin proprotein bicistronic cassette shown in Figure 2C; SEQ ID NO: 8.

Figure 35 shows nucleotide sequence of Homologous ZIKV del108C-prM-E & del108CZIKV-human furin proprotein bicistronic cassette shown in Figure 2B; SEQ ID NO: 9.

Figure 36 shows nucleotide sequence of ZIKV del108C-prM-E & human furin proprotein bicistronic cassette shown in Figure 4B; SEQ ID NO: 10.

Figure 37 shows nucleotide sequence of YFV del108C-prM-E & del108CYFV-human furin proprotein bicistronic cassette shown in Figure 2D; SEQ ID NO: 11.

Figure 38 shows nucleotide sequence of HCV del108C-E1-E2 & del108CHCV-human furin proprotein bicistronic cassette shown in Figure 2E; SEQ ID NO: 12.

Figure 39 shows nucleotide sequence of YFV del108C-prM-E & human furin proprotein bicistronic cassette shown in Figure 4C; SEQ ID NO: 13.

Figure 40 shows nucleotide sequence of HCV del108C-E1-E2-p7 & human furin proprotein bicistronic cassette shown in Figure 4D; SEQ ID NO: 14.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Vaccine candidates based on DENV VLPs have not been attempted because of insufficient VLP yields due to improper processing of DENV E and/or insufficient secretion of E. In general, flavivirus VLPs have proven to be difficult to make for similar reasons, although some, like Japanese encephalitis virus (JEV), work better than others [Kuwahara M and Konishi E, *Clinical and vaccine immunology*: CVI 17(10): 1560-6 (2010)]. For these reasons, the VLP platform was mostly abandoned for DENV vaccines and other flaviviruses; however, over the years different groups have tried to optimize DENV VLP expression cassettes to increase yields. Most recently, a new DENV VLP cassette was constructed that included a defensin A signal sequence, DENV prM and DENV E containing a JEV E transmembrane domain. Unfortunately, when analyzed in insect cells, yields were either still low or an E fusion loop mutation was needed to stabilize VLP production limiting vaccine potential [Charoensria N, et al., *Journal of virological methods* 205C: 116-23 (2014)]. Flaviviruses require furin to cleave prM which occurs in the low pH environment of the Golgi in both insect cells and mammalian cells [Lindenbach BD, et al., *Flaviviridae*: The viruses and their replication. In: Knipe DM, Howley PM, editors. *Fields Virology*. 5 ed. Philadelphia: Lippincott Williams & Wilkins; p. 1101-52 (2007); Gubler DJ. Flaviviruses. In: Knipe DM, Howley PM, editors. *Fields Virology*, Fifth Edition. Philadelphia, PA: Lippincott Williams & Wilkins; p. 1153-252 (2007)]. More specifically, low pH induces conformational changes in prM-E that expose the furin cleavage site within prM and cleavage is one of the final steps of virion maturation prior to budding [Pierson TC and Diamond MS, *Current opinion in virology* 2(2): 168-75 (2012)]. The exact factors that control the extent of virion maturation remain poorly understood and unfortunately for DENV VLPs produced using over expression systems, VLP maturation and budding are highly inefficient. Although slightly different than DENV, ZIKV and YFV, HCV E1-E2-p7 also require cleavage by host proteases and

numerous furin sites are present in the HCV structural gene. Although mature HCV virions are resistant to pH, the sensitivity of immature HCV particles to pH remains largely unknown as do the exact factors that control virion maturation [Falcón V., et al., *Virus Genes*, 53(2):151-164 (2017). Historically, like DENV, for HCV VLPs produced using over expression systems, VLP maturation and budding is highly inefficient [Baumert TF, et al., *J Virol*. 1998; 72(5): 3827-36].

We hypothesized that if we could somehow synchronize DENV prM-E or HCV E1-E2-p7 and furin expression and assure furin would always accompany DENV prM-E of HCV E1-E2-p7 as it traffics through the endoplasmic reticulum (ER) and Golgi, cleavage and maturation of DENV prM-E or HCV E1-E2-p7 and secretion of DENV E or HCV E1-E2 could be significantly improved. Further, we believed that maintaining DENV prM-E or HCV E1-E2-p7 and furin in close proximity would not just improve but would also maximize cleavage.

Definitions

Certain terms employed in the specification, examples and appended claims are collected here for convenience.

The term "subject" is herein defined as vertebrate, particularly mammal, more particularly human. For purposes of research, the subject may particularly be at least one animal model, e.g., a mouse, rat and the like. In particular, for treatment or prophylaxis of flavivirus infection and/or flavivirus-linked diseases, the subject may be a human.

The term "treatment", as used in the context of the invention refers to ameliorating, therapeutic or curative treatment.

The term "comprising" is herein defined to be that where the various components, ingredients, or steps, can be conjointly employed in practicing the present invention. Accordingly, the term "comprising" encompasses the more restrictive terms "consisting essentially of" and "consisting of."

A person skilled in the art will appreciate that the present invention may be practiced without undue experimentation according to the methods given herein. The methods, techniques and chemicals are as described in the references given or from protocols in standard biotechnology and molecular biology text books.

EXAMPLES

Materials and Methods

Tissue culture

Sf9 cells were purchased from Gibco (Grand Island, NY)) and maintained in Sf-900 II SFM (1X) supplemented with Gentamicin (Gibco) in a 28°C incubator.

SDS-PAGE and immunoblotting

Cell lysates, clarified supernatants, permeates and partially purified DENV2 and ZIKV VLP retentates were analyzed using SDS-PAGE and Western blot. Samples were separated (10-12% acrylamide gels), transferred to nitrocellulose membranes and probed with DENV and ZIKV E-specific mouse monoclonal antibodies or polyclonal human DENV or ZIKV patient serum. Bound mouse or human IgG was detected using horseradish peroxidase (HRP) conjugated anti-mouse or anti-human antibodies and TMB Membrane peroxidase substrates (KPL, Gaithersburg, Maryland, USA).

Electron microscopy

Partially purified DENV2 VLPS or clarified culture supernatants containing ZIKV VLPs were sent to Nanoimaging Services (San Diego, CA) for cryo-transmission electron microscopy (TEM). The DENV sample was imaged undiluted whereas the clarified supernatant containing ZIKV VLPs was pelleted using 35% sucrose and ultracentrifugation prior to imaging. The samples were preserved in vitrified ice supported by holey carbon films on 400-mesh copper grids. The samples were prepared by applying a 3µl drop of sample suspension to a cleaned grid, blotting away with filter paper, and immediately proceeding with vitrification in liquid ethane. Grids were stored under liquid nitrogen until transferred to the electron microscope for imaging. Electron microscopy was performed using an FEI Tecnai T12 electron microscope (serial number D1100), operating at 120keV equipped with an FEI Eagle 4k x 4k CCD camera. Vitreous ice grids were transferred into the electron microscope using a cryostage that maintains the grids at a temperature below -170 °C. Images of each grid were acquired at multiple scales to assess the overall distribution of the specimen.

Flavivirus Virus-like Particle (VLP) Expression Cassette Design:

Generation of novel bicistronic DENV and ZIKV VLP expression cassettes

We opted to examine DENV VLP feasibility using DENV2 due to low DENV2 vaccine performance in recent clinical trials [Halstead SB. *Lancet* 380(9853): 1535-6 (2012)]. The DENV2 polyprotein sequence (C-prM-E) used to make the bicistronic VLP expression

cassette was a consensus sequence derived from patient isolate sequences and is most closely related to a 1996 isolate (GenBank: KF744405.1). For ZIKV, the French Polynesian polyprotein sequence was used to construct the bicistronic VLP expression cassette and C-prM-E is identical in amino acid sequence to ZIKV recently isolated in Brazil [Song, B.-H., et al., *J. Neuroimmunol*. (2017), http://dx.doi.org/10.1016/j.jneuroim.2017.03.001]. For YFV, the Asibi polyprotein sequence was used to construct the bicistronic VLP expression cassette (GenBank: KF769016.1). For HCV, HC-TN was used to construct the bicistronic VLP expression cassette containing capsid-E1-E2-p7 (GenBank: EF621489).

General schematics of novel bicistronic flavivirus VLP expression cassettes are shown in Figures 1 and 3. Specific examples of the novel bicistronic DENV2, ZIKV, YFV and HVC VLP expression cassette are shown in Figures 2 and 4. Source materials for the DENV VLP cassette (DENV2 del108C-prM-E ORF-IRES and IRES-DENV2 del108C ORF-human furin signal peptide-human furin proprotein ORF) were synthesized by GenScript (Piscataway, NJ, USA) using DENV 2 structural protein coding sequences (capsid=C; membrane precursor=prM; envelope glycoprotein=E), an Encephalomyocarditis virus (EMCV) internal ribosome binding site (IRES) sequence and human furin signal peptide (hfsp) and human furin proprotein coding sequences. The DENV2 del108C ORF-hfsp-human furin proprotein ORF genetic fusion created during synthesis represents a highly novel feature of the bicistronic VLP cassette. The ORFs were codon optimized (Figures 5 and 7) for expression in insect cells prior to synthesis and each IRES contained an overlapping unique *BglI* restriction site to facilitate cloning. The DENV2 del108C-prM-E ORF-IRES synthesized DNA had a unique *BamHI* site upstream of the DENV2 del108C-prM-E ORF. The IRES-DENV2 del108C ORF-hfsp-human furin proprotein ORF synthesized DNA had a unique *EcoR1* site downstream of the human furin proprotein ORF. Each piece of synthesized DNA was cloned sequentially into a Gateway® entry vector (Invitrogen, Carlsbad, CA, USA) using *BamHI/BglI* and then *BglI/EcorRI* giving rise to the novel bicistronic DENV2 VLP expression cassette (DENV2 del108C-prM-E ORF-IRES-DENV2 del108C ORF-hfsp-human furin proprotein ORF). Expression clones containing the novel bicistronic DENV2 VLP expression cassette inserted downstream of the polyhedrin promoter were made from the entry clone and the destination vector pDEST8 according to the instructions in the Invitrogen Gateway Technology® manual (pIRA1). An example of the novel bicistronic DENV2 VLP expression cassette is shown in Figure 2A. The novel bicistronic ZIKV VLP expression cassettes were made similarly. The ZIKV del108C-prM-E ORF-IRES sequence was codon optimized for expression in insect cells (Figure 6) and synthesized by GenScript using ZIKV structural protein coding sequences (capsid=C; membrane precursor=prM; envelope glycoprotein=E) and the EMCV IRES sequence.

Synthesized DNA had a unique *BamHI* site upstream of the ZIKV del108C-prM-E ORF and an overlapping unique *KpnI* site within the IRES. The synthesized ZIKV del108C-prM-E ORF-IRES DNA was cloned into the expression clone containing the novel bicistronic DENV2 VLP expression cassette, replacing DENV2 del108C-prM-E ORF-IRES with ZIKV del108C-prM-E ORF-IRES; giving rise to pIRA3. An example of the first ZIKV VLP expression cassette is shown in Figure 2C. An IRES- ZIKV del108C-hfsp ORF genetic fusion was also synthesized by GenScript which contained an overlapping unique *KpnI* site within the IRES and an overlapping unique *NheI* site within hfsp. This fragment was cloned into the first bicistronic ZIKV VLP expression cassette using *KpnI* and *NheI*, replacing DENV2 del108C-hfsp with a ZIKV del108C-hfsp. The ZIKV del108C-hfsp-human furin proprotein ORF genetic fusion represents a highly novel feature of the second novel bicistronic ZIKV VLP expression cassette (pIRA5). An example of the second ZIKV VLP expression cassette is shown in Figure 2B. Similarly, an IRES- hfsp ORF genetic fusion was also synthesized by GenScript which contained an overlapping unique *KpnI* site within the IRES and an overlapping unique *NheI* site within hfsp. This fragment has been cloned into the bicistronic DENV2 or ZIKV VLP expression cassette using *KpnI* and *NheI*, replacing DENV2 or ZIKV del108C-hfsp with simply hfsp (pIRA2 and pIRA4, respectively). General and specific examples are shown in Figures 3 and 4.

Recombinant bacmids containing the novel bicistronic DENV2 and ZIKV VLP expression cassettes bacIRA1, bacIRA3, bacIRA4 and bacIRA5 were made from individual expression clones according to the instructions in the Invitrogen BAC-TO-BAC® manual.

Production and purification of DENV and ZIKV VLP

One µg of each recombinant bacmid (bacIRA1, bacIRA3, bacIRA4 or bacIRA5) was transfected into a T25 flask containing $2x10^6$ Sf9 cells using Effectene® Transfection Reagent kit (Qiagen) as per manufacturer's instructions to generate recombinant baculovirus stocks (passage 1). The passage 1 stocks were amplified to generate master virus stocks (passage 2) and working virus stocks (passage 3) with a titre of $>10^9$ plaque forming units (pfu)/ml. Virus titre for recombinant baculovirus was determined by plaque assay on Sf9 cells. Briefly, $8x10^5$ Sf9 cells were seeded into each well of a 6-well plate and incubated overnight at 28°C. Medium was removed from the well and 1 ml of virus inoculum in 10-fold serial dilution was added to the wells. After 1 h incubation at 28°C, 2 ml per well of overlay medium (Sf-900 II SFM (1X) supplemented with 1% HI-FBS, Gentamicin, and 1% SeaPlaque agarose (Lonza)) was added to the wells. After 6-10 days incubation at 28°C, wells were stained with neutral red and plaque numbers were manually counted after 1 day incubation at 28°C.

DENV2 and ZIKV recombinant secreted flavivirus structural proteins and/or VLPs were produced by infecting Sf9 cell suspension with recombinant baculoviruses (passage 3) at a multiplicity of infection (MOI) of 0.1-3. Cell pellets and virus supernatant were harvested on day 3 or 4, cell pellets were lysed and supernatants were clarified by centrifugation at 3,200xg for 10 min. Clarified supernatant was concentrated approximately 10-fold using 750, 500, 300 or 100 KDa MWCO Hollow Fiber Ultrafiltration Cartridge (GE Healthcare Life Sciences) and the retentate was subjected to further diafiltration.

Retentates containing DENV or ZIKV VLPs were centrifuged at 21,000xg for 3 hours. Supernatant was collected and stored at -86°C. Pellets containing VLPs were resuspended in NTE buffer (10mM Tris, 100 mM NaCl, 1 mM EDTA; pH=8.0) overnight. Pellets were overlaid on top of 15-60% discontinuous sucrose gradients and gradients were centrifuged at 17,000 rpm for 18 hours at 4C. Following centrifugation, 2 ml fractions were collected from the top of the gradient and analyzed by Western blot. Fractions containing DENV or ZIKV VLPs were pooled, diluted 1:10 with NTE (<10% sucrose final) and further buffer exchanged/concentrated using 100 kDA Centricon spin columns and centrifugation. Concentrated purified DENV or ZIKV VLPs were quantitated using BCA kits (Thermofisher) following the manufacturer's instructions.

DENV or ZIKV *in vitro* VLP potency ELISA

Potent vaccine candidates are known to contain antigenic epitopes that can elicit neutralizing antibodies in the host. In an effort to estimate the potency of the DENV and ZIKV VLP vaccine candidates *in vitro*, an ELISA was established to measure the presence of neutralizing epitopes within DENV and ZIKV VLPs. The indirect potency ELISA was based on standard methods [Hickey AC, et al., *Am J Trop Med Hyg*. 89(6): 1043-57 (2013)] with slight modification. Specifically, anti-human IgG was used to capture DENV or ZIKV-specific antibody from DENV or ZIKV patient sera. Subsequently, retentates containing DENV or ZIKV VLPs or purified VLPs, infectious ZIKV, inactivated DENV2 or an irrelevant insect cell antigen (C636) were added and plates were incubated overnight at 4°C. After washing, 1 non-neutralizing and 7 different DENV- or ZIKV-neutralizing monoclonal mouse antibodies were added individually and plates were incubated for 1 hr at room temperature. Following 3 washes, rabbit anti-mouse-HRP was added and plates were incubated for 1 hr at room temperature. Plates were washed and developed with TMB following standard protocols. Optical density was measured at 450 nm.

DENV and ZIKV VLP immunogenicity assessment

The hallmark of any vaccine candidate, including VLPs, is the ability to elicit neutralizing antibodies *in vivo*. Further, the use of adjuvant can greatly increase immune responses in vivo. To determine if DENV and ZIKV VLPs are immunogenic *in vivo*, purified VLPs were used to immunize mice. Antigen was prepared by infecting Sf9 cells with recombinant baculovirus expressing DENV2 or ZIKV-VLPs and harvesting culture supernatant on day 4 or 5. Supernatant was clarified by centrifugation and concentrated approximately 10-fold using 500, 300 or 100 kDa MWCO Hollow Fiber Ultrafiltration Cartridges (GE Healthcare Life Sciences) and retentates were subjected to further diafiltration. Following diafiltration, retentates were centrifuged at 21,000xg and pellets containing VLPs were resuspended and overlaid onto discontinuous sucrose gradients (15-60%). Following centrifugation, fractions containing VLPs were pooled, diluted, buffer exchanged and concentrated and VLPs were quantitated using BCA kits and assessed *in vitro* using the VLP potency ELISA.

Two groups of C57BL/6 mice were immunized with 1 or 2.5 µg ZIKV VLPs, respectively, and VLPs were premixed with adjuvant prior to immunizations (0.1% Alhydrogel (aluminum hydroxide); Brenntag Biosector, Denmark). An additional group of control mice received adjuvant alone. Each group of mice contained 10 animals and mice were immunized intramuscularly with 100 ul VLP containing adjuvant. Animals were boosted with an identical dose of VLPs containing adjuvant 3 weeks later (100 µl/animal administered intramuscularly). Blood was collected on day 0, day 21 and day 42 for analysis of antibody responses in mice.

The strength and specificity of the antibody response in vaccinated animals was evaluated using indirect ELISA according to standard methods with slight modification [Hickey AC, et al., *Am J Trop Med Hyg*. 89(6): 1043-57 (2013)]. Plates were coated with anti-human IgG and ZIKV patient sera was used to capture infectious ZIKV. Mouse sera were diluted 1:100 to 1:12800 for the assay.

Plaque assay and plaque reduction neutralization tests (PRNT) were done as previously described [Hickey AC, et al., *Am J Trop Med Hyg*. 89(6): 1043-57 (2013)]. For PRNT, the level of serum neutralizing antibody against ZIKV was determined for individual animals in each group and an average was also determined for each group.

ZIKV VLP efficacy assessment

Recently, a new highly lethal mouse model of ZIKV neuropathogenesis has been described [Smith DR, et al., *PLoS Negl Trop Dis* 11(1): e0005296. doi:10.1371/journal.pntd.0005296 (2017)] and this new mouse model was used to evaluate the efficacy of ZIKV-VLP vaccine candidates. All mice immunized with two doses of 1 or 2.5 µg ZIKV VLP-adjuvant or adjuvant alone, as described above, were inoculated with a lethal dose of ZIKV three weeks after boost. Animals were monitored for disease following challenge and percent weight change and survival curves were generated for each group of immunized mice.

Brief description of expression cassettes:

Single Promoter Bicistronic Expression Cassette

a. Bicistronic containing flavivirus structural genes and human furin proprotein. Both open reading frames (ORF) are in close proximity and transcribed simultaneously (Figs. 1A-D, 2A-J, 3A-D, 4A-H)

b. polyA signal sequence for RNA stabilization

c. Internal Ribosome Entry Site (IRES) for enhanced translation of the second ORF (human furin proprotein); (Figs. 1A-D, 2A-J, 3A-D, 4A-H).

d. Partial flavivirus capsid sequence fused in-frame upstream of flavivirus prM-Envelope (E) or E1-E2-p7 and human furin proprotein to serve as a signal sequence for protein trafficking and ER membrane anchor. The identical signal sequence will target both translated proteins to the same location, again keeping the viral structural proteins in close proximity to the protease, human furin (Figures 1 and 2).

    I. These constructs also contain the natural human furin proprotein signal peptide (hfsp) upstream of the human furin proprotein sequence for in case it is important for cleavage of the human furin proprotein to the active form of the furin protein.

    II. Some constructs have been designed with only the hfsp for simplicity (Figures 3 and 4).

e. Flavivirus structural genes and the human furin proprotein sequence were codon optimized for insect cell expression (Figures 5A-C, 6A-C, 7A-C, 8A-C). As optimization was extensive, protein translation should be significantly increased.

Dual promoter

a. Contains the bicistronic cassette described above as well as a second promoter upstream of a flavivirus nonstructural protein 1 (NS1) sequence (Figures 9-12).

b. A second polyA signal sequence downstream of the NS-1 sequence for RNA stabilization

c. The flavivirus NS-1 gene was codon optimized for insect cell expression (Figs. 13A-C). As optimization was extensive, protein translation should be significantly increased.

RESULTS

A recombinant baculovirus containing the DENV2 Single Promoter Bicistronic VLP Expression Cassette (Figure 2) was made and rescued. The recombinant virus was used to infect insect cells and the culture supernatants or cell lysates were collected at various time points post-infection and analyzed using SDS-PAGE and Western blot. Blots from non-reduced samples were probed with human seronegative or seropositive DENV reference serum or with DENV-specific monoclonal antibodies (mAbs).

As demonstrated in Figure 14A, pooled convalescent serum from DENV patients (PCS) and pooled high positive human DENV reference serum (HPR) both recognized a protein identical in size to DENV envelope (E). Moreover, a doublet was apparent which is consistent with non-reduced DENV2 E analyzed from infectious virus and suggests that DENV2 delC-prM-E was processed properly.

Of the 4 DENV-mAbs assayed, only the two specific for DENV E (DE8 and FE1) gave signal, confirming the presence of DENV E doublet (Figure 14B).

Importantly, Figure 14 also demonstrated that significant amounts of the DENV2 E were present in the culture supernatant, demonstrating the presence of secreted properly processed DENV2 E.

In an effort to determine if a virus-like particle was secreted into the culture supernatant, a large 1 liter culture of insect cells was infected with the recombinant baculovirus containing the DENV2 Single Promoter Bicistronic VLP Expression Cassette. The cell lysate and culture supernatant were harvested and the supernatant was processed using a 750 kDa ultrafiltration step. All materials were then analyzed by SDS-PAGE and

Western as described above; however, both reduced and non-reduced samples were analyzed but only human HPR and mAb FE1 were used to probe blots.

As demonstrated in Figure 15, mAb FE1 detected the DENV2 E doublet in non-reduced samples in the lysate, supernatant and ultrafiltration retentate; however amounts appeared quite low in the retentate (A). DENV2 E was only faintly detected in reduced supernatant samples (B); however, significant DENV2 E was present in the ultrafiltration retentate.

As demonstrated in Figure 16, DENV HPR detected the DENV2 E doublet in non-reduced samples in the lysate, supernatant and ultrafiltration retentate; and although amounts of secreted E appear to be low in the retentate, a high molecular weight secreted DENV2 E species is present at the very top of the blot (A). DENV2 E was only faintly detected in reduced supernatant samples (B); however, significantly, a high molecular weight secreted DENV2 E species was present in the ultrafiltration retentate.

Together the data in Figures 15 and 16 suggest a large molecule weight species of secreted E is generated by the DENV2 Single Promoter Bicistronic VLP Expression Cassette.

The experiment was repeated and the cell lysate and culture supernatant were harvested and the supernatant was processed using either a 100 or 300 kDa ultrafiltration step. All materials were then analyzed by SDS-PAGE and Western as described above; however, both reduced and non-reduced samples were analyzed but only human HPR was used to probe blots. Results are shown in Figure 17A-B and demonstrate a large molecular weight species of secreted DENV2 E, suggesting a virus-like-particle.

Similar experiments were done with the ZIKV VLP cassette. The recombinant baculovirus was used to infect insect cells and the culture supernatants or cell lysates were collected at day 4 post-infection and analyzed using SDS-PAGE and Western blot. Blots from non-reduced samples were probed seropositive DENV reference serum or with FE5, a flavivirus E-specific monoclonal antibody (mAb). Results are shown in Figures 18 (mAb) and Figure 19 (DENV reference serum). As demonstrated in Figure 18, FE5 mAb detected fully processed ZIKV E in non-reduced samples in the lysate and supernatant and the majority of E was in the supernatant (A). ZIKV E was only faintly detected in reduced supernatant samples (B). As demonstrated in Figure 19, DENV HPR recognized fully processed ZIKV E in non-reduced samples in the lysate and supernatant and the majority of processed ZIKV E was in the supernatant (A). Importantly, a high molecular weight secreted ZIKV E species was present at the very top of the blot. Secreted ZIKV E was only faintly detected in

reduced supernatant samples (B); however, significantly, a high molecular weight species of secreted ZIKV E was present.

In further efforts to determine if a virus-like particle was secreted into the culture supernatant, 1 liter and 0.4 liter cultures of insect cells were infected with the recombinant baculoviruses containing the DENV2 and ZIKV Single Promoter Bicistronic VLP Expression Cassettes, respectively. The cell lysates and culture supernatants were harvested and the supernatant was clarified by centrifugation and further processed using 300 or 100 kDa ultrafiltration and diafiltration, respectively. The retentates were initially analyzed by Western blot to verify that secreted DENV2 and ZIKV E were present (data not shown) and subsequently analyzed using cryo transmission electron microscopy (cryoTEM). Importantly, as demonstrated in Figure 20, baculovirus particles as well as enveloped virus-like-particles the approximate size of DENV2 (A, upper and lower panels) and ZIKV (B, upper and lower panels) (40-50 nm) were evident in electron micrographs of the retentates.

To further characterize VLP expression cassettes, a head-to-head comparison was undertaken using the three different ZIKV VLP expression cassettes (bacIRA3, bacIRA4 and bacIRA5). Each cassette has the same ZIKV del108C-prM-E upstream of the IRES, but bacIRA3 has the del108C from DENV2 genetically fused to the human furin signal peptide (hfsp), bacIRA4 has no del108C upstream of hfsp and bacIRA5 has the homologous del108C from ZIKV fused to the hfsp. For each, a 0.4 liter culture of insect cells was infected with the corresponding recombinant baculovirus. The cell lysates and culture supernatants were harvested and the supernatants were clarified by centrifugation and further processed using 300 kDa ultrafiltration and diafiltration. The cell lysates and retentates were analyzed by Western blot to compare the amounts of secreted ZIKV E generated by each ZIKV VLP expression cassette. As shown in Figure 21A-B, significant amounts of ZIKV E were present in the retentates for all three expression cassettes (lanes 3, 4 and 5). Importantly, as 300 kDa MWCO cartridges were used for ultrafiltration, the ZIKV E detected in retentates reflects a high molecular weight species of E and, together with the electron microscopy data, suggest significant amounts of ZIKV VLPs are present in all 3 retentates. Additionally, the ZIKV VLP expression cassette containing homologous ZIKV del108C upstream of human furin demonstrated the highest amounts of ZIKV E expression (lane 5).

To assess the antigenic potential of the high molecular weight species of ZIKV and DENV2 E generated using the ZIKV and DENV2 VLP expression cassettes, retentates from studies described above were assayed using an *in vitro* VLP potency ELISA as described in the methods. For these studies, three additional 1 liter cultures of insect cells were infected

with bacIRA1, bacIRA3 or bacIRA5 and cell lysates and culture supernatants were harvested as described above. However, following clarification by centrifugation, supernatants were processed using 500 or 750 kDa ultrafiltration and diafiltration and retentates were then assayed using the *in vitro* VLP potency ELISA. Eight E-specific mouse monoclonal antibodies are used in the VLP potency assay of which 7 neutralize ZIKV and DENV2 and one (FE2) is non-neutralizing. Importantly, as shown in Figure 22A, for all 5 retentates generated using ZIKV VLP expression cassettes, captured high molecular weight ZIKV E was bound by 6 of 7 E-specific neutralizing antibodies, demonstrating the presence of ZIKV E-specific neutralizing epitopes in the high molecular weight species of ZIKV E. Similarly, as shown in Figure 22B, for retentates generated using a DENV2 VLP expression cassette, captured high molecular weight DENV2 E was bound by all E-specific neutralizing antibodies, demonstrating the presence of DENV2 E-specific neutralizing epitopes in the high molecular weight species of DENV2 E.

To further determine if the high molecular weight species of ZIKV or DENV E seen in retentates was a VLP, classical sucrose gradient sedimentation studies were undertaken. The 7 retentates described in Figure 22 were used for these studies. Each was centrifuged at 21,000xg, supernatant was removed and the pellet was resuspended in NTE overnight and then overlaid on a discontinuous sucrose gradient (15-60%). After centrifugation, 2ml fractions were collected from the top of the gradient to the bottom and fractions were analysed by Western blot. Results from retentates made using bacIRA5 (ZIKV) and bacIRA1 (DENV2) and 500 kDa ultrafiltration and diafiltration are shown in Figure 23A. Importantly, ZIKV and DENV2 VLPs were predominantly detected in fractions 9-16, a distribution nearly identical to those published previously for flavivirus viral particles [Putnak R, et al., *J Infect Dis.* Dec;174(6): 1176-84, 1996]. Further, although only two representative gradients are shown for ZIKV and DENV E, all 7 retentates gave nearly identical results, suggesting that all VLP expression cassettes generated secreted ZIKV or DENV2 VLPs with a density similar to that seen for flavivirus particles. For each sucrose gradient, fractions containing ZIKV or DENV2 VLPs were pooled, diluted, buffer exchanged and then concentrated. Western blot analysis of pooled fractions and purified concentrated ZIKV or DENV2 VLPs are shown in Figure 23B. The highest yield of purified ZIKV VLPs was from the retentate generated using bacIRA5 (contains del108CZIKV upstream of human furin) and processed using 500 kDa ultrafiltration and diafiltration. The retentate generated using bacIRA1 (contains del108CDENV2 upstream of human furin) and processed using 500 kDa ultrafiltration and diafiltration demonstrated DENV2 VLP yields similar to the high ZIKV VLP yields generated using bacIRA5. The ZIKV VLPs generated with bacIRA5 were chosen for

immunogenicity and efficacy studies. Prior to starting *in vivo* studies, the purified ZIKV VLPs as well as the purified DENV2 VLPs were analysed using the *in vitro* VLP potency ELISA. As shown in Figure 24, purified ZIKV VLPs were bound by 6 or 7 E-specific mouse neutralizing monoclonal antibodies and purified DENV2 VLPs were bound by all E-specific mouse neutralizing monoclonal antibodies.

To assess VLP immunogenicity, purified ZIKV VLPs were used to immunize wildtype C57BL/6 mice. Two groups of mice (n=10 per group) were immunized with ZIKV VLPs. Group 1 animals received 1 µg ZIKV VLPs twice administered intramuscularly. Animals in Group 2 received 2.5 µg ZIKV VLPs twice i administered intramuscularly. For both groups, prime and boost were done 21 days apart and VLPs were mixed with adjuvant (0.1% Alhydrogel) just prior to immunization. A third group of animals (n=10) received adjvant alone on the same schedule. Serum was collected from all animals prior to prime (day 0), prior to boost (day 21) and 3 weeks after boost (day 42). ZIKV-specific immunoglobulins (Ig) in sera from immunized mice were measured using an indirect ELSIA as described in the methods and results are shown in Figure 25. Importantly, all animals that were immunized with ZIKV VLPs had detectable ZIKV-specific Ig in serum on day 21 and levels increased with dose and following boost (day 42). By comparison, ZIKV-specific Ig was not found in animals that received adjuvant alone. Mouse serum samples were also assayed in PRNT and ZIKV neutralizing antibodies were detected in all animals except one in Group 1 (Table 1) and adjuvant alone animals (data not shown).

Table 1. ZIKV-specific neutralizing antibody responses in mice immunized with ZIKV VLP[1]

Mouse #	Group 1 day 21	Group 1 day 42	Group 2 day 21	Group 2 day 42
1	28.6	10.8	34.9	13.8
2	-4.6	15.3	**60.7**	47.0
3	44.7	7.1	43.7	**53.1**
4	23.1	7.4	**53.0**	ND
5	36.9	**59.2**	42.9	**100***
6	29.6	**50.8**	**73.9**	**51.8**

7	8.5	45.6	**67.4**	39.2
8	12.4	10.6	27.3	**78.4**
9	36.1	30.3	19.0	**74.8**
10	7.6	11.4	49.4	42.3
Average	**22.3**	**24.9**	**47.2**	**55.6**

[1]Percent ZIKV neutralization at 1:16 serum dilution

Individual serum samples from group 1 animals demonstrated a range of ZIKV neutralization (7-59%) and as an averaged group demonstrated 22% neutralization on day 21 and 25% neutralization on day 42. Individual serum samples from group 2 animals also demonstrated a range of ZIKV neutralization (27-100%) and as an averaged group demonstrated 47% neutralization on day 21 and 56% neutralization on day 42. Importantly these data demonstrate that the purified ZIKV VLPs are immunogenic and elicit ZIKV-neutralizing antibodies in vivo.

Recently, a new highly lethal mouse model of ZIKV neuropathogenesis was described [Smith DR, et al., *PLoS Negl Trop Dis* 11(1): e0005296. doi:10.1371/journal.pntd.0005296 (2017)] that used immunocompetent wildtype C57BL/6 mice. This highly relevant mouse model was used to evaluate the efficacy of the new ZIKV VLP vaccine candidates. All mice from the ZIKV VLP immunogenicity studies (Groups 1, 2 and 3 described above) were inoculated with a lethal dose of ZIKV three weeks after boost (day 42). Animals were monitored for disease following challenge and survival curves were generated for each group of mice. As demonstrated in Figure 26, none of the mice in the adjuvant alone group survived. Conversely, 50% of the animals in Group 1 and 80% of the animals in Group 2 survived ZIKV challenge and survival seemed to correlate to neutralization activity higher than 15% (Table 1). Importantly, although the two doses of ZIKV VLP used were considered suboptimal, performance was significant even in the lowest dose group (1 µg). Most importantly, the data demonstrate that ZIKV VLP immunizations afford protection against lethal ZIKV challenge highlighting their exceptional vaccine potential.

DISCUSSION

A new bicistronic expression cassette for DENV VLP production that included both DENV prM-E and furin was designed. The new cassette also included key transcriptional and translational regulatory elements as well as a novel signal sequence strategy in an

attempt to co-locate DENV prM-E and furin during cell trafficking. Figure 2 details the features of the new DENV VLP expression cassette and includes two similar ZIKV VLP expression cassettes. After comparing the furin amino acid sequence from Sf9 cells to the human furin proprotein sequence, significant discrepancies, including whole domain differences were noted. As this could possibly account for the low cleavage of DENV prM in Sf9 cells, we opted to use the human furin proprotein sequence in the new VLP expression cassette. Further, human furin may have less toxic effects in Sf9 cells given the differences between the Sf9 and human furin sequences which should benefit VLP production. The open reading frames (ORFs) for DENV or ZIKV prM-E and human furin proprotein were codon optimized for insect cell expression and cloned downstream of a single promoter sequentially with an Internal Ribosome Entry Site (IRES) between the two ORFs. Such an approach allows DENV or ZIKV prM-E and furin to be transcribed on one mRNA, in temporal sync, but translated independently. Also, as excess furin could cause detrimental cleavage effects in the cell, the human furin proprotein ORF was inserted downstream of the IRES to dampen expression slightly. As DENV capsid (C) protein anchors in the membrane of the ER and contains the bonafide DENV signal sequence for prM-E, a partial fragment of DENV C (del108C) was used as an ER membrane anchor and signal sequence for both DENV prM-E and human furin. By targeting both proteins to the same region of the ER membrane normally targeted by DENV prM-E we hypothesized furin and prM-E would remain in close proximity. Further, the cellular signal peptidase that cleaves the membrane anchored DENV C will be the same for both proteins as the signal sequences were identical, eliminating another potential idiosyncrasy. A similar approach was used for the ZIKV VLP expression cassette; however, in addition to the ZIKA del108C-prM-E-IRES-ZIKA del108C-human furin, cassette an additional ZIKV VLP expression cassette was made with DENV2 del108C upstream of human furin. For comparison, DENV2 and ZIKV VLP expression cassettes that lack del108C sequences upstream of human furin have also been made. For YFV and HCV, expression cassettes were made that contained the viral del108C upstream of the flavivirus structural gene and human furin as well as expression cassettes that did not contain the viral del108C upstream of human furin. All cassettes have been designed so that the region between the IRES and furin can be changed easily. Further, all cassettes contain the normal human furin signal sequence (hfsp) in case it is important for cleavage of human furin proprotein to the active human furin protein.

For other medically important arboviruses, including some flaviviruses, baculovirus-insect cell expression systems have emerged as an ideal platform for producing recombinant antigens and VLPs [Metz SW, Pijlman GP. *J Invertebr Pathol* 107 Suppl: S16-30 (2011)].

Indeed this is not too surprising as all arboviruses actively replicate in arthropod cells. For these reasons we opted to pilot expression of our new DENV and ZIKV VLP expression cassettes using recombinant baculovirus and chose the polyhedrin promoter to drive bicistronic transcription (Figure 2). In initial experiments, Sf9 cells were infected with recombinant baculovirus expressing the bicistronic DENV VLP cassette. On day 3 and 4 post-infection cells and culture supernatants were harvested and analyzed using SDS-PAGE and Western blot and results are shown in Figure 14A-B. Two species of DENV E with approximate molecular weights between 50 and 60 kDa were present in cell lysates and culture supernantants, consistent with previous reports of properly processed DENV E analyzed under non-reducing conditions. Importantly, the supernatant contained the highest amount of both forms of DENV E.

To further assess secreted DENV E, a 1L Sf9 culture was employed and cells were infected with the recombinant baculovirus expressing the biscistronic DENV VLP cassette. On day 4 cells and culture supernatant were harvested and culture supernatants (VS) were freeze/thawed once (VS (1)), sterile filtered (VS (1) SF) and subjected to ultrafiltration using two different molecular weight cutoffs (100K and 300K kDA). The permeates (UF-P1) and retentates (UF-R) were collected and all materials were analyzed using SDS-PAGE under non-reduced (A) or reduced (B) conditions and Western blot using DENV HPR. Results are shown in Figure 17A-B. Interestingly, under reduced conditions, significant amounts of a high molecular weight species of secreted DENV E was found in the culture supernatants and ultrafiltration retentates but was not present in the lysate. The high molecular weight species of secreted DENV E was also present in the non-reduced culture supernatants and ultrafiltration retentates (at the very top of the blot). Importantly, electron microscopy studies using a ultrafiltration retentate containing secreted DENV E revealed the presence of virus-like-particles similar in size to DENV. Although further characterization needs to be done, we were excited by the data as significant amounts of secreted DENV E was produced using the recombinant baculovirus platform and secreted E appears to be processed properly and form high molecular weight virus-like-particles which could represent the first scalable recombinant DENV VLP. We have also rescued the recombinant baculovirus expressing the bicistronic ZIKV VLP cassette. Importantly, in initial experiments, the majority of ZIKV E was also secreted into the culture supernatant, processed properly and forms high molecular weight species analogous to DENV VS results (Figure 19A-B). Electron microscopy studies using a ultrafiltration retentate containing secreted ZIKV E revealed the presence of virus-like-particles similar in size to ZIKV (Figure 20B) which optimistically suggested a recombinant ZIKV VLP vaccine may be possible, which would be ideal for pregnant women.

We have continued to evaluate the novel VLP platform and have compared ZIKV VLP cassettes that contain heterologous, homologous or no del108C sequences upstream of human furin and have confirmed that the homologous combination leads to the highest levels of secreted ZIKV E in culture supernatants (Figure 21A-B). We have also determined that the homologous del108C combination in the DENV2 VLP cassette leads to maximum yields of secreted DENV2 E in culture supernatants. Further, we have demonstrated that the secreted ZIKV and DENV2 E in retentates are recognized by cross-reactive neutralizing monoclonal antibodies (Figure 22) and secreted ZIKV and DENV2 E densities are similar to flavivirus particle density (Figure 23A). Importantly, we have shown that purified ZIKV or DENV2 VLPs retain important neutralizing epitopes (Figure 24) and ZIKV VLPs can elicit neutralizing antibodies in the host (Figure 25). Of most significance, we have shown that ZIKV VLP immunizations protect animals from lethal ZIKV challenge (Figure 26).

In order to advance our understanding of potential flavivirus VLP vaccines, we believe it is essential to compare and contrast different viruses using our new flavivirus VLP expression cassette. We have prioritized DENV and ZIKV in our initial studies as recent large DENV2 vaccine trials have not produced desirable outcomes, opening the door for new vaccine ideas and ZIKV because a VLP vaccine would be ideal for pregnant women. Moreover, DENV and ZIKV co-circulate in many parts of the world and by studying the viruses and VLPs side by side we hope to determine the extent of serological cross-reactivity between the two.

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CLAIMS:

1. An expression cassette comprising;

i. a flavivirus structural gene,

ii. a furin gene, and

iii. a bicistronic expression element positioned between the flavivirus structural gene and the furin gene, wherein:

i) the flavivirus structural gene comprises a partial capsid protein (delC), a complete membrane precursor (prM) protein coding sequence and a complete envelope (Env) protein coding sequence, wherein the delC coding sequence comprises the C-terminal anchor containing the signal sequence for prM; or

ii) wherein the flavivirus structural gene comprises a partial capsid protein (delC), a membrane (p7) protein coding sequence and complete envelope (E1 and E2) protein coding sequences, wherein the delC protein coding sequence comprises the C-terminal anchor containing the signal sequence to E1.

2. The expression cassette according to claim 1, wherein the bicistronic expression element is selected from the group comprising an internal ribosome entry site (IRES) and F2A.

3. The expression cassette according to claim 1 or 2, wherein the furin gene comprises a furin signal peptide (fsp) and a furin proprotein coding sequence.

4. The expression cassette according to any one of claims 1 to 3, further comprising a partial capsid protein (delC) coding sequence, wherein the delC protein coding sequence comprises the C-terminal anchor sequence containing the signal sequence for prM or E1, fused in frame to the 5’ end of the furin signal peptide coding sequence.

5. The expression cassette according to any one of claims 1 to 4, wherein the delC protein does not contain the first 108 nucleotides of the capsid protein coding sequence.

6. The expression cassette according to any one of claims 1 to 5, comprising a promoter to drive transcription of the expression cassette.

7. The expression cassette according to any one of claims 1 to 6, comprising a polyA signal sequence positioned at the 3’ end of the furin gene.

8. The expression cassette according to claim 7, wherein the polyA signal sequence is a SV40 late polyA signal sequence.

9. The expression cassette according to any one of claims 1 to 8, wherein the furin gene is selected from the group comprising human, non-human mammal or insect furin gene.

10. The expression cassette according to claim 9, wherein the furin gene is human.

11. The expression cassette according to any one of claims 1 to 10, wherein the flavivirus structural gene and the furin gene are codon-optimised for expression in insect cells.

12. The expression cassette according to any one of claims 1 to 11, wherein the flavivirus is selected from at least one of the group comprising Dengue virus, Zika virus, Yellow fever virus, West Nile virus and Japanese encephalitis virus and serotypes thereof.

13. The expression cassette according to any one of claims 1 to 11, wherein the flavivirus is Hepatitis C virus.

14. The expression cassette according to claim 12, wherein the flavivirus is selected from at least one of the group comprising Dengue virus serotype 1, Dengue virus serotype 2, Dengue virus serotype 3, Dengue virus serotype 4 and Zika virus.

15. The expression cassette according to claim 14, wherein the Dengue virus serotype is DENV2.

16. The expression cassette according to any one of claims 4 to 15, wherein the cassette is homologous or heterologous with respect to the partial capsid protein delC.

17. The expression cassette according to claim 16, wherein the cassette is heterologous and comprises a Dengue virus delC coding sequence and a ZIKA virus delC coding sequence.

18. An expression cassette according to any one of claims 1 to 17, comprising a nucleic acid sequence selected from the group comprising SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9 SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13 and SEQ ID NO: 14.

19. A method for the production of recombinant secreted flavivirus structural proteins and/or VLPs comprising the steps: cultivating a eukaryotic cell that has been transfected with a plasmid containing an expression cassette as defined in any one of claims 1 to 18, and recovering the recombinant secreted virus structural proteins and/or VLPs from the cell or the cultivation medium.

20. The method according to claim 19, wherein the eukaryotic cell has been infected with a recombinant baculovirus expressing said expression cassette.

21. The method according to claim 19, wherein the eukaryotic cell is selected from:

i) a mammalian cell;

ii) a Chinese hamster ovary cell or human kidney cell;

iii) an insect cell, or

iv) a Sf9 insect cell from *Spodoptera frugiperda*.