DNA VACCINE EXPRESSING MATURE DENGUE VIRUS-LIKE PARTICLES AND SEROLOGICAL TESTS TO DISTINGUISH DENGUE AND ZIKA

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ABSTRACT

Our study investigated DNA vaccines expressing mature dengue virus (DENV) virus-like particles (VLPs) and developed serological tests to distinguish dengue and Zika. We showed that human-codon optimized DNA vaccines improved the expression and production of VLPs in vitro compared with non-codon optimized DNA vaccines. Human-codon optimized DNA vaccine expressing mature VLPs induced similar level of anti-DENV and neutralizing antibodies compared with that expressing mixed VLPs. Secondly, we found that DNA-prime and inactivated virion-boost regimens improved the immunogenicity in mice compared with DNA vaccine alone. Future challenge experiments are needed to show the protective effect of these vaccine candidates. Thirdly, we demonstrated that combination of three enzyme-linked immunosorbent assays based on non-structural protein 1 can distinguish DENV and Zika virus infections with high specificity and sensitivity. In light of the cross-reactivity of current serological tests, our findings have implications for serodiagnosis between DENV and Zika virus or other flaviviral infections.

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ABBREVIATIONS

Abs	Antibodies
ADE	Antibody dependent enhancement
C	Capsid
cp	Cardiopuncture
CR	Complex-reactive
DALYs	Disability-adjusted life years
DC	Dendritic cells
DENV	Dengue virus
DF	Dengue Fever
DHF	Dengue hemorrhagic fever
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
E	Envelope protein
ER	Endoplasmic reticulum
FL	Fusion loop
FRNT	Foci reduction neutralization test
GR	Group-reactive
IM	Intramuscular
Imm	Immature
IP	Intraperitoneal
IV	Inactivated virion
JEV	Japanese encephalitis virus
Kb	Kilo-base

m	Mature
M	Membrane
mAbs	Monoclonal antibodies
MOI	Multiplicity of infection
MVEV	Murray Valley encephalitis virus
NCR	Noncoding region
NS	Non-structural
NT	Neutralizing
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
pDENV	Primary dengue
prM	precursor membrane
PRNT	Plaque reduction neutralization test
pWNV	Primary West Nile Virus
pZIKV	primary Zika
rE	Recombinant E
RNA	Ribonucleic acid
rNS1	Recombinant nonstructural protein
rOD	Relative optical density
sDENV	Secondary dengue
SLEV	St. Louis encephalitis virus
SC	Subcutaneous
SM	Submandibular
TGN	Trans-Golgi network
TS	Type-specific

VLPs	Virus-like particles
WB	Western blot
WNV	West Nile virus
WT	Wild type
YFV	Yellow fever virus
ZIKV	Zika virus
ZIKVwpDENV	Zika with previous dengue

CHAPTER 1

INTRODUCTION

Flaviviruses

Flaviviruses are members of the Flaviviridae family and can be transmitted by arthropod vectors (Pierson and Diamond 2013). Several members of the flaviviruses are known to cause extensive morbidity and mortality globally and have become important to public health problems (Gubler 2012). These include encephalitic viruses such as Japanese Encephalitis Virus (JEV), West Nile Virus (WNV), Murray Valley Encephalitis Virus (MVEV), and St. Louis Encephalitis Virus (SLEV). Other flaviviruses such as Yellow Fever Virus (YFV) and Dengue Virus (DENV) can cause hemorrhagic fever (Pierson and Diamond 2013). The recently emerging Zika Virus (ZIKV) can cause neurological complications and birth defects such as microcephaly (Weaver, Costa et al. 2016).

Dengue virus

DENV is one of the most important vector-borne viral diseases in humans. Infection with any of the four DENV serotypes, DENV1, DENV2, DENV3, and DENV4 can cause a wide spectrum of disease (Guzman, Halstead et al. 2010, Gubler 2012). Inadequate sewage and waste management following urban growth in dengue endemic regions combined with the lack of effort to control the mosquito vector allowed epidemic dengue to expand globally (Gubler 2012). The amino acid sequence homology among the four serotypes ranges from 63% to 77% (Lindenbach, Murray et al. 2013). Co-circulation of multiple DENV serotypes has been reported in many endemic countries (Pierson and Diamond 2013).

Genome

DENV is a single-stranded, positive-sense RNA virus with a genome approximately 10.6 kb in length that contains a single open reading frame (ORF) flanked by 5' and 3' noncoding regions (NCR) encoding a polyprotein precursor (Lindenbach, Murray et al. 2013). This polyprotein is then cleaved by viral and cellular proteases into three structural proteins: the capsid (C), precursor membrane (prM), and envelope (E) protein as well as seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Murphy and Whitehead 2011).

Replication cycle

The DENV enters target host cells such as dendritic cells through clathrin-mediated endocytosis. Several cell surface receptors have been characterized to interact with DENV particles upon viral entry. Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrins (DC-SIGN) for example, acts as an attachment factor for dengue virus. Following endocytosis, the low pH of the endosome induces the conformational changes of the viral E protein and allows the fusion between virus and the host cell membrane, releasing the viral RNA from the endosome into the cell cytoplasm (Perera, Khaliq et al. 2008, Lindenbach, Murray et al. 2013, Guzman and Harris 2015). Translation of the genome then occurs in the endoplasmic reticulum (ER) followed by cleavage into three structural proteins and seven NS proteins. The virus then undergoes RNA replication in the invaginated membrane vesicles derived from ER (Guzman and Harris 2015). Virion assembly occurs on the ER membranes, close to the viral RNA replication site. Immature

virions, containing prM and E proteins on the surface, bud into the lumen of the ER and are transported through the secretary pathway. The virions mature as they go through the trans-Golgi network (TGN), where the low pH environment allows the cellular serine protease furin to cleave prM into pr peptide and membrane (M) protein. This results in the production of mature virions which contain M and E proteins on the surface (Perera, Khaliq et al. 2008). However, studies have shown that this cleavage is incomplete in DENV and several other flaviviruses, resulting in culture supernatants that consist of a mixed population of mature, immature, and partially immature virions (Junjhon, Edwards et al. 2010, Plevka, Battisti et al. 2011).

DENV virion

The DENV virion is spherical and enveloped with a lipid bilayer consisting of prM/M and E proteins present on the surface. Inside the envelope, there is a nucleocapsid consisting of C protein and RNA genome. Based on several cryo-electron microscopic (cryo-EM) studies (Kuhn, Zhang et al. 2002, Zhang, Chipman et al. 2003, Zhang, Corver et al. 2003, Yu, Zhang et al. 2008), mature virions have a smooth surface approximately 50 nm in diameter consisting of 90 E protein dimers, whereas immature virions have a spiky surface with approximately 60 nm in diameter and consists of 60 trimeric prM-E heterodimers (Perera, Khaliq et al. 2008).

E protein

The E glycoprotein on the surface of the virion is a major protein that mediates binding and fusion activities during virus infection and acts as a major target for

neutralizing (NT) antibodies (Abs). X-ray crystallography studies have shown that the N-terminal ectodomain of the E protein contains three domains: domain I (DI) which is located in the center, domain II (DII) which contains a fusion loop (FL) at the tip that is involved in membrane fusion, and domain III (DIII) which is involved in receptor binding (Modis, Ogata et al. 2003, Modis, Ogata et al. 2004).

During the maturation process of the immature virions, the prM and E proteins on the virion undergo a conformational change due to the low pH environment in the TGN, where the immature spiky particles become smooth, exposing the prM cleavage site. This site is recognized by the cellular serine protease furin, which then cleaves the pr peptide from the prM protein (Perera, Khaliq et al. 2008, Yu, Zhang et al. 2008), thus disrupting the trimer which gives the immature particles their characteristic spiky image. The E protein is rearranged to form a dimer and lays flat against the surface in the characteristic herringbone pattern seen on smooth, mature particles (Perera, Khaliq et al. 2008).

Epidemiology

The dengue virus causes a significant burden on both global public health and the economy with more than 100 countries endemic with dengue (Gubler 2012, Bhatt, Gething et al. 2013, WHO 2017). One report estimates that there are approximately 390 million infections per year, with about 500,000 of these cases resulting in dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Bhatt, Gething et al. 2013, WHO 2017). In addition to health concerns, reports show that 264 disability-adjusted life years (DALYs) per million population are lost due to infection with DENV (WHO 2015). These numbers however are most likely much

lower than the actual numbers because many cases are underreported or misdiagnosed (Suaya, Shepard et al. 2009).

Vectors

DENV is transmitted by two species of mosquitoes. *Aedes aegypti* is the predominant vector for DENV, although *Aedes albopictus* is also able to transmit the virus. *A. albopictus* was initially found as a secondary vector for DENV in Asia but has since spread to North America and Europe where its distribution now largely overlaps with that of *A. aegypti* (WHO 2015, Lessler, Chaisson et al. 2016).

Clinical Features

An estimated three billion humans are at risk of infection with dengue globally. The majority if DENV infections are asymptomatic or show only mild symptoms. However, of the approximate 390 million infections per year, 25% of these individuals present with apparent infections (Bhatt, Gething et al. 2013) with an incubation period that can range from 3 to 14 days, but is generally 4 to 7 days (Whitehead, Blaney et al. 2007). Most of the symptomatic cases present as dengue fever (DF), which includes mild febrile illness, rash, myalgia, arthralgia, headache, and retro-orbital pain. A smaller percentage of cases (about 5-10%) may develop more severe forms known as DHF and DSS, which are associated with acute vascular permeability which results in capillary leakage, thrombocytopenia, and hemorrhage (Guzman, Halstead et al. 2010, Peeling, Artsob et al. 2010). The loss of plasma fluids is the main contributing factor to the severity of the disease and may

ultimately lead to a life-threatening shock. Secondary infection with a heterologous serotype has been shown to cause DHF and DSS more frequently compared to a primary infection (Halstead 1988).

Immune Response

The adaptive immune response plays a major role in clearing the virus and providing protection from re-infection, but is also believed to be the main player in the development of severe disease seen in patients with DHF and DSS (Whitehead, Blaney et al. 2007). DENV is able to infect a variety of immune cells including monocytes, DCs, and macrophages, and upregulates MHC class I on the surface of these cells (Rothman 2011).

DENV Infection and Antibody Response

Primary infection with DENV confers lifelong protection against and correlates with type specific NT Abs against that same serotype. Following primary DENV infection, patients develop an early IgM response which is detected 5 or more days after onset of illness, followed by an IgG response which is detected from 10-15 days. Secondary DENV infection with a heterologous serotype does not provide protection and increases the risk of severe disease due to ADE. After a secondary infection, IgM appears earlier or in the same time frame as that of primary infection but usually at lower titers, compared to IgG which increases rapidly due to its presence from the previous infection (Sabin 1952, Halstead 1974, Guzman, Alvarez et al. 2007, Imrie, Meeks et al. 2007, Peeling, Artsob et al. 2010).

The *Flavivirus* genus is comprised of several serocomplexes, including the JEV, DENV, YFV, and TBEV serocomplexes (Lindenbach, Murray et al. 2013). Group reactive (GR) anti-E Abs are able to recognize members of different serocomplexes, complex reactive (CR) recognize members within the same serocomplex, and type specific (TS) can recognize a single member (Calisher, Karabatsos et al. 1989). Several epitope studies of mouse and human anti-E mAbs have shown that GR mAbs primarily recognize the FL of DII while TS and CR mAbs recognize DIII residues (Modis, Ogata et al. 2003, Modis, Ogata et al. 2004, Crill, Hughes et al. 2009, Brien, Austin et al. 2010, Shrestha, Brien et al. 2010, de Alwis, Beltramello et al. 2011, de Alwis, Smith et al. 2012, Teoh, Kukkaro et al. 2012, Costin, Zaitseva et al. 2013, Tsai, Lai et al. 2013). TS mAbs have also been shown to recognize the DI/DII hinge and quaternary interdomain epitope (de Alwis, Smith et al. 2012, Teoh, Kukkaro et al. 2012). In general, TS mAbs have greater neutralizing potency in vitro than that of CR mAbs, which is greater than that of GR mAbs (Gromowski and Barrett 2007, Sukupolvi-Petty, Austin et al. 2007, de Alwis, Smith et al. 2012, Teoh, Kukkaro et al. 2012). Thus, eliciting TS potent NT anti-E Abs is the main goal of current tetravalent dengue vaccines.

Infection with one serotype may generate an immune response against both homotypic and heterotypic serotypes. Studies of mAbs have shown that anti-FL and anti-prM mAbs cause ADE both in vitro (Huang, Yang et al. 2006, Goncalvez, Engle et al. 2007, Beltramello, Williams et al. 2010, Dejnirattisai, Jumnainsong et al. 2010, Rodenhuis-Zybert, van der Schaar et al. 2010, Rodenhuis-Zybert, Moesker et al. 2011) and in vivo (Goncalvez, Engle et al. 2007, Zellweger, Prestwood et al. 2010). Studies of polyclonal human sera have reported that anti-E Abs were predominantly GR and recognized FL residues in DII (4-63%), which were shown to be cross-

reactive weakly NT or non-NT, whereas only a minor proportion was TS that recognize DIII (1-8%), I/II hinge or quaternary epitopes and are potent NT Abs (Lai, Tsai et al. 2008, Crill, Hughes et al. 2009, Wahala, Kraus et al. 2009, Lai, Williams et al. 2013, Gallichotte, Widman et al. 2015, Nivarthi, Kose et al. 2017).

Antibody-Dependent Enhancement (ADE)

Individuals with a secondary infection have an increased risk of developing severe clinical outcomes such as DHF and DSS (Sangkawibha, Rojanasuphot et al. 1984, Burke, Nisalak et al. 1988, Guzman, Kouri et al. 1990) which is believed to be due to ADE (Halstead and O'Rourke 1977, Halstead 1988). ADE is thought to occur when a heterotypic weakly non-NT Ab from a previous DENV infection binds to the virus during a subsequent infection but is unable to neutralize the virus (Burke, Nisalak et al. 1988, Guzman, Kouri et al. 2000). The newly formed Ab-virus complex binds to Fc gamma receptors on monocytes, resulting in an increase in DENV entry into cells, replication and virus load and thus causing more severe disease outcomes (Murgue, Roche et al. 2000, Vaughn, Green et al. 2000).

Vaccines

Current strategies for a tetravalent vaccine aim to include all four serotypes as immunogens in order to provide full protection and prevent ADE and the severe diseases that are associated with it (Whitehead, Blaney et al. 2007, Murphy and Whitehead 2011). The ideal DENV vaccine should be free of reactogenicity, induce a level of protection that is seen during natural infection, avoid ADE, provide lifelong

protection against all four serotypes, and be safe in children and adults (Whitehead, Blaney et al. 2007, Guzman and Harris 2015). Development of DENV vaccines have been challenging due to the possibility of triggering ADE, difficulty in eliciting comparable levels of NT Abs against all four serotypes, conferring life-long protection, the lack of an animal model that is able to mimic clinical symptoms seen in humans, and the lack of well-defined immune correlates of protection (Guzman and Harris 2015).

Current DENV vaccine candidates include live-attenuated, recombinant protein, DNA, inactivated, virus-like particle (VLP), and virus vector vaccines (Guzman and Harris 2015). Dengvaxia (CYD-TDV) from Sanofi Pasteur is a chimeric live-attenuated vaccine that uses a recombinant infectious cDNA clone of the yellow fever 17D vaccine strain as a backbone and substitutes the prM and E genes with those of each four DENV serotypes (Guirakhoo, Weltzin et al. 2000, Guirakhoo, Pugachev et al. 2004, Villar, Rivera-Medina et al. 2013). Although Dengvaxia has been licensed in several dengue-endemic countries (Pang 2016), phase II and phase III clinical trials have shown varying efficacies between the four serotypes and only moderate overall efficacy (30-60%) despite the presence of NT Abs (Sabchareon, Wallace et al. 2012, Capeding, Tran et al. 2014, Villar, Dayan et al. 2015). Results from the phase III clinical trials of Dengvaxia showed that the vaccine efficacy was lower in individuals who were seronegative than those who were seropositive, with higher rates of hospitalizations in the vaccinated seronegative individuals, suggesting that the vaccine was aiding in the increase of non-NT infection-enhancing Abs (Capeding, Tran et al. 2014, Hadinegoro, Arredondo-Garcia et al. 2015, Villar, Dayan et al. 2015, Aguiar, Stollenwerk et al. 2016). This event was most notable amongst young children. Due to the increased risk of developing

severe dengue in the 2-5 year age group, the WHO Strategic Advisory Group of Experts (SAGE) has recommended that the vaccine not be administered to children under 9 years of age (Capeding, Tran et al. 2014, Villar, Dayan et al. 2015, Pang 2016). Thus, there is a need for a better understanding of NT Abs and the immune correlates of protection and a need for a better vaccine.

DNA vaccines are also being considered as a candidate for dengue and other flavivirus vaccines (Martin, Pierson et al. 2007, Beckett, Tjaden et al. 2011). Benefits of DNA vaccines include ease and flexibility of construction, stability, intracellular production of antigen, transient expression, no induction of antivector immunity, induction of humoral and cellular immunity, and low occurrence of reactogenicity (Martin, Pierson et al. 2007, Danko, Beckett et al. 2011, De Filette, Soehle et al. 2014).

Zika Virus (ZIKV)

Zika, which was first discovered in 1947, was characterized as causing only a mild or inapparent disease similar to dengue (Fauci and Morens 2016). However, ZIKV emerged as a major public health concern, first as a large outbreak on Yap Island in 2007 with over 70% of the population infected (Duffy, Chen et al. 2009), followed by another outbreak in French Polynesia from 2013-2014 infecting 66% of the population (Cao-Lormeau, Roche et al. 2014), and finally most recently in Central and South America starting in 2015 (WHO 2015, Fauci and Morens 2016, CDC 2017). Phylogenetics has shown that ZIKV is closely related to the DENV serocomplex (Calisher, Karabatsos et al. 1989, Lessler, Chaisson et al. 2016). The global spread of ZIKV starting from DENV endemic regions has raised concerns on

its co-circulation with other flaviviruses such as WNV, TBEV, JEV, and YFV (Lessler, Chaisson et al. 2016).

Genome

Like other members of the Flaviviridae family, ZIKV is a positive-sense single-stranded RNA virus with a genome encoding a single polyprotein which is then cleaved by viral and cellular proteases into three structural proteins: the C, prM, and E protein as well as seven NS proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Song, Qi et al. 2016).

Epidemiology

Since the first outbreak in 2007 on Yap Island (Lanciotti, Kosoy et al. 2008, Duffy, Chen et al. 2009), ZIKV has spread globally, with large outbreaks occurring in Brazil and the Americas (WHO 2015, Lessler, Chaisson et al. 2016, Weaver, Costa et al. 2016). Imported cases from travelers to endemic regions as well as local cases in United States territories have also been reported (CDC 2017). Accurate data on global case counts of Zika is currently not available, as Zika was not mandatory to report. Estimates from Brazil range between approximately 500,000 to 1,500,000 infections (Weaver, Costa et al. 2016). An increase in the number of infants born with the microcephaly, a form of congenital brain abnormality, in Brazil suggests its possible link to Zika infection (Kleber de Oliveira, Cortez-Escalante et al. 2016, Schuler-Faccini, Ribeiro et al. 2016). Although experts were initially reluctant to state a causal relationship between Zika and microcephaly, growing evidence from in vitro

(Chan, Yip et al. 2016, Onorati, Li et al. 2016, Pawitwar, Dhar et al. 2017), animal (Cugola, Fernandes et al. 2016, Li, Saucedo-Cuevas et al. 2016, Manangeeswaran, Ireland et al. 2016, Miner, Cao et al. 2016) and clinical studies (Brasil, Pereira et al. , Calvet, Aguiar et al. 2016, Driggers, Ho et al. 2016, Mlakar, Korva et al. 2016) have shown a strong association and supported a causal relationship (Rasmussen, Jamieson et al. 2016).

Vectors

Like DENV, ZIKV is also transmitted *A. aegypti* and *A. albopictus*. Both mosquito vectors are found in Asia, parts of North and South America, and Africa where its distribution overlaps with both DENV and ZIKV (WHO 2015, Lessler, Chaisson et al. 2016).

Clinical Features

After ZIKV infection, majority are inapparent, and only 20% of individuals presenting with symptoms that include a slight fever, conjunctivitis, rash, myalgia, joint pain, and muscle pain (Pawitwar, Dhar et al. 2017). Due to the non-specific nature and similarity of the symptoms to that of the co-circulating DENV, it is difficult to diagnose and differentiate between the two flaviviral infections. Infected adults may also develop Guillain-Barre Syndrome, as many countries have reported increases in the occurrence ranging from two to ten times than that before the ZIKV outbreaks (Cao-Lormeau, Blake et al. 2016, Dos Santos, Rodriguez et al. 2016, Petersen, Jamieson et al. 2016). Increasing evidence has also shown the

association between infants born with microcephaly from mothers infected with ZIKV during their pregnancy (Brasil, Pereira et al. , Mlakar, Korva et al. 2016, Rubin, Greene et al. 2016).

Diagnosis

Due to the nonspecific and similar clinical manifestations caused by ZIKV and DENV (Simmons , Farrar et al. 2012, Waggoner, Gresh et al. 2016), these flaviviruses can be misdiagnosed for each other. This is especially difficult for ZIKV and DENV because of their geographic overlap where co-infections and secondary infections are common.

Current CDC authorized tests for diagnosing a ZIKV infection including the Zika E-protein based immunoglobulin M capture enzyme-linked immunoabsorbent assay (MAC-ELISA) and Trioplex real-time reverse transcription-polymerase chain reaction assay (Oussayef, Pillai et al. 2017). Guidelines for laboratory diagnosis of ZIKV start with a two week period after a symptomatic or asymptomatic traveler returns from an area with ZIKV activity. Within this two week period, RT-PCR will be conducted on serum, saliva, urine or other body fluids for confirmatory results. Beyond this 2 week window, serum will be tested with the IgM MAC-ELISA. A test with negative results for ZIKV as well as DENV will indicate no recent DENV or ZIKV infection. However, a positive result for any of these two indicates a presumptive flavivirus infection but cannot confirm a ZIKV infection and will need to undergo further testing with a plaque reduction neutralization test (PRNT). Although a positive PRNT result for either DENV or ZIKV will indicate a recent primary infection, a positive for both cannot differentiate between the flavivirus infections (CDC 2016).

Difficulty with using the IgM MAC-ELISA may be due to the cross-reactivity between DENV and ZIKV E protein. An alternative to this is to use NS1 ELISAs, which have been reported to have high sensitivity and specificity (Steinhagen, Probst et al. 2016). This ZIKV-NS1 ELISA however does not address the question whether serum from secondary DENV infection can cross-react to ZIKV-NS1 and whether it can distinguish between primary ZIKV (pZIKV) and ZIKV with previous DENV infection (ZIKVwpDENV).

Hypothesis and specific aims

Specific Aim 1. Test if codon-optimized DNA vaccine expressing mature DENV virus-like particles (VLPs) induce better antibody responses than that expressing mixed VLPs.

Hypothesis: DNA vaccines expressing mature DENV particles induce less cross-reactive anti-FL Abs and no anti-prM Abs, compared with DNA vaccines expressing mixed DENV particles.

Rationale: Anti-FL and anti-prM mAbs are cross-reactive weakly or non-NT and cause ADE in vitro and in vivo (Huang, Yang et al. 2006, Goncalvez, Engle et al. 2007, Dejnirattisai, Jumnainsong et al. 2010, Rodenhuis-Zybert, van der Schaar et al. 2010, Rodenhuis-Zybert, Moesker et al. 2011). FL is exposed on immature and partially immature particles but poorly exposed on mature DENV particles, and pr is exposed on immature and partially immature particles but not on mature DENV particles (Kuhn, Zhang et al. 2002, Zhang, Chipman et al. 2003, Zhang, Corver et al. 2003, Li, Lok et al. 2008).

Experimental Plan: Expression and production of VLPs in vitro will be tested by Western blot (WB) analysis. Female 6-week-old C57/BL6 mice will be immunized intramuscularly (IM) with 3 doses of DNA vaccines expressing mature or mixed DENV VLPs of DENV1. Sera will be assayed for binding anti-DENV Abs by ELISA and neutralization by foci reduction neutralization test (FRNT) using wild type (WT) DENV1 VLPs (Crill, Hughes et al. 2012, Hughes, Crill et al. 2012).

Specific Aim 2. Test if DNA-prime and protein-boost strategy induces stronger antibody response than DNA alone.

Hypothesis: Heterologous DNA-prime and protein-boost vaccination elicits stronger antibody response compared to a DNA-only homologous vaccination strategy (Simmons, Porter et al. 2006, De Filette, Soehle et al. 2014).

Rationale: Although DNA vaccines are able to elicit cell-mediated immune responses, most DENV DNA vaccines in previous studies have only shown moderate NT Ab titers in mouse and non-human primate models. Several studies have shown that the use of a heterologous prime-boost strategy can induce higher antibody titers than a homologous vaccine strategy (Simmons, Porter et al. 2006, De Filette, Soehle et al. 2014).

Experimental Plan: Recombinant truncated E protein and inactivated DENV1 virion will be generated. 6-week-old female C57/BL6 mice will be immunized IM with 1 dose of DNA vaccines expressing mature or mixed DENV VLPs of DENV1, followed by two doses of either recombinant E protein or inactivated virion. Sera will be assayed for binding anti-DENV Abs by ELISA using wild type (WT), mature (m), or immature (Imm) DENV1 VLPs and neutralization by FRNT using WT DENV1 virions (Crill, Hughes et al. 2012, Hughes, Crill et al. 2012).

Specific Aim 3. To develop and validate a serological test to distinguish DENV and ZIKV.

Hypothesis: Recombinant NS1 (rNS1) based IgG and IgM ELISA can detect and differentiate between anti-NS1 Ab to DENV and ZIKV.

Rationale: Current IgM ELISA cannot confirm ZIKV infection due to cross-reactivity to E protein between ZIKV, DENV, and other flaviviruses (Lai, Tsai et al. 2008, Tsai, Durbin et al. 2015, CDC 2016, Oussayef, Pillai et al. 2017). Previous studies have shown that anti-NS1 Ab in human sera after DENV infection recognize DENV NS1 protein but not WNV NS1 protein, suggesting they are not cross-reactive between different flaviviral serocomplexes (Lai, Tsai et al. 2008, Tsai, Durbin et al. 2015). Another recent study has demonstrated that anti-NS1 mAbs derived from Zika cases are mostly not cross-reactive to DENV and vice versa, and polyclonal sera from Zika cases without previous dengue can distinguish DENV and ZIKV NS1 (Stettler, Beltramello et al. 2016).

Experimental Plan: Recombinant NS1 protein will be generated for ZIKV, DENV1-2, WNV, and YFV. RT-PCR confirmed sera of patients with pZIKV), ZIKVwpDENV and secondary DENV (sDENV) infections will be assayed by IgG and IgM ELISA using rNS1 proteins.

Significance and Innovation.

Compared with mixed DENV particles, vaccination with mature DENV particles can induce NT Abs, less cross-reactive weakly or non-NT Abs and no anti-prM Abs, thus reducing the risk of ADE and severe diseases. This represents a novel strategy for safe and effective DENV vaccines.

Current serological tests to diagnose Zika infections are unable to distinguish pZIKV and ZIKVwpDENV. Recent studies have shown that anti-DENV mAbs and polyclonal sera can enhance ZIKV infection in vitro through ADE, raising the possibility that previous DENV infection may increase the risk and severity of fetal microcephaly (Castanha, Braga et al. 2016, Dejnirattisai, Supasa et al. 2016, Priyamvada, Quicke et al. 2016, Stettler, Beltramello et al. 2016), further necessitating a serological test that can distinguish between pZIKV and ZIKVwpDENV and further our understanding of ZIKV pathogenesis and complications during pregnancy.

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CHAPTER 2

MATERIALS AND METHODS

Preparation of VLPs

HEK293T cells, prepared in a 10-cm culture dish at 5 x 10^5 cells per dish 1 day earlier, were transfected with 10 μg of plasmid DNA using the Lipofectamine 2000 method (Life Sciences). The plasmid DNA constructs expressed the prM/E proteins of DENV1 in the presence or absence of furin to produce mature or immature VLPs, respectively. At 48 h post-transfection, culture supernatants were collected, and cells were washed with phosphate-buffered saline (PBS) and treated with 1% NP40 lysis buffer (100 mM Tris [pH 7.5], 150 mM NaCl, 20 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, and protease inhibitors [Roche Diagnostics]), followed by centrifugation at 20,000 x *g* and 4°C for 30 min to obtain cell lysates (Hsieh, Zou et al. 2011).

Culture supernatants were separated by centrifugation at 1,250 x *g* and 4°C for 20 min, filtered through a 0.22-μm-pore-sized membrane (Millipore), layered over a 20% sucrose buffer, and ultracentrifuged at 65,000 x *g* and 4°C for 5 h to obtain pellets containing VLPs, which were re-suspended in 30 μL 1 x PBS buffer (Hsieh, Zou et al. 2011).

Western blot analysis

WB analysis was performed by first adding cell lysates or pellets to 4 x non-reducing buffer (2% SDS, 0.5 M Tris [pH 6.8], 20% glycerol, 0.001% bromophenol blue [final concentrations]) and subjected to 12% polyacrylamide gel electrophoresis (PAGE) using an apparatus (Bio Rad) at 80 – 120 V. The gel was then transferred at 1.3 A for 7 m (BIO-RAD Trans-Blot Turbo Transfer System) to a nitrocellulose

membrane (BIO-RAD). After blocking with blocking buffer (4% milk in wash buffer; 10 mM Tris [pH7.4], 150 mM NaCl, 0.2% Tween-20 [final concentrations]), the membrane was incubated at 4^{o}C overnight with primary antibody (anti-E mouse mAb at 1:200000; calnexin 1:10000). After washing three times with 1 x PBS at 10 min per wash on the following day, the membrane was incubated at room temperature (RT) for 1 h with secondary Ab (anti-mouse IgG at 1:10000, Pierce). After washing three times at 10 min per wash, the signals were detected by enhanced chemiluminescence reagents (Perkin Elmer Life Sciences) (Lai, Tsai et al. 2008, Hsieh, Zou et al. 2011). The intensities of mature and immature VLPs were compared.

Constructs of DNA vaccine

The prM/E expression plasmids of the D1 serotype (pCB-D1 constructs), were used as DNA vaccines (Hu, Hsieh et al. 2007). The prM/E expression plasmids containing furin, designated as pCB-F constructs, were generated by inserting IRES-furin-flag into the pCB-WT constructs. Human codon optimized prM/E expression plasmids were designated as pCB-D1h and pCB-D1hF. Insertions were verified by the patterns of restriction enzyme digestion and sequencing of the entire insert. DNA plasmids were amplified using DH5α competent cells and purified using an endotoxin-free plasmid DNA Purification Kit (Omega Biotek,Qiagen).

Constructs of recombinant E protein

Recombinant E (rE) protein expression plasmids of the four DENV serotypes were generated with pMT/Bip-based constructs containing prM and C-terminally truncated E for expression in Drosophila S2 cells. Constructs include C-terminal E truncations at amino acid residue position 395 (pMT/Bip-D1E80), 400 (pMT/Bip-D1E81, pMT/Bip-D2E81, pMT/Bip-D3E81, pMT/Bip-D4E81), 408 (pMT/Bip-D1E82), and 416 (pMT/Bip-D184). Insertions were verified by the patterns of restriction enzyme digestion and sequencing of the entire insert. DNA plasmids were amplified using DH5α competent cells and purified using an endotoxin-free plasmid DNA Purification Kit (OmegaBiotek, Qiagen).

Expression and purification of rE protein

Drosophila S2 cells were transfected with pMT/Bip-based rE constructs using Lipofectamine LTX (Life Sciences) to express rE protein and incubated at 27 ^{o}C. Stable clones were generated using hygromycin B selection in 10 ml of serum free medium, followed by expanding to 1200 ml for purification. Total volume of 1200 ml was purified through a 1 ml immunoaffinity column conjugated with anti-E mAb 4G2 using the AKTA system (GE). The purified rE proteins were concentrated into 2-3 ml volumes and stored in -80 ^{o}C. Concentrations of proteins were quantified using spectrophotometry.

Preparation of inactivated DENV for mouse immunization

Purified inactivated DENV1 viruses were kindly provided by Dr. Wen-Yang Tsai. Briefly, HEK293T cells and HEK293T-furin cells, prepared in a 75 mL flask at 7 x 10^6 cells per flask, were infected with DENV1 (Hawaii strain) at a multiplicity of infection (MOI) of 0.05 to generate mixed (DENV1mixed) and mature (DENV1m) virions, respectively. HEK293T cells infected with DENV1 were further treated with NH_4Cl to obtain immature (DENV1imm) virions. Culture supernatant was harvested when 50% cytopathic effect (CPE) was observed and subjected to ultracentrifugation (25,000 rpm, 5 h at 4^{o}C) to collect virus in the pellets. Purified viruses were inactivated by formalin (0.05%, 22 ^{o}C water bath, 10 days) (Putnak, Barvir et al. 1996) or H_2O_2 (3%, 22-24 ^{o}C, 2 h prior to performing mouse immunization) (Amanna, Raue et al. 2012).

Vertebrate Animals

This study was conducted according to the principles in the NIH Guide for the Care and Use of Laboratory Animals. All animal experiment protocol (Wang 12-1014-5) were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Hawaii at Manoa. Procedures comply with the USDA Animal Welfare Act Regulations, American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals: 2013 Edition, and the Guide for the Care and Use of Laboratory Animals 8th Edition (2011).

Mice immunization schedule

Mice were obtained from COBRE core facility (kindly provided by Dr. Vivek Nerurkar) and were maintained in the animal facility of John A. Burns School of Medicine, University of Hawaii at Manoa. Seven week-old female C57BL/6 mice (n = 5-7/group) were immunized with PBS, pCB-D1, pCB-D1F, pCB-D1h, or pCB-D1hF DNA vaccines at 0 week (wk), 3 wk, and 6 wk for the DNA only groups (DDD), or with formalin- or H_2O_2- inactivated D1WT or D1m virion (DVVformalin, DVV H_2O_2). Immunization with DNA was conducted by intramuscular (im) injection with 50 μL PBS or DNA vaccine (pCB-D1, pCB-D1h 50 μg/μl; pCB-D1F, pCB-D1hF 80 μg/μl) injected into the thigh of each mouse. Immunization with inactivated virion was conducted by either subcutaneous (sc) or intraperitoneal (ip) injection at 3 wk and 6 wk (5 μg). Mice were bled submandibularly (sm) at 0 wk, 2 wk, 5 wk, and via cardiopuncture (cp) at 10-12 wks.

Mouse Serum Collection

Whole blood collected from C57/BL6 mice were centrifuged at 2,500 rpm for 5 min; the supernatants (sera) were collected and then stored in -80^{o}C. Pooled sera of mice from each group as well as individual mice sera were tested for ELISA and FRNT assay.

Preparation of inactivated DENV for ELISA

Vero cells, prepared in a 75 mL flask at 7 x 10^6 cells per flask were infected with DENV1 (Hawaii strain) at a multiplicity of infection (MOI) of 0.05. Culture supernatant was harvested when 50% cytopathic effect (CPE) was observed and subjected to ultracentrifugation (25,000 rpm, 5 h at 4^{o}C) to collect virions in the pellets. Virions were inactivated by UV (Strategen, 600 V, twice, 5 min).

Three-layer virion ELISA

Three-layer ELISA was performed on collected sera of PBS and DNA vaccinated groups at various time points. 96-well flat-bottom plates were coated with vero-derived inactivated DENV1 virion or HEK293T-derived DENV1mixed, DENV1m, or DENV1imm virions and incubated at 4^{o}C overnight. Blocking buffer (Starting Block^{TM}, Thermo) was added the following day and the plate was incubated at room temperature for 1 h, followed by incubation of primary Ab (pooled mice sera or individual mice sera with serial dilutions) at 37^{o}C for 2 h and washing four times. Incubation of secondary Ab (goat anti-mouse Ab at 1:5000 dilution, or donkey anti-human Ab at 1:10000 dilution, Jackson Laboratory) was done at 37^{o}C for 1 h, followed by washing 6 times. TMB was added and incubated at room temperature for 15 min. Stop solution (2N H_2SO_4) was added promptly. OD was measured at a wavelength of 450 nm with reference wavelength of 650 nm using an ELISA plate reader (BioTek ELx50) (Tsai et al., 2013). O.D. results were analyzed by Graph Pad Prism 5.0 and endpoint titers were calculated.

Modified Focus Reduction Neutralization Test (FRNT)

Modified FRNT assays against DENV1 serotype were performed on collected pooled and individual sera to test the neutralization potency. Vero cells were seeded on a 96-well ELISPOT plate in 10% DMEM one day prior to virus infection and incubated at 37ºC. Two-fold serial dilutions of each serum were prepared and incubated with 50 focus-forming unites (ffu) of DENV1 virus serotype at 37ºC for 1 h. Virus-serum mixture was inoculated into the Vero cell monolayers and incubated at 37ºC for 90 min with gentle shaking every 15 min during the incubation. After 150 μL of overlay with 2% FBS DMEM, plates were incubated at 37ºC for 70 h. The plates were then washed with 200 μL of 1 x PBS 3 times, and fixed with 200 μL fixation buffer (30% methanol/70% acetone) for 20 min in -20 ºC. After removing the fixation buffer and drying at room temperature for 1 h, 200 μL of blocking solution (5% milk in 1 x PBS) was added and incubated on a shaker at room temperature for 30 min. After removing the blocking buffer, primary Ab (human Ab, Palau #8, 1:10,000 dilution) was added and incubated at 37ºC for 1 h. After washing 3 times with 1 x PBS, secondary Ab (anti-human Ab, 1:10,000 dilution, 800 nm; DRAQ5 fluorescent probe, 1:10,000, 700 nm) was added and incubated at 37ºC for 1 h. After washing 3 times with 1 x PBS, the plate was then washed with ddH_2O and dried overnight. Plates were read with LI-COR Odyssey and the titers of $FRNT_{50}$ were determined by a nonlinear regression analysis using Graph Pad Prism 5.0.

Constructs of recombinant NS1 protein

Recombinant NS1 (rNS1) protein expression plasmids of the DENV1 serotype, WNV, and ZIKV were generated with pMT/Bip-based constructs (pMT/Bip-D1-NS1, pMT/Bip-WNV-NS1, pMT/Bip-ZIKV-NS1) with His-tag at the C-terminus (Integrated DNA Technologies) containing codon-optimized NS1 gene (amino acids 1-352) for expression in Drosophila S2 cells. Insertions were verified by the patterns of restriction enzyme digestion and sequencing of the entire insert. DNA plasmids were amplified using DH5α competent cells and purified using an endotoxin-free plasmid DNA Purification Kit (OmegaBiotek, Qiagen).

Expression and purification of rNS1 protein

Serum-free adapted drosophila S2 cells (kindly provided by Dr. David Clements at the Hawaii Biotech) were co-transfected with pMT/Bip-based rNS1 constructs and selection plasmid hygromycin B using Lipofectamine LTX (Life Sciences) to express rNS1 and incubated at 27 ºC. Total volume of 600 ml was purified through a 1 ml His-trap column using the AKTA system (GE). The purified protein were concentrated into volumes of 2-3 ml and stored in -80ºC. Concentrations of proteins were quantified using spectrophotometry.

Three-layer rNS1 ELISA

Three-layer ELISA was performed on human serum or plasma samples from of ZIKV-, DENV1-, and WNV- infected individuals. 96-well ELISA plates were coated

with 16 ng/well with ZIKV-NS1, D1-NS1, or WNV-NS1 and incubated at 4ºC overnight. Blocking buffer (Starting Block™, Thermo) was added the following day and the plate was incubated at room temperature for 1 h, followed by incubation of primary Ab (mouse anti-His at1:5000 dilution, or human sera and plasma at 1:400 dilution) at 37ºC for 2 h and washing four times. Incubation of secondary Ab (goat anti-mouse Ab at 1:5000 dilution, or donkey anti-human Ab at 1:10000 dilution, Jackson Laboratory) was done at 37ºC for 1 h, followed by washing 6 times. TMB was added and incubated at room temperature for 15 min. Stop solution (2N H_2SO_4) was added promptly. OD was measured at a wavelength of 450 nm with reference wavelength of 650 nm using an ELISA plate reader (BioTek ELx50) (Tsai, Lai et al. 2013). NS1-IgM ELISA was performed similarly except that each sample was incubated with Gullsorb reagent (Meridian Bioscience), an IgG absorbent, for 10 min before adding to wells (100 ng NS1 per well) (Paldanius, Bloigu et al. 2003). O.D. results were analyzed using GraphPad Prism 6 and endpoint titers were calculated. Two-tailed Mann-Whitney test was used to determine the *P*-value comparing the relative OD (rOD) values between two groups.

Clinical samples

The study of coded serum or plasma samples was approved by Institutional Review Boards (IRB) of the University of Hawaii (CHS #17568, CHS#23786). Forty convalescent-phase samples from RT-PCR confirmed Zika cases that were DENV-naïve (n=20) or previously DENV-exposed (n=20), designated as primary ZIKV and ZIKV with previous DENV infection panels respectively, were kindly provided by Dr. Eva Harris at the University of California Berkeley from the Pediatric Dengue Cohort

Study in Managua, Nicaragua between July and September, 2016 (Kuan et al., 2009). The study was approved by the IRBs of the University of California, Berkeley, and Nicaraguan Ministry of Health.

Nineteen convalescent-phase samples from patients who presented with symptoms compatible with Zika and had detectable anti-DENV IgG antibodies, designated as probable ZIKV with previous DENV infection panel, were kindly provided by Dr. Carlos Brites at the Federal University of Bahia, Salvador, Brazil between November 2015 and May 2016. The study was approved by the IRB of Federal University of Bahia.

Convalescent-phase or post-convalescent-phase dengue samples were from RT-PCR confirmed dengue cases including 40 from Kaohsiung, Taiwan between 2001 and 2009 (Wang et al., 2006),18 from Nicaragua (three cases with sequential samples) between 2006 and 2008 (Lai et al., 2013), and 12 from the Big Island, Hawaii during the 2015 DENV1 outbreak. Flavivirus-naïve samples (n=12) were from previous studies (Lai et al., 2013). Six and four plasma samples from blood donors with RT-PCR confirmed West Nile virus infection and ZIKV infection, respectively, were kindly provided by Dr. Michael Busch at the Blood Systems Research Institute at San Francisco. The study was approved by the IRB of the University of California, San Francisco.

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CHAPTER 3

RESULTS

RESULTS

Specific Aim 1. Test if codon-optimized DNA vaccine expressing mature DENV VLPs induce better antibody responses than that expressing mixed VLPs.

Experimental plan

To test the immunogenicity of codon-optimized and non-codon optimized DNA vaccines expressing mature or immature DENV1 VLPs, four constructs were generated (Fig. 1). Expression and production of VLPs in vitro was first examined by WB analysis. Then 6-week-old female C57/BL6 mice were immunized im with 3 doses of DNA vaccines expressing mature or mixed DENV1 VLPs. Sera were assayed for binding by ELISA and neutralization by foci reduction neutralization test (FRNT) using DENV1 (Crill, Hughes et al. 2012, Hughes, Crill et al. 2012).

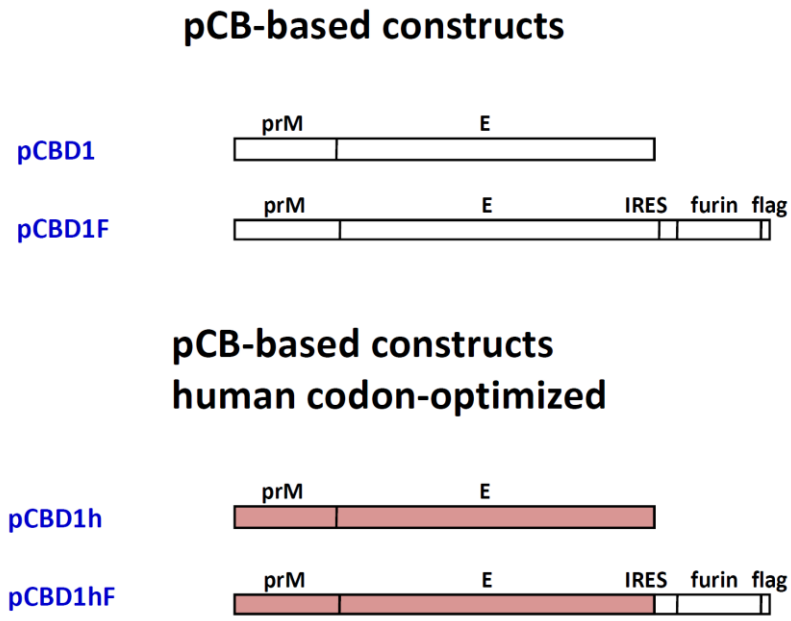


Fig. 1. Schematic drawing of the pCB-based constructs pCBD1 and pCBD1-Fand human codon-optimized constructs pCBD1h and pCBD1hF. Wild type constructs contain genes for the prM and E proteins. Furin constructs contain gene for furin and IRES and flag in addition to prM and E genes.

Expression of mature and mixed VLPs

Mature and immature VLPs were successfully produced by transfection of HEK293T cells with plasmid DNA constructs expressing the prM/E proteins of DENV1 in the presence or absence of furin (Fig.1). Expression and VLP production of DENV1 constructs were confirmed by WB analysis using anti-E mouse mAb to detect E proteins in cell lysates and VLP pellets derived from ultracentrifugation of culture supernatants of transfected cells (Fig. 2). E protein was expressed in all four constructs, with the codon-optimized furin construct (D1hF) showing higher expression than the non-optimized furin construct (D1F), and codon-optimized wild type construct (D1h) showing similar expression as the non-optimized wild type

construct (D1). Expression in the codon-optimized wild type construct (D1h) was higher than that of the codon-optimized furin construct (D1hF). VLPs were generated by all four DENV1 constructs, with higher production in in the codon-optimized constructs for both furin and wild type compared to their non-optimized counterparts. In the codon-optimized constructs, VLP production was higher in the wild type than the furin. These results show that human codon-optimized constructs with furin increase the expression and production of VLPs greatly compared with non-optimized constructs with furin.

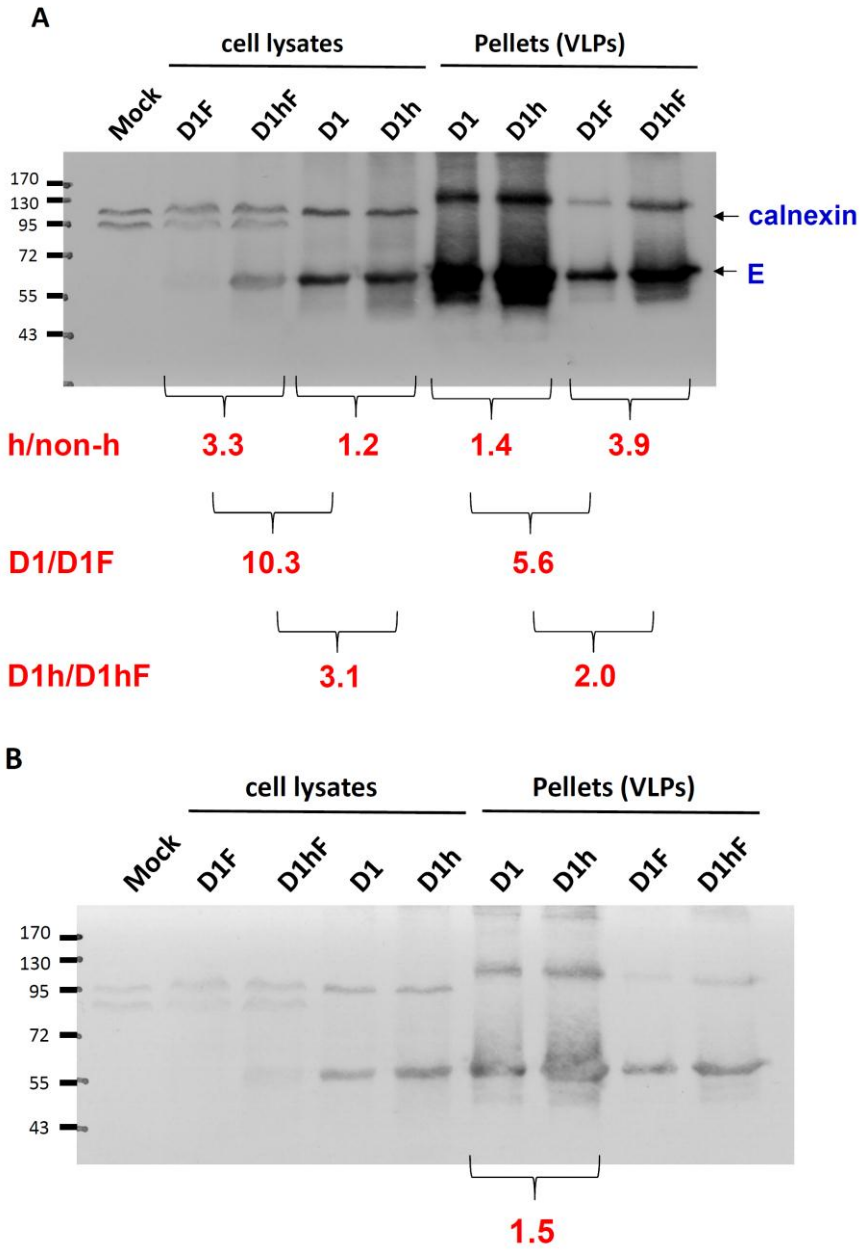


Fig. 2. Western blot analysis to examine E protein expression in cell lysates and VLP production. (A) Long exposure and (B) Short exposure of gels of cell lysates and VLP production from HEK293T cells transfected with pCBD1, pCBD1F, pCBD1h, and pCBD1hF constructs probed with anti-E mouse mAb (1:200000) and calnexin (1:10000).

Binding and neutralization of immunized mice sera

Since codon-optimized constructs (pCBD1h and pCBD1hF) expressed better than non-codon-optimized constructs, these two constructs were chosen for immunization experiment. Groups of 7 female C57BL/6 mice were immunized with a three dose regimen of DNA vaccine of pCBD1h designated as D1-WT DDD or pCBD1hF designated as D1-F DDD (Fig. 3). Individual sera collected from vaccinated mice was first tested for binding and then for NT Abs to DENV1 virion (Fig. 4). Though not significantly different, the ELISA Ab titers in the D1-WT DDD group are higher than the D1-F DDD (Fig. 4A). This is consistent with the higher expression levels of D1h than D1hF in vitro. The NT_{50} titers for both D1-WT DDD and D1-F DDD groups were low (Fig. 4B).

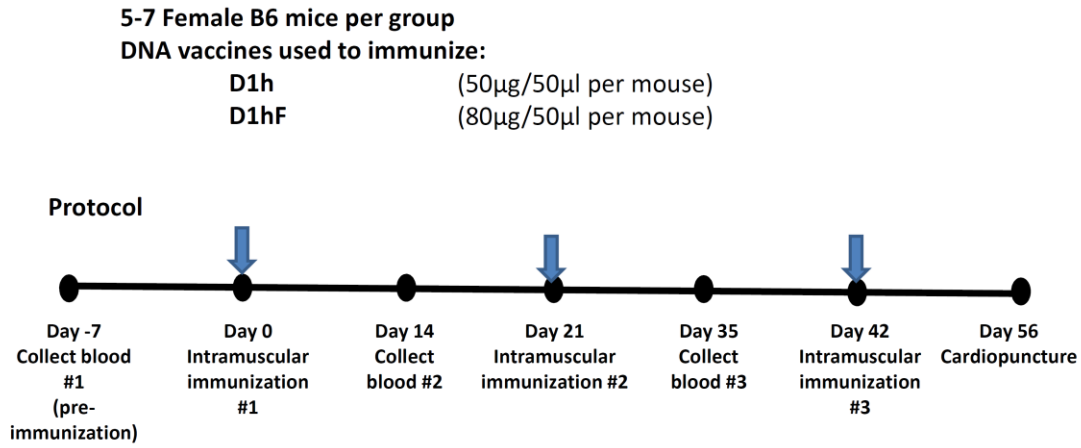


Fig. 3. Experimental protocol of mice immunization. Groups of 5 to 7 female C57BL/6 mice were immunized IM with 3 doses of DNA vaccines pCBD1h or pCBD1hF. Groups receiving virion-boost were immunized with 1 dose of DNA vaccines followed by two doses of inactivated virions by SC or IP route. Pooled and individual sera from each group were assayed for anti-DENV Abs and NT Abs.

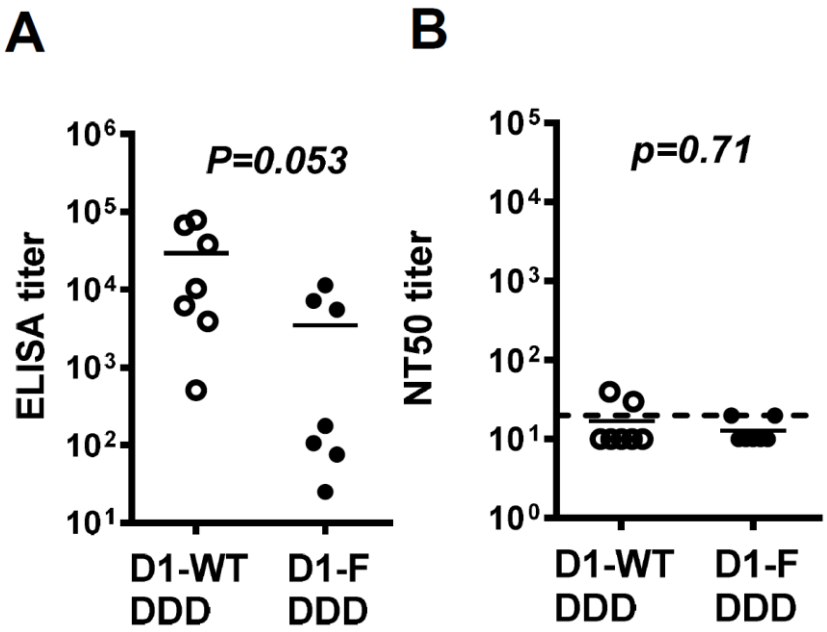


Fig. 4. Titers of anti-DENV Abs and NT Abs of individual sera from mice vaccinated IM with three doses of DNA vaccine of pCBD1h or pCBD1hF. (A) Individual sera of pCBD1-WT and pCBD1-F immunized mice collected at 8 wk (n=7, each group) were subjected to DENV1 virion-ELISA. The endpoint titers were determined. (B) Individual sera of mice immunized with pCBD1-WT and pCBD1-F collected at 8 wk (n=7, each group) were subjected to FRNT and the $FRNT_{50}$ titers were determined.

Specific Aim 2. Test if DNA-prime and virion-boost strategy induces stronger antibody response than DNA alone.

Experimental plan

To test the immunogenicity of DNA-prime and virion-boost, formalin- or H_2O_2-inactivated mature and mixed DENV1 virions were generated by colleague in the lab (from Dr. Wen-Yang Tsai). Then 6-week-old female C57BL/6 mice were immunized IM with 1 dose of DNA vaccines expressing mature or mixed DENV1 VLPs followed

by two doses of inactivated virion. Sera were assayed for binding by ELISA using mixed, mature (m), or immature (imm) DENV1 virions and neutralization by FRNT using mixed DENV1 virions (Crill, Hughes et al. 2012, Hughes, Crill et al. 2012).

Binding and neutralization activity in mice sera after DNA-prime and virion-boost immunization

Due to the low in vivo efficacy, a heterologous prime-boost regimen was implemented (Simmons, Porter et al. 2006, De Filette, Soehle et al. 2014). Mice were immunized with one dose of D1-WT DNA followed by two subcutaneous (SC) doses of formalin-inactivated mixed virion designated as D1-WT DVVsc, or one dose of D1-F DNA followed by two doses of formalin-inactivated mature virion designated as D1-F DVVsc. Individual sera were tested on DENV1 virion-ELISA (Fig. 5A). The ELISA titer of the D1-WT DVVsc group was significantly higher than that of the D1-F DVVsc group ($p=0.0262$) demonstrating the induction of higher binding Abs by the WT group. Individual sera were also tested for neutralization (Fig. 5B). Although there was no significant difference between the two groups, they both showed higher neutralization compared with DNA immunization alone (D1-WT DDD and D1-F DDD groups, Fig. 4B).

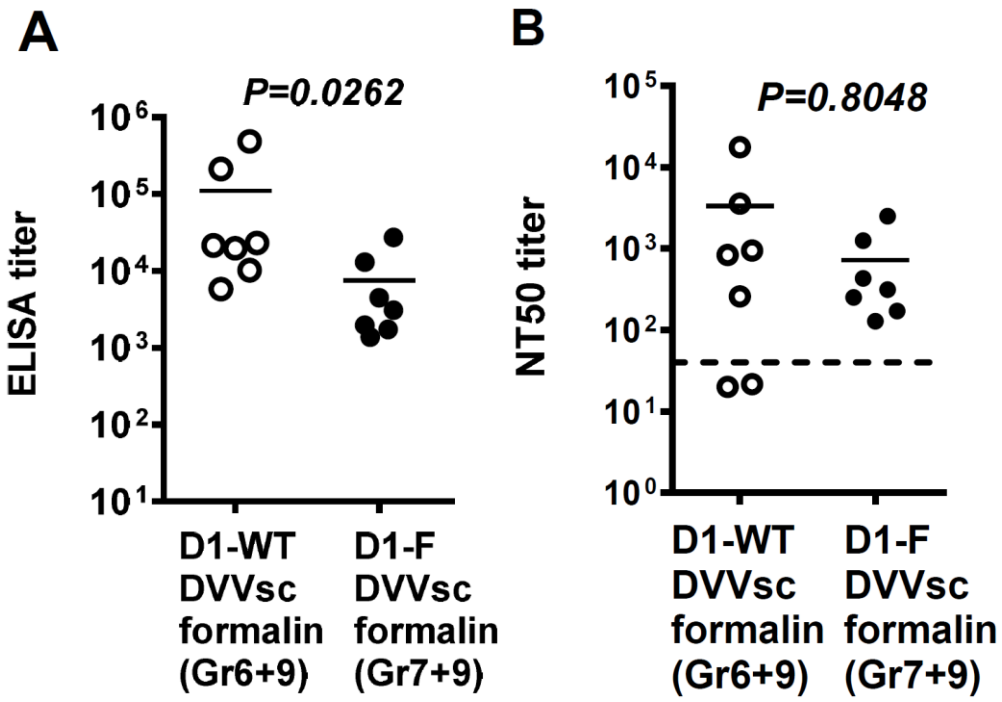


Fig. 5. Titers of anti-DENV Abs and NT Abs of individual sera from mice immunized IM with one dose of DNA vaccine of pCBD1h or pCBD1hF followed by two SC doses of formalin-inactivated virions. (A) Individual sera of D1-WT DVVsc and D1-F DVVsc groups collected at 10 wk (n=7, each group) were subjected to DENV1 virion-ELISA. The endpoint titers were determined. (B) Individual sera of m D1-WT DVVsc and D1-F DVVsc groups collected at 10 wk (n=7, each group) were subjected to FRNT and the $FRNT_{50}$ titers were determined.

Pooled sera of the D1-WT DVV and D1-F DVV groups were also tested for binding to DENV1 virions from different cells or with different maturation status. When tested with vero cell-derived mixed DENV1 virions, the WT group showed ELISA titer that was greater than one log compared to the F group (Fig. 6A). The WT group also showed better binding to HEK293T-derived mixed and immature DENV1 virions than the F group, but had similar ELISA titers when tested with the mature DENV1 virions (Fig. 6B). Taking the ratio of the ELISA titers of mature to immature

virions shows that the F group is much higher than the WT group (Fig. 6C). Consistent with this, the ratio of the NT_{50} over ELISA titers was higher in the F group than the WT group (*P*=0.0006, Fig. 6D), suggesting a better quality of Abs, based on higher binding to mature than immature virions and more potent NT activity, was induced by the F group compared with the WT group.

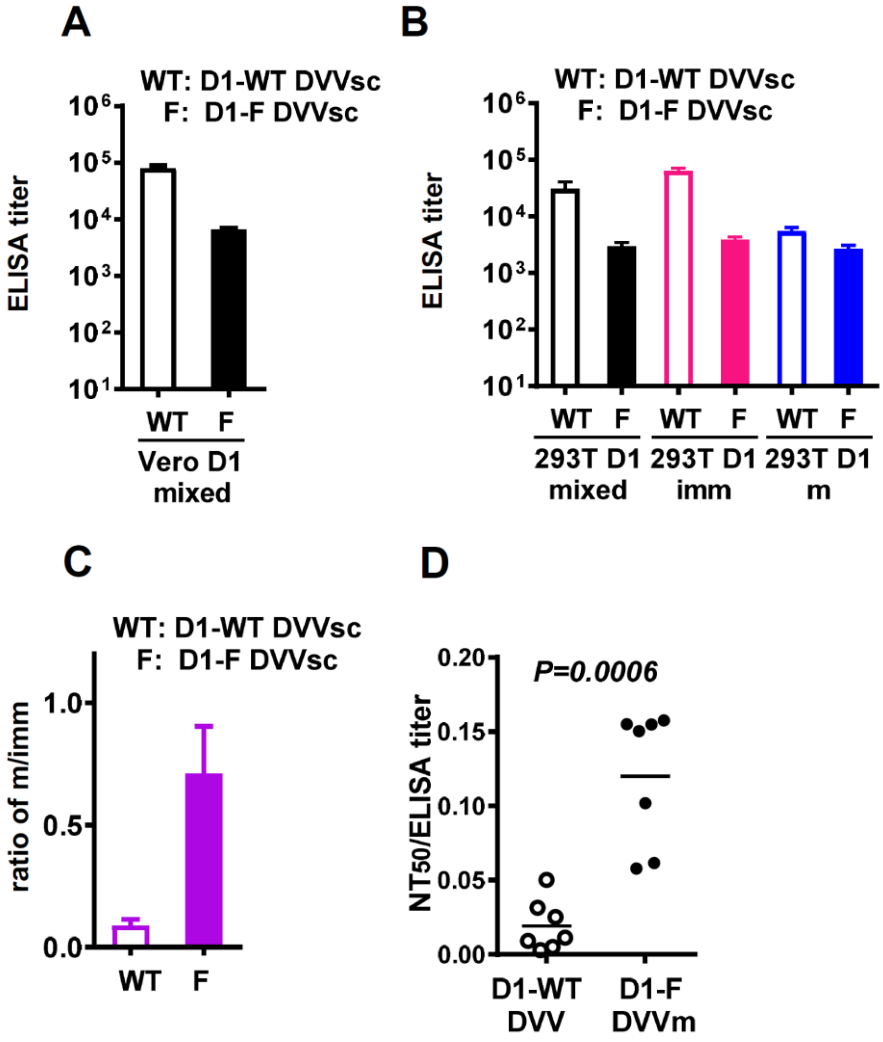
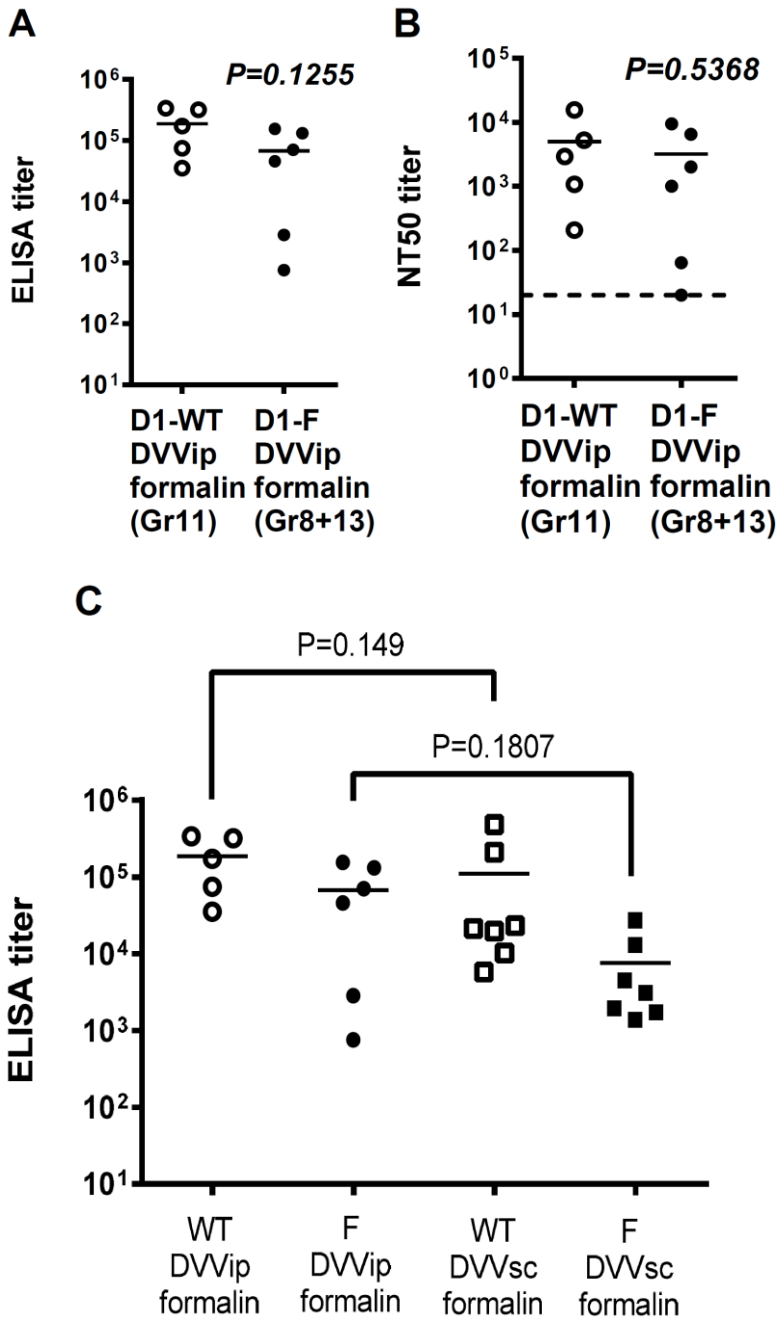


Fig. 6. Titers of anti-DENV Abs and NT Abs of pooled sera from mice vaccinated IM with three doses of DNA vaccine of pCBD1h or pCBD1hF followed by two doses of inactivated virion. (A) Pooled sera of D1-WT DVVsc and D1-F DVVsc groups were subjected to vero-derived DENV1 virion-ELISA. The endpoint titers were determined. (B) Pooled sera of D1-WT DVVsc and D1-F DVVsc groups were subjected to

HEK293T-derived DENV1 mixed, immature, and mature virion-ELISAs. The endpoint titers were determined (C) Ratio of mature to immature DENV1 virion ELISA titers. (C) Ratio of NT_{50} to ELISA titers of D1-WT DVV to D1-F DVVm groups.

To test the immunogenicity of different routes of immunization, groups of 5 to 7 female C57BL/6 mice were then immunized with one dose of D1-WT DNA followed by two intraperitoneal (ip) doses of formalin-inactivated mixed virion designated as D1-WT DVVip formalin, or one dose of D1-F DNA followed by two doses of formalin-inactivated mature virion designated as D1-F DVVip formalin. Individual sera from the WT (Gr 11) and F (Gr 8+13) groups did not show a significant difference but both had high ELISA (Fig. 7A) and NT_{50} titers (Fig. 7B). There was no difference in the ELISA and NT Ab titers between ip and sc routes of immunization (Figs. 7C-D).


A
B
P=0.1255
P=0.5368
ELISA titer
NT50 titer
D1-WT DVVip formalin (Gr11)
D1-F DVVip formalin (Gr8+13)
C
P=0.149
P=0.1807
WT DVVip formalin
F DVVip formalin
WT DVVsc formalin
F DVVsc formalin

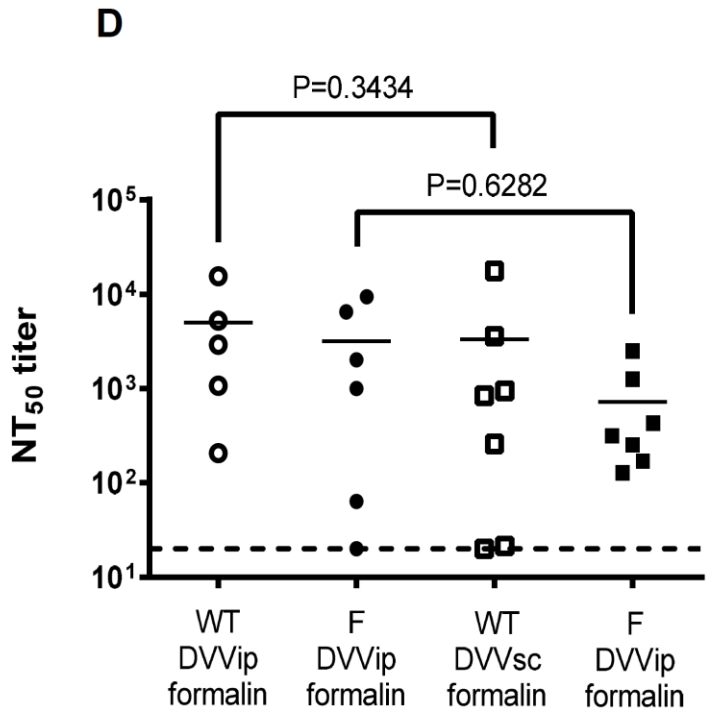
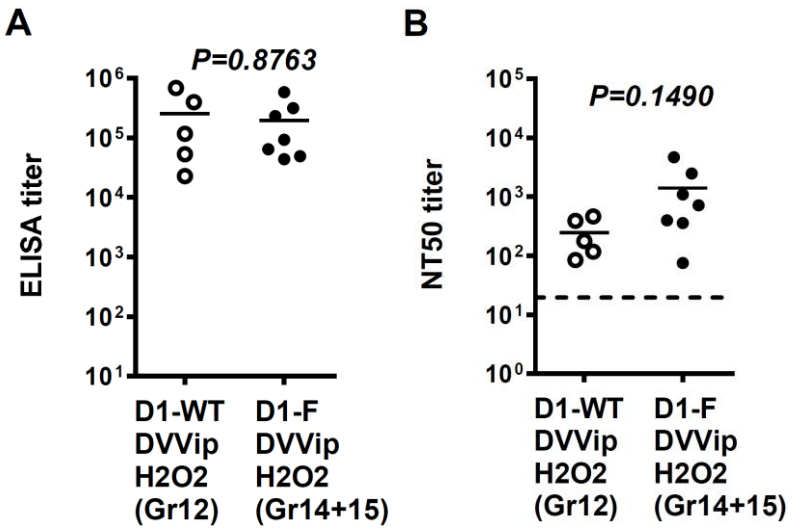
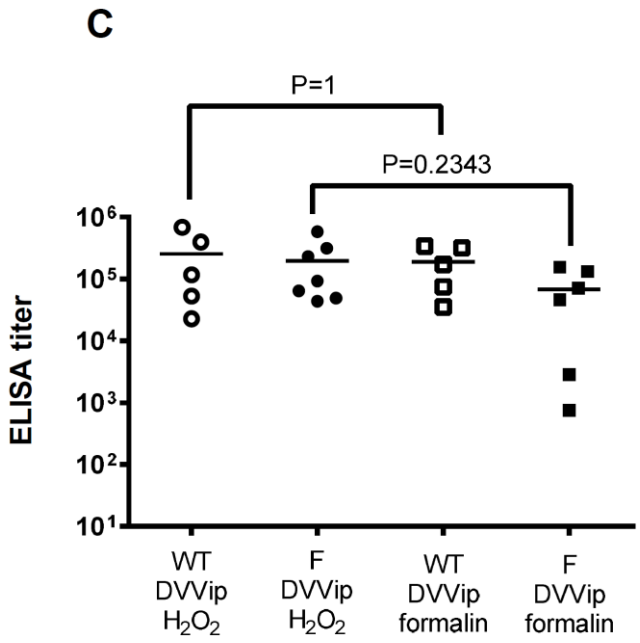


Fig. 7. Titers of anti-DENV Abs and NT Abs of individual sera from mice immunized IM with one dose of DNA vaccine of pCBD1h or pCBD1hF followed by two SC doses of formalin-inactivated virions. (A) Individual sera of D1-WT DVVip formalin (n=5) and D1-F DVVip formalin groups collected at 10 wk (n=7), were subjected DENV1 virion-ELISA. The endpoint titers were determined. (B) Individual sera of m D1-WT DVVip formalin (n=5) and D1-F DVVip formalin (n=7) groups collected at 10 wk were subjected to FRNT and the $FRNT_{50}$ titers were determined. (C) Comparison of ELISA titers between ip and sc routes of immunization in WT and F groups. (D) Comparison of NT_{50} titers between ip and sc routes of immunization in WT and F groups.

To test the immunogenicity of hydrogen peroxide (H_2O_2) inactivation, female C57BL/6 mice were immunized with one dose of D1-WT DNA followed by two ip doses of H_2O_2-inactivated mixed virions designated as D1-WT DVVip H_2O_2, or one dose of D1-F DNA followed by two doses of H_2O_2-inactivated mature virions

designated as D1-F DVVip H_2O_2. Both groups showed high ELISA titers (Fig. 8A). The F group (Gr12) showed slightly higher NT_{50} titers, though not significant, compared with the WT group (Gr 14+15); both had moderate neutralization activity (Fig. 8B). There was no difference in ELISA Ab titers between formalin and H_2O_2 inactivation (Fig. 8C). However, a higher NT Ab titer in the D1-WT DVVip formalin group was seen compared to its H_2O_2 counterpart.





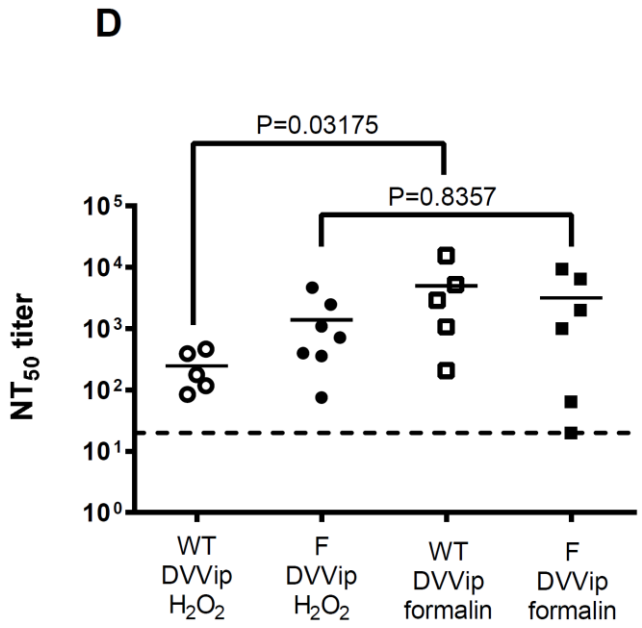


Fig. 8. Titers of anti-DENV Abs and NT Abs of individual sera from mice immunized IM with one dose of DNA vaccine of pCBD1h or pCBD1hF followed by two SC doses of formalin-inactivated virions. (A) Individual sera of D1-WT DVVip H_2O_2 (n=5) and D1-F DVVip H_2O_2 (n=7) groups collected at 10 wk were subjected DENV1 virion-ELISA. The endpoint titers were determined. (B) Individual sera of D1-WT DVVip H_2O_2 (n=5) and D1-F DVVip H_2O_2 (n=7) were collected at 10 wk were subjected to FRNT and the $FRNT_{50}$ titers were determined. (C) Comparison of ELISA titers between H_2O_2 and formalin inactivation in WT and F groups. (D) Comparison of H_2O_2 and formalin inactivation in WT and F groups.

Specific Aim 3. To develop and validate a serological test to distinguish DENV and ZIKV.

Experimental plan

To develop ELISA based on NS1 protein, recombinant NS1 (rNS1) proteins were generated for ZIKV, DENV1-2, WNV, and YFV. Then RT-PCR confirmed sera of patients with primary ZIKV (pZIKV), ZIKV infection with previous DENV (ZIKVwpDENV) and secondary DENV (sDENV) infections were assayed by IgG and IgM ELISA using rNS1 protein (Steinhagen, Probst et al. 2016).

E-based IgG ELISAs

Panels of serum or plasma samples from patients with primary DENV1 (pDENV1), primary WNV (pWNV), pZIKV, sDENV and ZIKVwpDENV infections were first tested with traditional E protein-based IgG ELISAs, including ZIKV-VLP, DENV1-inactivated virion (IV), and WNV-VLP IgG ELISAs. The ZIKV-VLP, DENV1-IV, and WNV-VLP ELISAs showed cross-reactivity across all serum and plasma samples. The ZIKV-VLP ELISA (Fig. 9A) showed positive for 5 out of 6 (83%) in pWNV, 16 out of 16 (100%) in pDENV1, 4 out of 4 (100%) in pZIKV, 22 out of 22 (100%) in sDENV, and 8 out of 8 (100%) in ZIKVwpDENV groups. Similarly, the DENV1-IV ELISA (Fig. 9B) showed positive for 5 out of 6 (83%) in pWNV, 16 out of 16 in pDENV1, 4 out of 4 in pZIKV, 22 out of 22 in sDENV, and 8 out of 8 in ZIKVwpDENVgroups. The WNV-VLP ELISA (Fig. 9C) showed positive for 6 out of 6 in pWNV, 16 out of 16 in pDENV1, 3 out of 4 (75%) in pZIKV, 22 out of 22 in sDENV, and 8 out of 8 in ZIKVwpDENV groups. Thus, the E-based IgG ELISAs are not able to distinguish between pWNV, pDENV1, pZIKV, sDENV, and ZIKVwpDENV infections.

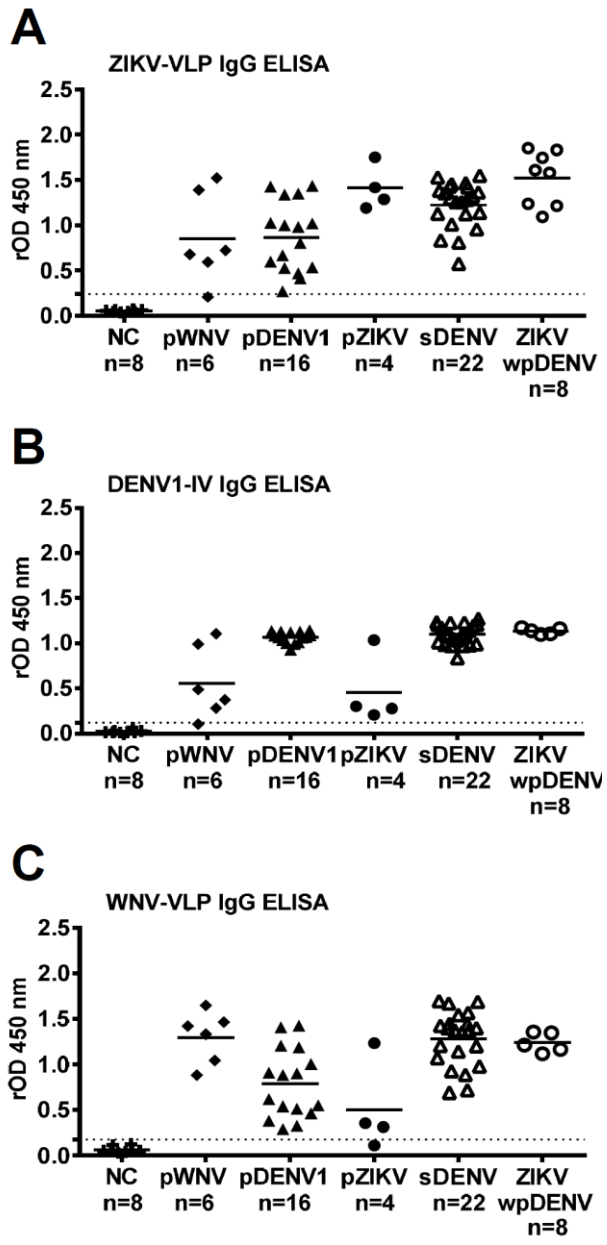


Fig. 9. Results of ZIKV-VLP, DENV1-IV, and WNV-VLP IgG ELISAs for pDENV, pZIKV, pWNV, sDENV, and ZIKVwpDENV panels. (A) ZIKV-VLP, (B) DENV1-IV, and (C) WNV-VLP IgG ELISAs in convalescent-phase samples from pDENV, pZIKV, pWNV, sDENV, and ZIKVwpDENV panels.

Expression and purification of rNS1 proteins

To produce rNS1 proteins of ZIKV, DENV1, DENV2, WNV, and YFV, five pMT-bip constructs were generated first (Fig. 10A). Stable clones were then established in Drosophila S2 insect cells and confirmed by Western blot analysis using anti-His Ab (Fig. 10B) and coomassie blue staining (Fig. 10C). The ZIKV-NS1 stable clones were most notably able to show the presence of the NS1 monomer and dimer. The supernatants were run through a His-trap column in a fast purification chromatography system to obtain pure NS1 protein. Coomassie blue staining shows purified ZIKV-NS1 protein in monomer and dimer forms (Fig. 10D), as well as monomers of the WNV, D1, D2, and YFV-NS1 proteins (Fig. 10E).

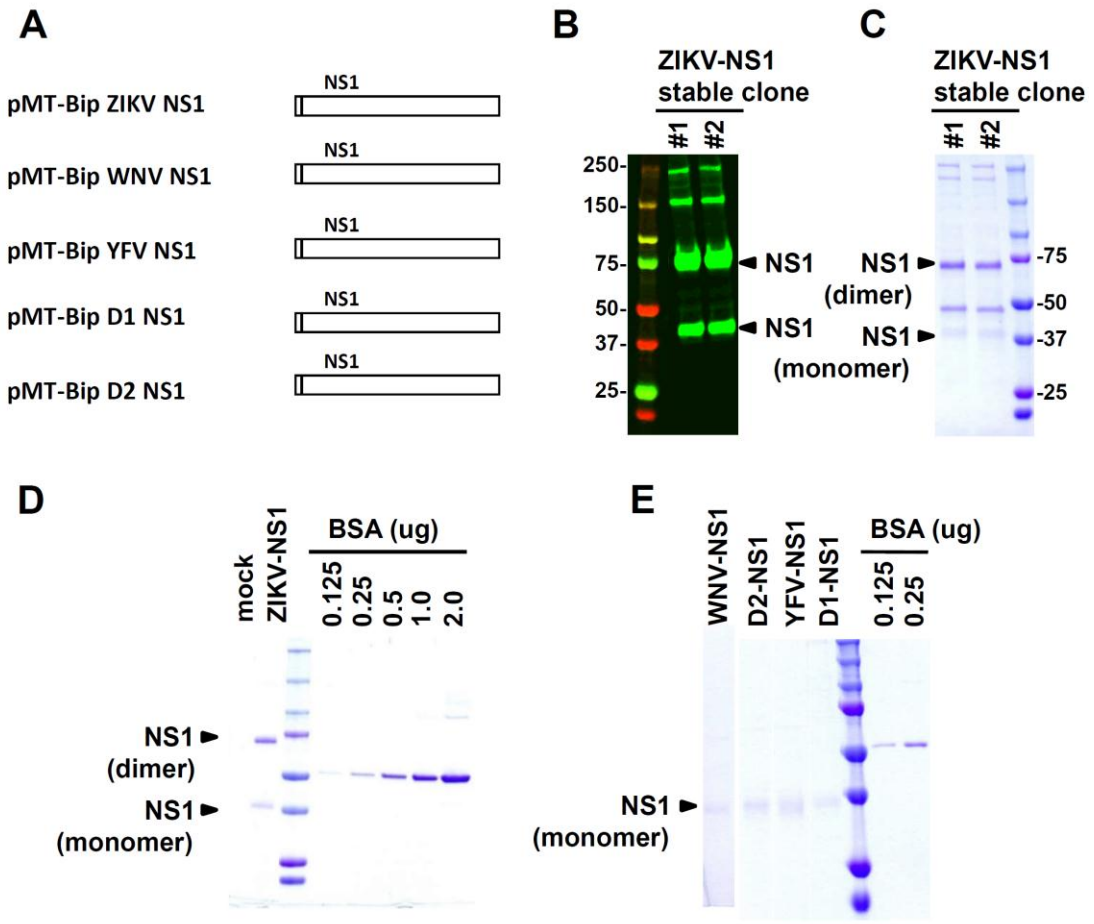


Fig. 10. (A) Schematic drawing of the pMT-based constructs. All constructs contain the N-terminal signal sequence from the insect Bip gene and a His-tag at the C-terminus. (B) Western blot analysis using anti-His Ab and (C) coomassie blue stained gel of ZIKV-NS1 stable clones. Purified NS1 proteins of (D) ZIKV, (E) WNV, YFV, DENV1, and DENV2.

NS1 IgG ELISAs for pDENV1, pWNV, and pZIKV infections

Panel of sera and plasma from patients with pDENV1, pWNV, and pZIKV infections were tested on ZIKV- (Fig. 11A), DENV1- (Fig. 11B), and WNV-NS1 IgG ELISAs (Fig. 11C). The ZIKV-NS1 ELISA showed no cross-reactivity with the pWNV (0 out of 6) and pDENV1 (0 out of 16) samples, while 3 out of 4 pZIKV samples were positive.

There was also no cross-reactivity seen on the DENV1-NS1 ELISA with pWNV and pZIKV samples while 13 out of 16 pDENV1 samples were positive. The WNV-NS1 ELISA was positive for 5 out of 6 (83%) of the pWNV samples and showed no cross-reactivity with pDENV1 and pZIKV samples (Table 1). Taken together, the NS1 IgG ELISA for primary infection panels had a sensitivity of 75% for ZIKV-NS1, 81.5% for DENV-NS1, and 83.3% for WNV-NS1 and 100% specificity for all three (Table 2). Thus, our ELISAs are able to distinguish between pDENV, pZIKV, and pWNV infections with high sensitivity and specificity.

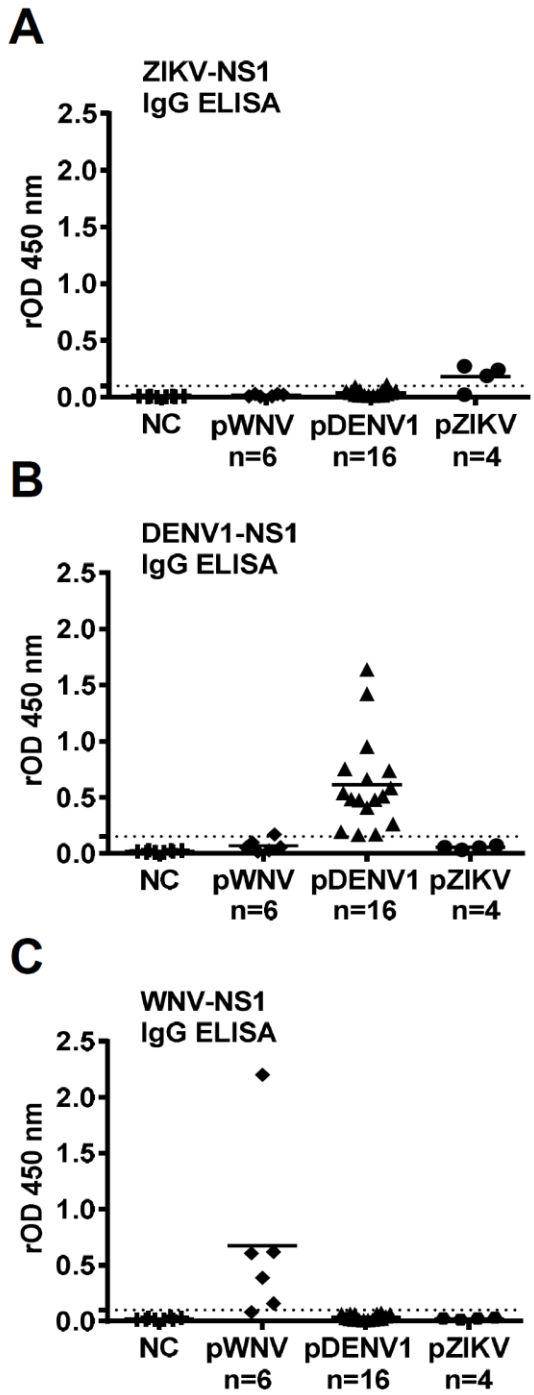


Fig. 11. Results of ZIKV-NS1, DENV1-NS1, and WNV-NS1 IgG ELISAs for pDENV, pZIKV, and pWNV panels. (A) ZIKV-NS1, (B) DENV1-NS1, and (C) WNV-NS1 IgG ELISAs in convalescent-phase samples from pDENV, pZIKV, and pWNV panels.

	Serum/plasma panels		
IgG ELISA	pWNV (n=6)	pDENV1 (n=16)	pZIKV (n=4)
ZIKV NS1 +	0/6 (0%)	0/16 (0%)	3/4 (75%)
DENV1 NS1 +	0/6 (0%)	13/16 (81%)	0/4 (0%)
WNV NS1 +	5/6 (83%)	0/16 (0%)	0/4 (0%)

.

Table 1. Positive rates of DENV1-, and ZIKV-, and WNV-NS1 IgG ELISAs in pDENV1, pZIKV, and pWNV panels.

	Primary infection	
IgG ELISA	Sensitivity	Specificity
ZIKV-NS1	75.0%	100%
DENV1-NS1	81.5%	100%
WNV-NS1	83.3%	100%

Table 2. Sensitivity and specificity DENV1-, and ZIKV-, and WNV-NS1 IgG ELISAs in pDENV1, pZIKV, and pWNV panels.

NS1 IgG ELISAs for sDENV, and ZIKVwpDENV infections

Previous studies have been able to show good sensitivity and specificity for serological tests on primary infection samples (Steinhagen, Probst et al. 2016) but have been able to clearly demonstrate the same results for patients who may have a secondary infection. Panel of serum and plasma samples from patients with sDENV and ZIKVwpDENV infections were tested on DENV1-NS1 and ZIKV-NS1 ELISAs. The sDENV samples were positive on the DENV-NS1 ELISA. ZIKVwpDENV

samples including 20 from Nicaragua and 19 from Brazil were positive on both DENV1- and ZIKV-NS1 ELISAs (Figs. 12A and 12B). Although there was some cross-reactivity of the sDENV samples on the ZIKV-NS1 ELISA, it was mostly able to distinguish between sDENV and ZIKVwpDENV (Fig. 12B). In order to further distinguish between sDENV and ZIKVwpDENV, the ratio of ZIKV-NS1 over DENV1-NS1 relative OD was taken with a cutoff value of 0.24 (Fig. 12C). The results of this show that the addition of the DENV1-NS1 ELISA can distinguish ZIKVwpDENV and sDENV infections. This was able to improve the sensitivity of the assay to 87.5% and the specificity to 81.3%.

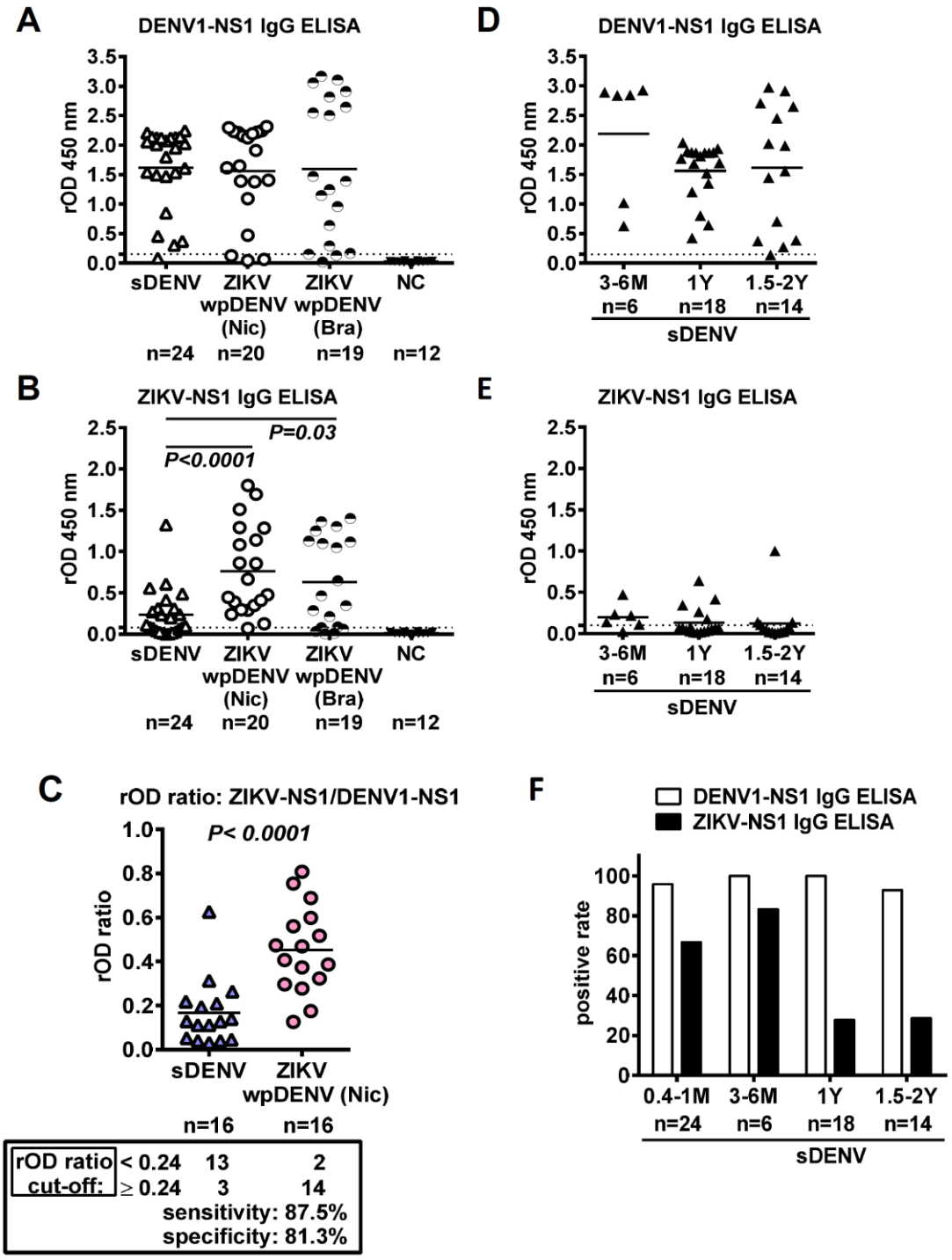


Fig. 12. Results of ZIKV-NS1 and DENV1-NS1 IgG ELISAs for sDENV and ZIKVwpDENV panels. (A) DENV1-NS1 and (B) ZIKV-NS1 IgG ELISAs in convalescent-phase samples from sDENV, ZIKVwpDENV (Nicaragua [Nic]) and probable ZIKVwpDENV (Brazil [Bra]) panels. (C) rOD ratio of ZIKV-NS1 to DENV1-NS1. The sensitivity and specificity are shown based on a cut-off rOD ratio at 0.24.

(D) DENV1-NS1 and (E) ZIKV-NS1 IgG ELISAs in post-convalescent-phase samples (3 months to 2 years POS) from sDENV panels. (F) Positive rates of DENV1- and ZIKV-NS1 IgG ELISAs in sDENV panels over time. Dotted lines indicate cut-off rOD values for ELISAs. Data are mean of two experiments (each in duplicates). Two-tailed Mann-Whitney test was used to compare two groups.

Cross-reactivity of sDENV panel to ZIKV-NS1 protein over time

To investigate whether the cross-reactivity of sDENV samples to ZIKV-NS1 is limited to the convalescent phase, 38 post-convalescent phase samples of sDENV cases ranging from 3 months to 2 years post infection were tested. Because the post-convalescent phase samples were collected at different time points, they were separated into different time ranges to determine if this affects the positivity rates. The rOD values for both DENV1-NS1 (Fig. 12D) and ZIKV-NS1 IgG ELISA (Fig. 12E) in these samples did not show a significant difference from those in the convalescent-phase samples. A decrease in the positivity rates of the sDENV panels in the ZIKV-NS1 IgG ELISA was observed, from 83.3% (3 to 6 months POS) to 27.7% (1 year POS) and 28.6% (1.5 to 2 years POS) (Fig. 12F).

Proposed algorithm to distinguish ZIKV and DENV infections

Based on the results of three ELISAs, the ZIKV-NS1 IgM, ZIKV-NS1 IgG and DENV-NS1 IgG ELISAs (as summarized in Tables 3, 4 and 5), we propose an algorithm to distinguish pZIKV, ZIKVwpDENV, and sDENV infections in dengue and Zika endemic regions, especially where they geographically overlap (Fig. 13).

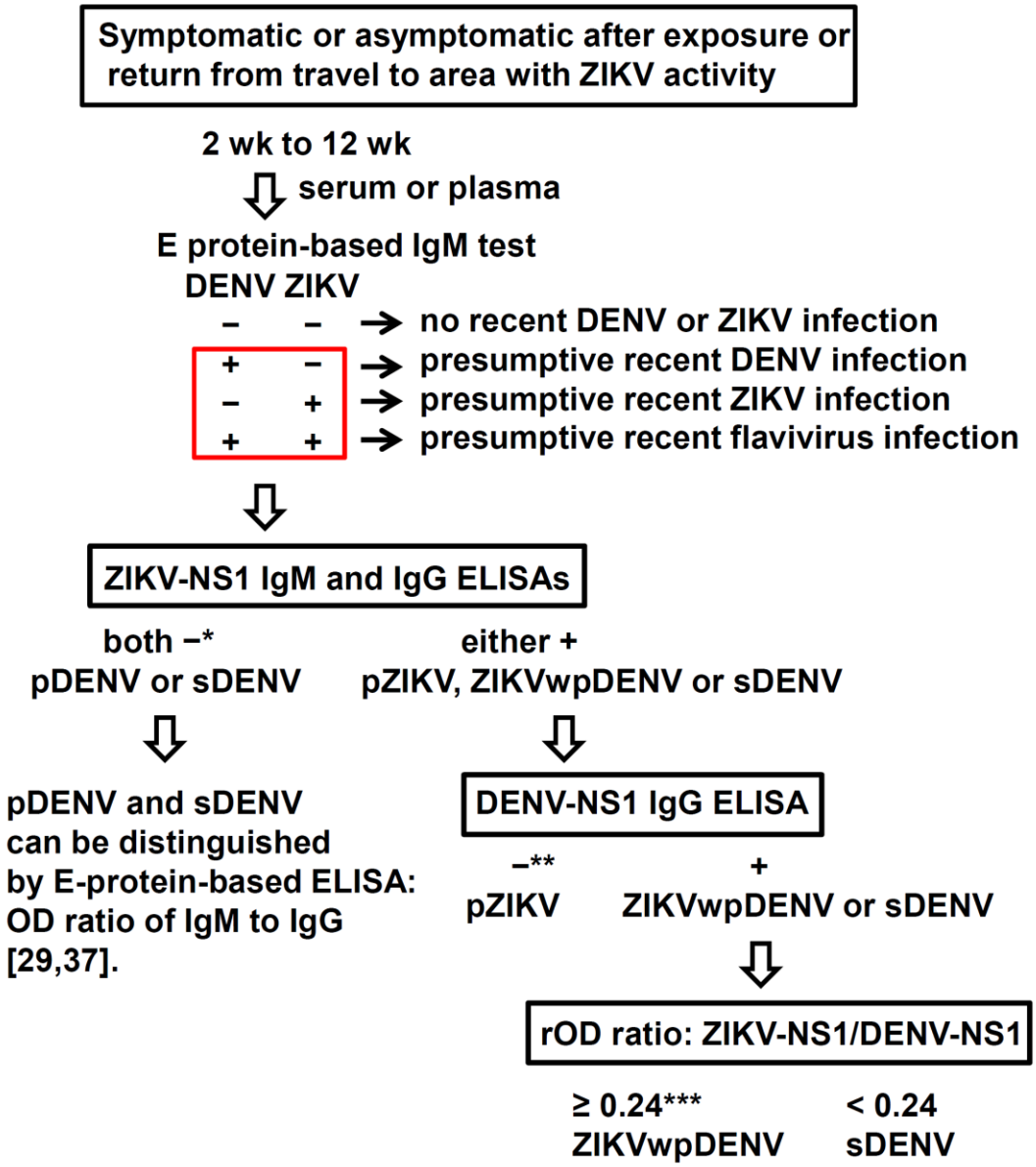


Fig. 13. Proposed algorithm of using three serological tests (without neutralization tests) to distinguish pZIKV, ZIKVwpDENV, pDENV, and sDENV infections in dengue and Zika endemic regions in the framework of CDC guidelines for laboratory diagnosis of ZIKV infection. *specificity=95%; **specificity=90.9%; ***sensitivity=87.5%, specificity=81.3% based on Figure 11 and Table 4.

	serum/plasma panels				
ELISA[a]	naïve n=12	pDENV1 n=16	pZIKV n=20	sDENV n=24	ZIKVwpDENV (Nic) n=20
ZIKV-NS1 IgM+	0/8 (0%)	0/9 (0%)[b]	18/20 (90%)	1/24 (4%)	11/20 (55%)
ZIKV-NS1 IgG+	0/12 (0%)	0/16 (0%)	1/20 (5%)	16/24 (67%)	19/20 (95%)
ZIKV-NS1 IgM+ or IgG+	0/8 (0%)	0/16 (0%)	18/20 (90%)	16/24 (67%)	20/20 (100%)
DENV1-NS1 IgG+	0/12 (0%)	13/16 (81.3%)	0/20 (0%)	23/24 (95.8%)	17/20 (85%)

Table 3. Results of ZIKV-NS1 IgM, ZIKV-NS1 IgG, and DENV1-NS1 IgG ELISAs in different serum and plasma panels. Abbreviations: naïve, flavivirus-naïve; pDENV1, primary DENV1 infection; pZIKV, primary ZIKV infection; sDENV: secondary DENV infection; ZIKVwpDENV, ZIKV infection with previous DENV infection; ELISA, enzyme-linked immunosorbent assay; Nic, Nicaragua. [a]Results were based on data from two experiments (each in duplicates) as described in Methods. [b]For pDENV1 panel, only those collected within 3 months post-onset of symptoms were tested for IgM.

	serum/plasma panels						
ELISA[a]	naïve n=12	pDENV1 n=16	sDENV n=24	DENV n=40	pZIKV n=20	ZIKVwp DENV (Nic) n=20	ZIKV n=40
ZIKV-NS1 IgM+	0	0	1	1	18	11	29
ZIKV-NS1 IgM−	8	9[b]	23	30	2	9	11
				31			40
ZIKV-NS1 IgG+	0	0	16	16	1	19	20
ZIKV-NS1 IgG−	12	16	8	24	19	1	20
				40			40
ZIKV-NS1 IgM+ or IgG+	0	0	16	16	18	20	38
ZIKV-NS1 IgM- and IgG−	8	16	8	24	2	0	2
				40			40
DENV1-NS1 IgG+	0	13	23	36	0	17	17
DENV1-NS1 IgG−	12	3	1	4	20	3	23
				40			40

Table 4. Numbers of positive and negative for ZIKV-NS1 IgM, ZIKV-NS1 IgG, and DENV1-NS1 IgG ELISAs and their combinations. Abbreviations: naïve, flavivirus-naïve; pDENV1, primary DENV1 infection; pZIKV, primary ZIKV infection; sDENV: secondary DENV infection; ZIKVwpDENV, ZIKV infection with previous DENV infection; ELISA, enzyme-linked immunosorbent assay; Nic, Nicaragua. [a]Results were based on data from two experiments (each in duplicates) as described in Methods. [b]For pDENV1 samples, only those collected within 3 months post-onset of symptoms were tested for IgM.

ELISA	Comparisons[a]	Sensitivity[b]	Specificity[b]
ZIKV-NS1 IgM+	ZIKV vs DENV	72.5%	96.8%
ZIKV-NS1 IgG+	ZIKV vs DENV	50%	60%
ZIKV-NS1 IgM+ or IgG+	ZIKV vs DENV	95%	60%
ZIKV-NS1 IgM− and IgG−	DENV vs ZIKV	60%	95%
DENV1-NS1 IgG+	DENV vs ZIKV	90%	57.5%
DENV1-NS1 IgG+	DENV and ZIKVwpDENV vs pZIKV	88.3%	100%
DENV1-NS1 IgG−	pZIKV vs ZIKVwpDENV and sDENV	100%	90.9%

Table 5. Sensitivity and specificity of three NS1 ELISAs and their combinations. Abbreviations: pZIKV, primary ZIKV infection; sDENV: secondary DENV infection; ZIKVwpDENV, ZIKV infection with previous DENV infection; ELISA, enzyme-linked immunosorbent assay. [a]ZIKV includes pZIKV and ZIKVwpDENV infections. DENV includes primary DENV1 and sDENV infections. [b]Data were based on Table 3 and Table 4. Data from flavivirus-naïve samples were not included in the analysis.

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CHAPTER 4

SUMMARY AND DISCUSSION

Overview

In this study, we first investigated the immunogenicity of human codon-optimized DNA vaccines expressing mature and mixed DENV particles by examining the characteristics of the Abs produced by immunized mice. We then tested a DNA-prime and virion-boost strategy to examine the Ab response and compare it to the Ab response of DNA alone. Finally, we aimed to develop a serological assay to distinguish DENV and ZIKV infections, which is critical to further our understanding of ZIKV pathogenesis and complications during pregnancy.

WB results from expression and VLP production of our D1 constructs (Figs. 1A-B) show that human codon-optimized constructs with furin increase the expression and production of VLPs greatly compared with non-optimized constructs with furin, whereas a mild increase is found in constructs without furin. This supports our hypothesis that human codon-optimized DNA constructs can improve the expression of VLPs as a better candidate vaccine for DENV.

To explore the immunogenicity of mature virions in vivo, we immunized one group of female C57BL/6 mice with pCBD1-WT or pCBD1-F, which express mixed or mature VLPs, respectively. Sera collected from the mice groups were subjected to ELISA and FRNT assays. Results of total anti-DENV Abs based on 3-layer ELISA and $FRNT_{50}$ titers are generally in agreement with the expression and VLP production of these D1 constructs based on WB analysis, in which there was a higher expression level of D1h than D1hF in vitro (Figs. 1A-B, Figs. 4A-B). Mice were able to produce anti-DENV Abs with similar titers induced by both DNA vaccines, suggesting that the vaccine producing mature VLPs (pCBD1-furin) is immunogenic compared with pCBD1-WT (Fig. 4A). Some variation in titers among individual mice

was observed (Fig. 4A), which could be due the technique of handling and vaccine administration. Both DNA vaccines induced low $FRNT_{50}$ titers (Fig. 4B). This may be due to the low immunogenicity of DNA vaccine compared with others and lack of or low in vivo efficacy that have been reported (Danko, Beckett et al. 2011, De Filette, Soehle et al. 2014).

As an approach to improve the efficacy of our DNA vaccine in vivo, we applied a DNA-prime and virion-boost immunization strategy. Introducing a virion-boost using formalin-inactivated D1 virions administered subcutaneously showed that mice were able to produce anti-DENV Abs with similar titers when compared to the DNA only groups, with the D1-WT DVVsc formalin group showing a higher ELISA titer than the D1-F DVVsc formalin group (Fig. 5A). $FRNT_{50}$ titers in these two groups were both higher than those of the DNA only groups but did not show difference between each other (Fig. 5B). In order to determine which route of administration would result in better titers, ip immunization of formalin-inactivated DENV1 virion was also tested. Although both routes of immunization showed high ELISA and $FRNT_{50}$ titers, there was no significant difference in the ELISA and $FRNT_{50}$ titers between the ip and sc routes of immunization (Fig. 7C-D), suggesting that both routes are effective in eliciting neutralizing Abs. Because the ip route showed slightly higher $FRNT_{50}$ titers, we continued to use this route for our future immunization groups. In addition to vaccine delivery routes, we also tested H_2O_2 as another method of virion inactivation. Although formalin is the more common method for inactivation, recent studies have shown that using H_2O_2 is promising (Amanna, Raue et al. 2012, Walker, Raue et al. 2012, Pinto, Richner et al. 2013). H_2O_2-inactivated WT and F groups showed higher ELISA (Fig. 8A) $FRNT_{50}$ titers (Fig. 8B) than the DNA-only groups. There was no significant difference between using formalin or H_2O_2 inactivation in the ELISA titer

(Fig. 8C). A higher NT_{50} titer in the D1-WT DVVip formalin group than the H_2O_2 group was seen (Fig. 8D), suggesting that formalin-inactivation may be more effective.

We further tested the titers of binding Abs from pooled sera to DENV from the DVV WT and F groups to mixed, immature, and mature DENV1 virions. As expected, the WT group was able to show higher ELISA titers than the F group when tested with vero DENV1 mixed virions and 293T DENV1 mixed and immature virions (Figs. 6A and 6B). When tested with DENV1 mature particles, the ELISA titers were lower compared with those to the mixed and immature DENV1 virions (for both the WT and F groups) (Fig. 6B), suggesting that the mature particles were less recognized by the Abs. Comparing the ELISA titers to immature DENV1 between the WT and F groups, there was more than 1 log difference, whereas comparing the ELISA titers to mature DENV1 between the WT and F groups, there was less than 0.5 log difference. In order to better compare the binding of Abs to mature and immature DENV1, we looked at the ratio of ELISA titer to mature to that of immature particles. The ratio was lower in the WT group, suggesting that they are able to better differentiate between the mature and immature particles.

Due to the recent ZIKV outbreak in DENV endemic areas, a serological diagnostic assay to differentiate the two has become increasingly important. We used ZIKV-VLP, DENV1-IV, and WNV-VLP IgG ELISAs to show that E-protein based ELISAs are unable to differentiate between pWNV, pDENV1, pZIKV, sDENV, and ZIKVwpDENV infections (Fig. 9). Our results demonstrate that our NS1 IgG ELISA is able to differentiate between pDENV, pZIKV, and pWNV convalescent samples (Fig. 11, Tables 1-2). We needed to further address secondary infections due to the geographic co-circulation of dengue and Zika. The cross-reactivity seen in the ZIKVwpDENV panel on the DENV1-IgG ELISA suggests that a single test is not

enough to accurately diagnose the infection (Fig. 12A). An additional assay using a ZIKV-NS1 ELISA was developed to test sDENV and ZIKVwpDENV panels (Fig. 12B). Using both the DENV1 and ZIKV-NS1 ELISAs in combination is able to differentiate sDENV and ZIKVwpDENV infections, and using the rOD ratio based on the two ELISAs with a cutoff of 0.24 is able to further separate this distinction (Fig. 12C). These results demonstrate a simple and relatively quick way to diagnose these two infections. Currently, using our proposal of three serological tests to distinguish pZIKV, ZIKVwpDENV, and sDENV infections is a novel method. The algorithm of using these three ELISAs can be applied to clinical diagnoses of these infections (Fig. 13). This is particularly relevant for pregnant women both residing and returning from dengue endemic regions, most of whom present with asymptomatic infection or might miss the golden period of detection by RT-PCR, good serological tests can help us to better understand and treat the ZIKV related complications during pregnancy. Because the algorithm calls for ELISAs, the testing can bypass the time that is needed to do PRNTs in the current CDC guidelines, thus improving the cost and time-efficiency.

Limitations and future plans

There are several limitations of this study. In Aim 1, one main limitation was the sample size of our mice. Also, there may have been variations in the amount DNA vaccine delivered and sera collected due to the handling of the mice. As this was the initial study conducted, there may be some differences in the quality of techniques compared to later mouse studies. Further studies using a larger number of mice is required.

There were also limitations in Aim 2. This study was conducted to look at the binding and neutralizing anti-DENV1 Abs. Further studies looking at T-cell responses, ADE, percent anti-FL Abs, anti-pr, and anti-E Abs should be done. Moreover, challenge experiments in AG129 mice model to test the protective efficacy of these vaccines or passive transfer experiments in AG129 mice are needed for vaccine development. These are the ongoing collaborative research with our collaborators at CDC Fort Collins. For this aim, we only looked at using inactivated virions as a form of protein-boost. For future experiments, recombinant E proteins can be generated to use in vaccinations.

For Aim 3, one limitation is that the sample size is small and future studies would require larger sample sizes to further validate the observations. Also, future studies using sequential samples from various time-points after different flaviviral infections are needed to further extend this approach and validate the assays. Finally, our assays used only the DENV1 serotype and ZIKV-NS1. Future studies should include the other DENV serotypes both individually and as a mixture, as well as other relevant flaviviruses such as JEV, YFV, WNV, and TBEV to further distinguish these from ZIKV.

Concluding summary

In summary, our study showed that human-codon optimized constructs increased the expression and production of VLPs. Using human codon-optimized DNA vaccines to test our hypothesis, we showed that DNA vaccine expressing mature VLPs can induce similar anti-DENV Abs and NT Abs compared with DNA vaccine expressing mixed VLPs. Using a DNA-prime and inactivated virion-boost regimen,

we showed improved ELISA and NT titers compared to DNA alone through different delivery and virion inactivation methods. Our study also showed that an algorithm using three ELISAs are a cost- and time-effective way to accurately differentiate ZIKV and DENV infections. Together, these three aims provide new strategies in dengue vaccine development to prevent DENV infection and the new methods for serodiagnosis of flaviviruses in endemic regions.

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