COMPUTATIONALLY OPTIMIZED BROADLY REACTIVE ANTIGEN (COBRA) DESIGN FOR A UNIVERSAL DENGUE VACCINE

by

NAOKO UNO

(Under the Direction of Ted M. Ross)

ABSTRACT

Developing a comprehensive vaccine for Dengue virus (DENV) has been challenging. Any potential vaccine needs to protect against strains from all four serotypes of the virus to avoid potential antibody dependent enhancement (ADE). ADE can occur when pre-existing antibodies to a virus from one DENV serotype do not neutralize, but enhance a heterotypic infection of a DENV from another serotype. Therefore, it is critical to produce a vaccine against viruses representing all four DENV serotypes without enhancing disease. In the current study, we developed and tested DENV subviral particle (SVP) vaccine targeting the envelope (E) glycoprotein by designing consensus sequences using computationally-optimized broadly reactive antigen (COBRA) methodology.

DENV E sequences were obtained from GenBank and a layered, consensus-building approach was used to derive four final COBRA DENV sequences. COBRA and wild-type SVPs were expressed from 293T cells using a mammalian expression vector encoding prM-E genes. Female C57BL/6 mice (age 6-8 weeks) were vaccinated intramuscularly three times at 4-week intervals with 100mg total SVP plus Imject Alum. Vaccines were prepared as individual or tetravalent SVP formulations. Immune sera were

collected and total IgG antibody titers to DENV E were analyzed by ELISA and the ability to prevent virus infection *in vitro* was assessed in a focus reduction neutralization test ($FRNT_{50}$) against a panel of 12 prototype and modern strains from all four serotypes.

Mice vaccinated with wild type DENV SVPs expressed anti-E IgG antibodies that were specific to strains in each homologous serotype. The elicited antibodies neutralized serotype specific viruses. COBRA DENV SVPs elicited a broader breadth of antibodies that neutralized various strains across all four serotypes. Similar results were seen in rhesus macaques (*Macca mulatta*) that were immunologically naïve to DENV or pre-immune with antibodies to DENV. One COBRA DENV E immunogen neutralized all 12 strains of DENV *in vitro*, comparable to tetravalent SVP vaccination. This is a promising vaccine candidate based on broad protection against strains representing all four serotypes of DENV in both naïve and pre-immune populations.

INDEX WORDS: Dengue virus, universal vaccine, cross-protection, pre-immunity

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DEDICATION

To my parents, for their unwavering support and patience in all of my endeavors.

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CHAPTER 1

INTRODUCTION

Dengue virus (DENV) is the most prevalent arbovirus worldwide, found in over 100 tropical and subtropical countries. Over half of the global population is at risk for DENV infection, with 100 million symptomatic cases being reported every year [1]. There are four serotypes of the virus, DENV1-4. In endemic countries, more than one serotype circulates [2]. Cross protective immunity is not long-lasting, and infection with more than one serotype can lead to severe disease and mortality [3]. Antibody dependent enhancement (ADE) is theorized to be the cause of severe disease after sequential infection with a different serotype. This occurs when pre-existing antibodies to one DENV serotype do not neutralize but enhance a heterotypic infection [4, 5]. The non-neutralizing antibody will bind to the virus and facilitate entry via Fcγ receptors (FcγR) on circulating monocytes. The virus alone cannot infect these FcγR-bearing cells; thus, viral replication and burden are increased as a result. It is critical to produce a vaccine against viruses representing all four DENV serotype without enhancing disease in both immunologically naïve or pre-immune populations. The envelope glycoprotein (E) is the main target for neutralizing antibodies and is responsible for receptor binding and fusion [6]. The membrane and pre-membrane (M and prM, respectively) proteins also make up the structural shell of the virus.

Currently, there is one licensed vaccine for DENV- Sanofi Pasteur's chimeric yellow fever virus (YFV) vaccine, Dengvaxia [7]. This vaccine inserts DENV prM-E into an attenuated YFV backbone. The overall efficacy is about 60% and it is recommended for

ages 9-45 in endemic countries [8]. Possible vaccine enhancement in younger children and seronegative individuals has caused debate over the safety and efficacy of this vaccine [9-11]. One theory to explain the low efficacy for the vaccines during recent clinical trials is that they utilize E gene inserts from wild-type viruses that did not match the currently circulating strains. Other leading candidates are also live viral vaccines. DENVax by Takeda consists of live attenuated DENV2 and chimeric DENV 1, 3, and 4 prM-E in live attenuated DENV2 backbones [12], and TV003 by the National Institute of Allergy and Infectious Disease consists of a tetravalent formulation with the four DENV1-4 strains attenuated by a 30 nucleotide deletion in the 3' end [13].

One theory for the low efficacy seen in Dengvaxia is that it utilizes E gene inserts from wild-type viruses that do not match the currently circulating strains. The other two candidates also use outdated strains: Dengvaxia strains are from Asian strains that were isolated between 1978 to 1988. DENVax uses Asian strains from 1964 to 1976. TV003 uses strains isolated from 1974 to 1978. With DENV spread dramatically increasing in the last decades, many of the strains and genotypes that had been regional are now global. This results in an increase of outbreaks when a new strain is introduced [2]. Studies that compared antigenic differences between circulating genotypes and strains used in Dengvaxia showed that vaccine efficacy decreased as amino acid sequence mismatch increased, especially in younger children [14, 15]. The strains that were circulating decades ago are not circulating in many countries [16, 17], though more virulent strains tend to last longer [18].

To address the effects of strain mismatch, novel DENV vaccines can be designed using multiple rounds of consensus building known as computationally-optimized broadly

reactive antigens (COBRA). DENV E immunogen can be produced that contain conserved epitopes spanning many genotypes. The E immunogen can be delivered via multiple platforms

The long-term goal of this project is to develop a safe and effective vaccine that protects against all four serotypes of DENV. The overall objective to attain this long-term goal is to construct a computationally optimized broadly reactive antigen (COBRA) for DENV envelope (E) glycoprotein that elicits broad breadth of protective immune response in both naïve and pre-immune populations. The central hypothesis is that DENV COBRA E vaccination will elicit neutralizing antibodies against all four serotypes of DENV virus in naïve and pre-immune populations. The rationale is that creating a broadly reactive vaccine against modern strains will be more efficient and cost effective while eliminating potential competition and bias seen in tetravalent formulations.

The contribution of the results will be significant because the vaccine would offer broad protection for current circulating strains from all four serotypes regardless of immune status. Furthermore, we will better our understanding of cross-protective epitopes, correlates of protection, and elucidate the complexities of vaccine elicited neutralization versus enhancement.

The proposed research is innovative, because it represents a substantive departure from the status quo by designing a monovalent vaccine for all four serotypes. This will eliminate competition between the four components as well as decrease production costs.

To test the COBRA vaccine, the following specific aims will be pursued:

Specific Aim 1: Design and construct DENV COBRA subviral particles (SVPs).

The working hypothesis is that DENV COBRA E immunogens can be constructed from consensus-based design and displayed on SVPs by transfecting mammalian cells with plasmids containing DENV prM-E constructs.

Specific Aim 2: Determine immunogenicity and breadth of immune response of mice vaccinated with DENV COBRA SVPs and wild type SVPs.

The working hypothesis is that DENV COBRA SVP vaccination will elicit more neutralizing antibodies against all four serotypes of DENV virus compared to wild type SVP vaccination in C57BL/6 mice. A broadly neutralizing monovalent COBRA candidate will be identified.

Specific Aim 3: Determine immunogenicity and breadth of immune response elicited by DENV COBRA SVP vaccination in naïve and pre-immune non-human primates.

The working hypothesis is that monovalent DENV COBRA vaccination will elicit broadly neutralizing antibodies in naïve and pre-immune populations comparable to tetravalent formulation in non-human primates.

CHAPTER 2

LITERATURE REVIEW[1]

[1]Uno, Naoko, and Ted M Ross. "Dengue virus and the host innate immune response." *Emerging microbes & infections* vol. 7,1 167. 10 Oct. 2018. Reprinted here with permission of the publisher.

Dengue Epidemiology

Dengue virus (DENV) is the most prevalent arbovirus worldwide, found in over 100 tropical and subtropical countries [19]. It is transmitted mainly by the *Aedes aegypti and Aedes albopictus* mosquitoes. Over half of the global population is at risk for dengue infection; approximately 400 million infections are reported annually, mainly in the Americas and Asia [20]. There are four genetically distinct serotypes of the virus, DENV 1-4 [21]. Due to the antigenic differences between the serotypes, infection with one serotype will confer long-lasting immune protection against that serotype only, while cross-protection against other serotypes are short-term. In endemic countries, more than one serotype of DENV circulates [2].

Primary infections may cause a rash and fever, but many infections are asymptomatic. Cross protective immunity is transient, with neutralizing antibody being detected up to 2 years after infection [22, 23]. Secondary infections, however, are known to cause severe disease, specifically after a heterotypic infection [24]. The exact cause of this is unknown, but the phenomenon of antibody-dependent enhancement (ADE) may cause increased pathogenicity and virulence [25]. ADE occurs when antibodies from a previous heterotypic infection do not neutralize a secondary infection with a different subtype but still bind to viral proteins. This creates a virus-antibody complex phagocytosed by cells that are not usually infected via Fcγ receptors, specifically monocytes via FcγIIa receptor [26]. This results in increased viremia and pathology. Severe disease is seen in only 1% of DENV cases, however mortality in severe cases can have a rate of up to 20% [24].

Surveillance and diagnosis for DENV is difficult because many endemic countries do not have the resources to provide accurate, rapid, and specific analysis, crucial for reducing mortality and spread [27]. Rapid diagnostic tests often cross-react with other flavivirus, such as Zika virus, but sensitive tests like RT-qPCR and neutralization assays are more costly and time-consuming [28]. There are no approved antivirals for DENV. Treatment for clinical manifestations of the acute disease are paracetamol for high fever and oral or intravenous fluid intake [27]. Asymptomatic dengue infections contribute to mosquito transmission- 25% of infectiousness occurs prior to onset of symptoms [29].

DENV Virion

DENV is part of the *Flaviviridae* family, which also includes Zika, Yellow Fever, Japanese Encephalitis, and West Nile viruses. They are enveloped and spherical, with a positive-sensed, single-stranded RNA (ssRNA) genome that encodes one open reading frame (ORF) with 3 structural (capsid, precursor membrane, and envelope) and 7 non-structural (NS) proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). The genome is approximately 11,000 kilobases in length, containing a type I cap at the 5' end and lacking a 3' poly(A) tail [30]. The virus structure consists of a well-organized outer shell with an icosahedral symmetry, a lipid bilayer, and a poorly ordered nucleocapsid core that encapsulates the RNA genome [31].

Out of the three structural proteins, the envelope glycoprotein (E) is the main target for neutralizing antibodies and is responsible for receptor binding and fusion [30]. It is a class II fusion protein, with 90 E dimers lying flat on the surface of the virion [31]. The dimer is found as the major component of the virus envelope, made up of three distinct

domains [32]. Domain I (DI) is a central β barrel, domain II (DII) contains the fusion peptide, and domain III (DIII) is an immunoglobulin-like segment [33]. Neutralizing antibodies have been found to bind to DIII [34, 35] and to the envelope dimer epitope [36, 37]

The membrane protein sits below the E protein on the surface of the mature virion. Immature virus particles, on the other hand, have a precursor membrane (prM) protein that forms protruding trimers with E, which create a "spiky" appearance instead of the smooth, icosahedral structure of the mature form [38]. The capsid protein is found below the outer protein shell and the lipid bilayer. It is not as well-ordered as the other structural proteins, and it is difficult to discern the viral RNA from the capsid during cryo-EM imaging [39].

The NS proteins are responsible for viral replication and host immune evasion. The exact roles of NS1 and the transmembrane proteins NS2a, NS2b, NS4a, and NS4b are not well characterized. NS1 is dimeric in early stages of infection and secreted in hexameric form in later stages [40, 41]. The NS1 dimer is located on the lumen side of the ER, yet it plays an essential role in viral RNA replication, since deletion of NS1 from the viral genome inhibits replication [42]. Through transmembrane interaction with NS4a and NS4b, NS1 will help form vesicles for virus replication, called the viral replication complex (RC), and colocalize with double-stranded RNA (dsRNA)[42, 43] (Fig 2.1). It also modulates infectious virus particle production by interacting with structural proteins prM/E [44]. NS4a plays a role in membrane alteration, in order to form the RC [45]. NS2a is crucial for viral RNA synthesis and virion assembly [46]. NS2b binds to NS3 and forms the functional NS3 protease [47]. NS4b interacts with the NS3 helicase domain [48].

NS3 and NS5 are the best characterized out of the NS proteins, both having enzymatic activity essential for viral replication. NS3 functions as the viral protease and helicase. The N-terminal domain of the NS3 is essential for protease activity [47], cleaving the viral polypeptide into structural and non-structural proteins. The C-terminal end containing the helicase domain [48], is necessary for unwinding the RNA duplex during replication. Cofactor NS2b is necessary for the protease to be functional. NS5 is the largest and most conserved of the *Flavivirus* proteins [30]. It functions as the viral RNA-dependent RNA polymerase (RdRp), and the N-terminal domain contains the methyltransferase (MTase), which is responsible for 5'-RNA capping of the new viral genomes [49].

DENV Life Cycle

DENV is spread to humans from an infected mosquito. The exact cell types and binding receptors on human cells are unknown. Many putative receptors have been proposed as candidates, including receptors such as heparan sulfate, glycosphingolipid nLc_4Cer, DC-SIGN, mannose, CD14, and HSP70/90 [50]. Cells that are permissive for DENV infection are dendritic cells (DCs), endothelial cells, fibroblasts, keratinocytes, macrophage, mast cells, and monocytes [51].

After receptor binding to the DENV E glycoprotein, the virus will enter the cell via clathrin-mediated endocytosis and a drop in endosomal pH triggers conformational change of the virion that leads to membrane fusion and release of viral genome into the cytoplasm [52]. Replication occurs in association with virus-induced cellular membrane structures that form the RC [53]. The positive-sensed RNA genome can be immediately translated to a polyprotein by the host ribosome [54]. The polyprotein is cleaved by host and viral

proteases, with structural prM and E proteins assembling in the ER lumen and the nonstructural (NS) proteins localizing in virus-induced membrane vesicles for RNA synthesis [55].

After protein translation and genome replication, the virus is assembled and transported to the Golgi apparatus, where the prM is cleaved by host furin protease to form the mature virion[55]. The mature, infectious virion is released by exocytosis [56]. However, prM cleavage is not efficient and immature and partially mature virions can be secreted [57]. Hexameric form of NS1 is also secreted [41] (Fig 2.1). Immature particles are non-infectious by themselves, though non-neutralizing antibodies to prM can aid in virus uptake in Fc-receptor bearing cells [58].

DENV Infection of Vector

DENV is spread to humans from an infected mosquito. The primary mosquito vectors for human DENV transmission are *Aedes aegypti* and *Aedes albopictus* [59]. The female mosquito takes a blood meal and the virus goes into the midgut, replicating in the midgut epithelium, disseminating, and reaching the salivary glands for transmission to a new host [60, 61]. There exact receptor for binding to mosquito cells is unknown [60]. DENV may bind to putative heat shock protein receptors on *Aedes albopictus* cell line C6/36 [62].

The mosquito immune system is divided into three compartments: The midgut, the hemocoele (body cavity), and salivary glands [63]. The virus disseminates into the hemocoele and can then infect secondary organs. Ultimately the virus infects and replicates

in the salivary glands, so it can be transmitted to a person when the mosquito takes another blood meal [60, 64, 65].

The mosquito immune system responds to a pathogen by antimicrobial peptide (AMP) produced in the fat body organ, melanization of the hemolymph, and phagocytosis by hemocytes [64, 66]. The viral PAMPs bind to insect PRRs (the exact cell receptors for DENV are unknown) and activate various signaling pathways. including Toll [67, 68], JAK-STAT [69], and RNA interference (RNAi) pathways [70-72]. These pathways induce immune responses to limit viral replication. The exact DENV PAMPs that induce immune responses are unknown, though transcriptomic analysis implicate NS3, NS5, and C proteins [73].

Innate Immune Response Against DENV

Viral Sensing

DENV is transmitted to people via a mosquito bite. Following infection, the virus initially replicates in skin cells, such as keratinocytes and Langerhans cells [51]. This will trigger a variety of host innate immune responses. Innate immune cells are the first to respond to infection by using pattern recognition receptor (PRR) recognizing pathogen-associated molecular patterns (PAMPs)[74, 75]. These immune cells include dendritic cells (DCs), macrophages, and monocytes. PRR recognition will trigger production of cytokines and chemokines which induce an antiviral state. The PRRs that are associated with DENV recognition are cytoplasmic retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), along with endosomal Toll like receptor 3

(TLR3) and TLR7[76, 77]. Activation of these receptors by DENV recognition induces type 1 interferon (IFN) responses.

RIG-I and MDA5 are RIG-I-like receptors (RLRs), located in the cytoplasm of a variety of cells, including myeloid, epithelial, and central nervous system. They sense phosphate- containing RNA in the cytoplasm and long dsRNA [74]. Thus, they are an essential part of the innate immune response against virus, sensing viral replication in the cytoplasm. The exact RNA ligands on DENVs that these receptors recognize are unknown. Following virus recognition, the RLRs translocate to the mitochondrial membrane and activate mitochondrial antiviral signaling (MAVS) protein found on the surface of the mitochondria via the caspase activation recruitment domains (CARD) of the RLRs and MAVS. This leads to activation of TANK binding kinase 1 (TBK1), IKB kinase-e (IKKe), phosphorylating interferon regulatory factors (IRF3) and IRF7, which enter the nucleus to induce production of type I IFNs such as IFN-β [78] (Fig 2.2).

Another type of PRR that recognizes viruses are the toll-like receptors (TLRs). Two TLRs critical in the innate response to DENV infection are TLR3 and TLR7. TLR3, which is the primary TLR for DENV, recognizes dsRNA in endosomal compartments and TLR7 recognizes ssRNA in DC endosomal compartments [79]. TLR3 activation causes phosphorylation of TIR-domain-containing adapter-inducing IFN-β (TRIF), interacting with TNF-receptor associated factor 3 (TRAF3) and TBK1/IKKe to induce IFN-α/β stimulating genes (ISGs) and chemokines [75]. TLR3 acts synergistically with RIG-I and MDA5 in producing an antiviral state against DENV infection [76]. TLR7 recognition of ssRNA, including DENV genomic fragments, uses the myeloid differentiation primary response gene 88 (MyD88)-dependent signal pathway to induce pro-inflammatory

cytokines by recruiting TRAF6 to activate inhibitor of nuclear factor-κB-kinase (IKK)α/IKKα/ IKKγ, and activate nuclear factor-kB(NF-kB)[77] (Fig 2.2).

The cyclic GMP-AMP synthase (cGAS) and stimulator of interferon gene (STING) pathway is also activated during DENV infection by the cGAS PRR, despite the fact that this pathway recognizes cytoplasmic DNA [80]. DENV damages the mitochondria by inducing swelling and other morphological changes [81] thus the cGAS-STING is activated by mitochondrial DNA (mtDNA) released in the cytosol [82]. After mtDNA sensing, the cGAS nucleotidyl transferase produces second-messenger cyclic GMP-AMP (cGAMP) that binds to STING, leading to activation of TBK1, phosphorylation of IRF3, and production of type I IFNs [83] (Fig 2.2). This release of mtDNA has been shown to activate TLR9, an endosomal PRR that recognizes DNA containing nonmethylated CpG motifs, in human DCs *in vitro* [84]

Type I IFN response

Production of type I interferons (IFNs) inhibits DENV infection of other monocytes [85]. These cytokines bind to IFNα/β receptors (IFNARs) on the surface of nearby or infected cells, activating the Janus Kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, and producing IFN-stimulated genes (ISGs) to further promote antiviral activity [86]. IFNα/βcytokines bind to IFNAR, activating JAK1 and tyrosine kinase 2 (TYK2), leading to the phosphorylation and dimerization of STAT 1 and STAT 2 which forms a complex with IRF9. The complex will translocate to the nucleus where they induce transcription of ISGs by the IFN-stimulated response element (ISRE) (Fig 2.3).

Complement system response

The complement system is also an important part of innate immune response to the virus. The mannose binding lectin (MBL) pathway induces neutralizing protection against DENV. MBL binds to surfaces containing mannose glycans, and this protein will recognize the surface of DENV [87]. Recognition of DENV by the MBL complex will induce cleavage of C4 and C2 by MBL-associated serine protease-2 (MASP-2) and deposit C4b and C2a on the surface of the virion, making the C3 convertase [88]. The following classical complement cascade includes the formation of C5 convertase and the C5b-9 membrane attack complex (MAC) to induce lysis, recruitment of phagocytes, and inflammation [89] (Fig 2.3b).

Other innate immune responses

RNA interference (RNAi) for antiviral defense in plants and invertebrates have been well characterized, including the DENV vector *Aedes aegypti* [70]. There is also evidence of vertebrate systems using the RNAi to inhibit DENV infection, though it is not as well understood [90, 91]. RNAi is a sequence-specific process to regulate gene expression mediated by small interfering RNA (siRNA) or microRNA (miRNA). Dicer will create siRNA from long dsRNA, while both Drosha and Dicer are needed to create miRNA. These small non-coding RNAs will associate with RNA-induced silencing complex (RISC) in the cytoplasm to target mRNA for inhibition or degradation by Argonaute (Argo) proteins. During viral infection of mammalian hosts, viral RNA is recognized by the miRNA-RISC assembly and targeted for silencing or degradation [92] (Figure 2.2). Knockdown of essential components of RNAi (Dicer, Drosha, Argo1, Argo2)

resulted increased DENV viral titer in human cell line Huh7, implicating RNAi as a regulator of DENV replication [93]. There have been a number of cellular miRNA that are modulated during DENV infection, such at let-c, miRNA-30e*, and miRNA-126-5p [94].

Autophagy is a natural cellular process to maintain homeostasis, regulating cell degradation usually in response to starvation [95] and disease [96]. Double membrane vacuoles, called autophagosomes, will engulf cytoplasmic material and fuse with the lysosome for degradation. Thus, autophagy is an important system to clearing the host of foreign pathogens such as viruses. Autophagy has been shown to be activated in DENV infections and has anti-viral or pro-viral activity depending on cell type. Autophagy inhibits replication in monocytes, specifically under ADE conditions which make these cells highly susceptible to infection [97]. Reticulophagy, a selective autophagy process for ER homeostasis, reduces DENV via its FAM134B receptor in endothelial cells [98]. In liver cells, autophagy has a pro-viral effect. DENV blocks the autophagosome from fusing with the lysosome, and instead uses the vacuoles for replication [99], assembly and maturation [100], and evading neutralizing antibodies during transmission [101].

Apoptosis is a highly-regulated process of self-destruction that cells undergo in response to stimuli such as redundant or dangerous cells like tumors or pathogen-infected cells. There are two main apoptotic pathways, the intrinsic (or mitochondrial) and extrinsic, though the two are linked and converge at the execution phase, where the cell undergoes DNA fragmentation, degradation of the cytoskeleton, and formation of apoptotic bodies that are ultimately engulfed by surrounding phagocytes [102]. DENV proteins have been shown to activate apoptosis inside infected cells. The capsid protein nuclear localization interacts with death-associated protein 6 (DAXX) and triggers Fas-mediated apoptosis in

liver cells [103]. The intrinsic pathway is activated by the DENV membrane protein ectodomain export from Golgi to plasma membrane [104]. The NS2b-NS3 protease precursor and NS3 protease induce apoptosis [105] most likely through the caspase-8 pathway [106] or NF-κB [107].

Innate Immunity Associated with Severe Disease

Some immune responses are implicated with disease severity. TLR4 recognition of NS1 leads to pro-inflammatory cytokine production that contribute to vascular damage [108] (Figure 2.3). NS1 will also exacerbate disease by binding to uninfected cells to initiate vascular leakage [109, 110]. Mast cells will produce soluble mediators, such as vascular endothelial growth factor (VEGF), during DENV infection to cause vascular leak [111], and anti-NS1 antibodies will reduce mast cell degranulation and disease severity in mice [112].

Activation of the alternative complement pathway is associated with disease severity [113]. Apoptosis may contribute to disease severity; apoptotic cells were found in liver, cerebral, and endothelial cells from autopsies of patients with DHF/DSS [114]. TLR2 expression on classic monocytes ($CD14^{++}$) was associated with development of severe disease in pediatric patients in Cambodia [115].

DCs are one of the first immune cells that encounter DENV following infection and send signals to recruit NK cells via type I IFN and TNF-α, resulting in perforin/granzyme activity, Fas/Fas ligand-dependent virus killing and IFNγ production [116]. It is unclear if increased number of NK cells contribute to increase in disease severity. KIR3DLI, an inhibitory receptor of the killer immunoglobulin-like receptors (KIRs) family that is

expressed on NK cells, binds to DENV NS1 [117] and is associated with development of DHF. However, acutely infected patients in Thailand showed no correlation between NK cell subsets and level of severity [118]. Genetic factors influence the role of NK cells in disease severity: recent studies from Thai patients show that certain polymorphisms in KIR and their cognate ligand human leukocyte antigen (HLA) are associated with disease severity [119]

DENV Evasion of Innate Immune System

DENV uses NS proteins to block or inhibit signaling pathways in the infected cells. It also can block pathways that alert nearby cells of infection. DENV can block PRR signaling and production of type I IFN response by targeting the RLR and TLR pathways mentioned above. NS5 2'-O-methylation of 5' prevents the virus from being sensed by RIG-I [120]. NS3 blocks RIG-I translocation to mitochondria by binding with the mitochondrial-targeting chaperone protein 14-3-3ε [121]. NS4a will also inhibit RIG-I interaction with MAVS by binding to the MAVS CARD-like domain and transmembrane domain [122]. NS2a and NS4b from DENV1, 2 , 4 and NS4a from DENV1 block the RIG-I/MAVS signaling pathway by preventing phosphorylation of TBK1/IRF, inhibiting IFNβ induction [123]. NS2b targets cGAS for autophagy-lysosome dependent degradation and prevents mitochondrial DNA sensing[82]. NS2b/3 protease inhibits IFN production by cleaving STING [124] (Figure 2.4). NS4b triggers elongation of mitochondria by inactivating mitochondrial fission factor Dynamin-Related Protein 1 (DRP1), resulting in altered mitochondria-associated membranes (MAMS), increased DENV replication [125],

and decreased IFN production- possibly by blocking activated RIG-I recruitment to MAMs [126].

Furthermore, along with inhibiting IFNα/β production, NS proteins block the IFNα/βreceptor pathways. NS2a, NS4a, NS4b complex is responsible for subverting the IFNα/β response by inhibiting STAT1 signaling after IFNα/βreceptor activation *in vitro* [127]. NS5 inhibits IFN-mediated response via STAT-2 by binding with the host ubiquitin protein ligase E3 component N-recognin 4 (UBR4) for proteasomal degradation of STAT2 [128] (Figure 2.5)

DENV will subvert apoptosis early in the life cycle to ensure viral replication. The PI3/Akt pathway plays an important role in cell survival, regulating proliferation and inhibiting apoptosis; many viruses modulate this pathway for survival during early, chronic, and latent infections [129]. DENV2 blocks caspase-dependent apoptosis in cells during early infection by activating the phosphatidylinositol 3 kinase (PI3K)/Akt pathway [130].

The virus evades the complement response using NS1. NS1 inhibits the formation of the classical pathway C3 convertase by binding to C4, C1s, and C4b [131]. NS1 also protects DENV from neutralization by binding to MBL[132]. To block the formation of MAC, NS1 will bind to complement regulators vitronectin (VN) or inhibit C9 polymerization [133]. NS1 binding to complement inhibitory factor clusterin (Clu) will also inhibit MAC formation [134] (Fig 2.5).

Other DENV non-structural proteins have been shown to have immune evasion activity. NS4b suppresses the RNAi pathway in mammalian cells by blocking Dicer processing via transmembrane domain 3 (TMD3) and TMD5, preventing the biogenesis of

mRNA; this is independent of the N-terminal domain that is associated with IFN suppression [93]. NS3 will also block RNAi by binding to heat shock cognate 70, an essential protein for creating RISC, and inhibit TRBP-Argonaute interaction and RISC formation [135] (Figure 2.4). NS2a may also suppress RNAi activity in both mosquito and mammalian cells [90]. DENV NS2b/3 protease inhibits reticulophagy (selective autophagy of the ER) by cleaving *FAM134B*, an essential receptor for reticulophagy [98].

Structural proteins have also been shown to aid in innate immune evasion. DENV C protein aids in inhibition of apoptosis in Huh7 cells by interacting with calcium modulating cyclophilin-binding ligand (CAML) an ER protein associated with cell survival by regulating Bim-dependent death[136]. DENV C also interacts with cellular protein DEAD (Asp-Glu-Ala-Asp) Box Helicase 3, X- linked (DDX3X) resulting in higher viral titers and down regulation of innate immune responses against DENV independent of the type I IFN pathway [137].

Adaptive Immune Response Against DENV

In order to control and resolve the infection, the adaptive immune response is necessary since it has been shown that DENV can persist for up to one week at the site of infection [138]. The DENV-specific B and T cell responses can be cross-reactive toward different serotypes, but long-lasting memory responses thought to be serotype specific. Primary and sequential homotypic infections usually do not result in severe disease, but secondary heterologous infections are thought to increase risk for DHF and DSS, as discussed below.

Primary DENV infection

At the site of infection, dermal DCs, and LCs can be infected by DENV, as well as monocyte-derived DCs that are recruited by chemokines released during innate immune response [138-140]. Activated DCs migrate to draining lymph nodes (LNs) in CCR7 and CCL21 dependent manner [141]. DENV can replicate in LNs and migrate to secondary lymphoid organs and progress to systemic infection [142-144]. However, LNs are also where antigen-presentation occurs to initiate the adaptive immune response (Fig 2.6). DCs upregulate co-stimulatory molecules like CD80 and CD86 for presentation to $CD8_+$ and $CD4_+$ T cells on the major histocompatibility complex (MHC) I and MHC II molecules, respectively [145]. CD 8_+ T cells mainly target epitopes on DENV NS3 and NS5, and $CD4_+$ T cells target DENV components that are also targeted by B lymphocyte (E, C, NS1) [146].

Activated $CD4_+$ T cells will differentiate to T helper type 1 (Th1) and follicular helper T (Tfh) cells to provide help to $CD8_+$ T and B cells [147, 148]. Activated $CD8_+$ T DENV-specific T cells will express chemokine receptors CXCR3 and CCR5 and cutaneous lymphocyte-associated antigen (CLA) to migrate to the site of infection to kill infected cells [149].

How naïve B cells are activated by DENV and DENV antigens in the B cell follicle are still unknown. Viral antigens may enter the follicles via small gaps in the floor of the LN sinus where they are bound by cognate B cell [150]. Macrophages of the LN may facilitate B cell activation by collecting and displaying viral antigens [151]. Early B cell response in primary DENV infections are GC-independent, due to the lack of somatic hypermutation in antibodies sampled from infected donors [152]. However, DENV viral RNA has been found in germinal centers [153].

IgM antibodies are produced early during onset of viremia (after 4-6 day incubation time from mosquito bite), and peak from 1- 2 weeks [27]. DENV-specific IgG will appear 4-7 days , and comprise 50% of neutralizing antibody response [154], remaining in the sera for at least several months. Cross-reactive neutralizing antibodies will wane after several months, while serotype-specific neutralizing antibodies will confer life-long protection [155].

Correlates of protection are still not clearly defined [156, 157]. Nonetheless, it is widely accepted that the humoral immune response is important for protection and eliminating disease [158], with neutralizing antibodies being crucial factors for protection against disease [159]. Neutralizing antibodies directed to the E protein inhibit viral entry into cells [160]. Broadly neutralizing E antibodies target quaternary structures such as the hinge region [161], lateral ridge[162], E dimer spanning epitopes (EDE) [163], and cryptic epitopes that are accessible from virus breathing [164]. Flaviviruses require around ~30 antibodies to be neutralized [165]. For weakly binding antibodies, it may take all 180 E proteins to be bound. Spatial arrangement of the flavivirus epitopes affect the differences in binding occupancy and stoichiometry between antibodies [166]. Antibody-dependent cell-mediated cytotoxicity (ADCC) also contributes to limiting DENV replication [167] (Figure 2.6).

The protective mechanisms of T cells during DENV infection are still unclear. Analyses of PBMCs from patients with asymptomatic versus clinical cases during acute infection have not been able to elucidate phenotypic or functional differences in $CD4^+$ [168] nor in $CD8^+$ T cells [169]. Human T cells can be infected by DENV, suggesting their role as viral reservoirs during acute stage of infection [170]. However, in an

immunosuppressed individual, DENV infection was not resolved until $CD8_+$ T cell counts increased, despite the presence of DENV antibodies, suggesting important role of $CD8_+$ T cells for virus clearance and sterilizing protection [171]. In Cambodian children, transcriptomic profiles of asymptomatic cases had increased T cell activation and apoptosis, which suggests protective role for T cells against clinical disease [172]. Regulatory T (T_{reg}) cells expanded significantly in milder cases [173], which could inhibit proinflammatory immune responses, leading to less severe clinical disease. However, another study showed that although T_{regs} are expanded in acute dengue, they are predominantly naïve, with poor suppressive capacity [174].

After primary infection, memory B cells and long-lived plasma cells [155] and memory T cells [175, 176] will provide rapid and robust response after a homologous infection. However, after a heterologous secondary infection, adaptive immune responses may contribute to disease exacerbation, as discussed below.

Adaptive Immunity Associated with Severe Disease

The exact mechanisms of how DENV cause severe disease resulting in DHF and DSS are still unclear. DHF and DSS occur after decline in viremia and increase in serum antibody and T cell response. Thus, cross-reactive adaptive immune responses have been implicated, specifically after secondary, heterologous infection [177]. Two main theories for severe disease due to secondary, heterologous infection are antibody dependent enhancement (ADE) and original antigenic sin (Fig 2.7).

ADE is thought to occur when pre-existing antibodies to one DENV serotype do not neutralize but enhance a heterotypic infection [4, 5]. The non-neutralizing antibody

will bind to the virus and facilitate entry via Fcγ receptors (FcγR) on circulating monocytes. The virus alone cannot infect these FcγR-bearing cells; thus, viral replication and burden are increased as a result [26, 178]. ADE has been shown in *in vitro* models [26, 179], *in vivo* mouse [180, 181] and non-human primate [182, 183] models, and in humans [157]. Enhancing antibodies from human sera haven been shown to bind to prM [58] and fusion loop [184]. Maternal DENV antibodies can also cause DHF in infants after a primary infection [185, 186]. The orientation of the antibody may also contribute to enhancement-perpendicular binding as opposed to lying flat along the surface may cause ADE instead of neutralization [187].

Original antigenic sin may occur when memory cells from a previous infection respond more rapidly than naïve cells during acute secondary infection, but they cannot effectively clear the infection due to suboptimal avidity [188]. This leads to increased production of proinflammatory cytokines (like TNFα) and immune cell activation, resulting in vascular permeability and damage [189]. Other cytokines that are correlated with severe disease in humans are IL-6, IL-10 IL-15, and MCP-1 [172, 190], while IFN-γ and IL-2 correlated with subclinical secondary disease [147, 191].

In humans, DENV-specific B cells tend to be more broadly reactive in secondary infections compared to primary infections [192]. High cross-reactive plasmablast responses from peripheral B cells during secondary infections are correlated with severe disease [193]. Increase in IL-10 is thought to contribute, since IL-10 is required for plasmablast differentiation of B cells stimulated by DENV-infected monocytes [172]. Similarly, higher follicular T helper cell expansion from cross-reactive memory $CD4^+$ T cells are observed in severe versus mild disease [194], contributing to high plasmablast

numbers (Fig 2.7). Non-neutralizing cross-reactive antibodies are not the sole cause of ADE. Suboptimal levels of neutralizing antibodies prior to secondary infection have shown to increase the risk of severe disease in humans [195].

It is unclear how cross-reactive memory T cell response in humans contribute to protection from or enhancement of DENV disease [147, 196]. In mice, memory $CD8^+$ T cells showed protection after heterologous infection [197, 198]. However, in humans, memory $CD8^+$ T cells that are cross-reactive to the NS3 epitopes have been shown to contribute to disease exacerbation [199]. Memory $CD8^+$ T cell activation in the skin (with no accumulation of DENV-antibodies) was associated with DSS [200]. On the other hand, memory $CD4^+$ T cell activation may contribute to protection in humans [172].

Current Vaccine Status

The exact roles the adaptive immune response plays in DENV protection versus disease enhancement are unknown. Thus, correlates of protection for DENV vaccines are still unclear [156]. It is widely accepted that the humoral immune response, particularly neutralizing antibodies, is important for protecting against viral infection and disease [159]. DENV vaccine candidates must be able to elicit robust neutralizing antibody titers against all four serotypes of DENV in order to avoid ADE. Suboptimal levels of pre-existing neutralizing antibody titers have been associated with increasing risk for severe DENV disease compared to no pre-existing antibody or high antibody levels [157]. ADE usually occurs two years after a primary infection, when most cross protective antibodies wane [201, 202]. Thus, it is also important for the vaccine to maintain robustness of neutralizing antibodies.

The T cell epitopes that elicit protection are still uncertain, though studies show live-attenuated vaccine candidates elicit memory T cell response against NS3 and NS5 proteins [203-205]. Vaccines that target NS1 protein have been shown to be protective in mice [206, 207]. However, recent human studies show that patients that developed DHF had significantly higher NS1 antibody titers than patients with mild DF [208]. *In vitro* experiments with human sera have also shown that NS1 is associated with endothelial damage [209]. More studies will be needed to determine the which of the NS1, 3, and 5 epitopes are associated with protection vs. original antigenic sin. Progress is hampered by the limited knowledge of HLA-restricted immunodominant epitopes that are commonly expressed in human populations [210, 211].

There is currently one licensed vaccine, Dengvaxia by Sanofi-Pasteur, which uses the prM-E Dengue sequence in a Yellow Fever Virus backbone. However, the administration of the vaccine has been widely criticized, since it has only about 60% efficacy [11]. In phase I clinical trials, DENV3 and DENV4 of the Dengvaxia vaccine had higher replication than DENV1 or DENV2, which later correlated to eliciting higher neutralizing antibody titers for DENV3 and DENV4 than DENV 1 or DENV 2 in phase III trials [212].

Other vaccines in clinical trials are a live attenuated vaccine by Takeda [12], which consists of live attenuated DENV2 and chimeric DENV 1, 3, and 4 prM-E in live attenuated DENV2 backbone; a live attenuated recombinant vaccine by the National Institute of Allergy and Infectious Disease [13], where the 4 strains are attenuated by a 30 nucleotide deletion in the 3' end; a subunit vaccine by Merck [213], where recombinant, truncated E proteins from four serotypes are made by removing the membrane anchor sequence to

improve secretion and immunogenicity; and a monovalent DNA vaccine by the US Naval Medical Research Center [214], where gene encoding prM-E of DV1 are in phase I clinical trials.

Dengvaxia has caused debate over its safety and efficacy due to possible vaccine enhancement in younger children and seronegative individuals [9-11]. The overall efficacy is about 60% and it is recommended for ages 9-45 in endemic countries [8]. One theory for low efficacy for the vaccines during recent clinical trials are that they utilize E gene inserts from wild-type viruses that did not match the currently circulating strains.

The other two candidates also use outdated strains: Dengvaxia strains came from Asian strains isolated between 1978 to 1988. DENVax by Takeda uses Asian strains from 1964 to 1976. TV003 by NIAID uses strains isolated from 1974 to 1978. The currently circulating strains of DENV differ substantially from the isolates chosen by the various vaccine manufacturers. Globalization has enabled regional strains to rapidly circulate around the world. New introductions lead to more outbreaks as new strains are introduced to new areas [2].

The amino acid sequence of the E glycoprotein varies between serotypes, with up to 40% of the amino acids are not identical. Even with a serotype there is high variation, and a 9% difference within one serotype is define as a different genotype [215]. To address the effects of strain mismatch, novel DENV vaccines can be designed using multiple rounds of consensus building known as computationally-optimized broadly reactive antigens (COBRA). DENV E immunogen can be produced that contain conserved epitopes spanning many genotypes.

Computationally optimized broadly reactive antigen (COBRA) methodology has been used for other viruses with high antigenic diversity, such as influenza, to produce virus-like particles (VLPs) that elicits neutralizing antibodies against the virus [216, 217]. Conserved epitopes of E can be captured and presented on the surface of subviral particles (SVPs). SVPs do not contain any genetic material and are a safer alternative to live viral vectors as a delivery platform. SVPs are produced naturally during DENV infection and replication, requiring only the pre membrane (prM)- envelope (E) structural proteins to undergo the same maturation process and display similar fusogenic activity as infectious particles [218]. This allows the SVP to be taken up into cells for processing and antigen presentation.

The drawbacks to the SVP delivery method are that they require multiple injection since they are unable to replicate. They also require an adjuvant. The E immunogen can be delivered via mRNA to produce the SVP *in vivo* to reduce the number of injections and eliminate the need for adjuvants [219, 220].

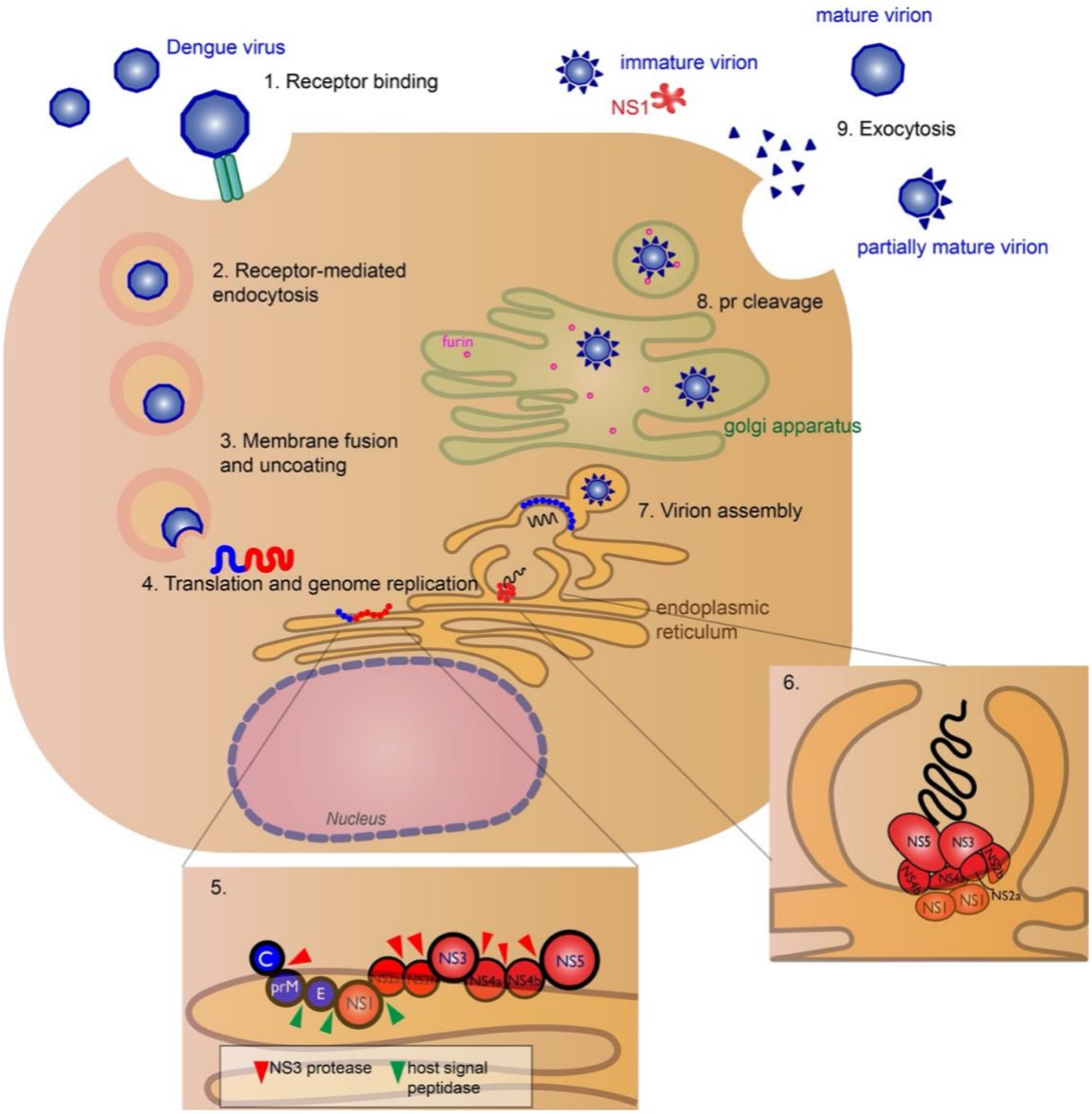


Figure 2.1 The viral life cycle of dengue virus (DENV). The virus binds to host cell receptors (exact receptors are unknown) (1) and enters the host cell (DENV permissive cells include keratinocytes, dendritic cells, endothelial cells, fibroblasts, macrophage, and mast cells), via receptor-mediated endocytosis (2). Acidification of the endosome induces conformational change of the E glycoprotein causing the virus to fuse with the endosomal membrane and release its genomic RNA material into the cytoplasm (3). DENV RNA translation and replication occur at the endoplasmic reticulum (ER) (4). The host ribosome

directly translates the genomic RNA into a polyprotein, where host and viral proteases cleave the nascent protein into structural (blue) and non-structural (red) proteins (5). RNA replication occurs in virus-induced membrane vesicles by the viral replication complex, with the transmembrane NS2a, NS2b, NS4a, and NS4b proteins acting as the scaffold (6). The viral genome is packaged into the immature virus particles during assembly (7). These particles are transported through the Golgi apparatus, where host furin-like proteases cleave the prM peptide (8), and the nascent viral particles exit the cell via exocytosis as fully mature virions (9). Some pr peptides are not cleaved resulting in immature, non-infectious virions or partially mature virions. The soluble NS1 hexamer is also secreted [41].

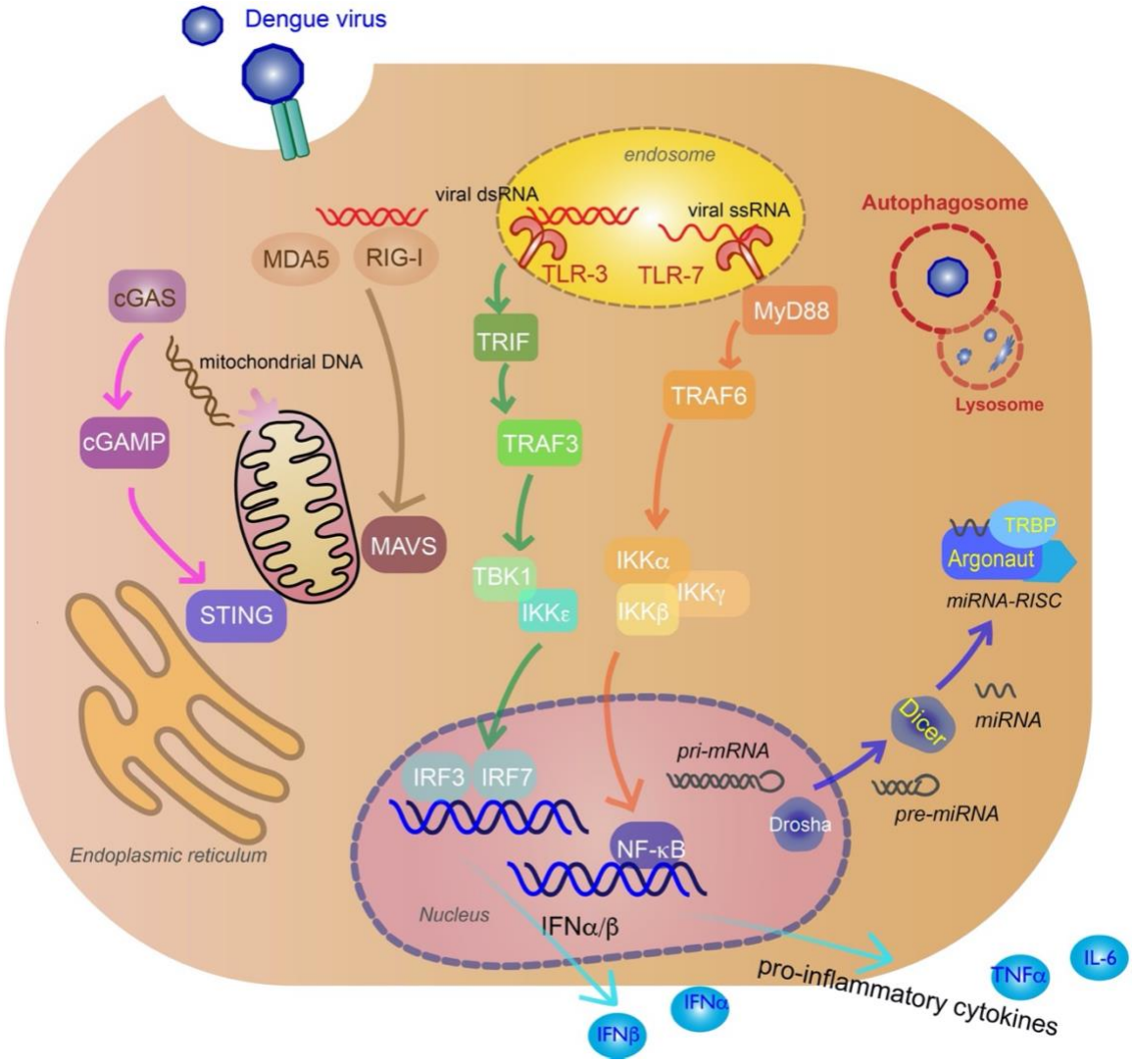


Figure 2.2 Innate immune response to DENV infection. Recognition of viral genomes by cytoplasmic retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) trigger mitochondrial antiviral signaling (MAVS) activation that lead to TANK binding kinase 1 (TBK1), IκB kinase-ε (IKKε) induction of interferon regulatory factor 3 (IRF3) and IRF7 (tan arrows). Viral genome recognition by endosomal toll-like receptor (TLR) 3 (green arrows) and TLR7 (orange arrows) will activate TIR-domain-containing adapter-inducing IFN β (TRIF) and myeloid differentiation primary response gene 88 (MyD88) signaling pathways, inducing

IRF3/IRF7 and inhibitor of nuclear factor-κB-kinase (IKK)α/IKKβ/IKKγ, and activate nuclear factor-κB (NF-κB) to produce IFN-α/β and pro-inflammatory cytokines. Virus-induced mitochondria damage activates the cyclic GMP-AMP synthase (cGAS) (pink arrows) and stimulator of interferon gene (STING) pathway to induce IFNα/β production via IRF3 and IRF7. MicroRNAs (miRNAs) associate with RNA-induced silencing complex (RISC) in the cytoplasm to target viral RNA for inhibition or degradation. miRNA biogenesis starts in the nucleus as pri-miRNA and processed by Drosha into pre-miRNA. Pre-miRNAs are transported to the cytoplasm and cleaved by Dicer to produce mature miRNAs[221]. Argonaute (Argo) and TAR RNA-binding protein (TRBP) are proteins essential to the formation of RISC (blue arrows). Double membrane vacuoles, called autophagosomes, will engulf foreign cytoplasmic material and fuse with the lysosome for degradation, inhibiting virus replication (red).

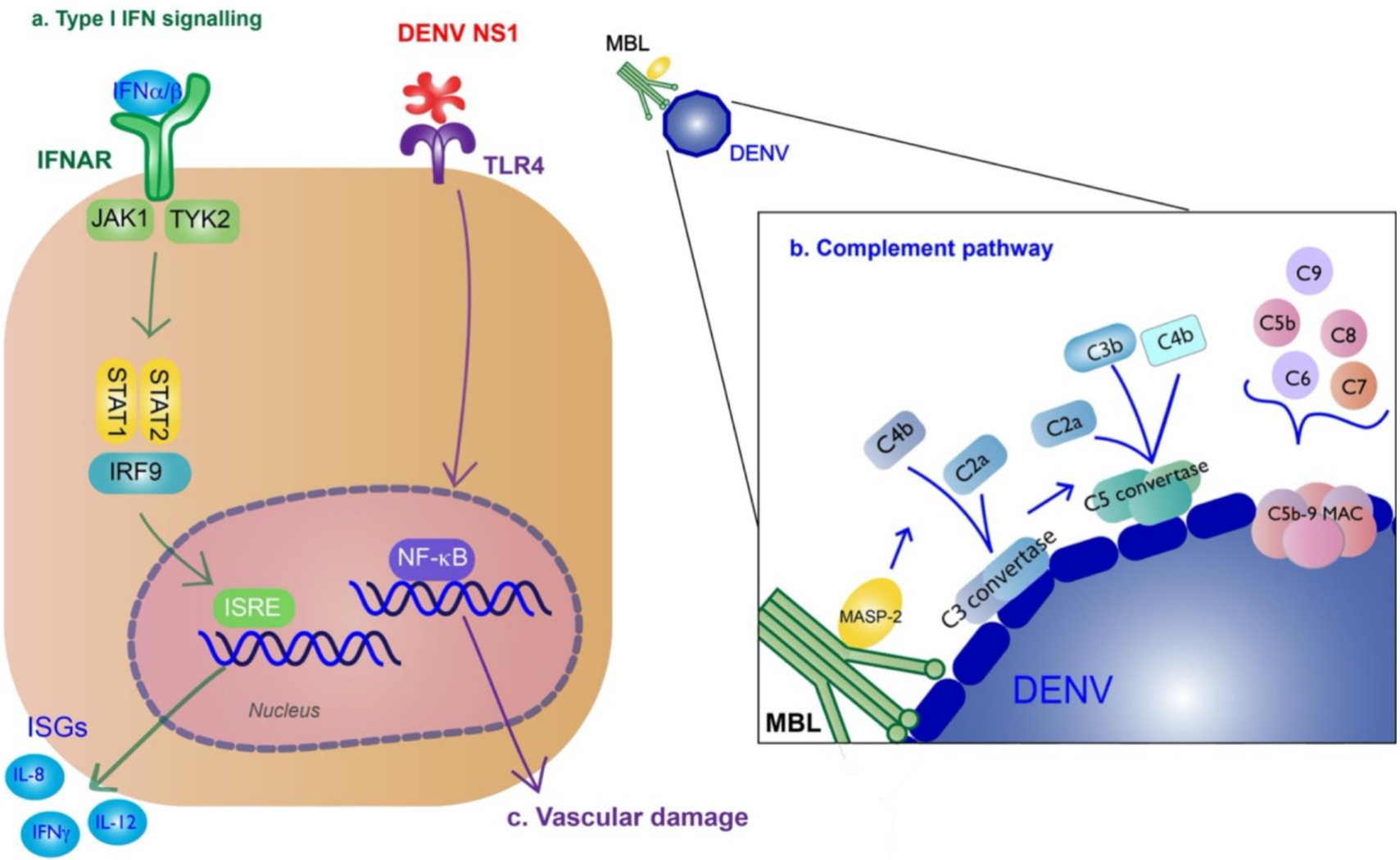

a. Type I IFN signalling
DENV NS1
MBL
IFNα/β
IFNAR
TLR4
DENV
JAK1
TYK2
STAT1
STAT2
IRF9
NF-κB
ISRE
Nucleus
ISGs
IL-8
IFNγ
IL-12
c. Vascular damage
b. Complement pathway
C9
C5b
C8
C6
C7
C3b
C4b
C2a
C4b
C2a
C5 convertase
C5b-9 MAC
C3 convertase
MASP-2
DENV
MBL

Figure 2.3 Type I IFN response and complement activation from DENV infection.

(a) Type I IFN signaling (green): Binding of type I IFN to IFNα/β receptors (IFNARs) stimulates IFN-stimulated gene (ISG) expression that results in antiviral activity. These cytokines bind to on the surface of nearby or infected cells, activating the Janus Kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. JAK1 and tyrosine kinase 2 (TYK2), lead to phosphorylation and dimerization of STAT 1 and STAT 2 which forms a complex with interferon regulatory factor 9 (IRF9). The complex will translocate to the nucleus where they induce transcription of ISGs by the IFN-stimulated response element (ISRE). (b) Complement pathway (blue): Recognition of DENV by the mannose binding lectin (MBL) complex will induce complement activation. Cleavage of C4 and C2 by MBL-associated serine protease-2 (MASP-2) make the C3 convertase and initiates the classical complement cascade, including the formation of C5 convertase and the C5b-9 membrane attack complex (MAC) to induce lysis, recruitment of phagocytes, and inflammation. (c) NS1 binding to TLR4 will induce vascular damage (purple).

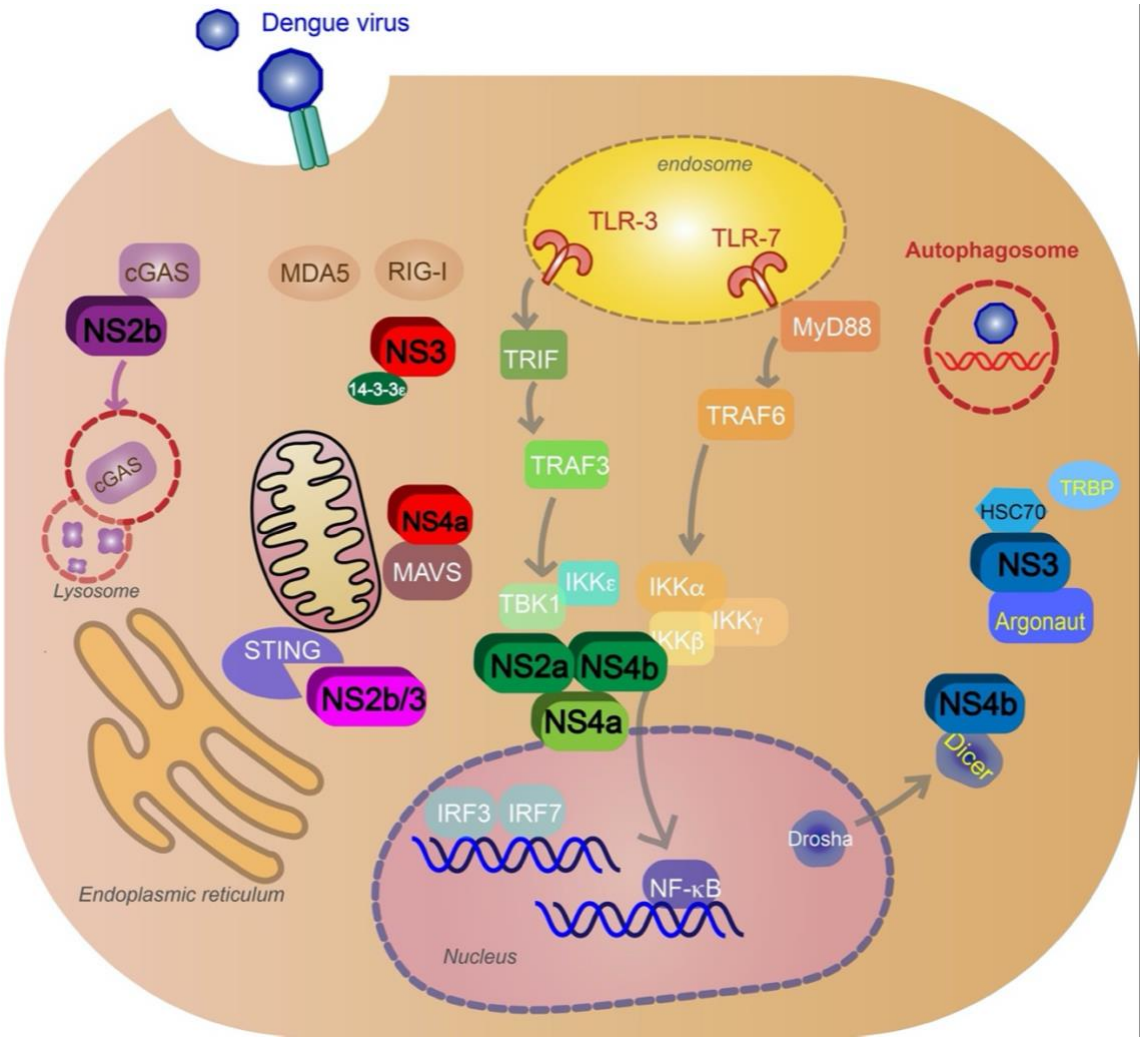


Figure 2.4 DENV evasion of innate immune response. Non-structural proteins block signaling pathways after virus recognition and inhibit type I IFN production. NS3 and NS4a block retinoic acid-inducible gene I (RIG-I) translocation to mitochondria (red). NS2a and NS4b from DENV-1, 2, 4 (green) and NS4a from DENV-1 (light green) inhibit the activation of TANK binding kinase 1 (TBK1), blocking the RIG-I/ mitochondrial antiviral signaling (MAVS) signaling pathway and IFNβ induction. NS2b targets cyclic GMP-AMP synthase (cGAS) for autophagy-lysosome dependent degradation and prevents mitochondrial DNA sensing (purple). NS2b/3 protease inhibits IFN production by cleaving stimulator of interferon gene (STING) (pink). NS4b inhibits RNAi by

binding to Dicer, preventing the biogenesis of miRNA. NS3 will also block RNAi by binding to heat shock cognate 70, an essential protein for creating RISC, and inhibit TRBP-Argonaute interaction and RISC formation (blue). DENV will exploit the autophagy pathway and use autophagosomes for replication, assembly and maturation, and evasion of neutralizing antibodies during transmission (red).

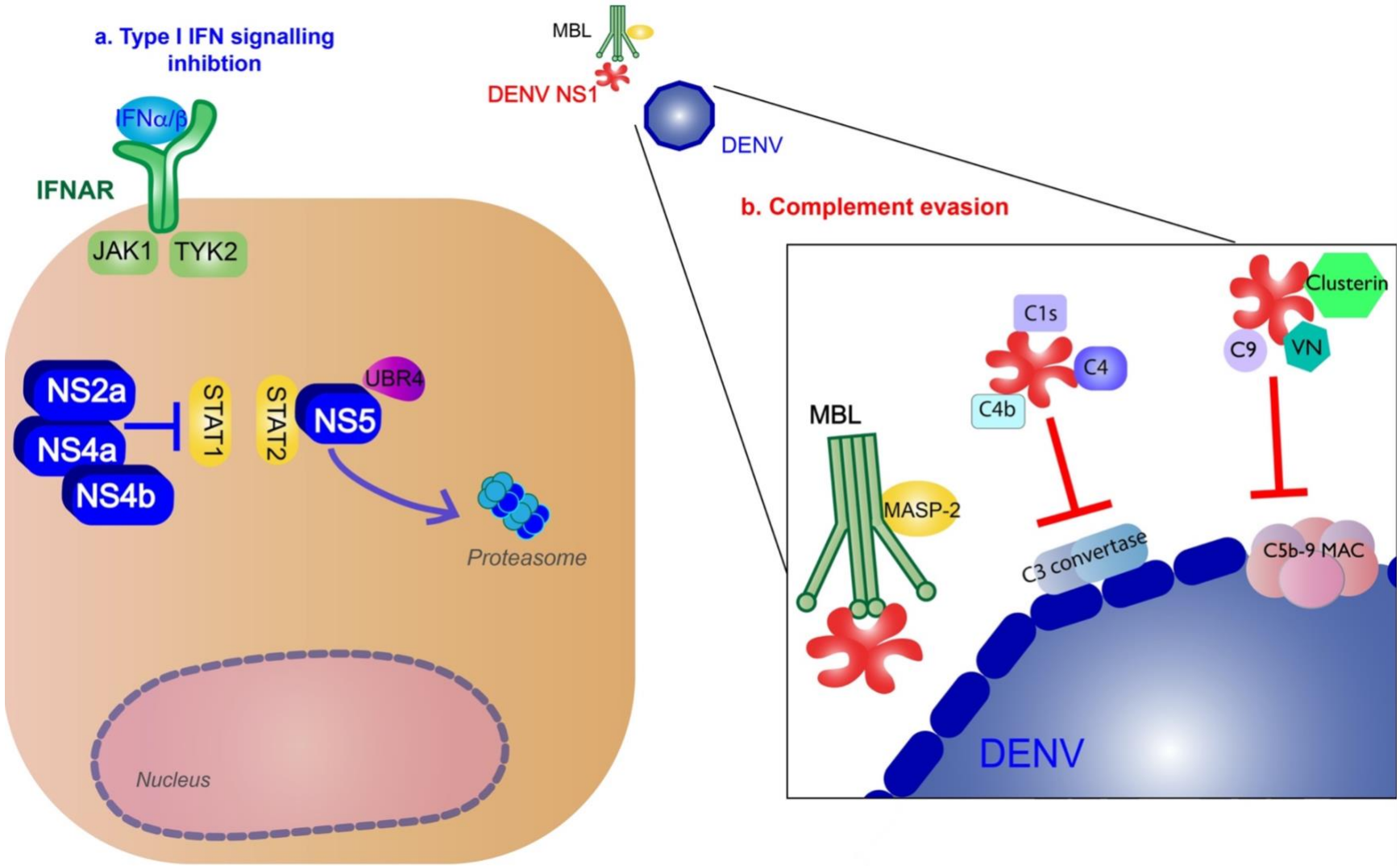

a. Type I IFN signalling inhibtion
IFNα/β
IFNAR
JAK1
TYK2
NS2a
NS4a
NS4b
STAT1
STAT2
UBR4
NS5
Proteasome
Nucleus
MBL
DENV NS1
DENV
b. Complement evasion
C1s
C4
C4b
MBL
MASP-2
C3 convertase
Clusterin
C9
VN
C5b-9 MAC
DENV

Figure 2.5 DENV inhibition of type I IFN response and complement pathways. (a) Type I IFN signaling inhibition (blue): NS2a, NS4a, NS4b complex inhibit signal transducer and activator of transcription 1 (STAT1) signaling after IFNα/β receptor activation. NS5 will cause proteasomal degradation of STAT2 by binding with the host ubiquitin protein ligase E3 component N-recognin 4 (UBR4). (b) Complement evasion (red): NS1 hexamer inhibits the formation of the classical pathway C3 convertase by binding to C4, C1s, and C4b. NS1 inhibits neutralization by the mannose binding lectin (MBL) pathway by binding to MBL. NS1 blocks membrane attack complex (MAC) formation by binding to complement regulators complement regulators vitronectin (VN), C9, or clusterin (Clu).

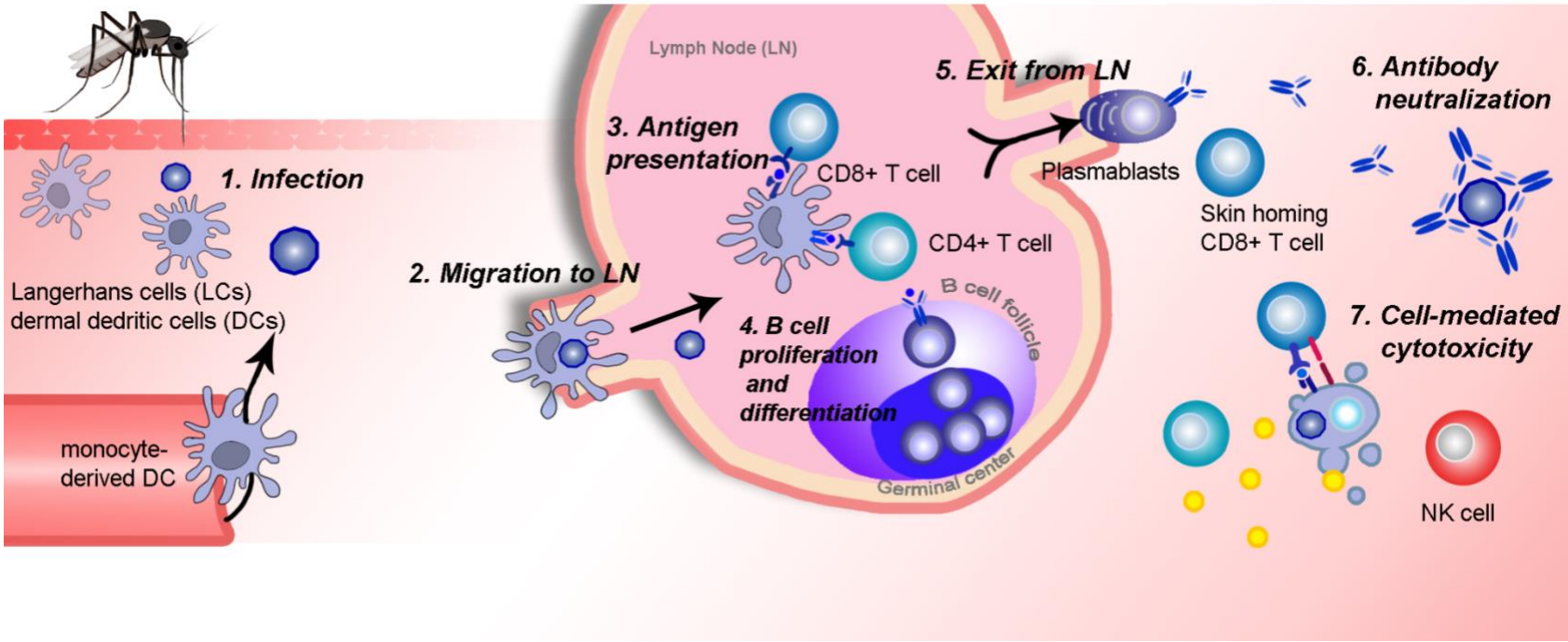

Lymph Node (LN)
1. Infection
Langerhans cells (LCs)
dermal dedritic cells (DCs)
monocyte-
derived DC
2. Migration to LN
3. Antigen
presentation
CD8+ T cell
CD4+ T cell
4. B cell
proliferation
and
differentiation
B cell follicle
Germinal center
5. Exit from LN
Plasmablasts
Skin homing
CD8+ T cell
6. Antibody
neutralization
7. Cell-mediated
cytotoxicity
NK cell

Figure 2.6 Adaptive immune response to DENV infection. 1) Sentinel immune cells of the skin, dermal dendritic cells (DC) and Langerhans cells (LCs) are infected by DENV. Monocyte-derived DCs are recruited to site of infection and are also susceptible to infection. 2) DCs migrate to the lymph node (LN), where DENV can amplify and disseminate to other secondary lymphoid organs. 3) DCs will activate $CD4_+$ and CD8+ T cells via antigen presentation on MHC I and MHC II molecules. 4) Activated B cells proliferate and turn in to plasmablasts that secret antibodies. $CD4_+$ T cells can help B cells undergo somatic hypermutation and affinity maturation in the germinal center to produce robust plasmablast and memory B cell response. 5) Effector cells exit the lymph node. 6) Neutralizing antibodies will inhibit DENV entry or fusion on host cell membrane. 7) Activated $CD8_+$ T cells will home to site of infection to kill infected cells. Antibodies aid in antibody-dependent cell cytotoxicity. $CD4_+$ T cells will release cytokines that activate and recruit phagocytes.

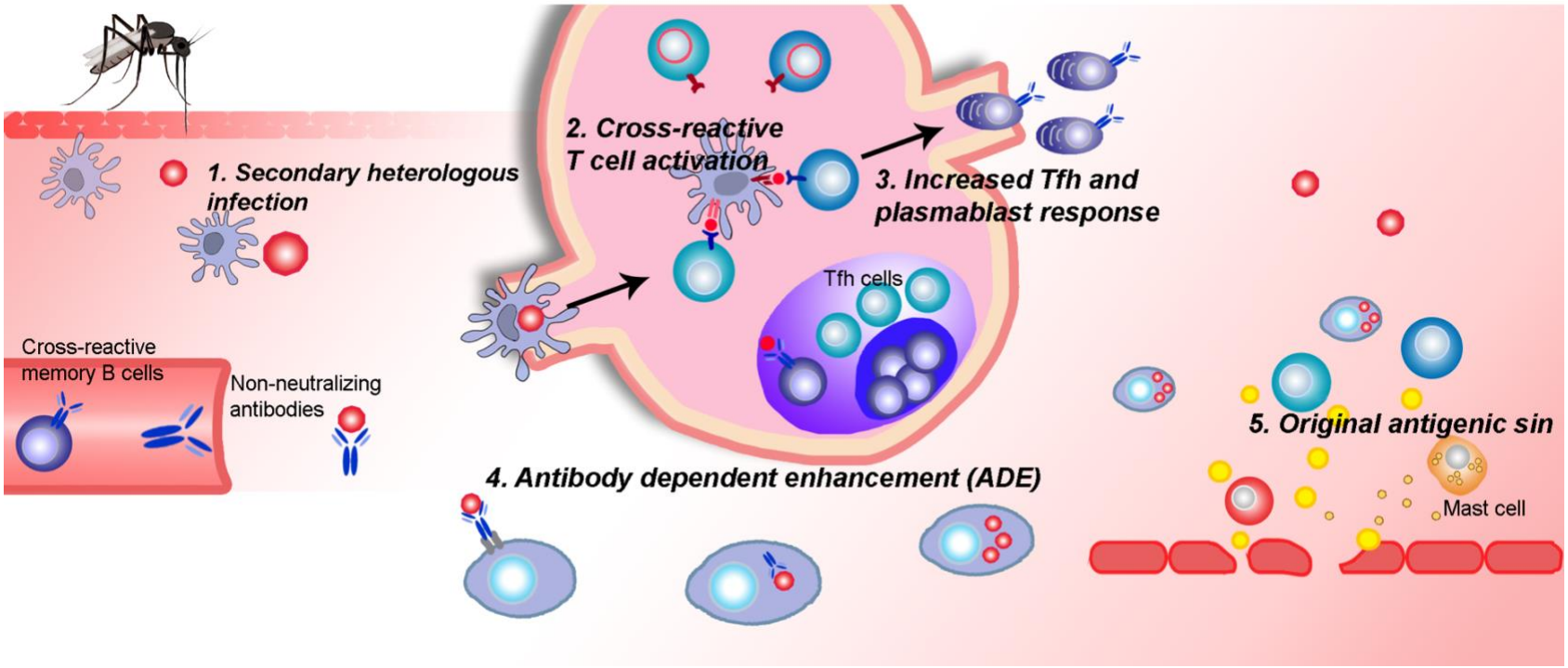

1. Secondary heterologous infection
2. Cross-reactive T cell activation
3. Increased Tfh and plasmablast response
Tfh cells
Cross-reactive memory B cells
Non-neutralizing antibodies
4. Antibody dependent enhancement (ADE)
5. Original antigenic sin
Mast cell

Figure 2.7 Adaptive immune response associated with severe disease. 1) Secondary infection with a different serotype of DENV. 2) Weakly binding cross-reactive memory T cells from previous infection outcompete naïve T cells that have higher avidity and specific to the current serotype. 3) Increase in number of memory-derived follicular T helper (Tfh) cells and subsequent increase in plasmablasts correlated with increased disease severity. 4) Antibody dependent enhancement (ADE) occurs when non-neutralizing antibodies bind to virion and facilitate infection of FcγR bearing cells not usually permissive to DENV, resulting in higher viral load. 5) Original antigenic sin occurs when activated cross-reactive T cells are not effective at clearing virus, causing cytokine storm and vascular damage resulting in DHF and DSS.

CHAPTER 3

DESIGN AND CONSTRUCT COMPUTATIONALLY OPTIMIZED BROADLY REACTIVE ANTIGEN (COBRA) DENV E AND EXPRESSION OF COBRA DENV PRM-E SUBVIRAL PARTICLES (SVPS)

Introduction

Developing a universal vaccine against dengue virus (DENV) has been difficult. The only licensed DENV vaccine is recommended for individuals between the ages of 9-45 years that live in endemic areas. Immunologically DENV-naïve children were at higher risk for severe DENV disease after vaccination. This may be due to the fact that the vaccine's live viral vector acted as a primary infection that exacerbated a subsequent natural infection, similar to the risk associated with secondary heterologous infection [222]. Seronegative individuals tended to have serotype-specific neutralizing antibodies, usually to DENV4, and an infection with a different serotype was theorized to cause severe disease due to antibody dependent enhancement (ADE) [15, 223]. A universal vaccine would need to be protective against all four serotypes of DENV and safe for all age groups regardless of serostatus.

Subviral particles (SVPs) do not contain any genetic material and are a safer alternative to live viral vectors as a delivery platform. SVPs are produced naturally during DENV infection and replication, requiring only the pre membrane (prM)-envelope (E) structural proteins to undergo the same maturation process and display similar fusogenic activity as infectious particles [218]. The E glycoprotein is the main target of neutralizing

antibodies, conferring long-term homologous immunity. Between the serotypes, there is a 40% difference in amino acids of the E glycoprotein, and a 9% difference within one serotype, resulting in different genotypes [215].

For other viruses with high antigenic diversity, such as influenza, computationally optimized broadly reactive antigen (COBRA) methodology has been used to produce virus-like particles (VLPs) that elicit neutralizing antibodies against the virus [216, 217]. Likewise, conserved epitopes in E can be captured and presented on the surface of SVPs using multi-layer consensus COBRA generation. Particle-based vaccinations have been shown to elicit both humoral and cellular responses [224, 225], both of which are important for DENV protection [25, 226, 227].

Materials and methods

Plasmid construction

To design COBRA DENV E, nucleotide sequences isolated from human infections from 1941 to 2006 were downloaded from the GenBank Database (www.ncbi.nlm.nih.gov/genbank) and the sequences were translated into protein sequences (100 from each serotype). DENV E amino acid sequences were group by phylogenetic clustering and geographic location per serotype. Multiple rounds of sequence alignments were performed to retain common epitopes within the sequence.

The full-length prM/E gene segments were cloned in the pTR600 expression plasmid, as previously described [228]. Briefly, the final amino acid COBRA E sequences, as well as the wild-type E sequences from prototype, wild-type (WT) DENV were reverse translated and optimized for expression in mammalian cells, including codon usage and

RNA optimization (Gene Art; Regensburg, Germany). Prototype virus E sequences from the Hawaii (DENV-1), NCG (DENV-2), H87 (DENV-3), and H241 (DENV-4); (accession numbers X76219, M29095, M93130, S66064) were optimized. Plasmids were amplified using QIAGEN® plasmid maxi kit, according to the manufacture's protocol. Plasmid were verified using restriction enzyme digestions and examination of specific DNA fragment patterns following resolution by electrophoresis on a 1% agarose gel with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA).

Transient transfection and purification of SVPs

Human embryonic kidney (HEK) 293T cells were transfected with 10μg of DNA using Lipofectamine 3000. After incubating for 4 days at 37°C, supernatants were collected and cell debris removed by centrifugation and vacuum filtration through a 0.22mM sterile filter. SVPs were collected via ultracentrifugation (100,000×*g* through 20% glycerol wt/vol) for 4h at 4°C. The concentrated SVP pellets were resuspended in phosphate buffered saline (PBS) and stored at -80°C. Protein concentration was determined by MicroBCA™ Protein Assay Reagent Kit (Thermo Fisher Scientific).

Verification of surface expression of DENV E

For ELISA, Nunc Maxisorp plates (Thermo Fisher Scientific) were coated overnight at 4°C with SVPs diluted in PBS (1mg/mL). Plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with 1% BSA in PBS for 1 hr at RT. Subsequently, pan-dengue monoclonal antibody (Acris antibody AM01108PU-N, OriGene Technologies, Inc. Rockville, MD, USA) were added to the plates for 2 h. After washing

3 times, peroxidase-conjugated anti-mouse IgG antibody was added for 1 h before developing the plates using an pNpp substrate (SeraCare, Milford, MA, USA). The optical density (O.D.) was determined at 405 nm. For immunoblotting, 1mg of purified SVPs were added to Bolt 4-12% Bis-Tris gel (Thermo Fisher Scientific) for SDS-PAGE under non-reducing conditions and electroblotted on a PVDF membrane. The samples were probed with pan-flavivirus mAb (5E284, Santa Cruz Biotechnology, Dallas TX, USA) using the iBind Western Device (Thermo Fisher Scientific). For the secondary antibody, goat anti-mouse IgG conjugated to HRP (Southern Biotech, Birmingham, AL, USA) was used. Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA) was used for chemiluminescent imaging.

Results

Using Computationally Optimized Broadly Reactive Antigen (COBRA) technology, four unique dengue virus (DENV) envelope (E) genes were designed using ~2,100 bp of full-length E sequences from human isolates (Fig. 3.1). For each COBRA antigen, multiple rounds of sequences were aligned to create a final COBRA E sequence. For the primary sequence alignment, DENV E nucleotide sequences from 100 human clinical isolates were downloaded from GenBank that span the many decades, from prototype to modern strains (1941-2006). Nucleotide sequences were translated into protein sequences using the standard genetic code. DENV E amino acid sequences were grouped by phylogenetic and geographic clustering. These clusters were aligned and 6 primary consensus sequences formed. Two secondary consensus sequences were formed

from the primary consensus sequences. From those two, a final COBRA E consensus sequence was created.

For Fig. 3.1, primary cluster 1 were isolates mainly from Cambodia and Thailand, cluster 2 were from Cambodia, cluster 3 were from Vietnam. The three primary consensus sequences from these three clusters subsequently formed an Asian-like secondary consensus sequence. Primary cluster 4 isolates were mainly from US and Venezuela, cluster 5 isolates were from Brazil, and cluster 6 isolates were from Nicaragua and Venezuela. The three primary consensus sequences from these three clusters subsequently formed an American-like secondary consensus sequence. The third and final consensus sequence yielded a COBRA E sequence.

Each COBRA E gene sequence was cloned into the vector pTR600 [216] in frame with a pre-membrane (prM) sequence to express a subviral particle (SVP). The prM sequences were derived from prototype strains. COBRA prM were not created since it is the E protein that is the main target of neutralizing antibodies; prM does not elicit neutralizing antibodies [229]. The plasmids were amplified and gene inserts were verified with gel electrophoresis following restriction enzyme digest (Fig 3.2). The prM-E insert can be visualized at the correct size (~2,100 bp) as well as the plasmid vector (~5,400 bp).

SVPs were expressed following transfection into HEK 293T cells and purified by ultracentrifugation. Each SVP efficiently expressed each COBRA E protein on the particle surface. ELISA testing showed that a pan-DENV monoclonal antibody can recognize and bind to E on the surface of the SVPs (Fig 3.3a) and western blot analysis showed the E to be at the correct molecular weight, ~55kDa. Pan-DENV monoclonal antibody recognized E on the surfaces of COBRA 1 and COBRA 2 SVPs compared to COBRA 3 or 4 SVPs.

This is due to the monoclonal antibody having higher binding to serotypes 1 and 2 comapred to 3 and 4, as stated by the manufacturer. As an alternative, silver staining can be done to show that the amount of E on the surface is the same amount for all SVPs.

Discussion

Consensus generation of vaccine immunogens have been used for other antigenically diverse viruses such as human immunodeficiency virus (HIV) and influenza viruses [230, 231]. These sequences minimize the mismatch between the vaccine and circulating strains. These immunogens can be incorporated onto the surface of virus like particles and elicit broadly reactive humoral and cellular responses [232].

COBRA E sequences were designed from multiple consensus alignments of hundreds of human clinical isolates. The consensus alignments we made from clusters based on phylogeny and geographic region. It has been shown that strains from different geographic regions are more virulent than others in the same serotype [233]. Increased global travel has allowed more virulent strains to spread and cause pandemics, displacing the previously circulating strain [234]. Furthermore, the major circulating serotype often changes in endemic countries [2]. Since currently circulating strains range from new genotypes to prototypes, depending on virulence[18], the strains used for sequencing also spanned from 1941 to 2006.

The COBRA E sequences were cloned into plasmid vectors in frame with prototype prM to be expressed on the surface of DENV SVPs after transient transfection. SVPs are more effective than recombinant E proteins as a vaccine candidate, since they still maintain

fusogenic properties of live virus to activate endogenous as well as exogenous antigen recognition pathways [225].

Linear epitopes are important for eliciting T cell response, and conformational epitopes elicit humoral responses. The exact epitopes that elicit protective T cell responses are unclear [235]. T cells mainly recognize epitopes from non-structural proteins, but have been shown to recognize epitopes from structural proteins as well [236, 237]. It has been shown that neutralizing antibodies target DIII [238, 239], hinge region [240], and fusion loop [241] of E glycoprotein. Additionally, E dimer spanning [36, 37, 163] and cryptic [242] epitopes elicit broadly neutralizing antibodies that are cross-reactive to different serotypes.

Compared to other DENV vaccine candidates that utilize just one strain per serotype, this multi-epitope approach will more likely recognize different DENV strains and serotypes to generate a broader immune response. Having a particle-based vaccine is preferable to a soluble protein, since COBRA SVPs will be able to elicit neutralizing antibodies that target epitopes found on quaternary structures (such as E dimer spanning and cryptic). The COBRA DENV SVPs can be expressed *in vitro* by transfection of mammalian cell line or *in vivo* by messenger RNA (mRNA) technology.

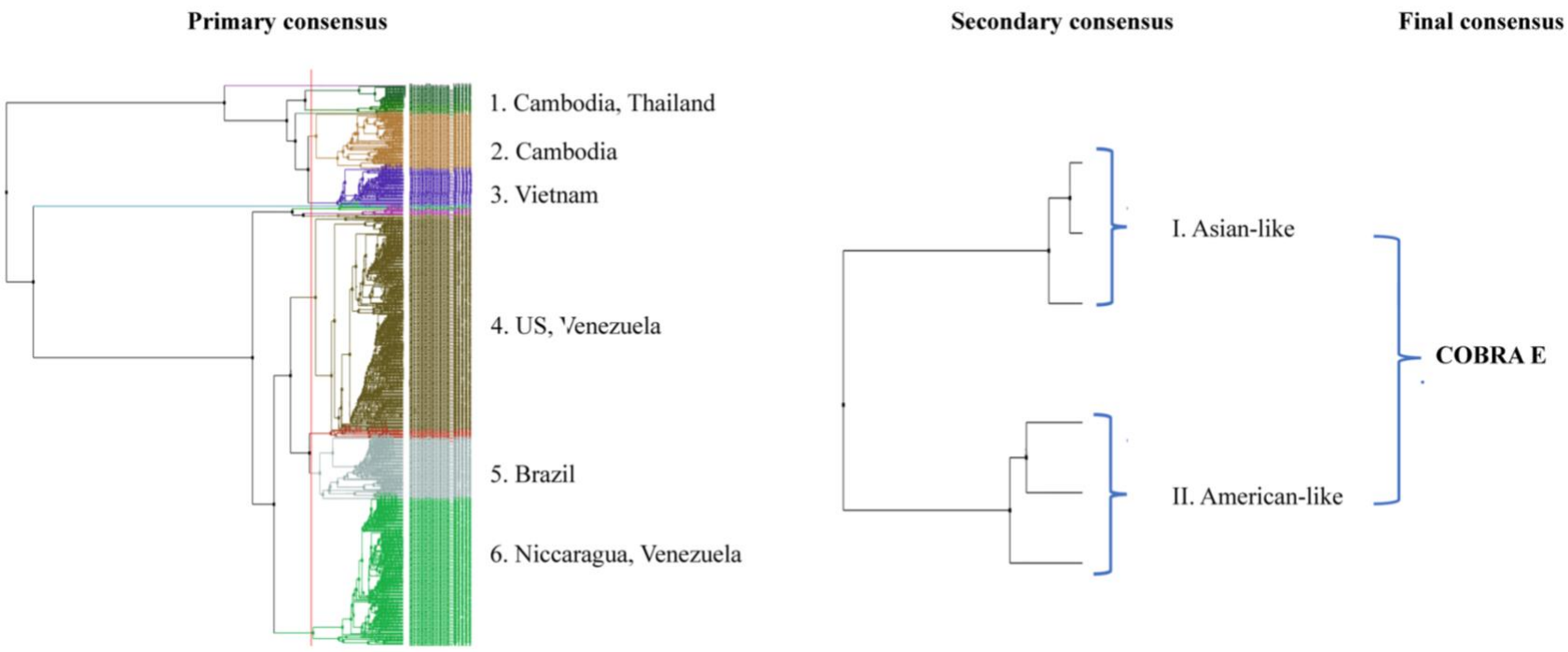


Figure 3.1 Schematic of COBRA design. Dengue virus (DENV) E nucleotide sequences isolated from human infections from 1941 to 2006 were translated into protein sequences (100 from each serotype) and groups by phylogenetic clustering and geographic location per serotype. Multiple rounds of sequence alignments were performed to retain common epitopes within the sequence. Primary alignment yielded 6 consensus sequences, secondary alignment yielded 2 consensus sequences, and final alignment produced COBRA E sequence.

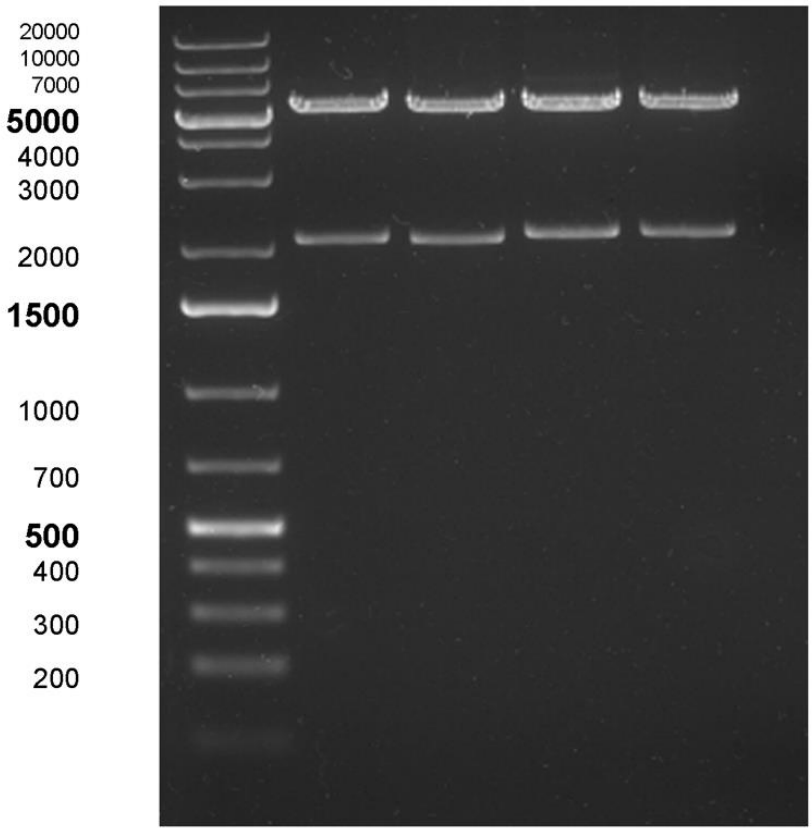


Figure 3.2 Gel of COBRA plasmids. Restriction enzyme digests were done on amplified plasmids and DNA fragments were examined following resolution by gel electrophoresis. DNA ladder is indicated on the left, in kilodaltons.

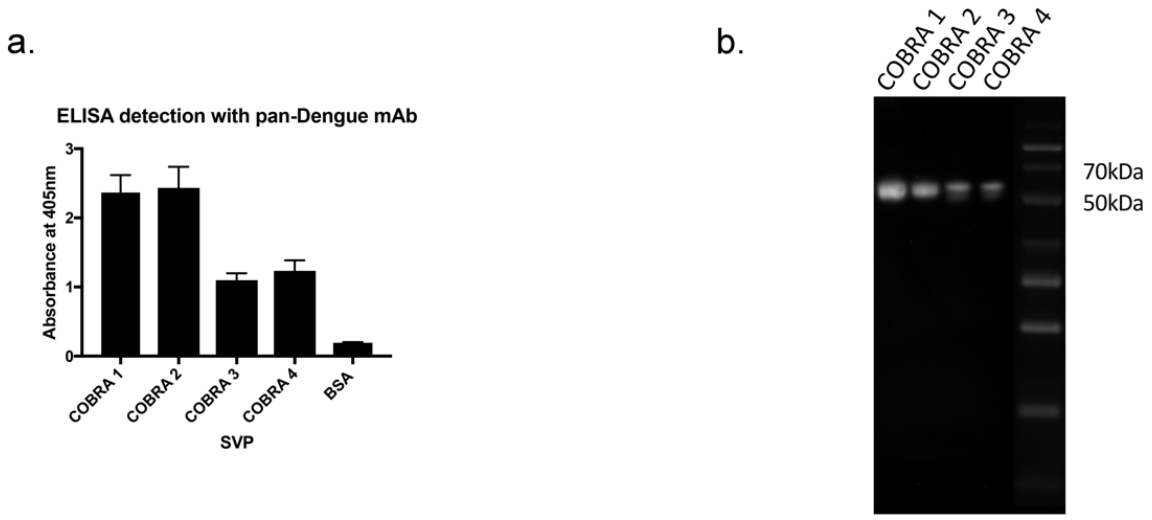


Figure 3.3 Verification of E expression on COBRA SVPs. a) Surface expression of DENV E was detected with Enzyme-linked Immunosorbent Assay (ELISA) using pan-Dengue monoclonal antibody. Absorbance was measured at 405nm. b) E glycoprotein was characterized with western blot using pan-flavivirus monoclonal antibody, protein ladder on right with relevant molecular weights indicated.

CHAPTER 4

DETERMINE IMMUNOGENECITY AND BREADTH OF IMMUNE RESPONSE OF MICE VACCINATED WITH DENV COBRA SVPS AND WILD TYPE SVPS[1]

[1]A version of this chapter was submitted to *Journal of Virology* 4/9/20.
"Uno, N and Ross, T. A Universal Dengue Vaccine Elicits Neutralizing Antibodies Against Strains from All Four Dengue Serotypes".

Abstract

Any potential dengue virus (DENV) vaccine needs to elicit protective immunity against strains from all four serotypes to avoid potential antibody dependent enhancement (ADE). In this study, four independent DENV envelope (E) glycoproteins were generated using wild-type E sequences from viruses isolated between 1943 to 2006 using computationally-optimized broadly reactive antigen (COBRA) methodology. COBRA and wild-type E antigens were expressed on the surface of subviral particles (SVPs). Four separate wild-type E antigens were used for each serotype. Mice vaccinated with wild-type DENV SVPs had anti-E IgG antibodies that neutralized serotype specific viruses. COBRA DENV SVPs elicited a broader breadth of antibodies that neutralized strains across all four serotypes *in vitro*, which was comparable to antibodies elicited by a tetravalent wild-type E SVP vaccination mixture. Therefore, using a single DENV COBRA E protein can elicit neutralizing antibodies against strains representing all four serotypes of DENV.

Introduction

Dengue virus (DENV) is the most prevalent arbovirus worldwide and is endemic in greater than 100 countries [19]. Over half of the global population is at risk for DENV infection with 100 million symptomatic cases being reported every year [1]. It is estimated that there are 400 million infections a year, mainly in the tropical and subtropical regions of the Americas and Asia [20]. There are four serotypes of the virus, DENV1-4 [21]. They are genetically and antigenically distinct. Between the serotypes, ~40% of the amino acids differ between the E glycoproteins and a 9% difference within one serotype, resulting in different genotypes [215]. In endemic countries, more than one serotype circulates [2]. Cross protective immunity is not long term, lasting about a year [22]. Infection with more than one serotype can lead to severe disease and mortality [3].

Dengue virus is a member of *Flavivirus* family and is transmitted by the *Aedes aegyptii and Aedes albopictus* mosquitoes. It is enveloped, spherical, with positive-sensed single stranded RNA genome that encodes one open reading frame (ORF) with 3 structural (capsid, matrix, and envelope) and 7 non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5)[30]. The envelope glycoprotein (E) is the main target for neutralizing antibodies and is responsible for receptor binding and fusion[6]. The membrane (M) and pre-membrane (prM) proteins are located on the surface envelope membrane of the virus. The immature prM form of the virus is usually found inside the host cell, where the prM protein acts as a chaperone for E protein folding in the endoplasmic reticulum [243]. Cellular furin proteases cleave the prM and the release the pr polypeptide. The remaining protein complex forms a mature, infectious virion that induces membrane fusion following increase in endosomal acidic pH [244, 245]. The

mature virions are released from the cell by exocytosis [246]. In addition to fully mature virions, non-infectious, immature, subviral particles (SVPs) are also secreted from infected cells during viral replication [247]. SVPs contain no genetic material and are produced by expression of prM-E gene cassette. These SVPs undergo the maturation process and acquire fusion activity [218, 248].

Currently, the only licensed DENV vaccine, Dengvaxia™, as well as two other leading candidates, express DENV prM-E from either an attenuated, chimeric yellow fever virus (YFV) vector [7] or chimeric DENV viruses. The overall efficacy is ~60% and the vaccine is recommended for individuals between the 9 to 45 years of age in endemic countries [8]. Dengvaxia™ has been associated with vaccine enhancement in younger children and seronegative individuals [9-11]. These vaccine candidates had low effectiveness during recent clinical trials that may be due to utilizing E gene inserts from wild-type viruses that did not match the currently circulating strains [156, 249].

To address the effects of genotype mismatch, novel DENV vaccines can be designed using multiple rounds of consensus building known as computationally-optimized broadly reactive antigens (COBRA). These results by DENV E immunogens that represent each DENV serotype spanning many genotypes [228]. To generate COBRA sequences, 100 full length E sequences from each DENV serotype 1-4, from human infections were obtained from GenBank and used for COBRA consensus sequence generations. The sequences were all unique. SVPs were produced from recombinant prM-E constructs. SVPs are non-infectious and a safer alternative to live-attenuated viral vaccines [250].

To determine immunogenicity and effectiveness of COBRA SVP vaccines female C57BL/6 mice (age 6-8 weeks) were vaccinated with DENV SVP intramuscularly individually or in a tetravalent mixture. Immune sera were collected and total IgG antibody titers to DENV E were analyzed by ELISA and the ability to prevent virus infection *in vitro* was assessed in a focus reduction neutralization test ($FRNT_{50}$) against a panel of 12 prototype and modern strains from all four serotypes. Animals vaccinated with wild type DENV SVPs expressed anti-E IgG antibodies that were specific to strains in each homologous serotype and elicited antibodies neutralized serotype specific viruses. COBRA DENV SVPs elicited a broader breadth of antibodies that neutralized various strains across all four serotypes. One COBRA DENV E immunogen neutralized all 12 strains of DENV *in vitro* comparable to tetravalent SVP vaccination.

Materials and methods

Mouse vaccination Female C57BL/6 mice, age 6-8 weeks (n=10) were intramuscularly vaccinated with purified monovalent or tetravalent wild type or COBRA SVPs (100 μg total protein per vaccination, with Imject adjuvant) three times at 4-week intervals. Control groups were given PBS (with Imject adjuvant) using the same vaccine regimen. Serum samples were collected at each vaccination and also at 4 and 20 weeks after final boost.

Transient transfection and purification of SVPs

Human embryonic kidney (HEK) 293T cells were transfected with 10μg of DNA plasmid encoding for COBRA or wild type prM-E using Lipofectamine 3000. After incubating for 4 days at 37°C, supernatants were collected and cell debris removed by centrifugation and

vacuum filtration through a 0.22µm sterile filter. SVPs were collected via ultracentrifugation (100,000×*g* through 20% glycerol wt/vol) for 4h at 4°C. The concentrated SVP pellets were resuspended in phosphate buffered saline (PBS) and stored at -80°C. Protein concentration was determined by MicroBCA™ Protein Assay Reagent Kit (Thermo Fisher Scientific).

Detection of anti-DENV E antibodies

Quantitative ELISA was performed to detect anti-SVP or anti-E antibodies elicited by vaccination. Nunc Maxisorp plates were coated (2ug/mL) overnight at 4°C with either subviral particles expressing one of the wild-type DENV E proteins or soluble DENV E (MyBiosource, Inc. San Diego, CA, USA). Plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with 1% BSA in PBS for 1 hr at RT. Subsequently, 1:100 dilution of sera samples were added to the plates for 2 h. Following three washes with PBS-T, peroxidase-conjugated anti-mouse IgG-Fc, IgG1, IgG2a, IgG2b, or IgG3 antibodies (Bethyl Laboratories, Montgomery, TX, USA) were added for 1 h before developing the plates using pNpp substrate. OD values were read at 405 nm.

Dengue viruses

Viruses used in this study were for DENV 1: DV1/US/Hawaii/1944, DV1/Vietnam/BID-V1792/2007, DV1/Costa Rica/BC89/1994; DENV 2: DV2/New Guinea/NGC/1944, DV2/Vietnam/BID-V1007/2006, DV2/US/BID-V594/2006; DENV 3: DV3/Philippines/H-87/1956, DV3/Sri Lanka/271242/1991, DV3/US/BID-V1619/2005,

and DENV 4: DV4/Philippines/H-241/1956, DV4/Malaysia/BC13/1997, DV4/US/BC258/1994.

Neutralization assay

Neutralization activity in collected antisera were determined using a focus reduction neutralization test (FRNT). Sera was heat inactivated at 56°C for 30 min and the serially diluted (4 fold). Sera was incubated with dengue viruses (100 FFU) representing each of the 4 serotypes for 60 min at 37°C. Three dengue viruses were used per each serotype that represented the prototype, modern Asian, and modern American strains. Each dengue virus was mixed with the serially diluted antisera and used to infect a single cell monolayer of Vero cell in 96-well plates for 90 min at 37°C that were plated. Cells were overlaid methylcellulose (1% [wt/vol]). Cells were incubated for 3 days at 37°C (2 days for DENV 4 viruses). Cells were washed with PBS and fixed with a mixture of acetone and methanol (Foci were stained using Vectastain ABC kit, following the manufacturer's protocols (Vector Laboratories, Burlingame, CA, USA). Anti-DENV E monoclonal antibodies were used to detect virally infected cells. For DENV 1 and 3 viruses, the antibody AM01108PU-N (OriGene Technologies, Rockville, MD, USA) was used and for DENV 2 the monoclonal antibody 9.F.10 (Santa Cruz Biotechnology, Dallas, TX, USA) was used and for DENV 4, the monoclonal antibody 4G2 (Millipore Sigma, Billerica, MA, USA) was used. Foci were imaged using CTL Immunospot (Cellular Technology Limited, Cleveland, OH, USA). $FRNT_{50}$ determined by calculating the point at which sera reduced dengue foci by 50% compared to serum-free, dengue virus control wells.

B cell ELISPOT

Mice were stimulated *in vivo* by intramuscular injection of homologous SVP (100µg) and spleens were harvested after 6 days. Multiscreen HTS (Millipore Sigma) plate was pre-wetted with 35% ethanol and coated with 5mg/mL of DENV1-4 peptides (My BioSource), anti-mouse IgG (Mabtech), or BSA. Splenocytes were added in serial dilutions starting at 300,000 cells/well in RPMI media supplemented with 55 µM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin,100 µg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and 1% MEM nonessential amino acids and incubated at 37° for 24hr. The cells were washed five times with PBS and anti-monkey IgG-biotin (Mabtech) added overnight at 4°C. The following day, plates were washed five times with PBS. Streptavidin-HRP (Mabtech) was added and incubated at room temperature for 2 hours. After washing five times with PBS, spots were developed with TMB solution (Vector Laboratories). Spots were imaged using CTL ImmunoSpot Analyzer and counted using ImmunoSpot software version 5.1.36 Professional SC.

Statistical analysis

To determine statistical significance of individual vaccine groups compared to PBS control group, one-tailed unpaired t-test were done, with confidence level at 95%. Vaccine groups were compared in ELISA antibody binding against PBS control group with soluble E from all four serotypes. P-values less than .05 were considered statistically significant. *ns* for $p > 0.05$, * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, **** for $p \leq 0.0001$.

See Appendix A for complete tables of ELISA statistical analysis.

Differences in IgG subclass response were analyzed by unpaired t-test with Welch's correction. Differences in neutralization titer were analyzed by paired student's t-test. Statistical analyses were performed using Prism (GraphPad Software, La Jolla, CA, USA). P-values less than .05 were considered statistically significant. *ns* for $p > 0.05$, * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, **** for $p \leq 0.0001$.

Results

C57BL/6 mice were vaccinated with the SVPs expressing COBRA E or wild-type E dengue protein individually (monovalent) or in a tetravalent mixture. The vaccine consisted of 100μg total SVP protein that was supplemented with Imject™ alum as an adjuvant. Four weeks after the final vaccination, sera were collected and tested for anti-E antibodies, as well as neutralization activity.

All mice vaccinated with SVPs expressing WT or COBRA E antigens had significant cross-reactive IgG binding antibodies to any of the dengue serotypes expressed as SVP (Fig. 4.1a). One-way ANOVA tests were completed to show statistical significance between the vaccinated groups and PBS control group (Appendix A Table A.1). However, each serum IgG sample only bound to commercially made soluble E protein that represented the serotype used in vaccination (homologous binding) (Fig 4.1b).

IgG subclasses that were analyzed including: IgG1, IgG2a, 2b, and 3. IgG1 elicits a type 2 T-helper (T_h2)-related response, while the other three are T_h1-related. IgG2a and 2b are generally considered to be the most potent for activating antiviral and effector [251, 252]. Mice vaccinated with SVP vaccines expressing COBRA 1 and 2 E antigens had high

IgG1 titers to E antigens (Table 4.1). COBRA 1 and 2 elicited IgG2c, which is associated with T-helper (Th) 1 response.

Sera were tested for the ability to neutralize a panel of 12 dengue viruses representing all 4 dengue serotypes (Table 4.2). Modern strains (American and Asian) were chosen along with prototype, since genotypes tend to separate by geographic location [253, 254].

Mice vaccinated with each of the four SVPs expressing a WT E antigen elicited antibodies that primarily neutralized the viruses from the homologous serotype (Fig. 4.3a-d). In contrast, mice vaccinated with SVPs expressing the COBRA E antigens had antibodies that neutralized all four serotypes of dengue virus (Fig. 4.3e-h). COBRA 1 and COBRA 2 were comparable to tetravalent formulations (Fig. 4.3i-j). COBRA tetravalent had more robust neutralizing antibody response compared to WT tetravalent.

To assess the longevity and breadth of response in the top COBRA candidates, sera were collected 20 weeks after final vaccination and splenocytes were collected 22 weeks after final vaccination. COBRA 1, 2, and tetravalent formulations maintained broadly neutralizing titers in mice 20 weeks after final vaccination (Fig 4.3). COBRA 1 SVP vaccinated group maintained neutralizing antibodies against all 12 strains, while COBRA 2 SVP group maintained neutralizing antibodies against 11 of the 12. COBRA 1 SVP vaccine groups had DENV-specific memory B cells that produced IgG antibodies against all four DENV E antigens comparable to tetravalent formulation (Fig 4.4). There was no significant difference between B cell memory response between COBRA 1 SVP and COBRA tetravalent SVP (Appendix A Table A.4).

Discussion

With increased global travel and the spread of mosquitoes due to climate change, the risk of dengue virus infection continues to spread worldwide, while new strains are introduced to endemic countries [2]. Any potential DENV vaccine would need to protect against all four serotypes and currently circulating strains from different parts of the globe. The COBRA methodology takes into account strains from various geographic locations and time periods in order to create a broadly protective immunogen. This technology has been successfully used to generate broadly-reactive influenza virus vaccines [216, 255]. Four COBRA E immunogens were designed and administered as SVP vaccinations to wild type C57BL/6 mice. COBRA 1 SVP elicited neutralizing antibodies against all four serotypes from different geographical regions and time periods

Correlates of protection against dengue virus are still not clearly defined that contributes to the difficulties in vaccine design and assessment [156]. Nonetheless, it is widely accepted that the humoral immune response, particularly neutralizing antibodies, is important for protecting against viral infection and preventing dengue-induced disease [159]. The COBRA E antigen may elicit cross-neutralizing antibodies that target quaternary structure of the E protein. COBRA SVP vaccinations elicited cross-reactive binding antibodies to both virus or virus-like particles expressing E proteins, but not to soluble monomer versions of E (Fig. 4.1). Other studies show that monoclonal antibodies that bind to quaternary structures are effective at eliciting cross-neutralizing antibodies [36, 161, 163].

COBRA E antigens are an innovative approach for a universal DENV vaccine. Current dengue vaccine strategies need to formulate four separate components based on

prM-E from a wild type strains of dengue that represent each dengue serotype. One of the challenges with this approach is the difficultly to achieve a balanced tetravalent formulation that may produce a skewed antibody response to one or more of the four dengue serotypes. In phase I clinical trials, DENV3 and DENV4 of the Dengvaxia vaccine replicated more efficiently than DENV1 or DENV2, which later correlated to eliciting higher neutralizing antibody titers for DENV3 and DENV4 than DENV 1 or DENV 2 in phase III trials [212]. In contrast to vaccines that utilize four vaccine components, the single COBRA E vaccine will be more cost effective to produce since it elicits similar cross-reactive antibodies as a four separate dengue component vaccine. Another advantage of using the dengue COBRA E-based vaccine is it can be used in many different types of delivery platforms, such as RNA [220], DNA, expressed from viral vectors, as recombinant protein, as well as live-attenuated viruses.

One area of concern for any dengue vaccine is antibody dependent enhancement (ADE). ADE occurs when pre-existing antibodies to one DENV serotype do not neutralize, but rather enhance a heterotypic infection [4, 179]. Suboptimal levels of pre-existing antibody against Dengue have been associated with severe DENV disease compared to no antibody or high antibody levels [157]. ADE usually occurs two years after a primary infection and any cross protective antibodies wane [201, 202]. Thus, it is important for a DENV vaccine to be protective against all four strains and maintain robustness of breadth in protection.

In mice, the longevity of neutralizing antibodies was maintained 20 weeks after vaccination, though there was a significant decrease against 2 of the 12 strains in our panel (Fig 4.3). Neutralizing antibody titers were still greater that 1:10, which is an accepted

correlate of protection for other flaviviruses such as Yellow Fever virus (YFV) [256] and Japanese Encephalitis virus (JEV) [257]. COBRA 1 SVP vaccination elicited robust DENV- specific memory B cell response toward all four serotypes (Fig 4.4).

Challenge studies and ADE assays were not done during this study. Wild type mice (such as C57BL/6) are not susceptible to DENV infection [258]. Immunocompromised mice have been used for DENV infection studies (such as IFN receptor deficient mice A129, AG129), but since they lack key innate immune components, they are not relevant models to study immune responses to vaccination [259]. *In vitro* assays for ADE are not ideal since they are discordant with *in vivo* results using immunocompromised mice [260, 261].

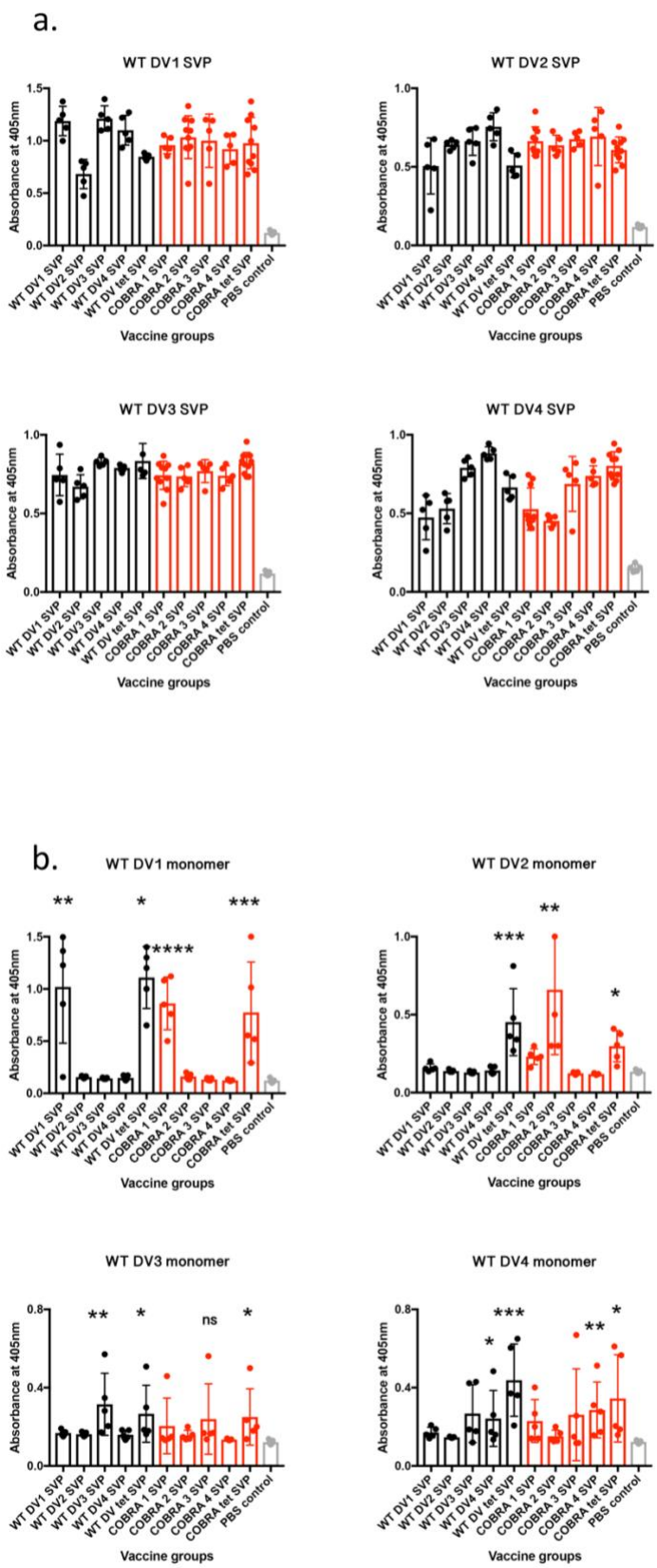

a.
WT DV1 SVP
WT DV2 SVP
WT DV3 SVP
WT DV4 SVP
Absorbance at 405nm
WT DV1 SVP
WT DV2 SVP
WT DV3 SVP
WT DV4 SVP
WT DV tet SVP
COBRA 1 SVP
COBRA 2 SVP
COBRA 3 SVP
COBRA 4 SVP
COBRA tet SVP
PBS control
Vaccine groups
b.
WT DV1 monomer
WT DV2 monomer
WT DV3 monomer
WT DV4 monomer
ns

Figure 4.1 Seroconversion of mice vaccinated with COBRA or wild type DENV SVPs. Each graph represents antibody binding to a) SVP or b) soluble E representing one of the four dengue serotypes. Vaccine groups are indicated on x-axis, with black bars representing wild type SVP vaccinations and red bars representing COBRA SVP vaccinations. Total IgG binding of sera (1:100 dilution) from individual mice were measured by their optical density (OD) values. Absorbance was measured at 405 nm.

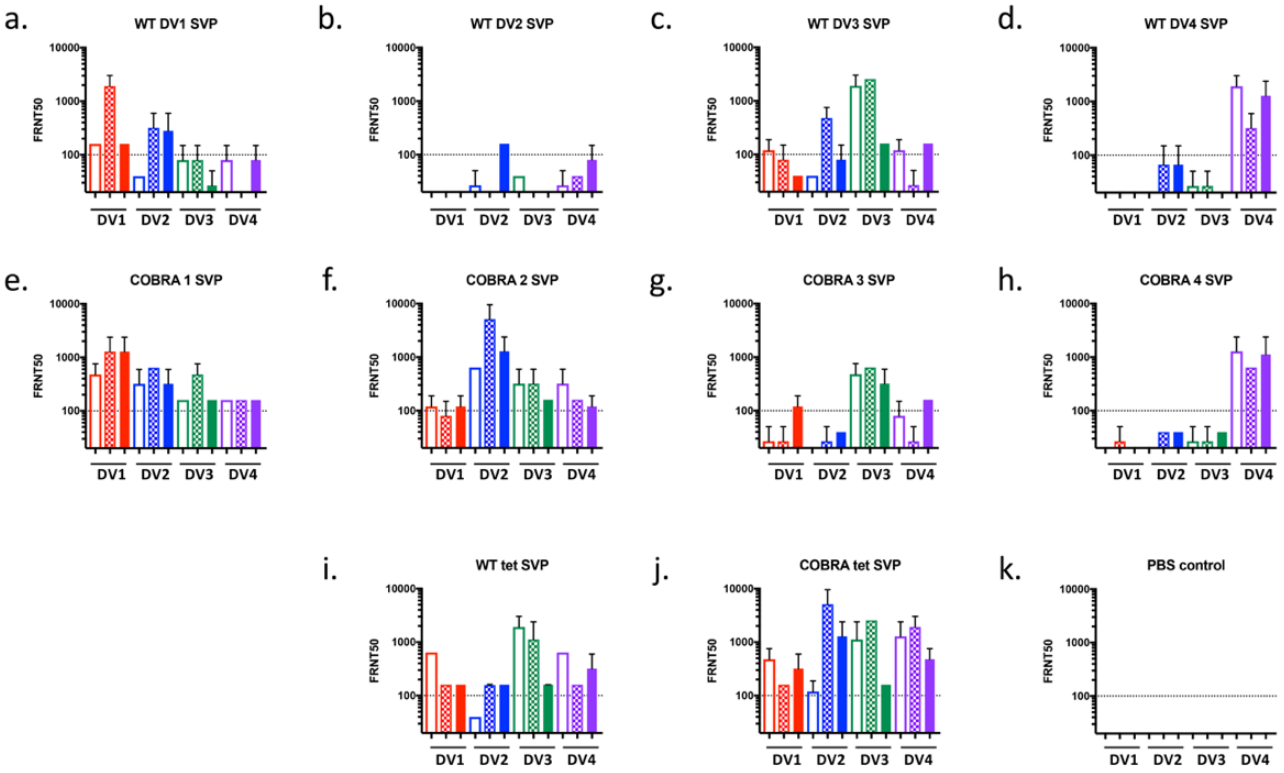


Figure 4.2 Neutralization ability of sera from mice vaccinated with COBRA or wild type DENV SVPs. Focus reduction neutralization test $(FRNT)_{50}$ of pooled sera from C57BL/6 mice vaccination with a) – d) monovalent wild type SVP, e) – h) monovalent COBRA SVP, i) – j) tetravalent SVP, or k) PBS control against a panel of prototype (unfilled), modern American (checkered), and modern Asian (filled) strains from each serotype. Dashed line indicates 1:100 titer.

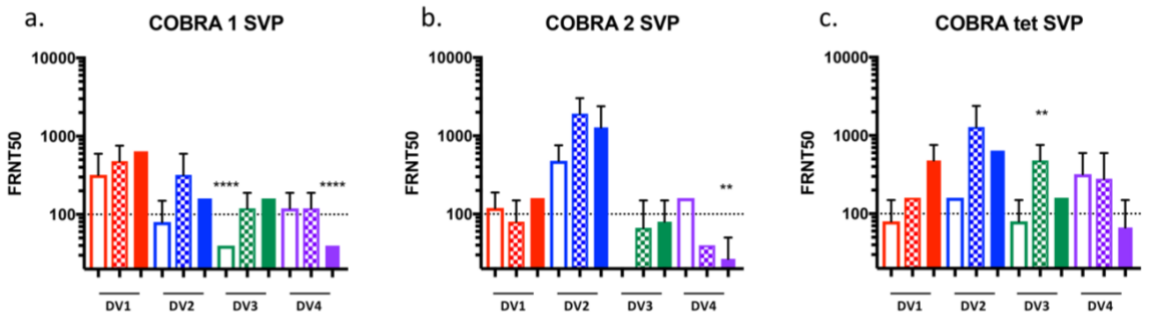


Figure 4.3 Longevity of neutralizing antibody titers of COBRA SVP vaccination in mice. $FRNT_{50}$ of pooled sera from C57BL/6 mice 20 weeks after vaccination with a) COBRA 1, b) COBRA 2, or c) COBRA tetravalent SVPs against a panel of prototype (unfilled), modern American (checkered), and modern Asian (filled) strains from each serotype. Dashed line indicates 1:100 titer. Statistical differences between 4 weeks and 20 weeks post vaccination calculated by paired t-test. ** for $P \leq 0.01$, **** for $P \leq 0.0001$.

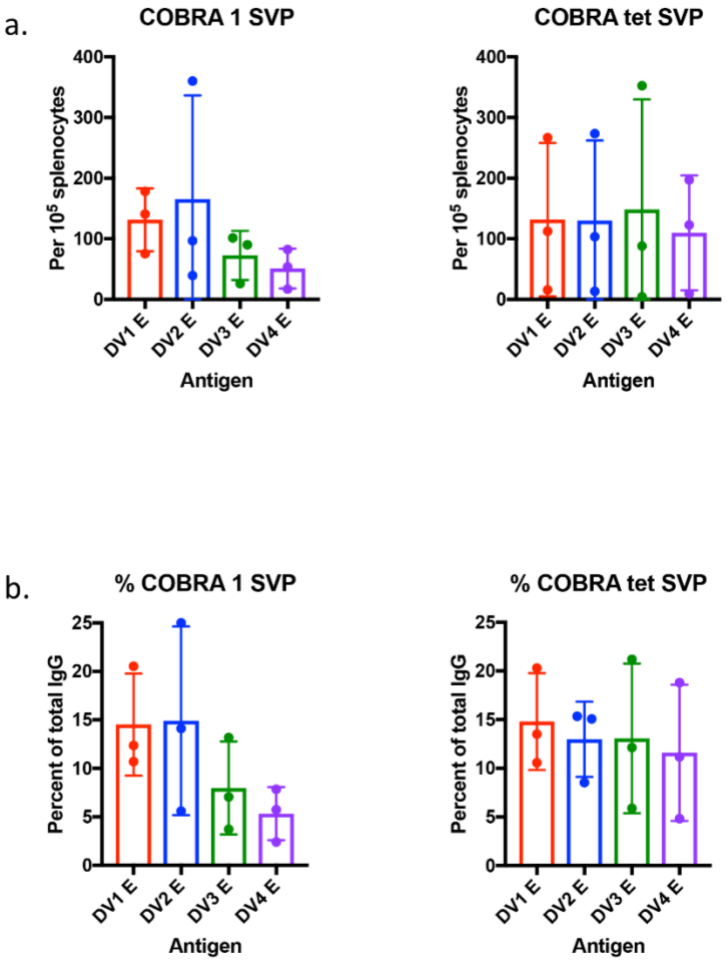


Figure 4.4 Memory B cell response of COBRA SVP vaccination in mice. 22 weeks after final vaccination, mice were stimulated *in vivo* with intramuscular injection of homologous SVP and spleens were harvested after 6 days. a) Each graph represents the total number of DENV-specific memory B cells (measured by their conversion to IgG producing cells) from a vaccine group. E antigens that represent each of the serotypes are on the x-axis. b) Each graph represents the percentage of DENV-specific memory B cells from a vaccine group. E antigens that represent each of the serotypes are on the x-axis. Percentages were calculated from total number of IgG secreting cells.

Table 4.1 IgG subclasses elicited by COBRA or wild type DENV SVPs. Each table shows IgG subclass binding of homologous monovalent WT SVP, WT tetravalent SVP, COBRA E SVP, and COBRA tetravalent SVP to commercial E antigen representing a) DENV1, b) DENV2, c) DENV3, d) DENV4. Asterisks represent statistical significance measured against PBS control group, measured with unpaired t-test with Welch's correction, with the color intensity increasing with the significance. *ns* for $P > 0.05$, * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, **** for $P \leq 0.0001$. In parentheses are the percentage of mice in each group that had IgG subclass binding (OD value) twice as greater that the average of the corresponding binding of the PBS control group.

p-value

n.s. ****

a.

DENV 1	IgG subclass			
Vaccine group	IgG1	IgG2b	IgG2c	IgG3
WT DV 1 SVP	* (80%)	* (80%)	ns (20%)	ns (20%)
WT DV tet SVP	* (80%)	** (60%)	ns (20%)	* (0%)
COBRA 1 SVP	**** (100%)	* (40%)	** (70%)	ns (0%)
COBRA tet SVP	* (50%)	* (60%)	** (60%)	ns (0%)

b.

DENV 2	IgG subclass			
Vaccine group	IgG1	IgG2b	IgG2c	IgG3
WT DV 2 SVP	**** (60%)	* (0%)	ns (0%)	ns (0%)
WT DV tet SVP	* (40%)	* (0%)	ns (0%)	ns (0%)
COBRA 2 SVP	**** (100%)	ns (20%)	* (20%)	ns (0%)
COBRA tet SVP	ns (10%)	* (20%)	ns (20%)	ns (0%)

c.

DENV 3	IgG subclass			
Vaccine group	IgG1	IgG2b	IgG2c	IgG3
WT DV 3 SVP	* (60%)	*** (0%)	ns (0%)	** (0%)
WT DV tet SVP	** (100%)	ns (20%)	ns (20%)	ns (0%)
COBRA 3 SVP	ns (20%)	ns (0%)	ns (0%)	**** (0%)
COBRA tet SVP	** (70%)	* (40%)	* (40%)	** (20%)

d.

DENV 4	IgG subclass			
Vaccine group	IgG1	IgG2b	IgG2c	IgG3
WT DV 4 SVP	ns (40%)	ns (0%)	ns (0%)	ns (0%)
WT DV tet SVP	* (60%)	ns (20%)	ns (20%)	* (0%)
COBRA 4 SVP	* (80%)	ns (0%)	ns (0%)	** (0%)
COBRA tet SVP	** (50%)	ns (20%)	ns (20%)	* (20%)

Table 4.2 DENV strains used for focus reduction neutralization test.

Serotype	Virus strain
DV1	Hawaii/1944 (prototype)
DV1	Costa Rica/BC89/1994 (modern American)
DV1	Vietnam/BID-V1792/2007 (modern Asian)
DV2	New Guinea/NGC/1944 (prototype)
DV2	US/BID-V594/2006 (modern American)
DV2	Vietnam/BID-V1007/2006 (modern Asian)
DV3	Philippines/H-87/1956 (prototype)
DV3	US/BID-V1619/2005 (modern American)
DV3	Sri Lanka/271242/1991 (modern Asian)
DV4	Philippines/H-241/1956 (prototype)
DV4	US/BC258/1994 (modern American)
DV4	Malaysia/BC13/1997 (modern Asian)

CHAPTER 5

IMMUNOGENICITY AND BREATH OF IMMUNE RESPONSE ELICITED BY DENV COBRA SVP VACCINATION IN NAÏVE AND PRE-IMMUNE NON-HUMAN PRIMATES[1]

[1]A version of this chapter was submitted to *Journal of Virology* 4/9/20.
"Uno, N and Ross, T. A Universal Dengue Vaccine Elicits Neutralizing Antibodies Against Strains from All Four Dengue Serotypes".

Abstract

Any potential dengue virus (DENV) vaccine needs to elicit protective immunity against strains from all four serotypes to avoid potential antibody dependent enhancement (ADE). Previously, four independent DENV envelope (E) glycoproteins were generated using wild-type E sequences from viruses isolated between 1943 to 2006 using computationally-optimized broadly reactive antigen (COBRA) methodology. COBRA E antigens were expressed on the surface of subvirion viral particles (SVPs). Two COBRA DENV vaccine candidates that elicited the broadest breadth of neutralizing antibodies in mice were used to vaccinate rhesus macaques (*Macca mulata*) that were either immunologically naïve to any DENV serotype or were had pre-existing antibodies to DENV. Antibodies elicited by COBRA DENV E immunogens neutralized all 12 strains of DENV *in vitro*, which was comparable to antibodies elicited by a tetravalent vaccination mixture. Therefore, using a single DENV COBRA E protein can elicit neutralizing antibodies against strains representing all four serotypes of DENV in both naïve and dengue pre-immune populations.

Introduction

Developing a vaccine for Dengue virus (DENV) has been difficult to achieve. There are four serotypes of the virus and any potential vaccine needs to protect against all four serotypes of the virus to avoid antibody dependent enhancement (ADE). ADE is thought to occur when pre-existing antibodies to one DENV serotype do not neutralize, but enhance a heterotypic infection. Thus, the vaccine needs to elicit antibodies that neutralize all four serotypes. Countries that are endemic to DENV, more than one serotype circulates, with all four often circulating.

Increases in global travel and the spread of mosquito vector due to climate change have also increased the amount of DENV naïve individuals at risk for infection. Potential DENV vaccines will need to be effective and safe for these groups as well. The only licensed vaccine, Dengvaxia, is only recommended for individuals aged 9-45 in endemic regions. It has been shown to act as a primary infection in some naïve individuals, increasing their risk for severe disease during an actual DENV infection.

Vaccine development has also been slow due to the lack of a relevant mouse model [262]. Non-human primates (NHPs) are natural hosts of the sylvatic DENV cycle, so they are more appropriate for vaccine studies [263]. Rhesus macaques have been widely used for studying immune response to DENV [264], as well as green monkeys, cynomolgus macaque, and marmosets [265-267]. Viremia can be detected from infection with human strains, though NHPs are asymptomatic [268]. Another limitation is the ability to analyze T cell responses, due to the lack of tetramers and known T cell epitopes [269]. However, rhesus macaques have immune responses that are qualitatively similar to human responses, therefore they are often used in DENV vaccine studies [265] .

In the current study, we developed and tested a DENV subviral particle (SVP) vaccine targeting the envelope (E) glycoprotein by designing consensus sequences using computationally-optimized broadly reactive antigen (COBRA) methodology in rhesus macaques. The vaccines were tested in both DENV naïve and pre-immune groups. COBRA DENV SVP vaccination elicited broadly neutralizing antibodies against all four serotypes regardless of previous serostatus.

Materials and methods

NHP vaccination

Immunologically naïve and pre-immune rhesus macaques (*Macca mulata*) were vaccinated with monovalent or tetravalent DENV COBRA SVP (1 g per vaccination with Imject adjuvant) twice at 6 weeks intervals. Pre-immune rhesus macaques were previously infected at the Walter Reed Army Institute of Research two years prior. Blood was collected at week 6 and 10, sera and peripheral mononuclear cells (PBMCs) were isolated using BD Vacutainer Cell Preparation Tubes (CPT) with Sodium Heparin (Becton Dickinson, Franklin Lakes, NJ, USA) following the manufacturer's protocol. Sera were frozen at -80°C. PBMCs were incubated in RPMI 1640 medium (Millipore Sigma, Burlington, MA, USA) containing 10% FBS, 55 µM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin,100 µg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and 1% MEM nonessential amino acids (all from Thermo Fisher Scientific).

Transient transfection and purification of COBRA SVPs

Human embryonic kidney (HEK) 293T cells were transfected with 10μg of DNA plasmid encoding for COBRA prM-E using Lipofectamine 3000. After incubating for 4 days at 37°C, supernatants were collected and cell debris removed by centrifugation and vacuum filtration through a 0.22mM sterile filter. SVPs were collected via ultracentrifugation (100,000×*g* through 20% glycerol wt/vol) for 4h at 4°C. The concentrated SVP pellets were resuspended in phosphate buffered saline (PBS) and stored at -80°C. Protein concentration was determined by MicroBCA™ Protein Assay Reagent Kit (Thermo Fisher Scientific).

Detection of anti-DENV E antibodies

Quantitative ELISA was performed to detect anti-SVP or anti-E antibodies elicited by vaccination. Nunc Maxisorp plates were coated (2ug/mL) overnight at 4°C with either subviral particles expressing one of the wild-type DENV E proteins or soluble DENV E (MyBiosource, Inc. San Diego, CA, USA). Plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with 1% BSA in PBS for 1 hr at RT. Subsequently, 1:100 dilution of each serum samples were added to the plates for 2 h. Following three washes with PBS-T, horse radish peroxidase (HRP)-conjugated anti-monkey IgG (H+L) antibody (Thermo Fisher Scientific) was added for 1 h before developing the plates using TMB substrate. OD values were read at 450 nm.

Dengue viruses

Viruses used in this study were for DENV 1: DV1/US/Hawaii/1944, DV1/Vietnam/BID-V1792/2007, DV1/Costa Rica/BC89/1994; DENV 2: DV2/New Guinea/NGC/1944, DV2/Vietnam/BID-V1007/2006, DV2/US/BID-V594/2006; DENV 3:

DV3/Philippines/H-87/1956, DV3/Sri Lanka/271242/1991, DV3/US/BID-V1619/2005, and DENV 4: DV4/Philippines/H-241/1956, DV4/Malaysia/BC13/1997, DV4/US/BC258/1994.

Neutralization assay

Neutralization activity in collected antisera were determined using a focus reduction neutralization test (FRNT). Sera was heat inactivated at 56°C for 30 min and the serially diluted (4 fold). Sera was incubated with dengue viruses (100 FFU) representing each of the 4 serotypes for 60 min at 37°C. Three dengue virus were used per each serotype that represented the prototype, modern Asian, and modern American strains. Each dengue virus was mixed with the serially diluted antisera and used to infect a single cell monolayer of Vero cell in 96-well plates for 90 min at 37°C that were plated. Cells were overlaid methylcellulose (1% [wt/vol]). Cells were incubated for 3 days at 37°C (2 days for DENV 4 viruses). Cells were washed with PBS and fixed with a mixture of acetone and methanol (Foci were stained using Vectastain ABC kit, following the manufacturer's protocols (Vector Laboratories, Burlingame, CA, USA). Anti-DENV E monoclonal antibodies were used to detect virally infected cells. For DENV 1 and 3 viruses, the antibody AM01108PU-N (OriGene Technologies, Rockville, MD, USA) was used and for DENV 2 the monoclonal antibody 9.F.10 (Santa Cruz Biotechnology, Dallas, TX, USA) was used and for DENV 4, the monoclonal antibody 4G2 (Millipore Sigma, Billerica, MA, USA) was used. Foci were imaged using CTL Immunospot (Cellular Technology Limited, Cleveland, OH, USA). $FRNT_{50}$ determined by calculating the point at which sera reduced dengue foci by 50% compared to serum-free, dengue virus control wells.

B cell ELISPOT

PBMCs were stimulated *ex-vivo* with IL-2 and R848 (Mabtech) for 5 days. Multiscreen HTS (Millipore Sigma) plate was pre-wetted with 35% ethanol and coated with 5mg/mL of DENV1-4 peptides (My BioSource), anti-monkey IgG (Mabtech), or BSA. Splenocytes were added in serial dilutions starting at 300,000 cells/well in RPMI media supplemented with 55 µM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin,100 µg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and 1% MEM nonessential amino acids and incubated at 37° for 24hr. The cells were washed five times with PBS and anti-monkey IgG-biotin (Mabtech) added overnight at 4°C. The following day, plates were washed five times with PBS. Streptavidin-HRP (Mabtech) was added and incubated at room temperature for 2 hours. After washing five times with PBS, spots were developed with TMB solution (Vector Laboratories). Spots were imaged using CTL ImmunoSpot Analyzer (Cellular Technology Limited, Cleveland OH, USA) and counted using ImmunoSpot software version 5.1.36 Professional SC.

Statistical analysis

To determine statistical significance between individual vaccine groups binding to DENV E, two-tailed unpaired t-test were done, with confidence level at 95%. Vaccine groups were compared in ELISA antibody binding against each other with soluble E from all four serotypes. For comparisons between NHP vaccine groups with different n, Welch's t-test was performed. P-values less than .05 were considered statistically significant. *ns* for $p > 0.05$, * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, **** for $p \leq 0.0001$.

See Appendix B for complete table of ELISA statistical analysis.

Results

To determine the effectiveness of these vaccine candidates in pre-immune populations, the top two monovalent COBRA E SVP vaccines were tested in rhesus macaques (*macaca mulatta*) that were antibody positive to Brazilian strains DENV-1 or DENV-2 serotypes (Figure 5.1). All pre-immune monkeys had cross reactive anti-E IgG antibodies both prior to– and 4 weeks post-vaccination (Fig. 5.2, 5.3). Immunologically naïve monkeys had little to no anti-IgG E antibodies prior to vaccination to any the DENV strains used in this study. However, 4 weeks post-boost, all monkeys had detectable titers of anti-E antibodies to all four serotypes. COBRA 2 SVP groups had statistically significant titers of anti-E compared to pre-vaccination for all four serotypes (Appendix Table B.1).

Comparing IgG binding to different dengue E, there was no significant difference in the pre-immune groups, except for COBRA 2 being significantly higher than COBRA tetravalent to dengue serotype 2 (Fig 5.2b). In naïve groups, binding titer to COBRA 1 was comparable to COBRA tetravalent, while they were significantly higher than COBRA 2 in binding to E from serotype 1 and 3. COBRA tetravalent binding was also higher than COBRA 2 to serotypes 1, 3, and 4 (Fig 5.2, 5.3 and Appendix Table B.2)

Monkeys vaccinated with COBRA 1 or 2 SVPs had neutralizing antibodies against all four serotypes and at least 11 of the 12 strains (Figs. 5.4-5.7). Pre-immune monkeys vaccinated with tetravalent COBRA SVPs elicited similar antibody titers as animals vaccinated with either COBRA SVP vaccine (Appendix B Table B.3). Neutralizing titers ($FRNT_{50}$) were increased after vaccination in pre-immune animals regardless of the vaccine

administered (Figure 5.4, 5.6). This was also seen in $FRNT_{80}$ of pre-immune groups (Figure 5.7)

In $FRNT_{50}$ of non-pre-immune monkeys, COBRA 1 SVP vaccines elicited neutralizing antibodies against all 12 strains, while COBRA 2 SVPs elicited neutralizing antibodies against 11 of the DENV strains (Fig. 5.5, 5.6). Neutralizing antibody titers elicited by COBRA 1 SVP vaccination were similar to COBRA tetravalent SVP vaccination, while COBRA 2 SVP vaccination elicited lower neutralizing antibody titers to COBRA tetravalent SVP vaccination (Appendix Table B.4)

To study memory response to Dengue E, PBMCs were collected 4 weeks post boost and stimulated *ex vivo* with Dengue full length E. The amount of antibody secreting cells (ASCs) were enumerated by ELISPOT. Pre-immune monkeys had high numbers of ASCs against DENV1 and 3 (Fig. 5.8a). These same vaccines administered to naïve animals resulted in fewer ASCs stimulated (Fig. 5.8b). Naïve monkeys vaccinated with COBRA 1 SVPs elicited the highest number of ASCs that correlated with the relatively high cross-reactive IgG and neutralizing antibody titers. COBRA 2 may have elicited fewer cells, but each cell produced more neutralizing antibodies. Overall, COBRA 1 SVP vaccination in both pre-immune and naïve groups elicited memory B cells against all four serotypes comparable to tetravalent formulation (Appendix Tables B.5, B.6).

Discussion

Any potential DENV vaccine needs to be safe and protective against all four serotypes in populations with different serostatus. With increased global travel and the spread of mosquitoes due to climate change, the risk of dengue virus infection spills into

new geographic areas, while circulating strains continue to change [2]. The COBRA methodology takes into account strains from various geographic locations and time periods in order to create a broadly protective immunogen. COBRA E immunogens were designed from human isolates from 1941-2006 spanning different regions of the world. This process resulted in the COBRA 1 SVP vaccine, a promising universal DENV vaccine candidate that elicits neutralizing antibodies against all four serotypes in both naïve and pre-immune populations.

Correlates of protection against dengue virus are still not clearly defined which contributes to the difficulties in vaccine design and assessment [156]. Nonetheless, it is widely accepted that the humoral immune response, particularly neutralizing antibodies, is important for protecting against viral infection and preventing dengue-induced disease [159]. COBRA SVP vaccinations elicited cross-reactive binding antibodies to both virus or virus-like particles expressing E proteins, but not to soluble monomeric E (Fig. 5.1). Other studies show that monoclonal antibodies that bind to quaternary structures are effective at eliciting cross-neutralizing antibodies [36, 161, 163]. The COBRA SVP may elicit cross-neutralizing antibodies that target quaternary structure of the E protein.

The COBRA SVP vaccine is a safer alternative to live viral vaccines since it contains no genomic material and still maintains fusogenic properties of mature virions [218]. Thus, it can still be taken up by host cells for processing and antigen presentation. The COBRA E also incorporates more relevant and comprehensive epitopes from strains representing multiple serotypes of dengue compared to other leading dengue vaccine candidates [270]. The Dengvaxia vaccine uses Asian strains that were isolated between

1978 to 1988. DENVax uses Asian strains from 1964 to 1976 and TV003 uses strains isolated from 1974 to 1978.

COBRA 1 SVP vaccines elicited robust neutralizing antibodies (>1:200) against multiple dengue viral strains representing all four serotypes in both pre-immune and non-pre-immune rhesus macaques. Though an exact correlate of protection for neutralizing antibodies to dengue viruses are unknown, when testing non-replicating DENV vaccines in rhesus macaques, neutralizing titers are recommended to be 1:200 [271]. In a human cohort study of DENV infections, neutralization test titers less than 1:100 were associated with severe disease [272].

COBRA 2 SVP groups had significant increase of antibodies that bound to soluble E representing all four serotypes, including naïve groups. Naïve monkeys given COBRA 1 SVP vaccination had significant seroconversion to DV1 and DV3 E. However, the naïve COBRA 2 SVP group did not elicit higher neutralizing antibodies than naïve COBRA 1 SVP group. This may be because COBRA 1 SVP elicited neutralizing antibodies that bind to quaternary DENV structures and not E monomers.

COBRA E design is an innovative approach for a universal DENV vaccine. Current dengue vaccine strategies need to formulate four separate components based on prM-E from a wild type strains of dengue that represent each dengue serotype. One of the challenges with this approach is the difficultly to achieve a balanced tetravalent formulation that may produce a skewed antibody response to one or more of the four dengue serotypes. In phase I clinical trials, DENV3 and DENV4 of the Dengvaxia vaccine replicated more efficiently than DENV1 or DENV2, which later correlated to eliciting higher neutralizing antibody titers for DENV3 and DENV4 than DENV 1 or DENV 2 in

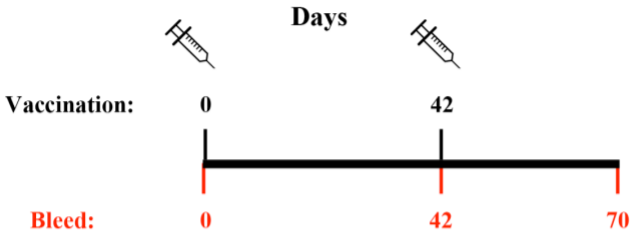
phase III trials [212]. In contrast to vaccines that utilize four vaccine components, the single COBRA E vaccine will be more cost effective to produce since it elicits similar cross-serotype antibodies as a four separate dengue component vaccine. Another advantage of using the DENV COBRA E-based vaccine is it can be used in many different types of delivery platforms, such as RNA, DNA, recombinant protein, as well as viral vectors [220].

One area of concern for any dengue vaccine is antibody dependent enhancement (ADE). ADE occurs when pre-existing antibodies to one DENV serotype do not neutralize, but rather enhance a heterotypic infection [4, 179]. Suboptimal levels of pre-existing antibody against Dengue have been associated with severe DENV disease compared to no antibody or high antibody levels [157]. ADE usually occurs two years after a primary infection and any cross protective antibodies wane [201, 202].

It is important for a DENV vaccine to be protective against all four strains and maintain robustness of breadth in protection. Dengvaxia reduced the risk of symptomatic disease by 60% during the first 2 years after the vaccine was introduced [212]. However, after the third year, preliminary data indicated that there was a higher risk of hospitalization. This trial will continue for another 6 years [273]. The pre-immune animals used in the current study had been infected two years prior, and they did not exhibit any adverse reactions from vaccination compared to the naïve groups. They elicited more robust neutralizing antibody titers and memory B cell response compared to naïve groups.

COBRA 1 SVP vaccination elicited DENV- memory B cells in naïve and pre-immune NHPs comparable to tetravalent formulation (Fig. 5.4). T cell responses were unable to be analyzed due to lack of reagents [269]. Challenge studies were not able to be performed for this study. Due to the lack of an ideal animal model for DENV, clinical

trials in humans to determine the safety and efficacy of COBRA vaccine will need to be tested.



Vaccine	Baseline Serology	N	Dose (ug)
COBRA 1 SVP	DENV1, 2^{+}	6	1000
COBRA 1 SVP	Naïve	3	1000
COBRA 2 SVP	DENV1, 2^{+}	6	1000
COBRA 2 SVP	Naïve	3	1000
COBRA tet SVP	DENV1, 2^{+}	5	1000
COBRA tet SVP	Naïve	3	1000

Figure 5.1 Schematic of non-human primate vaccine study.

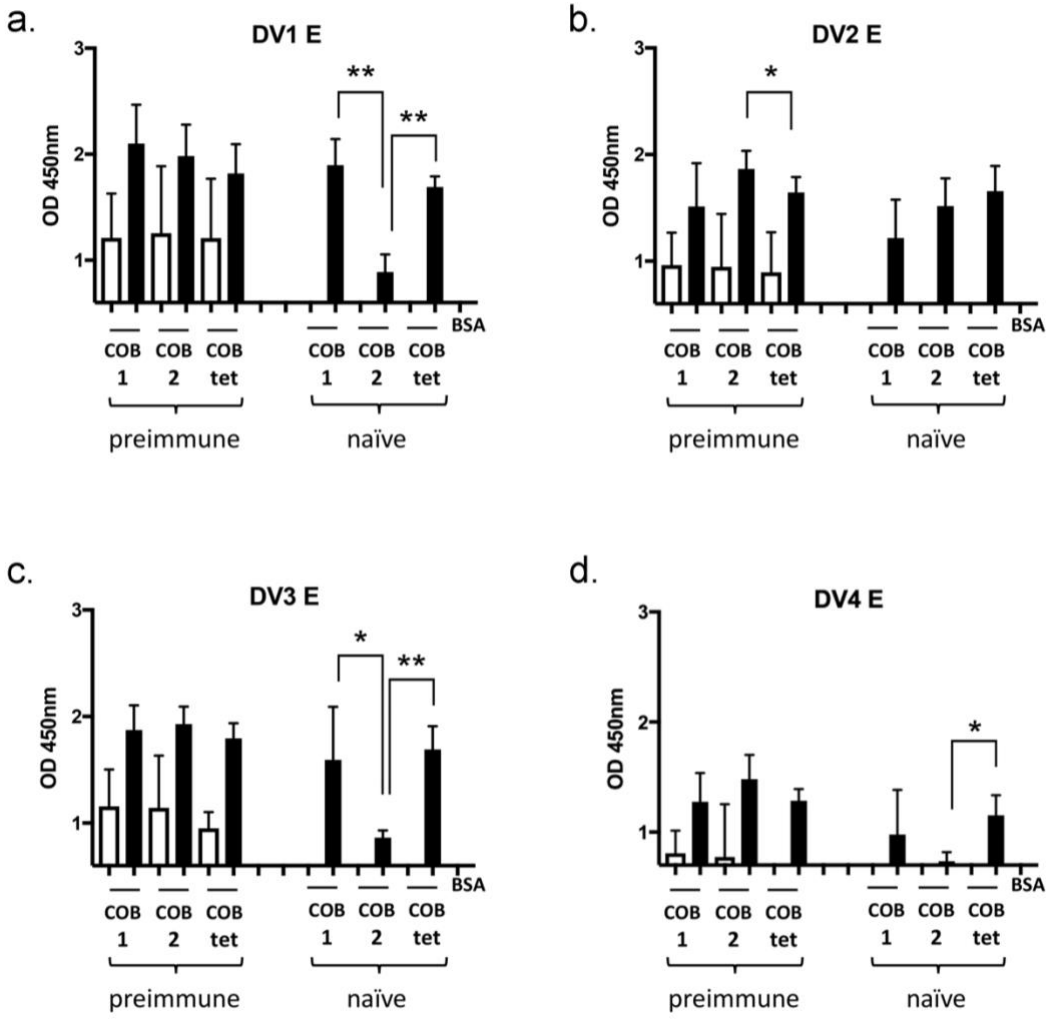
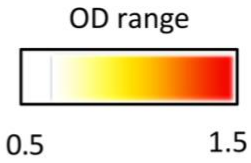


Figure 5.2 Seroconversion of naïve and pre-immune non-human primates vaccinated with COBRA DENV SVPs. ELISA for seroconversion after vaccination with DENV COBRA SVPs. a-d) Antibody binding to E of DV 1-4. Vaccine groups listed on x-axis. Total IgG binding of sera (1:100 dilution) from individual NHP were measured by their optical density (OD) values. Absorbance was measured at 405 nm. Unfilled bars are day 0 (pre-vaccination), filled bars are day 70 (four weeks after boost). Vaccine groups were compared in day 70 antibody binding against each other by two-tailed unpaired t-tests, with confidence level at 95%. For comparisons between NHP vaccine groups with

different n, Welch's t-test was performed. P-values less than .05 were considered statistically significant. *ns* for $p > 0.05$, * for $p \leq 0.05$, ** for $p \leq 0.01$.



COB 1 SVP		DV1		DV2		DV3		DV4	
		d0	d70	d0	d70	d0	d70	d0	d70
97-18	preimmune	1.8835	2.3705	1.4225	1.881	1.606	2.0855	1.061	1.527
98-18		0.8415	1.3825	1.0385	1.284	0.614	1.4295	0.557	0.845
99-18		1.3395	2.2505	0.605	1.102	0.927	1.8815	0.696	1.1345
100-18		1.1645	2.2165	0.677	1.152	1.1975	1.8735	0.663	1.31
101-18		1.33	2.311	0.908	1.5695	1.3685	1.989	1.025	1.541
103-18		0.702	2.0765	1.1315	2.0905	1.2285	1.9795	0.853	1.298
117-18	naïve	0.509	1.656	0.6195	1.4895	0.6566	1.852	0.7005	1.2525
118-18		0.5635	2.148	0.61	1.352	0.5345	1.9085	0.5065	1.1695
59-17		0.441	1.8855	0.5385	0.81	0.494	1.0205	0.5955	0.5135

COB 2 SVP		DV1		DV2		DV3		DV4	
		d0	d70	d0	d70	d0	d70	d0	d70
107-18	preimmune	0.9905	2.1535	0.275	1.6845	0.6385	1.9205	0.3325	1.3185
108-18		2.1435	2.164	1.649	2.1175	1.87	2.086	1.4605	1.827
109-18		1.827	2.213	1.1545	1.8415	1.6105	1.9975	1.1595	1.603
111-18		1.2755	2.1515	0.484	1.685	0.7685	2.095	0.284	1.513
112-18		0.4575	1.5335	1.039	1.87	0.9325	1.6905	0.4975	1.2035
121-18		0.835	1.6775	1.082	1.989	1.0365	1.786	0.9055	1.4195
62-17	naïve	0.3775	1.066	0.332	1.6955	0.2445	0.8325	0.3665	0.6915
65-17		0.4915	0.863	0.4415	1.2175	0.387	0.8155	0.334	0.683
66-17		0.174	0.74	0.161	1.638	0.423	0.9405	0.443	0.83

COB tet SVP		DV1		DV2		DV3		DV4	
		d0	d70	d0	d70	d0	d70	d0	d70
102-18	preimmune	1.3275	2.0005	0.5625	1.6365	0.9265	1.936	0.5375	1.2825
104-18		0.4845	1.519	1.0485	1.83	0.806	1.7395	0.446	1.24
105-18		1.8245	1.96	0.8095	1.6425	1.1665	1.9135	0.5945	1.368
106-18		1.6125	2.092	0.582	1.431	1.0385	1.8025	0.539	1.4005
120-18		0.8005	1.5185	1.4685	1.6895	0.8165	1.584	0.639	1.137
60-17	naïve	0.2285	1.7145	0.504	1.93	0.3965	1.49	0.222	1.14
61-17		0.189	1.777	0.183	1.5235	0.166	1.9235	0.155	1.3415
110-18		0.1335	1.582	0.1545	1.5165	0.1805	1.661	0.158	0.9805

Figure 5.3 Heatmap of ELISA data of NHP given COBRA SVP vaccination. Each table represents a COBRA SVP vaccine group, pre-immune monkeys at top and naïve monkeys at the bottom. Day 0 (d0) indicates pre-vaccination and d70 indicated 4 weeks after boost . Individual monkey IDs are listed at the left. Increase in red intensity correlate to increase in optical density (OD) values.

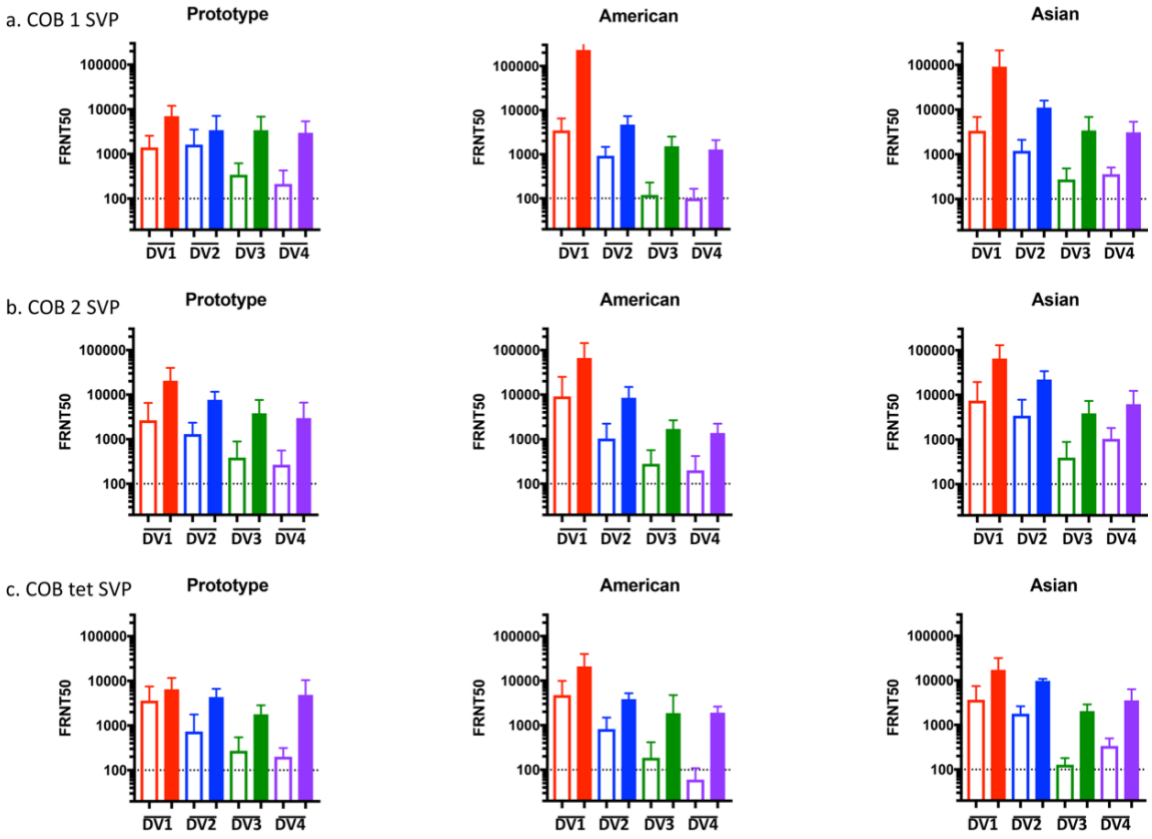


Figure 5.4 Neutralization ability of sera from pre-immune non-human primates vaccinated with COBRA DENV SVPs. $FRNT_{50}$ of individual DENV pre-immune NHPs given a) COBRA 1 SVP, b) COBRA 2 SVP, c) COBRA tetravalent SVP vaccinations. Each graph represents a different genotype. DENV serotypes indicated on x-axis. Dashed line indicate 1:100 titer. Unfilled bars are day 0, filled bars are day 70.

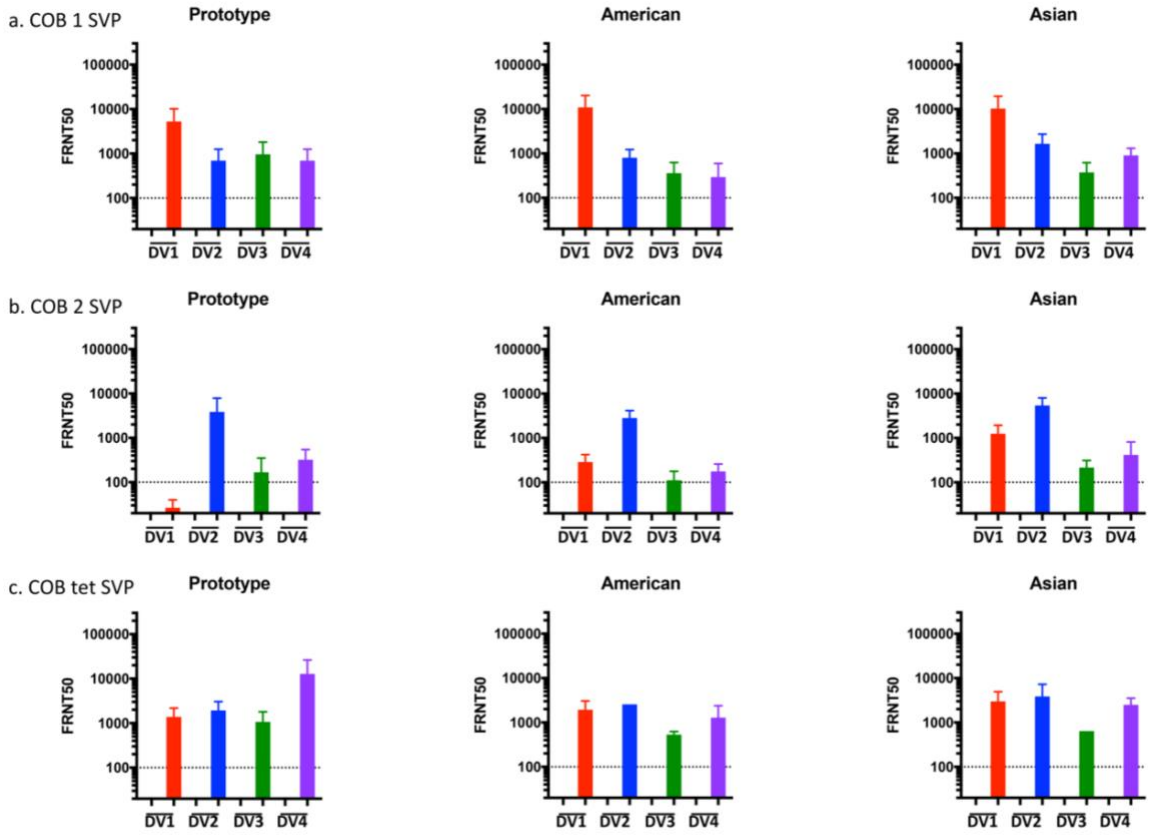


Figure 5.5 Neutralization ability of sera from naïve non-human primates vaccinated with COBRA DENV SVPs. $FRTN_{50}$ of individual naïve NHPs given a) COBRA 1 SVP, b) COBRA 2 SVP, c) COBRA tetravalent SVP vaccinations. Each graph represents a different genotype. DENV serotypes indicated on x-axis. Dashed line indicate 1:100 titer. Unfilled bars are day 0, filled bars are day 70.

			DV1				DV2				DV3				DV4			
			prototype		American		prototype		American		prototype		American		prototype		American	
			d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70
COBRA 1 SVP	Preimmune	97-18	2560	10240	7680	7E+05	7680	2E+05	2560	2560	1280	7680	2560	10240	640	2560	320	2560
		98-18	0	480	40	1920	120	1280	640	640	1280	3840	1920	10240	13	640	40	160
		99-18	2560	10240	2560	2E+05	1920	20480	120	1280	120	1280	160	7680	120	2560	40	1280
		100-18	1920	10240	5120	2E+05	2560	30720	640	640	320	5120	640	10240	480	2560	120	1920
		101-18	1280	10240	5120	2E+05	7680	2E+05	640	5120	1280	7680	1280	20480	640	10240	640	2560
		103-18	120	640	320	2560	160	5120	5120	10240	1280	2560	640	7680	160	1920	160	2560
	Naïve	117-18	0	10240	0	20480	0	7680	0	1280	0	1280	0	2560	0	640	0	320
		118-18	0	5120	0	10240	0	20480	0	160	0	640	0	1920	0	1920	0	640
		59-17	0	480	0	1920	0	2560	0	640	0	480	0	480	0	320	0	160
COBRA 2 SVP	Preimmune	107-18	640	10240	1280	40960	1280	30720	30	2560	120	2560	320	10240	40	1280	40	1280
		108-18	2560	40960	10240	2E+05	10240	2E+05	1280	10240	2560	20480	7680	30720	640	10240	640	10240
		109-18	10240	40960	40960	2E+05	30720	1E+05	2560	10240	2560	10240	10240	40960	1280	5120	640	5120
		111-18	2560	30720	2560	30720	1920	71680	120	2560	160	5120	320	10240	320	5120	80	2560
		112-18	13	640	40	1280	320	5120	1920	10240	640	7680	1280	20480	40	640	240	640
		121-18	0	160	40	640	120	2560	1920	10240	160	5120	640	20480	0	480	40	640
	Naïve	62-17	0	40	0	480	0	1920	0	2560	0	5120	0	7680	0	480	0	160
		65-17	0	13	0	160	0	1280	0	640	0	1920	0	1280	0	40	0	40
		66-17	0	13	0	320	0	480	0	10240	0	2560	0	5120	0	40	0	40
COBRA tet SVP	Preimmune	102-18	2560	10241	7680	40960	2560	30720	160	2560	320	2560	1280	10240	160	2560	80	640
		104-18	13	480	40	1280	240	2560	640	5120	1280	5120	1920	10240	40	640	40	640
		105-18	7680	10240	10240	30720	7680	20480	160	4480	640	5120	2560	7680	480	2560	160	640
		106-18	7680	10240	10240	40960	7680	30720	160	1920	640	2560	640	10240	640	2560	160	1280
		120-18	80	1120	160	10240	160	1920	2560	7680	120	2560	2560	10240	40	640	640	7680
	Naïve	60-17	0	1920	0	2560	0	5120	0	2560	0	2560	0	7680	0	1920	0	640
		61-17	0	480	0	2560	0	2560	0	640	0	2560	0	2560	0	640	0	480
		110-18	0	1760	0	640	0	1280	0	2560	0	2560	0	1280	0	640	0	480

			DV1				DV2				DV3				DV4			
			prototype		American		prototype		American		prototype		American		prototype		American	
			d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70
COBRA 1 SVP	Preimmune	97-18	2560	10240	7680	7E+05	7680	2E+05	2560	2560	1280	7680	2560	10240	640	2560	320	2560
		98-18	0	480	40	1920	120	1280	640	640	1280	3840	1920	10240	13	640	40	160
		99-18	2560	10240	2560	2E+05	1920	20480	120	1280	120	1280	160	7680	120	2560	40	1280
		100-18	1920	10240	5120	2E+05	2560	30720	640	640	320	5120	640	10240	480	2560	120	1920
		101-18	1280	10240	5120	2E+05	7680	2E+05	640	5120	1280	7680	1280	20480	640	10240	640	2560
		103-18	120	640	320	2560	160	5120	5120	10240	1280	2560	640	7680	160	1920	160	2560
	Naïve	117-18	0	10240	0	20480	0	7680	0	1280	0	1280	0	2560	0	640	0	320
		118-18	0	5120	0	10240	0	20480	0	160	0	640	0	1920	0	1920	0	640
		59-17	0	480	0	1920	0	2560	0	640	0	480	0	480	0	320	0	160
COBRA 2 SVP	Preimmune	107-18	640	10240	1280	40960	1280	30720	30	2560	120	2560	320	10240	40	1280	40	1280
		108-18	2560	40960	10240	2E+05	10240	2E+05	1280	10240	2560	20480	7680	30720	640	10240	640	10240
		109-18	10240	40960	40960	2E+05	30720	1E+05	2560	10240	2560	10240	10240	40960	1280	5120	640	5120
		111-18	2560	30720	2560	30720	1920	71680	120	2560	160	5120	320	10240	320	5120	80	2560
		112-18	13	640	40	1280	320	5120	1920	10240	640	7680	1280	20480	40	640	240	640
		121-18	0	160	40	640	120	2560	1920	10240	160	5120	640	20480	0	480	40	640
	Naïve	62-17	0	40	0	480	0	1920	0	2560	0	5120	0	7680	0	480	0	160
		65-17	0	13	0	160	0	1280	0	640	0	1920	0	1280	0	40	0	40
		66-17	0	13	0	320	0	480	0	10240	0	2560	0	5120	0	40	0	40
COBRA tet SVP	Preimmune	102-18	2560	10241	7680	40960	2560	30720	160	2560	320	2560	1280	10240	160	2560	80	640
		104-18	13	480	40	1280	240	2560	640	5120	1280	5120	1920	10240	40	640	40	640
		105-18	7680	10240	10240	30720	7680	20480	160	4480	640	5120	2560	7680	480	2560	160	640
		106-18	7680	10240	10240	40960	7680	30720	160	1920	640	2560	640	10240	640	2560	160	1280
		120-18	80	1120	160	10240	160	1920	2560	7680	120	2560	2560	10240	40	640	640	7680
	Naïve	60-17	0	1920	0	2560	0	5120	0	2560	0	2560	0	7680	0	1920	0	640
		61-17	0	480	0	2560	0	2560	0	640	0	2560	0	2560	0	640	0	480
		110-18	0	1760	0	640	0	1280	0	2560	0	2560	0	1280	0	640	0	480

Figure 5.6 Heatmap of $FRNT_{50}$ data for individual NHPs. COBRA vaccine groups are indicated on the left, with individual monkey ID #s and serostatus. Panel of DENV strains are indicated at the top, grouped by serotype. Gradient color bar indicates minimum (<1:40, white) and maximum (>1:5000, red) titer, with 1:40 titer (limit of detection) being yellow. Increase in red intensity indicates increase in neutralization titer. Each individual monkey's $FRNT_{50}$ titer is specified for day 0 (d0, pre-vaccination) and day 70 (d70, four weeks post-boost).

			DV1						DV2						DV3						DV4					
			prototype		American		Asian		prototype		American		Asian		prototype		American		Asian		prototype		American		Asian	
			d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70	d0	d70
COBRA 1 SVP	Preimmune	97-18	1280	10240	2560	20480	1920	10240	640	1280	640	2560	640	2560	160	640	160	640	160	640	160	640	40	640	120	1760
		98-18	0	240	0	160	0	160	640	640	320	640	640	1920	0	160	0	40	0	320	13.3	160	0	120	80	160
		99-18	640	2560	1280	7680	640	7680	40	160	40	640	80	640	40	640	13	160	80	640	80	640	13.3	160	87	640
		100-18	640	5120	1920	10240	1280	10240	160	640	160	640	480	2560	80	640	40	640	120	640	40	640	40	320	120	480
		101-18	640	10240	1920	20480	1920	20480	160	2560	320	2560	640	5120	160	2560	80	640	160	2560	160	2560	40	640	160	640
		103-18	40	320	80	640	40	1280	640	1280	480	2560	480	2560	40	640	40	160	40	640	40	640	40	320	120	1120
	Naïve	117-18	0	640	0	7680	0	2560	0	40	0	160	0	160	0	160	0	40	0	80	0	160	0	40	0	160
		118-18	0	640	0	2560	0	2560	0	160	0	160	0	640	0	160	0	160	0	320	0	160	0	120	0	320
		59-17	0	160	0	640	0	640	0	160	0	160	0	40	0	40	0	40	0	40	0	40	0	27	0	120
COBRA 2 SVP	Preimmune	107-18	160	2560	640	10240	640	7240	40	640	40	640	40	2560	40	640	13.3	320	40	640	13.3	480	0	160	320	1280
		108-18	1280	10240	5120	40960	2560	40960	640	2560	640	10240	2560	10240	160	2560	320	1920	640	2560	640	2560	160	1280	480	1280
		109-18	2560	10240	10240	10240	5120	20480	640	10240	640	2560	2560	10240	640	2560	320	640	160	2560	160	640	160	640	320	1920
		111-18	640	2560	640	7680	640	10240	40	1280	80	2560	160	2560	40	1920	40	640	40	1920	40	640	13.3	640	320	1120
		112-18	0	80	40	160	80	1280	640	2560	640	2560	640	5120	40	160	0	320	40	320	40	480	40	160	160	480
		121-18	0	80	0	160	40	640	640	10240	160	2560	320	17920	0	160	0	160	0	160	13.3	640	13.3	480	227	960
	Naïve	62-17	0	0	0	40	0	480	0	640	0	1280	0	2560	0	13.3	0	40	0	27	0	40	0	40	0	120
		65-17	0	0	0	13.3	0	80	0	320	0	480	0	480	0	0	0	0	0	27	0	40	0	40	0	67
		66-17	0	0	0	40	0	80	0	2560	0	640	0	2560	0	0	0	0	0	27	0	40	0	40	0	227
COBRA tet SVP	Preimmune	102-18	640	2560	2560	10240	1280	10240	160	640	106.7	1280	160	2560	160	640	40	640	80	640	40	640	40	640	40	1920
		104-18	0	80	13.3	320	27	640	320	2560	320	2560	640	2560	0	160	0	160	40	320	40	640	40	320	80	640
		105-18	2560	2560	2560	10240	2560	2560	160	640	160	640	320	2560	160	640	40	480	120	640	40	320	40	640	120	480
		106-18	2560	7680	2560	10240	2560	10240	160	640	160	640	160	2560	160	1120	40	640	160	640	40	640	40	640	120	320
		120-18	0	120	40	160	40	640	640	2560	13.3	1280	1280	2560	0	80	0	80	13	160	40	640	13	160	40	120
	Naïve	60-17	0	960	0	640	0	1120	0	640	0	1280	0	1280	0	320	0	160	0	320	0	2560	0	640	0	1120
		61-17	0	440	0	320	0	1280	0	160	0	640	0	1280	0	160	0	160	0	160	0	1280	0	320	0	1120
		110-18	0	80	0	640	0	640	0	640	0	480	0	1280	0	320	0	160	0	160	0	320	0	480	0	320

Figure 5.7 Heatmap of $FRNT_{80}$ data for individual NHPs. COBRA vaccine groups are indicated on the left, with individual monkey ID #s and serostatus. Panel of DENV strains are indicated at the top, grouped by serotype. Gradient color bar indicates minimum (<1:40, white) and maximum (>1:5000, red) titer, with 1:40 titer (limit of detection) being yellow. Increase in red intensity indicates increase in neutralization titer. Each individual monkey's $FRNT_{50}$ titer is specified for day 0 (d0, pre-vaccination) and day 70 (d70, four weeks post-boost).

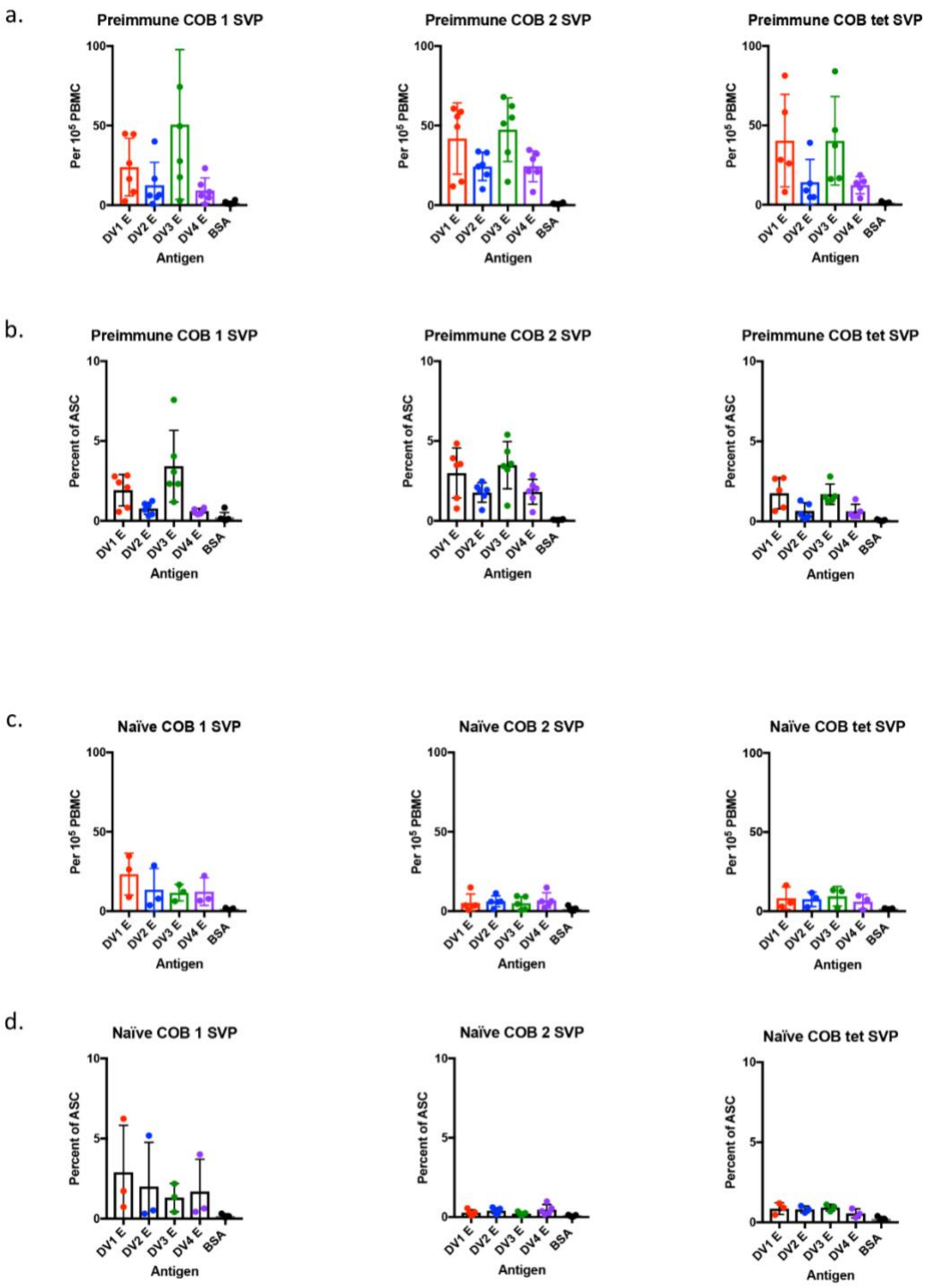


Figure 5.8 Memory B cell response of naïve and pre-immune non-human primates vaccinated with COBRA DENV SVPs. NHP PBMCs were stimulated *ex-vivo* with IL-2 and R848 for 5 days then incubated with 5µg/mL of DENV1-4 E protein, anti-monkey

IgG, or BSA. The total number of memory B cells (measured by their conversion to IgG producing cells) were counted for each DENV E or BSA (x-axis) in a) pre-immune and b) naïve NHP vaccination groups. The percentage of DENV E-specific cells of total IgG secreting cells were counted for each serotype or BSA (x-axis) in c) pre-immune and d) naïve NHP vaccination groups. Each graph represents a vaccination group.

CHAPTER 6

IMMUNOGENECITY AND BREADTH OF RESPONSE OF MICE VACCINATED WITH DENV COBRA MESSENGER RNA (MRNA)

Introduction

With half of the global population at risk for dengue virus (DENV) infection, attaining an effective, universal vaccine is imperative, yet enigmatic. An ideal candidate needs to protect against all four serotypes that are currently circulating in different geographic locations, and be safe for all age groups. Current top candidates are based on live viral vector platform consisting of a tetravalent formulation using prM-E sequences from past strains that are decades old.

To address the effects of genotype mismatch, novel DENV vaccines can be designed using multiple rounds of consensus building known as computationally-optimized broadly reactive antigens (COBRA). This results in DENV E immunogens that represent each DENV serotype spanning many genotypes. Since these antigens are designed as nucleotide gene inserts, these vaccines can be delivered on several types of vaccine platforms.

A safer alternative vaccine platform to live viral vectors are messenger RNA (mRNA). The only licensed DENV vaccine, a live viral vector, has caused controversy because vaccination in naïve individuals may mimic a primary infection, exacerbating disease after a natural infection due to antibody dependent enhancement [274]. This would

not happen with an mRNA vaccine, since it will be unable to replicate inside the host and only create the SVP *in vivo.* mRNA is an integral part of human biology and recently have been investigated for development of cancer therapeutics and infectious disease vaccines [219]. The genetic message of the recombinant mRNA encoding the antigen of interest is translated *in vivo* and the synthesized proteins can undergo secretion by the cell or surface presentation on MHC molecules to effector cells [220]. mRNA vaccinations require fewer doses than recombinant protein and do not require adjuvant.

Wild type mouse models are not ideal for vaccine studies since they are not susceptible to DENV infection. DENV NS proteins are unable to subvert antiviral responses and replicate in the murine system [258, 275]. Since immunocompetent mice cannot be infected by clinical human isolates, mouse-adapted viral strains [276] or immunodeficient mice (especially those deficient in IFNα/β signaling [277]) are used to study viral pathogenesis and ADE (reviewed in [259]). However, their lack of type I and II IFN response is not ideal for a vaccine study, since the effects on B and T cell priming are not evident [278]. Wild type mice can be made susceptible to other flaviviruses such as West Nile Virus and Zika Virus by treatment with IFNα/β receptor blocking monoclonal antibody (mAb) MAR1-5A3 [278, 279]. To establish a more relevant mouse model of DENV challenge after vaccination, immunocompetent C57BL/6 mice were treated with MAR1-5A3 to block early type I IFN response.

This study will assess the immunogenicity and breadth of COBRA mRNA vaccines in wild type mice and to test the efficacy of the vaccines by developing an immunologically relevant infection model in wild type mice. C57BL/6 were given 2 doses of monovalent or tetravalent DENV COBRA, tetravalent wildtype, or irrelevant mRNA vaccines. COBRA

mRNA vaccinations elicited robust neutralizing antibodies against DENV serotypes. COBRA 1 mRNA elicited robust neutralizing antibodies against all 4 serotypes when tested against the 12-strain panel *in vitro*, comparable to tetravalent formulations. Vaccine efficacy could not be tested *in vivo* due to insusceptibility of C57BL/6 mice despite treatment with MAR1-5A3.

Methods

Vaccines

COBRA mRNA vaccines were manufactured by Moderna Inc (Cambridge, MA, USA). COBRA 1-4 sequences from chapter 3 were used for monovalent and tetravalent mRNA formulations. Briefly, the mRNA was synthesized *in vitro* using T7 polymerase-mediated DNA-dependent RNA transcription where the UTP was substituted with 1-methylpseudoUTP, using a linearized DNA template, which incorporates 5' and 3' untranslated regions (UTRs) and includes a poly-A tail. A donor methyl group S-adenosylmethionine (SAM) was added to the methylated capped RNA (cap 0), resulting in a cap 1 structure to increase mRNA translation efficiency. The modified mRNAs encoded the signal sequences from JEV prM (MWLVSLAIVTACAGA) and the prM and E genes from the COBRA sequences.

Lipid nanoparticle (LNP) formulations were prepared by dissolving lipids in ethanol at molar ratios of 50:10:38.5:1.5 (ionizable lipid: DSPC: cholesterol: PEG-lipid). The lipid mixture was combined with a 50 mm citrate buffer (pH 4.0) containing mRNA at a ratio of 3:1 (aqueous:ethanol) using a microfluidic mixer (Precision Nanosystems, Vancouver, BC). Formulations were dialyzed against PBS (pH 7.4) in dialysis cassettes

for at least 18 hr. Formulations were concentrated using Amicon Ultra Centrifugal Filters (EMD Millipore, Billerica, MA), passed through a 0.22-mmfilter and stored at 4°C until use. All formulations were tested for particle size, RNA encapsulation, and endotoxin and were found to be between 80 to 100 nm in size, with greater than 90% encapsulation and < 1 EU/ml of endotoxin.

Mouse vaccinations

Female C57BL/6 mice, age 6-8 weeks (n=10) were given i.m. vaccinations with monovalent or tetravalent COBRA mRNA, tetravalent wild type mRNA, irrelevant mRNA, or PBS control twice at 4-week intervals. 2μg per vaccination were given for monovalent formulations, and 8μg per vaccination for tetravalent formulations. Serum samples were collected at each vaccination and at 4 and 20 weeks after boost. 22 weeks after boost, mice were given another vaccination in order to stimulate memory cells *in vivo*.

Detection of anti-DENV E antibodies

Quantitative ELISA was performed to detect anti-SVP or anti-E antibodies elicited by vaccination. Nunc Maxisorp plates were coated (2ug/mL) overnight at 4°C with either subviral particles expressing one of the wild-type DENV E proteins or soluble DENV E (MyBiosource, Inc. San Diego, CA, USA). Plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with 1% BSA in PBS for 1 hr at RT. Subsequently, 1:100 dilution of sera samples were added to the plates for 2 h. Following three washes with PBS-T, peroxidase-conjugated anti-mouse IgG-Fc, IgG1, IgG2a, IgG2b, or IgG3

antibodies (Bethyl Laboratories, Montgomery, TX, USA) were added for 1 h before developing the plates using pNpp substrate. OD values were read at 405 nm.

Flavivirus strains

Viruses used in this study were for DENV 1: DV1/US/Hawaii/1944, DV1/Vietnam/BID-V1792/2007, DV1/Costa Rica/BC89/1994; DENV 2: DV2/New Guinea/NGC/1944, DV2/Vietnam/BID-V1007/2006, DV2/US/BID-V594/2006; DENV 3: DV3/Philippines/H-87/1956, DV3/Sri Lanka/271242/1991, DV3/US/BID-V1619/2005; DENV 4: DV4/Philippines/H-241/1956, DV4/Malaysia/BC13/1997, DV4/US/BC258/1994; ZIKV: DAK-AR/44671.

Neutralization assay

Neutralization activity in collected antisera were determined using a focus reduction neutralization test (FRNT). Sera was heat inactivated at 56°C for 30 min and the serially diluted four-fold. Sera was incubated with dengue viruses (100 FFU) representing each of the 4 serotypes for 60 min at 37°C. Three dengue virus were used per each serotype that represented the prototype, modern Asian, and modern American strains. Each dengue virus was mixed with the serially diluted antisera and used to infect a single cell monolayer of Vero cell in 96-well plates for 90 min at 37°C that were plated. Cells were overlaid methylcellulose (1% [wt/vol]). Cells were incubated for 3 days at 37°C (2 days for DENV 4 or ZIKV viruses). Cells were washed with PBS and fixed with a mixture of acetone and methanol (Foci were stained using Vectastain ABC kit, following the manufacturer's protocols (Vector Laboratories, Burlingame, CA, USA). Anti-DENV E monoclonal

antibodies were used to detect virally infected cells. For DENV 1 and 3 viruses, the antibody AM01108PU-N (OriGene Technologies, Rockville, MD, USA) was used and for DENV 2 the monoclonal antibody 9.F.10 (Santa Cruz Biotechnology, Dallas, TX, USA) was used and for DENV 4, the monoclonal antibody 4G2 (Millipore Sigma, Billerica, MA, USA) was used. Foci were imaged using CTL Immunospot (Cellular Technology Limited, Cleveland, OH, USA). counted using ImmunoSpot® software version 5.1.36 Professional SC. $FRNT_{50}$ determined by calculating the point at which sera reduced dengue foci by 50% compared to serum-free, dengue virus control wells.

B cell ELISPOT

Mice were stimulated *in vivo* by intramuscular injection of homologous mRNA (2µg) and spleens were harvested after 6 days. Multiscreen HTS (Millipore Sigma) plate was pre-wetted with 35% ethanol and coated with 5mg/mL of DENV1-4 peptides (MyBiosource), anti-mouse IgG (Mabtech), or BSA. Splenocytes were added in serial dilutions starting at 300,000 cells/well in RPMI media supplemented with 55 µM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin,100 µg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and 1% MEM nonessential amino acids and incubated at 37° for 24hr. The cells were washed five times with PBS and anti-mouse IgG-biotin (Mabtech) added overnight at 4°C. The following day, plates were washed five times with PBS. Streptavidin-HRP (Mabtech) was added and incubated at room temperature for 2 hours. After washing five times with PBS, spots were developed with TMB solution (Vector Laboratories). Spots were imaged using CTL ImmunoSpot® Analyzer (Cellular

Technology Limited, Cleveland, OH, USA) and counted using ImmunoSpot® software version 5.1.36 Professional SC.

Mouse infection model

Female C57BL/6 mice (n=3 mice per group; aged 6–8 weeks) were given type I IFN blocking regimen similar to other studies for flavivirus challenge [280, 281]: 2 mg of MAR1-5A3 (BioXcell, New Lebanon, NH, USA) or MOPC-21 isotype control (BioXcell) was administered i.p at day -1, and .5 mg of the mAb on days +1 and +3. On day 0, the mice were challenged with 10_6, 10_7, or 10_8 FFU of DENV or 10_7 FFU ZIKV (Table 6.1) Blood was collected at day 2 and 4 to assess viral titer and day 28 to assess seroconversion. The blood samples were centrifuged at 6000 rpm for 10 min to separate the serum. Sera were analyzed fresh or frozen at -80°C.

Quantifying recovered viral titers

Viral titers were determined by serially diluting sera and incubating with Vero cell monolayers in 96-well plates for 90 min at 37 °C. An overlay containing 1% (wt/vol) methylcellulose was added to the cells. After a 3-day incubation at 37 °C (2 days for DENV4 and ZIKV), the cells were washed and fixed with a 1:1 mixture of acetone and methanol. Foci were stained using Vectastain ABC kit, following the manufacturer's protocols. Detection antibodies were Acris antibody AM01108PU-N (OriGene Technologies) for DENV1 and 3, 9.F.10 Dengue virus mAb (Santa Cruz Biotechnology, Dallas, TX, USA) for DENV2, and 4G2 mAb (Millipore Sigma, Billerica, MA, USA) for

DENV4 and ZIKV. Foci were imaged using CTL Immunospot and counted using ImmunoSpot® software version 5.1.36 Professional SC.

T-cell ELISPOT

Mouse IFN-γ/IL-4 Double-Color ELISPOT plates (Cellular Technology Limited, Cleveland, OH, USA) were pre-wetted with ethanol and IFN-γ/IL-4 capture media added to plates and incubated overnight at 4°C. Plates were washed with sterile PBS. Spleens were harvested from MAR1-5A3 treated mice (using the same regimen as above) 6 days after infection with DV1/VN/BID-V1792/2007 (10_8 FFU). Freshly isolated mouse splenocytes (300,000 cells/well) were incubated in complete media (RPMI supplemented with 10% FBS, pen/strep, and L-glutamine). Peptide pools (1μg/mL) representing DENV 1 E proteins were added to each plate and incubated at RT at 37°C for 24 h. Control stimulants were irrelevant peptide (Influenza A hemagglutinin), no stimulation, and positive stimulation (PMA and ionomycin). Plates were washed with PBS (2X) and then with 0.05% Tween-PBS (2X). IFN-γ/IL-4 detection solution was added and incubated overnight at 4°C. Plates were washed (3X) with 0.05% Tween-PBS. Tertiary solution was added and incubated at RT for 2 h. After washing with 0.05% Tween-PBS (2X), then with deionized (DI) water (2X), blue developer was added to each plate to detect IL-4 and red developer was added to detect IFN-γ. Spots were imaged using CTL ImmunoSpot® Analyzer (Cellular Technology Limited) and counted using ImmunoSpot® software version 5.1.36 Professional DC.

Statistical analysis

Statistically significant differences between seroconversion of vaccine groups to control group were determined by one-tailed unpaired t-test. Individual mRNA vaccine groups were compared to irrelevant mRNA control group, with confidence level at 95%. Definition of statistical significance was $p < 0.05$.

Differences in IgG subclass response were analyzed by unpaired t-test with Welch's correction. Differences in neutralization titer were analyzed by paired student's t-test. Statistical analyses were performed using Prism (GraphPad Software, La Jolla, CA, USA). P-values less than .05 were considered statistically significant. *ns* for $p > 0.05$, * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, **** for $p \leq 0.0001$.

Results

Six lipid nanoparticle (LNP) encapsulated mRNA vaccines encoding for DENV prM-E were produced. These included COBRA E sequence designs from the previous chapters. C57BL/6 mice were administered i.m. vaccinations of monovalent or tetravalent COBRA, or wild type tetravalent mRNA two times at 4-week intervals. Irrelevant mRNA vaccine was also administered as a negative control using the same regimen.

Four weeks after final vaccination, DENV mRNA vaccinations elicited sera IgG that bound to wild type SVPs and monomer E (Appendix Table C.1). All groups had sera IgG that bound to wild type SVPs representing all four serotypes (Fig 6.1a). IgG binding to soluble E, a monomer, was more serotype specific (Fig 6.1b). mRNA vaccinations elicited more IgG2 than IgG1 antibodies, indicating a Th1-biased response (Table 6.1).

Sera were tested for the ability to neutralize panel of 12 dengue viruses representing all 4 dengue serotypes. Three strains from each serotype were prototype, modern American and modern Asian, since genotypes tend to separate by geographic location [253, 254].

Mice vaccinated with monovalent COBRA mRNA vaccines elicited neutralizing antibodies against at least 11 of the 12 strains (Fig 6.2a-d). COBRA 1 mRNA vaccine groups had the most robust response within the monovalent groups, eliciting neutralizing antibodies against all 12 strains with at titers of at least 1:160 (Fig 6.2a). COBRA and WT tetravalent mRNA vaccinations elicited neutralizing antibodies against all 12 strains at titers of at least 1:640 (Fig 6.2e-f).

To evaluate vaccine efficacy *in vivo*, an infection model in immunocompetent mice first needed to be established. C57BL/c mice were treated with MAR1-5A3 mAb and infected with different strains representing all four serotypes (Table 6.2). Virus strains from all four serotypes were able to be grown to at least 10_6 FFU/mL. No significant weight loss was observed for the 14 days of the study (Fig 6.3), even in mice given high titers of DENV (Fig 6.3b). In contrast, MAR1-5A3 treated C57BL/6 mice infected with ZIKV all reached endpoint weight loss by day 7 (Fig 6.4).

At 2 and 4 post-infection, blood was collected and tested for virus recovery. Virus could not be recovered from sera of most DENV infected mice. Low viral titers were recovered in sera of two of the mice given the high doses of DENV infection dose DV1/Vietnam/BID-V1792, and high viral titers were recovered from ZIKV infected mice. (Table 6.3). 28 days after infection, all mice had antibodies that recognized the representative E from that serotype (Fig 6.5).

Subsequently, longevity of breadth of response was examined. COBRA tetravalent mRNA maintained broadly neutralizing titers in mice 20 weeks after final vaccination. WT tetravalent mRNA maintained broadly neutralizing titers as well, though there were significant decreases in neutralizing antibody titers to DV4 prototype and modern Asian strains. COBRA 1 mRNA maintained broadly neutralizing antibodies against all 12 strains, though titers to 2 strains decreased- DV1 prototype and DV4 modern Asian (Fig 6.6). Memory B cell response of mice after *in vivo* stimulation showed that COBRA 1 mRNA vaccination elicited memory response against all four serotypes, similar to tetravalent formulations (Fig 6.7, Appendix Table C.4).

Discussion

In this study, COBRA E immunogens were expressed on the surface of SVPs that were expressed *in vivo* via mRNA delivery. mRNA vaccine is more advantageous than SVP vaccination, because it requires fewer vaccinations and no adjuvant. COBRA mRNA vaccinations elicit antibodies that recognize homologous soluble E and heterologous virus like particles (Fig 6.1). Broadly neutralizing antibodies have been shown to bind to quaternary structures and not soluble E monomers [282].

The COBRA mRNA vaccination elicits robust neutralizing antibody titer, which allays concerns for ADE. At suboptimal levels even neutralizing antibodies can cause ADE [157]. Conversely, antibodies with weak avidity may be neutralizing at high enough levels [165]. COBRA 1 mRNA elicited robust response, comparable to tetravalent formulations. This is notable since the amount of monovalent vaccines given were a fourth of the tetravalent. The efficacy of the vaccines could not be examined in wild type mice. After

transient blockade of type I IFN signaling, DENV infection did not result in weight loss or viral recovery. A very low number of viral FFU were seen in sera from mice given the highest dose of DENV, but this amount would not be high enough for adequate statistical power in a vaccine study.

For other flaviviruses such as WNV and ZIKV, blocking the IFNα/β receptor is enough to render C57BL/6 mice susceptible to infection [280, 283]. In a recent study, HLA-I transgenic mice were able to be made susceptible to DENV [206], suggesting that in the absence of type I IFN, murine $CD8^+$ T cell response is sufficient to protect against DENV infection while human $CD8^+$ T cell response is not. Type I IFNs play a major role in controlling the $CD8^+$ T cell response to viral infection, notably by blocking the expansion of pre-existing non-specific memory T cells [284]. Type I IFNs also suppress NK cell cytotoxicity and protect antiviral T cells from elimination by NK cells [285, 286]. DENV-specific T cells and regulation of NK cells may not be as important in murine response to DENV infection compared to humans.

Accordingly, COBRA or WT mRNA vaccinated mice did not have significantly different T cell response after DV1 infection compared to irrelevant mRNA control group, despite being treated with MAR1-5A3 (supplemental Fig 6.8); all groups elicited IFNγ response to hinge region of E. No viral titers were recovered from any of the mice. In order to determine mRNA vaccine efficacy, human clinical trials will need to be done.

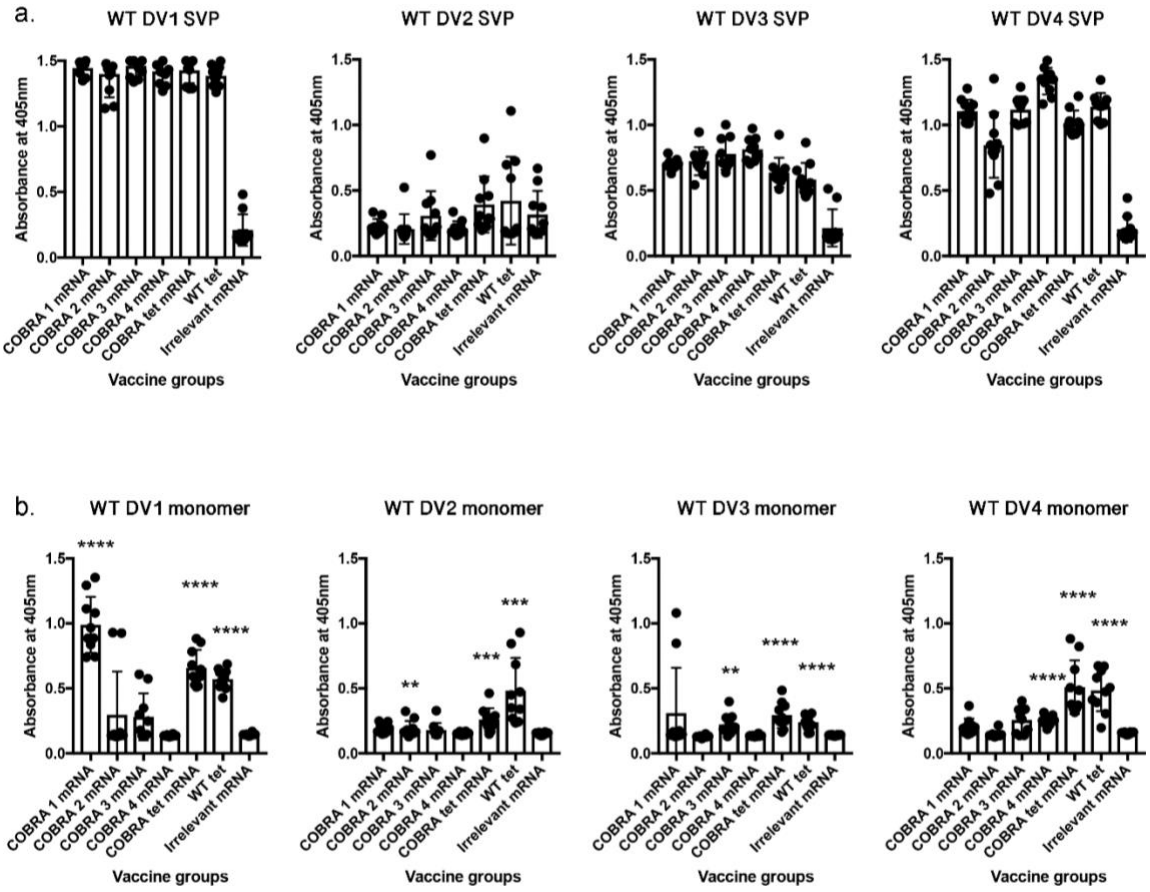


Figure 6.1 Seroconversion of mice vaccinated with COBRA or wild type DENV mRNA. Each graph represents antibody binding to a) SVP or b) soluble E representing one of the four dengue serotypes. Vaccine groups are indicated on x-axis. Total IgG binding of sera (1:100 dilution) from individual mice were measured by their optical density (OD) values. Absorbance was measured at 405 nm. Statistical significance was determined by one-tailed unpaired t-test. Individual mRNA vaccine groups were compared to irrelevant mRNA control group, with confidence level at 95%. Definition of statistical significance was $p < 0.05$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, **** for $p \leq 0.0001$.

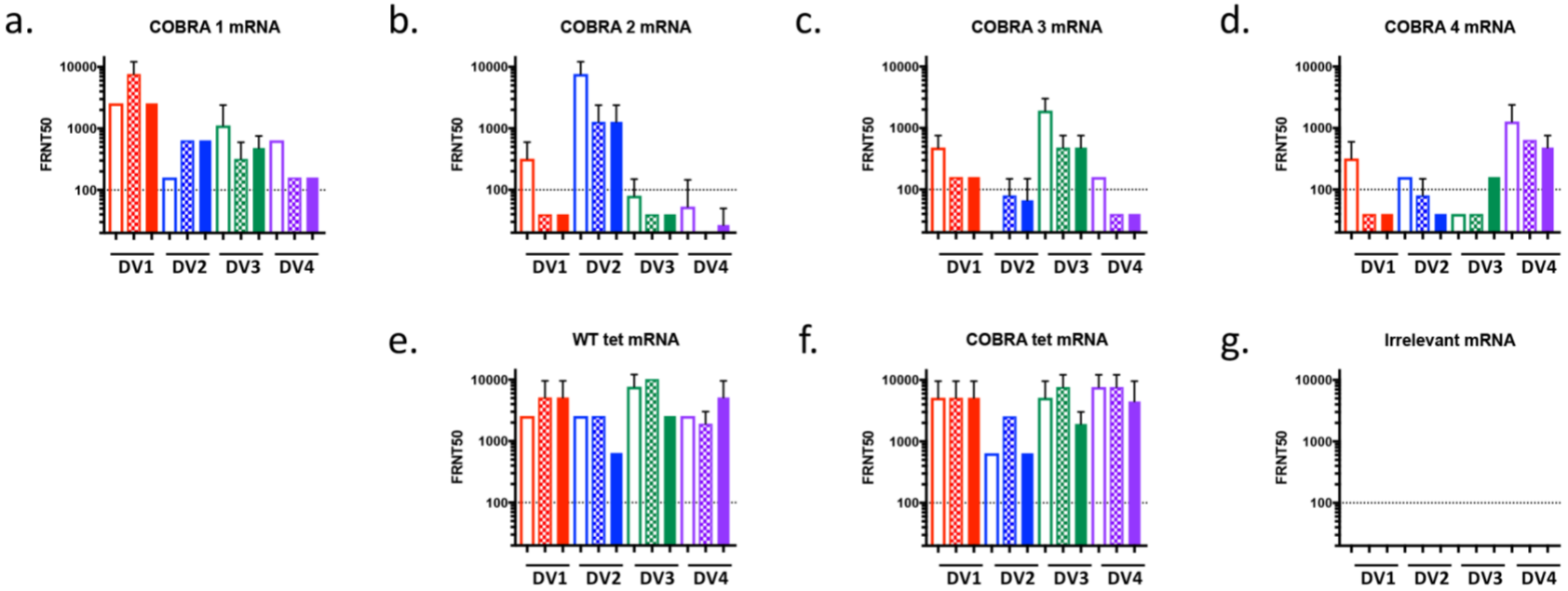


Figure 6.2 Neutralization ability of sera from mice vaccinated with COBRA or wild type DENV mRNA. Focus reduction neutralization test $(FRNT)_{50}$ of pooled sera from C57BL/6 mice vaccination with a) – d) monovalent wild type SVP, e) – h) monovalent COBRA SVP, i) – j) tetravalent SVP, or k) PBS control against a panel of prototype (unfilled), modern American (checkered), and modern Asian (filled) strains from each serotype. Dashed line indicates 1:100 titer.

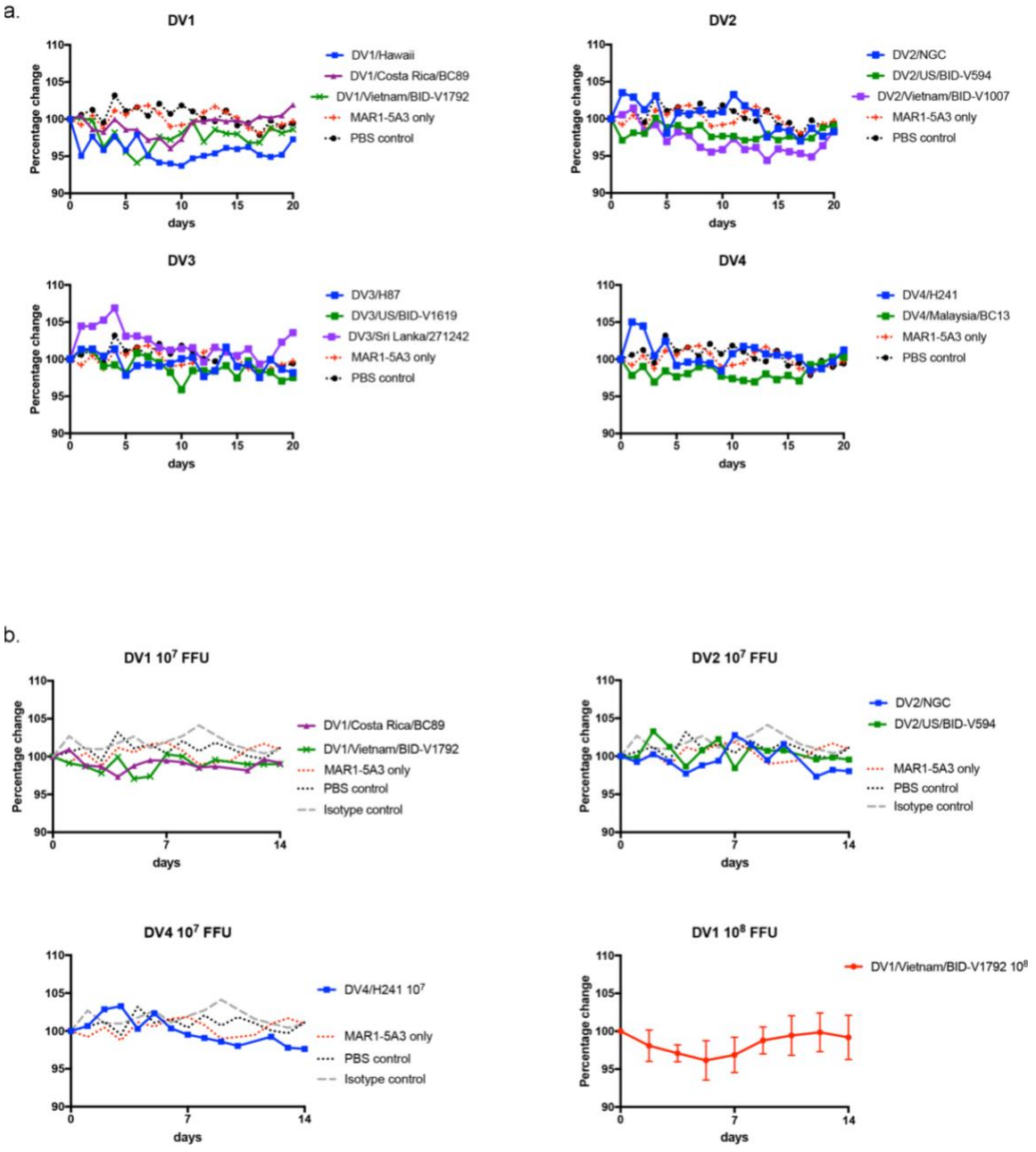


Figure 6.3 Weight loss of wild type mice infected with dengue virus strains. Female C57BL/6 mice (n=3 per group) were given MAR1-5A3 or isotype control i.p at day -1 (2mg), and days +1 and +3 (.5mg each day). On day 0, the mice were infected i.p. with DENV or mock control. a) Weight loss of mice infected i.p. with 10_6 FFU of DENV strains,

with each graph representing one of the four serotypes. b) Weight loss of mice infected i.p. with higher FFU of strains could be grown to higher viral titers.

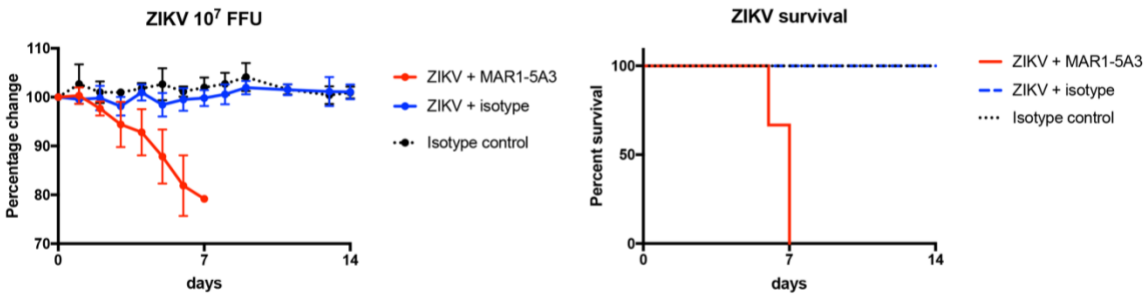


Figure 6.4 Weight loss and survival of wild type mice infected with Zika virus. Female C57BL/6 mice (n=3) were given MAR1-5A3 or isotype control i.p at day -1 (2mg), and days +1 and +3 (.5mg each day). On day 0, the mice were infected i.p. with 10_7 FFU of ZIKV DAK-AR 44671. All mice reached 80% weight cut off and had to be euthanized.

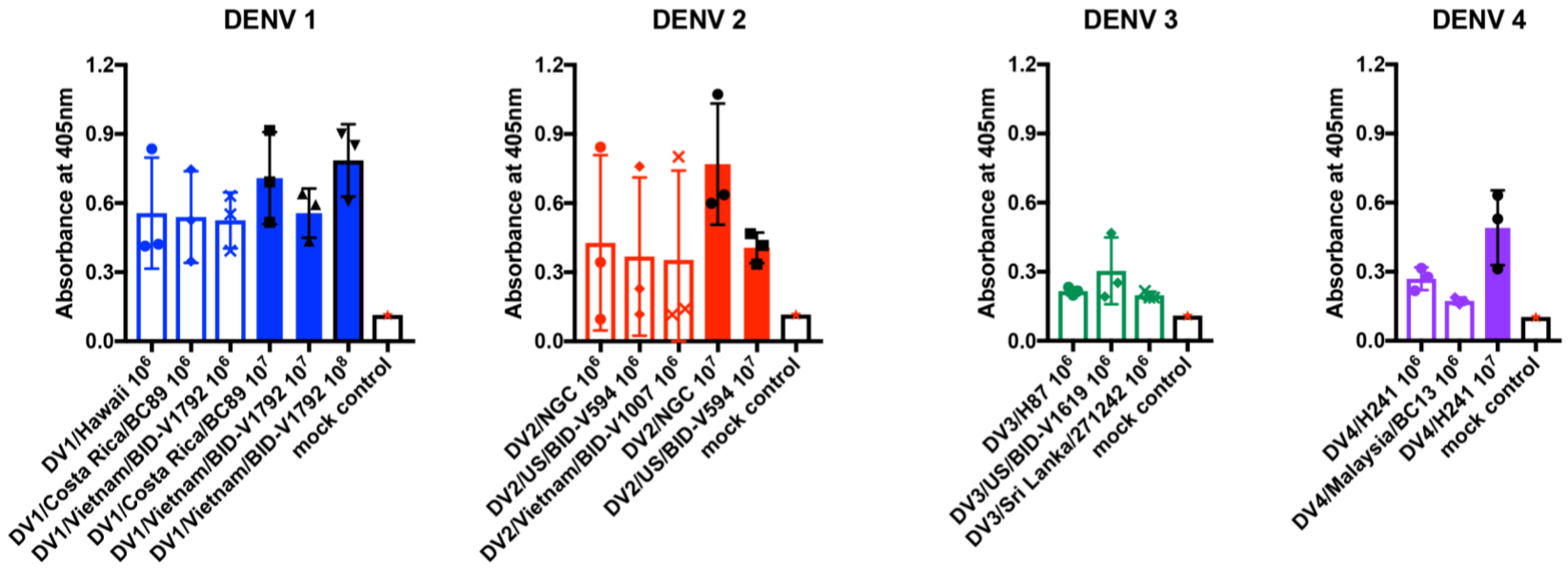


Figure 6.5 Seroconversion of wild type mice infected with dengue virus strains. Each graph represents ELISA plates were coated with commercial E antigen and incubated with sera from MAR1-5A3 treated mice after infection.

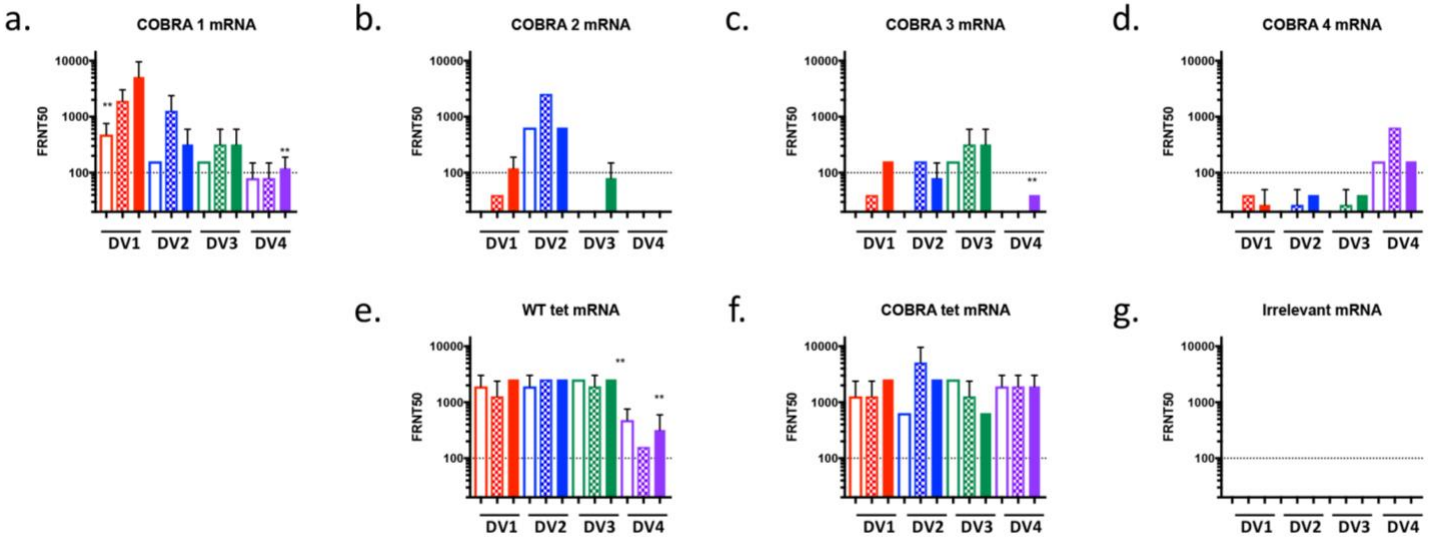


Figure 6.6 Longevity of neutralization ability of COBRA or wild type DEV mRNA vaccination in mice. $FRNT_{50}$ of pooled sera from C57BL/6 mice 20 weeks after vaccination with a) COBRA 1, b) COBRA 2, or c) COBRA tetravalent SVPs against a panel of prototype (unfilled), modern American (checkered), and modern Asian (filled) strains from each serotype. Dashed line indicates 1:100 titer. Statistical differences between 4 weeks and 20 weeks post vaccination calculated by two-tailed paired t-test. ** for $p \leq 0.01$, **** for $p \leq 0.0001$.

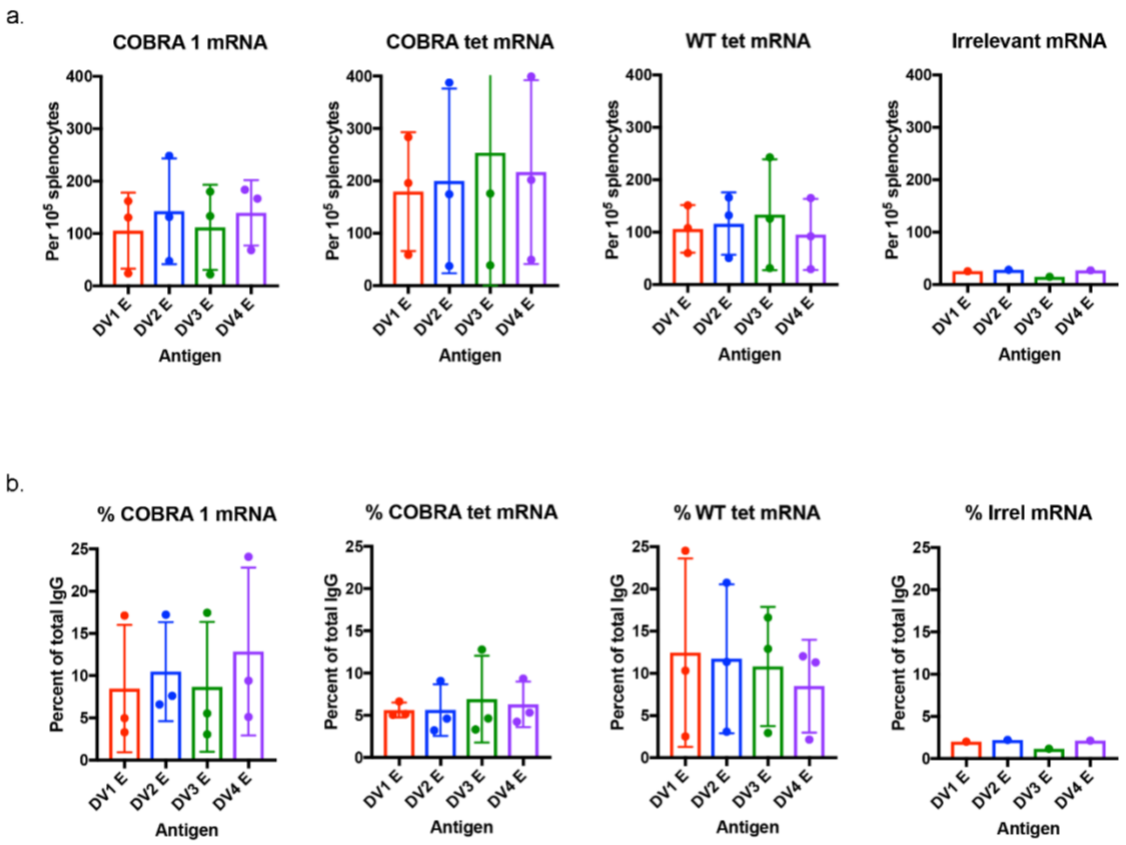
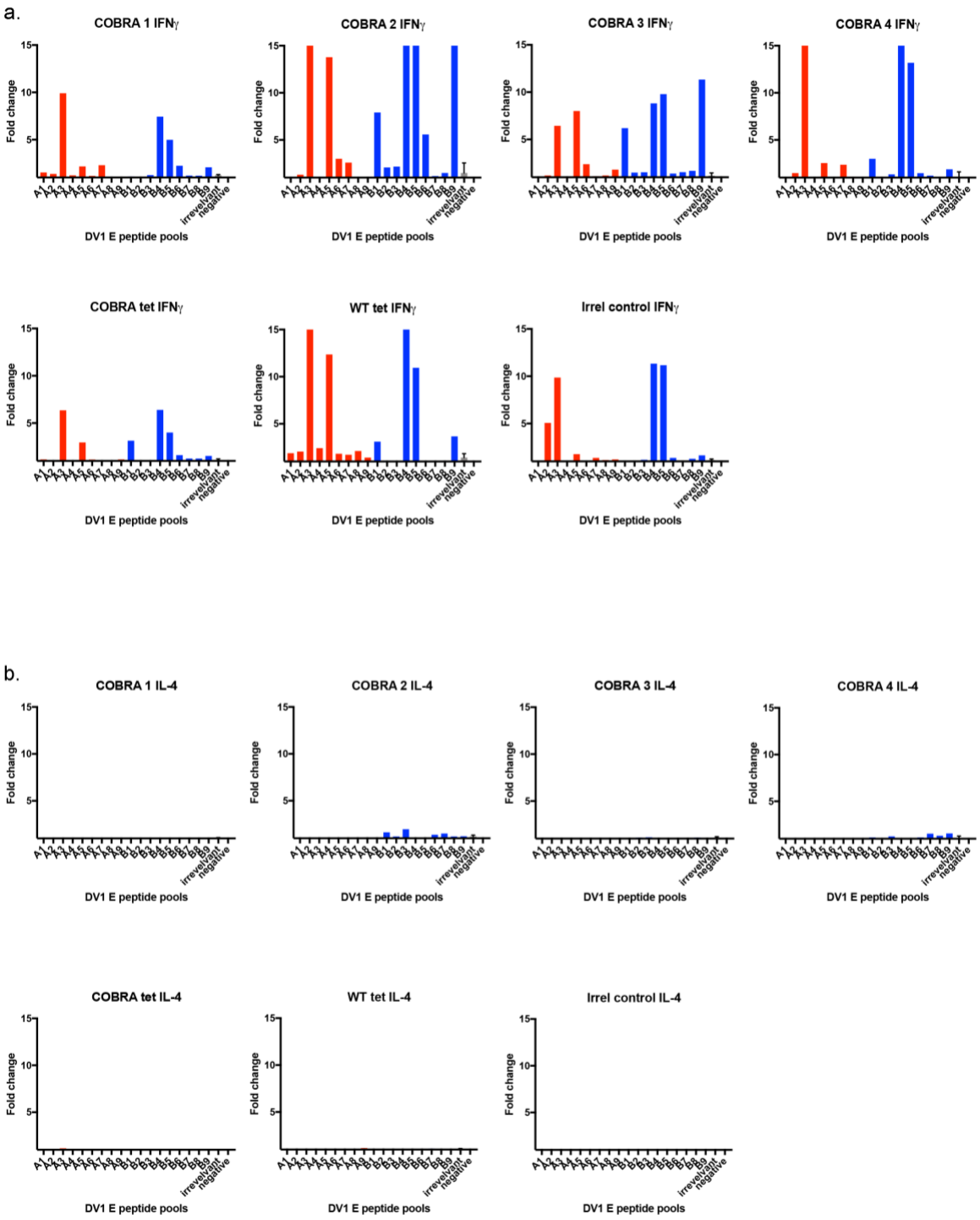


Figure 6.7 Memory B cell response of mice given COBRA or wild type mRNA vaccinations (n=3) or irrelevant mRNA (n=2). 22 weeks after final vaccination, mice were stimulated *in vivo* with intramuscular injection of homologous mRNA and spleens were harvested after 6 days. a) Each graph represents the total number of DENV-specific memory B cells (measured by their conversion to IgG producing cells) from a vaccine group. E antigens that represent each of the serotypes are on the x-axis. b) Each graph represents the percentage of DENV-specific memory B cells from a vaccine group. E antigens that represent each of the serotypes are on the x-axis. Percentages were calculated from total number of IgG secreting cells.



Supplemental Figure 6.8 IFNγ and IL-4 response from COBRA or WT mRNA vaccinated mice after DV1 stimulation. C57BL/6 mice previously vaccinated with COBRA or WT mRNA vaccines (n=1) were treated with MAR1-5A3 and infected with

DV1/VN/BID-V1792/2007 (10_8 FFU). Splenocytes harvested 6 days post infection were assayed via T cell ELISPOT for IFNγ (a) and IL-4 (b) response against peptide pools for DV1 E. X-axis indicates the two peptide pools, A (red) and B (blue). Y-axis indicated the fold change from no peptide stimulation.

Table 6.1 IgG subclasses elicited by COBRA or wild type DENV mRNA. Each table shows IgG subclass binding of sera from mice vaccinated with homologous monovalent COBRA mRNA or COBRA tetravalent, WT tetravalent, or COBRA 1 mRNA to commercial E antigen representing a) DENV1, b) DENV2, c) DENV3, d) DENV4. Asterisks represent statistical significance measured against PBS control group, measured with unpaired t-test with Welch's correction, with the color intensity increasing with the significance. *ns* for $P > 0.05$, * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, **** for $P \leq 0.0001$. In parentheses are the percentage of mice in each group that had IgG subclass binding (OD value) twice as greater that the average of the corresponding binding of the PBS control group.

p-value

n.s. ****

a.

DENV 1	IgG subclass			
Vaccine group	IgG1	IgG2b	IgG2c	IgG3
COBRA 1 mRNA	* (40%)	*** (100%)	**** (100%)	ns (10%)
COBRA tet mRNA	** (50%)	*** (100%)	**** (100%)	ns (20%)
WT tet mRNA	ns (0%)	** (80%)	**** (90%)	ns (20%)
Irrelevant mRNA	ns (0%)	* (40%)	* (40%)	ns (0%)

b.

DENV 2	IgG subclass			
Vaccine group	IgG1	IgG2b	IgG2c	IgG3
COBRA 2 mRNA	ns (0%)	ns (0%)	ns (20%)	ns (10%)
COBRA tet mRNA	ns (0%)	ns (30%)	*** (90%)	ns (0%)
WT tet mRNA	ns (10%)	ns (40%)	*** (90%)	ns (0%)
Irrelevant mRNA	ns (0%)	ns (0%)	ns (30%)	ns (0%)

c.

DENV 3	IgG subclass			
Vaccine group	IgG1	IgG2b	IgG2c	IgG3
COBRA 3 mRNA	ns (10%)	* (40%)	** (80%)	** (0%)
COBRA tet mRNA	ns (30%)	** (80%)	**** (100%)	ns (20%)
WT tet mRNA	ns (0%)	** (50%)	**** (100%)	ns (0%)
Irrelevant mRNA	ns (0%)	ns (10%)	ns (20%)	** (0%)

d.

DENV 4	IgG subclass			
Vaccine group	IgG1	IgG2b	IgG2c	IgG3
COBRA 4 mRNA	* (10%)	** (40%)	**** (100%)	* (0%)
COBRA tet mRNA	* (20%)	*** (70%)	**** (100%)	ns (10%)
WT tet mRNA	ns (0%)	* (30%)	**** (100%)	ns (0%)
Irrelevant mRNA	ns (0%)	ns (20%)	ns (30%)	** (0%)

Table 6.2 Dengue virus strains used for infection.

Serotype	Virus strain	FFU
DV1	Hawaii/1944	10^6
DV1	Costa Rica/BC89/1994	10^6 10^7
DV1	Vietnam/BID-V1792/2007	10^6 10^7 10^8
DV2	New Guinea/NGC/1944	10^6 10^7
DV2	US/BID-V594/2006	10^6 10^7
DV2	Vietnam/BID-V1007/2006	10^6
DV3	Philippines/H-87/1956	10^6
DV3	US/BID-V1619/2005	10^6
DV3	Sri Lanka/271242/1991	10^6
DV4	Philippines/H-241/1956	10^6 10^7
DV4	Malaysia/BC13/1997	10^6
ZIKV	DAK-AR/44671	10^7

Table 6.3 Viral recovery from sera of mice infected with DENV.

Challenge group	**Day 2** Average FFU	**Day 4** Average FFU
DV1/Vietnam 10^7	<1	4.5
DV1/Vietnam 10^8	3.5	8.5
ZIKV/DAK-AR 10^7	>$4x10^5$	$2.5x10^3$

CHAPTER 7

CONCLUSIONS

Dengue virus (DENV) infects millions of people living in the tropical areas of the world with an estimated 390 million infections every year [2], with 25% producing symptoms. DENV induced diseases can range from mild fever and rash to severe hemorrhage and death [4]. More than 120 countries have DENV circulating, and most endemic countries have all four serotypes circulating [20]. Primary infection generates long-lasting immunity to that serotype, but secondary, heterologous infection increases risk for severe disease [287]. In order to have an effective DENV vaccine, any potential candidate would need to neutralize viruses from all four serotypes of DENV in all individuals regardless of serostatus or geographic location. With increased global travel and spread of mosquito vectors due to climate change, almost half of the global population are at risk for infection [2], which include DENV naïve and pre-immune individuals in endemic and non-endemic countries.

The goal of this project was to develop a safe and effective vaccine that protects against all four serotypes of DENV. Computationally optimized broadly reactive antigen (COBRA) methodology was used to generate DENV envelope (E) glycoprotein to elicit broad breadth of protective immune response in both naïve and pre-immune populations. The hypothesis was DENV COBRA E subviral particle (SVP) vaccination will elicit neutralizing antibodies against all four serotypes of DENV virus in naïve and pre-immune populations.

To test this hypothesis, the following specific aims were investigated:

Specific Aim 1: Design and construct DENV COBRA subviral particles (SVPs). The working hypothesis is that DENV COBRA E immunogens can be constructed from consensus-based design and displayed on SVPs by transfecting mammalian cells with plasmids containing DENV prM-E constructs.

Four COBRA E sequences were created from hundreds of human clinical isolates that captured conserved epitopes from different strains from all over the world. They were cloned into an expression vector with prM to be successfully expressed on the surface of subviral particles after *in vitro* transfection. These results show that COBRA methodology can be used to produce particle-based DENV vaccine that will be a safer alternative to live viral vector vaccine that has a greater chance of neutralizing currently circulating strains.

Specific Aim 2: Determine immunogenicity and breadth of immune response of mice vaccinated with DENV COBRA SVPs and wild type SVPs. The working hypothesis is that DENV COBRA SVP vaccination will elicit more neutralizing antibodies against all four serotypes of DENV virus compared to wild type SVP vaccination in C57BL/6 mice. A broadly neutralizing monovalent COBRA candidate will be identified.

C57BL/6 mice were vaccinated with monovalent and tetravalent COBRA SVPs or their wild type counterparts. Immunogenicity of the SVPs vaccines were determined. Mice seroconverted and produced sera antibodies that recognized and bound to wild type E expressed on the surface of SVPs or in soluble form.

Vaccination also elicited antibodies that recognized and neutralized wild type virus strains. WT SVP vaccination elicited antibodies with a narrow breadth of reactivity.

COBRA SVP vaccination elicited more broadly reactive antibodies. Notably, COBRA 1 SVP vaccination elicited antibodies that neutralized a broad range of virus strains from all four serotypes, comparable to tetravalent formulations.

Since challenge studies could not be performed on wild type mice, longevity and memory response were evaluated. COBRA 1 SVP maintained broad breadth of protection 20 weeks after vaccination. COBRA 1 SVP vaccine groups had DENV-specific memory B cells that produced IgG antibodies against all four DENV E antigens 22 weeks after vaccination. These results show that antibodies elicited by monovalent COBRA DENV E immunogen neutralized all 12 strains of DENV *in vitro*, which was comparable to antibodies elicited by tetravalent SVP vaccination mixture.

Specific Aim 3: Determine immunogenicity and breadth of immune response elicited by DENV COBRA SVP vaccination in naïve and pre-immune non-human primates. The working hypothesis is that monovalent DENV COBRA vaccination will elicit broadly neutralizing antibodies in naïve and pre-immune populations comparable to tetravalent formulation in non-human primates

Rhesus macaques that were naïve to DENV or previously infected with wild type strains were vaccinated with COBRA 1, 2, or tetravalent SVP formulations.
Both naïve and pre-immune groups had antibodies that recognized DENV E proteins after vaccination. Furthermore, they both developed neutralizing antibodies against a panel of DENV strains representing all four serotypes.

Due to the animals being scheduled for other experiments, challenge studies nor longevity studies could not be done. However, vaccination produce memory B cells that

produced antibody secreting cells that recognized DENV E from all four serotypes after *ex vivo* stimulation. These results showed that COBRA 1 SVP vaccination elicited broadly neutralizing antibodies in naïve and pre-immune populations comparable to tetravalent formulation in non-human primates.

Additional experiments: Determine breadth of protection of COBRA mRNA vaccinations in wild type mice. The working hypothesis is that DENV COBRA mRNA vaccination will elicit protective antibodies against all four serotypes of DENV virus. C57BL/6 mice were vaccinated with monovalent and tetravalent COBRA 1-4 or tetravalent wild type, formulated as mRNA. Immunogenicity and breadth were able be determined in these groups. COBRA 1 mRNA vaccination elicited antibodies that neutralized all serotypes. This breadth was maintained in sera and in memory B cell response.

Efficacy could not be tested due to the inability of establishing an immunocompetent mouse model with IFNα/β receptor blocking mAb MAR1-5A3. Wild type mice may have innate-like lymphocyte (ILC) or unconventional T cell responses that inhibit DENV replication [288] even without type I IFN response.

The reason that COBRA 1 vaccination can neutralize strains from all four serotypes of DENV may be due to the virus like particle eliciting antibodies that bind to quaternary structures. Monoclonal antibodies that bind to quaternary structures of DENV have shown to neutralize all four serotypes [36, 37, 161, 163]. Antibodies that bind to E monomers tend to be serotype-specific [289, 290]. In ELISA data, COBRA 1 vaccination did not elicit robust cross-reactive binding to soluble, monomeric E. COBRA 1 vaccine groups also

maintained longevity of breadth. The vaccination was able to produce DENV-specific memory B cells that were cross reactive to all four serotypes.

Immune responses to DENV infection or vaccination are broad and complex. Neutralizing antibodies are understood to be necessary for protection. However, non-neutralizing antibodies are shown to exacerbate disease. Recent studies have shown that high antibody titers correlate with DENV vaccine efficacy, though low titers do not correlate to zero vaccine efficacy, concluding that other (unknown) factors play a role in protection [291]. A lack of an animal vaccine model exacerbates this uncertainty.

A major area of concern for any dengue vaccine is antibody dependent enhancement (ADE). ADE occurs when pre-existing antibodies to one DENV serotype do not neutralize, but rather enhance a heterotypic infection [4, 179]. Suboptimal levels of pre-existing antibody against Dengue have been associated with severe DENV disease compared to no antibody or high antibody levels [157]. Antibodies targeting prM of immature DENV have shown to cause enhanced disease *in vitro* and *in vivo* [58, 292]. However, similar to animal vaccine models, ADE models are inadequate. Cell-based ADE assays do not reflect ADE in immunocompromised mice [260, 261]. Recent studies showed that DENV circulating in humans are more mature and infectious than cell cultured viruses [293]. In order to fully understand the protective efficacy of COBRA E vaccines, human clinical trials will need to be conducted.

In conclusion, these results show that a broadly reactive immunogen designed with multiple consensus sequence alignment can elicit neutralizing antibodies against all four serotypes of DENV in naïve and pre-immune populations. The status quo as it pertains to DENV vaccine development is to formulate 4 separate components on a live attenuated

backbone. The COBRA E immunogen was expressed on the surface of SVPs and elicited broadly neutralizing antibodies against strains that are circulating in different parts of the world. This study shows that a monovalent vaccine is effective against all 4 serotypes, which eliminates competition and bias among four components and decrease production costs.

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APPENDIX A

STATISTICAL SIGNIFICANCE OF DATA FROM CHAPTER 4

All statistical analyses were performed using Prism (GraphPad Software, La Jolla, CA, USA). *P*-values indicated as asterisks.

ns for $p > 0.05$, * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, **** for $p \leq 0.0001$

ELISA data (Figure 4.1)

Table A.1 One-way analysis of variance (ANOVA) for seroconversion. This analysis was done to determine if there are any statistically significant differences between means in all mouse populations. Vaccine groups were compared in ELISA antibody binding to wild type SVPs (Fig 4.1a) or soluble E (Fig 4.1b) from all four serotypes. Additional follow up test was done to compare the mean rank of each vaccine group with the mean rank of control (PBS group) with Dunnett test to correct for multiple comparisons using statistical hypothesis testing. Family-wise significance and confidence level: .05 (95% confidence level).

Comparison of vaccine groups against SVP

	SVP coating			
Vaccine groups	**DV1**	**DV2**	**DV3**	**DV4**
WT SVPs	****	****	****	****
COBRA SVPs	****	****	****	****
All SVPs	****	****	****	****

Comparison of vaccine groups against soluble E

	soluble E coating			
Vaccine groups	**DV1**	**DV2**	**DV3**	**DV4**
WT SVPs	****	****	***	**
COBRA SVPs	****	****	**	*
All SVPs	****	****	**	**

Table A.2 Statistically significant differences between seroconversion of vaccine groups to PBS control group. One-tailed unpaired t-test were done to compare each individual vaccine group to PBS control group, with confidence level at 95%. One-tailed tests were done since there was an *a priori* expectation the means of the vaccine groups would be greater than the mean of the PBS control. Vaccine groups were compared in ELISA antibody binding against PBS control group with ELISA plates coated with wild type SVPs (top table, data from Fig 4.1a) or soluble E (bottom table, data from Fig 4.1b) from all four serotypes. Definition of statistical significance was $p < 0.05$.

	SVP coating			
Vaccine groups	DV1	DV2	DV3	DV4
WT 1	****	****	****	****
WT 2	****	****	****	****
WT 3	****	****	****	****
WT 4	****	****	****	****
WT tet	****	****	****	****
COBRA 1	****	****	****	****
COBRA 2	****	****	****	****
COBRA 3	****	****	****	****
COBRA 4	****	****	****	****
COBRA tet	****	****	****	****

	Soluble E coating			
Vaccine group	DV1	DV2	DV3	DV4
WT 1	**	ns	***	**
WT 2	*	ns	***	***
WT 3	ns	ns	**	*
WT 4	ns	ns	**	*
WT tet	****	***	*	***
COBRA 1	****	**	ns	*
COBRA 2	*	**	*	ns
COBRA 3	ns	ns	ns	ns
COBRA 4	ns	*	*	**
COBRA tet	***	**	*	*

Table A.3 Statistically significant differences between seroconversion of COBRA and WT SVP vaccine groups. Two-tailed unpaired t-tests were done. COBRA groups were compared in ELISA antibody binding against WT comparator groups with ELISA plates coated with wild type SVPs (top table, data from Fig 4.1a) or soluble E (top table, data from Fig 4.1b) from all four serotypes. Definition of statistical significance was $p < 0.05$.

	SVP coating			
Vaccine groups	DV1	DV2	DV3	DV4
COBRA 1 vs WT DV1	*	ns	ns	ns
COBRA 2 vs WT dV2	**	ns	ns	ns
COBRA 3 vs WT DV3	ns	ns	ns	ns
COBRA 4 vs WT DV4	ns	ns	ns	**
COBRA tet vs WT DV twt	*	ns	ns	ns

	Soluble E coating			
Vaccine groups	DV1	DV2	DV3	DV4
COBRA 1 vs WT DV1	ns	*	ns	ns
COBRA 2 vs WT dV2	ns	**	ns	ns
COBRA 3 vs WT DV3	ns	ns	ns	ns
COBRA 4 vs WT DV4	*	*	*	ns
COBRA tet vs WT DV tet	ns	ns	ns	ns

ELISPOT data (Figure 4.4)

Table A.4 Statistically significant differences in memory B cell response between COBRA 1 and COBRA tetravalent SVP vaccine group. Unpaired two-tailed t-tests were done to compare ELISPOT data between mice vaccinated with COBRA 1 and COBRA tetravalent SVPs (data from Fig 4.4). 1st row compared total number of DENV-specific memory B cells (data from Fig 4.4a) and 2nd row compares the percentage of DENV-specific B cells (data from Fig 4.4b) for all four serotypes. Definition of statistical significance was $p < 0.05$.

	ELISPOT coating			
Vaccine groups	DV1	DV2	DV3	DV4
COBRA 1 vs COBRA tet	ns	ns	ns	ns
% COBRA 1 vs COBRA tet	ns	ns	ns	ns

APPENDIX B

STATISTICAL SIGNIFICANCE OF DATA FROM CHAPTER 5

All statistical analyses were performed using Prism (GraphPad Software, La Jolla, CA, USA). *P*-values indicated as asterisks.

ns for $p > 0.05$, * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, **** for $p \leq 0.0001$

ELISA data (Figure 5.2, 5.3)

Table B.1 Statistically significant differences in antibody binding before and after vaccination with COBRA SVPs. Paired t- tests were done to compare day 0 and day 70 ELISA data from all 4 serotypes. Pre-immune non-human primate (NHP) groups (top rows) were two-tailed paired t-tests and naïve NHP groups (bottom rows) were one-tailed paired t-test. One-tailed tests were done since there was an *a priori* expectation the means of the naïve vaccine groups at day 70 would be greater than the means at day 0. Definition of statistical significance was $p < 0.05$.

		DV1	DV2	DV3	DV4
Preimmune	COBRA 1 SVP	**	**	**	***
	COBRA 2 SVP	*	*	*	*
	COBRA tet SVP	ns	*	***	***
Naïve	COBRA 1 SVP	*	ns	*	ns
	COBRA 2 SVP	**	**	**	**
	COBRA tet SVP	**	*	*	**

Table B. 2 Statistically significant differences in seroconversion between COBRA SVP vaccine groups. Unpaired two-tailed t-tests were done to compare ELISA data for pre-immune or naïve vaccination groups. For comparisons between NHP vaccine groups with different sample size (pre-immune COBRA 1 vs tetravalent and pre-immune COBRA 2 vs tetravalent), Welch's t-test was performed. Definition of statistical significance was $p < 0.05$.

		DV1	DV2	DV3	DV4
Preimmune	COBRA 1 vs COBRA 2	ns	ns	ns	ns
	COBRA 1 vs COBRA tet	ns	ns	ns	ns
	COBRA 2 vs COBRA tet	ns	*	ns	ns
Naïve	COBRA 1 vs COBRA 2	**	ns	*	ns
	COBRA 1 vs COBRA tet	ns	ns	ns	ns
	COBRA 2 vs COBRA tet	**	ns	**	*

FRNT data (Figures 5.4-5.7)

Table B.3 Statistically significant differences in neutralization titer before and after COBRA SVP vaccination. Paired t- tests were done to compare day 0 and day 70 FRNT data. Pre-immune NHP groups (top rows) were two-tailed paired t-tests and naïve NHP groups (bottom row) were one-tailed paired t-tests. One-tailed tests were done since there was an *a priori* expectation the means of the naïve vaccine groups at day 70 would be greater than the means at day 0. The virus strains used for FRNT is indicated at the top. Top table is $FRNT_{50}$, bottom table is $FRNT_{80}$. Definition of statistical significance was $p < 0.05$.

	$FRNT_{50}$	DENV virus strain panel											
		DV1 Prototype	DV1 American	DV1 Asian	DV2 Prototype	DV2 American	DV2 Asian	DV3 Prototype	DV3 American	DV3 Asian	DV4 Prototype	DV4 American	DV4 Asian
Preimmune	COBRA 1	*	***	****	ns	**	***	**	***	****	****	**	***
	COBRA 2	**	***	***	**	***	**	**	**	****	**	***	***
	COBRA tet	ns	**	**	**	**	**	**	****	****	***	****	**
Naïve	COBRA 1	**	**	**	**	***	**	**	**	**	**	**	***
	COBRA 2	**	**	**	**	***	**	*	**	**	**	**	**
	COBRA tet	**	**	**	**	****	**	**	***	****	**	**	***

	$FRNT_{80}$	DENV virus strain panel											
		DV1 Prototype	DV1 American	DV1 Asian	DV2 Prototype	DV2 American	DV2 Asian	DV3 Prototype	DV3 American	DV3 Asian	DV4 Prototype	DV4 American	DV4 Asian
Preimmune	COBRA 1	**	**	**	*	**	***	**	**	**	***	***	**
	COBRA 2	**	*	***	***	***	**	**	**	**	**	**	****
	COBRA tet	ns	**	*	***	*	**	*	**	***	****	****	*
Naïve	COBRA 1	**	**	**	**	****	*	**	**	**	**	**	**
	COBRA 2	ns	**	**	**	**	**	ns	ns	**	*	****	**
	COBRA tet	**	***	***	**	***	****	***	****	***	**	***	**

Table B.4 Statistically significant differences of neutralizing antibody titer between COBRA SVP groups. Unpaired two-tailed t-tests were done to compare FRNT data for pre-immune or naïve vaccination groups. For comparisons between NHP vaccine groups with different sample size (pre-immune COBRA 1 vs tetravalent and pre-immune COBRA 2 vs tetravalent), Welch's t-tests were performed. The virus strains used for FRNT is indicated at the top. Top table is $FRNT_{50}$, bottom table is $FRNT_{80}$. Definition of statistical significance was $p < 0.05$.

	$FRNT_{50}$	DENV virus strain panel											
		DV1 Prototype	DV1 American	DV1 Asian	DV2 Prototype	DV2 American	DV2 Asian	DV3 Prototype	DV3 American	DV3 Asian	DV4 Prototype	DV4 American	DV4 Asian
Preimmune	COBRA 1 vs tetravalent	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	COBRA 2 vs tetravalent	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	COBRA 1 vs 2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Naïve	COBRA 1 vs tetravalent	ns	ns	ns	ns	*	ns	ns	ns	ns	*	ns	*
	COBRA 2 vs tetravalent	**	*	ns	ns	ns	ns	ns	*	*	*	*	**
	COBRA 1 vs 2	**	*	ns	ns	*	ns	ns	ns	ns	ns	ns	*

	$FRNT_{80}$	DENV virus strain panel											
		DV1 Prototype	DV1 American	DV1 Asian	DV2 Prototype	DV2 American	DV2 Asian	DV3 Prototype	DV3 American	DV3 Asian	DV4 Prototype	DV4 American	DV4 Asian
Preimmune	COBRA 1 vs tetravalent	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	COBRA 2 vs tetravalent	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	COBRA 1 vs 2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Naïve	COBRA 1 vs tetravalent	ns	ns	ns	ns	**	ns	ns	ns	ns	*	*	ns
	COBRA 2 vs tetravalent	*	**	*	ns	ns	ns	*	*	*	*	***	*
	COBRA 1 vs 2	*	**	*	*	*	ns	ns	ns	ns	ns	ns	ns

ELISPOT data (Figure 5.8)

Table B.5 Statistically significant differences of memory B cell responses. Paired two-tailed t-tests were done to compare DENV-specific and non-specific (BSA) memory B cell response from ELISPOT data. Top table compares number of DENV-specific memory B cells vs. negative control (data from Fig 5.8a and c) and bottom row compares the percentage of DENV-specific B cells vs. negative control (data from Fig 5.8b and d) for all four serotypes. Definition of statistical significance was $p < 0.05$.

# of spots (vs. neg. control)

		ELISPOT coating			
	Vaccine groups	DV1	DV2	DV3	DV4
Preimmune	COBRA 1	**	*	**	*
	COBRA 2	****	****	****	****
	COBRA tet	***	**	***	***
Naïve	COBRA 1	**	*	**	*
	COBRA 2	*	**	*	**
	COBRA tet	*	*	*	ns

% of spots (vs. neg. control)

		ELISPOT coating			
	Vaccine groups	DV1	DV2	DV3	DV4
Preimmune	COBRA 1	**	**	**	*
	COBRA 2	****	****	****	****
	COBRA tet	***	**	***	***
Naïve	COBRA 1	**	*	*	*
	COBRA 2	*	**	*	**
	COBRA tet	*	*	*	ns

Table B.6 Statistically significant differences of memory B cell responses between vaccine groups. Unpaired two-tailed t-tests were done to compare percentage of DENV-specific antibody secreting cells (Figure 5.4b and d). For comparisons between NHP vaccine groups with different sample size (pre-immune COBRA 1 vs tetravalent and pre-immune COBRA 2 vs tetravalent), Welch's t-tests were performed. Top rows are comparisons in pre-immune monkeys (Fig 5.8b) and bottom rows are comparison in naïve monkeys (Fig 5.8d). Definition of statistical significance was $p < 0.05$.

		ELISPOT coating			
	Vaccine groups	DV1	DV2	DV3	DV4
Preimmune	COBRA 1 vs tetravalent	ns	ns	ns	ns
	COBRA 2 vs tetravalent	ns	*	ns	*
	COBRA 1 vs 2	ns	*	ns	**
Naïve	COBRA 1 vs tetravalent	ns	ns	ns	ns
	COBRA 2 vs tetravalent	*	ns	**	ns
	COBRA 1 vs 2	*	ns	*	ns

APPENDIX C

STATISTICAL SIGNIFICANCE OF DATA FROM CHAPTER 6

All statistical analyses were performed using Prism (GraphPad Software, La Jolla, CA, USA). *P*-values indicated as asterisks.

ns for $p > 0.05$, * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$, **** for $p \leq 0.0001$

ELISA data (Figure 6.1)

Table C.1 One-way analysis of variance (ANOVA) for seroconversion. This analysis was done to determine if there are any statistically significant differences between means in all mouse populations. Vaccine populations were compared in ELISA antibody binding to wild type SVPs (top row, Fig 6.1a) or soluble E (bottom row, Fig 6.1b) for all four serotypes. Additional follow up test was done to compare the mean rank of each vaccine group with the mean rank of control (irrelevant mRNA group) with Dunnett test to correct for multiple comparisons using statistical hypothesis testing. Family-wise significance and confidence level: .05 (95% confidence level).

ELISA coating	**DV1**	**DV2**	**DV3**	**DV4**
WT SVP	****	ns	****	****
soluble E	****	****	****	****

Table C.2 Statistically significant differences between seroconversion of vaccine groups to control group. One-tailed unpaired t-test were done to compare individual mRNA

vaccine groups to irrelevant mRNA control group, with confidence level at 95%. One-tailed tests were done since there was an *a priori* expectation the means of the vaccine groups would be greater than the mean of the irrelevant control. ELISA data from plates coated with wild type SVPs (top table, data from Fig 6.1a) or soluble E (bottom table, data from Fig 6.1b) were analyzed. Definition of statistical significance was $p < 0.05$.

	SVP coating			
Vaccine groups	DV1	DV2	DV3	DV4
COBRA 1 mRNA	****	ns	****	****
COBRA 2 mRNA	****	ns	****	****
COBRA 3 mRNA	****	ns	****	****
COBRA 4 mRNA	****	*	****	****
COBRA tet mRNA	****	ns	****	****
WT tet mRNA	****	ns	****	****

	Soluble E coating			
Vaccine groups	DV1	DV2	DV3	DV4
COBRA 1 mRNA	****	**	ns	ns
COBRA 2 mRNA	ns	*	**	ns
COBRA 3 mRNA	*	ns	**	**
COBRA 4 mRNA	*	ns	ns	****
COBRA tet mRNA	****	***	****	****
WT tet mRNA	****	***	****	****

Table C.3 Statistically significant differences between seroconversion of COBRA mRNA vaccine groups vs WT tetravalent mRNA group. Two-tailed unpaired t-tests were done to compare ELISA data from each COBRA mRNA vaccine groups against WT tetravalent mRNA vaccine group with ELISA plates coated with wild type SVPs (top table, data

from Fig 6.1a) or soluble E (top table, data from Fig 6.1b) from all four serotypes. Definition of statistical significance was $p < 0.05$.

	SVP coating			
mRNA vaccine groups	DV1	DV2	DV3	DV4
COBRA 1 vs WT tet	ns	ns	*	*
COBRA 2 vs WT tet	ns	ns	*	ns
COBRA 3 vs WT tet	*	ns	**	*
COBRA 4 vs WT tet	ns	ns	***	****
COBRA tet vs WT tet	ns	ns	ns	*

	Soluble E coating			
mRNA vaccine groups	DV1	DV2	DV3	DV4
COBRA 1 vs WT tet	****	**	ns	****
COBRA 2 vs WT tet	*	**	****	****
COBRA 3 vs WT tet	***	**	ns	**
COBRA 4 vs WT tet	****	***	****	***
COBRA tet vs WT tet	ns	*	ns	ns

ELISPOT data (Figure 6.7)

Table C.4 Statistically significant differences between memory B cell responses of mRNA vaccinations to control vaccination. Unpaired one-tailed t-tests with Welch's correction were done to compare memory B cell ELISPOT data from COBRA and WT mRNA groups to memory B cell ELISPOT data from irrelevant mRNA group. One-tailed

tests were done since there was an *a priori* expectation the means of the vaccine groups would be greater than the mean of the irrelevant control. Top rows compare total number of DENV-specific memory B cells (data from Fig 6.7a) and bottom rows compare the percentage of DENV-specific B cells (data from Fig 6.7b) for all four serotypes. Definition of statistical significance was $p < 0.05$.

		ELISPOT coating			
	Vaccine groups	DV1	DV2	DV3	DV4
# of IgG secreting cells	COBRA 1 mRNA	ns	*	ns	*
	COBRA tet mRNA	*	ns	*	ns
	WT tet mRNA	*	*	*	ns
% of total IgG secreting cells	COBRA 1 mRNA	*	*	*	*
	COBRA tet mRNA	**	*	*	*
	WT tet mRNA	ns	ns	*	ns