

DISSERTATION
INCREASING DENGUE VIRUS VACCINE SAFETY AND IMMUNOGENICITY BY
MANIPULATING ANTIGENIC DETERMINANTS OF THE FLAVIVIRUS
ENVELOPE PROTEIN

Submitted by

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ABSTRACT OF DISSERTATION
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Dengue virus (DENV), which exists as four closely related serotypes, is a mosquito-borne pathogen causing significant global disease burden, either as classic dengue fever (DF) or in its most severe manifestation, dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS). Severe dengue disease is often associated with secondary DENV infection and hypothesized to frequently be induced by cross-reactive, weakly neutralizing antibodies, a process referred to as antibody-dependent enhancement of infection (ADE). Due to the complex humoral immune response to DENV infection and the complexity of severe DENV disease, currently no licensed DENV vaccine is available.

The goal of this dissertation is to increase the safety and immunogenicity of DENV vaccination through a better understanding of the antigenic properties of the envelope protein. To these ends the objectives of this research were to: 1) use site-directed mutagenesis and monoclonal antibody mapping to identify possible cross-reactive epitopes of the DENV-2 E protein which could contribute to ADE (Chapter 2), 2) use B cell epitope modification to construct a serotype-specific DENV-2 vaccine with

reduced potential of vaccine-induced ADE (Chapter 3), and 3) identify potential dominant T cell epitopes in West Nile virus E which could act as immunological adjuvants for DENV-2 vaccines (Chapter 4).

Humoral immune responses to DENV infection are complex and can exacerbate pathogenicity, yet are essential for immune protection. DENV-2 E protein epitope-specific antigens were created and used to measure immunoglobulin responses to three distinct epitopes in serum samples from DENV-2 infected humans. Immunoglobulin responses to DENV-2 infection exhibited significant levels of individual variation. Antibody populations targeting broadly cross-reactive epitopes centered on the fusion peptide in structural domain II were large, highly variable, and greater in primary than in secondary sera from DENV-2 infected patients, confirming previous studies and identifying the fusion peptide as an immunodominant epitope. E protein domain III cross-reactive immunoglobulin populations were similarly variable and much larger in IgM than in IgG. DENV-2 specific domain III IgG formed a very small proportion of the antibody response, yet was significantly correlated with DENV-2 neutralization, suggesting that the highly protective IgG recognizing this epitope in murine studies plays a role in humans as well. These results begin to tease apart complex humoral immune responses to DENV infection and thus are important for improving our understanding of dengue disease and immunological correlates of protection relevant to DENV vaccine development and testing.

DENV vaccines must induce a balanced protective immunity to all four serotypes to reduce the possibility of cross-reactive antibody induced severe disease upon subsequent infection. By modification of immunodominant B cell epitopes of E, cross-

reactivity reduced (CRR) DENV-2 DNA vaccine candidates were developed and tested in mice for immunogenicity and potential reductions in developing vaccine induced ADE in mice. Unlike wild-type vaccine, CRR vaccine immunized mouse sera neutralized virus and did not enhance viral infection *in vitro*. Thus, reducing cross-reactivity in the envelope glycoprotein of DENV may provide a solution to increase vaccine safety and resolve the long-standing obstacle of immune enhancement in dengue vaccine development.

DNA vaccination is a highly pursued vaccine platform for its safety, stability, and ease of development, use and manufacturing. However, DNA vaccination is hindered by lower immunogenicity. Methods investigated to increase the immunogenicity of DNA vaccines have included the use of immunological adjuvants; however, few are approved for human use. A dominant, naturally occurring CD4 T cell epitope located in the transmembrane domain of West Nile virus was identified and its ability to increase the immunogenicity of heterologous flavivirus vaccines was investigated. The incorporation of the West Nile virus CD4 epitope into a DENV-2 DNA or protein vaccine significantly increased neutralizing antibody titers compared to parental vaccines without the CD4 epitope. Identifying differential antigenic properties of vaccines may alleviate concerns of imbalanced immunity associated with multivalent vaccinations.

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DEDICATION

This dissertation is dedicated to my best friend and husband, Aaron Janssen. Beyond encouragement, strength and love, he has provided constant solid grounding and comedic relief reminding me the most important things in life are often its simplest joys.

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Chapter 1

Literature Review

Introduction

Recent history has seen a global resurgence of viral diseases once thought to be under control such as dengue, Japanese encephalitis, and yellow fever, or viruses that have expanded their geographic distribution such as West Nile virus (Gubler, 2002b) and dengue. With the recent autochthonous transmission of dengue virus in Key West, Florida (CDC, 2010), a region that has not seen local dengue transmission in the past 60 years, the need for appropriate control measures for such viruses is evident.

Flaviviridae

The viruses composing the family *Flaviviridae* are single stranded RNA viruses with positive polarity divided into three genera: *Hepacivirus*, *Pestivirus* and *Flavivirus*. The *Hepacivirus* genus includes the blood borne hepatitis C virus causing persistent hepatotropic infections in humans, as well as GB virus B, in which the natural distribution and disease is not fully understood. The *Pestivirus* genus includes viruses causing disease in livestock such as bovine viral diarrhea virus, classical swine fever virus and border disease virus. The *Flavivirus* genus is composed of close to 70 viral species most, but not all, of which require a hematophagous arthropod vector to maintain the natural transmission cycle, including the prototype yellow fever virus (YFV). Historically flaviviruses, known as Group B arboviruses, were separated from Group A arboviruses, alphaviruses, based upon hemagglutination inhibition tests, which define the

broadest spectrum of antigenic differences (Casals and Brown, 1954). The group A and B arboviruses were placed in the family *Togaviridae* based on their mode of transmission and biochemical properties. Not until 1984, when the morphological, biochemical, and replicative differences of the flaviviruses were understood, was a new *Flaviviridae* family created (Westaway et al., 1985). Flaviviruses are widely distributed throughout the world with the exception of Antarctica. More than half of the known flaviviruses are associated with human disease, including the important human pathogens YFV, dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), and the Tick-borne encephalitis viruses (TBEV) (Vasilakis and Weaver, 2008). The most common outcome of human flavivirus infection is subclinical; however, clinical infections present as a flu-like disease with fever, arthralgia, myalgia, retro-orbital headache, maculopapular rash, leucopenia, vascular leakage, hepatitis and/or encephalitis.

Viruses are taxonomically grouped within the *Flavivirus* genus by their antigenic relationships, which correlate with their vector: tick-borne, mosquito-borne, and no known vector. There are eight antigenic complexes within flaviviruses that approximate the ecological characteristics of these viruses based on geographic distribution, vertebrate host, and arthropod vector (Calisher et al., 1989). Phylogeny of the flavivirus genus, based on a 1kb 3' terminal segment of the non-structural protein 5 (NS5) gene, also reveals taxonomic clustering predominantly coincident with their vector associations and antigenic relationships among them (Kuno et al., 1998). From the putative ancestor, two major branches are composed of the cluster of non-vector and vector-borne viruses. The vector-borne group then further divides into the tick-borne and mosquito-borne clusters. Within the tick-borne cluster the human pathogenic viruses such as TBEV, Powassan

(POWV) and Omsk hemorrhagic fever virus create a distinct clade from the seabird viruses. The mosquito-borne cluster divides into clades generally based on the principal vector, either *Aedes* or *Culex* mosquitoes (Kuno et al., 1998). Viruses vectored by *Culex* mosquitoes are generally encephalitic and include the JEV serogroup, where birds are the natural reservoir, while viruses vectored by *Aedes* mosquitoes are viscerotropic and include the DENV serogroup as well as YFV, where humans or primates serve as the natural reservoir (Gaunt et al., 2001; Sabin, 1959). The NS5 phylogenetic analysis supports the possibility of the non-vector viruses evolving first, followed by the tick-borne and finally the mosquito-borne clusters. This is further supported by the observation that some mosquito-borne viruses such as SLEV, WNV, and YFV have been isolated from ticks, while the only tick-borne virus to be isolated from mosquitoes is POWV (Kuno et al., 1998). The non-vector group is mainly isolated from bats or rodents and some cause a persistent infection in the vertebrate host (Baer and Woodall, 1966; Constantine and Woodall, 1964). Few of the non-vector viruses have been isolated from humans, but they commonly induce a febrile disease (Shope, 2003).

Flavivirus genome: The flavivirus RNA genome is single-stranded and approximately 11 kilobases in length (Rice et al., 1985). The genomic RNA is infectious and therefore of positive polarity. Genomic length RNA are the only viral mRNAs present in flavivirus infected cells (Boulton and Westaway, 1977). The genomic RNA has a type I cap at the 5' end (m⁷GpppAmp) and lacks a poly(A) tail on the 3' end (Wengler, Wengler, and Gross, 1978). A striking feature of the flavivirus genome is the presence of a single long open reading frame (ORF) that spans nearly the whole genome, encoding at least 10 proteins. The long ORF is flanked on each end by 5' and 3' non-

coding regions (NCR), respectively (Rice et al., 1985). The 5' NCR is not well conserved among the flaviviruses; however, a common stem-loop secondary structure is often shared (Brinton and Dispoto, 1988). This stem-loop structure influences translation, as antisense oligos to this structure inhibit translation and viral replication (Deas et al., 2005; Holden et al., 2006). Similarly, the 3' NCR is also highly variable among flaviviruses but a common stem-loop structure is present (Markoff, 2003). The 3' stem-loop is important in translation and replication, as several viral replicase proteins interact with the stem-loop (Chen et al., 1997). Upstream of the 3' stem-loop is a region that is complementary to the beginning of the capsid gene (Hahn et al., 1987), referred to as a cyclization sequence. These long-distance RNA-RNA interactions are vital for viral replication, but do not play a role in viral translation (Holden et al., 2006; Khromykh et al., 2001).

The structural proteins capsid (C), membrane (M; expressed in the precursor form prM), and envelope (E) are encoded on the 5' quarter of the genome while the non-structural (NS) proteins compose the remainder (Rice et al., 1985). The ORF generates a single polyprotein in the order 5'-C-prM/M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'. The polyprotein is post- and/or co-translationally cleaved into the individual proteins. Processing of the structural region requires membranes and an intact signal sequence, as it has been shown that proteolytic processing of TBEV polyprotein in Krebs ascites cells is not active when treated with detergent (Svitkin et al., 1984; Svitkin et al., 1981). Presumably, following translation of the C 3'-hydrophobic sequence, the flavivirus polysome becomes associated with the rough endoplasmic reticulum (ER) and translation of all the remaining viral proteins is likely to be membrane associated

(Chambers et al., 1990a). Host signal peptidase is responsible for the cleavage of the N termini of prM, E, NS1 and NS4B (von Heijne, 1984). A viral encoded serine protease cleaves intracellular C to create virion C, NS2B, NS3, NS4A and NS5 (Rice et al., 1985). The prM protein is cleaved into the pr peptide and mature M in the Golgi network by resident furin-like protease (Stadler et al., 1997). Processing of the flavivirus polyprotein results in the formation of 10 direct or indirect membrane-associated viral proteins: three structural proteins, seven non-structural proteins, and two polypeptide cleavage fragments.

Flavivirus proteins: The capsid (C) protein is a small protein, approximately 120 amino acids long, which constitutes the protein component of the nucleocapsid. The C protein is quite basic, probably acting to neutralize the negatively charged viral RNA in such a compact structure (Rice et al., 1985). C protein folds into a compact dimer, with each monomer containing four alpha-helices (Jones et al., 2003). A C-terminal hydrophobic region of the C protein (intracellular C), which functions as a signal peptide for ER translocation of prM, is cleaved from the mature C protein (virion C) by the NS3/NS2B viral serine protease (Lobigs, 1993).

The prM protein is the precursor glycoprotein to the structural protein M. The N terminal region of prM contains one to three N-linked glycosylation sites, one at Asn69 in DENV, (Chambers et al., 1990a) and six conserved cysteine residues, all of which contribute to disulfide bridging (Nowak and Wengler, 1987). The transmembrane domains of prM and E act as ER retention signals and may assist in heterodimer formation (Lin and Wu, 2005). prM undergoes a delayed cleavage to form the M protein, a process that is linked to viral budding/maturation to prevent immature virions from

fusing with host cell membranes (Li et al., 2008). Proteolytic cleavage of prM in prM/E heterodimers, results in dissociation, release of pr, and formation of E homodimers (Stiasny et al., 1996; Wengler and Wengler, 1989).

The E protein is the major surface glycoprotein and is glycosylated in some, but not all flaviviruses. E has 12 completely conserved cysteine residues, and all have been demonstrated to contribute to intramolecular disulfide bridges in WNV (Nowak and Wengler, 1987). The native form of E folds into an elongated structure rich in beta-sheets, forming head-to-tail homodimers. E homodimers lie parallel with regards to the virion surface and form a relatively smooth viral surface. The surface is composed of three sets of nearly parallel E dimers forming a herringbone pattern (Kuhn et al., 2002).

Through crystallographic analysis, each flavivirus E protein has been shown to be composed of three structural domains (Rey et al., 1995) labeled E domain I (EDI) to EDIII. EDI is the central beta-barrel domain, composed of 120 residues in three segments (1-51, 137-189, and 285-302). EDI acts as a flexible hinge region that is important in fusion. This domain is stabilized by two disulfide bridges, and has a conserved glycosylation site at Asn153, with an additional site at Asn 67 in EDII of DENV. These glycosylation sites do not have an effect on the antigenic recognition of the E protein by monoclonal antibodies (Adams et al., 1995; Winkler, Heinz, and Kunz, 1987). EDII is the dimerization domain composed of two segments (52-136, 190-284), and containing three disulfide bridges. EDII also contains an internal type II fusion loop at the distal end, which is packed against EDIII of the adjacent E protein, and undergoes a dramatic acid-mediated conformational change (Guirakhoo, Heinz, and Kunz, 1989). EDIII has an immunoglobulin (Ig)-like fold and is composed of the C terminal portion of

the E protein (303-395). EDIII is joined to EDI through 15 residues and anchored by one intra-monomer disulfide bridge. This interface is partly hydrophobic and partly polar. The Ig beta-barrel lies perpendicular to the virion surface and projects the furthest (Rey et al., 1995).

Flavivirus E is involved in several important biological functions. Flaviviruses mediate host cell entry through receptor mediated endocytosis (Barth, 1992; Krishnan et al., 2007), utilizing EDIII for interaction with cellular receptors (Chen, Maguire, and Marks, 1996; Huerta et al., 2008). Murine derived monoclonal antibodies (MAb) targeting EDIII, efficiently neutralize virus by blocking viral attachment (Crill and Roehrig, 2001). In addition, recombinant EDIII is able to bind vertebrate target cells through interactions with heparan sulfates (Hung et al., 2004; Thullier et al., 2001) and inhibit viral infection (Bhardwaj et al., 2001; Chu et al., 2005; Hung et al., 2004).

In addition to receptor binding, E is a type II fusion protein responsible for fusion of the viral envelope with the cellular endosome membrane (Lescar et al., 2001). After exposure to acidic pH, virions are no longer fusion-competent and therefore non-infectious (Corver et al., 2000; Heinz et al., 1994). Of particular importance regarding fusion is the presence of a highly conserved, hydrophobic region in EDII (Rey et al., 1995), which mutational analysis has shown to be an internal fusion peptide (FP) (Allison et al., 2001). In the native dimer, this fusion loop is buried by a hydrophobic pocket provided by EDI and EDIII of the adjacent monomer, shielding the fusion loop from interactions with membranes in its neutral-pH conformation (Modis et al., 2003). Upon exposure to acidic pH, E undergoes a complete oligomeric rearrangement forming trimers from dimers (Allison et al., 1995a), which are thermodynamically more stable

(Stiasny et al., 2001). The conversion of dimers to trimers requires the disintegration of the structure of the viral envelope, including the dissociation of E dimers into monomers. This leads to the exposure of the FP loop, allowing for its interaction with target membranes (Stiasny et al., 2002). The conformational rearrangement involves reorganization of E from a horizontally oriented anti-parallel dimer, into a perpendicularly oriented trimeric spike, where each monomer is arranged in parallel. Importantly, these dramatic transitions do not require refolding of the E polypeptide chain and the structural integrity of the three domains remains intact. Instead, the major conformational changes involve the reorientation of the domains with respect to each other, made possible by the flexible hinge regions between EDI and EDII and between EDI and EDIII (Stiasny and Heinz, 2006).

The NS1 protein contains two or three N-linked glycosylation sites and 12 conserved cysteines, all of which form disulfide bonds (Mason, 1989; Smith et al., 1970). Rapidly after synthesis, NS1 forms highly stable homodimers with a high affinity for membranes (Winkler et al., 1989; Winkler et al., 1988); however, due to the largely hydrophilic amino acid content and lack of a transmembrane domain, the nature of this membrane association is unclear. NS1 is largely retained within infected cells (Lindenbach and Rice, 2003) and plays an important, yet unclear role in RNA replication. Immunofluorescence analysis has shown that NS1 localizes to sites of RNA replication (Mackenzie, Jones, and Young, 1996) and mutation of the N-linked glycosylation sites has a dramatic effect on RNA replication and virus production (Muylaert et al., 1996). NS1 associates with the plasma membrane (Cardiff and Lund, 1976) and is expressed on the cellular surface, eliciting an antibody response resulting in complement-mediated

cytolysis (Schlesinger et al., 1990). NS1 antigen is also secreted from infected cells simultaneously with E protein, suggesting a role in virion transport or release (Lee, Crooks, and Stephenson, 1989). Additionally, anti-NS1 antibodies confer partial protection from virus challenge (Gould et al., 1986; Schlesinger et al., 1986).

NS2A is a relatively small, hydrophobic, multifunctional, membrane-associated protein involved in RNA replication (Chambers, McCourt, and Rice, 1989; Mackenzie et al., 1998). NS2A binds to replication proteins NS3 and NS5, as well as the 3' NCR of the genome RNA with high affinity (Mackenzie et al., 1998). This interaction allows for NS2A to be involved in virus assembly. Studies with YFV have shown mutation at amino acid position 190 efficiently blocks the production of infectious virus particles, although the secretion of empty, non-infectious prM/E virus-like particles (VLP) is unaffected (Kummerer and Rice, 2002). Additionally, substitutions at amino acid position 59 impaired production of infectious virus particles and the formation of virus-induced membrane formation (Leung et al., 2008). NS2A has also been shown to inhibit signaling of the host anti-viral interferon response by blocking phosphorylation of STAT-1 and inhibiting the subsequent nuclear translocation (Liu et al., 2005). In addition, mutation of a single amino acid at position 30 disables IFN antagonism and attenuates WNV virulence in mice (Liu et al., 2006).

NS2B is also a small membrane-associated protein (Clum, Ebner, and Padmanabhan, 1997). NS2B forms a stable complex with NS3 and acts as a cofactor for catalytic activity of the viral serine protease (Falgout et al., 1991). The NS2B increases the rigidity of NS3 leading to a remodeling of the protease that facilitates cleavage sequence recognition and substrate binding (Zuo et al., 2008).

NS3 is a large, multifunctional protein required for polyprotein processing and RNA replication. The N-terminal third of the NS3 protein is the catalytic domain of the NS2B-NS3 serine protease complex (Bazan and Fletterick, 1989; Chambers et al., 1990b; Gorbalenya et al., 1989), preferentially cleaving after dibasic residues (Chambers et al., 1990a). The NS2B-NS3 complex cleaves NS2B, NS3, NS4A and NS5 of the polypeptide (Rice et al., 1985) as well as generating the C-termini of mature C proteins (Amberg et al., 1994; Yamshchikov and Compans, 1994). The C-terminal region of the NS3 protein displays RNA-stimulated nucleoside triphosphatase (Wengler and Wengler, 1991) and RNA unwinding activities (Warrener, Tamura, and Collett, 1993). In addition to a role in replication, NS3 has been shown to induce apoptosis of mammalian cells in infection by Langkat virus, DENV, WNV and JEV (Prikhod'ko et al., 2002; Ramanathan et al., 2006; Shafee and AbuBakar, 2003; Yang et al., 2009) through activation of caspases 3 and 8 (Prikhod'ko et al., 2002; Yang et al., 2009).

NS4A/2K is a small hydrophobic protein that localizes to sites of RNA replication (Mackenzie et al., 1998) and interacts with NS1, which is essential for RNA replication (Lindenbach and Rice, 1999), suggesting a role in viral replication. NS4A has been implicated in the rearrangement of cytoplasmic membranes involved in replication (Roosendaal et al., 2006). NS4A associates with membranes through four internal hydrophobic regions, where the N-terminal third localizes to the cytoplasm while the C-terminus localizes in the ER lumen (Miller et al., 2007). This topology suggests a mechanism where NS4A induces membrane curvature toward the cytoplasm (Miller et al., 2007) that may be amplified by NS4A homo- or hetero-oligomerization (Mackenzie et al., 1998). Recent evidence suggests NS4A is a cofactor essential to NS3 helicase

performance, where NS4A regulates ATPase activity, allowing for conservation of energy and for NS3 to sustain RNA unwinding efficiency under ATP deficient conditions (Shiryayev et al., 2009). NS4A also displays the ability to interfere with JAK/STAT IFN signaling (Munoz-Jordan et al., 2003) through inhibiting phosphorylation of STAT-1 and STAT-2 (Lin et al., 2008b).

NS4B is the largest of the small hydrophobic NS proteins, consisting of 248 amino acids. NS4B resides in cytoplasmic foci originating from the ER, colocalizing with NS3 and double-stranded RNA, arguing that NS4B is a part of the membrane bound viral replication complex (Miller, Sparacio, and Bartenschlager, 2006). NS4B was found to not only colocalize with NS3, but to also bind the C-terminal region of NS3, dissociating it from ssRNA, enabling binding to a new duplex and enhancing the helicase activity of NS3 (Umareddy et al., 2006). Similar to NS2A and NS4A, NS4B is a potent IFN antagonist by inhibiting signaling (Munoz-Jordan et al., 2003). Additionally, coexpression of NS4A and NS4B enhances the inhibition of interferon-stimulated response element promoter activation in response to IFN α/β stimulation (Munoz-Jordan et al., 2005).

NS5 is the largest and most conserved of the flavivirus proteins, composed of approximately 900 amino acids. NS5 is essential in replication containing domains for RNA-dependent RNA polymerase (RdRp) and 5' capping. The original identification of NS5 as a capping enzyme was determined by a sequence motif conserved in Ado-Met-dependent methyl-transferases located in the N-terminal region of NS5 (Koonin, 1993). Subsequent examination showed purified N-terminal NS5 could transfer methyl groups from S-adenosyl-methionine to capped RNA substrates (Egloff et al., 2002).

Comparative sequence analysis found sequence motifs in the C-terminal region of NS5 conserved in other viral RdRp (Rice et al., 1985; Sumiyoshi et al., 1987). RdRp activity of NS5 without the presence of other viral or host cellular components was demonstrated using bacterially expressed NS5 and showing the ability to produce template-sized products of negative polarity (Tan et al., 1996). Subsequently, NS5 was demonstrated to initiate RNA syntheses *de novo* without the need of a primer (Ackermann and Padmanabhan, 2001). However, the conserved stem-loop structure in the 5' NCR is essential for binding of NS5 and the methyltransferase and RdRp activities (Dong et al., 2007a). NS5 forms a complex with NS3 (Cui et al., 1998; Kapoor et al., 1995) and stimulates the NTPase (Cui et al., 1998) and RTPase activity of NS3 (Yon et al., 2005). NS3 only complexes with NS5 when in the cytoplasmic hypophosphorylated form (Kapoor et al., 1995) and the complex is able to bind the 3' NCR stem-loop structure of the viral genome (Chen et al., 1997). As with other NS proteins, NS5 is able to antagonize the host IFN response in a flavivirus specific manner. Langkat virus NS5 inhibits IFN signaling by binding to IFN α/β receptor subunit IFNRRAR2 (Best et al., 2005). JEV NS5 may activate phosphatases to inhibit JAK/STAT signaling rather than directly interfering with signaling (Lin et al., 2006). In contrast, DENV-2 NS5 is able to induce interleukin (IL)-8 production and secretion (Medin, Fitzgerald, and Rothman, 2005).

Dengue virus

DENV serocomplex is composed of four distinct species within the flavivirus genus, which includes four genetically different, yet antigenically related serotypes (DENV-1, -2, -3, and -4) (Calisher et al., 1989). Among members of the flavivirus

genus, DENV are unique and most restricted in terms of their natural vertebrate host range, which only includes primates. All four serotypes circulate in urban environments of the tropics and subtropics between humans and the peridomestic *Aedes aegypti* mosquito. This distribution puts nearly a third of the global population at risk for DENV infection (Farrar et al., 2007). DENV-1,-2, and -4 also circulate in a sylvatic cycle in the forests of Asia between *Macaca* and *Presbytis* monkeys vectored by *Ae. nivues* (Peiris, Dittus, and Ratnayake, 1993). A sylvatic cycle of DENV-2 in west Africa (Rico-Hesse, 1990) occurs between *Erythrocebus patas* monkeys and *Ae. taylori*, *furficcer*, *viattus*, and *leutocephalus* mosquitoes (Traore-Lamizana et al., 1994). DENV-3 has yet to be isolated from a sylvatic cycle but DENV-3 antibody seroprevalance suggests the cycle exists (Rudnick, Marchette, and Garcia, 1967).

Dengue virus diversity: Within each of the four DENV serotypes, the viruses are further grouped into genotypes (Rico-Hesse, 1990). DENV-1 contains five genotypes, generally correlated with the geographic origin of the strains: genotype I contains strains from Southeast Asia, China, and east Africa; genotype II contains strains isolated from Thailand in the 1950s and 1960s; genotype III contains the sylvatic strains from Malaysia; genotype IV contains strains from the west Pacific Islands and Australia; and genotype V consists of isolates from the Americas, west Africa, and a few from Asia. (Goncalvez et al., 2002; Rico-Hesse, 1990; Wang et al., 2000). DENV-2 isolates are generally accepted to be subdivided into five genotypes: genotype I contains the Asian I strains from Malaysia and the Asian II strains from Vietnam, China, Taiwan, Sri Lanka and Philippines; genotype II contains Cosmopolitan strains; genotype III contains the American, Caribbean, Indian subcontinent isolates, and strains from the Pacific Islands

isolated in the 1950s and 1960s; genotype IV contains strains from Thailand, Vietnam, and the American isolates within the past 30 years; and genotype V contains the sylvatic strains from west Africa and Southeast Asia (Lewis et al., 1993; Rico-Hesse, 1990; Wang et al., 2000). DENV-3 can be divided into five genotypes: genotype I contains isolates from Indonesia, Malaysia, the Philippines and the South Pacific islands; genotype II contains isolates from Thailand; genotype III contains isolates from Sri Lanka, India, Africa and Samoa; genotype IV contains isolates from Puerto Rico and Tahiti in 1965 (Lanciotti et al., 1994); and genotype V contains isolates from China, Philippines and Malaysia that were originally grouped in genotype I (Wittke et al., 2002). DENV-4 can be divided into four genotypes: genotype I contains isolates from Thailand, Philippines, Sri Lanka, and Japan (isolated from a Japanese traveler); genotype II contains isolates from Indonesia, Malaysia, Tahiti, Caribbean and the Americas; genotype III contains isolates from Thailand; and genotype IV contains the sylvatic isolates (Lanciotti, Gubler, and Trent, 1997; Wang et al., 2000).

Dengue virus history and epidemiology: The first description of a dengue-like illness is found in Chinese literature during the Chin Dynasty (CE 265), Tang Dynasty (CE 610) and Northern Sung Dynasty (CE 992) (Gubler, 1997), describing a disease called “water poison” and the association with water-associated flying insects. A similar description of illness did not occur until 1635 and 1699 in the French West Indies and Panama (Gubler, 1997). An entire century later (1779-1788), the first reports of possible DENV pandemic in Indonesia, Egypt, North America, and Spain were described as the first evidence for wide-spread DENV distribution (Gubler, 1997). By 1920, DENV

disease behavior in Southeast Asia, India, and the Philippines had changed from the sudden onset of urban epidemics, to endemicity (Smith, 1956).

The events of World War II brought immense changes to DENV ecology, epidemiology, and disease. Destruction of water distribution systems leading to domestic water storage created an abundance of ideal habitats for *Ae. aegypti* larvae. In addition, transportation of troops and supplies resulted in the importation of susceptible hosts, and the movement of *Ae. aegypti* into new geographic regions (Vasilakis and Weaver, 2008). Between 1941 and 1945, DENV epidemics were raging among military personnel in East Africa, the Caribbean, and the Pacific theater (Sabin, 1952b), leading to a heightened awareness and DENV disease study. DENV-1 was first isolated in Japan, followed by the isolation of DENV-1 and DENV-2 from U.S. soldiers. Research also led to the identification of homotypic immunity following infection, and the development of hemmagglutination inhibition tests for serodiagnosis (Sabin, 1952b). By the end of the 1960s, all four serotypes were circulating in Southeast Asia and India.

Although the occurrence of severe and fatal hemorrhagic disease associated with DENV infection was reported in the 1780 Philadelphia outbreak, it was probably rare and did not pose a serious public health problem (Vasilakis and Weaver, 2008). The first well documented cases of DHF were associated with epidemics in Thailand and the Philippines in the 1950s and thought to be a new disease (Hammon et al., 1960). Through epidemiological studies, an association between secondary DENV infection and severe DENV disease was elucidated (Halstead et al., 1967; Russell, Udomsakdi, and Halstead, 1967; Sangkawibha et al., 1984). These observations led to the antibody-

dependent enhancement (ADE) theory of DHF pathogenesis (Halstead, 1970; Halstead, Chow, and Marchette, 1973).

The first DENV outbreak in the Americas occurred during the 1778 pandemic that included Philadelphia. DENV did not appear again until 1828, when an epidemic swept west across the Caribbean, Gulf, and Atlantic regions including the seaports of the Virgin Islands, Cuba, Jamaica, Venezuela, and the United States- Pensacola, Charleston, Savannah and New Orleans (Ehrenkranz et al., 1971). Outbreaks of DENV continually developed in these seaport cities throughout the Civil War and Reconstruction Era, including a large outbreak in New Orleans in 1873 where 40,000 people became ill. The westward movement of DENV into Texas began in 1885 involving the port cities of Houston and Galveston and spread inland along the rail-roads resulting in hemorrhagic manifestations (Ehrenkranz et al., 1971). Outbreaks of DENV continued in Texas into the 20th century when in 1922 epidemic DENV began in Galveston and swept east into Louisiana, Florida, Georgia, and into the Caribbean. A major DENV epidemic occurred in 1934 beginning in Miami and spreading throughout Florida into southern Georgia.

The last outbreak of DENV in the United States occurred in Texas in 1944 (Ehrenkranz et al., 1971), with an additional outbreak of DENV in Hawaii from 1943-44 (Gilbertson, 1945). In the 1940s the Pan American Health Organization began an *Ae. aegypti* eradication program to minimize urban epidemics of yellow fever. This program successfully eradicated the vector from 19 countries (Gubler, 1997); however, by 1970 the program was not sustained and reinfestation of the mosquito mimicked previous distributions. Between 1946 and the 1990s no cases of DENV acquired in the United States were reported. Since then few locally acquired DENV infections have occurred

along the Texas-Mexico border, associated with large DENV outbreaks in neighboring Mexican cities (CDC, 1996; CDC, 2007). In September of 2001, an outbreak of DENV-1 occurred on several of the Hawaiian islands, associated with a large outbreak in the French Polynesian islands (Effler et al., 2005). Sporadic cases of locally transmitted DENV disease occurred in these regions until 2009, when DENV infections in Key West, Florida residents without a history of travel were confirmed (CDC, 2010) with cases continuing into 2010, suggesting sustained transmission of the virus, possibly leading to endemicity.

A large Caribbean epidemic began in 1963. The introduction of an Asian genotype DENV-3 into Puerto Rico and Jamaica led to several epidemics in the Caribbean, northern Colombia, and Venezuela (Ehrenkranz et al., 1971; Morales et al., 1973; Russell et al., 1966; Spence, Jonkers, and Casals, 1969). The introduction of an Asian genotype DENV-2 into Cuba in 1981 was followed by an increase in disease severity in Cuban and Venezuelan epidemics (Kouri et al., 1983; Kouri et al., 1989; Uzcategui et al., 2001). The Cuban epidemic of 1981 led to the observations of the importance of genetics, gender, age, and secondary infection in the development of severe DENV disease (Guzman et al., 1991; Guzman et al., 2002; Guzman et al., 1984). The introduction of new genotypes and the increased circulation of multiple serotypes resulting in the increased disease severity in the Americas, mirroring the events that occurred in Southeast Asia in the 1950s and 1960s (Vasilakis and Weaver, 2008).

Dengue virus disease: The most common clinical disease caused by DENV infection is dengue fever (DF), a febrile illness of older children and adults. DF is commonly associated with 5-7 day duration of fever, headache, retroocular pain, myalgia,

arthralgia, and frequently a generalized maculopapular rash on the trunk. Treatment for DF is supportive and patients normally fully recover. Severe manifestations of DENV infection include dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Children under 16 are at the greatest risk for developing DHF/DSS. Severe disease and shock is frequently observed the day following defervescence and rarely lasts more than 48 hours. DHF/DSS is characterized by fulminant hepatitis, massive hemorrhage including positive tourniquet test, petechia, intestinal hemorrhage and hematemesis, organ failure, vascular leak with hypovolemic shock induced by plasma leakage, thrombocytopenia of less than 100,000 cells per mm³, elevated liver enzymes, and coagulation disorders including decreased fibrin and increased fibrin degradation products. DHF/DSS is divided into four grades (I-IV) ranging from mild hemorrhage in the form of petechia, to profound shock and circulatory collapse. DHF/DSS is managed through replacement of lost plasma and maintenance of circulatory volume. (WHO, 2009)

Dengue hemorrhagic fever pathogenesis

Much attention has been given to the pathogenesis of DHF/DSS since its first epidemic appearance in Thailand. The mechanisms leading to the development of severe DENV disease are not fully understood but are likely multifactorial. Several theories have been proposed to explain the manifestation of severe DHF/DSS.

Virulent viruses: The virus virulence hypothesis states that certain DENV strains are responsible for more severe disease. This is supported by the fact that the first outbreak of DHF in the Americas coincided with the introduction of Southeast Asian genotype DENV-2 into Cuba in 1981 (Guzman et al., 1995; Rico-Hesse, 1990).

Moreover, in a prospective study in Peru, no cases of DHF or DSS occurred when the infecting virus was an American genotype DENV-2 (Watts et al., 1999).

Complement: The complement system is one of the main humoral components of innate immunity. During the time of defervescence, high levels of complement activation products, C3a and C5a, are present in plasma, followed by reduction of complement components in DSS patients' sera (Churdboonchart, Bhamarapavati, and Futrakul, 1983; Nishioka, 1974; Shaio, Chang, and Hou, 1992). Heterotypic antibodies may bind to surface expressed NS1 and result in complement activation (Avirutnan et al., 2006). In addition, NS1 released from infected cells can activate complement in the fluid phase (Kurosu et al., 2007). The production of the C5b-C9 complex could trigger production of inflammatory cytokines associated with severe DHF/DSS (Avirutnan et al., 2006).

Autoantibodies: Antibodies produced during a DENV infection have been shown to cross-react with self-antigens. A portion of the DENV E protein (amino acids 100-110) shares sequence similarity with several clotting factors such as prothrombin, plasminogen, and tissue plasminogen factor, and antibodies against E protein are reactive with plasminogen (Huang et al., 1997; Markoff et al., 1991). Anti-NS1 antibodies cross-react with platelets causing thrombocytopenia and hemorrhage in mice (Lin et al., 2008a; Sun et al., 2007). These NS1 cross-reactive antibodies can also inhibit aggregation and lyse platelets, where deletion of the C-terminal portion of NS1 ablated this ability (Chen et al., 2009). Anti-NS1 IgM antibodies also cross-react with endothelial cells, inducing caspase-dependent apoptosis (Lin et al., 2003) and secretion of proinflammatory cytokines IL-6, IL-8, and MCP-1 (Lin et al., 2005).

Cross-reactive T cells: CD8 positive T cells are a dichotomy in DENV infection as they play a role in viral clearance and in immunopathogenesis (An et al., 2004). High avidity cross-reactive CD8⁺ T cells become activated and produce high concentrations of proinflammatory cytokines TNF α , IFN γ , and IL-13; however, these high avidity CD8⁺ T cells quickly undergo apoptosis and cannot be isolated from convalescent patients (Dong et al., 2007b). Alternatively, low avidity cross-reactive CD8⁺ T cells to the infecting virus can be preferentially expanded, known as original antigenic sin (Mongkolsapaya et al., 2003). These low avidity cross-reactive CD8⁺ T cells are able to secrete proinflammatory cytokines but have lost ability to secrete cytotoxic granules (Mongkolsapaya et al., 2006). Studies in mice have also revealed a preferential expansion of cross-reactive memory CD8⁺ T cells that secrete more TNF α during a secondary infection than during a primary infection (Beaumier et al., 2008). This massive activation of cross-reactive T cells leads to increased levels of inflammatory cytokines such as IFN γ , TNF α , IL-6, IL-8, and IL-1 β , which have been shown to exist in high levels in serum of DHF/DSS patients (Hober et al., 1993; Kurane et al., 1991; Raghupathy et al., 1998). In addition, cross-reactive memory T cells have been shown to display altered secondary responses compared to primary responses, secreting higher proportions of TNF than IFN γ , dependent upon the sequence of DENV infection (Beaumier et al., 2008; Beaumier and Rothman, 2009; Friberg et al., 2010), which could contribute to the varying degrees of disease outcomes in secondary DENV infection.

The participation of cross-reactive memory T cells in the development of severe DHF/DSS, the majority of which are restricted to non-structural proteins, has been widely documented (Beaumier et al., 2008; Beaumier and Rothman, 2009; Mongkolsapaya et al.,

2003). Studies using murine models (Rothman, Kurane, and Ennis, 1996) have identified CD8⁺ cytotoxic T lymphocyte (CTL) clones specific for prM, E, NS1/2a, and NS3. All DENV cross-reactive CTL clones identified targeted the non-structural proteins, while DENV specific CTL clones targeted structural proteins. Studies using human DENV immune sera have also identified a similar dominance in cross-reactive T cell repertoire. CTLs isolated from a DENV-4 infected patient were shown to lyse fibroblasts infected with all four DENV serotypes, in addition to lysing fibroblasts expressing non-structural proteins at higher efficiency than those expressing structural proteins (Bukowski et al., 1989). Further investigation of DENV CTLs has led to the identification of NS3 as a dominant protein for eliciting T cell responses during infection. Interestingly, DENV specific and cross-reactive CD8⁺ CTL clones isolated from DENV-4 immune patients were mapped to a single epitope on NS3, amino acids 500-508 (Livingston et al., 1995), which suggests CD8⁺ CTL clones can recognize the same epitope yet display distinctive specificities. In addition to NS3, NS1 and NS2a have been identified as eliciting DENV specific and cross-reactive CD8⁺ CTLs. Following vaccination with live attenuated monovalent DENV vaccines, CTL clones isolated from all patients lysed fibroblasts expressing NS3 or NS1/2a while only few CTL clones lysed fibroblasts expressing E (Mathew et al., 1996). This method also identified one donor who received DENV-4 live attenuated vaccine that elicited CTL clones that lysed prM expressing fibroblasts, marking the first description of CD8⁺ CTLs with prM specificity. A similar predominance of NS targeted CD8⁺ CTLs is seen following secondary infection. All CD8⁺ CTL clones isolated from Thai donors following secondary DENV infection were DENV cross-reactive and were targeted against NS1.2a and NS3 (Mathew

et al., 1998). In addition, a large proportion of the CTL clones isolated recognized amino acids 221-232 on NS3. Based upon these studies, the T cell response to DENV infection can best be described as highly variable, with multiple epitope specificities and a highly dominant recognition of NS3 and NS1.2a.

Despite the breadth of studies indicating the importance of cross-reactive T cells isolated from DENV immune individuals, a recent prospective study questions this role during active DENV infection. A kinetic study of activated CD8+ T cells in peripheral blood from primary and secondary DENV infected children suggests hemoconcentration and thrombocytopenia begin before the appearance of measurable levels of NS3 specific CD8+ T cells (Dung et al., 2010).

Antibody-dependent enhancement: Epidemiological evidence associates DHF/DSS with secondary DENV infections (Guzman et al., 1990; Halstead et al., 1967; Russell, Udomsakdi, and Halstead, 1967; Sangkawibha et al., 1984). The observations of Halstead (Halstead, 1970; Halstead, Nimmannitya, and Cohen, 1970) noted severe DHF/DSS peaked in two key groups.

The first peak is seen in infants experiencing primary DENV infection, for whom maternally derived antibodies declined to subneutralizing levels. Subsequent studies have revealed a correlation between maternal DENV neutralization titers and the age of the infant during the onset of DHF/DSS (Kliks et al., 1988); infants from mothers with high neutralizing titers were older at onset of illness than infants from mothers with low neutralizing titers. Furthermore, a prospective study showed maternally derived neutralizing anti-DENV IgG declined below measureable levels in infants by six months of age, while IgG reactive with DENV virions persisted until 12 months of age (Chau et

al., 2009), suggesting a timeframe when maternal anti-DENV IgG can bind but not neutralize virus, potentially resulting in ADE.

The second peak of severe DENV infection occurs in young children who have experienced a prior mild or subclinical DENV infection and were later infected with a different DENV serotype (Halstead, 1970; Halstead, Nimmannitya, and Cohen, 1970). These observations led to the conclusion that subsequent infection of preimmune individuals with a different DENV serotype could exacerbate disease. This phenomenon is thought to be due to cross-reactive antibodies and termed antibody-dependent enhancement (ADE) of infection (Halstead, 1970; Halstead, Chow, and Marchette, 1973). Additional studies revealed the essential features of ADE *in vitro*: first, non- or weakly-neutralizing antibodies are important as heterotypic DENV was only neutralized at low PRNT₅₀ titers compared to homotypic DENV; second, Fc receptors on target cells are essential since anti-DENV F(ab)₂ were unable to enhance infection; finally, the presence of immune complexes are crucial in the development of ADE as enhancing antibodies do not attach to mononuclear phagocytes before the formation of immune complexes (Halstead and O'Rourke, 1977).

The Fc receptors (FcR) can be divided into three classes, FcγRI – FcγRIII, and are present on several immune cells depending on receptor type. FcγRI is exclusively located on monocytes, while FcγRII is expressed on monocytes, neutrophils, eosinophils, platelets, and B cells (Unkeless, 1989). FcγRI and FcγRII have been shown to participate in ADE (Kontny, Kurane, and Ennis, 1988; Littaua, Kurane, and Ennis, 1990), while association of FcγRIII has not been directly investigated. Blocking the Fc receptor with anti-FcγR IgG or F(ab)₂ was able to diminish capability of monoclonal antibodies (MAb)

to enhance the infection of WNV (Peiris et al., 1981). In addition, modification of the Fc region ablates the ability of MAbs to induce ADE and severe disease *in vitro* and *in vivo* (Balsitis et al., 2010; Goncalvez et al., 2007), and Fc modification has the potential to work as an effective therapeutic, as these Fc modified MAbs result in reduced viremia and viral tissue burden in AG129 mice without inducing severe disease (Balsitis et al., 2010).

FcR-mediated ADE could exacerbate DENV infection and severe disease. ADE could lead to an increased infection rate of target cells or convert non-infectious, immature and/or structurally defective virions using FcR as an accessory viral receptor, resulting in the high viral load observed with DHF/DSS (Libraty et al., 2002; Vaughn et al., 2000; Wang et al., 2003; Wang et al., 2006). In addition to increasing the overall viral burden, FcR-mediated ADE modifies the innate and adaptive intracellular antiviral mechanisms. DENV infection via the FcR-mediated pathway was shown to suppress the transcription of proinflammatory cytokines IL-12, IFN γ , and TNF α , while up regulating the transcription of anti-inflammatory cytokines IL-6 and IL-10 (Chareonsirisuthigul, Kalayanaroj, and Ubol, 2007), suggesting FcR-mediated ADE may preferentially induce a Th2 driven T cell response. Moreover, DENV infection via ADE resulted in suppression of nitric oxide production by down regulating the activity of STAT-1 and IRF-1 (Chareonsirisuthigul, Kalayanaroj, and Ubol, 2007) and potentially inhibiting the innate antiviral defenses of host cells.

Although ADE of DENV infection is commonly associated with FcR, studies have displayed the ability of non-Fc cell surface molecules in inducing ADE. Original work by Halstead and colleagues determined IgG, but not IgM was responsible for ADE

(Halstead and O'Rourke, 1977); however, a role for IgM in ADE has been elucidated. Studies demonstrate that IgM has the ability to enhance DENV infection through complement receptor 3 (CR3) (Cardosa, Porterfield, and Gordon, 1983). It was demonstrated that IgM in the presence of sera that had been heat inactivated or treated with cobra venom factor, did not mediate ADE of DENV, while IgM in the presence of fresh serum did enhance DENV infection. In addition, blocking CR3 also ablated the ability of IgM and fresh sera to enhance DENV. These data show a direct role of IgM in the development of ADE and severe DENV diseases during a natural infection. Additional cell surface molecules have also been implicated in ADE *in vitro*. Through the use of bispecific MAbs containing Fab of DENV MAbs cross-linked to Fab of MAbs targeting cell surface molecules, the infection of DENV was increased when bispecific MAbs targeted β 2-microglobulin, CD15 or CD33 (Mady et al., 1991). These data suggest that FcR is not a unique requirement of ADE of DENV infection and the virus/antibody complexes formed during ADE may function as an accessory receptor to facilitate the interaction of DENV with its true cell surface receptor.

The ability of antibody to enhance the infection of flaviviruses was well established before an understanding of their viral antigenic determinants was developed. Through the use of hybridoma technology, type-specific and group cross-reactive MAbs capable of enhancing infection of WNV (Peiris, Porterfield, and Roehrig, 1982) and YFV (Schlesinger and Brandriss, 1983) were determined to be reactive with the E protein, while MAbs against NS1 were unable to enhance YFV infection (Schlesinger and Brandriss, 1983). Recent studies have also revealed the importance of anti-prM antibodies in the development of ADE. Confirming previous results, DENV infection

was enhanced by anti-E MAbs, and not enhanced by anti-NS1 or anti-C MAbs; however, novel anti-prM MAbs were shown to enhance DENV infection in a FcR-mediated fashion (Huang et al., 2006). Interestingly, anti-prM MAb could also enhance the infection of cells that did not bear FcR by binding to membrane proteins such as HSP60, suggesting that anti-prM antibodies have dual specificity and a unique method of ADE. Subsequently, anti-prM MAbs were shown to enhance the infection of immature DENV particles (Rodenhuis-Zybert et al., 2010) suggesting the ability of anti-prM antibodies to render non-infectious immature DENV particles infectious.

The evidence for a role of *in vivo* ADE and the contribution of ADE to human severe DENV disease is limited. Following sequential DENV infection, an enhanced viremia has been demonstrated in monkeys (Halstead, Shotwell, and Casals, 1973b). Additionally, passive transfer of DENV MAb to macaques resulted in an increased viremia titer (Goncalvez et al., 2007). However, in both studies enhanced viremia could not be associated with enhanced disease, as monkeys do not develop severe DENV disease. A recently described AG129 mouse model has shown the ability of DENV immune sera or cross-reactive MAb to enhance viremia and induce a DENV lethal disease similar to clinical disease in humans (Zellweger, Prestwood, and Shresta, 2010).

Evidence for the role of ADE in human cases of severe DENV infection remains circumstantial. Studies of both primary and secondary DHF/DSS have failed to reveal a link between ADE activity of plasma and severe disease. In a prospective study of infants, an association of ADE activity at illness onset and the development of severe DENV disease could not be established (Libraty et al., 2009). In addition, in a prospective study of Thai school children, preillness plasma could enhance the infection

of K562 cells; however, the enhancement did not correlate with clinical severity or viral burden (Laoprasopwattana et al., 2005).

Flavivirus envelope protein antigenicity

Flaviviruses exhibit three major antigenic classes: serotype-specific, complex cross-reactive, and flavivirus group cross-reactive (Clarke, 1960). Through diagnostic serology, flavivirus E protein has been shown to be responsible for several serological attributes of flavivirus infection, including hemagglutination inhibition (HI), complement fixation, and virus neutralization (Cardiff et al., 1971; Hammon and Price, 1966; Qureshi and Trent, 1973). The advent of murine MAbs allowed for dissection of the antigenic regions on E. The flavivirus E has three non-overlapping antigenic regions termed A, B, and C (Guirakhoo, Heinz, and Kunz, 1989; Heinz et al., 1983), which were subsequently found to correlate with the three structural domains EDII, EDIII, and EDI respectively (Rey et al., 1995). Through blocking studies of TBEV, MAbs mapping to antigenic domain A (EDII) were HI active, non-neutralizing, and flavivirus group cross-reactive. MAbs mapping to antigenic domain B (EDIII) were HI active, neutralizing, and contained complex cross-reactive as well as serotype specific epitopes (Heinz et al., 1983). MAbs mapping to antigenic domain C (EDI) are more variable in their HI and neutralization activity, and mostly sub-type specific (Guirakhoo, Heinz, and Kunz, 1989). Subsequent studies of DENV-2 confirmed the antigenic and biological activities of the three flavivirus antigenic domains (Roehrig, Bolin, and Kelly, 1998).

Domain A: Flavivirus E protein antigenic domain A (EDII) contains the internal fusion peptide. Antibodies directed against this domain are able to block pH-mediated virus-mediated membrane fusion in endosomal compartments (Gollins and Porterfield,

1986; Roehrig, Bolin, and Kelly, 1998). Subsequent studies have identified the highly conserved fusion peptide region as an important antigenic epitope determinant (Allison et al., 2001; Crill and Chang, 2004; Goncalvez, Purcell, and Lai, 2004; Oliphant et al., 2006). Site-directed mutational analysis at G104, G106, and L107 has shown that the fusion peptide is responsible for recognition of several cross-reactive MAbs including flavivirus group cross-reactive, and complex cross-reactive MAbs (Crill and Chang, 2004; Trainor et al., 2007). In addition, MAbs targeting the fusion peptide seem to recognize the region in a structurally specific manner. The first study to show this phenomenon used anti-peptide antibodies against DENV-2 and determined some anti-peptides defining the fusion peptide were more efficient at binding low-pH treated virus than neutral pH treated virus (Roehrig et al., 1990). Moreover, solubilization of WNV virions led to an increase in binding efficiency of fusion peptide targeted MAbs (Stiasny et al., 2006).

A recent structural study of immature WNV virions demonstrates non-neutralizing group cross-reactive MAbs bind with high affinity to spike structures found on immature and partially mature WNV virions but do not bind effectively to mature virions (Cherrier et al., 2009). This same study demonstrated immature and partially mature WNV and DENV virions are present in virus preparations. These data suggest broadly cross-reactive antibodies could promote ADE of infection by increasing infectivity of low infectious, partially mature virions.

Studies using flavivirus infected human polyclonal sera have furthered our understanding of the importance of the fusion peptide in the flavivirus antibody response. The first such study demonstrated DENV immune human sera displayed reduced ELISA

reactivity to TBEV recombinant sub-viral particles (RSP) containing substitutions in the fusion peptide, leading to the postulate that the fusion peptide is an immunodominant antigen (Stiasny et al., 2006). Subsequent studies of human sera have examined the relative proportion of antibodies targeting the fusion peptide through competitive binding assays, western blots, or epitope-specific ELISA. These studies reveal antibodies targeting the fusion peptide are cross-reactive, non-neutralizing toward heterologous serotypes, and make up a large, highly variable proportion of the antibody response in DENV infected human sera (Crill et al., 2009; Lai et al., 2008; Throsby et al., 2006). The immunodominant production of these flavivirus group cross-reactive antibodies has enabled the utilization of diagnostic antigens containing substitutions in the fusion peptide, which increase the accuracy of clinical flavivirus diagnosis (Chiou et al., 2008b; Roberson, Crill, and Chang, 2007a).

Domain B: The flavivirus antigenic domain B (EDIII) interacts with the putative cellular receptor and contains complex cross-reactive epitopes as well as serotype-specific and protective epitopes. Shortly after the passive transfer of MAbs was demonstrated to confer protection from challenge for several flaviviruses (Brandriss et al., 1986; Hawkes et al., 1988; Kaufman et al., 1987; Kimura-Kuroda and Yasui, 1988; Mathews and Roehrig, 1984), protective MAbs were determined to react with antigenic domain B (EDIII) (Cecilia et al., 1988). Using truncated DENV-2 E polypeptides, the binding of a potently neutralizing serotype-specific MAb was elucidated to be between amino acids 255 and 422 in EDIII, with a more precise mapping to amino acids 386 to 397 (Trirawatanapong et al., 1992). Further characterization of DENV serotype-specific neutralizing MAbs has shown the importance of DENV-2 serotype conserved residues

K305, P384 (Gromowski and Barrett, 2007) E383, P384 (Sukupolvi-Petty et al., 2007), and residues 307-312, 387, 389, and 391 of DENV-1 (Lisova et al., 2007). Studies of JEV E protein using phage display have found residues 307-309, 327-333, and 386-390 to bind with high affinity to JEV neutralizing MAbs (Wu and Lin, 2001). Analysis of WNV neutralization escape mutants reveals residues 307, 330, and 332 are important in neutralization of WNV by MAbs (Beasley and Barrett, 2002). Discrepancies between the exact locations of important serotype-specific residues could easily be due to differences in methods between phage display, RSPs, mutagenesis procedures, and different amino acid substitutions. Despite slight variation in the exact location of EDIII neutralizing epitopes, these studies suggest the importance of the lateral ridge of EDIII in eliciting serotype-specific neutralizing antibodies. Although EDIII plays a large role in the binding of murine MAbs, recent studies of EDIII depleted DENV infected human sera suggest antibodies against EDIII may play a smaller role in total DENV neutralization as the EDIII depleted sera retained a relatively high neutralization titer (Wahala et al., 2009). This could be due to domains outside EDIII being implicated in flavivirus neutralization. For example, human MAbs isolated from WNV infected human sera neutralize virus post attachment in a pH-sensitive manner and are inhibited by mutations in the EDII dimerization region and the EDII/EDI hinge region, demonstrating the binding of these MAbs to the EDII/EDI region (Vogt et al., 2009).

In addition to serotype-specific neutralizing antibodies, EDIII elicits serocomplex cross-reactive neutralizing antibodies. The complex-specific epitopes of DENV are localized to a similar overlapping region of EDIII: residues 306, 307, 308, 310, 311, 312, 330, 332, 364, 381, 387, 389, and 391 (Crill et al., 2009; Gromowski, Barrett, and

Barrett, 2008; Matsui et al., 2008; Rajamanonmani et al., 2009; Sukupolvi-Petty et al., 2007). Although these serocomplex cross-reactive sites cluster within the neutralization epitopes previously described, the critical binding residues are not identical (Matsui et al., 2008). Additionally, the serocomplex-specific MAbs have more variability in neutralization efficiency, and require a significantly higher occupancy level of available binding sites on the virion in order for neutralization to occur (Gromowski, Barrett, and Barrett, 2008).

Domain C: While a detailed mapping study of antigenic epitopes in antigenic domain C (EDI) has yet to be accomplished, several studies suggest the presence of serotype-specific or subtype-specific neutralizing antibodies. Neutralization escape mutants of YFV type-specific murine MAbs, led to the identification of important neutralization residues at residues 155 and 158 in EDI (Ryman et al., 1997) acting as part of a conformational epitope in conjunction with amino acid 71 in EDII (Daffis et al., 2005). Similar neutralization escape mutants of DENV-4 identified residues 174 and 176 in EDI as important serotype-specific neutralizing epitopes (Lai et al., 2007).

Dengue virus vaccines

The current means for DENV disease prevention are education and vector control. Despite over 70 years of effort, a licensed DENV vaccine has been unobtainable, unlike other flaviviruses such as YFV, JEV, and TBEV. DENV vaccine research suffers several obstacles in the successful development of a licensed vaccine. A main obstacle in vaccine development is the lack of an animal model that reproduces human disease.

Among the most widely used animal models in preclinical DENV vaccine studies includes the use of mouse models. Human isolates do not replicate well, or cause

pathology in immune competent mice; therefore, different immune-compromised mouse models have been developed. Humanized mice have been developed by irradiated immune-compromised newborn mice and reconstituting the immune system with a xenograft of human CD34⁺ hematopoietic stem cells. Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, reconstituted with CD34 engraftment, develop viremia, fever, rash, thrombocytopenia, and elevated liver enzymes following infection with human DENV isolates; however, few mice developed anti-DENV antibodies (Bente et al., 2005). NOD/SCID/IL2R γ ^{null} mice, which are NOD/SCID mice lacking the IL-2 receptor gamma chain, also develop viremia, rash, fever, and thrombocytopenia following infection, in addition to low levels of DENV IgG (Mota and Rico-Hesse, 2009). RAG2^{-/-} γ c^{-/-} mice do not produce B, T, or NK cells. After engraftment, they have a relatively longer life than other humanized mice. Following DENV infection, they develop viremia, fever and DENV specific human IgM and IgG (Kuruvilla et al., 2007). Though humanized mice are advantageous by developing a human antibody response to DENV infection, the high cost and labor-intensive procedure has limited the extensive use of this model.

Interferon-receptor deficient mice (AG129) can permit for the replication of all four DENV serotypes. Though the tissue tropism of DENV in these mice is similar to that of human infections, infection with DENV-2 typically induces a paralytic phenotype (Johnson and Roehrig, 1999) and mouse adaptation is required to induce a vicerotropic infection (Shresta et al., 2006). The phenomenon of ADE was also observed in AG129 mice through passive transfer of either immune sera or MAbs followed by infection with mouse adapted DENV-2 (Balsitis et al., 2010). This model is attractive due to the tissue

tropism and ability of DENV infection to induce a disease similar to that of humans, in addition to these mice being commercially available. However, this model is limited because infection does not induce the full spectrum of immune responses since these mice lack interferon receptors. Additionally, DENV-2 infection only results in a disease characteristic of humans after viral adaptation. Similar animal models in less immunocompromised hosts would be more desirable.

The use of non-human primates (NHP) for preclinical research has also been widely used. NHP can be infected with human clinical isolates and allow for viral replication without the need of adaptation; however, NHP do not develop overt disease following infection that resembles DF or DHF/DSS (Halstead, Shotwell, and Casals, 1973a; Halstead, Shotwell, and Casals, 1973b). NHP have been utilized to evaluate live attenuated vaccine candidates, however, they have not been able to predict the immunogenicity and reactogenicity of live attenuated vaccines in humans. Additionally, this vaccine model is hindered by high costs and limited availability to the research community.

In addition to the lack of a DENV animal model, obstacles include the need for development of separate vaccines for each of the four DENV serotypes and the risk of vaccine induced severe disease upon subsequent natural infection if neutralizing antibody titers to one of the four serotypes should decrease over time. These concerns have led to the developmental philosophy that DENV vaccines should induce long-lasting, protective immunity against all four serotypes (Whitehead et al., 2007). Despite these difficulties, DENV vaccine development has made considerable advances in recent decades, with two live attenuated candidates currently in Phase II clinical trials, several live candidates in

Phase I clinical trials, and many subunit, vectored, and DNA vaccine candidates in preclinical development (Durbin and Whitehead, 2010).

Live attenuated vaccines: Live attenuated vaccines are among the most rigorously pursued methods for DENV vaccination. Biologically-derived live attenuated vaccines were the earliest candidates for live attenuated DENV vaccines during World War II (Hotta, 1952; Sabin, 1952a). The intracerebral serial passage of DENV-1 or DENV-2 in mouse brain resulted in a mouse adapted DENV strain with reduced pathogenicity in humans. Sabin observed that after the seventh mouse passage, DENV-1 vaccine provided protection from exposure to DENV-1 infected *Aedes aegypti* mosquitoes (Sabin, 1952a). The vaccination with mouse adapted DENV resulted in mild DENV symptoms usually accompanied by rash, but systemic symptoms were absent or negligible. Hotta concurrently demonstrated mouse-brain passaged DENV-1 lost human pathogenicity, induced neutralizing antibody titers, and protected volunteers from highly viremic DENV infected human serum (Hotta, 1952). Additionally, vaccinated volunteers remained well during the Osaka epidemic of 1945.

In the 1980s the Center for Vaccine Development at Mahidol University in Bangkok, Thailand and the Walter Reed Army Institute of Research (WRAIR) in Washington, DC began the development of live attenuated DENV vaccines implementing tissue-culture derived live vaccines. The original viruses were isolated from DENV infected patients and serially passaged in primary dog kidney (PDK) cells or primary green monkey kidney (PGMK) cells. Passage of DENV-2 in PDK cell culture leads to accumulations of mutations associated with an attenuated phenotype that have been shown to occur in the 5' NCR, NS1 and NS3 genes (Butrapet et al., 2000). The Mahidol

live attenuated vaccine candidates were tested as monovalent, bivalent, trivalent and tetravalent formulations in adult flavivirus-naïve Thai and American volunteers (Bhamarapavati and Sutee, 2000; Bhamarapavati and Yoksan, 1989; Bhamarapavati et al., 1987). These vaccines were found to be generally safe, with fever, rash and elevated liver enzymes being the most common side effects. The vaccines elicited seroconversion rates between 90 and 100% when tested as mono, bi, or trivalent formulations. The seed viruses were licensed to Sanofi Pasteur for further clinical tetravalent evaluations. In tetravalent formulations, there was increased reactogenicity when compared to monovalent formulations (Kanasa-thasan et al., 2001). In addition, DENV-3 was preferentially replicated and induced the highest neutralization titers. To avoid interference from DENV-3 replication, different dose formulations were evaluated, as well as a two-dose regimen (Sabchareon et al., 2002). The first dose was most reactogenic, producing fever, headache, myalgia, eye pain or rash, with 71% tetravalent seroconversion after two-doses. Tetravalent formulas in a three-dose regimen were tested in school age Thai children (Sabchareon et al., 2004). After one dose fever, headache, rash, and elevated liver enzymes were reported. These manifestations were reduced after second and third-dosages. After three-doses 89-100% of the children developed tetravalent seroconversion. A final Phase 1b trial of two different formulations was conducted on Australian adults; however, the trial was stopped by the sponsor after only one dose due to reactogenicity and problems with formulations related to DENV-3 interference (Kitchener et al., 2006).

WRAIR has developed several live attenuated DENV vaccines through serial PDK passage. Despite attenuation characteristics in preclinical studies, several of these

candidates were found to be unacceptably reactogenic in human trials, or over attenuated and non-immunogenic and therefore no longer pursued (Bancroft et al., 1984; Bancroft et al., 1981; Eckels et al., 1984; Innis et al., 1988; McKee et al., 1987; Scott et al., 1983). Increasing PDK cell passage of monovalent DENV vaccine candidates resulted in increased attenuation for volunteers, yet decreased immunogenicity, leading to the unavoidable difficulty of selection of appropriate attenuated serotype viruses for tetravalent formulations (Kanesa-Thanan et al., 2003). Initial tetravalent studies confirmed appropriate reactogenicity in volunteers, as well as increased seroconversion and neutralization titers following three doses (Sun et al., 2003). The selected monovalent vaccines were tested in 16 formula combinations including low and high dose formulations (Edelman et al., 2003). All formulations were considered safe with variable reactogenicity after the first-dose and nearly non-reactogenicity following the second and third doses. The most common side effects were low-grade fever, rash, and temporary leukopenia. Reactogenicity was positively correlated with immunogenicity. The similar serotype specific seroconversion rate between monovalent and tetravalent formulations suggested a lack of heterologous interference between serotypes. A Phase I trial evaluated the safety and immunogenicity of this vaccine in seven Thai children aged six to nine years old (Simasathien et al., 2008). A two-dose regimen six months apart was implemented. The vaccine was well tolerated with only one volunteer experiencing fever. The vaccine, however, was poorly immunogenic after one-dose, with five of six volunteers developing tetravalent seroconversion following two-doses. Phase 2 trial of the PDK vaccines was successful in decreasing the reactogenicity by increasing DENV-1 passage, and increasing DENV-4 immunogenicity by decreasing PDK passage, resulting

in a formulation selected for further clinical evaluation (Sun et al., 2008). This vaccine has been licensed by GlaxoSmithKline and is currently in Phase 2 clinical trials.

Scientists at the National Institutes of Health engineered specific mutations in the 3' NCR that resulted in an attenuated phenotype. The 3' NCR contains nucleotide sequence predicted to form secondary structures essential in flavivirus replication; however, this region is not well conserved. Upstream of the secondary structure is a highly conserved sequence that was the target of attenuation studies. Deletion mutations into this region of the 3' NCR of DENV-4 cDNA clones resulted in a range of attenuated phenotypes (Men et al., 1996). These deletion mutants were restricted in growth in LLC-MK2 cells, produced small plaques on C6/36 cells, and were immunogenic in monkeys without producing a viremia. Of particular interest was the deletion mutation from nucleotides 172-143 from the 3' end, later referred to as $\Delta 30$. The DENV-4 $\Delta 30$ vaccine performed well in human volunteers eliciting neutralizing antibodies and low reactogenicity (Durbin et al., 2001; Durbin et al., 2005). A single-dose of vaccine resulted in 95-100% seroconversion. The most common side effect was rash; however no recipient developed systemic illness or dengue-like symptoms. In the wake of success from the DENV-4 $\Delta 30$ vaccine, the $\Delta 30$ mutation was engineered into the remaining DENV serotypes. DENV-1 $\Delta 30$ resulted in an attenuated vaccine similar to DENV-4 $\Delta 30$ (Whitehead et al., 2003). DENV-2 $\Delta 30$ resulted in only moderate attenuation (Blaney et al., 2004b), while DENV-3 $\Delta 30$ failed to attenuate the virus and resulted in viremia levels similar to wild-type DENV-3 (Blaney et al., 2004a). Chimeric DENV-3/DENV-4 $\Delta 30$ and DENV-2/DENV-4 $\Delta 30$ vaccines, replacing the prM and E genes of DENV-4 with DENV-3 or DENV-2, were developed (Blaney et al., 2004a; Durbin et al., 2006).

Chimeric DENV-3/DENV-4 Δ 30 vaccine displayed attenuated phenotype in cell culture and elicited DENV-3 neutralizing antibody in monkeys. Chimeric DENV-2/DENV-4 Δ 30 was well tolerated in dengue naïve adults and resulted in high DENV-2 neutralizing antibody titers for the entire study (Durbin et al., 2006). Transient rash was the most common side effect, and RNA recovered from blood revealed the Δ 30 mutation was stable. Further attenuation studies of DENV-3 have revealed two deletions in the 3' UTR of DENV-3, or replacement of DENV-3 3' UTR with DENV-4 3' UTR that result in infection without detectable viremia and protect monkeys from viral challenge (Blaney et al., 2008). Phase 1 evaluation of these new candidates has been initiated.

Similar to the chimeric strategy described above, live attenuated tetravalent chimeric DENV vaccine has been developed utilizing YFV 17D as a genetic backbone. Four DENV vaccines were created where the prM and E proteins of YFV were replaced by those of DENV 1-4 (ChimeriVax-DENV1-4) (Guirakhoo et al., 2001; Guirakhoo et al., 2000). Preclinical trials in non-human primates demonstrate that ChimeriVax induces low level, detectable viremia in YFV immune and non-immune monkeys when compared to monkeys given wild-type DENV. Neutralizing antibodies to all four DENV serotypes were demonstrated in post immunization sera; however, DENV-2 appeared to be immunodominant (Guirakhoo et al., 2001). Lowering the dose of ChimeriVax DENV-2 in the tetravalent formulation resulted in a more balanced neutralizing antibody response to DENV-1, -2, and -3 with modestly higher titers to DENV-4 (Guirakhoo et al., 2002). Testing four dose formulations of ChimeriVax DENV1-4 in a non-human primate challenge resulted in nearly complete protection from viremia and displayed decreased neurovirulence when compared to YF-Vax (Guirakhoo et al., 2004). Phase I clinical

trials of ChimeriVax DENV-2 in flavivirus naïve and YFV immune volunteers resulted in mild side effects, similar to that of YF-Vax (Guirakhoo et al., 2006). All volunteers vaccinated with high dose ChimeriVax DENV-2 seroconverted to DENV-2, and no volunteers seroconverted to DENV-1, -3, -4, or YFV. In addition, YFV immunity did not interfere with ChimeriVax DENV-2 immunization and increased heterologous DENV seroconversion. In an analysis of potential replicative or immune interference between the four serotypes, it was demonstrated DENV-1 and DENV-4 were dominant in neutralizing antibody titers regardless of dose or route of administration (Guy et al., 2009). When tetravalent ChimeriVax was given to non-human primates or adult volunteers, a longer time span between primary immunization and boost increased the seroconversion rates, suggesting interference could be prevented with a longer dose schedule (Morrison et al., 2010). ChimeriVax, licensed by Sanofi Pasteur, is currently being evaluated in a three-dose format in Phase 2 studies in DENV endemic regions.

A third chimeric method includes the use of the DENV-2 PDK-53 virus as the genetic backbone, which had a low infectious dose, was strongly immunogenic, and produced no clinical symptoms in humans. The attenuation of the DENV-2 PDK-53 vaccine has been attributed to substitutions in the 5' NCR, NS1 and NS3 genes (Butrapet et al., 2000). Chimeric DENV-2 PDK-53/DENV-1, where the prM and E genes of DENV-1 replaced those of DENV-2 PDK-53, elicited higher DENV-1 neutralizing antibody titers in mice when compared to the DENV-1 PDK-13 vaccine (Huang et al., 2000a). In a tetravalent formulation of DENV-2 PDK-53 and chimeric DENV-2 PDK-53/DENV-1, -3, and -4, immunization of mice resulted in neutralizing antibody titers to all four DENV serotypes with DENV-2 as the dominant response and DENV-4 as the

lowest response (Huang et al., 2003). In addition, vaccination of monovalent chimeric DENV-2 PDK-53/DENV-1 protected 100% of mice in an AG129 challenge model. Further preclinical evaluation of the tetravalent vaccine in non-human primates is completed. The monovalent vaccines are currently undergoing Phase I clinical trials in flavivirus naïve adults.

Live attenuated DENV vaccines have by far been the most rigorously pursued vaccine format, including several vaccines in human clinical trials. In general, live replicating vaccine viruses have been shown to induce high levels of protective antibodies and also induce protective cytotoxic T lymphocytes, making these vaccines highly desirable. However, plausible risks of vaccination with live attenuated viruses hold high concerns. Though these vaccine viruses have been attenuated, biologically or engineered, to replicate at lower efficiency than the parental strains, these vaccines are not appropriate to administer to immune compromised individuals. In addition, replicative multivalent vaccines suffer from replicative and/or immune interference (Guy et al., 2009). Vaccine interference hinders the ability of multivalent vaccines to induce a balanced immune response, which is thought to be detrimental in DENV vaccination as there is a threat of vaccine-induced ADE. Biologically derived live attenuated viruses are also at the risk of under attenuation, or reversion back to wild-type virulence, as seen with oral poliovirus vaccine (Minor, 2009). Caution is also warranted in the use of chimeric live attenuated viruses as a risk of inter- and intraspecies recombination exists. Evidence of interspecies recombination of flaviviruses in nature has been controversial (Baillie et al., 2008; Twiddy and Holmes, 2003). However, evidence of intraspecies recombination in DENV has been documented (Aaskov et al., 2007) where a single

patient harbored two genotypes of DENV-1 as well as a DENV-1 strain with inter-genomic recombination events between the two genotypes. Although many researchers believe the benefits of DENV vaccination outweigh the theoretical risks of recombination (Monath et al., 2005), the risk of intraspecies recombination in DENV is still a concern that should not be overlooked.

Whole virus inactivated vaccines: Whole virus inactivated vaccines have perceived advantages over live attenuated vaccines. These vaccines cannot go through reversion mutations to attain the virulent phenotype, they do not replicate and therefore cannot interfere with other strains in a tetravalent format, and they can be administered to immune compromised individuals. However, these vaccine formats are hindered by lower immunogenicity than their live attenuated counterparts and therefore require more vaccinations to reach protective levels, and require booster immunizations since neutralizing antibody titers wane over time.

A purified, inactivated (PIV) DENV-2 vaccine was developed by WRAIR in 1996 (Putnak et al., 1996a; Putnak et al., 1996b). Sucrose gradient purified virus was inactivated with formalin and tested for protective efficacy in mice or macaques. Vaccinated mice and macaques developed high titers of DENV-2 neutralizing antibody and were partially protected from viral challenge. In macaques, the neutralizing antibody response did not remain stable over a prolonged period, regardless of adjuvant used (Putnak et al., 2005) suggesting higher doses of vaccine or additional boosting may be required to maintain protective antibody levels.

Recombinant subunit protein vaccines: In addition to whole virus inactivated vaccines, recombinant subunit protein vaccines have also been investigated as an

alternative to live attenuated vaccines. Similar to whole virus inactivated vaccines, subunit protein vaccines are also hindered by lower immunogenicity and required administration with an adjuvant, few of which have been approved for human use.

Recombinant DENV proteins can be expressed in a variety of systems including, baculovirus, *Escherichia coli*, yeast, vaccinia virus, drosophila cells and mammalian cells and then purified for use as subunit vaccines. As described earlier, the flavivirus E protein contains the major antigenic and protective epitopes; however, proper folding of E ensures antigenic stability and requires the coexpression of prM (Allison et al., 1995b). Full-length E without prM is not secreted and fails to induce neutralizing antibodies in mice (Fonseca et al., 1994). In addition, the coexpression of prM/E can induce the formation of flavivirus-like particles (VLP) (Sugrue et al., 1997). Flavivirus VLPs are immunogenic and elicit neutralizing and protective antibodies in mice (Hunt, Cropp, and Chang, 2001; Konishi and Fujii, 2002; Konishi et al., 1992).

The deletion of the C terminal 20% of the E gene resulted in increased extracellular secretion while maintaining antigenicity and inducing protective antibodies in mice (Deubel et al., 1991; Men, Bray, and Lai, 1991). Recombinant subunit vaccine of 80% DENV-2 E and prM (r80), expressed in *Drosophila* cells, was tested using four different adjuvants in macaques (Putnak et al., 2005). Regardless of adjuvant used, these vaccines elicited high levels of DENV neutralizing antibodies; however, after viral challenge there was evidence of viral replication by qRT-PCR analysis and virus isolation, indicating there was not sterilizing immunity. In a tetravalent formulation in macaques, 5µg of each serotype DENV r80 soluble E elicited serotype specific neutralizing titers, with DENV-2 as the predominant response and DENV-1 and DENV-4

with lowest responses (Clements et al., 2010). Low doses of tetravalent r80 resulted in detectable viremia in only one-of-four animals following viral challenge.

An alternative subunit protein vaccine method is the use of recombinant EDIII, expressed mainly from *E. coli* or yeast. Early attempts at EDIII vaccination involved fusing recombinant EDIII to various proteins. Fusion of tetravalent EDIII with trpE from *E. coli* elicited antibodies binding EDIII that were non-neutralizing (Fonseca et al., 1991). Fusing DENV-2 EDIII to staphylococcal protein A resulted in increased levels of DENV-2 neutralizing antibody that protected against DENV-2 challenge; however, staphylococcal protein A has high affinity for human IgG and unsuitable for vaccination (Srivastava et al., 1995). Alternatively, fusion of DENV EDIII with maltose binding protein (MBP) from *E. coli* induces neutralizing antibodies and protection upon challenge (Simmons et al., 1998). In a tetravalent formulation, DENV-MBP vaccine elicited equivalently high titers of DENV neutralizing antibodies for DENV-1, -2, and -3, and significantly lower levels for DENV-4 in mice (Simmons, Murphy, and Hayes, 2001). The carrier capacity and safety of MBP has not been evaluated for use in humans. P64K protein from *Neisseria meningitidis* has also been evaluated as a fusion carrier for EDIII vaccines. DENV-2 EDIII expressed from *E. coli* fused with P64K maintained antigenicity based upon reactivity with MAbs, elicited DENV-2 neutralizing antibodies, and partially protected mice from challenge (Hermida et al., 2004b). Immunogenicity of monovalent DENV-1, -3, and -4 EDIII fused with P64K have each been confirmed in mice (Hermida et al., 2004a; Lazo et al., 2008; Zulueta et al., 2006). DENV-2 EDIII fused to P64K administered with Freund's adjuvant was found to elicit neutralizing antibodies and protected macaques from viremia (Hermida et al., 2006), and a recent

study found polysaccharide A of *N. meningitidis* was a suitable adjuvant eliciting neutralizing antibodies and partially protecting macaques from challenge (Valdes et al., 2008).

Several studies have explored the possibility of vaccination with recombinant EDIII without a fusion protein. Using *E. coli* expressed DENV EDIII without a fusion partner (Jaiswal, Khanna, and Swaminathan, 2004), vaccination with bivalent DENV-2 and DENV-4 EDIII linked by a flexible amino acid linker region maintained antigenic properties of the respective monovalent EDIII vaccines, and elicited neutralizing antibodies to both DENV-2 and DENV-4 (Khanam et al., 2006). In a tetravalent formulation, DENV-1-4 EDIII expressed from yeast elicited neutralizing antibody titers in mice to all four serotypes, with DENV-4 the predominant response (Etemad et al., 2008). In addition, T cells from vaccinated mice proliferated and secreted IFN γ and IL-4 in response to all four serotypes.

An alternative approach for EDIII vaccination was investigated using DENV consensus sequences. *E. coli*-expressed recombinant EDIII was developed using DENV1-4 consensus sequences (cEDIII) (Leng et al., 2008). When vaccinated with cEDIII, mice developed neutralizing titers to all four DENV. Titers ranged from 1:128 to 1:8 in a 40% plaque reduction assay. DENV-4 elicited the lowest level of neutralizing antibodies, related to DENV-4 having the lowest similarity to the cEDIII consensus sequence. Further optimization of the consensus EDIII is needed to increase and equilibrate neutralizing antibody titers.

Virus-vectored vaccines: Recombinant poxviruses and adenoviruses that express antigens from heterologous viruses have been shown to induce humoral and

cellular immune responses against several human pathogens. These viruses infect cells and express the antigen of interest *de novo* within the cell. The genes are translated, leading to induction of an MHC I restricted immune response, and the antigens are processed and glycosylated naturally. This vaccine format is advantageous due to the live nature of the vaccine; however, preexisting immunity to the vector can hinder vaccine efficacy. Although a plethora of possible adenovirus vectors exists, continually tailoring the vaccine vector to the individual's immune status is costly and inefficient.

Early attempts at developing a vaccinia-vectored DENV vaccine elicited variable results. Using prM/E/NS1/NS2a expression, vaccinia-vectored DENV-4 failed to induce an anti-E immune response in cotton rats (Zhao et al., 1987) and E protein accumulated intracellularly. However, when vaccinia-vectored DENV-4 full-length structural, or full-length E protein were used to vaccinate mice, all mice survived intra cerebral challenge despite low level detection of DENV-4 antibodies to the structural proteins (Bray et al., 1989). Although all mice survived challenge, mice vaccinated with full-length E did display encephalitic disease symptoms. Interestingly, when the virion capsid, membrane and envelope structural protein genes of DENV-2 were cloned into a vaccinia-vector, DENV-2 antibody could not be detected in vaccinated mice, hamsters or monkeys (Deubel et al., 1988). In addition, vaccinated monkeys developed levels of viremia after challenge similar to the control animals. To improve the immunogenicity of vaccinia-vectored DENV vaccines, the 20% C terminal region was deleted from the E protein of DENV-2 and DENV-4 (Men et al., 2000). DENV E was detected in extracellular fractions of infected cell cultures; in addition, these vaccines elicited neutralizing

antibody titers in mice. When macaques were vaccinated with three doses, all monkeys were protected from challenge as shown by the absence of viremia (Men et al., 2000).

Replication defective adenovirus-based vectors have also been studied for DENV vaccination. Insertion of the DENV-2 E ectodomain coding region into the E1 early region of the adenovirus genome resulted in secretion of E from virus infected cells, elicited DENV-2 neutralizing antibodies following a booster immunization comparable to inactivated DENV-2 vaccine, and induced IFN γ secreting CD4 T cells (Jaiswal, Khanna, and Swaminathan, 2003). Using two complex adenovirus vectors, expressing the prM/E of DENV-1 and DENV-2 (cAdVaxD1-2) or DENV-3 and DENV-4 (cAdVaxD3-4), a bivalent DENV vaccine was administered to mice (Holman et al., 2006). These mice developed DENV cross-reactive antibodies as well as DENV serotype-specific neutralizing antibodies, in addition to IFN γ secreting CD4 T cells. To test this two-vaccine system in a tetravalent format (cAdVax-DenTV), macaques were vaccinated with adenovirus-vectors, cAdVaxD1-2 and cAdVaxD3-4, by combining the two bivalent vaccine constructs (Raviprakash et al., 2008). Vaccinated macaques exhibited neutralizing antibody titers ranging from 200 (DENV-2) to 937 (DENV-3) following a booster vaccination. All animals were protected from viremia from DENV-1 and DENV-3; however, viremia was detected in one animal challenged with DENV-2 and all animals challenged with DENV-4. Interestingly, both DENV-2 and DENV-4 prM/E were introduced into the E4 region of the adenovirus vector of their respective construct. Further investigation and optimization is warranted.

In addition to full-length E protein, viral vectors have been investigated to express EDIII. Expression of DENV-2 EDIII in recombinant adenovirus resulted in induction of

DENV-2 specific neutralizing antibodies in mice using either an adenovirus-DENV-2 prime/plasmid expressing DENV-2 EDIII boost, or a plasmid prime/adenovirus boost (Khanam, Khanna, and Swaminathan, 2006). This method elicited both Th1 and Th2 T cells, with a predominant Th1 response. Alternatively, the pediatric measles vaccine was engineered to express EDIII of DENV-1 fused with the ectodomain of M (Brandler et al., 2007). The immunization of mice susceptible to measles virus resulted in the production of DENV-1 neutralizing antibodies. In addition, the presence of ectoM in the vaccine acted as an adjuvant and stimulated the maturation of dendritic cells and the secretion of cytokines when compared to a vaccine that does not contain ectoM. The possibility of a single vaccination against both DENV and measles virus makes this an interesting strategy. Additionally, as with adenovirus vectors, the effect of previous immunity to measles virus on vaccine immunogenicity should be evaluated.

DNA vaccines: DNA vaccines represent a novel means of expressing antigens *in vivo* for generation of humoral and cellular immunity. The DNA vaccines typically consist of a bacterial plasmid with a strong promoter, gene of interest, and polyadenylation/termination sequence. The plasmids do not contain an origin of replication functional in eukaryotic cells, therefore plasmids cannot replicate in mammalian cells. Common safety concerns of DNA vaccines include the potential integration of plasmid DNA into the host chromosome and the production of autoimmune anti-DNA antibodies. However, studies have shown no demonstrable evidence of integration of plasmid into the host genome and no production of anti-DNA antibodies (Arrode-Bruses et al., 2010; Nichols et al., 1995). DNA is advantageous as a vaccine

platform because it is stable for long periods of time, is resistant to temperature extremes, is less reactogenic and can be administered to immune compromised individuals.

Using DNA as a DENV vaccine platform was first investigated by the Naval Medical Research Institute in Bethesda, Maryland. Using a eukaryotic expression plasmid, the prM and 92% E of DENV-2 were shown to be expressed in tissue culture with proper size and folding when compared to naturally infected DENV-2 cell cultures (Kochel et al., 1997). When Balb/C mice received three-doses, the DNA elicited DENV-2 neutralizing antibodies in all mice vaccinated 22 weeks post vaccination. However, these vaccinated mice were not protected from challenge at 6 weeks of age, likely due to the lack of a neutralizing antibody response 3 weeks post vaccination. When mice received two vaccinations before challenge at 6 weeks of age, 60% of mice survived intracerebral inoculation (Porter et al., 1998). These mice, however, still had lower neutralizing antibody titers prior to challenge compared to neutralizing titers from mice that received three-doses of DNA. When DNA expressing DENV-1 prM and full length E were inoculated into *Aotus* monkeys and rhesus macaques, all monkeys developed DENV-1 neutralizing antibody titers following two boost vaccinations and most were completely protected from viremia upon viral challenge. Monkeys that developed viremia had a shorter duration than the control monkeys (Kochel et al., 2000; Raviprakash et al., 2000).

Konishi and colleagues also developed a DENV DNA vaccine consisting of DENV-2 prM/E. When mice were inoculated with 100 µg of plasmid, all mice developed DENV-2 neutralizing antibodies, and titers rapidly increased following viral challenge, indicating that the mice developed an anamnestic antibody responses (Konishi

et al., 2000). When evaluated in a tetravalent formula containing 25 µg of each DENV plasmid, mice developed neutralizing antibodies to all four DENV serotypes and an anamnestic response to challenge (Konishi, Kosugi, and Imoto, 2006). In addition, neutralizing antibody titers prior to challenge were similar in magnitude to neutralizing antibody titers elicited from the monovalent vaccines, indicating the tetravalent DNA vaccine is not impeded by interference as is common with live attenuated vaccines.

Chang and colleagues developed DENV-2/JEV chimeric prM/E DNA vaccines to increase the biosynthesis and secretion of DENV-2 VLP. Plasmids expressing DENV-2 prM/E were constructed containing 10% or 20% C of the JEV E C terminus compared to 100% DENV-2 E to identify possible retention sequences in DENV-2 E (Chang et al., 2003). All three plasmids caused release of DENV-2 VLP into tissue culture media; however, the plasmid containing 80% DENV-2/20% JEV E secreted significantly higher levels of VLPs, suggesting an unidentified retention sequence in the DENV-2 E between amino acids 397 and 436. These VLPs were determined by MAb reactivity to express authentic DENV-2 E protein epitopes, suggesting that the JEV E C terminal 20% had no effect on antigenicity. When mice were vaccinated with two doses of 100 µg of plasmids, neutralizing antibody titers correlated with the ability of plasmids to secrete VLPs into tissue culture medium. In addition, 11 out of 12 pups from mothers vaccinated with plasmid containing 80% DENV-2/20% JEV E survived intracranial DENV-2 challenge while no pups from unvaccinated mothers survived.

Several groups have investigated methods to increase the immunogenicity of DNA vaccines. When DENV-2 plasmid expressing prM/E was coadministered with recombinant DENV-2 EDIII (Simmons et al., 2001) or with DENV-2 VLP (Konishi,

Terazawa, and Imoto, 2003), immunized mice developed significantly higher neutralizing antibody titers than when vaccinated with DNA or protein alone. A similar trend was also observed in vaccinated macaques (Simmons et al., 2006). Incorporation of bacterial CpG motifs into the DNA plasmid, or incorporation of the GM-CSF gene into the plasmid has also been shown to increase the immunogenicity of DENV-1 prM/E DNA vaccines in *Aotus* monkeys (Raviprakash et al., 2003). The antibody titers elicited by these vaccines were increased and 87% of challenged monkeys were completely protected from viremia compared to 33% who received the prM/E plasmid alone. Methods to increase the immunogenicity of DNA vaccines for non-DENV pathogens include the simultaneous gene expression of interleukins or other cytokines.

Summary

Dengue is the most common arthropod-borne viral disease of humans and the virus exists as four closely related serotypes. Dengue is a threat to global public health as it continues its rapid spread throughout the tropics and subtropics, leaving an estimated 3.6 billion people at risk for dengue virus infection (PDVI, 2009). Dengue disease presents as a wide range of clinical symptoms from subclinical, to the classic febrile disease, dengue fever, to the most severe manifestation and life threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Current treatments of severe manifestations of dengue disease include maintaining circulatory volumes and electrolyte balances. This form of dengue is mainly seen in children and is the leading cause of pediatric hospitalizations in endemic countries (Hombach, 2007). To control dengue virus disease, emphasis should be placed on prevention, including vector control and education as there is no licensed dengue virus vaccine.

Dengue vaccine development has been hindered by the complexity of disease pathogenesis. Epidemiological evidence associates severe dengue disease with secondary infections (Guzman et al., 1990; Sangkawibha et al., 1984). Infection with any one dengue serotype induces life-long protection, though heterologous protection from the other serotypes lasts only months (Sabin, 1952b). Though the mechanism of severe dengue disease is not fully understood, the pathogenesis is complex and most likely multifactorial. One hypothesis to describe DHF is antibody-dependent enhancement (ADE) (Halstead, Chow, and Marchette, 1973), which occurs when cross-reactive weakly-neutralizing antibodies bind to viruses and form antibody-virus complexes that enhance infection of Fc γ receptor bearing cells, resulting in an increase in virus load associated with severe disease (Wang et al., 2003). The virion envelope (E) glycoprotein is responsible for eliciting the majority of the protective antibody response. Recent studies suggest broadly cross-reactive, weakly-neutralizing antibodies directed against the fusion peptide (FP) form a major proportion of the antibody response in DENV patients (Crill et al., 2009; Lai et al., 2008; Stiasny et al., 2006; Throsby et al., 2006). The dominant production of high avidity antibodies could compete with neutralizing antibodies recognizing overlapping epitopes through steric interference (Ndifon, Wingreen, and Levin, 2009) and promote severe disease through ADE. For these reasons, DENV vaccines must induce a balanced protective immunity to all four serotypes to reduce the possibility for cross-reactive antibody induced severe disease upon subsequent infection.

Current DENV vaccines in clinical development include live-attenuated vaccines. Live-replicating vaccines are susceptible to replicative and/or immune interference

between different virus serotypes. The current solution to this problem has been to utilize a two- or three-dose vaccination schedule with an extended resting time period between primary immunization and boost. The replicative and/or immune interference associated with these vaccines has resulted in a large proportion of non-responders following one- and two-doses (Morrison et al., 2010; Sun et al., 2008) with 100% tetravalent seroconversion requiring three doses spanning a year (Morrison et al., 2010). The time required for full seroconversion spans the epidemic season, leaving a significant portion of vaccinated individuals without tetravalent protective immunity who are at risk for vaccine-induced severe disease following natural infection. In order to control DENV human disease, new vaccine formats need to be investigated, taking into account the need for a balanced immune response and the importance of the cross-reactive antibody response in potentiating severe DHF through ADE.

Chapter 2

Humoral Immune Responses of Dengue Patients using Epitope-Specific Serotype-2

Virus-like Particle Antigens

Introduction

Dengue virus (DENV) causes the quintessential 21st century re-emerging infectious disease. Advances in post exposure treatment, epidemiological understanding, and vector control did much to reduce dengue disease burden in the post WWII era. However, in the last three decades, DENV has spread epidemically dramatically increasing in disease severity and range, with overlapping co-circulation of the four DENV serotypes spreading into geographic regions containing other pathogenic flaviviruses (Gubler, 2006; Mackenzie, Gubler, and Petersen, 2004; Solomon and Mallewa, 2001). Approximately 40% of the world's population, over 2.5 billion people, live at risk of infection in DENV-endemic areas, resulting in an estimated 50-100 million infections yearly (Farrar et al., 2007; Normile, 2007). Significant effort and resources have been applied toward DENV vaccine development over the last 30 years, yet in spite of promising vaccine candidates in development and/or early-phase trials, a safe and efficacious vaccine appears to still be years away (Normile, 2007; Whitehead et al., 2007).

DENV consist of four closely related viral serotypes (DENV-1, -2, -3, and -4) and as with the other flaviviruses, infection with any single virus appears to provide life-long immunity, however cross-protection to other DENV serotypes is limited and transient

(Calisher et al., 1989; Kuno et al., 1998; Sabin, 1952b; Tesh et al., 2002). Human infections with DENV result in a range from asymptomatic to acute self-limiting febrile illness known as dengue fever (DF) or with increasing frequency, a life-threatening hemorrhagic fever and circulatory shock known as dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Gubler, 2006). The DENV genome is a positive-sense single-stranded RNA molecule, approximately 11 kb in length. It is translated into a single polyprotein encompassing three structural proteins, capsid (C), premembrane/membrane (prM/M) and envelope (E) proteins, and seven non-structural proteins (Lindenbach and Rice, 2003). Mature virions contain an ER derived lipid bilayer covered with a dense lattice of membrane-bound prM/M and E proteins, organized into dimers on its surface (Kuhn et al., 2002). The E protein elicits the majority of the protective immune response and contains a highly conserved internal fusion peptide and the cellular receptor-binding motifs, both essential for viral infectivity via receptor-mediated endocytosis (Allison et al., 2001; Kuhn et al., 2002; Lindenbach and Rice, 2003; Pokidysheva et al., 2006). DENV and all flavivirus E proteins contain three structural and functional domains (Modis et al., 2003; Modis et al., 2005; Rey et al., 1995). The epitope specificity and biological characteristics of antibody responses to the E protein are mostly known from murine MAb studies. E protein domain I (EDI) is the central domain and contains both virus-specific and cross-reactive, predominantly non-neutralizing epitopes; EDII, the dimerization domain, contains the internal fusion peptide that forms the epicenter of a series of overlapping immunodominant cross-reactive epitopes stimulating predominantly non- or weakly neutralizing antibodies; EDIII has an immunoglobulin-like fold, contains the primary cellular receptor-binding motifs and in

mice elicits serotype-specific, highly protective neutralizing antibodies and DENV complex cross-reactive antibodies (Chiou et al., 2008a; Crill and Chang, 2004; Crill and Roehrig, 2001; Gromowski and Barrett, 2007; Gromowski, Barrett, and Barrett, 2008; Roehrig, Bolin, and Kelly, 1998; Stiasny et al., 2006; Sukupolvi-Petty et al., 2007).

Infection with any DENV serotype thus produces a wide spectrum of anti-E immunoglobulins varying from broadly cross-reactive, recognizing all flaviviruses, to those recognizing only subsets of viruses in different serocomplexes, to those recognizing only DENV-complex viruses to DENV serotype-specific antibodies. The broadly cross-reactive antibodies stimulated from the overlapping immunodominant epitopes surrounding the EDII fusion peptide are predominantly non-neutralizing as evidenced by the general lack of cross-protection between the flaviviruses (Calisher et al., 1989; Kuno et al., 1998; Oliphant et al., 2007a; Sabin, 1952b; Stiasny et al., 2006; Tesh et al., 2002). DENV complex and sub-complex cross-reactive antibodies stimulated from overlapping epitopes in EDIII vary in their neutralizing capabilities (Gromowski, Barrett, and Barrett, 2008; Roehrig, Bolin, and Kelly, 1998; Sukupolvi-Petty et al., 2007) and these antibodies can be protective, as evidenced by occasionally documented transient cross-protection between DENV serotypes (Sabin, 1952b). Secondary infection with a heterologous DENV serotype stimulates a broadly cross-reactive anamnestic immune response, which although not efficiently cross-protective, can cause serious immune mediated disease. The presence of cross-reactive anti-E immunoglobulins in sera from recovered humans can create difficulty for serodiagnosis and especially for estimates of disease burden in regions with multiple co-circulating pathogenic flaviviruses. In clinical serodiagnosis detecting IgM, this cross-reactivity is most problematic with secondary infections due to

the difficulty of differentiating antibodies elicited by primary infections from those elicited in a secondary infection (Kuno, 2003; Martin et al., 2004; Roberson, Crill, and Chang, 2007a). For example, in Puerto Rico since 1997 all four DENV serotypes were co-circulating and the vast majority of patients were secondary DENV infections. With the arrival of West Nile virus (WNV) into the Caribbean and apparent DENV infections with neurological manifestations, clinical serodiagnosis has become especially complicated (CDC Dengue Branch pers com., (2008; Barrera et al., 2008). Serosurvey estimates of flavivirus disease burden are even more problematic due to cross-reactive IgG responses accumulated over a lifetime of multiple virus or vaccination exposures.

The presence of cross-reactive anti-E immunoglobulins may enhance the pathogenicity of flavivirus infections via the process of antibody-dependent enhancement (ADE)(Takada and Kawaoka, 2003). Although ADE has been demonstrated in vitro for encephalitic as well as hemorrhagic flaviviruses (Wallace et al., 2003) it is of greatest in vivo concern with DENV infections where the severe pathogenic manifestations dengue hemorrhagic fever and shock syndrome (DHF/DSS) are correlated with secondary infections and sub-neutralizing levels of heterologous anti-E immunoglobulins (Gubler, 2002a; Guzman et al., 2007; Halstead, 1989). Concern over ADE and its role in DHF/DSS has instilled a belief in the necessity for tetravalent DENV vaccines stimulating balanced immune responses across DENV serotypes to reduce this concern (Chang et al., 2004; Monath, 2007; Whitehead et al., 2007). However, multivalent DENV candidate vaccine trials have been severely hampered by difficulties in inducing balanced serotype-specific immune responses in vaccinees (Chang et al., 2004).

An important research agenda necessary to successfully address the DENV global public health challenge is to improve our understanding of humoral immune responses to DENV infection, specifically the E-protein epitope-recognition of the immunoglobulin response and the relative quantities of epitope-specific antibody populations after viral exposure and vaccination in humans, their infection neutralizing and/or enhancing capabilities of these immunoglobulin populations, and their role as correlates of protection in vaccine efficacy studies (Farrar et al., 2007; Goncalvez et al., 2007; Halstead, 1989; Hombach et al., 2007; Rigau-Perez, 2006; Senior, 2007; Sukupolvi-Petty et al., 2007). As an initial step toward this goal, in this report we developed a series of E-protein epitope-specific knock-out virus-like particles (VLPs) and use these antigens to measure humoral immune responses in sera from DENV-2 infected humans from Puerto Rico and Taiwan. We quantified immunoglobulin populations targeting four distinct E-protein antigenic regions: the most broadly cross-reactive EDII fusion peptide epitopes, EDIII serocomplex cross-reactive epitopes, epitopes incorporating determinants from either or both of these domain regions, and EDIII DENV-2 type-specific epitopes. Humoral immune responses in DENV-2 infected patient sera were highly variable between individual sera and immunoglobulin class. Immunoglobulin populations recognizing dominant cross reactive epitopes centered on the EDII fusion peptide were large and highly variable, averaging approximately 50% and 30% in primary and secondary DENV-2 infected sera respectively. Immunoglobulin recognizing DENV complex cross-reactive epitopes in EDIII exhibited similar trends but the magnitudes were smaller overall and the EDIII cross-reactive IgM response was much greater than IgG in both primary and secondary DENV-2 infected sera. Immunoglobulin populations

recognizing EDIII DENV-2 specific epitopes were very small, though larger in IgM than IgG. IgG recognizing this epitope averaged only 1% of the immunoglobulin response yet the magnitudes of EDIII virus specific IgG were significantly correlated with DENV-2 specific neutralization. The results presented in this report extend our understanding of the diverse nature of humoral immune responses to human DENV infection and we discuss their relevance in the context of understanding DENV disease and the development and testing of safe and efficacious DENV vaccine candidates.

Results

Monoclonal Antibody Epitope Mapping

We examined the effects of substitutions in two distinct antigenic regions of the E-glycoprotein, EDII and EDIII, on MAb reactivities. The highly conserved fusion peptide located in EDII is an immunodominant region containing multiple overlapping epitopes typically eliciting weakly neutralizing and widely cross-reactive antibodies against viruses from several major serocomplexes (Crill and Chang, 2004; Crill, Trainor, and Chang, 2007; Goncalvez, Purcell, and Lai, 2004; Stiasny et al., 2006; Trainor et al., 2007). DENV EDIII elicits predominantly complex and sub-complex cross-reactive and serotype-specific antibodies, some of which can be potently neutralizing (Gromowski, Barrett, and Barrett, 2008; Roehrig, Bolin, and Kelly, 1998; Sukupolvi-Petty et al., 2007). Previous studies of flavivirus epitope mapping have demonstrated that different substitutions of an E-protein residue can have distinct effects on both MAb reactivities and VLP secretion, and that a specific substitution's effect on MAb binding can vary between flaviviruses (Allison et al., 2001; Crill, Trainor, and Chang, 2007; Oliphant et al., 2006; Stiasny et al., 2006; Trainor et al., 2007). For this reason we chose first to

determine the effect of individual E-protein amino acid substitutions on MAb reactivity and VLP secretion, then to combine substitutions in an attempt to maximize both MAb reactivity reductions and DENV-2 VLP secretion.

In the fusion peptide of EDII we examined two, five and six different substitutions at Gly104, Gly106 and Leu107 respectively (Table 2.1). As previously observed with different substitutions and in different viruses, G104 substitutions dramatically reduced VLP secretion from transiently transformed Cos-1 cells, thus G104 substitutions were excluded from further analysis (Crill and Chang, 2004; Crill, Trainor, and Chang, 2007; Trainor et al., 2007). Individual substitutions for G106 or L107 residues reduced the reactivities predominantly against group-cross-reactive MAbs that recognize all flavivirus E proteins (data not shown). Some of the group-reactive MAbs exhibited reactivity reductions for substitutions at either G106 or L107 (4G2), others only for G106 (6B6C-1 and 4A1B-9) or L107 (23-1), and one group-reactive MAb (23-2) was not affected by any of the individual substitutions. Additionally, DENV subcomplex reactive MAb 1B4C-2 exhibited dramatic reductions in reactivity for all individual G106 and L107 mutants. Based upon these observations we combined three different substitutions at G106 with two at L107 to create six double-mutant constructs (Tables 2.1 and 2.2). As expected, the observed effects of the single substitutions on MAb reactivities and VLP secretion typically combined additively into the double mutants. However, the pattern of reductions in 4G2 reactivity for single substitutions at either G106 or L107 did not completely extend to the G106/L107 double mutant DENV-2 VLPs. G106W/L107K and G106D/L107D VLPs exhibited no reductions in MAb 4G2 recognition relative to WT DENV-2 even though the individual substitutions all

Table 2.1. Nucleotide sequences of mutagenic primers used and % VLP secretion from resultant plasmids relative to wild-type (100%).

Primer	Primer Sequence (5'-3') ¹	Nucleotide Substitution	Amino Acid Substitution	% VLP Secretion ²
D2-G104E	TTTCCAAATAGTCCACATTCATTTCCCCATCCTCTGTCTACC	GGA-GAA	Gly-Glu	<50
D2-G104H	CCCTTTCCAAAT6AGTCCACAG T GATTTCCCCATCCTCTGTCTACC	GGA-CAC	Gly-His	<50
D2-G106M	GCCTCCCTTTCCAAATAG C ATACATCCATTTCCCCATCC	GGA-ATG	Gly-Met	100
D2-G106R	GCCTCCCTTTCCAAATAG A CGACATCCATTTCCCCATCC	GGA-CGT	Gly-Arg	100
D2-G106D	GCCTCCCTTTCCAAATAG A T C ACATCCATTTCCCCATCC	GGA-GAT	Gly-Asp	150
D2-G106W	GCCTCCCTTTCCAAATAG C CAACATCCATTTCCCCATCC	GGA-TGG	Gly-Trp	<50
D2-G106Q	GCCTCCCTTTCCAAATAG T T G ACATCCATTTCCCA	GGA-CAA	Gly-Gln	150
D2-L107Q	GCCTCCCTTTCCAAAT T CTCCACATCCATTTCCCCATCC	CTA-GAA	Leu-Gln	150
D2-L107D	GCCTCCCTTTCCAAA A T T CTCCACATCCATTTCCCCATCC	CTA-GAT	Leu-Asp	<<50
D2-L107M	GCCTCCCTTTCCAAA C ATTCCACATCCATTTCCCCATCC	CTA-ATG	Leu-Met	50
D2-L107G	GCCTCCCTTTCCAAA A C T CCACATCCATTTCCCCATCC	CTA-GGT	Leu-Gly	100
D2-L107K	GGTCACAATGCCTCCCTTTCCAAAT T TTCCACATCCATTTCCCC	CTA-AAA	Leu-Lys	75
D2-L107F	GCCTCCCTTTCCAAA A A A TCCACATCCATTTCCCCATCC	CTA-TTT	Leu-Phe	150
D2-G106D/L107K	CACAATGCCTCCCTTTCCAAAT T T G T C ACATCCATTTCCCCATCC	GGA-GAC CTA-AAA	Gly-Asp Leu-Lys	67
D2-G106D/L107D	CACAATGCCTCCCTTTCCAA A G T C G T C ACATCCATTTCCCCATCC	GGA-GAC CTA-GAC	Gly-Asp Leu-Asp	<5
D2-G106R/L107D	CACAATGCCTCCCTTTCCAA A G T C G C G ACATCCATTTCCCCATCC	GGA-CGC CTA-GAC	Gly-Arg Leu-Asp	8
D2-G106R/L107K	CACAATGCCTCCCTTTCCAA A C T T G C G ACATCCATTTCCCCATCC	GGA-CGC CTA-AAG	Gly-Arg Leu-Lys	85
D2-G106W/L107K	CACAATGCCTCCCTTTCCAA A C T T C CAACATCCATTTCCCCATCC	GGA-TGG CTA-AAG	Gly-Trp Leu-Lys	17
D2-G106W/L107D	CACAATGCCTCCCTTTCCAA A G T C C CAACATCCATTTCCCCATCC	GGA-TGG CTA-GAC	Gly-Trp Leu-Asp	67
D2-K305E	TATTTCCCTTCACA A CTTTAA A CT T CTCCTGTGCACATAGAG	AAG-GAG	Lys-Glu	6
D2-K305N	TATTTCCCTTCACA A CTTTAA A G T TTCCCTGTGCACATAGAG	AAG-AAC	Lys-Asn	13
D2-V308N	CCATGTTGTGTTTCTGCTATTT C CTT C ACAT T TTTAA A CTTT C CTGT	GTT-AAT	Val-Asn	19
D2-K310E	TGTGTTTCTGCTATTT C CT C CA A CTTTAA A CTTT C CTGT	AAG-GAG	Lys-Glu	25
D2-K310Q	TGTGTTTCTGCTATTT C CT G CA A CTTTAA A CTTT C CTGT	AAG-CAG	Lys-Gln	38
D2-E311R	CCATGTTGTGTTTCTGCTAT T C G CT T CA A CTTTAA A CTTT C CTG	GAA-CGA	Glu-Arg	100
D2-E311K	CCATGTTGTGTTTCTGCTAT T T C TT C CA A CTTTAA A CTTT C CTG	GAA-AAA	Glu-Lys	38
D2-E311Y	CCATGTTGTGTTTCTGCTAT G T A CT T CA A CTTTAA A CTTT C CTG	GAA-TAC	Glu-Tyr	6
D2-K310E/E311R	GTGTTTCTGCTAT T C G CT C CA A CTTTAA A CTTT C CTGTGC	AAG-GAG GAA-GGA	Lys-Glu Glu-Arg	25

D2-R323E	CCGTCCCCTTCATATTGCACT TC GATAACTATTGTTCCAT	AGA-GAA	Arg-Glu	6
D2-R323M	CCGTCCCCTTCATATTGCACCATGATAACTATTGTTCCAT	AGA-ATG	Arg-Met	17
D2-K361D	TATGTTGACTGGGCTATCAT CTT CTGTCACAATTGGGTTG	AAA-GAT	Lys-Asp	38
D2-P364R	AGGTTCTGCTTCTATGTTGACCCGGCTATCTTTTTCTGTC	CCA-CGG	Pro-Arg	25
D2-P364E	GGAGGTTCTGCTTCTATGTTGACT TC GCTATCTTTTTCTGTC	CCA-GGA	Pro-Glu	25
D2-K388D	GCTTCCTTTCTTAAACCAGTTGAGAT CC AGTTGTCCCG	AAG-GAT	Lys-Asp	50
D2-K388E	GCTTCCTTTCTTAAACCAGTTGAGTT CC AGTTGTCCCG	AAG-GAA	Lys-Glu	50

¹ Mutated nucleotides are shown in bold.

² Average of triplicate experiments of mutant VLP secretion from transiently transformed Cos-1 cells, standardized against the wild-type DENV-2 plasmid VLP secretion.

significantly reduced the binding by this MAb. These two G106/L107 mutant VLPs also exhibited the most limited reductions in overall group cross-reactive MAb recognition of the six DENV-2 double mutants examined.

We also observed significant reductions in reactivity of G106/L107 mutants for MAbs unaffected by the individual fusion peptide substitutions. Two of six double mutants exhibited reduced reactivity for group-reactive MAb 23-2. The reactivity of MAb 5-1, a MAb generated against Japanese encephalitis virus (JEV) and recognizing only DENV-2 and JEV, was reduced by all six double mutants, although the single G106 or L107 substitutions did not reduce the binding of this MAb. We also observed reactivity reductions for DENV complex and subcomplex reactive MAbs D3-5C9-1, 9D12, and 10A4D-2. In general reactivity reductions to these MAbs were less pronounced than those observed with the more broadly cross-reactive MAbs. However, MAb 10A4D-2, which was unaffected by the individual fusion peptide substitutions, did show significant reductions against three of the six double mutations (Table 2.2, some data not shown). Similarly, previous studies in JEV, WNV and Saint Louis encephalitis virus (SLEV) demonstrated that the reactivity of group cross-reactive MAb 23-2 and JEV complex cross-reactive MAbs were only abrogated by combining G106 and L107 substitutions (Chiou et al., 2008a; Crill, Trainor, and Chang, 2007; Trainor et al., 2007).

Thus, combinations of substitutions acting as epitope determinants in the EDII fusion peptide have a greater effect than do individual substitutions on disrupting the binding of some antibodies in this region. Moreover, in DENV-2, fusion peptide residues act as epitope determinants for antibodies with diverse patterns of cross reactivity, this

Table 2.2. MAb reactivities for DENV-2 VLP mutants¹.

	MAb:	MHIAF	4G2	6B6C-1	4A1B-9	23-1	23-2	20	5-1	5-2	1B7-5	D3-5C9-1	1A1D-2	9D12	10A4D-2	1B4C-2	3H5
	CR²:	Poly-clonal	grp	grp	grp	grp	grp	Sub grp. JEV	Sub grp. D2	Sub grp. JEV	comp. D3	comp. D4	Sub comp. D2	Sub comp. D1	Sub comp. D2	Sub comp. D2	Type spec. D2
	Virus:	D2	D2	SLEV	MVEV	WNV	JEV	JEV	D2	JEV	D3	D4	D2	D1	D2	D2	D2
VLP construct																	
	WT DENV-2 ³	≥6.0	≥6.0	≥6.0	5.1	≥6.0	≥6.0	≥6.0	≥6.0	≥6.0	≥6.0	5.1	≥6.0	≥6.0	≥6.0	>4.2	≥6
	G106R	25	<0.1	0.8	0.4	100	100	100	100	100	25	100	25	25	100	3	100
	L107D	100	<0.1	25	25	<0.1	25	100	100	100	100	100	50	100	100	3	100
	G106R/L107D	100	<0.1	<0.1	<3	0.2	0.2	100	3	100	100	25	100	100	6	nd	50
	K305E	100	Nd ⁴	nd	nd	nd	nd	100	100	100	100	50	3	100	100	5	<0.1
	K310E	100	nd	nd	nd	nd	nd	100	100	100	100	100	<3	<3	100	100	100
	E311R	100	nd	nd	nd	nd	nd	<0.1	<0.1	100	100	50	3	100	100	2.5	100
	P364R	100	nd	nd	nd	nd	nd	100	100	100	25	50	6	50	25	2.5	100
	K388D	100	nd	nd	nd	nd	nd	100	100	100	100	100	6	100	100	100	100
	K310E/E311R/ P364R	100	100	100	150	100	100	<0.1	<0.1	100	100	100	0.2	0.1	100	nd	100
	G106R/L107D/ K310E/E311R/ P364R	100	<0.1	0.2	<0.8	<0.1	0.2	<0.1	<0.1	100	100	1.5	0.1	0.4	100	<1.3	100

¹Percent of wild-type (WT) DENV-2 VLP reactivity for MAbs exhibiting varying levels of cross-reactivity (CR) and selected from different flaviviruses.

² MHIAF is polyclonal murine ascitic fluid; group CR antibodies recognize all viruses of the four major pathogenic flavivirus serocomplexes; sub-group CR MAbs recognize all or some members of two or more different flavivirus serocomplexes (e.g., MAbs 20, 5-1 and 5-2 recognize DENV-2 and JEV, JEV and DENV-2 and JEV, DENV-1 and -2 respectively); comp. and sub-comp CR MAbs recognize all four DENV complex viruses or a subset thereof respectively, and type MAbs recognize only DENV-2.

³ MAb reactivities for wild-type (WT) DENV-2 VLP are presented as inverse log₁₀ Ag-capture ELISA endpoint values and all mutant VLPs as percent of remaining reactivity compared to WT.

⁴ nd denotes not determined.

included predominantly flavivirus group cross-reactive antibodies, but also for MAbs recognizing only individual viruses from different flavivirus serocomplexes to DENV complex cross-reactive MAbs. This appears to be a general phenomenon, as recent reports in other flaviviruses demonstrate that antibodies exhibiting varying levels of cross-reactivity recognize epitopes including the highly conserved fusion peptide residues (Chiou et al., 2008a; Crill, Trainor, and Chang, 2007; Oliphant et al., 2006; Stiasny et al., 2006; Trainor et al., 2007).

We next examined the effects of 15 amino acid substitutions at eight different residues in EDIII alone and in combinations (Table 2.1). Because a major objective of this work is to determine the antigenic structure of E glycoprotein cross-reactive epitopes and differential immune responses to these epitopes, we selected EDIII residues most likely to be incorporated into such epitopes for mutagenesis. The EDIII substitutions examined primarily altered the reactivity of DENV complex MAbs (Table 2.2). The residues that had the greatest effects on MAb reactivities were K305, K310, E311 and P364 (Table 2.2). The MAbs affected by substitutions at these four residues exhibited three different reactivity patterns: those recognizing some but not all viruses in both the JEV and DENV serocomplexes (MAbs 20 and 5-1), MAbs recognizing only viruses within the DENV complex (1A1D-2, 9D12, and 10A4D-2) and DENV-2 type specific MAbs (3H5). The K305 substitutions (K305E and K305N) were the only EDIII substitutions that significantly affected DENV-2 type-specific antibody recognition. Interestingly, K305 substitutions also affected DENV complex MAb 1A1D-2 in addition to the prototype DENV-2-specific MAb 3H5. There were no significant effects of the K305 substitutions for other DENV complex MAbs. In spite of K305 acting as an

epitope determinant for DENV complex MABs, because substitutions at this residue had such strong effects on type-specific MAb recognition, K305 substitutions were not incorporated into the multiple EDIII complex cross-reactive epitope-knock-out constructs.

Some of the same antibodies exhibiting reactivity reductions from the fusion peptide substitutions were also affected by EDIII substitutions (Table 2.2). For example, MAb 5-1 had reduced reactivity to all six fusion peptide double mutant VLPs and also for one of three E311 substitutions. VLPs containing the charge reversal substitution E311R lost all measurable ELISA recognition by MAb 5-1 yet VLPs containing the alternative charge reversal substitution E311K or E311Y, were unchanged. Similarly, DENV subcomplex MAb 10A4D-2 exhibited reactivity reductions for VLPs containing substitutions in either DIII or the DII fusion peptide. These observations suggest and extend previous observations that some cross-reactive MABs might recognize inter-E dimer epitopes spatially overlapping EDII and EDIII structural domains of independent monomers (Goncalvez, Purcell, and Lai, 2004). MABs 5-1 and 20 have the same cross-reactivity, recognizing only DENV-2 and JEV; however, MAb 20 was raised against DENV-2 whereas 5-1 was raised against JEV. Although MAb 20 was not affected by fusion peptide substitutions and MAb 5-1 was, VLPs containing charge reversal substitutions at E311 reduced the reactivity of both of these MABs to similarly undetectable levels. K310 and P364 substitutions alone did not alter these MAB reactivities; however, all VLPs containing combinations of these substitutions, with or without E311 substitutions showed similar complete losses of reactivity by both MAb 20 and 5-1.

The third class of antibodies recognizing epitope determinants in EDIII were the DENV complex and subcomplex MAbs. The antibody panel contained six DENV complex cross-reactive MAbs, three raised from DENV-2 and one each from the other three serotypes. The two MAbs exhibiting the greatest decreases in EDIII reactivity were 1A1D-2 and 9D12, subcomplex reactive MAbs raised from DENV-2 and -1 respectively (Table 2.2). 1A1D-2 reactivity was reduced by single substitutions at all DIII residues investigated with the exception of K361, consistent with recent crystal structure results of the 1A1D-2 antigen-binding fragment complexed with recombinant DENV-2 EDIII (Tables 2.1, 2.2); (Lok et al., 2008). MAb 9D12 reactivity was only affected by the individual K310 and P364 substitutions as well as multiple combinations of EDIII substitutions. However, EDIII substitutions, both individual and in combination did not affect the reactivity of DENV complex reactive MAbs 1B7 or D3-5C9-1, antibodies raised against DENV-3 and DENV-4 respectively (Table 2.2).

Substitutions from the EDII fusion peptide and from EDIII were combined into the same plasmid construct in an attempt to maximize reductions in cross-reactivity from these two disparate structural regions. The general effect on MAb reactivities of combining substitutions was predictably additive. In general there was little effect of DIII substitutions on the group cross-reactive MAbs, and limited effect of fusion peptide substitutions on most complex and subcomplex MAbs. When substitutions were combined together the resulting VLPs exhibited reduced reactivities to both classes of antibodies (Table 2.2). We also observed significant reductions in the reactivity of DENV-4 raised complex cross-reactive MAb D3-5C9-1. This was unexpected since we did not observe reductions in D3-5C9-1 reactivity for VLPs containing substitutions in

either DII or DIII alone. By combining epitope determinants from both domains we were able to construct mutant VLPs exhibiting significantly reduced or ablated reactivity to the majority of MAbs in the panel representing four distinct reactivity classes: group, subgroup, complex and subcomplex cross-reactive antibodies (Table 2.2). Thus, this series of differential cross-reactivity reduced VLPs can be utilized as serodiagnostic antigens to describe the epitope-specific targeting of the polyclonal human immunoglobulin response to DENV infection.

Epitope-specific Humoral Immune Responses of DENV-2 Infected Patients

We selected a number of well-characterized sera from DENV-2 infected humans to examine the epitope-specific proportions of the polyclonal E protein-specific immune response using mutant VLP antigens described in the previous section of this report. All serum specimens (6-18 days post onset of symptoms) were collected from acute DENV-2 infected individuals suffering from dengue fever and were viral RNA positive as determined by a DENV-2 specific reverse transcriptase-PCR (RT-PCR) assay (Table 2.3). Primary and secondary sera, as defined by the IgM to IgG ratio, from DENV-2 infected humans were selected from both Puerto Rico and Taiwan (Table 2.3). IgM and IgG end-point titers were determined using six distinct DENV-2 VLP antigens to estimate the proportions of immunoglobulin recognizing four distinct E-protein antigenic regions (Table 2.2). Wild-type (WT) DENV-2 VLP was used to determine total anti-E IgM and IgG titers. Immunoglobulin proportions targeting EDII fusion peptide epitopes were determined with EDII fusion peptide knock-out mutant G106R-L107D (RD). Immunoglobulin recognizing cross-reactive epitopes in EDIII was determined with the EDIII cross-reactive knock-out mutant K310E-E311R-P364R (ERR) and total

Table 2.3. Serological characterization of DENV-2 infected sera examined in this study.

Sera #	Country of Origin	DPO ¹	1°/2° ²	IgM P/N ³	IgG P/N	M/G OD-N ⁴
4	Taiwan	17	1°	46.0	20.4	1.65
5	Taiwan	17	1°	27.8	10.3	2.53
12	Taiwan	14	1°	33.7	29.0	1.19
16	Taiwan	18	1°	5.02	3.59	1.25
8882	Puerto Rico	6	1°	44.2	5.04	11.1
0078	Puerto Rico	10	1°	65.0	12.1	4.23
9	Taiwan	14	2°	35.6	24.4	0.91
10	Taiwan	18	2°	15.6	24.1	0.41
17	Taiwan	14	2°	6.1	24	0.21
0169	Puerto Rico	6	2°	29.2	35.4	0.65
9608	Puerto Rico	13	2°	33.4	39.8	0.78
8867	Puerto Rico	7	2°	53.6	54.2	1.04

¹ DPO denotes days post onset of symptoms

² denotes primary (1°) or secondary (2°) DENV-2 infection.

³ OD of positive sera/OD of negative sera

⁴ This column denotes the ratio of the optical density (OD) for IgM/divided by IgG. Each value is corrected by subtracting 2x the negative OD value.

immunoglobulin proportions recognizing all cross-reactive epitopes incorporating determinants in either or both EDII fusion peptide and/or EDIII were determined with an EDII-III combination of these two knock-out mutants (RDERR). Finally the proportion of EDIII DENV-2 specific immunoglobulin was determined as the difference between the EDIII K305E and K388D antigen reactivities. End-point titers of each serum specimen were determined concurrently in IgM and IgG antibody-capture ELISA with each DENV-2 antigen and a negative antigen. As described in the Materials and Methods section, immunoglobulin end-points were calculated for the different VLP antigens using asymptotic covariance matrix V method by regression analysis with four variables: antigen class, immunoglobulin class (IgM, IgG), primary or secondary infection status, and the resident country of infected patients (Puerto Rico or Taiwan); and a general least squares (GLS) approach was applied to determine endpoints. Because the mutant antigens are epitope knock-outs, the domain-associated, epitope specific immunoglobulin percentages were determined by dividing the endpoint titer determined with a knock-out antigen by the titer of the WT antigen, subtracting this value from 1.0 and multiplying by 100. DENV-2 specific immunoglobulin percentages were determined as the difference between the percent recognizing the K305E and the K388D antigens.

Results from ANOVA on the GLS regression indicated that the three-way interaction among antigen, immunoglobulin type and geographic origin was not statistically significant ($p=0.68$; Table 2.4). This result suggests that the patterns observed in mean endpoints among the different antigens, within the same immunoglobulin class are similar for sera collected from two different geographic locations, Taiwan or Puerto Rico. The two-way interactions between immunoglobulin

Table 2.4. Analysis of variance table of calculated DENV-infected serum end-point data.

<u>Variable</u>	<u>F-value</u>	<u>p-value</u>
Antigen	370	<0.0001
Immunoglobulin type	239	<0.0001
1° or 2° Infection	362	<0.0001
Geographic origin of Sera	3.36	0.071
Antigen : Immunoglobulin type	17.7	<0.0001
Antigen : Geographic origin of Sera	2.91	0.0189
Antigen : 1° or 2° Infection	30.8	<0.0001
Immunoglobulin type : 1° or 2° Infection	118	0.4367

type and primary or secondary infection status ($p < 0.01$), between antigen class and geographic origin ($p = 0.019$), and between antigen class and immunoglobulin type ($p < 0.01$) were statistically significant (Table 2.4). There were highly significant effects of three of the four individual variables on epitope-specific end-point titers ($p < 0.01$); the only individual variable not having a significant effect on antigen endpoints was the geographic origin of the sera. In other words, there were no significant differences in total endpoint titer averaged across the different antigens, between the sera from Puerto Rico and from Taiwan (Table 2.4). The significant interaction between antigen class and geography does, however, indicate that there could be significant geographical effects on the magnitudes of individual epitope-specific immunoglobulin populations. Such differences could arise either from differential immune status correlated with geography and/or from the different genetic backgrounds of patients from Taiwan and Puerto Rico.

Because the ANOVA results suggest no generalized effect of geographic origin of test sera, the source of sera are not specified unless indicated. End-point titers of E-protein specific immunoglobulins in sera from DENV-2 infected (measured with the WT DENV-2 antigen) ranged from 21,400-1,510,000 (mean=299,000) for IgM and from 12,400-11,000,000 for IgG (mean=2,400,000; Table 2.5). Primary DENV-2 infected

Table 2.5. Epitope Specific Proportions of Envelope Protein Specific IgM and IgG from DENV-2 Infected Human Sera.

DENV-2 Antigen ¹	Epitope Specific Target	Mean Endpoint Titer ²	Endpoint Titer Range	Range of Percent Response ³	Mean (Median) Percent Epitope Specific Response
<i>IgM</i>					
WT	All	2.99x 10 ⁵	2.14 x 10 ⁴ – 1.51 x 10 ⁶	100	100
RD	EDII _{FP} ⁴	1.66 x 10 ⁵	6.07 x 10 ² – 4.79 x 10 ⁵	<1 - 97	44 (34)
ERR	EDIII _{CR} ⁵	1.71 x 10 ⁵	1.59 x 10 ⁴ – 8.32 x 10 ⁵	<1 - 90	43 (39)
RDERR	EDII _{FP} /EDIII _{CR} ⁶	1.26 x 10 ⁵	5.00 x 10 ² – 3.98 x 10 ⁵	<1 - 91	58 (67)
K305E-K388D	EDIII _{TS} ⁷	1.79 x 10 ⁴	2.19 x 10 ² – 7.98 x 10 ⁴	<1 - 20	6 (4.5)
<i>IgG</i>					
WT	All	2.40 x 10 ⁶	1.24 x 10 ⁴ – 1.10 x 10 ⁷	100	100
RD	EDII _{FP}	1.54 x 10 ⁶	7.37 x 10 ² – 4.79 x 10 ⁶	<1 - 94	36 (36)
ERR	EDIII _{CR}	2.46 x 10 ⁶	1.16 x 10 ⁴ – 1.05 x 10 ⁷	<1 - 26	5.8 (13)
RDERR	EDII _{FP} /EDIII _{CR}	6.76 x 10 ⁵	4.74 x 10 ² – 1.70 x 10 ⁶	<1 - 96	72 (64)
K305E-K388D	EDIII _{TS}	2.40 x 10 ⁴	1.24 x 10 ² – 4.50 x 10 ⁵	<1 - 8	1 (<1)

¹ The mutant antigens used are all knock-out mutants. The substitutions represented by RD, ERR, and RDERR antigens and MAb reactivities of all antigens are shown in Table 2.

² The endpoint titer determined with the knock-out antigen, thus representing immunoglobulins recognizing epitopes not targeted with the knock-out antigen.

³ Because the mutant antigens knock-out antibody recognition of specific epitopes, the percent of immunoglobulin recognizing a particular epitope was determined for each individual sera by calculating the percent reactivity measured with a mutant antigen relative to that determined with the WT antigen and subtracting this value from 1.0;

$$(1 - [\text{Endpoint}_{\text{mutant}}/\text{Endpoint}_{\text{WT}}]) \times 100; \text{ for the percent DENV-2 specific reactivity we used } (1 - [(\text{Endpoint}_{\text{K305E}}/\text{Endpoint}_{\text{WT}}) - (\text{Endpoint}_{\text{K388D}}/\text{Endpoint}_{\text{WT}})]) \times 100.$$

⁴ EDII_{FP}, antibodies recognizing E-protein structural domain II fusion peptide epitopes do not bind this antigen.

- ⁵ EDIII_{CR}, antibodies recognizing the E-protein structural domain III cross-reactive epitopes do not bind to this antigen.
- ⁶ EDII_{FP}/EDIII_{CR}, antibodies recognizing either EDII fusion peptide or EDIII cross-reactive epitopes do not bind to this antigen.
- ⁷ EDIII_{TS}, antibodies recognizing only the DENV-2 type-specific EDIII epitope are represented by the reactivity difference between the K305E and K388D antigens.

patient sera exhibited similar IgM and IgG titers for individuals and as a class these sera had IgM and IgG titers ranging from 21,400-1,510,000 (mean=408,000) and 12,400-1,020,000 (mean=376,000) respectively (Table 2.6). Secondary DENV-2 infected patient sera exhibited a distinctly different pattern with IgG titers ranging from seven to over 100 times greater than IgM titers for individual sera (21,900-447,000; mean=189,000 for IgM and 2,290,000-11,000,000; mean=4,430,000 for IgG; Table 2.6). The larger magnitude IgG response in secondary DENV infections could result from the rapid and strong memory B- and T-cell response and the reduced frequency of IgM responses in secondary DENV infections (Kuno, 2003). Although all serum specimens were late acute to early convalescent phase (6-18 dpo) this is sufficient time for typically rapid and strong anamnestic IgG responses.

Cross-reactive immunoglobulin populations from two overlapping antigenic regions form the majority of the immune response and are greater in magnitude for IgM than for IgG.

Immunoglobulin proportions targeting cross-reactive EDII fusion peptide epitopes were highly variable yet tended to be large, and were distinctly greater in primary than in secondary DENV-2 infected patients. Immunoglobulin responses specific for EDII fusion peptide ranged from undetectable levels to 97% of IgM and from undetectable to 94% of IgG. The mean and median values were 44% and 34% of IgM and 36% and 36% of IgG respectively (Table 2.5). The larger magnitude fusion peptide-specific immunoglobulin response in primary versus secondary DENV-2 infections was greater in IgM than in IgG (54% vs. 24% and 44% vs. 35% in IgM and IgG respectively (Table 2.6). EDII fusion peptide-specific IgM comprised a greater percentage of the total IgM

Table 2.6. Epitope Specific Proportions of Envelope Protein Specific IgM and IgG from Primary and Secondary DENV-2 Infected Human Sera.

Infection Status	DENV-2 Antigen¹	Epitope Specific Target	Mean End-point Titer²	Endpoint Titer Range	Mean Percent Epitope Specific Response³
<i>IgM</i>					
Primary	WT	All	4.08×10^5	$2.14 \times 10^4 - 1.51 \times 10^6$	100
	RD	EDII _{FP} ⁴	1.89×10^5	$6.07 \times 10^2 - 4.79 \times 10^5$	54
	ERR	EDIII _{CR} ⁵	2.57×10^5	$1.59 \times 10^4 - 8.32 \times 10^5$	37
	RDERR	EDII _{FP} /EDIII _{CR} ⁶	1.53×10^5	$5.00 \times 10^2 - 3.47 \times 10^5$	63
	K305E-K388D	EDIII _{TS} ⁷	1.70×10^4	$1.07 \times 10^3 - 2.74 \times 10^4$	4
Secondary	WT	All	1.89×10^5	$2.19 \times 10^4 - 4.47 \times 10^5$	100
	RD	EDII _{FP}	1.44×10^5	$1.66 \times 10^4 - 4.07 \times 10^5$	24
	ERR	EDIII _{CR}	8.55×10^4	$1.95 \times 10^4 - 2.34 \times 10^5$	55
	RDERR	EDII _{FP} /EDIII _{CR}	9.91×10^4	$1.32 \times 10^4 - 3.98 \times 10^5$	48
	K305E-K388D	EDIII _{TS}	1.48×10^4	$2.19 \times 10^2 - 3.66 \times 10^4$	8
<i>IgG</i>					
Primary	WT	All	3.76×10^5	$1.24 \times 10^4 - 1.02 \times 10^6$	100
	RD	EDII _{FP}	2.10×10^5	$7.37 \times 10^2 - 6.92 \times 10^5$	44
	ERR	EDIII _{CR}	3.60×10^5	$1.16 \times 10^4 - 8.91 \times 10^5$	7.5 ⁸
	RDERR	EDII _{FP} /EDIII _{CR}	1.68×10^5	$4.74 \times 10^2 - 4.90 \times 10^5$	55
	K305E-K388D	EDIII _{TS}	3.76×10^3	$1.24 \times 10^2 - 1.02 \times 10^4$	<1
Secondary	WT	All	4.43×10^6	$2.29 \times 10^6 - 1.10 \times 10^7$	100
	RD	EDII _{FP}	2.87×10^6	$1.45 \times 10^6 - 4.79 \times 10^6$	35
	ERR	EDIII _{CR}	4.57×10^6	$2.00 \times 10^6 - 1.05 \times 10^7$	4.0
	RDERR	EDII _{FP} /EDIII _{CR}	1.18×10^6	$7.08 \times 10^5 - 1.70 \times 10^6$	73
	K305E-K388D	EDIII _{TS}	8.86×10^4	$2.29 \times 10^4 - 4.50 \times 10^5$	2

¹ The mutant antigens used are all knock-out mutants. The substitutions represented by RD, ERR, and RDERR antigens and MAb reactivities of all antigens are shown in Table 2.

- ² The endpoint titer determined with the knock-out antigen, thus representing immunoglobulins recognizing the epitopes not targeted with the knock-out antigen.
- ³ Because the mutant antigens knock-out antibody recognition of specific epitopes, the percent of immunoglobulin recognizing a particular epitope was determined for each individual sera by calculating the percent reactivity measured with a mutant antigen relative to that determined with the WT antigen and subtracting this value from 1.0;
- (1 - [Endpoint_{mutant}/Endpoint_{WT}]) x 100; for the percent DENV-2 specific reactivity we used (1 - [(Endpoint_{K305E}/Endpoint_{WT}) - (Endpoint_{K388D}/Endpoint_{WT})]) x 100.
- ⁴ EDII_{FP}, antibodies recognizing E-protein structural domain II fusion peptide epitopes do not bind this antigen.
- ⁵ EDIII_{CR}, antibodies recognizing the E-protein structural domain III cross-reactive epitopes do not bind to this antigen.
- ⁶ EDII_{FP}/EDIII_{CR}, antibodies recognizing either EDII fusion peptide or EDIII cross-reactive epitopes do not bind to this antigen.
- ⁷ EDIII_{TS}, antibodies recognizing only the DENV-2 virus specific EDIII epitope are represented by the reactivity difference between the K305E and K388D antigens.
- ⁸ Two of six primary and three of six secondary infected DENV-2 sera had IgG endpoint titers with the ERR antigen greater than or equal to those with the WT antigen thus producing ERR epitope specific percentages that would have been negative. The percent ERR epitope specific IgG was thus set to 0.0 for these three sera and that value was used to compute the average value shown.

response in the sera from DENV-2 infected patients from Taiwan (51%) than it did for the sera from Puerto Rican patients (33%). This was the only epitope-specific immunoglobulin subclass where there was a dramatic percent difference between serum specimens from the two geographic regions. The significance of these differences is supported by the ANOVA results: although there was non-significant trend towards an effect of geographic origin of the sera alone on the total immunoglobulin titers ($p=0.071$) there was a significant two-way interaction between epitope-specific antigen classes and the geographic origin of the sera on these end-points ($p=0.040$; Table 2.4).

Immunoglobulins targeting cross-reactive EDIII epitopes showed similar trends as did EDII fusion peptide immunoglobulins but their variability and magnitudes were smaller and there was an even greater reduction in IgG titers relative to IgM. EDIII cross-reactive immunoglobulin ranged from <1% to 45% in primary infections and <1% to 90% in secondary infections (Table 2.5). IgM recognizing cross-reactive EDIII epitopes averaged 37% and 55% in primary and secondary infections respectively, whereas EDIII cross-reactive IgG averaged only 7.5% and 4% of primary and secondary DENV-2 infected patient sera (Table 2.6). Thus, there was a much greater EDIII cross-reactive IgM response than in IgG and for IgM this response was greater in secondary than in primary infections.

In addition to the EDII fusion peptide and the EDIII cross-reactive epitope knock-out antigens, serum specimens were screened with an EDII-EDIII knock-out antigen (RDERR) combining the same substitutions from the individual mutant antigens in these two antigenic regions (Table 2.2). Using the RDERR antigen, immunoglobulins recognizing epitopes in either of -or overlapping- these structural domain regions

averaged 58% of IgM and 72% of the IgG present (median=67% and 64% respectively; Table 2.5). There was a greater total cross-reactive IgM response in primary versus secondary DENV-2 infected sera (63% and 48% respectively). However, this pattern was reversed in IgG where 55% and 73% recognized these cross-reactive epitopes in primary and secondary DENV-2 infected sera respectively (Table 2.6). Interestingly, the percent cross-reactive immunoglobulin as determined with the RDERR antigen was not the same as estimated by adding the percents of immunoglobulin recognizing the EDII fusion peptide (determined with the RD antigen) and cross-reactive EDIII epitopes (determined with the ERR antigen, Tables 2.5 and 2.6). There were exceptions to this general observation for IgM or IgG for some individual serum specimens. The total proportion of cross-reactive immunoglobulins determined with the RDERR antigen was greater than that estimated by adding the percentages determined with the RD and ERR antigens for IgG (72% and 42% respectively), yet the opposite pattern was observed for IgM (RDERR=58%; RD + ERR= 87%; Table 2.5). These data suggest that there are differences in the epitope specificity of cross-reactive antibody responses between IgM and IgG. In the MAb screening we observed significant reactivity reductions with DENV complex cross-reactive MAb D35C-9-1 for the inter-domain RDERR VLP, yet no reactivity reductions of this MAb for either the RD or the ERR VLPs alone (Table 2.2). These results suggest that a proportion of human IgG might recognize epitopes similar to murine MAb D35C-9-1 or that they might recognize epitopes not represented in our MAb panel, yet that similar to D35C-9-1 exhibit reduced recognition for the RDERR antigen and not for either the RD or ERR antigens alone.

Immunoglobulin populations targeting EDIII DENV-2 specific epitopes are small, yet of greater magnitude in IgM than in IgG and in secondary than in primary DENV-2 infected sera.

To determine the proportion of immunoglobulin recognizing EDIII DENV-2 type-specific epitopes identified as potently neutralizing in murine studies we used two different EDIII mutant antigens. K305E knocks out the reactivity of MAb 3H5 (Table 2.2) the prototype DENV-2 type-specific MAb recognizing this epitope. K305 has been demonstrated to be essential for the binding of MAb 3H5 and six other potently neutralizing DENV-2 specific MAbs (Gromowski and Barrett, 2007). However, K305E also reduced the reactivity of subcomplex cross-reactive neutralizing MAb 1A1D-2 (Table 2.2). The K388D antigen only reduced the reactivity of MAb 1A1D-2 - to similar levels as K305 E - and both of residues have been identified as 1A1D-2 binding residues in a recent Fab-DENV-2 structural determination with this MAb (Lok et al., 2008). The amount of EDIII DENV-2 specific immunoglobulin was therefore determined as the difference between the percent reduction in endpoint titer relative to WT, between the K305E and K388D antigens.

EDIII DENV-2 type-specific immunoglobulin formed very small percentages of the antibody response and was greater in IgM than in IgG. In contrast to the cross-reactive immunoglobulins however, EDIII virus-specific antibody populations averaged twice as large in secondary as in primary DENV-2 infected sera (Tables 2.5 and 2.6). EDIII DENV-2 specific IgM averaged 6% across sera, 4% and 8% in primary and secondary DENV-2 infections, and ranged from <1% to 20% of the IgM response. EDIII DENV-2 specific IgG averaged only 1% across all sera, was <1% and 2% of IgG in

primary and secondary serum samples, and ranged from <1% to 8% of total IgG.

Interestingly, there was no correlation between the magnitude of the IgM and IgG response to this epitope for individual DENV-2 infected patient sera.

Neutralizing antibody responses of primary and secondary DENV-2 infected patient sera: EDIII DENV-2 specific IgG titer is significantly correlated with DENV-2 neutralization.

Focus-reduction microneutralization (FR_μNT) assays were conducted with the sera to see if homologous/heterologous virus neutralization correlated with differential percentages or titers of virus-specific or cross-reactive immunoglobulins across the sera. All sera were screened against five different viruses, DENV-1, -2, -3, -4, and JEV; the Puerto Rican sera were additionally screened against WNV. Serum samples were diluted two-fold from 1:50 to 1:1600 and the 50%, 75%, and 90% FR_μNT end-point titers were determined. Predicted 90% endpoint titers were also determined from a non linear regression of the FR_μNT data using a variable slope sigmoidal dose-response formula. The 90% FR_μNT of sera from primary DENV-2 infected patients ranged from 1:200 to 1:800 and all were greater than or equal to four-fold higher than for that of the next highest virus titer (Table 2.7). Sera from secondary DENV-2 infected patients had much higher DENV-2 90% FR_μNT titers ranging from 1:400 to >1:1600 (predicted 90% range: 431->19,584 Table 2.8). None of the sera from secondary DENV-2 infected patients had the highest 90% FR_μNT for DENV-2. This observation could have resulted from not diluting the sera beyond 1:1600; however, serum specimen 9608 with a DENV-2 90% FR_μNT of 1:400 also had a DENV-4 90% FR_μNT of 1:400. The period of neutralizing antibody dominance to the primary infecting virus after secondary infection with a

Table 2.7. Focus reduction microneutralization test (FR μ NT) titers for primary DENV-2 infected serum samples from dengue fever patients.

Serum #	Country of Origin	DPO ¹	Virus	FR μ NT Titers			Pred. 90% ²
				50%	75%	90%	
4	Taiwan	17	DENV-1	200	50	<50	17
			DENV-2	≥ 1600	800	400	373
			DENV-3	100	50	<50	18
			DENV-4	200	50	<50	29
			JEV	<50	<50	<50	7
5	Taiwan	17	DENV-1	50	<50	<50	3
			DENV-2	≥ 1600	≥ 1600	800	706
			DENV-3	50	50	<50	10
			DENV-4	100	<50	0	16
			JEV	<50	<50	<50	4
12	Taiwan	14	DENV-1	400	200	50	57
			DENV-2	≥ 1600	≥ 1600	400	604
			DENV-3	200	100	50	50
			DENV-4	4700	50	<50	45
			JEV	50	<50	<50	18
16	Taiwan	18	DENV-1	50	<50	<50	4
			DENV-2	≥ 1600	800	400	412
			DENV-3	<50	<50	<50	7
			DENV-4	50	<50	<50	13
			JEV	<50	<50	<50	3
8882	Puerto Rico	6	DENV-1	50	<50	<50	9
			DENV-2	800	400	200	144
			DENV-3	100	<50	<50	15
			DENV-4	200	<50	<50	24
			WNV	<50	<50	<50	4
JEV	<50	<50	<50	3			
0078	Puerto Rico	10	DENV-1	100	<50	<50	16
			DENV-2	800	400	200	145
			DENV-3	200	50	<50	31
			DENV-4	800	50	<50	64
			WNV	50	<50	<50	12
JEV	<50	<50	<50	3			

¹ days post onset of symptoms

² predicted actual 90% FR μ NT titers based up a nonlinear regression of the FR μ NT data using a variable slope sigmoidal dose-response model.

Table 2.8. Focus reduction microneutralization test (FR μ NT) titers for secondary DENV-2 infected serum samples from dengue fever patients.

Serum #	Country of Origin	DPO ¹	Virus	FR μ NT Titers			Pred. 90% ²
				50%	75%	90%	
9	Taiwan	14	DENV-1	≥ 1600	≥ 1600	≥ 1600	na ³
			DENV-2	≥ 1600	≥ 1600	≥ 1600	na
			DENV-3	≥ 1600	≥ 1600	≥ 1600	4095
			DENV-4	≥ 1600	≥ 1600	≥ 1600	1778
			JEV	800	100	<50	83
10	Taiwan	18	DENV-1	≥ 1600	≥ 1600	≥ 1600	na
			DENV-2	≥ 1600	≥ 1600	≥ 1600	na
			DENV-3	≥ 1600	≥ 1600	≥ 1600	na
			DENV-4	≥ 1600	≥ 1600	800	991
			JEV	≥ 1600	100	50	103
17	Taiwan	14	DENV-1	≥ 1600	≥ 1600	≥ 1600	na
			DENV-2	≥ 1600	≥ 1600	≥ 1600	na
			DENV-3	≥ 1600	≥ 1600	≥ 1600	na
			DENV-4	≥ 1600	≥ 1600	≥ 1600	1795
			JEV	200	<50	0	31
0169	Puerto Rico	6	DENV-1	≥ 1600	400	200	307
			DENV-2	≥ 1600	≥ 1600	≥ 1600	19,584
			DENV-3	≥ 1600	≥ 1600	800	934
			DENV-4	≥ 1600	400	200	167
			WNV	400	50	<50	59
			JEV	50	<50	<50	17
9608	Puerto Rico	13	DENV-1	400	200	50	79
			DENV-2	≥ 1600	800	400	431
			DENV-3	800	200	100	95
			DENV-4	≥ 1600	800	400	526
			WNV	400	100	50	74
			JEV	200	<50	<50	25
8867	Puerto Rico	7	DENV-1	≥ 1600	800	200	366
			DENV-2	≥ 1600	≥ 1600	≥ 1600	5149
			DENV-3	800	200	100	135
			DENV-4	≥ 1600	≥ 1600	800	1341
			WNV	≥ 1600	200	50	122
			JEV	200	<50	<50	38

¹ days post onset of symptoms

² predicted actual 90% FR μ NT titers based up a nonlinear regression of the FR μ NT data using a variable slope sigmoidal dose-response model.

³ these sera had either total virus neutralization (zero foci) at the 1600 dilution or too few foci to form a reliable curve to predict actual 90% FR μ NT titers.

heterologous DENV serotype has been shown to vary from less than a week to multiple weeks, so presumably this early convalescent phase patient serum has not yet switched to DENV-2 dominance (Kuno, 2003). Two of the remaining five sera from secondary DENV-2 infected patients had next highest 90% FR_μNT of 1:800 for another DENV serotype, and the remaining three sera from secondary DENV-2 infected patients had 90% FR_μNT of $\geq 1:1,600$ for three or four DENV serotypes. All of the sera from primary DENV-2 infected patients had JEV 50% FR_μNT titers <50 , whereas all sera from secondary DENV-2 infections had JEV 50% FR_μNT titers ≥ 50 . This was not surprising for the three Taiwanese sera since they were also IgG ELISA positive against JEV VLP antigen (Chiou et al., 2008a). We were surprised that the three sera from secondary DENV-2 infected patients from Puerto Rico exhibited neutralizing antibodies against JEV, as the titers appeared to be greater than expected from DENV infected cross-reactivity. Because the Puerto Rico sera were collected in 2007 when WNV was known to be circulating on the island we decided post hoc to test these sera for neutralizing antibodies against WNV. Two of these three sera from secondary DENV-2 infected patients had WNV 50% FR_μNT titers of 1:400 and a third (8867) was $\geq 1:1,600$; these three serum samples had predicted 90% FR_μNT WNV titers of 59, 74, and 122 (Table 2.8). Sera from Primary DENV-2 infected patients from Puerto Rico had 50% FR_μNT titers $<1:50$ for both JEV and WNV, and all four sera from primary DENV-2 infected patients from Taiwan had JEV 50% FR_μNT titers $\leq 1:50$. Taken together these results suggest that there is limited cross-neutralization from infection by viruses in the DENV complex to viruses in the JEV serocomplex. Thus, for at least one, and possibly all three of the sera from secondary DENV-2 infected Puerto Rican patients, the presence of JEV

neutralizing antibody is consistent with JEV complex-cross-reactivity occurring from actual WNV exposure. We did not observe any significant correlations between the proportions or the total magnitude of cross-reactive immunoglobulin populations and heterologous neutralization to other DENV serotypes or to JEV or WNV, implying that the large cross-reactive antibody populations were either weakly or non-neutralizing or that there was large variation in the neutralizing capabilities of these immunoglobulins between serum specimens.

To investigate if EDIII virus specific epitopes stimulate strongly neutralizing and protective immunoglobulin in humans as they do in mice, we regressed EDIII DENV-2-specific IgM or IgG titers on the percent DENV-2 neutralization (calculated at the highest serum dilution of 1:1600) and performed an analysis of variance on the regression. For IgM, the regression slope was actually negative and not significantly different from 0 ($m = -0.127$, $p = 0.233$), suggesting that IgM recognizing EDIII DENV-2 specific epitopes in these sera is not strongly neutralizing (Table 2.9). However, when EDIII DENV-2 specific IgG titers were regressed on percent DENV-2 neutralization the slope was positive and significant ($m = 0.182$, $p = 0.0482$) indicating that increasing EDIII DENV-2 specific IgG is correlated with increasing neutralization (Table 2.9). These data suggest that the very small proportion of EDIII DENV-2 specific IgG is responsible for a significant proportion the DENV-2 specific neutralization, and that the potentially neutralizing EDIII virus specific epitopes identified in mice also play a role in DENV-2 specific neutralization in humans.

Table 2.9. Analysis of variance of the slope determined from EDIII DENV-2 specific IgM and IgG titers each individually regressed on DENV-2 specific neutralization.

Dependent Variable	Explanatory Variable	Slope	p-value
EDIII DENV-2 specific IgM ¹	DENV-2 specific Neutralization ²	-0.127	0.233
EDIII DENV-2 specific IgG	DENV-2 specific Neutralization	0.128	0.048

¹IgM and IgG titers were log transformed prior to analysis so that they would not violate assumptions of normality in the linear regression model.

²DENV-2 neutralization was expressed as the percent plaque reduction at the maximum serum dilution of 1:1600.

Discussion

MAb Epitope Mapping

Previous studies have indicated that the EDII fusion peptide region contains multiple, overlapping, broadly cross-reactive, immunodominant epitopes and that both serotype-specific and DENV complex cross-reactive epitopes are located in EDIII (Chiou et al., 2008a; Crill and Chang, 2004; Crill and Roehrig, 2001; Crill, Trainor, and Chang, 2007; Goncalvez, Purcell, and Lai, 2004; Gromowski and Barrett, 2007; Gromowski, Barrett, and Barrett, 2008; Lisova et al., 2007; Oliphant et al., 2006; Roehrig, Bolin, and Kelly, 1998; Stiasny et al., 2006; Sukupolvi-Petty et al., 2007; Trainor et al., 2007). The mutagenesis-based epitope mapping presented here supports and extends these results. For example, in a previous paper examining cross reactive epitope determinants in DENV-2 we demonstrated that flavivirus group cross-reactive MAb 4G2 lost recognition for VLPs containing either G106Q or L107K substitutions, whereas similar group-cross-reactive MAb 6B6C-1 only exhibited reduced binding for VLPs containing the G106Q substitution (Crill and Chang, 2004). More extensive and detailed mutagenesis of these

two E-protein residues in JEV, WNV and SLEV extend this pattern: substitutions at either G106 or L107 ablate MAb 4G2 recognition, however only substitutions at G106 interfere with MAb 6B6C-1 recognition (Crill, Trainor, and Chang, 2007; Trainor et al., 2007). This pattern was upheld in this study with the exception of L107F, which did not significantly reduce the binding of 4G2 or of any other group cross-reactive MAbs (data not shown). However in JEV, WNV, SLEV, and tick-borne encephalitis virus (TBEV) E proteins the L107F substitution has been shown to reduce cross-reactive antibody recognition, and L107F TBEV recombinant subviral particles have been shown to reduce the immunoglobulin recognition by polyclonal DENV infected human serum (Allison et al., 2001; Crill, Trainor, and Chang, 2007; Stiasny et al., 2006; Trainor et al., 2007). In spite of the invariant nature of most fusion peptide residues, L107F does occur in a number of flaviviruses: Powassan and Deer tick viruses, JEV vaccine strain SA-14-14-2, and DENV-2 strain POU-280 (Blok et al., 1989; Kuno et al., 2001; Nitayaphan et al., 1990).

One of the novel findings of the DENV-2 epitope mapping is that substitutions in different structural domains of adjacent E-protein monomers can act as epitope determinants for a single antibody, suggesting that some antibodies might recognize inter-monomer epitopes including the disparate structural domains EDII and EDIII. Perhaps the best example of this was DENV-4 raised DENV complex cross-reactive MAb D35C-9-1. This MAb exhibited only minor reactivity reductions (50-25%) for all six G106/L107 fusion peptide mutants and for two individual EDIII mutants, E311R and P364R (50%). However, when the EDII fusion peptide and EDIII substitutions were combined into a single VLP construct, D35C-9-1 reactivity was dramatically reduced to

only 1.5% of WT DENV-2 reactivity. Such synergistic effects of combining substitutions on antibody reactivity could occur when residues do not play a critical binding role within the antibody paratope and thus the introduction of single substitutions might not interfere with antibody recognition, yet when two or more substitutions of little to no individual effect are combined together they either decrease K_a or increase K_d to the point where a significant reduction in antibody recognition is observed. Other examples of EDII-EDIII inter-monomer epitope determinants occurred with DENV subcomplex MAb 1B4C-2 and subgroup cross-reactive MAbs 5-1, and 5-2. MAb 1B4C-2 is a DENV-2 raised non-neutralizing antibody recognizing a surface accessible epitope originally assigned to EDI on the basis of biophysical mapping (Roehrig, Bolin, and Kelly, 1998). A previously published mutagenesis study identified EDII fusion peptide residues that could act as epitope determinants affecting the binding of this MAb (Crill and Chang, 2004), and this study confirms the effect of both fusion peptide substitutions and EDIII substitutions acting as 1B4C-2 epitope determinants. Similar to MAb D35C-9-1, the greatest decreases in binding of 1B4C-2 occurred for VLPs combining substitutions from EDII and EDIII.

MAb 5-1 is a JEV-raised antibody that recognizes only JEV and DENV-2. All VLPs containing G106/L107 substitutions exhibited reduced binding by this MAb, as were E311R VLPs. Again, combinations of the EDII substitutions and EDIII E311R had the most dramatic effect, ablating our ability to measure any MAb recognition for this VLP at all. MAb 20 is similar to MAb 5-1; it was raised against DENV-2 and also recognizes only JEV and DENV-2. Although fusion peptide substitutions did not alter the binding of this MAb, just as with MAb 5-1, all mutant VLPs containing combinations

of E311 substitutions exhibited significant reductions in MAb 20 binding. E311 is conserved across the DENV complex yet this residue is an asparagine in the JEV complex viruses (Fig. 2.1); adjacent residue K310 is almost completely conserved across the mosquito borne flaviviruses, however K310 substitutions did not alter the binding of either MAb 20 or 5-1. Thus other shared residues between DENV-2 and JEV, possibly including fusion peptide residues, must be responsible for the identical cross-reactivity patterns of these two antibodies. MAb 5-2 is another JEV-raised subgroup cross-reactive MAb, recognizing JEV, DENV-1 and DENV-2. This study did not identify any substitutions in DENV-2 VLPs that disrupted the binding of this MAb. However, in JEV and in DENV-1, substitutions in both the fusion peptide and in EDIII significantly reduced the binding of this MAb (Chiou et al., 2008a) and unpublished results).

The only other published report of similar inter-monomer EDII-EDIII epitopes is for chimpanzee MAb 1A5, a flavivirus group cross-reactive MAb raised against DENV-2 (Goncalvez, Purcell, and Lai, 2004). G106 and H317 were independently identified by neutralization escape from MAb 1A5 and thus identified as epitope determinants. G106V significantly reduced antibody binding and neutralization whereas H317Q did not affect binding but did reduce neutralization by this MAb. Although structurally close to G106 (~15Å) H317 lies buried on the bottom membrane side of the E dimer in native virions, consistent with its lack of effect on 1A5 binding. The EDIII residue we identified as cooperating with fusion peptide residues G106 and L107 as a cross-reactive epitope determinant was E311. E311 is highly exposed on the outer surface of the dimer

		300	310	320	330	340	
DENV-2	16681	:	KGMSYS	CTGKFKIVKEIAETQ	GTIVIRVQYEGDGS	CKIPFEIM-DLEKR	:
DENV-1	16007	:V.....S	...E.....Q.K...TDA.....SSQ-.EKGV	:	
DENV-3	H87	:A..LNT	.V.K.....S.....K.EDA.....STE-.G.G.	:	
DENV-4	H241	:S.D.....T...K...A.A.....I..R-.N.E	:		
JEV	SA-14	:	..T..G...E..SFA	.NP.D.G.....E.S.S.SDG.....IVS.AS.NDM	:		

		350	360	370	380	390				
DENV-2	16681	:	HVLGRLITVNP	IVTEKDS	P--VNIEAEP	PPFGDSYIIIGV	EF	GQLKLNW	FKKG	:
DENV-1	16007	:	TQN.....A.....D	.EK.--.....E.....AGEKA...S.....	:					
DENV-3	H87	:	AHN.....A.....K	.EE.--.....E.N.....GDKA.....	:					
DENV-4	H241	:	K.....ST..AENT..V--T...L.....GNSA..T.H.....	:						
JEV	SA-14	:	TP.....F.ATSSANSK	.L..M.....RGDK..NHH.H.A.	:					

Figure 2.1. EDIII Alignment of Representative Strains of the Four DENV Serotypes and JEV. Single letter amino acid abbreviations are shown for EDIII of DENV-2 using DENV-2 numbering. Amino acids conserved relative to DENV-2 in the other serotypes are shown as dots, and single letter abbreviations for non-conserved amino acids are depicted. Colored residues depict epitope-specific determinants as determined in this report and previously published reports. DENV-2 residues demonstrated to be involved in DENV complex and subcomplex cross-reactive epitopes are highlighted in yellow, DENV-2 specific residues are highlighted in red, and residues from the region of overlap between these epitopes and hence affecting DENV complex and DENV-2 virus specific epitopes are highlighted green (Gromowski and Barrett, 2007; Gromowski, Barrett, and Barrett, 2008; Lok et al., 2008; Sukupolvi-Petty et al., 2007). The mutated EDIII residues incorporated into antigens in this study are marked in black for the other DENV serotypes: DENV EDIII complex cross-reactive knock out mutants incorporated K310D, E311R, P364R, and K388D; K305E was utilized to determine EDIII DENV-2 specific immunoglobulin response

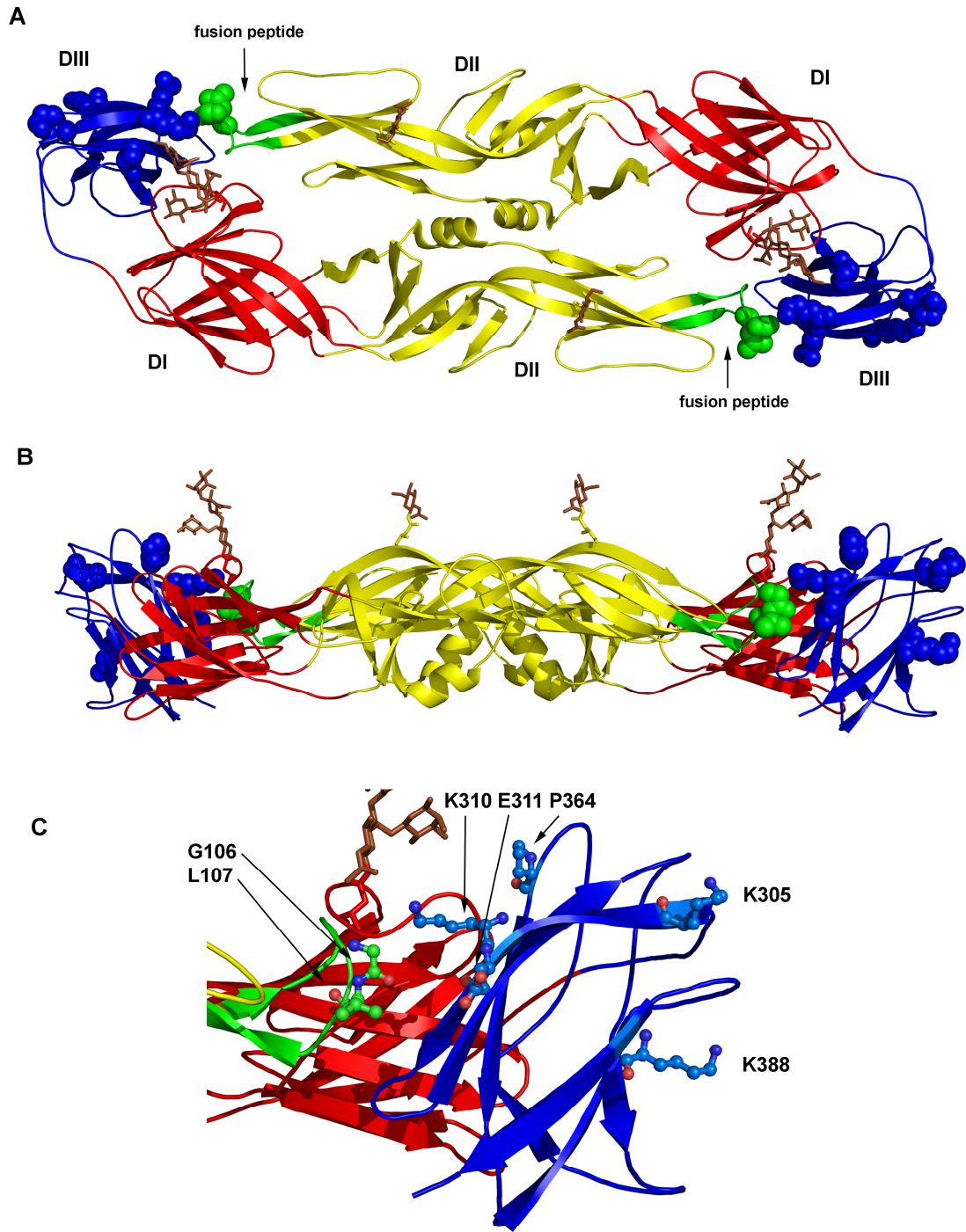


Figure 2.2. Structural Locations of Envelope Protein Epitope-specific Knock-out

(A) Crystal structure of the DENV-2 E dimer (Modis et al., 2003) as it appears from above in mature virions and depicted as a ribbon diagram. The structural domains are colored red (EDI), yellow (EDII), and blue (EDIII). The fusion peptide residues are colored green and the glycans in EDI (N153) and EDII (N67) are depicted as ball and stick representation and colored brown. Epitope specific knock-out substitutions in the fusion peptide and EDIII are depicted as space filling representations and colored magenta.

(B) Side view of the same representation of the E protein dimer in mature virions with all structural depictions and colors the same as in panel A.

(C) An enlarged view from panel B of the interface between the EDII fusion peptide of one monomer and EDIII of the alternate monomer of the E dimer. Residue locations of epitope-specific knock out substitutions are depicted as ball and stick representations. Substitutions of Gly106 and Leu107 in the EDII fusion peptide knock out the binding of broadly cross-reactive immunoglobulins, those recognizing viruses in the DENV complex and other flavivirus complexes. Substitutions of Lys310, Glu311, Pro364, and Lys388 in EDIII knock out the binding of immunoglobulins recognizing all or subsets of the four serotypes of DENV, but do not interfere with DENV-2 specific immunoglobulin recognition. Substitutions of Lys305 knock out the binding of DENV-2 serotype specific MABs.

and is only 6-9Å from EDII fusion peptide residues G106 and L107 of the alternate monomer, well within the binding area of a typical Fab footprint (Fig. 2.2). Competitive binding assays with DENV-2 identified three distinct MAb epitope binding clusters and the cluster recognized by the majority of the antibodies included MAbs recognizing epitopes from all three E structural domains. This cluster not only included MAbs recognizing fusion peptide epitopes such as 6B6C-1, but also included EDII-EDIII binding MAb 1B4C-2 (Roehrig, Bolin, and Kelly, 1998). These results suggest that such inter-domain epitopes could be more common than previously recognized. It has been noted in this report and elsewhere that there is a diversity of overlapping epitopes containing fusion peptide residues recognized by antibodies with variable patterns of cross-reactivity (Crill, Trainor, and Chang, 2007; Trainor et al., 2007). Due to the size of an antibody Fab footprint, it is expected that such epitopes will include residues outside of, and less conserved than those in the fusion peptide itself and that binding to these more variable residues must account for the different patterns of cross-reactivity observed for antibodies recognizing epitopes in this region. Thus, it is not unexpected that there are antibody epitopes incorporating EDII fusion peptide residues and residues within the structurally adjacent EDIII of the alternate monomer. These results and interpretations suggest a point of caution for studies attempting to map antibody epitopes using only recombinant EDIII (rEDIII) protein or soluble E-protein monomers.

Some of the general and specific epitope mapping results presented here are supported by recently published studies. A number of studies have mapped DENV-complex and sub-complex cross-reactive epitopes to the lateral surface of EDIII (Gromowski, Barrett, and Barrett, 2008; Lisova et al., 2007; Lok et al., 2008; Modis et al., 2003; Modis et al., 2005;

Sukupolvi-Petty et al., 2007). MAb 1A1D-2 is a DENV-2 raised subcomplex cross-reactive antibody that recognizes DENV-1, -2, and -3 (Roehrig, Bolin, and Kelly, 1998). We identified six residues located on three different β -strands in EDIII acting as epitope determinants for this antibody. Four of these residues are in β -strand A (Lys305, Val308, Lys310, and Glu311), and one each in β -strand B (Arg323) and β -strand G (Lys388) (Modis et al., 2003). The seventh residue identified as an epitope determinant for this MAb, Pro364, is the last residue of the loop just prior to β -strand E. Recently published analyses using yeast surface display mutagenesis of DENV-2 rEDIII also identified MAb 1A1D-2 epitope determinants in β -strand A (Gly304, Lys305, Lys 307, and Lys310); (Sukupolvi-Petty et al., 2007). In fact, a crystal structure of MAb 1A1D-2 Fab complexed with rEDIII was recently solved that confirms the principal involvement of EDIII β -strand A in 1A1D-2 binding (Lok et al., 2008). Another recent study examined five DENV subcomplex MAbs with cross-reactivity profiles the same as 1A1D-2 but not including this MAb. All five of these MAbs recognized a single antigenic site including residues in β -strands A, B, and G and centered on K310 (Gromowski, Barrett, and Barrett, 2008). It therefore appears that the well-characterized epitope recognized by MAb 1A1D-2 could be representative of most subcomplex antibodies recognizing DENV-1, -2, and -3.

MAb 9D12 was raised against DENV-1 and recognizes DENV-1, -2, and -4. This MAb appears to recognize an overlapping, yet distinct epitope from MAb 1A1D-2. Two of the residues we identified as epitope determinants for 1A1D-2 had similar effects for 9D12 reactivity, K310 and P364. Yeast display mutagenesis of rEDIII also identified the involvement of K310, but they did not examine the effect of substitutions at P364 for

either 1A1D-2 or 9D12 binding (Sukupolvi-Petty et al., 2007). There was one direct conflict between the 9D12 results presented here and those from a previous study. In this report there was no effect of either of the substitutions examined at K305 on 9D12 reactivity, whereas a previous study using rEDIII found significant reductions in 9D12 binding for a K305E mutant (Sukupolvi-Petty et al., 2007). One possibility is that there could be subtle differences in EDIII conformational structure between VLPs and rEDIII. For example, there could be differences in local dynamic motions, and hence minor conformational perturbations of the K305E substitution when introduced into rEDIII compared to E protein dimers as displayed on the VLP surface. Such local dynamic motions have recently been characterized in a recombinant EDIII system and demonstrated to be associated with specific substitutions that correlate with reductions in MAb recognition and neutralization (Maillard et al., 2008). DENV complex cross-reactive MAb 4E11, which we did not examine, has been studied extensively in DENV-1 and also binds to a discontinuous EDIII epitope centered primarily around four residues in β -strand A, including K310 (Lisova et al., 2007).

DENV type-specific antibodies, specifically potentially neutralizing murine MAbs, recognize epitopes in EDIII (Gromowski and Barrett, 2007; Hiramatsu et al., 1996; Lin et al., 1994; Lok, Ng, and Aaskov, 2001; Rey et al., 1995; Roehrig, Bolin, and Kelly, 1998; Sukupolvi-Petty et al., 2007; Trirawatanapong et al., 1992). Although we purposefully selected amino acids that were likely to be incorporated into cross-reactive epitopes for this study we did identify one residue, K305 that reduced the binding of the DENV-2-specific neutralizing MAb 3H5. 3H5 appears to be typical of many DENV-2 specific neutralizing MAbs, recognizing similar overlapping epitopes centered around EDIII

residues K305 and P384 (Gromowski and Barrett, 2007; Sukupolvi-Petty et al., 2007). Interestingly, in the same rEDIII yeast surface display mutagenesis study, P384 was identified as an important DENV-2 specific epitope determinant, yet not K305; despite the use of MAb 3H5 and K305 mutants in both the previous and this current study (Sukupolvi-Petty et al., 2007). Sukupolvi-Petty et al. did identify G304 as a major DENV-2 specific neutralizing MAb epitope determinant and G304 substitutions were not examined in this study or by Gromowski and Barrett, and given its close proximity to K305, G304 could be included as a part of this epitope. A clear consensus result from all of these studies is that there exist two different overlapping epitopic regions on the lateral surface of EDIII, one stimulating antibody with varying levels of cross-reactivity in the DENV serocomplex and second stimulating virus-specific antibodies (Fig. 2.2). These results all stem from studies of murine MAbs and a major still to be resolved issue is whether the outer lateral surface of EDIII contains protective virus-specific neutralizing epitope determinants for humans as well as mice (Lai et al., 2007).

Characterization of Serum from DENV-2 Infected Patients

Recently there has been increased interest and capability to examine and dissect complex polyclonal human immune responses to flavivirus infection. The results presented in this study compliment and add to this nascent body of work with detailed epitope-specific antibody assignments of IgM in addition to IgG. Stiasny et al. (Stiasny et al., 2006) measured total E protein-specific IgG titers from six sera from DENV-2 infected patients that ranged in IgG titers from 10^4 to 10^6 ; similar magnitudes and variation as determined in this study. Another recent study of WNV epitope-specific immune responses found much smaller and less variable E-specific IgG titers in sera

from WNV-infected patients (10^3 - 10^4) (Oliphant et al., 2007a). These sera from WNV-infected patients were collected 4-7 months post onset of symptoms, although a few sera were less than one month post onset that had similar IgG titers. The sera from DENV-2 infected patients examined by Stiasny et al had low to no IgM, consistent with their being convalescent phase sera. These observations suggest that the different magnitudes of E-specific IgG between sera from DENV-2 and WNV infected patients in these studies could be virus specific phenomena. DENV viremia in humans lasts approximately 4-10 days, whereas WNV viremia is more transient, lasting only 1-3 days. Thus the larger magnitude IgG response in DENV-2 infected patients compared to WNV-infected patients could result from differences in the length of viremia between these two viruses. Clearly, more studies will be needed to determine if this is a general phenomenon. In this study, E-specific IgM titers averaged across primary and secondary infected sera were also in the $10^4 - 10^6$ range (Table 2.5); although IgM titers were not presented in the WNV study cited above, they would be expected to be lower based on the late convalescent timing of the serum collections.

The importance of the EDII fusion peptide as an immunodominant antigenic region containing a series of overlapping epitopes stimulating broadly cross-reactive antibodies has been well established in mice (Crill and Chang, 2004; Crill, Trainor, and Chang, 2007; Oliphant et al., 2007a; Stiasny et al., 2006; Trainor et al., 2007). The results presented in this report and other recent studies confirm the extension of this observation to humans as well (Lai et al., 2008; Oliphant et al., 2007a; Stiasny et al., 2006). In this study IgG recognizing EDII fusion peptide epitopes ranged from undetectable to 94% of the E-specific IgG response and averaged 36% (Table 2.5). Stiasny et al also found

variable levels of EDII fusion peptide recognizing IgG in DENV-2 infected sera; they did not quantify this variation but estimated the proportion of IgG recognizing these epitopes to average about 30% and confirmed the limited neutralizing capability of these antibodies. Because their study examined convalescent phase sera, the similar percentage estimates of IgG targeting EDII fusion peptide epitopes in these studies suggest that the high concentrations of these cross-reactive antibodies are long-lived. This observation is supported by flavivirus serodiagnostic studies identifying high levels of cross-reactivity in IgG assays (Chiou et al., 2008a; Johnson et al., 2000; Kuno, 2003). Interestingly, although 4-7 months is not particularly long, the continued persistence of large populations of weakly or non-neutralizing cross-reactive antibody is relevant to antibody-dependent enhancement of infection and its potential role in increasing DENV disease severity leading to DHF/DSS (Gonzalez et al., 2005; Guzman et al., 2007; Halstead, 2003). Because antibody stimulated from EDII fusion peptide epitopes tends to be broadly cross-reactive, it is not surprising that the immunodominant nature of EDII fusion peptide epitopes is also supported by recent studies of WNV-infected human sera (Oliphant et al., 2007a; Throsby et al., 2006).

We examined both primary and secondary DENV-2 infected patient serum and found EDII fusion peptide specific IgG was greater in primary than in secondary infections (mean= 44% and 35% respectively; Table 2.6). Lai et al. (Lai et al., 2008) recently used western blots to examine late acute-early convalescent phase sera from DENV-2 infected patients from Taiwan. They also observed that the majority of the cross-reactive antibody response targeted epitopes in the highly conserved EDII fusion peptide. Moreover, Lai et al found that the EDII-fusion peptide specific antibody

response was greater in primary than in secondary DENV-2 infected patients, although they assayed total immunoglobulin and did not distinguish between IgM and IgG in their assays. The results presented in this report suggest that the increased cross-reactivity observed in primary relative to sera from secondary DENV-2 infected patients results more from IgM than from IgG. IgM populations targeting these cross-reactive epitopes in primary infections were larger than IgG populations, and averaged twice as large as EDII fusion peptide specific IgM in sera from secondary infected patients (54% and 24% of IgM in primary and secondary infections respectively, Table 2.6).

IgM responses to cross-reactive EDIII epitopes were similar in variability and magnitude as those for the EDII fusion peptide, but the proportion of IgG recognizing these epitopes was much smaller (Table 2.5 and 2.6). Flavivirus immune responses targeting EDIII have not been examined in humans to the extent that they have for EDII fusion peptide epitopes, and neither of the DENV-2 infected patient sera studies cited above examined immune responses to EDIII. Oliphant et al. (Oliphant et al., 2007a) recently studied epitope-specific immune responses in convalescent phase sera from WNV-infected patients, examining both total EDIII IgG and EDIII WNV-specific IgG, the latter epitope being homologous to that examined for DENV-2 in this report. Total IgG recognizing EDIII epitopes in these WNV-infected sera averaged 7.3% and ranged from 0.6% to 50.5%, suggesting that the relatively small yet variable magnitude cross-reactive EDIII IgG response we estimated from DENV-2 infected sera (mean=5.8%, range <1% - 26%) could be a generalized response to flavivirus infection. More studies are needed to determine if the larger magnitude cross-reactive IgM response relative to IgG and the greater cross-reactive EDIII IgM in secondary relative to primary DENV-2

infections found in this study are typical for dengue or other flaviviruses. If so, the use of IgM titers or P/Ns (OD value of positive sera/OD value of negative sera) alone to attempt to assign current infecting serotype in secondary DENV infections could be highly misleading. Recent detailed IgM and IgG serodiagnostic analyses of a large number of primary and secondary DENV infected serum specimens confirm this observation (Chang et al, unpublished results).

Lai et al (Lai et al., 2008) examined late acute to early convalescent serum specimens from DENV-2 infected patients from Taiwan, similar to the Taiwanese serum specimens from this study. Using western blot to assay total immunoglobulin they estimated the DENV-2 specific anti-E protein response in primary DENV-2 infected sera to range from 0% to 8.6% with a single specimen estimated at 21.5%. They did not attempt to identify the epitopes recognized by these DENV-2 specific antibodies. Our results in sera from primary DENV-2 infected patients were similar (<1% - 9%) and indicate that the more of the DENV-2 specific immunoglobulin is IgM, with little IgG (mean = 4% and <1% respectively). We found the same pattern in sera from secondary DENV-2 infected patients, but the magnitude of IgM and IgG responses averaged twice as large as in primary infections. Oliphant et al (Oliphant et al., 2007a) also found small proportions of EDIII recognizing virus specific IgG in WNV infected sera (mean = 1.6%, range 0% - 6.8%), they did not, however, investigate WNV-specific epitopes outside EDIII.

We were able to demonstrate for the first time that there is a significant and positive correlation between the magnitude of EDIII DENV-2 specific IgG titer and the percent of DENV-2 specific neutralization in humans. In WNV infections, however,

there was no correlation between overall levels of EDIII WNV-specific IgG and clinical outcome or measurable difference in neutralization profiles between WT and EDIII WNV-specific knock-out reporter virus particles, suggesting a limited role for EDIII virus-specific IgG in WNV protective neutralization (Oliphant et al., 2007a). One possible explanation for this apparent discrepancy between the importance of EDIII virus specific IgG as a correlate of protection in DENV-2 and WNV is the large titer differences between these two antibody populations in these studies, despite averaging about 1% of the IgG response against each virus. Total DENV-2 IgG titers, and hence EDIII-DENV-2 specific IgG, ranged from 10-100-fold greater than WNV IgG in the primary DENV-2 infected sera and up to 1000-fold greater in sera from secondary DENV-2 infected patients. A definitive test to see if these minor IgG populations are indeed protective would be to examine homologous DENV-2 (or WNV) secondary sera and determine if there is a marked anamnestic increase in this antibody population and if it is associated with increased protection. Alternatively, there could simply be a difference between DENV-2 and WNV in the importance of EDIII virus specific IgG and neutralization or protection. Although by no means definitive, our results suggest that the EDIII virus-specific epitopes identified as strongly neutralizing and protective in mice could play a similar role against DENV-2 infection in humans. The conclusions and questions stemming from the results presented in this report begin to disentangle the complex polyclonal humoral immune responses to primary and secondary DENV infections and point a direction for future studies in this field that will be essential both for improving our understanding of DENV pathogenesis and for the development and testing of candidate DENV vaccines.

Methods

Cell culture, construction of plasmids and virus-like particle (VLP) production.

COS-1 cells (ATCC CRL 1650; Manassas, VA) were grown at 37°C with 5% CO₂ on Dulbecco's modified Eagle's minimal essential medium (D-MEM, GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT), 110 mg/l sodium pyruvate, 0.1 mM nonessential amino acids, 2mM L-glutamine, 20 ml/l 7.5% NaHCO₃, 100U/ml penicillin, and 100ug/ml streptomycin. We used the recombinant expression plasmid pVAXD2i derived from pCBD2-2J-2-9-1, which has been previously characterized and described in detail (Chang et al., 2003; Crill and Chang, 2004). This derivation is the same as same as described for constructing pVJE from pCBE previously (Chiou et al., 2008a). COS-1 cells were electroporated with WT pVAXD2i and mutant plasmids using the protocol described in (Chang, Hunt, and Davis, 2000). Electroporated cells were recovered in 50 ml DMEM, seeded into three separate 75 cm² culture flasks for VLP expression and incubated at 28°C with 5% CO₂. Tissue-culture medium was harvested 4-5 days post transformation for VLP antigen characterization including triplicate measurements of secretion levels and MAb reactivity screening.

Selection and introduction cross-reactive epitope residue substitutions. Cross-reactive epitope residues selected for substitution in the fusion peptide were originally identified by Crill and Chang, (Crill and Chang, 2004) with numerous subsequent publications emphasizing the importance of overlapping immunodominant epitopes in this region eliciting cross-reactive antibodies (Crill, Trainor, and Chang, 2007; Goncalvez, Purcell, and Lai, 2004; Oliphant et al., 2007b; Oliphant et al., 2006;

Roberson, Crill, and Chang, 2007b; Stiasny et al., 2006; Trainor et al., 2007). E protein domain III (EDIII) prospective cross-reactive epitope residues were also selected based on previously described procedural algorithms (Crill and Chang, 2004; Trainor et al., 2007). Using this structure-based design approach we identified 22 different probably EDIII cross-reactive epitope residues. We modeled the effects of side-chain substitutions at eight of these residues using the DENV-2 pdb E-protein structural coordinates and the swiss-model workspace selecting those with the highest probability of disrupting antibody binding without altering E-glycoprotein structural conformation, particle formation, or secretion (Modis et al., 2003) (<http://us.expasy.org/spdbv/>). Applying these criteria we selected 15 amino acid substitutions to introduce at these eight EDIII residue positions (Table 2.1).

Site-specific mutations were introduced into the DENV-2 E gene using the Stratagene Quick Change® multi site-directed mutagenesis kit (Stratagene, La Jolla, CA) and pVAXD2i as DNA template following the manufacturer's recommended protocols. The sequences of the mutagenic primers used for all constructs are listed in Table 1. Structural gene regions and regulatory elements of all plasmids were sequenced entirely upon identification of the correct mutation. Automated DNA sequencing was performed using a Beckman Coulter CEQ™ 8000 genetic analysis system (Beckman Coulter, Fullerton, CA) and analyzed using Beckman Coulter CEQ™ 8000 (Beckman Coulter) and Lasergene® software (DNASTAR, Madison, WI).

Characterization of wild-type (WT) and mutant pVAXD2i secreted VLP antigen.

Antigen-capture ELISA (Ag-ELISA) was used to detect and quantify secreted antigen from the mutagenized and WT pVAXD2i transformed COS-1 cells. Secreted antigen

was captured in the inner 60 wells of Immulon II HB flat-bottom 96-well plates (Dynatech Industries, Inc., Chantilly, VA) with polyclonal rabbit anti-DENV-2 WT VLP sera, incubated overnight at 4°C, and wells were blocked with 300µl of StartBlock blocking buffer (Pierce, Rockford, Ill.) according to the manufacturer's recommended protocol. Antigen was diluted 2-fold in PBS, incubated for 2 hr at 37°C and detected with murine hyper-immune ascitic fluid (MHIAF) specific for DENV-2 diluted in 5% milk/PBS. MHIAF was detected using horseradish peroxidase conjugated goat anti-mouse IgG (Jackson ImmunoResearch, Westgrove, PA) in 5% milk/PBS and incubated for 1 hr at 37°C. Bound conjugate was detected with 3,3',5,5'-tetramethylbenzidine substrate (TMB; Neogen Corp., Lexington, KY), the reaction was stopped with 2N H₂SO₄ and measured at A₄₅₀ using a Synergy HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VA). WT and mutant antigens were screened against the MAb panel using the same ELISA protocol as above with the exception that 2-fold dilutions of the specific MAb replaced the anti-DENV-2 MHIAF and antigens were used at a single standardized concentration producing an optical density (OD) of 1.0 in the secretion ELISA. Standardized concentrations of WT and mutant VLP antigens were analyzed in Ag-ELISA to determine MAb end point reactivities (Roehrig, Bolin, and Kelly, 1998).

Monoclonal antibodies. MAbs 4G2, 6B6C-1, 4A1B-9, 1B7, D3-5C9-1, 1A1D-9, 9D12, 10A4D-2, 1B4C-2, 9A3D-8, and 3H5 were obtained from hybridomas in the collection of the Arbovirus Diseases Branch, Division of Vector-borne Infectious Diseases, US Centers for Disease Control and Prevention (CDC). Many of these MAbs originated from the work of John Roehrig, 4G2, 1B7, 9D12, and 3H5 hybridomas were originally

obtained by the CDC from the Walter Reed Army Institute (Henchal et al., 1985). MAbs 23-1, 23-2, 20, 5-1 and 5-2 were provided by Dr. L.-K. Chen of Tzu Chi University, Hualien, Taiwan.

Serological characterization of sera from DENV-2 infected humans. DENV-2 infected human sera from dengue fever patients were obtained from the Dengue Branch, US Centers for Disease Control and Prevention, San Juan, Puerto Rico; and also from the Taiwan Center for Disease Control. The Puerto Rican DENV-infected sera were collected from Puerto Ricans infected locally during the 2007 transmission season and the Taiwanese sera were from Taiwanese residents who contracted DENV-2 while traveling in SE Asia outside of Taiwan in 2005. All sera were confirmed DENV-2 positive by either virus isolation or RT-PCR either from these specimens or from paired acute-phase sera (data not presented and collected in either Puerto Rico or Taiwan).

Sera were assayed for the presence of E-specific immunoglobulins with both IgM and IgG antigen capture ELISAs (MAC- and GAC-ELISA). All VLP antigen concentrations were standardized using the same anti-DENV-2 polyclonal rabbit sera capture and MHIAF detection ELISA and methods described above in antigen characterization. Antigen concentrations were standardized at an OD of 1.4, within the region of antigen excess near the upper asymptote of the sigmoidal OD curve. MAC- and GAC-ELISA were performed as previously described with some modifications (Roberson, Crill, and Chang, 2007b). Briefly, Immulon II HB flat-bottom 96-well plates were coated overnight at 4°C with goat anti-human IgM or IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and blocked with StartBlock. DENV-2 infected patient sera and positive and negative control sera were diluted 1:1000 in wash buffer, added to

wells and incubated at 37°C for 90min. WT DENV-2 and negative control antigens were diluted appropriately in wash buffer tested against each serum sample in triplicate and incubated overnight at 4°C. DENV-2 virus-infected MHIAF was diluted in 5% milk/PBS and incubated for 1h at 37°C. Horseradish-peroxidase conjugated goat anti-mouse IgG was diluted in 5% milk/PBS, 50µl, added to wells and incubated for 1h at 37°C. Bound conjugate was detected with TMB substrate and plates incubated at room temperature for 8 min. The reaction was stopped with 2N H₂SO₄ and OD was measured at A₄₅₀.

P/N ratios were calculated as previously described with test validation utilizing internal positive and negative control sera on each plate (Martin et al., 2002). Positive values were determined as the average OD for the patient serum sample reacted with WT DENV-2 antigen and negative values as the average OD of the normal human control serum with WT DENV-2 antigen. The primary or secondary DENV infection status of each specimen was determined by calculating the ratio of IgM/IgG OD each subtracted by two times the corresponding negative value; ratios ≥ 1.0 are indicative of primary infections and ratios < 1.0 of secondary infections, Table 2.3).

Determination of E protein epitope-specific immunoglobulin populations in sera from DENV-2 infected humans. To quantify the epitope-specific humoral immune response following DENV-2 infection we used the same MAC- and GAC-ELISA format described above and serially diluted sera from 1×10^3 to 1×10^8 . In addition to WT DENV-2 VLP antigen we utilized a series of epitope-specific knock-out VLPs characterized in this study (Table 2.2). Relative concentrations of WT and epitope-specific knock-out antigens were standardized using the same rabbit sera-capture, MHIAF-detection ELISA format described in the P/N analysis above. Sera were diluted

and tested with WT, five different epitope-specific knock-outs, and negative antigen in duplicate at each serum dilution. The five mutant DENV-2 VLP antigens used were an EDII fusion peptide G106R-L107D cross-reactive knock-out mutant (RD), an EDIII K310E-E311R-P364R complex cross-reactive knock-out mutant (ERR), EDII-EDIII combination of these two mutants G106R-L107D-K310E-E311R-P364R (RD-ERR) and two single EDIII mutants K305E and K388D (Table 2.2).

The resulting OD data were modeled as a nonlinear function of the \log_{10} dilution using a four-parameter logistic model. In a four-parameter logistic curve, the lower horizontal asymptote is not constrained to zero. This model was chosen over the three-parameter model (which does constrain the lower asymptote to zero) because although the sera can be diluted to the point where there is no remaining immunoglobulin, the OD values are not expected to reach zero. The model was fit with the S-plus (v8.0) function `nls` that uses least squares to estimate the parameters. Sera, immunoglobulin type, and antigen type were included as covariates. After fitting the model, endpoints were computed by finding where the OD curves for each antigen type crossed the OD curve for two times the negative control values at each serum dilution. The asymptotic covariance matrix V for the endpoints was computed using the multivariate delta method (Lehmann, 1998). Using V , general least squares was used to regress endpoints on antigen type, IgM/IgG, primary/secondary infection status, and Taiwan/Puerto Rico geographic origin class. Statistically significant main effects and two- and three-way interactions were investigated further with multiple comparisons using Scheffe's method.

The model could not satisfactorily fit the data for Taiwan sera #16 into a logistic curve, due to its very low positive OD signal, since our highest concentration serum

dilution (1:1000) only captured the tail end of the actual curve of signal vs. antibody concentration. Nevertheless, for both WT and all three EDIII mutant antigens there was positive OD signal greater than two times the negative value at the higher serum concentrations. For this serum sample we therefore used the following linear interpolation of the data to estimate the endpoint cut-offs, endpoint = (mean OD of the antigen of interest at the last positive serum dilution divided by two times the negative antigen OD at the same serum dilution) multiplied by the last positive dilution. Hence this is the percent remaining positive signal at the last positive dilution times that last positive dilution.

Epitope-specific IgM and IgG percentages were calculated by dividing the immunoglobulin end-point value obtained with a specific knock-out antigen by that obtained with the WT antigen on the same sera, subtracting this value from 1.0 and multiplying by 100. The two EDIII single-mutant antigens were included to examine human immune responses to the protective EDIII virus-specific neutralizing epitope indentified in murine studies and were analyzed slightly differently. K305E knocks out reactivity to neutralizing DENV-2 type-specific MAbs (e.g. 3H5 in this study) and complex cross-reactive MAbs such as 1A1D-2, Table 2.2 (Gromowski and Barrett, 2007; Lok et al., 2008). K388 is not incorporated into the DENV-2-specific epitope typified by MAb 3H5 and the K388D substitution knocks out only complex cross-reactivity exemplified by MAb 1A1D-2 (Table 2, Fig. 2; (Lok et al., 2008). Thus, the percent immunoglobulin recognizing the DENV-2 specific EDIII neutralizing murine epitope was calculated as $100 \times 1.0 - [(K305 \text{ endpoint}/WT \text{ endpoint}) - (K388D \text{ endpoint}/WT \text{ endpoint})]$. In a few cases the titer measured with a mutant antigen was the same or

greater than that with the WT antigen. We interpreted these cases as undetectable levels of antibody recognizing this epitope and the percent serum antibody recognizing this epitope for further analysis was conservatively set to 1%.

Focus-Reduction Microneutralization Assays. We utilized an immunostaining micro-neutralization assay to measure the neutralizing capability of the human serum samples against all four DENV serotypes and JEV and WNV. 2.47×10^4 Vero cells in DMEM were added to 96 well black, clear flat bottom plates and incubated 16hr overnight at 37°C and 5% CO₂. Serum specimens were diluted 1:50 in BA-1 and heat inactivated at 56°C for 30 minutes, diluted 2-fold to a final dilution of 1:1600 and 320 virus pfu was added to each serum dilution. Plates were then incubated for 1hr at 37°C, 5% CO₂. After incubation, serum and virus suspensions were transferred back to Vero cell monolayer containing plates. These Vero cell plates were incubated at 37°C, 5% CO₂ for 45 minutes rocking every 5 minutes to allow for virus infection. Barry's Ye Lah overlay media containing 6% sodium bicarbonate and 1% Carboxymethylcellulose sodium salt (Fluka biochemical) was added and plates were incubated at 37°C, 5% CO₂. Incubation times were as follows: WNV (NY-99), JEV (SA-14-14-2): 24 hr; DENV-2 (16681) and DENV-4 (H241): 48 hr; DENV-1 (56BC94/95), DENV-3 (116RC1396): 70 hr. Following incubation plates were washed and fixed with 3:1 acetone, then decanted and plates were allowed to dry overnight. Immunostaining was performed by adding virus-specific MHIAFs diluted in PBS and incubated at 37°C for 30 min, washing and adding goat anti-mouse HRP diluted in 5% milk/PBS and similarly incubated for 30 min. and washed. Infected virus foci were visualized using Vector-VIP peroxidase substrate kit SK-4600 as per manufacturers' instructions. Foci were counted using Zeiss KS300

microscope and Axiovision software version 4.6. 50%, 75% and 90% FR μ NT dilution titers were calculated for each virus relative to the back that virus back-titration in BA-1. The predicted 90% FR μ NT titers were determined using Graph pad Prism version 4 (Graph Pad Software, San Diego, CA) sigmoidal dose response (variable slope) formula. All reported values are the average of two independent replicates.

Chapter 3

Dengue vaccine candidates reduce potential antibody-dependent enhancement

Introduction

Dengue virus (DENV) is a mosquito-borne flavivirus of global public health concern as two-fifths of the world's population live in DENV endemic/epidemic regions (Farrar et al., 2007). Infection with any one of the four closely related dengue virus (DENV) serotypes (DENV-1 to -4) can result in a wide range of clinical symptoms from subclinical, to classic dengue fever (DF), to life threatening dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), characterized by a plasma leakage syndrome. Epidemiological evidence indicates severe DHF is associated with secondary DENV infection (Guzman et al., 1990; Sangkawibha et al., 1984). Although the mechanism leading to severe disease is not fully understood, it is thought to be multifactorial. Two non-mutually exclusive immunopathological mechanisms to describe the events leading to DHF/DSS are antibody-dependent enhancement of infection (ADE) (Halstead and O'Rourke, 1977), and the involvement of cross-reactive memory T cells (Mongkolsapaya et al., 2003; Rothman and Ennis, 1999), known as original antigenic sin (Mongkolsapaya et al., 2003). ADE occurs when cross-reactive, weakly-neutralizing antibodies bind to viruses and form antibody-virus complexes that enhance infection of Fcγ receptor bearing cells, resulting in an increase in virus load associated with severe disease (Wang et al., 2003). The hypothesis of original antigenic sin involves proinflammatory cytokine secretion by cross-reactive memory T cells. These cross-reactive T cells from a primary

infection are of low-avidity to the secondary infecting virus serotype but are preferentially expanded during a secondary infection, leading to the release of proinflammatory cytokines.

The envelope (E) glycoprotein is responsible for eliciting the majority of the protective antibody response. The E protein covers the virion surface, arranged in anti-parallel dimers containing three structural and antigenic domains (Rey et al., 1995). E protein domain I (EDI), the central domain, contains virus specific and cross-reactive, predominantly non-neutralizing epitopes; EDII, the dimerization domain, contains the internal fusion peptide (FP) and overlapping immunodominant, broadly cross-reactive, weakly-neutralizing epitopes; EDIII has an immunoglobulin-like fold that is involved with receptor binding, and contains serocomplex cross-reactive and serotype specific potent neutralizing epitopes (Crill and Chang, 2004; Crill and Roehrig, 2001; Lisova et al., 2007; Matsui et al., 2008; Roehrig, Bolin, and Kelly, 1998; Sukupolvi-Petty et al., 2007). Recent studies suggest that broadly cross-reactive antibodies directed against the fusion peptide (FP) form a major proportion of the antibody response in DENV patients (Crill et al., 2009; Lai et al., 2008; Oliphant et al., 2007a; Stiasny et al., 2006). The dominant production of high avidity, weakly-neutralizing antibodies could compete with neutralizing antibodies, recognizing overlapping epitopes through steric interference (Ndifon, Wingreen, and Levin, 2009) and promote severe disease through ADE. Structural studies of immature West Nile virus virions suggest cross-reactive weakly-neutralizing antibodies recognizing the FP bind with high affinity to immature and partially mature virions but do not bind effectively to mature virions (Cherrier et al.,

2009). These data suggest that broadly cross-reactive antibodies could promote ADE of DENV infection by increasing infectivity of low infectious, partially mature virions.

DENV vaccine development has been a WHO priority for over thirty years. Despite all efforts, a licensed DENV vaccine is not yet available. A DENV vaccine must induce a balanced, protective, tetravalent immunity to reduce the possibility of vaccine-related severe disease upon subsequent infection. We have designed DENV-2 DNA vaccine candidates, pVD2i, based upon a previously described DENV-2 DNA vaccine expressing prM and E proteins, which was shown to protect mice from DENV-2 challenge (Chang et al., 2003) and contains an intron modification to increase antigen secretion. These candidates are engineered with specific substitutions in immunodominant E protein B cell epitopes that reduce the recognition by a panel of murine monoclonal antibodies (MAbs) (Crill et al., 2009). By introducing specific substitutions into the FP (at G106 and L107) and into serocomplex cross-reactive epitopes of EDIII (at K310, E311 and P364), we have constructed cross-reactivity reduced (CRR) DENV-2 DNA vaccine candidates in an attempt to dampen or eliminate the induction of cross-reactive, enhancing antibodies recognizing weakly or non-neutralizing epitopes.

Results and Discussion

The immunologic profile stimulated by the wild-type vaccine pVD2i (WT) in mice is compared to that of CRR vaccines: pVD2iG106R/L107D (RD) containing substitutions in EDIIFP; pVD2iK310E/E311R/P364R (ERR) containing substitutions in EDIII; and pVD2iG106R/L107D/K310E/E311R/P364R (RDERR) a combination of both FP and EDIII substitutions. Mice were immunized at 0-week and 5-weeks. Ten weeks

post vaccination we evaluated the immunogenicity of WT and CRR vaccines using a focus-reduction micro-neutralization assay (FR μ NT) against DENV-2 (Figure 3.1a). All vaccinated mice exhibited high levels of DENV-2 specific neutralizing antibody (Nt-Ab) with mean reciprocal 50% endpoint titers ranging from 211 to 378. Although there was a significant difference in DENV-2 Nt-Ab titer in mice between the vaccine treatments as determined by an analysis-of-variance (p=0.03), a Tukey's post-test revealed only RD vaccinated sera to be significantly different from RDERR vaccinated sera (p=0.02). In

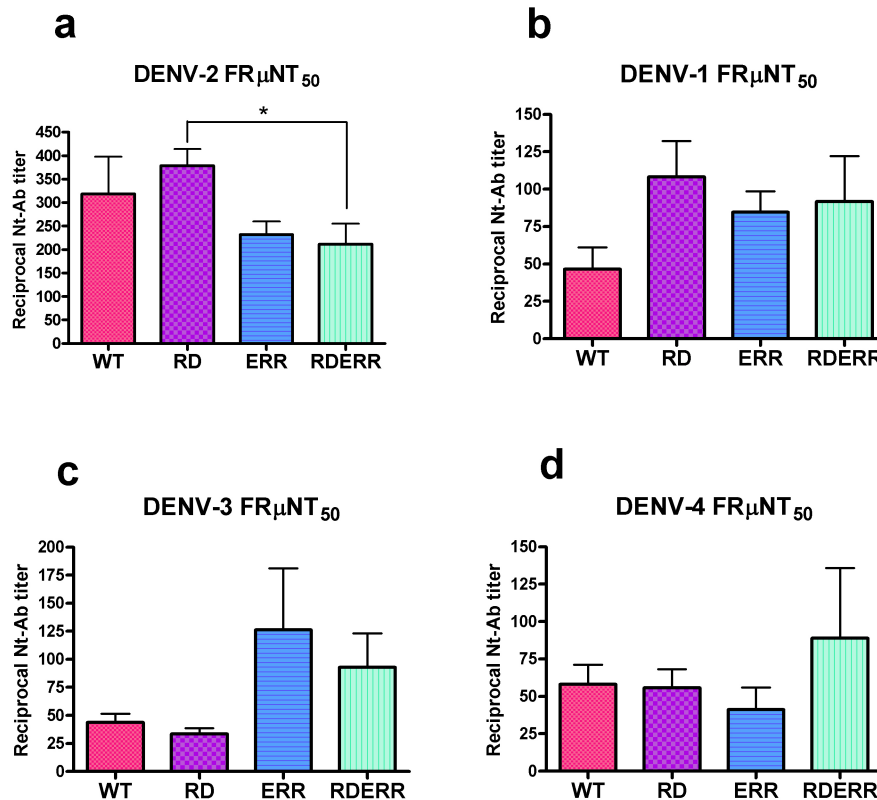


Figure 3.1. CRR vaccines elicit neutralizing IgG. **a)** DENV-2 neutralizing-antibody (Nt-Ab) titers elicited by WT and CRR vaccines as determined by 50% FR μ NT. **b)** DENV-1 Nt-Ab titers. **c)** DENV-3 Nt-Ab titers. **d)** DENV-4 Nt-Ab titers. Data are expressed as mean \pm s.e.m. for n=10. ANOVA were performed on log transformed data for each neutralization followed by a Tukey's post-test; p<0.05 were considered significant; single asterisk, p<0.05.

CRR vaccines containing substitutions in EDIII, ERR and RDERR, there was a trend toward a decrease in neutralization relative to WT. This supports the observation that antibodies against the FP are weakly neutralizing, while antibodies against EDIII can be more potent (Heinz et al., 1983). Interestingly, CRR vaccines displayed a trend to increase the capability to neutralize heterologous DENV-1 ($p=0.07$), DENV-3 ($p=0.27$), and DENV-4 ($p=0.47$) (Figure 3.1 b-d).

The FP of EDII is immunodominant and stimulates a large proportion of the total E protein antibody response, with antibodies that have limited neutralization capabilities (Stiasny et al., 2006). These antibodies have been implicated in ADE (Cherrier et al., 2009) and could block the binding of neutralizing antibodies through steric interference (Ndifon, Wingreen, and Levin, 2009). Recent studies demonstrate the capability of FP recognizing antibodies to induce severe DENV disease similar to DHF via ADE in a mouse model (Zellweger, Prestwood, and Shrestha, 2010). Studies with immunotoxin and other therapeutic proteins have shown amino acid substitutions can dampen the immunogenicity of B cell epitopes (Onda, 2009; Onda et al., 2008). We utilized an epitope-specific IgG ELISA (Crill et al., 2009) to determine if substitutions in the EDIIFP reduced or eliminated its immunodominance, (Table 3.1). Similar to studies examining DENV infected human sera (Crill et al., 2009; Lai et al., 2008; Oliphant et al., 2007a; Stiasny et al., 2006), the WT vaccine elicited a large proportion of the total E response directed against the EDIIFP epitope (average 67%), which was highly variable between individual outbred mouse sera. The response against the serocomplex cross-reactive epitopes of EDIII (EDIICR) made up a much smaller proportion (average 6%), and the response towards epitopes outside EDIIFP and EDIICR was a moderate

Table 3.1. WT and CRR vaccine epitope-specific IgG antibody responses.

Vaccine	Specific Epitope ¹	Endpoint mean ²	Endpoint range	% response mean ³	% response range ³
pVD2i	E	1.77x10 ⁵	8.60x10 ⁴ -2.91x10 ⁵	100	100
	EDIIFP	5.95x10 ⁴	6.95x10 ³ -1.16x10 ⁵	67	45-94
	EDIICR	1.73x10 ⁵	1.02x10 ⁵ -2.71x10 ⁵	6	<1-16
	Remaining	5.02X10 ⁴	1.42X10 ⁴ -1.87X10 ⁵	20	<1-76
RD	E	4.26x10 ⁵	7.14x10 ⁴ -1.24x10 ⁶	100	100
	EDIIRD	1.61x10 ⁵	2.91x10 ² -9.77x10 ⁵	67	<1-99
	EDIICR	1.41x10 ⁵	1.19x10 ² -7.61x10 ⁵	26	<1-98
	Remaining	1.4X10 ⁵	1.2X10 ² - 7.1X10 ⁵	34	<1-100
ERR	E	2.19X10 ⁵	1.03X10 ² -5.71X10 ⁵	100	100
	EDIIFP	9.09x10 ⁴	1.29x10 ² -1.78x10 ⁵	81	59-100
	EDIIIERR	2.00x10 ⁵	1.08x10 ² -5.16x10 ⁵	5	<1-28
	Remaining	9.10x10 ⁴	1.02X10 ² -1.78X10 ⁵	31	<1-64
RDERR	E	4.23X10 ⁵	7.68X10 ⁴ -1.29X10 ⁶	100	100
	EDIIRD	7.14x10 ⁵	1.16x10 ⁵ -2.25x10 ⁶	73	32-86
	EDIIIERR	9.06X10 ⁴	2.1X10 ⁴ - 2.75X10 ⁵	<1	<1
	Remaining	1.14X10 ⁵	2.9X10 ⁴ - 3.4X10 ⁵	38	21 -68

¹ Epitopes targeted by specific antibody populations. E denotes antibodies recognizing all E-protein epitopes. EDIIFP denotes broadly cross-reactive WT epitopes of the E domain II fusion peptide. EDIIRD denotes the epitope of the RD FP substitutions. EDIICR denotes WT serocomplex cross-reactive epitopes in EDIII. EDIIIERR denotes the epitope containing the ERR substitutions in EDIII. Remaining refers to the epitopes outside EDIIFP and EDIICR investigated in this study.

‡ Endpoint titers determined with knock-out antigens, thus representing immunoglobulins not targeted by the knock-out antigen.

* Percentages below limit of detection are expressed as <1.

proportion (average 20%) and also highly variable between mice. CRR vaccines containing substitutions in the FP (RD and RDERR) did not elicit antibodies that recognized epitopes encoded by the WT EDIIFP (Figure 3.2) and should therefore not induce ADE. A large proportion of the RD and RDERR vaccinated IgG response, however, recognized the novel EDIIRD FP antigenic region. The average proportion of

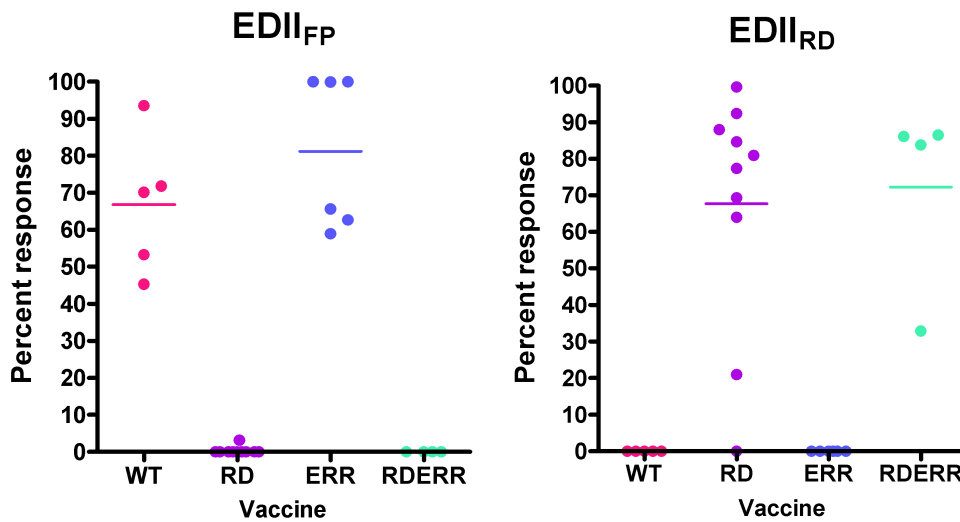


Figure 3.2. RD and RDERR vaccines do not elicit IgG recognizing the WT FP epitope. Percent of total anti-E antibody response that recognizes the epitopes of the EDIIFP WT epitopes or antibodies that recognize the EDIIRD substituted epitope.

antibodies recognizing the EDIIRD FP epitope were 67% and 73%, in RD and RDERR vaccinated mouse sera respectively, a proportion similar to that recognizing WT EDIIFP in WT vaccinated mice. This suggests that the substitution of G106R and L107D did not dampen or eliminate the immunodominance of the WT EDIIFP but created a novel epitope with distinct specificity. These findings are in agreement with Rajamani et.al. (Rajamani et al., 2004), who identified arginine as a main anchor residue in several protein-protein interactions. These data suggest instead of dampening the recognition of the EDIIRD FP epitope by B cell receptors, the substitution of G106R allowed anchoring of the B cell receptor and the subsequent production of antibodies. ERR vaccine, with substitutions in the serocomplex cross-reactive epitopes of EDIII, also elicited antibodies directed against the EDIIIERR epitope (average 5%) in proportions similar to that of WT vaccine recognizing EDIIICR. The response against the EDIIIERR epitope had a greater

variability than did the WT response against EDIIICR. Sera from mice vaccinated with RDERR did not contain antibodies directed against the ERR epitope. This suggests that the combination of EDIIFP and EDIII substitutions was able to reduce the antigenicity of the EDIII cross-reactive epitopes. Consistent with this, RDERR vaccinated sera displayed the greatest increase to stimulate antibodies recognizing E epitopes outside EDIIFP and EDIIICR (Table 3.1). This could be due to the lack of steric interference from cross-reactive antibodies, allowing for better binding of antibodies to E epitopes outside those we investigated.

Severe DHF is associated with secondary DENV infections in older children and adults, or primary infections in infants. We tested CRR vaccines to determine whether altered cross-reactive epitopes can increase vaccine safety by reducing the potential for ADE *in vitro*. FcR2 bearing human K562 cells were infected with DENV alone, with DENV-immune complexes formed in the presence of vaccinated mouse sera, or with DENV-immune complexes formed in the presence of EDIIFP recognizing MAb 4G2 (positive enhancing control). Sera from mice vaccinated with WT significantly enhanced the infection of homologous DENV-2 at a peak titer of 1:1,250 compared to RD ($p=0.01$), ERR ($p=0.021$), and RDERR ($p=0.01$) (Figure 3.3a), while the average 50% neutralization titer of WT vaccinated sera was 1:318. These results were interpreted to mean that as the IgG concentration decreases, the proportion of potentially neutralizing antibodies becomes insufficient to neutralize virus, allowing for ADE. This interpretation is evident in primary DHF in infants, where maternal DENV neutralizing antibody titers were correlated to infant age at onset of severe disease (Kliks et al., 1988) when maternally derived neutralizing anti-DENV IgG maintained reactivity with whole

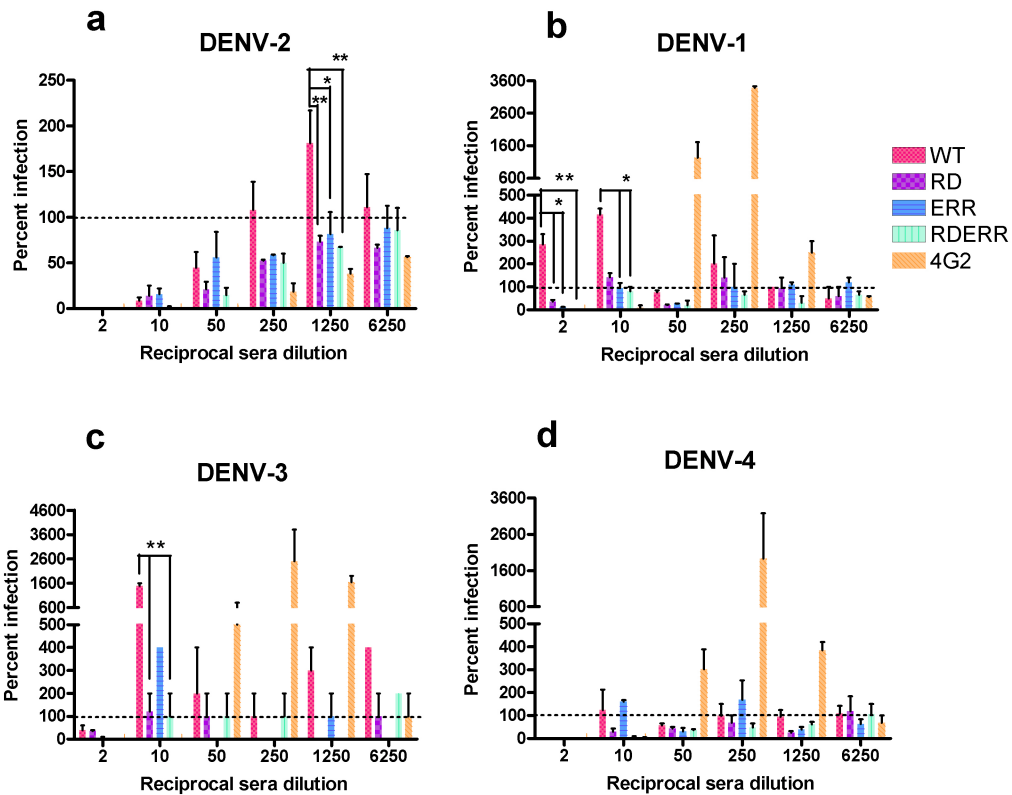


Figure 3.3. CRR vaccinated sera have a reduced potential to participate in ADE *in vitro* compared to WT vaccinated sera. Enhanced infection of K562 cells as determined by focus assay of cultured supernatants compared to the virus-only infection control (dotted line) **a)** DENV-2 enhancement reported as percent infection compared to the virus input control. **b)** DENV-1 enhanced percent infection. **c)** DENV-3 enhanced percent infection. **d)** DENV-4 enhanced percent infection. All data represent the mean \pm s.e.m. of two independent experiments. Two-way ANOVA were performed on square-root transformed data with a Bonfferoni post test; $p < 0.05$ were considered significant; single asterisk, $p < 0.05$; two asterisks, $p < 0.01$.

DENV virions but could not neutralize virus (Chau et al., 2009). This situation sets the stage for ADE and severe disease in infants. In contrast to antibodies in WT vaccinated mice, none of the CRR vaccinated sera enhanced homologous DENV-2 infection at any concentration. This could be due to the lack of cross-reactive antibodies that recognize WT enhancing epitopes (i.e. FP eptiopes). In heterologous ADE assays, sera from vaccinated with WT significantly enhanced DENV-1 infection at the lowest dilution

tested (1:2) compared to RD ($p=0.04$), ERR ($p=0.02$), and RDERR ($p=0.01$), and also enhanced DENV-1 infection at a 1:10 dilution compared to ERR ($p=0.04$) and RDERR ($p=0.03$). WT vaccinated sera also significantly enhanced DENV-3 infection at a dilution of 1:10 compared to RD ($p=0.01$) and RDERR ($p=0.01$) (Figure 3.3c), while no CRR vaccinated sera significantly enhanced any heterologous DENV at any dilution tested. Only RD and RDERR vaccinated sera significantly reduced DENV-3 enhancement, whereas the 400% increase seen in ERR vaccinated sera was not significantly different from WT vaccinated sera. This suggests a role of the immunodominant FP targeting antibody response in the enhancement of severe disease, because RD and RDERR vaccines do not produce antibodies that recognize WT EDIIFP. None of the serum from vaccinated mice significantly enhanced DENV-4 infection (Figure 3.3d). This agrees with the findings of Nisalak et al. (Nisalak et al., 2003) where DENV-4 was not associated with DHF outbreaks in Thailand, although 97% of DENV-4 isolates were from secondary infections.

In addition to reducing the circulating cross-reactive antibody response following vaccination, we investigated the potential role of memory B cells in secondary infection to increase the proportion of cross-reactive, potentially enhancing antibodies. Swiss Webster mice were vaccinated with 100 μ g of pVD2i WT or RDERR vaccines at week 0, boosted with 100 μ g DENV-1 DNA vaccine, pVD1i, on week 4 to simulate a secondary immune response, and sacrificed on week 12. The immune response to secondary stimulation by pVD1i was measured by epitope specific ELISA. Mice who were vaccinated with pVD2i WT followed by pVD1i elicited a large proportion of the antibody response toward the DENV-2 EDIIFP (average 90%) (Table 3.2) while mice

Table 3.2. WT and RDERR EDIIFP-specific antibody responses following secondary immunization with DENV-1.

Vaccine	Specific Epitope ¹	Endpoint mean ²	Endpoint range	% response mean	% response range ³
WT	DENV-2 EDIIFP	2.7X10 ²	1.0X10 ² -3.8X10 ²	90	76-98
	DENV-1 EDIIFP	8.0X10 ²	1.0X10 ² -1.8X10 ³	72	27-97
RDERR	DENV-2 EDIIRDFP	2.3X10 ³	7.5X10 ² -6.3X10 ³	46	14-70
	DENV-1 EDIIFP	2.6X10 ³	1.4X10 ² -7.6X10 ³	45	<1-91

¹ Epitopes targeted by specific antibody populations. E denotes antibodies recognizing all E-protein epitopes. EDIIFP denotes broadly cross-reactive WT epitopes of the E domain II fusion peptide. EDIIRD denotes the epitope of the RD FP substitutions.

²Endpoint titers determined with knock-out antigens, thus representing immunoglobulins not targeted by the knock-out antigen.

³ Percentages below limit of detection are expressed as <1.

who were vaccinated with pVD2i RDERR followed by pVD1i elicited a significantly lower percentage toward the cognate DENV-2 FP, EDIIFPRD (average 46%) (p=0.03) (Figure 3.4). Moreover, mice vaccinated with pVD2i WT followed by pVD1i elicited a large proportion of the antibody response that also recognizes the DENV-1 EDIIFP (average 72%) while mice vaccinated with pVD2i RDERR followed by pVD1i elicited a lower antibody response toward the DENV-1 EDIIFP (average 45%), though this difference was not significant due to the highly variable nature of the immune response toward the EDIIFP (Crill et al., 2009). Of particular interest is that mice vaccinated with pVD2i RDERR followed by pVD1i elicited a nearly identical proportion of antibodies directed against either the cognate DENV-2 antigen, EDIIFPRD, or the DENV-1 EDII antigen, suggesting that these mice developed a greater primary response toward

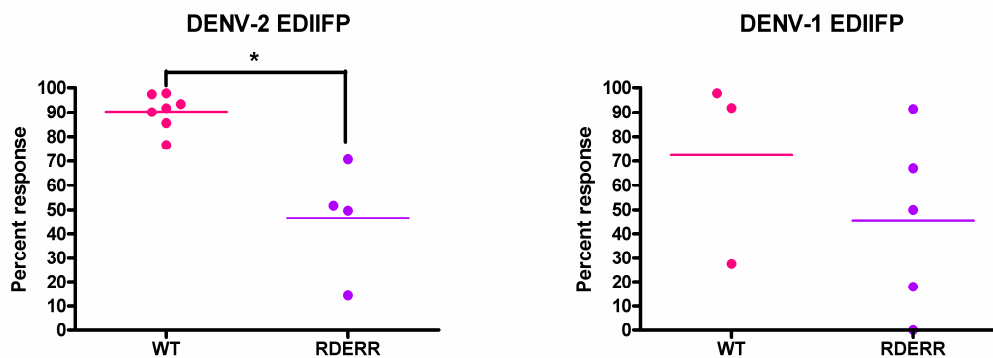


Figure 3.4. RDERR vaccine does not elicit memory B cells that recognize the EDIIFP. Mice were immunized with DENV-2 WT or RDERR at week 0 and reimmunized with WT DENV-1 DNA vaccine at 4 weeks. Sera were collected at week 12 and the percent of total anti-E antibody response that recognizes either the cognate DENV-2 EDIIFP (EDIIFP or EDIIFPRD) antigens or the DENV-1 EDIIFP antigen was determined. All graphed data represent the mean \pm s.e.m. A Student's t-test with Satterthwaite correction was used to make the appropriate comparisons. $p < 0.05$ were considered significant; single asterisk, $p < 0.05$.

stimulation with DENV-1. Together, these data suggest mice vaccinated with RDERR do not elicit memory B cells that can recognize the highly conserved EDIIFP and potentially act to reduce the potential of vaccine-induced ADE from the rapid expansion of memory B cells in a secondary infection.

Several studies have identified CD4⁺ and CD8⁺ T cell epitopes on DENV proteins, many of which have been identified on the non-structural proteins while few have been identified in E (Mathew et al., 1996; Roehrig et al., 1994; Rothman, Kurane, and Ennis, 1996; Wen, Duan, and Jiang). Many of the T cell epitopes identified in E have mapped to EDIII. To determine whether mice that received CRR vaccines had a reduced memory T cell response as compared to the response of WT vaccinated mice, mice were splenectomized 10 weeks post vaccination and single cell suspensions were made. The mixed leukocyte reactions (MLR) were stimulated with 2 μ g of inactivated

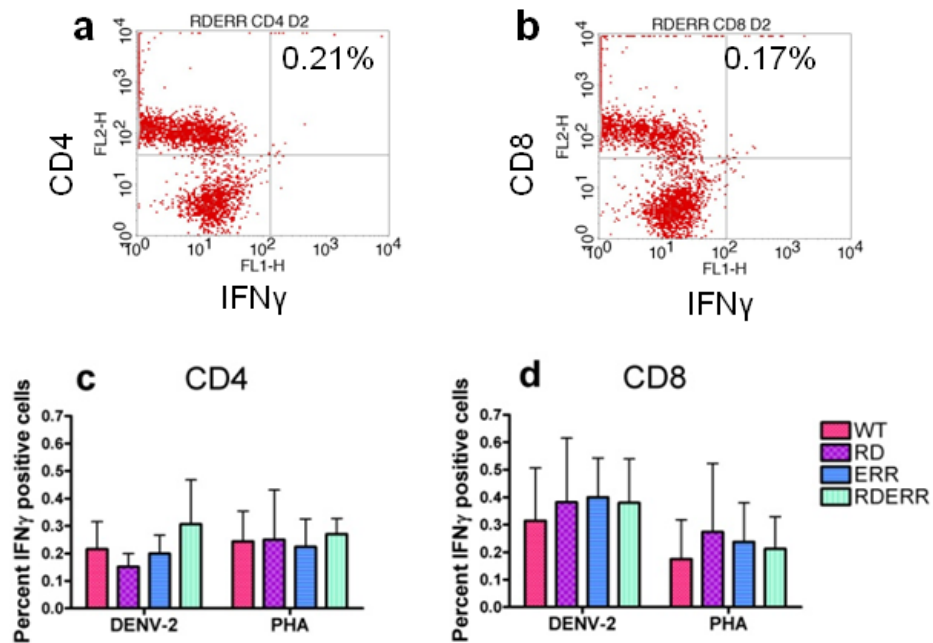


Figure 3.5. CRR vaccines elicit virus-specific memory CD4+ and CD8+ T cells similar to WT vaccine. **a)** Representative dot plot of CD4+ and IFN γ positive splenocytes. **b)** Representative dot plot of CD8+ and IFN γ positive splenocytes. **c)** Percent of CD4+, IFN γ positive gated splenocytes stimulated *ex vivo* with inactivated DENV-2 or PHA. **d)** Percent of CD8+ and IFN γ positive splenocytes. All data reported as mean \pm s.e.m. of four independent experiments. ANOVA was performed; $p < 0.05$ were considered significant.

DENV-2 or phytohemagglutinin (PHA) mitogen, the positive control, for 6 hours in the presence of brefeldin A and stained for CD4 or CD8 and interferon γ (IFN γ) (Figure 3.5).

Based upon flow cytometric analysis, splenocytes from CRR vaccinated mice elicited IFN γ producing CD4+ and CD8+ T cells in proportions similar to WT vaccinated splenocytes ($p > 0.05$) suggesting the substitutions introduced into E did not interfere with the CRR vaccine's ability to elicit DENV-2 specific memory T cells.

While DENV serotype specific CD8+ T cells have been shown to play a role in viral clearance in mice (Yauch et al., 2009), the primary focus of DENV T cell research has been to elucidate the immunopathogenesis induced by DENV cross-reactive T cells.

During secondary DENV infections, cross-reactive low-affinity CD4⁺ (Beaumier and Rothman, 2009) and CD8⁺ T cells (Beaumier et al., 2008; Mongkolsapaya et al., 2003) expand preferentially and secrete proinflammatory cytokines (Dong et al., 2007b). Moreover, DHF patients' sera have increased levels of tumor necrosis factor α (TNF α), interleukin-6 (IL-6) (Hober et al., 1993), and IL-10 (Green et al., 1999). To determine the response of CRR vaccinated splenocytes to DENV stimulation, MLR were stimulated with 2 μ g of UV inactivated DENV-1, -2, -3, or -4 for 18 hours. Supernatants were clarified and analyzed with a BDTM cytometric bead array (CBA) (Figure 3.6). MLR from WT vaccinated mouse splenocytes secreted high levels of TNF α , MCP-1, and IL-6 in response to DENV while CRR vaccinated mice secreted levels similar to those of the naïve control. RD vaccinated splenocytes secreted significantly reduced levels of IL-6 ($p=0.04$) and reduced levels of TNF α , and MCP-1 ($p>0.05$) compared to WT vaccinated splenocytes when stimulated with DENV-4. These results suggest CRR vaccines have a reduced potential to stimulate secretion of proinflammatory cytokines upon secondary stimulation with heterologous DENV. IL-6 increases vascular permeability (Avirutnan et al., 1998; Huang et al., 2000b), enhances the production of anti-platelet auto-antibodies, elevates levels of tissue plasminogen activator, and causes a deficiency of coagulation factor XII, (Lei et al., 2001) all of which could lead to the plasma leakage and bleeding seen in severe DENV infection. It has been observed that serum IL-6 levels correlate with human mortality from DSS (Suharti et al., 2002). All vaccinated splenocytes in our studies showed marked decreases in the production of IL-12p70 when compared to naïve animals. IL-12p70 has a profound effect on the

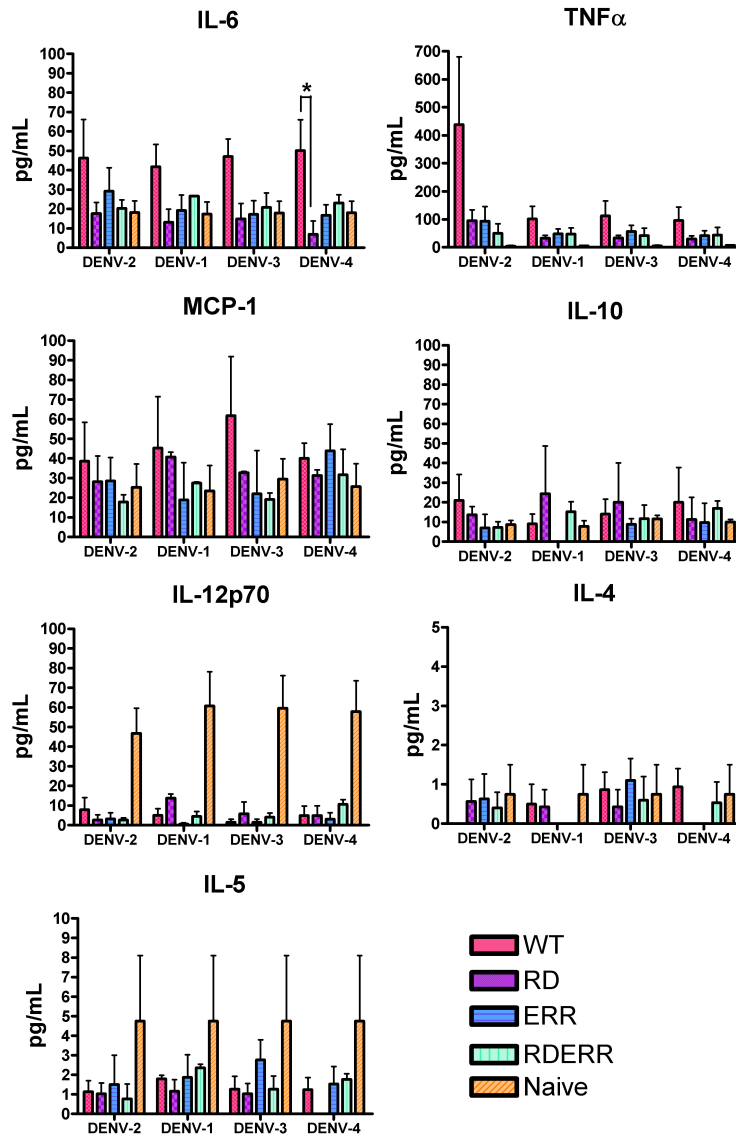


Figure 3.6. CRR vaccinated splenocytes secrete reduced levels of proinflammatory cytokines compared to WT vaccine. Secretion of cytokines into tissue culture medium by splenocytes from vaccinated mice stimulated *ex vivo* with inactivated DENV. All data reported as mean \pm s.e.m. of two or three independent experiments. Two-way ANOVA were performed on log transformed data with a Bonfferoni post test; p<0.05 were considered significant; single asterisk, p<0.05.

maintenance of a Th1 driven response and the switch from a Th1 to a Th2 driven response has been shown to associate with severe DHF/DSS (Chaturvedi et al., 2000).

None of the splenocytes harvested from any vaccinated mouse in this study produced levels of IL-4 or IL-5, Th2 driven cytokines, above levels of naïve animals (Figure 3.6).

To our knowledge, this is the first description of an approach to increase vaccine safety by limiting the production of cross-reactive antibodies. The presented data demonstrate the direct involvement of vaccine induced cross-reactive, weakly-neutralizing antibodies in the development of ADE *in vitro*. In addition, this same strategy may be expandable to other vaccine formats and other viruses with the potential to exacerbate disease through ADE such as HIV (Takeda, Tuazon, and Ennis, 1988). Unfortunately, this approach may not be applicable to DENV live-attenuated vaccines, since substitutions in the FP can be lethal (Huang et al., 2009).

The lack of an appropriate animal model has made it difficult to study the contribution of ADE in the development of severe disease *in vivo*. The immunopathology of DHF/DSS in regards to ADE has been difficult to fully elucidate and most studies have relied on *in vitro* systems. We have shown that the CRR vaccinated murine immune response, in regards to immunodominant epitopes, is similar in magnitude and proportion to studies of immunodominant epitopes in DENV infected humans (Crill et al., 2009; Lai et al., 2008; Oliphant et al., 2007a; Stiasny et al., 2006). We have demonstrated the importance of these eptiopes in inducing ADE *in vitro*. With the recent demonstration of ADE induced severe DENV disease in AG129 mice(Zellweger, Prestwood, and Shresta, 2010), our ongoing studies include increasing vaccine immunogenicity by introduction of dominant CD4 epitopes, the expansion of this

approach to the remaining three DENV serotypes, and the evaluation of protective efficacy and safety of CRR vaccines to prevent ADE in this new DENV disease model.

Materials and Methods

Vaccines. Characterization of DENV-2 DNA plasmids with substitutions in the EDIIFP and EDIII have been described and characterized previously (Crill et al., 2009).

Selection of vaccine candidates was based on virus-like particle secretion and reductions in reactivity to the MAb panel (Crill et al., 2009). Vaccines were manufactured by Aldevron.

Mice. Swiss Webster mice from the DVVID colony (n=10) were vaccinated with 100 µg intramuscularly at 0 and five weeks. Sera were collected 10 weeks post vaccination.

Swiss Webster mice (Charles River) (n=10) were similarly vaccinated with WT or RDERR vaccines at 0-week and similarly boosted with 100 µg pVD1i at 4-weeks. Sera were collected 12 weeks post vaccination.

C57BL/6J mice (Jackson laboratory) (n=5) were similarly vaccinated. 10 weeks post vaccination mice were splenectomized and single cell suspensions were made. Animal experiments were approved by Institutional Animal Care Use Committee.

Antibody-dependent enhancement. Heat inactivated mouse sera were pooled, diluted, and titrated. 5×10^3 pfu of virus was added to each sera dilution and incubated for 1 hour at 37°C. K562 cells (MOI=0.5) were added to the antibody-virus complexes and incubated 2 hours at 37°C. After infection, cells were centrifuged, supernatants removed, resuspended in RPMI media with 10% FBS and plated on 24-well plates. DENV

infection alone was used as virus control; MAb 4G2 was used as a positive enhancing antibody control.

Focus assay. Supernatants from DENV infected K562 cells were collected 48 hours post infection, clarified by centrifugation, and serially titrated. 2.47×10^4 Vero cells in DMEM (Gibco) with 10% FBS (Atlas) were added to 96-well black, clear flat bottom plates (Corning/Costar), and were infected with each titration in duplicate. Vero plates were incubated at 37°C, for 45 min, and overlaid with media containing 1% Carboxymethylcellulose sodium salt (Fluka BioChemika). Plates were incubated, cells acetone fixed and immunostained according to the FR μ NT assay procedure. Virus foci were counted using Zeiss KS300 microscope and Axiovision version 4.6 (Carl Zeiss) and pfu/mL of each original sera dilution calculated and compared to DENV infection alone as percent infection.

Virus neutralization. A focus reduction microneutralization (FR μ NT) technique was utilized. Briefly, 2.47×10^4 Vero cells in DMEM (Gibco) supplemented with 10% FBS (Atlas) were added to 96-well black, clear bottom plates (Corning/Costar) and incubated 16 hours overnight at 37°C. Vaccinated mouse sera (n=10) were diluted 1:10 and heat inactivated at 56°C for 30 min, titrated 2-fold to the volume of 40 μ L, and 320 virus pfu/40 μ L was added to each serum dilution. Plates were incubated 1 hour at 37°C. After incubation, 25 μ l of serum and virus suspension (100 pfu) was transferred to the Vero cell monolayer containing plates. Vero plates were incubated at 37°C for 45 min, overlaid with media consisting of 1% Carboxymethylcellulose sodium salt (Fluka BioChemika), Earle's balanced salt solution without phenol red, 6.6% Ye-lah media, 2% FBS (Atlas), 6% sodium bicarbonate (Gibco), and incubated at 37°C. Incubation times

were as follows: DENV-2 (16681) and DENV-4 (H241) 48 hours; DENV-1 (56BC94/95) and DENV-3 (116RC1396) 70 hours. Following incubation, plates were washed and fixed using 3:1 Acetone in PBS, decanted and allowed to dry. Immunostaining was performed by adding virus-specific mouse hyperimmune ascitic fluid diluted in PBS and incubated at 37°C for 30 min, washing and adding goat anti-mouse HRP conjugated (Jackson ImmunoResearch) secondary antibody diluted in 5% skim milk/PBS and similarly incubated for 30 min and washed. Infected virus foci were developed using Vector-VIP peroxidase substrate kit SK-4600 (Vector Laboratories) as per manufacturer's instructions. Foci were counted using Zeiss KS300 microscope and Axiovision software version 4.6 (Carl Zeiss). FR_μNT titers were calculated for each virus relative to the virus back titration. Exact FR_μNT titers were modeled using Graph Pad Prism version 4 (Graph Pad Software) sigmoidal dose response (variable slope) formula. Values are the average of two independent replicates and reported as 50% reduction.

Epitope-specific IgG ELISA. To quantify the epitope-specific IgG in vaccinated mouse sera, the same IgG-capture ELISA protocol as described previously (Crill et al., 2009) was used with a few modifications. Vaccinated mouse sera (n=10) were diluted 1:100 and serially titrated. In addition to WT DENV-2 virus-like particle antigen, we utilized previously characterized epitope-specific knock-out antigens (Crill et al., 2009). The resulting OD values were modeled as a non-linear function of the log₁₀ sera dilution using a Gaussian non-linear regression in Graph Pad Prism version 4.0 (Graph Pad Software) and the endpoint dilutions determined as the titer where the OD value was equal to two-times the OD value of the test serum reacted against COS-1 normal antigen.

The EDII and EDIII epitope-specific IgG percentages were calculated as described (Crill et al., 2009) with minor modification for CRR sera. Briefly, the EDII and EDIII epitope specific IgG percentages for WT vaccinated mice were calculated by dividing the IgG endpoint titer obtained with specific knock-out antigens by the endpoint titer obtained by WT antigen on the same sera, subtracting this value from 1.0 and multiplying by 100. Since CRR sera contained antibodies that do not recognize WT antigen (WT antigen acts as the knock-out antigen) but recognize epitopes of the knock-out antigens, the cognate knock-out antigen was used to determine 100% E reactivity (i.e. RDERR sera reacted on RDERR antigen represents 100% reactivity). CRR vaccinated epitope-specific percentages were calculated as $100 \times [1.0 - (\text{epitope knock-out antigen} / \text{RDERR antigen})]$.

The DENV-2 or DENV-1 EDIIFP specific IgG responses following secondary stimulation were calculated similarly with minor modification. Specific IgG percent for WT vaccinated mice followed by DENV-1 were determined utilizing DENV-2 EDIIFP knock-out antigen and DENV-1 EDIIFP knock-out antigen (Chang et al. unpublished data). Specific DENV-2 or DENV-1 EDIIFP percentages were calculated as $100 \times [1.0 - (\text{EDII knock-out antigen endpoint} / \text{DENV-2 WT antigen endpoint} + \text{DENV-1 WT antigen endpoint})]$. Mice vaccinated with RDERR followed by DENV-1, EDIIFP percentages were calculated utilizing DENV-2 EDIICR knockout antigen and DENV-1 EDIIFP knock-out antigen: $100 \times [1.0 - (\text{DENV-2 EDIICR knock-out antigen or DENV-1 EDIIFP knock-out antigen endpoints} / \text{RDERR} + \text{DENV-1 WT antigen endpoints})]$.

Mixed leukocyte reactions. Single cell suspensions made from freshly harvested spleens using a 0.7 μm cell strainer (BD Falcon) and red blood cells lysed with lysis

buffer (BD Biosciences). 1×10^6 cells in culture media, RPMI (Gibco) supplemented with 20% FBS (Atlas), were plated in 96-well plates for intracellular cytokine staining. 1×10^7 pooled splenocytes in culture media were plated in 24-well plates for CBA. Splenocytes were stimulated with 2 μ g of UV inactivated DENV; PHA (Roche Diagnostics) was used as a positive control and naïve splenocytes in cell culture medium as a negative control. 24-well plates were incubated 18 hours at 37°C, supernatants harvested, and clarified by centrifugation. 96-well plates were incubated 2 hours at 37°C, and 1 μ g of Golgi plug (BD Biosciences) was added to each well, incubated for an additional 4 hours before intracellular cytokine staining.

Intracellular cytokine staining. Stimulated splenocytes were centrifuged, washed with BD Stain buffer (BD Biosciences), 0.5 μ g mouse BD Fc block (clone 2.4G2, BD Biosciences) was added to each well for 20 min at 4°C, and washed twice. Splenocytes were labeled with 0.2 μ g phycoerythrin (PE)-conjugated anti-CD4 (clone GK1.5, BD Biosciences) or 0.25 μ g PE-conjugated anti-CD8 (clone 53-6.7, BD Biosciences), incubated for 30 min at 4°C, washed twice, and fixed with BD cytofix/cytoperm buffer (BD Biosciences) for 20 min at 4°C. Cells were washed twice with BD perm/wash buffer (BD Biosciences) and labeled with 0.1 μ g fluorescein isothiocyanate (FITC) anti-IFN γ (clone XMG1.2, BD Biosciences) per well for 30 min at 4°C, washed twice, and resuspended in BD Stain buffer. Fluorescence was detected with BD FACSCalibur and Cell quest software (BD Biosciences). The lymphocyte population was gated on a FSC and SSC plot, and 20,000 gated events were collected and analyzed for FITC and PE positive cells. The number of double positive cells from the negative control was

subtracted from each sample before statistical analysis. Values are averages of four independent experiments. Dot plots are representative of a single replicate.

Cytometric bead array. Supernatants from splenocytes stimulated for 18 hours were used in detection of cytokines in mouse Th1/Th2 CBA (BD Biosciences) and mouse Inflammatory CBA (BD Biosciences) as per manufactures protocol. Fluorescence was detected on BD FACSCalibur and analyzed with CBA software (BD Biosciences).

Values are averages of two or three independent experiments.

Statistical analysis. All graphed original values are means \pm s.e.m. Data were natural-log transformed to achieve homogenous variances (Leven's test) and normality (Kolmogorovo-Smirnov test). Transformed data was analyzed with a student's t-test with Satterthwaite correction when necessary, or ANOVA and Tukey's post-test or Bonferroni post-test as indicated. Statistical analysis performed with SAS 9.2. $p < 0.05$ were considered significant.

Chapter 4

A West Nile virus CD4 T cell epitope improves the immunogenicity of dengue virus serotype 2 vaccines

Introduction

Dengue virus (DENV), which exists as four closely related serotypes, is a single-stranded RNA virus in the *flavivirus* genus. With the global resurgence of DENV infections, including the DENV-1 outbreak in Key West, Florida (CDC, 2010), dengue has evolved into one of the world's most important arboviral disease. DENV infection causes either mild dengue fever, or severe life-threatening dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS). Severe dengue is a common occurrence in children residing in hyperendemic countries and is strongly associated with secondary heterotypic infections (Sangkawibha et al., 1984). Currently, vector control and education programs are all that are available for dengue prevention; and the development of dengue vaccination has been hindered by concerns of waning or imbalanced tetravalent immunity leading to vaccine induced DHF/DSS. However, a handful of vaccines are in the early stages of clinical trials (Durbin and Whitehead, 2010).

DNA vaccination has become a fast growing field in vaccine technology since the 1990s following the first reports of plasmid DNA inducing an immune response to plasmid-encoded antigen (Tang, DeVit, and Johnston, 1992). Although DNA vaccines are considered by some to be one of the most important discoveries in the field of vaccinology (Mor, 1998), DNA vaccination in most cases is hampered by low

immunogenicity and efficacy. Thus various strategies to improve the immune response following DNA vaccination have been developed. Earliest attempts to increase DNA vaccine immunogenicity have included optimization of route, dosage, and timing of administration; DNA encoded or exogenously administered co-stimulatory molecules and cytokines; and prime-boost regimens (Leitner, Ying, and Restifo, 1999).

We have previously described the development and demonstrated the protective efficacy of a West Nile virus (WNV) DNA vaccine directing the expression of pre-membrane and envelope (prM/E) proteins. A single 100 µg intramuscular (im) injection of WNV DNA vaccine in mice induced a high level of WNV neutralizing antibodies and protected 100% of mice challenged by either intraperitoneal (ip) or mosquito inoculation (Davis et al., 2001). In addition, a single im injection of WNV DNA vaccine protected vaccinated horses from viremia by mosquito inoculation. In comparison, our previously described chimeric DENV-2 DNA vaccine, expressing prM and 80% DENV-2 E/ 20% JEV E to enhance the secretion of virus like particles, required two im vaccinations of 100 µg to elicit a high enough neutralizing antibody titer to passively protect neonatal mice from challenge (Chang et al., 2003). Both DNA vaccines contain identical enhancer, promoter, translational control element and JEV signal sequence (Chang, Hunt, and Davis, 2000); however, the difference in immunogenicity of the two vaccines is quite striking. Moreover, three days post vaccination of 100 µg of WNV DNA vaccine, 100% of mice were protected from virus challenge (Chang et. al. unpublished data), suggesting a rapid cell mediated and innate immune response to the vaccine. These observations led to the hypothesis that there are differential antigenic determinants between the WNV and DENV-2 DNA vaccines, potentially involving the

cellular mediated arm of the immune system. Here, we describe the identification and application of a potent WNV CD4 positive T cell epitope to increase the immunogenicity of DENV-2 DNA and virus-like particle (VLP) vaccines.

Results

Strong CD4 positive epitope in transmembrane domain of WNV. In order to better understand the nature of the cellular immune response to pVWN, we developed a peptide library scanning the entire prM and E protein coding sequences of pVWN and pVD2i to determine differential immune responses between the two vaccine constructs. We initially screened the library using 23 peptide pools (data not shown). Each peptide consisted of 15 amino acids, with 10 amino acid overlapping regions for both pVWN and pVD2i. C57BL/6J mice were immunized with pVWN or pVD2i, boosted at three weeks and sacrificed at 6 weeks. Spleens were homogenized and used in a mixed leukocyte reaction for determination of positive peptide pools by FACS analysis. From positive pools we identified individual peptides for further analysis. Splenocytes from vaccinated mice were incubated with 2 μ g of each individual peptide and stained for CD4 or CD8 and IFN γ for determination of positive peptides. We identified weak CD4 positive epitopes in DENV-2 E protein amino acids 1-20, 31-50, and 356-385. Weak CD4 positive epitopes to both pVWN and pVD2i vaccinated splenocytes were identified in the conserved fusion peptide region of E from amino acids 99-110, and also in E domain II amino acids 186-215. Interestingly, a very strong CD4 positive epitope was identified in WNV E at amino acids 466-495, that was present, but much weaker in 20% JEV E (Figure 4.1). This CD4 epitope of WNV E is located in two transmembrane domain (TMD) alpha helices. When comparing the transmembrane domain of WNV E and JEV

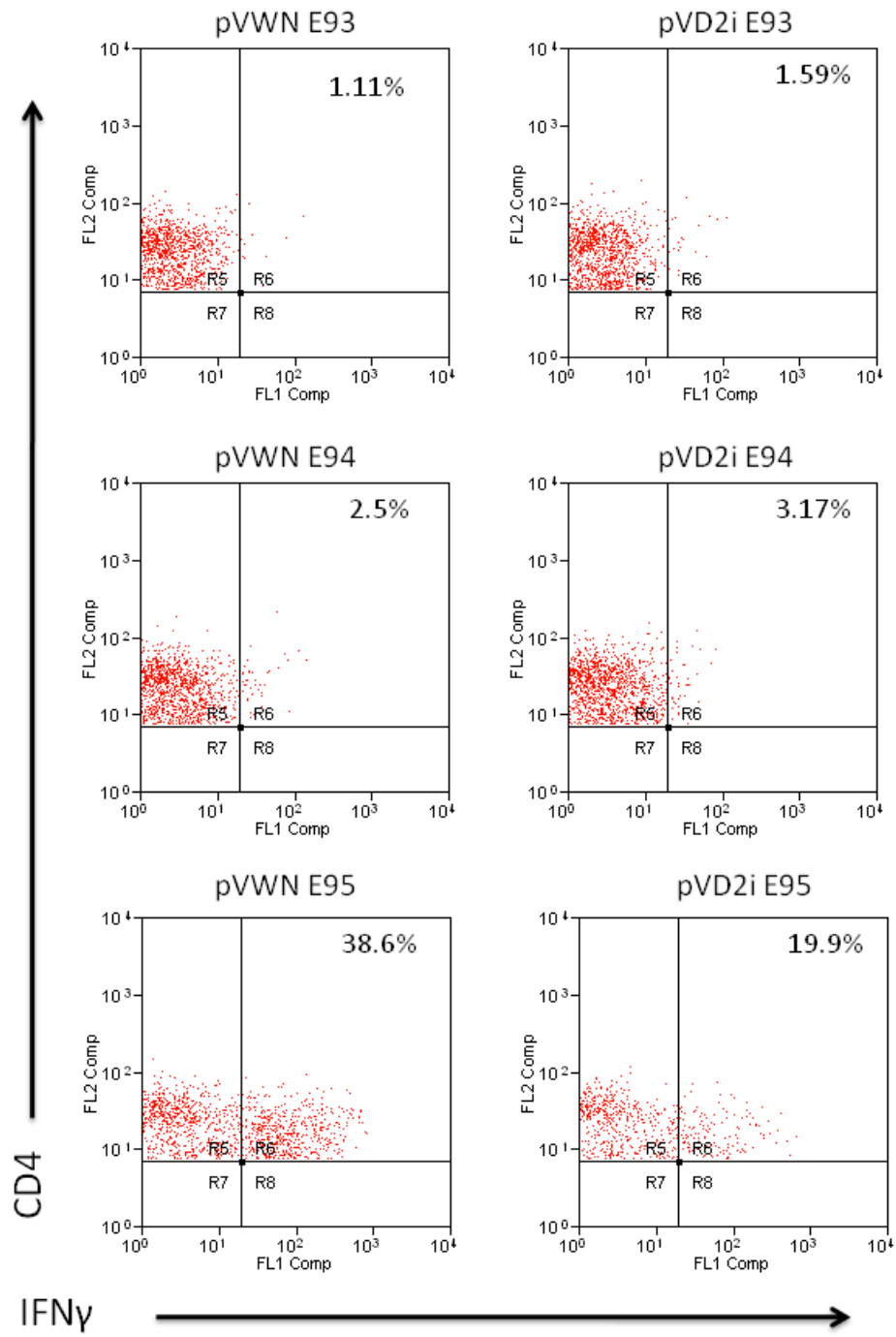


Figure 4.1. WNV transmembrane domain region contains a strong CD4 epitope not present in JEV. Splenocytes from mice vaccinated with pVWN or pVD2i (containing C terminal 20% JEV E (Chang et al., 2003)) were stimulated *ex vivo* with 2 μ g of envelope peptide (pVWN E93-E95 or pVD2i E93-E95). Cells were stained for CD3, CD4 and IFN γ and 10,000 of the CD3/CD4 gated population was counted. While E peptide 95 of both pVWN and pVD2i elicited IFN γ producing CD4 T cells, the response to pVWN E95 was greater than that of pVD2i E95. Dot plots are representative of a single experiment.

E, amino acid alignment reveals the transmembrane domain amino acids 466-495 are entirely conserved between WNV and JEV with the exception of four amino acids at 474, 484, 488, and 493 (Figure 4.2). Using ProPed I to predict CD8 epitopes and ProPed to predict CD4 epitopes revealed that the transmembrane region of WNV contains promiscuous CD8 and CD4 epitopes with potential to bind to several human HLA alleles (Table 4.1). Though the important 9 core amino acids (Table 4.1, highlighted blue) for each allele varies, the core frequently utilizes one or more of the four amino acids we have identified, suggesting the incorporation of this epitope may increase the immunogenicity of DENV-2 DNA vaccination in an outbred population.

Incorporation of WNV CD4 epitope to pVD2i increases vaccine immunogenicity.

The identification of a strong CD4 epitope in the WNV transmembrane domain, and its ability to potentially be a promiscuous CD4 and CD8 epitope led us to investigate the ability of this transmembrane domain (TMD) epitope to increase the immunogenicity of our DENV-2 DNA vaccine, pVD2i. Using site-directed mutagenesis, the four amino acids at the C-terminal 20% JEV E in pVD2i were sequentially changed to the corresponding amino acids in pVWN. These changes produced the plasmids pVD2iV474I (pVD2i-I), pVD2iV474I/A484T (pVD2i-IT), pVD2iV474I/A484T/T488V (pVD2i-ITV), and pVD2iV474I/A484T/T488V/V493L (pVD2i-ITVL). To first investigate increased immunogenicity of the WNV CD4 TMD epitope, we utilized the

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      *           440           *           460           *           480           *           500
WN  : LGDTAWDFGSSVGGVETS SVGKAVHQVFGGAFRSLEFGMSWITQGLLGALLLMMCI NARDRSIALTFLAVGGVLI FLSVNVHA : 501
JE  : ..... I.....N..I.....T.....M.....V.....A...T....V..AT.... : 500
MVE : ..... N..I.....T.....SP.....V...K...A...T....AT.... : 501
SLE : ..... I.....N..I.....T.....LQ.....S..L..T..I..I..ATS..Q. : 501
DEN2 : .....L.....I...L...AIYGAA.S.V..TMKI..I..VIIT..I..M..S..ST..LSVSLVL..V.T.Y..GAM..Q. : 495
DEN1 : ..... I.....LI..I..T..YGV..S.V..TMKIGI..I..T..L..L..S..ST..LSM..CI...M.T.Y..G.M..Q. : 495
DEN3 : .....LN..L..M..I..S..YTA..S.V...MKIGI..V..T..I..L..SKNT..MSFSCI..I..IIT..Y..G.V..Q. : 493
DEN4 : ..E.....L..L.....SVYTTM...V..MIRI..I..F.V..I..T..S..NT..M.M.CI...IT...GFT..Q. : 495
YF  : M..A...S..A..F...GI..T...S..QG...LN...KVIM...V..I..V...T..NMTMSMSMIL..VIMM...LG..G. : 493
TBE : I..EH.....A...FLS..I...T..L...N..I...VGFLPKL...VA..A..L..L..M..NPTMSMS...LA...LV..AMTLG..G. : 496
      I           T           V           KI           L S NT MS S           G           Q

```

Figure 4.2. The transmembrane domain region is well conserved between WNV and JEV. Single letter amino acid abbreviations are shown for the transmembrane domain of WNV E protein using WNV numbering. Amino acids conserved relative to WNV in the other viruses are shown as dots, and single letter abbreviations for non-conserved amino acids are depicted. Four amino acids differ between residues 466 and 495 of WNV and JEV and are highlighted green.

Table 4.1. WNV TMD CD4 epitope may bind to several human HLA alleles.

Restriction	Allele	WNV TMD Positive Peptide Sequences ^{1,2}	
CD4	DRB1_0101	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0102	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0306	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0307	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0308	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0311	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0401	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0404	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0405	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0408	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0410	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0423	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0426	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0701	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0703	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0804	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0806	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_0813	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_1102	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_1107	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_1114	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_1121	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_1304	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_1307	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_1321	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_1322	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_1323	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_1501	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_1502	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB1_1506	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB5_0101	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	DRB5_0105	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
	CD8	HLA-A*0201	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA
		HLA-A*0205	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA
		HLA-A24	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA
HLA-A3		GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
HLA-A*3101		GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
HLA-A*3302		GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
HLA-A2.1		GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
HLA-B14		GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
HLA-B*2702		GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
HLA-B*2705		GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
HLA-B*3501		GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
HLA-B*3701		GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
HLA-B*3901		GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA	
HLA-B*5101	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA		

HLA-B*5102	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA
HLA-B*5103	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA
HLA-B*5401	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA
HLA-B*51	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA
HLA-B*5801	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA
HLA-B7	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA
HLA-B*0702	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA
HLA-B8	GALLLWMGINARDRSIALTFLAVGGVLLFLSVNVHA

¹WNV TMD sequence shown includes E amino acids 466-501

²Positive ProPed nanomers highlighted in blue and obligatory P1 binding residues in red

inbred mouse strain where the epitope was initially described. C57BL/6J mice were immunized with each construct, boosted on week-4 and sacrificed on week-8. To compare differential immunogenicity between the constructs, focus reduction microneutralization (FR_μNT) was performed on individual mouse sera. Eight weeks post vaccination pVD2i-I did not appear to increase the neutralizing antibody titer compared to pVD2i (p=0.20) (Figure 4.3). However, pVD2i-IT (p=0.02), pVD2i-ITV (p=0.11), and pVD2i-ITVL (p=0.04) elicited increased levels of neutralizing antibodies compared to pVD2i vaccine. This suggests that amino acid 474, located in the first transmembrane helix, may not be an integral part of the CD4 epitope, as vaccines with the subsequent amino acid substitutions at WNV E positions 484, 488, and 493 elicited higher levels of neutralizing antibodies compared to pVD2i. This also suggests that the second transmembrane helix may be more important in the CD4 epitope since amino acid 474 lies in the first transmembrane helix while amino acids 484, 488, and 493 all lie within the second transmembrane helix.

We previously described the development of cross-reactivity reduced DENV-2 DNA vaccines that limit the production of cross-reactive antibodies and vaccine induced antibody-dependent enhancement (Hughes, Crill et al 2010, submitted). These novel

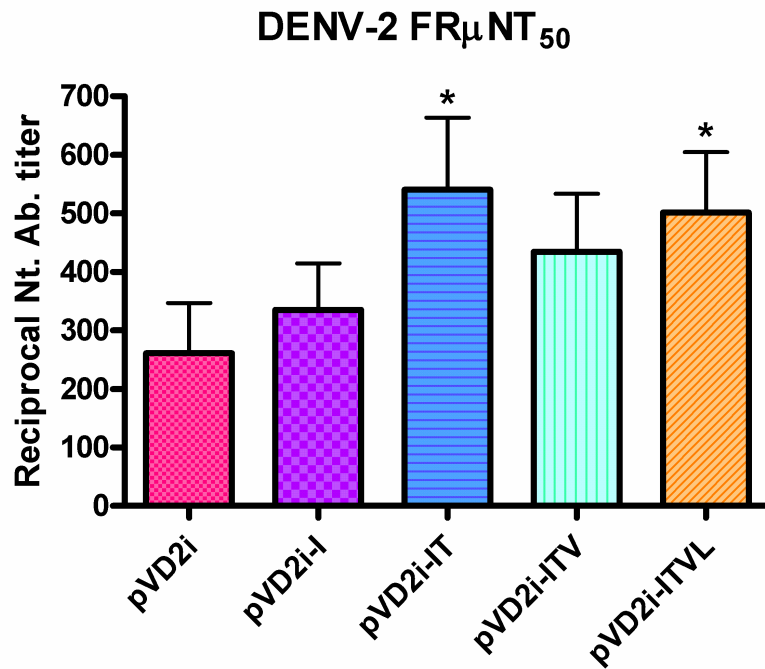


Figure 4.3. The incorporation of the WNV TMD CD4 epitope increases the immunogenicity of the pVD2i vaccine. DENV-2 neutralizing antibody (Nt. Ab.) titers 8 weeks post vaccination of C57BL/6 mice vaccinated with pVD2i with sequential addition of the WNV TMD amino acids (pVD2i-I: pVD2iV474I; pVD2i-IT: pVD2iV474I/A484T; pVD2i-ITV: pVD2iV474I/A484T/T488V; pVD2i-ITVL: pVD2iV474I/A484T/T488V/V493L). Data are expressed as mean \pm s.e.m. for n=5. ANOVA and Tukey's post test was performed on transformed data, p<0.05 were considered significant: single asterisk, p<0.05.

vaccines, however, showed a reduced but not significant, neutralizing antibody titer when compared to the wild type pVD2i vaccine (WT). To determine if the incorporation of the WNV TMD CD4 epitope could increase the immunogenicity of our DENV-2 cross-reactivity reduced vaccine, we introduced the WNV CD4 epitope V474I/A484T/T488V/V493L (TMD) into our cross-reactivity reduced DENV-2 DNA vaccine pVD2iG106R/L107D/K310E/E311R/P364R (RDERR) to create pVD2iG106R/L107D/K310E/E311R/P364R/V474I/A484T/T488V/V493L (RDERR-TMD) and compared the immunogenicity of pVD2i (WT), pVD2i

V474I/A484T/T488V/V493L (WT-TMD), RDERR, and RDERR-TMD by FR μ NT. Outbred Swiss Webster mice were immunized, boosted at 4 weeks, and sacrificed at 12 weeks. DENV-2 neutralizing antibody titers at 4 and 8 weeks post vaccination were not significantly different when comparing vaccines without TMD modification to vaccines with TMD modification, however there was a visible trend for WT-TMD and RDERR-TMD to elicit increased neutralizing antibody titers compared to WT and RDERR respectively (Figure 4.4). Twelve weeks post vaccination WT-TMD ($p=0.008$) and RDERR-TMD ($p=0.02$) elicited significantly more DENV-2 neutralizing antibodies compared to WT and RDERR respectively. Moreover, with the incorporation of the WNV TMD CD4 epitope, there was no significant difference in neutralizing antibody titers between WT-TMD and RDERR-TMD ($p=0.71$). These data suggest the addition of the WNV TMD CD4 epitope significantly increases the immunogenicity of both WT and cross-reactivity reduced DENV-2 DNA vaccines.

In addition to determining the neutralizing antibody response elicited by vaccines containing the WNV TMD CD4 epitope, we also investigated the epitope specificity of the total antibody response. In order to accomplish this, we utilized an epitope-specific IgG ELISA previously described and detailed in the previous section. WT-TMD vaccine elicited a large proportion of the total anti-E antibody response toward the wild type fusion peptide, EDIIFP_{WT} (mean 66%) (Table 4.2), while RDERR-TMD elicited a large antibody response against the novel FP epitope, EDIIFP_{RD} (mean 57%). Unfortunately, the mice immunized with WT and RDERR vaccines did not elicit an anti-E IgG antibody response in a magnitude that could be detected by our methods. This correlates with the overall lower neutralizing antibody titers observed in these two groups. However, these

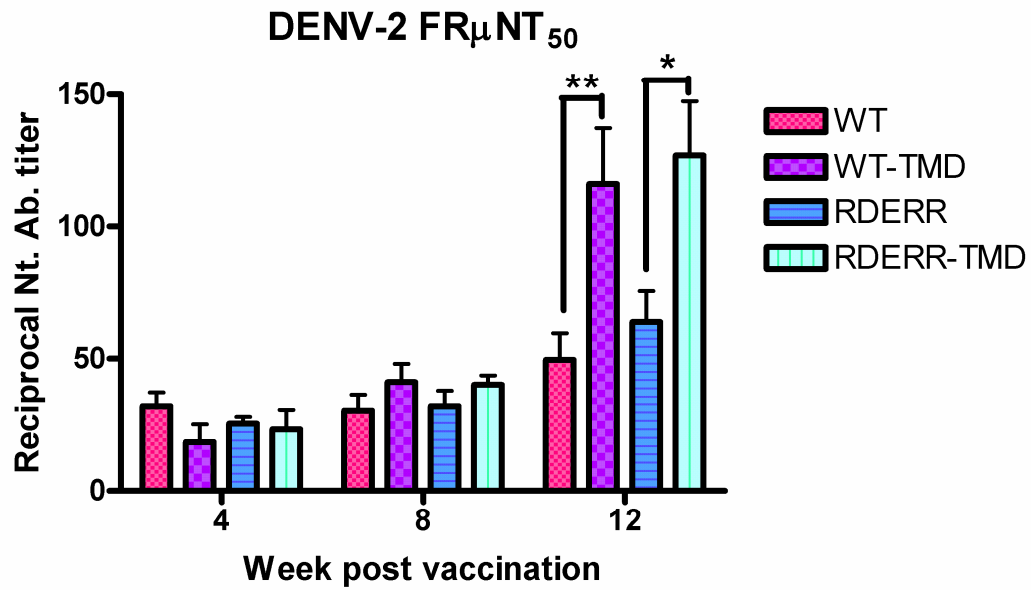


Figure 4.4. The incorporation of WNV TMD CD4 epitope increases the immunogenicity of DENV-2 CRR vaccines. DENV-2 Nt. Ab. titers of Swiss Webster mice 4, 8, and 12 weeks post vaccination. Data are expressed as mean \pm s.e.m. for n=10. Student's t-test with Satterthwaite correction was used to compare vaccine treatments as indicated. p<0.05 were considered significant: single asterisk, p<0.05, double asterisk p<0.01.

Table 4.2. WT-TMD and RDERR-TMD epitope-specific IgG responses.

Vaccine	Specific Epitope ¹	Endpoint average ²	Endpoint range	% response mean ³	% response range ³
WT-TMD	E	2.2X10 ³	1.7X10 ² -4.8X10 ³	100	
	EDIIFP	3.0X10 ²	2.5X10 ¹ -1.3X10 ³	66	<1-99
	EDIIICR	4.8X10 ²	7.6X10 ¹ -1.8X10 ³	71	48-93
	Remaining	1.8X10 ²	2.5X10 ¹ -8.3X10 ²	18	<1-74
RDERR-TMD	E	1.2X10 ³	1.1X10 ² -2.0X10 ³	100	
	EDIIRD	4.3X10 ²	5.7X10 ¹ -1.6X10 ³	57	<1-95
	EDIIIERR	1.7X10 ³	1.0X10 ² -3.1X10 ³	18	<1-83
	Remaining	1.2X10 ³	1.7X10 ² -2.9X10 ³	63	5-99

¹Epitopes targeted by specific antibody populations. E denotes antibodies recognizing all E-protein epitopes. EDII_{FP} denotes broadly cross-reactive WT epitopes of the E domain II fusion peptide. EDII_{RD} denotes the epitope of the RD FP substitutions. EDIII_{CR} denotes WT serocomplex cross-reactive epitopes in EDIII. EDIII_{ERR} denotes the epitope containing the ERR substitutions in EDIII. Remaining refers to the epitopes outside EDII_{FP} and EDIII_{CR} investigated in this study.

²Endpoint titers determined with knock-out antigens, thus representing immunoglobulins not targeted by the knock-out antigen.

³ Percentages below limit of detection are expressed as <1.

anti-FP proportions are similar in magnitude to what we observed previously in mice, and also similar to recent studies using DENV infected human sera (Crill et al., 2009; Lai et al., 2008; Oliphant et al., 2007a; Stiasny et al., 2006). Interestingly, the proportion of antibodies recognizing the cross-reactive epitopes of EDIII, EDIII_{CR}, were greatly increased in WT-TMD (mean 71%) than what we have observed in non-TMD vaccinated mice or than that of infected human sera (mean 5%). Moreover, the proportion of antibodies recognizing E epitopes other than EDII_{FP} or EDII_{ICR}, denoted ‘remaining’, were also higher in RDERR-TMD (mean 63%) than WT-TMD (mean 17%). A similar trend to what we have previously reported in mice though RDERR-TMD elicited higher

proportions than previous RDERR mice (mean 21%). These data suggest the WNV TMD CD4 epitope may be enhancing or redirecting the production of antibodies to previously non-dominant epitopes.

WNV CD4 epitope upregulates CD154 on CD4 T cells. Cell to cell interactions play a pivotal role in the regulation of the immune response. CD154 is expressed on a variety of cells, including but not limited to, activated CD4 T cells, CD8 T cells, mast cells, basophils, and eosinophils (Grewal and Flavell, 1998). CD154 is the ligand for CD40, which is expressed on B cells, antigen presenting cells, epithelium cells and endothelial cells among others. The binding of CD154 and CD40 results in T cell activation, B cell activation, and APC activation, and extravasation. The expression of CD154 and CD40 on a variety of immune cells suggests the importance of these molecules in both the initiation and effector functions of these cells. Studies in mice blocking the CD154 and CD40 interaction reveal a primary role of CD154 in regulating B cell proliferation, production of immunoglobulins (Ig), Ig class switching, germinal center formation, and generation of memory B cells (Clark, Foy, and Noelle, 1996). The incorporation of WNV TMD CD4 epitope into pVD2i resulted in an increase of neutralizing antibody titers in vaccinated mice. To better understand the mechanism of this antibody increase we investigated the potential of the WNV TMD CD4 epitope to increase the expression of CD154 on CD4 T cells. C57BL/6J mice were vaccinated im with 100 μ g of WT, WT-TMD, RDERR, or RDERR-TMD. One and two weeks post vaccination three mice from each group were sacrificed, spleens were homogenized and stimulated in a mixed leukocyte reaction with 2 μ g of UV inactivated DENV-2, pVWN peptide 95, which contains the CD4 TMD epitope, or pVD2i peptide 95 (containing the JEV TMD

sequence, Figure 4.2). The extracellular expression of CD4 and CD154 was measured by FACS analysis. One week post vaccination WT-TMD and RDERR-TMD vaccinated splenocytes displayed higher extracellular expression of CD154 compared to WT and RDERR (Figure 4.5) when stimulated with UV inactivated DENV-2 or pVWN peptide 95, while WT and RDERR vaccinated splenocytes expressed higher levels of CD154 when stimulated with pVD2i peptide 95, although these trends are evident, the differences were not statistically significant. Two weeks post vaccination, there were no similar trends of CD154 expression between vaccine groups (data not shown), suggesting the WNV TMD CD4 epitope may elicit the early activation of CD154 expressing CD4 T cells and play a role in the activation of B cells to produce higher neutralizing antibody titers.

ProPed promiscuous epitope predictions revealed the WNV TMD CD4 epitope may be a promiscuous epitope binding to several human HLA alleles (Table 4.1). To validate the incorporation of this epitope into the DENV-2 DNA vaccines, outbred Swiss Webster mice were vaccinated with 100 μ g of WT, WT-TMD, RDERR or RDERR-TMD on weeks-0 and 4. Five mice per group were sacrificed one-week post vaccination, and the remaining 10 mice were sacrificed 12 weeks post vaccination. Freshly harvested spleens were homogenized and stimulated in a MLR with 2 μ g of UV inactivated DENV-2. Extracellular expression of CD4 and CD154 was measured by FACS analysis. One week post vaccination WT-TMD vaccinated splenocytes elicited significantly higher levels of CD154 expressing T cells ($p=0.013$) compared to WT vaccinated splenocytes, while RDERR-TMD vaccinated splenocytes also displayed a trend for increased CD154 expression, the difference compared to RDERR vaccinated splenocytes was not

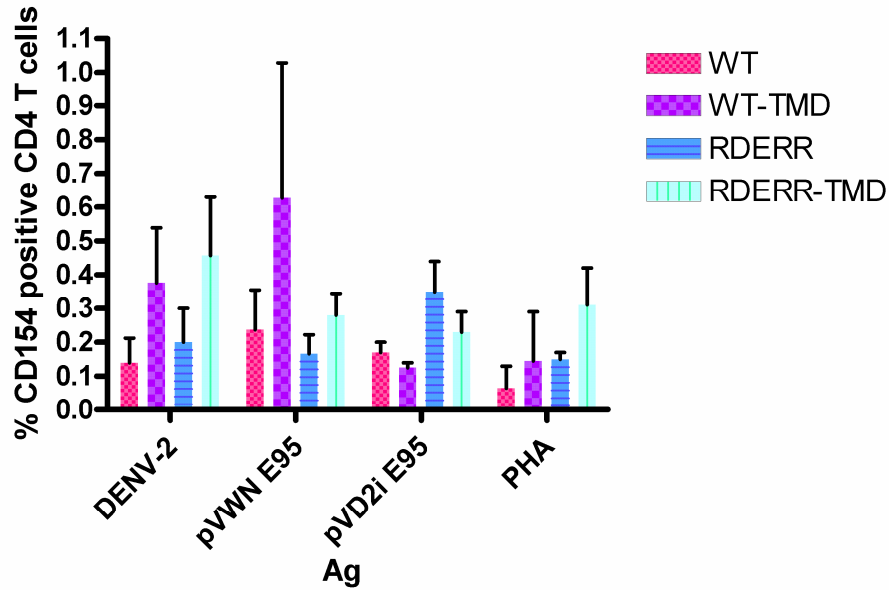


Figure 4.5. WNV TMD CD4 epitope may increase CD154 expression of CD4 T cells. C57BL/6 splenocytes were stimulated *ex vivo* with 2 μ g of indicated antigen. CD154 expression of CD4 T cells was analyzed by FACS. Vaccines containing WNV TMD CD4 epitope respond with higher expression of CD154 when stimulated with inactivated DENV-2, pVWN peptide E95, pVD2i peptide E95, or PHA one week post vaccination. Data are expressed as mean \pm s.e.m. for n=3. Student's t-test with Satterthwaite correction was used to compare vaccine treatments as indicated or ANOVA and Tukey's post test was performed on transformed data. $p < 0.05$ were considered significant.

significant (Figure 4.6a). This data suggests the WNV TMD CD4 epitope may act early in the immune response to up regulate CD154 on CD4 T cells, potentially increasing the neutralizing antibody titers as observed in C57BL/6J mice. In addition, stimulated splenocytes one-week post vaccination elicited a higher percentage of IL-4 positive CD4 T cells (Figure 4.6c) than IFN γ positive CD4 T cells (Figure 4.6e) suggesting the predominance of a Th2 driven T cell response. All vaccinated splenocytes elicited similar levels of IL-4 ($p=0.1$) and IFN γ positive T cells one-week post vaccination.

Twelve weeks post vaccination, all stimulated splenocytes are eliciting a balanced Th1 and Th 2 driven T cell response (Figure 4.6). RDERR and RDERR-TMD elicited

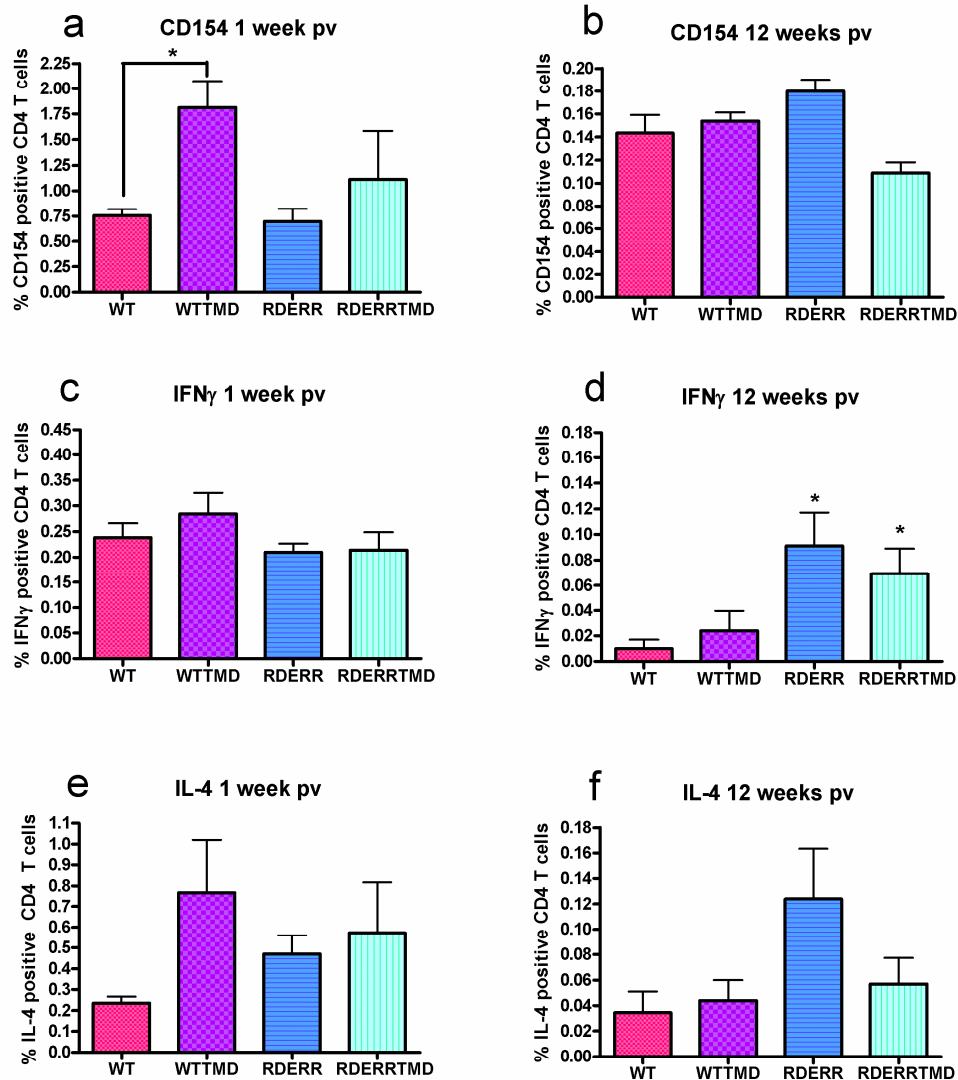


Figure 4.6. WNV TMD CD4 epitope induces early CD154 expression of CD4 T cells and a predominant Th2 driven response. FACS analysis of T cell responses of vaccinated Swiss Webster mice. Splenocytes were stimulated *ex vivo* with 2 μ g inactivated DENV-2 1 and 12 weeks post vaccination (pv). CD154 expression of CD4 T cells **a**) one and **b**) 12 weeks pv. Th1 driven T cell responses of vaccinated mice **c**) one and **d**) 12 weeks post vaccination. Th2 driven T cell responses **e**) one and **f**) 12 weeks pv. Data are expressed as mean \pm sem for n=5 (1 week pv) or n=10 (12 weeks pv). Student's t-test with Satterthwaite correction was used to compare vaccine treatments as indicated. p<0.05 were considered significant: single asterisk, p<0.05

higher levels of Th1 driven CD4⁺/IFN γ ⁺ T cells than WT or WT-TMD vaccines (p=0.05) (Figure 4.6d). All vaccines elicited similar levels of Th2 driven CD4⁺/IL-4⁺ T cells (p=0.19) (Figure 4.6f). Additionally, 12 weeks post vaccination there were no significant differences in CD154 expression on CD4 T cells between WT and WT-TMD or RDERR and RDERR-TMD (Figure 4.6b), also suggesting WNV TMD CD4 epitope induces CD154 expression as an early event in the immune response toward the vaccines.

WNV CD4 epitope increases immunogenicity of DENV VLP vaccine. Protein vaccines are a commonly used platform for several pathogens. Using protein as a vaccine alleviates common concerns of DNA vaccine formats such as potential integration into the host chromosome and the development of anti-DNA autoantibodies. To investigate the potential benefit of WNV CD4 epitope to alternative vaccine formats, we immunized Swiss Webster mice with 1 μ g of purified WT, WT-TMD, RDERR, RDERR-TMD VLPs, or β -propiolactone inactivated DENV-2, all formulated with 8% Alum. Mice were boosted at 4 weeks and sacrificed at 8 weeks. The neutralizing antibody titer of WT-TMD VLP (p=0.055) and RDERR-TMD VLP (p=0.015) was increased compared to the WT VLP and RDERR VLP vaccines respectively (Figure 4.7). These results were similar to that observed for the DENV-2 DNA vaccines (Figure 4.4) and suggest the incorporation of the WNV TMD CD4 epitope can increase vaccine immunogenicity of additional vaccine formats and not solely functional in DNA vaccine formats.

In addition to determining the antibody response toward the VLP vaccines, we investigated any potential differential T cell responses between the vaccine treatments. Splenocytes from individual mice were used in a mixed leukocyte reaction and stimulated

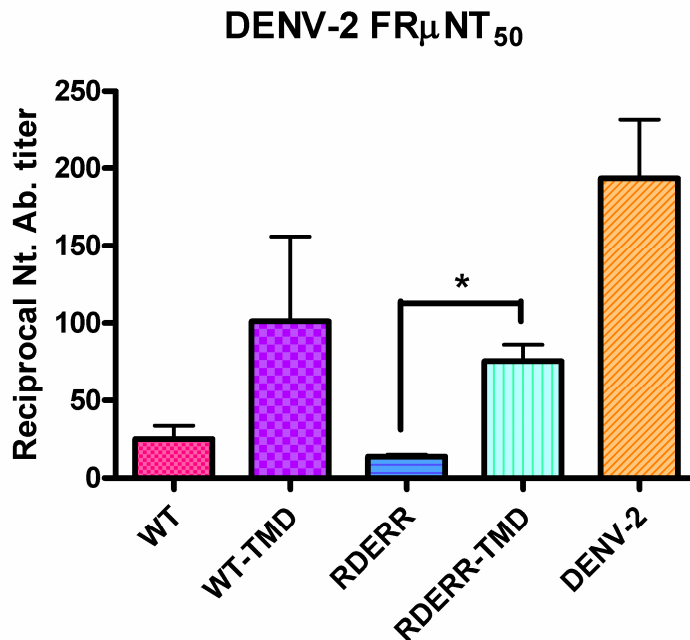


Figure 4.7. WNV TMD CD4 epitope increases immunogenicity of DENV-2 VLP vaccines. DENV-2 Nt. Ab. titers 8 weeks post vaccination of Swiss Webster mice. Data are expressed as mean \pm s.e.m. for n=5. Student's t-test with Satterthwaite correction was used to compare vaccine treatments as indicated. $p < 0.05$ were considered significant: single asterisk, $p < 0.05$

ex vivo with 2 μ g inactivated DENV-2. Cells were labeled for CD3, CD4 or CD8, and IFN γ or IL-4 and analyzed by FACS. Mice immunized with VLP vaccines elicited a predominant Th2 driven response as all groups had measurable levels of CD4 $^{+}$ /IL-4 $^{+}$ T cells (Figure 4.8a). VLP vaccinated mice also elicited low levels of CD4 $^{+}$ /IFN γ $^{+}$ T cells with the exception of RDERR VLP that did not elicit measurable levels of CD4 $^{+}$ /IFN γ $^{+}$ T cells (Figure 4.8b). The helper T cell responses between vaccine treatments were not significantly different based on ANOVA. Interestingly, the VLP vaccines elicited CD8 $^{+}$ /IFN γ memory T cells in all groups tested (Figure 4.8c). WT-TMD, RDERR, and RDERR-TMD VLP vaccines elicited significantly higher amounts of CD8 $^{+}$ /IFN γ $^{+}$ T cells than WT or inactivated DENV-2 ($p=0.05$). This phenomena has been observed for

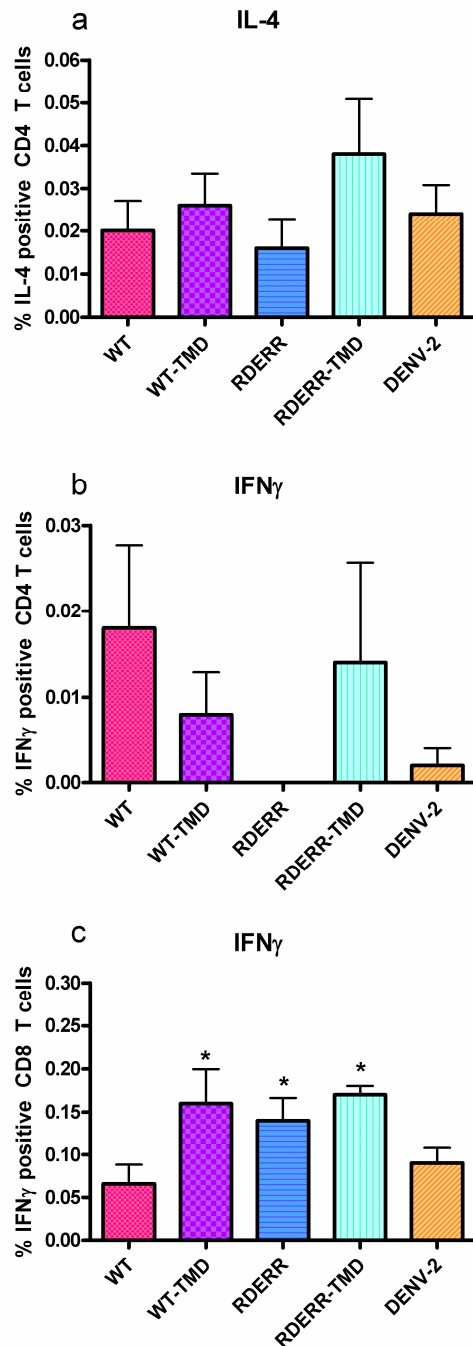


Figure 4.8. DENV-2 VLP vaccines elicit a predominant Th2 driven response and a Th1 independent CD8 memory response. FACS analysis of vaccinated Swiss Webster splenocytes stimulated *ex vivo* with 2 μ g inactivated DENV-2. **a)** Percent CD4 positive IL-4 producing Th2 driven T cells. **b)** Percent CD4 positive IFN γ producing Th1 driven T cells. **c)** Percent CD8 positive IFN γ producing memory cells. Data are expressed as mean \pm s.e.m. for n=5. ANOVA and Tukey's post test was performed on transformed data, p<0.05 were considered significant: single asterisk, p<0.05.

other intracellular pathogens such as simian immunodeficiency virus (Tsukamoto et al., 2009) and *Listeria monocytogenese* (Grenningloh et al., 2008), and is described by the “licensing” model (Lanzavecchia, 1998) where activated dendritic cells are able to activate CTL cells without the direct involvement of CD4 T cells. These data suggest the VLP vaccines, like the DNA vaccines, induce a predominant Th2 driven T helper cell response and yet also induce the development of CD8+ memory T cells without a strong Th1 driven response.

Discussion

We analyzed potential differences in CD4 T cell epitopes in the pVWN E and pVD2i chimeric-E protein. Several T cell epitopes have been described for the flaviviruses using T cell clones, many of which are localized to the non-structural proteins while fewer epitopes have been identified in the E protein using this approach (Rothman, Kurane, and Ennis, 1996). Using pVWN or pVD2i vaccinated splenocytes *ex vivo* stimulation with an overlapping peptide scanning library, we identified the conserved fusion peptide of E domain II harbored CD4 T cell epitope that stimulated CD4 T cell proliferation and IFN γ secretion in both WNV and DENV-2 vaccinated mice. This observation is in agreement with Roehrig et al. (Roehrig et al., 1994) where after immunizing mice with synthetic peptides, peptide 4-6 corresponding to the highly conserved fusion peptide elicited anti-DENV antibodies that only bound to virus exposed to low pH. The fusion peptide has previously been identified as an immunodominant B cell epitope eliciting a large proportion of the total anti-E antibody response (Crill et al., 2009; Lai et al., 2008; Oliphant et al., 2007a; Stiasny et al., 2006) directing the immune response to produce weakly-neutralizing, broadly cross-reactive antibodies that have been

implicated in antibody-dependent enhancement and exacerbate DENV disease. The immunodominant nature of the FP in eliciting cross-reactive antibodies may be a result of a potential CD4 T cell epitope in the same region. It was previously shown that a CD4 T cell epitope can increase the immunogenicity of homologous and heterologous peptides, however the response was significantly greater for the homologous peptide. In addition, the response was greatest when the T-B cell epitopes were collinearly synthesized (Roehrig et al., 1992).

The participation of cross-reactive memory T cells in the development of severe DHF/DSS has also been widely documented (Beaumier et al., 2008; Beaumier and Rothman, 2009; Mongkolsapaya et al., 2003), although the majority of these cross-reactive T cells are restricted to non-structural proteins. Our results, along with Roehrig et al. (Roehrig et al., 1994), indicate the presence of a T cell epitope in this conserved region in WNV and DENV-2. Though cross-reactivity was not measured in our or previous works, the highly conserved nature of this epitope warrants further investigation of the fusion peptide as a potential cross-reactive T cell epitope participating in the development of severe DENV disease. The cross-reactivity reduced DENV-2 DNA vaccine, RDERR, was specifically designed to reduce the production of cross-reactive antibodies and limit vaccine induced ADE. With the potential CD4 T cell epitope in the FP, the ability of the RDERR to increase vaccine safety by limiting the induction of memory CD4 T cells that recognized the highly conserved FP deserves further investigation.

We also identified a strong CD4 T cell epitope in the transmembrane domain (TMD) of WNV, and a much weaker CD4 T cell epitope in the corresponding region of

our DENV-2 DNA vaccine. To our knowledge, this is the first description of a T cell epitope present in this region of any flavivirus E protein. This epitope spanning amino acids 466-495 encompasses the two transmembrane helices of the E protein, is highly conserved between the pVWN and pVD2i with the exception of four amino acids. The first amino acid, 474 lies in the first transmembrane helix while amino acids 484, 488, and 493 all lie within the second transmembrane helix. Helical wheel projections indicate amino acids 484 and 488 in the second transmembrane helix all reside on the same side of the alpha helix while amino acid 493 lies on the opposite side of the helix. When looking at the entire peptide, amino acids 466-495, amino acids 474, 484, and 488 are all on the same side of the alpha helix. In contrast, amino acid 493 lies on the opposite side of the helix along with N475 and R479, which are predicted to be important anchor residues in MHCII binding. These projections indicate the probable involvement of amino acids 474, 484, and 488 in the CD4 T cell receptor epitope that correlates nicely with the neutralization data (Figure 4.3) showing an increase in neutralizing antibody titer following the incorporation of the first two amino acid residues into pVD2i.

Increasing the immunogenicity of DNA vaccines has been an active area of investigation as some DNA vaccines are hindered by low immunogenicity and efficacy. The incorporation of the WNV TMD CD4 epitope significantly increased the immunogenicity of DENV-2 DNA vaccine (WT, pVD2i) and also the cross-reactivity reduced DENV-2 DNA vaccine, RDERR. This increased immunogenicity appears to be due in part to an early increase in activated CD154 CD4 T cells. Traditional methods to increase the immunogenicity of DNA vaccination have included the use of genetic adjuvants where immunostimulatory molecules are encoded into the DNA vaccine, such

as CpG motifs, cytokines, chemokines, GM-CSF, and ubiquitin (Chiarella et al., 2008). Immunostimulatory epitopes have also been investigated in several vaccine fields. One such method includes the fusion of a foreign universal T cell epitope sequence to the target gene of interest. Zhu et al. fused the FrC fragment of tetanus toxin to a DNA vaccine containing the sequence for the protective epitope of PorA protein of *Neisseria meningitides* (Zhu et al., 2008). The incorporation of the tetanus epitope significantly increased the immunogenicity of the DNA vaccine and induced bacteriocidal antibodies. In a similar approach, a polyepitope DNA vaccine was constructed using the strong CD8 immunostimulatory properties of the hepatitis B small surface antigen simultaneously encoding cytotoxic T lymphocyte epitopes from six different viruses (Chen et al., 2010). The new plasmid using the HBsAg epitopes as an adjuvant resulted in significant development of CTL responses to all six viruses compared to the polyepitope DNA without the HBsAg. One concern with this method is the presence of HBsAg antibody present in human sera due to HBV vaccination potentially interfering with vaccination efficacy or unbalanced HBV immune responses.

Although DNA vaccination in clinical trials has been proven safe, concerns about tumorigenic potential due to DNA integration and the development of DNA autoimmunity remain. The incorporation of the WNV TMD CD4 epitope into the DENV-2 VLPs resulted in a significant increase in neutralizing antibody titers compared to DENV-2 VLPs without the WNV TMD CD4 epitope. Similar to strategies of DNA vaccination, the use of T helper epitopes to increase the immunogenicity of protein vaccines has also been investigated utilizing a universal T cell epitope. Lu et. al. (Lu et al., 2009) were able to increase the anti-tumor ability of a HSP60-fused gastrin-release

peptide DNA vaccine by heterologously boosting with a recombinant protein that also contained a foreign T helper epitope of HSP70. Similar to use of tetanus toxin, HBsAg universal T helper epitopes, the potential development of anti-carrier immunity hindering vaccination should be addressed.

Our approach to increase the immunogenicity of the DENV-2 DNA and VLP vaccines utilizes a naturally occurring flavivirus CD4 epitope in contrast to previous studies using foreign universal T cell epitopes. There is limited concern of immunity toward the WNV TMD CD4 epitope from previous exposure interfering with the vaccine efficacy. The transmembrane domain of flaviviruses is either of low B cell antigenicity or antigenically inert, as vaccination with DENV-2 DNA plasmid containing 80% DENV-2 E and the C terminal 20% JEV E did not elicit any measurable antibody response against JEV (Chang et al., 2003) and no B cell epitopes have previously been identified. Utilizing this WNV TMD CD4 epitope to increase vaccine immunogenicity has additionally advantageous since the transmembrane domain does not affect the proper antigenic folding of E.

Incorporating a naturally occurring dominant CD4 epitope may offer advantages in additional flavivirus vaccination formats. The variable yet highly conserved nature of the flavivirus transmembrane domain (Figure 4.2) suggests potential differential T cell antigenicity across the flaviviruses, which can be readily manipulated as demonstrated in this study. With the global resurgence and expansion of flaviviruses, there will be increasing demand and utility for multivalent flavivirus vaccines. Multivalent vaccine interference is a frequently observed phenomenon that may be caused by competition for resources in the lymphnodes, changes in the Th1/Th2/Th0 balance, induction of

regulatory T cells, and replicative interference (Dagan, Poolman, and Siegrist, 2010; Guy et al., 2009). These data show the incorporation of the strong WNV CD4 epitope into DENV-2 vaccines of different immunogenicity produces similar monovalent antibody titers. Moreover, the incorporation of this natural flavivirus epitope into live attenuated vaccines may alleviate previous observations of interference (Guy et al., 2009) and induce a more balanced immune response. We are currently examining the potential use of this flavivirus epitope to help balance tetravalent DENV vaccine immunogenicity and its application to multivalent flavivirus vaccines beyond DENV deserves further investigation.

The incorporation of naturally occurring dominant CD4 epitopes in one component of a multivalent vaccine to increase vaccine immunogenicity of a weaker component may be a possible generalized strategy for multivalent vaccines hindered by imbalanced immunogenicity or interference. For example, serotype specific immune interference affecting vaccine immunogenicity of multivalent human papilloma virus (HPV) VLP vaccine was recently demonstrated (Zhang et al.). Licensed tetravalent vaccine containing VLP for HPV serotypes 16/18/6/11 elicited a balanced serotype specific neutralizing antibody response, while the trivalent HPV 16/18/58 displayed significant decreases in type specific neutralizing antibodies to serotype 58. Identifying and manipulating different immunogenic elements such as the CD4 T cell epitope identified here, and/or altering cross-reactive B-cell epitopes - as with the pVD2i-RDERR vaccine candidate in this study (Crill et al., 2009; Miller, 2010) could prove the strategy to understanding and alleviating the antigenic differences between virus serotypes interfering with multivalent vaccine safety and efficacy.

Materials and Methods

Vaccines. West Nile plasmid, pVWN, was previously constructed and described (Chang et al., 2007; Davis et al., 2001). Construction and characterization of DENV-2 DNA plasmid optimized with C terminal 20% JEV has been previously described (Chang et al., 2003). Characterization of DENV-2 plasmids, pVD2i, with substitutions in the E domain II fusion peptide (EDIIFP) and EDIII have been described previously (Crill et al., 2009). pVWN, pVD2i (WT) and pVD2iG106R/L107D/K310E/E311R/P364R (RDERR) were manufactured by Aldevron (Fargo, ND). pVD2i with substitutions in the transmembrane domain, pVD2iV474I, pVD2iV474I/A484T, pVD2iV474I/A484T/T488V, and pVD2iV474I/A484T/T488V/V493L (WT-TMD), were generated by using Quick change site-directed mutagenesis kit (Stratagene) sequentially with the primers (Operon):

3'CGGGATGACGAGACCTACCCGTATTTGCGTGCTCTG5',

3'GCTAGTTACGAACTGGAAGAATCGGTGTCCCCCACAC5',

3'CTGGAAGAATCGGCATCCCCCACACGAGCACAAG5', and

3'CCCCCACACGAGGACAAGAATCGCTGGTTACACG5'.

pVD2iG106R/L107D/K310E/E311R/P364R/V474I/A484T/T488V/V493L (RDERR-TMD) was generated by restriction enzyme cloning by digesting

pVD2iV474I/A484T/T488V/V493L (WT-TMD) with KpnI and StuI (New England Biolabs), and Quick ligation (New England Biolabs) of the transmembrane domain

region into the pVD2iG106R/L107D/K310E/E311R/P364R backbone. WT-TMD and RDERR-TMD were grown in *E. coli* XL1 Blue cells and DNA purified for vaccination by Endofree Plasmid Maxi-prep Kit (Qiagen) as per manufacturer's instructions.

Structural gene elements and regulatory elements of all plasmids were sequenced entirely

upon identification of the correct mutation. Automated DNA sequencing was performed using a Beckman Coulter CEQ 8000 genetic analysis system (Beckman Coulter) and analyzed by using Beckman Coulter CEQ 8000 (Beckman Coulter) and Lasergene software (DNASTAR).

Virus-like particles (VLPs) were generated by transformation of COS-1 cells as previously described (Chang, Hunt, and Davis, 2000) and secreted VLPs were harvested from serum and animal product free media [Sfm4megavir (Hyclone) supplemented with L-glutamine, non-essential amino acids, penicillin-streptomycin, sodium pyruvate, and cholesterol (Gibco)]. Tissue culture media was harvested 4 days post transformation, pelleted by ultracentrifugation at 19,000rpm, concentrated 100-fold in TN buffer, pelleted by 20% sucrose cushion, and resuspended in 1/100 of original volume in TN buffer.

Protein concentration was determined by Bradford Assay (BioRad) as per manufacturer's instructions. Final VLP vaccines consisted of 1 µg protein in 8% Alum (Thermo Scientific). DENV-2 strain 16681 was propagated in C6/36 cells as described previously. Virion particles were similarly purified using 20% sucrose cushion, inactivated by 0.01% of β-propiolactone and formulated with 8% Alum.

Mice. C56BL/6J mice (Jackson Laboratory) were immunized im with 100µg of DNA at weeks 0 and 4, sacrificed on week8, splenectomized and serum collected. Swiss Webster mice (Charles River) were similarly vaccinated, sacrificed on week 12, splenectomized and serum collected. Swiss Webster mice (Charles River) were immunized im with 1µg of VLPs or 1µg of BPL inactivated purified DENV-2 virions formulated with 8% Alum (Thermo Scientific) on weeks 0 and 4, sacrificed on week 8, splenectomized and serum collected.

Peptide scanning library. A library of 15 amino acid peptides with 5 amino acid overlaps was designed to cover either the envelope (E) protein or the pre-membrane (prM) region of WNV or DENV-2. (AC Scientific, Inc.) Peptides were arranged in pools as described previously (Betts et al., 2001; Hoffmeister et al., 2003; Kern et al., 2000; Maecker et al., 2001). Single peptides were used at a concentration of 1 μ g/ml with the total concentration of each pool being no greater than 10 μ g/ml (Betts et al., 2001; Kern et al., 2000; Maecker et al., 2001). Pool volumes were diluted in such a manner that the DMSO concentration is no greater than 1% of v/v (Hoffmeister et al., 2003). Individual peptides were identified by pool overlap. The selected individual peptides went through a second round of screening as described above. Positive peptides were determined by *ex vivo* stimulation of vaccinated splenocytes and demonstrated CD4+ and/or CD8+ and levels of IFN- γ expression.

Epitope prediction of positive peptides was accomplished using online prediction engines ProPedI (<http://www.imtech.res.in/raghava/propred1/>) and ProPed (<http://www.imtech.res.in/raghava/propred/>) setting the threshold to the most stringent 1%. Helical wheel projections of peptides were generated using BioEdit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>).

Mixed leukocyte reactions. Single cell suspensions were made from freshly harvested C57BL/6J or Swiss Webster spleens and plated in 96-well plates for extracellular surface antigen and intracellular cytokine staining (ICS). Splenocytes were stimulated with peptides as described above or 2 μ g of UV inactivated DENV; PHA (Roche Diagnostics) was used as a positive control, and naïve splenocytes in cell culture medium as negative controls. 96-well plates were incubated 2 hours, and 1 μ g of Golgi plug (BD Biosciences)

was added to each well, incubated an additional 4 hours before extracellular and intracellular staining.

Flow cytometry. The antibodies mouse BD Fc block, phycoerythrin (PE)-conjugated anti-CD4, PE-conjugated anti-CD8, PE-conjugated anti-CD154, PE-Cy5-conjugated anti-CD3, fluorescein isothiocyanate (FITC) anti-IFN γ , FITC-conjugated anti-IL-4, and FITC-conjugated anti-CD4 were purchased from BD Biosciences. Stimulated splenocytes were centrifuged, washed with BD Stain buffer (BD Biosciences), Fc blocked, and labeled for CD3, CD4 and CD154, or CD8. Splenocytes were fixed with BD cytofix/cytoperm buffer (BD Biosciences) and labeled for IFN γ or IL-4. Fluorescence was detected with BD FACSCalibur and Cell quest software (BD Biosciences). The lymphocyte population was gated on a FSC and SSC plot, and further gated on CD3 and CD4 or CD8 and 40,000 gated events were collected and analyzed for FITC and PE positive cells. Double-positive cells from the negative control were subtracted from each sample before statistical analysis. Dot plots are representative of a single replicate.

Neutralization assay. A focus reduction microneutralization (FR μ NT) technique was utilized as previously described (Crill et al., 2009) with few modifications. Vaccinated mouse sera were diluted 1:10, heat inactivated, titrated 2-fold to the volume of 40 μ L, and 320 virus pfu/40 μ L was added to each dilution. FR μ NT titers were calculated for each virus relative to a back titration. Exact FR μ NT titers were modeled by the sigmoidal dose response with variable slope using Graph Pad Prism version 4. Values are the average of two independent replicates.

Epitope-specific ELISA. An IgG ELISA protocol described previously (Crill et al., 2009) was used with a few modifications. Vaccinated mouse sera (n=10) were diluted 1:100 and serially titrated. We utilized previously characterized epitope-specific knock-out antigens (Crill et al., 2009). OD values were modeled as non-linear functions of the \log_{10} sera dilutions using a Gaussian non-linear regression in Graph Pad Prism version 4.0 and endpoint dilutions determined as the titer where the OD value equaled two-times the OD value of the test serum reacted against normal antigen.

EDII and EDIII epitope-specific IgG percentages were calculated as described (Crill et al., 2009) with minor modifications. Briefly, the EDII and EDIII epitope specific IgG percentages for WT vaccinated mice were calculated by dividing the IgG endpoint titer obtained with specific knock-out antigens by the endpoint titer obtained by WT antigen on the same sera, subtracting this value from 1.0 and multiplying by 100. Since cross-reactivity reduced (CRR) sera contained antibodies that do not recognize WT antigen (WT antigen acts as the knock-out antigen) but recognize epitopes of the knock-out antigens, the cognate knock-out antigen was used to determine 100% E reactivity (eg. RDERR sera reacted on RDERR antigen represents 100% reactivity). CRR vaccinated epitope-specific percentages were calculated as $100 \times [1.0 - (\text{WT antigen endpoint} / \text{epitope knock-out antigen endpoint})]$.

Statistical analysis. All graphed original values are means \pm s.e.m. Data were natural-log transformed to achieve homogenous variances (Leven's test) and normality (Kolmogorovo-Smirnov test). Transformed data was analyzed with a Student's t-test with Satterthwaite correction when necessary, or ANOVA and Tukey's post-test as indicated. Statistical analysis performed with SAS 9.2. $p < 0.05$ were considered significant.

Chapter 5

Conclusions

The results of this dissertation confirm and expand previous observations of flavivirus envelope protein antigenicity. The results also provide new insights into the dengue virus humoral immune response, and suggest strategies for B cell and T cell epitope modification to reduce the potential of vaccine-induced ADE and increase vaccine immunogenicity.

The second chapter, entitled “Humoral Immune Responses of Dengue Patients using Epitope-Specific Serotype-2 Virus-like Particle Antigens,” focuses on the mapping of cross-reactive epitopes of the DENV-2 envelope protein and the use of these VLPs to better understand the human humoral immune response following primary and secondary DENV infection. The flavivirus E domain II (EDII) has previously been shown to contain multiple overlapping, broadly cross-reactive, immunodominant epitopes (Crill and Chang, 2004; Crill, Trainor, and Chang, 2007; Lai et al., 2008; Oliphant et al., 2007a; Stiasny et al., 2006; Trainor et al., 2007) and this study confirmed that substitutions in the fusion peptide at G106 and L107 ablated the recognition of group cross-reactive MABs.

A novel finding of chapter two is the possibility of inter-monomer epitopes. For example, the reactivity of MAb D35C9-1 was only moderately reduced when tested against substitutions in the FP or EDIII. However, when substitutions in FP and EDIII were combined into a single antigen, the reactivity of MAb D35C9-1 was greatly reduced compared to WT antigen reactivity. This suggests MABs may recognize inter-monomer

epitopes from disparate domains. This observation heeds cautions for antigenic mapping studies relying on soluble E or recombinant EDIII, as the entire MAb footprint could be contained in multiple domains on multiple monomers.

Chapter two presents results from experiments that begin to dissect the complexities of the human polyclonal immune response to DENV infection. The results of this chapter confirm and extend previous reports on the EDII FP as an immunodominant region made of a series of overlapping epitopes stimulating broadly cross-reactive antibodies (Lai et al., 2008; Oliphant et al., 2007a; Stiasny et al., 2006). The presence of these highly cross-reactive antibodies with limited neutralizing capabilities is presumably long-lived as many of these studies utilize convalescent sera. This observation has immediate implications in the theory of ADE as this immunodominant epitope could be exacerbating secondary DENV disease. Interestingly, the total proportion of Ig recognizing EDII FP was higher in primary infections than secondary infections, and also higher for IgM than IgG. In addition, IgM was also found to target the cross-reactive EDIII epitopes in greater magnitude than IgG and also in greater magnitude in secondary infections. This suggests the use of IgM in attempt to assign serotype to secondary DENV infections may be highly misleading.

The results of chapter two suggest a novel approach for DENV vaccination. With the highly immunodominant nature of the EDII FP, removal of this region through mutagenesis could eliminate the production of highly cross-reactive antibodies and improve vaccine safety by limiting vaccine-induced ADE. Moreover, the removal of immunodominant cross-reactive epitopes could enhance vaccine efficacy by redirecting

the immune system as a small proportion of IgG recognizing EDIII was correlated with neutralization.

Chapter three entitled “Dengue vaccine candidates reduce potential antibody-dependent enhancement,” further expands on the issue of DENV vaccination safety by investigating the potential to engineer cross-reactivity reduced DENV vaccines by dampening the immune responses to these epitopes and redirect the response to less dominant protective epitopes. Though antibodies targeting the EDII FP and cross-reactive EDIII epitopes can be weakly neutralizing (Stiasny et al., 2006) and generally require a higher occupancy than potentially type-specific neutralizing antibodies (Gromowski, Barrett, and Barrett, 2008), the removal of these epitopes from a DENV DNA vaccines still elicited a high neutralizing antibody response that did not differ significantly from vaccination with WT, unmodified, vaccine.

In vitro enhancement of DENV infection was significantly lower when the cross-reactive E protein epitopes were removed. The replicative and/or immune interference associated with live attenuated vaccines, has resulted in a large proportion of non-responders following one- and two-doses (Morrison et al., 2010; Sun et al., 2008) with 100% tetravalent seroconversion requiring three doses spanning a year (Morrison et al., 2010). The time required for full seroconversion spans the epidemic season, leaving a significant portion of vaccinated individuals without tetravalent protective immunity at risk for vaccine-induced severe disease following natural infection. These results demonstrate the feasibility of engineering DENV vaccines with enhanced safety and limited potential of vaccine-induced ADE. However, this approach may not be

applicable to DENV live-attenuated vaccines since many substitutions in the FP can be lethal (Huang et al., 2009).

An interesting observation in chapter three was that removal of the EDII FP resulted in more dramatic decreases in enhancement of DENV infection than removal of EDIII cross-reactive epitopes alone. These data suggest immunodominant FP may play a vital role in the development of ADE and severe DENV disease seen in secondary DENV infection and EDIII sero-complex cross-reactive epitopes may not be as important. This is intriguing due to the fact severe secondary disease is seen in DENV infection, one could presume the sero-complex cross-reactive antibody response would be important in ADE.

The highly conserved nature of the EDII FP could suggest ADE would be a factor in additional flaviviruses. The phenomenon of ADE is, indeed, not an exclusive mechanism of DENV. MAbs have been demonstrated to enhance the infection of additional flaviviruses including WNV, TBEV and YFV *in vitro* (Peiris and Porterfield, 1979; Peiris, Porterfield, and Roehrig, 1982; Phillipotts, Stephenson, and Porterfield, 1985; Schlesinger and Brandriss, 1983). *In vivo* enhancement of flaviviruses other than DENV has also been demonstrated in mice with enhancement of neurovirulence of YFV and JEV by the passive transfer of E reactive MAbs or immune sera (Gould and Buckley, 1989). In addition, passive transfer of JEV immune sera at subneutralizing levels resulted in increased viremia titers and mortality of Murray Valley encephalitis virus in mice (Wallace et al., 2003). Although the possible enhanced disease of heterologous flaviviruses has been demonstrated with a handful of viruses in mouse models, it is reasonable to surmise with the conserved nature of the EDII FP and the global expansion

and cocirculation of many flaviviruses, there should continued vigorous studies to identify the risks of exacerbated human disease in secondary flavivirus infections. With the possible exception of a single case report of severe TBEV disease in a patient with previous WNV exposure (Ferenczi et al., 2008) the phenomena of severe human disease in secondary infection is exclusive of DENV.

The substitution of the EDII FP at G106 and L107 was able to eliminate the cross-reactivity of the elicited antibodies and reduce the potential of vaccine induced ADE. The results of the chapter two mapping study in addition to previous works (Crill and Chang, 2004; Crill, Trainor, and Chang, 2007; Trainor et al., 2007) have identified these two amino acids as key residues in MAb recognition of the EDII FP. However, the substitution of G106 with arginine and L107 with aspartic acid did not eliminate the dominant nature of this epitope. Instead, the substitutions elicited antibodies with a unique specificity that do not recognize the wild-type EDII FP. This suggests two possibilities. First, the large, charged amino acids created new binding residues for the B cell receptor, or second, G106 and L107 are not the essential amino acids in the dominance of the EDII FP. Redirection of the immune response away from the EDII FP to less dominant epitopes could still be accomplished through a detailed mapping study of this region utilizing substitutions with glycine or alanine. Although a large proportion of the antibody response to the modified vaccines were elicited against the new amino acid substitutions, the fact these antibodies have a unique specificity limited their ability to participate in ADE.

Interestingly, the modified vaccines were able to increase the recognition of the polyclonal immune response to epitopes outside EDIIFP and the cross-reactive epitopes

of EDIII. This may suggest the ability of these vaccines to redirect the immune response away from the immunodominant EDII FP and EDIII, to epitopes that have yet to be classified. Detailed mapping studies of the E protein have been described exhaustively for EDII FP and EDIII, however, a detailed mapping study of the antigenicity of EDI has yet to be accomplished. Mapping studies have made use of murine MAbs, and although the results of chapter two would agree the murine type-specific MAbs can be characteristic of the potentially type-specific human polyclonal response, recent studies implicate the importance of additional domains in human infection. Characterization of a potentially neutralizing type-specific chimpanzee MAb recognizing EDI has been recently described (Lai et al., 2007). In addition, anti-EDIII depleted human sera still maintained high neutralizing ability, suggesting EDIII antibodies may play a smaller role in virus neutralization in humans (Wahala et al., 2009). Moreover, murine MAbs recognizing DENV-3 EDIII were not able to effectively neutralize different genotypes of DENV-3 (Brien et al., 2010), a phenomena yet to be demonstrated in human sera. This suggests domains outside EDIII may be important in the human polyclonal response to flavivirus protection. With the ability of modified CRR vaccines to increase antibody recognition of epitopes outside EDII FP and EDIII and yet maintain high neutralizing titers, also suggests a role for additional epitopes or domains in neutralization.

Chapter four entitled, “A West Nile virus CD4 T cell epitope improves the immunogenicity of dengue virus serotype 2 vaccines,” investigates the ability of a naturally occurring flavivirus CD4 T cell epitope to act as an immunological adjuvant for heterologous flavivirus vaccines. The results of chapter four reveal a dominant CD4 epitope in the transmembrane domain alpha helices of WNV. Interesting, this region

between pVWN and the DENV-2/JEV chimeric pVD2i is entirely conserved with the exception of four amino acids; however the pVWN peptide stimulated a stronger CD4 response than did the pVD2i peptide. In addition, substitution of the four amino acids in pVD2i for the pVWN sequence resulted in a significant increase in neutralizing antibody titers of DENV-2 DNA and VLP vaccines.

The use of a naturally occurring flavivirus CD4 epitope as a vaccine adjuvant is beneficial for several reasons. First, the transmembrane domain of flaviviruses does not elicit an antibody response (Chang et al., 2003) therefore previous exposure to WNV would not adversely affect the efficacy of the CD4 epitope. This is in contrast to current methods to increase vaccine immunogenicity through use of universal T help epitopes such as tetanus toxin or hepatitis B small antigen. Previous vaccine exposure to these epitopes may elicit an antibody response and therefore render the T helper epitopes inert. Conversely, the use of these universal epitopes could also elicit or increase the antibody response against these epitopes and potentially hinder booster vaccinations against the parental tetanus or HBV vaccines. In addition, the transmembrane domain does not affect the proper folding and antigenicity of the E protein because removal of the C terminal 20% of E elicits soluble E monomers with intact antigenicity (Deubel et al., 1991; Men, Bray, and Lai, 1991).

The transmembrane domain region of the flaviviruses though is well conserved, is highly variable in the alpha helices with the presence of mutational “hot spots” (Lewis et al., 1993). The incorporation of the WNV CD4 epitope could enhance the immunogenicity of additional heterologous flavivirus vaccines. In addition, the standard use of this epitope in tetravalent live attenuated DENV vaccines may be the key to

eliminating the road-block of replicative and/or immune interference and inducing a balance immune response and deserves further evaluation.

The goal of this dissertation research was to construct safer DENV vaccines by mapping the DENV-2 E protein, manipulating B cell epitopes to limit vaccine-induced ADE, and manipulating T cell epitopes to increase vaccine immunogenicity. Through this work, we have gained a better insight into the antigenic properties of the envelope protein of flaviviruses and demonstrated a viable approach through B and T cell epitope manipulation to increase the safety and immunogenicity of DENV vaccines. Although there remains much to learn about the antigenicity and immunodominance of the envelope protein, with the global expansion of DENV and increasing disease burden it is hoped these studies have contributed to the growing body of knowledge and will aid in the future control of DENV disease.

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