MECHANISMS OF ANTIBODY MEDIATED NEUTRALIZATION OF DENGUE VIRUS

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ABSTRACT

YANG ZHOU: Mechanisms of Antibody Mediated Neutralization of Dengue Virus
(Under the direction of Aravinda de Silva)

Dengue virus (DENV) is the most significant arthropod-borne virus in this world, affecting 2.5 billion people. Pre-existing sub-neutralizing antibodies (Abs) can enhance virus infection, leading to severe disease. Thus a better understanding of the interaction between humoral immunity and dengue virus is imperative. In this thesis I characterized 37 human monoclonal Abs (hMAbs) cloned from human PBMC. The majority of these MAbs were broadly cross-reactive and capable of enhancement of infection *in vitro*; few exhibited serotype-specific neutralizing activity. I next studied the mechanism and stoichiometry of Ab mediated neutralization of DENV and found that MAbs displaying threshold stoichiometry all neutralize post-attachment steps while MAbs neutralizing pre-attachment step display linear stoichiometry, indicating that different neutralization mechanisms are associated with different stoichiometric models respectively. I also studied the neutralization using multiple MAbs mixed together and demonstrated that the neutralization two MAbs are independent of each other in the mixture.

It was previously found that MAb 8A1 displays variable neutralizing activity against different DENV-3 genotypes. My results demonstrated that EDIII mutations at 301 and 383 alter the ability of 8A1 to bind and neutralize different genotypes of DENV-3.

Besides genetic mutation, non-genetic variation such as maturation state of the dengue virion can also modulate neutralization. I found that monocytic cell derived dengue virions were more mature than mosquito cell derived virions. Using a panel of MAbs and sera, I demonstrated that maturation reduces virus sensitivity to neutralization mediated by weakly neutralizing cross-reactive MAbs or sera but it does not affect sensitivity to type-specific and strongly neutralizing MAbs or sera. Most interestingly, maturation enhanced virus sensitivity to neutralization of certain MAbs and sera. Further study indicated that preferential binding of immature virions by weakly neutralizing MAbs and furin cleavage of partially mature virions inside endosome are responsible for the maturation dependent differential neutralization. These results offer new insight into how humoral immunity neutralizes dengue viruses and how viruses evade the immunity. These discoveries may also shed light on rational design of dengue vaccine and antibody therapy.

ACKNOWLEDGEMENTS

I first want to thank my mentor, Dr. Aravinda de Silva, for providing me with the opportunity and freedom to explore the scientific world through hypothesis driven scientific research. With his enthusiasm and his inspiration, he helped me to go through the process of discovering interesting phenomenon, proposing hypothesis, designing and carrying out experiments and learning how to become an independent scientist. I am indebted to all of my committee members for their continued support and guidance. I am grateful for their helpful advices and encouragement in the last three years. I would like to thank both present and past members of the de Silva lab whose continued support drives me to finish the experiments that I presented in this thesis. I especially want to thank Dr. Wahaha Padmasriri Wahala who taught me doing experiments and provided technical support for my research. I also want to thank Anne Broadwater, lab manager of the de Silva lab, for the wonderful support she provided throughout my graduate career. I want to extend my gratitude to all our collaborators for providing necessary materials and technique support for my projects. I want to express my greatest gratitude to all my family back in China as their love and encouragement give me strength and brace me up in the past years.

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LIST OF ABBREVIATIONS

AA	amino acid
Ab	antibody
ADE	antibody dependent enhancement
ATCC	American type culture collection
C	capsid protein
CO_2	Carbon dioxide
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
DENV	dengue virus
DF	dengue fever
DHF	dengue hemorrhagic fever
DSS	dengue shock syndrome
E	envelope protein
EDI	envelope protein domain I
EDI/II	envelope protein domain I and domain II
EDII	envelope protein domain II
EDIII	envelope protein domain III
ELISA	enzyme-linked immunosorbent assay
FRNT	Focus Reduction Neutralization Assay
HCV	hepatitis C virus
HIV	human immunodeficiency virus
IFN	interferon
IgG	immunoglobulin G
IgM	immunoglobulin M
JEV	Japanese encephalitis virus
M	membrane protein

MOI	multiplicity of Infection
MAb	monoclonal antibody
MBP	maltose binding protein
nm	nanometer
NS	non-structural protein
OD	optical density
PCR	polymerase chain reaction
PFU	plaque forming units
prM	pre-membrane protein
PRNT	plaque reduction neutralization test
rEDIII	recombinant EDIII
RTPCR	reverse transcription–PCR
TBEV	tick-borne encephalitis virus
WNV	West Nile virus

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 General Introduction

1.1.1 Emerging virus: Dengue virus (DENV) is a re-emerging arth2ropod-borne virus. In the last 50 years, dengue incidences increased 30 fold and its geographical distribution expanded to most tropical and subtropical areas. As a result, dengue is becoming increasingly recognized as one of the world's most threatening infectious diseases. However, despite decades of research, we still do not have a vaccine to prevent its transmission or feasible post-exposure therapy to treat the disease.

1.1.2 Dengue epidemiology: Dengue is endemic in more than 110 countries in tropical and subtropical areas such as Southeast Asian, Latin America, and Sub-Sahara Africa. These areas are all heavily populated and every year 50 - 100 million people are infected with Dengue, leading to half a million hospitalization and 12,500 - 25,000 deaths (WHO 2009; Whitehorn and Farrar 2010). Most people can recover from dengue infection without any adverse consequences. Overall, the mortality rate is very low, but in cases of severe disease, the mortality rate roars up to 26% without supportive therapy (WHO 2009; Ranjit and Kissoon 2011).

Dengue is transmitted by mosquitoes through a human-mosquito-human cycle. Mosquitoes of species *A. aegypti* and *A. albopictus,* infect humans by injecting virus containing saliva while taking a blood meal. An infected patient will have viremia lasting

for about 4 days and mosquitoes taking blood meal from this patient will get infected and transmit the virus to other humans living in the neighborhood, leading to localized outbreak. In the last 50 years, due to a combination of urbanization, population growth and increasing international travel, dengue became a global epidemic, putting 2.5 billion people worldwide in risk (WHO 2009).

1.1.3 Dengue virology: Dengue is a single-stranded, positive sense RNA virus of the family *Flaviviridae*, genus *flavivirus*. It is closely related to several other important human pathogens such as Japanese encephalitis virus (JEV), West Nile virus (WNV), Yellow fever virus (YFV) and Tick-borne encephalitis virus (TBEV). The DENV genome encodes 10 genes and is translated into a single polyprotein and then cleaved into structural proteins (C-prM-E) and non-structural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) (Figure 1.1). The C (capsid) protein forms a virion scaffold that encapsidates the RNA genome. The prM (membrane) protein is important in the assembly of virion and subsequent maturation. The E protein (envelope) is the major surface exposed antigen and the principal target of host antibody response. The 7 NS proteins have diverse roles such as protease, RNA-dependent RNA polymerase or immune regulation proteins participating in evading immune response.

Serology and phylogenetic evidences indicated that there are four serotypes of Dengue: DENV1, DENV2, DENV3 and DENV4, sharing about 70% sequence homology between different serotypes (Fields Virology). The remaining 30% sequences variation results in antigenic, transmission and virulence variation between different serotypes. Each serotype can be further divided into genotypes by phylogeny and geographic distribution, for example, DENV3 consists of four genotypes (I, II, III, IV) (Villabona-Arenas, Miranda-Esquivel et al. 2009); DENV2 consists of 4 genotypes too and among them two genotypes are associated with very different virulence: the Southeast Asian

genotype causes severe disease epidemics whereas the American genotype has only been linked to mild or subclinical epidemics (Rico-Hesse 2003).

The E glycoprotein has been crystallized and the structure has been solved (Modis, Ogata et al. 2004; Nayak, Dessau et al. 2009). E protein forms a homodimer on the mature DENV virion. The virus envelope consists of 90 E glycoprotein homodimers. Three dimers are arranged in a herringbone pattern to form a raft-like structure on the virion surface (Figure 1.2). The ectodomain of E protein has three domains: domain I, II and III (EDI-EDIII). Specifically, EDII contains fusion loop, which mediates membrane fusion and EDIII belongs to IgG superfamily and is believed to participate in cell receptor binding (Crill and Roehrig 2001).

The Envelope protein is involved in critical steps of virus infection such as attachment and endocytosis. E protein mediates virus attachment to cells through DC-SIGN molecule, heparan sulfate or mannose (Kroschewski, Allison et al. 2003; Navarro-Sanchez, Altmeyer et al. 2003). After entry into cells of mononuclear phagocyte lineage through unknown cell receptors via endocytosis, the low pH in the endosome triggers dissociation of the E homodimers and exposes the hydrophobic fusion peptide to the target membrane (Stiasny, Allison et al. 2002) (Figure 1.3). Subsequently insertion of the fusion peptide into the endosome membrane induces assembly of E trimers and finally leads to fusion of the endosome membrane and virus membrane, resulting in the entry of virus nucleocapsid into cell cytoplasm. After uncoating of nucleocapsid, the viral RNA genome is released to cytoplasm, where it functions as an mRNA to direct the translation of the viral polyprotein. The translated polyprotein is further cleaved into three structural proteins (C, prM, E) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The E glycoprotein is glycosylated at amino acid residue Asn67 and Asn153 to assure proper folding of the protein (Modis, Ogata et al. 2003; Bryant, Calvert

et al. 2007). Then the NS proteins initiate replication of viral genomes and subsequently the newly synthesized RNA is packaged by C protein to form a nucleocapsid (Figure 1.3). Through unknown mechanisms, the nucleocapsid is encapsulated by ER membrane rafts covered with prM/E trimers and buds into the ER to form immature virion. The immature virion contains 180 prM/E dimers that project vertically outward from viral surface as 60 trimeric spikes (Zhang, Chipman et al. 2003; Zhang, Corver et al. 2003) (Figure 1.4). The immature virions are transported through trans-Golgi network, in which the low pH environment triggers structural reorganization of glycoproteins (Figure 1.3). The prM/E heterodimers dissociate and form 90 E dimers lying flat on the surface of the particle, with prM capping the fusion peptide of the E protein. This exposes the cleavage site for cellular endoprotease furin to cleave "pr" peptide that covers the fusion peptide (Stadler, Allison et al. 1997; Yu, Zhang et al. 2008; Zybert, van der Ende-Metselaar et al. 2008). This process is called maturation. It has been shown that the maturation process is not efficient in certain cell lines, resulting in the production of partially mature or totally immature virions (Lok, Kostyuchenko et al. 2008; Dejnirattisai, Jumnainsong et al. 2010; Junjhon, Edwards et al. 2010).

1.1.4 Pathogenesis: Dengue infection with any serotype results in long term protection against the same serotype but not the other serotypes. Majority of all dengue infections are asymptomatic. The symptomatic cases include mild dengue fever, or severe disease like dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS) (WHO 2009). The symptoms of DHF/DSS include rash, internal bleeding, thrombocytopenia, plasma leakage and collapse of the circulation system which is fatal. Majority of DHF/DSS cases occurred in secondary infection by dengue serotype that is different from the primary infection (Halstead, Nimmannitya et al. 1970; Kliks, Nimmannitya et al. 1988). For infants with waning maternal dengue immunity, their chances of developing

DHF/DSS are much higher than infants born to mothers who are naïve to dengue infection (Halstead, Nimmannitya et al. 1970). These evidences strongly suggested that pre-existing heterotypic immunity is critical for development of DHF/DSS. Halstead proposed Antibody Dependent Enhancement (ADE) as an underlying mechanism of DHF/DSS (Halstead 1970). The ADE hypothesis stated that during secondary infection, the pre-existing antibodies from a primary infection fails to neutralize heterotypic virus but forms infectious immune complex with the virus, and the immune complexes bind to and infect monocytic cells expressing Fc-ɣ receptors via the Fc portion of the antibody. This leads to increased total infected cell mass and enhanced virus replication and is hypothesized to be responsible for the severe disease outcome. Other competing hypothesis such as dengue strain-dependent virulence or host factors had been proposed but none of them have been extensively studied like the ADE hypothesis (Halstead, Rojanasuphot et al. 1983; Mongkolsapaya, Dejnirattisai et al. 2003). Numerous studies have confirmed the ADE phenomenon *in vitro* or *in vivo* with animal models but the detailed mechanisms of how ADE leads to DHF/DSS are still unknown.

1.1.5 Vaccine development: Despite numerous approaches that have been tried in the last sixty years, an effective dengue vaccine remains elusive. The ideal dengue vaccine must be protective against all four serotypes and cost-effective. Various groups have tried many vaccine development strategies, including live attenuated tetravalent vaccines (Guirakhoo, Pugachev et al. 2001; Sun, Edelman et al. 2003), chimeric tetravalent vaccines based on attenuated dengue virus or Yellow Fever 17D (Durbin, Kirkpatrick et al. 2011;Guy, Barrere et al. 2011; Osorio, Huang et al. 2011), recombinant DNA vaccines based on flavivirus and non-flavivirus vectors or VEE Replicon Particles (White, Sariol et al. 2013) and Recombinant protein vaccines (Putnak, Coller et al. 2005). Among these efforts, alphavirus vector based tetravalent dengue vaccine shows

promising potential compared to other vaccine candidates. The major concern with any dengue vaccine is to avoid eliciting cross-reactive and non-neutralizing antibodies which may enhance subsequent virus infection instead of preventing it. To address this problem, the goal of vaccines in development is to elicit robust and simultaneous protective immunity to all 4 serotypes. In addition, we need to know exactly which virus epitopes induce type-specific/neutralizing or cross-reactive/non-neutralizing antibodies. My work will help to shed some light on this question.

1.2 Antibody Neutralization of Dengue Virus

1.2.1 Neutralizing antibody: Most of my work in this dissertation is about antibody neutralization of dengue virus. The study of dengue neutralization is critical for understanding dengue virology and pathogenesis and advancing vaccine development. Type-specific antibodies are believed to be the major neutralizing component in dengue polyclonal serum and depletion study by de Alwis *et al.* demonstrated this hypothesis (de Alwis, Smith et al. 2012). So the questions are where do these type-specific antibodies bind and where do most human dengue-reactive antibodies bind? Studies using mouse MAbs indicated that most neutralizing antibody epitopes are on EDIII (Cockburn, Navarro Sanchez et al. 2012) but Wahala and colleagues noted that EDIII is not the main target of human neutralizing Abs (Wahala, Kraus et al. 2009). The generation of large cohorts of human anti-dengue MAbs from PBMC revealed the existence of cross-reactive Ab response decades after infection (Smith, Zhou et al. 2012). Other reports also found that the cross-reactive MAbs make majority of the cloned MAbs and type-specific and strongly neutralizing MAbs are rare (Beltramello, Williams et al. 2010; de Alwis, Beltramello et al. 2011). Recent reports further suggested that most of these cross-reactive E antibodies target fusion loop (Lai, Tsai et al. 2008; Costin, Zaitseva et al. 2013). Further characterization of the few strongly neutralizing MAbs revealed one

particular group of antibodies binding to conformational epitopes on virion surface which appear to be the major neutralizing epitopes targeted by Abs in polyclonal sera (de Alwis, Smith et al. 2012). These Abs bind to whole virus but do not bind to soluble E glycoprotein which suggests that the epitope exists only on virus surface but not E protein monomer. Whether these Abs bind to tertiary structure of E trimers on the immature virion surface or E dimmers of the mature virion surface is still unknown.

1.2.2 Mechanisms of neutralization: Many DENV neutralizing MAbs had been characterized up to today but the mechanisms and stoichiometry of neutralization are still largely elusive. Pierson and colleagues suggested that antibody neutralization of flavivirus is a multiple hit phenomenon in which the number of antibodies binding to the virion must exceed certain threshold for neutralization to happen (Pierson, Xu et al. 2007). This model has been demonstrated in West Nile virus and the threshold is estimated to be around 30 antibodies. This stoichiometry model explains the MAb's neutralization potency as occupancy of accessible epitopes: for strongly neutralizing MAb, its accessible epitopes are much more than 30, so it takes small occupancy of binding (percentage of the total available epitopes that are bound by antibodies) to exceed the threshold; otherwise for weakly neutralizing MAb, its total available epitopes are less than 30, then even maximum occupancy (at highest concentration) are still below threshold. However, this model has several flaws as it ignores the possibility that location and function of epitope itself may affect the threshold requirement as binding to different epitopes may exert different effect on virus entry, fusion, replication steps and thus the stoichiometry of neutralization may vary. Secondly, it does not take into consideration the cases of different MAbs binding to the same virion and composition of these bound antibodies may alter the threshold requirement of neutralization. In this

work, I tried different experimental design and proposed additional stoichiometry model to address these flaws.

1.3 Methods to study dengue Virus

1.3.1 Generation of Infectious Viruses: Most previous DENV neutralization and ADE studies have used virus derived from mosquito cell line C6/36. The C6/36 cell monolayer is infected at low MOI and kept in 28 ºC incubator for 6 days before the supernatants are harvested as infectious virus stocks. Occasionally, people may use Vero cell derived viruses for their work, but seldom did anyone uses human monocytic cells derived viruses as infectious virus. As mentioned above, viruses derived from different cells lines may vary in glycosylation and maturation. It has been shown that mosquito cells and mammalian cells have variation in processing prM Cleavage (Dejnirattisai, Jumnainsong et al. 2010). Our experience suggested even using the same method of virus production such as C6/36 methods mentioned above, the virus stocks produced at different batches still vary in neutralization assays, not only the titer varies in logs magnitude but also the sensitivity to neutralization. In this thesis, the mechanisms of this variation will be illustrated. But it is important for the field to recognize the existence of this pitfall and take serious effort to standardize infectious virus stock between different labs.

More attention should be paid to human monocytic cell derived virus in the future. After mosquito biting and injecting the virus to human blood, the viruses in the primary infection site are mosquito cell derived but after one replication cycle, the progeny viruses now turn into be human monocytic cell line derived for the rest of the viremia. So in general, it is the human cell derived viruses that challenged and shaped our immune response to dengue infection. Therefore the human cell derived viruses are more

relevant in pathogenesis than the mosquito cell derived viruses. However, so far there were not any reports using human monocytic cell derived viruses for dengue neutralization or ADE study. Considering the lack of knowledge about these viruses, it is imperative and indispensable for us to study human monocytic cell line derived viruses and its neutralization.

1.3.2 Neutralization Assays: Focus Reduction Neutralization Assays (FRNT) is currently the most widely used assay for dengue neutralization study. It is derived from the focus assay, which is also the standard method for titration of virus (Okuno, Igarashi et al. 1978). Compared to traditional Plaque Reduction Neutralization Assay (PRNT), FRNT uses Vero cell monolayer and antibody staining to detect the virus infection focus instead of plaque formation and this improvement provides better accuracy, higher out-put and the ability to use virus strains that do not plaque well. Based on this assay, a more sophisticated high-throughput assay called micro-neutralization test (MNT) has been developed. This assay utilizes 96 well plates and automated counting of focus using ELISpot reader (Taketa-Graham, Powell Pereira et al. 2010).

However, since dengue virus infects monocytic cell *in vivo*, the Vero cell based neutralization has limitations in representing biologically relevant neutralization. Our collaborator Mark Heise developed a human monocytic cell line U937 expressing DC-SIGN molecule and created dengue permissive cell line U937-DC-SIGN. Using this cell line, a FACS based neutralization assay was developed in our lab (Lambeth, White et al. 2005). Instead of counting focus formations, this method counts single cell infection (stained with Alexa-488 conjugated Dengue specific antibodies) using flow-cytometry. It is a high volume automated method and the results are more relevant in estimating the neutralization potency of antibodies or sera *in vivo*.

Both FRNT and FACS based neutralization assay were employed in this research. For most of the neutralization assay, FACS based assay using U937-DC-SIGN is preferred for its efficiency and relevance, especially in experiment when only C6/36 derived viruses are used. But when U937-DC-SIGN derived viruses are used as infection agents, the traditional FRNT is preferred because monocytic cell line derived viruses show a very low efficiency in infecting other monocytic cell lines such as U937-DC-SIGN (data not shown), a phenomenon already reported by Dejnirattisai (Dejnirattisai, Webb et al. 2011).

1.3.3 Factors Modulating *in vitro* Neutralization: DENV neutralization by antibodies *in vivo* is critical for host to clear virus infection. While using animal models for dengue neutralization study is expensive and time-consuming, researchers are forced to rely on *in vitro* neutralization assays that can mimic *in vivo* neutralization. Dengue infection of cell *in vitro* is a complicate process involving viruses, cells, temperatures, pH, incubation time, antibodies and other serum components such as complement. Any variation in these factors can alter virus infection and affect antibody mediated virus neutralization. However, most of these factors are understudied. But they must be taken into consideration when interpreting neutralization data.

Many cell lines have been found permissive to dengue viruses and several of them (Vero, U937-DC-SIGN, Raji-DC-SIGN and BHK) have been used in neutralization assay. These cell lines are known to use different surface molecule as attachment molecule for dengue virus (Kroschewski, Allison et al. 2003; Navarro-Sanchez, Altmeyer et al. 2003). The different usage of attachment molecule is a factor that must be considered when comparing neutralization data among assays using different cell lines. Besides, the endosome furin level may also vary between cell lines, and this will affect virus maturation both during virus secretion out of cell and virus entry to the cell. Apart

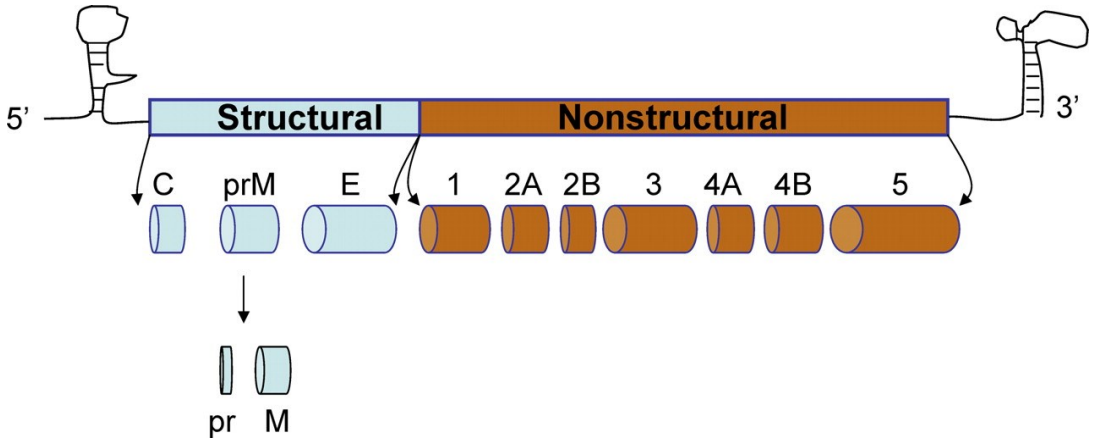
from cellular factors, viral factors such genetic mutation and non-genetic variation (such as maturation or glycosylation) affect neutralization even more. A lot has been studied about virus genetics and indicated how mutations increase or decrease neutralization sensitivity. This part is well elucidated and explained in this dissertation. There are also some non-genetic factors may affect neutralization, including maturation and glycosylation.

1.4 Objectives of Study

One important question in DENV studies is that the humoral immunity fails to protect heterologous secondary infection. In order to elucidate this question, we aim to investigate the long term antibody response *in vivo* and characterize the neutralizing and enhancing monoclonal antibodies. Strongly neutralizing MAbs have the potential of treating DENV infection as therapeutic antibodies. To promote development of DENV antibody therapy, we aim to study mechanisms and stoichiometry of dengue-antibody interaction and to illustrate the mechanisms of virus escaping neutralization through natural genetic mutations. The monocytes are major targets of DENV infection *in vivo* but monocytic cell derived virus was seriously understudied in previous neutralization and ADE studies. To fill in this gap, we aim to determine the maturation of viruses derived from monocytes, and study how this may affect virus sensitivity to antibody mediated neutralization. In general, the objective of this study is to get better understanding of interaction between antibody and virus, and provide clues for DENV antibody therapy and vaccine development.

Figures and Legends

Figure 1.1



Figures 1.1: Genome structure of dengue virus and the 11 virus proteins (adapted from *Exp Biol Med* April 2008 vol. 233 no. 4 401-408)

Figure 1.2

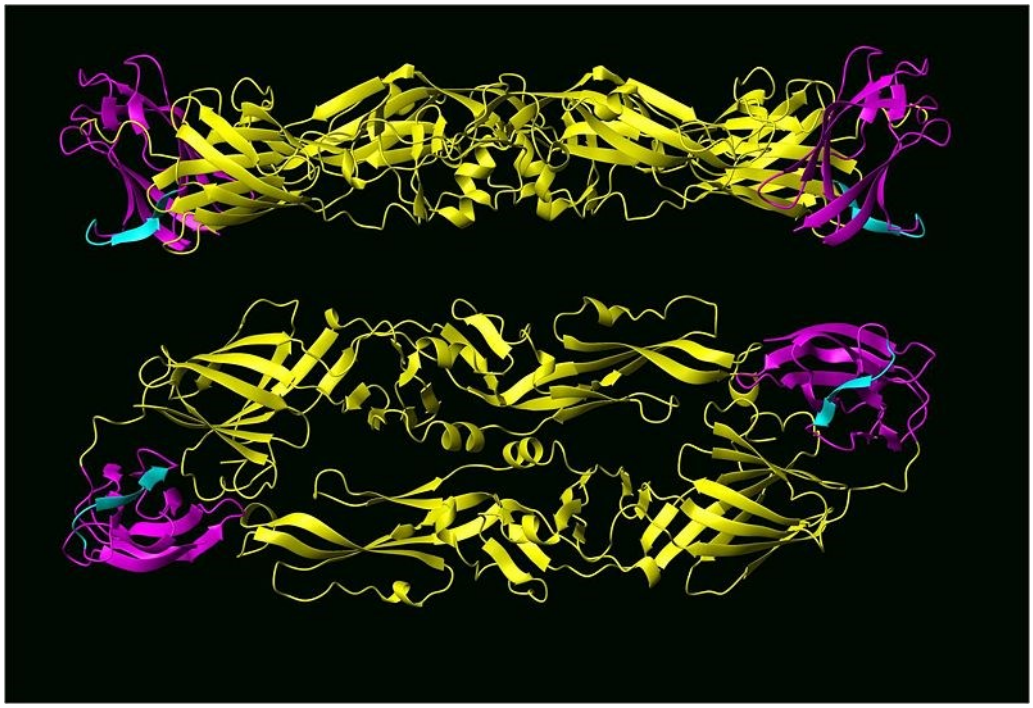


Figure 1.2: Top and side view of the Dengue virus envelope protein dimer structure with domain III hi-lighted in magenta and cyan (Adapted from Volk based coordinates from Harrison et al, RSCB protein data bank)

Figure 1.3

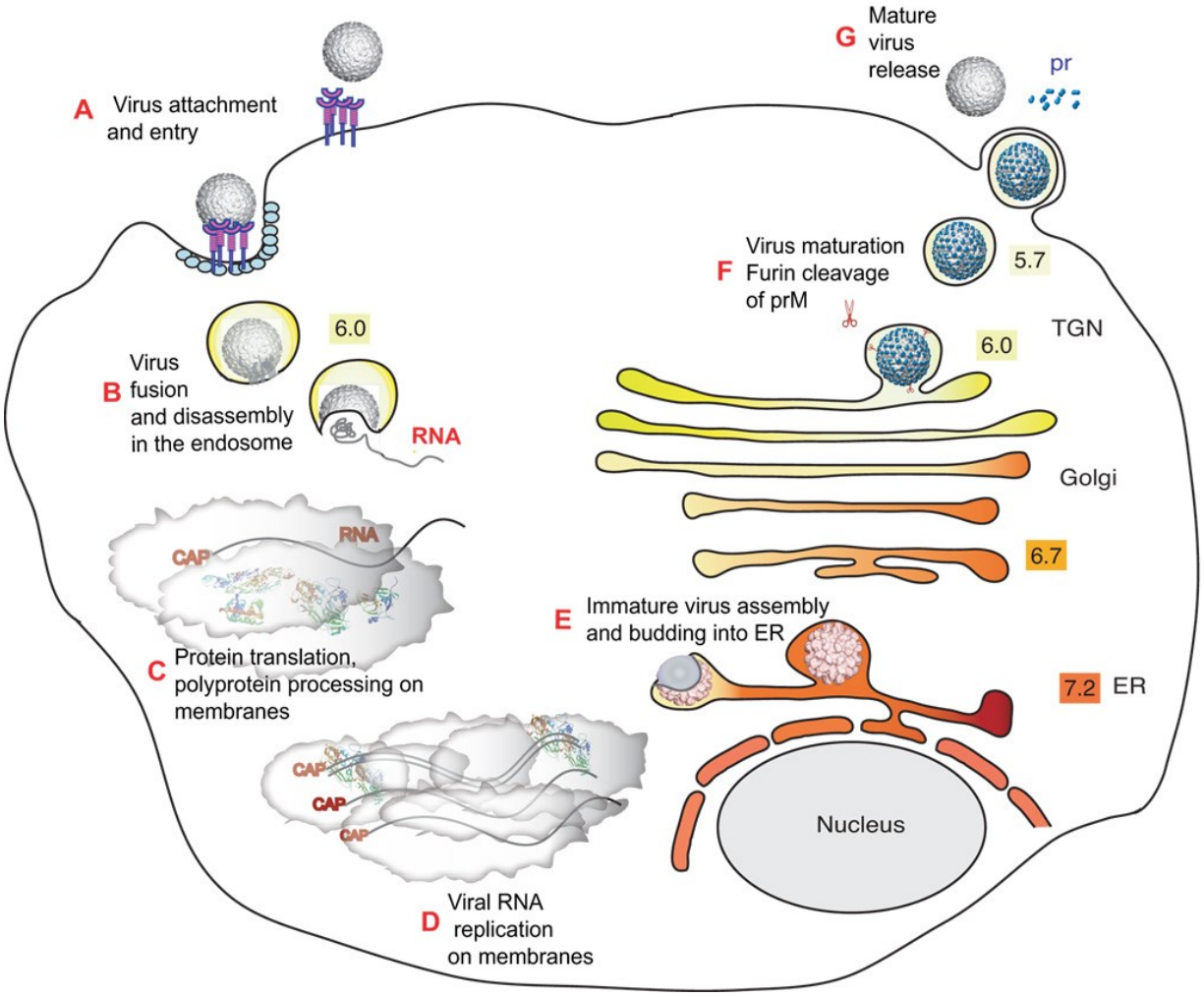


Figure 1.3: The flavivirus life cycle. A - G represents each step of flavivirus life cycle (adapted from Perera et al, *Antiviral Res.* 2008 Oct; 80(1):11-22. doi: 10.1016/j.antiviral.2008.05.004.)

Figure 1.4

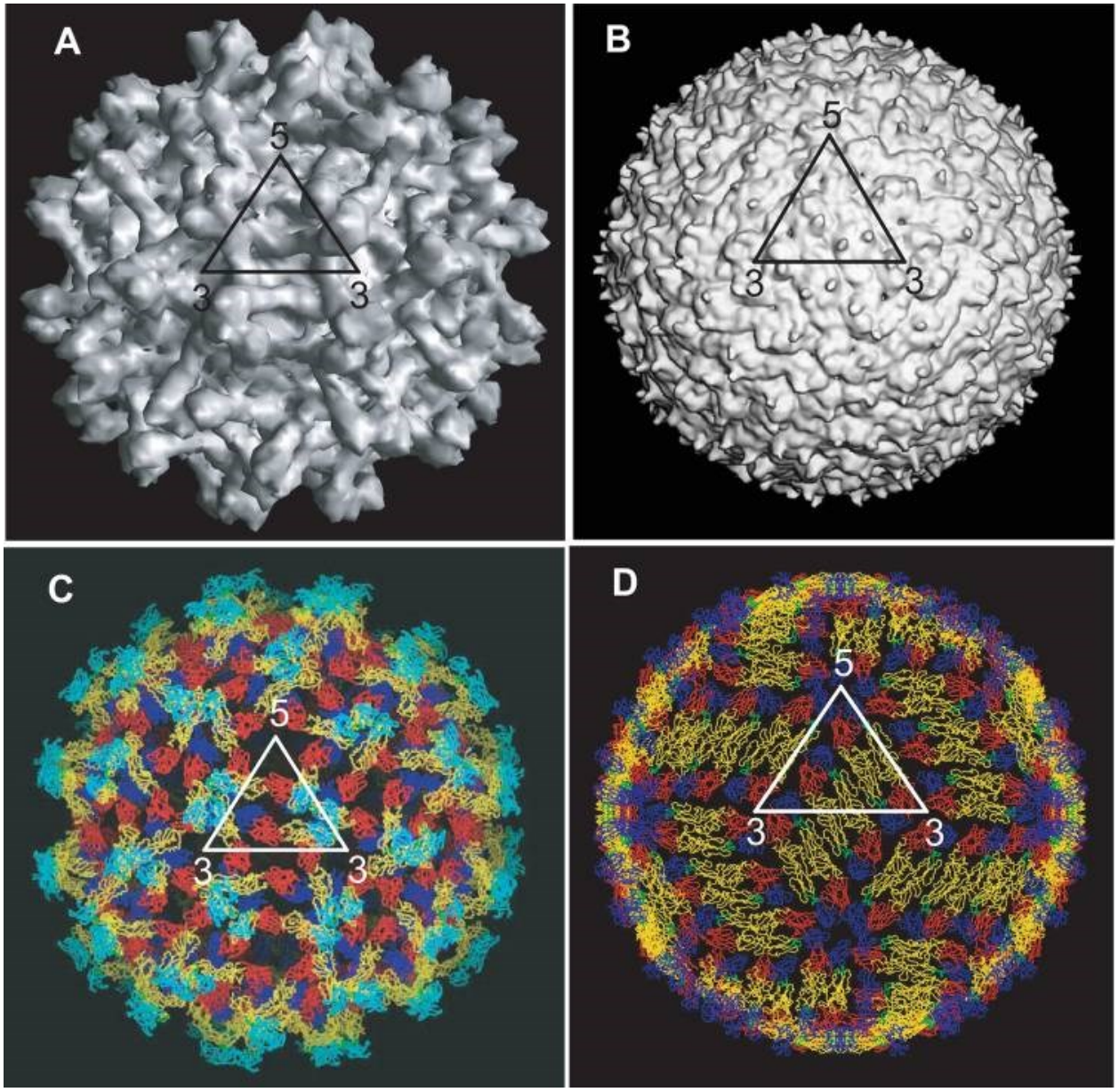


Figure 1.4: Structure of mature virion and immature virion. **A.** A surface shaded view of the cryo-EM reconstruction of immature DENV-2. **B.** A surface shaded view of the cryo-EM reconstruction of mature DENV-2. **C.** Arrangement of the E proteins on the surface after fitting of the atomic coordinates of the E protein Cα residues into the immature virus and **D.** mature virus. The 3 vertices of the triangle and number 3, 3, 5 represent different symmetry points in an icosahedral unit (adapted from Izabela A. Rodenhuis-Zybert, *trends in microbiology*, Volume 19, Issue 5, May 2011, Pages 248–254).

1.5 References

Beltramello, M., K. L. Williams, et al. (2010). "The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity." Cell Host Microbe **8**(3): 271-283.

Bryant, J. E., A. E. Calvert, et al. (2007). "Glycosylation of the dengue 2 virus E protein at N67 is critical for virus growth in vitro but not for growth in intrathoracically inoculated Aedes aegypti mosquitoes." Virology **366**(2): 415-423.

Cockburn, J. J., M. E. Navarro Sanchez, et al. (2012). "Mechanism of dengue virus broad cross-neutralization by a monoclonal antibody." Structure **20**(2): 303-314.

Costin, J. M., E. Zaitseva, et al. (2013). "Mechanistic study of broadly neutralizing human monoclonal antibodies against dengue virus that target the fusion loop." J Virol **87**(1): 52-66.

Crill, W. and J. Roehrig (2001). "Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells." **75**: 7769-7773.

de Alwis, R., M. Beltramello, et al. (2011). "In-depth analysis of the antibody response of individuals exposed to primary dengue virus infection." PLoS Negl Trop Dis **5**(6): e1188.

de Alwis, R., S. A. Smith, et al. (2012). "Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions." Proc Natl Acad Sci U S A **109**(19): 7439-7444.

Dejnirattisai, W., A. Jumnainsong, et al. (2010). "Cross-reacting antibodies enhance dengue virus infection in humans." Science **328**(5979): 745-748.

Dejnirattisai, W., A. I. Webb, et al. (2011). "Lectin switching during dengue virus infection." J Infect Dis **203**(12): 1775-1783.

Halstead, S. B. (1970). "Observations related to pathogensis of dengue hemorrhagic fever. VI. Hypotheses and discussion." Yale J Biol Med **42**(5): 350-362.

Halstead, S. B., S. Nimmannitya, et al. (1970). "Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered." Yale J Biol Med **42**(5): 311-328.

Halstead, S. B., S. Rojanasuphot, et al. (1983). "Original antigenic sin in dengue." Am J Trop Med Hyg **32**(1): 154-156.

Heise, M. T., A. Whitmore, et al. (2009). "An alphavirus replicon-derived candidate vaccine against Rift Valley fever virus." Epidemiol Infect **137**(9): 1309-1318.

Junjhon, J., T. J. Edwards, et al. (2010). "Influence of pr-M cleavage on the heterogeneity of extracellular dengue virus particles." J Virol **84**(16): 8353-8358.

Kroschewski, H., S. L. Allison, et al. (2003). "Role of heparan sulfate for attachment and entry of tick-borne encephalitis virus." Virology **308**(1): 92-100.

Lai, C. Y., W. Y. Tsai, et al. (2008). "Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II." J Virol **82**(13): 6631-6643.

Lambeth, C. R., L. J. White, et al. (2005). "Flow cytometry-based assay for titrating dengue virus." J Clin Microbiol **43**(7): 3267-3272.

Lok, S. M., V. Kostyuchenko, et al. (2008). "Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins." Nat Struct Mol Biol **15**(3): 312-317.

Modis, Y., S. Ogata, et al. (2003). "A ligand-binding pocket in the dengue virus envelope glycoprotein." Proc Natl Acad Sci U S A **100**(12): 6986-6991.

Modis, Y., S. Ogata, et al. (2004). "Structure of the dengue virus envelope protein after membrane fusion." Nature **427**(6972): 313-319.

Mongkolsapaya, J., W. Dejnirattisai, et al. (2003). "Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever." Nat Med **9**(7): 921-927.

Navarro-Sanchez, E., R. Altmeyer, et al. (2003). "Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses." EMBO Rep **4**(7): 723-728.

Nayak, V., M. Dessau, et al. (2009). "Crystal structure of dengue virus type 1 envelope protein in the postfusion conformation and its implications for membrane fusion." J Virol **83**(9): 4338-4344.

Okuno, Y., A. Igarashi, et al. (1978). "Neutralization tests for dengue and Japanese encephalitis viruses by the focus reduction method using peroxidase-anti-peroxidase staining." Biken J **21**(4): 137-147.

Pierson, T. C., Q. Xu, et al. (2007). "The stoichiometry of antibody-mediated neutralization and enhancement of West Nile virus infection." Cell Host Microbe **1**(2): 135-145.

Putnak, R., J. Fuller, et al. (2003). "Vaccination of rhesus macaques against dengue-2 virus with a plasmid DNA vaccine encoding the viral pre-membrane and envelope genes." Am J Trop Med Hyg **68**(4): 469-476.

Ranjit, S. and N. Kissoon (2011). "Dengue hemorrhagic fever and shock syndromes." Pediatr Crit Care Med **12**(1): 90-100.

Rico-Hesse, R. (2003). "Microevolution and virulence of dengue viruses." Adv Virus Res **59**: 315-341.

Smith, S. A., Y. Zhou, et al. (2012). "Persistence of circulating memory B cell clones with potential for dengue virus disease enhancement for decades following infection." J Virol **86**(5): 2665-2675.

Stadler, K., S. L. Allison, et al. (1997). "Proteolytic activation of tick-borne encephalitis virus by furin." J Virol **71**(11): 8475-8481.

Stiasny, K., S. L. Allison, et al. (2002). "Membrane interactions of the tick-borne encephalitis virus fusion protein E at low pH." J Virol **76**(8): 3784-3790.

Taketa-Graham, M., J. L. Powell Pereira, et al. (2010). "High throughput quantitative colorimetric microneutralization assay for the confirmation and differentiation of West Nile Virus and St. Louis encephalitis virus." Am J Trop Med Hyg **82**(3): 501-504.

Villabona-Arenas, C. J., D. R. Miranda-Esquivel, et al. (2009). "Phylogeny of dengue virus type 3 circulating in Colombia between 2001 and 2007." Trop Med Int Health **14**(10): 1241-1250.

Wahala, W. M., A. A. Kraus, et al. (2009). "Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody." Virology **392**(1): 103-113.

White, L. J., C. A. Sariol, et al. (2013). "An alphavirus vector-based tetravalent dengue vaccine induces a rapid and protective immune response in macaques that differs qualitatively from immunity induced by live virus infection." J Virol **87**(6): 3409-3424.

Whitehorn, J. and J. Farrar (2010). "Dengue." Br Med Bull **95**: 161-173.

WHO (2009). "Dengue and dengue haemorrhagic fever." WHO Fact Sheets **117**.

Xie, H., A. R. Cass, et al. (1998). "Yellow fever 17D vaccine virus isolated from healthy vaccinees accumulates very few mutations." Virus Res **55**(1): 93-99.

Yu, I. M., W. Zhang, et al. (2008). "Structure of the immature dengue virus at low pH primes proteolytic maturation." Science **319**(5871): 1834-1837.

Zhang, W., P. R. Chipman, et al. (2003). "Visualization of membrane protein domains by cryo-electron microscopy of dengue virus." Nat Struct Biol **10**(11): 907-912.

Zhang, Y., J. Corver, et al. (2003). "Structures of immature flavivirus particles." EMBO J **22**(11): 2604-2613.

Zybert, I. A., H. van der Ende-Metselaar, et al. (2008). "Functional importance of dengue virus maturation: infectious properties of immature virions." J Gen Virol **89**(Pt 12): 3047-3051.

CHAPTER 2

CHARACTERIZATION OF MONOCLONAL ANTIBODIES ISOLATED FROM PBMC OF DENGUE IMMUNE PATIENTS

2.1 Introduction

Dengue viruses (DENV) are expanding globally with an estimated 50-100 million cases of dengue infection worldwide annually, and more than 20,000 deaths. It is now estimated that approximately one fifth of the world population is at risk of infection by DENV (Guzman, Halstead et al. ; Gibbons and Vaughn 2002; Gubler 2002). Dengue is also threatening the continental US. More than 25 cases of locally acquired infection were reported in Key West, Florida in 2009 and 2010 . Symptomatic dengue disease ranges in severity from an influenza-like illness to life threatening hemorrhagic fever or shock. Understanding the pathogenesis of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) is of central importance in the rationale development of antiviral for treatment and vaccines to prevent dengue disease (Green and Rothman 2006; Whitehead, Blaney et al. 2007).

The DENV complex consists of 4 serotypes. Infection with a single serotype leads to antibody responses that cross-react with all serotypes (Halstead 2002). Despite the cross-reactivity, individuals only develop protective immunity against the serotype responsible for infection (Halstead 2002; Rothman 2004). Human studies have established clearly that the risk of progressing to DHF is greater during secondary than during primary infection (Halstead 2003). A prevailing theory that explains severe dengue during secondary infection is that pre-existing, non-neutralizing dengue-specific

antibodies bind to virions and enhance entry and replication in Fc-receptor-bearing cells, which leads to a higher viremia and release of cytokines and vasoactive mediators that increase vascular permeability (Halstead 2003). The molecular process of antibody-dependent enhancement (ADE) of infection has been demonstrated to occur with DENV and antibodies using cells in culture and animal models (Halstead and O'Rourke 1977). A better understanding of the molecular, genetic, and structural basis for recognition of dengue viruses by human antibodies is greatly needed, and could lead to the rational design of vaccines that enhance the induction of neutralizing antibodies while lowering the risk of DHF/DSS.

Dengue viruses are enveloped viruses of the *Flaviviridae* family that display pseudo-icosahedral symmetry, with 180 copies of the envelope (E) glycoprotein and 180 copies of the membrane (M) protein in the lipid bilayer membrane. Previous structural studies have shown that the dengue virus E protein is arranged into 30 rafts of three parallel dimers in mature virion particles (Kuhn, Zhang et al. 2002). Envelope glycoproteins form dimers, and crystal structures of the dimeric E protein have been determined for three DENV serotypes (DENV1, DENV2 and DENV3) (Modis, Ogata et al. 2003; Zhang, Zhang et al. 2004; Modis, Ogata et al. 2005; Nayak, Dessau et al. 2009). The E glycoprotein monomer possesses three principal domains, designated domains I, II, and III (DI, DII, DIII). DIII is likely the recognition domain for the principal cell receptor (Rey, Heinz et al. 1995; Bhardwaj, Holbrook et al. 2001; Crill and Roehrig 2001; Hung, Hsieh et al. 2004). DII possesses the fusion loop (Modis, Ogata et al. 2004) and an N-linked glycan, which can contribute to cell binding by interaction with DC-SIGN, a dendritic cell-specific adhesion receptor that is a C-type lectin (Pokidysheva, Zhang et al. 2006). Extensive characterization of neutralizing epitopes on dengue virus E protein has occurred over the past 10 years, using monoclonal antibodies (MAbs) generated in mice

inoculated multiple times with dengue viruses (Brien, Austin et al. ; Shrestha, Brien et al. ; Roehrig, Bolin et al. 1998; Chen, Huang et al. 2007). Mapping of epitopes recognized by strongly neutralizing mouse MAbs has identified several major antigenic sites on the E protein (Pierson, Fremont et al. 2008). Studies have shown that sites in the region of the fusion peptide, located at the tip of E protein DII (Rey, Heinz et al. 1995; Oliphant, Nybakken et al. 2006) and near the E protein hinge and linker regions between DI and DII, are recognized by cross-reactive, moderately neutralizing mouse MAbs. The most potent neutralizing antibodies are serotype-specific, however, and bind epitopes on the lateral surface of DIII of the E protein (Modis, Ogata et al. 2005; Oliphant, Engle et al. 2005). Investigators also have started to uncover specific mechanisms of antibody neutralization of flaviviruses. Some flavivirus antibodies that neutralize probably do so by sterically blocking attachment of the virus to the cell receptor. Other neutralizing antibodies block a step after cell attachment and such antibodies possibly neutralize by preventing protein conformational changes required for viral fusion in endosomes (Nybakken, Oliphant et al. 2005; Kaufmann, Nybakken et al. 2006). Some antibodies (for example the DENV MAb1A1D-2) appear to bind to hidden epitopes that are transiently exposed on the particle and promote a cascade of E protein rearrangements on the particle (Lok, Kostyuchenko et al. 2008). These studies have been instrumental in understanding mechanisms for flavivirus neutralization, however, since the antibodies were generated artificially in mice, the ability to translate this information to humans is limited.

The ability of flavivirus MAbs to enhance dengue virus infection in cell culture and animal models has been studied extensively (Balsitis, Williams et al. ; Dejnirattisai, Jumnainsong et al. ; Halstead and O'Rourke 1977; Goncalvez, Engle et al. 2007). ADE of infection has been shown to occur with antibodies binding to E or prM proteins

(Dejnirattisai, Jumnainsong et al. ; Kou, Lim et al. ; Huang, Yang et al. 2006). This phenomenon results in as much as a 1,000-fold increase in infectivity and is Fc-mediated (Balsitis, Williams et al. ; Littaua, Kurane et al. 1990). Recent work by Pierson *et al*., using mouse MAbs against WNV, has shed light on the stoichiometry of this process (Pierson ; Pierson, Xu et al. 2007). For each antibody studied, a threshold number of MAbs docked to the virion dictated whether neutralization or enhancement took place. The type of functional activity was determined not only by the location of the epitope, but also by the antibody affinity and epitope accessibility. An additional related ADE mechanism has been demonstrated to occur with anti-prM antibodies. Using either mouse or human MAbs, investigators have shown that the presence of anti-prM antibodies causes immature viral particles that have prM on the surface, which are normally noninfectious, to acquire the ability to infect cells efficiently through Fc-mediated pathways (Dejnirattisai, Jumnainsong et al. ; Rodenhuis-Zybert, van der Schaar et al. ; Rodenhuis-Zybert, Wilschut et al.). The role that ADE and anti-prM antibody-mediated enhanced infectivity of immature virus particles play in the pathogenesis of dengue infection is of significant interest. Characterization of antibodies obtained from humans following natural infection could prove to be an important tool for further investigation.

Until very recently, the isolation of naturally occurring human MAbs has been challenging. Most of our knowledge of the human antibody response to dengue has come from studies using polyclonal sera of naturally infected patients. Using antibody depletion experiments, we have previously demonstrated that E protein DIII binding antibodies make up a small fraction of the anti-dengue binding and neutralization activity in immune sera (Wahala, Kraus et al. 2009). Crill *et al.* confirmed that serotype-specific DIII antibodies formed a very small proportion of the polyclonal response, however,

these investigators showed a significant correlation between the presence of such antibodies and dengue virus neutralizing activity (Crill, Hughes et al. 2009). Taken together, these studies suggest that very rare serotype-specific potent neutralizing antibodies may be the primary determinants of protection against severe disease in humans.

Recently, several groups have generated panels of human dengue-specific MAbs using B cells from people exposed to natural DENV infection, to investigate the humoral response to dengue infection. Schieffelin *et al.* generated three E protein reactive human MAbs and showed that all three where at least partially cross-reactive and two lacked neutralizing activity (Schieffelin, Costin et al.). A panel of six human anti-preM monoclonal antibodies was developed and shown to be entirely cross-reactive and devoid of significant neutralization activity (Dejnirattisai, Jumnainsong et al.). Other investigators have isolated larger panels of human MAbs to dengue (Beltramello, Williams et al. ; de Alwis, Beltramello et al.). These studies indicate that most antibodies are serotype cross reactive and weakly to non-neutralizing. Fewer than 5% of the antibodies displayed moderate to strong neutralization of one or more serotypes.

In the study presented here, we use a high efficiency optimized method to generate human hybridomas to make a large panel of MAbs to dengue viruses derived from peripheral blood cells of travelers following natural primary or secondary infections.

2.2 Materials and Methods

Viruses and recombinant proteins used in study. DENV1 WestPac-74, DENV2 S-16803, DENV3 CH-53489, and DENV4 TVP-360, provided by Dr. Robert Putnak (Walter Reed Army Institute of Research, Silver Spring, MD) were used in the present study. These viruses were used to prepare infectious stocks and purified DENV antigens as

previously described (Wahala, Kraus et al. 2009). A pure virus antigen mixture containing equal quantities of each of the 4 serotypes were used to screen and characterize human hybridomas. Recombinant proteins containing fragments of E or prM were used to determine antigens and domains recognized by human antibodies. Recombinant envelope (rE) proteins (80% of E protein) from the 4 DENV serotypes were purchased from Hawaii Biotech, Inc (Modis, Ogata et al. 2003). This antigen binds to conformational MAbs and X-ray crystallography studies have demonstrated that these proteins retained a native-like structure (Modis, Ogata et al. 2003; Modis, Ogata et al. 2005). DIII of envelope from each of the 4 serotypes was expressed as fusion protein with maltose binding protein (MBP) in E. coli and purified as previously described (Modis, Ogata et al. 2003). These proteins also bind to conformational MAbs and retain native-like structure (Modis, Ogata et al. 2003). Purified DENV2 proteins containing DI and II of envelope and pr peptide of prM were kindly provded by Margaret Kielian and A. Zheng from Albert Einstein College of Medicine, New York (Liao, Sanchez-San Martin et al. ; Zheng, Umashankar et al.).

Human subjects and peripheral blood cell isolation. We identified a panel of 12 dengue immune subjects in North Carolina by screening volunteers who suspected exposure during past travel to dengue endemic regions. In most cases we were able to pinpoint the year and country of infection by taking a detailed travel and clinical history. From dengue immune subjects approximately 100 mL of blood was collected by veinpuncture and immediately processed to isolate peripheral blood mononuclear cells (PBMCs) by density gradient separation on Ficoll. The cells were immediately cryopreserved and stored in liquid nitrogen. The protocol for recruiting and collecting blood samples from people was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill.

Generation of human hybridomas. Previously cryopreserved samples were thawed rapidly in a 37 ºC water bath and washed once in 10 mL pre-fusion medium (Stemcell Technologies, ClonaCell®-HY 03801). Cells were counted and viability assessed with trypan blue staining (Gibco 15250-061). For every 4 million viable cells, the following then was added: 13 mL warmed pre-fusion medium, 20 μL CpG stock (2.5 mg/mL)(ODN 2006; Invivogen #tlrl-hodnb-5), 20 μL cyclosporin A stock (1 mg/mL in ethanol; Sigma C1832), 20 μL Chk2i stock (10 mM; Sigma #C3742), 4.5 mL clarified supernatant from cultures of B95.8 cells (ATCC VR-1492) containing Epstein-Barr virus. The mixture then was plated into 384-well plates (NUNC 164688) at 50 μL/well, and plates were incubated at 37 ºC with 5% CO_2 for 10 days, prior to screening for antigen specific cell lines with ELISA. Cells from wells with supernatants reacting in dengue virus-specific ELISA then were expanded by collecting all cells in the well and transferring them to a 96 well flat bottom plate (Falcon 353072) in 200 μL pre-fusion medium containing irradiated human PBMCs, as follows: 20 mL pre-fusion media, 20 μL CpG stock, 20 μL Chk2i stock, and 8 million heterologous healthy donor PBMCs that had been gamma-irradiated with 3,000 rad. Plates were incubated at 37 ºC for 4 days before exchanging 100 μL of medium with fresh pre-fusion medium. Plates then were incubated for an additional 3-4 days prior to fusion with HMMA2.5 non-secreting myeloma cells (kindly provided by Dr. Marshall Posner).

Screening ELISA. Gradient-purified DENV prepared in carbonate binding buffer was used to coat ELISA plates (Nunc 242757), then UV-inactivated using a calibrated UV light source (Stratalinker; Stratagene) for 10 min, prior to incubation at 4 °C overnight. Plates then were blocked with 50 μL/well blocking solution and incubated at room temperature for 1 hr in block, consisting of 10 g powdered milk, 20 mL goat serum, 100 mL of 10X PBS, 0.5 mL Tween (Sigma 7949), mixed to 1L final volume with dH_2O.

Plates were washed x 4 with PBS, and 5 μL of supernatant was transferred from one well of a 384-well plate containing EBV-transformed B cell lines, using a pin-tool device (V&P Scientific), into 25 μl/well of block. Plates were incubated at room temperature for 1 hr prior to washing x 4 with PBS. Secondary antibody (goat anti-human Fc; Meridian Life Science, W99008A) was applied at a 1:5,000 dilution in blocking solution using 25 μl/well, and plates again were incubated at room temperature for 1 hr. Following repeat PBS washing x 4, phosphatase substrate solution (1 mg/mL phosphatase substrate in 1 M Tris aminomethane) (Sigma, S0942) was added at 25 μL/well and plates were incubated at room temperature for 3 hr before reading the optical density at 405 nm on a Molecular Devices plate reader.

Electrofusion of EBV-transformed B cells with myeloma fusion partner. HMMA2.5 cells were counted and suspended as 10 million cells/mL in a microcentrifuge tube in 1 mL warmed cytofusion media prepared as follows: 300 mM sorbitol (Fisher, BP439-500), 0.1 mM calcium acetate (Fisher, AC21105-2500), 0.5 mM magnesium acetate (Fisher, AC42387-0050), 1.0 mg/mL BSA (Sigma, A2153). Following 7 days of expansion in 96-well plates, cells from EBV-transformed B cell wells were pipetted gently into microcentrifuge tubes containing 1 mL of warmed cytofusion medium. Transformed B cells and HMMA2.5 cells were centrifuged at 900 x *g* for 4 min, supernatants were decanted and the pellets resuspended in 1 mL of cytofusion medium. This process was repeated three times to ensure equilibration to cytofusion medium. Cytofusion medium then was decanted gently from each sample tube such that approximately 100 μL remained, and the pellet was retained. HMMA2.5 cells then were resuspended in cytofusion medium to achieve a 10 million cells/mL concentration. One hundred μL of HMMA cell suspension then was mixed with each sample tube and the mixture of HMMA and EBV transformed B cells was pipetted into cytofusion cuvettes (BTX, 450125). The

cuvette then was placed in a modified cytofusion device, using a BTX cuvette holder (BTX Safety stand, Model 630B) and Cyto-Pulse Sciences generator (Cyto-Pulse Sciences, PA-4000/PA-101) and the electrical discharge program run with following settings: pre-fusion AC current of 70 volts for 40 sec, followed by a DC current pulse of 300 volts for 0.04 msec, then post-fusion AC current of 20 volts for 30 sec. Following fusion, cuvettes were incubated at 37 °C for 30 min. The content of each cuvette then was added to 20 mL of HAT medium containing ouabain, composed of the following: 500 mL post-fusion medium (Stemcell Technologies, 03805), one vial 50X HAT (Sigma, H0262), and 150 μL of a 1 mg/mL stock of ouabain (Sigma, 013K0750). Fusion products then were plated at 50 μL/well into 384-well plates and incubated at 37 °C for 21 days before screening hybridomas for antibody production by ELISA.

MAb production and purification. Wells containing hybridomas producing dengue virus-specific antibodies were cloned biologically by two rounds of limited dilution plating. Once clonality was achieved, each hybridoma was expanded in post-fusion medium (Stemcell Technologies, 03805) until 50% confluent in 75 cm^2 flasks (Corning, 430641). For antibody expression, the cells in 75 cm^2 flasks were collected with a cell scraper; the hybridomas were washed in serum free media (GIBCO Hybridoma-SFM from Invitrogen, 12045084), and split equally amongst four 225 cm^2 flasks (Corning, 431082) containing 250 mL serum free media. Flasks were incubated for 21 days before medium was clarified by centrifugation and 0.2 μm sterile filtered. Antibodies were purified from clarified medium by protein G chromatography (GE Life Sciences, Protein G HP Columns, 17-0404-03).

Neutralization assay. The neutralizing potency of antibodies was measured using a flow cytometry-based neutralization assay with the U937 human monocytic cell line

stably transfected with DC-SIGN, as previously described (Kraus, Messer et al. 2007).

ADE assays. The ability of antibodies to enhance DENV was measured using U937 cells that had not been engineered to express DC-SIGN in the absence of the virus attachment factor; these Fc receptor bearing cells are only susceptible to infection in the presence of dengue-specific antibodies. Test antibodies were diluted to different concentrations and mixed with viruses containing 10^4 FFU in 96-well round bottom plates, and incubated at 37°C for 45 minutes. Then 2 x 10^4 U937 cells were added to the mixture and incubated for 2 hrs at 37°C. The cells were washed to remove unbound virus/ antibody mixtures and incubated with fresh medium at 37°C. The cells were harvested 24 hrs later, fixed and processed for flow cytometric analysis to determine the percent of infected cells. ADE activity was expressed as the percent increase of infected cells in the dengue-specific antibody treated sample compared to the sample treated with a control antibody.

2.3 Results

Electrofusion technique for generation of human hybridomas. For generation of human hybridomas using PBMCs from individuals who had recovered from natural infection, we used an established electrofusion method, but made several alterations to the previously published protocol (Yu, McGraw et al. 2008). First, we found that the kinetics and efficiency of transformation of human B cells using EBV with CpG stimulation was improved inhibiting EBV-associtated apoptosis with the addition of a pharmacologic inhibitor of CHK2, a protein kinase that is activated in response to DNA damage and is involved in cell cycle arrest (Nikitin, Yan et al.). Also, following EBV transformation of PBMCs and identification of a well producing a dengue antibody of interest, expansion of EBV transformed B cells in the presence of irradiated PBMCs,

CpG, and CHK2 inhibitor greatly increased transformed B cell numbers, resulting in the greater likelihood of generating the hybridoma of interest by electrofusion. This added step improved the overall efficiency of isolation of hybridomas by at least two-fold (data not shown). Additional improvements in throughput and efficiency were achieved by adapting a different electroporation cuvette system (BTX cat no. 45-0207, Model 630B) from that previously described for use with a PA-4000/PA-101 electrofusion device (Cyto-Pulse Sciences). This cuvette system allows for the use of standard disposable electroporation cuvettes, permitting cytofusion of two samples at one time. In addition to the convenience of the disposable cuvette, a moderate improvement in the efficiency of hybridoma generation was observed when compared to the Cytopulse cuvette system (data not shown). Finally, improvement in consistency was seen with the use of a commercial hybridoma cell culture medium, typically used for generation and growth of mouse hybridomas (ClonaCell-HY Medium, STEMCELL Technologies). Together these modifications resulted overall in a greater than ten-fold increase in the number of human hybridomas that could be generated using PBMCs from immune individuals, compared to historical data in our laboratory.

Demographics and screening of traveler subjects. To better our understanding of the human antibody response to DENV following infection, we initiated a study to identify and obtain blood from people living in North Carolina who had been exposed to DENV during travel or previous residence in endemic regions. Subjects with a past clinical history compatible with DENV infection were invited to volunteer. We confirmed a past DENV infection by identifying the presence of dengue virus neutralizing antibodies in donor serum, testing against each of the 4 serotypes of DENV (Table 2.1). An individual whose serum mainly neutralized a single serotype was classified as having a past history of infection with a single serotype (“primary”) (Table 2.1). Individuals whose serum

contained antibodies that broadly neutralized two or more serotypes were considered to have had two or more previous infections ("secondary") (Table 2.1). For secondary cases, it was not possible to use the neutralization titers to identify the specific serotypes causing infection or the sequences of the infecting viruses, since these sera typically recognized all four serotypes. All DENV immune subjects filled out a detailed questionnaire about previous travel and clinical history, and this information was used to determine the year and location of likely infection, as well as the interval between infection and sample collection for the current study (Table 2.1).

To produce dengue specific human hybridomas, cryopreserved PBMC samples from each subject were thawed and inoculated with EBV in the presence of CpG, CHK2 inhibitor, and cyclosporine A, as described in the Materials and Methods section. As shown in Table 2.1, between 744 and 2,976 EBV transformed B cell cultures were generated from samples from each of the twelve subjects, who had suffered primary DENV 2 (2 subjects) or DENV 3 (4 subjects) infection, or secondary infection (6 subjects). Screening for the presence of antibodies to dengue virus in the supernatant of transformed B cell cultures showed that between 0.4 and 5% of wells in the 384-well transformations were positive. Based on the number of positive wells and the number of lymphocytes tested, the frequency of DENV-specific B cells in circulation was estimated for each subject (Table 2.1). Frequency was based on the total number of B cells plated (estimated as 5% of total PBMCs) with an estimated 10% EBV transformation efficiency (based on average colony counts in transformed wells). A total of thirty-seven human anti-dengue virus hybridomas was generated from twelve different subjects, with a yield ranging from 1 to 10 hybridomas per subject. Twenty-eight hybridomas were derived from individuals who had primary infection and nine from secondary infection. Remarkably, five hybridomas were derived from cells of subjects who had been infected

with dengue 20 years or more prior to PBMC collection. Antibodies secreted by each of the thirty-seven human hybridoma cell clones were purified and characterized to determine their dengue virus protein specificity. Twenty-nine MAbs bound the E protein, while eight bound the M protein.

Characteristics of E protein binding human MAbs. Results of the characterization of 29 purified human anti-E MAbs from anti-dengue hybridomas are shown in Table 2.2 Hybridomas derived from subjects 3, 19, 103 and 105 comprise the majority (23 of the 29 anti-E MAbs generated). Cells were obtained from these subjects following a primary infection. The remaining six anti-E antibodies were derived from six individuals following either primary or secondary infection. Three anti-E MAbs (designated 4G9, 2C2, and 3H4) were IgG2 molecules, while the rest were of the IgG1 isotype. Of the anti-E MAbs generated, 12 used kappa and 17 used lambda light chains.

As can be seen in Table 2, 29 of the 37 human anti-dengue MAbs bound to E protein in either ELISA or western blot assay. Of the 29 anti-E antibodies characterized, all but 3 were fully cross-reactive, binding all four dengue serotypes in ELISA at the lowest concentration tested (1 ng/ml). Two antibodies, MAbs 2J20 and 2D7, were considered partially cross-reactive, as they bound to more than one but less than four serotypes. The DENV 2 serotype-specific MAb 2D22 bound only to purified DENV 2 virus in ELISA. Next, we determined the binding of these MAbs to recombinant E protein in ELISA. All but 5 MAbs bound recombinant E protein in ELISA. E protein-specific MAbs that did not bind recombinant E protein were shown to bind E protein using western blotting, with the exception of 2D22. DENV 2 serotype-specific antibody 2D22 did not bind to structural proteins in western blot or to recombinant E protein constructs in ELISA. Finally, we evaluated the ability of the twenty-nine anti-E MAbs to bind domain I/II or domain III fragments of E protein. Five antibodies bound the domain III portion of E

protein – 1C17, 1M23, 2J20, 1B23 and 1M19. Antibodies that did not bind recombinant domain III of E protein were tested in ELISA for binding to domain I/II of E. All of the anti-E protein antibodies that did not bind domain III (except for 2D22; *i.e.*, 23 out of 29 anti-envelope antibodies) bound DI/II.

The ability of these 29 human anti-E protein MAbs to neutralize representative viruses of the four dengue serotypes is shown in Table 2.2. Interestingly, six antibodies exhibited little or no neutralizing activity ($neut_{50}$ > 10μg/ml) against any of the four dengue serotypes. Most MAbs, 13 out of 29, were found to neutralize viruses weakly from three or four serotypes, with equivalent potency against those viruses. Seven antibodies were found to neutralize only one or two serotypes weakly. Of the 29 antibodies determined to bind E protein, only three MAbs, 2D22, 5J7, and 2J20, were determined to have moderate to strong ($neut_{50}$ < 1μg/ml) neutralizing activity against at least one serotype.

In Figure 2.1, the concentration-dependent neutralization activity of two strongly neutralizing human anti-E protein MAbs is shown. The percent neutralization for the four dengue serotypes is shown in panel A for MAb 5J7. This MAb, which was isolated from an individual who recovered from a primary DENV 3 infection, cross reacted with all 4 serotypes and bound to an epitope on DI/II of E protein (Table 2.1). Despite the serotype cross reactivity in binding assays, MAb 5J7 neutralized only serotype 3 virus. In panel C, the concentration-dependent neutralization activity of MAb 5J7 is shown in more detail, using a broader range of halving dilutions. MAb 5J7 neutralized 50% of serotype 3 virus at a concentration of 0.1μg/ml. The percent neutralization for MAb 2D22 against viruses from the four dengue serotypes is shown in Figure 2.1 panel B and D. This antibody, which was isolated from a subject exposed to a primary DENV2 infection, bound to DI/II of envelope protein of DENV2 only (Table 2.1). In panel B, MAb 2D22 is shown to have neutralizing activity only to serotype 2. MAb 2D22 exhibited 50% neutralization of DENV

2 at a concentration of 0.08 μg/ml (panel D). Interestingly, as can be seen in Figure 2.1, the maximum percentages of virus neutralization for MAbs 5J7 and 2D22 were very different. The maximum percentage of neutralization for MAb 5J7 was nearly 100%, whereas MAb 2D22 could neutralize only about 60% of virus in the assay at the highest concentration of antibody tested.

The ability of the 29 human anti-E MAbs to enhance infection *in vitro* is shown in Table 2. ADE assays were performed at a concentration of 1 μg/ml against a virus from each dengue serotype. Four antibodies did not exhibit enhancing activity (*i.e.*, caused < 5 fold enhancement) to three or even four dengue serotypes. Most MAbs, 15 out of 29, were found to enhance infection moderately (5–25 fold) for viruses from two to four dengue serotypes. Of the 29 antibodies tested, 17 had strong (> 25 fold) enhancing activity against virus from at least one serotype. Interestingly, MAb 2J20 strongly neutralized DENV 3 virus but strongly enhanced DENV 2 infection.

In Figure 2.2, neutralization or enhancement activity against a heterologous DENV 4 virus is shown for several representative human anti-E protein MAbs. Flow cytometric neutralization assays were performed using U937-DCSIGN cells and enhancement assays were performed using U937 cells lacking DCSIGN, as described in the Materials and Methods section. In Figure 2.2 panel A, data for fully cross-reactive, weakly neutralizing and moderately enhancing MAbs 2A15, 1L5, and 2A10 are shown. All three MAbs neutralized 50% of DENV 4 at a concentration of approximately 10μg/ml. These antibodies enhanced dengue infection moderately, resulting in a 5-25 fold peak increase in titer of DENV 4. In Figure 2.2 panel B, fully cross-reactive, non-neutralizing and non-enhancing MAbs 3F13, 1A15, and 3H9 are shown. All three antibodies bound DENV 4 but they did not neutralize or enhance virus infection effectively. Together, these two principal categories of anti-E protein antibodies represented the majority of the

human MAbs we isolated from donors with history of previous primary or secondary dengue infection.

Characteristics of membrane (M) protein binding human MAbs. A significant number of the MAbs we isolated were specific for M protein. Results of the characterization of purified M protein-specific human MAbs from anti-dengue hybridomas are shown in Table 2.3. Hybridomas derived from subjects 27 and 105 comprise 4 of the 8 anti-M MAbs generated. The remaining 4 antibodies were derived from cells from subjects 3, 5, 15, and 110. In contrast to the findings with anti-E protein MAbs, an equal number of anti-M protein MAbs was generated from subjects following primary or secondary infection. All of the anti-M protein MAbs were of the γ1 isotype. Of the anti-M protein MAbs generated, 4 had kappa light chains and 4 had lambda light chains.

As can be seen in Table 2.3, eight of the 37 human anti-dengue MAbs did not bind E protein in initial analysis, but instead bound to M protein in western blot. Of the eight anti-M protein antibodies characterized, all were fully cross-reactive, binding all four dengue serotypes in ELISA at the lowest concentration tested, 1μg/ml. To further characterize these human anti-M protein antibodies, we tested their ability to bind the recombinant “pr” portion of the membrane protein of DENV2 in ELISA. All eight MAbs bound recombinant “pr” in ELISA (Table 2.3). We tested these 8 human anti-M protein MAbs for ability to neutralize viruses from each of the four dengue serotypes (Table 2.3). Interestingly, each exhibited some weak neutralizing activity against virus from at least one of the four dengue serotypes. Surprisingly, MAbs 2M2 and 2H12 showed moderate to strong neutralization activity against virus of a single serotype.

The ability of the eight human anti-M protein MAbs to enhance infection *in vitro* is shown in Table 2.3. ADE assays were performed at a concentration of 1μg/ml against

representative viruses from each dengue serotype. All but one of the eight MAbs had strong (> 25-fold) enhancing activity for virus from at least one serotype. Interestingly, anti-M protein MAb 5L20 was nearly devoid of enhancing activity for viruses of any of the dengue serotypes in this assay.

Concentration-dependent neutralization and ADE assays were performed for each of the human anti-M protein MAbs against viruses from each of the four dengue serotypes. Results for several representative MAbs are shown in Figure 2.3. The ability of each anti-M protein antibody to neutralize and/or enhance infection with DENV 1 (panel A), DENV 2 (panel B), DENV 3 (panel C), or DENV 4 (panel D) is shown. MAbs 4F8, 1G6, and 4E9, each exhibited similar concentration-dependent neutralization curves, with $neut_{50}$ concentrations of > 10μg/ml against viruses from each dengue serotype. The concentration-dependent neutralization of MAb 5L20, however, differed, exhibiting almost no neutralizing activity. ADE occurred with MAbs 4F8, 1G6, and 4E9 (as well as 5E6, 2M2, 2H12, and 5G22 - data not shown). Overall, all but one anti-M protein MAb exhibited enhancing activity. The degree of enhancement varied between 2- and 126-fold, depending on the MAb and the serotype of the virus tested. Interestingly, anti-M protein MAb 5L20 did not enhance infection in this assay at any concentration tested.

2.4 Discussion

These studies reveal that the human B cell response to DENV infection is dominated by cross-reactive antibodies with low or no neutralizing potency and significant potential to enhance infectivity by Fc-mediated mechanisms. Antibodies with enhancing activity were isolated that were directed to both E and M proteins, thus conferring the ability to enhance infectivity not only with mature infectious particles but

also with partially or fully immature particles displaying prM protein that otherwise would be poorly infectious or non-infectious. Remarkably, B cells encoding these types of antibodies persisted in the circulation for decades after infection.

Interestingly however, a few rare, potently neutralizing antibodies that are nearly devoid of enhancing activity were produced naturally by humans in response to infection. Mapping of the epitopes recognized by naturally-occurring human dengue virus specific antibodies is essential in determining whether these enhancing and neutralizing activities can be separated. Understanding the epitopes and activity of these neutralizing antibodies will be critical for vaccine development, as vaccines that induce high potency neutralizing antibodies that lack enhancing activity are desirable. Ideally, the reactivity of epitopes bound by enhancing antibodies should be reduced or eliminated in candidate antigens during the rational development of a dengue vaccine, so as to discourage such dominant recognition of these antigenic features by the humoral immune response. The goal is that such molecular information could be used in the rational design of dengue vaccines that enhance the induction of protective neutralizing antibodies and reduce the risk of development of severe disease.

DENV E protein was the most common protein target of circulating human memory B cells in this study. Over three-quarters (29 of 37) of the human anti-dengue antibodies identified in this study bound to E protein. We screened for virus-specific antibodies using purified whole virus preparations that contained both immature and mature virus particles and all of the virus structural proteins [including a complete (non-truncated) E protein]. Therefore, these results confirm that the bulk of the human B cell response to exposed structural antigens is directed toward the E protein. The majority of the anti-E protein antibodies, 23 of 29, was obtained from subjects following primary infection. The E protein has been thought to be the dominant antigen following primary

and secondary dengue infection, when evaluated by western blotting studies using soluble antibodies in polyclonal sera from immune subjects (Lai, Tsai et al. 2008). Our studies show that the pattern of specificity of circulating B cells also reflects this predominance. Characterization of the E protein-specific antibodies showed that most were of the IgG isotype. This distribution is typical of the profile of most virus-specific responses in humans and also reflects the predominance of the IgG1 isotype in the circulating human memory B cell population. Light chain usage was split almost evenly at 12 kappa light chains and 17 lambda light chains. Both isotype and light chain usage suggest that isolation of clones using the hybridoma method is unbiased, as expected. Thus, the panel of human antibodies presented here likely reflects the frequency and specificity of the anti-dengue B memory cell pool present in circulation following recovery from primary or secondary infection.

The most striking feature of the anti-E protein antibodies was the predominance of cross-reactivity. Of the 29 anti-E protein antibodies characterized, all but three were fully cross-reactive, binding all four dengue serotypes. Considerable attention has been place on the DIII portion of the E protein for dengue and other flaviviruses (Beasley and Barrett 2002; Oliphant, Engle et al. 2005; Sanchez, Pierson et al. 2005; Sukupolvi-Petty, Austin et al. 2007). The importance of the human antibody response to DIII has been studied extensively, originally using polyclonal sera and more recently using human MAbs (Beltramello, Williams et al. ; Wahala, Kraus et al. 2009). However, it is clear that epitopes across other regions of the E protein contribute significantly to human protection (Oliphant, Nybakken et al. 2006; Wahala, Kraus et al. 2009). The data here provides additional information, specifically a better understanding of the frequency of these antibodies in circulation. Only 5 of the 29 E protein reactive antibodies bound the DIII portion of E protein. All the DIII antibodies were cross-reactive, and only one (2J20) had

neutralizing activity. Characterization of the 26 fully cross-reactive anti-E protein MAbs showed that most, but not all, were directed toward the DI/II region.

E protein specific human B cells specifying strongly neutralizing antibodies are rare. Of the 29 human anti-envelope antibodies generated, only three (MAbs 5J7, 2J20 and 2D22) met our definition of moderate to strongly neutralizing ($neut_{50}$ < 1.0μg/ml) – two following primary and one following secondary infection. Two of the three antibodies, MAbs 5J7 and 2J20 exhibited cross-reactive binding to all four dengue viruses in ELISA, yet strongly neutralized virus of only one serotype. 2D22 was DENV2 type-specific both for binding and neutralization. MAb 2J20 bound to DIII and 5J7 to DI/II of E protein. 2D22 was particularly interesting because it bound to the virus particle but not to any of the recombinant protein constructs used in the current study. There are several plausible explanations for these observations. The 2D22 epitope may be located on a region of the E that is not included in the recombinant constructs, given that the membrane proximal regions were removed in the design of these constructs in order to make them soluble. Additionally, the conformation of the epitope may be altered during preparation for ELISA or western blot. Alternatively, the epitope recognized by this very interesting human antibody might be very complex, involving the interface of neighboring E proteins. Indeed human MAbs that only bind a West Nile virus E protein epitope that is preserved on the intact virion but not recombinant protein have recently been described (Vogt, Moesker et al. 2009). The maximum percentage of virus neutralization for MAbs 5J7 and 2D22 differed greatly. The maximum percentage of neutralization for MAb 5J7 was nearly 100%, whereas MAb 2D22 could neutralize only about 60% at the highest concentration of antibody tested. This finding not only suggests that the MAbs recognize different epitopes, but also probably means that they use different mechanisms of neutralization.

ADE activity, not neutralization, was the dominant functional activity noted for the E-protein specific antibodies in this study. All but two anti-E protein antibodies enhanced infection of at least one serotype at the concentration tested (1µg/ml). Strongly neutralizing serotype-specific MAb 2D22 and fully cross-reactive weakly neutralizing MAb 3H4 were the only MAbs that had no detectable ability to enhance dengue infection at this initial concentration. Interestingly, further dilution of 2D22, well below the concentration where enhancement occurred with all of the other MAbs, demonstrated enhancement of infection (data not shown). Maximum ADE activity (25-fold) was seen at a concentration of 0.025µg/ml, as compared to between 0.1 and 1.0µg/ml, seen for other MAbs in our panel. Sixteen antibodies met our definition for mediating strong (> 25-fold) enhancement of at least one serotype, including the DIII-binding strongly neutralizing antibody 2J20. Taken together, these data show that potency of neutralization does not always inversely correlate with the strength of enhancement, as strongly neutralizing antibodies also were strongly enhancing at lower concentrations, while MAb 3H4 that was weakly neutralizing did not display enhancing activity. It is important to note that the human antibody response to dengue infection seems to rarely include antibodies that are able to separate neutralization from enhancement, as exemplified by MAb 2D22. This MAb possesses potent serotype-specific neutralization activity without the ability to enhance infection at the typical concentration range seen with weakly neutralizing cross-reactive MAbs. Understanding the specificity of these rare but very important antibodies is critical for the rational design of a dengue vaccine.

Nearly one-quarter, eight of 37, of the human anti-dengue antibodies identified in this study were M protein-specific. When compared to the number of anti-E protein antibodies obtained following primary versus secondary infection (24 following primary, 5 following secondary), there appeared to be a greater percentage of anti-M protein MAbs

generated from subjects following secondary infection (17 % anti-E, 50 % anti-M). This finding suggests that the human antibody response to M protein may be increased relative to the response to E protein following a secondary infection. This observation has been suggested previously by others using western blotting methods and dengue immune sera (Lai, Tsai et al. 2008). The most prominent characteristic noted in the panel of human anti-M MAbs here was the degree of cross-reactivity. All eight MAbs were fully cross-reactive, binding all four dengue serotypes in ELISA at the lowest concentration tested (1µg/ml) using whole virus as antigen. All but one M protein-specific MAb met our definition for strong (> 25-fold) enhancement of infection. Further characterization of these eight MAbs showed that all were directed toward the "pr" portion of the M protein, as they bound to recombinant "pr" fragment protein in ELISA. Interestingly, however, two anti-M protein MAbs met our definition for moderate to strongly neutralizing activity ($neut_{50}$ < 1.0µg/ml) – MAbs 2H12 and 2M2. These two pr-specific MAbs demonstrated serotype-specific neutralizing activity despite being fully cross-reactive. The physiologic significance of potent neutralization of dengue by pr-specific antibodies that should recognize only immature particles is uncertain, but bears further exploration.

In summary, the human B cell response encoding dengue-specific antibodies is complex. Human antibody-mediated neutralizing activity is directed to several proteins and diverse epitopes in those proteins. Most of the E protein specific antibodies exhibit ADE activity, while only rare clones possess potent neutralizing activity. The antibodies that do neutralize exhibit features that suggest differing mechanisms of neutralization. Most intriguingly, the most potent E protein specific neutralizing antibodies recognize occult epitopes that are present in virus particles, but not represented in recombinant antigens. Discovery of the structure and nature of the epitopes for these unique MAbs

could point the way toward better rational design of dengue vaccine antigens. Unexpectedly, some pr-specific antibodies exhibit relatively potent neutralizing activity.

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Table 2.1. Subject demographics, serologies, and hybridoma yields from dengue immune travelers

Type of infection	Serotype	Subject #	Geographic location of infection	Year	Years since infection	Reciprocal serum antibody 50% neutralization titer, to indicated DENV serotype				# DENV positive EBV wells per total wells tested	Estimated frequency of DENV+ B cells in circulation (x 10^{-4})	# of hybridomas obtained
						DEN V1	DEN V2	DEN V3	DEN V4			
Primary	2	19	Thailand	1997	8	95	>	20	105	79/1,860	9.3	10
		31	South Pacific	1997	8	20	320	40	20	9/2,232	1.8	1
	3	3	Thailand	2001	4	30	87	338	<	37/ 744	9.2	7
		5	Puerto Rico	2001	4	<	<	>	<	21/1,860	5.2	1
		103	Nicaragua	1995	14	<	<	278	<	12/1,860	2.5	3
		105	Thailand	2002	7	<	<	210	<	25/2,232	4.5	6
Secondary	Multiple	8	Haiti	1982-84	21	640	611	213	319	7/ 744	2.0	1
		9	India/Sri Lanka	2000	5	>	>	290	393	23/2,976	4.2	1
		15	West Indies	72-82	23	371	320	288	>	12/2,232	1.6	1
		27	Thailand/Cambodia	1981	24	>	>	>	285	33/1,488	6.3	3
		110	Kuala Lumpur	1998-99	10	205	>	153	96	49/2,232	9.5	2
		115	Sri Lanka	1974-97	12	90	134	330	165	6/1,488	1.7	1

> indicates a titer > 1:1,280; < indicates a titer < 1:20. Frequency was estimated based on the total number of B cells plated (an average of 5 % of total PBMCs) with an estimated 10% EBV transformation efficiency, based on colony counts in the transformation plates.

Table 2.2. Characteristics of envelope protein binding human mAbs

Type of infection	Serotype	Subject #	mAb	IgG subclass	□ or □	Binding to whole virus in ELISA, for indicated serotype at 1 ng/mL				Binding to rE protein in ELISA	Binding to E protein (western blot)	Binding to E protein DIII fragment (ELISA)	Binding to E protein DI/II fragment (ELISA)	50% neutralization concentration (□ g/mL) against indicated serotype				Fold enhancement of infection, for indicated serotype at 1 □ g/mL			
						D1	D2	D3	D4					D1	D2	D3	D4	D1	D2	D3	D4
Primary	2	19	1C16	1	□	+	+	+	+	+	ND	-	+	10	9	9	-	9	18	**53**	11
			5M22	1	□	+	+	+	+	+	ND	-	+	7	9	5	-	11	11	**42**	11
			1M19	1	□	+	+	+	+	+	ND	+	ND	-	-	10	-	6	8	**37**	15
			4C23	1	□	+	+	+	+	+	ND	-	+	-	7	6	7	15	20	**28**	11
			2A10	1	□	+	+	+	+	+	ND	-	+	7	7	5	8	11	8	**52**	18
			4G9	2	□	+	+	+	+	+	ND	-	+	9	10	7	-	9	5	18	6
			5J22	1	□	+	+	+	+	-	+	-	+	-	10	10	-	6	6	22	6
			3F13	1	□	+	+	+	+	-	-	-	+	-	-	-	-	-	-	**37**	-
			2D22	1	□	-	+	-	-	-	-	-	-	-	**0.08**	-	-	-	-	-	-
			1B23	1	□	+	+	+	+	+	ND	+	ND	4	5	2	9	21	11	**67**	7
		31	4N23	1	□	+	+	+	+	-	+	-	+	-	-	-	-	-	-	19	5
	3	3	2A15	1	□	+	+	+	+	+	ND	-	+	7	7	5	10	10	6	16	5
			1A15	1	□	+	+	+	+	+	ND	-	+	-	-	-	-	-	-	7	-
			1C17	1	□	+	+	+	+	+	ND	+	ND	-	-	7	-	**27**	**26**	**43**	15
			1I17	1	□	+	+	+	+	+	ND	-	+	10	8	8	-	7	12	**27**	7
			1M23	1	□	+	+	+	+	+	ND	+	ND	-	-	7	-	9	13	**51**	7
			1L5	1	□	+	+	+	+	+	ND	-	+	10	10	2	10	18	24	**34**	23
		103	2D7	1	□	+	+	+	-	+	ND	-	+	3	-	3	-	-	25	12	-
			2C2	2	□	+	+	+	+	+	ND	-	+	10	1	-	1	6	5	6	-
			2D17	1	□	+	+	+	+	+	+	-	+	-	-	-	-	13	5	**27**	-
		105	5J20	1	□	-	-	+	+	+	ND	-	+	-	-	8	-	-	-	**122**	15
			3I6	1	□	+	+	+	+	+	ND	-	+	-	-	7	-	10	-	15	13
			5J7	1	□	+	+	+	+	+	ND	-	+	-	-	**0.1**	-	-	-	23	-
			6K5	1	□	+	+	+	+	+	ND	-	+	7	5	10	-	**40**	5	**29**	13
Secondary	Multiple	8	2J20	1	□	+	+	+	+	+	ND	+	ND	6	-	**0.5**	-	7	**28**	22	9
		9	2C7	1	□	+	+	+	+	+	ND	-	+	4	1	4	6	22	5	5	24
		27	3H9	1	□	+	+	+	+	+	ND	-	+	-	-	-	-	-	-	5	-
		110	6B22	1	□	+	+	+	+	-	-	-	+	-	-	-	-	12	20	**36**	10
		115	3H4	2	□	+	+	+	+	+	ND	-	+	10	10	2	10	-	-	-	-

If the antibody did not bind to recombinant E (rE) protein, western blotting was performed to determine E protein binding as an alternate method. Binding to recombinant domain III or recombinant domain I/II also are shown. The concentration (μg/mL) at which 50% of virus was neutralized ($neut_{50}$) is shown for each dengue serotype: a dash indicates $neut_{50}$ value > 10 μg/mL, $neut_{50}$ values between 1.0-10.0 μg/mL are shown, $neut_{50}$ values < 1.0 μg/mL are bold and underlined. ADE assays were performed for each human antibody (at a concentration of 1 μg/mL) against each dengue serotype and shown as fold enhancement: a dash indicates < 5 fold enhancement, 5–25 fold enhancement values are shown, > 25 fold enhancement values are bold and underlined.

Table 2.3. Characteristics of membrane protein binding human mAbs

Type of infection	Serotype	Subject #	mAb	IgG subclass	□ or □	Binding to whole virus in ELISA, for indicated serotype at 1 ng/mL				Binding to r-prM protein in ELISA	Binding to M protein (western blot)	50% neutralization concentration (□ g/mL), against indicated serotype				Fold enhancement of infection, for indicated serotype at 1 □ g/mL			
						D1	D2	D3	D4			D1	D2	D3	D4	D1	D2	D3	D4
Primary	3	3	**2H12**	1	□	+	+	+	+	+	ND	-	-	**0.1**	10	14	7	**35**	9
		5	**5L20**	1	□	+	+	+	+	+	+	-	-	-	-	-	-	5	-
		105	**4E9**	1	□	+	+	+	+	+	+	-	-	1	-	7	-	**30**	21
			5G22	1	□	+	+	+	+	+	+	-	-	6	-	**40**	12	**94**	**46**
Secondary	Multiple	15	**4F8**	1	□	+	+	+	+	+	+	-	-	2	5	13	15	**57**	24
		27	**2M2**	1	□	+	+	+	+	+	+	1	**0.5**	2	1	17	8	8	**48**
			1G5	1	□	+	+	+	+	+	+	-	-	5	-	19	20	**70**	**38**
		110	**5E6**	1	□	+	+	+	+	+	+	-	-	6	-	23	15	**36**	13

The pattern of virus binding to purified virus in ELISA at the lowest concentration tested (1 ng/mL) is shown for each serotype. Binding of each antibody to recombinant "pr" fragment of the membrane protein in ELISA along with binding membrane protein by western blot is indicated. Neutralization assays performed for each antibody are shown against each dengue serotype: a dash indicates $neut_{50}$ > 10 µg/mL, $neut_{50}$ values between 1.0-10.0 µg/mL are shown, $neut_{50}$ values < 1.0 µg/mL are bold and underlined. ADE assays were performed for each human antibody (at a concentration of 1 µg/mL) against each dengue serotype and shown as fold enhancement: a dash indicates < 5-fold enhancement, 5–25 fold enhancement values are shown, > 25-fold enhancement values are bold and underlined.

Figures and Legends

Figure 2.1.

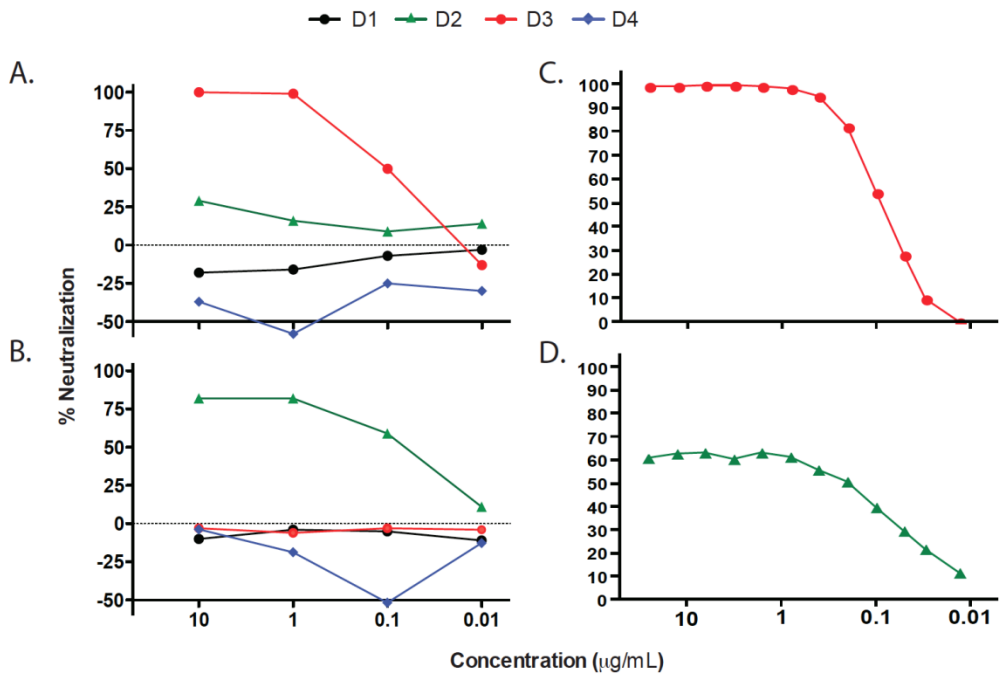


Figure 2.1: Serotype-specific strongly neutralizing E protein binding human MAbs **A.** The ability of purified human MAb 5J7 to neutralize the four serotypes of dengue virus is shown over a concentration range. **B.** The ability of purified human MAb 2D22 to neutralize the four serotypes of dengue virus is shown over a concentration range. **C.** Purified human MAb 5J7 neutralization of serotype 3 virus over a detailed, broader range of halving dilutions. **D.** Purified human MAb 2D22 neutralization of serotype 2 virus over a detailed range of halving dilutions. Flow cytometric neutralization assays were performed using U937-DCSIGN cells.

Figure 2.2

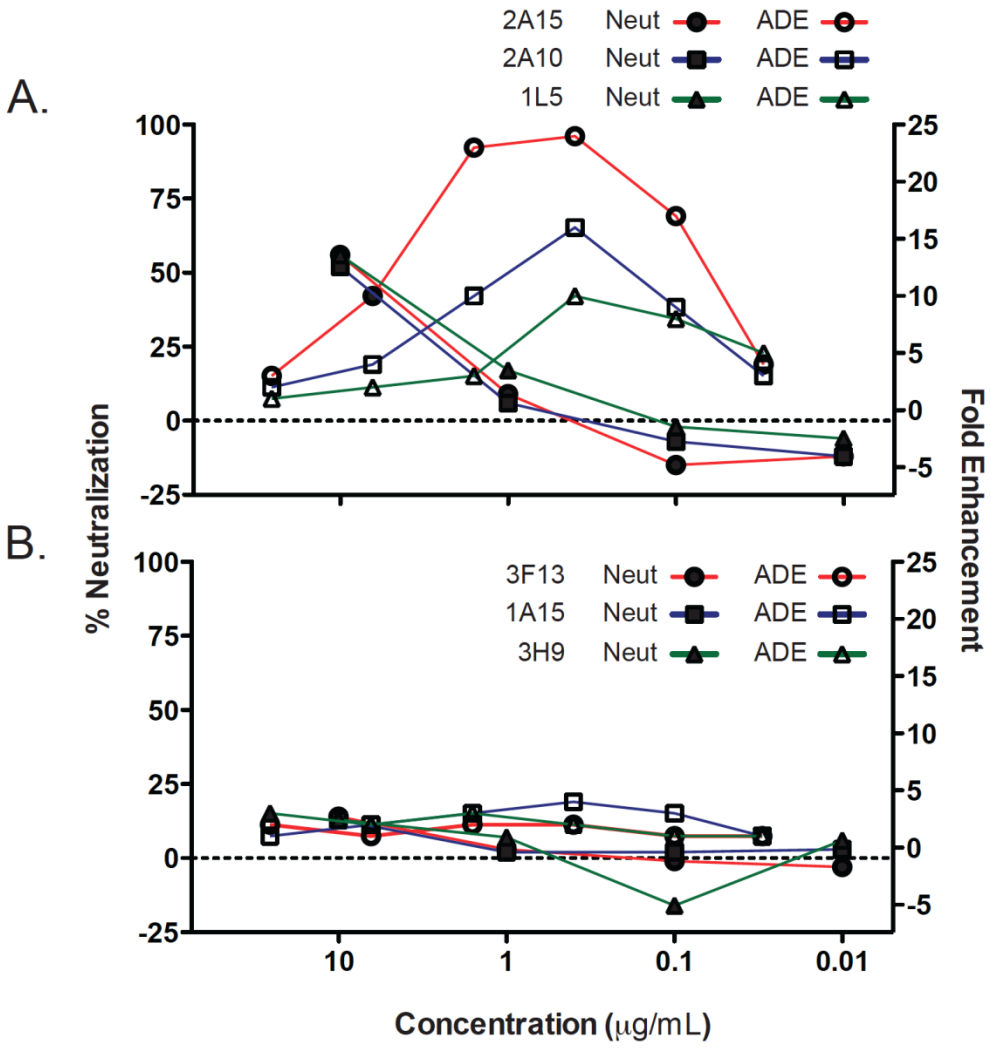


Figure 2.2: Cross-reactive poorly neutralizing E protein binding human MAbs
A. The ability of purified human MAb 2A15, 2A10, or 1L5 to neutralize or enhance DENV 4 is shown over a concentration range. These MAbs represent a class of fully cross-reactive, weakly neutralizing and moderately enhancing antibodies. **B.** The ability of purified human MAb 3F13, 1A15, or 3H9 to neutralize or enhance DENV 4 is shown over a concentration range. These MAbs represent a class of fully cross-reactive, non-neutralizing and non-enhancing antibodies. Flow cytometric neutralization assays were performed using U937-DCSIGN cells. ADE assays were performed using U937 Fc receptor expressing cells.

Figure 2.3

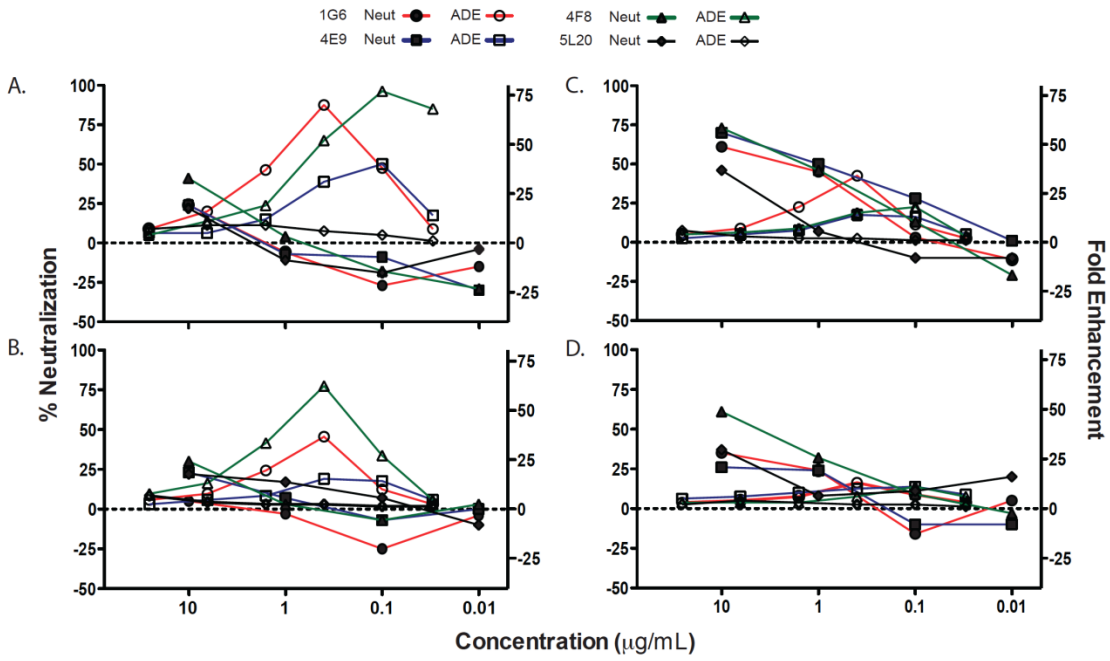


Figure 2.3: Cross-reactive poorly neutralizing PrM protein binding human MAbs
The ability of purified human MAb 1G6, 4E9, 4F8, or 5L20 to neutralize or enhance DENV 1 (panel A), DENV 2 (panel B), DENV 3 (panel C), or DENV 4 (panel D) is shown over a concentration range. Flow cytometric neutralization assays were performed using U937-DCSIGN cells. ADE assays were performed using U937 Fc receptor expressing cells.

2.6 References

"Locally acquired Dengue--Key West, Florida, 2009-2010." MMWR Morb Mortal Wkly Rep **59**(19): 577-581.

Balsitis, S. J., K. L. Williams, et al. "Lethal antibody enhancement of dengue disease in mice is prevented by fc modification." PLoS Pathog **6**(2): e1000790.

Beasley, D. W. and A. D. Barrett (2002). "Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein." J Virol **76**(24): 13097-13100.

Beltramello, M., K. L. Williams, et al. "The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity." Cell Host Microbe **8**(3): 271-283.

Bhardwaj, S., M. Holbrook, et al. (2001). "Biophysical characterization and vector-specific antagonist activity of domain III of the tick-borne flavivirus envelope protein." J Virol **75**(8): 4002-4007.

Brien, J. D., S. K. Austin, et al. "Genotype-specific neutralization and protection by antibodies against dengue virus type 3." J Virol **84**(20): 10630-10643.

Chen, Y. C., H. N. Huang, et al. (2007). "Generation and characterization of monoclonal antibodies against dengue virus type 1 for epitope mapping and serological detection by epitope-based peptide antigens." Clin Vaccine Immunol **14**(4): 404-411.

Crill, W. D., H. R. Hughes, et al. (2009). "Humoral immune responses of dengue fever patients using epitope-specific serotype-2 virus-like particle antigens." PLoS One **4**(4): e4991.

Crill, W. D. and J. T. Roehrig (2001). "Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells." J Virol **75**(16): 7769-7773.

de Alwis, R., M. Beltramello, et al. "In-depth analysis of the antibody response of individuals exposed to primary dengue virus infection." PLoS Negl Trop Dis **5**(6): e1188.

Dejnirattisai, W., A. Jumnainsong, et al. "Cross-reacting antibodies enhance dengue virus infection in humans." Science **328**(5979): 745-748.

Gibbons, R. V. and D. W. Vaughn (2002). "Dengue: an escalating problem." BMJ **324**(7353): 1563-1566.

Goncalvez, A. P., R. E. Engle, et al. (2007). "Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention." Proc Natl Acad Sci U S A **104**(22): 9422-9427.

Green, S. and A. Rothman (2006). "Immunopathological mechanisms in dengue and dengue hemorrhagic fever." Curr Opin Infect Dis **19**(5): 429-436.

Gubler, D. J. (2002). "Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century." Trends Microbiol **10**(2): 100-103.

Guzman, M. G., S. B. Halstead, et al. "Dengue: a continuing global threat." Nat Rev Microbiol **8**(12 Suppl): S7-S16.

Halstead, S. B. (2002). "Dengue." Curr Opin Infect Dis **15**(5): 471-476.

Halstead, S. B. (2003). "Neutralization and antibody-dependent enhancement of dengue viruses." Adv Virus Res **60**: 421-467.

Halstead, S. B. and E. J. O'Rourke (1977). "Antibody-enhanced dengue virus infection in primate leukocytes." Nature **265**(5596): 739-741.

Huang, K. J., Y. C. Yang, et al. (2006). "The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection." J Immunol **176**(5): 2825-2832.

Hung, J. J., M. T. Hsieh, et al. (2004). "An external loop region of domain III of dengue virus type 2 envelope protein is involved in serotype-specific binding to mosquito but not mammalian cells." J Virol **78**(1): 378-388.

Kaufmann, B., G. E. Nybakken, et al. (2006). "West Nile virus in complex with the Fab fragment of a neutralizing monoclonal antibody." Proc Natl Acad Sci U S A **103**(33): 12400-12404.

Kou, Z., J. Y. Lim, et al. "Human antibodies against dengue enhance dengue viral infectivity without suppressing type I interferon secretion in primary human monocytes." Virology **410**(1): 240-247.

Kraus, A. A., W. Messer, et al. (2007). "Comparison of plaque- and flow cytometry-based methods for measuring dengue virus neutralization." J Clin Microbiol **45**(11): 3777-3780.

Kuhn, R. J., W. Zhang, et al. (2002). "Structure of dengue virus: implications for flavivirus organization, maturation, and fusion." Cell **108**(5): 717-725.

Lai, C. Y., W. Y. Tsai, et al. (2008). "Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II." J Virol **82**(13): 6631-6643.

Liao, M., C. Sanchez-San Martin, et al. "In vitro reconstitution reveals key intermediate states of trimer formation by the dengue virus membrane fusion protein." J Virol **84**(11): 5730-5740.

Littaua, R., I. Kurane, et al. (1990). "Human IgG Fc receptor II mediates antibody-dependent enhancement of dengue virus infection." J Immunol **144**(8): 3183-3186.

Lok, S. M., V. Kostyuchenko, et al. (2008). "Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins." Nat Struct Mol Biol **15**(3): 312-317.

Modis, Y., S. Ogata, et al. (2003). "A ligand-binding pocket in the dengue virus envelope glycoprotein." Proc Natl Acad Sci U S A **100**(12): 6986-6991.

Modis, Y., S. Ogata, et al. (2004). "Structure of the dengue virus envelope protein after membrane fusion." Nature **427**(6972): 313-319.

Modis, Y., S. Ogata, et al. (2005). "Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein." J Virol **79**(2): 1223-1231.

Nayak, V., M. Dessau, et al. (2009). "Crystal structure of dengue virus type 1 envelope protein in the postfusion conformation and its implications for membrane fusion." J Virol **83**(9): 4338-4344.

Nikitin, P. A., C. M. Yan, et al. "An ATM/Chk2-mediated DNA damage-responsive signaling pathway suppresses Epstein-Barr virus transformation of primary human B cells." Cell Host Microbe **8**(6): 510-522.

Nybakken, G. E., T. Oliphant, et al. (2005). "Structural basis of West Nile virus neutralization by a therapeutic antibody." Nature **437**(7059): 764-769.

Oliphant, T., M. Engle, et al. (2005). "Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus." Nat Med **11**(5): 522-530.

Oliphant, T., G. E. Nybakken, et al. (2006). "Antibody recognition and neutralization determinants on domains I and II of West Nile Virus envelope protein." J Virol **80**(24): 12149-12159.

Pierson, T. C. "Modeling antibody-enhanced dengue virus infection and disease in mice: protection or pathogenesis?" Cell Host Microbe **7**(2): 85-86.

Pierson, T. C., D. H. Fremont, et al. (2008). "Structural insights into the mechanisms of antibody-mediated neutralization of flavivirus infection: implications for vaccine development." Cell Host Microbe **4**(3): 229-238.

Pierson, T. C., Q. Xu, et al. (2007). "The stoichiometry of antibody-mediated neutralization and enhancement of West Nile virus infection." Cell Host Microbe **1**(2): 135-145.

Pokidysheva, E., Y. Zhang, et al. (2006). "Cryo-EM reconstruction of dengue virus in complex with the carbohydrate recognition domain of DC-SIGN." Cell **124**(3): 485-493.

Rey, F. A., F. X. Heinz, et al. (1995). "The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution." Nature **375**(6529): 291-298.

Rodenhuis-Zybert, I. A., H. M. van der Schaar, et al. "Immature dengue virus: a veiled pathogen?" PLoS Pathog **6**(1): e1000718.

Rodenhuis-Zybert, I. A., J. Wilschut, et al. "Partial maturation: an immune-evasion strategy of dengue virus?" Trends Microbiol.

Roehrig, J. T., R. A. Bolin, et al. (1998). "Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica." Virology **246**(2): 317-328.

Rothman, A. L. (2004). "Dengue: defining protective versus pathologic immunity." J Clin Invest **113**(7): 946-951.

Sanchez, M. D., T. C. Pierson, et al. (2005). "Characterization of neutralizing antibodies to West Nile virus." Virology **336**(1): 70-82.

Schieffelin, J. S., J. M. Costin, et al. "Neutralizing and non-neutralizing monoclonal antibodies against dengue virus E protein derived from a naturally infected patient." Virol J **7**(1): 28.

Shrestha, B., J. D. Brien, et al. "The development of therapeutic antibodies that neutralize homologous and heterologous genotypes of dengue virus type 1." PLoS Pathog **6**(4): e1000823.

Sukupolvi-Petty, S., S. K. Austin, et al. (2007). "Type- and subcomplex-specific neutralizing antibodies against domain III of dengue virus type 2 envelope protein recognize adjacent epitopes." J Virol **81**(23): 12816-12826.

Vogt, M. R., B. Moesker, et al. (2009). "Human monoclonal antibodies against West Nile virus induced by natural infection neutralize at a postattachment step." J Virol **83**(13): 6494-6507.

Wahala, W. M., A. A. Kraus, et al. (2009). "Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody." Virology **392**(1): 103-113.

Whitehead, S. S., J. E. Blaney, et al. (2007). "Prospects for a dengue virus vaccine." Nat Rev Microbiol **5**(7): 518-528.

Yu, X., P. A. McGraw, et al. (2008). "An optimized electrofusion-based protocol for generating virus-specific human monoclonal antibodies." J Immunol Methods **336**(2): 142-151.

Zhang, Y., W. Zhang, et al. (2004). "Conformational changes of the flavivirus E glycoprotein." Structure **12**(9): 1607-1618.

Zheng, A., M. Umashankar, et al. "In vitro and in vivo studies identify important features of dengue virus pr-E protein interactions." PLoS Pathog **6**(10): e1001157.

CHAPTER 3

THE STOICHIOMETRY MODELS AND MECHANISMS OF ANTIBODY MEDIATED VIRUS NEUTRALIZATION

3.1 Introduction

Dengue is an emerging flavivirus and it is endemic in tropical and subtropical areas putting 2.5 billion people in risk of infection every year (WHO 2009). Dengue infection can cause either mild flu like disease called dengue fever (DF) or a severe life threatening disease called Dengue Hemoharrgic Fever or Dengue Shock Syndrome (DHF/DSS). Four serotypes of dengue viruses co-circulate world-widely and immunity against one serotype is unable to protect virus against re-infection from another serotype (WHO 2009). Epidemiological evidence further suggests that the sub-neutralizing heterotypic immunity in secondary infections can lead to enhancement of the infection, which is called Antibody Dependent Enhancement (ADE) (Halstead and Simasthien 1970). ADE occurs when Fc-γ receptors of monocytic cells serve as additional attachment molecules, helping antibody-coated dengue viruses to infect cells (Halstead and O'Rourke 1977). ADE poses the greatest obstacle to the development of a safe dengue vaccine. Therefore understanding the mechanisms of dengue neutralization is critical for preventing ADE and the subsequent severe disease.

The dengue virus genome is a positive-sense RNA of 11kb, encoding 3 structure proteins (C-prM-E) and 7 non-structure proteins. The mature DENV particle is composed of 90 E glycoprotein dimers in an icosahedra scaffold. The E glycoprotein is the primary

surface antigen inducing protective immunity. It is essential for membrane fusion and binding to cellular receptors. The E protein ecto-domain consists of three domains: EDI, EDII and EDIII, each connected by flexible hinge regions. EDIII belongs to the IgG superfamily and it binds to viral receptors, and many strongly neutralizing MAbs were found to bind to EDIII (Roehrig 2003; Brien, Austin et al. 2010; Shrestha, Brien et al. 2010; Sukupolvi-Petty, Austin et al. 2010). However, later research suggests that EDIII plays a minor role in dengue neutralization by polyclonal sera (Wahala, Kraus et al. 2009). EDI/II contains the fusion peptide and recent studies have suggested that fusion loop is the major target of neutralizing antibodies (Lai, Tsai et al. 2008; Lin, Tsai et al. 2012; Costin, Zaitseva et al. 2013). As shown in Figure 1.3, to infect a cell, the virus will go through attachment, internalization, transformation and membrane fusion before it can release the RNA genome into the cell cytoplasm. Specifically, attachment to a cell involves E glycoprotein binding to the DC-SIGN molecule (or heperan sulfate) or through antibody bridged binding of the Fc- γ receptors. Then, the putative dengue cellular receptor will bind to E and mediate the uptake and internalization of virus into the endosome, where the low PH environment activates the virus envelope to go through a conformational change (transformation), during which the 90 glycoprotein E dimers dissociate, and re-associate to form 60 trimers (Yu, Zhang et al. 2008). The transformation exposes the fusion peptide for subsequent fusion. The hydrophobic force between the fusion loop and cellular membrane enables insertion of the fusion loop into the membrane, starts structure rearrangement of the envelope and drives the virus membrane to fuse with the cellular membrane, resulting in the release of virus genome RNA into the cell cytoplasm (Kuhn, Zhang et al. 2002; Modis, Ogata et al. 2004).

Antibodies neutralize dengue viruses through binding E protein and blocking the steps mentioned above. In the last decade, cloning and characterization of both murine

MAbs and human MAbs have enabled detailed study of antibody neutralization mechanisms. Particularly, a function assay has been developed to differentiate neutralization at the attachment step and post-attachment step. At 37°C, endocytosis of viruses follows spontaneously and immediately after virus attachment. The function assay utilizes cold temperatures to enable virus attachment without subsequent endocytosis and thus allows us to separate the attachment step out. Apart from attachment, all the following virus infection steps, such as receptor-mediated internalization, transformation and membrane fusion, are labeled as post-attachment steps in the function assay. So far, less than 50 DENV MAbs have been studied using this function assay and these MAbs were found to block mostly post-attachment steps (Rajamanonmani, Nkenfou et al. 2009; Teoh, Kukkaro et al. 2012; Costin, Zaitseva et al. 2013). Other complicated assays, such as the liposome coflotation assay, have been adapted to identify the MAbs' capability to block membrane fusion (Yu, Holdaway et al. 2009).

The multi-hit model of virus neutralization argues that neutralization is the cumulative effect of multiple antibodies binding to a virion simultaneously (Della-Porta and Westaway 1978). Recent advances in West Nile virus neutralization research secured the multi-hit model as a dominant model in flavivirus neutralization. Pierson and colleagues examined the stoichiometry of antibody-mediated neutralization of WNV and demonstrated that a threshold of antibody binding is required for neutralization to occur (Pierson, Xu et al. 2007). This model is named as the Threshold Model and it implies that antibody neutralization potency is determined by interplay between antibody affinity and epitope accessibility. As of the present day, the threshold model has not been tested with DENV. Gromowski and colleagues studied the stoichiometry of several mouse MAbs and did find evidence supporting the threshold model, though they also reported a

linear correlation between antibody binding of EDIII and the neutralization of infectivity (Gromowski and Barrett 2007).

It is unknown whether antibodies that neutralize through different mechanisms vary in binding/neutralization stoichiometry, but such knowledge is invaluable for both understanding dengue neutralization and antibody therapy or vaccine design. To fill in this gap, I collected a panel of well characterized MAbs and determined their binding/neutralization stoichiometry using the FACS-based neutralization assay and ELISA. The neutralization mechanism of each MAb is determined through FACS-based function assay. Our results demonstrated that there is an association between the stoichiometry model and neutralization mechanisms.

3.2 Materials and Methods

Cells. Mosquito *Aedes albopictus* C6/36 cells were maintained in MEM (Gibco) media at 28°C. Human monocyte lymphoma cell line U937cells expressing DC-SIGN (U937 DC-SIGN) were maintained in RPMI-1640 (Gibco) at 37°C supplemented with 50mM beta mercaptoethanol. Vero-81 cells were maintained in DMEM at 37°C. All media used were also supplemented with 5% FBS, 100U/mL penicillin, 100mg/mL streptomycin, 0.1mM non-essential amino acids (Gibco) and 2mM glutamine and all cells were incubated in the presence of 5% CO_2. The 5% FBS was reduced to 2% to make infection media for each cell line.

Antibodies, Infectious Viruses and Antigens. A total of 10 monoclonal antibodies were obtained from multiple sources and stored in -20°C (Table 3.1). 80% confluent C6/36 cells were infected with viruses at an MOI of 0.05 and incubated at 29°C 5% CO_2

for 6 days. Supernatants were harvested, clarified of cell debris, aliquoted and stored at -80°C for future use as infectious virus stocks. 80% confluent vero-81 cells were infected with viruses at an MOI of 0.05 and incubated at 37°C for 6 days. Supernatants were harvested, cleared of cell debris, and ultra-centrifuged at 80,000 x g for 4 hrs with a 20% sucrose cushion to pellet down virions (Beckman Countler ultracentrifuge, Optima^{TM} L-90K). Virion pellets were resuspended in PBS and further purified by density gradient ultracentrifugation at 100,000 x g for 2 hrs (OptiPrep® Density Gradient Medium, Sigma). The gradient was fractioned immediately after ultracentrifugation and each fraction was measured for virus content using reducing protein gel. Pure antigen containing fractions were pooled, diluted in PBS and ultra-centrifuged again. The final pellets were resuspended in PBS, titrated for concentration using Bradford assay and stored at -80^{o}C for future use as virus antigen.

ELISA. high binding ELISA plates (Corning Life Sciences) were coated with 50 ng of purified virus antigen per well in 0.1 M carbonate buffer. Plates were incubated at 4˚C overnight, washed three times (all washes were performed three times with tris-buffered saline containing 0.2% Tween-20), then blocked with dilution buffer on a shaker at 37˚C for 1hr. 50 µL of antibodies prepared in Tris-buffered saline containing 0.05% Tween-20 and 3% Normal Goat Serum were added to the wells and incubated on a shaker at 37˚C for 1 hr. Unbound primary antibodies were washed off and 1:1000 alkaline phosphatase conjugated diluted goat anti-mouse or anti-human secondary antibody added; the plate was then incubated on a shaker at 37˚C for 1 hr. Unbound secondary antibodies were washed off, pNPP substrate added, and absorbance readings were taken at 405 nm in an Epoch Microplate Spectrophotometer (Biotek Instruments Inc. Winooski, VT).

FACS Based Neutralization Assay. Antibodies were diluted in RPMI 1640 media with 2% FBS buffered with 20mM HEPES (infection media). Infectious virus stocks were diluted

in infection media to make 20, 000 PFU/mL. 40 µl of diluted antibodies and 40 µL of diluted viruses were added to a 96 well round bottom plate (Corning Life Science, Lowell, MA) and incubated in a 37˚C incubator for 45 minutes. 20 µL of U937-DC-SIGN cells at a density of 10^6 cells/mL were added to each well and incubated for 2 hrs. Unbound viruses and antibodies were washed off by pelleting cells at 1500 rpm for 5 minutes and the cells were resuspended in 200 µL infection media. After 24 hrs of incubation, the cells were pelleted, washed with PBS and fixed with Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA) at room temperature for 10 minutes. After washing twice with permeablization buffer (BD Biosciences, San Jose, CA), cells were stained with Flexor-488 conjugated 2H2 antibody for 1 hr at 37^{o}C. The unbound conjugated antibodies were washed off and the plates were read using Guava system flow cytometry (Millipore Inc. Billerica, MA).

FACS-based Function Assay. The Function Assay is modified from the FACS-based Neutralization Assay described above. Specifically, for the pre-attachment assay, U937-DC-SIGN cells were pre-chilled at 4°C. Viruses were incubated with serial diluted MAbs at 4°C for 1 hr and then the cells were added and kept in 4°C incubation for another 1 hr. Unbound antibodies and viruses were washed off with 4°C washing media and then moved to a 37°C incubator for 24 hrs. For the post-attachment assay, the viruses were added to pre-chilled cells and incubated at 4°C for 1 hr. Unbound viruses were washed off using chilled media. Then diluted MAbs were added to the cells and incubated at 4°C for 1 hr. After washing off unbound antibodies, the plate was moved to 37°C and incubated for 24 hrs. All operations were done on ice surface.

3.3 Results

Variation in antibody binding affinity and neutralization potency

The 10 MAbs studied here vary in neutralization potency, isotype, subclass and specificity (Table 3.1). Most of the MAbs (4G2, 9F16, 2Q1899, 5C36, DVC 3.7, DVC25.5, 1A1D-2, 8A1 and 14A4) had been characterized in previous studies while 9D1 was characterized for the first time (Littaua, Kurane et al. 1990; Gromowski and Barrett 2007; Lok, Kostyuchenko et al. 2008; Wahala, Donaldson et al. 2010; de Alwis, Beltramello et al. 2011). Among these MAbs, 8A1, 14A4 and 9D1 were DENV3 type-specific; all others were either DENV2 type-specific or cross-reactive (in this study they were tested against DENV2 viruses). To study the stoichiometry between binding and neutralization, MAbs were tested for binding affinity using ELISA and for neutralization potency using FACS-based Neutralization Assay. Each MAb was tested at 12 concentrations (2-fold dilutions) for a complete sigmoidal shape dose response curve (Figure 3.2). The EC_{50} of a MAb represents a concentration at which the virus is half occupied by this MAb and it indicates the MAb's binding affinity to the virus. $Neut_{50}$ represents a concentration at which half of the virus' infectivity is inhibited by the MAb and it indicates the neutralization potency of the MAb. Both EC_{50} and $Neut_{50}$ were calculated based on the sigma-dose response curve fitting (Table 3.1). Comparison of the two values of each MAb indicated that for some MAbs (4G2, 9F16, 5C36, 2Q1899, 9D1 and DVC 3.7), the two values are very close (Figure 3.2A, 3.2B and Table 3.1) while for some other MAbs (1A1D-2, DVC 25.5, 8A1, 14A4 and 9D1) the two values have 10 fold differences (Figure 3.2C, 3.2D, and Table 3.1). This discrepancy suggests that the stoichiometry of binding/neutralization may be different between the two groups of MAbs.

Two Stoichiometry Models of Binding/Neutralization

To better contrast the stoichiometry difference between the two groups of MAbs, I plotted each MAb's percentage neutralization against its corresponding ELISA OD value at each concentration. This plot is adapted from a similar plot drawn by Growmoski but is improved here by replacing rEDIII protein with purified virus antigen (Gromowski and Barrett 2007; Gromowski, Barrett et al. 2008). The results indicated that the MAbs with small $Neut_{50}/EC_{50}$ ratio (4G2, 9F16, 5C36, 2Q1899 and DVC3.7) show a good linear correlation between antibody binding and neutralization, as shown by representative MAb 4G2 in Figure 3.2A. On the other hand, the MAbs showing large $Neut_{50}/EC_{50}$ ratio (8A1, 14A4, 1A1D-2 and DVC 25.5) indicated a threshold pattern of neutralization (Figure 3B). The hallmark of the threshold model is that there is no significant neutralization until the ELISA binding exceeds a certain threshold, however, once binding reaches the threshold, incremental binding will enable the neutralization jump to 100% (Figure 3.2B). However, MAb 9D1 is an exception as it indicated a decent linear stoichiometry (Figure 3.2A) with an R^2 value of 0.9 while its $Neut_{50}/EC_{50}$ ratio is close 10. For this MAb, the plot is more convincing as it is based on the whole sigma dose curve rather than its middle point ($Neut_{50}/EC_{50}$). Generally stoichiometry model results are consistent with the $Neut_{50}/EC_{50}$ results, as for MAbs with the linear model, half of total occupancy means half of total neutralization, but for MAbs with the threshold model, half of total occupancy did not enable neutralization until it reaches the threshold, which is close to saturation.

The neutralization mechanisms: pre-attachment or post-attachment

To demonstrate that the distinct stoichiometry models of binding/neutralization are associated with different neutralization mechanisms, the function assay was

employed to identify the neutralization mechanisms of each MAb. Though this assay can only differentiate blocking attachment from blocking post-attachment steps, it can still provide evidence supporting or disproving my hypothesis. For pre-attachment neutralization, antibodies were added before viruses' absorption to cells at cold temperature (4°C) and this exposed viruses to antibody-mediated neutralization before and after attachment. For post-attachment neutralization, the antibody was added to viruses that were already absorbed to cells, which allowed antibody neutralization at the post-attachment steps only. Pre-attachment neutralization and post-attachment neutralization of each MAb were measured at serial MAb dilutions, allowing the neutralization mechanism to be identified by comparing the pre-attachment and post-attachment neutralization curves (Figure 3.3A and 3.3B). For MAbs 9D1, 9F16, 5C36 and 2Q1899, the function assay results indicated that these MAbs block the attachment step and exert little inhibition on post-attachment steps (Figure 3.3C). MAbs added at pre-attachment step induced neutralization while antibodies added post-attachment induced insignificant neutralization. For MAbs 4G2, DVC3.7, 8A1, 14A4, 1A1D-2 and DVC25.5, the majority of neutralization is contributed by inhibition of the post-attachment steps, as the antibodies added post-attachment induced neutralization almost equal to the neutralization resulting from adding the antibody at pre-attachment step (Figure 3.3C).

3.4 Discussion

In this study I systematically investigated the stoichiometric relationship between antibody binding and virus neutralization. Comparison of MAb EC_{50} and $Neut_{50}$ clearly suggested that there might be two different stoichiometric models among the studied

MAbs. Plotting the percentage neutralization against ELISA OD value further confirmed the two distinct stoichiometric models. Simply put, 6 MAbs displayed a linear model, as their neutralization was proportional to antibody binding, while 4 MAbs revealed a threshold model as only antibody binding exceeding a threshold can lead to neutralization. None of the MAb characteristics such as isotype, type specificity, binding epitope or neutralization potency were found to be correlated with the stoichiometry model (Table 1). Therefore I hypothesized that different neutralization mechanisms are associated with the two distinct stoichiometric models respectively. Function assay was employed to identify the neutralization mechanism of MAbs and the results suggested a correlation between stoichiometry models and neutralization mechanisms.

As shown in Figure 3.3, the 4 MAbs 9F16, 5C36, 9D1 and 2Q1899 all indicate neutralization at the attachment step but not at the post-attachment steps, and most intriguingly, all four MAbs display linear stoichiometry of binding/neutralization. This suggests that MAb neutralization by blocking the attachment step is associated with linear stoichiometry of binding/neutralization (Figure 3.4).

It is commonly believed that only total abolishing of virus infectivity via antibody binding can lead to neutralization of virus. But flavivirus has a limited life-span *in vivo* or *in vitro* and for DENV, the half-life is about 2 hr to 3 hrs *in vitro* in neutral pH at 37 ºC (unpublished data). When only a few antibodies bind to the functional E protein of the virus and block these E proteins from “attaching” to the cell (but leave all other E proteins intact and fully functional), it will not result in total loss of virus infectivity, but will definitely lead to a decrease of chances of infection within the virus’ limited life-span, and ultimately resulted in the decrease of total infection. Obviously a 50% decrease of chances of infection by antibody binding within the life-span will lead to a 50% decrease of infection of the total virus population, which is 50% neutralization by definition. The

feature of this neutralization mechanism is that with more E proteins blocked, there is less chance of attachment (and less infection). Put simply, the more antibody binding, the more E proteins blocked from attachment function, and the more neutralization. Statistically, neutralization is linearly correlated to antibody binding (the linear model of stoichiometry). Since in this scenario, neutralization occurs without the requirement of total abolishing of the virus' infectivity, there is no "threshold" requirement. The evidence of 9F16, 5C36, 9D1, and 2Q1899 supports this hypothesis and indicates that the neutralization mechanism of blocking attachment is associated with linear stoichiometry of binding/neutralization (Figure 3.4).

On the other hand, the 6 MAbs identified as neutralizing post-attachment steps do not all display the threshold model: MAb 4G2 and DVC3.7 displayed the linear model of stoichiometry. Specifically, MAb 4G2 is identified as blocking membrane fusion (Crill and Chang 2004). The membrane fusion mediated by fusion peptide is subject to antibody blocking and the fusion event itself is similar to attachment, depending on the function of individual fusion peptides. In other words, the chance of successful fusion is determined by the quantity of available functional fusion peptides (free from antibody occupation). This explains why MAb blocking the fusion step also displays linear stoichiometry of binding/neutralization (Figure 3.4). Similar to blocking attachment and fusion, blocking the cellular receptor also depends on the quantity of occupied individual E proteins, so it is very likely that MAbs with this neutralization mechanism also follow a linear stoichiometry model (Figure 3.4). I speculate that EDIII-binding MAb DVC 3.7 may be candidate MAb for blocking receptor binding and it may prove useful in future research involving identification of the dengue cellular receptor.

Besides receptor binding and fusion, another post-attachment step prone to antibody mediated neutralization is the pH-triggered conformational change of envelope

(E dimers transform to trimers). During this transformation, an obvious fact is that at least 3 dimers co-dissociated all together can make 2 integrate trimers. Considering the compact organization of 90 E dimers of the virion and the close inter-connection between adjacent E proteins (Figure 1.4), the 3 dimers do not act alone but mobilize transformation of neighboring dimers, initiating a chain reaction and finally a global transformation. Therefore I speculate that the envelope transformation is a “global” conformational change that involves all 180 E proteins simultaneously. If this is the case, it is either a “global” transformation or no transformation and no “partial” transformation. Therefore only occupation of enough number of E simultaneously is able to inhibit this “global” transformation, which suggests that antibody binding needs to pass a threshold quantity to be effective. Put in other words, this implies that neutralization by blocking transformation is associated with a threshold stoichiometry. Interestingly, in this dimer to trimer transformation, 6 neighboring E proteins are one minimal unit for this kind of change and since there are 30 such units in one virus, theoretically, to inhibit all 30 units simultaneously, a minimum number of 30 MAbs are required, which is exactly what Pierson estimated the threshold number to be (Pierson, Xu et al. 2007). According to this hypothesis, the 4 MAbs (8A1, DVC 25.5, 1A1D-2 and 14A4) showing the threshold stoichiometry model should all neutralize DENV by blocking transformation. Though it is beyond this thesis scope to prove, the function assay results indicated that they all block the post-attachment step and this is consistent with my hypothesis (Figure 3.4).

In general, I hypothesized that the neutralization mechanisms of blocking attachment (A), blocking cellular receptor binding (B), and blocking fusion peptide (C) are associated with linear stoichiometry model; blocking conformational change (D) should follow a threshold stoichiometric model (Figure 3.4). The limited evidence from

the function assay supported hypotheses A and C, and is consistent with hypothesis D while hypothesis B cannot be proved or disproved with current knowledge.

This is the first systematic study to explore the relationship between binding-neutralization stoichiometry and mechanisms of neutralization. Though due to the limitation of experiment techniques, the results could not provide complete and conclusive evidence for the hypothesis, it nevertheless provides new insights into our understanding of how antibodies neutralize viruses. A statistical perspective of "neutralization" is introduced and discussed here. It suggests that neutralization of the virus does not necessarily means the virus is completely deprived of any chance of infecting a target cell. For example, if we observe a 50% reduction of neutralization, it may suggest half of the virus population is completely sterilized while the other half is unhampered in infectivity, or it could be that on average, each virus's chance to infect a cell is decreased by half (due to antibody binding). In other words, 50% neutralization is a statistical average of all viruses as a whole and there is no clear demarcation of "dead" and "live" virus at all. In this scenario, no threshold of neutralization is needed and neutralization increases proportionally to antibody binding. Experiments shall be designed to demonstrate this concept of neutralization in future. Though methods are limited to directly differentiate blocking receptor binding, blocking fusion, or blocking conformational change, by plotting post-attachment neutralization only against ELISA binding, we may be able to differentiate these post-attachment steps of neutralization and test if blocking fusion and blocking receptor binding follows linear model while blocking conformational change follows threshold model of stoichiometry.

Tables:

Table 3.1: MAbs and their EC50 of binding and Neutralization

name	subclass	specificity	Source	Epitope	EC_{50}	$Neut_{50}$	$Neut_{50}/EC_{50}$ Ratio
4G2*	mIgG2a	Flavivus-group	ATCC	EDI/II	2.07	1.51	0.75
9D1	mIgG1	DV3 type-specific	Dr. Mary Ann Accavitti-Loper	Unknown[#]	0.0539	0.732	**13.6**
14A4	mIgG1	DV1and DV3	Dr. Putnak	EDIII	0.027	2.79	**10**
8A1	mIgG1	DV3 type-specific	Dr. Putnak	EDIII	0.045	0.61	**14**
9F16	mIgG1	DV2 type-specific	U.S. Biological	EDIII	3.9	6.31	1.6
2Q1899	mIgG1	DV2 type-specific	U.S. Biological	EDIII	1.5	3.20	2.1
5C36	mIgG1	DV2 type-specific	U.S. Biological	EDIII	1.47	3.15	2.1
1A1D-2	mIgG1	Dengue group	Dr. Look	EDIII	0.0099	0.26	**26**
DVC3.7	hIgG1	DV2 type-specific	Dr. Lanzavecchia	EDIII	0.0232	0.0133	0.6
DVC25.5	hIgG1	DV2 type-specific	Dr. Lanzavecchia	EDIII	0.0084	3.17	**377**

*: Except the DENV3 type-specific MAbs, all other MAbs were tested against DENV2 virus for ELISA and Neutralization.

[#]: ELISA results indicated 9D1 have high binding affinity to DENV3 viruses but it showed negative binding to either EDIII or E protein or EDI/II protein. This is similar to the conformational epitope binding MAb 2D22 (Chapter 6) but this is not proved yet.

Figures and Legends

Figure 3.1

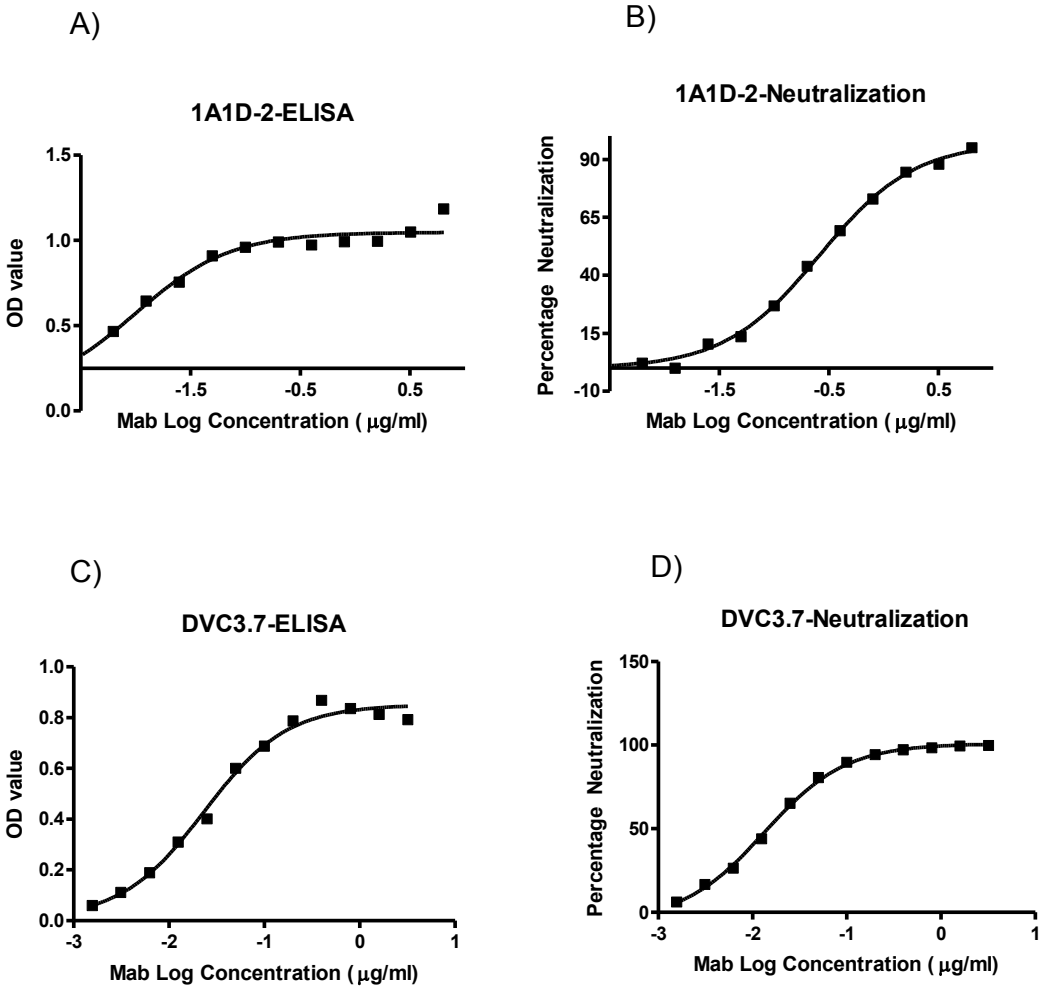
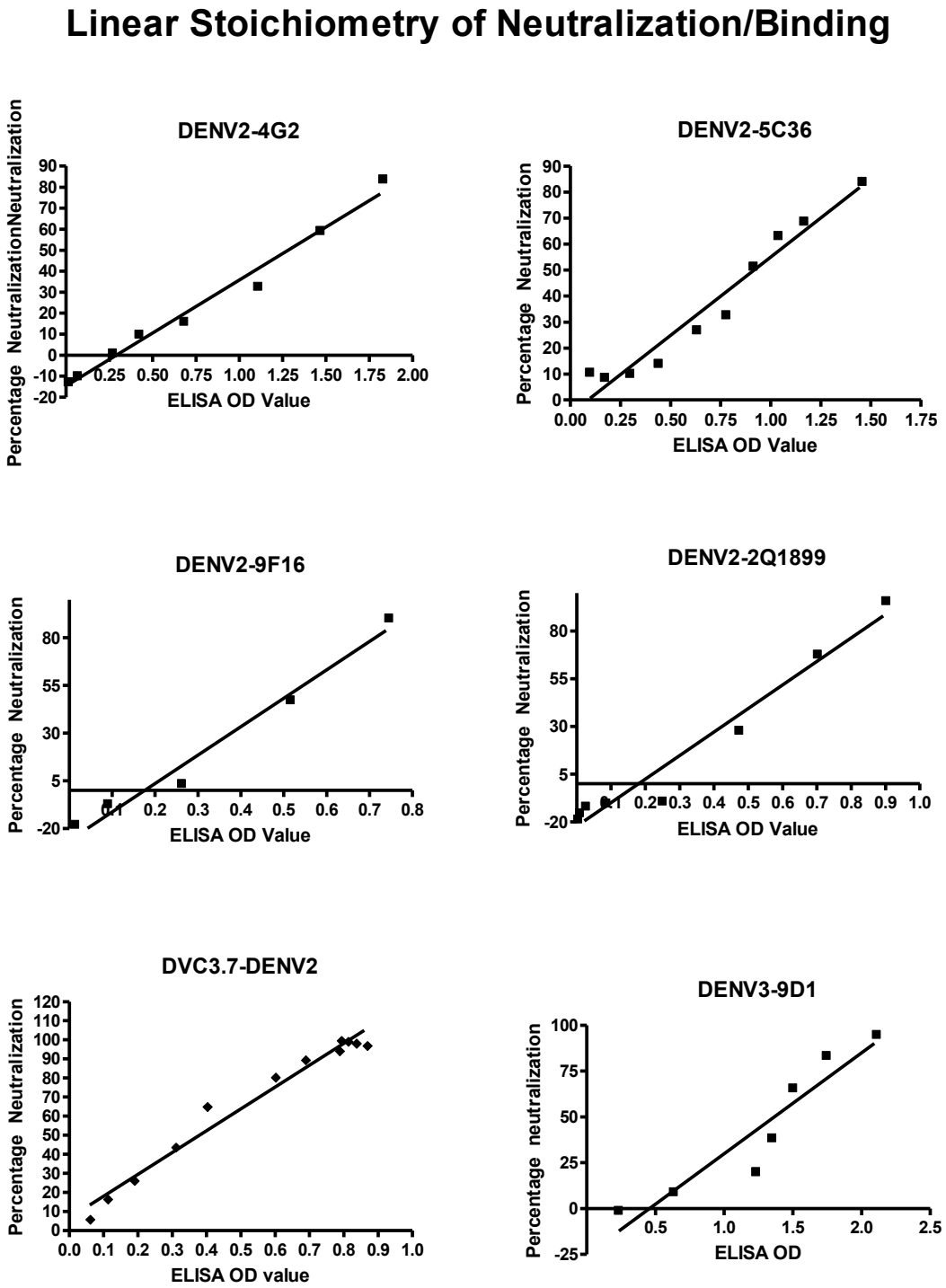


Figure 3.1: ELISA Binding Curve and Neutralization Curves of representative MAbs 1A1D-2 (2A, 2B) and DVC3.7 (2C, 2D).

Figure 3.2

A)


Linear Stoichiometry of Neutralization/Binding
DENV2-4G2
Percentage NeutralizationNeutralization
ELISA OD Value
DENV2-5C36
Percentage Neutralization
ELISA OD Value
DENV2-9F16
Percentage Neutralization
ELISA OD Value
DENV2-2Q1899
Percentage Neutralization
ELISA OD Value
DVC3.7-DENV2
Percentage Neutralization
ELISA OD value
DENV3-9D1
Percentage neutralization
ELISA OD

B)

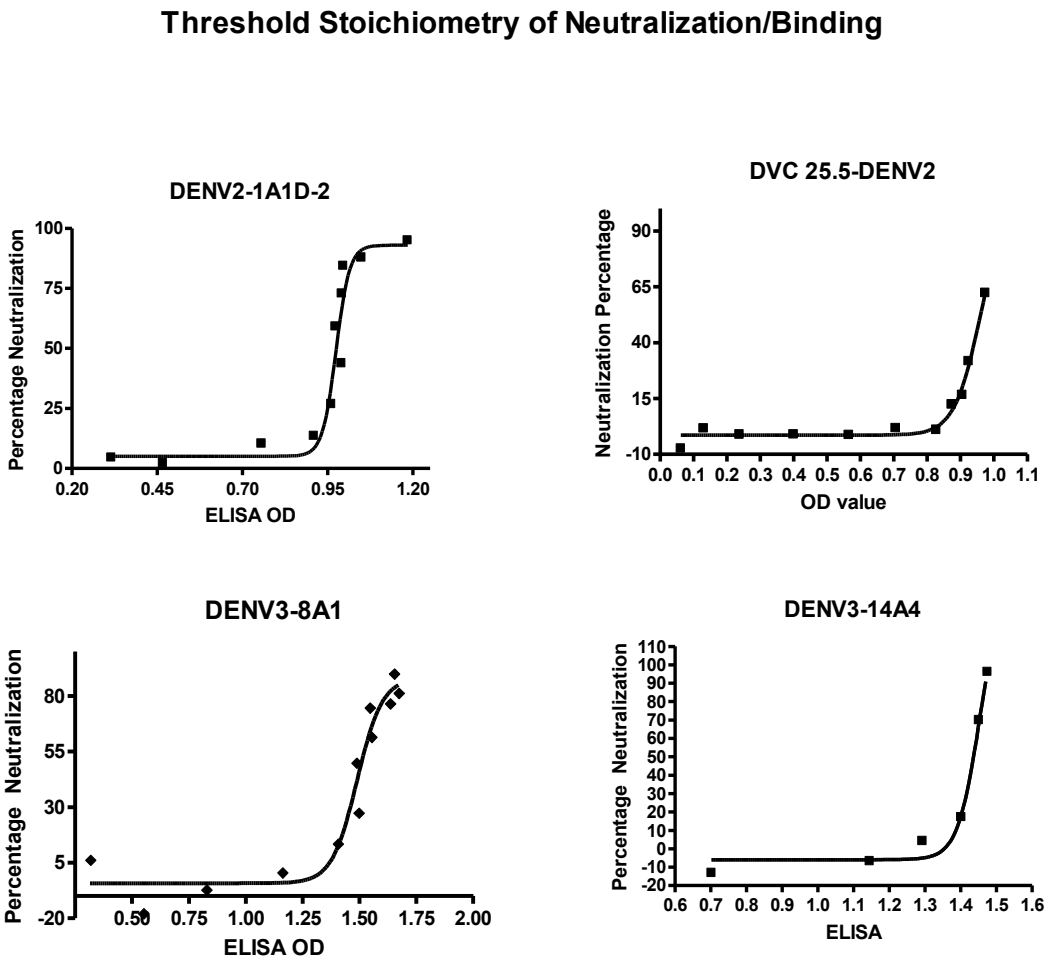


Figure 3.2: Neutralization/Binding Plots of 6 MAbs showing **A)** linear stoichiometry and 4 MAbs showing **B)** threshold stoichiometry.

Figure 3.3

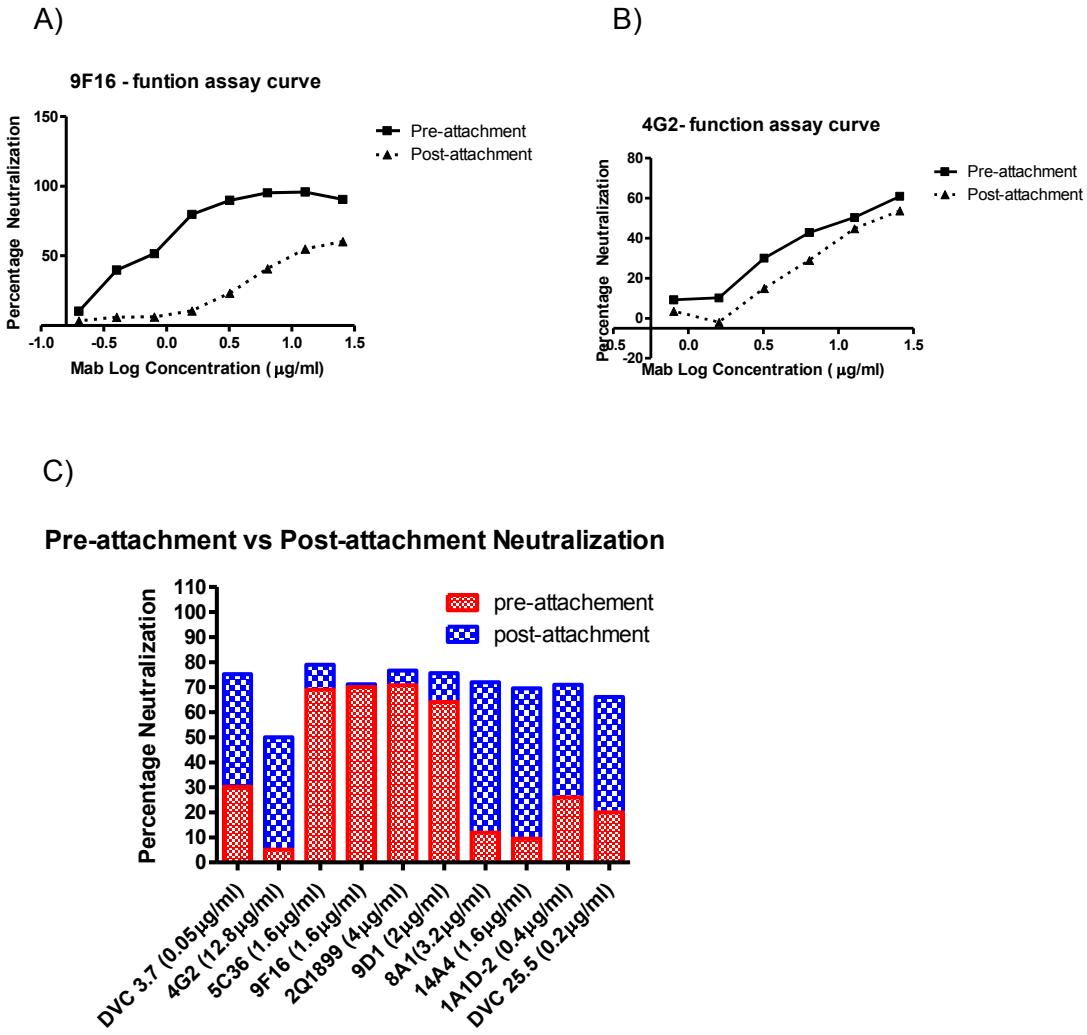


Figure 3.3: Function assay results of all 10 MAbs. **A)** MAb 9F16 represents MAbs neutralize at attachment step only; **B)** MAb 4G2 represents MAbs neutralize at post-attachment only; **C)** Each MAb were tested at several different concentrations but to simplify the comparison among the 10 MAbs, a concentration at which neutralization ranged from 50% to 80% was chosen as representative to contrast the pre-attachment and post-attachment neutralization results. The pre-attachment neutralization in this Bar graph is calculated by deducting the post-attachment neutralization from the total neutralization (which is neutralization measured with MAb added at the pre-attachment step in the function assay). The concentration of each MAb at µg/ml was included in the MAb tag.

Figure 3.4

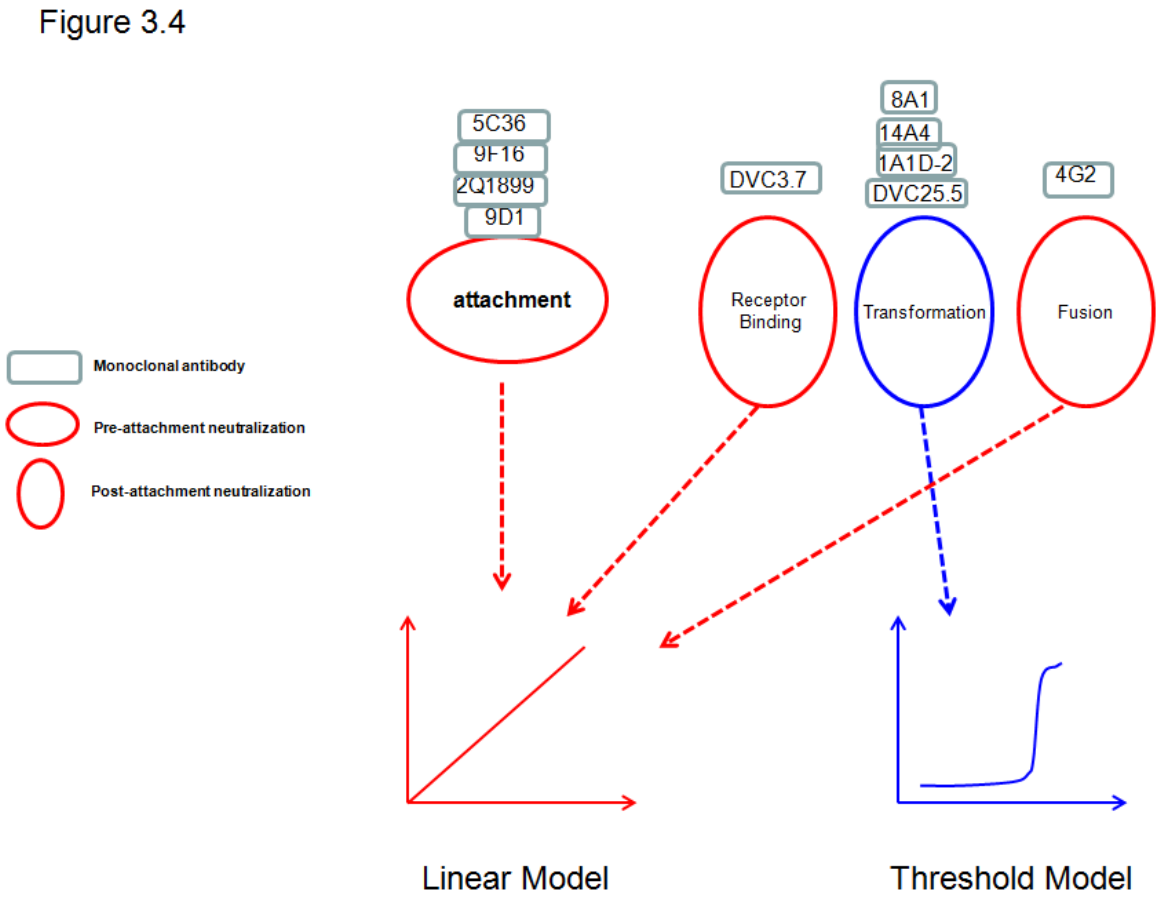


Figure 3.4: Hypothetical relationship between neutralization mechanisms and stoichiometry models and the MAbs assumed to associate with each scenario.

3.5 References

Brien, J. D., S. K. Austin, et al. (2010). "Genotype-specific neutralization and protection by antibodies against dengue virus type 3." J Virol **84**(20): 10630-10643.

Costin, J. M., E. Zaitseva, et al. (2013). "Mechanistic study of broadly neutralizing human monoclonal antibodies against dengue virus that target the fusion loop." J Virol **87**(1): 52-66.

Crill, W. D. and G. J. Chang (2004). "Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes." J Virol **78**(24): 13975-13986.

de Alwis, R., M. Beltramello, et al. (2011). "In-depth analysis of the antibody response of individuals exposed to primary dengue virus infection." PLoS Negl Trop Dis **5**(6): e1188.

Della-Porta, A. J. and E. G. Westaway (1978). "A multi-hit model for the neutralization of animal viruses." J Gen Virol **38**(1): 1-19.

Gromowski, G. D. and A. D. Barrett (2007). "Characterization of an antigenic site that contains a dominant, type-specific neutralization determinant on the envelope protein domain III (ED3) of dengue 2 virus." Virology **366**(2): 349-360.

Gromowski, G. D., N. D. Barrett, et al. (2008). "Characterization of dengue virus complex-specific neutralizing epitopes on envelope protein domain III of dengue 2 virus." J Virol **82**(17): 8828-8837.

Halstead, S. B. and E. J. O'Rourke (1977). "Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody." J Exp Med **146**(1): 201-217.

Halstead, S. B. and P. Simasthien (1970). "Observations related to the pathogenesis of dengue hemorrhagic fever. II. Antigenic and biologic properties of dengue viruses and their association with disease response in the host." Yale J Biol Med **42**(5): 276-292.

Kuhn, R. J., W. Zhang, et al. (2002). "Structure of dengue virus: implications for flavivirus organization, maturation, and fusion." Cell **108**(5): 717-725.

Lai, C. Y., W. Y. Tsai, et al. (2008). "Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II." J Virol **82**(13): 6631-6643.

Lin, H. E., W. Y. Tsai, et al. (2012). "Analysis of epitopes on dengue virus envelope protein recognized by monoclonal antibodies and polyclonal human sera by a high throughput assay." PLoS Negl Trop Dis **6**(1): e1447.

Littaua, R., I. Kurane, et al. (1990). "Human IgG Fc receptor II mediates antibody-dependent enhancement of dengue virus infection." J Immunol **144**(8): 3183-3186.

Lok, S. M., V. Kostyuchenko, et al. (2008). "Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins." Nat Struct Mol Biol **15**(3): 312-317.

Modis, Y., S. Ogata, et al. (2004). "Structure of the dengue virus envelope protein after membrane fusion." Nature **427**(6972): 313-319.

Pierson, T. C., Q. Xu, et al. (2007). "The stoichiometry of antibody-mediated neutralization and enhancement of West Nile virus infection." Cell Host Microbe **1**(2): 135-145.

Rajamanonmani, R., C. Nkenfou, et al. (2009). "On a mouse monoclonal antibody that neutralizes all four dengue virus serotypes." J Gen Virol **90**(Pt 4): 799-809.

Roehrig, J. T. (2003). "Antigenic structure of flavivirus proteins." Adv Virus Res **59**: 141-175.

Shrestha, B., J. D. Brien, et al. (2010). "The development of therapeutic antibodies that neutralize homologous and heterologous genotypes of dengue virus type 1." PLoS Pathog **6**(4): e1000823.

Sukupolvi-Petty, S., S. K. Austin, et al. (2010). "Structure and function analysis of therapeutic monoclonal antibodies against dengue virus type 2." J Virol **84**(18): 9227-9239.

Teoh, E. P., P. Kukkaro, et al. (2012). "The structural basis for serotype-specific neutralization of dengue virus by a human antibody." Sci Transl Med **4**(139): 139ra183.

Wahala, W. M., E. F. Donaldson, et al. (2010). "Natural strain variation and antibody neutralization of dengue serotype 3 viruses." PLoS Pathog **6**(3): e1000821.

Wahala, W. M., A. A. Kraus, et al. (2009). "Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody." Virology **392**(1): 103-113.

WHO (2009). "Dengue and dengue haemorrhagic fever." WHO Fact Sheets **117**.

Yu, I. M., H. A. Holdaway, et al. (2009). "Association of the pr peptides with dengue virus at acidic pH blocks membrane fusion." J Virol **83**(23): 12101-12107.

Yu, I. M., W. Zhang, et al. (2008). "Structure of the immature dengue virus at low pH primes proteolytic maturation." Science **319**(5871): 1834-1837.

CHAPTER 4

MODELING COMBINED NEUTRALIZATION AND ENHANCEMENT OF DNEGUE VIRUS BY MULTIPLE MONOCLONAL ANTIBODIES

4.1 Introduction

Dengue (DENV) is a re-emerging flavivirus that affects 2.5 billion people worldwide (WHO 2009). The number of reported severe DENV cases has been increasing at an alarming rate in the past several decades, while an effective vaccine is still not available (WHO 2009). DENV is divided into four serotypes (DENV1-4) based on their antigenicity. Immunity to one serotype does not protect against re-infection with different serotypes (Guzman, Kouri et al., 2000). In most cases, dengue infection results in a mild flu-like disease called dengue fever (DF). However, secondary infection with a heterologous serotype can lead to life-threatening Dengue Hemoharrgic Fever or Dengue Shock Syndrome (DHF/DSS). It was found that pre-existing heterologous antibodies are sub-neutralizing and can bridge viruses with Fc-ɣ receptors of non-permissive cells, assisting virus entry into these cells (Halstead et al., 1984). This was referred to as Antibody Dependent Enhancement (ADE) and evidences strongly suggested that ADE leads to DHF/DSS (Halstead 2003). ADE has seriously hindered the development of dengue vaccine. In the absence of effective dengue vaccine, therapeutic antibody is another option for treating dengue infection and many groups are working on screening candidate monoclonal antibodies (MAbs).

Both broadly cross-neutralizing and type-specific strongly neutralizing MAbs have been characterized as potential therapeutic antibodies (He, Innis et al. 1995; Lai, Goncalvez et al. 2007; Lok, Kostyuchenko et al. 2008; Cockburn, Navarro Sanchez et al. 2012; de Alwis, Smith et al. 2012). Most of the type-specific MAbs are mapped to EDIII. Though it was found that unlike in mouse models, EDIII reactive antibodies constitute a small fraction in human immune polyclonal serum and play an insignificant role in neutralization (Batra, Raut et al. 2010; Wahala, Kraus et al. 2009). Nevertheless, EDIII recognizing type-specific MAbs are still the most promising candidate for therapeutic MAbs. However, single MAb is known for its potential to drive escaping mutation, especially for a RNA virus with high mutagenesis rate. The generation of escape mutant not only risks spreading treatment resistance virus but also inducing ADE. Therefore, a cocktail of MAbs is necessary to achieve maximum neutralization and minimal risk of escape mutation.

It has been shown that the cross-reactive antibodies poses a risk of ADE during secondary infections though type-specific neutralizing MAbs can mediate ADE too when diluted to sub-neutralization level (Brandt, McCown et al. 1982). It has been reported that majority of anti-E antibody responses *in vivo* were cross-reactive against domain II (Lai, Tsai et al. 2008). PrM antibodies are also non-neutralizing, cross-reactive and enhancing (Huang, Yang et al. 2006). A recent report revealed that prM MAbs comprise a significant fraction in the MAbs cloned from human dengue convalescent PBMC (Dejnirattisai, Jumnainsong et al. 2010). Other reports revealed that cross-reactive E MAbs are the majority of cloned dengue reactive MAbs (Beltramello, Williams et al. 2010;Smith, Zhou et al. 2012). These cross-reactive MAbs must be taken into consideration when therapeutic antibodies were to be used to treat patients with pre-

existing immunity. Understanding their interaction with therapeutic MAbs is critical for designing safe and effective antibody therapy.

Understanding how various antibodies in polyclonal serum interact with each other in neutralization and ADE is another important medical question. Both *in vitro* and *in vivo* experiments have shown that DENV neutralizing polyclonal serum, diluted to sub-neutralizing concentration, can enhance dengue infection of monocytic origin cells (Cardosa 1987; Morens, Halstead et al. 1987; Morens, Larsen et al. 1987; Morier, Kouri et al. 1987; Littaua, Kurane et al. 1990; Morens and Halstead 1990; Burke and Kliks 2006). Polyclonal serum is a complicated mixture of various fractions of antibodies, such as type-specific and cross-reactive, neutralizing and non-neutralizing, prM antibodies and E antibodies, EDIII and EDI/II reactive antibodies, and antibodies of different isotypes and subclasses. It was found that these antibodies vary in neutralization potency and ADE potential, and specifically, different isotype or subclass are associated with different neutralization and ADE potential (Littaua, Kurane et al. 1990; Thein, Aaskov et al. 1993; Schlesinger and Chapman 1999; Shu, Chen et al. 2000; Mehlhop, Ansarah-Sobrinho et al. 2007; Rodrigo, Block et al. 2009). It is unclear how different fractions of polyclonal antibodies interact with each other in neutralization or enhancement. Studying the mixture of MAbs should be the first step to investigate the subtle interactions between different fractions of the polyclonal serum.

In this project, we mixed well-characterized MAbs together and studied their neutralization potency and ADE potential using neutralization assay and ADE assay. We established a model of combined neutralization and ADE between two different MAbs and developed empirical mathematic equations to describe them. These results offered insights into understanding how various antibody subsets in polyclonal serum interact with each other and provided guidelines for further therapeutic antibody formulation.

4.2 Materials and Methods

Cells. Vero cells (American Type Culture Collection, CCL-81) were maintained in Dulbecco's Modified Eagle Medium (DMEM). A cell line designated U937-DC-SIGN was maintained in RPMI-1640 (Gibco) supplemented with 50 mM beta mercaptoethanol. This human monocyte lymphoma cell line was derived from U937 cells but also expresses Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin or DC-SIGN, also known as CD209 (Cluster of Differentiation 209). Mosquito *Aedes albopictus* C6/36 cells were maintained in *Minimum Essential Medium* (MEM; Gibco) at 28°C. All media used also were supplemented with 5% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.1 mM non-essential amino acids (Gibco) and 2 mM glutamine, and all cells were incubated in the presence of 5% CO_2 at 37°C, unless otherwise specified. The 5% FBS was reduced to 2% to make infection medium for each cell line.

Serum and MAbs. Murine MAbs 2Q1899, 9F16 and 5C36 were purchased from US Biological (Swampscott, MA). MAbs 14A4, 2H2, 1A1D-2 and 8A1 were kindly provided by Dr. Robert Putnak (Walter Reed Army Research Institute, MD) (Table 4.1).

Virus suspension and virus antigen. C6/36 cells at 80% confluency were infected at MOI of 0.05 for 2 hrs followed by addition of 25 mL of infection medium. After 6 days of incubation, the supernatants were harvested, clarified by 30 minutes ultracentrifugation at 18,000 x g, aliquoted into cryovials and frozen at -80°C as working virus stock. Vero cells were inoculated and its supernatants were harvested in the same way as C6/36 cells. Viruses from the supernatants were pelleted at 70,000 x g through 20% sucrose cushion. The pelleted viruses were further purified using *OptiPrep*™ Density Gradient Medium (Sigma-Aldrich) and the virus containing fractions were collected, pelleted,

resuspended and frozen at -80°C for further use as purified virus antigens. All the viruses used were WHO reference strains of each serotype.

ELISA. high binding ELISA plates (Corning Life Sciences) were coated with 50 ng of purified virus antigen per well in 0.1 M carbonate buffer. Plates were incubated at 4˚C overnight, washed three times (all washes were performed three times with tris-buffered saline containing 0.2% Tween-20), then blocked with dilution buffer on a shaker at 37˚C for 1hr. 50 µL of antibodies prepared in Tris-buffered saline containing 0.05% Tween-20 and 3% Normal Goat Serum were added to the wells and incubated on a shaker at 37˚C for 1 hr. Unbound primary antibodies were washed off and 1:1000 alkaline phosphatase conjugated diluted goat anti-mouse secondary antibodies added; the plate was then incubated on a shaker at 37˚C for 1 hr. Unbound secondary antibodies were washed off, pNPP substrate added, and absorbance readings were taken at 405 nm in an Epoch Microplate Spectrophotometer (Biotek Instruments Inc. Winooski, VT).

FACS Based Neutralization Assay. Antibodies were diluted in RPMI 1640 media with 2% FBS buffered with 20mM HEPES (infection media). Infectious virus stocks were diluted in infection media to 20, 000 PFU/mL. Then 40 µL of the MAb dilutions or MAb mixtures and 40 µL of the diluted viruses were added together into each well in a 96-well round bottom plate (Corning Life Science, Lowell, MA) according to experiment design and incubated in 37˚C incubator for 45 minutes for antibody-virus binding. U937-DC-SIGN cells were concentrated to 1,500,000 cells/mL density and 20 µL of the cells were added to each well and incubated for 2 hrs for infection. The cells were washed twice with infection media and incubated for 24 hrs. The rest of the experiment is the same as described in previous literatures (Lambeth, White et al., 2005)

FACS Based ADE Assay. The FACS Based ADE assay protocol is same as the FACS Based Neutralization assay except that 1) infectious virus stock is diluted to 500,000

PFU/mL in infection media and 2) the U937-DC-SIGN cell line is replaced with U937 cells.

4.3 Results

Independent neutralization of two MAbs in mixture

Study of neutralization by a homogeneous population of MAbs has been well described in many previous reports and was further explored in Chapter 3. However, it is still unclear how neutralization by heterogeneous antibody binding of DENV using a mixture of MAbs might work. To study this question, the combined neutralization of two MAbs was assayed by mixing MAbs together in the FACS-based neutralization test. We first studied two DENV2-neutralizing EDIII-binding MAbs, 1A1D-2 and 2Q1899 (Table 4.1). As shown in figure 4.1A, the neutralization curve of serially diluted MAb 1A1D-2 is a typical sigma dose response curve. When MAb 2Q1899 at a 1.6ng/μL concentration (neutralizing 40% of the DENV2 infection) was added to each dilution of 1A1D-2, the combined neutralization increased, but the increment varied over different concentrations of 1A1D-2. After fitting the data, we found a simple equation to describe the numerical relationship between neutralization of the mixture and that of the single MAb: $Neut_{combined} \approx Neut_{1A1D-2} + Neut_{2Q1899} - Neut_{1A1D-2} \times Neut_{2Q1899}$

Based on this equation, we predicted combined neutralization and found it fitting well with the measured value (Figure 4.1A). We further tested two MAbs bound to different E domains: 4G2 (EDII) and 1A1D-2 (EDIII). Their combined neutralization is also a close approximation to what was predicted by the equation. Other combinations of MAbs such as mouse MAbs (5C36 or 1A1D-2) and human MAb (1E4), or MAbs with overlapping binding epitopes (5C36 and 9F16) were also tested (Figure 4.1A). Besides

DENV2, we also tried combinations of DENV3 neutralizing MAbs: type-specific 9D1 and 8A1, and cross-neutralizing MAb 1A1D-2 in various dilution schemes (Figure 4.1B). All of these combinations demonstrated the accuracy and robustness of this equation.

Additive ADE of two MAbs in mixture

Sub-neutralizing antibodies can enhance dengue virus infection by bridging the virus to the Fc-γ receptors of monocytic cells. To assess how different antibodies interact during this process, MAbs of different subclasses or binding epitopes were mixed together and tested in ADE assay. Previous reports suggested that high antibody concentrations inhibit ADE through neutralization, as indicated by the bell-shaped ADE curve (Morens, Halstead et al. 1987). To avoid such complication, the MAbs were used at sub-neutralizing concentrations.

Both 4G2 and 2H2 belong to the mouse IgG2a subclass, but they recognize E and prM protein respectively. MAbs 4G2, 2H2 and their mixture were assayed to evaluate the potential synergistic enhancement effect. Our results indicated that their combined ADE is precisely the addition of each MAb ADE alone (Figure 4.2A). This suggests that ADE mediated by the prM antibodies and E antibodies are additive to each other in antibody cocktail and there is no detectable synergistic effect. Next we tested if MAbs belonging to different subclasses may have a synergistic effect. It was shown that different subclasses of IgG bind to different Fc-ɣ receptors, RI and RII, which regulate different cell functions and modulate neutralization and ADE (Indik, Park et al. 1995). So two E MAbs: 4G2 (IgG2a) and 2Q1899 (IgG1), were mixed and tested in ADE assay. The results suggested that their combined enhancement is also the addition of that of each MAb ADE and no synergistic effect was detected (Figure 4.2B). In summary, combination experiments of various MAbs mixtures demonstrated that ADE is additive at sub-neutralizing concentrations.

Neutralizing MAb inhibits ADE mediated by Enhancing MAb

Numerous works have revealed how neutralizing antibodies inhibit direct infection of cells (via DC-SIGN molecule or heperan sulfate) using various neutralization assays. But little has been done to study how neutralizing antibodies inhibit ADE infection of cells (via Fc-ɣ receptor). To fill in this gap, we mixed one enhancing MAb with one neutralizing MAb and assessed their combined ADE. MAb 2H2 represents the cross-reactive prM antibodies that play a major role in promoting ADE in secondary infections (Beltramello, Williams et al. 2010; Dejnirattisai, Jumnainsong et al. 2010). As shown in Figure 4.3A, 2H2 at a 0.0125 µg/mL concentration mediated 11 fold enhancement of infection. After the strongly neutralizing MAb 1A1D-2 was added, the 2H2 mediated ADE infection was reduced to base level. Moderately neutralizing MAb 9F16 (Table 3.1) can also inhibit most of the ADE infection (Figure 4.3B). We further found that even the weakly neutralizing MAb 4G2, at neutralizing concentrations, can inhibit 2H2 mediated enhancement (Figure 4.3C). In general, our data indicated that neutralizing MAbs can inhibit Fc-ɣ receptor mediated ADE infection as well as they inhibit direct infection of cells.

Non-neutralizing Non-enhancing MAbs Modulate ADE infection

EDIII binding MAbs 12C1 and 13A5 are cross-reactive with all four serotypes but do not neutralize any serotype, nor do they contribute to neutralization when mixed with neutralizing MAbs (Figure 4.4A – B and unpublished data). Surprisingly, they lack the capability to mediate ADE infection as well (Figure 4.4C – D). To test whether they contribute to ADE when mixed with other enhancing MAbs, each of them was mixed with MAb 4G2 in ADE assay. Interestingly, when MAb 12C1 was mixed with 4G2, the combined ADE increased significantly compared to that of 4G2 alone. The increase

becomes larger as the concentration of 12C1 goes up (Figure 4.4C). Even more interestingly, MAb 13A5 displays just the contrary effect: It inhibits the ADE when mixed together with 4G2 and the ADE is totally abolished as 13A5 concentration increases to 2μg/mL(Figure 4.4D). Although MAbs like 12C1 and 13A5 does not neutralize or enhancement infection by themselves, our data suggests that they do play a role in regulating the ADE mediated by other enhancing antibodies.

4.4 Discussion

Significant progress has been made in searching for candidate MAbs for dengue post-exposure therapy (He, Innis et al. 1995; Lai, Goncalvez et al. 2007; Lok, Kostyuchenko et al. 2008; Sukupolvi-Petty, Austin et al. 2010; Cockburn, Navarro Sanchez et al. 2012; de Alwis, Smith et al. 2012). However, using a single MAb for therapy could be problematic due to two reasons: 1) a single MAb, especially a type-specific MAb, may not be broadly neutralizing against diverse dengue strains (Wahala, Donaldson et al. 2010; Zhou, Austin et al. 2013); and 2) a single MAb may drive dengue evolution to generate neutralization-escaping mutations, leading to ADE. Using a cocktail of different MAbs would be ideal to avoid these problems, with potential synergistic effect as a bonus. Therefore, knowledge about how the neutralization of cocktail MAbs work will be critical for the development of dengue antibody therapy. The synergistic effect in neutralization has been observed in HIV and Adenovirus antibody neutralization experiments (Tilley, Honnen et al. 1992; Potts, Field et al. 1993; Mascola, Louder et al. 1997; Gahery-Segard, Farace et al. 1998; Verrier, Nadas et al. 2001), but unfortunately for the dengue MAbs tested here, there is no evidence of such synergistic neutralization. Instead, our results suggested that for dengue, MAb neutralizations are

independent of each other and the combined neutralization follows this equation:

$$Neut_{A\&B} \approx Neut_A + Neut_B - Neut_A \times Neut_B$$

This equation may be of interests to the medicine industry. Considering the dose response of antibody neutralization, this equation provides a guideline for the formulation of therapeutic MAbs to get maximum neutralization at the lowest possible cost. This equation also holds interesting implications for neutralization mechanisms. The above equation can be transformed to the following equation:

$$(1 - Neut_{A\&B}) \approx (1 - Neut_A) \times (1 - Neut_B)$$

If we define $1 - Neut$ as the percentage of non-neutralized infectivity, or rate of survival, the above equation can be further transformed to:

$Survival_{A\&B} \approx Survival_A \times Survival_B$. This simple equation directly implies that the viruses have to survive the neutralization of MAb A and MAb B independently in the MAbs cocktail. It implies that each antibody neutralizes viruses independently and there is no synergistic neutralization. One explanation for this independence is that the two MAbs tested here are neutralizing the viruses through different mechanisms. Take the pair 5C36 and 4G2 as an example, 5C36 neutralizes viruses at the attachment step while 4G2 neutralizes viruses at the post-attachment step (Chapter 3), and the two MAbs work at different “time points” of the virus infection sequence.

Pierson and colleagues proposed that flavivirus neutralization follows the “threshold model”, which states that viruses bound with MAbs exceeding the threshold will get neutralized. This model implies a synergistic neutralization of the virus. For example, a virus was bound by MAb A and MAb B simultaneously and each MAb alone is below the neutralization threshold, but together they are above the threshold

requirement. Based on the threshold model, the virus should now be neutralized, which is known as synergistic neutralization (two MAbs at non-neutralizing concentrations are mixed together and become neutralizing). The threshold model predicted the existence of the synergistic effect, but our study failed to identify any. This result questions the threshold model as the universal model for flavivirus neutralization. Instead, the independent neutralization found here is compatible with the lack of synergistic effect and can also explain this equation. In other words, this result implies that the threshold model is not the universal model of flavivirus neutralization and there are other stoichiometry models.

The results of the combined ADE of MAb mixtures also revealed no synergistic effect between MAbs of different subclasses, or MAbs recognizing different antigens (prM and E). The ADE of MAbs at sub-neutralizing concentrations are additive to each other when mixed together, implying that in the absence of neutralizing antibodies, ADE potential is proportional to the quantity of enhancing antibodies bound to viruses. By mixing neutralizing MAbs with enhancing MAbs, our results revealed that neutralizing MAbs can inhibit the ADE mediated by enhancing MAbs. The mechanism of inhibiting ADE is understudied, but it has been suggested that antibodies blocking the attachment step are not able to inhibit ADE infection because in ADE infection the virus-antibody complex does not utilize attachment molecules such as DC-SIGN but instead use of the Fc gamma receptors of the cell (Nybakken, Oliphant et al. 2005; Chan, Zhang et al. 2011). 4G2 and 1A1D-2 were shown to block post-attachment steps (Chapter 3) and as expected they were able to block ADE infection (Figure 4.3A and 4.3C). However, MAb 9F16 blocks mostly the attachment step (Chapter 3) and yet it was also able to block ADE infection efficiently (Figure 4.3B). This may suggest that 9F16 might utilize mechanisms other than blocking attachment to inhibit 2H2 mediated ADE infection.

Since circulation of enhancing antibodies is very common in dengue infected patients, the results presented here are critical to optimize usage of neutralizing MAbs to minimize ADE risk in therapy.

Apart from these neutralizing or enhancing MAbs, the non-neutralizing non-enhancing MAbs make up a significant fraction of cloned human MAbs yet they received little attention at all (Smith, Zhou et al. 2012). Usually, these MAbs are considered “idle”. However, our results indicated these “idle antibodies” are not really “idle” as we found the unexpected ADE modulation function of MAbs 12C1 and 13A5. The mechanisms of their inhibitory or synergistic effect in ADE is still unknown. Work should be done to investigate these mechanisms, as our recent human MAb cohort study also reveals quite abundant existence of this kind of “idle antibody” in human sera (Smith, Zhou et al. 2012) and their role in dengue pathogenesis may deserve more attention.

By studying the neutralization and ADE of MAbs cocktail, we were able to reveal some medically important discoveries of dengue neutralization and ADE: neutralizations mediated by various antibodies are mostly independent from each other and ADE mediated by various sub-neutralizing antibodies are additive to each other. Both discoveries have important applications for medicine industry and academic field. Another important discovery is the ADE modulation by non-neutralizing non-enhancing antibodies.

Tables

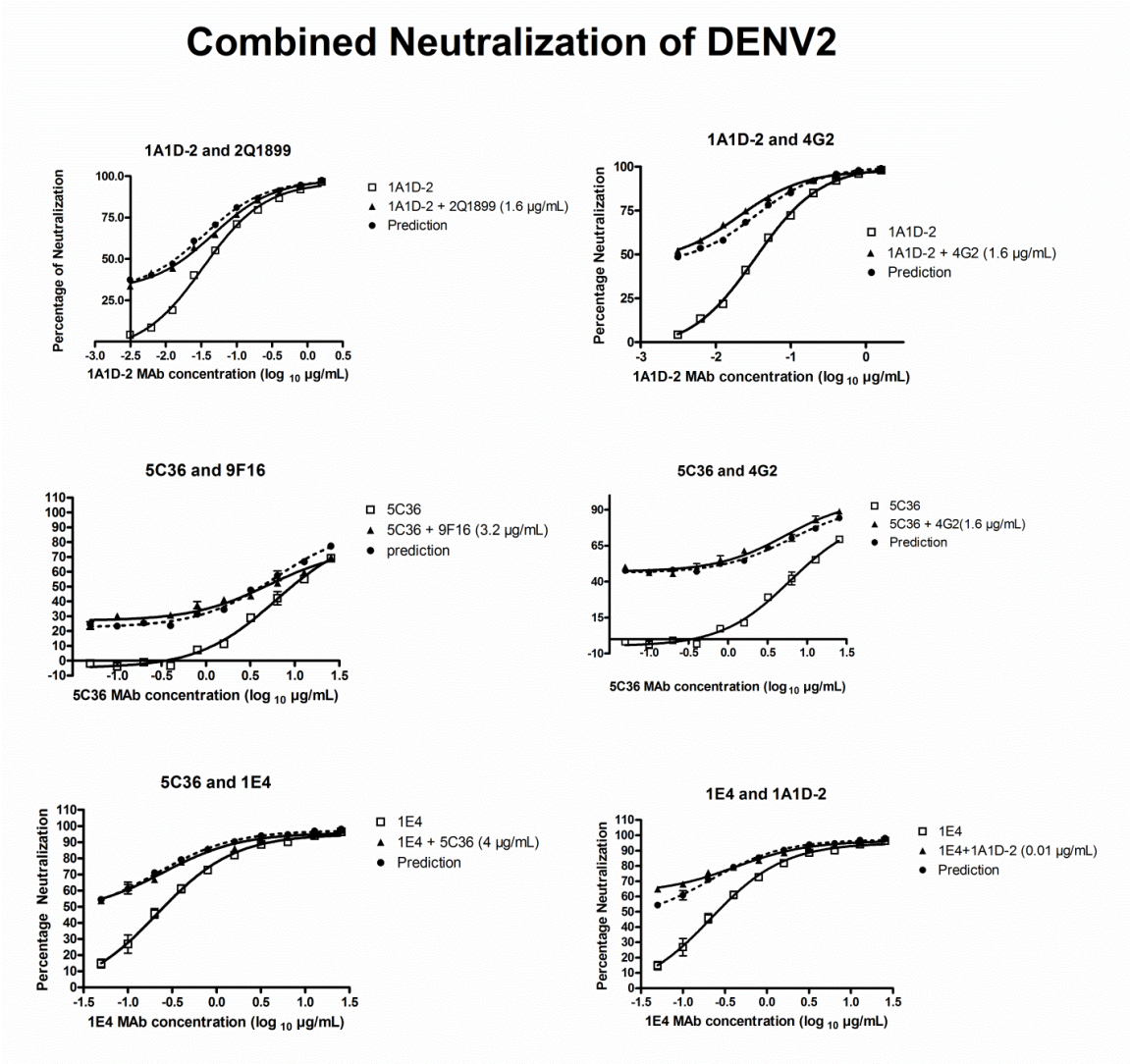
Table 4.1: Panel of mouse MAbs used in this project.

Name	Subclass	Specificity	Source	Epitope
4G2	IgG2a	Flavivus-group	ATCC	EDII
12C1	IgG1	Dengue group	Dr. Accavitti-loper	EDIII
13A5	IgG1	Dengue group		EDIII
14A4	IgG1	Dengue sub-complex (DV1and DV3)	Dr. Putnak	EDIII
2H2	IgG2a	Dengue group	Dr. Putnak	prM
8A1	IgG1	DV3 type-specific	Dr. Putnak	EDIII
9D1	IgG1	DV3 type-specific	Dr. Accavitti-loper	unknown
9F16	IgG1	DV2 type-specific	U.S. Biological	EDIII
2Q1899	IgG1			EDIII
5C36	IgG1			EDIII
1A1D-2	IgG1	Dengue group	Dr. Putnak	EDIII

Figure and Legends

Figure 4.1

A)



B)

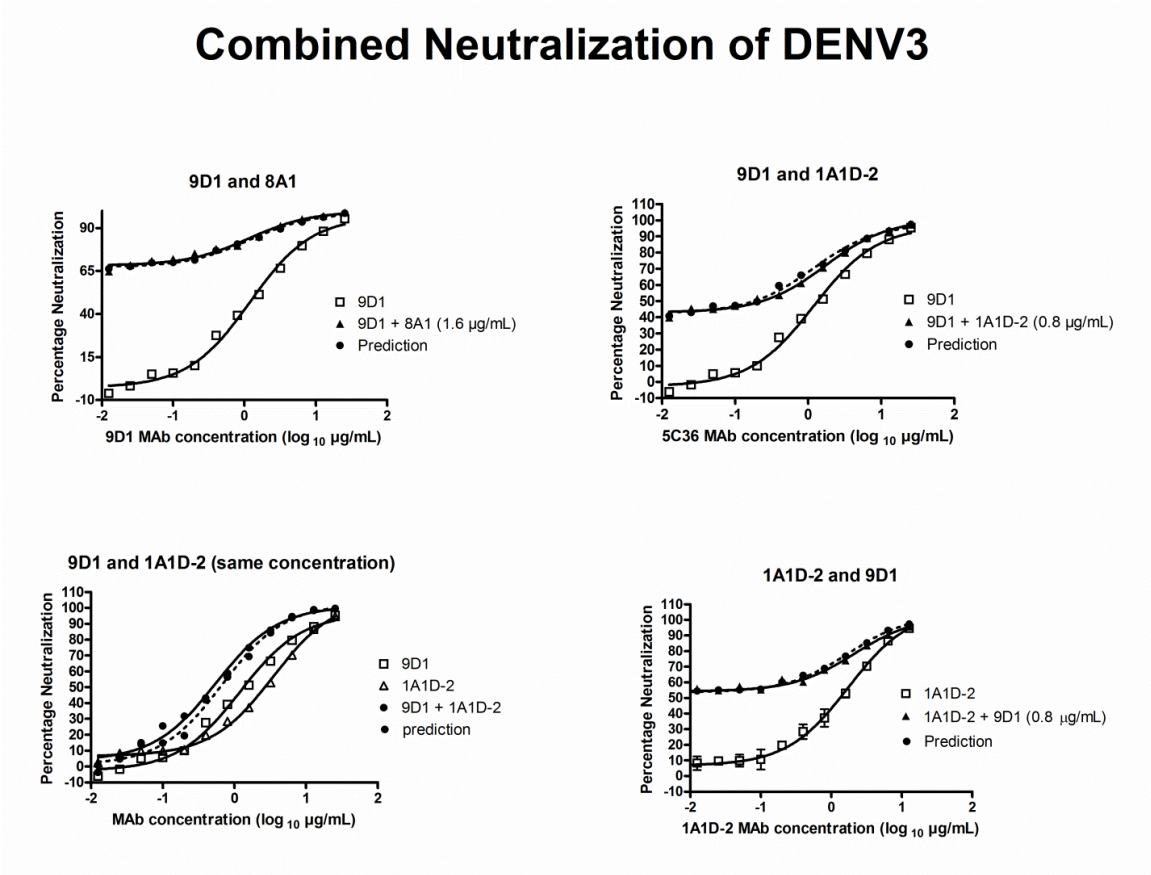


Figure 4.1: Combined Neutralization of DENV by Two MAbs. A) Combined neutralization of DENV2 virus by two MAbs. **B)** Combined neutralization of DENV3 virus by two MAbs. For each experiment, one MAb was serially diluted to different concentrations and mixed with another MAb at the $Neut_{50}$ concentration. Dashed lines are predicted results. All the neutralization assays in the same figure were done side by side in one experiment. All the experiments were repeated 3 times and a representative result is presented here.

Figure 4.2

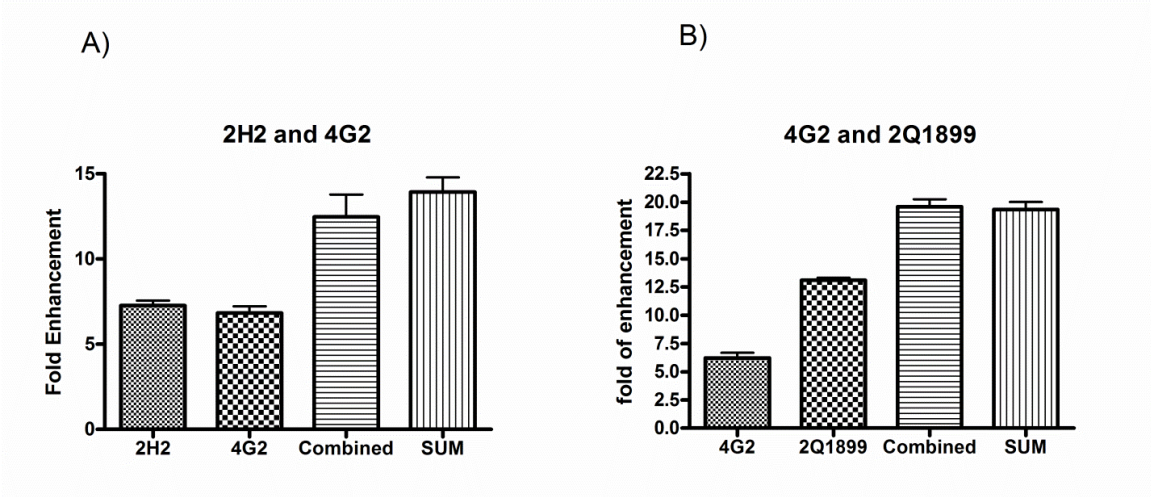


Figure 4.2: Combined ADE of two MAbs at Non-neutralizing concentrations. A) E MAb 4G2 and prM MAb 2H2, each at non-neutralizing concentration of 0.0125µg/mL, were tested for ADE in U937 cells alone or mixed together. **B)** IgG2a subclass MAb 4G2 and IgG1 subclass MAb 2Q1899, each at non-neutralizing concentration of 0.0125µg/mL, were tested for ADE in U937 cells alone or mixed together.

Figure 4.3

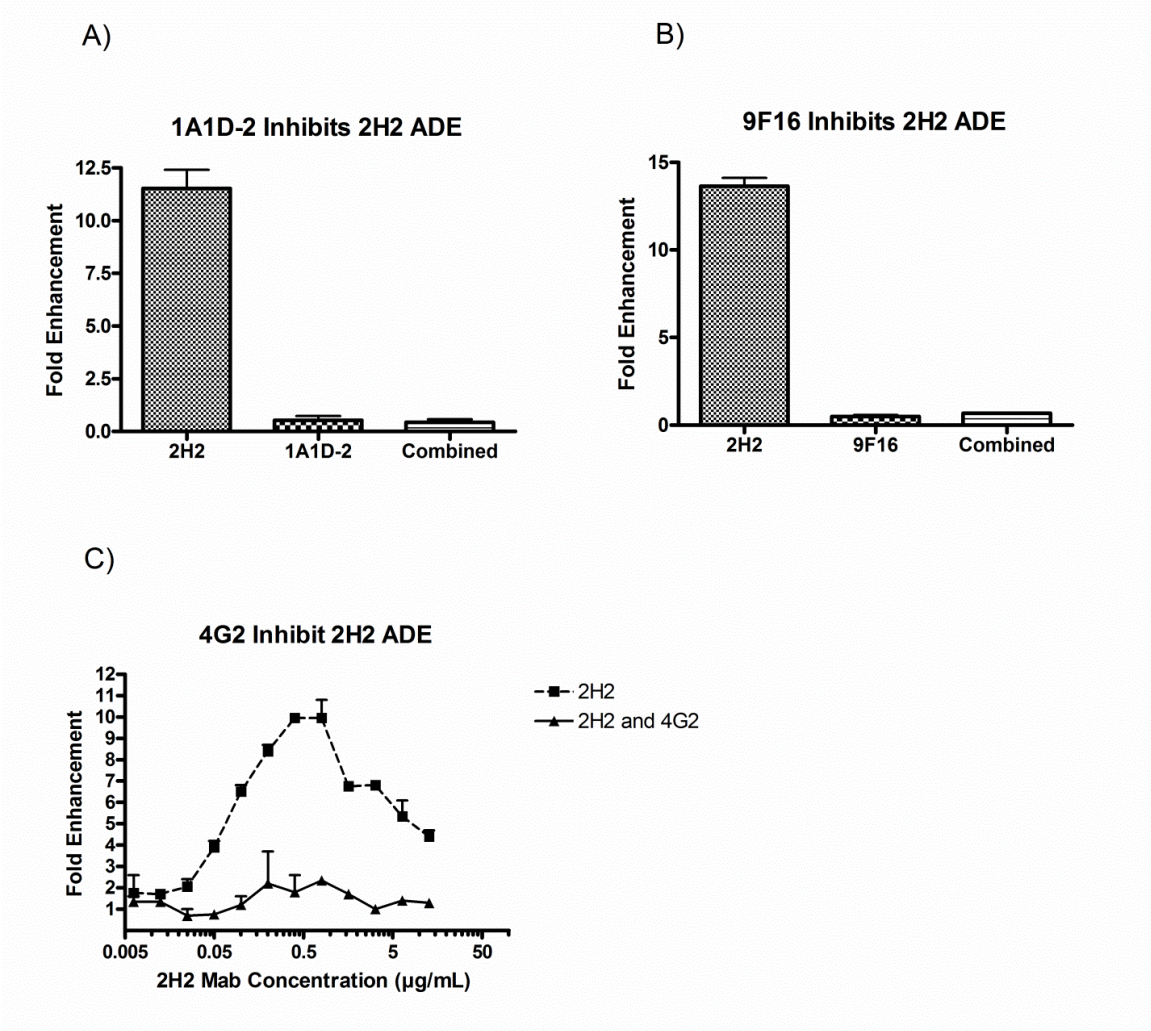


Figure 4.3: Inhibition of ADE by Neutralizing MAbs. A) MAb 1A1D-2 (1.6 µg/mL, 95% neutralization potency in U937-DC-SIGN cells) was added to enhancing MAb 2H2 (0.2 µg/mL) and totally inhibited the 11 fold enhancement mediated by 2H2. **B)** 9F16 (12.5 µg/mL, 95% neutralization potency in U937-DC-SIGN cells) totally inhibited 2H2 mediated ADE. **C)** Adding 6.4µg/mL of 4G2 to 2H2 can inhibit the 2H2 mediated ADE at all concentrations.

Figure 4.4

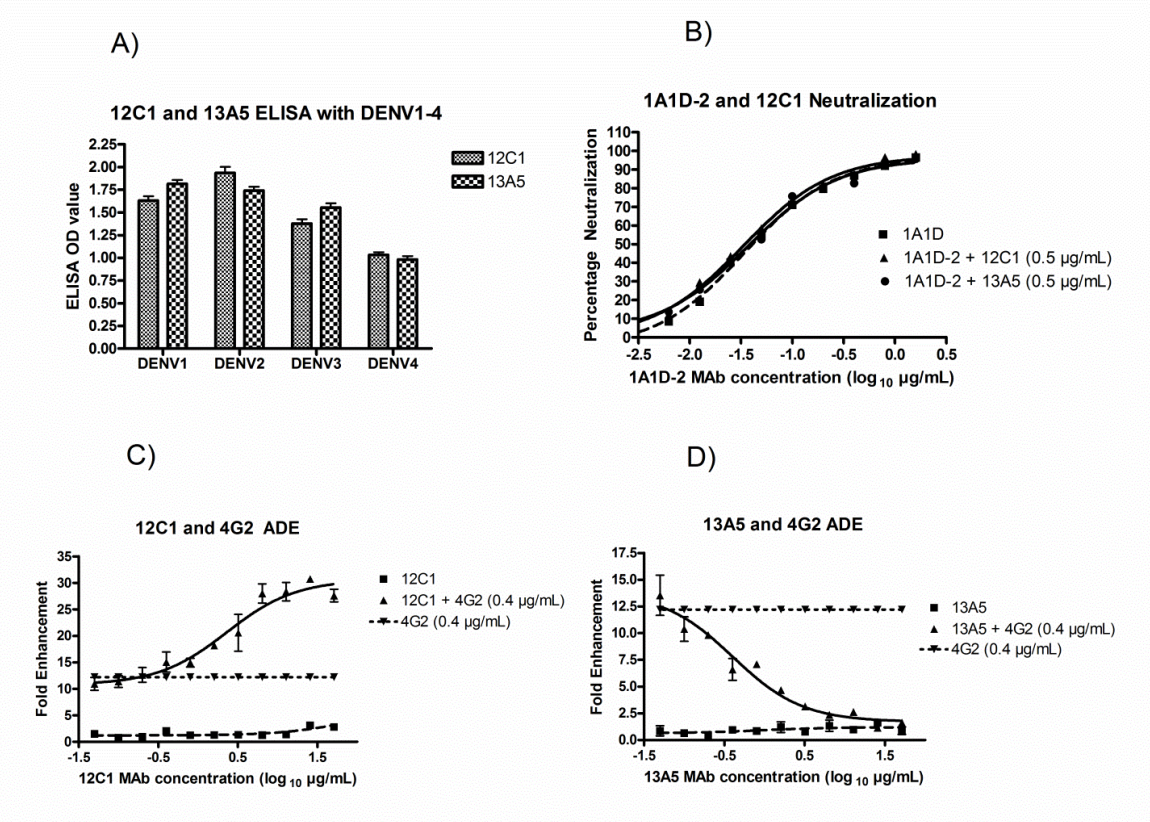


Figure 4.4: Cross-reactive and non-neutralizing MAb 12C1 and 13A5 can modulate the ADE mediated by MAb 4G2. A) Both MAb 12C1 and 13A5 both are cross-reactive MAbs against four serotypes. **B)** 0.5 µg/mL of 12C1 or 13A5 was added to 1A1D-2 MAb and there was no increase of neutralization of DENV2 at any concentration of 1A1D-2. **C)** Increased concentrations of 12C1 were added to 4G2 (0.4µg/mL) and the DENV2 infection enhancement mediated by 4G2 increased gradually. **D)** Increasing concentrations of 13A5 were added to 4G2 (0.4µg/mL) and the DENV2 infection enhancement mediated by 4G2 decreased gradually.

4.5 Reference

Batra, G., R. Raut, et al. "Pichia pastoris-expressed dengue virus type 2 envelope domain III elicits virus-neutralizing antibodies (2010)." J Virol Methods **167**(1): 10-16.

Beltramello, M., K. L. Williams, et al. "The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity." Cell Host Microbe **8**(3): 271-283.

Beltramello, M., K. L. Williams, et al. (2010). "The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity." Cell Host Microbe **8**(3): 271-283.

Brandt, W. E., J. M. McCown, et al. (1982). "Infection enhancement of dengue type 2 virus in the U-937 human monocyte cell line by antibodies to flavivirus cross-reactive determinants." Infect Immun **36**(3): 1036-1041.

Burke, D. S. and S. Kliks (2006). "Antibody-dependent enhancement in dengue virus infections." J Infect Dis **193**(4): 601-603; author reply 603-604.

Cardosa, M. J. (1987). "Dengue virus isolation by antibody-dependent enhancement of infectivity in macrophages." Lancet **1**(8526): 193-194.

Cockburn, J. J., M. E. Navarro Sanchez, et al. (2012). "Mechanism of dengue virus broad cross-neutralization by a monoclonal antibody." Structure **20**(2): 303-314.

de Alwis, R., S. A. Smith, et al. (2012). "Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions." Proc Natl Acad Sci U S A **109**(19): 7439-7444.

Dejnirattisai, W., A. Jumnainsong, et al. "Cross-reacting antibodies enhance dengue virus infection in humans." Science **328**(5979): 745-748.

Gahery-Segard, H., F. Farace, et al. (1998). "Immune response to recombinant capsid proteins of adenovirus in humans: antifiber and anti-penton base antibodies have a synergistic effect on neutralizing activity." J Virol **72**(3): 2388-2397.

Halstead, S. B. (2003). "Neutralization and antibody-dependent enhancement of dengue viruses." Adv Virus Res **60**: 421-467.

He, R. T., B. L. Innis, et al. (1995). "Antibodies that block virus attachment to Vero cells are a major component of the human neutralizing antibody response against dengue virus type 2." J Med Virol **45**(4): 451-461.

Huang, K. J., Y. C. Yang, et al. (2006). "The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection." J Immunol **176**(5): 2825-2832.

Indik, Z. K., J. G. Park, et al. (1995). "Structure/function relationships of Fc gamma receptors in phagocytosis." Semin Immunol **7**(1): 45-54.

Lai, C. J., A. P. Goncalvez, et al. (2007). "Epitope determinants of a chimpanzee dengue virus type 4 (DENV-4)-neutralizing antibody and protection against DENV-4 challenge in mice and rhesus monkeys by passively transferred humanized antibody." J Virol **81**(23): 12766-12774.

Lai, C. Y., W. Y. Tsai, et al. (2008). "Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II." J Virol **82**(13): 6631-6643.

Littaua, R., I. Kurane, et al. (1990). "Human IgG Fc receptor II mediates antibody-dependent enhancement of dengue virus infection." J Immunol **144**(8): 3183-3186.

Lok, S. M., V. Kostyuchenko, et al. (2008). "Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins." Nat Struct Mol Biol **15**(3): 312-317.

Mascola, J. R., M. K. Louder, et al. (1997). "Potent and synergistic neutralization of human immunodeficiency virus (HIV) type 1 primary isolates by hyperimmune anti-HIV immunoglobulin combined with monoclonal antibodies 2F5 and 2G12." J Virol **71**(10): 7198-7206.

Mehlhop, E., C. Ansarah-Sobrinho, et al. (2007). "Complement protein C1q inhibits antibody-dependent enhancement of flavivirus infection in an IgG subclass-specific manner." Cell Host Microbe **2**(6): 417-426.

Morens, D. M. and S. B. Halstead (1990). "Measurement of antibody-dependent infection enhancement of four dengue virus serotypes by monoclonal and polyclonal antibodies." J Gen Virol **71 (Pt 12)**: 2909-2914.

Morens, D. M., S. B. Halstead, et al. (1987). "Profiles of antibody-dependent enhancement of dengue virus type 2 infection." Microb Pathog **3**(4): 231-237.

Morens, D. M., L. K. Larsen, et al. (1987). "Study of the distribution of antibody-dependent enhancement determinants on dengue 2 isolates using dengue 2-derived monoclonal antibodies." J Med Virol **22**(2): 163-167.

Morier, L., G. Kouri, et al. (1987). "Antibody-dependent enhancement of dengue 2 virus in people of white descent in Cuba." Lancet **1**(8540): 1028-1029.

Potts, B. J., K. G. Field, et al. (1993). "Synergistic inhibition of HIV-1 by CD4 binding domain reagents and V3-directed monoclonal antibodies." Virology **197**(1): 415-419.

Rodenhuis-Zybert, I. A., H. M. van der Schaar, et al. "Immature dengue virus: a veiled pathogen?" PLoS Pathog **6**(1): e1000718.

Rodrigo, W. W., O. K. Block, et al. (2009). "Dengue virus neutralization is modulated by IgG antibody subclass and Fcgamma receptor subtype." Virology **394**(2): 175-182.

Schlesinger, J. J. and S. E. Chapman (1999). "Influence of the human high-affinity IgG receptor FcgammaRI (CD64) on residual infectivity of neutralized dengue virus." Virology **260**(1): 84-88.

Shu, P. Y., L. K. Chen, et al. (2000). "Dengue NS1-specific antibody responses: isotype distribution and serotyping in patients with Dengue fever and Dengue hemorrhagic fever." J Med Virol **62**(2): 224-232.

Smith, S. A., Y. Zhou, et al. (2012). "Persistence of circulating memory B cell clones with potential for dengue virus disease enhancement for decades following infection." J Virol **86**(5): 2665-2675.

Sukupolvi-Petty, S., S. K. Austin, et al. "Structure and function analysis of therapeutic monoclonal antibodies against dengue virus type 2." J Virol **84**(18): 9227-9239.

Thein, S., J. Aaskov, et al. (1993). "Changes in levels of anti-dengue virus IgG subclasses in patients with disease of varying severity." J Med Virol **40**(2): 102-106.

Tilley, S. A., W. J. Honnen, et al. (1992). "Synergistic neutralization of HIV-1 by human monoclonal antibodies against the V3 loop and the CD4-binding site of gp120." AIDS Res Hum Retroviruses **8**(4): 461-467.

Verrier, F., A. Nadas, et al. (2001). "Additive effects characterize the interaction of antibodies involved in neutralization of the primary dualtropic human immunodeficiency virus type 1 isolate 89.6." J Virol **75**(19): 9177-9186.

Wahala, W. M., A. A. Kraus, et al. (2009). "Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody." Virology **392**(1): 103-113.

WHO (2009). "Dengue and dengue haemorrhagic fever." WHO Fact Sheets **117**.

CHAPTER 5

THE MECHANISM OF DIFFERENTIAL NEUTRALIZATION OF DENGUE SEROTYPE 3 STRAINS BY MONOCLONAL ANTIBODY 8A1

5.1 Introduction

Dengue virus (DENV) is a mosquito borne virus that is endemic in most of the tropical areas in the world, putting 2.5 billion people in risk (WHO 2009). Four serotypes of DENV co-circulate and infection with one serotype does not provide life-long immunity against other serotypes (Halstead 1988). Many DENV infections are asymptomatic, while symptomatic disease can manifest as classical Dengue Fever (DF), or can develop into more severe form of disease called Dengue Hemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS) (Shepard, Suaya et al. 2004). It is estimated that DHF/DSS leads to 10,000 – 15,000 deaths annually (WHO 2009). Epidemiologic data suggests that pre-existing antibodies, either from previous heterotypic DENV infection or, in the case of newborns and infants, maternally acquired anti-DENV antibodies, are associated with development of the more severe disease (Halstead and O'Rourke 1977). This phenomenon, known as Antibody-Dependent Enhancement (ADE), has been demonstrated *in vitro* using sub-neutralizing concentration of antibodies to facilitate infection of otherwise non-permissive cells such as monocytes via Fc-γ receptor mediated endocytosis (Halstead and O'Rourke 1977). This particular feature of DENV potentially confounds vaccine implementation and design strategies.

DENV is a single-stranded, positive-sense RNA virus in the family *Flaviviridae*, genus flavivirus. It is closely related to several other important human pathogens such as Japanese encephalitis (JEV), West Nile (WNV), Yellow fever (YFV) and Tick-borne encephalitis (TBEV). The DENV genome is translated into a single polyprotein and then cleaved into structural proteins (C-prM-E) and non-structural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5). The Envelope protein (E) is the major surface exposed antigen and the principal target of host antibody response. The envelope proteins of different DENV serotypes have been crystallized and their structures characterized (Rey, Heinz et al. 1995; Modis, Ogata et al. 2003; Zhang, Zhang et al. 2004; Modis, Ogata et al. 2005; Cockburn, Navarro Sanchez et al. 2012). On the mature DENV virion, E forms a homodimer and is arranged on the virion surface in a herringbone pattern with dimers arranged in sets of three rafts. The ectodomain of E protein has three domains: domain I, II and III (EDI-EDIII). Importantly, EDIII is believed to be involved in cell receptor binding (Crill and Roehrig 2001). EDIII belongs to the IgG superfamily and is composed of several beta-strands linked by loops (Figure 5.1A, 5.1B). A subset of these loops form a surface exposed structure called the lateral ridge, which is the target of many monoclonal antibodies that strongly neutralize DENV (Modis, Ogata et al. 2005; Pierson and Diamond 2008).

EDIII is a potent immunogen that elicits type-specific antibodies in various animal models (Schmitz, Roehrig et al. 2011). Consequently, recombinant EDIII based vaccines have been under development with the hypothesis that EDIII type-specific antibodies will be protective but less likely to enhance dengue virus infection. Many type-specific monoclonal antibodies against EDIII target the most diversified/non-conserved region – the lateral ridge and hence mutations within this region may have important immunological consequences.

There are four distinct DENV-3 genotypes: I, II, III and IV (Lanciotti, Lewis et al. 1994). Currently genotype I and II are circulating in Asia , genotype III is circulating in the Indian subcontinent, Africa and Latin America, while genotype IV appears to have been displaced but occurred throughout the Caribbean in the 1960s and 70s (Messer, Gubler et al. 2003; Araújo, Nogueira et al. 2009). Wahala *et al.* revealed difference of sensitivity between DENV-3 genotypes to certain type-specific neutralizing MAbs (Wahala, Donaldson et al. 2010). Other researchers have also shown that genotypes play a role in antibody neutralization and protection (Brien, Austin et al. 2010; Shrestha, Brien et al. 2010; Sukupolvi-Petty, Austin et al. 2010; Pitcher, Gromowski et al. 2012), including the finding that intra-genotypic variations can elicit different immune response that fail to effectively neutralize virus of the same serotype (Wong, Abd-Jamil et al. 2007). Since multiple genotypes co-circulate worldwide (Nogueira, Stella et al. 2008; Jiang, Yu et al. 2012), it becomes imperative to understand how viral genotypic variation affects neutralization and define its mechanism. The constant evolution of dengue viruses further justifies studying how mutations influence interactions with antibodies (de Mora, Andrea et al. 2009; Kukreti, Mirtal et al. 2010; Ramirez, Fajardo et al. 2010).

To better understand the role of genotypic variation in DENV-3 neutralization, we tested the mouse monoclonal antibody 8A1 against a panel of recombinant DENV-3 viruses that expressed complete envelope genes from each of the four genotypes. We then constructed additional mutant recombinant viruses containing single or multiple amino acid mutations to identify the residues critical to 8A1 neutralization of DENV-3. We found that the sensitivity of genotype I and II, compared to resistant genotype III, are attributed to only two amino acid differences in EDIII region. Further study revealed that the amino acids work independently to confer the sensitivity to 8A1. Variation at two amino acid positions led to different on and off rates of epitope/antibody binding and thus

different affinity. Our studies provided insights into neutralization mechanism and how binding kinetics affect virus sensitivity to different antibodies.

5.2 Methods and materials

Cells. Mosquito *Aedes albopictus* C6/36 cells were maintained in MEM (Gibco) media at 28°C. Human monocyte lymphoma cell line U937 expressing DC-SIGN (U937 DC-SIGN) were maintained in RPMI-1640 (Gibco) at 37°C supplemented with 50mM beta mercaptoethanol. Vero-81 cells were maintained in DMEM at 37°C. All media used were also supplemented with 5% FBS, 100U/ml penicillin, 100mg/ml streptomycin, 0.1mM non-essential amino acids (Gibco) and 2mM glutamine and all cells were incubated in the presence of 5% CO_2. The 5% FBS was reduced to 2% to make infection media for each cell line.

DENV-3 Molecular Clone Strategy. The four fragment cloning strategy for the DENV-3 clone was recently described (Messer, Yount et al. 2012). In brief, plasmids containing the four DENV fragments DNAs (A-D) were propagated in *E. coli*, purified, restriction enzyme digested and directionally ligated to create a full-length cDNA of the dengue viral genome. The cDNAs were subsequently transcribed with T7 polymerase (Ambion). This RNA produced infectious dengue virus when electroporated into Vero E6 cells. E variant clones have been previously characterized (Messer, Yount et al. 2012). Briefly, E genes from representative DENV-3 isolates (D3/InJ-16-82, D3/D95-0400, D3/Cuba21/02, UNC3001, D3/1339, each from genotype I, II, III, III, IV) were synthesized and introduced into the parent clone A and B fragments using unique Type IIS restriction enzymes. The resulted constructs were each named as Indonesia '82 (I), Thailand '95 (II), Cuba '02 (III),

Sri Lanka '89 (III), Puerto Rico '77 (IV), to indicate the isolates' location, year of isolation and genotype.

To generate A fragments with EDIII variants, synthesized EDIII constructs were amplified with the DenBglI #1+ (5'-gaagccaagagggcgcaatgcataccgcactg-3') and the Den2kb- (nnnnncgtctcgcctcaatattgacaggctcc) primers. These amplicons were digested with BglI and ligated to another amplicon which had been made using Den 985+ (5'-nnnnnnacccagaaggtggtcattttcatactac-3') and DenBglI#2- (5'-gcgccctcttggcttccaaggacgactacttcttg-3') and had been similarly digested. The ligated products were gel isolated and cloned into the pCR-XL TOPO cloning vector. Consensus clones were digested with BstEII and BsmBI and ligated into the parent A plasmid which had also been digested with BstEII and BsmBI. Variants at position 383 were generated either using parent B plasmid (N383) or Thailand '95 B plasmid (K383). The B plasmid sequences were otherwise identical.

Recombinant Virus Recovery. The virus clones were recovered following a protocol that was recently described by Messer *et al.* (Messer, Yount et al. 2012). Briefly, each plasmid was transformed, propagated, cloned to *E. coli* and expanded in LB media. Plasmid purified (Qiagen Mini-Spin Kit) and digested as follows according to manufacturer's instructions. Fragments were gel-isolated (Qiagen Gel Extraction Kit) on 0.8% agarose gel, mixed in equivalent copy number and ligated with T4 ligase (NEB) overnight at 4°C. Full-length transcripts of DENV-3 cDNA constructs were generated in vitro and mixed with Vero cells trypsinized and resuspended in RNAse free PBS in an electroporation cuvette. After electroporation, the Vero cells were then incubated at 37°C for 4 days. Supernatant from transfected Vero cells were further passaged to Vero cells and these supernatants were harvested at day 7 as working virus stocks.

Virus Titration and Focus Reduction Neutralization Test (FRNT). The FRNT procedure is based on a method previously described (Durbin, Karron et al. 2001). Briefly, twenty-four well plates were seeded with Vero cells and grown to 80% confluence in incubator (37˚C, 5% CO_2 unless otherwise specified). For virus titration, virus stocks were serially diluted, added to wells and after 1 hr incubation at 37˚C overlaid with 0.8% methylcellulose in Opti-MEM (Gibco) supplemented with 2% FBS, antibiotic mix (Gibco) and non-essential amino acids (Gibco). Following incubation for 5 days, the overlay was removed, the wells washed twice with PBS and cells fixed in 80% methanol/PBS. Fixed monolayers were either stored at -80˚C or developed for foci visualization immediately. Briefly, wells were blocked for 10 minutes with 5% non-fat milk in 1x PBS, followed by 1 hr incubation with anti-flavivirus MAb 4G2 diluted in blocking buffer at 37˚C, then washed, incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse Ab (Sigma) dilutions, washed again and foci developed by the addition of TrueBlue HRP substrate (KPL). Foci were counted on a light box and viral titers calculated by standard methods. For FRNT, MAbs were serially diluted, mixed with approximately 600 focus forming units (ffu)/ml of virus to a final volume of 400 ul, The virus-MAb mixes were incubated for 1 hr and added in triplicate (100 ul volumes) to 24-well plates and incubated and developed as described above.

Monoclonal Abs. Purified MAbs 8A1 (IgG1), 7.5 mg/ml and 14A4 (IgG1), 9.6 mg/ml were kindly provided by Robert Putnak (Walter Reed Army Institute of Research, MD) (Matsui, Gromowski et al. 2010; Wahala, Donaldson et al. 2010). Hybridoma cells producing MAb 1H9 (IgM) were kindly provided by John Aaskov (Queensland University of Technology, Australia) (Serafin and Aaskov 2001).

Software and statistics. FRNT counts were entered into GraphPad Prism (Version 5.00 for OSX, GraphPad Software, San Diego California USA, www.graphpad.com).

$FRNT_{50}$ values were calculated by sigmoid dose-response curve fitting with upper and lower limits of 100 and 0 respectively. All error bars show 95% confidence intervals unless otherwise specified. Mean log values were compared by one-way ANOVA followed by Tukey HSD multiple comparison test with significance level alpha (P) set at <0.05. EDIII structure figure was made using The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.(http://www.pymol.org). Alignment of the DENV-3 genotype DIII sequences was prepared using ALINE (Bond and Schuttelkopf 2009).

ELISA. High binding 96-well ELISA plates were coated with 300 ng human monoclonal antibody 3H4 (which binds E protein, but not EDIII) per well in 0.1 M carbonate buffer. Plates were incubated at 4˚C overnight, washed three times (All washes were performed three times with Tris-buffered saline containing 0.2% Tween-20), then blocked with dilution buffer on a shaker at 37˚C for 1hr. Antigen was captured by adding virus in Tris-buffered saline containing 0.05% Tween-20 and 3% Normal Goat Serum to wells and incubating on a shaker at 37˚C for 1 hr. Unbound antigen was washed off, primary mouse monoclonal antibody (either 8A1 or 1H9) was serially diluted and added to wells, and plates were incubated on a shaker at 37˚C for 1 hr. Unbound primary antibody was washed off and 1:1000 alkaline phosphatase conjugated diluted human sera-adsorbed, goat anti-mouse secondary antibody added; and plate was incubated on a shaker at 37˚C for 1 hr. Unbound secondary antibody was washed off, pNPP substrate added, and absorbance readings were taken at 405 nm in Epoch Microplate Spectrophotometer (Biotek Instruments Inc. Winooski, VT).

Mutagenesis and production of rEDIII. Recombinant EDIII constructs were created as described by Wahala *et al.* (Wahala, Donaldson et al. 2010). E gene fragments encoding EDIII each genotype of viruses were engineered into pMAL c2X vector to generate recombinant EDIII-MBP fusion protein construct. Individual or combinations of mutations

at E301 and E383 were further introduced to the rEDIII-MBP construct using QuikChange multi kit (Stratagene, LaJolla, CA) according to manufacturer's instructions and were confirmed by sequencing. The recombinant EDIII-MBP constructs, including the mutants, were expressed in *E. coli* DH5a (Invitrogen) and purified using amylose resin affinity chromatography (NEB) as instructed by manufacturer.

Surface Plasmon Resonance (SPR). Kinetic information on the interaction between anti-dengue antibodies and DIII variants was obtained using a Biacore T100 instrument. Anti-mouse capture antibodies (GE Life Sciences) were immobilized at high density using amine coupling to a Series S CM5 chip. The anti-dengue antibodies or control (WNV E16) antibody were flowed over the surface of the chip and captured at 700 RUs (Response Units) or less. A two-fold dilution series of the DENV-DIII MBP fusion protein variants were injected over the chip at a flow rate of 65 ul/minute for 180 seconds and allowed to dissociate for 450 seconds. The flow cell was regenerated with 10 mM glycine pH 1.7 for 180 seconds, followed by a second pulse for 30 seconds, at 30ul/min. Data was processed using the Biacore Evaluation Software (Version 1.1.1) by double referencing (control antibody and buffer alone subtraction) and a global 1:1 Langmuir fit of the curves. Results were generated from at least three independent experiments.

5.3 Results

Mouse Monoclonal antibody (MAb) neutralization against four DENV-3 isogenic clones containing E protein from different genotypes

It was commonly believed that only inter-serotype variation matters in virus-antibody interactions. However, several recent studies challenged this assumption by showing that intra-serotype variation plays a role in this interaction too (Wong, Abd-Jamil et al. 2007; Brien, Austin et al. 2010; Wahala, Donaldson et al. 2010). To study this

problem we used a DENV-3 reverse genetic platform to capture naturally occurring variation in DENV-3 envelope glycoprotein. Because each variant E gene is expressed from an isogenic genome backbone, the recombinant viruses allow us to probe the impact of micro-variation on the antigenic properties of variant E glycoproteins. Focus Reduction Neutralization Test (FRNT) for the parent and isogenic clones with variant E genes were performed against a panel of mouse monoclonal antibodies (14A4, 1H9 and 8A1) and $FRNT_{50}$ values were calculated for each of the MAbs against each of the clones. Mouse MAb 14A4 is a DENV-3 specific IgG1 MAb that has been mapped to the A-strand of DENV-3 E domain III (Matsui, Gromowski et al. 2010; Wahala, Donaldson et al. 2010). 14A4 is known to neutralize all four genotypes within DENV-3 and would be expected to neutralize all of the isogenic clones (Wahala, Donaldson et al. 2010). As expected, 14A4 neutralized the entire recombinant DENV-3 panel at equivalent concentrations (Figure 5.2A). Calculated $FRNT_{50}$ values were not significantly different and ranged from 12 ug/ml for genotype I to 34 ug/ml for genotype III (Cuba ’02) (Figure 5.2A).

Mouse MAb 1H9 is an IgM antibody that is specific to DENV-3 and has been previously mapped to the lateral ridge of domain III of the E glycoprotein (Wahala, Donaldson et al. 2010). Serafin *et al*. demonstrated that mutation from lysine to asparagine in position E386 enabled DENV-3 prototype strain H87 to escape neutralization from 1H9 (Serafin and Aaskov 2001). Subsequently, Wahala *et al.* demonstrated by both binding and FRNT that clinical isolate genotype IV PR ’77, via a naturally occurring K to R mutation at 386, also allows for escape from neutralization by 1H9 (Wahala, Donaldson et al. 2010). 1H9 was tested against the parent and isogenic clones using FRNT. As expected, the genotype IV was not neutralized by 1H9 (Figure 5.2B), consistent with the wild type PR ’77 phenotype. In contrast, genotype I and

genotype II were neutralized at very low antibody concentrations - 0.046 ug/ml and 0.061 ug/ml respectively (Figure 5.2B). Genotype III clones Cuba ’02 and SL ’89 were also effectively neutralized by 1H9, but at antibody concentrations significantly greater - 1 log - than genotype I and II clones (P <0.01) (Figure 5.2B). 1H9 had essentially three neutralization phenotypes - an escape phenotype (IV), an intermediately sensitive phenotype (III), and a highly sensitive phenotype (I and II).

Mouse MAb 8A1 is a DENV-3 specific IgG1 MAb that binds EDIII, via lateral ridge residues 301, 302, 380 and 386, similar to that observed for MAb 1H9 (Wahala, Donaldson et al. 2010). The DENV-3 clones recapitulate genotypic variation at those residues. Purified 8A1 was tested for ability to neutralize parent and isogenic clones (Figure 5.2C and Table 5.1). Previous studies have demonstrated that MAb 8A1 binds EDIII from DENV-3 genotypes I, II and III but not genotype IV (Wahala, Donaldson et al. 2010). As predicted from these binding studies, the genotype IV clone was extremely resistant to neutralization, with a calculated $FRNT_{50}$ of 413 ug/ml, which was 2-3 logs greater than values recorded for the other isogenic recombinant viruses (P <0.01). $FRNT_{50}$s for genotype III recombinant viruses Cuba ’02 and SL ’89 were 13.5 ug/ml and 9.9 ug/ml, respectively, virtually identical and forty-fold less than genotype IV (Table 5.1). The remaining genotype I and II clones were again significantly more sensitive to neutralization by 8A1, with calculated $FRNT_{50}$s of 0.2 ug/ml and 0.37 ug/ml respectively, 800-fold more sensitive to neutralization than genotype IV (P <0.01). For MAb 8A1, as with MAb 1H9, a distinct pattern of neutralization was observed; genotype IV requires a high concentration of antibody for neutralization, genotype III requires intermediate concentration of antibody and finally genotype I and II are neutralized by low concentrations of antibody (Figure 5.2C).

Amino Acid Variations in EDIII lateral ridge

Wahala *et al.* previously demonstrated that 8A1 bound to DENV-3 genotypes I, II and III but not to IV. Moreover, Wahala *et al.* mapped loss of 8A1 binding to genotype IV to a K386R (lysine to arginine) mutation on the lateral ridge of DENV-3 genotype IV (Wahala, Donaldson et al. 2010). We analyzed EDIII sequence differences between DENV-3 genotypes I, II and III to explore why genotypes I and II were more sensitive to neutralization than genotype III, despite the antibody binding to all three genotypes. The EDIII sequence differences between the different DENV-3 genotypes are summarized in Figure 5.1B. The more sensitive genotype I and genotype II clones have two common non-conservative variations compared to the less-sensitive genotype III clones: T301L and N383K. These two sites are located in adjacent loops of the lateral ridge, with position 301 at the apex of the N-terminal linker and position 383 in the FG loop. Genotype I and genotype III (Cuba '02) viruses also each have variations at 303 and 329 in the lateral ridge (Figure 5.1A), but as these variations are not conserved within sensitive genotypes (I and II) or within genotype III they are not considered critical to the phenotype. We hypothesized that variations at one or both sites (301, 383) are responsible for the sensitivity difference of MAb 8A1 and our reverse genetics system provided us a chance to test this hypothesis and further study the mechanism of this differential neutralization at molecular level.

To test our hypothesis residues T301 and N383 were mutated from the parental DENV-3 genotype III background both individually and in combination to amino acids - T301L and N383R respectively, recapitulating the residues in the sensitive genotype I and II strains. FRNTs for the parent and mutant variants against 8A1 were done and $FRNT_{50}$ values calculated. We found that both mutations were required to increase sensitivity to neutralization (Figure 5.3). Single mutations led to partial increases in

neutralization sensitivity but only the double mutant was as sensitive to neutralization as the DENV-3 genotype I and II sensitive strains. This suggests that the two amino acids at 301 and 383 both confer sensitivity to MAb 8A1, with double mutations at these two positions conferring the greatest effect.

The Mutations Altered Binding Affinity

To further explore the mechanism of neutralization sensitivity by the individual and combined effect of 301 and 383 site mutations, we studied the binding affinity difference of mutant viruses to MAb 8A1. ELISA plates were coated with equal quantities of purified antigen from DENV-3 genotype II and III for binding studies with MAb 8A1. The binding curve indicated that genotype II has higher binding affinity to 8A1 than genotype III. The EC50 concentration for DENV-3 genotype III virus and genotype II virus are 0.098 ug/ml, and 0.050 ug/ml respectively. This EC50 difference is consistent with the observed $FRNT_{50}$ differences. We further studied the binding affinity of 8A1 to genotype II, genotype III and mutant virus variant using capture ELISA. After optimization, equal amount of virus was captured and 8A1 concentration at 0.2 ug/ml was chosen for comparison for its sensitivity of detecting affinity variation. As shown in Figure 5.4, the sensitive genotype II strain had 50% increase of OD value compared to non-sensitive genotype III in the capture ELISA and introduction of single mutation T301L can increase the OD value to the same level of genotype II, suggesting the mutation at position 301 fully restores binding affinity. Introducing N383K alone increased the OD value only slightly, indicating that N383K mutation failed to increase binding affinity. This is consistent with previous observation that mutation at this site did not cause change of 8A1 binding of EDIII recombinant protein (Wahala, Donaldson et al. 2010). In other words, our ELISA data demonstrated that site 301 determines the differential binding affinity between genotype II and genotype III.

8A1 affinity is determined by off-rate

We next employed surface plasmon resonance (SPR) to better understand how specific residues affect binding affinity and kinetics of 8A1 to the EDIII protein from each genotype. Recombinant EDIII proteins from genotypes I, II and III were engineered as fusion proteins with maltose binding protein (MBP), produced and purified as described and validated by Wahala *et al.* (Wahala, Donaldson et al. 2010). The recombinant EDIII proteins differ at two critical sites: position 301 and 383 (Table 5.1). The two EDIIIs from potently neutralized genotypes (I and II) display distinct binding curves relative to those of the poorly neutralized genotype III EDIII (Figure 5.5A-C). EDIII from genotype I and genotype II had a half-life of 60.5 seconds and 59.0 seconds respectively (Table 5.1). These values contrast with those of the genotype III EDIII-MBP, which had a half-life of 16.6 seconds (Table 5.1). The association rate clearly cannot explain the half-life difference or neutralization sensitivity variation. The dissociation rate data are consistent with the neutralization data and half-life data as slower dissociation results in the longer half-life of binding, and subsequently a higher 8A1 occupancy of the virus at any given moment, which ultimately leads to neutralization (Pierson, Xu et al. 2007). Generally, our data suggests that the discrepancies of 8A1 binding affinity for DENVs EDIIIs are primarily due to distinct dissociation rates.

EDIII Residues at Site 301 Alone Determines Dissociation Rate and Half-Life

Expanding our kinetic data characterizing 8A1 binding to EDIII, we sought to determine the role of specific residues involved in this interaction. Our neutralization experiments suggested that sites 301 and 383 both contribute to neutralization sensitivity. To study how each site contributes to the kinetics of 8A1 binding, especially dissociation rate, EDIII-MBP from the genotype III background was mutated to the

corresponding residues found in the genotype II virus (L301 or K383) and tested by SPR as above. Mutation of position 301 from a threonine to a leucine resulted in a half-life of 68.2 seconds (Figure 5.5D and Table 5.1), comparable to that of the wild-type genotype II EDIII. In contrast, mutation of position 383 from an asparagine to a lysine resulted in a decrease of only association rate and a half-life comparable to parental genotype III EDIII (11.9 seconds; Figure 5.5E and Table 5.1). These results suggest that the dissociation rate is determined by position 301 alone as T301 leads to short half-life (12-16 seconds) while L301 leads to long half-life (50-68 seconds).

5.4 Discussion

How natural strain variation within each DENV serotype influences virus-antibody interactions has important implications for rational vaccine design and virus evolution (Wong, Abd-Jamil et al. 2007; Brien, Austin et al. 2010; Wahala, Donaldson et al. 2010). We and others have recently reported genotype dependent variable neutralization within serotypes using both MAbs and human DENV-3 sera (Wahala, Donaldson et al. 2010; Messer, Yount et al. 2012). Despite the accumulated evidence, the detailed mechanism of genotype dependent variable neutralization remains unknown. To better understand this mechanism, we employed a DENV-3 reverse genetic clone system to capture the amino acid variation in E glycoprotein of all the four genotypes into the otherwise isogenic background of genotype III. The resulting isogenic viruses recapitulated the previously reported sensitivity differences of the DENV-3 genotypes to these MAbs (Wahala, Donaldson et al. 2010). Using these isogenic viruses, we mapped the mutations critical to MAb 8A1 neutralization to two sites - 301 and 383 - on the lateral ridge region of EDIII. We found that both mutations are needed to alter neutralization by

MAb 8A1. Study of antibody-antigen affinity of whole virus suggested the distinct neutralization sensitivity is mainly attributed to binding affinity differences between different residues at these two positions. Using SPR and recombinant EDIII protein, we further found that binding differences are determined mainly by the dissociation rate and mutation at site 301 alone affects the dissociation rate.

Our finding that only two residues are responsible for differential neutralization between DENV-3 genotypes I, II, III and IV sheds light on our understanding of how neutralization is modulated by viral genetic variations. Consistent with previous research, our ELISA data suggests that binding affinity differences cause neutralization sensitivity variation (Gromowski and Barrett 2007; Lisova, Hardy et al. 2007; Gromowski, Barrett et al. 2008; Gromowski, Roehrig et al. 2010). However, mutant T301L restores fully binding affinity but it restores only partially the neutralization sensitivity. This suggests that site 383 also contributes to 8A1 neutralization sensitivity via a mechanism other than binding affinity. The evidence is that mutant N383K increased neutralization sensitivity about 4 times, without increasing binding affinity (Figure 5.4) or half-life of binding (Table 5.1). It is even more interesting to find that after introducing mutation N383K into the mutant virus T301L to make it double mutant/genotype I or II, the neutralization sensitivity increased about 3-5 times from mutant T301L, comparable to the effect of introducing N383K into the parental genotype III (Table 5.1). We can find a similar pattern for mutation T301L as introducing this mutation to genotype III increased sensitivity for about 10 times and introducing this mutation into mutant N383K also increased sensitivity for about the same extent. This pattern strongly argues that both sites contribute to 8A1 neutralization sensitivity through different and independent mechanisms. We speculate that N383K could alter neutralization by affecting virus structural dynamics so that each 8A1 antibody binding may exert more constraints on

virus post-attachment steps such as conformational change for fusion, as MAb 8A1 neutralizes virus by blocking post-attachment steps (unpublished data). It has been shown that flavivirus dynamic equilibrium (breathing) can regulate antibody neutralization (Lok, Kostyuchenko et al. 2008; Dowd, Jost et al. 2011; Cockburn, Navarro Sanchez et al. 2012). It is also possible that the 383 mutation affects binding when the virion is in alternate conformation which can't be detected with current ELISA methods.

SPR has been proven a powerful tool in studying and viewing antigen-antibody interactions from a kinetic and structure perspective (Bedouelle, Belkadi et al. 2006). The correlation between the virus binding affinity data (Figure 5.4) and the half-life data (Table 5.1) suggests that 8A1 binding affinity is determined by off-rate. We further found that both residues 301 and 383 can affect association rate as mutation at either residue changed the association rate (Table 5.1). However, only residue 301, not residue 383, affects dissociation rate, and also half-life (Table 5.1). These data are consistent with previous mapping data of 8A1 showing that mutation of residue 383 did not affect 8A1 binding (Wahala, Donaldson et al. 2010).

The association rate of genotype II is aberrant and extremely low, consequently the resulted unexpectedly high KD value contradicts with both genotype II EC50 and 8A1 neutralization titer (Table 5.1). This anomaly could be either due to a) instability of this particular construct or b) non-specific interaction between the DIII and MBP fusion partner that occludes recognition of the 8A1 epitope. Another factor we must take into consideration is that there is essential difference between the affinity measured using EDIII-MBP fusion protein against immobilized 8A1 antibody (SPR) and the avidity measured using immobilized whole virus and 8A1 antibody (EC50). The two values may not necessarily corroborate each other. Thus, caution must be taken when using the protein-antibody SPR results to interpret virus binding and neutralization.

In sum, these data established a causal relationship between binding kinetics, binding affinity and neutralization. Site 301 determines dissociation rate to 8A1 between the genotypes, which dominates binding kinetics difference, and subsequently determines binding affinity variation, and contributes 2/3 of neutralization differences. The other 1/3 is attributed to mutation at site 383, through a neutralization mechanism different and independent from binding affinity variation. Structural investigation is underway to resolve this problem.

Our work demonstrates how two naturally occurring mutations altered binding kinetics, leading to change of affinity and then neutralization sensitivity. Understanding how single or multiple mutations alter the viral neutralization profile is critical for further understanding of how different virus strains elicit immunity after infection, and how potentially protective antibodies differentially interact with virus infection in a secondary infection with a homologous serotype. More importantly, for a vaccine to elicit immunity that fully neutralize all DENV-3 genotypes with minimal risk of ADE, studies like this one will help to understand which epitopes among the inter-genotype variable sites should be targeted. This knowledge is especially helpful considering the high diversity in the lateral ridge in DENV-3 and other serotypes. Our results identifying how mutations in this region lead to neutralization alteration through changing association and dissociation rates will help identify the most critical neutralization epitope of dengue virus. Future studies of antibodies with intra-serotype or intra-genotype neutralization variation employing this approach should define additional important neutralization epitopes and the mechanism of differential neutralization.

Table 5.1 : Kinetics of DENV-3 DIII-MPB variants with 8A1.

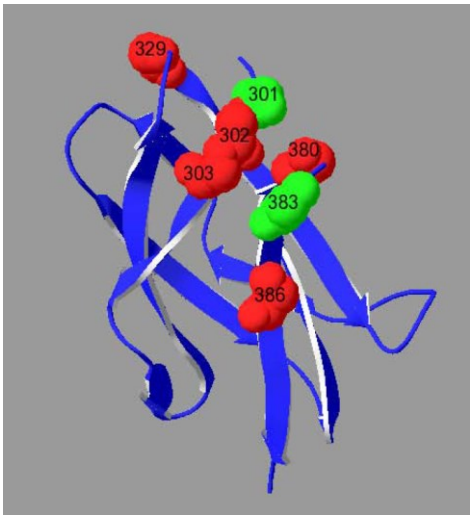
DIII Variant	301	383	ka ($10^5 M^{-1}s^{-1}$)	kd ($10^{-3}s^{-1}$)	*K*D (nM)	*t* ½ (sec)	$FRNT_{50}$ (ug/ml)
III	T	N	2.176	43.51	200.9	16.6	13.5*
I	L	K	1.124	11.51	103.2	60.5	0.20
II	L	K	0.268	12.62	470.9	59.0	0.37
T301L	L	N	0.6892	10.15	155.3	68.2	1.08
N383K	T	K	0.823	59.8	718.2	11.9	3.50
IV	S	K	NA	NA	NA	NA	413.1

* There are two genotype III virus clones: SL ’89 and Cuba ’02. $FRNT_{50}$ of SL ’89 (III) was indicated in this table and $FRNT_{50}$ of Cuba ’02 (III) is 9.9 ug/ml. 8A1 binding was not detected for genotype IV DIII-MBP at concentrations of 5000 nM.

Figures and Legends

Figure 5.1

A)



B)

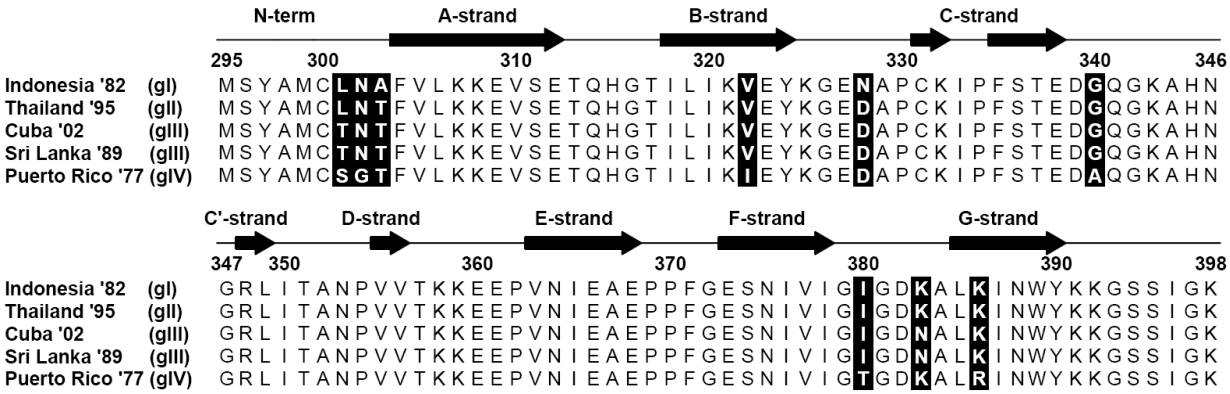
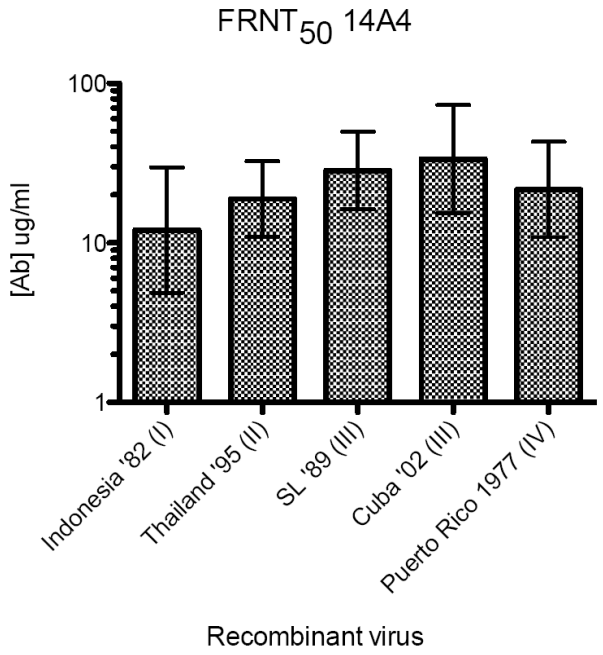


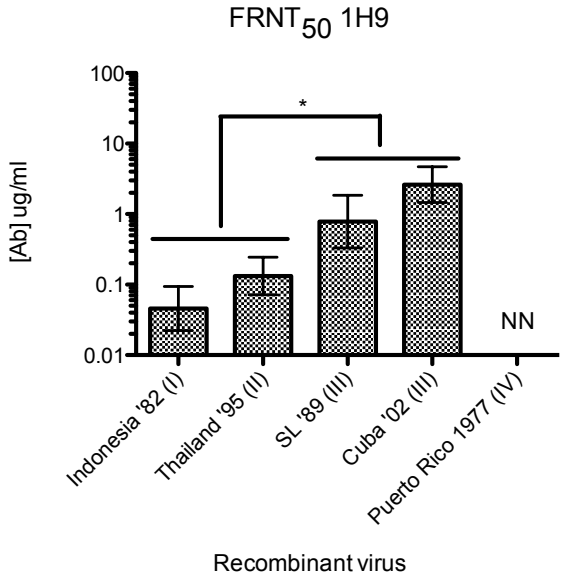
Figure 5.1: A) Cartoon of DENV3 EDIII. Lateral ridge EDIII variable sites for the recombinant clones are color labeled: 301 and 383 (red); 302, 303, 329, 380 and 386 (green). Secondary structure features are labeled. PDB ID: 3VTT. **B)** Alignment of EDIII amino acid sequences. Beta-strands are labeled as arrows and genotypic variation is highlighted according to Fig 1A.

Figure 5.2

A)


FRNT50 14A4
100
10
1
[Ab] ug/ml
Indonesia '82 (I)
Thailand '95 (II)
SL '89 (III)
Cuba '02 (III)
Puerto Rico 1977 (IV)
Recombinant virus

B)


FRNT50 1H9
100
10
1
0.1
0.01
[Ab] ug/ml
*
NN
Indonesia '82 (I)
Thailand '95 (II)
SL '89 (III)
Cuba '02 (III)
Puerto Rico 1977 (IV)
Recombinant virus

C)

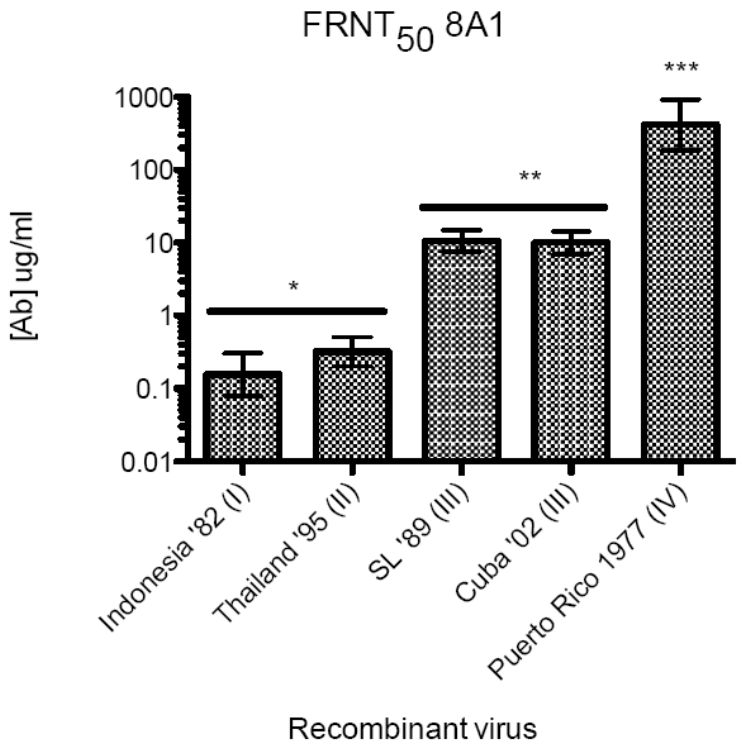


Figure 5.2: Calculated $FRNT_{50}$ values for mouse MAbs against the recombinant virus clones. Error bars show 95% confidence intervals. **A)** Calculated $FRNT_{50}$ values for 14A4 did not differ (P >0.05 ANOVA). **B)** Calculated $FRNT_{50}$ values for 1H9 against Indonesia '82 (I) and Thailand '95 (II) were significantly lower than SL '89 (III) and Cuba '02 (III) (P<0.05 ANOVA followed by Tukey's HSD). NN = not neutralized. **C)** Calculated $FRNT_{50}$ values for 8A1 against Indonesia '82 (I) and Thailand '95(II) were significantly lower than SL '89 (III) and Cuba '02 (III) and Puerto Rico 1977 $FRNT_{50}$ was significantly greater than against the other clones (P <0.05, ANOVA followed by Tukey's HSD).

Figure 5.3

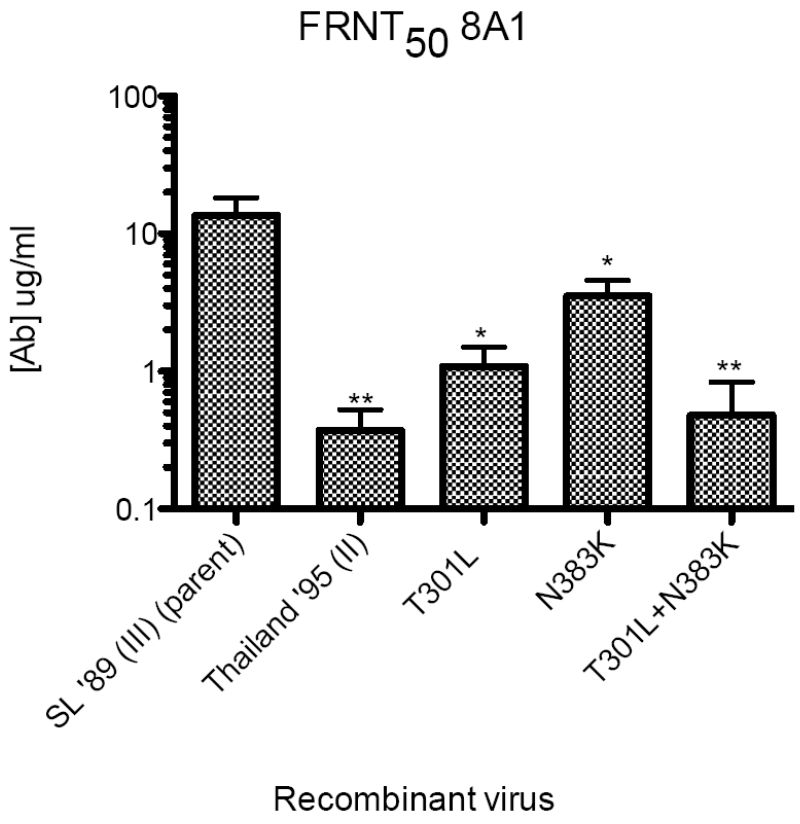


Figure 5.3: Calculated $FRNT_{50}$ values for MAb 8A1 against the parent SL'89 clone, Thailand '95 clone and point mutant clones T301L, N383K and the combined mutant T301L+N383K clone. Error bars show 95% confidence intervals. Calculated $FRNT_{50}$ for 8A1 against Thailand '95 (II), mutant clones T301L, N383K and combined mutant T301L+N383K clone were significantly lower than parent SL '89 clone (III) ($P<0.05$ ANOVA followed by Tukey's HSD). Calculated $FRNT_{50}$ against Thailand '95 clone (II) do not differ significantly from combined mutant T301L + N383K clone ($P>0.05$ ANOVA followed by Tukey's HSD).

Figure 5.4

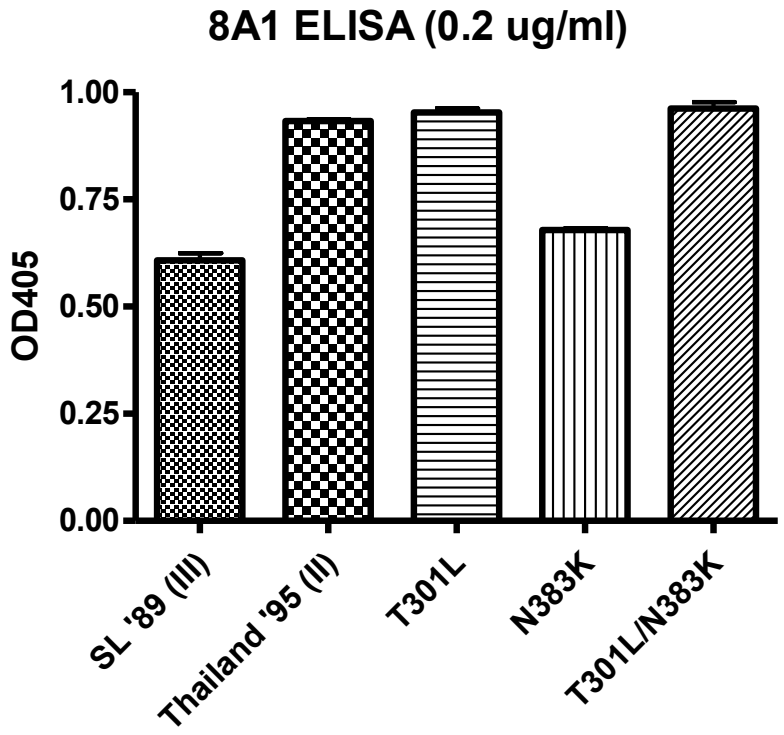


Figure 5.4: Results of ELISA using mouse MAb 8A1 (0.2 ug/ml) against captured whole parent virus SL '89, recombinant clone Thailand '95, and mutant clones T301L, N383K and T301L+N383K. Error bars show 95% confidence intervals. Whole virus was captured with human MAb 1M19, a cross-reactive EDIII binding antibody. The virus captured were quantified to be equal amount using 12C1 (cross-reactive EDIII binding mouse MAb) as control.

Figure 5.5

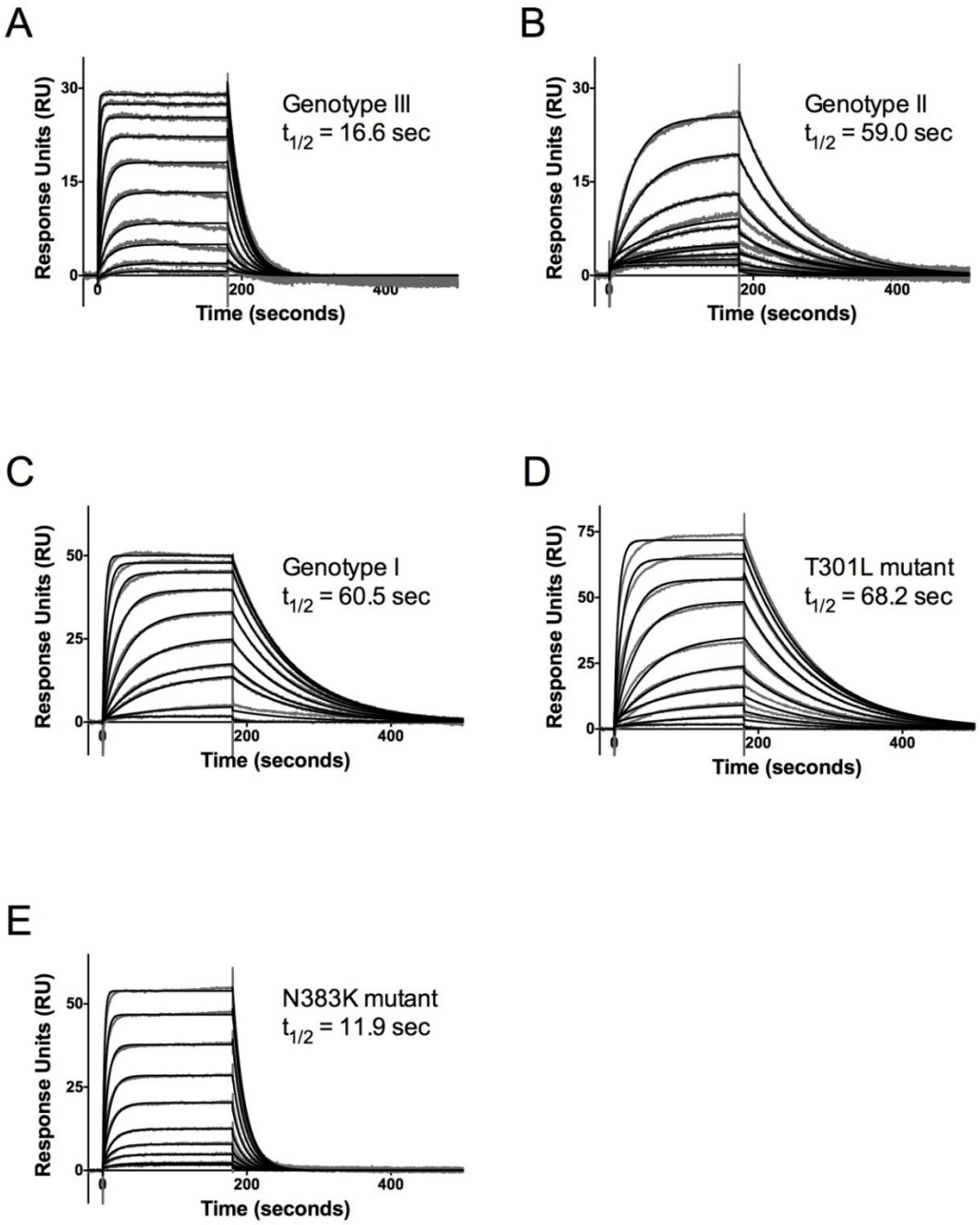


Figure 5.5: Kinetic analysis of 8A1 interaction with DENV-3 EDIII-MBP genotypes and mutants. MAb 8A1 was captured and recombinant EDIII-MBP proteins of genotype I, II, III or mutants were flowed over at two fold serial diluted concentrations to measure the kinetic parameters of the interaction. SPR sensorgrams and traces are presented as MAb 8A1 interacting with A) genotype III, B) genotype II, C) genotype I, D) mutant T301L, E) mutant N383K.

5.5 Reference

Araújo, J., R. Nogueira, et al. (2009). "Phylogeography and evolutionary history of dengue virus type 3." Infect Genet Evol **9**(4).

Bedouelle, H., L. Belkadi, et al. (2006). "Diversity and junction residues as hotspots of binding energy in an antibody neutralizing the dengue virus." FEBS J **273**(1): 34-46.

Bond, C. S. and A. W. Schuttelkopf (2009). "ALINE: a WYSIWYG protein-sequence alignment editor for publication-quality alignments." Acta Crystallogr D Biol Crystallogr **65**(Pt 5): 510-512.

Brien, J. D., S. K. Austin, et al. (2010). "Genotype-Specific Neutralization and Protection by Antibodies against Dengue Virus Type 3." Journal of Virology **84**(20): 10630-10643.

Cockburn, J. J., M. E. Navarro Sanchez, et al. (2012). "Mechanism of dengue virus broad cross-neutralization by a monoclonal antibody." Structure **20**(2): 303-314.

Cockburn, J. J., M. E. Navarro Sanchez, et al. (2012). "Structural insights into the neutralization mechanism of a higher primate antibody against dengue virus." EMBO J **31**(3): 767-779.

Crill, W. and J. Roehrig (2001). "Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells." **75**: 7769-7773.

de Mora, D., L. D. Andrea, et al. (2009). "Evidence of diversification of dengue virus type 3 genotype III in the South American region." Archives of Virology **154**(4): 699-707.

Dowd, K. A., C. A. Jost, et al. (2011). "A dynamic landscape for antibody binding modulates antibody-mediated neutralization of West Nile virus." Plos Pathogens **7**(6): e1002111.

Durbin, A. P., R. A. Karron, et al. (2001). "Attenuation and immunogenicity in humans of a live dengue virus type-4 vaccine candidate with a 30 nucleotide deletion in its 3'-untranslated region." American Journal of Tropical Medicine and Hygiene **65**(5): 405-413.

Gromowski, G. and A. Barrett (2007). "Characterization of an antigenic site that contains a dominant, type-specific neutralization determinant on the envelope protein domain III (ED3) of dengue 2 virus." **366**: 349-360.

Gromowski, G. D., N. D. Barrett, et al. (2008). "Characterization of dengue virus complex-specific neutralizing epitopes on envelope protein domain III of dengue 2 virus." Journal of Virology **82**(17): 8828-8837.

Gromowski, G. D., J. T. Roehrig, et al. (2010). "Mutations of an antibody binding energy hot spot on domain III of the dengue 2 envelope glycoprotein exploited for neutralization escape." Virology **407**(2): 237-246.

Halstead, S. B. (1988). "Pathogenesis of dengue: challenges to molecular biology." Science **239**(4839): 476-481.

Halstead, S. B. and E. J. O'Rourke (1977). "Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody." J Exp Med **146**(1): 201-217.

Jiang, T., X. D. Yu, et al. (2012). "Co-circulation of two genotypes of Dengue virus serotype 3 in Guangzhou, China, 2009." Virology Journal **9**(1): 125.

Kukreti, H., V. Mirtal, et al. (2010). "Continued Persistence of a Single Genotype of Dengue Virus Type-3 (DENV-3) in Delhi, India Since its Re-emergence Over the Last Decade." Journal of Microbiology Immunology and Infection **43**(1): 53-61.

Lanciotti, R., J. Lewis, et al. (1994). "Molecular evolution and epidemiology of dengue-3 viruses." Journal of General Virology **75**.

Lisova, O., F. Hardy, et al. (2007). "Mapping to completeness and transplantation of a group-specific, discontinuous, neutralizing epitope in the envelope protein of dengue virus." Journal of General Virology **88**(Pt 9): 2387-2397.

Lok, S. M., V. Kostyuchenko, et al. (2008). "Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins." Nat Struct Mol Biol **15**(3): 312-317.

Matsui, K., G. D. Gromowski, et al. (2010). "Characterization of a dengue type-specific epitope on dengue 3 virus envelope protein domain III." Journal of General Virology **91**(Pt 9): 2249-2253.

Messer, W. B., D. J. Gubler, et al. (2003). "Emergence and global spread of a dengue serotype 3, subtype III virus." Emerging Infectious Diseases **9**(7): 800-809.

Messer, W. B., B. Yount, et al. (2012). "Development and characterization of a reverse genetic system for studying dengue virus serotype 3 strain variation and neutralization." PLoS Negl Trop Dis **6**(2): e1486.

Modis, Y., S. Ogata, et al. (2003). "A ligand-binding pocket in the dengue virus envelope glycoprotein." **100**: 6986-6991.

Modis, Y., S. Ogata, et al. (2005). "Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein." Journal of Virology **79**(2): 1223-1231.

Nogueira, M. B., V. Stella, et al. (2008). "Evidence for the co-circulation of dengue virus type 3 genotypes III and V in the Northern region of Brazil during the 2002-2004 epidemics." Mem Inst Oswaldo Cruz **103**(5): 483-488.

Pierson, T. C. and M. S. Diamond (2008). "Molecular mechanisms of antibody-mediated neutralisation of flavivirus infection." Expert Rev Mol Med **10**: e12.

Pierson, T. C., Q. Xu, et al. (2007). "The stoichiometry of antibody-mediated neutralization and enhancement of west nile virus infection." Cell Host & Microbe **1**(2): 135-145.

Pitcher, T. J., G. D. Gromowski, et al. (2012). "Conservation of the DENV-2 type-specific and DEN complex-reactive antigenic sites among DENV-2 genotypes." Virology **422**(2): 386-392.

Ramirez, A., A. Fajardo, et al. (2010). "Evolution of Dengue Virus Type 3 Genotype III in Venezuela: Diversification, Rates and Population Dynamics." Virology Journal **7**.

Rey, F., F. Heinz, et al. (1995). "The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution." **375**: 291-298.

Schmitz, J., J. Roehrig, et al. (2011). "Next generation dengue vaccines: a review of candidates in preclinical development." Vaccine **29**(42): 7276-7284.

Serafin, I. L. and J. G. Aaskov (2001). "Identification of epitopes on the envelope (E) protein of dengue 2 and dengue 3 viruses using monoclonal antibodies." Archives of Virology **146**(12): 2469-2479.

Shepard, D. S., J. A. Suaya, et al. (2004). "Cost-effectiveness of a pediatric dengue vaccine." Vaccine **22**(9-10): 1275-1280.

Shrestha, B., J. Brien, et al. (2010). "The development of therapeutic antibodies that neutralize homologous and heterologous genotypes of dengue virus type 1." **6**: e1000823.

Sukupolvi-Petty, S., S. Austin, et al. (2010). "Structure and function analysis of therapeutic monoclonal antibodies against dengue virus type 2." **84**: 9227-9239.

Wahala, W., E. Donaldson, et al. (2010). "Natural strain variation and antibody neutralization of dengue serotype 3 viruses." **6**: e1000821.

WHO (2009). "Dengue and dengue haemorrhagic fever." WHO Fact Sheets **117**.

Wong, S. S., J. Abd-Jamil, et al. (2007). "Antibody neutralization and viral virulence in recurring dengue virus type 2 outbreaks." Viral Immunology **20**(3): 359-367.

Zhang, Y., W. Zhang, et al. (2004). "Conformational changes of the flavivirus E glycoprotein." **12**: 1607-1618.

CHAPTER 6

DENGUE VIRUS MATURATION MODULATES SENSITIVITY TO ANTIBODY MEDIATED NEUTRALIZATION

6.1 Introduction

Dengue is a mosquito borne re-emerging virus that affects 2.5 billion people worldwide (WHO 2009). There are four DENV serotypes (DENV1-4) co-circulating in tropical and subtropical areas (Guzman, Kouri et al. 2000). Most symptomatic dengue infections result in mild dengue fever (DF) with flu like symptoms. Some people exposed to DENVs develop a severe disease known as Dengue Hemoharrgic Fever or Dengue Shock Syndrome (DHF/DSS). The risk of severe disease is greater in people exposed to a second infection with a different serotype from the original infection (Sangkawibha, Rojanasuphot et al. 1984). Severe plasma leakage and circulatory collapse are the hallmarks of DHF/DSS and these symptoms may put patients at risk of death without timely and proper supportive treatment. The number of reported severe DENV cases has been increasing at an alarming rate in the past several decades while an effective vaccine is still not available (WHO 2009). The goal of the current study is to explore how maturation state of DENVs influences the ability of antibodies (Abs) to neutralize the virus.

DENV belongs to family *Flaviviridae* genus *flavivirus*. The virus has a single positive genome of 11kb encoding 3 structural proteins [capsid (C), pre-Membrane (prM) and envelope (E)], and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B,

NS5). The mature DENV particle is about 50 nm in diameter, and its envelope is formed by an icosahedral scaffold of E protein and membrane (M) protein. A crystal structure of a soluble form of the extracellular region of E glycoprotein reveals that it is composed of three domains (I, II, and III) each connected by flexible hinges (Modis, Ogata et al. 2005).

DENV assembles on the endoplasmic reticulum (ER) and buds into ER lumen as immature virions. Sixty trimeric spikes composed of prM and E heterodimers form the rough surface of the immature virion. During secretion of the virion from the cell, a furin-like protease in the Golgi apparatus cleaves prM protein to separate the pr peptide from M protein. The cleavage of prM leads to dissociation of the trimeric spike to form an E protein dimer that lies flat on the viral surface of a smooth mature virion (Li, Lok et al. 2008; Yu, Zhang et al. 2008). Incomplete cleavage may result in partially mature DENV particles being released from infected cells. DENVs grown in standard laboratory cell lines mainly consist of partially mature particles containing considerable proportion of uncleaved prM protein (Dejnirattisai, Jumnainsong et al. 2010; Junjhon, Edwards et al. 2010). Consequently, most studies of antibody-mediated neutralization of DENVs have been conducted using virus stocks containing heterogeneous mixtures of immature and partially mature particles.

Human monocyte-derived cells are the main target of DENV infection in people. The goal of this study was to compare the maturation state and neutralization phenotypes of DENV particles produced by human monocytic cells or other non-human cell lines commonly used to propagate DENVs in laboratories. We report here that the maturation state of DENVs varies depending on the cells used to grow the virus. We also report that the ability of some Abs to neutralize DENV was strongly influenced by maturation state, whereas other Abs neutralized DENVs irrespective of maturation state.

6.2 Methods and Materials

Cells. Vero cells (American Type Culture Collection, CCL-81) were maintained in Dulbecco's Modified Eagle Medium (DMEM). A cell line designated U937 DC-SIGN (9) was maintained in RPMI-1640 (Gibco) supplemented with 50 mM beta mercaptoethanol. This human monocyte lymphoma cell line was derived from U937 cells but also expresses Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin or DC-SIGN, also known as CD209 (Cluster of Differentiation 209). Mosquito *Aedes albopictus* C6/36 cells were maintained in Minimum Essential Medium (MEM; Gibco) at 28°C. All media used also were supplemented with 5% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.1 mM non-essential amino acids (Gibco) and 2 mM glutamine, and all cells were incubated in the presence of 5% CO_2 at 37°C, unless otherwise specified. The 5% FBS was reduced to 2% to make infection medium for each cell line.

Serum and MAbs. Murine MAbs 9F16 and 5C36 were purchased from US Biological and MAB 8A1 was kindly provided by Dr. Robert Putnak, Walter Reed Army Research Institute, MD. The human MAbs used in the current study (DVC3.7, DVC25.5, DVC10.16, 2D22, 2A10 and 1M19 have been described previously (de Alwis, Beltramello et al. 2011; de Alwis, Smith et al. 2012). Polyclonal DENV immune sera were collected from people living in North Carolina who had acquired DENV previously during foreign travel. The protocol for collecting blood was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill.

C6/36 cell derived virus. C6/36 cells at 80% confluency were inoculated at MOI of 0.05 for 2 hrs followed by two washings and addition of 25 mL of infection medium. After 6

days of incubation, the supernatants were harvested, clarified by 30 minutes ultracentrifugation at 18,000 x g, aliquoted into cryovials and stored at -80°C as working virus stocks. Virus used were DENV2 (S16803) and DENV3 (CH 53498) low passage.

U937-DC-SIGN cell derived virus. Monolayer cultures of U937-DC-SIGN cells were inoculated using C6/36 insect cell line derived virus at MOI of 0.1 in infection medium for two hrs, followed by washing to remove all unbound C6/36 cell line derived virus and replenishing with 25 mL fresh infection medium. After 36 hrs of incubation, the medium was harvested, and clarified by 30 minutes of ultracentrifugation at 10,000 x g. The virus-containing medium was concentrated further ~10-fold using 100 kDa Amicon Ultra Centrifugal Filters (Millipore, Billerica, MA) and frozen at -80°C as working virus stock.

Immature virus. C6/36 insect cell culture monolayers at 80% confluence were inoculated using C6/36 insect cell line derived virus at MOI of 0.2 for 2 hrs, followed by washing to remove all C6/36 cell line derived virus and replenishing with 25 mL fresh infection medium. Twelve hrs post infection, NH_4Cl was added to the medium at a final concentration of 20mM. The infected cell were incubated for a further 12 hrs before the supernatant was harvested and the virus concentrated by ultra-centrifugation at 100,000 x g for 3 hrs.

Virus titration and Focus Reduction Neutralization Test (FRNT). The FRNT procedure is based on a method previously described by Messer *et al*. (Messer, Yount et al. 2012). Briefly, twenty-four well plates were seeded with Vero cells to grow till 80% confluence in incubator (37°C, 5% CO_2). For virus titration, virus stocks were diluted serially to 100 µL, added to wells and incubated for 1 hr before adding overlay. Plates were incubated for 3 days, washed with PBS, then fixed in 80% methanol, blocked for 10 minutes with 5% instant milk in PBS for 1 hr. Anti-flavivirus MAb 4G2 (for DENV2) or

MAb 8A1 (for DENV3) diluted in blocking buffer at 37°C, was added to each well and incubated at 37°C for 1 hr. After washing, the plates were incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse Ab (Sigma) dilutions, washed again, and foci developed by the addition of TrueBlue HRP substrate (Kirkegaard & Perry Laboratories). Foci were counted on a light box by visual inspection and viral titers calculated by standard methods. For FRNT, MAbs were diluted serially, mixed with approximately 30 focus forming units (ffu) of virus to a final volume of 200 µL, incubated for 1 hr and added to 24 wells plates and processed as above. FRNT ffu counts were entered into Graphpad Prism (Version 5.00 for OSX, GraphPad Software, San Diego California USA, www.graphpad.com). $FRNT_{50}$ values were calculated by sigmoid dose-response curve fitting with upper and lower limits of 100 and 0 respectively. All error bars show 95% confidence intervals unless otherwise specified. Mean $FRNT_{50}$ values were compared by one-way ANOVA followed by the Tukey HSD multiple comparison test, with significance level alpha (P) set at <0.05.

Function Assay. For pre-attachment neutralization, viruses were incubated with antibodies at 4˚C for 1 hr before being added to Vero cells and incubated at 4˚C for 1 hr. After washing off unbound virus and antibodies with chilled media, the cell culture was moved to 37˚C incubator. For post-attachment neutralization, Vero cells were incubated with viruses for 1 hr at 4˚C for virus absorption, followed by two washings to wash off unbound viruses, then antibodies were added to cell culture and incubated at 4˚C for 1 hr, followed by washing and moving to 37˚C incubator. All the operations are done on ice before moving to 37˚C incubator. The rest of the experiment was the same as described in the FRNT assay.

Furin Inhibition Experiment. Furin inhibitor I, also known as peptidyl chloromethylketone (Decanoyl-RVKR-CMK) is purchased from Calbiochem (Millipore,

Billerica, MA). Furin inhibitor I was dissolved in DMSO at 1mM concentration. For each infection assay or FRNT assay, the furin inhibitor was added at concentration of 25 μM.

Western blot. Samples were mixed with loading dye without DTT, heated at 95°C for 5 minutes, then loaded into a 12% acrylamide gel, and run at 70V until markers fully separated. Proteins in the gel then were transferred onto a nitrocellulose membrane (Amersham) in a Semi-dry Blotting Unit (Fisher Scientific, FB-SOB-2020) at 0.30 A for 50 minutes. The membrane then was blocked with 5% skim milk for 1 hr, and stained with prM MAbs or MAb 4G2 in an orbital shaker at room temperature for 2 hrs, followed by 3 washings with 0.2% Tween-TBS buffer with 5 minutes intervals. Goat-anti-mouse (or -human) HRP conjugated secondary antibody (Sigma) was diluted 1:1,000 and incubated with membrane for 1 hr before three washings. Finally, the membrane was developed with ECL Prime Western Blotting Detection Reagent (GE Healthcare). The film (Hyblot CL, Denville Scientific) were exposed to the membrane in a dark room and then developed using a Konica SRX-101 film processer (Konica Minolta, Tokyo, Japan).

Capture ELISA. High binding 96-well ELISA plates were coated with 400 ng of human MAb 1M19 per well in 0.1 M carbonate buffer. Plates were incubated at 4°C overnight, washed three times (All washes were performed three times with Tris-buffered saline containing 0.2% Tween-20), then blocked with blocking buffer (3% goat serum in Tris-buffered saline containing 0.05% Tween-20) on a shaker at 37°C for 1 hr. Antigen was captured by adding virus in blocking buffer to wells and incubating on a shaker at 37°C for 1 hr. Unbound antigen was washed off, primary mouse MAbs were diluted to desired concentrations and added to wells, and plates were incubated on a shaker at 37°C for 1 hr. Unbound primary antibody was washed off and 1:1,000 diluted alkaline phosphatase conjugated human sera-adsorbed, goat anti-mouse secondary antibody (Sigma) added; plate was incubated on a shaker at 37°C for 1 hr. Unbound secondary antibody was

washed off, pNPP substrate added, and absorbance readings were taken at 405 nm in an Epoch Microplate Spectrophotometer (Biotek Instruments Inc. Winooski, VT). In cases of testing human primary MAb, murine MAb 5C36 or 8A1 (EDIII binding type-specific) was used as the coating antibody and alkaline phosphatase-conjugated diluted mouse sera-adsorbed, goat anti-human secondary antibody (Sigma) was used as secondary antibody.

MAb Epitope Mapping. A DENV-4 Env expression construct (341750 strain) was subjected to high-throughput alanine scanning mutagenesis to generate a comprehensive mutation library. Each residue within prM/E was mutated to alanine, while alanine codons were mutated to serine. In total, 660 DENV-4 mutants were generated (>97% coverage), sequence confirmed, and arrayed into 384-well plates. Each Env mutant was transfected into HEK-293T cells and allowed to express for 22 hrs. Cells were fixed in 4% (vol/vol) paraformaldehyde (Electron Microscopy Sciences) and permeabilized with 0.1% (wt/vol) saponin (Sigma-Aldrich) in PBS plus calcium and magnesium (PBS++). Cells were stained with purified 2A10 antibody (0.8 ug/ml) diluted in 10% normal goat serum (NGS) (Sigma)/0.1% saponin, pH9. Antibody binding was detected using 3.75 µg/ml AlexaFluor488-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) in 10% NGS/0.1% saponin. Cells were washed 3 times with PBS++/0.1% saponin followed by 2 washes in PBS. Mean cellular fluorescence was detected using the Intellicyt high throughput flow cytometer (HTFC, Intellicyt). Antibody reactivity against each mutant Env clone was calculated relative to wild-type Env protein reactivity by subtracting the signal from mock-transfected controls and normalizing to the signal from wild-type Env-transfected controls. Antibodies that bind the fusion loop (1C3, 1I16, 1K16, 1L6, 1N5, 1N8, 3B4, 3D18, 4E8, and 5E6, unpublished data) or domain II (1A15, 1D6, 1D8, and 5C8, unpublished data) were used for reactivity

comparisons. Critical amino acids that affected antibody binding were visualized on the DENV2 Env crystal structure (PDB ID #1OAN) (Modis, Ogata et al. 2003).

6.3 Results

The human monocytic cell line U937-DC-SIGN produced mostly mature DENV

In humans, DENVs infect cells derived from the myeloid lineage such as monocytes and dendritic cells. However, most cell culture studies of DENVs have been conducted using viruses grown in insect cells or non-human, mammalian cells of epithelial origin. We began by comparing the maturation of state of DENV2 produced by a human monocytic cell line expressing a DENV attachment factor (U937-DC-SIGN) and non-human cell lines (mosquito C6/36 cells and monkey kidney epithelial Vero cells) commonly used to propagate DENV in laboratories. As a control, we obtained virus from C6/36 cells maintained in the presence of NH_4Cl, which raises intracellular pH and blocks furin processing of prM protein to the cleaved state. The maturation state of the viruses was determined using an antigen capture ELISA to measure the ratio of prM to E protein in the virus preparation. As shown in Figure 6.1A, the NH_4Cl-treated immature virus control had a prM/E ratio close to 1. Vero cell-derived viruses were similar to immature viruses in prM level. In comparison, C6/36 insect cell derived viruses had a prM/E ratio of 0.4, indicating that about half of the prM on these particles remained uncleaved. Viruses produced in U937-DC-SIGN cells were mostly mature, as indicated by a prM/E ratio of 0.15. This finding of variation in maturation state that depended on the type of infected cell was confirmed by studies using western blot (Figure 6.1B). Similar to the findings with DENV2, human monocytic cell line derived DENV3 particles also exhibited a predominantly mature phenotype (Figure6. 1C).

Maturation state of DENV modulates neutralization sensitivity to some MAbs

Eight human and mouse MAbs of differing DENV serotype-specificity and neutralization potency were chosen to test whether variation in virion particle maturation influenced the ability of Abs to neutralize these viruses (Table 1). U937-DC-SIGN cell line derived mature viruses and C6/36 insect cell line derived partially mature viruses were chosen for comparison as they represent the main cell types that are relevant to the DENV transmission cycle involving human and mosquito hosts. The sensitivity of these different suspensions of virus to antibody-mediated neutralization was measured using FRNT (Durbin, Karron et al. 2001) (Figure 6.2 and Table 6.1). Cross-reactive MAbs with weak to moderate neutralizing potency (MAbs 4G2, 2A10 and DVC10.16) demonstrated decreased neutralization potency against mature virus, obtained from the U937-DC-SIGN cell line (Figure 6.2A and Table 6.1) compared to the partially mature virus, obtained from the C6/36 insect cell line. Five type-specific MAbs with moderate to strong neutralizing potency neutralized mature or partially mature viruses equally well (MAbs 9F16, 5C36 and 2D22) or neutralized mature viruses better than partially immature viruses (MAbs DVC3.7 and DVC25.5) (Figures 6.2B, 2C and Table 6.1). In summary, these results demonstrated that the maturation state of DENV virion particles modulates the sensitivity to neutralization mediated by MAbs.

Epitopes recognized by maturation state sensitive MAbs

As depicted in Table 6.1, with the exception of 2A10, all the MAbs used in the current study have been previously mapped to specific residues or epitopes on E protein. Previous studies had mapped the 2A10 epitope to E protein domains I or II (Smith, Zhou et al. 2012). We used alanine scanning mutagenesis to further map the binding site of 2A10. Alanine mutations at residues C74 and C105 and an alanine to serine mutation at

position 245 greatly decreased binding of 2A10 to E protein (Figure 6.3). These results suggest that 2A10 binds an epitope at or next to the fusion loop on EDII (Figure 6.3B). Next, we compared the maturation-dependent neutralization pattern and epitope location of each MAb to test if neutralization patterns were linked to Ab binding to specific regions or epitopes on E protein. MAbs that neutralized partially mature virions better than mature virions were serotype-cross reactive and bound to epitopes at the fusion loop (4G2 and 2A10) of EDII or A strand of EDIII (DVC10.16). MAbs that neutralized partially and fully mature virions equally well or neutralized mature virions better than partially mature virions were serotype-specific and bound to the lateral ridge of EDIII (DVC3.7, DVC25.5, 5C36 and 9F16) or the hinge region between EDI and II (2D22) (Table 6.1).

Binding of MAbs to mature or immature virions

We next compared the ability of MAbs to bind to mature or immature virions to test if the ability to bind to viruses of different maturation state correlated with the observed variation in potency of neutralization. An antigen capture ELISA was used to compare the ability of each MAb to bind to mature virions, derived from U937-DC-SIGN cells, or immature virions, obtained from NH_4Cl-treated C6/36 insect cell line supernatants. The MAb's binding preference is indicated as a mature to immature (M/I) ratio, which was calculated using the optical density (OD) in the ELISA for MAb binding to the mature virus suspension divided by the OD for MAb binding to the immature virus suspension. As shown in Figure 6.4, the prM-specific MAb 2H2 (negative control) exhibited an M/I ratio close to zero, consistent with the fact that prM MAbs do not bind to mature virus. Most interestingly, the three cross-reactive MAbs 4G2, 2A10, DVC10.16, which neutralized partially immature virions better than mature virions, had low M/I ratio (0.2 - 0.4) compared to the other five MAbs, which did not show preferential neutralization of partially immature virions. This finding suggests that the more potent

neutralization of partially mature virus by the three cross-reactive MAbs was due to better binding to immature compared to mmature virions. The two MAbs DVC3.7 and DVC25.5 that neutralized mature virus more potently than partially immature virions did not show an M/I ratio higher than that of the MAbs 9F16, 5C36 and 2D22 that neutralized both viruses equally well (Figure 6.4). This observation suggests that the enhanced neutralization of mature virus by DVC3.7 and DVC25.5 is due to mechanisms other than preferential binding to mature virions.

Human immune sera from dengue patients displayed maturation dependent differences in neutralization potency

We tested a panel of 10 well-characterized DENV immune human sera (Table 6.2) for ability to neutralize DENV3 in the mature form (derived from human U937+DC-SIGN cells) or partially mature form (derived from mosquito C6/36 cells). Similar to the findings with MAbs above, these polyclonal sera also exhibited different patterns of particle maturation-dependent neutralization (Figure 6.5). Six sera (designated 105, 129, 133, 115, 116 and 121) neutralized partially mature virions better than mature virions (Figure 6.5A, Table 6.2). The remaining 4 sera neutralized both mature and partially mature viruses equally well (103 and 132) or neutralized mature virions better than partially mature virions (110 and 130) (Figures 6.5B and 6.5C, Table 6.2). Thus the three dominant patterns of neutralization observed with MAbs also were observed with polyclonal DENV immune sera.

Endosome furin cleavage is involved in modulating antibody mediated neutralization

It has already been shown that endosome furin cleavage is involved in conferring infectivity to immature DENV (Zybert, van der Ende-Metselaar et al. 2008; Rodenhuis-Zybert, Moesker et al. 2011). So we speculated that furin may play a role in partially

mature DENV infection of Vero cells too. To test this hypothesis, furin inhibitor peptidyl chloromethylketone was employed to inhibit furin cleavage in endosome before or after virus infection. As shown in Figure 6.6, furin inhibitor added after virus infection failed to block the infectivity of any viruses. But with furin inhibitor added before virus infection, there is a 90% reduction of partially mature virus infectivity while the mature virus infectivity is only slightly inhibited (Figure 6.6A). This contrast indicated that partially mature virus is very sensitive to furin inhibition during the infection process while the mature virus is not. The same pattern was observed for the mature and partially mature virus of DENV3 and DENV1 (Figure 6.6B and 6.6C). The indispensable role of furin in partially mature virus infection of Vero cells suggested that partially mature virus will undergo further furin cleavage in endosome before infection can occur.

Previous function study of DENV MAbs suggested that many strongly neutralizing MAbs neutralize DENV at post-entry steps in endosome (Nybakken, Oliphant et al. 2005; Lok, Kostyuchenko et al. 2008; Rajamanonmani, Nkenfou et al. 2009; Deng, Dai et al. 2011; Teoh, Kukkaro et al. 2012; Costin, Zaitseva et al. 2013). The furin cleavage induces conformational rearrangement of immature patches of virion and this may lead to dissociation of MAbs and subsequently reduce their neutralization potency if these two MAbs neutralize virus in endosome (post-attachment neutralization). We speculate that this may be the mechanism of the reduced neutralization of partially mature DENV by MAb DVC3.7 and DVC25.5. To test this hypothesis, we employed function assay and demonstrated that DVC3.7 and DVC25.5 neutralize virus mostly at post-attachment steps, supporting out hypothesis that the furin cleavage in endosome may intervene with their neutralization (Figure 6.7). Finally, to demonstrate that furin cleavage is responsible for MAb's enhanced neutralization of mature virus, I compared DVC3.7 neutralization in the presence or absence of furin inhibitor. The results

suggested that without furin cleavage, DVC3.7 neutralizes mature virus and partially mature virus equally well (Figure 6.8A). Using furin inhibitor in 4G2 neutralization experiments further demonstrated that this furin cleavage inhibition does not affect neutralization potency of cross-reactive MAbs like 4G2 and this is consistent with my argument that 4G2 differential neutralization is attributed to preferential binding (Figure 6.8B).

6.4 Discussion

We characterized the maturation state of DENVs produced in non-human cell lines commonly used to grow the viruses in laboratories and a human monocytic cell-line expressing a DENV attachment receptor. We found that the human monocyte cell-derived viruses were more mature than viruses derived from non-human cells. We also demonstrated that the ability of human or mouse Abs to neutralize DENV was influenced significantly by the maturation state of virions. The ability of Abs to bind to virions of different maturation state correlated with neutralization efficiency. We further found that furin cleavage of partially mature virus is critical for virus infectivity and this may contribute to increased neutralization resistance of the partially mature virus.

The long-accepted view of the assembly of DENV and other flaviviruses is that immature, non-infectious virions produced in the ER are proteolytically processed in the Golgi apparatus and secreted from cells as mature, infectious particles. Recent studies have demonstrated, however, that many individual virions produced in cell culture contain envelopes that possess a mixture of E protein in the immature (trimeric) and mature (dimeric) forms (Cherrier, Kaufmann et al. 2009; Junjhon, Edwards et al. 2010). Recent studies also have demonstrated that these partially mature virions and even

almost completely immature virions can be infectious in cell culture under some conditions (Zybert, van der Ende-Metselaar et al. 2008; Rodenhuis-Zybert, van der Schaar et al. 2010). These observations have led investigators to speculate that immature virions play a previously unrecognized role in DENV pathogenesis, including the possibility of Abs directed against uncleaved prM protein playing a role in Ab-enhanced infection and disease. However, the true biological relevance of immature or partially mature virions is unknown because we do not know the maturation state of virions that are produced within infected people.

The observations reported here that a human monocytic cell line produced virions that are mostly mature suggests that the monocytic cells in infected people that are a principal target of infection also may produce predominantly mature virions. This conclusion is also supported by recent findings by Dejnirattisai *et al*. that virions produced by primary human dendritic cells are mainly mature compared to viruses grown in non-human cultured cell lines (Dejnirattisai, Jumnainsong et al. 2010). Thus, while mature particles are undoubtedly of significance *in vivo*, further studies are needed to assess the significance of immature particles in pathogenesis of naturally-acquired infection. As some of the *in vivo* cellular targets of DENV remain unknown, it is conceivable that some human cells produce partially mature particles. Moreover, the proportion of mature to immature virions *in vivo* may depend on factors such as the strain of infecting virus, primary versus secondary infection and inherent variation between different people. A major area for future work is to determine if the high level of production of partially and fully immature dengue virions in some cell culture lines is merely an *in vitro* artifact or if these less mature forms of virion particles play a significant role during natural human infection.

We currently do not understand why DENVs produced in different cell lines display differences in maturation state. Cell line dependent variation in furin or furin-like protease levels is one possible explanation. Alternatively, the quantity of virus produced might differ between cell lines, and cells that produce a non-physiologic burst size of virus might overwhelm the ability of the levels of furin-like proteases in infected cells to process DENVs. We favor the latter explanation because we have observed an inverse correlation between the total quantity of viral antigen secreted by cells and the efficiency of prM processing (data not shown).

Our studies with a panel of 8 MAbs revealed that serotype cross-reactive weakly neutralizing MAbs (the murine MAb 4G2 and human MAb 2A10) and serotype cross-reactive moderately neutralizing MAb (MAb DVC10.16) neutralized partially mature virions better than mature virions. MAbs 4G2 and 2A10 have been mapped to conserved epitopes at or close the fusion loop on domain II (Figure 4) (Trainor, Crill et al. 2007). These results are concordant with elegant studies done by Nelson *et al.* who demonstrated that MAbs binding to epitopes around the fusion loop neutralized partially mature West Nile virus (WNV) better than totally mature virus (Nelson, Jost et al. 2008; Vogt, Moesker et al. 2009). These investigators demonstrated that in WNV the fusion loop region is more exposed in immature virions compared to mature virions, which explains the increased sensitivity of partially immature virions to neutralization by fusion loop MAbs (Cherrier, Kaufmann et al. 2009).

hMAb DVC10.16, which also neutralized partially immature virions better than mature virions, is a serotype cross-reactive MAb that binds to the A strand epitope of EDIII (de Alwis, Beltramello et al. 2011). In fact, studies with DENV cross-reactive murine MAb 1A1D-2 have demonstrated that the EDIII A strand epitope is hidden in the mature virus particle (Lok, Kostyuchenko et al. 2008). Studies by Lok *et al*. indicate that

this hidden epitope becomes exposed transiently and available for Ab binding as the virus particle flexes at elevated temperatures. Another study by Cockburn *et al.* indicated that the A strand-specific MAb 4E11 neutralizes virus by disrupting architecture of the mature virion and inducing premature fusion loop exposure (Cockburn, Navarro Sanchez et al. 2012). Based on our findings with MAb DVC10.16, we propose that the exposure of the EDIII A strand epitope also changes as a function of virus maturation state.

The observation here that fusion loop-specific Abs neutralize partially immature DENVs better than mature viruses has important implications for characterizing polyclonal antibody profiles in human immune sera following natural infection or experimental DENV vaccination. Following exposure to natural DENV infections, the fusion loop is a major target of the total Ab response (Lai, Tsai et al. 2008; Costin, Zaitseva et al. 2013). The fusion loop targeting Abs can exhibit both strongly neutralizing and infection-enhancing phenotypes (Sukupolvi-Petty, Austin et al. 2010; Deng, Dai et al. 2011; Rodenhuis-Zybert, Moesker et al. 2011; Lin, Tsai et al. 2012; Williams, Sukupolvi-Petty et al. 2013). In sera dominated by fusion loop Abs, the overall neutralization titer is likely to vary widely depending on the maturation state of virions used to infect cells in the assay. We observed that 6 out of 10 human DENV immune sera neutralized partially immature virions better than mature viruses. When reference panels of DENV immune sera have been tested in different laboratories for neutralizing Abs, the actual titers vary widely between laboratories for the same serum sample. We propose that variation in the maturation state of viruses used in the neutralization assay is a major source of this variability. The current standard used to evaluate immunogenicity as a correlate of immunity for experimental DENV vaccine candidates is the serum neutralization assay. The increased susceptibility of partially immature virions to neutralization by fusion loop Abs will complicate the assessment of vaccine responses.

While fusion loop- and EDIII A strand-specific MAbs neutralized partially mature virions better than mature virions, the five strongly neutralizing, serotype-specific MAbs in our panel neutralized partially mature and mature virions equally well (MAbs 2D22, 5C36 and 9F16) or neutralized mature virions better than partially mature virions (MAbs DVC25.5 and DVC3.7). Human MAb 2D22 is a strongly neutralizing Ab that binds to a quaternary epitope centered around the hinge between domains I and II (Li, Lok et al. 2008). We also have tested EDI/II hinge Abs that bind to DENV1 or 3 and observed equal neutralization of partially mature and fully mature DENVs (data not shown). Recent studies have pinpointed the EDI/II as the major target of strongly neutralizing Abs in people after natural infection with DENV or WNV (de Alwis, Smith et al. 2012; Teoh, Kukkaro et al. 2012). We propose that EDI/II hinge antibodies, which are likely to be responsible for the solid long-term protection observed after natural exposure to DENV, do not discriminate between DENVs of different maturation state.

All EDIII lateral ridge-specific MAbs in our panel neutralized both classes of mature and partially mature viruses equally well (MAbs 5C36 and 9F16) or neutralized mature particles better than partially mature virions (MAbs DVC3.7 and DVC25.5). We also observed preferential neutralization of mature DENVs by two of the DENV immune sera (samples DT110 and DT130).

The lower M/I ratio of all the three cross-reactive MAbs suggests that these MAbs bind immature virions with higher affinity, or higher accessibility of cross-reactive MAb epitopes in immature virions, and this is consistent with Cryo-electron microscopy study suggested (Cherrier, Kaufmann et al. 2009). The association between cross-reactive MAbs and immature virion is intriguing. It implies a possible critical role of immature virion in eliciting cross-reactive antibody response, and also a critical role of immature virion in ADE mediated by cross-reactive MAbs. If both hypotheses were verified, it may

suggest immature and partially mature virions may play a bigger role than mature virions *in vivo* for dengue pathogenesis.

Our results suggested that the preferential neutralization of mature virus by MAbs DVC3.7 and DVC25.5 is caused by endosome furin cleavage of the partially mature virus. The role of endosome furin in flavivirus infection is controversial as it was shown that WNV infection of Raji-DC-SIGN B cell lines is insensitive to furin inhibition (Mukherjee, Lin et al. 2011) and our results corroborated this by showing that partially mature dengue virus infection of monocytic cell line U937-DC-SIGN is also insensitive to furin inhibition (data unpublished). Nevertheless, our evidence suggested that, at least in the case of Vero cells, partially mature dengue virus might need to become fully mature post-entry in endosomes. It is possible that as cleavage processing of virus occurs to virus-antibody complex, the antibodies in proximity of cleavage site might be inevitably affected, leading to decreased neutralization of the partially mature virus. Most interestingly, in the presence of furin inhibitor, the differential neutralization of MAb DVC3.7 is abolished while the differential neutralization of 4G2 is still evident, suggesting this post-entry furin cleavage contributed to the differential neutralization of type-specific MAb DVC3.7 but not cross-reactive MAbs like 4G2. This also implies that inefficient cleavage of virus particles in virus production may have important evolutionary significance as it may serve as a means of immune evasion.

Besides these two mechanisms discussed, other mechanisms may also contribute to the differential neutralization between the two viruses. One possibility is the structure kinetic difference (breathing) or stability difference between the mature virus and partially mature virus. The viral structure difference is very likely lead to variation in structure dynamic and stability. Another possibility is glycosylation. It has been shown that mosquito cell line is different from human cells in Env protein glycosylation (Hacker,

White et al. 2009). Glycosylation variation can alter antibody binding and affect neutralization sensitivity. Future works will include experiments to explore the role of glycosylation in MAb's differential neutralization of mosquito derived and human monocytic cell derived virus.

In summary, our studies demonstrate that the maturation state of DENVs produced in the laboratory depends to a large extent on the cell type used to produce the viruses. Moreover, the ability of some Abs to neutralize DENVs was influenced heavily by the maturation state of particles used in the neutralization assay, while other Abs were insensitive to maturation differences. The ability of fusion loop-specific Abs to neutralize partially immature virions preferentially is likely to be major source of variation in the use of the current standard neutralization test and likely confounds the reproducible analysis of vaccine serum samples. On the other hand, DENV serotype-specific strongly neutralizing Abs, including those linked to long-term homotypic protection neutralized DENVs irrespective of maturation state.

Table 6.1: **Monoclonal Antibodies used in this work**

Mab	Isotype	Specificity	Binding Epitope	Reference	$FRNT_{50}$ of DENV2 Neutralization		
					C6/36 (μg/ml)	U937-DC-SIGN (μg/ml)	Ratio *
DVC3.7	human IgG1	DV2 specific	EDIII	de Alwis et al	0.0264	0.002802	9.4
DVC25.5	human IgG1	DV2 specific	EDIII	de Alwis et al	0.1371	0.06272	2.1
2D22	human IgG1	DV2 specific	Hinge region	de Alwis et al	0.1648	0.1239	Insignificant^
5C36	mouse IgG1	DV2 specific	Lateral Ridge	Growmoski et al	0.1465	0.1444	Insignificant
9F16	mouse IgG1	DV2 specific	Lateral Ridge	Growmoski et al	0.4849	0.7523	Insignificant
2A10	human IgG1	cross-reactive	EDI/II	Smith et al	0.9421	2.617	0.36
4G2	mouse IgG2	Cross-reactive	EDI/II	NA	1.835	7.667	0.23
DVC10.16	human IgG1	DV subcomplex	EDIII	de Alwis et al	0.1764	0.7300	0.24

Note: * Ratio of $FRNT_{50}$ is calculated as: ($FRNT_{50}$ of C6/36 virus) / ($FRNT_{50}$ of U937-DC-SIGN virus).

^ The difference between the two $FRNT_{50}$ are not significant from each other based on the 95% confidence interval calculated using sigma dose response model of non-linear fitting in GraphPad-prism.

Table 6.2: **Sera used in this work and the differential neutralization of two viruses**

Serum ID	Serotype specificity & Infection history	$FRNT_{50}$ of DENV3 Neutralization		
		C6/36	U937-DC-SIGN	Ratio
103	DENV 3 primary	258.6*	193.8	Insignificant
105	DENV 3 primary	291.3	88.16	3.3
129	DENV 3 primary	615.0	296.0	2.1
132	DENV 3 primary	1391	933	Insignificant
133	DENV 3 primary	2805	1082	2.6
110	secondary	135.6	1238	0.11
115	secondary	293.2	63.92	4.6
116	secondary	1582	835.9	1.9
121	secondary	1073	263	4.1
130	secondary	476	2246	0.21

Note: * dilutions of serum

Figure 6.1

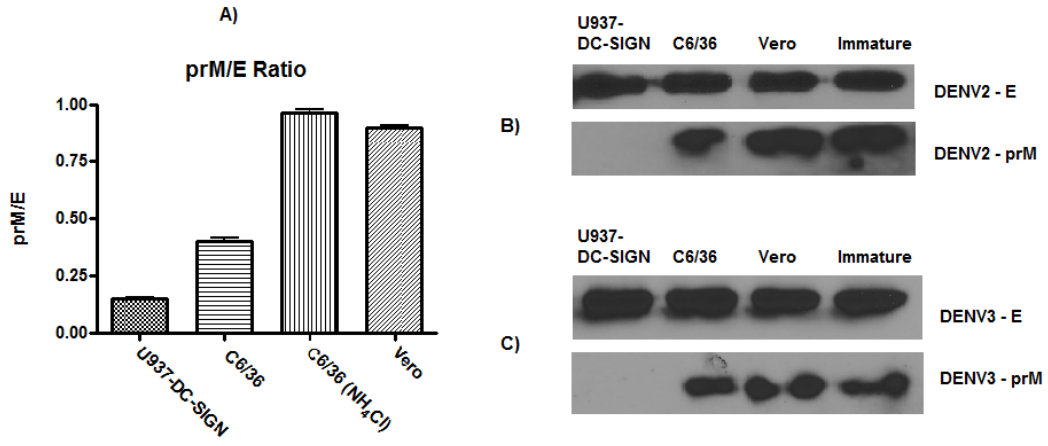


Figure. 6.1: Maturation state of DENVs produced by different cell lines. A) Murine EDIII-binding MAbs were used to capture DENVs from various sources onto 96-well ELISA plates. The amount of prM and E protein on the captured virions was determined by ELISA using human prM MAbs and E MAbs and alkaline phosphatase conjugated anti-human IgG secondary antibody. The prM/E ratio was calculated using the optical density (OD) signal of human prM MAbs divided by the signal for human E MAbs. **B)** Western blot detection of prM and E protein levels of DENV2 virus samples from various cell lines. **C)** Western blot detection of prM and E protein levels of DENV3 virus samples from various cell lines.

Figure 6.2

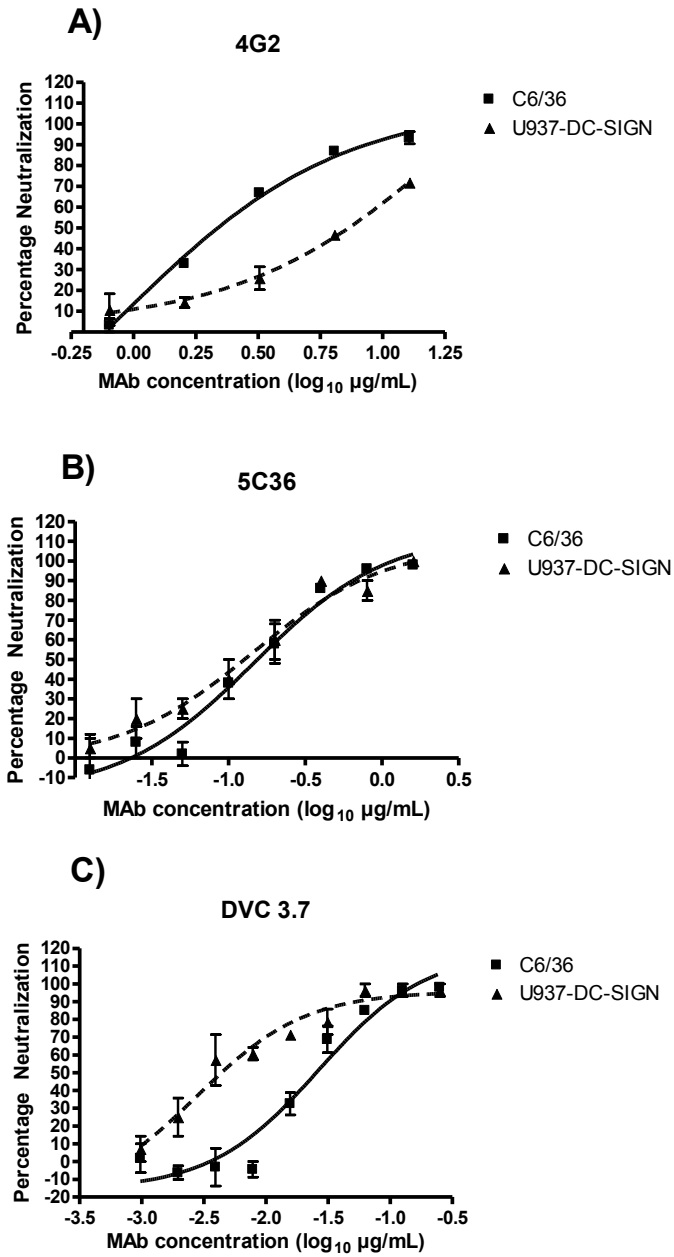


Figure. 6.2: Maturation-dependent neutralization of DENVs by MAbs. A) MAb 4G2 is representative MAbs that neutralized partially mature virions (C6/36-derived) better than fully mature virions (U937-DC-SIGN-derived). **B)** MAb 5C36 is representative of MAbs showing equal neutralization of partially and fully mature virions. **C)** MAb DVC3.7 is representative of MAbs that neutralized fully mature virions better than partially mature virions.

Figure 6.3

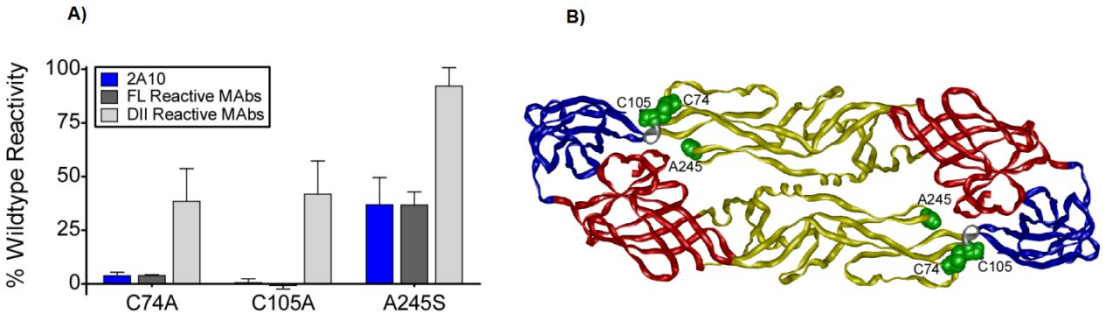


Figure. 6.3: DENV Envelope Residues that Affect 2A10 Binding. A) Mammalian cells expressing mutant DENV envelope proteins were immunostained with MAb 2A10 and detected by flow cytometry. 2A10 binding assays were performed 2 times and ranges are shown. The mean reactivity of control MAbs that bind the fusion loop (10 Abs) or Domain II (4 Abs) are shown for comparison. **B)** Mutations that reduced 2A10 reactivity were mapped onto the DENV E protein structure (PDB ID # 1OAN) (33). DENV E protein domains I, II, and III are depicted in red, yellow, and blue, respectively. The fusion loop is shown in silver.

Figure 6.4

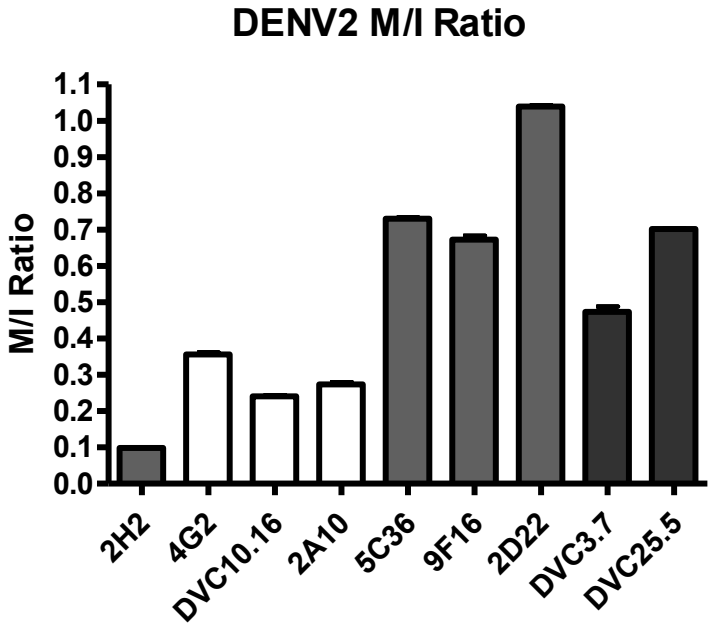


Figure. 6.4: Relative binding of MAbs to fully and immature DENVs. Equal amount of mature virus and immature virus were captured onto 96-well plates. The ability of each MAb to bind to mature and immature virions was detected by ELISA. Mature to immature (M/I) binding ratio was calculated as (OD of binding mature virus) / (OD of binding immature virus).

Figure 6.5

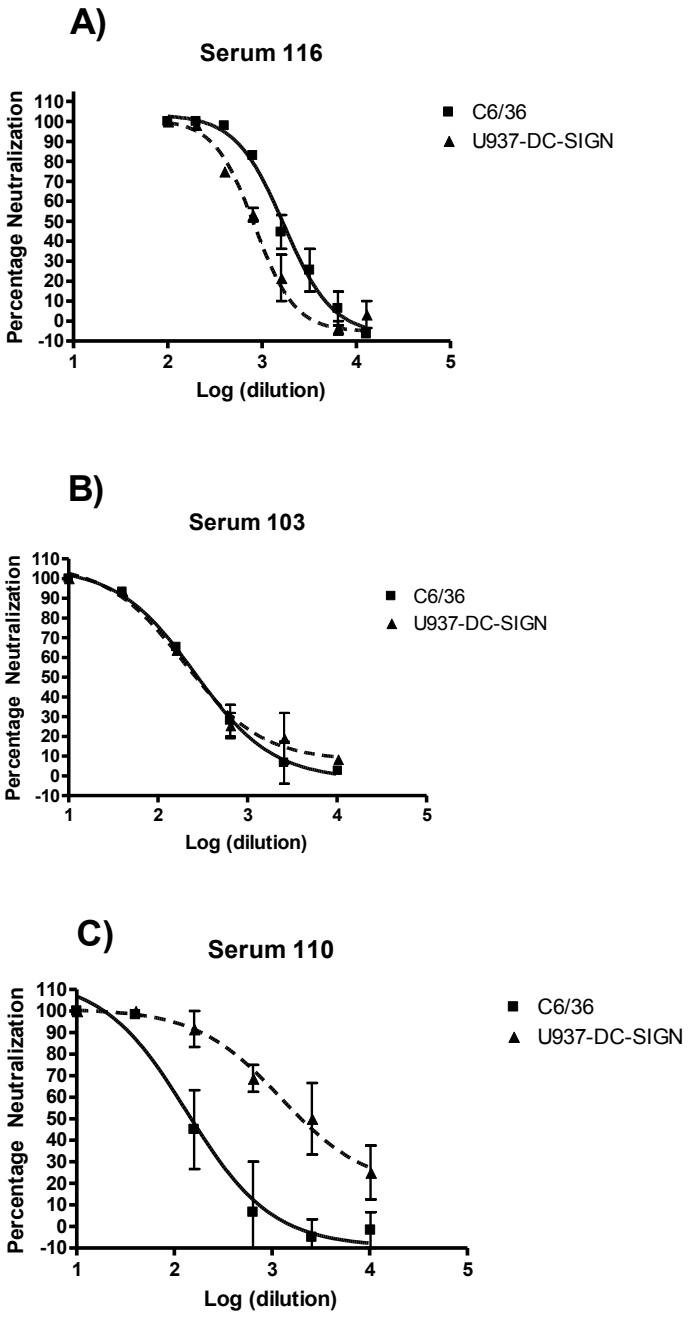
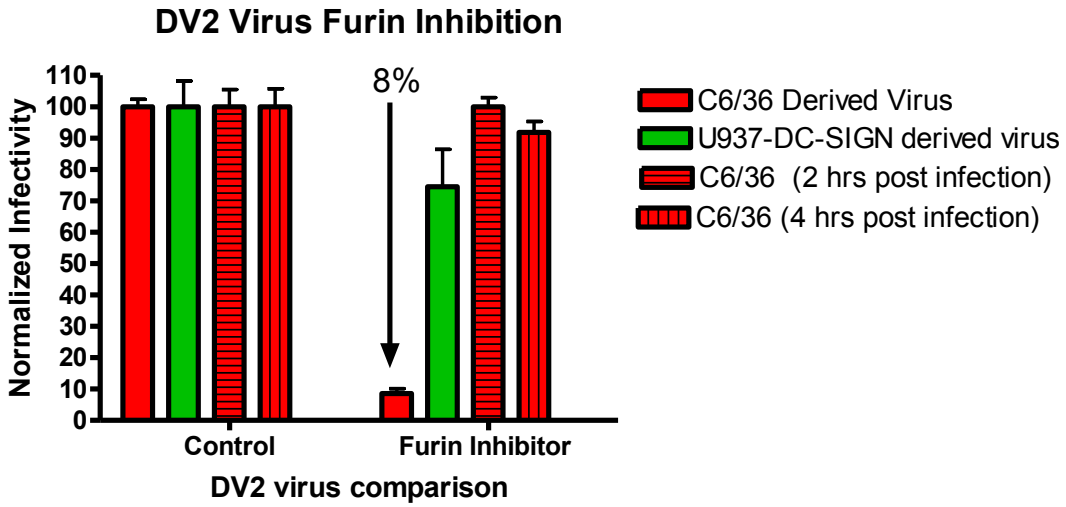


Figure. 6.5: Maturation-dependent neutralization of DENVs by human dengue immune sera. **A)** Serum 116 represents sera displaying better neutralization of partially mature compared to fully mature DENV3. **B)** Serum 103 represents sera displaying equal neutralization of partially and fully mature DENV3. **C)** Serum 110 represents sera displaying increased neutralization of mature compared to partially mature DENV3.

Figure 6.6

A)



B)

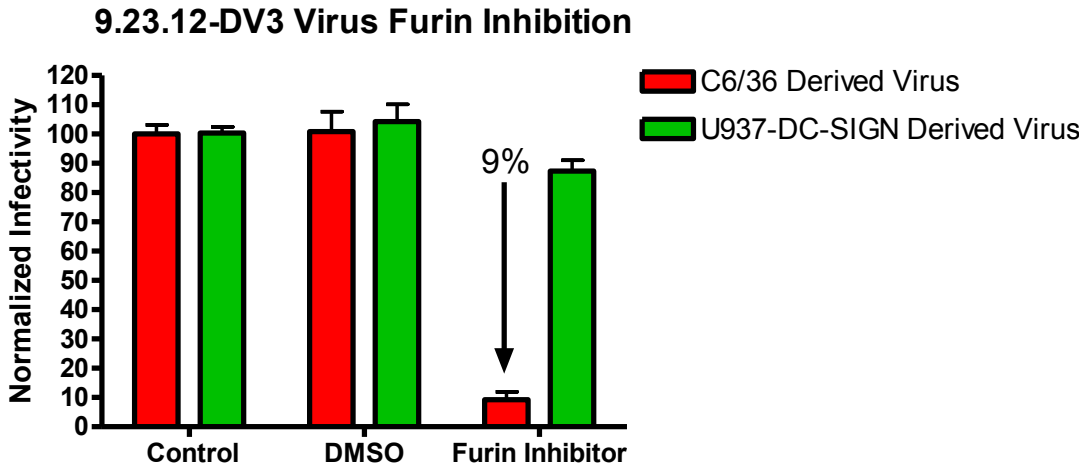
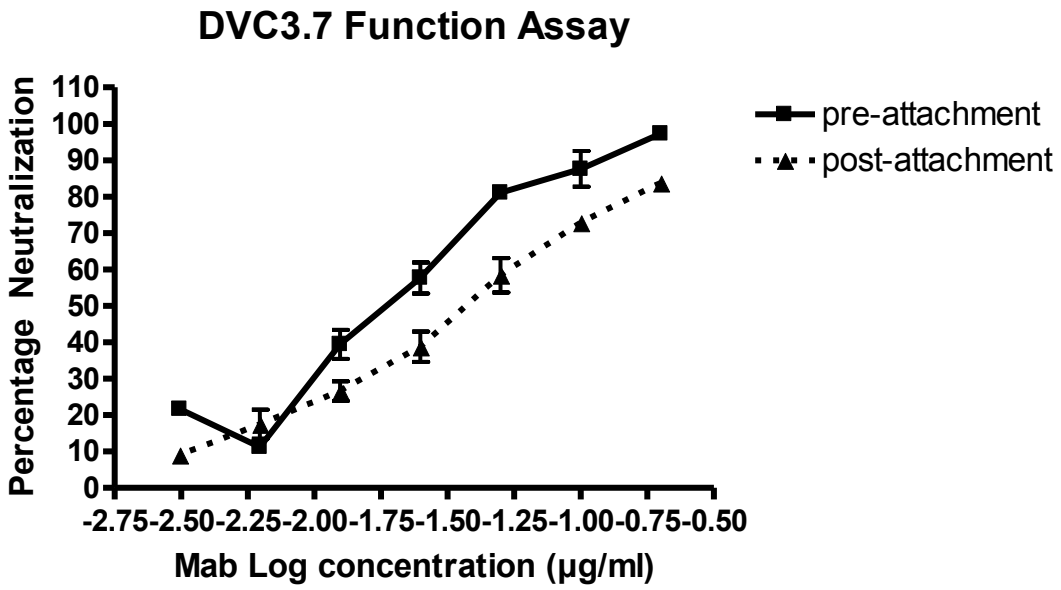


Figure 6.6: Furin cleavage inhibition abolishes majority of C6/36 virus infectivity but not that of U937-DC-SIGN derived virus. A) Furin inhibitor was added to U937-DC-SIGN virus before infection and was added to C6/36 derived virus before or 2 hrs or 4 hrs after infection; **B)** Furin inhibitor was added before infection of both viruses and DMSO, the solvent of furin inhibitor was added to cells alone as control.

Figure 6.7

A)



B)

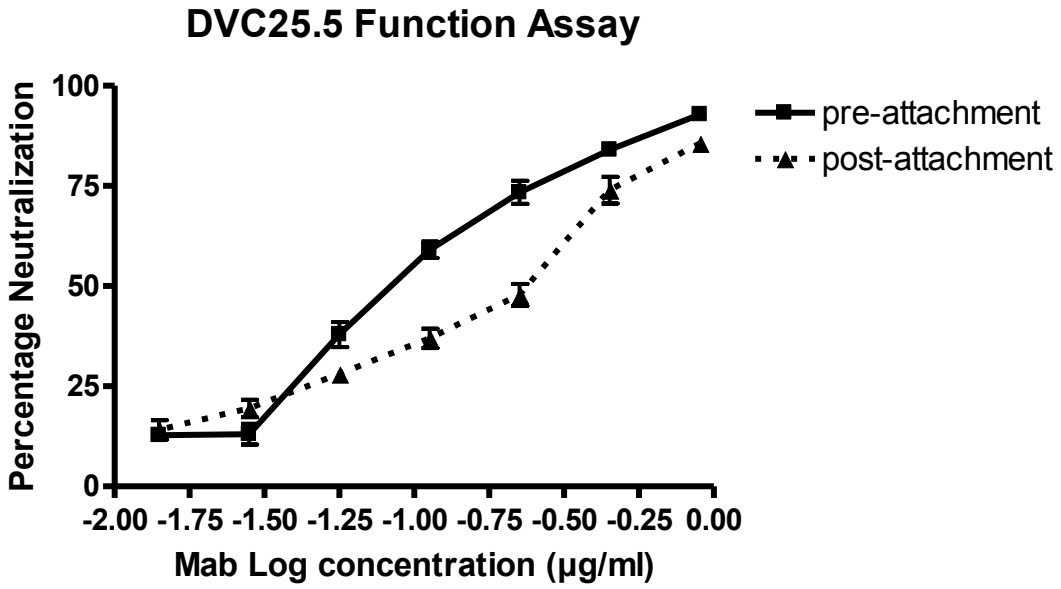
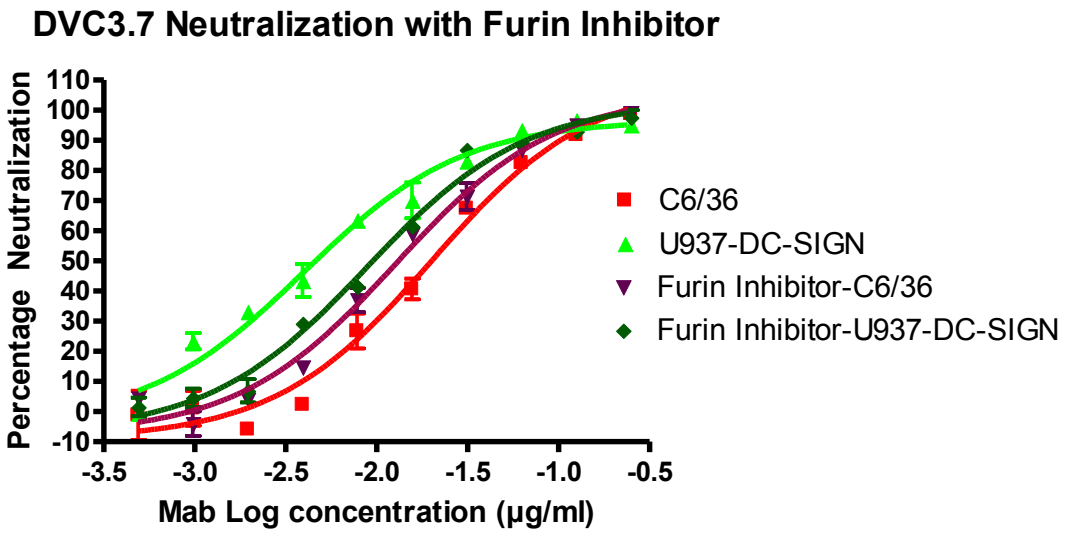


Figure 6.7: Function Assay of MAb **A)** DVC3.7 and **B)** DVC 25.5 revealed that these MAbs mainly neutralize post-attachment step of infection. MAbs were tested at concentrations covering 10% to 100% neutralization.

Figure 6.8

A)



B)

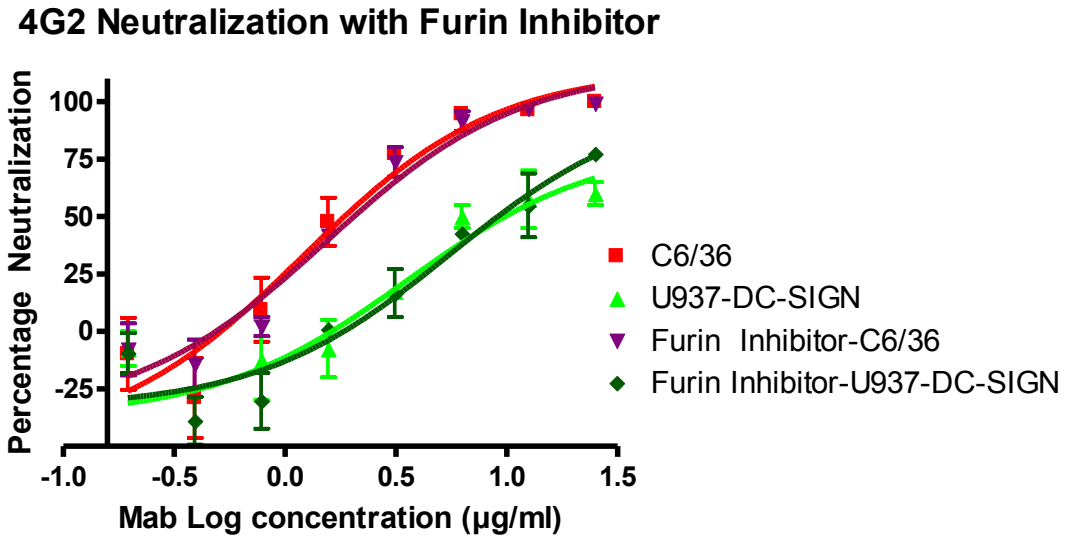


Figure 6.8: Furin Inhibition Experiment with MAb DVC3.7 and 4G2. A) Furin inhibition abolished the differential neutralization of MAb DVC3.7. **B)** 4G2 neutralization of mature and partially mature virus is unchanged in the presence of furin inhibitor.

6.5 References

Cherrier, M. V., B. Kaufmann, et al. (2009). "Structural basis for the preferential recognition of immature flaviviruses by a fusion-loop antibody." EMBO J 28(20): 3269-3276.

Cockburn, J. J., M. E. Navarro Sanchez, et al. (2012). "Mechanism of dengue virus broad cross-neutralization by a monoclonal antibody." Structure **20**(2): 303-314.

Costin, J. M., E. Zaitseva, et al. (2013). "Mechanistic study of broadly neutralizing human monoclonal antibodies against dengue virus that target the fusion loop." J Virol 87(1): 52-66.

de Alwis, R., M. Beltramello, et al. (2011). "In-depth analysis of the antibody response of individuals exposed to primary dengue virus infection." PLoS Negl Trop Dis 5(6): e1188.

de Alwis, R., S. A. Smith, et al. (2012). "Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions." Proc Natl Acad Sci U S A 109(19): 7439-7444.

Dejnirattisai, W., A. Jumnainsong, et al. (2010). "Cross-reacting antibodies enhance dengue virus infection in humans." Science 328(5979): 745-748.

Deng, Y. Q., J. X. Dai, et al. (2011). "A broadly flavivirus cross-neutralizing monoclonal antibody that recognizes a novel epitope within the fusion loop of E protein." PLoS One 6(1): e16059.

Durbin, A. P., R. A. Karron, et al. (2001). "Attenuation and immunogenicity in humans of a live dengue virus type-4 vaccine candidate with a 30 nucleotide deletion in its 3'-untranslated region." Am J Trop Med Hyg 65(5): 405-413.

Elshuber, S., S. L. Allison, et al. (2003). "Cleavage of protein prM is necessary for infection of BHK-21 cells by tick-borne encephalitis virus." J Gen Virol 84(Pt 1): 183-191.

Gromowski, G. D. and A. D. Barrett (2007). "Characterization of an antigenic site that contains a dominant, type-specific neutralization determinant on the envelope protein domain III (ED3) of dengue 2 virus." Virology 366(2): 349-360.

Gromowski, G. D., N. D. Barrett, et al. (2008). "Characterization of dengue virus complex-specific neutralizing epitopes on envelope protein domain III of dengue 2 virus." J Virol 82(17): 8828-8837.

Gromowski, G. D., J. T. Roehrig, et al. (2010). "Mutations of an antibody binding energy hot spot on domain III of the dengue 2 envelope glycoprotein exploited for

neutralization escape." Virology 407(2): 237-246.

Guzman, M. G., G. Kouri, et al. (2000). "Epidemiologic studies on Dengue in Santiago de Cuba, 1997." Am J Epidemiol 152(9): 793-799; discussion 804.

Hacker, K., L. White, et al. (2009). "N-linked glycans on dengue viruses grown in mammalian and insect cells." J Gen Virol 90(Pt 9): 2097-2106.

Junjhon, J., T. J. Edwards, et al. (2010). "Influence of pr-M cleavage on the heterogeneity of extracellular dengue virus particles." J Virol 84(16): 8353-8358.

Lai, C. Y., W. Y. Tsai, et al. (2008). "Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II." J Virol **82**(13): 6631-6643.

Li, L., S. M. Lok, et al. (2008). "The flavivirus precursor membrane-envelope protein complex: structure and maturation." Science 319(5871): 1830-1834.

Lin, H. E., W. Y. Tsai, et al. (2012). "Analysis of epitopes on dengue virus envelope protein recognized by monoclonal antibodies and polyclonal human sera by a high throughput assay." PLoS Negl Trop Dis **6**(1): e1447.

Lisova, O., F. Hardy, et al. (2007). "Mapping to completeness and transplantation of a group-specific, discontinuous, neutralizing epitope in the envelope protein of dengue virus." J Gen Virol 88(Pt 9): 2387-2397.

Lok, S. M., V. Kostyuchenko, et al. (2008). "Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins." Nat Struct Mol Biol 15(3): 312-317.

Messer, W. B., B. Yount, et al. (2012). "Development and characterization of a reverse genetic system for studying dengue virus serotype 3 strain variation and neutralization." PLoS Negl Trop Dis 6(2): e1486.

Mukherjee, S., T. Y. Lin, et al. (2011). "The infectivity of prM-containing partially mature West Nile virus does not require the activity of cellular furin-like proteases." J Virol 85(22): 12067-12072.

Nelson, S., C. A. Jost, et al. (2008). "Maturation of West Nile virus modulates sensitivity to antibody-mediated neutralization." PLoS Pathog 4(5): e1000060.

Nybakken, G. E., T. Oliphant, et al. (2005). "Structural basis of West Nile virus neutralization by a therapeutic antibody." Nature 437(7059): 764-769.

Rajamanonmani, R., C. Nkenfou, et al. (2009). "On a mouse monoclonal antibody that neutralizes all four dengue virus serotypes." J Gen Virol 90(Pt 4): 799-809.

Rodenhuis-Zybert, I. A., B. Moesker, et al. (2011). "A fusion-loop antibody enhances the infectious properties of immature flavivirus particles." J Virol 85(22): 11800-11808.

Sangkawibha, N., S. Rojanasuphot, et al. (1984). "Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak." Am J Epidemiol 120(5): 653-669.

Smith, S. A., Y. Zhou, et al. (2012). "Persistence of circulating memory B cell clones with potential for dengue virus disease enhancement for decades following infection." J Virol 86(5): 2665-2675.

Sukupolvi-Petty, S., S. K. Austin, et al. (2010). "Structure and function analysis of therapeutic monoclonal antibodies against dengue virus type 2." J Virol **84**(18): 9227-9239.

Teoh, E. P., P. Kukkaro, et al. (2012). "The structural basis for serotype-specific neutralization of dengue virus by a human antibody." Sci Transl Med 4(139): 139ra183.

Trainor, N. B., W. D. Crill, et al. (2007). "Mutation analysis of the fusion domain region of St. Louis encephalitis virus envelope protein." Virology **360**(2): 398-406.

Vogt, M. R., B. Moesker, et al. (2009). "Human monoclonal antibodies against West Nile virus induced by natural infection neutralize at a postattachment step." J Virol 83(13): 6494-6507.

WHO (2009). "Dengue and dengue haemorrhagic fever." WHO Fact Sheets 117.

Williams, K. L., S. Sukupolvi-Petty, et al. (2013). "Therapeutic efficacy of antibodies lacking FcgammaR against lethal dengue virus infection is due to neutralizing potency and blocking of enhancing antibodies." PLoS Pathog **9**(2): e1003157.

Yu, I. M., W. Zhang, et al. (2008). "Structure of the immature dengue virus at low pH primes proteolytic maturation." Science 319(5871): 1834-1837.

Zhou, Y., S. K. Austin, et al. (2013). "The mechanism of differential neutralization of dengue serotype 3 strains by monoclonal antibody 8A1." Virology.

Zybert, I. A., H. van der Ende-Metselaar, et al. (2008). "Functional importance of dengue virus maturation: infectious properties of immature virions." J Gen Virol 89(Pt 12): 3047-3051.

CHAPTER 7

DISCUSSION

The results presented in this thesis focused on two general topics: The first topic was to understand the antibody responses following dengue infection; the second topic was about how genetic and maturation variations of DENV virion influence the ability of Abs to neutralize DENV. The significance of these findings and possible future directions had been discussed in each chapter respectively. In this chapter, all the results will be discussed under one unifying question: how dengue viruses evade the immune response. Based on the synthesis analysis of all the data presented, new hypothesis of dengue evasion of immune response will be proposed and discussed.

7.1 The misled humoral immune response

Humoral immunity is one of the critical host defenses against dengue virus infection and ironically it is also regarded as the major risk factor of the severe disease DHF/DSS. To solve the myth behind this paradox, a lot of studies have been dedicated to cloning and screening of MAbs from human PBMCs isolated during or post infection, including the results presented in Chapter 2 (Beltramello, Williams et al. 2010; Dejnirattisai, Jumnainsong et al. 2010; de Alwis, Beltramello et al. 2011; Smith, Zhou et al. 2012). A consensus of all these studies is the rarity of type-specific strongly neutralizing MAbs. The majority of the cloned dengue reactive human MAbs are cross-reactive, weakly neutralizing and enhancing. To be more specific, EDIII harbors more

type-specific epitopes yet it plays a minor role in inducing antibody response in human despite its strong immunogenicity in mouse models (Wahala, Kraus et al. 2009; Cockburn, Navarro Sanchez et al. 2012); the majority of humoral immunity is directed against EDI/II but these two domains are more conserved than domain III between serotypes and are more likely to induce cross-reactive antibody response (Costin, Zaitseva et al. 2013). In general, human humoral immune response against dengue is far from being the "perfect" or "ideal" immune response. This dilemma implies that human immune response against dengue infection is "misled" to produce a "dysfunctional" humoral response that poses a risk of ADE for subsequent infections.

This should not be a surprise as most successful viruses had adapted various strategies to evade the host's immune response. The best known example is the evolution of HIV: The long infection period and high mutation rate of the HIV genome enable the virus to "outrun" the immune system and eventually crush it. However, for a virus like dengue, the viremia is short-lasting (4-5 days) and thus the virus does not have enough time to generate significant genetic and antigenic drift/shift *in vivo*. Based on the argument presented below, I hypothesized that DENV utilizes several particular features of its life cycle to mislead the immune system, resulting in a "dysfunctional" humoral response.

The first feature (1) is the maturation variation of the dengue virus. As shown in Chapter 6, cell type switch can lead to maturation variation of the progeny viruses. More specifically, immediately after virus injection through a mosquito bite, the human immune system is primed by the mosquito derived partially mature virus while later the viremia causing the disease is comprised of mostly monocytic cell-derived mature virus. The drastic difference of surface protein structures between mature and immature virus suggests that they have different antigenicity. Therefore, the immune response primed

by the partially mature virions may not be optimized to recognize and neutralize the mature virions. Though it is still unknown how much the mosquito-derived viruses shape the immune response compared to the monocytic cell-derived viruses do in the 4-6 days of viremia, it is reasonable to speculate that the maturation variation of the dengue virus may be an evolutionary strategy of virus to evade/mislead the immune response. In terms of changing DENV surface antigenicity to escape neutralization, maturation variation may have advantages over genetic mutation: First, virus maturation changes can be done without losing or changing the genetic information of the genome, allowing more freedom of evolution; second, the change is triggered by alteration of the cell type the virus replicates in, which is faster than natural selection of genetic mutation.

The second feature (2) is using different glycosylation. Hacker in our lab demonstrated that the glycosylation of viruses derived from mosquito cells and mammalian cells are not all the same (Hacker, White et al. 2009). Glycosylation variation may lead to antigenic differences between mosquito cell derived viruses and monocytic cell derived viruses thus the same argument above can be applied here, too.

What we observed in our lab supports the above arguments. First, the cross-reactive antibodies, which make up to 75% of the humoral immune response (de Alwis, unpublished data), have reduced neutralization against monocytic cell derived mature viruses. Secondly, 6 out of 10 polyclonal sera I tested have reduced neutralization against monocytic derived viruses (Chapter 6). Both observations support the argument that the immune response primed by C6/36 derived viruses is optimized to neutralize C6/36 derived viruses, not monocytic cell derived viruses. Both maturation and glycosylation may contribute to this evasion strategy.

The third feature (3) is the virion conformational change in the endosome. DENV will undergo low pH triggered conformational change after entry into the endosome. Antibody neutralization at this stage is called post-attachment neutralization. Therefore virions inside the endosome are most likely to have E in trimeric form instead of dimeric form. However, during the infection, the virions present themselves in pre-conformational change form for B cell to recognize thus the immune response is optimized to recognize the virus in pre-conformational change form, not post-conformational change form. As a result, the humoral immune response may not bind the post-transform virions as well as pre-transform virions.

The fourth feature (4) is closely related to the third one: the peculiar environment inside the endosome, such as low pH and the presence of furin, is very different from the extra-cellular environment. It is known that pH is critical for protein binding affinity and the change of pH will affect antibody binding to virus. Similar to the argument I made in feature 3, the B cells were primed by dengue virions in close to neutral pH in the blood plasma, not the low pH in the endosomes, so it is expected that Abs that are optimized to recognize the viruses in physiological neutral pH may not be able to bind and neutralize the virus very well in the endosomes. In other words, the low pH environment helps DENV to evade the immune response that is optimized to recognize DENV at near-neutral pH. This may explain why certain MAbs (such as 12C1 or 13A5 in Chapter 4) that bind virus strongly in a normal pH ELISA are not neutralizing at all in the neutralization test, since perhaps they just do not bind to the virus so well in the low pH conditions. Measuring antibody binding to viruses in the low pH environment shall be carried out to test this hypothesis. Besides the pH, the presence of proteases in the endosome may also interfere with antibody binding and affect neutralization, as indicated in Chapter 6.

What makes feature 3 and 4 ideal immune evasion strategies is that the flavivirus' most important steps, such as envelope conformational change and membrane fusion, all occur inside the endosome. However, these post-attachment steps of the virus life cycle are hidden from the B cell repertoire and the B cells are trained to recognize viruses before or during attachment. It is expected that humoral response would recognize and neutralize the virus at pre-attachment better than at post-attachment. It has been shown that antibodies that block virus attachment to cells are a major component of the human neutralizing antibody response against the dengue virus (He, Innis et al. 1995). This supports my hypothesis that the immune system is optimized to neutralizing the virus outside the endosome, at its attachment step, which is not the most efficient way to neutralize the virus as the virus has other ways of attachment to the cell (such as Fc-gamma receptor). More importantly, the most efficient stage to neutralize the virus is at the post-attachment steps, as the function data in Chapters 3 and 6 suggested that most of the strongly neutralizing MAbs are post-attachment neutralizing. To illustrate this point, I made a table of the previous identified strongly neutralizing MAbs that have been studied for pre-attachment or post-attachment neutralization. As shown in Table 7.1, out of 28 studied MAbs, there are only 5 blocking attachment while the rest 23 MAbs, especially the strongly neutralizing MAbs, all neutralize at the post-attachment steps. This is not conclusive evidence but it strongly implies that the post-attachment steps are the vulnerable steps of virus infection and can be neutralized with high efficiency. In summary, my argument is: the most vulnerable stage of virus infection is at post-attachment stage, yet the virus at this stage is only inside the endosome, where the low pH environment and virus transformation can shield the virus from a humoral immune response that is optimized to recognize the virus outside the endosome.

In general, our interpretation of dengue humoral immune responses and antibody neutralization data pointed out one new hypothesis: The dengue virus utilizes cell type switch dependent maturation/glycosylation and endosome related transformation and low pH to evade the humoral immune response and this is why the majority of the cloned MAbs are weakly neutralizing and strongly enhancing. More data are needed to further support all the above hypotheses. If they are proved, it may shed light on dengue vaccine design. For example, the immunogen can be designed to mimic the post-transformation virus to induce the most potent neutralizing antibodies. Furthermore by studying how affinity changes in low pH conditions, we may be able to design vaccines that induce antibodies that bind better in low PH environment.

7.2 The gap between antibody binding and virus neutralization

In Chapter 3, the association between neutralization mechanisms and stoichiometry models was discussed. In Chapter 4, monoclonal antibodies were tested for their neutralization potency in MAb mixtures, and we found independent neutralization instead of a synergistic effect. I propose that to obtain a synergistic effect, the two MAbs should have overlapping neutralization mechanism and have no competitive binding. In Chapter 5, 8A1 binding and neutralization differences were tested and compared and we observed neutralization variations not due to binding differences. In Chapter 6 and this chapter, the effects of low pH, furin cleavage and maturation on binding and neutralization were discussed. Factors like antibody diversity, epitope accessibility, virus structure dynamic, pH, temperature, stoichiometry and cell type all play a role in modulating the antibody mediated neutralization. But these factors have been either understudied or neglected in previous research. Thus, all the points made in these chapters can boil down to one main goal: to elucidate the gap between antibody binding and virus neutralization. Each chapter solved a small piece of the big question

but by putting all the data and conclusion together, we may be able to get a better view of the overall landscape of virus binding-neutralization, and new directions of research can be planned accordingly.

First, identifying and elucidating synergistic neutralization of MAb mixtures would greatly benefit future development of cocktail antibody therapy for dengue infection. It would also offer a good chance to understand how polyclonal serum works in neutralization. To do that, in Chapter 4, I proposed starting with a pair of MAbs with threshold stoichiometry and binding to non-overlapping epitopes. As my hypothesis predicts, MAbs with threshold stoichiometry should be able to block conformational change of the virus (Chapter 3). The MAb function assay data in Chapter 3 will be useful for designing such an experiment and it suggests that MAbs 8A1 and 1A1D-2 would be a good candidate pair. They both neutralize DENV3 and display threshold stoichiometry. 8A1 binds to the EDIII lateral ridge and 1A1D-2 binds to the A-strand of EDIII, so the possibility of competitive binding is limited. Future experiments with the two MAbs should be done to identify a synergistic effect.

Second, it would be quite revealing to compare the neutralization of virus during normal infection with that of the ADE infection. Elucidating their common neutralization mechanisms could be important for preventing ADE. It has been hypothesized that MAbs blocking attachment should not be able to neutralize ADE because ADE does not use a regular attachment molecule but rather use Fc-ɣ receptors and the Fc portion of the binding antibody. But our results in Chapter 4 disproved this hypothesis, as we found 2Q1899 and 9F16 are very competent in inhibiting 2H2-mediated enhancement (Chapter 4) and both MAbs neutralize by blocking attachment (Chapter 3). There are two explanations for this paradox. One is that 9F16 inhibits ADE by aggregating multiple virions into an immune complex to reduce infectious unit numbers and also by increasing

the complex size to cross-link the inhibitive Fc-ɣ receptors. This is consistent with the function assay results as aggregation of viruses occurs at pre-attachment steps. I propose to modify the current function assay to test the neutralization mechanism of ADE infection. The modification will include using the MAb-virus complex to replace the virus and using K562 or U937 cells to replace Vero cells. Comparing the results of this modified assay with the results of the original function assay will reveal the neutralization mechanism differences between normal infection and ADE infection.

The third point is to systematically study how factors such temperature and pH affect antibody-mediated neutralization. Variation in temperature may alter the virus structure dynamic, stability, antibody binding affinity, and subsequently neutralization potency or the neutralization mechanism (Dowd, Jost et al. 2011). The function assay uses the temperature 4°C and it may not be an accurate estimation of the MAb neutralization mechanism at 37°C. Besides, in the life cycle of the dengue virus there are at least three temperature switches: 28°C or even lower inside the mosquito, 37°C inside the human and up to 40°C during the fever stage. Therefore, understanding how temperature affects the virus and its interaction with antibodies is indispensable. PH variation has the same effect on virus and antibody interaction. The significance of understanding how pH affects the virus and neutralization is presented in detail in section 7.1.

In summary, the research presented in this dissertation revealed the persistence of cross-reactive antibodies with the potential for dengue virus disease enhancement decades after infection. The antibody's stoichiometry and mechanisms of neutralization were explored and the MAbs' combined neutralization and ADE were also measured and modeled using neutralization assay and ADE assay. The differential neutralization of DENV3 strains by MAb 8A1 was studied in detail and the mechanisms of this differential

neutralization were revealed. Finally, the differential neutralization patterns of the mature and partially mature dengue viruses were investigated and the mechanisms of differential neutralization were identified. The discoveries made and mechanisms revealed in this study will not only shed light on our understanding of how antibodies neutralize dengue viruses, but also provide insight into how dengue viruses evade the immune response. This study offers a rationale for new strategy of dengue vaccine design and provides guidelines for antibody therapy of dengue disease.

Table 7.1 neutralization mechanisms of Monoclonal antibodies

MAbs	Class[#]	Specificity	Potency*	Mechanism^	Source
1A1D-2	mIgG1	cross-reactive	strong	post	Chapter 3
8A1	mIgG1	DENV3	strong	post	Chapter 3
14A4	mIgG1	DENV3	strong	post	Chapter 3
9D1	mIgG1	DENV3	strong	post	Chapter 3
9F16	mIgG1	DENV2	moderate	pre	Chapter 3
2Q1899	mIgG1	DENV2	moderate	pre	Chapter 3
5C36	mIgG1	DENV2	moderate	pre	Chapter 3
2D22	hIgG1	DENV2	strong	post	de Alwis, unpublished data
1F4	hIgG1	DENV1	strong	post	de Alwis, unpublished data
5J7	hIgG1	DENV3	strong	post	de Alwis, unpublished data
DVC3.7	hIgG1	DENV2	strong	post	Chapter 6
DVC25.5	hIgG1	DENV2	strong	post	Chapter 6
DVC10.16	hIgG1	Sub-complex	strong	post	Zhou, Unpublished data
14c10	hIgG1	DENV1	strong	pre	(Teoh, Kukkaro et al. 2012)
9F12	mIgG1	cross	moderate	pre	(Rajamanonmani, Nkenfou et al. 2009)
2A10G6	mIgG1	cross	moderate	post	(Deng, Dai et al. 2011)
4.8A	hIgG1	cross	moderate	post	(Costin, Zaitseva et al. 2013)
D11C,	hIgG1	cross	moderate	post	(Costin, Zaitseva et al. 2013)
1.6D.	hIgG1	cross	moderate	post	(Costin, Zaitseva et al. 2013)
1A5	Fab	cross	moderate	fusion	(Goncalvez, Purcell et al. 2004)
5H2	cIgG1	DENV4	strong	post	(Lai, Goncalvez et al. 2007)
DV2-44, DV2-58, DV2-76, DV2-77, DV2-87, DV2-104.	mIgG1	NA	strong to moderate	post	(Sukupolvi-Petty, Austin et al. 2010)

Notes: [#]: mIgG means mouse origin IgG, hIgG means human origin IgG, cIgG means chimpanzee origin IgG.

* : Strong neutralization is defined as neutralizing at picomolar concentrations. Moderate neutralization is defined as neutralizing at nanomolar concentrations

^ : pre means pre-attachment; post means post-attachment

7.3 References

Armstrong, P. M. and R. Rico-Hesse (2001). "Differential susceptibility of Aedes aegypti to infection by the American and Southeast Asian genotypes of dengue type 2 virus." Vector Borne Zoonotic Dis **1**(2): 159-168.

Beltramello, M., K. L. Williams, et al. (2010). "The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity." Cell Host Microbe **8**(3): 271-283.

Cockburn, J. J., M. E. Navarro Sanchez, et al. (2012). "Mechanism of dengue virus broad cross-neutralization by a monoclonal antibody." Structure **20**(2): 303-314.

Costin, J. M., E. Zaitseva, et al. (2013). "Mechanistic study of broadly neutralizing human monoclonal antibodies against dengue virus that target the fusion loop." J Virol **87**(1): 52-66.

de Alwis, R., M. Beltramello, et al. (2011). "In-depth analysis of the antibody response of individuals exposed to primary dengue virus infection." PLoS Negl Trop Dis **5**(6): e1188.

Dejnirattisai, W., A. Jumnainsong, et al. (2010). "Cross-reacting antibodies enhance dengue virus infection in humans." Science **328**(5979): 745-748.

Deng, Y. Q., J. X. Dai, et al. (2011). "A broadly flavivirus cross-neutralizing monoclonal antibody that recognizes a novel epitope within the fusion loop of E protein." PLoS One **6**(1): e16059.

Goncalvez, A. P., R. H. Purcell, et al. (2004). "Epitope determinants of a chimpanzee Fab antibody that efficiently cross-neutralizes dengue type 1 and type 2 viruses map to inside and in close proximity to fusion loop of the dengue type 2 virus envelope glycoprotein." J Virol **78**(23): 12919-12928.

Hacker, K., L. White, et al. (2009). "N-linked glycans on dengue viruses grown in mammalian and insect cells." J Gen Virol **90**(Pt 9): 2097-2106.

Halstead, S. B. (2007). "Dengue." Lancet **370**(9599): 1644-1652.

He, R. T., B. L. Innis, et al. (1995). "Antibodies that block virus attachment to Vero cells are a major component of the human neutralizing antibody response against dengue virus type 2." J Med Virol **45**(4): 451-461.

Lai, C. J., A. P. Goncalvez, et al. (2007). "Epitope determinants of a chimpanzee dengue virus type 4 (DENV-4)-neutralizing antibody and protection against DENV-4 challenge in mice and rhesus monkeys by passively transferred humanized antibody." J Virol **81**(23): 12766-12774.

Lai, C. Y., W. Y. Tsai, et al. (2008). "Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II." J Virol **82**(13): 6631-6643.

Leitmeyer, K. C., D. W. Vaughn, et al. (1999). "Dengue virus structural differences that correlate with pathogenesis." J Virol **73**(6): 4738-4747.

Lok, S. M., V. Kostyuchenko, et al. (2008). "Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins." Nat Struct Mol Biol **15**(3): 312-317.

Messer, W. B., U. T. Vitarana, et al. (2002). "Epidemiology of dengue in Sri Lanka before and after the emergence of epidemic dengue hemorrhagic fever." Am J Trop Med Hyg **66**(6): 765-773.

Munoz-Jordan, J. L. (2010). "Subversion of interferon by dengue virus." Curr Top Microbiol Immunol **338**: 35-44.

Neumann-Haefelin, C., H. E. Blum, et al. (2005). "T cell response in hepatitis C virus infection." J Clin Virol **32**(2): 75-85.

O'Beirne, J., J. Mitchell, et al. (2009). "Inhibition of major histocompatibility complex Class I antigen presentation by hepatitis C virus core protein in myeloid dendritic cells." Virology **389**(1-2): 1-7.

Pape, G. R., T. J. Gerlach, et al. (1999). "Role of the specific T-cell response for clearance and control of hepatitis C virus." J Viral Hepat **6 Suppl 1**: 36-40.

Rajamanonmani, R., C. Nkenfou, et al. (2009). "On a mouse monoclonal antibody that neutralizes all four dengue virus serotypes." J Gen Virol **90**(Pt 4): 799-809.

Smith, S. A., Y. Zhou, et al. (2012). "Persistence of circulating memory B cell clones with potential for dengue virus disease enhancement for decades following infection." J Virol **86**(5): 2665-2675.

Sukupolvi-Petty, S., S. K. Austin, et al. (2010). "Structure and function analysis of therapeutic monoclonal antibodies against dengue virus type 2." J Virol **84**(18): 9227-9239.

Teoh, E. P., P. Kukkaro, et al. (2012). "The structural basis for serotype-specific neutralization of dengue virus by a human antibody." Sci Transl Med **4**(139): 139ra183.

Waggoner, S. N., C. H. Hall, et al. (2007). "HCV core protein interaction with gC1q receptor inhibits Th1 differentiation of CD4+ T cells via suppression of dendritic cell IL-12 production." J Leukoc Biol **82**(6): 1407-1419.

Wahala, W. M., A. A. Kraus, et al. (2009). "Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody." Virology **392**(1): 103-113.