QUALITIES OF THE PROTECTIVE ANTIBODY RESPONSE AGAINST DENGUE VIRUS INFECTION

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ABSTRACT

Cameron R. Adams: Qualities of the protective antibody response against dengue virus infection
(Under the direction of Aravinda de Silva)

Dengue viruses (DENV1-4) cause 390 million annual infections across the globe. Primary DENV infection elicits an antibody response that is thought to provide lifelong protection against re-infection with the same serotype. A protective antibody response provided by vaccination would be the best tool available to prevent DENV related disease but the currently licensed DENV vaccine had variable efficacy. We still have large knowledge gaps that need to be addressed surrounding qualities of protective antibodies.

My thesis defines three properties of the protective antibody response to DENV infection and vaccination. Isolation and characterization of potently neutralizing monoclonal antibodies identified the majority of these antibodies bind across multiple DENV E-proteins (quaternary epitopes) on the virion surface. For my thesis, I defined the epitope of a potently neutralizing antibody to the closely related Zika virus (ZIKV) and used its structure to guide questions about the mechanism of potent neutralization. I identify that targeting a quaternary epitope is essential for potent neutralization. Recently, a DENV vaccine that induced neutralizing antibodies did not demonstrate protection in DENV naïve individuals. To understand qualities of a protective neutralizing antibody response, I compared the antibody response elicited by WT DENV infection to the antibody response elicited in DENV vaccinated individuals who subsequently experience infection. I observed a specific fraction of the neutralizing antibody response correlated with protection. While antibody response to primary infection protects against clinically significant disease, it is unclear if the antibody response provides sterilizing immunity as rare, homotypic reinfections are reported. To understand if antibody response to primary infection provides sterilizing immunity, I used a unbalanced DENV vaccine as a human challenge model. The majority of individuals

respond to the vaccine, regardless of DENV serostatus. My results help guide future development of DENV vaccines to achieve a protective, balanced vaccine.

ACKNOWLEDGEMENTS

I am a product of the environment Aravinda de Silva has fostered in his laboratory. I came to UNC as part of the UNC MSTP program. My focus was to ask questions in translational science where we could apply our findings directly to clinical medicine. My previous work informed me that vaccinology, specifically antibody response to vaccines and infection, was the route I wanted to pursue. I was lucky to have a friend recommend the de Silva lab to me during my introductory BBSP group. Aravinda was the perfect fit in a mentor to develop skills to pursue my goals of integrating clinical and research practices to perform research from bench to bedside. He is a patient teacher who is willing to stay after the workday has ended to ensure I understand the topic at hand. His support is greatly appreciated over the last four years.

Members of the de Silva laboratory have played a large role in my development as a scientist. My co-mentor, Premkumar Lakshmanane, helped me hone skills in dissecting antibody structure and asking structure guided questions. Laura White has taught me how to organize a large cohort and design experiments to ask specific questions. Sandra Henein provided support and a partner in crime throughout my PhD. Ramesh Jadi helped to complete the enormous task of understanding the dengue virus serostatus of our Cebu cohort. Usha Nivarthi often would help me to address flaws in my experimental designs. Emily Freeman, Elizabeth Adams, and Demetri Samaras provided much needed and appreciated technical support in the last year of this PhD. I appreciated the insight and support both Devina Thiono and Caitlyn Molloy gave throughout my PhD. I am a product of the time I have accrued with all of these individuals and I deeply appreciate the effort they put into helping me develop as a scientist.

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Most of all, I am a product of the two people that raised me. I'd like to thank both of my parents for the support they provided all the way across the United States. Thank you for helping me along this journey and for putting me in a position to succeed. You two are the best.

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LIST OF ABBREVIATIONS

DENV- Dengue virus

ZIKV- Zika virus

JEV- Japanese encephalitis virus

YFV- Yellow fever virus

E-protein- envelope protein

DSS- dengue shock syndrome

DHF- dengue hemorrhagic fever

prM- premembrane

ER- endoplasmic reticulum

EDI- envelope domain 1

EDII- envelope domain 2

EDIII- envelope domain 3

NS1- nonstructural protein 1

ADE- antibody dependent enhancement

CYD-TDV- Dengvaxia

mAb- monoclonal antibody

Fab- fragment, antigen binding

FRNT- focus reduction neutralization test

CDRH- complementarity determining region heavy chain

FWRH- framework region heavy chain

Nab- neutralizing antibody

TS- type-specific antibody

CR- cross-reactive antibody

NHS- normal human sera

FcyR- Fcy receptor

CHAPTER 1: INTRODUCTION

1.1 Summary:

Dengue virus serotypes 1-4 (DENV1-4) and Zika virus (ZIKV) are members of the *Flaviviridae* family that infect several hundred million people living in tropical regions of the world (*1*). A primary DENV or ZIKV infection stimulates antibodies that prevent re-infection by the same serotype (*2-4*) but these antibodies can also enhance the replication of other serotypes and increase the risk of severe DENV disease (*5, 6*). While several live attenuated DENV vaccines are at different stages of clinical development, vaccine trials have demonstrated that antibodies stimulated by leading vaccines can be protective or pathogenic depending on the vaccine and challenge DENV serotype (*7*). The goal of my thesis was to define the properties of flavivirus protective antibodies induced by wild type virus infection or vaccination. Definition of the properties of protective antibodies will aid in development of more efficacious vaccines. This chapter summarizes the current understanding of antibody responses to flavivirus infection and vaccination.

1.2 DENV and ZIKV Overview:

ZIKV and DENV1-4 are mosquito borne members of the *Flaviviridae* family. Other notable, mosquito borne members of this family include two well described neurotropic viruses, West Nile virus and Japanese encephalitis virus (JEV), as well as yellow fever virus (YFV). There is currently a highly protective vaccine for both JEV and YFV. DENV has the highest global health burden of the members of the *Flaviviridae* family with an estimated 390 million (96 million symptomatic) annual DENV infections across the globe (*1*). In contrast, ZIKV has caused sporadic outbreaks in Asia and the Pacific Islands (*8-10*) before causing a large epidemic beginning in 2015 in the Americas(*11, 12*). The vast majority (~80%) of DENV and ZIKV infections are asymptomatic. Acute symptomatic DENV and ZIKV infection results in febrile illness alongside myalgia, headache, and, in case of ZIKV, a maculopapular rash. Rare but severe

clinical manifestations of DENV infection include dengue hemorrhagic fever (DHF) and dengue shock syndrome(DSS) (*6, 13*). In the case of DENV, severe disease is more common during secondary DENV infection with a new serotype. DENV cross-reactive and non-neutralizing antibodies induced by a first infection bind to the DENV envelope (E) protein of the infecting second DENV serotype to provide an independent entry pathway to increase replication and disease severity (*5, 6, 13, 14*). This process is known as antibody dependent enhancement (ADE). Additionally, the DENV non-structural protein 1 (NS1) plays a role in triggering hyperpermeability of endothelial cells by destroying the endothelial glycocalyx layer (*15*). Severe ZIKV outcomes are described most often in pregnant women who were infected with ZIKV during the first or second trimester (*12*). ZIKV was identified as the first novel viral teratogen in the last half century. Clinical manifestations in the newborns of pregnant women infected with ZIKV include microcephaly and ocular malformations. Newer evidence suggests prior ZIKV infection can increase likelihood of severe DENV illness (*6*).

1.3 Flavivirus Entry and Replication:

Flaviviruses are positive sense, ssRNA viruses **(Figure 1.1A)** (*16*). Flaviviruses attach to the cell surface and enter through clathrin-mediated endocytosis (**Figure 1.1B)** (*17, 18*). In response to the acidic pH of the early endosomes, the flavivirus E-protein undergoes rearrangement on the virion surface required for viral fusion **(Figure 1.2).** E-protein dimers on the virion surface rearrange to fusogenic E-protein trimers. These E-protein trimers facilitate fusion of viral membrane with host cell membrane to release flavivirus genomic material for initiation of viral replication. The 11kb, capped, positive sense, ssRNA is translated on the cytoplasmic side of the endoplasmic reticulum (ER) to produce a ER associated transmembrane polyprotein. The polyprotein is cleaved by host and viral proteases into the three structural proteins: Capsid, E- and preMembrane (prM). The polyprotein also is cleaved into non-structural proteins that assist in producing new viral RNA. Viral RNA is incorporated into assembling viral particles that bud into the ER. Immature, viral particles are transported through the secretory pathway to the cell surface. During egress, immature virions are acted upon by the host Furin enzyme that cleaves the prM protein, resulting in a fully mature virion released from the infected cell.

1.4 DENV Protective Antibodies:

The human antibody response against primary DENV infection protects against reinfection by the same serotype (*2-4*). Conversely, cross-reactive antibodies promote severe DENV disease outcomes through ADE (*5*). In an effort to prevent DENV infection, multiple live attenuated tetravalent vaccines are in development. A recently licensed live attenuated tetravalent chimeric vaccine, Dengvaxia (CYD-TDV), demonstrated a neutralizing antibody response against DENV1-4 but had little to no efficacy in naïve individuals (*7*). The focus of my thesis was to understand the properties and mechanisms of the protective neutralizing antibody response to flavivirus WT infection and vaccination. The subsequent chapters of my thesis address the mechanism of potent neutralizing antibodies, identify an antibody immune correlate of protection, and understand the role of primary immunity in providing sterilizing immunity.

1.5 Flavivirus Virion Structure:

To understand how antibodies neutralize flaviviruses and provide protection, it is necessary to review the structure of flavivirus virions. The outer shell on mature flavivirus virions consist of 180 copies of the E-protein. The E-protein is a major target of the human antibody response to flaviviruses. The E-protein consists of three domains, envelope domain I (EDI), envelope domain II (EDII), and envelope domain III (EDIII) **(Figure 1.3)** (*19, 20*). EDIII contributes to binding to the host cell(*21, 22*) while EDII contains the hydrophobic fusion loop necessary to initiate flavivirus fusion. E-proteins are organized in a head to tail orientation to form E-dimers on the viral surface (*23, 24*). Three of these E-dimers form an antigenic raft and 30 dimer rafts come together to form a herringbone pattern. Murine polyclonal neutralizing antibody responses heavily target EDIII(*25, 26*) while the human antibody response targets epitopes that bind across multiple E-proteins(*25*). This repeating structure offers 180 potential binding sites for each antibody on the surface of a single virion but all sites are unlikely to be bound because of steric effects. A “multiple-hit” model of antibody neutralization was proposed where a minimum threshold of occupied antibody binding sites is needed to neutralize the infecting virus (*27*). This model identifies both antibody affinity and epitope accessibility as factors in antibody neutralization.

1.6 Flavivirus Processing and Maturation:

prM/E-protein heterodimers are incorporated into the immature virus particle that is transported through the secretory pathway to exit the cell. During this process, the prM protein is implicated in preventing the E-protein from undergoing premature conformational changes due to the acidic conditions of the trans-Golgi network (*28*). Intracellular flaviviruses remain in the immature form until they are cleaved by the host enzyme Furin **(Figure 1.1B)**. Furin cleaves the prM protein prior to virion release into the extracellular environment to convert the bumpy, immature virion to a fully mature, smooth virion (*23, 29*) Vero-81 cell culture lines used to traditionally produce DENV stocks lack the Furin enzyme, resulting in partially or fully immature virus stocks that are not reflective of virions circulating in humans (*30*). Comparisons between immature virus stocks produced in traditional Vero-81 cell lines and fully mature virus stocks grown in a Furin over-expressing Vero-81 cell lines demonstrated that cell culture stocks are more easily neutralized in in-vitro neutralization tests. The use of immature stocks may overestimate the neutralizing antibody response and confound analysis that rely upon identification of neutralizing antibody populations.

1.7 Antibody Response to Primary Flavivirus Infection:

Infection by flaviviruses elicits a robust antibody response (*25, 31*). Primary DENV infection elicits a neutralizing antibody that provides lifetime protection in the majority of individuals against reinfection by the same serotype and a transient cross-protective antibody response that protects against other DENV serotypes. As this transient cross-protective antibody response wanes, the risk of DHF and DSS increase (*5*). In rare cases, reinfection with the same serotype is documented (*32*). Only a small subset of the antibody response to primary DENV infection can potently neutralize the infecting virus (*4, 33*). To further characterize the potently neutralizing portion of the polyclonal antibody response, multiple potently neutralizing monoclonal antibodies (mAbs) were isolated from memory B-cells and plasmablasts. Early in primary DENV infection, naïve B-cells activate and proliferate into antibody secreting cells known as plasmablasts. Plasmablasts contribute to the early antibody response to infection by secreting antibodies specific to the infecting serotype as well as cross-reactive antibodies that can bind all four DENV serotypes (*34*). This mixed population of serotype specific and cross-reactive antibodies in polyclonal

sera is maintained after primary infection by DENV specific plasma cells (*35, 36*). Additionally, DENV specific memory B-cells continue to circulate after resolution of the initial DENV infection and provide a line of defense against re-infection.

Multiple independent groups isolated and characterized potently neutralizing mAbs against DENV (*37-40*). The majority of these potently neutralizing mAbs are specific in binding and neutralization to a single DENV serotype and demonstrate protection in a murine DENV challenge model (*37, 39*). Epitopes of these mAbs defined through Cryo-electron microscopy and X-ray crystallography revealed these antibodies bind to the DENV E-protein across different E-protein domains. **Figure 1.4** displays the structurally defined epitopes of potently neutralizing antibodies against DENV1-4 as well as other epitopes targeted by antibodies that are specific to JEV, ZIKV and YFV (*37-43*). What the majority of potently neutralizing human mAbs share is they target an epitope that spans 2 or more E-proteins (quaternary epitope). As E-proteins rearrange to achieve a fusogenic state to complete viral entry, quaternary epitope targeting antibodies are suggested to block pH induced rearrangement of the E-protein surface (*43, 44*). Analysis of polyclonal sera from primary infection demonstrates the serotype specific antibody fraction provides the majority of in vitro neutralization (*45*) and identified quaternary epitopes are heavily represented in polyclonal sera after DENV infection (*46-49*).

Alongside potently neutralizing antibodies, other cross-reactive antibodies were isolated after primary infections (*50, 51*). Mapping of individual cross-reactive monoclonal antibodies revealed the majority target either the prM protein or the highly conserved fusion loop of the DENV E-protein. Only a small fraction of the persistent cross-reactive antibody population (*51*) after primary infection demonstrates in vitro neutralizing activity while most were identified to contribute to antibody dependent enhancement (*52, 53*). Studies in murine challenge models have illuminated weakly neutralizing antibodies are less protective than their serotype specific counterparts (*50*).

Thus far, we understand that a neutralizing antibody response is protective against reinfection by the same serotype and antibodies targeting quaternary epitopes are important for potent neutralization. To develop and, more importantly, evaluate the next generation of DENV vaccines, we need to understand the molecular mechanism that underscores potent antibody neutralization. Chapter two of my

thesis identified this mechanism by using a novel, potently neutralizing ZIKV specific monoclonal antibody as a model for antibodies targeting quaternary epitopes.

1.8 Alternative Mechanisms of Antibody Protection:

Potently neutralizing antibodies can directly block flavivirus replication by inhibiting viral attachment to target cells or inhibiting viral membrane fusion. Possible additional mechanisms of antibody mediated protection include activation of Fcy-receptor (FcyR) dependent functions and inhibiting flavivirus NS1 pathogenesis. Antibody responses to other viral infections demonstrated non-neutralizing antibodies can interact with FcyR on targeted immune cells to initiate a cascade resulting in antibody dependent cellular cytotoxicity and phagocytosis. (*54-56*) Some subclasses of FcyR can inhibit DENV infection (*57*) while other FcyR subclasses interactions are proposed to contribute to the ADE mechanism. (*58, 59*) Previous studies demonstrated the polyclonal antibody response to flavivirus infection targets NS1. (*60*) Antibody binding to NS1 blocks both NS1 induced pathology(*61*) and severe outcomes of ZIKV infection.(*62, 63*) Specific epitope targeting to DENV NS1 is suggested to correlate with disease outcomes in humans. (*64*)

1.9 Antibody Response to Secondary DENV Infection:

Secondary infection with a different DENV serotype carries an increased risk of DHF and DSS. Upon secondary infection, the DENV-specific plasmablast compartment expands (*65, 66*) to produce hypermutated and cross-neutralizing antibodies that can neutralize all four DENV serotypes (*67*). This broadly neutralizing antibody response protects against subsequent infections from DENV, even to serotypes that have never infected the host as tertiary DENV infections are rarely detected (*68*). Only two classes of potently cross-neutralizing monoclonal antibodies from secondary infection have been isolated. One class anchors in the EDI/EDII region of the DENV E-protein and cross neutralizes ZIKV(*69, 70*). Another recently defined class lacks a high resolution structure but alanine scanning studies using the DENV E-protein suggests the antibodies are anchored in EDI(*71*). Currently the model of developing cross-neutralizing antibody response after secondary infection is low affinity, cross-reactive secreting B-

cells from primary infection further evolve to secrete cross-neutralizing antibodies but further evidence is needed to support this (*33*).

1.10 Current State of DENV Vaccines and Lessons from CYD-TDV:

With the significant burden of disease, multiple DENV vaccines are in development (*72*). Two live-attenuated tetravalent vaccines, TAK-003 and TV003/TV005, are currently completing phase III clinical trials. TAK-003 was developed by Takeda. TV003/TV005 was developed by NIAID. An interim analysis at 18 months post-vaccination demonstrated TAK-003 was well tolerated and efficacious against symptomatic DENV regardless of DENV serostatus prior to vaccination (*73*). TV003/TV005 is still in the process of phase III clinical trials(NCT02406729) but was safe, well tolerated and immunogenic in participants after a single dose (*74*).

CYD-TDV manufactured by Sanofi Pasteur (SP) is the only DENV vaccine that has completed phase III clinical trials. CYD-TDV is a chimeric formulation that inserted the antigenic prM and E-proteins of DENV1-4 separately into the YFV 17D vaccine backbone. Prior to licensure, CYD-TDV was tested in 26 clinical trials (*75*) culminating in two large, phase III clinical trials, CYD14(*76*) and CYD15(*73*). CYD14 (located in Asia, subjects 2-14 years old) and CYD15 (Latin America, 9-16 years old) enrolled pediatric patients to receive the three dose regimen of CYD-TDV over the course of 1 year. Both trials demonstrated neutralizing antibody response after vaccination. Vaccine efficacy was calculated 13 months after the final dose and CYD14/15 reported similar efficacy against DENV1-4 (CYD14 = 60.8% (95% CI 52-68) CYD15 = 56.5% (43.8-66.4)). The differences in antibody response and clinical outcome after WT DENV infection require stratifying DENV vaccine performance based upon pre-vaccination DENV serostatus. After completion of CYD14/15, SP performed a retrospective analysis on the vaccinated individuals during their phase III clinical trials to understand CYD-TDV vaccine performance stratified by pre-vaccination DENV serostatus. SP collected a serum sample prior to vaccination in only a small subset of individuals. To establish pre-vaccination DENV serostatus, SP developed a DENV NS1 ELISA (*77*) to identify individuals with prior DENV immunity. SP demonstrated in individuals who were DENV naïve prior to vaccination, the vaccine had no efficacy against DENV1-3 during the phase III clinical trials (*7*). Additionally, DENV naive individuals had a higher risk of hospitalization due to DENV

infection during the extended follow up period. Tragically, these results were released after the start of a school based immunization program in the Philippines wherein 830,000 children received a single dose of CYD-TDV (*75*). The vaccination campaign and later identification of increased risk of severe disease after vaccination is an ongoing a public health disaster in the Philippines. Additionally, the CYD-TDV story has lowered overall vaccine confidence and the Philippines has experienced a rise in identified cases of vaccine preventable diseases (*78, 79*). While age has been suggested as a factor influencing vaccine efficacy, it is more likely baseline serostatus plays a role in immunological and clinical outcome (*80*). Currently the WHO SAGE group recommends a pre-vaccination screening strategy among countries considering a DENV vaccination program within the target age group of 9 to 45 years (*81, 82*).

The differential protection afforded by CYD-TDV offers an opportunity to better understand mechanisms of antibody protection. Two other successful flavivirus vaccines, SA-14-14-2 and YFV-17D, are licensed and prevent disease caused by JEV and YFV respectively. Neutralizing antibody response in both vaccines are identified as a correlate of protection (*83-87*). CYD14, CYD15, and a phase IIb clinical trial (CYD23) demonstrated a neutralizing antibody response was elicited by the vaccine but also found variable efficacy against DENV1-4. CYD23 was a phase IIb clinical trial conducted in Thailand (*88*). Mirroring findings in CYD14/15, CYD-TDV elicited a neutralizing antibody response in CYD23 but had low efficacy (30.2% (-13.4-56.6)) against DENV1-4 measured at 25 months. These three trials highlight that neutralizing antibodies alone are not the sole predictor of DENV vaccine efficacy. Chapter three of my thesis defines the qualities of protective neutralizing antibodies by using CYD14/15 vaccinated individuals who experienced a DENV1 or DENV3 infection during the phase III clinical trial.

It is unknown if DENV antibodies provide sterilizing immunity. A monotypic or unbalanced DENV vaccine is likely to operate as a primary infection, carrying risks of developing severe disease with the elicitation of a cross-reactive antibody population. A fully balanced, tetravalent DENV vaccine where each individual component replicates equally would potentially elicit a balanced immune response to provide protection against DENV1-4. Evidence emerged CYD-TDV was an unbalanced vaccine weighted towards the DENV4 component. A phase II clinical trial that measured viremia after a single dose of the tetravalent formulation demonstrated DENV4 was detected more frequently than the other three components (*89*). Analysis of antibody response after the final dose of CYD-TDV in healthy, DENV naïve

individuals identified the antibody response was heavily weighted towards the DENV4 component of the vaccine (*90*). Together, these data suggest CYD-TDV is similar to a DENV4 primary infection. This is further supported by high efficacy against DENV4 and the increased risk of hospitalization in vaccinated naïve individuals(*7*). DENV human challenge models to understand if antibodies provide sterilizing immunity are cost-intensive but CYD-TDV offers the opportunity to perform a challenge model as it operates mostly as a primary DENV4 infection. Chapter 4 of my thesis uses the unbalanced CYD-TDV to answer questions about DENV antibodies and sterilizing immunity.

1.11 Questions Remaining About Protective Antibodies Answered in My Dissertation:

Chapter 2: What is the mechanism of potent neutralization by flavivirus specific monoclonal antibodies?

Chapter 3: What is the DENV immune correlate of protection in DENV vaccinated naïve individuals?

Chapter 4: Do DENV antibodies provide sterilizing immunity?

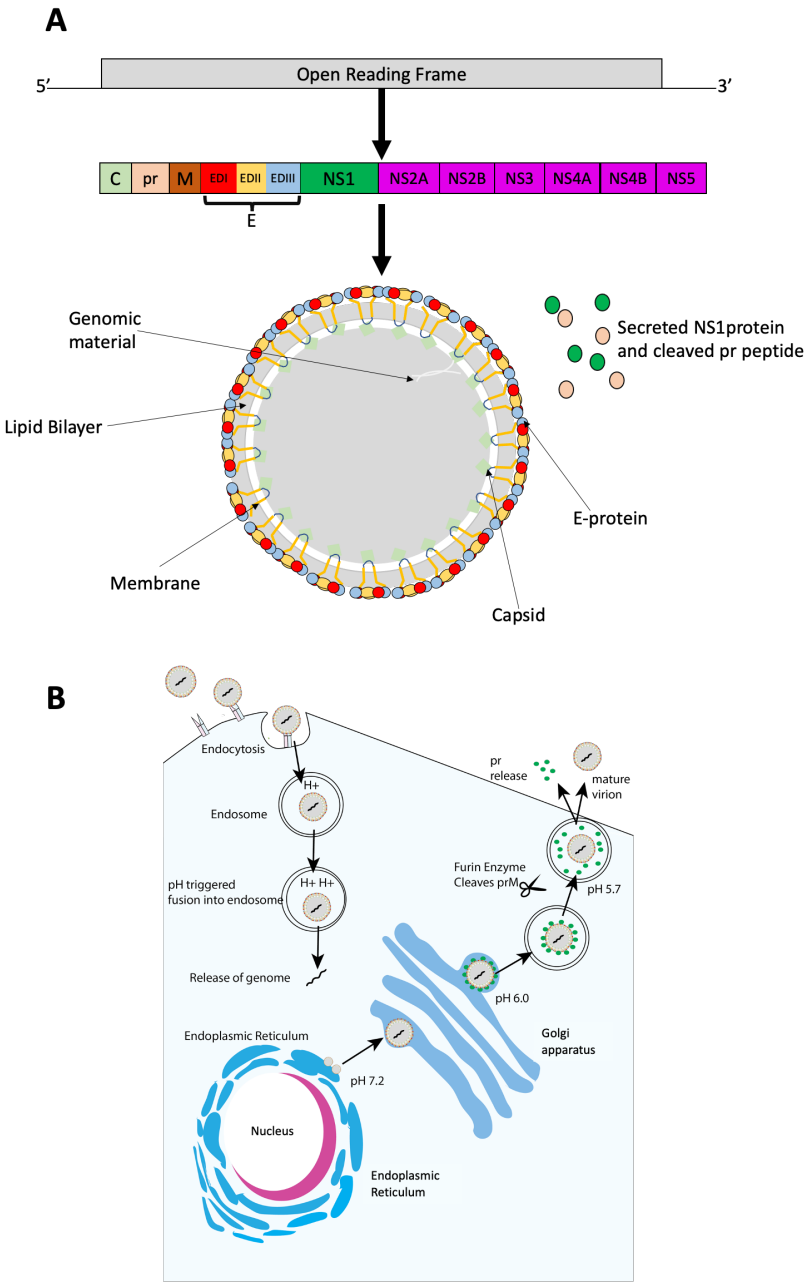


Figure 1.1 Schematic of Flavivirus polyprotein, entry, and egress

A) Flavivirus genome encodes a polyprotein that is cleaved and processed into capsid (C), prM(prM), envelope (E), non-structural protein 1 (NS1) and other non-structural proteins. **B)** Flaviviruses enter through the endosomal pathway and replicates on the cytoplasmic side of the ER. As flaviviruses leave the host cell, host enzyme Furin cleaves prM to produce a mature, infectious particle.

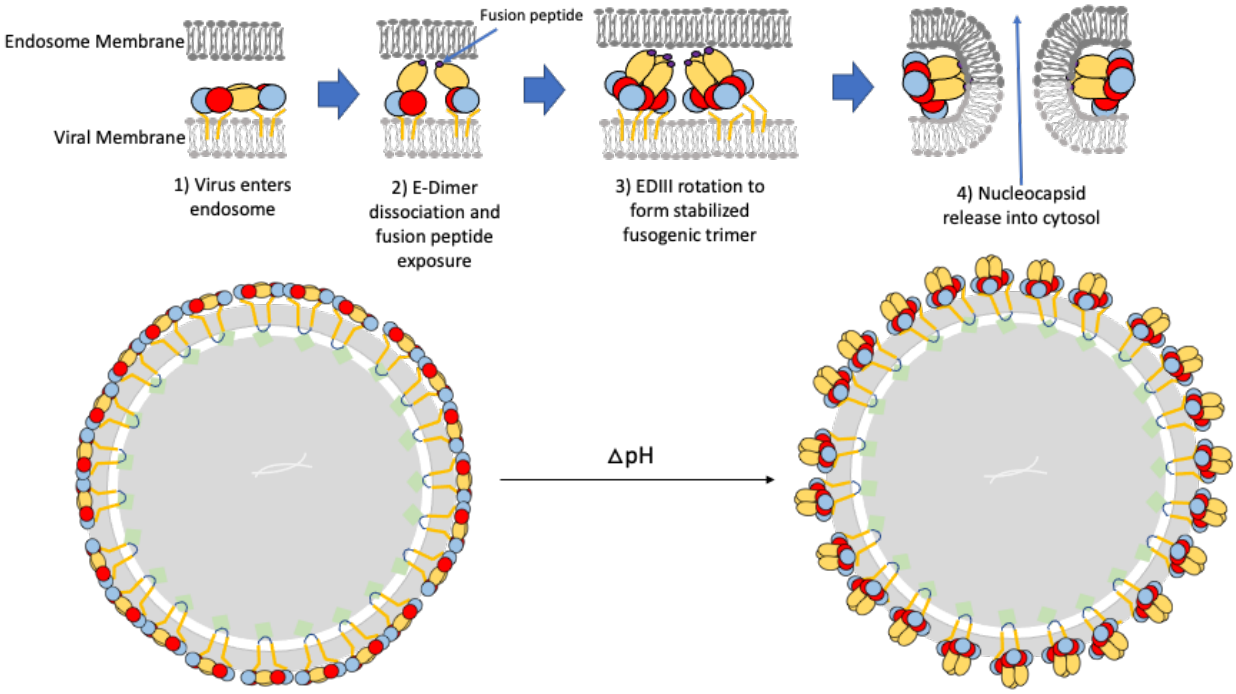


Figure 1.2. Change in E-protein during viral entry.

During endosomal entry, E-protein reacts to changing pH to rearrange into fusogenic trimer. The highly conserved fusion peptide inserts into endosomal membrane to complete viral entry

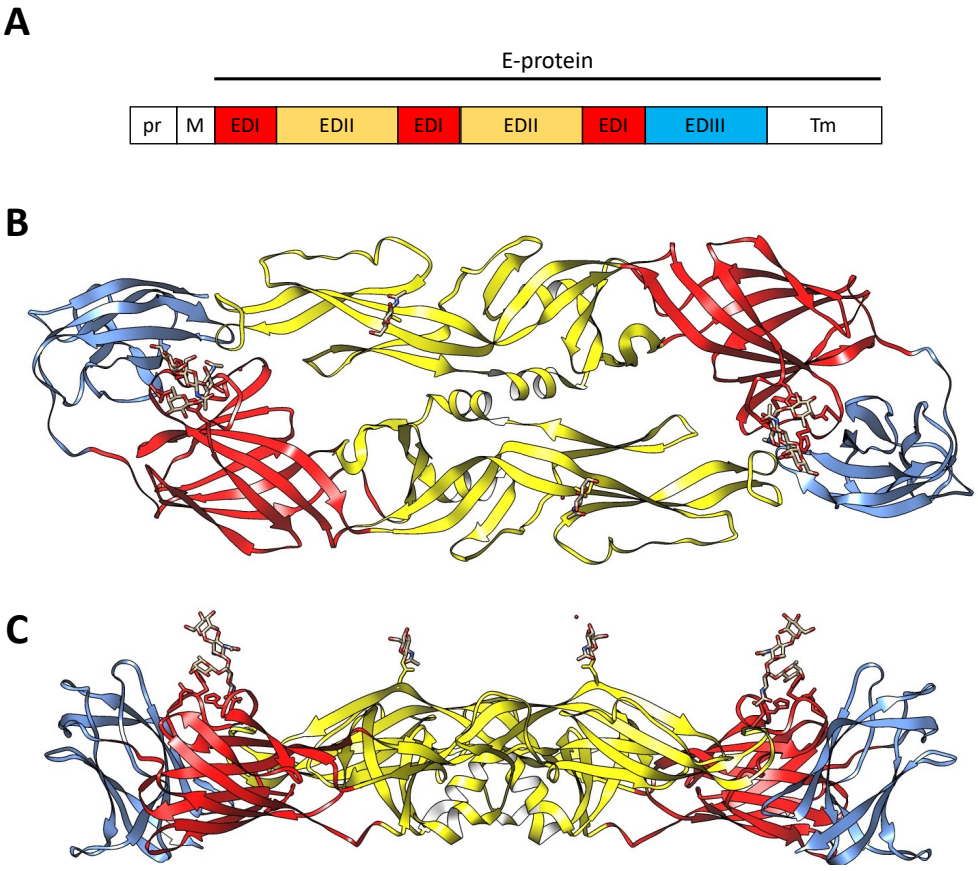


Figure 1.3. DENV E-protein structure.

A) Schematic of DENV E-protein. DENV E-protein comprises three domains, domain 1 (red), domain 2 (yellow), domain III (blue). **B)** DENV2 E-protein with domains colored. **C)** Side view of DENV2 E-protein with domains colored. Glycosylation at amino acid positions 67 and 154 are represented by ball and stick model. E-protein images are generated using DENV2 crystal structure (PDB: 1OAN)

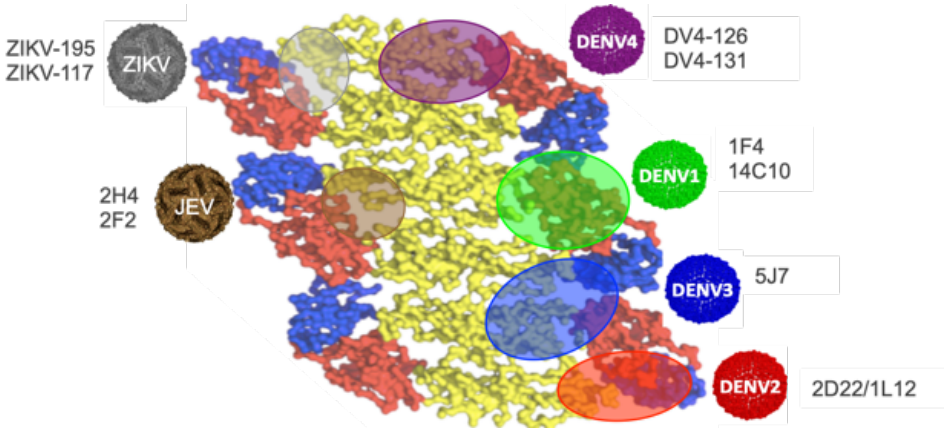


Figure 1.4 Epitopes of potently neutralizing antibodies on flavivirus E-protein.

Potently neutralizing mAbs target the Flavivirus E-protein. Ovals delineate epitope targeted by isolated, potently neutralizing, virus specific mAbs. Potently neutralizing mAbs target epitopes that span multiple E-proteins.

CHAPTER 2: HIGH-RESOLUTION STRUCTURE AND NEUTRALIZATION MECHANISM OF A HUMAN ANTIBODY TARGETING A COMPLEX EPITOPE ON ZIKA VIRUS

2.1 Overview:

We currently have a poor understanding of why only a fraction of human antibodies that bind to flaviviruses block infection of cells. Here we describe the structure of a strongly neutralizing human monoclonal antibody (mAb G9E) in complex with Zika virus (ZIKV) E protein at 3.4 Å resolution. G9E bound to a quaternary structure epitope that spanned two E molecules on the viral surface. G9E neutralized ZIKV by blocking a step after viral attachment to cells, most likely viral membrane fusion. We tested if the neutralization mechanism of G9E was dependent on the mAb cross-linking E molecules, which would block conformational changes required for fusion. By introducing targeted mutations to the G9E paratope, we demonstrate that virus neutralization is mainly due to antibody mediated E protein cross-linking. We propose that, beyond antibody binding strength, the ability of human antibodies to cross-link E-proteins is a critical determinant of flavivirus neutralization potency.

2.2 Introduction:

Zika virus (ZIKV) is one of several medically important flaviviruses transmitted by mosquitos or ticks to humans (*91-94*). Flaviviruses are positive-sense RNA viruses of approximately 50 nm in diameter with a lipid bilayer containing the envelope (E) and premembrane (prM) glycoproteins (*16*). The ectodomain of E protein is composed of two non-continuous domains (EDI and EDII) and a continuous immunoglobulin-like domain (EDIII). E monomers assemble into stable homodimers and 90 homodimers assemble to form a smooth protein envelope with icosahedral symmetry (*95*).

The E glycoprotein is essential for viral attachment and entry into host cells (*95*). The antibody response in individuals exposed to flaviviruses heavily targets the E protein but some E protein binding antibodies strongly neutralize flaviviruses, while other antibodies fail to neutralize at all (*2*). For dengue viruses, non-neutralizing antibodies are implicated in enhanced viral replication and more severe disease (*6*). We currently have an incomplete understanding of why some E protein specific antibodies potently neutralize flaviviruses, while others fail to do so. To better define the structural basis and mechanism of antibody neutralization, here we focus on human monoclonal antibody (mAb) G9E isolated from a Zika immune individual. G9E strongly neutralizes ZIKV, protects mice from a lethal ZIKV challenge, and binds to a region on the virus commonly targeted by other human antibodies(*96*).

2.3 Results:

To define the footprint of G9E bound to ZIKV E protein, we expressed and purified the ectodomain of ZIKV E and the antigen binding (Fab) fragment of G9E. The G9E Fab retained neutralizing activity against ZIKV (strain H/PF/2013) in a cell culture focus reduction neutralization test (FRNT) **(Figure 2.1).** The ZIKV-E/G9E-Fab complex was purified by size exclusion chromatography, and the crystal structure of the complex was determined by molecular replacement. The resulting electron density map resolved the polypeptide chain and revealed the fine details of E protein interaction with G9E at 3.4 Å resolution **(Figure 2.2, Figure 2.3)**

The crystallographic asymmetric unit contains a hetero-hexameric subunit formed by two G9E Fab fragments and one ZIKV E protein homodimer **(Figure 2.2A)**. Both Fabs exhibited a similar mode of binding to one E protein dimer. Structural alignment between the two copies of the E proteins or the two copies of the heavy or light chains revealed that they are highly similar, judged by the low root mean square deviations (0.34–0.53 Å for equivalent Ca atoms). While overall E protein structure was largely retained upon binding to G9E Fab, superimposition of E monomers in the G9E Fab complex and the previously determined ZIKV E protein structure (PDB ID 5JHM) showed global domain shifts in EDII and EDIII. This included inward movements of the fusion loop region by 2 Å, and the movement of EDIII by 3 Å toward the highly ordered EDI N154 glycan loop, causing an increase in the E homodimer interface by 120 $Å^2$ **(Figure 2.4).**

The ZIKV-E/G9E-Fab complex structure revealed that each Fab fragment cross-linked the E homodimer by binding to a quaternary structure epitope spanning the homodimer **(Figure 2.2B)**. The G9E Fab footprint covers a buried surface area (BSA) of 988 $Å^2$, of which 73% of the BSA (709 $Å^2$) comprises the majority of EDII of one E protein **(Figure 2.2B, Site 1)**. The smaller BSA is formed on the adjacent homodimer E protein involving the EDI N154 glycan loop and the EDI-EDII hinge region **(Figure 2.2B, Site 2)**. In each Fab, the heavy chain variable domain binds to both site1 and 2 and contributes ~74% (731 $Å^2$) to the BSA. In comparison, the light chain variable domain binds only to site 1, contributing ~ 26% (257 $Å^2$) to the BSA.

The G9E footprint on ZIKV E-protein overlaps with the binding sites of a previously characterized ZIKV neutralizing human mAbs, ZIKV-117 (*41*), ZIKV-195 (*42*), and Z20 (*97*) **(Figure 2.2C and 2.2 D).** G9E neutralization potency (5 ng/ml) is similar to ZIKV-117 (6 ng/ml), 15-fold greater than ZIKV-195 (77-600 ng/mL), and >60-fold better than the Z20 (370 ng/ml). Both ZIKV-117 and Z20 were shown to recognize quaternary epitopes on EDII covering BSA of 1132 and 797 $Å^2$, respectively. While G9E and Z20 binding sites are entirely comprised within the E-protein homodimer of the E protein, the ZIKV-117 binding site is defined to have both E homodimer and E dimer-dimer interface, covering three E proteins in the raft. Therefore, each icosahedral E protein coat on the virion can potentially bind up to 180 copies of the G9E or Z20 Fabs, but only 60 copies of ZIKV-117 Fab **(Table 2-1)**. Moreover, our modeling of the ZIKV E/G9E structure indicates that G9E IgG molecules can occupy all 180 Fab binding sites on the virus without steric hindrance **(Figure 2.5)**.

G9E binds to ZIKV but not to the closely related dengue virus (DENV) serocomplex (*96*). ZIKV E protein has a single N-linked glycan on EDI, whereas DENVs have two N-linked glycans, one on EDI and a second glycan at N67 on EDII. By comparative sequence and structural analysis of DENV and ZIKV E proteins, we predicted that introducing an N-linked glycan at position 67 on EDII of ZIKV would block the G9E epitope and prevent antibody binding **(Figure 2.6A)**. To test this hypothesis, we used an infectious clone of ZIKV to generate rZIKV-D67N with the Asp at position

67 changed to Asn thereby introducing a N-linked glycosylation site in ZIKV E analogous to the N67 site in DENV. The recombinant virus was viable and confirmed to have a second glycan at N67 by western blot **(Figure 2.7)**. Next, we compared the ability of different ZIKV-specific mAbs to bind and neutralize WT ZIKV and rZIKV-D67N. As predicted, G9E was unable to bind or neutralize rZIKV-D67N **(Figure 2.6B and 2.6C)**. Among the other ZIKV neutralizing mAbs with epitopes that overlap with G9E, Z20 failed to neutralize rZIKV-D67N, whereas our humanized version of ZIKV-117 retained the neutralizing activity against rZIKV-D67N. Human mAbs that bind to EDIII (ZKA190), EDI (A9E), and the E dimer dependent epitope conserved between DENVs and ZIKV (EDE C8) bound and neutralized both WT and rZIKV-D67N **(Figure 2.6B and 2.6C)**. These results establish that a site on ZIKV EDII targeted by neutralizing human mAbs is blocked by adding a glycan at position N67, a site generally glycosylated in the four DENV serotypes(*98*).

To evaluate if the ZIKV EDII neutralization site defined by mAb G9E was a target of serum neutralizing antibodies, we compared the ability of convalescent sera from ZIKV patients to neutralize WT ZIKV and rZIKV-D67N. The addition of the glycan at position 67 on EDII reduced the neutralization potency of 7 of 10 ZIKV immune sera tested **(Figure 2.6 D)**. Our results demonstrate that mAb G9E defines an antigenic region centered on EDII that is a major target of serum neutralizing antibodies in ZIKA patients.

We performed studies to define the mechanism of G9E mediated neutralization of ZIKV. In the mature virus, the E protein remains flat on the surface of the virion, burying the fusion loop within the head-to-tail E homodimer. Following viral attachment and entry into cells, the low pH environment within endosomes triggers the rearrangement of E proteins from homodimers to trimers leading to viral envelope fusion with endosomal membranes and the release of the viral nucleocapsid-RNA complex into the cytoplasm (*99-102*). As G9E cross-links E molecules forming a single homodimer, the mAb is likely to neutralize ZIKV by blocking conformational changes required for viral membrane fusion within endosomes. We performed studies to further test this hypothesis.

If G9E mainly neutralizes ZIKV by blocking viral membrane fusion and not viral attachment to cells, the mAb should be able to neutralize ZIKV after the virus attaches to the cell surface **(Figure 2.8A)**. Initially,

we assessed the ability of ZIKV to bind to the cell surface in presence of G9E. We incubated ZIKV with increasing concentrations of G9E and then incubated the virus/mAb mixture with Vero-81 cells at 4 °C to allow viral attachment to the surface but not entry. After washing the cells to remove any unbound virus, we measured levels of cell-associated ZIKV RNA by qPCR. In this pre-attachment assay, G9E reduced the relative amount of ZIKV RNA associated with Vero-81 cells by approximately 50% at a concentration of 200 ng/ml **(Figure 2.8B)**. We subsequently performed the same protocol but now placed virus/mAb/cell mixture at 37°C to measure infection by FRNT. At 200ng/ml under this condition, the mAb completely blocked the ability of the virus to infect the cells **(Figure 2.8C)**. These results indicate that G9E is able to block attachment and steps after attachment required for infection because the virions that bound to the virus in the presence of the mAb were unable to infect cells. To more directly assess if G9E was able to block infection after attachment, we preincubated ZIKV with Vero-81 cells at 4 °C. After washing the cells to remove any unbound virus, we added increasing quantities of mAb for 1 hour at 4 °C and then measured levels of cell-associated ZIKV RNA or ZIKV infection. Under these conditions, the mAb was unable to displace virions already bound to cells but mAb was able to block productive infection of cells at concentrations >20ng/ml **(Figure 2.8B and 2.8C)**. These results demonstrate that G9E is able to block infection at a step after viral attachment to cells.

Our hypothesis that G9E neutralizes ZIKV by cross-linking dimers and preventing fusion implies that a single Fab binding to both sites 1 and 2 is required for the potent neutralizing activity of the mAb. To gain deeper insights into specific G9E-ZIKV E interactions and their contribution to virus neutralization, we analyzed the paratope-epitope interface regions by PISA (Protein Interfaces, Surfaces, and Assemblies).

This analysis revealed that G9E binding to ZIKV E is predominately driven by hydrophilic and electrostatic interactions involving the heavy chain complementarity-determining regions (CDRH)3, CDRH2, and framework region (FWRH)3 of the heavy chain and the light chain CDRL1 and CDRL3 of light chain with the lateral ridge region of EDII (site 1), the EDI 154-glycan loop and

KL-hairpin and FG loop at the EDI-EDII hinge region (site 2) **(Figure 2.9A)**. Four regions on the heavy chain contact site 1 and site 2 residues on the E dimer. First, the CDRH3 (N107, W109, E111) connects to the exposed edge of the B strand (D67, M68, S70, S72, and R73) of EDII by four backbone and two side-chain hydrogen bonds, thereby distinctively extending to the BDC β-sheet on the EDII lateral ridge. Second, the side chains of D53, D54, and S56 of CDRH2 form a salt bridge and hydrogen bonding interactions with R252 from the J strand of EDII. Notably, we previously reported that R252 is also critical for G9E interaction by alanine scan screening (*96*). Third, CDRH2 mediates additional contacts involving the side-chains of D57, Q58 with D278, and K209 from KL-hairpin and FG-loop at the EDI-EDII hinge region (site 2). Lastly, K76 from the FWRH3 forms an electrostatic interaction with E159 on the 154-glycan loop **(Figure 2.9A and 2.9B)**. In comparison, the light chain contacts involve only site 1 mediated by four hydrogen-bonding interactions between G31, Y32, Y34, and Y93 (from CDRL loops L1 and L2) and S66, D67, and K84 (from stands B and E of EDII) (Figure 4A).

To understand the significance of G9E cross-dimer interactions with site2 for ZIKV neutralization, we generated three G9E paratope mutants. These mutants were designed to perturb the ZIKV E G9E interactions in site 1 (G9E-S1), site 2 (G9E-S2) or site 1 and 2 (G9E-S1/2). G9E-S1 has two mutations in the CDR H3 (W109A, E111A) that were expected to disrupt the beta-strand addition to the BDC β-sheet on the EDII lateral ridge. G9E-S2 contains three mutations (D57S, Q58A, K76S) that eliminate the weak interactions between G9E and site 2. G9E-S1/2 has the same mutations as in G9E-S2 and additional mutations to reduce interaction with R252 in site 1 (D53A, D54A, D57S, Q58A, K76S). G9E-S1 entirely lost both binding and neutralization activity, demonstrating that site 1 is the primary binding site, and in the absence of site 1 interaction, site 2 completely lacks functional or binding activity **(Figure 2.9B and 2.9C)**. G9E-S2 showed no difference in binding, whereas its neutralization activity sharply decreased by greater than 100-fold compared to G9E **(Figure 2.9C)**. G9E-S1/2 demonstrated a 6-fold loss of binding affinity compared to G9E and a reduction in neutralization potency that was comparable to G9E-S2 alone **(Figure 2.9B and 2.9C).** These data suggest that G9E interaction with E homodimer via site 2 plays a critical role in enhancing the potency of neutralizing activity, even though its isolated contribution is minimal to the

overall binding of G9E to ZIKV E protein. We propose that G9E neutralization is strongly dependent on the mAb cross-linking ZIKV E-homodimers.

2.4 Discussion:

While people infected with flaviviruses develop robust and long-lived MBC and circulating antigen-specific antibody responses, only a small fraction of these antibodies are responsible for functional virus neutralization and protection (*103*). While the structure of human antibodies bound to flaviviruses or E protein complexes is consistent with antibody cross-linking of E proteins as a neutralization mechanism, direct experimental support for this is lacking (*39, 45, 104*). The nature of the ZIKV G9E footprint, consisting of a single dominant binding site on one E monomer and weaker peripheral contacts extending to the second E monomer, provided us a unique opportunity to test the significance of the minor intra-dimer contacts to virus neutralization. Our results strongly support that antibody mediated crosslinking of E proteins plays a critical role in the mechanism of virus neutralization. While we did not directly measure the impact of G9E binding on membrane fusion, our results establish a step after viral attachment requiring E protein crosslinking as the major mechanism of neutralization. Recent studies demonstrated that many human mAbs that strongly neutralize flaviviruses bind to complex quaternary structure epitopes that span 2 or more E molecules on the viral surface (*41, 45, 47, 103, 104*). Our experimental data linking the G9E mediated E protein cross-linking to functional neutralization is likely to be broadly applicable to how other potent human antibodies block flavivirus infections.

Among the well-studied pathogenic flaviviruses, ZIKV is most closely related to the DENV complex. The antigenic region on EDII defined by G9E is likely the target of ZIKV-specific neutralizing and protective antibodies because the corresponding site on DENVs is masked by a N-linked glycan at position N67. Among pathogenic flaviviruses, the DENV complex is unique in having a N-linked glycan at N67. We predict that the region defined by G9E is likely to be a major target of neutralizing antibodies against other flaviviruses that do not belong to the DENV complex. While individuals exposed to sequential DENV serotypes often develop durable cross-neutralizing

antibodies to all 4 serotypes, this response does not reliably extend to ZIKV (*6, 31, 105*). Individuals sequentially infected with DENV followed by ZIKV also do not reliably develop durable DENV-ZIKV cross-neutralizing and cross-protective Ab responses (*6, 105*). Instead, these individuals develop distinct type-specific neutralizing Ab responses to the original DENV serotype and to ZIKV (*105*). While not directly addressed here, the presence of an N-linked glycan at position N67 in the 4 DENV serotypes but not on ZIKV may be key to understanding antibody neutralization patterns between DENVs and ZIKV.

In conclusion, a hallmark of flavivirus infections is the induction of rare but potent, quaternary epitope directed neutralizing antibodies that are correlated with long-term protection. We have identified an antigenic region on ZIKV that is a major target of type-specific neutralizing antibodies produced by plasma cells and MBCs at late convalescence (>6 months after infection). Our findings highlight the importance of antibody-mediated cross-linking of E proteins in the pre-fusion conformation as a mechanism for neutralizing flaviviruses.

2.5 Materials and Methods:

Expression and purification of recombinant ZIKV E proteins and antibodies. G9E sequence was obtained during initial characterization of mAb(*96*). Z-20 and ZKA190 mAb sequences were obtained from PDB entries 5GZO, and 5Y0A respectively. A humanized ZIKV117 in IgG1 format was generated using the sequence obtained from PDB 5UHY. A codon optimized synthetic gene encoding for WT or mutant heavy or light chain mAb or Fab was cloned into a mammalian expression plasmid pAH. A human serum albumin secretion signal sequence was included at the 5'-end of each construct to enable secretion into the culture medium. The Fab heavy chain constructs also contained a 6xHistidine tag at the 3'-end. Recombinant Fab or mAb was expressed in Expi293 mammalian expression by co-transfection of heavy and light chain plasmids at 1:1 ratio. Recombinant Fab proteins were purified from the culture supernatant by nickel-nitrilotriacetic acid agarose (Qiagen). Recombinant mAbs were affinity purified by MabSelect resin (Cytiva, #17543802). Recombinant ZIKV E-protein and a cysteine cross-linked stable ZIKV E-protein dimer (A264C) with C-terminal 6x His-tag were expressed in the Expi293 cells and purified as described before(*106*). Anti-flavivirus MAbs 2H2 (ATCC HB-114) and 4G2 (ATCC HB-112) were produced in hybridoma cell line by the

UNC Protein Expression and Purification Core Facility. Purified protein products were verified by SDS-PAGE reducing gel. (Supplementary Figure 6)

ZIKV/G9E Fab complex crystallization and structure determination. ZIKV E/G9E Fab complex was formed by mixing purified recombinant ZIKV E-protein and G9E Fab in solution at 1:1.2 ratio at room temperature for 30 min. The ZIKV E/G9E Fab complex was purified by Superdex 200-increase size exclusion chromatography column. Crystallization screening and optimization of ZIKV E/G9E Fab complex were performed in mosquito robots at the UNC's Center for Integrative Chemical Biology and Drug Discovery using the sitting-drop vapor-diffusion method. Crystals of ZIKV E/G9E Fab complex were grown by mixing 150 ul protein solution at 2.5 mg ml−1 and 150 ul crystallant solution consisting of 100 mM HEPES pH 7.5, 10%(w/v) PEG 8000. X-ray diffraction data were recorded on a MAR-225 CCD detector at the APS SER-CAT 22-BM beamline. Reflections were processed and scaled in HKL2000. Phases were obtained by molecular replacement using the structures of ZIKV E protein (PDB ID: 5JHM) and Fab (PDB ID: 4NKI) as templates. An initial search using the complete PDB coordinates of ZIKV E protein or Fab as a model was unsuccessful. Instead, four fragments of template structures encompassing ZIKV EDI-EDII (1-301 aa), ZIKV EDIII (302-406 aa), heavy and light chains of Fab molecules were used to phase the structure of ZIKV E/G9E Fab complex using Phaser. Refinement was performed using PHENIX and Coot. Molecular figures were generated in PyMOL and interaction analysis were performed in PISA. The data collection and refinement statistics are given in supplementary table 2. The refined model of ZIKV E/G9E Fab complex had six protomers in the asymmetric unit.

ZIKV infectious clone mutagenesis. We used a previously described infectious clone of ZIKV strain H/PF/2013(*107, 108*). Site-directed mutagenesis was used to introduce a glycosylation motif (N-X-S/T) at position 67 of the envelope protein (GAC ATG GCT > AAC ACG ACA). The resulting purified plasmids were digested (New England BioLabs), ligated, *in vitro* transcribed (mMachine T7 Ultra transcription kit from Ambion), and electroporated into Vero-81 cells as previously described(*109*). Supernatants from electroporated Vero-81 cells were harvested after 6 to 7 days and passaged once on Vero-81 cells to

generate virus stocks. Virus stocks were titered by FFA on Vero-81 cells. Envelope protein glycosylation status was confirmed by size shifts on Western Blots as previously described(*108*).

Pre and Post-attachment assay. Pre and post-attachment assays were done as previously described(*37*). Briefly, pre-attachment conditions added varying concentrations of G9E mAb to 60-80 foci of H/PF/2013 ZIKV and incubated 1 hour at 4°C. The virus mixed with antibody solution was added to the confluent layer of Vero-81 cells and incubated for 1 hour at 4°C. Post-attachment conditions added 60-80 foci of H/PF/2013 ZIKV to the confluent layer of Vero-81 cells for 1 hour at 4°C. Cells were washed of excess ZIKV with ice-cold DMEM/F12 media supplemented with 20 mM HEPES buffer. Varying concentrations of mAb were added to Vero-81 surface-bound ZIKV at 4°C. For both conditions, cell-associated viral RNA was harvested by adding trizol directly to the confluent cell layer and purifying RNA through QiaAMP viral mini kit. RNA was converted to cDNA by iScript Reverse Transcriptase Supermix (Biorad, #1708841) and detected using Sybr Green (Thermo, 4309155) system with primers specific for ZIKV E-protein (F: CCGCTGCCCAACACAAG, R: CCACTAACGTTCTTTTGCAGACAT) adapted from a previous publication(*10*). In separate plates, the focus-forming assay was proceeded by heating the attached complex to 37°C and harvesting after 40 hours. Foci were detected by immunostaining with pan-Flavi antibody 4G2.

ZIKV E-dimer capture ELISA. 96 well-high-binding titer plate (Greiner, 655061) was coated at 200ng/well with anti-His mAb (ProteinTech, 66005-1-Ig) in tris-buffer saline, pH 7.4 (TBS). The wells were blocked with TBS containing 3% skim milk and 0.05% Tween-20 for 1 hour at 37C. In parallel, ZIKV E-dimer (A264C) was incubated with varying concentrations of G9E WT or mutant mAb for 1 hour at 37°C. The above solution was added to the wells coated with anti-His mAb and incubated for 1 hour at 37°C. Plates were washed with TBS containing 0.2% Tween and incubated for 1 hour at 37°C with Goat anti-human IgG conjugated to alkaline phosphatase (Sigma, A9544). Plates were washed, developed with p-Nitrophenyl phosphate substrate (Sigma, N1891), and absorbance was measured at 405 nm.

Focus Reduction Neutralization Test (FRNT). ZIKV FRNT assay was performed as described(*110*). Briefly, mAbs were serially diluted in DMEM (Life Technologies, 11330032) media supplemented with 2% Fetal Bovine Serum (Sigma, TMS-013-B), 1% L-glutamine (Life Technologies 25030081), 1% penstrep (Mediatech, 30002CI), and 1% sodium bicarbonate (Life Technologies, 25080094) and incubated with H/PF/2013 ZIKV for 1 hour at 37°C. Antibody and virus mixture was added to the confluent layer of Vero-81 cells in 96-well flat-bottom plate and incubated for 1 hour at 37°C. Excess mAb/virus mixture was flicked off the plate, and 180 uL of Optimum (Life technologies, 31985070) supplemented with 2% methylcellulose was added to individual wells. The plate was fixed with 4% PFA and stained for ZIKV foci with flavivirus specific mAb after 40-hour incubation.

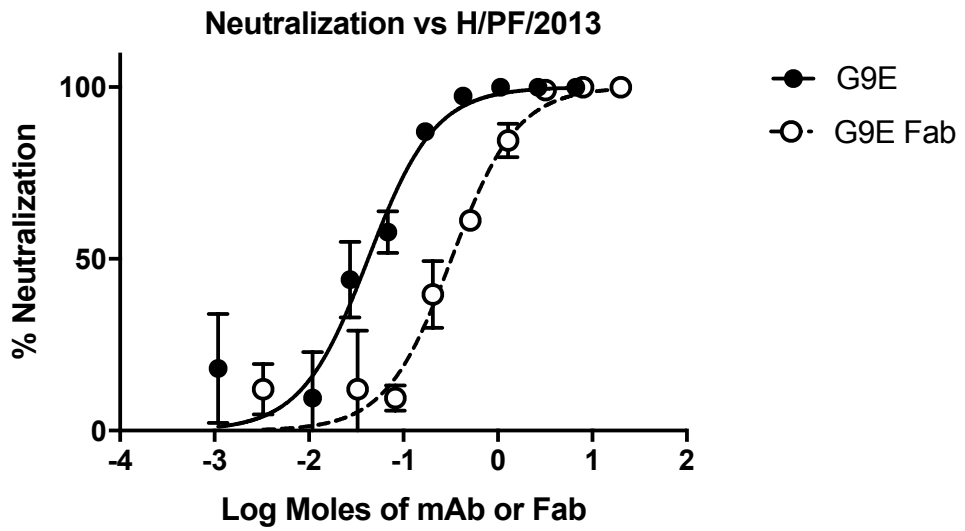


Figure 2.1. Neutralization of ZIKV H/PF/2013 by G9E mAb and G9E Fab.

G9E mAb and G9E Fab were expressed in Expi293 mammalian cells and purified from the cell culture medium by MabSelect resin or nickel-nitrilotriacetic acid agarose resin, respectively. G9E Fab retains neutralization activity against ZIKV, albeit lower than the G9E mAb.

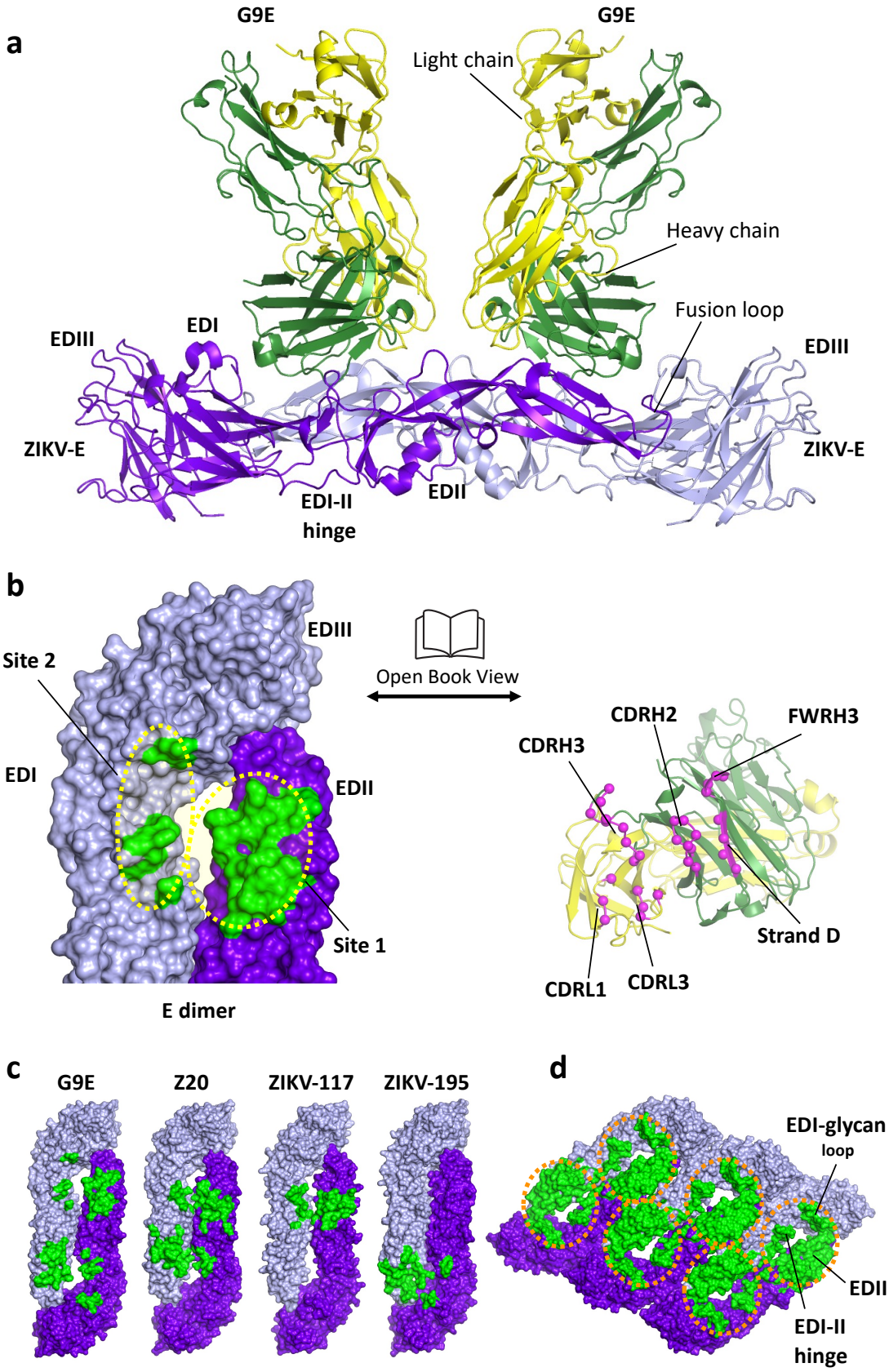

a
G9E
G9E
Light chain
Heavy chain
Fusion loop
EDIII
EDI
EDIII
ZIKV-E
ZIKV-E
EDI-II
hinge
EDII
b
EDIII
Site 2
EDI
EDII
Site 1
E dimer
Open Book View
CDRH2
FWRH3
CDRH3
Strand D
CDRL1
CDRL3
c
G9E
Z20
ZIKV-117
ZIKV-195
d
EDI-glycan
loop
EDII
EDI-II
hinge

Figure 2.2. G9E targets an immunodominant quaternary epitope on ZIKV E-protein.

A. Structure of ZIKV E in complex with G9E. The structure reveals that two G9E Fab fragments bind the E-dimer in a similar mode. G9E Fab fragments and E-dimer are shown in cartoon representation (green - Fab heavy chain; gold - Fab light chain; ZIKV E dimer (protomer 1 - purple; protomer 2 - lavender). **B.** G9E binds a quaternary epitope formed on E-dimer. Open book representation of the interface formed between E-dimer (green) and G9E (pink) are shown. The quaternary epitope comprises a major (site 1) and a minor (site 2) site on E-dimer. The paratope comprises heavy and light chain CDRs. **C.** G9E targets an immunodominant epitope centered on EDII. G9E footprint on ZIKV E-dimer overlaps with previously described potently neutralizing human mAbs ZIKV-117, Z20, and ZIKV-195 isolated from patients infected with ZIKV. The quaternary epitopes targeted by the respective neutralizing mAbs are shown in green. **D.** Combined EDII targeting antibody epitope on ZIKV E-raft. Combined epitope comprised of G9E, Z20, ZIKV-117, and ZIKV-195 is shown within the orange circle, including EDII, EDI-II hinge, and the EDI glycan loop.

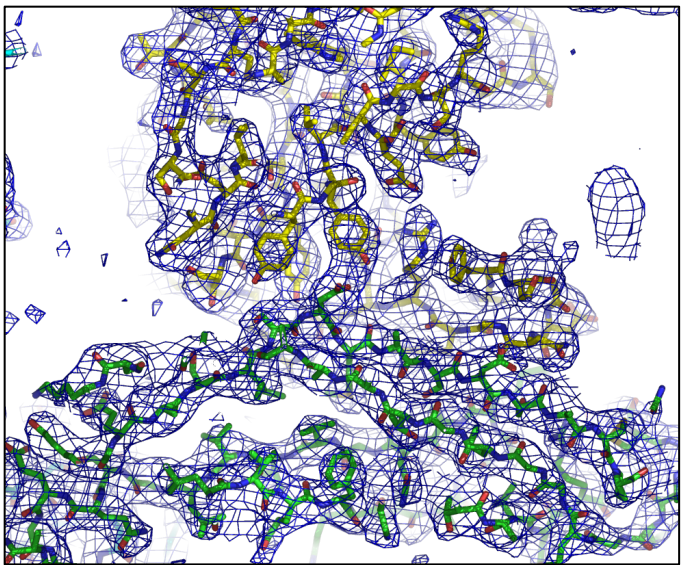


Figure 2.3. Electron density map of G9E Fab/ZIKV E complex.

An initial 2Fo-Fc electron density map (contour 1.0 sigma) of the ZIKV E/G9E complex illustrates that the starting phases obtained by molecular replacement were of excellent quality to reveal the nature of the interaction between G9E and ZIKV-E-protein. ZIKV E-protein (green) G9E Fab (yellow) are shown as sticks.

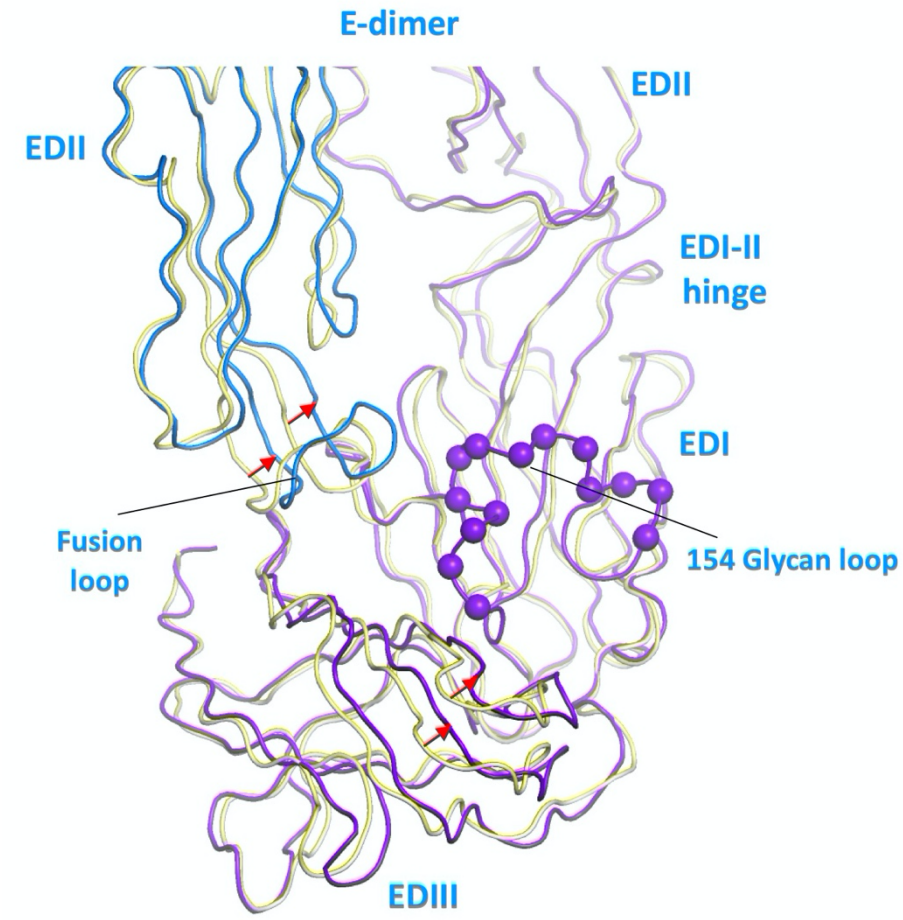


Figure 2.4. G9E binding induces a small domain motion in ZIKV E-dimer.

Structural superposition of the E-protein conformation of the template structure used for molecular replacement (PDB ID: 5JHM, yellow) and the E-protein conformation observed in complex with G9E (protomer 1-blue; protomer 2 - purple). G9E induces a 2 Å inward movement of the fusion loop (blue strand, notated by red arrows) towards the EDI glycan loop (purple spheres) of the neighboring E-protein. G9E also causes a 3 Å inward movement of EDIII (purple strand, notated by the red arrow) towards its EDI glycan loop (purple spheres). These movements cause an increase in the E-dimer interface

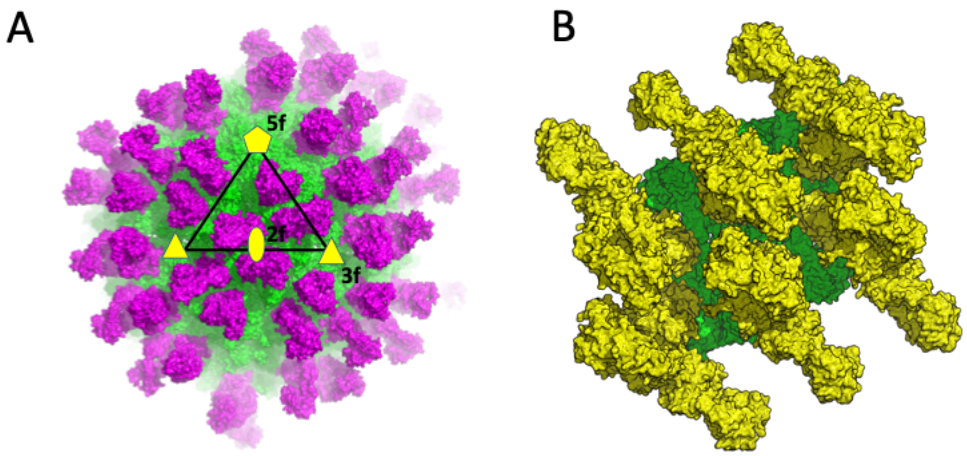


Figure 2.5. Molecular modeling of G9E Fab on ZIKV virion and G9E mAb on ZIKV E-protein raft. **(A)** G9E Fab (magenta) was modeled on to cryoEM structure of ZIKV (green; PDB ID 6CO8). The 2-fold (oval), 3-fold (triangle), and 5-fold (pentangle) icosahedral axis of symmetry are shown. **(B)** G9E mAb (yellow) was modeled on to ZIKV E-protein raft (green). G9E mAb was built by analogy with the structure of anti-PD-1 (PDB: 5DK3).

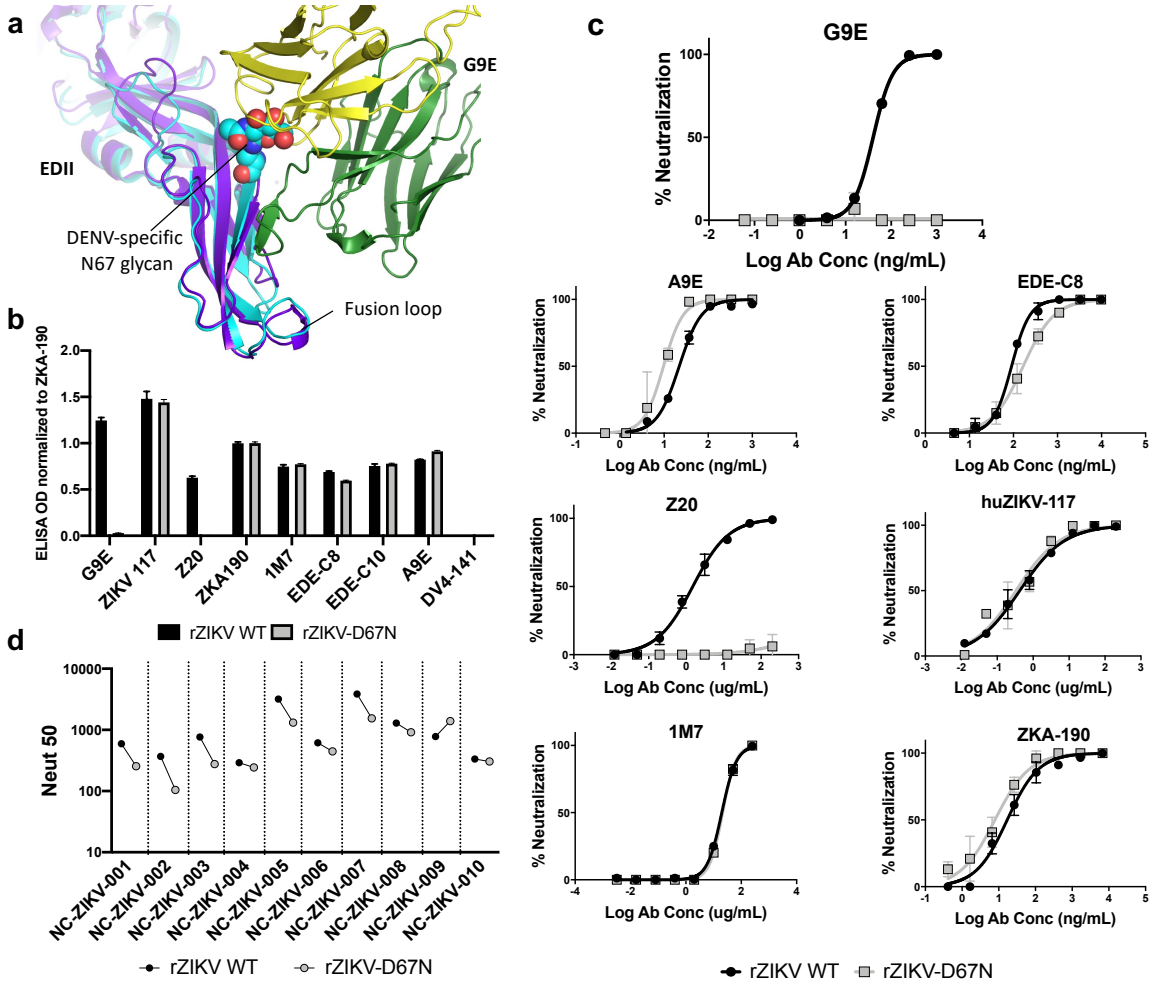


Figure 2.6. An engineered N67 glycan on ZIKV E protein blocks G9E binding and neutralization. **A.** Structural overlay of ZIKV E/G9E complex on to DENV2 E-protein. Introducing a 'DENV-like' N-67 glycan on ZIKV E protein was predicted to block G9E binding. DENV E (cyan, PDB 1OAN), ZIKV E (purple), G9E heavy-chain (green), and G9E light-chain (gold) are shown in cartoon representation. DENV N-67 glycan is shown as spheres. **B.** G9E does not bind to a recombinant ZIKV with N-linked glycan on EDII. Comparison of ZIKV mAbs binding to ZIKV H/PF/2013 and ZIKV H/PF/2013 with engineered glycan at position 67 (rZIKV-D67N). Antibody binding was evaluated on a ZIKV capture ELISA. The binding was normalized to ZIKV EDIII targeting mAb ZKA-190. G9E, Z20, and ZIKV-117 target quaternary structure epitopes on EDII. 1M7 targets the conserved fusion loop on E-protein. EDE-

C8 and EDE-C10 target quaternary structure epitopes formed by E-dimer, covering the conserved fusion loop region on E-protein and the EDIII of the neighboring E-protein. DV4-141 is a DENV4 specific mAb used as a negative control. **C.** Comparison of virus neutralization against ZIKV and rZIKV-D67N by a panel of ZIKV mAbs. While the integrity of the epitopes targeted by ZIKV mAbs is maintained, G9E failed to neutralize r-ZIKV-D67N. **D.** Patients sera isolated from primary ZIKV infection show reduced neutralization against rZIKV-D67N. Convalescent sera from people with primary ZIKV infection was tested against ZIKV and rZIKV-D67N in Vero-81 virus neutralization assay.

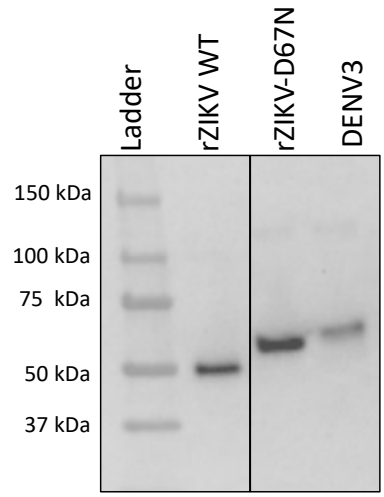


Figure 2.7. Western blot of rZIKV and rZIKV-D67N.

WT rZIKV retains a glycosylation site amino acid position 154 on the envelope protein while DENV3 has two glycosylation sites at amino acid positions 67 and 154. rZIKV-D67N was created through an infectious clone system that introduced a glycosylation motif (N-X-S/T) at position 67. To indirectly measure glycosylation status, E-protein from WT rZIKV, DENV3 and rZIKV-D67N were measured in western blot using flavivirus mAb 4G2 as primary antibody followed by HRP-conjugated goat anti-mouse IgG as secondary antibody. rZIKV-D67N demonstrated shift in size consistent with double glycosylated DENV3 when compared to WT rZIKV.

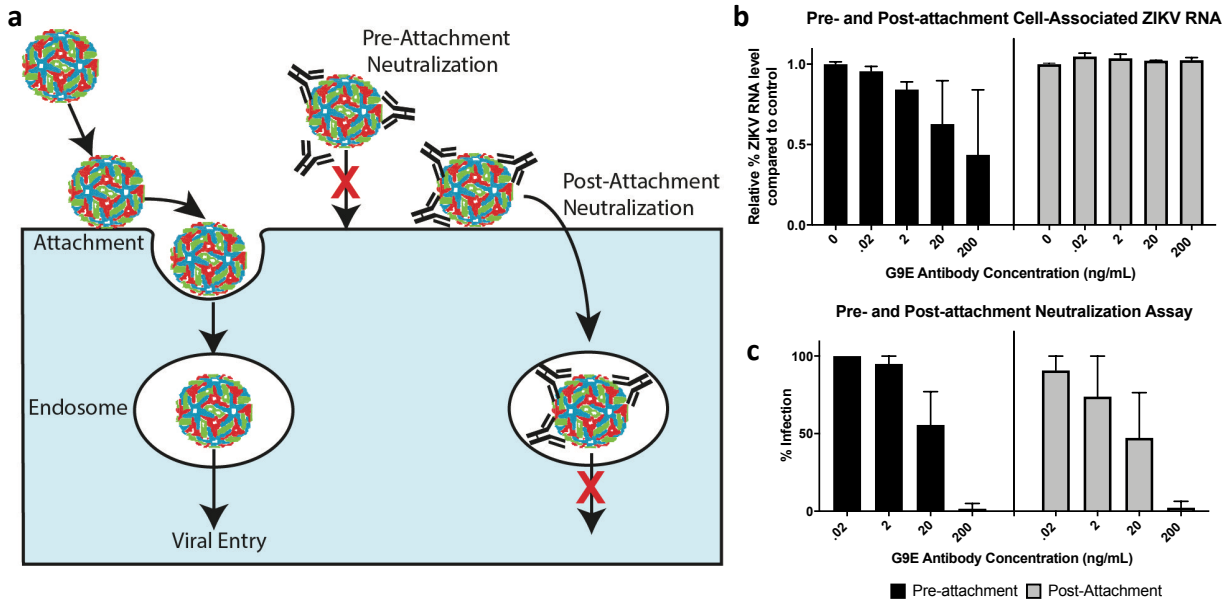
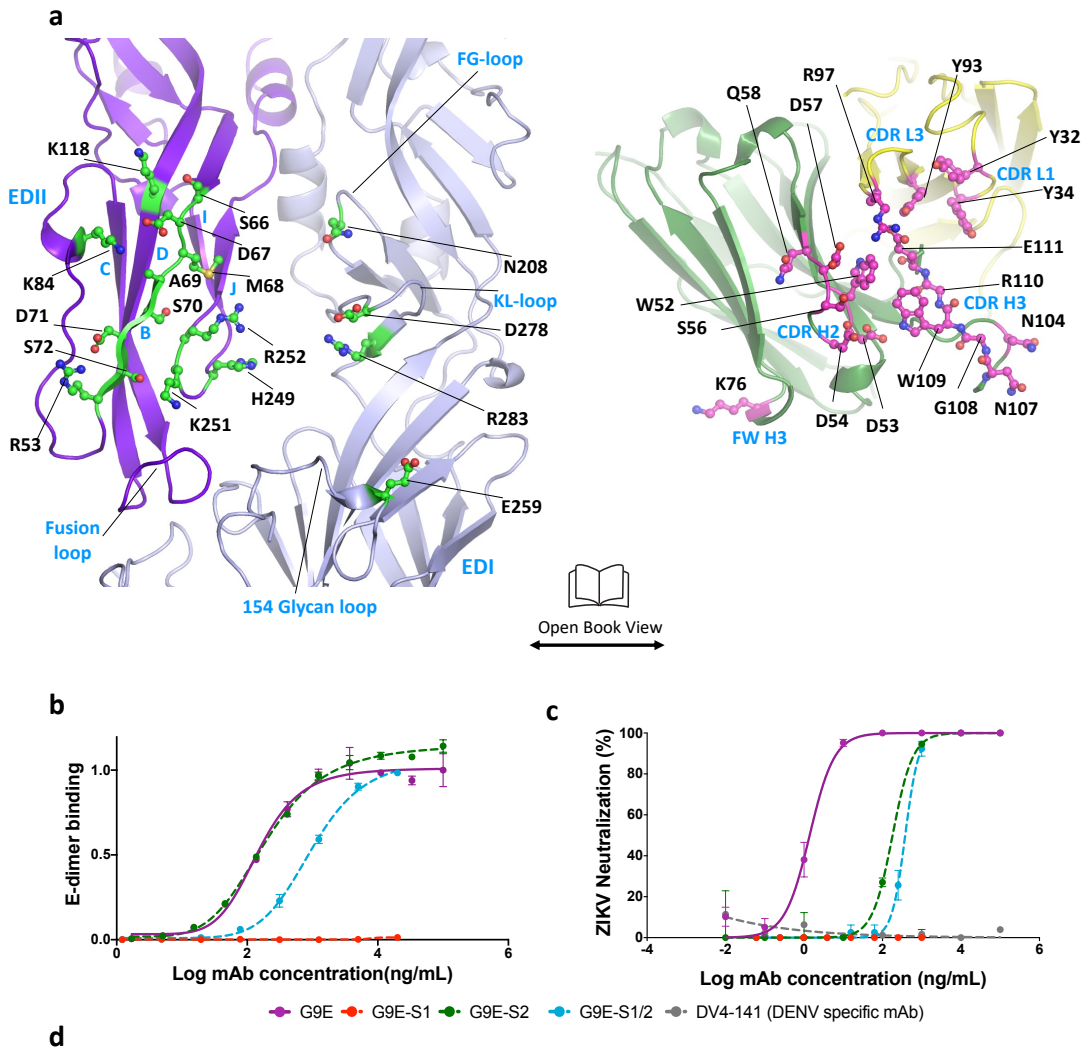


Figure 2.8. G9E blocks post-attachment steps of viral entry.

A. Schematic of ZIKV entry into the target cell. ZIKV initially attaches to the cell surface, internalizes, and traffics through the endosomal pathway to complete the viral entry. Neutralizing mAbs can either block before (pre-attachment neutralization) or after (post-attachment neutralization) virus attachment to the cell surface. **B.** G9E can block viral attachment to the cell surface. The mechanism of G9E neutralization was assessed by pre-and post-attachment assay. In the pre-attachment condition, G9E and ZIKV were mixed before adding to the Vero-81 cells (black). In the post-attachment condition, ZIKV was allowed to attach to the cell surface, and G9E was added subsequently (grey). **C.** G9E was able to block infection after the virus has attached to the cell surface. Measurements of cell-associated RNA and % infection was performed as described in the methods.



d

G9E mAbs	G9E paratope mutations	Interacting epitope residues	Binding50 ng/mL (95% CI)	Neut50 ng/mL (95% CI)
WT	N/A		148.3 (117 to 187.5)	1.3 (1.1 to 1.8)
Site 2 mutant	D57S, Q58A, K76S	N208, D278, R283, E159	195.0 (172.6 to 221.3)	180.6 (173.6 to 187.7)
Site 1 and Site 2 mutant	D53A, D54A, K76S, Q58A, D57S	R252, N208, D278, R283, E159	981.7 (849.2 to 1161.4)	379.4 (345.7 to 415.8)
Site1 mutant	W109A, E111A	B-strand	No binding	

Figure 2.9. G9E cross-dimer binding underlies potent neutralization of ZIKV.

A. Close up view of the interactions between ZIKV E-dimer and G9E is shown in an "open book" representation. The interfacing residues between ZIKV E-dimer and G9E were identified by Protein Interfaces, Surfaces, and Assemblies program (PISA). Contact residues on E protein (green) and G9E (pink) are shown in ball and stick representations. **B.** Comparison of ZIKV E-dimer binding with G9E WT and G9E paratope mutants, designed to disrupt site-1 (G9E-S1), or site 2 (G9E-S2) or site1/2 (G9E-

S1/2). **C.** Comparison of ZIKV neutralization with G9E WT and G9E paratope mutants, G9E-S1, or G9E-S2, or G9E-S1/2. **D.** Characteristics of E-dimer binding and ZIKV neutralization by G9E WT and paratope mutants.

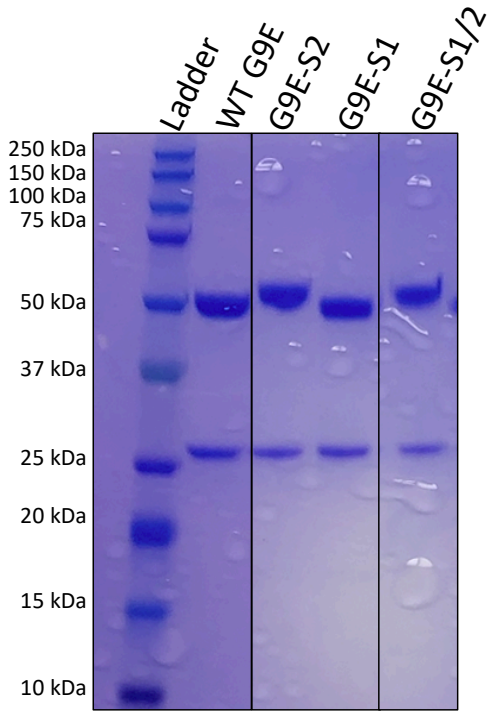


Figure 2.10. SDS-PAGE analysis of the purified G9E mAbs.

G9E WT and paratope mutants were expressed in Expi293 mammalian cells and purified by MabSelect resin. Coomassie-stained SDS-PAGE run under reduced condition shows the band corresponding to heavy (50 kDa) and light (25 kDa) chains of G9E mAbs.

mAb	G9E	Z20	ZIKV-117	ZIKV-195
Neutralization 50% (ng/mL) vs H/PF/2013	6.3	370	5.4	600
Estimated sites per virion	180	180	60	180
In-vivo protection	Yes	Not tested	Yes	Yes

Table 2-1. Comparison of isolated ZIKV mAb targeting EDII.

Z20(*97*), ZIKV-117(*41*), and ZIKV-195(*42*) are previously reported human mAbs isolated from patients who experienced ZIKV infection. G9E, ZIKV-117, and ZIKV-195 protected against ZIKV infection in a murine model. The estimated binding sites on the ZIKV virion and the virus neutralization titer are tabulated.

Data collection statistics	
Wavelength (Å)	1.00000
Resolution	50 - 3.38 (3.46 - 3.38)
Space group	P 1 21 1
Unit cell (Å)	95.0 133.6 105.1 90 106.5 90
Total reflections	262931
Unique reflections	35195
Multiplicity	7.5
Completeness (%)	99.4 (94.7)
Mean I/sigma(I)	12.9 (1.4)
Wilson B-factor	95.67
R-merge	0.177 (1.4)
R-pim	0.07 (0.59)
CC1/2 in shell	.62
Refinement statistics	
Resolution range	45.57 - 3.38 (3.50 - 3.38)
Reflections used in refinement	35167 (3352)
Reflections used for R-free	1689 (171)
R-work	23.2 (34.5)
R-free (%)	25.9 (35.5)
Number of non-hydrogen atoms	12838
Protein residues	1695
RMS(bonds)	0.002
RMS(angles)	0.62
Ramachandran favored (%)	91.21
Ramachandran allowed (%)	8.67
Ramachandran outliers (%)	0.12
Rotamer outliers (%)	0.07
Clashscore	5.02
Average B-factor	120.7

Table 2-2. Data collection and refinement statistics of ZIKV E/G9E complex structure.

Statistics for the highest-resolution shell are shown in parentheses.

CHAPTER 3: IDENTIFYING THE ROLE OF DENV SEROTYPE SPECIFIC ANTIBODY IN VACCINE INDUCED PROTECTION[1]

3.1 Overview:

The four serotypes of dengue virus (DENV1-4) are mosquito-borne flaviviruses that infect humans. Live attenuated tetravalent DENV vaccines are at different phases of clinical testing. DENV vaccine developers have relied on neutralizing antibodies (NAbs) as a correlate of protection. A leading tetravalent vaccine (Dengvaxia) stimulated NAbs to the 4 DENV serotypes, yet overall vaccine efficacy was low in children who were DENV seronegative at baseline before vaccination. We compared the properties of 1) NAbs induced by wild type DENV1 or 3 infections, which are strongly correlated with protection from repeat infections, and 2) NAbs induced by Dengvaxia in individuals who subsequently experienced DENV1 or DENV3 breakthrough infections. Wild type infections induced NAbs that recognized epitopes unique (type-specific) to each serotype, whereas the vaccine stimulated qualitatively different NAbs that recognized epitopes conserved (cross-reactive) between serotypes. Our results indicate that among children who were DENV seronegative at baseline, unbalanced replication of the DENV type 4 vaccine component in the tetravalent vaccine stimulates Abs capable of cross neutralizing DENV1 and 3 in vitro but not protect in vivo. In DENV seronegative individuals who are vaccinated, we propose that type specific NAbs are a better correlate of protection than total levels of NAbs.

3.2 Introduction:

The four dengue virus serotypes (DENV1-4) are mosquito-transmitted flaviviruses estimated to infect over 100 million people every year.(*1*) Wild type DENV infections stimulate neutralizing antibodies

[1] This chapter previously appeared as an article in the Journal of Clinical Investigation. The original citation is as follows: Henein S, Adams C, Bonaparte M, Moser JM, Munteanu A, Baric R, de Silva AM. Dengue vaccine breakthrough infections reveal properties of neutralizing antibodies linked to protection. J Clin Invest. 2021 Jul 1;131(13):e147066. doi: 10.1172/JCI147066. PMID: 34003796; PMCID: PMC8245170.

(NAbs) binding to epitopes on viral envelope (E) protein that are strongly correlated with durable protection. DENV vaccines are being developed as tetravalent formulations to stimulate protective responses to all 4 serotypes (*111-113*). While the clinical development of DENV vaccines was guided by the presence of NAbs as a correlate of protection, recent data from leading dengue vaccines (Dengvaxia developed by Sanofi Pasture and TAK-003 developed by Takeda Vaccines) demonstrated that the presence of NAbs to the four serotypes after vaccination is not a reliable correlate of protection in subjects who were dengue seronegative at baseline (*73, 114, 115*). The goal of the current study was to compare the properties of NAbs induced by wild type DENV serotype 1 and 3 infections and Dengvaxia to improve our understanding of the properties of protective Abs.

A person infected with DENV for the first time (a primary infection) develops a durable serotype-specific (TS) NAb response that is correlated with resistance to repeat infections by the same serotype (*3, 111-113*). After a primary infection, people are susceptible to second infections with new serotypes. DENV serotype cross-reactive (CR) Abs induced by primary infections were linked to enhanced viral replication and more severe disease during secondary infections (*116*). Individuals who recovered from secondary DENV infections develop new populations of serotype CR NAbs that are correlated with durable serotype cross protective immunity (*68, 113, 117*) To minimize the risk of DENV vaccines inducing Abs that enhance DENV infections, leading vaccines are based on tetravalent formulations to induce balanced protective immunity to all 4 serotypes.

Dengvaxia is a live attenuated chimeric tetravalent Dengue Vaccine (CYD-TDV) that was developed by modifying the yellow fever 17D live attenuated vaccine to contain the E and pre-membrane proteins of each DENV serotype (*75*). The safety and efficacy of Dengvaxia was tested in a phase 2b trial (CYD23) in Thailand and two large phase 3 trials (CYD14 and CYD15) in Asia Pacific and Latin America respectively (*7, 88, 118, 119*). Overall vaccine efficacy was high in children with pre-existing immunity to DENVs who received the vaccine. In DENV-naïve children, the vaccine reliably stimulated NAbs to all 4 serotypes, yet overall efficacy was low (*7, 114*). More recently, preliminary efficacy data from another clinical trial (TAK-003 developed Takeda) also indicate that the presence of vaccine induced NAbs is not a

reliable indicator of protection in naïve children who were vaccinated (*73*).These results established that the presence of NAb alone is not a reliable correlate of protection in individuals who are dengue seronegative at baseline. We hypothesize that TS NAbs are a better correlate of protection because these Abs bind to epitopes that are exposed on the mature infectious virus (*120, 121*) and require replication of the matched vaccine component. To test this hypothesis, we characterized Dengvaxia induced Ab responses in baseline seronegative individuals who subsequently experienced symptomatic breakthrough infections with DENV1 or DENV3. As controls, we characterized the properties of Abs in baseline seronegative vaccine recipients who did not experience a breakthrough infection and in individuals exposed to primary wild type DENV1 or 3 infections, who are protected from repeat infections by the same serotype.

3.3 Results and Discussion:

We characterized the specificity of NAbs in individuals who experienced primary DENV1 (n=11) or DENV3 (n=8) infections (**Table 3-1**) because wild type infections are known to induce durable protection from clinically symptomatic reinfection with the same serotype (*111, 113*). To measure levels of DENV1 or 3 TS Abs in the specimens, we incubated the samples with beads coated with DENV serotypes not responsible for infection to deplete serotype CR Abs. The Ab depleted samples no longer bound to DENV4 by ELISA confirming removal of CR Abs **(Figure 3.1)**. The depleted samples bound to DENV1 or DENV3, although at reduced levels compared to control depleted samples, demonstrating the presence of TS Abs to each serotype responsible for infection **(Figure 3.1)**. The CR Ab depleted samples neutralized the homologous serotype of infection (DENV1 or DENV3) at levels comparable to control depleted samples demonstrating that TS Abs were mainly responsible for functional neutralization (**Figure 3.2A and 3.2B**). These results are consistent with previous studies demonstrating that primary DENV infections stimulate durable NAbs to unique epitopes on the infecting serotype (*111, 120*).

Most children with no immunity to DENVs who received 3 doses of Dengvaxia developed NAbs to DENV1 and 3, yet vaccine efficacy against these two serotypes was low (*114*). We characterized the properties of vaccine induced Abs in baseline seronegative children who subsequently experienced DENV1 (n=15 subjects) or 3 (n=18 subjects) breakthrough infections **(Table 3-2)**. Children who experienced DENV1

breakthrough infections had comparable levels of Abs binding to DENV1 and DENV4 after vaccination (**Figure 3.3A**). When the sera were depleted using a mixture of DENV3 and 4 virions, nearly all binding to DENV1 was lost indicating that most Abs bound CR epitopes conserved between serotypes (**Figure 3.3A**). When the specimens were depleted with DENV1 and 2 virions, all 15 samples retained binding to DENV4, demonstrating the presence of TS Abs to DENV4 **(Figure 3.3A)**. A similar pattern was observed when the Ab depleted samples were tested for neutralization of DENV1 and 4. While all 15 subjects who experienced DENV1 breakthrough infections had developed DENV1 NAbs after vaccination (GMT=97), only 4/15 subjects had DENV1 TS NAbs (GMT = 2.5) (**Figure 3.4A**). While levels of DENV4 NAbs also dropped after removal of CR Abs, 10/15 children had DENV4 TS NAbs (GMT = 15) (**Figure 3.4B**). The level of DENV4 TS NAb was higher than the level of DENV1 TS NAb in these children who were susceptible to DENV1 infections (**Figure 3.4C and 3.4D**).

Among the 18 children who experienced DENV3 breakthrough infections, 14 had DENV3 NAbs (GMT =22) and all 18 had DENV4 NAbs (GMT = 122) (**Figure 3.5A**). After Ab depletion to remove CR Abs, only 4/18 subjects had DENV3 TS NAbs (GMT= 2) and 14/18 subjects had DENV4 TS NAbs (GMT = 19) (**Figure 3.5B**). The level of DENV4 TS NAb was higher than the level of DENV3 TS NAb in these children who were susceptible to DENV3 breakthrough infections (**Figure 3.5C and 3.5D**). These results demonstrate that Dengvaxia mainly stimulated DENV1 and 3 CR NAbs in children who subsequently experienced DENV1 or 3 breakthrough infections. Most of these children susceptible to DENV1 and 3 infections had DENV4 TS NAbs.

Next, we characterized the properties of DENV1, 3 and 4 NAbs in baseline seronegative vaccine recipients (n = 11) who did not experience a breakthrough infection during the clinical trial. These subjects had variable NAb levels to the three serotypes tested, with the highest response to DENV4 followed by DENV3 then DENV1 (GMT= 103, 67 and 60 respectively) **(Figure 3.6**). When the sera were subjected to Ab depletions to estimate levels of TS NAbs, we observed that 2/11 had DENV1 TS NAbs, 3/11 had DENV3 TS NAbs and 5/11 had DENV4 TS NAbs **(Figure 3.6)**. As the breakthrough and non-breakthrough cases had similar Ab profiles, we combined the results from all the children tested to define properties of DENV1,

3 and 4 Abs stimulated by the vaccine in children who were seronegative at baseline. Although nearly all children (42/44) had DENV1 NAbs, only 12 (27%) had DENV1 TS NAbs (**Figure 3.7A**). Similarly, 24/29 children tested had DENV3 NAbs but only 7 (24%) had DENV3 TS NAbs (**Figure 3.7B**). In contrast to DENV1 and 3, 29/44 (66%) children tested had TS NAbs to DENV4 (**Figure 3.7C**). These results indicate that in children who were DENV seronegative at baseline, Dengvaxia stimulated low levels of TS NAbs to DENV1 or 3 in about a quarter of the children, whereas the majority of children had higher levels of TS NAbs to DENV4 (**Figure 3.7D and 3.7E**).

The aim of our study was to identify improved Ab correlates of protection against DENV1 and 3 by comparing the properties of Abs in vaccine recipients who experienced breakthrough infections and individuals exposed to primary DENV 1 and 3 infections. The clinical observation that Dengvaxia stimulated NAbs in baseline seronegative individuals that were not correlated with protection was unexpected because NAbs are correlated with the success of other flavivirus vaccines. Our results demonstrate that both wildtype infection and vaccinated individuals had NAbs to DENV1 and 3 but wildtype virus infections targeted epitopes unique to DENV1 or DENV3 whereas the vaccine mainly induced NAbs to epitopes conserved between serotypes. Studies in animal models and humans established that the DENV4 component in Dengvaxia replicates to higher levels than the other three components (*75, 122*). In fact, we observed that most, but not all, children had DENV4 TS NAbs after vaccination. We propose that the DENV4 vaccine component stimulates serotype CR Abs that neutralize DENV1 and 3 in cell culture, but these Abs are not protective or correlated with protection in vivo in seronegative subjects.

At first glance, our conclusion that total levels of NAbs are a poor correlate of vaccine efficacy in baseline seronegative individuals appears to be inconsistent with recent publications demonstrating that levels of NAbs induced by Dengvaxia are correlated with vaccine efficacy (*115, 123*). However, these reports are based on a pooled analysis of baseline seropositive and seronegative children that mainly consisted of seropositive children. When the analysis was stratified by baseline serostatus, the correlation was weak and imprecise in baseline seronegative children (*115, 123*). These two populations require separate evaluation when evaluating immune correlates, vaccine efficacy and safety given fundamental

differences in how adaptive immunity is activated in individuals who are seronegative and seropositive at the time of vaccination.

Recent studies provide explanations for why some Abs might neutralize DENVs in cell culture but not protect in vivo (*30*). DENV stocks produced using laboratory cell lines consist of virions at different stages of maturation with low specific infectivity (*30*). DENV1 virions circulating in humans were observed to be more mature and infectious than cell culture derived virus used in cell culture neutralization assays (*30*). DENV1 TS monoclonal Abs and sera from people exposed to primary DENV1 infections neutralized both plasma and cell culture produced DENV1 efficiently (*30*). DENV serotype CR MAbs and heterotypic immune sera from people exposed to primary DENV2 or DENV3 infections efficiently neutralized cell culture produced DENV1 and poorly neutralized or failed to neutralize human plasma derived DENV1(*30*). Most human DENV serotype-cross reactive antibodies target conserved epitopes at or near the fusion loop at the tip of domain II of E protein. This region of E protein is partially exposed and accessible to antibody binding in immature virions, whereas in mature virions the fusion loop is buried and not readily accessible to antibody binding(*124, 125*). DENV neutralizing Ab assays utilizing partially mature DENV stocks will measure NAbs to exposed and hidden epitopes on mature virions and overestimate levels of protective Abs.

Typical studies to identify vaccine immune correlates rely on clinical trials that establish partial vaccine efficacy where individuals who experienced breakthrough infections demonstrate a different response than individuals who did not experience breakthrough infections. For Dengvaxia, no significant efficacy against DENV1 or 3 was observed in dengue seronegative children who were vaccinated and monitored for 5-6 years (*114*). Therefore, our observation that baseline seronegative vaccinated individuals had similar Ab profiles in the breakthrough and non-breakthrough groups is not surprising. In the absence of subgroups within the seronegative vaccinated population for identifying immune correlates, we compared Ab responses in individuals exposed to primary DENV1 and 3 infections and individuals who were vaccinated to identify improved correlates. While not the perfect comparison, our finding that DENV1 and 3 TS NAbs are readily detected after natural infection and rarely in baseline seronegative vaccinated

children who experienced breakthrough infections, indicates that TS NAbs are a more reliable correlate than total levels of NAbs to guide the development of DENV vaccines for use in this population.

We note that a few vaccinated individuals who developed low levels of TS NAbs to DENV1 or DENV3 experienced breakthrough infections with the matched serotype. Therefore, the presence of TS NAbs alone may not always be sufficient for protection. Individuals with TS NAb responses may be susceptible to infections caused by natural variant strains that differ at critical TS Ab epitopes from the vaccine strain. Recent studies established that the DENV4 response induced by leading tetravalent vaccines, including Dengvaxia, is more effective against the vaccine matched DENV4 genotype II viruses compared to genotype I viruses (*120, 126, 127*). Further studies are also needed to understand alternate mechanisms of vaccine induced immunity that may augment or replace Ab mediated protection.

Dengvaxia was efficacious in children with pre-existing immunity to DENVs who were vaccinated, and our study was not designed to identify vaccine correlates and mechanisms of protection in this population. Our results are applicable to understanding the performance of tetravalent DENV vaccines in subjects who are seronegative at baseline. We note that the second leading DENV vaccine TAK-003 developed by Takeda is poorly balanced and dominated by the DENV2 vaccine component that replicates better than the other three components (*128, 129*). Preliminary vaccine efficacy results in baseline seronegative children demonstrate high efficacy against DENV2 and lower efficacy against the other serotypes (*73*). In individuals with no baseline immunity to DENVs, we propose that TS NAbs are a better correlate than the current standard for evaluating vaccines.

3.4 Materials and Methods:

Statistics: Comparisons of percentage serotype specific and absolute serotype specific neutralization titer were done by Wilcoxon signed ranks test (Figure 2 and 3) and Kruskal Wallis Test (Figure 4).

Study approval: The CYD14 (ClinicalTrials.gov ID NCT01373281) and CYD15 (ClinicalTrials.gov ID NCT01374516) trial protocols have been approved by all relevant ethics review boards (*76, 130*). The

Institutional Review Board of the University of North Carolina at Chapel Hill reviewed and approved the receipt and analysis of anonymized CYD14 and CYD15 clinical specimens at the University of North Carolina at Chapel Hill (protocol-08-0895).

Serum Sample Collection Dengvaxia phase III clinical trials in Asia (CYD14) and Latin America (CYD15) were previously described(*119, 130*). In brief, CYD14 was conducted in five countries in Asia where 2-14 years old healthy children were enrolled, while CYD15 was conducted in four countries in Latin America and Puerto Rico, enrolling 9-16 years old healthy children. Both studies were randomized 2:1 where participants received three doses of either the vaccine or placebo at months 0, 6 and 12 of the study. Samples serostatus prior to vaccination was determined by NS1 or PRNT when available. Sera was drawn in month 13 of clinical trial, one month after completion of three dose Dengvaxia regimen. The natural infection serum samples were collected from US residing healthy adults, who experienced a dengue infection while traveling to a dengue endemic country.

Antibody Depletion Depletion studies were performed as previously described.(*120*) Dynabeads were covalently linked to anti-E MAb 1M7 for 18 hrs at 37°C. Complex was subsequently blocked with 1% BSA in PBS solution at 37°C and washed with 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer. Beads were incubated with purified virus serotypes separately. Virion/bead/MAb complex was then washed with PBS. DENV specific antibodies were depleted from sera by incubating virus/bead complex with sera diluted at 1:10 in PBS for 1 hr at 37°C with end over end mixing for three sequential rounds of depletion.

Enzyme-Linked Immunosorbent Assay (ELISA) To confirm successful depletion of a certain DENV specific population of Abs, IgG binding ELISA was conducted as previously described. (*111*) Purified DENV was either directly coated or captured using a DENV specific monoclonal Ab on a 96-well ELISA plate, the plate was then blocked using either 3% Normal Goat Serum (NGS) or 3% Non-Fat Dry Milk (NFDM) respectively to eliminate any nonspecific binding. The depleted serum sample is then added at 1:20 dilution and incubated at 37°C for an hour, then washed off. Binding was evaluated by 1 hour incubation with secondary anti-human-alkaline phosphatase conjugated Ab at 37°C, which is then washed

off. *P*-nitrophenyl phosphate substrate is then added and the Optical Density (OD) is measured at 405nm. In a successful depletion of a dengue experienced serum sample, the OD in the control depleted sample should be high (≥1) and the OD in the homologous depleted sample should be close to background or Normal Human Serum (NHS) level. If that sample has Abs that are specific to a particular serotype, the OD in the heterologous depleted sample should be higher than the NHS and background level. Limit of detection was defined as average of normal human sera + 3x standard deviation.

Focus Reduction Neutralization Test (FRNT) The neutralization tests were conducted in 96-well format on Vero-81 cells. $2x10^4$ cells were seeded overnight. On the day of the assay, virus stocks were diluted in dilution media (Dulbecco modified Eagle medium (DMEM) with 2% Fetal Bovine Serum (FBS)) to achieve 60-80 foci/well in the virus + cells only wells. Separately the serum samples to be tested were serially diluted three folds starting at 1:10 in the same dilution media. The diluted virus is then added to the serum in a 1:1 ratio making the final starting dilution of serum at 1:20 and the complex is then incubated at 37°C for 1 hour before being added to the cells and incubated for another hour at 37°C. The cells were then washed with the dilution media and Opti-MEM (Gibco) supplemented with 2% FBS, 1% Anti-Anti (Gibco) and 1% Carboxymethylcellulose is added and cells are incubated at 37°C for 45-52 hours before fixing using 4% PFA. The reported EC_{50} values were calculated using variable slope-sigmoidal dose response equation using GraphPad Prism 8. All reported results were subjected to our quality control parameters of $R^2 \geq 7.5$, a hill slope of $|\geq 5|$ and the calculated EC_{50} value should be within the range of the assay. All values that did not meet these standards were assigned the baseline value.

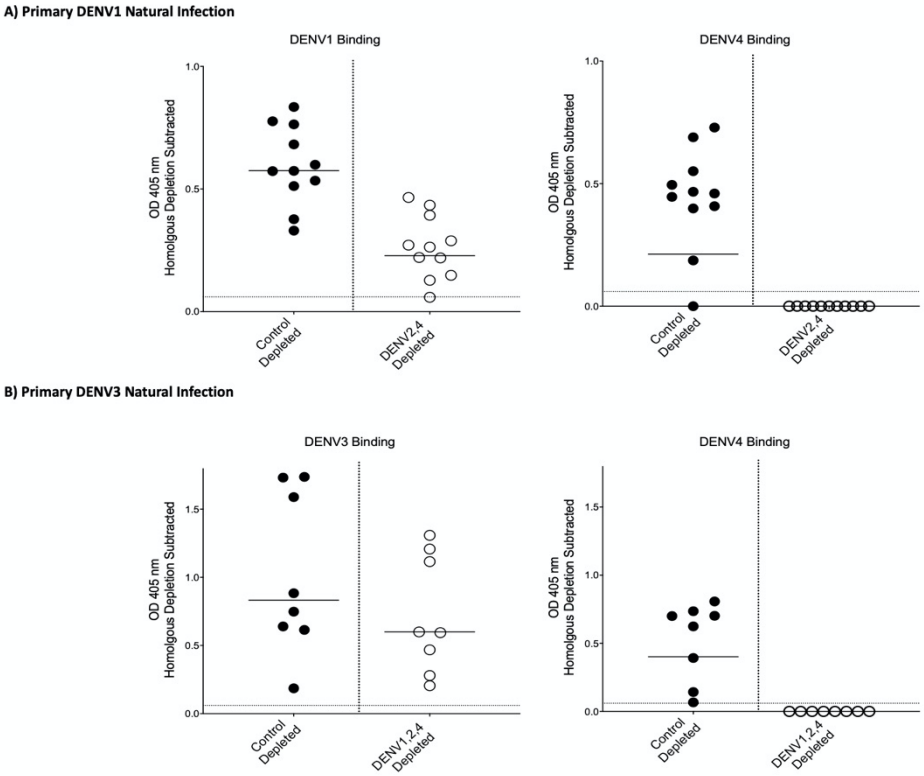


Figure 3.1. Depletion of DENV serotype cross-reactive antibodies in sera from people exposed to primary DENV1 or DENV3 infections.

Convalescent sera from people exposed to primary (A) DENV1 or (B) DENV3 infections were incubated beads coated with heterologous DENV serotypes to remove serotype CR Abs. The depleted sera were tested by ELISA for Abs binding to a heterologous serotype (DENV4) to confirm removal of CR Abs. The depleted sera were tested by ELISA for Abs binding to the homologous serotype (DENV1 or DENV3) to estimate levels of TS binding Abs. Limit of detection (dashed dotted line) was defined as average of normal human sera + standard deviation multiplied by 3.

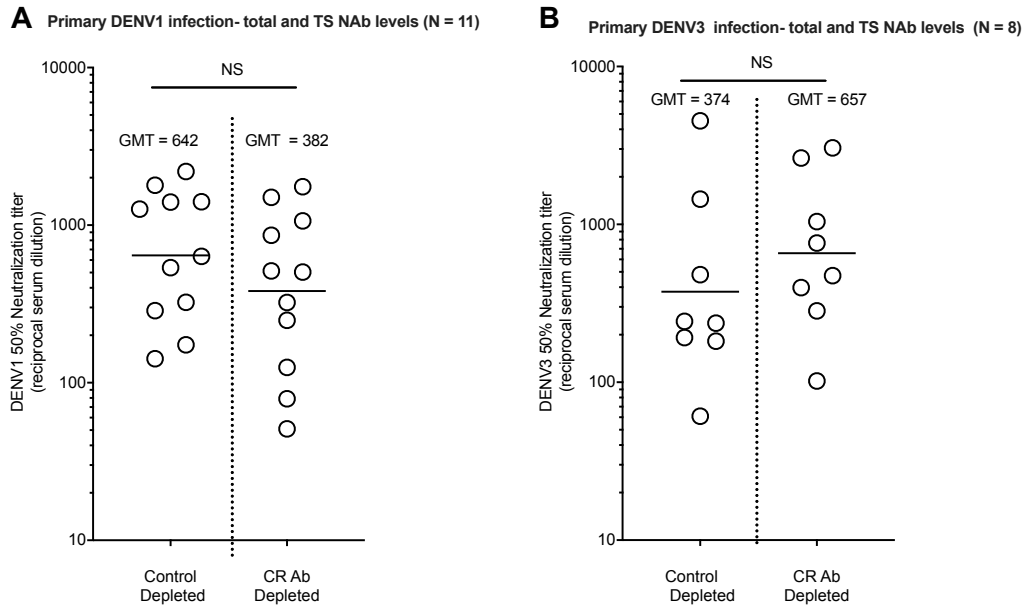


Figure 3.2. Antibody responses following primary DENV1 or DENV3 infection.

Convalescent sera from people exposed to primary (A) DENV1 or (B) DENV3 infections were tested for NAb before (control depletion) and after removal of serotype CR Abs. The levels of NAbs were not reduced after removal of CR Abs. Wilcoxon signed ranks test was used to establish statistical significance.

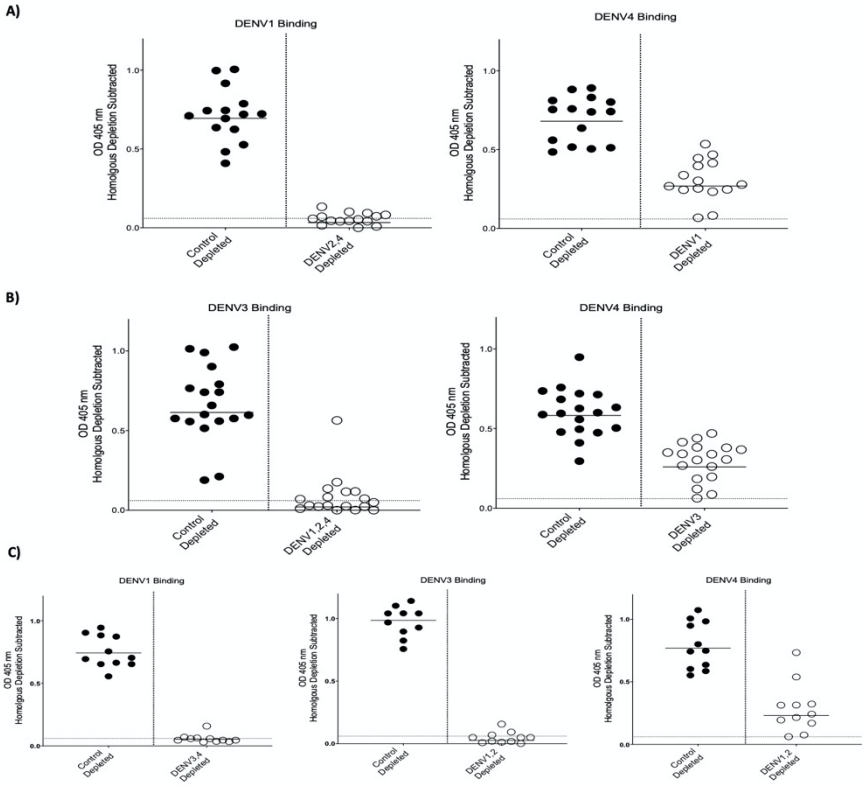


Figure 3.3. Depletion of specific antibody populations from immune sera collected from dengue naïve children who received Dengvaxia.

Vaccine immune sera from children who subsequently experienced DENV1 breakthrough infections (A), DENV3 breakthrough infections (B) or no DENV infections (C) were incubated with beads coated with dengue virions to remove specific Ab populations. The depleted sera were tested by ELISA to estimate levels of TS and CR binding antibodies to DENV1, DENV3 and DENV4. Limit of detection (dashed dotted line) was defined as average of normal human sera + standard deviation multiplied by 3.

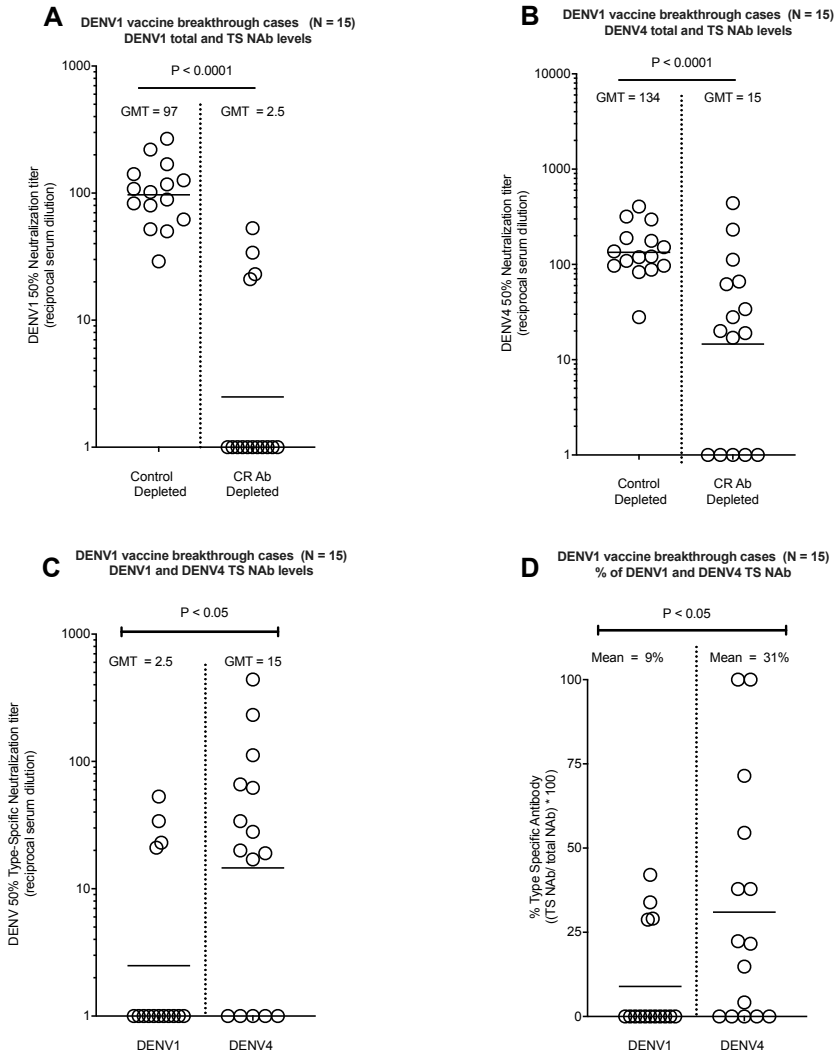


Figure 3.4. Vaccine induced Ab responses in children who experienced DENV1 breakthrough infections.

Vaccine responses were analyzed in baseline seronegative children who received Dengvaxia and subsequently experienced DENV1 (n = 15) breakthrough infections. The sera were tested to measure levels of total and TS (CR Ab depleted) DENV1 (A) and DENV4 (B) NAbs. The data were also analyzed to compare absolute level (C) and percentage (D) of DENV1 and DENV4 TS NAbs in the children after vaccination. Wilcoxon signed ranks test was used to establish statistical significance.

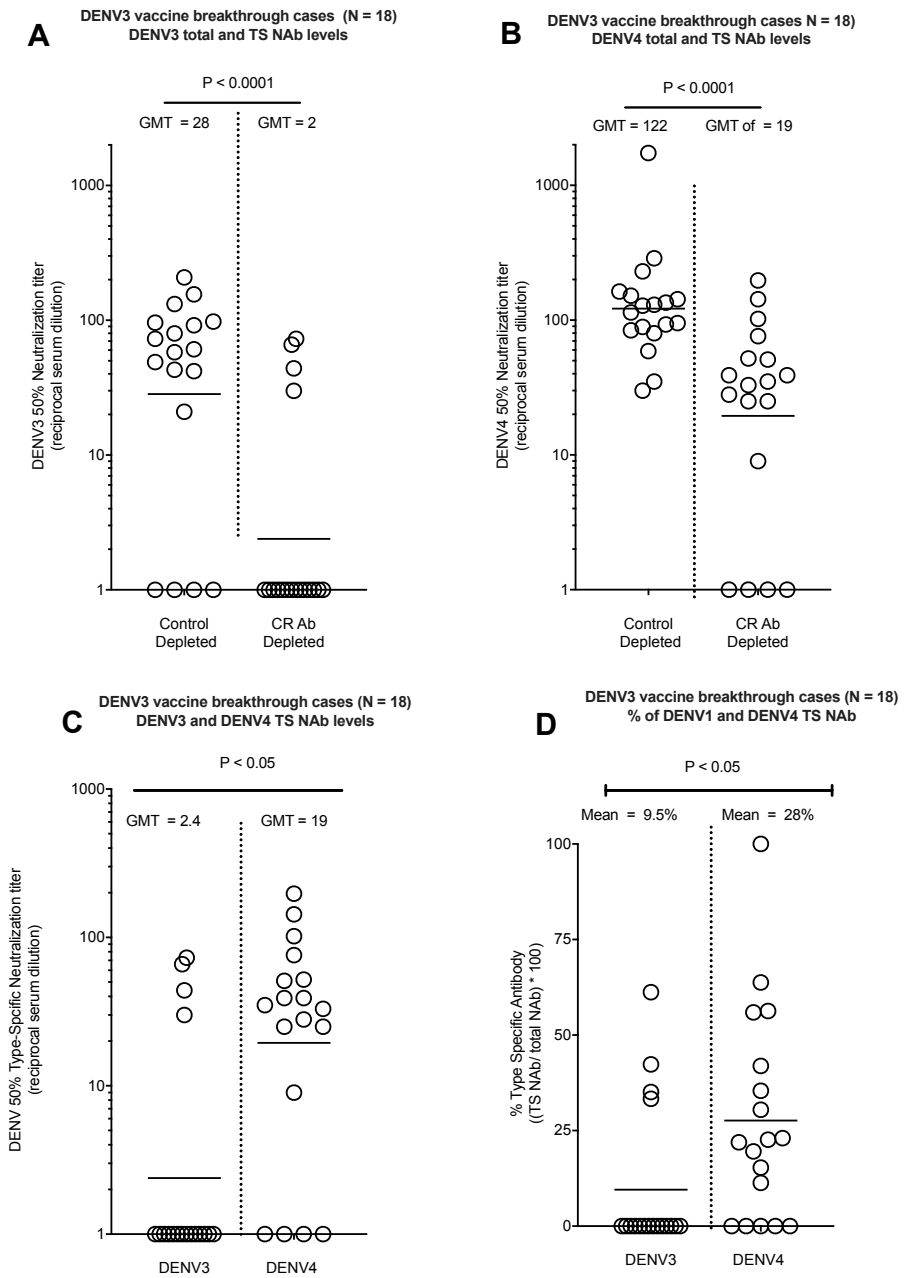


Figure 3.5. Vaccine induced Ab responses in children who experienced DENV3 breakthrough infections.

Vaccine responses were analyzed in baseline seronegative children who received Dengvaxia and subsequently experienced DENV3 (n = 18) breakthrough infections. The sera were tested to measure levels of total and TS (CR Ab depleted) DENV3 (A) and DENV4 (B) NAbs. The data were also analyzed to compare absolute level (C) and percentage (D) of DENV3 and DENV4 TS NAbs in the children after vaccination. Wilcoxon signed ranks test was used to establish statistical significance.

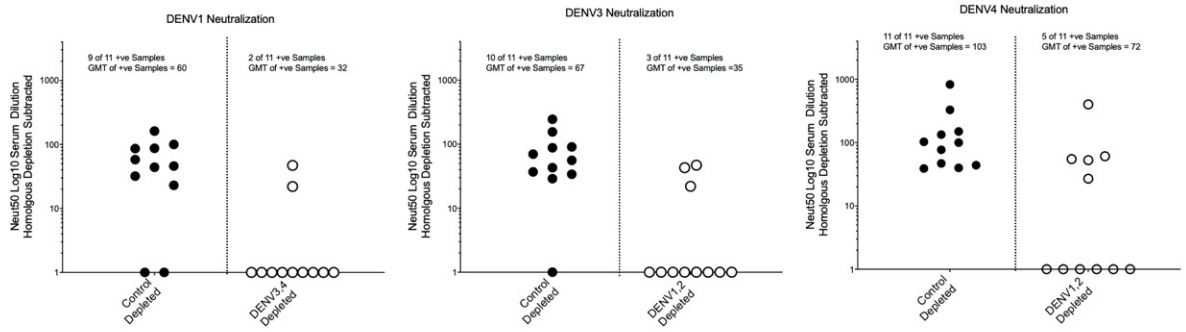


Figure 3.6. Vaccine induced Ab responses in children with no documented DENV breakthrough infections.

Vaccine responses were analyzed in baseline seronegative children (n = 11) who received Dengvaxia and did not experience breakthrough infections during the clinical trial. The sera were tested to measure levels of total and TS NAbs to DENV1 (A), DENV3 (B) and DENV4(C).

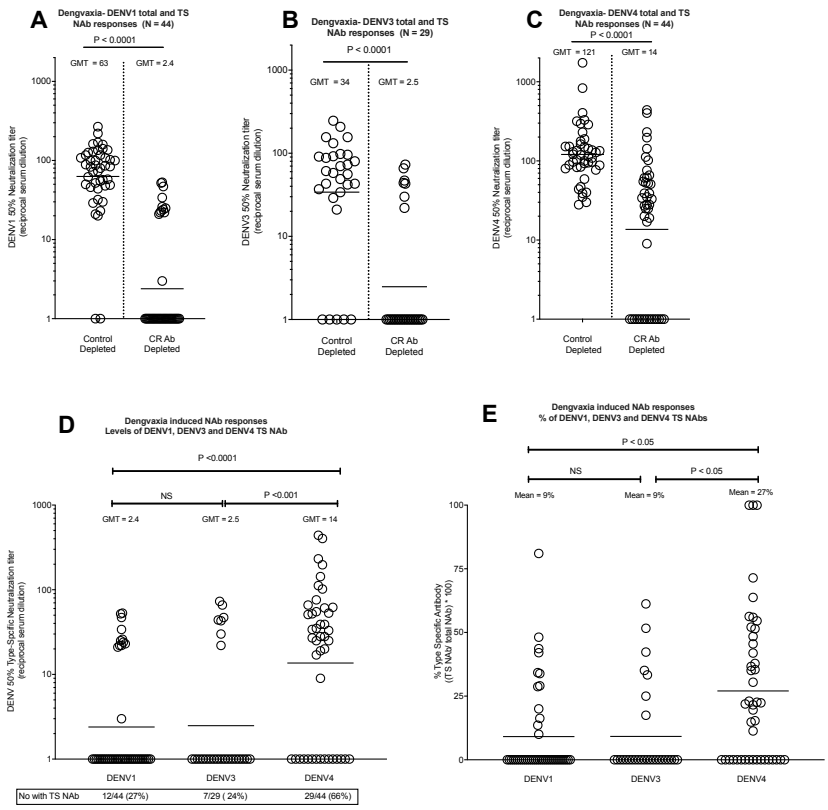


Figure 3.7. Dengvaxia induced Ab responses in children with no pre-existing immunity to DENVs.

Dengvaxia recipients irrespective of subsequent outcome during CYD-TDV clinical trials were tested to determine levels of total and TS DENV1 (A), DENV3 (B) and DENV4 (C) NAbs. Wilcoxon signed ranks test was used to establish statistical significance. The data were also analyzed to compare absolute level (C) and percentage (D) of vaccine induced DENV1, DENV3 and DENV4 TS NAbs. Kruskal Wallis test was used to compare Ab responses between serotypes.

Supplementary Table 1. Dengvaxia Breakthrough Infection Specimens				
Sample	Country of Origin	Genotype	Infecting Virus	Number of days between last dose of vaccine and breakthrough infection
DV1-1	Colombia	V	DENV1	157
DV1-2	Colombia	V		112
DV1-3	Philippines	IV		1108
DV1-4	Philippines	IV		130
DV1-5	Philippines	IV		308
DV1-6	Philippines	IV		336
DV1-7	Thailand	I		972
DV1-8	Thailand	I		994
DV1-9	Mexico	V		46
DV1-10	Mexico	V		714
DV1-11	Mexico	V		263
DV1-12	Mexico	V		243
DV1-13	Vietnam	I		502
DV1-14	Vietnam	I		264
DV1-15	Vietnam	I		1003
DV3-1	Colombia	III	DENV3	213
DV3-2	Colombia	III		150
DV3-3	Colombia	III		74
DV3-4	Colombia	III		58
DV3-5	Colombia	III		339
DV3-6	Colombia	III		93
DV3-7	Colombia	III		155
DV3-8	Colombia	III		359
DV3-9	Colombia	III		662
DV3-10	Colombia	III		47
DV3-11	Honduras	III		411
DV3-12	Honduras	III		337
DV3-13	Honduras	III		359
DV3-14	Philippines	I		1103
DV3-15	Thailand	III		316
DV3-16	Thailand	III		960
DV3-17	Thailand	III		1047
DV3-18	Vietnam	II		398

Table 3-1. Dengvaxia breakthrough infections specimens.

Sample	Country of Infection	Infecting Virus	Year of Infection
WT DV1-1	India	DENV1	2007
WT DV1-2	Ecuador		2006
WT DV1-3	Bolivia		2012
WT DV1-4	India		1991
WT DV1-5	Unknown		Unknown
WT DV1-6	Unknown		Unknown
WT DV1-7	Virgin Islands		1982-1995
WT DV1-8	Brazil		1998
WT DV1-9	Dominican Republic		2004
WT DV1-10	Guyana		2010
WT DV1-11	Malaysia		2008
WT DV3-1	Unknown	DENV3	Unknown
WT DV3-2	Unknown		Unknown
WT DV3-3	Nicaragua		1995
WT DV3-4	Thailand		2002
WT DV3-5	Sri Lanka		2008
WT DV3-6	Nicaragua		2009
WT DV3-7	Sri Lanka		2011
WT DV3-8	Nicaragua		1998

Table 3-2. WT DENV1 and DENV3 infection specimens

Subject	Breakthrough Infection	Dep Strategy	% TS-Ab-DV1	% TS-Ab-DV3	% TS-Ab-DV4
C-1	None	BSA, DV1/2, DV3/4	0	25	0
C-2		BSA, DV1/2, DV3/4	0	0	35
C-3		BSA, DV1/2, DV3/4	0	0	6
C-4		BSA, DV1/2, DV3/4	0	0	46
C-5		BSA, DV1/2, DV3/4	0	0	0
C-6		BSA, DV1/2, DV3/4	0	0	37
C-7		BSA, DV1/2, DV3/4	0	0	51
C-8		BSA, DV1/2, DV3/4	0	17	6
C-9		BSA, DV1/2, DV3/4	14	0	48
C-10		BSA, DV1/2, DV3/4	0	52	0
C-11		BSA, DV1/2, DV3/4	81	0	0
DV3-1	DENV3	BSA, DV3, DV1/2/4	NT	61	35
DV3-2		BSA, DV3, DV1/2/4	NT	0	11
DV3-3		BSA, DV3, DV1/2/4	NT	0	42
DV3-4		BSA, DV3, DV1/2/4	NT	0	0
DV3-5		BSA, DV3, DV1/2/4	NT	0	100
DV3-6		BSA, DV3, DV1/2/4	NT	0	56
DV3-7		BSA, DV3, DV1/2/4	NT	0	30
DV3-8		BSA, DV3, DV1/2/4	NT	42	23
DV3-9		BSA, DV3, DV1/2/4	NT	0	64
DV3-10		BSA, DV3, DV1/2/4	NT	0	30
DV3-11		BSA, DV3, DV1/2/4	NT	0	0
DV3-12		BSA, DV3, DV1/2/4	NT	0	15
DV3-13		BSA, DV3, DV1/2/4	NT	35	20
DV3-14		BSA, DV3, DV1/2/4	NT	0	56
DV3-15		BSA, DV3, DV1/2/4	NT	0	22
DV3-16		BSA, DV3, DV1/2/4	NT	0	0
DV3-17		BSA, DV3, DV1/2/4	NT	0	0
DV3-18		BSA, DV3, DV1/2/4	NT	33	22
DV1-1	DENV1	BSA, DV1, DV2/4	0	NT	8
DV1-2		BSA, DV1, DV2/4	0	NT	0
DV1-3		BSA, DV1, DV2/4	0	NT	0
DV1-4		BSA, DV1, DV2/4	0	NT	38
DV1-5		BSA, DV1, DV2/4	0	NT	4
DV1-6		BSA, DV1, DV2/4	0	NT	100
DV1-7		BSA, DV1, DV2/4	7	NT	22
DV1-8		BSA, DV1, DV2/4	29	NT	15
DV1-9		BSA, DV1, DV2/4	0	NT	0
DV1-10		BSA, DV1, DV2/4	8	NT	22
DV1-11		BSA, DV1, DV2/4	0	NT	71
DV1-12		BSA, DV1, DV2/4	0	NT	55
DV1-13		BSA, DV1, DV2/4	29	NT	52
DV1-14		BSA, DV1, DV2/4	34	NT	100
DV1-15		BSA, DV1, DV2/4	42	NT	0

Table 3-3. Percentage of serotype specific antibodies in Dengvaxia recipients.

CHAPTER 4: THE ROLE OF STERILIZING IMMUNITY IN ANTIBODY PROTECTION AGAINST DENV INFECTION[2]

4.1 Overview:

Antibody response to dengue virus (DENV) primary infection provides long-term protection from disease caused by repeat infections by the same serotype. The extent to which these antibodies provide durable virus sterilizing immunity against repeat infections is unknown. Dengvaxia (CYD-TDV) is a tetravalent live attenuated chimeric vaccine with DENV prM and E-proteins inserted into a YFV-17D backbone. Recent studies demonstrated CYD-TDV is unbalanced, with high level replication of the DENV4 component and little to no replication of the other 3 serotypes. In this chapter, I used the unbalanced CYD-TDV vaccine as a DENV4 human live DENV challenge model to assess if antibodies stimulated by the primary infections provide sterilizing immunity. In CYD-TDV clinical trials, I predicted that individuals with DENV4 primary immunity will sterilize the DENV4 vaccine component more frequently than individuals with primary immunity to DENV1, 2 or 3. My results do not support this hypothesis.

4.2 Introduction:

An estimated 96 million symptomatic DENV infections occur annually (*1*). DENV1-4 often co-circulate in endemic regions where majority of individuals will experience two or more DENV infections before teenage years (*131*). Antibody response to the first DENV infection is comprised of neutralizing antibodies specific to infecting serotype as well as cross-reactive non-neutralizing antibodies that can

[2] A portion of this chapter was previously published in Lancet Global Health. The original citation is as follows: Lopez AL, Adams C, Ylade M, Jadi R, Daag JV, Molloy CT, Agrupis KA, Kim DR, Silva MW, Yoon IK, White L, Deen J, de Silva AM. Determining dengue virus serostatus by indirect IgG ELISA compared with focus reduction neutralisation test in children in Cebu, Philippines: a prospective population-based study. Lancet Glob Health. 2021 Jan;9(1):e44-e51. doi: 10.1016/S2214-109X(20)30392-2. Epub 2020 Nov 16. PMID: 33212030.

bind other DENV serotypes (primary serostatus). Secondary infection with a heterologous serotype results in development of a cross-neutralizing antibody response that can neutralize all four DENV serotypes (*33*).

Previous studies demonstrated neutralizing antibodies derived from a primary infection can inhibit disease when challenged with the homotypic serotype (*3, 4*). Yet it is unclear if neutralizing antibodies provides sterilizing immunity as increasing evidence suggests rare homotypic reinfections are possible (*32, 132, 133*). While human challenge models provide a direct avenue to assess sterilizing immunity,(*134*) they are limited in size and cost. Live, attenuated vaccines provide an alternative to challenge models as measurement of immunologic outcomes after vaccination can serve as a surrogate for vaccine replication (*135*).

CYD-TDV is a live attenuated tetravalent chimeric vaccine produced by Sanofi Pasteur (SP) that comprises of all four DENV pr/E proteins inserted into a YFV 17-D backbone. CYD-TDV is currently approved for clinical use for individuals aged 9 through 16 with previously laboratory-confirmed DENV infection and living in endemic areas. CYD-TDV demonstrated efficacy against DENV1-4 in individuals with prior DENV immunity but failed to provide protection against DENV1-3 infection in naïve individuals (*7*). Preclinical evaluations demonstrated CYD-TDV is a unbalanced live attenuated tetravalent vaccine that is weighted heavily towards the DENV4 component (*89, 90*). Based upon initial efficacy in phase III clinical trials, CYD-TDV was licensed in 20 countries but most prominently used in a short lived school based immunization program in the Philippines where individuals received a single dose of CYD-TDV. In this chapter, I use specimens from a cohort of 2,996 children aged 9-14 in the Cebu province of the Philippines enrolled in a Dengvaxia study to determine the impact of pre-existing antibodies on the replication of the DENV4 vaccine component. I developed a novel algorithm combining a simplified neutralization assay and a commercial ELISA to define DENV serostatus of the entire cohort before vaccination (*131*). To test my hypothesis, I identified a subset of vaccinated individuals with primary immunity to DENV1, 2, 3 or 4 prior to vaccination and compared vaccine induced antibody responses before and after vaccination.

4.3 Results:

830,000 children in the Philippines were enrolled in a CYD-TDV school based immunization program (*75*). To study the vaccine effectiveness of CYD-TDV in a phase IV clinical trial, the Philippines NIH recruited a cohort of 2,996 children, 65% of which received a single dose of CYD-TDV. To understand the vaccine's clinical outcome based upon prior DENV immune status, the Philippines NIH measured DENV binding antibodies in pre-vaccination serum sample by a dengue IgG antibody indirect ELISA (Panbio, Brisbane, Australia, Cat #: E-DEN01G). They identified 13% of individuals had no DENV binding antibody (n=395), 87% of individuals had DENV binding antibody (n=2595), and six individuals were unable to be categorized due to an equivocal reading. This commercial dengue IgG antibody indirect ELISA was previously shown to be highly specific (100%) for DENV seronegative individuals and highly sensitive for individuals (97.9%) with DENV multiypic immunity but had low sensitivity for primary serostatus individuals (33%) (*136*). To identify individual's pre-vaccination serostatus, the gold standard assay is a 8 dilution focus reduction virus neutralization test (FRNT) but this assay is unwieldy in context of large cohorts. To remedy this and identify the DENV serostatus of each child in the cohort, I designed an algorithm that incorporated the commercial DENV ELISA as an initial screen and subsequently tested remaining samples by a simplified version of the gold standard FRNT. I simplified the gold standard neutralization test by performing a single dilution virus neutralization test at 1:40. The single dilution FRNT with a assay cutoff value of 70% neutralization was 95% specific and 93% sensitive compared to the gold standard 8 dilution FRNT(*31*) **(Figure 4.1).** To reduce the number of samples to be tested by 1:40 FRNT, I tested a subset of individuals at the extremes of ELISA index value range to assess if the ELISA index values correlates with FRNT phenotype. Individuals with an ELISA index value < 0.2 (n=79) were 98.7% DENV naïve by 1:40 FRNT. Individuals with ELISA index value >3.0 (n=370) were 98.9% multitypic DENV serostatus by 1:40 FRNT **(Table 4-1).** Of 2,996 samples, 7.8% (n=233) had a DENV IgG ELISA index value less than 0.2. Based upon our analysis, I categorized these individuals as DENV naïve. 41.8% had a value greater than 3.0 (n=1,251) and were categorized as DENV multitypic. For the remainder of the samples with DENV IgG ELISA index value between .2 and 3.0 (n=1,512), I performed the 1:40 dilution FRNT to determine DENV serostatus prior to vaccination. Individuals who demonstrated neutralizing antibody at 1:40 >70% to only 1 DENV serotype were categorized as DENV primary.

Individuals who demonstrated neutralizing antibody at 1:40 >70% to two or more DENV serotypes were classified as DENV multitypic. Individuals without neutralizing antibody to any DNV serotype >70% were classified as DENV naïve. From this algorithm, I categorized our cohort as 10.6% DENV naïve (n=320), 9.6% primary DENV immune (n=292), and 79.6% multitypic DENV immune prior to vaccination (n=2384) **(Figure 4.2)**.

After identifying individuals with primary DENV immunity prior to vaccination, I compared pre- and post-vaccination neutralizing antibodies. To perform this analysis, I aimed to use viruses that best represented clinical strains that infect individuals in the Philippines. Cell culture adapted viruses grown in standard cell lines contain structural properties that are not suggested to reflect virions circulating within humans. (*30*) As DENV egresses from the cell, DENV virion undergoes a maturation process catalyzed by the human enzyme, Furin. Furin acts in a pH dependent manner to cleave the pr peptide from the prM proteins, allowing production of a smooth, fully mature, infectious virus particle. In common laboratory cell lines used to create DENV stocks, inefficient prM processing creates a heterogenous mixture of immature, partially mature, and fully mature virions. Additionally, recent studies on laboratory adapted DENV demonstrated the viral envelope is more flexible under higher temperatures. This viral breathing is attributed to cell culture adaptions accumulating from repeated passages in cell culture lines. Collectively, these factors result in more easily neutralized DENV when compared to cell culture isolates. To understand the vaccine response, I identified a panel of DENV clinical isolates reflective of currently circulating genotypes **(Figure 4.3)** (*137, 138*) and grew the clinical isolate viruses in a modified Vero-81 cell line over-expressing the Furin enzyme.

To test my prediction that individuals exposed to primary DENV4 infections will sterilize the DENV4 vaccine component better than individuals exposed to primary infections with the other three serotypes, I compared the neutralizing antibody titers in 56 children 12-24 months after a receiving a single dose of CYD-TDV to pre-vaccination neutralizing antibodies titers. In DENV1 (n=14), DENV2 (n=22), and DENV3 (n=4) primary serostatus children, the majority of individuals mount a secondary response (62.5%, 25/40) **(Figure 4.4A).** 43.7% of the DENV4 primary serostatus individuals (7/16) demonstrated a change in neutralizing antibody titer to a heterologous serotype **(Figure 4.4B).** The proportions of DENV4 primary serostatus individuals mounting a secondary response against the

proportion of DENV1, DENV2, DENV3 primary serostatus mounting a secondary response did not reach statistical significance (p-value=.24) **(Table 4-2).** These results suggest that DENV4 primary serostatus did not significantly reduce replication of CYD-TDV when compared to the other three DENV primary serostatus.

The antibody response to DENV infection is focused primarily on the E, prM, and NS1 proteins (*25, 64*). Dengvaxia is a chimeric live attenuated tetravalent vaccine with the DENV1-4 E and prM proteins inserted into a YFV 17-D backbone that includes YFV NS1. YFV is not prominent in Asia (*139*) and YFV 17D vaccination is only recommended for individuals traveling to Africa or South America(*140*). To provide another independent gauge of vaccine response in the primary serostatus children, we developed a YFV NS1 ELISA. Prior to vaccination, the majority of our subjects did not react with YFV NS1 **(Figure 4.5)**. Initially, we compared YFV NS1 reactivity in a subset of both DENV4 primary serostatus individuals and DENV1,DENV2, and DENV3 serostatus individuals. Difference among the two groups was statistically insignificant. (p-value .10) **(Figure 4.6A).** We subsequently compared change after vaccination in YFV reactivity among children who developed a secondary neutralizing antibody response to individuals who did not **(Figure 4.6B)**. Differences among the two groups was statistically insignificant (p-value = .12).

4.4 Discussion:

I utilized the unbalanced CYD-TDV live attenuated vaccine as a DENV4 human challenge model to understand if DENV primary immunity provides sterilizing immunity. I compared neutralizing antibody levels and YFV NS1 specific antibody levels in individuals with DENV1, DENV2, or DENV3 immunity prior to vaccination to individuals with primary DENV4 immunity prior to vaccination. I found no significant difference among DENV4 primary immunity individuals compared to DENV1, DENV2, or DENV3 in either neutralizing or YFV NS1 antibody levels.

Our study has several limitations. Previously, the Baric laboratory demonstrated the effect DENV2 and DENV4 genotype have on neutralizing antibody levels in both natural infection and vaccinated individuals (*120, 141*). Juraska et. al. described CYD-TDV vaccine efficacy decreased significantly in for younger subjects in the phase III clinical trial when measured against DENV4 genotype

1 compared to DENV4 genotype II, identifying a sieve effect (*126*). Here we did not account for this genotypic effect by measuring neutralization against different genotypes of DENV4. CYD-TDV is a unbalanced tetravalent live attenuated DENV vaccine that is weighted towards the DENV4 component(*90*) but other serotypes were still detected by PCR after initial dose in other studies (*89*). Unpublished data from our laboratory suggests the DENV2 component replicates in DENV naïve individuals who received the three dose regiment during phase III clinical trial (data now shown). My data suggests that individuals with DENV2 serotype have a higher frequency of boost to their primary DENV serotype after vaccination compared to other serotypes, suggesting replication of the DENV2 component of the vaccine **(Figure 4.7)** but further analysis is needed to identify what component of the vaccine replicated in these individuals. I originally identified 292 individuals who were primary immune prior to vaccination. 145 vaccinated individuals returned for follow-up post-vaccination blood draw who were vaccinated. At time of writing, we present data for 56 individuals but will complete the remaining 89 individuals to better support our conclusions. A better measure of sterilizing immunity would be detection of replicating vaccine virus shortly after vaccination. I used immunological surrogates of vaccine replication in this analysis as an indirect measure of vaccine replication, but detection of these surrogates could be influenced by the timing of collection of post-vaccine sample. Antibody response to a single dose of CYD-TDV was recently suggested to wane over time (*142, 143*).

SP retrospective analysis was limited by the broad categorization of individuals into naïve and DENV pre-immune subjects (*7*). This study was sufficient to identify the CYD-TDV risks in DENV naïve individuals but insufficient to understand vaccine response differences among primary and multitypic serostatus individuals. Follow up studies on individuals who received CYD-TDV have produced new estimates of vaccine efficacy over time. Vaccine efficacy calculated over 13 months since final vaccination dose in primary or multitypic immune individuals revealed an efficacy of 77.4% (56.4-88.2) and 89.2% (71.5-95.9) respectively (*144*). Continued follow-up up to five years after final dose estimated an lower efficacy of 49.2% (19.4-68.0) in seropositive individuals (*145*). Our cohort suggests 45% of children who received a single dose of CYD-TDV did not demonstrate a secondary neutralizing antibody response 1 year after receiving vaccination **(Figure 2).** This is surprising considering that the majority of immunity from other live attenuated flavivirus vaccines occurs after a single dose (*146, 147*). SP recently

identified two doses (instead of three) was sufficient for elicitation and detection of an antibody response but a single dose had a waning antibody response after 1 year (*142*). The Philippines NIH will continue to follow this cohort for another three years to document their DENV clinical outcome and link it to the post-vaccination response to understand if the CYD-TDV single dose affords protection.

My analysis of the post-vaccination response in primary serostatus individuals suggested DENV4 primary immunity did not afford sterilizing immunity when challenged with a single dose of the unbalanced, DENV4 weighted, CYD-TDV. My study was limited by the sample size and time of sampling. The low rate of conversion to secondary neutralizing antibody phenotype in primary individuals was surprising but continued monitoring for DENV infection in the cohort is required to link to clinical outcome.

4.5 Materials and Methods:

Study Participants: This study is part of a longitudinal cohort study conducted in Bogo and Balamban, two semi-urban areas in the Cebu province of the Philippines, to understand the efficacy of a single CYD-TDV dose. In June 2017, a community-based mass dengue vaccination program vaccinated children aged 9–14 years in the Cebu province of the Philippines but was abruptly discontinued after one dose. Post-vaccination samples were collected 12-24 months after receiving single CYD-TDV dose.

PanBio ELISA: The University of the Philippines Manila National Institutes of Health (Manila, Philippines) performed the dengue IgG antibody indirect ELISA (PanBio; Brisbane, QLD, Australia) following the manufacturer's instructions (*136*). The manufacturer's recommended index cutoff values were as follows: less than 0·9 indicated seronegativity (no evidence of previous dengue infection), 0·9–1·1 indicated an equivocal serostatus, and more than 1·1 indicated seropositivity (presence of detectable IgG antibodies, indicating a previous dengue infection).

Virus Strains and Amino Acid Alignment: Initial, pre-vaccination characterization used West Pac 1974 (DENV1, Accession number: 4GT0_B), S16803 (DENV2, GU289914), CH53489 (DENV3, DQ863638), TVP376 (DENV4, KC963424) grown in C636 cell line (ATCC# CRL-1660). Comparison of pre- and post-vaccination neutralizing antibody used GS0736 (DENV1, (*30*)), GS1816 (DENV2,

MK579857), UNC3001 (DENV3, JQ411814), and Sri Lanka 1992a (DENV4, FJ225466). Stains used in CYD-TDV vaccine used in comparison: Thailand PUO-359/TVP-1140 (DENV1, AF425630),Thailand PUO-218 (DENV2, AF038402), Thailand PaH881/88 (DENV3, AF349753), and Indonesia 1228/TVP-980 (DENV4, JN022608). Amino acid sequences were aligned using Multiple Sequence Alignment (MUSCLE) program in Seaview (*148*).

Focus Reduction Neutralization Test: To measure neutralizing antibody titers to DENV 1–4, we used a micro FRNT adapted to a 96-well plate format as described previously (*31*). 96 well plates were seeded with 20,000 cells per well 24 hours prior to performing assay. For the full FRNT, serial three-fold dilutions of each serum were mixed with 50–100 focus-forming units of virus in Dulbecco's modified Eagle's medium (Gibco; Grand Island, NY, USA) with 2% fetal bovine serum. The virus-antibody mixtures were incubated for 1 hour at 37°C and then transferred to the confluent monolayer of Vero-81 cells on the 96-well plates. Following an additional 1 hour incubation at 37°C, the monolayers were overlaid with Opti-MEM (Gibco) containing 2% fetal bovine serum and 1% (weight per volume) carboxymethyl cellulose (Sigma; St Louis, MO, USA). 50% effective concentration (EC50) values were calculated by graphing percentage neutralization versus serum dilution and fitting a sigmoidal dose response (variable slope) using Prism 8 (GraphPad Software; San Diego, CA, USA). Neutralizing antibody titers (Neut50) represent the dilution at which the serum neutralizes 50% of the infection. Criteria to accept values to be reported were an R^2 of more than 0.75 and a Hill Slope absolute value of more than 0.5. The simplified version of the FRNT performed the FRNT at a single 1:40 dilution. Individuals who crossed the 70% neutralization threshold at 1:40 were considered positive. For samples tested by FRNT, the neutralization of a single dengue serotype was classified as a primary or monotypic response, indicating one previous dengue infection. Neutralization of two or more dengue serotypes was defined as a multitypic response, indicating two or more previous dengue infections; these samples were included in full-curve FRNT.

YFV NS1 ELISA: Purified YFV NS1 was purchased from the Native Antigen Company. (YFV-NS1-500) YFV NS1 was coated overnight in 1x TBS at 2 ug/mL on 96 well high-binding plates (VWR #82050-720). Coated plate was washed 3x with 0.2% TBS-Tween20 (TBS-T) then subsequently blocked

for 1 hour at 37C with 3% non-fat dried milk in .05% TBS-T. Sera diluted 1:20 was added in duplicate and detected at 405 nm using 1:2500 Goat Anti-Human IgG (FC) AP conjugated antibody (Sigma Cat #: A9544). A pooled normal human sera control and a confirmed YFV vaccinee were run on every ELISA plate. NHS values were subtracted from raw OD value of samples and reading was normalized to the confirmed YFV vaccinee reading. Limit of detection was calculated as average NHS value + 2x standard deviation.

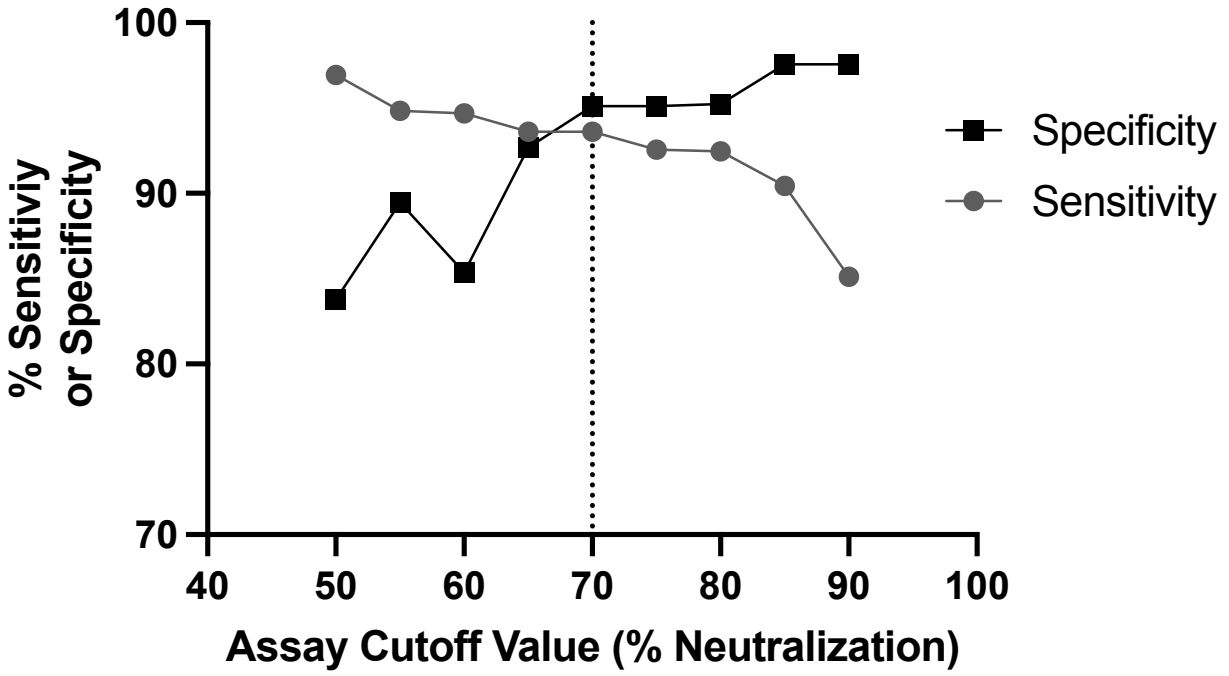


Figure 4.1. Sensitivity and specificity of 1:40 FRNT vs 8 dilution FRNT phenotype.

Pre-vaccination samples were concurrently tested in 1:40 and 8 dilution FRNT format against DENV1-4 WHO strains. Neutralizing phenotype in 8 dilution FRNT was assigned by previously validated algorithm(*31*). 1:40 dilution phenotype was defined by number of serotypes neutralized (no serotypes = naïve, one serotype = primary, two or more = multitypic). 70% neutralization at 1:40 is 95% specific and 93% sensitive compared to phenotype defined by 8 dilution FRNT.

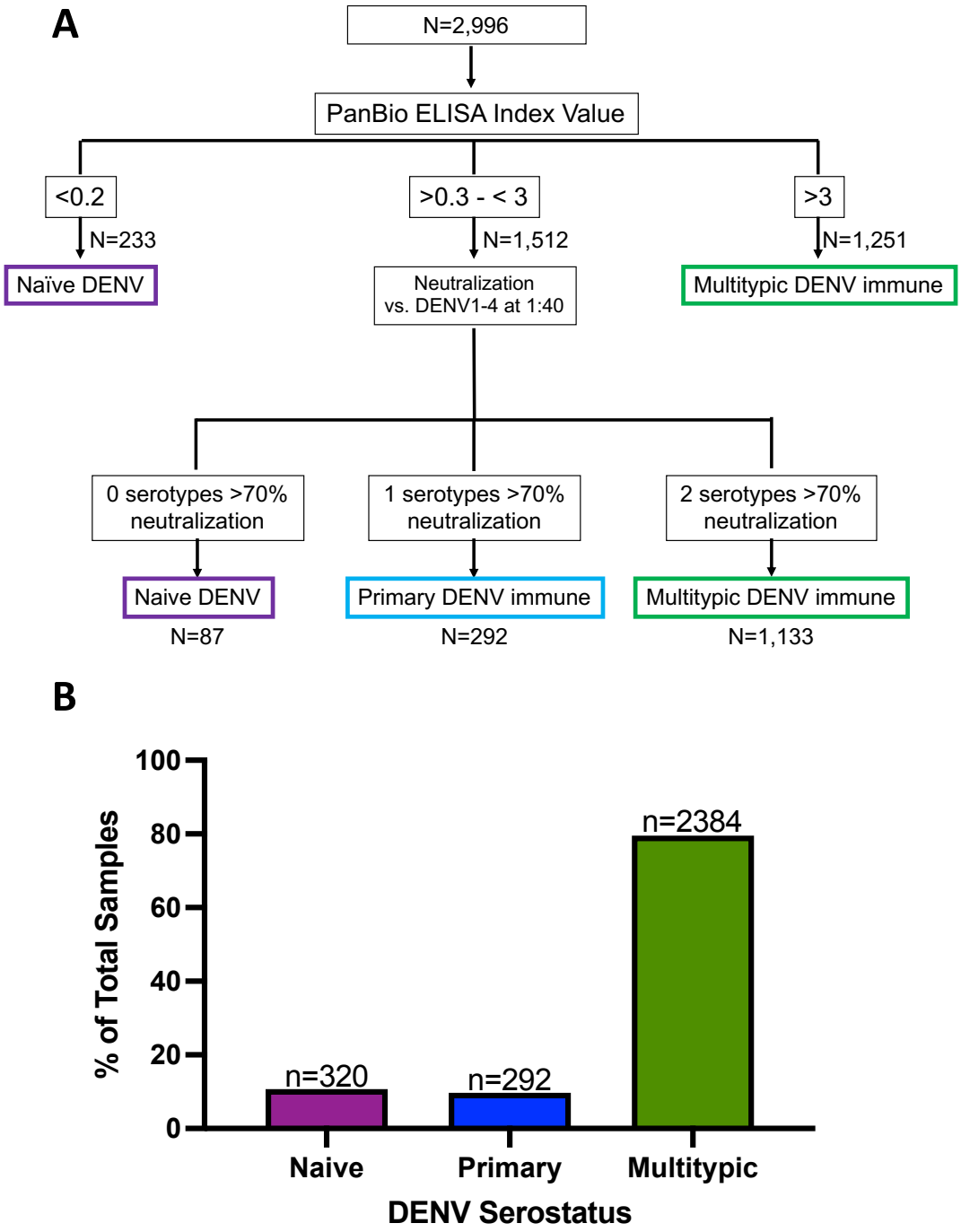


Figure 4.2. Categorization of serostatus prior to vaccination.

Philippines NIH recruited 2,996 pediatric subjects aged 9-14. **A)** To establish pre-vaccination serostatus, 2,996 subjects were tested by dengue IgG antibody indirect ELISA. (Panbio, Brisbane, Australia).

Individuals with ELISA index value <0.2 were considered DENV naïve and ELISA index value >3.0 were considered DENV multitypic. Individuals with index value between .2-3.0 were tested by single dilution FRNT at 1:40 against DENV1-4 WHO strains in Vero-81 focus reduction neutralization test. **B)** Individuals were categorized using commercial DENV ELISA and 1:40 FRNT into naïve, primary, and multitypic serostatus. 10.7% of cohort is DENV naïve, 9.7% DENV primary, and 79.6% DENV multitypic.

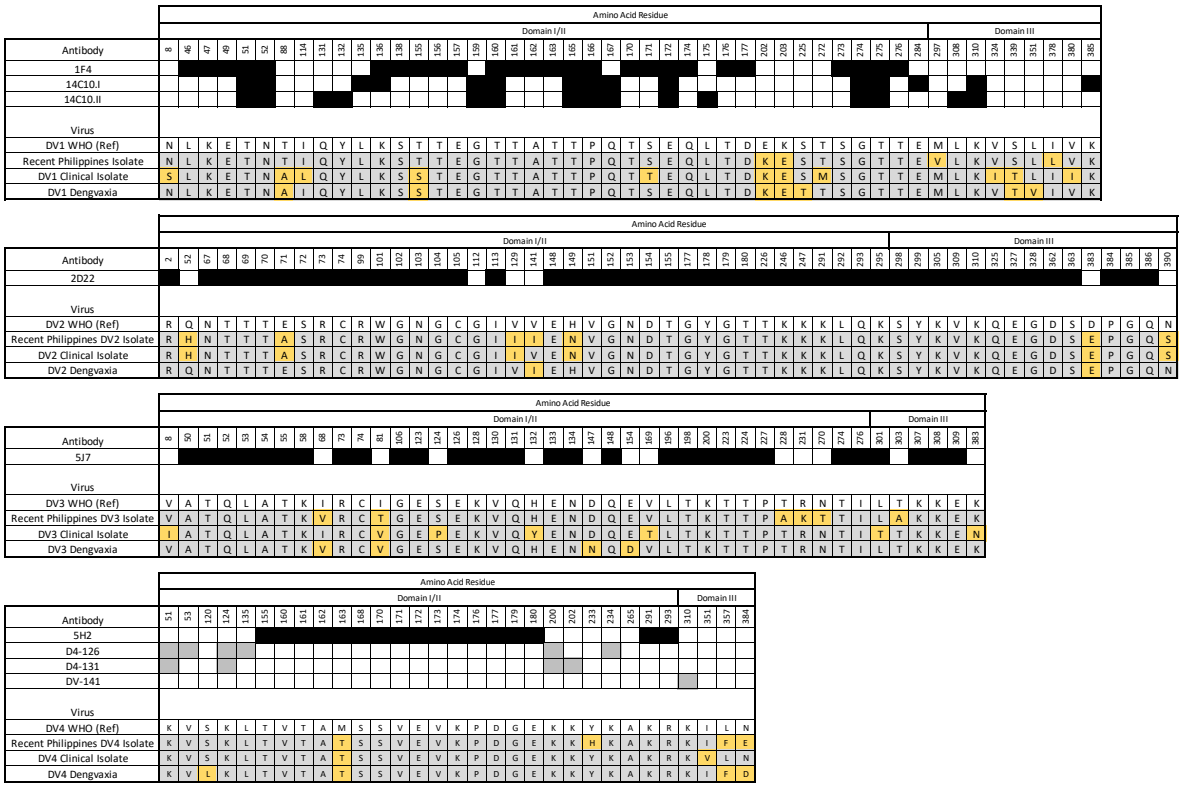


Figure 4.3 Comparison of E-protein amino acid sequence for DENV WHO, recent Philippines isolate, DENV clinical isolate and CYD-TDV strains.

Antibody contact sites previously identified from high resolution structure of potently neutralizing specific antibody with DENV-E protein are indicated by black boxes. Key residues for antibody binding identified by shotgun mutagenesis are indicated by grey boxes. Variant residues (in reference to WHO strain) are highlighted in orange. Amino acid sequences were aligned using Multiple Sequence Alignment (MUSCLE) program in Seaview (*148*).

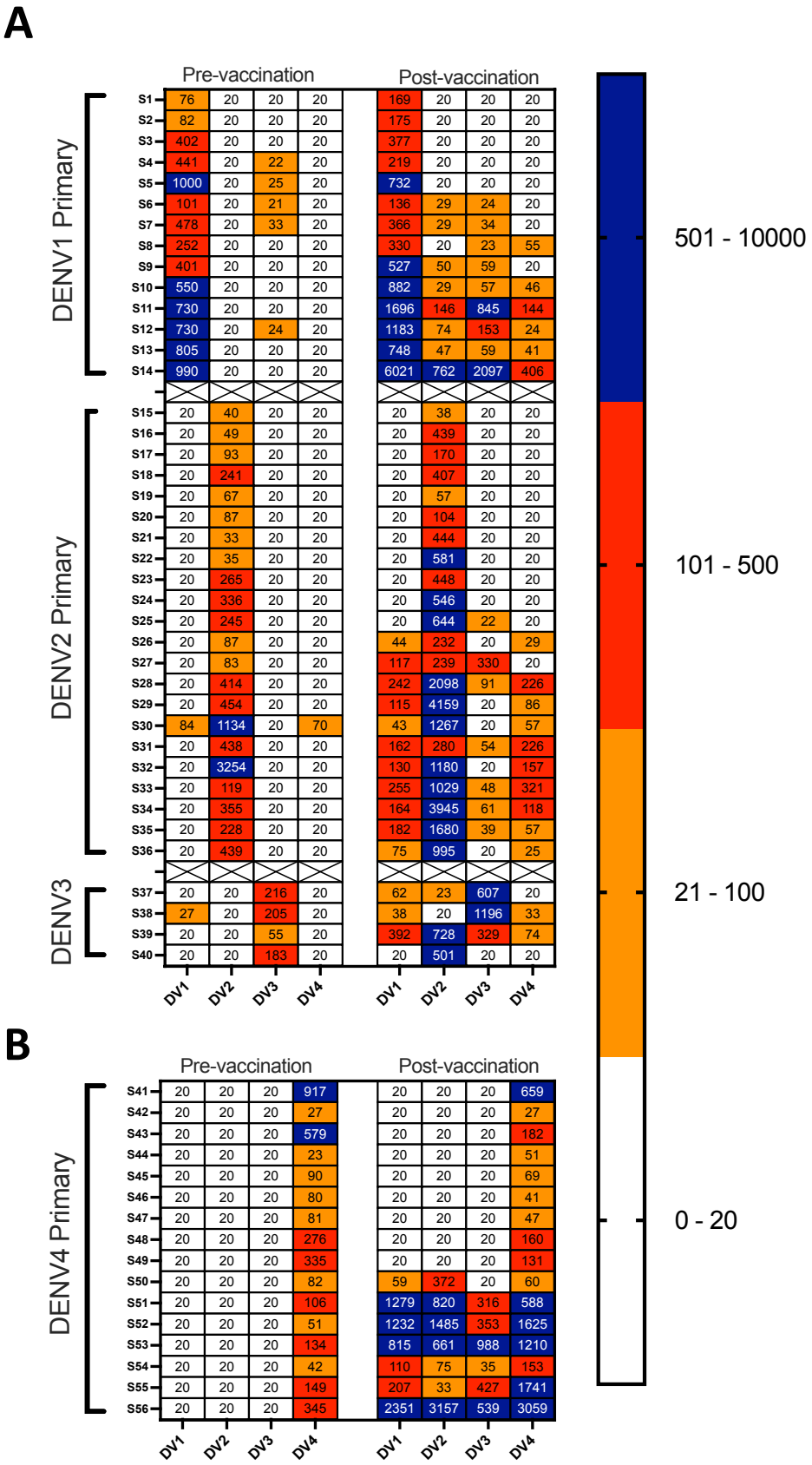

A
Pre-vaccination
Post-vaccination
DENV1 Primary
DENV2 Primary
DENV3
S1 76 20 20 20 169 20 20 20
S2 82 20 20 20 175 20 20 20
S3 402 20 20 20 377 20 20 20
S4 441 20 22 20 219 20 20 20
S5 1000 20 25 20 732 20 20 20
S6 101 20 21 20 136 29 24 20
S7 478 20 33 20 366 29 34 20
S8 252 20 20 20 330 20 23 55
S9 401 20 20 20 527 50 59 20
S10 550 20 20 20 882 29 57 46
S11 730 20 20 20 1696 146 845 144
S12 730 20 24 20 1183 74 153 24
S13 805 20 20 20 748 47 59 41
S14 990 20 20 20 6021 762 2097 406
S15 20 40 20 20 20 38 20 20
S16 20 49 20 20 20 439 20 20
S17 20 93 20 20 20 170 20 20
S18 20 241 20 20 20 407 20 20
S19 20 67 20 20 20 57 20 20
S20 20 87 20 20 20 104 20 20
S21 20 33 20 20 20 444 20 20
S22 20 35 20 20 20 581 20 20
S23 20 265 20 20 20 448 20 20
S24 20 336 20 20 20 546 20 20
S25 20 245 20 20 20 644 22 20
S26 20 87 20 20 44 232 20 29
S27 20 83 20 20 117 239 330 20
S28 20 414 20 20 242 2098 91 226
S29 20 454 20 20 115 4159 20 86
S30 84 1134 20 70 43 1267 20 57
S31 20 438 20 20 162 280 54 226
S32 20 3254 20 20 130 1180 20 157
S33 20 119 20 20 255 1029 48 321
S34 20 355 20 20 164 3945 61 118
S35 20 228 20 20 182 1680 39 57
S36 20 439 20 20 75 995 20 25
S37 20 20 216 20 62 23 607 20
S38 27 20 205 20 38 20 1196 33
S39 20 20 55 20 392 728 329 74
S40 20 20 183 20 20 501 20 20
DV1 DV2 DV3 DV4 DV1 DV2 DV3 DV4
501 - 10000
101 - 500
21 - 100
0 - 20
B
Pre-vaccination
Post-vaccination
DENV4 Primary
S41 20 20 20 917 20 20 20 659
S42 20 20 20 27 20 20 20 27
S43 20 20 20 579 20 20 20 182
S44 20 20 20 23 20 20 20 51
S45 20 20 20 90 20 20 20 69
S46 20 20 20 80 20 20 20 41
S47 20 20 20 81 20 20 20 47
S48 20 20 20 276 20 20 20 160
S49 20 20 20 335 20 20 20 131
S50 20 20 20 82 59 372 20 60
S51 20 20 20 106 1279 820 316 588
S52 20 20 20 51 1232 1485 353 1625
S53 20 20 20 134 815 661 988 1210
S54 20 20 20 42 110 75 35 153
S55 20 20 20 149 207 33 427 1741
S56 20 20 20 345 2351 3157 539 3059
DV1 DV2 DV3 DV4 DV1 DV2 DV3 DV4

Figure 4.4. Pre and post-vaccination neutralization50 titers against DENV1-4 in pediatric subjects who received a single dose of CYD-TDV.

Samples are sorted by DENV primary serostatus prior to vaccination. **A)** DENV1, DENV2, or DENV3 serostatus **B)** DENV4 serostatus. Limit of detection in virus neutralization test is 20. Dilution at 50% neutralization (neutralization50) was calculated against DENV1-4 clinical isolates grown in Furin overexpressing Vero-81 cell line.

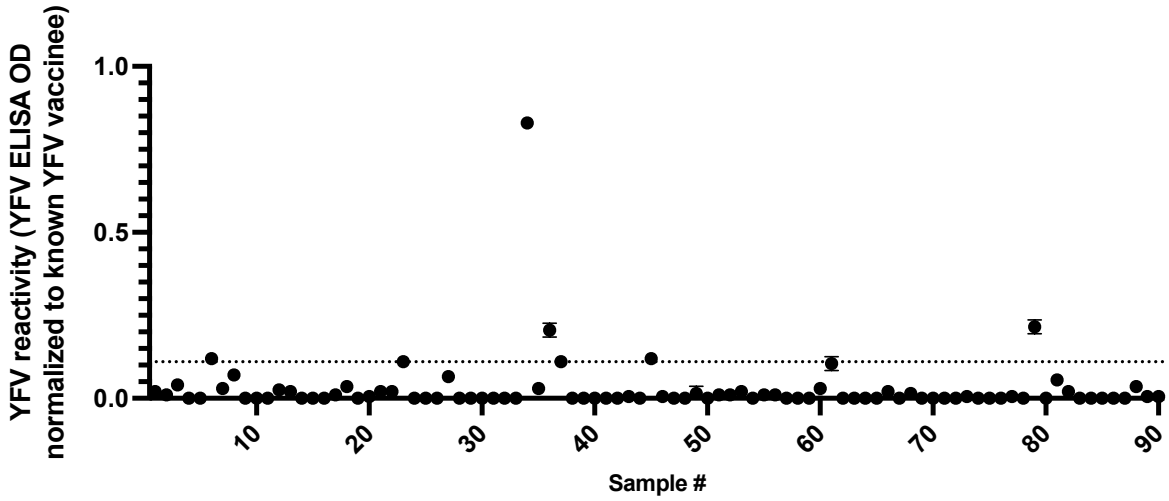


Figure 4.5. YFV NS1 reactivity prior to CYD-TDV vaccination.

Reactivity to YFV NS1 was assessed in pre-vaccination sera samples from primary serostatus individuals (n=90). Majority of pre-vaccination sera do not react with YFV NS1. Dotted line indicates 3x standard deviation of normal human sera control.

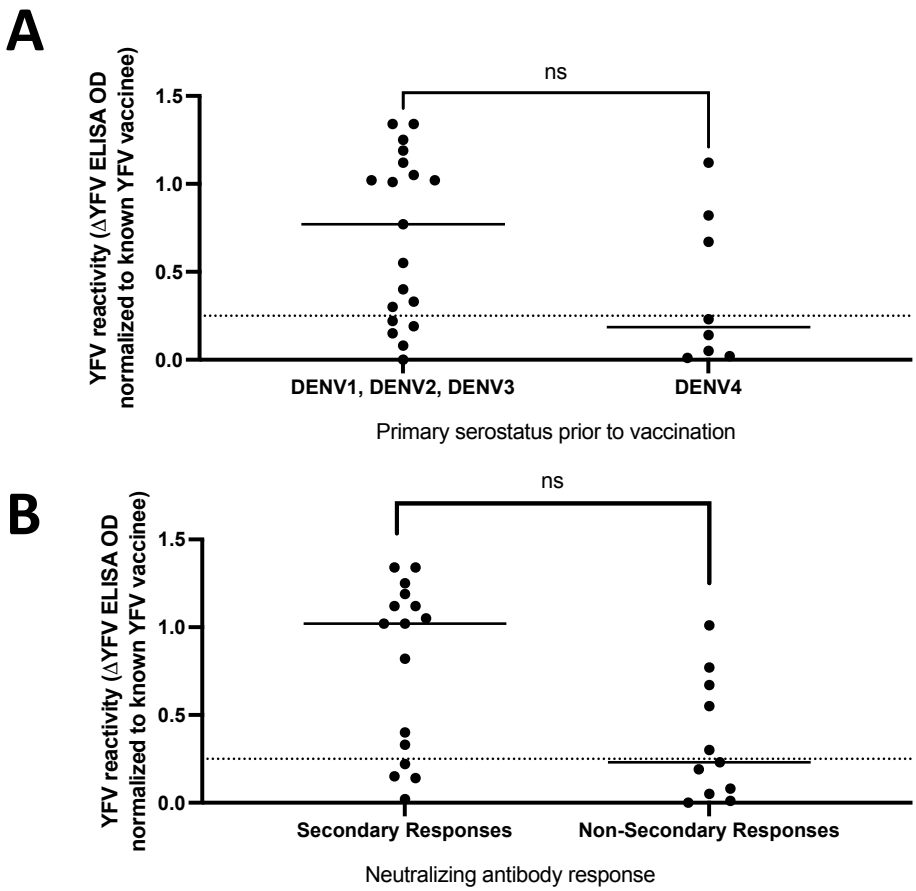


Figure 4.6. Change in YFV OD after receiving single dose of CYD-TDV.

Pediatric subjects(n=27) who received a single dose of CYD-TDV were tested for YFV NS1 binding antibodies pre- and post-vaccination. Duplicate YFV NS1 ELISA OD values were normalized to known YFV vaccinee and difference calculated between pre- and post-vaccination sample. Dotted line indicates LOD. LOD calculated as average of non-vaccinated individuals + 2x standard deviation.

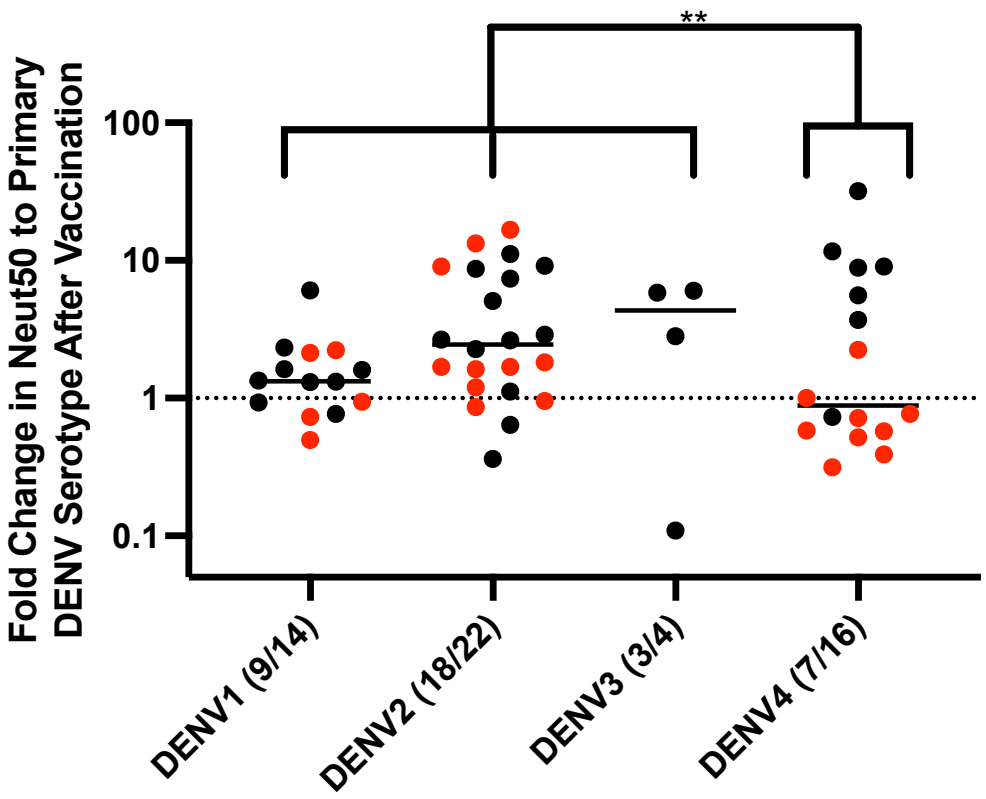


Figure 4.7. Fold change in Neut50 to primary DENV serotype after vaccination.

Serum dilution at 50% neutralization was calculated for pre- and post-vaccination samples of 56 DENV1,DENV2,DENV3, or DENV4 primary serostatus individuals. Values greater than 1 (above dotted line) indicate a boost to original serotype in Neut50 value. Red indicates subjects who did not have a change in heterotypic DENV neut50 value. Fisher's exact test compared proportions of DENV1,DENV2, and DENV3 with values greater than 1 to proportion of DENV4 (p-value .0019).

	Neutralization Categorization by 1:40 FRNT		
ELISA OD Value Range	Naïve	Primary	Multitypic
<0.2 (n=79)	78 (98.7%)	0 (0%)	1 (1.3%)
>3.0 (n=370)	0 (0%)	4 (1.1%)	366 (98.9%)

Table 4-1. PanBio ELISA OD vs Neutralization Categorization by 1:40 FRNT.

Assay cutoff threshold was 70% neutralization.

Primary Serostatus	Secondary Response	Non-Secondary Response	P-value (Fisher's exact test)
DV1,2,3 (n=40)	25 (62.5%)	15 (37.5%)	.2410
DV4 (n=16)	7 (44%)	9 (56%)	

Table 4-2. Summary of Response to CYD-TDV by Primary Serostatus.

Proportions of individuals with secondary vs non-secondary response was measured by Fisher's exact test.

CHAPTER 5: DISCUSSION

5.1 Identifying the mechanism of potently neutralizing antibodies

The primary immune response to DENV infection consists of a robust antibody response that is comprised of antibodies targeting unique epitopes of the infecting serotype (serotype specific) and cross-reactive antibodies that can bind to multiple DENV serotypes (*33, 111*). Investigators have identified human serotype specific mAbs that potently neutralize DENV in-vitro (*37-43*). Studies that have mapped epitopes targeted by these mAbs have revealed a common theme. The majority of potently neutralizing mAbs target quaternary epitopes that stretch across multiple E-proteins. This theme of quaternary epitopes applies to potently neutralizing mAbs specific to other flaviviruses including ZIKV (*42, 43*). While investigators have proposed that quaternary epitope-directed antibodies lock E-protein on the viral surface and block conformational changes required for viral entry, there is little direct experimental evidence to support such a mechanism. In my thesis, I sought to determine the contribution of quaternary epitope binding to potent neutralization. I defined the quaternary epitope of the previously isolated, ZIKV-specific, potently neutralizing mAb, G9E (*96*). Through an antibody paratope mutagenesis strategy, I altered the antibody paratope to convert a quaternary epitope that stretched across the E-dimer to an epitope contained within one E-monomer. The mutated antibody demonstrated a significant loss in neutralization without a loss in binding. My findings support the hypothesis that quaternary epitope binding is more important than binding avidity for neutralization.

The goal of DENV vaccine developers is the development of a vaccine that stimulates balanced and durable protection against all 4 serotypes. Balance has been difficult to achieve with tetravalent live attenuated virus vaccines in clinical testing because of the unbalanced replication of different vaccine components. Dengvaxia (CYD-TDV), a leading candidate, is a live attenuated unbalanced vaccine that increased the risk of DENV hospitalization when administered to DENV naïve people(*7*). Defining characteristics of functional antiviral antibody responses can contribute to better DENV vaccine design.

My data suggests quaternary epitope binding is an important component of neutralizing and protective antibodies that develop after flavivirus infection. This conclusion is further supported by recent studies that defined properties of human polyclonal serum antibodies the neutralize DENV (*45-49*). Two popular subunit strategies have emerged as alternatives to DENV live attenuated tetravalent vaccine formulations. One strategy aims to minimize reactivity to a major cross-reactive epitope, the fusion loop, by generating stabilized E-dimers (*149, 150*). The other strategy is to immunize with the EDIII of the DENV E-protein to generate a strongly neutralizing, serotype specific antibody response (*151, 152*). My findings support the use of subunit vaccines that elicit a quaternary antibody response as antibodies targeting this type of epitope provide the most potent avenue to neutralize the virus.

5.2: A novel correlate of protection against DENV infection

Neutralizing antibody is an accepted correlate of protection for other live attenuated flavivirus vaccines (*83-87*). CYD-TDV clinical trials (CYD14, CYD15, and CYD23) demonstrated that in children with no pre-existing immunity to DENV who were vaccinated, a DENV neutralizing antibody response vaccine did not correlate with protection (*7*). This evidence challenges the previously accepted notion that neutralizing antibody response is a correlate of protection for DENV vaccines. Analysis of serum samples from vaccinated children who subsequently experienced breakthrough infections allows us to directly link clinical outcome to vaccine response. My studies utilized sera from individuals exposed to natural DENV infections and post-vaccination sera from children with breakthrough infections to determine what component of the neutralizing antibody response directly correlates with protection. I compared antibody response induced by CYD-TDV in DENV1 and DENV3 breakthrough infections to antibody response induced in WT DENV infection. After they were depleted of cross-reactive antibodies, I observed that neutralizing antibody response after WT DENV1 and DENV3 infection targeted serotype specific epitopes while antibody response after CYD-TDV vaccination demonstrated a mostly cross-reactive antibody response to DENV1 and DENV3. This observation paired with low efficacy against DENV1 and DENV3 in phase III clinical trials supported serotype specific neutralizing antibody as a better correlate of protection than the current standard of total neutralizing antibody.

Two additional live attenuated tetravalent DENV vaccines are nearing completion of phase III clinical trials and my finding that serotype specific antibody is a correlate of protection can assist in evaluation for licensure. Both TAK-003 and TV003 elicit a neutralizing antibody response to all four DENV serotypes as measured in a conventional neutralization assay (*73, 153*). Using serum depleted of cross-reactive antibodies, TAK-003 demonstrated a serotype specific response weighted towards one component of the tetravalent formulation, mirroring CYD-TDV (*154*). TV003 demonstrated a more balanced serotype specific neutralizing antibody response compared to TAK-003 and CYD-TDV, with majority of subjects developing serotype specific antibodies to 3 or 4 DENV serotypes(*153*). CYD-TDV was a public health disaster that resulted in lowered vaccine confidence in the Philippines (*78*). Licensing additional unbalanced live attenuated tetravalent DENV vaccines may increase vaccine hesitancy among target populations.

5.3 Sterilizing immunity as measured by DENV4 human challenge model

Primary DENV infections provide lifelong immunity against clinically apparent reinfection by the same serotype. Investigators have documented rare cases of reinfections with the same serotype (*32*). We currently do not know the frequency with which homotypic re-infections occur. In chapter 4, I attempted to answer this question by testing how pre-existing primary DENV immunity to each serotype influenced response to the unbalanced tetravalent CYD-TDV. I assessed the ability of CYD-TDV to replicate by measuring DENV neutralizing antibody response and antibody response to YFV NS1 after vaccination. The results supported that prior DENV4 immunity does not provide sterilizing immunity.

These findings have direct implications on epidemiological modeling of DENV transmission. Due to limitations of both surveillance and DENV diagnostics, it is possible homotypic reinfection is a widespread phenomenon that has gone undetected. Waggoner et. al. identified instances of rare reinfection by examining medical cases that were not defined by classic DENV symptoms (*32*). Additional epidemiological evidence supports widespread homotypic DENV2 reinfection 15 years after primary infection in Peru(*133*). My findings support that immune response after primary infection does not provide sterilizing immunity, supporting the possibility of homotypic reinfection. In context of vaccines, a human challenge model 6 months after vaccination with TV003 demonstrated sterilizing immunity in 21/21

subjects (*153*). Further studies are required to understand how genotype and time between primary infection and homotypic challenge effect the ability of antibodies to provide sterilizing immunity. Epidemiological modeling of DENV considers the four distinct co-circulating DENV serotypes and interactions due to cross-reactive human antibody response and different DENV genotypes. The possibility of homotypic reinfections further complicates these models.

5.4 Future directions for flavivirus vaccine research

My conclusions in this dissertation expand knowledge about the properties of human antibodies that are protective against DENV and other flaviviruses. I demonstrate that quaternary epitope targeting gives rise to potent neutralization. In the context of a phase III clinical trial, my work has demonstrated that in baseline seronegative children who receive a DENV vaccine, neutralizing antibodies recognizing epitopes unique to each serotype are a better correlate of protection than the current standard of total antibodies that neutralize each serotype. Additionally, I provide evidence from a DENV human challenge model that primary antibody response after infection is not sterilizing. Based on my thesis, the field can apply this knowledge to both direct and evaluate future DENV vaccines.

DENV vaccines are complex and continued development requires addressing significant gaps in knowledge. DENV vaccines need to elicit an antibody response that protects against DENV clinical disease without eliciting a pathogenic antibody response that causes significant disease through antibody dependent enhancement. The results from CYD-TDV clinical trials highlighted need for further analysis of protective antibody responses. While my thesis adds to this knowledge, significant gaps in knowledge remain. Unlike other fields who have elucidated the molecular mechanisms behind developing quality antibodies from germline to final mAb (*155-157*), we still do not know the finer details behind the immunologic process of protective antibody development in DENV WT infection or DENV vaccination. CYD-TDV vaccine efficacy provided protection in individuals with previous DENV immunity(*7, 76, 130*), but detailed analysis is needed to identify a refined correlate of protection in these individuals. Waning vaccine immunity was detected in individuals with DENV immunity prior to CYD-TDV vaccination (*144, 145*). This observation yields more questions surrounding longevity of antibody response to vaccine, the impact of genotypic variation of serotypes on vaccine immunity and the overall design of phase III clinical

trials. Other antibodies that target key DENV proteins and other components of the immune response assist in a protective response, but we need to describe the exact mechanisms of their role.

We have accrued a large volume of knowledge surrounding antibody response to DENV WT infection and vaccination but more work is to be done. Without a suitable animal model, continued support of longitudinal human cohort studies that can understand antibody responses after infection are necessary. Continued collaboration with pharmaceutical companies to tease apart finer details of protective immune responses is required. With this in addition to other research exploring epidemiology, transmission, and other clinical manifestations, we can continue to add to our knowledge base and develop better DENV vaccines.

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