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2011

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Protective and Pathogenic Effects of Dengue Virus Antibodies *in vitro* and *in vivo*

by

Katherine Lindsay Williams

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Infectious Diseases and Immunity

in the

Graduate Division

of the

University of California, Berkeley

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Professor Eva Harris, Chair

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Fall 2011

ABSTRACT

Protective and Pathogenic Effects of Dengue Virus Antibodies *in vitro* and *in vivo*

by

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Doctor of Philosophy in Infectious Diseases and Immunity

University of California, Berkeley

Professor Eva Harris, Chair

The mosquito-borne dengue virus (DENV) is endemic in 100 countries and annually threatens over half of the world's population. Any of the four serotypes of DENV (DENV1-4) can cause a wide range of disease, ranging from inapparent illness to classic dengue fever to severe disease characterized by vascular permeability and hypotensive shock (dengue hemorrhagic fever/dengue shock syndrome; DHF/DSS). The complex interactions between epidemiological, viral, host genetic, and immunological factors make dengue pathogenesis difficult to dissect. While both the humoral and cellular arms of the adaptive immune response have been reported to contribute to the development of severe dengue disease, antibodies alone are sufficient to enhance DENV disease *in vitro*, a concept referred to as "antibody-dependent enhancement". Under this hypothesis, antibodies generated against the first DENV infection recognize but do not neutralize the second DENV serotype and instead permit increased entry into Fc receptor (FcR)-bearing target cells. The development of small animal models to study DENV pathogenesis has greatly facilitated dissection of the role of individual immune components critical to both protection and enhancement of DENV disease. Here, we characterize a novel mouse model of lethal DENV disease and further demonstrate that antibodies alone are sufficient to enhance a sub-lethal DENV infection. Hallmarks of the antibody-enhanced, lethal phenotype include increased viral load, reduction in platelet counts, elevated pro-inflammatory cytokines and vascular permeability, all of which are considered defining features of severe human dengue disease. We further demonstrate that genetically modified non-FcR-binding anti-DENV monoclonal antibodies (mAbs) can be used to treat lethal disease caused by both high viral inoculum (virus-only) and enhancing amounts of antibody. While prophylactic ability appears dependent upon the neutralizing titer of the mAb, therapeutic efficacy following an antibody-enhanced, lethal infection is unique to highly avid mAbs that target the fusion loop and envelope protein Domain III (EDIII) A strand epitopes. Further investigation into the protective role of antibodies examined the role of anti-EDIII antibodies in mediating serotype-specific protection and enhancement *in vivo*. While previous studies of murine mAbs suggested that anti-EDIII antibodies were predominantly serotype-specific and highly neutralizing, our work demonstrated that they only constitute between 15% and 35% of the *in vitro* neutralizing potency of human and mouse serum, respectively, and are not required for protection *in vivo*. Further analysis of specific antibody subsets revealed that serotype-specific antibodies (targeting all E domains) contribute predominantly to maintaining serotype-specific protection, while cross-reactive antibodies drive disease enhancement. Taken together, our *in vivo* studies using a small animal

model of DENV disease have validated *in vitro* observations and identified specific roles for antibody subsets in mediating both protection and enhancement.

Dedicated to my grandfather, William James Happel
Feb 8, 1922- May 11, 2007

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ACKNOWLEDGEMENTS

I would like to start by thanking my parents, who never stopped encouraging me – to not only work hard, but to take time for myself. I would also like to especially thank my younger sister, Liz, for her kind words of support, and continued re-affirmation that “This was your choice, don’t take it out on me!”

Since moving to Berkeley, I have developed the most wonderful network of friends. I must thank my very first friend in Berkeley, Zoe Harris, for being my companion on amazing, crazy nights at the Graduate, through marathon law library study sessions and on frozen yogurt breaks. To Stef, Mel and Alissa, you have been the most supportive and encouraging friends- you’ve listened when I needed to vent, given advice when I needed a second opinion, and been excellent companions on crazy adventures – whether it has been a crazy night in San Francisco, back-packing in the Sierra Nevadas, or skiing in Tahoe. Shawna, you have been my grounding force- never more than a phone call away, and the voice of reason throughout my hysteria. I have been exceptionally lucky to join a triathlon training team – to M2, you gave me the ability to complete an ironman- and to my training teammates – Heather, Lena, Kelly, Rachel, Pavel and Ryan, I could not have made the journey without you! My SF ladies crew- my weekends would never be the same without you. I owe a special thanks to Julie Donofrio, who over the course of my dissertation writing, called me to make sure I had made it outside at least once every day and had at least one fun activity to look forward to – especially my study date/ wedding planning dates with Heather. And of course, I must thank my best friends, Jenna and Corinna, who never stopped encouraging me, even from far, far away.

Most importantly, I owe my greatest thanks to my wonderful fiancée, Rob, whose constant support (even from China) and understanding ear – even when I’m acting irrational and saying absolutely non-sensical and absurd things, have been a constant strength. Thank you for being such a patient, loving person.

I must also thank the many people I have worked with over the course of the past five and a half years – firstly, to Scott Balsitis, my first mentor in the lab- you taught me how to think like a scientist- “yes, you COULD do that, but SHOULD you do that”. I am forever grateful. To Molly, who despite hating antibodies and mice, was an amazing sounding board for my crazy ideas and –sometimes- even crazier data. To Susana, for being a great friend and great work partner. I rely more on you than you will ever know. I must also thank Robert – for not letting me fly off the handle and telling me when I’m absolutely full of it and Josefina for being a great shoulder and huge support system. I would also like to extend a large thanks to two important collaborators, Mike Diamond and Aravinda de Silva – its been a wonderful learning experience to work with you both. And of course, I must thank Eva. You have given me the most amazing opportunities- the past five years have been an incredible experience. But most importantly, you taught me how to love science. Your enthusiasm and energy has been truly inspirational and it will stay with me forever.

CHAPTER 1
INTRODUCTION

Dengue disease pyramid and clinical manifestations of disease

Dengue virus (DENV) is a mosquito-borne virus of the *Flaviviridae* family and is related to other well-known viruses that cause West Nile Encephalitis, Japanese Encephalitis and Yellow Fever [1,2,3,4]. Endemic to the majority of the tropical and subtropical regions of the world, there are currently over 3.5 billion people at risk for DENV infection [5], making DENV the most medically important arthropod-borne virus worldwide and a major public health challenge. There are an estimated 50 million cases of dengue fever (DF) annually accompanied by 250,000–500,000 cases of the potentially fatal dengue hemorrhagic fever/ dengue shock syndrome (DFH/DSS), most often affecting children [4,6]. DENV is transmitted through the bite of either *Aedes aegypti* [7], the predominant DENV vector, or *Aedes albopictus* [8] mosquitoes. There are four serotypes of DENV, and infection with any of the four can cause a wide range of clinical symptoms in humans, from asymptomatic infection to DF to the more severe illness, DHF/DSS. Between 50-90% of all DENV infections are asymptomatic [9,10,11]; of the symptomatic infections, a large percentage are unreported [6,12]. Classic DF generally lasts 2-7 days, beginning with high fever and accompanied by headache, retro-orbital pain, nausea, vomiting, muscle, joint, and bone aches and laboratory diagnosis of neutropenia, thrombocytopenia (low platelet count) and elevated liver enzymes [3,4]. Only 0.5-1% of all DENV infections develop into severe disease; however, without proper care, the fatality rate can reach 20% [6,13] (Figure 1). The clinical course of DHF/DSS is initially quite similar to the clinical description of DF; however, following defervescence, DHF/DSS patients rapidly deteriorate into life-threatening conditions characterized by hemorrhagic manifestations, thrombocytopenia and shock (DSS) and can die within 24-48 hours [3,4]. The inability to differentiate DF from DHF/DSS at early stages of infection contributes to the difficulty in treating the disease. Both prospective and retrospective epidemiological analysis of dengue epidemics suggest the greatest risk factor for development of DHF/DSS is prior infection with a different DENV serotype [10,14,15,16,17].

Replication of the DENV virion

DENV contains a small, positive-sense RNA genome (~10.7 kb) composed of three structural proteins (capsid, C; pre-membrane/membrane, prM/M; envelope, E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4b NS5) proteins flanked by 5' and 3' untranslated regions (UTRs) [18]. The surface of the virion consists of 180 copies of the E protein; in the mature conformation of the virion, the E protein is arranged in 90 homodimers that lay flat across the surface of the lipoprotein membrane [19].

Infection with the mature virion is a receptor-mediated, endocytic process that is followed by acidification of the endosomal compartment [20,21]. Acidification of the endosomal compartment triggers rearrangement of the E protein into homotrimers, such that the fusion loop of each E protein is located at the same end and can insert jointly into the endosomal membrane [22,23]. Approximately 18 hours following initial infection, new virions assemble in the endoplasmic reticulum (ER) and bud into the lumen [24]. During this stage, cryo-electron images have depicted an icosahedral arrangement of 60 trimeric spikes [25,26] where the prM protein covers the fusion peptide on the E protein to prevent inactivation as the virion progresses through the secretory pathway [27,28]. The prM portion of the membrane protein is subsequently cleaved by a furin-like protease, releasing the “pr” portion of prM [29,30] and allowing the now mature form of the virion to settle into a homodimeric conformation of 90 E proteins that lay flat against

the surface of the virion [19]. The E protein thus forms three distinct geometries throughout the virus maturation and infection process [31].

As a result of the flexibility of the E protein, particular epitopes may be more or less accessible depending on the chemical environment and geometric conformation of the virion [32,33,34]. Further, different host cells can affect the efficiency of the maturation process, such that virus preparations may be composed of different ratios of mature and immature virions [35,36,37]. Non-infectious, immature virion particles have been shown to become infectious when incubated with antibodies that target epitopes unique to the immature particle such as the fusion loop and prM [38]. Further, antibodies targeting the EDI lateral ridge and EDII fusion loop demonstrate a 30-40% reduced ability to neutralize fully mature virion preparations as compared to a heterogeneous population composed of mature and immature virions [36].

Development and use of monoclonal antibodies to study DENV structure and biology

Monoclonal antibodies (mAbs) have been instrumental in mapping the structure and surface of the DENV virion. Early breakthroughs in hybridoma production helped to facilitate systematic advances in our understanding of the conformation and structure of the virion. More recent observations using mAbs obtained from DENV-immune human B cells have provided novel insights into the mechanisms of DENV neutralization, and have further advanced our knowledge of the composition of the polyvalent antibody repertoire. Innovations in hybridoma technology facilitated the isolation of the first monoclonal antibodies targeting the DENV virion in 1982. An initial panel of 22 mAbs, including 3H5, was isolated from mouse ascites fluid developed through multiple rounds of infection with either purified virus or mouse suckling-brain passaged New Guinea C (NGC) virus [39]. This panel, as well as a second panel of mAbs, including 4G2 and 2H2 [40], were subsequently used to classify mAbs by flavivirus cross-reactivity (group-reactive; dengue complex-specific; dengue sub-complex-specific; dengue serotype-specific) [41]. Initial mapping studies using these mAb panels identified several novel DENV E protein epitopes and suggested that the E protein was composed of three domains [42,43,44,45,46]. Ultimately, using tick-borne encephalitis (TBE) virus as a model, Mandl *et al* [47] proposed a structural model of the E protein composed of three non-overlapping, antigenically distinct domains, A, B and C [47]. Three-dimensional imaging of the crystal structure of the TBE E protein confirmed these antigenically-derived hypotheses, and clearly identified three domains, termed I (EDI), II (EDII) and III (EDIII) corresponding to previously-labeled domain C, A and B, respectively [48].

Roehrig *et al* [49,50] pioneered the early DENV mapping studies using a model based on TBE. Epitope mapping using 11 peptides identified four distinct antigenic regions by ELISA; these regions were further identified as discontinuously linked protein sequences corresponding to domains A, B and C [49,50]. Further refinement and identification of new epitopes [51,52] using a series of differently-sized linear peptides resulted in the conclusion that most neutralizing antibodies, such as 3H5, recognize discontinuous epitopes [53,54]. In-depth characterization of the virion surface, including identification of relative areas of chemical stability, flavivirus group sensitivity, neutralization, surface accessibility, hemagglutination and fusion was further facilitated by the development of a second large panel of antibodies through immunization of Balb/C mice with either high- or low-pH virus emulsified in Freund's adjuvant [55].

The early mAb studies identified distinguishing characteristics between mAbs targeting different domains. While mAbs targeting the EDIII lateral ridge were type-specific and potently neutralizing, mAbs targeting EDI/II were much more cross-reactive and less potent *in vitro* [56].

Using both forward (chimeric DENV2 viruses) and reverse genetics (viral escape variants) Hiramatsu *et al* [57] identified that the strongly-neutralizing [55], type-specific [41] MAb 3H5 bound directly to residues 383, 384 and 385, and that these three amino acids were the main residues conferring type specificity within EDIII [57]. Supporting these studies, Beasley and Barrett (2002) mapped a similar type-specific epitope to the lateral ridge in West Nile Virus (WNV) [58]. The prominence of the lateral ridge epitope was highlighted through a series of studies examining the therapeutic efficacy of a highly potent WNV-specific mAb, E16, that bound to the EDIII lateral-ridge [34,59,60]. Derived following repeated immunization of Balb/C mice with insect-cell produced E protein and boosted with purified E protein, E16 proved to be potently neutralizing *in vitro* and therapeutically efficacious when given even up to five days following WNV infection *in vivo* [59].

The prophylactic and therapeutic ability conferred by WNV mAb E16 spurred new investigations to discover similarly potent DENV-specific mAbs that targeted EDIII. In-depth analysis of a panel of 14 antibodies targeting DENV2 EDIII identified 8 that were type-specific, 4 that were subcomplex- or complex-reactive, and 2 that were broadly cross-reactive. In this study, all of the mAbs categorized as type-specific were considered either strongly or moderately neutralizing. Further investigation of the two subcomplex-reactive mAbs revealed that both were moderately to strongly neutralizing *in vitro* and bound to a new epitope (K305, K307, K310, P384) that mapped to the A strand of EDIII [61]. Additional fine mapping studies of DENV2 EDIII using a panel of 12 EDIII-specific mAbs ascribed similar residues (K310, I312, P332, L389, W391) to a similarly complex-reactive epitope [62]. Further functional analysis comparing the traditional type-specific mAbs to the new complex-reactive mAbs demonstrated that the type-specific mAbs required a lower occupancy for neutralization and were generally more potently neutralizing [61,62,63]. Concurrent with this in-depth analysis of previously existing mAbs, two studies were undertaken to produce potently neutralizing, DENV1-specific [64] and DENV2-specific mAbs [65] in the hope of generating mAbs of comparable potency to WNV mAb E16. In both studies, immunization of interferon $\alpha/\beta R^{-/-}$ C57BL/6 (AG-B6) mice was required to facilitate adequate DENV replication [66]. To produce the mAbs, AG-B6 mice were immunized twice with either DENV1 or DENV2, and the mice with high antibody titers were boosted with purified EDIII protein. From the DENV1 study, one MAb, E105, bound strongly to the EDIII lateral ridge and demonstrated therapeutic efficacy in a paralytic infection model in mice [64]. In the DENV2 study, 24 mAbs were produced that targeted a number of different epitopes previously defined on the surface of WNV, including the EDI lateral ridge, EDII dimer interface, lateral ridge and fusion loop, and EDIII lateral ridge, C-C' loop and A strand. In comparison to the DENV1 study, no potently neutralizing, lateral ridge-specific mAbs were identified in the DENV2 mAb study [65]. Given the strongly neutralizing and predominantly type-specific response associated with EDIII mAbs, multiple groups have begun testing vaccination strategies using recombinant EDIII protein [67,68,69,70].

Despite the strong focus on EDIII-specific mAbs, concurrent studies in both WNV and DENV were being conducted to further explore the binding and functional properties of EDI/II MAbs. Fine-mapping studies of the fusion loop region in DENV identified distinct epitopes that bind a number of group- and complex-reactive mAbs of moderate neutralizing potency [32,71]. A study of 89 mAbs binding to EDI or EDII of WNV identified six novel epitopes as well as the previously-characterized fusion loop epitope. While not as potent as most EDIII-specific mAbs, fusion loop mAbs nonetheless were shown to protect against WNV infection *in vivo* [72]. Recent investigations have highlighted the importance of mAbs with unique properties, including non-

neutralizing mAbs that are protective *in vivo* [37], strongly neutralizing mAbs that bind to an epitope conformationally dependent upon the breathing of the virus [73,74,75], and a small class of mAbs that do not bind to recombinant E, but rather to heterodimeric complexes of the folded E protein [76].

Mechanisms of antibody-mediated neutralization

Antibody-mediated neutralization of viral infection can be described as acting through either a single or multi-hit mechanism. A single-hit mechanism requires a lone antibody-binding event to render the virus non-infectious, while multi-hit mechanisms require engagement of multiple antibodies and epitopes to ensure effective viral neutralization (reviewed in Parren and Burton [77]). Neutralization of flaviviruses requires a distinct ‘multi-hit’ mechanism (reviewed in Pierson *et al* [78]). Antibody-mediated neutralization is mechanistically defined as the number of antibodies bound to a virion required to exceed a given threshold [79]. MAb affinity and epitope accessibility are the two critical components that define whether a mAb can stoichiometrically exceed this threshold [80]. Theoretically, the most potently neutralizing mAbs would be those of the highest affinity that bind to highly accessible epitopes. MAbs binding to partially occluded epitopes may only be able to neutralize a viral infection under super-saturating conditions, while mAbs binding to obscured epitopes may be incapable of neutralization, or only under specific conditions [36,73,75,80]. As an example of these hypotheses, Pierson *et al* [79] mathematically derived that 30 of the 120 available binding sites must be bound for the highly potent, WNV-specific mAb E16 [34,59] to neutralize 50% of virus particles *in vitro* [79]. Similar data examining functional characteristics of EDIII-specific mAbs supports this observation and identifies a relationship between neutralization potency and mAb avidity [63]. Antibody-mediated neutralization of DENV can interfere with DENV infection by direct virus neutralization, FcγR-mediated clearance [37], complement-mediated lysis of infected cells [81,82], or antibody-dependent cellular cytotoxicity [83,84,85,86]. Direct, virus-mediated neutralization can occur through a number of different mechanisms, including prohibiting viral attachment, internalization and/or fusion [78]. Recent evidence has suggested that the most potently neutralizing DENV mAbs inhibit infection via a post-attachment mechanism [65].

The adaptive immune response to DENV

Host factors, including major histocompatibility complex (MHC) polymorphisms and prior T- and B-cell immunity, are key determinants of susceptibility to severe dengue disease [87]. Type I (α/β) and Type II (γ) interferons induce resistance to DENV disease [66,88], and both arms of the adaptive immune response have been demonstrated to play an important role in both protection [89] and enhancement [14,16,90,91] of dengue disease. In general, most individuals infected with DENV for the first time (primary infection) experience inapparent infection, undifferentiated fever, or DF [2]. Following primary infection, individuals are considered to be immune for life against re-infection with the same serotype [88,89,92]. Repeat infection with the same serotype resulting in illness are rare, if it exists at all [93]. Early studies conducted by Sabin (1952) [89] using human volunteers determined serotype-specific immunity to be protective against re-infection with the same serotype for at least 18 months following primary infection, but for only 1-2 months against a different serotype [89]. In contrast, epidemiological data and human studies suggest that the greatest risk factor for development of DHF/DSS is prior infection with a different DENV serotype [88]. While secondary infections are significantly associated with an increased risk of severe disease, progression to DHF/DSS is

quite rare, indicating that the cross-reactive immune response can also provide protection [94,95]. Indeed, Zompi *et al* [96] have recently shown a role for both cross-reactive antibodies and cross-reactive T cells in mediating heterotypic protection *in vivo* [96]. Further, recent evidence using statistical modeling suggests that a serotype cross-reactive immune response may provide protection for up to two years [97].

Two non-mutually exclusive hypotheses have been proposed to explain the role of the adaptive immune response in contributing to the development of DHF/DSS. The first implicates antibodies in mediating enhanced disease. In hallmark studies, Halstead and O'Rourke [98] demonstrated that DENV did not replicate in peripheral blood leukocytes (PBL) of non-immune primates but did replicate in PBL isolated from immune primates; however, non-immune PBL could be infected by adding anti-DENV antisera. This illustrates a phenomenon termed "antibody-dependent enhancement" (ADE) [88], in which non-neutralizing anti-DENV antibodies facilitate entry of the virus into Fcγ receptor (FcγR)-bearing cells [88,99]. This increase in infected cells directly contributes to the higher viremia levels associated with DHF/DSS [11,100], and the infection of target monocyte/macrophage cells leads to activation, ultimately promoting the "cytokine storm" that characterizes DHF/DSS [101]. Additional evidence for ADE *in vivo* is derived from the observation that most severe cases in primary DENV infections occur in infants in endemic areas, where DENV-specific antibodies are transferred transplacentally to infants from their dengue-immune mothers [102]. These antibodies wane over time until they can enhance DENV infection [102,103], suggesting that pre-existing antibodies alone are sufficient to promote severe DENV illness [88]. The second hypothesis explaining the immunopathology underlying severe secondary DENV infection implicates T cells [91]. Specifically, low-affinity, cross-reactive CD4⁺ and CD8⁺ memory T cells resulting from a primary infection are over-activated during secondary DENV infection with a different serotype, and these serotype cross-reactive T cells produce high levels of cytokines, such as TNF-α, which contribute to increased vascular permeability [87,104,105]. While *in vitro* and *ex vivo* experiments provide insight into individual contributions of the humoral and cell-mediated immune response, an *in vivo* model is required to mechanistically define their contribution to protection and enhancement of DENV infection and disease.

Evidence for the role of pre-existing immunity and the development of severe disease in infant and pediatric cohort studies

The largest burden of dengue disease is found in infants and children/adolescents [106,107]. Multiple research groups have designed prospective pediatric cohorts in dengue-endemic regions, including both Central and South America and South and Southeast Asia in an effort to facilitate immunologic and epidemiologic investigation of the complex interactions between DENV immunity and disease. Specifically, two main questions have been the subject of intense research. In 1988, Kliks *et al* [102] hypothesized that infants born to DENV-immune mothers are at increased risk of severe dengue disease as their passively acquired anti-DENV antibody titers wane over time. Thus, the first question addressed in a subset of these cohort studies has been to assess the impact of passively acquired antibodies and the risk of severe dengue disease in infants. The burden of disease in dengue-hyperendemic countries generally falls on the pediatric population; severe dengue disease is rarely seen in adult populations, as these individuals are often to completely immune following repeated exposures over the course of their lifetime. For example, in Nicaragua, by the age of 2, between 22-40% of children demonstrate evidence of immunity to one serotype, and by 9, over 95% are DENV-immune [9].

During this period, the risk for development of severe disease is the highest in children 5-9 [106,108]. Thus, the second topic addressed in these cohort studies has been a more thorough analysis of the incidence and prevalence of inapparent and symptomatic infection and of severe disease and the identification of immune correlates of either protection or enhancement in pediatric populations.

Infant studies

In 1979, Marchette *et al* [109] reported that cord blood from infants born to DENV-immune mothers contained an enhancing factor that decays with time. Indeed, passive transfer of antibodies from mother to infant is commonplace in dengue-endemic countries. An estimated 94.7% of mothers enrolled in a Thai cohort had anti-DENV antibodies at delivery, and a direct correlation was found to exist between maternal and infant antibody titers [110] as well as between the maternal antibody titer at birth and infant age at onset of disease [111]. The actual antibody titer at the onset of disease in infants is difficult to measure but can be predicted using the maternal antibody titer at birth and the decay rate associated with measles IgG [102]. Based on these assumptions, multiple studies have estimated that infants <4 months of age possess highly neutralizing, protective titers of antibodies >1:1000 that decline to an estimated range of 1:5 to 1:100 when the infant is between 5 and 9 months of age. By 12 months of age, the antibody titers have declined such that they are generally not measurable and may afford neither protection nor enhancement to the infants [112,113]. Supportive of this observation is the age distributions of symptomatic DENV infection, where the ratio of DHF: DF is the lowest before 4 months and after 8 months of age but almost equal at 5-6 months of age [111,114]. In a study of DHF in 75 Vietnamese infants, Simmons *et al* [113] demonstrated that 65% of infants with severe disease had plaque reduction neutralization test (PRNT) titers <1:20, and suggested PRNT to be a good, but not absolute correlate for disease severity. In support of this observation, Libraty *et al* [111] similarly found that all Philippino infants had anti-DENV3 titers of <1:50 at the time of symptomatic DENV3 infection. However, no difference in either PRNT titer or the virus output generated through a K562-cell based enhancement assay was correlated with disease severity when comparing cord blood collected from infants with DHF to either hospitalized or non-hospitalized infants with DF in this study [111]. Taken together, the literature has universally identified a correlation between poorly neutralizing antibody titers, the age of the infant, and an increased risk of severe disease. However, further evidence supporting a causal relationship between poorly neutralizing antibodies and the development of DHF/DSS is lacking. While difficult to do, further studies should focus on comparing infant anti-DENV neutralizing titers between asymptomatic and symptomatic DENV cases to clarify whether antibody titers truly affect the onset of DENV disease in otherwise DENV-naïve infants.

Pediatric studies

Studies conducted in DENV-endemic regions of both the Americas and Asia have documented a direct relationship between DENV immunity with age, such that most children living in DENV endemic regions are considered immune to at least one serotype by age 9 (67% estimated in Indonesia [17], 95% estimated in Nicaragua [9]). Multiple factors, including co-circulation of multiple DENV serotypes [88], population immunity [95], population genetics [115,116] and viral factors [117,118], can affect estimates of incidence of dengue. In general, estimates of DENV infection incidence are thought to be higher in Asian populations than in Latin America, resulting most likely from the difference in the force of infection. In a

Nicaraguan pediatric cohort, the incidence of all DENV infections was estimated to be between 5.8 and 11.1% [9] or 343-1,759 cases/100,000 person-years [12], while in Indonesia, the incidence was estimated at 38.9% in seronegative children [14]. Similarly, the ratio of inapparent to symptomatic infections varies dramatically by region and changes between primary and secondary infections. Even within the same region, ratios can vary widely from year to year. For example, in Nicaragua, between 2004 and 2008, the ratios of primary inapparent: symptomatic infection ranged from 5.3:1 (2007-08) to 11.3:1 (2006-07) and for secondary infection, from 2:1 (2007-08) to 37.4:1 (2004-05) [9].

The development of severe disease displays a bi-modal distribution, with infants and children at the greatest risk [3,108]. Efforts to understand pre-existing immunity and its role in mediating disease severity following a primary infection have been complex -- even basic *in vitro* correlates of severe disease have been controversial. For example, while some studies have identified a significant relationship between viremia levels and disease severity [93,100], others have failed to support the association [119]. As mentioned previously, conclusions about the role of pre-existing antibodies and disease severity have also been controversial. While some evidence exists in support of an inverse relationship between neutralizing titers against the infecting serotype and severe disease [93], this correlation has been refuted in other studies [119]. Similarly, studies examining the enhancing activity (measured using assays using K562 cells that are only permissive to DENV infection in the presence of antibody) of DENV-immune serum have failed to support a relationship with either viremia or disease severity [119]. In conclusion, epidemiological investigations have identified interesting, but complicated questions that would greatly benefit from in depth investigation in a controlled, laboratory environment with a small animal model capable of investigating relevant components of both protection and enhancement.

Development of a mouse model for DENV infection and disease

A small animal model for studying dengue disease is of critical importance to furthering many areas of dengue research, including host immunity, disease pathogenesis and drug and vaccine development. Non-human primate (NHP) models, including the rhesus macaque, were originally used to study antibody-dependent enhancement *in vivo* [98]. Recent observations have validated the rhesus macaque model by demonstrating that both protection [120] and enhancement [121] can be achieved with passive transfer studies *in vivo*. While an intriguing model, non-human primates are severely limiting for large scale *in vivo* experimentation. Although many attempts were made over the past several decades to develop an animal model for studying DENV, most animal species proved to be resistant to DENV infection [122]. Over the past 7 years, our group and others [123,124,125] have developed and characterized the AG129 mouse (IFN- α/β , and γ receptor-deficient) as a tool for studying dengue pathogenesis and immunology.

Initial attempts to develop a mouse model for dengue in immunocompetent mice required high doses of virus and were hindered by the inability to recapitulate several important aspects of human DENV infection, including replication in peripheral tissues and development of the hallmark symptoms of dengue disease [122]. As another approach, humanized mice developed by engrafting severe combined immunodeficient (SCID) mice with either human peripheral blood lymphocytes or a variety of DENV-susceptible tumor cells, including human K562 erythroleukemic cells [126] and human liver HepG2 [127] and Huh-7 [128] cells were explored as potential DENV mouse models. Improvement of the humanized mouse model has included

the development and characterization of nonobese diabetic (NOD)/SCID mice [129] and RAG2^{-/-} γ ^{-/-} mice [130] engrafted with CD34⁺ human cord blood hematopoietic stem cells. Interestingly, the reconstituted NOD/SCID mice developed viremia as well as clinical signs of dengue, including fever, rash and thrombocytopenia following a subcutaneous (sc) infection with DENV2 [129]. However, the interaction between human cells and the mouse immune system may not completely reproduce a functional immune system, limiting the study of the adaptive immune response to DENV infection. Additionally, these mice are difficult and expensive to generate, thus hindering large-scale studies.

Severely immunocompromised strains, BALB/c athymic nu/nu mice [131] and RAG1^{-/-} mice [66], both succumb to infection with DENV, but death results from paralysis instead of a defined vascular leak phenotype. Given the importance of IFNs in controlling viral infections, studies were conducted in IFN- α/β , and - γ receptor deficient [56,66] and STAT1-deficient mice [132,133,134]. Johnson and Roehrig [56] initially established the utility of AG129 mice to study primary DENV infection and vaccine challenge. Comparison between mice lacking either the IFN- α/β or IFN γ receptor or both suggested that both knockouts are required for early viral replication in peripheral tissues and subsequent disease [66]. Degradation of the transcription factor STAT2 was identified by Ashour *et al* [135] to be a critical factor underlying murine resistance to DENV; while human Stat2 can be degraded by DENV, murine Stat2 is refractory. Perry *et al* [134] further supported the role of Stat2 in protection against early disease in a STAT1-independent manner by infecting mice deficient in either or both of the transcription factors. While STAT1^{-/-} and STAT2^{-/-} mice demonstrated similar phenotypes following DENV infection, STAT1^{-/-} x STAT2^{-/-} mice demonstrated an early death phenotype, indicating that STAT2 could drive a STAT1-independent, protective mechanism [134]. Despite the immunodeficiency, the AG129 mouse is one of the only models that can be infected by four serotypes of DENV and supports replication in relevant cell and tissue types, thus allowing for investigation of tropism and pathogenesis in context of a functional adaptive immune system.

Characterization of the AG129 mouse model of disease

Over the past several years, multiple studies have characterized the tissue and cellular tropism of DENV in the AG129 model [94,136], demonstrating significant parallels with human infection [136,137,138]. Specifically, initial tropism studies using the AG129 model demonstrated that clinical isolates from all four DENV serotypes replicate efficiently in spleen, lymph node, bone marrow and muscle. Negative-strand viral RNA was detected in dendritic cells and macrophages of the lymph node and spleen [94]. Similarly, antibodies directed against the non-structural NS3 DENV protein indicated active viral replication in macrophages, dendritic cells, hepatocytes and bone marrow-derived myeloid cells in infected AG129 mice [136]. Both of these studies coincide with tropism data from human autopsy studies [136,138] and flow cytometry analysis of infected human peripheral blood mononuclear cells [137], where the infected cells were predominantly of the myeloid lineage. Importantly, the subcutaneous route of infection and 10²-10⁵ pfu inoculum used in these murine tropism studies are compatible with the natural route and viral inoculum found in a mosquito bite [139]. In addition, AG129 mice exhibit thrombocytopenia inversely related to viral load and develop high levels of soluble NS1 during DENV infection comparable to levels in humans [124].

Characterization of the AG129 immune response revealed a functional adaptive immune response to DENV infection. Specifically, antibodies elicited by infection are a mixture of serotype-specific and serotype-cross-reactive antibodies, including long-lasting neutralizing

antibodies [140], and the distribution of IgG isotypes among DENV-specific antibodies include IgG1, IgG2a and IgG2b in ratios similar to the ratios of isotypes elicited by viral infection of wild-type 129 mice [141] (KW, EH unpublished data). Sequential infections of one DENV serotype followed 4-52 weeks later by another serotype demonstrated reduction in viral load in the second infection as compared to naïve mice experiencing a primary infection [140]. Passive transfer studies demonstrated that transfer of high doses of mAbs directed against different epitopes on the E protein (EDII fusion loop or EDIII lateral ridge; see below) or anti-DENV polyclonal sera 24 hours prior to DENV infection protect against viral challenge with either the same or a different serotype as measured by a reduction in viral load in spleen, lymph node and serum [140] (data not shown). Additionally, studies examining the T cell response to DENV infection in IFN α / β R^{-/-} C57BL/6 mice determined that CD8⁺ T cells release IFN γ and TNF α and have cytotoxic effects *in vivo* [123]. Mapping studies identified twelve immunodominant epitopes that mapped to six DENV proteins, and vaccination with four of these epitopes supported clearance of viral proteins [123]. In contrast, depletion of CD4⁺ T cells was shown to neither effect viral load following infection, nor induction of a neutralizing antibody response. However, vaccination with CD4 epitopes helped reduce viral load following challenge, indicating that CD4⁺ T cells may participate in viral clearance [142]. Zompi *et al* [96] recently supported a role for T cells in mediating cross-reactive protection in a heterotypic model of DENV infection [96]. Taken together, these results support the role of the mouse model in studying the role of the humoral immune response in mediating both serotype-specific and cross-reactive protection and enhancement.

To further investigate the role of dengue pathogenesis *in vivo*, a virulent, mouse peripherally-adapted strain of DENV2, D2S10, was generated by alternately passaging the virus through mice and mosquito cells ten times, harvesting mouse serum in each cycle [143]. A high inoculum of D2S10 administered to AG129 mice results in a lethal “vascular-leak” syndrome within 4-5 days. D2S10 has only two mutations, N124D and K128E, in the E protein that differentiate it from the parental clinical isolate PL046. These mutations decrease heparin sulfate binding and consequently reduce clearance of the virus, thus increasing viremia and contributing to the lethal disease phenotype [144]. A triple plaque-purified clone of D2S10, S221, has an additional mutation in NS1 and causes the same phenotype as D2S10 [123]. The DENV2 D2S10 virus has been used to investigate the role of antibodies in mediating both protection against and enhancement of disease in Chapters 2,3,4 and 5.

Unraveling the polyvalent antibody response to DENV: a complex interplay between neutralizing and enhancing DENV antibodies

While MAb studies demonstrated that anti-EDIII mAbs were the most potently neutralizing, ELISA binding studies examining the composition of the polyvalent antibody repertoire comparing humans with neuro-invasive and non-neuroinvasive WNV disease suggested that a very small percentage of the total human antibody repertoire targeted EDIII [60]. In a separate DENV-specific study, antibodies targeting EDIII were depleted from primary DENV-immune serum. Comparison of the binding and functional ability of control-depleted and EDIII-depleted serum similarly suggested that polyvalent DENV-immune serum contained only a small proportion of EDIII-specific antibodies, and that these antibodies functionally did not contribute to either serotype-specific or serotype-cross-reactive neutralization *in vitro* [145,146]. Binding studies using ablated EDIII epitopes supported these observations and suggested that >80% of the antibodies in both primary and secondary human cases bound to cross-reactive epitopes on

either the EDII fusion loop [147,148] or EDIII A strand [147]. Two independent, high throughput studies using alternate screening methods to analyze B cells from infected humans both isolated pools of mAbs that were largely cross-reactive [149,150]. Interestingly, a dominant focus of the immune response appears to target prM [149]. A third, recently-published study screened serum using whole virus rather than recombinant proteins and found, surprisingly, that only ~35% of antibodies bound to recombinant E protein, as compared to the whole virion [151]. These data indicate that the target of the humoral immune response may be to epitopes that have only recently become the subject of intense investigation [73].

Thesis objectives

Given the recent, conflicting observations about the role of different components of the immune response, the objective of this dissertation research was to use a small animal model system to dissect and identify the contribution of different components of the humoral immune response to mediating protection and enhancement following DENV infection. In Chapter 2, we discuss the development and characterization of a mouse model to study lethal, antibody-enhanced DENV disease *in vivo*. The third and fourth chapters of this dissertation use this model to investigate the relevant subsets of the humoral immune response that contribute to both protection and enhancement of DENV infection *in vivo*. In Chapter 4, we analyze the role of EDIII-specific antibodies from both human as well as murine DENV-immune serum. In Chapter 3, we examine the role of aglycosylated mAbs that cannot bind to the FcγR in mediating therapeutic protection following a lethal, antibody-enhanced DENV infection. The fifth chapter examines the hypothesis that different pools of antibodies (serotype-specific versus serotype-cross-reactive) mediate protection and enhancement of DENV or whether the same antibodies can be both protective and enhancement, but at different dilutions. As vaccine studies progress through Phase III trials [152], a more thorough understanding of the contribution of the humoral immune response to both protection and enhancement is essential. Further identification of the immunogenic and highly protective antibodies will be critical to development of a safe, long-lasting and efficacious vaccine.

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CHAPTER 2

LETHAL ANTIBODY ENHANCEMENT OF DENGUE DISEASE IN MICE IS PREVENTED BY FC MODIFICATION

INTRODUCTION

The four serotypes of dengue virus (DV) are mosquito-borne flaviviruses responsible for 50-100 million human infections annually. Primary infection in individuals over the age of one year with any DV serotype is usually asymptomatic or results in self-limited dengue fever (DF), but secondary infection with another DV serotype carries an increased risk of severe disease, including life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) [1,2]. Fatal disease is characterized by increased vascular permeability leading to hemoconcentration and hypovolemic shock [3]. The increased severity of secondary infections is believed to result, at least in part, from antibody-dependent enhancement (ADE) of DV infection, in which FcγR engagement by antibody-virus immune complexes facilitates virus entry into susceptible myeloid cell types [4]. A role for ADE in human dengue pathogenesis is supported by observations that maternally-derived anti-DV antibodies increase the risk of DHF in infants during primary infection with DENV2 [5,6]. Antibody-mediated increases in DV viremia have been demonstrated in macaques, but a limited number of antibody conditions were examined, and exacerbation of dengue disease by passively transferred antibodies was not observed [7,8]. Consequently, fundamental questions about the immunology and pathogenesis of ADE have remained unanswered, and small animal models for testing antiviral interventions in the context of ADE have not been available.

Recently, we derived a mouse-adapted DV2 strain, D2S10, that produces a TNF- α -dependent fatal vascular permeability syndrome in interferon- α and γ -receptor-deficient (AG129) mice 4-5 days after intravenous (iv) infection with 10^7 plaque forming units (pfu) of virus [9]. DV infection in AG129 mice reproduces important features of human DV infection, including similar tissue and cellular tropism, viremia, vascular leakage, and elevated serum cytokine levels [9,10,11,12]. Antibodies elicited by DV infection are a mixture of serotype-specific and serotype-cross-reactive antibodies, including long-lasting neutralizing antibodies. Memory immune responses are present after primary DV infection, and serotype cross-protective immunity was observed in three different sequential infection scenarios [13]. Thus, we utilized the AG129 model to examine the effects of serotype cross-reactive antibodies on DV2 infection *in vivo*. In this report, we demonstrate lethal enhancement of DV infection and disease by both polyclonal and monoclonal antibodies. We also show that ADE functions to increase the viral burden in blood and tissues, resulting in a vascular permeability syndrome that is similar to that seen in mice with a higher inoculum in the absence of immune antibody and shares clinical features of human dengue disease. Finally, we confirm the critical role of FcγR interaction in ADE *in vivo* and provide proof-of-principle for a pre- and post-exposure treatment strategy utilizing genetically engineered monoclonal antibodies that can no longer bind FcγR.

RESULTS

Lethal enhancement of dengue disease by anti-DV serum.

Serum containing anti-DV1 antibodies was collected from AG129 mice 8 weeks after subcutaneous (s.c.) inoculation with 10^5 pfu of DV1 strain 98J. Heat-inactivated anti-DV1 serum exhibited a 50% neutralizing titer (NT₅₀) against DV2 D2S10 of 1:296 and against DV1 98J of 1:1,069 using a flow-based neutralization assay [14], peak enhancement titers of 1:75 against DV2 D2S10 (fold enhancement 14.8%) and 1:225 against DV1 98J (fold enhancement 10.7%) in

an *in vitro* ADE assay with FcγR-bearing human K562 cells, and ELISA titers of 400 and 3200 against purified DV2 and DV1, respectively (data not shown). In addition, no residual DV1 could be isolated following inoculation into C6/36 mosquito cells (data not shown). The effects of anti-DV1 serum on DV2 infection were investigated after intraperitoneal (i.p.) injection of 100ml of either naïve mouse serum (NMS) or anti-DV1 serum, followed 24 hours later by i.v. challenge with 10^4 - 10^6 pfu of DV2. Lethal infection controls received 10^7 pfu of DV2, and all mice were monitored for mortality for 10 days. While no mortality was observed in NMS-recipient mice infected with 10^6 pfu or less of DV2, 92-100% of anti-DV1 recipients died after inoculation with 10^5 - 10^6 pfu of DV2 (**Figure 2.1A** and **Table 2.1**) between 4 and 5 days post-infection. In both the 10^7 pfu infection controls and anti-DV1 recipients infected with 10^5 or 10^6 pfu, lethal disease was accompanied by fluid accumulation in visceral organs characteristic of the vascular permeability syndrome induced by D2S10 [9] (**Figure 2.1B**). Mice administered anti-DV1 serum and challenged with D2S10 also experienced significant increases in serum TNF-α ($p < 0.01$) and IL-10 ($p < 0.01$) and greater platelet depletion ($p < 0.02$), as compared to NMS-recipient controls (**Figure 2.1C-F**); each of these disease parameters also correlates with dengue severity in humans [15,16,17].

Anti-DV antibody increases systemic viral burden.

Viral burden was subsequently compared between anti-DV1 and NMS-recipient mice infected with 10^5 or 10^6 pfu DV2. Viral burden was systemically increased in anti-DV1 versus NMS-recipient mice, with a 20-fold increase ($p < 0.02$) in viremia accompanied by significant 3- to 12-fold increases in viral load in multiple tissues ($p \leq 0.04$) including peripheral blood mononuclear cells, liver, small intestine, lymph node, and bone marrow (**Figure 2.2A**); non-significant increases in the large intestine and spleen ($p > 0.08$) and lungs (data not shown) were observed. No statistically significant differences were observed in tested disease parameters, viral burden, or tissue tropism between 10^7 pfu of D2S10 infection in the absence of antibody and antibody-enhanced infection with 10^5 pfu of D2S10. Notably, anti-DV antibodies also enhanced infection with non-adapted low-passage human DV isolates DV1 Western Pacific-74 (**Figure 2.2B**) and DV2 TSV01 (**Figure 2.2C**), as determined by significant increases ($p \leq 0.04$) in viral burden in the liver and small intestine for both viruses, and in serum for DV2 TSV01. Although mortality was not observed, a subset of animals infected with DV1 Western Pacific-74 under antibody-enhanced conditions displayed fluid accumulation in visceral organs and gross morphology similar to but less pronounced than that observed with enhanced DV2 D2S10 disease.

Increased infection of FcγR-bearing cells during ADE.

ADE is predicted to facilitate infection of FcγR-bearing cell types such as tissue macrophages and dendritic cells [4]; therefore, we examined the cellular tropism of DV2 in mice by immunostaining for the viral NS3 protein, which is only present during active replication of virus. As found in humans [11,18], infected cells with morphology and location consistent with tissue macrophages or dendritic cells [19,20] were detected in lymph node, small intestine, large intestine, and bone marrow under all infection conditions, and NS3⁺ cells with endothelial and/or phagocyte morphology were also observed in liver (**Figure 2.3** and data not shown). Infection in myeloid cells was confirmed by co-staining of serial sections and bone marrow aspirates for NS3 and the myeloid/macrophage marker F4/80 (data not shown). Furthermore, using flow

cytometry, DV NS3 and E protein were detected in bone marrow cells expressing myeloid markers CD11b, CD11c, and F4/80, and in liver DV infection was primarily in CD31⁺CD45⁻ sinusoidal endothelial cells, which also express FcγR (**Figure S2.1**). Notably, significantly greater numbers of NS3⁺ cells ($p < 0.05$) were present in tissues of anti-DV1 recipient mice compared to naïve serum recipient controls infected with the same dose of DV2, supporting the hypothesis that ADE functions to increase the viral burden in cells and tissues.

Effect of antibody dose on ADE *in vivo*.

While serotype cross-reactive immunity is implicated in the pathogenesis of severe dengue, serotype-specific immunity typically protects against re-infection with the same DV serotype [2]. However, *in vitro* studies suggest that all antibodies that neutralize infection are capable of ADE at some lower concentration [21]; therefore, we examined the effects of anti-DV1 and anti-DV2 sera on DV2 D2S10 infection in mice over a range of doses. While the highest dose (400 μl) of anti-DV1 serum lethally enhanced infection (**Figure 2.4A** and **Table 2.2**), recipients of 400 μl of anti-DV2 serum developed no signs of illness and lacked detectable viremia (**Figure 2.4B and C**; **Table 2.2**), confirming that serotype-specific antibodies can provide robust protection in this model. However, lower doses of both anti-DV1 and anti-DV2 serum caused lethal enhancement, showing that serotype-specific as well as serotype-cross-reactive antibodies can also enhance infection *in vivo* in a dose-dependent manner (**Figure 2.4A and B**). To assess the ability of the BHK PRNT₅₀ assay to predict *in vivo* protection and enhancement in this mouse model, neutralizing activity was measured in the sera of mice immediately prior to infection with D2S10. Serum was collected approximately 18 hours post-transfer of anti-DV antibodies, and 4 hours prior to infection. Similar to human studies [22], lethal enhancement occurred even in mice that had detectable neutralizing antibodies, although no lethality was observed in mice with PRNT₅₀ values of >200.

To further define the characteristics of enhancing antibodies, we examined the ability of monoclonal antibodies (MAbs) to enhance DV disease in mice. Mice were inoculated with DV2 D2S10 24 hours after transfer of increasing amounts of the flavivirus cross-reactive, neutralizing MAb 4G2 (**Figure 2.4D**). 4G2 caused lethal enhancement at doses of 0.062-4mg/kg (1.25-80 μg/mouse), but no mortality occurred in mice receiving 20mg/kg (400 μg/mouse) or in IgG2a isotype control antibody recipients (**Figure 2.4D** and **Table 2.2**). 4G2, anti-DV1 serum, and anti-DV2 serum all enhanced infection and disease over a ~60-fold range in concentration.

ADE requires FcγR interaction *in vitro* and *in vivo*.

Since FcγR engagement is required for ADE *in vitro* [23], we hypothesized that eliminating the ability of antibodies to bind to FcγRs would prevent ADE *in vivo*. To test this, we first generated F(ab')₂ fragments of 4G2. These fragments were indistinguishable from intact 4G2 in their ability to bind to DV2 E protein by ELISA (**Figure S2.2A**), but were unable to enhance DV infection of the human FcγR-bearing cell line K562 (**Figure 2.5A**). *In vivo*, F(ab')₂ fragments have a shorter serum half-life than intact IgG, thus it was necessary to identify a dosing regimen that would maintain serum concentration of F(ab')₂ fragments within the known enhancing range for intact IgG antibodies. Serum F(ab')₂ levels were measured one and 24 hours after i.v transfer of 20μg of F(ab')₂ by E protein ELISA; this dose maintains E-reactive antibodies at levels within the range where IgG causes enhancement for 24 hours (**Figure S2.2C**). To examine the effects of intact IgG and F(ab')₂ *in vivo*, we compared the enhancing

effects of a single dose of 4G2 MAb with daily 20 μ g doses of 4G2 F(ab')₂ (**Figure 2.5B**). Whereas significant mortality was observed in 4G2 MAb recipients ($p \leq 0.04$) no illness occurred in 4G2 F(ab')₂ or IgG2a isotype control recipients (**Figure 2.5C**). Viremia measured at 3.5 days post-infection in F(ab')₂ recipients was significantly reduced ($p < 0.03$) compared to isotype control antibody recipients (**Figure 2.5D**), suggesting that loss of Fc γ R interaction not only diminished enhancement but also promoted neutralization to reduce viral load.

We followed up these studies using a MAb genetically engineered to eliminate Fc γ R binding. MAb E60 is a flavivirus cross-reactive neutralizing mouse IgG2a antibody that, similar to 4G2, binds to an epitope in the fusion peptide of domain II on the E protein [24,25]. This MAb was cloned and the constant regions replaced [26] with those from human IgG1 to create an E60-chimeric human IgG1 (E60-hIgG1). In addition, an asparagine to glutamine variant at position 297 in human IgG1 was engineered (E60-N297Q), as this mutation abolishes Fc γ R binding without altering the half-life of the antibody in mouse serum [27]. Affinity measurements conducted by surface plasmon resonance indicated that E60-mouse IgG2a (E60-mIgG2a), E60-hIgG1, and E60-N297Q all exhibited similar binding to purified E protein (**Figure S2.3A**) and DV2-infected cells by flow cytometry (data not shown), as well as similar neutralizing activity against DV2 by both PRNT₅₀ assay (0.23, 0.25, and 0.42 μ g/ml, respectively) and a neutralization assay using DC-SIGN-expressing human target cells (**Figure S2.3B**). However, as expected, E60-mIgG2a and E60-hIgG1 enhanced DV2 infection of K562 cells *in vitro* whereas E60-N297Q did not (**Figure 2.6A**).

To test the ability of the E60-N297Q variant to enhance DV infection *in vivo*, mice were administered 20 μ g of E60-mIgG2a, E60-hIgG1, and E60-N297Q 24 hours prior to infection with 10⁶ pfu of D2S10. Whereas both E60-mIgG2a and E60-hIgG1 consistently caused antibody-dependent mortality 4 to 5 days post-infection, equivalent doses of E60-N297Q caused neither morbidity nor mortality (**Figure 2.6B**). Instead, viremia and tissue viral burden in E60-N297Q recipients were substantially reduced, demonstrating that the N297Q mutation converted the *in vivo* effect of E60 on viral burden from enhancement to neutralization (**Figure 2.6C**, data not shown).

The N297Q mutation also abolishes binding to complement component C1q [27]. Consequently, we generated a second variant antibody, E60-A330L, to assess whether the loss of C1q binding or the loss of Fc γ R binding explained the inability of E60-N297Q to mediate ADE. E60-A330L does not bind C1q but retains binding to Fc γ R [28], and we confirmed this by surface plasmon resonance (data not shown). E60-A330L exhibited similar binding and neutralization activity to E60-hIgG1, enhanced DV infection *in vitro* in K562 cells, and lethally enhanced a DV2-D2S10 infection *in vivo* (**Figure S2.3B, C, and D**, and data not shown). Thus, C1q interaction was not required for ADE *in vitro* or *in vivo*, and a loss of C1q binding does not explain the inability of E60-N297Q to enhance DV infection.

An antibody that cannot bind Fc γ R has both prophylactic and therapeutic potential.

The experiments above suggested that an N297Q variant antibody against DV could have potential as an antiviral intervention. To assess this, 20 μ g of E60-hIgG1 or E60-N297Q was administered concurrently with 25ml of anti-DV1 serum 1 day prior to infection with DV2. E60-N297Q protected mice against any signs of illness, whereas all recipients of anti-DV1/E60-hIgG1 succumbed to infection (**Figure 2.7A**). Post-exposure therapeutic application of E60-N297Q was evaluated by administering 25ml anti-DV1 serum to mice, followed by infection

with DV2 the next day, and i.v. administration of E60-N297Q or E60-hIgG1 on day 1 or 2 post-infection. While all mice treated with E60-hIgG1 succumbed to infection, lethality was completely prevented by a single 20 μ g dose of E60-N297Q on day 1 (**Figure 2.7B** and data not shown), and E60-N297Q treatment significantly decreased viremia, tissue viral burden, and serum TNF- α levels as measured 3.5 days post-infection (**Figure S2.4**, $p < 0.04$). Moreover, 20 or 50 μ g doses of E60-N297Q administered on day 2 resulted in 40% and 80% survival, respectively, demonstrating therapeutic efficacy for this antibody in mice (**Figure 2.7B**).

MAbs 82.11 and 87.1 complexed with either mouse Ig γ 2A or human Ig γ 1 Fc regions are equivalently enhancing *in vivo*.

We next wanted to determine whether human aglycosylated antibodies could be used to treat lethal, antibody-enhanced disease. Before beginning these studies, we first needed to determine that mAbs with the same F(ab) $'$ 2 but with either a human or mouse Fc γ constant region would be able to interact equivalently with mouse Fc γ R. To ask this question, we created chimeric mAbs using human mAb 82.11 and 87.1, where each mAb was engineered with either mouse Ig γ 2A or human Ig γ 1. 5 μ g of either the wildtype and mouse chimeric MAbs were subsequently administered *in vivo* 24 hours prior to a sublethal 10⁵ pfu DENV2 D2S10 infection. Mice receiving either mouse or human 87.1 demonstrated 33% and 67% survival, respectively, while mice receiving either variant of 82.11 all succumbed to a lethal infection (**Figure 2.8**). This data allows us to conclude that mAbs with human Ig γ 1 can interact with mouse FcR, and that human and mouse mAbs with the same F(ab) $'$ 2 fragment are equivalently enhancing *in vivo*.

Human MAbs that cannot bind Fc γ R do not enhance DENV infection and show therapeutic efficacy *in vivo*.

We next tested two strongly neutralizing non-FcR-binding (LALA) human MAbs to determine whether these MAbs would be capable of similarly preventing enhancement or acting therapeutically following an antibody-enhanced infection. Unmodified or LALA variant MAbs were transferred into AG129 mice 24 hours prior to a sub-lethal infection with DENV2 strain D2S10. While mice pretreated with 1 or 5 μ g unmodified MAbs DV87.1 and DV82.11 showed enhanced lethal DENV infection, mice receiving the same amounts of LALA variants did not succumb to infection (1 or 5 μ g 87.1 LALA, $p < 0.0253$; 1 or 5 μ g 82.11 LALA, $p < 0.0455$ as compared to mice which received PBS in place of MAb) (**Figure 2.9A**) or show signs of illness. In contrast, mice receiving 5 μ g of the mouse MAb 4G2 (pan-flavivirus, E DI/II-specific), succumbed to an enhanced, lethal D2S10 infection, whereas 5 of 6 mice receiving PBS alone survived infection (**Figure 2.9A**).

To determine whether survival following transfer of the DV87.1 LALA and DV82.11 LALA variants was associated with reduced viral load, we measured the viral burden in serum and tissues 3.5 days following D2S10 antibody-enhanced infection. Serum viremia and tissue viral load measured in liver, small intestine and lymph node were significantly decreased in mice receiving 5 μ g of either LALA variant as compared to mice receiving 5 μ g of the parent unmodified MAbs (**Figure 2.10A-D**).

To explore whether the LALA variants could serve as a possible therapy following DENV infection, we administered 50 μ g of the LALA variants or unmodified MAbs to mice 24 hours after infection with DENV2 D2S10 under enhancing conditions (24 hours after transfer of heterotypic anti-DENV1 serum. Mice receiving either the DV87.1 or DV82.11 LALA variant

survived the normally lethal infection, whereas mice receiving the unmodified parent MABs succumbed to infection, as did mice receiving non-binding isotype control (DV22.3 LALA MAB that only recognizes DENV4) or PBS (82.11 LALA, $p=0.011$; 87.1 LALA, $p=0.0015$ as compared to non-treated control mice) (**Figure 2.9B**). In summary, these results demonstrate that engineering of the LALA mutation on strongly neutralizing MABs abrogates the capacity for ADE and confers a protective phenotype as a post-exposure therapy in mice [29].

DISCUSSION

Understanding the immunopathogenesis of DV infection has been severely hampered by the lack of a small animal disease model. Thus, studies of ADE have been limited to epidemiological observations and *in vitro* experimentation. Here, we present the first model of antibody-enhanced lethal dengue disease *in vivo*. This work describes a long-sought mouse model for investigation of dengue pathogenesis, characterizes a clinically important mechanism of immunopathogenesis, has implications for vaccine development, and identifies a possible antibody-based antiviral strategy to treat life-threatening DV infection.

Numerous attempts have been made to establish a mouse model of dengue disease and have been limited by the relatively low susceptibility of mice to DV infection. Previous models have included intracerebral inoculation of DV or injection of very high ($>10^9$ PFU) doses of virus into immunocompetent mice [30,31]; infection of SCID [32,33,34,35] or NOD/SCID or RAG2g(c)^{-/-} mice [36,37] implanted with human cells or cell lines; and use of various immunodeficient strains of mice [38,39]. The most common outcome is neurovirulent disease, with a few recent exceptions [36,37]. Of these, the AG129 mouse model has proven both useful and tractable, as it is permissive to infection with all four DV serotypes, displays relevant tissue and cellular tropism, produces long-lasting serotype-specific and serotype-cross-reactive anti-DV antibodies of a balanced isotype ratio, and generates a vascular leakage syndrome that in many respects resembles human dengue disease [9,10,11,12,13]. Nonetheless, we acknowledge that the lack of IFN receptors limits reproduction of some facets of human disease, especially in relation to cytokine profiles or infection conditions that are modulated by IFNs. However, the many similarities with specific features of human DV infection and the critical role for FcγR in ADE *in vivo* that we demonstrate here support the use of the AG129 model for specific avenues of dengue research. Interestingly, IFN-receptor deficient mice (A129) have recently been successfully adapted for other mosquito-borne viruses, including both Chikungunya and yellow fever [40,41].

In vivo ADE models have also been established for other viruses including Yellow Fever Virus (YFV), Murray Valley Encephalitis Virus (MVEV), Japanese Encephalitis Virus (JEV), and Feline Infectious Peritonitis Virus (FIPV) [42,43,44,45,46,47], in which passive transfer of antibody increases viral titers and/or mortality. These models show several parallels with our model of antibody-enhanced DENV infection: with FIPV, immune sera are able to enhance macrophage infection and disease during subsequent challenge with the same FIPV serotype in kittens [41, 46], and with MVEV, JEV, and YFV, enhanced mortality was observed in mice administered flavivirus cross-reactive polyclonal antibodies or non-neutralizing YFV-specific monoclonal antibodies [42-45]. However, none of these pathogens are associated with antibody-enhanced disease in humans. By modelling ADE with a pathogen known to cause antibody-enhanced disease in humans and using a model that displays a variety of relevant disease phenotypes, this report extends previous work on ADE to the ability to model human disease

parameters and aid in the development of therapeutics.

In vivo evidence of ADE of DV infection was first described by Halstead *et al* [8] following the passive transfer of antibodies in the rhesus macaque. Similar data was recently obtained by Gonçalves *et al* [7], where passive transfer of the serotype-cross-reactive MAb 1A5 enhanced DV4 viremia over a ~30-fold concentration range (0.22-6 mg/kg). While both of these studies described elevated viremia, neither resulted in a clinical phenotype with parallels to human disease. Here, we describe enhancement of a mouse-adapted strain of DV2 by serotype-specific and cross-reactive sera as well as by monoclonal antibodies. Importantly, characterization of antibody-dependent dengue disease in the AG129 mouse maintains several parallels with severe disease in humans. Hallmark features of human DHF/DSS are vascular leak, higher viral burden, increased levels of serum cytokines such as TNF- α and IL-10, and platelet depletion [48]. All of these features were observed in our mouse model of ADE. Moreover, the magnitude of DV enhancement also mimics that seen in humans and non-human primates. We observed a 20-fold increase in viremia triggered by ADE; DV viremia in humans is reported to be 10- to 100-fold higher in DHF cases than in DF cases [49,50], and ADE in macaques increases viremia 5-100 fold [7,8]. Interestingly, in all of the disease parameters we examined, there was no apparent difference between lethality resulting from antibody-enhanced infection with a sublethal viral dose and lethality resulting from direct inoculation with a 100-fold higher viral dose. Thus, this model did not reveal any fundamental difference in the mechanisms of pathogenesis between antibody-enhanced and non-enhanced infection; rather, lethality here appears to be a result of higher viral burden, regardless of how such a burden was achieved.

To ensure that enhanced disease in the AG129 model was not solely a feature of the mouse-adapted strain, mice were infected with clinical isolates DV1 Western Pacific-74 and DV2 TSV01 in the presence of anti-DV antibodies, and enhanced viremia was observed in both cases. The lack of mortality in infections with these viruses is likely a result of the lower viral burden established by non-adapted strains even in the presence of enhancing antibody. Interestingly, mild fluid accumulation was also observed in the gastrointestinal organs in a subset of mice. As only a small fraction (0.5%) of human secondary DV infections results in severe disease, and some DV strains are more virulent than others based on genetic differences [51], the observed spectrum in disease severity is not surprising, but rather parallels the human condition. Immunohistochemical characterization of the cellular tropism associated with ADE using NS3-specific antibodies indicated infection in cells with morphology consistent with dendritic cells and tissue macrophages in the lymph node, small intestine, large intestine and bone marrow. Further characterization by flow cytometry supported the IHC data and demonstrated infection, as evidenced by both anti-E and anti-NS3 staining, in cells with surface markers of monocytes and macrophages in the bone marrow and sinusoidal endothelial cells in the liver. By both methodologies, the infected cell types identified in the murine model agree with those cells defined as the natural targets of DV in the human host [11,18]. Interestingly, the infected cell types did not change between an enhanced and non-enhanced DV infection; rather, quantification by both IHC and flow cytometry indicated an increase in the number of infected cells. Taken together, antibody-enhanced disease appears to result in increased infection in the natural targets of DV infection and resulting pathogenesis that does not significantly differ from the disease that results when a 100-fold higher dose of DV is used in the absence of enhancing antibody.

In human infants who have acquired maternal anti-DV antibody, severe dengue can occur

even when calculated neutralizing antibody titers against the secondary infecting serotype are >1:100 [52]. Similarly, children with detectable neutralizing antibody against the infecting virus strain can develop DHF during secondary DV infections [22]. These studies indicate that the *in vitro* neutralization assay using BHK21 cells is not a consistent correlate of protection in humans. Similarly, our PRNT₅₀ assays performed on serum samples from mice after antibody transfer but before virus challenge demonstrated that despite *in vitro* neutralizing activity at the time of infection, anti-DV1 sera, anti-DV2 sera, and 4G2 all enhanced infection *in vivo*. Enhanced disease was consistently observed in antibody-recipient mice with pre-infection neutralizing titers of <1:200, but not greater. Thus, substantial neutralizing antibody levels appear to be required to prevent severe disease in this model. Of note, the passive transfer and primary infection scheme used does not examine anamnestic B and T cell immune responses, and thus, more accurately models DHF/DSS in infants with primary DV infection rather than secondary DV infections.

In vitro evidence had previously indicated that an interaction between the Fc portion of the antibody and the FcγR was necessary for ADE [7]; however, this hypothesis had never been corroborated *in vivo*. Using two different reagents – F(ab)₂ fragments of 4G2 and the N297Q variant of hE60-IgG1, we demonstrate that binding of the Fc portion of the antibody to the FcγR is required for ADE-induced disease. Further analysis with F(ab)₂ or the N297Q variant showed a reduction in viral titer below the level in mice receiving PBS in place of MAb. Thus, under conditions where the antibody cannot bind the FcγR, the F(ab) portion of the antibody can neutralize infection. These data also demonstrate that antibodies directed to the fusion loop in E domain II are capable of neutralizing DV infection independently from effector functions mediated by FcγR and C1q. Because the N297Q mutation also ablated the C1q binding site, we tested a second hE60 variant, hE60-A330L, that contained a mutation disrupting the complement C1q receptor binding site, but not the FcγR interaction. Mice receiving the hE60-A330L variant succumbed to an enhanced DV infection. This confirms that interaction of the anti-DENV MAb with the FcγR, and not binding of C1q, is essential for ADE *in vivo*.

Given the promising data with the hE60-N297Q variant, we tested the prophylactic and therapeutic efficacy of this antibody. When given as prophylaxis together with an enhancing amount of anti-DV1 serum, hE60-N297Q was completely protective. Although interesting, a DENV prophylactic is not likely to be a clinically useful reagent. However, when given 24 hours after an enhanced DENV infection, E60-N297Q completely protected against mortality; likewise, tissue viral load and systemic TNF-α levels in these mice at 3.5 days post-infection were significantly reduced. Two different doses of E60-N297Q, 20 and 50 μg, were administered 48 hours post-infection and resulted in 50% and 80% survival, respectively. Given the condensed timeframe of DENV pathogenesis in the AG129 model, E60-N297Q or similar therapeutic MAbs may have a broader time window for intervention and efficacy in humans or other animal models that display more protracted kinetics of DV infection.

In summary, we report the first animal model of lethal antibody-mediated enhancement of DV infection, describe virologic and pathologic changes induced by ADE, and define antibody conditions for protection and ADE in passive antibody transfer recipients. Furthermore, we show that antibodies engineered to prevent FcγR interaction exhibit prophylactic and therapeutic efficacy against DV infection, and thus have potential as a novel antiviral strategy against DV.

Acknowledgements. The authors thank Ritela Gonzalez, Hector Nolla, Simona Zompi, Soila Sukupolvi, and Anja Fuchs for experimental assistance, James Brien for figure preparation, and Sondra and Milton Schlesinger for scientific input in preparing the manuscript.

MATERIALS AND METHODS

Ethics Statement. All experimental procedures were pre-approved by the UC Berkeley Animal Care and Use Committee and were performed according to the guidelines of the UC Berkeley Animal Care and Use Committee.

Viruses, cell lines and monoclonal antibodies. DV was propagated in the *Aedes albopictus* cell line C6/36 (American Type Culture Collection [ATCC]) as described elsewhere [53]. DV2 strain D2S10 (passaged 4 times in C6/36 cells) was derived in our laboratory [9] from the parental DV2 PL046 Taiwanese isolate as previously described [9]. The DV1 strain 98J was isolated in our laboratory from a patient from Guyana in 1998 [54] and passaged 7 times in C6/36 cells. The DV1 strain Western Pacific 74, originally isolated in Nauru in 1974, was obtained from the National Institutes for Biological Standards and Control (Hertfordshire, UK) and passaged 3 times in C6/36 cells. The DV2 strain TSV-01, isolated in Townsville, Australia, in 1993 was obtained from W. Schul, passaged ~10 times in C6/36 cells (Novartis Institute for Tropical Diseases, Singapore) [10]. Virus titers were obtained by plaque assay on baby hamster kidney cells (BHK21, clone 15) as described [53]. For mouse experiments, virus was concentrated by centrifugation at 53,000 x g for 2 hours at 4°C and resuspended in cold PBS with 20% FBS (HyClone, Thermo Scientific). U937 DC-SIGN cells were obtained from A. de Silva (University of North Carolina, Chapel Hill) and grown in RPMI media (Invitrogen) at 37°C in 5% CO₂. K562 cells were used for all enhancement assays and grown in RPMI media (Invitrogen) at 37°C in 5% CO₂. The hybridoma of MAb 4G2 was purchased from ATCC, grown in serum-free medium (Invitrogen), and purified using protein G affinity chromatography (Thermo Scientific).

Mouse mAbs Mouse mAb E60 and human E60-IgG1 (hE60), were obtained from M. Diamond, and hE60-N297Q was obtained from S. Johnson (MacroGenics, Inc.) The mouse E60 IgG2a mAb was originally generated against WNV E protein, reacts with an epitope in the fusion peptide in DII, and cross-reacts with DV E proteins [25]. The generation of a chimeric human-mouse E60 with the human IgG1 constant regions and the mouse VH and VL was performed as described previously [26]. Point mutations in the Fc region that abolish FcγR and C1q binding (N297Q) or C1q binding alone (A330L) were introduced by QuikChange mutagenesis (Stratagene). All recombinant antibodies were produced after transfection of HEK-293T cells, harvesting of supernatant, and purification by protein A affinity chromatography.

Human mAbs Human mAbs 82.11 and 87.1 were obtained from F. Sallusto and A. Lanzavecchia (Institute for Research in Biomedicine, Bellinzona Switzerland) and are human in origin. Peripheral blood mononuclear cells were isolated and cryopreserved from donors who had been diagnosed with DENV infection. Previously sorted IgG⁺ memory B cells were immortalized using 20 cells/well in multiple cultures using EBV with CpG oligodeoxynucleotide 2006 (Microsynth) and irradiated allogeneic PBMCs. The culture supernatants were subsequently screened for DENV-specific Mabs, and the positive cultures were cloned by limiting dilution. Domain-specific binding patterns were determined by ELISA using a yeast

display assay provided by M. Diamond. The LALA variants were created by sequencing and cloning the variable regions of heavy-chain and light-chain genes by PCR. Leucine-to-alanine (LALA) mutations at positions CH2 1.3 and 1.2 of Igγ1 were introduced by site-directed mutagenesis (GenScript). Recombinant antibodies were produced in HEK-293T cells and purified by protein A affinity chromatography and size-exclusion chromatography.

Infection of AG129 mice. AG129 mice [55] were originally obtained from M. Aguet (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland) and were bred in the University of California (UC) Berkeley Animal Facility. All experimental procedures were pre-approved and were performed according to the guidelines of the UC Berkeley Animal Care and Use Committee. **Generation of mouse anti-DV sera.** 6-8 week-old AG129 mice were infected subcutaneously with 10^5 pfu of either DV1 strain 98J or DV2 strain PL046 or PBS. Mice were sacrificed by terminal bleed six to eight weeks after infection. Serum was separated from whole blood by centrifugation, heat-inactivated, and frozen at -80°C . **Enhancement of DENV in vivo.** Mice were injected intraperitoneally with MAb, PBS, or anti-DV sera in a total volume of 400 μl , then infected 20-24 hours later with DV by intravenous (iv) injection into the tail vein in a total volume of 100ml. In some experiments, 50-100 ml of blood was obtained via retro-orbital bleed approximately 18 hours post-transfer (i.e., 4-6 hours prior to DV infection) and processed into serum as above.

Measurement of cytokines and platelet counts. Cytokines were measured using commercially available ELISA kits (EBioscience). Platelet counts were obtained by diluting 20 μl of anticoagulated blood into Unopette reservoirs (BD) and counting on a hemocytometer.

Quantitation of virus in tissues by plaque assay. Viral load was determined in the indicated tissues as previously described [53], and expressed as either pfu/g (all solid tissues) or pfu/ 10^9 cells (bone marrow and PBMCs). To obtain PBMCs, 200-300 μl of whole blood was collected into EDTA-coated microtainer tubes (Becton Dickinson) after cardiac puncture. Samples were washed 3 times in red blood cell lysis buffer (eBioscience) and once in cold PBS, and resuspended in 250ml alpha-MEM with 5% fetal bovine serum (FBS, Hyclone), 10mM HEPES (Invitrogen) and 100U penicillin/100 μg streptomycin (P/S; Invitrogen).

Quantitation of virus in serum by quantitative RT-PCR. Viral RNA was extracted from 60ml serum aliquots using Qia-Amp Viral RNA recovery kit (Qiagen). Quantitation of viral RNA utilized Taqman reagents (One Step RT-PCR Kit, Applied Biosystems, Foster City, CA) and an ABI PRISM 7700 sequence detection system as described [56]. Viremia is expressed as plaque-forming unit equivalents/ml, which was calculated by dividing the genomic RNA copy number in each sample by the genome:pfu ratio of C6/36-derived virus as determined by plaque assay and qRT-PCR.

Immunohistochemistry. Tissues were collected at day 3.5 (n=3-6 mice per group), formalin-fixed, and processed into paraffin sections. Serial sections from each tissue were stained for NS3 using MAb E1D8 or an isotype control as previously described [11]. For quantification of NS3⁺ cells, at least ten visual fields were counted for each sample except bone marrow, where four

fields from four independent sections were counted due to the small area of mouse bone cross-sections. All pairwise comparisons were performed by two-sided Wilcoxon Rank Sum tests.

Flow Cytometry. *Bone marrow aspirates* were collected by perfusing two femurs with cold, complete RPMI media (Invitrogen) containing 10% FBS (Hyclone), 10mM Hepes (Invitrogen) and 100U penicillin/100 µg streptomycin (P/S; Invitrogen). Resuspended cells were washed once in red cell lysis buffer and once in D-PBS (Invitrogen). The cells were subsequently resuspended in flow cytometry buffer containing D-PBS, 2.0% bovine serum albumin (BSA; Fisher Scientific) and 0.02% sodium azide (Sigma-Aldrich) and plated in a 96-well U-bottom plate (Becton- Dickinson) at 1×10^6 cells/ well. Cells were blocked with 5% normal rat serum (Jackson Laboratories) diluted in flow cytometry buffer. Bone marrow cells were stained extracellularly using CD11b-PeCy7 (eBioscience), CD11c-PE (eBioscience), and F4/80-TC (Caltag) or isotype control, and then fixed in 2% paraformaldehyde (Ted Pella, Inc.), washed and permeabilized with 0.1% saponin (Sigma-Aldrich), followed by intracellular staining with human anti-DV E MAb 87.1 (F. Sallusto and A. Lanzavecchia, Institute for Research in Biomedicine, Bellinzona, Switzerland) or isotype control MAb hIgG1 WNV-E16 (M.S. Diamond) followed by secondary goat anti-human IgG conjugated to Alexa488 (Invitrogen). *Livers* were harvested into 10mL cold, complete RPMI media and subsequently digested using 20mg/mL collagenase VII (Sigma-Aldrich), washed, and the digested tissue passed over a 70-µm cell strainer (Fisher). The resulting cells were centrifuged over an Optiprep gradient (14.7%/ 22.2%), washed once with D-PBS, and plated in a 96-well plate at 1×10^6 cells/well. The cells were stained extracellularly using CD31-PE (eBioscience), fixed in 2% PFA, permeabilized with 0.1% saponin, and stained intracellularly with anti-NS3 MAb E1D8 conjugated to Alexa488 (Invitrogen) or isotype control (mIgG2a-Alexa488 (Invitrogen)). Data was collected using either an LSR II (BD) or FC-500 (BD) flow cytometer and analyzed using FlowJo v8.8.6 software (TreeStar).

Surface plasmon resonance Monoclonal antibodies at a concentration range of 12.5 to 200 nM were injected over the surface of a Biacore 3000 instrument with immobilized E protein (~300 RU) at a flow rate of 30ml/min for 120 seconds and a dissociation time of 180 seconds. Binding curves at concentration zero were subtracted as blank. Kinetic parameters were calculated by fitting binding curves to a bivalent analyte binding model. The kinetic parameters were similar for binding of both MAb variants to E protein, as the difference between affinities is less than two-fold.

Preparation of F(ab)² fragments and ELISA: 4G2 F(ab)² fragments were generated using the F(ab)² Preparation kit (Pierce) according to the manufacturer's instructions. To ensure that the F(ab)² fragments did not contain residual Fc portions, the 4G2 F(ab)² proteins were diluted in SDS-PAGE loading dye, boiled, and electrophoresed on a 10-20% Tris-glycine gel (Bio Rad) and stained with Colloidal Blue (Invitrogen) overnight. To measure the stability of F(ab)² fragments *in vivo*, sera from mice given different amounts of F(ab)² were tested by ELISA for DV2 E protein binding. In brief, ELISA plates (Fisher Scientific) were coated with 2mg/ml of recombinant DV2 E protein (Hawaii Biotech Inc.) in carbonate coating buffer, pH 9.6 overnight at 4°C. The plate was blocked for 1 hour at room temperature in 5% nonfat dry milk and 5% donkey serum (Jackson Laboratories) in PBS-0.5% Tween 20. After washing, 50ml of serum containing intact 4G2 or F(ab)² 4G2 diluted 1:10 in blocking buffer was added to the plates. After washing, 100 µl of either goat anti-mouse anti-F(ab)² (Jackson Laboratories) or goat anti-

mouse anti-Fc (Jackson Laboratories) diluted 1:1000 in PBS-T was added as secondary antibody. Biotinylated mouse anti-goat antibody (Jackson Laboratories) was added as a tertiary antibody, followed by streptavidin-alkaline phosphatase (Zymed). P-Nitrophenyl phosphate (PnPP; Sigma Aldrich) was added as the substrate, and the reaction was stopped 3M NaOH and read in an ELX-808 ultra microplate reader (Bio-Tek Instruments) at 405 nm.

Neutralization assay using U937 DC-SIGN cells and plaque reduction neutralization test. Serial 3-fold dilutions of antibodies were mixed with DV2 D2S10 virus at a multiplicity of infection (MOI) generating 7-15% infection of U937 DC-SIGN cells in a 96-well U bottom plate as described previously [14]. After infection for 24 hours, the cells were subsequently washed once with flow cytometry buffer, and fixed in 2% PFA for 10 minutes at room temperature. The cells were then permeabilized in FACS buffer with 0.1% saponin (Sigma Aldrich) and stained with 2.5 μ g/mL 4G2-Alexa 488 (Invitrogen). The cells were washed twice, and percent infection determined by flow cytometry on a Beckman Coulter EPICS XL flow cytometer. The resulting raw data was expressed in GraphPad Prism 5.0 software as percent infection versus log₁₀ of the serum dilution, and a sigmoidal dose-response curve with a variable slope was applied to determine the antibody titer coinciding with a 50% reduction in infection as compared to the no-serum control (NT₅₀). The plaque reduction neutralization test (PRNT) was performed in duplicate as described previously [13].

***In vitro* measurement of ADE.** Serial 3-fold dilutions of antibody were mixed with DV2 D2S10 virus in duplicate for 45 min at 37°C, then mixed with K562 cells at MOI of 1 for 48 hours [23] in a 96-well plate. The cells were subsequently washed once with FACS buffer and fixed in 2% PFA for 10 minutes at room temperature. To stain, the cells were permeabilized in FACS buffer with 0.1% saponin (Sigma Aldrich), and then stained with 2.5 μ g/mL 4G2-Alexa 488 (Invitrogen). The cells were washed twice, and percent infection was determined by flow cytometry on a Beckman Coulter EPICS XL flow cytometer. The resulting data was expressed as percent cellular infection versus log₁₀ of the serum dilution in Microsoft Windows Excel.

Statistical analysis. Kaplan-Meier survival curves were used to display mortality data, and log rank analyses were used to determine statistical significance between experimental groups. Non-parametric analyses using the two-sided Wilcoxon rank sum tests were used for pairwise comparisons of viral load, cytokines, and platelet counts. A Fisher's exact test was used to examine survival on day 4 post-infection in F(ab')₂ experiments because the instability of F(ab')₂ fragments necessitated comparison at a single time point. Calculations were performed in GraphPad Prism 5.0 software.

FIGURES

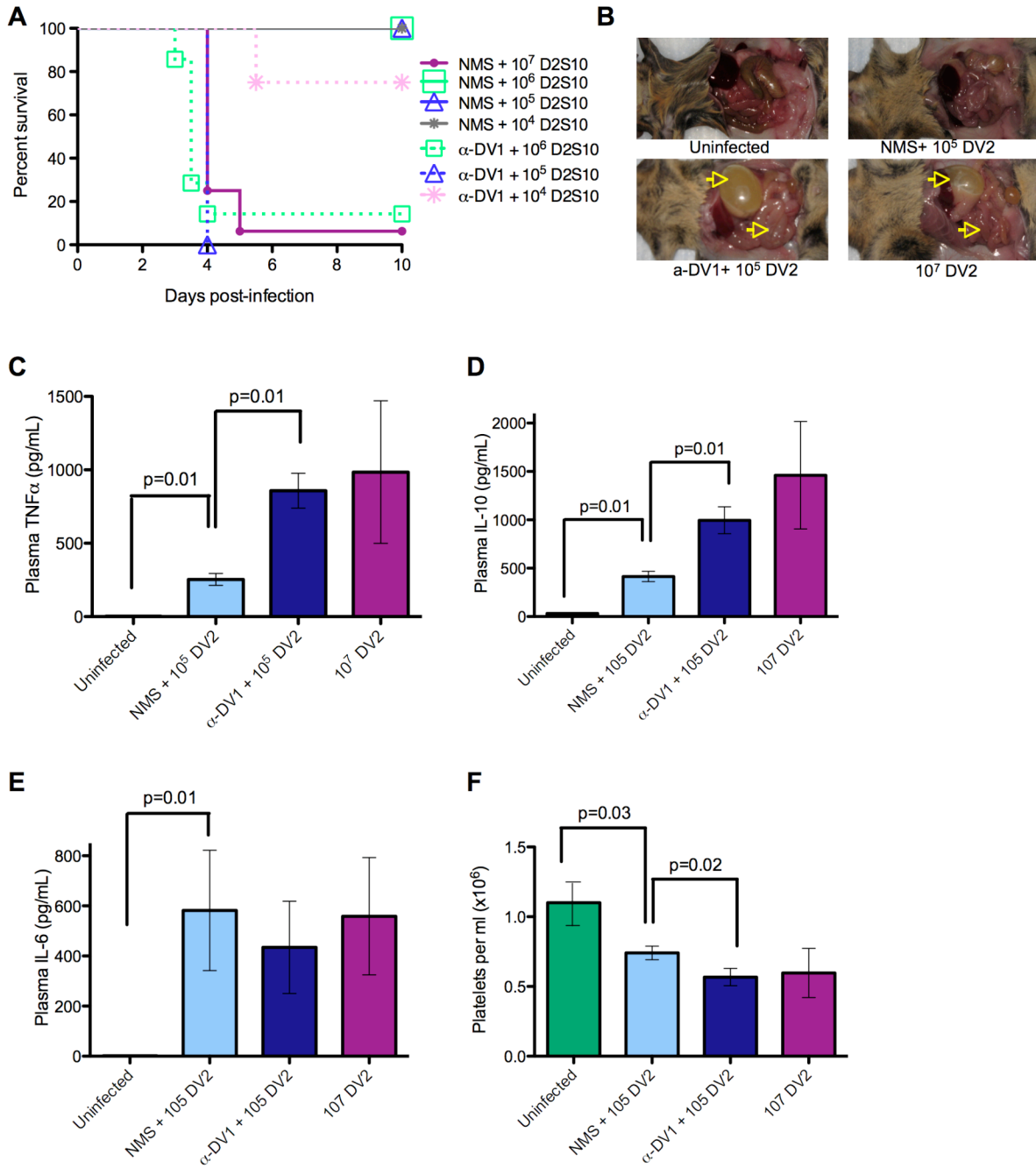


Figure 2.1 Lethal enhancement of dengue disease by anti-DV sera. **A.** Mice were administered 100ml naïve mouse serum (NMS) or anti-DV1 serum (a-DV1), challenged i.v. with the indicated dose of DV2 strain D2S10 24 hours later, and monitored for survival. Kaplan-Meier survival curves are shown; see Supplementary Table 1 for p-values. The numbers of mice per group are as follows: NMS + 10^7 D2S10, 15; NMS + 10^6 D2S10, 8; NMS + 10^5 D2S10, 11; NMS + 10^4 D2S10, 4; α 98J + 10^6 D2S10, 12; α 98J + 10^5 D2S10, 4; α 98J + 10^4 D2S10, 4. **B-F.** Disease parameters were compared in mice receiving no virus, 10^7 pfu DV2, or 10^5 pfu DV2 after transfer of naïve or anti-DV1 serum. **B.** Vascular leak-associated fluid accumulation in visceral organs at day 3.5 post-infection (p.i.). **C-E.** Serum levels of TNF- α (c), IL-10 (d), and IL-6 (e) in infected mice at day 3.5 p.i. were measured by ELISA (eBioscience). **F.** Platelet counts in mice at day 3.5 p.i. were determined using a hemacytometer. In (c-f), n=4 in all groups except the uninfected group in panel f, where n=12. Error bars represent standard deviations, and two-sided Wilcoxon rank sum tests were used to determine statistical significance

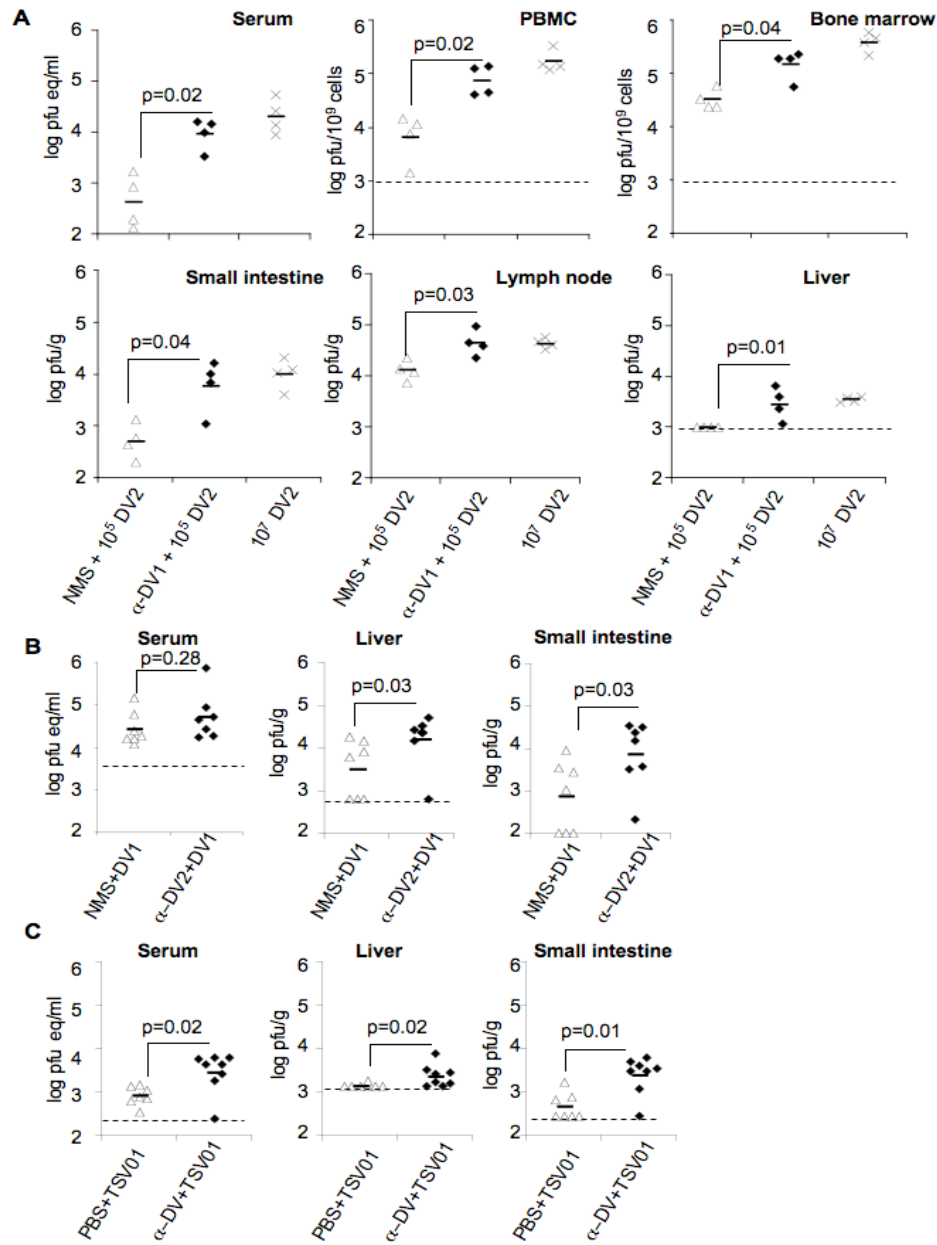


Figure 2.2 Enhancement by heterologous anti-DV antibodies increases systemic viral burden of mouse-adapted and clinical isolates of DV. **A.** Mice were administered 100 μ l NMS or anti-DV1 serum i.p. and infected 24 hours later with 10^5 pfu DV2 D2S10 i.v.; lethal infection controls were infected i.v. with 10^7 pfu DV2 D2S10. Viral burden was measured in the indicated tissues at day 3.5 p.i. by qRT-PCR (serum) or plaque assay (all other tissues) as described in Materials and Methods. **B.** Mice were administered 100 μ l NMS or anti-DV2 serum i.p. and infected the next day with 3×10^6 pfu DV1 Western Pacific-74 i.v.. Virus burden was measured in the indicated tissues at day 3.5 p.i. by qRT-PCR (serum) or plaque assay (all other tissues) as described in the Materials and Methods. **C.** Mice were administered 20 μ g anti-DV MAb 4G2 or PBS i.p. and infected the next day with 10^6 pfu DV2 TSV01 i.v.. Virus burden was measured as in (A). Symbols indicate values in individual mice. Limits of detection are represented by dashed lines when present, or the horizontal axes. All pairwise comparisons were performed by two-sided Wilcoxon Rank Sum tests.

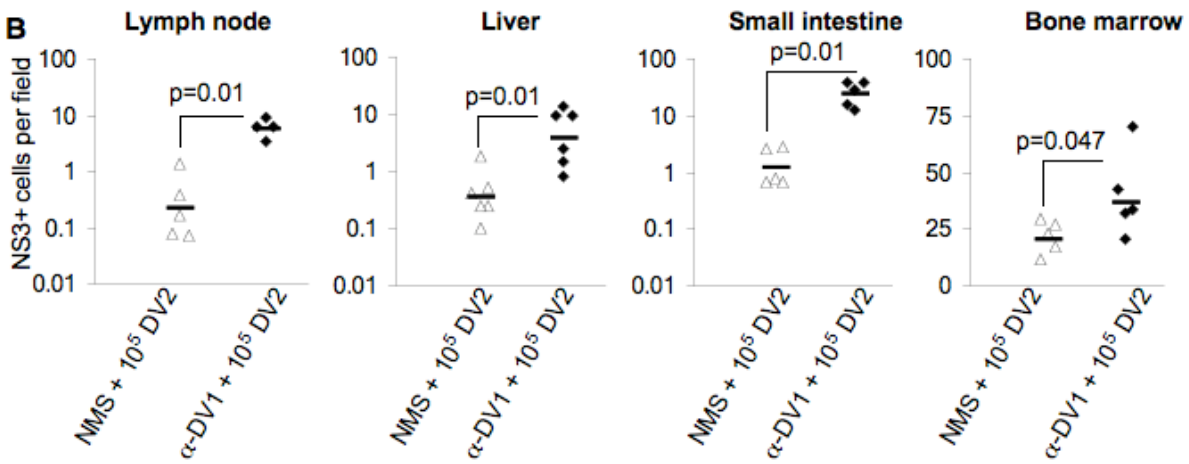
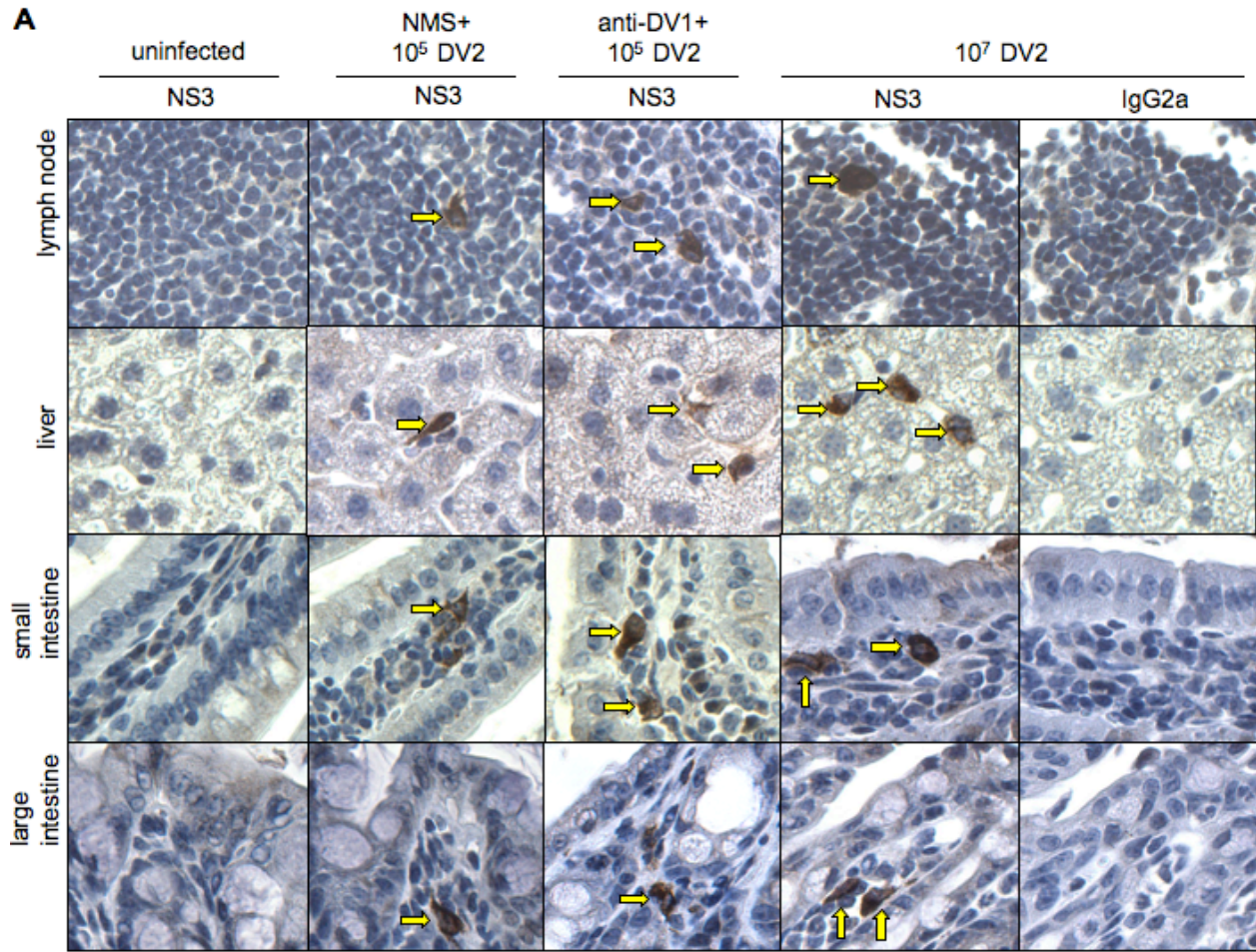


Figure 2.3 Detection and quantification of DV-infected cells with or without antibody-dependent enhancement. Mice were administered naïve serum (NMS) or anti-DV1 serum and infected i.v. with 10^5 pfu DV2 the following day. Controls were mock-infected or infected with 10^7 pfu DV2 i.v. **A.** Tissues were collected from all mice (n=3-6 per group) at day 3.5, formalin-fixed, and processed into paraffin sections. Serial sections from each tissue were stained with anti-DV NS3 antibody E1D8 (NS3) or an isotype control mouse IgG2a (IgG2a data not shown), and multiple sections of each tissue type were thoroughly examined for staining. Positive staining for NS3 is brown while hematoxylin counterstain is blue. Strong cytoplasmic staining observed with E1D8, but not IgG2a control antibody, was considered DV-specific when observed in infected mice but not uninfected controls. NS3⁺ cells in lymph node, small intestine, and large intestine exhibited morphology and location consistent with tissue macrophages under all infection conditions (arrowheads). In liver, NS3⁺ cells were consistent with tissue macrophages and/or endothelial cells. Serial sections showed the F4/80 macrophage marker staining in the same locations where infected cells were present in lymph nodes, small intestine, large intestine, and bone marrow (data not shown). **B.** NS3⁺ cells per visual field were quantified. At least ten visual fields were counted for each sample except bone marrow, where four fields from four independent sections were counted due to the small area of mouse bone cross-sections. All pairwise comparisons were performed by two-sided Wilcoxon Rank Sum tests.

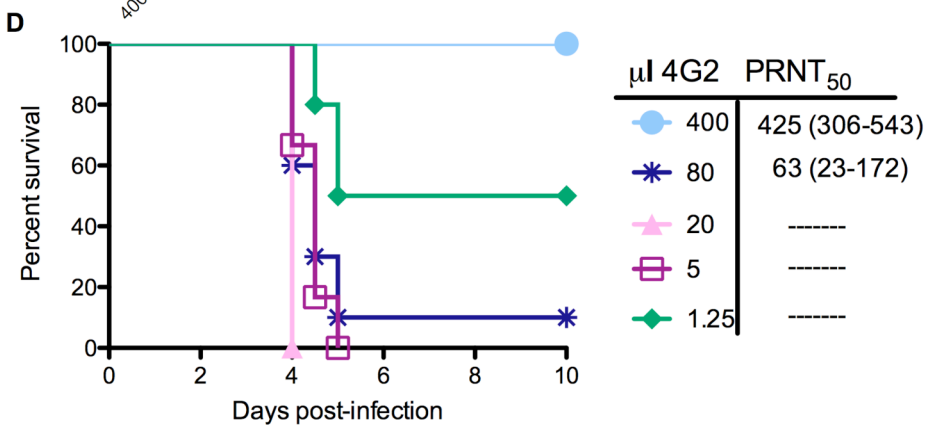
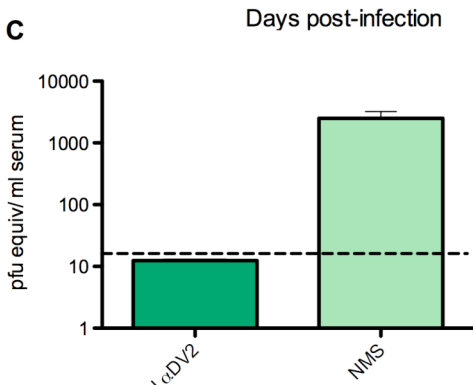
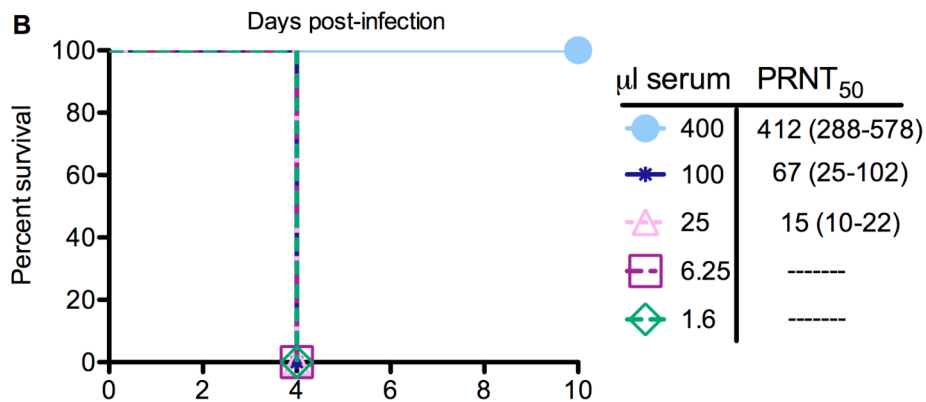
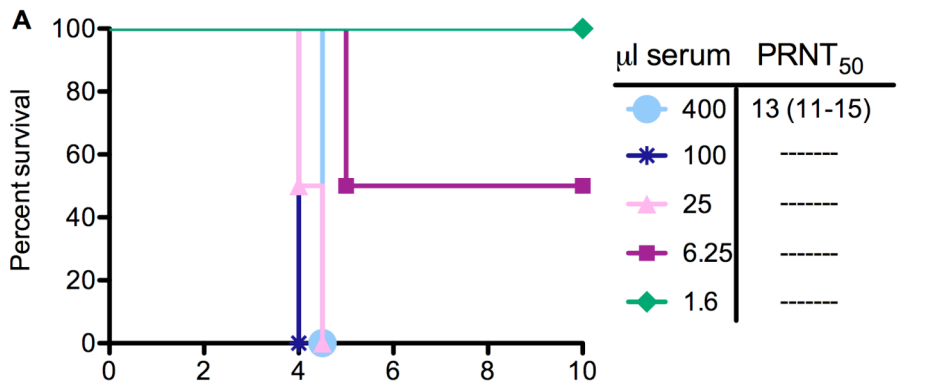


Figure 2.4 Antibody conditions for enhancement of DV infection. **A.** Mice were administered 1.6-400 μ l anti-DV1 serum i.p., and pre-infection serum samples were collected the next day. Mice were then infected with 10^5 pfu DV2 i.v. and monitored for survival. Neutralizing activity of each pre-infection serum was determined in duplicate by PRNT₅₀ assay on BHK21-15 cells. For each serum dose, PRNT₅₀ results are displayed as the average of 3 to 4 mice, with the range in brackets. **B.** Serum transfers, bleeds, virus challenges, and PRNT assays were performed as in (A), but using anti-DV2 serum generated in AG129 mice. **C.** Viremia was measured in naïve serum controls (n=4) and recipients of 400 μ l anti-DV2 serum (n=3) on day 4 p.i. by qRT-PCR. **D.** Mice were administered 1.25-400 μ g of anti-DV monoclonal antibody 4G2, and pre-infection bleeds, challenges, and PRNT assays were performed as in (A).

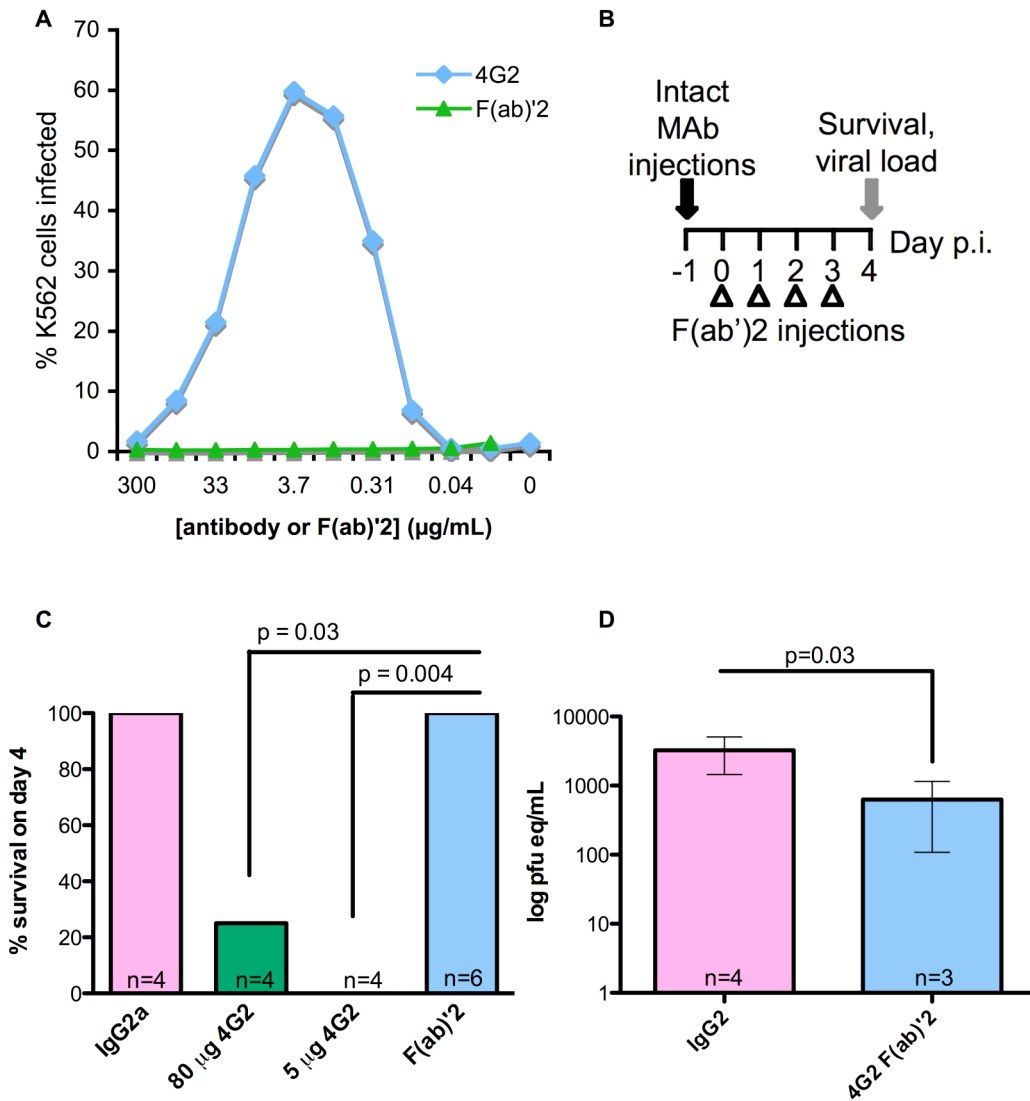


Figure 2.5 Antibodies that lack the Fc region fail to mediate ADE and instead protect against lethal challenge. **A.** Infection of K562 cells by DV2 in the presence of 4G2 MAb or 4G2 F(ab')₂ fragment was determined 48 hours p.i. by staining with Alexa488 anti-DV E protein MAb followed by flow cytometry. Average infection without antibody was 0.74%. **B.** Dosing scheme used to compare the in vivo effects of F(ab')₂ and intact MAb. Mice were administered intact 4G2 or IgG2a control MAb on day -1, or 20µg doses of F(ab')₂ every 24 hours beginning 1 hour prior to infection, and were then challenged with 10⁵ pfu of DV2 i.v. **C.** Survival in mice from **(B)** receiving the indicated antibodies was scored on day 4 post-infection, and a two-sided Fisher's exact test was used. **D.** Viremia at day 4 p.i. in surviving mice from **(C)**, measured by qRT-PCR. Error bars represent standard deviations, and pairwise comparisons were performed by two-sided Wilcoxon rank sum tests.

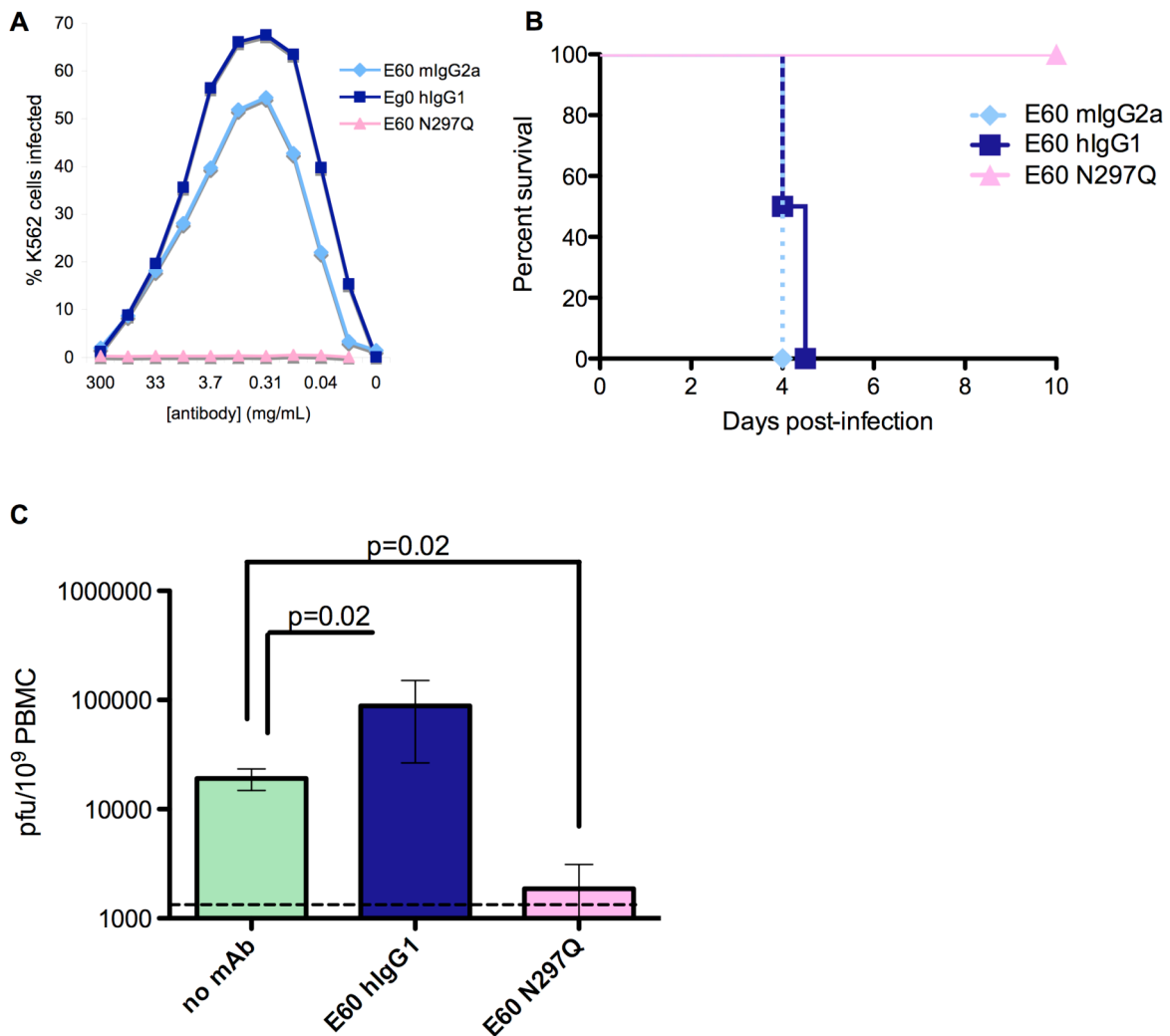


Figure 2.6. Antibodies with a mutated Fc γ R binding site cannot enhance DV infection *in vitro* or *in vivo*. **A.** Infection of K562 cells by DV2 in the presence of E60-mIgG2a, E60-hIgG1, E60-N297Q. **B.** Mice were administered 20 μ g of the indicated E60 MAbs, challenged 24 hours later with 10^6 pfu DV2 i.v., and monitored for survival (n=4 mice per group). $p=0.02$ for E60-hIgG1 versus E60-N297Q. **C.** Mice were administered E60 MAbs and virus as in (B), and viral burden in peripheral blood cells was measured by plaque assay (n= 4 mice per group).

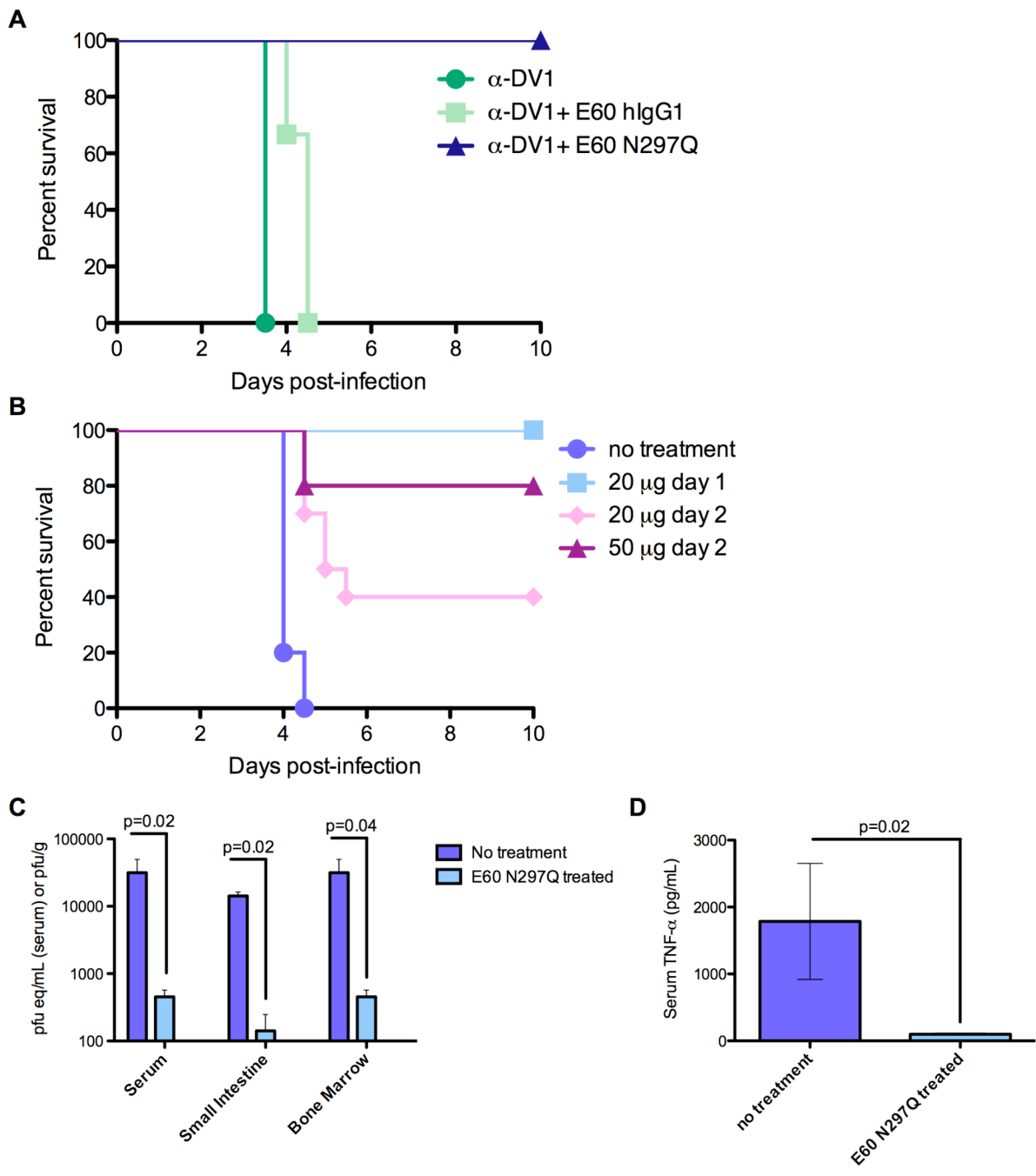


Figure 2.7 Antibodies with a mutated Fc γ R binding site have both prophylactic and therapeutic potential and reduce viral load and serum TNF- α in DV-infected mice. **A.** Mice were simultaneously administered 25 μ l anti-DV1 serum and 20 μ g of the indicated E60 MAbs i.p., challenged 24 hours later with 2×10^5 pfu of DV2 i.v., and monitored for survival (n=5 mice per group). p=0.009 for E60-hIgG1 versus E60-N297Q recipients. **B.** Mice were administered 25 μ l anti-DV1 serum i.p., challenged 24 hours later with 10^5 pfu of DV2 i.v., treated by i.v. administration of E60-N297Q at the indicated doses and days p.i., and monitored for survival. p-values compared to untreated controls (n=9) were: p=0.008 for 20 μ g E60-N297Q on day 1 p.i. (n=5); p=0.005 for 20 μ g E60-N297Q on day 2 p.i. (n=10); and p=0.02 for 50 μ g E60-N297Q on day 2 p.i. (n=5). Survival differences were compared using logrank tests. **C.** Mice were administered 25 μ l of anti-DV1 serum i.p. and infected i.v. the next day with 10^5 pfu of DV2. Mice were injected i.v. with either PBS (untreated) or 20 μ g E60-N297Q 24 hours later. On day 3.5 p.i, mice were euthanized and serum and tissues were collected. Viral burden in serum, small intestine, and bone marrow were measured by qRT-PCR for serum and plaque assay for solid tissues. **D.** Serum TNF- α was measured by ELISA. n=4 mice per group in all analyses. For viral load and TNF- α values, error bars represent standard deviations, and pairwise comparisons were performed by two-sided Wilcoxon rank sum tests.

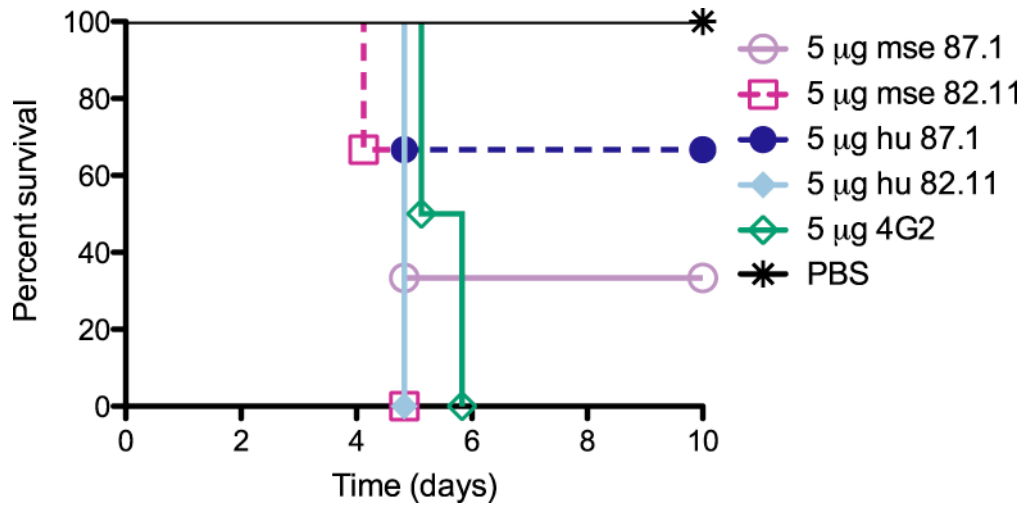


Figure 2.8 F(ab)² fragments with either mouse Igγ2A or human Igγ1 enhance a sublethal DENV2 D2S10 infection equivalently. AG129 mice (n = 3/ group) were administered 5 µg of mAb 87.1 or 82.11 F(ab)² with either mouse (mse) Igγ2A or human (hu) Igγ1 24 hours prior to a sub-lethal DENV2 D2S10 infection. Animals were monitored for morbidity and mortality for 10 days following infection.

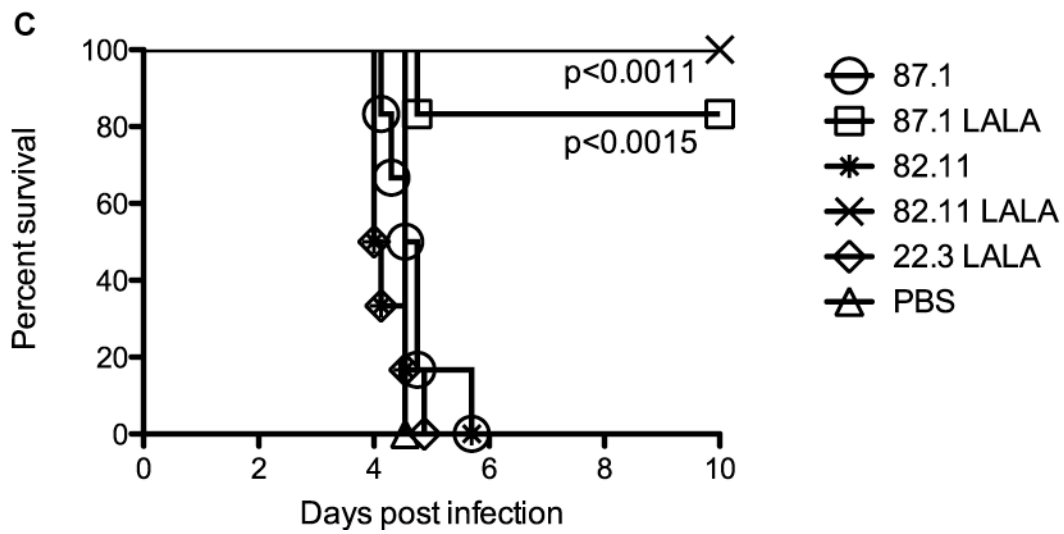
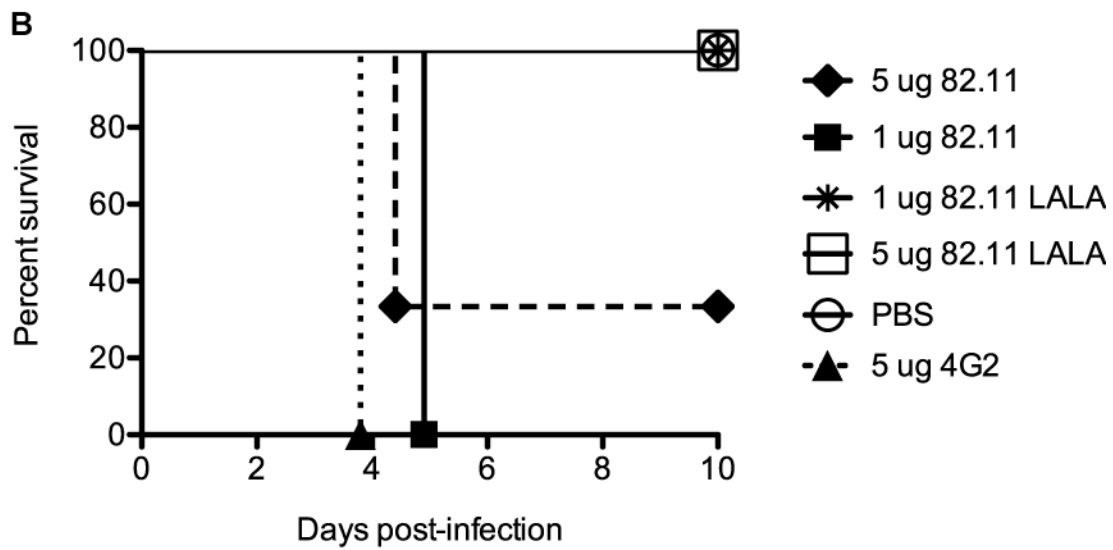
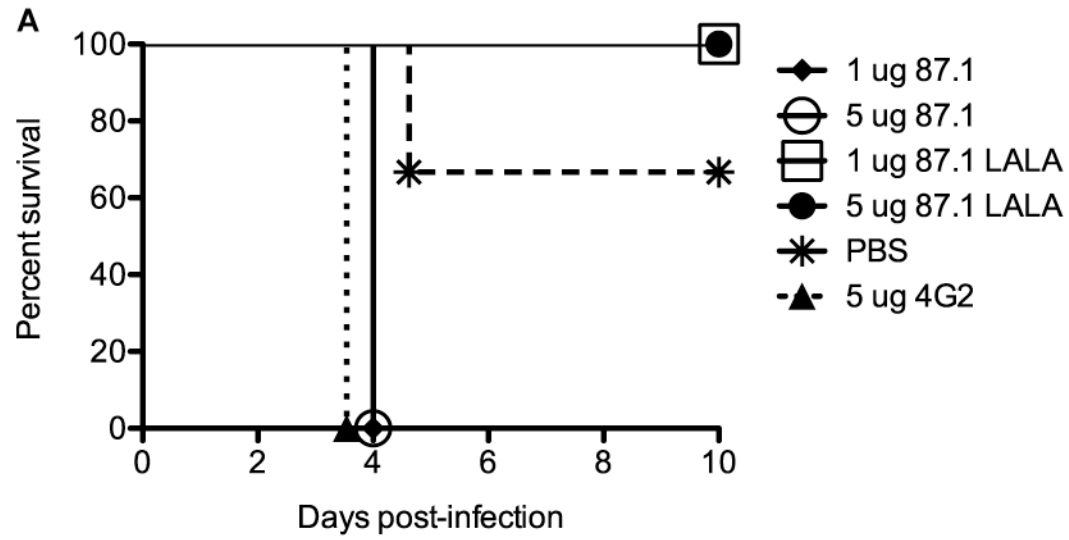


Figure 2.9 LALA variants do not enhance DENV *in vivo* and demonstrate postexposure therapeutic efficacy **A.** (Top) 1 or 5 μg of DV87.1, DV87.1 LALA variant, 4G2, or PBS were transferred i.p. in 200 μl volume into AG129 mice ($n = 3$ per group). The mice were infected 18–24 hr later with 106 pfu DENV-2 strain D2S10. (Bottom) 1 or 5 μg of DV82.11, DV82.11 LALA variant, 4G2, or PBS were transferred i.p. in 200 μl volume into AG129 mice ($n = 3$ per group). The mice were subsequently infected 18–24 hr later with 106 pfu DENV-2 D2S10. All mice receiving LALA-variant MAb survived as compared to mice receiving a lethal, antibody-enhanced infection (1 or 5 μg DV87.1 LALA, $p < 0.0253$; 1 or 5 μg DV82.11 LALA, $p < 0.0455$ compared to 4G2 MAb positive control). **B.** AG129 mice were administered 35 μl anti-DENV-1 serum i.p. and were infected 24 hr later with 105 pfu of DENV-2 D2S10 i.v. At 24 hr after infection, the mice were treated with 50 μg of either DV87.1, $n = 6$; DV87.1 LALA, $n = 6$; DV82.11, $n = 6$; DV82.11 LALA, $n = 5$; DV22.3 LALA, $n = 6$; or PBS, $n = 6$ i.p. In all cases, mortality was monitored daily for 10 days. Mice treated with either 50 μg DV87.1 LALA ($p < 0.0015$) or DV82.11 LALA ($p < 0.011$) 24 hr post infection survived significantly longer than mice receiving 50 μg of nonbinding LALA variant MAb DV22.3.

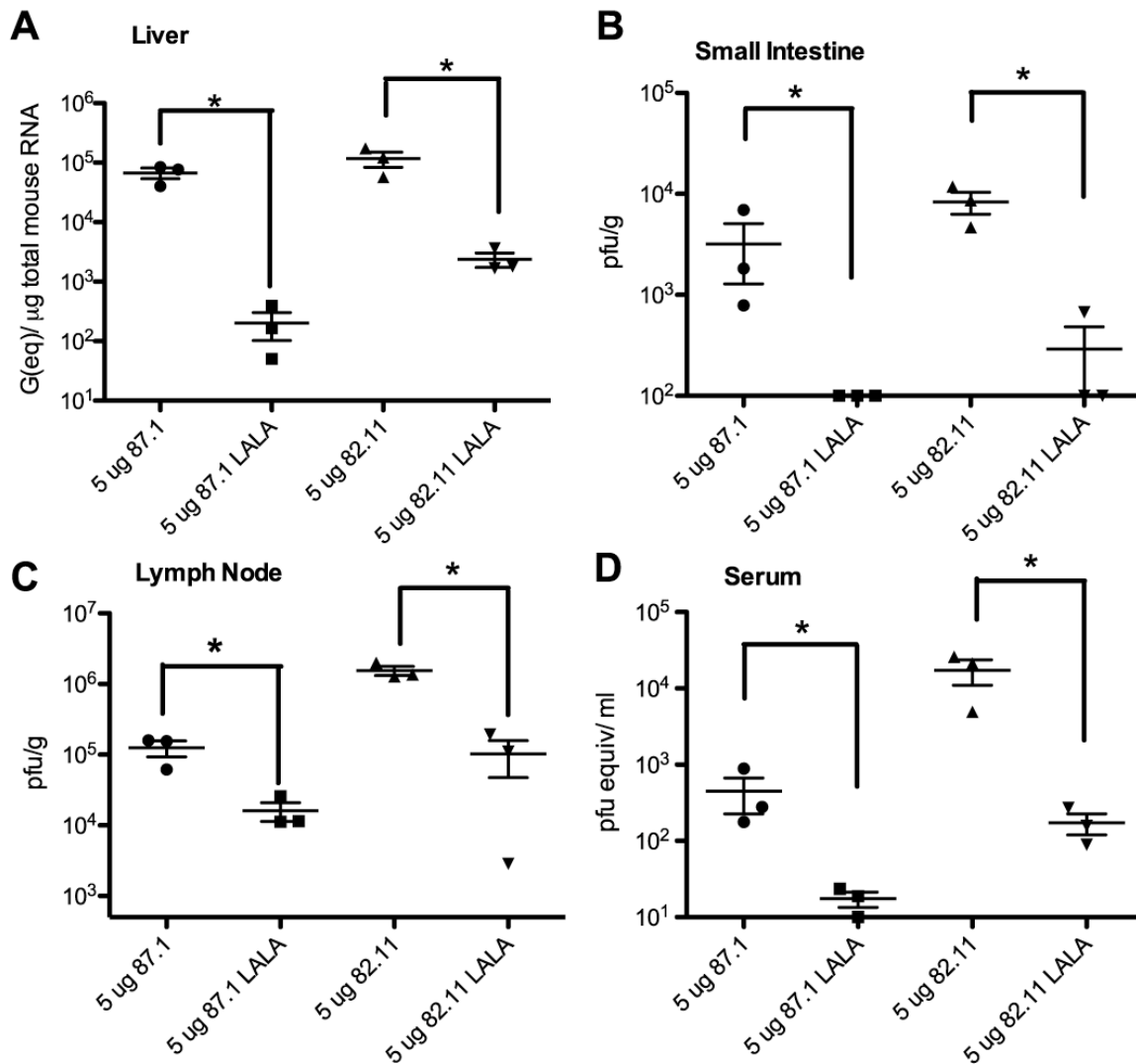


Figure 2.10 LALA variants protect against DENV2 infection by reducing viral load. AG129 mice (n=3 per group) were administered 5 g of either DV87.1, DV87.1 LALA, DV82.11 or DV82.11 LALA i.p. and infected 18-24 h later with 106 pfu DENV-2 D2S10 i.v. The animals were sacrificed at day 3.5. (A-D) Virus burden was measured in the indicated tissues by either qRT-PCR (liver and serum) or plaque assay (small intestine and lymph node). Bars indicate mean SEM. The limit of detection for each panel is the x-axis. A two sample Wilcoxon rank sum analysis was used to compare viral load values between MAb variants. An asterisk indicates a p-value <0.05 (p<0.0495 for comparisons between MAb variants in the liver, sera and lymph; p=0.0369 for comparison between DV87.1 MAb variants in the small intestine, and p=0.0463 between DV82.11 MAb variants).

Table 2.1 Effect of anti-DV1 serum and viral dose on morbidity and mortality.

Serum ^a	DV2 dose (pfu)	morbidity ^b	morbidity p-value vs. NMS control ^c	mortality	mean survival time \pm s.d. (days) ^d	mortality p-value vs. NMS control ^e
NMS ^f	10 ⁷	15/15		14/15	4.2 \pm 0.4	
NMS	10 ⁶	6/7		0/7	n.a. ^g	
α -DV1 ^h	10 ⁶	8/8	0.5	7/8	3.6 \pm 0.3	0.003
NMS	10 ⁵	0/11		0/11	n.a.	
α -DV1	10 ⁵	4/4	0.03	4/4	4.0 \pm 0.0	0.001
NMS	10 ⁴	0/4		0/4	n.a.	
α -DV1	10 ⁴	4/4	0.03	1/4	5.5	0.3

^a100 μ l of the indicated antiserum was given to all mice in each group.

^bMice were scored as morbid if hunched posture, ruffled fur, and lethargy were simultaneously present at any time during the first ten days post-infection.

^cResult of two-sided Fisher's exact test comparing α -DV1 recipients to NMS recipients at the same viral dose.

^dMean survival time of mice who succumbed to infection during 10-day timecourse.

^eResult of logrank analysis comparing anti-DV1 recipients to NMS recipients at the same viral dose.

^fNaive mouse serum.

^gNot applicable. No mortality occurred in these groups.

^hAnti-DV1 serum collected 8 weeks after infection of AG129 mice with 10⁵ pfu DV1.

Table 2.2 Morbidity and mortality with 10⁵ pfu DV2 inoculation under varying antibody conditions.

antiserum or antibody	antiserum or antibody dose ^a	morbidity ^b	morbidity p-value vs. control ^c	mortality	mean survival time \pm s.d. (days) ^d	mortality p-value vs. NMS control ^e
NMS ^f	100	0/11		0/11	n.a. ^g	
α -DV1 ^h	400	4/4	0.001	4/4	4.5 \pm 0.0	0.001
α -DV1	100	4/4	0.001	4/4	4.0 \pm 0.0	0.001
α -DV1	25	4/4	0.001	4/4	4.2 \pm 0.3	0.001
α -DV1	6.25	4/4	0.001	2/4	5.0 \pm 0.0	0.02
α -DV1	1.25	0/4	1.0	0/4	n.a.	1.0
α -DV2 ⁱ	400	0/4	1.0	0/4	n.a.	1.0
α -DV2	100	4/4	0.001	4/4	4.0 \pm 0.0	0.001
α -DV2	25	4/4	0.001	4/4	4.0 \pm 0.0	0.001
α -DV2	6.25	4/4	0.001	4/4	4.0 \pm 0.0	0.001
α -DV2	1.25	4/4	0.001	4/4	4.0 \pm 0.0	0.001
IgG2a	20	0/8		0/8	n.a.	
4G2	400	0/7	1.0	0/7	n.a.	1.0
4G2	80	10/10	0.00002	9/10	4.4 \pm 0.4	0.001
4G2	20	5/5	0.0008	5/5	4.0 \pm 0.4	0.005
4G2	5	6/6	0.0003	6/6	4.4 \pm 0.4	0.0005
4G2	1.25	5/6	0.003	3/6	4.8 \pm 0.3	0.03

^aDose of antiserum administered in μl or dose of MAb administered in μg .

^bMice were scored as morbid if hunched posture, ruffled fur, and lethargy were simultaneously present at any time during the first ten days post-infection.

^cResult of two-sided Fisher's exact test comparing antiserum recipients to NMS recipients, or 4G2 recipients to IgG2a recipients.

^dMean survival time of mice who succumbed to infection during 10-day timecourse.

^eResult of logrank analysis comparing anti-DV1 recipients to NMS recipients at the same viral dose.

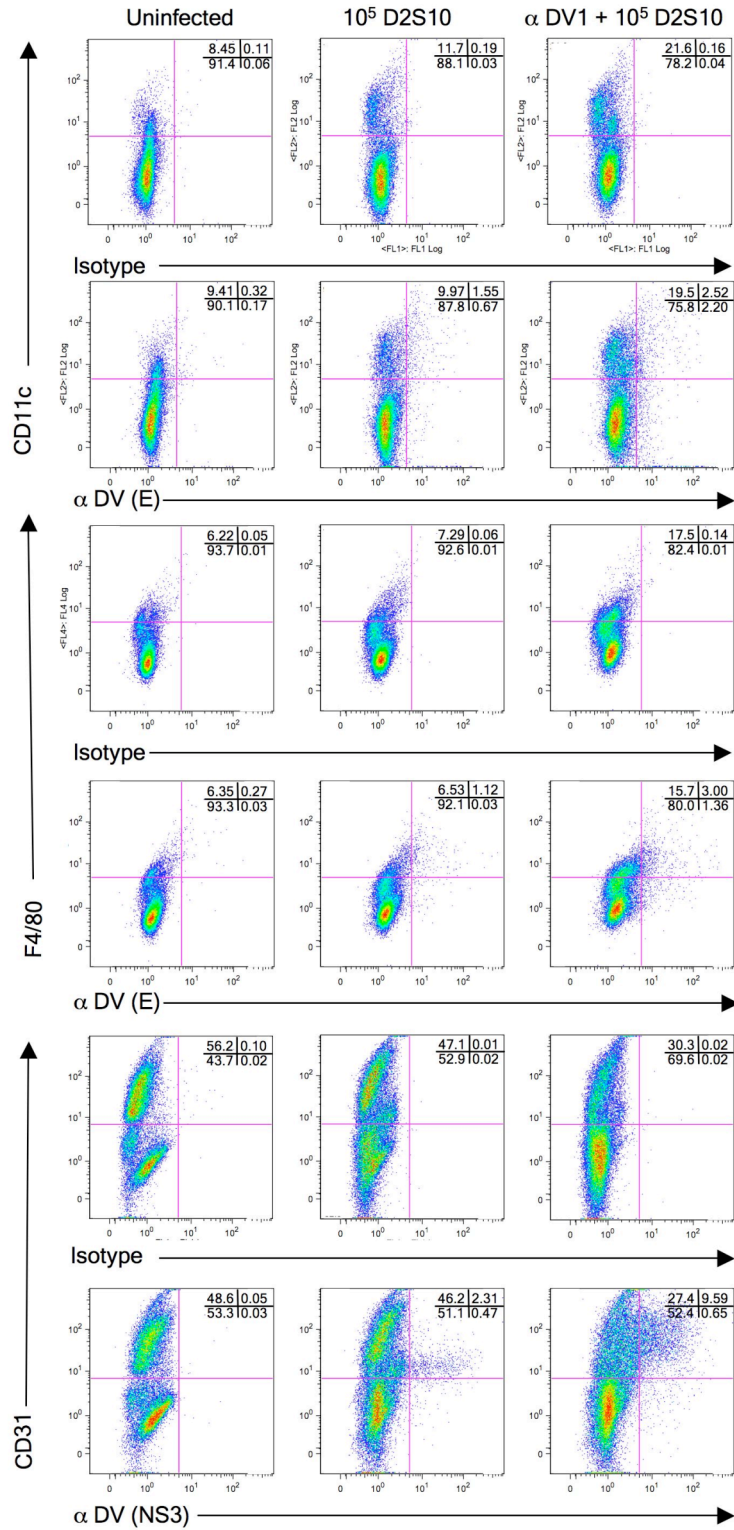
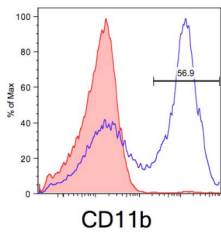
^fNaive mouse serum.

^gNot applicable. No mortality occurred in these groups.

^hAnti-DV1 serum collected 8 weeks after infection of AG129 mice with 10^5 pfu DV1 strain 98J.

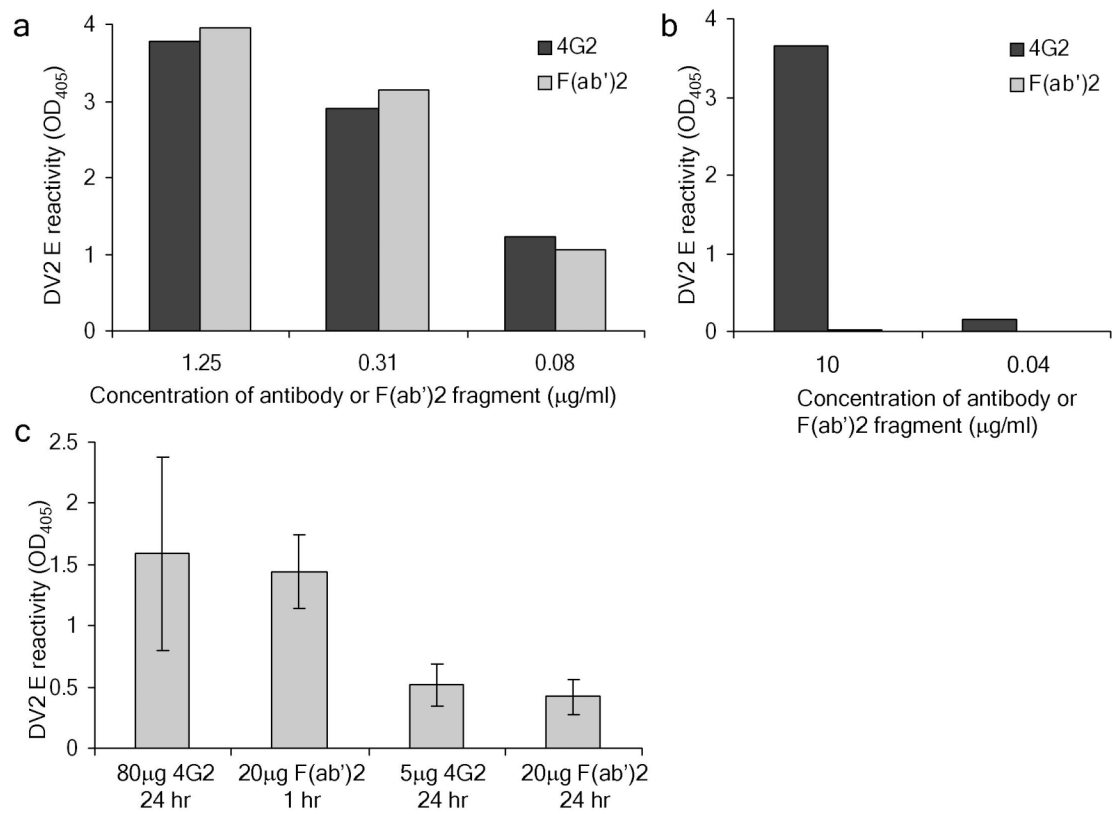
ⁱAnti-DV2 serum collected 8 weeks after infection of AG129 mice with 10^5 pfu DV2 strain PL046

a

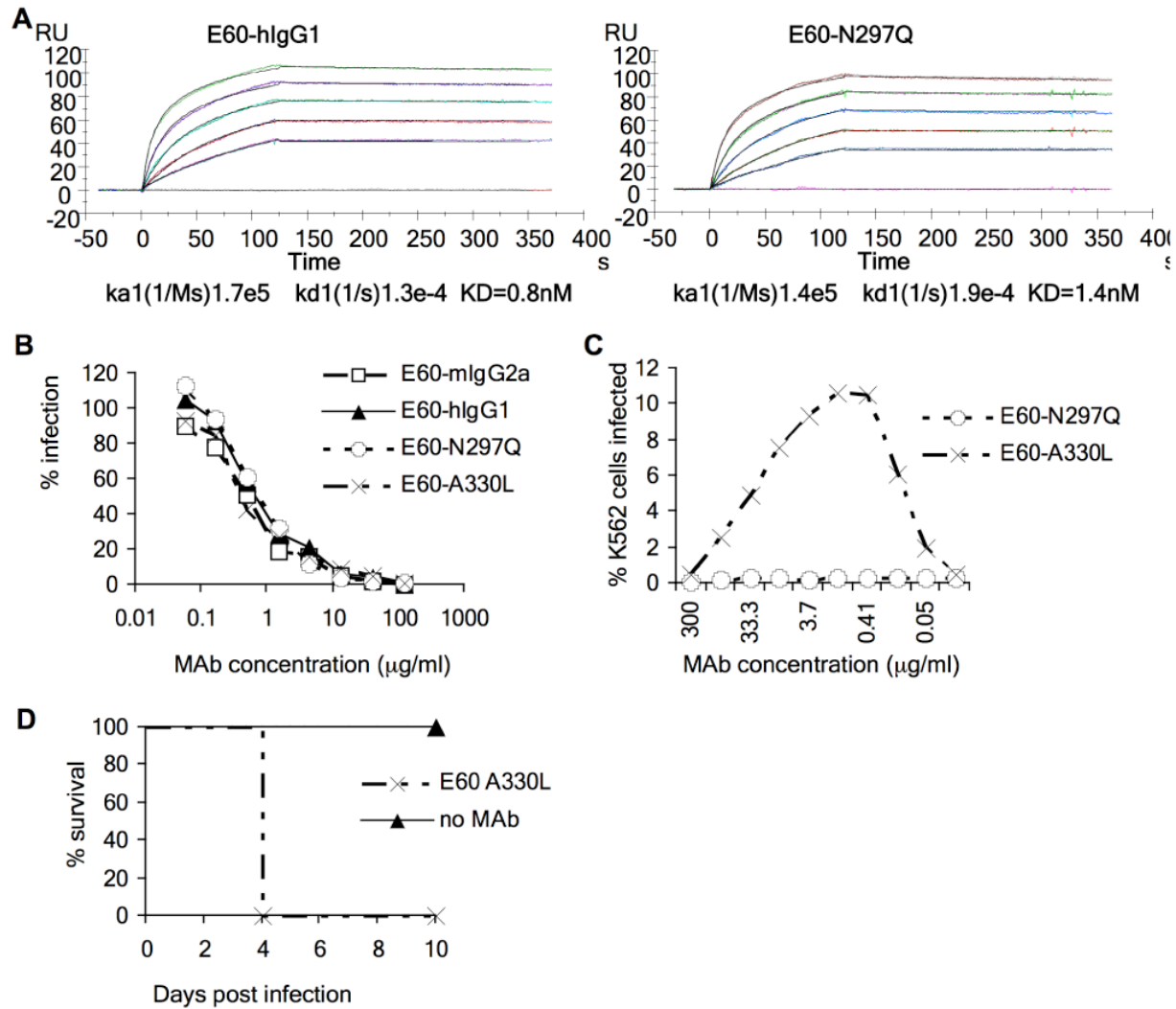


b

Supplementary Figure 2.1 Phenotyping of DV-infected cell types in bone marrow and liver under non-ADE and ADE conditions. Mice were administered naïve serum (NMS) and 24h later injected i.v. with either PBS (uninfected) or 10^5 DV2 D2S10 (non-ADE) or were infected with 10^5 DV2 D2S10 24h after receiving anti-DV1 serum (ADE). Bone marrow aspirates and livers were collected on day 3.5 p.i. **A.** The bone marrow cells were stained and collected as described in the materials and methods. The majority of DV⁺ cells were CD11b⁺ (65%); thus, cells were initially gated on CD11b. The isotype control for CD11b is depicted in pink in the initial histogram. Scatterplots of CD11b⁺ cells stained with anti-DV E or isotype control and either CD11c or F4/80 are shown for one representative animal out of six. Similar results were obtained using anti-DV NS3 MAb E1D8: of CD11b⁺ cells, 0.33%, 0.96% and 3.03% were CD11c⁺NS3⁺ in uninfected, non-ADE, and ADE conditions, respectively; and 0.39%, 0.96%, and 3.34% were F4/80⁺NS3⁺ in uninfected, non-ADE, and ADE conditions, respectively. **B.** Livers were processed and stained as described in the materials and methods. Data collection and analysis was performed as in (a). Scatterplots of cells stained with CD31⁺ and anti-DV NS3 or isotype control are shown for one representative animal out of six. Similar results were obtained with human anti-DV E.



Supplementary Figure 2.2 Characterization of F(ab')₂ fragments of 4G2. MAb 4G2 was processed into F(ab')₂ fragments using the Immunopure F(ab')₂ kit (Pierce). **A.** Intact 4G2 and purified F(ab')₂ fragments were tested for reactivity against purified DV2 E protein (Hawaii Biotech Inc.) by ELISA and detected with anti-F(ab')₂-specific antibody. **B.** ELISA was performed as in (A), but with detection antibody specific for the Fc portion of mouse IgG. **C.** Mice were administered 4G2 i.p. at doses shown to enhance infection *in vivo* (5 or 80 µg), and serum was collected 24 hours later. 4G2 F(ab')₂ fragments were administered to mice i.v. and serum collected 1 and 24 hours later. Serum levels of intact 4G2 and F(ab')₂ fragment were measured by reactivity to DV2 E protein by ELISA using anti-F(ab')₂-specific antibody.



Supplementary Figure 2.3 Further characterizations of E60 antibody variants.

A. Monoclonal antibodies at a concentration range of 12.5-200nM were injected over the surface of a Biacore 3000 with immobilized E protein (~300RU) at a flow rate of 30ml/min for 120sec and a dissociation time of 180 sec. Binding curves at concentration zero were subtracted as blank. Kinetic parameters were calculated by fitting binding curves to a bivalent analyte binding model. The kinetic parameters are similar for binding of both MAb variants to E protein, as the difference between affinities is less than two-fold. **B.** Neutralizing activity of E60 variants on DC-SIGN-expressing U937 cells. DV2 was incubated with the indicated concentrations of each E60 variant MAb, applied to U937 cells expressing the DV attachment receptor DC-SIGN, and the percentage of cells infected with DV was assessed 24 hours later by flow cytometry staining with Alexa488-labelled anti-DV E protein MAb. **C.** E60 A330L enhances DV infection *in vitro*. Enhancement assays were performed as in Figure 2.6A using E60-A330L. E60-N297Q is shown for comparison. **D.** E60 A330L enhances DV infection *in vivo*. Mice were administered 20 μg E60-A330L or PBS (no MAb) i.p, then infected 24 hours later with 10⁶ pfu DV2 i.v. Survival was monitored for 10 days. n=4 for E60-A330L recipients.

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CHAPTER 3

MECHANSTIC ANALYSIS OF THERAPEUTIC EFFICACY OF AGLYCOSYLATED ANTIBODIES FOLLOWING LETHAL DENGUE VIRUS INFECTION

INTRODUCTION

Dengue, caused by the four serotypes of dengue virus (DENV1-4) is a mosquito-borne infectious disease found predominantly in tropical and sub-tropical regions of the world [1,2]. Symptoms associated with DENV infection can range from inapparent infections to classic dengue fever, a debilitating self-limited disease, to dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), characterized by vascular permeability and hypotensive shock [3]. In some Southeast Asian countries with high rates of co-circulation of different DENV serotypes, dengue is a major cause of pediatric hospitalization. In the absence of supportive care, fatality rates can reach 20-30% [1,4,5,6]. While several tetravalent vaccines [4,6,7] are currently in early stages of clinical evaluation, no vaccine has been licensed to date and no anti-viral therapeutic has been approved to treat DENV-induced disease in a clinical setting.

Epidemiological analysis has identified a previous DENV infection as the greatest risk factor for the development of severe disease upon subsequent infection with a different DENV serotype [8,9,10,11,12]. Infection with one serotype provides life-long protection against re-infection with the same serotype but may not protect against re-infection with a different serotype [13,14]. Only 60-70% amino acid homology exists among the four DENV serotypes [15]. Thus, while the adaptive immune response responds to a second DENV infection, it may lack the specificity to adequately neutralize the second infection, as it was originally developed against the serotype causing the first infection. This theory, termed “original antigenic sin”, offers an explanation for the increased risk of severe disease during secondary DENV infections [16,17]. Due to a number of factors including geographic expansion of the DENV vectors *Aedes aegypti* and *Aedes albopictus* and increased global urbanization, trade and travel, [18,19], there has been a substantial increase in not only the incidence of new dengue epidemics but also co-circulation of the four DENV serotypes in the same region [20]. Over the past 35 years, the co-circulation of all four serotypes of DENV has expanded to 60 countries and has resulted in an increased number of epidemics of severe disease in dengue-endemic regions previously known for outbreaks of only mild disease [1,21,22,23,24].

DENV is a member of the flavivirus genus and a close relative of notable arboviral pathogens such as West Nile Virus and Yellow Fever Virus [15,25]. DENV has a 10.7-kb, positive-sense RNA genome composed of 3' and 5' untranslated regions that flank a polyprotein encoding three structural and seven non-structural proteins [26]. Of the three structural proteins, both the pre-membrane (prM/M) protein and the envelope protein (E) have been identified as the main antigenic targets of the humoral immune response in humans [27,28,29]. The E protein is composed of three structural domains, Domains I (EDI), II (EDII) and III (EDIII) [30,31,32,33], where EDII contains the fusion peptide [34] and EDIII contains viral receptor binding sites [35,36]. Recent investigations have identified a number of new epitopes on the E protein, the most potently neutralizing of which have been mapped to EDIII lateral ridge and A strand epitopes [37,38].

While both the humoral and cellular arms of the adaptive immune response have been reported to contribute to the development of severe dengue disease, antibodies alone are sufficient to enhance DENV disease *in vitro*, a concept referred to as “antibody-dependent enhancement” (ADE) [39]. In an *in vivo* model of ADE, passive transfer of both monoclonal antibodies as well as polyvalent serotype-cross-reactive serum, when administered at sub-neutralizing concentrations, is sufficient to enhance a sub-lethal dose of a mouse-adapted strain of DENV2, D2S10, in interferon α/β and γ -receptor deficient (AG129) mice [40,41]. The

resulting lethal antibody-enhanced disease is characterized by significantly increased viremia and tissue viral load, elevated pro-inflammatory cytokine levels, and vascular permeability resulting in fluid accumulation in the stomach and small intestine three to five days following infection [40,41]. Recently, our group reported that aglycosylated monoclonal antibodies (mAbs) targeting both the EDII fusion loop (E60 N297Q [40]) and the EDIII A strand (87.1 LALA and 82.11 LALA [27]) that were genetically engineered to eliminate the glycosylation site required for FcR binding, when administered 24 hours following an antibody-enhanced DENV2 infection were sufficient to therapeutically prevent lethal disease and significantly reduce viral load *in vivo*. In this chapter, we expand upon these results and evaluate the ability of a panel of aglycosylated monoclonal antibodies targeting different epitopes on the E protein to act therapeutically following both a virus-only as well as an antibody-enhanced lethal infection. To this end, we identify relevant antibody characteristics that correlate with the ability of aglycosylated antibodies to act therapeutically, and we develop an *in vitro* assay to predict therapeutic efficacy following an antibody-enhanced DENV infection *in vivo*.

RESULTS

Wildtype mAbs act prophylactically to prevent antibody-enhanced lethal disease.

Severe forms of DENV infection, including dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) can be fatal if not properly treated. Supportive fluid treatment is the recommended standard of care for treating DHF/DSS patients, and no therapeutic is currently licensed for use. In this study, we extended previous observations of the prophylactic and therapeutic efficacy of mAb E60 (anti-EDI/II fusion loop-specific mAb) [40] by studying a larger panel of monoclonal antibodies targeting additional epitopes located on the surface of the E protein homodimer, including the fusion loop and dimer interface on EDII and the C-C' loop and A strand on EDIII (**Figure 3.1**).

Previous studies indicated that mAb E60 targeting the fusion loop was prophylactic in the presence of an antibody-enhanced lethal infection as a non-modified mAb [40]. To test whether this efficacy was unique to mAb E60, we assessed the prophylactic efficacy of a number of mAb targeting different epitopes and spanning a wide range of neutralizing potency (**Table 3.1**). When administered at a dose of 50 µg concurrently with an enhancing amount of DENV1-immune mouse serum, most non-modified mAb were prophylactic with the exception of 82.11, which only protected 50% of animals tested (Table 2). When 20 µg of wildtype mAb were tested prophylactically, mice receiving E28 were not protected, while mice receiving E44, E76, E87, E60 and 87.1 were protected. As a control, we also tested mAb 4G2, a group-reactive mAb that is modestly neutralizing *in vitro* (393 ng/mL) and can be enhancing *in vivo* over a range of concentrations from 2-80 µg. When administered concurrently with an enhancing dose of polyvalent DENV1-immune serum, 100 µg of 4G2 was completely protective, while a two-fold lower dose, 50 µg, was not protective (**Table 3.2**). These data demonstrate that even moderately neutralizing mAb can be prophylactically protective in the presence of an enhancing volume of DENV-immune serum and possibly imply a synergistic interaction with the antibodies found in DENV-immune serum. These *in vivo* data support the *in vitro* hypothesis [42,43] that antibodies bind and prevent infection when present at a stoichiometry sufficient to neutralize the flavivirus infection, even in the presence of enhancing concentrations of antibody. Indeed, neutralizing potency appears to directly correlate with prophylactic efficacy, as only moderately neutralizing

mAb (NT₅₀ of ≥ 150 ng/mL) failed to protect when administered at 20 or 50 μ g ($p < 0.0253$) as compared to strongly neutralizing mAb (NT₅₀ < 150 ng/mL). While an important validation of *in vitro* stoichiometrical observations, prophylactic prevention of disease is not a viable option when considering human DENV infection. Therefore, we next studied the therapeutic ability of the panel of mAbs when administered following lethal DENV challenge.

Aglycosylated mAbs universally prevent virus-only lethal disease but not antibody-enhanced lethal disease

Although epidemiological studies have identified a prior infection with a different DENV serotype as the greatest risk factor for the development of severe disease, severe clinical manifestations have also been reported following a primary infection [44]. Previous data have indicated that wildtype mAbs cannot act therapeutically following an antibody-enhanced, lethal disease [40]. Therefore, we focused solely on the ability of aglycosylated mAb to act as a therapeutic agent. In order for a non-FcR-binding mAb to be a versatile therapeutic, these mAb must be functionally therapeutic following both a virus-only and an antibody-enhanced lethal DENV infection. To assess the ability of each mAb to protect against a lethal DENV infection, mice were infected with a lethal dose of DENV2 D2S10 and 24 hours later, administered 20 μ g of each non-FcR-binding mAb. All of the non-FcR-binding mAbs tested prevented development of overt disease and protected against death (**Figure 3.2A, Table 3.3**). We subsequently tested whether the same mAb would be able to protect against an antibody-enhanced lethal DENV infection. To this end, we passively administered anti-DENV1 serum 24 hours prior to a sub-lethal infection of DENV2 D2S10 and treated the animals 24 hours following infection with 20 μ g of each non-FcR-binding mAb. Of all the mAb tested, only E60 N297Q ($p < 0.001$ as compared to non-treated mice) and 87.1 LALA ($p < 0.01$ as compared to non-treated mice), targeting the fusion loop and A-strand, respectively, prevented mortality (**Figure 3.2B and Table 3.3**). MAb E76 N297Q ($p < 0.05$ as compared to PBS-treated mice) and 82.11 LALA ($p = 0.189$ as compared to PBS-treated mice) resulted in 67% and 50% survival, respectively. MAb E44 N297Q and E87 N297Q did not prevent the development of lethal disease (**Figure 3.2B, Table 3.3**).

Not all mAbs targeting the fusion loop demonstrate therapeutic efficacy following antibody-enhanced lethal disease.

To determine why certain non-FcR-binding mAbs were efficient therapeutically following a virus-only lethal infection but not an antibody-enhanced infection, we examined a number of different *in vitro* characteristics of each mAb, including epitope specificity, neutralization potency and mAb avidity. As E60 N97Q has previously been demonstrated to be completely therapeutic 24 hours following DENV infection and protective in a dose-dependent fashion when administered 48 hours following infection [40], we hypothesized that the fusion loop epitope might be an important target for therapeutic mAbs. Therefore, we tested two additional aglycosylated mAbs, E18 and E28 that also target the fusion loop to determine their therapeutic efficacy following both a lethal and an antibody-enhanced DENV infection. Whereas all of the animals treated with mAb E18 and E28 after a virus-only lethal infection survived ($p < 0.01$ for both E18 N297Q and E28 N297Q as compared to PBS-treated mice, **Figure 3.3A and Table 3.4**), none of the animals treated with aglycosylated mAbs E18 and E28 following an antibody-enhanced infection demonstrated any difference from the PBS-treated control group (**Figure 3.3B, Table 3.4**). These data demonstrate that while all mAbs targeting the fusion loop

can be effective following a virus-only lethal infection, additional antibody characteristics other than epitope specificity must determine the ability of these mAbs to act therapeutically following an antibody-enhanced lethal infection.

Neutralization potency correlates with therapeutic efficacy within but not across epitopes.

Neutralization potency was next examined as a potentially critical characteristic determining *in vivo* therapeutic potential of aglycosylated mAbs. The neutralization titer of each of the mAbs was assessed via a flow cytometry-based neutralization assay using human monocytic U937 cells expressing the DC-SIGN DENV attachment factor [45] against the infecting DENV2 D2S10 serotype, and the neutralization potency was expressed as the mAb concentration required to neutralize 50% *in vitro* infection (NT₅₀). While E60 N297Q demonstrated an NT₅₀ titer of 72 ng/mL, E18 N297Q and E28 N297Q were 5 to 8-fold less potent, with NT₅₀ titers of 371 and 544 ng/mL, respectively (Table 3). Thus, examining solely mAbs targeting the fusion loop epitope, neutralization potency appeared to be an important correlate of therapeutic efficacy. However, comparison of NT₅₀ titers between effective and non-effective therapeutic mAbs targeting different epitopes failed to demonstrate a clear relationship between neutralizing potency and therapeutic efficacy for antibody-enhanced lethal infections (**Tables 3.1 and 3.4**). Indeed, the NT₅₀ titer of E60 N297Q was 72 ng/mL, while the non-therapeutic aglycosylated mAbs E44 and E87 had comparable NT₅₀ titers of 68 ng/mL and 59 ng/mL, respectively (**Tables 3.1 and 3.3**). Analysis of the neutralizing potency of aglycosylated variants did not support a relationship between NT₅₀ titer and survival ($p = 0.101$) after administration in the context of a lethal antibody-enhanced DENV infection.

MAB avidity correlates with neutralization potency but not therapeutic efficacy *in vivo*.

The strength of binding between a single F(ab) region and its ligand, defined as a mAb's affinity, are critical components of a mAb's neutralizing potency. Therefore, we next hypothesized that mAb avidity, the strength of binding between multiple ligands and bivalent antibodies, may contribute to therapeutic efficacy following an antibody-enhanced, lethal infection. To test this hypothesis, we performed biochemical analysis using a direct, virion-coated ELISA to determine the dissociation rate (K_d) as a measure of a mAb's avidity. We then analyzed whether a correlation existed between the K_d and therapeutic efficacy of each mAb. Initial analysis of mAbs targeting the fusion loop identified a potential relationship between dissociation rate and therapeutic efficacy, as MAb E18 N297Q and E28 N297Q, neither effective therapeutically, demonstrated higher dissociation rates than the therapeutically effective MAb E60 N297Q that also targeted the fusion loop. However, further analysis of the dissociation rates of mAbs targeting alternative epitopes did not support a clear relationship between K_d and therapeutic efficacy (**Table 3.1**, $p = 0.83$).

Aglycosylated mAbs E60 and 87.1 neutralize DENV via different mechanisms

Antibodies can inhibit DENV infection by blocking prior to the virus attaching to the cell (pre-attachment), or after the virus has already bound to the cell (post-attachment) [46]. To determine whether the therapeutically effective mAbs neutralized DENV in a similar fashion, we performed pre- and post-attachment neutralization assays [35,47,48] using U937 cells expressing DENV attachment factor DC-SIGN. A concentration of mAb (10 µg/mL), at least 20-fold greater than the least potent mAb NT₅₀ titer, was selected for this experiment. In the pre-attachment assay, mAb and virus were incubated together at 4°C prior to infection of U937 DC-SIGN cells

at 37°C. In the post-attachment assay, virus and cells were first incubated together at 4°C and then incubated with mAb at 4°C for an additional hour. A significant decrease in the neutralizing ability of the serum between the pre- and post-attachment assays would suggest that the mAb must be able to interact with the virion prior to incubation with cells to successfully neutralize the infection. If there is no change observed between the two assays, this could be interpreted as the mAb can bind to its epitope even after the virus has attached to cells and begun to fuse with the endosomal membrane and still prevent infection. Comparison to the standard neutralization protocol completed normally at 37°C was used as an internal control. While none of the mAbs demonstrated large changes comparing neutralization at 4°C or 37°C in the pre-attachment assay, neutralization by three mAbs was eliminated in the post-attachment assay. MAb E44, 82.11 and E60 targeting the dimer interface (E44) and fusion loop (82.11 and E60), were no longer able to neutralize infection when virus and cells were pre-incubated. While all three of these mAbs bind to epitopes on EDII and appear to share a pre-attachment neutralization mechanism, they nonetheless exhibited very different levels of therapeutic efficacy. In contrast, neutralizing potency of mAb 87.1 was only slightly impaired in the post-attachment assay, indicating that this mAb neutralizes through a post-attachment mechanism. Taken together this data suggests that therapeutically effective mAbs can neutralize DENV via different mechanisms (**Figure 3.4**).

***In vitro* competition assay correlates with *in vivo* efficacy following murine polyclonal serum-enhanced infection**

As only some mAbs demonstrated therapeutic efficacy following an antibody-enhanced lethal infection but all mAbs tested were efficacious following a virus-only lethal infection, we next hypothesized that the polyvalent serum passively administered to promote enhancement was interfering with the neutralizing ability of the non-therapeutically effective mAb E44 and E87. To determine if polyvalent serum was out-competing aglycosylated antibodies for binding to the virion, we designed an assay to mimic the interaction of DENV-immune serum and non-FcR binding mAbs *in vivo*. Using murine DENV1-immune serum, we generated enhancement curves in K562 cells, an erythroleukemic cell line that expresses FcγRIIA and is not naturally permissive for DENV infection in the absence of anti-DENV antibodies, and identified the serum dilution responsible for peak enhancement (**Figure 3.5A**). We next tested the ability of aglycosylated mAbs to reduce enhancement *in vitro* by first incubating the virus with mouse serum at its peak enhancing titer for 30 minutes, followed by addition of a dilution series of each aglycosylated mAb for 30 minutes, followed by incubation with K562 cells for 48 hours. Importantly, the concentrations of both immune serum and virus were comparable between the *in vitro* system and *in vivo* infections. The volume of anti-DENV1 serum administered *in vivo* is 0.025 mL, resulting in an approximate dilution of 1:110, assuming the total blood volume in a 25g-AG129 mouse to be 2.5 mL. In comparison, the two dilutions of serum used to generate the peak enhancement were 1:180 and 1:540. Similarly, the concentration of virus used *in vivo* is estimated to be 4×10^4 pfu/mL, while the virus concentration used *in vitro* was 8.3×10^4 pfu/mL.

Aglycosylated mAbs were tested beginning at 2000 ng/mL, a concentration at least 4-fold higher than the NT₅₀ titer of the least potently neutralizing mAb. At concentrations of both 2000 ng/mL and 1000 ng/mL, the aglycosylated mAbs that were moderately to strongly therapeutic *in vivo* (>60% protection) were significantly more efficient at blocking enhancement *in vitro* than mAbs that were weakly to completely ineffective as therapeutics ($p < 0.03$ for both 2000 ng/mL and 1000 ng/mL concentrations) (**Figure 3.5B**). Specifically, the three most effective therapeutic

mAbs, 87.1 LALA, E60 N297Q and E76 N297Q, demonstrated 86%, 75% and 70% reduction of enhancement, respectively, at the highest concentration of aglycosylated antibody tested against an enhanced infection with 1:180 dilution of mAb. In contrast, mAbs 82.11 LALA, E44 N297Q and E87 N297Q reduced enhancement by 35%, 37% and 52%, respectively under the same experimental conditions (**Figure 3.5B**). This data was recapitulated when the DENV1-immune serum was used at a dilution of 1:540 (**Figure 3.5C**).

Aglycosylated mAb can prevent enhancement by human DENV-immune serum *in vitro*.

We next tested whether the *in vitro* competition assay would be able to predict the efficacy of therapeutic mAbs following an antibody-enhanced infection using human DENV-immune serum *in vivo*. To test this hypothesis, we selected a primary DENV1 (**Figure 3.6A, 3.4B**) and primary DENV3 (**Figure 3.6A, 3.6E**) human immune serum sample, both collected 3 months following infection, and a primary DENV4 (**Figure 3.6A, 3.6D**) human immune serum sample collected years after infection and tested the ability of the aglycosylated mAbs to reduce enhancement *in vitro*. We first identified the peak enhancement titer for each primary immune serum sample (**Figure 3.6A**), and then used that serum dilution to test the ability of aglycosylated mAbs to reduce enhancement *in vitro*. As with the mouse immune serum, we identified a dilution of 1:180 and 1:540 as the two dilutions of serum yielding peak enhancement. Initial observations indicated that the two aglycosylated mAbs previously identified as therapeutic following a murine serum-enhanced infection, E60 N297Q and 87.1 LALA, both demonstrated similar potency in reducing enhancement *in vitro* following an enhanced infection generated with human immune serum. An additional antibody, 82.11 LALA, was also identified as protective against an infection enhanced with human DENV-immune serum *in vitro*. While moderately protective both *in vitro* and *in vivo* against infections enhanced with murine DENV-immune serum, mAb E76 N297Q did not appear to be protective against an infection enhanced with human immune serum. MAb E18 N297Q, E28 N297Q, E44 N297Q and E87 N297Q displayed minimal therapeutic efficacy following an infection enhanced with human immune serum. In summary, mAbs E60 N297Q, 87.1 LALA, and 82.11 LALA were significantly more efficient at preventing enhancement against both the DENV1, DENV3 and DENV4 immune serum-enhanced infections than mAbs E18, E28, E76, E44 or E87 N297Q at concentrations of 2000 ng/mL ($p < 0.03$) and 1000 ng/mL ($p < 0.03$) (**Figure 3.6B, 3.6C and 3.6D**).

Aglycosylated mAbs predicted by the *in vitro* competition assay are therapeutic following a DENV infection enhanced by human immune serum *in vivo*.

To determine whether the *in vitro* competition model is predictive of protection *in vivo*, we administered enhancing volumes of anti-DENV4 human immune serum 24 hours prior to a sub-lethal infection with DENV2 D2S10 and tested *in vivo* the three antibodies (E60 N297Q, 87.1 LALA and 82.11 LALA) predicted to be the most potent therapeutics. Whereas the animals receiving PBS in place of aglycosylated mAb all succumbed four to five days following infection, animals treated therapeutically with any of the three selected aglycosylated mAbs survived infection with minimal indications of disease (**Figure 3.7**). This *in vivo* data supports the competition model as a relevant *in vitro* predictor of *in vivo* outcome following therapeutic administration of aglycosylated mAbs.

Aglycosylated mAbs are not dominant over intact mAbs in *in vitro* assays or as a therapeutic *in vivo*.

To further characterize the stoichiometry underlying the interaction between non-modified and aglycosylated mAbs, we wanted to determine whether the intact or aglycosylated variants (assuming the same affinity for both mAbs) acted dominantly *in vitro* and whether this relationship would be reproducible *in vivo*. To ask this question, we selected the two most therapeutically efficient mAbs, E60 N297Q and 87.1 LALA, and mixed different ratios of non-modified and aglycosylated mAb together including the following conditions: 100% non-modified mAb, 90% non-modified and 10% aglycosylated mAb, 75% non-modified and 25% aglycosylated mAb, 50% of each mAb, 25% non-modified and 75% aglycosylated mAb, 10% non-modified and 90% aglycosylated mAb, and 100% aglycosylated mAb. The enhancing ability of each mAb mixture was then tested in a standard K562 assay enhancement assay. The only combination of non-modified E60/aglycosylated E60 that was not enhancing *in vitro* was the 10% WT/90% aglycosylated mAb combination, indicating that the majority of the antibody mixture must be aglycosylated in order to prevent enhancement of DENV infection (**Figure 3.8A**). In contrast, the enhancement profiles of the combination of non-modified mAb 87.1 and mAb 87.1 LALA demonstrated a complete reduction in enhancement with ratio of 25% non-modified/75% aglycosylated mAb (**Figure 3.8B**).

Using combinations of non-modified E60 and E60 N297Q, we then tested whether the *in vitro* requirement that 90% of the mAb mixture be aglycosylated was similarly required for therapeutic treatment *in vivo*. The same percentages of non-modified and aglycosylated mAbs tested *in vitro* were mixed together in a total of 20 µg and administered 24 hours following an antibody-enhanced DENV2 infection of AG129 mice. As predicted by the *in vitro* analysis, 90% of the administered mixture was required to be aglycosylated in order to provide therapeutic protection *in vivo* (**Figure 3.8C**). Aglycosylated and non-modified mAbs that were combined in a ratio less than 10% WT/90% aglycosylated mAb reduced therapeutic efficacy to 33% or less (**Figure 3.8C**).

Displacement of enhancing antibodies may be critical to therapeutic efficacy following an ADE infection

Antibody-mediated neutralization is achieved when a sufficient number of antibodies bound to the virion exceed a pre-determined threshold. Epitope availability and mAb avidity are the two most crucial biochemical factors that will determine if the stoichiometric requirements for neutralization can be met. All of the mAbs tested demonstrated neutralizing ability when tested *in vitro* and were therapeutically effective following a virus-only, lethal DENV infection. However, two aglycosylated mAbs, E44 N297Q and E87 N297Q, despite having similar dissociation rates (K_d) as E60 N297Q, were not therapeutically effective following an antibody-enhanced, lethal infection. Because our *in vitro* competition assay accurately predicted *in vivo* therapeutic efficacy, we concluded that interactions with the polyvalent immune serum used for enhancement were interfering with the ability of the aglycosylated mAb to neutralize DENV infections. In order to further investigate the mechanism underlying why E60 and 87.1 were effective therapeutically and E87 and E44 were not, we propose two related, non-mutually exclusive hypotheses. The first hypothesis relates to epitope accessibility and suggests that the antibodies comprising a polyvalent serum mixture could bind to the virion and block the epitope to which E44 and E87 bind. If the dimer interface (E44) and C-C' loop (E87) were not well-exposed (few epitopes available for binding) and their accessibility was further compromised in

the presence of polyvalent serum, these aglycosylated mAbs could be unable to bind in sufficient concentrations to neutralize the DENV infection. In contrast, if the fusion loop (E60 target epitope) and the A stand (87.1 target epitope) were highly exposed, the aglycosylated variants of these mAbs could bind easily and quickly, establish a sufficient stoichiometry to neutralize the viral infection, and ultimately prevent enhancement. Alternatively, the therapeutic mechanism may be due to a displacement phenomenon, the focus of the second hypothesis. In both murine and human polyvalent serum, non-neutralizing, cross-reactive antibodies have been hypothesized to largely target the fusion loop [49] and to a lesser extent, the A strand epitope on EDIII [50], such that the effectiveness of mAbs E60 and 87.1 would be dependent upon their ability to displace antibodies from the polyvalent serum of lesser avidity that bind to the same epitope. E44 and E87 may be ineffective therapeutics simply because few antibodies in the polyvalent serum repertoire target the dimer interface and C-C' loop. It may also be possible that both hypotheses are true; that certain epitopes in the presence of polyvalent serum are obscured, and, of the epitopes that have a large number of available binding sites, therapeutically successful mAbs must be able to displace antibodies of lesser avidity.

To attempt to differentiate between the two hypotheses (accessibility vs displacement), we tested whether aglycosylated mAbs would yield different *in vivo* phenotypes when tested in the presence of enhancing concentrations of mAb 4G2, an anti-fusion loop mAb that enhances DENV2 D2S10 over a wide range of concentrations (2 μ g to 80 μ g) and is moderately neutralizing (NT₅₀ titer of 393 ng/mL). First, we hypothesized that if the accessibility hypothesis were true, antibodies such as E44 and E87 would demonstrate improved therapeutic efficacy, 87.1 would similarly remain potent, but E60 and 82.11 (mAbs targeting the fusion loop) would lose potency. In contrast, if the displacement hypothesis were true, we would hypothesize that E44 and E87 would not demonstrate improved efficacy, E60 and 82.11 would still retain efficacy, but 87.1 would lose efficiency. As in the previous studies, animals were administered 20 μ g of mAb 4G2, infected with 10⁵ pfu of DENV2 D2S10, and administered 20 μ g of aglycosylated mAb on Days -1, 0 and +1, respectively. Mice receiving E60 N297Q demonstrated neither morbidity nor mortality ($p < 0.01$ as compared with PBS-treated mice), and mice treated with mAb 82.11 LALA demonstrated an improved survival rate of 67% ($p < 0.01$ as compared to PBS-treated mice). Mice treated with either E44 or E87 N297Q under these conditions did not demonstrate improved survival rates, similarly supporting the displacement hypothesis (**Figure 3.9**). The latter conclusion assumes that binding of 4G2 does not inhibit or obscure the ability of E44 or E87 to bind to the virion. These two pieces of data support the displacement hypothesis in that the aglycosylated mAbs that displace enhancing, non-modified antibodies from their binding sites can establish therapeutic dominance, but those that do not displace enhancing mAbs cannot act therapeutically. Interestingly, animals receiving 87.1 LALA were also protected and displayed a survival rate of 83% ($p < 0.05$ as compared to PBS-treated mice) (**Figure 3.9**); this survival rate is not significantly different than that observed in animals administered polyvalent serum as an enhancing agent and treated with 87.1 LALA (**Figure 3.2**). These data imply that if the binding site of a potentially neutralizing aglycosylated mAb is accessible such that a stoichiometrically sufficient number of the aglycosylated variant can bind, it can also establish therapeutic dominance even in the presence of intact antibodies targeting a different epitope, thus supporting the accessibility hypothesis. Together these data provide supporting evidence for both the displacement and the accessibility hypotheses.

DISCUSSION

In this manuscript, we study a panel of nine monoclonals that bind to a number of different epitopes on the dengue virion, including the fusion loop and dimer interface on Domain II of the E protein, and the A strand and C-C'loop on EDIII. Here we determine that critical differences exist between the ability of aglycosylated mAbs to act therapeutically following a virus-only lethal infection as compared to an antibody-enhanced lethal infection. Comparison of mAb characteristics such as neutralization potency, avidity and neutralization mechanism did not clearly identify a strong *in vitro* correlate explaining the differences in therapeutic efficacy *in vivo*. Rather, an assay designed to measure the potential for competition of the aglycosylated mAb with both mouse and human anti-DENV enhancing immune serum proved a statistically valid correlate. Further examination of this competition theory suggests that aglycosylated mAbs must be able to displace mAbs originating from polyvalent serum that are binding to the same epitope in order to exceed the stoichiometric requirements for neutralization and act therapeutically.

Prophylactic efficacy of DENV infection appears to be directly correlated with mAb neutralizing potency. While mAbs of moderate neutralizing potency failed to protect when administered at between 20-50 μg , potently neutralizing mAbs were completely protective when tested at 20 μg . Further investigation is required to ascertain how important binding specificity may be in mediating prophylactic potency. Pierson *et al* (2008) [51] suggests that neutralization is a dynamic interaction between epitope availability and binding strength and has mathematically derived a requirement of 25% binding, or 30 of 120 available binding sites for mAb E16, a mAb targeting the EDIII lateral ridge on WNV [42]. Similarly for DENV, binding requirements for potent neutralization vary depending upon the epitope of interest [43]. In the presence of enhancing concentrations of polyvalent DENV immune serum, binding of individual mAbs becomes more complicated. Epitope availability may decline due to antibodies in the polyvalent serum either competing for binding sites or sterically hindering the aglycosylated mAbs from binding to their respective epitopes. Thus, one could hypothesize that antibodies binding to obscure epitopes (epitopes that are poorly immunogenic *in vivo* and are thus are not a predominant target of the DENV immune response) that maintain a low occupancy requirement for *in vitro* neutralization (partially reflected in the *in vitro* NT₅₀ titer) would be the best *in vivo* prophylactic antibodies. While the data presented here support this hypothesis, further experimentation comparing mAbs with similar *in vitro* neutralizing potencies and avidities but with different binding epitopes could provide critical insight into this question.

While prophylactic efficacy appears strongly correlated with neutralizing potency, therapeutic efficacy requires that additional criteria be met. Following lethal, virus-only infections, aglycosylated mAbs appear robustly protective, irrespective of neutralizing potency, binding avidity or epitope specificity, whereas only some mAbs are therapeutic following an antibody-enhanced lethal infection. While the reasons for this difference are most likely due to the competition between the polyvalent serum and aglycosylated mAbs, differences in viral burst size following a virus-only, as compared to antibody-enhanced, lethal infection could also be contributing. Recent evidence has supported that DENV antibody-viral complexes, upon binding to and complexing Fc γ RI and Fc γ RIIA, activate the antiviral pathway RIGI/MDA5, decreasing Type I interferon production and upregulating production of interleukin 10 [52,53]. While both diseases are lethal 4-5 days following infection, it is possible that the initial burst size following the first one to two rounds of infection might be larger in an antibody-enhanced, versus lethal

virus-only infection [54] due to interactions with the FcγR and downstream signaling effects, thus initially producing a greater amount of viral progeny that is more difficult to neutralize.

Differences in aglycosylated mAb efficacy following an antibody-enhanced, lethal infection raise fundamental questions about the interaction between neutralizing and enhancing antibodies. The competition assay we designed *in vitro* underscores a basic requirement for the therapeutic mAb to be able to compete off antibodies from the polyvalent serum for binding. These data thus suggest that multiple mechanistic components are required in order for an aglycosylated mAb to serve as an effective therapeutic following an antibody-enhanced DENV infection. Firstly, the studies examining the interaction between the non-modified and aglycosylated mAb variants determined that a large majority (25% for 87.1/87.1 LALA and 90% for E60/E60 N297Q) of the mAb mixture must be aglycosylated to prevent enhancement *in vitro*. This experiment has assumed that the avidity of both the non-modified and aglycosylated mAbs is identical, such that they can bind and dissociate from the virion equivalently. While our *in vitro* ELISA assay suggests this may be the case, additional studies with surface plasmon resonance with mAb E60 and its N297Q variant have indicated that while E60 has a slightly stronger affinity, the difference is not greater than two-fold [40]. *In vivo* analysis of this observation supported our *in vitro* conclusions, in that as a therapeutic mAb mixture, a majority of the antibody repertoire competing for binding sites on the virion must be aglycosylated to be effective. Comparison of non-modified 87.1 and 87.1 LALA suggest that the avidity of the aglycosylated MAb may be slightly higher than its wildtype variant, perhaps supporting the observation that 75% of the antibody mixture, as opposed to 90% with the E60/E60 N297Q mixture, is required to be aglycosylated to prevent enhancement. Although it is possible that such affinity differences could contribute to this observation, it nonetheless appears that antibodies targeting the same epitope with comparable affinities require a substantial portion of the mAb mixture to be incapable of binding the FcR.

Secondly, a successful therapeutic mAb must be strongly neutralizing (NT₅₀ <100 ng/mL). As mentioned before, neutralization capacity is a reflection of epitope availability and mAb avidity. Three mAbs targeting the fusion loop, E60, E18 and E28 bind comparable residues within the fusion loop epitope, but E18 and E28 dissociate from the virion 2-3 times more rapidly than E60. Additionally, neither E18 nor E28 are therapeutic, while E60 is robustly protective, lending credence to the hypothesis that mAb avidity is an important component of therapeutic efficacy *in vivo*. Similarly, when comparing aglycosylated mAbs targeting the A strand, mAb 87.1 LALA was completely protective, was strongly neutralizing and had a low K_d. MAb E76 N297Q was also a potent neutralizer, but had a 2-fold higher off-rate and was only 67% protective *in vivo*. Taken together, examination of mAbs that are directed to the same epitope suggests a strong relationship between antibody avidity and therapeutic efficacy. Similar to the conclusions drawn from the analysis of neutralization potency, examination of avidity measurements across different epitopes suggests that additional factors are clearly important contributors to mediating mAb therapeutic efficacy against dengue disease.

Epitope specificity is the second of two biochemical components critical to establishing a mAb's neutralization potency. However, in the context of therapeutic mAbs, both the epitope specificity of the aglycosylated mAb and the dominant epitope targets of the polyvalent anti-DENV serum must be taken into account. The composition of mouse and human antibody repertoires is different; thus, it was somewhat surprising to us that the same two antibodies, E60 and 87.1, both targeting different epitopes on EDII and EDIII, respectively, would be effective against both murine and human DENV-immune serum enhanced infections. Mice generate a

strongly neutralizing antibody response against EDIII [31,35,38,43,55] that can be both serotype-specific [37,43,56] as well as cross-reactive in nature [37,38,43,56]. In contrast, humans make very little neutralizing EDIII-specific antibody [16,57,58]. Rather, non-biased screens of human anti-DENV-immune serum suggest that a majority of the non-neutralizing, cross-reactive antibody response targets mostly the fusion loop and to some extent, the A strand [49,50]. Within the context of our displacement hypothesis, E60 N297Q and 87.1 LALA may be the most effective therapeutic mAbs because they are highly avid and can displace antibodies generated from polyvalent serum that target the EDII fusion loop and EDIII A strand, bind to the virion in sufficient quantities, and prohibit infection. When we used mAb 4G2 (moderately neutralizing, fusion-loop-specific mAb) to enhance DENV2 D2S10, we found that E60 N297Q still worked effectively as a therapeutic, which supported our hypothesis that aglycosylated mAbs must dislodge enhancing antibodies bound to the same epitope to be therapeutic. Interestingly, 87.1 LALA was significantly therapeutically effective, despite the fact that 87.1 LALA and 4G2 bind to distinctly different epitopes. This observation is most likely due to the fact that the A strand is highly accessible and 87.1 LALA potently neutralizing. Finally, neither mAb E44 nor E87 N297Q was able to protect in the presence of mAb 4G2. These latter data can be explained in that the two epitopes targeted by these mAbs, the C-C' loop and dimer interface, are not predominant targets of the murine or human anti-DENV immune response. Under circumstances where the aglycosylated mAb cannot dislodge other antibodies targeting the same epitope, the aglycosylated mAb fails to be protective. Further experiments are underway to use additional mAbs that target other epitopes to enhance sub-lethal D2S10 infections and test the displacement hypothesis with aglycosylated mAbs that target that same epitope. Furthermore, it is encouraging that E60 N297Q, 87.1 LALA and 82.11 LALA all reduced *in vitro* enhancement generated with human DENV1, DENV3 and DENV4- derived cross-reactive enhancement. This work lends credence to the idea that the cross-reactive antibodies are indeed the major component of the human antibody response driving enhancement.

Further examination of the utility of aglycosylated mAbs in treatment of DENV infections is warranted. As these mAbs are effective following either a virus-only or ADE-derived severe infection, they could be conceivably be used in a clinical setting. As prM-specific mAbs have also been found to constitute a large proportion of the human polyvalent antibody response [27,28], testing of aglycosylated mAbs in the presence of prM-specific mAbs would be of interest. Further studies examining synergistic interactions between promising aglycosylated mAbs could be informative in identifying even more effective cocktails to treat antibody-enhanced, lethal DENV disease.

MATERIALS AND METHODS

Viruses and cell lines. All viruses were propagated in *Aedes albopictus* cell line C6/36 (American Type Culture Collection) and titered by plaque assay on baby hamster kidney cells (BHK21, clone 15) [59]. DENV2 D2S10 was derived as previously described [60]. All *in vitro* neutralization assays and *in vivo* infections were performed with non-concentrated virus. DENV2 D2S10 virus was concentrated by ultra-centrifugation and used in the virion direct capture ELISA. U937-DC-SIGN (gift from A. de Silva, University of North Carolina, Chapel Hill) and K562 cells were used for flow cytometry-based *in vitro* neutralization and enhancement assays, respectively. Both cell lines were grown in RPMI medium (Invitrogen) and supplemented with

5% fetal bovine serum (Denville Scientific), 1% penicillin/ streptomycin and 1% hepes at 37°C in 5% CO₂.

Generation and purification of mouse anti-DENV wildtype and aglycosylated mAbs. Mouse mAb E60, E18, and E28 [61] and E44, E76 and E87 [38] were obtained from M. Diamond (Washington University School of Medicine, St. Louis, MO). Briefly, mouse mAb E60, E18 and E28 were generated against WNV E protein, but are cross-reactive with DENV E protein [61]. Anti-DENV2 mAb E44, E76 and E87 were generated by two sequential infections of IFN- $\alpha\beta$ R^{-/-} C57BL/6 mice with 10⁵ pfu of a 1:1 mixture of DENV2 strain 16681 and DENV2 strain New Guinea C (NGC). Following the second dose, the mice were boosted with purified EDIII (from strain 16681) i.v. three days before splenocyte harvest [38]. In both experiments, splenocytes were fused to P3X63Ag8.53 myeloma cells using polyethylene glycol 1500 [62]. All hybridomas producing anti-DENV2 mAb were subcloned by limiting dilution, isotyped using an ELISA Kit (Southern Biotech) and purified by protein A affinity chromatography (Invitrogen, Carlsbad, CA). Each mAb was further screened for epitope-specific binding patterns using yeast surface display [37,57].

The generation of a chimeric human-mouse E60 mAb with the human IgG1 constant region and the mouse VH and VL region was performed as described previously [57]. Point mutations in the Fc region (N297Q) that abolish FcR and C1q binding were introduced by QuikChange mutagenesis (Stratagene). All recombinant antibodies were produced after transfection of HEK-293T cells, harvesting of supernatant, and purification by protein A affinity chromatography.

Generation and purification of human anti-DENV wildtype and aglycosylated mAb. MAb 87.1 and 82.11 were obtained from F. Sallusto and are human in origin [27]. The generation of these mAbs has been discussed in detail in Beltramello *et al*, 2010 [27]. Briefly, peripheral blood mononuclear cells were isolated and cryopreserved from donors who had been diagnosed with DENV infection. Previously sorted IgG⁺ memory B cells were immortalized using 20 cells/well in multiple cultures using EBV with CpG oligodeoxynucleotide 2006 (Microsynth) and irradiated allogeneic PBMCs. The culture supernatants were subsequently screened for DENV-specific MAb, and the positive cultures were cloned by limiting dilution. Domain-specific binding patterns were determined by ELISA using a yeast display assay provided by M. Diamond. Production of the LALA variants is described in Beltramello *et al*, 2010 [27]. Briefly, variable regions of heavy-chain and light-chain genes were sequenced and cloned by PCR and leucine-to-alanine mutations at positions CH2 1.3 and 1.2 of Igy1 were introduced by site-directed mutagenesis (GenScript). Recombinant antibodies were produced in HEK-293T cells and purified by protein A affinity chromatography and size-exclusion chromatography.

Clinical Serum Samples from Dengue Patients. The DENV1 and DENV3 samples tested *in vitro* in this study were serum samples obtained from patients 6 months to 14 years of age who were admitted to the National Pediatric Reference Hospital, Hospital Infantil Manuel de Jesús Rivera in Managua, Nicaragua, and, after meeting the inclusion criteria, were enrolled in a dengue study [63,64]. A primary DENV infection was defined by antibody titer of <1:10 (acute) or antibody titer of <1:2560 (convalescent), and a secondary DENV infection was defined by anti-DENV antibody titer of \geq 1:10 (acute) or \geq 1:2560 (convalescent), as determined by an Inhibition ELISA [65] [66]. The serum samples used in this study were collected three months

post-symptom onset. The Nicaraguan hospital-based dengue study was approved by the Institutional Review Board at UC Berkeley and the Nicaraguan Ministry of Health. The DENV4 serum sample used for *in vitro* and *in vivo* experiments was obtained from A. deSilva (University of North Carolina, Chapel Hill). In a study approved by the Institutional Review Board of the University of North Carolina at Chapel Hill, serum was collected from volunteers who had experienced natural DENV infections during prior travel abroad.

AG129 Mouse Infections. AG129 mice [67] were bred at the University of California, Berkeley, Northwest Animal Facility. All procedures were pre-approved and conducted according to UC Berkeley Animal Care and Use Committee guidelines.

Production of mouse anti-DENV serum. AG129 mice were infected intra-peritoneally (i.p.) with 10^5 pfu of DENV1 448. Six to eight weeks post-infection, mice were sacrificed and whole blood collected by terminal cardiac puncture. Serum was isolated from whole blood by centrifugation, heat inactivated, and stored at -80°C .

In vivo therapeutic experiments.

DENV2 D2S10 enhanced disease: AG129 mice were administered either 25 μl mouse anti-DENV1 immune serum (in 200 μl final volume) or 200 μl human anti-DENV4 immune serum i.p. 24 hours prior to infection with an intra-venous (i.v.) sub-lethal, 10^5 pfu dose of DENV2 D2S10. **DENV2 D2S10 virus-only, lethal disease:** AG129 mice were infected i.v. with 4×10^6 pfu of DENV2 D2S10. **Prophylactic treatment:** Mice were concurrently administered either 20 μg or 50 μg of non-modified mAb with 25 μl of DENV1 immune serum in a final volume of 200 μl i.p. **Therapeutic treatment:** Animals were administered 25 μl of DENV1 immune serum on Day -1, 10^5 pfu DENV2 D2S10 on Day 0 and 20 μg of non-FcR-binding mAb in a final volume of 100 μl i.v. 24 hours following infection (Day +1). All animals were monitored carefully for morbidity and mortality following infection by checking status four times per day.

DENV neutralization assay. The neutralization titer of each wildtype mAb and its non-FcR-binding variant was measured using the U937-DC-SIGN flow cytometry-based neutralization assay as described in Kraus *et al* (2006) [68]. In brief, mAb were diluted to a starting dilution of 2000 ng/mL and eight 3-fold serial dilutions were performed. The diluted mAb were mixed with DENV2 D2S10 at an MOI of 0.0125, incubated together for 45 minutes at 37°C , and subsequently used to infect OR incubated with 5×10^4 U937-DC-SIGN cells. Two hours following infection, the cells were washed and resuspended in RPMI medium containing 5% FBS (Denville Scientific), 1% penicillin/ streptomycin and 1% hepes. Twenty-four hours following infection, the cells were washed, fixed in 2% paraformaldehyde (Ted Pella, INC), permeabilized with saponin (Sigma Aldrich), and stained intracellularly with 4G2-Alexa488 (Invitrogen). Percent infection was determined by flow cytometry using the BD LSR- Fortessa flow cytometer. Relative infection was calculated using the last duplicate dilution of each mAb series (1:0.19 ng/mL) as its own denominator to minimize variation. NT_{50} titers were calculated as described in Balsitis & Williams *et al*, 2010 [40]. Variations on this assay include incubation with dilutions of DENV-immune serum and virus at 4°C or pre-incubation with virus and cells at 4°C prior to the addition of serial dilutions of mAb [35,38].

DENV enhancement assay

For each of the serum samples (murine DENV1-immune and human DENV1-, DENV3- and DENV4-immune serum samples), the peak enhancement titer (PET) was calculated. Eight 3-fold serial dilutions beginning at a starting dilution of 1:20 were mixed with DENV2 D2S10 virus at an MOI of 0.1. The serum dilution and virus were mixed together at a 1:1 ratio. 50,000 K562 cells were added after a 45-minute incubation of the virus and serum at 37°C. Two hours following infection, the cells were washed and resuspended in RPMI media. After a 48-hour incubation period, the cells were washed, fixed in 2% paraformaldehyde (Ted Pella, INC), permeabilized with saponin (Sigma Aldrich), and stained intracellularly with 4G2-Alexa488 (Invitrogen). The peak enhancement titer (PET) was calculated by plotting raw percent infection on the y-axis and the log-reciprocal serum dilution on the x-axis. A Gaussian distribution was used to fit each enhancement curve, and the amplitude was identified. This data point was then used to derive the log-reciprocal serum dilution corresponding to the amplitude and was reported as the PET.

Steric hindrance assay DENV-immune serum was diluted to the concentration previously identified to cause the greatest enhancement (PET) in an *in vitro* K562 assay. DENV2 D2S10 virus at an MOI of 0.1 and serum were mixed together in equal volumes for 30-45 minutes at 37°C. Aglycosylated mAb were subsequently prepared in five 2-fold dilutions beginning at 2000 ng/mL and were added to the polyvalent serum/virus mixture and allowed to incubate for an additional 30-45 minutes prior to the addition of 50,000 K562 cells. The cells were washed two hours following infection and fixed and stained as above in the neutralization assays. Baseline infection in K562 cells was determined in each assay to be between 1% and 2%, while peak infection ranged from 40-60%, depending on the serum used for enhancement. Relative infection was expressed as the average percent infection for each duplicate divided by the percent infection measured in the presence of the dilution of DENV-immune serum selected for each assay. A hatched line indicates the limit of detection for each assay.

Direct capture virion ELISA. DENV2 D2S10 virus was prepared by ultra-centrifugation at 53,000xg for 2 hours at 4°C and resuspended in cold PBS with 20% FBS (HyClone, Thermo Scientific). This protocol was obtained from Gromowski *et al* (2007) with minor modifications [43]. Concentrated virus was diluted to 5×10^4 pfu in carbonate coating buffer, pH 9.6, and 50 μ l was added to each well of a 96-well flat-bottomed plate (xxx). The plate was coated overnight at 4°C and washed thoroughly with 1X phosphate-buffered saline with 0.1% Tween-20 (PBS-T) prior to blocking in 50 μ l of blocking buffer (5% milk w/v in PBS-T) for one hour. Both the non-modified and aglycosylated mAb were diluted to a starting concentration of 120 μ g/mL in blocking buffer and titrated two-fold for a total of 12 serial dilutions, and 100 μ l of each mAb dilution was added in duplicate to the coated plate for one hour. The plate was thoroughly washed with PBS-T, and 100 μ l of 1:2500 dilution of alkaline phosphatase (AP)-conjugated goat anti-human secondary Ab (Meridian) was added for one hour. The plates were again washed with PBS-T, and AP substrate PNPP (Sigma Aldrich) was added. The reaction was developed for 45 minutes, and the absorbance was read at 405 nm on a UV-plate reader (xxx) using KC Junior software.

Statistical Analysis All graphs were produced using Prism 5 software (La Jolla, CA). Statistical analysis was performed using Stata v10 (College Station, Texas). Comparison of survival

functions was conducted using a non-parametric logrank equality test. Aglycosylated mAb that resulted in a statistically improved survival rate following an antibody-enhanced infection as compared to non-treated controls were considered ‘moderately to strongly therapeutic’ and compared to aglycosylated mAb that did not significantly improve survival. Wilcoxon Rank-Sum analysis was used to further evaluate potential differences in mAb characteristics including NT₅₀ titer, mAb avidity (as estimated by the K_d), and reduction in *in vitro* enhancement (competition assay).

FIGURES

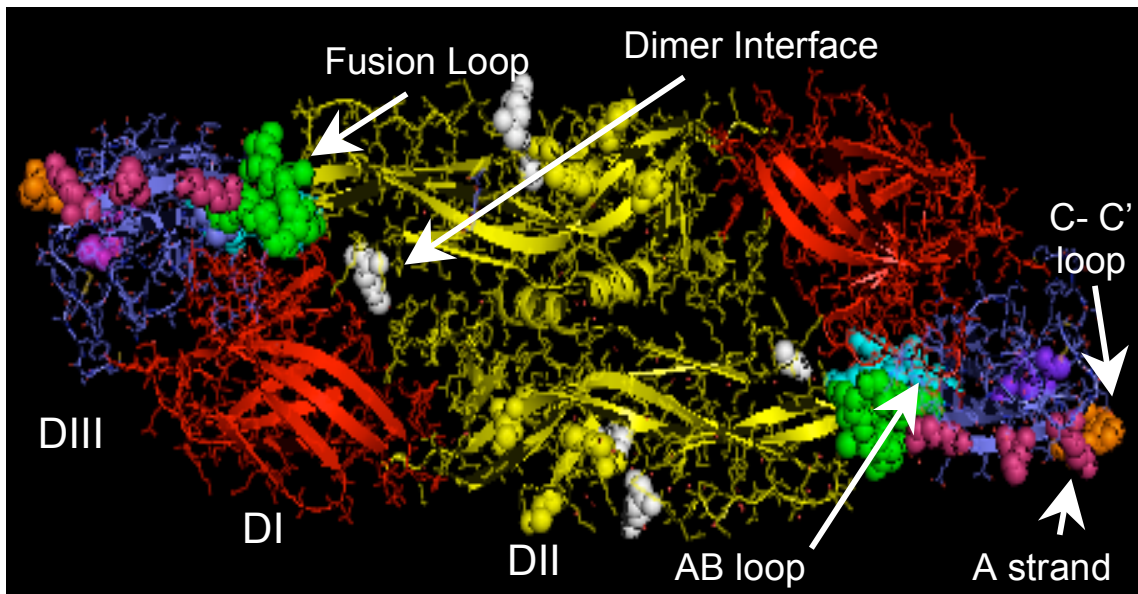


Figure 3.1 Anti-DENV mAbs target a number of different epitopes on the E protein. Ribbon diagram of a homodimer of the E protein constructed from DENV2 16681 sequence [32]. Domain I is red, Domain II is yellow and Domain III is blue. The epitopes targeted by mAbs in Table 1 include the fusion loop (green), dimer interface (white), C-C' loop (orange) and A strand (magenta).

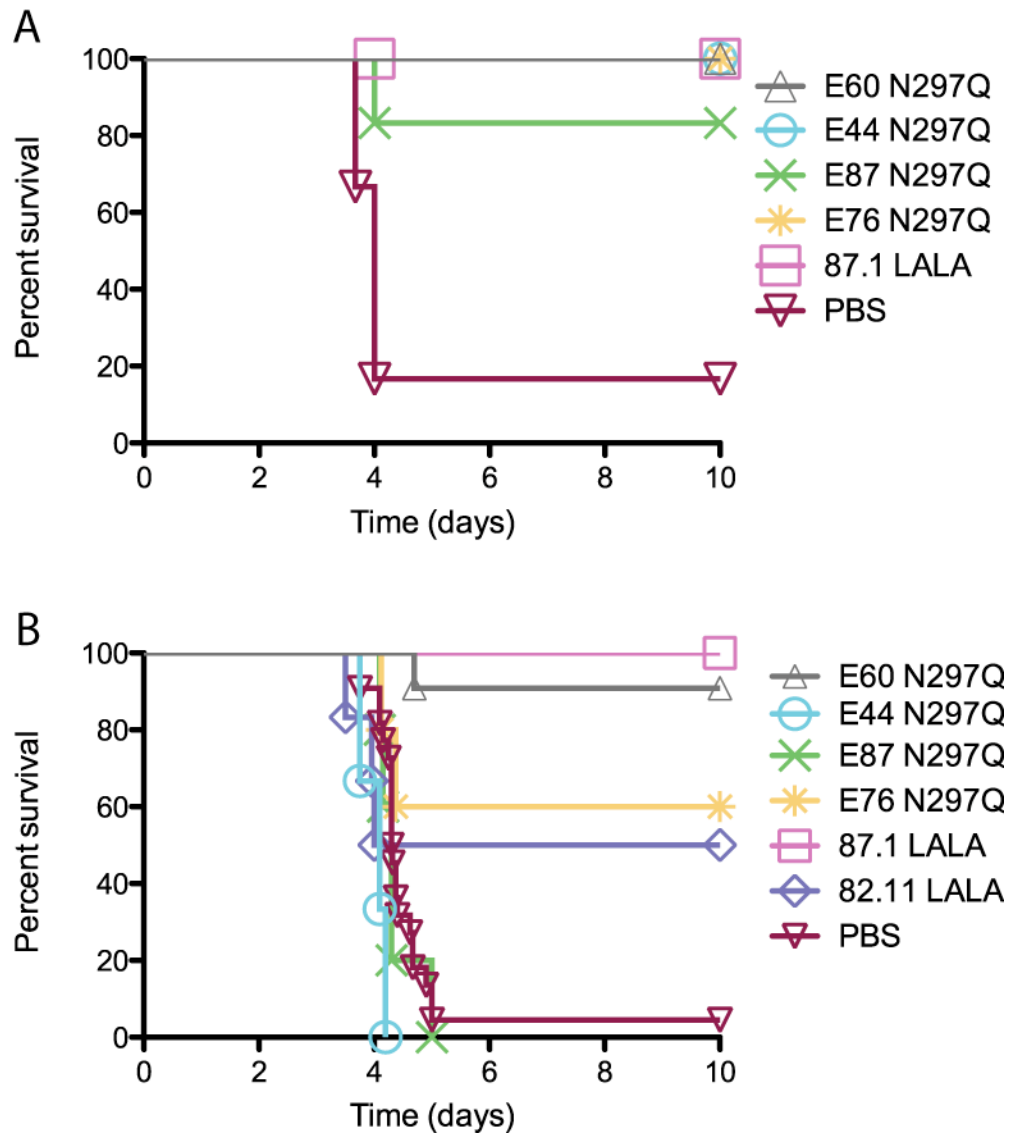


Figure 3.2 Therapeutic prevention of lethal and antibody-enhanced disease by non-FcR-binding mAbs. **A)** AG129 mice were administered a lethal inoculum of DENV2 D2S10 and subsequently treated with 20 μg of non-FcR-binding MAb. **B)** AG129 mice were administered a sub-neutralizing amount of anti-DENV1 polyvalent mouse serum 24 hours prior to a sub-lethal dose (10^5 pfu) of DENV2 D2S10. Mice were therapeutically administered 20 μg of non-FcR-binding MAb 24 hours following infection and monitored for 10 days for morbidity and mortality.

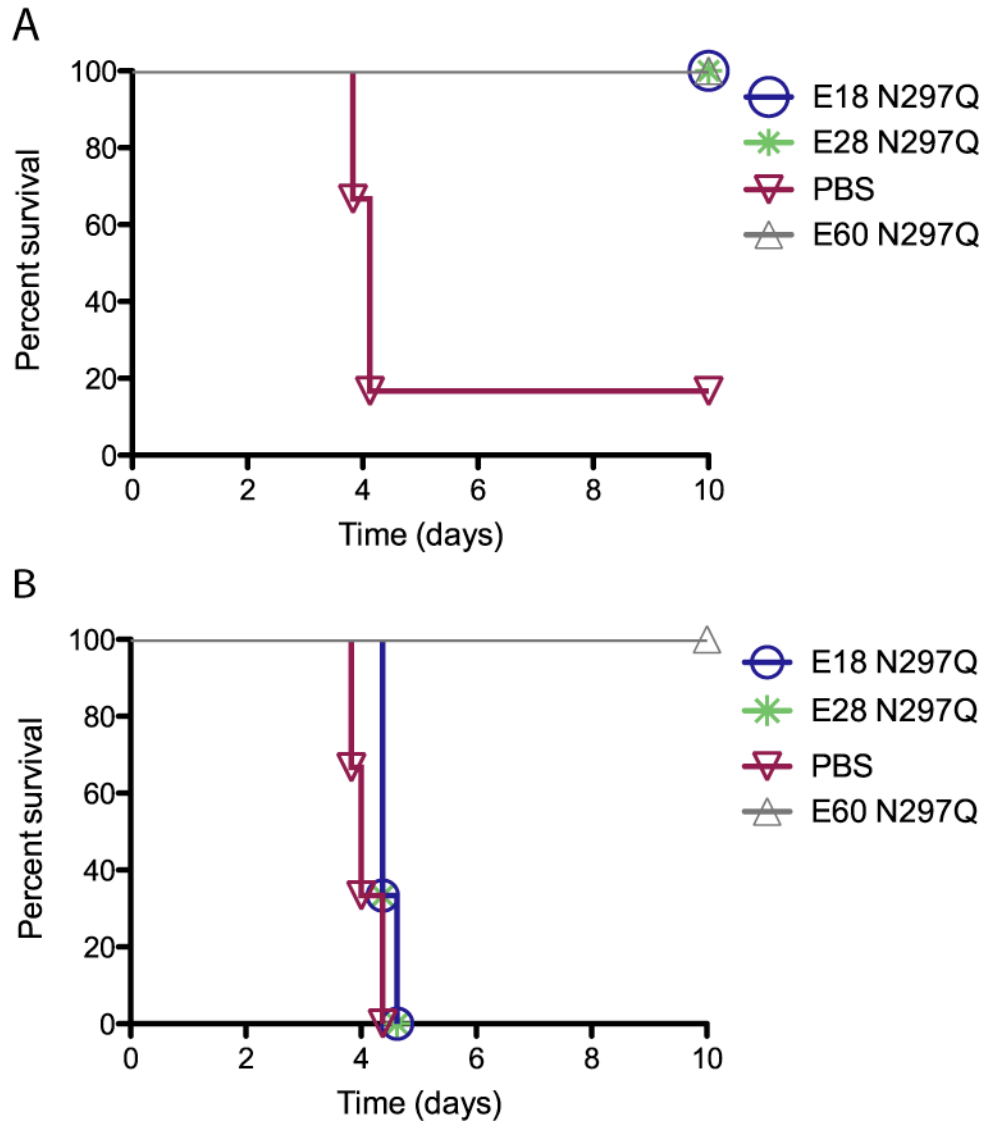


Figure 3.3 Therapeutic prevention of lethal and antibody-enhanced dengue disease by non-FcR-binding anti-fusion loop mAbs. A) Mice (n = 3 per group) were administered a lethal inoculum of DENV2 D2S10 and subsequently treated with 20 μ g of non-FcR-binding mAb. B) Mice (n = 3-5 per group) were administered a sub-neutralizing amount of anti-DENV1 polyvalent serum 24 hours prior to a sub-lethal dose of DENV2 D2S10. Mice were therapeutically administered 20 μ g of non-FcR-binding mAb 24 hours following infection and monitored for 10 days for morbidity and mortality.

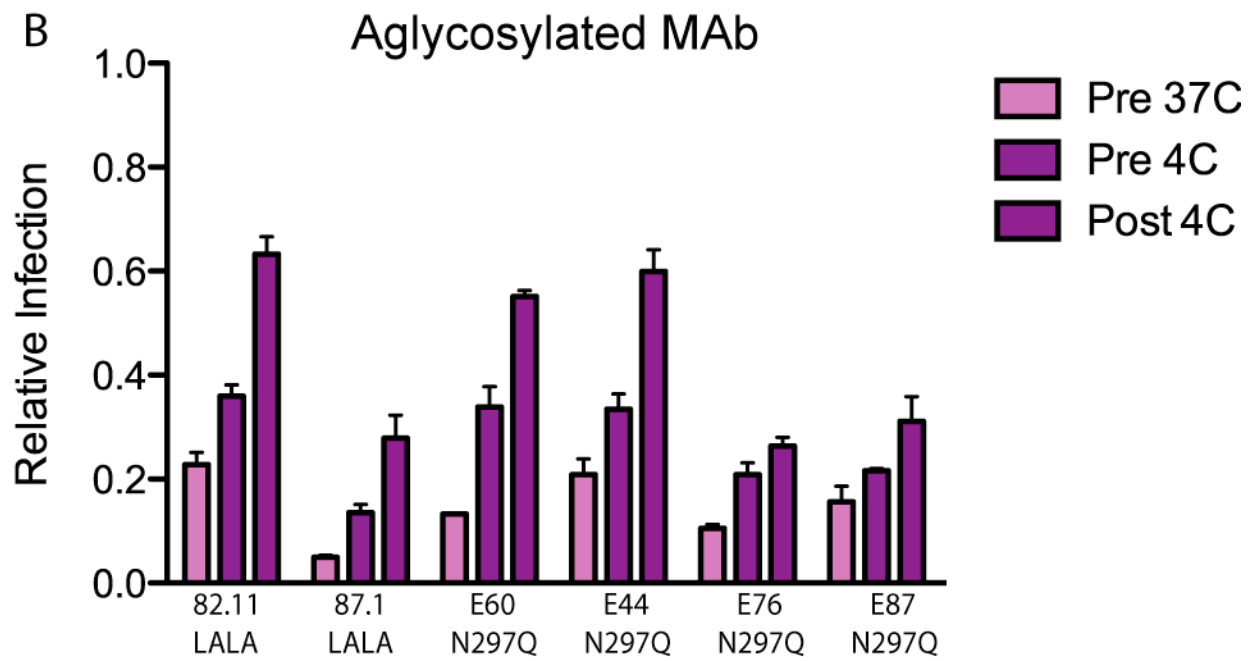
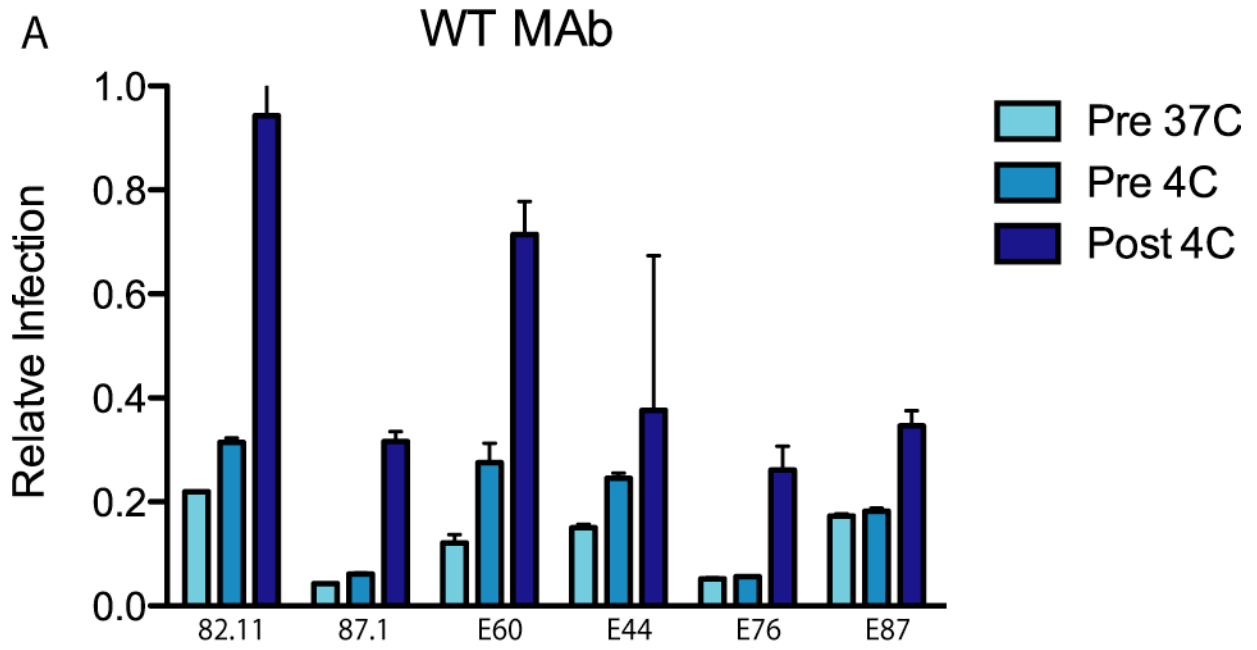


Figure 3.4. E60 N297Q and 87.1 LALA mAbs neutralize via different mechanisms. Pre-attachment (**A**) and post-attachment (**B**) experiments were performed to determine whether mAbs blocked prior to, or after, viral attachment. In the pre-attachment assay, mAbs were tested at saturating conditions (10 $\mu\text{g/ml}$) and incubated with 625 DENV2 D2S10 particles at either 4°C or 37°C for one hour; the resulting antibody-virus mixture was then used to infect U937 DC-SIGN cells. In the post-attachment assay, virus and U937 DC-SIGN cells were initially incubated together for 1 hour at 4°C prior to incubation with antibody for an additional hour at 4°C. In both assays, percent infection was measured 24 hours later by flow cytometry.

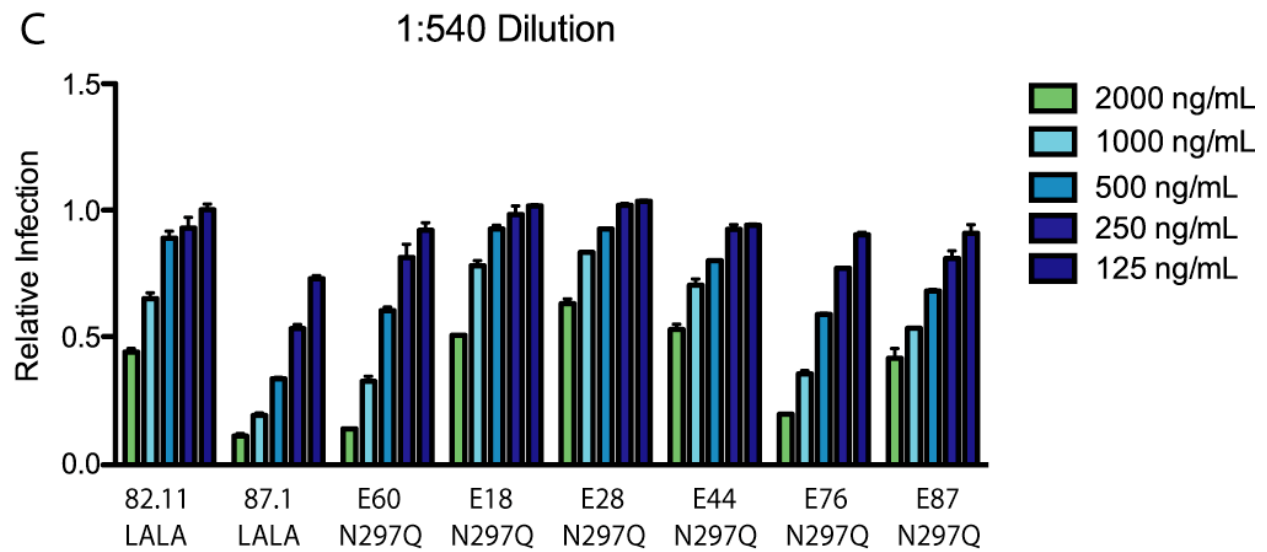
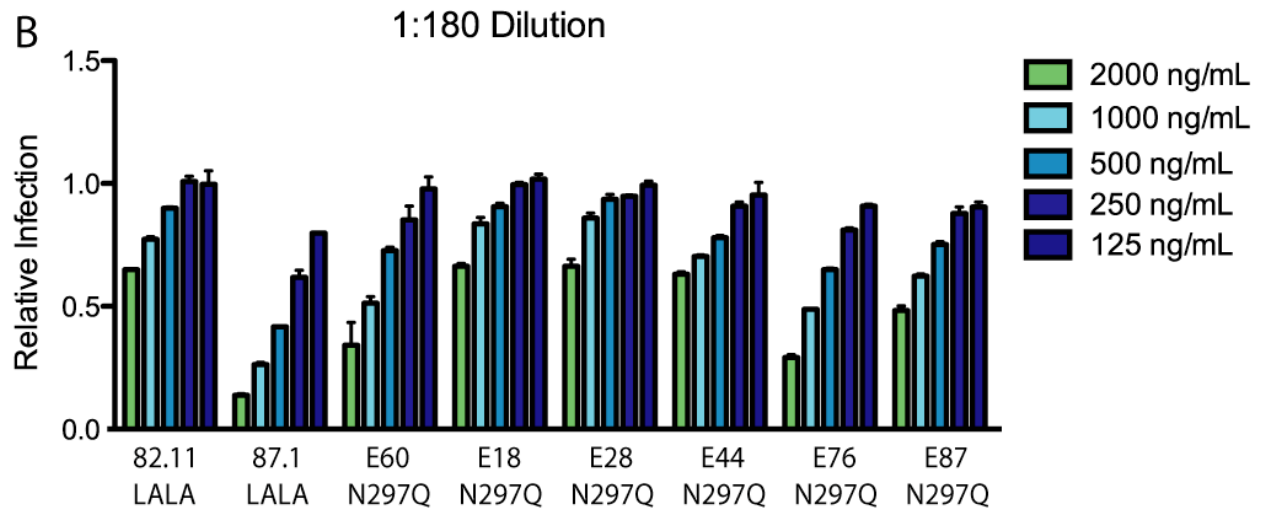
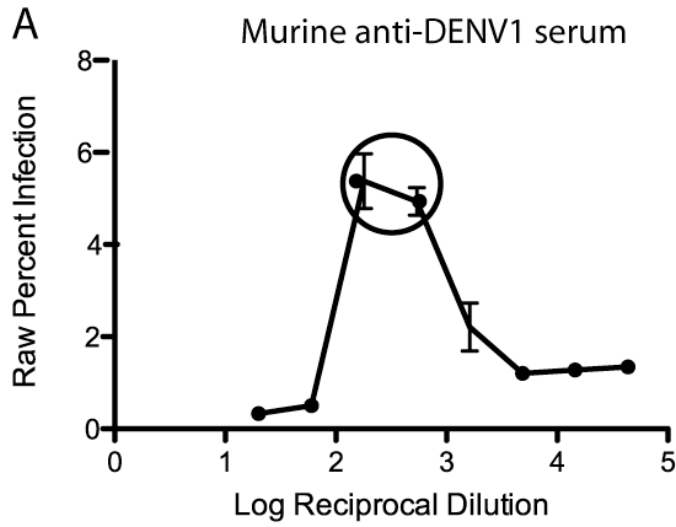


Figure 3.5 MAbs E60 N297Q and 87.1 LALA reduce enhancement with mouse DENV1-immune serum *in vitro*. **A)** DENV1-immune mouse serum was diluted 8-fold beginning at 1:10 and mixed with equal volumes of DENV2 D2S10 for 45 min before infection of K562 cells. Forty-eight hours following infection, cells were stained with 4G2-Alexa488, and percent infection was assessed by flow cytometry. The dilutions yielding peak enhancement, 1:180 and 1:540, are circled. **B) and C)** Mouse DENV1-immune serum diluted 1:180 (**B**) or 1:540 (**C**) was pre-incubated with DENV2 D2S10 for 30 min before addition of six 2-fold dilutions of aglycosylated mAb beginning at 2000 ng/mL. K562 cells were added 30 min following incubation of immune serum, virus and aglycosylated mAb. The cells were fixed 48 hours following infection and stained intracellularly with 4G2-Alexa488, and the percent infection was obtained by flow cytometry.

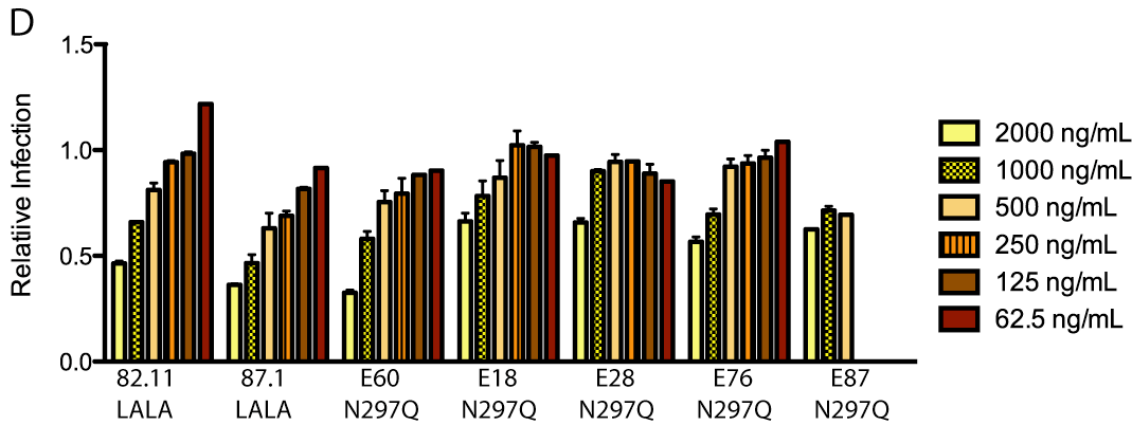
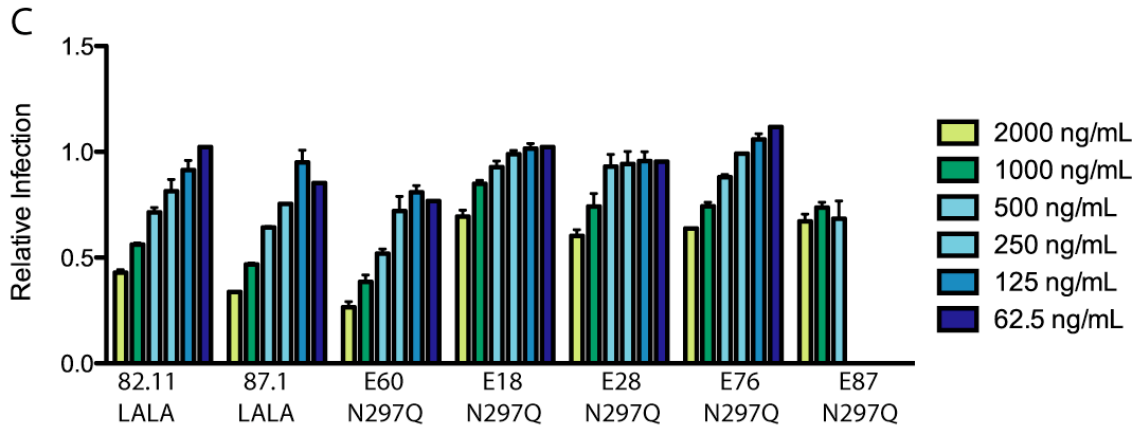
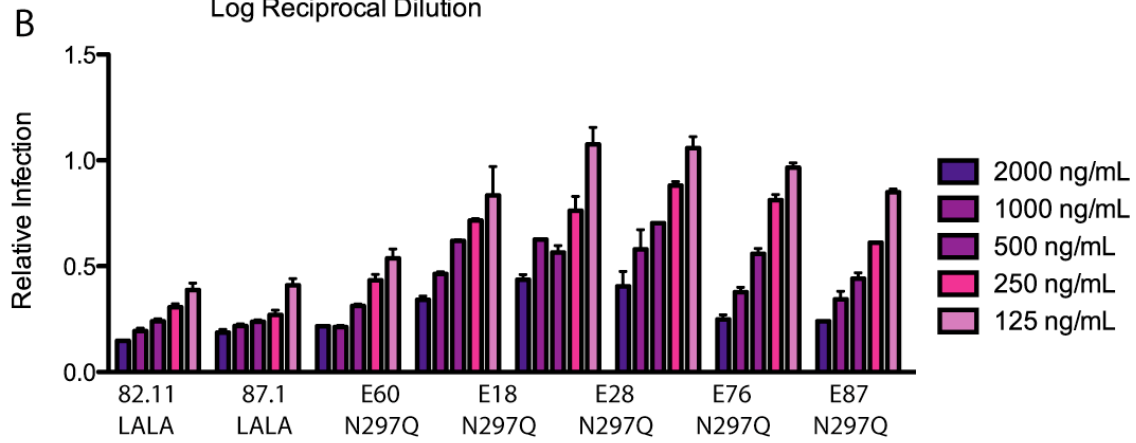
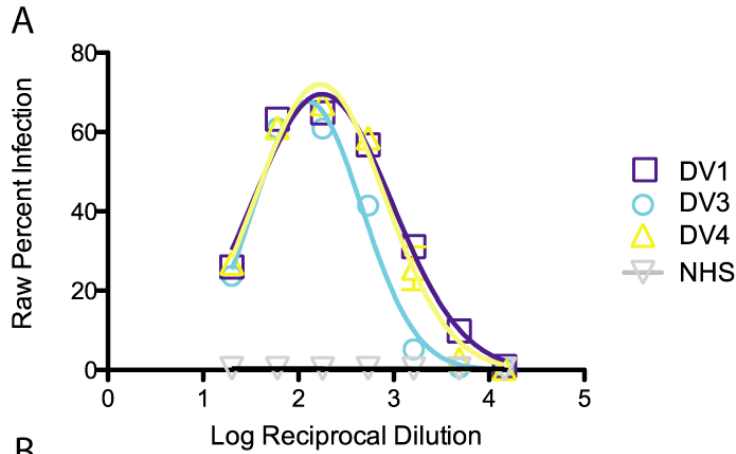


Figure 3.6 MAbs E60 N297Q, 82.11 LALA and 87.1 LALA reduce enhancement with human DENV-immune serum *in vitro*. **A)** DENV1- and DENV3-immune human serum (obtained 3 months following primary infection) and DENV4-immune human serum (obtained years following DENV infection) were diluted 8-fold starting at 1:10 and mixed with equal volumes of DENV2 D2S10 for 45 min before infection of K562 cells. Forty-eight hours following infection, cells were stained with 4G2-Alexa488, and percent infection was assessed by flow cytometry. Peak dilutions for all three samples were 1:180 and 1:540. **B), C) and D)** Serum diluted 1:180 was pre-incubated with DENV2 D2S10 for 30 min before addition of serial dilutions of aglycosylated mAb at the specified concentrations. K562 cells were added 30 minutes following incubation of immune serum, virus and aglycosylated mAb. The cells were fixed 48 hours following infection and stained intracellularly with 4G2-Alexa488, and the percent infection was obtained by flow cytometry.

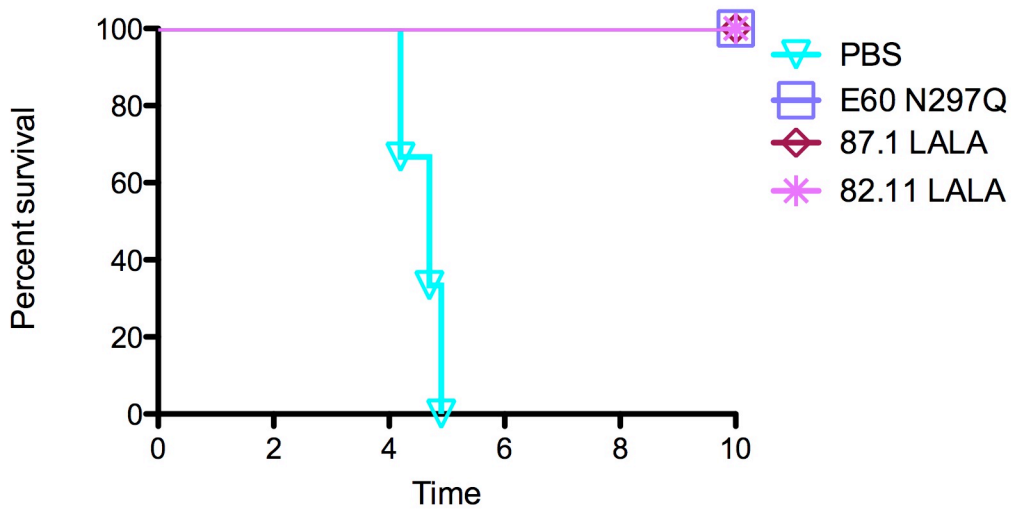


Figure 3.7 Aglycosylated mAbs predicted by the *in vitro* competition assay therapeutically protect against a lethal, human immune-serum-enhanced infection. AG129 mice (n = 3-5 per group) were administered 200 μ l of DENV4 human immune serum on Day -1, infected with 10^5 pfu of DENV2 D2S10 on Day 0 and administered 20 μ g of either E60 N297Q, 87.1 LALA and 82.11 LALA on Day +1, 24 hours following infection. Animals were monitored for morbidity and mortality for 10 days following infection.

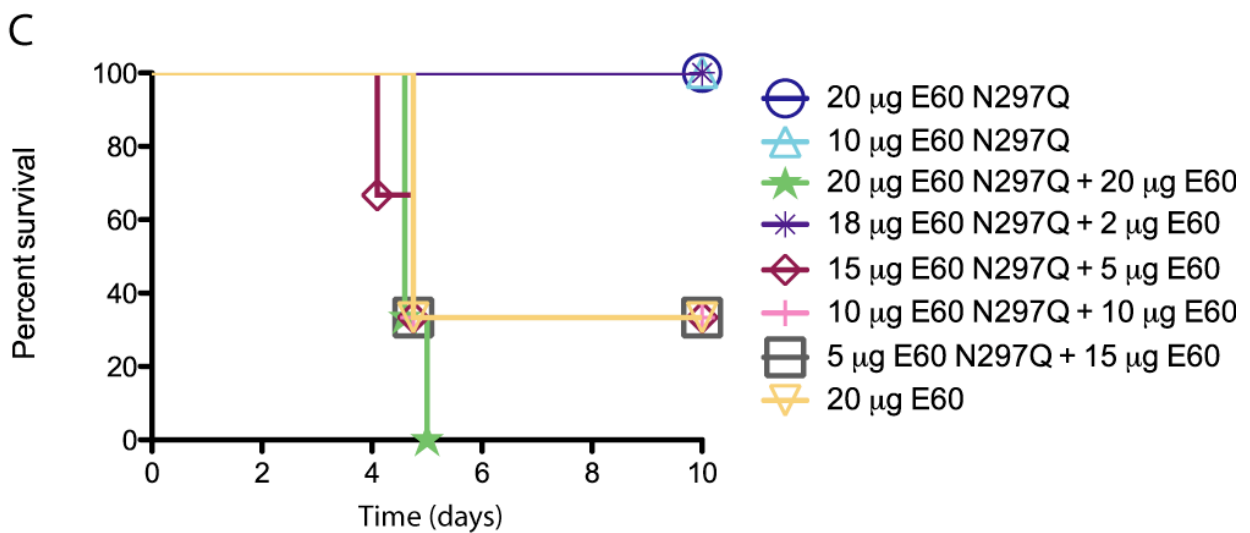
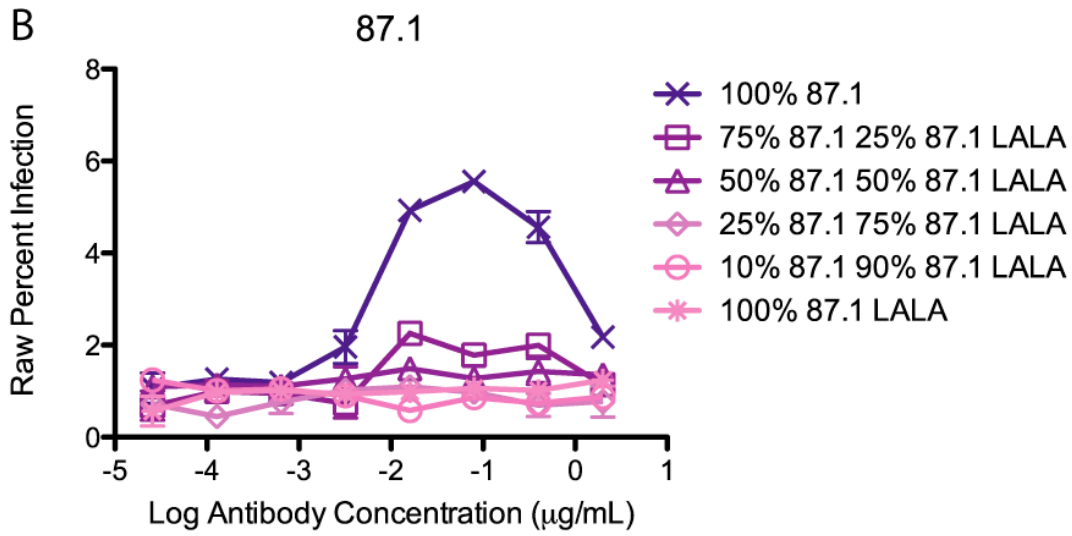
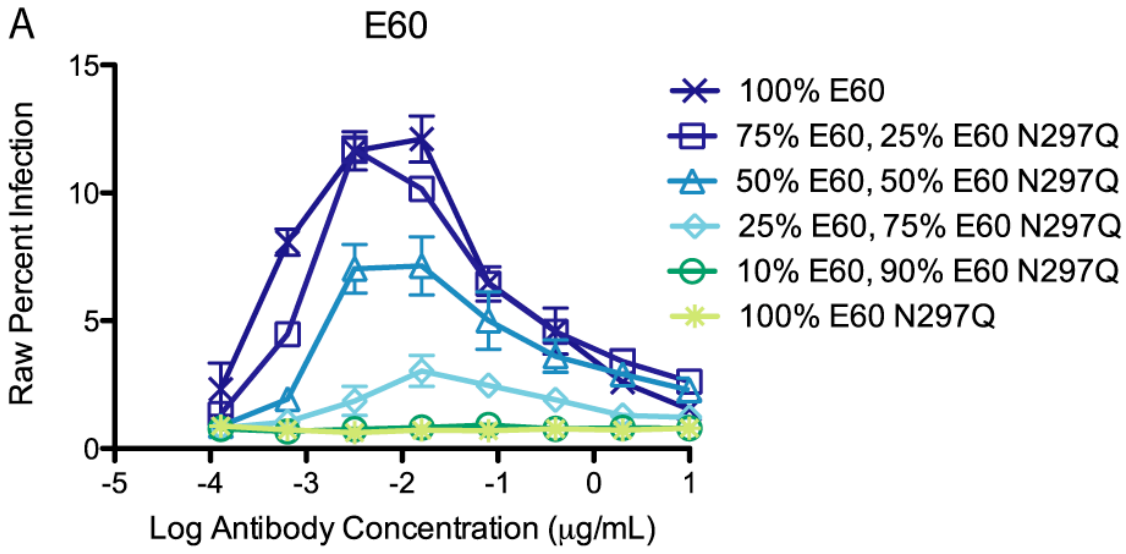


Figure 3.8 Non-modified mAbs are dominant over aglycosylated mAbs *in vitro* and *in vivo*.

A and B) Mixtures of non-modified and aglycosylated antibodies E60/E60 N297Q (**A**) and 87.1/87.1 LALA (**B**) were pre-mixed at ratios of 90%:10%, 75%:25%, 50%:50%, 25%:75% and 10%:90% before incubation with DENV2 D2S10. The antibody/virus mixture was then used to infect K562 cells. The cells were subsequently stained 48 hours later intracellularly using 4G2-Alexa-488, and percent infection was measured by flow cytometry. **C)** AG129 mice (n = 3 per group) were administered 25 μ l anti-DENV1 mouse immune serum, infected with 10^5 pfu of DENV2 D2S10 and administered a total of 20 μ g of E60/E60 N297Q in the same *in vitro* combinations tested in **A)** on Days -1, 0 and +1, respectively. Animals were monitored for morbidity and mortality for 10 days following infection.

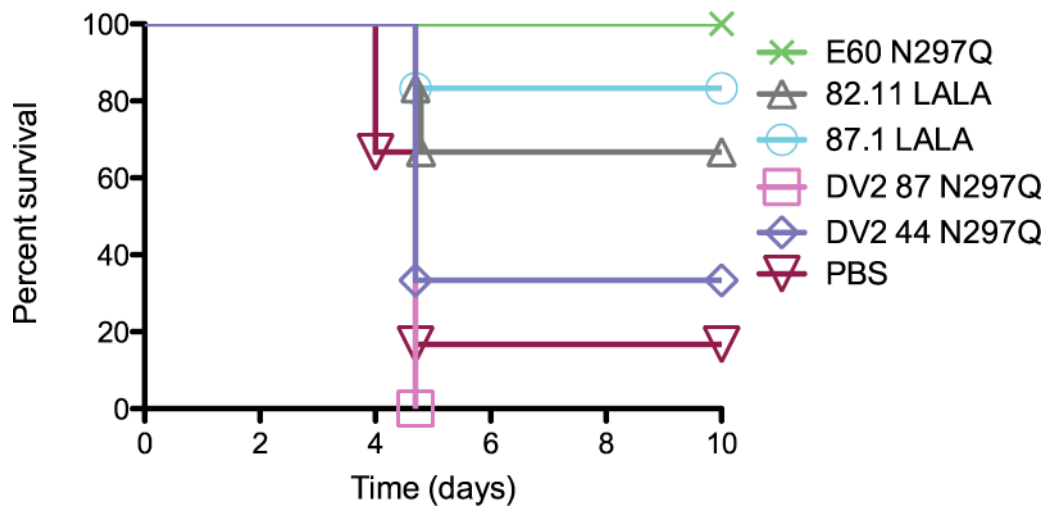


Figure 3.9 Displacement of enhancing antibodies may be an important mechanism for successful therapeutic mAbs. AG129 mice were administered 20 μg mAb 4G2 (anti-fusion loop) on Day -1, infected with 10^5 pfu DENV2 D2S10 on Day 0, and then administered 20 μg of either 82.11 LALA (n = 6), 87.1 LALA (n = 6), E60 N297Q (n = 6), E44 N297Q (n = 3), or E87 N297Q (n = 3) or PBS (n = 6) on Day +1, 24 hours following infection. Animals were monitored for morbidity and mortality for 10 days following infection.

Table 3.1 Defining *in vitro* characteristics of monoclonal antibodies

MAB	Origin	Epitope	Cross-reactivity	Isotype	Average NT ₅₀ (ng/ml), non-modified ^a	Average NT ₅₀ (ng/mL), FcR-modified ^a	Kd, non-modified mAb (ng/mL) ^b	Kd, FcR-modified mAb (ng/mL) ^b
E60	Diamond	E-DII Fusion loop	DV1,2,3,4	hIgG1	49	72	192	185
82.11	Lanzavecchia	E-DI/II	DV1,2,3,4	hIgG1	142	99	172	136
E18	Diamond	E-DII Fusion Loop	DV1,2,3,4	hIgG1	244	371	411	519
E28	Diamond	E-DII Fusion Loop	DV1,2,3,4	hIgG1	502	544	517	432
87.1	Lanzavecchia	E-DIII A strand	DV1,2,3	hIgG1	23	24	97	55
E76	Diamond	E-DIII A strand	DV2 only	hIgG1	11	27	146	165
E87	Diamond	E-DIII LR- C strand	DV2 only	hIgG1	66	59	184	214
E44	Diamond	E-DI/II Dimer interface	DV2 only	hIgG1	51	68	27	92
E111	Diamond	anti-DENV1	DV1 only	hIgG1	NN ^c	NN	NB ^d	NB

^a The data presented is the average of two to five replicates of duplicate measures

^b The data presented is the average of two to four replicates of duplicate measures

^c "NN" indicates that the mAb did not neutralize DENV2 D2S10

^d "NB" indicates that the mAb did not bind to DENV2 D2S10 by direct capture ELISA

Table 3.2 Prophylactic efficacy of a non-modified mAb

Wildtype mAb	Dose of mAb ^a	Morbidity	Mortality
82.11	20 µg	ND	ND
	50 µg	1/2	1/2
87.1	20 µg	0/2	0/2
	50 µg	0/2	0/2
E28	20 µg	2/2	2/2
	50 µg	ND	ND
E44	20 µg	0/2	0/2
	50 µg	0/7	0/7
E60	20 µg	1/2	0/2
	50 µg	0/2	0/2
E76	20 µg	0/2	0/2
	50 µg	0/7	0/7
E87	20 µg	0/2	0/2
	50 µg	0/2	0/2
4G2	50 µg	2/2	2/2
	100 µg	0/2	0/2
PBS	---	4/4	4/4

^a MAb administered concurrently with an enhancing dose (25 µl) of DENV-1-immune mouse serum 24 hours prior to infection with a sublethal dose (10^5 pfu) of DENV2 D2S10.

Table 3.3 Therapeutic efficacy of aglycosylated mAb variants targeting different epitopes

Aglycosylated mAb	Virus-only Mortality	Virus-only Mortality	ADE Mortality	ADE Mortality
82.11 LALA	ND	ND	3/6	0.1890
87.1 LALA	0/6	0.0054	0/6	0.0002
E44 N297Q	0/3	0.0373	3/3	0.0053
E60 N297Q	0/6	0.0054	0/9	0.0001
E76 N297Q	0/8	0.0017	2/5	0.0398
E87 N297Q	1/6	0.0125	5/5	0.7923
PBS	5/6	---	18/19	---

^a p-value vs PBS-treated mice

^b p-value vs PBS-treated mice

Table 3.4 Therapeutic efficacy of aglycosylated mAb variants targeting the fusion loop

Aglycosylated mAb	Virus-only Mortality	Virus-only Mortality	ADE Mortality	ADE Mortality
E18 N297Q	0/6	0.0054	4/5	0.2074
E28 N297Q	0/6	0.0054	5/5	0.7607
E60 N297Q	0/3	0.0373	0/3	0.0110
PBS	5/6	---	8/9	---

^a p-value vs PBS-treated mice

^b p-value vs PBS-treated mice

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CHAPTER 4

ANTIBODIES TARGETING E-DOMAIN III ARE NOT REQUIRED FOR SEROTYPE-SPECIFIC PROTECTION *IN VIVO*

INTRODUCTION

Placing up to half of the world's population at risk, dengue virus (DENV) is the most important emerging arboviral pathogen affecting humans [1]. Transmitted by the *Aedes aegypti* and *Ae. Albopictus* mosquitoes [2,3], DENV is comprised of 4 serotypes, DENV1-4 [4]. A large percentage of the estimated 100 million annual infections are clinically asymptomatic; symptomatic infections range from the self-limited but debilitating dengue fever (DF) to the potentially life-threatening dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) [5,6].

The DENV envelope (E) protein is the major antigenic target on the surface of the virion [7]. Initial monoclonal antibody (MAb) mapping studies (reviewed in Roehrig, 1998) identified the E protein to be composed of three distinct antigenic regions: A, B and C [7]. Subsequent analysis of the crystal structure of DENV E glycoprotein revealed three domains- I, II and III- that were directly comparable with the previously defined antigenic regions [8]. Studies with mouse monoclonal antibodies (MAbs) have determined that antibodies targeting Domains I/II (EDI/II) are generally more cross-reactive among serotypes and of low to moderate neutralizing potency [9,10]. In contrast, mouse MAbs binding Domain III (EDIII) are serotype-specific and highly neutralizing [11,12,13,14,15], although both cross-reactive and non-neutralizing MAbs binding to newly identified EDIII epitopes have been identified [15,16,17].

A person exposed to a primary DENV infection develops a polyclonal antibody response that neutralizes the homologous serotype responsible for infection, while leaving the subject susceptible to a second infection with a different serotype [18,19]. Pre-existing cross-reactive antibodies may enhance a second DENV infection and lead to more severe disease [20]. Investigators are now beginning to study the binding and functional properties of human antibodies and to compare the human response to the well-studied mouse response. Studies of human sera and MAbs indicate that multiple viral antigens including E protein, pre-membrane (prM/M) protein and non-structural protein 1 (NS1) are recognized by human antibodies [21,22,23,24,25,26,27]. Moreover, most DENV-specific human antibodies are cross reactive and weakly neutralizing, and a minor population of antibody appears to be responsible for the ability of immune sera to strongly neutralize the homologous serotype [21,22,23,24,25,26,27]. Based on studies with mouse MAbs, investigators had assumed that human antibodies that potently neutralize DENV also bind to EDIII. However, recent data has suggested that EDIII-specific antibodies do not constitute a large percentage of the human anti-DENV antibody repertoire and do not contribute substantially to *in vitro* neutralization of DENV [26,28,29]. As the *in vitro* properties of antibodies do not always correlate with *in vivo* properties, here we compare the contribution of EDIII-specific antibodies in mouse and human immune sera to DENV neutralization and enhancement using a mouse model of dengue developed by our laboratory.

RESULTS

Human serum depleted of anti-EDIII antibodies reduces viral load *in vivo*

Previous studies have demonstrated that humans infected with DENV develop low levels of anti-EDIII antibodies and that these antibodies make only a minor (<15%) contribution to the neutralization potency of the human immune serum in *in vitro* assays [26,29]. Thus, we first asked whether anti-EDIII antibodies in human serum contribute to protection *in vivo* using a mouse model of DENV infection and disease we had previously developed [30,31]. We obtained

a convalescent serum sample from a person exposed to a primary DENV2 infection and depleted the serum of anti-EDIII antibodies using a recombinant EDIII-maltose binding protein (MBP) fusion protein or MBP alone as previously described [26] (**Figure 4.1A**). The neutralizing and enhancing abilities of the MBP-depleted and EDIII-depleted serum were assessed using U937 DC-SIGN and K562 flow-cytometry based assays, respectively. EDIII depletion led to a 15% reduction in homologous neutralization titer as compared to the MBP-depleted control (**Figure 4.1B**) and did not significantly affect serotype cross-reactive neutralization (**Supplementary Figure 4.1A, B and C**). Both the MBP-depleted and EDIII-depleted samples demonstrated similar peak enhancement titers against DENV2 (422 and 359), respectively (**Figure 4.1C**). Taken together, this data supports previously published work [26,29] and indicates that depletion of anti-EDIII antibodies from human serum does not drastically alter either the *in vitro* neutralization or enhancement profiles.

While depletion of anti-EDIII antibodies only decreased the *in vitro* neutralization titer of the human immune serum by 15%, it is conceivable these antibodies might be required for protection *in vivo*. To test this possibility, experiments were performed using a recently developed mouse model of DENV infection and disease [30,31]. Eighty μ l of MBP-depleted or EDIII-depleted serum was transferred into AG129 mice (n=5/group) after accounting for the 15% reduction in neutralization titer associated with the depletion 24 hours prior to a 10^3 pfu DENV2 D2S10 infection, a dose at which morbidity is not observed but protective effects on viremia and tissue viral load can be measured (**Figure 4.2A**). Non-terminal bleeds were collected 4-6 hours prior to infection to measure circulating antibody titers. As demonstrated in Figure 2B, the average NT₅₀ titer for MBP-depleted and EDIII-depleted groups were not significantly different from each other, but both were significantly higher than the normal human serum (NHS) recipient mice. Serum viremia and tissue viral load levels were subsequently measured four days post-infection by either plaque assay or quantitative RT-PCR. Mice receiving either MBP-depleted or EDIII-depleted serum displayed viremia levels that were at or slightly above the limit of detection for the assay (1 pfu(eq)/mL) and were significantly lower than the NHS-recipient group (**Figure 4.2C**). Viral load levels were further measured in primary and secondary lymphoid organs; significant reductions in the spleen and bone marrow were observed in both experimental groups, as compared to the NHS control (**Figure 4.2D and 4.2E**). Importantly, across all organs tested, there was no difference in serum viremia or tissue viral load between the MBP-depleted and EDIII-depleted groups. These data indicate that anti-EDIII antibodies in human serum were not required for protection observed *in vivo*.

Mice infected with DENV develop anti-EDIII antibodies that contribute to neutralization but not enhancement *in vitro*

Previous data has suggested that most mouse MAbs that strongly neutralize DENV bind to EDIII [15]. Thus, we next examined whether mice exposed to DENV infection develop a polyclonal neutralizing antibody response that is mainly directed against epitopes on EDIII. We depleted pooled anti-DENV2 mouse serum with EDIII protein using the same approach described for human immune sera [26]. We confirmed depletion of anti-EDIII antibodies using an EDIII-specific ELISA and demonstrated that there was little measurable binding to the EDIII protein in depleted serum (**Figure 4.3A**).

Having removed the anti-EDIII antibodies from the anti-DENV2 serum, we measured the contribution of anti-EDIII antibodies to homologous and heterologous neutralization and enhancement *in vitro*. A 34% reduction in homologous neutralization titer was observed

comparing EDIII-depleted serum to MBP-depleted serum (Figure 3B). We subsequently assessed the effect of anti-EDIII depletion on serotype cross-reactive neutralization of DENV1 strain 448, DENV3 strain CH53489 and DENV4 strain TVP30 (**Supplementary Figure 4.1D-F**) and observed comparable differences in neutralization titer that were similar in magnitude to those observed when testing homologous neutralization. The MBP-depleted and EDIII-depleted serum demonstrated similar peak enhancement titers (304 and 295, respectively) against DENV2 (**Figure 4.2C**). Our data indicate that mice make EDIII-specific antibodies that contribute comparably to both serotype-specific, as well as serotype cross-reactive neutralization. Moreover, the data also indicate that more than half the neutralizing antibodies in mice are not directed against EDIII.

EDIII-depleted anti-DENV2 mouse serum can protect against DENV2 infection.

As our *in vitro* analysis of the EDIII-depleted serum suggested that EDIII-specific mouse antibodies contributed 34% to the overall neutralizing potency of DENV2-immune serum, we next asked whether EDIII-depleted serum would be as protective *in vivo* as control-depleted serum. Similarly to the experiments described using human immune serum, anti-DENV immune mouse serum was transferred 24 hours prior to a sublethal 10^3 pfu infection and non-terminal bleeds were taken 4-6 hours prior to infection to measure the neutralizing titer of the circulating antibodies present at the time of infection (**Figure 4.4A**). We first transferred equivalent volumes of MBP-depleted (MBP) and anti-EDIII-depleted (EDIII-A) serum (n= 4-6 per group) such that the average neutralization titer of the transferred serum *in vivo* was 67.4 and 47, respectively (**Figure 4.4B**) and significantly higher than NMS-recipient mice ($p < 0.01$ for both comparisons). As the difference between the MBP-depleted and EDIII-depleted serum was 34% *in vitro*, we expected to see a comparable difference following transfer *in vivo*; indeed, comparing the average NT_{50} titers between the MBP (67) and EDIII-A (47) groups yielded a difference of 42%. Serum viremia levels in mice administered either MBP or EDIII-A serum were significantly reduced as compared to NMS-recipient mice ($p < 0.015$, MBP and $p < 0.015$, EDIII-A) (**Figure 4.4C**); however, the viremia levels were not significantly different from each other. In the primary and secondary lymphoid organs, the mice administered either MBP or EDIII-A immune serum showed significant reductions in viral load as compared to the NMS-recipient groups. In the lymph node, the animals receiving EDIII-A serum had statistically elevated titers as compared to MBP group ($p = 0.0285$) while both groups demonstrated reduced viral loads as compared to NMS-recipient mice (**Figure 4.4D-F**).

We next assessed whether EDIII-depleted serum transferred at equivalent neutralizing titer (EDIII-B) as MBP-depleted serum (MBP) would be more protective than EDIII-depleted serum of lower *in vivo* neutralizing titer (EDIII-A). Thus, we transferred EDIII-depleted (EDIII-B) serum such that the average NT_{50} titer of the circulating antibodies was 63 (**Figure 4.4B**) and comparable to animals receiving MBP-depleted serum (MBP, NT_{50} average, 67). Animals receiving MBP-B serum had significantly reduced viral loads as compared to NMS-recipient mice, and a reduction in viral load was observed in the lymph node ($p = .055$; **Figure 4.4E**) and bone marrow ($p = 0.13$, **Figure 4.4F**) as compared to EDIII-A recipient mice. There was no difference in viral load comparing the EDIII-B to the MBP-A recipient mice. Taken together, this data indicates that when anti-EDIII antibodies are present, they confer no additional benefit than serum depleted of anti-EDIII antibodies, but of the same neutralizing potency. While the 30% reduction in neutralization titer observed in the EDIII-A recipient mice resulted in a significant increase in lymph node viral load as compared to both MBP-A and EDIII-A recipient

mice, it did not result in increased viral load changes in other organs. Thus, this data demonstrates that even in the absence of EDIII antibodies, homologous serum is still potently neutralizing and capable of serotype-specific protection, despite the 30% loss in *in vitro* neutralizing potency.

Neutralization titer, rather than polyvalent serum composition may be a better indicator of potential for enhancement *in vivo*

Infection with one serotype of DENV is expected to confer life-long immunity against the same serotype [18,32]. In contrast, a second infection with a different serotype is the greatest risk factor associated with the development of severe disease [20]. As the anti-EDIII antibodies do not appear to contribute substantially to *in vivo* protection, we next hypothesized that they may be a key component in preventing homologous enhancement. To test this hypothesis, AG129 mice (3-6 per group) were administered equivalent volumes of either MBP-depleted or EDIII-depleted serum or NMS. Pre-infection bleeds were taken 4-6 hours prior to infection to measure the circulating antibody titers by neutralization assay (NT₅₀ titer). As in the protection experiment, the difference in the average NT₅₀ titer between the MBP (73.5) and EDIII-A (53.8) groups was 37%. A fourth group of mice received EDIII-depleted serum of equivalent neutralizing potency (EDIII-B, NT₅₀ average 75) as the MBP group. The control group of mice receiving NMS did not have a measurable NT₅₀ titer (**Figure 4.5A, Table 4.1**).

Mice were infected 24 hours following serum transfer with 10⁴ pfu DENV2 D2S10, a sublethal dose that can be lethal under enhancing antibody conditions, and were followed for morbidity and mortality. Only 1/5 animals receiving the MBP-depleted serum developed significant disease and succumbed and a second developed transient signs of disease (0.5 days in duration). In contrast, 3/4 animals receiving the same volume of EDIII-depleted serum (EDIII-A) all developed severe signs of disease and died by Day 5 (p<0.05 as compared to MBP-depleted serum control) (**Figure 4.5B, Table 4.1**). These data suggest that anti-EDIII antibodies are protecting against enhancement *in vivo*.

To determine whether an increase in neutralization titer of the EDIII-depleted serum would be sufficient to abrogate this enhancement response, we transferred sufficient volumes of EDIII-depleted serum such that the average NT₅₀ titer of the transferred serum *in vivo* was 75 (EDIII-B) and not significantly different from the MBP-depleted serum recipient mice (MBP) (**Figure 4.5A, Table 4.1**). Following a 10⁴ sub-lethal D2S10 infection, none of the three animals receiving the increased volume of EDIII-depleted serum demonstrated signs or symptoms of illness (p<0.075 as compared to the EDIII-A group) (**Figure 4.5B, Table 4.1**). These data suggest that murine anti-EDIII antibodies actively contribute to protection against enhancement when present but anti-EDI/II antibodies, when transferred at sufficiently high neutralizing titers comparable in potency to control MBP-depleted serum, will similarly no longer enhance a sub-lethal DENV2 infection.

DISCUSSION

Many mouse MAbs that strongly neutralize DENV and other flaviviruses bind to well-defined epitopes on domain III of the viral envelope protein. The objective of this study was to determine whether anti-EDIII antibodies were responsible for potent neutralizing activity in immune sera from mice and people exposed to DENV infections. Here, the role of anti-EDIII

antibodies in mouse and human immune sera were studied using *in vitro* cell culture models and an *in vivo* mouse model of DENV infection and disease.

Our initial observations studying human serum confirm previously published results that anti-EDIII antibodies constitute between 5-15% of the serotype-specific neutralizing titer measured *in vitro*. As suggested by the *in vitro* neutralization studies, anti-EDIII-depleted human serum was as protective as control-depleted serum when tested *in vivo*. To further investigate the role of anti-EDIII antibodies in modulating protection *in vivo*, we turned to anti-DENV-immune mouse serum, where we found that anti-EDIII antibodies contributed approximately 34% to *in vitro* neutralization. When murine serum depleted of EDIII antibodies was tested at the same volume *in vivo* as control-depleted serum, we observed a significant increase in replicating virus in the lymph node and a marginal increase in bone marrow, but little difference in other organs. The lack of difference between the two groups is not entirely surprising, as the difference in neutralizing potency between the two sera following *in vivo* transfer was ~ 34%, and this difference was not sufficient to result in a significant loss in protection. When EDIII-depleted serum was transferred at an increased volume (400 μ l) to compensate for the marginal loss in neutralizing titer, protection was observed in that viral titers as in MBP-depleted controls. Taken together, this data indicates that *in vitro*, mouse anti-EDIII antibodies are responsible for ~ 34% of *in vitro* serum neutralizing potency. This reduction in neutralization potency confers only a small loss of protection *in vivo*, and robust protection can be re-established following transfer of an increased volume of additional EDIII-depleted serum, indicating that anti-EDIII antibodies *per se* are not required for homologous protection *in vivo*.

These results indicate that while the neutralizing antibody response may be more skewed towards generating EDIII-specific antibodies in mice than in humans, the anti-EDIII antibodies are not critical for *in vivo* protection. This finding is intriguing, as MAb studies have frequently isolated strongly neutralizing, EDIII-specific antibodies from mice [7,13,14,15,16,17], whereas such MAbs are present but rare in humans [21,25]. While binding studies clearly suggest that mice make a potent anti-EDIII directed response, it appears that a large percentage of these antibodies are non-neutralizing both *in vitro* and *in vivo*. Although the observation that non-EDIII antibodies can potentially neutralize DENV may appear surprising, investigators have recently described murine and human antibodies that bind outside EDIII that potentially neutralize dengue and other flaviviruses [16,33]. Confirmation that recombinant EDIII protein was properly folded was confirmed using a panel of MAbs directed to critical EDIII epitopes [26]. However, it is possible that some antibodies may bind to oligomeric epitopes that straddle either EDII and EDIII or EDI and EDIII when the virion is in its mature confirmation.

Although a 34% difference in the average NT₅₀ titers was observed between the mice receiving MBP and EDIII-A depleted serum, this difference was not statistically significant. Despite this, there was a clear phenotypic change in *in vivo* outcome when the animals were challenged with an enhancing dose of DENV2 in that 75% of animals receiving EDIII-A serum succumbed, while only 17% of mice receiving MBP control serum died. These data suggest that both antibody composition and neutralizing potency of transferred serum may be important factors in development of enhancement. An increase in the NT₅₀ titer of the EDIII-depleted serum (EDIII-B) to an average titer identical to control, MBP-depleted serum eliminated enhancement *in vivo*, implicating that neutralizing potency of the transferred serum, rather than antibody composition, is a more important correlate of enhancement *in vivo*. Observations similar to this in maternal-infant clinical studies have similarly suggested a relationship between reduced neutralization titers against the infecting serotype and development of severe disease

[34,35,36]. Additional experiments exploring synergistic interactions between EDIII-specific MAbs (anti-lateral ridge or A strand) and other epitopes may provide further insight into the role of EDIII antibodies in the context of polyvalent immune responses. Studies exploring the composition of the antibody repertoire and how the ratio of antibodies targeting different epitopes changes with time following the primary exposure to DENV may also be of interest, especially in cases of waning primary immune responses where years have elapsed since initial exposure [24,25]. Given the potential role for anti-EDIII antibodies to prevent homologous enhancement and their minimal contribution to serotype cross-reactive interaction, the proposal to use EDIII protein as a vaccine is quite intuitive. Indeed, Block *et al* (2010) have initially tested this hypothesis using EDIII-based protein vaccines and found that vaccination of mice with tetravalent EDIII protein produced antibodies that were both non-neutralizing and neutralizing [37]. When tested for enhancement activity *in vitro*, these antibodies were less enhancing over a much smaller range of serum dilutions than comparable polyvalent mouse serum raised against DENV virions.

Using a murine DENV disease model, we analyzed the relationship between immune DENV serum and disease severity in the absence of additional contributing factors such as long-lived plasma cells, memory B cells, or the cell-mediated immune response. We have demonstrated that anti-EDIII antibodies contribute only marginally to *in vivo* protection, such that the remaining components of the antibody repertoire (anti-EDI/II or anti-virion antibodies) are protective at a high neutralizing titer. While neither mice nor humans appear to make large amounts of functional anti-EDIII antibodies in response to natural infection, these data suggest that when present, anti-EDIII antibodies contribute to protection against enhancement. Vaccines designed to skew the immune response towards highly neutralizing and serotype-specific epitopes on the surface of EDIII would be predicted to be quite efficient in protecting against homotypic re-infection. Further evaluation of the correlation between neutralization titer and disease severity in the context of prospective pediatric and maternal-antibody cohort studies will be most beneficial to the long-term safety and efficacy of tetravalent DENV vaccines.

MATERIALS AND METHODS

Viruses and cell lines. All viruses was propagated in *Aedes albopictus* cell line C6/36 (American Type Culture Collection) and titered by plaque assay on baby hamster kidney cells (BHK21, clone 15) [38]. DENV2 D2S10 was derived as described in [39]. DENV1 West-Pac, DENV3 CH53489 and DENV4 TVP360 were obtained from the National Institutes for Biological Standards (Hertfordshire, UK). U937 DC-SIGN (A. de Silva, University of North Carolina, Chapel Hill) and K562 cells were used for neutralization and enhancement assays, respectively. Both cells lines were grown in RPMI media (Invitrogen) at 37°C in 5% CO₂.

DENV immune human Serum Convalescent DENV immune sera were obtained from volunteers who had experienced natural DENV infections during travel abroad. The protocol for recruiting and collecting blood samples from people was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill. Written informed consent was obtained from all subjects before collecting blood.

AG129 Mouse Infections. AG129 mice [40] were bred at the University of California, Berkeley. All procedures were pre-approved and conducted according to UC Berkeley Animal Care and Use Committee.

Production of mouse anti-DENV serum. AG129 mice were infected intra-peritoneally with 10^5 pfu DENV2 PL046. Six to eight weeks post infection, mice were sacrificed and whole blood collected by terminal cardiac puncture. Serum was isolated from whole blood by centrifugation, heat inactivated, and stored at -80°C .

In vivo protection experiments. AG129 mice were administered either mouse or human anti-DENV2 immune serum in a final volume of 400-500 μl 24 hours prior to infection with an intravenous (i.v.) sub-lethal, 10^3 pfu dose of DENV2 D2S10.

In vivo enhancement experiments. AG129 mice were administered either mouse anti-DENV2 immune serum in a final volume of 500-900 μl 24 hours prior to infection with an iv sub-lethal, 10^4 pfu dose of DENV2 D2S10.

Depletion of mouse and human DENV2 immune serum.

Recombinant EDIII protein fused to maltose binding protein (MBP-EDIII) was made as previously described using dengue virus type 2 strain S16803 (1) for human DENV-immune serum and strain 16681 for mouse DENV-immune serum. EDIII antibodies from mouse and human sera were depleted according to Wahala *et al* (2010) with minor modifications (2). Briefly, 250 μl of amylose resin was coated with 350 μg of EDIII or MBP control protein in column buffer. After washing, the resin was blocked with 8% NHS or NMS and undiluted DENV2 immune sera were incubated with MBP-EDIII resin for 1.5 hours. Repeated depletion cycles were performed until all of the EDIII-reactive antibodies were removed. The same serum was depleted in parallel with MBP coated resin as a control. ELISA confirmed complete depletion of EDIII reactive antibodies as previously described (2).

Quantification of virus in tissue by plaque assay and quantitative RT-PCR. Tissue viral load was measured by plaque assay as previously described [38] and was expressed as pfu/g from in solid tissue and as pfu/ 10^9 in bone marrow cells. Samples of all tissue were saved in RNA later (Ambion) and RNA extracted using an RNeasy Mini kit (Qiagen). Serum was extracted from whole blood by centrifugation, and RNA extracted using a Qia-Amp Viral Recovery RNA kit (Qiagen). Serum viremia levels and tissue viral load were measured as described in Balsitis *et al* (2010)[31]. Viral load was expressed as either plaque-forming equivalents/ μg GAPDH (tissue) or pfu (eq)/ mL (serum).

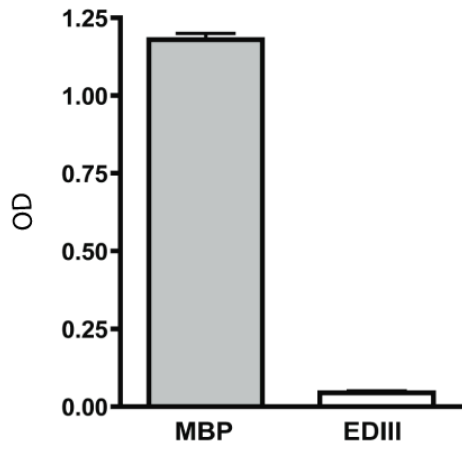
In vitro Neutralization and Enhancement Assays. Four to six hours prior to D2S10 infection, retro-orbital eye bleeds were performed and 100 μl of whole blood obtained and processed for serum as described above. Pre-transfer serum or serum obtained from the retro-orbital eye bleeds was subsequently analyzed for neutralization potency using U937 DC-SIGN cells as described and enhancing activity using K562 cells. In both assays, serum was diluted either 1:5 (retro-orbital bleeds) or 1:10 (pre-transfer serum) and titrated in 8, 3-fold dilutions prior to the addition of DENV2 D2S10 virus followed by a 45 minute incubation. U937 or K562 cells were then infected for 2 hours, washed and resuspended in RPMI media. Twenty-four hours (U937 DC-SIGN cells) or 48 hours (K562 cells) following infection, the cells were washed, fixed in 2% paraformaldehyde (Ted Pella, INC), permeabilized with saponin (Sigma Aldrich) and intracellularly stained with 4G2-Alexa488 (Invitrogen). Percent infection was determined by

flow cytometry using the BD LSR- Fortessa flow cytometer. Relative infection was calculated using the last dilution of each serum sample (1:21,870) as its own denominator to minimize variation with staining. NT₅₀ titers were calculated as described in Balsitis, 2010[31]. Peak enhancement titer (PET) were calculated by plotting raw percent infection on the y-axis, and log serum reciprocal dilution on the x-axis. A Gaussian distribution was used to fit each enhancement curve, and the amplitude identified. This data point was then used to derive the log-reciprocal serum dilution corresponding to the amplitude and reported as the PET.

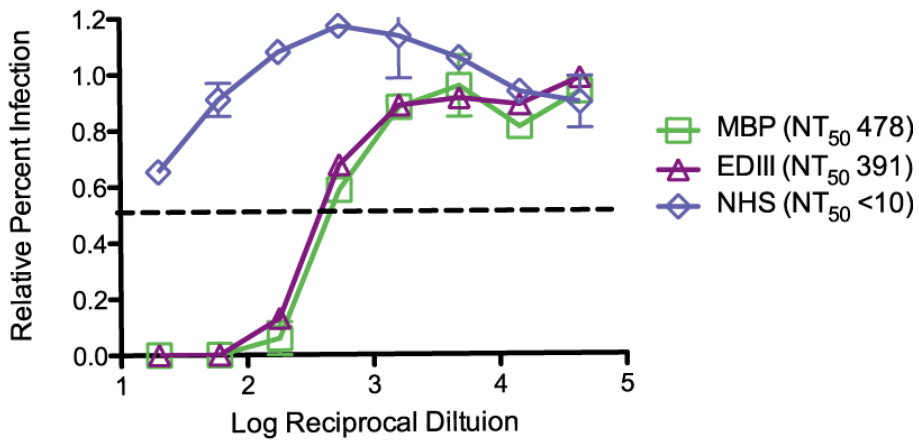
Statistical Analysis Nonparametric analysis of neutralization titers, PET, tissue viral load and serum viremia data were compared using a Wilcoxon ranksum analysis. Time to onset of morbidity was assessed using a logrank analysis. All data was analyzed in Stata v10 (College Station, Texas).

FIGURES

A ELISA



B Neutralization



C Enhancement

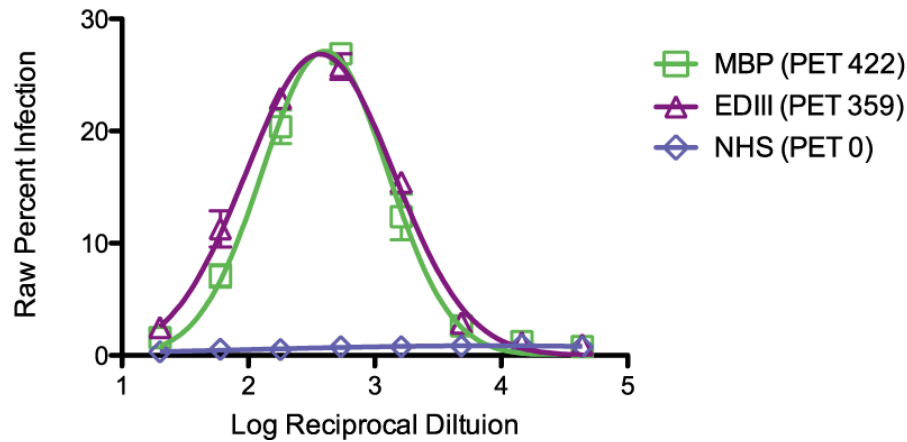
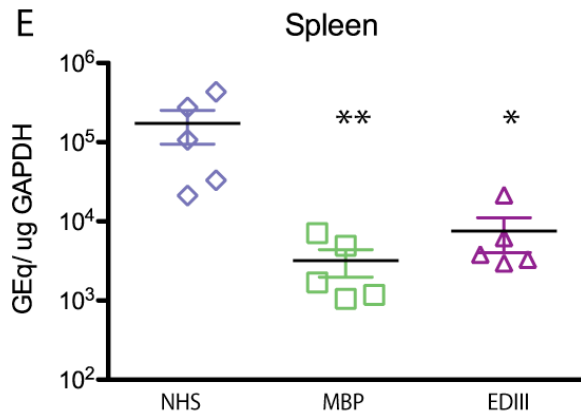
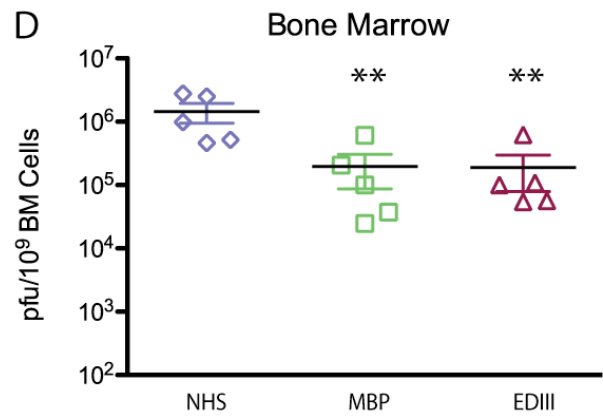
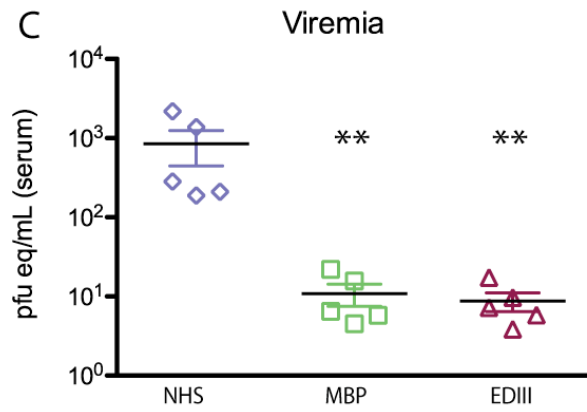
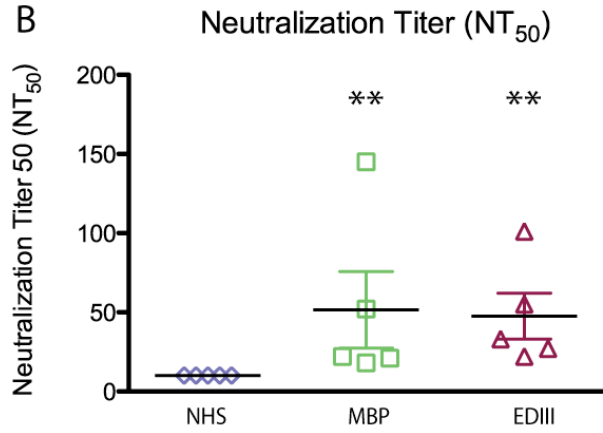
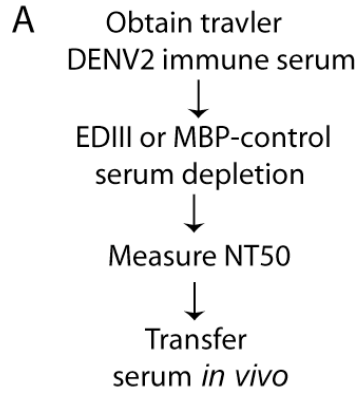


Figure 4.1 Human E Domain III (EDIII) antibodies constitute a small portion of the polyvalent serum neutralizing antibody response. Human DENV2 immune serum was obtained from a DENV-immune individual enrolled in the University of North Carolina dengue traveler study and was subsequently depleted of anti-EDIII antibodies using either EDIII or MBP-control beads. **A)** ELISA plates were coated with DV2 EDIII or MBP protein (negative control). Undiluted EDIII- and MBP-depleted human sera were tested for reactivity against with DENV2 EDIII, and absolute OD value at 405 nm is shown on the Y axis. **B)** Neutralization against the DENV2 D2S10 strain used for *in vivo* infections was measured by a neutralization assay using human U937 DC-SIGN cells. Relative percent infection is shown on the x-axis and log reciprocal dilution of the serum is shown on the y-axis. **C)** Enhancement against the same DENV2 D2S10 strain was measured using K562 cells, where raw percent infection is measured on the y-axis and log reciprocal serum dilution is on the x-axis. Each panel is representative of two or three separate experiments.



* $p < 0.05$ as compared to NHS
 ** $p < 0.01$ as compared to NHS

Figure 4.2 Human anti-EDIII antibodies are not required for protection *in vivo*. AG129 mice (n=5 per group) were administered either human non-depleted (“DENV2”), MBP-depleted (“MBP”) or EDIII-depleted (“EDIII”) DENV2 immune serum corrected for the dilution factor associated with the depletion procedure, non-immune human serum (“NHS”) 24 hours prior to infection with 10^3 pfu D2S10 iv. **A)** Schematic of the anti-DENV2 EDIII-depletion and *in vivo* transfer experimental design. **B)** Retro-orbital eye-bleeds were taken 4-6 hours prior to DENV infection, and circulating neutralizing antibodies were measured against the infecting virus. **C-E)** Animals were sacrificed four days post-infection, and viral load was measured in the serum (**C**), bone marrow (**D**) and spleen (**E**). The data points presented here are combined from two separate experiments.

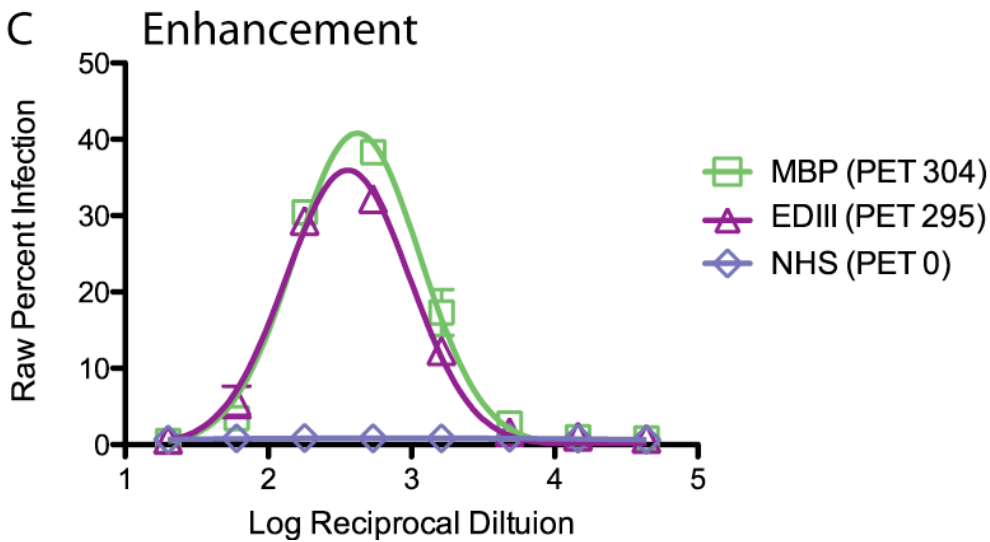
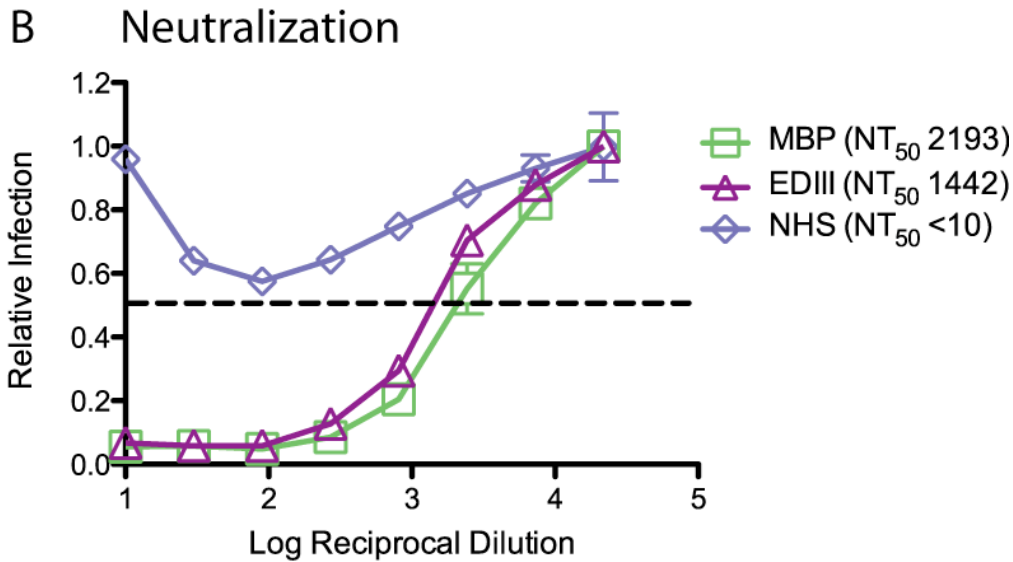
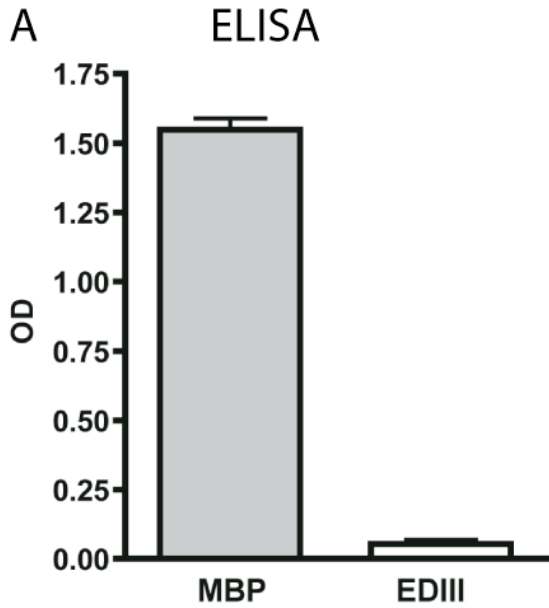
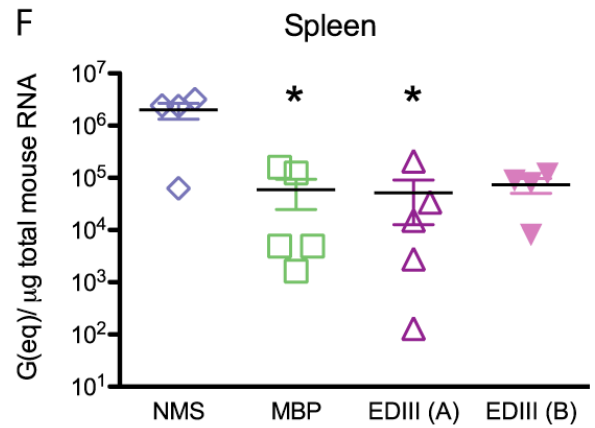
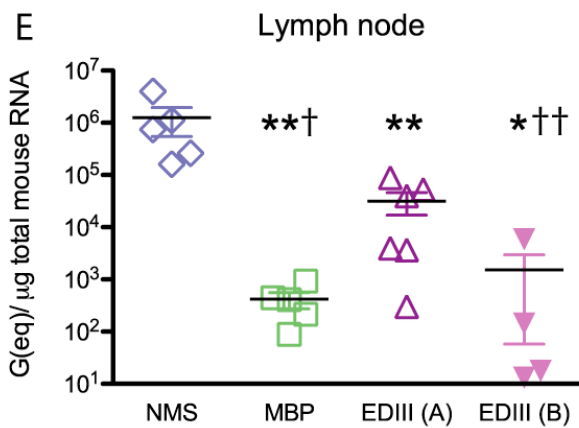
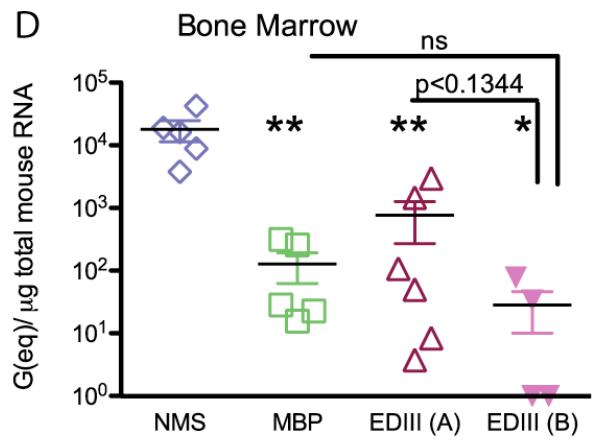
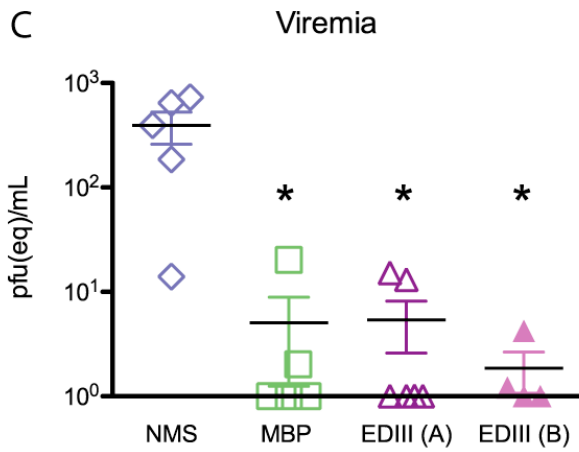
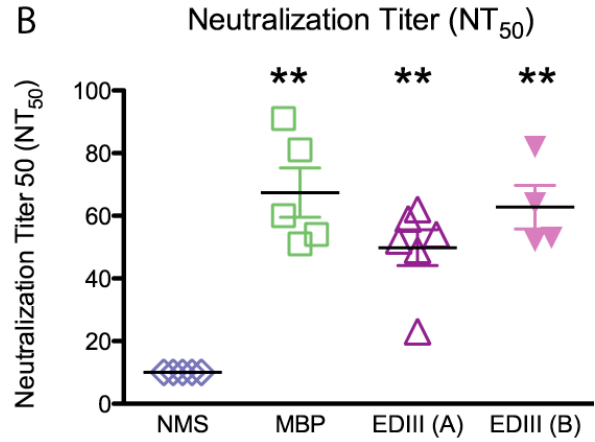
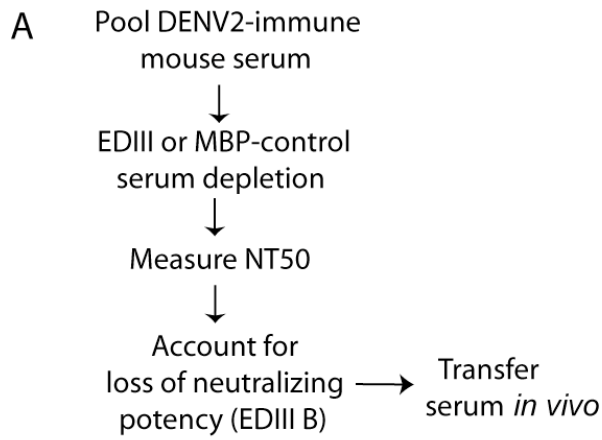


Figure 4.3 Mouse anti-DENV EDIII antibodies contribute to serotype-specific neutralization but not enhancement *in vitro*. Pooled anti-DENV2 PL046 serum was collected 8-10 weeks following intra-peritoneal infection of AG129 mice and was depleted of anti-EDIII antibodies using EDIII or control MBP protein. **A)** ELISA plates were coated with DV2-EDIII and MBP protein (negative control). Undiluted EDIII- and MBP-depleted mouse sera were tested for reactivity with DENV2 EDIII, and absolute OD value at 405 nm is shown on the Y axis. **B)** Neutralization against the DENV2 D2S10 strain used for *in vivo* infections was measured by neutralization assay in U937 DC-SIGN cells. Relative percent infection is shown on the x-axis, the log reciprocal dilution of the serum is shown on the y-axis and a dashed line indicates 50% infection. **C)** Enhancement against DENV2 D2S10 was measured in K562 cells, where raw percent infection is shown on the y-axis and log reciprocal serum dilution is shown on the x-axis. Each panel is representative of two or three separate experiments.



* p<0.05 as compared to NMS
 ** p<0.01 as compared to NMS
 † p<0.05 as compared to EDIII(A)
 †† p<0.055 as compared to EDIII(A)

Figure 4.4 Murine EDIII-specific antibodies contribute only marginally to protection *in vivo*. AG129 mice (n=3-5/group) were administered either 250 μ l (MBP-A) of MBP-depleted serum or 250 μ l (EDIII-A) or 400 μ l (EDIII-B) of EDIII-depleted serum or 400 μ l non-immune mouse serum (NMS) 24 hours prior to infection with 10^3 pfu D2S10 iv. **A)** Schematic of the anti-DENV2 EDIII-depletion and *in vivo* experimental design. **B)** Sub-mandibular bleeds were taken 4-6 hours prior to infection, and circulating neutralizing antibodies were measured against the infecting virus. **C-E)** Animals were sacrificed four days post-infection, and viral load was measured in the serum (**C**), bone marrow (**D**), lymph node (**E**), and spleen (**F**). The data points presented here are combined from two separate experiments.

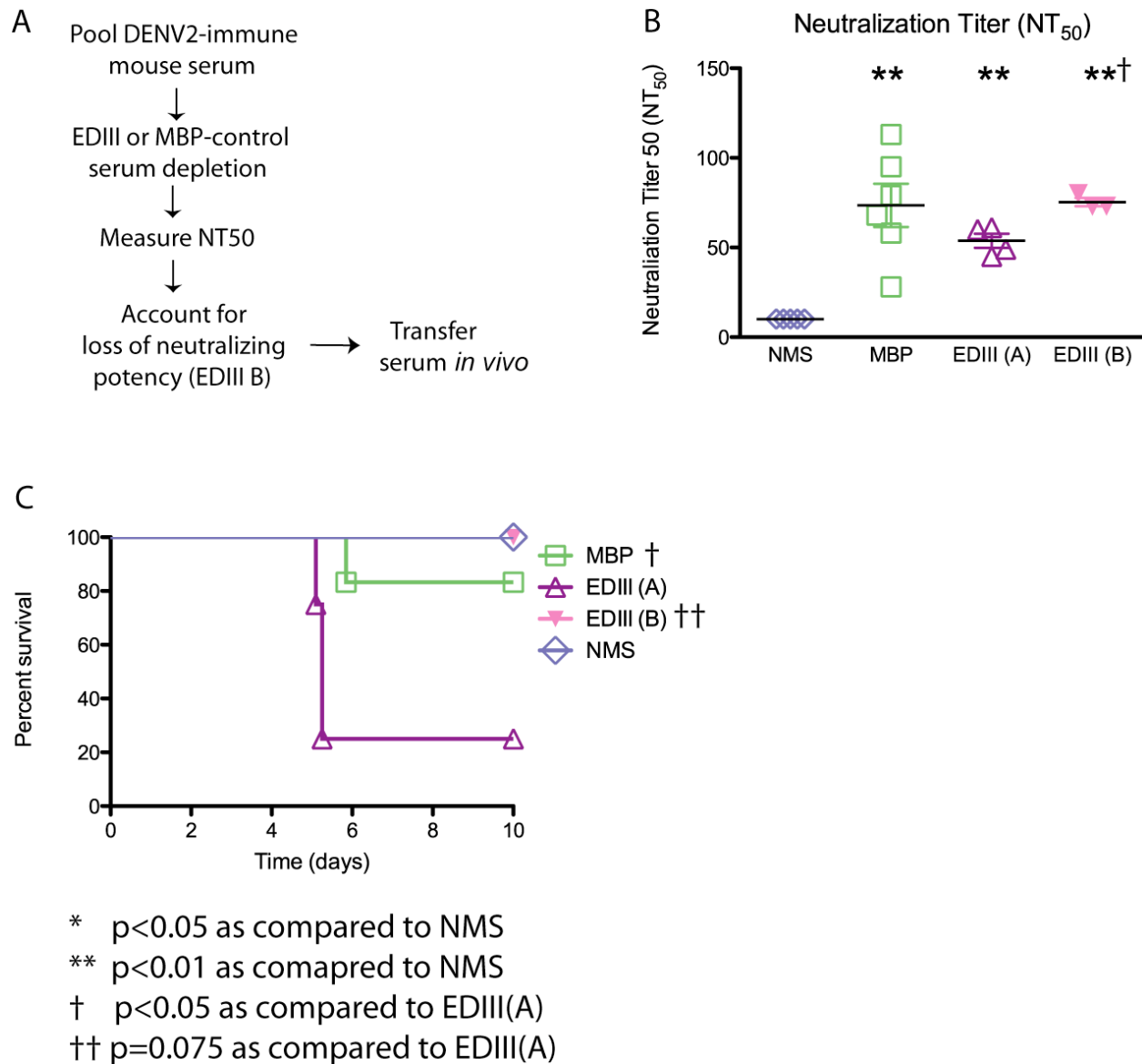


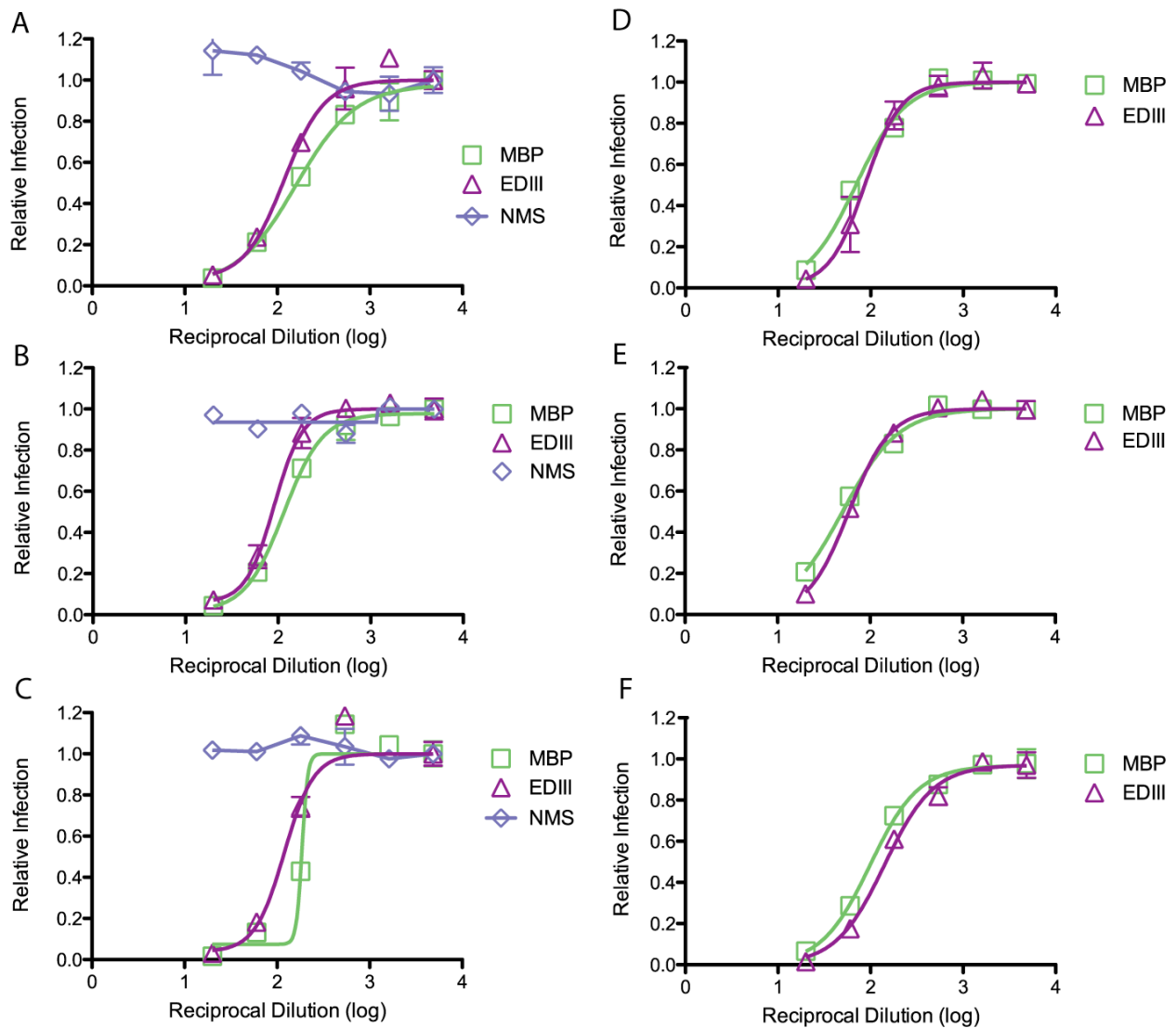
Figure 4.5 Anti-EDIII antibodies prevent enhancement *in vivo*

AG129 mice ($n=3-6$ /group) were administered either 50 μ l (DENV2-A) of non-depleted DENV2 serum, 250 μ l of MBP-depleted serum (MBP) or 250 μ l (EDIII-A) or 400 μ l (EDIII-B) or 400 μ l of non-immune mouse serum (NMS) prior to a sublethal 10^4 pfu DENV2 D2S10 iv infection. **A)** Schematic of the anti-DENV2 EDIII-depletion and *in vivo* transfer scheme. **(B)** Twenty-four hours prior to infection, sub-mandibular bleeds were taken and the serum was used to measure neutralization in U937-DCSIGN cells. **(C)** Animals were followed for morbidity and mortality for 10 days following infection. Survival outcome is depicted in a Kaplan-Meier survival curve. The data points presented here are combined from two separate experiments, except for the EDIII-B condition, which could only be tested once.

Table 4.1

	Neutralizing Titer (NT ₅₀ Mean ± sd)	Morbidity	Mortality	Mortality p-value*
NMS	10	0/5	0/5	na
MBP	74.5	2/5	1/5	---
EDIII-A	53.8	3/4	3/4	.0389
EDIII-B	75	0/3	0/3	.075

* p-value calculated in comparison to MBP



	Human DENV2-immune serum		Mouse DENV2-immune serum	
	MBP-depleted	EDIII-depleted	MBP-depleted	EDIII-depleted
DENV1	69	86	164	115
DENV3	50	59	116	87
DENV4	102	147	184	121

Supplementary Figure 4.1 Mouse (A-C) and human (D-F) DENV2-immune serum was tested for heterotypic neutralization *in vitro*. Neutralization titer against DENV1 strain West-Pac (A,D), DENV3 strain CH53489 (B,E), or DENV4 strain TVP-360 (C,F) was assessed using U937-DCSIGN cells.

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CHAPTER 5

THE ROLE OF SEROTYPE-SPECIFIC AND SEROTYPE CROSS-REACTIVE ANTIBODIES IN MEDIATING PROTECTION AND ENHANCEMENT *IN VIVO*

INTRODUCTION

Mouse monoclonal antibody studies of flaviviruses, begun in 1982 [1,2,3], have contributed greatly to our current understanding of the structure of the DENV virion [4] and the critical epitopes targeted by the humoral immune response [5,6,7,8]. Only in the past few years have researchers begun to dissect the human polyclonal immune response to DENV. In contrast to the conclusions drawn with mouse polyvalent immune serum, the human antibody repertoire is largely cross-reactive [9,10,11,12], and targets epitopes such as prM/M [10], the EDII fusion loop, and EDIII A strand epitope [13]. One major difference between the antibody repertoire in mice versus humans appears to be the predominance of serotype-specific, neutralizing antibodies targeting EDIII in murine serum [14]. It remains unclear what epitopes induce a potent neutralizing antibody response in humans [15], and whether these antibodies are serotype-specific or serotype-cross-reactive in nature. Another major unanswered question is whether antibodies that induce protection and those that induce enhancement are separate pools of antibodies or whether the same antibodies induce protection and enhancement (i.e., at different dilutions).

Original antigenic sin suggests that a complex interaction occurs between the adaptive immune response primed against the first DENV infection and the distinct DENV serotype responsible for the subsequent infection [16,17]. Upon secondary infection, the sub-complex and complex-specific antibodies capable of binding the new virus may be insufficient to neutralize the viral infection, and may instead facilitate enhanced viral uptake through FcR and contribute to the development of more severe disease [18]. As candidate live attenuated and subunit vaccines proceed through early clinical trials, it becomes even more imperative to understand how different components of the immune response both prevent and facilitate DENV infection [19,20,21,22,23]. In the context of a vaccine, we must understand how the antibody response contributes to protection, but of equal importance, how to prevent antibody-dependent enhancement. Understanding *in vitro* correlates of *in vivo* outcome is a critical step towards determining whether an immune response generated against a natural infection, or vaccination, is potent enough to neutralize infection and to prevent enhancement *in vivo*. Further elucidation of both monotypic and polytypic immune responses is necessary to counter well-placed fears associated with DENV vaccination. In this chapter, we attempt to dissect the serotype-specific and serotype-cross-reactive components of the human polyvalent humoral immune response and identify their contribution to both protection and enhancement of DENV infection *in vivo* using our mouse model of DENV infection and disease.

RESULTS

DENV2 homotypic but not heterotypic anti-DENV3 serum reduces viral load *in vivo* despite equivalent *in vitro* neutralizing titers

We first wanted to determine whether there were measurable differences in the ability of human homotypic, as compared to heterotypic polyvalent serum, to protect against DENV2 infection. To that end, we administered 10-fold different doses of either α DENV2 or α DENV3 immune serum such that the neutralization capacity of the serum (measured using the U937 DC-SIGN neutralization assay) from the two groups was not significantly different (**Figure 5.1A**). Animals were subsequently infected with a 10^3 pfu dose of DENV2 D2S10 and followed for four days, after which they were sacrificed and serum viremia levels measured. Despite circulating

neutralization titers being comparable between the two groups of mice, only the group having received the α DENV2-immune serum demonstrated reduced serum viremia levels ($p < 0.05$, **Figure 5.1B**). This data allowed us to conclude that qualitative differences existed between the serotype-specific and cross-reactive antibody response.

Depletion of serotype-cross-reactive antibodies does not affect protective capacity of serotype-specific, anti-DENV immune serum *in vivo*

Having determined that homotypic α DENV-immune serum was capable of robust protection, while heterotypic serum was not, despite equivalent circulating antibody titers *in vitro*, we next wanted to determine what fraction of the polyvalent serum may be contributing to the protection observed *in vivo*. In collaboration with Dr. Aravinda de Silva's group at the University of North Carolina, Chapel Hill, we depleted human α DENV2-immune serum of cross-reactive antibodies using DENV3 virions (DENV2-B), of serotype-specific antibodies targeting the DENV2 virion (DENV2-C), or of non-specific antibodies using BSA coated to beads (DENV2-A). These depleted serum samples were initially evaluated *in vitro* and then further analyzed *in vivo*. ELISA assays performed by the de Silva laboratory demonstrated that the DENV3-depleted serum had lost all ability to bind to DENV1, DENV3, and DENV4 virions (hereafter referred to as "DENV1,3,4-depleted serum") but retained the ability to bind to DENV2. In contrast, DENV2-depleted serum lost the ability to bind to the DENV2 virion, as measured by ELISA assay (data not shown). Initial testing in our laboratory of the neutralization capacity of control and DENV1,3,4-depleted serum demonstrated no change in the NT₅₀ titer, whereas depletion with DENV2 virions resulted in an 85%-90% loss in serum neutralizing potency (**Figure 5.2A and 5.3A**).

We next tested whether the phenotypes observed with the DENV1,3,4-depleted and DENV2-depleted serum *in vitro* would yield a similar phenotype in an *in vivo* protection experiment. To that end, we transferred equivalent volumes of either control-depleted NHS (NHS-A), DENV1,3,4-depleted NHS (NHS-B), control-depleted α DENV2 serum, DENV1,3,4-depleted α DENV2 serum, or DENV2-depleted α DENV2 serum into AG129 mice ($n = 3/\text{group}$) 24 hours prior to a 10^3 pfu DENV2 D2S10 infection. Retro-orbital eye-bleeds were collected 4-6 hours prior to infection, and the serum was used to measure circulating antibody titers against the infecting virus. Mice administered either NHS-A or NHS-B serum did not demonstrate measurable neutralizing titers, and two of three animals receiving α DENV2-C serum (DENV2-depleted α DENV2 serum) similarly displayed neutralizing titers below the limit of detection. Surprisingly, serum from the third animal was capable of neutralizing DENV2 D2S10 *in vitro*. Animals receiving either the α DENV2-A (control-depleted α DENV2 serum) or α DENV2-B serum (DENV1,3,4-depleted α DENV2 serum) demonstrated measurable neutralizing titers against DENV2 D2S10 that were significantly greater than either the NHS-A or NHS-B groups (**Figure 5.2B**). Four days following infection, animals receiving either the NHS-A or NHS-B serum demonstrated robust viremia and DENV infection in the bone marrow and spleen. In contrast, animals receiving either α DENV2-A or α DENV2-B serum demonstrated a complete reduction in serum viremia, a 3-log reduction in the spleen, and a 1-2 log-fold reduction in bone marrow viral load (**Figure 5.2C-E**). Although the *in vitro* neutralization data suggested that the mice receiving the α DENV2-C serum (DENV2-depleted α DENV2 serum) would not be protected, we observed moderate, but significant reduction in serum viremia levels and tissue viral load in the spleen and bone marrow (**Figure 5.2C-E**).

As the DENV2-depleted α DENV2 serum was surprisingly protective *in vivo*, we repeated the experiment with a second human α DENV2-immune sample, and again the serum was either control-depleted (α DENV2-A) or depleted using DENV2 virions (α DENV2-C). As before, DENV2-depleted α DENV2 serum demonstrated a 90% reduction in neutralizing potency as compared to control-depleted serum *in vitro* (**Figure 5.3A**). When the serum was tested for protective capacity *in vivo*, the control-depleted α DENV2 serum was completely protective, whereas the animals receiving DENV2-depleted α DENV2 serum again displayed intermediate viremia and spleen viral load levels. To eliminate the possibility that the human DENV2-immune serum was simply extremely neutralizing *in vivo*, we diluted the control-depleted α DENV2 serum 1:10, such that it retained only 10% of the original neutralizing capacity to compare with the DENV2-depleted α DENV2 serum (α DENV2-C) that exhibited 10% residual neutralizing potency against DENV2 D2S10. Thus, a third group of animals (α DENV2-D) was administered control-depleted serum diluted 1:10 in NHS. Two of three animals administered α DENV2-D serum did not have measurable neutralization titers following *in vivo* transfer, but all three animals displayed comparable viremia and spleen viral load levels to the NHS-A animals (**Figure 5.3D**), suggesting that control-depleted α DENV2 serum diluted 1:10 was not protective *in vivo*. Taken together, this data suggests that depletion of α DENV2-immune serum with DENV2 virions reduces, but does not eliminate, neutralizing potency *in vitro*. Additionally, the 10% residual neutralizing potency found in the DENV2-depleted α DENV2 serum is significantly more neutralizing than 10% of the control-depleted serum alone, indicating that an important component of the protective capacity of DENV2-immune serum, as measured by *in vitro* neutralization assays, is not eliminated following depletion of antibodies binding to the DENV2 virion. Further, the *in vitro* neutralizing potency estimated for this remaining fraction of antibodies does not correlate with *in vivo* protection as has been previously observed. Identification of the target of this residual protective component in DENV2-depleted α DENV2 serum is currently underway.

***In vitro* assessment of both neutralization (NT₅₀) and enhancement (PET) can predict *in vivo* outcome**

Monoclonal antibody (MAb) studies have suggested that the 50% neutralization titer (NT₅₀) of a mAb correlates with the peak enhancement titer (PET) against the same virus. To determine whether a similar relationship exists with human monotypic sera, we determined the NT₅₀ and PET of four monotypic sera (one serum sample from a primary DENV infection with each serotype) against DENV2. In this experiment, the three cross-reactive sera (α DENV1, α DENV3 and α DENV4) displayed moderate neutralization of DENV2, with NT₅₀ titers between 44 and 82 in a neutralization assay using U937 cells with the DC-SIGN DENV attachment factor. The PET was subsequently measured in K562 cells, a cell line that displays Fc γ RIIA on the surface, but is not naturally permissive for DENV infection in the absence of anti-DENV antibodies. One NT₅₀/PET serum pair had identical NT₅₀ and PET titers (α DENV3), while the other two PET titers were 2.5-3.5 times greater in magnitude than the NT₅₀ titer. The α DENV2 serum sample was strongly neutralizing and had comparable NT₅₀ and PET titers. Comparing the α DENV2 serum to α DENV1, α DENV3 or α DENV4 serum, the α DENV2 serum was ~20-40 fold more neutralizing and 10-40 fold less enhancing *in vitro* (**Figure 5.4A-D**). Combined, these data support the observation that serotype-specific serum must be significantly diluted in order to be enhancing. In contrast, serotype-cross-reactive serum is much more enhancing and requires

substantially less dilution. Further, the relationship between NT₅₀ and PET appears to be relatively consistent in both serotype-specific, and serotype-cross-reactive scenarios.

Having observed that serotype-specific serum is 10-40 fold less enhancing *in vitro* than serotype cross-reactive serum and must be substantially diluted in order to become enhancing, we next tested whether a similar relationship could be observed *in vivo*. To test this hypothesis, we transferred α DENV2 human immune serum at dilutions of 1:12.5 (200 μ l transferred), 1:100 (25 μ l transferred) or 1:400 (6.25 μ l transferred) (assuming the total blood volume of an AG129 mouse to be 2.5 mL) 24 hours prior to a sub-lethal infection with DENV2 D2S10. In support of the hypothesis derived from our *in vitro* data, mice receiving a 1:12.5 dilution of α DENV2 serum did not develop enhanced disease, whereas mice receiving a 10-fold (1:100 dilution) or 40-fold (1:400 dilution) more diluted dose demonstrated between 40% ($p < 0.075$ as compared to 200 μ l α DENV2) and 50% survival, respectively (**Figure 5.4E**). We next tested whether cross-reactive serum, identified as more enhancing *in vitro*, required less dilution than serotype-specific serum in order to develop enhanced disease. As suggested by the *in vitro* enhancement data, α DENV3 serum was the most enhancing, meaning that it was diluted the least to yield peak enhancement (PET: 44, **Figure 5.4C**). In agreement with this observation *in vitro*, α DENV3 serum diluted 1:25 (100 μ l) was not enhancing *in vivo*, while α DENV3 serum diluted 1:12.5 (200 μ l) was 50% lethal (**Figure 5.4E**). As this particular serum was limited in quantity, we were not able to test less dilute/higher volumes of the serum to establish whether these concentrations would be even more enhancing *in vivo*. The remaining two cross-reactive human α DENV-immune sera, α DENV1 and α DENV4, were 6-fold and 2.5-fold less enhancing *in vitro*, respectively, than the α DENV3 serum. In agreement with this, when tested at a dilution of 1:12.5 (200 μ l) *in vivo*, each serum was potently enhancing ($p < 0.05$ for α DENV1 and $p < 0.01$ for α DENV4 as compared to mice receiving NHS) (**Figure 5.4F**). Further analysis is needed to determine whether more dilute doses such as 1:25 would still be enhancing *in vivo*. As it stands, these *in vivo* data support a strong relationship between relative *in vitro* PET (and NT₅₀) and *in vivo* enhancement.

Depletion of cross-reactive anti-DENV antibodies eliminates enhancement *in vivo*

Having studied the components of the immune response required for *in vivo* protection, we next wanted to determine whether serotype cross-reactive antibodies were the main contributors to *in vivo* enhancement. To ask this question, we depleted α DENV3 serum of all cross-reactive antibodies targeting the DENV2 virion, with serum depleted using a control protein analyzed in parallel. When tested *in vitro*, DENV2-depleted α DENV3 serum displayed a complete loss in binding to the three other virions when tested by ELISA and no change in neutralizing potency as compared to the control or non-depleted α DENV3 serum (**Figure 5.5B**), but was no longer capable of neutralizing DENV2 (**Figure 5.5A**). When transferred prior to a sub-lethal, enhancing (10^5 pfu) dose of DENV2 D2S10, mice receiving the DENV2-depleted α DENV3 sera did not develop enhanced disease, whereas animals receiving control-depleted and non-depleted serum displayed 40% and 80% mortality rates (non-depleted serum, $p < 0.05$ as compared to DENV2-depleted α DENV3 serum), respectively (**Figure 5.5C**). While only 2/5 control-depleted α DENV3 mice succumbed to a lethal infection, all developed measurable signs of illness. In contrast, none of the mice receiving the DENV2-depleted α DENV3 immune serum developed any signs of disease ($p < 0.05$ as compared to DENV2-depleted α DENV3) (**Figure 5.5D**). This data suggests that two different antibody populations indeed exist in polyvalent

serum, one that contributes largely to neutralization and protection, while a second that is mainly enhancing and contributes to the development of severe disease *in vivo*.

DISCUSSION

Understanding relevant *in vitro* correlates of *in vivo* outcome is an essential component for development of a safe and effective DENV vaccine. In this chapter, we have identified robust *in vitro* correlates of both protection and enhancement in a mouse model of DENV infection and disease using monotypic human α DENV immune serum. As these observations were made using only monotypic serum, it would be worthwhile to extend these observations to include an analysis of polytypic responses following two or three DENV infections. Further, longitudinal analysis following individuals living in endemic countries over years would be instrumental in quantifying how the neutralization potency and enhancement capacity of serum changes over time and after secondary DENV infection. Also of interest would be studying serum from individuals vaccinated with either monovalent or tetravalent candidate dengue vaccines to determine whether either neutralization potency or enhancement capacity could be correlated with *in vivo* outcome in our mouse model, ideally to demonstrate that the humoral immune response after vaccination would not promote an enhanced infection in mice.

The depletion of cross-reactive anti-DENV antibodies from DENV2-immune serum, by definition, refers to the removal of all sub-complex and complex-specific antibodies capable of binding to DENV1, DENV3 and DENV4 virions. This will also result in the removal of cross-reactive antibodies that could bind to the DENV2 virion, including, for example, EDII fusion loop and EDIII A strand antibodies. Following depletion, the only antibodies remaining in the serum were serotype-specific antibodies targeting only DENV2. Surprisingly, the cross-reactive depletion did not reduce the neutralization potency of the serum, nor did it negatively affect *in vivo* protection. Further analysis of the depleted serum to determine if all fusion loop and A strand (two predominantly cross-reactive epitopes) antibodies were removed should be completed to further validate the depletion strategy.

As a negative control for the cross-reactive antibody depletion experiment, depletion of serotype-specific antibodies (all antibodies targeting DENV2) was included to demonstrate that depletion of this subset of antibodies, in turn, did effectively eliminate both *in vitro* neutralization and *in vivo* protection. While effective at reducing *in vitro* neutralization potency by 85%-90%, the DENV2 depletion produced serum that was still moderately protective *in vivo*, and this observation was repeated following depletion of a second sample from a different individual. An initial assumption we have made following these experiments is that the antibodies contributing to *in vivo* protection are the same antibodies contributing to the residual 10% neutralization observed *in vitro*. To eliminate the option that human immune serum is highly neutralizing and can be protective even at extremely low titers, we controlled for the *in vitro* 10% neutralization capacity by diluting control-depleted serum 10-fold, but were not able to recapitulate the same protective phenotype *in vivo*. These data suggest that the antibodies remaining in the DENV2-depleted α DENV2 serum, while not neutralizing by traditional *in vitro* neutralization assays, confer measurable protection *in vivo*. An initial hypothesis attempting to identify these antibodies was that they targeted a rarely exposed, “breathing” form of the virion, such that when the virion is in a static state (as it is during the depletion with fixed virions), the epitope(s) are not exposed and the antibodies targeting these epitopes cannot be depleted. To test this hypothesis, additional experiments were performed that included incubating the serum with

DENV virions at higher temperatures and for longer time periods, but these experiments did not eliminate the residual 10% of the antibody population that contributed to *in vitro* neutralization. As this is human immune serum transferred into a mouse model, it is also possible that aberrant interactions between human Fc and mouse FcR could be augmenting the ability of the residual antibodies to mediate protection. Finally, we are currently in the process of testing whether the residual antibody in DENV2-depleted α DENV2 serum targets the DENV NS1 protein, which is secreted from infected cells and generates a small, but measurable antibody response. If it does, we will further deplete DENV2-depleted α DENV2 serum with NS1 protein to determine whether we can eliminate the residual 10% *in vitro* neutralization and remaining *in vivo* protection. This data is consistent with data obtained in our laboratory where mice immunized with NS1 protein and Sigma adjuvant plus CPG were protected against a lethal DENV challenge, while animals vaccinated with BSA, SIGMA adjuvant and CPG were not (SZ, SO, PRB, EH, unpublished data).

Combining observations from both the protection and enhancement experiments, we have been able to prove that depletion of cross-reactive antibodies does not alter the homotypic, protective capacity of α DENV-immune serum, but beneficially eliminates enhancement *in vivo*. Specifically, α DENV3-immune serum depleted of cross-reactive antibodies targeting the DENV1, DENV2 and DENV4 virions eliminated the ability of the serum to neutralize DENV2 virus *in vitro* and abrogated enhancement *in vivo*. These data demonstrate that serotype-cross-reactive antibodies are fundamentally responsible for DENV enhancement and are separable from the serotype-specific antibodies that contribute to neutralization and protection. Within this context, it appears as though serotype-specific antibodies strongly contribute to homotypic neutralization, while a separate category of cross-reactive antibodies mainly facilitate enhancement. We are also in the process of examining whether or not the antibody immune response contributes to serotype cross-reactive protection, and if so, what subsets of antibodies may play important roles in mediating this protective capacity. In summary, we have validated a mouse model of DENV infection and disease to quantify the contribution of different components of the humoral immune response in mediating protection and enhancement *in vivo*. Further studies determining whether antibodies targeting recombinant E protein or alternative epitopes on the virion (including those binding to heterodimeric complexes formed between E protein dimers) will be critical to identifying the main components of the humoral immune response contributing to both protection and enhancement *in vivo*.

MATERIALS AND METHODS

Viruses and cell lines. All viruses were propagated in *Aedes albopictus* cell line C6/36 (American Type Culture Collection) and titered by plaque assay on baby hamster kidney cells (BHK21, clone 15) [24]. DENV2 D2S10 was derived as previously described [25]. DENV1 448 was a gift from Dr. Susie Kliks and DENV3 CH53489 and DENV4 TVP360 were obtained from the National Institute for Biological Safety and Control (Hertfordshire, England). All *in vitro* neutralization assays and *in vivo* infections were performed with non-concentrated virus. U937-DC-SIGN (gift from A. de Silva, University of North Carolina, Chapel Hill) and K562 cells were used for flow cytometry-based *in vitro* neutralization and enhancement assays, respectively. Both cell lines were grown in RPMI medium (Invitrogen) and supplemented with 5% fetal bovine serum (Denville Scientific), 1% penicillin/ streptomycin and 1% hepes at 37°C in 5% CO₂.

Clinical Serum Samples from Dengue Patients. All human DENV-immune serum samples used in these experiments were obtained from A. deSilva (University of North Carolina, Chapel Hill). In a study approved by the Institutional Review Board of the University of North Carolina at Chapel Hill, serum was collected from volunteers who had experienced natural DENV infections during prior travel abroad.

Virion depletion of human DENV-immune serum Human monotypic α DENV-immune serum was depleted using polystyrene microsphere beads adsorbed with purified Vero-grown dengue virus or using beads alone in presence of bovine serum albumin (used as a blocking agent). Three rounds of 2-hour depletions at 37°C on a rocking platform were completed to ensure complete depletion. Efficient depletion was measured using a virion-coated ELISA as described in de Alwis 2011 [12].

AG129 Mouse Infections. AG129 mice [26] were bred at the University of California, Berkeley, Northwest Animal Facility. All procedures were pre-approved and conducted according to UC Berkeley Animal Care and Use Committee guidelines.

In vivo protection experiments AG129 mice were administered 50 μ l DENV-immune serum diluted into a final volume of 250 μ l i.p. 24 hours prior to infection with an intra-venous (i.v.) sub-lethal, 10^3 pfu dose of DENV2 D2S10. A retro-orbital eye bleed was performed four to six hours prior to infection to measure circulating antibody titers. Animals were sacrificed four days following infection and organs and serum were taken to measure viral load.

In vivo enhancement experiments AG129 mice were administered different volumes of serum (specified in figure legend) diluted into a final volume of 400 μ l i.p. 24 hours prior to infection with an intra-venous (i.v.) sub-lethal, 10^4 pfu dose of DENV2 D2S10. Retro-orbital eye-bleeds were taken 4-6 hours prior to infection. Animals were monitored for morbidity and mortality for 10 days following infection.

Quantification of virus in tissue by plaque assay and quantitative RT-PCR. Tissue viral load was measured by plaque assay as previously described [24] and was expressed as pfu/g from in solid tissue and as pfu/ 10^9 in bone marrow cells. Samples of all tissue were saved in RNA later (Ambion) and RNA extracted using an RNeasy Mini kit (Qiagen). Serum was extracted from whole blood by centrifugation, and RNA extracted using a Qia-Amp Viral Recovery RNA kit (Qiagen). Serum viremia levels and tissue viral load were measured as described in Balsitis *et al* (2010) [27]. Viral load was expressed as either plaque-forming equivalents/ μ g GAPDH (tissue) or pfu (eq)/ mL (serum).

DENV neutralization assay. The neutralization titer of each serum sample was measured using the U937-DC-SIGN flow cytometry-based neutralization assay as described in Kraus *et al* (2006) [28]. In brief, serum was diluted 1:10 and eight 3-fold serial dilutions were performed. The diluted serum were mixed with DENV2 D2S10 at an MOI of 0.0125, incubated together for 45 minutes at 37°C, and the mixture incubated with 5×10^4 U937-DC-SIGN cells. Two hours following infection, the cells were washed and resuspended in RPMI medium containing 5% FBS (Denville Scientific), 1% penicillin/ streptomycin and 1% hepes. Twenty-four hours following infection, the cells were washed, fixed in 2% paraformaldehyde (Ted Pella, INC), permeabilized with saponin (Sigma Aldrich), and stained intracellularly with 4G2-Alexa488 (Invitrogen). Percent infection was determined by flow cytometry using the BD LSR- Fortessa

flow cytometer. Relative infection was calculated using the last duplicate dilution of each serum sample (1:2430) as its own denominator to minimize variation. NT₅₀ titers were calculated as described in Balsitis & Williams *et al*, 2010 [27].

DENV enhancement assay

For each of the serum samples, the peak enhancement titer (PET) was calculated. Eight 3-fold serial dilutions beginning at a starting dilution of 1:10 were mixed with DENV2 D2S10 virus at an MOI of 0.1. The serum dilution and virus were mixed together at a 1:1 ratio. 50,000 K562 cells were added after a 45-minute incubation of the virus and serum at 37°C. Two hours following infection, the cells were washed and resuspended in RPMI media. After a 48-hour incubation period, the cells were washed, fixed in 2% paraformaldehyde (Ted Pella, INC), permeabilized with saponin (Sigma Aldrich), and stained intracellularly with 4G2-Alexa488 (Invitrogen). The peak enhancement titer (PET) was calculated by plotting raw percent infection on the y-axis and the log-reciprocal serum dilution on the x-axis. A Gaussian distribution was used to fit each enhancement curve, and the amplitude was identified. This data point was then used to derive the log-reciprocal serum dilution corresponding to the amplitude and was reported as the PET.

Statistical Analysis All graphs were produced using Prism 5 software (La Jolla, CA). Statistical analysis was performed using Stata v10 (College Station, Texas). Comparison of survival functions was conducted using a non-parametric logrank equality test.

FIGURES

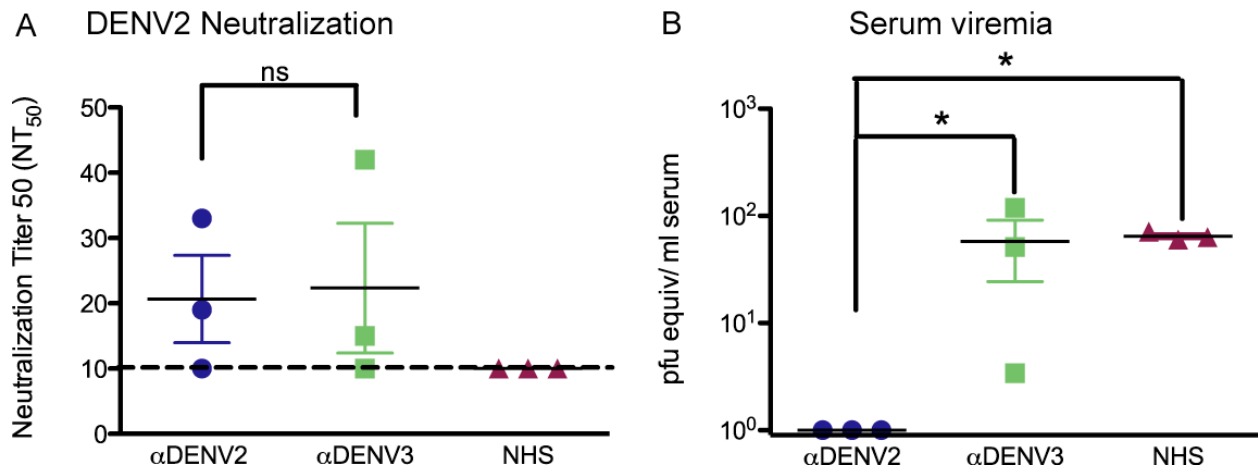


Figure 5.1 Homotypic anti-DENV immune serum is more protective *in vivo* than heterotypic serum of equivalent neutralizing titer. AG129 mice (n=3 / group) were administered either 25 μ l of α DENV2 serum or 250 μ l of α DENV3 serum 24 hours prior to a sub-lethal, 10^3 pfu DENV2 D2S10 infection. **A)** Retro-orbital eyebleeds were taken 4-6 hour prior to infection to measure circulating antibody titers using the U937 DC-SIGN neutralization assay. **B)** Animals were sacrificed four days following infection and serum viremia levels measured using quantitative RT-PCR with primers directed against the NS5 protein.

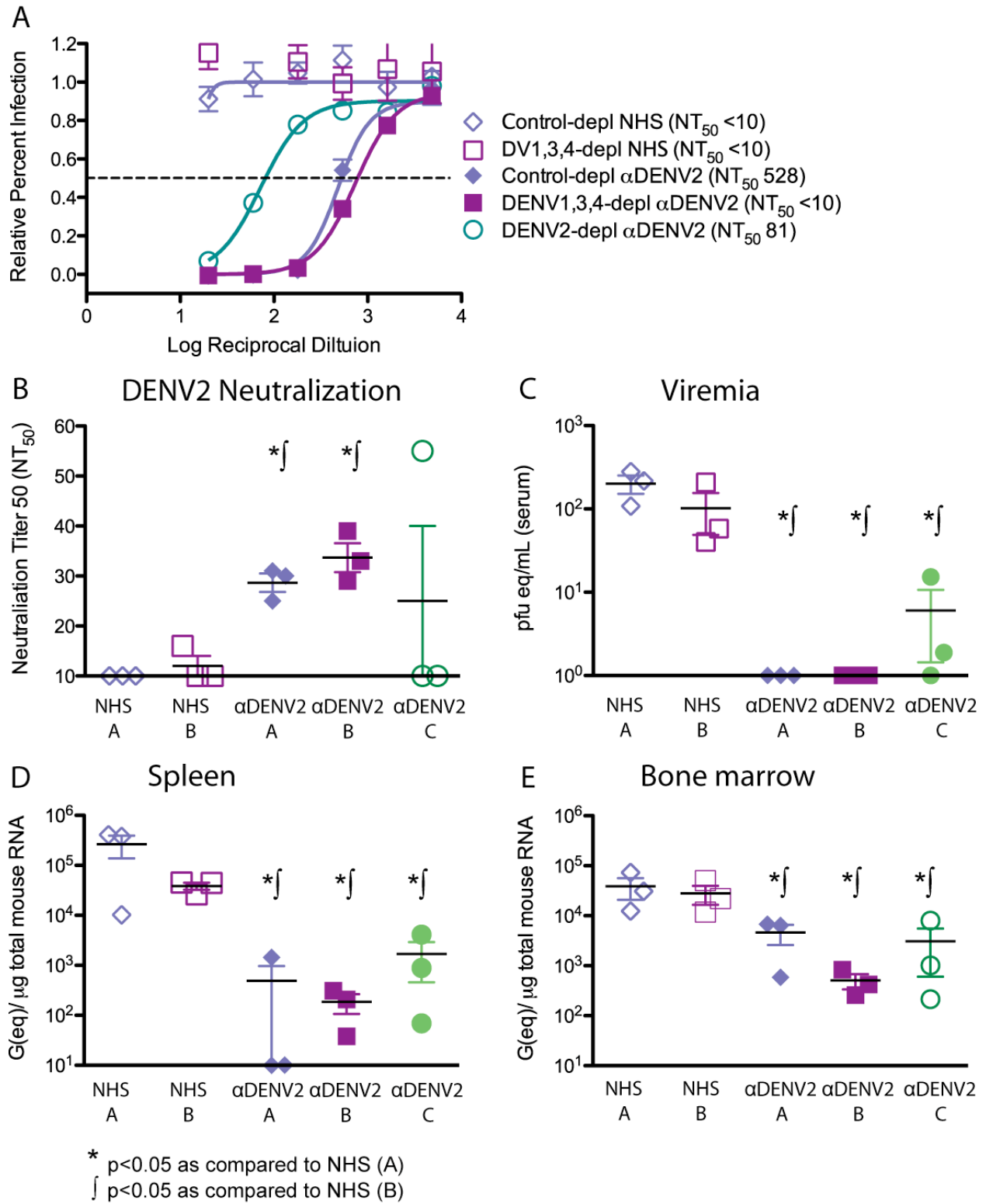
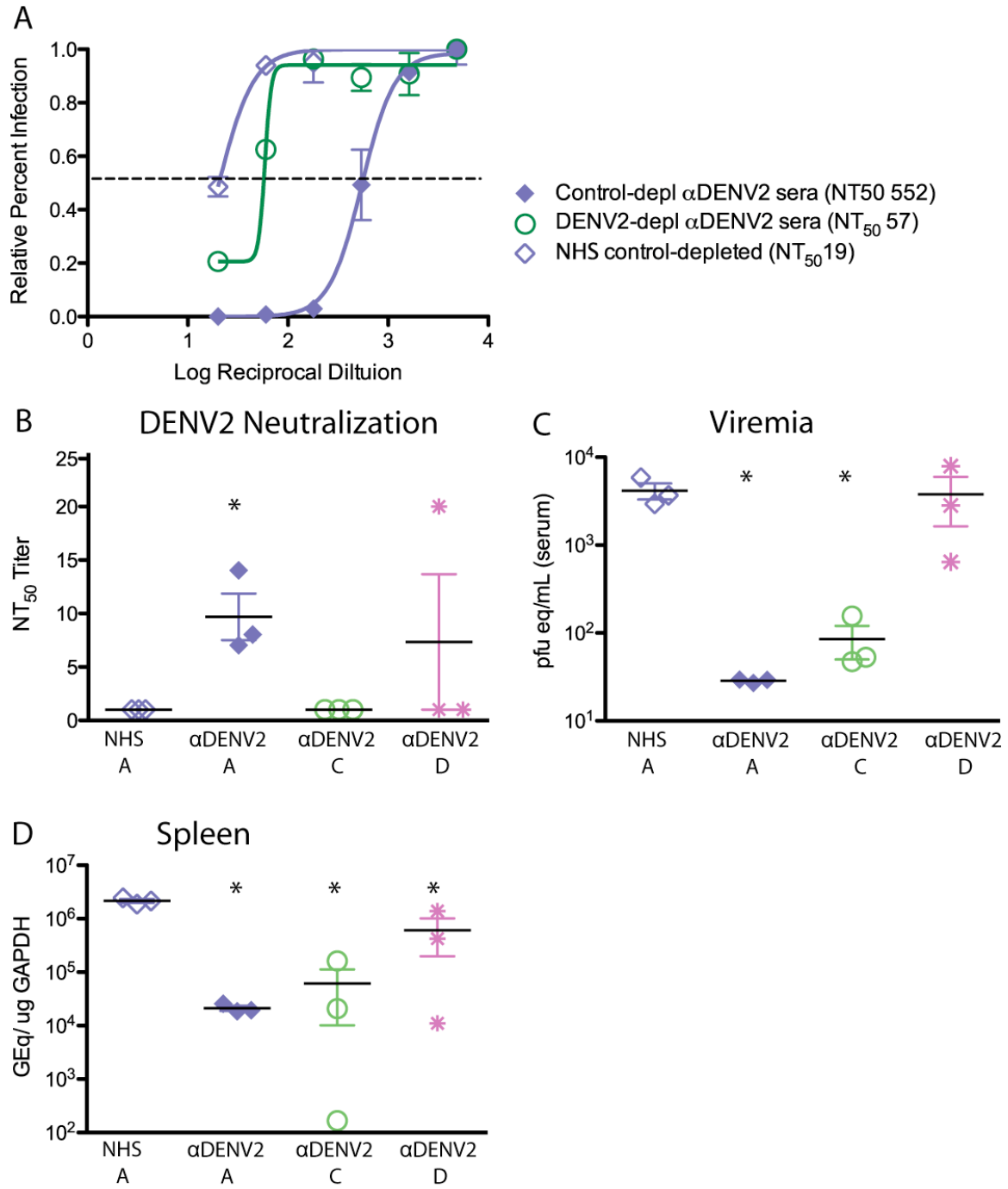
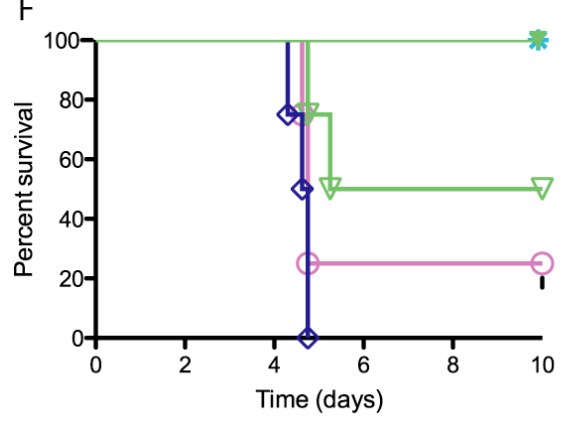
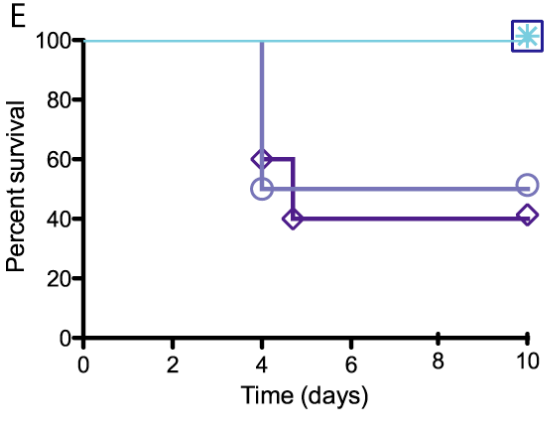
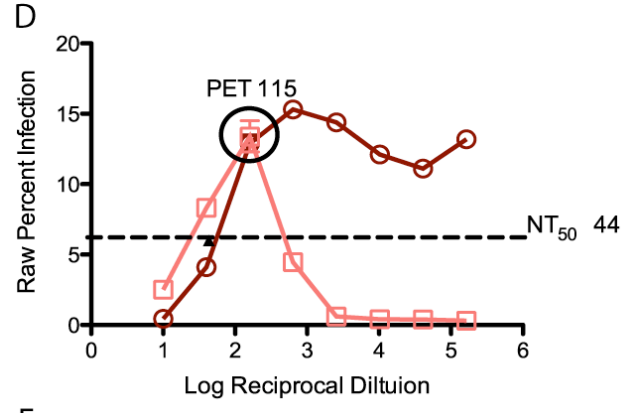
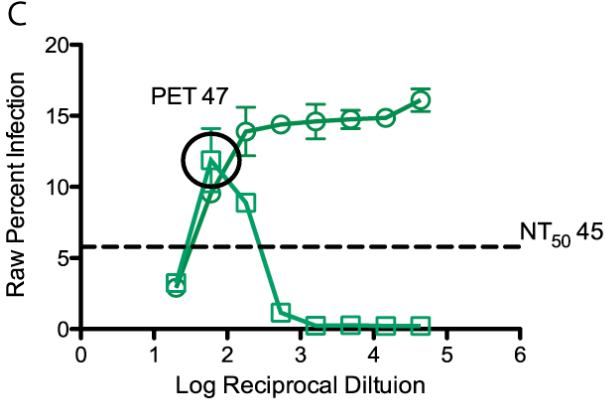
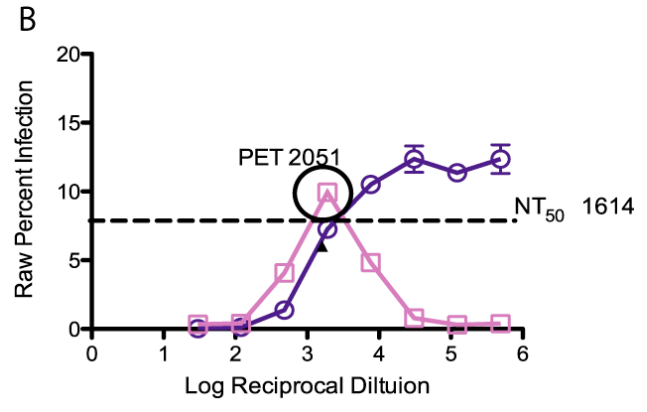
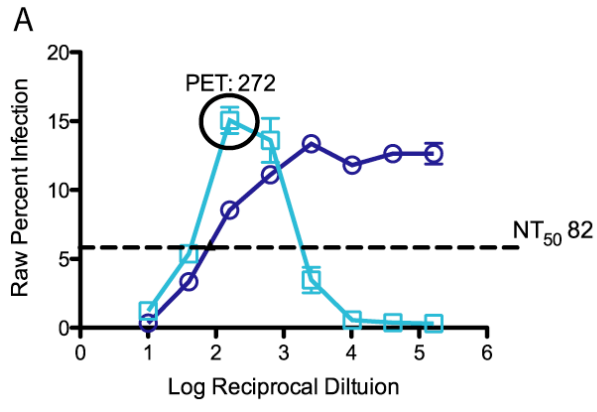


Figure 5.2 Sera depleted of serotype-specific and serotype cross-reactive antibodies both confer protection *in vivo* despite having different neutralization titers *in vitro*. A DENV2-immune serum sample (α DENV2) or normal human serum (NHS) sample was either control-depleted (NHS-A, α DENV2-A), depleted of all cross-reactive antibodies targeting the DENV1, DENV3 and DENV4 virions (NHS-B, α DENV2-B), or depleted of all serotype-specific antibodies targeting the DENV2 virion (α DENV2-C). **A)** The *in vitro* neutralization titer was measured for each of these depleted serum using U937 DC-SIGN cells. Log reciprocal dilution is shown on the x-axis, relative infection is shown on the y-axis, and the dashed line indicates 50% neutralization (NT_{50}). The DENV2-depleted serum demonstrated a 85% reduction in NT_{50} titer. **B-E)** AG129 mice ($n = 3/\text{group}$) were administered 80 μl of each serum, and infected with 10^3 pfu DENV2 D2S10 24 hours later. Four to six hours prior to infection, a retro-orbital eye-bleed was taken and the serum used to measure neutralizing antibodies circulating at the time of infection (**B**). Animals were sacrificed 4 days following infection, and viremia (**C**) and tissue viral load in spleen (**D**) and bone marrow (**E**) was measured by qRT-PCR.



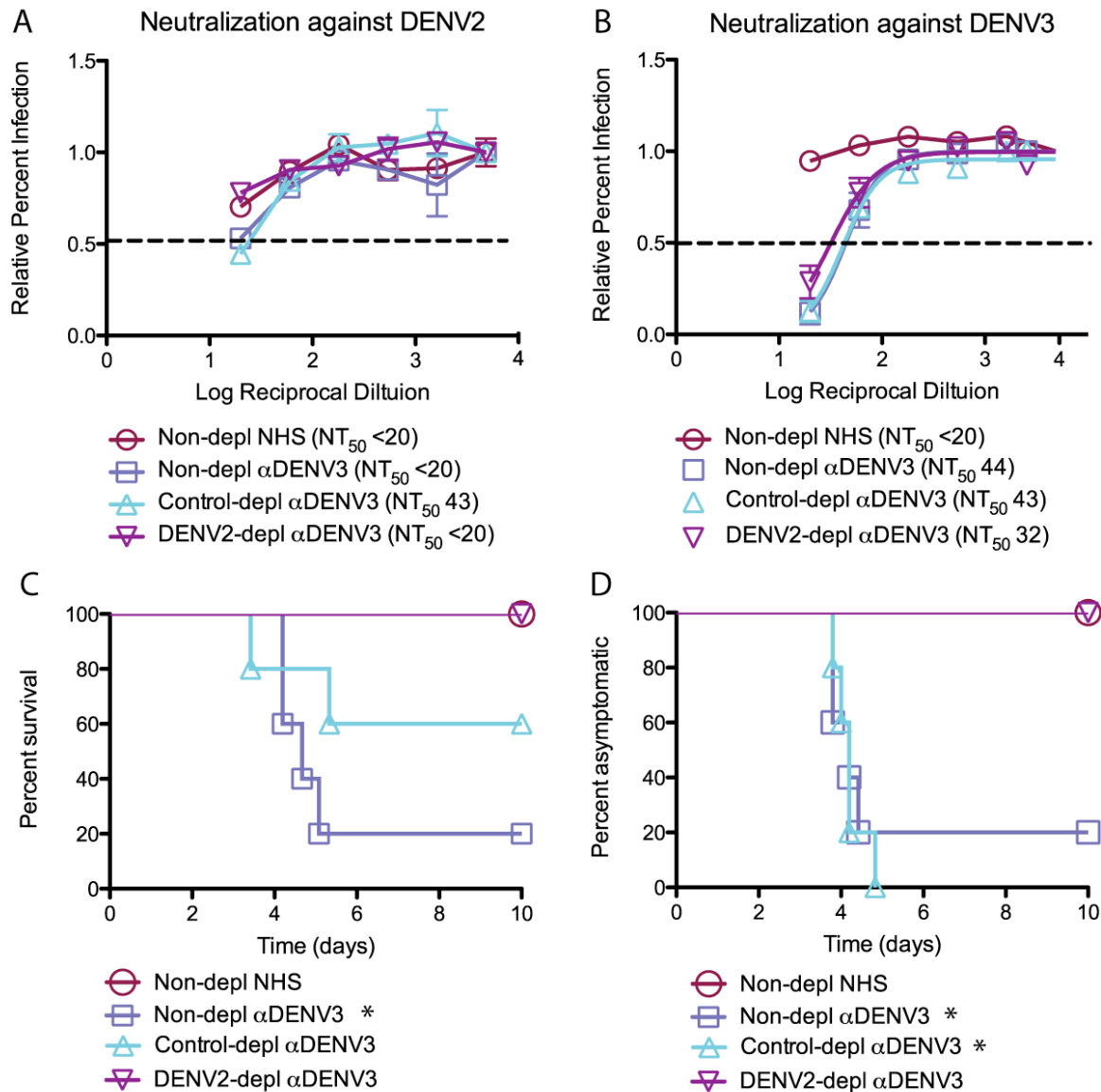
* p-value < 0.05 as compared to NHS (A)

Figure 5.3 DENV2-virion depletion removes 85% of homotypic neutralizing antibodies *in vitro*, but still protects *in vivo*. A second DENV2-immune serum sample (α DENV2) or normal human serum (NHS) sample was either control-depleted (NHS-A, α DENV2-A), or depleted of all serotype-specific antibodies targeting the DENV2 virion (α DENV2-C). Control-depleted α DENV2 serum was diluted to 10% of the original neutralizing titer of the α DENV2 control-depleted serum (α DENV2-D). **A)** The *in vitro* neutralization titer was measured for each of the depleted sera using U937 DC-SIGN cells. Log reciprocal dilution is shown on the x-axis, relative infection is shown on the y-axis, and the dashed line indicates 50% neutralization (NT_{50}). The DENV2-depleted serum demonstrated a 90% reduction in NT_{50} titer. **B-D)** AG129 mice ($n = 3/\text{group}$) were administered 80 μl of each serum and infected with 10^3 pfu of DENV2 D2S10 24 hours later. Four to six hours prior to infection, a retro-orbital eye-bleed was taken and the serum used to measure neutralizing antibodies circulating at the time of infection (**B**). Animals were sacrificed 4 days following infection, and viremia (**C**) and tissue viral load in spleen (**D**) was measured by qRT-PCR.



- ◆ 25 µl αDENV2
- 6.25 µl αDENV2 *f*
- 200 µl αDENV2
- * NHS
- * p-value <0.05 as compared to NHS
- ** p-value <0.01 as compared to NHS
- f* p-value = 0.075 as compared to 200 µl αDENV2
- 200 µl αDENV1 *
- ▽ 200 µl αDENV3
- ▽ 100 µl αDENV3
- ◆ 200 µl αDENV4 **
- * NHS

Figure 5.4 Serotype-specific human α DENV2 monotypic sera is 10-fold less enhancing than serotype cross-reactive sera both *in vitro* and *in vivo*. Human DENV-immune sera was tested for neutralization and enhancing ability *in vitro*, and the data used to predict *in vivo* outcome. **A,B,C,D)** For each serum sample, a neutralization curve generated using U937 DC-SIGN cells (open circle) is superimposed on an enhancement curve generated using K562 cells. A dashed line indicates 50% neutralization (NT₅₀) for each serum sample and a circle denotes the peak enhancement titer (PET). **E)** Either 25 μ l of human α DENV2 serum (n = 5) or 6.25 μ l (n = 5) of human α DENV2 serum was transferred into AG129 mice 24 hours prior to a 10³ pfu DENV2 D2S10 infection. Mice were monitored for morbidity and mortality for 10 days following infection. **F)** 200 μ l of human anti-DENV1, DENV3 or DENV4 sera was transferred into AG129 mice (n = 4/group) 24 hours prior to injection with 10⁵ pfu of DENV2 D2S10. Mice were monitored for morbidity and mortality for 10 days post-infection.



* p-value <0.05 as compared to DENV2-depl αDENV3

Figure 5.5 Depletion of cross-reactive antibodies from DENV3-immune sera minimizes neutralization of DENV2 *in vitro* and eliminates enhancement of DENV2 *in vivo*. A DENV3-immune human serum sample was either control-depleted or depleted of all antibodies that bind to the DENV2 virion. **A, B)** The *in vitro* neutralization titer against DENV2 (**A**) and DENV3 (**B**) was measured for each of these depleted serum samples using U937 DC-SIGN cells. Log reciprocal dilution is shown on the x-axis, relative infection is shown on the y-axis, and the dashed line indicates 50% neutralization (NT₅₀). **C, D)** AG129 mice (n = 4-5/group) were administered 200 μl of the specified serum and infected with 10⁵ pfu of DENV2 D2S10 24 hours later. Animals were monitored for morbidity (**D**) and mortality (**C**) for 10 days following infection.

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CHAPTER 6

CONCLUSION

Project Summary One of the foremost goals underlying my dissertation research was to identify robust *in vitro* correlates of *in vivo* outcome. As shown in chapters 2,4 and 5, we found that the neutralization titer of anti-DENV antibodies following passive transfer of DENV-immune serum correlates well in the context of both protection and enhancement experiments. Protection experiments suggest that there is an inverse relationship between the increasing titer of passively transferred immune serum and decreasing levels of viral replication in the serum and lymphoid organs. Similarly, enhancement experiments suggest that increasing dilutions of homotypic serum will reduce the neutralizing potency and ultimately render the serum enhancing. However, cross-reactive enhancement, as suggested in the Chapter 5, may be more complicated. Further analysis of the subsets of serum that contribute to enhancement *in vivo*, and whether standard *in vitro* assays can accurately capture these properties still remains unclear.

Initial observations in Chapter 2 suggested that E60 N297Q [1] and human mAbs 87.1 and 82.11 [2] were potently therapeutic and acted by neutralizing the viral infection even when administered 24 hours following infection. This demonstrates that these mAbs can reduce DENV viral load even in the absence of effector functions provided by the Fc portion of the antibody, possibly acting differently than anti-WNV fusion loop mAbs [3]. Our initial hypothesis studying a larger panel of therapeutic mAbs was that the more neutralizing the monoclonal, the more potently therapeutic. While this mechanism appears to support prophylactic potency, it does not appear to explain therapeutic efficacy. Indeed, a complex interaction between epitope specificity, displacement ability and mAb avidity appears to determine therapeutic efficacy, such that only certain antibodies targeting the fusion loop and A strand epitopes are therapeutically protective. Within one epitope, aglycosylated mAbs must have a high avidity (estimated using K_d) to be therapeutically efficacious. However, across epitopes, it appears that the ability of the aglycosylated antibodies to displace enhancing antibodies present in polyvalent immune serum and ultimately dominate binding over the surface of the virion is critical for therapeutic success. Through the experiments in Chapters 2 and 3 we also validated the ability of both human IgG1 mAbs and human polyvalent immune serum to enhance sublethal DENV2 infections. This model of human antibody-enhanced disease was subsequently used to validate the ability of E60 N297Q, 87.1 LALA and 82.11 LALA to therapeutically protect against lethal disease *in vivo*. Further investigations into synergistic or additive interactions between fusion loop and A strand antibodies are underway and might identify even more potently therapeutic combinations.

The investigation into the role of anti-EDIII antibodies in polyvalent serum emerged from the observation that EDIII-specific mAbs isolated following murine DENV infection are potently neutralizing [4,5]. The derivation of these mAbs, however, resulted from *in vivo* infection schemes that involved at least one boost with recombinant EDIII protein. Thus, while these antibodies are indeed generated, the development of them may have been influenced by artificial boosting conditions, and may not have been completely reflective of the mouse's natural immune response to DENV infection. Nonetheless, depletion of anti-EDIII antibodies from mouse immune serum resulted in a 35% loss in both homotypic as well as heterotypic neutralization titers, suggesting that the anti-EDIII antibodies that mice produce following one infection are both serotype-specific and serotype cross-reactive and potently neutralizing. *In vivo* protection studies demonstrated that while depletion of anti-EDIII antibodies reduced the neutralizing titer of the serum transferred *in vivo*, it did not result in a significant drop in viral load in all tissues. When examined in the context of enhancement, EDIII-depleted serum similarly reduced the neutralization titer of the transferred serum, but also resulted in a lethal phenotype in 75% of animals tested, indicating that anti-EDIII antibodies may be playing a role in preventing

homologous enhancement. In contrast, humans generate very few EDIII-specific antibodies, and further *in vivo* protection studies suggested that they are not required for a potentially neutralizing antibody response. In conclusion, these data suggest that anti-EDIII antibodies are not the main source of *in vivo* protection. Rather, it is likely that novel classes of antibodies recently identified, such as those targeting heterodimeric complexes and that bind to the virion, but not to recombinant E protein [6,7,8], may be important components of the protective antibody response in humans.

The studies dissecting specific antibody subsets into serotype-specific and serotype-cross-reactive antibodies argues strongly in favor of the concept that there are two separate classes of antibodies that independently mediate protection and enhancement. Depletion of serotype-specific antibodies significantly reduced neutralization titer *in vitro*, and decreased protection *in vivo*, while depletion of cross-reactive antibodies (those binding all cross-reactive epitopes including fusion loop and A strand) appeared not to affect *in vivo* protection at all. In contrast, depletion of the same cross-reactive antibodies completely eliminated enhancement *in vivo*. Further investigation into this hypothesis, and evaluation of antibodies binding to recombinant E will be critical to identifying the components of the α DENV immune response required for mediating protection and enhancement *in vivo*.

The development of a small animal model to study antibody-enhanced DENV disease provided concrete evidence that “antibody-dependent enhancement” can indeed result in clinically severe disease *in vivo*. Characterization of the AG129 model of ADE has revealed a number of characteristics similar to severe human disease including increased viremia and tissue viral load, elevated pro-inflammatory cytokines, thrombocytopenia and most importantly, vascular permeability [1,9]. While an important contribution to studying lethal DENV disease, other requirements for the model are subject to criticism. Severe antibody-enhanced murine disease develops rapidly (over the course of 24 hours) and is lethal by five days following infection. In contrast, the kinetics associated with human lethal DENV disease are more protracted. Similarly, the development of DHF/DSS in humans during or after defervescence, and does not correlate with peak viremia, as it appears to in mice [10]. Finally, the lack of both Type I and Type II interferon receptors limits the scope of this model and prevents analysis of the IFN response *in vivo*. The ideal small animal model would be one that maintains an intact interferon response, but is infectable with all four strains of DENV. The main limitation of the murine model is that mouse Stat2, unlike human Stat2, is resistant to cleavage by DENV NS5 protein [11]. Thus, a new mouse model would require that mouse Stat2 be replaced with human Stat2, or a new mouse-adapted strain of DENV with mutations in the coil-coil region of NS5 be introduced to effectively eliminate this concern.

Future studies Several experiments are currently underway to follow up the observations presented in my dissertation. First, new studies investigating the combined role of fusion loop and A strand aglycosylated mAbs are in progress to determine whether there is any synergistic interaction afforded when combining therapeutic agents following an antibody-enhanced DENV2 infection. Secondly, a series of experiments testing the hypothesis that the residual subset of antibodies contributing to ~10% *in vitro* neutralization but substantial *in vivo* protection target DENV NS1 are currently underway. Finally, a new study testing potential differences in the protective capacity of classes of antibodies (those that bind to prM/M, EDI/II, or EDIII, those that bind to the whole virus but not recombinant E protein) is currently being planned using a newly generated panel of human mAbs from DENV-immune Sri Lankan and Nicaraguan individuals.

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