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Mapping the neutralizing antibody response to natural dengue virus infection

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Mapping the Neutralizing Antibody Response to Natural Dengue Virus Infection

By

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Committee in charge:

Professor Eva Harris, Chair
Professor Lee Riley
Professor Laurent Coscoy
Professor Ellen Robey

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Abstract

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Professor Eva Harris, Chair

Dengue virus (DENV) is the most common mosquito-borne human viral disease worldwide, and infection with any of the four serotypes (DENV1-4) can result in a range of disease outcomes, from inapparent infection to classical dengue fever (DF) to the most severe forms, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). The four DENV serotypes can cause repeat infections in a single individual, each time with a new serotype. While primary infection generates what is thought to be life-long neutralizing antibodies to the infecting serotype, secondary DENV infections elicit cross-neutralizing antibodies to two or more serotypes, as well as type-specific antibodies to the infecting serotype. While natural infection studies have provided valuable insights into the immune response to DENV infection, the complex antibody response needs further elucidation, especially in the light of recent vaccine trial results. In this dissertation, the functional properties and epitope specificity of the antibody response in primary and secondary natural DENV infections are explored.

Recent advances in the characterization of human antibody responses to primary DENV infection indicate that highly neutralizing type-specific antibodies target quaternary epitopes on the DENV envelope protein. Among such type-specific antibodies is 5J7, a human monoclonal antibody (hmAb) isolated from a traveler infected with DENV3. Using two recombinant chimeric DENV4 viruses containing differently sized transplants of the 5J7 epitope, we measured the levels and kinetics of neutralizing antibodies targeting this epitope in post-primary DENV3 infection plasma in a large number of individuals in Nicaragua. We found that DENV3 neutralizing antibodies in post-primary DENV3 infections track with the 5J7 epitope to varying degrees and can be detected even years after infection. Some individuals, however, presented little to no recognition of this epitope even in the expanded transplant version, which suggested the existence of additional epitopes within the DENV3 repertoire that required further investigation. In addition to measuring the prevalence of antibodies directed to the 5J7 epitope in a population in a dengue-endemic area, this study validated and helped define the footprint of a quaternary highly neutralizing epitope. Having demonstrated that chimeric viruses are a powerful tool for mapping type-specific neutralizing antibody responses, we carried out a similar approach to measure type-specific neutralizing antibody responses to the epitope bound by 1F4, a DENV1 type-specific hmAb. This time, we analyzed post-primary DENV1 infection antibody response in individuals from two endemic regions -- Asia and the Americas -- where different genotypes of DENV1 are

circulating. The 1F4 epitope was prevalent in both areas, although a higher proportion was found in the Asian population, supporting the hypothesis that intra-serotype diversity can affect neutralization potential. Our second finding was that both populations presented a bimodal pattern of recognition of the 1F4 epitope, suggesting that additional epitopes within the DENV1 repertoire may contribute to type-specific neutralization of the DENV1 serotype.

After a secondary DENV infection, the neutralizing antibody response gains breadth, and potent broadly neutralizing antibodies may be elicited. In an attempt to investigate the molecular pathways that generate broadly neutralizing antibodies, together with collaborators at Vanderbilt University, we isolated and sequenced hmAbs from EBV-transformed PBMCs collected after secondary DENV infection. Subsequently, we performed deep sequencing of the immunoglobulin (Ig) genes of B cells in matched PBMCs after the first DENV infection of the same individual at time-points prior to the second infection. Although the limited sample size from our pediatric subjects did not allow the necessary depth for tracing the clonal lineages of broadly neutralizing antibodies, we gained substantial knowledge about the molecular and functional properties of hmAbs isolated from memory B cells (MBC) after secondary DENV infection. In our panel of hmAbs, we observed a predominance of serotype cross-neutralizing and somatically hypermutated clones. In fact, we observed a higher degree of somatic hypermutation (SHM) in cross-reactive clones compared to type-specific clones. Notably, we identified a number of potent DENV3 type-specific neutralizing hmAbs isolated after secondary DENV infection. In accordance with our previous study that predicted additional epitopes within the DENV3 repertoire, we mapped three additional novel antigenic sites for DENV3 neutralization, as well as one epitope that overlapped with the already characterized 5J7 site. Strikingly, the sequence of infecting serotypes may modulate the epitope and potency of DENV3 type-specific hmAbs. Moreover, the newly identified DENV3 hmAbs are protective against DENV3 challenge in a mouse model of DENV infection.

As an increasing number of natural infection and immunization studies underscore the importance of the quality as well as the quantity of the antibody response, we investigated the contribution of type-specific versus cross-reactive antibodies to polyclonal neutralization after a first, second and third DENV infection in the same individual. Through depletion of antibody subpopulations in collaboration with collaborators at the University of North Carolina at Chapel Hill, we concluded that after the first infection, type-specific antibody populations drive a substantial proportion of the polyclonal neutralization. Following second and third infections, the cross-reactive populations dominate, although type-specific populations to each of the infecting serotypes can still be detected. Strikingly, in one case, we observed an increase in the proportion of the type-specific antibody population to the first infecting serotype and no evidence of a type-specific response to another serotype in a subsequent inapparent infection, suggesting homotypic reinfection. Depletion of antibody subpopulations enables detection of non-typical DENV infections and assessment of the frequency of heterotypic infections, homotypic reinfections and heterologous boosts, providing critical information regarding the epidemiology and long-term immunity to DENV.

Taken together, the work included in this dissertation has made significant contributions to the field's efforts to decipher the specificity and functional properties of the neutralizing antibody response following primary and secondary natural DENV infections.

Dedication

To my dearest parents, Marcia and Luis,
for their unconditional love and support

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Acknowledgments

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As I finish this challenging and rewarding chapter of my life, the feeling is of accomplishment and eager desire to go for bigger dreams. Once again, my sincerest thanks to everyone who was in this journey with me.

Curriculum Vitae

Education

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Graduate Student Researcher 2010-2012
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- Investigated immunoprofiles associated with disease progression of Chagas disease.

Research Fellow 2011
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1. **Andrade DV**, Katzelnick LC, Widman DG, Balmaseda A, de Silva AM, Baric RS, Harris E. Analysis of Individuals from a Dengue-Endemic Region Helps Define the Footprint and Repertoire of Antibodies Targeting Dengue Virus 3 Type-Specific Epitopes. MBio. 2017 Sep 19;8(5). pii: e01205-17. doi: 10.1128/mBio.01205-17.

2. **Andrade DV**, Harris E. Recent advances in understanding the adaptive immune response to Zika virus and the effect of previous flavivirus exposure. Virus Res. 2017 Jun 26. pii: S0168- 1702(17)30462-8. doi: 10.1016/j.virusres.2017.06.019.

3. **Andrade DV**, Gollob KJ, Dutra WO. Acute Chagas disease: new global challenges for an old neglected disease. PLoS Negl Trop Dis. 2014; 8(7):e3010 doi: 10.1371/journal.pntd.0003010

Publications in Submission, Revision, or Preparation

1. **Andrade DV**, Young E, de Silva AM, Baric RS, Harris E. Tracking the polyclonal neutralizing response to a DENV1 serotype-specific epitope across two populations in Asia and the Americas.

2. Andrade P**, Andrade DV,** Gimblet- Ochieng C, Montoya M, Henein S, Katzelnick LC, de Silva AM, Harris E. Contribution of type-specific versus cross-reactive antibodies to neutralization following repeat dengue virus infections.

3. Young E, **Andrade DV,** Espinosa D, Crowe J, Harris E, Baric R. Isolation of novel DENV3 type-specific human monoclonal antibodies after secondary dengue virus infection with potent neutralization and protective effects in a mouse model of dengue infection.

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CHAPTER 1

INTRODUCTION

Dengue virus infection: epidemiology and clinical manifestations

The dengue virus (DENV) complex belongs to the *Flavivirus* genus in the *Flaviviridae* family and is comprised of four antigenically similar serotypes, DENV1-DENV4[1]. This mosquito-borne infection is endemic to the majority of the tropical and subtropical regions of the world. With up to an estimated 390 million infections and 96 million cases annually and 3.5 billion people at risk of infection[2], dengue is a major public health challenge. Transmitted via the bite of either *Aedes aegypti* or *Aedes albopictus* mosquitoes[3], infection with any of the DENV serotypes can lead to a wide range of clinical manifestations in humans, including asymptomatic infection, dengue fever (DF) and the most severe disease, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS)[4]. In 2009, the WHO published a revised classification scheme for disease severity, consisting of Dengue without Warning Signs, Dengue with Warning Signs, and Severe Dengue, to facilitate clinical management[5]. During primary infection, the serotypes DENV2 and DENV4 appear to be less associated with severe disease than DENV1 and DENV3[6]. Asymptomatic cases account for 5-90% of all DENV infections[7–12] and represent a problem for disease control and dengue surveillance[13,14]. DF is characterized by the sudden onset of fever, which is accompanied by a variety of nonspecific symptoms, such as retro-orbital pain, body aches, muscle and joint pain, frontal headache, nausea and vomiting, weakness and rash[15]. The more severe forms of disease, DHF and DSS, are characterized by fever, thrombocytopenia, coagulopathy and increased vascular permeability[4], which may lead to shock syndrome and multiorgan failure[16]. The pathophysiology of DHF/DSS is attributed to the malfunction of vascular endothelial cells triggered by cytokines and chemical mediators, including the viral protein NS1[16,17]. While DHF/DSS may occasionally occur during primary infection, patients experiencing a second infection with a heterologous DENV serotype have a significantly higher risk of DHF/DSS pathogenesis[18]. Initially reported in only five countries in Southeast Asia, DHF/DSS has now been documented in more than 60 countries[4], where it causes ~25,000 annual deaths. The spread of the vector, introduction of multiple DENV serotypes into endemic areas, and the rapid and increased urbanization and migration have contributed to the spread and persistence of dengue in most of the tropical and subtropical areas in the world[19,20]. Dengue is now classified as an emerging infectious disease and efforts to develop an effective vaccine have intensified.

Dengue virus structure

Dengue virus is a positive-stranded RNA flavivirus with a 10.7kb genome that encodes an envelope protein (E), a pre-membrane/membrane (prM/M) protein, a capsid protein and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5)[21]. The envelope glycoprotein is the major protein on the surface of the virion, where it forms head-to-tail homodimers that lie parallel on the surface of mature viruses[21–23]. Across serotypes, the amino acid homology of the E protein is in the range of 60-70%[24]. The E protein ectodomain is the major target of neutralizing antibodies and consists of three domains: EDI, EDII and EDIII[25–27]. As shown in **Figure 1.1**, EDI is formed into an eight-stranded B-barrel and comprises the central region of the E monomer. EDII

contains a highly conserved fusion loop that facilitates fusion of the virus with endosomal membranes under mildly acidic conditions. EDIII has an immunoglobulin-like fold and is involved in receptor binding to the host cell[28].

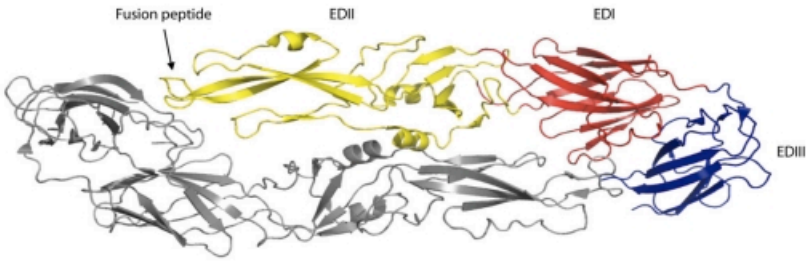

Figure 1.1. Structure of dengue virus (DENV) envelope protein (E). Each subunit within the E protein homodimer contains three domains named EDI (red), EDII (yellow) and EDIII (blue). Figure adapted from Wahala and de Silva[29].

DENV enters the host cell via receptor-mediated endocytosis[21,23]. While the definitive internalization receptor for DENV is yet to be described, a number of important attachment factors that aid DENV infection *in vitro* have been identified, namely the C-type lectin DC-SGN (dendritic-cell specific ICAM-3 grabbing non-integrin)[30], glycosaminoglycans (heparin sulphate, manose receptor in macrophages)[31–33] and phosphatidylserine receptors[34]. During the endosomal pathway, the acidic pH environment generates conformational changes on the E protein from dimers lying flat on the virion surface to trimer spike-like forms[23,35]. These events lead to viral fusion with the host membrane, where the fusion loop at the tip of EDII is inserted into the inner endosomal membrane leaflet. During the membrane fusion process, the EDI-EDII hinge also undergoes structural changes[27]. Once the viral RNA is released into the cytoplasm, the hijacked host protein machinery translates the (+)ssRNA DENV genome into a polyprotein in vesicle packets[36] in the rough endoplasmic reticulum (ER), where virion assembly occurs. In the ER of the infected host cell, the newly assembled immature DENV virions present an icosahedral structure formed by quasi-trimers of E and prM proteins[35,37,38]. The immature virions bud into the lumen of the ER and are transported through the secretory pathway[35]. In the mildly acidic compartments of the secretory pathway, the virion is covered with prM-E heterodimers. The prM protein, crucial for preventing the premature fusion of the E protein with host cell membranes and for chaperoning the folding and assembly of the E protein[39], is cleaved by furin protease into pr peptide and M protein[35] in the trans-Golgi network. The trimer is then disrupted and the E proteins are rearranged into the dimeric form, characteristic of the mature particle[40]. However, DENV prM cleavage is inefficient, generating a heterogeneous population of mature, partially immature and immature DENV particles in tissue culture[41,42]. In humans, virions have been shown to predominantly mature particles in primary DENV1 infections[43].

Cellular adaptive immune response to natural DENV infection

The protective role of T cells in viral infections is generally mediated by $CD8^+$ T cells via direct cytotoxicity and inflammatory cytokines such as IFN-γ and TNF-α, while $CD4^+$ T cells enhance B cell responses and secrete inflammatory cytokines[44,45]. In primary DENV infection, antigen-specific T cells proliferate, secrete IFN-γ and lyse infected target cells[46–48]. Several lines of evidence suggest a protective role of T cells against homologous reinfection or heterologous infection. Notably, higher frequencies of DENV-specific T cells secreting IFN-γ were found in children manifested inapparent DENV infection in secondary infection[49].

DENV-specific $CD8^+$ T cells in DENV1, 2, and 4 infections predominantly target the non-structural proteins, with most of the $CD8^+$ T cells directed towards NS3 and NS5, while DENV3-reactive $CD8^+$ T cells target structural and nonstructural proteins equally[50–54]. It was once postulated that cross-reactive NS3 targeting T cells were associated with severe clinical disease (DHF) by eliciting a "cytokine storm"[55,56]. However, further studies indicated that the timing of the T cell response does not correspond with plasma leakage, a key feature of DHF/DSS[57]. Furthermore, the levels of DENV NS3-specific memory T cell responses were not differentiated across those with different disease outcomes[58]. While earlier studies supported a pathogenic role for T cells during DENV infection[50,59,60] more recent and ongoing studies point to a protective role for T cells[61–63]. Using blood samples from donors in two dengue-endemic areas (Nicaragua and Sri Lanka), Weiskopft et al suggested an HLA-linked protective role for $CD8^+$ T cells[50]. Strikingly, the same patterns of HLA-associated responses to DENV infection were observed in a different cohort of donors from Nicaragua[64]. DENV $CD8^+$ T cells associated with protective HLA alleles produced both IFN-γ and TNF-α, and presented a memory phenotype ($CD45RA^-CCR7^-$ and $CD45RA^+CCR7^-$ subsets) with high levels of programmed death 1 (PD-1) receptor[50,64].

In contrast with the $CD8^+$ T cell response, the $CD4^+$ T cell response targets mainly structural proteins (C and E)[51]. In secondary DENV infection, expansion of the $CD45RA^+CCR7^-$ subset of $CD4^+$ T cells was reported, particularly in individuals who display the HLA alleles associated with protection from severe disease[63]. Based on the expression of Granzyme B, perforin, CD107a and *ex vivo* cytolytic activity, it is suggested that this population is likely cytotoxic effector, a finding similar to $CD8^+$ T cells. In addition to the functional mechanisms of T cells in DENV infection, another interesting area of research is focused on evaluating the repertoire of epitopes spanning the most common HLA types. Via megapools that can detect T cell immune responses *ex vivo*[62,65], Grifoni et al[66] identified 431 epitopes restricted by different HLA-DRB1 alleles that are highly reactive across DENV donors worldwide, such as Nicaragua, Brazil, Singapore and Sri Lanka. Importantly, this broad cross-reactivity was also seen in samples from individuals immunized with the NIH Tetravalent Dengue Live-Attenuated Vaccine (TV005)[66].

Taken together, these ongoing studies point to a protective role of T cells in DENV infection. However, the nonstructural proteins targeted by T cells were not present in the

recently licensed Dengvaxia recombinant live-attenuated tetravalent yellow-fever chimeric virus vaccine, which could be a factor that at least partially explains the low efficacy levels[67]. Hence, in addition to eliciting neutralizing antibodies, efforts at designing a DENV vaccine that stimulates T cells should also be prioritized.

Panels of mAbs isolated from memory B cells (MBCs) indicate that the DENV-specific B cell repertoire can be detected even years after infection, with responses targeted to both the E and the prM proteins[68,69]. Similar to the polyclonal antibody response, the MBC response is predominantly serotype-specific after primary infection, while a more predominant cross-reactive profile is seen after secondary DENV infection[70]. In the context of a polyclonal antibody response, the activation of MBCs selected by a primary infection has been implicated in the predominance of neutralizing antibodies to the first infecting serotype to the detriment of neutralizing antibodies specific to the second infecting serotype[71]. This so-called original antigenic sin (OAS) could favor ADE by outcompeting DENV-specific B cell clones to the second infecting serotype. Understanding the B cell responses to DENV infection and how they relate to serological memory is fundamental for the analysis of protective mechanisms mediated by the adaptive immune response.

Activation of pre-existing MBCs contributes to substantial plasmablast response during secondary DENV infection[72–74], which in turn results in an increase in neutralizing titers[75]. Compared to primary DENV infection, the plasmablast response in secondary infection is presented rapidly and at a substantially higher magnitude[76]. Indeed, DENV-specific plasmablasts peak at a time associated with the onset of severe disease symptoms and display varying degrees of cross-reactivity[72]. Notably, mAbs isolated from plasmablasts in the acute phase of secondary DENV infection have high somatic hypermutation (SHM) frequencies, at levels even higher than found in MBCs[77]. The high levels of SHM, coupled with the presence of clonal expansion, are strong evidence that DENV plasmablasts in secondary infection derive from reactivated MBCs from the first infection. However, plasmablasts activated during acute DENV infection circulate only for a relatively short time frame, after which they undergo contraction or migrate to tissues[72]. A relevant question that needs to be better addressed is whether DENV-specific plasmablasts that survive are further modified in the memory phase and precisely how encounter with a heterologous serotype modifies their breadth and neutralization potential.

Taken together, these studies imply that the mechanisms of B cell activation and affinity maturation are important elements determining the subsequent infection and disease outcome.

Antibody response to primary dengue virus infection

Following a first infection with any of the DENV serotypes, IgM antibody responses arise within 4-5 days of fever onset. The IgM titers peak about 2 weeks and remain detectable up to 2-3 months after infection[78]. Upon class switching recombination, DENV-specific IgG antibodies (IgG1 and IgG3 subclasses) rise rapidly and can be

detected within 1-2 weeks of illness. The initial polyclonal IgG response contains different antibody populations, including serotype-specific neutralizing antibodies, serotype cross-reactive neutralizing antibodies and serotype cross-reactive non-neutralizing antibodies[79]. Cross-reactive antibodies react to all four serotypes and with the flavivirus complex[80,81]. In fact, the dominance of this broad cross-reactivity precludes the employment of antibody-binding assays to identify the infecting flavivirus. Thus, neutralizing assays are more specific and informative for identification of the flavivirus and DENV serotype causing the infection.

Early serological studies conducted by Sabin (1952)[1] demonstrated for the first time that type-specific antibodies confer long-term protection against the homologous serotype. More recent approaches that enable the depletion of antibody populations have provided evidence that after primary DENV infections, neutralization is primarily driven by serotype-specific antibodies[82,83]. The population of cross-reactive antibodies elicited by primary infection, in contrast, can bind and neutralize the heterologous serotypes for only a transient period of time[84]. Further epidemiological studies have established that this transient cross-protection may last up to 6-12 months, when it is postulated that individuals are protected against DENV infection of any serotype[85]. Dogma in the field dictates that in the absence of subsequent DENV infections, the cross-neutralizing antibody titers wane over time[86–88]. This large population of weakly cross-neutralizing antibodies that initially mediates cross-protection may later enhance infection in a secondary heterologous DENV infection[89–92].

The long-standing dogma of life-long protection to reinfection with the homologous serotype and the waning of cross-neutralizing titers after primary infection has been recently challenged. First, homotypic re-infections confirmed by RT-PCR and virus isolation have been reported in Nicaragua[93]. In addition, an epidemic of severe DENV2 in a population with pre-existing DENV2 neutralizing antibodies was reported in Peru[94]. Second, studies in dengue-endemic areas indicate that cross-neutralizing antibody titers appear to be maintained over time after primary infection[87,95]. A topic oftentimes neglected, the impact of genotypic differences on antibody neutralization dynamics could be an underlying factor associated with breakthrough infections. In fact, recent data points to genotypic mismatch of the component in a tetravalent dengue vaccine (genotype II) and the circulating genotype (genotype I) as a factor associated with lower vaccine efficacy against DENV4[96]. With regard to the maintenance of cross-reactivity after primary infection, natural boosts in neutralizing titers (<4 fold increase) have been reported in two dengue-endemic areas (Nicaragua and Thailand)[87,95]. Of note, the difference in the quality of cross-reactive antibodies after primary versus secondary DENV infections may provide substantial clues to the protective mechanisms mediated by these antibody populations.

Antibody response to secondary dengue virus infection

After secondary infection, a quicker and higher magnitude IgG antibody response is elicited. In addition to activation of naïve B cells during the second infection, MBCs from the first infection are also activated. Such stimulation leads to a rapid rise of IgG

antibodies specific to DENV and at much higher titers compared to primary infections. As a matter of fact, the first antibodies that appear after the secondary infection better neutralize the infecting serotype of the primary infection, a phenomena termed " original antigenic sin"[71,97].

Over time, the breadth of the neutralizing antibody response increases and then becomes directed not only against the previously infecting serotypes, but also against serotypes to which they have not yet been exposed[98]. Methods for depletion of antibody populations in polyclonal sera of individuals with well-characterized infection history with two DENV serotypes indicate that type-specific antibodies to both of the infecting serotypes are generated, although it is the cross-reactive population that drives most of the neutralization after secondary infection[83]. Following a heterotypic secondary infection, it has been observed that a considerable portion of the serum antibodies are directed to prM, present on immature or partially immature dengue virions[99,100]. prM antibodies are highly cross-reactive and poorly neutralizing, allowing immature viruses to be taken up into cells via Fc-receptor-mediated endocytosis[101]. Conversely, strongly cross-neutralizing antibodies characterized so far bind to an epitope near the bc-loop on EDII of the E protein monomer[102,103] or to a complex quaternary epitope across the E dimer (EDE)[104].

Individuals experiencing a secondary dengue infection are at a greater risk of developing the severe forms of the disease (DHF/DSS)[18]. These clinical observations led to the hypothesis that pre-existing immunity can exacerbate disease due to antibody-dependent enhancement (ADE). ADE has been demonstrated *in vitro*[105] and recently at a population level[92,106]. In this immunopathological phenomenon, pre-existing weakly neutralizing antibodies from the first infection bind to the second infecting serotype, yet do not mediate neutralization. Instead, they form immune complexes with the viruses, which in turn attach to Fcγ gamma receptors (FcR) on FcγR-bearing myeloid cells, such as monocytes and macrophages[105], and are taken-up via FcR-mediated endocytosis[107]. ADE is a concentration-dependent phenomenon that can even be triggered by neutralizing mAbs at very low concentrations[68,99,108]. Thus, induction of potent and durable neutralizing antibodies to all four serotypes has been a goal of DENV vaccine developers.

In contrast with second DENV infections implicated in increased risk of disease severity, third and fourth infections are rare, and when they occur, are mostly mild or inapparent[109] and present lower rates of hospitalization[11,85,110]. As such, broadly cross-neutralizing antibodies elicited after a second DENV infection may have a protective role. Despite the relevance of understanding the evolution of cross-neutralizing antibodies from expanded MBCs, the molecular mechanisms that lead to their generation are still largely unknown.

Neutralizing antibodies and considerations for dengue vaccine design

While the development of a highly effective vaccine for flaviviruses such as yellow fever and Japanese encephalitis viruses was shown to be feasible, the development of a dengue vaccine is complicated given the presence of four serotypes and the risk of

disease enhancement. In this scenario, where the antibody response is a double-edged sword that may protect or enhance disease, a strategy for dengue vaccines has been to immunize with all four DENV serotype antigens in an attempt to induce a balanced type-specific response to each of the four serotypes[111].

CYD-TDV (Dengvaxia) is a live attenuated tetravalent DENV vaccine licensed on a three-dose schedule in 20 countries in Asia and Latin America and recommended to a population aged 9-45 years[112]. In children aged 2-5 years, safety concerns arose as increased hospitalization rates compared to placebo controls were reported during the long-term follow-up phase of the Phase 3 efficacy trial[112]. Despite moderate efficacy (56-80%) in Phase 2b and 3 clinical trials, *post hoc* analysis indicated unequal efficacy in different populations[113]. In particular, lower efficacy among dengue-naïve vaccine recipients was observed compared to dengue-immune individuals (35.5-43.2 vs. 74.3-83.7%)[113]. These results reveal the impact of immunological memory on vaccine performance. In individuals without DENV infection history (DENV-naïve), a first natural infection following vaccination would behave as a secondary infection, which in turn could place individuals at *increased* risk of severe disease. Conversely, a subsequent infection in vaccinated dengue-immune individuals would act as a tertiary infection, which in turn is associated with lower risk of severe disease. Although not tested yet, the hypothesis behind the superior performance of Dengvaxia in the dengue-immune group is that MBCs induced initially by natural infection would expand and acquire neutralization breadth upon immunization. These somatically mutated higher-affinity clones would then acquire capacity to broadly neutralize multiple DENV serotypes.

A second important lesson from the Dengvaxia vaccine trial stems from data indicating that seroconversion of neutralizing antibody titers does not necessarily translate to protection[114]. While longitudinal cohort studies have demonstrated a correlation between neutralizing antibody titers and protection from symptomatic infection[87], breakthrough infections after vaccination with the licensed dengue vaccine was reported in individuals who had *in vitro* titers against the infecting serotype[115,116]. The discrepancies between *in vitro* neutralization and protection could also be affected by the parameters of the assays performed. Many DENV strains used in vaccines and for research do not represent strains currently in circulation and/or have undergone large numbers of passage in laboratories. For example, a DENV1 strain included as a vaccine antigen belongs to an extinct genotype and has acquired mutations *in vitro* that reduce sensitivity to neutralization[117]. Indeed, an antigenic cartography study demonstrated that this DENV1 strain does not group with other DENV1 strains, further indicating its atypical features[118].

A second factor directly involved in antigenic reactivity differences is the variability in the extent of prM cleavage, a virus strain-specific property[119]. In most cell lines used to propagate DENV, the cleavage of prM is variable, which leads to the release of a heterogeneous population of dengue virions, which may be fully mature, immature or partially mature[120]. The binding and neutralizing ability of antibodies is directly affected by the maturation state of DENV[121]. For example, on partially immature virions, the highly conserved fusion peptide is exposed on E, which results in enhanced binding and

neutralization by abundant cross-reactive antibodies in polyclonal sera[122]. In addition to assessing the heterogeneity of DENV virions *in vitro*, it is critical that further studies demonstrate the maturation properties of virions circulating during infection in humans. In a like manner, antigenic variation within serotypes may present differential sensitivity to temperature and may have different maturation states[123]. Differences at the amino acid level reflects on differences in virion breathing, which in turn allows cryptic epitopes to be revealed during temperature and time-dependent changes in the virion structure[124]. As we describe the immunity of neutralizing antibodies may not be life-long[93] nor sterilizing[94], it is crucial to understand the quality of the antibody response.

Structural properties of DENV neutralizing antibodies

Isolation of human mAbs from DENV-immune B cells has been instrumental in mapping the structure of the dengue virion and the antigen-binding sites of neutralizing antibodies. Employed as an approach to further decipher the neutralizing viral epitopes targeted by DENV-immune individuals, human mAbs (hmAbs) have been isolated using a range of technologies, including EBV-immortalization of B cells and single-cell expression cloning of plasmablasts[125] from both natural infection or vaccination[68,73,77,100,102,104,108,126]. Similarly to the serum antibody repertoire, a predominance of cross-reactive mAbs were isolated from individuals after primary and secondary DENV infection, whereas type-specific mAbs have been isolated mostly from individuals after primary infection.

While type-specific mouse mAbs targeting the EDIII were described to be highly neutralizing[127], further studies showed that only a small percentage of the human antibody repertoire targets this region[128]. In fact, depletion of EDIII-reactive antibodies from human polyclonal sera followed by binding and neutralization assays did not affect the readout, which suggests that these antibodies do not play a major role in serotype-specific or cross-reactive neutralization[127]. These findings were followed by a seminal study that demonstrated that DENV virions of the homologous serotype, rather than recombinant E protein (recE), could deplete the serotype-specific neutralizing antibodies[126]. This indicates that the human polyclonal antibody response that drives the majority of the type-specific neutralizing response after primary infection targets quaternary structure E protein epitopes[126]. Packing of the E proteins on the virion surface creates these complex quaternary epitopes, where two or more adjacent E proteins interact in specific conformations. Similar quaternary epitopes have also been described for several other viruses, including West Nile Virus (WNV)[129], HIV[130] and Zika virus (ZIKV)[131]. Escape mutants were used to elucidate the epitope of three potent type-specific DENV hmAbs: 1F4, 2D22 and 5J7, which neutralize DENV1, DENV2 and DENV3 serotypes, respectively. Analysis of the escape mutants revealed their epitopes on the EDI/EDII hinge region or on EDIII[126]. Further cryo-EM studies allowed the fine mapping of these epitopes and established that each of these hmAbs have contact points on different regions of a monomer or across a dimer, as shown on **Figure 1.2**. For instance, the 1F4 epitope is located within an E protein monomer and binds to EDI/EDII hinge region and EDI and is sensitive to the hinge angle, only recognizing the conformation in the context of the intact virion[132]. In contrast with 1F4, the 2D22 and 5J7

hmAbs bind across neighboring E proteins. While the 2D22 epitope is located on EDIII and the fusion loop in EDII[123], the 5J7 binds to an epitope centered around the EDI/EDII hinge region, and its footprint includes amino acid residues from three different E molecules within a single raft[133]. In the context of vaccine design, these breakthrough findings suggest that soluble E protein subunits would not be the appropriate vaccine immunogen, as they lack higher protein structure and epitopes exclusively displayed on the intact virion.

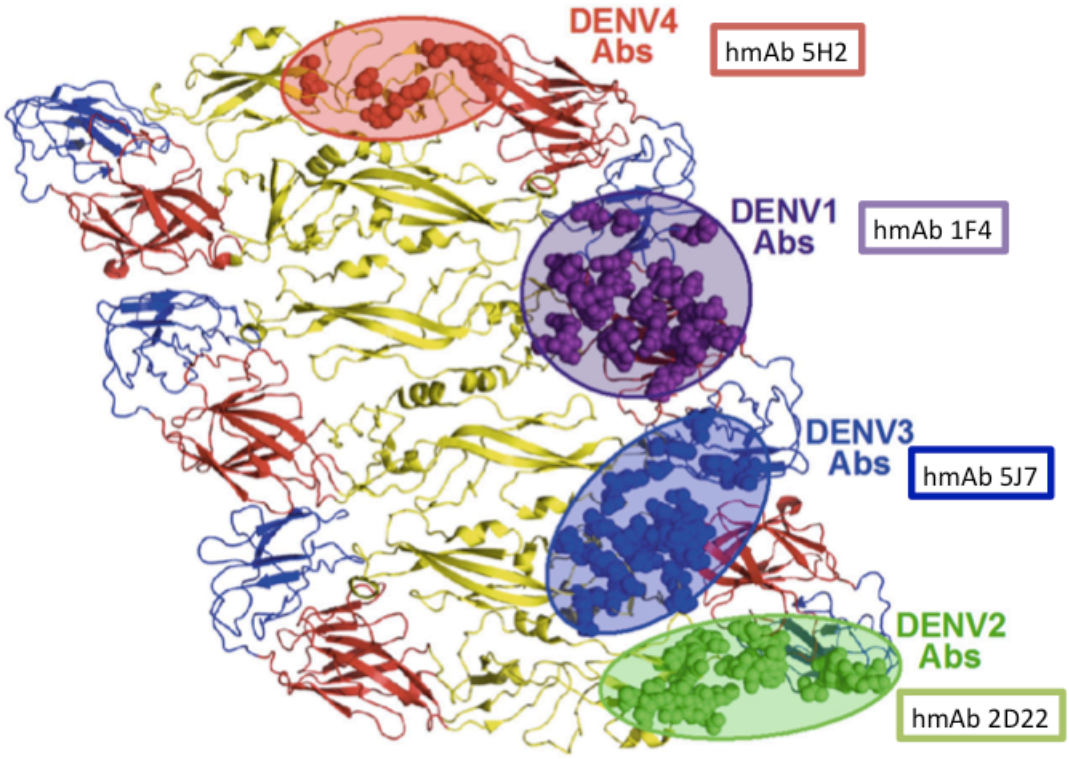

Figure 1.2 Antigenic sites on DENV targeted by potent type-specific neutralizing human mAbs. Epitope mapping of human mAbs isolated after primary DENV infection indicate the specific binding sites on the E protein monomer (1F4) or across E protein dimers (2D22, 5J7). Figure adapted from Gallichotte and de Silva, 2018[134].

The isolation and characterization of complex type-specific neutralizing hmAbs was followed by elegant studies that employed reverse genetics and structure-guided design to create chimeric viruses, in which epitope residues on the E protein were transplanted onto DENV backbones of a different serotype. Forty amino acid residues in the EDIII region where the 2D22 hmAb binds were inserted into a DENV4 backbone (rDENV4/2). Analysis of a limited number of individuals who experienced natural primary DENV2 infection revealed that polyclonal sera tracked with the 2D22 epitope transplanted into the DENV4 backbone. Interestingly, no loss of neutralization in primary DENV4 was observed, suggesting that the DENV2 and DENV4 type-specific epitopes do not overlap[135]. Using a chimeric DENV4/3 virus containing 29 amino acids residues from the 5J7 epitope, Andrade *et al* revealed that this epitope is recognized by primary DENV3 sera up to four years post-infection in a large number of individuals in a dengue-

endemic area[136]. Strikingly, inclusion of additional residues transplanted into the DENV4 backbone did not result in gain of neutralization in most of the individuals who were partially or fully refractory to the 5J7 mAb, which strongly suggested that the DENV3 repertoire is more diverse than anticipated. In fact, novel DENV3 type-specifc neutralizing epitopes have just been isolated and are being characterized (Chapter 4). Another recent study employed a chimeric virus where 25 EDI/EDII hinge residues from DENV3 were incorporated into a DENV4 backbone and resulted in substantial loss of neutralization in 3 of 6 primary DENV4 sera and all four vaccinees who received a live-attenuated DENV4 vaccine. This suggests that these residues within the hinge region contribute to DENV4 neutralizing in natural infection and vaccination[137]. All together, these studies have made significant contribution to defining the antigenic determinants of type-specific neutralizing responses after primary DENV infection. However, to fully define the boundaries of the polyclonal response, additional isolation and characterization of hmAbs is imperative.

After a secondary DENV infection, a predominantly cross-reactive response is described in polyclonal sera of DENV-infected individuals. Compared to cross-reactive mAbs isolated after a first infection, DENV cross-reactive mAbs isolated after secondary DENV infection had higher avidity and neutralization activity[103]. Coupled with depletion studies that showed that cross-reactive antibodies contribute to most of the polyclonal neutralization after secondary DENV infections[83], these findings provide the basis for the hypothesis that cross-neutralizing antibodies evolved from low avidity and poorly neutralizing after primary infection to high avidity and potent neutralizing after secondary infection.

Epitope mapping revealed that several cross-neutralizing mAbs map to an epitope in the bc loop near the fusion loop in EDII of the E protein monomer[102,103]. Additionally, a novel class of potent cross-reactive neutralizing mAbs was isolated from plasmablasts collected in the early convalescent phase after secondary DENV infection[104]. Similarly to the potent type-specific neutralizing mAbs described above, these cross-neutralizing mAbs bind to quaternary epitopes on the E protein. More specifically, these mAbs bind to EDI or EDIII of one monomer and reach over to the adjacent dimer where they binds to EDII, hence the name envelope dimer epitope (EDE) antibodies. The discovery of dimer epitopes as a target of broadly neutralizing antibodies demonstrates that inducing a balanced immune response to all four DENV serotypes simultaneously could be feasible. However, designing stable recombinant E dimers is a complex and is the subject of active investigation. An additional uncertainty about the EDE antibodies is the fact that they were isolated from plasmablasts during acute secondary DENV infection and have not yet been identified in the MBC pool. Given the high relevance of broadly neutralizing antibodies, the molecular mechanisms that lead to their generation is a highly relevant area of study.

Thesis objectives

The mechanisms by which the host immune response to DENV infection either provides protection or induces enhancement of subsequent infection is an intriguing question that

has hindered the development of an effective dengue vaccine for populations with different levels of pre-existing immunity. In view of the need for a deeper understanding of the specificity of the antibody response to DENV, the objective of this dissertation is to determine the neutralizing antibody epitopes alongside molecular and functional characterization of the neutralizing antibody response in both primary and secondary natural DENV infection. In Chapter 2, we use chimeric viruses and our well-characterized longitudinal samples from community-based cohort and hospital-based studies of dengue in Nicaragua to measure the prevalence and kinetics of the neutralizing DENV3 type-specific response that tracks with a specific quaternary DENV3 epitope. The third chapter of this dissertation employs a similar approach to evaluate the DENV1 epitope repertoire in populations living in two geographically distinct dengue-endemic areas, Asia and the Americas. In chapter 4, we focus on the molecular and functional characterization of broadly neutralizing monoclonal antibodies and DENV3 type-specific monoclonal antibodies isolated from memory B cells after secondary DENV infection. Finally, the contribution of type-specific versus cross-reactive antibodies to polyclonal neutralization after sequential DENV infections is studied in Chapter 5. Altogether, our findings improve the understanding of antigenic determinants of type-specific DENV antibodies, as well as the breadth and diversity of antibody responses after secondary DENV infection.

REFERENCES

1. SABIN, A. B. The dengue group of viruses and its family relationships. *Bacteriol. Rev.* **14,** 225–32 (1950).
2. Bhatt, S. *et al.* The global distribution and burden of dengue. *Nature* **496,** 504–507 (2013).
3. Gubler, D. J. Dengue and dengue hemorrhagic fever. *Clin. Microbiol. Rev.* **11,** 480–96 (1998).
4. *Dengue haemorrhagic fever Diagnosis, treatment, prevention and control SECOND EDITION Contents*. (1997).
5. *DENGUE GUIDELINES FOR DIAGNOSIS, TREATMENT, PREVENTION AND CONTROL TREATMENT, PREVENTION AND CONTROL TREATMENT, PREVENTION AND CONTROL*.
6. Fried, J. R. *et al.* Serotype-Specific Differences in the Risk of Dengue Hemorrhagic Fever: An Analysis of Data Collected in Bangkok, Thailand from 1994 to 2006. *PLoS Negl. Trop. Dis.* **4,** e617 (2010).
7. Balmaseda, A. *et al.* High seroprevalence of antibodies against dengue virus in a prospective study of schoolchildren in Managua, Nicaragua. *Trop. Med. Int. Health* **11,** 935–42 (2006).
8. Burke, D. S., Nisalak, A., Johnson, D. E. & Scott, R. M. A prospective study of dengue infections in Bangkok. *Am. J. Trop. Med. Hyg.* **38,** 172–80 (1988).
9. Balmaseda, A. *et al.* Trends in Patterns of Dengue Transmission over 4 Years in a Pediatric Cohort Study in Nicaragua. *J. Infect. Dis.* **201,** 5–14 (2010).
10. Gordon, A. *et al.* The Nicaraguan Pediatric Dengue Cohort Study: Incidence of Inapparent and Symptomatic Dengue Virus Infections, 2004–2010. *PLoS Negl. Trop. Dis.* **7,** e2462 (2013).
11. Montoya, M. *et al.* Symptomatic Versus Inapparent Outcome in Repeat Dengue Virus Infections Is Influenced by the Time Interval between Infections and Study Year. *PLoS Negl. Trop. Dis.* **7,** e2357 (2013).
12. Endy, T. P. *et al.* Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng Phet, Thailand. *Am. J. Epidemiol.* **156,** 40–51 (2002).
13. Chatchen, S., Sabchareon, A. & Sirivichayakul, C. Serodiagnosis of asymptomatic dengue infection. *Asian Pac. J. Trop. Med.* **10,** 11–14 (2017).
14. Pongsiri, A. *et al.* Differential Susceptibility of Two Field Aedes aegypti Populations to a Low Infectious Dose of Dengue Virus. *PLoS One* **9,** e92971 (2014).
15. Hayes, E. B. & Gubler, D. J. Dengue and dengue hemorrhagic fever. *Pediatr. Infect. Dis. J.* **11,** 311–7 (1992).
16. Halstead, S. B. Dengue. *Lancet* **370,** 1644–1652 (2007).
17. Glasner, D. R., Puerta-Guardo, H., Beatty, P. R. & Harris, E. The Good, the Bad, and the Shocking: The Multiple Roles of Dengue Virus Nonstructural Protein 1 in Protection and Pathogenesis. *Annu. Rev. Virol.* **5,** 227–253 (2018).
18. Halstead, S. B. Pathogenesis of dengue: challenges to molecular biology. *Science* **239,** 476–81 (1988).
19. Kyle, J. L. & Harris, E. Global Spread and Persistence of Dengue. *Annu. Rev.*

Microbiol. **62,** 71–92 (2008).
20. Wilder-Smith, A. & Gubler, D. J. Geographic Expansion of Dengue: The Impact of International Travel. *Med. Clin. North Am.* **92,** 1377–1390 (2008).
21. Lindenbach, B. D., Thiel, H.-J. & Rice, C. M. *33 Flaviviridae: The Viruses and Their Replication*. (2006).
22. Kuhn, R. J. *et al.* Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* **108,** 717–25 (2002).
23. Stiasny, K. & Heinz, F. X. Flavivirus membrane fusion. *J. Gen. Virol.* **87,** 2755–2766 (2006).
24. Westaway, K. E., Blok, W. J. & Westaway, R. M. Taxonomy and evolutionary relationships of flaviviruses. (1997).
25. Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc. Natl. Acad. Sci.* **100,** 6986–6991 (2003).
26. Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. *J. Virol.* **79,** 1223–31 (2005).
27. Zhang, Y. *et al.* Conformational changes of the flavivirus E glycoprotein. *Structure* **12,** 1607–18 (2004).
28. Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C. & Harrison, S. C. The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* **375,** 291–298 (1995).
29. Wahala, W. M. P. B. & De Silva, A. M. The Human Antibody Response to Dengue Virus Infection. *Viruses* **3,** 2374–2395 (2011).
30. Tassaneetrithep, B. *et al.* DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J. Exp. Med.* **197,** 823–9 (2003).
31. Chen, Y. *et al.* Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat. Med.* **3,** 866–71 (1997).
32. Dalrymple, N. & Mackow, E. R. Productive Dengue Virus Infection of Human Endothelial Cells Is Directed by Heparan Sulfate-Containing Proteoglycan Receptors. *J. Virol.* **85,** 9478–9485 (2011).
33. Miller, J. L. *et al.* The mannose receptor mediates dengue virus infection of macrophages. *PLoS Pathog.* **4,** e17 (2008).
34. Meertens, L. *et al.* The TIM and TAM families of phosphatidylserine receptors mediate dengue virus entry. *Cell Host Microbe* **12,** 544–57 (2012).
35. Yu, I.-M. *et al.* Structure of the immature dengue virus at low pH primes proteolytic maturation. *Science* **319,** 1834–7 (2008).
36. Welsch, S. *et al.* Composition and Three-Dimensional Architecture of the Dengue Virus Replication and Assembly Sites. *Cell Host Microbe* **5,** 365–375 (2009).
37. Li, L. *et al.* The Flavivirus Precursor Membrane-Envelope Protein Complex: Structure and Maturation. *Science (80-.).* **319,** 1830–1834 (2008).
38. Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. Structure of the dengue virus envelope protein after membrane fusion. *Nature* **427,** 313–319 (2004).
39. Lorenz, I. C., Allison, S. L., Heinz, F. X. & Helenius, A. Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. *J. Virol.* **76,** 5480–91 (2002).

40. Mukhopadhyay, S., Kuhn, R. J. & Rossmann, M. G. A structural perspective of the flavivirus life cycle. *Nat. Rev. Microbiol.* **3,** 13–22 (2005).
41. Cherrier, M. V *et al.* Structural basis for the preferential recognition of immature flaviviruses by a fusion-loop antibody. *EMBO J.* **28,** 3269–3276 (2009).
42. Junjhon, J. *et al.* Influence of pr-M cleavage on the heterogeneity of extracellular dengue virus particles. *J. Virol.* **84,** 8353–8 (2010).
43. Raut, R. *et al.* Dengue type 1 viruses circulating in humans are highly infectious and poorly neutralized by human antibodies. *Proc. Natl. Acad. Sci.* 201812055 (2018). doi:10.1073/pnas.1812055115
44. Remakus, S. & Sigal, L. J. in *Advances in experimental medicine and biology* **785,** 77–86 (2013).
45. Sant, A. J. & McMichael, A. Revealing the role of CD4 $^{+}$ T cells in viral immunity: Figure 1. *J. Exp. Med.* **209,** 1391–1395 (2012).
46. Kurane, I., Hebblewaite, D., Brandt, W. E. & Ennis, F. A. Lysis of dengue virus-infected cells by natural cell-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. *J. Virol.* **52,** 223–30 (1984).
47. Mathew, A. *et al.* Dominant recognition by human CD8+ cytotoxic T lymphocytes of dengue virus nonstructural proteins NS3 and NS1.2a. *J. Clin. Invest.* **98,** 1684–1691 (1996).
48. Kurane, I., Meager, A. & Ennis, F. A. Dengue virus-specific human T cell clones. Serotype crossreactive proliferation, interferon gamma production, and cytotoxic activity. *J. Exp. Med.* **170,** 763–75 (1989).
49. Hatch, S. *et al.* Intracellular Cytokine Production by Dengue Virus–specific T cells Correlates with Subclinical Secondary Infection. *J. Infect. Dis.* **203,** 1282–1291 (2011).
50. Weiskopf, D. *et al.* Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. *Proc. Natl. Acad. Sci.* **110,** E2046–E2053 (2013).
51. Rivino, L. *et al.* Differential targeting of viral components by CD4+ versus CD8+ T lymphocytes in dengue virus infection. *J. Virol.* **87,** 2693–706 (2013).
52. Duangchinda, T. *et al.* Immunodominant T-cell responses to dengue virus NS3 are associated with DHF. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 16922–7 (2010).
53. Simmons, C. P. *et al.* Early T-Cell Responses to Dengue Virus Epitopes in Vietnamese Adults with Secondary Dengue Virus Infections. *J. Virol.* **79,** 5665–5675 (2005).
54. Weiskopf, D. *et al.* Immunodominance Changes as a Function of the Infecting Dengue Virus Serotype and Primary versus Secondary Infection. *J. Virol.* **88,** 11383–11394 (2014).
55. Mongkolsapaya, J. *et al.* Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat. Med.* **9,** 921–927 (2003).
56. Mangada, M. M. *et al.* Dengue-Specific T Cell Responses in Peripheral Blood Mononuclear Cells Obtained prior to Secondary Dengue Virus Infections in Thai Schoolchildren. *J. Infect. Dis.* **185,** 1697–1703 (2002).
57. Dung, N. T. P. *et al.* Timing of CD8+ T Cell Responses in Relation to Commencement of Capillary Leakage in Children with Dengue. *J. Immunol.* **184,** 7281–7287 (2010).

58. Jeewandara, C. *et al.* Functionality of Dengue Virus Specific Memory T Cell Responses in Individuals Who Were Hospitalized or Who Had Mild or Subclinical Dengue Infection. *PLoS Negl. Trop. Dis.* **9,** e0003673 (2015).
59. Gagnon, S. J., Ennis, F. A. & Rothman, A. L. Bystander target cell lysis and cytokine production by dengue virus-specific human CD4(+) cytotoxic T-lymphocyte clones. *J. Virol.* **73,** 3623–9 (1999).
60. Kurane, I. *et al.* Human T cell responses to dengue virus antigens. Proliferative responses and interferon gamma production. *J. Clin. Invest.* **83,** 506–513 (1989).
61. Xie, H., Nie, J., Chen, Q., Huang, W. & Wang, Y. Comparison of the genotypic and phenotypic properties of HIV-1 standard subtype B and subtype B/B′ env molecular clones derived from infections in China. *Emerg. Microbes Infect.* **7,** 90 (2018).
62. Weiskopf, D. *et al.* HLA-DRB1 Alleles Are Associated With Different Magnitudes of Dengue Virus–Specific CD4 $^{+}$ T-Cell Responses. *J. Infect. Dis.* **214,** 1117–1124 (2016).
63. Weiskopf, D. *et al.* Dengue virus infection elicits highly polarized CX3CR1 $^{+}$ cytotoxic CD4 $^{+}$ T cells associated with protective immunity. *Proc. Natl. Acad. Sci.* **112,** E4256–E4263 (2015).
64. de Alwis, R. *et al.* Immunodominant Dengue Virus-Specific CD8 $^{+}$ T Cell Responses Are Associated with a Memory PD-1 $^{+}$ Phenotype. *J. Virol.* **90,** 4771–4779 (2016).
65. Weiskopf, D. *et al.* Human CD8 $^{+}$ T-Cell Responses Against the 4 Dengue Virus Serotypes Are Associated With Distinct Patterns of Protein Targets. *J. Infect. Dis.* **212,** 1743–1751 (2015).
66. Grifoni, A. *et al.* Global Assessment of Dengue Virus-Specific CD4+ T Cell Responses in Dengue-Endemic Areas. *Front. Immunol.* **8,** 1309 (2017).
67. Guy, B. *et al.* From research to phase III: Preclinical, industrial and clinical development of the Sanofi Pasteur tetravalent dengue vaccine. *Vaccine* **29,** 7229–7241 (2011).
68. Beltramello, M. *et al.* The Human Immune Response to Dengue Virus Is Dominated by Highly Cross-Reactive Antibodies Endowed with Neutralizing and Enhancing Activity. *Cell Host Microbe* **8,** 271–283 (2010).
69. Schieffelin, J. S. *et al.* Neutralizing and non-neutralizing monoclonal antibodies against dengue virus E protein derived from a naturally infected patient. *Virol. J.* **7,** 28 (2010).
70. Mathew, A. *et al.* B-Cell Responses During Primary and Secondary Dengue Virus Infections in Humans. *J. Infect. Dis.* **204,** 1514–1522 (2011).
71. Midgley, C. M. *et al.* An In-Depth Analysis of Original Antigenic Sin in Dengue Virus Infection. *J. Virol.* **85,** 12100–12100 (2011).
72. Wrammert, J. *et al.* Rapid and Massive Virus-Specific Plasmablast Responses during Acute Dengue Virus Infection in Humans. *J. Virol.* **86,** 2911–2918 (2012).
73. Xu, M. *et al.* Plasmablasts Generated during Repeated Dengue Infection Are Virus Glycoprotein-Specific and Bind to Multiple Virus Serotypes. *J. Immunol.* **189,** 5877–5885 (2012).
74. Balakrishnan, T. *et al.* Dengue Virus Activates Polyreactive, Natural IgG B Cells after Primary and Secondary Infection. *PLoS One* **6,** e29430 (2011).

75. Toh, Y. X. *et al.* Dengue Serotype Cross-Reactive, Anti-E Protein Antibodies Confound Specific Immune Memory for 1 Year after Infection. *Front. Immunol.* **5,** 388 (2014).
76. Garcia-Bates, T. M. *et al.* Association between Magnitude of the Virus-Specific Plasmablast Response and Disease Severity in Dengue Patients. *J. Immunol.* **190,** 80–87 (2013).
77. Priyamvada, L. *et al.* B Cell Responses during Secondary Dengue Virus Infection Are Dominated by Highly Cross-Reactive, Memory-Derived Plasmablasts. *J. Virol.* **90,** 5574–5585 (2016).
78. Vaughn, D. W. *et al.* Rapid serologic diagnosis of dengue virus infection using a commercial capture ELISA that distinguishes primary and secondary infections. *Am. J. Trop. Med. Hyg.* **60,** 693–8 (1999).
79. Coloma, J. & Harris, E. Broad and strong: the ultimate antibody to dengue virus. *Nat. Immunol.* **16,** 135–137 (2015).
80. Koraka, P. *et al.* Kinetics of Dengue Virus-Specific Serum Immunoglobulin Classes and Subclasses Correlate with Clinical Outcome of Infection. *J. Clin. Microbiol.* **39,** 4332–4338 (2001).
81. Mansfield, K. L. *et al.* Flavivirus-induced antibody cross-reactivity. *J. Gen. Virol.* **92,** 2821–2829 (2011).
82. Henein, S. *et al.* Dissecting antibodies induced by a chimeric yellow fever-dengue, live-attenuated, tetravalent dengue vaccine (CYD-TDV) in naïve and dengue exposed individuals. *J. Infect. Dis.* **215,** jiw576 (2016).
83. Patel, B. *et al.* Dissecting the human serum antibody response to secondary dengue virus infections. *PLoS Negl. Trop. Dis.* **11,** e0005554 (2017).
84. SABIN, A. B. Research on dengue during World War II. *Am. J. Trop. Med. Hyg.* **1,** 30–50 (1952).
85. Gibbons, R. V *et al. Analysis of Repeat Hospital Admissions for Dengue to Estimate the Frequency of Third or Fourth Dengue Infections Resulting in Admissions and Dengue Hemorrhagic Fever, and Serotype Sequences*. (2007).
86. Puschnik, A. *et al.* Correlation between Dengue-Specific Neutralizing Antibodies and Serum Avidity in Primary and Secondary Dengue Virus 3 Natural Infections in Humans. *PLoS Negl. Trop. Dis.* **7,** e2274 (2013).
87. Katzelnick, L. C., Montoya, M., Gresh, L., Balmaseda, A. & Harris, E. Neutralizing antibody titers against dengue virus correlate with protection from symptomatic infection in a longitudinal cohort. *Proc. Natl. Acad. Sci.* **113,** 728–733 (2016).
88. Guzman, M. G. *et al.* Neutralizing antibodies after infection with dengue 1 virus. *Emerg. Infect. Dis.* **13,** 282–6 (2007).
89. Kliks, S. C., Nisalak, A., Brandt, W. E., Wahl, L. & Burke, D. S. Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. *Am. J. Trop. Med. Hyg.* **40,** 444–51 (1989).
90. Sangkawibha, N. *et al.* Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. *Am. J. Epidemiol.* **120,** 653–69 (1984).
91. Halstead, S. B. In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. *J. Infect. Dis.* **140,** 527–33 (1979).
92. Katzelnick, L. C. *et al.* Antibody-dependent enhancement of severe dengue

disease in humans. *Science* **358,** 929–932 (2017).
93. Waggoner, J. J. *et al.* Homotypic Dengue Virus Reinfections in Nicaraguan Children. *J. Infect. Dis.* **214,** 986–993 (2016).
94. Forshey, B. M. *et al.* Incomplete Protection against Dengue Virus Type 2 Re-infection in Peru. *PLoS Negl. Trop. Dis.* **10,** e0004398 (2016).
95. Clapham, H. E. *et al.* Dengue Virus (DENV) Neutralizing Antibody Kinetics in Children After Symptomatic Primary and Postprimary DENV Infection. *J. Infect. Dis.* **213,** 1428–35 (2016).
96. Gallichotte, E. N. *et al.* Genetic Variation between Dengue Virus Type 4 Strains Impacts Human Antibody Binding and Neutralization. *Cell Rep.* **25,** (2018).
97. Halstead, S. B., Rojanasuphot, S. & Sangkawibha, N. Original antigenic sin in dengue. *Am. J. Trop. Med. Hyg.* **32,** 154–6 (1983).
98. Corbett, K. S. *et al.* Preexisting Neutralizing Antibody Responses Distinguish Clinically Inapparent and Apparent Dengue Virus Infections in a Sri Lankan Pediatric Cohort. *J. Infect. Dis.* **211,** 590–599 (2015).
99. Dejnirattisai, W. *et al.* Cross-Reacting Antibodies Enhance Dengue Virus Infection in Humans. *Science (80-.).* **328,** 745–748 (2010).
100. Lai, C.-Y. *et al.* Antibodies to Envelope Glycoprotein of Dengue Virus during the Natural Course of Infection Are Predominantly Cross-Reactive and Recognize Epitopes Containing Highly Conserved Residues at the Fusion Loop of Domain II. *J. Virol.* **82,** 6631–6643 (2008).
101. Smith, S. A. *et al.* Dengue Virus prM-Specific Human Monoclonal Antibodies with Virus Replication-Enhancing Properties Recognize a Single Immunodominant Antigenic Site. *J. Virol.* **90,** 780–789 (2016).
102. Smith, S. A. *et al.* The potent and broadly neutralizing human dengue virus-specific monoclonal antibody 1C19 reveals a unique cross-reactive epitope on the bc loop of domain II of the envelope protein. *MBio* **4,** e00873-13 (2013).
103. Tsai, W.-Y. *et al.* High-Avidity and Potently Neutralizing Cross-Reactive Human Monoclonal Antibodies Derived from Secondary Dengue Virus Infection. *J. Virol.* **87,** 12562–12575 (2013).
104. Dejnirattisai, W. *et al.* A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus. *Nat. Immunol.* **16,** 170–177 (2015).
105. Halstead, S. B. Neutralization and antibody-dependent enhancement of dengue viruses. *Adv. Virus Res.* **60,** 421–67 (2003).
106. Salje, H. *et al.* Reconstruction of antibody dynamics and infection histories to evaluate dengue risk. *Nature* **557,** 719–723 (2018).
107. Halstead, S. B. in *Antibodies for Infectious Diseases* **2,** 249–271 (American Society of Microbiology, 2014).
108. Teoh, E. P. *et al.* The Structural Basis for Serotype-Specific Neutralization of Dengue Virus by a Human Antibody. *Sci. Transl. Med.* **4,** 139ra83-139ra83 (2012).
109. Olkowski, S. *et al.* Reduced Risk of Disease During Postsecondary Dengue Virus Infections. *J. Infect. Dis.* **208,** 1026–1033 (2013).
110. Bhoomiboonchoo, P. *et al.* Sequential dengue virus infections detected in active and passive surveillance programs in Thailand, 1994–2010. *BMC Public Health*

15, 250 (2015).
111. Murphy, B. R. & Whitehead, S. S. Immune Response to Dengue Virus and Prospects for a Vaccine. *Annu. Rev. Immunol.* **29,** 587–619 (2011).
112. Wilder-Smith, A. *et al.* Deliberations of the Strategic Advisory Group of Experts on Immunization on the use of CYD-TDV dengue vaccine. *Lancet Infect. Dis.* (2018). doi:10.1016/S1473-3099(18)30494-8
113. Hadinegoro, S. R. *et al.* Efficacy and Long-Term Safety of a Dengue Vaccine in Regions of Endemic Disease. *N. Engl. J. Med.* **373,** 1195–1206 (2015).
114. Guy, B. & Jackson, N. Dengue vaccine: hypotheses to understand CYD-TDV-induced protection. *Nat. Rev. Microbiol.* **14,** 45–54 (2016).
115. Rothman, A. L. & Ennis, F. A. Dengue Vaccine: The Need, the Challenges, and Progress. *J. Infect. Dis.* **214,** 825–827 (2016).
116. Guy, B. *et al.* A recombinant live attenuated tetravalent vaccine for the prevention of dengue. *Expert Rev. Vaccines* **16,** 671–684 (2017).
117. Dowd, K. A., DeMaso, C. R. & Pierson, T. C. Genotypic Differences in Dengue Virus Neutralization Are Explained by a Single Amino Acid Mutation That Modulates Virus Breathing. *MBio* **6,** (2015).
118. Katzelnick, L. C. *et al.* Dengue viruses cluster antigenically but not as discrete serotypes. *Science (80-.).* **349,** 1338–1343 (2015).
119. Mukherjee, S. *et al.* Mechanism and Significance of Cell Type-Dependent Neutralization of Flaviviruses. *J. Virol.* **88,** 7210–7220 (2014).
120. Plevka, P., Battisti, A. J., Sheng, J. & Rossmann, M. G. Mechanism for maturation-related reorganization of flavivirus glycoproteins. *J. Struct. Biol.* **185,** 27–31 (2014).
121. Pierson, T. C. & Diamond, M. S. Degrees of maturity: the complex structure and biology of flaviviruses. *Curr. Opin. Virol.* **2,** 168–75 (2012).
122. Cherrier, M. V *et al.* Structural basis for the preferential recognition of immature flaviviruses by a fusion-loop antibody. *EMBO J.* **28,** 3269–3276 (2009).
123. Fibriansah, G. *et al.* Cryo-EM structure of an antibody that neutralizes dengue virus type 2 by locking E protein dimers. *Science (80-.).* **349,** 88–91 (2015).
124. Dowd, K. A., Jost, C. A., Durbin, A. P., Whitehead, S. S. & Pierson, T. C. A Dynamic Landscape for Antibody Binding Modulates Antibody-Mediated Neutralization of West Nile Virus. *PLoS Pathog.* **7,** e1002111 (2011).
125. Wilson, P. C. & Andrews, S. F. Tools to therapeutically harness the human antibody response. *Nat. Rev. Immunol.* **12,** 709–719 (2012).
126. de Alwis, R. *et al.* Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions. *Proc. Natl. Acad. Sci.* **109,** 7439–7444 (2012).
127. Wahala, W. M. P. B., Kraus, A. A., Haymore, L. B., Accavitti-Loper, M. A. & de Silva, A. M. Dengue virus neutralization by human immune sera: Role of envelope protein domain III-reactive antibody. *Virology* **392,** 103–113 (2009).
128. Oliphant, T. *et al.* Induction of epitope-specific neutralizing antibodies against West Nile virus. *J. Virol.* **81,** 11828–39 (2007).
129. Kaufmann, B. *et al.* Neutralization of West Nile virus by cross-linking of its surface proteins with Fab fragments of the human monoclonal antibody CR4354. *Proc. Natl. Acad. Sci.* **107,** 18950–18955 (2010).

130. Spurrier, B. *et al.* Structural Analysis of Human and Macaque mAbs 2909 and 2.5B: Implications for the Configuration of the Quaternary Neutralizing Epitope of HIV-1 gp120. *Structure* **19,** 691–699 (2011).
131. Stettler, K. *et al.* Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection. *Science (80-.).* **353,** 823–826 (2016).
132. Fibriansah, G. *et al.* A potent anti-dengue human antibody preferentially recognizes the conformation of E protein monomers assembled on the virus surface. *EMBO Mol. Med.* **6,** 358–71 (2014).
133. Fibriansah, G. *et al.* A highly potent human antibody neutralizes dengue virus serotype 3 by binding across three surface proteins. *Nat. Commun.* **6,** 6341 (2015).
134. Gallichotte, E. N., Baric, R. S. & de Silva, A. M. in *Advances in experimental medicine and biology* **1062,** 63–76 (2018).
135. Gallichotte, E. N. *et al.* A new quaternary structure epitope on dengue virus serotype 2 is the target of durable type-specific neutralizing antibodies. *MBio* **6,** e01461-15 (2015).
136. Andrade, D. V *et al.* Analysis of Individuals from a Dengue-Endemic Region Helps Define the Footprint and Repertoire of Antibodies Targeting Dengue Virus 3 Type-Specific Epitopes. *MBio* **8,** (2017).
137. Nivarthi, U. K. *et al.* Mapping the Human Memory B Cell and Serum Neutralizing Antibody Responses to Dengue Virus Serotype 4 Infection and Vaccination. *J. Virol.* **91,** e02041-16 (2017).

CHAPTER 2

ANALYSIS OF INDIVIDUALS FROM A DENGUE-ENDEMIC REGION HELPS DEFINE THE FOOTPRINT AND REPERTOIRE OF ANTIBODIES TARGETING DENGUE VIRUS 3 TYPE-SPECIFIC ANTIBODIES

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Summary

The four dengue virus serotypes (DENV1-4) cause dengue, a major public health problem worldwide. Individuals exposed to primary DENV infections develop serotype-specific neutralizing antibodies, including strongly neutralizing antibodies targeting quaternary epitopes. To date, no studies have measured the levels and kinetics of serum antibodies directed to such epitopes among populations in dengue-endemic regions. Here, we use a recombinant DENV4 (rDENV4/3-M14) displaying a major DENV3 type-specific quaternary epitope recognized by human monoclonal antibody 5J7 to measure the proportion, magnitude and kinetics of DENV3 type-specific neutralizing antibody responses targeting this epitope. Primary DENV3 sera from 30 individuals in a dengue hospital-based study in Nicaragua were studied 3, 6, 12, and 18 months post-infection, alongside samples collected annually 1-4 years post-primary DENV3 infection from 10 individuals in a cohort study in Nicaragua. We found substantial individual variation in the proportion of DENV3 type-specific neutralizing antibody titers attributed to the 5J7 epitope (range 0-100%), with the mean significantly increasing from 22.6% to 41.4% from 3 to 18 months. We extended the transplanted DENV3 5J7 epitope on the virion (rDENV4/3-M16), resulting in increased recognition in several individuals, helping define the footprint of the epitope. However, 57% and 13% of the subjects still showed little to no recognition of the 5J7 epitope at 3 and 18 months, respectively, indicating that one or more additional DENV3 type-specific epitopes exist. Overall, this study demonstrates how DENV-immune plasma from endemic populations, when coupled with structurally-guided recombinant viruses, can help characterize the epitope-specific neutralizing antibody response in natural DENV infections, with direct implications for design and evaluation of dengue vaccines.

INTRODUCTION

Dengue is the most prevalent mosquito-borne viral disease in humans, caused by infection with four antigenically distinct serotypes of dengue virus (DENV1-DENV4), an enveloped, positive-sense RNA flavivirus. A range of clinical manifestations can occur during DENV infection, from undifferentiated illness and classic dengue fever (DF), to the most severe forms, Dengue Hemorrhagic Fever/Dengue Shock Syndrome (DHF/DSS), characterized by plasma leakage, shock and potentially death[1]. One of the hallmarks of dengue is the fact that sequential infections with different serotypes can be either protective or pathogenic. While antibodies elicited by primary infection usually confer life-long protection against the homologous serotype, cross-reactive neutralizing responses can wane over time. In the event of a secondary DENV infection, it is thought that the risk of severe disease may be increased via pre-existing cross-reactive weakly neutralizing or non-neutralizing antibodies that facilitate virus entry into target Fcg receptor-bearing cells[2–5]. Given this risk factor, it is imperative that vaccines induce protective immunity to all 4 serotypes simultaneously.

The DENV envelope (E) protein is the major target for neutralizing antibodies[6], and its ectodamain is comprised of three domains, EDI, EDII and EDIII. E facilitates virus attachment to host cells and subsequent fusion with the endosomal membrane. Recent findings have shown that potently neutralizing human monoclonal antibodies (hmAbs) are directed to complex quaternary epitopes present only on the E protein as assembled on the dengue virion, not as the recombinant monomer (recE)[7–10]. Within this class of highly neutralizing hmAbs is 5J7, which was isolated from a donor with a history of primary DENV3 infection[7]. Cryo-electron microscopy (Cryo-EM) studies of the 5J7 hmAb showed that one single Fab molecule binds three distinct E proteins on the surface of the virion: the EDI/EDII hinge region of one E monomer and domains II and III of two adjacent E proteins, although it is less clear whether contact points in all three monomers are essential for neutralization potency[9]. Subsequent studies employed a reverse genetics approach to transplant the core of the quaternary epitope targeted by 5J7 into a DENV1 backbone to create a rDENV1/3 chimeric virus, which was partially sensitive to neutralization by the hmAb 5J7[11]. Importantly, when administered prophylactically, 5J7 was shown to reduce viral load in a mouse model infected with DENV3 or rDENV1/3[11]. Based on the characterization of the DENV2 type-specific mAb 2D22, a different chimera, rDENV4/2, was created by transplanting the DENV2 EDIII onto a DENV4 backbone[12]. The DENV2-specific antibody response in polyclonal sera of individuals who experienced a natural primary DENV2 infection or were immunized with a monovalent live attenuated DENV2 vaccine tracked with DENV2 type-specific quaternary structure epitope(s) on the rDENV4/2 virus[12]. These observations demonstrate the utility of chimeric DENV viruses as tools for tracking epitope-specific neutralizing antibody responses in immune sera following DENV infection or vaccination.

In this chapter, we used two recombinant DENV4 strains with expanded transplants of the 5J7 quaternary epitope to measure the level and kinetics of neutralizing antibodies directed to this epitope following natural primary DENV3 infection in Nicaragua[13] up to four years post-infection. Our results show that the 5J7 epitope is either moderately or highly targeted by DENV3 type-specific neutralizing antibodies by 63 and 87% of the individuals analyzed at 3 and 18 months, respectively, and the proportion of the DENV3 neutralizing response attributable to the partial 5J7 epitope increases over time. Our results also suggest that the DENV3 antibody repertoire contains other type-specific neutralizing epitope(s) yet to be characterized. Our analysis of the recognition of a quaternary DENV epitope in a population living in a dengue-endemic region serves as a model for measuring the fine specificity of antibodies to DENV after natural infection or vaccination.

RESULTS

Study participants

Thirty patients who were enrolled for suspected dengue between 2010 and 2011 at the National Pediatric Reference Hospital (HIJMR) were selected for analysis of plasma collected 3, 6, 12 and 18 months post-infection. All the patients were laboratory-confirmed as DENV3-positive by RT-PCR and/or virus isolation. All experienced a primary DENV infection, with 20/30 (67%) manifesting disease as DF and 10/30 (33%) classified as DHF. In addition, 10 individuals participating in a long-term pediatric cohort study who experienced a primary DENV3 infection between 2009 and 2010 and manifested disease as DF were chosen to analyze plasma samples 1-4 years post-illness.

Longitudinal analysis of DENV-specific neutralizing antibodies following primary DENV3 infection

Following primary DENV infection, a durable serotype-specific neutralizing response can be detected years after exposure[14]. To determine whether the 5J7 epitope is recognized by the DENV3 type-specific antibody response in a dengue-endemic setting, neutralization assays were performed with primary DENV3 plasma collected 3 and 18 months post-infection and the rDENV4/3-M14 virus displaying the partial 5J7 epitope (**Figure 2.1a**). Sigmoidal-dose response curves representative of one primary DENV3 infection shows neutralization of the rDENV4/3-M14 virus at 3 and 18 months post-infection at levels close to parental DENV3 at the later time-point (**Figures 2.1c and 2.1d**). As expected, in all subjects, primary DENV3 plasma strongly neutralized DENV3, with titers significantly higher than to DENV4 virus (**Figure 2.1e**). The neutralizing titers to the parental strain UNC 3001 and the infecting DENV3 Nicaraguan strain were similar at both time-points (**Figure 2.S1**), as both are members of DENV3 genotype III[15]. Plasma from most individuals neutralized the rDENV4/3-M14 virus, and some neutralized it at levels similar to DENV3 (**Figure 2.1e**). This indicates that DENV3 type-specific neutralizing antibodies in human DENV3-immune plasma recognized the 5J7 amino acid residues transplanted into the DENV4 backbone. The longitudinal analysis showed a significant increase of the neutralizing titers to the rDENV4/3-M14 at 18 months ($p<0.001$), while the neutralizing titers to the parental viruses did not show significant variation between the time-points analyzed (**Figure 2.1e**).

Correlation between neutralizing antibody titers to rDENV4/3-M14 and DENV3 at 3 and 18 months post-primary DENV3 infection

Following primary infection, a type-specific response to the infecting serotype and a cross-reactive response to heterologous DENV serotypes is developed[7]. In individuals who experienced a primary DENV3 infection, the correlation between the DENV3 NT_{50} titers and the DENV4 cross-reactive NT_{50} titers in plasma was not significant at 3 and 18 months post-infection (**Figures 2.2a and 2.2b**). To investigate how the rDENV4/3-M14 virus neutralizing antibody titers track with the DENV3 response, correlation analyses

between rDENV4/3-M14 and DENV3 NT_{50} values were performed at 3 and 18 months post-infection. At both time-points analyzed, this correlation was significant (**Figures 2.2c and 2.2d**). Thus, the increased positive correlation between DENV3 and rDENV4/3-M14, compared to the correlation between DENV3 and DENV4, is due to inclusion of the 5J7 epitope transplant in the DENV4 backbone.

Proportion and kinetics of the DENV3 type-specific neutralizing response attributable to the 5J7 epitope

The proportion of the DENV3 type-specific response attributable to the 5J7 epitope recognition was measured as (rDENV4/3-M14 NT_{50} - DENV4 NT_{50})/(DENV3 NT_{50} - DENV4 NT_{50}), the ratio between rDENV4/3-M14 titers and DENV3 NT_{50} titers from which the cross-reactive component (DENV4 NT_{50} titers) had been subtracted. The mean proportion of DENV3 type-specific response targeted to the 5J7 epitope was 22.6% at 3 months post-infection, and at 18 months post-infection, the proportion significantly increased to 41.4% ($p=0.0184$) (**Figure 2.3a**). The proportion was not significantly different between individuals who experienced DF versus DHF (**Figure 2.S2**).

To visualize the proportions of the DENV3 neutralizing antibody response attributable to cross-reactive neutralization (DENV4 NT_{50} titers) or the 5J7 epitope (rDENV4/3-M14 NT_{50} titers not explained by DENV4 NT_{50} titers), we plotted the ratio of DENV4 NT_{50}/DENV3 NT_{50} and rDENV4/3-M14 NT_{50}/DENV3 NT_{50}, where the percent of the DENV3 neutralizing antibody response is represented on the Y-axis (**Figures 2.3b and 2.3c**). The thirty individuals are aligned in an ascending order of proportion of the DENV3 response explained by rDENV4/3-M14 at 18 months. It is evident that, at both 3 and 18 months, there was extensive variability in the amount to which the DENV3 response is explained by 5J7 or cross-reactive neutralization. Further, there was variation by individual in changes in neutralizing antibody responses over time, with most individuals having an increasing proportion of the DENV3 response attributable to the 5J7 epitope, while several lost 5J7 recognition (**Figure 2.3c**).

As the prevalence of this type-specific epitope among individuals living in dengue-endemic regions is an important guide for vaccine design, the individual variation of the proportion was further analyzed. The proportion of the DENV3 neutralizing antibody response directed to the 5J7 epitope ranged from 0 to 97% at 3 months and from 6 to 100% at 18 months post-infection across the thirty individuals studied (**Figure 2.3a-2.3c**). The proportion of the DENV3 neutralizing response attributable to the 5J7 epitope was defined as low (0-10%), moderate (10-30%) and high (above 30%). At 3 months, 57% (17/30) showed inexistent to low recognition of the 5J7 epitope, 13% (4/30) displayed moderate recognition, and 30% (9/30) displayed high recognition of the 5J7 epitope. At 18 months, 13% (4/30), 47% (14/30) and 40% (12/30) of the individuals displayed low, moderate and high recognition of the 5J7 epitope, respectively. Matched pair analysis enables assessment of the change of the proportion in each individual over time (**Figure 2.3d**). Using one standard deviation of the average of the proportion at 3 and 18 months as the criterion to define change, 50% (15/30) of the individuals

displayed an increase in the proportion over time, while 37% (11/30) showed no alteration and 13% (4/30) decreased (**Figure 2.3e**).

Next, we analyzed the data using antigenic cartography, where the NT_{50} titer was treated as a 'distance' between antisera and rDENV4/3-M14 as well as its parental viruses. A convergent pattern over time was observed, with rDENV4/3-M14 and DENV3 moving towards each other at 18 months post-primary DENV3 infection and DENV4 virus staying about the same distance from the primary DENV3 anti-sera (**Figure 2.3f**). This indicates greater antigenic similarity between DENV3 and rDENV4/3-M14 as recognized by the primary infection antisera at the later time point, with the antibody response becoming increasingly specific to the 5J7 epitope over time.

Trajectories of the rDENV4/3-M14, DENV3 and cross-reactive neutralizing responses

While the cross-reactive neutralizing antibody response has been described to wane over time[7], recent studies in dengue-endemic settings have described the maintenance of cross-reactive titers, potentially due to "boosting" by homotypic or heterotypic re-infection that fell short of the antibody threshold for a new infection[16,17]. To investigate whether the constant, increased or decreased proportion of the neutralizing antibody titers targeting the 5J7 epitope between 3 and 18 months is associated with the change in magnitude of homologous and heterologous titers, samples collected at 6 and 12 months of a subset of individuals (n=12) were used for neutralization assays to the rDENV4/3-M14 and all DENV serotypes (DENV1-DENV4) (**Figure 2.4 and Figure 2.S3**). In this analysis, in addition to DENV4, the cross-reactive titer as an average of anti-DENV1, DENV2 and DENV4 titers were plotted. As an example of a constant proportion between 3 and 18 months, one subject (Figure 4A) displayed rDENV4/3-M14 titers that followed similar kinetics to the DENV3 NT_{50} titers at 3, 6, 12 and 18 months, while the cross-reactive titers decayed over time. This pattern was observed in 42% (5/12) of the individuals analyzed. As an illustration of an increased proportion, subject 1228 (**Figure 2.4b**) showed an increase in DENV3 and rDENV4/3-M14 titers between 3 and 6 months in parallel with a slight decrease of cross-reactive titers; one case (8%) with this pattern was seen. Another example of increased titers from 3 to 18 months is shown with subject 1231 (**Figure 2.4c**), where the increase of DENV3 and rDENV4/3-M14 titers is observed at the later time-points – between 12 and 18 months. This pattern, observed in 33% (4/12) of the subjects, suggests a possible homotypic boost, whereby re-infection with DENV3 may have occurred [1], as the effect is only seen in the homologous titers (**Figure 2.4c**). Finally, the pattern of decreased proportion is observed in subject 1513 (**Figure 2.4d**), where an increase in neutralizing titers to the heterologous serotypes is seen at the later time-points – between 12 and 18 months – which could indicate heterotypic boosting. This pattern was observed in 17% (2/12) of the individuals analyzed.

Proportion of the DENV3 neutralizing response targeted to the 5J7 epitope years after infection

To test whether the durability of the DENV3 neutralizing antibody response targeted to the 5J7 epitope is maintained beyond 18 months post-infection, primary DENV3 plasma samples collected up to four years after infection in a long-term cohort study were analyzed. While the rDENV4/3-M14 neutralizing titers did not change across the years (**Figure 2.5a**), the proportion trended towards an increase between year 1 and year 2 after infection (**Figure 2.5b**). From year 1 through year 4, the mean proportion was 8, 25, 29 and 34% respectively across the ten individuals analyzed (**Figure 2.5c**). The individual trajectories of the proportion indicate a variation of 0-0.26, 0-0.80, 0-0.87 and 0-0.89 at years 1 through 4 (**Figure 2.5c**). The long-term analysis of the titers to the rDENV4/3-M14 virus indicates that the response to the 5J7 epitope is stable and durable.

rDENV4 virus containing a larger footprint of the 5J7 epitope region

To test whether some individuals were refractory to the rDENV4/3-M14 virus due to the inclusion of the 5J7 epitope transplant with contact sites on 2 DENV3 E monomers, as opposed to 3 monomers[18], a second chimeric virus was generated (Widman et al., unpublished). This chimeric virus, designated here rDENV4/3-M16, contains the expanded 5J7 epitope transplant to encompass all three monomers (**Figure 2.1b**). Plasma samples from all subjects were tested against the new rDENV4/3-M16 virus to determine whether the entire structurally defined footprint of the 5J7 epitope confers a gain of neutralization compared to the rDENV4/3-M14 virus (**Figure 2.6**). The neutralizing antibody titers due to rDENV4/3-M16 were slightly higher than rDENV4/3-M14 at 3 and 18 months post-infection, but not significantly so (**Figures 2.6a**). The expansion of the quaternary 5J7 epitope in the rDENV4/3-M16 virus in individuals who presented a moderate/high degree of recognition of the rDENV4/3-M14 virus (n=30) did not result in a significant increase of the proportion of the DENV3 response attributable to the 5J7 epitope at either time-point analyzed (**Figures 2.6b**). However, in individuals who displayed low neutralization of the rDENV4/3-M14 virus at 3 months (n=17), a gain of neutralization to the rDENV4/3-M16 virus was observed in 6 individuals at 3 months post-infection (**Figure 2.6c**). At 18 months, 4 individuals who displayed low neutralization of rDENV4/3-M14 (n=8) gained neutralization to the rDENV4/3-M16 virus (**Figure 2.6d**). However, the fact that a number of individuals still did not recognize the 5J7 epitope at 3 months (n=11) and 18 months (n=4) even in an expanded structure suggests that other DENV3 type-specific neutralizing epitope(s) contribute to the DENV3-specific epitope repertoire.

DISCUSSION

Defining the antibody repertoire in natural DENV infections and vaccination is critical to understanding the quality of neutralizing antibody responses *in vivo*. Isolating hmAbs from DENV-immune individuals is one approach, which has greatly advanced the field[7–10,19,20]; however, the next critical step is studying human polyclonal sera from individuals living in dengue-endemic regions to analyze the prevalence of specific epitopes of interest over time. Our findings on the prevalence and durability of a DENV3 type-specific epitope in a dengue-endemic population up to four years after infection indicates that the 5J7 is an important epitope within a larger DENV3 repertoire that requires further study. In addition, our study provides insights regarding the footprint of the epitope and demonstrates that chimeric viruses represent a powerful tool for mapping type-specific responses following natural infection at the population level.

The recognition of a quaternary DENV2 epitope transplant in a chimeric virus by polyclonal sera has been previously demonstrated in rhesus macaque DENV-immune sera and human DENV-immune sera from travelers and vaccinees[12]. The successful transplantation of the amino acids residues contained within the 5J7 epitope into a DENV4 backbone provides an important tool for identification of antigenic sites specific to polyclonal neutralizing antibodies following natural DENV3 infection. A recent study demonstrated that the 5J7 mAb neutralizes the rDENV4/3-M16 virus more efficiently than rDENV4/3-M14 and that returned travelers' sera partially tracked with the rDENV4/3-M14 and showed increased neutralizing antibody responses to the rDENV4/3-M16 virus (Widman et al., unpublished). Here, we show that large numbers of human plasma samples from a population living in a dengue-endemic region validate the footprint of the 5J7 epitope.

Following a primary DENV infection, an increasingly type-specific response over time is described in sera of individuals living in non-endemic areas[21] and returned travelers who experienced DENV infection[7]. We found that while the parental DENV3 titers did not change over time, the neutralizing response to the rDENV4/3-M14 virus increased from 3 to 18 months. This suggests that the DENV3 type-specific response recognizing the 5J7 epitope is enriched/selected months after infection. Supporting evidence that the proportion of the DENV3 neutralizing response directed to the 5J7 epitope increases over time is that antigenic cartography maps show convergence of the rDENV4/3 and parental DENV3 viruses from 3 to 18 months.

This increase in specificity could reflect the evolution and diversity of the antibody repertoire due to stochastic events in the germinal center and mutations during affinity maturation following infection. Memory B cells and long-lived plasma cells develop from naïve B cells and undergo affinity maturation during the several months after infection[22,23]. The improved recognition of the 5J7 epitope at 18 months could suggest the presence of viral antigens in peripheral organs that may sustain affinity maturation of the epitope over time. However, contrary to Zika virus[24], West Nile virus[25], Japanese encephalitis virus[26], tick-borne encephalitis virus[27], and Ebola virus[28], to our knowledge no studies have detected DENV in peripheral sites months after infection. Therefore, the

gain of neutralization to the 5J7 epitope from 6 to 18 months may not be attributed to ongoing affinity maturation months after infection triggered by persistence of DENV antigens.

The levels of cross-reactive antibodies are thought to decay over time in non-endemic regions[21]. However, the trajectory of cross-reactive titers at 3, 6,12 and 18 months post-infection revealed an increase in NT_{50} values in a subset of plasma samples analyzed. This finding is consistent with a previous study by our group demonstrating a modest increase in the magnitude and cross-reactivity of the neutralizing antibody response following primary DENV infection in Nicaragua[16]. The observation of "boosts" in neutralizing antibody titers suggests that re-infection with the homologous serotype or possibly low-level heterotypic infection may have occurred. These findings are consistent with another longitudinal study in Thailand, which also demonstrated an increase in cross-reactive neutralizing antibody titers over time[17]. In the light of these findings, re-exposure to DENV may be an important factor modulating the long-term antibody response. As such, it may also affect the serotype-specific epitope repertoire following DENV infection. For instance, re-exposure to homotypic virus, consistent with the epidemiology of DENV in our cohort study in Nicaragua, where one serotype predominantly circulates for several years at a time, could help explain the increase in the proportion of the neutralizing antibody response to the 5J7 epitope over time. On the other hand, boosting of cross-reactive titers between 6 and 18 months could explain the loss of rDENV4/3 recognition at 18 months observed in a few individuals.

While only a small fraction (<3%) of the DENV-specific memory B cell population produces potent neutralizing antibodies[29], we showed that the 5J7 epitope is a substantial target of neutralizing antibodies in DENV3-immune plasma months and up to four years post-infection. As the 5J7 epitope transplant was expanded to include contact sites on 3 rather than 2 E monomers, the number of individuals who recognized the 5J7 epitope increased. Overall, as most individuals recognized the epitope either partially or fully, this provides evidence that the 5J7 epitope is an important component of the DENV3 type-specific neutralizing repertoire in human populations. Furthermore, these studies demonstrate how analysis of human populations can help define the footprint of an epitope of interest.

However, the remaining primary DENV3 plasma samples that did not gain neutralization to even the chimeric virus displaying a larger footprint of the 5J7 epitope suggests that neutralizing antibodies may target other DENV3 type-specific epitopes or an epitope overlapping with the 5J7 site. Further, the abundant cross-reactive weakly neutralizing antibodies in polyclonal serum may compete with a minor subpopulation of potently neutralizing antibodies targeting these quaternary epitopes through steric interference. Our data provide compelling evidence that the DENV3 type-specific neutralizing antibody repertoire contains additional epitopes. In fact, multiple type-specific neutralizing epitopes exist for other DENV serotypes, as reported for DENV1[10,20] and DENV4[30]. In addition, in the context of a polyclonal response, binding of antibodies to one specific epitope may result in changes in the accessibility of a different epitope due to alternative conformations of the E protein[31]. As previously shown in EDIII[32], variations

in the E sequence across DENV3 strains may also affect recognition and neutralization of the 5J7 epitope. Similarly, a high degree of individual variation in antibody epitope repertoire has also been observed in sera of individuals naturally infected or vaccinated with yellow fever virus[33] and tick-borne encephalitis virus[34].

In summary, the differential recognition of the 5J7 epitope among individuals could suggest (1) variability of the antibody repertoire within the host; (2) the critical need for additional amino acid residues spanning the three E protein monomers contained within the 5J7 epitope that were not transplanted into the original chimeric rDENV4/3-M14 virus or even a greater expansion of the epitope(s) (rDENV4/3-M16); and/or (3) the existence of other epitopes in the DENV3 type-specific repertoire. This is the first large-scale study demonstrating that human plasma from a population living in a dengue-endemic area can validate and help define the footprint of a quaternary epitope. Further, our results strongly suggest that 5J7 epitope is an important component of a larger DENV3 type-specific neutralizing epitope repertoire. Moreover, the use of chimeric viruses as a molecular tool to decipher the neutralizing response in a human population has direct impact in guiding vaccine development and measurement of type-specific response following DENV infection.

EXPERIMENTAL PROCEDURES

Ethics statement

The Dengue Hospital-based Study and the Pediatric Dengue Cohort Study were approved by the Institutional Review Boards of the University of California, Berkeley, and the Nicaraguan Ministry of Health. Parents or legal guardians of the subjects enrolled in these studies provided written informed consent, and participants 6 years of age and older provided assent.

Dengue Hospital-based study

Study enrollment took place in the Nicaraguan National Pediatric Reference Hospital, Hospital Infantil Manual de Jesús Rivera (HIIMJR). Children between six months and 14 years old suspected of dengue (<7 days of illness) were eligible to participate in the hospital study, as described previously[35]. Laboratory-confirmed dengue cases were classified by severity using a computerized algorithm that compiled all clinical data meeting each criterion, as detailed in the 1997 WHO guidelines[1,35] (Dengue Fever, DF; Dengue Hemorrhagic Fever, DHF; or Dengue Shock Syndrome, DSS). Based on these guidelines, 20 individuals were classified as DF, while 10 experienced DHF. Plasma samples were collected in the acute (days 1-6) and convalescent (days 14-28) phases, as well as 3, 6, 12 and 18 months post-onset of illness.

Pediatric Dengue Cohort Study

The Nicaraguan Pediatric Dengue Cohort Study (PDCS; 2004 to present) is a community-based prospective study of approximately 3,500 children 2 to 14 years of age in Managua, Nicaragua[36]. Healthy annual blood samples collected from 10 participants 1, 2, 3, and 4 years after experiencing a primary DENV3 infection were used.

Laboratory tests

DENV infection was confirmed by: RT-PCR for detection of viral RNA[37,38]; isolation of DENV on C6/36 cells[37] and/or seroconversion by IgM ELISA or a ≥4-fold increase in total antibody titer as measured by Inhibition ELISA in paired acute and convalescent-phase samples. In the hospital study, primary dengue cases were determined by Inhibition ELISA[39,40], where antibody titers <2560 in the convalescent-phase sample (day 14-28 post-onset of symptoms) define primary infection status[13]. In the cohort study, a primary DENV infection was classified by seroconversion (a titer of <1:10 to 1≥1:10 as determined by Inhibition ELISA) in paired consecutive annual samples[36].

Cell and viruses

U937 cells expressing DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin), a known DENV attachment factor, were maintained as suspension cell cultures at 37°C with 5% CO_2 in RPMI 1640 (Gibco) supplemented with 1% non-essential amino acids, 1% penicillin and streptomycin, and 5% fetal bovine serum (FBS; HyClone). *Aedes albopictus* C6/36 cells were grown at 32°C in 5% CO_2 and were used for propagation of the Nicaraguan strains DENV1 (N1265), DENV2 (N172) and DENV3 (N7236), parental strains DENV3 UNC 3001, DENV4 Haiti 74, and recombinant viruses rDENV4/3-M14, and rDENV4/3-M16. The rDENV4/3-M14 virus contains 29 amino acid residues within the 5J7 epitope, spanning across two E protein monomers. The rDENV4/3-M16 virus contains 36 amino acids residues from the 5J7 epitope spanning three E protein monomers (Widman et al.,unpublished).

DENV neutralization assay

A flow cytometry-based neutralization assay was used to measure DENV-specific neutralizing antibodies, as previously described[41]. Briefly, DENV-immune sera at an initial dilution of 1:5 were serially diluted 3-fold 8 times in RPMI supplemented with 2% FBS. An amount of virus that infects 15% of the cells (previously determined by virus titration) was added to the plasma dilutions and incubated 45 minutes (min) at 37°C. After 24 hours (h), the cells were centrifuged at 252 x g for 5 min and resuspended in 100 µl of RPMI medium. Next, cells were fixed in 4% paraformaldehyde, incubated for 10 min at room temperature (RT) and centrifuged at 252 x g for 5 min. Then cells were blocked in permeabilization buffer (0.1% Saponin, 5% bovine serum albumin in 1X PBS) for 30 min at RT. Cells were subsequently incubated with anti-E mAb 4G2 conjugated to Alexa 488, diluted 1:1000 in blocking buffer (0.5% bovine serum albumin and 0.02% sodium azide in 1X PBS), for 25 min at RT. Cells were washed and resuspended in PBS. The percentage of infected cells was determined using a Guava Flow Cytometer (EMD Millipore) by gating Alexa 488-positive cells. The plasma dilution that reduced viral infection by 50% (NT_{50}) was calculated by a nonlinear, 4-parameter dose-response regression analysis with Prism Software (GraphPad), which is expressed as the reciprocal serum dilution. To eliminate the effect of non-specific cross-reactivity in human plasma, DENV-naïve titers (average of 10 DENV-naïve individuals enrolled in the PDCS) were subtracted from DENV-immune titers. Quality control criteria for the sigmoidal dose-response regression fit included an absolute sum of squares <0.2 and the coefficient of determination (R^2) >0.9.

Antigenic cartography map

Antigenic maps were generated from NT_{50} against DENV3 UNC 3001, DENV4 Haiti 74, and rDENV4/3 obtained from antisera collected at 3 and 18 months post-infection, as previously described[42]. The measured antigenic distance D_{ij} between virus *i* and antiserum *j* was estimated as $D_{ij}=log_2(b_j)-log_2(N_{ij})$, where b_j is the NT_{50} titer for the virus best neutralized by each antiserum *j*, and N_{ij} is the NT_{50} for virus *i* and antiserum *j*. Error

function $E=\sum_{ij} e(D_{ij},d_{ij})$ was minimized (5000 independent optimizations) to estimate coordinates for viruses and antisera that produced antigenic maps with the least disagreement between Euclidean distances d_{ij} and measured antigenic distances D_{ij}. Error was defined as $e(D_{ij},d_{ij})=(D_{ij}-d_{ij})^2$ when the NT_{50} was within the limit of detection of the assay and $e(D_{ij},d_{ij})=(D_{ij}-1-d_{ij})^2(1/(1+e^{-10(Dij-1-dij)}))$ for NT_{50} titers below the assay limit of detection. The minimum error antigenic map is shown on a grid indicating antigenic distances as two-fold dilutions, in any direction, on the antigenic map. The shift in virus position from the 3- to 18-month maps is shown with arrows.

Statistical analysis

Statistical analysis was conducted using Prism Graph Pad 5.0 (La Jolla, CA) and R (Version 3.1.3). Two-way ANOVA was used to compare the NT_{50} titers to rDENV4/3, DENV3 and DENV4 viruses at 3 and 18 months post-illness. Linear regression analysis with Pearson coefficient was used to evaluate the correlation between rDENV4/3 virus and DENV3 or DENV4 at 3 and 18 months post-illness. The paired t-test was used for comparing the proportion of the DENV3 neutralizing antibody response attributable to the 5J7 epitope between samples collected 3 and 18 months post-illness. A p-value of <0.05 was accepted as statistically significant.

FIGURES

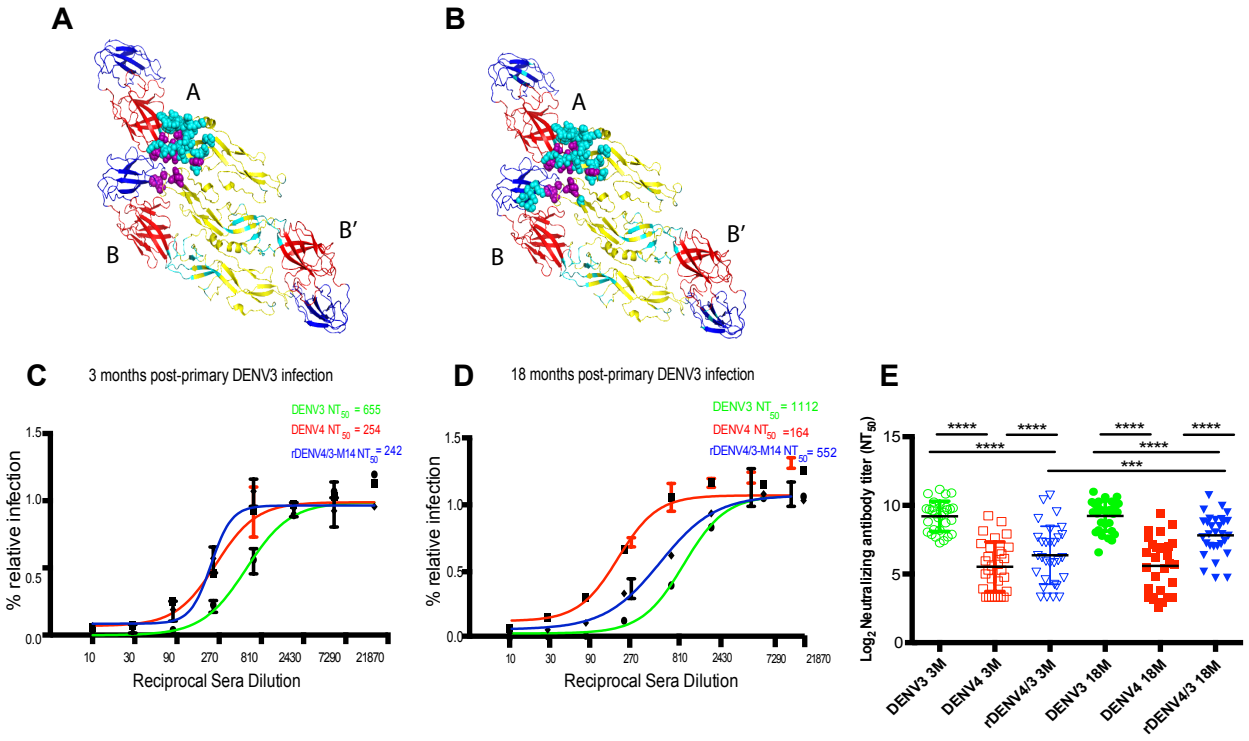


Figure 2.1. Neutralizing antibody titers to the chimeric rDENV4/3-M14 virus and parental DENV3 and DENV4 viruses 3 and 18 months post-primary DENV3 infection. (A) Ribbon diagram of the DENV E trimer (domains I, II, and III in red, yellow, and blue, respectively), showing amino acid residues within the 5J7 epitope spanning the A, B, and B′ monomers (purple and teal spheres); the residues represented by the teal spheres were transplanted into a DENV4 backbone, creating the rDENV4/3-M14 virus. (B) As in panel A, but the residues represented by the teal spheres were transplanted into a DENV4 backbone to create the rDENV4/3-M16 virus. (C and D) The raw antibody titration data were fitted with a four-parameter sigmoidal dose-response curve to estimate the 50% neutralizing antibody titer (NT50) to the rDENV4/3-M14 and parental viruses DENV3 and DENV4 in plasma samples 3 and 18 months postinfection, respectively. (E) Geometric mean of the NT50 values to the rDENV4/3-M14 and parental viruses of DENV3 and DENV4 in plasma samples 3 and 18 months postinfection. Data are representative of two independent experiments processed in duplicate for each plasma sample. The NT50 values were compared by two-way ANOVA (n = 30). ***, P < 0.001; ****, P < 0.0001.

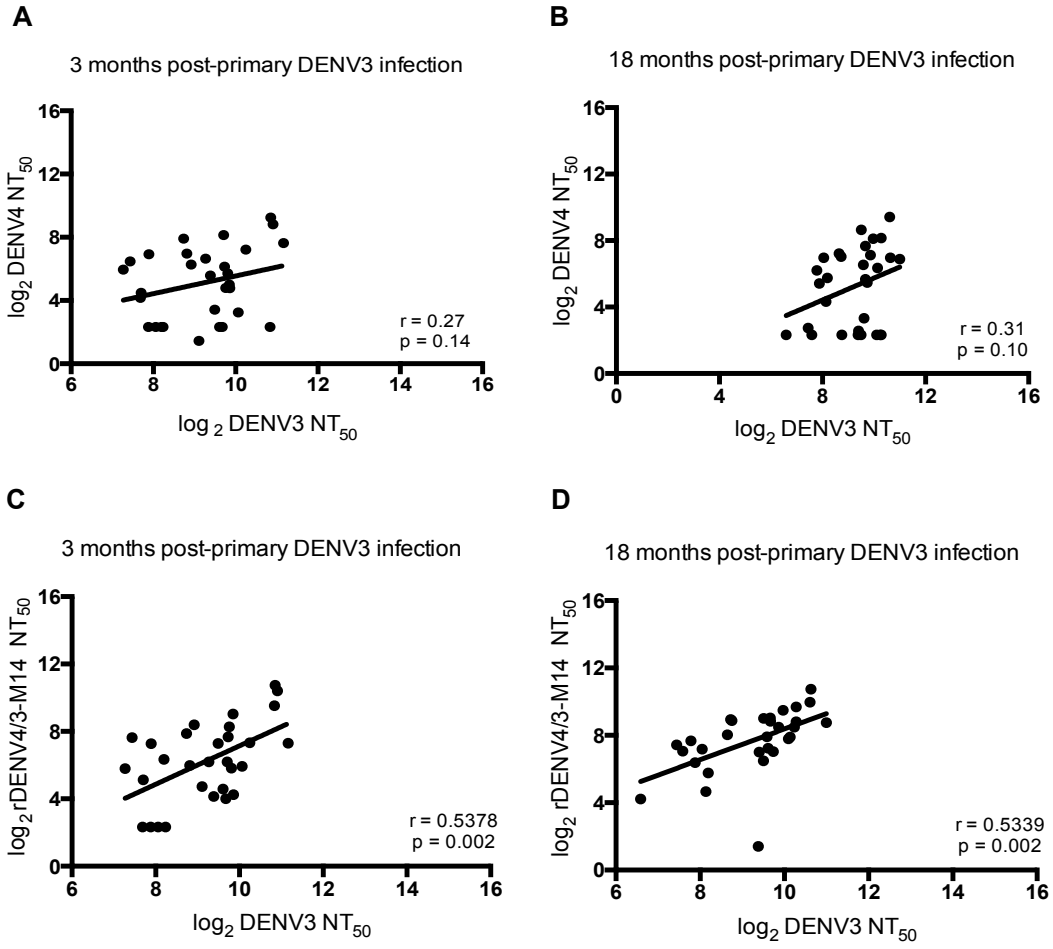


Figure 2.2. Correlation analysis between DENV3 and DENV4 or rDENV4/3-M14 neutralizing antibody titers over time. (A and B) Correlation between DENV3 NT50 and DENV4 NT_{50} at 3 and 18 months following primary DENV3 infection, respectively. (C and D) Correlation between DENV3 NT_{50} and rDENV4/3-M14 NT_{50} at 3 and 18 months following primary DENV3 infection, respectively. Data are representative of two independent experiments performed in duplicate for each plasma sample (n = 30). Pearson correlation coefficients (r) were calculated between the pairs of NT_{50} values. Linear regression lines are shown.

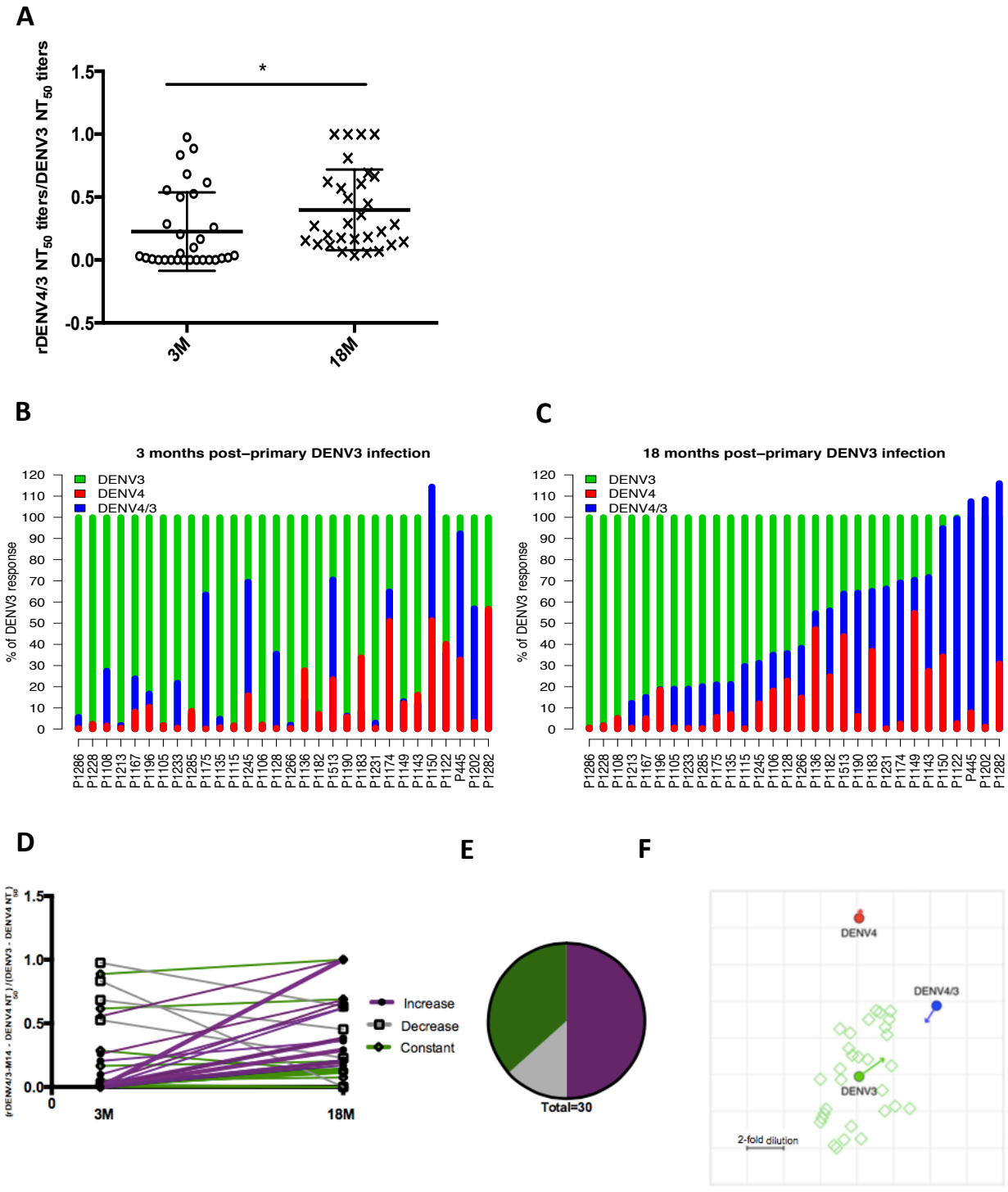

A
rDENV4/3 NT50 titers/DENV3 NT50 titers
1.5
1.0
0.5
0.0
-0.5
*
3M
18M
B
3 months post–primary DENV3 infection
% of DENV3 response
DENV3
DENV4
DENV4/3
P1286 P1228 P1108 P1213 P1167 P1196 P1105 P1233 P1285 P1175 P1135 P1115 P1245 P1106 P1128 P1266 P1136 P1182 P1513 P1190 P1183 P1231 P1174 P1149 P1143 P1150 P1122 P445 P1202 P1282
C
18 months post–primary DENV3 infection
% of DENV3 response
DENV3
DENV4
DENV4/3
P1286 P1228 P1108 P1213 P1167 P1196 P1105 P1233 P1285 P1175 P1135 P1115 P1245 P1106 P1128 P1266 P1136 P1182 P1513 P1190 P1183 P1231 P1174 P1149 P1143 P1150 P1122 P445 P1202 P1282
D
3M
18M
Increase
Decrease
Constant
E
Total=30
F
DENV4
DENV4/3
DENV3
2-fold dilution

Figure 2.3. Proportion and kinetics of the DENV3 type-specific neutralizing antibody response attributable to the 5J7 epitope. (A) The proportion of the DENV3 type-specific response directed to the 5J7 epitope obtained with 3- and 18-month post-infection samples. Results for 3 and 18 months (n = 30) were compared by paired t test, where * represents $P < 0.05$. (B and C) Proportion of the rDENV4/3-M14 and DENV4 neutralizing antibody response relative to DENV3 NT_{50}, where the y-axis represents 100% of the DENV3 NT_{50} values at 3 and 18 months post-infection, respectively. Individual codes are ordered by the proportion of DENV3 attributable to rDENV4/3-M14 observed at 18 months. (D) Individual trajectories of the proportion of the DENV3 type-specific response directed to the 5J7 epitope at 3 and 18 months (n = 30). (E) Pie chart representing the percentage of individuals who displayed a constant, decreased, or increased proportion of the rDENV4/3-M14 DENV3 response between 3 and 18 months post-primary DENV3 infection (n = 30). (F) Antigenic cartography map generated from NT_{50}. The antigenic cartography map positions viruses (closed circles [rDENV4/3-M14, DENV3, and DENV4]) and plasma (open squares) as points, with the distance between each virus and plasma derived from its respective neutralization titer (see Materials and Methods. Each grid square corresponds to a 2-fold dilution in the neutralization titer. Each open square represents one of the 30 plasma samples analyzed.

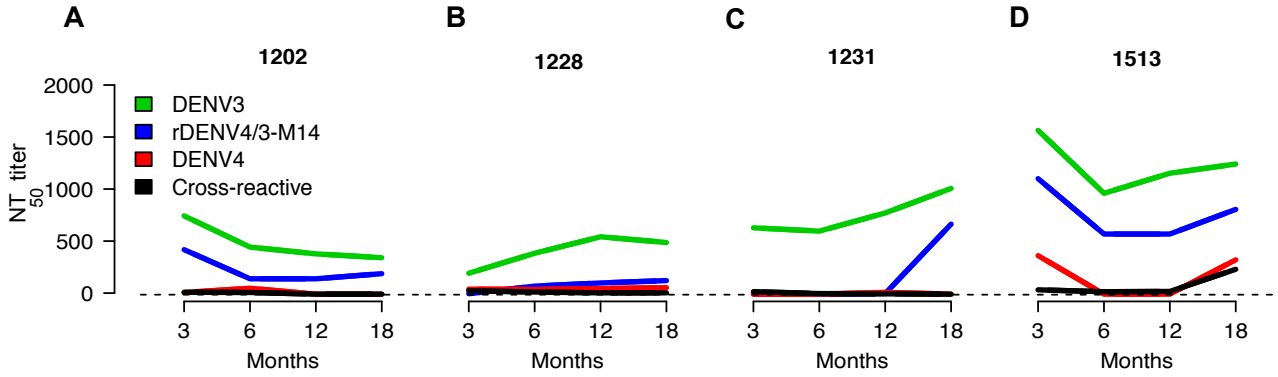


Figure 2.4. Trajectories of neutralizing antibody responses 3, 6, 12, and 18 months post-primary DENV3 infection. The neutralizing antibody titers to rDENV4/3-M14, DENV3, DENV4, and the overall cross-reactive titers (geometric mean of DENV1, DENV2, and DENV4 NT50 values) from 12 individuals were analyzed. (A) rDENV4/3-M14 titers follow similar trajectory to DENV3 titers in an individual with a constant proportion of the 5J7 response. (B) Increase of rDENV4/3-M14 titers between 3 and 6 months, with parallel decay of cross-reactive titers, indicates affinity maturation in an individual who displayed an increased proportion of the 5J7 response. (C) Increase in rDENV4/3-M14 titers between 12 and 18 months indicates possible homotypic reexposure in an individual with an increased proportion of the 5J7 response. (D) Increase in cross-reactive titers between 12 and 18 months indicates possible heterotypic reexposure in an individual who displayed a decreased proportion of the 5J7 response over time.

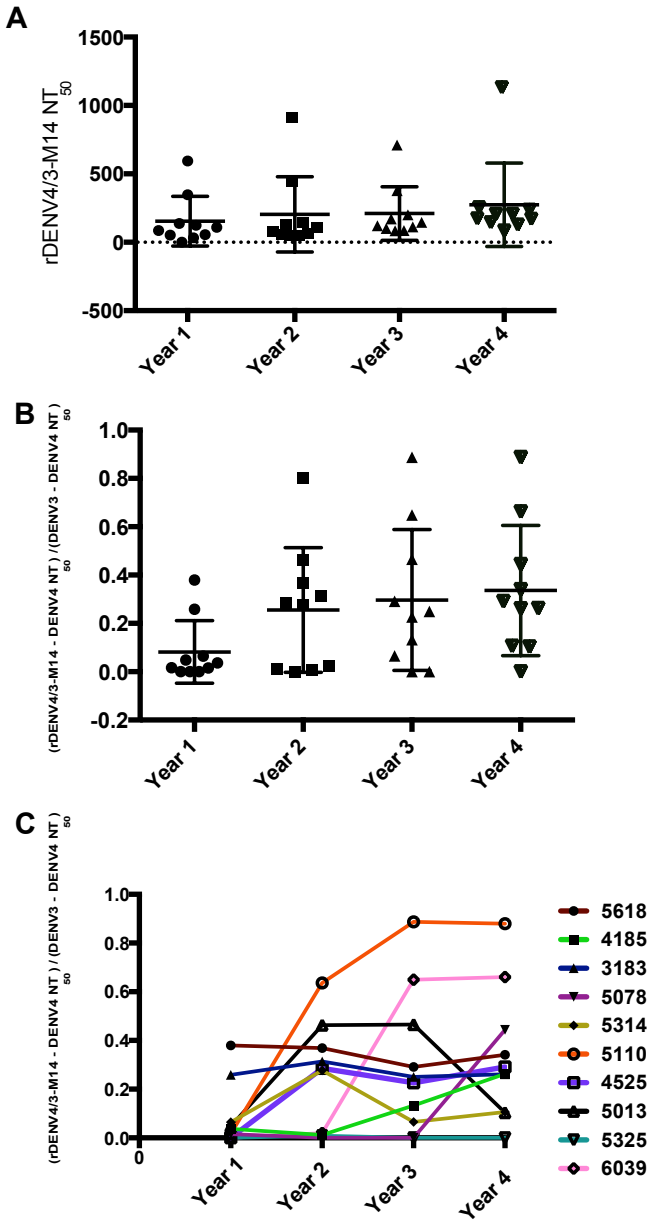


Figure 2.5. Proportion of the DENV3 type-specific neutralizing response targeted to the 5J7 epitope up to 4 years after infection in a cohort study design. (A) Neutralizing titers to the rDENV4/3-M14 virus from year 1 through year 4 post-primary DENV3 infection in a cohort study setting. (B) The ratio of the 5J7 epitope neutralizing antibody response (rDENV4/3-M14) to that of the parental DENV3 was analyzed from 1 to 4 years after primary DENV3 infection. (C) Individual trajectories of the DENV4/3-M14/DENV3 proportions from year 1 through year 4 post-primary DENV3 infection. Data are representative of two independent experiments processed in duplicate for each plasma sample. The NT50 and the proportion of the 5J7 response were compared by one-way ANOVA (n = 10); no significant differences were observed.

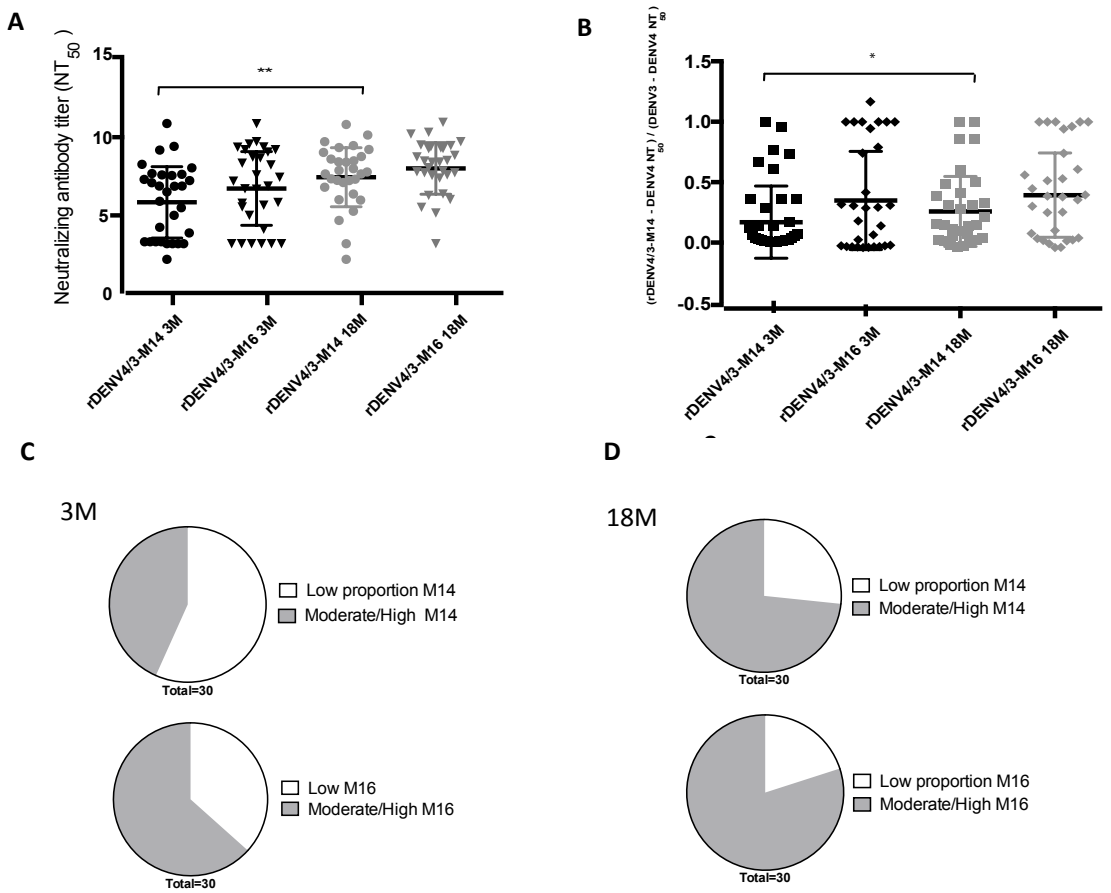


Figure 2.6. Neutralizing titers and proportion of the DENV3 response attributable to the 5J7 amino acid residues contained in the rDENV4/3-M14 and rDEV4/3-M16 viruses 3 and 18 months post-primary DENV3 infection. (A) Neutralizing antibody titers to rDENV4/3-M14 and to the chimeric virus with a larger footprint of the 5J7 epitope (rDENV4/3-M16) in primary DENV3 plasma 3 and 18 months postinfection (n = 30). (B) Proportion of the DENV3 neutralizing response attributable to the 5J7 amino acid residues transplanted into the DENV4 backbone in the rDENV4/3-M14 and rDENV4/3-M16 viruses 3 and 18 months post-primary DENV3 infection (n = 30). (C-D) Representation of the number of individuals who strongly recognize the rDENV4/3-M14 and rDENV4/3-M16 viruses or individuals who are refractory to rDENV4/3-M14 virus and only recognize the rDENV4/3-M16 virus at 3 and 18 months postinfection. The criteria for "low rDENV4/3-M14" or "low rDENV4/3-M16" was 0 to 10% of the DENV3 neutralizing response due to the 5J7 epitope, whereas "moderate/high rDENV4/3-M14" or "moderate/high rDENV4/3-M16" was defined as a proportion higher than 10%. The NT50 and the proportion of the 5J7 response were compared by two-way ANOVA (n = 30), where * represents $P < 0.05$ and ** represents $P < 0.01$.

SUPPLEMENTARY MATERIAL

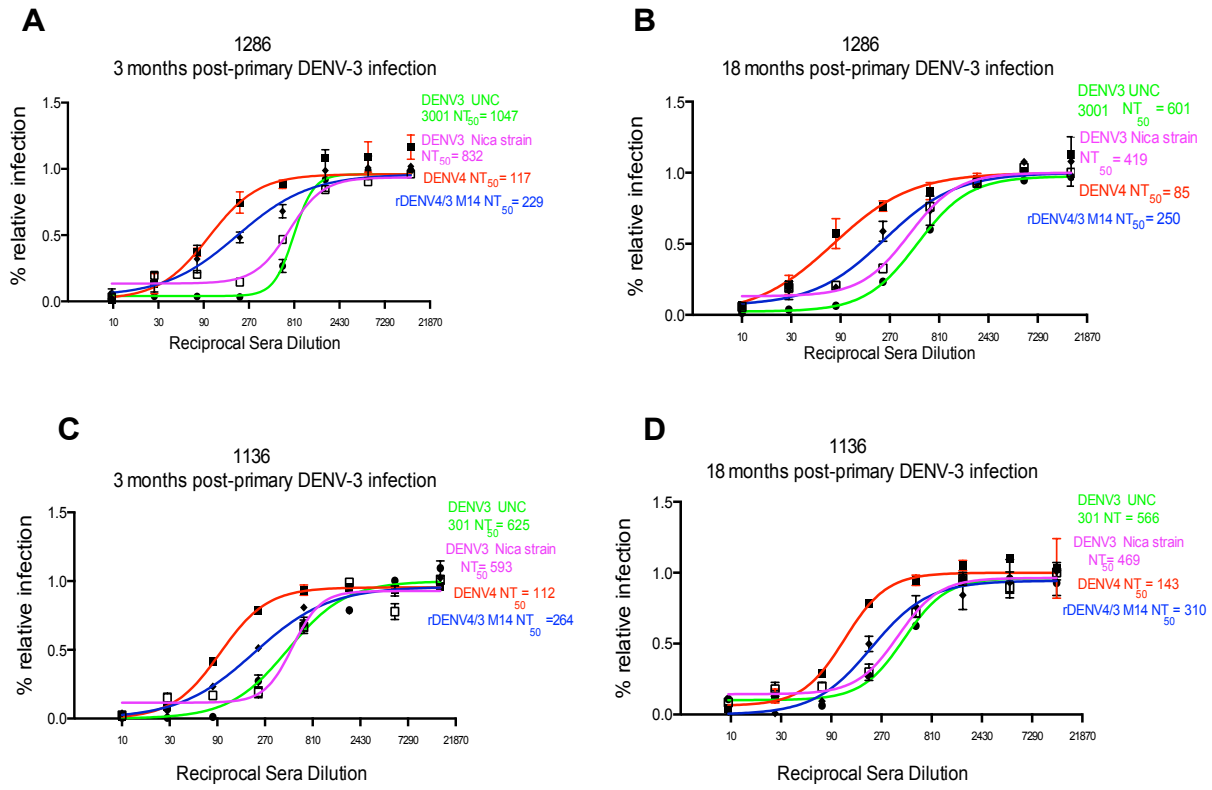


Figure 2.S1. Neutralizing antibody titers of primary DENV3 plasma against the parental DENV3 UNC 3001 strain and the DENV3 Nicaraguan strain. Neutralization assays were performed in parallel with the UNC 3001 and Nicaraguan DENV3 strains circulating during the 2010-2011 epidemics for plasma samples collected 3 and 18 months post-primary DENV3 infection (n = 2).

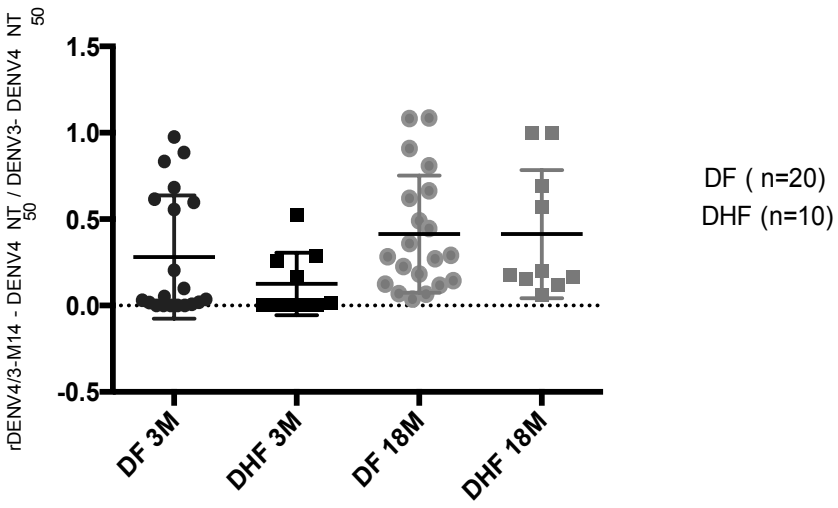


Figure 2.S2. Proportion of the DENV3 neutralizing antibody response attributable to the 5J7 epitope in individuals with different disease severity classifications. The analysis of the proportion of the DENV3 neutralizing antibody response attributable to the 5J7 epitope in individuals who experienced DF (n = 20) and DHF (n = 10) at 3 and 18 months post-infection was tested for significance with a two-way ANOVA and was found to be nonsignificant.

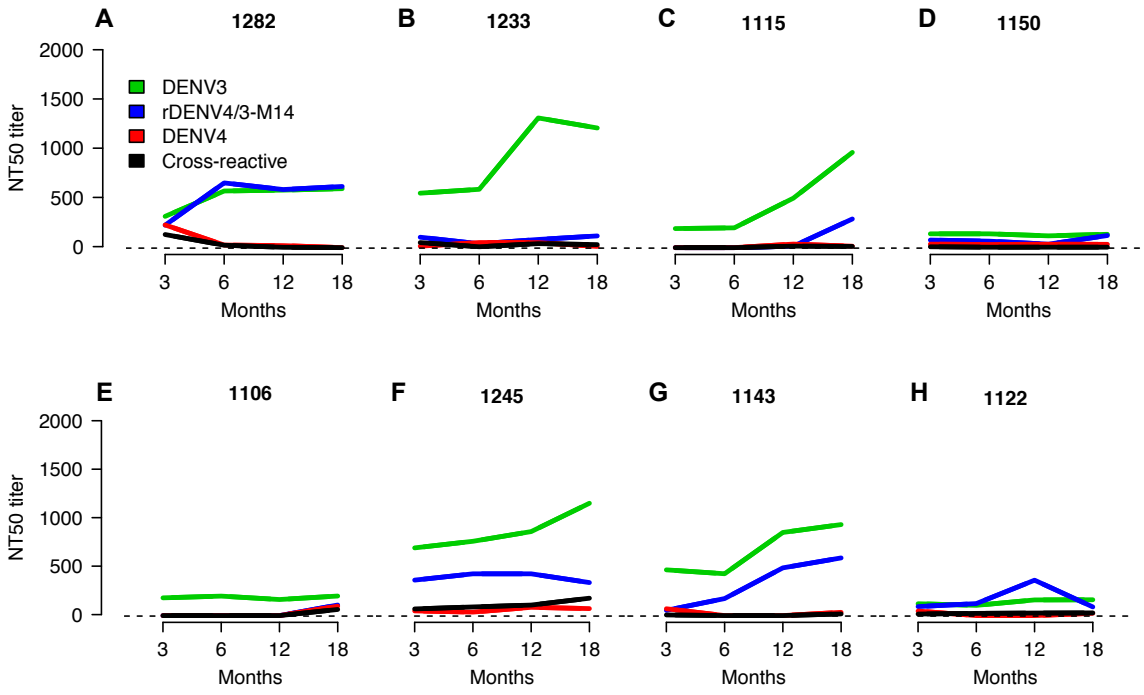
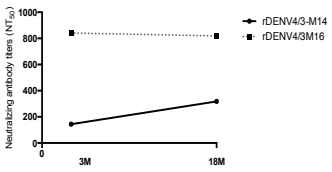


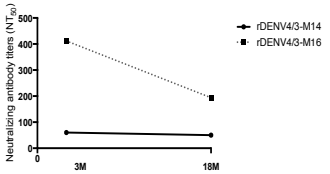
Figure 2.S3. Trajectories of neutralizing antibody responses at 3, 6, 12, and 18 months of a subset of 8 individuals in the hospital study. In the top panel, each line represents the NT50 for each time point analyzed (3, 6, 12, and 18 months) for DENV3 (green), rDENV4/3-M14 (blue), DENV4 (red), or the mean of cross-reactive titers (DENV1, DENV2, and DENV4) (black). (A) Trajectory of rDENV4/3-M14 and DENV3 neutralizing antibody titers between 3 and 18 months indicates affinity maturation in the early time points post-infection. (B to D) Increase of rDENV4/3-M14 at the later time points (6 or 12 months postinfection) indicates possible homotypic boosting. (E to H) Increase of cross-reactive neutralizing antibody titers at the later time points post-infection indicates possible heterotypic reexposure.

A — 1106



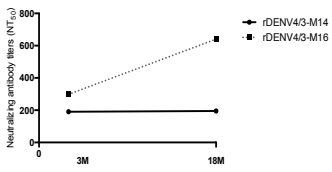
1106	3M	18M
rDENV4/3-M14	0.02	0.18
rDENV4/3-M16	1	1

B — 1136



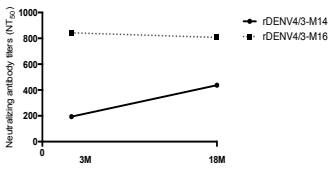
1136	3M	18M
rDENV4/3-M14	0.07	0.07
rDENV4/3-M16	1	0.45

C — 1228



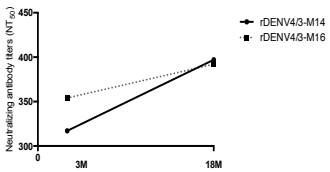
1228	3M	18M
rDENV4/3-M14	0.06	0.05
rDENV4/3-M16	0.15	0.43

D — 1282



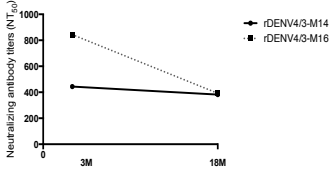
1282	3M	18M
rDENV4/3-M14	0.06	0.66
rDENV4/3-M16	1	1

E — 1174



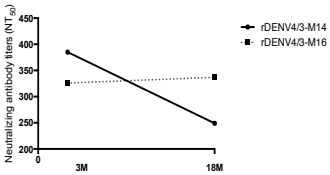
1174	3M	18M
rDENV4/3-M14	0.14	0.75
rDENV4/3-M16	0.47	0.74

F — 1175



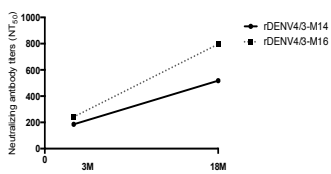
1175	3M	18M
rDENV4/3-M14	0.15	0.05
rDENV4/3-M16	0.36	0.06

G — 1108



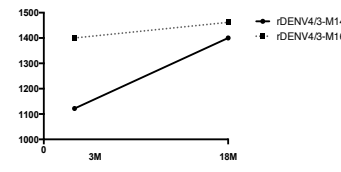
1108	3M	18M
rDENV4/3-M14	1	0.19
rDENV4/3-M16	1	1

H — 1182



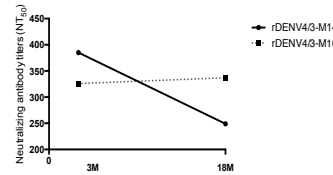
1182	3M	18M
rDENV4/3	0.01	0.16
rDENV4/3-2	0.02	0.5

I — 1105



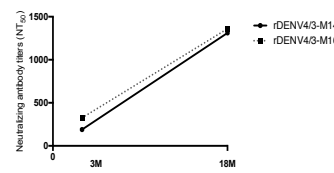
1105	3M	18M
rDENV4/3	0	0.18
rDENV4/3-2	0	0.15

J — 1108



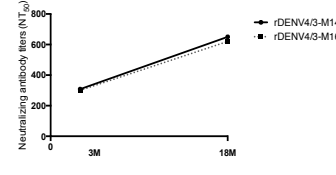
1108	3M	18M
rDENV4/3-M14	1	0.19
rDENV4/3-M16	1	1

K — 1115



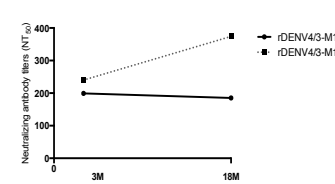
1115	3M	18M
rDENV4/3-M14	0.61	1
rDENV4/3-M16	1	1

L — 1128



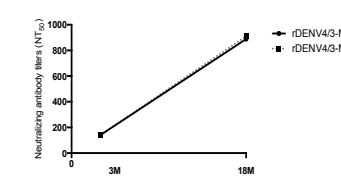
1128	3M	18M
rDENV4/3-M14	0.36	0.39
rDENV4/3-M16	0.35	0.36

M — 1135



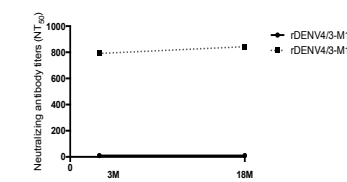
1135	3M	18M
rDENV4/3-M14	0.03	0.01
rDENV4/3-M16	0.11	0.14

N — 1143



1143	3M	18M
rDENV4/3-M14	0.02	0.56
rDENV4/3-M16	0.02	0.58

O — 1149



1149	3M	18M
rDENV4/3-M14	0.01	0.44
rDENV4/3-M16	0.01	0.45

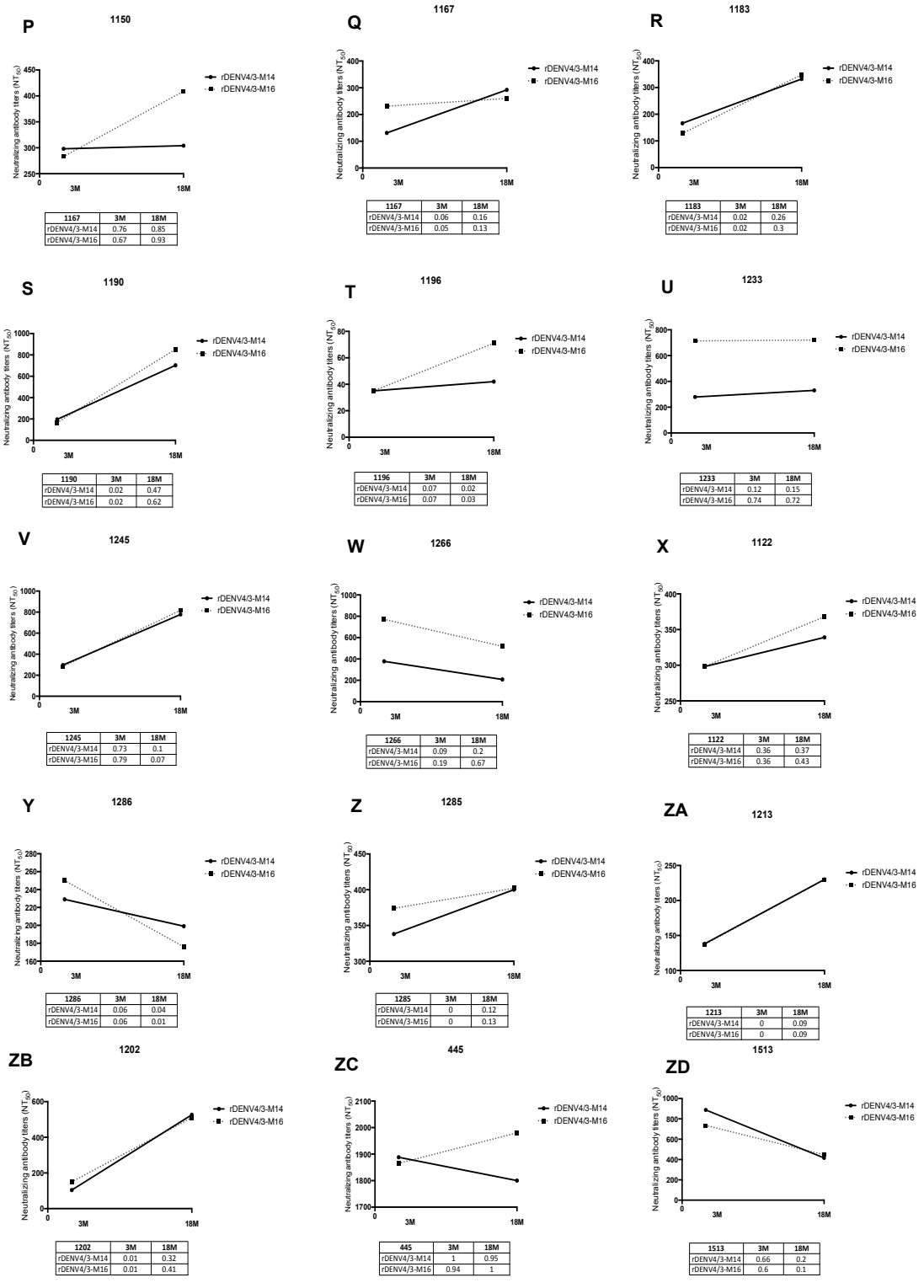


Figure 2.S4 Neutralizing antibody titers to rDENV4/3-M14 and rDENV4/3-M16 and respective proportion analysis at 3 and 18 months post-infection. (A to L) Individuals with higher recognition of the rDENV4/3-M16 compared to the rDENV4/3-M14 virus at 3 and/or 18 months post-infection. (M to ZD) Individuals with similar recognition of the rDENV4/3-M16 compared to the rDENV4/3-M14 virus at 3 and/or 18 months post-infection.

REFERENCES

1. *Dengue haemorrhagic fever Diagnosis, treatment, prevention and control SECOND EDITION Contents*. (1997).
2. SABIN, A. B. Research on dengue during World War II. *Am. J. Trop. Med. Hyg.* **1,** 30–50 (1952).
3. Halstead, S. B. Observations related to pathogensis of dengue hemorrhagic fever. VI. Hypotheses and discussion. *Yale J. Biol. Med.* **42,** 350–62 (1970).
4. Sangkawibha, N. *et al.* Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. *Am. J. Epidemiol.* **120,** 653–69 (1984).
5. Burke, D. S., Nisalak, A., Johnson, D. E. & Scott, R. M. A prospective study of dengue infections in Bangkok. *Am. J. Trop. Med. Hyg.* **38,** 172–80 (1988).
6. Roehrig, J. T. Antigenic structure of flavivirus proteins. *Adv. Virus Res.* **59,** 141–75 (2003).
7. de Alwis, R. *et al.* Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions. *Proc. Natl. Acad. Sci.* **109,** 7439–7444 (2012).
8. Fibriansah, G. *et al.* Cryo-EM structure of an antibody that neutralizes dengue virus type 2 by locking E protein dimers. *Science (80-.).* **349,** 88–91 (2015).
9. Fibriansah, G. *et al.* A highly potent human antibody neutralizes dengue virus serotype 3 by binding across three surface proteins. *Nat. Commun.* **6,** 6341 (2015).
10. Fibriansah, G. *et al.* A potent anti-dengue human antibody preferentially recognizes the conformation of E protein monomers assembled on the virus surface. *EMBO Mol. Med.* n/a-n/a (2014). doi:10.1002/emmm.201303404
11. Messer, W. B. *et al.* Functional Transplant of a Dengue Virus Serotype 3 (DENV3)-Specific Human Monoclonal Antibody Epitope into DENV1. *J. Virol.* **90,** 5090–5097 (2016).
12. Gallichotte, E. N. *et al.* A new quaternary structure epitope on dengue virus serotype 2 is the target of durable type-specific neutralizing antibodies. *MBio* **6,** e01461-15 (2015).
13. Gutierrez, G. *et al.* Unusual Dengue Virus 3 Epidemic in Nicaragua, 2009. *PLoS Negl. Trop. Dis.* **5,** e1394 (2011).
14. Imrie, A. *et al.* Antibody to Dengue 1 Detected More Than 60 Years after Infection. *Viral Immunol.* **20,** 672–675 (2007).
15. Messer, W. B. *et al.* Development and characterization of a reverse genetic system for studying dengue virus serotype 3 strain variation and neutralization. *PLoS Negl. Trop. Dis.* **6,** e1486 (2012).
16. Katzelnick, L. C., Montoya, M., Gresh, L., Balmaseda, A. & Harris, E. Neutralizing antibody titers against dengue virus correlate with protection from symptomatic infection in a longitudinal cohort. *Proc. Natl. Acad. Sci.* **113,** 728–733 (2016).
17. Clapham, H. E. *et al.* Dengue Virus (DENV) Neutralizing Antibody Kinetics in Children After Symptomatic Primary and Postprimary DENV Infection. *J. Infect. Dis.* **213,** 1428–35 (2016).
18. Fibriansah, G. *et al.* A highly potent human antibody neutralizes dengue virus

serotype 3 by binding across three surface proteins. *Nat. Commun.* **6,** 6341 (2015).
19. Smith, S. A. *et al.* The potent and broadly neutralizing human dengue virus-specific monoclonal antibody 1C19 reveals a unique cross-reactive epitope on the bc loop of domain II of the envelope protein. *MBio* **4,** e00873-13 (2013).
20. Teoh, E. P. *et al.* The Structural Basis for Serotype-Specific Neutralization of Dengue Virus by a Human Antibody. *Sci. Transl. Med.* **4,** 139ra83-139ra83 (2012).
21. Guzman, M. G. *et al.* Neutralizing antibodies after infection with dengue 1 virus. *Emerg. Infect. Dis.* **13,** 282–6 (2007).
22. McHeyzer-Williams, L. J., Cool, M. & McHeyzer-Williams, M. G. Antigen-specific B cell memory: expression and replenishment of a novel b220(-) memory b cell compartment. *J. Exp. Med.* **191,** 1149–66 (2000).
23. Smith, K. G., Light, A., Nossal, G. J. & Tarlinton, D. M. The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. *EMBO J.* **16,** 2996–3006 (1997).
24. Driggers, R. W. *et al.* Zika Virus Infection with Prolonged Maternal Viremia and Fetal Brain Abnormalities. *N. Engl. J. Med.* **374,** 2142–2151 (2016).
25. Roehrig, J. T. *et al.* Persistence of virus-reactive serum immunoglobulin m antibody in confirmed west nile virus encephalitis cases. *Emerg. Infect. Dis.* **9,** 376–9 (2003).
26. Ravi, V. *et al.* Persistence of Japanese encephalitis virus in the human nervous system. *J. Med. Virol.* **40,** 326–329 (1993).
27. Günther, G., Haglund, M., Lindquist, L., Sköldenberg, B. & Forsgren, M. Intrathecal IgM, IgA and IgG antibody response in tick-borne encephalitis. Long-term follow-up related to clinical course and outcome. *Clin. Diagn. Virol.* **8,** 17–29 (1997).
28. Kreuels, B. *et al.* A Case of Severe Ebola Virus Infection Complicated by Gram-Negative Septicemia. *N. Engl. J. Med.* **371,** 2394–2401 (2014).
29. Smith, S. A. *et al.* Persistence of circulating memory B cell clones with potential for dengue virus disease enhancement for decades following infection. *J. Virol.* **86,** 2665–75 (2012).
30. Nivarthi, U. K. *et al.* Mapping the Human Memory B Cell and Serum Neutralizing Antibody Responses to Dengue Virus Serotype 4 Infection and Vaccination. *J. Virol.* **91,** e02041-16 (2017).
31. Pierson, T. C., Fremont, D. H., Kuhn, R. J. & Diamond, M. S. Structural Insights into the Mechanisms of Antibody-Mediated Neutralization of Flavivirus Infection: Implications for Vaccine Development. *Cell Host Microbe* **4,** 229–238 (2008).
32. Wahala, W. M. P. B. *et al.* Natural Strain Variation and Antibody Neutralization of Dengue Serotype 3 Viruses. *PLoS Pathog.* **6,** e1000821 (2010).
33. Vratskikh, O. *et al.* Dissection of Antibody Specificities Induced by Yellow Fever Vaccination. *PLoS Pathog.* **9,** e1003458 (2013).
34. Jarmer, J. *et al.* Variation of the Specificity of the Human Antibody Responses after Tick-Borne Encephalitis Virus Infection and Vaccination. *J. Virol.* **88,** 13845–13857 (2014).

35. Narvaez, F. *et al.* Evaluation of the Traditional and Revised WHO Classifications of Dengue Disease Severity. *PLoS Negl. Trop. Dis.* **5,** e1397 (2011).
36. Kuan, G. *et al.* The Nicaraguan Pediatric Dengue Cohort Study: Study Design, Methods, Use of Information Technology, and Extension to Other Infectious Diseases. *Am. J. Epidemiol.* **170,** 120–129 (2009).
37. Balmaseda, A., Sandoval, E., Pérez, L., Gutiérrez, C. M. & Harris, E. Application of molecular typing techniques in the 1998 dengue epidemic in Nicaragua. *Am. J. Trop. Med. Hyg.* **61,** 893–7 (1999).
38. Lanciotti, R. S., Calisher, C. H., Gubler, D. J., Chang, G. J. & Vorndam, A. V. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J. Clin. Microbiol.* **30,** 545–51 (1992).
39. Fernández, R. J. & Vázquez, S. Serological diagnosis of dengue by an ELISA inhibition method (EIM). *Mem. Inst. Oswaldo Cruz* **85,** 347–51
40. Balmaseda, A. *et al.* High seroprevalence of antibodies against dengue virus in a prospective study of schoolchildren in Managua, Nicaragua. *Trop. Med. Int. Health* **11,** 935–42 (2006).
41. Kraus, A. A., Messer, W., Haymore, L. B. & de Silva, A. M. Comparison of Plaque- and Flow Cytometry-Based Methods for Measuring Dengue Virus Neutralization. *J. Clin. Microbiol.* **45,** 3777–3780 (2007).
42. Katzelnick, L. C. *et al.* Dengue viruses cluster antigenically but not as discrete serotypes. *Science (80-.).* **349,** 1338–1343 (2015).

CHAPTER 3

TRACKING THE POLYCLONAL NEUTRALIZING ANTIBODY RESPONSE TO A DENV1 SEROTYPE-SPECIFIC EPITOPE ACROSS TWO POPULATIONS IN ASIA AND THE AMERICAS

Summary

The four dengue virus (DENV) serotypes cause the most prevalent mosquito-borne human viral disease worldwide. Recent advances in the characterization of the humoral response to primary dengue virus (DENV) infection indicate that highly neutralizing serotype-specific antibodies target conformation-dependent epitopes on the DENV envelope (E) protein. Within this class of type-specific neutralizing antibodies is 1F4, a human monoclonal antibody (hmAb) isolated from a donor with history of primary DENV1 infection. We engineered a recombinant DENV2 virus containing the 1F4 epitope (rDENV2/1) via structure-guided immunogen design and reverse genetics. Here, we use the rDENV2/1 chimeric virus to measure the proportion and kinetics of DENV1 neutralizing antibody response targeting the 1F4 epitope in individuals living in two dengue-endemic areas – Asia and the Americas – where different DENV1 genotypes were circulating. Primary DENV1 plasma samples from 20 individuals in a dengue hospital-based study in Nicaragua were analyzed 3 and 18 months post-infection, alongside 5 samples from individuals in a cohort study collected up to four years post-primary DENV1 infection. Furthermore, we studied convalescent samples from Sri Lankan individuals who experienced primary DENV1 infection. We found that the 1F4 epitope is prevalent to varying degrees across the two populations living in dengue-endemic areas regardless of the DENV1 genotype circulating and can be detected as early as 60 days and as late as four years post-primary DENV1 infection. Additionally, both populations displayed what appears to be a bimodal pattern of recognition, where individuals had either high or low proportions of recognition of the 1F4 epitope. Our findings indicate that additional epitopes within the DENV1 repertoire, such as the target of the 14C10 mAb, may contribute to type-specific neutralization of DENV1 serotype. Altogether, we show that analysis of the epitope-specific neutralizing antibody responses in natural DENV infections can substantiate the repertoire of antibodies directed to DENV serotype-specific sites, with implications for vaccine development.

INTRODUCTION

The four dengue virus serotypes (DENV1-DENV4) are positive-sense RNA arboviruses in the family *Flaviviridae.* Widely distributed across many geographical regions, DENV causes the most prevalent human mosquito-borne viral disease worldwide, with over one third of the world's population at risk of infection[1]. Although asymptomatic infection occurs frequently, infection with DENV may cause a spectrum of disease manifestations, ranging from classical dengue fever (DF) to more severe outcomes, dengue hemorrhagic fever (DHF) and shock syndrome (DSS)[2,3]. While severe manifestations may be reported during primary infection, second infection with a heterologous DENV serotype is the main risk factor for DHF/DSS pathogenesis[4].

Neutralizing antibodies are a correlate of protection against symptomatic DENV infection[5–7]. After a first natural infection with any of the four DENV serotypes, serotype-specific neutralizing antibodies directed to the homologous serotype are elicited. In addition to these type-specific antibodies, which are thought to be life-long, a large population of cross-neutralizing antibodies is generated, which in turn may be protective or may enhance infection/disease with heterologous serotypes[8–10]. Given this risk, it is imperative that vaccines elicit a balanced and protective immunity to all four DENV serotypes simultaneously.

The envelope (E) glycoprotein is the major protein on the surface of the virion, where it forms parallel head-to-tail homodimers that lie in rafts on the surface of mature viruses[11–13]. Across serotypes, the amino acid homology of the E protein is in the range of 60-70%[14]. The E protein ectodomain is the major target of neutralizing antibodies and consists of three domains: EDI, EDII and EDIII[11,15–17]. EDI is formed into an eight-stranded β-barrel and comprises the central region of the E monomer. EDII contains a highly conserved fusion loop that facilitates fusion of the virus with endosomal membranes under mildly acidic conditions[18]. EDIII has an immunoglobulin-like fold and is involved in receptor binding to the host cell[19].

Isolation of human monoclonal antibodies (hmAbs) from individuals with a history of DENV infection has been fundamental for elucidating the specificity and mechanisms of the neutralizing antibody response to DENV infection. One of the key findings demonstrated that potent type-specific hmAbs target epitopes that require the intact E protein assembled on the DENV virion[20–22]. One such potent type-specific antibody is 1F4, a DENV1 type-specific hmAb[20,23]. However, unlike other hmAbs that recognize quaternary epitopes, 1F4 does not bind across neighboring E proteins. Instead, the footprint of 1F4 located within an E monomer and spans mostly across EDI, although interaction on the EDI/EDII hinge region is also described[23]. The conformation of the E protein adopted in the context of the virion is essential for binding to the 1F4 hmAb[23].

Reverse genetics approaches have been employed to transplant the core of quaternary epitopes targeted by DENV type-specific hmAbs into a DENV backbone to create chimeric viruses[24–26]. We have previously shown that primary DENV3 polyclonal sera of

a large number of individuals from a dengue-endemic area track to varying degrees with the 5J7 epitope transplanted into a DENV4 backbone[27]. The DENV2-specific neutralizing antibody response was also shown to track with the 2D22 quaternary epitope in samples from individuals who experienced natural DENV infection[28]. Similarly, chimeric viruses have been successfully used to track the antibody response of individuals who were immunized with a monovalent live attenuated DENV2 vaccine[26] or tetravalent dengue vaccine candidates[29,30]. All together, these studies support the pertinence of chimeric DENV viruses as tools for dissecting the specificity of the polyclonal neutralizing antibody response and helping validate the footprint of novel epitopes.

In this chapter, we used recombinant DENV2 virus with transplant of the 1F4 conformational epitope to quantify the proportion and kinetics of neutralizing antibodies directed to this epitope in individuals who experienced primary DENV1 infection. Of note, we analyzed individuals in two dengue-endemic regions where different genotypes of DENV1 are circulating – in Asia and in the Americas – at various time-points post-infection, which informs about the prevalence and durability of this epitope in the light of genotypic variations within the DENV1 serotype. Our results show that the 1F4 is prevalent in individuals from the two endemic areas analyzed and can be found in polyclonal plasma as early as ~60 days and as late as four years post-infection. Importantly, the proportion of the DENV1 type-specific response to this target shows a bimodal pattern, which suggests that the DENV1 repertoire is comprised of more than one immunodominant epitope. In sum, our findings validate the 1F4 epitope as an important component of the DENV1 type-specific neutralizing response in two dengue-endemic regions globally. Moreover, it reiterates the relevance of further studies aimed at characterizing major antigenic sites on the DENV1 serotype.

RESULTS

Study participants

Twenty patients enrolled in the Nicaraguan dengue hospital-based study were selected for analysis of the proportion and kinetics of the DENV1 response directed to the 1F4 epitope. We used plasma samples collected 3 and 18 months post-primary DENV1 infection. All cases were confirmed to be positive for DENV1 by reverse-transcription PCR (RT-PCR) and/or virus isolation. All individuals experienced only one primary infection with DENV1, with 16 manifesting disease as DF and 4 as DHF. For analysis of the 1F4 response for up to four years post-illness, we selected 5 individuals enrolled in the Pediatric Dengue Cohort Study (PDCS) who experienced one infection with the DENV1 serotype between 2011 and 2012. All 5 individuals manifested disease as DF. To analyze the response to the 1F4 epitope in a population in Sri Lanka, we selected 12 samples collected approximately 60 days post-primary DENV1 infection. The DENV1 genotype V was circulating in Nicaragua, while DENV genotype I was circulating in Sri Lanka.

Polyclonal response post-primary DENV1 infection tracks with a DENV1 type-specific conformational epitope transplanted into a heterologous DENV backbone

Recent findings reveal that the most potent DENV neutralizing hmAbs recognize epitopes that are only present on the assembled virion[21–23,31]. One such hmAb is 1F4, a conformation-dependent DENV1 type-specific hmAb isolated from an individual who experienced primary infection with DENV1[20]. The 1F4 epitope footprint is located on EDI and the EDI/EDII hinge region within an E protein monomer and consists of 26 amino acids, represented by red and yellow spheres in **Figure 3.1a**[23]. Thirty amino acid residues of the 1F4 epitope were transplanted onto a DENV2 backbone, creating the rDENV2/1 virus (**Figure 3.1b**). To determine whether DENV1 type-specific antibodies target the 1F4 epitope in the polyclonal sera of individuals who experienced a primary infection with DENV1, neutralization assays were performed with plasma samples collected at different time-points post-illness (3 and 18 months) against the rDENV2/1 virus displaying the 1F4 epitope and its parental DENV1 and DENV2 strains. Representative sigmoidal dose-response curves displaying neutralizing antibody titers of plasma samples from individual 1495 indicate that antibodies in polyclonal sera recognize the 1F4 epitope transplanted into the DENV2 backbone at both 3 and 18 months post- infection (**Figures 3.1c and 3.1d**). In addition, a shift in the rDENV2/1 curve indicating increasing DENV1-specific neutralizing antibody titers is observed at the later time point.

Neutralizing antibody titers to the rDENV2/1 virus are maintained at 3 and 18 months post-infection

The kinetics of the DENV-specific antibody response after a first infection is characterized by the appearance of IgM antibodies, followed by a durable IgG response. The early IgG response contains diverse antibody populations, including

cross-neutralizing antibodies and serotype-specific neutralizing antibodies. While some cross-reactive antibodies are transient, type-specific antibodies are believed to be life-long and can be detected years after infection[32]. We used the rDENV2/1 virus to specifically determine how the primary DENV1 antibody response tracks with the 1F4 conformational epitope in multiple individuals. As above, we performed neutralization assays with primary DENV1 plasma against the rDENV2/1 virus and its DENV1 and DENV2 parental viruses. As expected, in all subjects, primary DENV1 plasma strongly neutralized the parental DENV1 virus at both timepoints post-infection (**Figure 3.2a** and 3.**2b**). Also as expected, the NT_{50} values to the homologous serotype DENV1 were significantly higher than to the heterologous serotype DENV2 (**Figure 3.2a** and **Figure 3.2b**). At both time points, the NT_{50} values to the rDENV2/1 were significantly higher than the parental DENV2 titers and significantly lower than the parental DENV1 titers. Paired analysis of longitudinal samples at 3 and 18 months revealed significant decay of DENV1 NT_{50} values (**Figure 3.2c**), while DENV2 and rDENV2/1 titers remain constant (**Figure 3.2d and Figure 3.2e**). All together, this longitudinal analysis suggest that the antibodies targeting the 1F4 epitope are maintained over time despite the decay of DENV1 NT_{50} values, a first indication of the durability of this epitope-specific response.

The proportion of the DENV1 neutralizing response attributable to the 1F4 epitope varies across the Nicaraguan population

Next, we determined how much of the DENV1 type-specific response is directed to the 1F4 conformational epitope. Using the following formula (rDENV2/1 NT_{50} – DENV2 NT_{50})/(DENV1 NT_{50} – DENV2 NT_{50}), where the cross-reactive (DENV2 NT_{50}) has been subtracted from both the DENV2/1 and DENV1, we calculated the proportion of the DENV1 neutralizing antibody response attributable to the 1F4 epitope. Across the 20 individuals enrolled in the hospital-based study in Nicaragua, we observed that the mean proportion of the DENV1 type-specific response targeted to the 1F4 epitope was 24.9% at 3 months post-infection and 38.3% at 18 months post-infection (**Figure 3.3a**). This analysis shows a bimodal pattern in the degree to which DENV1 NT_{50} titers track with the 1F4 epitope: most individuals contained either high or low proportions to the 1F4 epitope. At 3 months post-primary DENV1 infection, the proportion ranges from 0-91%, while at 18 months it ranges from 0-100% (**Figure 3.3a**). Pairwise analysis showed that most individuals either gained response towards the 1F4 epitope (8/20) or retained the response to the 1F4 epitope (10/20) (**Figure 3.3b and 3.3c**), which is a strong indication of a long-lived pool of antibodies specific to this epitope. To estimate the antigenic similarity between DENV1 and rDENV2/1, we employed the antigenic cartography method[33]. The neutralizing antibody titers of each plasma sample at 3 and 18 months was treated as a measure of the distance between the plasma and the viruses. We observed that the rDENV2/1 is fairly close to DENV1 at 3 months, and the rDENV2/1 virus moves toward DENV1 at 18 months post-primary DENV1 infection (**Figure 3.3d**). This analysis indicates that rDENV2/1 and DENV1 share greater similarity at the later time-point, when the antibody response becomes increasingly targeted to the 1F4 epitope.

Individual variation exists in the DENV1 neutralizing antibody response directed to the 1F4 epitope

To better understand the different patterns illustrated in the individual trajectory analysis in **Figure 3.3b**, we grouped the individuals who displayed increased, constant or decreased proportions of DENV1 neutralizing antibodies attributable to 1F4 over time and concomitantly analyzed their neutralizing antibody titers to the rDENV2/1 virus and the DENV1 and DENV2 parental viruses. As seen in **Figure 3.4a**, individuals who displayed an increase in the 1F4 proportion (Group A) exhibit decay in DENV1 NT_{50} values, while only a slight increase in the rDENV2/1 in NT_{50} values is observed. In the subset of individuals who maintained constant proportion over time, we observe the same pattern, although the magnitude of DENV1 NT_{50} values decay is lower than in Group A (**Figure 3.4b**). Finally, the individuals who displayed a decreased proportion over time presented a rise in the DENV1 and DENV2 antibody titers (**Figure 3.4c**). In all the groups, the DENV2/1 neutralizing antibody titers remain relatively constant.

Antibodies directed to the 1F4 epitope can be detected years after primary DENV1 infection

While we demonstrated that DENV1 type-specific antibodies track with the 1F4 epitope in a substantial number of Nicaraguan individuals out to 18 months post-infection, we wanted to evaluate whether this response is maintained over an extended period of time. To test the long-term durability of the DENV1 response directed to the 1F4 epitope, we analyzed samples collected from 1 to 4 years post-primary DENV1 infection in a long-standing dengue cohort study in Nicaragua. Overall, the average NT_{50} value to the rDENV2/1 virus and both of the parental viruses was maintained from year 1 to year 4 (**Figures 3.5a-c**). With regard to the analysis of the proportion of 1F4, we observed that from year through year 4, the 1F4 epitope accounts on average for 47, 48, 52 and 47% of the DENV1 neutralizing response, respectively (**Figure 3.5d**). However, similar to the population enrolled in the Nicaraguan hospital study, we observed substantial variability among individuals in the amount of the DENV1 type-specific response directed to this conformational epitope over time (**Figure 3.5d and 3.5e**).

The 1F4 epitope is prevalent in a dengue-endemic area in Asia

The traditional classification of DENV into four genetically distinct serotypes appears to underestimate the impact of genotypic variation on neutralizing antibody response[33]. Recent data point to a clear effect of genetic variation among genotypes on neutralization capacity[34,35]. Given the efforts to design a vaccine that is effective for populations exposed to DENV strains of different genotypes, we evaluated whether the polyclonal response from individuals living in a dengue-endemic area where a distinct gentoype of DENV1 (genotype I) was circulating also tracks with the 1F4 epitope. From a Sri Lankan hospital study, we selected 12 individuals who experienced laboratory-confirmed primary DENV1 infection, from whom convalescent phase blood samples (approximately 60 days post-infection) were collected. We performed a similar analysis as above and found that the highest titers were found to the DENV1 homologous

serotype, while cross-neutralizing titers to the heterologous DENV2 serotype were significantly lower (**Figure 3.6a)**. While the NT_{50} values to the rDENV2/1 virus varied across the individuals, the average was comparable to the DENV1 NT_{50} values but significantly higher than the DENV2 NT_{50} values (**Figure 3.6a**). On average, 52% of the DENV1 neutralizing antibody response targeted the 1F4 epitope across the 12 individuals in Sri Lanka (**Figure 3.6b**), higher than in the Nicaraguan samples. Similar to the Nicaraguan population, we also observed what appears to be a bimodal pattern of recognition of the 1F4 epitope (**Figure 3.6b**). Finally, the antigenic cartography map again supports the antigenic similarity between rDENV2/1 and DENV1, as indicated by close distance between these viruses and the primary DENV1 sera (**Figure 3.6c**). Notably, the parental DENV1 West Pac 74' is in Genotype IV, which is more closely related to Genotype I circulating in Sri Lanka than to Genotype V circulating in Nicaragua[36–38].

DISCUSSION

While the homology of the E protein is around 60-70% across serotypes, the footprint of type-specific epitopes targeted by hmAbs appears to be located in distinct regions of the virion. As shown by structural studies, the footprint of the DENV3 type-specific hmAb 5J7 is located around the EDI/EDII hinge region and it spans across three different molecules within a single raft[22]. The DENV2 type-specific hmAb 2D22, on the other hand, targets mostly EDIII and EDII including the fusion loop[21,26], whereas the DENV1 type-specific 1F4 epitope is centered around EDI and extends to the EDI/EDII hinge region[23]. As recent studies highlight, polyclonal sera of naturally DENV infected individuals or recipients of dengue vaccines track to varying degrees with complex quaternary epitopes of the 5J7 and 2D22 hmAbs[27,28]. To date, two potent DENV1 type-specific mAbs have been isolated and characterized, namely 1F4[23] and 14c10[39]. Sera from recipients of a tetravalent vaccine were shown to track with the 1F4 epitope transplanted in a DENV3 backbone[29]. Here, we employ our chimeric virus rDENV2/1 displaying the 1F4 epitope to measure the prevalence of antibodies targeting this epitope across two populations in Asia and in the Americas. Of note, this is the first time chimeric viruses are used to measure a type-specific epitope in areas affected by distinct genotypes, hence providing a deeper analysis of the DENV1 repertoire landscape.

Results of the Phase IIb clinical trial of the Dengvaxia vaccine demonstrated reduced protection against DENV2 disease, despite high seroconversion rates and geometric mean neutralization titers to DENV2[40]. One plausible explanation is that the vaccine did not induce type-specific neutralizing antibodies but instead, a large population of cross-reactive neutralizing antibodies[30]. While it has been shown that serotype-specific antibodies drive most of the neutralization in post-primary sera[30,41,42], the viral epitopes targeted by this antibody in a larger population remains to be examined. Therefore, approaches that dissect the specificity of the polyclonal antibody response elicited by natural infection are relevant and needed. In our longitudinal analysis of the neutralizing antibody titers at 3 and 18 months post-primary DENV1 infection, we observed low levels of neutralization of the DENV2 heterologous serotype. These plasma samples gained neutralization capacity when amino acid residues of the DENV1 type-specific 1F4 epitope were transplanted into a DENV2 backbone, indicating that this epitope is specifically recognized by a DENV1 type-specific antibody population in polyclonal sera post-primary DENV1 infection.

In the Nicaraguan population, the proportion of the DENV1 neutralizing antibody response attributable to the 1F4 epitope varied substantially across individuals. Strikingly, this variation appears to be bimodal, with one group of individuals displaying very high levels of the 1F4 epitope, and a second group with nonexistent or very low levels of the DENV1 antibody response directed to this conformational epitope. Such findings suggest the existence of other immunodominant epitopes within the DENV1 repertoire. Indeed, another DENV1 type-specific hmAb, designated 14c10, has been described[39]. In contrast with the 1F4, the 14c10 hmAb binds across two E proteins.

Furthermore, 14c10 extends to EDIII, in addition to EDI and EDI/EDII hinge region[39]. Structural studies revealed that both 14c10 and 1F4 hmAbs bind to overlapping regions on the ED/EDII hinge, which could indicate that type-specific neutralizing antibodies preferentially target this region on the E protein. While the prevalence of the 14c10 in primary DENV1 sera is still unknown, one hypothesis is that binding of 14c10 antibodies to the hinge region may alter the accessibility of the epitope recognized by the 1F4-like antibody population. In fact, binding of the 1F4 Fab fraction has been shown to be sensitive to the hinge angle between EDI and EDII[39].

Besides the heterogeneous prevalence of the 1F4 epitope in the Nicaraguan population, we observed different patterns of gain or loss of the neutralizing response attributable to this epitope between 3 and 18 months post-infection. A large subset of individuals retained the same proportion of the DENV1 type-specific response attributable to the 1F4 epitope at 3 and 18 months. Interestingly, a number of individuals gained DENV1 neutralization directed to the 1F4 epitope at the later time-point. In this subset, we observed a decay of overall DENV1 NT_{50} values and a slight decrease of DENV2 titers, which could be attributed to waning of cross-reactive titers[20,43]. Thus, in individuals who became more serotype-specific over time, 1F4-like antibodies accounted for a larger proportion of the DENV1 polyclonal neutralization at the later time point. Conversely, a very small subset of individuals lost response to the 1F4 epitope at 18 months. Similar to our previous findings with the 5J7 epitope in primary DENV3 cases[27], we observed a rise in the cross-reactive antibody titers in this small subset of individuals who lost recognition of the type-specific epitope. In all three groups, rDENV2/1 neutralizing antibody titers remained relative constant over time.

Numerous studies substantiate neutralizing titers (NT_{50}) as correlates of protection for dengue diseaase[5–7]. Nonetheless, homotypic reinfections in Nicaragua[47] and Peru[46] have been reported, as well as breakthrough infections in individuals who seroconverted to the serotype in question following immunization[47,48]. One hypothesis for such observations is the genotypic variation within each serotype that may lead to neutralization escape. An increasingly number of studies show the impact of genotypic variation on neutralizing responses[34, 49–53]. Importantly, recent results from a DENV vaccine clinical trial pointed to a higher efficacy when the vaccine DENV4 genotype matched the one circulating in the area where immunization took place[47]. To compare the prevalence of the 1F4 epitope in areas affected by different DENV1 genotypes, we included samples from Sri Lanka, where genotype I circulates, in contrast with Nicaragua, where genotype V is found. Similar to the Nicaraguan population, the levels of 1F4 neutralization varied across the Sri Lankan individuals analyzed. However, the Sri Lankan population presented a higher proportion of the DENV1 neutralizing response to the 1F4 than the Nicaraguan population, and the closer antigenic similarity between DENV1 and rDENV2/1 virus was more evident in the antigenic cartography analysis of the Sri Lankan plasma samples. Interestingly, the parental DENV1 virus used for the chimeric virus is the West Pac 74, within genotype IV. As evidenced by phylogenetic analysis, DENV1 strains of genotypes IV are more closely related to genotype I (circulating in Sri Lanka) than to genotype V (circulating in Nicaragua), which could explain the higher proportion of this epitope in the Sri Lankan population. Taken

together, our analyses highlight the importance of capturing intra-serotype genotypic variations when analyzing novel neutralizing epitopes.

In sum, we demonstrated that the 1F4 epitope is an important component of the DENV1 epitope type-specific repertoire in a large number of individuals who experienced natural DENV1 infection. The differential recognition of this epitope across populations exposed to different DENV1 genotypes is evidence that intra-serotype amino acid variations leads to variation in neutralization of type-specific antibodies. Importantly, the bimodal pattern of 1F4 epitope recognition in both Nicaraguan and Sri Lankan populations suggest additional epitopes within the DENV1 repertoire also drive type-specific neutralization and deserve further investigation.

EXPERIMENTAL PROCEDURES

Ethics statement

The protocols for the Pediatric Dengue Cohort Study (PDCS) and the Pediatric Dengue Hospital-based Study (PDHS) in Nicaragua were reviewed and approved by the Institutional Review Boards of the University of California, Berkeley and the Nicaraguan Ministry of Health. Parents or legal guardian of the subjects enrolled in these studies provided written informed consent, and participants 6 years of age and older provided assent.

Study population

Study enrollment took place at Hospital Infantil Manuel de Jesús Rivera, the national pediatric reference hospital. Children ages between 6 months and 14 suspected of DENV infection (<7 days since onset of symptoms) were eligible to participate in the hospital study, as described previously[54].Laboratory-confirmed cases were classified by disease severity according to the 1997 WHO guidelines[3] using a computerized algorithm that compiled all clinical data meeting all criterion for dengue fever (DF), dengue hemorrhagic fever (DHF), or dengue shock syndrome (DSS)[54]. Plasma samples were collected in the acute (days 1 to 6 of illness) and convalescent (days 14 to 28 post-onset of symptoms) phases, as well as 3, 6, 12, and 18 months after illness. (ii) Pediatric Dengue Cohort Study (PDCS). The PDCS is an ongoing prospective dengue cohort study that follows approximately 3,700 children ages 2-14 in District II of Managua, Nicaragua[55]. Healthy annual blood samples collected from 5 participants from year 1 through 4 post-primary DENV1 infection were used.

Laboratory tests

In the Nicaraguan studies, DENV infection was identified by serotype-specific RT-PCR for detection of viral RNA, isolation of DENV on C6/36 cells[56], and/or seroconversion by IgM enzyme-linked immunosorbent assay (ELISA)[57] or a $\geq$4-fold increase in total antibody titer as measured by inhibition ELISA in paired acute- and convalescent-phase samples[8,58]. In the hospital study, primary dengue cases were determined by inhibition ELISA, where antibody titers of <2,560 in days 14-28 post-onset of symptoms (early convalescent phase) defined primary infection status[54]. In the cohort study, primary infection with DENV was detected by seroconversion (a titer of <1:10 to $\geq$1:10 as determined by Inhibition ELISA) in paired consecutive annual samples[55].

Cells and viruses

U937 cells expressing DC-SIGN (dendritic cell-specific intracellular adhesion molecule-3-grabbing nonintegrin), a known DENV attachment factor, were used for the neutralization assays. The U937 cells were maintained as suspension cell cultures at 37°C with 5% $C0_2$ in RPMI 1640 (Gibco) supplemented with 1% non-essential amino

acids, 1% penicillin and streptomycin, and 5% fetal bovine serum (FBS, HyClone). Propagation of the parental DENV1 (West Pac 74) and DENV2 (S16803) viruses and recombinant virus rDENV2/1 was performed in *Aedes albopictus* C6/36 cells grown at 32°C in 5% $C0_2$.

DENV neutralization assay

To measure DENV-specific neutralizing antibodies, we employed a flow cytometry-based assay, as previously described[59]. Briefly, DENV-immune plasma samples at an initial dilution of 1:5 were serially diluted 3-fold 8 times in RPMI supplemented with 2% FBS. A dilution of virus that infects between 8-15% of the U937 cells (previously determined by virus titration) was added to the plasma dilutions and incubated for 1h at 37°C. Following the incubation time, the cells were centrifuged at 252 x g for 5 minutes (min) and resuspended in 100 µL RPMI medium. Next, cells were fixed in 4% paraformaldehyde, incubated for 10 min at room temperature (RT), and centrifuged at 252 x g for 5 min. Subsequently, cells were blocked in permeabilization buffer (0.1% saponin, 5% bovine serum albumin in 1X phosphate-buffered saline [PBS] for 30 min at RT. Then, cells were incubated with anti-E mAb 4G2 conjugated to Alexa 488, diluted in blocking buffer (0.5% bovine serum albumin and 0.02% sodium azide in 1X PBS) for 25 min at RT. Finally, cells were washed and resuspended in PBS. Acquisition of the infected cells was performed with a Guava flow cytometer (EMD Milipore) by gating Alexa 488-positive cells. The neutralizing antibody titer that reduced the infection by 50% (NT_{50}) was calculated by a nonlinear, 4-parameter dose-response regression analysis with Prism software (GraphPad), which is expressed as the reciprocal serum dilution. Data generated had to fit the quality control criteria, where the sigmoidal dose response regression an absolute sum of squares of <0.2 and a coefficient of determination (R^2) of >0.9.

Statistical analysis

Statistical analysis was performed using Prism Graph Pad 5.0 (La Jolla, CA). One-way analysis of variance (ANOVA) was used to compare the NT_{50} values to the chimeric virus and parental viruses at early convalescent, 3 and 18 months post-illness. Paired t test was used to compare the proportions of the DENV1 type-specific neutralizing response attributable to the 1F4 epitope between samples collected between 3 and 18 months post-infection. Statistical difference was considered significant when p-value <0.05.

FIGURES

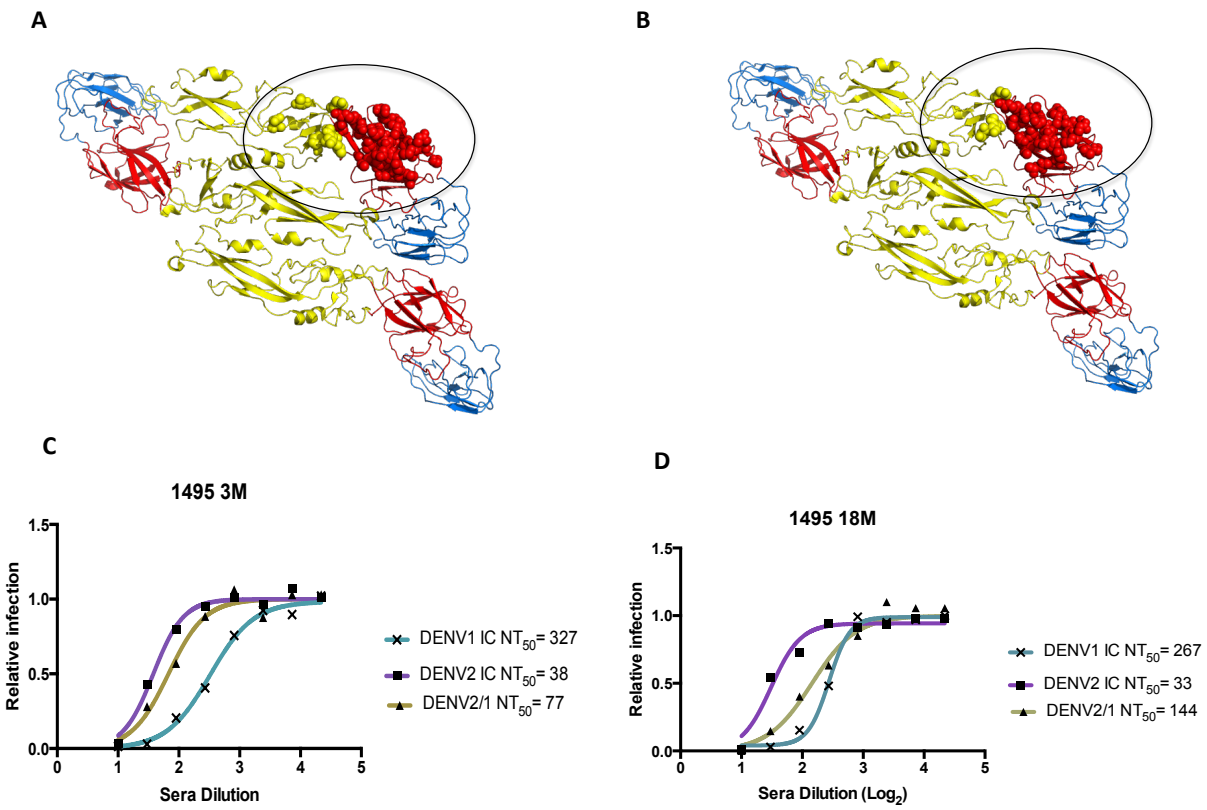


Figure 3.1. The 1F4 epitope amino acid residues in EDI and EDI/EDII hinge region of an E protein monomer were transplanted to a DENV2 backbone and analyzed by neutralization assay. (A) Diagram of the DENV E dimer (domains I, II and III in red, yellow and blue, respectively) with yellow and red spheres representing the 1F4 epitope footprint. (B) The amino acid residues within the 1F4 epitope represented in red and yellow spheres were transplanted into a DENV2 backbone, creating the rDENV2/1 virus. (C and D) Representative sigmoidal dose-response curves used to estimate the 50% neutralization titer (NT_{50}) to the rDENV2/1 and parental DENV1 and DENV2 viruses in plasma samples from patient 1495 at 3 (C) and 18 months (D). A greater titer to DENV2/1 in the polyclonal sera post-primary DENV1 infection is observed at the later time-point.

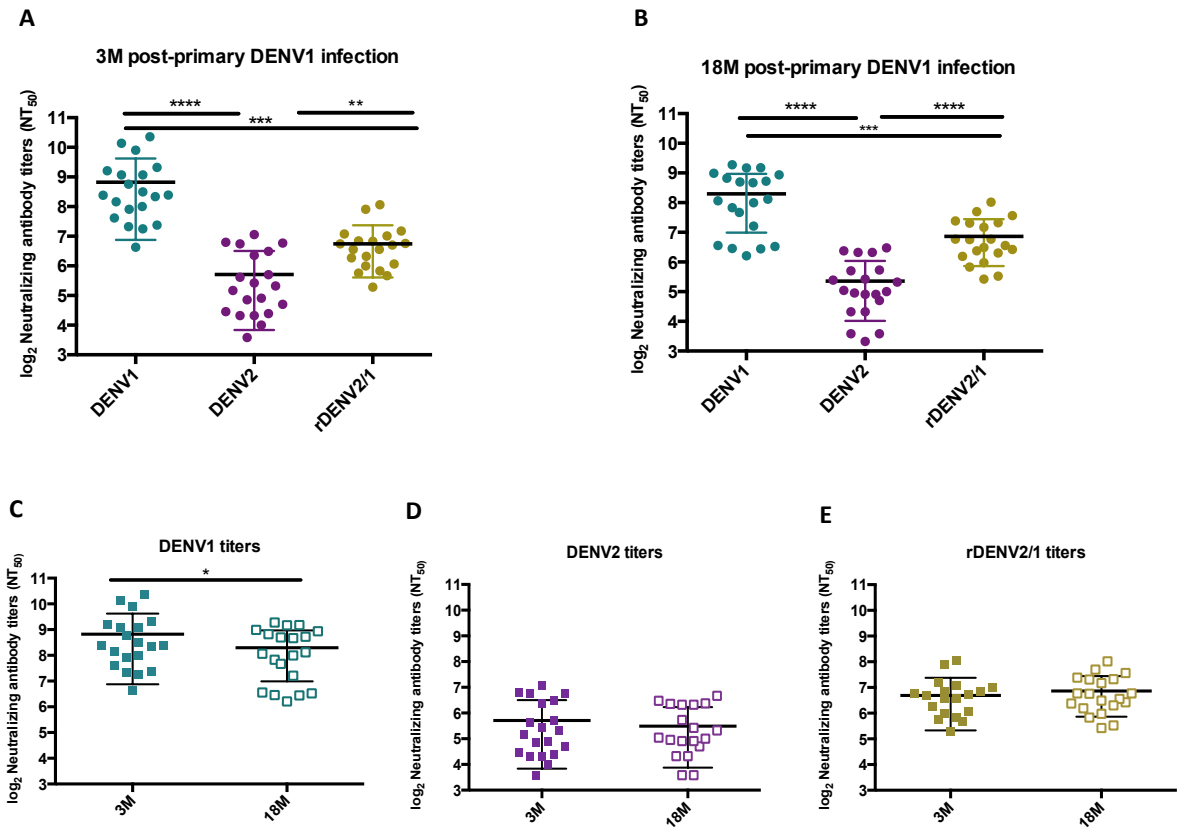


Figure 3.2. Inclusion of the 1F4 amino acid residues in a DENV2 backbone results in gain of neutralization against rDENV2/1 chimeric virus by polyclonal sera post-primary DENV1 infection. (A and B) Primary DENV1 plasma from 20 individuals in the Nicaraguan hospital-based study strongly neutralizes the parental DENV1 virus and gains neutralization capacity against a DENV2 backbone containing the 1F4 amino acid residues (DENV2/1) at 3 and 18 months post-infection. The NT_{50} values to the rDENV2/1 virus and the DENV1 and DENV2 parental viruses were compared by one-way ANOVA (n=20). (C-E) Paired analysis of the longitudinal samples at 3 and 18 months shows a significant decay of DENV1 NT_{50} values, whereas DENV2 and rDENV2/1 titers are maintained constant over time. The t-test was used to compare the neutralizing antibody titers to each serotype at 3 and 18 months post-infection. Data are representative of two independent experiments, and samples were processed in duplicate for each plasma sample. **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

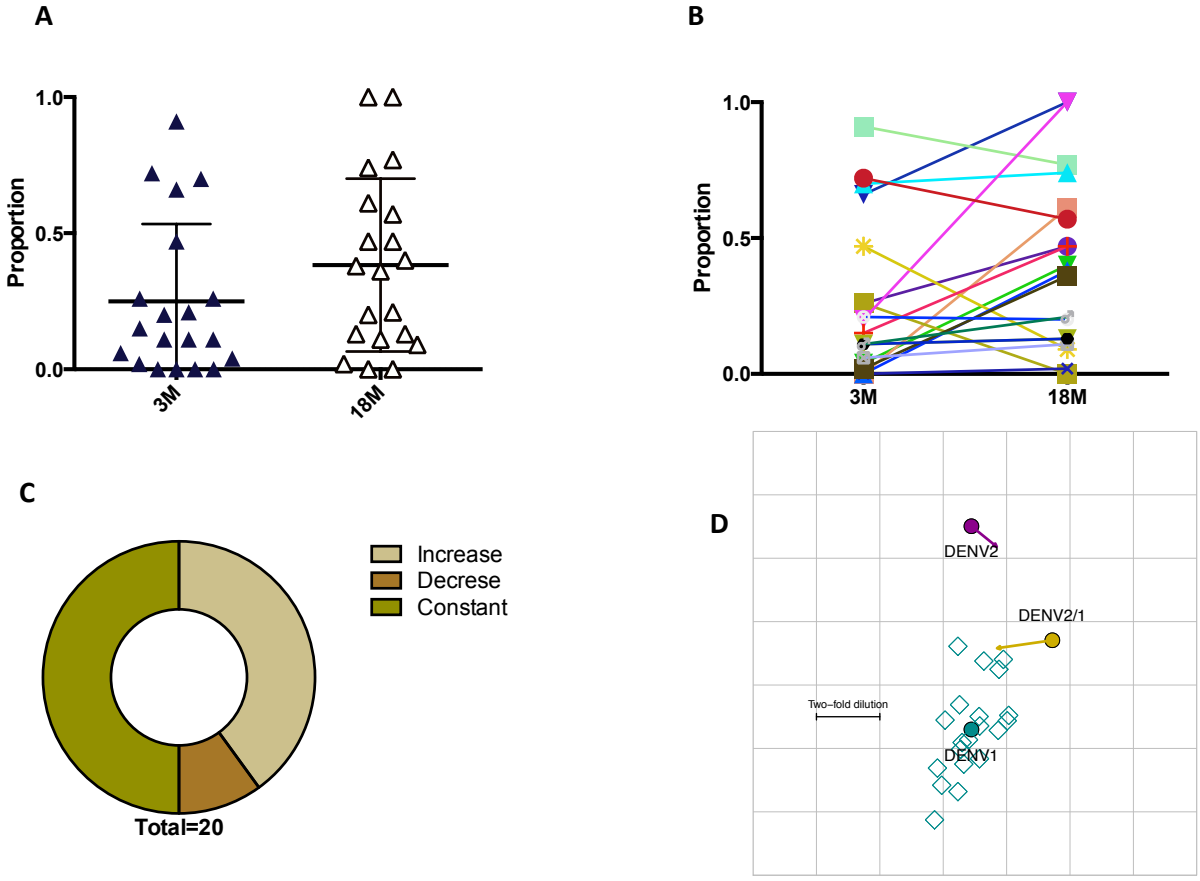


Figure 3.3. The proportion of the DENV1 type-specific neutralizing antibody response targeted to the 1F4 epitope varies across the Nicaraguan population in the hospital-based study and presents a bimodal pattern at 3 and 18 month post-infection. (A) Analysis of the proportion of the DENV1 type-specific response attributable to the 1F4 epitope at 3 and 18 months indicates a bimodal pattern, where most individuals either have high or low proportions of the DENV1 response directed to the 1F4 epitope. (B) Paired analysis where each individual was assigned a color and a symbol to enable visualization of the trajectory of the DENV1 type-specific response to the 1F4 epitope at 3 and 18 months post-infection. (C) Distribution of individuals who gained recognition of the 1F4 epitope between 3 and 18 months (beige), lose recognition (brown) or remain constant (green). (D) The antigenic cartography map positions viruses (DENV1, DENV2 and rDENV2/1 in teal, purple and yellow, respectively) and plasma (open teal squares), with the distance between each virus and plasma derived from its respective neutralizing antibody titer. Each grid square corresponds to a 2-fold dilution in the NT_{50}. From 3 to 18 months post-primary DENV1 infection, the DENV1 and rDENV2/1 titers converge, as indicated by the rDENV21 arrow pointing towards DENV1.

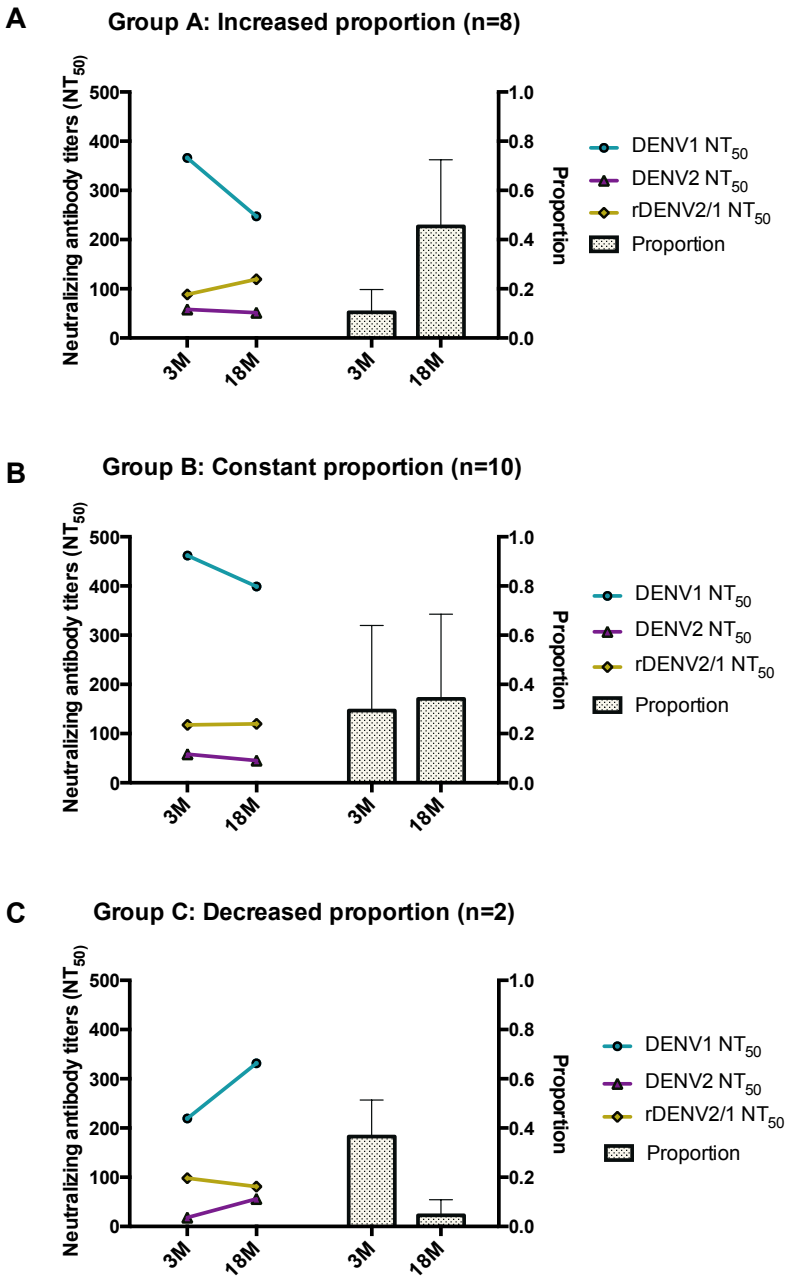


Figure 3.4. The proportion of the DENV1 neutralizing antibody response directed to the 1F4 epitope is affected by the levels of homotypic and heterotypic titers. (A-C) To better understand the different patterns of the proportion observed in the individual trajectory analysis, we grouped the individuals who displayed increased (A), constant (B) or decreased (C) proportion over time (right Y-axis) and concomitantly analyzed their NT_{50} values (left Y-axis) to the parental DENV1 (teal line) and DENV2 viruses (purple line) and the rDENV2/1 chimeric virus (yellow line). In group A, the increase in antibodies targeting the 1F4 epitope is associated with a decay in the DENV1 NT_{50} values and concomitant maintenance of the rDENV2/1 titers. In group B, the constant proportion between 3 and 18 months is accompanied by changes in the magnitude of neutralizing antibody titers to the parental viruses, while in Group C, the loss of recognition of the 1F4 epitope is associated with an increase in the NT_{50} values to DENV1 and DENV2 viruses.

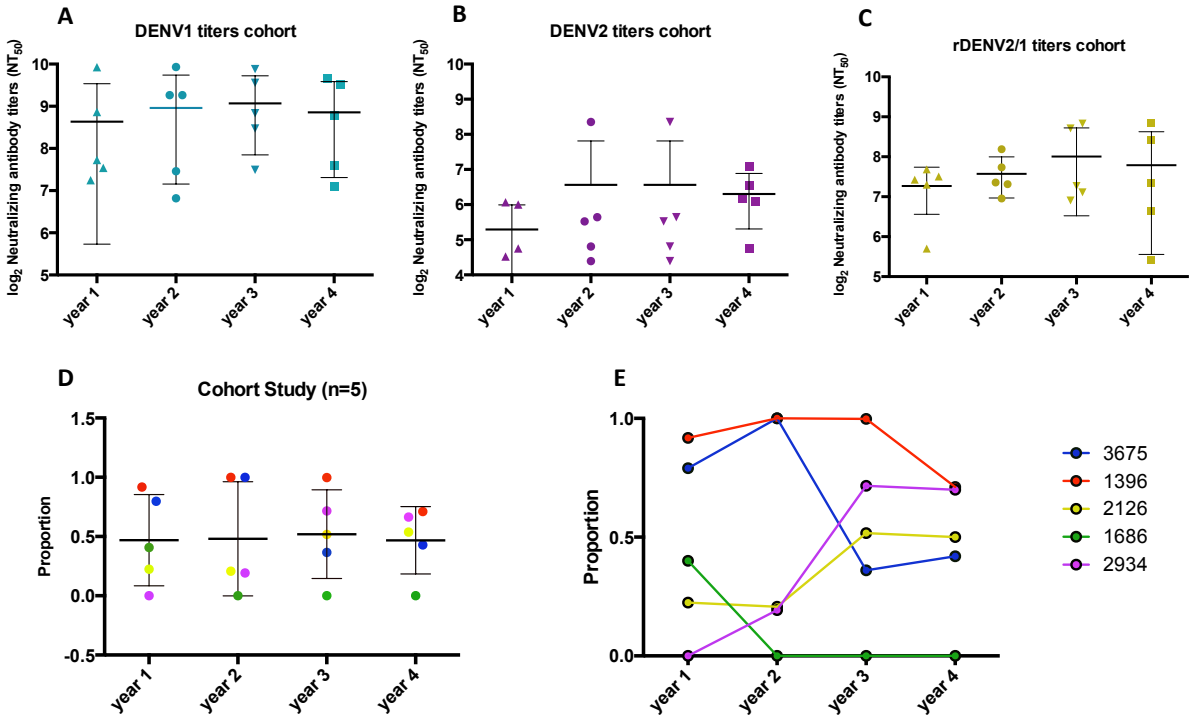


Figure 3.5. Primary DENV1 plasma samples collected up to four years post-infection track to varying degrees with the 1F4 epitope in the Pediatric Dengue Cohort Study in Nicaragua. (A-C) The mean of he neutralizing antibody titers to the rDENV2/1 and parental viruses DENV1 and DENV2 did not change significantly over four years post-infection. The NT_{50} values were compared by one-way ANOVA analysis (n=5). (D) From year 1 to year 4 post-primary DENV1 infection, the 1F4 epitope accounts for 47, 48, 52 and 47% of the DENV1 type-specific response, respectively. The proportions were compared by one-way ANOVA analysis (n=5). (E) Individual trajectory analysis over four years post-infection shows varying degrees of the DENV1 response attributed to the 1F4 epitope across the individuals in the cohort study.

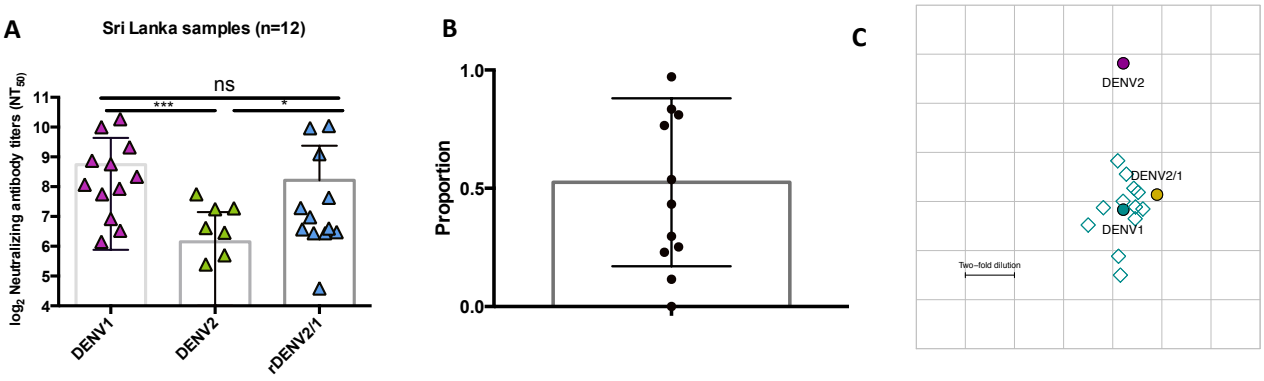


Figure 3.6. The 1F4 epitope is recognized by polyclonal sera from Sri Lankan individuals in the convalescent phase post-primary DENV1 infection. (A**)** In the convalescent phase (~60 days post-illness), the NT_{50} values to the DENV1 infecting serotype and the rDENV2/1 is significantly higher than the NT_{50} values to the DENV2 serotype in Sri Lankan dengue patients. (B) The proportion of the DENV1 type-specific response attributable to the 1F4 epitope displays a bimodal pattern and averages 53% across 12 Sri Lankan individuals. (C) The antigenic cartography map positions viruses (DENV1, DENV2 and rDENV2/1 in teal, purple and yellow, respectively) and plasma (12 open teal squares) and indicates a close proximity between DENV1 and rDENV2/1 viruses. Each grid square corresponds to a 2-fold dilution in the neutralization titer. The NT_{50} values were compared by one-way ANOVA analysis (n=12). *, $p< 0.05$, ***, $p<0.001$.

REFERENCES

1. Bhatt, S. *et al.* The global distribution and burden of dengue. *Nature* **496,** 504–507 (2013).
2. Halstead, S. B. Dengue. *Lancet* **370,** 1644–1652 (2007).
3. *Dengue haemorrhagic fever Diagnosis, treatment, prevention and control SECOND EDITION Contents*. (1997).
4. Halstead, S. B. Pathogenesis of dengue: challenges to molecular biology. *Science* **239,** 476–81 (1988).
5. Katzelnick, L. C. *et al.* Immune correlates of protection for dengue: State of the art and research agenda. *Vaccine* **35,** 4659–4669 (2017).
6. Katzelnick, L. C., Montoya, M., Gresh, L., Balmaseda, A. & Harris, E. Neutralizing antibody titers against dengue virus correlate with protection from symptomatic infection in a longitudinal cohort. *Proc. Natl. Acad. Sci.* **113,** 728–733 (2016).
7. Buddhari, D. *et al.* Dengue Virus Neutralizing Antibody Levels Associated with Protection from Infection in Thai Cluster Studies. *PLoS Negl. Trop. Dis.* **8,** e3230 (2014).
8. Katzelnick, L. C. *et al.* Antibody-dependent enhancement of severe dengue disease in humans. *Science* **358,** 929–932 (2017).
9. Salje, H. *et al.* Reconstruction of antibody dynamics and infection histories to evaluate dengue risk. *Nature* **557,** 719–723 (2018).
10. Kliks, S. C., Nisalak, A., Brandt, W. E., Wahl, L. & Burke, D. S. Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. *Am. J. Trop. Med. Hyg.* **40,** 444–51 (1989).
11. Lindenbach, B. D., Thiel, H.-J. & Rice, C. M. *33 Flaviviridae: The Viruses and Their Replication*. (2006).
12. Kuhn, R. J. *et al.* Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* **108,** 717–25 (2002).
13. Stiasny, K. & Heinz, F. X. Flavivirus membrane fusion. *J. Gen. Virol.* **87,** 2755–2766 (2006).
14. Westaway, K. E., Blok, W. J. & Westaway, R. M. Taxonomy and evolutionary relationships of flaviviruses. (1997).
15. Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc. Natl. Acad. Sci.* **100,** 6986–6991 (2003).
16. Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. *J. Virol.* **79,** 1223–31 (2005).
17. Zhang, Y. *et al.* Conformational changes of the flavivirus E glycoprotein. *Structure* **12,** 1607–18 (2004).
18. Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. Structure of the dengue virus envelope protein after membrane fusion. *Nature* **427,** 313–319 (2004).
19. Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C. & Harrison, S. C. The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* **375,** 291–298 (1995).

20. de Alwis, R. *et al.* Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions. *Proc. Natl. Acad. Sci.* **109,** 7439–7444 (2012).
21. Fibriansah, G. *et al.* Cryo-EM structure of an antibody that neutralizes dengue virus type 2 by locking E protein dimers. *Science (80-.).* **349,** 88–91 (2015).
22. Fibriansah, G. *et al.* A highly potent human antibody neutralizes dengue virus serotype 3 by binding across three surface proteins. *Nat. Commun.* **6,** 6341 (2015).
23. Fibriansah, G. *et al.* A potent anti-dengue human antibody preferentially recognizes the conformation of E protein monomers assembled on the virus surface. *EMBO Mol. Med.* **6,** n/a-n/a (2014).
24. Gallichotte, E. N. *et al.* Human dengue virus serotype 2 neutralizing antibodies target two distinct quaternary epitopes. *PLoS Pathog.* **14,** e1006934 (2018).
25. Messer, W. B. *et al.* Functional Transplant of a Dengue Virus Serotype 3 (DENV3)-Specific Human Monoclonal Antibody Epitope into DENV1. *J. Virol.* **90,** 5090–5097 (2016).
26. Gallichotte, E. N. *et al.* Epitope Addition and Ablation via Manipulation of a Dengue Virus Serotype 1 Infectious Clone. *mSphere* **2,** (2017).
27. Andrade, D. V *et al.* Analysis of Individuals from a Dengue-Endemic Region Helps Define the Footprint and Repertoire of Antibodies Targeting Dengue Virus 3 Type-Specific Epitopes. *MBio* **8,** (2017).
28. Gallichotte, E. N. *et al.* A new quaternary structure epitope on dengue virus serotype 2 is the target of durable type-specific neutralizing antibodies. *MBio* **6,** e01461-15 (2015).
29. Swanstrom, J. A. *et al.* Analyzing the Human Serum Antibody Responses to a Live Attenuated Tetravalent Dengue Vaccine Candidate. *J. Infect. Dis.* **217,** 1932–1941 (2018).
30. Henein, S. *et al.* Dissecting antibodies induced by a chimeric yellow fever-dengue, live-attenuated, tetravalent dengue vaccine (CYD-TDV) in naïve and dengue exposed individuals. *J. Infect. Dis.* **215,** jiw576 (2016).
31. Dejnirattisai, W. *et al.* A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus. *Nat. Immunol.* **16,** 170–177 (2015).
32. Imrie, A. *et al.* Antibody to Dengue 1 Detected More Than 60 Years after Infection. *Viral Immunol.* **20,** 672–675 (2007).
33. Katzelnick, L. C. *et al.* Dengue viruses cluster antigenically but not as discrete serotypes. *Science (80-.).* **349,** 1338–1343 (2015).
34. Gallichotte, E. N. *et al.* Genetic Variation between Dengue Virus Type 4 Strains Impacts Human Antibody Binding and Neutralization. *Cell Rep.* **25,** (2018).
35. Messer, W. B. *et al.* Development and characterization of a reverse genetic system for studying dengue virus serotype 3 strain variation and neutralization. *PLoS Negl. Trop. Dis.* **6,** e1486 (2012).
36. Villabona-Arenas, C. J. & Zanotto, P. M. de A. Worldwide Spread of Dengue Virus Type 1. *PLoS One* **8,** e62649 (2013).
37. Pyke, A. T. *et al.* Highly divergent dengue virus type 1 genotype sets a new distance record. *Sci. Rep.* **6,** 22356 (2016).

38. Tissera, H. A. *et al.* New dengue virus type 1 genotype in Colombo, Sri Lanka. *Emerg. Infect. Dis.* **17,** 2053–5 (2011).
39. Teoh, E. P. *et al.* The Structural Basis for Serotype-Specific Neutralization of Dengue Virus by a Human Antibody. *Sci. Transl. Med.* **4,** 139ra83-139ra83 (2012).
40. Sabchareon, A. *et al.* Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. *Lancet* **380,** 1559–1567 (2012).
41. Patel, B. *et al.* Dissecting the human serum antibody response to secondary dengue virus infections. *PLoS Negl. Trop. Dis.* **11,** e0005554 (2017).
42. Nivarthi, U. K. *et al.* Mapping the Human Memory B Cell and Serum Neutralizing Antibody Responses to Dengue Virus Serotype 4 Infection and Vaccination. *J. Virol.* **91,** e02041-16 (2017).
43. Guzman, M. G. *et al.* Neutralizing antibodies after infection with dengue 1 virus. *Emerg. Infect. Dis.* **13,** 282–6 (2007).
44. SABIN, A. B. Research on dengue during World War II. *Am. J. Trop. Med. Hyg.* **1,** 30–50 (1952).
45. Waggoner, J. J. *et al.* Homotypic Dengue Virus Reinfections in Nicaraguan Children. *J. Infect. Dis.* **214,** 986–993 (2016).
46. Forshey, B. M. *et al.* Incomplete Protection against Dengue Virus Type 2 Re-infection in Peru. *PLoS Negl. Trop. Dis.* **10,** e0004398 (2016).
47. Rabaa, M. A. *et al.* Genetic epidemiology of dengue viruses in phase III trials of the CYD tetravalent dengue vaccine and implications for efficacy. *Elife* **6,** (2017).
48. Juraska, M. *et al.* Viral genetic diversity and protective efficacy of a tetravalent dengue vaccine in two phase 3 trials. *Proc. Natl. Acad. Sci.* **115,** E8378–E8387 (2018).
49. ŠANTAK, M. *et al.* Antigenic differences between vaccine and circulating wild-type mumps viruses decreases neutralization capacity of vaccine-induced antibodies. *Epidemiol. Infect.* **141,** 1298–1309 (2013).
50. Burton, D. R., Poignard, P., Stanfield, R. L. & Wilson, I. A. Broadly Neutralizing Antibodies Present New Prospects to Counter Highly Antigenically Diverse Viruses. *Science (80-.).* **337,** 183–186 (2012).
51. Zhang, P. *et al.* Hepatitis C virus epitope-specific neutralizing antibodies in Igs prepared from human plasma. *Proc. Natl. Acad. Sci.* **104,** 8449–8454 (2007).
52. Lindesmith, L. C. *et al.* Antigenic Characterization of a Novel Recombinant GII.P16-GII.4 Sydney Norovirus Strain With Minor Sequence Variation Leading to Antibody Escape. *J. Infect. Dis.* **217,** 1145–1152 (2018).
53. Chua, C.-L., Sam, I.-C., Merits, A. & Chan, Y.-F. Antigenic Variation of East/Central/South African and Asian Chikungunya Virus Genotypes in Neutralization by Immune Sera. *PLoS Negl. Trop. Dis.* **10,** e0004960 (2016).
54. Narvaez, F. *et al.* Evaluation of the Traditional and Revised WHO Classifications of Dengue Disease Severity. *PLoS Negl. Trop. Dis.* **5,** e1397 (2011).
55. Kuan, G. *et al.* The Nicaraguan pediatric dengue cohort study: study design, methods, use of information technology, and extension to other infectious diseases. *Am. J. Epidemiol.* **170,** 120–9 (2009).
56. Balmaseda, A., Sandoval, E., Pérez, L., Gutiérrez, C. M. & Harris, E. Application

of molecular typing techniques in the 1998 dengue epidemic in Nicaragua. *Am. J. Trop. Med. Hyg.* **61,** 893–7 (1999).
57. Balmaseda, A. *et al.* Diagnosis of dengue virus infection by detection of specific immunoglobulin M (IgM) and IgA antibodies in serum and saliva. *Clin. Diagn. Lab. Immunol.* **10,** 317–22 (2003).
58. Balmaseda, A. *et al.* High seroprevalence of antibodies against dengue virus in a prospective study of schoolchildren in Managua, Nicaragua. *Trop. Med. Int. Health* **11,** 935–42 (2006).
59. Kraus, A. A., Messer, W., Haymore, L. B. & de Silva, A. M. Comparison of Plaque- and Flow Cytometry-Based Methods for Measuring Dengue Virus Neutralization. *J. Clin. Microbiol.* **45,** 3777–3780 (2007).

CHAPTER 4

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF MONOCLONAL ANTIBODIES ISOLATED FROM MEMORY B CELLS AFTER SECONDARY DENGUE VIRUS (DENV) INFECTION

Summary

The adaptive immune response is involved in both protection and enhancement of DENV infection. While primary infection with any of the four DENV serotypes is thought to confer long-term protection to the homologous serotype, the exact immune determinants involved in this protection are yet to be fully determined. Despite representing the major risk factor for severe disease, secondary infection with a heterologous DENV serotype causes asymptomatic or results in only mild disease in the majority of the cases. The immune mechanisms underlying protection against a heterologous secondary infection are not completely understood. Overall, recent studies of natural infection and clinical trials provide striking evidence that the quality of the antibody response to DENV infection is a strong component of immune correlates of protection. Identification and characterization of new DENV human monoclonal antibodies (hmAbs) isolated from individuals with a well-characterized infection history represent a powerful approach to better define the determinants of antibody neutralization of DENV. Furthermore, investigating the molecular pathways involved in the generation of potent neutralizing antibodies is an important approach to improve understanding of the antibody repertoire following DENV infection. In this study, we isolated and characterized a panel of hmAbs isolated from EBV-transformed PBMCs collected after secondary DENV infection. In an attempt to investigate the ontogeny of selected hmAbs displaying broad and strong neutralization potential, we performed deep sequencing of the immunoglobulin (Ig) genes of B cells in matched PBMCs after previous DENV infection of the same individual at time-points prior to the secondary infection. We successfully isolated 74 new DENV hmAbs from three donors, and our data reveal a predominance of cross-neutralizing hmAbs after secondary DENV infection. Analysis of the somatic hypermutation (SHM) of the variable heavy and light genes indicates that most hmAbs are highly mutated, suggesting a memory B cell, as opposed to naïve B cell, origin. In addition to cross-reactive hmAbs, we also describe a number of DENV type-specific hmAbs, especially to the DENV3 serotype. Further characterization reveals the identification of novel DENV3 epitopes, adding invaluable knowledge to the definition of antigenic landscape. All together, our findings contribute significantly to understanding the molecular and functional determinants of the B cell response to secondary DENV infection.

INTRODUCTION

The DENV genome consists of a positive-sense RNA that encodes 3 structural proteins -- capsid (C), membrane (prM/M) and envelope (E) -- and 7 nonstructural viral proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5)[1,2]. The glycoprotein shell consists of 180 copies of the E protein, which associate differentially with the prM protein in immature and mature DENV particles. In the immature virion, prM and E protein heterodimers form 60 trimeric spikes on the virion surface. In mature viruses, this arrangement consists of 90 homodimers that lie flat on the virion surface. Three E dimers form dimer rafts, which in turn assemble in an icosahedral orientation with both three-fold and five-fold axes of symmetry[3]. The ectodomain of the E protein consists of three distinct domains: EDI, EDII and EDIII, involved in attachment to the host cell and membrane fusion. All three domains are connected by hinge regions, which allow rearrangement of domains during virus assembly, maturation and infection[4]. The DENV E protein is the main antigenic target on the surface of the virion and, as such, has been extensively investigated in monoclonal antibody (mAb) mapping studies[5].

Mouse monoclonal antibodies (mAbs) have been largely employed to study the functional and structural characteristics of DENV neutralizing epitopes. The specificity and neutralization properties of these mAbs have been traditionally determined using binding assays to recombinant DENV proteins (rE and rEDIII). While neutralizing DENV mAbs map to all three E domains, mouse type-specific mAbs with potent neutralization capacity predominantly map to EDIII, which is unique to each serotype[6–8]. Unlike mouse DENV type-specific mAbs, flavivirus cross-reactive mouse mAbs that bind to EDIII show moderate to weakly neutralizing properties[9,10]. In humans, the polyclonal response to DENV generates EDIII-reactive antibodies following primary and secondary DENV infection. However, this population of EDIII-binding antibodies drives only a small percentage of the total neutralization to DENV, as shown by depletion of EDIII-binding antibodies in human sera[11] and recombinant DENVs with mutations in EDIII epitopes[12]. Collectively, these findings suggest that human polyclonal sera contain neutralizing antibodies that bind to epitopes other than the ones located on EDIII, emphasizing the relevance of in-depth analysis of the antibody repertoire induced after primary and secondary DENV infections.

The isolation of DENV human monoclonal antibodies (hmAbs) has generated refined tools to define the molecular specificity of antibody responses that mediate type-specific neutralization. Recent findings indicate that strongly neutralizing hmAbs isolated post-DENV infection target epitopes displayed on the intact virions but not on the ectodomain of recombinant soluble E proteins[13]. Serotype-specific hmAbs recognize unique conformations of the E monomer on the virion or quaternary structure epitopes that span different E proteins (on E dimers or rafts) on the viral surface. In fact, the location of serotype-specific epitopes appears to be different between serotypes, despite the high degree of E protein structural similarity[14]. For instance, DENV1 type-specific 1F4 binds to an E monomer, mainly to EDI and the hinge between EDI and EDII, and is sensitive to the hinge angle between EDI and EDII[15]. The DENV3 hmAb 5J7 recognizes

an epitope centered around the EDI/EDII hinge region, and its footprint includes amino acid residues from three different E molecules within a single raft[16]. The DENV type-specific hmAb 2D22, on the other hand, targets an epitope centered on EDIII and EDI of one E protein, in addition to EDII including the fusion loop of another E protein[17]. In human DENV-immune sera, antibodies recognizing complex epitopes contribute to a high proportion of the polyclonal neutralization[13,18,19]. While isolation of hmAbs has helped define the footprint and repertoire of antibodies directed to DENV epitopes, only a handful of strongly neutralizing DENV hmAbs have been isolated so far. Thus, the antigenic landscape of each serotype must be further explored.

After a secondary DENV infection, individuals present serotype-specific neutralizing antibodies to the infecting serotypes and a new population of serotype-cross-neutralizing antibodies that also recognize serotypes not encountered yet[20]. de Silva and colleagues depleted antibody populations and demonstrated that with each successive DENV infection, cross-reactive antibodies constitute a larger proportion in polyclonal sera[21]. At the mAb level, it has been shown that cross-reactive mAbs derived from individuals who experienced secondary DENV infection display higher binding affinity and neutralization potency compared with mAbs derived from individuals who only experienced one DENV infection[22]. Furthermore, the reactivity profile of hmAbs isolated after secondary DENV infection shows a predominance of cross-neutralization[23–25]. Collectively, these observations suggest that cross-neutralizing antibodies evolved from low avidity and poorly neutralizing to high avidity and potent neutralizing following secondary DENV infection. As indicated by cohort studies in dengue-endemic areas, tertiary infections are nearly always mild or inapparent, implicating a protective role for these broadly cross-neutralizing antibodies that develop after a second DENV infection[26].

Similar to the strategy applied to understanding serotype-specific epitopes, hmAbs have been fundamental for determining the properties of broadly neutralizing antibodies. Functional characterization studies of isolated hmAbs indicate that the human response to DENV generates a large proportion of antibodies directed to the E and prM proteins that are predominantly cross-reactive yet weakly neutralizing[13,23,25,27,28]. Weakly cross-reactive antibodies target the fusion loop (FL) epitope on EDII of the E protein[29,30], although mAbs with moderate to strong neutralization potential have also been shown to target this region[22]. This abundant antibody population has been shown to cause ADE activity *in vitro*[31–33], and *in vivo*[34–37]. Despite the low frequency of potent broadly neutralization antibodies, studies have successfully isolated this interesting class of hmAbs. Among them is 1C19, a serotype cross-neutralizing hmAb that recognizes a novel conserved site known as the bc loop. In addition to high neutralization potency (EC_{50}= 0.06 µg/mL), 1C19 displays additional features such as competitive binding advantage over weakly neutralizing FL antibodies[38]. Interestingly, the 1C19 hmAb was isolated from a donor who experienced secondary DENV infection, suggesting that sequential DENV infections may generate broadly neutralizing antibodies[38].

Another successful hmAb isolation study identified a new class of broadly neutralizing antibodies (bnAbs) from acute-phase plasmablasts in the peripheral blood of secondary

DENV cases[39]. These bnAbs display 50% neutralization titer (NT_{50}) as low as 3.3 x 10-11 M and are maturation stage insensitive, as demonstrated by the capacity to bind to DENV virions with varying levels of prM content. Designated envelope dimer epitope (EDE) antibodies, these hmAbs map to quaternary epitopes on the E homodimer[39,40] that span EDI or EDIII of one monomer and EDII of the adjacent monomer. While the discovery of the novel EDE antibodies is promising and may provide unprecedented knowledge for the design of an effective DENV vaccine, some gaps still need to be addressed. For instance, it is still undetermined if EDE antibodies isolated from plasmablasts during acute secondary infection undergo affinity maturation and populate memory B cell (MBC) and long-lived plasma cell (LLPCs) populations. Other pressing questions still need to be addressed, such as the prevalence of EDE antibodies across dengue-immune individuals, whether infection history influences the generation of EDE antibodies, and how the B cell response specific to this epitope is shaped over time. Given the high relevance of bnAbs for vaccine design, understanding the molecular mechanisms leading to the evolution of this antibody population following secondary DENV infection is urgently needed.

The pathways involved in the development of bnAbs from their precursor lineages have been largely investigated in the HIV and influenza fields[41–43]. These studies have consistently shown that a common feature of bnAbs is a high level of somatic hypermutation (SHM). This affinity maturation process introduces mutations in the variable (V), Diversity (D) and Joining (J) germline sequences of B cells that have encountered an antigen. Analysis of the number of mutations observed in the V and J gene sequences is a benchmark for segregating sequences from either naïve or affinity-matured B cells[44,45]. These mutations are mostly found in the complementary-determining regions (CDR) in V_H genes rather than in the framework region (FR). For instance, bnAbs isolated from HIV chronically infected individuals presented on average 20% divergence from the putative germinline nucleotide sequence for the variable heavy chain (V_H and J_H) region[46–49]. During acute secondary DENV infection, expanded plasmablasts can account for approximately 30% of the lymphocyte population shortly after fever onset, and they show high degrees of somatic hupermutation (SHM)[24,50–52]. In fact, the SHM frequencies per patient VH ranged from 14.5 and 21.7[24] which are comparable to influenza infection and vaccination. The high levels of SHM suggest that DENV plasmablasts may be reactivated MBCs. Furthermore, the sequences obtained from all donors indicated clonal expansions that represented over 20% of the total of sequences analyzed, also supporting the hypothesis that these clones derive from the MBC pool[24]. A longitudinal study by our group analyzed PBMC samples from 44 individuals during acute symptomatic DENV infection at days 2-7 post symptom onset (dpo), convalescent at 7-47 dpo, and post-convalescent (around 180 dpo) and identified highly prevalent CDR3 signatures in acute DENV infection[53]. Using the closest reference V germline sequence in the International Immunogenetic (IMGT) database, it was shown that the median amount of somatic hypermutation in the V gene associated with all convergent CDR3s varied from 4.4 to 6.9%. Such high mutation rate suggests that these CDR3s were likely derived from the MBC pool, as opposed to naïve B cells. Moreover, the significantly higher prevalence of convergent CDR3 in acute secondary DENV infection compared to acute primary infection also supports the evidence of a

recall response of MBCs during acute secondary DENV infection[53]. All together, these studies advanced the understanding of B cell responses in acute DENV infection. However, it is still largely unknown which molecular mechanisms are involved in the generation and evolution of bnAbs following sequential DENV infections.

In this chapter, we isolated and characterized the functional and molecular properties of selected broadly neutralizing antibodies via deep sequencing of the immunoglobulin genes of B cells in PBMCs collected longitudinally. Moreover, we successfully isolated high neutralizing DENV3 type-specific hmAbs after secondary DENV infection that bind to novel epitopes and are protective against DENV3 challenge in a mouse model of DENV infection. Taken together, our findings have made significant contribution to understanding the breadth and functional properties of DENV neutralizing antibodies after secondary DENV infection.

RESULTS

DENV infection history of selected Pediatric Dengue Cohort Study participants

We selected participants enrolled in the Nicaraguan Pediatric Dengue Cohort Study (PDCS; 2004 to present), a community-based prospective study of approximately 3,700 children 2 to 14 years of age in Managua, Nicaragua[54]. Healthy blood samples collected annually were used to reconstruct the DENV immune history of the participants based on a Reporter Viral Particle (RVP) flow cytomety-based DENV neutralization assay, as previously described[55,56]. The infecting serotypes in the inapparent infections were identified according to the highest fold-change in the NT_{50} values for each serotype, as highlighted on **Table 4.1**. Individuals 985 and 1791 experienced a first inapparent infection with DENV2 serotype, followed by a second symptomatic DENV3 infection. Individual 3243, on the other hand, experienced a first symptomatic infection with DENV3 serotype, followed by a second inapparent DENV1 infection (**Table 4.1**).

Frequency of DENV-specific B cells and isolation of hmAbs from PBMCs post-secondary infection

We collaborated in the context of an NIH Program Project directed by Dr. Harris with the group of Dr. James Crowe at Vanderbilt University for hmAbs isolation. PBMCs were transformed by infection with Epstein-Barr virus (EBV), as previously described[23,57]. Isolation of potent DENV neutralizing hmAbs is difficult due to the low frequency of antigen-specific B cell clones in the human response[58] and the complex conformation of epitopes recognized by strongly neutralizing antibodies[13]. To circumvent these intrinsic challenges, the supernatants from transformed B cells were screened for binding to DENV virions by ELISA and only cells reactive to DENV were electrofused with HMMA2.5 myeloma cells. Based on the number of positive wells and the number of transformed B cells (determined by average colony counts in transformed wells), the frequencies of DENV-specific B cells were estimated to be 1.43, 1.50 and, 2.35% for individuals 985, 1791, and 3243, respectively (**Table 4.2**). Across the three individuals, the frequency of DENV-specific B cells was similar despite differences in serum titers and infection history (**Table 4.1**). Following cytofusion with the myeloma cells, the fused cells were selected by growth in a hypoxanthine-aminopterin-thymidine (HAT) medium containing oubain. Hybridomas were screened for antibodies reactive to DENV via binding ELISA and cloned biologically by single-cell fluorescent-activated cell sorting (FACS). A total of 33,7 and 34 hmAbs were obtained from donors 875, 1791 and 3243, respectively (**Table 4.2**).

Predominance of partially or fully cross-reactive hmAbs isolated from MBCs

Previous findings have demonstrated that DENV-specific MBCs secrete predominantly poorly neutralizing antibodies, and that only a small fraction (<5% of DENV-specific B cells) secretes bnAbs[13,23,28]. To first characterize the binding properties of the 74 hmAbs we successfully isolated, we screened the hmAbs in ELISA-binding assays using DENV1-4 virions. Of the total 74 hmAbs, 15 (20.2%) bound to only one serotype

(serotype-specific), while the remaining 50 (67.6%) were cross-reactive to all four serotypes, 5 (6.8%) to three serotypes or 4 (5.4%) to only two serotypes (**Figure 4.1a**). To determine the ability of our hmAb panel to neutralize DENV1-DENV4, we used a flow cytometry-based neutralization assay with Nicaraguan viruses. A majority of the hmAbs in our panel showed neutralization activity *in vitro* (65/74; 87.8%) (**Figure 4.1b**). However, in contrast to the binding profile, the frequency of cross-reactive antibodies that neutralized all four serotypes did not represent the majority of the hmAb panel (67.6% binding vs. 24% neutralization). Ten hmAbs (13.5%) cross-neutralized 3 serotypes, with DENV4 being the least neutralized serotype. We observed that of the 25 type-specific hmAbs, 17 were monotypic to DENV3 (17/25), 6 to DENV1 (6/25) or and 2 to DENV2 (2/25). Type-specific hmAbs were generated to the first and second infecting serotypes and corroborated the infection history inferred by RVP neutralization assays. With regard to immunoglobulin (IgG) subclasses, we observed that the majority of the hmAbs were IgG1, in accordance with previous studies describing the predominance of this IgG subclasss in patients with secondary DENV infection[59]. Dominance of IgG1 has also been shown in HIV [60,61] and Hepatitis C[62] infections (**Figure 4.1c**).

Human mAbs isolated after secondary DENV infection present diverse patterns of DENV binding

To investigate the breadth and magnitude of the binding activity of the 74 isolated hmAbs, we performed ELISAs with DENV1-DENV4 virions. The EC_{50} values for binding to DENV1-DENV4 of hmAbs isolated from individuals 985 (purple line), 1791 (teal line) and 3243 (yellow line) are represented in this heat map. The color gradient indicates the range of EC_{50} values, with the darkest tones indicating high binding activity and the lightest tones indicating low binding activity. The EC_{50} values of hmAbs isolated from individual 985 displays a wide range of binding capacity, with some clones binding efficiently to only one serotype (e.g. hmAbs 443, 437, 415, 404, 419 and 406), while others bind efficiently to all four serotypes (e.g. 330,444). hmAs isolated from individual 1791 either had strong binding capacity to DENV3 (hmAbs 297, 286,290, 298, 354) or to all serotypes (282 and 362). In contrast, most hmAbs isolated from individual 3243 bound to all four DENV serotypes and displayed had a wide range of EC_{50} values (**Figure 4.2**).

Predominance of cross-neutralizing hmAbs isolated from MBCs after secondary DENV infection

The DENV E protein is the major antigenic target for neutralizing antibodies[25,28]. The sigmoidal dose-response curves in **Figure 4.3** illustrate four different hmAbs that have neutralization activity against all four DENV serotypes. The majority of the hmAbs were fully or partially cross-neutralizing, consistent with previous resports[24,28,63]. Based on the number of serotypes neutralized, the hmAbs were classified as serotype-specific (one serotype), XR 2 serotypes (cross-neutralizes 2 serotypes), XR 3 serotypes (cross-neutralizes 3 serotypes) or broad specificity (cross-neutralizes all 4 serotypes). As expected, we observed a range of potency, including hmAbs with poor (EC_{50} >10,000

ng/mL), moderate (EC_{50} 100-1000 ng/mL) or potent (EC_{50} ng/mL ≤ 100 ng/mL) neutralization efficacy (**Table 4.3**). While the majority of the hmAbs isolated were shown to neutralize DENV serotypes with different degrees of reactivity and potency, a number of hmAbs (12/74; 16.2%) lacked neutralization activity.

Human mAbs isolated after secondary DENV infection are genetically diverse

The diversity of the heavy chain antibody repertoire is mainly achieved during ontogeny by random recombination of the variable (V), diversity (D) and joining (J) segments and by modification of the V_HDJ_H junctions[64]. Following activation with an antigen, sequence diversification is further achieved through somatic hypermutation (SHM). To understand the diversity of the DENV-specific antibody repertoire in our panel of mAbs, we analyzed the heavy chain variable region sequences using the ImMunoGeneTics (IMGT) database to determine the V and J usage. We observed a preference for the VH3 family across all three individuals, in accordance with previous findings[52] (**Figure 4.4a**). In particular, we observed a preferential usage of the alleles 23 and 30 within the VH3 family (**Figure 4.4b**). Regarding the J segment, we observed a high frequency of JH4 (**Figure 4.4c**), corroborating previous findings describing a predominance of JH4 families in the MBC repertoire after DENV infection[65]. Within the JH4 family, we observed a predominance of the allele 02 (**Figure 4.4d**).

Functional properties of expanded V_H families

We next further investigated the functional characteristics of the three most expanded V_H families, namely, VH3-23, VH3-30 and VH1-69 (**Figure 4.5a**). As shown in **Figure 4.5b**, the mAbs derived from each V_H family displayed a range of neutralization potency and breadth. Despite containing comparable numbers of mutations in the V sequence compared to VH3-23 and VH1-69 families (**Figure 4.5c**), we observed that the VH3-30 hmAbs tended towards a broader and more potent neutralization capacity than the other two predominant families (**Figures 4.5d and 4.5e**). Conversely, VH3-23 hmAbs were show to be predominantly serotype-specific and presented a range of neutralization potency, from very potent to low neutralization activity (**Figures 4.5d and 4.5e**).

Human mAbs isolated after secondary DENV infection are highly mutated

To test the hypothesis that broadly neutralizing antibodies derive from MBC precursors that have undergone further SHM and affinity maturation, we analyzed the number of mutations in the V_H gene of our panel of hmAbs isolated after secondary DENV infection. The mutation frequency was calculated over the V gene as nucleotides or amino acid differences from the putative germline sequence. Of the total 69 hmAbs sequenced, the average number of mutations per V_H segment is 17.7 nucleotide acid changes, while V_L is 11.3. As expected, the frequency of mutations was significantly higher in the V_H than V_L (**Figure 4.6a**). The average levels of SHM were comparable across three individuals, ranging from 4 to 34 mutations per sequence and averaging 17.7 mutations overall (**Figure 4.6b**). While hmAbs isolated from individuals 985 and 3243 had a wide range of mutations per sequence, hmAbs isolated from 1791

presented very similar number of mutations per V sequence. In the V_L genes, the levels of SHM were significantly higher in individual 985. The V_L mutations ranged from 1 to 25 per sequence and averaged 11.3 mutations overall (**Figure 4.6c**). In humans, the CDRH3 length is typically between 8 to 16 amino acids, and bnAbs with longer sequences have been described[66,67]. Here, we determined CDR3 lengths according to the IMGT definition and observed that the CDRH3 length was comparable across the three individuals, ranging from 5 to 24 amino acids with an average of 16.0 amino acids (**Figure 4.6d**).

Cross-neutralizing hmAbs present overall higher degrees of SHM than type-specific hmAbs

To determine whether higher SHM levels are associated with neutralization breadth, we compared the number of mutations per sequence in type-specific versus cross-reactive antibodies (**Figure 4.7a**). We observed a trend towards higher degrees of SHM in cross-neutralizing antibodies, although the analysis did not reach statistical difference. In fact, we also observed similar pattern when we subdivided the cross-reactive clones according to their reactivity to two, three or four serotypes (**Figure 4.7b**). Interestingly, we observed one type-specific hmAb (115) with strong neutralization potential to DENV3 that presents a very high degree of SHM (31 amino acid changes per V sequence). When this outlier was removed, the comparison of V mutations in type-specific versus cross-reactive antibodies was significant ($p<0.05$) (**Figure 4.7c**). Moreover, comparison of the mutations in type-specific versus the cross-reactive subgroups indicated a significant trend towards higher degrees of SHM in the clones reactive to more serotypes when this outlier was removed (**Figure 4.7d**).

Precursor lineages of broadly neutralizing antibodies were not identified

To reconstruct the lineage generation of bnAbs, we performed high throughput sequence analysis of the total B cell repertoire from PBMCs collected at prior time-points (post-primary infection and pre-secondary infection) from the same individuals from whom the mAbs were isolated, as indicated in **Table 4.4.** B cell clonal lineages were defined by heavy chain rearrangements that shared V and J gene segment usage (referred to hereafter as V_H and J_H) and encoded CDR3 regions of equal length and similar CDR3 nucleotide sequence, clustering at a 90% identity threshold using single linkage clustering. However, given the insufficient sampling depth inherent to the nature of pediatric samples (average 5 million PBMCs per time-point), we were unable to identify the precursor clones that generated broadly neutralizing antibodies (**Table 4.5**).

DENV3 type-specific hmAbs isolated after secondary DENV infection have potent neutralization activity against diverse genotypes

Depletion of antibody populations from plasma samples from sequential DENV infections indicate that in addition to cross-neutralizing antibodies, populations of type-specific antibodies to the infecting serotypes are also elicited[21]. From our panel of hmAbs isolated from MBCs after secondary infection, we noted that while the majority of

hmAbs are either partially or fully cross-neutralizing, numerous type-specific hmAbs are also seen. Interestingly, all donors from whom the hmAbs were isolated had experienced a symptomatic DENV3 infection. In fact, the highest number of type-specific antibodies is to the DENV3 serotype (14 mAbs), and they ranged in EC_{50} from 2.77 to 402 ng/mL (**Figure 4.8a**). Given that previous studies have shown genotypic variation in DENV3 neutralization[68–71], in collaboration with Drs. Ellen Young and Ralph Baric at UNC Chapel Hill, we tested our panel of DENV3 type-specific hmAbs against DENV3 strains representative of genotypes I (DENV3 Indonesia), genotype II (DENV3 Thailand 1995), genotypes III (Cuba 2002 and Sri Lanka 1989) and genotype IV (Puerto Rico 1977). As a reference, we also used 5J7 and EDE hmAbs. Similar to 5J7, hmAbs 144, 286, 298, 354, 404, 408, 437 and 443 successfully neutralized all DENV3 genotypes tested in our panel. Conversely, hmAbs 115, 66, 236, 297 lacked neutralization against genotype IV, while hmAb 415 did not neutralize genotype III (**Figures 4.8c and 4.8d**).

Newly identified DENV3 type-specific hmAbs bind to novel epitopes on the E protein

Currently, the only DENV3 type-specific neutralizing hmAb is 5J7, which recognizes a complex quaternary epitope that spans 3 monomers of the E glycoprotein[15]. Previous findings by Andrade and colleagues concluded that the 5J7 epitope is an important epitope within the DENV3 repertoire, but other epitopes are predicted to exist[19]. To map the epitopes targeted by the DENV3 type-specific hmAbs we isolated, we used chimeric viruses to identify residues associated with the hmAbs epitopes, an approach that has been previously shown to be successful[72]. We show that indeed, the newly identified mAbs map to novel epitopes on the E protein (**Figure 4.9**). The newly identified hmAb 144, for instance, overlaps with the 5J7 epitope but reaches further into the EDIII of the adjoining subunit. On the other hand, hmAbs 115 and 290 bind primarily to EDII, while hmAb 66 mainly binds to EDIII. Interestingly, a large number of hmAbs (236, 297, 298, 354, 404, 406, 415, 437 and 443) bind to EDI (**Table 4.6**). Strikingly, all these hmAbs were isolated from individuals who shared the same infection history of inapparent DENV2 followed by symptomatic DENV3 (**Table 4.4. and Table 4.6**), a first indication that the order of the infecting serotypes may influence the location of epitope binding. Thus, we have identified novel DENV3 epitopes using hmAbs isolated from individuals in a dengue-endemic area, a finding that improves the understanding of the DENV3 neutralizing repertoire.

Novel DENV3 hmAbs are protective in a mouse model of DENV infection

DENV type-specific hmAbs that bind to complex epitopes on the E protein have been shown to confer protection in DENV animal models[15,17,73]. Here, we used the α/β and γ interferon receptor-deficient AG129 mouse strain to test whether the novel DENV3 hmAbs are protective prophylactically *in vivo* against DENV3 infection. Groups of AG129 mice were treated with 50 µg of hmAb 66, 115, 144 or 443, which were chosen based on the different regions on the E protein to which they bound. Control groups were treated with 50 µg of 5J7 as a positive control, and PBS or IgG1 isotype as

negative controls. After 24 hours, the mice were challenged with DENV3 strain UNC 3009, and viremia was measured by qRT-PCR 3 days post-challenge. Compared to PBS and IgG1 controls, the viral load normalized to GAPDH was significantly lower in the groups treated with hmAbs 115, 443 and 66 ($p<0.0001$) and hmAb 144 ($p<0.001$). This *in vivo* study demonstrates a protective role of newly identified DENV3 type-specific hmAbs in a mouse model of DENV infection.

DISCUSSION

While secondary infection is the major risk factor for severe dengue disease, cohort studies in dengue-endemic areas indicate that tertiary infections are mostly mild or inapparent, thus implicating a protective role mediated by cross-neutralizing antibodies elicited after a second DENV infection[26]. Furthermore, in light of results from DENV vaccine trials that demonstrated efficacy in populations pre-exposed to DENV but not in naïve populations[74], it is relevant to understand how cross-neutralizing responses develop after sequential DENV infections. Diverse studies show that cross-reactive antibodies after secondary DENV infection display more potent neutralization activities and higher binding avidities than antibodies isolated after primary DENV infection[22,75–77]. Moreover, depletion of antibody populations in samples of sequential DENV infections indicates that cross-reactive populations drive most of the polyclonal neutralization after secondary infections[21]. Collectively, these findings allow us to hypothesize that DENV cross-reactive MBCs derived from primary infections undergo somatic hypermutation and acquire greater neutralization breadth and potency after each subsequent DENV exposure.

While previous studies have shown a frequency of 0.1 to 0.2% DENV-specific B cells in the MBC pool following DENV infection[58,78], our study generated a high frequency of DENV-specific transformed B cells (1.4-2.4%). In addition to the screening strategy, our higher frequencies may be explained by the optimization of the EBV-mediated B cell transformation, with addition of the TLR9 agonist CpG[79], cyclosporine, and ChK2 inhibitor[80]. Despite differences in the polyclonal DENV neutralizing antibody titers and infection history, the frequency of DENV-specific B cells was comparable across the three individuals analyzed in this study. This observation is not surprising given the fact that serum neutralizing antibodies derive from long-lived plasma cells (LLPCs) in the bone marrow and not the MBC compartment. In fact, previous studies have demonstrated a weak correlation between DENV neutralizing antibody titers (NT_{50}) and frequency of DENV-specific MBCs in circulation[81,82].

The specificity of the human antibody response to DENV has been largely elucidated via isolation and characterization of hmAbs. The functional characterization of hmAb panels indicate that a large fraction is cross-reactive and weakly neutralizing[13, 23, 24, 27, 28, 59, 61]. Indeed, our panel of 74 hmAbs isolated from MBCs after secondary DENV infection is predominantly partially or fully cross-neutralizing, although a number of potent type-specific hmABs were identified (see below). Similar to the polyclonal response, which contains a mixture of DENV-specific antibodies that are neutralizing or non-neutralizing, our hmAb panel contains hmAbs with a range of neutralization potential. While most cross-reactive clones displayed NT_{50} values in the range of 100-1000 ng/mL, we also detected 12 potent broadly neutralizing antibodies that neutralized at least three serotypes. The frequency of B cell clones with broad and potent DENV neutralization is reportedly rare in several studies to date, which derived from adult PBMC donors[23]. Nevertheless, our panel of hmAbs isolated from pediatric samples contained a relatively abundant population of broadly neutralizing antibodies. The higher frequency of potent broadly neutralizing antibodies observed in our panel could

potentially be attributed to the fact that we used pediatric PBMC samples, in contrast with most studies, which have employed samples from adults. Supporting this hypothesis, a number of studies have documented the early rise of broadly neutralizing antibodies and higher diversity of epitopes in HIV-infected children compared to HIV-infected adults, which could indicate fundamental age-related differences in the ability of PBMCs to generate B cell lineages to viruses[84–87].

In addition to neutralizing hmAbs with varies degrees of neutralization potential, we observed a small fraction of DENV-binding hmAbs that lacked neutralization activity. Supported by previous studies that indicated an abundance of prM and NS1-reactive MBCs in convalescent dengue patients[23,25,28,81] , the minor group of non-neutralizing hmAbs found in our study could be NS1-reactive. Alternatively, these hmAbs may represent a population of antibodies that binds to the virion but do not present neutralization activity. Another alternative hypothesis is the observation that the landscape for antibody binding and neutralization of the virus might be affected by the existence of cryptic epitopes, which are transiently exposed at higher temperatures[88,89]. In polyclonal sera, DENV antibodies have been shown to target these cryptic epitopes, which means that the virus is neutralized only under specific conditions that allow exposure of these epitopes. Finally, it should be noted that beyond the canonical neutralization capacity, Fc region-mediated functions of non-neutralizing antibodies may also help control viral burden, as shown in HIV[90], Ebola[91,92] and human cytomegalovirus (HCMV)[93].

Clonal expansion and somatic hypermutation (SHM) in B cells undergoing germinal center reaction provides the basis for the selection of high affinity clones, a hallmark of the adaptive immune response. Most clones in the MBC pool have undergone SHM and present, on average, 10-26 nucleotide mutations from precursor genes[94]. Our analysis of the variable heavy gene sequences indicated that most hmAbs carry a high degree of SHM, with a number of clones (26/69) that display more than 21 mutations, indicating a memory origin of these lineages. Conversely, a minority (11/69) of clones presented a very limited number of mutations per variable heavy chain gene sequence, which suggests a *de novo* B cell response induced by second infection. As we compared the number of mutations in the variable region sequence of hmAbs reactive to one serotype (type-specific) versus to two or more serotypes (cross-reactive), we observed a trend towards higher degree of SHM in the latter group. Further, this trend is maintained as we subdivided the hmAbs by cross-reactivity to 2, 3 or 4 serotypes. Despite the limitation of not being able to trace the development of these hmAbs within each individual, this observation allows us to hypothesize that after secondary DENV infection, somatically hypermutated clones gain neutralization breadth.

The high degree of SHM observed in a variety of broadly neutralizing antibodies suggests that multiple rounds of affinity maturation may be a prerequisite for a broad functional profile[46,47,95–98]. While somatically hypermutated hmAbs have been isolated from plasmablasts after secondary DENV infection[24,39] the implications of SHM for neutralization breadth and potency have not been established yet. From our hmAb panel, we identified clones presenting low degrees of SHM despite broad neutralizing

response. Regarding neutralization potential, we observed cross-reactive clones that achieved potent neutralization despite low SHM rates, which has been reported in ZIKV[99], VSV[100], influenza[101] and HIV infections[102]. However, since our study was limited in the ability to trace the lineage development of broadly neutralizing antibodies, we were unable to test the hypothesis that DENV broadly neutralizing antibodies expand from clones generated in the first infection that acquire neutralization potency upon SHM in a subsequent DENV infection. The limited depth inherent to the very small sample volume – and hence low numbers of B cells – in pediatric samples was a limiting factor in our study. As pointed out by Briney and colleagues[103], PBMC samples containing 5-10 million cells is an extremely small fraction of the antibody diversity encoded by a very abundant B cell repertoire.

Despite not identifying clonally related hmAbs, we did find a number of clones that shared the same heavy chain gene allele. In fact, we noticed the predominance of three families in particular, namely VH3-30, VH1-69 and VH3-23. Preferential usage of VH1-69 has been previously reported in natural infection with DENV[104], as well as influenza[105] and hepatitis C viruses[106]. Notably, IGVH1-69 was the predominant V_H family in plasmablast-derived mAbs isolated from individuals vaccinated with a tetravalent DENV vaccine[107]. While the prevalence of VH3-23 is not surprising given its previously reported high frequency in the human repertoire[108], it is interesting to note that clones of VH3-23 family were found to be expanded in response to ZIKV infection in individuals who had a prior history of DENV1 infection[109]. In a similar manner, most of the hmAbs within the VH3-23 family were serotype-specific to either DENV1 or DENV3. While no expansion of VH3-30 alleles have been reported so far in DENV-infected individuals, we observed a high expansion of this family, and expansion of VH3-30 is commonly found in the human immunoglobulin repertoire[110]. Interestingly, despite sharing on average the same number of mutations with the other two expanded families, VH3-30 clones tended to have greater neutralization breadth and potency. Since V_H bias to other pathogens has also been reported[111–113], it is plausible that the V_H repertoire may impact the humoral immunity in DENV natural infection and vaccination.

While all potent type-specific hmAbs isolated so far derive from MBCs sampled after primary DENV infection[15,16,114], our hmAb panel isolated after secondary DENV infection successfully identified new DENV3 highly potent type-specific antibodies targeting novel epitopes. Notably, all three individuals experienced symptomatic DENV3 infection either as a first infection (individual 3243) or as a second infection (individuals 985 and 1791). Thus, we conclude that type-specific hmAbs to the second infecting serotype can also be elicited. These findings corroborate depletion studies of secondary DENV infections, which demonstrate that populations of type-specific antibodies contribute to polyclonal neutralization after second infection, albeit comprising a small proportion of the overall neutralizing titer[21]. To date, only one DENV3 type-specific hmAb has been described[16], despite strong evidence that the DENV3 repertoire comprises additional epitopes[19]. Mapping of the newly identified DENV3 hmAbs defined three novel regions, as well as an overlapping region with the 5J7, for binding of potent DENV3 neutralizing hmAbs. Strikingly, all hmAbs that bound to EDI derived from the two donors who shared the

same infection history (inapparent DENV2 infection followed by symptomatic DENV3 infection), which allows us to hypothesize that MBCs from the first DENV2 infection may be recalled during the secondary infection with DENV3. In fact, the EDI is the site of the epitope targeted by mAb 3F9, which is a DENV2 type-specific hmAb that tracks with the polyclonal sera of individuals who experienced primary DENV2 infection[18]. Another important feature of the novel DENV3 epitopes is the predominance of genotype insensitive neutralization profiles. In the light of recent data showing the impact of genotypic variation in neutralization activity[115], the identification of antibodies that are potent neutralizers of all DENV3 genotypes is highly relevant. As such, further studies employing structure-guided chimeric viruses can help define how much of the DENV3 type-specific neutralizing response in polyclonal sera tracks with the novel epitopes identified here should be carried out, as previously done by our group and our collaborators in the NIH Program Project[19,78,116].

In conclusion, our study has elucidated important functional and molecular characteristics of hmAbs isolated after secondary DENV infection, namely (i) predominance of cross-neutralizing and somatically hypermutated clones, (ii) higher degrees of SHM in cross-reactive clones versus type-specific clones, (iii) preferential usage of certain V_H families in the MBC pool, (iv) generation of potent type-specific antibodies to the second infecting serotype, (v) identification of novel DENV3 epitopes, and (vi) the sequence of infecting serotypes as a potential determinant of sites targeted by antibodies. By investigating the immunoglobulin repertoire during sequential DENV infections, the field may gain valuable information on how SHM diversifies clonal lineages and associated[1] functional properties. Another important concept that needs further investigation is how independent B cell lineages converge to a common epitope following SHM. Overall, comprehensive studies aimed at defining the molecular basis of affinity maturation in DENV infection may lead to more robust correlates of protection and can be exploited for immunogen design.

EXPERIMENTAL PROCEDURES

Ethics statement

The human subject protocol for the Pediatric Dengue Cohort Study (PDCS) in Nicaragua was reviewed and approved by the Institutional Review Boards of the University of California, Berkeley and the Nicaraguan Ministry of Health. Parents or legal guardian of the subjects enrolled in the PDCS provided written informed consent, and participants 6 years of age and older provided assent.

Study population

The PDCS is an ongoing prospective dengue cohort study with approximately 3,700 children ages 2-14 in District II of Managua, Nicaragua[54]. Participants with suspected dengue, as defined by the traditional WHO dengue guidelines[117] and undifferentiated febrile illness are worked up for DENV infection, which is identified by serotype-specific RT-PCR for detection of viral RNA, isolation of DENV on C6/36 cells, and/or seroconversion by IgM enzyme-linked immunosorbent assay (ELISA) or a ≥4-fold increase in total antibody titer as measured by inhibition ELISA in paired acute- and convalescent-phase samples[36,54,118–121]. Inapparent DENV infections were identified through serological testing of paired annual healthy blood samples, where seroconversion or a 4-fold or greater increase in total DENV-specific antibody titer by Inhibition ELISA (IE) or DENV neutralizing antibody titers indicates an inapparent DENV infection[36,54,56,122].

Reporter Viral Particle-based neutralization assay

The neutralizing antibody titers (NT_{50}) of healthy annual samples are determined using a flow cytometry-based assay with reporter (GFP) viral particles (RVPs) representing the four serotypes and Raji cells expressing the DENV attachment factor DC-SIGN[55,56]. Briefly, RVPs were prepared according to working dilution previously determined to result in 8-15% infection of Raji-DC-SIGN cells in a final volume of 25 µl of RPMI pH 8.0 complete medium. RVPs were then mixed with an equal volume of serum (eight 3-fold serial dilutions in RPMI pH 8.0 complete medium starting at 1:5, tested in duplicate) in 96-well plates and incubated on a shaker for 1 hour at room temperature. Following that, 50 µl of diluted RVPs were added to 40,000 Raji DC-SIGN cells in a total volume of 100 µl RPMI media and incubated for 48h at 37°C in 5% CO_2. After incubation, cells were fixed in 2% paraformaldehyde. The percentage of infected, GFP-positive cells for each serum concentration was plotted as percent infection versus $\log_{10}$ of the reciprocal serum dilution using Prism 5.0 (GraphPad). A sigmoidal dose response curve with a variable slope was then generated to determine the 50% neutralization titer, or NT_{50} – the serum dilution at which a 50% reduction in infection was observed compared to the positive (no-serum) control. Background GFP levels were subtracted from all measurements using a negative control (no-RVP).

PBMC isolation

For peripheral blood mononuclear cell (PBMC) isolation, whole blood samples were collected in Vacutainer tubes with EDTA anticoagulant reagent. In the Nicaraguan National Virology Laboratory, 5 mL of blood was transferred into a Leucosep tube containing 3 mL of Ficoll Histopaque and centrifuged at 500 x g for 20 minutes (min) at room temperature. The PBMC fraction was then collected and transferred to a 15-mL conical tube containing 9 mL of PBS with 2% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were washed 3 times in this solution by centrifugation at 500 x g for 10 min and resuspended in 10 mL of complete medium. Prior to the third wash, an aliquot of 500 uL was used to obtain a cell count using a hemacytometer. Following the third wash, cells were suspended in a final concentration of 10^7 cell/mL in freezing medium consisting of 90% FBS and 10% dimethyl sulfoxide and then aliquotted. Cryovials containing the cell suspension were temporarily stored in isopropanol containers at -80°C overnight and then transferred to liquid nitrogen for permanent storage.

Generation of hmAbs to DENV (performed in the laboratory of Dr. James Crowe)

PBMCs collected in the annual sampling immediately after the secondary DENV infection were used for mAb isolation. In the laboratory of Dr. James Crowe, the cryopreserved samples were transformed with Epstein-Barr virus (EBV), as previously described[23,57]. Transformed B cells were tested for binding to DENV antigen and the reacting cells were expanded, followed by cytofusion with HMMA2.5 nonsecredting myeloma cells[38]. Following fusion, the hybridomas were selected by growth in a hypoxanthine-aminopterin-thymidine (HAT) medium containing oubain. Hybridomas were screened for antibodies reacting to DENV1-DENV4 and cloned biologically by single-cell fluorescent-activated cell sorting (FACS). Secreted antibodies were purified by protein G chromatography[38]. For the sequence analysis of antibody variable region genes, cellular RNA was extracted from the generated clonal hybridomas, and RT-PCR was performed using a cocktail of primers designed to amplify all heavy chain or light chain antibody variable regions and then sequenced by Sanger sequencing[57]. The heavy and light chain antibody region sequences were analyzed using the IMGT/V-Quest software, as previously described[123,124].

Viruses

DENV1 Thailand 1964, DENV2 Thailand 1964, DENV3 Philippines 1964 and DENV4 Columbia 1982 used for binding ELISAs were propagated in *Aedes albopictus* C6/36 cells at 32°C in 5% CO_2. For the neutralization assays, Nicaraguan strains of DENV1 (N1265), DENV2 (N1272), DENV3 (N7236) and DENV4 (N703-99) propagated in *Aedes albopictus* C6/36 cells at 37°C in 5% CO_2 were used.

U937-DC SIGN-based neutralization assay

The neutralizing potency of the hmAbs was measured using a flow cytometry-based neutralization assay with the U937 human monocytic cell line stably transfected with DC-SIGN[125]. At an initial concentration of 15,000 ng/mL, hmAbs were serially diluted 3-fold 12 times in RPMI supplemented with 2% FBS. A dilution of virus that infects between 8-15% of the U937 cells (previously determined by virus titration) was added to the hmAb dilutions and incubated for 1 hour at 37°C. Following incubation, the cells were centrifuged at 252 x g for 5 min and resuspended in 100 µL RPMI medium. Next, cells were fixed in 4% paraformaldehyde, incubated for 10 min at room temperature, and centrifuged at 252 x g for 5 min. Following this, cells were blocked in permeabilization buffer (0.1% saponin, 5% bovine serum albumin in 1X phosphate-buffered saline [PBS]) for 30 min at room termperature. Then, cells were incubated with anti-E mAb 4G2 conjugated to Alexa 488, diluted in blocking buffer (0.5% bovine serum albumin and 0.02% sodium azide in 1X PBS) for 25 min at room temperature. Finally, cells were washed and resuspended in PBS. Acquisition of the infected cells was performed with a Guava flow cytometer (EMD Milipore) by gating Alexa 488-positive cells. The data were analyzed using a nonlinear, 4-parameter dose-response regression analysis with Prism software (GraphPad). The NT_{50} was determined as the concentration of the hmAb dilution that achieved a 50% reduction of the infection compared to infection control. Data generated had to meet the quality control criteria, whereby the sigmoidal dose-response regression fit had to include an absolute sum of squares of <0.2 and a coefficient of determination (R^2) of >0.9.

In vivo studies

This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All procedures were approved by the UC Berkeley Animal Care and Use Committee guidelines. AG129 mice[126] were bred in the Animal Facilities at UC Berkeley. Mice aged 6 to 9 weeks of age were administered 50 µg of the hMAbs of interest, with hmAb 5J7 as a positive control and an isotype control (IgG1) or PBS as negative controls. A total volume of 200 µL was injected intraperitoneally 24 hours prior to challenge with 5 $x10^6$ PFU of the mouse-adapted DENV3 virus UNC 3009 administered intraveneously (i.v) in a total volume of 100 µL. Three days post-inoculation, the mice were sacrificed, and spleen was harvested and stored in Trizol.

RNA extraction and qRT-PCR

Stored spleens were homogenized with silica beads followed by RNA extraction with Trizol reagent. To quantify the virus genome, a real-time quantitative reverse transcription-PCR (qRT-PCR) method was used. Primers for the E gene were designed to quantify DENV3 UNC 3009 copies in each mouse and were used at a final concentration of 10 nM. The sequences for the forward and reverse primers were as follows: 5'GGACTGGATACACGCACCCA,

and 5'CATGTCTCTACCTTCTCGACTTGTCT, respectively. A Taq-man probe with the following sequence was used for detection: FAM-5' Z-BHQ-1 ACCTGGATGTCGGCTGAAGGAGCTTG. The SuperScript III One-Step RT-PCR System (Invitrogen) was used for reverse transcription of viral RNA and subsequent amplification of viral complementary DNA (cDNA). Viral RNA was reverse-transcribed (52°C for 15 min), and the resulting cDNA was amplified via one cycle of denaturation (94°C for 2 min), and 45 cycles of denaturation (94°C for 15 s), annealing (55°C for 40 s), and extension (68°C for 10 s). For quantification of DENV3 copies, a 4-point standard curve (8.0, 6.0, 4.0, and 2.0 $\log_{10}$ copies/µl of eluate) was used. The concentration of RNA in the eluate (expressed as $\log_{10}$ copies/µL of eluate) was calculated from the linear regression equation for the standard curve. Viral load in $\log_{10}$ copies/g of spleen was then calculated from this value, accounting for volumes used in extraction, as previously described[127].

FIGURES

985

NT50	2004	2005	2006	2007	2008	2009	2010	2011
DENV1	<10	<10	16	26	61	52	25	304
DENV2	<10	<10	2150	1347	947	1061	466	1586
DENV3	<10	<10	32	41	58	35	37	887
DENV4	<10	<10	<10	<10	<10	<10	<10	<10
Infecting serotype			DENV2					DENV3
Infection outcome			Inapparent					Symptomatic

1791

NT50	2004	2005	2006	2007	2008	2009	2010	2011
DENV1	<10	<10	30	22	47	<10	47	373
DENV2	<10	353	500	304	334	286	243	475
DENV3	<10	74	10	33	97	57	36	849
DENV4	<10	30	18	32	39	65	31	551
Infecting serotype		DENV2						DENV3
Infection outcome		Inapparent						Symptomatic

3243

NT50	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
DENV1						<10	65	75	166	814
DENV2						<10	11	26	56	71
DENV3						<10	762	838	1890	6258
DENV4						<10	43	23	49	523
Infecting serotype							DENV3			DENV1
Infection outcome							Symptomatic			Inapparent

Table 4.1. Longitudinal analysis of neutralizing antibody titers in selected cohort participants. Annual serum/plasma samples collected from each of the three participants in this study were used for neutralization assays using Reporter Virus Particles (RVP). A ≥4-fold increase in DENV-specific neutralizing antibody titers (NT_{50}) in paired annual samples indicates a DENV infection during the study year, as highlighted for DENV1 (orange), DENV2 (yellow) and DENV3 (green). All three donors experienced one symptomatic DENV3 infection, which was also confirmed by RT-PCR/virus isolation.

Participant ID	DENV-specific B cell frequency	Number of hmAbs
985	1.43%	33
1791	1.50%	7
3243	2.35%	34

Table 4.2. Frequency of DENV-specific in EBV-transformed B cells. For each PBMC donor, the frequency of DENV-specific transformed B cells was estimated based on the number of wells positive for DENV antigen binding divided by the number of transformed B cells. Thirty-three, 7 and 34 hmAb hybridomas were successfully generated via myeloma fusion with EBV-transformed B cells from individuals 985, 1791 and 3243, respectively. EBV transformation, fusion, hybridoma recovery, and screening by ELISA were performed and data analyzed by the laboratory of collaborator Dr. James Crowe at Vanderbilt University.

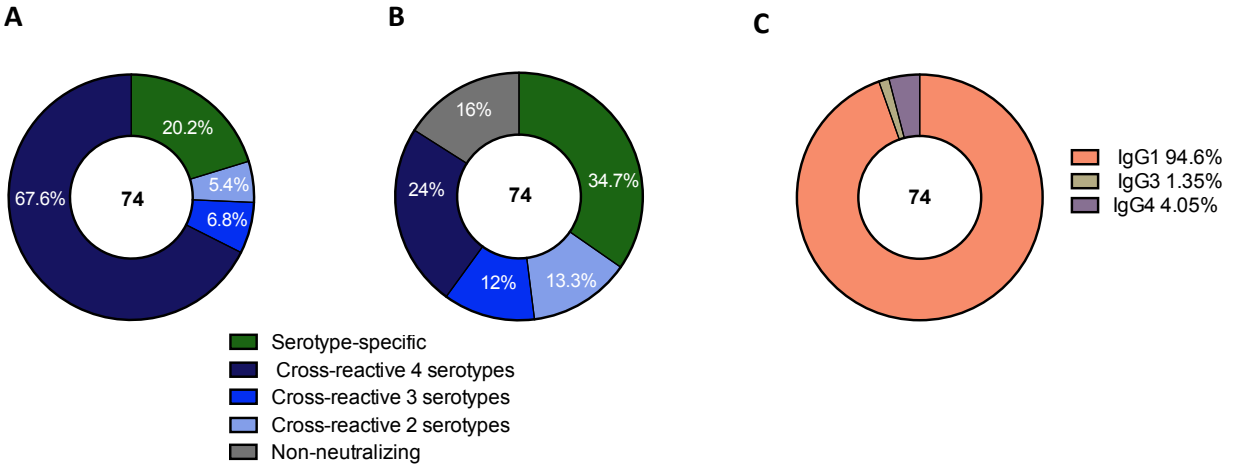


Figure 4.1. Human mAbs isolated after secondary DENV infection are predominantly cross-reactive. Based on the reactivity to the four DENV serotypes, hmAbs that bind (A) or neutralize (B) one or more than one DENV serotypes were classified as type-specific or cross-reactive, respectively. (C) The percentage of each category is also shown. The immunoglobulin (IgG) isotype distribution of all hmAbs was determined and shown to be predominantly IgG1. The number in the center of the donut indicates the total number of hmAbs characterized by binding and neutralization assays. Binding ELISA and IgG subclass data were generated by the Crowe Laboratory and analyzed by D. Andrade.

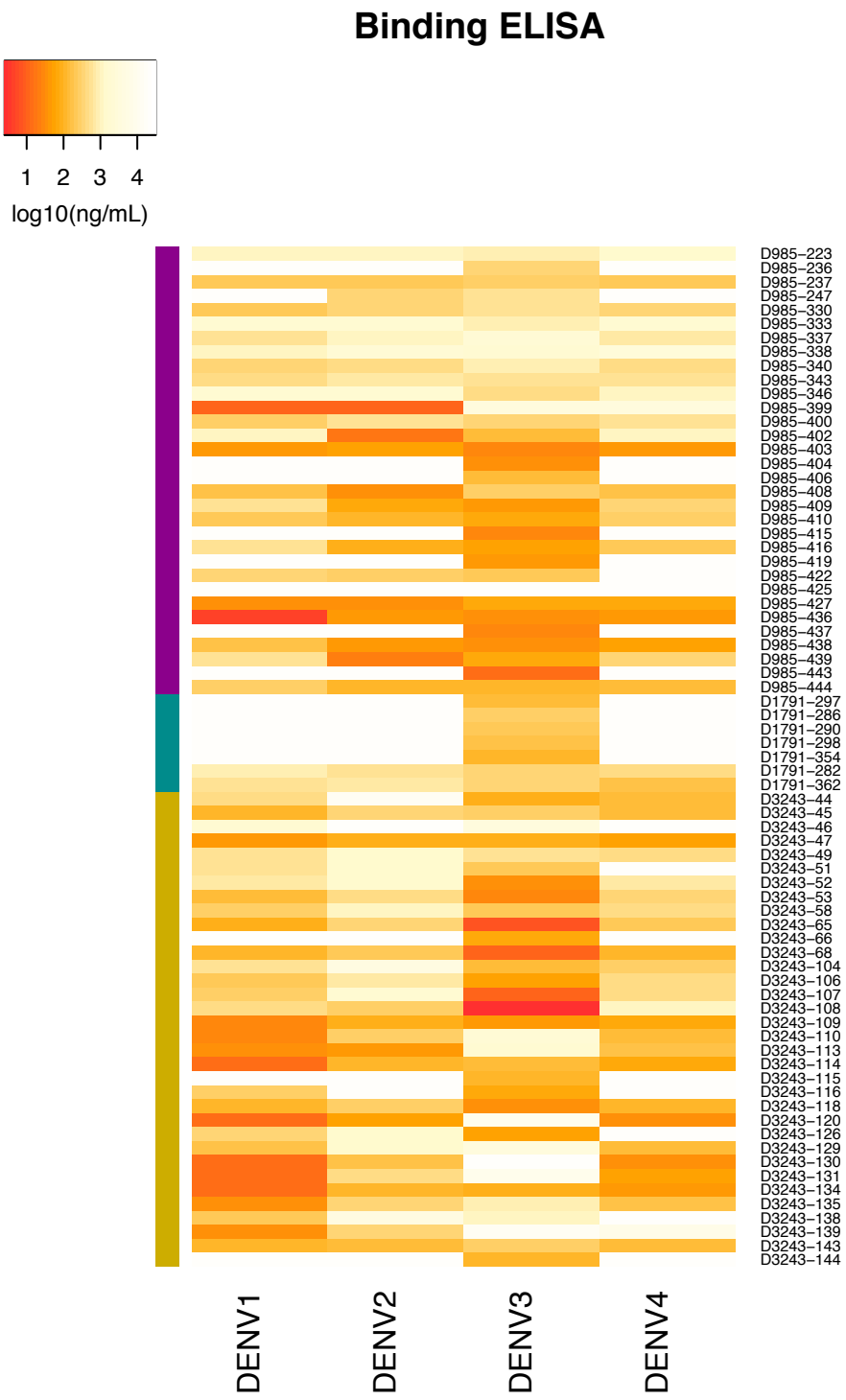


Figure 4.2. Human mAbs isolated from MBCs after secondary infection bind DENV serotypes with variable EC_{50} concentrations. Each hmAb was tested against all four DENV serotypes for values. The log 10-transformed half maximum effective concentration (EC_{50}) of each hmAb isolated from individuals 985 (purple line), 1791 (yellow line) and 3243 (teal line) is represented in this heat map. The color gradient ranges from red to white indicating high and low binding activities, respectively. Binding ELISA data generated by the Crowe Laboratory and analyzed by D. Andrade.

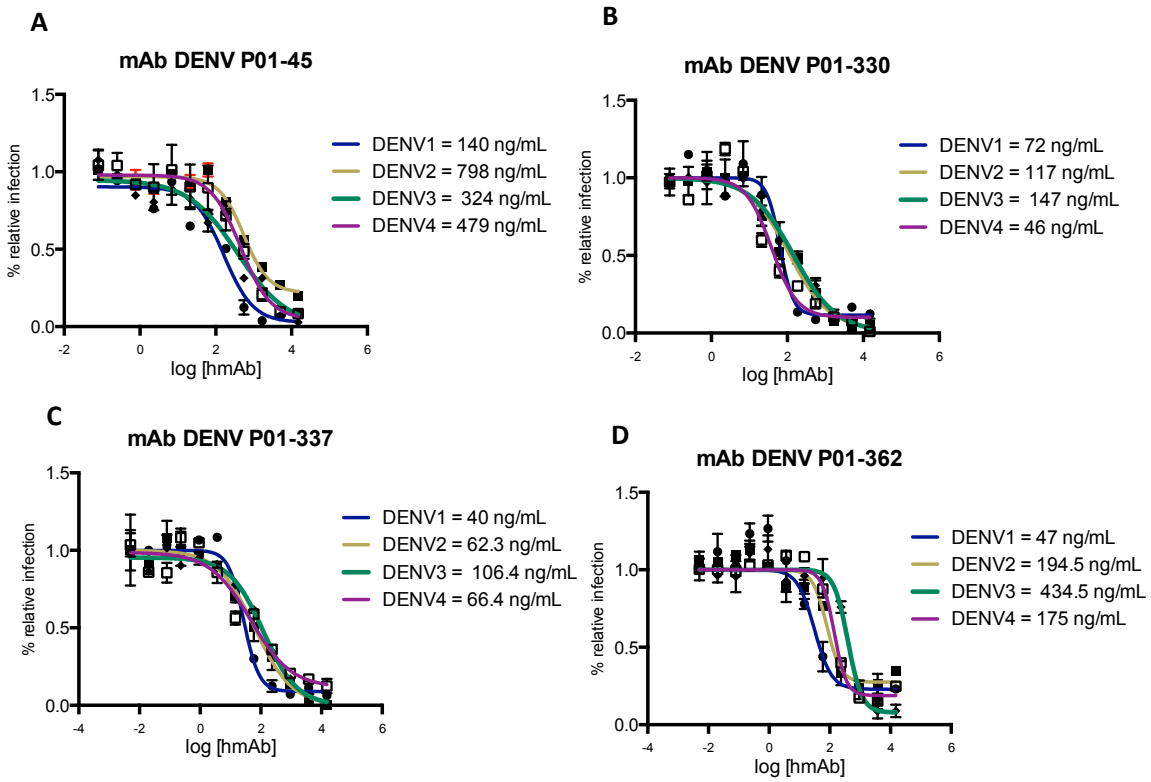


Figure 4.3 Representative sigmoidal dose-response curves of broadly neutralizing hmAbs. U937-DC-SIGN-based neutralization assays were performed using Nicaraguan DENV strains with each of the hmAbs isolated. The raw antibody titration data were fitted with a four-parameter sigmoidal dose-response curve to estimate the 50% concentration (EC_{50}) for each hmAb against each DENV serotype, and values are indicated in ng/mL. Error bars represent the range of values. Graphs show one representative experiment of 2 independent experiments.

	mAb Clone	Donor ID	NEUT-UCB (ng/ml) DV1 1265-4	DV2 N172-06	DV3 N2845-09	DV4 N703-99
DENV serotype-specific	443	985	NN	NN	57	NN
	437	985	NN	NN	207	NN
	404	985	NN	NN	25	NN
	419	985	NN	NN	2.77	NN
	66	3243	NN	NN	402.3	NN
	115	3243	NN	NN	54.05	NN
	144	3243	NN	NN	39.35	NN
	406	985	NN	NN	190	NN
	297	1791	NN	NN	14.5	NN
	286	1791	NN	NN	16.5	NN
	236	985	NN	NN	12.6	NN
	131	3243	NN	NN	127.4	NN
	129	3243	NN	NN	123.9	NN
	290	1791	NN	NN	74	NN
	298	1791	NN	NN	100	NN
	354	1791	NN	NN	71	NN
	403	985	N/A	N/A	4159	NN
	138	3243	260.6	NN	NN	NN
	130	3243	153	NN	NN	NN
	53	3243	1225	NN	NN	NN
	444	985	1064	NN	NN	NN
	120	3243	76.11	NN	NN	NN
	49	3243	447	NN	NN	NN
	338	985	NN	6039	NN	NN
	402	985	NN	1738	NN	NN
XR 2 serotypes	109	3243	137.1	475.3	NN	NN
	118	3243	106	3684	NN	NN
	436	985	681	NN	3266	NN
	438	985	1122	NN	11158	NN
	408	985	354	NN	729	NN
	46	3243	381	NN	1135	NN
	106	3243	4.67	NN	3, 273	NN
	134	3243	1016.2	NN	807.2	NN
	47	3243	396	684	NN	NN
	237	985	34.07	N/A	4083	NN
XR 3 serotypes	126	3243	62.75	65.99	42.16	NN
	51	3243	39.71	11534	2098	NN
	110	3243	72.11	NN	946.2	2, 290
	52	3243	923	3864	1107	NN
	427	985	614	230	2786	NN
	410	985	3105	NN	18.96	1050
	44	3243	315	5998	2163	NN
	104	3243	146.89	NN	1361	5,470
	346	985	500	2911	119	NN
Broad Specificity	108	3243	27.77	799	584.2	2958
	65	3243	1534	3213	765.6	1119.4
	68	3243	172.45	4,326	96.5	71.1
	114	3243	372.7	150.45	122	142.45
	58	3243	225.9	7,500	16	86.1
	143	3243	36.11	19.575	46.46	9045
	45	3243	103	500.6	179.4	542
	282	1791	1535	4920	2128	1581
	362	1791	86	183.55	455.25	501.5
	343	985	339	86	198.8	473.25
	139	3243	58.25	197.45	24.05	8710
	135	3243	415	215	1148	2218
	337	985	415	215	1148	2218
	333	985	99	77	271	157
	340	985	33.45	74.65	51.45	299.2
	330	985	66.225	75.825	161.225	228.1
	223	985	879	1216	698.5	1024.25
	116	3243	355.45	2083.35	199.55	8809.5
Non-neutralizing	409	985	NN	NN	NN	NN
	416	985	NN	NN	NN	NN
	439	985	NN	NN	NN	NN
	400	985	NN	NN	NN	NN
	399	985	NN	NN	NN	NN
	425	985	NN	NN	NN	NN
	156	1035	NN	NN	NN	NN
	415	985	NN	NN	NN	NN
	107	3243	NN	NN	NN	NN
	247	985	NN	NN	NN	NN
	422	985	NN	NN	NN	NN
	107	3243	NN	NN	NN	NN

Table 4.3. Panel of hmAbs isolated after secondary DENV infection showing type-specific as well as partially or fully cross-neutralizing hmAbs. Each hmAb was tested for neutralization activity against DENV Nicaraguan viruses at least twice in two independent experiments using a U937-DC-SIGN based neutralization assay. Based on their reactivity to the four DENV serotypes, the hmAbs are classified type-specific (neutralizes only one serotype), cross-reactive (XR) to 2 or 3 serotypes, broad specificity (neutralizes all four serotypes) and non-neutralizing. The EC_{50} values were categorized as ≤10 ng/mL (green), 10-100 ng/mL (yellow), 100-1000 ng/mL (orange) and >1000 ng/mL (red). Non-neutralizing hmAbs are designated as NN. Data are representative of the average of two independent experiments.

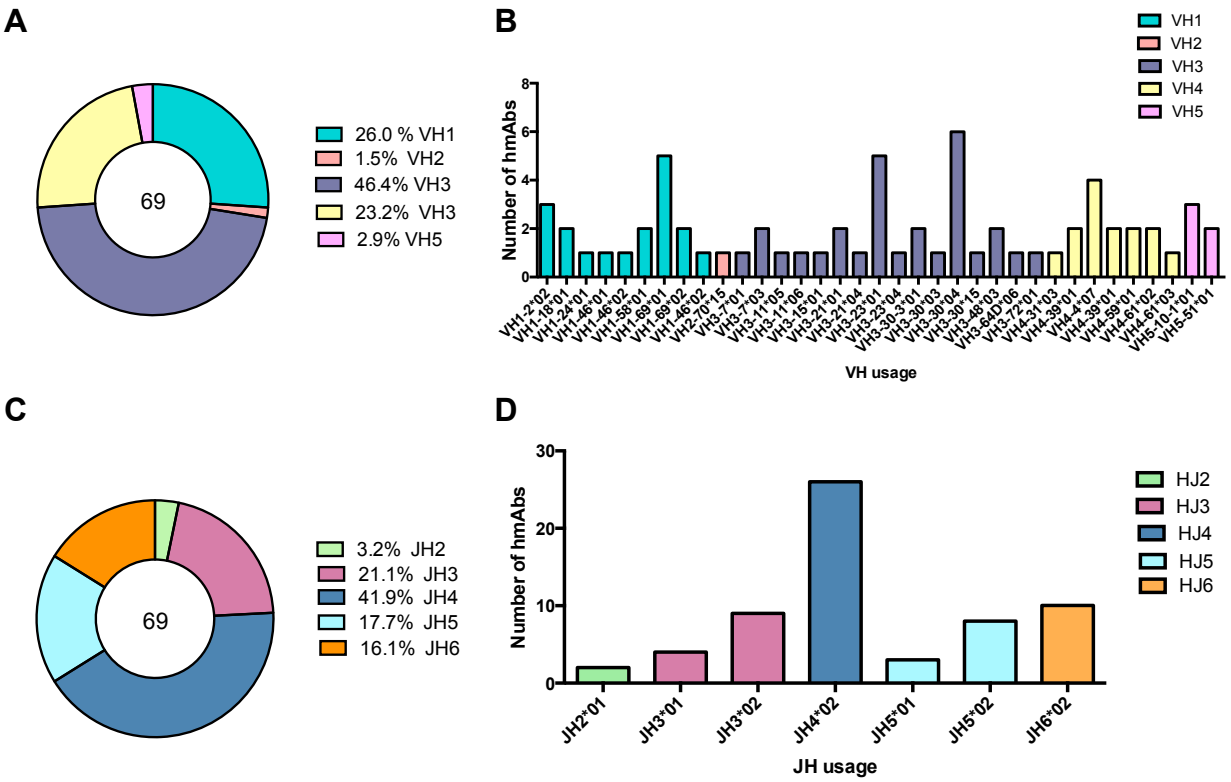
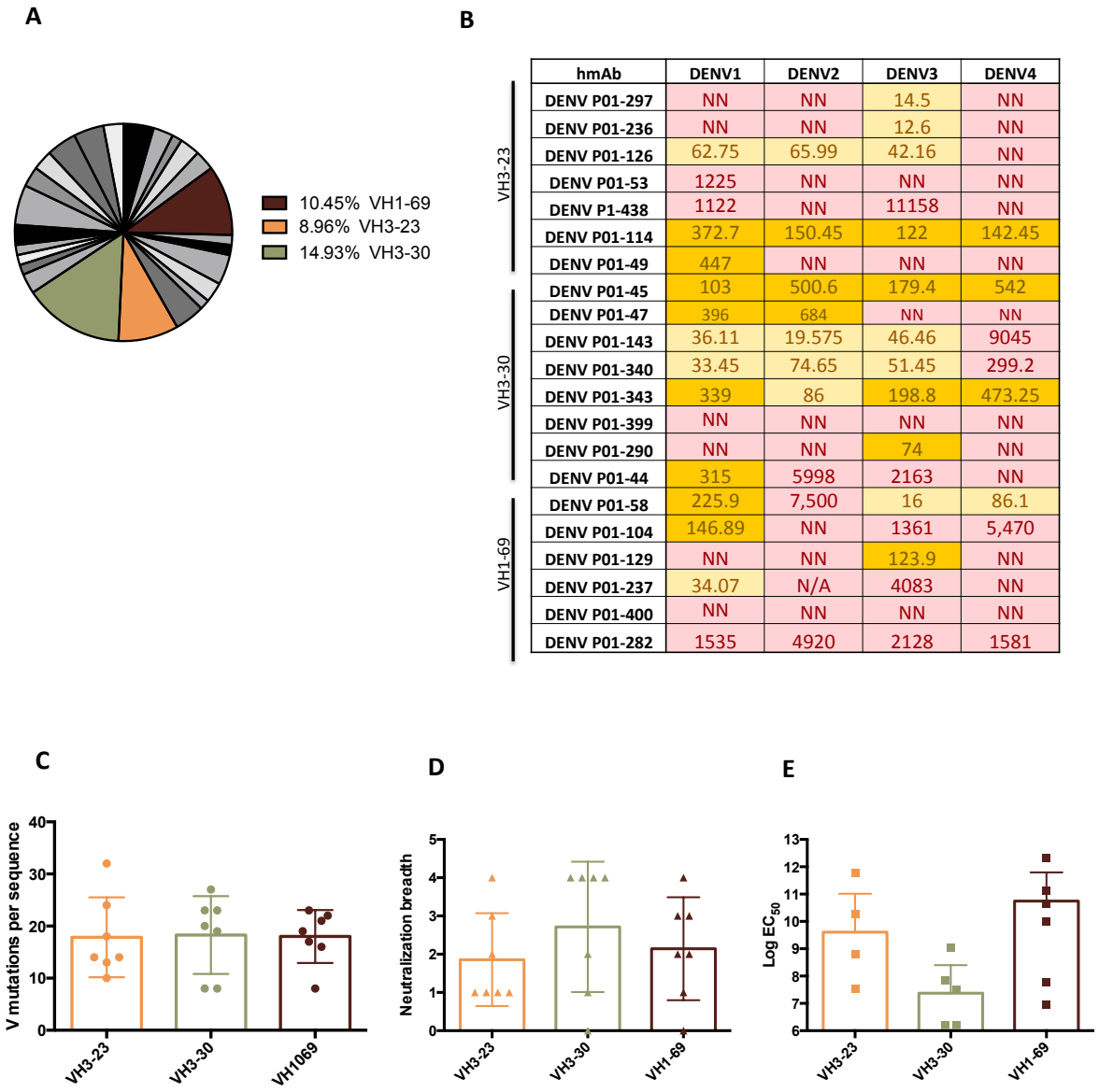


Figure 4.4 The variable (V) and joining (J) genes of the heavy chain of hmAbs isolated from MBCs after secondary DENV infection are genetically diverse. The heavy chain region sequences were analyzed using the ImMunoGeneTics (IMGT) database to determine the V and J usage. (A) Analysis of the V_H gene family usage and (B) distribution of alleles in hmAbs derived from MBCs collected after secondary DENV infection is shown. Preferential usage of the VH3 family, specifically VH3-23, VH3-30, and VH1-69 is observed. (C) JH family usage and (D) distribution of alleles of hmAbs derived from MBCs after secondary DENV infection is shown. Bias towards HJ4 family, particularly HJ4*02, is seen. The number at the center of each donut represents the number of heavy-chain sequences analyzed. Sequence data generated by Dr. Crowe's laboratory and analyzed by D. Andrade.



	hmAb	DENV1	DENV2	DENV3	DENV4
VH3-23	DENV P01-297	NN	NN	14.5	NN
	DENV P01-236	NN	NN	12.6	NN
	DENV P01-126	62.75	65.99	42.16	NN
	DENV P01-53	1225	NN	NN	NN
	DENV P1-438	1122	NN	11158	NN
	DENV P01-114	372.7	150.45	122	142.45
	DENV P01-49	447	NN	NN	NN
VH3-30	DENV P01-45	103	500.6	179.4	542
	DENV P01-47	396	684	NN	NN
	DENV P01-143	36.11	19.575	46.46	9045
	DENV P01-340	33.45	74.65	51.45	299.2
	DENV P01-343	339	86	198.8	473.25
	DENV P01-399	NN	NN	NN	NN
	DENV P01-290	NN	NN	74	NN
	DENV P01-44	315	5998	2163	NN
VH1-69	DENV P01-58	225.9	7,500	16	86.1
	DENV P01-104	146.89	NN	1361	5,470
	DENV P01-129	NN	NN	123.9	NN
	DENV P01-237	34.07	N/A	4083	NN
	DENV P01-400	NN	NN	NN	NN
	DENV P01-282	1535	4920	2128	1581

Figure 4.5 Predominant VH3-23, VH3-30 and VH1-69 families have similar degrees of SHM but present different degrees of neutralization breadth and potency. (A) Of the total of 69 variable region gene sequences obtained, we observed a preferential usage of VH3-23, VH3-30 and VH1-69 families, represented in orange, green and dark brown, respectively. (B) The EC_{50} values for each hmAb and its respective V_H family are indicated. Boxes highlighted in yellow represent EC_{50} values between 10-100 ng/mL, while boxes in orange and pink represent EC_{50} values of 100-1000 ng/mL and above 1000 ng/mL, respectively. Non-neutralizing hmAbs are designated as NN. (C) Each dot represents the number of V_H mutations per hmAb sequence in the VH3-23 (orange), VH3-30 (green) and VH1-69 (dark brown) families. (D) The neutralization breadth on the Y-axis represents the number of serotypes neutralized by each hmAb in the VH3-23 (orange), VH3-30 (green) and VH1-69 (dark brown) families. (E) The geometric mean of the EC_{50} values against all four serotypes is represented on a log_2 scale on the Y-axis. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey's multiple comparison test to determine the statistical differences between the groups. Data obtained by Dr. Crowe's group and analyzed by D. Andrade

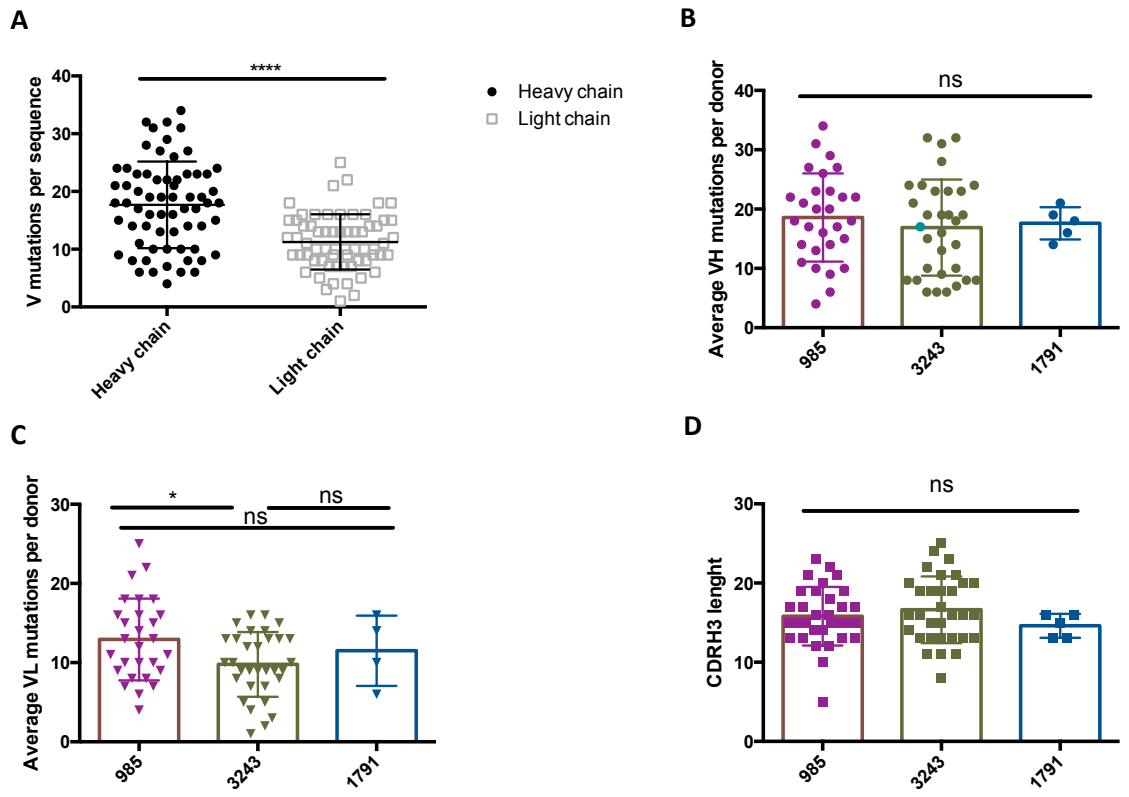


Figure 4.6 Human mAbs derived from MBCs after secondary DENV infection display high levels of SHM. (A) The number of mutations per V heavy chain sequence (black dots) versus the number of mutations per V light chain sequence (gray squares) in relation to germline sequence was compared by unpaired t-test. (B and C) The average mutations in the variable region of the heavy chain (B) and light chain (C) of hmAbs isolated from donors 985, 3243 and 1791 were compared by an ordinary one-way ANOVA with Tukey's multicomparison test. (D) The CDR3 of the heavy chain (CDRH3) of hmAbs isolated from each donor was compared by an ordinary one-way ANOVA with Tukey's multiple comparison test. *, $p<0.05$; ****, $p<0.001$; ns, not significant. Sequence data generated by Dr. Crowe's laboratory and analyzed by D. Andrade.

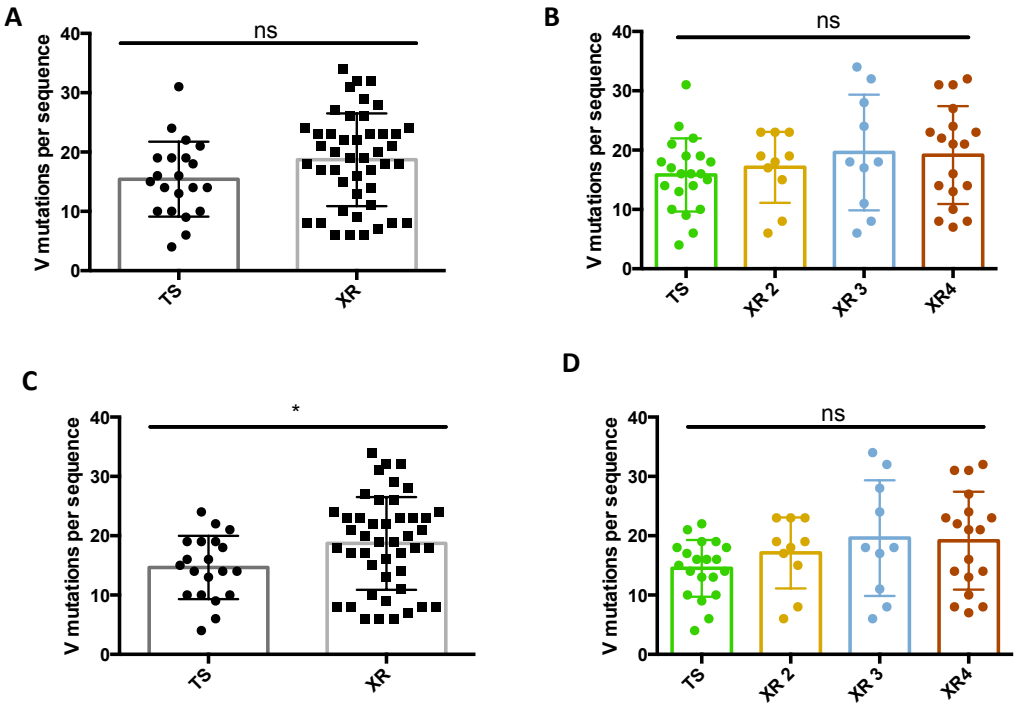


Figure 4.7. Cross-neutralizing hmAbs have higher degrees of SHM compared to type-specific hmAbs. (A) The number of mutations in the V region of the heavy chain of cross-reactive (XR) hmAbs compared to type-specific (TS) hmAbs was analyzed and indicates a trend of greater SHM in XR compred to TS hmAbs (p=0.09). Statistical analysis was performed using an unpaired t-test. (B) XR hmAbs were further classified as cross-reactive to 2 (XR 2), 3 (XR 3) or 4 (XR 4) serotypes. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test and indicated no significant differences (ns). (C) Exclusion of a type-specific outlier in the analysis of V gene mutations results in a statistical difference of XR compared to TS groups (p=0.03). (D) Exclusion of TS outlier indicates a trend towards a higher number of mutations as the neutralization breadth increases (p=0.16). Data generated by Dr. Crowe's laboratory and analyzed by D. Andrade.

985											
	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Annual PBMC sample					✔	✔	✔	X			
Infecting serotype			DENV2					DENV3			
Infection outcome			Inapparent					Symptomatic			

1791											
	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Annual PBMC sample				✔	✔	✔	✔	X			
Infecting serotype		DENV2						DENV3			
Infection outcome		Inapparent						Symptomatic			

3243											
	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Annual PBMC sample								✔	✔	X	
Infecting serotype							DENV3			DENV1	
Infection outcome							Symptomatic			Inapparent	

Table 4.4. Longitudinal PBMC samples used for sequence analysis of the B cell repertoire in natural DENV infections. Annual PBMC samples collected immediately after secondary infection with DENV3 (individuals 985 and 1791) or DENV1 (individual 3243) were used to isolate hmAbs via EBV transformation and fusion (indicated by **X**). Resulting hmAbs were sequenced by Sanger sequencing. PBMCs collected from the same individuals after their first DENV infection but prior to their second DENV infection (indicated by ✔) were sequenced by NGS to search for lineage precursors of hmAbs isolated after second DENV infection.

Participant ID	Number monoclonals	B cell number	Number of unique colonotypes
985	33	170,100	41,349
1791	7	144,800	30,826
3243	34	149,800	34,096

Table 4.5 NGS sequence analysis of the total B cell repertoire from longitudinal PBMCs was unable to identify precursor clones of broadly neutralizing antibodies. To reconstruct the lineage development of the isolated hmAbs, NGS sequencing of the immunoglobulin genes of B cells in PBMCs after previous DENV infections of the same individual was performed. From each donor, the number of B cells and unique clonotypes was determined. Data analysis and table from Dr. Crowe's laboratory.

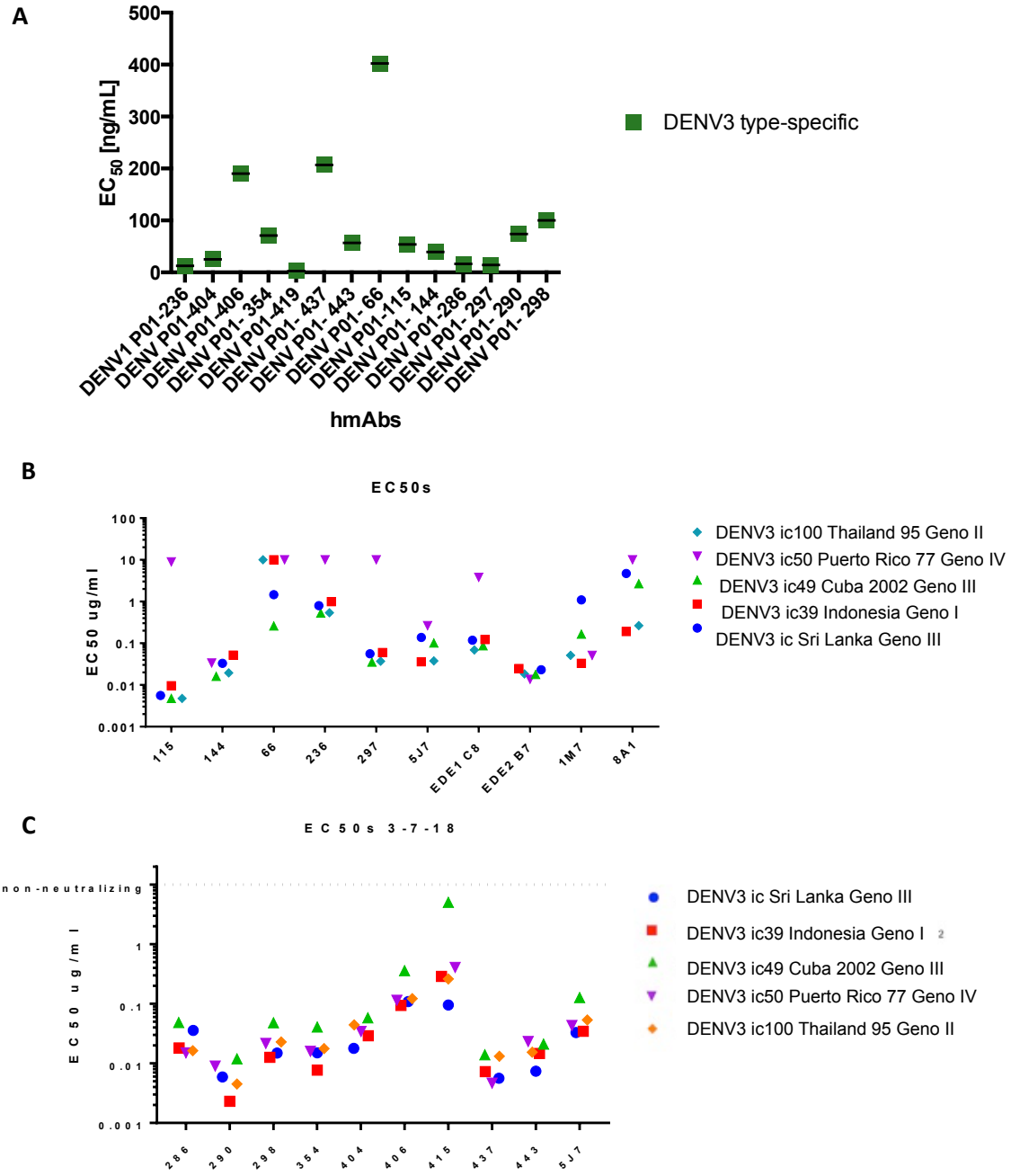


Figure 4.8 DENV3 type-specific hmAbs isolated from MBCs after secondary DENV infection have potent neutralization activity and are predominantly insensitive to genotypic variation. (A) From the panel of 74 hmAbs characterized via U937-DC-SIGN-based neutralization assay against Nicaraguan DENV1-DENV4 strains, we identified 14 hmAbs that exclusively neutralized DENV3. The EC_{50} values for each DENV3 type-specific hmAb is plotted in ng/mL. Data generated and analyzed by D. Andrade. (A). The same 14 hmAbs were tested for neutralization using a Vero microneutralization assay against an isogenic set of chimeric viruses expressing the prM/M and E proteins of DENV3 Indonesia (Genotype I), DENV3 Thailand (Genotype II), DENV3 Sri Lanka (Genotype III), DENV3 Cuba (Genotype III) and DENV3 Puerto Rico (Genotype IV). Based on the EC_{50} values calculated in μg/mL, the majority of the hmAbs efficiently neutralizes this panel of isogenic DENV3 strains. In B and C, data was generated and analyzed by Dr. Ellen Young and Dr. Ralph Baric.

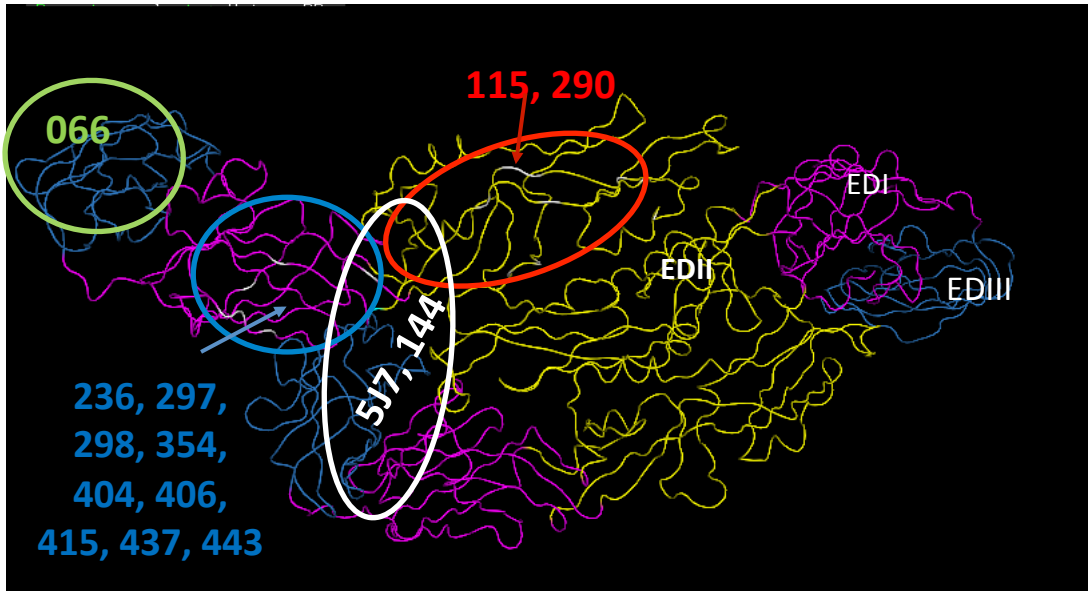


Figure 4.9 Newly identified DENV3 type-specific hmAbs bind to novel epitopes on the DENV E protein. Using chimeric viruses, the binding regions of the newly isolated hmAbs were shown to be distinct from the 5J7 epitope. The hmAbs clustered in blue (236, 297, 354, 404, 406, 415, 437 and 44) primarily bind to EDI, while the hmAbs in red (115, 290) bind mainly to EDII. The hmAb in white (144) overlaps with the 5J7 epitope but reaches further into the EDIII of the adjoining subunit, while the hmAb in green (66) primarily bind to EDIII. Figure generated by Drs. E. Young and R. Baric.

E domain	mAbs	Infection history
EDI	236,297,354,404,406,415,437,443	DENV2 ➔DENV3
EDIII	144	DENV3 ➔DENV1
EDIII	66	DENV3 ➔DENV1
EDII	115 290	DENV3 ➔DENV1 DENV2 ➔DENV3

Table 4.6 The regions bound by the novel DENV3 type-specific monoclonal antibodies may be influenced by the order of infecting serotypes. The hmAbs that bind to each of the E domains were grouped with their respective sequence of infecting serotypes.

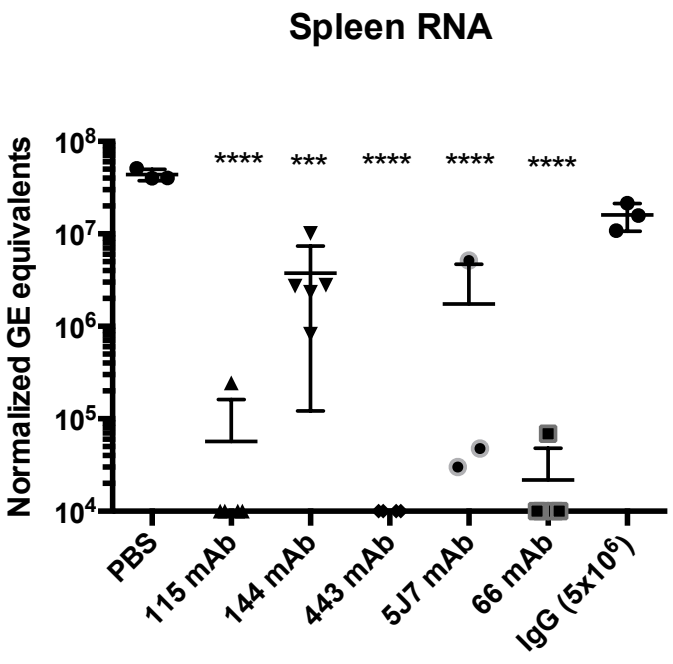


Figure 4.10 Newly identified DENV3 type-specific hmAbs are protective against DENV3 infection in a mouse model. AG129 mice 6 to 9 weeks old were inoculated with 50 µg of the newly identified DENV3 hmAbs 115 (n=5), 144 (n=5), 443 (n=5) or positive control 5J7 (n=3). Negative control groups were treated with PBS (n=3) or an isotype control antibody (IgG1) (n=3). After 24 hours, the mice were challenged with 5 x10^6 PFU DENV3 UNC 3009. Three days post-inoculation, mice euthanized and spleens were harvested. RNA was extracted, and viral load was measured by real-time quantitative reverse transcription-PCR (qRT-PCR). Viral load in log10 genome-equivalence (GE) is shown on the y-axis normalized to cellular GAPDH mRNA, and the hmAbs and control groups are indicated on the x-axis.

REFERENCES

1. Sim, S. & Hibberd, M. L. Genomic approaches for understanding dengue: insights from the virus, vector, and host. *Genome Biol.* **17,** 38 (2016).
2. Gebhard, L. G., Filomatori, C. V & Gamarnik, A. V. Functional RNA elements in the dengue virus genome. *Viruses* **3,** 1739–56 (2011).
3. Kuhn, R. J. *et al.* Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* **108,** 717–25 (2002).
4. Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc. Natl. Acad. Sci.* **100,** 6986–6991 (2003).
5. Roehrig, J. T., Bolin, R. A. & Kelly, R. G. Monoclonal Antibody Mapping of the Envelope Glycoprotein of the Dengue 2 Virus, Jamaica. *Virology* **246,** 317–328 (1998).
6. Crill, W. D. & Roehrig, J. T. Monoclonal Antibodies That Bind to Domain III of Dengue Virus E Glycoprotein Are the Most Efficient Blockers of Virus Adsorption to Vero Cells. *J. Virol.* **75,** 7769–7773 (2001).
7. Sukupolvi-Petty, S. *et al.* Type- and Subcomplex-Specific Neutralizing Antibodies against Domain III of Dengue Virus Type 2 Envelope Protein Recognize Adjacent Epitopes. *J. Virol.* **81,** 12816–12826 (2007).
8. Sukupolvi-Petty, S. *et al.* Structure and Function Analysis of Therapeutic Monoclonal Antibodies against Dengue Virus Type 2. *J. Virol.* **84,** 9227–9239 (2010).
9. Oliphant, T. *et al.* Induction of epitope-specific neutralizing antibodies against West Nile virus. *J. Virol.* **81,** 11828–39 (2007).
10. Sánchez, M. D. *et al.* The neutralizing antibody response against West Nile virus in naturally infected horses. *Virology* **359,** 336–348 (2007).
11. Wahala, W. M. P. B., Kraus, A. A., Haymore, L. B., Accavitti-Loper, M. A. & de Silva, A. M. Dengue virus neutralization by human immune sera: Role of envelope protein domain III-reactive antibody. *Virology* **392,** 103–113 (2009).
12. Wahala, W. M. P. B., Huang, C., Butrapet, S., White, L. J. & de Silva, A. M. Recombinant dengue type 2 viruses with altered e protein domain III epitopes are efficiently neutralized by human immune sera. *J. Virol.* **86,** 4019–23 (2012).
13. de Alwis, R. *et al.* Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions. *Proc. Natl. Acad. Sci.* **109,** 7439–7444 (2012).
14. Fields, B. N., Knipe, D. M. (David M. & Howley, P. M. *Fields virology*. (Wolters Kluwer Health/Lippincott Williams & Wilkins, 2007).
15. Fibriansah, G. *et al.* A potent anti-dengue human antibody preferentially recognizes the conformation of E protein monomers assembled on the virus surface. *EMBO Mol. Med.* **6,** n/a-n/a (2014).
16. Fibriansah, G. *et al.* A highly potent human antibody neutralizes dengue virus serotype 3 by binding across three surface proteins. *Nat. Commun.* **6,** 6341 (2015).
17. Fibriansah, G. *et al.* Cryo-EM structure of an antibody that neutralizes dengue virus type 2 by locking E protein dimers. *Science (80-.).* **349,** 88–91 (2015).

18. Gallichotte, E. N. *et al.* Human dengue virus serotype 2 neutralizing antibodies target two distinct quaternary epitopes. *PLoS Pathog.* **14,** e1006934 (2018).
19. Andrade, D. V *et al.* Analysis of Individuals from a Dengue-Endemic Region Helps Define the Footprint and Repertoire of Antibodies Targeting Dengue Virus 3 Type-Specific Epitopes. *MBio* **8,** (2017).
20. Corbett, K. S. *et al.* Preexisting Neutralizing Antibody Responses Distinguish Clinically Inapparent and Apparent Dengue Virus Infections in a Sri Lankan Pediatric Cohort. *J. Infect. Dis.* **211,** 590–599 (2015).
21. Patel, B. *et al.* Dissecting the human serum antibody response to secondary dengue virus infections. *PLoS Negl. Trop. Dis.* **11,** e0005554 (2017).
22. Tsai, W.-Y. *et al.* High-Avidity and Potently Neutralizing Cross-Reactive Human Monoclonal Antibodies Derived from Secondary Dengue Virus Infection. *J. Virol.* **87,** 12562–12575 (2013).
23. Smith, S. A. *et al.* Persistence of circulating memory B cell clones with potential for dengue virus disease enhancement for decades following infection. *J. Virol.* **86,** 2665–75 (2012).
24. Priyamvada, L. *et al.* B Cell Responses during Secondary Dengue Virus Infection Are Dominated by Highly Cross-Reactive, Memory-Derived Plasmablasts. *J. Virol.* **90,** 5574–85 (2016).
25. Dejnirattisai, W. *et al.* Cross-Reacting Antibodies Enhance Dengue Virus Infection in Humans. *Science (80-.).* **328,** 745–748 (2010).
26. Olkowski, S. *et al.* Reduced Risk of Disease During Postsecondary Dengue Virus Infections. *J. Infect. Dis.* **208,** 1026–1033 (2013).
27. de Alwis, R. *et al.* In-Depth Analysis of the Antibody Response of Individuals Exposed to Primary Dengue Virus Infection. *PLoS Negl. Trop. Dis.* **5,** e1188 (2011).
28. Beltramello, M. *et al.* The Human Immune Response to Dengue Virus Is Dominated by Highly Cross-Reactive Antibodies Endowed with Neutralizing and Enhancing Activity. *Cell Host Microbe* **8,** 271–283 (2010).
29. Lai, C.-Y. *et al.* Antibodies to Envelope Glycoprotein of Dengue Virus during the Natural Course of Infection Are Predominantly Cross-Reactive and Recognize Epitopes Containing Highly Conserved Residues at the Fusion Loop of Domain II. *J. Virol.* **82,** 6631–6643 (2008).
30. Lin, H.-E. *et al.* Analysis of Epitopes on Dengue Virus Envelope Protein Recognized by Monoclonal Antibodies and Polyclonal Human Sera by a High Throughput Assay. *PLoS Negl. Trop. Dis.* **6,** e1447 (2012).
31. Halstead, S. B. & O'Rourke, E. J. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. *J. Exp. Med.* **146,** 201–17 (1977).
32. Boonnak, K. *et al.* Role of dendritic cells in antibody-dependent enhancement of dengue virus infection. *J. Virol.* **82,** 3939–51 (2008).
33. Halstead, S. B. Neutralization and antibody-dependent enhancement of dengue viruses. *Adv. Virus Res.* **60,** 421–67 (2003).
34. de Alwis, R. *et al.* Dengue Viruses Are Enhanced by Distinct Populations of Serotype Cross-Reactive Antibodies in Human Immune Sera. *PLoS Pathog.* **10,** e1004386 (2014).

35. Williams, K. L. *et al.* Therapeutic Efficacy of Antibodies Lacking FcγR against Lethal Dengue Virus Infection Is Due to Neutralizing Potency and Blocking of Enhancing Antibodies. *PLoS Pathog.* **9,** e1003157 (2013).
36. Katzelnick, L. C. *et al.* Antibody-dependent enhancement of severe dengue disease in humans. *Science* **358,** 929–932 (2017).
37. Halstead, S. B. In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. *J. Infect. Dis.* **140,** 527–33 (1979).
38. Smith, S. A. *et al.* The potent and broadly neutralizing human dengue virus-specific monoclonal antibody 1C19 reveals a unique cross-reactive epitope on the bc loop of domain II of the envelope protein. *MBio* **4,** e00873-13 (2013).
39. Dejnirattisai, W. *et al.* A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus. *Nat. Immunol.* **16,** 170–177 (2015).
40. Rouvinski, A. *et al.* Recognition determinants of broadly neutralizing human antibodies against dengue viruses. *Nature* **520,** 109–113 (2015).
41. Wu, N. C. *et al.* In vitro evolution of an influenza broadly neutralizing antibody is modulated by hemagglutinin receptor specificity. *Nat. Commun.* **8,** 15371 (2017).
42. Ekiert, D. C. *et al.* Antibody Recognition of a Highly Conserved Influenza Virus Epitope. *Science (80-.).* **324,** 246–251 (2009).
43. Dreyfus, C., Ekiert, D. C. & Wilson, I. A. Structure of a classical broadly neutralizing stem antibody in complex with a pandemic H2 influenza virus hemagglutinin. *J. Virol.* **87,** 7149–54 (2013).
44. Wrammert, J. *et al.* Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. *Nature* **453,** 667–671 (2008).
45. Glanville, J. *et al.* Naive antibody gene-segment frequencies are heritable and unaltered by chronic lymphocyte ablation. *Proc. Natl. Acad. Sci.* **108,** 20066–20071 (2011).
46. Walker, L. M. *et al.* Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature* **477,** 466–70 (2011).
47. Wu, X. *et al.* Rational Design of Envelope Identifies Broadly Neutralizing Human Monoclonal Antibodies to HIV-1. *Science (80-.).* **329,** 856–861 (2010).
48. Walker, L. M. *et al.* Broad and Potent Neutralizing Antibodies from an African Donor Reveal a New HIV-1 Vaccine Target. *Science (80-.).* **326,** 285–289 (2009).
49. Wu, X. *et al.* Focused Evolution of HIV-1 Neutralizing Antibodies Revealed by Structures and Deep Sequencing. *Science (80-.).* **333,** 1593–1602 (2011).
50. Wrammert, J. *et al.* Rapid and Massive Virus-Specific Plasmablast Responses during Acute Dengue Virus Infection in Humans. *J. Virol.* **86,** 2911–2918 (2012).
51. Garcia-Bates, T. M. *et al.* Association between Magnitude of the Virus-Specific Plasmablast Response and Disease Severity in Dengue Patients. *J. Immunol.* **190,** 80–87 (2013).
52. Xu, M. *et al.* Plasmablasts Generated during Repeated Dengue Infection Are Virus Glycoprotein-Specific and Bind to Multiple Virus Serotypes. *J. Immunol.* **189,** 5877–5885 (2012).
53. Parameswaran, P. *et al.* Convergent Antibody Signatures in Human Dengue. *Cell Host Microbe* **13,** 691–700 (2013).
54. Kuan, G. *et al.* The Nicaraguan Pediatric Dengue Cohort Study: Study Design,

Methods, Use of Information Technology, and Extension to Other Infectious Diseases. *Am. J. Epidemiol.* **170,** 120–129 (2009).
55. Mattia, K. *et al.* Dengue Reporter Virus Particles for Measuring Neutralizing Antibodies against Each of the Four Dengue Serotypes. *PLoS One* **6,** e27252 (2011).
56. Montoya, M. *et al.* Symptomatic Versus Inapparent Outcome in Repeat Dengue Virus Infections Is Influenced by the Time Interval between Infections and Study Year. *PLoS Negl. Trop. Dis.* **7,** e2357 (2013).
57. Gilchuk, P. *et al.* Multifunctional Pan-ebolavirus Antibody Recognizes a Site of Broad Vulnerability on the Ebolavirus Glycoprotein. *Immunity* **49,** 363–374.e10 (2018).
58. Smith, S. A. *et al.* Isolation of dengue virus-specific memory B cells with live virus antigen from human subjects following natural infection reveals the presence of diverse novel functional groups of antibody clones. *J. Virol.* **88,** 12233–41 (2014).
59. Posadas-Mondragón, A. *et al.* Indices of anti-dengue immunoglobulin G subclasses in adult Mexican patients with febrile and hemorrhagic dengue in the acute phase. *Microbiol. Immunol.* **61,** 433–441 (2017).
60. Broliden, P. A., Morfeldt-Månsson, L., Rosen, J., Jondal, M. & Wahren, B. Fine specificity of IgG subclass response to group antigens in HIV-1-infected patients. *Clin. Exp. Immunol.* **76,** 216–21 (1989).
61. Banerjee, K. *et al.* IgG Subclass Profiles in Infected HIV Type 1 Controllers and Chronic Progressors and in Uninfected Recipients of Env Vaccines. *AIDS Res. Hum. Retroviruses* **26,** 445–458 (2010).
62. Netski, D. M. *et al.* Humoral Immune Response in Acute Hepatitis C Virus Infection. *Clin. Infect. Dis.* **41,** 667–675 (2005).
63. Dejnirattisai, W. *et al.* Cross-Reacting Antibodies Enhance Dengue Virus Infection in Humans. *Science (80-.).* **328,** 745–748 (2010).
64. Rajewsky, K. Clonal selection and learning in the antibody system. *Nature* **381,** 751–758 (1996).
65. Appanna, R. *et al.* Plasmablasts During Acute Dengue Infection Represent a Small Subset of a Broader Virus-specific Memory B Cell Pool. *EBioMedicine* **12,** 178–188 (2016).
66. Yu, L. & Guan, Y. Immunologic Basis for Long HCDR3s in Broadly Neutralizing Antibodies Against HIV-1. *Front. Immunol.* **5,** 250 (2014).
67. Wu, T. Te, Johnson, G. & Kabat, E. A. Length distribution of CDRH3 in antibodies. *Proteins Struct. Funct. Genet.* **16,** 1–7 (1993).
68. Messer, W. B. *et al.* Development and characterization of a reverse genetic system for studying dengue virus serotype 3 strain variation and neutralization. *PLoS Negl. Trop. Dis.* **6,** e1486 (2012).
69. Wahala, W. M. P. B. *et al.* Natural Strain Variation and Antibody Neutralization of Dengue Serotype 3 Viruses. *PLoS Pathog.* **6,** e1000821 (2010).
70. Brien, J. D. *et al.* Genotype-Specific Neutralization and Protection by Antibodies against Dengue Virus Type 3. *J. Virol.* **84,** 10630–10643 (2010).
71. Zulueta, A. *et al.* Amino acid changes in the recombinant Dengue 3 Envelope domain III determine its antigenicity and immunogenicity in mice. *Virus Res.* **121,** 65–73 (2006).

72. Gallichotte, E. N. *et al.* Epitope Addition and Ablation via Manipulation of a Dengue Virus Serotype 1 Infectious Clone. *mSphere* **2,** (2017).
73. Messer, W. B. *et al.* Functional Transplant of a Dengue Virus Serotype 3 (DENV3)-Specific Human Monoclonal Antibody Epitope into DENV1. *J. Virol.* **90,** 5090–5097 (2016).
74. Guy, B. *et al.* From research to phase III: Preclinical, industrial and clinical development of the Sanofi Pasteur tetravalent dengue vaccine. *Vaccine* **29,** 7229–7241 (2011).
75. Matheus, S. *et al.* Discrimination between Primary and Secondary Dengue Virus Infection by an Immunoglobulin G Avidity Test Using a Single Acute-Phase Serum Sample. *J. Clin. Microbiol.* **43,** 2793–2797 (2005).
76. Tsai, W.-Y., Lin, H.-E. & Wang, W.-K. Complexity of Human Antibody Response to Dengue Virus: Implication for Vaccine Development. *Front. Microbiol.* **8,** 1372 (2017).
77. Zompi, S., Montoya, M., Pohl, M. O., Balmaseda, A. & Harris, E. Dominant Cross-Reactive B Cell Response during Secondary Acute Dengue Virus Infection in Humans. *PLoS Negl. Trop. Dis.* **6,** e1568 (2012).
78. Nivarthi, U. K. *et al.* Mapping the Human Memory B Cell and Serum Neutralizing Antibody Responses to Dengue Virus Serotype 4 Infection and Vaccination. *J. Virol.* **91,** e02041-16 (2017).
79. Bernasconi, N. L., Traggiai, E. & Lanzavecchia, A. Maintenance of Serological Memory by Polyclonal Activation of Human Memory B Cells. *Science (80-.).* **298,** 2199–2202 (2002).
80. Smith, S. A. & Crowe, J. E. Use of Human Hybridoma Technology To Isolate Human Monoclonal Antibodies. *Microbiol. Spectr.* **3,** AID-0027-2014 (2015).
81. Friberg, H. *et al.* Analysis of human monoclonal antibodies generated by dengue virus-specific memory B cells. *Viral Immunol.* **25,** 348–59 (2012).
82. Mathew, A. *et al.* B-Cell Responses During Primary and Secondary Dengue Virus Infections in Humans. *J. Infect. Dis.* **204,** 1514–1522 (2011).
83. Xu, M. *et al.* Plasmablasts generated during repeated dengue infection are virus glycoprotein-specific and bind to multiple virus serotypes. *J. Immunol.* **189,** 5877–85 (2012).
84. Ditse, Z. *et al.* HIV-1 Subtype C-Infected Children with Exceptional Neutralization Breadth Exhibit Polyclonal Responses Targeting Known Epitopes. *J. Virol.* **92,** e00878-18 (2018).
85. Goo, L., Chohan, V., Nduati, R. & Overbaugh, J. Early development of broadly neutralizing antibodies in HIV-1–infected infants. *Nat. Med.* **20,** 655–658 (2014).
86. Muenchhoff, M. *et al.* Nonprogressing HIV-infected children share fundamental immunological features of nonpathogenic SIV infection. *Sci. Transl. Med.* **8,** 358ra125-358ra125 (2016).
87. Simonich, C. A. *et al.* HIV-1 Neutralizing Antibodies with Limited Hypermutation from an Infant. *Cell* **166,** 77–87 (2016).
88. Goo, L., VanBlargan, L. A., Dowd, K. A., Diamond, M. S. & Pierson, T. C. A single mutation in the envelope protein modulates flavivirus antigenicity, stability, and pathogenesis. *PLOS Pathog.* **13,** e1006178 (2017).
89. Dowd, K. A., DeMaso, C. R. & Pierson, T. C. Genotypic Differences in Dengue

Virus Neutralization Are Explained by a Single Amino Acid Mutation That Modulates Virus Breathing. *MBio* **6,** e01559-15 (2015).
90. Lewis, G. K. Role of Fc-mediated antibody function in protective immunity against HIV-1. *Immunology* **142,** 46–57 (2014).
91. Schmaljohn, A. & Lewis, G. K. Cell-targeting antibodies in immunity to Ebola. *Pathog. Dis.* **74,** ftw021 (2016).
92. Schmaljohn, A. L. Protective antiviral antibodies that lack neutralizing activity: precedents and evolution of concepts. *Curr. HIV Res.* **11,** 345–53 (2013).
93. Bootz, A. *et al.* Protective capacity of neutralizing and non-neutralizing antibodies against glycoprotein B of cytomegalovirus. *PLoS Pathog.* **13,** e1006601 (2017).
94. Tiller, T. *et al.* Autoreactivity in Human IgG+ Memory B Cells. *Immunity* **26,** 205–213 (2007).
95. Klein, F. *et al.* Somatic Mutations of the Immunoglobulin Framework Are Generally Required for Broad and Potent HIV-1 Neutralization. *Cell* **153,** 126–138 (2013).
96. Scheid, J. F. *et al.* Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. *Nature* **458,** 636–640 (2009).
97. Sok, D. *et al.* The Effects of Somatic Hypermutation on Neutralization and Binding in the PGT121 Family of Broadly Neutralizing HIV Antibodies. *PLoS Pathog.* **9,** e1003754 (2013).
98. Zhou, T., Lynch, R. M., Mascola, J. R. & Kwong, P. D. Structural Repertoire of HIV-1-Neutralizing Antibodies Targeting the CD4 Supersite in 14 Donors. *Cell* **161,** 1280–1292 (2015).
99. Magnani, D. M. *et al.* A human inferred germline antibody binds to an immunodominant epitope and neutralizes Zika virus. *PLoS Negl. Trop. Dis.* **11,** e0005655 (2017).
100. Kalinke, U. *et al.* The role of somatic mutation in the generation of the protective humoral immune response against vesicular stomatitis virus. *Immunity* **5,** 639–52 (1996).
101. Thornburg, N. J. *et al.* H7N9 influenza virus neutralizing antibodies that possess few somatic mutations. *J. Clin. Invest.* **126,** 1482–1494 (2016).
102. Simonich, C. A. *et al.* HIV-1 Neutralizing Antibodies with Limited Hypermutation from an Infant. *Cell* **166,** 77–87 (2016).
103. Briney, B., Le, K., Zhu, J. & Burton, D. R. Clonify: unseeded antibody lineage assignment from next-generation sequencing data. *Sci. Rep.* **6,** 23901 (2016).
104. Godoy-Lozano, E. E. *et al.* Lower IgG somatic hypermutation rates during acute dengue virus infection is compatible with a germinal center-independent B cell response. *Genome Med.* **8,** 23 (2016).
105. Avnir, Y. *et al.* Molecular Signatures of Hemagglutinin Stem-Directed Heterosubtypic Human Neutralizing Antibodies against Influenza A Viruses. *PLoS Pathog.* **10,** e1004103 (2014).
106. Chan, C. H., Hadlock, K. G., Foung, S. K. & Levy, S. V(H)1-69 gene is preferentially used by hepatitis C virus-associated B cell lymphomas and by normal B cells responding to the E2 viral antigen. *Blood* **97,** 1023–6 (2001).
107. Magnani, D. M. *et al.* Potent Plasmablast-Derived Antibodies Elicited by the National Institutes of Health Dengue Vaccine. *J. Virol.* **91,** (2017).

108. Arnaout, R. *et al.* High-Resolution Description of Antibody Heavy-Chain Repertoires in Humans. *PLoS One* **6,** e22365 (2011).
109. Robbiani, D. F. *et al.* Recurrent Potent Human Neutralizing Antibodies to Zika Virus in Brazil and Mexico. *Cell* **169,** 597–609.e11 (2017).
110. Wu, Y.-C. *et al.* High-throughput immunoglobulin repertoire analysis distinguishes between human IgM memory and switched memory B-cell populations. *Blood* **116,** 1070–1078 (2010).
111. Guo, K. *et al.* Immunoglobulin VH gene diversity and somatic hypermutation during SIV infection of rhesus macaques. *Immunogenetics* **67,** 355–70 (2015).
112. Racanelli, V. *et al.* Antibody Vh Repertoire Differences between Resolving and Chronically Evolving Hepatitis C Virus Infections. *PLoS One* **6,** e25606 (2011).
113. Chin, S. T. *et al.* Comparative study of IgA V_H 3 gene usage in healthy TST^- and TST^+ population exposed to tuberculosis: deep sequencing analysis. *Immunology* **144,** 302–311 (2015).
114. Teoh, E. P. *et al.* The Structural Basis for Serotype-Specific Neutralization of Dengue Virus by a Human Antibody. *Sci. Transl. Med.* **4,** 139ra83-139ra83 (2012).
115. Gallichotte, E. N. *et al.* Genetic Variation between Dengue Virus Type 4 Strains Impacts Human Antibody Binding and Neutralization. *Cell Rep.* **25,** (2018).
116. Gallichotte, E. N. *et al.* A new quaternary structure epitope on dengue virus serotype 2 is the target of durable type-specific neutralizing antibodies. *MBio* **6,** e01461-15 (2015).
117. *Dengue haemorrhagic fever Diagnosis, treatment, prevention and control SECOND EDITION Contents*. (1997).
118. Lanciotti, R. S., Calisher, C. H., Gubler, D. J., Chang, G. J. & Vorndam, A. V. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J. Clin. Microbiol.* **30,** 545–51 (1992).
119. Balmaseda, A., Sandoval, E., Pérez, L., Gutiérrez, C. M. & Harris, E. Application of molecular typing techniques in the 1998 dengue epidemic in Nicaragua. *Am. J. Trop. Med. Hyg.* **61,** 893–7 (1999).
120. Balmaseda, A. *et al.* Diagnosis of dengue virus infection by detection of specific immunoglobulin M (IgM) and IgA antibodies in serum and saliva. *Clin. Diagn. Lab. Immunol.* **10,** 317–22 (2003).
121. Balmaseda, A. *et al.* High seroprevalence of antibodies against dengue virus in a prospective study of schoolchildren in Managua, Nicaragua. *Trop. Med. Int. Health* **11,** 935–42 (2006).
122. Katzelnick, L. C., Montoya, M., Gresh, L., Balmaseda, A. & Harris, E. Neutralizing antibody titers against dengue virus correlate with protection from symptomatic infection in a longitudinal cohort. *Proc. Natl. Acad. Sci.* **113,** 728–733 (2016).
123. Brochet, X., Lefranc, M.-P. & Giudicelli, V. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. *Nucleic Acids Res.* **36,** W503–W508 (2008).
124. Giudicelli, V., Brochet, X. & Lefranc, M.-P. IMGT/V-QUEST: IMGT Standardized Analysis of the Immunoglobulin (IG) and T Cell Receptor (TR) Nucleotide Sequences. *Cold Spring Harb. Protoc.* **2011,** pdb.prot5633-pdb.prot5633 (2011).

125. Kraus, A. A., Messer, W., Haymore, L. B. & de Silva, A. M. Comparison of Plaque- and Flow Cytometry-Based Methods for Measuring Dengue Virus Neutralization. *J. Clin. Microbiol.* **45,** 3777–3780 (2007).
126. van den Broek, M. F., Müller, U., Huang, S., Zinkernagel, R. M. & Aguet, M. Immune defence in mice lacking type I and/or type II interferon receptors. *Immunol. Rev.* **148,** 5–18 (1995).
127. Waggoner, J. J. *et al.* Viremia and Clinical Presentation in Nicaraguan Patients Infected With Zika Virus, Chikungunya Virus, and Dengue Virus. *Clin. Infect. Dis.* **63,** 1584–1590 (2016).

CHAPTER 5

CONTRIBUTION OF TYPE-SPECIFIC VERSUS CROSS-REACTIVE ANTIBODIES TO POLYCLONAL NEUTRALIZATION FOLLOWING REPEAT NATURAL DENGUE VIRUS (DENV) INFECTIONS

Summary

The four dengue virus (DENV) serotypes can cause repeat infections in a single individual, each time with a new serotype. While primary infection generates life-long neutralizing antibodies to the infecting serotype, secondary DENV infections elicit cross-neutralizing antibodies to two or more serotypes. As recent vaccine trial results and natural DENV infection studies highlight, the quality of the neutralizing response, as well as the quantity, is critical for protection and is a complex matter that needs further investigation. One important question to be addressed is the contribution of type-specific and cross-reactive antibody populations to polyclonal neutralization after each sequential DENV infection. Antibody depletion methods developed previously provide a tool to specifically evaluate the proportion of these antibody populations in polyclonal sera after each repeat DENV infection. In this chapter, we analyzed sera from two subjects enrolled in a long-term prospective pediatric cohort study in Nicaragua, which allowed us to longitudinally evaluate the neutralizing antibody populations over each sequential infection in the same individual. We found that after a first infection, a substantial proportion of type-specific antibodies contribute to the neutralizing response, and after a second infection, the cross-reactive response dominates, although type-specific antibodies to both infecting serotypes can be detected. Interestingly, in one subject, the proportion of type-specific neutralizing antibodies to the first infecting serotype increased in a subsequent inapparent infection, suggesting a homotypic reinfection. This unique set of samples from individuals with repeat infections in a dengue-endemic area enables dissecting the anti-DENV serological responses at the individual level and in an epidemiological context. Altogether, this study underscores the importance of methods that allow detection of non-typical DENV infections, such as homotypic reinfections and heterologous boosts that could be erroneously interpreted as heterologous infections with traditional neutralization assay methods. Further studies with a larger number of samples from dengue-endemic areas are pivotal for the assessment of the frequency of repeat heterotypic infections, homotypic reinfections and heterologous boosts, with impact on models of disease transmission and ultimately vaccine design.

INTRODUCTION

The four antigenically related dengue virus (DENV) serotypes infect up to 390 million people worldwide[1]. Although the majority of cases manifest as inapparent infection, around 25% of infections cause acute febrile illness and in some cases, may progress to the severe forms of disease, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)[2,3]. Following primary DENV infection, an antibody response that is specific to the infecting serotype (type-specific) is elicited and potently neutralizes the homologous serotype. This antibody population is thought to be life-long and believed to confer protection against reinfection with the same serotype[4]. In addition to type-specific antibodies, populations of cross-reactive antibodies are also generated after primary DENV infection, which include serotype cross-neutralizing and serotype cross-reactive non-neutralizing antibodies[4]. At the individual level, the serotype cross-neutralizing antibodies confer protection against heterologous symptomatic infection for about 1.5-2 years and against severe disease for up to 2.5 years[5–7]. Low levels of cross-reactive antibodies may facilitate virus entry into target cells expressing Fc receptors and consequently enhance viral replication[8]. This mechanism, called antibody-dependent enhancement (ADE) has been associated with severe disease in dengue-endemic areas[9,10]. In addition to natural infection, ADE may increase risk of severe disease in dengue-naïve recipients of the tetravalent DENV vaccine CYD-TDV, recently licensed as Dengvaxia[11].

Following second heterologous DENV infection, type-specific antibodies to the infecting serotype are elicited, alongside cross-reactive antibodies that may neutralize serotypes not yet encountered. Compared to cross-reactive antibodies elicited after primary infection, this cross-reactive antibody population generated in secondary infection has higher avidity and neutralization potential both in polyclonal sera[12] and at the monoclonal level[13–15]. Cross-neutralizing antibodies elicited in secondary DENV infection are also thought to be more protective than the ones elicited after first infection, as indicated by the low frequency of hospitalized cases during third and fourth DENV infection[6,16–18]. The role of cross-neutralizing antibodies in protection has been shown, and a recent study in Nicaraguan individuals concluded that pre-infection neutralizing antibody titers to the second infecting serotype, as opposed to the first infecting serotype, reduces probability of secondary symptomatic infection[5,7,18–21]. The impact of previous DENV immunity on the quality of the antibody population elicited in a subsequent infection has also been evidenced in a live attenuated tetravalent dengue vaccine efficacy trial, which indicated higher efficacy in the dengue-immune group compared to the DENV naïve group[22–25].

Given that cross-reactive antibodies may be enhancing or protective, understanding the magnitude of this response over sequential DENV infections is important for a comprehensive understanding of the serological response to DENV infection. In fact, a number of recent studies in dengue- endemic regions challenge the dogma in the field that after primary infection, cross-reactive antibody titers decay over time[26]. Analysis of the neutralizing antibody trajectory of children enrolled in a dengue cohort study in

Nicaragua revealed that the magnitude of the neutralizing antibody titers actually increased between primary infection and subsequent infection. Additionally, the breadth of the response was expanded[19]. In a hospital study in the same region, the magnitude of the neutralizing titers was shown to decline between 2 weeks and 6 months post-infection, but a rise between 6 and 18 months was reported for a subset of the individuals studied[27]. Similarly, Andrade and colleagues observed boosts of heterologous titers in samples collected post-primary DENV3 infection[28] in the hospital-based study in Nicaragua. In a dengue-endemic area in Asia, boosts of neutralizing titers have also been reported[29]. Collectively, these studies indicate increases in the breadth and magnitude of the antibody response post-primary DENV infection. These observations could be attributed to reexposure to DENV of the same infecting serotype Notably, RT-PCR-confirmed re-infections have been documented in Nicaragua[30], which is also consistent with epidemiological data showing the predominance of a particular serotype for multiple years at a time in this region[31]. Furthermore, an epidemic of severe DENV2 in a population with pre-existing neutralizing antibodies to DENV2 has been reported in Peru[32].

In addition to homotypic reinfections, heterotypic boosting may also maintain the neutralizing antibody titers after primary infection[19,28,29]. As a number of studies in dengue-endemic areas show evidence of boosts of neutralizing titers, it becomes relevant to distinguish events associated with heterotypic repeat infection, homotypic reinfection or heterotypic boosting. However, given the cross-reactivity between serotypes, traditional methods that measure the rise in neutralizing titers between paired samples are insufficient to infer the exact history of infections. Depletion of antibody subpopulations enables dissecting the contribution of type-specific versus cross-reactive antibodies to polyclonal neutralization, and such methods may indicate what populations of antibodies are elicited in heterologous boosts or homotypic reinfections. Using this method, Patel and colleagues studied serum samples from travelers who experienced secondary DENV infection and showed that some people had a predominant serotype cross-neutralizing response, whereas others presented a mixture of type-specific and cross-reactive neutralizing antibody populations[33]. Of note, these convalescent samples were collected at a single time-point and varied across the individuals. Using the same depletion strategy, samples of individuals enrolled in a pediatric cohort study Nicaragua who experienced two sequential infections were analyzed. In these individuals, a mixture of type-specific and cross-reactive neutralizing to both the first and second infecting serotypes was observed[33]. While this study provided important evidence of the role of type-specific and cross-reactive antibody populations in polyclonal neutralization after secondary infection, it lacked the capacity to evaluate in the same individual how these antibody population dynamics shift after each repeat infection.

In this chapter, we depleted plasma samples of two individuals who experienced repeat DENV infections and determined how the contribution of type-specific versus cross-reactive antibodies shifts after each subsequent infection. We found that after a first infection (symptomatic or inapparent), type-specific antibodies to the homologous

serotype drive most of the neutralization. In one individual, no type-specific antibodies to the second infecting serotype were elicited, whereas a small population was seen for the third infection serotype. Interestingly, in another individual, we observed an increase in the proportion of the type-specific population to the first infecting serotype upon a subsequent inapparent infection, which suggests a homotypic reinfection. Taken together, our findings illustrate the use of depletion of antibody populations as a method to accurately capture reexposures in dengue-endemic areas that are otherwise classified as heterotypic inapparent infections. A larger number of longitudinal samples are required to make more solid conclusions, and the impact of DENV reexposure on the long-term immunity should be further investigated.

RESULTS

DENV infection history of selected Pediatric Dengue Cohort Study participants

We selected participants enrolled in the Nicaraguan Pediatric Dengue Cohort Study (PDCS; 2004 to present), a community-based prospective study of approximately 3,700 children 2 to 14 years of age in Managua, Nicaragua[34]. Healthy blood samples collected annually were used to reconstruct the DENV immune history of the participants based on a Reporter Viral Particle (RVP) flow cytomety-based DENV neutralization assay, as previously described[6,35]. The infecting serotypes in the inapparent infections were identified according to the highest fold-change in the NT_{50} values for each serotype, as highlighted on **Table 5.1**, with the assumption widely held to date that individuals do not experience homotypic infections. According to these guidelines, the infection history for individual 836 is characterized by inapparent DENV2 infection in 2004-5, followed by inapparent second infection with DENV4 in 2006-7 and a subsequent symptomatic DENV3 infection in 2009-10 (**Table 5.1**). The infection history for individual 3112 is characterized by symptomatic primary DENV1 infection in 2004-5, followed by inapparent DENV2 infection in the year right after (2005-6), a third infection with DENV3 in 2010-11 and potentially a subsequent inapparent infection with DENV4 (2011-12). This final infection is not convincing, however, since the neutralizing antibody titers to the other 3 go down rather than up from 2011 to 2012.

DENV-specific binding to the infecting serotypes after removal of homologous and heterologous antibody populations in subject 3112

To characterize the antibody populations that drive polyclonal neutralization after each sequential DENV infection, our collaborators in Dr. Aravinda de Silva's laboratory at the University of North Carolina at Chapel Hill depleted plasma samples from longitudinal annual samples from the individuals of interest using polystyrene beads coated with BSA (control), purified DENV2 or a mixture of purified DENV1, DENV3, and DENV4. ELISAs were performed to assess successful depletion of antibodies and to measure binding activity to each of the DENV serotypes. Depletion of primary DENV1 sera with heterologous DENV2 is expected to remove all cross-reactive antibodies, while depletion of the same sera with beads coated with DENV1,3,4 is expected to remove both type-specific and cross-reactive antibodies. As indicated in **Figure 5.1a**, depletion of cross-reactive antibodies in the post-primary DENV1 sera (3112.2) did not change binding activity to DENV1 compared to the control sera. The same sample depleted of both type-specific and cross-reactive antibodies loses binding compared to the control group. These data indicate that type-specific antibodies account for the majority of the binding to DENV1, the infecting serotype causing the first infection. However, depletion of primary DENV1 sera with heterologous serotypes caused complete loss of binding to DENV2, 3 and 4 (**Figures 5.1b-d**). Based on the criteria of a ≥4-fold increase in neutralizing antibody titers (**Table 5.1**), this individual experienced a subsequent inapparent DENV2 infection (3112.3). Depletion of the heterologous serotypes caused loss of binding to DENV2 (**Figure 5.1b**), indicating that cross-reactive antibodies contribute to binding to DENV2 after secondary infection. After a third infection with

DENV3 (3112.8), depletion of heterologous antibodies (DENV2) did not abrogate binding to DENV3, indicating a predominant type-specific antibody binding response (**Figure 5.1c**). Finally, even though RVP data indicates a potential fourth inapparent infection with DENV4 (**Table 5.1**), the binding levels of the control depleted sample to DENV4 were very comparable to the limit of detection (**Figure 5.1d**).

Shifts in neutralizing antibody titers after depletion of homologous and heterologous antibody populations in individual 3112

Next, we measured the contribution of DENV type-specific and cross-reactive antibodies to polyclonal neutralization after each sequential DENV infection. Here, we used a U937-DC-SIGN-based neutralization assay with Nicaraguan DENV1-4 viruses. For each time-point, plasma samples were depleted with beads coated with BSA (left column), DENV2 (middle clolumn) or DENV1,3,4 (right column**)**. After primary infection (3112.2), depletion with DENV1,3,4 results in complete abrogation of DENV1 neutralization, as expected since both homologous and heterologous antibodies were depleted (**Figure 5.2c**). Conversely, depletion with only the heterologous serotype (DENV2) causes reduction in the NT_{50} (**Figure 5.2b**) that is about half of the NT_{50} value in the BSA-depleted sample (**Figure 5.2a**). This indicates that most of the neutralization is driven by DENV1 type-specific neutralizing antibodies. In the subsequent inapparent infection (3112.3), depletion of the heterologous antibodies (DENV1,3,4 coated beads) caused loss of neutralization to DENV2 (**Figure 5.2e**), indicating that cross-reactive antibodies primarily drive the neutralization to secondary DENV2 infection. After a third inapparent infection (3112.8), depletion of cross-reactive antibodies (DENV2-coated beads) does not completely abrogate neutralization of DENV3 (**Figure 5.2h**), suggesting that a population type-specific antibodies to the third infecting serotype is elicited. In a subsequent potential inapparent infection with DENV4, depletion of cross-reactive antibodies caused loss of neutralization, indicating the response is entirely cross-reactive (**Figure 5.2k**). After the fourth infection, modest NT_{50} values specific to DENV4 are seen after depletion of heterologous antibodies, which may suggest a type-specific population (**Figure 5.2m-5.2o**).

Predominance of cross-reactive antibody populations driving polyclonal neutralization after each sequential DENV infection in subject 3112

Following the neutralization assays that allowed us to determine the NT_{50} values to each serotype in depleted sequential infection samples, we calculated the proportion of type-specific and cross-reactive antibodies to polyclonal neutralization. In the sample collected after symptomatic DENV1 infection (3112.2), we observe that over 50% of the neutralization is driven by DENV1 type-specific neutralizing antibodies (**Figure 5.3a**). Over time, the levels of this antibody population fluctuate, as seen by a drop in the following time-point (**Figure 5.3b**) and a slight increase at the following time-points (**Figures 5.3c-5.3e**). Despite an indication of inapparent infection in the subsequent year (3112.3), no type-specific antibody neutralization is seen for DENV2, DENV3 or DENV4 serotypes. Following a third infection (3112.8), a small proportion of DENV3 type-specific population is observed, in accordance with the binding data and the RT-PCR-confirmed symptomatic infection with DENV3. Although the RVP data indicates an inapparent

infection with DENV4 in 2012 (**Table 5.1**), our depletion data indicate a slight increase in the proportion of DENV3 type-specific neutralization (**Figure 5.3d**), while a DENV4 type-specific population is only seen two years later in time-point 3112.11 (**Figure 5.3e**).

DENV-specific binding to the first infecting serotype increases in a subsequent inapparent infection in subject 836

To confirm depletion of antibody populations and measure the binding activity to DENV1-DENV4 for subject 836, we performed ELISAs, as described above. In the sample collected after primary DENV2 infection (836.2), depletion of cross-reactive antibodies (DENV1,3,4-coated beads) did not result in reduction of binding to DENV2, suggesting that binding is predominantly mediated by a DENV2 type-specific population (**Figure 5.4b**). In the subsequent infection (836.4), when a DENV4 inapparent infection may have occurred (**Table 5.1**), depletion of cross-reactive antibodies results in complete loss of binding (**Figure 5.4d**), which indicates that no type-specific antibodies were generated to DENV4. Instead, we observed a slight increase in the binding to DENV2, the infecting serotype of the first infection (**Figure 5.4b**). Following a third symptomatic infection with DENV3 (836.7), we observed binding to DENV3 after removal of cross-reactive antibodies (DENV2), indicating that this infection generated a population of type-specific binding antibodies (**Figure 5.4c**), which were also maintained 2 years later (836.9). Type-specific DENV1 binding antibodies were not detected at any time-point (**Figure 5.4a**).

Neutralizing antibody titers of samples depleted with homologous and heterologous serotypes in individual 836

We next performed neutralization assays to determine the neutralizing antibody titers after depletion of plasma samples from subject 836 with beads coated with BSA (control), DENV2 or DENV1,3,4. Following primary infection with DENV2, the highest neutralizing antibody titers are to the infecting serotype (**Figure 5.5a**). Depletion with the heterologous serotypes resulted in a drop of neutralizing antibody titers, but did not result in complete loss of neutralization activity (**Figure 5.5c**). According to the RVP NT_{50} data, this individual may have experienced a subsequent inapparent DENV4 infection (**Table 5.1**), and the increase in overall titers is also seen in the control-depleted plasma sample (**Figure 5.5c**). Interestingly, the DENV2 type-specific neutralizing antibody response was sustained even after depletion with the heterologous serotypes (DENV1,3,4) (**Figure 5.5d**). No DENV4 type-specific neutralizing antibodies were detected (**Figures 5.5c and 5.5d**). After symptomatic DENV3 infection (836.7), depletion of the heterologous serotypes (DENV2-coated beads) did not cause complete loss of neutralization, indicating the presence of DENV3 type-specific neutralizing antibodies (**Figures 5.5g-5.5i**). Although the neutralizing antibody response is predominantly cross-neutralizing 2 years after infection, DENV2 and DENV3 type-specific neutralization is still seen after depletion with heterologous serotypes (**Figures 5.5j-5.5l**).

Evidence of homotypic DENV2 reinfection in subject 836

Based on neutralization titers of the depleted samples, we calculated the contribution of type-specific versus cross-reactive populations to polyclonal neutralization, as previously done for individual 3112. In accordance with previous findings[36], type-specific neutralizing antibodies accounted for a great proportion of the polyclonal neutralization after primary infection (**Figure 5.6a**). While the antibody response is reportedly broader after a secondary infection[33], we observed not only maintenance of the DENV2 type-specific population but an increase, suggesting a homotypic reinfection with DENV2. Unlike the RVP infection history data, we did not observe any evidence of DENV4 infection (**Figure 5.6b**); however, the RVP neutralization data was somewhat inconclusive to begin with. Following a third infection with DENV3, the cross-neutralizing response predominated, but a DENV3 type-specific population was also elicited and contributes to some of the polyclonal neutralization (**Figure 5.6c**). Albeit at low levels, type-specific antibodies to the both DENV2 and DENV3 are maintained even two years after the last infection (**Figure 5.6d**).

DISCUSSION

Studies in regions that are not endemic for dengue have revealed maintenance of type-specific neutralizing antibody titers, which are linked to durable antibody neutralizing responses believed to be protective against reinfection with the same serotype[4,26]. The cross-reactive antibody populations, on the other hand, are postulated to confer cross-protection to heterotypic infection only for a limited period of time, with subsequent waning of the neutralizing antibody titers[26]. At the epitope level, type-specific antibodies in humans with strong neutralization potential target quaternary or conformation-dependent epitopes displayed on the intact virion[37–40]. After secondary infection, a key feature is the broadening of the antibody response and long lasting neutralizing activity against multiple serotypes. The epitopes targeted by human mAbs isolated from people exposed to secondary DENV infection are located on EDII, with many targeting the fusion loop, as well as quaternary epitopes that span across two E proteins forming a dimer[41–43].

Here, we analyzed longitudinal samples from the same individuals after repeat DENV infection to determine the contribution of type-specific versus cross-reactive antibodies to polyclonal neutralization after each sequential infection. The first subject analyzed (3112) experienced primary DENV1 infection, after which DENV1 type-specific neutralizing antibodies were shown to drive a significant portion of the polyclonal neutralization, in accordance with previous findings[33,36]. Not surprisingly, we also observed a small DENV3 type-specific population, which could be a result of the DENV subcomplexes that target shared epitopes[44,45]. While the trajectory of the neutralizing antibody titers defined by the RVP neutralization assay point to a subsequent inapparent DENV2 infection, no type-specific population was observed for DENV2 at both the binding and neutralization levels in depleted samples. Following an inapparent DENV3 infection, DENV3 type-specific antibodies were elicited. However, the majority of the polyclonal neutralizing antibody response is attributable to cross-reactive antibodies, as previously described in depleted samples after natural secondary DENV infection[33] and vaccinees after secondary immunization[13]. In this individual (subjet 3112), we conclude that polyclonal antibodies after sequential DENV infections recognize serotype-specific and cross-reactive epitopes. Interestingly, our group recently isolated human monoclonal antibodies (hmAbs) after secondary DENV infection and described type-specific hmAbs to the first and also the second infecting serotypes, in addition to an abundant number of cross-reactive hmAbs (Chapter 4). The higher magnitude and breadth of the antibody response after repeat DENV infections is likely due to rapid expansion of MBCs that recognize conserved DENV epitopes and generate higher-avidity and affinity-matured cross-reactive neutralizing antibodies[12,46–48].

An increasingly number of studies in dengue-endemic areas challenge the concept of a predominantly type-specific response and reveal stable levels of cross-reactive neutralizing antibodies after primary infection[19,29]. In addition, also contrary to long-standing dogma, homotypic reinfection with the same serotype can occur, as recently reported in Nicaragua, where patients developed viremia 1-2 years after a DENV

infection of the same serotype[30]. It should also be noted that homotypic reinfections have also been suggested by previous serological studies, but in the absence of virological confirmation[20,49,50]. Indeed, homotypic reinfection can result in a symptomatic outcome, which is compelling evidence that challenges the concept of sterile immunity following primary infection[30]. Current methods in our cohort study to identify inapparent infections rely on serological tests on paired annual samples that either seroconverted (a titer of <1:10 to ≥1:10) or a ≥4 fold increase in antibody titer[34,51]. However, due to high cross-reactivity, analysis of NT_{50} values alone is insufficient to determine whether boosts of NT_{50} values were caused by heterologous boosts or resulted from homotypic reinfection. Using depletion of antibody populations in sequential DENV infection samples, we identified a case strongly suggestive of DENV2 homotypic reinfection. Occurrence of homotypic reinfection is also supported by epidemiological data in Nicaragua that indicates a dominant circulating serotype for several seasons, as opposed to Asia, which is hyperendemic for all four DENV serotypes[31,52,53]. Although epidemics occurs during the rainy season (August-January) in Managua, low levels of cases can occur throughout the year[52]. In fact, DENV2 was the dominant serotype in Nicaragua during the period individual 836 experienced two infections with DENV2[54].

In dengue-endemic areas, the incidence of inapparent infections is high, as reported in cohort studies[55–58]. In the light of our findings, homotypic reinfections may be more frequent than anticipated and may be erroneously classified as heterotypic infections. Therefore, implementation of the methods described here to a larger number of longitudinal samples collected during periods in which homotyoic reinfections may have occurred will help characterize DENV exposures in endemic areas. In search of patterns associated with repeat infections, homotypic reinfections and boosted infections, simpler methods based on ELISA assays may reflect the patterns observed using more sophisticated and time-intensive techniques and can be used as a proxy to quantify the frequency of such infection events in a larger number of cohort study participants. These findings may provide unprecedented understanding of the complex immune response to natural DENV infections, with implications for disease transmission and vaccine design.

EXPERIMENTAL PROCEDURES

Ethics statement

The protocols for the Pediatric Dengue Cohort Study (PDCS) in Nicaragua were reviewed and approved by the Institutional Review Boards of the University of California Berkeley and the Nicaraguan Ministry of Health. Parents or legal guardian of the subjects enrolled in these studies provided written informed consent, and participants 6 years of age and older provided assent.

Study population

Pediatric Dengue Cohort Study (PDCS) is an ongoing prospective dengue cohort study with approximately 3,700 children ages 2-14 in District II of Managua, Nicaragua[34]. Participants with suspected dengue, as defined by the traditional WHO dengue guidelines[59] and undifferentiated febrile illness are worked up for DENV infection, which is identified by serotype-specific RT-PCR for detection of viral RNA, isolation of DENV on C6/36 cells, and/or seroconversion by IgM enzyme-linked immunosorbent assay (ELISA) or a ≥4-fold increase in total antibody titer as measured by inhibition ELISA in paired acute- and convalescent-phase samples[9,34,51,60–62] Inapparent DENV infections were identified through serological testing of paired annual healthy blood samples, where seroconversion or a 4-fold or greater increase in total DENV-specific antibody titer by Inhibition ELISA (IE) or DENV neutralizing antibody titers indicates an inapparent DENV infection[6,9,19,34].

Viruses

For the depletion and binding ELISA, WHO reference strains DENV1 (West Pac 74'), DENV2 (D-16803), DENV3 (CH-53489) and DENV4 (TVP-376) propagated in *Aedes albopictus* C6/36 cells at 37°C in 5% $C0_2$ were used. DENV were purified by density gradient and ultracentrifugation as previously described[33]. For the U937 DC-SIGN-based neutralization assay, Nicaraguan strains of DENV1 (N1265), DENV2 (N1272), DENV3 (N7236) and DENV4 (N703-99) propagated in *Aedes albopictus* C6/36 cells at 37°C in 5% $C0_2$ were used.

Reporter Viral Particle-based neutralization assay

The neutralizing antibody titers (NT_{50}) of healthy annual samples are determined using a flow-cytometry-based assay with reporter (GFP) viral particles (RVPs) representing the four serotypes and Raji cells expressing the DENV attachment factor DC-SIGN[6,35]. Briefly, RVPs were prepared according to working dilution previously determined to result in 8-15% infection of Raji-DC-SIGN cells in a final volume of 25 µl of RPMI pH 8.0 complete medium. RVPs were then mixed with an equal volume of serum (eight 3-fold serial dilutions in RPMI pH 8.0 complete medium starting at 1:5, tested in duplicate) in 96-well plates and incubated on a shaker for 1 hour at room temperature. Following that, 50 µl of diluted RVPs were added to 40,000 Raji DC-SIGN cells in a total volume of

100 µl RPMI media and incubated for 48h at 37°C in 5% CO_2. After incubation, cells were fixed in 2% paraformaldehyde. The percentage of infected, GFP-positive cells for each serum concentration was plotted as percent infection versus $\log_{10}$ of the reciprocal serum dilution using Prism 5.0 (GraphPad). A sigmoidal dose response curve with a variable slope was then generated to determine the 50% neutralization titer, or NT_{50} – the serum dilution at which a 50% reduction in infection was observed compared to the positive (no-serum) control. Background GFP levels were subtracted from all measurements using a negative control (no-RVP).

Whole virus depletion of DENV-specific antibodies from human immune sera (performed in the laboratory of Dr. Aravinda de Silva)

For the depletion of DENV-specific antibody subpopulations, plasma samples were incubated with virus absorbed beads for 45 min at 37°C, as previously described[33]. To confirm successful depletion of DENV-specific antibodies and test for binding activity, ELISAs were performed[33].

U937 DC-SIGN-based neutralization assay

To measure DENV-specific neutralizing antibodies, we employed a flow-cytometry-based neutralization assay with the U937 human monocytic cell line stably transfected with DC-SIGN, as previously described[63]. Briefly, DENV-immune plasma at an initial dilution of 1:10 were serially diluted 3-fold 8 times in RPMI supplemented with 2% FBS. A dilution of virus that infects between 8-15% of the U937 cells (previously determined by virus titration) was added to the plasma dilutions and incubated for 1h at 37°C. Following the incubation time, the cells were centrifuged at 252 x g for 5 min and resuspended in 100 µL RPMI medium. Next, cells were fixed in 4% paraformaldehyde, incubated for 10 min at room temperature, and centrifuged at 252 x g for 5 min. Following this, cells were blocked in permeabilization buffer (0.1% saponin, 5% bovine serum albumin in 1X phosphate-buffered saline [PBS] for 30 min at room temperature. Then, cells were incubated with anti-E mAb 4G2 conjugated to Alexa 488, diluted in blocking buffer (0.5% bovine serum albumin and 0.02% sodium azide in 1X PBS) for 25 min at room termperature. Finally, cells were washed and resuspended in PBS. Acquisition of the infected cells was performed with a Guava flow cytometer (EMD Milipore) by gating Alexa 488-positive cells. The data were analyzed using a nonlinear, 4-parameter dose-response regression analysis with Prism software (GraphPad). The NT_{50} was determined as the concentration of the hmAb dilution that achieved a 50% reduction of the infection compared to infection control. Data generated had to meet the quality control criteria, whereby the sigmoidal dose-response regression fit had to include an absolute sum of squares of <0.2 and a coefficient of determination (R^2) of >0.9.

Calculating the proportion of type-specific and cross-reactive neutralizing antibodies

Following neutralization assays with samples depleted with BSA, DENV2 or DEN1,3 4, the percentage of type-specific neutralizing antibodies were calculated using the following formula

% DENV type-specific neutralizing antibodies
= (1 –(NT_{50} value after heterologous depletion)/(NT_{50} value after BSA depletion)) * 100

% DENV cross-reactive neutralizing antibodies
= 1 –(% type-specific neutralizing antibodies)

FIGURES

3112

NT50	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
DENV1	<10	393	3334	2547	1102	1698	1231	7721	834	383
DENV2	<10	331	2458	1500	758	920	291	1569	36	32
DENV3	<10	194	1228	795	265	498	302	4730	148	152
DENV4	<10	<10	<10	<10	<10	<10	<10	<10	822	889
Infecting serotype	DENV1		DENV2				DENV3	DENV4		
Infection outcome	Symptomatic		Inapparent				Inapparent	Inapparent		

836

NT50	2004	2005	2006	2007	2008	2009	2010	2011	2012
DENV1	<10	<10	27	86	40	57	150	521	316
DENV2	<10	182	133	342	211	262	176	325	106
DENV3	<10	27	86	228	70	73	289	211	78
DENV4	<10	30	28	120	61	31	63	199	643
Infecting serotype	DENV2		DENV4			DENV3			
Infection outcome	Inapparent		Inapparent			Symptomatic			

Table 5.1. Longitudinal analysis of neutralizing antibody titers in selected cohort participants. Annual plasma samples collected from each of the two participants in this study were used for neutralization assays using Reporter Virus Particles (RVP). A ≥4-fold increase in DENV-specific neutralizing antibody titers (NT_{50}) in paired annual samples indicates a DENV infection during the study year, as highlighted for DENV1 (orange), DENV2 (yellow), DENV3 (green) and DENV4 (blue). Symptomatic infection was also confirmed by RT-PCR/virus isolation.

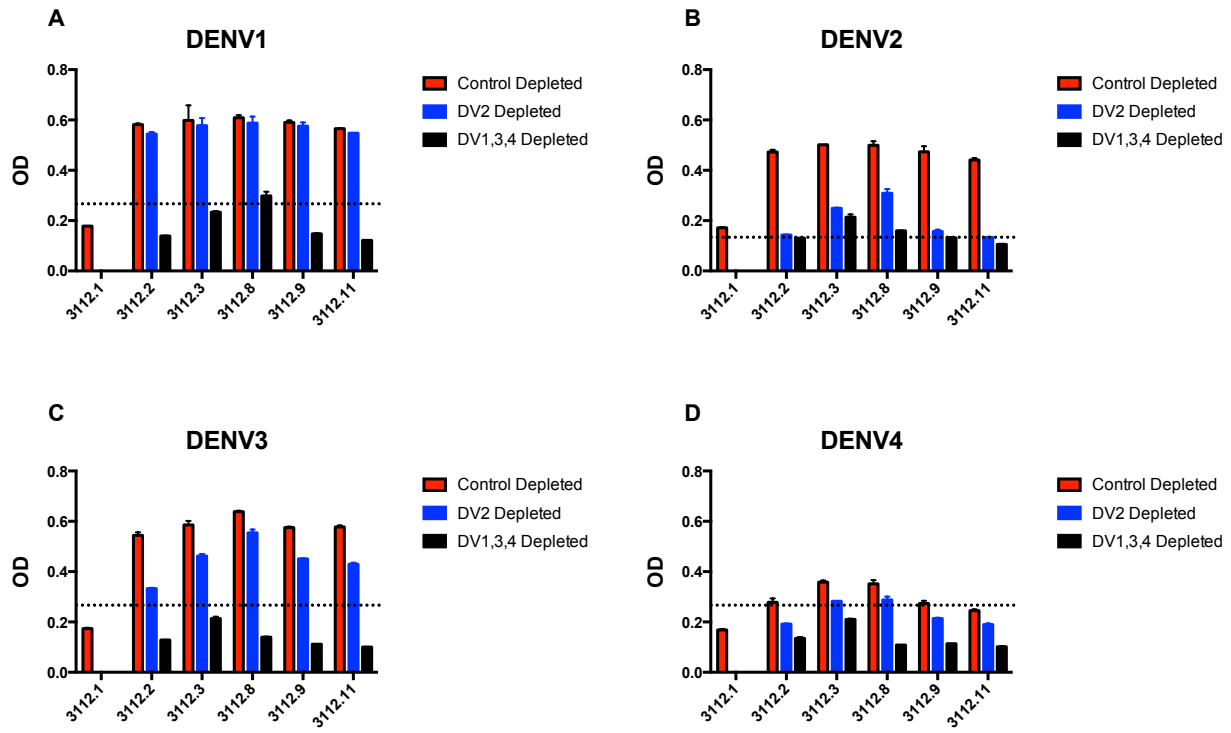


Figure 5.1. Binding activity against DENV1-DENV4 in samples depleted of antibody populations in each sequential DENV infection. Polystyrene beads coated with BSA, purified DENV2 or a mixture of purified DENV1, 3,4 were used to deplete longitudinal sera samples collected prior to DENV infection (3112.1), after symptomatic primary infection (3112.2) and after subsequent inapparent infections (3112.3, 3112.8, 3112.9 and 3112.11). Following depletion, ELISAs were performed to measure the binding activity of depleted sera against DENV1 (A), DENV2 (B), DENV3 (C) and DENV4 (D) antigens. Erros bars indicate Standard Error of the Mean (SEM). ELISAs were performed and data analyzed by the laboratory of collaborator Dr. Aravinda de Silva at the University of North Carolina at Chapel Hill.

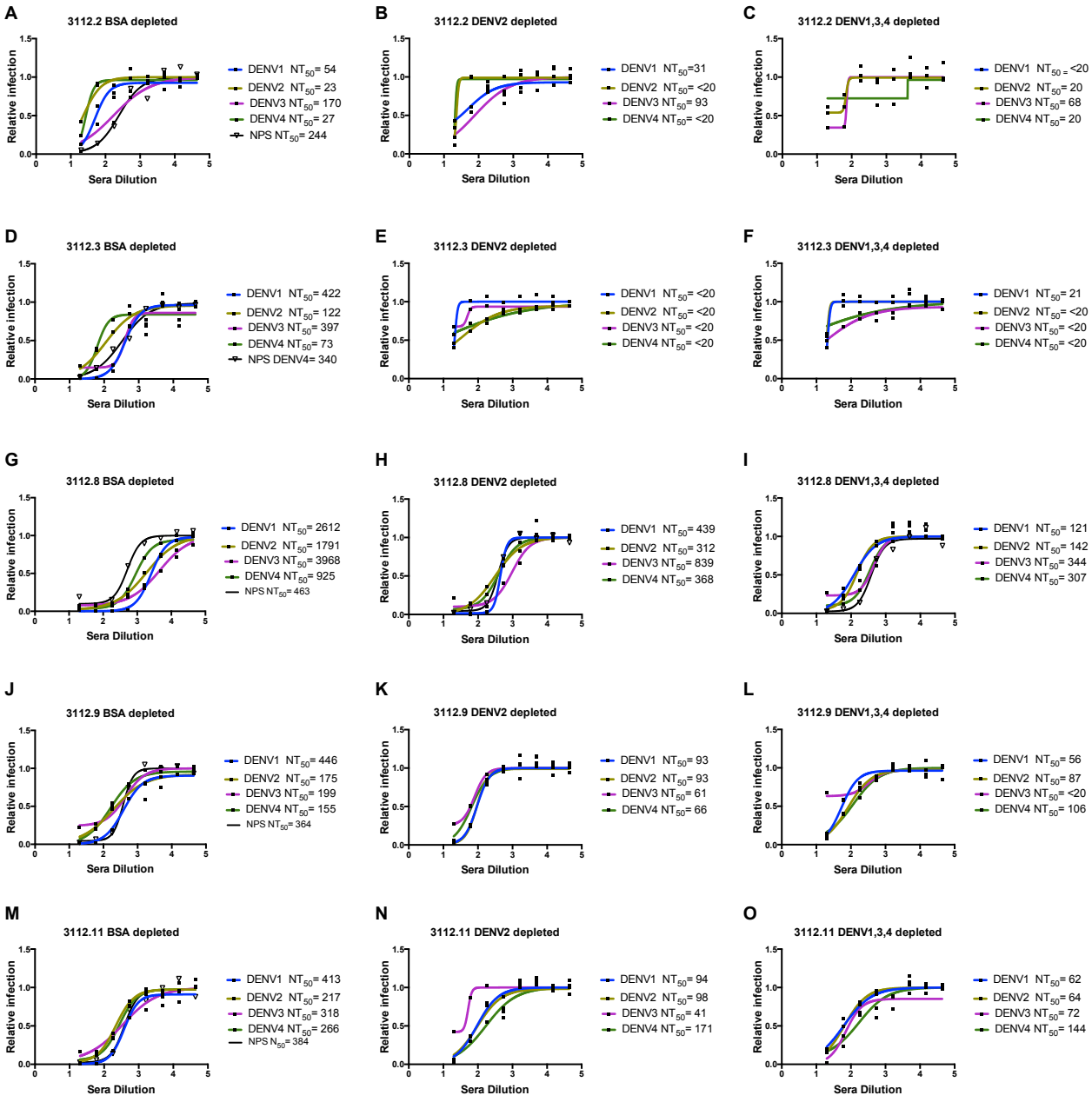


Figure 5.2. Sigmoidal dose-response curves representative of neutralization assays performed after depletion of longitudinal plasma samples. Control-depleted, DENV2-depleted and DENV1,3,4-depleted plasma samples were tested for neutralization in a U937-DC-SIGN-based neutralization assay with Nicaraguan viruses after a first symptomatic infection (A-C), second inapparent infection (D-F), third inapparent infection (G-I), fourth inapparent infection (J-L) and two years afterwards (M-O).

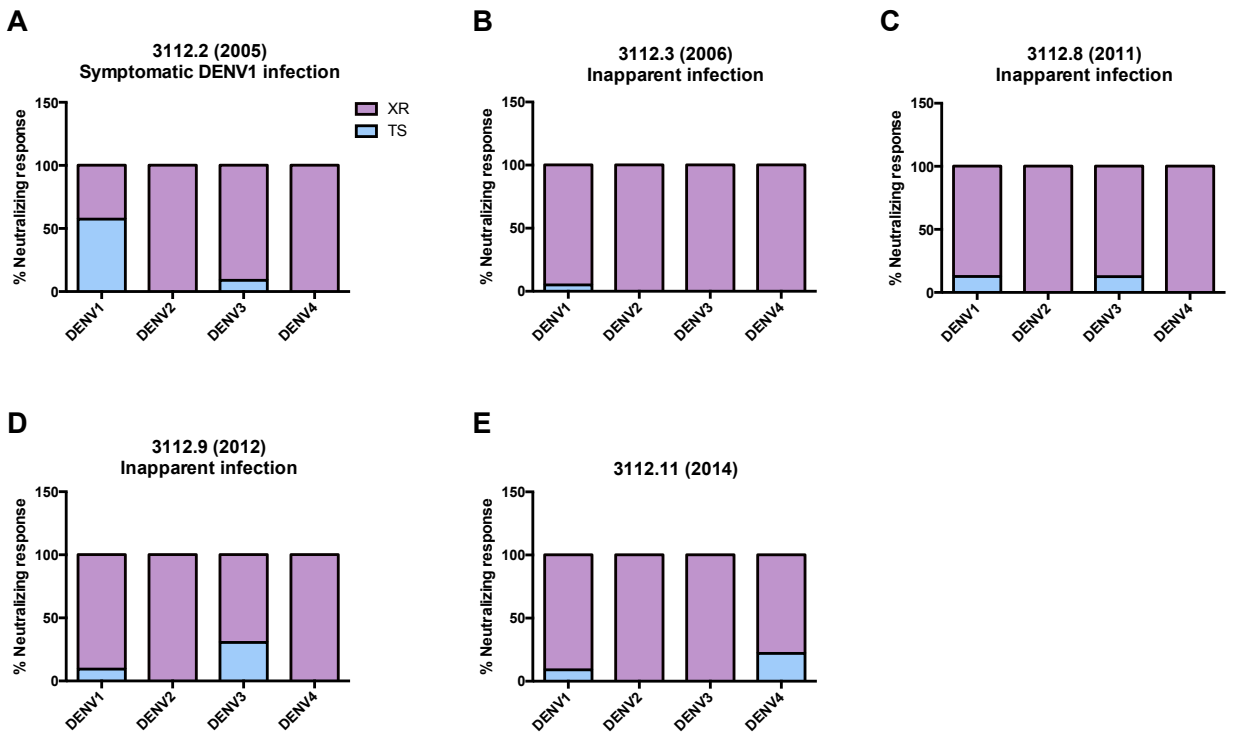


Figure 5.3. Proportion of type-specific versus cross-reactive neutralizing antibodies after each sequential DENV infection in subject 3112. After depletion of homologous and heterologous antibodies, neutralization assays were performed. The levels of type-specific (blue) and cross-reactive (purple) neutralizing antibodies were determined after a first symptomatic infection with DENV1 (A), subsequent inapparent infection with DENV2 (C), followed by inapparent infection with DENV3 (D) and subsequent inapparent infection with DENV4 (D). The proportion of each antibody population to polyclonal neutralization was also measured two years later (E).

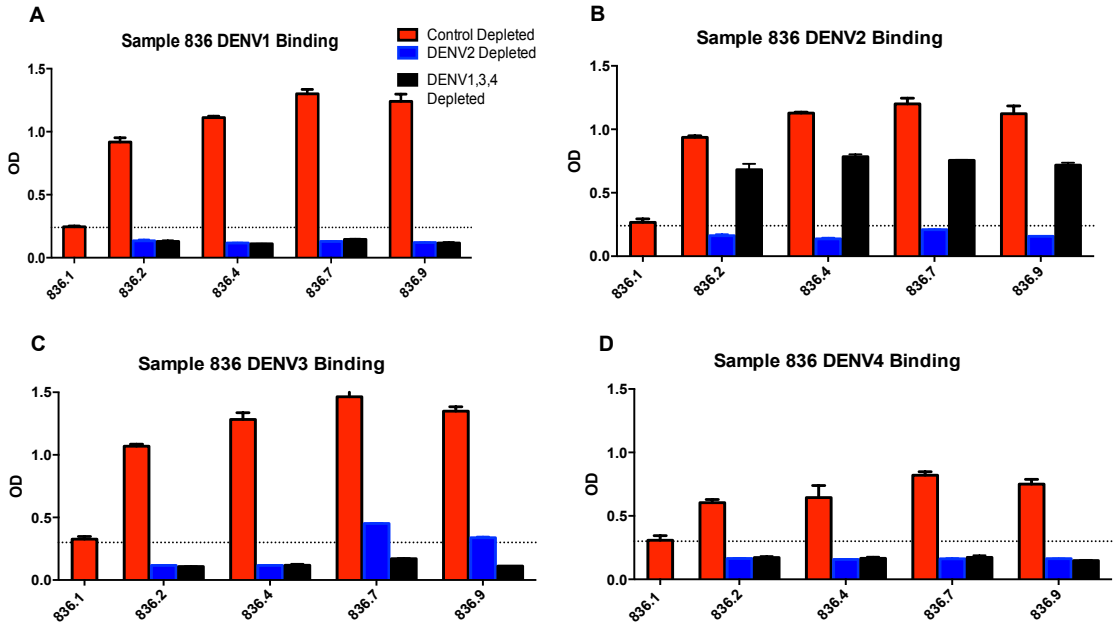


Figure 5.4. Binding of type-specific antibodies to the first infecting serotype slightly increase after subsequent inapparent infection and is sustained over time in subject 836. Polystyrene beads coated with BSA (red bars), purified DENV2 (blue bars), or a mixture of purified DENV1, 3, and 4 (black bars) were used to deplete longitudinal sera samples collected prior to DENV infection (836.1), after inapparent DENV2 infection (836.2), subsequent inapparent infection (836.4), and after symptomatic DENV3 infection (836.7). A sample collected two years after the last infection was also examined (836.9). Following depletion, ELISAs were performed to measure the binding activity of depleted sera against DENV1 (A), DENV2 (B), DENV3 (C) and DENV4 (D) antigens. ELISAs were performed and data analyzed by the laboratory of collaborator Dr. Aravinda de Silva at the University of North Carolina at Chapel Hill.

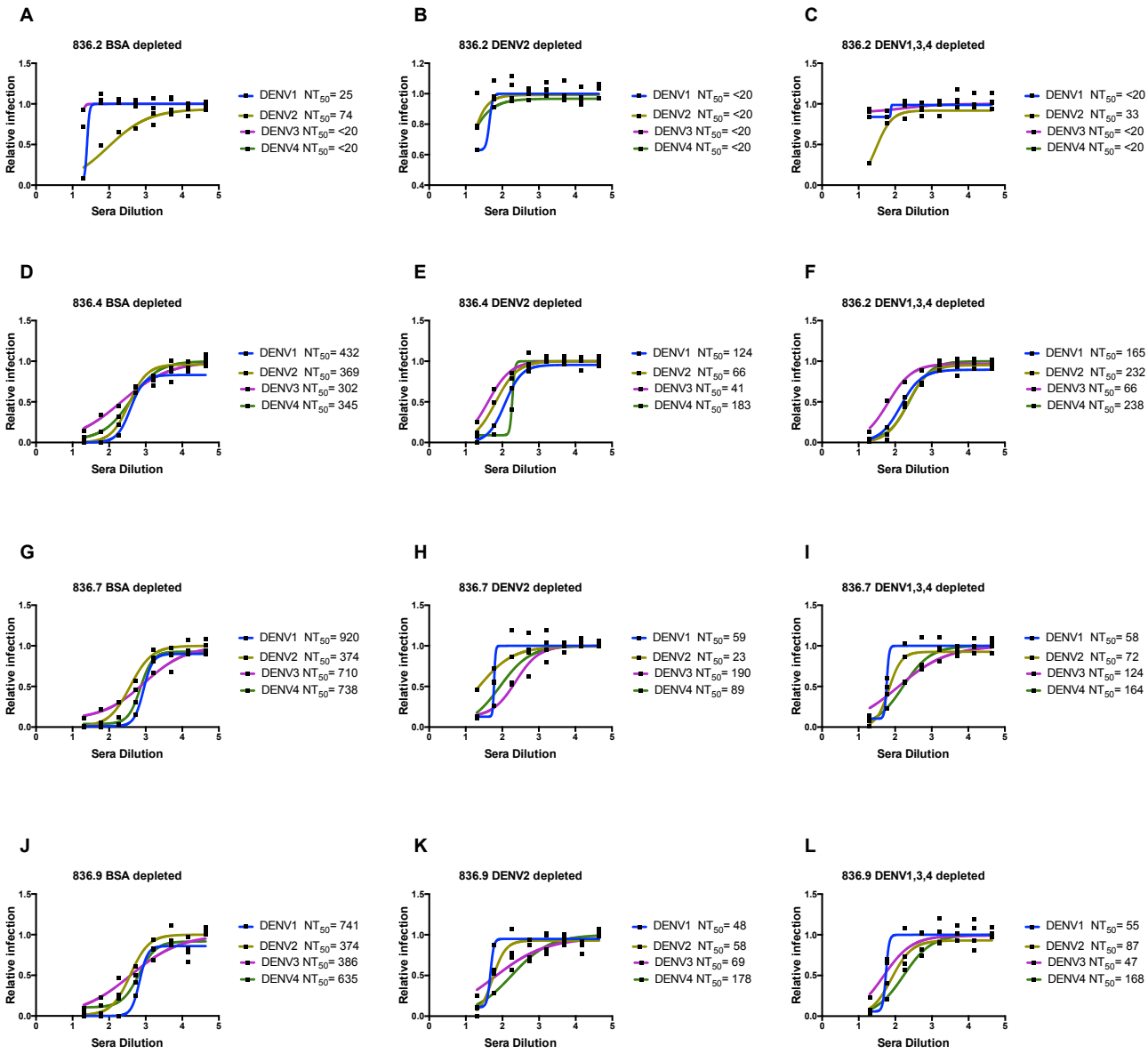


Figure 5.5. Sigmoidal dose-response curves representative of neutralization of depleted samples from individual 836. Control-depleted, DENV2-depleted and DENV1,3,4-depleted plasma samples were tested for neutralization in a U937-DC-SIGN-based neutralization assay with Nicaraguan viruses after a first inapparent infection (A-C), second inapparent infection (D-F), third symptomatic infection (G-I), and two years afterwards (J-L).

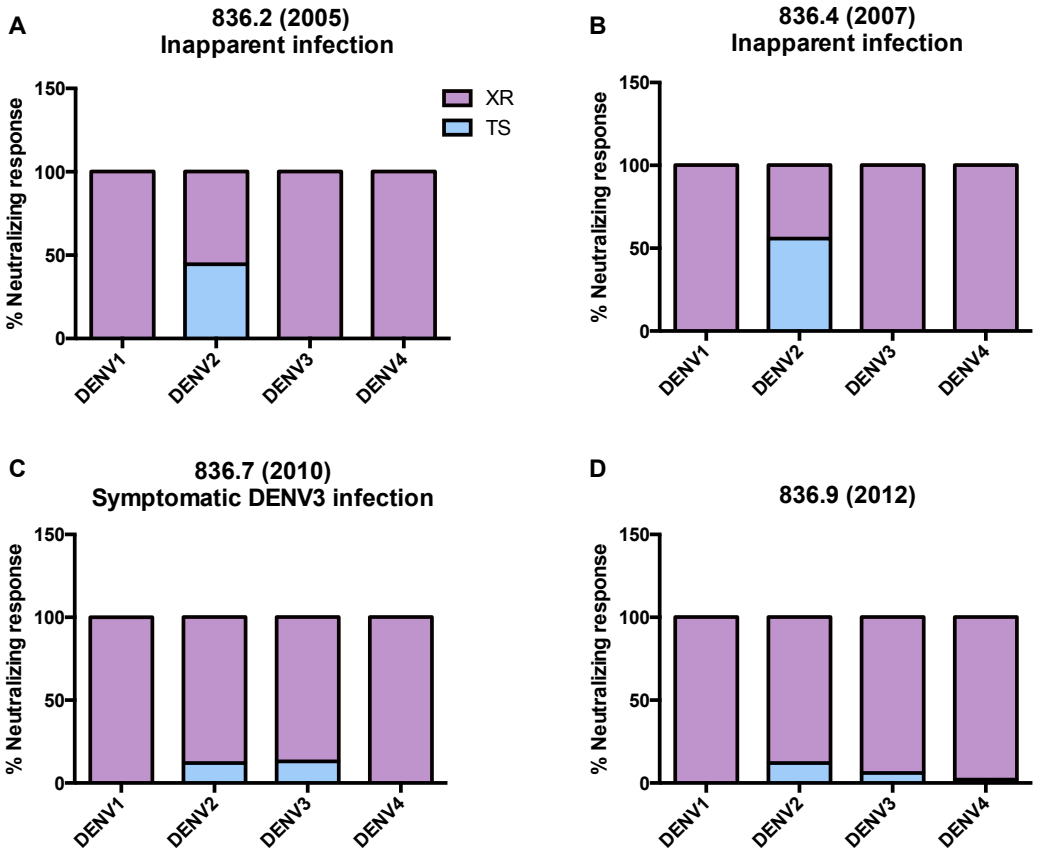


Figure 5.6. Depletion of antibody populations provides evidence of DENV2 homotypic reinfection. After depletion of homologous and heterologous antibodies, neutralization assays were performed. The levels of type-specific (blue) and cross-reactive (purple) neutralizing antibodies were determined after a first symptomatic infection with DENV2 (A), subsequent inapparent infection with DENV2 (B), followed by symptomatic infection with DENV3 (C) and another sample two years post-infection (D).

REFERENCES

1. Guzman, M. G. & Harris, E. Dengue. *Lancet* **385,** 453–465 (2015).
2. Hayes, E. B. & Gubler, D. J. Dengue and dengue hemorrhagic fever. *Pediatr. Infect. Dis. J.* **11,** 311–7 (1992).
3. Gubler, D. J. Dengue and dengue hemorrhagic fever. *Clin. Microbiol. Rev.* **11,** 480–96 (1998).
4. SABIN, A. B. Research on dengue during World War II. *Am. J. Trop. Med. Hyg.* **1,** 30–50 (1952).
5. Reich, N. G. *et al.* Interactions between serotypes of dengue highlight epidemiological impact of cross-immunity. *J. R. Soc. Interface* **10,** 20130414 (2013).
6. Montoya, M. *et al.* Symptomatic Versus Inapparent Outcome in Repeat Dengue Virus Infections Is Influenced by the Time Interval between Infections and Study Year. *PLoS Negl. Trop. Dis.* **7,** e2357 (2013).
7. Anderson, K. B. *et al.* A Shorter Time Interval Between First and Second Dengue Infections Is Associated With Protection From Clinical Illness in a School-based Cohort in Thailand. *J. Infect. Dis.* **209,** 360–368 (2014).
8. Halstead, S. B. In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. *J. Infect. Dis.* **140,** 527–33 (1979).
9. Katzelnick, L. C. *et al.* Antibody-dependent enhancement of severe dengue disease in humans. *Science* **358,** 929–932 (2017).
10. Salje, H. *et al.* Reconstruction of antibody dynamics and infection histories to evaluate dengue risk. *Nature* **557,** 719–723 (2018).
11. Sridhar, S. *et al.* Effect of Dengue Serostatus on Dengue Vaccine Safety and Efficacy. *N. Engl. J. Med.* **379,** 327–340 (2018).
12. Zompi, S., Montoya, M., Pohl, M. O., Balmaseda, A. & Harris, E. Dominant Cross-Reactive B Cell Response during Secondary Acute Dengue Virus Infection in Humans. *PLoS Negl. Trop. Dis.* **6,** e1568 (2012).
13. Tsai, W.-Y. *et al.* Complexity of Neutralizing Antibodies against Multiple Dengue Virus Serotypes after Heterotypic Immunization and Secondary Infection Revealed by In-Depth Analysis of Cross-Reactive Antibodies. *J. Virol.* **89,** 7348–7362 (2015).
14. Matheus, S. *et al.* Discrimination between Primary and Secondary Dengue Virus Infection by an Immunoglobulin G Avidity Test Using a Single Acute-Phase Serum Sample. *J. Clin. Microbiol.* **43,** 2793–2797 (2005).
15. de Souza, V. A. U. F. *et al.* Sensitivity and specificity of three ELISA-based assays for discriminating primary from secondary acute dengue virus infection. *J. Clin. Virol.* **39,** 230–233 (2007).
16. Gibbons, R. V *et al. Analysis of Repeat Hospital Admissions for Dengue to Estimate the Frequency of Third or Fourth Dengue Infections Resulting in Admissions and Dengue Hemorrhagic Fever, and Serotype Sequences*. (2007).
17. Bhoomiboonchoo, P. *et al.* Sequential dengue virus infections detected in active and passive surveillance programs in Thailand, 1994–2010. *BMC Public Health* **15,** 250 (2015).

18. Olkowski, S. *et al.* Reduced Risk of Disease During Postsecondary Dengue Virus Infections. *J. Infect. Dis.* **208,** 1026–1033 (2013).
19. Katzelnick, L. C., Montoya, M., Gresh, L., Balmaseda, A. & Harris, E. Neutralizing antibody titers against dengue virus correlate with protection from symptomatic infection in a longitudinal cohort. *Proc. Natl. Acad. Sci.* **113,** 728–733 (2016).
20. Endy, T. P. *et al.* Relationship of Preexisting Dengue Virus (DV) Neutralizing Antibody Levels to Viremia and Severity of Disease in a Prospective Cohort Study of DV Infection in Thailand. *J. Infect. Dis.* **189,** 990–1000 (2004).
21. Corbett, K. S. *et al.* Preexisting Neutralizing Antibody Responses Distinguish Clinically Inapparent and Apparent Dengue Virus Infections in a Sri Lankan Pediatric Cohort. *J. Infect. Dis.* **211,** 590–599 (2015).
22. Capeding, M. R. *et al.* Clinical efficacy and safety of a novel tetravalent dengue vaccine in healthy children in Asia: a phase 3, randomised, observer-masked, placebo-controlled trial. *Lancet* **384,** 1358–1365 (2014).
23. Hadinegoro, S. R. *et al.* Efficacy and Long-Term Safety of a Dengue Vaccine in Regions of Endemic Disease. *N. Engl. J. Med.* **373,** 1195–1206 (2015).
24. Sabchareon, A. *et al.* Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. *Lancet* **380,** 1559–1567 (2012).
25. Villar, L. *et al.* Efficacy of a Tetravalent Dengue Vaccine in Children in Latin America. *N. Engl. J. Med.* **372,** 113–123 (2015).
26. Guzman, M. G. *et al.* Neutralizing antibodies after infection with dengue 1 virus. *Emerg. Infect. Dis.* **13,** 282–6 (2007).
27. Puschnik, A. *et al.* Correlation between Dengue-Specific Neutralizing Antibodies and Serum Avidity in Primary and Secondary Dengue Virus 3 Natural Infections in Humans. *PLoS Negl. Trop. Dis.* **7,** e2274 (2013).
28. Andrade, D. V *et al.* Analysis of Individuals from a Dengue-Endemic Region Helps Define the Footprint and Repertoire of Antibodies Targeting Dengue Virus 3 Type-Specific Epitopes. *MBio* **8,** (2017).
29. Clapham, H. E. *et al.* Dengue Virus (DENV) Neutralizing Antibody Kinetics in Children After Symptomatic Primary and Postprimary DENV Infection. *J. Infect. Dis.* **213,** 1428–35 (2016).
30. Waggoner, J. J. *et al.* Homotypic Dengue Virus Reinfections in Nicaraguan Children. *J. Infect. Dis.* **214,** 986–993 (2016).
31. OhAinle, M. *et al.* Dynamics of Dengue Disease Severity Determined by the Interplay Between Viral Genetics and Serotype-Specific Immunity. *Sci. Transl. Med.* **3,** 114ra128-114ra128 (2011).
32. Forshey, B. M. *et al.* Incomplete Protection against Dengue Virus Type 2 Re-infection in Peru. *PLoS Negl. Trop. Dis.* **10,** e0004398 (2016).
33. Patel, B. *et al.* Dissecting the human serum antibody response to secondary dengue virus infections. *PLoS Negl. Trop. Dis.* **11,** e0005554 (2017).
34. Kuan, G. *et al.* The Nicaraguan Pediatric Dengue Cohort Study: Study Design, Methods, Use of Information Technology, and Extension to Other Infectious Diseases. *Am. J. Epidemiol.* **170,** 120–129 (2009).
35. Mattia, K. *et al.* Dengue Reporter Virus Particles for Measuring Neutralizing Antibodies against Each of the Four Dengue Serotypes. *PLoS One* **6,** e27252

(2011).
36. de Alwis, R. *et al.* Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions. *Proc. Natl. Acad. Sci.* **109,** 7439–7444 (2012).
37. Fibriansah, G. *et al.* A potent anti-dengue human antibody preferentially recognizes the conformation of E protein monomers assembled on the virus surface. *EMBO Mol. Med.* **6,** n/a-n/a (2014).
38. Teoh, E. P. *et al.* The Structural Basis for Serotype-Specific Neutralization of Dengue Virus by a Human Antibody. *Sci. Transl. Med.* **4,** 139ra83-139ra83 (2012).
39. Fibriansah, G. *et al.* Cryo-EM structure of an antibody that neutralizes dengue virus type 2 by locking E protein dimers. *Science (80-.).* **349,** 88–91 (2015).
40. Fibriansah, G. *et al.* A highly potent human antibody neutralizes dengue virus serotype 3 by binding across three surface proteins. *Nat. Commun.* **6,** 6341 (2015).
41. Lai, C.-Y. *et al.* Antibodies to Envelope Glycoprotein of Dengue Virus during the Natural Course of Infection Are Predominantly Cross-Reactive and Recognize Epitopes Containing Highly Conserved Residues at the Fusion Loop of Domain II. *J. Virol.* **82,** 6631–6643 (2008).
42. Beltramello, M. *et al.* The Human Immune Response to Dengue Virus Is Dominated by Highly Cross-Reactive Antibodies Endowed with Neutralizing and Enhancing Activity. *Cell Host Microbe* **8,** 271–283 (2010).
43. Dejnirattisai, W. *et al.* A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus. *Nat. Immunol.* **16,** 170–177 (2015).
44. Gromowski, G. D. *et al.* Mutations of an antibody binding energy hot spot on domain III of the dengue 2 envelope glycoprotein exploited for neutralization escape. *Virology* **407,** 237–246 (2010).
45. Lok, S.-M. *et al.* Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins. *Nat. Struct. Mol. Biol.* **15,** 312–317 (2008).
46. Mathew, A. *et al.* B-Cell Responses During Primary and Secondary Dengue Virus Infections in Humans. *J. Infect. Dis.* **204,** 1514–1522 (2011).
47. Wrammert, J. *et al.* Rapid and Massive Virus-Specific Plasmablast Responses during Acute Dengue Virus Infection in Humans. *J. Virol.* **86,** 2911–2918 (2012).
48. Xu, M. *et al.* Plasmablasts generated during repeated dengue infection are virus glycoprotein-specific and bind to multiple virus serotypes. *J. Immunol.* **189,** 5877–85 (2012).
49. Margolis, H. S., Hunsperger, E., Sharp, T. M., Tomashek, K. M. & Muñoz-Jordán, J. L. Sequential Episodes of Dengue—Puerto Rico, 2005–2010. *Am. J. Trop. Med. Hyg.* **91,** 235–239 (2014).
50. Sirivichayakul, C., Sabchareon, A., Limkittikul, K. & Yoksan, S. Plaque reduction neutralization antibody test does not accurately predict protection against dengue infection in Ratchaburi cohort, Thailand. *Virol. J.* **11,** 48 (2014).
51. Balmaseda, A., Sandoval, E., Pérez, L., Gutiérrez, C. M. & Harris, E. Application of molecular typing techniques in the 1998 dengue epidemic in Nicaragua. *Am. J. Trop. Med. Hyg.* **61,** 893–7 (1999).

52. Balmaseda, A. *et al.* Trends in Patterns of Dengue Transmission over 4 Years in a Pediatric Cohort Study in Nicaragua. *J. Infect. Dis.* **201,** 5–14 (2010).
53. Gubler, D. J. & Clark, G. G. Dengue/dengue hemorrhagic fever: the emergence of a global health problem. *Emerg. Infect. Dis.* **1,** 55–7 (1995).
54. Gutierrez, G. *et al.* Unusual Dengue Virus 3 Epidemic in Nicaragua, 2009. *PLoS Negl. Trop. Dis.* **5,** e1394 (2011).
55. Gordon, A. *et al.* The Nicaraguan Pediatric Dengue Cohort Study: Incidence of Inapparent and Symptomatic Dengue Virus Infections, 2004–2010. *PLoS Negl. Trop. Dis.* **7,** e2462 (2013).
56. Burke, D. S., Nisalak, A., Johnson, D. E. & Scott, R. M. A prospective study of dengue infections in Bangkok. *Am. J. Trop. Med. Hyg.* **38,** 172–80 (1988).
57. Endy, T. P. *et al.* Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng Phet, Thailand. *Am. J. Epidemiol.* **156,** 40–51 (2002).
58. Yoon, I.-K. *et al.* Underrecognized Mildly Symptomatic Viremic Dengue Virus Infections in Rural Thai Schools and Villages. *J. Infect. Dis.* **206,** 389–398 (2012).
59. *Dengue haemorrhagic fever Diagnosis, treatment, prevention and control SECOND EDITION Contents*. (1997).
60. Lanciotti, R. S., Calisher, C. H., Gubler, D. J., Chang, G. J. & Vorndam, A. V. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J. Clin. Microbiol.* **30,** 545–51 (1992).
61. Balmaseda, A. *et al.* Diagnosis of dengue virus infection by detection of specific immunoglobulin M (IgM) and IgA antibodies in serum and saliva. *Clin. Diagn. Lab. Immunol.* **10,** 317–22 (2003).
62. Balmaseda, A. *et al.* High seroprevalence of antibodies against dengue virus in a prospective study of schoolchildren in Managua, Nicaragua. *Trop. Med. Int. Health* **11,** 935–42 (2006).
63. Kraus, A. A., Messer, W., Haymore, L. B. & de Silva, A. M. Comparison of Plaque- and Flow Cytometry-Based Methods for Measuring Dengue Virus Neutralization. *J. Clin. Microbiol.* **45,** 3777–3780 (2007).

CHAPTER 6

CONCLUSION

Project summary

The overall goal of my dissertation was to investigate the functional and structural properties of the neutralizing antibody response to primary and secondary natural DENV infections. In Chapter 2, I conducted the first large-scale study of the neutralizing antibody response post-primary DENV3 infection in a dengue-endemic area, which validated and helped define the footprint of the 5J7 hmAb quaternary epitope. In spite of the large number of individuals whose DENV3 neutralizing antibody response tracked with the 5J7 epitope to varying degrees, a number of subjects showed little to no recognition of this epitope. This observation provided the first evidence that the DENV3 neutralizing antibody repertoire is larger than initially anticipated, as the 5J7 had been the only DENV3 type-specific hmAb isolated. Remarkably, our group successfully isolated and characterized DENV3 potently neutralizing hmAbs that bind to three novel regions on the E protein, in addition to an region overlapping with the 5J7 epitope, as discussed in Chapter 4. Together, both studies generated substantial new knowledge about the fine specificity of the DENV3 neutralizing repertoire, with direct implications for vaccine design.

Similar to the 5J7 hmAb, the 1F4 DENV1 type-specific hmAb binds to an epitope that requires the intact E protein assembled on the DENV virion. As we had previously validated chimeric viruses as a tool to track the DENV type-specific neutralizing antibody response after primary infection, in Chapter 3 we employed a similar approach to evaluate the prevalence of the 1F4 neutralizing antibody response following primary DENV1 infection. Importantly, we analyzed individuals in two dengue-endemic countries -- Nicaragua and Sri Lanka -- where different genotypes of DENV1 are circulating. Interestingly, we observed higher levels of 1F4 epitope recognition and closer antigenic similarity between DENV1 and the chimeric virus in the Sri Lankan population. Such findings provide evidence that intra-serotype amino acid variations can affect neutralization potential. Moreover, both geographic populations displayed a bimodal pattern of 1F4 epitope recognition, which strongly suggests that additional epitopes within the DENV1 repertoire exist and contribute to DENV1 type-specific neutralization. Collectively, both studies enabled better understanding of the fine specificity of the type-specific neutralizing antibody response to DENV1 and DENV3 infections, with direct implications for design and evaluation of dengue vaccine candidates.

In the context of secondary DENV infection, we attempted to study the molecular pathways leading to the generation of potent broadly neutralizing antibodies, as discussed in Chapter 4. Although we were unable to identify precursor lineages of broadly neutralizing antibodies because of the limited sample size of our pediatric subjects, we gained substantial knowledge regarding the functional and molecular properties of hmAbs isolated from MBCs after secondary DENV infection. As expected, serotype cross-neutralizing hmAbs were predominant in our panel. Overall, the hmAbs were highly mutated, suggesting an MBC, as opposed to naïve B cell, origin. We also observed trends indicating that cross-reactive hmAbs are more somatically hypermutated than type-specific hmAbs, which suggests potential gain of neutralization breadth through SHM. As discussed above, newly identified DENV3 type-specific

hmAbs were isolated after secondary DENV infection, an interesting observation given that all type-specific DENV hmAbs isolated to date have been isolated after primary infection. Since our study had the strength of using PBMC samples from well-characterized PBMC infections, we were able to infer that the order of the infecting serotypes may shape the antigenic sites bound by the newly identified DENV3 hmAbs. Lastly, we demonstrated the protective effects of the newly isolated DENV3 hmAbs against DENV3 challenge in a mouse model of DENV infection. While more studies focused on the panel of hmAbs are ongoing, our findings contribute significantly to understanding the specificity and molecular properties of the neutralizing antibody response elicited after secondary DENV infection.

In contrast to long-standing dogma in the field, we observed evidence for long-term maintenance of the levels of cross-reactive antibodies after primary DENV infection, as shown in a subset of Nicaraguan hospital-study participants in Chapters 2 and 3. A second long-standing belief that we found evidence against is life-long protection against re-infection with the same serotype. As shown by depletion of antibody subpopulations, we observed an increase in the proportion of type-specific neutralizing antibodies to the first infecting serotype upon subsequent inapparent infection. Despite the limited sample size of our study, we demonstrated that depletion of antibody subpopulations followed by binding and neutralization assays is a method that enables accurate identification of atypical DENV infections, such as homotypic reinfections and heterotypic boosts. Applying this method to a larger sample set is critical in order to establish patterns that can be recapitulated by simpler proxy methods that then can be used to estimate the frequency of repeat heterotypic reinfections, homotypic reinfections and heterologous boosts in the larger cohort population and relate these to infection outcome. The synergistic approach of understanding the immunological response in an epidemiological context has important implications for models of disease transmission and vaccine design.

In conclusion, the work presented in this dissertation contributes substantially to an in-depth understanding of the antigenic determinants of DENV type-specific neutralizing antibody response, as well as the breadth and functional properties of DENV neutralizing antibodies after secondary DENV infection.

Future directions

The antibody response to DENV is a fascinating and complex topic of research with many pressing questions that remain to be addressed. As our findings point out, the antigenic landscape within each serotype is far from being completely understood. Isolation of new hmAbs will provide valuable tools for elucidation of the specificity and properties of DENV type-specific repertoires. Following up on our findings, controlling for the sequence of infecting serotypes may inform how antigenic sites are generated and recognized in sequential DENV infections. As evidenced by our study and others, genotypic variations have an impact on neutralization potential both in natural infection and vaccine efficacy. Therefore, further studies with panels of isogenic clones and

chimeric viruses enable a more in-depth analysis of the intra serotype variation and its interplay with neutralization escape from type-specific neutralizing antibodies.

Previously isolated potent broadly neutralizing hmAbs map to EDII, with a subset targeting residues near the fusion loop, as well as quaternary epitopes that span across two E proteins forming a dimer. As the mapping of our newly identified potent broadly neutralizing hmAbs is underway, we may learn new antigenic sites for this class of antibodies and their mechanisms of action. Deciphering the molecular pathways that generate potent broadly neutralizing antibodies is pivotal for vaccine design and as such, cutting-edge sequencing technologies of the antibody repertoire may provide the necessary resolution for addressing this question.

The long-standing dogma of waning of cross-reactive antibodies following primary infection and life-long protection to the homologous serotype have been recently challenged. Therefore, development of simpler methods that enable accurate identification of atypical infections in larger number of individuals in dengue-endemic areas is urgently needed, in addition to determining the impact on infection outcome.

As we continue to gain more insight into the immunology of DENV infection, the cross-talk between natural infection studies and vaccine trials is paramount for countering a disease of such public health importance as dengue.