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Determinants Of Antibody Immunity In Flavivirus Infections And Immunization

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DETERMINANTS OF ANTIBODY IMMUNITY IN *FLAVIVIRUS* INFECTIONS AND IMMUNIZATION

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ABSTRACT

The most significant arthropod-borne viral infections in modern history are caused by species of the *Flavivirus* (FV) genus due to their global transmission. While immunization is effective at decreasing the burden imposed by some FV, vaccines for other FV such as Dengue virus (DENV) and Zika virus (ZIKV) are still in development or not widely available. A better understanding of the protective immune response, specifically the antibody response, to DENV and ZIKV will advance the vaccine development endeavors for these viruses. Utilizing longitudinal samples from controlled human immunization/challenge studies and natural infections, we investigated the antibody response to these viruses in both the cellular and serological compartments.

Primary DENV or ZIKV infection results in an antibody response specific to the infecting virus with little cross-reactivity to other related FV. This specificity profile is observed in the immune serum, and both the acute plasmablast and convalescent memory B cell sub-populations. Neutralizing antibodies were identified in these compartments of the antibody response, suggesting their functional association with homologous immunity to the infecting virus. In contrast to primary infection, the antibody response induced by immunization with the tetravalent live attenuated vaccine TV003 is cross-reactive in both the serological and cellular compartments. Interpretation of these findings reveals an intimate and complex relationship between B cell sub-populations and serum antibodies, and contributes to our knowledge of various differentiation routes amongst these B cells. Further analysis using monoclonal antibodies reveals structural vulnerabilities of FV. We identified domain III of the viral envelope glycoprotein to be one of the immunodominant and neutralizing targets for DENV- and ZIKV-specific antibodies. Further studies will continue to elucidate the connections between different B cell sub-populations engaged in FV infections, and identify specific subsets that either confer protection or pose a disease risk in the secondary antibody response invoked by multiple infections. Characterization of antibodies produced by these B cell subsets will further define the determinants of antibody-mediated protection.

Application of these findings will benefit the endeavor to combat the burden of FV. Better understanding of the structural-functional relationship between viral epitopes and neutralizing antibodies will create avenues for antibody-based therapies as well as vaccine design. Leveraging virus-specific antibodies induced by primary exposures will improve the specificity of DENV or ZIKV diagnosis to differentiate infection by each of these viral species or serotypes. Accurate diagnoses will lead to proper treatments to improve disease outcomes. Furthermore, accurate diagnoses will aid in sero-surveillance efforts to ultimately inform vaccine strategies for endemic populations.

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LIST OF ABBREVIATIONS

ADE:	Antibody-dependent enhancement
C:	Capsid protein
CR:	Cross-reactive
DENV:	Dengue Virus
E:	Envelope protein
ED(I/II/III):	E protein domain (I/II/III)
EDE:	E dimer epitope
FV:	*Flavivirus*(es)
JEV:	Japanese Encephalitis Virus
LLPC:	Long-lived plasma cell(s)
M:	Membrane protein
mAb:	Monoclonal antibody(-ies)
MBC:	Memory B cell(s)
NS:	Non-structural protein(s)
PAHO:	Pan American Health Organization
PB:	Plasmablast(s)
PC:	Plasma cell(s)
prM:	Pre-membrane protein
TS:	Type-specific
UTR:	Untranslated region(s)
WHO:	World Health Organization
WNV:	West Nile Virus
YFV:	Yellow Fever Virus
ZIKV:	Zika Virus

CHAPTER I – COMPREHENSIVE LITERATURE REVIEW

Introduction

The *Flaviviridae* family comprises three genera of viral pathogens that cause debilitating diseases and mortality in humans and animals. Its largest genus - *Flavivirus* (FV) - contains more than 70 members that are divided into sub-complexes according to their antigenicity and phylogenetics[1]. *Flaviviridae* was named after the Yellow Fever Virus (YFV) whose infections often cause yellowing of the skin due to liver failure – the Latin root "Flavus" translates to "yellow". However, not all FV infections cause liver disease; there is a wide range of symptoms associated with distinct etiologies. FV holds great significance in modern history due to its continuous and widespread transmission causing large outbreaks across many global regions[2]. YFV causes over 100,000 cases every year in the African and American tropics[3]. Zika virus (ZIKV), during its reemergence in 2015-2016, caused over 600,000 cases worldwide, with highest incidence in Central and South American countries (PAHO). The most prevalent FV, the four Dengue virus serotypes (DENV1-4), infect upward of 400 million people every year in the American and Asian tropics[4]. These global-scale FV epidemics impose a massive burden on the healthcare and economic structures of afflicted countries[4].

Immunization is a key strategy that has been implemented to hinder FV transmission and control disease at the population level. For instance, YFV and Japanese Encephalitis virus (JEV) immunization initiatives can markedly reduce transmission of these viruses in endemic countries, and protect populations from these viral diseases[5]. Immunization of livestock can limit transmission of West Nile virus (WNV)[6]. However, most vaccines for other FV such as DENV and ZIKV are still in development.

Meanwhile, globalization, coupled with changes in climate patterns and social dynamics (e.g., encroachment into mosquito habitats), have created favorable conditions that perpetuate existing epidemics and introduce new FV into naive populations. With constant potential for global outbreaks, the need to understand the biology of FV, and their interplay, with the human immune response and the human population, is urgent. These understandings will inform the development of immunization and therapeutic strategies to limit FV transmission, treat illness, and strengthen population immunity.

This review highlights the fundamentals of the human humoral immune response shaped by two FV species: DENV and ZIKV. Virus-specific antibodies have conventionally been considered a hallmark for protective immunity following infections and immunization for some FV such as YFV and JEV[7]. Defining a similar correlate of protection for DENV and ZIKV has been more complicated, owing to the potential for cross-reactive (CR) antibodies. The antibody response to infection by any FV species has an inherent potential to cross-react to other members of the genus due to shared antigenicity; this is particularly true amongst the four DENV serotypes and ZIKV. The four DENV serotypes share 60-80% amino acid sequence homology with one another, and up to 60% with ZIKV. CR antibodies are thought to predispose a DENV-immune subject for severe disease upon subsequent heterotypic exposures[8, 9, 10, 11, 12]. However, CR antibodies with potent neutralizing capability have also been reported[13]. We are constantly reminded of the ease and shortcomings of inducing a less-than-perfect antibody response by immunization, which may put the vaccinated subject at risk of severe disease upon subsequent natural FV exposures. A comprehensive view of how the antibody response is generated and honed by DENV and ZIKV infections will enrich our

understanding of how the human immune system responds to viral infections, while highlighting the nuances specific to these viruses. Ultimately, these collective findings are informative for the process of vaccine development, specifically in designing immunogens to effectively elicit a protective antibody response that is representative across the human population.

DENV and ZIKV epidemiology

Historical documents of dengue-like illness are dated as early as the middle of the 18th century[14]. However, not until around World War II did investigators establish more accurate and comprehensive records of the disease, including its viral etiology[15, 16, 17]. Since then, dengue has continued to be the most important mosquito-borne viral disease worldwide, with highest prevalence in the global tropics. The Global Burden of Disease study of 2013 suggested the annual incidence of dengue more than doubled every 10 years, from 8 million clinical cases in the 1990s to approximately 50 million cases by the 2010s, opposite to the global declining trends of other communicable diseases[18]. Other studies have estimated 50-100 million clinical dengue cases every year[19]. Currently without effective preventive and therapeutic measures, dengue continues to be a threat for populations in endemic regions, which account for two thirds of the global population.

The emergence of ZIKV as an epidemic was more recent compared to that of DENV. The virus was first isolated from sentinel monkeys in the Ugandan Zika forest in 1947[19, 20]. While first isolated in humans in 1952, ZIKV did not become associated with large-scale epidemics until the outbreak on the island of Yap (2007), followed by the French Polynesian (2013) and South American (2015-2016) outbreaks[20, 21, 22, 23, 24]. The number of zika cases in the Americas peaked in early 2016, totaling over 600,000 cases,

and markedly decreased in 2017, possibly due to development of herd immunity (WHO, PAHO). Despite the decreasing zika incidence, there is on-going concern warranted by several factors. DENV and ZIKV are co-endemic and cause similar nonspecific flu-like symptoms, complicating diagnosis. Misdiagnosis in antibody testing is also possible because of shared antigenicity between the two viruses. Additionally, antibodies induced in infection by either virus have the potential to facilitate antibody-dependent enhancement (ADE) of infection, which may result in more severe disease upon secondary infections by the heterologous virus.

The disease and economic burdens of FV infections, particularly of DENV, are not likely to diminish over the next few decades given the current dynamics of its mosquito vector and the human population, coupled with the lack of effective prevention[25]. The warming global climate has expanded the geographical distribution for the viruses' primary vector, the *Aedes aegypti* mosquitoes. The high rate of urbanization in hyper-endemic countries such as India or the Philippines remains the main stimulus for the proliferation of *A. aegypti*, since this species is adapted to urbanized settings. Furthermore, increased international traveling and mass emigration contribute to the global transmission of FV. Together, these environmental and social changes increase the potential of FV outbreaks both in endemic regions and in naive human populations.

Viral life cycle

DENV and ZIKV transmission in humans is mediated by mosquito vector. Upon the infection, the virus gains access to the host's cellular machinery and resource to produce progeny viruses (**Fig. 1**). DENV and ZIKV infect their target cells via interaction between the major viral surface glycoprotein – the envelope protein (E), and cellular

receptors. Studies from natural infections and immunization have shown that both DENV and ZIKV target cells of the myeloid lineage, specifically monocytes and dendritic cells[26, 27, 28, 29, 30, 31, 32, 33]. Several cellular surface molecules have been predicted or verified as receptors for FV, including T cell immunoglobulin mucin domain (TIM) receptor family, and several lectin-type proteins (**Fig. 1A**)[34]. Amongst these receptors, the C-type lectin dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) has been widely used in *in vitro* applications to support FV infection in mammalian cell lines that are otherwise non-permissive[26, 35, 36]. Receptor-bound virus is internalized via the clathrin-mediated pathway during which endosomal acidification induces rearrangement of viral glycoproteins to facilitate fusion of the viral and endosomal membranes (**Fig. 1B-C**)[37, 38]. The uncoated viral positive-sense RNA genome (**Fig. 1D**) immediately serves as a template for translation of viral proteins required for genome replication and new viral assemby[39]. Assembly of viral particles occurs in the endoplasmic reticulum network, producing immature particles (**Fig. 1E**). Maturation of new viruses occurs as they progress through the golgi transport pathway, before release at the plasma membrane (**Fig. 1F-G**)[40].

Alternatively, DENV and ZIKV can gain entry to target cells via interaction between opsonizing non-neutralizing antibodies and the corresponding Fc receptors[41, 42, 43, 44]. In this scenario, the immune complexes aggregate Fc receptors, particularly FcγRIIA and FcγRIIIA, which relay their activating signal via the SRC kinase pathway, ultimately resulting in cytoskeletal rearrangement-mediated phagocytosis of the opsonized virus[45]. This mode of attachment/entry perhaps is the most efficient for the virus to gain access to its target because Fc receptors are highly expressed on the surface

of monocytes and dendritic cells. Antibody-mediated entry, or ADE of infection, is thought to be the underpinning of increased viral replication *in vitro* and *in vivo* in the presence of non-neutralizing antibodies.

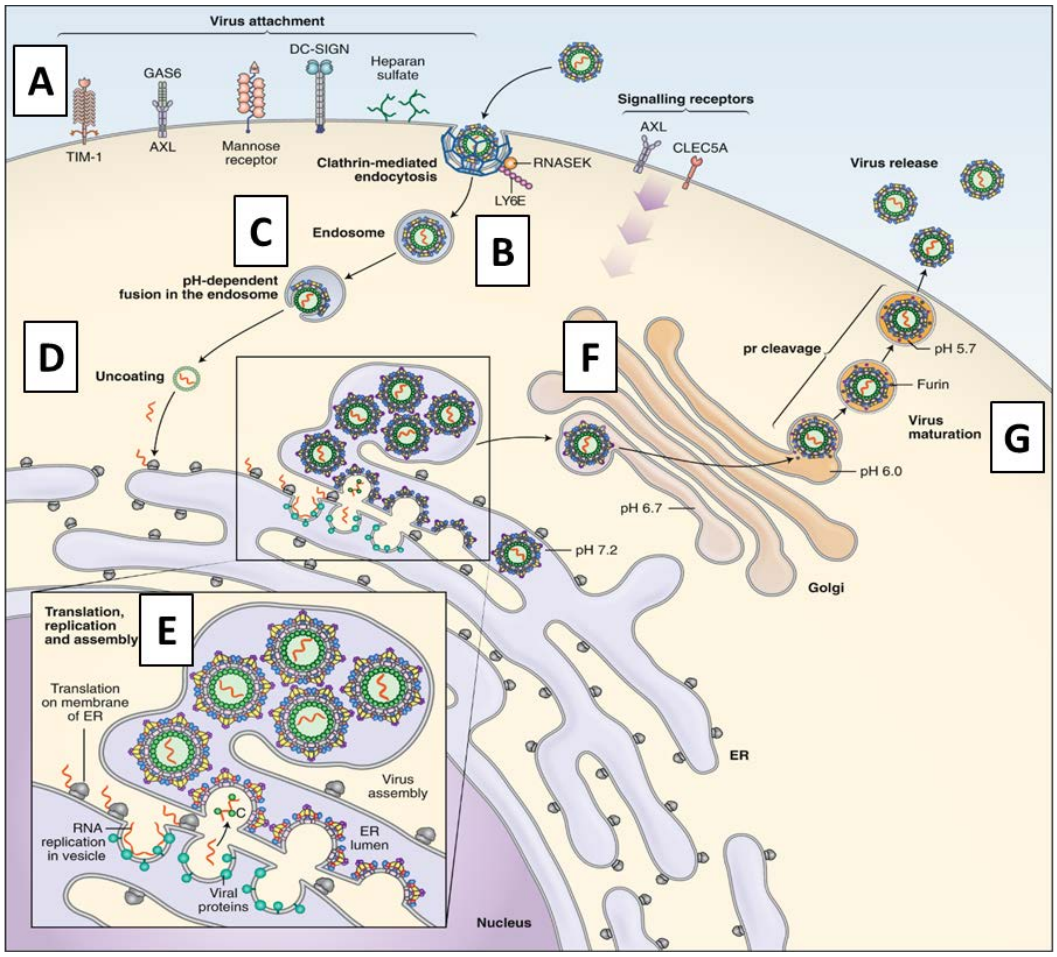

Figure 1. Flavivirus life cycle. (**A**) Several cellular surface molecules act as receptors for FV. The virus is endocytosed via the clathrin-mediated pathway (**B**), during which the viral membrane fuses with the endosomal membrane (**C**) and viral genome uncoating occurs (**D**). Viral production occurs in the endoplasmic reticulum network (**E**) and new viral particles progress towards the cellular plasma membrane through the golgi trafficking compartments (**F**). Viral maturation occurs as host furin cleaves viral pre-membrane proteins, yielding mature infectious particles to be released at the cellular plasma membrane (**G**). Figure cited[2].

Viral genome

The FV positive-sense single-stranded RNA genome is approximately 11kb. The RNA molecule is 5'-capped, but not 3'-polyadenylated (**Fig. 2**)[46, 47]. Being positive sense, the FV genomic RNA can initiate translation following transfection into suitable host cells. Using reverse genetics approaches, investigators have taken advantage of this feature of the viral genome to generate mutant viral isolates to define the function of viral proteins, map antibody epitopes, and develop live attenuated vaccine candidates.

The genome's single open reading frame is flanked by 5' and 3' untranslated regions (UTRs) (**Fig. 2**). While sequence variation exists amongst FV species, common secondary structures have been identified in these UTRs and in their negative-sense complementary RNA regions[48, 49, 50]. These secondary structures help maintain the integrity of the RNA genome, and regulate RNA replication and protein translation. Both viral and host factors interact with these UTRs to orchestrate genome replication and protein synthesis[51]. Such coordination is necessary considering the material demand for viral assembly, in which a single particle requires hundreds of proteins but only one RNA genome. Given their regulatory functions, modification of these UTRs to reduce viral replication has become a way to attenuate the virus in vaccine development[52, 53, 54, 55].

Viral proteins

The FV genome encodes a polyprotein that is cleaved into ten proteins (**Fig. 2**)[56]. The 5' terminal genes encode three structural proteins that assemble with viral RNA to become a new viral particle. The remaining genes (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) encode non-structural proteins including replicases, proteases, and other enzymes and cofactors that manufacture a supply of viral RNA and proteins for viral assembly.

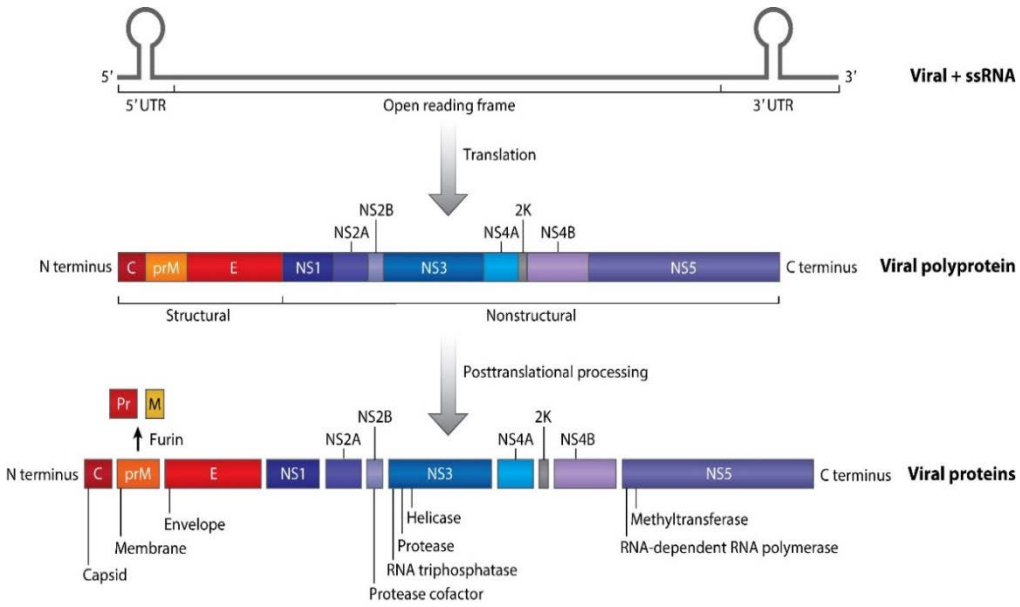

Figure 2. Flavivirus genome and encoded proteins. The 11kb positive sense single-stranded RNA genome contains one open reading frame, flanked by untranslated regions that form conserved secondary structures. The open reading frame is translated into one single polyprotein that is further processed to ten proteins for viral production. The three structural proteins (Capsid, pre-Membrane, and Envelope) are required for viral assembly. The nonstructural proteins (NS) along with host proteases perform a variety of function including RNA processing and synthesis, and post-translational modification. Figure cited[71].

The first to be translated, the capsid protein (C) mediates RNA interaction thanks to its highly charged termini[57, 58]. Complete translation of C guides the elongating polyprotein to the endoplasmic reticulum where viral assembly subsequently takes place. Here, a host signal peptidase cleaves C from the precursor (pre-membrane or prM) of the membrane protein (M)[59, 60]. Upon its synthesis, prM folds rapidly and forms a heterodimer with the envelope glycoprotein (E) while acting as its chaperone[61]. The E glycoproteins encase the viral particle, and mediate attachment and entry to target cells. One hundred and eighty prM/E dimers assemble with one C-bound genome to form an immature viral particle. In the newly assembled particle, prM/E dimers adopt a trimeric arrangement, forming sixty spikes extending radially from the viral surface (**Fig. 3A**).

The pr fragment is thought to prevent E from rearranging into its fusogenic form in the acidic environment of early trafficking compartments[62, 63]. During trafficking, a host furin cleaves the pr fragment from prM, flattening the configuration of E, yielding a mature particle with a smooth surface (**Fig. 1G, 3A**)[64]. Depending on the cell types from which viral production takes place, the maturation state of FV is generally variable in regard to prM : M content ratio, rendering a heterogeneous population of fully mature viruses and partially mature, or mosaic, viruses[44, 65].

On the mature viral particle's surface, one hundred and eighty copies of E form ninety homodimers, with a head-to-toe, or anti-parallel configuration (**Fig. 3**)[66]. Symmetry is also seen in the form of three E dimers lying flat and parallel to one another, forming thirty parallelogram rafts on the viral surface. These rafts are organized around three-fold and five-fold axes by their obtuse and acute corners, respectively (**Fig. 3B**)[67]. Due to its extensive coverage on the particle's surface, the domains and configurations of E are the major targets for the host's antibody response[68, 69, 70].

The FV genome encodes several non-structural (NS) proteins responsible for viral replication (**Fig. 2**). NS1 plays an important role of FV replication, pathology, and immune response. This protein provides the scaffold for the early stage of viral RNA replication, and is thought to augment infectivity of virus transmitted to the mosquito vector during a blood meal[71, 72, 73]. During FV infection, NS1 accumulates in the subject's serum, and therefore can be used as a biomarker for diagnosis[74]. The high level of NS1 secreted from infected cells sequesters antibodies and helps the virus evade the immune response. Nevertheless, the high level of NS1 in the serum also means that NS1 can induce a strong antibody response during acute infection. NS2A is also involved in early

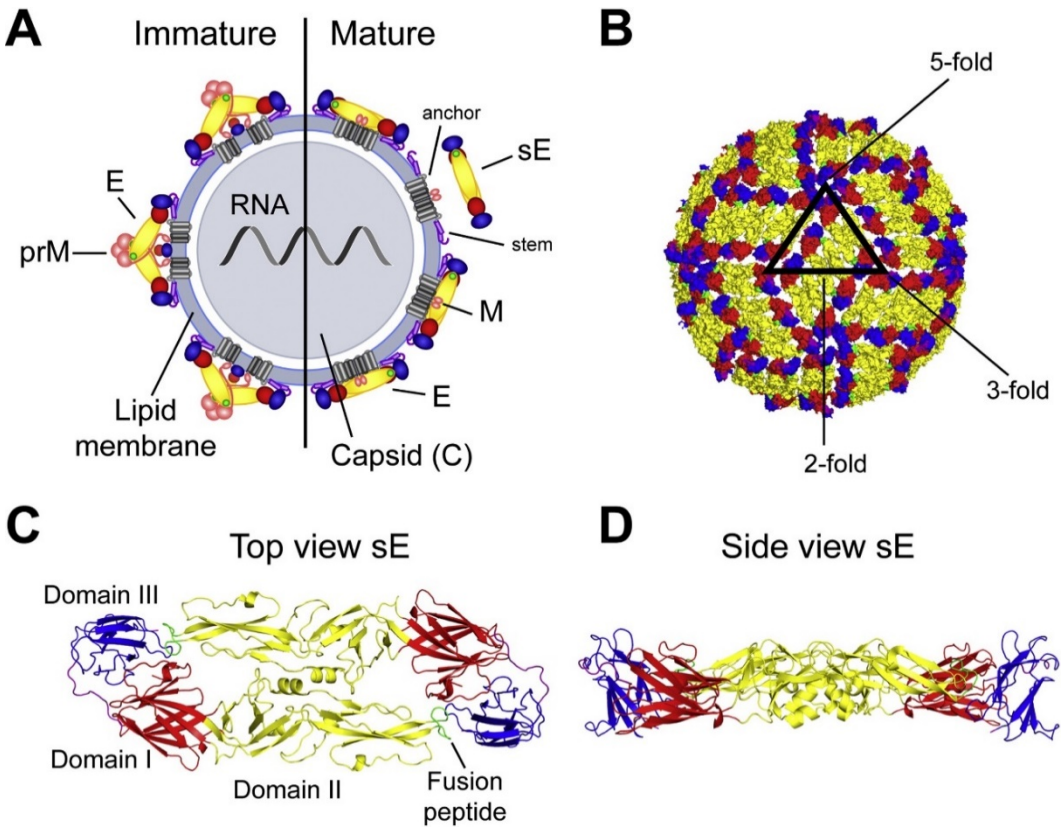

Figure 3. Flavivirus structure. (A) Presentation of immature and mature viral particles. Immature particle is encased in sixty trimeric spikes of prM/E dimers. The proteolytic cleavage of prM re-configures E where the protein adopts a homodimeric arrangement. Ninety E dimers organize in a herringbone pattern, yielding a smooth-surface particle. sE: soluble form of E lacking the membrane anchor and an adjacent stem element. M: proteolytic cleavage product of prM. **(B)** Configurations of E on the surface of mature viral particle determined by cryo-electron microscopy. The triangle indicates 2-, 3-, and 5-fold symmetry axes. **(C & D)** Ribbon presentations of the sE homodimer. Printed in grey-scale version, the E domains (ED) are displayed in shades of grey (EDIII darkest, EDII lightest). The fusion peptide at the tip of EDII, proximal from EDI and adjacent to EDIII of the second monomer. In full color version, EDI red, EDII yellow, EDIII blue, fusion peptide green. Figure cited, legend adapted[68].

viral RNA replication, and both NS1 and NS2A have been shown to induce vascular permeability, contributing to pathogenesis in the host[75, 76, 77]. NS2B acts as a cofactor for the NS2B/NS3 complex, which carries out helicase function in RNA replication, and proteolytic function on the viral polyprotein[78, 79]. NS4A appears to direct the translocation of the adjacent NS4B, and effectively the NS3 replication complex, into the endoplasmic

reticulum lumen, since NS4B interacts with this complex to regulate RNA replication[80]. Both NS4A and NS4B, along with NS2A, help subvert the host antiviral response by antagonizing interferon (IFN) signaling[81]. Finally, NS5 is an RNA-dependent RNA polymerase with 5' cap modification function[82, 83].

Pathology

In humans, FV infection causes a wide range of symptoms, from mild fever and malaise to severe encephalitis and hemorrhagic fever. Severe cases can result in fatality without prompt and sufficient medical attention. All four serotypes of DENV are capable of causing disease. However, about 75% of DENV infections are asymptomatic. Still, approximately 100 million infections manifest clinically every year. The most common form of disease, dengue fever (DF), is a mild flu-like syndrome characterized by rapid onset of fever in combination with headache, arthralgia, myalgia, retro-orbital pain, and a rash. The more severe form of disease, dengue hemorrhagic fever, shows all DF symptoms in combination with thrombocytopenia, coagulopathy, and plasma leakage, which can lead to hypotension and circulatory collapse (WHO).

Like DENV infection, ZIKV infection is often asymptomatic, and clinical cases present with flu-like illness, characteristic of many FV infections. During the 2015-2016 outbreak, it became more apparent that ZIKV infection was associated with the autoimmune disorder Guillain-Barré syndrome in adults, characterized by extremely rapid onset of muscle weakness, motor function disturbance, and ascending paralysis following a minor viral or bacterial infection[84]. ZIKV infection is also associated with neurological abnormalities in neonates of infected mothers. The recent outbreak saw an

increased frequency of neonatal congenital malformation, including microcephaly, which resulted in fetal death in some cases[22, 85, 86, 87, 88, 89, 90, 91, 92].

Innate immunity

Upon transmission from an infected mosquito bite, DENV and ZIKV initially infect myeloid cells in the skin, followed by infection of monocytes and macrophages in the peripheral blood, triggering an antiviral response mediated primarily by these myeloid cells[29, 93]. Viral sensing by numerous extra- and intracellular pattern recognition receptors (e.g., Toll-like receptors) results in production of cytokines and chemokines that induce an inflammatory state. The interferon (IFN) system, particularly type I and II (IFN-α, IFN-β; and IFN-γ, respectively), is the primary defense mechanism during this inflammatory period[69, 93, 94, 95, 96, 97, 98, 99, 100]. These cytokines act in a positive feedback loop on immune cells to induce proliferation, recruitment, and differentiation amongst other immune functions. *In vitro* infection models showed that type I IFN rendered cell lines non-permissive to DENV and ZIKV infections and had the potential to set up an antiviral response. In other studies, upon DENV infection of monocytes *ex vivo*, these cells upregulated expression of proinflammatory cytokines and their receptors, and differentiated into dendritic cells to increase mobility and antigen presentation ability, bridging the adaptive immune response[100]. Similarly, increased expression of type I and II IFN, apoptotic factors, and other proinflammatory cytokines was observed in peripheral blood mononuclear cells of acutely DENV and ZIKV infected subjects, compared to healthy controls[94, 99, 100, 101, 102]. Fittingly, mice deficient of type I IFN response (e.g., *Stat2-/-*, *Ifnα-/-*) have been used as models for experimental DENV and ZIKV infections[103]. These findings emphasize the engagement of the innate immune

response during DENV and ZIKV exposures, which in some individuals restrains the infection to self-limiting disease.

Cellular mediated immunity

The involvement of the T cell response in FV infections and immunization have been demonstrated for both CD4+ and CD8+ T cells[104, 105, 106, 107, 108, 109, 110, 111, 112]. Transcriptomic and phenotypic analyses of T cells during DENV infection showed upregulation of genes involved in activation, proliferation, migration, and toxicity[112, 113, 114]. Responding T cells exhibit clonal expansion, production of proinflammatory cytokines, and cytotoxic killing of infected cells. T cells appear to be important in protection from disease because in DENV- or ZIKV-infected mice, depletion of T cells resulted in enhanced infection, which can be reversed by adoptive transfer of virus-specific memory T cells[107, 115, 116]. In humans, a robust T cell response has been correlated with development of neutralizing antibodies and protection from dengue disease[104, 117, 118]. Additionally, data from dengue endemic cohorts revealed an association between certain HLA alleles and the amplitude of DENV-specific T cells, and protection from severe disease[104, 106, 119, 120]. These findings support the notion that the T cell response can control FV infection and contribute to protection from disease symptoms.

Viral peptide antigen presentation mediated by class I and II HLA molecules on antigen presenting cells drives the virus-specific T cell response. Recent studies mapping the T cell epitopes have revealed immunodominant target viral proteins and shown strong concordance between the epitope profiles of naturally DENV infected subjects and subjects immunized with a live attenuated DENV vaccine[104, 105, 106, 109, 121]. Both the CD4+ and CD8+ T cell responses showed a strong preference towards NS proteins,

although reactivity against peptides from structural proteins was also observed. This trend was consistent in ZIKV-infected subjects, particularly for the CD8+ T cell response, while the CD4+ T cell response targeted both structural and NS proteins equally[122]. Cumulatively, these results suggest that protective T cell immunity in FV infections relies on exposure to both viral structural and NS proteins. As will be discussed below in the context of live attenuated vaccine design, ensuring robust T cell responses to peptide antigens throughout the viral genome (especially NS proteins) during vaccination is likely to be critical to vaccine success.

On the other hand, T cell-mediated pathology has been reported in DENV and ZIKV infections. In line with the original antigenic sin principle, in which pre-existing immunity to a related pathogen (adversely) affects the response to a new infection, CR T cells from a primary DENV infection can be recalled during secondary heterotypic infection. Given enough genetic divergence between the two infecting viral serotypes, these CR memory T cells are predicted to exhibit low affinity to the viral peptides in the secondary infection and therefore are ineffective at controlling viremia. However, these responding cells may produce excess proinflammatory cytokines, leading to cytokine release syndrome. The magnitude of activated T cells has been correlated with severe dengue disease outcome, particularly in secondary infections[123, 124]. Additionally, mouse studies have shown a link between excess infiltration of both CD4+ and CD8+ T cells, and cytotoxic tissue damage in infections by multiple FV species[125, 126].

These findings point to a critical balance in the T cell response to mobilize antiviral functions while limiting immunopathology during DENV and ZIKV infections. Robust proinflammatory cytokine production during acute infection can orchestrate

immune functions to control viral replication, but high levels of these factors present a risk for symptomatic disease in both primary and, especially, secondary exposures.

Humoral immunity

Antibodies are a hallmark of the adaptive immune response and are often associated with protection following viral immunization. In many cases, presence of virus-specific antibodies is an indication of protective immunity against any particular virus[7, 127, 128]. However, the antibody response generated in DENV infection is complex and does not always imply protection. While infection with a single DENV serotype often results in lifelong homotypic immunity against that specific serotype, a secondary heterotypic DENV exposure is often associated with symptomatic disease. This association is thought to be the result of increased viremia due to antibody-mediated viral entry into target cells, or ADE of infection. In ADE, antibodies raised from the primary infection cross-react but are unable to neutralize the heterologous virus in the secondary infection, and promote viral entry via Fc receptor-mediated phagocytosis (**Fig. 4**). Multiple *in vitro* and *in vivo* studies have demonstrated ADE, evident in increased viral output in the presence of reactive but non-neutralizing monoclonal antibodies (mAb), or immune sera at sub-neutralizing dilutions[41, 42, 43, 129, 130, 131, 132]. Due to the shared antigenicity amongst DENV serotypes (60-80% sequence identity), antibodies induced by one serotype has the potential to cross-react with others, implicating ADE risk. The recent reemergence of ZIKV prompted concerns of ADE occurring between DENV and ZIKV since these viruses co-circulate in some endemic regions and share up to 60% sequence identity.

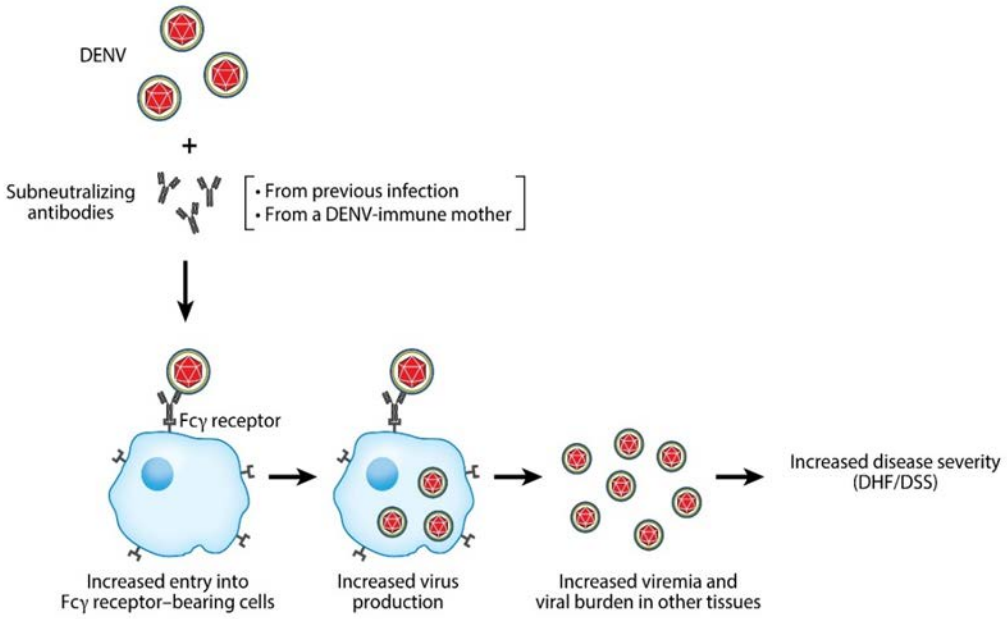

Figure 4. Antibody-dependent enhancement of infection. Binding of antibodies at sub-neutralizing concentration, or of non-neutralizing antibodies, promotes viral infection of target cells. Opsonized virus binds to target cells via Fc receptors, which are abundant on monocytes and dendritic cells. Enhancement of infection results in increased viral replication, which is associated with increased disease severity. Figure cited[71].

Understanding the composition of the antibody response in DENV infection is necessary to understand protection against versus risk for secondary dengue. During a primary DENV infection, the induced antibody repertoire comprises serotype-specific (or type-specific, TS) clones, and CR clones that bind to multiple serotypes, and in some cases, other FV species (**Fig. 5**). Bulk analyses of mAb and immune sera have indicated that CR antibodies were the dominant component following primary DENV infections[68, 133, 134, 135, 136, 137, 138, 139, 140, 141]. However, recent studies from our groups and others have shown, using sensitive serum antibody depletion assays, that there is high prevalence of TS antibodies in primary infections, and that *in vitro* serum neutralization activity strongly correlates with TS antibodies[134, 142, 143, 144]. Several factors should be considered in interpreting the discrepancies between these findings: (1) whether the locales of these

infections were endemic for DENV, which may determine (2) the number of exposures the subjects may have experienced, (3) the timing of the diagnoses, which may influence the proportion of CR and TS antibodies, and (4) the methodologies and availability of reagents, which may impose a limit on experimentation and data interpretation. Nevertheless, emerging evidence suggests that the antibody response generated in primary DENV infections on a FV-naïve background is generally focused on the infecting virus, with high prevalence of TS antibodies.

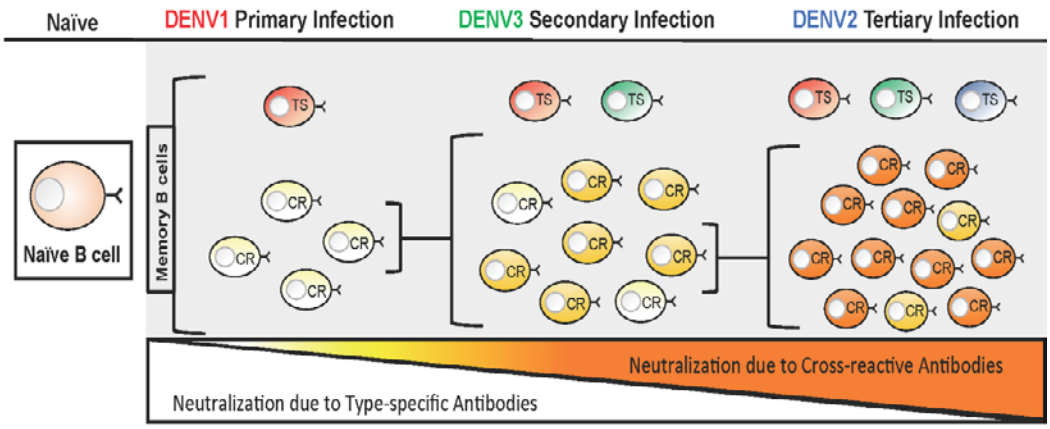

Figure 5. Proposed dynamics of the antibody response in DENV infections. The antibody repertoire following primary DENV infection comprises both TS and CR clones. TS antibodies are thought to confer homotypic protection upon repeated infection of the same serotype of the virus. During secondary heterotypic infection, CR B cell clones in the memory compartment are mobilized in the recall response while new TS clones are added to the repertoire. Post-secondary DENV infection sees high prevalence of CR clones, which are thought to provide cross-protection thereafter. Figure cited[138].

We and others have shown that TS antibodies are often neutralizing, and therefore are thought to confer homotypic protection[68, 133, 134, 142, 145]. The durability of DENV TS antibodies and their function have been shown through the ability of late convalescent sera to neutralize the corresponding virus, even decades after the infection[146, 147]. In chapter II, we provide evidence to support this significance of TS antibodies with the

analysis of the antibody response following primary DENV2 infection. These findings emphasize the role of TS antibodies as a parameter applicable not only in serological testing but also in building and maintaining homotypic immunity.

The roles of CR antibodies are more controversial. Following primary DENV infection, some individuals experience a cross-protection period lasting up to one year[16, 17, 41, 148, 149, 150, 151]. This suggests that CR antibodies may offer short-term protection in conjunction with other immune mechanisms, such as T cells. However, the risk of symptomatic dengue associated with secondary infection increases as serum antibodies wane. Indeed, sub-neutralizing serum antibody titers in endemic cohorts were associated with higher risk for severe secondary dengue[152]. Furthermore, infants born to DENV-immune mothers were at higher risk of symptomatic dengue upon their first exposures, as maternal DENV-specific antibodies waned[153]. In this scenario, non-neutralizing CR antibody clones possibly dominate the recall response and can enhance the secondary infection[136, 137, 143, 154, 155, 156, 157].

There is also evidence suggesting that CR antibodies may confer protection. Epidemiological surveys and serological studies in endemic populations suggest multiple DENV exposures broaden antibody cross-reactivity, increase affinity, and likely improve its protective function against symptomatic dengue (**Fig. 5**)[143, 158, 159, 160]. Older individuals in endemic regions are likely to have experienced multiple infections, and are generally protected from symptomatic disease in subsequent infections. Additionally, CR, neutralizing mAb isolated from peripheral blood of people living in endemic areas have been identified, and shown to have potent activity against all four DENV serotypes as well as other FV species such as ZIKV[161, 162, 163, 164, 165, 166, 167].

These findings suggest the characteristics of the antibody response to DENV infections can be influenced by prior FV exposures and serostatus, but how these characteristics may apply to other FV species, such as ZIKV, is also of interest for vaccine design and understanding development of natural protection. We and others have shown that ZIKV infection in FV-naïve subjects induced an antibody response specific to ZIKV both at the cellular and serological levels[167, 168, 169]. On the other hand, ZIKV infection in FV-experienced subjects induced a more CR antibody response[169, 170]. Conversely, DENV infection in FV-naïve subjects resulted in little antibody cross-reactivity against ZIKV[171]. ZIKV cross-reactivity in immune sera of secondary dengue cases appeared to follow a temporal pattern, in which acute sera were more CR than convalescent sera[171, 172, 173]. These findings suggested that CR antibody clones from previous DENV infections were involved in the recall response during ZIKV infection. The level of CR antibodies depended on the time interval between the two exposures. In the laboratory setting, DENV infection-derived CR antibody-mediated neutralization and ADE for ZIKV have been observed[130, 174, 175, 176], but increased incidence of zika in dengue-endemic populations (possibly due to ADE) has not been reported. In fact, pre-existing DENV immunity may protect against ZIKV infection. One recent epidemiological study showed an association between high DENV antibody titers and reduced risk of ZIKV infection and symptoms, suggesting preexisting FV immunity may influence the infection outcomes for individuals and local transmission for the virus[177].

These findings emphasize the importance of dissecting the antibody composition following DENV and ZIKV infections. Continuous efforts have shed light on the evolving dynamics of both TS and CR antibodies following one and multiple infections

by these viruses. This understanding will be beneficial for development of vaccine strategies, for both endemic populations and for FV-naive subjects. Both TS and CR antibodies can play significant roles in providing protection. Therefore, eliciting the proper clones of these antibodies, with preferred specificity, will be important in building immunity, particularly skewing the antibody quantity and quality towards neutralization and away from ADE. More than ever, investigators are paying attention to the epitopes and lineages of neutralizing antibodies to broaden our understanding of the response, while refining vaccine immunogens that will induce a more focused antibody-repertoire against neutralizing epitopes.

Epitopes of DENV- and ZIKV-specific antibodies

Because antibodies have been shown to have both protective and infection-enhancing roles in FV infections, mapping specific epitopes is important to understand their structural-functional relationship. Antibodies generated from FV infections target both viral structural and NS proteins. The major targets for FV antibodies are the E and prM proteins due to their coverage on the viral surface, and NS1 protein due to its abundant expression in, and secretion from, infected cells[67].

Each E monomer is composed of three β sheet-rich domains (EDI/II/III) (**Fig. 3C)**[178]. EDI forms a β barrel that functions as a hinge between EDII and EDIII. The elongated EDII lies parallel to the surface of the mature viral particle (**Fig. 3**). Concealed at EDII's tip, distal from EDI, is the fusion peptide that facilitates fusion between the viral and host endosomal membranes. EDIII is an immunoglobulin-like protein domain responsible for cellular receptor binding. Anti-E antibodies have been mapped to epitopes on all three domains of the protein (**Fig. 6A**). EDIII-specific antibodies have been shown

to be strongly neutralizing, although they are rare in DENV-immune human sera[68, 145, 163, 179, 180, 181, 182, 183, 184, 185, 186]. On the other hand, EDIII-specific antibodies are abundant after ZIKV infection, and exhibit high binding and neutralizing titers[167, 187]. As EDIII is the least conserved domain across FV species, EDIII-specific antibodies are usually TS to the infecting virus or serotype[68, 145].

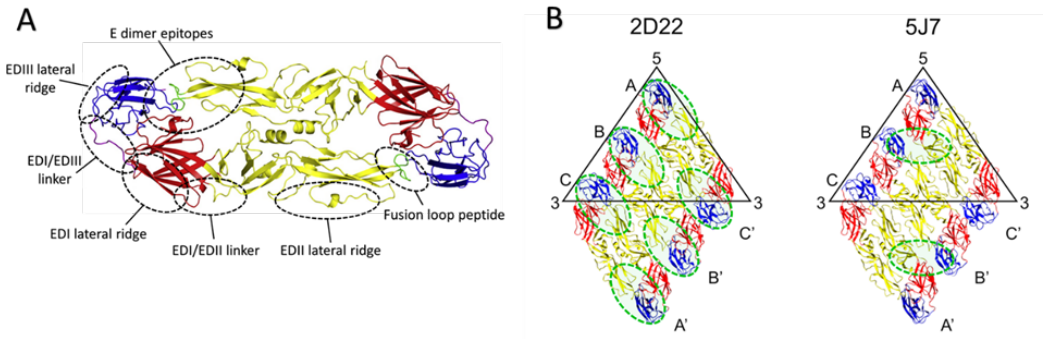

Figure 6. Antigenic determinants of the antibody response. (**A**) Footprints of different antibody specificities on monomeric and dimeric E are depicted with dotted ovals. (**B**) Specific epitopes (green dotted ovals) of DENV-specific mAb 2D22 and 5J7 spanning two and three E monomers in the icosahedral asymmetric units (black triangle) within an E raft. Numbers indicate symmetry axes; letters indicate E monomers in asymmetric units. Figure adapted and cited[70].

The more conserved fusion loop is another immunodominant epitope, but its accessibility is dependent on the maturation state of the virus. Accessibility of this epitope increases by the presence of uncleaved prM on the viral surface[188, 189]. On the surface of mature virus, the fusion loop is buried in the E-dimer interface, making the epitope less accessible to antibodies (**Fig. 6A**)[190, 191, 192]. Therefore, antibodies against the fusion loop, while usually CR, are generally poorly neutralizing, especially against mammalian cell-produced virus from which the content of prM is lower than in insect cell-produced virus[65]. In chapters IV and V, we have expanded the paradigm of ZIKV TS mAb with our discovery of highly potent clones that bind across multiple E domains.

While many neutralizing antibodies have been mapped to specific domains of the E protein, the footprints of E-specific antibodies can expand beyond a single E monomer (**Fig. 6**). Virus binding of these colloquially termed "quaternary antibodies" generally requires the E protein to adopt its quaternary form on the surface of intact virus[134, 142, 168, 193, 194, 195]. The DENV2-specific mAb 2D22 binds to the E dimer interface and has substantial contact with EDIII of one of the monomers (**Fig. 6B**)[195, 196]. The DENV3-specific mAb 5J7 binds three adjacent E monomers and engages three E domains, one from each monomer (**Fig. 6B**)[197]. Recently, a class of CR potently neutralizing antibodies was discovered, which target conserved quaternary E epitopes across EDI/EDIII of one monomer and EDII of the other in an E dimer (named E-dimer epitope or EDE) (**Fig. 6A**)[44, 171, 198, 199]. The footprints of these antibodies span across the prM/E interface and the conserved fusion loop, explaining why this group of antibodies is CR. And while their binding footprint covers the fusion loop, binding of these antibodies is not influenced by the maturation status of the virus, as EDE antibodies neutralize viruses produced from both insect cells and primary dendritic cells.

Antibodies against the prM protein account for a substantial fraction of the response in humans. While most prM antibodies are CR, they are poorly neutralizing[44, 140, 44]. One explanation for their weak neutralization activity is that many viruses produced by mammalian cells have low to no uncleaved prM content due to the host cells' furin activity. Increase in uncleaved prM content (in insect cell-derived virus), which results in increased accessibility to the fusion loop, drives the generation of the two groups of CR, poorly neutralizing antibodies (e.g., anti-prM and anti-fusion loop)[200].

Thus, these antibodies can be considered an immune evasion mechanism that also helps increase viral replication via ADE.

Finally, antibodies to NS1 protein are also a major component of the antibody response in humans[67]. NS1 are found on the surface of infected cells, as well as released from these cells, and therefore can induce antibodies during acute infection. Recent findings have implicated the role of NS1 in pathogenesis, where these proteins directly cause weakening of endothelial tight junctions, leading to vascular permeability[201, 202, 203]. Antibodies against NS1 have been shown to protect mice from lethal DENV and ZIKV challenges[201]. As NS1 is not accessible on the surface of the virus, anti-NS1 antibodies' mechanisms of action are not inhibition of the virus per se, but sequestration of soluble NS1 and mediating cytotoxic killing of infected cells by binding to cell surface NS1[204, 205, 206].

By revealing the immunodominant antigens and vulnerabilities of the virus, epitope mapping studies have made a strong case for understanding the nuances and the complexity of the antibody response to DENV and ZIKV infections. Defining antibody-virus functional interactions can help us to understand the roles of these antibodies in protection versus infection enhancement. This work will be critical for the process of designing vaccine immunogens, to selectively induce specific groups of antibodies that will promote protection. On the other hand, the existence of immunodominant antigens implies evolutionary interaction between the virus and the human antibody repertoire, highlighting the existence of neutralizing antibody clones that have common specificity in the human population[168, 207]. As single-cell analytic methodologies have become available to study antibody repertoire, there is interest in diving into the human

immunome to study common B cell lineages that may have inherent neutralizing attributes against the virus and confer protection.

Evolution of humoral immunity

Durable antibody-mediated protection against virus relies on a repertoire of B cells expressing immunoglobulin (Ig) with high viral antigen affinity that persists over many years to ensure a rapid and robust response upon reinfection. Extensive studies have demonstrated the involvement of the antibody response by describing the dynamics of different B cell sub-populations from acute to convalescent phase following viral infections[134, 208, 209, 210]. These studies have provided a broad perspective on how the antibody response engages, evolves, and persists for years after the infections.

During acute infection, naive B cells traversing through secondary lymphoid organs are primed by antigens binding to surface Ig. Primed B cells proliferate and differentiate following a number of routes (**Fig. 7**). The earliest B cell response to virus involves short-lived, proliferating plasmablasts (PB) that secrete antibodies, forming the first wave of the antibody response[100, 210, 211, 212]. We and others have defined the kinetics of this transient PB response in DENV infection (Chapter II) and vaccination (Chapter III), and in other viral infections[134, 135, 137, 154, 213, 214, 215, 216]. We found that DENV2 induced PB within two weeks after infection, and live attenuated vaccination induced PB within three weeks. The PB response is stimulated by viral replication, and therefore comprises a great percentage of virus-specific clones[134, 213, 216, 217]. Interestingly, recent study has shown that PB are not the only early B cell responders during acute infection. Following influenza vaccination and Ebola infection, another sub-population of B cells arose, termed activated B cells (ABC), with phenotype and temporal kinetics distinct

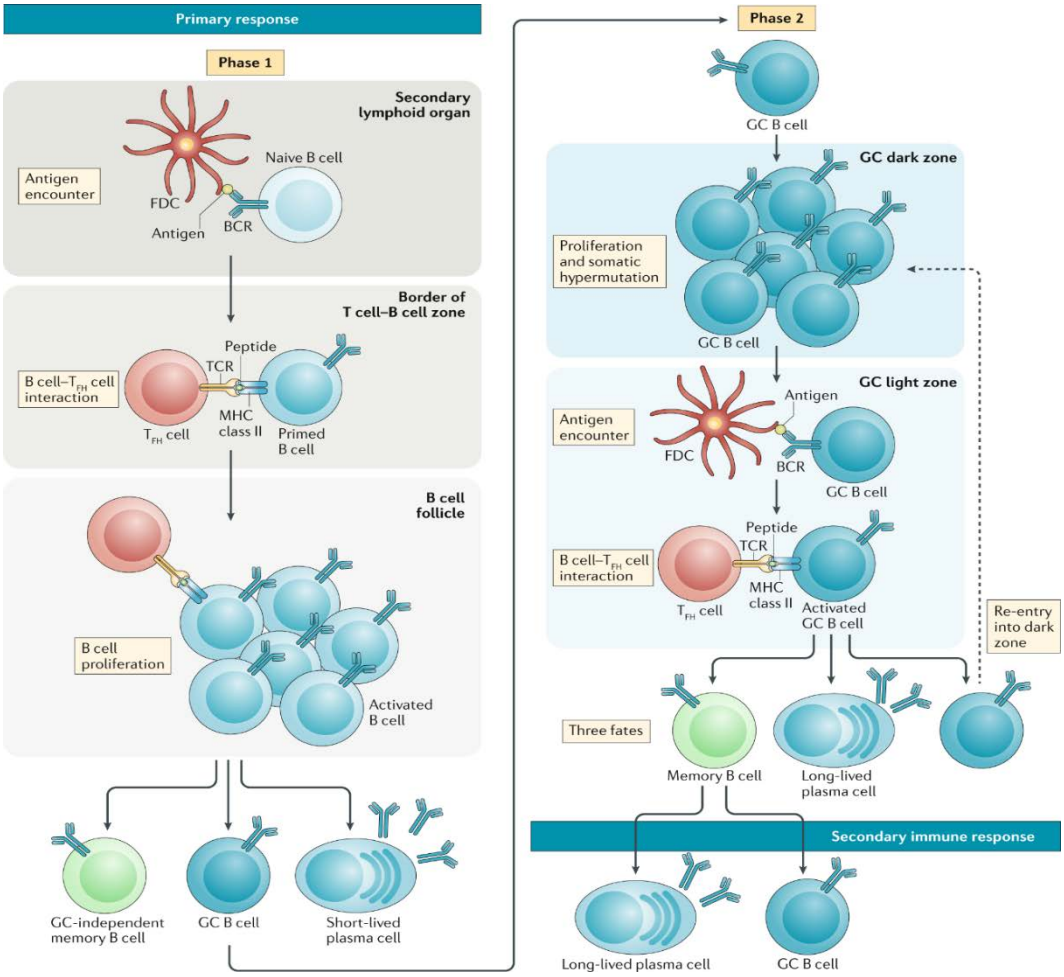

Figure 7. Two phases of the acquisition of B cell memory. In phase 1 of the primary response (left), naive B cells exit the circulation, enter B cell follicles in the secondary lymphoid organ and survey the environment for antigen. Antigens encountered on follicular dendritic cells (FDCs) activate B cells through the B cell receptor (BCR), and the antigens are processed and presented to T cells at the T cell–B cell border, driving naive B cells to proliferate and differentiate into three main cell types: germinal center (GC)-independent MBC, GC B cells or short-lived PC. In phase 2 of the primary response (right), newly differentiated GC B cells form GCs and undergo proliferation and somatic hypermutation in the dark zone before exiting to the light zone, where the GC B cells encounter antigen on FDCs, present the antigen to T follicular helper cells (T_{FH} cells) and undergo three main fates: namely, differentiation into MBC, differentiation into LLPC or re-entry into the GC dark zone. In the secondary response (bottom right), MBC respond to antigen and differentiate into LLPC or GC B cells that undergo GC reactions. TCR, T cell receptor. Figure and legend cited[225].

from those of PB[210]. Transcriptomic and clonal analyses proposed that PB differentiated into long-lived plasma cells (LLPC), and ABC into memory B cells (MBC)[210]. These studies raise the curiosity about the precursors and the fates of these acute B cells, and beg the questions whether there are other unidentified B cell sub-populations engaged during an infection and how much they contribute to the humoral response.

Through the course of the infection, a portion of primed B cells migrate to the B cell follicles within the secondary lymphoid organs to establish germinal centers (GC) (**Fig.7**). Here, primed B cells interact with follicular dendritic cells and helper T cells, undergo extensive proliferation, cycle through multiple rounds of somatic hypermutation (SHM), and switch their isotypes from IgM to others such as IgG or IgA. Virus-specific IgE antibodies to DENV or ZIKV are not known. The GC reaction produces B cells whose Ig have acquired high affinity for the infecting virus and functionally relevant isotypes[218, 219, 220, 221, 222, 223]. GC survival B cells differentiate into antibody-secreting plasma cells (PC) and MBC. PC home to the bone marrow niche and become LLPC, while MBC populate secondary lymphoid organs and circulate in the peripheral blood. The formation of these two memory compartments establishes two layers of the humoral defense: (1) high-affinity antibodies from sessile LLPC readily opsonizing virus upon reinfection, and (2) MBC re-instigating SHM to diversify and boost other virus-experienced B cell populations[224]. Both of these defense layers are established in DENV and ZIKV infections. Virus-specific MBC in peripheral blood are detected within a few months following the infection, and can persist, along with serum antibodies, for years afterward[138, 140, 146, 147]. Understanding of their characteristics and connections is critical to understand how the antibody response changes with every FV infection, and to

decipher whether the changing specificity of the antibody repertoire may result in protection or become a risk.

A different portion of primed B cells appear to bypass the GC reaction to become GC-independent (GCI) MBC earlier than classic GC MBC (**Fig. 7**)[225, 226, 227]. The majority of GCI MBC are germline-encoded, thus low-affinity, and maintain IgM isotype. Upon reinfection, they have the potential to establish GC, although their contribution to the secondary antibody response following homologous infection appears minimal. Using a prime/boost model in mice, Mesin and colleagues showed that upon IgY rechallenge, the sub-population of these presumably GCI MBC was largely untapped to seed new GC[228]. Instead, a small number of GC MBC clones with high affinity for IgY populated the new GC during the recall response, alongside likely-naive B cells which occupied more than 90% of the new GC. This finding showed that the antibody-secreting cell population recalled from humoral memory may only represent a small fraction of the memory B cell breadth. This notion helps explain why there was little clonal overlap between the PB and MBC populations following DENV infections in human subjects[214]. In fact, germline-like mAb have been identified in DENV and ZIKV infections, and shown to have high affinity and potent neutralization capability despite having little SHM[163, 229, 230]. The significance of these GCI MBC warrants further exploration. One speculation is that their high diversity and low affinity may pre-authorize these cells to be promptly mobilized, and readily undergo SHM upon infection with variants of the virus[226].

For viruses with multiple variants such as DENV, sequential heterotypic infections can influence the quality of the antibody response. The antibody response to

DENV generally becomes more CR following sequential heterotypic infections (**Fig. 5**) [44, 137, 143, 154, 214, 216, 231]. Similarly, ZIKV infection on a DENV-immune background saw a broadened CR antibody repertoire[162, 167, 169, 232]. These observations can be explained by new TS B cell clones joining the memory compartments following each new infection. Alternatively, but not exclusively, each new heterotypic infection can boost the existing CR clonal pool and hone the antibody repertoire to include more of these clones, consistent with the original antigenic sin principle. Longitudinal studies using immune sera and mAb isolated from naturally infected subjects suggested the latter scenario may be the paradigm. Evidently, upon ZIKV and DENV infections, FV-experienced subjects had a higher level of PB with higher rate of SHM compared to FV-naïve subjects, suggesting memory origin of these cells[137, 169]. In either case, new PC will need to populate the bone marrow niche to maintain durable serological response and potentially alter the existing clonal repertoire[211, 233, 234]. However, the bone marrow niche for LLPC is limited, with these cells making up less than 1% of the total cellular component[235]. Thus, there must be a regulatory mechanism that maintains the existing LLPC population while accommodating new incoming clones. Hofer and colleagues mathematically posited that such a mechanism relies on the number and magnitude of PB influx or invasions as a percentage of resident LLPC. Nevertheless, studies on tetanus and measles immunization indicated that it is likely that LLPC residence could withstand hundreds of PB invasions, which would extend beyond a human lifespan, assuming an average of four infections a year, before any significant reduction in the existing virus-specific antibody response[233]. If this premise holds true, then the bone marrow niche can accommodate both new TS clones and expanded existing CR clones in repeated FV infections.

Controlled human infection studies utilizing attenuated DENV are underway to answer the specific question whether new TS B cell clones are invoked and join the memory compartment or whether the existing CR MBC clones are recalled and expand during the secondary response.

These lines of evidence emphasize the intimate relationship between different B cell sub-populations through the course of a viral infection (**Fig. 8**). They added nuances to the conventional understanding of priming and boosting. This current understanding,

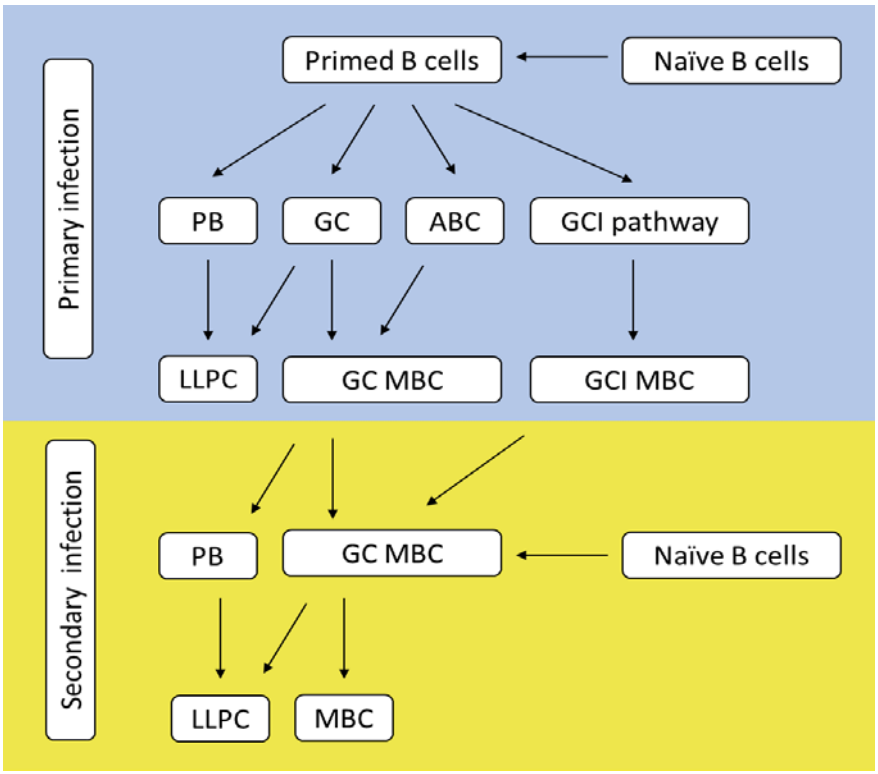

Figure 8. Proposed dynamics of B cell sub-population in sequential infections of viral variants. In acute primary viral infection, primed B cells follow several routes of differentiation to become short-live PB (PB), germinal center B cells (GC), activated B cells (ABC) and germinal center-independent B cells (GCI pathway). At convalescence, the acute B cell sub-populations become long-live plasma cells (LLPC) and GC MBC which have high affinity for the primary virus and switched isotypes, and IgM+ GCI MBC with low affinity. Upon a secondary infection with a different variant of the virus, classic GC MBC can quickly differentiate into (recalled) PB, which can further enrich the LLPC pool. Both GC MBC and GCI MBC can seed the secondary germinal centers along with naïve B cells to further enrich the LLPC and MBC repertoires.

while useful to establish a mechanistic paradigm of humoral response, is restrictive considering the viral or antigenic heterogeneity. Such extension of our understanding will guide further exploration of the antibody response in FV infections, and also other diverse viral genera. For FV, we have only begun to unpack the complexity of the antibody response in DENV infection in recent years with the availability of controlled human infection models and improved specificity in diagnostic testing. Further investigation is necessary to fully understand how each heterotypic infection drives the antibody response, or how vaccination with all four serotypes configures the response.

Neutralization mechanism of DENV- and ZIKV- specific antibodies

The paradoxical roles of FV antibodies raise an interesting question - what are the factors that determine the activity or function of the antibodies? For DENV- and ZIKV-specific antibodies, their activities can generally be predicted by their epitopes, as discussed previously in the case of EDIII-targeting antibodies being both TS and neutralizing. Therefore, depending on their epitopes, neutralizing antibodies probably have different mechanisms of action - inhibiting the virus at different steps in the viral life cycle. As more mAb are added to the FV research arsenal, researchers have begun to examine how neutralization occurs, especially for DENV and ZIKV, when the line between neutralization and ADE is still unclear.

For DENV and ZIKV, sub-neutralizing dilutions of immune sera or concentrations of mAb can promote ADE of infection, suggesting a stoichiometry threshold for neutralization[152, 236, 237, 238, 239, 240]. In that case, it is possible that the neutralizing antibodies may inhibit contact between the virus and its cellular receptors. Neutralizing antibodies against EDIII, which is involved in cellular receptor binding,

have been shown to block viral adsorption in cell culture. He and colleagues suggested attachment blockade may be a major mechanism of serum antibody neutralization of DENV[241]. A recent study reported viral aggregation as another mode of pre-viral-attachment neutralization. The CR mAb SIgN-3C bound to an epitope spanning the interface of two E dimers and neutralized ZIKV by aggregation[242]. Aggregation of viral particles effectively reduces the infectivity load in the cellular milieu, and probably causes steric hindrance to inhibit binding of the virus to its cellular receptors[243, 244].

In other scenarios, neutralizing antibodies can exert their functions at post-attachment or post-entry steps during the viral life cycle[243, 244]. The DENV CR EDE mAb C10 (epitope depicted in **Fig. 6A**) locks the quaternary conformation of E proteins as they are on the viral surface, preventing E rearrangement at lower pH[245]. By stabilizing the flat configuration of E on the viral surface, C10 likely inhibits fusion between viral and host endosomal membranes. The ZIKV mAb ZKV-116, while targeting a much simpler epitope on the EDIII lateral ridge (epitope depicted in **Fig. 6A**) compared to C10 epitope, also inhibits membrane fusion[246]. Alternatively, post-attachment neutralizing antibodies can function to inhibit genome uncoating, and perhaps even viral release as demonstrated in other viral infections[243, 244].

Several studies have demonstrated that certain mAb can neutralize virus through multiple modalities. The C10 mAb effectively neutralizes DENV and ZIKV both pre- and post-attachment at incredibly low concentrations. The mAb SIgN-3C neutralized DENV2 by preventing membrane fusion, while neutralizing ZIKV by aggregation[242]. From the pathogen-centric perspective, this mechanistic flexibility of the mAb SIgN-3C signified an “escape” by the ZIKV species since neutralization potency against ZIKV was

about 10-fold lower than that against DENV2[242]. However, since SIgN-3C arose from a DENV infection, it is likely that the humoral response had cast a wide net in anticipation of a future variant infection.

These finer mechanistic details remain a topic of intense investigation for FV. Current efforts integrating structural and immunological studies will continue to define the modes of action for DENV- and ZIKV-specific neutralizing antibodies. These studies will continue to reveal whether superior neutralization activity can be correlated to specific epitopes, which will in turn inform immunogen design in vaccine development. In addition, it is important to recognize that antibody-mediated neutralization in a physiological context is the result of polyclonality and therefore synchronization or cooperation between different antibody clones is realistic and should be considered[247].

The significance of understanding antibody mechanisms of action lies in the mechanisms themselves as well as in their therapeutic potential. Recent studies have explored strategies to reinforce or boost mAb functionality with multi-specificity recombination[248, 249, 250]. Such an approach can ensure that the virus is countered at different conformational states as it progresses through the infection cycle. Incorporating the principle of polyclonality in the form of multi-specific antibodies can also significantly diminish the stochastic probability of escape mutants. Wang and colleagues showed the bi-specific ZIKV antibody FIT-1 effectively neutralized ZIKV, protected mice from lethal challenge, and prevented ADE and escape mutation[248]. Shi and colleagues showed improved neutralization capability of the bi-specific antibody DVD-1A1D-2A10 against all four DENV serotypes. DVD-1A1D-2A10 improved protection for mice in lethal challenges, compared to administration of individual clones or

combination of both[250]. Furthermore, the Fc regions of these antibodies can be engineered to improve half-life, but also abrogate ADE. A double amino acid substitution (L234A/L235A) in the Fc domain reduced binding of the engineered mAb to Fcγ receptors and therefore prevented ADE[198, 251, 252, 253, 254]. A triple amino acid substitution (M252Y/S254T/T256E) in the Fc domain of the mAb MEDI8897* (targeting respiratory syncytial virus) improved the mAb half-life by 3-fold in the physiological condition[255]. Detailed knowledge of neutralizing antibody epitopes and mechanisms of action will promote more antibody-based therapies such as FIT-1 and DVD-1A1D-2A10, and serve as refined markers of vaccine immunogenicity.

Towards safe and efficacious vaccines

Development of a safe and efficacious DENV vaccine is a complex challenge. A vaccine must induce durable immunity, and a balanced immune response across all four serotypes of the virus. An unbalanced vaccine that favors a response towards a single serotype could mimic a primary infection and predispose a FV-naïve subjects to vaccine-enhanced disease, akin to secondary dengue caused by ADE. Therefore, the consensus is to include all four DENV serotypes in the formulation. Multiple vaccine platforms, including DNA, subunits, inactivated and live attenuated vaccines, are at different stages of development[5, 256].

The most advanced candidates are tetravalent live attenuated vaccines, which are in or beyond phase III clinical trials in endemic countries (**Fig. 9**). CYD-TDV (Dengvaxia) is a licensed chimeric tetravalent formula built on the genomic backbone of the efficacious YFV 17D vaccine[257]. Each DENV component in Dengvaxia comprises the coding sequences for DENV E and prM proteins. In clinical trials, Dengvaxia induced

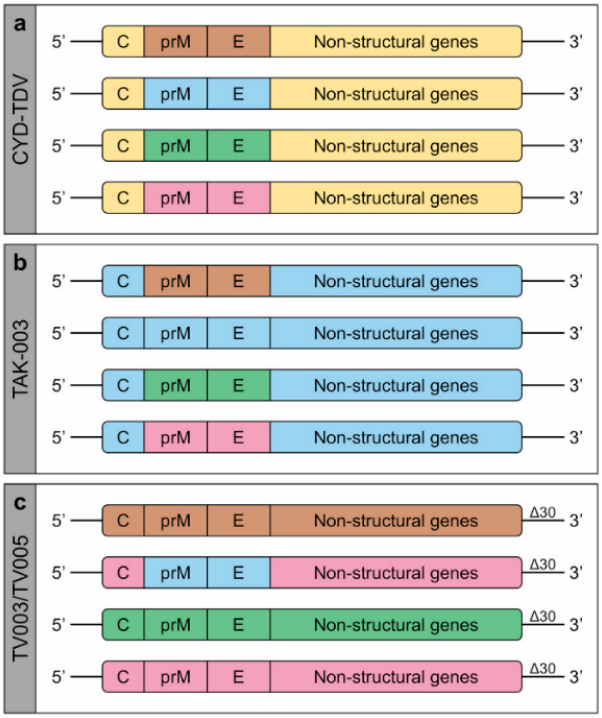

Figure 9. Live attenuated Dengue vaccines. (A) CYD-TDV employs the YFV-17D vaccine viral genomic backbone to express DENV structural proteins. **(B)** TAK-003 uses a DENV2 genome chimerized with genes encoding structural proteins of other serotypes. **(C)** TV003/TV005 are attenuated by30-nucleotide deletion in the genomic 3'-UTR. For sufficient attenuation, DENV2 component is a chimeric virus on DENV4 genomic backbone, with DENV2 structural genes. Figure cited[257].

modest, but stronger protection against disease by DENV3 and DENV4, and the least protection against DENV2[258, 259, 260, 261, 262]. The vaccine had higher efficacy in subjects with pre-existing DENV immunity, suggesting immunization may have broadened existing immunity rather than eliciting complete protection[263]. In DENV-naive subjects, who tended to be younger but are found across all age groups, the Dengvaxia-induced antibody response was highly skewed towards that of a primary monovalent DENV4 infection. For these reasons, Dengvaxia is recommended for limited age groups (>9 years of age) living in dengue endemic areas and with evidence of pre-existing DENV immunity in order to avoid adverse illness. The other two live vaccine candidates are in phase III clinical trials and have shown promising results. Similar to Dengvaxia, the second candidate, TAK-003, was developed using an attenuated DENV2 genomic backbone chimerized with structural components of other serotypes. Efficacy of TAK-003 against symptomatic disease and hospitalization varied by serotypes but was the

highest against DENV2, consistent with the bias in serum neutralizing antibody response towards DENV2 [264, 265]. Longer term follow-up will reveal whether TAK-003 promotes vaccine-enhanced dengue for DENV1, 3, or 4, akin to Dengvaxia. Currently in phase III trial, the third candidate, TV003 (with which we are working), comprises three heterotypic components of attenuated DENV1, 3 and 4, and a chimeric DENV4 backbone with DENV2 structural components. Early results indicate that TV003 induces a balanced antibody response and protects immunized subjects from symptomatic illness in a controlled DENV2 challenge[266, 267, 268, 269].

In addition to live vaccines, subunit and other inactivated formulations continue to be of interest due to simpler logistics (relaxed refrigeration and stability requirements), and the lack of genetic reversion risk[5]. These strategies focus on the viral E protein as the immunogen because the protein contains epitopes for neutralizing antibodies[180, 270180, 270]. The caveat is that the antibody repertoire generated from natural infection is much more diverse and complex, and targets E in its multiple configurations. Indeed, a tetravalent subunit vaccine even with an adjuvant induces modest serum neutralizing antibodies in humans[271], but boosts antibody levels in subjects previously vaccinated with the live attenuated tetravalent vaccine candidates TV003 or TV005[272]. Optimization of vaccine platforms focusing on soluble E as immunogen can greatly benefit from epitope mapping and structural studies to theoretically restrict the antibody response to only neutralizing epitopes. Other formulations with inactivated virus, or particle-based approaches can produce a broader antibody repertoire that better resembles the response in a natural infection. However, there is argument to be had as to whether a vaccine-induced broad antibody repertoire is necessary. Compared to live vaccines, the shortcoming of

these approaches is a minimal or absent T cell response, which is thought to be important in providing protection, as previously discussed.

Most investigations of ZIKV vaccines are in early stages. Generally, the efforts are similar to those used for DENV vaccine development, and in some cases built upon the DENV vaccine platforms. One of the more advanced candidates, the purified inactivated ZIKV vaccine ZPIV with alum adjuvant, has shown encouraging results in phase I clinical trials[273, 274]. ZPIV induced robust neutralizing antibodies although the response was not durable. The vaccine also broadened the antibody repertoire in subjects with pre-existing DENV immunity, producing potent CR neutralizing antibodies against the two viruses[162, 273]. Most other candidates, while promising, are still in preclinical stage and will need to overcome the challenge of adapting from animal models to human subjects. Since ZIKV and DENV are closely related and co-endemic, another obstacle to overcome for clinical evaluation of the vaccines is to deconvolute the CR antibody response. Development of more specific assays are underway to circumvent this issue. ZIKV vaccine development, therefore, is benefiting from the many lessons in DENV vaccine research. With ZIKV having only one serotype and little antigenic shift, global protection against this virus is feasible once an efficacious vaccine is produced.

From the host-centric perspective, recent studies have provided evidence of convergent evolution in the antibody response to DENV and ZIKV infections. (1) Investigators continue to uncover immunodominant epitopes targeted by neutralizing antibodies that are representative of the population's response[168]. (2) Antibody repertoire analyses and characterization of neutralizing mAb have identified common clonal lineages amongst human subjects[134, 187, 207, 208, 230, 246]. These neutralizing mAb utilized the

same IGVH/IGVL genes, suggesting an evolutionary defense against the virus, focusing on conserved epitopes. It is however necessary to identify and establish a pool of these lineages to achieve good coverage for the population as the antibody repertoire in humans is exceptionally diverse. Recent discoveries of germline-like CR neutralizing mAb strengthen the case that good population coverage is achievable[163, 229, 230]. These findings point to the possibility of immunogen designs that can engage these common B cell lineages to produce neutralizing antibodies. This endeavor can benefit from predecessor studies in HIV vaccine design[275, 276, 277, 278]. Steichen and colleagues reported on a methodology of HIV immunogen design to target common germline antibody lineages with the hope of inducing broadly neutralizing clones after affinity maturation has occurred[277, 278]. This integrative approach offers new opportunities for general vaccine development and holds a promise that perhaps a pan-FV vaccine is possible in the future.

Aims of the dissertation

Understanding the complexity of the antibody response generated in FV infections, particularly by DENV and recently ZIKV, has been a major research objective. This complexity challenges the conventional paradigm of using antibodies as a correlate of protection following infection and immunization of these viral pathogens. In these contexts, antibodies can provide protection as well as confer disease risk. To expand the current state of knowledge and apply new discoveries, the FV community has expanded the focus from not only understanding the general components of antibody immunity, but also to identifying neutralizing antibodies with particular specificities. The ability to design vaccine immunogens to elicit such antibodies, or to develop these

antibodies into therapeutics, will move us closer to gaining control of DENV and ZIKV transmission, and to effectively treating acute diseases.

In the following chapters, I provide a comprehensive view of how different components of antibody immunity are shaped by DENV infection and immunization, and by ZIKV infection. These studies expand on the definition of "antibody response" by exploring the cellular components that produce antibodies, and examining these components over time. These studies deconvolute the complex concept of antibody immunity to identify some of the determinants of the response such as PB and TS antibodies; and moreover, determinants associated with antibody neutralization and disease protection. In studying primary DENV (Chapter II) and ZIKV (Chapter IV) infections, we determined a strong focus of the antibody response on the infecting virus. In studying tetravalent DENV immunization, we determined that multivalent exposures result in an antibody response with broadened specificity against the viral variants, and defined a potential cellular determinant (PB) for vaccine-induced protection from dengue disease. In Chapters IV and V, we determined some of the antibody determinants contributing to virus neutralization at the molecular level. These studies contribute to almost a century of research endeavor to enrich our collective knowledge of how immunity against FV is established. They provide several considerations in appreciating the evolution of antibody immunity from exposure to convalescence, and also reveal insights into how novel immune correlates such as PB or the proportions of TS/CR antibodies may be useful in understanding vaccine efficacy.

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CHAPTER II – LONGITUDINAL ANALYSIS OF ACUTE AND CONVALESCENT B CELL RESPONSES IN A HUMAN PRIMARY DENGUE SEROTYPE 2 INFECTION MODEL

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Abstract

Acute viral infections induce a rapid and transient increase in antibody-secreting plasmablasts (PB). At convalescence, memory B cells (MBC) and long-lived plasma cells (LLPC) are responsible for long-term humoral immunity. Following an acute viral infection, the specific properties and relationships between antibodies produced by these B cell compartments are poorly understood. We utilized a controlled human challenge model of primary Dengue virus serotype 2 (DENV2) infection to study acute and convalescent B cell responses. The level of DENV2 replication was correlated with the magnitude of the PB response. Functional analysis of PB-derived monoclonal antibodies (mAb) showed that the DENV2-specific response was dominated by cells producing DENV2 serotype-specific (TS) antibodies. DENV2-neutralizing antibodies targeted quaternary structure epitopes centered on domain III of the viral envelope protein (EDIII). Functional analysis of MBC and serum antibodies from the same subjects at 6-month post-challenge revealed maintenance of the TS response in both compartments. The serum response mainly targeted DENV2 TS epitopes on EDIII. Our data suggest overall functional alignment of DENV2-specific responses from the PB, through the MBC and plasma cell compartments following primary DENV2 inflection. These results provide enhanced resolution of the temporal and specificity of the B cell compartment in viral infection, and serve as framework for evaluation of B cell responses in challenge models.

Introduction

Dengue viruses (DENV) transmitted by mosquito vectors are the etiological agents of dengue fever and dengue hemorrhagic fever[1, 2]. It is estimated that DENV cause 390 million infections annually, mostly in the tropical and subtropical regions of the world[3]. With more than 100 million apparent cases, DENV infections are the leading cause of pediatric hospitalization in many afflicted countries.

The DENV complex consists of four closely related serotypes (DENV1-4). Following a natural primary DENV infection, high levels of circulating homotypic neutralizing antibodies, or serotype-specific (TS) antibodies, against the infecting serotype are elicited[4] and maintained for decades[5], suggesting a contribution to lifelong homotypic protection. Serotype cross-reactive (CR) antibodies also elicited by primary infection are thought to provide transient cross-protection against secondary heterotypic infection with a new serotype[6]. More remote (years) secondary heterotypic infections are associated with a greater risk of developing severe dengue hemorrhagic fever[7, 8, 9, 10]. The increased risk of severe disease in secondary cases appears to be driven, at least in part, by CR, non-neutralizing antibodies that can enhance viral infection[11]. The temporal relationship between early and late B cell responses, and maintenance of serotype specificity following primary DENV infection are incompletely understood.

Plastmablasts (PB) are an antibody-secreting B cell population present at low levels in healthy peripheral blood but can proliferate dramatically following viral infection or vaccination[12, 13, 14, 15]. High levels of PB have been documented during acute DENV infection[16, 17, 18]. Later after recovery, long-term protective antibody responses are maintained for many years by antigen-specific memory B cells (MBC) and long-lived

plasma cells (LLPC)[19, 20]. Approximately 0.1–0.5% of the MBC in peripheral blood produce DENV-specific antibodies[21, 22]. Previous studies have shown that most DENV-specific MBC produce CR and poorly neutralizing antibodies. A small fraction of DENV-specific MBC encodes for TS and strongly neutralizing antibodies[23, 24, 25]. Most strongly neutralizing antibodies bind to complex conformational epitopes of the viral envelope protein (E) that are efficiently displayed on intact virion but not on E monomer[21, 26, 27, 28]. The polyclonal LLPC-derived serum antibody response contains both poorly neutralizing CR antibodies and a small fraction of strongly neutralizing TS antibodies[21, 23, 29].

In contrast to the MBC and LLPC responses, the early PB response to primary DENV infection is less studied. Several groups have characterized the early PB response following secondary DENV infections[16, 17, 30, 31]. DENV-specific PB accounted for 30-50% of circulating B cells during an ongoing secondary infection[16, 30, 32, 33]. In secondary infections, the magnitude of the PB response has been positively correlated with disease severity[30]. The vast majority of the PB pool in secondary DENV infection is CR[33, 34] and not maintained into convalescence[35]. Here we aim to characterize the kinetics, magnitude, and specificity of the PB response in primary DENV infection.

To longitudinally define the functionality of the PB, MBC, and serum compartments following a primary DENV infection, we analyzed DENV-specific responses in each of these compartments in the same individuals following infection with a DENV2 challenge virus[36]. In this controlled infection model, a primary DENV2 infection stimulated a rapid PB response wherein a large fraction of these cells produced TS antibodies to DENV2. The dominance of DENV2 TS antibodies was also observed at

late convalescence (6 months after infection) in the MBC and serum compartments. Overall, the properties of antigen-specific antibodies in the early phase are also reflected in the late convalescent phase.

Materials and methods

Study participants

For this study, subjects received 10^3 focus-forming units of the partially attenuated virus rDEN2Δ30 (Tonga/74 strain) by subcutaneous injection and followed for six months. Ten subjects were participants in a stand-alone trial for the safety and immunogenicity of rDEN2Δ30/Tonga/74 (CIR286, Clinicaltrials.gov NCT01931176[37]) and nine were participants in the placebo/challenge arm of a Phase II vaccine/ challenge clinical trial to test the safety and immunogenicity of the tetravalent live attenuated dengue vaccine TV003 (CIR287, Clinicaltrials.gov NCT02021968[36]). All subjects were serologically confirmed as *Flavivirus* (FV)-naïve at the time of rDEN2Δ30 challenge, as described previously[36]. Studies were approved by the Institutional Review Boards at the University of Vermont and Johns Hopkins University. Informed consent was obtained in accordance with federal and international regulations (21CFR50 and ICHE6). External independent monitoring was performed by the National Institute of Allergy and Infectious Diseases Data Safety Monitoring Board every 6 months.

Clinical procedures and collection of samples

At study visits, blood was collected by venipuncture into serum separator tubes for analysis of viremia and serology, and into EDTA tubes for isolation of peripheral blood mononuclear cells (PBMC). Serum was isolated and frozen at –20^{o}C until use. PBMC were isolated by Ficoll-paque density gradient separation, counted and frozen in culture medium with 10% DMSO/40% fetal bovine serum (FBS), and cryopreserved in liquid nitrogen vapor phase.

Virus Quantification and Serologic Response in Subjects

Serum samples collected at follow-up visits every other day through day 16 were tested for infectious virus by direct titration on Vero-81 cells (American Type Culture Collection; CCL-81, RRID: CVCL_0059), and foci were detected with TS monoclonal antibodies as described[38]. Neutralizing antibody responses against DENV1 (WestPac/74), DENV2 (New Guinea C and Tonga/74), DENV3 (Sleman/78), and DENV4 (Dominica/81) were measured by focus reduction neutralization test (FRNT), using the lowest serum dilution that gave a 50% reduction in viral foci (FRNT50) as described[38]. Unless otherwise specified, these viruses were used for determination of DENV2 serotype-specificity and cross-reactivity of antibodies derived from the PB, MBC, and serum in ELISA or neutralization assays.

Analysis of Plasmablasts and memory B cells

PB phenotyping. PBMC at days 0, 4, 8,14, 21, 28, 56, 90, 150 and 180 after rDEN2Δ30 challenge were stained with: anti-CD19 (clone HIB19, PE-Dazzle 594), anti-CD20 (2H7, PE-Cy7), anti-CD27 (O323, Brilliant Violet 510), anti-CD38 (HIT2, AlexaFluor 647), anti-CD3 (UCHT1, FITC), and anti-CD14 (HCD14, FITC). Antibodies were purchased form BioLegend. PB were phenotyped by gating for CD3-CD14-CD19+ CD20-CD27++CD38++ live blasting cells. Data was collected on a BD LSRII and data were analyzed with FlowJo v10 (TreeStar).

PB sorting. PBMC at days 8, 14, and 21 after rDEN2Δ30 challenge were stained with: anti-CD19 (HIB19, Brilliant Violet 421, BioLegend), anti-CD20 (2H7, PE-Cy7, BioLegend; or L27, PerCP-Cy5.5, BD Biosciences), anti-CD27 (O323, Brilliant Violet 510, BioLegend), anti-CD38 (HIT2, Alexa647), anti-IgA (IS11-8E10, FITC, Miltenyi),

anti-IgM (MHM-88, FITC, BioLegend), anti-CD3 (UCHT1, FITC, BioLegend), and anti-CD14 (HCD14, FITC, BioLegend). Flow cytometry sorting for IgG^+ PB was performed, gating for CD3-CD14-IgA-IgM-CD19+CD20-CD27++CD38++ cells using a BD FACSJazz and BD FACS Diva software. Single cells were sorted into 96-well PCR plates containing lysis buffer (10 mM Tris-HCl, pH 7.6), with 2 mM dNTPs (New England Biolabs), 5 μM oligo(dT)20VN, and 1 unit/μl of RiboLock RNase Inhibitor (ThermoFisher Scientific). Single-cell sorted plates were stored at −80°C until use for reverse transcription (RT).

Reverse transcription, PCR, barcode assignment, sequence assembly, V(D)J assignment, and identification of mutations were performed as described previously[39, 40] with the following modifications: biotinylated oligo(dT) was used for reverse transcription, cDNA was extracted using Streptavidin C1 beads (Life Technologies), DNA concentrations were determined using qPCR (KAPA SYBR® FAST qPCR Kit for Titanium, Kapabiosystems), and a minimum coverage of 10 reads was required from each chain assembly to be included in the sequence repertoires. V(D)J assignment and mutation identification were performed using an implementation of SoDA[41]. Paired H- and L-chain sequences from each subject's PB repertoire were assigned to the same lineage if the H-chain V-gene usage, CDRH3 length, L-chain V-gene usage, and CDRL3 length were identical. H- and L-chain CDRs were identified by aligning amino acid sequences to a hidden Markov model[42]. Sequences were further separated into putative lineages based on the degree of identity of the CDRH3 and CDRL3 sequences.

Selection, cloning of antibody genes and expression of monoclonal antibodies from PB. The different antibody lineages were ranked based on evidence for infection-

driven expansion and convergence across subjects as described[43]. Briefly, the criteria used to rank the lineages were (1) the number of distinct PB clones within each lineage indicative of expansion or biased response to the infection, (2) the number of mutations suggestive of affinity maturation, (3) overlap of lineages across the three subjects suggestive of convergent evolution, and (4) clonal lineages with apparent sequence similarity amongst the lineage's members, indicative of sharing common progenitors. From each of the 96 highest priority lineages, we selected one clonal member for recombinant expression and purification. Selected sequences were either from the PB clone in the lineage with the highest identity to the consensus sequence of the lineage, or from the clone expressed by the greatest number of PB in the lineage.

The 96 antibody H and L chain gene pairs were cloned into mammalian expression vectors (Lake Pharma, Belmont, California). Each complete construct was confirmed by sequencing; a small scale (0.01 L) transient production was done in HEK293 cells. For each antibody, both the heavy- and light-chain encoding DNA constructs were transiently co-transfected into cells. The cells were maintained as a batch-fed culture until the end of the production. The proteins were purified using Protein A purification. The conditioned media from the transient production run was harvested and clarified by centrifugation and filtration. The supernatant was loaded into a Protein A column pre-equilibrated with binding buffer. Washing buffer was passed through the column until the OD_{280} value (NanoDrop, ThermoScientific) was measured to be zero. The target protein was eluted with a low pH buffer; fractions were collected and filtered through a 0.2 μm membrane filter. The antibodies were in 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH7.0 buffer. Protein concentration was calculated from the

OD_{280} value and the calculated extinction coefficient. The average yield was 0.117 mg and the median yield was 0.08 mg. Ninety-two of the 96 selected IGH/IGL pairs yielded sufficient protein for functional testing.

Memory B cell isolation and immortalization. Switched memory B cells (MBC) were isolated from cryopreserved PBMC collected on day 180 following rDEN2Δ30 challenge. After thawing, PBMC viability was >80% as assessed by negative DAPI staining (4, 6-diamidino-2-phenylindole, 5 µg per sample in PBS – analyzed by flow cytometry on a Miltenyi VYB auto-sampler). B cells were enriched by labeling PBMC with microbead-conjugated anti-CD22 antibody (Miltenyi, catalog no. 130-046-401) followed by magnetic field separation (Miltenyi MS columns) to an average purity of 85% of B cells in the eluted sample. Switched MBC were purified from CD22-enriched B cells by labeling with anti-CD3 (UCHT1, FITC), anti-CD19 (HIB19, PE-Dazzle594), anti-CD27 (O323, PE-Cy7), and anti-IgM (MHM-88, PerCP-Cy5.5). Antibodies were purchased from BioLegend. Cells were sorted into complete culture medium (see below) by FACS from the live lymphocyte gate for CD3-CD19+CD27+ IgM- cells on a BD FACSAriaIII using the BD FACSDiva software. Post-sort purity was $\geq$ 95%.

Purified MBC were immortalized with the introduction of BCL-6 and Bcl-xL using retroviral transduction as previously described[44]. Briefly, purified MBC were activated in a 24-well tissue culture-treated plate with 1 mL of Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, ThermoFisher) containing 8% FBS (Atlanta Biologicals), penicillin (100 units/mL, Gibco), streptomycin (100 µg/mL, Gibco), 1 x 10^5 irradiated (50 Gy) human CD40L-expressing mouse fibroblasts (L cells) and 50 ng/mL recombinant human interleukin (rhIL)-21 (Peprotech) for 36-48 hours at 37°C, 5% CO_2.

Cells were then suspended in 0.25 mL of serum-free IMDM into 24-well non-tissue culture-treated plates coated with 30 ng/mL retronectin (Takara) and blocked with 2% human serum albumin. The suspension of activated B cells in serum-free medium was mixed with equal volume of retrovirus (in DMEM/F-12 with 8% FBS). Plates were centrifuged at room temperature for one hour at 700 × g and then incubated at 37^{o}C for at least 6 hours-to-overnight. After incubation, cells were washed with complete IMDM and maintained with rhIL-21 and CD40L-L cells. On culture day 7-10, GFP expression was used as a marker of transduction. We observed subject-specific transduction efficiency ranging 25-70%. CD19+GFP+ B cells were then sorted to polyclonal cultures at 50 cells/well into 96-well plates containing rhIL-21 and 1×10^4 CD40L- L cells in complete IMDM using a BD FACSAriaIII (using the "single cell sort mask" mode, which provides the highest possible accuracy (<4% variance) in dispensing cells. After maintained for 2-3 weeks, polyclonal cultures were screened for DENV-binding IgG by ELISA (see below). Recovery of sorted polyclonal cultures (viable cultures) was 100% and secretion of IgG into culture supernatant (mean of 110 ng/mL) was confirmed by ELISA (Capture antibody: AffiniPure goat anti-human IgG (Fcγ), Jackson Immuno-research cat no. 109-005-008; detection: Peroxidase AffiniPure Goat Anti-Human IgG, Fcγ fragment specific, Jackson Immunoresearch cat no.109-035-008).

Monoclonal antibody cloning from immortalized MBC. Selected DENV2-specific polyclonal cultures were single-cell sorted into monoclonal cultures, grown on CD40L and IL-21, and later screened as above after three weeks. DENV-specific monoclonal cultures were further qualitatively tested for DENV neutralization by incubation with virus prior to addition to Vero cells; neutralization activity was assessed with

microneutralization assay (see below). From frozen (DENV-specific) cell pellets of monoclonal cultures we isolated RNA, performed nested PCR for IGH and IGL genes, and sequenced using specific primers as described[45, 46]. Sequences were input into IgBLAST (https://www.ncbi.nlm.nih.gov/igblast/) and compared to germline to determine variable heavy and light chain usage, V-(D)-J gene usage, somatic hypermutation, complementary determining region (CDR)3 sequence, and IgG subtype. We cloned the VH into human IgG1 (Genbank FJ475055) and VL into Ig-λ or Ig-κ expression vectors (FJ517647, FJ475056, respectively). Heavy- and light chain-encoding plasmids were verified by sequencing and co-transfected into HEK-293F cells (RRID: CVCL_6642) to produce mAb as described[45, 46].

rDENV epitope-mapping viruses

Recombinant viruses are constructed using a four-cDNA cloning strategy as described previously[47]. Briefly, the rDENV4/2 was created by introducing the envelope protein (E) domain III (EDIII) residues from DENV2 into the DENV4 A subclone (Sri Lanka 1992; GenBank KJ160504.1), and replacing E nucleotides 900 – 1179 with the corresponding nucleotides encoding variant DENV2 amino acids[47]. rDENV2/1 was also obtained in a similar fashion on a DENV2 S16803 infectious clone backbone. The human monoclonal antibody (mAb) 1F4 is a strongly neutralizing DENV1 TS antibody. Using cryo-electron microscopy, the footprint of mAb 1F4 has been mapped to a quaternary epitope that spans the E monomers of a dimer near the envelope domain I/II hinge region. The DENV1 1F4 epitope was transplanted into the DENV2 (S16803) infectious clone by changing 30 amino acids in DENV2 to their counterparts in DENV1. The transplantation results in a virus, rDENV2/1, which is sensitive to neutralization by the DENV2 TS mAb

2D22, and fully gains sensitivity to neutralization by the DENV1 TS mAb 1F4. The full-length cDNAs were transcribed into genome-length RNAs using T7 polymerase, as previously described[47, 48]. These transcripts were electroporated into C6/36 cells (RRID:CVCL_Z230), and cell culture supernatant containing viable virus was harvested. Virus was passaged twice on C6/36 cell monolayer cultures and stored at -80°C. Passage 3 virus was used as our working stock.

ELISAs to detect virus, recombinant E and recombinant EDIII–binding antibodies

Non-chimeric viruses used in the ELISA screens and neutralization assays were grown in Vero-81 cells at 37°C, as previously described[22]. Recombinant envelope protein (recE) from WHO reference strain DENV2 S16803 was produced in our laboratory or purchased from Hawaii Biotech, Inc[49]. Recombinant EDIII protein (rEDIII) from DENV2 was produced in our laboratory as described previously[50]. For detection of IgG reactive to whole DENV by ELISA, each batch of each serotype was titrated separately by ELISA using a fixed amount of highly CR serum sample (DT000) to achieve OD_{405} = 1.0, normalizing amongst serotypes and amongst batches in ELISA assays. Equivalent quantities of DENV virus was captured by plate-bound mouse anti-E mAb 4G2 overnight at 4°C. Recombinant E proteins were directly coated (rE - 100 ng/well; rEDIII - 200 ng/well) on ELISA plates overnight at 4°C[47]. Plates were blocked with 3% (vol/vol) normal goat serum (Gibco - ThermoFisher, USA), in Tris-buffered saline (TBS) containing 0.05% (vol/vol) Tween 20 (blocking buffer). PB -derived IgG1 mAb were tested at a fixed concentration of 20 µg/ml. 50 µL of polyclonal supernatant from immortalized MBC was tested. Following four washes, antigen-antibody binding was detected with alkaline-phosphatase conjugated secondary anti-human IgG antibodies and

p-nitrophenyl phosphate substrate. Reaction color changes at OD_{405} were quantified by spectrophotometry.

Determination of quality of serum neutralizing antibodies by antibody depletion of using DENV viruses

To determine whether DENV2 TS or CR antibodies contribute to neutralization activity of immune serum, we performed antibody depletion studies as previously described[22]. The WHO reference strains for purified viral antigens are as follows: DENV1 (American genotype; strain West Pac74), DENV2 (Asian genotype; strain S16803), (provided by Robert Putnak, Walter Reed Army Institute of Research, Silver Spring, MD). These viral antigens were used to deplete DENV-specific antibodies from immune sera, and further used for confirmation ELISA and neutralization assays.

Purified DENV was adsorbed onto 4.5-μm-diameter polystyrene microspheres (Polysciences, Inc.) at a bead (μL) to ligand (μg) ratio of 5:2. Polystyrene beads were washed three times with 0.1 M borate buffer (pH 8.5) and incubated with the relevant purified DENV (DENV2 for homotypic depletions and DENV1 for heterotypic depletions) overnight at room temperature (RT). Control beads were incubated overnight with an equivalent amount of bovine serum albumin (BSA). The control and virus-adsorbed beads were blocked with 10 mg/ml BSA–borate buffer for 30 min at RT three times and washed four times with phosphate-buffered saline (PBS). DENV2 immune sera from the three selected subjects were depleted of virus-specific antibodies by incubating the samples with virus-adsorbed beads for 1 hour at 37°C with end-over-end mixing. Samples were subjected to at least three sequential rounds of depletions before successful removal of the respective antibodies was confirmed by ELISA. The ability of the

depleted samples to neutralize viruses of all of the four serotypes was tested after the confirmation ELISA. Neutralization capacity of the depleted DENV2 immune sera were measured using a flow cytometry-based neutralization assay with U937+DC-SIGN cells as previously described[29].

Vero microneutralization assays

We adapted the focus reduction-neutralization test (FRNT) as previously described[47] to a 96-well format[51]. 96-well plates were coated with 2×10^4 Vero-81 cells/well and incubated at 37°C for 24 hours. Neutralization titers were determined by three-fold serial dilutions of sera mixed with 50-100 focus forming units per well in DMEM/F-12 supplemented with 2% FBS. Neutralization activity of mAb was tested similarly over a range of 1-1000 ng/mL. The antibody-virus mixtures were incubated at 37°C for 1 hour before transferring to the 96-well plates containing confluent Vero-81 monolayers. Following an additional 1-hour incubation at 37°C, the monolayers were overlaid with Opti-MEM (Gibco, ThermoFisher) containing 2% FBS and 1% (wt/vol) carboxymethyl cellulose (Sigma). Infected plates were incubated for 2 days at 37°C with 5% CO_2, followed by fixation with 50µl 4% paraformaldehyde for each well. Fixed cells were then permeabilized and blocked with non-fat dried milk in permeabilization buffer. Then the plates were incubated with a mix of 4G2 and 2H2 primary antibodies for an hour at 37°C, followed by incubation with horseradish peroxidase (HRP)- conjugated anti-mouse IgG (KPL) antibody for an hour at 37°C, and developing with TrueBlue peroxidase substrate (KPL). The reaction was stopped with water and the plates were air dried before counting the foci on an Immunospot® S6 analyzer (Cellular Technology Limited) using the Immunospot® double count software. We calculated IC_{50} values by

using the sigmoidal dose response (variable slope) equation of Prism 6 (Graph Pad Software). Reported values were required to have an R^2 >0.75, a hill slope >0.5, and an IC_{50} within the range of the dilutions.

Results

We utilized a human DENV infection model to analyze the B cell and serum antibody responses to DENV infection. The human infection model is based on challenging subjects with a partially attenuated strain of DENV2 (rDEN2Δ30/Tonga/74). A complete description of the virologic and clinical features of this model has been published[36, 37]. Healthy, FV-naïve subjects infected with rDEN2Δ30 were followed over a 6-month period (**Figure S1**).

PB response to rDEN2Δ30 in FV-naïve subjects

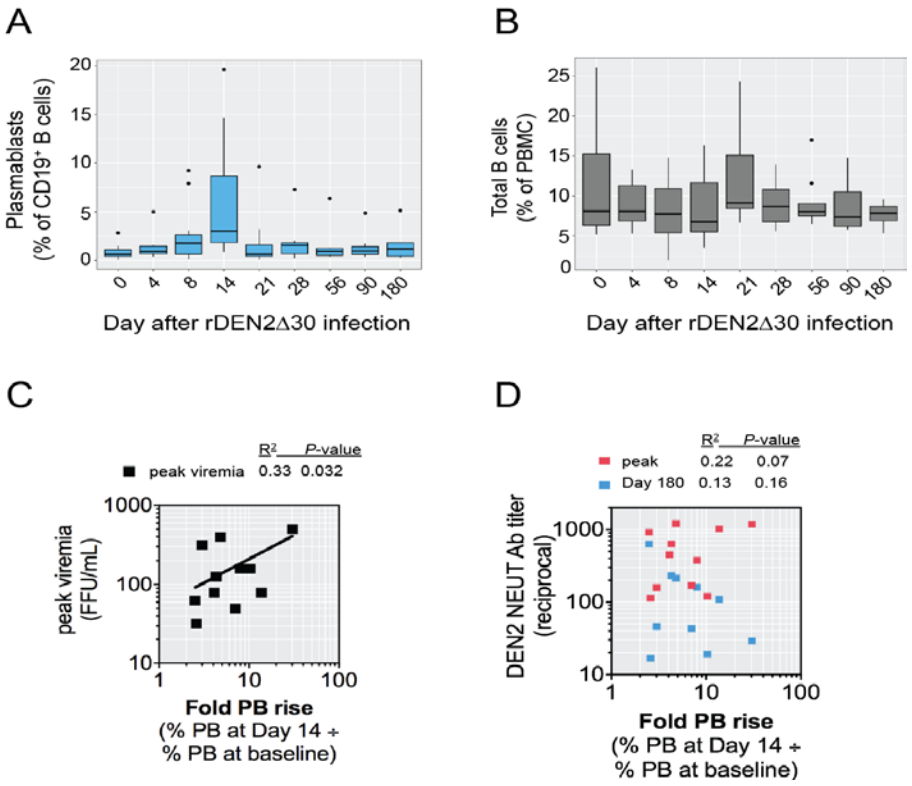


Figure 1. Kinetics of the PB response to primary DENV2 infection. Flow cytometric analysis of (**A**) CD3–CD19+CD20–CD27++CD38++ PB and (**B**) total CD19+ B cells in PBMC from subjects infected with rDEN2Δ30. Box and whiskers plots (Tukey) are shown (n = 12). (**C, D**) The fold-rise of PB on Day 14 versus baseline was plotted against (**C**) peak DEN2 viral load and (**D**) peak DEN2 neutralizing antibody titers after infection (Days 28-56) or at Day 180 after infection. Pearson's R-squared correlation coefficients and P-values are shown.

We first focused on PB, a population of antibody-secreting B cells that transiently increases in the blood early after viral infections[12] including DENV[16, 17, 30]. To determine whether a PB increase was induced in the primary DENV2 infection model, we assessed the frequencies of CD19+CD20-CD27++CD38++ cells in cryopreserved PBMC of twelve subjects infected with rDEN2Δ30. PB levels increased beginning at day 8 post-infection, peaked at day 14 and waned by day 21 (**Fig. 1A, Supplemental Fig. S2A**), ranging 1.5-20% of the total CD19+ B cell population amongst subjects, and expanding on average 8.7–fold (range 2.5 – 30-fold) from day 0 to peak response (**Supplemental Fig. S2A**). We assessed the percentage of peripheral B cells in total PBMC to determine whether change in PB percentage was a function of total B cells. Generally, the percentage of peripheral B cells remained stable before and throughout the 6-month period following the infection (**Fig. 1B, Supplemental Fig. S2B and S3**).

In people exposed to natural DENV infections, PB levels have been correlated with peak viral titer[16, 52]. Similarly, in this challenge model, the peak viral load was positively correlated with the PB increase (**Fig. 1C**). We observed a weak correlation between changes in the PB levels and peak serum neutralizing antibody titer (**Fig. 1D**), although the degree of correlation did not reach significance (P = 0.07).

The PB response to primary DENV2 infection is clonally expanded, and exhibits convergent evolution across subjects

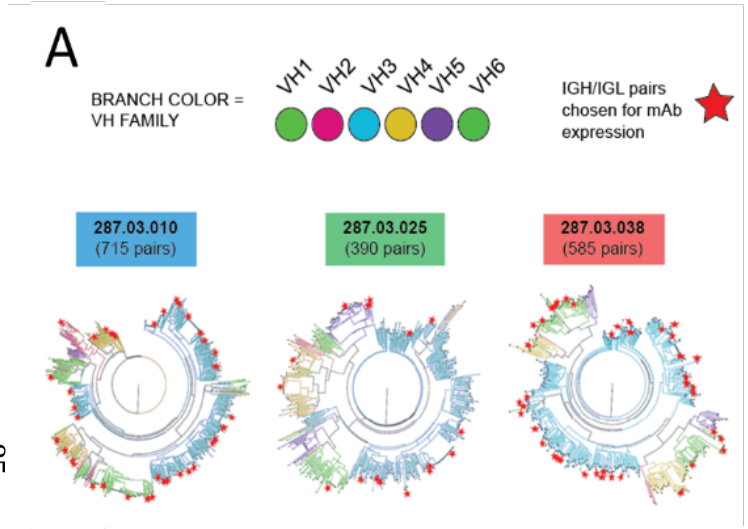
For three individuals across a range of rDEN2Δ30 viremia and PB dynamics (**Table 1**), we sought to perform in-depth longitudinal assessment of PB, MBC and serum antibodies in the same subjects over time. To characterize the PB antibody repertoire, PB from each individual were single-cell sorted into 96-well plates on the basis of cell-

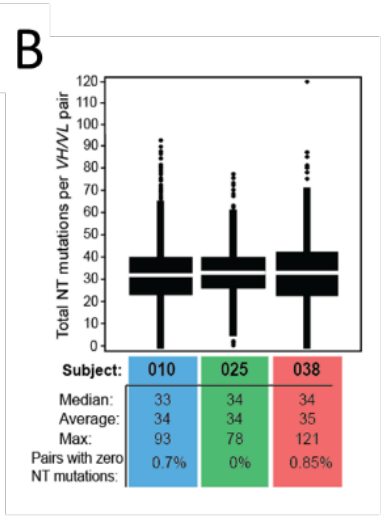
Table 1: Viremia and PB in three rDEN2Δ30 recipients

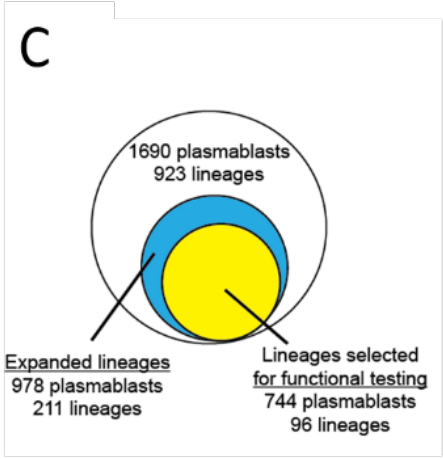
Subject ID	DENV2 viremia			DENV2-induced PB					
	Onset (day)	Peak titer (log_{10}PFU/mL)	Peak day	% Baseline	% day 14	Fold-rise day 14	Peak day	% peak	Fold-rise peak
010	8	1.5	10	0.5	1.3%	2.6	21	2.7%	5.4
025	6	2.1	6	1.5	9.6%	6.5	14	9.6%	6.5
038	4	2.9	4	0.3	3.1%	10.3	14	3.1%	10.3

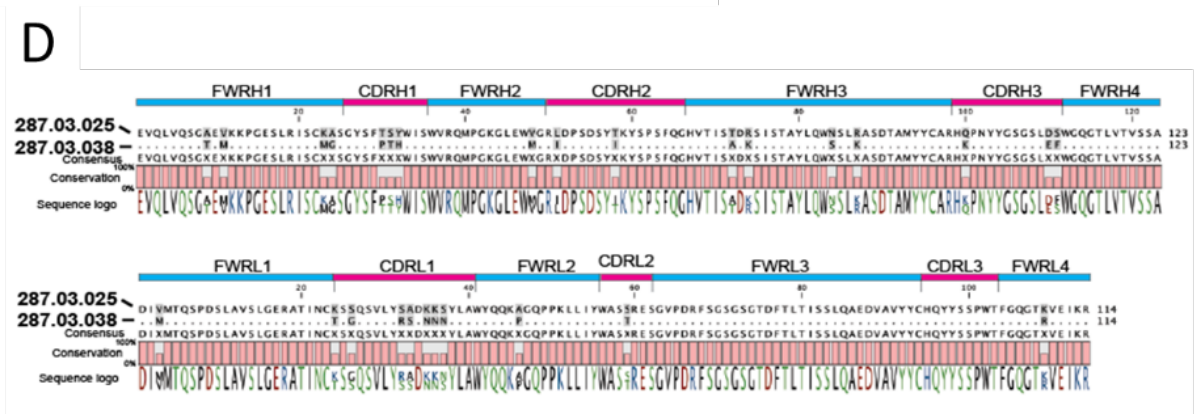
surface staining for CD19+CD20-CD27++CD38++IgA-IgM-**.** A DNA barcoding method was utilized to obtain the paired heavy- and light-chain antibody sequences from a total of 1,690 individual cells from the three subjects **(Table S1)**[39]. Phylogenetic trees of the PB repertoires from the subjects were generated based on sequence homology of the paired heavy- and light-chain sequences (colored branches indicate heavy chain variable gene usage, **Fig. 2A**). Only 0.6% of these cells expressed naïve, unmutated antibodies (data not shown). The remaining PB exhibited an average rate of 34 nucleotide mutations per VH and VL pair (**Fig. 2B**), resulting in an average of 21 amino acid replacements across VH and VL per cell per subject (not shown).

Examples of clonally expanded PB lineages were observed in all three subjects. The 1,690 IGH/IGL pair sequences represented 923 lineages based on nucleotide sequence (clonal) similarity (**Fig. 2C**). A quarter of the lineages (211 of 923) were represented by at least two PB and were considered to have undergone proliferative expansion (i.e. *in vivo* selection). While the majority of blood PB sequenced (978/1690, 58% of total) belonged to expanded lineages, the remainder of the cells (712/1690, 42% of total) belonged to lineages comprising only one cell (singletons) (**Table S2**). of the expanded lineages, we identified 48 lineages exhibiting convergent evolution. Members of convergent lineages shared germline IGHV/IGLV genes, CDRH3 and CDRL3 lengths, and exhibited ≥75% identity across the mature VH or VL peptide. An example of these convergent IGH/IGL pairs is shown in **Figure 2D**. Taken together, these data show that


A
BRANCH COLOR =
VH FAMILY
VH1
VH2
VH3
VH4
VH5
VH6
IGH/IGL pairs
chosen for mAb
expression
287.03.010
(715 pairs)
287.03.025
(390 pairs)
287.03.038
(585 pairs)


B
Total NT mutations per VH/VL pair
Subject: 010 025 038
Median: 33 34 34
Average: 34 34 35
Max: 93 78 121
Pairs with zero NT mutations: 0.7% 0% 0.85%


C
1690 plasmablasts
923 lineages
Expanded lineages
978 plasmablasts
211 lineages
Lineages selected
for functional testing
744 plasmablasts
96 lineages


D
FWRH1
CDRH1
FWRH2
CDRH2
FWRH3
CDRH3
FWRH4
287.03.025
287.03.038
Consensus
Conservation
Sequence logo
FWRL1
CDRL1
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CDRL2
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CDRL3
FWRL4

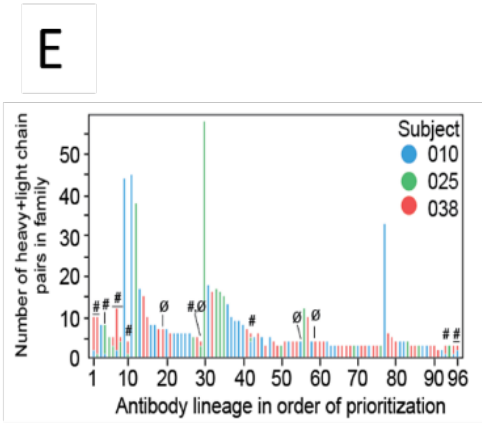
E
Number of heavy+light chain pairs in family
Subject
010
025
038
Antibody lineage in order of prioritization

Figure 2. Evidence of somatic hypermutation and diversity in the PB response to rDEN2Δ30. PB from the peak response time points from three rDEN2Δ30 recipients were single-cell sorted and IGH/IGL sequencing was performed. Natively paired IGH/IGL sequences were compared to germline sequences and (**A**) phylograms were constructed to visualize IGH/IGL diversity. The distance from center is proportional to paired VH/VL somatic hypermutation rate compared to germline. VH gene usage is shown as colored branches. The number of unique PB sequenced from each donor is indicated. Stars indicate antibody VH/VL pairs selected for recombinant IgG1 expression. **(B)** Median, average, and maximal values for sum of nucleotide mutations across paired VH and VL regions in all PB is shown for each subject. (**C**) Venn diagram of the total number of PB sequenced (with corresponding number of lineages), the number of cells contained within lineages showing evidence of expansion (i.e. lineages represented by at least 2 cells), and the number of cells within the expanded lineages that were selected (n = 96), and from which mAb were recombinantly expressed. Note that the expressed mAb represented 76% of the cells from lineages with evidence of expansion and 43% of the overall sequenced PB repertoire. (**D**) High amino acid sequence identity across IGH and IGL variable regions from single PB from two different subjects challenged with rDEN2Δ30. Gray shading in aligned sequence indicates mismatch. Sequence logo coloring: green, polar/neutral; black, nonpolar/neutral; red, polar/acidic; blue, polar/basic. (**E**) Based on sequence and repertoire features (see methods), 96 lineages were prioritized for generation of an antibody screening library. A single antibody clone from the first 96 of these lineages was selected for recombinant IgG expression, including 12 pairs that were convergent (#) across two or more subjects (note the different colors representing different subjects in stacked bars). Four mAb did not express as protein in sufficient quantity (noted with Ø), leaving 92 mAb (including 11 convergent) for functional testing.

primary DENV2 infection elicited a diverse pool of PB exhibiting clonal expansion, somatic hypermutation, and population of convergent lineages shared amongst subjects.

Binding properties of PB-derived antibodies

To estimate the extent to which PB that expanded following primary DENV2 infection were able to recognize viral antigen, we selected 96 IGH/IGL pairs for recombinant expression and testing for DENV binding and neutralization (**Fig. 2C).** This number was chosen as a balance between adequate coverage of the expanded lineages (96 of 211, or 45%), resources, and compliance with a microplate-based cloning procedure. Within this group of expanded lineages selected for functional testing, we identified 12 convergent lineages (**Fig. 2E, Table S3**). Ninety-two sequences yielded

sufficient protein for further testing. These 92 lineages contained a total of 741 cells, which is 76% of the total number of cells in the 211 expanded lineages (978 cells) in the dataset (**Table S2**). Although all 92 antibodies were recombinantly expressed as IgG1, they were derived from native antibodies that were either IgG1 (83/92, 90%), IgG2 (3/92, 3%), or IgG3 isotypes (6/92, 7%). The final testing selection included mAb from all three subjects, with 11 convergent sequences shared between at least two subjects (**Fig. 2E**). Forty-five percent (41/92) of the recombinantly expressed antibodies bound to DENV2 Tonga/74 antigen (**Fig. 3A**). of the DENV-binding antibodies, 32% were DENV serotype CR while 68% bound only to DENV2 (TS) (**Fig. 3B**). of the 11 convergent sequences, 6 antibodies (55%) bound DENV2 antigen and one was neutralizing (**Table S3**). In addition, for each subject, we determined the percentage of PB in lineages producing DENV-binding antibodies amongst the total PB in lineages selected for recombinant expression (**Table S2)**. In subjects 010, 025, and 038, the proportions of the PB in DENV-positive lineages were 32%, 70%, and 60% respectively. On the whole, the lineages we tested for binding that contained DENV2 reactive clones represented 54% of the PB amongst PB in all tested lineages.

Next, we compared the binding of the DENV2 TS mAb to different strains of DENV2, including Tonga/74 (challenge strain, American genotype), New Guinea C (Asian genotype II), S16803 (Asian genotype I), and Nicaragua 694 (Asian/American genotype). Fifty-six percent (14/25) of the tested DENV2 TS antibodies bound exclusively to Tonga/74 strain, while the remaining antibodies bound to at least two DENV2 strains (**Fig. 3B**). These results demonstrate that the early PB response to primary DENV2 infection is a mixture of DENV TS and CR antibodies. Furthermore,

nearly half the TS antibodies are strain-specific and recognize epitopes displayed on the DENV2 Tonga/74 virus but not other closely related DENV2 strains.

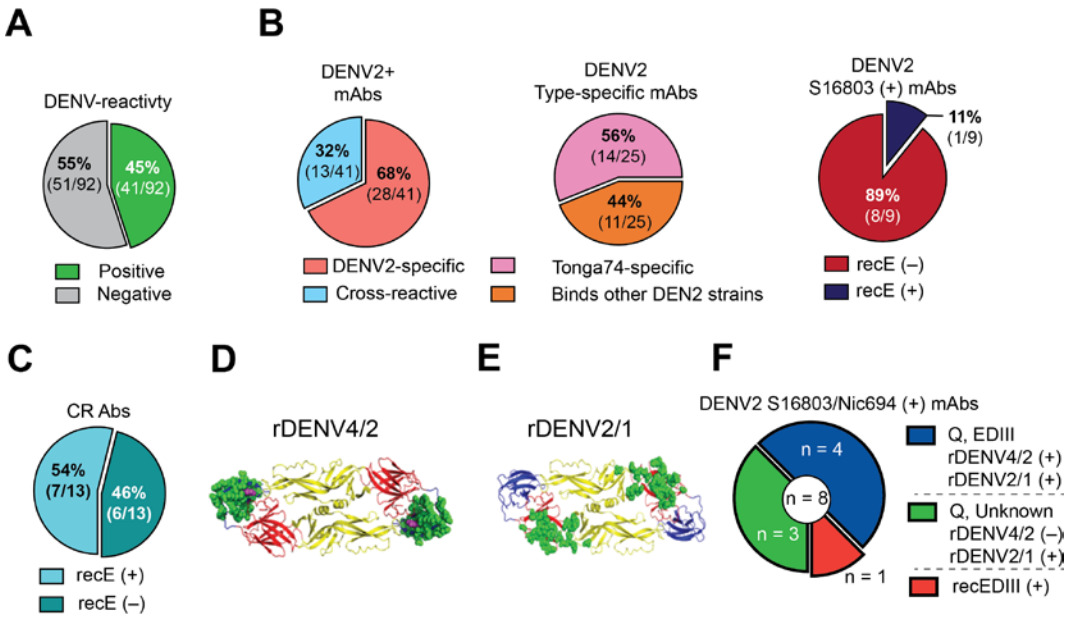

Figure 3. Binding properties of PB-derived antibodies. (**A**) Fraction of PB-derived DENV reactive hits and non-reactive antibodies are shown. (**B**) Serotype-specificity of DENV2-positive mAb (left) and indicated DENV2 strain-specificities (middle). Of those mAb that bound to the WHO reference S16803 strain, the proportion that bound to S16803-derived recE are shown (right). (**C**) Proportion of CR mAb which bind to recE are depicted. (**D**) Structural model of DENV4 E protein dimer, with swapped residues from EDIII of DENV2 (Nicaguara-694) colored in green to represent the rDENV4/2 E glycoprotein, and the DENV2 TS mAb 2D22 escape mutations highlighted in magenta. (**E**) Structural model of DENV2 (S16803) E protein dimer (with DENV1-derived EDI and EDI/II hinge residues colored in green to represent the rDENV2/1 virus E glycoprotein. (**F**) Eight DENV2-specific mAb binding to S16803 and Nicaguara-694 DENV2 viruses were tested for binding to recombinant EDIII (derived from S16803) as well as rDENV4/2 and rDENV2/1 chimeric viruses. The number and proportion of mAb that bound to the indicated antigens is shown. Q: quaternary epitope-targeting mAb.

Mapping epitopes recognized by DENV-specific PB-derived antibodies

We performed more detailed mapping studies with the DENV2 TS antibodies. Using recombinant proteins and chimeric virus reagents based on the DENV2 S16803 strain, we mapped the epitopes of 9 DENV2 TS antibodies that bound to S16803 (out of the 25 TS clones that bound to DENV2 Tonga/74). The majority (8/9) of these TS mAb did not bind to recombinantly expressed E protein (recE) monomers derived from the same strain, indicating that quaternary structure E epitopes displayed on intact virion are likely targets of these antibodies (**Fig. 3B**). The one TS antibody that bound recE recognized a simple epitope on EDIII (data not shown). We also tested the 13 DENV CR antibodies for binding to recE. Fifty-four percent (7/13) antibodies bound to recE demonstrating that some CR antibodies bound to simple epitopes on monomeric E protein, while others recognized prM protein or quaternary E protein epitopes displayed only on intact virion (**Fig. 3C**).

To further map epitopes targeted by the DENV2 TS antibodies, we performed binding assays using recombinant chimeric DENV viruses with mutated E glycoproteins. rDENV4/2 is a DENV4 strain in which the entire DENV4 EDIII has been replaced with EDIII from DENV2-Nicaguara 694 (**Fig. 3D**). This recombinant virus has been used to map the binding of TS antibodies that target simple or complex epitopes centered on DENV2 EDIII[47]. rDENV2/1 is a DENV2 (S16803) virus in which residues in EDI and the hinge region between EDI and EDII have been mutated to display an epitope for the DENV1 TS and neutralizing human antibody 1F4 (**Fig. 3E**). We therefore selected 8 mAb that recognize both the S16803 and Nicaguara-694 strains for analysis. Four of the 8 mAb directed at DENV2-specific quaternary epitopes (**Fig. 3B**) showed binding to

rDENV4/2 and showed no appreciable loss of binding to the rDENV2/1 strain, establishing quaternary epitopes centered on DENV2 EDIII as the main target of binding (**Fig. 3F**, shown in blue). One mAb was DENV2 recE-reactive and targeted simple monomeric epitopes on recombinant EDIII (**Fig. 3F**, indicated in red). The remaining three mAb bound to rDENV2/1 but not to rDENV4/2, indicating that residues on EDIII or the EDI/II hinge region were unlikely to be critical for binding (**Fig. 3F**, shown in green). Overall, these results demonstrate that most PB –derived CR mAb targeted simple epitopes on recE protein, whereas the TS mAb recognize quaternary structure epitopes centered on EDIII, including novel regions that do not map to known DENV2-specific mAb epitopes such as that of 2D22[53].

Functional neutralization of DENV2 by PB–derived mAb

We tested all DENV-binding mAb in a DENV2 neutralization assay and identified five neutralizing mAb, with potencies (assessed by IC_{50} concentrations) ranging 50-1700 ng/mL (**Fig. 4A**). Four of five were DENV2 TS (**Table 2**), including mAb 1662, with a potency that rivals 2D22, a strong DENV2-neutralizing mAb[29]. These 5 neutralizing mAb were not DENV2 Tonga74 strain-specific. Four of five mAb bound to all the DENV2 strains/genotypes tested in the study. Only one mAb (1615) did not bind to S16803 strain but bound to all other strains. Moreover, the three TS neutralizing mAb that bound to epitopes on whole DENV2 virion and not to recE showed gain of binding to rDENV4/2 virus and did not lose binding to rDENV2/1. These results showed that the three TS neutralizing mAb targeted quaternary epitopes in the EDIII region. In **Fig. 4B-D**, we mapped all DENV2 TS, CR, and neutralization hits from each of the three

subjects to the phylogenic PB trees but were unable to discern particular VH usage or clonal lineage characteristics predictive of functional activity.

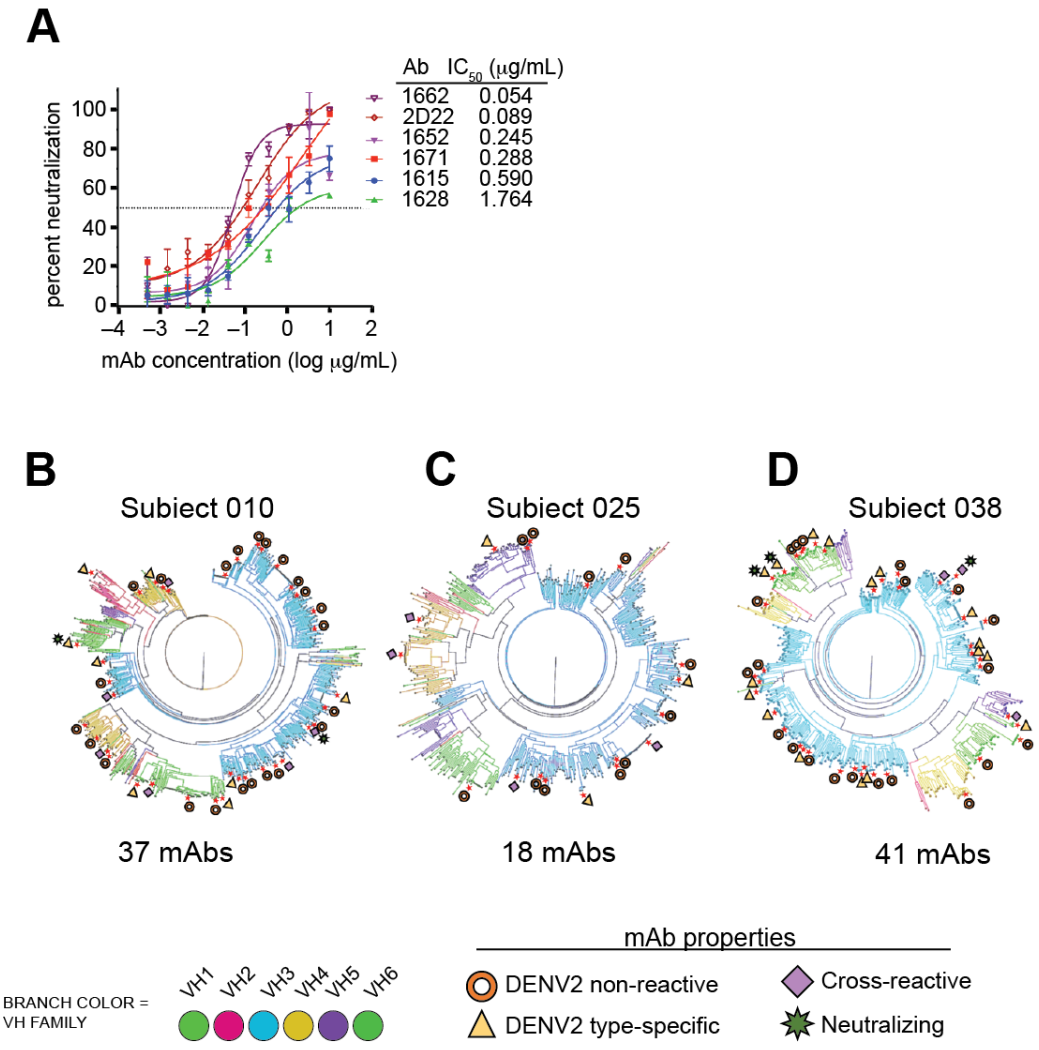

Figure 4. **DENV2 neutralizing antibodies from PB.** (**A)** Vero micro neutralization assays were conducted with all the DENV2 reactive hits to determine the neutralization of DENV2 Tonga 74. Neutralization curves for five neutralizing antibodies are shown. Results are from 2 technical replicates in an experiment. Each point represents the mean neutralization value from the two replicates, and the error bars depict the standard deviations. Neutralization experiments were repeated at least twice. (**B-D**) Phylogenetic trees depicting the properties of the antibodies tested in all the three subjects is shown.

Table 2. Epitope mapping and functional characterization of PB-derived DENV2 neutralizing mAb

mAb	Subject	Binds DEN2 Tonga[a]	recE/Q[b]	rEDIII binding[c]	TS/CR[d]	$NEUT_{50}$ (μg/mL)[e]
1662	010	+	Q	–	TS	0.054
1652	038	+	recE	+	CR	0.245
1671	038	+	Q	NB	TS	0.28
1615	010	+	ND	ND	TS	0.59
1628	038	+	Q	NB	TS	1.764

[a] Whole virus ELISA, "+" = O.D. ≥ 2-fold above blank

[b] binding to recombinant E from DENV2 (recE). If positive, noted as "recE". If negative, noted as quaternary (Q) epitope. ND, not determined as this antibody does not bind to the parental WHO S16803 strain from which recE is derived.

[c] binding to recombinant E domain III from DENV2 (rEDIII). NB, no binding; ND, not determined.

[d] whole virus ELISA. TS: binding to DENV2 only; CR: binding to DENV2 & at least one other serotype.

[e] microneutralization assay using Vero cells, NEUT50 is concentration of mAb required for 50% reduction in infection compared to negative control.

Memory B cells following DENV2 primary infection

Our next objective was to evaluate the properties of memory B cells (MBC) six months after primary DENV2 infection in these three subjects. We utilized a previously described genetic reprogramming approach based on retroviral transduction of the germinal center master regulator B cell lymphoma (BCL)-6 and the anti-apoptotic protein BCL-xL (together known as 6XL) to immortalize MBC from the three subjects[44]. To estimate the fraction of the MBC producing DENV-binding antibodies, we screened immunoglobulin-containing culture supernatants from 6XL-transduced cells by DENV capture ELISA (**Supplemental Fig. S4, Table S4)**[22, 29]. The frequencies of DENV-specific MBC were calculated using the number of positive cultures and the total number of transduced B cells sorted. We estimated an average 0.40% (219/52800) of total MBC to be DENV-specific, with the highest responder, subject 287.03.025, exhibiting a frequency of 0.74% (89 positive cultures out of 12,000 total $6XL^+$ cells plated) DENV2-

reactive MBC followed by 287.03.010 with a frequency of 0.34% (98 positives out of 28,800 $6XL^+$ cells), and 287.03.038 with a frequency of 0.28% (32 positives of 12,000 $6XL^+$ cells) (**Fig. 5A**).

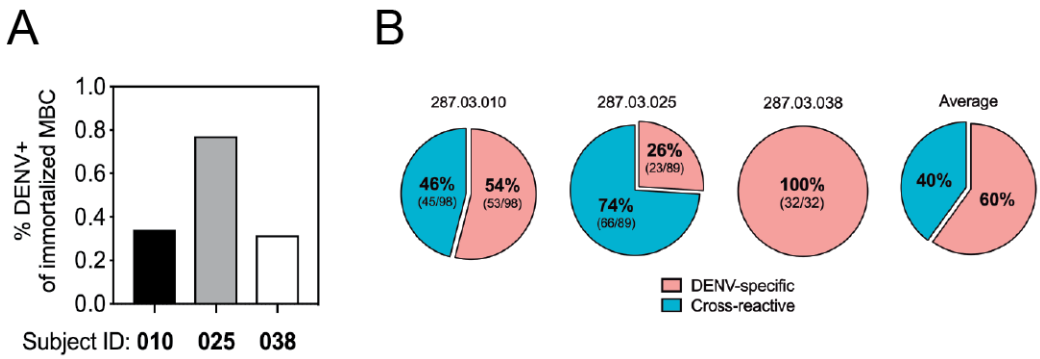

Figure 5. Elicitation of DENV-specific MBC by rDEN2Δ30 infection. (**A**) Total CD19+IgM-CD27+ switched MBC were isolated six months after rDEN2Δ30 infection in the same three subjects (287.03.010, 287.03.025, and 287.03.038) from which the PB analyses were conducted. MBC from each subject were immortalized with a retrovirus encoding BCL6, Bcl-xL (6XL) and the transduction marker green fluorescent protein (GFP). 6XL-immortalized MBC were sorted by FACS for GFP and cultured at 50 cells/well; supernatants were screened for IgG binding to DENV2 Tonga/74. For each subject, frequency of DENV-reactive MBC was calculated from the number of positive wells divided by the total number of 6XL+ cells cultured per subject and based on the presence of an average one positive clone yielding the positive signal per 50 cell input. (**B**) From each subject, DENV2-binding MBC were screened for reactivity to other serotypes by ELISA. The proportions (and numbers) of 219 MBC cultures that were DENV2 TS or CR with at least one other serotype (CR) are presented. The average proportions of TS versus CR cultures across the three subjects is also shown.

Binding specificity of MBC-derived antibodies

We screened the 219 DENV2-binding MBC culture supernatants from these three subjects to determine the extent to which these antibodies cross-reacted with other DENV serotypes. We found both TS MBC and CR MBC with bi-, tri-, and tetravalent reactivity. The TS fraction of the DENV2-reactive MBC response was donor-dependent; and across three subjects, an average of 60% of DENV2-reactive MBC was DENV2 TS and 40% was CR with DENV2 and at least one other serotype (**Fig. 5B**).

From one of the subjects (287.03.010) we isolated four DENV2-specific mAb, one of which was neutralizing, that represented four distinct lineages (**Table S5**). None of these paired IGH/IGL sequences was identified in the PB sampled from this subject. This lack of clonal overlap between the two B cell compartments has been observed in a prior study of secondary dengue[35]. Taken together, these data demonstrated the functional maintenance of TS humoral response in convalescence following primary DENV infection.

Quality of the convalescent serum neutralizing antibodies following DENV2 infection

Next, we characterized the properties of convalescent (6 months after infection) serum neutralizing antibodies. All three subjects developed neutralizing antibodies that neutralized DENV2 but not DENV1, 3, and 4 (**Fig. 6A)**. The immune sera efficiently neutralized the infection DENV2 strain (Tonga/74) as well as two other strains belonging to different DENV2 genotypes (NGC and S16803) (**Fig. 6B, Table S6**). We performed antibody depletion studies to further define levels of DENV2 TS and CR neutralizing antibodies. Polystyrene beads coated with the homotypic (DENV2) or heterotypic (DENV1) serotypes were incubated to deplete specific populations of antibodies. Depletion of antibodies was confirmed by ELISA before using the samples in neutralization assays (data not shown). Depleting the day 180 serum samples with the homotypic DENV2 antigen led to the removal of nearly all the DENV specific (DENV2 TS and CR) antibodies in the samples as well as a significant loss of neutralizing activity. When only the DENV serotype CR antibodies were depleted in each sample (depletion n), there was minimal reduction in neutralization of DENV2 (**Fig. 6C-E**). On average, ~89% of the serum neutralizing activity tracked with DENV2 TS antibodies (**Fig. 6F**).

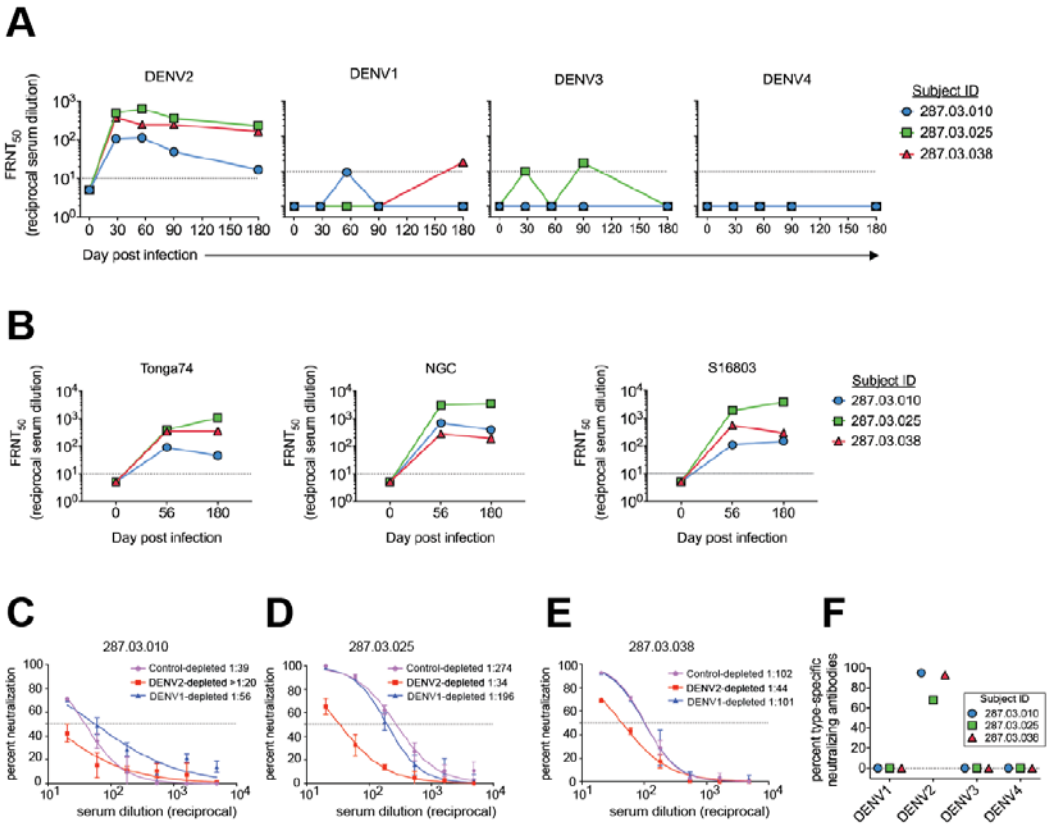

Figure 6. The properties of convalescent serum neutralizing antibodies following DENV2 infection. (**A**) 6 months after infection, sera from the three subjects were tested against DENV1-4 in a neutralization assay. (**B**) Sera from time points at day 0, 56, and 180 from the three subjects were tested for ability to neutralize DENV2 Tonga/74 (challenge strain, American genotype) and New Guinea C (Asian genotype II) in a microneutralization assay. For A and B, dotted lines indicate the lowest serum dilution factor used (1:10) in the neutralization assays and also the threshold above which a serum titer is considered positive. (**C-E**) 6-month post infection sera were depleted of antibodies binding to DENV2 antigen or DENV1 antigen. Control depletions were performed using bovine serum albumin as an antigen. Results presented here are from 2 technical replicates in one experiment. Neutralization curves are against DENV2 antigen for each subject. Each point in the neutralization curves represents the mean neutralization value from the two replicates, and the error bars depict the standard deviation. Dotted lines depict 50% neutralization. (**F**) All the three subjects develop DV2 TS antibodies that mainly contribute to DENV2 neutralization.

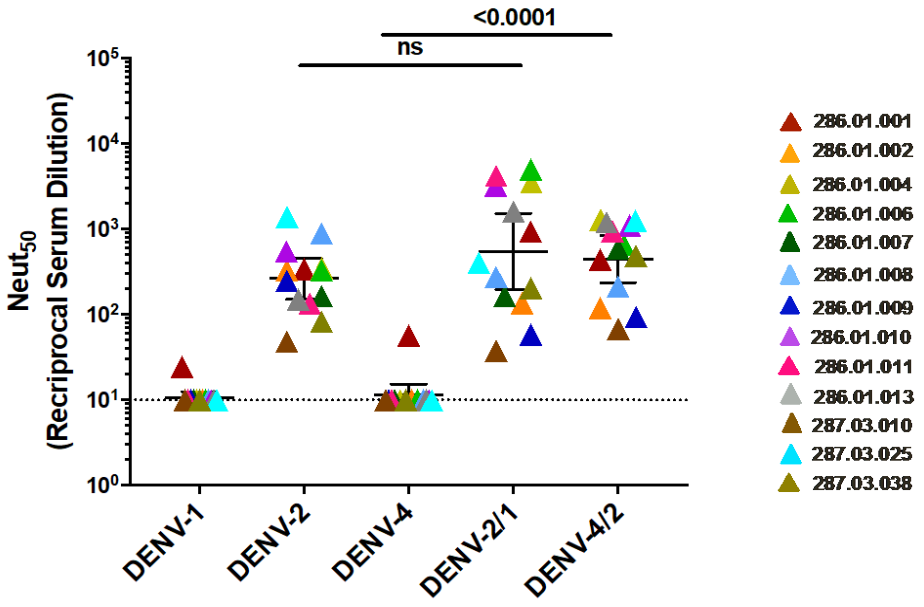

Figure 7. The DENV2 TS neutralizing antibodies target epitopes centered in the EDIII region. Neutralization assays were performed using a panel of polyclonal sera from subjects who received the Tonga 74 DENV2 strain using the rDENV4/2 and rDENV2/1 chimeric and wild type viruses. Each color represents a single sample, and the neutralization against each virus (x-axis). Bars indicate the geometric mean titers and 95% confidence intervals of the grouped samples. P-values were calculated by Kruskal-Wallis followed by Dunn's post-hoc test.

To further characterize the epitopes targeted by the DENV2 TS neutralizing antibodies elicited by the rDEN2Δ30 infection, we performed neutralization assays using the rDENV2/1 and rDENV4/2 viruses. We also performed the assays with an additional panel of 10 serum samples collected from individuals who received the rDEN2Δ30 challenge. Samples collected 180 days after infection were tested for neutralization capacity against the rDENV2/1 and rDENV4/2 and parental viruses **(Fig. 7).** All of the tested samples neutralized DENV2 well, and DENV1 and 4 poorly. The rDENV2/1 virus displaying the DENV1 1F4 epitope was neutralized at the same level as DENV2, demonstrating that the EDI region on DENV2 disrupted by the DENV1 1F4 epitope transplant was not a major target of neutralizing antibodies induced in people infected

with the rDEN2Δ30. The neutralizing antibody response induced by rDEN2Δ30 was mainly directed to epitopes centered on EDIII because rDENV4/2 displaying EDIII from DENV2 was neutralized to the same level as the parental DENV2.

Discussion

Strong neutralizing antibodies play an essential role in viral clearance and thereby contribute to long-term protection in DENV infection[29]. However, the evolution of these rare neutralizing antibodies is poorly understood. Using a unique human DENV2 infection model, we have provided a longitudinal analysis of the continuum of the B cell response to primary DENV infection. Overall, we found that the functionality in terms of DENV2 serotype-specificity is conserved from the acute PB response through the convalescent MBC and serological compartments (likely reflecting LLPC-derived antibodies). We propose that this framework to track the development of DENV-specific B cells, including those that produce TS antibodies, will be useful for evaluation of live attenuated DENV vaccine candidates, and to aid in deconvoluting the complex serology often found in natural infection.

During an acute secondary DENV infection, the PB response can comprise a substantial fraction (36-95%) of the total CD19+ B cell population[16, 30]. In our DENV2 primary infection model, we observed an average PB peak of 6.0% of the total B cell population. Our results are similar to a recent study reporting a PB peak of 2.5% of peripheral B cells amongst people exposed to Zika virus as a primary FV infection[54]. In another study of 84 patients with laboratory-confirmed primary or secondary DENV infections, the magnitude of PB response was substantially greater in secondary compared to primary infections[30]. These studies suggest that primary FV infections elicit a lower PB response compared to secondary infection. The higher response in secondary DENV infection may be due to a recall response upon encounter with similar DENV antigens. Another possibility arising from the association of the PB response and viremia,

shown here and elsewhere, suggests viral replication as a putative deterministic regulator of the B cell response[16]. It will be essential to determine whether low replication level of a tetravalent live attenuated dengue vaccine viruses compared to the rDEN2Δ30 challenge virus[36, 55] predicts a modest PB response. It is also possible, however, that the simultaneous administration of four viruses elicits a PB response distinct from natural infection or primary challenge with rDEN2Δ30.

PB expansion to DENV2 infection may be the manifestation of multiple immune activation mechanisms acting on the B cell compartment. During acute DENV infection, myeloid cells such as monocytes stain positive for the NS3 antigen, which marks replicating DENV[56]. Activated CD14+CD16+ monocytes have been connected to PB differentiation and proliferation[52]. Additionally, direct interaction between DENV and B cells without surface B cell receptor/immunoglobulin engagement could also lead to global activation of B cells. Serum neutralizing antibodies against DENV2 developed in all subjects following the transient PB response, suggesting an antigen-specific activation of the B cell response. In line with this, nearly half the PB lineages tested produced antibodies that bound to DENV2. Moreover, the paired IGH/IGL repertoire analysis of the PB response revealed that over half of blood PB belong to lineages exhibiting evidence of clonal expansion. We also observed evidence of expanded convergent lineages amongst three subjects. The overall level of DENV2-binders identified, the clonality, and shared lineages across subjects suggested the PB proliferation was driven by cognate DENV antigens besides by purely bystander effects.

To estimate the degree of antigen-specificity of the PB response, we characterized a panel of mAb derived from a broad array of lineages comprising large, small, and

convergent lineages. Using DENV binding ELISA we determined that 45% of the produced mAb were DENV-specific. There are some limitations to our analysis. First, our approach did not fully cover the PB landscape. The selected 92 lineages for antibody expression, though representing about 76% of the PB in all expanded lineages (741 of 978 cells), represented 44% of all PB sequenced. We reasoned that expanded lineages (representing 978 of 1690 total cells) would have a high likelihood of exhibiting DENV-specific activity when expressed. However, it is possible that some of the singleton lineages (n = 712) could be DENV-specific. Finally, only one antibody clone from each of these 92 lineages was selected for analysis. Continued effort is underway to characterize additional clones from these expanded and singleton PB lineages to further refine our estimation of antigen-specificity in the PB response. Since we tested mAb from lineages representing 76% of those with evidence of expansion, and found that 45% of these bound DENV, our calculation suggests that at least 34% of the PB in lineages that expanded in response to primary rDEN2Δ30 infection are antigen-specific. Other studies in natural secondary dengue infection have reported virus-specific frequencies in the PB pool ranging from 36-95%[16, 18].

We further refined the specificity, antigenic regions, and functional properties of our mAb panel derived from primary rDEN2Δ30 infection. Screening for virus binding revealed that 68% of the PB-derived mAb recognized the infecting DENV2 serotype exclusively, and the remaining fraction showed cross-reactivity to DENV2 and at least one other serotype. Most of the TS antibodies targeted quaternary epitopes on intact virion, rather than simple epitopes on the viral E protein. This is contrary to a report on PB-derived antibodies following secondary dengue infections in which these antibodies

were mainly CR and targeting simple E monomer epitopes and not complex epitopes[35]**.** We found DENV2-specific mAb with functional activity similar to the mAb 2D22 isolated from natural infection[53, 57, 58, 59] but also mAb whose specificities could not be mapped to these epitopes or even to EDIII, suggesting the possibility that our panel of mAb target uncharacterized DENV2 specificities. Five out of 41 DENV-reactive mAb neutralized DENV2, indicating that neutralizing activity observed in serum may rely on only a small fraction of DENV-specific B cells despite the broad binding activity of the total repertoire. Identification and characterization of these novel DENV2-specific mAb augment our repertoire of DENV2-specific reagents, and provide new insights to DENV2 neutralization mechanisms in addition to the few currently known DENV2-specific neutralizing mAb[57, 58, 59]. We recently showed that DENV1 passaged by laboratory cell lines were structurally immature and hypersensitive to neutralization by human antibodies, particularly those that are heterotypic, as compared with DENV1 circulating in human subjects[60]. With the caveat that the same notion remains to be tested for DENV2, we posit that the TS DENV2 mAb found here will neutralize circulating DENV2, while CR DENV2-binding antibodies may not.

It was striking that a high level of PB-derived DENV-specific mAb exhibited DENV2 strain specificity (towards DENV2 Tonga/74). At the time of the epitope mapping work, only the recombinant antigens based on the WHO DENV2 strain (S16803) were available, which directed our analyses to mAb that bound S16803 strain. Based on recent publications regarding differential neutralization abilities of antibodies against different genotypes or strains within a serotype, and failure of vaccines against genotypic variants[22, 61], it is important to understand the epitopes targeted by these strain-

specific antibodies and now this may change over time. However, this DENV2 strain-specificity was not seen in MBC (data not shown) (**Fig. 6B**), indicating that the memory response is broader than the PB response. These observations suggest that it could be an intrinsic property of the PB response to focus on the infecting DENV strain, while MBC may evolve to maintain broader specificity. Two limitations of our study are the small number of subjects in which we characterized the PB and memory response to primary rDEN2Δ30 infection and that rDEN2Δ30 is a model of primary infection. Nonetheless, similar differences in the breadth of PB and MBC compartments have been reported from a mouse model of West Nile virus infection[62].

The presence of antigen-specific MBC in circulation is one of the markers of long-term antiviral adaptive immunity[63]. To further interrogate the B cell response generated by primary DENV2 infection, we analyzed the DENV-specific repertoire of immortalized MBC 6-months post-infection. Across the three subjects, we estimated an average of 0.4% of the immortalized MBC pool to be DENV-specific compared to 0.5-8.1% reported for secondary DENV infections[35]. Our estimate was also similar to a reported frequency (0.39%) of DENV-specific cells in the MBC pool following primary natural DENV infection, and higher than the estimate from vaccination (0.08%)[64]. This level of antigen specificity in the MBC pool compared to natural infection supports the applicability of rDEN2Δ30 for use as a physiologically relevant, but safe challenge virus[36, 37].

In studies of natural infection, the DENV-specific MBC response has been characterized as overwhelmingly CR[23, 65, 66, 67]. We find here that, similar to the PB response, the MBC response to primary rDEN2Δ30 infection was biased towards the

infecting virus. A possible explanation for this discrepancy may be the time points after infection at which MBC were studied – we immortalized MBC six months after the infection, whereas previous studies utilized natural infection samples collected several years to decades post-infection. Also, definitive determination of DENV exposure status in natural infection may be inexact. It is also possible that the MBC and serological response further converge after many years due to population of the LLPC compartment being replenished with MBC-derived clones over time[35].

Regarding the possibility of clonal overlap between the PB and MBC compartments, we performed IGH/IGL sequencing on MBC from one of the subjects and identified four distinct lineages based on CDR3 sequences. We did not find these lineages in the PB repertoire from the same subject. One possibility can be attributed to the limited number of unique MBC sequences isolated from that subject. Another possibility is that there is might be little sharing of clones between the PB and the MBC arms, implicating different sources of these clones during B cell development and differentiation. Indeed, it has been reported that PB during acute secondary DENV infection represent a small subset of the MBC pool[35]. Additionally, recent work indicated that the activated B cell subset was phenotypically, transcriptionally, and functionally distinct from PB and contributes to the MBC pool to a greater extent than does the PB pool[14].

The predominance of the TS response observed in the PB repertoire was also corroborated in the serological data. Within 4 weeks following the infection, our subjects developed serum neutralizing antibodies against DENV2 but not DENV 1, 3, and 4, indicating that primary DENV infection in FV-naïve subjects resulted in a homotypic neutralizing response focused on the infecting serotype. This is consistent with our work

and those of others showing the importance of TS antibodies and their contribution in serum neutralization activity in natural infection[6, 22, 68]. This serological response was sustained into convalescence at 6-month post-infection despite the short-lived nature of the PB population. The unique insight afforded by the use of challenge model specimens was that the early B cell response to DENV infection contributes to the pool of long-lived plasma cells that produce serum antibody

In summary, we defined the temporal and specificity characteristics of the early B cell response to primary DENV2 infection and showed that these early antibodies produced by acute stage PB functionally overlap with those produced in the long-term MBC and LLPC compartments. With the caveat that this study was conducted using a small number of subjects, our results help to further validate the rDEN2Δ30 human challenge model of primary DENV infection, as has also been done by analysis of the T cell response[69]. Next, our data linking PB peak response with viremia may also provide a secondary means of indirectly monitoring viral replication *in vivo*, which will be important in the continued development of live attenuated dengue vaccines. In the context of both natural infections and live attenuated vaccines, by profiling the acute stage PB response, it may be possible to link viral replication with early PB and identify protective responses at the cellular level. We propose that the framework presented here may be useful for its capacity to identify novel immune response patterns, many of which phenocopy events seen in natural infections, and to predict the quality and durability of long-term antibody responses to vaccination.

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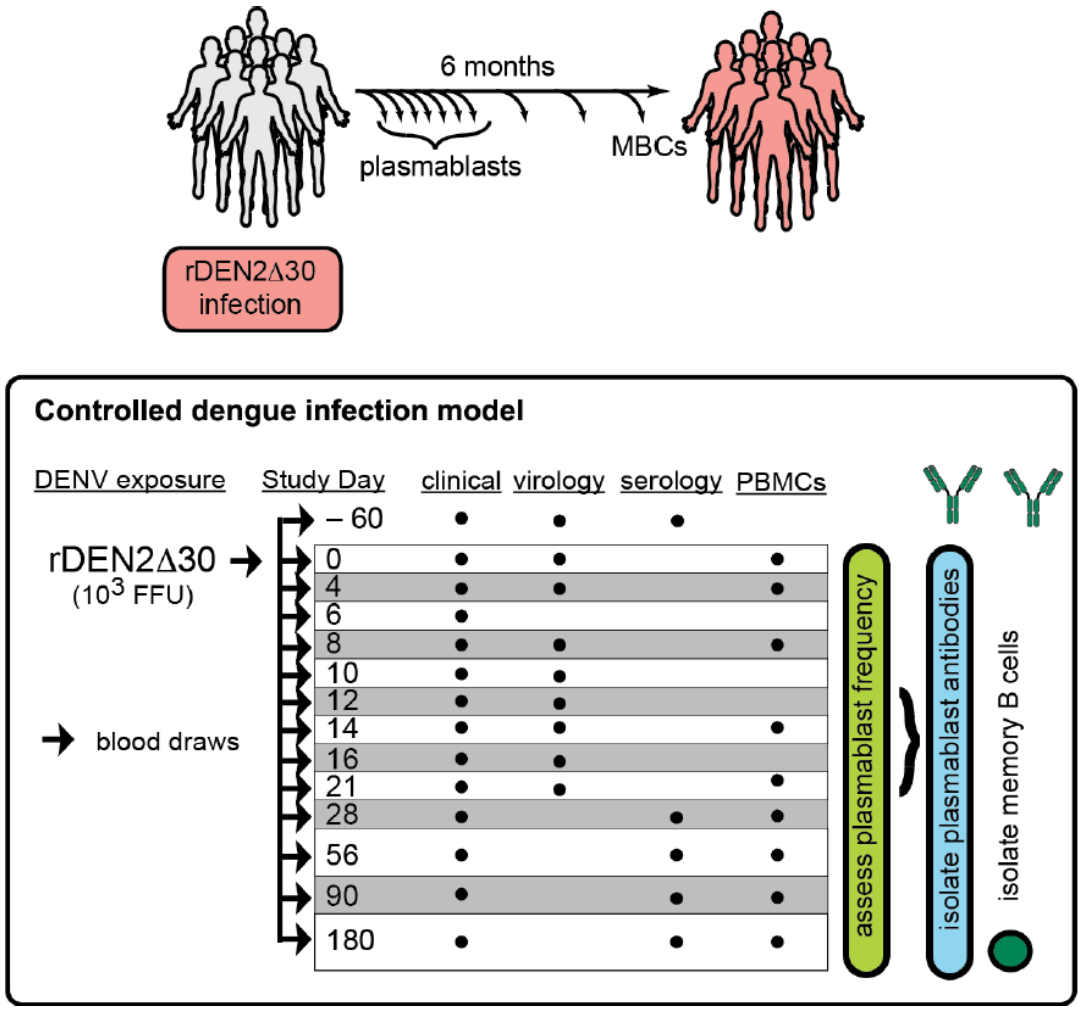
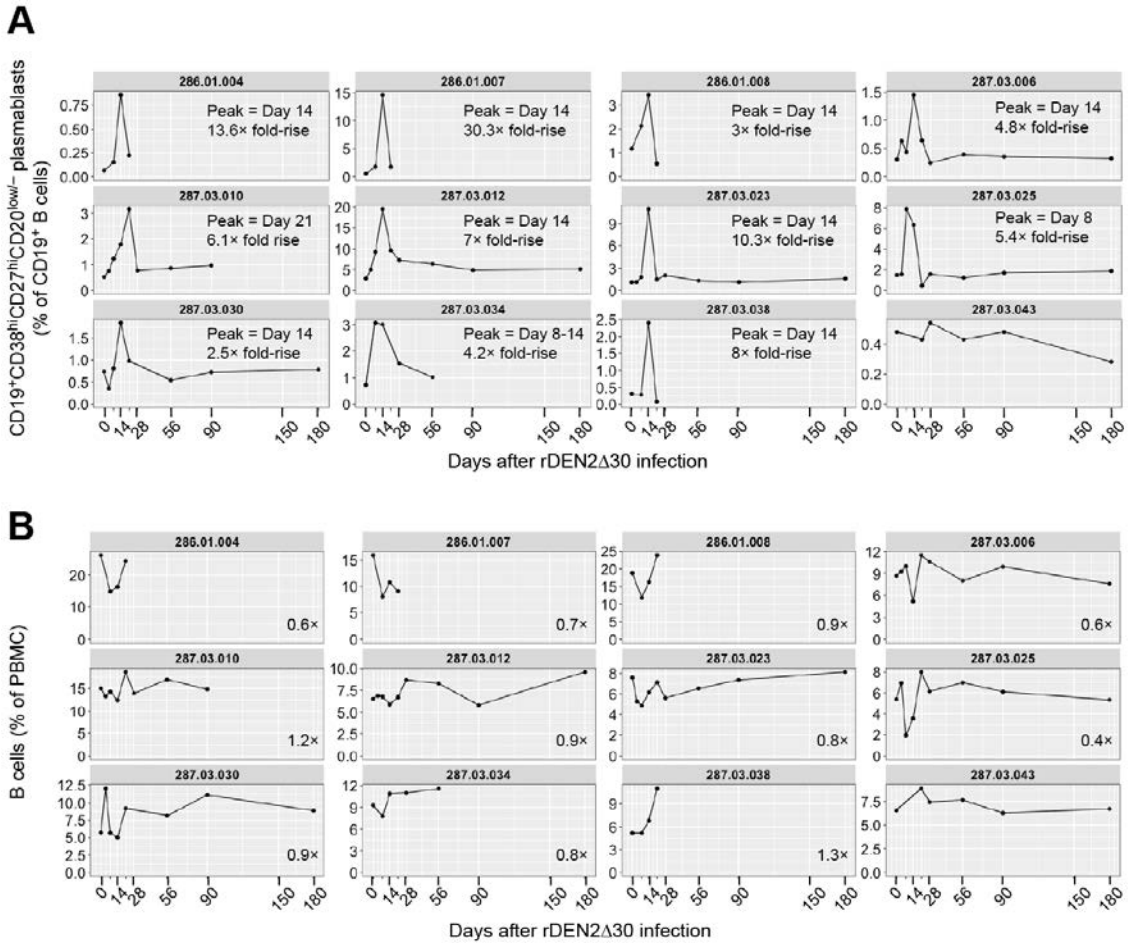
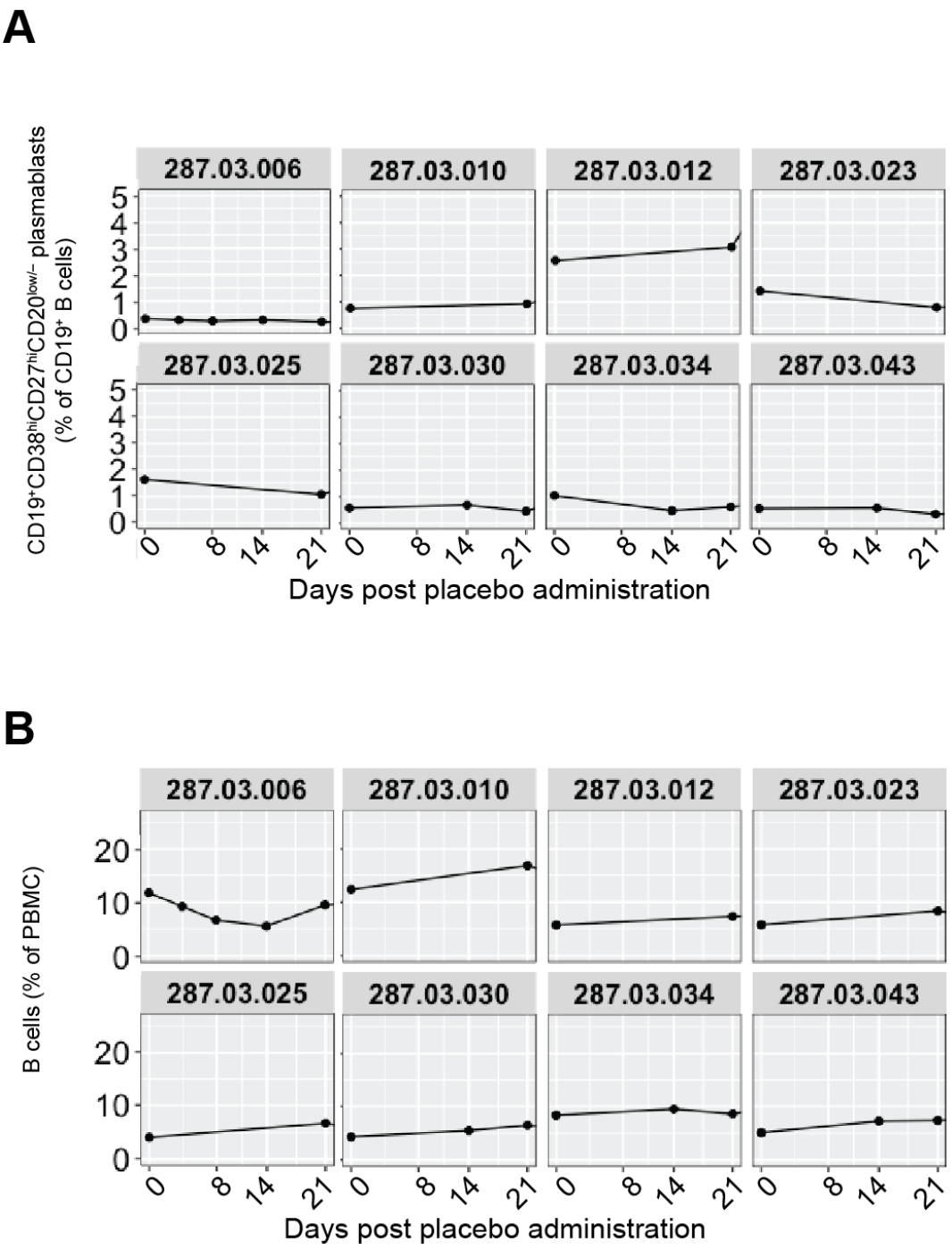
Supplemental information



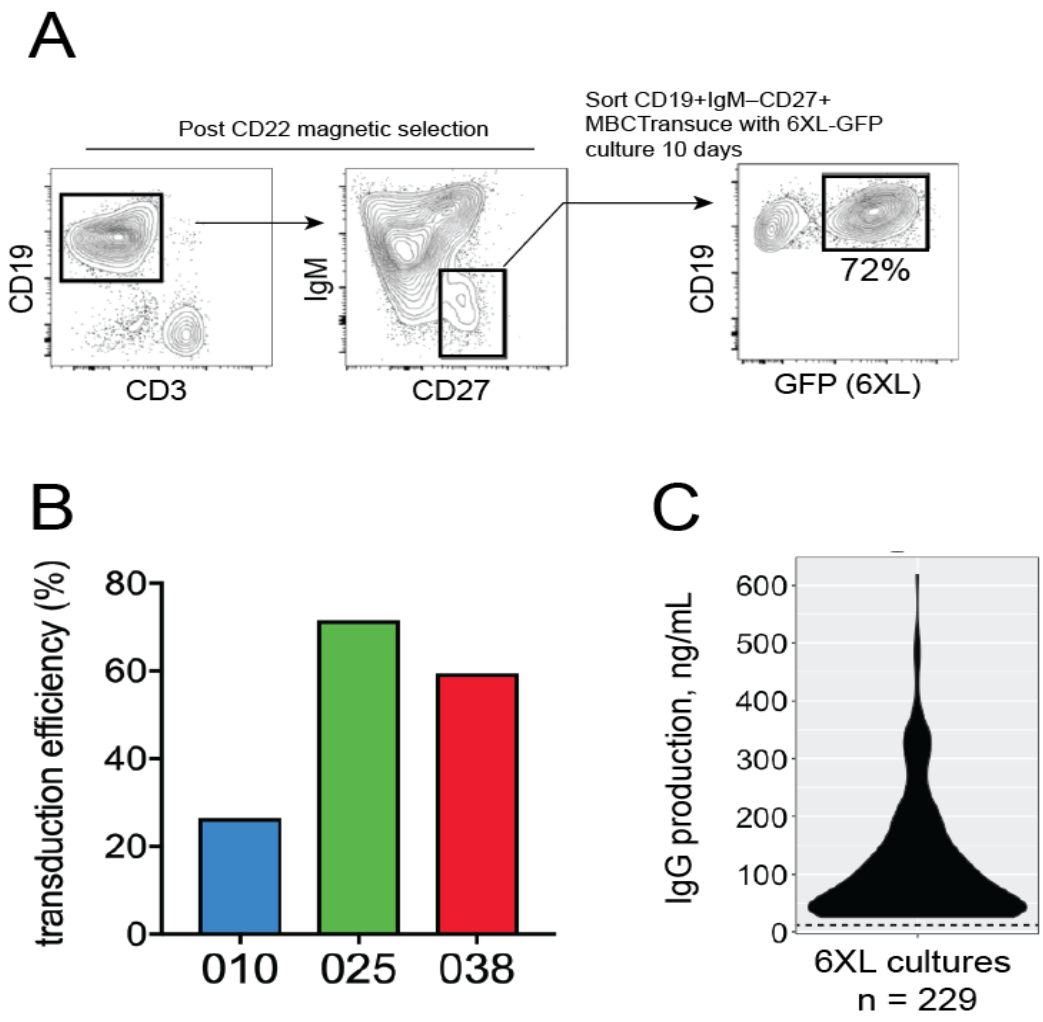
Supplemental Figure S1. Study design of controlled dengue 2 human infection. FV-naïve subjects were infected with rDEN2Δ30 as the placebo arm of a vaccine/challenge trial[41]. All subjects were viremic with mean peak titer of 2.3 $\log_{10}$ FFU/mL and all subjects seroconverted to DENV2.



Supplemental Figure S2. Subject-specific PB frequencies (as percent of total B cells) and overall B cell frequencies (as percent of live PBMC) to primary rDEN2Δ30 infection. (A) Day of peak PB response and fold-rise compared to baseline (Day 0) is noted in inset (average 8.7–fold, range = 2.5–30-fold rise in PB frequencies at peak). Fold-rise indicates the fold change in PB frequencies measured at Day 0 to frequencies measured on day of peak response. (% PB at day of peak response divided by the % PB at Day 0). (**B**) B cell frequencies were not affected by rDEN2Δ30 infection (average 1.3-fold).



Supplemental Figure S3. Pre-challenge baseline PB (A) and total B cell (B) frequencies in eight subjects prior to rDEN2Δ30 challenge. Subjects received a placebo (virus diluent) and PB and B cell frequencies were monitored for a three-week period. These subjects were then later challenged with rDEN2Δ30 (in Figure S2).



Supplemental Figure S4. Isolation and immortalization of switched peripheral blood MBC (**A**) representative fluorescence -assisted cell sorting of CD19+CD3-IgM- CD27+ MBC that are then transduced with BCL6+Bcl-xL (6XL) retroviral vector containing a green fluorescence protein (GFP) marker which is used to assess transduction efficiencies by flow cytometry of CD19+ B cells 10 days after transduction. (**B**) transduction efficiencies of IgM-CD27+ MBC from subjects 287.03.010, 287.03.025, and 287.03.038. (**C**) IgG production by stably-transduced 6XL+ MBC. Dashed line, limit of detection, 5 ng/mL.

Supplemental Table S1. Lineage traits of PB sequenced from three subjects infected with rDEN2Δ30.

Subject	010	025	038	Total
PB sequenced (pairs)	715	390	585	1690
Lineages detected	379	188	356	923
Lineages detected with >1 PB clone ("expanded")	78	35	98	211
PB in expanded lineages	414	237	327	978
Percent of all PB that are in expanded lineages	58%	61%	56%	58%

Supplemental Table S2. Lineage mapping of DENV2-binding mAb in individual rDEN2Δ30-induced PB repertoires

mAb	010	025	038	Total or average[1]
No. of PB in repertoire	715	390	585	1690
No. of PB in expanded lineages	414	237	327	978
No. of PB in tested lineages	335	202	204	741
Fraction of PB in expanded lineages that were in tested lineages	81%	85%	62%	76%
No. of PB in DENV2+ lineages	106	141	123	370
Fraction of PB in tested lineages that are DENV2+	32%	70%	60%	54%[1]

Supplemental Table S3. Sequence & functional characteristics of IGH/IGL pairs convergent in two or more rDEN2Δ30 infected subjects.

Priority #[a]	mAb ID	Subject IDs	IGH-V			IGL-V			DENV2 binding[d]	DENV2 neutralizing[e]
			VH gene	CDRH3 length (AA)	% Identity[c]	VL gene	CDRL3 length (AA)	% Identity[c]		
1	1579	**010 038**	IGHV3-30/33[b]	17	80	IGKV1D-39	10	86	+	–
2	1652	**025 038**	IGHV3-74	12	80	IGLV3-9	10	86	+	+
4	1618	**010 025**	IGHV3-30/33	15	77	IGLV1-51	12	81	–	ND
6	1581	**025 038**	IGHV5-51	16	83	IGKV4-1	10	91	–	ND
7	1619	**010 038**	IGHV1-18	17	84	IGLV2-11	11	87	–	ND
8	1582	**025 038**	IGHV3-30/33	14	80	IGKV1D-39	10	84	–	ND
10	1636	**025 038**	IGHV3-49	13	84	IGLV4-69	10	91	+	–
29	1639	**025 038**	IGHV3-30.33	18	83	IGLV1-51	12	89	ND[f]	NA[g]
42	1641	**010 025 038**	IGHV3-21	20	94	IGKV3-21	12	99	+	–
93	1674	**010 038**	IGHV1-18	12	94	IGLV2-23	11	97	+	–
95	1616	**010 038**	IGHV3-30/33	15	75	IGLV3-11	10	89	+	–
96	1617	**010 038**	IGHV3-30/33	15	98	IGLV4-1	10	97	–	ND
Summary (average or proportion)				15.3	84.3%		10.7	89.8%	**6/11 (55%)**	**1/11 (9%)**

a, From Figure 2E.
b, IGHV30 and IGHV33 germline genes have a high degree of sequence identity and are not called separately
c, BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) for entire IGHV or IGLV mature peptides between subjects
d, Binding to DENV2 by ELISA (see methods)
e, Neutralization of DENV2 by FRNT50 assay (see methods)
f, ND, not done
g, NA, not available

Supplemental Table S4. Frequencies of total B cells and IgM-CD27+ MBC in subjects infected six months prior with rDEN2Δ30 and the transduction efficiencies of these IgM-CD27+ MBC with 6XL.

Subject	% B cells in PBMC	% IgM–CD27+ MBC of B cells	% GFP+ after 6XL transduction
010	12.9%	23.5%	38.9%
025	7.1%	17.8%	89.6%
038	10.1%	10.6%	78.7%

Supplemental Table S5. Summary of Variable (V) gene usage, CDRH3 sequence, and somatic hypermutation of four monoclonal DENV2-binding mAb derived from MBC at 6 months after rDEN2Δ30 infection.

Poly-clonal culture ID	Mono-clonal culture ID	Heavy / Light chain	IGH/IGL gene usage	CDRH3 length (# of aa)	CDRH3 sequence (aa)	Mutation rate compared to germline		$NEUT_{50}$ DENV2 (ng/mL)
						SHMs (H+L)	non-silent (H+L)	
F6	11 clones	IgG1 Ig-κ	IGHV4-59/23 IGKV3-20.3D/10	23	ARGAARSPRRRGTLYSYPYMDVW	40 (25+15)	35 (20+15)	Not done
B10	B10	IgG1 Ig-λ	IGHV3-30.33/12 IGLV2-14/13	12	ARGLGLVPGAYW	34 (19+15)	29 (16+13)	Negative
F11	B7	IgG4 Ig-κ	IGHV3-23/20 IGKV3-20.3D/9	20	VRGPSGGFWSGYYIGAFDSW	44 (27+17)	41 (25+16)	Negative
F5	E6	IgG1 Ig-λ	IGHV1-69/17 IGLV2-11/11	17	AISGAAAASPYYYLDVW	28 (23+5)	26 (21+5)	35-100

Supplemental Table S6. Functional properties of PB-, MBC-, and serum-derived antibodies in three subjects infected with rDEN2Δ30.

		PB	MBC	Plasma (Neutralizing antibodies)		
				Tonga	NGC	S16803
Frequencies of DENV specific B cell clones	287.03.010	38%	0.32%			
	287.03.025	44%	0.73%			
	287.03.038	51%	0.27%			
Strain specific neutralization titers for serum	287.03.010			45	410	152
	287.03.025			1254	3734	3925
	287.03.038			361	194	304
TS	287.03.010	57%	54%			
	287.03.025	43%	26%			
	287.03.038	85%	100%			
CR	287.03.010	43%	46%			
	287.03.025	57%	74%			
	287.03.038	15%	0%			
% TS Neutralizing antibodies in serum	287.03.010					100%
	287.03.025					68%
	287.03.038					98%
% CR Neutralizing antibodies in serum	287.03.010					0%
	287.03.025					32%
	287.03.038					2%

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CHAPTER III – STIMULATION OF B CELL IMMUNITY IN *FLAVIVIRUS*-NAÏVE INDIVIDUALS BY THE TETRAVALENT LIVE ATTENUATED DENGUE VACCINE TV003

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Abstract

The tetravalent live attenuated dengue vaccine candidate TV003 induces neutralizing antibodies against all four dengue virus serotypes (DENV1-4) and protects against experimental DENV2 challenge in humans. Protection occurred with or without a secondary post-challenge rise in DENV2-specific neutralizing antibodies. Here we combined high-resolution temporal sampling and analysis of antigen-specific responses after vaccination and challenge to determine the cellular underpinnings of the B cell response in relation to vaccine viremia and serum antibodies. TV003 vaccine-related viremia was associated with an acute plasmablast (PB) response that correlated with the development of serum neutralizing antibodies. At 6-month following immunization, subjects had developed DENV2-specific memory B cells (MBC) including serotype-specific (TS) and multi-valent responders. DENV2 challenge of vaccinees did not induce a post-challenge PB response, though stronger and earlier post-vaccine PB responses were associated with sterile humoral protection from DENV2 challenge (i.e., a lack of DENV2 antibody boosting). Our findings demonstrate that TV003 vaccine triggers a durable B cell response containing PB and MBC, which functionally link early vaccine viremia and the serum antibody responses.

Introduction

The four serotypes of the dengue virus (DENV1-4) cause an estimated 390 million infections every year, with approximately 100 million clinically apparent cases[2]. Dengue disease ranges from non-specific febrile illness with rash and body aches to more severe symptoms including hemorrhagic fever or shock syndrome[3]. Infection by one serotype can confer life-long protection against subsequent symptomatic homotypic infections. After a short window of cross-protection following the primary infection, a heterotypic secondary infection can be associated with severe dengue disease[4, 5]. Thus, it is critical that a dengue vaccine promotes robust and balanced immunity to all DENV serotypes to provide maximum protection and minimize the risk for secondary infection-associated disease.

The most clinically advanced dengue vaccine candidates are tetravalent live attenuated vaccines[6]. Each includes the structural genes coding for prM (pre-membrane) and E (envelope) proteins from each of the four DENV serotypes. The E protein, which decorates the surface of the viral particle and mediates particle attachment and entry into host cells, is thought to be the major target of the anti-DENV neutralizing antibody response. Indeed, serum neutralizing antibodies have been the primary metric for evaluation of dengue vaccine immunogenicity[6]. Serum neutralizing antibodies correlate with protection for other *Flavivirus* (FV) vaccines against yellow fever and Japanese encephalitis viruses[7], but may be inadequate to explain protection for all dengue vaccines. The chimeric yellow fever/dengue (CYD) vaccine (i.e., yellow fever virus backbone with the prM and E proteins of DENV) induced a high rate of seropositivity (as assessed by serum neutralizing antibodies) to multiple serotypes, but protection varied widely across

serotypes and occurred mainly in subjects that were dengue seropositive at time of vaccination[8, 9, 10, 11]. Similarly, baseline serostatus appears to influence serum neutralizing antibody levels to the DENV2-based tetravalent live attenuated chimeric dengue vaccine TAK-003[12].

The tetravalent live attenuated dengue vaccine candidate TV003 from the National Institute of Allergy and Infectious Diseases has progressed though phase 1 and 2 clinical studies, including in endemic areas, where it has proven to be safe and able to induce neutralizing antibodies against all four DENV serotypes[12, 13, 14, 15]. TV003 is now in Phase 3 (NCT02406729) studies in endemic areas. TV003 completely protected FV-naïve adult subjects from clinical symptoms and dengue viremia, as measured by virus culture and RT-PCR, following challenge with a recombinant heterotypic DENV2, known as rDEN2Δ30[16]. Nearly half of the vaccinated cohort exhibited a rise in DENV2 serum neutralizing antibodies after challenge, indicating sterilizing immunity in some subjects. The major goal of our study was to evaluate the kinetics and phenotypic profile of plasmablasts (PB) and memory B cells (MBC) in the setting of protective human DENV vaccination.

Viral antigen exposure during infection or vaccination induces a population of highly proliferative antibody-secreting B cells known as PB[17, 18, 19, 20]. In our human DENV2 primary infection model, we showed that peripheral blood PB peaked at two-week post-infection, with a preponderance of these cells producing antibodies specific to DENV2[21]. Further, this DENV2-specificity was maintained in the MBC compartment at 6-month post-infection[21]. However, very little is known about the B cell responses to dengue immunization, or how these different cellular populations correlate with other

vaccine-associated parameters such as vaccine viremia and elicitation of serum antibodies. Here we utilized longitudinal serum and peripheral blood samples collected from subjects immunized with TV003 and subsequently challenged with DENV2 to ask how key features of the B cell response may correlate with vaccine immunogenicity and protection from challenge. We show TV003 vaccine viremia corresponded with a rise in PB that were associated with development of DENV serum neutralizing antibodies and a broad, durable DENV-specific MBC response.

Materials and methods

Study participants

Subjects in this study were participants of phase I studies to evaluate the safety and immunogenicity (trial CIR268/Clinicaltrials.gov NCT01072786[13]), and efficacy (trial CIR287/Clinicaltrials.gov NCT02021968[22]) of the tetravalent live attenuated dengue vaccine TV003. All subjects were serologically confirmed as FV-naïve at the time of immunization. Studies were approved by the Institutional Review Boards at the University of Vermont and the Western Institutional Review Board (Johns Hopkins University). Informed consent was obtained in accordance with federal and international regulations (21CFR50 and ICHE6). External monitoring was performed by the National Institute of Allergy and Infectious Diseases Data Safety Monitoring board every 6 months.

Clinical sample procurement

At study visits, blood was collected by venipuncture into serum separator tubes for analyses of viremia and serology, and into EDTA tubes for isolation of peripheral blood mononuclear cells (PBMC). Serum was frozen at –20ºC until use. PBMC were isolated by Ficoll-paque density gradient separation, counted, and frozen in cell culture medium with 10% dimethyl sulfoxide (DMSO) and 40% fetal bovine serum (FBS), and cryopreserved in liquid nitrogen vapor phase.

Viruses

DENV1 West Pacific 74, DENV2 New Guinea C, DENV3 Sleman/78, and DENV4 Dominica/81 were propagated in Vero-81 cells (American Type Culture Collection; CCL81, RRID:CVC_0059). These viruses were used both in DENV binding

ELISA and neutralization tests. Titers of virus stocks were determined by serial dilution and infection of Vero-81 cell monolayers on 24 well plates. Optimal dilution for use in ELISA was determined by serial dilution of stocks in DENV binding ELISA (see below) using a DENV CR mAb 1M7[22] at 1 μg per well to achieve an OD_{405} = 1.0 for each individual serotype to normalize amongst serotypes and assays. For the depletion assays, the Vero-81 derived purified WHO references strains, DENV1 (American genotype; strain West Pac74), DENV2 (Asian genotype; strain S-16803), DENV3 (Asian genotype; strain CH-53489), and DENV4 (American genotype; strain TVP-376) were used as described previously[23].

Serological analyses

Sera collected at follow-up visits every other day up to day 16 following TV003 immunization, and up to day 196 following DENV2 challenge, were tested for infectious virus by titration and infection of Vero-81 cells, and foci were detected with TS monoclonal antibodies as previously described[24]. Serum neutralizing antibody titers against DENV1-4 were determined by plaque reduction neutralization test (PRNT), using the lowest serum dilution that yielded a 50% reduction in viral plaques ($PRNT_{50}$), as previously described[16].

PB phenotyping

PBMC were surface-stained with the following fluorophore-conjugated monoclonal antibodies: anti-CD19 (HIB19, PE-Dazzle 594), anti-CD3 (UCHT1, Pacific Blue), anti-CD14 (HCD14, Pacific Blue), anti-CD20 (2H7, PE-Cy7), anti-CD27 (O323, Pacific Orange), anti-CD38 (HIT2, AlexaFluor 647), and 4’,6-diamidino-2-phenylindole (DAPI, Invitrogen). Anti-IgG (M1310G05, PE) and anti-IgM (MHM-88, PerCP-Cy5.5)

were also included to assess expression of surface immunoglobulin. All flow cytometry antibodies were purchased from BioLegend. PB were defined as CD3-CD14-D19+CD20-CD27++CD38++, from DAPI- lymphocytes. Data were acquired on a BD LSRII and analyzed using FlowJo version 10 (TreeStar).

Memory B cell isolation and immortalization

B cells were enriched from PBMC using positive magnetic selection with microbead-conjugated anti-CD22 antibodies (Miltenyi, catalog no. 130-046-401). CD22-enriched B cells were labeled with fluorophore-conjugated monoclonal antibodies: anti-CD3 (UCHT1, FITC), anti-CD19 (HIB19, PE-Dazzle 594), anti-CD27 (O323, PE-Cy7) and anti-IgM (MHM-88, PerCP-Cy5.5), and DAPI (Invitrogen). All flow cytometry antibodies were purchased from BioLegend. IgM-CD27+CD19+CD3- memory B cells (MBC) were sorted from the live (DAPI-) lymphocyte singlets into complete Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 8% FBS (Atlanta Biologicals), 100 units/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco) on a FACSAriaIII using the BD FACSDiva software.

Purified MBC were immortalized with BCL-6 and Bcl-xL via retroviral transduction, as previously described[21, 25]. MBC were first activated with 1×10^5 irradiated (50Gy) human CD40L-expressing mouse fibroblasts and 50 ng/mL recombinant human interleukin 21 (rhIL-21) (Peprotech and Shenandoah Biotech) on tissue culture-treated 24-well plates for 36-48 hours at 37°C, 5% CO_2. Following activation, cells were suspended in 0.25 mL serum-free IMDM and mixed with equal volume of retrovirus. Cells/virus mixture was added onto *non-tissue culture-treated* 24-well plate coated with 30 ng/mL retronectin (Takara, catalog no. T202) and blocked with

2% human serum albumin in phosphate buffered saline. Plates were centrifuged at room temperature for 1 hour at 700 × g followed by incubation at 37°C in 5% CO_2 for 6 hours to overnight. Cells were then washed and maintained in complete IMDM with 50 ng/mL rhIL-21 and CD40L-expressing cells on tissue culture-treated 24-well plate. After approximately two weeks of culture, MBC expressing CD19 and GFP (a marker for transduction) were sorted to polyclonal cultures at 50 cells/well onto 96 well plates containing complete IMDM supplemented with rhIL-21 and 2.5×10^4 CD40L-expressing cells using BD FACSAriaIII. Polyclonal cultures were maintained for three weeks before IgG-containing supernatant was collected to screen for DENV-reactivity. The average IgG concentration of polyclonal supernatant was 100 ng/mL at time of collection, as measured with IgG ELISA (Capture antibody: AffiniPure goat anti-human IgG (Fcγ), Jackson Immunoresearch catalog no. 109-005-008; detection antibody: Peroxidase AffiniPure goat anti-human IgG (Fcγ), Jackson Immunoresearch catalog no. 109-035-008; substrate: SureBlue, KPL, catalog no. 52-00-00).

ELISA to detect DENV-specific antibodies

The assays were performed using microplates (ThermoFisher, catalog no. 44-2404-21) as previously described[23]. Briefly, virus was captured by plate-adsorbed mouse CR anti-DENV envelope protein (E) monoclonal antibody 4G2. IgG-containing polyclonal supernatant was then added and positive DENV binding was detected by alkaline phosphatase-conjugated goat anti-human IgG (Fc) antibody (Sigma, catalog no. SAB3701277) and *p*-nitrophenyl phosphate substrate (Sigma, catalog no. N2770). Reaction color change, indicating DENV-binding, was measured by spectrophotometry as OD_{405}.

Antibody depletion from immune sera

Depletion of CR or TS antibodies from TV003 immune sera was performed as described previously[23]. Purified DENV was adsorbed onto microbeads (Polybead Microspheres, Polysciences Inc, catalog no. 17135-5; or Dynabeads M-280 Tosylactivated, ThermoFisher, catalog no. 14204). Beads adsorbed with bovine serum albumin (BSA) were used as a control. Human sera were diluted 1:10 in 1× PBS and incubated with BSA- or virus-adsorbed beads for 1 hour at 37°C with end-over-end mixing. Depleted sera were then tested for binding to the target serotype by ELISA as described above. For estimating the relative amount of DENV2 TS antibodies, beads incubated with a heterologous mixture containing an equivalent amount of DENV1,3,4 antigens were used. To estimate the % TS antibodies against DENV1, DENV3, or DENV4, beads incubated with DENV2 antigen were used. The percentage of CR and TS IgGs against each serotype were calculated using the following formulas:

$$\%\ \text{CR binding antibody} = \left(\frac{\text{OD after heterotypic depletion} - \text{BSA depletion}}{\text{OD after homotypic depletion} - \text{BSA depletion}}\right) \text{x } 100\%$$

$$\%\ TS\ antibody\ binding = 100\% - \%\ CR\ antibody\ binding$$

Statistical analyses

Differences in PB or CD19+ B cell levels at different time points were assessed using ANOVA and the post-hoc Tukey's Honestly Significantly Different (HSD) test for multiple comparisons in R (version 3.6.1). Correlation analyses between viremia, PB levels, and serum neutralizing antibody titers were performed using nonparametric Spearman correlation tests using GraphPad Prism (Version 8.0.2). To determine if/how the pattern of PB induction post-vaccination or post challenge was related the mode of protection (boosted = nonsterile versus not-boosted = sterile) over multiple time points

we used a linear mixed effects model[26] that allows for parametric repeated-measures testing incorporating error to account for data limitations such as sporadic missing samples (due to missed subject visit or sample viability). This was done using the lme R package in RStudio (R 3.6.1). ANOVA testing was then performed on LME models containing “boosting” and “day” versus “boosting” alone as interaction terms to evaluate significant difference in the model outputs. *P*-values are indicated.

Results

Kinetics of PB formation following TV003 vaccination

Acute DENV infection has been shown to induce a population of highly proliferative antibody-secreting B cells known as plasmablasts (PB); up to 70% of which are virus-specific[27, 28, 29, 30]. We previously showed that the increased level of PB in primary DENV2 infection correlated with viral load and neutralizing antibodies[21]. We therefore hypothesized that the kinetics and size of the PB pool would be related to vaccine viremia and serum antibodies in the context of vaccination with live attenuated dengue vaccines. To investigate this, we assessed CD38++CD27++ PB in PBMC of healthy FV-naïve adults following immunization with the tetravalent live attenuated dengue vaccine TV003 (**Fig. 1A**). TV003 induced peak PB frequencies ranging from 0.4 – 8.3% of B cells (median of ~2%) and occurring at days 4, 8, 14, 21, or 28 after immunization (**Supplemental Fig S1A**). Cumulatively, the data showed that PB were significantly induced on days 14 and 21 after immunization (**Fig. 1B**). Our samples were from two different phase 1 trials of TV003, from 2010 and 2013-2014, but PB kinetics or magnitude did not differ between the trials (**Supplemental Fig. S2A**). The increase in PB frequency after TV003 immunization was not due to changes in total CD19+ B cells as these remained stable after vaccination (**Fig. 1C, Supplemental Fig. S1B**).

Relation of PB and TV003 vaccine virus replication

We then focused on PB in the context of a TV003 vaccination and heterotypic DENV2 challenge (CIR287) to determine whether if PB dynamics reflected vaccine viral replication or protection from challenge. TV003 vaccine viremia across any serotype was detected by day 4 post-immunization and persisted up to one week and

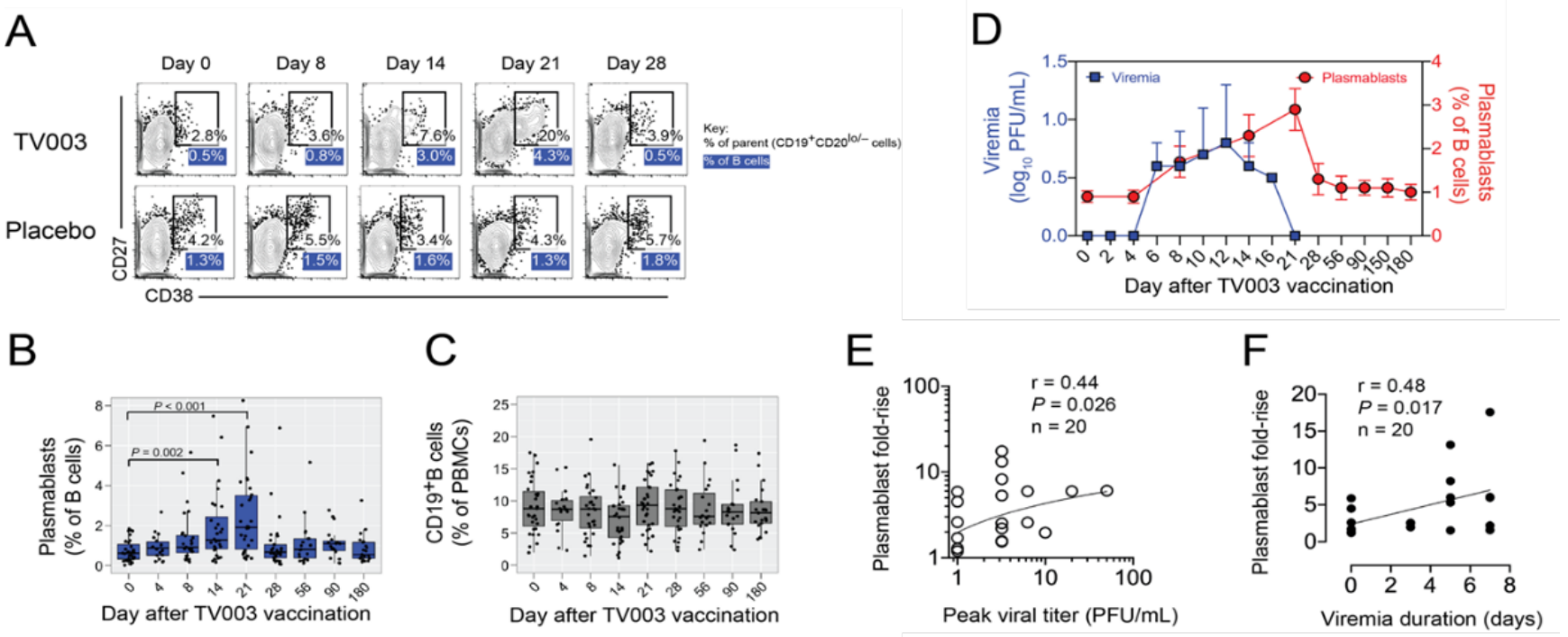

Figure 1. **Induction of PB by a live attenuated tetravalent DENV vaccine.** (**A**) PB were defined at the indicated times after vaccination by flow cytometry as CD38++CD27++ cells within the CD19+CD20- B cell population in live CD3-CD14- PBMCs from TV003 vaccinees. A representative vaccine and placebo sample are shown. Values in gate refer to percent of the CD19+CD20- parent population and the blue shaded values under the gate indicate the percent PB of total CD19+ B cells. (**B**) Frequencies of PB as a percentage of (**C**) total CD19+ B cells after vaccination with TV003 (n = 30). (**D**) Mean PB frequencies (red circles) and vaccine-associated viral titers (blue squares) are plotted as a function of day after vaccination with TV003 (n = 20). Correlation of day 21 PB fold-rise (defined as PB frequency at day 21 divided by the frequency at day 0) with (**E**) duration of viremia and (**F**) peak viral titer. Spearman R correlation coefficients and *P*-values are reported.

waned before the peak PB response was detected (**Fig. 1D**). Point-in-time PB frequencies at days 14 and 21 after vaccination did not correlate with vaccine peak viremia (**Supplemental Fig S3A, B**) or vaccine viremia duration (**Supplemental Fig. S3D, E**). To account for subject-level variation in baseline and post-vaccination PB responses (**Supplemental Fig. S1A**), we calculated the ratio (i.e., fold-rise) of peak to baseline frequencies. Given that day 21 after vaccination was the prevailing peak PB response time point (in 17 of 30 vaccinees, **Table S1**), we calculated fold-rise at day 21 for each subject. Day 21 PB induction positively correlated with peak viral titer (**Fig. 1E**) and with viremia duration (**Fig. 1F**). We also found positive correlations between any post-vaccination peak PB induction (**Table S1**) with peak viral titer (**Supplemental Fig. S3C**), and viremia duration (**Supplemental Fig. S3F**). Taken together, these data showed that PB induction positively correlated with TV003 vaccine viral replication.

TV003-induced PB and serum neutralizing antibodies

All TV003 vaccinees developed serum neutralizing antibodies to at least three DENV serotypes, and 92% developed a tetravalent response through day 180 after a single dose[16]. Serum neutralizing antibodies to all four serotypes were present at day 28 post-vaccination and were maintained for at least 6 months (**Fig. 2A**). We found a positive correlation (R = 0.55, $P = 0.006$) between day 21 PB fold-rise and overall DENV1-4 serum neutralizing antibody titers (**Figure 2B**) and titers for DENV3 and DENV4, while the relationship to antibody titers for DENV2 and DENV1 was weaker (**Figure 2C-F**). Point-in-time PB frequencies at days 14 or 21 after vaccination did not correlate with neutralizing antibodies (**Supplemental Figure S4A, B**). Peak PB induction (fold-rise at any point within 28 days after vaccination compared to baseline)

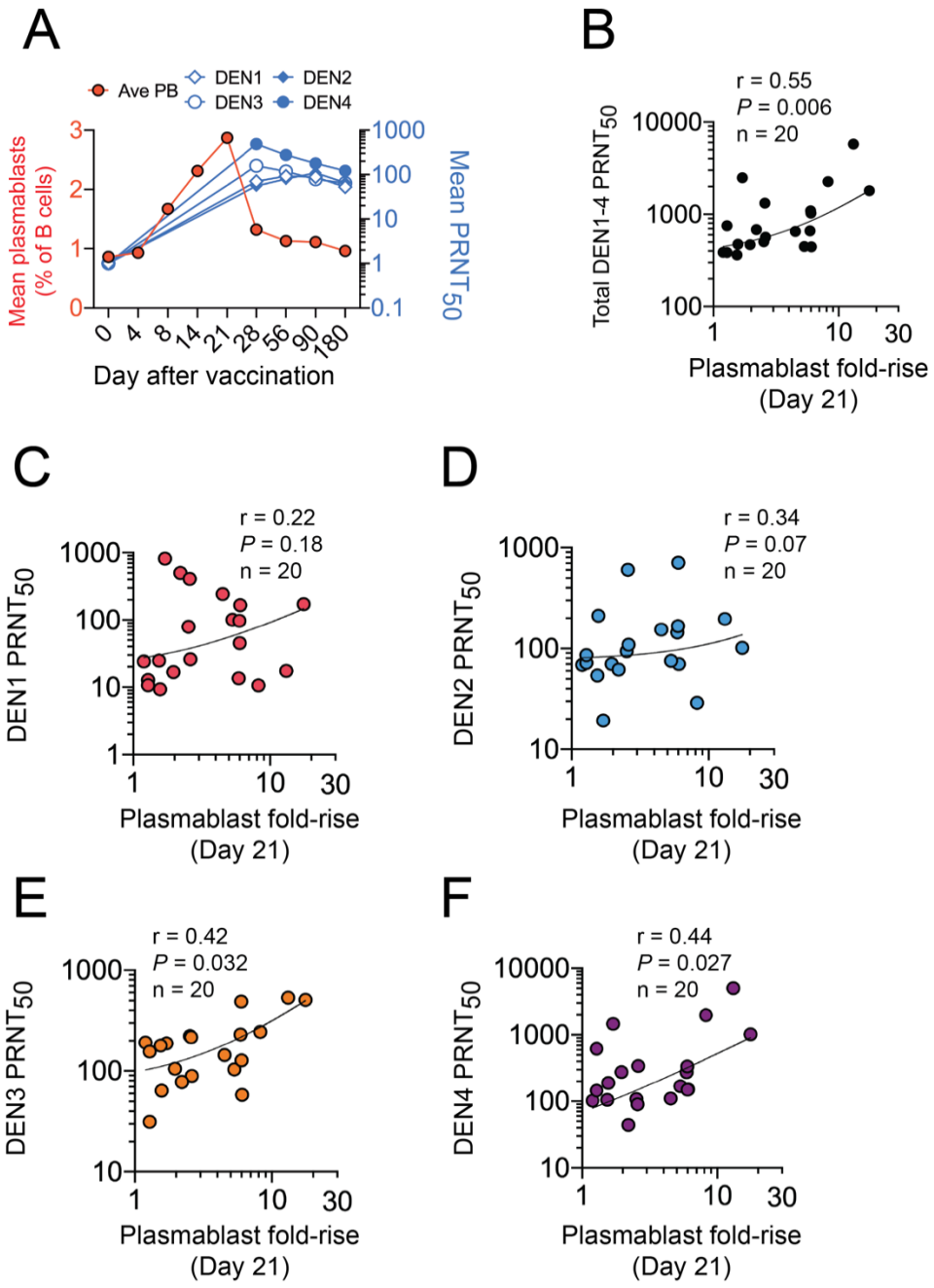

Figure 2. Induction of PB and serum neutralizing antibodies by TV003. (**A**) Kinetics of PB (red, left y-axis) and DENV1-4 neutralizing antibody responses (right y-axis, $PRNT_{50}$) after vaccination with TV003. Mean responses in 21 subjects are shown. (**B-F**) PB responses (Day 21 fold-rise) are plotted against the (**B**) sum of peak DENV1-4 PRNT50 titers or against individual (**C**) DENV1, (**D**) DENV2, (**E**) DENV3, or (**F**) DENV4 peak PRNT50 values induced within 180 days after TV003. Spearman correlation coefficients and *P*-values are reported.

was positively correlated with DENV3 neutralizing antibody titers, and less so with other antibody titers for other serotypes or with overall DENV serum neutralizing antibody titers (**Figure S4C**). Thus, the magnitude of the PB response to TV003 immunization correlated with peak DENV serum neutralizing antibody titers.

TV003-induced T cell response and acute B cell response

Activated CD4+ T cells can aid in the generation of broad antiviral antibody responses, prompting us to investigate whether TV003 induced a CD4+ T cell response in association with B cell responses. Though cytokine-independent assays have been used to detect antigen-specific cells in natural dengue[31], we directly identified TV003-induced DENV-specific CD4+ T cells by *ex vivo* stimulation of PBMC with DENV-derived MHC class II peptide megapools, as described[24, 32, 33]. We previously showed that TV003 induced a robust DENV-specific T cell response in the same CIR287 cohort, with peak response on day 21 post- immunization[32]. For subjects with matching T cell, PB, and neutralizing antibody data, we determined the day 21 fold-rise for CD4+ T cells to be consistent with the day 21 PB induction metric. We observed a positive, though not statistically significant, trend of DENV-specific CD4+ T cell activation and PB induction after TV003 vaccination (**Supplemental Fig. S5A**), but found a significant correlation (R=0.86, $P < 0.001$) between DENV-specific day 21 CD4 T cell fold-rise and overall peak DENV neutralizing antibody titers (**Supplemental Fig. S5B**). These results implicate acute CD4+ T cell response and the development of serum antibodies following TV003 immunization.

Post-vaccination and post-challenge PB dynamics

Although PB were elicited after primary DENV2 infection of FV-naïve subjects[21], there was no change in the PB frequencies following DENV2 challenge in subjects vaccinated with TV003 (**Fig. 3A**). Comparison between pre- and post-challenge DENV2 serum antibody titers across-subjects revealed that the titers were boosted (≥ 4-fold increase) upon DENV2 challenge in 9 of 21 subjects (boosted group), but not in the remaining subjects (non-boosted group) (**Fig. 3B**). In the non-boosted group, we observed a more robust and earlier (day 8-14) PB increase compared to the boosted group (**Fig. 3C**). Similarly, PB fold-rise was earlier and higher at days 8 and 14 post-vaccination in the non-boosted subjects versus boosted subjects (**Fig. 3D**). Day 21 PB trended higher in non-boosted vaccinees but did not discriminate between protection modes (**Fig. 3C, 3D**).

We then assessed post-challenge PB in both groups of vaccinees, reasoning that recall of TV003-elicited memory B cells by DENV2 challenge may generate a cellular response consistent with PB induction in boosted subjects. However, post-challenge PB frequencies and fold-induction were overall low and did not differ in magnitude or kinetics by mode of protection (**Fig. 3E, 3F**). Taken together, our results suggested that early and robust PB induction occurring at 1-2 weeks after TV003 vaccination was consistent with sterilizing humoral immunity to subsequent DENV2 challenge.

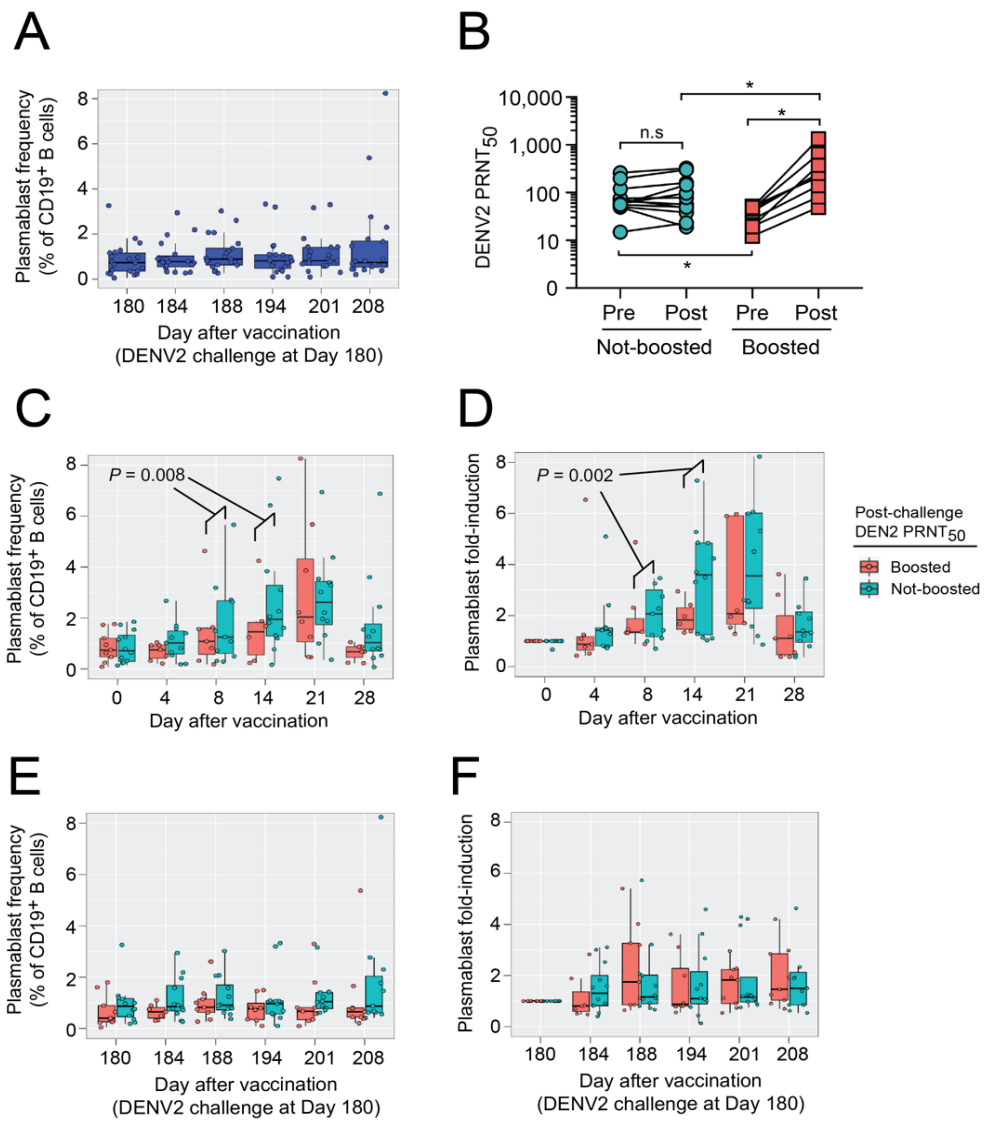

Figure 3. Post-TV003 and post-challenge PB induction is associated with mode of protection against challenge with rDEN2Δ30. (**A**) PB frequencies were determined at intervals after DENV2 challenge (day 180) in subjects previously vaccinated with TV003 (n = 20). (**B**) DENV2 $PRNT_{50}$ titers in TV003 vaccinees challenged with DENV2 are plotted for day 180 after vaccination (i.e. at time of challenge) and at peak response after challenge. Subjects were classified as "Boosted" or "Not-Boosted" depending on a ≥ 4-fold rise in DENV2 $PRNT_{50}$ titers post-challenge compared to pre-challenge titers. (**C**) PB frequency or (**D**) fold induction was assessed post-TV003 vaccination as a function of the subjects' booster response. Data were fit to linear mixed effect models and a significant interaction between "boosted" and "day" on days 8 and 14 after vaccination was found by ANOVA analysis of the model containing both variables versus that with just the 'boosted' term. (**E**) PB frequency or (**F**) fold induction was assessed post-DENV2 challenge as a function of the subjects' booster response.

Breadth of DENV-specific memory B cells induced by TV003 immunization

A key goal of live attenuated tetravalent dengue vaccines is the generation of durable memory. To determine whether TV003 elicited a durable DENV-specific MBC response, we employed genetic reprogramming[25] to immortalize class-switched (IgM-) memory (CD27+) CD19+ B cells from TV003 vaccinees at 6-month after immunization and screened their secreted IgG for reactivity to DENV antigen. We screened MBCs for DENV2 reactivity because this was a tetravalent vaccine/ DENV2 challenge study, and this would allow us to compare DENV2-specific MBC frequencies to our previous study that estimated DENV2-binding MBC frequencies after primary DENV2 infection[21]. MBC transduction efficiency (as indicated by GFP transduction marker positivity) averaged at 67% (range 26 - 94%, **Supplemental Table S2**) across the 11 subjects, indicating broad repertoire coverage. To reduce the complexity of the transduced polyclonal MBC population, we cultured GFP+ MBC at 50 cells/well and screened for DENV2-reactive IgG by ELISA. We estimated the frequencies of DENV2-reactive cells in the MBC pool by dividing the number of DENV-binding-positive wells by the total number of GFP^+ cells screened, as we have done previously[21, 34].

We estimated an average 0.15% of the MBC repertoire to be DENV2-reactive (ranged 0.03%-0.40%) at 6-month post TV003 vaccination (**Fig. 4A**). This frequency of DENV2-specific MBC post-vaccination was lower than that found for primary DENV2-infected subjects at the same time point (**Fig. 4A**)[21]. Amongst the TV003 group, DENV2-specific MBC frequencies did not discriminate between subjects that subsequently did or did not boost DENV2 serum antibody titers after challenge (**Fig. 4B**). We also assessed whether the endpoint of post-vaccination DENV2-specific MBC was

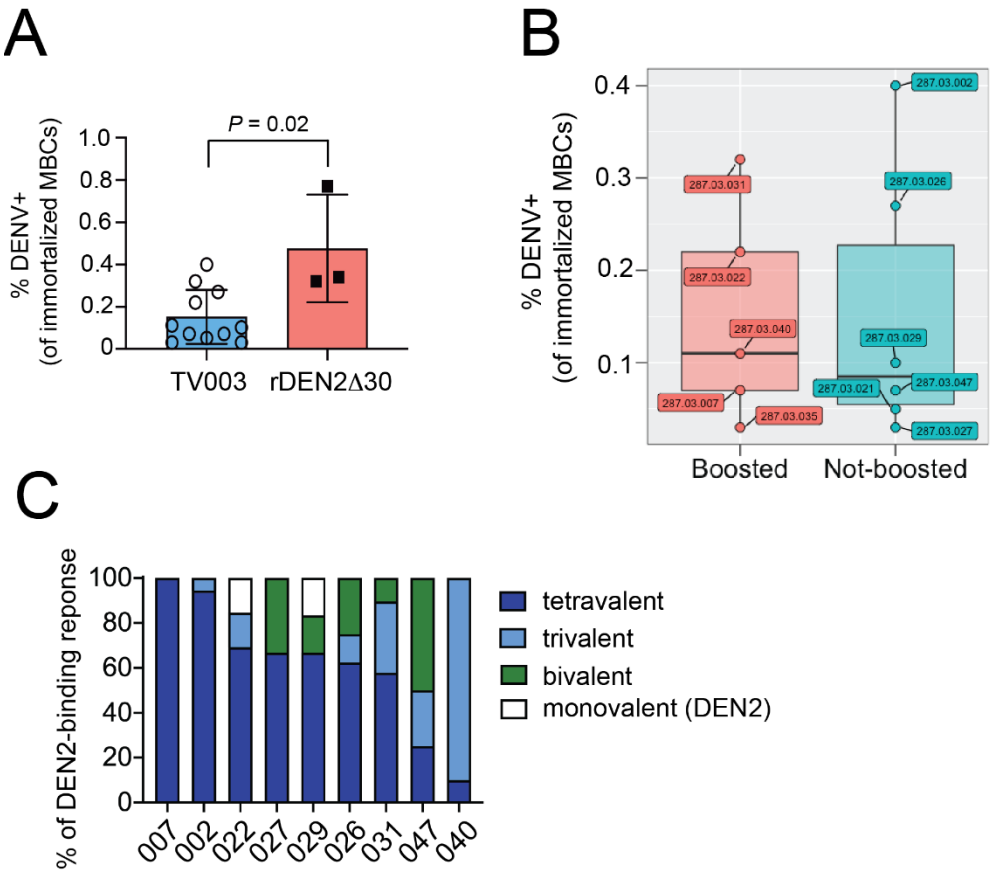

Figure 4. TV003 vaccination induces DENV-specific memory B cells (MBC). (**A**) IgM-CD27+ MBC were isolated six months after TV003 immunization (n = 11 subjects) or after primary DENV2 infection (n = 3 subjects), immortalized with BCL6 + Bcl-x_L (6XL), and supernatants from polyclonal (50 cells/well) cultures of 6XL-immortalized cells were screened for IgG binding to DENV2 (the challenge virus). The frequency of DENV2-reactive MBC was estimated from the total number of cells screened, and based on the average of 1-2 reactive clones in a positive polyclonal culture as previously shown. Statistical significance was determined using the non-parametric Mann-Whitney test. (**B**). Frequency of DENV-specific MBC at time of DENV2 challenge (Day 180) in TV003 vaccinees with non-sterile protection (i.e. Boosted post-challenge DENV2 neutralizing antibody levels) and those with sterile protection from DENV2 infection (i.e. Not-boosted DENV2 antibody levels). (**C**) DENV2-reactive MBC cultures from 9 TV003-vaccinated subjects (subject IDs on x-axis) were screened individually for binding to DENV1, DENV3, and DENV4 virions by ELISA and the proportions of monovalent (DENV2-only); bivalent (DENV2 + one other DENV); trivalent (DENV2 + two other DENV); and tetravalent (DENV1-4) are expressed as percentage of total response.

reflective of the vaccine-induced PB response or serum antibody titers (data not shown). We did not find significant correlation of day 180 DENV2-specific MBC frequencies with post-TV003 plasmablasts at days 14 or 21 or overall serum neutralizing antibodies (**Table S3**). We therefore conclude that TV003 induces DENV2-reactive MBCs at 6 months after TV003 vaccination, but this metric was not sufficient to predict sterile immunity from DENV2 challenge in a subset of TV003 vaccinees.

Humoral immunity to DENV is a complex mixture of serum antibodies that bind to DENV in a TS or CR manner. We next asked the extent to which DENV-reactive IgG elicited by TV003 vaccination were TS or CR at the cellular level (i.e., MBC). Since this was a TV003/DENV2 challenge study we screened the DENV2-binding MBC-derived IgG for binding to DENV1, -3, and -4 antigens. Amongst the MBC that were immortalized, we found a broad range of responses from DENV2-TS to bi-, tri-, and tetravalent responses in TV003 vaccinees (**Fig. 4C**). Our results demonstrate that TV003 induces a broad array of DENV2-reactive MBC that are readily found 6 months after vaccination.

Multivalent serum neutralizing antibody responses induced by TV003

To qualitatively explore the composition of total TS and CR binding antibodies for each DENV serotype at 6-months post-TV003, we used an established virus depletion approach[23, 35, 36]. To assess DENV2- TS and CR binding activity, we depleted TV003 vaccine serum samples with microbeads coated with DENV1, 3, 4 antigens (heterotypic depletion). For other serotypes, we incubated serum with beads coated with DENV2 (homotypic depletion). For all serotypes, we found that between 50-80% of total binding activity was due to CR antibodies (**Fig. 5A**). In agreement with our MBC results for

DENV2, we found a broad span of serum DENV2 binding activity with TS:CR ratios ranging from 0-0.7 (**Fig. 5B**).

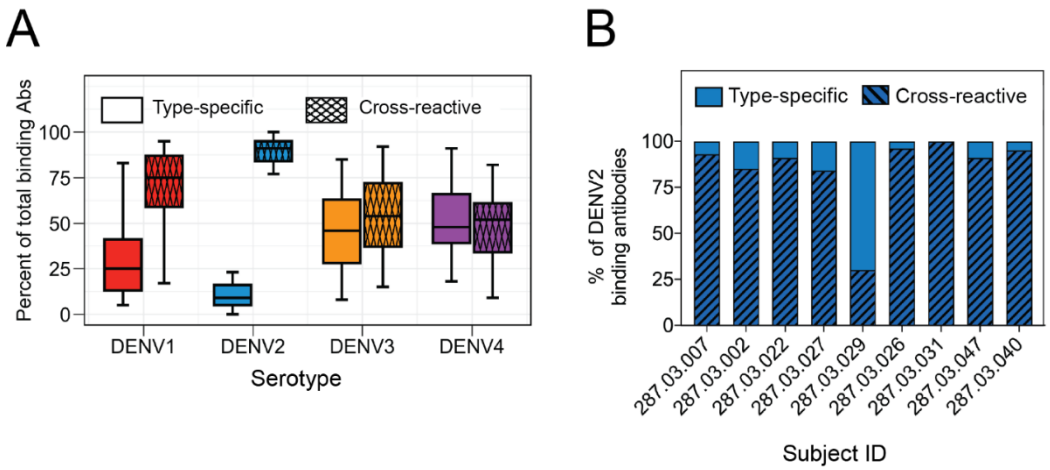

Figure 5. Properties of the DENV-reactive serum antibody response in FV-naive subjects who received the live attenuated tetravalent DENV vaccine TV003. The properties of the serum DENV-binding response in convalescent (6 months after vaccination) TV003 vaccinees was determined by virus depletion and DENV-binding ELISAs. (**A**) TS and CR binding to each serotype was determined. Box plots represent the 25-75th percentiles with whiskers showing the 5-95th percentiles with the horizontal bar showing the median of the fraction of each type of antibody measured across subjects (n = 21 subjects). (**B**). The proportion of DENV2-reactive serum antibodies were classified as TS (open bars) and CR (hatched bars) was determined for a subset of 9 subjects with memory B cell reactivity data (See Figure 4).

Overall, our results demonstrate that TV003 elicited an early PB response, the kinetics of which appeared to correlate with subject-specific modes of protection from DENV2 challenge. TV003 also induced a tetravalent response in both serum and memory B cells, suggesting durable immunity across multiple humoral compartments.

Discussion

We have examined the underpinnings of the B cell response over time in relation to vaccine viremia and serum antibodies in the context of TV003-mediated protection from DENV2 challenge. We and others have found a positive correlation between PB induction and viremia during acute DENV infection[21, 27]. Here we extended this relationship to immunization with the live attenuated vaccine TV003. Whereas primary DENV2 infection led to peak viremia titers of 2-3 $\log_{10}$ PFU/mL and a peak PB frequency of 2-20% (of B cells) on day 14 after infection[21], the more attenuated TV003 vaccine virus generated lower peak viremia titers (0.5 – 1.7 $\log_{10}$ PFU/mL), and lower PB peak frequencies (2-7%) at a later time point (days 14-21) after vaccination. These results suggest that, in the context of both primary DENV infection or vaccination with a live attenuated vaccine, viral load drives the magnitude and kinetics of the PB response, which predominantly occurs within one month after inoculating. High PB levels were detected outside of the 28-day window post-vaccination in two subjects (015 and 018, **Supplemental Fig. S1A**). We do not know if this increase was induced by the vaccine, but we posit that this may not be the case due to clearance of viremia by this point.

A recent report on the PB response following TV003 immunization in a DENV-experienced subject showed a massive 70-fold PB increase[37] – possibly the manifestation of an anamnestic response induced by immunization. We previously showed that TV003 vaccination generated higher DENV serum neutralizing antibodies in subjects who were FV-experienced at the time of vaccination, compared to FV-naïve subjects[38]. Future work will determine how FV experience influences the PB response to TV003 vaccination.

DENV-specific PB are highly induced in natural dengue infection[27, 30]. We recently isolated over 40 DENV2-specific monoclonal antibodies – several of which were neutralizing – from PB induced by primary DENV2 infection[21]. We found here that PB correlated also with TV003 serum neutralizing antibodies. Since PB responded concordantly with both viremia and antibodies, our results suggest that these cells act as an important mechanistic cellular link between replication of virus and the development of neutralizing antibody responses.

To evaluate how PB kinetics may explain TV003-associated modes of protection, we examined the PB induction in subjects experiencing sterile and non-sterile protection from DENV2 challenge. An earlier and more robust vaccine-induced PB response was associated with higher DENV2 antibody titers at the time of challenge, and with sterilizing immunity against rDEN2Δ30. Meanwhile, a weaker vaccine-induced PB response was associated with non-sterilizing immunity upon DENV2 challenge. We posit that an earlier and stronger vaccine-induced PB expansion may generate a higher neutralizing antibody baseline associated with sterilizing immunity. Additionally, there may be subject-specific clonal repertoire differences in DENV2-specific B cells that produce highly potent TS neutralizing antibodies such as 2D22[39, 40] or broadly CR neutralizing antibodies such as those that target E-dimer epitopes[29].

Our data show that TV003 induced antigen-specific MBC at 6-month post vaccination. This finding extends previous work by us and others showing the presence of antigen-specific MBC after natural infection with DENV[21, 41, 42, 43], DENV1 monovalent vaccination[44], Zika virus infection[34], hepatitis C infection[45, 46], or respiratory syncytial virus exposure[25]. Furthermore, by comparing the proportion of DENV2-specific

MBC at 6-month after primary DENV2 infection and TV003 vaccination, these results suggest that early viremia after infection or vaccination may help to "set" antigen-specific MBC frequencies in early convalescence.

Given that TV003 induced durable MBCs but distinct post-challenge DENV2 antibody responses, we hypothesized that secondary exposure to DENV2 antigens would recall TV003-elicited MBCs into a germinal center-like phenotype that may produce PB. However, secondary PB were not observed after challenge. One potential explanation is that the lack of DENV2 viremia following challenge may translate to a lack of antigenic stimulation for a PB response. Another possibility is that recalled MBC may exhibit a distinct phenotype compared to that of PB. We and other have previously shown there was limited clonal overlap between the PB and the MBC repertoires in DENV infections, suggesting these cells may follow distinct differentiation paths[21, 47]. To address this possibility, we also performed a correlation analysis of vaccine-induced DENV2-specific MBC frequencies versus vaccine-induced PB levels and serum neutralizing antibodies, and failed to find statistically significant relationships. These data suggested that post-vaccination precursor MBC frequencies may not be related to the boost response in the subset of DENV2-challenged TV003 vaccinees. In agreement with previous and current findings[21], others have shown that the MBC repertoire has limited overlap with PB[48]. Moreover, MBC exhibited substantial overlap with the CD71+ "activated B cell" (ABC) response, which engaged later than the PB response[48]. Though we did not capture the ABC response in our study, it is possible that DENV2 challenge re-activated quiescent vaccine-associated MBCs to assume an ABC-like phenotype and contribute to the "boost" response.

TV003 immunization elicits neutralizing antibodies against all four DENV serotypes[13, 14, 16, 49] and protection against DENV2 challenge[16], but the specificities of the DENV-specific antibody responses are unclear. Serum antibody neutralization in natural DENV infection[50, 51] and monovalent or tetravalent DENV vaccination[35, 52, 53] rely considerably on TS clones. However, broadly neutralizing CR antibodies have been isolated from individuals with multiple DENV exposures[29]. In our study, DENV2 reactivity was attributable to CR antibodies in both the MBC and serum compartments. The formation of this CR response probably reflected the higher stoichiometry of conserved versus heterotypic epitopes present in this tetravalent vaccine. Our findings are congruent with previous studies showing a preponderance of CR antibodies in DENV infections and monovalent vaccination, suggesting there may be fewer TS antibodies clones compared to CR clones[43, 44].

With our DENV2-focused screening strategy we did not investigate TS and CR responses for the other serotypes, which would provide a broader view of the humoral landscape generated by TV003. Although we readily detected DENV-specific MBC 6 months after TV003 vaccination, it will also be important to determine how long these cells persist beyond this period and whether this is akin to tetanus- or smallpox-reactive B cells that have been shown to persist for decades[54, 55]. Another limit to our study was that IgG produced by MBC cultures was insufficient for neutralization assays in order to probe the functionality of MBC-derived antibodies. Furthermore, we recovered a relatively low number of DENV2-reactive MBC, presumably due the limited replication potential of the inoculum. Nonetheless, our MBC studies show that TV003 elicits a broad

panel of virus-specific MBC producing of TS and CR antibodies that are durable at 6-month after vaccination.

In addition to humoral immunity, we and others have found that durable CD4+ and CD8+ T cell responses are induced by tetravalent live attenuated dengue vaccination[24, 32, 56]. Thus, it is also possible that T cells activated by TV003 protected against DENV2 challenge in concert with B cells in subjects exhibiting sterilizing humoral immunity. Indeed, our data indicated a positive relationship between DENV-specific CD4+ T cell response and serum DENV neutralizing antibody titers following TV003 immunization.

In sum, early and robust PB responses to TV003 vaccination correlate with sterilizing humoral immunity to DENV challenge. Maintenance of durable balanced activity against all DENV serotypes elicited by TV003 may involve both MBC and plasma cells producing potently neutralizing TS and CR antibodies. We propose that analyzing the different cellular components of the humoral response to vaccination may be an important approach to provide new ways to assess vaccine performance and immunogenicity.

Acknowledgments

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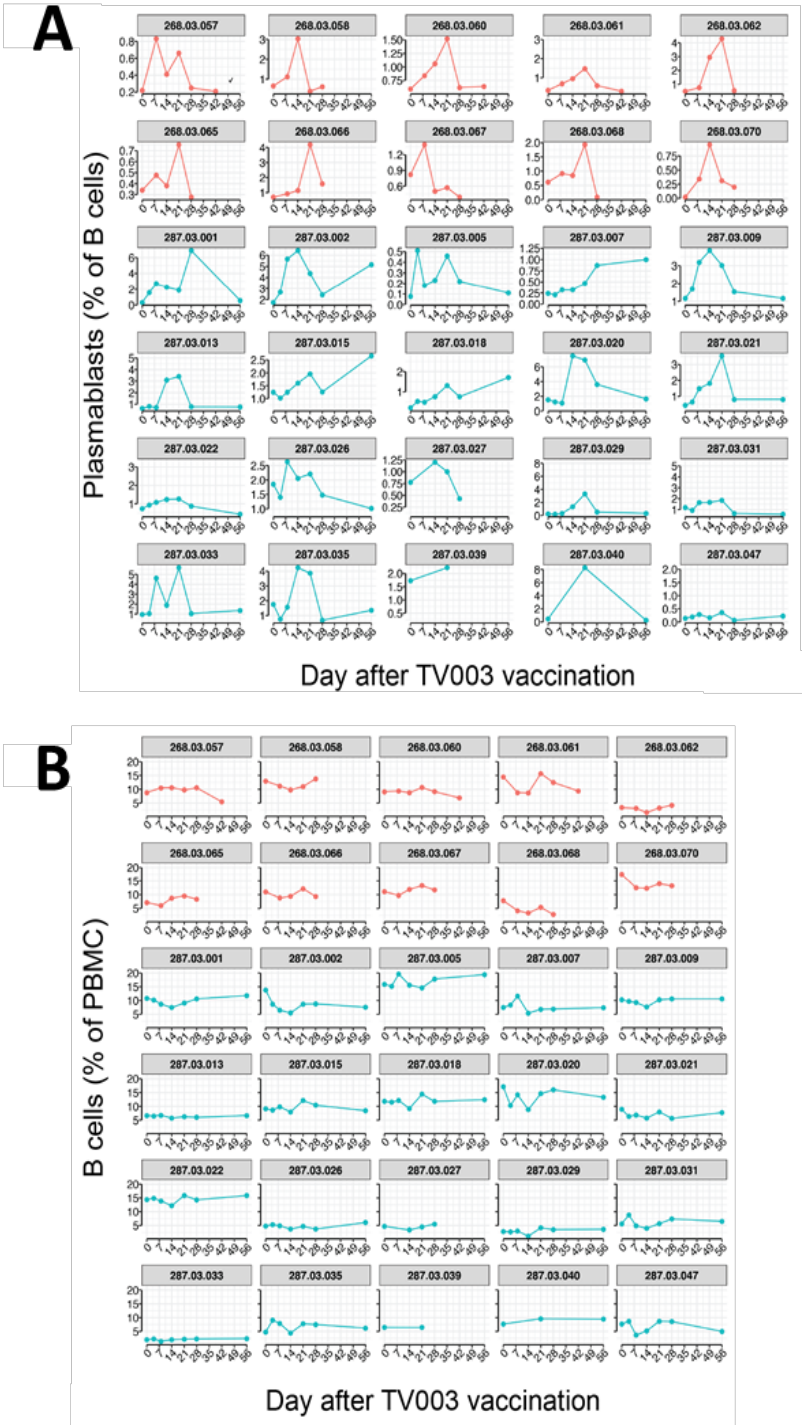
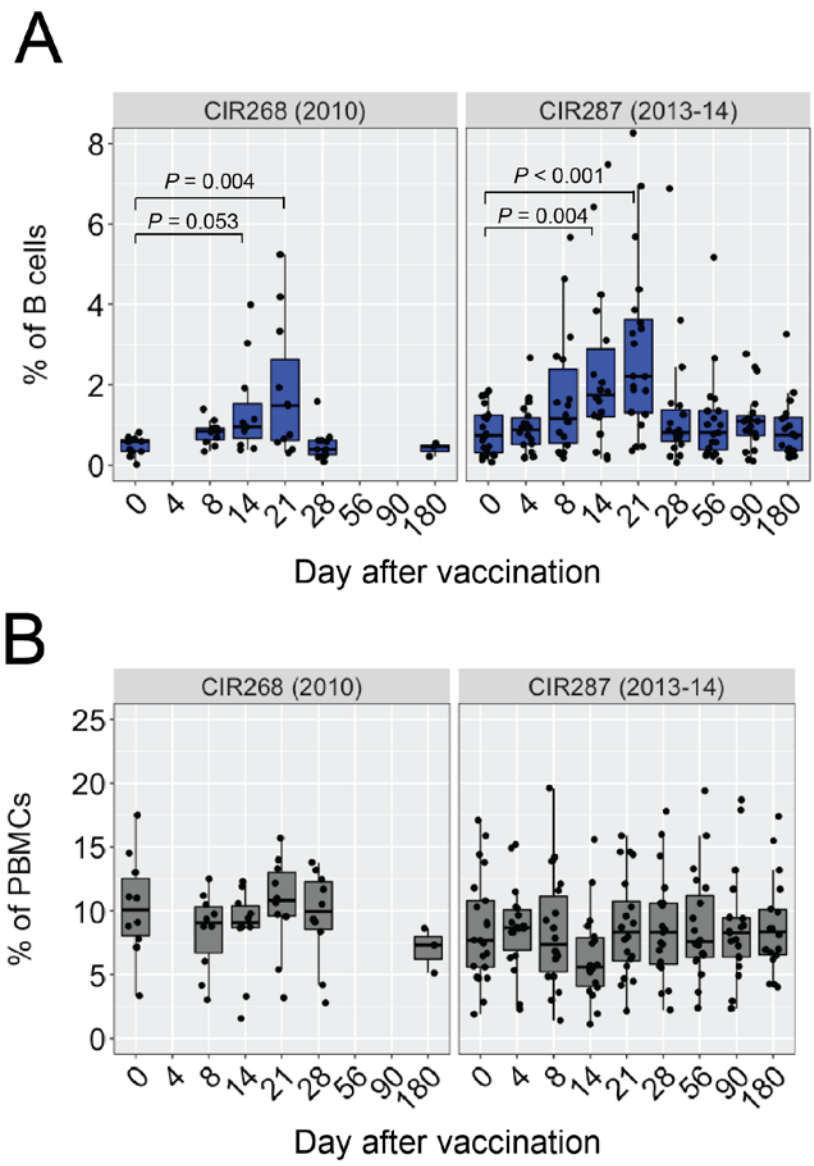
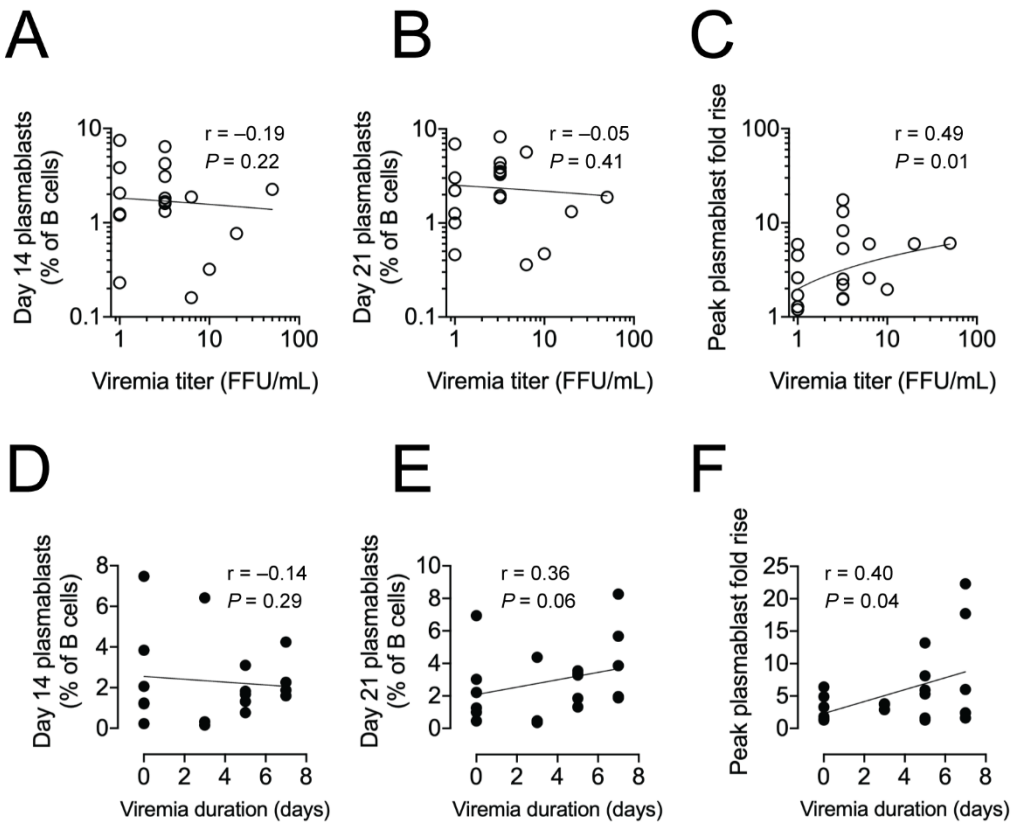
Supplemental information



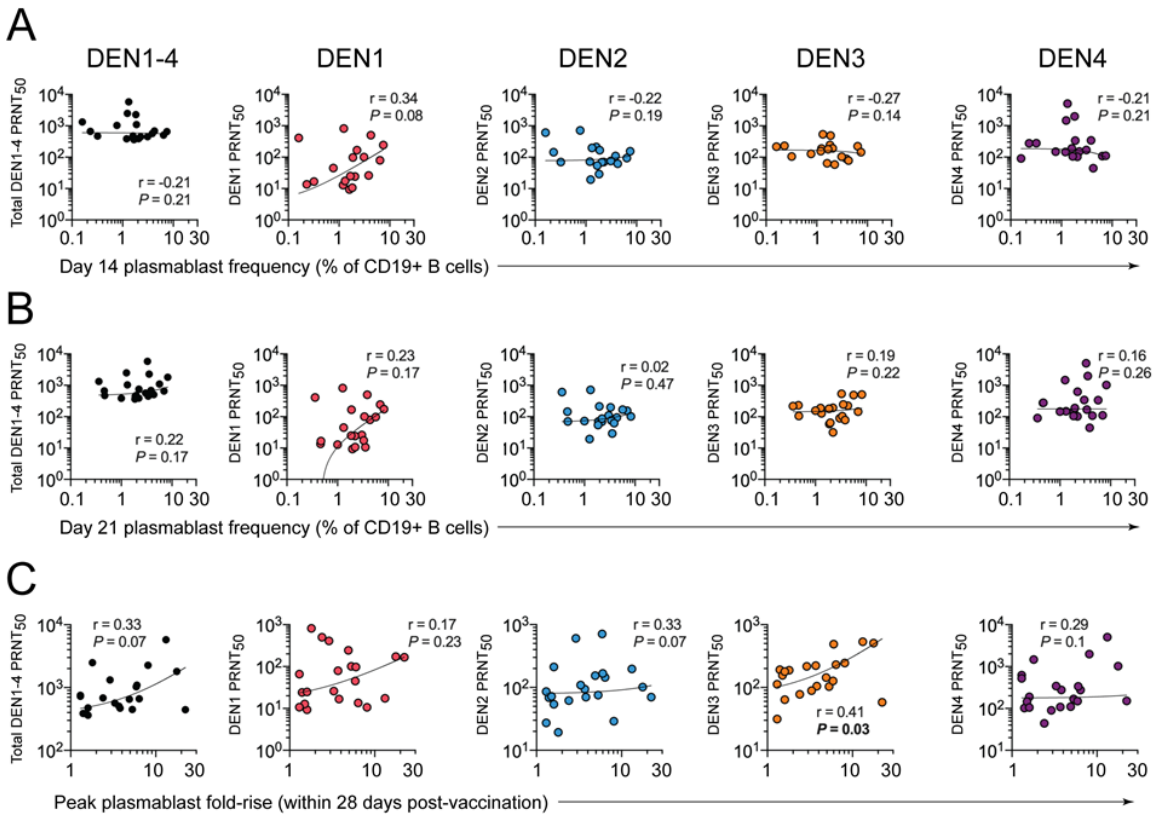
Supplemental Figure S1. Patient-specific PB and B cell % after TV003 vaccination. The frequency of (**A**) CD19+CD20-CD38++CD27++ PB and (**B**) total CD19+ B cell levels after vaccination with the tetravalent live attenuated dengue vaccine TV003. Coded subject IDs with "268.03._" refer to the 2010 CIR268 trial and those with "287.03._" are from the 2013-14 CIR287 vaccine/challenge trial.



Supplemental Figure S2. PB and B cell frequencies after TV003 vaccination across two different clinical trials. The TV003 vaccine trials CIR268 and CIR287 were conducted in 2010 and in 2013-14, respectively. (**A**) PB and (**B**) total B cell frequencies from each trial are summarized in boxplots with 25-75th percentiles as boxes, whiskers are 5-95th percentiles, and line is median. The PB data were compared across the trials by linear means ANOVA with Tukey post-test, CIR268, n = 10 subjects per time point; CIR287, n = 21 subjects per time point).



Supplemental Figure S3. Peak PB induction and TV003 vaccine viral replication. The relationship between peak vaccine virus titer and (**A**) Day 14 or (**B**) Day 21 PB frequencies or (**C**) peak PB (Days 0-28) after TV003 vaccination are shown. Correlation analyses of vaccine viremia duration with (**D**) peak PB fold-rise, (**E**) Day 14 and (**F**) Day 21 PB frequencies. Spearman correlation coefficients and *P*-values are reported.



Supplemental Figure S4. Relationship of PB and the DENV neutralizing antibody response after TV003 vaccination. PB frequencies (as % of B cells) at (**A**) day 14 or (**B**) day 21 after vaccination are plotted against peak serum neutralizing titers (as $PRNT_{50}$) induced within 180 days after vaccination for total DEN1-4 and individual serotypes as indicated. (**C**) Peak PB response (expressed as fold-rise at peak within 28 days after vaccination compared to baseline) is plotted against DEN neutralizing antibody titers. Spearman correlation coefficients and *P*-values are reported. CIR287 trial data (n = 20 subjects).

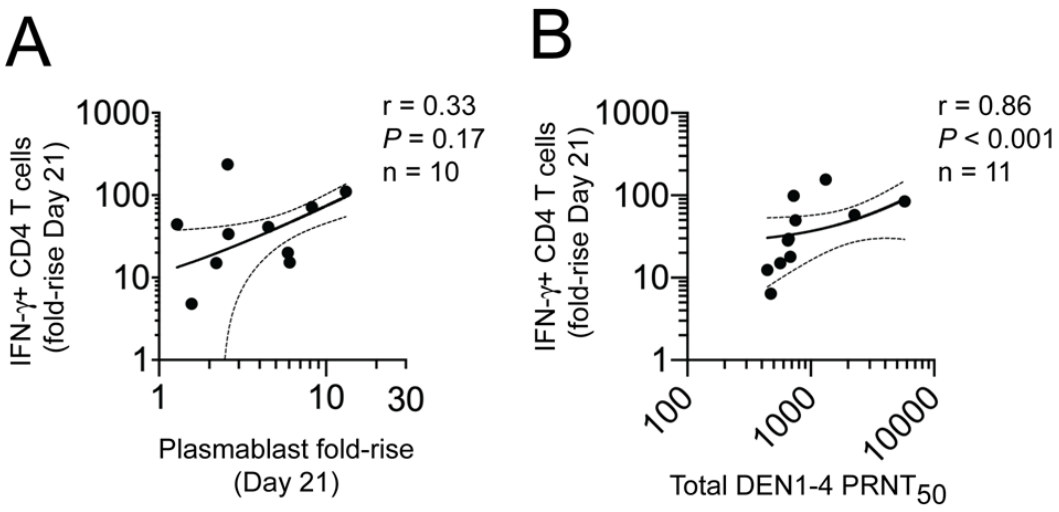


Figure S5. Relationships between TV003-induced IFN-γ+ CD4+ T cells, plasmablasts, and serum neutralizing antibodies after TV003 vaccination. (A) Total plasmablasts and DENV-specific IFN-γ+ CD4+ cells were measured ex vivo in subjects vaccinated with TV003. The fold rise in plasmablasts at day 21 after vaccination versus baseline is plotted against the day 21 fold-rise in DENV-specific IFN-γ+ CD4+ T cells. **(B)** The DENV-specific overall peak DENV serum neutralizing antibody titers (day 28 – 180 post-vaccination) are plotted against day 21 IFN-γ+ CD4+ T cell fold increase. Spearman correlation coefficients and *P*-values are reported. 95% confidence intervals shown in dashed lines. CIR287 trial data.

Supplemental Table S1. Peak PB responses to TV003.

			PB fold-rise from day 0 through day 28	
	Subject	**Peak frequency**	**Day 21**	**Peak (day)**
CIR268 (2010)	268.03.057	0.8%	3.5×	4.5× (8)
	268.03.058	3.0%	≈	6.0× (14)
	268.03.060	1.5%	3.0×	3.0× (21)
	268.03.061	1.5%	4.4×	4.4× (21)
	268.03.062	4.2%	8.6×	8.6× (21)
	268.03.065	0.75%	2.3×	2.3× (21)
	268.03.066	4.2%	6.0×	6.0× (21)
	268.03.067	1.4%	≈	2.0× (8)
	268.03.068	2.0%	3.0×	3.0× (21)
	268.03.070	1.0%	17.0×	47.0× (14)
CIR287 (2013-14)	287.03.001	7%	6.1×	22.3× (28)
	287.03.002	7%	2.5×	3.7× (14)
	287.03.005	0.5%	5.9×	6.4× (4)
	287.03.007	0.9%	2.0×	3.8× (28)
	287.03.009	4%	2.6×	3.3× (14)
	287.03.013	3.5%	5.3×	5.3× (21)
	287.03.015	2.0%	1.6×	1.6× (21)
	287.03.018	1.8%	6.0×	6.0× (21)
	287.03.020	5.5%	4.5×	4.9× (14)
	287.03.021	3.5%	8.2×	8.2× (21)
	287.03.022	1.3%	1.8×	1.8× (21)
	287.03.026	2.6%	1.2×	1.4× (8)
	287.03.027	1.2%	1.3×	1.5× (14)
	287.03.029	3.3%	13.2×	13.2× (21)
	287.03.031	1.9%	1.6×	1.6× (21)
	287.03.033	5.7%	6.0×	6.0× (21)
	287.03.035	4.2%	2.2×	2.4× (14)
	287.03.039	2.2%	1.3×	1.3× (21)
	287.03.040	8.3%	17.6×	17.6× (21)
	287.03.047	0.4%	2.6×	2.6× (21)
Summary			# of subjects	
	day 4 peak		1	
	day 8 peak		3	
	day 14 peak		**7**	
	day 21 peak		**17**	
	day 28 peak		2	

Supplemental Table S2. Memory B cell 6xL immortalization efficiency

Subject ID	# MBC transduced	Transduction efficiency % 6XL-GFP+ (of CD19+)	Immortalized cells sorted	# DENV2-positive cultures	% DENV-specific MBC (of 6XL+ MBC)
287.03.002	5,775	67%	9,000	36	0.40%
287.03.007	9,806	65%	9,000	6	0.07%
287.03.021	9,359	94%	6,000	3	0.05%
287.03.022	20,461	93%	6,000	13	0.22%
287.03.026	13,084	54%	6,000	16	0.27%
287.03.027	6,236	58%	6,000	2	0.03%
287.03.029	9,004	26%	6,000	6	0.10%
287.03.031	9,419	756%	6,000	19	0.32%
287.03.035	3,429	96%	6,000	2	0.03%
287.03.040	5,576	67%	9,000	10	0.11%
287.03.047	17,916	42%	6,000	4	0.07%
Average	10,006	67%	6,818	11	0.15%

Table S3. Correlation matrix of post-TV003 DENV2-specific MBC frequencies with PB and serum neutralizing antibodies (Spearman correlation).

Freq. DENV2+ MBC vs:	PB						Serum neutralizing antibodies				
	Frequency (% of B cells)			Fold-rise (vs. baseline)			Peak post- TV003 (days 0-180)				
	Day 14	Day 21	Peak	Day 14	Day 21	Peak	DV1	DV2	DV3	DV4	Total
R	0.32	0.22	0.22	–0.11	–0.12	–0.10	0.26	–0.03	0.32	0.01	-0.12
P-value	0.37	0.51	0.51	0.76	0.72	0.78	0.43	0.94	0.33	0.98	0.72
n	10	11	11	10	11	11	11	11	11	11	11

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CHAPTER IV - HUMAN ANTIBODY RESPONSE TO ZIKA TARGETS TYPE-SPECIFIC QUATERNARY STRUCTURE EPITOPES

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Abstract

The recent Zika virus (ZIKV) epidemic in the Americas has revealed rare but serious manifestations of infection. ZIKV has emerged in regions endemic for dengue virus (DENV), a closely related mosquito-borne FV. Cross-reactive (CR) antibodies confound studies of ZIKV epidemiology and pathogenesis. The immune responses to ZIKV may be different in people depending on their DENV immune status. Here, we focus on the human B cell and antibody response to ZIKV as a primary *Flavivirus* (FV) infection to define the properties of neutralizing and protective antibodies generated in the absence of pre-existing immunity to DENV. The plasma antibody and memory B cell response is highly ZIKV type-specific (TS), and ZIKV neutralizing antibodies mainly target quaternary structure epitopes on the viral envelope. To map viral epitopes targeted by protective antibodies, we isolated two TS monoclonal antibodies (mAb) from a ZIKV case. Both mAb were strongly neutralizing *in vitro* and protective *in vivo*. The mAb recognize distinct epitopes centered on domains I and II of the envelope protein. We also demonstrate that the epitopes of these mAb define antigenic regions commonly targeted by plasma antibodies in individuals from endemic and non-endemic regions who have recovered from ZIKV infections.

Introduction

Zika virus (ZIKV) became a prominent international concern in 2015 when it caused a large epidemic in the Americas, linked to thousands of birth defects, miscarriages, and stillbirths, as well as cases of Guillain-Barré syndrome in several countries[1]. These severe phenotypes are in contrast to the majority of infections that are asymptomatic or cause only a self-limiting illness. ZIKV is only one example of many expanding, emerging, or re-emerging mosquito-borne *Flavivirus* (FV)[2]. In locales where yellow fever vaccine is not routinely administered, sporadic outbreaks result in 100,000 severe cases with tens of thousands of deaths each year[3], and dengue virus (DENV) continues to pose a threat to two-thirds of the world's population with more than 300 million new infections every year[4]. To successfully deal with emerging FV, multifaceted and coordinated response efforts are required. Central to these endeavors is a comprehensive knowledge of human immune responses to these pathogens, which directly supports development of vital public health tools such as vaccines and diagnostics. Indeed, vaccination against yellow fever, tick-borne encephalitis, and Japanese encephalitis has demonstrated the potential public health benefit made possible by developing effective vaccines against FV[5].

ZIKV has an 11kb positive-sense, single-stranded RNA genome, which encodes seven nonstructural (NS) proteins and three structural proteins: capsid (C), pre-membrane (prM), and envelope (E)[1]. While other components of the adaptive immune system such as T cells are likely important for long-term immunity[6], a large body of work has supported a central role for neutralizing antibody responses. E protein is the main target of neutralizing and protective antibodies elicited in people exposed to FV infections or

vaccines[7, 8, 9]. The FV E protein is a class II viral fusion protein that mediates attachment to cellular receptors, and low-pH triggered membrane fusion within endosomes required for viral entry into cells. The E protein monomer contains three distinct domains designated EDI, EDII, and EDIII[10]. The surface of the FV virion is covered by 90 E protein homodimers, which are tightly packed to form a viral envelope with icosahedral symmetry[11]. For DENV and West Nile virus, FV that are closely related to ZIKV, human neutralizing antibodies often target complex or quaternary epitopes, with antibody binding footprints that include residues on multiple adjacent E monomers on the intact virion[12, 13, 14, 15].

Particularly for the four DENV serotypes, studies have demonstrated that humans exposed to primary FV infections develop type-specific (TS) neutralizing antibodies and memory B cells (MBC) that are strongly correlated with long-term protection from re-infection by the same virus[8, 16]. Understanding humoral immunity to ZIKV is complicated in that most ZIKV transmission occurs in areas where DENV (and potentially other FV) are endemic, with DENV seroprevalence as high as 90% by early adulthood[4, 17]. Antibody cross-reactivity at the level of binding and neutralization is a well-known phenomenon amongst FV in general and between DENV and ZIKV in particular, which can confound serologic assays[18]. The impact of antibody cross-reactivity on clinical outcomes for ZIKV infections in DENV-immune hosts (and *vice versa*) remains an active area of investigation[19, 20, 21]. Extensive cross-reactivity is expected given considerable conservation in amino acid sequence of DENV and ZIKV E (approximately 50%)[22]. Furthermore, B cell and antibody responses to a second DENV infection are skewed by preferential activation of pre-existing cross-reactive (CR) memory B cells. Indeed, recent

studies suggest that a similar phenomenon may occur when ZIKV infects a DENV-immune person[23, 24, 25, 26]. However, we[27] and others[24, 25] have observed that ZIKV TS antibody responses develop in humans even in the presence of immunity to prior DENV infection.

Here, we focused on understanding the molecular determinants of the human neutralizing antibody response to primary ZIKV infection. To date, most ZIKV infections have occurred in DENV-immune individuals, but as ZIKV becomes endemic throughout Latin America and the Caribbean, more individuals will experience ZIKV as their first FV infection. To learn how ZIKV may affect immunity to subsequent FV infection, or how secondary FV infection could alter existing immunity to ZIKV, we must first understand the immune response to ZIKV in the absence of other (particularly DENV) FV exposures. Additionally, understanding the properties and mechanisms underlying the induction of neutralizing antibodies in naïve individuals is directly relevant to developing vaccines for use in FV-naïve populations living in countries with endemic DENV and ZIKV transmission.

Materials and methods

Human subjects and bio specimen collection

UNC travelers: Plasma was collected from North Carolina residents with history of or risk for arbovirus infection based on travel to endemic areas and self-reported symptoms and medical history. Plasma samples were tested by virus capture ELISA. DENV– or ZIKV–reactive plasma was further characterized by neutralization assays on Vero cells (see below) to verify prior FV infection. Plasma that neutralized one DENV serotype or ZIKV with minimal neutralizing activity to other viruses were defined as primary FV infections (meaning that the FRNT50 for a single DENV serotype or ZIKV is at least 4-fold higher than any other virus tested). In our ZIKV cases, the travel history of the subject corroborated the primary ZIKV immune status. Secondary FV infections were defined by the highest two or more FRNT50 values being separated by less than 4-fold. Existing plasma with known FV neutralization profiles were used as controls in several experiments: Primary (1^o) DENV neutralized a single DENV serotype and not ZIKV; Secondary (2^o) DENV neutralized at least 2 DENV serotypes and not ZIKV.

Nicaraguan subjects: Patients seeking medical attention for fever, rash, and/or nonsuppurative conjunctivitis in León, Nicaragua, were recruited to a prospective cohort study (ZIKA-TS) in which ZIKV cases were identified by RT-PCR testing on site and confirmed serologically at UNC. ZIKV cases were sampled by blood draw at presentation and at weeks 2, 3, 4, 8, 12, and 24 post symptom onset.

Sri Lankan subjects: During a DENV1 epidemic in Sri Lanka in 2014, suspected symptomatic DENV cases were enrolled for prospective sampling. Cases were confirmed by RT-PCR. All subjects were enrolled within 4 days of symptom onset and a

convalescent blood sample was obtained (ranging from 16-29-days post onset of symptoms).

Viruses and cells

The MR766 and Dakar 41525 strains of ZIKV were obtained from the World Reference Center for Emerging Viruses and Arboviruses (Dr. Robert Tesh, University of Texas Medical Branch)[28, 29]. ZIKV strains H/PF/2013 and PRVABC59 were provided by the US Centers for Disease Control and Prevention[30]. ZIKV/2012/PHL (Genbank: KU681082), ZIKV/2014/TH (Genbank: KU681081.3), and ZIKV/2015/Paraiba (Genbank: KX280026.1) were obtained from Dr. Stephen Whitehead (NIAID). DENV WHO reference strains DENV1 West Pac 74, DENV2 S-16803, DENV3 CH54389, and DENV4 TVP-360 were initially obtained from Dr. Robert Putnak (Walter Reed Army Institute of Research). DENV2 NGC, DENV2/1974/Tonga (Genbank: AY744147.1), DENV3/1978/Sleman (Genbank: AY648961.1), DENV4/1981/Dominica (Genbank: AF326573.1) were used in neutralization experiments and were obtained from Dr. Stephen Whitehead. To perform culture-based experiments and maintain virus stocks, C6/36 *Aedes albopictus* cells (ATCC# CRL-1660) or Vero (*Cercopithecus aethiops*) cells (ATCC# CCL-81) were used. C6/36 cells were grown at 32°C with 5% CO_2 in MEM supplemented with 10% fetal bovine plasma, L-glutamine, non-essential amino acids, and HEPES buffer. Vero cells were grown at 37ºC with 5% CO_2 in DMEM supplemented with 5% fetal bovine plasma and L-glutamine. Virus stocks were titrated on Vero cells by plaque assay or focus-forming assay. All studies were conducted under biosafety level 2 containment.

Human monoclonal antibody generation and identification

From one primary ZIKV case (DT168), mAb were generated as previously described using the 6XL method[31]. Briefly, total cryopreserved peripheral blood mononuclear cells (PBMC) were thawed and memory B cells isolated by magnetic purification for CD22+ B cells and flow cytometric sorting for CD19+CD27+IgM– class-switched memory B cells (MBC). We then transduced MBC with 6XL retrovirus (encoding both Bcl-6 and Bcl-x_L) and activated the cells with CD40L-expressing L cells and interleukin IL-21, which together support proliferation and secretion of soluble antibody[32]. Transduced cells are sorted by GFP expression (transduction marker) into polyclonal cultures at 50 GFP+ cells/well on 96-well plates using fluorescence-activated cell sorting on BD FACSAria III using the "single cell sort mask" mode, which provides the highest possible accuracy (<4% variance) in dispensing cells. Supernatants from polyclonal cultures were tested for the presence of IgG targeting ZIKV by capture ELISA. ZIKV-specific supernatants were further screened for cross-reactivity to DENV in capture ELISA, and for ZIKV E80 binding in direct antigen coating ELISA. Selected ZIKV-specific polyclonal cultures were single-cell sorted into monoclonal cultures using flow cytometry on a BD FACSAria, grown on CD40L and IL-21 and then screened as above after four weeks. ZIKV-specific monoclonal cultures were further qualitatively tested for neutralization of ZIKV by incubation of ZIKV with 30 µL of culture supernatants prior to infection of Vero cells and assessment of neutralizing activity by microneutralization assay (**Supplemental Fig. S3**).

From frozen cell pellets of monoclonal cultures we isolated RNA, performed nested PCR for *IGH* and *IGL* genes, and sequenced using specific primers as described[33, 34]. Sequences were input into IgBLAST (https://www.ncbi.nlm.nih.gov/igblast/) and

compared to germline to determine variable heavy and light chain usage, V-(D)-J gene usage, somatic hypermutations, complementary determining region (CDR)3 sequence, and IgG subtype. Since sequencing of both of the potently neutralizing mAb revealed IgG1 isotype and Ig-λ light chain usage, we then used described methods[33, 34] to clone IGH into human IgG1 (Genbank FJ475055) and Igλ expression vectors (FJ517647), respectively. Heavy and light chain vectors were verified by sequencing and co-transfected into HEK-293F cells, and mAb were produced as described[33, 34].

ELISA

Binding of mAb or human plasma IgG to DENV or ZIKV was measured by capture ELISA as previously described[35]. Briefly, DENV or ZIKV virion were captured by the anti-E protein mouse mAb 4G2, blocked with 3% nonfat dry milk (LabScientific, Inc), and incubated with mAb or human plasma at indicated dilutions at 37ºC for 1 hour, and binding was detected with an alkaline phosphatase-conjugated goat anti-human IgG secondary antibody (Sigma) and *p*-nitrophenyl phosphate substrate (Sigma). Absorbance at 405 nm (optical density, OD) was measured on Epoch or Cytation3 plate reader systems (BioTek). ELISA assays to measure recombinant antigen binding (ZIKV E80, ZVEDI, ZVEDIII) or used to confirm depletion were performed as above with the exception that 50 ng purified antigen was coated directly to the plate at 37ºC for 1 hour. ELISA data are reported as OD values that are the average of technical replicates unless otherwise indicated in figure legends. The average OD for technical replicates using naïve human plasma (NHS) at the same dilution factor as test samples serves as the negative control in ELISA assays. In depletion experiments, the OD of depleted sample is expressed as percentage of control from same plasma for some graphs as indicated. For

IgG binding to ZVEDI and ZVEDIII, which are expressed as fusion proteins with an MBP tag, the OD values reported are background subtracted for each plasma individually (OD to ZIKV antigen – OD to MBP).

Blockade of binding (BOB) assay

Assays for blockade of binding were performed as described previously[36]. Briefly, ZIKV was captured using mouse anti-E mAb 4G2 and plates were blocked as described above for ELISA. Serial dilutions of plasma were added to plates in duplicate and incubated at 37°C for 1 hour. After plates were washed, 100 ng/well of alkaline phosphatase-conjugated G9E or A9E were added, and plates were incubated at 37°C for 1 hour. P-nitrophenyl phosphate substrate was added, and reaction color changes were quantified by spectrophotometry. Percentages of blockade of binding were calculated as follows: [100 − (optical density of sample/optical density of control) × 100].

Neutralization assays

Neutralization titers were determined by 96-well microFRNT[27, 37]. Serial dilutions of mAb or plasma were mixed with approximately 50-100 focus-forming units of virus in DMEM with 2% FBS. The virus-antibody mixtures were incubated for 1 hour at 37°C and then transferred to a monolayer of Vero cells for infection for 2 hours at 37°C. OptiMEM overlay media (Gibco, 31985) supplemented with 2% FBS, 1% Antibiotic Antimycotic (Sigma A5955) and 1% Carboxymethylcellulose (Sigma, C-5013) was then added, and cultures were incubated for 40 hours (ZIKV), 48 hours (DENV2 and DENV4), or 52 hours (DENV1, DENV3). Cells were fixed with 70 µL of 4% paraformaldehyde (Thermo, 28908) for 30 minutes. 100 µL of permeabilization buffer was added for 10 minutes followed by 100 µL of blocking buffer (3% normal goat

plasma, Sigma G-9023 in permeabilization buffer) and left overnight at 4°C. Fifty microliters of a mixture of primary antibodies 4G2 and 2H2[38] (ATCC, HB-114; 2H2 not used for ZIKV) were added to the plates and incubated for 1 hour at 37°C. Cells were washed with a microplate washer (BioTek, ELx405) followed by the addition of 50 µl of 1:1900 horseradish peroxidase-conjugated goat anti-mouse secondary antibody (KPL ,074-1806) for 1 hour at 37°C. Foci were visualized with 60 µL of True Blue (KPL, 5510-0030) and counted with a user-supervised automated counting program on 2x-magnified images of micro-wells obtained on a CTL ELISPOT reader. Two naïve human plasma (NHS) controls were included on every plate to define 100% infection.

Antibody depletions

ZIKV recombinant E protein was purified as previously described[39] and conjugated to HisPur Ni-NTA magnetic beads (Thermo Scientific) per manufacturer's instructions. Control beads were incubated with an equal amount of His-tagged human myelin basic protein (His-MBP). For depletion, plasma was diluted 1:20 and incubated with 30 µg ZIKV E80 or His-MBP control split over 2 rounds for 1 hour at 37°C each round. Depletion efficiency was confirmed by a ZIKV E80 binding ELISA. Plasma were also depleted of all ZIKV binding antibodies using ZIKV VLPs as previously described[40]. ZIKV VLPs were provided by The Native Antigen Company, Kidlington, UK and produced by transiently expressing ZIKV prM and E proteins in suspension culture adapted HEK-293 cells. Supernatants were cleared by centrifugation and concentrated by tangential flow filtration. The VLPs were purified by discontinuous sucrose gradient, ion exchange chromatography, and size exclusion chromatography, which also provided exchange of buffers to storage buffer. Purified VLPs were stored in

10 mM sodium phosphate, 20 mM sodium citrate, 154 mM sodium chloride, pH 7.4 at -80°C until further use.

Escape mutant selection and sequence analysis

ZIKV-PRVABC59 was incubated for 1 hour at 37°C with various concentrations of mAb – at two-fold the FRNT50 of each mAb for initial escape selection. The mAb concentration was increased every 3-6 passages up to a maximum concentration of 1000× the FRNT50. Vero cell monolayers in 6-well tissue culture plates were infected with ZIKV-mAb mixture at a MOI of 0.01 for 2 hours at 37°C. Vero cells were washed three times with PBS, and media with the same concentration of selecting mAb was replaced. Cultures were incubated up to 96 hours and checked daily for cytopathic effect. Virus growth in the presence of antibody was monitored by quantitative RT-PCR and by immunofluorescent detection of ZIKV antigens in cell monolayers. WT ZIKV-PRVABC59 was passaged in media alone alongside virus undergoing mAb selection. The E gene of stock, WT passaged, and escape mutants were sequenced and aligned in Vector NTI. Mutations resulting in changes in predicted amino acids were visualized in topographical models using PyMOL.

Epitope mapping

Alanine scanning mutagenesis was carried out by Integral Molecular on an expression construct for ZIKV prM/E (strain ZikaSPH2015; UniProt accession # Q05320). Residues were mutagenized to create a library of clones, each with an individual point mutant[41]. Residues were changed to alanine (with alanine residues changed to serine). The resulting ZIKV prM/E alanine-scan library covered 100% of target residues (672 of 672). Each mutation was confirmed by DNA sequencing, and

clones were arrayed into 384-well plates, one mutant per well. Cells expressing each ZIKV E mutant were immunostained with the mAb to be mapped and control mAb to normalize for protein expression levels. Mean cellular fluorescence was detected using an Intellicyt flow cytometer. If no critical mutations were identified in the initial screen, mAb was converted to Fab and rescreened. This was done for G9E. Mutations within clones were identified as critical to the mAb epitope if they did not support reactivity of the mAb, but did support reactivity of conformation-dependent control mAb. This counter-screen strategy facilitates the exclusion of Env mutants that are globally or locally misfolded or that have an expression defect. Validated critical residues represent amino acids whose side chains make the highest energetic contributions to the mAb-epitope interaction.

Mouse protection experiments

Five week old male and female *Ifnαr1*$^{-/-}$ mice (C57BL/6 background) received 200µg of A9E, G9E, or IgG1 isotype control by intraperitoneal injection 1 day prior to infection with 1000 FFU of ZIKV (H/PF/2013) by subcutaneous footpad inoculation[42]. Weight and lethality were monitored daily for 14 days.

Statistics

FRNT50 values were determined in neutralization assays by using the sigmoidal dose response (variable slope) equation of Prism 6 (GraphPad Software, San Diego, CA, USA). Dilution curves for plasma antibody and monoclonal antibody binding were generated using the same equation. Reported FRNT50 values were required to have an R^2 >0.75, a hill slope >0.5, and an FRNT50 falling with the range of the dilution series. Kaplan-Meier curves were used to establish survival differences in mouse

challenge experiments. An unpaired students t-test was performed to compare between groups of plasma tested in BOB experiments.

Study approval

All donations at theUniversity of North Carolina (UNC) at Chapel Hill were collected in compliance with the UNC Institutional Review Board (IRB) of the (protocol 08-0895). Informed consent/assent or parental consent was obtained for all Nicaraguan subjects under approval of the Ethics Committee of the Universidad Nacional Autónoma de Nicaragua-León (Acta 37,2017) and UNC IRB (protocol 16-0541). The hospital-based study in Sri Lanka was approved by the Ethical Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka, and de-identified specimens were shared with UNC for further analysis. All mouse experiments were conducted under the approval of the UNC IACUC, in AAALAC-accredited facilities.

Results

Human subjects with primary ZIKV

Table 1. Demographic and serologic characteristics of subjects

Subject ID	YFV vaccine	Symptoms	FRNT50				
			DENV1	DENV2	DENV3	DENV4	ZIKV
DT168	Unknown	F, R, C, HA, AR, GI	<20	<20	28	<20	3,931
DT172	No	F, R, C, AR	<20	<20	<20	<20	5,267
DT206	No	F, R, C, HA, AR	<20	<20	<20	<20	5,048
DT244	No	R	<20	<20	<20	<20	1,845

F, fever; R, rash; C, conjunctivitis; HA, headache; AR, arthralgia; GI, gastrointestinal distress

To understand the antibody response in people experiencing ZIKV as a primary FV infection, we identified four US residents that acquired ZIKV infection during foreign travel (**Table 1).** All subjects were infected in Latin America between 2015-2016 and experienced uncomplicated, self-limiting, symptomatic infections that resolved within 1 week. As we were mainly interested in the long-term MBC and plasma antibody responses, only late convalescent blood samples collected 6 months or more after infection were analyzed for the current study. Subjects DT206 and DT244 reported positive ZIKV PCR testing during clinical evaluation following travel, and we detected anti-ZIKV IgM in plasma samples obtained from those two subjects within 12 weeks of infection but not in the late convalescent samples used in these studies (data not shown). All four subjects had strong TS neutralizing antibody responses to ZIKV (FRNT50 titer range 1845-5267) (**Table 1, Fig. 1C**).

Human antibody responses to primary Zika

Plasma from all four ZIKV cases exhibited positive IgG binding in antigen capture ELISA using ZIKV or a mix of DENV1-4 virus as antigens, over a range of

plasma dilutions (**Fig. 1A**). The binding signal for DENV decayed more rapidly than for ZIKV, reaching assay limit of detection between the 1:500-1:1000 dilution. IgG binding to ZIKV was readily detectable over background for all four primary ZIKV plasma samples at the highest dilution (1:1000), indicating higher IgG titers to ZIKV. All four plasma samples contained IgG antibodies that also bound ZIKV recombinant E (ZIKV E80) and E domain I and III (ZVEDI and ZVEDIII) (**Fig. 1B**). Consistent with the serologic diagnostic criteria used here to confirm ZIKV cases, the plasma from these four travelers strongly neutralized ZIKV by focus reduction neutralization tests (FRNT) and exhibited minimal to no cross-neutralization of DENV serotypes 1-4 (**Table 1, Fig. 1C**).

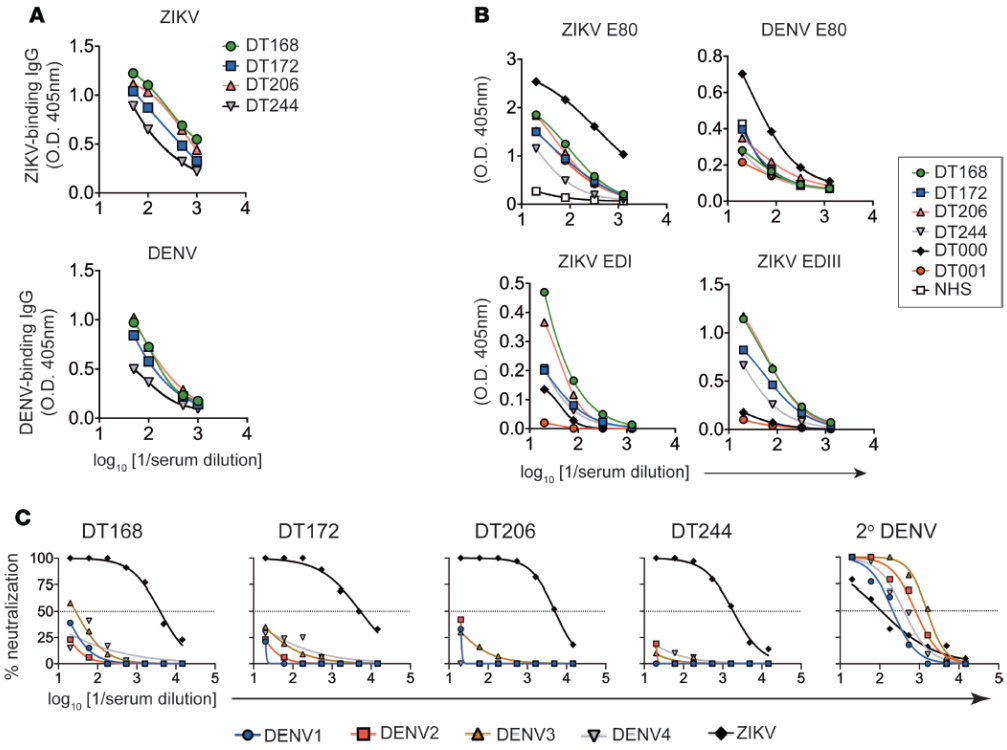


Figure 1. Serological response to ZIKV. (**A**) Plasma from four primary ZIKV cases (DT168, 172, 206, and 244) were tested for IgG binding to ZIKV (top) and DENV (bottom). (B) Primary ZIKV plasma, primary (DT001), and secondary (DT000) DENV plasma were tested for IgG binding to ZIKV recombinant E (ZIKV E80), DENV recombinant E (DENV E80), ZVEDI, and ZVEDIII. (**C**) FRNT were performed for each primary ZIKV plasma and a secondary DENV plasma control. NHS = normal human plasma, a negative binding control for ELISA.

Contribution to neutralization activity by distinct antibody subsets

Strongly-neutralizing antibody responses to DENV often target E protein quaternary epitopes displayed on the virion but not on recombinantly-expressed monomeric E protein[12, 13, 35]. We tested whether plasma antibodies in people infected with ZIKV recognized simple or quaternary epitopes on E protein. A recombinantly expressed monomeric ectodomain of ZIKV E protein (ZIKV E80) was purified, immobilized onto beads and used to deplete all ZIKV E80-binding antibody from the plasma of primary ZIKV cases. Confirmation ELISA demonstrated loss of ZIKV E80 binding activity (**Fig. 2A**), but retained IgG binding to intact virions (**Fig. 2B**), albeit at variably reduced levels compared to the un-depleted specimens. Compared to control depleted plasma, neutralization activity of ZIKV E80-depleted plasma was unaffected for DT172 and DT244, but exhibited a partial reduction for DT168 and DT206 (**Fig. 2C, Table 2**). To confirm that the majority of ZIKV neutralization is attributable to antibody against quaternary epitopes, plasma from each of the four primary ZIKV cases were depleted with ZIKV virus-like particles (VLP), which present all conformational epitopes of the intact virion. Marked loss of neutralization activity was observed (**Fig. 2D, Table 2**). Taken together, these results indicate that primary ZIKV infection elicits a complex

Table 2. Neutralization titers in ZIKV-immune plasma following ZIKV antigen

Subject ID	Un-depleted	Control	ZIKV E80	Un-depleted	Control	ZIKV VLP
DT168	2,985	2,191	535	676	538	<50
DT182	992	1,572	964	1,023	1,787	70
DT206	2,884	2,473	413	1,513	2,742	94
DT244	630	538	617	717	1,218	73

Numbers are FRNT50 values expressed as the calculate plasma dilution factors.

antibody response that includes populations of antibodies that are CR but non-neutralizing to DENV, as well as ZIKV TS neutralizing antibodies. Furthermore, ZIKV neutralizing antibodies target quaternary epitopes, though a minor fraction of neutralizing activity is attributable to antibodies that target epitopes largely contained on the E monomer in some individuals.

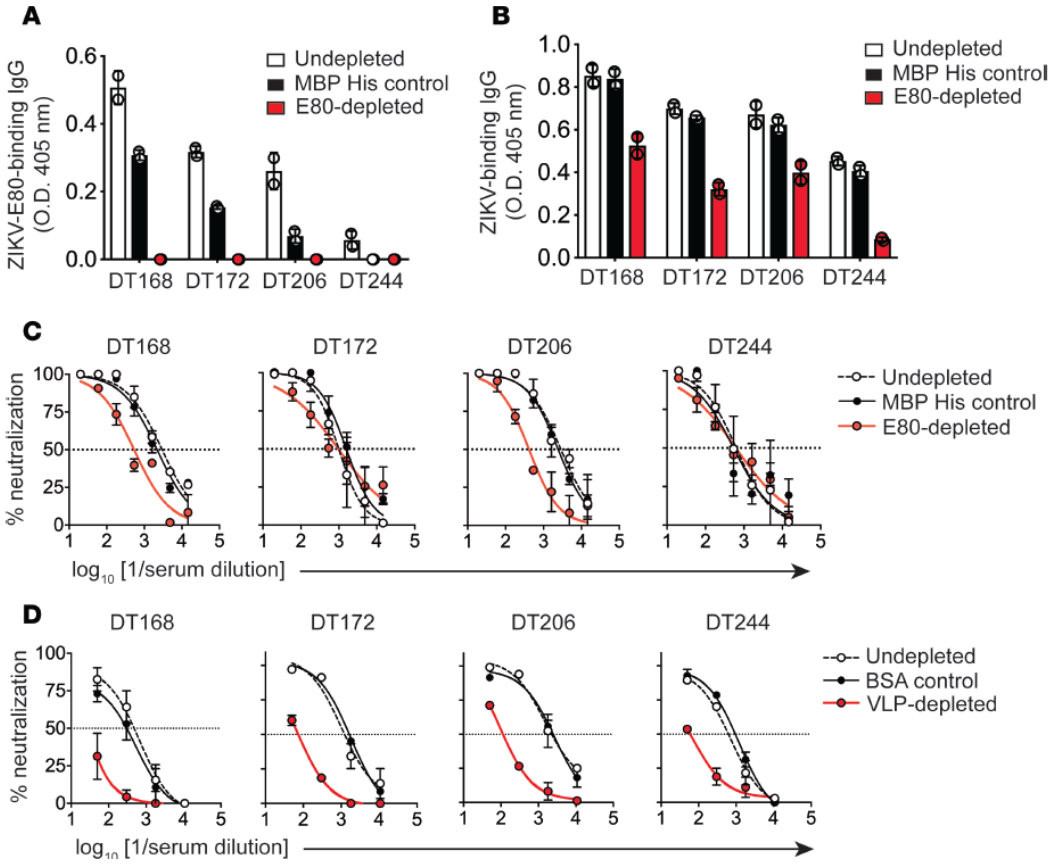


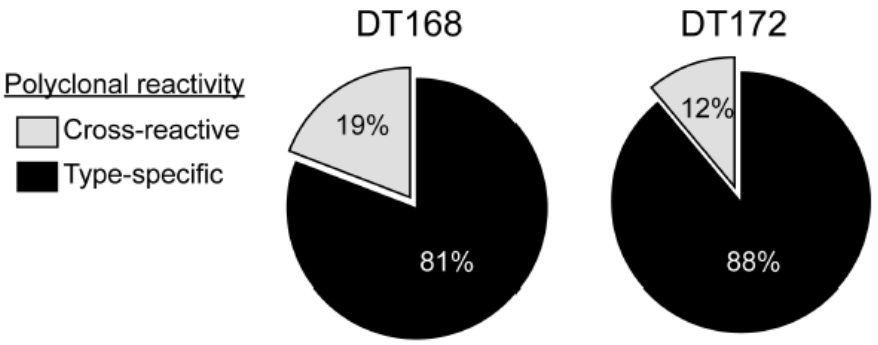
Figure 2. Antibodies against quaternary epitopes are the predominant mediators of ZIKV neutralization. (**A**) Depletion of ZIKV E80–binding IgG in primary ZIKV plasma was confirmed by direct antigen-coating ELISA comparing ZIKV E80–binding IgG in depleted (red bars) to MBP-control-depleted (white bars) or un-depleted (black bars) plasma. Mean optical density values from FV-uninfected plasma (background) are subtracted from each group. (**B**) IgG binding to ZIKV in depleted plasma was tested by antigen-capture ELISA. Mean optical density values from FV-uninfected plasma (background) are subtracted from each group. (**C**) FRNT assays were performed for ZIKV E80–depleted plasma and controls using ZIKV H/PF/2013. (**D**) FRNT assays were performed for ZIKV VLP–depleted plasma and controls using ZIKVH/PF/2013.

The MBC response to primary ZIKV infection

To assess the MBC repertoire in individuals following primary ZIKV infection, we immortalized memory B cells (MBC), as previously described[31], from the first two recruited subject, DT168 and DT172. Immortalized MBC were sorted by fluorescence-activated cell sorting (FACS) for GFP expression into polyclonal cultures on 96-well plates at 50 cells/well (<4% error). Supernatants of polyclonal cultures were then screened for IgG binding to ZIKV and DENV1-4. The frequencies of antigen-specific MBC with respect to total 6XL-transduced cells (to account for transduction efficiencies) was estimated using the number of ELISA positive cultures divided by the total number of immortalized MBC cultured. This calculation was based on the assumption of stochastic sampling during sorting and the average presence of one unique clone in the originating 50-cell polyclonal culture capable of producing IgG that yielded a positive ELISA signal. Across 480 polyclonal cultures (24,000 MBC clones), 267 cultures were ZIKV-reactive. The vast majority of these culture supernatants showed exclusive specificity to ZIKV (n = 222, 83%), and a minority were CR to ZIKV and DENV (n = 45, 17%) (**Fig. 3**). For DT168, the frequency of ZIKV-specific MBC was 1.2% of total MBC, with 1.0% ZIKV TS and 0.2% ZIKV/DENV CR. DT172 was similar with 0.9% ZIKV-reactive MBC, comprising 0.8% ZIKV TS and 0.1% ZIKV/DENV CR.

Potent ZIKV-specific neutralizing monoclonal antibody following primary ZIKV infection

To better understand the molecular determinants of ZIKV neutralization, we sought to isolate neutralizing monoclonal antibodies (mAb) and use these as tools for more detailed studies of virion-antibody interactions. Using single-cell sorting, we established monoclonal MBC cultures from ten polyclonal cultures with positive ZIKV



	DT168	DT172
ZIKV+ polyclonal wells	176	91
ZIKV-TS polyclonal wells	142	80
ZIKV-CR polyclonal wells	34	11
Total immortalized MBCs screened	14,400	9,600
ZIKV-specific frequency (of immortalized MBC)	1.2%	0.9%

Figure 3. Frequency of ZIKV-specific and cross-reactive MBC. MBC were transduced using the 6XL method and culture supernatants assessed for ZIKV- and DENV-binding IgG. Pie charts show the % of ZIKV TS and CR wells for 2 donors with primary ZIKV infection. The table below delineates the raw numbers used to calculate the % shown in pie charts and the total % of ZIKV-reactive MBC for each donor. ZIKV-TS: IgG binding for ZIKV and negative for DENV antigen. ZIKV-CR: IgG positive for both ZIKV and DENV antigens.

ELISA binding signals for subject DT168 (**Table 3**). We recovered about 40% of the monoclonal cultures as proliferating, IgG-producing cultures. Supernatants from monoclonal cultures were then screened for ZIKV-specific IgG. Half of the polyclonal cultures yielded ZIKV-reactive monoclonal cultures. Amongst monoclonal cultures from a given polyclonal progenitor culture, we found multiple positive wells. For some monoclonal cultures (from polyclonal cultures E3, H10, G11), all of the positive wells exhibited an extremely narrow range of values for ZIKV-binding (as assessed by optical density (OD) in ELISA) suggesting clonality, which for E3 was confirmed by sequencing all six ZIKV-specific subclones. This is consistent with the assumption that one clone in the original polyclonal culture was responsible for the initial positive signal. For E4- and

Table 3. Derivation of polyclonal and monoclonal memory B cells from DT168

Polyclonal Cultures		Monoclonal Cultures								Notes
Well ID	Positive O.D.[A]	# cells sorted	# viable cultures	% recovery	# ZIKV+ wells	Positive OD[A] (range)	unique clones[B]	% unique positive clones in polyclonal	# neutralizers (% inhibition)[C]	IGH/IGL sequencing
E3	0.60	120	12	10%	6	0.32 ± 0.01 (0.3 – 0.35)	1	2% (1/50)	1 (84%)	("A9E"): 6 identical clones
D1	0.49	120	77	64%	13	0.34 ± 0.04 (0.30 – 0.40)	2	4% (2/50)	2 (83, 53%)	1 of 2 clones sequenced (G9E)[D]
H10	0.49	120	53	44%	5	0.25 ± 0.01 (0.25 – 0.27)	1	2% (1/50)	1 (58%)	N.D.
G11	0.42	120	53	44%	9	0.19 ± 0.02 (0.14 – 0.22)	1	2% (1/50)	1 (58%)	N.D.
E4	0.54	180	89	49%	68	0.23 ± 0.03 (0.20 – 0.30)	2	4% (2/50)	1 (51%)	N.D.
F11	0.51	120	20	17%	0	–	–	–	–	N.D.
B3	0.46	120	18	15%	0	–	–	–	–	N.D.
D10	0.44	120	60	50%	0	–	–	–	–	N.D.
C3	0.42	120	52	43%	0	–	–	–	–	N.D.
F9	0.42	120	26	22%	0	–	–	–	–	N.D.
Mean± SD or Totals	0.48 ± 0.06	1260	460	37%	101	0.27 ± 0.06	7	2.8% (1.4/50)	6	

[A] Mean ± SD of OD for ZIKV-binding ELISA (background = 0.1–0.15). [B] Equals 1 if SD ≤ 10% of mean, equals 2 if SD > 10% of mean, confirmed by sequencing of D1 and E3 clones. [C] Number positive is defined as ≥50% neutralization of ZIKV; % neutralization is presented in parentheses. [D] Clone G9E with 83% neutralization was sequenced. "A9E" full name is DT168 (A)-E3-(A)-E9. "G9E" full name is DT168 (A)-D1-(G)-E9. N.D., not determined.

D1-derived clones, positive monoclonal wells fell into two categories of OD values differing by >20%, each with very narrow (<10%) intra-group OD value range, suggesting two clones may have been present in the polyclonal culture. We confirmed this for D1 by sequencing one of the two potential clones and found two identical subclones of the "G9E" monoclonal culture. Taken together, these results support our assumption that, on average, one clone in the 50-cell polyclonal culture produces a positive signal, thus validating the calculation for estimating ZIKV-specific MBC frequencies as above. Occasionally, multiple reactive clones may exist in the polyclonal culture. This would be more likely to happen when the frequency of antigen-specific MBC is higher, and it would lead to potential underestimation of the antigen-specific MBC frequency. ZIKV IgG-positive reactive supernatants were then screened for neutralization activity (**Supplemental Fig. S1** and data not shown). Again, subclones with tight OD values exhibited near-identical neutralization values.

We then isolated RNA from monoclonal cultures producing the two most potent ZIKV-neutralizing mAb (A9E and G9E) and assessed IgG isotype, heavy and light chain pairing, V gene usage, CDR3 sequence, and somatic hypermutations (SHM) by sequencing of Ig heavy and light chain gene products (**Table 4**) as described[43]. We recovered two distinct mAb – both were IgG1, used Ig-λ light chain, and exhibited high level of non-silent SHM in their CDR regions compared to framework regions across IGH and IGL. The two mAb were distinct in heavy chain V(D)J gene usage and CDR3 sequence. These unique mAb IGH and IGL sequences were inserted into IgG1/Igλ expression vectors, respectively, and produced in HEK-293F cells as described[33, 34].

Binding dynamics and epitope mapping

Both the A9E and G9E human mAb bound ZIKV virions in an antigen capture ELISA, but did not bind to the four DENV serotypes (**Fig. 4A**), in accordance with the initial characterization of the polyclonal cultures from which these mAb were derived. Surprisingly, both mAb bound to recombinant ZIKV E80, and A9E bound to ZVEDI (EC50 = 2500 ng/mL), albeit at higher concentrations compared to ZIKV E80 (EC50 = 40 ng/mL). Neither mAb bound to ZVEDIII (**Fig. 4B**). Both mAb were unable to bind DENV1-4 when testing each serotype individually, confirming ZIKV specificity (**Supplemental Fig. S2**). To approximate the location of the epitope recognized by each mAb, competition assays were performed (hereafter referred to as blockade of binding (BOB)). A panel of six FV CR and six ZIKV-TS mAb were competed with A9E and G9E in BOB assays. DENV-specific mAb were used as a control to establish 100% binding. As a positive control, unlabeled A9E or G9E mAb was competed with itself and showed a high level of auto-blockade (**Fig. 4C**). None of the DENV TS controls decreased the OD signal of A9E or G9E binding. Most FV CR mAb and ZIKV-specific mAb failed to appreciably reduce the binding of A9E or G9E, with two notable exceptions. Both EDE1 mAb C8 and C10[44], which bind across domain II of E molecules paired in a homodimer, showed partial blockade of G9E. Additionally, ZKA190, a human ZIKV-specific mAb known to bind to the EDI-III linker and lateral ridge of EDIII[45] strongly blocked A9E with a similar EC50 as A9E against itself. Neither of the two novel mAb exhibited BOB activity against the other (data not shown), indicating the two mAb target distinct, non-overlapping epitopes.

Table 4. Sequence characteristics of ZIKV-neutralizing mAb

Clone	Isotype	Heavy chain gene usage			HCDR 1-2-3 lengths (AA)	Non-silent SHM[A]	Non-silent: Silent SHM rates		HCDR3 AA sequence
		V	D	J			FR	CDR	
A9E	IgG1,λ	V3-23*01	D3-3*01	J6*03	8-8-17	23	3.25	10	ARSDFWRSGRYYYYMDV
G9E	IgG1,λ	V3-23*01	D1-14*01	J4*02	8-8-21	13	0.83	8	VGGSSAYNGDNGWREAASLDD
		Light chain gene usage			**LCDR 1-2-3 lengths(AA)**	**Non-silent SHM[A]**	**Non-silent: Silent SHM rates**		**LCDR3 AA sequence**
		V	**J**				**FR**	**CDR**	
A9E		V2-14*01	J2*01		9-3-11	18	0.86	4	SSYSISSTLLV
G9E		V2-14*01	J3*02		9-3-10	11	1.2	1.7	SSYTSRRTWV

"A9E" full name is DT168 (A)-E3-(A)-E9. "G9E" full name is DT168 (A)-D1-(G)-E9. [A] SHM in nucleotide sequences assessed compared to germline across FR1-CDR1-FR2-CDR2-FR3-CDR3 using IgBLAST (https://www.ncbi.nlm.nih.gov/igblast/).

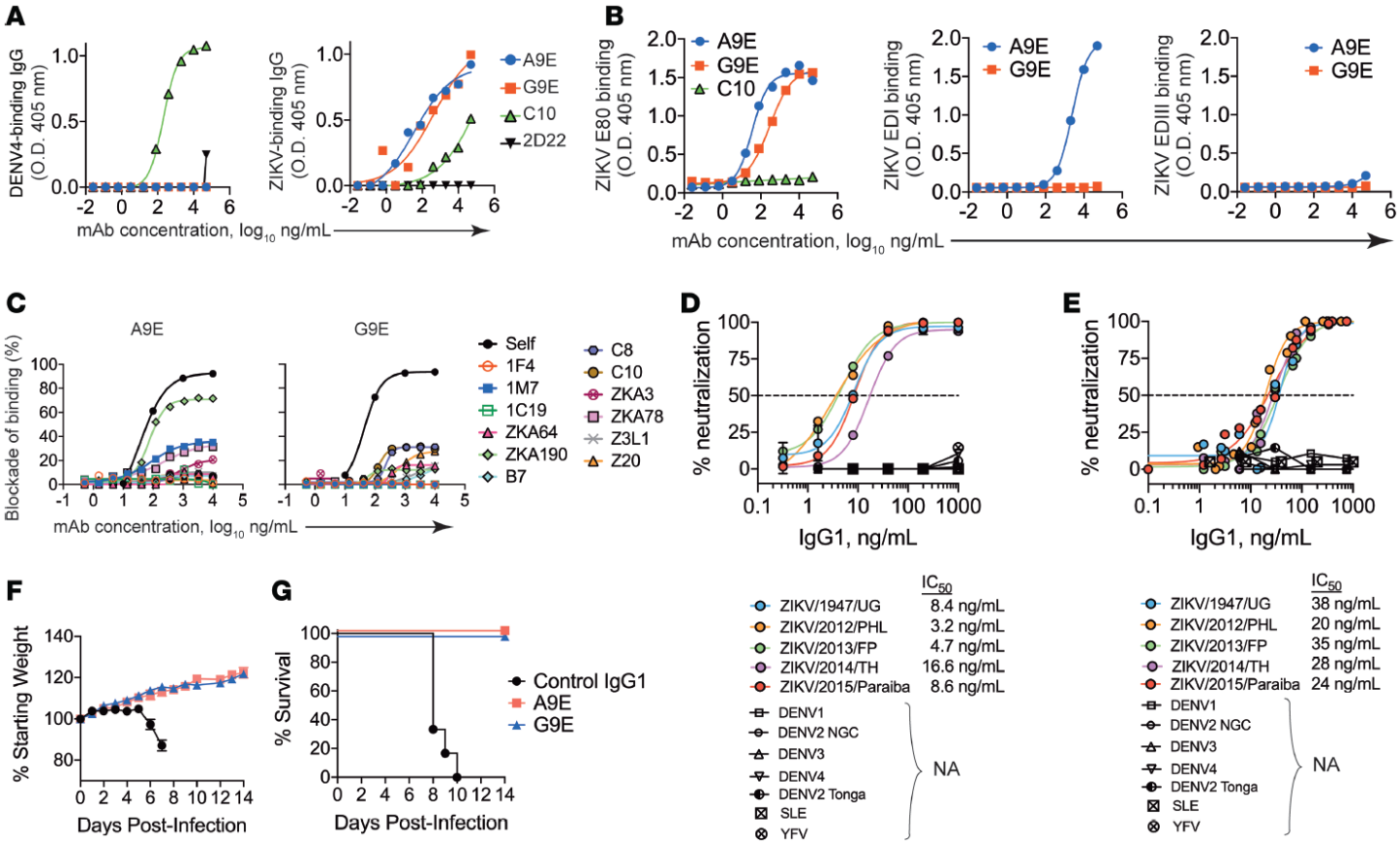


Figure 4. **mAb A9E, G9E potently neutralize ZIKV and protect *in vivo*.** (**A**) Antigen binding of A9E, G9E, & control mAb (C10: ZIKV/DENV CR; 2D22: DENV2 TS) against DENV4 (left) & ZIKV (right). (**B**) mAb binding to ZIKV recE, EDI & EDIII. (**C**) Binding competition assays to define A9E & G9E epitopes. (**D-E**) FRNT against different ZIKV strains & related FV with A9E (**D**) & G9E (**E**). (**F-G**) Four- to 6-week-old *Ifnar*–/– mice treated with 200μg of A9E, G9E, or negative control human IgG (day –1) & challenged with 1,000 FFU of ZIKV (day 0). Weight loss (**F**) & mortality (**G**) were monitored for 14 days after infection. Results represent 2 experiments (6 to 7 mice/group). Weights are shown as mean ± SEM & were censored upon the first death in the group. NA, not applicable.

Both A9E and G9E strongly neutralized Asian and African-lineages of ZIKV **(Fig. 4D-E** and **Supplemental Fig. S3**), but exhibited no activity against any of the four DENV serotypes, St. Louis encephalitis virus, or yellow fever virus. A9E and G9E exhibited mean FRNT50 concentrations of 8.3 and 29 ng/mL across all ZIKV strains tested. Finally, to investigate the potential of these mAb to protect against ZIKV infection *in vivo*, we treated 5-week-old *Ifnαr-/-* mice[42] with 200µg of A9E, G9E, or isotype control IgG by intraperitoneal injection 1 day prior to subcutaneous foot pad infection with 1000 FFU ZIKV (H/PF/2013). Control mice lost weight and succumbed to infection by 8-10 days whereas all mice that received A9E or G9E gained weight and did not succumb to infection (**Fig 4F-G**).

Escape mutations and further epitope mapping

To investigate the determinants for neutralizing mAb binding in a biologically-relevant model system, ZIKV (PRVABC59) was passaged under increasing concentrations of A9E and G9E mAb on Vero cells. No escape virus that could tolerate increasing concentrations of G9E was isolated, even when beginning the process with concentration of G9E as low as 20.6 ng/ml. In contrast, for A9E, an escape virus was isolated after three rounds of passage that could be propagated in the presence of 35,800 ng/mL A9E (approximately 780× FRNT50). Viral isolates were plaque purified to generate clonal stocks. Two viral isolates were tested for binding by mAb and plasma (**Fig. 5A**) and for neutralization escape (**Fig. 5B**). Isolate nomenclature is as follows: passage 4 from experiment 1 = A9E ZV 4.1 and passage 3 from experiment 2 = A9E ZV 3.2. A9E lost binding to escape mutants, whereas binding was retained by G9E, 1M7, and ZKA190 as well as by all four primary ZIKV polyclonal plasma. A9E failed to neutralize

both of the isolated escape mutants compared to potent neutralization of the WT positive control. However, G9E and two polyclonal primary ZIKV-immune plasma neutralized A9E escape mutants similarly to WT virus. Mutant viruses were sequenced and aligned to WT, revealing two mutations, one in EDIII (V364I) and the other in EDI (G128D) as depicted in **Figure 5.**

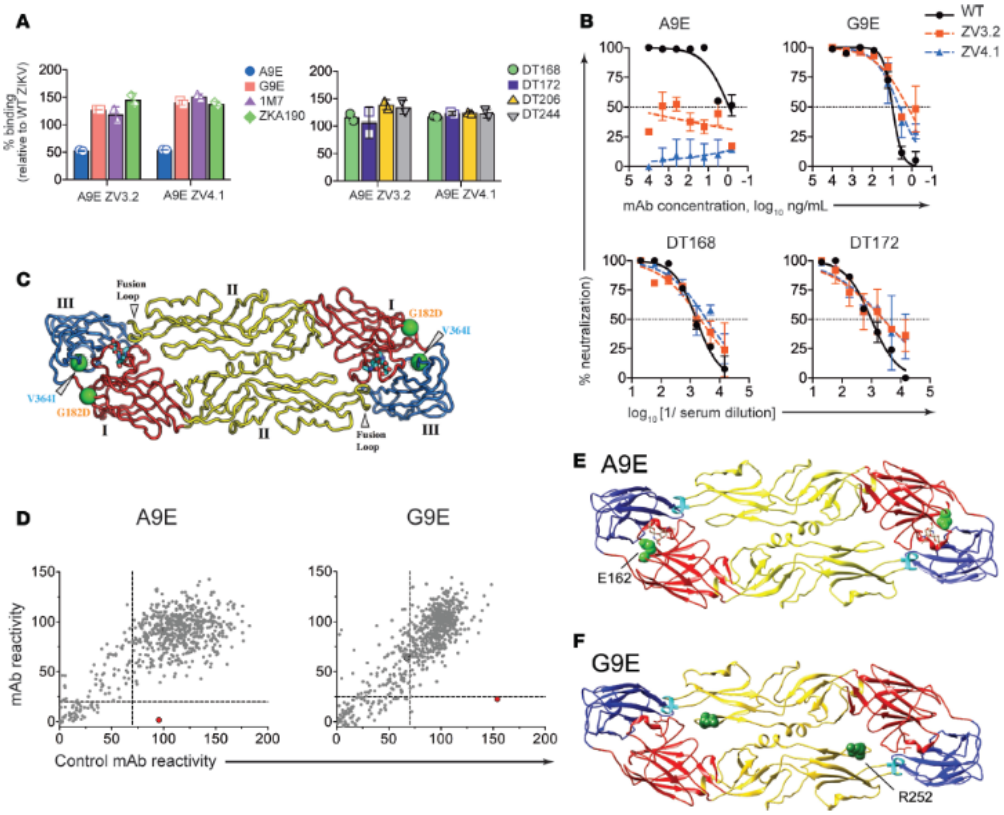


Figure 5. Epitope mapping of ZIKV-neutralizing mAb. (**A**–**C**) Escape mutants for A9E were generated from PRVABC59. (**A**) Binding of indicated mAb (left) and plasma (right) against A9E escape mutants from 2 independent experiments is shown. (**B**) Neutralization of 2 A9E escape mutants from 2 independent experiments by indicated mAb (top) and plasma (bottom) is shown. (**C**) ZIKV E homodimer with escape mutations indicated. (**D**) Amino acid residues critical for A9E mAb and G9E Fab binding were determined by alanine scanning shotgun mutagenesis. Plots show the binding of A9E and G9E versus control mAb. The data point in red corresponds to the alanine mutant that significantly reduces probe mAb binding compared with loading control mAb. (**E**) Critical residues (green spheres) discovered in alanine mutagenesis mapping are represented on a 3-dimensional model from a ZIKV cryo-EM structure (PDB ID: 5IRE). The fusion loop of E domain II is in cyan, domain I is in red, domain II is in yellow, and domain III is in blue.

To map the epitopes engaged by neutralizing human mAb by a complementary approach, both A9E and G9E were epitope mapped using alanine scanning shotgun mutagenesis as previously described (**Fig. 5D-E**)[41]. This approach compares mAb binding to a library of prM/E proteins with distinct point mutations to binding of control mAb that normalizes for target protein expression and folding. One critical amino acid that significantly reduced binding was detected for each mAb. For A9E, loss of binding was observed with mutation of E162, which is within EDI, proximal to the glycan at N154 residue. This result is consistent with the A9E escape mutant containing alterations in EDI and the partial binding of this mAb to ZVEDI. For G9E, mutation of residue R252 resulted in loss of G9E Fab binding.

Representation of A9E and G9E in ZIKV-infected subjects

Based on escape mutations and alanine scanning mutagenesis, A9E and G9E recognize distinct epitopes contained on ZIKV E. To test whether the epitopes engaged by A9E and G9E are frequently targeted by polyclonal plasma antibodies in natural ZIKV infection and whether DENV infection could elicit CR antibodies that bind similar epitopes present on ZIKV, a set of DENV- and/or ZIKV-immune plasma were competed against each mAb in BOB assays. The sources of plasma included US travelers, PCR and serology-confirmed ZIKV cases from León, Nicaragua, and subjects from a Sri Lankan hospital-based cohort with PCR-confirmed DENV infection. The majority of DENV-immune plasma failed to block mAb binding to ZIKV at a level greater than 20% (**Fig. 6A**). The samples collected from DENV-immune plasma that showed greater than 40% blockade were collected during early convalescence when CR antibodies are higher **(Supplemental Fig. S4).** Plasma specimens from ZIKV-infected individuals were further

analyzed by dividing them into primary versus secondary FV infection (**Fig. 6B**) and there was no difference in the level of blockade between the two groups. Plasma from DT168 exhibited greater than 70% blockade for each mAb; this was the highest level of activity amongst the 4 primary ZIKV-immune traveler plasma as expected, given that both mAb were derived from DT168 PBMCs (**Supplemental Fig. S5**). Interestingly, when testing multiple specimens from the same donor at different times, the later specimen tended to have higher BOB activity. DT206 and DT244 exhibit negligible BOB against A9E early (even through FRNT50 titers are high), but begin to show blockade (~30%) by 6-month post-infection **(Supplemental Fig. S5)**. This suggests that BOB activity of plasma may be affected by changes in the specificities represented in the antibody repertoire, not just the amount of IgG being produced. To further test this hypothesis, paired samples from ZIKV cases in Nicaragua were analyzed at 21 days and 6-month post infection and the trend for 8 out of 10 specimens was an increase in BOB at the later time (**Fig. 6C).** Taken together, these findings indicate that, following natural ZIKV infection, antibody responses targeting the same antigenic region of the potent ZIKV-specific neutralizing clones we isolated are maintained into late convalescence.

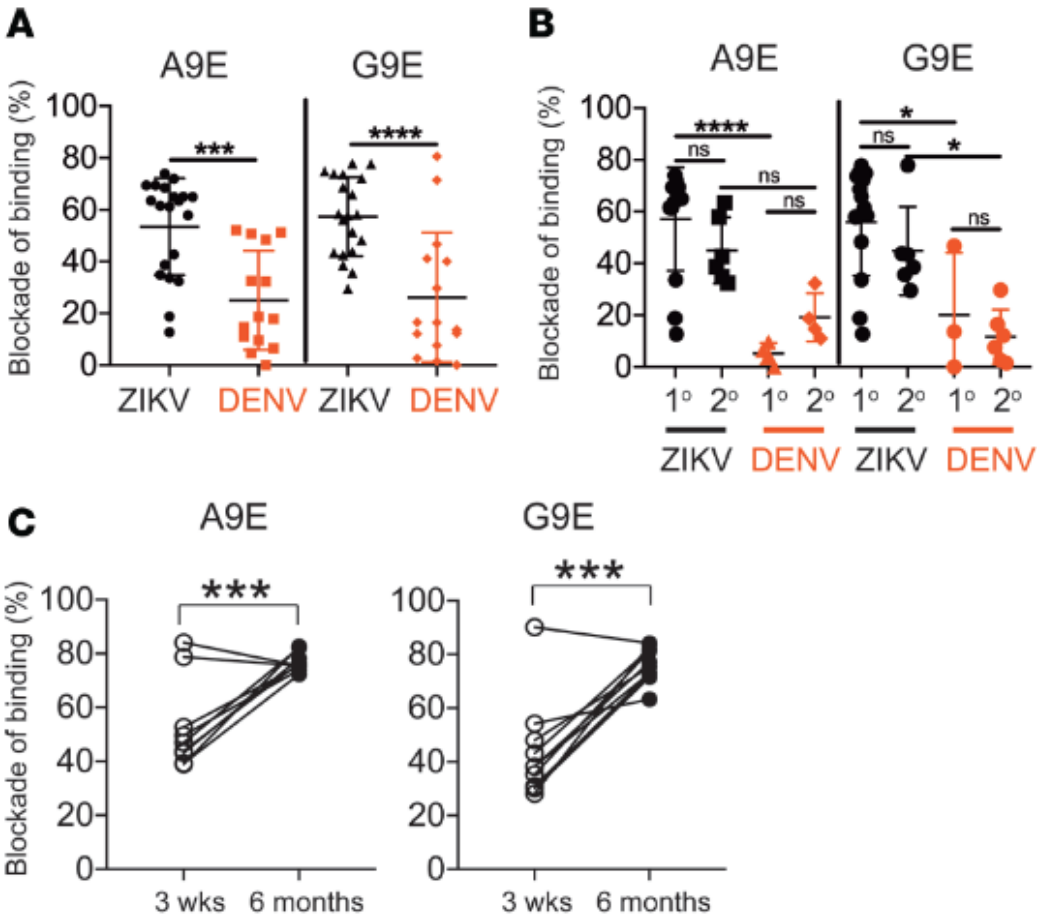


Figure 6. A9E and G9E epitope-binding IgGs are widely represented in polyclonal plasma following natural ZIKV infection. (**A**) Blockade of binding (BOB) against A9E and G9E was tested amongst plasma at a 1:20 dilution from ZIKV and DENV cases from the UNC Traveler's study, Nicaragua, and Sri Lanka, as was done for mAb in Figure 4C. (**B**) The ZIKV cases were subdivided into primary (1°) and secondary (2°) ZIKV (ZIKV infection in a DENV-immune host). (**C**) Paired plasma specimens at 1:10 dilution from symptomatic ZIKV cases in Nicaragua were analyzed by BOB at early (day 21 after symptom onset) versus late (6 months after symptom onset) convalescence. An unpaired Student's *t* test was performed to determine differences in means between groups as indicated by bars. ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. ns, not significant.

Discussion

This study shows that the polyclonal antibody response in ZIKV-infected individuals comprises a complex mixture of antibodies that recognize quaternary epitopes present on intact virion, and epitopes present on the recombinant ZIKV envelope protein monomer (simple epitopes). Furthermore, our data indicate that the majority of neutralizing activity in the four primary ZIKV plasma specimens is attributable to antibody that recognize quaternary epitopes. Recent studies with other FV, particularly the four DENV serotypes, have reached similar conclusions, suggesting that the importance of antibody targeting complex structural epitopes is a generalizable feature of the human antibody response to this genus of viruses[12, 13, 15, 35, 46]. However, we observed in two of the four subjects that antibodies targeting simple epitopes also contributed to plasma neutralizing activity (**Fig. 2C**). Similarly, other studies have identified ZIKV TS mAb, which target simple epitopes on recombinant envelope proteins, particularly on EDIII, and neutralize the virus at variable potency[23, 25, 47, 48]. Recently, our group has also found that epitopes on EDI and EDIII are frequently targeted by ZIKV-specific antibodies[18]. In DENV, EDIII-directed antibody generally constitute a minor component of the human neutralizing antibody response[49]. We hypothesize the same is likely true for ZIKV, but this is yet to be formally demonstrated. Taken together, these findings emphasize the contribution and protective role of quaternary-epitope antibodies in ZIKV neutralization following primary infection.

To analyze humoral immunity in greater detail and elucidate the molecular determinants of neutralization, we examined the memory B cell population from two subjects, isolated two distinct potently neutralizing mAb from one of the subjects,

mapped their key binding determinants, and assessed the representation of these two mAb specificities in a more general population. Approximately 1 ± 0.04% of immortalized MBC were ZIKV-reactive. One caveat to this estimate is the transduction efficiency of MBC by 6XL, though this is accounted for by expressing frequency estimates as a function of immortalized cells. Another caveat is the possibility according to the Poisson distribution that not every well will have exactly 50 cells dispensed and cells have grown equally. While the latter is difficult to assess, we have found up to a 4% variance in observed versus expected cell dispensing after sorting. Given these limitations, our frequency estimates are within the expected range for antigen-specific MBC responses to DENV[16] and ZIKV[50], suggesting adequate sampling of the memory B cell pool.

The presence of replacement mutations in the framework regions (FWR) and complementary-determining regions (CDR) of *IGVH/L* genes of MBC clones from which A9E and G9E were derived suggested that these MBC have undergone somatic mutation. In these clones, the CDRs contained more replacement mutations than the FWR. This is consistent with the low tolerability of FWR replacement mutations compared to CDR replacement mutations[51]. This is because FWRs are essential to maintain variable region structural integrity[52, 53] and positive selection of clones[54]. Interestingly, A9E exhibited a positive ratio of replacement to neutral FWR mutations in *IGHV* akin to potent anti-HIV mAb[55]. Functionally, we found the vast majority of antigen-specific MBC clones isolated from primary ZIKV cases to be ZIKV-specific and not CR to DENV. Others have clearly shown that ZIKV infection in a DENV-immune host activates pre-existing, CR MBC responses[23, 24, 25], which means the repertoire selected when ZIKV is a primary versus

secondary FV infection could be distinct and have consequences for virus control, clinical outcome, and transmission. Our results also raise the question of how the minor population of DENV CR MBC generated following primary ZIKV infection will contribute to the humoral response to subsequent DENV infection.

Identifying targets of the long-lived neutralizing antibody response is a fundamental requirement for vaccine development, as these may guide further antigen design as well as assessment of vaccine-induced immunity. For DENV[12, 13, 35, 46] and other FV[15], it has been observed that strongly-neutralizing, TS antibodies often target quaternary epitopes, and our data support the same conclusion for primary ZIKV-immune individuals (**Fig. 2D**). Interestingly, the two potently neutralizing mAb isolated in our study bound to recombinant ZIKV envelope protein monomer. Depletion experiments (**Fig. 2**) are consistent with subject DT168 having a neutralizing antibody response against ZIKV that recognizes both simple and complex structural epitopes. An expanded set of mAb from DT168 and other ZIKV cases is being isolated for comparative study. It is highly likely that mAb against quaternary ZIKV epitopes will be isolated from DT168. Additionally, binding of the ZIKV E monomer does not preclude that residues on adjacent E proteins be included in the mAb footprint nor that these potential residues be critical for the mechanism of neutralization by A9E and G9E. Thus, binding experiments such as those performed here are an important part of mAb characterization but are limited in that structure and function cannot be inferred from results. Therefore, additional work is needed to fully understand the properties of these two mAb as well as the primary antibody response to ZIKV in general.

We found that A9E and G9E recognize distinct epitopes based on lack of competitive binding by each other and on different critical binding residues identified by complementary epitope mapping approaches. A9E binding was blocked by ZKA190, whose epitopes span the lateral ridge of EDIII and residues in the EDI/EDIII linker region[45]. EDI likely contains part but not all of the A9E footprint based on ZKA190 competition and the weaker binding of EDI versus ZIKV E80 exhibited by A9E. It was not possible to generate an escape mutant to G9E, perhaps because the footprint of G9E includes at least one critical residue essential for viral fitness. G9E appears to bind residues primarily in EDII as mutagenesis revealed loss of binding with R252A, and this mAb did not bind monomeric EDI or EDIII. Moreover, BOB by EDE1 antibodies (C8 and C10) supports an epitope in EDII. Taken together, our data suggest that the epitopes of these two antibodies do not overlap. Ongoing crystallography and cryo-electron microscopy studies will refine the antigenic determinants of these two potent ZIKV-specific mAb.

Antigen-specific responses arise under the influence of a variety of host- and pathogen-specific features, which leads to certain responses being particular of an individual ("private") while others are more broadly represented in populations ("public"). The latter would need to be true for tracking of an antigen-specific response to be a useful tool for vaccine development. In general, plasma antibodies from ZIKV-immune individuals (including those with and without prior DENV infection) competed with A9E and G9E for ZIKV virion binding. DENV-immune plasma seldom blocked binding of A9E and G9E to ZIKV, or it did so with substantially less efficiency. Interestingly, ZIKV-immune plasma from later times (> 1 month and typically 6-month

post infection) exhibited a greater degree of blocking activity. Overall neutralization titers typically peak and decline before 6 months, which suggests that this effect is not simply due to total amount of IgG present in the plasma, but may involve ongoing shaping of specificities maintained in the antibody repertoire for months following acute infection. While these results do not prove that the exact epitope of either mAb is widely targeted in people with ZIKV infection, it does indicate that the region of E surrounding the A9E and G9E epitopes appears to be highly immunogenic in human ZIKV infection.

It is important to note that *in vitro* selection of escape-mutants is a tool for epitope mapping and does not indicate that these same escape mutants are likely to arise in nature. The experimental conditions used in the lab do not model natural infection: the immunologic pressure exerted by a naturally-infected host is unlikely to be entirely against a single epitope, a point recently illustrated by a ZIKV glycosylation mutant that confers escape to a specific mAb but not to polyclonal plasma[56]. Additionally, FV are subject to purifying selection due to both mammalian and insect hosts in their life cycle[57].

The primary goal of this work was to study the primary antibody response to ZIKV in detail, but we identified two ZIKV mAb with potential for further development for therapeutic[41, 45] or diagnostic[58] purposes. The FRNT50 values of A9E (3-17 ng/mL) and G9E (20-38 ng/mL) are amongst the most potent reported for native human ZIKV mAb. Multiple strains of ZIKV, representing African and Asian lineages, were effectively neutralized, consistent with the idea that ZIKV exists as a single serotype[59]. Additionally, A9E and G9E both fail to bind or neutralize DENV, and both protected against murine lethal ZIKV challenge *in vivo*. Finally, these two mAb appear to define epitopes that are consistently targets of the antibody response to natural ZIKV infection

as evidenced by the BOB studies with our initial set of human plasma from ZIKV-infected individuals. Additional experiments, including more detailed *in vivo* work to assess various timing and dose schedules and for breakthrough or persistent viremia, are being pursued to further explore the prophylactic and therapeutic potential of the mAb.

Because people exposed to primary DENV infections also develop antibodies CR to heterologous DENV serotypes with the potential to enhance viral replication and disease during a secondary heterotypic DENV infection[60, 61, 62], potential enhancement of zika disease by CR antibodies elicited by prior DENV infection was a major concern early in the recent ZIKV epidemic. However, the grounds for this hypothesis is limited to *in vitro* experiments[22, 25, 63, 64, 65] and mouse models[66], but has not borne out in non-human primate studies[20, 67] or epidemiologically in humans[19]. It is also plausible that ZIKV infection may elicit CR antibodies that could later enhance a subsequent DENV infection[21, 25, 68]. The mAb we have identified here do not bind DENV, precluding the possibility of causing antibody-dependent enhancement (ADE) of DENV infection. Enhancement of ZIKV infection is a theoretical possibility, but very unlikely as there is no evidence of ADE occurring in natural ZIKV infection of humans. Additionally, in contrast to DENV, all ZIKV strains constitute a single serotype[59], and we have shown that A9E and G9E potently neutralize different ZIKV strains from the Asian and African lineages. We further show that A9E- and G9E-like ZIKV-specific binding is present in a substantial portion of primary ZIKV cases and such activity increases over time. This suggests a narrowing of the repertoire towards ZIKV-specificity typified by A9E and G9E-like reactivity, indicating limited potential for DENV enhancement by antibodies of this type.

In conclusion, this work provides a foundation for further definition of the molecular determinants of ZIKV neutralization and the durable humoral responses that will contribute to protection against infection. The tools and approaches used here will be useful in assessing antibody and MBC responses elicited by candidate ZIKV vaccines and the further study of antibody responses elicited by sequential, heterologous FV infection.

Acknowledgements

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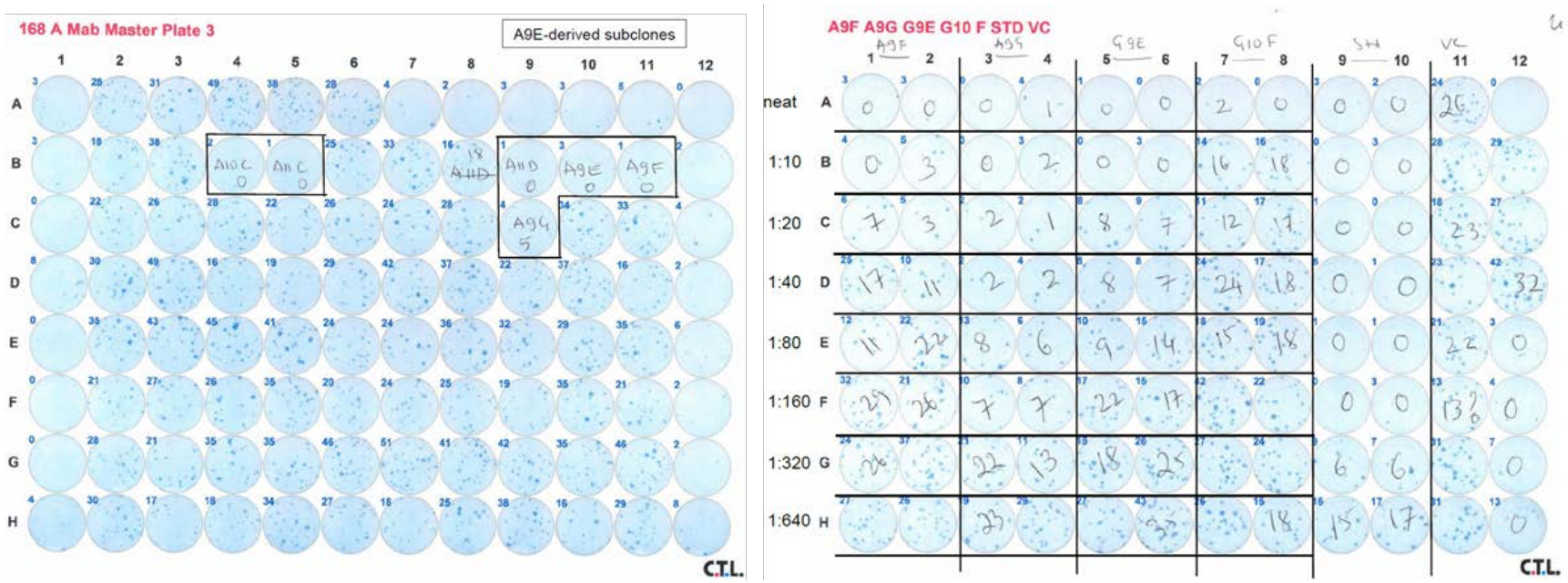
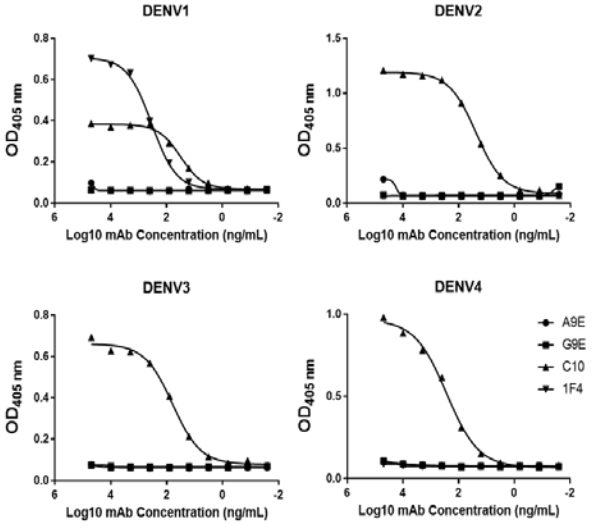
MHC and HT contributed equally. MHC wrote the manuscript with input from the other authors. MHC, HT, CGO, AMS, and SAD designed the study and interpreted findings. HT generated transformed polyclonal and monoclonal MBC libraries. HML performed *in vivo* challenge experiments, provided ZIKV reagents, and edited the manuscript. CGO generated and tested escape mutants. HT, RJ, AL, BDM, SWM, AT, and CGO performed experiments and analyzed data. BD and ED performed epitope mapping. FB, NMB, SBD, and YR led Nicaraguan cohort study and provided clinical specimens.

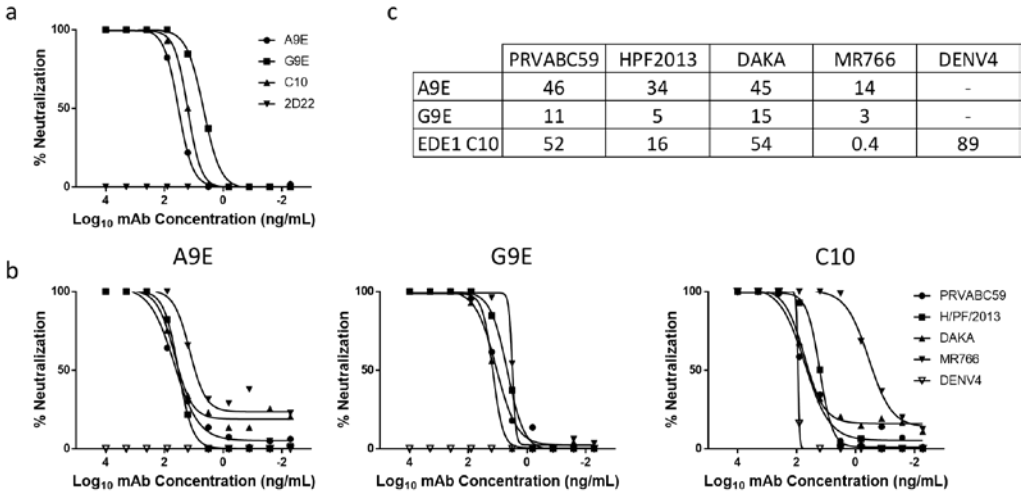
Supplemental information



Supplemental Figure S1. Selecting Clones exhibiting ZIKV neutralization. (**left panel**) Supernatants from monoclonal culture wells with IgG binding to ZIKV but not DENV were screened for ZIKV neutralization activity. 30µL of supernatant was mixed directly (neat) with 2x infectious virus stock of H/PF/2013 ZIKV, which was then used to infect Vero cells. A few supernatants strongly inhibited ZIKV infection, see well B10 for example; this well is labeled "A9E" to designate the corresponding monoclonal culture. (**right panel**) Supernatants with neutralization activity when undiluted were selected to perform FRNT assay over a dilution series as a crude estimate of neutralization potency of mAb. Note the "G9E" is run in replicate in columns 5 and 6 and exhibits greater than 50% neutralization until at least the 1:40 dilution. The strongly-neutralizing mAb C10 was run in columns 9 and 10 as a positive control.



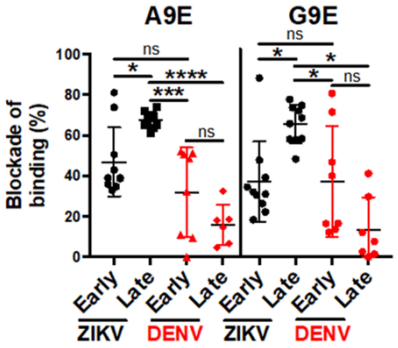
Supplemental Figure S2. A9E and G93 do not bind to DENV E80. Both A9E and G9E were tested for binding to recombinant E80 of each indicated DENV serotype over the range of concentrations listed on the x-axes. C10 was run as a control for DENV virus binding and a DENV1 TS mAb (1F4) was included as a control for type specific binding.



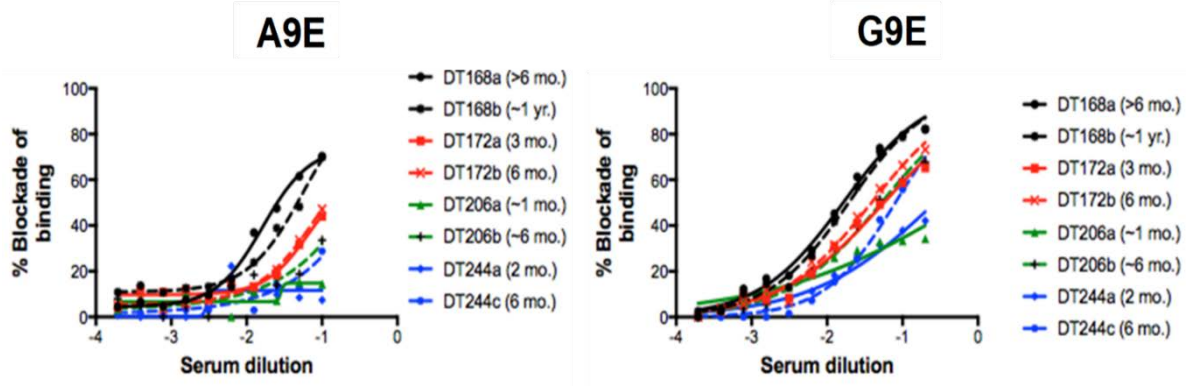
c

	PRVABC59	HPF2013	DAKA	MR766	DENV4
A9E	46	34	45	14	-
G9E	11	5	15	3	-
EDE1 C10	52	16	54	0.4	89

Supplemental Figure S3. Neutralization of African and Asian strains of ZIKV by human mAb. (**a**) The relative potency of candidate ZIKV mAb versus C10 mAb was determined with FRNT assays using H/PF/2013. (**b**) The indicated mAb in each panel was tested for neutralization activity against a panel of viruses representing ZIKV strains ranging from the original African isolate (MR766) to the recent Latin American outbreak (PRVABC59, from Puerto Rico). (**c**) The inset shows the $FRNT_{50}$ (ng/mL) to each virus for A9E, G9E and C10.



Supplemental Figure S4. Greater BOB cross-reactivity in early versus late DENV cases. BOB activity was assessed in immune plasma from PCR-confirmed ZIKV cases in Nicaragua and DENV cases in Sri Lanka were divided as Early (≤1 month) and Late (≥1month post symptom onset).



Supplemental Figure S5. Plasma from primary ZIKV cases block binding of ZIKV mAb. Blockade of binding against A9E and G9E was tested amongst paired plasma obtained at the indicated time points from primary ZIKV cases amongst Traveler. Data are depicted as dilution curves for each subject at the time indicated in legend.

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CHAPTER V – DEFINING THE MECHANISM OF ACTION OF A NOVEL MONOCLONAL ANTIBODY AGAINST ZIKA VIRUS

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Abstract

The mosquito borne Zika virus (ZIKV) belongs to the *Flavivirus* (FV) genus, which is widely transmitted in many tropical regions. The reemergence of ZIKV in recent years resulted in global outbreaks affecting hundreds of thousands of individuals, and emphasized the urgent needs for sensitive and specific diagnostics, and efficacious therapeutics and vaccines. The antibody response generated in ZIKV infection has been an important topic of investigation as virus-specific antibodies can have application in diagnostics, therapeutics, and immunization. In studying the antibody response to primary ZIKV infection, we isolated ZIKV-specific monoclonal antibodies (mAb) with potent neutralization activity against the virus, making these mAb promising candidates for further downstream clinical development. In this study, we characterized the mAb B11F, which was isolated from the memory B cell (MBC) pool of a primary ZIKV-infected subject. B11F exhibited strong neutralization activity against ZIKV by inhibiting viral attachment to target cells. Epitope mapping results showed that B11F targeted a quaternary epitope spanning domain I and III of the viral envelope glycoprotein. These results contributed to our understanding of the determinants of antibody neutralization by uncovering potential connections between the mAb' specific epitopes and their neutralization modalities.

Introduction

Zika virus (ZIKV) belongs to the *Flavivirus* (FV) genus of the Flaviviridae family, the transmission of which is widespread in many tropical regions[1]. The reemergence of ZIKV in recent years resulted in global scale epidemics, with over 600,000 confirmed cases during the 2015-2016 outbreak (WHO). While ZIKV infection usually results in self-limiting febrile illness, severe cases can manifest as neurological disorders including Guillain-Barre syndrome in adults, and congenital microcephaly in infants of infected mothers[2, 3, 4, 5, 6, 7, 8, 9, 10].

ZIKV transmission relies on the *Aedes* mosquito vector, which is the primary vector for the related Dengue virus (DENV), and therefore the two FV species can share geographical distribution. This distribution overlap, and that infections by these viruses often result in non-specific febrile illness (WHO), pose challenges for clinical diagnosis. Furthermore, ZIKV and DENV share up to 60% sequence identity, which complicates antibody testing due to antibody cross-reactivity to both viruses. However, studies utilizing immune plasmas and monoclonal antibodies (mAb) have determined that the antibody repertoire induced by ZIKV infection is shaped not only by exposure to the viral antigen, but also by the FV serostatus of the individuals at the time of the infection. Primary ZIKV infection (on FV seronegative background) results in an antibody response that is focused on ZIKV, with little cross-reactivity against DENV[11, 12, 13]. We have previously shown this specificity profile of the antibody response in primary ZIKV-infected subjects, and further demonstrated that this antibody response mainly targeted epitopes contingent on the quaternary structure of the virus[12].

Here we investigated the determinants for virus neutralization of a novel mAb isolated from one of the subjects (subject DT 172) in our aforementioned study[12]. The mAb B11F displayed exclusive ZIKV-binding activity and exhibited potent *in vitro* neutralization activity. Epitope mapping for B11F revealed the footprint of the mAb covers both domain I (EDI) and domain III (EDIII) of the viral envelope glycoprotein (E), and required E to adapt its dimeric configuration as on the quaternary viral structure. As the B11F epitope involved EDIII which is critical for cellular receptor binding, we proposed that B11F inhibited ZIKV infection by preventing viral attachment to target cells. We demonstrated that B11F more efficiently neutralized ZIKV before the virus attached to its target cells by blocking viral binding. These results help elucidate the structural-functional relationship of neutralizing mAb given their specific epitopes, and reveal the structural vulnerabilities of the virus that are targets for neutralizing antibodies.

Materials and methods

Human subjects and specimen collection

Whole blood and plasma samples were obtained from consented donors with self-reported risk or diagnosis of ZIKV infection during their travels. Plasmas were used to verify the infection in ZIKV and DENV binding ELISA. If antibody binding occurred, plasma neutralization titers were determined with 50% focus reduction neutralization test (FRNT50)[12]. Plasmas that neutralized ZIKV with minimal activity against DENV and other FV were classified as primary FV infections (defined as 4-fold or higher FRNT50 titer for ZIKV compared to for other viruses). Secondary ZIKV infections were defined by less than 2-fold difference between titers for different FV. Previously characterized plasmas were used as controls for ELISA and FRNT50.

Virus and cells

ZIKV strain H/PF/2013 was obtained from the U.S. Center for Disease Control and Prevention. The virus was propagated in Vero cells (ATCC 81) with MOI 0.01 and harvested at 72-hour post-infection. Vero cell cultures were maintained at 37ºC and 5% CO_2 in DMEM supplemented with 5% fetal bovine serum, L-glutamine and penicillin/streptomycin. Virus stocks were titrated on Vero cell using plaque assay on 6-well plate format for PFU/mL titers, and using focus forming assay on 96 well plate format for FFU/mL titers.

Human mAb identification and expression

Memory B cells (MBC) from subject DT172 were immortalized using the previously described 6xL methods[14]. Briefly, MBC was isolated from PBMC by magnetic enrichment for total CD22+ B cells (Miltenyi; Cat. No.130-046-401), followed

by flow cytometry sorting for class-switched IgM-CD27+CD19+ MBC on a BD FACS Aria. Purified cells were activated with recombinant human interleukin IL-21 (Peprotech; Cat. No. 200-21) and human CD40L-expressing mouse fibroblasts (L cells) for 36-48 hours before spin transduction with retroviral vector containing coding sequences for Bcl-6, Bcl-xL, and the selectable marker GFP. Transduced cells expressing GFP were subsequently sorted to polyclonal cultures at 50 cells/well. Transduced cells were maintained in IMDM supplemented with 8% FBS, pen/strep, IL-21, and L cells. Polyclonal cultures producing antibodies reactive to ZIKV in capture ELISA were further sorted into monoclonal cultures and screened to identify B cell clones specific to the virus.

Monoclonal cultures specific to ZIKV were expanded before cells were processed for RNA isolation. IGH and IGL genes were obtained using nested PCR with degenerate primers, and sequenced with specific primers, as previously described[15]. Obtained sequences were assessed with IgBLAST to determine variable gene usage, SHM rate, CDR characteristics, and IgG subtypes. Since sequencing of the mAb B11F revealed IgG1 isotype and Ig-λ gene usage, we cloned the IGH and IGL sequences into corresponding expression vectors. Sequence-verified heavy and light chain vectors were co-transfected into HEK-293F expression system and mAb were purified as described.

Capture ELISA

ZIKV reactivity was determined for immune plasmas, MBC culture supernatant, and mAb using sandwich ELISA capturing whole virus. Virus was captured with the mouse anti-FV envelope protein 4G2. Primary antibody binding (in plasmas, supernatant, or as mAb) was detected with goat anti-human IgG antibody conjugated with alkaline

phosphatase, and p-Nitrophenyl phosphate substrates. 4G2 coating was in 0.1M carbonate buffer, for at least 2 hours at either room temperature or 4ºC. In other incubation steps, reagents were diluted in blocking buffer (3% natural goat serum and 0.05% Tween in TBS) and incubated at 37ºC for 1 hour. Following each incubation period, plates were washed 6 times with wash buffer (0.2% tween in TBS). Optical density 405nm absorbance was measured on a Biotek Cytation 3 platform.

Neutralization assay

50% focus reduction neutralization test (FRNT50) concentration for mAb was determined on 96-well plates, as previously described[12]. Briefly, dilutions of the mAb were mixed with 100-200 focus-forming viral units and diluted in Vero cell medium. The virus-antibody mixtures were incubated at 37ºC for 1 hour before being added to Vero cell monolayers. After 1 hour of incubation, the monolayers were overlaid with OptiMEM supplemented with 1% methylcellulose and 2% FBS. Cultures were incubated for 40-48 hours. Subsequently, cells were fixed with Acetone: Methanol (50%:50%) at 4ºC for 30 minutes. Viral proteins were detected with 4G2, visualized with rabbit anti-mouse IgG conjugated with horseradish peroxidase, and TrueBlue TMB substrate. In incubation steps, reagents were diluted in PBS (with 5% non-fat dry milk powder). Following each incubation period, plates were washed with PBS. Foci were counted and images obtained on a CTL ELISpot reader. 50% inhibitory concentration (IC50) was obtained by interpolation of standard curves in GraphPad Prism.

Pre- and post-attachment neutralization assay

Pre-attachment: dilutions of mAb were mixed with 100-200 FFU of virus and held at 4ºC for 1 hour before incubation with chilled Vero cell monolayers for an

additional 1 hour at 4ºC. The monolayers were then washed 3 times with chilled PBS and overlay medium was added. Post-attachment: 100-200 FFU of virus was added to chilled Vero cell monolayers and held at 4ºC for 1 hour. Monolayers were then washed 3 times with chilled PBS to remove unbound virus before dilutions of mAb were added. Following 1 hour of incubation at 4ºC, monolayers were washed 3 times with chilled PBS and overlay medium was added. The following incubation step and immunostaining for viral proteins were the same as following neutralization assay. IC50 was obtained by interpolating standard curves in GraphPad Prism.

Blockade of attachment and qPCR analysis

$2x10^5$ Vero cells per well were plated on 24-well plates for 24 hours. Dilutions of mAb were incubated with $2x10^5$ FFU of virus at 4ºC for 1 hour before being added to chilled cell monolayers. Following an additional 1-hour incubation at 4ºC, cell monolayers were washed 3 times with chilled PBS. Total RNA from samples were extracted using RNAEasy kit (Qiagen; Cat. No. 74106), and first-strand cDNA was synthesized with SuperScript III Reverse Transcriptase kit (Thermo Fisher; Cat. No. 18080993), according to manufacturers' protocols. Quantitation of viral genome was performed on the QuanStudio6 platform using reporter dye/quencher Taqman probe system (Applied Biosystems), with the following primers:

ZIKV Envelope Forward 5'-CCGCTGCCCAACACAAG-3',

Reverse 5'-CCACTAACGTTCTTTTGCAGACAT-3",

Probe 5'-AGCCTACCTTGACAAGCAATCAGACACTCAA-3';

Primate GAPDH primer/probe set (Applied Biosystems; Cat. No. 4331182) was used as a housekeeping gene to normalize ZIKV envelope gene measurement. Results were

analyzed using the comparative ΔΔCt method. Dunn's multiples comparisons test was used for statistical analysis in GraphPad Prism.

Results

The human mAb B11F potently neutralizes ZIKV

We isolated mAb to better characterize the antibody response to ZIKV, probing their specificity, neutralization potency, and their modes of virus inhibition. The mAb B11F, derived from MBC of a primary ZIKV-infected subject, showed reactivity against ZIKV (**Fig. 1A**), but not the four serotypes of DENV as demonstrated in capture ELISA (data not shown). B11F exhibited potent neutralizing activity *in vitro* with IC50 at approximately 60 ng/mL, similar to other previously described mAb (G9E: IC50 50 ng/mL) (**Fig. 1B**)[12, 16]. It is important to note that the *in vitro* IC50 of mAb were dependent on viral input as seen in this study's experiments (100-200 FFU/sample), in comparison to the FRNT50 reported in Chapter IV (50-100 FFU/sample)[12].

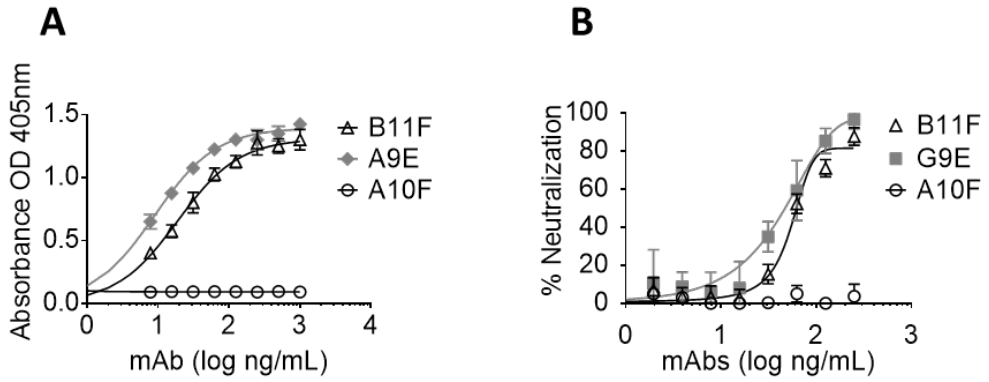

Figure 1. ZIKV binding and neutralization activity of the mAb B11F. (**A**) Binding of B11F to ZIKV demonstrated in capture ELISA, with A9E as a positive control and A10F as a negative isotype control. (**B**) B11F neutralized ZIKV *in vitro* at potency comparable to the mAb G9E. Values are representative of at least two experiments with at least three replicates for each sample. Error bars represent SEM.

B11F neutralizes ZIKV by preventing the virus from attaching to target cells

To determine the mechanisms by which B11F inhibits ZIKV infection, we compared neutralization activity of this mAb before and after the virus has bound to target cells (pre- versus post-attachment). In the pre-attachment assay, we allowed B11F to bind to ZIKV before the immune complex wass added to Vero cells. In the post-attachment assay, the mAb was added after the virus had bound to the cells. In both conditions, experiments were held at 4°C to prevent internalization of virus before final incubation at 37°C to allow viral entry and replication. Comparison between the two conditions showed that B11F neutralized ZIKV more efficiently when the antibody is allowed to bind the virus before inoculation, with the IC50 at approximately 220 ng/mL (**Fig. 2A**). In contrast, B11F failed to reduce 50% of the infection in post-attachment condition even at a concentration 10-fold higher than the pre-attachment IC50. These results suggested that B11F mainly neutralized ZIKV pre-attachment.

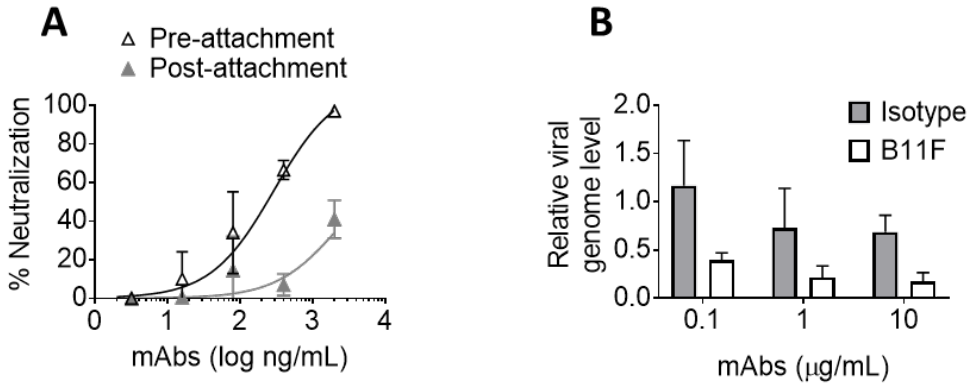

Figure 2. B11F inhibits ZIKV infection *in vitro* before the virus attaches to the target cells. (**A**) B11F neutralized ZIKV more efficiently in pre-attachment condition in which the antibody binds to the virus before the virus attaches to the target cells. (**B**) Incubation of B11F with ZIKV prior to adding the mixture to Vero cells effective reduced the levels of viral genomes associated with the cells. A10F was used as a negative isotype control. Dunn's multiple comparisons test was used to assess difference in viral genome levels amongst groups. Values are representative of at least three experiments with at least three replicates for each sample, error bars indicated SEM.

Since B11F neutralized ZIKV pre-attachment, we determined whether the antibody inhibited the infection by blocking viral attachment to target cells. Dilutions of B11F were incubated with ZIKV before adding to Vero cell monolayers and additional incubation. Experiments were held at 4°C to prevent viral internalization. After extensive washing to remove unbound virus, total cellular and viral RNA was extracted to quantify the level of viral genome associated with the monolayer in the presence of BIIF, or non-specific control isotype. While the results were not statistically significant, increasing concentrations of B11F reduced the levels of viral genomes associated with the cells, compared to isotype controls', suggesting B11F prevented the virus from binding to target cells (**Fig. 2B)**. This result was consistent with the ability of B11F to neutralize ZIKV before the virus gains access to the cells.

B11F recognizes a quaternary epitope spanning the ZIKV Envelope EDI/EDIII

To map the specific epitope of B11F, we used this mAb in ELISA capturing different recombinant antigens of the ZIKV envelope protein, including the ZVrecE, EDI, and EDIII[17, 18]. ZVrecE is a recombinant protein comprising 80% of the viral E protein (ectodomain without the membrane anchor). ZVrecE exists in solution at an equilibrium favoring monomers over dimers. EDI and EDIII are recombinant domains of the E protein. B11F showed modest binding to ZVrecE and no binding to EDI or EDIII, suggesting the mAb's footprint spanned multiple E domains or that binding required the quaternary conformation of E (**Fig. 3A**). Additionally, B11F showed higher binding to ZVrecE dimer engineered with cysteine residues for stabilized dimeric conformation, compared to monomeric ZVrecE (**Fig. 3B**)[18]. These results indicated that B11F targeted quaternary E epitope on the ZIKV particles.

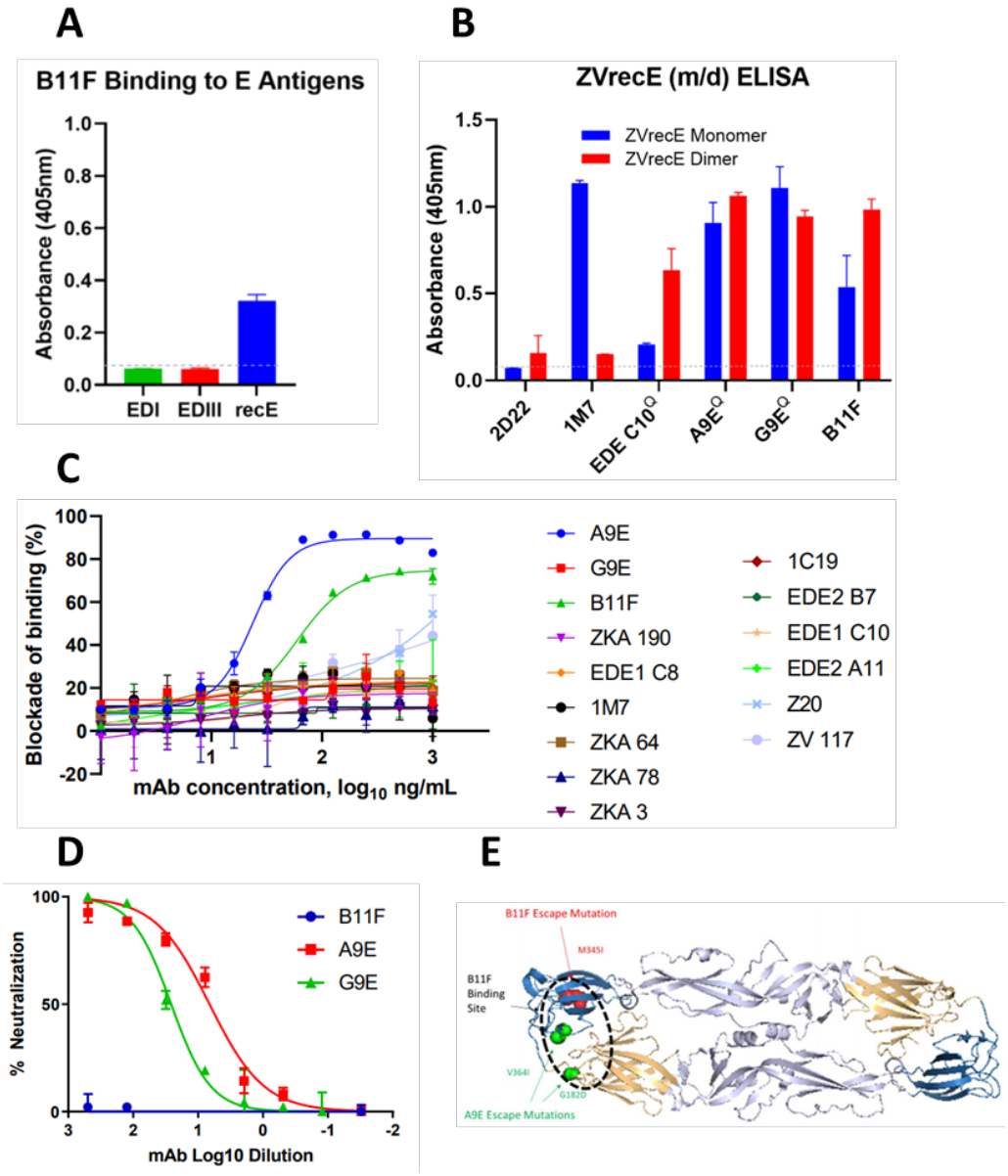

Figure 3. Epitope mapping for B11F. (A) B11F bound to recombinant ZIKV envelope protein ZVrecE but not to single domains of E (EDI, EDIII) as demonstrated by capture ELISA. **(B)** B11F binding to recombinant E dimer was higher than to E monomers. **(C)** Blockade of binding activity of a panel of characterized mAb against B11F. **(D)** B11F loss of B11F binding to escape mutant generated in the presence of the mAb. The escape mutant retained epitopes recognized by other two mAb A9E and G9E. **(E)** Proposed epitope for B11F (dotted oval). Escape mutation for B11F binding is indicated in red; Escape mutations for A9E are indicated in green. Control mAb: 2D22 (DENV2-specific); 1C19 (DENV cross-reactive); 1M7, C10, C8, B7, A11, ZKA78, ZKA3 (ZIKV and DENV cross-reactive); A9E and G9E, ZKA64, ZKA190, Z20, Z3L1, ZV117 (ZIKV-specific). Data & figure courtesy of Graham SD.

Next, we utilized a blockade of binding assay in which binding of alkaline phosphatase-conjugated B11F was competed against other unlabeled mAb with known epitopes. Reduction in phosphatase activity measured by decreasing optical density of metabolized substrate indicated blockade of B11F binding to ZIKV antigen. All but one of the tested mAb failed to reduce B11F binding to ZIKV antigen even at high concentrations, suggesting B11F targeted an epitope distinct from those previously described for these mAb. The mAb A9E effectively blocked B11F binding to ZIKV at approximately EC50 30 ng/mL, and unlabeled B11F blocked labeled B11F binding at approximately EC50 100 ng/mL (**Fig. 3C**). We previously showed that A9E bound to recombinant EDI and ZVrecE, and targeted an epitope involving residues on both EDI and EDIII[12]. These results suggested that B11F likely targeted an epitope overlapping A9E epitope, spanning the junction between ZIKV EDI/EDIII.

To identify specific amino acid residues involved in the B11F epitope, we generated escape mutants by propagating ZIKV in increasing concentrations of B11F. Wild-type ZIKV (H/PF/2013 strain) was initially passaged in IC50 of B11F, followed by increasing concentrations of this mAb after a 3-day incubation period. After 3 passages, we observed better growth of the virus in the presence of B11F, evident by cytopathic effect. Comparison of sequences from wild-type virus and virus propagated with B11F revealed the M345I mutation, buried in the core of ZIKV EDIII, suggesting a conformational disruption of the epitope abrogated B11F neutralizing activity (**Fig. 3D, E**). FRNT50 was performed with the mutated virus and B11F to validate the escape mutation. As predicted, the mutant virus was resistant to B11F while still neutralized by A9E and another positive control mAb G9E (**Fig. 3D)**.

Discussion

To better understand the determinants of antibody neutralization against ZIKV, we characterized the specificity and neutralization activity of ZIKV-specific mAb isolated from the MBC pool of ZIKV-infected subjects. This study focused on the characteristics of the novel mAb B11F. Isolated from a primary FV-infected subject[12] (ZIKV as the first FV infection), B11F showed exclusive binding to ZIKV, and not cross-reactivity against other serotypes of DENV. This result was consistent with our previous finding that the MBC repertoire of primary FV infection was generally focused on the infecting virus[11, 12, 13, 19].

Epitope mapping effort revealed that the B11F footprint spanned across EDI and EDIII of the E protein and overlapped the EDI/EDIII linker epitope of the mAb A9E[12]. Whereas A9E bound to recombinant EDI and not EIII[12], B11F was unable to bind neither suggesting its binding required E to adapt its quaternary monomeric conformation, and its dimeric configuration as on the viral surface. Sequencing of B11F viral escape mutant revealed that the mAb's binding was dependent on the fine contour of EDIII since the M345I mutation in the core of the domain likely did not alter the charges of the epitope. Further investigation of the B11F epitope using alanine scanning or structural methods such as crystallography will offer more insight on how this structural determinant influences the activity of the mAb.

As EDIII is involved in cellular receptor attachment, mAb binding to epitopes involving EDIII likely inhibit this interaction between the virus and its target cells. Our results supported this hypothesis that B11F inhibited ZIKV infection by blocking viral attachment to target cells. Addition of B11F pre- versus post-attachment showed the mAb inhibited the infection at steps prior to viral attachment, which was elaborated with

blockade of attachment qPCR data. Increasing concentrations of B11F reduced levels of viral genomes associated with target cells, indicating that the mAb prevented ZIKV from binding to its target. While we did not determine whether B11F could prevent endocytosis of the virus, it is reasonable to eliminate this route of neutralization since such a mechanism would likely allow viral attachment and would not reduce cell-associated viral genome level at increasing antibody concentrations. Alternatively, we did not explore the possibility of B11F affecting the structural integrity of ZIKV, rendering the virus unable to attach to its target or non-infectious. These possible pre-attachment mechanisms of neutralization can be investigated, alongside structural studies, to better understand how B11F specifically inhibits the infection.

Several studies have enriched our knowledge of determinants of ZIKV-, and DENV-specific neutralizing mAb[20, 21, 22, 23, 24, 25]. Depending on their epitopes, mAb likely neutralize the virus using different mechanisms. The cross-reactive mAb C10 binds to conserved quaternary epitope spanning across E domain II (EDII) and EDIII in an E dimer, and blocks rearrangement of these proteins into their fusogenic form in acidic environment, thus preventing fusion between viral and host endosomal membranes[26]. mAb targeting EDIII such as B11F are also found to be highly neutralizing[11, 23, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36]. Our understanding of these mAb's neutralization mechanisms and their epitopes will elucidate how the structural interactions between mAb and the viral proteins dictate their functions. Further studies will continue to validate the connections between epitopes and neutralization mechanisms, and establish whether certain mechanisms are associated with specific mAb' superior neutralization activity. Such understandings can inform vaccine development, especially in immunogen design to specifically elicit

neutralizing antibodies. Additionally, strongly neutralizing mAb such as B11F have the potential to be developed into therapeutic strategies to treat ZIKV infection, or to be used as a prophylactic during a ZIKV outbreak.

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HT and SDG contributed equally. HT generated transformed polyclonal and monoclonal MBC libraries. BDM cloned the antibodies into expression vector. HT designed and performed neutralizing mechanism experiments with in consultation with SAD and Emily Bruce (Vermont Center for Immunobiology and Infectious Diseases). SDG performed epitope mapping work under guidance of AMS and AJM. HT, SDG analyzed data and HT wrote the manuscript with input from the other authors.

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CHAPTER VI – CONCLUDING REMARKS

FV continue to impose a health and economic threat to the global community. While several vaccines such as those for YFV and JEV can help control transmission and decrease the burden imposed by these viral infections, vaccines for other FV such as DENV and ZIKV are still in development or not widely available[1]. Furthering our understanding of the complexity of the antibody responses to these viruses will help to advance the development of these vaccines. Leveraging longitudinal samples from controlled infection/vaccination studies[2], and from natural infections[3], we provided a comprehensive view of the antibody responses induced by DENV and ZIKV. The studies in this dissertation expand the definition of "antibody response" by exploring the evolution of this response over time, and examining different cellular components that contribute to this humoral arm of the immune system.

One major focus of FV research has been to study the specificity of the antibody response to these viral infections to define determinants of protective immunity[4]. Studies of naturally infected subjects in endemic areas found a prevalence of CR antibodies in DENV infections, and demonstrated the potential of these antibodies to facilitate ADE *in vitro*[2, 5, 6, 7, 8, 9, 10, 11, 12, 13]. However, our findings suggest that primary infection of either DENV (Chapter II) or ZIKV (Chapter IV) on FV-seronegative background result in a more TS antibody response, which is also consistent with more recent studies[2, 14, 15, 16]. While our studies do not discount the existence of CR antibodies, ADE, and the possibility of secondary dengue disease, they provide the basis to understand how the antibody response is shaped in primary infections. Delineating the determinants of this

TS antibody repertoire has provided more insights on how these antibodies may confer homotypic protection.

We found TS antibodies to be highly neutralizing and often target epitopes involving EDIII of the viral E glycoprotein. Consistent with other studies[6, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26], our findings support the notion that EDIII may be the Achilles's heel of the virus, and therefore a promising target for neutralizing antibodies. This premise offers an advantage for vaccine development by providing a specific target for immunogen design. Given the fact that EDIII is the least conserved E domain[6, 17], one possible strategy for vaccine development is to combine heterologous EDIII immunogens into a tetravalent DENV vaccine formula, or perhaps a pentavalent formula to include ZIKV, to induce multiple subsets of TS antibodies for each viral serotype. Such an approach potentially minimizes the generation of CR antibodies that may aggravate ADE risks.

Serotype-specificity was observed in multiple antibody compartments in primary DENV and ZIKV infections, from acute phase (PB) to convalescent phase (MBC and serum)[2, 3]. From the immunological perspective, the formation of a TS antibody repertoire is likely associated with homotypic immunity, especially with the presence of neutralizing clones, as observed in the subjects' cellular and serological compartments. Continuous monitoring of these subjects will provide a better understanding of the antibody response's durability. It is possible that this response persists for many years as observed in other studies using convalescent sera collected decades following the infections[27, 28]. Importantly, follow-up studies will reveal how the TS and CR antibody ratio changes over time, whether the subjects remain unexposed or have other subsequent FV exposures (e.g., infection or vaccination, homotypic versus heterotypic).

Of particular interest, the changing quantity and quality of CR antibodies at late convalescence of the primary infection may hold the answer to how these antibodies may functionally switch from cross-protection to becoming ADE risk. One FV research priority is dissecting the B cell components to understand the origins and functionalities of CR clones invoked during secondary infections. Our studies have begun to address this topic by linking the PB dynamics to serum antibodies. Future investigation should extend beyond this relationship to identify other sub-populations of antibody-secreting cells, their kinetics, and clonal evolution, in order to appreciate how these cells may influence the serological features. Exploration in this area will also answer (1) what are the requirements for the persistence of TS antibodies and homotypic immunity, and (2) how do multiple infections alter the TS antibody repertoire while shaping the characteristics of the CR clonal pool?

TS antibodies generated from primary DENV and ZIKV infections represent an opportunity to improve the specificity of diagnosing these acute viral infections. Current testing practices include quantitative PCR that detects viral genomes, and lateral flow rapid tests detecting viral NS1 protein and virus-specific antibodies[4]. Despite their shortcomings, current testing protocols can provide a quick diagnosis for clinicians to proceed with proper care to improve disease outcomes. The PCR platform, while highly sensitive and specific, requires technical expertise and instrumentation, and therefore not suitable for mass testing at the national level during an outbreak. Other forms of rapid tests, while affordable and simple to perform, are less specific due to cross-reactivity of virus-specific antibodies. Leveraging the presence of TS antibodies following these viral infections and incorporating their corresponding antigens (e.g., heterologous DENV

EDIII) will then deconvolute the complexity of polyclonal serum to differentiate infection by each DENV serotype, and differentiate DENV (or ZIKV) infection from other FV infections. The rationale for this approach relies on the assumptions that TS antibodies are in circulation at some level after one or multiple heterotypic DENV infections, and that these TS antibody clones are commonly found in the immunome of the population[3]. Additional longitudinal studies are necessary to validate these assumptions as previously discussed, and to define a convergent antibody response of the human population to these viruses.

More pragmatically, the diagnostic improvement provided by TS-antibody-defined antigens will aid in sero-surveillance efforts, which will inform immunization strategies. Several DENV vaccine candidates such as Dengvaxia or TV003 have shown to induce a stronger antibody response in DENV-experienced subjects[29, 30]. On the other hand, Dengvaxia immunization in DENV-naïve subjects is associated with severe disease upon a subsequent natural infection, because the vaccine-induced antibody response resembles that of a primary infection[29, 31, 32, 33, 34, 35]. Therefore, it is critical to understand the serostatus of the population when determining vaccine schedules and rolling out a vaccine campaign.

In the context of dengue immunization, the gold standard serum antibody titers obtained from plaque (or focus) reduction neutralization tests appear to be inadequate in translating laboratory data to outcomes on the population level. The reason for this disconnect is that these conventional assays do not consider the nuances of the antibody response. Our study of the antibody response generated from TV003 immunization addressed this issue by dissecting different components of the response. With our

longitudinal time course and systematic sampling, we shed light on the intimate relationship between different immune correlates that occurred in a sequential order following immunization. Therefore, it is possible to predict how the antibody response may evolve given certain characteristics of early immune correlates (i.e., PB). More systematic studies are necessary to validate the correlations observed in our studies. However, if it holds true that the PB response is indeed predictive of the serological dynamics[36], PB may be used as an early indicator of vaccine response. Since the PB increase occurs shortly following vaccination, the use of this metric to evaluate vaccine performance will significantly reduce the amount of time required for immunogenicity/ efficacy clinical trials, which can take years.

In studying TV003-induced antibody responses, we found a relatively high level of CR antibodies in both the cellular and serological compartments. Future functional studies of these CR antibodies will reveal whether and how they contribute to protection. Potently neutralizing CR antibodies exist, and have been found in subjects who experience multiple exposures in DENV endemic regions[37]. An interesting pursuit will be to determine whether these antibodies are (1) common in the human population, and (2) induced by TV003 immunization.

It is important to recognize that simultaneous immunization with all four heterotypic attenuated DENV components is not the only option in order to induce a balanced immune response. Investigators are exploring other strategies such as sequential heterotypic immunization, or prime/boost schedule with different vaccine formulas[38, 39]. Considering the concern of ADE and CR antibodies, perhaps a model priming with TV003 and boosting with a subunit vaccine comprising TS-antibody-defined

immunogens may help induce both cellular and humoral immunity while building up the repertoire of TS antibodies – if these antibodies are indeed protective in the context of immunization. Whereas these endeavors can be perceived as a race to produce an effective vaccine, they also contribute to our collective knowledge of how the antibody response is shaped in different modalities. The scientific community will gain a deeper understanding of how a superior antibody response to viruses with multiple variants is formed, and the global community will benefit from the vaccines and drugs that are the translation of this knowledge.

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