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Lalita Priyamvada Date

Dengue plasmablast responses and
their cross-reactivity to Zika virus: A single-cell analysis

By

Lalita Priyamvada
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Microbiology and Molecular Genetics

_____
Jens P. Wrammert, Ph.D.
Advisor

_____
Joshy Jacob, Ph.D.
Committee Member

_____
Tracey J. Lamb, Ph.D
Committee Member

_____
Martin L. Moore, Ph.D.
Committee Member

_____
Mehul S. Suthar, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

# Dengue plasmablast responses and their cross-reactivity to Zika virus: A single-cell analysis

By

Lalita Priyamvada
B.S., St. Lawrence University, 2012

Advisor: Jens P. Wrammert, Ph.D.

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## Abstract

Dengue plasmablast responses and
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By Lalita Priyamvada

Antibodies are critical to viral clearance and protection in dengue virus (DENV) infections. While antibody titers to the infecting serotype are long-lived, cross-protective immunity wanes over time, leaving DENV-exposed individuals susceptible to secondary heterotypic infections. Studies have linked secondary dengue with more severe symptoms, implicating pre-existing immunity as a risk factor for increased disease severity. Previously, we showed that secondary dengue patients mount a massive DENV-specific plasmablast response early after fever onset. The appearance of plasmablasts in blood overlaps with a decrease in viremia but also with the onset of severe clinical symptoms. To better understand how plasmablasts contribute to dengue protection/immunopathology, we isolated plasmablasts from four secondary DENV2-infected patients, and generated monoclonal antibodies (mAbs) by *in vitro* expression cloning. By focusing on plasmablasts and not memory B cells, we avoided antigen-based cell screening for mAb synthesis, therefore performing unbiased analyses of patient B cell responses. We found that the plasmablasts were largely memory-derived, and that most mAbs were envelope-specific and neutralized multiple DENV serotypes. Although a majority of mAbs neutralized the infecting serotype, DENV2, several mAbs from two patients neutralized DENV1 more potently. This neutralization bias towards a heterologous serotype was reminiscent of original antigenic sin (OAS), and could potentially dampen protective immunity against the infecting virus. In view of the antigenic and structural similarities between DENV and Zika virus (ZIKV), we next examined whether the cross-reactive phenotype of dengue-induced antibodies could extend to ZIKV. Using the same mAbs characterized above, and acute and convalescent sera from additional dengue patients, we conclusively demonstrated immunological cross-reactivity between DENV and ZIKV. All dengue sera bound and neutralized ZIKV, and cross-reactive binding was broad at the mAb level. However, few dengue mAbs also neutralized ZIKV, thus displaying an OAS-like bias towards DENV. ZIKV-reactive dengue sera and mAbs also enhanced ZIKV infection in an FcγR-dependent manner. Overall, our findings suggest that pre-existing, cross-reactive dengue antibodies may modulate protective/pathological immune responses against future heterotypic DENV, or ZIKV infections. Our single-cell approach revealed the vast heterogeneity in the binding, neutralization and enhancement patterns of dengue antibodies, differences that might have remained concealed in serum-based analyses. These data are relevant to future vaccine efforts, given the high propensity of secondary DENV infections, and the overlapping distribution of ZIKV in DENV-endemic regions.

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# Chapter 1: Introduction

Sections of this chapter have been submitted to *Emerging Microbes and Infections* as part of a review.

Priyamvada L, Hudson WH, Ahmed R, Wrammert J. (2017) Humoral cross-reactivity between Zika and dengue viruses: Implications for protection and pathology. Accepted at *Emerg Microbes Infect*.

## Part I: The human antibody response after dengue virus infection

Dengue is a systemic mosquito-borne infection caused by the four dengue virus (DENV) serotypes DENV1-4. As a result of its rapid and extensive geographic spread over the past several decades (1), DENV has established endemic and epidemic transmission cycles in over 100 countries globally (2). Over half of the world's population, 3.9 billion people, are currently living in areas of DENV circulation and are therefore at risk of viral exposure (2). Recent studies have estimated that 390 million infections occur every year (3), including 96 million symptomatic cases (3) and 10,000 deaths (4). Additionally, dengue prevention, treatment and control costs amount to an estimated $8.9 billion annually (5). Given the high incidence of asymptomatic infections (3), low hospitalization rates and under-reporting by hospitals (6), these figures, although significant, may actually underestimate the true global impact of dengue disease. Consequently, dengue has emerged as one of the most important viral diseases in the world, and it is becoming increasingly critical to tackle the global threat of this neglected tropical disease.

### DENV classification and virion structure

DENV is a member of the flavivirus genus of the *Flaviviridae* virus family. There are 4 DENV serotypes, DENV1-4, each of which are further subdivided into genotypes based on genetic relatedness. Overall, DENV1-4 share a considerable sequence identity of 65-70% at the amino acid level (7). The positive sense, single-stranded RNA genome of DENV is contained within a nucleocapsid core, which is surrounded

by an outer lipid envelope made up of 180 units each of two structural proteins: envelope (E) and pre-membrane (prM) (8). E is involved in viral fusion and entry (9) and is the main antigenic target for humoral responses in DENV infections (10-13). Like other flaviviral E proteins, DENV E contains three domains: a central β-barrel-shaped domain I (EDI), a finger-like domain II (EDII), and a C-terminal immunoglobulin-like domain III (EDIII) (reviewed in (14)) (**Figure 1c**). The viral fusion peptide is located at the tip of EDII, and lies shielded by EDI and EDIII from the other monomeric subunit within the E protein dimer. The prM protein, comprised of the pr peptide and the M protein, assists in the proper folding of E (15). The pr peptide blocks the rearrangement of E to its fusogenic form prior to budding out of the host cell (16), and is cleaved off by the host protease furin during the viral maturation process (17). Viral maturation is often inefficient, resulting in the release of a heterogeneous population of viruses with different states of maturity, including particles that exclusively express cleaved M, or both prM and cleaved M (18). While the prM/M surface ratio of a viral particle can affect its infectious properties, partially or even fully immature viral particles have been shown to remain infectious (19).

Cryo-EM reconstructions of DENV reveal that the E proteins on the viral surface rearrange to different conformations based on the maturation status of the particle (17, 20). As shown in **Figure 1b**, mature DENV particles appear to have a smooth outer surface. Here, E proteins are arranged as dimers in a head-to-tail herringbone pattern, giving the particle icosahedral symmetry (20). In contrast, immature DENV

particles can have a smooth or spiky appearance, depending on their stage of life cycle after viral entry. Within an infected cell, the neutral pH of the endoplasmic reticulum triggers the trimerization of E-prM heterodimers. There are 60 such trimer spikes on the viral surface that project outward (21) causing the particle to have a larger diameter (60 nm) compared to that of mature flavivirus virions (50 nm) (16, 17, 22) (**Figure 1a**). When the viral particle reaches the trans-Golgi network, the neutral pH of the environment results in the rearrangement of heterodimers from the trimer spikes to the herringbone pattern also seen on mature virions, causing a smooth appearance (17).

Inside the nucleocapsid, the RNA genome is found complexed with the capsid (C) protein (reviewed in (8)). Besides the three structural proteins E, prM and C, the DENV genome encodes 7 additional proteins that altogether make up one single polyprotein. These 7 proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, are non-structural and are only produced after viral infection. The polyprotein is processed and cleaved by host and viral proteases, releasing the 10 DENV proteins. The DENV non-structural proteins carry out various functions related to RNA replication, packaging, translation and polyprotein processing, and are primarily found contained within virally infected cells. A major exception is NS1, which translocates to the cell surface, is secreted by infected cells (23) and can be detected in significant quantities in serum (up to 50 μg/mL) within a few days after DENV infection (24, 25). Consequently, in addition to the surface glycoproteins E and prM,

NS1 is also an important antigenic determinant in the dengue antibody response (10, 26).

DENV reservoir and tissue tropism

DENV is a vector-borne virus that maintains two transmission cycles: human and sylvatic (27). In the former, DENV transmits to humans through the bite of infected mosquitos of the *Aedes* genus, primarily *Aedes aegypti and Aedes albopictus* (28). Several studies suggest that DENV may transmit vertically in mosquitos (29-32). In comparison, human-to-human DENV transmission has rarely been reported to date. The sylvatic cycle of DENV involves transmission between non-human primates and arboreal *Aedes* mosquitos. Evidence for sylvatic dengue has been well documented in the forests of Southeast Asia and West Africa (reviewed in (27)).

Upon inoculation through a mosquito bite, DENV infects Langerhans cells local to the site of infection (33). These infected dendritic cells carry the virus to draining lymph nodes, following which DENV is believed to replicate and spread to various organs within the body resulting in a systemic infection. Although the primary *in vivo* cellular targets of DENV are widely thought to be cells of the myeloid lineage, such as macrophages, monocytes, and dendritic cells, few studies have demonstrated that these cells support active DENV replication in humans. Instead, a majority of data on DENV tropism comes from immunohistochemical staining-based techniques that identify sites of DENV localization by detecting DENV proteins in fixed cells/tissues. Using human autopsy/biopsy samples, a few groups have

reported the presence of DENV NS3 and E in lymph nodes and splenic phagocytes, alveolar macrophages, perivascular cells in the brain, liver hepatocytes and splenic endothelial cells (34-37).

Clinical presentation of disease

The vast majority of dengue infections are asymptomatic (3, 38). When symptomatic infection does occur, patients can present with a wide range of clinical symptoms (reviewed in (39, 40)). While most clinical dengue infections are self-limiting and resolve after mild disease, some cases progress to the more severe forms of dengue, characterized by plasma leakage and hemorrhage. Based on clinical presentation, cases were previously categorized by the World Health Organization (WHO) in increasing order of severity as undifferentiated fever, dengue fever (DF), and dengue hemorrhagic fever (DHF). The DHF category was further subdivided into four grades of severity, with dengue shock syndrome (DSS) ranking in at grades III and IV on this scale (1). However, difficulties in diagnosing severely ill patients on this strict four-grade scale and the changing epidemiology of dengue, among other factors, resulted in the revision of dengue case classifications (41, 42). In 2009, the WHO published new guidelines, recommending that infections be grouped as: dengue without warning signs, dengue with warning signs and severe dengue (1). The symptoms associated with each of these categories are shown in **Figure 2.**

Dengue infection begins with an incubation period that lasts between 3 to 14 days (28). During this time, DENV is replicating and circulating within the body, without the appearance of observable symptoms. The end of the incubation period is marked by fever onset. The febrile phase of dengue can last for 2-7 days, and other symptoms such as myalgia, arthralgia, headache, nausea and skin erythema are also reported during this time (43-45). Dengue patients may also have increased hematocrit levels and a positive tourniquet test. Highly elevated hematocrit can also be an indication that the patient might progress to severe dengue (43).

While most patients recover shortly after abatement of fever (defervescence), others go on to acquire more severe symptoms. The critical phase of dengue typically lasts for 24-48 hours and is marked by abnormal blood cells counts due to leukopenia and acute thrombocytopenia, and increased capillary permeability (1). The latter causes plasma leakage, which without timely and suitable fluid replacement therapy can result in excessive plasma loss leading to shock. A prolonged period of shock can in turn cause multiple organ failure and hemorrhage. In the absence of appropriate medical interventions, such severe illness may lead to death. Cases that survive the critical phase without progression to severe dengue usually recover over the next 48-72 hours. During this time, hematocrit levels normalize and white blood cells and platelets reconstitute blood. Overall, myalgia and general malaise subside, plasma levels stabilize and patients enter the convalescent phase. Although currently there is no defined cure for dengue, prompt triage and supportive care can greatly improve the clinical outcome of disease (46).

Human immune response to DENV

As with other viral infections, the human immune response to DENV begins with the activation of innate pathways, followed by adaptive immunity. Dengue patients have high serum levels of type I IFN and pro-inflammatory cytokines such as IFN-α, TNF-α, IL-6, IL-8, and IFN-γ during the acute phase of infection (47-49). The early induction of these cytokines is mediated by host pattern recognition receptors (PRRs) that sense viral proteins and genetic material in virus-infected cells. The endosomal Toll-like receptors (TLRs) TLR3 and TLR7/8, and the cytoplasmic RIG-I, MDA5 and LGP2, are key PRRs in the flavivirus innate immune response (50). Several *in vitro* studies have demonstrated that these receptors are involved in the inhibition of DENV replication and induction of type I IFN production (51-54). In DENV-infected rhesus macaques, administration of TLR3 and TLR7/8 agonists decreased viremia and boosted anti-viral inflammatory and humoral immune responses (55).

Although the induction of pro-inflammatory cytokines is associated with an anti-viral state, several studies have suggested that excessive inflammation may be linked to increased dengue disease severity (56, 57). Activated T cells have been implicated in exacerbating disease by creating a "cytokine storm" of pro-inflammatory cytokines (reviewed in (58)). On the other hand, several mouse studies have demonstrated a protective role for T cells in the dengue immune response. In one study, depletion of $CD8^+$ T cells in IFNAR mice prior to infection caused higher viral loads compared to non-depleted mice (59). $CD8^+$ T cells were

shown to have *in vivo* cytotoxic activity and bound to epitopes on non-structural and structural DENV proteins. The same group demonstrated that virus-specific $CD4^+$ T cells producing IFN-γ, TNF-α, and IL-2 are induced in response to DENV infection in mice (60). Depleting $CD4^+$ T cells prior to infection did not affect viral loads or the induction of the $CD8^+$ T cell response. However, immunization with $CD4^+$ T cell epitopes prior to infection reduced viral loads, suggesting that $CD4^+$ T cells may also play a part in DENV clearance. In humans, elevated numbers of DENV-specific $CD8^+$ T cells are present in peripheral blood early after dengue (61, 62). Epitope mapping efforts have revealed that cells directed to non-structural proteins epitopes (NS3, NS4b and NS5) typically dominate the dengue $CD8^+$ T cell response (62, 63) and that IFN-γ $CD8^+$ T cell response in dengue may vary based on the infecting serotype (63). Studies have also indicated that the breadth of DENV epitopes identified by $CD8^+$ T cells in dengue and the magnitude of these $CD8^+$ T cell responses might be HLA-dependent, suggesting a link between HLA type and the protective role of T cells during the dengue immune response (64-66).

In addition to innate and cell-mediated immunity, humoral immunity plays an important role in the human immune response in dengue. B cell responses are crucial to viral clearance and long-term immunity, and neutralizing antibody titers are an important correlate of protection against symptomatic infection (67). After DENV infection, DENV-specific B cells are activated and proliferate rapidly, and antibody secreting cells (ASCs) can be detected in circulation in high numbers early after fever onset (68). **Figure 4** describes the properties of the various B cell types

involved in the dengue immune response. Shortly after infection, short-lived plasma cells, also known as plasmablasts, secrete DENV-specific antibody in response to antigen exposure (68). These contribute to the rise in serum antibody titers seen early after DENV infection. A key feature of adaptive immunity is immunological memory, and two key players in providing long-term immunity after DENV-exposure are long lived plasma cells and memory B cells. As with other viral infections, long-lived plasma cells are believed to reside in the bone marrow and secrete DENV-specific antibody even in the absence of circulating antigen. As a result, dengue antibody titers may exist in serum for decades after infection (69). In addition to ASCs such as plasmablasts and plasma cells, DENV infection leads to the generation of DENV-specific memory B cells (MBCs) (70, 71), which can re-activate and may provide long-lived protection in the face of future DENV exposures.

Humoral responses: primary vs. secondary infection

The progression of clinical symptoms after DENV infection temporally overlaps with the induction and expansion of humoral immune responses (1, 72, 73). As shown in **Figure 3**, following a primary DENV infection, DENV-specific antibody titers are detectable in patient serum within the first week of fever onset (74). The IgM response is followed by a surge in serum IgG by the second week of disease (74). These antibodies are either cross-reactive, i.e. recognize shared epitopes on multiple DENV serotypes owing to the antigenic similarities between DENV1-4, or are serotype-specific in DENV-reactivity. After resolution of infection, serum titers decrease but remain detectable long-term (72). IgM can be detected for up to 3

months after a primary DENV infection, and IgG may persist for years to decades in serum (69).

Several studies have shown that a large proportion of the dengue antibody response is cross-reactive to multiple DENV serotypes (70, 71, 75-77). The magnitude and functional quality of these responses can, however, depend on a patient's prior dengue disease/ exposure history, among other factors. A human challenge study conducted by Sabin in 1952 demonstrated that individuals infected with a specific DENV serotype remained protected from re-infection with the same virus for up to 18 months. In contrast, individuals challenged with a heterologous serotype were only protected for 2-3 months. Beyond the 3-month window, a heterologous DENV challenge resulted in considerable viremia and clinical symptoms (78). These data, together with more recent studies illustrating the longevity of type-specific antibodies (79), suggest homotypic immunity may persist long-term. However, the exact duration of long-term sterilizing immunity is unknown, and homotypic re-infections can cause symptomatic disease (80, 81). In addition, cross-neutralizing antibody titers may wane over time resulting in only temporary protection against future heterologous infections. Consequently, DENV pre-exposed individuals are susceptible to repeat infections, which are referred to as secondary DENV infections.

Secondary dengue infections are followed by a less significant IgM, but an amplified IgG response, compared to primary infections (68, 74). The IgG response is also more rapid than in primary infections, with plasmablasts appearing in high

frequencies in blood within a few days of fever onset (11, 68, 82). In case of secondary heterotypic dengue, studies have shown that B cells secreting cross-reactive antibodies are boosted in response to infection, and can be detected in sera or the MBC pool long after convalescence (71, 77, 83). What remains less well understood, however, is whether the frequency of these cells and the quantity of antibodies they secrete cross the minimum threshold required to protect against multiple DENV serotypes.

Antigenic determinants of dengue antibodies

Studies analyzing dengue antibodies, shortly after infection as well as months to years after exposure, indicate that E is the dominant antigenic target in the dengue antibody response (10-13). Since it plays a key role in viral fusion and entry, E is also the main antigenic determinant for neutralizing antibody responses in DENV infections. Among the E protein domains, EDIII is the least conserved between the four DENV serotypes and has been shown to be an important target for type-specific potently neutralizing antibodies in mice (84, 85). However, serum depletion experiments reveal that EDIII-specific antibodies make up a small proportion of human DENV-immune serum and play a minor role in neutralization (13, 86). In contrast, the highly conserved fusion loop region containing the fusion loop epitope (FLE) is more immunodominant and is a target for moderate-potently neutralizing and highly cross-reactive antibodies (12).

Another target of significant interest is termed the envelope dimer epitope (EDE) (12). EDE antibodies, directed towards epitopes that span across the E dimer interface, have been shown to be conformationally-sensitive and broadly neutralizing. These dimer-dependent epitopes are part of a novel and growing class of complex, quaternary epitopes that are only present in the intact virion and not the monomeric form of E (12, 87-89). A study demonstrated that serum antibody binding titers to recombinant E protein ectodomain only represented 10-35% of the total DENV-specific antibody titer, suggesting that virion-dependent epitopes, like the ones described above, may constitute a significant proportion of the dengue antibody response (70).

In addition to E-specific titers, dengue patient serum also contains prM and NS1-specific antibodies (10, 26). DENV prM and NS1-specific MBCs have been also detected in the blood of convalescent patients by several groups (71, 75, 77, 90). Antibodies targeting prM largely exhibit poor or moderate neutralizing potency *in vitro,* and can be highly cross-reactive to the four DENV serotypes (75). The highly cross-reactive phenotype of prM antibodies is not surprising, given the substantial sequence identity of prM between DENV1-4. The highly cross-reactive but poorly neutralizing nature of prM antibodies has linked them to antibody dependent enhancement (ADE; discussed on page 15-17) of DENV infection (75), a potential mechanism of DENV pathogenesis.

Antibodies directed to NS1 are not typically associated with viral neutralization since they target a non-structural protein. However, mouse studies in which immunization with NS1 protected mice against lethal DENV challenge, point to a potentially protective role of these antibodies during dengue disease (91). In the same breath, NS1-specific antibodies have also been hypothesized to contribute to dengue immunopathology through molecular mimicry (92, 93). Homologous amino acid sequences have been identified between NS1 and self-antigens on platelets, clotting factors and endothelial cells, suggesting that NS1 antibodies may cross-react with cellular antigens, potentially leading to damage and dysfunction of these cell types (93). Additional studies are required to fully clarify the role of NS1 antibodies in dengue pathogenesis. Lastly, antibodies to NS3, NS5 and C proteins have also been identified in dengue patients (10, 94). However their contribution to overall serum reactivity and protection or pathology in dengue remains less well characterized.

Viral neutralization by antibody

Antibodies can impede flavivirus infection by blocking attachment of the virion to the target cell (88, 89). Alternatively, antibodies may block infection at the "post-attachment" stage, by inhibiting viral fusion (95, 96). The binding of antibody to the viral surface may hinder the rearrangement of E proteins required for fusion with the endosomal membrane. It is generally believed that the antibody-dependent neutralization of DENV and other flaviviruses follows the "multiple-hit" model (reviewed in (97, 98)). According to this model, viral neutralization requires the

binding of antibody with a stoichiometry that exceeds a necessary threshold, as opposed to a single binding event by antibody at a "critical site". Through studies using antibodies to an EDIII WNV epitope, one group calculated the number of antibodies required for flavivirus neutralization to be ~30 (99, 100). However, whether this figure applies broadly to all epitopes and in the context of polyclonal sera remains unclear.

An important consideration for viral neutralization is antibody affinity (101) – high affinity antibodies can occupy more sites on a virion than low affinity antibodies directed to the same epitope. Studies have shown that the same antibodies can exhibit different *in vitro* neutralization potencies against viruses of the same DENV serotype, suggesting that changes in antibody affinity due to genotypic variations can affect neutralization (102, 103). The stoichiometry of antibody binding is also affected by the number of accessible viral epitopes. A highly accessible epitope might require a lower occupancy rate by antibodies than a cryptic epitope for neutralization. For instance, monoclonal antibody E53 binds the relatively inaccessible FLE, buried between DI and DIII of the adjacent E protein monomer (18). In order to neutralize virus, such antibodies may have to bind every single available epitope on the viral surface to exceed the stoichiometric requirement of antibody-virus engagement (104, 105). The maturation status of the viral particle may also impact epitope accessibility, thereby affecting neutralization. As mentioned earlier, viruses released from DENV infected cells can vary in their surface expression of prM, resulting in a heterogeneous population of structurally

different virions. In this context, mAbs like the FLE-binding E53, which bind epitopes more easily accessible in immature particles, may neutralize prM-expressing virus but not the mature virions (18, 104).

Antibody dependent enhancement

Alongside their protective potential, antibody responses in dengue and other flaviviral infections have also been implicated in exacerbating disease. Epidemiological studies associate secondary DENV infections with greater disease severity than primary infections, suggesting that prior DENV exposure and pre-existing immunity may be risk factors for severe disease (38, 106-109). ADE is one of several hypotheses proposed to explain the increase in disease severity associated with repeat heterotypic infections (110, 111). Studies have shown that infecting cells expressing Fcγ-receptors (FcγR) in the presence of antibodies from flavivirus-immune donors can increase their rate of infection (112). ADE is thought to occur when cross-reactive antibodies present at sub-neutralizing concentrations facilitate the uptake of virions by permissive cells, thereby enhancing infection. Rather than inhibiting viral infection, the immune complexes formed between such antibodies and viral particles attach to cells and are internalized more efficiently via FcγR-engagement (112). ADE of DENV infection has been demonstrated by multiple groups using sera and mAbs from primary and secondary dengue patients (71, 75, 113). Both neutralizing and non-neutralizing mAbs have been shown to greatly enhance DENV infection *in vitro* (71, 75, 77). The results of these studies indicate that antibodies are capable of enhancing infection at any concentration higher than

the minimum number of molecules required to attach virus to cells via FcγR-engagement, but lower than the stoichiometric requirement for neutralization (reviewed in (97)).

The ADE hypothesis has also been tested *in vivo,* in non-human primate and mouse models. In one study involving rhesus macaques, administering sub-neutralizing concentrations of a DENV-reactive mAb led to higher viremia, although no differences in severity of symptoms were reported (114). In AG129 mice, treatment with DENV-specific mAbs or serum prior to DENV infection resulted in greater pathology compared to untreated DENV-infected mice (115). The mice had higher systemic levels of virus, elevated cytokines and hematocrit, and thrombocytopenia, all characteristics reminiscent of human DHF/DSS. A second study with similar results determined that the increased viral replication observed was dependent on the interaction between virus-immune complexes and FcγR, thus supporting the ADE mechanism of dengue immunopathology (116). The studies above utilized immuno-compromised mice and a non-human primate model that did not show signs of clinical disease. Given these limitations, the physiological relevance of their findings to human DENV infection is subject to uncertainties and requires further investigation.

**Introduction to Chapter 2**

As outlined in **Figure 3**, within a few days following dengue symptom onset, DENV-specific serum antibody levels begin to rise. This occurs due to the rapid activation

and expansion of the acutely-induced, antibody secreting subset of B cells known as plasmablasts. Our lab and other groups have demonstrated that plasmablasts represent a significant proportion of circulating B cells during the febrile phase of dengue (68, 82). We previously reported that in a cohort of secondary dengue-infected Thai patients, plasmablasts reached frequencies up to 30% of the total circulating lymphocyte compartment and constituted greater than 50% of the peripheral B cell population in some patients (68) (**Figure 5a, b**). **Figure 5c** provides context to the magnitude of these responses, showing the striking difference between plasmablast induction in secondary dengue infection vs. vaccination with influenza and yellow fever virus (YFV) vaccine.

The plasmablast numbers returned to baseline levels, comparable to frequencies observed in healthy donors without recent antigen exposure, within a month after infection (**Figure 5b**). Additionally, plasmablast numbers strongly correlated with time after fever onset, with counts increasing steadily from day 2 till day 6-8 (**Figure 5d**). ELISPOT analyses revealed that these antibody-secreting cells were largely DENV-specific, and predominantly secreted IgG, followed by IgA (**Figure 5e, f**). Since the study cohort comprised almost entirely of secondary dengue patients, the high frequency of class-switched cells and low IgM responses were not entirely unexpected.

The sheer magnitude of the antibody response, as well as its temporal overlap with the onset of clinical symptoms prompted questions about the origin and functional

role of plasmablast responses in dengue: Are plasmablast responses primarily naïve or of memory-origin? What is the functional quality of the plasmablast response in secondary dengue? Which antigenic targets dominate the acute phase ASC response? Can plasmablast-derived mAbs enhance DENV infection? To address these questions and improve our current understanding of the acute phase B cell response during DENV infection, we isolated plasmablasts from four secondary DENV2-infected patients and generated a panel of mAbs by cloning and expressing the plasmablast immunoglobulin genes. We quantified levels of somatic hypermutation and examined the VDJ repertoire of dengue plasmablasts to gauge the origin and breadth of these responses. Plasmablast sequence analysis revealed high levels of somatic hypermutation and the presence of clonal expansions, strongly supporting an MBC origin for these acutely-induced cells.

In addition, we determined binding targets and measured *in vitro* neutralization activity of the mAbs. We found that a majority of mAbs were E-specific and cross-neutralizing. Several DENV-neutralizing mAbs bound to whole DENV virion but not recombinant E protein, suggesting that they might target epitopes that are conformationally-sensitive or virion-dependent. Several mAbs neutralized a heterologous serotype (DENV1) more potently than the infecting serotype (DENV2), and in two of the four patients, a neutralization bias to DENV1 was observed. This bias was echoed at the serum level, with the same two patients showing higher serum neutralizing titers against DENV1 compared to DENV2. This phenotype was reminiscent of original antigenic sin, given that all four patients were experiencing

secondary DENV infection at the time of the study. Lastly, we quantified the ADE capacity of the mAb panel in U937 cells. We observed that a large proportion of DENV-specific mAbs enhanced infection of the FcγR-bearing cell line, irrespective of neutralization potency. The experimental methods and results of this study are described in **Chapter 2**.

## Part II: Humoral cross-reactivity between Zika and dengue viruses

### Zika virus emergence and changing clinical features

Zika virus (ZIKV) is a mosquito-borne flavivirus that was first discovered in 1947 in the Zika forest in Uganda (117). The virus was isolated from a febrile rhesus macaque through a yellow fever surveillance network in the area. A year later, ZIKV was also isolated from *Aedes* mosquitoes from the same forest, pointing to a potential sylvatic transmission cycle involving non-human primates and mosquitoes (117, 118). The first confirmed human ZIKV case was a laboratory-acquired infection reported in Uganda in 1964 (119) following which sporadic cases of natural human infection were identified in Nigeria (120, 121) and Indonesia (122). However, serological data suggests a wider geographical distribution, as seroprevalence for ZIKV antibodies has been documented in several additional countries spanning South and Southeast Asia and Africa (123, 124), as well as in Uganda as early as 1952 (118).

The first significant human outbreak of ZIKV outside these areas occurred on the Yap Island of Micronesia in 2007, which was marked by 49 confirmed infections, 131 probable or suspected cases and an estimated ZIKV infection rate of 73% (125, 126). Thereafter in 2013, the virus caused a larger epidemic in French Polynesia, with estimates ranging between 8,500 and 19,000 suspected infections (127, 128). Until 2013, symptomatic ZIKV infections were primarily associated with mild dengue-like illness involving fever, rash, myalgia, arthralgia, and conjunctivitis (123,

125). However, during the French Polynesia outbreak, many ZIKV patients presented with severe clinical manifestations including Guillain-Barré Syndrome, which required hospitalization and medical interventions (129, 130).

In 2015, ZIKV was discovered to have spread to Brazil (131-133), which initiated the largest ZIKV epidemic known to date. Since its emergence in Brazil, cases of autochthonous ZIKV transmission have been reported in nearly 50 additional countries and territories in the western hemisphere (134), including the United States (135). In addition, ZIKV infections in the Brazilian outbreak have been linked to complications in pregnancy, and severe ocular and neurological deformities in neonates born to ZIKV-infected mothers including microcephaly (136-138). Besides the striking increase in the incidence of microcephaly reported concurrently with the ZIKV outbreak (139), the presence of ZIKV in brain tissues of aborted microcephalic fetuses (140, 141), as well as in the amniotic fluid of pregnant mothers of microcephalic fetuses (142) demonstrate a causal relationship between ZIKV infection and this devastating developmental defect (143).

Accordingly, ZIKV has now emerged as one of the most critical arboviruses and is a significant public health concern worldwide. Given the overlapping presence of DENV in a majority of ZIKV epidemic regions (3, 134, 144), there is a pressing need to better understand the extent and characteristics of DENV-ZIKV immunological cross-reactivity. Further, the potential impact of this cross-reactivity on the

protective efficacy of ZIKV-induced antibody responses warrants careful investigation.

Viral transmission and tissue tropism

The main mode of transmission of ZIKV to humans is via the bite of infected mosquitos of the *Aedes* genus. While *Aedes aegypti* and *Aedes albopictus* are the principle vectors for ZIKV transmission, ZIKV has been isolated from other mosquito species in various studies conducted in West Africa (reviewed in (145)). ZIKV may also transmit from human to human, either vertically from an infected mother to her fetus during pregnancy (140, 141) or horizontally through sexual contact (146-148). Additionally, cases of possible ZIKV transmission through blood and platelet transfusion have also been reported (149, 150).

In addition to its unique ability to transmit independent of mosquito vectors, ZIKV also appears to have a broader cellular and tissue tropism than other flaviviruses like DENV. ZIKV RNA has been detected in human brain and placental tissue (151), sperm and semen (152, 153), vaginal secretions (154), cervical muscous (155) and conjunctival fluid (156). In several of the aforementioned cells and bodily fluids, ZIKV RNA was found to persist for weeks to months beyond the viremic period. *In vitro,* human neural progenitor cells (157), placental Hofbauer cells, trophoblasts and endothelial cells (158, 159), skin and uterine fibroblasts (160, 161), have all been shown to be permissive to ZIKV infection. The persistence of ZIKV in a broad range of body tissues and fluids is an important consideration for ZIKV surveillance

and diagnosis, and may pose unique challenges to future efforts aimed towards controlling ZIKV spread.

Genetic and immunological relatedness between ZIKV and DENV

ZIKV, like DENV, is a member of the *Flaviviridae* virus family. The cryo-EM structure of ZIKV reveals that the virus has a nearly identical organizational structure to DENV, including the characteristic herringbone arrangement of E protein head-to-tail homodimers on the virus surface (162, 163). In addition to structural similarities between the viral particles, the main targets for antibody responses in dengue infections, namely E, prM and the non-structural protein NS1, share substantial amino acid sequence identity between ZIKV and DENV (162, 164-166) (**Figure 6**). The considerable structural and genetic relatedness between ZIKV and DENV has been hypothesized to cause immunological cross-reactivity between these closely related viruses, which may make diagnosing patients challenging as well as potentially impact protective/pathologic immune responses to these infections.

During the Yap State outbreak, suspected ZIKV cases were tested for serum binding and neutralizing (PRNT90) titers to ZIKV and other flaviviruses including DENV (125, 126). Most patients tested were categorized as flavivirus pre-immune due to the presence of cross-reactive IgG in their acute phase (<10 days after symptom onset) sera. A majority of these presumed secondary flavivirus cases showed measurable DENV PRNT90 serum titers (126). Although ZIKV was the only

detectable circulating virus during the outbreak (as stated in (126)), DENV infections had been previously reported on the island (167). Similarly during the French Polynesia outbreak, cross-reactive serum titers against DENV were observed in ZIKV-probable cases (130). This may have been in part due to the co-circulation of DENV1 and DENV3 in French Polynesia at the time of its ZIKV outbreak (128). In addition, the country has experienced several dengue epidemics (168-170), and moreover, serological surveys in 2011-2013 indicated that nearly 80% of the adult population was DENV-seropositive (171). Consequently, given the possibility of prior/concomitant exposure to DENV, the presence of cross-reactive antibody titers in patients from the Oceania outbreaks was not entirely unexpected.

The studies described above provide a premise for cross-reactivity between ZIKV infection sera and DENV. However such analyses of polyclonal sera alone may not reveal the origin of these DENV or ZIKV-reactive antibody responses, or the relative proportion of type-specific vs. cross-reactive antibodies. Especially in the flavivirus-experienced populations described above, the possibility of multiple independent pools of antibodies contributing to the apparent serum cross-reactivity, rather than one common pool that recognizes both DENV and ZIKV also cannot be easily ruled out.

**Introduction to Chapter 3**

To address the issue of immunological cross-reactivity between DENV and ZIKV, we tested sera and mAbs obtained from RT-PCR confirmed Thai dengue patients

against ZIKV. Sera collected at various acute and convalescent time points after DENV infection were tested for binding to ZIKV antigens. In addition, their *in vitro* neutralization potency against ZIKV was determined. Both acute and convalescent sera exhibited high ZIKV binding and neutralization titers.

However, since the possibility of prior ZIKV exposure could not be ruled out in these patients, we also tested the plasmablast-derived mAbs described in **Chapter 2** against ZIKV to conclusively demonstrate DENV-ZIKV cross-reactivity at the single-cell level. We found that mAbs generated from *in vivo* activated, single-cell sorted plasmablasts isolated during ongoing DENV infection can bind and neutralize ZIKV. As the source of the mAbs analyzed was plasmablasts activated in response to DENV infection, our findings indicated that dengue-induced antibodies can cross-react to a heterologous virus, ZIKV. Although a majority of mAbs cross-reacted to DENV antigens, only a few also neutralized virus *in vitro*. These data suggest that a small proportion of antibodies may contribute to the considerable cross-neutralization potency of dengue serum against ZIKV.

Lastly, we assessed whether dengue antibody responses could enhance ZIKV infection by the ADE mechanism. We found that both sera and mAbs enhanced ZIKV infection of FcR-bearing cells, and that the range of enhancing concentrations depended to some degree on the neutralization range of the serum sample/ mAb tested. The experimental methods and results of this study are described in **Chapter 3**.

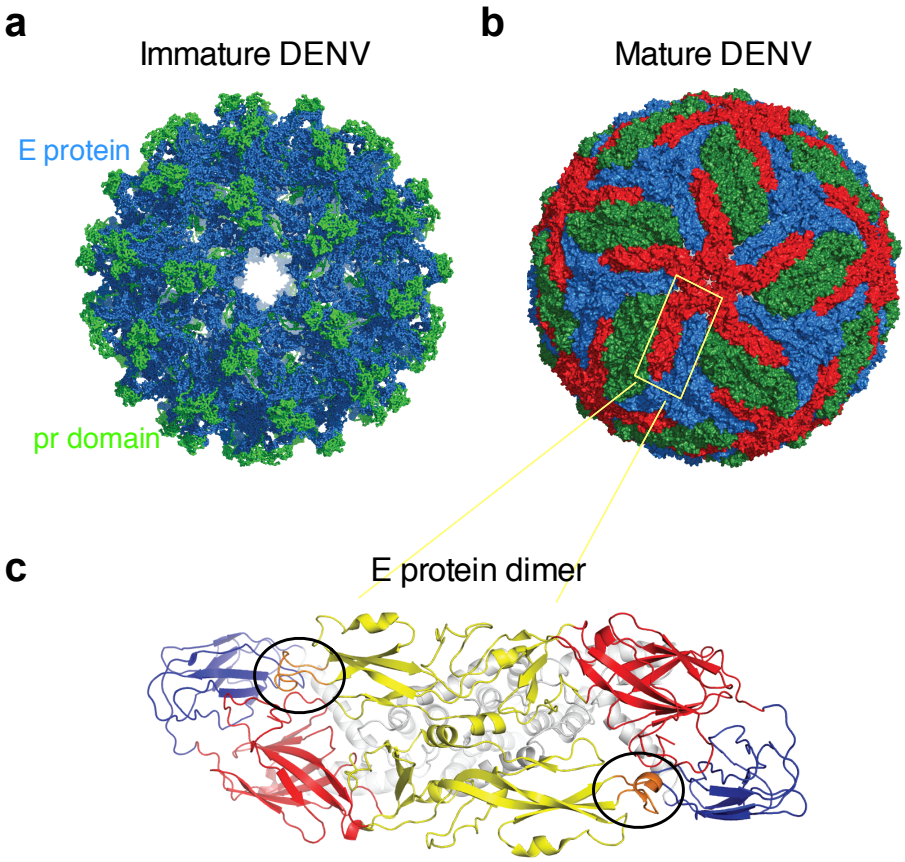


**Figure 1. The DENV particle and E protein dimer.** Cryo-EM surface structures of a) immature (PDB 4B03) (172) and b) mature (PDB 3J27) (20) DENV. The E protein homodimer is highlighted in yellow. c) The DENV E protein dimer colored by its domain, EDI: red, EDII: yellow and EDIII: blue. The fusion loop (orange) is circled in black. All structural figures in (a-c) were created using PyMol (Schrödinger LLC).

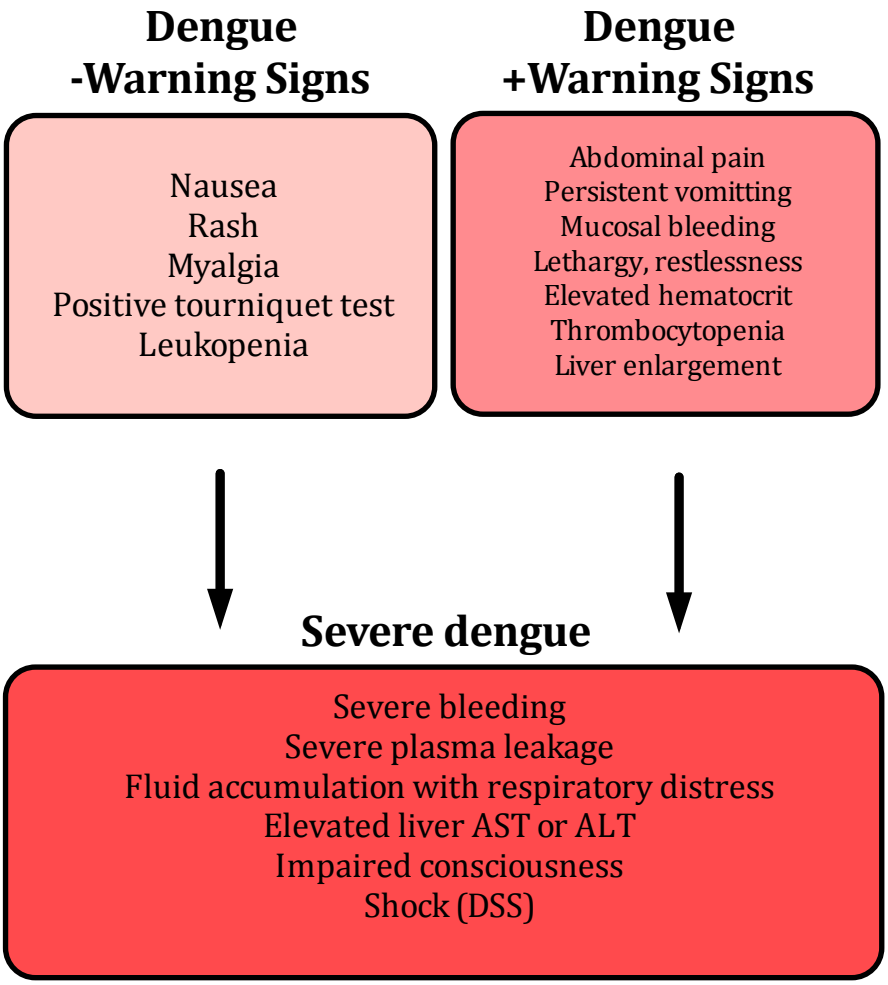


**Figure 2. Symptoms associated with dengue with or without warning signs and severe dengue.** Suggested dengue disease classification and clinical features associated with each, as per WHO (1).

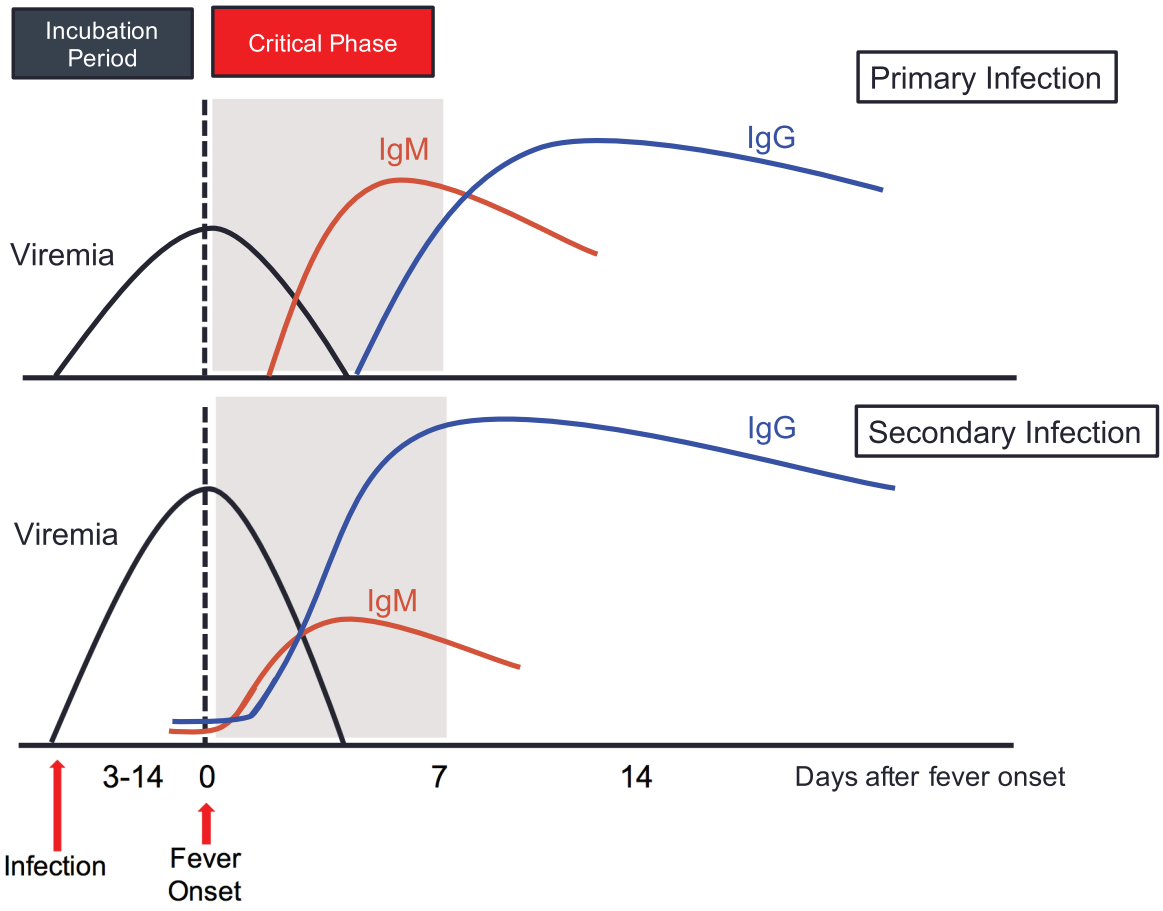


**Figure 3. Kinetics and relative magnitude of antibody responses after primary and secondary DENV infection.** Temporal overlap between rise in serum antibody levels and onset of clinical symptoms after primary or secondary DENV infection. Curves depict trends, are not drawn to scale, and are subject to variations. Adapted from a report published by WHO (1) and other studies (68, 72-74).

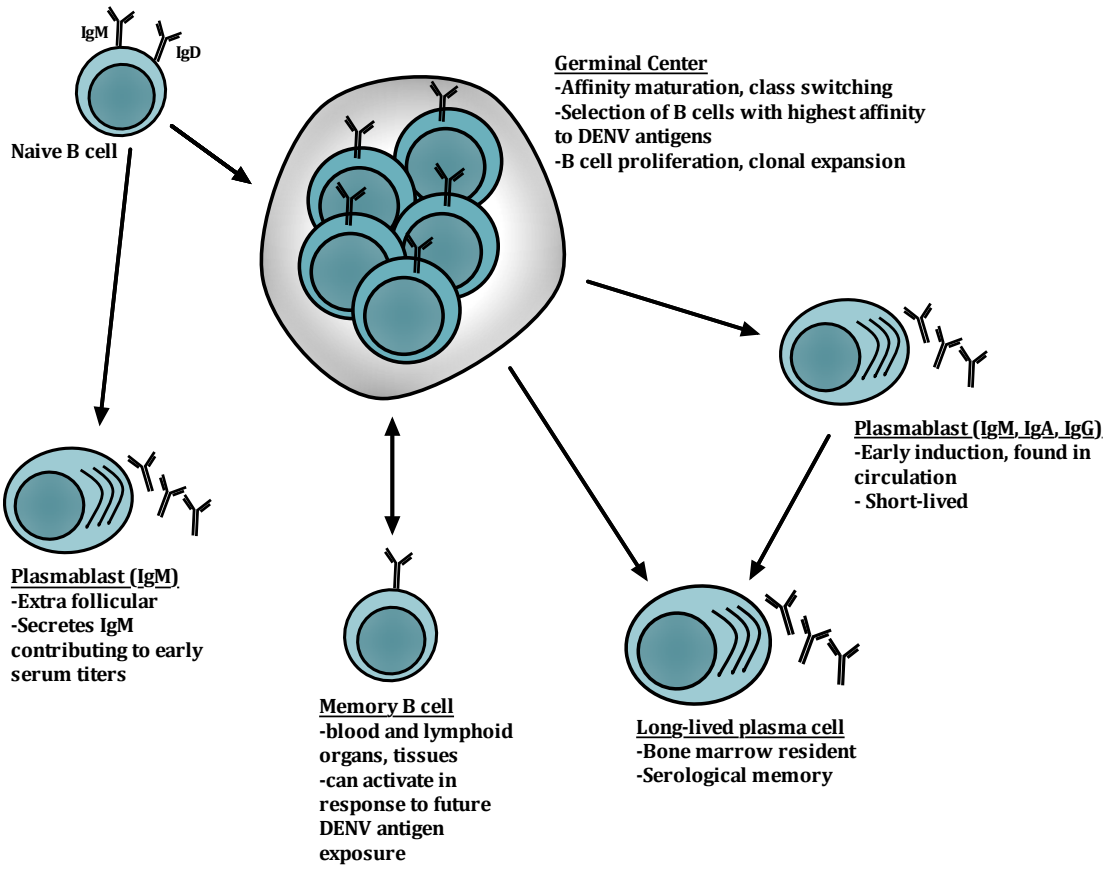


**Figure 4. B cell types in the dengue immune response.** Schematic illustration of cell subsets involved in the human B cell response to DENV infection.

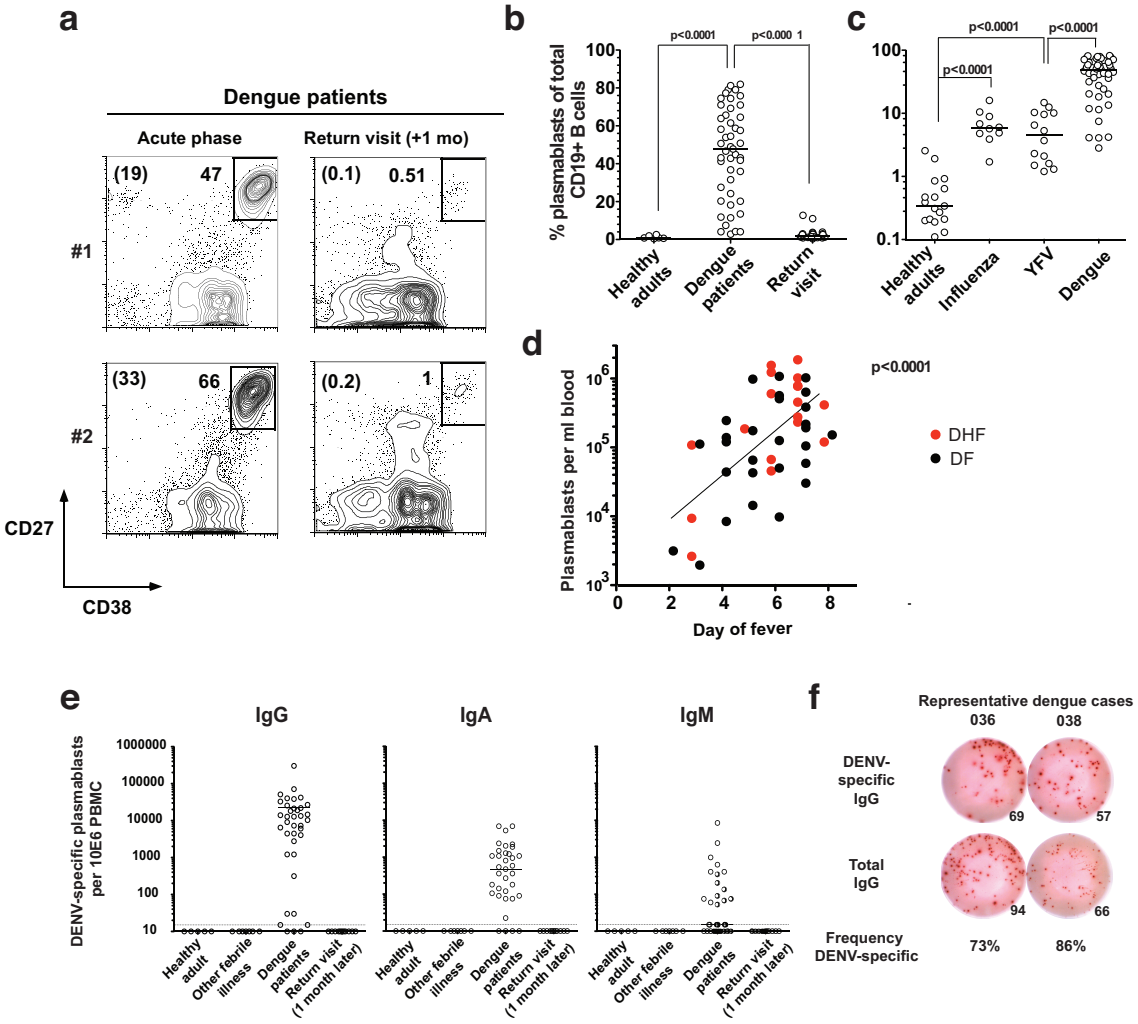


**Figure 5. Robust DENV-specific plasmablast response early after infection in secondary dengue patients.** a) Representative flow cytometric analysis of the plasmablast frequency in two patients at an acute vs. convalescent (1 month later) time point. The numbers in the plots represent the frequency of plasmablasts in the peripheral B cell compartment, defined as $CD3^-$ $CD20^{-/low}$ lymphocytes, while numbers in parentheses indicate percentage per all lymphocytes. b) Frequency of plasmablasts per total $CD19^+$ B cells in healthy adults, patients with ongoing dengue

virus infection, and dengue patients at 1 month after the acute sample. c) Comparison of plasmablast responses after DENV infection vs. vaccination with either the inactivated influenza virus vaccine or yellow YFV vaccine. Statistical analysis in parts b and c was done using an unpaired, two-tailed *t* test. d) Number of plasmablasts per ml blood as a function of number of days post fever onset. Spearman's correlation coefficient test revealed a significant correlation of $p<0.0001$. e) Number of DENV-specific IgG-, IgA-, or IgM-secreting plasmablasts per million PBMCs. f) Representative IgG-specific ELISPOT analysis of two dengue patients showing total and DENV-specific IgG, and frequency of DENV-specific plasmablasts per total IgG-secreting ASCs. This figure is a compilation of previously published data (68).

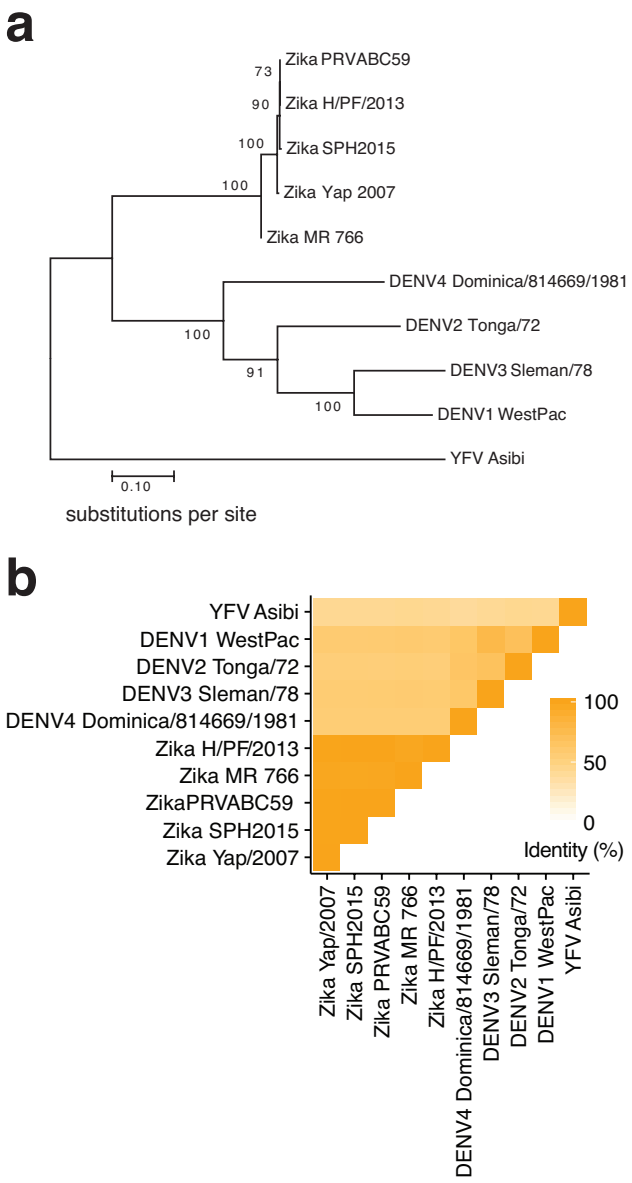


**Figure 6. ZIKV and DENV E proteins share considerable sequence identity.** a) Phylogenetic tree, showing relatedness based on E protein sequence, created using MEGA7 (173). The evolutionary history between the viruses was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (174). The percentage of trees in which the associated viral sequences clustered together is shown next to the branches. Branches are drawn to scale, with lengths measured in

the number of substitutions per site. b) Heatmap showing E protein sequence identity, generated with ggplot2 in R (175). Sequences were aligned in Geneious version 6.1(176). For (a) and (b), the ZIKV strains analyzed and their GenBank accession numbers are: PRVABC59: KU501215, MR766: AY632535, H/PF/2013: KJ776791, Yap/2007: EU545988 and SPH2015: KU321639. The DENV strains are DENV1 WestPac: U88535, DENV2 Tonga/72: AY744147.1, DENV3 Sleman/78: AY648961.1 and DENV4 Dominica/814669/1981: AF326573.1. In addition, the YFV strain Asibi: KF769016.1 was also included as an outgroup in the sequence analyses above.

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# Chapter 2: B cell responses during secondary dengue infection are dominated by highly cross-reactive, memory-derived plasmablasts

Lalita Priyamvada, Alice Cho, Nattawat Onlamoon, Nai-Ying Zheng, Min Huang, Yevgeniy Kovalenkov, Kulkanya Chokephaibulkit, Nasikarn Angkasekwinai, Kovit Pattanapanyasat, Rafi Ahmed, Patrick C. Wilson, Jens Wrammert

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**Abstract**

Dengue virus (DENV) infection results in the production of both type-specific and cross-neutralizing antibodies. While immunity to the infecting serotype is long-lived, heterotypic immunity wanes a few months after infection. Epidemiological studies link secondary heterotypic infections with more severe symptoms, and cross-reactive poorly neutralizing antibodies have been implicated in this increased disease severity. To understand the cellular and functional properties of the acute dengue B cell response and its role in protection and immunopathology, we characterized the plasmablast response in four secondary DENV2 patients. Dengue plasmablasts had high degrees of somatic hypermutation, with a clear preference for replacement mutations. Clonal expansions were also present in each donor, strongly supporting a memory origin for these acutely-induced cells. We generated 53 mAbs from sorted patient plasmablasts and found that DENV-reactive mAbs were largely envelope-specific and cross-neutralizing. Many more mAbs neutralized DENV than reacted to envelope protein, emphasizing the significance of virion-dependent B cell epitopes and the limitations of envelope protein-based antibody screening. A majority of DENV-reactive mAbs, irrespective of neutralization potency, enhanced infection by ADE. Interestingly, even though DENV2 was the infecting serotype in all four patients, several mAbs from two patients neutralized DENV1 more potently than DENV2. Further, half of all type-specific neutralizing mAbs were also DENV1-biased in binding. Taken together, these findings are reminiscent of original antigenic sin (OAS) given the patients had prior dengue exposures. These data describe the ongoing B cell response in

secondary patients, and may further our understanding of the impact of antibodies in dengue pathogenesis.

**Importance**

In addition to their role in protection, antibody responses have been hypothesized to contribute to the pathology of dengue. Recent studies characterizing memory B cell (MBC)-derived mAbs have provided valuable insight into the targets and functions of B cell responses generated after DENV exposure. However in case of secondary infections, such MBC-based approaches fail to distinguish acutely induced cells from the pre-existing MBC pool. Our characterization of plasmablasts and plasmablast-derived mAbs provides a focused analysis of B cell responses activated during ongoing infection. Additionally, our studies describe evidence of OAS in the acute phase dengue immune response, providing basis for future work examining the impact of OAS-phenotype antibodies on protective immunity and disease severity in secondary infections.

## Introduction

Dengue viruses (DENV) cause an estimated 390 million infections worldwide every year (1). With as many as 500,000 cases of severe dengue-related hospitalizations per year, dengue has emerged as one of the most critical arboviral diseases in the world today (2). There are four serotypes of dengue viruses (DENV1-4), and each can cause acute infection with a wide spectrum of symptoms (3). Clinical disease can range from self-limiting mild febrile illness, to hemorrhagic fever (DHF), to the fatal dengue shock syndrome (DSS) (3-5). Individuals infected with dengue generate serum antibody titers that provide long term protection against future homotypic infections (6). However in case of heterotypic infections, several sero-epidemiological studies suggest that prior DENV exposure and pre-existing antibody may be risk factors for severe disease (7-11). Furthermore, severe DENV infections typically evolve into DHF/DSS 3-7 days after fever onset (3), a time associated with a decline in viremia but a rise in serum antibody levels (12, 13). Consequently, in addition to its role in viral clearance, the humoral immune response has also been hypothesized to contribute to viral pathogenesis and immunopathology (14, 15).

Several hypotheses have been proposed over the past few decades to explain the increased disease severity associated with DHF and DSS cases. These include excessive T cell responses leading to elevated cytokine levels ("cytokine storm") as well as antibody dependent enhancement (ADE) (16-20). The latter implicates pre-existing sub-neutralizing, cross-reactive antibodies in increasing viral uptake, thereby enhancing DENV infection (21, 22). Of the studies that have

investigated the involvement of B cells in DENV infection, the majority focuses on serum antibody, or memory B cell (MBC) responses in dengue patients a few months to years after viral clearance. Such studies have shown that B cell responses elicited after infection are primarily directed to the structural proteins E and prM and are cross-reactive to multiple serotypes, with a minor proportion exhibiting serotype-specific activity (17, 23-25). While serotype-specific protection is believed to be long term, cross-neutralizing serum titers have been reported to peak a few weeks after infection and wane within a year (26).

The cellular aspects of the B cell response induced during infection remain less well characterized. We and other groups have shown that a rapid and massive expansion of plasmablasts occurs during the acute phase of human DENV infection (27-29). Plasmablasts can account for as many as 30% of all peripheral lymphocytes in patients a few days to a week post fever onset (27). This rapidly expanding B cell population, made up almost entirely of DENV-specific IgG secreting cells, peaks at a time associated with the onset of severe disease symptoms (27). Recently, two groups have investigated plasmablast responses during DENV infection by deriving monoclonal antibodies (mAbs) from patient antibody secreting cells (ASCs). These studies have shown that E is the primary target of acute phase B cell responses, and that a majority of dengue plasmablasts display varying degrees of cross-reactivity. However, questions regarding the origin and the functions of plasmablasts during the dengue immune response still remain.

Here we provide a detailed analysis of the plasmablast response induced during ongoing secondary DENV2 infection in four patients. The acute phase B cell repertoire contained highly affinity matured, antigenically selected plasmablasts. Clonal expansions were observed in each of the four donors. We generated a panel of 53 mAbs from sorted patient plasmablasts and found that the majority of DENV-reactive mAbs were E-specific. Antibodies that cross-reacted to more than one DENV serotype in both binding and neutralization dominated the mAb panel. A majority of DENV-reactive mAbs, irrespective of neutralization capacity, enhanced DENV infection of U937 cells indicating that the potential for ADE is not limited to weakly neutralizing antibodies. Interestingly, a large number of mAbs in the panel, including half of all mono-neutralizing mAbs, preferentially neutralized DENV1 suggesting that original antigenic sin (OAS) may play a role in the dengue immune response. These characterizations of plasmablast-derived mAbs give insight into the specificity and function of early antibody responses in dengue infection at a single cell level, and may further our understanding of the role of antibodies in the pathology of dengue.

## Materials and Methods

**Patient Cohort.** As part of a larger clinical study of dengue patients admitted at Siriraj Hospital in Bangkok, Thailand, four patients were selected for an in-depth analysis of the dengue plasmablast response. The serotype of ongoing infection was determined by serotype-specific RT-PCR as previously described (27, 30). Based on dengue-specific serum IgG titers and an IgM/IgG ratio lower than 1.7, all four patients were classified as having secondary infections (31). Patient details including age, sex, date of sampling, and information related to their clinical diagnosis are outlined in Table 1. These studies were approved by the institutional review boards at Siriraj Hospital and Emory University.

**Dengue virus and viral antigens.** Dengue virus (DENV1 WP, DENV2 Tonga/74, DENV3 Sleman/78 and DENV4 Dominica/84) were graciously gifted by Dr. Whitehead (NIH). Viral stocks were made by infecting vero cells (ATCC; CRL-1586) in Opti-Pro SF media (Invitrogen; 12309019). Purified PFA-inactivated DENV2 particles were purchased from Microbix Biosystems Inc. (DENV2 16681; EL-22-02). DENV1-4 recombinant envelope proteins were purchased from CTK Diagnostics (A2301: DENV1 VN/BID-V949/2007, A2302: DENV2 GWL39 IND-01, A2303: DENV3 US/BID-V1090/1998, A2304: DENV4 341750).

**Flow cytometry, PBMC isolation and sorting of plasmablasts.** Staining for analytical flow cytometry of plasmablasts was performed as described previously (27, 32, 33). Briefly, 300 µL of whole blood was stained with the antibodies CD19-FITC, CD38-PE, CD3-PerCP, CD20-PerCP and CD27-APC

followed by RBC lysis. The percentage of plasmablasts, defined as $CD19^{+}$ $CD3^{-}CD20^{-/low}$ $CD27^{high}$ $CD38^{high}$ cells, of total $CD19^{+}$ B cells was determined. Flow cytometry data were analyzed using FlowJo software. The remaining whole blood sample was processed to isolate PBMCs for use in ELISPOT assays and for plasmablast single cell sorting (32). The plasmablasts were single cell sorted into 96-well PCR plates containing RNase-inhibitor using FACSAria III and were frozen immediately on dry ice (32, 33).

**ELISPOT assay.** The numbers of DENV-specific and total IgG-, IgM-, and IgA-secreting plasmablasts were determined using an enzyme-linked immunosorbent spot (ELISPOT) assay as previously described (27, 32, 34). In brief, 96-well ELISPOT assay filter plates (Millipore; MAHA N4510) were coated overnight with either purified DENV2 virions (10 μg/mL) or goat anti-human Ig (Jackson ImmunoResearch; 109-005-064) diluted in PBS. PBMCs isolated from whole blood were washed thoroughly, added to the plates and serially diluted. After an overnight incubation at 37°C, biotinylated anti-human IgG (Invitrogen; H10015), anti-human IgA (Invitrogen; H14015), or anti-human IgM (Invitrogen; H15015) was added to the plates for 1.5 h at room temperature followed by avidin D-horseradish peroxidase conjugate (Vector Laboratories; A-2004) for 1 h. Finally, the plates were developed using 3-amino-9-ethyl-carbazole (AEC) substrate (Sigma; A5754). Developed ELISPOT plates were scanned and analyzed using an ELISPOT counter (CTL, Cellular Technologies Ltd.).

**Generation of monoclonal antibodies from plasmablasts.** The generation of mAbs from single cell sorted plasmablasts was performed as described

previously (32, 33, 35). Briefly, the Ig gene rearrangements of IgG heavy and kappa and lambda light chains of the sorted plasmablasts were identified and amplified by reverse transcription and PCR. Instead of the one-step RT-PCR described previously, cDNA was synthesized using random hexamers followed by PCR steps. The first round of PCR was performed using a cocktail of primers that cover all families of variable (V) and joining (J) genes, followed by a nested PCR to determine the sequence of the V and J genes of the heavy and light chains. VDJ/VJ regions were then amplified by a second PCR step, using highly specific primers containing restriction sites for subsequent cloning. The heavy and light chain sequences were cloned into separate plasmid vectors containing human constant gene and expressed in HEK293 cells. The mAbs were purified using affinity chromatography with sepharose-A beads (33).

**Analysis of clonality and somatic hypermutation of $V_H$ genes.** $V_H$ sequences generated from single cell sorted plasmablasts were analyzed to examine repertoire breadth. The IMGT database was used to identify the V and J gene families for all sequences. To determine frequency of clonal expansions, all plasmablast-derived $V_H$ sequences (including mAb $V_H$ sequences) were analyzed (n=29, 32, 33, 24 for Pt 31, Pt. 32, Pt. 33 and Pt 39 respectively). For somatic hypermutation analysis, the $V_H$ mutation frequency refers to the total number of silent and replacement mutations between and including FR1 and CDR3. Mutation frequencies of naïve (n=5), IgG memory (n=7) and IgG GC (n=8) B cells, and ASCs during influenza infection (n=6) were obtained from previously published data (36).

**ELISA.** For envelope ELISA, plates were coated overnight at 4°C with DENV1-4 recombinant envelope proteins at 0.25 μg/mL. Virion ELISA was performed with DENV2 purified virion at 1 μg/mL. Plates were washed with PBS with 0.5% Tween (PBS-T) and blocked with PBS containing 10% FCS and 0.2% Tween (PBS-T-FBS) for 1.5 h. Monoclonal antibodies generated from all four patients were serially diluted in PBS-T-FBS and added to the plates for 1 h. After incubating with a peroxidase-conjugated anti-human IgG antibody (Jackson ImmunoResearch; 109-036-098) for 1.5 h, the plates were developed using *o*-phenylenediamine substrate. For virus capture ELISA, plates were coated overnight at 4°C with a mouse anti-flavivirus monoclonal antibody 4G2 (Millipore; MAB10216) diluted 1/1000 in carbonate binding buffer. After blocking with PBS-T-FBS for 1 h, 50 μL of virus containing supernatant from infected vero cells was added to each well. Plates were incubated at room temperature for half an hour, washed 10 times with PBS-T-FBS and serially diluted mAbs were then added. The addition of the secondary antibody and developing steps were performed as described above. For all ELISA experiments, antibody concentrations were plotted versus their respective OD values at 490 nm. The minimum effective concentration for binding was determined as the concentration required for three times the signal as obtained by plain blocking buffer or an irrelevant mAb.

**Neutralization assay.** A focus-forming assay was used to determine the neutralization potential of mAbs or serum. Serially diluted mAbs/serum were incubated with 100 ffu DENV1-4 at 37°C for 1 h. The mixture was added on to vero cell monolayers for 1 h at 37°C. A 2% methylcellulose (Sigma; M0512-2506)

overlay was added to the infected cells for 72 hours at 37°C. After the 3 day incubation, cells were fixed with a 1:1 mixture of methanol and acetone, and processed for staining. Infected foci were stained using 4G2 for 2 h followed by HRP-linked anti-mouse IgG (Cell Signaling; 7076S) for 1 h. Foci were developed using TrueBlue Peroxidase substrate (KPL; 50-78-02) and imaged and counted on an ELISPOT reader.

**Antibody dependent enhancement assay.** A flow-cytometry based assay was used to determine the infection enhancement capacity of mAbs. First, 5000 ffu of DENV1-4 was mixed with an equal volume of mAb at 1 μg/mL for 1 h at 37°C. The virus and mAb mixture was then added to a 96 well plate containing U937 cells (ATCC; CRL-1593.2) at 20,000 cells/well. After a 24 h incubation at 37°C, infected cells were fixed with Fix/Perm Solution (BD; 51-2090KZ), permeabilized with Perm/Wash Buffer (BD; 51-2091KZ) and stained for 1 h using 4G2 followed by anti-mouse IgG Alexa Fluor 488 (Life Technologies; A11029) for 1 h. The number of infected cells was determined using flow cytometry, and fold enhancement was calculated as the relative percent infection with virus + dengue mAb treatment compared to percent infection with virus alone.

## Results

**Potent plasmablast induction in patients with secondary DENV2 infection.** To characterize the dengue plasmablast response in depth, four patients with confirmed DENV2 infection were sampled during acute disease. All patients were clinically diagnosed with DHF based on WHO definitions (37) and were classified as having secondary infections based on serum IgM/IgG ratios (Table 1). Blood samples were collected 3-6 days post fever onset to determine the magnitude of plasmablast responses. Plasmablasts represented a large percentage of the peripheral B cell population in all four donors, ranging between 51%-80% of all peripheral B cells (Fig. 1A). The cells were primarily producing IgG (data not shown) and a large proportion of the total IgG response was DENV-specific in all patients (Fig. 1B and C). These observations related to the timing, magnitude, isotype usage and specificity of plasmablast responses in dengue patients were all consistent with our previous findings (27). Additionally, serum neutralization assays revealed potent titers towards DENV2, mimicking the ELISPOT data showing high DENV2-reactivity (Fig. 1C and D). Interestingly, serum DENV1 titers in all four patients were also high at this time point.

**A majority of patient plasmablast-derived mAbs were DENV-reactive.** To investigate the role of plasmablasts and the antibodies they produce during ongoing DENV infection, we generated and analyzed antibodies produced by this effector B cell population. We single cell sorted plasmablasts into 96-well microtiter plates and PCR amplified their Ig heavy and light chain variable region as we have previously reported (32, 38). These VDJ/VJ sequences were

subsequently cloned into IgG expression vectors and expressed as mAbs. We generated a panel of 53 mAbs, with 10-15 mAbs from each patient (Table 1). For the initial mAb screen, we performed indirect ELISAs using formalin-inactivated DENV2 whole virions, the same antigen used in the ELISPOT assays. A majority of the mAbs reacted to DENV2 virion, confirming that the mAbs generated were largely DENV-specific (Fig. 1E).

**The plasmablast response during secondary DENV infection is highly affinity matured and contains clonal expansions.** Although plasmablasts have previously been shown to dominate the B cell response during ongoing dengue infection, the origin of these antibody-secreting cells remains unclear. We hypothesized that the plasmablast response during secondary infection is mainly MBC-derived given that a majority of these acute-phase cells are class-switched, secrete IgG and appear in circulation rapidly after fever onset. To test the same, we compared variable gene somatic hypermutation (SHM) frequencies in our dengue mAb panel with previously published data from other B cell populations (36). We found that plasmablasts from dengue patients had high SHM frequencies, with per patient $V_H$ mutation averages ranging between 14.5 and 21.7 (Fig. 2A). These levels were comparable to those seen in influenza infection (Fig. 2A) and vaccination (32). The number of $V_H$ mutations in dengue patient plasmablasts was significantly higher than mutations in GC $IgG^+$ B cells, and higher, though not statistically significant, than $IgG^+$ memory B cells (Fig. 2A). The levels of SHM were comparable between the four dengue patients, ranging from 5-39 mutations per sequence and averaging 18.1 mutations overall (Fig. 2B, Table 2).

Additionally, we examined replacement (R) vs. silent (S) $V_H$ mutations frequencies in all the mAb sequences in our panel for signs of selection. A preference for R mutations in the CDRs (R/S>2.9) suggests antigenic selection. All four patients had an average CDR R/S ratio >2.9 (Fig. 2C) suggesting that the plasmablasts making the mAbs were antigenically selected as a response to DENV infection. We also analyzed the VDJ/VJ gene usage of the plasmablast heavy and light chain sequences for the presence of clonal expansions. An analysis of plasmablast clonality is shown in Fig. 2D. Clonal relatedness between the plasmablast sequences from each patient averaged 23% overall. In three of the four patients, clonal expansions represented more than 20% of all sequences analyzed, reaching as high as 28% in Pt. 32.

**Highly cross-reactive, E-specific mAbs dominate the secondary dengue plasmablast response.** Previous studies have shown that the viral structural protein E is a major antigenic target for human humoral responses to DENV (17, 23, 25, 29). We tested the mAb panel for reactivity to E by performing ELISAs with DENV1-4 recombinant envelope (rE) proteins. We found that a majority of mAbs bound to rE (31/53) of at least one DENV serotype and all rE-specific mAbs were either partially (2-3 serotypes) or fully (4 serotypes) cross-reactive (Fig 3). No serotype-specific rE binding was observed. Interestingly, we noted striking differences in rE binding patterns between patients. While a majority of Pt. 31 and Pt. 39 mAbs reacted strongly to rE, most mAbs from Pt. 32 and Pt. 33 failed to bind to rE of any serotype (Fig 3A). The mAbs that did not bind to rE were tested for DENV-reactivity by Western blot using DENV2 lysate. An

additional 6 mAbs were found to be E-specific (data not shown), making E the target for 70% of the mAbs in the panel.

The mAbs were tested for neutralization against DENV1-4 by FFA (Fig. 3 and Fig 4A). A majority of mAbs in the panel (46/53) exhibited neutralizing activity *in vitro*, of which 34 (74%) were either partially or fully cross-neutralizing. The cross-neutralizing mAbs from each patient displayed a range of phenotypes, varying in the permutations of serotypes neutralized as well as in the potency of neutralization of each serotype. Serotype-specific neutralizing mAbs represented 23% of the overall mAb panel. Most of these mono-neutralizing mAbs reacted either to DENV1 (5/12) or DENV2 (6/12) (Fig 3A, Fig 4 B). Overall, DENV4 was the least neutralized serotype, as only 20 mAbs showed DENV4 neutralizing activity. As with binding trends, the neutralization patterns of mAbs also differed between patients. Whereas DENV2 was neutralized most potently by a majority of Pt. 31 and Pt. 39 mAbs, DENV1 was the preferred serotype of neutralization for most Pt. 32 and Pt. 33 mAbs (Fig 3, 4A).

For most mAbs, neutralization patterns against DENV1-4 did not mirror rE binding activity. For instance, 10/12 mAbs from Pt. 31 had comparable affinities to the four DENV rE proteins by ELISA, but displayed varied neutralization activities against DENV1-4 by FFA (Fig 3A). While 26 mAbs in the panel fully cross-reacted to DENV1-4 rE, only 6 of these also neutralized all four DENV serotypes (Fig. 3A). A large number of these fully cross-reactive mAbs were instead shown to neutralize only 2-3 serotypes *in vitro*. Additionally, 15 mAbs

that did not react to rE by ELISA exhibited neutralizing activity by FFA (Fig. 3A, 4B).

In addition to their neutralizing titers we also tested the ability of the mAbs to enhance DENV infection using a human histiocytic lymphoma cell line, U937. These monocytic cells express Fcγ receptors (FcγR) and show significantly higher levels of DENV infection in the presence of enhancing antibodies (39). The mAbs were screened for ADE potential against all four DENV serotypes at a concentration of 1 μg/mL. We found that 45/53 mAbs either moderately (5-25 fold) or potently (>25 fold) enhanced DENV infection (Table 3). A majority of mAbs that tested positive for DENV binding and/or neutralization also exhibited ADE activity. Cross-reactive neutralizing mAbs on average caused greater fold enhancement of infection than mono-neutralizing mAbs. However, a majority of mono-neutralizing mAbs also enhanced DENV infection. In fact, the mAb 31.3F03, which neutralized only DENV2 *in vitro*, exhibited the highest cross-serotypic enhancing activity in the entire mAb panel.

**Evidence of OAS in secondary dengue immune response.** As noted previously, a large number of mAbs in the panel neutralized DENV1 more potently than DENV2 (Fig. 4A). This DENV1-biased phenotype was primarily observed in Pt. 32 and Pt. 33 mAbs. The neutralization bias towards DENV1 for Pt. 32 and Pt. 33 was also reflected at the serum level (Fig. 1D). We also noticed that a majority of DENV1-biased mAbs failed to react to any of the four DENV serotypes by rE ELISA. Conversely, the mAbs that neutralized DENV2 more potently mostly exhibited binding activity to all DENV rE proteins (Fig 3, 4B).

These distinct phenotypes in binding and recognition of rE were also observed in serotype-specific mAbs. Besides 31.3G04 which efficiently neutralized DENV4, all mono-neutralizing mAbs clustered as either DENV1- or DENV2-specific for neutralization. Notably, the 5 DENV1-specific mAbs exhibited stronger neutralizing activity against their preferred serotype (median $FRNT_{50}$ = 0.16 μg/mL) than DENV2-specific mAbs (median $FRNT_{50}$ = 1.2 μg/mL) even though DENV2 was the infecting serotype in all four patients. While the DENV2-specific mAbs bound to all 4 rE proteins, all DENV1-specific mAbs tested negative for rE binding. We suspected that this lack of binding could be due to the absence of critical conformational epitopes in the rE proteins, and therefore attempted a capture ELISA using unfixed, intact virus to probe for binding activity. The capture ELISA confirmed the fully cross-reactive binding phenotype of DENV2-specific mAbs. More interestingly however, it revealed that the mAbs that primarily neutralized DENV1 also showed a clear preference to DENV1 in binding. Low levels of binding to DENV2 were also recorded for the DENV1-specific mAbs, potentially explaining why the plasmablasts making these mAbs were activated in response to the ongoing DENV2 infection.

## Discussion

Until recently, B cell responses in dengue patients were largely studied after convalescence, months to years after the resolution of infection. These studies provide valuable insight into the epitopes and varied binding, neutralization and ADE phenotypes of dengue-specific antibodies. However, they fail to capture plasmablasts, the B cell subset responsible for the peak in serum antibody observed during an ongoing dengue infection (27). In an effort to understand the origin and functional properties of dengue plasmablasts, we isolated plasmablasts from four Thai patients experiencing secondary DHF-grade DENV2 infection and generated a panel of mAbs by single cell PCR and cloning of plasmablast Ig genes. Our key findings were the following: (i) Secondary dengue infection induces highly affinity matured, antigenically selected plasmablasts that exhibit several clonal expansions. (ii) The dengue plasmablast response is predominantly E-specific and cross-reactive with regard to both binding and neutralization. (iii) Most dengue-reactive mAbs were capable of enhancing infection *in vitro*, irrespective of neutralization potency or degree of cross-reactivity. (iv) A large number of mAbs demonstrated preferential neutralization of a heterologous serotype, reminiscent of OAS and strongly suggesting an MBC origin for the secondary dengue plasmablast response.

All four patients mounted potent DENV-specific plasmablast responses, echoing the findings of our previous study in an independent Thai cohort (27). The plasmablasts were primarily secreting IgG, merely a few days after fever onset. The magnitude and rapidity of antibody production suggested that these

plasmablasts may have been induced as a recall response. To gain insight into the origin of the dengue plasmablast response in our patient cohort, we analyzed mAb SHM frequencies. We found that dengue plasmablasts had a high SHM frequency, comparable to levels seen after annual influenza vaccination (32) or infection (38). SHM levels in the four dengue patients were higher than those seen after true primary infection or vaccination, such as in acute infection HIV (AHI) patients or primary vaccinia vaccinees (38, 40), as well as SHM rates in memory B cells. The high levels of somatic mutation suggest that dengue plasmablasts may be reactivated MBCs. A preference for replacement mutations in the CDRs of plasmablast-derived mAbs, together with the high frequency of antigen-specific antibodies indicates that the ASCs were functionally selected. As the plasmablasts used for mAb synthesis were not enriched based on antigen specificity, but rather collected based on *in vivo* activation, we were able to examine the repertoire of the plasmablast response during the ongoing infection with minimal bias. An analysis of the plasmablast immunoglobulin repertoire revealed clonal expansions in all four donors. Surprisingly, 23% of all dengue patient-derived Ig sequences analyzed were clonal. The levels of clonal relatedness, although lower than those observed after influenza vaccination (32), are notably higher than those in naïve B cells or MBCs (41). The presence of clonal expansions in all four patients also further supports an MBC-origin of the plasmablast responses.

In the past few years, several groups have generated panels of human mAbs from MBCs of dengue-infected patients and shown that multiserotype-reactive mAbs dominate the dengue immune response (17, 23-25, 42). The surface glycoprotein

E has been described as an important target in the human immune response to DENV infection (23, 25, 42, 43). More recently, two groups derived mAbs from dengue patient ASCs and demonstrated that E contains major epitopes for antibody binding and viral neutralization during ongoing infection (29, 44). As expected, a majority of mAbs (31/53) in our panel bound to rE. All rE reactive mAbs displayed cross-reactivity to two or more DENV serotypes, and a large number of mAbs also neutralized more than one DENV serotype *in vitro*. Given that all patients were previously exposed to DENV and that the four DENV serotypes share high genomic sequence homology (5), we were not surprised by the large number of fully cross-reactive mAbs in our panel. A recently published report examining secondary responses in dengue vaccinees ably demonstrates the importance of cross-reactive antibodies and the impact of their depletion on serum neutralization and binding titers (45). Our data showing the large abundance of cross-reactive mAbs during natural dengue infection reiterates the significance of broadly neutralizing responses to vaccine design efforts.

For a majority of mAbs, the patterns of binding and neutralization did not fully overlap. Of the 26 mAbs that fully cross-reacted to all four DENV rE proteins, 6 mAbs were serotype-specific for neutralization. The remaining 20 neutralized 2-3 serotypes by FFA. This data suggests that the recognition of conserved epitopes by antibodies is not sufficient for effective neutralization of multiple DENV serotypes. Conversely, several mAbs that were DENV-specific by capture virus ELISA and/or neutralized at least one DENV serotype failed to react to rE. This could be explained by potential antigenic differences between the laboratory-adapted strains used in our *in vitro* assays and the clinical strains the

patients were infected with at the time of the study. Another possibility is the absence of critical conformational epitopes in rE, that are found only in the context of the whole intact virion. Recent studies have shown that human mAbs with potent DENV neutralization ability can recognize conformational epitopes positioned on the E dimer interface (44, 46). These mAbs do not bind to monomeric rE proteins as the epitopes they recognize are unique to the E dimer. It is possible that the mAbs in our panel that do not bind rE exhibit similar conformation-sensitivity or bind to epitopes expressed only on an intact virion.

An alternative hypothesis is that some of the mAbs in the panel are actually prM-specific, or in the case of non-neutralizing mAbs, NS1-specific. Previous studies have shown the abundance of prM- and NS1-reactive memory B cells in convalescent dengue patients (17, 23-25). Western blots using DENV2 lysate revealed that none of the mAbs had reactivity to these proteins (data not shown). The absence of binding to NS1 and prM in our assays could be due to antigen-based limitations, and therefore the possibility that some of these mAbs bind non-E epitopes cannot be completely ruled out at this time. Future binding and epitope mapping experiments testing the rE non-reactive mAbs will be useful in determining where these antibodies bind.

While all four patients were diagnosed with DENV2 at the time of the study, the neutralization patterns of the mAbs generated varied greatly between patients. A large proportion of Pt. 32 and Pt. 33 mAbs neutralized DENV1 more potently than DENV2, echoing the DENV1 bias also observed at the serum level for these two patients. Given all patients were experiencing secondary infection, we

suspected that the neutralization bias towards DENV1 in the two patients could be due to a previous DENV1 exposure. Screening the DENV1 mono-neutralizing mAbs by captured virus ELISA revealed that these mAbs had a clear preference for DENV1 in binding in addition to neutralization. The low levels of binding detected towards DENV2 validated our hypothesis that these plasmablasts resulted from the reactivation of MBCs from a past DENV1 exposure, rather than as a part of a naïve response to the current DENV2 infection. OAS has been well described for antibody responses to repeated influenza exposures (47-50), and a few reports have demonstrated this phenomenon in the secondary dengue B cell response as well (29, 51, 52). Our data strongly suggests that OAS may play a role in the secondary dengue immune response, and result in decreased binding affinity and neutralization potency against the infecting serotype. However the physiological relevance of OAS and its impact on protective B cell responses in dengue needs to be examined further, both in animal models and in humans.

In case of patients with a history of DENV exposures, epidemiological data suggest that progression to the more severe forms of disease is often associated with secondary heterotypic infection (22, 53, 54). ADE is one of several hypotheses proposed to explain his observed increase in dengue disease severity (53, 55). To address whether plasmablast-derived antibodies were capable of infection enhancement, we determined the fold increase in DENV infection of FcγR-bearing cells in the presence of the mAbs in our panel. With the exception of 32.2F04 and 39.3G03, every DENV-reactive mAb demonstrated enhancing ability *in vitro*. Regardless of the degree of cross-reactivity or potency of neutralization, a majority of mAbs increased DENV infection at 1 μg/ml.

Although these mAbs had the capacity to enhance DENV infection, the likelihood that plasmablast responses contribute to ADE during the current infection is low. This is because viremia in dengue patients starts subsiding before the peak in B cell responses (12, 13, 53, 56). The generation of plasmablast responses with ADE potential is likely more critical for future dengue exposures, as enhancing antibodies may already be present in serum at the time of viral infection.

In conclusion, we have shown that the B cell response rapidly generated after secondary DENV infections contains plasmablasts with memory origin. The plasmablasts induced are highly affinity matured and produce antibodies that are largely cross-reactive, E-specific and capable of potent DENV neutralization *in vitro*. However in addition to the cross-reactive mAbs, we report the presence of several type-specific neutralizing mAbs, including a subset that more potently neutralized a heterotypic strain than the infecting serotype. Varying degrees of cross-reactivity and neutralization were observed across the panel, showcasing the vast heterogeneity in the plasmablast response of secondary dengue patients. Future epitope mapping experiments with the most potently neutralizing mAbs may reveal important targets for prophylactic and therapeutic interventions. Moreover, focused analyses of the DENV1 vs. DENV2 mono-neutralizing mAbs may provide valuable information about the different epitopes recognized by these mAbs, and how they impact DENV serotype specificity and neutralization. Additionally, a relatively less explored yet interesting avenue is the comparison of primary and secondary plasmablast responses. A study comparing MBC-derived mAbs from primary and secondary infected patients suggested notable differences in cross-reactivity, avidity,

neutralization potency and mechanism of neutralization between primary and secondary infection (42). Future clinical studies involving comparisons of secondary vs. primary cases or patients from non-endemic areas may provide added insight into the biology of plasmablast responses and their role during the acute dengue immune response.

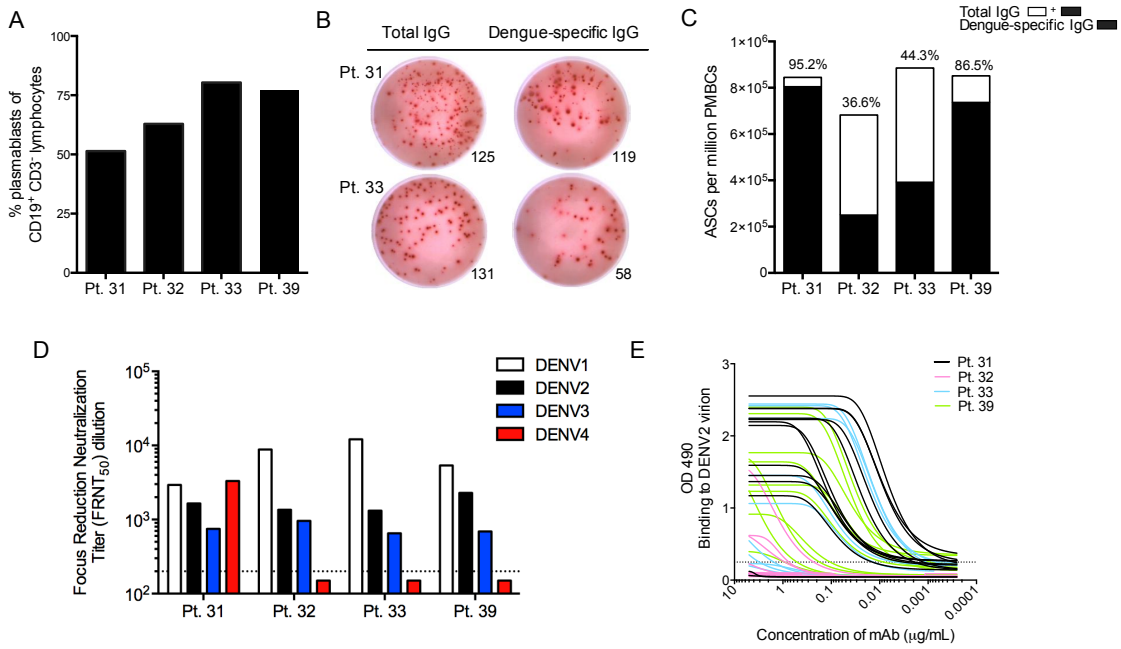


**Figure 1. Generation of mAbs from plasmablasts of patients with secondary DENV2 infection.** (A) The percentage of plasmablasts (defined as $CD19^{+}CD3^{-}CD27^{hi}CD38^{hi}$ lymphocytes) of total $CD19^{+}$ B cells determined by flow cytometry. (B) Representative ELISPOT analysis showing total and DENV-specific IgG secreting cells for patient (Pt.) 31 and Pt. 33. Each well shown contains 222 PBMCs. Numbers below each well indicate spot count. (C) Total and dengue-specific IgG secreting cells/ million PBMCs for all patients. (D) Serum neutralization titers were determined by focus forming assay (FFA) and focus reduction neutralization titer ($FRNT_{50}$) values are shown. FFAs were performed on serum samples collected the same day the plasmablasts were sorted for mAb synthesis. Values are from two or more independent experiments, with the mean $FRNT_{50}$ plotted. The dotted line represents the initial dilution factor of serum

tested (1:200). Each serum sample was tested against all four DENV serotypes in FFA: DENV1 (white), DENV2 (black), DENV3 (blue) and DENV4 (red). (E) Antibodies were tested for binding using the same antigen as the ELISPOT assay.

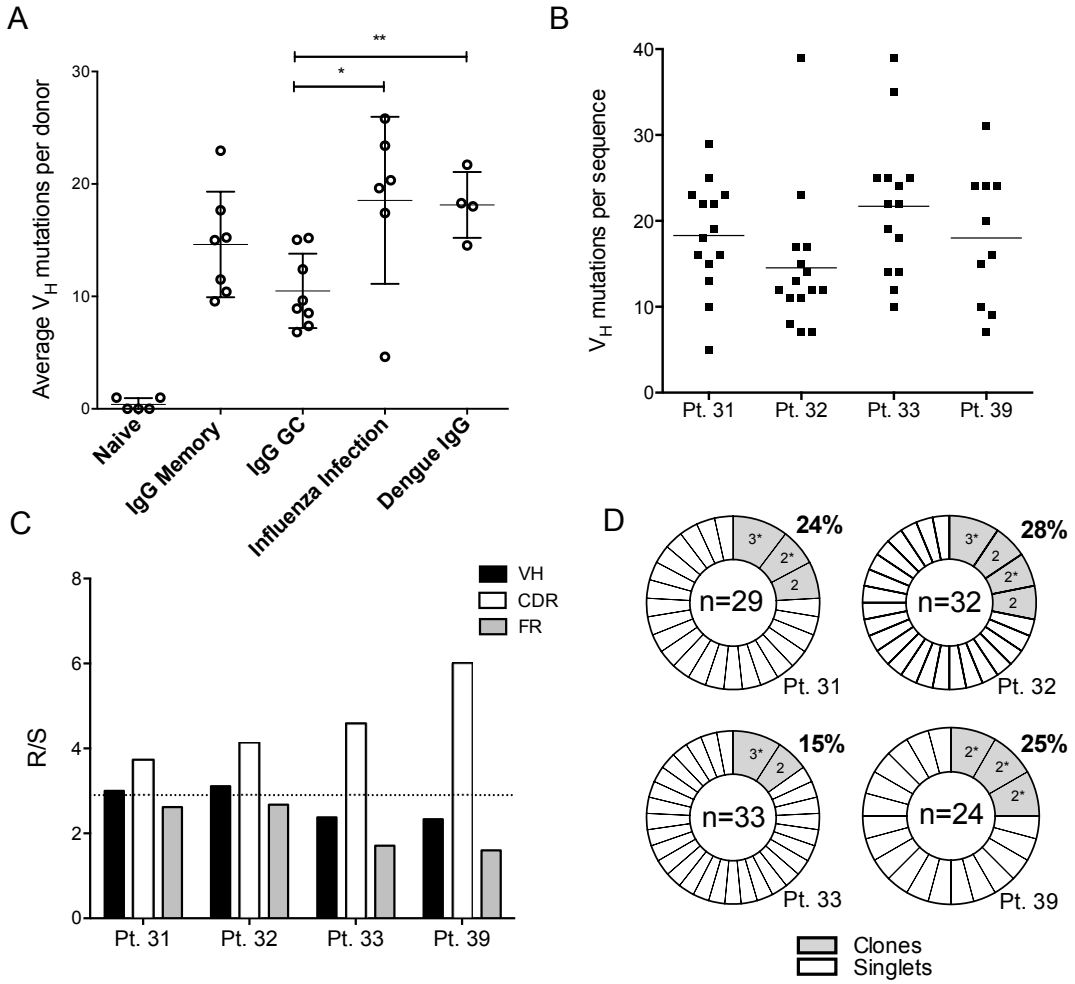


**Figure 2. Plasmablasts induced during secondary DENV infection are highly affinity matured and are clonally related.** (A) $V_H$ mutation frequency in the dengue patient cohort compared to historical data. Each circle represents the average number of $V_H$ nucleotide mutations per donor. Somatic hypermutation frequencies in naive, memory, germinal center and influenza-specific peripheral B cells were derived from previously published data (36). Statistical analyses were performed using an unpaired, two-tailed t test. * $p < 0.05$, ** $p < 0.005$ (B) The range of $V_H$ mutation frequencies in all four dengue patients. Each square represents the number of $V_H$ mutations per mAb sequence. (C) Replacement (R) versus silent (S) mutation ratios in the entire $V_H$ gene (black), CDRs (white) and

framework regions (gray). Ratios based on average number of R and S mutations for all mAb sequences per patient. A ratio above 2.9 (dotted line) suggests antigenic selection. (D) Clonality in plasmablast-derived heavy chain sequences for all patients. White sections indicate singlets while gray sections represent clones. The number at the center of each pie chart denotes the total number of heavy chain sequences analyzed (mAb sequences + unpaired $V_H$ sequences that were not pursued for mAb synthesis). The asterisk indicates a clonal group for which a mAb exists in our panel. The percentage of clonal sequences is also shown.

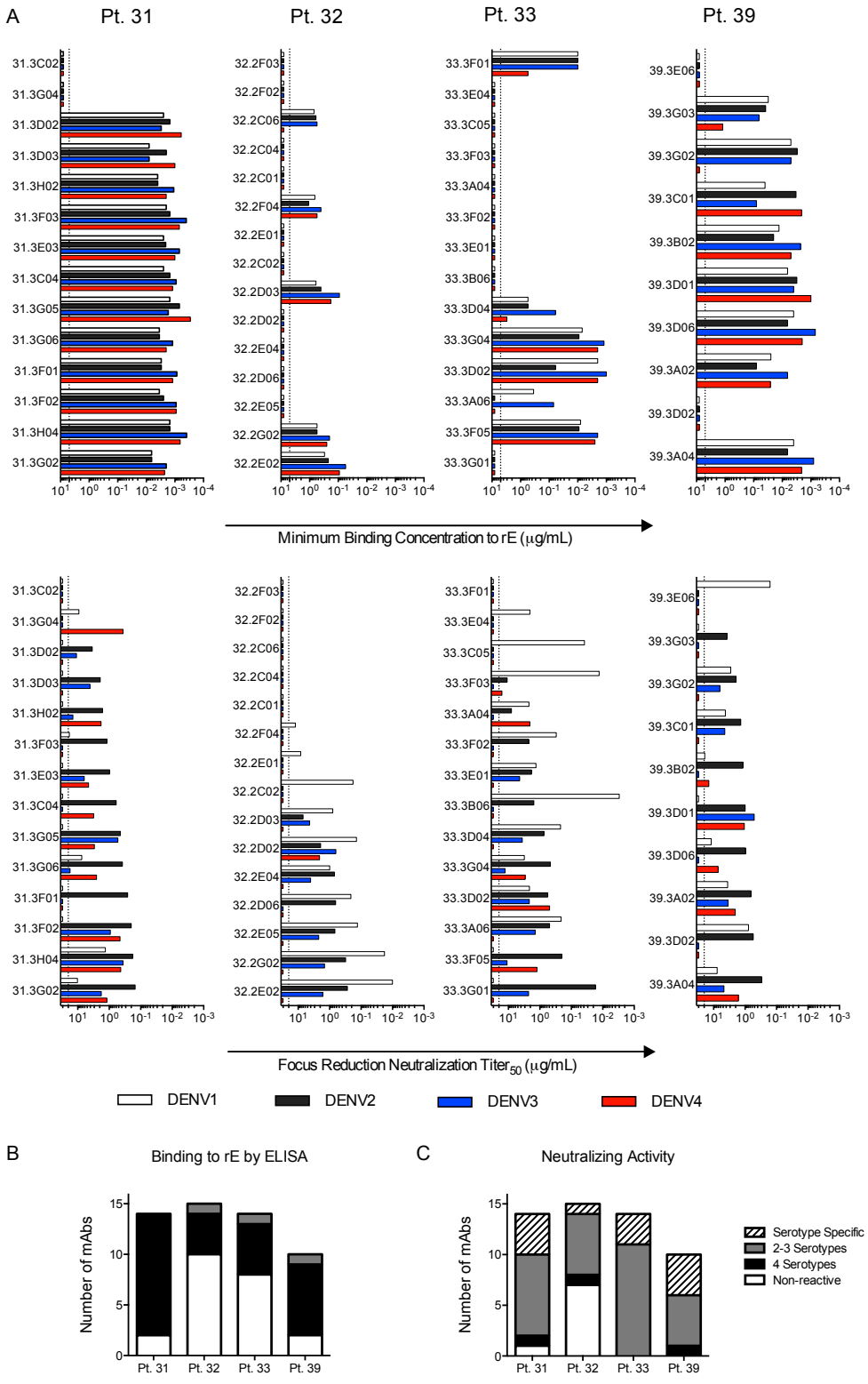


**Figure 3. Patient plasmablast-derived mAbs display highly heterogeneous binding and neutralization activities.** (A) Each mAb was tested against all four dengue serotypes for binding and neutralization activity: DENV 1 (white),

DENV2 (black), DENV3 (blue) and DENV4 (red). To test binding, ELISAs were performed with recombinant envelope (rE) proteins from DENV1-4. Values plotted represent the minimum concentration required for three times the background signal with plain blocking buffer. Neutralization activity was determined by FFA and focus reduction neutralization titer ($FRNT_{50}$) values are shown. FFAs were performed with duplicates in two or more independent experiments, and the mean value is plotted. The dotted lines represent the maximum concentration of mAb tested in ELISA: 5 μg/mL and FFA: 20 μg/mL. (B) Graph summarizing rE binding patterns of mAbs as determined by ELISA. Each bar represents one patient and sections within the bar indicate extent of cross reactivity: serotype specific (shaded), 2-3 serotypes (gray), fully cross-reactive (black) and non-reactive (white). (C) Summary graph depicting DENV neutralization patterns of mAbs as determined by FFA. $FRNT_{50}$ values ≤5 μg/mL were considered a positive result for neutralization. Bars color coded as in (B).

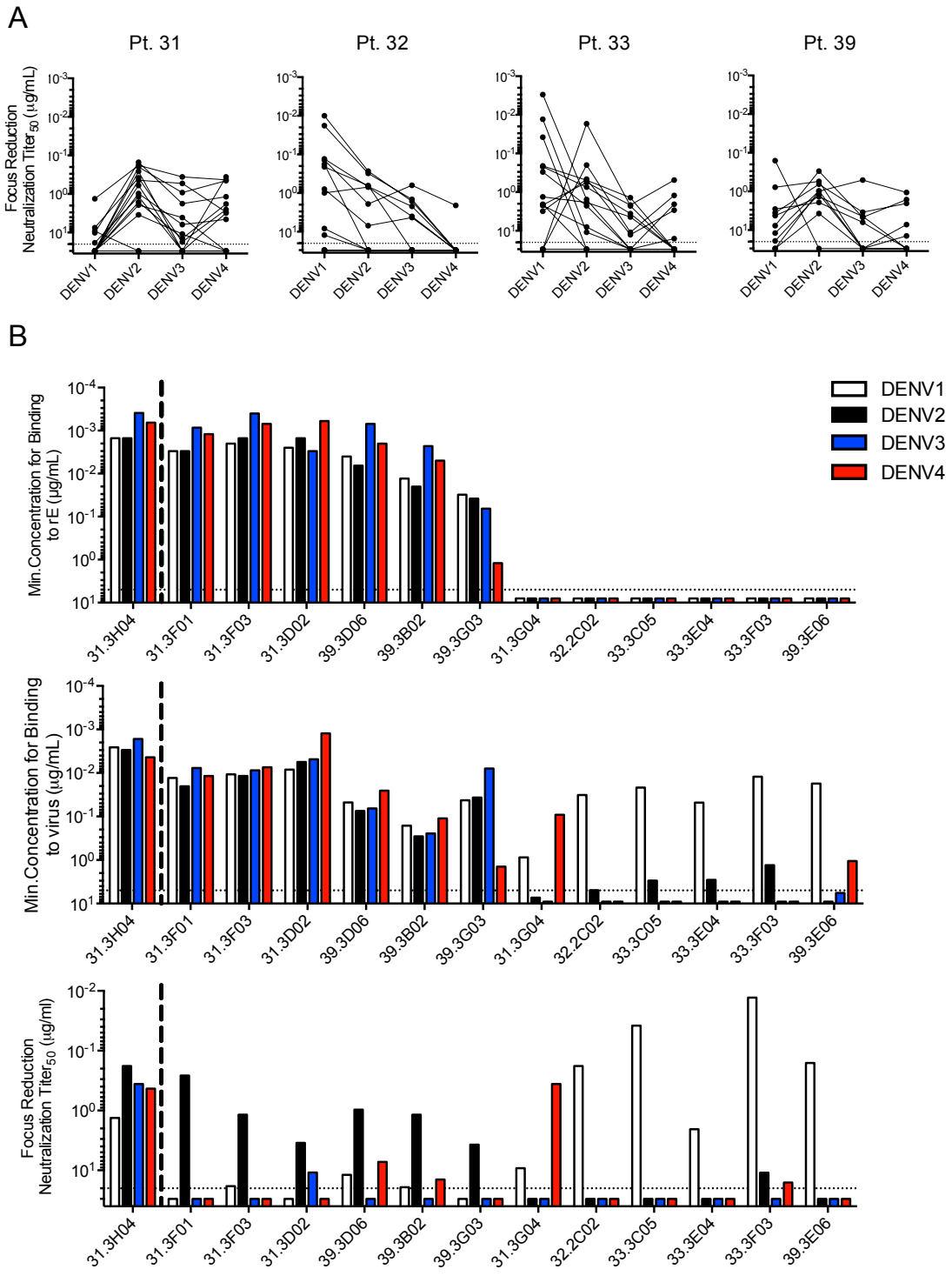


**Figure 4. Secondary DENV2 infection induced the activation of DENV1-specific plasmablasts in patients.** (A) Graphs depicting mAb focus reducation neutralization titer ($FRNT_{50}$) values against DENV1-4, as determined by FFA. Each circle represents one mAb and the solid lines connect the same mAb across

serotypes. The dotted line represents the highest concentration of mAbs tested in the FFA: 20 μg/ml. Antibodies below the dotted line failed to neutralize virus by 50% at 20 μg/mL. (B) Comparison of binding vs. neutralization activities of mono-neutralizing mAbs. Binding was tested by rE ELISA and capture virus ELISA. 31.3H04, shown to the left of the dotted line, is provided as a contrasting example of a mAb with full cross-reactivity. Each mAb was tested against all four DENV serotypes in ELISA and FFA: DENV1 (white), DENV2 (black), DENV3 (blue) and DENV4 (red).

TABLE 1. Patient information

| Patient | Age | Gender | Clinical Diagnosis[1] | Dengue Serotype[2] | Fever Day of Sample[3] | mAbs per Patient |
|---|---|---|---|---|---|---|
| 31 | 58 | Female | DHF | 2 | 4 | 14 |
| 32 | 37 | Male | DHF | 2 | 3 | 15 |
| 33 | 14 | Male | DHF | 2 | 5 | 14 |
| 39 | 19 | Male | DHF | 2 | 6 | 10 |

1. Final clinical diagnosis based on available clinical data according to WHO definitions.
DHF = Dengue Hemorrhagic Fever
2. Dengue serotype determined by RT-PCR.
3. Number of days post fever onset at which blood was collected.

TABLE 2. Heavy and light chain characteristics of dengue patient-derived monoclonal antibodies

| Pt. | Mab | Heavy Chain | | CDR3 Length | # Mutations | S/R | Light Chain | | | CDR3 Length | # Mutations | S/R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | V gene | J gene | (aa) | | | K/L | V gene | J gene | (aa) | | |
| 31 | 3C02 | 1-69 | 4 | 20 | 10 | 4/6 | Lambda | 2-23 | 2 | 10 | 7 | 2/5 |
| | 3C04 | 1-2 | 4 | 16 | 16 | 3/13 | Kappa | 3-20 | 4 | 9 | 11 | 5/6 |
| | 3D02 | 3-23 | 4 | 11 | 23 | 6/17 | Kappa | 3-20 | 3 | 9 | 14 | 5/9 |
| | 3D03 | 4-4 | 3 | 21 | 5 | 0/5 | Kappa | 1-5 | 2 | 10 | 7 | 3/4 |
| | 3E03 | 3-43 | 4 | 12 | 19 | 5/14 | Lambda | 7-43 | 2 | 9 | 14 | 6/8 |
| | 3F01 | 3-30 | 4 | 11 | 23 | 8/15 | Kappa | 2-30 | 4 | 8 | 11 | 2/9 |
| | 3F02 | 3-30 | 4 | 13 | 29 | 3/26 | Kappa | 2-30 | 2 | 8 | 2 | 1/1 |
| | 3F03 | 3-43 | 4 | 12 | 22 | 7/15 | Lambda | 7-43 | 2 | 9 | 19 | 8/11 |
| | 3G02 | 3-13 | 4 | 11 | 15 | 3/12 | Kappa | 2-30 | 4 | 8 | 6 | 2/4 |
| | 3G04 | 3-9 | 6 | 20 | 13 | 2/11 | Lambda | 1-40 | 3 | 10 | 9 | 2/7 |
| | 3G05 | 3-23 | 4 | 11 | 25 | 7/18 | Kappa | 3-20 | 3 | 9 | 16 | 5/11 |
| | 3G06 | 3-30 | 4 | 19 | 18 | 6/12 | Lambda | 2-23 | 2 | 12 | 19 | 4/15 |
| | 3H02 | 3-30-3 | 4 | 7 | 22 | 6/16 | Kappa | 3-11 | 2 | 9 | 9 | 5/4 |
| | 3H04 | 4-30-2 | 4 | 17 | 16 | 4/12 | Kappa | 1-12 | 1 | 9 | 13 | 4/9 |
| 32 | 2C01 | 4-59 | 4 | 12 | 7 | 1/6 | Kappa | 3-15 | 2 | 10 | 5 | 3/2 |
| | 2C02 | 3-30 | 4 | 16 | 15 | 5/10 | Kappa | 1-33 | 4 | 10 | 9 | 2/7 |
| | 2C04 | 3-48 | 6 | 19 | 13 | 5/8 | Kappa | 1D-12 | 4 | 10 | 17 | 5/12 |
| | 2C06 | 3-72 | 5 | 16 | 8 | 1/7 | Kappa | 1-5 | 1 | 3 | 2 | 1/1 |
| | 2D02 | 4-59 | 4 | 14 | 7 | 0/7 | Lambda | 3-21 | 2 | 12 | 16 | 3/13 |
| | 2D03 | 3-21 | 3 | 21 | 12 | 5/7 | Kappa | 1-5 | 2 | 5 | 20 | 5/15 |
| | 2D06 | 1-46 | 5 | 19 | 12 | 3/9 | Lambda | 3-21 | 2 | 11 | 6 | 1/5 |
| | 2E01 | 3-72 | 4 | 14 | 39 | 14/25 | Kappa | 1-6 | 4 | 9 | 15 | 8/7 |
| | 2E02 | 1-46 | 6 | 21 | 11 | 1/10 | Kappa | 2-28 | 2 | 9 | 3 | 0/3 |
| | 2E04 | 1-46 | 6 | 21 | 17 | 4/13 | Kappa | 2-28 | 2 | 10 | 5 | 1/4 |
| | 2E05 | 1-46 | 6 | 20 | 14 | 2/5 | Kappa | 2-28 | 3 | 9 | 2 | 1/1 |
| | 2F02 | 3-64 | 4 | 13 | 17 | 1/16 | Kappa | 3-20 | 1 | 9 | 9 | 1/8 |
| | 2F03 | 3-48 | 6 | 16 | 23 | 6/17 | Kappa | 3-20 | 4 | 9 | 23 | 4/19 |
| | 2F04 | 1-46 | 5 | 12 | 11 | 0/11 | Kappa | 1D-17 | 4 | 10 | 4 | 1/3 |
| | 2G02 | 1-46 | 5 | 19 | 12 | 3/9 | Lambda | 3-21 | 2 | 11 | 6 | 1/5 |
| 33 | 3A04 | 1-69 | 4 | 19 | 19 | 9/10 | Kappa | 3-11 | 5 | 9 | 14 | 6/8 |
| | 3A06 | 4-61 | 5 | 18 | 35 | 13/22 | Lambda | 1-44 | 3 | 11 | 18 | 6/12 |
| | 3B06 | 1-46 | 6 | 21 | 25 | 6/19 | Lambda | 3-21 | 3 | 9 | 11 | 2/9 |
| | 3C05 | 1-69 | 5 | 20 | 14 | 5/9 | Lambda | 1-40 | 3 | 11 | 11 | 2/9 |
| | 3D02 | 3-15 | 4 | 14 | 12 | 1/11 | Kappa | 3-20 | 2 | 9 | 8 | 3/5 |
| | 3D04 | 3-49 | 5 | 20 | 22 | 1/21 | Kappa | 1-39 | 1 | 9 | 15 | 0/15 |
| | 3E01 | 1-69 | 4 | 14 | 39 | 17/22 | Kappa | 3-15 | 5 | 10 | 13 | 5/8 |
| | 3E04 | 3-21 | 5 | 18 | 22 | 6/16 | Kappa | 3-15 | 2 | 10 | 8 | 6/2 |
| | 3F01 | 3-21 | 6 | 13 | 25 | 4/21 | Kappa | 3-20 | 3 | 9 | 7 | 1/6 |
| | 3F02 | 5-10-1 | 3 | 11 | 24 | 7/17 | Lambda | 1-47 | 3 | 11 | 17 | 3/14 |
| | 3F03 | 1-69 | 5 | 17 | 25 | 7/18 | Kappa | 3-15 | 5 | 9 | 10 | 4/6 |
| | 3F05 | 3-11 | 4 | 10 | 10 | 4/6 | Kappa | 1-33 | 1 | 9 | 10 | 3/7 |
| | 3G01 | 3-23 | 4 | 21 | 18 | 5/13 | Kappa | 3-11 | 3 | 10 | 3 | 0/3 |
| | 3G04 | 3-21 | 4 | 11 | 14 | 5/9 | Lambda | 1-44 | 3 | 11 | 6 | 1/5 |
| 39 | 3A02 | 3-7 | 3 | 21 | 31 | 11/20 | Lambda | 2-14 | 2 | 10 | 23 | 8/15 |
| | 3A04 | 4-39 | 4 | 11 | 24 | 5/19 | Lambda | 2-14 | 2 | 10 | 12 | 1/11 |
| | 3B02 | 3-21 | 4 | 13 | 7 | 1/6 | Lambda | 1-47 | 3 | 11 | 12 | 0/12 |
| | 3C01 | 1-69 | 6 | 19 | 24 | 8/16 | Lambda | 1-51 | 2 | 12 | 8 | 3/5 |
| | 3D01 | 3-21 | 4 | 13 | 20 | 4/16 | Lambda | 1-47 | 3 | 9 | 13 | 4/9 |
| | 3D02 | 4-59 | 5 | 20 | 9 | 3/6 | Lambda | 1-44 | 3 | 11 | 12 | 3/9 |
| | 3D06 | 7-4-1 | 4 | 9 | 16 | 6/10 | Lambda | 7-43 | 3 | 12 | 32 | 8/24 |
| | 3E06 | 1-69 | 5 | 16 | 15 | 5/10 | Lambda | 2-11 | 1 | 13 | 8 | 2/6 |
| | 3G02 | 3-30 | 4 | 12 | 24 | 7/17 | Kappa | 1-9 | 4 | 10 | 21 | 7/14 |
| | 3G03 | 3-7 | 4 | 16 | 10 | 4/6 | Kappa | 1-39 | 2 | 9 | 8 | 2/6 |

TABLE 3. Antibody dependent enhancement (ADE) activity of monoclonal antibodies

Fully cross-reactive (4 serotypes) binding to rE

| Mab | ADE Fold Increase at 1 µg/mL DENV1 | DENV2 | DENV3 | DENV4 |
|---|---|---|---|---|
| 31.3D02 | - | 5 | 5 | 5 |
| 31.3F01 | 28 | 35 | 25 | 36 |
| 31.3F03 | 21 | 40 | 65 | 40 |
| 39.3B02 | 7 | 15 | 14 | 11 |
| 39.3D06 | 14 | 34 | 19 | 46 |
| 39.3G03 | - | - | - | - |
| 31.3D03 | - | 4 | 5 | 2 |
| 31.3E03 | 11 | 28 | 12 | 26 |
| 31.3F02 | 23 | 53 | 24 | 36 |
| 31.3G02 | 9 | 31 | 16 | 161 |
| 31.3G05 | - | 3 | 5 | 2 |
| 31.3G06 | 10 | 18 | 9 | 22 |
| 31.3H02 | 10 | 20 | 13 | 24 |
| 32.2D03 | 14 | 4 | 5 | 6 |
| 32.2E02 | 15 | 67 | 39 | 9 |
| 32.2G02 | 3 | 84 | 57 | - |
| 33.3D02 | 21 | 20 | 32 | 20 |
| 33.3D04 | 3 | 38 | 28 | - |
| 33.3F01 | 3 | 10 | 39 | 6 |
| 33.3F05 | 13 | 17 | 41 | 17 |
| 33.3G04 | 12 | 21 | 35 | 22 |
| 39.3A04 | 9 | 27 | 13 | 23 |
| 39.3C01 | 3 | 4 | 3 | 6 |
| 39.3D01 | - | 2 | 4 | 9 |
| 31.3H04 | 24 | 35 | 54 | 49 |
| 39.3A02 | 19 | 35 | 19 | 30 |
| 32.2F04 | - | - | - | - |

Partially cross-reactive / Non-reactive by rE ELISA

| Mab | ADE Fold Increase at 1 µg/mL DENV1 | DENV2 | DENV3 | DENV4 |
|---|---|---|---|---|
| 33.3A06 | 3 | 23 | 17 | 4 |
| 39.3G02 | - | 4 | 3 | - |
| 32.2C06 | - | 2 | - | 2 |
| 31.3G04 | 16 | 3 | - | 4 |
| 32.2C02 | 2 | 7 | - | 2 |
| 33.3C05 | 2 | 16 | 4 | - |
| 33.3E04 | 18 | 5 | - | - |
| 33.3F03 | 2 | 27 | 6 | - |
| 39.3E06 | 8 | 3 | - | 6 |
| 32.2D06 | 14 | 42 | 39 | 3 |
| 32.2E04 | 20 | 48 | 50 | 6 |
| 32.2E05 | 11 | 57 | 48 | 45 |
| 33.3A04 | 14 | 2 | 2 | 14 |
| 33.3B06 | 24 | 66 | 11 | - |
| 33.3E01 | 18 | 12 | 15 | - |
| 33.3F02 | 24 | 8 | - | - |
| 33.3G01 | 3 | 10 | 8 | - |
| 39.3D02 | 23 | 33 | 5 | - |
| 32.2D02 | 8 | 19 | 31 | 30 |
| 31.3C02 | 4 | - | - | 13 |
| 32.2C01 | - | - | - | - |
| 32.2C04 | - | - | - | - |
| 32.2E01 | - | - | - | - |
| 32.2F02 | 28 | 21 | - | - |
| 32.2F03 | - | - | - | - |

Serotype-specific in neutralization | Partially-cross neutralizing | Fully cross-neutralizing | Non-reactive

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## Chapter 3: Human antibody responses after dengue virus infection are highly cross-reactive to Zika virus

Lalita Priyamvada, Kendra M. Quicke, William Hudson, Nattawat Onlamoon, Kovit Pattanapanyasat, Kulkanya Chokephaibulkit, Patrick Wilson, Rafi Ahmed, Mehul S. Suthar, Jens Wrammert

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**Abstract**

Zika virus (ZIKV) is an emerging mosquito-borne flavivirus of significant public health concern. ZIKV shares a high degree of sequence and structural homology with other flaviviruses, including dengue virus (DENV), resulting in immunological cross-reactivity. Improving our current understanding of the extent and characteristics of this immunological cross-reactivity is important, as ZIKV is presently circulating in areas that are highly endemic for dengue. To assess the magnitude and functional quality of cross-reactive immune responses between these closely related viruses, we tested acute and convalescent sera from nine Thai patients with PCR-confirmed DENV infection against ZIKV. All sera tested were cross-reactive with ZIKV, both in binding and in neutralization. To deconstruct the observed serum cross-reactivity in depth, we also characterized a panel of DENV-specific plasmablast-derived monoclonal antibodies (mAbs) for activity against ZIKV. Nearly half of the 47 DENV-reactive mAbs studied bound to both whole ZIKV virion and to ZIKV lysate, of which a subset also neutralized ZIKV. In addition, both sera and mAbs from the dengue infected patients enhanced ZIKV infection of FcγR-bearing cells *in vitro*. Taken together, these findings suggest that pre-existing immunity to DENV may impact protective immune responses against ZIKV. In addition, the extensive cross-reactivity may have implications for ZIKV virulence and disease severity in DENV-experienced populations.

**Significance Statement**

In this study, we address the issue of cross-reactivity between dengue virus (DENV) and Zika virus (ZIKV) by testing sera and plasmablast-derived monoclonal antibodies from dengue patients against ZIKV. We show that both acute and convalescent dengue sera potently bind and neutralize ZIKV, and that this cross-reactivity is also evident at the monoclonal level. We also demonstrate in vitro antibody dependent enhancement of ZIKV infection in the presence of dengue-induced antibodies. Our findings strongly suggest that pre-existing dengue antibodies may modulate immune responses to ZIKV infection. These data are timely and highly relevant from a public health standpoint given that a majority of regions currently experiencing Zika virus epidemics are endemic for dengue.

## Introduction

Zika virus (ZIKV) is a mosquito-borne virus belonging to the *Flaviviridae* family of single-stranded positive-sense RNA viruses. First isolated in Uganda in 1947 (1), this virus remained largely dormant for the next six decades until it re-emerged as the cause of an epidemic on Yap Islands, Micronesia in 2007 (2). ZIKV has since then been linked with several outbreaks in the Pacific and Americas, along with sporadic human cases in Africa and Asia (3, 4). Until its appearance in French Polynesia in 2013 and more recently in Brazil in 2015, ZIKV infection was primarily associated with mild self-limiting illness, with symptoms similar to and often milder than dengue virus (DENV) or Chikungunya virus (CHIKV) infections (2-4). However, the more recent outbreaks have caused severe neurological complications including Guillain-Barré Syndrome in adults and an increase in congenital microcephaly and other adverse birth outcomes in Brazil (5-7). The Pan American Health Organization has reported that as of May 2016, local transmission of ZIKV had spread to over 38 countries or territories in the Americas. In addition, a recent WHO report states that 44 new countries are experiencing their first ZIKV outbreak since 2015. Despite the improving surveillance of the virus, accurate diagnosis has been challenging given the similarities in the clinical presentation of ZIKV to other arboviral infections endemic in these regions, among other factors.

During the viremic period, ZIKV can be found in patient blood, saliva, urine and other bodily fluids early after symptom onset (8-10). During the Yap Islands

epidemic in 2007, anti-ZIKV IgM ELISAs and ZIKV plaque reduction neutralization tests (PRNT) were performed to confirm infection in RT-PCR negative cases (2, 8). However as these studies showed, the cross-reactivity between ZIKV and other flaviviruses makes confirmation of infection difficult, especially when patients may have had flavivirus exposures prior to their suspected ZIKV infection (2, 8). Given the overlapping presence of DENV and other flaviviruses in a majority of ZIKV epidemic regions (11), there are great challenges in serology-based testing of flavivirus immune patients (12).

The DENV envelope (E) protein, considered a major imunodominant target for antibody responses in dengue patients (13-15), bears greater than 50% homology to ZIKV E protein (16). In addition to complicating the serology-based diagnosis of ZIKV infection, this raises an interesting question about the biological implications of the cross-reactivity on protection, virulence and immunopathology of ZIKV infections. At present, the effect of pre-existing immunity to DENV or other flaviviruses on immune responses induced by ZIKV infection is unknown. To this end, we were interested in determining the degree to which dengue-induced antibodies cross-react with ZIKV in terms of binding, virus neutralization and antibody dependent enhancement (ADE) of ZIKV infection, both at the serum and single cell level.

In this study, we provide an analysis of the cross-reactivity of acute and convalescent dengue immune sera against ZIKV. The sera were collected from nine patients admitted to Siriraj Hospital in Bangkok, Thailand with confirmed DENV infection. Both acute and convalescent sera showed high binding titers to ZIKV lysate, and could also neutralize ZIKV *in vitro.* To understand the origin and characteristics of these cross-reactive serum responses we also analyzed a panel of plasmablast-derived DENV-reactive monoclonal antibodies (mAbs). Of the 47 mAbs tested nearly half (22/47) bound to ZIKV lysate, and an additional four to whole virus. Seven of these mAbs also neutralized ZIKV *in vitro*. Five sera and a subset of the mAbs were also tested for ADE activity using the FcγR-bearing monocytic U937 cell line. All sera and ZIKV-reactive mAbs tested enhanced infection *in vitro,* while two DENV-specific but ZIKV non-reactive mAbs did not. The data presented here have important implications for clinical diagnosis given that the current ZIKV outbreak in the Americas and the Caribbean is largely ongoing in dengue endemic areas. Equally important, these findings set the stage for more in-depth studies that explore how pre-existing flavivirus immunity may shape immune responses to ZIKV infection.

## Materials and Methods

**Patient samples:** The dengue serum samples in this study were collected at Siriraj Hospital in Bangkok, Thailand. All patients were diagnosed with DENV infection by serotype-specific RT-PCR (17) and serum samples were collected during acute infection and/or convalescence. From four of these patients, a panel of mAbs was derived from single cell sorted plasmablasts (13). Two flavivirus-naïve sera were also included as controls. These studies were pre-approved by the Faculty of Medicine at Siriraj Hospital and the Emory institutional review board (IRB) #IRB00015730.

**Viruses and viral antigens:** ZIKV PRVABC59 (KU501215.1) was passaged by infecting Vero cells (ATCC; CRL-1586) at an MOI of 0.1 in serum-free MEM (Life Technologies Gibco). After a 1 h infection at 37°C, MEM supplemented with 10% FBS and 1% antibiotic/antimycotic (Corning) was added to the cells and virus inoculum. Upon observation of severe CPE, supernatants were collected and spun down at 2000 rpm for 10 min at 4°C. Supernatant containing virus was supplemented with an additional 10% FBS before freezing at -80°C. The titer of the passaged virus was determined by plaque assay. To prepare ZIKV lysate, the remaining adherent cells and cell pellet from the virus-containing supernatant were washed twice with PBS and then re-suspended in RIPA Buffer (10mM Tris, 150mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, pH 7.4) supplemented with protease inhibitor (Thermo Fisher Scientific; 87785) and phosphatase inhibitor

(Biovision; K275-1). Mock lysate was prepared in a similar fashion with uninfected cells. Bradford assay was performed to quantitate total protein yield.

DENV2 Tonga/74 (AY744147.1) was gifted by Dr. Stephen S. Whitehead: NIH/NIAID, Bethesda, MD. DENV2 viral stocks were made by infecting Vero cells at an MOI of 0.01 in Opti-Pro SF media (Invitrogen; 12309019). Virus-containing supernatant was collected at day 5 post infection after appearance of CPE and frozen after addition of 10% SPG stabilizer as previously described (18). Viral stocks were titrated by FFA prior to use.

**Preparation of 4G2 antibody:** A hybridoma expressing a pan-flavivirus mouse monoclonal (D1-4G2-4-15; ATCC HB-112) was grown in RPMI supplemented with 2% FBS, antibiotics and L-glutamine until terminal density. Clarified supernatant was filtered through a 0.2 μm filter, purified over a protein G column according to manufacturer recommendations, and stored in PBS with sodium azide.

**Sequence and structure alignment:** To visualize structural similarity between the DENV and ZIKV E proteins, their structures (19, 20) (PDB accession codes 3J27 and 5IRE, respectively) were aligned and secondary structure assigned in Chimera (21). Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco. Structural figures were made using the PyMOL Molecular Graphics System, Version 1.7 (Schrödinger, LLC). Sequences were aligned using Geneious (Biomatters, Ltd.) using GenBank accession

numbers AY744147.1 and KU501215.1 for DENV and ZIKV E proteins, respectively. Envelope domains (ED) I-III (22), the hinge (23), fusion loop (24), and transmembrane helices (25) were designated as previously described. For the fusion loop alignment, GenBank accession numbers used were EF623988.1 (JEV), M12294.2 (West Nile), KF769016.1 (Asibi), JX949181.1 (17D).

**Western blot:** ZIKV and mock lysate samples (20 μg per lane) were prepared with BME containing loading buffer and boiled for 15 min at 95°C. Lysates were run by SDS-PAGE on a 10% polyacrylamide gel and transferred to nitrocellulose membrane. Blots were blocked for 30 min in 5% milk in PBS with 0.1% Tween and probed for ZIKV E protein using the mouse anti-flavivirus 4G2 primary antibody for 30 min. Blots were washed and incubated with HRP-conjugated goat anti-mouse secondary antibody (Southern Biotech; 1030-05) for 10 min. Blots were developed using SuperSignaling West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific; 34096) on a Bio-Rad Molecular Imager ChemiDoc XRS+.

**ELISA**: For lysate ELISA, NUNC Maxisorp plates (eBioscience; 44-2404) were coated overnight at 4°C with ZIKV or mock lysates diluted in PBS. Plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with PBS with 10% FBS and 0.05% Tween (PBS-T-FBS) for 1.5 h. Subsequently, mAbs or serum was serially diluted in PBS-T-FBS and added to the plates for 1 h. A peroxidase-conjugated anti-human IgG antibody (Jackson ImmunoResearch; 109-036-098) was added for 1.5 h

before developing the plates using an *o*-phenylenediamine substrate (Sigma; P8787).

For virus capture ELISA, plates were coated overnight at 4°C with 4G2 at a concentration of 0.25 μg/well. After blocking with PBS-T-FBS for 1.5 h, ZIKV was added for 1 h. Plates were washed with PBS-T, and serially diluted mAbs or serum was added. The addition of the secondary antibody and developing steps were performed as described above. For all ELISA experiments, the serum dilution factor or mAb concentration was plotted versus their respective OD values at 490 nm. The endpoint titer/ minimum effective concentration was determined as the concentration required for three times the background signal of flavivirus-naive serum/ irrelevant mAb.

**Viral neutralization assay**: The neutralization potential of mAbs and serum samples was determined by a focus reduction neutralization test (FRNT) as previously described (13) with select modifications. Serially diluted mAbs or heat inactivated sera were incubated with a previously titrated amount of virus (60-100 focus forming units) of ZIKV or DENV2 for 1 h at 37°C. Vero cell monolayers in 96 well plates were subsequently infected with the mixture for 1 h at 37°C. An overlay containing 2% methylcellulose (Sigma; M0512-2506) was added to the cells. After a 3 day incubation at 37°C, the cells were washed, and fixed with a 1:1 mixture of acetone and methanol. Foci were stained using 4G2 for 2 h followed by HRP-linked anti-mouse IgG (Cell Signaling; 7076S) for 1 h, and developed using TrueBlue

Peroxidase substrate (KPL; 50-78-02). Foci were imaged using a CTL-Immunospot S6 Micro Analyzer. FRNT50 was determined as the concentration or dilution factor of sample required for 50% neutralization of virus.

**Antibody dependent enhancement assay.** Serially diluted sera or mAbs were incubated with $10^4$ ffu ZIKV for 1 h at 37°C. The virus and serum/mAb mixture was then added to a 96 well plate containing $2x10^4$ U937 cells (ATCC; CRL-1593.2) per well in RPMI containing 10% FBS, antibiotics and L-glutamine. Cells were infected for 24 h at 37°C. Infected cells were washed, and then fixed/ permeabilized using BD intracellular staining reagents (Fix/Perm Solution (BD; 51-2090KZ) and Perm/Wash Buffer (BD; 51-2091KZ)) according to the manufacturer's protocol. Cells were stained using 4G2 for 1 h followed by anti-mouse IgG Alexa Fluor 488 (Life Technologies; A11029) for 25 min. The frequency of infected cells was determined using flow cytometry, defined as the percentage of 4G2+ cells.

## Results

**Sera from DENV infected patients are highly cross-reactive to ZIKV lysate.** A recently published study reported high structural similarity between the E proteins of ZIKV and other flaviviruses including DENV (16). We compared the ZIKV and DENV2 strains used in our study, ZIKV PRVABC59 and DENV2 Tonga/74, to determine the homology between their E proteins and identify potential targets for cross-reactive immune responses. The DENV2 and ZIKV E proteins share an extremely similar, superimposable structure (RMSD 1.1 Å; Fig. 1A and B), with an overall 53.9% amino acid sequence identity (Fig. S1A and C). Envelope domain I (EDI) and EDII exhibit slightly higher conservation (59.1 and 56.6% identity, respectively), including the fusion loop of EDII, which is perfectly conserved between the two proteins (Fig. S1B and C). To assess the degree of cross-reactivity of DENV-specific B cell responses against ZIKV, mock- and ZIKV-infected Vero cell lysates were generated for use in binding assays. The lysates were tested by Western blot and probed for the presence of E protein using the mouse pan-flavivirus antibody 4G2. A band consistent with the size of ZIKV E protein was observed in ZIKV lysate, and absent in the mock lysate (Fig. 2A). We then measured binding of both acute and convalescent dengue sera, as well as naive sera, using the ZIKV lysate by IgG ELISA (Fig. 2B and C, Table S1).

The nine dengue patients in this study were all confirmed for DENV infection by RT-PCR. Serum samples were collected once during the acute phase (n=9) and for five

patients, a second time at convalescence (n=5) (Table S1). Sera from two flavivirus-naïve donors were also included in our analyses as a comparison to dengue sera (Table S1). All 14 dengue serum samples showed high ZIKV-specific IgG endpoint dilution titers, with median values of 177,400 and 125,000 for acute and convalescent samples respectively (Fig. 2B and C, Table S1). All sera showed negligible titers against mock lysate (endpoint dilution < 250). The flavivirus-naïve samples were essentially negative against both the ZIKV-infected and the mock lysates (Fig. 2C, Table S1). These data illustrate that ZIKV cross-reactive antibodies can be readily detected in the serum of dengue patients living a highly dengue endemic country like Thailand.

**Dengue immune sera exhibit high neutralization potency against ZIKV.** To determine whether the dengue sera could also neutralize ZIKV *in vitro*, we performed focus reduction neutralization tests (FRNT) on all 14 dengue sera against ZIKV. A representative example of the ZIKV neutralization assay with two dengue acute sera (#33 and #39) and one flavivirus-naïve serum sample (#21) is shown in Fig. 3A. The ZIKV FRNT50 titers of the acute dengue samples ranged from 60 (#60) to 23,109 (#79), with a median value of 770. The convalescent dengue sera ranged in FRNT50 titers from 126 (#60R) to 50,346 (#79R), with a median titer of 350. While neutralization titers increased between the acute and convalescent bleeds for three patients, convalescent titers for patients #55 and #67 were lower than their acute titers (Fig. 3B, Table S1). Of note, the convalescent samples for these two

donors were obtained at a much later time-point after fever onset (61-100 days) than the other three convalescent sera (Table S1). These data show that dengue immune sera are can neutralize ZIKV *in vitro*. The impact of these neutralizing titers on either protective immunity or disease severity after ZIKV infection remains to be defined.

**Monoclonal antibodies derived from dengue-induced plasmablasts are highly cross-reactive to ZIKV.** While analysis of polyclonal sera from the dengue patients clearly illustrates ample cross-reactivity of dengue immune sera against ZIKV, serum analyses alone cannot determine the origin of these cross-reactive antibodies. In other words, whether the serum cross-reactivity was caused by two individual pools of antibodies, one DENV-specific and the other ZIKV-specific, or by antibodies that recognize both viruses, can only be conclusively determined by analyzing functional cross-reactivity at the monoclonal level. To dissect the cross-reactivity between DENV infection-induced antibodies and ZIKV, we characterized the binding and neutralization activity of a panel of plasmablast-derived mAbs against ZIKV. These mAbs were generated from *in vivo* activated, single cell sorted plasmablasts isolated during ongoing infection from four DENV2 patients, and were previously shown to be DENV-reactive either in binding, or in both binding and neutralization (13).

Of the 47 mAbs tested, 22 bound with high affinity to ZIKV lysate (Fig. 4A). An additional four ZIKV cross-reactive mAbs were identified using a whole virus capture ELISA (Fig. 4B, Table S2). A majority of the ZIKV-specific mAbs (20/26) came from the plasmablasts of donors #31 and #39. Only a handful of mAbs from donors #32 and #33 cross-reacted with ZIKV, with several of these recognizing only whole ZIKV. While nearly half of all DENV-reactive mAbs bound ZIKV lysate or whole virus, only seven of the mAbs neutralized ZIKV *in vitro* (Fig. 4C and 4D, Table S2). Six of these seven mAbs exhibited moderate neutralizing activity against ZIKV, with FRNT50 titers ranging between 5 μg/mL and 1 μg/mL. In contrast, mAb 33.3A06 was highly potent in ZIKV neutralization with a ZIKV FRNT50 titer of 0.03 μg/mL. Interestingly, despite the overall lower frequency of ZIKV-binding mAbs isolated from #32 and #33, half of all ZIKV neutralizing mAbs in the panel, including the three most potently neutralizing mAbs, were derived from these two patients. Repertoire analysis of the cross-reactive mAbs showed showed broad VJ gene usage and junctional diversity. The cross-reactive cells were also highly mutated, illustrating that these responses were likely the result of multiple previous DENV exposures (Table S2).

**Dengue-induced antibodies can enhance ZIKV infection of an FcγR-bearing monocytic cell line *in vitro*.** We tested the ability of five dengue sera and 11 plasmablast-derived mAbs to enhance ZIKV infection using a human FcγR-bearing monocytic cell line, U937. The U937 cell line is widely used to study ADE of DENV

infection, and it is not typically permissive to high levels of DENV infection in the absence of enhancing antibodies (26). The five dengue sera tested were all acute samples from DENV2-infected patients, including patients #31, #32, #33 and #39 from whose plasmablasts the mAbs in this study were derived. The mAbs tested included seven ZIKV-neutralizing mAbs, of which six were intermediate in neutralization (ELISA$^{+}$/Neut$^{int}$) and one potent (ELISA$^{+}$/Neut$^{++}$), two ZIKV-reactive but non-neutralizing mAbs (ELISA$^{+}$/Neut$^{neg}$), and two mAbs that bound DENV, but did not cross-react with ZIKV (ELISA$^{-}$/Neut$^{neg}$). In addition to the dengue sera and mAbs, one flavivirus-naïve serum sample (#21) and two irrelevant mAbs (cholera and influenza-specific) were also tested for ZIKV ADE activity. A representative example of the flow cytometry-based assay showing ADE activity of mAb 31.3F01 is provided in Fig. 5A. Each of the five dengue sera tested was able to enhance ZIKV infection of U937 cells, with peak percent infection between 27% (#31) to 66% (#55). The bell shaped ADE curves observed with this assay generally seemed to shift to lower dilutions as the neutralizing potency of the serum sample increased (Fig. 5B, Table S1), presumably due to complete neutralization of the virus at higher concentrations. The flavivirus-naïve serum sample did not enhance ZIKV infection of U937 cells (Fig. 5B).

The six ELISA$^{+}$/Neut$^{int}$ mAbs enhanced ZIKV infection at the maximum concentration tested (10 μg/mL), while the potent neutralizer 33.3A06 exhibited minimal ADE above 2 μg/mL, again potentially due to complete viral neutralization.

At lower concentrations, however, the mAb facilitated the infection of U937 cells, reaching a maximal percent infection of 81% (Fig. 5C). The two ZIKV ELISA$^{+}$/Neut$^{neg}$ mAbs also enhanced ZIKV infection, similar to the neutralizing mAbs. Two mAbs that were previously shown to be DENV1-specific (13) and were ZIKV non-reactive (Fig. 4) did not enhance ZIKV infection (Fig. 5C). These data demonstrate that ZIKV-reactive antibodies can potentiate infection of FcγR-bearing human monocytic cells *in vitro* and that both maximal infection and the effective concentration range of individual antibodies varies significantly.

**Discussion**

The emerging ZIKV shares a high degree of sequence and structural homology when compared to other flaviviruses, including DENV (16). For the current outbreak in the Americas and the Caribbean this is of major public health concern. It is not clear how pre-existing antibody titers to other flaviviruses might affect the quality of immune responses generated to ZIKV infection, and equally important, whether such cross-reactive antibodies provide protective immunity or impact disease severity in infected adults (27). In the study presented here, we have determined the degree by which dengue-induced antibodies cross-react with ZIKV, both at a serum level as well as at a single cell level.

We characterized the ZIKV binding and neutralization potential of sera obtained from PCR-confirmed dengue patients sampled during acute disease and at convalescence. Both acute and convalescent sera had high IgG binding titers to ZIKV and potently neutralized the virus *in vitro* (Fig 2C and 3B, Table S1). While no obvious correlation was observed between DENV2- and ZIKV-specific neutralization titers in the same patients (Table S1), it is evident that a significant proportion of serum antibodies present after DENV infection cross-react with ZIKV. Although a majority of the dengue sera tested neutralized DENV2 more potently than ZIKV, sera from patients #55 and #79 had higher FRNT50 titers to ZIKV compared to DENV2 (Table S1). For patient #79, the lower dengue titers could simply be attributed to the mismatch between the serotype of infection (DENV1) and the virus tested

(DENV2). For patient #55, this could have been caused by genetic differences between the lab-adapted DENV2 strain used in our study and the infecting DENV2 strain. An alternative possibility is that these patients were previously exposed to ZIKV, and thus had ZIKV-reactive antibodies in their sera as a result. In fact, in the past few years, isolated cases of ZIKV transmission in Thailand have been reported (10, 28, 29). Although there is no evidence of previous ZIKV epidemics in Thailand, the possibility that the patients in our study are ZIKV-immune, and that the extensive cross-reactivity of their sera against ZIKV is due to pre-existing ZIKV-induced antibodies cannot be formally ruled out. To definitively conclude that antibodies induced by DENV infection cross-react with ZIKV, it is important to demonstrate this cross-reactivity at the monoclonal level as well. In addition, from the serum data it is unclear whether the observed cross-reactivity is caused by a small number of highly potent, cross-reactive antibodies, or if this is the result of a broader, low level cross-reactivity.

To deconstruct the cross-reactivity observed at the serum level, we analyzed the ZIKV binding and neutralization activities of plasmablast-derived mAbs generated from four acutely infected DENV2 patients. We found that over half of the DENV-reactive mAbs bound with high affinity to ZIKV (Fig. 4A and B). At least 23 of the 26 ZIKV cross-reactive mAbs were E protein specific, as they were previously shown to bind recombinant DENV E protein (13). Although cross-reactive binding was abundant, and all 26 ZIKV reactive mAbs neutralized DENV2, less than a third

neutralized ZIKV *in vitro*. Furthermore, of the seven ZIKV-neutralizing mAbs, only one displayed potent neutralization activity (Fig. 4C). Therefore, even though a large number of dengue patient mAbs were able to bind viral epitopes, the capacity to cross-neutralize ZIKV was restricted to a select few. Additionally, a majority of these DENV-reactive mAbs were previously shown to neutralize more than one DENV serotype (Table S2) (13). Hence, for a large proportion of our mAb panel, the ability to cross-neutralize virus did not extend beyond the DENV species to ZIKV. Lastly, no obvious patterns in terms of VH gene usage or dominant clones were observed for the ZIKV-reactive mAbs (Table S2) (13). Thus, the cross-reactivity observed at the serum level, at least for these four patients, appears to be caused by a diverse repertoire of B cells.

The ZIKV E protein shares a high degree of homology with the E protein of other flaviviruses including DENV (16). We compared the E proteins of the ZIKV and DENV2 strains used in our study and found an overall sequence identity of 54% (Fig. S1A and C). EDI and EDII were relatively more conserved than EDIII, which had a lower sequence identity of 44.6% (Fig. S1C). Notably, the fusion loop is 100% conserved between the two viruses, and also when compared to other flaviviruses including yellow fever virus, West Nile virus, and Japanese encephalitis virus (Fig. S1B). The fusion loop has been described as a target for broadly cross-reactive antibodies against DENV (30, 31) as well as other flaviviruses (32-34), and could be one of the epitopes targeted by the cross-reactive antibodies described in our study.

In addition, despite the amino acid differences between the DENV2 and ZIKV E proteins compared, the two proteins share nearly identical structures (Fig 1A). This could have important implications for antibodies against conformationally sensitive epitopes, which depend on the quaternary structure of the E protein for recognition and binding (35-37). In fact, 4 out of 7 ZIKV neutralizing antibodies characterized in this study bound to whole virus, but failed to bind ZIKV lysate, suggesting that they recognize a conformational epitope. Efforts to map some of the antibodies described above are ongoing, focused especially on the potent ZIKV neutralizer 33.3A06. Identifying potential targets for broadly cross-neutralizing antibody responses could inform the design of vaccines or antibody-based therapies in the future.

Since the current ZIKV outbreak is largely localized within dengue endemic areas, the potential for pre-existing dengue-induced antibodies to enhance ZIKV infection is of concern. ADE is hypothesized to contribute to the increased disease severity often observed in secondary DENV infections (38). ADE is thought to occur when pre-existing cross-reactive antibodies form virus-antibody complexes that then facilitate the infection of FcγR-bearing cells (39). This may increase the number of infected cells and cause higher serum viral loads, which have been shown to positively correlate with higher disease severity (40, 41). To determine whether dengue antibodies can enhance ZIKV infection *in vitro*, we infected the FcγR-bearing U937 monocytic cell line in the presence of five acute sera and 11 dengue mAbs. All five sera and the nine ZIKV-reactive mAbs tested enhanced ZIKV infection *in vitro*

(Fig. 5B and 5C). Two DENV1-specific mAbs that did not react to ZIKV by binding or neutralization assays failed to enhance ZIKV infection in this system (Fig. 5C). These data clearly illustrate that ZIKV-cross reactive antibodies induced after DENV infection can enhance ZIKV infection *in vitro*. However, it is important to point out that the physiological relevance of this mechanism must be carefully examined *in vivo* to determine its importance in the context of ZIKV infection of flavivirus-immune patients.

Our findings raise important questions regarding the role of cross-reactive antibodies in protective immunity, as well as their potential impact on ZIKV pathogenesis and disease severity. The data presented suggest that ZIKV infection may have the potential to reactivate cross-reactive dengue-induced memory responses in patients with prior DENV exposures. There may thus be interesting differences between the immunological responses of DENV-immune patients vs. those of a flavivirus-naïve individual to ZIKV. To address these issues, ongoing comparative studies of immune responses, disease severity and clinical outcomes in ZIKV infected patients both in flavivirus-endemic and non-endemic areas are required. One of the most critical aspects of the current ZIKV virus outbreak is the ability of the virus to cause congenital microcephaly (6, 7). It will be essential to determine if the pre-existing cross-reactive antibodies may be involved in the context of maternal-fetal transmission of ZIKV. Equally important, studying cross-reactivity against multiple ZIKV isolates, derived from both recent and previous

epidemics, might shed light on the cause for the increased disease severity observed in the current outbreak. Finally, as additional ZIKV-reactive human plasmablast and memory B cell-derived mAbs are identified, characterizing their *in vivo* properties in murine and macaque models will be an important step in generating potential prophylactic/therapeutic treatments. Such studies will also improve our understanding of the immunobiology of ZIKV infection and how pre-existing antibodies to DENV or other flaviviruses might modulate the ZIKV immune response.

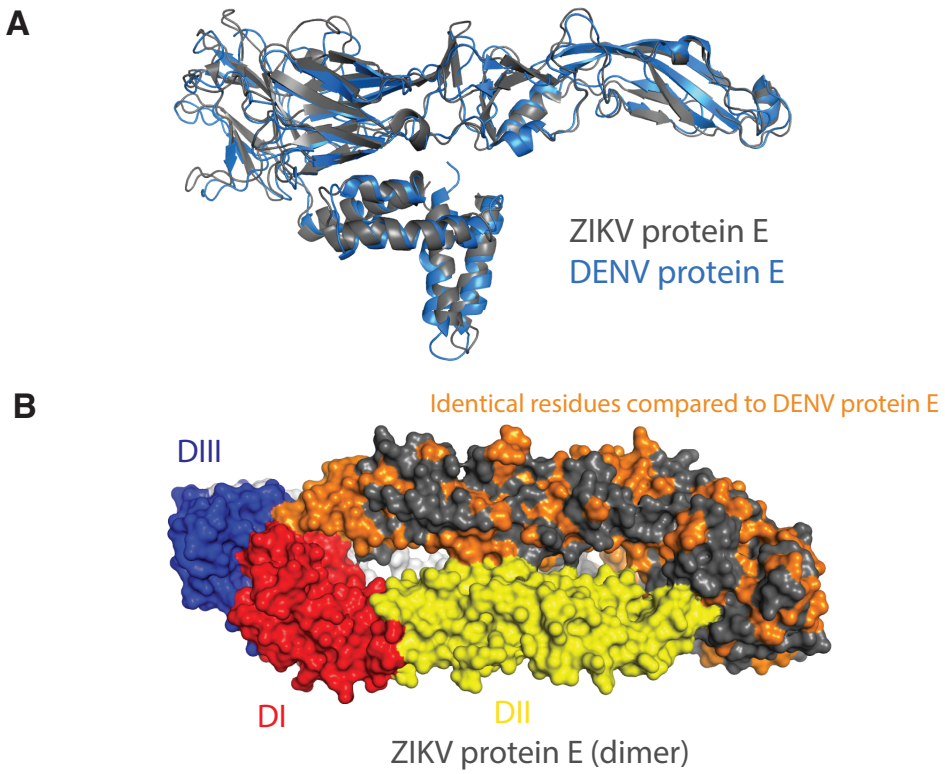



**Figure 1: The DENV2 and ZIKV E proteins share a highly similar fold and 54% sequence identity**. A) Overlay of DENV2 (blue) and ZIKV (gray) E protein structures (16, 19). B) Structure of the ZIKV E protein dimer. The left monomer is colored by its domain structure. At right, amino acids conserved between the ZIKV PRVABC59 and DENV2 Tonga/74 envelope proteins are colored orange on a gray ZIKV backbone.

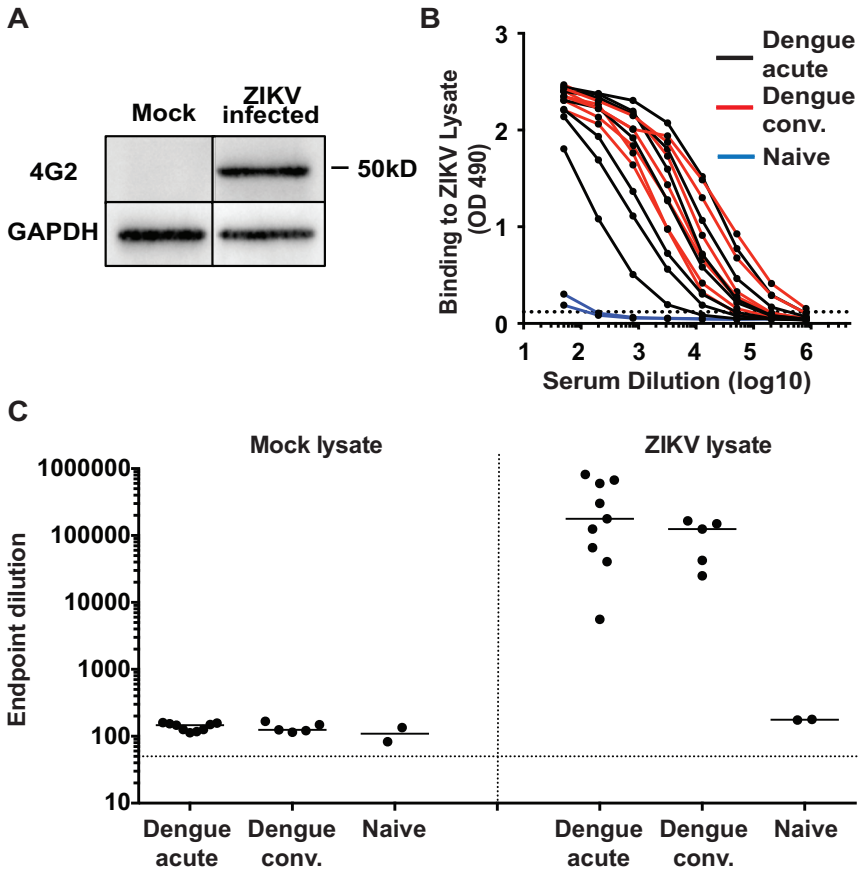



**Figure 2. Sera from patients with secondary DENV infection exhibit potent cross-reactivity against ZIKV.** A) Western blot of lysates from mock- or ZIKV-infected Vero cells. The pan-flavivirus reactive mAb 4G2 was used to probe for E protein. B) Binding of acute (black) and convalescent (red) dengue immune and flavivirus-naïve (blue) sera to ZIKV lysate. Dotted line represents three times the background signal of plain blocking buffer. C) Summary of binding of serum samples to lysates from mock- or ZIKV-infected Vero cells determined by ELISA. Acute (n=9) and convalescent (n=5) dengue and two control sera were tested. Median endpoint

IgG titers for each set of sera are indicated. The dotted line represents the initial serum dilution (1/60). The binding data shown in panels B and C are the result of two independent experiments and the mean value is plotted.

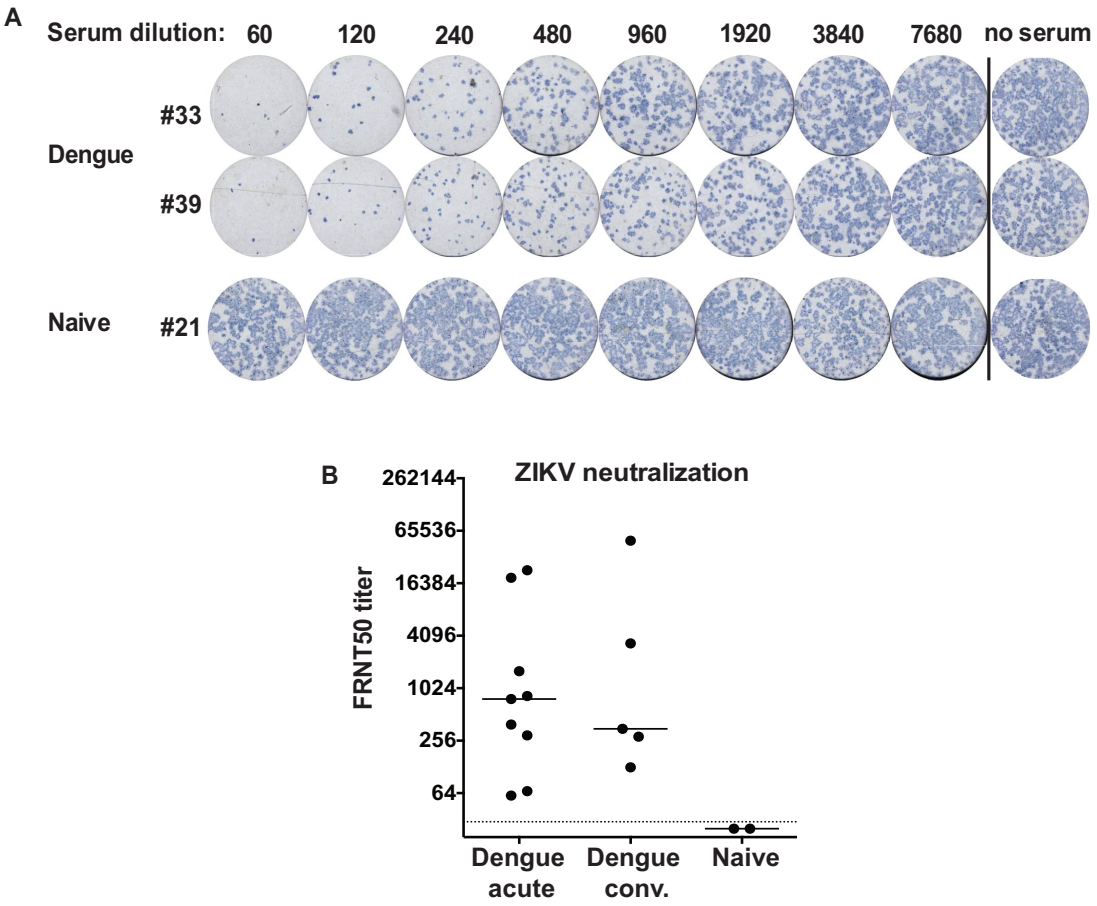



**Figure 3. Sera from acute and convalescent dengue patients can neutralize ZIKV.** A) Representative panel of FRNT assay showing neutralization of ZIKV by acute dengue sera (#33, #39) and one flavivirus-naïve serum sample (#21). B) Neutralization activity of serum samples against ZIKV. The FRNT50 titers of flavivirus-naïve (n=2) sera and acute (n=9) and convalescent (n=5) dengue sera were determined by FRNT assay as previously described (13). The FRNT assay for each sample was repeated in two or more independent experiments. The solid line represents median FRNT50 value, and dotted line represents the initial serum dilution (1/30).

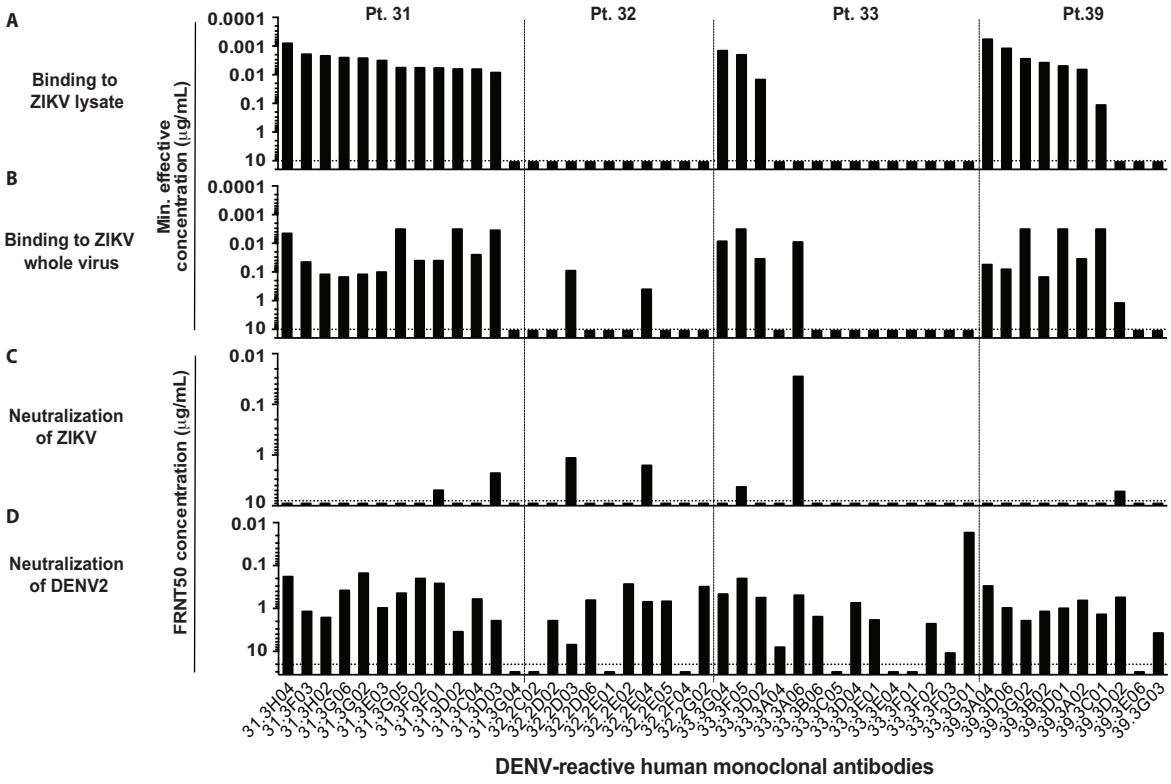



**Figure 4**. **A subset of DENV-specific plasmablast-derived mAbs cross-react to ZIKV both by binding and neutralization**. Binding of DENV-reactive mAbs (n=47) to (A) ZIKV lysate or (B) whole ZIKV. The mAbs are grouped by patient (Pt.). Values plotted represent the minimum concentration required for three times the background signal from an irrelevant mAb. Dotted line represents the maximum concentration of mAb tested in ELISA: 10 μg/mL. FRNT50 of DENV-reactive mAbs against ZIKV (C) and DENV2 (D). Dotted line represents the maximum concentration of mAb tested: 8 μg/mL (ZIKV FRNT) and 20 μg/mL (DENV FRNT). The DENV2 neutralization data in D has been adapted from previously published data (13). All experimental data shown is the result of two or more independent experiments.

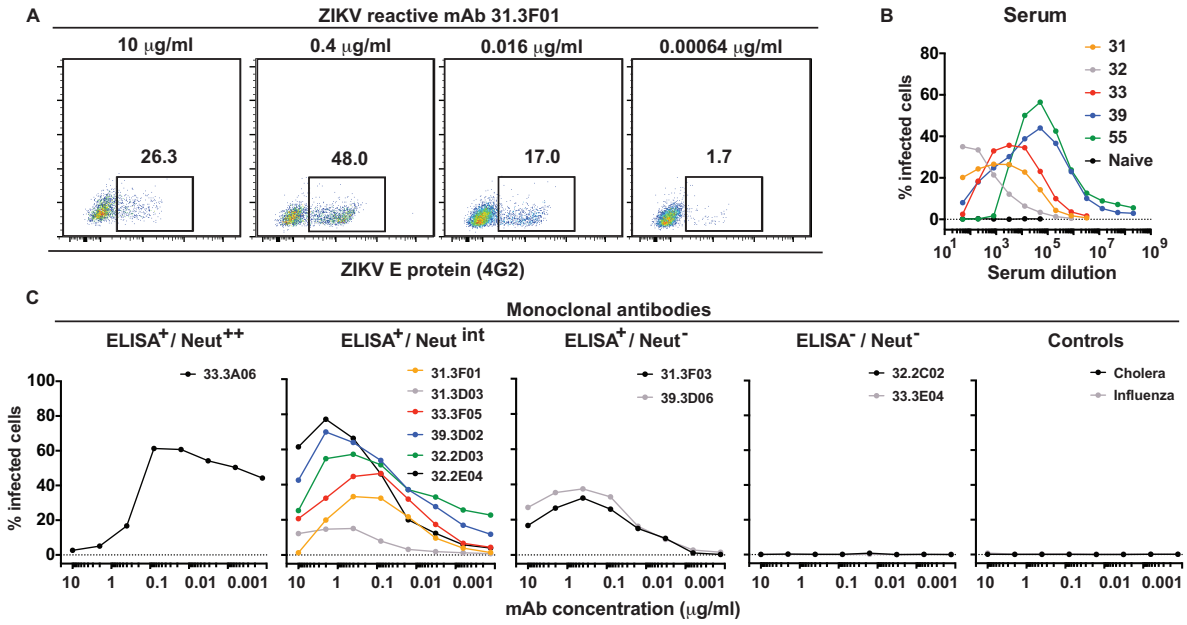
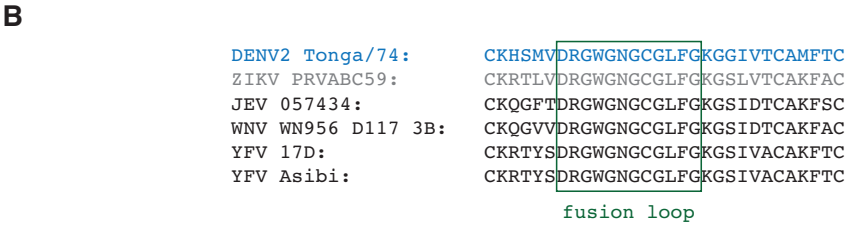



**Figure 5. Sera and mAbs from DENV-infected patients can enhance ZIKV infection of U937 cells.** A) Representative flow cytometry panel of mAb 31.3F01 showing percent infection at a range of mAb concentrations. B) ADE activity of five dengue sera and one flavivirus-naïve serum sample. C) ADE activity of dengue patient-derived (n=11) and control (n=2) mAbs. The antibodies are grouped by ZIKV cross-reactivity phenotype. ELISA$^{+/-}$ refers to binding activity to ZIKV by capture virus ELISA while Neut$^{++/+/neg}$ refers to ZIKV neutralization activity. Infected cells were identified by 4G2 staining. The dotted line in panels B and C represents percent infection in the absence of antibody (virus only). Data shown is representative of two or more independently performed experiments.


**C**

| E protein region | Sequence identity |
|---|---|
| Total E protein | 53.9% |
| EDI | 59.1% |
| EDII | 56.6% |
| EDIII | 44.6% |
| TM | 51.5% |
| Fusion loop | 100% |

**Figure S1. ZIKV and DENV2 E proteins share high sequence identity, especially in the fusion loop.** A) Sequence alignment of the ZIKV (gray) and DENV2 (blue) E proteins, which share 53.9% identical amino acids. The fusion loop and hinge regions are shown in green and magenta, respectively. B) The fusion loop is

perfectly conserved among other flaviviruses. DENV2 (blue) and ZIKV (gray) have been compared to isolate 057434 of JEV, the WNV strain WN 956 D117 3B and the 17D and Asibi strains of YFV. C) Sequence identity of the ZIKV and DENV2 E protein sequences. TM: trans-membrane domain.

## Table S1. Summary of serum binding and neutralization

| Sample | Day after fever onset* | Infection type | ZIKV endpoint dilution† | FRNT50‡ | |
|---|---|---|---|---|---|
| | | | | DENV2 | ZIKV |
| 31 | 4 | DENV2 | 600,000 | 1,653 | 392 |
| 32 | 3 | DENV2 | 5,600 | 1,355 | 294 |
| 33 | 5 | DENV2 | 40,600 | 1,318 | 1,602 |
| 39 | 6 | DENV2 | 819,200 | 2,286 | 770 |
| 55 | 4 | DENV2 | 675,000 | 1,373 | 18,940 |
| 55R§ | 100 | | 25,000 | 218 | 3,344 |
| 60 | 6 | DENV3 | 302,400 | 7,614 | 60 |
| 60R§ | 40 | | 150,000 | 14,807 | 126 |
| 67 | 6 | DENV1 | 125,000 | 2,858 | 832 |
| 67R§ | 61 | | 42,500 | 1,880 | 285 |
| 79 | 4 | DENV1 | 65,600 | 528 | 23,109 |
| 79R§ | 37 | | 165,000 | 470 | 50,346 |
| 86 | 6 | DENV3 | 177,400 | 322 | 67 |
| 86R§ | 41 | | 125,000 | 263 | 350 |
| 21 | | HC‹ | 175 | <30 | <30 |
| 22 | | HC‹ | 179 | <30 | <30 |

*Number of days post-fever onset at which blood was collected.

†IgG ELISA measuring endpoint titer for binding to ZIKV-infected lysate. The signal from mock lysate was <250 for all samples.

‡50% focus reduction neutralization titer.

§Convalescent sample.

‹Healthy control.

## Table S2. Summary of characteristics of mAbs

| | | | | | | | | | | | Rearrangement | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Binding to ZIKV[†] | | Neutralization—FRNT50 titer[‡], μg/mL | | | | | Ig isotype | | Heavy chain | | | Light chain | | |
| Patient* | Ab | Lysate | Whole virus | ZIKV | DENV1 | DENV2 | DENV3 | DENV4 | Heavy chain[§] | Light chain | V | J | Mutations | V | J | Mutations |
| 31 | 3H04 | + | + | — | 1.3 | 0.2 | 0.4 | 0.4 | IgG1 | Igκ | 4–30 | 4 | 16 | 1–12 | 1 | 13 |
| | 3F03 | + | + | — | — | 1.2 | — | — | IgG1 | Igλ | 3–43 | 4 | 22 | 7–43 | 2 | 19 |
| | 3H02 | + | + | — | — | 1.6 | — | 1.8 | IgG1 | Igκ | 3–30 | 4 | 22 | 3–11 | 2 | 9 |
| | 3G06 | + | + | — | 7.5 | 0.4 | — | 2.5 | IgG1 | Igλ | 3–30 | 4 | 18 | 2–23 | 2 | 19 |
| | 3G02 | + | + | — | — | 0.2 | 1.8 | 1.2 | IgG1 | Igκ | 3–13 | 4 | 15 | 2–30 | 4 | 6 |
| | 3E03 | + | + | — | — | 1.0 | 6.2 | 4.5 | IgG1 | Igλ | 3–43 | 4 | 19 | 7–43 | 2 | 14 |
| | 3G05 | + | + | — | — | 0.4 | 0.5 | 3.0 | IgG1 | Igκ | 3–23 | 4 | 25 | 3–20 | 3 | 16 |
| | 3F02 | + | + | — | — | 0.2 | 0.9 | 0.5 | IgG1 | Igκ | 3–30 | 4 | 29 | 2–30 | 2 | 2 |
| | 3F01 | + | + | 5.0 | — | 0.3 | — | — | IgG1 | Igκ | 3–30 | 4 | 23 | 2–30 | 4 | 11 |
| | 3D02 | + | + | — | — | 3.5 | — | — | IgG1 | Igκ | 3–23 | 4 | 23 | 3–20 | 3 | 14 |
| | 3C04 | + | + | — | — | 0.6 | — | 3.1 | IgG1 | Igκ | 1–2 | 4 | 16 | 3–20 | 4 | 11 |
| | 3D03 | + | + | 2.3 | — | 1.9 | 4.1 | — | IgG1 | Igκ | 4–4 | 3 | 5 | 1–5 | 2 | 7 |
| 32 | 2D03 | — | + | 1.1 | 0.8 | 7.0 | 4.3 | — | IgG1 | Igκ | 3–21 | 3 | 12 | 1–5 | 2 | 20 |
| | 2E04 | — | + | 1.6 | 1.0 | 0.7 | 4.0 | — | IgG1 | Igκ | 1–46 | 6 | 17 | 2–28 | 2 | 5 |
| 33 | 3G04 | + | + | — | 3.1 | 0.5 | — | 2.9 | IgG1 | Igλ | 3–21 | 4 | 14 | 1–44 | 3 | 6 |
| | 3F05 | + | + | 4.3 | — | 0.2 | — | 1.2 | IgG1 | Igκ | 3–11 | 4 | 10 | 1–33 | 1 | 10 |
| | 3D02 | + | + | — | 2.1 | 0.6 | 2.2 | 0.5 | IgG1 | Igκ | 3–15 | 4 | 12 | 3–20 | 2 | 8 |
| | 3A06 | — | + | 0.03 | 0.2 | 0.5 | 1.4 | — | IgG1 | Igλ | 4–61 | 5 | 35 | 1–44 | 3 | 18 |
| 39 | 3A04 | + | + | — | 7.7 | 0.3 | 4.7 | 1.6 | IgG1 | Igλ | 4–39 | 4 | 24 | 2–14 | 2 | 12 |
| | 3D06 | + | + | — | — | 1.0 | — | 7.2 | IgG1 | Igλ | 7–4 | 4 | 16 | 7–43 | 3 | 32 |
| | 3G02 | + | + | — | 2.9 | 1.9 | 6.3 | — | IgG1 | Igλ | 3–30 | 4 | 24 | 1–9 | 4 | 21 |
| | 3B02 | + | + | — | — | 1.2 | — | — | IgG1 | Igλ | 3–21 | 4 | 7 | 1–47 | 3 | 12 |
| | 3D01 | + | + | — | — | 1.0 | 0.5 | 1.1 | IgG1 | Igλ | 3–21 | 4 | 20 | 1–47 | 3 | 13 |
| | 3A02 | + | + | — | 3.6 | 0.7 | 3.5 | 2.1 | IgG1 | Igλ | 3–7 | 3 | 31 | 2–14 | 2 | 23 |
| | 3C01 | + | + | — | 4.2 | 1.4 | 4.4 | — | IgG1 | Igλ | 1–69 | 6 | 24 | 1–51 | 2 | 8 |
| | 3D02 | — | + | 5.2 | 0.8 | 0.6 | — | — | IgG1 | Igλ | 4–59 | 5 | 9 | 1–44 | 3 | 12 |

*All patients were infected with DENV2.
[†]Maximum mAb concentration tested = 10 μg/mL.
[‡]FRNT50 values below 8 μg/mL are shown.
[§]All mAbs were cloned into heavy chain expression vectors containing the IgG1 constant region.

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# Chapter 4: Conclusions

Sections of this chapter have been submitted to *Emerging Microbes and Infections* as part of a review.

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**Part I**

In **Chapter 2**, we dissected the cellular and functional properties of the acute phase B cell response in four Thai patients experiencing secondary DENV2 infections. We isolated plasmablasts from patient blood early after fever onset, cloned and expressed Ig rearrangements *ex vivo* and generated a panel of mAbs. Patient plasmablasts had high rates of somatic hypermutation, with a preference for replacement mutations. Clonal expansions were detected in each of the four donors. These data taken together suggested that the plasmablast response in secondary dengue may arise in part from the reactivation of MBCs generated during a previous DENV exposure.

A total of 53 mAbs were generated from the plasmablasts of the four patients. The mAb panel was almost entirely DENV-specific and a majority neutralized two or more DENV serotypes. A large proportion of mAbs bound rE (31/53) and additional mAbs were found to be E-specific by Western blot, indicating that E was the main target for plasmablast responses in these patients. Several mAbs that did not react to rE bound whole virus and also neutralized DENV. We suspect that some if not all of these DENV-neutralizing mAbs target E protein epitopes that are absent in soluble monomeric rE but are present in the context of the whole virion. Over the past few years, several groups have identified E-specific antibodies with a similar non rE-binding phenotype and have determined the complex virion-dependent epitopes they target (1-5).

Both type-specific and cross-reactive mAbs have been shown to bind quaternary-structure epitopes. The highly potent DENV3-neutralizing mAb 5J7 was found to bind an epitope that spans across three E proteins (5). The epitope includes the EDI-EDII hinge of one protein, EDIII of the second and the tip of EDII of the third E protein. The DENV1-specific mAbs 14c10 and 1F4 both bind to the EDI-EDII hinge region (2, 4). While the epitope of 1F4 is contained within one E protein monomer (4), 14c10 also binds DIII on a second E protein (2). Like 14c10, the DENV2-specific mAb 2D22 also binds within E dimers, engaging EDIII and EDI of one E protein, and EDII of the second (3). Other complex epitope-binding mAbs have been shown to be broadly neutralizing. These include E-dimer dependent (EDE) antibodies that bind epitopes across the E protein dimer interface (1). There are two types of EDE antibodies, EDE1 and EDE2, both of which bind similar epitopes that involve the fusion loop and DII of one monomer and DI and DIII of the second. The major difference between the two is the sensitivity of EDE2 antibodies to changes at E protein residues 153 and 155 that disrupt an N-linked glycosylation site.

The mAbs described above range in *in vitro* DENV neutralization potency from moderate to highly potent. The mechanisms of viral neutralization vary: while 5J7 was shown to inhibit viral attachment to cells, 2D22 is hypothesized to lock E dimers and prevent conformational changes required for viral fusion post attachment. Virion-dependent epitopes are increasingly gaining interest as important targets for highly neutralizing antibodies. What remains unclear, however, is the contribution of the antibodies that bind such complex epitopes to

overall serum neutralization titers. One study demonstrated that depleting primary dengue sera of rE-binding antibodies had little effect on overall serum neutralization activity (6), suggesting quaternary structure-dependent epitopes drive a majority of the neutralizing antibody response. Additional studies systematically examining primary vs. secondary dengue sera at both acute and convalescent time points are required to fully address this issue. A complementary approach is to deplete sera of antibodies targeting specific complex epitopes. This will allow us to better understand the individual contribution of each type of epitope to neutralizing titers at the serum level.

A subset of the mAbs in our panel exhibited serotype-specific neutralization activity. These mAbs clustered into two groups based on their neutralization and binding properties. The first group neutralized DENV2 but cross-reacted in binding to all four DENV serotypes. The second group was DENV1-specific in neutralization, and preferentially bound to DENV1 as well, with low levels of cross-reactivity to DENV2. Furthermore, even though all four patients had DENV2 infection at the time of the study, over half of the mAbs generated from two donors neutralized DENV1 more potently than DENV2. This neutralization bias towards DENV1 was also observed at the serum level for the same two patients. Although the disease history of the four donors is unknown, we hypothesize that these two patients had previously been exposed to DENV1 and were mounting recall responses that are cross-reactive to the current serotype of infection (DENV2) but more potently neutralize the original infecting serotype (DENV1).

This DENV1-biased phenotype of the mAbs was reminiscent of original antigenic sin (OAS). OAS is said to occur when memory-derived immune responses to an ongoing infection are skewed towards previously encountered antigens (reviewed in (7)). Due to the significant genetic similarities between the four DENV serotypes, cross-reactive memory B and T cells generated after a primary DENV exposure may get reactivated and dominate the immune response during secondary heterotypic DENV infection. This reactivation of cross-reactive memory responses has indeed been observed in the context of both T and B cell immunity to dengue (8, 9).

Although the T cell literature comparing primary and secondary dengue infections is relatively sparse, studies have shown that T cells induced after primary infection are largely type-specific (10), while cross-reactive epitopes dominate the secondary dengue $CD8^+$ T cell response (9-11). At present, whether such cross-reactive T cells are functionally protective or exacerbate dengue is not well defined. One group reported that cross-reactive $CD8^+$ T cells induced after secondary DENV infection are functionally impaired (low CD107a expression), but produce elevated levels of TNF-α (9), a pro-inflammatory cytokine that has been associated with increased dengue disease severity (12). In contrast, a different study reported no significant differences in the CD107a expression, cytokine profiles or avidity between type-specific and cross-reactive CD8+ T cells after secondary dengue (10), challenging the idea that OAS impairs CD8+ T cell-mediated protective responses. The contradictory results of these two studies might have been due to differences in study design. While the former study was performed using *in vitro* stimulated PBMCs from

secondary dengue patients shortly after infection, the latter was focused on *ex vivo* T cell analyses in healthy donors with previous dengue exposures. Given the inconsistent findings, additional human studies are required to clarify the impact of OAS on T cell mediated immunity in DENV infections.

In terms of humoral immunity, we and others have shown that cross-reactive antibodies dominate the B cell response after secondary DENV infections (1, 13, 14). These antibodies are generated, in part, due to the reactivation of cross-reactive MBCs that target epitopes shared between the previous and current DENV serotypes of infection. As MBCs are highly affinity matured, they are preferentially selected for expansion and cause the rapid production of cross-reactive antibodies early after infection. In contrast, naïve B cell responses specific to the serotype of infection might be delayed and lower in magnitude, resulting in relatively lower type-specific antibody titers in the serum. Previous studies have shown that secondary dengue immune sera have higher avidity (15, 16) and neutralizing titers (8) to a heterologous DENV serotype than the current serotype of infection. What remains unclear is whether the presence of such OAS phenotype antibodies diminishes the overall protective capacity of cross-reactive serum titers against ongoing and future DENV infections. In influenza, OAS has been implicated in reducing the effectiveness of sequential vaccination (17, 18). If the same is true for dengue, OAS may be an important consideration for ongoing and future vaccine design efforts given the widespread endemicity of dengue and high frequency of secondary DENV infections.

Cross-reactive poorly neutralizing antibodies have been hypothesized to contribute to the increased disease severity often associated with secondary dengue infections. One of the several mechanisms by which pre-existing antibody titers are implicated in exacerbating dengue disease is ADE. There are two types of ADE – the first, extrinsic ADE, refers to an increase in the number of DENV-infected cells in the presence of antibodies. Virus-antibody immune complexes can engage with FcγRs on the cell surface of susceptible cells, enhancing viral uptake (19). The second, intrinsic ADE, refers to the subversion of immune pathways within the infected cells that leads to a higher production of virus per cell (reviewed in (20)). The FcγR-dependent uptake of cells may suppress the anti-viral state of infected cells, resulting in greater viral replication (21-23).

In order to gauge whether plasmablast responses could potentially enhance viral infection through ADE, we tested the ability of the plasmablast-derived mAb panel to cause extrinsic ADE of DENV infection *in vitro.* We found that a large majority of DENV-reactive mAbs in our panel enhanced DENV infection of the FcγR-bearing U937 cell line. The physiological relevance of this data, however, is unclear. Among the several limitations of our approach, we used a human monocytic cell line as opposed to more relevant *in vitro* systems such as primary cells to study ADE. In addition, we only tested infection enhancement at a single antibody concentration. While this gave us a clue as to whether or not the mAbs could enhance DENV infection, it did little to explain the differences in ADE activities of mAbs with similar binding and neutralization profiles. A more thorough approach to study the ADE

capacity of mAbs would have been to determine enhancement at a range of concentrations. Lastly, our analysis was limited to examining the extrinsic ADE potential of the mAbs. To better understand how plasmablast-derived antibodies might participate in pathology, it would have been useful to determine whether infection in the presence of mAbs alters the immune status of infected cells. Nonetheless, the kinetics of viremia vs. induction of plasmablast responses leads us to believe that plasmablasts likely do not participate in ADE during an ongoing infection. As shown in **Figure 3**, viremia typically subsides before the peak in antibody responses in dengue. Therefore we hypothesize that the presence of high cross-reactive antibody titers in the serum, due to rapid the activation of plasmablasts, may contribute to ADE during future infections.

As described above, antibody responses in DENV infections play an important role in protection, but may also participate in disease pathology. In **Chapter 2**, we examined plasmablast responses in four secondary dengue patients in order to better understand the role of these cells and the antibodies they make during acute infection. By generating a panel of mAbs from patient plasmablasts, we determined the origin and functional characteristics of the acute phase B cell response to dengue. Our single-cell approach allowed us to dissect the properties of individual mAbs and appreciate the vast heterogeneity in their binding and neutralization patterns. In patients infected with the same DENV serotype who were experiencing similar levels of disease severity, such differences in the functional properties of the antibodies might have remained hidden in serum-based analyses. In addition, by focusing on plasmablasts as opposed to MBCs, we avoided the antigen-based

screening of cells for mAb synthesis and were therefore able to perform an unbiased analysis of the ongoing B cell response. Our findings provide a window into the B cell response during secondary dengue infection, and serve as a basis for future studies exploring the functional involvement of plasmablasts in dengue disease.

**Part II**

ZIKV is a re-emerging mosquito-borne flavivirus that has recently caused extensive outbreaks in Central and South America and the Caribbean. Given its association with Guillain-Barre Syndrome in adults and neurological and ocular malformities in neonates, ZIKV has become a pathogen of significant public health concern worldwide. ZIKV shares a considerable degree of genetic identity and structural homology with other flaviviruses, including DENV. The genetic relatedness between ZIKV and DENV may drive the generation or re-activation of cross-reactive antibody responses that target epitopes conserved between the two viruses. Studies of human sera from previous ZIKV outbreaks have provided a premise for ZIKV-DENV immunological cross-reactivity, by showing the presence of DENV-reactive titers in the sera of ZIKV patients. However, the serum-based approaches used in these studies could not rule out the possibility of prior or concurrent DENV exposure in the ZIKV patients. Furthermore, these studies failed to reveal the origin of DENV-reactive titers and the relative frequency and functional quality of virus-specific vs. cross-reactive responses.

In **Chapter 3**, we addressed the abovementioned issues and conclusively demonstrated that dengue-induced antibodies cross-react with ZIKV. We tested sera and a panel of plasmablast-derived mAbs obtained from patients with secondary dengue infection for functional cross-reactivity against ZIKV. We found that both acute and early convalescent dengue sera potently bind and neutralize ZIKV. Cross-recognition of ZIKV was evident at the monoclonal level as well, as a majority of the

DENV-reactive mAbs tested (26/47) bound to ZIKV antigens. Interestingly, despite high cross-neutralization titers at the serum level, only 7 of the 26 ZIKV-reactive mAbs also neutralized the virus *in vitro.* In addition, we showed that ZIKV-reactive dengue antibodies can enhance ZIKV infection of an FcγR-expressing cell line by ADE. The presence of dengue sera or mAbs enhanced ZIKV infection of U937 cells, and peak enhancement was observed at sub-neutralizing concentrations of the samples tested. The cross-recognition and neutralization of ZIKV by dengue antibodies, together with their capacity to enhance ZIKV infection, strongly suggest that pre-existing dengue immunity may modulate antibody responses to ZIKV infection.

Our findings vis-à-vis recent literature

In the past year, several other groups have characterized ZIKV and DENV induced antibodies in efforts to better understand the basis for and implications of immunological cross between these closely related viruses. Just as dengue sera can cross-react with ZIKV (24-26), sera from both DENV-naïve and DENV pre-immune ZIKV patients were shown to strongly bind DENV, with cross-reactive antibodies targeting both E and NS1 proteins (27). In addition, just as dengue plasmablast or MBC-derived mAbs bind and neutralize ZIKV (24-27), mAb panels generated from MBCs of DENV-naïve primary ZIKV patients have also been tested against DENV antigens to demonstrate ZIKV-DENV dual-reactivity at the single cell level (27). Therefore, the immunological cross-reactivity between DENV and ZIKV extends both ways.

Functional studies of mAbs have also allowed us to identify important targets for type-specific vs. cross-reactive antibody responses. In the ZIKV patient study described above, MBC-derived mAbs that bound NS1 were largely ZIKV-specific despite the high DENV NS1-reactivity exhibited by sera from the same donors. In addition, mAbs that bound EDIII, or whole virus but not E protein, were highly ZIKV-specific and potently neutralizing *in vitro*. In contrast, mAbs that were presumably EDI/EDII-specific, evident from their lack of binding to EDIII but recognition of the complete E protein, displayed cross-reactive binding to DENV but poorly neutralized ZIKV (27).

Studies analyzing dengue MBC or plasmablast-derived mAbs have shown that while binding to the virus is more ubiquitous, the potent cross-neutralization of ZIKV by dengue-induced mAbs appears to be a relatively restricted phenotype (24, 25, 28). Antibodies directed to the highly conserved and immunodominant FLE poorly neutralized ZIKV *in vitro* (28). In contrast, the dimer dependent EDE-specific mAbs were found to neutralize ZIKV potently (24, 26, 28). The recognition and potent neutralization of ZIKV by EDE antibodies suggests that quaternary and other complex epitopes may be important antibody targets in the ZIKV immune response. Mapping the epitopes of additional ZIKV-neutralizing mAbs, like 33.3A06 and other mAbs in our panel, may reveal novel antigenic sites critical for protection and also inform future vaccine development efforts.

We and others have demonstrated that the ADE capacity of dengue-induced antibodies can also extend to ZIKV (24, 25). These studies are important from an epidemiological perspective, as the vast majority of regions that have reported ZIKV cases also experience DENV outbreaks. While timely, the findings of these studies are not entirely surprising, given the significant biological similarities and abundant epitopes shared between the two viruses. In one of these studies, EDE mAbs, shown to potently neutralize all 4 DENV serotypes (1) as well as ZIKV (24, 28), also enhanced ZIKV infection *in vitro* by ADE. However, incubating neutralizing concentrations of specific EDE mAbs with enhancing concentrations of polyclonal dengue sera reduced infection of FcγR bearing cells. In contrast, the presence of poorly neutralizing FLE mAbs did not abrogate the enhancement of infection by serum antibodies (24). These data suggest that the neutralization potential of antibodies targeting certain epitopes, such as EDE, may impede the ADE effect of enhancing antibodies, emphasizing the possible advantages of epitope-based vaccine design.

In addition to the *in vitro* analyses described above, the protective capacity of several murine and human ZIKV-reactive mAbs has also been assessed *in vivo*. The fusion loop-specific murine mAb 2A10G6 was found to confer *in vivo* protection from ZIKV infection, albeit at a suboptimal dose of 500 μg (29). In another study, the EDIII lateral ridge-specific murine mAbs ZV-54 and ZV-67 protected mice from lethal challenge (30). Unlike 2A10G6, the EDIII mAbs were ZIKV-specific and did not

bind to DENV *in vitro*. Such ZIKV-specific mAbs may possess a selective advantage over broadly-reactive mAb in their inability to induce ADE of DENV infection.

The human MBC-derived mAb ZIKV-117 was evaluated for its prophylactic and therapeutic efficacy in a pregnant and non-pregnant mouse model. In addition to reducing mortality in wild type adult mice, in the fetal transmission model, administering ZIKV-117 decreased placental injury, reduced ZIKV infection of placenta and fetal tissue, and improved fetal outcome overall. Epitope mapping of ZIKV-117 suggested that the mAb binds a quaternary epitope on the E protein dimer-dimer interface (31). Another group generated a panel of mAbs from a convalescent ZIKV patient and of the mAbs isolated, Z23 and Z3L1 demonstrated potent ZIKV-specific *in vitro* neutralization and protected mice from weight loss and mortality after ZIKV infection. While Z23 was mapped by cryo-EM to bind to EDIII, Z3L1 appeared to make contact primarily with EDI residues (32). Unlike the above mAbs, EDE-specific mAb C10 was isolated from a DENV-experienced but presumably ZIKV-naive donor, and was also shown to protect mice from lethal challenge (26). While these results are immensely promising, additional studies are required to dissect the mechanism of neutralization of these various mAbs. Moreover, several of these aforementioned studies were performed in immunocompromised mice, and therefore a more physiologically relevant characterization of their prophylactic and/or therapeutic potential merits further investigation in macaque models.

Sero-epidemiological data from historical ZIKV outbreaks as well as the findings of recent human serum-based studies may also provide added insight to the protective potential of cross-reactive B cells against ZIKV infection. As demonstrated by *in vitro* studies, secondary dengue patient sera can strongly neutralize ZIKV while primary DENV sera exhibit limited cross-neutralization activity (25, 26). This could be explained by the differences in longevity of type-specific vs. cross-reactive antibodies after DENV infection. Type-specific responses after primary infection are believed to be long-lived, while cross-protective immunity can wane months after infection. Secondary heterotypic DENV exposures, on the other hand, may boost cross-reactive antibody production by reactivating cross-reactive MBCs, potentially resulting in broader, albeit short-lived, neutralization capacity.

Additionally, while acute or early convalescent (≤100 days after symptom onset) secondary sera potently neutralize ZIKV (as shown in **Chapter 3**), other studies report that late convalescent sera exhibit poor to moderate ZIKV neutralization (24-26). These data suggest that while recently DENV-exposed individuals may maintain protective antibody titers against ZIKV, DENV-seropositive individuals exposed to ZIKV years after their dengue exposure may not benefit from effective cross-protection. These findings are consistent with the health outcomes of past and current ZIKV outbreaks. French Polynesia and Brazil, both countries with high DENV seroprevalence, experienced significant ZIKV epidemics with adverse clinical presentations. Cases of birth abnormalities and severe disease have also been reported in numerous DENV-endemic areas (33-35) suggesting that prior DENV

exposure may not protect against future ZIKV infections. Future studies comparing naïve vs. recall human responses may help clarify the role of pre-existing cross-reactive antibodies during ZIKV disease, shedding more light on the protective/pathological potential of DENV-immunity in the context of ZIKV infection.

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## Appendix

### Studies on dengue B cell response and pathogenesis in children from India

**Analysis of B cell immunity in dengue virus infected children from India**

Anmol Chandele, Kaustuv Nayak, Mohit Singla, Sivaram Gunisetty, Siddhartha Kumar Bhaumik, **Lalita Priyamvada**, HareKrushna Panda, Pragati Sharma, Elluri Seetharamireddy, Charu Agarwal, Guruprasad R Medigeshi, Hasan Ahmed, Rustom Antia, Aftab Ansari, Rakesh Lodha, Sushil Kabra, Rafi Ahmed, Kaja Murali-Krishna

This manuscript is being prepared for submission.

LP contributed to this study by performing the serum FRNT50 neutralization assays and analyzing the data. LP also assisted with writing and reviewing the manuscript.

**Abstract**

India suffers disproportionately with highest human dengue infections worldwide. However, immune responses in these patients are not well understood. Numerous studies show antibody-types indicative of second dengue-infection (IgG alone or IgM/IgG < 1.2) associate with severe disease compared to antibody-types indicative of primary dengue (neither IgG nor IgM or IgM / IgG > 1.2). This study in dengue-infected children from India would have made exactly identical conclusion if we were to ignore time of illness in the analysis. However, we revise our interpretation because our B cell immunity studies showed patients with undetectable humoral antibodies at the beginning of illness, and hence would have been classified as primary dengue, can rapidly elicit a massive plasmablast expansion and convert to an IgG dominated humoral profile; and hence would have to be classified as secondary a day or two later. We show that dengue case frequencies were virtually independent of humoral IgM/IgG ratios; instead increased with time after illness concomitant with evolution of effector B cell response. Our study calls for a re-evaluation of the impact of B cell immunity on dengue pathogenesis in a time dependent and region-specific manner.

**Introduction**

Dengue is the most common and fastest-spreading mosquito-borne viral disease that is becoming a global epidemic. Nearly forty percent of the world population is living under the risk of transmission by one or more of the four dengue virus (DENV) serotypes. Approximately 390 million human dengue infections are estimated to occur annually with 100 million clinical disease cases, the consequences of which range from asymptomatic infection, fever, dengue warning signs, and much more severe manifestations of dengue disease that includes dengue hemorrhage fever (DHF), dengue shock syndrome (DSS) and or death, especially among children.

Numerous studies suggest that people with secondary heterologous dengue infections are at an increased risk of developing these severe forms of dengue disease (1-8), although exceptions exist (9-14). Studies from all over the world show antibody-types indicative of second dengue-infection (IgG alone or IgM/IgG < 1.2) associate with severe disease compared to antibody-types indicative of primary dengue (neither IgG nor IgM or IgM / IgG > 1.2). Enhancement of infection by pre-existing antibody induced by prior exposure to a heterologous dengue viral serotype has been proposed as a major mechanism to explain this increased disease severity in secondary infections, but other mechanisms such as host or viral factors, inflammation through Fc receptor engagement and T-cell mediated cytokine storm are also being investigated.

Although many South East Asian countries have reported dengue epidemics for more than a century, dengue is relatively recent in India (15). India, the home for nearly 1/5$^{th}$ world population, experienced the first major dengue epidemic only in 1996 (16)and rapidly acquired the status of the country with highest number of dengue infections worldwide in this short time. Recent epidemiological studies provide an estimate of 30 million annual human dengue infections in India (17). Despite the many elegant studies from across the globe, there is minimal understanding of immunological process in these patients (18, 19). Therefore, studies in India are important not only for understanding this rapidly growing public health problem, but also for gaining an insight on human immunological processes occurring in geographical locations that have started experiencing dengue relatively recent. This study describes our understanding of B cell responses in the dengue infected children in New Delhi, India, studied in years 2012-2016.

## Methods

**Dengue patient recruitment and blood collection:** The clinical site for this study is Pediatric Department of the All India Institute of Medical Sciences (AIIMS), New Delhi, India. The study was approved by the institutional ethical committee, and Informed consent was obtained from the study participants. Patients included in the study were negative for malaria antigen, and chkungunya IgM. The WHO 1997 classification was used to characterize disease grade as dengue infection (DI), dengue warning signs (DW) and severe dengue (SD). Samples from young adults (18-25 years) were used as controls.

**PBMC and plasma Isolation:** Approximately 1 – 5 ml of blood sample was collected in Vacutainer CPT tubes (Becton Dickenson) and immediately transported in a secondary container to the laboratory. The PBMC's and plasma were separated according to the manufacturer's instructions. The plasma was frozen at -80$^{o}$C in aliquots and later used for Dengue-specific ELISA's and neutralization assays. The PBMC's were washed extensively in 1% complete RPMI (RPMI containing 1% FCS, 1X Pen/Strep, 1X Glutamine) and used immediately for analytical flow cytometry and *ex vivo* Dengue-specific ELISPOT assays.

**Flow cytometry staining and analysis:** Extensively washed PBMC's were stained with fluorochrome labeled antibodies and fixable viable dye eFluor 780 (ebioscience) for live dead exclusion. For plasmablast analysis, cells were stained with CD19 (BD, 562294), CD20 (BD, 347647), CD38 (BD, 562288) and CD27

(Ebioscience, 17-0249). Plasmablast staining was analyzed using Flow Jo software (TreeStar Inc) starting with a lymphocyte gate that included the blasting cells and plasmablasts were described as cells that are $CD3^-$, $CD20^-$, $CD19^{+/Int}$, $CD27^+$ and $CD38^+$.

**Dengue-specific ELISpot assay:** Dengue-specific ELISpot assay was performed as previously described (20). Briefly, 96-well ELISpot plates (Millipore, MAHA N4510) were coated overnight with either Dengue 2 fixed virus antigen (Microbix, EL-22-02) or polyvalent total immunoglobulin (Invitrogen, H1700). Before adding PBMC's, the plates were washed thoroughly and blocked for 2 hrs at 37$^o$C with RPMI containing 10% FBS. Extensively washed $0.1X10^6$ PBMC's were then added to the plate and a 3-fold dilution was performed till the last well had a cell number of 45 cells and incubated overnight at 37$^o$C undisturbed. The cells were removed; plates were washed with 1X PBS+0.05% Tween 20 and developed with Goat anti-human IgG Biotin (Invitrogen A18809) and Avidin HRP (ebioscience, 18-4100). The spots were developed with AEC substrate (BD, 551951) and scanned using an automated ELISpot counter (CTL, Cellular Technologies LTD).

**Dengue virus neutralization by focus forming assay:** The neutralization capacity of plasma samples against DENV1-4 was determined by a focus forming assay (FFA). Frozen plasma was thawed at room temperature and heat inactivated at 56$^o$C for 30 min. Serially diluted plasma (1:100-1:102400) was incubated with 100 focus forming units of virus for 1h at 37$^o$C. Vero cell monolayers were infected with the

serum-virus mixture for 1 h at 37$^{o}$C. An overlay containing 2% methylcellulose was added to the infected cells. After 72 h, cells were fixed and stained with anti-flavivirus mAb 4G2 (Millipore; MAB10216) followed by HRP-linked anti-mouse IgG (Cell Signaling; 7076S). Foci were developed using TrueBlue Peroxidase (KPL; 50-78-02). $FRNT_{50}$ was calculated where 50% reduction in foci were observed.

**Dengue-specific ELISA for IgM and IgG:** Dengue IgM and IgG antibodies were detected by capture ELISA using the Dengue capture Elisa kits (Alere, 01PE10 / 01PE20) in patient sera at 1:100 dilution. As per WHO guidelines, Patients were categorized as primary dengue infections if they did not show any detectable levels of serum IgM and IgG (seronegative) or had IgM/IgG ≥ 1.2. Patients with serum IgM/IgG ≤ 1.2 were classified as secondary infections.

**Direct ELISA for detection of Dengue / Flavi-reactive IgG antibodies:** Dengue IgG endpoint titers were determined using direct ELISA method. Microtiter plates were coated over night at 4$^{o}$C with Dengue fixed virus antigen (Microbix, EL-22-02-001). The plates were washed extensively with 1XPBS+0.1% Tween 20 and serially diluted patient plasma was added to the plates and further incubated at RT for 1 hr. After incubation, the plates were washed and dengue specific IgG was detected by anti-human IgG biotin (Ebioscience 13-4998-83) followed by Avidin HRP (Ebioscience 18-4100-51) and TMB as substrate (Sigma T4444).

**Statistical Analysis:** All data was tabulated using MS Excel and analyzed using

GraphPad prism software. For analysis of groups, unpaired two-tailed t test was used to determine statistical significance and p values were interpreted as * p=0.05; ** p=0.01; *** p=0.001 and **** p=0.0001.

**Results and Discussion**

We analyzed a total of 154 children recruited at All India Institute of Medical Sciences (New Delhi, India). Patient characteristics are described in **Table 1**. Infants below 1.2 years were excluded from the study. All the children included in this analysis have been confirmed as dengue cases by positive test for plasma dengue NS1 and or dengue-specific IgM/ PCR. Viral serotype could be determined in 102 patients and a vast majority of these (>92%) were dengue virus (DENV)-serotype-2. Blood samples were collected on the day of first reporting to clinic. Clinical diagnosis at the time of first reporting showed 22% patients had febrile fever induced by dengue infection (DI), 32% had moderate disease indicated by dengue warnings (DW), and 44% had severe dengue (SD) including hemorrhage fever grade 2/ 3 and or shock (SD). Eight of these 154 children (5.19%) died. In some patients a second sample was collected 48-72hr later, but clinical evaluation at the time of second sampling was not considered for this analysis because the disease progression under clinical care is not likely reflective of natural setting.

To determine the status of dengue-specific humoral immunity, we performed capture ELISAs **(Figure 1A)**. The antibody index values varied substantially from patient to patient. As per WHO guidelines nearly half of the patients fell into primary infection category ($IgM^-IgG^-$, henceforth referred as seronegative, SN, or IgM/IgG >1.2, henceforth referred as IgM >IgG) and the other half fell into secondary infection category (IgM/IgG < 1.2, referred henceforth as IgG >IgM).

Unexpectedly, we discovered that there is an inverse relation in the distribution of these primary versus secondary classified cases when analyzed as a function of day of illness **(Figure 1B, left graph).** If the primary and secondary dengue infections in these patients are truly half and half (as indicated by capture ELISAs described above), one would expect that nearly 50% of the patients analyzed day 2 or earlier should also be IgG alone or IgG > IgM humoral profile. But this was not the case. Only 3 out of the 25 (12%) patients at day 2 or earlier showed IgG > IgM profile; whereas the frequency of these cases increased with time (32%, 56%, 64%, 70%, 90% by days 3, 4, 5, 6 and 7-11 respectively). Interestingly, this time dependent rise in IgG > IgM cases was also in parallel with a rise of IgM > IgG cases, but only up to day 5, afterwards the IgG dominating cases seems to overtake the IgM dominating cases (Frequency of IgM > IgG cases 4%, 21%, 28%, 32%, 18%, 9% at days <2, 4, 5, 6 and 7-11 respectively). Further analysis showed that most of these primary phenotype cases that segregate to earliest days of illness were SN **(Figure 1B, right graph)**. The shift from SN to IgM> IgG as well as IgG> IgM cases was rather dramatic between 2$^{nd}$ and 3$^{rd}$ day suggesting something is happening very fast in this window of time.

In order to explain this unexpected phenomenon, we considered the possibility that younger children, who are expected to enrich for primary infections (4, 6, 21, 22), may be preferentially reporting to the clinic at early days of fever. However, age distribution was not strikingly different as a function of the day of illness **(Figure S1).**

To address whether the IgG dominated humoral profiles that we preferentially see in these later day cases is a reflection of their ongoing effector B cell response or simply a reflection of humoral memory induced by prior infections, we analyzed blood circulating plasmablasts of these patients (**Figure 2A**)**.** In the SN patients, the plasmablast frequencies were very similar to those of healthy subjects irrespective of the day of illness **(Figure 2B top panel**); and these plasmablasts from these SN patients were secreting neither dengue specific IgM nor IgG, with an exception of two patients in who a very low frequency of IgG ASC were seen (**Figure 2C top panel).** By contrast, in both the IgM > IgG or IgG > IgM humoral cases, the plasmablasts showed massive expansion **(Figure 2B middle and lower panels)**. The expansion was preferentially higher in the patients analyzed at later days, peaking between day 4-5, and these plasmablasts were actively secreting dengue-specific immunoglobulins **(Figure 2C middle and lower panels).** Thus, the humoral immunity detected preferentially in these later day patients is indeed a reflection of their ongoing immune response. Interestingly, we found that even in the patients with humoral IgM > IgG profile, there were more of the dengue specific IgG ASC than IgM ASC; suggesting that, perhaps, these patients IgM > IgG humoral profile may be poised to convert to an IgG dominated humoral profile eventually.

These intriguing time dependent inverse distribution of the primary versus secondary phenotypes, and the rise of both IgG > IgM and IgM > IgG profiles increasing in parallel at later days, prompted us to ask what kind of B cell response

actually evolves from these SN patients that preferentially segregate to early days of illness. Serum capture ELISAs showed that seven of the 22 of these SN patients remain SN 48 - 72 h later (here after referred as SN→SN) (**Figure 3A**), six of the 22 of these SN patients convert to an IgM > IgG humoral phenotype (here after referred as SN→IgM > IgG) **(Figure 3B)**, and nine of the 22 of these SN patients convert to an IgG > IgM humoral phenotype (here after referred as SN→IgG> IgM) **(Figure 3C).** Wherever the IgG dominated humoral response is evolved, dengue neutralizing antibodies were also visible **(Figure 3A, B, C middle panels).** There was no preferential evolution of NT Abs against the infecting serotype, and the plasmablast expansion was more pronounced irrespective whether the patient converted to an IgM > IgG or IgG > IgM humoral profiles. We did not have sufficient cells to perform paired $1^{st}/2^{nd}$ bleed ASC assays for every patient presented in **Figure 3**, but ASC assays in 6, 3 and 7 patients in each of the SN→SN, SN→IgM> IgG and SN→IgG> IgM groups respectively **(Table 2),** confirmed that that the dengue-specific ASC numbers remain low in the second bleed of the SN→SN group except in one patient in who the IgG ASC increased dramatically, whereas the ASC increase in the second bleed of both the SN→IgM> IgG and SN→IgG> IgM groups. Interestingly, we found that usually the IgG ASC were more than IgM ASC irrespective of whether the shift in humoral profile is SN→IgG > IgM or in SN→IgM > IgG. This suggested that these plasmablasts in the SN→IgM > IgG patient might be already conditioning the host towards conversion into an IgG dominated humoral profile. From these data, we conclude that many of these patients with an undetectable humoral IgM and IgG in the early days of fever, and hence would have been classified as primary dengue, can

elicit a rapid and massive plasmablast expansion whose ASC functions are already shifting to an IgG dominated isotype, and, as a consequence, these patients, rapidly convert to an IgG dominated humoral profile. If we did not know these results, we would have wrongly classified the same patients as primary at onetime point, an as secondary 48-72hr later, depending upon when the patient is examined.

In numerous situations an IgG dominated humoral profile is the default expectation for a recall humoral responses. However, there are situations in humans where an IgG dominated response can occur during first time infection. Vigorous immune response in people infected with yellow fever virus, vaccinia virus or ebola viruses can induce an IgG dominated response (23-25). One would not consider these as secondary infections simply because there is an IgG dominated response. Dengue researchers, however, heavily rely on these measures. Our results above cautions against solely depending upon these measures without taking the time of illness into consideration. Moreover, the rules for an IgG dominated versus IgM dominated response may also change depending upon the infecting viral serotype or the geographical region (13). It is possible that these SN patients in India that would convert to an IgG > IgM humoral phenotype may have been truly exposed to dengue or other related flavivirus, but the circulating memory antibodies induced by such prior infections may not have been picked up by the capture ELISAs. Our experiments to address this showed low levels of dengue/ flavi binding antibodies in some but not all of these SN patients (**Figure 4**). However, no particular pattern was seen with regards to presence of these flavi-reactive antibodies and the

evolution of IgG dominated or IgM dominated humoral profiles in the second bleed. The first bleed of only five of the nine SN→ IgG> IgM group showed these flavi binding antibodies, albeit only marginally higher than the levels detected in flavi-negative healthy subjects. Interestingly, we found that, the first bleeds of two of the six SN→ IgM> IgG group also had these antibodies.

Considering the above data showing time dependent alterations of the humoral profiles in these patients, it is of interest to evaluate how disease severity behaves as a function of time and as a function of humoral profiles. If we perform the analysis without time consideration and by classifying all the SN and IgM > IgG cases combined as primary and all the IgG > IgM cases as secondary, as was done by numerous studies from all over the world that concluded severe disease association with secondary infections (1-8), our data would lead to an exactly identical conclusion (**Figure 5A).** However, considering that a vast majority of the SN cases segregate to early days of fever, we analyzed these data by grouping the patients as SN, IgM > IgG and IgG > IgM (**Figure 5B**). Surprisingly, by this analysis, we find that the frequency of severe disease cases was lowest only in the SN group (a majority of who segregate to earliest times of illness), but strikingly higher in both IgM > IgG and IgG > IgM groups. More importantly, the severe disease case frequency was virtually identical between these IgM > IgG and IgG > IgM groups. By further stratifying the patients based on the day of illness, we find that the frequency of severe disease cases increases as a function of the day of illness (**Figure 5C**). Thus, time after infection is more important than IgM/IgG ratios for severe dengue

disease in these patients. Analysis of the effector B cell response as a function of time and disease severity showed that the severe disease cases tend to have higher plasmablasts and the consequent humoral antibody at early days of fever (**Figure 5D)**. This is particularly evident on day 3 of illness where in the B cell responses are just beginning to pick up in these patients. However, at later times (day 3-5) when these responses begins to peak, these differences become moderate. One must use caution against over interpreting these results, especially at day 2 or at beyond day 4 because the DW/ SD cases were preferentially fewer at very early days and the DI cases become fewer at late days. Moreover, several host viral interactions, including viral antigen, innate and adaptive CD4, CD8 T cell responses as well as B cell responses dynamically change over time; and many of these are likely to be playing a role to influence disease outcome depending on the time of illness. Hence, further in depth studies are needed gain a holistic understanding of these.

The scientific research for understanding of dengue epidemiology, pathogenesis and human immunity has picked up since the 1780 dengue outbreak in Philadelphia. Sixty five years ago, Sabin, by experimentally infecting humans, demonstrated that people infected with one dengue serotype can be protected against re-infection by all the four dengue serotypes. But this protections wanes over a period of time, and these people that were immune to dengue can indeed be infected with a heterologous but not homologous dengue serotype (26). Although Sabin's experiments did not show any increased disease severity in this situation of this experimental heterologous secondary dengue infection of the humans, subsequent

epidemiological studies in Thailand during the early 1960s when dengue epidemics were becoming more frequent there, indicated that children experiencing a secondary dengue infection are more likely to experience severe dengue disease (2, 6). Elegant studies from Halstead et al and Russel et al, in early 1960s showed that patients with dengue specific hemagglutinin inhibiting/ neutralizing or IgG antibodies, and hence considered to be likely to be second dengue infections, tend to have more severe dengue disease (4, 27). These observations, combined with laboratory studies indicating that antibodies can enhance dengue infection *in vitro* (28), and the epidemiological studies suggesting that dengue infected children with maternally transmitted dengue antibodies tend to show more severe disease (although there are conflicting reports regarding on this), led to the hypothesis that pre-existing memory antibodies induced by a previous heterologous dengue exposure can enhance infection in vivo there by leading to severe dengue disease in the secondary dengue infections. In parallel to these studies, immense efforts were made for developing capture ELISAs to distinguish dengue specific serum IgM/IgG. World Health Organization provided guidelines for classifying primary and secondary dengue infections based on these serum IgG/ IgM ratios measured using the capture ELISAs. Using these measures, till date, over 100 studies from all over the world (9, 11), some from India (29), analyzing tens of thousands of patients, strengthened this hypothesis and led to the concept that severe dengue disease is really associated with secondary dengue infections (29-35). The current dengue vaccination strategies and their implementation efforts are largely dependent upon these concepts. Basing upon these concepts and the geographical differences in the

frequency of primary/ secondary infections that were measured based on the serum IgM/ IgG ratios, most recent community modeling studies provide guidelines for altering vaccine implementation policy (9); which if implemented will have important real life consequences globally. Considering these points, this study in dengue infected children from India is important and timely. In these patients, we show that time of after illness is a cofounding factor in distinguishing primary secondary dengue infections using serum IGM/ IgG ratios, these IgG/ IgM ratios have no relation to dengue disease severity, it is the time after illness that determines the frequency of severe disease cases, and that the effector B cell response and humoral immunity that evolves as a function of time is a risk factor for severe dengue disease, although other immune mechanisms that also evolve as a function of time likely to play a role. We do not know whether these phenomenon that we observed from the patients from India applies to patients in other geographical regions of the world. Interestingly, we noticed that even the 1960s study in which Halstead first proposed the association of severe dengue in secondary infections, there was data suggesting preferential segregation of primary dengue cases to early days of fever, although Halstead has not taken this factor into consideration in interpreting the results, probably because the methods to distinguish IgM and IgG were lacking at that time (36). Considering these factors, we urge the global dengue research community to carefully consider our observations and re-evaluate the preferential association of dengue with antibody types indicative of second infection.

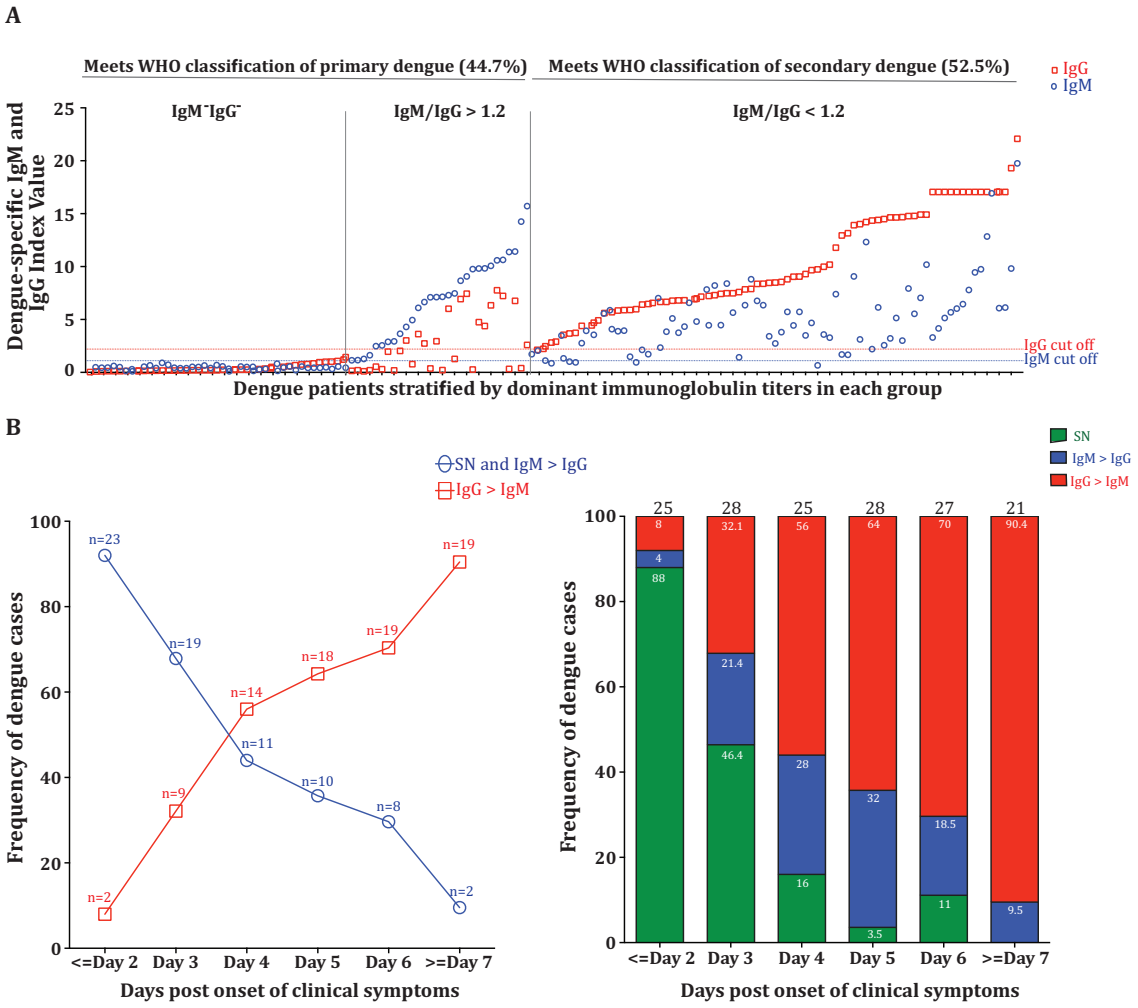


*Figure 1*

**Figure 1: Characterization of humoral immunity in dengue patients from India.**

**A.** Plasma samples derived from pediatric patients at the time of reporting to the clinic were analyzed for dengue-specific IgM and IgG by commercial capture ELISA. Patients are stratified based on the levels of Dengue-specific IgM (blue open circle) and IgG (red open square) within each group defined by WHO guidelines into primary infection category ($IgM^-IgG^-$, henceforth referred as

seronegative, SN, or IgM/IgG >1.2, henceforth referred as IgM>IgG) and secondary infection category (IgM/IgG < 1.2, referred henceforth as IgG>IgM).

**B.** Line graph on the left shows frequency of dengue cases presenting as primary dengue infection (blue open circle & blue line) and secondary dengue infection (red open square and red line) as a function of time post onset of clinical symptoms.

Stacked bar graph on right shows frequency of dengue cases that presented to the clinic as SN (green bar), IgM > IgG (blue bar) and IgG > IgM (red bar) as a function of time post onset of clinical symptoms. Numbers within each stack indicate frequency of cases.

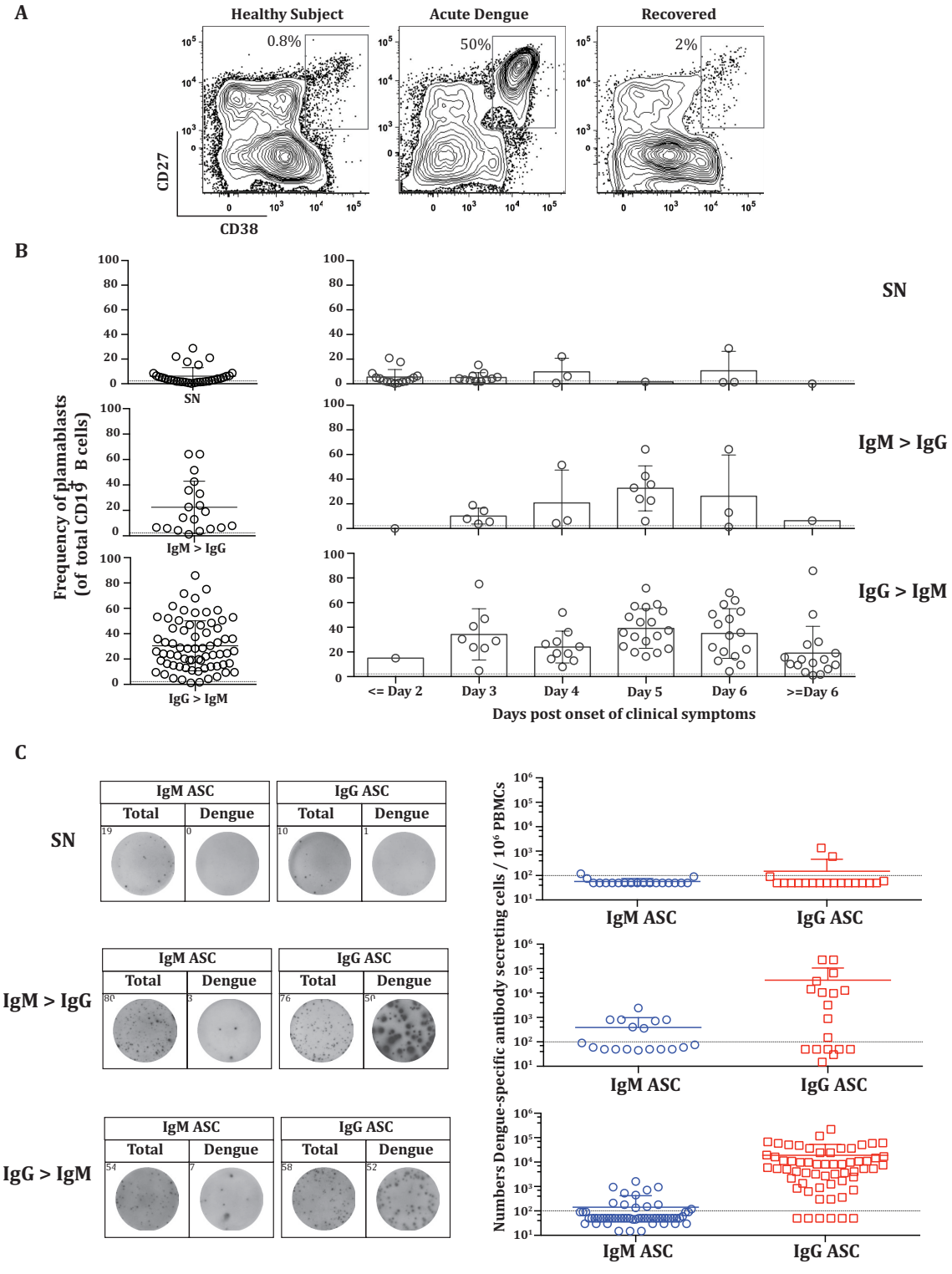


*Figure 2*

**Figure 2: Analysis of the blood circulating plasmablasts and antibody secreting cells in dengue patients with different humoral profiles.**

**A.** Dot plots show flow cytometry analysis of blood circulating plasmablasts in a healthy subject (left), dengue patient (middle) and dengue recovered (right).

**B.** Scatter plots show cumulative plasmablast response (left) and as a function of time (right) in dengue patients with SN (top), IgM > IgG (middle) and IgG IgM (bottom) humoral profiles.

**C.** Left panel shows representative ELISpot pictures and right graphs show absolute numbers of dengue IgM (blue circles) and IgG (red squares) antibody secreting cells in patients with humoral profile of SN (top), IgM > IgG (middle) and IgG > IgM (bottom).

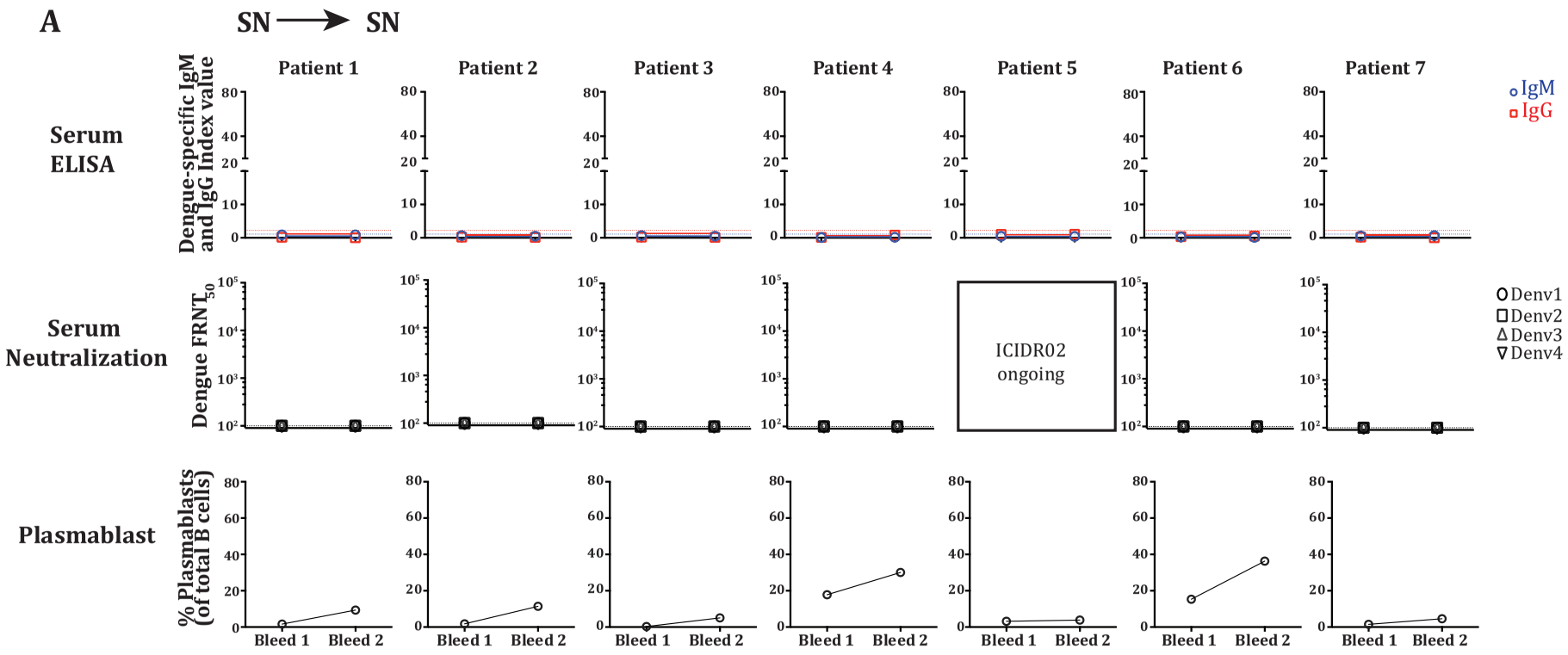
A
SN → SN
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Patient 6
Patient 7
Serum ELISA
Dengue-specific IgM and IgG Index value
IgM
IgG
Serum Neutralization
Dengue FRNT50
ICIDR02 ongoing
Denv1
Denv2
Denv3
Denv4
Plasmablast
% Plasmablasts (of total B cells)
Bleed 1
Bleed 2

*Figure 3A*

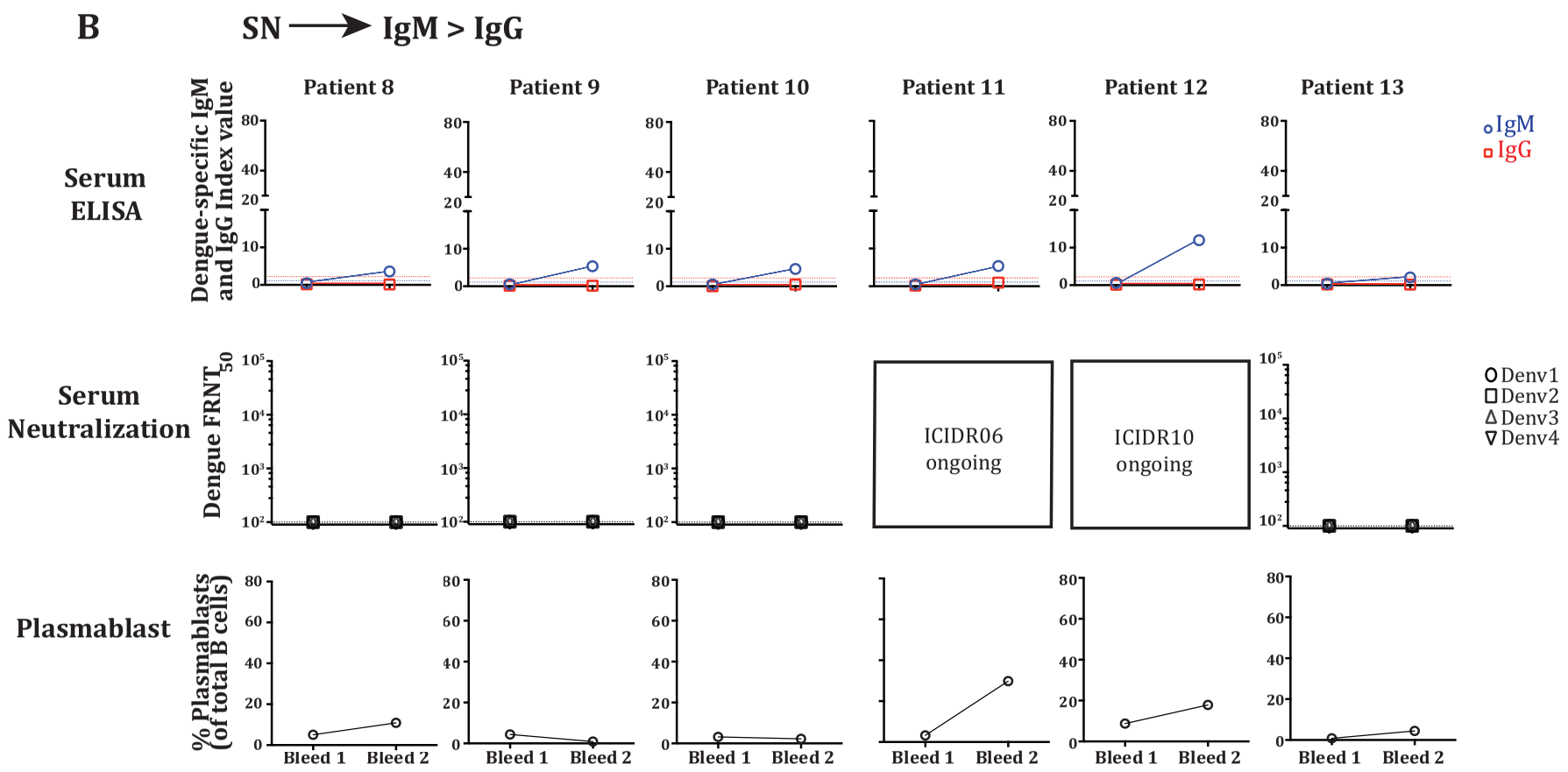
B
SN ⟶ IgM > IgG
Patient 8
Patient 9
Patient 10
Patient 11
Patient 12
Patient 13
Serum ELISA
Dengue-specific IgM and IgG Index value
IgM
IgG
Serum Neutralization
Dengue FRNT50
ICIDR06 ongoing
ICIDR10 ongoing
Denv1
Denv2
Denv3
Denv4
Plasmablast
% Plasmablasts (of total B cells)
Bleed 1
Bleed 2

*Figure 3B*

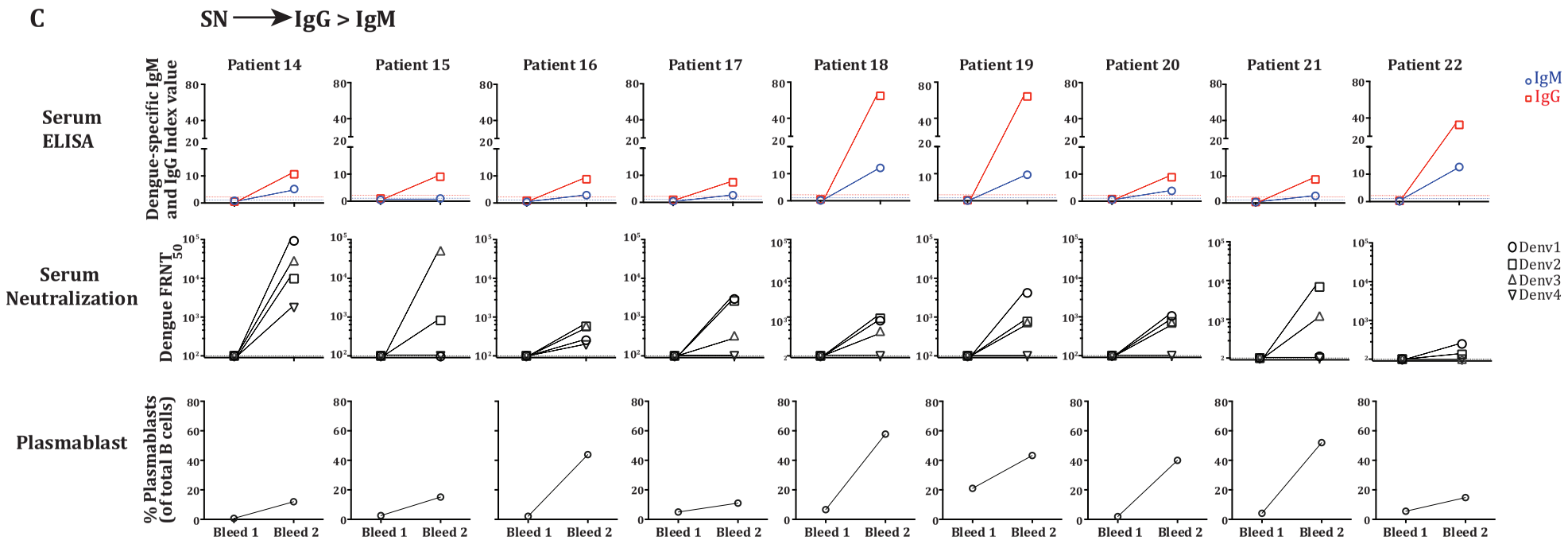
C
SN ⟶ IgG > IgM
Patient 14
Patient 15
Patient 16
Patient 17
Patient 18
Patient 19
Patient 20
Patient 21
Patient 22
Serum ELISA
Dengue-specific IgM and IgG Index value
IgM
IgG
Serum Neutralization
Dengue FRNT50
Denv1
Denv2
Denv3
Denv4
Plasmablast
% Plasmablasts (of total B cells)
Bleed 1
Bleed 2

*Figure 3C*

**Figure 3: Time is a confounding factor in classification of primary and secondary dengue infections.** We analyzed a subset of patients that presented as SN at a second time point within 48-72 hrs of the first sample collection. Serum capture ELISA (top row), neutralizing antibody responses (middle row) to Denv 1 (open circle); Denv 2 (open square); Denv3 (open triangle); Denv 4 (open inverted triangle) and plasmablast response (bottom row) is shown in patients that on the second sampling either remained SN **(A);** or seroconverted to IgM > IgG **(B)** or to IgG > IgM **(C).**

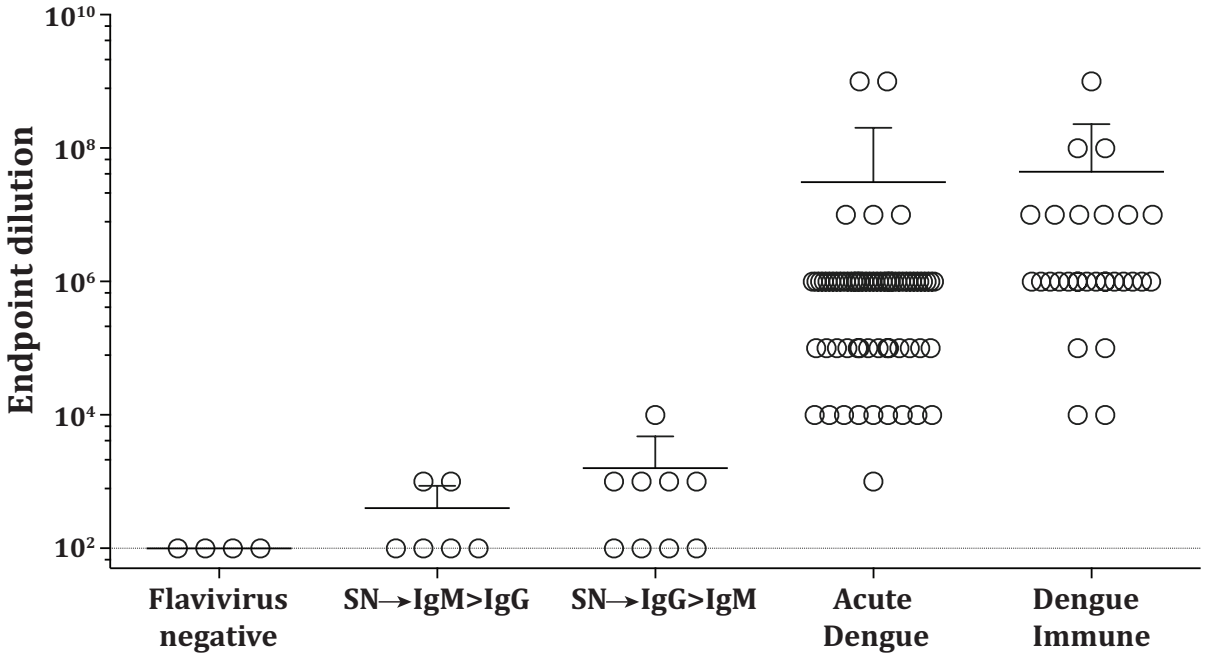


*Figure 4*

**Figure 4: Analysis of Flavi-reactivity in dengue patients.** Scatter plot shows endpoint dilution by indirect ELISA with fixed virus dengue antigen in groups that seroconverted to IgM > IgG or IgG > IgM and is compared to flavi-naïve healthy individuals, acute dengue patients and dengue immune individuals.

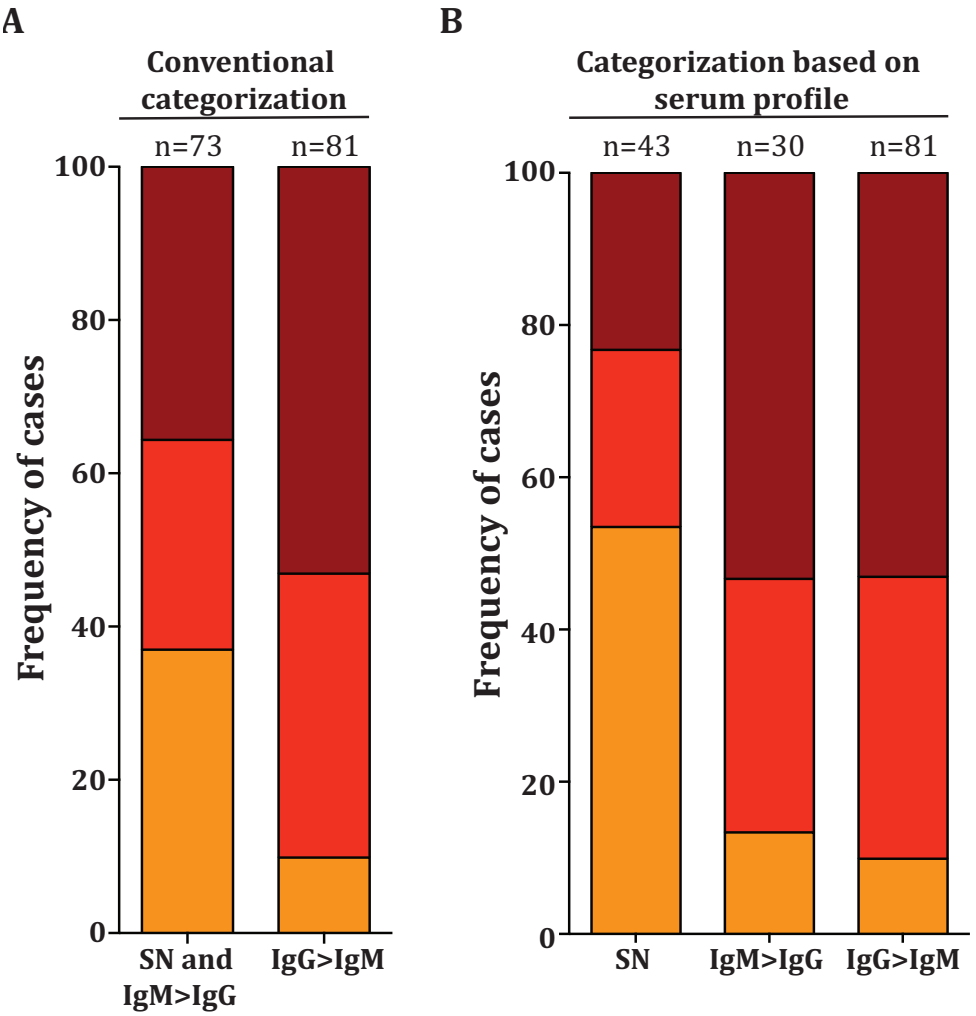


*Figure 5A, B*

**Figure 5: Evolution of severe disease during dengue is a function of time**

**A.** Stacked bar graphs show frequency of cases with dengue infection (orange bar); dengue with warning (red bar) and severe dengue (maroon bar) in SN +

**B.** IgM > IgG that are classified as primary infections and IgG > IgM that are classified as secondary based on WHO guidelines.

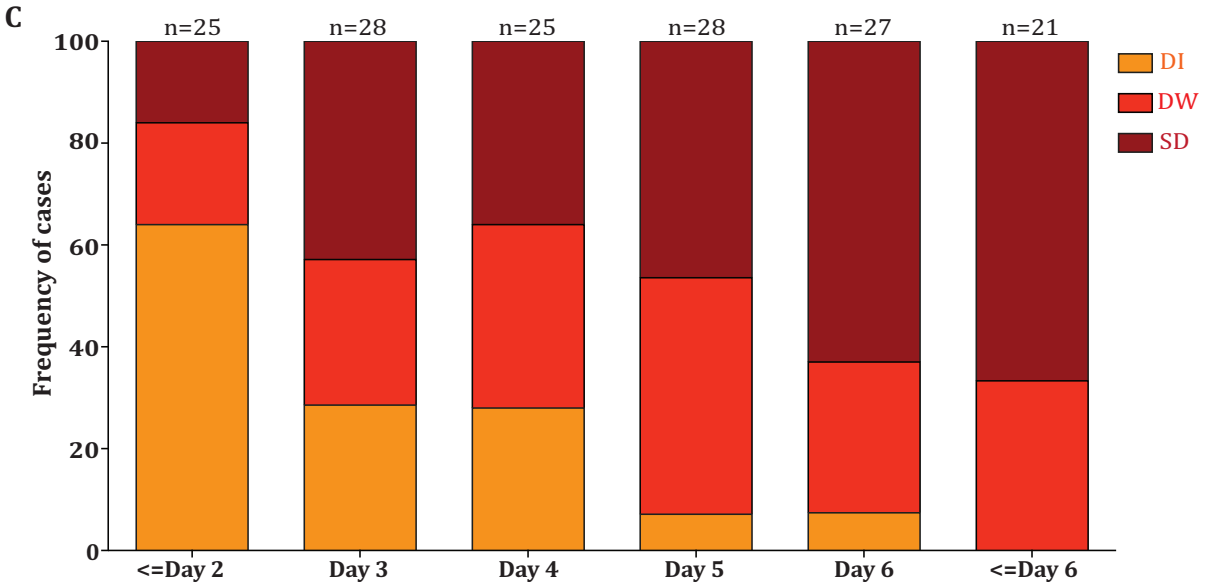


*Figure 5C*

**C**. Stacked bar graphs show frequency of cases with dengue infection (orange bar); dengue with warning (red bar) and severe dengue (maroon bar) after separating primary dengue infections from the previous analysis into SM, IgM > IgG and comparing to IgG > IgM as indicated on the x-axis. This data reveals that only SN group has very few severe dengue cases, however there is no difference in presentation of severe disease after sero-conversion to either IgM > IgG or IgG > IgM that are otherwise typically regarded to result in more severe dengue.

**D**

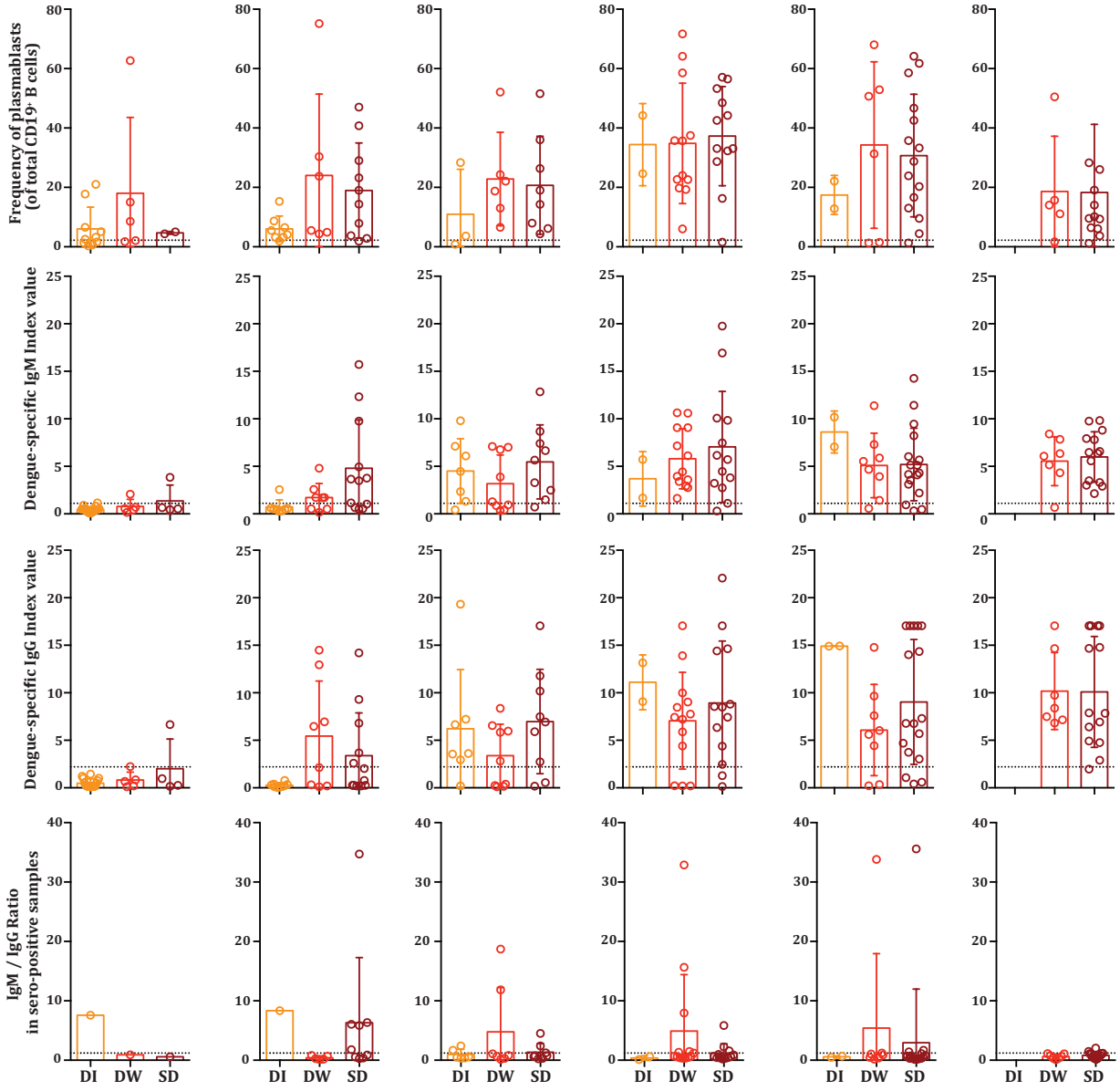


*Figure 5D*

**D.** The box and scatter graphs below aligned to each day post onset of clinical symptoms, show the serum IgM (1st row); IgG (2nd row); IgM/IgG ratios (3rd row) and frequency of plasmablasts (4th row) in DI, DW and SD cases as indicated.

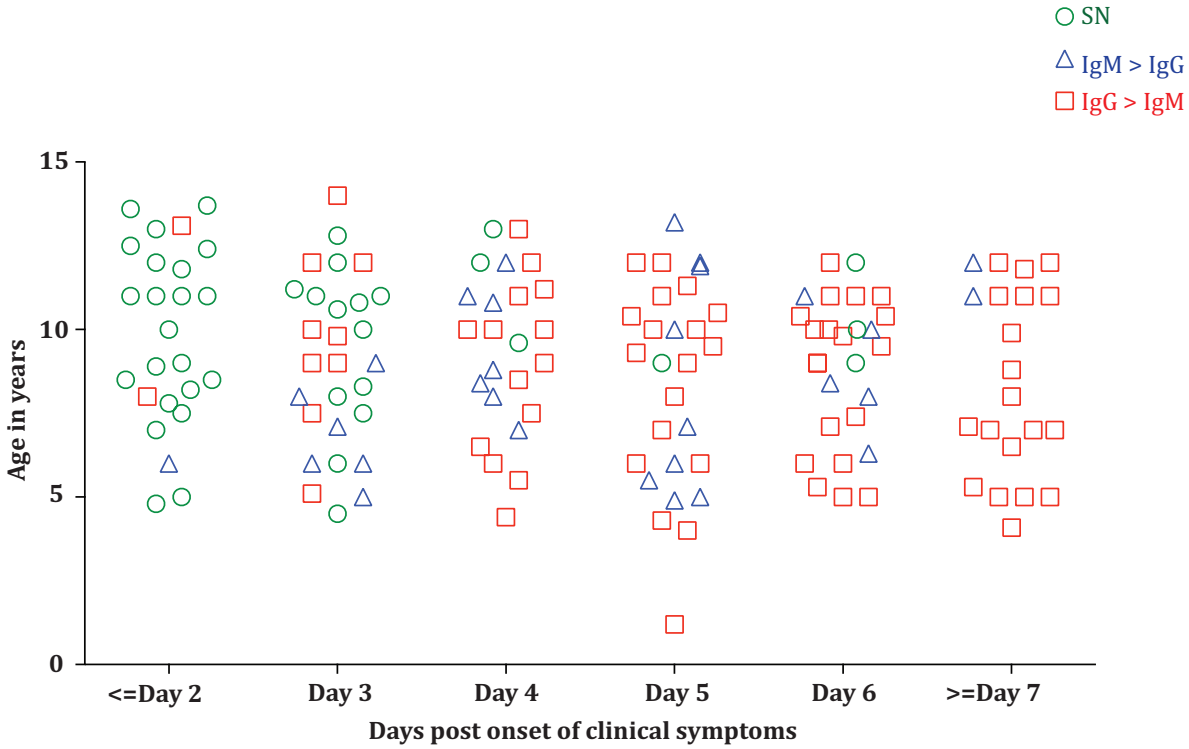


*Figure S1*

**Supplementary figure 1: Age distribution of dengue patients enrolled in the study.** Scatter graph shows age distribution of patients with humoral profile of SN (green circle); IgM > IgG (blue triangle) and IgG > IgM (red square) as a function of time post onset of clinical symptoms.

**Table 1: Patient demographic enrolled during the years of 2012 – 2016.** Characteristics of dengue confirmed children between the ages of 1.2 – 14 years enrolled in this study at the clinical site of All India Institute of Medical Sciences, New Delhi, India between the years 2012 – 2016 is tabulated.

| Summary of dengue patients analyzed in this study from 2012 - 2016 | |
|---|---|
| **Parameter** | **Value** |
| Number of patients analyzed in this study | 154 |
| Number of Males / Females | 85 / 69 |
| Age in years [range(average)] | 9 years (1.2 - 14 years) |
| Number of days post onset of clinical symptoms [range(average)] | 4.4 days (2 - 7 days) |
| Number of patients serotyped | 102 |
| Dengue 1 | 4 |
| Dengue 2 | 93 |
| Dengue 3 | 2 |
| Dengue 4 | 0 |
| Co-Infection Dengue 1+2 | 2 |
| Co-infection Dengue 2+4 | 1 |
| Number of patients with classified disease grade (2009, WHO) | 154 |
| Dengue Infection (DI) | 35 |
| Dengue with warning signs (DW) | 50 |
| Severe Dengue (SD) | 69 |

*Table 1*

**Table 2: Antibody secreting cell response from seronegative patients at a second blood sampling**. Antibody secreting cell response of seronegative patients that remained SN or seroconverted to IgM > IgG or IgG > IgM at a second blood sampling 48 – 72 hrs later.

| Humoral Profile | Patient Number | Bleed 1 | | Bleed 2 | |
|---|---|---|---|---|---|
| | | Dengue IgM ASC | Dengue IgG ASC | Dengue IgM ASC | Dengue IgG ASC |
| SN to SN | Patient 1 | 0 | 0 | 0 | 0 |
| | Patient 2 | 0 | 0 | 0 | 0 |
| | Patient 3 | 0 | 60 | 0 | 0 |
| | Patient 5 | 0 | 0 | 0 | 0 |
| | Patient 6 | 0 | 0 | 0 | 24330 |
| | Patient 7 | 0 | 0 | 0 | 0 |
| Humoral Profile | Patient Number | Bleed 1 | | Bleed 2 | |
| | | Dengue IgM ASC | Dengue IgG ASC | Dengue IgM ASC | Dengue IgG ASC |
| SN to IgM > IgG | Patient 11 | 0 | 0 | 60 | 1290 |
| | Patient 12 | 75 | 90 | 540 | 120 |
| | Patient 13 | 0 | 0 | 45 | 0 |
| Humoral Profile | Patient Number | Bleed 1 | | Bleed 2 | |
| | | Dengue IgM ASC | Dengue IgG ASC | Total IgM ASC | Dengue IgG ASC |
| SN to IgG > IgM | Patient 15 | 0 | 0 | 0 | 1350 |
| | Patient 16 | 0 | 0 | 300 | 14598 |
| | Patient 17 | 0 | 1350 | 0 | 60 |
| | Patient 18 | 0 | 0 | 0 | 34063 |
| | Patient 19 | 0 | 0 | 0 | 4862 |
| | Patient 21 | 0 | 0 | 1080 | 19464 |
| | Patient 22 | 0 | 0 | 210 | 300 |

*Table 2*

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