Longitudinal analysis of antibody cross-neutralization following Zika and dengue virus infection in Asia and the Americas

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Running title: Zika and dengue antibody neutralization

Summary: We find while DENV infection elicits cross-neutralizing antibodies to ZIKV that may be maintained for months to years, ZIKV lies antigenically outside the DENV serocomplex. Furthermore, traditional neutralization testing can be useful in certain contexts to distinguish between infecting flaviviruses.

ABSTRACT

Background: The four dengue virus serotypes (DENV1-4) and Zika virus (ZIKV) are related mosquito-borne flaviviruses of major importance globally. While monoclonal antibodies and plasma from DENV-immune donors can neutralize or enhance ZIKV *in vitro* and in small animal models, and *vice versa,* the extent, duration, and significance of cross-reactivity remains unknown, particularly in flavivirus-endemic regions.

Methods: We studied neutralizing antibodies to ZIKV and DENV1-4 in longitudinal serologic specimens through 3 years post-infection from people in Latin America and Asia with laboratory-confirmed DENV infections. We also evaluated neutralizing antibodies to ZIKV and DENV1-4 in Zika patients through 6 months post-infection.

Results: In Zika patients, the highest neutralizing antibody titers were to ZIKV, with low-level cross-reactivity to DENV1-4 that was greater in DENV-immune individuals. We found in primary and secondary DENV infections, neutralizing antibody titers to ZIKV were markedly lower than to the infecting DENV and heterologous DENV serotypes. Cross-neutralization was greatest in early convalescence, then ZIKV neutralization decreased, remaining at low levels over time.

Conclusions: Patterns of antibody cross-neutralization suggest ZIKV lies outside the DENV serocomplex. Neutralizing antibody titers can distinguish ZIKV from DENV infections when all viruses are analyzed simultaneously. These findings have implications for understanding natural immunity and vaccines.

Keywords: Dengue virus, Zika virus, neutralizing antibodies, cross-reactivity, Latin America, Asia, flavivirus, longitudinal, Nicaragua

BACKGROUND

The four dengue virus serotypes (DENV1-4) and Zika virus (ZIKV) are mosquito-borne flaviviruses of global health importance. Dengue is the most prevalent arboviral disease of humans, with up to 390 million infections annually [1]. ZIKV expanded dramatically throughout Latin America in 2015-2016 [2]. Though the majority of infections are mild or asymptomatic, ZIKV infection during pregnancy is linked with devastating birth defects, including microcephaly, and Guillain-Barré Syndrome in adults [3,4]. Because of shared *Aedes* mosquito vectors, ZIKV spreads in geographic areas where DENV is endemic [5]. Since ZIKV circulates in Africa and Asia [6,7], billions of people are at risk of sequential or concurrent DENV and ZIKV infections.

The envelope protein (E) is a major target of the human antibody response to flaviviruses. The ectodomain of E comprises three domains (EDI, EDII, and EDIII), with distinct roles in virus attachment, entry, and membrane fusion. DENV and ZIKV are closely related (~55% E amino acid identity) [8–10], giving rise to a high degree of structural and antigenic similarity [8,9]. Therefore, it is not surprising that extensive antibody cross-reactivity is observed.

In fact, serologic cross-reactivity among flaviviruses has long been appreciated and used to categorize flaviviruses into serocomplexes and subcomplexes [11,12]. With DENV, cross-reactive antibodies elicited by the infecting serotype bind and neutralize heterologous DENV serotypes, corresponding to a transient period of cross-protection or disease attenuation following primary DENV infection. In the absence of subsequent DENV infections, the neutralizing antibody (nAb) response typically narrows in non-endemic regions, conferring long-

term immunity only to the infecting DENV serotype [13,14]. However, cross-reactive nAb responses appear to be maintained over time in dengue-endemic regions [15]. Serologic cross-reactivity between DENV and ZIKV has been clearly demonstrated [8,10,16,17]. Some monoclonal antibodies (mAbs) derived from DENV-immune patients cross-neutralize ZIKV [18] and protect against lethal ZIKV challenge in a mouse model [19]. Human plasma collected ≤100 days after RT-PCR-confirmed DENV infection also binds and cross-neutralizes ZIKV [8]. However, late-convalescent plasma from DENV-immune travelers does not harbor durable, high levels of cross-neutralizing antibodies against ZIKV [20]. Thus, substantial deficiencies exist in our understanding of cross-neutralizing antibody responses among individuals with prior DENV exposure, particularly how these responses evolve over time and in various transmission contexts in flavivirus-endemic countries. Here, we characterized the extent of anti-DENV and anti-ZIKV neutralization in individuals from Latin America and Asia over months to years following molecularly-confirmed DENV and ZIKV infections.

METHODS

Ethics statement

All studies were approved by the relevant Institutional Review Boards (IRBs) at the participating institutions (see Supplementary Methods for details).

Study site and sample selection

Nicaragua. Samples were from two prospective studies of pediatric dengue and Zika in Managua, Nicaragua. In the hospital study (1998-present), study enrollment occurs in the

Nicaraguan Hospital Infantil Manual de Jesús Rivera. Children six months to 14 years of age suspected of dengue or Zika (<7 days of illness) are eligible [21]. All suspected dengue and Zika cases are confirmed by DENV serotype-specific and ZIKV RT-PCR and serologic methods [22,23]. Samples from dengue cases for this study (n=180) were collected at the acute (days 1-6) and convalescent (days 14-28) phases as well as 3, 6, 12, and 18 months following primary and secondary symptomatic DENV infections (DENV1, DENV2, and DENV3) (Table 1). Primary and secondary dengue cases were defined by an Inhibition ELISA [24,25] titer of <2,560 or ≥2,560, respectively, in the convalescent-phase sample [23]. Samples from the 14-year ongoing community-based Pediatric Dengue Cohort Study (2004-present) included annual healthy blood samples collected 1-3 years following secondary DENV1, DENV2, DENV3, and DENV4 infections (n=42). All dengue samples were collected prior to the introduction of ZIKV into Nicaragua in 2016. RT-PCR-confirmed Zika cases from the Hospital-based study [22] included those with (n=7) and without (n=10) prior DENV immunity sampled at acute, convalescent, 3-, and 6-month time-points (Table 1). All blood samples were collected in 5 mM EDTA, and plasma was separated by centrifugation, aliquoted, and stored at -80°C.

Sri Lanka. Cohort study: Archived dried blood spots (DBS) stored at 4°C on protein saver cards from the Sri Lankan Pediatric Dengue Vaccine Initiative cohort [26] were processed with validated methods as previously described [26,27]. From November 2008 to January 2010, a surveillance study of ~800 children ≤12 years of age was conducted by obtaining baseline, one-year and two-year follow-up DBS specimens, which were tested for DENV-specific IgG. Children experiencing a febrile illness (temperature ≥38°C for ≤7 days) gave blood specimens at

presentation and convalescence ≥10 days after symptom resolution. Diagnostic RT-PCR was performed on acute specimens [26]. The baseline dengue serostatus of each child was used to classify subsequent RT-PCR confirmed infection as primary or secondary [26]. Sri Lankan hospital study: Individuals presenting with acute febrile illness suspected of dengue based on clinical expertise of local clinicians were enrolled. Serum was collected at presentation, and convalescent serum specimens were obtained ≥10 days later. In hospital cases, the presence of DENV-specific IgG in the acute samples (days 1-5 after onset of symptoms) was used to define primary or secondary cases [27]. Only RT-PCR-confirmed cases were used in both these studies.

Thailand. Suspected dengue cases were identified on presentation to the Khon Kaen Hospital. Patients with acute febrile illness clinically suspected of dengue were enrolled. Only cases that showed dengue symptoms confirmed by DENV RT-PCR were included in this study. Samples collected during hospitalization were considered acute samples. Convalescent samples were collected at 2-4 weeks and again at 5-7 months. The infecting DENV serotype was determined by RT-PCR [28]. Secondary DENV infection was classified based on a ratio of anti-DENV IgM/IgG <1.8 as measured by IgM and IgG capture ELISA [29]. During the acute phase, the day of defervescence was defined as day 0, the day before as day -1, the day after as day 1, etc. Blood samples were collected in 5 mM EDTA, and plasma was separated by centrifugation, aliquoted, and stored at -80°C.

Cell culture and virus production

Vero-81, C6/36, and U937 cells were cultured as described in Supplemental Methods. *UCB.* All four DENV serotypes (DENV1 N1265-04, DENV2 N172-06, DENV3 N7236-98 and DENV4 N703-

99) and ZIKV Nica 1-16 were isolated in Nicaragua and propagated (low passage: 2-3) in C6/36 cells [30]. All viral stocks were *Mycoplasma*-free, and viral titers were determined by focus-forming assays on Vero cells. *UNC.* ZIKV strain H/PF/2013 was provided by the US Centers for Disease Control and Prevention [31]; DENV WHO reference strains (DENV1 West Pac 74, DENV2 S-16803, DENV3 CH54389 and DENV4 TVP-360) were obtained from Dr. R. Putnak (Walter Reed Army Institute of Research). Virus stocks were prepared in C6/36 *Aedes albopictus* cells (ATCC# CRL-1660). All DENV strains used were passage 4 and ZIKV was passage 3. *ICL*. ZIKV strain PF13/251013-18 (PF13) from French Polynesia in 2013 was provided by Dr. V.-M. Cao-Lormeau (Institut Louis Malardé). DENV1 (Hawaii), DENV2 (16681), and DENV3 (H87) were gifts from AFRIMS. DENV-4 (1-0093) was isolated from a DENV4-infected patient. Virus-containing supernatants were collected and stored at -80°C. Viral titers were determined by focus-forming assays on Vero cells. All cell lines and virus stocks were free from *Mycoplasma* contamination.

Focus reduction neutralization test

A focus reduction neutralization test (FRNT) was performed at UCB, UNC, and ICL using Vero cells and all four DENV serotypes and ZIKV in parallel (see Supplementary Methods for experimental details, calculation of nAb titers, and quality control measures).

Antibody-dependent enhancement (ADE) assay

Serially diluted plasma samples were incubated with virus at an MOI of 2 for one hour at 37°C before adding to U937 cells. After incubation for 2 (ZIKV) or 3 (DENV) days, supernatants were harvested and viral titers determined by focus-forming assays as described in Supplemental

Methods. Fold-enhancement was calculated by comparison to viral titers in the presence/absence of plasma.

RESULTS

Immune plasma from ZIKV infections with and without prior DENV immunity have high ZIKV nAb titers.

We selected Nicaraguan children from our hospital-based study who were RT-PCR-confirmed with symptomatic Zika with (n=7) or without (n=10) evidence of prior DENV immunity and measured nAb titers against DENV1-4 and ZIKV in samples collected at the acute and early convalescent phases and at 3 and 6 months post-ZIKV infection. First, assay background was confirmed as undetectable by testing uninfected controls (Supplementary Figure 1). We found that peak nAb titers against ZIKV following ZIKV infection were higher than any $FRNT_{50}$ titer to DENV1-4. Interestingly, the magnitude of the ZIKV nAb response ($FRNT_{50}$) at the peak (convalescence) and 3- and 6-month follow-up was similar regardless of pre-existing DENV immunity (Figure 1A,B). ZIKV infection elicited detectable cross-neutralization to DENV1-4 at all time-points after infection. As expected, samples from DENV-immune children with ZIKV infection exhibited greater neutralization to the four DENV serotypes than those who were DENV-naïve (Figure 1A,B).

DENV infection elicits low but sustained cross-neutralizing antibody responses to ZIKV in Nicaraguan children.

To determine the extent and duration of cross-neutralization of ZIKV by DENV-immune plasma, longitudinal samples collected at acute and convalescent phases, and 3, 6, 12 and 18 months

post-infection from primary and secondary DENV1, DENV2 and DENV3 cases (prior to the introduction of ZIKV into Nicaragua) in our hospital study were tested for nAb activity against all four DENV serotypes (DENV1-4) and ZIKV by FRNT. As expected, plasma from primary dengue cases showed the highest nAb titers to the infecting serotype and lower $FRNT_{50}$ values to heterologous DENV serotypes (Figure 2A). However, nAb titers to heterologous DENV strains were consistently higher than the $FRNT_{50}$ to ZIKV (Figure 2A). A peak in nAb titers for DENV and ZIKV was observed at the convalescent phase, and $FRNT_{50}$ titers were relatively stable from 3-18 months. Similarly, individuals with secondary DENV infections showed the highest nAb titers to the DENV serotypes, although not necessarily the secondary infecting serotype, and the lowest titers to ZIKV (Figure 2B). The kinetics of nAb responses in secondary dengue cases were comparable to those for primary dengue cases, with a peak in $FRNT_{50}$ titers at convalescence and more stable titers from 3-18 months, although there was more gradual antibody waning than in primary cases between 3-18 months. Primary and secondary dengue cases had similar levels of nAb titers to ZIKV over time.

To examine the kinetics of the nAb response along a longer time-frame, we evaluated longitudinal annual samples from children with secondary DENV infections in our community-based cohort study. Overall, these revealed the same trend, in that the highest titers were observed to the first DENV infection, which in most cases was DENV2, and the next highest titers were against the infecting serotype, followed by the other heterologous DENV serotypes and ZIKV (Figure 2C). Anti-DENV nAb titers, as well as low levels of cross-reactivity against ZIKV, were maintained 1-3 years post-infection.

Antigenic cartography places ZIKV outside the DENV serocomplex

To estimate the antigenic relationships among DENV1-4 and ZIKV, we used antigenic cartography [15,32] to analyze neutralization titers in plasma following primary DENV1-3 and ZIKV infection from the Nicaraguan hospital-based study. Each neutralization titer is treated as a measure of distance between a plasma sample and virus, and simultaneous fitting of these distances produces an antigenic map. In the acute-phase, DENV1-4 and ZIKV form a single cluster (Fig. 3A), but ZIKV separates from the DENV1-4 cluster by convalescence, and this is maintained out to 6 months post-infection (Figure 3B).

ZIKV cross-neutralization is variable and inferior to heterologous DENV neutralization in plasma from Sri Lankan subjects

To investigate the magnitude and kinetics of cross-neutralizing antibody responses to ZIKV following DENV infection in another geographical region, we examined archived serologic specimens from two sources in Sri Lanka (Table 1, Supplementary Table 1). From a community-based study of dengue conducted in 2008-2010 [26], we selected 12 subjects (6 primary and 6 secondary DENV infections) who experienced a laboratory-confirmed, symptomatic DENV infection and had at least one additional specimen available after incident DENV infection; the total number of samples varied depending on how many febrile episodes a child reported. From a Sri Lankan hospital-based study conducted in 2014, we selected 13 subjects (7 primary and 6 secondary DENV infections), from whom convalescent blood samples collected between 11 and 77 days post-infection were available.

We grouped specimens tested by primary versus secondary DENV infection and time after symptom onset. Neutralization profiles ($FRNT_{50}$) to each virus at each time-point are shown in Supplementary Table 1. In primary cases (Figure 4A), DENV1 was the most common infecting serotype, reflected in the higher neutralization titers to DENV1. The $FRNT_{50}$ for DENV2-4 clustered at averages below those for DENV1 at each time-point, while the ZIKV $FRNT_{50}$ was approximately 10-fold lower than DENV1 $FRNT_{50}$. ZIKV neutralization persisted beyond 120 days post-infection (128-217 days) at substantial levels, but subordinate to DENV titers.

In secondary DENV infections (Figure 4B), ZIKV $FRNT_{50}$ titers were again the lowest of all groups. ZIKV cross-neutralizing titers rose later in convalescence (31-90 days post-infection) of secondary cases to levels approximating the mean $FRNT_{50}$ of several DENV serotypes. At time-points >120 days post-infection (range 123-202 days) in secondary cases, the mean $FRNT_{50}$ for ZIKV declined markedly and began to further separate from that of the DENV serotypes. Thus, ZIKV cross-neutralization is detected after primary and secondary DENV cases, but typically with an $FRNT_{50}$ at least 4-fold lower than to the infecting DENV serotype. Notably, most Sri Lankan hospital samples were not tested beyond a dilution of 1:1280 and many DENV1-4 $FRNT_{50}$ values are >1:1280; thus, the difference in magnitude of ZIKV vs DENV $FRNT_{50}$ is underestimated.

Transient cross-neutralization of ZIKV following DENV infections observed in a Thai cohort

In a third country, we studied samples collected from Thai children (ages 6-14) admitted to the hospital with acute DENV infection between 2001-2003, when ZIKV was not reported in the area. Samples were collected on each day after hospital admission and at convalescent time-

points of 2 weeks, 2 months and 6 months following discharge (Table 1). Neutralization of DENV2 and ZIKV was measured for plasma samples from 5 secondary DENV2-infected cases (Figure 5A, Supplementary Figure 2 and Table 2). In general, neutralization titers for both DENV and ZIKV peaked between day 0 and day 14 and declined thereafter. Five of five cases showed DENV neutralization ($FRNT_{50}$>50% at plasma dilution 1:50), which was maintained until the 6-month time-point. Five of six cases also showed neutralization of ZIKV at early time-points, but this decayed more rapidly than the titers to DENV at 6 months. To validate these findings in a larger sample set representing infection by all 4 DENV serotypes, 15 additional secondary DENV cases were tested (Supplementary Table 3). These again showed strong neutralization of all DENV serotypes at all time-points, whereas nAb titers against ZIKV were lower during early time-points and at 6 months (Figure 5B).

DENV-immune plasma transiently enhances DENV and ZIKV infection *in vitro*

Finally, we found that DENV2-immune plasma promoted ADE of both DENV and ZIKV infection *in vitro* at a similar peak enhancement titer, although the magnitude of enhancement was greater for DENV2 (Supplementary Figure 3).

DISCUSSION

In this study, we characterized the nAb response against DENV and ZIKV over time following primary and secondary DENV infections and draw two main conclusions: 1) cross-neutralizing antibody responses to ZIKV are consistently detected after DENV infection, but they occur at

magnitudes notably less than to the infecting DENV serotype and generally less than to heterologous DENV serotypes, and 2) the magnitude and kinetics of ZIKV nAb responses are unaffected by previous DENV infection. Both of these findings are consistent with ZIKV and DENV belonging to a distinct serocomplex.

Across multiple well-characterized DENV infection cohorts representing populations in Latin America and Asia, we consistently observe cross-neutralizing activity against ZIKV, but ZIKV $FRNT_{50}$ tended to be 4-10 fold lower than DENV $FRNT_{50}$. Cross-neutralization to ZIKV variably declines over time, being maintained at low levels in children months to years after infection. We have previously seen that high levels of ZIKV cross-neutralizing antibodies are not maintained into late convalescence (≥6 months post infection) in DENV-infected travelers [20], raising the possibility that ongoing DENV exposure in endemic regions may drive maintenance of cross-neutralization of ZIKV following DENV infection [15]. Most studies reporting strong cross-neutralization between ZIKV and DENV have used early convalescent sera [8] or mAbs derived from plasmablasts at very early time-points after DENV infection [18,19]. Using antigenic maps, we show that DENV1-4 and ZIKV group closely when acute-phase primary DENV and ZIKV infection plasma are analyzed; however, by convalescence and out to 6 months post-infection, DENV1-4 group as a serocomplex, with ZIKV clearly outside of the DENV antigenic cluster.

Overall, it appears that ZIKV-cross-neutralizing antibodies are frequently induced by DENV infection at a fraction of the magnitude of DENV-neutralizing antibodies, with anti-ZIKV titers

reaching a steady-state level by 6-12 months. Furthermore, neutralization antibody assays can distinguish ZIKV from DENV infections when all viruses are analyzed simultaneously, particularly if relative titers of ZIKV versus DENV serotypes rather than specific cut-offs for positivity are considered [33], and thus may be useful in surveillance, research, and potentially some clinical settings outside of the acute phase of illness [20].

One assumption is that none of the dengue subjects included in the study were infected by ZIKV. While it is plausible that Zika in Asia is grossly underreported due to a high rate of asymptomatic infections or attribution of Zika cases to other causes of acute febrile illness such as dengue [6,34,35], this is highly unlikely in our study. Samples from Asian subjects did not exhibit high $FRNT_{50}$ at the earliest time-points interrogated after DENV infection nor display maintenance of ZIKV $FRNT_{50}$ titers into convalescence, which would be expected after a true prior ZIKV infection [12,13]. The Nicaraguan dengue samples were collected years before ZIKV was reported or projected to be circulating in Central America [36].

The neutralization assay has been an important tool in flavivirology, though lab-to-lab variations have been recognized [37]. Here we observed some differences in overall titer magnitude but reached similar conclusions across the three sites. This variation may be due to unique features of study populations or details such as virus strain used in the assay, DENV infection order, intensity of local transmission, and strain of DENV exposure, which were not systematically analyzed here.

The phenomenon of ADE, whereby cross-reactive non-neutralizing antibodies facilitate infection of FcγR-bearing cells and lead to higher levels of viremia and more severe disease manifestations is a well-established concept in dengue [13,38,39] and has recently been established in human populations [40]. Though clear evidence of enhancement of ZIKV infection in DENV-immune humans is still lacking [41], it remains a major concern. While DENV and other flaviviruses are readily enhanced by antibodies in cell culture ADE assays with FcγR-bearing cell lines, such assays have not been useful for predicting severe DENV disease in people [42]. Here, we observed that Thai individuals who had recovered from DENV2 infections enhanced DENV2 better than ZIKV in cell culture assays, further highlighting the challenges of extrapolating human flavivirus disease risk from *in vitro* ADE assays. The enhancing activity peaked at acute and convalescent phases and declined thereafter, mirroring the curve of neutralizing antibody profiles.

Alternatively, ZIKV cross-neutralizing antibodies elicited by DENV may mitigate ZIKV infection, in line with claims that a single vaccine against DENV1-4 and ZIKV could be developed [8,12,18]. Pre-infection nAb titers have been associated with decreased probability of symptomatic secondary DENV infection [27], and nAb correlates of protection exist for other flavivirus infections [43]. However, no specific correlate of protection has been identified for DENV or ZIKV, and many individuals who seroconverted by neutralization testing remained susceptible to DENV infection in vaccine studies [44,45]. Interestingly, DENV-immune non-human primates are readily infected with ZIKV with no observation of enhancement or protection [46,47].

Ongoing cohort studies relating known sequence and timing of DENV infections or prevalent DENV serostatus to ZIKV infection outcome will help address these critical questions.

Since 2015, many individuals in Latin America have experienced ZIKV as a primary or secondary flavivirus infection, raising the question of how ZIKV infection will affect immunity to DENV. A dramatic reduction in dengue cases throughout Latin America has been observed in 2017 [48], suggesting that ZIKV infection may induce at least temporary immune cross-protection from DENV. Our data demonstrate that ZIKV infection clearly elicits cross-neutralizing antibodies to DENV and may boost DENV $FRNT_{50}$ in a DENV-immune host. Whether higher or lower rates of DENV infection occur in ZIKV-immune individuals will be determined in longitudinal cohort studies. Interestingly, we found that the magnitude of anti-ZIKV nAbs was similar regardless of prior DENV infection, which may be inconsistent with recent reports [49,50] that prior DENV infection (at least by certain serotypes) may potentiate the nAb response to ZIKV. We also noted that nAb titers to ZIKV post-ZIKV infection were higher than post-DENV infection nAb titers were to the infecting DENV serotype.

Overall, ZIKV appears to be antigenically related but distinct from the DENV serotypes. Our study provides a framework for comprehensive assessment of the serologic immune responses to multiple flaviviruses that is needed to inform vaccine development and epidemiologic studies aimed at reducing the burden of disease caused by these important pathogens in endemic areas.

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Potential conflicts of interests : Aravinda de Silva has consulted for Takeda, Glaxo Smith Kline and Merck pharmaceutical companies on dengue vaccines; he is also an inventor on patent applications on flavivirus vaccines and diagnostics. The other authors declare no conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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Figure Legends

Figure 1. Longitudinal neutralizing antibody responses against DENV1-4 and ZIKV in post-ZIKV infection samples. $FRNT_{50}$ titers for RT-PCR-confirmed ZIKV cases without (n=10) **(A)** or with (n=7) **(B)** evidence of prior DENV infection. Acute, convalescent, 3-month and 6-month post-infection plasma samples were tested by FRNT against all four DENV serotypes (Nicaraguan DENV1-4) and a Nicaraguan ZIKV isolate. All samples were processed in duplicate. Data for each sample set represents the geometric mean ± standard deviation.

Figure 2. Longitudinal neutralizing antibody responses after primary and secondary DENV infections from the Nicaraguan hospital and cohort studies against DENV1-4 and ZIKV. Longitudinal plasma samples from **(A)** primary (n=5/serotype) and **(B)** secondary (n=5/serotype) dengue cases from the Nicaraguan hospital study collected at acute phase, convalescent phase, and 3, 6, 12, and 18 months post-infection. **(C)** Longitudinal samples from the Nicaraguan cohort study collected 1, 2 and 3 years after secondary infection with DENV1 (n=4), DENV2 (n=4), DENV3 (n=3), and DENV4 (n=3). FRNT was performed against Nicaraguan DENV1-4 and ZIKV strains, and the $FRNT_{50}$ was caclulated and plotted. All samples were processed in duplicate. Data for each sample represents the geometric mean ± standard deviation.

Fig. 3. Antigenic relationships among DENV1-4 and ZIKV based on neutralizing antibody titers in plasma from primary DENV and ZIKV infections. Antigenic maps were generated by fitting $FRNT_{50}$ titers for primary DENV and ZIKV infection plasma (light grey diamonds) from the Nicaraguan hospital-based study against DENV1-4 and ZIKV (colored circles) as distances in

Euclidean space. Each grid-square corresponds to a two-fold dilution in the $FRNT_{50}$ titer. Arrows show shifts in antigenic relationships between DENV1-4 and ZIKV Nicaraguan viruses based on how they were recognized by plasma drawn at different time-points post-infection.

Figure 4. Cross-neutralization of ZIKV following DENV infection in Sri Lanka

Neutralization titers were determined for DENV1-4 and ZIKV. Samples were grouped by time after laboratory-confirmed DENV infection and by **(A)** primary DENV infection and **(B)** secondary DENV infection. Points represent group mean ± SEM.

Figure 5. Longitudinal neutralizing antibody responses to DENV and ZIKV from Thai hospital study. (A) Mean $FRNT_{50}$ from neutralization assay of plasma from secondary DENV-infected subjects (n=5) to infecting DENV serotype (DENV2) and ZIKV strain PF13 during the course of acute infection and at convalescence (2 weeks), 2 months and 6 months post-infection. Data are presented as the percentage of neutralized virus and representative of one experiment with 5 samples. **(B)** Mean $FRNT_{50}$ of longitudinal specimens from 15 Thai subjects with secondary infection by any DENV serotype against DENV1-4 and ZIKV strain PF13. Results are shown as mean ± SD.

Supplementary Figure 1. Neutralization activity in flavivirus-naïve samples. $FRNT_{50}$ titers for DENV- and ZIKV-naïve samples (n=5) were determined against each virus indicated on the x-axis to ensure that there was no non-specific inhibition of viral infection in the assay.

Supplementary Figure 2. Longitudinal neutralizing antibody responses to DENV and ZIKV in the Thai hospital study. Neutralization assay of plasma from secondary DENV2-infected subjects (n=5) to infecting DENV serotype (DENV2) and ZIKV strain PF13 during the course of acute infection and at convalescence (2 weeks), 2 months and 6 months post-infection. Data are presented as the percentage of neutralized virus and representative of one experiment with 5 samples.

Supplementary Figure 3. Longitudinal ADE responses to DENV and ZIKV from Thai hospital study. (A) Infection of U937 cells with ZIKV (strain PF13) or DENV2 in the presence of serially diluted plasma from secondary DENV2-infected subjects (n=5) during the acute and convalescent phase of infection. Data are presented as the ratio of viral titer in the presence of plasma to viral titer in the absence of plasma and representative of one experiment with 5 samples. **(B)** Peak fold enhancement and **(C)** peak enhancement titer of infection of U937 with ZIKV and DENV. Results are expressed as mean ± SD of 5 samples.

Table 1. Samples included in the study.

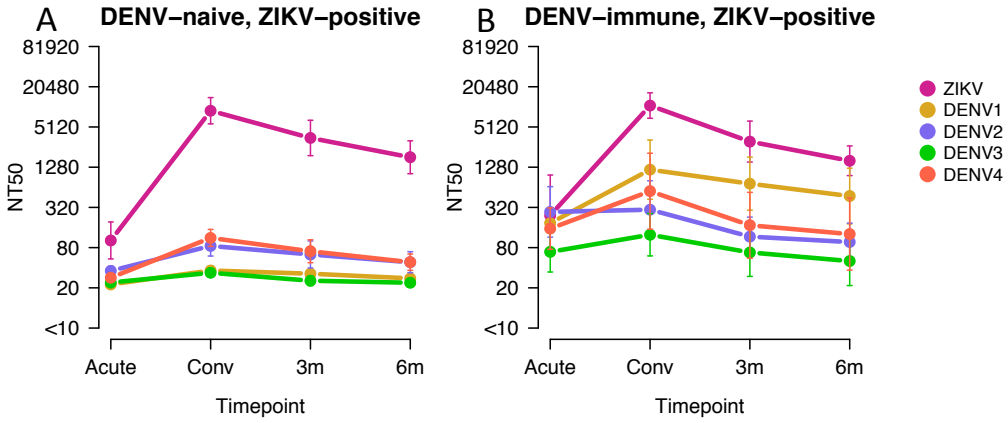
Nicaraguan samples	**Total**			
Hospital Study	**codes**	**Immune status**	**Time-points**	**Total samples**
DENV1	10	5 1°[a];5 2°[b]	Acute, Conv, 3, 6, 12, 18 m[c]	60
DENV2	10	5 1°;5 2°	Acute, Conv, 3, 6, 12, 18 m	60
DENV3	10	5 1°;5 2°	Acute, Conv, 3, 6, 12, 18 m	60
ZIKV	10	DENV-naïve	Acute, Conv, 3, 6 m	40
	7	DENV-immune	Acute, Conv, 3, 6 m	28
Cohort Study				
Negative Zika/dengue	5			5
DENV1	4	2°	1, 2, 3 years p.i.[d]	12
DENV2	4	2°	1, 2, 3 years p.i.	12
DENV3	3	2°	1, 2, 3 years p.i.	9
DENV4	3	2°	1, 2, 3 years p.i.	9

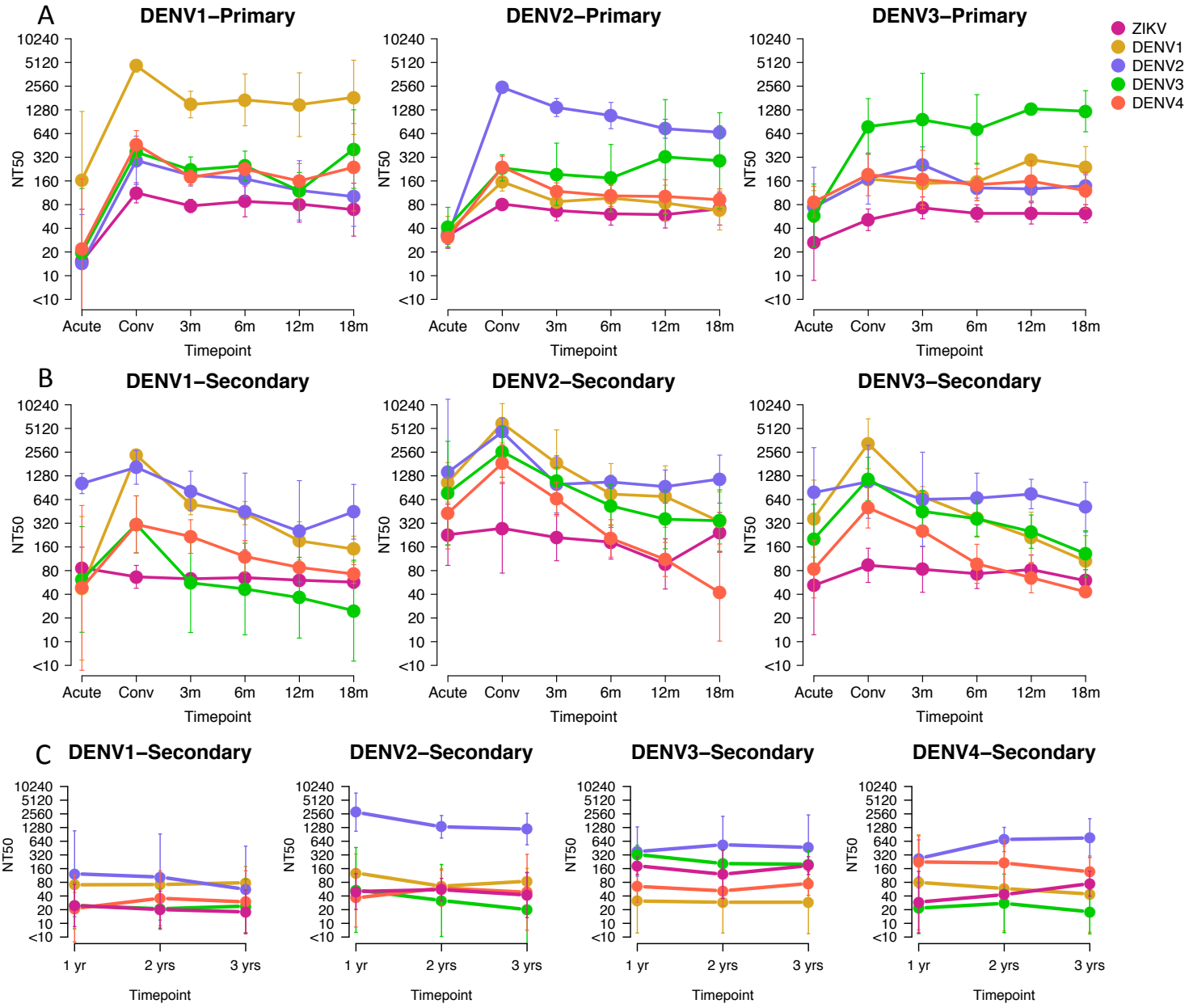
Sri Lankan samples	**Total codes**	**Immune status**	**Time-points**	**Total samples**
DENV1	18	10 1°; 8 2°	Variable, pre – 217 days p.i.	41
Other DENV	7	3 1°; 4 2°	Variable, pre – 202 days p.i.	16

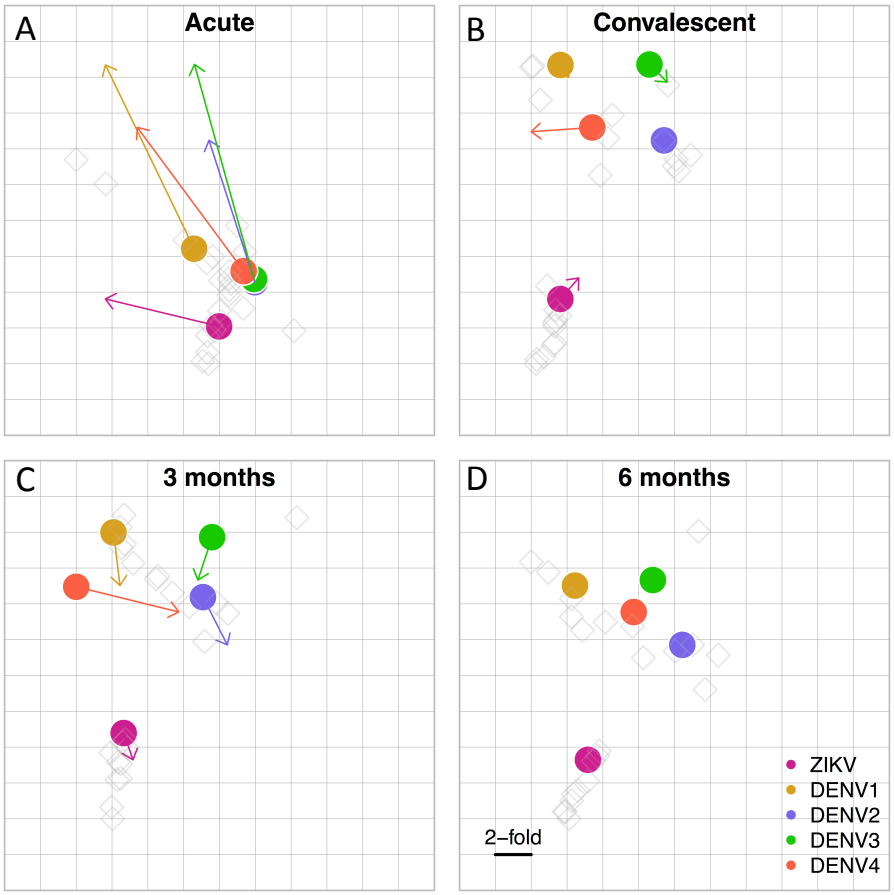
Thai samples	**Total codes**	**Immune status**	**Time-points**	**Total samples**

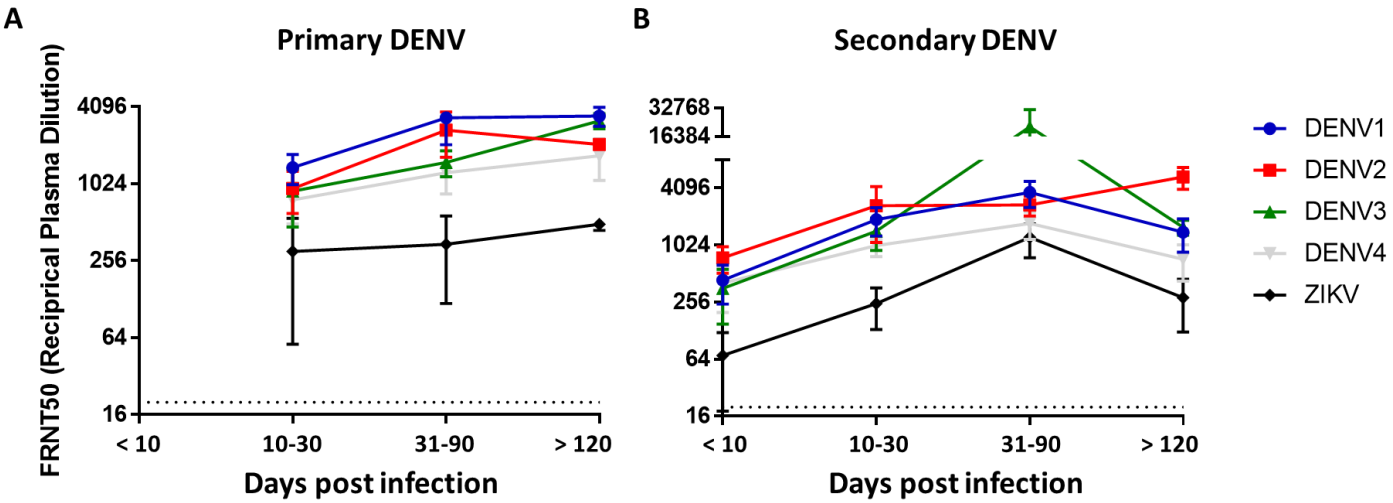
DENV1	6	2°	Acute, 2-4 weeks, 6 m	18
DENV2	11	2°	Acute, 2-4 weeks, 2m, 6 m	51
DENV3	1	2°	Acute, 2-4 weeks, 6 m	3
DENV4	2	2°	Acute, 2-4 weeks, 6 m	6

[a]1°, primary; [b]2°, secondary; [c]m, months; [d]p.i., post-infection.









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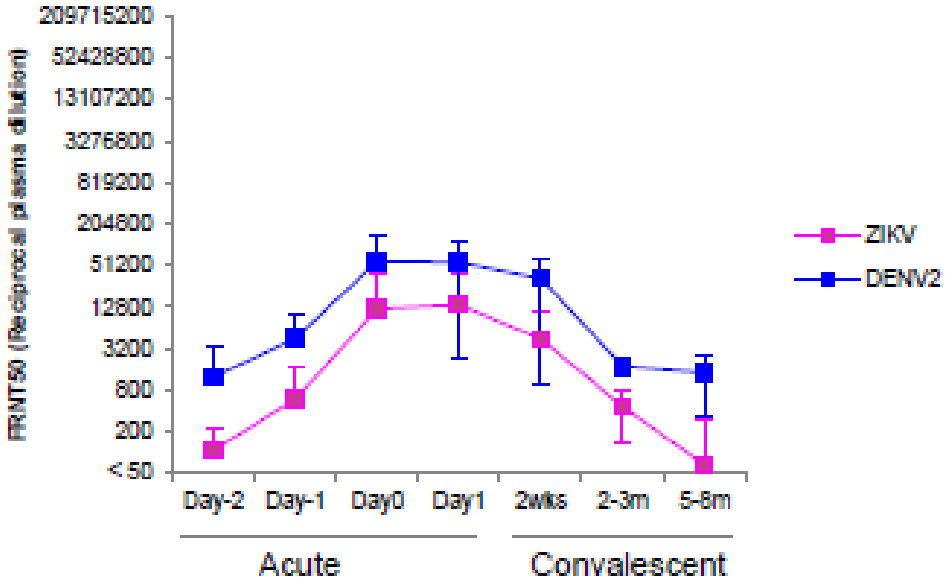

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