

Supporting Information

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SI Materials and Methods

Study Design and Participants.

Samples from Nicaragua. A total of 75 children RT-PCR-positive for ZIKV who experienced signs and symptoms of Zika between January 18 and September 20, 2016, from the Nicaraguan PDCS were included. The PDCS is a community-based prospective study of children 2–14 y of age that has been ongoing since August 2004 in Managua, Nicaragua (13). Participants present at the first sign of illness to the Health Center Sócrates Flores Vivas, and study physicians use standardized forms to collect clinical data. Subjects are followed daily during the acute phase of illness. In 2014, CHIKV was added to the PDCS, and in January 2016, ZIKV screening was initiated of participants meeting the clinical definition of dengue and/or chikungunya or presenting with undifferentiated fever. In February, the case definition was expanded to include those presenting with signs and symptoms of Zika, but without fever. Acute and convalescent (~14 d after onset of symptoms) blood samples are drawn for dengue, chikungunya, and Zika diagnostic testing. If confirmed ZIKV-positive subjects presented subsequently with another febrile episode, a blood sample was collected for clinical management and stored; these samples were also included in the current study. In the PDCS, a healthy blood sample is collected annually from participants; anti-DENV antibody titers are measured in paired annual samples using an inhibition ELISA (29, 30), and infections are defined by seroconversion or a fourfold or greater rise in anti-DENV titers. In this study, confirmed ZIKV cases were classified as DENV-naive if they entered the cohort study with no detectable anti-DENV antibodies (as measured by inhibition ELISA) and had no documented DENV infections (symptomatic or inapparent) during their time in the cohort, or were classified as DENV-immune if they either entered the cohort with detectable anti-DENV inhibition ELISA antibodies or entered the cohort study with no detectable anti-DENV antibodies and had one or more documented DENV infections during their time in the cohort. We also included 37 patients with Zika from the Nicaraguan National Surveillance System who become ill between January 26 and May 11, 2016, and were confirmed by RT-PCR by the National Virology Laboratory of the Ministry of Health. Late convalescent samples were collected weeks to months after onset of symptoms and were included in this study as well. All suspected Zika cases were confirmed by RT-PCR in serum and/or urine, using triplex assays that simultaneously screen for DENV and CHIKV infections [ZCD assay (20), CDC Triplex (21)], or in some cases the CDC ZIKV monoplex assay (8) in parallel with a DENV-CHIKV multiplex assay (22).

As a control group, a total of 146 patients with dengue confirmed by RT-PCR (23, 24) between 2005 and 2013 were included, of which 115 (41 primary cases and 74 secondary cases) belonged to the PDCS and 31 (18 primary cases and 13 secondary cases) were participants in the Nicaraguan Hospital-based Dengue Hospital at the National Pediatric Reference Hospital in Nicaragua (25), which has been ongoing since 1998. Primary DENV infection was defined by an antibody titer by inhibition ELISA of <2,560 in convalescent-phase samples (31). During the peak dengue season (August–January), children between 6 mo and 14 y of age with suspected dengue disease who present to the hospital within 7 d of symptom onset are eligible to participate; in 2014, chikungunya was added, and in 2016, Zika was included in the study. Blood samples are collected at enrollment, during the acute phase, and 2–4 wk after symptom onset (convalescent phase) for clinical laboratory tests and dengue diagnostic tests. Subjects also can consent to partici-

pate in a longitudinal arm of the study, in which a medical examination is conducted and blood samples are collected 3, 6, 12, and 18 mo postillness. Samples from acute, convalescent, and 3-mo visits were included in this study. The PDCS and the Dengue Hospital-based study were approved by the institutional review boards of the Nicaraguan Ministry of Health and the University of California, Berkeley. Parents or legal guardians of all subjects provided written informed consent, and subjects ≥ 6 y old provided assent.

Samples from Italy. The diagnostic assessment for DENV, ZIKV, CHIKV, WNV, and YFV included the following: detection of anti-DENV IgM and IgG antibodies in serum samples (using dengue virus IgM Capture DxSelect and dengue virus IgG DxSelect, Focus Diagnostics, United States), detection of ZIKV IgM and IgG antibodies [anti-Zika virus ELISA (IgM) and anti-Zika virus ELISA (IgG); Euroimmun], identification of CHIKV IgM and IgG using specific immunofluorescence tests [anti-chikungunya virus IFA (IgG), anti-chikungunya virus IFA (IgM); Euroimmun], detection of WNV IgM and IgG using specific tests [NovaLisa™ Dengue IgM and IgG ELISAs (NovaTec Immunodiagnostic GmbH); and WNV IgM Capture DxSelect and WNV IgG DxSelect (Focus Diagnostics)], and detection of YFV IgM and IgG with anti-yellow fever virus IFA (IgG) and anti-yellow fever virus IFA (IgM; Euroimmun). Serology results were confirmed by specific viruses neutralization assays (26). Detection of DENV RNA, ZIKV RNA, and WNV RNA in plasma and urine samples was performed using a pan-flavivirus heminested RT-PCR assay targeting a conserved region of the NS5 gene (27), as well as virus-specific real-time RT-PCR tests, targeting a conserved region in the 3' untranslated region of DENV1–DENV4 (32), a portion of the envelope protein gene of ZIKV (8), a portion of the E1 structural protein region of CHIKV (33), and a conserved region of the 5'-UTR and a part of the capsid gene of WNV lineages 1 and 2 (34). Sequencing was performed on positive pan-flavivirus amplicons to type DENV and WNV viruses. This retrospective analysis was performed according to guidelines of the institutional review board of the Fondazione IRCCS Policlinico San Matteo on the use of biologic specimens for scientific purpose in keeping with Italian law (art.13 D.Lgs 196/2003). The personnel of the Molecular Virology Unit participated in the collection of these samples, which were anonymized on collection. The local ethics committee consent was not required because according to a Regional Surveillance and Preparedness Plan (DGR 12591, December 27, 2012), diagnostic detection of arbovirus infections in the Lombardy Region was centralized at the Regional Reference Laboratory (Molecular Virology Unit, Fondazione IRCCS Policlinico San Matteo, Pavia). Informed consent was not necessary because patients with suspected arbovirus infections were included in a regional diagnostic protocol. Prospective samples (serum, cerebrospinal fluid, and urine) were collected by clinicians and handled by Molecular Virology Unit personnel; data were analyzed anonymously according to a Regional Surveillance and Preparedness Plan (DGR 12591, December 27, 2012).

Production and Labeling of the ZKA35 mAb Probe. The V_H and V_L sequences of ZKA35 human mAb were cloned into human Igy1, Igh expression vectors (kindly provided by Michel Nussenzweig, Rockefeller University), essentially as previously described (28). Recombinant ZKA35 mAb was produced by transient transfection of EXP1293 cells (Invitrogen) purified by protein A chromatography (GE Healthcare), and desalted against PBS. ZKA35 IgG1 mAb was biotinylated using the EZ-Link NHS-PEG solid phase biotinylation kit (Pierce). Labeled ZKA35 was tested for

binding to ZIKV NS1 to determine the optimal concentration to achieve 70% maximal binding.

NS1 BOB Assay. Polystyrene plates (Nunc MaxiSorp) were coated overnight with 1 $\mu\text{g}/\text{mL}$ ZIKV NS1 (MR766 strain; Meridian or Native Antigen Company, Inc.) in coating buffer (PBS) at 4 $^{\circ}\text{C}$. Plates were blocked for 1 h with PBS containing 1% BSA. Plasma or serum (1:10 dilution in PBS/1% BSA) were added to NS1-coated ELISA plates. After 1 h, an equal volume of biotinylated anti-NS1 ZKA35 at 20 ng/mL was added, and the mixture was incubated at room temperature for 15 min (final dilution of the plasma in the mixture, 1:20). Plates were washed with PBS-T (PBS plus 0.05% Tween 20), and alkaline-phosphatase-conjugated streptavidin was

added for 30 min. Plates were washed again with PBS-T, and the substrate (p-NPP, Sigma) was added for 30–60 min. Plates were read in an ELISA reader at 405 nm. The percentage of inhibition was calculated as follows: $[1 - (\text{OD sample} - \text{OD neg ctr})/(\text{OD pos ctr} - \text{OD neg ctr})] \times 100$.

Statistical Analysis. Sensitivity was calculated as the proportion of samples from ZIKV-infected individual identified as positive by the assay (percentage of inhibition $>50\%$), using one sample per individual (the latest time available, and only for samples collected >10 d after symptom onset). Specificity was calculated as the proportion of negative test results obtained among the different control groups used.

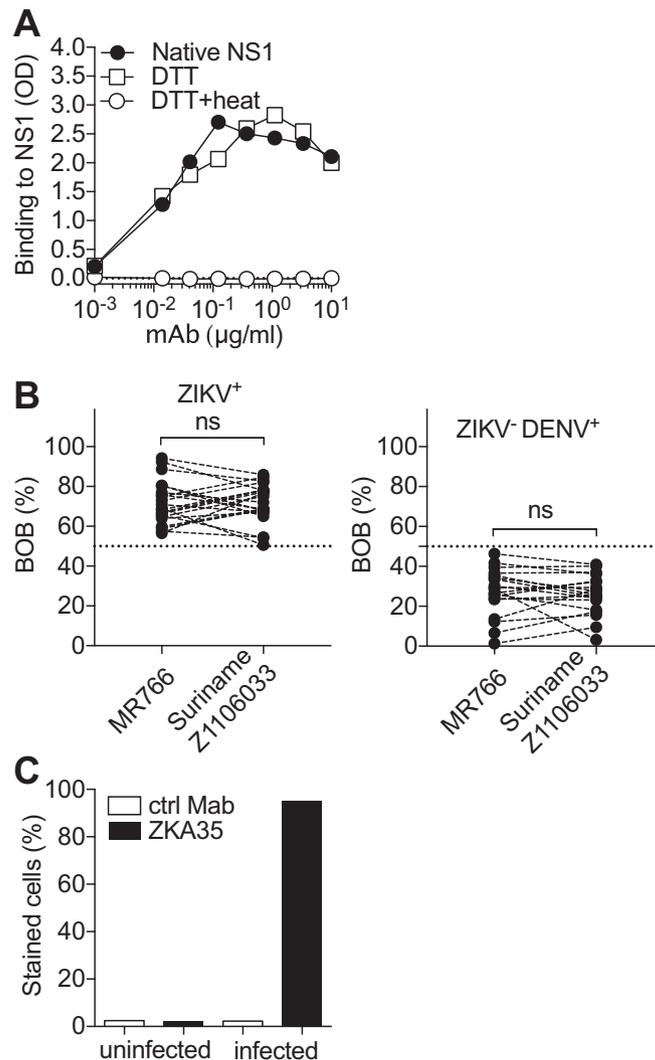


Fig. S1. ZKA35 binds to a conformational and highly conserved site on ZIKV NS1. (A) Binding of ZKA35 mAb to native, DTT-treated, or heated and DTT-treated NS1 as determined by ELISA. (B) BOB assay of ZIKV and DENV plasma samples from Nicaraguan studies, using NS1 from Uganda (MR766) and Suriname (Z1106033; both from Native Antigen Company, Inc.). Plotted is the percentage of ZKA35 mAb binding inhibition by 21 ZIKV-positive (Left) and 20 DENV-positive, ZIKV-negative (Right) plasma samples tested in parallel, using NS1 from MR766 and Suriname. The DENV-positive samples included DENV1 ($n = 9$) and DENV2 ($n = 11$) infections, as well as primary ($n = 4$) and secondary ($n = 16$) infections. Statistical analysis performed using Wilcoxon matched-pairs signed rank test: ns, not significant. The average inhibition in ZIKV-positive patients was 71.7 versus 71.6, and in DENV-positive, ZIKV-negative patients, it was 23.5 versus 24.3 for Uganda and Suriname NS1, respectively. (C) Binding of ZKA35 mAb to Vero cells infected with H/PF/2013 ZIKV strain. ZKA35 and a control mAb (MPE8 directed to RSV F protein) were tested at 0.3 $\mu\text{g}/\text{mL}$, using mock- or ZIKV-infected cells 2 d postinfection. Cells were permeabilized with 0.5% saponin, and binding was determined using an anti-human IgG Alexa488-conjugated antibody in the presence of the DraQ5 counterstain. Fluorescence was read using a high-throughput fluorometric microvolume assay technology (Mirrorball, TPP Labtech).

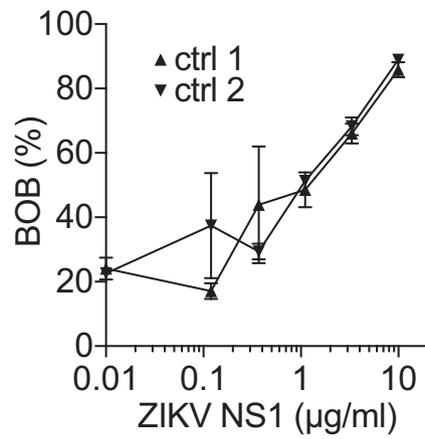


Fig. S2. Soluble NS1 inhibits ZKA35 binding to solid-phase ZIKV NS1. Plotted is the percentage of ZKA35 mAb binding inhibition by two plasma samples from Swiss blood donor controls (ZIKV- and DENV-negative) that were spiked with increasing amounts of ZIKV NS1. The addition of exogenous NS1 to negative control plasma samples resulted in ZKA35 mAb binding inhibition when the concentration of NS1 exceeded 1 µg/mL.