TITLE: T cell responses to nonstructural protein 3 distinguish infections by Dengue and Zika viruses

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ABSTRACT

The 2015-16 Zika virus (ZIKV) epidemic in the Americas and the Caribbean demonstrates that clinical assays to detect, distinguish, and characterize immune responses to flaviviral infections are needed. ZIKV and dengue virus (DENV) are mosquito-transmitted flaviviruses sharing overlapping geographical distribution and have significant sequence similarity that can increase the potential for antibody and T cell cross-reaction. Using nonstructural protein 1-based enzyme-linked immunosorbent assays (ELISAs), we determine the serostatus of individuals living in a DENV- and ZIKV-endemic region in Brazil, identifying individuals with primary DENV (pDENV) and ZIKV (pZIKV), ZIKV with primary DENV (ZIKVwpDENV), and secondary DENV (sDENV) infections; pDENV and pZIKV were further confirmed by neutralization tests. Development of an enzyme-linked immunospot (ELISPOT) assay for DENV and ZIKV structural and nonstructural (NS) protein antigens enables us to distinguish infections by these viruses based on T cells and to characterize those responses. We find that IFN-γ and TNF-α T cell responses to NS3 differentiates DENV and ZIKV infections with 94% sensitivity and 92% specificity. In general, we also show that pDENV and sDENV cases and pZIKV and ZIKVwpDENV cases elicit similar T cell response patterns, and that HIV-infected individuals have T cell responses that are lower in magnitude compared to HIV-negative individuals. These results have important implications for DENV and ZIKV diagnostic and vaccine development and provide critical insights into the T cell response in individuals with multiple flaviviral infections.

IMPORTANCE

The potential for antibody and T cell cross-reaction to DENV and ZIKV, flaviviruses that co-circulate and can sequentially infect individuals, has complicated diagnostic and vaccine development. Our serological data show that antibodies to nonstructural protein 1 can distinguish sequential human infections by DENV and ZIKV. The development of a simple and inexpensive assay also enables the differentiation of DENV and ZIKV infections based on the characterization of T cell responses. Our T cell data reveals strong response patterns that are similar in nature in individuals with one or multiple DENV infections and in individuals with only primary ZIKV infection and ZIKV-infected individuals with previous DENV exposure. The characterization of T cell responses in a serologically-validated group of individuals is of relevance to the development of vaccines and immunotherapeutics against these global threats.

INTRODUCTION

Aedes mosquitoes transmit globally relevant flaviviruses including dengue virus (DENV) and Zika virus (ZIKV). DENV exists as four antigenic serotypes, DENV1 to DENV4 (1). These viruses have a wide geographic distribution with approximately 390 million infections annually and more than a quarter of the world's population at risk (2). Prior to 2015, ZIKV was considered obscure and known to circulate in Africa and Southeast Asia as two separate viral lineages, African and Asian (3). While most asymptomatic, the clinical presentation of ZIKV infection resembles that of dengue including fever, rash, conjunctivitis, arthralgia, and myalgia (4). In early 2015, thousands of Asian ZIKV cases appeared in northeast Brazil, with accompanying reports of severe neuropathology including congenital microcephaly and Guillain-Barré syndrome (5, 6). In February 2016, the World Health Organization declared ZIKV a public health emergency of international concern (7). By June 2016, autochthonous transmission of ZIKV had been reported in 40 countries and territories throughout South and Central America and the Caribbean (8).

The emergence of ZIKV in DENV-endemic regions is of particular concern and relevant for diagnostic and vaccine development. The cocirculation of these genetically similar viruses can result in co-infection or sequential exposure, which has been shown to potentiate cross-reactive immunity at both the antibody and T cell levels (9-12). The envelope (E) protein is the major target of the antibody response in humans during flaviviral infection (1). Antibody-based assays were found to detect extensive cross-reactivity to ZIKV E protein with other flaviviruses, requiring confirmation by plaque

reduction neutralization tests (PRNTs) (11, 13-16). These tests, however, are challenged in their ability to confirm infection in individuals with multiple flaviviral infections especially during the acute and early convalescent phases. Several studies have also shown that most dengue-immune sera or DENV E monoclonal antibodies cross-react to ZIKV, but contain limited cross-neutralization activity and can instead enhance ZIKV infection, known as antibody-dependent enhancement (ADE) (17-22). In contrast, recent studies reported antibodies to ZIKV nonstructural protein 1 (NS1) were able to discriminate infections by these viruses (23, 24). We previously showed that combinations of DENV and ZIKV NS1-based enzyme-linked immunosorbent assays (ELISAs) were capable of distinguishing confirmed cases with past and present flaviviral infections including primary DENV (pDENV) and ZIKV (pZIKV), ZIKV with primary DENV (ZIKVwpDENV), and secondary DENV (sDENV) infections (12). These ELISAs are applicable for routine serological tests for DENV and ZIKV as well useful in retrospective studies to identify individuals with primary and multiple flaviviral infections.

Pre-existing T cell responses to DENV have also been shown to react to peptides encoded throughout the ZIKV proteome. DENV-naïve mice challenged with ZIKV developed ZIKV-specific $CD8^+$ T cells, whereas DENV-immune mice challenged with ZIKV elicited cross-reactive $CD8^+$ T cells that reduced infectious ZIKV (25). A study in humans infected with Asian ZIKV demonstrated that DENV serostatus influences the T cell response to ZIKV (10). DENV-immune individuals elicited $CD4^+$ and $CD8^+$ T cell responses to ZIKV more rapidly and of greater magnitude compared to DENV-naïve ZIKV-infected individuals. In addition, different patterns of immunodominant T cell responses were observed in the case of DENV and ZIKV infections. While $CD8^+$ T cell

139 responses against DENV target nonstructural (NS) proteins such as NS3, NS4B, and
140 NS5, ZIKV-specific $CD8^+$ T cell responses target the structural proteins, capsid (C),
141 premembrane (prM), and E (10, 26). We previously developed a modified anthrax toxin
142 (LFn)-based enzyme-linked immunospot (ELISPOT) assay, which revealed long-term T
143 cell responses that were ZIKV- and DENV-specific to NS3 protease but cross-reactive
144 to NS3 helicase in individuals infected with DENV and African ZIKV (27). The impact of
145 cross-reactive immune responses in protection or development of ZIKV-mediated
146 neuropathology remains unclear.

147 In this study, we utilized our NS1-based ELISAs to determine the DENV and
148 ZIKV serostatus of individuals from Salvador, Brazil, a DENV-hyperendemic region with
149 one of the highest incidence rates of ZIKV during the 2015-16 epidemic (28). We then
150 tested the ability of our LFn ELISPOT assay to distinguish infections by DENV and
151 Asian ZIKV based on T cells and to characterize those responses.

152

153 **RESULTS**

154

155 **NS1-based ELISAs and neutralization test determine DENV and ZIKV serostatus**

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157 During the ZIKV outbreak in Salvador, Brazil, acute-phase blood samples were
158 collected from hundreds of suspected ZIKV-infected patients attending HIV outpatient
159 clinics between November 2015 and May 2016. Serological testing for ZIKV-NS1 IgG
160 and DENV-E IgG was performed, revealing a high incidence of ZIKV infection in
161 presumed DENV-immune and -naïve individuals (28). Fifty of these patients were

included in the present study. Their median age was 43 (range: 23-72), 49% female, and 76% were human immunodeficiency virus (HIV)-infected. All HIV-infected individuals were on antiretroviral therapy; more than 92% had undetectable viral loads and normal CD4 counts. Patient characteristics and acute serology data are summarized in Table S1 and Table 1, respectively.

In order to determine the DENV and ZIKV serostatus among the study participants who had potentially been dual exposed, we collected late convalescent-phase blood samples and employed our previously developed ZIKV-NS1 and DENV-NS1 IgG ELISAs (22). For samples positive for DENV-NS1, we calculated the ratio of relative optical density (rOD) of ZIKV-NS1 to that of DENV-NS1 and used the rOD ratio < or ≥ 0.24 to determine sDENV or ZIKVwpDENV infection, respectively (22). Twelve ZIKVwpDENV and 21 sDENV infections were identified (Table 1, Fig. 1A to C). Five samples with ZIKV-NS1 positive and DENV-NS1 negative were pZIKV. Since these samples were collected more than one year post-infection, some anti-NS1 antibodies may have declined to levels below detection, we further tested with ZIKV and DENV E protein-based ELISAs and identified four samples negative for both ZIKV and DENV in all four ELISAs tested (Table 1). Based on the difference in rOD of ZIKV and DENV E proteins (ΔrOD=rOD of ZIKV – rOD of DENV), we identified four pZIKV (ΔrOD ≥0.17) and four pDENV (ΔrOD <–0.17) infections (Table 1, Fig. 1D to E). The negative, pZIKV and, pDENV samples were further confirmed by micro-neutralization tests to DENV1-4 and ZIKV; all four negative samples had NT_{90} titers <10 to DENV1-4 and ZIKV, and the five pZIKV and four pDENV samples showed monotypic neutralization pattern to ZIKV and to one of the four DENV serotypes, respectively (Table 1). For the remaining 33

samples, microneutralization tests to ZIKV, DENV1 and/or DENV2 or 3 were performed to show that all 12 ZIKVwpDENV samples neutralize (NT_{90} titers ≥10) ZIKV plus at least one DENV serotype, whereas all 21 sDENV samples neutralize at least two DENV serotypes or DENV plus ZIKV; these patterns were compatible with unspecified flavivirus infection according to CDC guidelines (16). An additional three samples (ZK1004, 1005, 1008), which had ΔrOD between –0.17 and 0.17, positive ZIKV-NS1 IgG at acute-phase sera but negative at late convalescent-phase, were classified as undetermined (Table 1). Another sample (ZK0981), for which DENV acute-phase serology was not performed, was negative for ELISAs using late convalescent-phase serum and was also classified as undetermined.

T cell responses to NS3 distinguish DENV and ZIKV infections

We recently reported the development of a LFn ELISPOT assay based on NS3 protease and helicase to distinguish DENV and African ZIKV human infections (27). To assess the ability of the assay to distinguish infection by DENV and Asian ZIKV, we performed DENV and ZIKV homologous and heterologous LFn-NS3 protease and helicase stimulation of date-matched late convalescent-phase PBMCs in an IFN-γ and TNF-α ELISPOT among the serological-validated pDENV, pZIKV, sDENV, and ZIKVwpDENV, and undetermined cases. Using a NS3 protease to helicase ratio cutoff of 1.05 for the IFN-γ ELISPOT, pDENV and sDENV cases and pZIKV, ZIKVwpDENV and the 3 out of the 4 serologically undetermined cases appeared to group together (Fig. 2A). From the undetermined cases, 3 out of 4 (ZK1004, 1005, 1008) grouped with

the ZIKV-exposed individuals, while ZK0981 grouped with the DENV-infected individuals. Using a ratio cutoff of 1.048 for the TNF-α ELISPOT, similar groupings were observed (Fig. 2B). We were unable to distinguish sequential infections based on T cell responses to NS3 protease and helicase.

Test data were further analyzed to define sensitivity (identifying true positives; individuals who had been infected by DENV versus ZIKV) and specificity (true negatives; DENV- or ZIKV-uninfected individuals). We evaluated sensitivity and specificity as functions of the IFN-γ and TNF-α cutoff values, above which a sample was considered positive and below which a sample was considered negative. We grouped pDENV and sDENV and pZIKV and ZIKVwpDENV cases together based on the clustering observed and excluded the 4 serologically undetermined cases from the analysis. Receiver Operating Characteristic (ROC) curves and corresponding numerical values illustrate the performance of the ELISPOTs as a function of the discrimination threshold, plotted as sensitivity versus 1 – specificity. The areas of the ROC curves represent test performance, where 1 represents a perfect test, and 0.5 represents a random predictor. We measured areas of 0.96 and 0.97 for the IFN-γ and TNF-α ELISPOTs, respectively (Table 2, Fig. 2 C-D). Using the cutoff values, the test sensitivity and specificity for both the IFN-γ and TNF-α ELISPOTs were 94% and 92%, respectively.

LFn-DENV and -ZIKV structural and nonstructural proteins elicit robust T cell responses and prior DENV exposure does not affect the response

231 To assess the magnitude of T cell responses among the study participants, we
232 stimulated late convalescent-phase PBMCs in IFN-γ and TNF-α ELISPOTs using the
233 following six LFn fusion proteins: LFn-DENV-NS3-Protease (LFn-DV NS3-P), LFn-
234 DENV-NS3-Helicase (LFn-DV NS3-H), LFn-ZIKV-Capsid (LFn-ZV C), LFn-ZIKV-
235 premembrance (LFn-ZV prM), LFn-ZIKV-NS3-Protease (LFn-ZV NS3-P), LFn-ZIKV-
236 NS3-Helicase (LFn-ZV NS3-H). Individuals with pDENV and sDENV infections elicited
237 similar IFN-γ and TNF-α T cell response patterns (Fig. 3 A-B). These individuals had T
238 cell responses to LFn-DV NS3-H and LFn-ZV NS3-H that were stronger in magnitude
239 than to LFn-DV NS3-P and LFn-ZV NS3-P, respectively. Additionally, T cell responses
240 to LFn-DV NS3-P and NS3-H were stronger compared to LFn-ZV NS3-P and NS3-H.
241 The amount of T cell cross-reaction to the ZIKV structural proteins (LFn-ZV C and LFn-
242 ZV prM) was limited, compared to high cross-reactivity to LFn-ZV NS3-P and NS3-H.
243 Furthermore, individuals with pZIKV and ZIKVwpDENV infections elicited T cell
244 responses to LFn-ZV NS3-H and LFn-DV NS3-H that were stronger in magnitude
245 compared to LFn-ZV NS3-P and LFn-DV NS3-P, respectively (Fig. 3 C-D). While
246 individuals with pZIKV and ZIKVwpDENV infections had stronger IFN-γ T cell responses
247 to LFn-ZV NS3-H than to the ZIKV structural proteins, TNF-α responses to the ZIKV
248 structural proteins were stronger than to LFn-ZV NS3-P.
249 We further evaluated the impact of DENV immunity on the magnitude of T cell
250 responses. We compared the magnitude of the IFN-γ and TNF-α T cell responses
251 between individuals with pDENV and sDENV infections and pZIKV and ZIKVwpDENV
252 infections. In all cases, T cell responses in individuals with prior DENV exposure were
253 not significantly higher compared to individuals with a primary DENV or ZIKV infection

(Fig. 3 A-D). While IFN-γ and TNF-α responses appeared stronger to the ZIKV structural proteins in individuals with sDENV infections than to individuals with pDENV infections, these differences were not statistically significant. Similarly, individuals with ZIKVwpDENV infection had comparable IFN-γ and TNF-α T responses to those with pZIKV infections (Fig. 3 C-D).

HIV influences the T cell response in DENV-exposed individuals

We also compared the magnitude of the IFN-γ and TNF-α T cell responses in DENV-exposed (grouping individuals with pDENV and sDENV infections together), pZIKV, and ZIKVwpDENV individuals who were HIV-negative or HIV-infected. DENV-exposed HIV-negative individuals had stronger IFN-γ responses to LFn-ZV C, -ZV NS3-P, and -ZV NS3-H compared to HIV-infected individuals. IFN-γ responses to LFn-DV NS3-P and NS3-H appeared to be stronger in the HIV-infected individuals, although these differences were not statistically significant ($p=0.61$ and $p=0.13$, respectively) (Fig. 4 A). A similar pattern of responses was observed for TNF-α (Fig. 4 D). In general, ZIKVwpDENV HIV-negative individuals had stronger IFN-γ and TNF-α responses compared to individuals that were HIV-infected (Fig. 4 B and E). In contrast, there was largely no difference in the IFN-γ and TNF-α responses in pZIKV HIV-negative and HIV-infected individuals (Fig. 4 C and F). There was an exception where the TNF-α response to LFn-ZV NS3-H was stronger in individuals that were HIV-negative.

DISCUSSION

We report on the characterization of late convalescent-phase antibody and T cell responses in individuals from Salvador, Brazil, a DENV-hyperendemic region that was burdened by the 2015-2016 ZIKV outbreak. Our study presents three major findings in a serologically-validated group of DENV and/or ZIKV infected individuals. First, IFN-γ and TNF-α T cell response ratios of ZIKV NS3 protease to DENV NS3 protease can discriminate infections in individuals exposed to these viruses. Second, individuals with pDENV and sDENV infections have similar T cell response patterns, with extensive cross-reactivity to ZIKV NS3 helicase, whereas individuals with pZIKV and ZIKVwpDENV infections have strong responses to both ZIKV structural and nonstructural proteins, with high cross-reaction to DENV NS3 helicase. Third, HIV-infection is associated with responses that are lower in magnitude in DENV exposed individuals.

Our previous study of NS1-based ELISAs on convalescent-phase sera from RT-PCR confirmed cases with pZIKV, pDENV, sDENV and ZIKVwpDENV infections showed that sDENV infection panel cross-react to ZIKV-NS1 and the rOD ratio of ZIKV-NS1 to DENV-NS1 in IgG ELISA can distinguish sDENV and ZIKVwpDENV infections (22). Since anti-NS1 antibodies may decline over time and become undetectable especially for those with primary infection, we further tested these samples with E protein-based IgG ELISAs and identified four negative samples, five pZIKV and four pDENV infections. All these 13 samples have been verified by neutralization test using NT_{90} ≥10 as cutoff based on the CDC guidelines (16), suggesting that ΔrOD based on

ZIKV-E and DENV-E IgG ELISAs can distinguish pZIKV and pDENV infections; this could potentially be a useful tool for epidemiology and pathogenesis study in endemic regions. However, the sample size is small and the ΔrOD of 0.17 was based on a single serum dilution of 1:800, future studies involving larger sample size and different dilutions or end-point titers are needed to further validate these observations.

The degree of amino acid sequence identity between DENV and ZIKV structural and nonstructural proteins is 49% and 51%, respectively (10). Multiple sequence alignment and homology determination of DENV and ZIKV NS3 demonstrates high amino acid sequence identity of 67%, with protease and helicase homology of 58% and 72%, respectively, consistent with the higher degree of DENV/ZIKV cross-reaction in NS3 helicase (Table 3, Fig. 5). Our recent characterization of acute- and convalescent-phase T cells collected from individuals infected with DENV and African ZIKV in Senegal, West Africa, revealed sustained DENV- and ZIKV-specific responses to NS3 protease and cross-reactive responses to NS3 helicase (27). Our findings in individuals infected with DENV and Asian ZIKV are in agreement with our previous observations. Although we were unable to distinguish sequential exposure, the LFn NS3 protease ELISPOT differentiates infections between DENV- and ZIKV-infected individuals with high sensitivity and specificity of 94% and 92%, respectively.

A relatively large body of epidemiological and laboratory-based evidence has suggested that severe and often fatal forms of dengue disease occurs most commonly during a secondary infection by a heterotypic DENV serotype (29, 30). Another phenomenon, known as original antigenic sin (OAS), has been observed in antibody as well as T-cell responses, in which less-effective T cells generated in response to a

primary DENV infection predominate during a subsequent infection with a different DENV serotype, resulting in an inappropriate response and predisposing individuals to severe disease (31, 32). The OAS hypothesis was challenged by a study in Sri Lankan individuals infected with DENV, which showed that the phenomenon does not generate less functional responses, but instead correlates with protective responses to conserved viral sequences (26). Unexpectedly, we did not observe T cell responses that were significantly higher in magnitude in individuals with prior DENV exposure. These results are in contrast to our data on African ZIKV infections, which showed that previous flavivirus exposure was associated with enhanced T cell responses (27). One possibility is that the proportion of HIV infection among those with prior DENV exposure was higher compared with DENV-naïve in this study (90.5% versus 50% comparing sDENV and pDENV cases; 83.3% versus 60% comparing ZIKVwpDENV and pZIKV cases). Nevertheless, as co-circulation of DENV, ZIKV, and other flaviviruses occurs throughout many parts of the world, it is critical to continue to develop tools to better understand T cell immunity in individuals exposed to multiple flaviviruses.

A recent study using human leukocyte antigen (HLA) transgenic mice infected with DENV2 and Asian and African ZIKV strains revealed cross-reactive T cell responses to HLA-restricted epitopes (25). Out of 8 ZIKV NS3 epitopes computationally predicted to bind HLA class I molecules, only 3 epitopes elicited DENV2/Asian ZIKV cross-reactive T cell responses. Of note, the cross-reactive epitopes were all positioned within the helicase domain of NS3, further supporting our observations of high DENV/ZIKV NS3 helicase T cell cross-reaction. Another study demonstrated ZIKV-specific and ZIKV/DENV cross-reactive T cell responses in humans (10). T cell

responses generated in response to prior DENV exposure recognized peptides sequences located throughout the ZIKV proteome. DENV serostatus also influenced T cell immunity to ZIKV. DENV-naïve ZIKV-positive individuals had predominant CD8 T cell responses directed against structural proteins. In contrast, a majority of CD8 T cells responses were directed against nonstructural proteins in DENV-immune ZIKV-positive individuals, suggesting that previous DENV exposure can alter the T cell response.

While the above studies used peptide stimulation to characterize the T cell response, there are concerns around this approach (33). Some of the HLA-predicted peptides may fail to stimulate strong T cell responses as expected. Longer and shorter peptides have also been shown to elicit different types of responses (34-36). An alternative to peptide stimulation is the anthrax LFn, which has the capability to deliver full length antigen into the cytosol for native processing via the MHC pathways, and to elicit better T cell responses compared to peptides in some cases (37-42). Our adaptation of the LFn ELISPOT, not only allowed detection of human DENV and ZIKV infections, but also characterization of the associated T cell responses to structural and nonstructural proteins. We demonstrated that individuals with pDENV and sDENV infections had similar IFN-γ and TNF-α T cell response patterns with high cross-reactivity to ZIKV NS3 helicase, but low cross-reactivity to the ZIKV structural proteins. A small number of individuals with sDENV infections had cross-reactive T cell responses to ZIKV structural proteins. Interestingly, however, individuals with pZIKV and ZIKVwpDENV infections had similar IFN-γ and TNF-α T cell responses patterns, with strong responses to structural and nonstructural proteins. It is noteworthy that we observed comparably strong T cell responses to the structural proteins in pZIKV and

ZIKVwpDENV cases, in contrast to Grifoni et al., suggesting that the most recent infection may dictate the T cell response (10). Another possibility that cannot be excluded is the differences in T cell stimulation strategies, which may be contributing to the observed differences. Additionally, due to the limited collection of blood samples from each patient, we were unable to characterize CD4- and CD8-specific responses. Future characterization studies using the LFn delivery system on CD4 and CD8 T cells will be important.

Our study of ZIKV seroprevalence in West Africa demonstrated continued human transmission of the virus in HIV- and malaria-infected individuals (43). The co-infection of flaviviruses with HIV or malaria could potentially impact pathophysiological mechanisms, induce different clinical and laboratory findings, and interfere with treatment. Previous studies have shown a suppression of HIV-1 replication during acute DENV infection (44, 45). In this study, we demonstrated that DENV-exposed individuals who are HIV-infected had T cell responses that were significantly lower in magnitude compared to HIV-negative individuals except in individuals with pZIKV infections. We also observed that DENV-exposed HIV-infected individuals have T cell responses that were lower in magnitude to ZIKV proteins compared to DENV-exposed HIV-negative individuals. Whether HIV infection in DENV-exposed individuals reduces the ability to induce cross-reactive T cell responses has important implications. More studies with larger sample sizes are needed to increase our limited understanding of the epidemiological and immunopathogenesis interactions of flavivirus exposure in individuals with HIV and other comorbidities.

391 In summary, despite high sequence homology between DENV and ZIKV,
392 diagnostic assays based on antibodies to NS1 and T cell responses to NS3 protease
393 are effective at distinguishing human infections by these viruses. The LFn ELISPOT
394 assay has enabled direct comparison of T cell characterization in DENV, Asian and
395 Africa ZIKV human infections. As vaccines against DENV and ZIKV are currently being
396 developed, the information generated from these characterization studies are of high
397 relevance. The results of these characterization studies may contribute to the design
398 and development of DENV and ZIKV vaccines and T cell based diagnostics.
399
400 **MATERIALS AND METHODS**
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402 **Clinical samples and ethical statement**
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404 Fifty late convalescent-phase blood samples were obtained from patients at
405 Professor Edgard Santos University Hospital, Federal University of Bahia, Salvador,
406 Brazil. These individuals were suspected ZIKV-infected during the 2015-2016 ZIKV
407 epidemic and their acute-phase sera were screened for ZIKV and DENV antibodies.
408 Late convalescent-phase peripheral blood mononuclear cells (PBMCs) were separated
409 from whole blood in EDTA tubes by Ficoll-Hypaque gradient density (Sigma-Aldrich, St.
410 Louis, MO, USA) and cryopreserved in freezing media (10% dimethyl sulfoxide [DMSO],
411 Sigma-Aldrich, St. Louis, MO, USA) at -80°C overnight prior to transfer to liquid
412 nitrogen. Convalescent-phase serum was aliquoted and immediately transferred to -
413 80°C.

The Federal University of Bahia Institutional Review Board (IRB), the Harvard T.H. Chan School of Public Health IRB, and the University of Hawaii IRB approved the primary studies under which the samples and data were collected. All patients provided informed consent for the collection of samples. Excess samples and corresponding data were banked, coded prior to analyses, and stored at the Federal University of Bahia.

ELISAs

For acute-phase sera, commercial ZIKV-NS1 and DENV-E based IgG ELISAs (Euroimmun, Luebeck, Germany) were performed (27). For late convalescent-phase sera, ZIKV- and DENV1-NS1 IgG ELISAs were performed as described previously (22). Briefly, purified NS1 proteins (16 ng per well) were coated onto 96-well plates overnight, followed by blocking and incubation with primary (serum at 1:400 dilution) and secondary (anti-human IgG conjugated with HRP, Jackson) antibodies (22). The OD at 450 nm was read with a reference wavelength of 650 nm. Each ELISA plate included two positives (two confirmed-Zika or confirmed-dengue samples for ZIKV- and DENV-NS1 ELISAs, respectively), four negatives (4 flavivirus-naïve sera), and tested samples (all in duplicates). The OD values were divided by the mean OD value of positive controls to calculate the rOD values. The cut-off was defined by the mean rOD value of negatives plus 12 standard deviations as described previously (22). For samples positive for both ZIKV- and DENV-NS1 ELISAs, the ratio of rOD (=rOD of ZIKV-NS1/rOD of DENV-NS1) was calculated; rOD ratio $<$ or $\geq$ 0.24 indicated sDENV or ZIKVwpDENV infection, respectively (22).

E protein-based IgG ELISAs using DENV1 virion or ZIKV (MR766 strain) virus like particles (VLP) were also tested for late convalescent-phase sera (46). Briefly, DENV1 virions or ZIKV-VLP derived from ultracentrifugation of culture supernatants of virus-infected Vero cells or pENTR-ZIKV prME plasmid-transfected 293T cells, respectively, were UV inactivated (for virions) and coated on 96-well plates at 4°C overnight, followed by blocking and incubation with primary (serum at 1:800 dilution) and secondary antibodies as above. The rOD and cut-off rOD values were similarly calculated. The difference in rOD of ZIKV and DENV E proteins (ΔrOD=rOD of ZIKV – rOD of DENV) was determined; ΔrOD ≥ 0.17 or < –0.17 was classified as pZIKV or pDENV infection, respectively.

Neutralization test

PRNT was performed on acute-phase sera to detect neutralization antibody to ZIKV as reported previously (47). For late convalescent-phase sera, a previously described micro-neutralization test was performed (48). Briefly, flat-bottom 96-well plates were seeded with Vero cells (3×10^4 cells per well) 24 h prior to infection. Four-fold serial dilutions of serum (starting from 1:10) were mixed with 50 focus-forming units of DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (CH53489), DENV4 (H241 strain) or ZIKV (PRVABC59 strain) at 37°C for 1 h. The mixtures were added to each well followed by incubation for 48 h (except 70 h for DENV1), removal of medium, and fixation as described previously (46). After adding murine mAb 4G2 and secondary antibody mixture (IRDye® 800CW-conjugated goat anti-mouse IgG at 1:10000 and

460 DRAQ5™ Fluorescent Probe at 1:10000), the signal (800 nm/700 nm fluorescence) was
461 detected by Li Cor Odyssey classic (LiCor Biosciences) and analyzed by Image Studio
462 software to determine percent neutralization at different concentrations and NT_{90} as
463 described previously (46, 48).
464
465
466 **LFn fusion protein design**
467
468 Commercially synthesized gene fragments encoding the NS3 protease and
469 helicase of DENV2 and C, prM, and NS3 protease and helicase of Asian ZIKV and were
470 cloned into the LFn expression vector (pET15bLFn). The pET15bLFn vector contains a
471 T7 promoter, histidine tag (His_6), and the terminal domain of the anthrax lethal factor
472 (LFn; 255 amino acids). The pET15bLFn containing the coding sequences of the DENV
473 and Asian ZIKV proteins were transformed into *E. coli* BLR (DE3) (Millipore, Medford,
474 MA, USA). Selected clones were sequences to verify the reading frame, and clones
475 containing the correct sequence were used for protein expression.
476 The LFn-DENV and -ZIKV fusion proteins and the LFn control were expressed
477 upon isopropylthiogalactoside ([IPTG], Sigma-Aldrich, St. Louis, MO, USA) induction in
478 5L Luria broth containing carbenicillin and chloramphenicol for 2-4 hours. Cells were
479 pelleted by centrifugation and resuspended in imidazole (1mM) binding buffer
480 (Novagen, Madison, WI, USA) in the presence of a protease inhibitor cocktail (Thermo
481 Fisher Scientific, Rockford, IL, USA). Cell pellets were sonicated, centrifuged at 4°C,
482 and the supernatants were loaded in an equilibrated nickel-charged column for affinity

purification. The bound proteins were eluted in 100-200 mM imidazole, desalted with a Sephadex G-25M column (Sigma-Aldrich, St. Louis, MO, USA), and eluted in PBS (Sigma-Aldrich, St. Louis, MO, USA). The PBS-eluted proteins were passed through Detoxi-Gel (Thermo Fisher Scientific, Rockford, IL, USA). Protein concentrations were determined samples were stored at -80°C.

ELISPOT assay

Ex vivo ELISPOTs were performed as previously described. Briefly, 96-well polyvinylidene difluoride (PVDF)-backed MultiScreen$_{HTS}$ (MSIP) microtiter plates (Millipore, Medford, MA, USA) were treated with 100ul of 90% ethanol for 30 seconds and washed 5 times with sterile PBS. Plates were coated with 100ul of capture antibodies (Abs) in PBS. Plates containing capture Abs were incubated overnight at 4°C. Plates were then blocked with 1% bovine serum albumin ([BSA], Sigma-Aldrich, St. Louis, MO, USA) in PBS and washed 6 times with PBS. Cryopreserved PBMCs were thawed in R10 medium and incubated overnight at 37°C. PBMCs were washed 2 times with PBS and seeded at 2 x 10^5 cells/well in a final volume of 100ul/well. LFn-DENV and -ZIKV proteins were added to each well. As a positive control, PBMCs were stimulated with phytohemagglutinin ([PHA], Sigma-Aldrich, St. Louis, MO, USA). As a negative control, wells received LFn. After incubation for 24-28 hours at 37°C in 5% CO_2, the cells were discarded and plates were washed 3 times with PBS and 3 times with PBS with 0.05% Tween-20 ([PBST], Bio Rad Technologies, Hercules, CA, USA) to remove cells. The detection antibodies were added and plates were incubated overnight

at 4°C. Plates were then washed 6 times with PBST, then incubated for 2 hours at room temperature with mixtures containing the enzymatic conjugates. To develop spots, plates were washed 4 times with PBST, three times with PBS, and 1 time with water. Vector Blue substrate solution (Vector Laboratories, Burlingame, CA, USA) was added for 5-15 mins before rinsing with water and air-drying. Digitized images were analyzed for spots using CTL ImmunoSpot reader (Cellular Technology Limited, Cleveland, OH, USA). DENV and ZIKV spots were calculated by subtracting the mean of the negative control value from the mean value of the specific stimulation. Positive responses had to be greater than 4 times the mean background, 3 standard deviations above the background, and ≥55 spot-forming cells per (SFC)/10^6 PBMCs.

ROC analysis

The ELISPOTs were validated using PBMCs from individuals that were confirmed DENV- and/or ZIIV-infected by ELISA and/or neutralization tests. DENV and ZIKV NS3 protease to helicase values were calculated, resulting in normalized test ratios (ZIKV NS3 protease divided by DENV NS3 protease) ranging from 0.15-2.95. On the basis of these data, we determined the optimal cutoffs between 0.15 and 2.95 by calculating the sensitivity (number of true positives divided by total confirmed positive values) and specificity (number of true negatives divided by the total confirmed negatives) at increasing 0.05 to the theoretical cutoffs. After calculating the sensitivity and specificity values, the optimal cutoffs were defined as the highest sum of sensitivity and specificity, such that the optimal cutoff values reflected the optimal sensitivity and

specificity. The optimal cutoffs obtained for the IFN-γ and TNF-α was 1.05 and 1.048, respectively (Prism 7, GraphPad Software, San Diego, CA, USA).

Multiple sequence alignment and percent homology analysis

Multiple sequence alignment of DENV1-4 and ZIKV NS3 was performed using the Clustal Omega program (EMBL-EB, Cambridgeshire, UK). Averages of DENV and ZIKV NS3 protease and helicase proteins were calculated using the ExPASy Bioinformatics Resource Portal (Swiss Institute of Bioinformatics, Lausanne, Switzerland) and based on averages of the different homology values in the four DENV serotypes and ZIKV. Average conservation was determined on a per-residue basis for NS3 protease, helicase, and full-length protein.

Statistical analysis

Statistical analysis was performed using Prism 7 (GraphPad Software, San Diego, CA, USA). Where appropriate, data were expressed as geometric positive means on box whisker and bar graphs ± standard deviation. Data comparisons were conducted using the Wilcoxon rank sum test. A threshold of $p<0.05$ was considered statistically significant.

Data Availability

All relevant data has been included in the manuscript. We will provide any additional data upon request.

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TABLES

Table 1 Results of Serological tests

	Acute-phase sera			Late convalescent-phase serum						
ID	ZIKV-NS1 IgG[a]	DENV-E IgG[a]	PRNT test[b]	ZIKV-NS1 IgG[c]	DENV-NS1 IgG[c]	rOD ratio[c]	ZIKV-E IgG[d]	DENV-E IgG[d]	NT test[f] D1/D2/D3/D4/ZIKV	Interpretation
ZK0978	-	-	-	-	-	NA	-	-	<10/<10/<10/<10/<10	negative
ZK0982	-	-	-	-	-	NA	-	-	<10/<10/<10/<10/<10	negative
ZK0987	-	-	-	-	-	NA	-	-	<10/<10/<10/<10/<10	negative
ZK0999	-	-	-	-	-	NA	-	-	<10/<10/<10/<10/<10	negative
ZK0979	+	+		+	-	NA	+[e]	+	<10/<10/<10/<10/>160	pZIKV
ZK0993	+	+		+	-	NA	+[e]	+	<40/<40/<10/<10/>160	pZIKV
ZK0998	+	+		+	-	NA	+[e]	+	<10/<10/<40/<10/>160	pZIKV
ZK1006	+	+		+	-	NA	+[e]	+	<80/<40<10/<40/640	pZIKV
ZK0996	+	-	+	+	-	NA	+[e]	+	<10/<10/<10/<10/>160	pZIKV
ZK0966	-	+	-	-	-	NA	+	+[e]	<40/160/<10/<40/<10	pDENV
ZK0980	-	+		-	-	NA	+	+[e]	<40/<40/>160/<40/<10	pDENV
ZK0995	-	+		-	-	NA	+	+[e]	>640/160/<10/<10/<10	pDENV
ZK0997	ND	ND		-	-	NA	+	+[e]	<40/>160/<40/<40/<10	pDENV
ZK0972	+	+		+	+	≥0.24	+	+	>40/ND/ND/ND/>80	ZIKVwpDENV
ZK0975	+	+		+	+	≥0.24	+	+	>40/ND/ND/ND/>80	ZIKVwpDENV
ZK0989	+	+	+	+	+	≥0.24	+	+	>40/ND/ND/ND/>80	ZIKVwpDENV
ZK0991	+	+	+	+	+	≥0.24	+	+	>40/ND/ND/ND/>80	ZIKVwpDENV
ZK1000	+	+		+	+	≥0.24	+	+	>40/ND/ND/ND/>40	ZIKVwpDENV
ZK1009	+	+	+	+	+	≥0.24	+	+	>10/ND/ND/ND/>80	ZIKVwpDENV
ZK1011	+	+		+	+	≥0.24	+	+	>40/ND/ND/ND/>80	ZIKVwpDENV
ZK1012	+	+	+	+	+	≥0.24	+	+	>40/ND/ND/ND/>80	ZIKVwpDENV
ZK1014	+	+	+	+	+	≥0.24	+	+	>40/ND/ND/ND/>80	ZIKVwpDENV
ZK1015	+	+	+	+	+	≥0.24	+	+	>40/ND/ND/ND/>80	ZIKVwpDENV
ZK0968	+	ND		+	+	≥0.24	+	+	>40/ND/ND/ND/>80	ZIKVwpDENV
ZK0984	+	+		+	+	≥0.24	+	+	>320/>1280/<80/>1280/>320	ZIKVwpDENV
ZK0976	-	+		+	+	<0.24	+	+	>40/ND/ND/ND/<10	sDENV
ZK0986	-	+		+	+	<0.24	+	+	>40/ND/ND/ND/>80	sDENV

ZK0967	+	+		+	+	<0.24	+	+	>40/ND/ND/ND/>80	sDENV
ZK0969	+	+		+	+	<0.24	+	+	>40/ND/ND/ND/>40	sDENV
ZK0971	+	+		+	+	<0.24	+	+	>40/>40/ND/ND/<10	sDENV
ZK0977	+	+		+	+	<0.24	+	+	>40/ND/ND/ND/>80	sDENV
ZK0983	+	+	+	+	+	<0.24	+	+	>40/ND/ND/ND/>10	sDENV
ZL0985	+	+	+	+	+	<0.24	+	+	>40/ND/ND/ND/>40	sDENV
ZK0988	+	+		+	+	<0.24	+	+	>40/ND/ND/ND/>40	sDENV
ZK0992	+	+	+	+	+	<0.24	+	+	>40/ND/ND/ND/>80	sDENV
ZK0994	+	+	-	+	+	<0.24	+	+	>40/ND/ND/ND/>40	sDENV
ZK1001	+	+		+	+	<0.24	+	+	>40/>40/ND/ND/<10	sDENV
ZK1010	+	+		+	+	<0.24	+	+	>40/ND/ND/ND/>80	sDENV
ZK1013	+	ND		+	+	<0.24	+	+	>40/ND/ND/ND/>10	sDENV
ZK0973	-	ND		-	+	<0.24	+	+	>40/<80/>80/ND/>320	sDENV
ZK0974	-	+		-	+	<0.24	+	+	>40/>320/>80/ND/>10	sDENV
ZK1002	-	+	-	-	+	<0.24	+	+	>40/>80/<80/ND/<10	sDENV
ZK1003	-	+	-	-	+	<0.24	+	+	>40/>80/<80/ND/<10	sDENV
ZK1007	-	+		-	+	<0.24	+	+	>40/>80/>80/ND/<10	sDENV
ZK1016	-	+		-	+	<0.24	+	+	>40/>1280/>1280/ND/>10	sDENV
ZK0990	-	+	-	-	+	<0.24	+	+	>40/>1280/>80/ND/<10	sDENV
ZK1004	+	+		-	+	NA	+	+	ND/ND/ND/ND/ND	unknown
ZK1005	+	ND		-	+	NA	+	+	ND/ND/ND/ND/ND	unknown
ZK1008	+	+		-	-	NA	+	+	ND/ND/ND/ND/ND	unknown
ZK0981	-	ND		-	-	NA	-	-	ND/ND/ND/ND/ND	unknown

pZIKV: primary ZIKV infection; pDENV: primary DENV infection; sDENV: secondary DENV infection; ZIKVwpDENV: ZIKV infection with previous DENV infection. ND: not determined; NA not applicable.

[a]Euroimmun ZIKV-NS1 and DENV-E IgG ELISAs were performed on acute-phase sera (28).

[b]PRNT was performed on acute-phase sera to detect neutralization antibody to ZIKV (47).

[c]ZIKV-NS1 and DENV-NS IgG ELISAs were described previously (12). The rOD ratio (ZIKV-NS1/DENV-NS1) < or ≥0.24 was classified as sDENV or ZIKVwpDENV (12).

[d,e]ZIKV-E and DENV-E IgG ELISAs utilized ZIKV VLP and DENV virions, respectively (46). ΔrOD (rOD of ZIKV–rOD of DENV) ≥ 0.17 or < –0.17 was classified as pZIKV or pDENV infection, respectively.

[f]Mico-neutralization test (NT) were performed (with NT_{90} titers shown) to confirm none, pZIKV or pDENV infection (46, 48).

Table 2 Numerical values of ROC analysis and sensitivity and specificity results

	IFN-γ$^+$ DENV vs. ZIKV	TNF-α$^+$ DENV vs. ZIKV
AUC[a]	0.96	0.97
95% CI[b]	0.91-1.02	0.92-1.01
Cutoff	1.055	1.048
Sensitivity	94%	94%
Specificity	92%	92%

[a]Area under the curve, [b]Confidence interval

Table 3 Sequence homology of DENV and Asian ZIKV NS3[a]

	% homology to ZIKV		
Serotype	NS3 protease	NS3 helicase	Full-length
DENV1	55%	71%	66%
DENV2	58%	72%	67%
DENV3	58%	72%	67%
DENV4	59%	71%	67%

[a]Homology analysis between Asian ZIKV (GenBank accession number: NC_035889.1) and DENV1 (ACO06157.1), DENV2 (JN819419.1), DENV3 (ACY70771.1), and DENV4 (AEW50183.1).

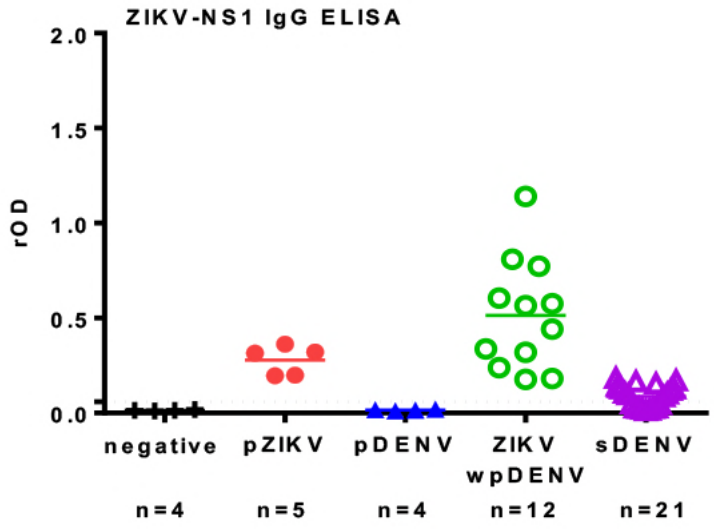
794 **Figure 1. ZIKV and DENV NS1-based and E-based IgG ELISAs.** (A) ZIKV-NS1, (B)
795 DENV-NS1 IgG ELISAs and (C) rOD ratio. (D) ZIKV-E, (E) DENV-E and (F) ΔrOD=rOD
796 of ZIKV–rOD of DENV. Dots lines indicate cut-off values, 0.24 for rOD ratio and 0.17 for
797 ΔrOD. pZIKV: primary ZIKV infection; pDENV: primary DENV infection; sDENV:
798 secondary DENV infection; ZIKVwpDENV: ZIKV infection with previous DENV infection.
799 NA not applicable.
800
801 **Figure 2. T cell responses to NS3 protease and helicase and ROC analysis of the**
802 **ELISPOT test.** Late convalescent-phase PBMCs from DENV- and/or ZIKV-infected
803 individuals were treated with homologous and heterologous LFn-DENV and -ZIKV NS3
804 protease and the specific IFN-γ and TNF-α T cell responses were detected by *ex vivo*
805 ELISPOTs. (A) Scatter plot of the ratios of ZIKV NS3 protease to DENV NS3 protease
806 IFN-γ responses versus ratios of helicase. (B) ROC analysis for the IFN-γ ELISPOT. (C)
807 Scatter plot of the ratios of ZIKV NS3 protease to DENV NS3 protease TNF-α T cell
808 responses versus ratios of helicase. (D) ROC analysis for the TNF-α ELISPOT. The
809 dashed line on (A) represents the optimal cutoff value of 1.05 and the dashed line on
810 (C) represents the optimal cutoff value of 1.048. Individual colored dots represent
811 serologically-validated DENV- and/or ZIKV-infected individuals and the undetermined
812 cases.
813
814 **Figure 3. T cell responses to ZIKV and/or DENV structural or nonstructural**
815 **proteins among subgroups with different DENV and ZIKV serostatus.** Late
816 convalescent-phase PBMCs from DENV- and/or ZIKV-infected individuals were treated

817 with homologous and/or heterologous LFn-DENV and -ZIKV capsid (ZV C),
818 premembrane (ZV prM), NS3 protease (DV or ZV NS3-P) and NS3 helicase (DV or ZV
819 NS3-H) and the specific IFN-γ and TNF-α T cell responses were detected by *ex vivo*
820 ELISPOTs. IFN-γ and TNF-α spot forming cells (SFC) were detected, counted, and
821 expressed as box plots with mean and standard deviations. Comparison of late
822 convalescent-phase (A) IFN-γ and (B) TNF-α T cell responses between individuals with
823 pDENV and sDENV infections. Comparison of late convalescent-phase (C) IFN-γ and
824 (D) TNF-α T cell responses between individuals with pZIKV and ZIKVwpDENV
825 infections. Individual colored plots represent serologically-validated DENV- and/or ZIKV-
826 infected individual. *, $p<0.05$.
827
828 **Figure 4. Impact of HIV status on the T cell response.** Comparison of mean
829 convalescent-phase IFN-γ T cell responses expressed as bars and standard deviation
830 between (A) HIV-negative (open black bars) and HIV-infected (shaded black bars)
831 individuals with pDENV and sDENV infections grouped together, (B) HIV-negative
832 (open dark grey bars) and HIV-infected (shaded dark grey bars) individuals with
833 ZIKVwpDENV infections, and (C) HIV-negative (open light grey bars) and HIV-infected
834 (shaded light grey bars) individuals with pZIKV infections. Comparison of mean
835 convalescent-phase TNF-α T cell responses expressed as bars and standard deviation
836 between (D) HIV-negative (open black bars) and HIV-infected (shaded black bars)
837 individuals with pDENV and sDENV infections grouped together, (E) HIV-negative
838 (open dark grey bars) and HIV-infected (shaded dark grey bars) individuals with

ZIKVwpDENV infections, and (F) HIV-negative (open light grey bars) and HIV-infected (shaded light grey bars) individuals with pZIKV infections. *, $p<0.05$.

Figure 5. Clustal Omega generated amino acid sequence alignment of DENV serotypes 1 to 4 and Asian ZIKV. The residues in yellow represent the NS3 protease domain (amino acids 1-169) and the residues in orange represent the helicase domain (amino acids 179-619). *, single, fully conserved residue. :, conservation between groups of strongly similar properties – scoring > 0.5 in the Gonnet PAM 250 matrix. ., conservation between groups of weakly similar properties – scoring =< 0.5 in the Gonnet PAM 250 matrix.

A



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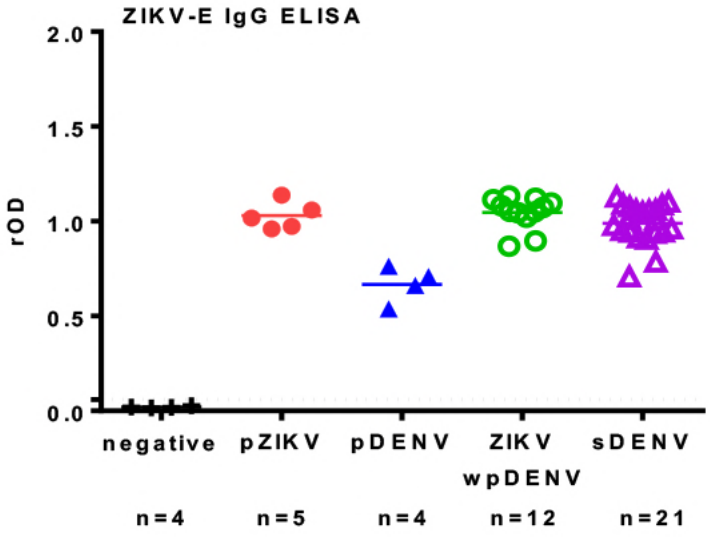

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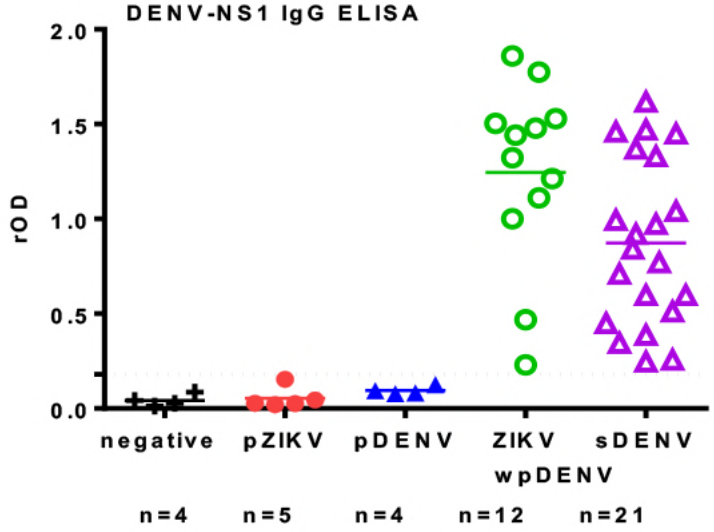

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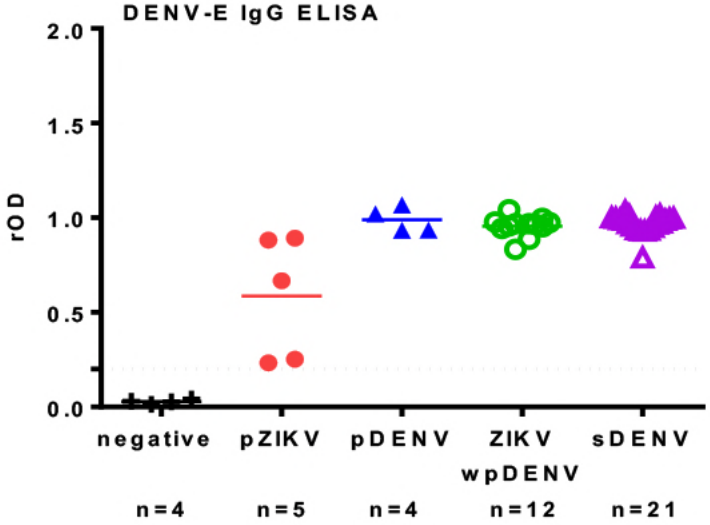

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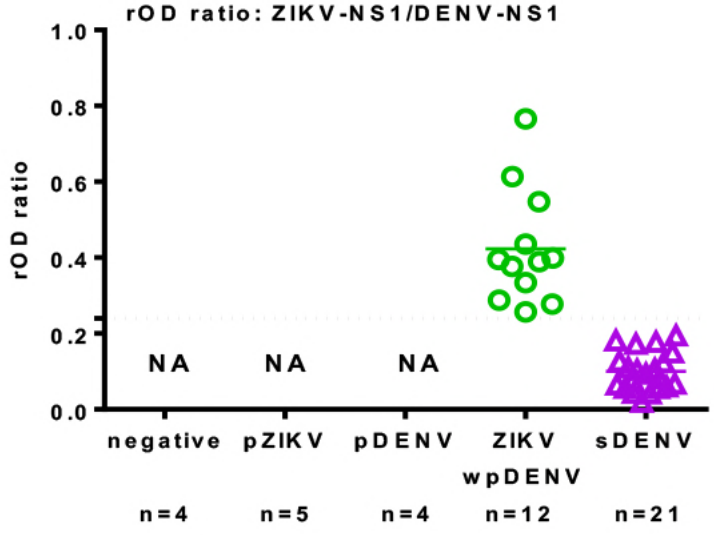

F