Antibody Responses to Zika Virus Infections in Environments of Flavivirus Endemicity

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ABSTRACT
Zika virus (ZIKV) infections occur in areas where dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV), and other viruses of the genus Flavivirus cocirculate. The envelope (E) proteins of these closely related flaviviruses induce specific long-term immunity, yet subsequent infections are associated with cross-reactive antibody responses that may enhance disease susceptibility and severity. To gain a better understanding of ZIKV infections against a background of similar viral diseases, we examined serological immune responses to ZIKV, WNV, DENV, and YFV infections of humans and nonhuman primates (NHPs). Using printed microarrays, we detected very specific antibody responses to primary infections with probes of recombinant E proteins from 15 species and lineages of flaviviruses pathogenic to humans, while high cross-reactivity between ZIKV and DENV was observed with 11 printed native viruses. Notably, antibodies from human primary ZIKV or secondary DENV infections that occurred in areas where flavivirus is endemic broadly recognized E proteins from many flaviviruses, especially DENV, indicating a strong influence of infection history on immune responses. A predictive algorithm was used to tentatively identify previous encounters with specific flaviviruses based on serum antibody interactions with the multispecies panel of E proteins. These results illustrate the potential impact of exposure to related viruses on the outcome of ZIKV infection and offer considerations for development of vaccines and diagnostics.

KEYWORDS
Zika, cross-reactivity, flavivirus, humoral immunity, protein microarray

Zika disease is spread to humans by transfer of Zika virus (ZIKV) primarily through the bites of infected Aedes aegypti or Aedes albopictus mosquitoes (1, 2) and secondarily by sexual (3) or vertical (4–6) transmission. The majority of ZIKV infections are asymptomatic or mild with low-grade fever, arthralgia, conjunctivitis, and rash (7), while a lower frequency of cases may result in congenital microcephaly via in utero infections in infants and Guillain-Barré syndrome in adults (4–6, 8). Prior to the first reported outbreaks in the Pacific Islands in 2007 (9) and 2013 to 2014 (10, 11), only sporadic human cases of ZIKV were documented in Africa and Southeast Asia (10, 11). However, the number of confirmed human cases has increased dramatically over the past 9 years as ZIKV has spread to regions with naive populations, leading to the current epidemic in Brazil and another 58 countries with ongoing ZIKV transmission (12). The single-stranded (plus-strand) genomic RNA of ZIKV and other Flavivirus species (flaviviruses) encodes a nonsegmented open reading frame that is cleaved during and
after translation into three structural proteins (capsid [C], envelope [E], and membrane [M] proteins) that are incorporated into the virus, and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) that are necessary for replication. The overall molecular organization of the mature ZIKV from cryo-electron microscopy (cryo-EM) structures (13, 14) is very similar to the closely related dengue virus (DENV) and West Nile virus (WNV) (15, 16), as well as more distantly related tick-borne encephalitis virus (TBEV) and Japanese encephalitis virus (JEV) (17, 18). However, infectious particles also exhibit structural heterogeneity from immature to mature forms (19) within species, which may affect the protective potency of antibodies. Heterodimers of E and M proteins displayed on the outer surfaces of the virus (13, 14) undergo extensive conformational changes that facilitate infection of cells, and these proteins are primary targets for circulating antibodies (20). Further, the E proteins of many flavivirus strains harbor a potentially glycosylated, four-residue loop, and deletions of this feature in ZIKV are selected against in vivo (21). The NS1 protein, which is secreted by infected cells (22), is another important antigen that may be involved in immune evasion and pathogenesis. The specificities of antibodies interacting with NS1 are likely to be affected by a balance of surface features that are conserved among flaviviruses as well as the diverse electrostatic characteristics (23).

The four DENV serotypes (DENV serotype 1 [DENV1] to DENV4) are loosely categorized by cross-neutralization with polyclonal antibodies (24). Serological immune responses protect from reinfection with the same (homotypic) virus, while cross-reactive antibodies generated from previous infections with another (heterotypic) DENV serotype may enhance disease outcomes (25–28). Although most infections are mild and self-limiting, dengue disease can progress to hemorrhagic fever, capillary leakage, and dengue shock syndrome (27, 28). Secondary heterotypic infection can in some cases lead to increased risk of severe dengue disease, possibly because antibodies to one serotype may enhance infections with heterologous serotypes (antibody-dependent enhancement [ADE]) by promoting viral entry and infection through Fc receptor-expressing cells (27–30). The extent of disease enhancement in human ZIKV infections due to preexisting flavivirus antibodies is not well documented. While there is a higher risk of more-severe disease from secondary DENV infections, among flaviviruses, severe neurological pathologies may be uniquely associated with ZIKV infections during fetal development, with considerable uncertainty remaining regarding potential long-term health effects.

Beyond the potential role of antibodies in exacerbating disease, there are great challenges for developing accurate serological tests for ZIKV infections. Assays were recently developed and approved by the FDA under emergency use authorization for the detection of viral RNA or specific IgM and neutralizing antibodies in the biological fluids from patients with suspected Zika disease (31, 32). However, negative results for viremia do not exclude ZIKV infection, as circulating virus levels are highest several days before the onset of symptoms and begin to decline early in the acute phase of infection (33). In addition, interpretation of ZIKV antibody test results is complicated due to suspected cross-reactivity with other flaviviruses, especially for individuals who have had previous flavivirus exposures. Infections caused by DENV, WNV, and yellow fever virus (YFV) are spread by common mosquito vectors, circulate in similar areas, and present early disease symptoms that are identical to those of ZIKV infections (25–28). Infected individuals may have high levels of antibodies to multiple flaviviruses that hinder conclusive determination of the virus responsible for the most recent infection (29). Thus, laboratory methods that can better differentiate clinical infections and facilitate accurate disease surveillance are integral to an effective public health response to the current Zika disease epidemic and to future outbreaks. Toward this goal, we examined serological immune responses to commonly encountered infections by using a microarray of viruses and isolated protein antigens representing major phylogenetic lineages of flaviviruses. Our results demonstrate that primary human and rhesus macaque antibody responses to infection are highly specific for envelope proteins from the etiological agent, while responses to whole viruses are most cross-
reactive. We further show that antibody recognition of isolated viral antigens can be used to resolve complex infection histories.

RESULTS

Antibody responses to ZIKV. To examine serological immune responses, we developed a flavivirus-focused microarray comprising mature and immature forms (34) of DENV1 to DENV4 and ZIKV (five Asian isolates and six African lineage isolates), along with recombinant protein antigens from 15 isolates (Table 1 and Fig. 1A). Equivalent densities of recombinant proteins or viruses were deposited by inkjet printing on microarrays surfaces coated with a thin layer of nitrocellulose. The printed antigens were evaluated with mouse antisera against 12 flaviviruses, representing antibodies produced by a noninfectious route. The E antigens from all flaviviruses except DENV3 were detected by mouse antibodies (IgG) that were produced in response to the corresponding virus, as shown in Fig. 1B, with evidence of significant E cross-reactivity for JEV antisera.

The NS1 proteins from all except DENV1 to DENV4 were detected by the mouse antivirus sera, whereas the M antigen was only weakly recognized at best. We utilized the microarray to examine sera from nonhuman primates (NHPs) (Macaca mulatta) that were challenged subcutaneously with either African or Asian isolates of ZIKV (Table 1; see Fig. S1A in the supplemental material) (33) (Zika Open-Research Portal [https://zika.labkey.com]). The African and Asian lineages of ZIKV share /H11011 95% of E-protein amino acid sequences (35) or about the same level of similarity found among E proteins of individual DENV serotypes. Serum antibody binding to virus particles and E antigens of ZIKV, as measured by the microarray, was substantially elevated (Fig. 2A and B) 21 to 28 days postinfection (dpi), while no antibody recognition was observed

### TABLE 1 Flavivirus strains used for production of whole viruses and recombinant proteins

| Virus | ID | Isolate | Country | Yr | Host | GenBank accession no. |
|-------|----|---------|---------|----|------|-----------------------|
| ZIKV  |    | H/PF/2013c | French Polynesia | 2013 | Human | KU776791 |
|       | 1  | SV0127/14 | Thailand | 2014 | Human | KU681081 |
|       | 2  | CPC-0740d | Philippines | 2012 | Human | KU681082 |
|       | 3  | VABC59 | USA (Puerto Rico) | 2015 | Human | KU501215 |
|       | 4  | SPH2015 | Brazil | 2015 | Human | KU321639 |
|       | 5  | YAPd | Micronesia | 2007 | Human | EU545988 |
|       |    | MR-766c,d | Uganda | 1947 | Macaca mulatta | KU955594 |
|       | 2  | IBH30656 | Nigeria | 1968 | Human | HQ234500 |
|       | 3  | DAKAR41525 | Senegal | 1984 | Aedes africanus | KU955591 |
|       | 4  | DAKAR 41662 | Senegal | 1984 | A. africanus | KU955592 |
|       | 5  | ARB7701 | Central Africa | 1976 | A. africanus | KF268950 |
|       | 6  | ArD_41519 | Senegal | 1984 | A. africanus | HQ234501 |
| DENV1 |    | HAWAI1 | USA | 1944 | Human | KM204119 |
| DENV2 |    | NGC | New Guinea | 1944 | Human | KM204118 |
| DENV3 |    | H87 | Philippines | 1956 | Human | M91310 |
| DENV4 |    | H241 | Philippines | 1956 | Human | AY947539 |
| WNV   |    | NY99 | USA | 1999 | Owl | NC_009942 |
| YFV   |    | 17-D-204 | USA | 1985 | Vaccine | JX503529 |
| JEV   |    | SA14-14-2 | South Korea | 2006 | Vaccine | JN604986 |
| SLEV  |    | PARTON | USA | 1933 | Human | EF158070 |
| MVEV  |    | 1-51 | Australia | 1952 | Human | NC_000943 |
| ROCV  |    | SPH34675 | Brazil | 1975 | Human | AY632542 |
| POWV  |    | LB | Canada | 1958 | Human | NC_003687 |
| TBEV-E|    | SOFJIN-HO | Russia | 1937 | Human | AB062064 |
| TBEV-EUR|    | NEUDOERFL | Austria | 1971 | Ixodes ricinus | NC_001672 |

*Virus abbreviations: ZIKV, Zika virus; DENV, dengue virus; WNV, West Nile virus; YFV, yellow fever virus; JEV, Japanese encephalitis virus; SLEV, St. Louis encephalitis virus; MVEV, Murray Valley encephalitis virus; ROCV, Rocio virus; POWV, Powassan virus; TBEV-E, tick-borne encephalitis virus, Eastern strain; TBEV-EUR, tick-borne encephalitis virus, European strain.*

*The identification (ID) number corresponds to the ZIKV strain labels shown in Fig. 2A and B.*

*Representative antigens are shown in Fig. 2C to 4 and Fig. S2 to S4 (virus for CPC-0740; E protein for YAP).*
for NS1 or M proteins (Fig. 2B), suggesting that anti-NS1 and anti-M antibodies represent a small proportion of the humoral immune response to infection during the time points examined. Antibody recognition was more robust for mature virus particles (~2-fold higher) than for immature virus particles, which may display different conformations of E and M proteins (13). Within the assay, the highest antibody interactions were detected by ZIKV E proteins in comparison to mature ZIKV (Fig. 2A and B). However, a direct quantitative relationship between virus and recombinant protein cannot be determined by these results, because the complex nature of the native virus precludes printing equal molar amounts of available antigen. Only minor differences were observed in antibody responses to individual African and Asian lineage ZIKV antigens for both virus and E protein (Fig. 2), consistent with the conserved amino acid sequences and a single ZIKV serotype, as recently reported by others (36). The E-protein-specific IgM responses were detected by 3 dpi, coinciding

**FIG 1** Phylogenetic relationships and recognition of microarrayed antigens by virus-specific antibody standards. (A) The phylogenies of flaviviruses examined in this study were inferred from an alignment of amino acid sequences from envelope (E) proteins. (B) Microarrays of E, nonstructural protein 1 (NS1), and premembrane (pM) proteins probed with mouse polyclonal antibodies generated against each virus shown (centered labels above each row of bar graphs). Antibody binding data are shown as $\log_{10}$-transformed mean fluorescence intensities ($\pm$ standard errors of the means [SEM] [error bars]), and the arrows indicate the virus-specific antigens. Heterologous antigens that exhibit increased recognition compared to the virus-specific antigen are labeled with an asterisk ($P < 0.05$, one-way ANOVA with Tukey’s range test). Virus abbreviations: YFV, yellow fever virus; SLEV, St. Louis encephalitis virus; DENV, dengue virus; DENV1, dengue virus serotype 1; POWV, Powassan virus; TBEV-E, tick-borne encephalitis virus, Eastern strain; TBEV-EUR, tick-borne encephalitis virus, European strain; MVEV, Murray Valley encephalitis virus; WNV, West Nile virus; ZIKV, Zika virus; ZIKV-AFR, ZIKV from Africa; ZIKV-AS, ZIKV from Asia; JEV, Japanese encephalitis virus; ROCV, Rocio virus.
with the rise of virus, peaked by 11 dpi, and subsided thereafter (Fig. 2C). The corresponding IgG responses were delayed compared to IgM, consistent with a naive immune response, and displayed increasing levels through 28 dpi (Fig. 2C). Further, there were no apparent differences in the magnitude or kinetics of humoral immune responses to the different amounts of virus used for challenges (Fig. S2), suggesting that levels of IgG and IgM were increasing in tandem with virus replication.

**Cross-reactivity of antibodies from primary flavivirus infections.** The E proteins of ZIKV and DENV have a high degree of structural similarity that may contribute to shared antibody epitopes. We examined NHPs (*M. mulatta*) challenged independently with DENV1 to DENV4 (Fig. S1A) (37). For virus, antibodies (30 dpi) from NHPs infected with any DENV serotype were highly cross-reactive to heterologous DENV serotypes and ZIKV (Fig. 3A and Table 1). Further, DENV2 and DENV3 antibodies displayed substantially higher reactivity with the heterologous ZIKV, while IgG from ZIKV-challenged NHPs was more specific for ZIKV at the virus level, with a lower overall level of cross-reactivity toward DENV1 to -4 (Fig. 3A). In contrast with viruses, the E proteins presented antibody recognition profiles that were very specific for the challenge virus (Fig. 3A) and minimal antibody recognition of E proteins from 10 more distantly related flaviviruses (Fig. 4A). DENV-challenged NHPs exhibited the highest antibody binding to the E protein from the DENV challenge serotype, and antibodies from ZIKV-challenged NHPs essentially bound only to ZIKV E antigens. A principal-component analysis (PCA)
of antibody bound by DENV and ZIKV E antigens differentiated serotype-specific DENV and ZIKV infection sera due to the higher degree of homotypic E recognition (Fig. 3B). In contrast to the E-antigen results, PCA based on IgG recognition of virus only enabled distinction of ZIKV- from DENV-challenged sera, whereas DENV serotype-specific clusters were not evident (Fig. 3B). Furthermore, antibodies from NHPs challenged with African or Asian lineage ZIKV were not differentiated by E protein or virus (Fig. 2 and 3B). We also considered YFV, both as a nearest neighbor of ZIKV and DENV (Fig. 1A) and because vaccination against yellow fever is common in many countries with a high prevalence of dengue. While serum antibodies from NHPs vaccinated with the 17D YFV strain (Fig. S1A) (38) predominantly recognized the E antigen of YFV (Fig. 4A), a modest level of cross-reactivity was evident with several other E proteins, including those of ZIKV and tick-borne encephalitis virus (TBEV).

**FIG 3** Differentiation of nonhuman primates challenged with ZIKV or DENV by specific IgG binding to E antigens. (A) Binding of convalescent-phase serum antibodies from nonhuman primates (NHPs) challenged with either an Asian (H/PF) \((n = 3)\) (red) or African (MR-766) \((n = 3)\) (royal blue) lineage ZIKV, or DENV \((n = 4\) each for the DENV1 [black], DENV2 [green], DENV3 [orange] groups; \(n = 3\) for the DENV4 group [magenta]) to whole viruses \((144\) h) and E proteins. Values shown are antibody binding signals relative to the virus used for challenge \((\pm\) SEM). (B) Principal-component analyses of relative IgG binding to E proteins and viruses \((144\) h) by NHP antibodies. Individual data points and virus-specific clusters are colored according to the challenge virus as in panel A. PC1, principal component 1.
The animals in the ZIKV, DENV, and YFV infection studies we examined were domestically bred in isolation from most infectious diseases. Therefore, it was important to compare results from the naive backgrounds of animal disease models with primary infections of humans without documented prior exposures to flaviviruses.

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**FIG 4** Antibody specificity of primary and secondary flavivirus infections. Relative binding (±SEM) of convalescent-phase serum antibodies from nonhuman primate (NHP) and human flavivirus infections to 15 flavivirus E proteins is shown. (A) Sera from primary infections are indicated by color as follows: gray, DENV-challenged NHPs (individual data for each NHP group are overlaid in a scatter plot; \( n = 4 \) each for the DENV1 [black], DENV2 [green], and DENV3 [orange] groups and \( n = 3 \) for the DENV4 group [magenta]); green, human (Hu) rDEN2Δ30 (\( n = 8 \)) (primary infection); red, pooled African and Asian lineage ZIKV NHPs (\( n = 6 \)); white, YFV-vaccinated NHPs (\( n = 3 \)). (B) Sera from confirmed human flaviviral infections with unknown infection histories are indicated by color as follows: gray, DENV (individual data are overlaid in a scatter plot; the colors correspond to the most recent DENV infection); green, DENV2 (\( n = 5 \)); orange, DENV3 (\( n = 2 \)); red, ZIKV (\( n = 4 \)); white, YFV vaccination (\( n = 13 \)); cyan, WNV (\( n = 20 \)). (C) Predicted infection histories of human secondary DENV (gray in panel B) and primary ZIKV (red in panel B) infections, based on a supervised SVM classifier. Individual human sera are shown at the bottom (Z for ZIKV, D2 for DENV2, and D3 for DENV3; virus followed by serum identification [ID] number), with probability values for each viral class (left) gradient colored from low to high (white to royal blue) (right). Predicted infection histories are designated by colored bars above serum ID (DENV1 [black], DENV4 [magenta], no prediction [no bar]).
Dengue human infection models were recently developed to assess the efficacy of live attenuated DENV vaccines (39). Human challenges with the attenuated DENV2 strain rDEN2Δ30 (40) result in a mild disease, with viremia, rash, and neutropenia. We examined sera collected from flavivirus-naive subjects 28 days after challenge (10^3 PFU) with rDEN2Δ30 by subcutaneous injection (Fig. S1B) (40). Among the extended panel of E proteins (Table 1), human antibody responses to rDEN2Δ30 resulted in specific recognition of the E protein from DENV2 and to a lesser degree from DENV4 (Fig. 4A and Fig. S3A), which is most similar to DENV2 among all other flaviviruses (Fig. 1A). Low levels of neutralizing antibodies against other DENV serotypes were previously reported for individuals challenged with rDEN2Δ30 (41). For viruses, extensive human antibody cross-reactivity was again noted for other DENV serotypes and ZIKV strains (Fig. S3A). These results indicated that the NHP DENV challenge model replicated the antigen specificity profile of human antibody responses to primary infection.

Antibody cross-reactivity between flaviviruses could be influenced by homology of sequences and structures, as well as the abundance and degree of cross-reactive antibodies in polyclonal sera. For example, cross-reactivity could be due to a small population of antibodies that exhibit high levels of specificity for heterologous E proteins, or it may be due to a larger population of antibodies that exhibit broad cross-reactivity. Although the highest recognition of the homologous E protein was common for antibodies from primary infections, we observed differences in the amount of total antibody across virus species (Fig. 5). Comparing results obtained with all E proteins, cross-reactive antibodies were not detected for ZIKV, while DENV1 and DENV2 antibodies recognized other DENV serotypes. Antibodies from DENV3-infected and YFV-vaccinated NHPs exhibited the lowest binding to the respective E proteins, while a high level of DENV2 E-specific antibodies interacted with the DENV2 E protein (Fig. 5).

The lower levels of DENV3 and YFV antibodies that were specific for the cognate E protein, compared to DENV2 for example, contributed to the appearance of an overall higher level of background cross-reactivity (Fig. 3A and 4A). Vaccination with the live-attenuated 17D strain results in low levels of viremia that mimic a true YFV infection, and titers of specific antibodies are also lower than those in wild-type YFV infections (42). In addition, antibodies from NHPs challenged with ZIKV (33) and DENV
exhibited neutralizing antibody titers (33, 43) that directly correlated ($R^2 > 0.99$) with the E-antibody recognition pattern we observed (Fig. 5).

**Secondary flavivirus infections.** The antibodies of flavivirus-naive NHPs and humans to primary flavivirus infection were highly specific to the E protein of the challenge virus. Because increased levels of antibody cross-reactivity would be expected for flavivirus-primed individuals with secondary flavivirus infection, we next examined human sera after one or more flavivirus exposures. In contrast to results obtained with primary infections, IgG from DENV2 or DENV3 infections occurring in Peru prior to the Zika epidemic (here defined as secondary DENV infections) (Fig. S1C) collectively interacted with several E proteins, including those from ZIKV (Fig. 4B), suggesting that antibodies from previous infections, possibly DENV4 (based on the amount of IgG bound), dominated immune responses to other flaviviruses. Despite expectations, sera from secondary DENV2 infections did not correlate with primary DENV2 infections (Fig. S3B), providing additional evidence of previous dengue infection in these samples. Moreover, although principally recognizing the E protein of YFV, antibodies from human 17D vaccinations (Fig. S1D) were less specific than those from primary NHP vaccinations, as E proteins from DENV4 and several other flaviviruses were also targeted (Fig. 4B). It is possible that the less-specific YFV responses were a result of declining antibody titers, as the sera were collected up to 118 days after vaccination. We further noted that serological responses from WNV infections that occurred in North America (Fig. S1D), a region with only a small incidence of dengue, exhibited elevated antibody interactions with E proteins from WNV and a few other flaviviruses, but only a low level of interactions with DENV antigens (Fig. 4B). Finally, we examined primary ZIKV infections from the Dominican Republic (Fig. S1C), a Caribbean country where dengue is endemic. Antibodies from ZIKV infections interacted to a greater extent with E proteins from DENV than from ZIKV (Fig. 4B and Fig. S3A) and also recognized E proteins from several other flaviviruses. It is important to note that levels of total E-specific antibodies from all human flavivirus exposures were significantly reduced compared to levels observed in primary infections (Fig. 5). While maximum E-specific antibody abundance never exceeded the low levels of binding observed for primary YFV and DENV3 exposures, these results suggested that serum levels of anti-E antibodies were predominantly driven by infection histories, and it is conceivable that at least one DENV infection preceded each clinical disease examined with sera from secondary infections.

Given the complexity of the human antibody response from primary ZIKV and secondary DENV infections (Fig. S1C), we attempted both to estimate the probability of previous flavivirus exposures and to identify the likely antecedent virus. We used a supervised machine learning method to classify sera by features of antibody binding to the extended panel of 15 E proteins (Table 1). The support vector machine (SVM) classifier was trained on a positive set of E-specific antibody binding signals from primary flavivirus infections and a negative set of background signals from flavivirus-naive sera. The performance of the SVM was evaluated using a 10-fold cross-validation resampling method, which readily differentiated infected from naive sera and different primary infections, resulting in a total model accuracy of 98.5%. Using a probability cutoff value of $\geq 0.5$, the classifier was used to predict flavivirus exposures that occurred prior to the secondary DENV and ZIKV infections. Four secondary DENV2 sera were predicted to have had a previous DENV4 infection, while high probability for two primary ZIKV sera suggested a previous DENV1 infection (Fig. 4C), which was consistent with clustering based on correlated antibody binding (Fig. S3B). Lower overall probabilities for single virus infections were observed for the remaining secondary DENV and ZIKV samples, and classification to a single group was therefore not possible (Fig. 4C). For example, a secondary DENV3 serum had comparable probability values for DENV2 (0.28) and DENV4 (0.27), suggesting a previous infection with either virus. The inclusion of more-extensive training data sets for primary ZIKV and other viral infections will be important for refining the predictive power of the described SVM method.
DISCUSSION

The coincidence of dengue in areas where there is a Zika virus epidemic limits the reliability of current serological assays and complicates vaccination strategies. The study described here examined the specificity of humoral immune responses to flaviviruses by using microarrays of 11 native viruses and recombinant E proteins from 15 species or lineages of flaviviruses that are pathogenic to humans. Antibodies from the first exposures of nonhuman primates and humans to ZIKV, DENV, WNV, and YFV were predominantly directed toward the E surface antigen from the infecting virus and enabled differentiation of infections. Whereas isolated human monoclonal antibodies that were cross-reactive for E antigens have been described (44), our results with polyclonal antibodies present a global analysis of the composite B-cell response. In contrast to the high specificity observed with E antigens, whole viruses exhibited significant levels of cross-reactivity with serum antibodies from primary ZIKV and DENV infections. Antibodies from human ZIKV or DENV infections that occurred in regions where dengue is endemic recognized heterotypic E antigens and exhibited decreased recognition of the homotypic E protein, consistent with higher levels of IgG from previous flavivirus exposures than from the most recent infection. The high degree of antibody specificity for E protein with sera from primary ZIKV and DENV infections. Antibodies from human ZIKV or DENV infections that occurred in regions where dengue is endemic recognized heterotypic E antigens and exhibited decreased recognition of the homotypic E protein, consistent with higher levels of IgG from previous flavivirus exposures than from the most recent infection. The high degree of antibody specificity for E protein with sera from primary ZIKV and DENV infections.

Clinical management of suspected Zika cases that test negative for viral RNA can be guided by laboratory evidence of ZIKV-specific antibodies (32), particularly to differentiate infections in late convalescence and beyond, as viral RNA is typically no longer detected. However, results from some in vitro assays will be difficult to extrapolate to human cases. Only weak antibody neutralization of ZIKV was reported for sera from DENV-infected patients that exhibited a high degree of cross-reactivity with ZIKV-infected Vero cell lysates (46), while other studies observe enhancement of ZIKV infections in cell culture by anti-DENV antibodies (44). Our results illustrate the application of high-throughput antigen microarrays for the study of antibody responses to ZIKV and other flaviviruses. In addition, printed microarrays provide a high-throughput means for evaluating the performance of many test antigens in the same assay.
example, by including both recombinant proteins and viruses in the same microarray, we determined that E proteins were the most effective probes for detecting serological immune responses. In agreement with previous reports that used antigen preparations from whole virus (9, 31, 46), we observed a high level of antibody cross-reactivity between DENV and ZIKV isolates. Although the precise reason for high cross-reactivity between viruses is unknown, possible mechanisms may include antibodies that interact with additional quaternary and glycosylated epitopes that were not present on the recombinant antigens or other indeterminate factors (14, 47). Further, the results presented here emphasize the value of determining total antibody recognition of E proteins for distinguishing between infections caused by different species of flaviviruses. While virus neutralization assays measure a functional subset of antibodies and provide an important indicator of antiviral immunity, the best correlate of protection against viremia in DENV infection may be total polyclonal antibody titers, rather than neutralizing antibody titers (43). Antibodies that are weakly neutralizing in cell culture assays can contribute to physiologically important non-ADE mechanisms of virus clearance that are facilitated by receptor-mediated uptake and effector cells (48).

A more detailed understanding of the interrelationships of antibody responses across flaviviruses is imperative because infections by one species or serotype are known to influence disease susceptibility and severity for infections caused by other related viruses (25–28). New techniques are also needed to guide accurate diagnosis of emerging infections, especially for flavivirus-immune individuals, as antibodies persist at levels that are detectable long after disease resolution (38, 42, 49, 50). Although the length of time from previous exposures may influence detection of responses to new infections, our results demonstrate that antibody recognition patterns from secondary infections can be used to estimate infection histories (Fig. 6). Importantly, since severe dengue is linked to secondary infections with a heterotypic DENV (25–28), it is possible that dengue virus-primed populations are more prone to ZIKV infections, and perhaps the associated severe neurological disorders of Guillain-Barré syndrome (8) and microcephaly (4–6). However, there is currently no evidence of enhanced severity, increased ZIKV loads, or increased incidence of Zika disease in countries with widespread immunity to dengue. Our results indicate that it should be possible to develop protein-based serological assays that are sensitive enough to differentiate flavivirus infections in individuals with preexisting immunity. Based on the assumption that multiple independent antibody binding events were measured for each clinical specimen collected from a region where dengue is endemic, data from primary infections can be used to train machine learning methods for classification of sera from unknown infection histories. The predictive algorithm that we developed for E recognition patterns may find useful applications in disease surveillance for inference of infection histories in both primary and secondary flavivirus encounters. As diagnostic methods by necessity focus only on the current disease, the general approach described here will also be important for addressing any causal relationships between Zika disease and previous infections.

MATERIALS AND METHODS

**Viruses.** The ZIKV and DENV presented in Table 1 were propagated and prepared as previously described (34), with some modifications. Briefly, HEK293T cells were maintained in Dulbecco modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. The cells were seeded in T125 flasks to 60% confluence and cultured for 12 h. The cells were infected (2 h) with 5 ml of suspended virus stock diluted 1:25 with fresh culture medium. Infectious suspensions were replaced for virus propagation, and culture supernatants were harvested at early (48-h) and late (144-h) time points to obtain immature and mature virions, respectively, while adding fresh HEK293T cells 72 h postinfection to ensure the presence of enough viable cells for sustained proliferation to generate mature virus. Culture supernatants were filtered using prewashed (Super G blocking buffer [Grace Bio-Labs] followed by sterile phosphate-buffered saline (PBS)) 45-μm syringe filters, and precipitated for 12 h (4°C) in PBS containing 8% polyethylene glycol 8000 (PEG8000) (vol/vol). Precipitates of viruses were pelleted by centrifugation (14,000 × g, 1 h, 4°C), resuspended in 300 μl sterile PBS (∼100-fold concentration by volume), snap-frozen in a dry ice-ethanol bath, and stored at −80°C.

**Viral proteins.** Viral RNA for preparation of protein-expressing plasmids was obtained from the following sources: American Type Culture Collection (DENV1 to DENV4), Integrated BioTherapeutics, Inc.
Purified pellets were stored at \(4^\circ\)C. Purified proteins were stored at \(-80^\circ\)C. Flavivirus and control proteins were printed onto microporous nitrocellulose-coated slides (Oncyte SuperNOVA; Grace Bio-Labs, Inc.) in replicates \((n = 6)\) with gene-specific primers (final concentration of \(1\) pmol/\(\mu\)l total) with gene-specific primers (final concentration of \(2.5\) pmol/\(\mu\)l) and \(2\)X Phusion high-fidelity PCR master mix with HF buffer (New England Biolabs Inc.). PCR-amplified genes were purified using QiAquick spin column PCR purification kit (Qiagen). NS1 and PM were produced as full-length open reading frames (ORFs), and E genes were truncated to exclude transmembrane domains, as predicted by analysis of amino acid sequences using TMHMM server v.2.0 (Center for Biological Sequence Analysis) (51, 52). Purified DNAs were TOPO cloned into the pENTR/TEV/D-TOPO vector (Gateway Technology, Life Technologies). Sequence-verified entry clones were shuttled into expression vectors by recombination reactions using LR clonase II (Life Technologies). Specifically, the ZIKV-MR766-PM ORF was shuttled into an N-terminal His-labeled maltose-binding protein (HisMBP)-tagged vector (53), while all other flaviviral ORFs were shuttled into the N-terminal 6\(\times\)His-tagged dEST17 (Life Technologies). All flavivirus constructs were expressed in \(E.\ coli\) BL21(DE3), propagating pellets in media containing Luria broth (300 ml) supplemented with 100 \(\mu\)g/ml ampicillin and 0.1% glucose. Proteins were induced at mid-log phase with 1 mM isopropyl-\(\beta\)-thiogalactopyranoside (IPTG) (EMD Chemicals). Induction conditions were optimized for each protein, and bacteria were grown at either \(30^\circ\)C (2 to 4 h) or \(18^\circ\)C (12 h) prior to harvest by centrifugation. Bacterial pellets were lysed in B-Per reagent (Thermo Scientific) containing EDTA-free \(3\)X Halt protease inhibitor cocktail (Thermo Scientific), 0.2 mg/ml \(\alpha\)-lysozyme, 250 U DNase I (Thermo Scientific), and 1 mM IPTG. Protein expression was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining, and Western blotting using a mouse anti-His-horseradish peroxidase (HRP)-conjugated polyclonal antibody (Abcam). For purification of insoluble proteins, inclusion body pellets were washed as previously described, with minor modifications (54). Briefly, buffer containing 50 mM Tris-HCl (pH 7.4), 1 M urea, and 1% Triton X-100 was used to wash pellets three times, followed by two washes with wash buffer (Tris-HCl (pH 7.4), with centrifugation at 15,000 \(\times\) \(g\) for 7 min between each wash, and purified pellets were stored at \(\sim\) 80°C. Purified inclusion bodies were solubilized in 50 mM HEPES (pH 7.3), 140 mM NaCl, 2 mM dithiothreitol (DTT), and 1% SDS, followed by incubation at 99°C with gentle mixing (5 to 15 min) and centrifugation to remove remaining insoluble protein. Solubilized proteins were analyzed by SDS-PAGE with Coomassie blue staining and by Western blotting using anti-His-HRP-conjugated polyclonal antibody (Abcam). The protein concentration and purity of flavivirus proteins were measured using the Agilent Protein 230 kit and Bioanalyzer 2100 instrument (Agilent Technologies). Purified proteins were stored at \(-20^\circ\)C in solubilization buffer, with a final concentration of 25% glycerol.

Microarrays of flavivirus antigens. Recombinant proteins were diluted to 200 ng \(\mu\)l\(^{-1}\) in microarray printing buffer (50 mM HEPES, 140 mM NaCl, 2 mM DTT [pH 7.3]) with glycerol added to a final concentration of 40%. Flavivirus and control proteins were printed onto microporous nitrocellulose-coated slides (Onycyte SuperNOVA; Grace Bio-Labs, Inc.) in replicates \((n = 6)\) using a noncontact inkjet microarray printer (ArrayJet, Glasgow, United Kingdom). The virus preparations were printed with printing buffer containing 50% glycerol, and preliminary experiments were performed with printed virus to optimize antibody binding signals. Frozen virus stocks were gamma irradiated (6 megarads) for inactivation and visualized by electron microscopy to assess quality and quantity. Concentrated virus and a dilution series of bovine serum albumin (BSA) (Sigma-Aldrich) were subjected to SDS-PAGE and stained with Coomassie blue to determine the relative amounts of viral proteins in each preparation. The final printing parameters were established by comparison of virus gradients printed onto nitrocellulose-coated slides, where deposited material was quantified against both an IgG and a BSA standard gradient by SYPRO Ruby staining (Thermo Scientific), and by comparing signal strength with a pan-flavivirus polyclonal rabbit antisera specific to an E-domain II peptide that is highly conserved among flaviviruses. The deposited protein antigens were similarly evaluated using SYPRO Ruby, an anti-N-terminal 6\(\times\)His monoclonal antibody (Sigma-Aldrich), and the pan-flavivirus rabbit antisera described above. Printed microarrays were desiccated (12 h) and stored frozen \((-20^\circ\)C) until use.

Microarray assays. All microarray processing steps were performed at 22°C, protected from light. For IgM detection assays, serum IgG was inactivated using GullSORB (Meridian) prior to performing microarray manipulations. NHP (1:50) and human (1:150) sera, diluted in probe buffer (1 \(\times\) PBS [pH 7.4], 0.1% Tween 20, 1% BSA), were precleared by incubating (1 mg ml\(^{-1}\)) with \(E.\ coli\) lysate (Promega) with gentle agitation, followed by centrifugation (17,000 \(\times\) \(g\), 5 min) to remove the pelleted immunoprecipitates. Microarrays were blocked with Super G blocking buffer (Grace Bio-Labs) for 1.5 h at 22°C and washed three times (for 5 min each time) in wash buffer (1 \(\times\) PBS, 0.2% Tween 20, 1% BSA). The microarrays were incubated for 2 h with \(E.\ coli\)-cleared serum, washed five times for 5 min each time, and incubated for 1 h with either Alexa Fluor 647-conjugated goat anti-human \(\gamma\)-specific IgG (1:1,000) or goat anti-human \(\mu\)-specific IgM (1:250) secondary antibody (Southern Biotech) diluted in probe buffer. Microarrays were washed three times with wash buffer, rinsed twice with filtered deionized water to remove any residual salts, and dried.

Data acquisition and analysis. Microarray slides were scanned at 635 nm using a confocal laser scanner (GenePix 4400A scanner; Molecular Devices) using settings below signal saturation. Background-subtracted pixel counts were determined with GenePix Pro 7 software, and outliers among data points were identified and removed. The data were normalized by log2 transformation and quantile normalization. The data were analyzed using limma and Bioconductor packages in R (55).
replicates, identified using a modified Z-score (median absolute deviation of >3.5), were removed. Pixel counts from replicate spots were averaged to obtain mean fluorescence intensity (MFI) and used for subsequent analysis. Relative binding signals were calculated as RB = (x/x0)(100) where x is the MFI originating from microarrayed antigens and x0 is the infecting virus species. Relative binding signals were used in hierarchical clustering analyses (average-linkage Pearson correlation) performed using MeV v4.8.1 within the TM4 software suite (55). Student’s t tests, polynomial curve fitting, principal-component analyses, and one-way analysis of variance (ANOVA) with Tukey’s posthoc honestly significant difference (HSD) test were performed using OriginPro v9.0 (Origin Lab Corporation).

**Machine learning.** The support vector machine (SVM) method LIBSVM ([https://www.csie.ntu.edu.tw/~cjlin/libsvm/](https://www.csie.ntu.edu.tw/~cjlin/libsvm/)), available in the R package e1071 ([https://www.csie.ntu.edu.tw/~cjlin/libsvm/](https://www.csie.ntu.edu.tw/~cjlin/libsvm/)), was used for predictions of infection histories with quantile normalized microarray data. An optimal separating hyperplane between data classes was determined with the SVM by maximizing the margin between the closest points and minimizing the classification error. All binary subclassifiers were fitted to the model, and the correct class was identified by a voting mechanism (i.e., the class with the highest probability). We used a radial basis function (RBF) as the kernel function, which is defined by \( K(u,v) = \exp(-\gamma ||u - v||^2) \), where \( u \) and \( v \) are two data vectors and \( \gamma \) (set at 0.001) is a training parameter that makes the decision boundary smoother as the value becomes smaller. The regularization factor \( C \), set at 100, controls the trade-off between a low training error and a large margin. A grid search was used for selection of \( C \) (1 to 1,000) and \( \gamma \) (0.0001 to 1) using 10-fold cross-validation of the training data set and the built-in “tune” function of e1071. The final SVM model was generated using the optimal parameters with complete training data sets. To evaluate the performance of the model, a 10-fold cross-validation was implemented on the training data set, which consisted of a positive set of E-specific antibody binding signals from primary flavivirus infections \((n = 32 \text{ human and NHP})\) and a negative set of background signals from flavivirus-naive sera \((n = 34 \text{ human and NHP})\). Based on one-way ANOVA followed by Tukey’s range test, the overall antibody binding patterns of DEN2-challenged NHPs and humans were not statistically significantly different \((P > 0.05)\). Therefore, the positive training set consisted of data from both DEN2-challenged humans and NHPs. The training data set was randomly divided into 10 equal parts, and each run of cross-validation was comprised of 1/10 as the independent test data set and the remaining 9/10 as the training data set. The performance of the model was calculated as accuracy = \( (TP + TN) / (TP + TN + FP + FN) \).

**E-protein molecular phylogeny.** A phylogenetic tree was generated based on E-protein amino acid sequences (Asian-YAP/2007 and African-MR-766/1947 lineage ZIKV selected as representative strains). CLUSTAL W2 ([57](#)) was used to generate three multiple-sequence alignments (MSAs), each with a different gap opening penalty (5, 10, 25), Blosum62 as the protein weight matrix, and all other options left as default. T-Coffee Combine ([58](#), [59](#)) was then used to generate a single alignment that had the best agreement of all three MSAs. gBlocks ([60](#), [61](#)) with relaxed settings (small blocks allowed, gap positions allowed within final blocks, and less-strict flanking positions) was used to eliminate poorly aligned positions and divergent regions in the combined alignment, and 202 conserved columns within the alignment were retained. A molecular phylogeny was generated using the maximum likelihood method implemented in the PhyML program (v3.0 a LRT) ([62](#)). The Blosum62 substitution model and four gamma-distributed rate categories were selected to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (\( \gamma = 1.564 \)). Tree topology and branch length were optimized for the starting tree and subtree pruning and regrafting selected for tree improvement.

**Animal and human sera.** (i) Animal use statements. All macaque monkeys used in this study were cared for by the staff at the Wisconsin National Primate Research Center (WNPRC) in accordance with the regulations and guidelines outlined in the Animal Welfare Act, the *Guide for the Care and Use of Laboratory Animals* ([63](#)), and the recommendations of the Weatherall report ([64](#)). This study was approved by the University of Wisconsin—Madison Graduate School Institutional Animal Care and Use Committee (Animal Care and Use Protocol G005401). For all procedures (i.e., physical examinations, virus inoculations, ultrasonad examinations, blood and swab collection), animals were anesthetized with an intramuscular dosage of ketamine (10 ml kg of body weight \(^{-1}\)). Blood samples were obtained using a Vacutainer system or needle and syringe from the femoral or saphenous vein.

(ii) Human use statements. Research on human subjects was conducted in full compliance with the U.S. Department of Defense (DoD), National Institutes of Health (NIH), federal, and state statutes and regulations relating to the protection of human subjects and adheres to principles identified in the *Belmont Report* ([65](#)). All specimens, data, and human subject research were gathered and conducted for this publication under institutional review board (IRB)-approved protocols.

**ZIKV.** Three groups of Indian origin *Macaca mulatta* (three individuals per group) were challenged subcutaneously with a different dose (10\(^6\), 10\(^5\), or 10\(^4\) PFU) of either an Asian (study identification [ID] ZIKV001 and ZIKV004) or African (study ID ZIKV002) lineage ZIKV ([Table 1](#); see Fig. S1A in the supplemental material). Sera were collected prior to ZIKV challenge (day 0) and daily for 10 days (all cohorts), followed by two to three times a week from 11 to 28 days postinfection (dpi) (ZIKV001 and ZIKV002 only) ([33](#) Zika Open-Research Portal [https://zika.labkey.com]). Human sera from ZIKV infections were collected from four female patients in the Dominican Republic that developed symptoms of ZIKV (fever, joint pain, headache, conjunctivitis, rash, and muscle pain) in January 2016 ([Fig. S1C](#)). Three patients were PCR confirmed for ZIKV infection by the CDC within the first 2 weeks of symptom onset, whereas the remaining patient tested positive for the presence of anti-ZIKV IgG by a microplate enzyme-linked immunosorbent assay (ELISA) (Euroimmun, Inc.), as performed by BocaBiolistics (Pompano Beach, FL), and sera were collected 12 to 31 days after the onset of symptoms.
DENV. Sixteen healthy, flavivirus naive rhesus macaques (*M. mulatta*) were subcutaneously injected with 10^3 PFU of DENV1 (West Pac 74), DENV2 (S16803), DENV3 (CH53489), or DENV4 (341750) (*n* = 4 per challenge group; Fig. S1A and 4), derived from low-passage, near-wild-type virus isolates. Sera were collected prior to infection and 30 dpi (37, 43). Sera from human primary DENV2 infections (*n* = 10; Fig. S1B) were collected as part of a DENV human challenge model originally developed by the Laboratory of Infectious Diseases at the U.S. National Institutes of Health. Sera from participants that had no history or serological evidence of flavivirus infection were collected prior to challenge and 28 days postchallenge with 10^3 PFU of rDENVΔ30. rDENVΔ30 induced viremia in all participants by 5 dpi (39, 40). Two individuals exhibited elevated binding to flavivirus E prior to challenge with rDENVΔ30 (Fig. S4) and were further excluded from our analysis. Convalescent DENV sera (*n* = 7) were collected in Peru by the U.S. Naval Medical Research Unit No. 6 (NAMRU-6) between February 2011 and November 2013. Subjects had febrile illness for 5 days or less and were confirmed to have DENV infections (DENV2, *n* = 5; DENV3, *n* = 2; Fig. S5) by testing during the acute phase of infection. Sera were collected 14 to 24 days after confirmation of acute infection.

YFV. Early immune yellow fever virus antisera from three NHPs (Fig. S1A; NR-29335, NR-29337, and NR-29338; BEI Resources, NIAID, NIH), immunized by subcutaneous injection of 0.5 ml of live, attenuated YFV vaccine (strain 17D [38]), were collected 30 days after vaccination. Human sera from 17D-vaccinated individuals (seven primary and six boosted [Fig. S1D]), collected 14 to 118 days after vaccination, were obtained from the Department of Defense Serum Repository (Silver Spring, MD).

**WNV.** Confirmed WNV-infected human sera (*n* = 20; Fig. S1D) were collected between 2009 and 2011 at FDA-approved blood donor locations within the United States in accordance with a surveillance protocol administered by the National Heart, Lung, and Blood Institute (NHBLI) Biologic Specimen and Data Repository Information Coordinating Center (BioLINCC) (66). Sera were identified as WNV positive by nucleic acid testing, indicating a current WNV infection at the time of blood donation. WNV-positive donors were then contacted for study enrollment, at which point subjects completed symptom questionnaires and provided subsequent blood samples at several weekly and monthly visits after the initial donation. Each specimen tested positive for the presence of WNV-specific IgM and IgG antibodies (67).

**Control sera.** Sera collected by SeraCare Life Sciences, Inc., from healthy U.S. donors (*n* = 5) were used for negative controls. These sera were selected based on no detected antibodies to human immunodeficiency virus type 1 and type 2, hepatitis A and B viruses, and all flaviviruses used in the microarray.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/CVI.00036-17.

**SUPPLEMENTAL FILE 1,** PDF file, 1.0 MB.

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We declare that we have no competing interests.

R.G.U., C.L.P., S.L.K., J.L.S., and S.M.R.J. designed the study. D.M.D. and A.P.D. collected and organized sera. C.L.P., J.L.S., and S.M.R.J. performed the experiments. S.L.K., C.L.P., J.L.S., S.M.R.J., D.H.O., R.D.H, and R.G.U. analyzed the data. S.L.K., C.L.P., J.L.S., S.M.R.J., and R.G.U. wrote the manuscript. All authors reviewed and edited the manuscript.

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