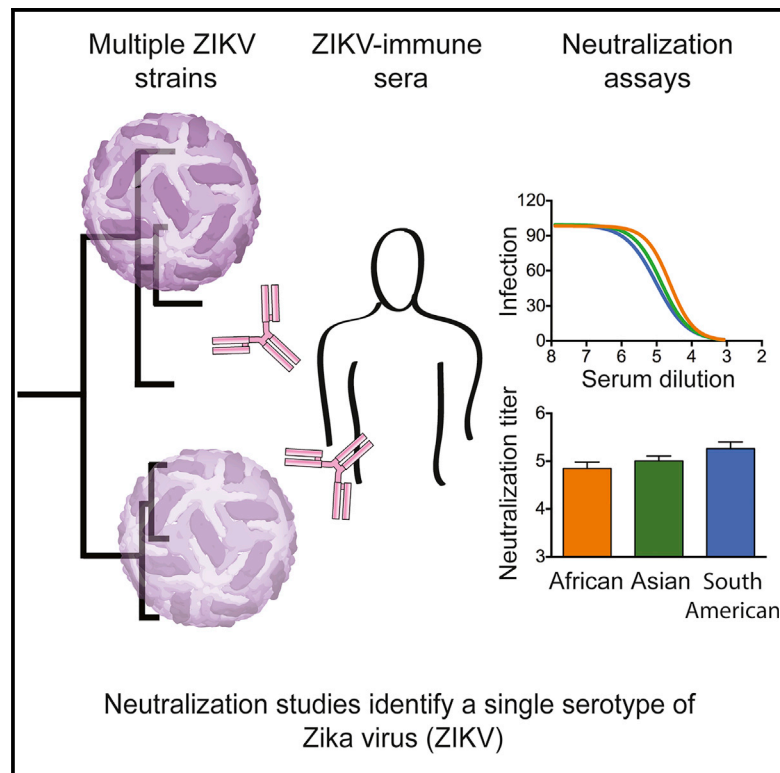


Broadly Neutralizing Activity of Zika Virus-Immune Sera Identifies a Single Viral Serotype

Graphical Abstract



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In Brief

Dowd et al. investigate the breadth of the neutralizing antibody response to ZIKV. They demonstrate that contemporary South American, Asian, and early African ZIKV strains are similarly sensitive to neutralization by ZIKV-confirmed convalescent human serum.

Highlights

- Neutralization studies with convalescent ZIKV-immune sera identify a single serotype
- Infection with a single ZIKV strain elicits broadly neutralizing antibodies
- Strain selection may not be a critical parameter for ZIKV vaccine development



Broadly Neutralizing Activity of Zika Virus-Immune Sera Identifies a Single Viral Serotype

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SUMMARY

Recent epidemics of Zika virus (ZIKV) have been associated with congenital malformation during pregnancy and Guillain-Barré syndrome. There are two ZIKV lineages (African and Asian) that share >95% amino acid identity. Little is known regarding the ability of neutralizing antibodies elicited against one lineage to protect against the other. We investigated the breadth of the neutralizing antibody response following ZIKV infection by measuring the sensitivity of six ZIKV strains to neutralization by ZIKV-confirmed convalescent human serum or plasma samples. Contemporary Asian and early African ZIKV strains were similarly sensitive to neutralization regardless of the cellular source of virus. Furthermore, mouse immune serum generated after infection with African or Asian ZIKV strains was capable of neutralizing homologous and heterologous ZIKV strains equivalently. Because our study only defines a single ZIKV serotype, vaccine candidates eliciting robust neutralizing antibody responses should inhibit infection of both ZIKV lineages, including strains circulating in the Americas.

INTRODUCTION

Zika virus (ZIKV) is a mosquito-transmitted flavivirus that has emerged from relative obscurity to cause an epidemic of great public health concern. During the half-century that followed its discovery, ZIKV was rarely linked to disease in humans, despite considerable transmission (Dick, 1953; Petersen et al., 2016). The emergence of epidemic ZIKV was first reported in Yap island in 2007, followed by outbreaks in French Polynesia in 2013 and 2014 and regularly thereafter in other islands of the Pacific. ZIKV was introduced into the western hemisphere in 2014–2015 and spread rapidly to 40 or more countries and territories. Historically, symptomatic ZIKV infection of humans was

described as a self-limiting mild febrile illness associated with rash, arthralgia, and conjunctivitis (Petersen et al., 2016). However, recent ZIKV infections also have been associated with neurological complications, including Guillain-Barré syndrome and meningoencephalitis (Brasil et al., 2016a, 2016b; Cao-Lor-meau et al., 2016; Oehler et al., 2014). Of greatest concern, ZIKV infection is now linked causally to microcephaly and intra-uterine growth retardation in the fetuses of women infected with the virus while pregnant (Hazin et al., 2016).

Flaviviruses are spherical virus particles that incorporate two structural proteins, premembrane/membrane (prM/M) and envelope (E), into their lipid envelope. High-resolution structures of the mature ZIKV virion and ectodomain of the E protein have been solved (Dai et al., 2016; Kostyuchenko et al., 2016; Sirohi et al., 2016). Similar to other flaviviruses, mature ZIKV virions are relatively smooth particles that incorporate 180 copies each of the E and M proteins. Neutralizing antibodies play a critical role in protection against flaviviruses and bind epitopes located in all three E protein structural domains (Heinz and Stiasny, 2012). Additionally, potentially neutralizing flavivirus antibodies have been isolated that bind surfaces composed of more than one domain or E protein (Screaton et al., 2015). Because neutralizing antibody titers correlate with protection by licensed vaccines for Japanese encephalitis virus (JEV), yellow fever virus (YFV), and tick-borne encephalitis virus (TBEV) (Belmusto-Worn et al., 2005; Heinz et al., 2007; Mason et al., 1973; Monath et al., 2002), eliciting neutralizing antibodies is a desired feature of candidate vaccines for related flaviviruses, including ZIKV.

Flaviviruses circulate as genetically distinct genotypes or lineages. ZIKV strains have been grouped into two lineages, African and Asian, which differ by <5% at the amino-acid level, including within the E protein gene (Haddow et al., 2012). The African lineage includes the historical MR-766 strain originally identified in 1947, whereas virus strains from the Asian lineage have been implicated in the recent outbreaks in Yap, French Polynesia, and the Americas. Understanding how sequence variation among ZIKV strains impacts antibody recognition is of particular importance to vaccine development. DENV, for example, circulates as four distinct serotypes that differ by 25%–40% at the

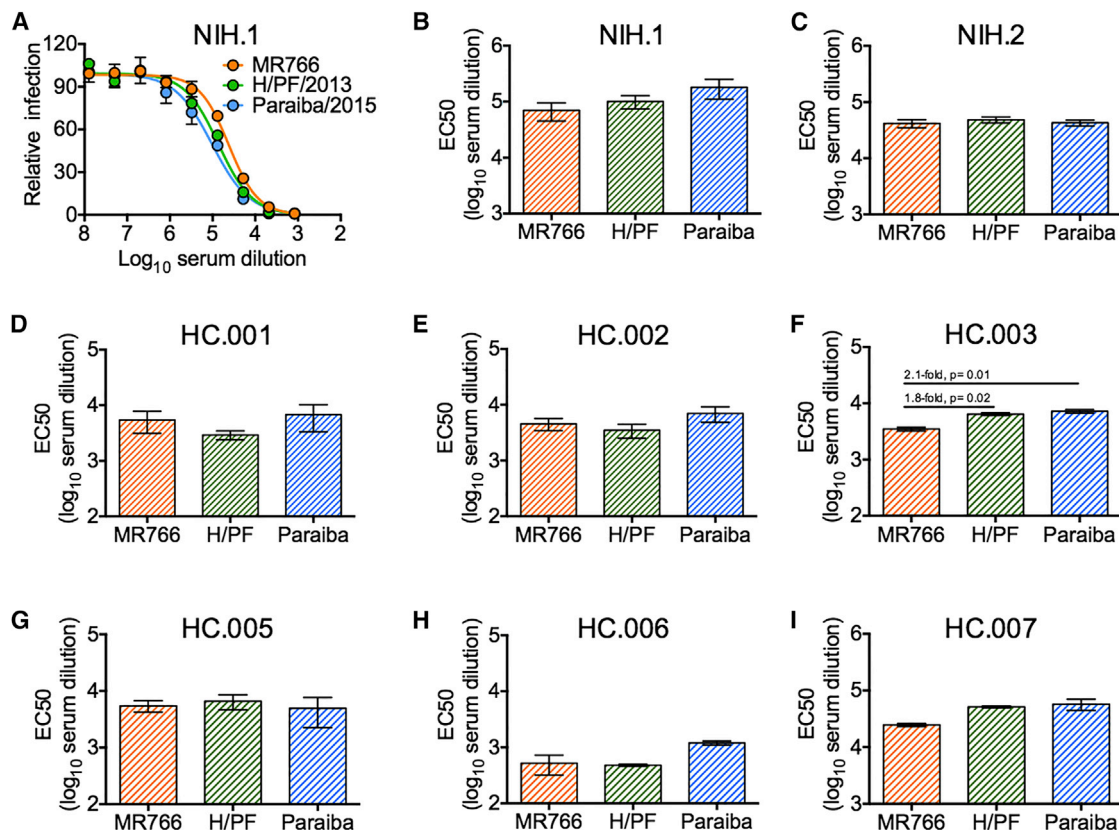


Figure 1. Neutralization of Multiple Strains of Infectious ZIKV by Immune Human Sera

The sensitivity of three ZIKV strains to neutralization by sera from ZIKV-infected individuals was compared. Stocks of MR-766, H/PF/2013, and Paraiba/2015 ZIKV strains were produced in Vero cells and used in neutralization experiments. ZIKV was mixed with serial 4-fold dilutions of serum for 1 hr at 37°C prior to being added to Raji-DCSIGNR cells. Infection was measured 20 hr post-infection by staining cells for intracellular E protein expression followed by flow cytometry. The dilution of sera at half-maximal neutralization of infection (EC_{50}) was estimated by non-linear regression analysis.

(A) Neutralization curves for a representative experiment are shown for serum NIH.1. Error bars represent the range of duplicate technical replicates.

(B–I) The average EC_{50} neutralization titers obtained from independent neutralization studies for eight ZIKV-immune convalescent sera are presented. Error bars reflect the range or SE of two to three experiments. Statistical differences in mean EC_{50} were identified using ANOVA with a multiple comparisons correction; the fold difference in sensitivity relative to MR-766 and multiplicity-adjusted p values are displayed when significant (only F).

amino acid level within the E protein. The challenges of eliciting a protective neutralizing antibody response against all four DENV serotypes have delayed vaccine development significantly (Guy et al., 2016). Desirable ZIKV vaccine candidates should provide equivalent protection against both Asian and African lineages.

In this study, we investigated the breadth of the humoral immune response elicited by ZIKV infection. The ability of eight convalescent ZIKV-immune human serum or plasma samples collected during the current outbreak to neutralize multiple ZIKV strains was evaluated. Our results demonstrate that antibodies elicited after infection with contemporary Asian lineage strains potentially inhibit infection of both homologous Asian and heterologous African strains. Similarly, immune sera from mice infected with either Asian or African lineages inhibited infection of homologous and heterologous ZIKV strains. Our studies indicate that the different lineages of ZIKV exist as a single serotype. These findings will be of particular importance in the ongoing effort to rapidly develop a ZIKV vaccine.

RESULTS

Broadly Neutralizing Activity of ZIKV-Immune Sera

To evaluate the breadth of the neutralizing antibody response elicited by ZIKV infection, sera or plasma were obtained from eight ZIKV-infected individuals (Table S1). We first evaluated the neutralization activity of each sample with Vero-cell-derived stocks of three different ZIKV strains representing both African and Asian lineages. The African lineage strain MR-766 used in this study was originally isolated in Uganda in 1947. Contemporary Asian lineage isolates were collected during the 2013 French Polynesian outbreak (H/PF/2013) (Baronti et al., 2014) and the recent Brazilian epidemic (Paraiba/2015). Amino acid variation among viruses is detailed in Tables S2 and S3. Dose-dependent inhibition curves with virus and human-immune sera and plasma were generated using Raji cells expressing DC-SIGNR. Neutralization curves of all three viruses revealed a similar profile with serum from subject NIH.1 (Figure 1A). The EC_{50} values from independent neutralization studies for serum NIH.1 (Figure 1B)

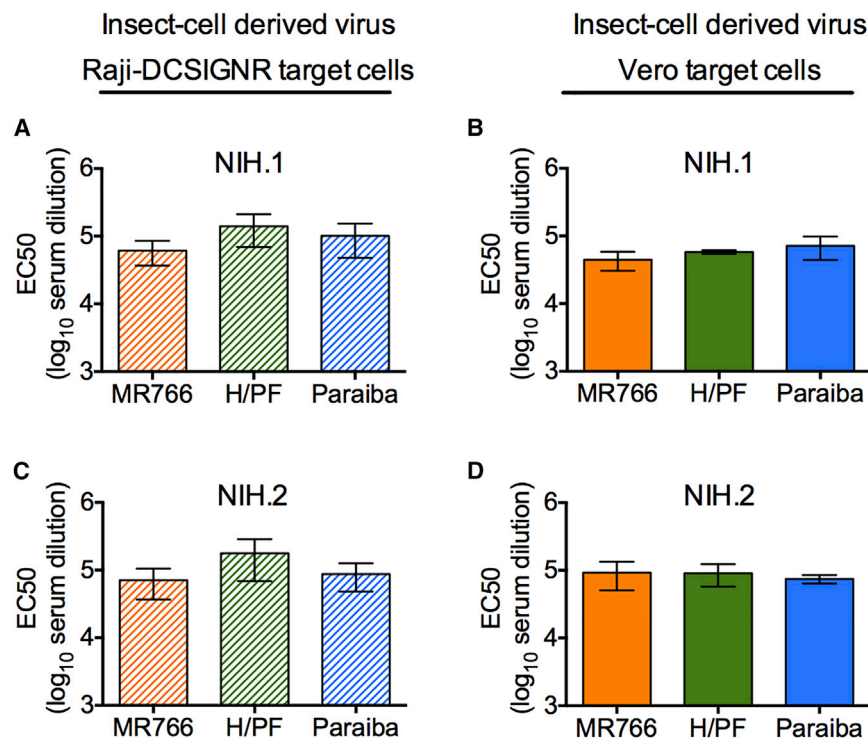


Figure 2. Varying the Cellular Source and Cellular Substrate in ZIKV Neutralization Assays

The sensitivity of ZIKV strains MR-766, H/PF/2013, and Paraiba/2015 produced in C6/36 insect cells to neutralization by sera NIH.1 (A and B) and NIH.2 (C and D) was assessed. ZIKV was mixed with serial 4-fold dilutions of serum for 1 hr at 37°C prior to being added to Raji-DCSIGNR (A and C) or Vero (B and D) cells. Infection was measured 20 hr post-infection by staining cells for intracellular E protein expression followed by flow cytometry. The dilution of sera at half-maximal neutralization of infection (EC₅₀) was estimated by non-linear regression analysis. The average EC₅₀ neutralization titers obtained from independent neutralization studies are presented. Error bars reflect the range of two experiments. No statistical differences in mean EC₅₀ were identified using ANOVA with a multiple comparisons correction.

and seven additional ZIKV convalescent samples (Figures 1C–1I) confirmed that all three ZIKV strains were similarly sensitive to inhibition by sera or plasma derived from individuals infected with contemporary ZIKV strains. Differences in the mean neutralization titer were uniformly less than 4-fold and, with one exception (Figure 1F), failed to reach statistical significance.

The efficiency of the virion maturation process varies among cell types used to produce viruses. For example, flaviviruses grown in insect cells are less efficiently processed than mammalian cell-derived viruses (Dejirattisai et al., 2015; Vogt et al., 2011). For many antibodies, inefficient cleavage of the prM structural protein results in increased neutralization potency due to enhanced accessibility of epitopes on partially mature virions (Pierson and Diamond, 2012). The prM content of virions also contributes to cell-type-dependent patterns of antibody neutralization (Mukherjee et al., 2014a). To determine whether the cellular substrate used for virus production impacts neutralization sensitivity among the ZIKV strains studied, we repeated neutralization studies with viruses produced in C6/36 insect cells (Figure 2). When assayed on Raji-DCSIGNR cells, neutralization titers obtained with these insect cell-derived stocks were similar among different ZIKV strains (Figures 2A and 2C) and to titers obtained with the same virus strains propagated in Vero cells (Figures 1B and 1C). The effect of using Vero cells as targets in neutralization assays also was evaluated and found to be minimal; the EC₅₀ values were virtually unchanged in comparison to experiments performed with Raji-DCSIGNR cells (Figures 2B and 2D versus 2A and 2C, respectively). Taken together, these data suggest that the similar sensitivity of multiple ZIKV strains to serum neutralization was not dependent on assay format.

structural genes provided *in trans*. We and others have used RVPs extensively to study the functional properties of neutralizing monoclonal antibodies (Pierson et al., 2007; Shrestha et al., 2010; Wang et al., 2016) and to evaluate the immunogenicity of candidate flavivirus vaccines in humans (Martin et al., 2007; VanBlargan et al., 2013). To confirm and extend the results of our studies with infectious ZIKV, we created RVPs that incorporate the structural proteins of five ZIKV strains representing African (MR-766 and ArB7701) and Asian (H/PF/2013, PHL/2012, and THA/2014) lineages. Studies with serum from subject NIH.2 revealed all five RVPs were neutralized equivalently (Figure 3A). More comprehensive studies were performed with MR-766 and H/PF/2013 RVPs and the entire panel of sera. The average EC₅₀ neutralization titers for multiple independent experiments are shown (Figures 3B–3I). These data confirm studies with fully infectious virus, demonstrating that strain-dependent differences in neutralization sensitivity are small, if they are present at all. Furthermore, comparison of the mean EC₅₀ for all samples evaluated with both RVPs and infectious virus revealed remarkable agreement (Figure S1). Limited studies with RVPs produced with the structural proteins of DENV and WNV revealed cross-reactive neutralization to varying degrees by ZIKV immune sera (Figure S2).

Analysis of ZIKV Strain-Dependent Neutralization in Mice

Mice lacking key components of the type I interferon signaling system have been defined as useful models of lethal ZIKV infection or *in utero* transmission (Aliota et al., 2016; Dowall et al., 2016; Lazear et al., 2016; Miner et al., 2016; Rossi et al., 2016).

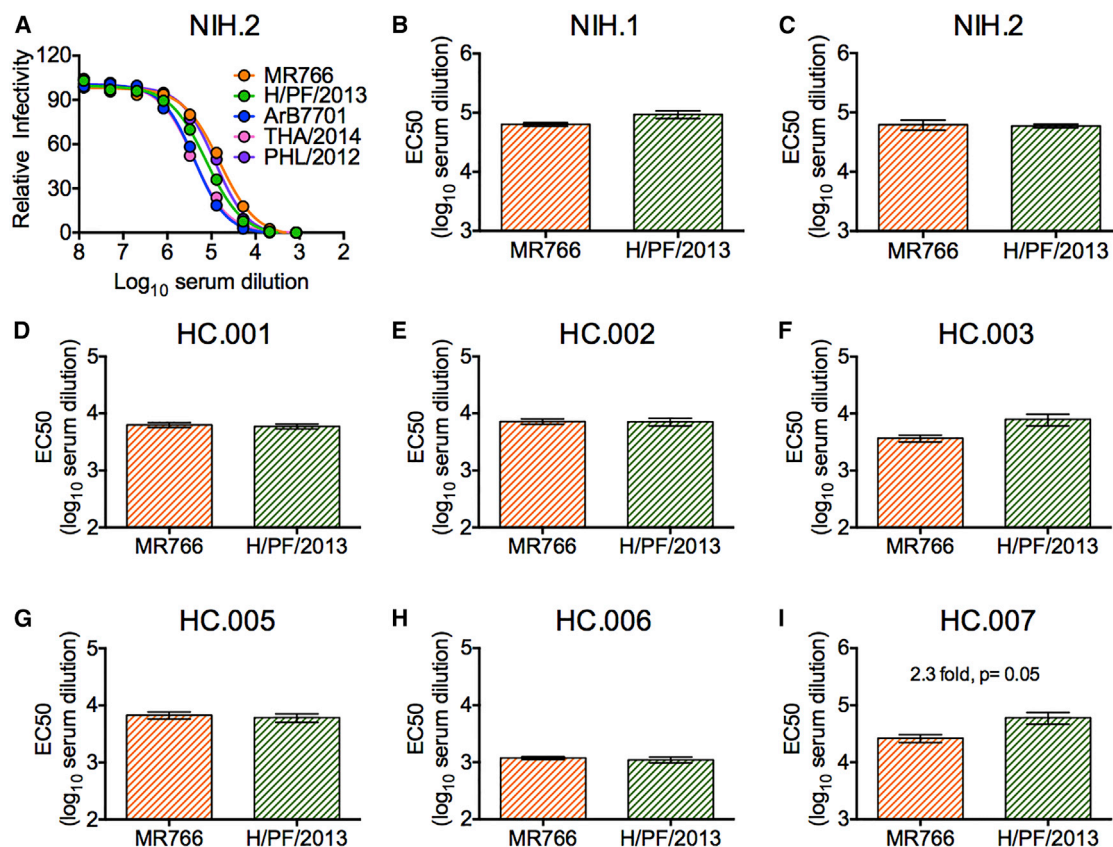


Figure 3. Neutralization of ZIKV RVPs by ZIKV-Immune Human Sera

ZIKV RVPs were produced by complementation of a GFP-expressing WNV replicon with a plasmid encoding the structural proteins of ZIKV strains MR-766, H/PF/2013, ArB7701, THA/2014, and PHL/2012. RVPs were mixed with serial 4-fold dilutions of serum for 1 hr at 37°C prior to being added to Raji-DCSIGNR cells. After 48 hr, GFP-positive infected cells were detected by flow cytometry. The dilution of sera at half-maximal neutralization of infection (EC₅₀) was estimated by non-linear regression analysis.

(A) Neutralization curves for a representative experiment (of three independent assays) are shown for serum NIH.2 against all five ZIKV RVPs. Error bars denote the range of duplicate technical replicates (present even when not visible due to low variation).

(B–I) The average EC₅₀ neutralization titers obtained from independent neutralization studies for eight ZIKV-immune convalescent sera measured against MR-766 and H/PF/2013 RVPs are shown. Error bars reflect the SE of 5–10 experiments. Statistical differences in the mean EC₅₀ values were identified using an unpaired t test; the fold difference and p values are displayed when significant (only I).

Because these systems may have value in preclinical vaccine studies, we investigated whether mice, like humans, mount an antibody response to ZIKV infection capable of equivalently neutralizing multiple strains. Studies in mice additionally allowed for assessment of the neutralizing activity of ZIKV-immune sera without any possibility of prior flavivirus exposure or pre-existing cross-immunity. *Irf3*^{-/-} mice, which allow ZIKV replication yet survive infection (Lazear et al., 2016), were infected with ZIKV strains MR-766 or H/PF/2013. Sera were collected and evaluated for neutralization using ZIKV RVPs displaying the homologous or heterologous structural proteins (Figures 4A and 4B). Sera from mice infected with either Asian or African ZIKV strains equivalently neutralized MR-766 and H/PF/2013 RVPs. These data confirm the broadly neutralizing activity of ZIKV-immune sera and suggest that strain selection or multi-antigen formulations will not be a critical parameter for the design of a ZIKV vaccine as long as strongly neutralizing antibody responses are elicited.

DISCUSSION

The rapid spread of ZIKV throughout South America and its linkage to birth defects in infants born to women infected during pregnancy has created a public health emergency that could be mitigated by vaccination. Vaccines have proven effective at blunting the impact of flavivirus infection on public health, most notably for YFV. The antiviral activity of convalescent and vaccine-induced antibodies reflects their capacity to bind to and neutralize virion infectivity, as well as mediate other effector functions (Burton, 2002; Nimmerjahn and Ravetch, 2008). Serum-neutralizing activity has proven to be a useful correlate of protection following vaccination against the related flaviviruses YFV, JEV, and TBEV. Phylogenetic analyses of ZIKV reveal that African and Asian lineages share >95% identity in amino acid sequences encoding the structural protein E targeted by neutralizing antibodies (Haddow et al., 2012). How amino acid variation among ZIKV strains impacts immunogenicity and

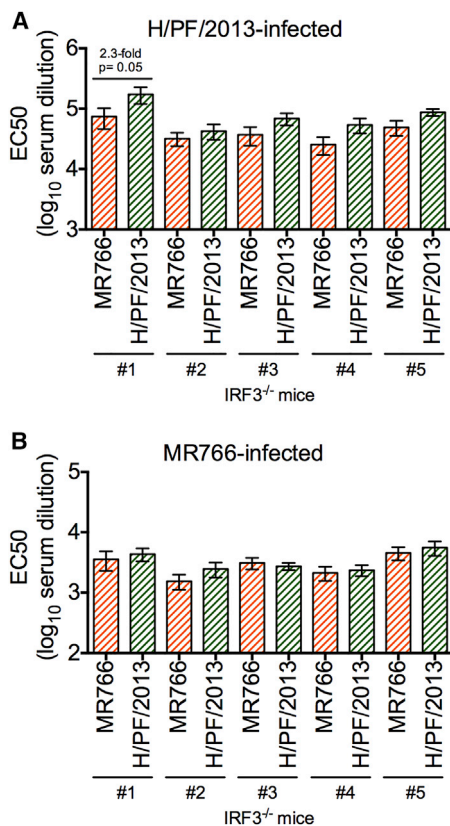


Figure 4. Neutralization of ZIKV RVPs with Sera from Mice Infected with ZIKV Strains H/PF/2013 or MR-766

Irf3^{-/-} mice were infected with 10^3 FFU of ZIKV strain MR-766 or H/PF/2013. Neutralization studies with mouse sera and both H/PF/2013 and MR-766 RVPs were performed as described for Figure 3. The average EC₅₀ neutralization titers obtained from independent neutralization studies of five mice infected with strain H/PF/2013 (A) and MR-766 (B) are shown. Error bars reflect the SE of three to five experiments. Statistical differences in mean EC₅₀ were identified for each mouse using ANOVA with a multiple comparisons correction; the fold difference and multiplicity-adjusted p values are displayed when significant (only A, mouse #1).

whether all ZIKV strains are sensitive to neutralization by antibodies elicited by heterologous antigens remained unknown.

In this study, we investigated the breadth of the neutralizing antibody response elicited by natural infection with ZIKV. Convalescent human sera or plasma obtained from individuals infected during the recent ZIKV epidemic were tested for their ability to neutralize infection of multiple strains of infectious ZIKV or ZIKV RVPs. This panel of human-immune sera or plasma neutralized contemporary Asian and historical African ZIKV strains equivalently, which suggests that ZIKV circulates as a single serotype. To extend these findings, we also investigated how the infecting ZIKV strain impacted the specificity of neutralizing antibodies. We demonstrate that mice infected with strain MR-766 or H/PF/2013 produced antibodies that neutralized both strains with equivalent potency. Our findings suggest antigens produced from African lineage viruses, such as the inactivated MR-766 vaccine candidate developed by Bharat Biotech, will

elicit antibodies capable of neutralizing contemporary circulating strains.

Although the existence of a single serotype was suggested by conservation of E protein sequences among ZIKV strains, factors that define the sensitivity of flaviviruses to neutralization remain incompletely understood (Pierson and Diamond, 2015). A recent study of the functional complexity of DENV vaccine-immune sera demonstrated that changes at only two amino acids were sufficient to render DENV1 and DENV2 RVPs equally sensitive to neutralization by monovalent DENV1 immune sera (VanBlargan et al., 2013). These findings suggest the extent of amino acid variability required for distinct viral serotypes cannot be predicted, and may be modest in number and dependent on viral background, an area that merits further study (Katzelnick et al., 2015). Beyond variation in amino acids in direct contact with antibody molecules, E protein variation may impact both structural heterogeneity and conformational dynamics of the virus particle, resulting in considerable changes to its overall antigenic structure (Kuhn et al., 2015; Pierson and Diamond, 2012). Although additional studies are required to obtain a detailed understanding of the antigenic structure of infectious ZIKV, our data suggest that differences in sequence, structural heterogeneity or dynamics among ZIKV strains have a minimal impact on sensitivity to the mixture of antibodies present in ZIKV-immune sera from convalescent patients or mice.

Overall, our results define a single ZIKV serotype and suggest that infection or vaccination with a single ZIKV strain can elicit broadly neutralizing antibodies against multiple strains, providing a direct path for the development of an effective vaccine.

EXPERIMENTAL PROCEDURES

Clinical Samples

ZIKV convalescent sera or plasma were collected with informed consent at the NIH Vaccine Research Center and the Hope Clinic of the Emory Vaccine Center. Additional details and clinical protocol numbers related to the collection of these samples are provided in Table S1 and the Supplemental Experimental Procedures.

Reporter Virus Particle Production

RVPs were produced by complementation of a WNV replicon with the structural proteins of WNV, DENV, or ZIKV using previously described methods. The ZIKV structural gene constructs described here were produced by cloning C-prM-E sequences from infectious virus or gene synthesis as described in the Supplemental Experimental Procedures as well as Tables S2 and S3.

Virus Neutralization Studies

Detailed protocols for neutralization studies with viruses and RVPs are provided in the Supplemental Experimental Procedures and Figures 1–3 and have been described in detail recently (Mukherjee et al., 2014b). Serial dilutions of sera or plasma were mixed with virus or RVPs and incubated for 1 hr at 37°C in order to ensure steady-state binding. Immune complexes were added to cells and incubated for one or two days for infectious viruses and RVPs, respectively. RVP infection was scored as a function of GFP expression, whereas infectious virus detection was measured by intracellular staining with an E-protein-specific mAb. The resulting data were analyzed by non-linear regression to estimate the dilution of sera required to inhibit 50% of infection. The lower limit at which neutralization could be measured with confidence was determined to be a 1/60 dilution of sera based on experiments with normal human sera collected prior to the ZIKV outbreak in South America (n = 29).

Mouse Studies

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH, and protocols were approved by the Institutional Animal Care and Use Committee at the Washington University School of Medicine (assurance no. A3381-01). ZIKV-immune sera used in neutralization studies were generated by infection of *Irf3*^{-/-} mice with a non-lethal 10³ FFU dose of ZIKV strain H/PF/2013 or MR-766. Additional details can be found in the [Supplemental Experimental Procedures](#).

Statistical Methods

Statistical analyses were performed using GraphPad Prism software. Mean EC₅₀ values were compared using an unpaired t test or ANOVA with Tukey's method for multiple comparisons correction and reported as p values or multiplicity-adjusted p values, respectively, when significant (≤ 0.05).

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures, two figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.07.049>.

AUTHOR CONTRIBUTIONS

K.D., C.D., R.P., S.S., A.S., L.G., and D.P. performed the experiments. K.D., M. D., and T. P. designed the experiments. J. M., B. G., M. M., and J. L. provided key reagents. K.D. and T.P. wrote the initial draft of the manuscript, and the other authors contributed to editing the final version.

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