Antibody Cross-Reactivity Between Zika Virus, Kedougou Virus, and Spondweni Virus

By

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Abstract

Zika virus (ZIKV), dengue virus (DENV), Kedougou virus (KEDV), and Spondweni virus (SPOV) are closely-related, mosquito-borne flaviviruses. It is well-established that ZIKV and DENV both cause fever, rash, and joint pain, in addition to more severe disease states such as microcephaly and hemorrhagic fever, respectively. However, little is known about KEDV or SPOV infections because they have not yet caused large human outbreaks. Given that cross-reactive antibodies between ZIKV and DENV complicate serodiagnostic tests in regions where both viruses circulate, it is crucial to understand and develop serological applications for other flaviviruses, such as KEDV and SPOV, which are even more closely related to ZIKV than DENV is. In addition, identifying a molecular basis for the cross-neutralization of ZIKV, KEDV, and SPOV can help inform the development of neutralizing antibodies and broadly protective vaccines. The objective of this project was to assess the neutralization of ZIKV (strain H/PF/2013), KEDV (D14701), and SPOV (SA Ar 94) by mouse polyclonal immune sera (anti-ZIKV, anti-KEDV, and anti-SPOV) and human convalescent sera (ZIKV-immune and DENV-immune). Another goal was to determine whether a panel of monoclonal antibodies (mAbs), specific to particular epitopes on the flavivirus E protein (domain I, domain II, domain III, fusion loop), broadly neutralized ZIKV, KEDV, and SPOV. Here, we found that anti-ZIKV mouse immune sera cross-neutralized KEDV and SPOV, though anti-KEDV and anti-SPOV sera did not cross-neutralize ZIKV (FRNT≤25). We observed that several mouse and human mAbs targeting the fusion loop bound ZIKV, KEDV, and SPOV. However, these fusion loop mAbs neutralized SPOV but not ZIKV, suggesting that virion maturation state could impact cross-neutralization. In addition, we identified two mAbs—G9E and EDEC8—that cross-neutralized both ZIKV and

SPOV by targeting the E protein cross-dimer interface, including domains I and II. These findings allow for subsequent studies on the cross-neutralization of ZIKV, KEDV, and SPOV by an expanded panel of ZIKV-immune and DENV-immune human convalescent sera samples, as well as other mAbs that cross-neutralize these viruses. This would facilitate both vaccine design and serodiagnostics in the event that a new flavivirus emerges in ZIKV/DENV co-endemic areas.

Introduction

Zika virus (ZIKV), dengue virus (DENV), Kedougou virus (KEDV), and Spondweni virus (SPOV) are closely-related mosquito-borne flaviviruses. Since 2007, ZIKV outbreaks have occurred in the South Pacific, Central and South America, and the Caribbean. DENV infection is widespread throughout much of the world, with an estimated 400 million new infections caused annually by the four serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) (Bhatt et. al, 2013). ZIKV and DENV both cause fever, rash, and joint pain. More significantly, ZIKV infection during pregnancy can cause birth defects, and DENV infection can cause hemorrhagic fever and shock.

Mature flavivirus virions are comprised of three structural proteins: capsid (C), membrane (M), and envelope (E) (Mukhopadhyay et. al, 2005). The flavivirus E protein dimer, with each subunit comprised of domains I, II, and III, facilitates receptor-mediated endocytosis into the host cell. In addition, it has been identified as a major target of protective and neutralizing antibodies in humans (Metz et. al, 2019). For instance, several ZIKV cross-reactive monoclonal antibodies (mAbs) that target conserved E protein epitopes, such as the fusion loop, have been isolated from DENV-infected patients (Barba-Spaeth et. al, 2016; Dussupt et. al, 2021). Since DENV serotype-specific antibodies, which are often weakly cross-neutralizing, can

enhance flavivirus infection during a secondary exposure to a heterologous strain (i.e.-antibody-dependent enhancement), it is critical to identify a molecular basis for potent cross-neutralization among closely related flaviviruses, such as KEDV and SPOV (Wahala et. al, 2011). This could inform the design of a vaccine that broadly cross-neutralizes these viruses.

Previous studies have found that DENV-immune and ZIKV-immune human sera cross-react with ZIKV and DENV, respectively (Premkumar et. al, 2018; Priyamvada et. al, 2017). These and other antigenically related flaviviruses co-circulate in tropical regions, complicating serodiagnostic assays. Therefore, it is essential to understand polyclonal immune sera cross-reactivity between ZIKV and other flaviviruses, such as SPOV and KEDV, that are more closely related than DENV is. Although SPOV infections have historically been limited to sporadic cases of mild febrile illness in the African subcontinent, it was recently detected by sequencing in *Culex quinquefasciatus* mosquitoes in Haiti (White et. al, 2018). Given that SPOV and ZIKV belong to the same serogroup, there is potential for the misidentification of a circulating SPOV strain in ZIKV endemic areas on the basis of serology alone (De Madrid et. al, 1974). Since there have been no confirmed human cases of KEDV to date, previous research studies have not assessed the cross-reactivity of ZIKV- and/or DENV-specific mAbs and convalescent polyclonal immune sera to KEDV (Weissenböck et. al, 2010). In the event of KEDV emergence in tropical regions, determining the extent of cross-reactivity between these viruses would prove beneficial for serodiagnostics (Robin et. al, 1978).

Here, we assessed the neutralization of ZIKV (strain H/PF/2013), KEDV (D14701), and SPOV (SA Ar 94) by mouse (anti-ZIKV, anti-KEDV, and anti-SPOV) and human (primary Zika, secondary Zika, and secondary dengue) polyclonal immune sera. Flaviviruses were classically

grouped by serologic cross-reactivity, which would suggest the existence of similar neutralization profiles among the three viruses (Collins et. al, 2017). Therefore, we hypothesized that the anti-ZIKV, anti-KEDV, and anti-SPOV mouse sera would cross-neutralize the closely related ZIKV, KEDV, and SPOV since the viruses share significant amino acid identity (Salazar et. al, 2019). In addition, we predicted that primary and secondary ZIKV-immune human sera would cross-neutralize KEDV and SPOV. Another goal of this research project was to reveal the molecular basis of mAb cross-neutralization between ZIKV, KEDV, and SPOV. This entailed (i) assessing the role of mAb cross-dimer binding across the flavivirus E protein as a mechanism for the potent cross-neutralization of ZIKV, KEDV, and SPOV and (ii) testing a panel of E protein-specific mouse (E60, ZV67, 4G2, ZV13) and human mAbs (Z20, ZIKV117, EDEC8, G9E, A9E, B11F, 1M7) for binding and neutralization of the viruses.

Materials and Methods

Viruses and Cells

Vero-81 cells were maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 5% heat-inactivated fetal bovine serum (FBS) and L-glutamine. ZIKV (H/PF/2013) was obtained from the United States Centers for Disease Control and Prevention (Division of Vector-borne Diseases, Fort Collins, CO). Both SPOV (SA Ar 94) and KEDV (D14701) were received from the World Reference Center for Emerging Viruses and Arboviruses (UTMB, Galveston, TX). ZIKV, KEDV, and SPOV virus stocks were grown in Vero-81 cells, supplemented with 2% FBS and 10 mM HEPES, and titrated by focus-forming assay (FFA). Confluent Vero cells were infected with serial dilutions of virus in DMEM+2% FBS+HEPES infection media and fixed with 2% paraformaldehyde at either 40 hours (ZIKV) or 48 hours

(SPOV and KEDV) post-infection. Infected foci were detected with the mouse monoclonal antibody (mAb) E60 and stained with HRP-conjugated anti-mouse secondary mAb and TrueBlue Peroxidase substrate. Foci were then counted at either the 100-, 1,000-, or 10,000-fold dilution factor to obtain the viral titer.

Human Sera Samples

Human serum samples were obtained through an IRB-approved research study from North Carolina residents who self-reported DENV or ZIKV infection following recent travel to endemic regions. DENV and ZIKV exposure history was confirmed by serologic assays in the laboratory of Dr. Aravinda de Silva. Sera were de-identified prior to use in our study. Primary ZIKV-immune sera (DT168 and DT172) were defined on the basis of having an FRNT-50 value for ZIKV that was ≥4-fold greater than that of any other flavivirus that was tested (Collins et. al, 2017). On the other hand, secondary ZIKV-immune (DT165 and DT166) and secondary DENV-immune (DT000) sera displayed significant neutralization of ≥ 2 flaviviruses (Collins et. al, 2017).

Anti-ZIKV Mouse Polyclonal Immune Serum

Anti-ZIKV polyclonal immune serum was generated by MD/PhD student Cesar Lopez of the Lazear Lab (Department of Microbiology and Immunology, UNC-Chapel Hill). *Ifnar1*$^{-/-}$ mice were first challenged by footpad with ZIKV PRVABC59. Following 1 month, the mice were challenged intravaginally with ZIKV H/PF/2013 and subsequently re-challenged 2 months later with ZIKV PRVABC59. The mice were then bled, and serum was pooled and heat-inactivated at 56^{o}C for 30 minutes.

Anti-KEDV Mouse Polyclonal Immune Serum

Anti-KEDV polyclonal immune serum was generated by Dr. Helen Lazear (Department of Microbiology and Immunology, UNC-Chapel Hill). Wild-type mice were infected by footpad with KEDV 14701 and boosted with the same strain by footpad 3 weeks later. Following 2 weeks, the mice were re-challenged with KEDV 14701 by intraperitoneal injection. The mice were then bled, and serum was pooled and heat-inactivated at 56^{o}C for 30 minutes.

Anti-SPOV Mouse Polyclonal Immune Serum

Anti-SPOV polyclonal immune serum was generated by Dr. Helen Lazear (Department of Microbiology and Immunology, UNC-Chapel Hill). Wild-type mice were first infected by footpad with SPOV SA Ar 94. Following 1 month, the mice were boosted with SPOV SA Ar 94 by footpad and subsequently re-challenged 2 weeks later with the same strain by intraperitoneal injection. The mice were then bled, and serum was pooled and heat-inactivated at 56^{o}C for 30 minutes.

Monoclonal Antibodies (mAbs)

The mouse mAbs ZV-13, ZV-67, and E60 were generated by hybridoma technology and screened for binding to the ZIKV E protein by ELISA (Oliphant et. al, 2005; Zhao et. al, 2016). The mAb 4G2 was similarly isolated from a mouse hybridoma (Priyamvada et. al, 2016). The mAb 1M7 was obtained from a human hybridoma producing DENV-specific antibodies (Smith et. al, 2013). On the other hand, the human mAbs Z20, ZIKV-117, A9E, G9E, and B11F were generated by cloning and expressing the variable regions of B cells specific for ZIKV into an igG1 constant region expression vector (Collins et. al, 2019; Graham et. al, 2021; Hasan et. al, 2017; Wang et. al, 2016). Similarly, the DENV-specific mAb EDEC8 was generated from

plasmablasts, following the amplification of heavy and light chain genes and cloning into an expression vector (Dejnirattisai et. al, 2015).

Enzyme-Linked Immunosorbent Assays (ELISAs)

ELISAs were used to assess the binding of several mouse mAbs (E60, ZV67, 4G2, ZV13) to KEDV and ZIKV. ELISA plates were first coated with 100 ng/well of the human mAb 1M7 (diluted in 0.1 M carbonate buffer) and refrigerated at 4^{o}C overnight. Wells were then washed with 1X PBST, blocked with 3% non-fat milk (diluted in 1X TBS with 0.05% tween), and incubated for 1 hour at 37^{o}C. Next, 50 µL of KEDV or ZIKV virus diluted in an equal volume of blocking buffer were added to the ELISA plates, followed by a 1-hour incubation period at 37^{o}C. Wells were then washed 3 times with 1X PBST buffer, and ten-fold dilutions of the primary mouse mAbs—E60, ZV67, 4G2, and ZV13—were added to the ELISA plates in duplicate, starting at an initial mAb concentration of 20 µg/mL. After another 1-hour incubation period and 3 washes with 1X PBST buffer, 50 µL of a 1:1000 dilution of alkaline phosphatase (AP)-conjugated anti-mouse secondary antibody in blocking buffer were added to the ELISA plates. Finally, 50 µL/well of AP substrate were added to the wells, whose absorbance values were then recorded at 405 nm by a spectrophotometer (Epoch Microplate Spectrophotometer).

Focus-Forming Assays

Focus-forming assays (FFAs) were used to qualitatively evaluate the binding of several mAbs (ZV67, 4G2, ZV13, 1M7) to ZIKV, KEDV, and SPOV infected cells. The viruses were first diluted to 2,000 FFU/mL in DMEM+2% FBS+HEPES infection media, and 50 µL of virus (100 FFU/well) were then added to 96-well plates seeded with confluent Vero cells. The wells were incubated at 37^{o}C for 1 hour and overlaid with 125 µL of 1% methylcellulose. Following an

incubation period of either 40 hours (ZIKV) or 48 hours (KEDV and SPOV) at 37°C, infected cells were fixed with 100 µL of 2% paraformaldehyde and incubated at room temperature for 1 hour. Next, the plates were washed 3 times with 1X PBST, and infected foci were detected with primary mAb (i.e.-human mAb 1M7 for mouse immune sera and mouse mAb E60 for human immune sera) overnight at 4°C. Following 3 washes with 1X PBST, a 1:2,500 dilution of HRP-conjugated anti-human or anti-mouse secondary mAb was added to the wells and incubated for 1 hour at room temperature. The plates were then washed with 1X PBST, and foci were visualized with TrueBlue Peroxidase substrate.

Neutralization Assays

Focus-reduction neutralization tests (FRNTs) were used to determine the neutralization of ZIKV, KEDV, and SPOV by mouse polyclonal immune sera (anti-ZIKV, anti-KEDV, and anti-SPOV), human polyclonal immune sera (primary Zika, secondary Zika, and secondary dengue), and a panel of human mAbs that target specific domains of the E dimer (Z20, ZIKV117, EDEC8, G9E, A9E, B11F, 1M7). Five-fold dilutions of mouse polyclonal immune sera/mAbs were first prepared, starting at an initial dilution of 1:12.5 in DMEM+2% FBS+HEPES media. Next, 30 µL of virus at a concentration of 2,000 FFU/mL were added to 30 µL of the polyclonal immune sera/ mAb serial dilutions, followed by a 1-hour incubation period at 37°C. Confluent Vero cells were infected with 50 µL of the serially diluted 1:1 sera:virus mixture, incubated for 1 hour, and overlaid with 125 µL of 1% methylcellulose. The plates were then incubated at 37°C for either 40 hours (ZIKV) or 48 hours (KEDV and SPOV). Infected foci were stained using the same protocol as the focus-forming assay outlined above.

Data Analysis of Neutralization Assays

Relative infection (%) was determined by normalizing the foci counts in the experimental wells against the naive sera (negative control) wells. This entailed averaging the foci counts, in duplicate, and dividing by the number of foci in the naive sera wells. The sigmoidal dose response inhibition equation (variable slope—4 parameters) of Prism (GraphPad Software) was then used to calculate FRNT-50 values. An FRNT-50 value represents the serum dilution at which 50% of the maximum foci counts are reduced. Reported values were required to fulfill three criteria: $R^2 > 0.75$, Hill Slope > 0.5, and an FRNT-50 within the range of the assay.

Results

Polyclonal Immune Sera Neutralization of KEDV, SPOV, ZIKV, and DENV4

Although we had access to convalescent serum samples from humans infected with DENV and/or ZIKV, no such human sera were available from documented cases of SPOV or KEDV infection. Given the current lack of KEDV and SPOV infections in humans, we challenged wild-type mice with KEDV and SPOV to generate anti-KEDV and anti-SPOV polyclonal immune sera. To identify the serological cross-reactivity of anti-ZIKV, anti-KEDV, and anti-SPOV mouse polyclonal immune sera to ZIKV, KEDV, and SPOV, we carried out focus-reducing neutralization assays. We found that anti-ZIKV immune serum cross-neutralized SPOV (FRNT-50 = 783) and KEDV (FRNT-50 = 351), though anti-KEDV and anti-SPOV mouse sera did not cross-neutralize ZIKV (FRNT-50 ≤ 25) **(Figure 1A, Figure 1B, Figure 1C)**. In addition, we observed that anti-SPOV mouse serum cross-neutralized KEDV (FRNT-50 = 135), and anti-KEDV mouse serum cross-neutralized SPOV (FRNT-50 = 184) **(Figure 1A and Figure 1B)**.

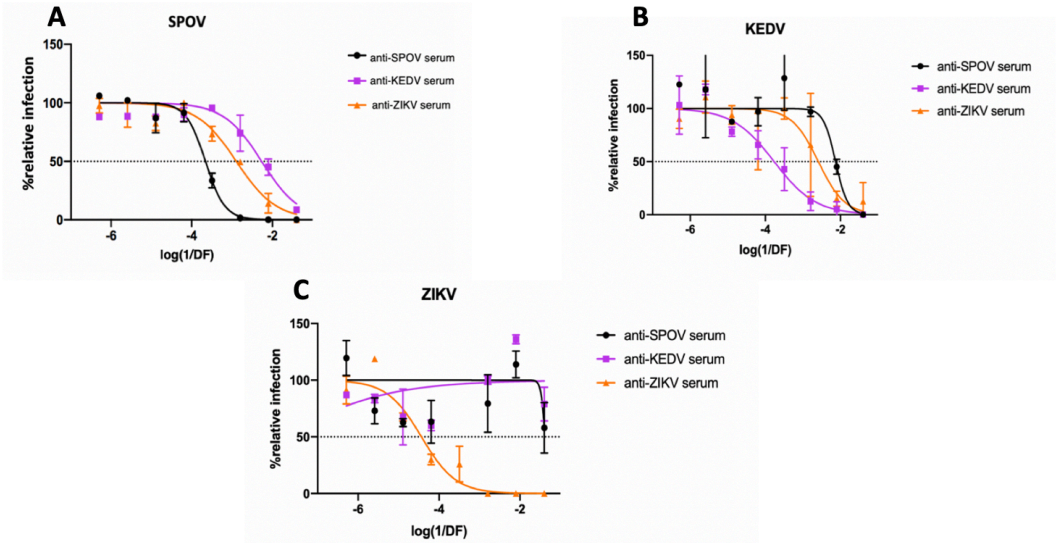

Figure 1. Focus-reducing neutralization assays were carried out to assess the neutralization of SPOV, KEDV, and ZIKV by anti-SPOV, anti-KEDV, and anti-ZIKV mouse polyclonal immune sera. Five-fold serial dilutions of the sera samples were added to an equal volume of virus at a concentration of 2,000 FFU/mL. Foci were stained with the primary human mAb 1M7, secondary HRP-conjugated anti-human mAb, and TrueBlue Peroxidase substrate. Relative infection (%) was obtained by normalizing foci counts against naïve human sera.

To evaluate if the cross-reactivity between polyclonal immune sera and these viruses also applies to human antibodies, we tested the neutralization of KEDV, SPOV, and DENV4 by a panel of late convalescent human sera samples obtained from people infected with ZIKV or DENV. Specifically, this panel was comprised of primary ZIKV-immune human sera samples (DT168 and DT172), secondary ZIKV-immune human sera samples (DT165 and DT166), and a secondary DENV-immune human serum sample (DT000) (Collins et. al, 2017). We found that both primary ZIKV-immune sera samples cross-neutralized SPOV. However, one of these, DT168, more potently neutralized SPOV (FRNT-50 = 180), compared to DT172 which was near the limit of detection (FRNT-50 = 44) **(Figure 2A)**. Both secondary ZIKV-immune sera samples, DT165 and DT166, neutralized SPOV to higher levels (FRNT-50 values of 105 and 375, respectively), compared to the primary ZIKV sera. We also observed that the secondary DENV-immune serum sample, DT000, neutralized SPOV (FRNT-50 = 255) **(Figure 2A)**.

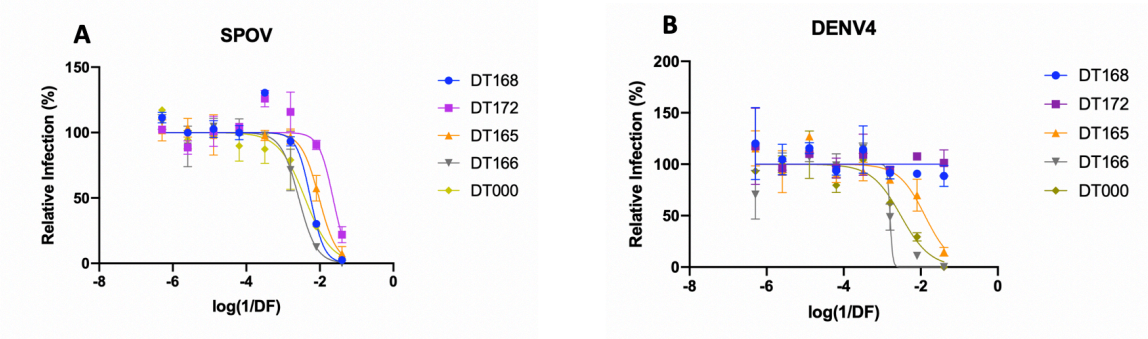

Figure 2. Focus-reducing neutralization assays were carried out to assess the neutralization of SPOV and DENV4 by primary ZIKV-immune (DT168 and DT172), secondary ZIKV-immune (DT165 and DT166), and secondary DENV-immune (DT000) human sera samples. Five-fold serial dilutions of the sera samples were added to an equal volume of virus at a concentration of 2,000 FFU/mL. Foci were stained with the primary mouse mAb E60, secondary HRP-conjugated anti-mouse mAb, and TrueBlue Peroxidase substrate. Relative infection (%) was obtained by normalizing foci counts against naïve human sera.

As expected, the secondary DENV-immune serum sample, DT000, neutralized DENV4 (FRNT-50 = 329) **(Figure 2B)**. Although the primary ZIKV-immune sera samples (DT168 and DT172) did not cross-neutralize DENV4, the secondary ZIKV-immune sera samples (DT165 and DT166) did, consistent with their previous exposure history **(Figure 2B)**. The neutralization of KEDV by this panel of late convalescent human sera samples was inconclusive (not shown).

Identification of a Molecular Basis for the Cross-Neutralization of ZIKV, KEDV, and SPOV

To determine the importance of binding across the E protein dimer as a mechanism for the potent cross-neutralization of ZIKV, KEDV, and SPOV, we used a monoclonal antibody (DNSK) generated by Cameron Adams of Dr. Aravinda de Silva's lab (UNC-Chapel Hill, Department of Microbiology and Immunology). DNSK is a paratope mutant of the mAb G9E, with mutations that ablate the binding site across the E protein dimer **(Figure 3)**. G9E is a potent ZIKV-neutralizing mAb isolated from DT168 (Collins et. al, 2019). We assessed the neutralization of ZIKV, KEDV, and SPOV by both the G9E mAb and its paratope mutant DNSK. We found that compared to the parental G9E mAb, which neutralized both ZIKV

(IC50=0.0044 µg/mL) and SPOV (IC50=0.88 µg/mL), the G9E paratope mutant less potently neutralized both ZIKV (IC50=0.15 µg/mL) and SPOV (IC50=160 µg/mL) **(Figure 4A and Figure 4B)**.

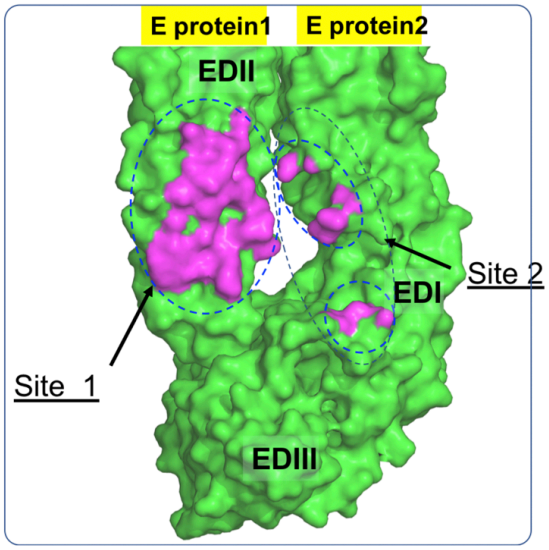

Figure 3. The G9E mAb paratope mutant, DNSK, was generated by knocking out the binding sites across the E protein dimer (Site 2 knock-out).

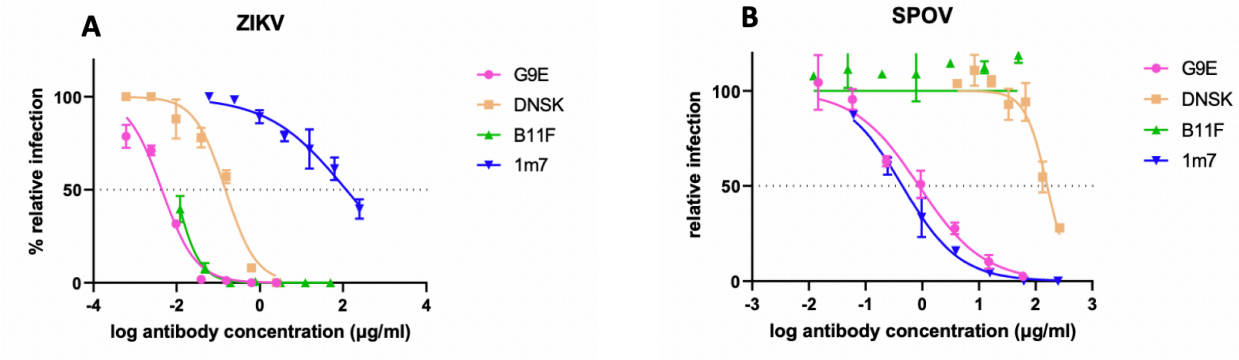

Figure 4. Neutralization of ZIKV and SPOV by a panel of human monoclonal antibodies. The mAb DNSK is the paratope mutant of the wild-type mAb G9E.

To identify other E protein target sites that cross-neutralize KEDV, SPOV, and ZIKV, we tested the binding and neutralization of these viruses by a panel of mouse and human mAbs. We observed that the mouse mAbs E60, 4G2, and ZV13, which target the conserved fusion loop of the E protein, bound both KEDV and ZIKV by ELISA **(Figure 5A and Figure 5B)**. The mAb ZV13 bound especially well to KEDV **(Figure 5A)**. On the other hand, the mAb ZV67, which targets domain III of the ZIKV E dimer, only bound ZIKV **(Figure 5B)**.

Next, we assessed whether the mouse mAbs ZV13, 4G2, and ZV67, along with the human mAb 1M7, bound to KEDV, ZIKV, and SPOV infected cells by focus-forming assay (FFA). We found that the fusion loop mAbs ZV13, 4G2, and 1M7 bound and stained KEDV, ZIKV, and SPOV infected cells by FFA **(Figure 6)**.

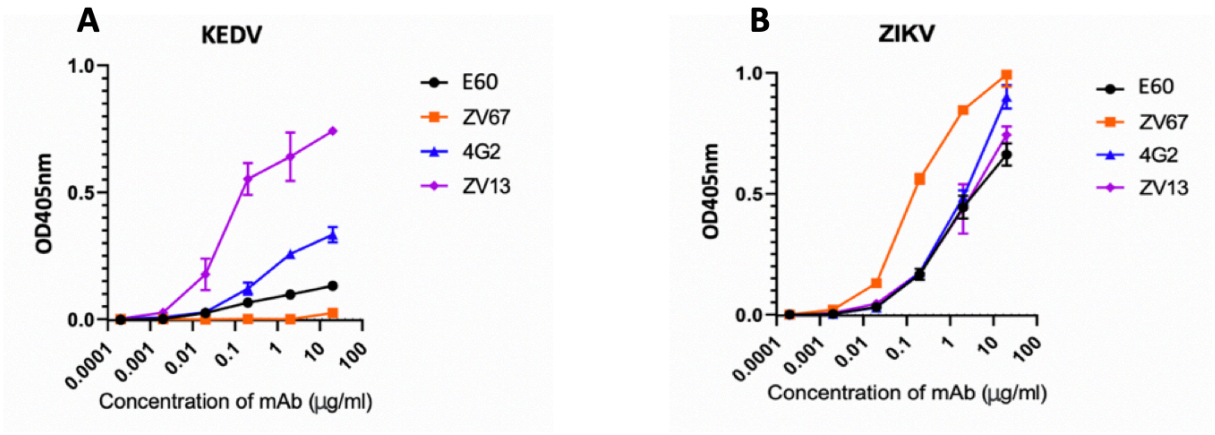

Figure 5. ELISA binding of KEDV and ZIKV by the mouse mAbs E60, ZV67, 4G2, and ZV13. Absorbance (OD) values were measured at a wavelength of 405 nm.

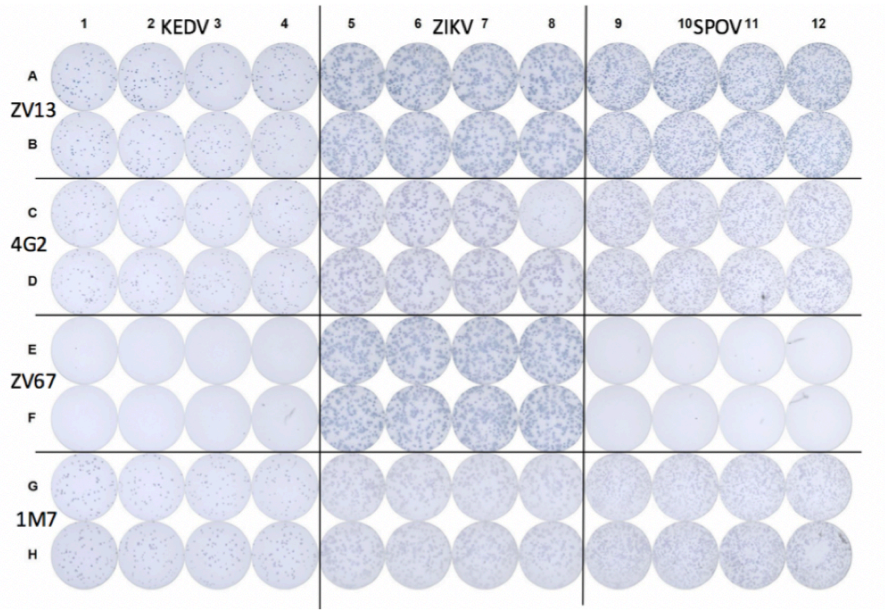

Figure 6. Columns 1-4 were infected with 2,000 FFU/mL of KEDV. Columns 5-8 were infected with 2,000 FFU/mL of ZIKV, and columns 9-12 were infected with 2,000 FFU/mL of SPOV. The primary mAbs ZV13 (Rows A-B), 4G2 (Rows C-D), ZV67 (Rows E-F), and 1M7 (Rows G-H) were then added to the wells at a concentration of 1 µg/mL. A goat anti-mouse or anti-human secondary igG antibody was subsequently added and stained with TrueBlue peroxidase substrate.

Given that neutralizing antibodies are generated differently among animal species, we recognized the importance of testing the cross-neutralization of ZIKV, SPOV, and KEDV by human mAbs (Zhao et. al, 2016). To accomplish this, we expanded our panel to include human mAbs (A9E, B11F, EDEC8, Z20, and ZIKV117) that target specific epitopes on the E protein **(Table 1)**. We found that the mAbs targeting domains I and II of the flavivirus E protein more potently neutralized ZIKV, compared to SPOV and KEDV. However, one of these mAbs, EDEC8, neutralized both ZIKV and SPOV **(Table 1)**. Furthermore, the mAbs EDEC8 and Z20 differentially neutralized the closely related SPOV (IC-50 = 0.153 µg/mL and 124.5 µg/mL, respectively) and KEDV (IC-50 = 2.119 µg/mL and >200 µg/mL, respectively) **(Table 1)**. The mAb 1M7, which targets the conserved fusion loop of the flavivirus E protein, neutralized SPOV

but did not neutralize ZIKV **(Figure 4A and Figure 4B)**. This was similarly observed with other human fusion loop mAbs (data not shown).

mAb	Target: Domain of E Protein	IC-50 (μg/mL)		
		ZIKV	SPOV	KEDV
A9E	EDI	0.022	>200	>200
B11F	EDI	0.081	>50	>50
EDEC8	EDI/EDII	0.090	0.153	2.119
Z20	EDII	1.490	124.500	>200
ZIKV117	EDII	0.424	>200	>200

Table 1. Focus-reducing neutralization assays were carried out to assess the neutralization of ZIKV, SPOV, and KEDV by human mAbs isolated from donors infected with ZIKV or DENV. These mAbs bind to different epitopes on the E protein, as shown above. Lower IC-50 values indicate more potent neutralization.

Because fusion loop mAb binding can be affected by flavivirus maturation state, we began to investigate whether maturation state affects the neutralization and binding ability of these antibodies on mature and immature virions (Cherrier et. al, 2009). To identify a suitable reagent that would allow for the assessment of SPOV and/or KEDV maturation state in a Western blot, we tested whether ZIKV prM-specific rabbit serum binds KEDV and SPOV infected cells in a focus-forming assay (FFA). We found that ZIKV anti-prM rabbit serum did not bind KEDV or SPOV, indicating ZIKV specificity.

Discussion

Since KEDV and SPOV are more closely related to ZIKV than DENV is, we expected that anti-ZIKV, anti-KEDV, and anti-SPOV mouse polyclonal immune sera would cross-react with the three viruses. However, we observed that anti-ZIKV mouse polyclonal immune sera cross-neutralized SPOV and KEDV, though anti-KEDV and anti-SPOV mouse sera did not cross-neutralize ZIKV. This may indicate an intriguing unilateral direction in flavivirus neutralization by mouse polyclonal immune sera. KEDV and SPOV exposure could result in primarily virus-specific antibodies in mice, though further experiments with similar infection routes and doses are required to definitively make this claim. E protein domain-specific antibodies could then be isolated from serum samples, which would render insight into how serum composition affects the neutralization of ZIKV, KEDV, and SPOV.

Both primary (DT168 and DT172) and secondary (DT165 and DT166) ZIKV-immune sera neutralized SPOV, corroborating previous reports of serological cross-reactivity between these viruses. Collins et. al, 2017 previously found that secondary DENV-immune serum (subject DT000) weakly neutralizes ZIKV (IC-50 = 29), compared to titers against DENV-2 (IC-50 = 744) and DENV-4 (IC-50 = 612). Given that SPOV belongs to same serogroup as ZIKV (Salazar et. al, 2019), we hypothesized that secondary DENV-immune serum would comparably neutralize SPOV. However, we observed that secondary DENV-immune serum (subject DT000), in fact, potently neutralized SPOV (FRNT-50 = 255), compared to the positive control DENV-4 (FRNT-50 = 329). Together, our data suggest that although ZIKV and SPOV have historically been grouped on the basis of overlapping serological cross-reactivity, there may be some notable differences in extent of cross-reactive epitopes between these two viruses.

Although we tested these primary ZIKV-immune (DT168 and DT172), secondary ZIKV-immune (DT165 and DT166), and secondary DENV-immune (DT000) sera samples against KEDV, no conclusions about cross-neutralization could be drawn because there were not enough KEDV foci in each well for the experiment to be valid. It is crucial to expand this panel of primary and secondary ZIKV- and DENV-immune sera samples in future studies to assess whether they differentially cross-neutralize the closely related KEDV and SPOV. This could have potential implications for serological applications and diagnostics, given that flaviviruses co-circulate in co-endemic areas and generate cross-neutralizing antibody responses in humans.

The fusion loop peptide has previously been identified as a conserved epitope of the flavivirus E protein and a target of the human antibody response (Cherrier et. al, 2009; Wahala et. al, 2011). However, it is also known that since fusion loop antibodies are broadly cross-reactive, they tend to be weakly neutralizing (Wahala et. al, 2011). Weakly neutralizing antibodies are typically associated with antibody-dependent enhancement (ADE), whereby antibodies generated in response to a primary viral infection cross-react but do not sufficiently neutralize either different strains of the same virus or closely related viruses during a secondary infection (Dejnirattisai et. al, 2016). These antibodies can, in turn, facilitate DENV infection of FcγR-expressing cells, resulting in severe disease outcomes, such as dengue hemorrhagic fever. Our data suggest that both mouse and human fusion loop mAbs (E60, 4G2, ZV13, 1M7) bind KEDV, ZIKV, and SPOV by ELISA and/or FFA **(Figures 5 and 6)**. This confirmed the cross-reactive nature of the conserved fusion loop epitope.

Immature flavivirus virions contain precursor membrane (prM) peptides, which have not yet undergone cleavage by the protease furin during the exocytic pathway typically associated

with maturation (Füzik et. al, 2018). Cherrier et. al, 2009 found that fusion loop antibodies preferentially recognize immature flavivirus virions because the target epitope is more readily exposed to solvent, compared to mature virions. Since our fusion loop mAb 1M7 neutralized SPOV but not ZIKV, consistent with data using other fusion loop mAbs (data not shown), we proposed that SPOV is more immature than ZIKV. Our ZIKV anti-prM rabbit serum did not bind KEDV or SPOV infected cells by FFA, which suggested that the prM epitope is not highly conserved among these closely related flaviviruses. Therefore, prM could potentially be a useful antigenic target to discriminate against ZIKV, KEDV, and SPOV immune serum. However, given the specificity of ZIKV anti-prM rabbit serum for ZIKV, alternative approaches are necessary to assess SPOV maturation state. To further assess this maturation hypothesis, we intend to produce fully mature KEDV and SPOV by growing them in Vero cells overexpressing the furin enzyme (Mukherjee et. al, 2016) and assessing whether the fusion loop mAbs differentially neutralize mature and immature KEDV and SPOV. This would implicate the role of virion maturation state in mAb cross-neutralization. Another potential explanation for our observations is that the incubation temperature at 37^{o}C affected the flexibility of the flavivirus envelopes, which caused the SPOV and ZIKV E protein epitopes to be differentially exposed—a phenomenon termed "breathing" (Goo et. al, 2016). This would account for the ability of the fusion loop mAbs to differentially neutralize SPOV and ZIKV.

Since the DNSK paratope mutant less potently neutralized both ZIKV and SPOV, compared to the parental G9E mAb (obtained from subject DT168 with primary ZIKV infection), it appears that cross-dimer binding across the E protein is a mechanism for the potent neutralization of these closely related viruses. We also found that this ablation in neutralization

potency occurred when the DNSK paratope mutant was tested against other more distantly related flaviviruses, such as West Nile virus and Japanese encephalitis virus (courtesy of Cameron Adams of Dr. Aravinda de Silva's lab). In addition, we found that the mAbs A9E, B11F, Z20, and ZIKV117, which target either domain I or domain II of the E protein, neutralized ZIKV to a greater extent, compared to KEDV and SPOV. This can potentially be explained by (a) differences in the amino acid composition of domains I and II in ZIKV and KEDV/SPOV or (b) the differential presentation of domain I and II epitopes in ZIKV and KEDV/SPOV. Nonetheless, two of the mAbs that target domains I/II—G9E and EDEC8—neutralized both ZIKV and SPOV, which raises the possibility that certain epitopes on these domains may be conserved between the viruses. Together, our findings suggest that these epitopes, along with cross-dimer interactions across the E protein, may be the target sites of potently cross-neutralizing mAbs. As previously discussed, several antibodies that target the fusion loop are weakly cross-neutralizing. Therefore, there is great value in instead isolating other E domain I/II and/or E protein cross-dimer mAbs that cross-neutralize ZIKV, KEDV, and SPOV. In addition to this, blockade-of-binding experiments would be useful to test whether these mAbs bind proximal epitopes. This can help inform vaccine design, which would ideally elicit an antibody response that cross-neutralizes these viruses, in the event of KEDV and/or SPOV emergence in co-endemic areas.

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