Article

Post-Vaccination Yellow Fever Antibodies Enhance Zika Virus Infection in Embryoid Bodies

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Abstract: Zika virus (ZIKV) is a flavivirus that originated in Africa but emerged in Latin America in 2015. In this region, other flaviviruses such as Dengue (DENV), West Nile, and Yellow Fever Virus (YFV) also circulate, allowing for possible antigenic cross-reactivity to impact viral infections and immune responses. Studies have found antibody mediated enhancement between DENV and ZIKV, but the impact of YFV antibodies on ZIKV infection has not been fully explored. ZIKV infections cause congenital syndromes, such as microcephaly, necessitating further research into ZIKV vertical transmission through the placental barrier. Recent advancements in biomedical engineering have generated co-culture methods that allow for in vitro recapitulation of the maternal: fetal interface. This study utilized a transwell assay, which is a co-culture model utilizing human placental syncytiotrophoblasts, fetal umbilical cells, and a differentiating embryoid body to replicate the maternal: fetal axis. To determine if cross reactive YFV vaccine antibodies impact the pathogenesis of ZIKV across the maternal fetal axis, maternal syncytiotrophoblasts were inoculated with ZIKV or ZIKV incubated with YFV vaccine anti-sera, and viral load was measured 72 hours post inoculation. Here we report that BeWo and HUVEC cells are permissive to ZIKV and that the impact of YFV post-vaccination antibodies on ZIKV replication is cell line dependent. Embryoid bodies are also permissive to ZIKV and the presence of YFV antibodies collected 1 to 6 months post vaccination enhances ZIKV infection. Our data show that each of the cell lines and EBs have a unique response to ZIKV complexed with post-vaccination serum suggesting there may be cellspecific mechanisms that impact congenital ZIKV infections. Since ZIKV infections can cause severe congenital syndromes, it is crucial to understand any potential enhancement or protection offered from cross-reactive, post-vaccination antibodies.

Keywords: Zika virus; yellow fever virus; flavivirus; cross-reactivity; neutralization; enhancement; congenital infections, Zika congenital syndrome, stem cell

1. Introduction

Zika Virus (ZIKV) and Yellow Fever Virus (YFV) are both part of the flavivirus family, with an enveloped, single-stranded positive-sense RNA genome. Both ZIKV and YFV are vectored by *Aedes* mosquitoes. Both YFV and ZIKV originated in Africa and have been found to co-circulate within the same regions of Latin America [1].. ZIKV first appeared in the Western Hemisphere in 2015 [2,3]. YFV however, has been circulating in the Americas since the African slave trade era and is endemic in many tropical regions such as Brazil, Columbia, Venezuela, and Peru to name a few [4]. In the 1930s, a live attenuated vaccine for YFV, 17D, was developed, and in its almost 80 years of use, has proven to have a significant impact on controlling YFV outbreaks [4,5]. Multiple countries have mass vaccination programs and some countries, where YFV is endemic, have the YFV-17D vaccine included in the national recommended childhood immunization schedule. Particularly, Bolivia, Brazil, Columbia, Ecuador and Venezuela all recommend the vaccine to children 9-12 months of age within the entire country, not just in known endemic regions [6]. Despite these recommendations,

recent surveys showed that little more than half of the population in these regions are vaccinated for YFV [7]. With ongoing vaccination campaigns in these areas, there are a spectrum of post-vaccination YFV antibodies, some which might enhance infection of other flaviviruses.

With many flaviviruses co-circulating in the same areas in Central and Southern America, there is the possibility of antigenic cross reactivity, especially since some YFV endemic areas have reported seroprevalence rates of ZIKV as high as 63% [4,8]. Antigenic cross reactivity and antibody mediated enhancement frequently occurs between flaviviruses. Cross-reactive Dengue virus (DENV) and West Nile virus antibodies have already been known to enhance ZIKV pathogenesis [9-11]. However, only limited studies have been conducted on the potential cross-reactive nature of YFV antibodies. One study, using commercial ELISA detection kits for DENV and ZIKV, found there to be minimal cross reactivity between YFV antibodies and DENV detection, and no cross-reactivity in ZIKV detection [12,13]. While these studies were very informative, they do not represent the actual immunological landscape as Souza et al [12] used post vaccination serum from 9-month old infants which have an undeveloped immune system, and the CDC MAC-ELISA for ZIKV was validated using a sample size of less than 10 with individuals of an unknown exposure history [13]. Further, South America, especially Brazil, has a high incidence of measles which can affect immunological memory in recovered persons [14].

This, however, does not indicate possible in vivo interactions as several reports indicate that flaviviral neutralization is complex and dependent upon many factors [15]. It has also been shown that antibodies that neutralize in vitro, like with neutralization assays, often do not neutralize in vivo suggesting that complex immunological interactions occur for neutralization [16-18]. In regions where ZIKV has a high prevalence, a large portion of the population also has YFV antibodies, not only from the childhood schedule of immunizations but also from ongoing vaccination campaigns that inoculate adults and provide boosters for pregnant women, HIV-infected persons, and other immunocompromised populations [19]. With a spectrum of YFV antibodies present in this population, it is important to understand any possible cross-reactivity, antibody mediated enhancement, or antibody mediated neutralization possible.

Studies have reported that vaccination of pregnant women with YFV occurs during vaccination campaigns [20,21]. While several studies have shown vaccination with YFV during pregnancy is safe, the development of protective immunity is reduced indicating that there may be increased non-neutralizing, cross-reactive antibodies [19-21]. Whether YFV vaccination occurs in childhood, adulthood, or during pregnancy, cross-reactive antibodies that complex with other flaviviruses could be a source for enhancement of infection. Further, non-neutralizing antibodies have been shown to contribute significantly to antibody dependent enhancement [22-24].

Since ZIKV infections can cause severe congenital syndromes, it is crucial to understand any potential enhancement or protection offered from cross-reactive antibodies [8]. Studying the vertical transmission of ZIKV has posed some challenges to researchers. Results produced in mice models are difficult to translate directly to a human or non-human primate model since mice placentas are structurally different [25-27]. Ovine and non-human primate models have proved to be promising, but these too have their limitations, such as increased costs, small sample sizes, and being labor intensive [28,29]. To address these roadblocks, recent advances in biotechnology have generated co-culture models that use primary human cell lines and stem cells to replicate cellular interfaces. Co-culture models have been used to simulate the blood brain barrier, the pulmonary barrier, and the maternal fetal axis in nanoparticle translocation studies [30-34].

The transwell co-culture model was utilized in this study to determine if the cross reactivity of YFV antibodies could impact ZIKV pathogenesis in utero during early pregnancy. This in vitro model offers multiple benefits, such as reproducibility, standardization, and excels in simulating the physiological boundary of the maternal fetal axis [35,36]. Maternal syncytiotrophoblasts and fetal

umbilical vein cells (BeWo and HUVEC) were used in our transwell co-culture, following established placental models [34,35,37,38]. The BeWo cell line was derived from a human placenta and best simulates the structure and function of the syncytiotrophoblasts layer of the placenta that forms the continuous outer layer to the placenta [39,40]. These cells directly contact maternal blood and regulate the exchange of nutrients and particles to a developing fetus [25,41,42]. Any virus or antibodies moving across the placental barrier would first have to cross the syncytiotrophoblast layer to reach a fetus, and previous studies have determined translocation rates across a BeWo layer to replicate the rates found in ex vivo placental perfusions [27,43]. Further, we employed a ZIKV isolate derived from human placenta which we felt would be more relevant than utilizing a lab adapted strain with unknown tissue tropism in humans.

We followed Campagnolo et al. (2018) by including a differentiating embryoid body (EB) in the basolateral chamber of our transwell co-culture which mimics an early stage developing embryo [38]. An EB is generated by inducing stem cells to differentiate and self-organize in the three germ layers, endoderm, mesoderm, and ectoderm [44-46]. By including an EB in the co-culture model, we hoped to determine if there were differences in the translocation of virus and/or virus: antibody complexes that cross the placental barrier and infect an EB. Here we describe a co-culture model (Figure 1) which can be utilized to study enhancement or neutralization of virus by maternal antibodies at the maternal fetal axis. We show that YFV post-vaccine antibodies can enhance ZIKV infection of an EB which could impact the development of congenital syndromes.



Figure 1. Experimental model of the trans-well co-culture assay modified from Campagnolo et al. (2018) [38]. Co-cultures of BeWo, HUVEC, and embryoid body (EB) were apically infected with either Zika virus (ZIKV) or ZIKV+YFV (Yellow Fever Virus) antibody.

2. Materials and Methods

2.1. Cell Culture and Virus Propagation

Primary Human Umbilical Vein Endothelial Cells; Normal, Human, Pooled (HUVEC) (ATCC PCS-100-013) were cultured in EndoGRO-MV-VEGF media (MilliporeSigma, Burlington, MA, USA) containing 5% fetal bovine serum (FBS). To promote microvasculature phenotypes commonly expressed in the first trimester with placental expansion and throughout pregnancy, a variety of factors were used including rh VEGF, rh EGF, rh FGF, rh IGF, ascorbic acid, hydrocortisone hemisuccinate, heparin sulfate, and 1X Glutamax per manufacturer's instructions [47,48]. Additionally, human placental cells BeWo (ATCC CCL-98) were cultured in Ham's F-12K (Kaighn's) Medium containing 10% FBS, 1X non-essential amino acids, 1X Glutamax, and 1mM HEPES. Lastly,

Cercopithecus aethiops kidney cell line Vero E6 (ATCC CRL-1586) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, supplemented with penicillin/streptomycin, 1X nonessential amino acids, 1X Glutamax, and 1mM HEPES. All cell lines were incubated at 37°C/ 5% CO₂. ZIKV R103451 was obtained from BEI Resources (Cat. # NR-50355) and expanded once in Vero cells. All Yellow Fever Antiserums were obtained through BEI Resources, (BEIresources.org). Pre-Immune Antiserum (Cat #NR-41782) was taken from a non-human primate prior to immunization, Early Immune Yellow Fever Virus Antiserum (Cat #NR-29335) was collected from a non-human primate 30 days post inoculation with live attenuated yellow fever virus vaccine (17D), Late-Immune Yellow Fever Virus Antiserum (Cat #NR-42576) was collected from non-human primate at 30 day intervals between 120-420 days post inoculation with live attenuated yellow fever virus vaccine (17D) and then pooled, and lastly, Very Late-Immune Yellow Fever Virus Antiserum (Cat #NR-43206) was comprised of pooled serum from non-human primate 420-570 days post inoculation with live attenuated yellow fever Virus Antiserum (Cat #NR-43206) was comprised of pooled serum from non-human primate 420-570 days post inoculation with live attenuated yellow fever Virus Antiserum (Cat #NR-43206) was comprised of pooled serum from non-human primate 420-570 days post inoculation with live attenuated yellow fever Virus Antiserum (Cat #NR-43206) was comprised of pooled serum from non-human primate 420-570 days post inoculation with live attenuated yellow fever Virus Antiserum (Cat #NR-43206) was comprised of pooled serum from non-human primate 420-570 days post inoculation with live attenuated yellow fever Virus Antiserum (Cat #NR-43206) was comprised of pooled serum from non-human primate 420-570 days post inoculation with live attenuated yellow fever Virus Antiserum (Cat #NR-43206) was comprised of pooled serum from non-human primate 420-570 days post inoculation with live attenuated yellow feve

2.2. Embryoid Body Formation and Imaging

Human Induced Pluripotent Stem Cells (ATCC ACS-1019) were cultured in mTeSR1 media (StemCell Technologies, Vancouver Canada) on plates coated with vitronectin XF (Stemcell Technologies, Vancouver, Canada). 100 μ L of undifferentiated ACS-1019 cells were seeded in each well of a 96-well round bottom ultra-low attachment plate at a density of 90,000 cells/mL using a multichannel pipettor to ensure uniformity and reproducibility of Embryoid Body (EB) formation. EB formation media (StemCell Cat#05893) was supplemented with 10 μ M Y-27632 and used for formation as well as culture during the transwell assay. The 96-well plate was incubated at 37°C/5% CO₂ for 48h without being disturbed. On day two and day four of formation, 100 μ L of EB formation medium was gently added to each well. On day five, EBs were observed for uniformity, each with a diameter between 400-600 μ m, and smooth round edges prior to being harvested and proceeding to the transwell assay.

In order to visualize ZIKV infection of EBs, EBs were rinsed in PBS, fixed with Paraformaldehyde Solution 4% in phosphate buffered saline (PBS) (ThermoScientific CAT# J19943-K2) and blocked in 5% lamb serum. Primary antibody staining using microtubule-associated protein 2 (MAP2) (Novus Biologicals, Littleton, CO, USA) to visualize cell structure and Monoclonal Anti-Flavivirus Group Antigen, Clone D1-4G2-4-15 (BEIresources NR-50327) was conducted overnight at 4°C. EBs were rinsed in PBS then incubated with secondary antibodies at room temperature for 1 hour. Secondary antibodies included AlexaFluor 647 and Alexafluor 488 (Invitrogen #A21235 and A11001). EBs were rinsed again and then placed on slides and mounted in ProLong Gold Antifade Reagent with DAPI (Cell Signaling Technology, Danvers, MA, USA catalog #8961S). Cover slips were placed on the slides and then gently pressed down to flatten the EB. Slides were incubated overnight at 4°C. Images were taken with an Olympus Fluoview 3000 confocal microscope and processed using the Olympus Fluoview FV10-ASW 4.1 software package. All images were obtained on the same day using the same imaging parameters (zoom, gain, offset, slices, etc).

2.3. Monolayer Infection and Imaging

Prior to proceeding with the co-culture assay, we verified the permissiveness of BeWo and HUVEC cells to ZIKV as no reports were available to document the susceptibility of these cell lines to flaviviral infection. BeWo and HUVEC monolayers were infected with 1000 infectious units per well. After 48 hrs, samples were fixed with Paraformaldehyde Solution 4% in phosphate buffered saline (PBS) (ThermoScientific CAT# J19943-K2) and blocked in 5% lamb serum. Primary antibody staining with microtubule-associated protein 2 (MAP2) (Novus Biologicals, Littleton, CO, USA) was used to visualize cell structure and anti-flavivirus group antigen, Clone D1-4G2-4-15 4G2 (BEIresources NR-50327) to visualize ZIKV. Staining was conducted overnight at 4°C. Cells were rinsed in PBS then incubated with secondary antibodies at room temperature for 1 hour. Secondary antibodies included AlexaFluor 647 and Alexafluor 488 (Invitrogen #A21235 and A11001). Cells were rinsed and then coverslips were mounted with ProLong Gold Antifade Reagent with DAPI (Cell Signaling Technology, Danvers, MA, USA catalog #8961S) and incubated overnight at 4°C. Images were taken

with an Olympus Fluoview 3000 confocal microscope and processed using the Olympus Fluoview FV10-ASW 4.1 software package.

2.4. Transwell Co-Culture

We followed methods previously described elsewhere **[49,50]**. Briefly, HUVEC cells were seeded on the basolateral side of the Corning 12 mm Trans-well®-COL Collagen-Coated 3.0 µm Pore PTFE Membrane Insert (Corning, NY, USA catalog# 3494) at a concentration of 1.0×10^5 cells per 200µl. The HUVEC monolayer on the basolateral side was achieved using methods described by Aengenheister et al. (2018) **[35]**. Briefly, inserts were inverted into 6 well plates, with 1mL of PBS in one well to ensure enough humidity. Rubber spacers (approximately 1.5mm thick) were placed on the corner of the 6 well plate to lift the lid slightly and prevent direct contact of the lid with the inverted insert. After the basolateral side was seeded with HUVECs and the lid was replaced, there was slight adhesion between the lid and the media. HUVEC seeded inserts were then incubated at 37°C/5% CO2 for 2hrs, and afterwards the inserts were placed back into the 12 well plate containing fresh HUVEC Media. After an insert was replaced in the 12 well plate the apical layer of the membrane was seeded with BeWo cells at a density of 1.5x105 cells per 500µl. Co-cultures were incubated for 72hrs with the media being changed every 48hrs until a 100% confluent layer was observed.

2.5. Transwell Neutralization Assay

Prior to infection, the media in each basolateral chamber was replaced with 1/2 HUVEC media 1/2 EB Formation media. Three EBs were added to the bottom of each well (figure 1). Virus assays using YFV serum were performed using a 1:200 dilution of serum in PBS. 1,000 infectious units of virus in PBS were incubated with serum for 1 hour at 37°C after which BeWo cells were inoculated with the mixture in the apical chamber. Assay controls included treatments of mock infection with PBS, Pre-Immune Antiserum, Early-Immune Antiserum, Late-Immune Antiserum, Very Late-Immune Antiserum, and virus only. To obtain neutralization data, supernatant from BeWo and HUVEC cells was taken at 72 hrs post inoculation and all 3 replicates per cell line were pooled to obtain a single combined solution which was titrated on Vero E6 cells (Figure 2). EBs were separated from the HUVEC supernatant by centrifugation at 400xg for 5 minutes.

EBs were washed with PBS before a second centrifugation at 400xg for 5 minutes, after which they were gently resuspended in PBS. 3 EBs from each replicate were pooled for a total of 9 EBs per treatment for each independent trial. Results of the titration assay are expressed as an average between two independent trials with three replicates for each treatment. A Student's t-test was used for pairwise comparisons between the treatments. All virus:antibody assays were compared against the ZIKV only assay.



Figure 2. Transwell neutralization assay. Supernatant from BeWo and HUVEC cells was taken at 72 hrs post inoculation and all 3 replicates per cell line were pooled to obtain a single combined solution which was titrated on Vero E6 cells. EBs were separated from the HUVEC media and washed in PBS. 3 EBs from each replicate were pooled for a total of 9 EBs per treatment for each independent trial. This figure was modified based on the original illustration by Campagnolo et al. 2018 [38].

Plaque assays were performed using pooled supernatant samples from each treatment following a method described previously [51]. EBs were separated as described above, and vigorously triturated to disassociate cells. Briefly, serial dilutions of culture supernatant or EBs in PBS were inoculated onto confluent Vero E6 cells and covered with 0.25% methylcellulose overlay. Overlay was removed after three days and cells were fixed and stained with 5% acetic acid, 43% ethanol, 50% methanol and 0.2% wt/vol Coomassie Brilliant Blue R-250 prior to counting plaques. Pairwise comparisons between virus:antibody treatments and ZIKV only were performed using a Student's t-test.

3. Results

3.1. Maternal and Fetal Placental Cells are Permissive to ZIKV Infection



Figure 3. Monolayer infection of BeWo and HUVEC cells with ZIKV viewed under 20x magnification. (A) BeWo monolayer stained 48 hrs post inoculation with ZIKV. (B) HUVEC monolayer stained 48 hrs post inoculation. (Blue=DAPI, Green=MAP2, Pink= ZIKV: 4G2).

Clinical reports indicate that placental pathology is minimal for ZIKV congenital infections and primarily involves reduced placental weight [52-54]. Reports have shown that ZIKV causes autophagy, apoptosis and other forms of CPE in HUVEC cells which is contrary to clinical data [55,56]. ZIKV has been detected in BeWo

cells also but investigations regarding CPE was limited [57,58]. Further these studies did not employ the placental isolate used here which was derived from the placenta of an infant with microcephaly which could impact observations [59]. The purpose of this experiment was to determine if our ZIKV strain was permissive to HUVEC and BeWo cells and that it would not induce excessive cytopathic effects that might interfere with the experiment. Monolayers of both BeWo and HUVEC cell lines were infected with ZIKV and infection visualized using the anti-ZIKV D1-4G2-4-15 antibody. Staining determined that both BeWo and HUVEC cell lines are permissive to ZIKV infection (Figure 3). This aligns with previous research showing the placental trophoblasts and endothelial cells to be permissive to ZIKV infection [60-63] There were no noticeable cytopathic effects within BeWo and HUVEC cell lines when infected with ZIKV (Figure 3).

3.2. Transwell Neutralization Assay

At 72hrs post inoculation (p.i.), ZIKV was detected via viral plaque assay. Very Late Antiserum significantly increased the amount of infectious ZIKV present in BeWo cells at 72hrs post inoculation as compared to when no Anti-serum was present in the transwell assay (p=0.033) (Table 1, Figure 4). The presence of YFV antibodies, regardless of time the antiserum was taken post vaccination, did not have any significant impact on ZIKV viral titers or Ct-values in the HUVEC cell line (Table 1). EBs had significantly increased levels of infectious ZIKV at 72hrs p.i. when Early and Late YFV Anti-serum were used as when compared to no anti-serum present (p=0.0294, p=0.000762) (Table 1). Imaging studies of the EB verified that ZIKV could be detected throughout the EB and CPE was inapparent in all treatments (Figure 4). The variation in size observed reflects that normal variance of size when EBs are generated in the manner we employed [64].

	ZIKV	ZIKV+Pre	ZIKV+Early	ZIKV+Late	ZIKV+Very Late
BeWo	$2.02^{\log^5} \pm 2.4^{\log^4}$	$2.13 \log^5 \pm 4.3 \log^4$	$2.3^{\log^5} \pm 3.7^{\log^4}$	$2.19^{\log^5} \pm 1.4^{\log^4}$	$2.34 \log^5 \pm 2.2 \log^4$
HUVEC	$2.11 \log^{5} \pm 1.6 \log^{3}$	$1.11 \log^5 \pm 6 \log^4$	$1.61 \log^5 \pm 8 \log^4$	$1.84^{\log{5}} \pm 5.5^{\log{4}}$	$1.98 \log^5 \pm 6.8 \log^4$
EB	$1.3 \log^3 \pm 3.9 \log^2$	$6.9 \log^3 \pm 5.3 \log^3$	$9.8 \log^3 \pm 8.8 \log^2$	$2.2^{\log^4} \pm 4.1^{\log^2}$	$1.2^{\log^4} \pm 3.2^{\log^3}$



Table 1. Viral titration of ZIKV in BeWo, HUVEC, and EBs 72hr p.i. Data provided signifies average \log titer ± standard error of 2 independent trials with 3 replicates each. Significant increases in ZIKV titers were seen in BeWo cells when Very Late YFV anti-serum was present when compared to ZIKV only (p=0.0334), and in EBs

when Early (p= 0.0294) and Late (p=0.000762) YFV anti-serum was present when compared to ZIKV only. Bold font indicates statistical significance.

Figure 4. Immunofluorescence of EBs. (Blue=DAPI, Green=MAP2, Red= ZIKV: 4G2). EBs were stained with D1-4G2-4-15 antibody and mounted on glass slides. Coverslips were pressed down on the EBs to flatten them, producing a ring effect in most images. Scale bar represents 100uM.

4. Discussion

Here, we present data showing that ZIKV derived from the human placenta can infect both BeWo and HUVEC cells and replicate without out causing excessive CPE. ZIKV has been known to infect placental tissue in humans as well as in animal models, and it can cross the placental barrier to infect a developing fetus to cause congenital syndromes **[11,28,65-67]**. The data in this study shows that within 72hrs p.i., ZIKV effectively crosses two monolayers, a basement membrane of placental collagen, and infects an EB. The detection of ZIKV in cells located on each side of the membrane as a model for the maternal: fetal axis supports reports of isolation of ZIKV from placentas and fetuses **[28,53,68,69]**. However, the permissiveness of cells observed in this study may not reflect the cellular tropism of ZIKV in actual placentas **[70-73]**.

Pre-Vaccination sera was collected prior to vaccination with the 17D YFV vaccine, Early serum was collected 30 days p.i., Late serum was collected at 30 days intervals between 120-420 days p.i., and lastly, Very Late serum was comprised of pooled serum from 420-570 days p.i.. It has been shown that broadly neutralizing IgM antibodies quickly appear after vaccination with the 17D vaccine, typically 4-7 days p.i., and have been found to circulate anywhere between 2-11 years p.i. [74,75]. IgM antibodies are broadly reactive while IgG antibodies are associated with a higher antigenic specificity and better immune responses. IgG antibodies take longer to appear after vaccination, usually 28-42 days p.i. with the 17D vaccine, IgM is present in significantly higher titers than IgG [75]. This could explain why there was a significant enhancement of ZIKV infection in EBs when Early and Late-Immune YFV-Antiserum were present. These differences are important to consider when analyzing why some sera in this study enhanced while others did not. IgG antibodies have Fc regions and can readily cross the placenta due the FcRn receptor on placental syncytiotrophoblasts and endothelial cells and have already been found to be endocytosed by the BeWo cell line [76-79]. By the time Early and Late antiserum was collected, both IgM and IgG antibodies would have been abundant, suggesting why ZIKV enhancement was observed in BeWo and EBs.

Anti-DENV antibodies have been shown to enhance ZIKV infection in multiple model systems and case studies [9,11,79-81]. Due to the genetic similarities between flaviviruses it would be expected that antibodies for other flaviviruses, such as YFV, may also contain the ability to enhance ZIKV infection. In this study, EBs had enhanced ZIKV infection along with lower viral loads as compared to the placental cell lines. This supports reports of ZIKV titers in fetal and placental tissues in vivo [28,68,69]. Since macrophages or other monocytes were not used in this study, the enhancement we observed was likely due to antibody mediated enhancement (AME). Like ADE, AME occurs when antibodies bind to virus particles forming complexes. These complexes interact with cell surface receptors and promote entry into host cells leading to increased levels of viral replication via suppression of innate immune processes and inflammatory cascades [23,82]. While this process is associated with Fc receptor-bearing monocytes, it is also possible for these virus–antibody complexes to infect other cell types and suppress innate immunity [23,82].

A limitation to this study was the omission of Hofbauer cells from the model. Hofbauer cells have been found to play a role in ZIKV transmission through the placental barrier [79,83-85]. They are placental macrophages that also have Fc receptors, play a role in early angiogenesis within trophoblast cells, and have been found to transfer viral particles into the fetal endothelial cells and blood supply [77,86-88]. Since they have been found to further facilitate vertical transmission of ZIKV,

not including them in the transwell co-culture assay limits the findings within this study. DENV antibodies have already been found to enhance ZIKV infection within Hofbauer cells, and as such not including them could have impacted the results [79].

5. Conclusions

Due to the already established cross reactive nature of flavivirus antibodies, it is crucial to understand the interactions of neutralizing and enhancing antibodies as vaccine research continues for many of these viruses [9,10,89,90]. The 17D vaccine for YFV has been included in the recommended vaccinations in most Latin American countries and ongoing vaccination campaigns are vaccinating more adults, creating a population of people with potentially cross-reactive antibodies [4-6,12,75]. Additionally, in regions of Latin America where these viruses co-circulate due to a common vector, *Aedes* mosquitos, many people have the potential to become co-infected or infected by different flaviviruses within their lifetime [1,4,8,84]. Further research about the cross-reactive nature of flavivirus antibodies is needed, especially because vertically transmitted viral infections can lead to congenital syndromes. More studies are also needed to better understand possible antibody passage through the transwell co-culture model, and to better understand possible antibody mediated enhancement of ZIKV by YFV antibodies produced after receiving the 17D vaccine especially in pregnant women who may be receiving a booster or receiving vaccination during campaigns.

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