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 innoculated with ZIKV or ZIKV incubated with YFV vaccine anti-sera, and viral load was measured 72 hours post inoculation. The data show that the impact of YFV post-vaccination antibodies on ZIKV replication is cell line dependent. In differentiating embryoids, the presence of YFV antibodies enhanced ZIKV infection. Since viral pathogenesis, and the impact of antigenic cross-reactive antibodies, is cell line specific at the maternal-fetal axis, this suggests there may be discreet mechanisms that impact congenital ZIKV infections. Since ZIKV infections can cause

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severe congenital syndromes, it is crucial to understand any potential enhancement or protection offered from cross-reactive antibodies

Keywords: Zika virus, yellow fever virus, flavivirus, cross-reactivity, neutralization, enhancement, congenital infections

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* mosquitos. These viruses have been found to co-circulate within the same regions of Latin America [1]. Both YFV and ZIKV originated in Africa. 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 effect 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 has YFV antibodies

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not only from the childhood schedule of immunizations but also 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 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.

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 [22-24]. 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 [25, 26]. 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 [27-31].

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 [32, 33]. Maternal syncytiotrophoblasts and fetal umbilical vein cells (BeWo and HUVEC) were used in our transwell co-culture, following established placental models [31, 32, 34, 35]. 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 [36, 37]. These cells directly contact maternal blood and regulate the exchange of nutrients and particles to a developing fetus [22, 38, 39]. 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 [24, 40].

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We also 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 [35]. An EB is generated by inducing stem cells to differentiate and self-organize in the three germ layers, endoderm, mesoderm, and ectoderm [41-43]. By including a differentiating 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 vaccine antibodies can enhance ZIKV infection of an EB which could impact the development of congenital syndromes.

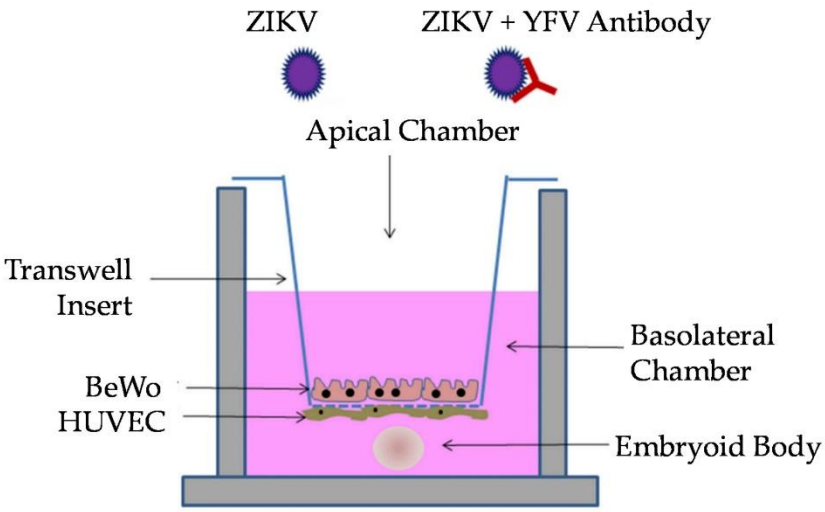

Figure 1. Experimental model of the trans-well co-culture assay modified from Campagnolo et al. (2018) [35]. Co-cultures of BeWo, HUVEC, and embryoid body (EB) were apically infected with either Zika virus (ZIKV) or ZIKV+YFV (Yellow Fever Virus) antibody.

2. Material 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 [44, 45]. Additionally, human placental cells BeWo (ATCC CCL-98) were cultured in Ham's F-

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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 non-essential amino acids, 1X Glutamax, and 1mM HEPES. All cell lines were incubated at 37°C/ 5% CO_2. 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 vaccine (17D).

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). 100µL 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 10µM Y-27632 and used for formation as well as culture during the transwell assay. The 96-well plate was incubated at 37°C/ 5% CO_2 for 48h without being disturbed. On day two and day four of formation, 100µL of EB formation medium was gently added to each well. On day five, EBs were observed for uniformity, each with a diameter between 400-600µm, and smooth round edges prior to being harvested and proceeding to the transwell assay.

72 hrs after infection, images were taken of each EB *in situ* to document changes in growth when infected. The area (μm^2) of each EB was determined using the ECHO REBEL microscope at 4X magnification with the integrated software. The areas of all technical replicates were pooled, and a Student's t-test was performed to determine significance between treatments.

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 MAP2 (Novus Biologicals, Littleton, CO, USA) and ZIKV/flavivirial monoclonal 4G2 was conducted overnight at 4°C. EBs were rinsed in PBS then incubated with secondary antibodies at room temperature for 1 hour. 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 cured overnight at 4°C. Images were taken with an Olympus Fluoview 3000 confocal microscope and processed using the Olympus

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Fluoview FV10-ASW 4.1 software package. Average relative fluorescent units (RFU) were calculated by averaging 5 randomly generated 250X250 pixel boxes over the image.

2.3. Monolayer Infection and Imaging

Monolayers of BeWo and HUVEC cells 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 using MAP2 (Novus Biologicals, Littleton, CO, USA) and ZIKV/flavivirial monoclonal 4G2 was conducted overnight at 4°C. Cells were rinsed in PBS then incubated with secondary antibodies at room temperature for 1 hour. Cells were rinsed and then coverslips were mounted with ProLong Gold Antifade Reagent with DAPI (Cell Signaling Technology, Danvers, MA, USA catalog #8961S) and cured 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

Corning 12 mm Trans-well®-COL Collagen-Coated 3.0 µm Pore PTFE Membrane Insert (Corning, NY, USA catalog# 3494) were seeded with HUVEC cells on the basolateral side of the insert at a concentration of $1.0x10^5$ cells per 200µl, and BeWo cells were seeded on the apical side of the insert at a density of $1.5x10^5$ cells per 500µl. The HUVEC monolayer on the basolateral side was achieved using methods described by Aengenheister et al. (2018) [32]. 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. 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. Multiple EBs were added to the bottom of each well (figure 1). Virus enhancement 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. Supernatant (BeWo and

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HUVEC) and EB samples were taken at 72 hrs post inoculation and pooled amongst technical replicates. EBs were separated from HUVEC supernatant by centrifugation at 400xg for 5 minutes. The supernatant was aspirated and EBs were washed with PBS before a second centrifugation at 400xg for 5 minutes, after which they were gently resuspended in PBS. Results are expressed as an average between two independent trials with three replicates for each treatment. A Student's t-test with a Turkey post-hoc test was used for pairwise comparisons between relative treatments.

2.6. Viral Quantification

Plaque assays were performed using pooled supernatant samples from each treatment following a method described previously [46]. EBs were separated as described above, and vigorously titurated 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. After three days, the overlay was removed, 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. Viral RNA was extracted using a kit in accordance to the manufacturer's instructions (Zymo Quick Viral RNA kit #R1034). Quantitative real-time PCR, performed on all supernatant samples taken from all treatments at each time point using Verso One-Step RT-qPCR Kit, SYBR Green, ROX (Thermo Fisher), and primers designed against the envelope gene [47]. A non-template control was used to normalize the RT-qPCR results. Pairwise comparisons between treatments were performed using raw Ct values with Student's t-test with a Tukey post-hoc test.

3. Results

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

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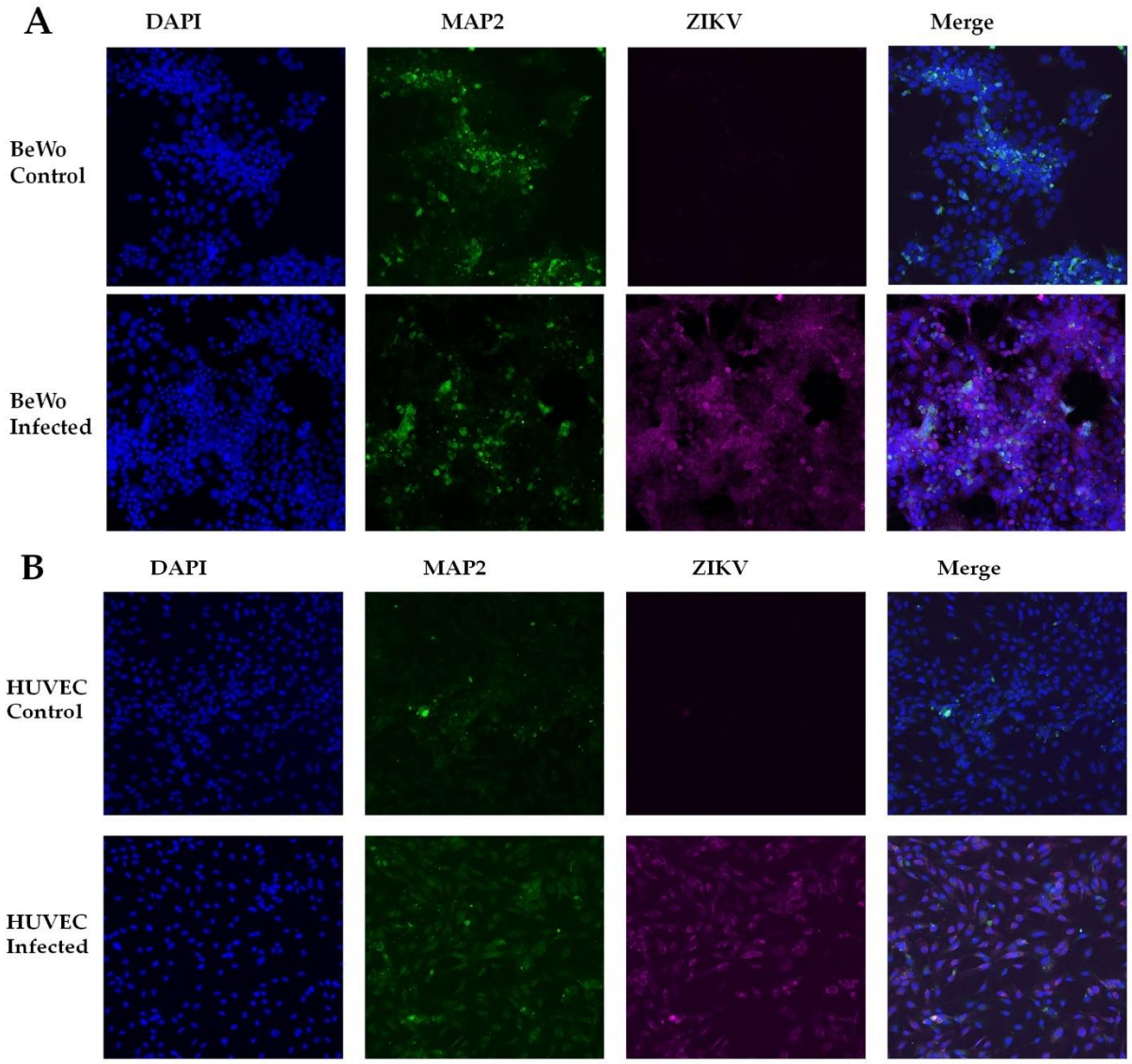

Figure 2. Monolayer infection of BeWo and HUVEC cells with ZIKV. (**A**) BeWo monolayer stained 48 hrs post inoculation with ZIKV. (**B**) HUVEC monolayer stained 48 hrs post innoculation. (Blue=DAPI, Green=MAP2, Pink= ZIKV).

Monolayers of both BeWo and Huvec cell lines were infected with ZIKV and infection visualized using the anti-ZIKV 4G2 antibody. Staining determined that both BeWo and HUVEC cell lines are permissive to ZIKV infection (Figure 2). This aligns with previous research showing the placental trophoblasts and endothelial cells to be permissive to ZIKV infection [48-51] There were no noticeable cytopathic effects within BeWo and HUVEC cell lines when infected with ZIKV (Figure 2).

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3.2. Transwell Neutralization Assay

At 72hrs post inoculation (p.i.), ZIKV was detected via RT-PCR and viral plaque assay. Very Late Antiserum significantly increased the amount of infectious ZIKV present in BeWo cells at 72hrs post innoculation as compared to when no Anti-serum was present in the transwell assay (p=0.033) (Figure 3A). However, there were no other siginificant differences in the amount of infectious ZIKV or ZIKV genomic material present in BeWo cells (Figure 3B). 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 (Figure 3). EBs had significantly incresed 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)(Figure3A). When evaluated by RT-PCR, there was significantly more ZIKV RNA when Pre-Vaccination YFV antibodies were present when compared to ZIKV only (p=0.00084) (Figure 3B). When Early YFV antibodies were present there was a significant decrease in ZIKV RNA as opposed to when there was no Anti-serum present (p=0.0398) (Figure 3B). In all three cell lines there was no correlation between viral titers and the amount of ZIKV RNA detected by RT-PCR for any of the YFV antibody treatments.

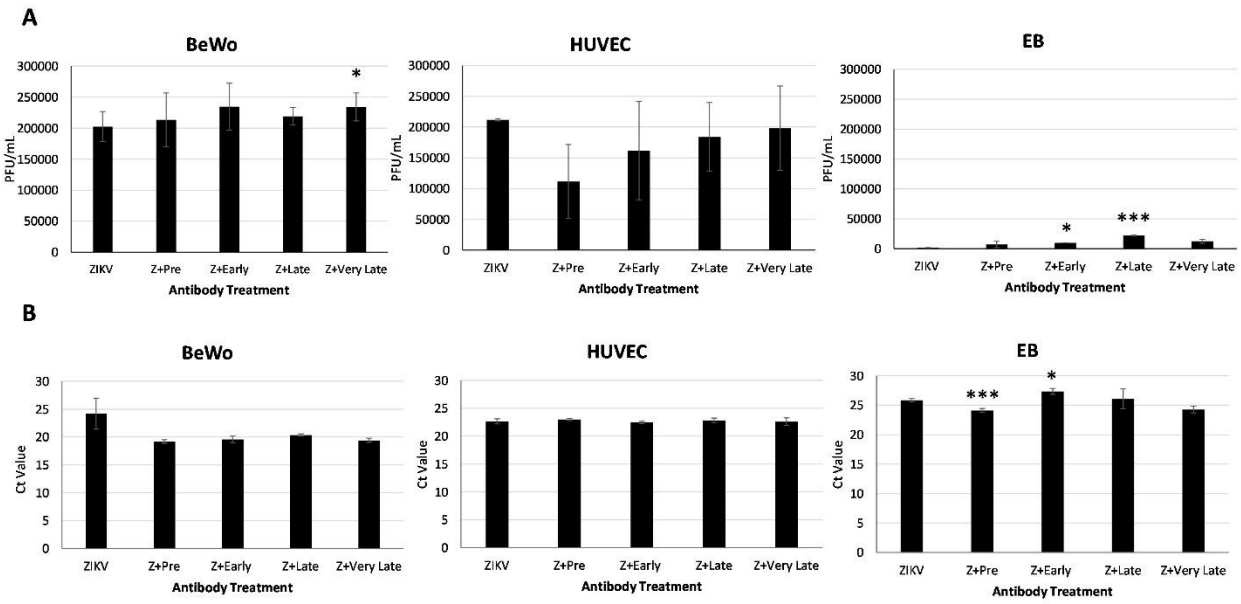

Figure 3. Viral quantification by viral plaque assay and RT-PCR of ZIKV in BeWo, HUVEC, and EB co-cultured in the transwell. (**A**) Viral titration of ZIKV in BeWo, HUVEC, and EBs cell lines 72hr p.i.. Significant increases in ZIKV titers were seen in BeWo cells when VL YFV anti-serum was used when compared to ZIKV only (p=0.0334), and in EBs when Early (p= 0.0294) and Late (p=0.000762) YFV anti-serum was used. (**B**) A significant increase in ZIKV genomic material was decteded by RT-PCR in EBs when Pre-vaccination antibodies were present when compared

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with ZIKV only (p=0.00084). Significant decrease in ZIKV RNA occurred in EBs in the presence of Early YFV anti-serum as compared to ZIKV only (p= 0.0398).

When immunofluorescence staining was conducted on EBs, it was observed that there were significantly more RFU for ZIKV in EBs 72hrs p.i. when Early, Late, and Very-Late YFV antiserum was present as compared to when no anti-serum was present (p=0.015, p=0.009, p=0.004) (Figure 4). RFU for ZIKV when pre-vaccination antibodies were present were comparable to ZIKV only (p= 0.308).

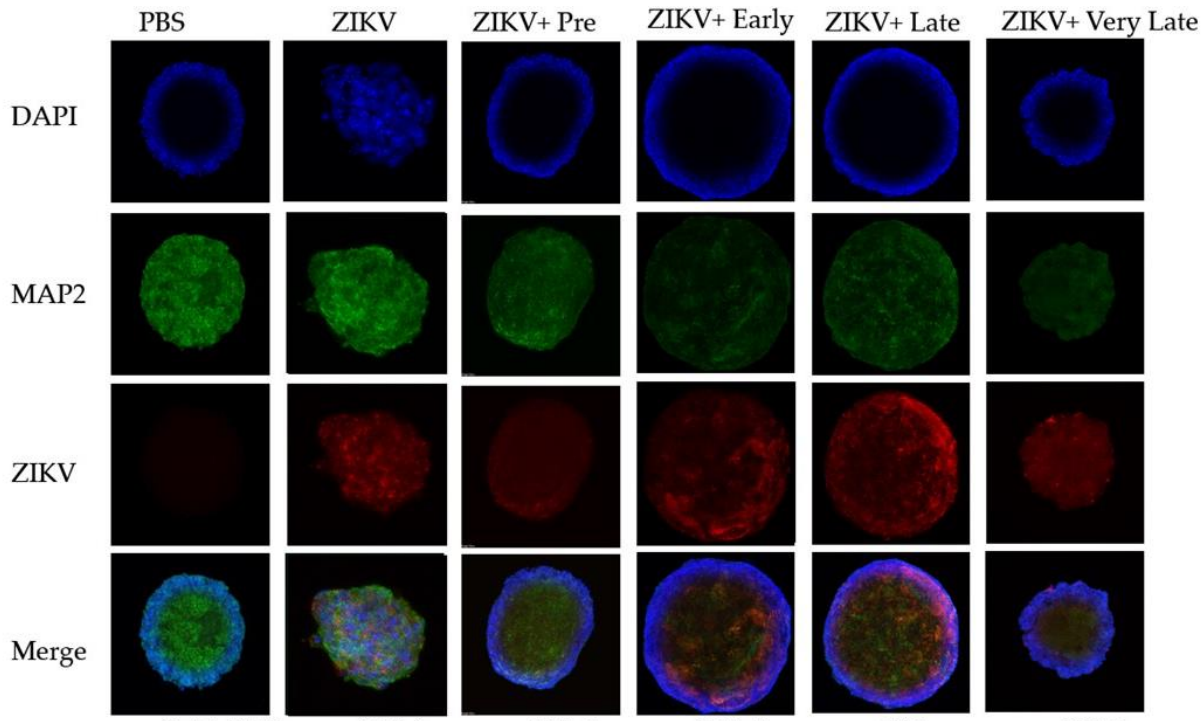

Figure 4. Immunofluorescence of EBs. (Blue=DAPI, Green=MAP2, Red= ZIKV). EBs were stained with 4G2 antibody and mounted on glass slides. Coverslips were pressed down on the EBs to flatten them, producing a ring effect in most images. RFU: Relative flourescence units.

4. Discussion

ZIKV can infect both maternal placental cells (BeWo), as well as umbilical endothelial cells (HUVEC), and EBs. 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, 25, 52-54]. 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

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fetuses [25, 55-57]. However, the permissiveness of cells observed in this study may not reflect the cellular tropism of ZIKV in actual placentas [58-61]. The data in this study, obtained using a transwell-co culture model, shows that within 72hrs p.i., ZIKV effectively crosses two monolayers and a basement membrane of placental collagen and infects an EB. Whether infection of the basolateral side of the membrane was due to cell: cell contact, or virus escape into the basolateral media is not known. Clearly, more investigation into the kinetics involved with ZIKV crossing these barriers needs to occur.

ZIKV was detected at 72 hrs p.i. by viral titration and RT-PCR for each cell line involved in the transwell co-culture when YFV antibodies were present. Four different YFV-Anti-sera were used to determine if the time post-vaccination with the 17-D YFV vaccine impacted ZIKV infection. The impact the YFV anti-serum had on ZIKV propagation was cell line dependent and dependent on the YFV anti-serum type. ZIKV infection was enhanced at 72hrs p.i. in maternal syncytiotrophoblasts when Very Late YFV Anti-serum was present. ZIKV infection was also enhanced in EBs 72hrs p.i. when Early and Late YFV anti-aerum was used. Viral titration results did not correlate with the RT-PCR data, likely because having just one more RNA copy can cause significant differences between Ct values due to exponential increases during amplification cycles. For example, there was no significant difference in ZIKV genomic copies in EBs when Late YFV Anti-serum was present, yet there was significant enhancement displayed in viral titers.

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. [62, 63]. 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, and for 28-42 days p.i. with the 17D vaccine, IgM is present in significantly higher titers than IgG [63]. This could explain why there was a significant enhancment 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 [64-67]. By the time Early and Late antiserum was collected, both IgM and IgG antibodies would have been abundant, suggesting why ZIKV enhancment 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, 67-69]. 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 [25, 55, 57]. 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 [70, 71]. 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 [70, 71].

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[67, 72-74]. 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 [65, 75-77]. 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 [67].

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, 78, 79]. 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, 63]. 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, 73]. Further research about the cross-reactive nature of flavivirus antibodies is needed, especially because vertically transmitted viral infections lead to congenital syndromes. More studies are also needed to better understand the kinetics of 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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Authors' contributions

KLB and ERS conceived and designed the experiments. KLB, ERS, and TJJ performed experiments and analyzed data. ERS drafted the manuscript and all other authors edited and approved the text.

Conflict of Interest

The sponsors had no role in the design, execution, interpretation, or writing of the study.

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