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Zika virus-related neurotropic flaviviruses infect human placental explants and cause fetal demise in mice

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Abstract

Although Zika virus (ZIKV) infection in pregnant women can cause placental damage, intrauterine growth restriction, microcephaly, and fetal demise, these disease manifestations only became apparent in the context of a large epidemic in the Americas. We hypothesized that ZIKV is not unique among arboviruses in its ability to cause congenital infection. To evaluate this, we tested the capacity of four emerging arboviruses (West Nile virus, WNV; Powassan virus, POWV; chikungunya virus, CHIKV; and Mayaro virus, MAYV) from related (flavivirus) and unrelated (alphavirus) genera to infect the placenta and fetus in immunocompetent, wild-type mice. Whereas all four viruses caused placental infection, only infection with the neurotropic flaviviruses (WNV and POWV) resulted in fetal demise. WNV and POWV also replicated efficiently in second-trimester human maternal (decidua) and fetal (chorionic villi and fetal membrane) explants whereas CHIKV and MAYV replicated less efficiently. In mice, RNA *in situ* hybridization and histopathological analysis revealed that WNV infected the placenta and fetal central nervous system, causing injury to the developing brain. In comparison, CHIKV and MAYV did not cause

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substantive placental or fetal damage despite evidence of vertical transmission. Based on the susceptibility of human maternal and fetal tissue explants and pathogenesis experiments in immunocompetent mice, other emerging neurotropic flaviviruses may share with ZIKV the capacity for transplacental transmission, and subsequent infection and injury to the developing fetus.

One Sentence Summary:

West Nile and Powassan viruses cause fetal demise in mice and replicate in human placental tissue.

INTRODUCTION

Zika virus (ZIKV) is a neurotropic flavivirus that belongs to the *Flaviviridae* family of RNA viruses, which includes several mosquito- (*e.g.*, Japanese encephalitis (JEV) and West Nile (WNV) viruses) and tick- (*e.g.*, Powassan (POWV, also known as Deer Tick virus) transmitted viruses. Although ZIKV was first identified more than 70 years ago (1), its ability to cause congenital infection and fetal malformations was discovered only after the explosive 2015 epidemic in South America, where more than 1.5 million people were infected (2). Some have speculated that genetic evolution of ZIKV may have contributed to some of the new clinical manifestations identified in the recent outbreaks (3, 4). Indeed, the emergence of a mutation in the prM gene (S139N) in post-epidemic strains was associated with greater infectivity and injury of neuroprogenitor cells (5). Another view is that congenital infection by ZIKV may have occurred historically but that the association between infection and microcephaly could be detected only in the context of a large epidemic (6).

We and others previously demonstrated that ZIKV infection can cause intrauterine growth restriction, microcephaly, and fetal demise in mice (7–10). Because ZIKV replicated inefficiently in immunocompetent wild-type (WT) mice, most studies in pregnant mice have used animals with genetic or acquired deficiencies in type I interferon (IFN) receptor signaling (7, 8, 10). Alternatively, injection of WT dams via intravenous or intrauterine routes with high doses of ZIKV can bypass peripheral immunity, resulting in transplacental transmission and fetal injury (9, 11). In comparison, there have been sporadic reports of congenital WNV infection in humans (12–14), and WNV is reportedly transmitted transplacentally in mice (15), although fetal demise, pathology and viral tropism have not been assessed. Moreover, WNV has not been compared directly to other arboviruses in human placental models or in mouse models of congenital infection.

WNV and POWV can cause severe neuropathology and death in humans (16, 17). The enzootic cycle of WNV is between *Culex* species of mosquitos and birds, with humans being incidental dead-end hosts. WNV has caused annual outbreaks in the United States since 1999, but these have been relatively small in comparison to the 2015 ZIKV epidemic. For example, there have been only ~50,000 documented human cases of WNV in the last 17 years, with an estimated seroprevalence of ~2 to 3 million (18). POWV is an emerging tick-borne flavivirus that is endemic to the Western Hemisphere. As with WNV, humans are a

dead-end host for POWV, which circulates in enzootic cycles between *Ixodes* ticks and rodents. POWV infections in humans are relatively rare with only ~100 encephalitis cases reported in the United States since 2003 (19). However, there are concerns about the potential for increasing numbers of infections in humans, given the growing numbers of deer testing positive for POWV in the United States (20, 21), a phenomenon that may be due to an expanding tick population (22).

Alphaviruses are divided into New World Alphaviruses, which typically cause encephalitis (e.g., Venezuelan equine encephalitis virus) and Old World Alphaviruses (e.g., chikungunya (CHIKV), Ross River (RRV), and Mayaro (MAYV) viruses), which cause inflammatory arthritis. Arthritogenic Old World alphaviruses also can cause encephalitis, but this occurs primarily in neonates (23). Between March 2005 and December 2006, a widespread outbreak of CHIKV occurred on the La Réunion Island in the Indian Ocean, infecting over one-third of the island population, causing at least 9 confirmed cases of neonatal encephalitis (23). Although some reports suggested that perinatal transmission of CHIKV may have occurred in the birth canal (24, 25), the presence of CHIKV RNA in the human placenta was confirmed in one case (26), although the cell type(s) targeted by the virus was not evaluated. MAYV is another emerging arthritogenic alphavirus that was first identified in forest workers in Trinidad in 1954 (27) but is now spreading in South and Central America (28). The capacity of CHIKV and MAYV to infect fetuses has not been studied in any experimental model to the best of our knowledge.

Here, we tested whether WNV, POWV, CHIKV, and MAYV cause congenital infection and placental and fetal pathology *in utero* in immunocompetent mice. Whereas all four viruses caused varying degrees of placental infection and subsequent transmission to the fetus, only WNV and POWV, the ZIKV-related neurotropic flaviviruses, resulted in significant fetal injury and demise. *Ex vivo* viral replication studies in second trimester human placental explants revealed similarly efficient replication of WNV, POWV, and ZIKV in both maternal and fetal tissues, whereas CHIKV and MAYV replicated much less efficiently. Thus, multiple neurotropic flaviviruses appear to have the capacity to infect human mid-gestation maternal and fetal tissues and to cause fetal demise in mice.

Results

WNV and POWV cause transplacental infection and fetal demise in mice

We chose to study the potential for congenital infection by two encephalitic flaviviruses (WNV and POWV) and two alphaviruses (CHIKV and MAYV), which have emerged in the Western Hemisphere. CHIKV and MAYV were selected because they have caused outbreaks in regions affected by ZIKV. Furthermore, CHIKV can cause perinatal encephalitis in newborns (24, 25), suggesting a possibility of fetal and/or perinatal transmission. To determine whether these arboviruses can cause congenital infection in mice, immunocompetent wild-type (WT) C57BL/6J dams were inoculated subcutaneously at embryonic day 6.5 (E6.5) with WNV (10^2 focus-forming units, FFU), POWV (10^3 FFU), CHIKV (10^3 FFU), or MAYV (10^3 FFU) and euthanized at E13.5 or E18.5 as previously described (7). Since WNV and POWV can cause morbidity and mortality in some mice by day 10 after infection (**Fig. S1A**), we harvested fetuses only from healthy-appearing dams

and euthanized any pregnant dams exhibiting hunched posture or lethargy. Similar to studies of congenital ZIKV infection (7, 29), we inoculated mice on E6.5 to allow time for peripheral viral replication so that peak viremia would coincide with placentation. However, to confirm that congenital infection occurs even after placentation, we also performed separate experiments with the neurotropic flaviviruses (WNV and POWV) where we inoculated dams subcutaneously on E9.5 (**Fig. S1B-D**).

On E13.5 after inoculation on E6.5, we observed viral RNA (vRNA) from all four arboviruses in the placenta and fetal head, with the most severe infection of fetal tissues occurring with WNV (~23 to 1,500-fold higher vRNA levels than POWV, CHIKV, and MAYV in the placenta, $P < 0.05$ and ~3 to 16,000-fold higher vRNA levels than POWV, CHIKV, and MAYV in the fetal head, $P < 0.01$) (Fig. 1A and B). In comparison, in pregnant dams, we detected similar viral titers ($P > 0.1$) in the maternal spleen on E13.5 (Fig. 1C), suggesting that the differences in placental and fetal infection among viruses were unlikely to be related to differences in maternal viral burden. At E18.5, we saw fetal demise in approximately 50% of flavivirus-infected animals (WNV and POWV), whereas no fetal lethality was observed in dams infected with the alphaviruses (CHIKV and MAYV) (Fig. 1D). Thus, both ZIKV-related neurotropic flaviviruses caused fetal demise, whereas the alphaviruses caused no gross fetal pathology despite a similar magnitude of infection in the placenta, at least for CHIKV. Consistent with the range of vRNA levels in WNV-infected fetuses (Fig. 1B), we observed variability in fetal size and resorption, even among fetuses from the same pregnant dam (Fig. 1E). Subcutaneous inoculation with WNV or POWV at a later gestational date (E9.5) similarly resulted in transplacental transmission (**Fig. S1**). WNV and POWV infection induced IFN-stimulated genes (ISGs, e.g., *Ifit1*, *Ifit2*, and *Ifitm3*) in fetuses and placentas to a greater extent than CHIKV and MAYV (~10 to ~100-fold higher expression of *Ifit1* and *Ifit2* with WNV and POWV than CHIKV, $P < 0.01$) (Fig. 1F), suggesting a more robust fetal immune response, possibly due to the greater viral burden or differences in viral mechanisms of immune evasion. Collectively, these results suggest that ZIKV is not unique among flaviviruses in its capacity to be transplacentally transmitted at different gestational stages to elicit fetal demise in mice.

Treatment of ZIKV-infected pregnant dams with a neutralizing human monoclonal antibody (mAb) protects the developing fetus from infection and demise (30). To determine whether therapeutic mAbs analogously protect against congenital WNV infection, we treated dams at day E8.5, two days after inoculation of WNV, with a neutralizing humanized anti-WNV mAb (E16) that binds the envelope protein (31). Therapy with E16 reduced viral titers in the placenta (~25-fold, $P < 0.05$) and fetal head (~1,000-fold, $P < 0.001$) compared to the isotype control mAb (Fig. 1G) and prevented fetal demise (62% vs 100% survival, $P < 0.0001$) (Fig. 1H), demonstrating that transplacental WNV infection and fetal death can be prevented by therapeutic mAbs.

WNV infects and damages the placenta and developing fetal brain

Histopathological analysis revealed that WNV-infected placentas were smaller than mock-infected or CHIKV-infected placentas (Fig. 2A-D), similar to that seen during congenital ZIKV infection (8). However, we did not observe evidence of severe damage, necrosis, or

loss of structural integrity, as the junctional and labyrinth layers remained intact in CHIKV- or WNV-infected placentas (Fig. 2A-C). This finding contrasts with the more severe placental damage observed after congenital ZIKV infection of *Ifnar1*^{-/-} dams, which may reflect ZIKV-specific effects on the placenta or greater infection of the immunodeficient *Ifnar1*^{-/-} maternal decidua (7).

Congenital ZIKV infection causes apoptosis of neural progenitor cells within the developing fetal brain (32). To determine whether CHIKV or WNV congenital infection caused fetal brain injury, we performed histopathological analysis of fetuses on E18.5. Fetuses from CHIKV-infected dams exhibited no evidence of brain damage compared to mock-infected control animals (Fig. 3A and B). In contrast, WNV caused substantial damage to the developing brain, resulting in hypocellularity of the cerebral cortex in mild cases and frank necrosis of the fetal brain in severe cases (**Fig. 3C and D, necrosis indicated by red arrows**).

We next evaluated the tropism of WNV in the mouse placenta and fetus using RNA *in situ* hybridization (ISH) on E13.5. WNV RNA staining was apparent throughout the junctional zone and umbilical cord, with less prominence in the maternal decidua and labyrinth layer of the placenta (**Fig. 4A and B**). In the fetus, we found high levels of WNV RNA staining in the forebrain, hindbrain, and spinal cord of congenitally infected animals (Fig. 4C-F). Consistent with a range in severity of viral burden measured by qRT-PCR (Fig. 1B), some fetuses were more severely infected than others. In two of six fetuses, we observed WNV RNA in the heart, liver, and gastrointestinal tract of the developing fetus (**Fig. 4C and G, black arrows**). In the remaining four fetuses, which were less severely infected, WNV RNA was limited mostly to the brain and spinal cord (**Fig. 4C, middle panel**).

Neurotropic flaviviruses replicate efficiently in human mid-gestation placental explants

As congenital arbovirus infection in mice might be species-specific in nature, we determined whether our observations had relevance to human tissues. To address this issue, we compared the ability of different arboviruses to replicate in maternal and fetal tissue explants obtained between 16 and 23 weeks of gestation from healthy human donors. Maternal-derived decidua and fetal-derived chorionic villi and fetal membrane were isolated, and infection of arboviruses was assessed. As previously reported, ZIKV replicated in human placental villi, decidua, and fetal membranes (Fig. 5A-C). We detected similarly efficient replication of POWV and WNV in chorionic villi and maternal decidua (Fig. 5A-B). In contrast, CHIKV did not replicate efficiently in villi or decidua (Fig. 5D and E), although we did observe limited replication in fetal membranes (Fig. 5F). MAYV replicated in all regions of the placental explants, but only in one of the three donor samples (Fig. 5D-F).

Immunofluorescence microscopy for dsRNA intermediates produced during viral replication revealed that all the neurotropic flaviviruses including ZIKV propagated efficiently in maternal-derived decidua and/or extravillous trophoblasts that remained associated with anchoring villi, with little replication detected within floating chorionic villi, including the syncytial layer (**Fig. 6, left and middle panels**). However, one difference between the two neurotropic flaviviruses was observed in fetal membranes. The fetal membrane is a complex multilayered tissue consisting of the amnion and chorion, with the inner amnion composed a single layer of cuboidal epithelial cells that directly contacts the amniotic fluid and the outer

chorion composed of a reticular layer, basement membrane, and trophoblasts. We found that ZIKV and WNV replicated in both the amniotic epithelium and the sub-epithelial layers of the fetal membrane whereas POWV replicated only in the sub-epithelial layer, which would not contact the amniotic fluid or fetal compartment directly (Fig. 6). We observed low-level infection with CHIKV exclusively in the sub-epithelial layer, whereas MAYV replicated in both layers of the fetal membrane (**Fig. 6, right panels**). Thus, multiple neurotropic flaviviruses, but not arthritogenic alphaviruses, can replicate efficiently in organoid explants derived from mid-gestation human placentas and are capable of infecting maternal decidua and fetal membranes.

Discussion

The 2015 epidemic prompted an intensive study of the biology of ZIKV and its potential for teratogenicity. Some have speculated that ZIKV is unique among flaviviruses in its capacity to cross the placental barrier to cause congenital malformations (33). Our data showing that WNV and POWV replicate efficiently in human placental explants and cause similar levels of placental infection, transplacental transmission, and fetal demise in immunocompetent mice suggests that ZIKV may not be the only flavivirus with the ability to cause congenital disease. In contrast, the arthritogenic alphaviruses CHIKV and MAYV did not cause fetal demise in mice and replicated much less efficiently in the villi and decidua of human placental explants.

Although congenital infection of mice with neurotropic flaviviruses was described decades ago, no studies have performed comparative analysis of multiple arboviruses in the same model. Experiments in the 1970s showed that intravenous inoculation of WT mice with St. Louis encephalitis virus (SLEV) resulted in severe neurological deficits in the surviving progeny, including encephalocele formation, hydrocephalus, brain necrosis, and memory impairment (34, 35). Vertical transmission of JEV during pregnancy also has been established in mice and in swine (36–38). In 1980, a case series was published describing course of pregnancy of five women infected with JEV; two of the women experienced spontaneous abortions, and JEV was isolated from the fetal brain, liver, and placenta (39). In the 1990s, administration of a yellow fever virus vaccine during pregnancy resulted in human fetal infections, revealing yet another flavivirus with the capacity to cause congenital infection (40–42). After WNV spread to the Western Hemisphere in 1999, congenital infection of WNV was modeled in mice (15) and in mouse trophoblast stem cells (43), although these studies did not assess the clinical or pathological impact on the placenta or fetus. Our study shows that WNV exhibits a broad tissue tropism in the fetus, and that congenital infection results in damage to the placenta and developing mouse brain, leading to fetal demise. Our results, along with the previously published studies in mice and humans, suggest that many flaviviruses (WNV, POWV, JEV, SLEV, YFV, and ZIKV) can cause fetal disease, with congenital infection in humans documented for four of these viruses (WNV, JEV, YFV, and ZIKV). It is possible that flavivirus infections induce pregnancy complications and congenital malformation more often than currently appreciated.

Remarkably, several prior studies examined small cohorts of women infected with WNV during pregnancy and their offspring (14, 44, 45), including one study of 77 pregnant

women (14). Among infants born to WNV-infected mothers, two had microcephaly, one had lissencephaly, and eight exhibited abnormal postnatal growth (14), suggesting a microcephaly rate of 2.6% in this group of WNV-infected neonates. Given the small numbers, a statistically significant link between congenital WNV infection and microcephaly was not established. It is worth noting that the association of WNV infection with congenital malformation in this study resembles that observed from a larger retrospective analysis of ZIKV-induced congenital diseases during the 2013 outbreak in French Polynesia where there were 95 cases of microcephaly in 10,000 pregnancies (rate of 0.95%) and the seroprevalence was 50% (46, 47). Thus, it is possible that the rate of flavivirus-induced congenital malformations could be similar for WNV and ZIKV, but the overall impact of congenital ZIKV infection is much greater because of the comparatively low seroprevalence rate of 1–5% for WNV (48, 49).

Since CHIKV RNA has been detected in the human placenta in one case (26), we speculated that arthritogenic alphaviruses (CHIKV and MAYV) also might be transmitted vertically to cause fetal demise resembling congenital ZIKV syndrome. However, we observed no evidence of fetal death or injury after congenital infection with CHIKV or MAYV in immunocompetent mice. Why transplacental infection with the arthritogenic alphaviruses failed to cause fetal death will require further study, but it is important to note that the route(s) of transplacental transmission of known teratogenic viruses such as ZIKV remain poorly defined (reviewed in (50)). These differences in the capacity to cause fetal demise may reflect the lack of susceptibility of mouse fetal cells to infection, differences in cell tropism or expression patterns of alphavirus receptors and attachment factors on maternal (decidua) and/or fetal cells, or the inability of these viruses to reach the intra-amniotic compartment. Neurotropic alphaviruses (e.g., Venezuelan equine encephalitis virus), which we did not test, could have greater potential to cause fetal infection and death than CHIKV and MAYV. Alternatively, the immune response may have a greater capacity to clear alphaviruses from the placenta and other fetal tissues. Alphaviruses might be more vulnerable to maternal antibody-dependent cell-mediated cytotoxicity by macrophages, natural killer cells, or neutrophils since their envelope proteins are displayed on the plasma membrane (51), in contrast to flaviviruses, which assemble in the endoplasmic reticulum (52). In human placental explants from the second trimester, both CHIKV and MAYV replicated in fetal membranes but did not replicate efficiently in the maternal-derived decidua or fetal-derived chorionic villi. Both alphaviruses failed to replicate in a layer of the fetal membrane subjacent to the amniotic epithelial layer that would directly contact amniotic fluid. In contrast, ZIKV and WNV infected the amniotic epithelial layer of the fetal membrane whereas POWV did not. Thus, in the context of human pregnancy, the amniotic epithelial layer of the fetal membrane would be permissive to ZIKV or WNV infection once these viruses enter the amniotic fluid (18), thereby generating an additional reservoir of infectious particles that could reach the fetus. These findings are consistent with the hypothesis that CHIKV causes neonatal encephalitis in the context of perinatal transmission (24, 25) rather than transplacental infection. Failure of CHIKV to replicate efficiently in human maternal and fetal tissues of the placenta may explain why congenital CHIKV infection has not been described despite large outbreaks affecting millions of individuals. Nevertheless, our *in vitro* studies of human placental explants are limited by a small donor

pool, as well as by gestational age and human genetic variability. Thus, larger studies and experiments in pregnant non-human primates are needed to confirm the physiological relevance of our studies.

One of the limitations of modeling congenital ZIKV syndrome is that ZIKV does not replicate efficiently in immunocompetent mice, and thus dams with deficiencies in type I IFN signaling are commonly used for congenital infection experiments when virus is introduced into the skin (7, 8, 10). In contrast, WNV and POWV readily disseminate in immunocompetent WT mice, leading to infection of the placenta and transmission to the fetus.

Our observation that human mid-gestation maternal and fetal tissues support efficient replication of multiple neurotropic flaviviruses suggests that congenital flavivirus infections could occur on a sporadic, undetected basis in humans. These cases may largely go unnoticed due to inadequate prenatal healthcare available to women in many regions of the world most affected by flavivirus outbreaks. Our findings presented here, coupled with prior literature associating other neurotropic flavivirus infections with congenital disease, indicate that greater surveillance of pregnancy complications and congenital anomalies associated with arbovirus infections is warranted, particularly in regions of the world where these viruses are endemic.

Materials and Methods

Study design

This study was initiated in order to compare the capacity of multiple arboviruses (WNV, POWV (also known as Deer Tick virus), CHIKV, MAYV, and ZIKV) to replicate in human placental explants and to cause transplacental infection in mice. Two of the viruses (WNV and POWV) are flaviviruses, like ZIKV, whereas the others are alphaviruses (CHIKV and MAYV). The flaviviruses were selected because they are clinically relevant emerging arboviruses that are related to ZIKV, which causes congenital infection. The arthritogenic alphaviruses were selected because they are clinically relevant, and also because CHIKV is known to cause neonatal encephalitis. However, mechanisms of CHIKV transmission in the perinatal period are not well understood. Our initial observation was that WNV caused more severe placental and fetal infection in mice than the other viruses, so we focused our subsequent experiments (histology and RNA ISH) on a comparison between a flavivirus (WNV) and an alphavirus (CHIKV). Based on the understanding that studies in mice may not always resemble disease in humans, we also tested the capacity of each of these viruses to replicate in mid-gestation human placental explants. Sample sizes and endpoints were selected on the basis of our experience with the ZIKV congenital infection model and prior studies in human placental explants. Investigators were blinded for measurements of placental diameter and other histological assessments, but not when measuring vRNA burden or ISG expression. Primary data are located in **Table S1**.

Viruses and cells

ZIKV (Paraiba) was isolated in Brazil in 2015 and propagated in Vero cells (American Type Culture Collection, ATCC). WNV (3000.0259, passage 2) was isolated from a human patient sample in New York in 2000 and propagated in C6/36 (*Aedes albopictus*) cells. POWV (lineage 2, Spooner strain, passage 3) was originally isolated from adult deer ticks and passaged in Vero cells (53). CHIKV (OPY1 p142, passage 2) was isolated in La Reunion Island in 2006 and propagated in Vero cells. MAYV (BeH407, passage 2) was provided by the World Reference Center for Arboviruses and propagated in Vero cells.

Human placental explants

Human mid-gestation placental tissue (from 16, 19, 22, or 23 weeks of gestation) from elective terminations of normal pregnancies was obtained from the University of Pittsburgh Health Sciences Tissue Bank through an honest broker system after approval from the University of Pittsburgh Institutional Review Board and in accordance with the University of Pittsburgh anatomical tissue procurement guidelines. Decidua, fetal membrane, or chorionic villi were isolated from intact placental tissue and cultured in DMEM/F12 (1:1) supplemented with 10% FBS, penicillin/streptomycin, and amphotericin B.

Focus-forming assays

Vero cells were plated in DMEM supplemented with 10% FBS (HyClone™, GE Healthcare Life Sciences) in a 96-well flat-bottomed plate at 4×10^4 cells/well the evening before inoculation. On the day of infection, medium was removed and replaced with serial dilutions of the virus stock, and the plate was incubated for 2 h at 37°C. Following incubation, a methylcellulose overlay (1:1 1% (w/v) methylcellulose and 1X MEM supplemented with 2% FBS) was added and incubated at 37°C for the following time periods based on standard protocols for titering these viruses: ZIKV, 40 h; WNV, 18 to 22 h; POWV, 50 to 60 h; CHIKV, 18 to 22 h; MAYV, 18 to 22 h. After incubation, the cell monolayers were fixed with 1% PFA in PBS for 1 h. After fixation, wells were emptied and washed four times with PBS, followed by addition of a mouse anti-virus primary monoclonal antibody (200 ng/mL of E60 for WNV (54), ZV-13 for ZIKV and POWV (55), CHK-11 for CHIKV, CHK-48 (cross-reactive anti-CHIKV mAb) for MAYV (51)) in PermWash (0.1% saponin + 0.1% BSA in 1X PBS, diluted 1:5 in ddH₂O) for 2 h. Cells then were washed three times with PermWash, and subsequently a horseradish peroxidase (HRP)-conjugated secondary antibody (500 ng/mL, Sigma-Aldrich) in PermWash was added for 1 h, followed by three washes with PermWash. Finally, TrueBlue (Kirkegaard and Perry Laboratories) substrate was added, and plates were rocked gently at room temperature for 2 to 15 min or until spots were defined with minimal background. Foci were counted manually by ELISPOT.

Viral growth curves and imaging of human placental explants

Second trimester human placental tissues (age 16–23 weeks) were cultured in DMEM/F12 and plated on the day of infection. Explants were incubated with 5×10^6 focus-forming units (FFU) of WNV, ZIKV, POWV, CHIKV, or MAYV for 2.5 h, and then medium was removed. Tissues then were washed in 40 ml of PBS for 15 min followed by DMEM/F12. At 3, 12,

24, 36, and 48 h after infection, an aliquot was removed and frozen at -80°C . The wells were supplemented with DMEM/F12 with 10% FBS after aliquot removal at each time point. Aliquots were titrated by focus-forming assay. At the end of the 48-h growth curve, tissues were placed into 4% PFA for histological analysis. For imaging studies, tissue was fixed in 4% paraformaldehyde (PFA) overnight, permeabilized with 0.25% Triton X-100 for 30 min with gentle agitation at room temperature, and then incubated with the following antibodies: recombinant mouse monoclonal anti-dsRNA (provided by Abraham Brass, University of Massachusetts) as described previously (56), rabbit anti-cytokeratin-19 (Abcam, ab52625), and/or counterstained with Alexa Fluor 633 conjugated phalloidin (Invitrogen, A22284). Tissue was mounted in Vectashield containing DAPI (Vector labs, H-1200) and imaging performed using a Zeiss 710 laser scanning microscope. Images were adjusted for brightness and contrast and/or pseudocolored using Fiji (Image J) or Adobe Photoshop CC.

Mouse experiments

C57BL/6J WT mice (stock #000664) were purchased from Jackson Laboratories and housed in a specific pathogen-free mouse facility at the Washington University School of Medicine in St. Louis (IACUC Protocol Number: 20160288; Assurance Number: A-3381-01). Mice were set up for timed matings and at embryonic day E6.5, pregnant dams were inoculated via footpad injection using 10^2 FFU (WNV) or 10^3 FFU (POWV, CHIKV, or MAYV) diluted in phosphate buffered saline (PBS). In some experiments, mice were treated two days after WNV infection (E8.5) with a single 300 μg dose of humanized E16 (31) or isotype control mAb (anti-CHIKV, 4N12) (57) diluted in PBS through an intraperitoneal route. At E13.5 mice were sacrificed and maternal spleens, fetal heads, and placentas were harvested. Four fetal heads and placenta per dam were analyzed for viral burden, and the remaining fetuses and placentas were used for histological analysis.

Measurement of vRNA burden *in vivo*

Maternal spleens, fetal heads, and placentas were harvested and homogenized with zirconia beads in a MagNA Lyser instrument (Roche Life Science) in 250 or 500 μL PBS and stored at -80°C . RNA was extracted using the RNeasy Mini Kit (Qiagen). Viral RNA levels were measured by one-step quantitative reverse transcriptase PCR (qRT-PCR) on an ABI 7500 Fast Instrument using standard cycling conditions. Viral burden was expressed on a $\log_{10}$ scale as vRNA equivalents per gram after comparison with a standard curve produced using serial 10-fold dilutions of WNV, POWV, CHIKV, or MAYV RNA. The primer-probe sets for the tested viruses are as follows: WNV; Fwd, 5'-TCAGCGATCTCTCCACCAAAG-3'; Rev, 5'-GGGTCAGCACGTTTGTTCATTG-3'; Probe, /56-FAM/TGC CCG ACC ATG GGA GAA GCT C/36-TAMSp/: POWV; Fwd, 5'-GCAGCACCATAGGTAGAATGT-3'; Rev, 5'-CCACCCACTGAACCAAAGT-3'; Probe, /56-FAM/TC TCA GTG G /Zen/T TGG AGA ACA CGC AT /3IABkFQ/: CHIKV; Fwd, 5'-TCG ACG CGC CCT CTT TAA-3'; Rev, 5'-ATC GAA TGC ACC GCA CACT-3'; Probe, /56-FAM/ACC AGC CTG /ZEN/CAC CCA TTC CTC AGA C /3IABkFQ/: MAYV; Fwd, 5'-GTGGTCGCACAGTGAATCTTTC-3'; Rev, (5'-CAAATGTCCACCAGGCGAAG-3'); Probe, /56-FAM/-5'-ATGGTGGTAGGCTATCCGACAGGTC-3'-/36-TAMRA/).

RNA *in situ* hybridization

After fetal heads and placentas were harvested from dams at E13.5 or E18.5, they were sectioned and RNA ISH was performed with RNAscope® 2.5 (Advanced Cell Diagnostics) according to the manufacturer's instructions, using virus-specific probes for WNV as previously described (58).

Statistical analysis

Unless otherwise specified, all data were analyzed using GraphPad Prism software by Mann-Whitney or ANOVA as specified in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. ZIKV-related flaviviruses cause fetal demise in mice that can be prevented by mAb treatment.

Wild-type pregnant dams were inoculated subcutaneously on E6.5 by footpad injection with 10^2 FFU of WNV or 10^3 FFU of POWV, CHIKV, MAYV, or PBS (mock) and tissues were harvested on either E13.5 or E18.5 as indicated. (A-C) qRT-PCR analysis of vRNA burden on E13.5 in the placenta (A), fetal head (B) and maternal spleen (C). (D) Fetal survival as assessed on E18.5. (E) Photograph of littermate mock- and WNV-infected fetuses at E18.5. (F) IFN-stimulated gene (ISG) mRNA expression as measured by qRT-PCR from placenta and fetal head on E13.5 after E6.5 infection with WNV, POWV, CHIKV or MAYV. (G-H) Pregnant mice were inoculated subcutaneously with WNV (10^2 FFU) via footpad injection on E6.5, then treated on E8.5 with a single intraperitoneal injection of 300 μ g anti-WNV mAb (humanized E16) or isotype control mAb (anti-CHIKV human mAb 4N12) mAb at E8.5. Fetal heads and placentas were harvested on E13.5 for viral titer measurement by qRT-PCR (G), or on E18.5 to assess fetal survival (H). Antibody treatment experiments are pooled from 3 independent experiments with 1 to 2 pregnant female dams per experiment. vRNA burden and ISG expression data represent the mean $\pm$ SEM for at least $n = 6$ tissues per group from 4 or 5 infected dams in at least 2 independent experiments. Each data point represents a biological replicate. In (A-C) and (F), data were analyzed by one-way ANOVA.

In (G), data were analyzed by Mann-Whitney test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$). The n for survival data is indicated above each bar. Survival data were analyzed by Chi-squared test (**** $P < 0.0001$).

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Fig. 2. Placental histology on E18.5 after infection with WNV and CHIKV.

Wild-type pregnant mice were infected with 10^2 FFU of WNV or 10^3 FFU of CHIKV or PBS (mock) via footpad injection on E6.5, and placentas were harvested on E18.5 for histological analysis. Hematoxylin and eosin (H & E) staining of placentas was subsequently performed on mock- (A), CHIKV- (B), and WNV-infected fetuses (C). Top row = lower magnification views. Bottom two rows = higher magnification views of the decidua (D, middle row) and labyrinth (L, bottom row). SpT indicates spongiotrophoblasts within the junctional zone. (D) Placental diameter of mock-, CHIKV-, and WNV-infected placentas. Data are representative of 2 independent experiments with $n = 3$ tissue samples for histological analysis and $n = 7$ fetuses from 2 or 3 infected dams for placental diameter measurements. Scale bars = 200 μ m in top row and 50 μ m in bottom two rows. Data in (D) were analyzed by ANOVA test (** $P < 0.01$; *** $P < 0.001$).

Fig. 3. Fetal brain histology on E18.5 after infection with WNV and CHIKV.

Wild-type pregnant mice were infected with 10^2 FFU of WNV or 10^3 FFU of CHIKV or PBS (mock) via footpad injection on E6.5, and fetuses were harvested on E18.5 for histological analysis. Hematoxylin and eosin (H & E) staining was performed on mock- (A), CHIKV- (B), and WNV-infected fetuses (C and D). Red arrows indicate necrosis of the brain in a severe case of WNV fetal disease. Data are representative of 2 independent experiments with $n = 3$ fetuses each. Scale bars = 200 μm in top rows and 100 μm in bottom rows.

Fig. 4. WNV tissue tropism in the mouse placenta and fetus.

Wild-type pregnant mice were infected with 10^2 FFU of WNV or PBS (mock) via footpad injection on E6.5, and fetuses were harvested on E13.5 for analysis by RNA in situ hybridization (ISH). (A-B) Low and high-magnification images of RNA ISH staining of mock- and WNV-infected placenta on E13.5. D = decidua, JZ = junctional zone, L = labyrinth, UC = umbilical cord. Scale bars = 1 mm in (A) and 100 μ m in (B). (C-G) Low and high-magnification images of RNA ISH staining of mock- and WNV-infected fetuses on E13.5. WNV RNA ISH staining in a severely infected fetuses (C), right panel and D-G),

including the forebrain (**D**), hindbrain (**E**), spinal cord (**F**), and myocardium (**G**). Data are representative of 2 independent experiments with $n = 3$ placentas or fetuses each. Scale bars = 1 mm in (**C**) and 100 μm in (**D-G**).

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Fig. 5. Viral growth curves in human placental explants.

(A-F) Human placenta villi, decidua, and fetal membrane explants from three donors (age 21–23 weeks gestation) were inoculated with 5×10^6 FFU of ZIKV, WNV, POWV (A-C), CHIKV, or MAYV (D-F), incubated at 37°C for 2.5 h, washed, and then cultured in DMEM/F12. Small aliquots of cell explant supernatants were collected at 3, 12, 24, 36, and 48 h and analyzed by focus-forming assay. Data represent the mean $\pm$ SEM from 3 or 4 organoid explants per experiment from 3 separate donors in each condition.

Fig. 6. Immunofluorescence staining of virus-infected human placental explants.

Confocal micrographs of human placental tissues (chorionic villi, decidua, and fetal membrane) from four donors (age 16–23 weeks gestation) that were infected with the indicated viruses (mock (PBS), ZIKV, WNV, POWV, CHIKV, and MAYV) for 48 h. Immunofluorescence microscopy was performed for vRNA (using anti-dsRNA antibody, green) and tissue counterstained for cytokeratin-19 (a marker of epithelial-derived cells, including trophoblasts) or actin (in red, as indicated below each panel). DAPI-stained nuclei are shown in blue. White boxes denote zoomed regions shown at bottom right. Images are

representative of 4 explants per experiment from 4 separate donors in each condition. Scale bar = 50 μm .

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