Maternal immunity and antibodies to dengue virus promote infection and Zika virus–induced microcephaly in fetuses

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Zika virus (ZIKV), an emergent flaviviral pathogen, has been linked to microcephaly in neonates. Although the risk is greatest during the first trimester of pregnancy in humans, timing alone cannot explain why maternal ZIKV infection leads to severe microcephaly in some fetuses, but not others. The antigenic similarities between ZIKV and dengue virus (DENV), combined with high levels of DENV immunity among ZIKV target populations in recent outbreaks, suggest that anti-DENV maternal antibodies could promote ZIKV-induced microcephaly. We demonstrated maternal-to-fetal ZIKV transmission, fetal infection, and disproportionate microcephaly in immunocompetent mice. We show that DENV-specific antibodies in ZIKV-infected pregnant mice enhance vertical ZIKV transmission and result in a severe microcephaly-like syndrome, which was dependent on the neonatal Fc receptor, FcRN. This novel immune-mediated mechanism of vertical transmission of viral infection is of special concern because ZIKV epidemic regions are also endemic to DENV.

INTRODUCTION
Zika virus (ZIKV) belongs to the family of Flaviviridae, which includes other arboviruses, such as dengue virus (DENV), Japanese encephalitis virus (JEV), and West Nile virus (WNV). ZIKV unexpectedly surfaced recently as a major public health concern because of the ongoing and spreading epidemic in South and Central America and the realization that it could cause birth defects and neurological complications (2, 3). In a recent study, the rate of neurologic and ocular defects in fetuses born to ZIKV-infected mothers was calculated to be ~7% (4). Rarely, adults experience ZIKV-induced Guillain-Barré syndrome (5), but the congenital Zika syndrome in infants includes a spectrum of neurologic and microvascular defects, including microcephaly, which is the most devastating and pressing aspect of ZIKV infection (6). It has been suggested that the risk of microcephaly is higher in the fetus when mothers are exposed to ZIKV during the first trimester of pregnancy (3, 4). Some studies have reported the direct effects of ZIKV infection on neuronal tissue damage (7, 8). However, not all ZIKV infections during pregnancy appear to result in brain abnormality during embryonic development, and the mechanisms that lead to microcephaly in some fetuses but not others are not yet understood.

ZIKV epidemic regions are often also endemic for other Flaviviruses infections, particularly DENV. Because of the structural similarities between ZIKV and DENV (9, 10), antibodies raised against one of the viruses are able to cross-react with the others (10, 11), possibly leading to antibody-dependent enhancement (ADE) of infection (12). In vitro assays (11) have demonstrated that the binding of human anti-DENV antibodies to ZIKV does not neutralize ZIKV and enhances ZIKV infection in cell cultures. These kinds of studies have raised the possibility that DENV antibodies might also induce ADE of ZIKV in vivo, in DENV-immune patients infected with ZIKV. Thus far, however, the evidence for ADE of ZIKV infection in animal models has been less clear. ADE is apparent in immunocompromised mice (13), but experiments on primates showed conflicting results. There is no enhancement, or only very moderate enhancement, of ZIKV infection in DENV-immune primates (14–15), although ZIKV immunity has been shown to cause ADE of DENV both in primates and in vitro (16–18). However, it has not been experimentally addressed whether DENV cross-reactive antibodies can influence vertical transmission of ZIKV infection. Because maternal antibodies are also passed from mother to fetus during pregnancy in a mechanism dependent on the neonatal Fc receptor (FcRN) (19, 20), which is expressed on the embryonic yolk sac, the fetal vascular endothelium, and syncytiotrophoblast cells (21, 22), we hypothesized that DENV antibodies might enhance translocation of the virus to the developing fetus.

RESULTS
Using the ZIKV strain H/PF/2013 (23), we characterized ZIKV infection in female C57BL/6 mice to determine whether immunocompetent mice would experience replicating ZIKV infection with this strain. After intraperitoneal infection of mice with ZIKV, ZIKV RNA could be detected within 24 hours in the cells of the peritoneum (Fig. 1A) and in lymphoid tissues, including the spleen (Fig. 1B) and mesenteric and iliac lymph nodes (Fig. 1, C and D). Detection of ZIKV persisted beyond 7 days at the site of infection and in lymphoid tissues of immunocompetent mice (Fig. 1, A to D). Infectious ZIKV was also detected in the serum of mice by using a focus-forming assay that allows detection of ZIKV non-structural antigens in target cells exposed ex vivo to serum from ZIKV-infected animals (Fig. 1E and fig. S1). We also observed splenic hypertrophy, consistent with an inflammatory response (Fig. 1F). However, in adult mice, we did not detect any ZIKV RNA in the brain (Fig. 1G), which is consistent with our understanding that ZIKV causes only a mild febrile illness in most adult human patients (1). Our polymerase chain reaction (PCR) data showing that ZIKV RNA could be detected at the site of infection and in lymphoid tissues for at least 1 week supported the view that ZIKV infection persists long enough in immunocompetent mice to allow assessment of its influence on fetal development during pregnancy.

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Fig. 1. Sustained ZIKV infection in female immunocompetent mice. ZIKV infection was quantified in peripheral tissues in (A) the cells isolated from the peritoneal cavity, (B) the spleen, (C) the mesenteric lymph node (LN), and (D) iliac lymph nodes, each day for 1 week following infection of female mice with \(1 \times 10^6\) plaque-forming units (PFU) of ZIKV, strain H/PF/2013. (E) Serum from uninfected or ZIKV-infected dams was harvested 72 hours after infection and absorbed onto BHK-21 cells for focus-forming assays to show replicating virus. Many cells appear infected, staining above baseline levels of uninfected serum, with ZIKV-infected serum diluted 1:2. Individual foci of infection (red circles) surrounded by a few single infected cells can be observed at higher 1:5 dilution of ZIKV serum. Additional images from multiple time points are provided in fig. S1. (F) ZIKV genome copies in the spleen are plotted with the splenic mass. (G) ZIKV was not detected (ND) in the brain of any animals by PCR. \(n = 5\) female mice per group. Error bars represent the SEM.
To address the question of whether maternal DENV immunity could increase embryonic neural complications, we infected mice of varying Flavivirus-immune status during pregnancy at embryonic day 7 (E7) with ZIKV (Fig. 2A). Although mouse and human developmental time points differ, this day was chosen because of its relative equivalence to the first trimester of fetal development in humans and because it represents a time point when the mouse placenta has begun to develop, which starts around E5.5 (24, 25). We also assumed that using a time point for inoculation, where the placenta had not completely formed, might facilitate vertical transmission of infection, while the fact that decidualization has begun by this time point (26) would allow us to address the potential of fetal endothelial cells to promote antibody transport into the fetus. DENV induces a mild replicating infection in immunocompetent mice that induces Flavivirus cross-reactive antibodies (27, 28). Pregnant dams were either naïve (to both ZIKV and DENV), immune to DENV2 after clearing infection that preceded pregnancy by 3 weeks, or passively transferred the monoclonal antibody 4G2, which was raised against DENV2 but is Flavivirus cross-reactive (29). Passive transfer of 4G2 was used to identify the contributions of preexisting cross-reactive antibodies alone (as a component of immunity) to ZIKV pathogenesis. DENV-immune mice were verified by enzyme-linked immunosorbent

Fig. 2. Maternal DENV immunity increases ZIKV microcephaly in fetuses. (A) Schematic of the experimental time course showing that female naïve mice or mice that were infected with DENV2 3 weeks before or mice that were passively transferred the monoclonal antibody 4G2 (50 µg, intraperitoneally) were each crossed to male mice. Dams were infected on E7 after conception, and the fetal development was assessed on E18. (B) ELISAs were performed against equivalent concentrations of DENV or ZIKV antigen using serum isolated 21 days after DENV infection. Serum from DENV-immune animals binds significantly more to DENV than to ZIKV. Graph represents the geometric mean titer twofold over naïve controls, and the error bars represent the SEM. Significance was determined by comparing endpoint dilutions by Student’s paired t test (P = 0.001). IgG, immunoglobulin G. (C) Representative images of fetal mice on a 1-mm² grid are provided to show that the DENV2-immune and 4G2-injected groups were visually smaller than controls. (D) Cross-sections of embryos stained with toluidine blue to reveal the reduced size of fetuses of naïve, DENV2-immune, and 4G2-injected ZIKV-infected dams. Additional representative images are presented in fig. S3. (E) Mass and (F) head circumference of fetal mice on E18 are presented. (G) Incidence of mice having microcephaly, defined as a head size in the third percentile or less, calculated from the SD of fetuses from naïve dams without ZIKV infection. For (E) and (F), ***P < 0.001, ****P < 0.0001, and by one-way analysis of variance (ANOVA) with Holm-Sidak’s multiple comparison test. Error bars represent the SEM. Variances do not differ significantly. For (E), n = 22 (naïve-uninfected), n = 28 (naïve-ZIKV), n = 15 (DENV2-ZIKV), n = 18 (4G2-ZIKV). For (F), n = 17 (naïve-uninfected), n = 18 (naïve-ZIKV), n = 15 (DENV2-ZIKV), n = 11 to 18 (4G2-ZIKV). Groups contain the combined data from all fetuses of three independent dams. (Photo credit: Wilfried A. A. Saron, Duke-NUS Medical School)
assay (ELISA) to have DENV-specific antibodies that bind weakly to ZIKV (Fig. 2B), although virus neutralization tests showed that they were only neutralizing for DENV and not ZIKV (fig. S2). At E18, near full term, dams were euthanized and the fetal mice were examined. A gross analysis of the fetuses showed that mice that were previously infected with DENV had pups with stunted growth (Fig. 2, C and D, and fig. S3). Quantification of the fetal mass showed a significant decrease in the mass of fetuses of naïve ZIKV-infected dams compared to those of naïve uninfected controls (Fig. 2E). This reduced body size was consistent with previous reports of ZIKV infection in mice (30, 31). However, ZIKV-infected DENV2-immune and 4G2-injected dams had fetuses that were even smaller than the naïve ZIKV-infected dams (Fig. 2, C to E). We examined the circumference of the fetal head, the primary measure used to define microcephaly in humans, and observed that DENV-naïve ZIKV-infected dams had fetuses with slightly but significantly smaller heads compared to healthy controls (Fig. 2F). However, 4G2-injected and DENV2-immune dams that were infected with ZIKV had fetuses with head sizes that were substantially smaller than both naïve uninfected mice and DENV-naïve ZIKV-infected mice (Fig. 2F). No significant differences in the range of phenotypes were observed litter to litter within experimental groups, and fetal demise was not observed. Additional control experiments were also performed concurrently, showing that no abnormalities were observed after control injection of 4G2 before pregnancy without maternal ZIKV infection and that isotype control (IC) antibodies did not influence fetal size or head circumference in ZIKV-infected dams (fig. S4).

Furthermore, DENV-immune dams with no ZIKV infection have normal pups without any reported fetal abnormalities (32, 33), and we did not observe any reduction in fetal size when 4G2-injected dams were given a challenge of DENV2 rather than ZIKV at E7 (fig. S4). Next, we looked at the frequency of reduced head circumference in the fetuses born to ZIKV-infected dams (defined as a head size in the third percentile or less for normal fetuses) and noted that, while 30% of fetuses from naïve mice infected at E7 with ZIKV could be assessed as having a phenotype consistent with microcephaly, >90% of the fetuses of DENV2-immune or 4G2-injected dams qualified as having microcephaly (Fig. 2G). These data support the hypothesis that maternal immunity or Flavivirus cross-reactive antibodies can enhance the severity and incidence of reduced head size during ZIKV infection.

Because of the prominence of interferon (IFN)–deficient models for studying Flaviviruses, we also investigated whether IFN-deficient mice could be used to study ZIKV infection at time points developmentally analogous to the first trimester of human infection. However, even with a 2-log lower inoculating dose than we used for wild-type (WT) mice, the infection was too severe in both the naïve and DENV-immune dams. Most dams died before E18 (fig. S5A), and the surviving mouse had fetuses displaying early developmental arrest (fig. S5B). This supported that our immunocompetent model more clearly recapitulated the outcomes of human ZIKV maternal and fetal infection than the IFN-deficient mouse model system.

To further characterize the impact of ZIKV infection, with and without maternal antibodies on the development of the fetal brain in

**Fig. 3. Impaired cortical development in ZIKV fetuses is enhanced by maternal DENV immunity.** (A) Images of embryonic brain sections showing reduced cortical thickness in ZIKV-infected embryos. Tissue sections were cut to 15-μm thickness and Nissl-stained using cresyl violet before imaging by light microscopy. Labels indicate the lateral ventricle (LV) and cortical regions: ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ), cortical plate (CP), and marginal zone (MZ). Additional representative images are included in fig. S6. (B) Quantification of cortical thickness from images. Error bars represent the SEM of average cortical thickness for individual litter mates, where n = 5 (naïve-uninfected), n = 6 (naïve-ZIKV), n = 7 (DENV2-ZIKV), and n = 8 (4G2-ZIKV). (C) Normal ratio of cortical thickness to body mass. Additional controls are provided in fig. S7. (D) Relative expression of transcriptional factors critical for neuronal growth in the fetal mouse brains (normalized to Actin) as determined by real-time reverse transcription PCR (RT-PCR). Error bars represent the SEM for n = 5 fetuses per group. For (B) to (D), *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001, as determined by one-way ANOVA with Holm-Sidak’s multiple comparison test. Variances do not differ among groups.
the WT mouse model, we examined the brains by histology (Fig. 3A and fig. S6). The DENV2-immune group showed a profound reduction in cortical thickness and loss of integrity of the expected cortical layers (Fig. 3A). In particular, there appeared to be reductions in the size of the ventricular zone, intermediate zone, and cortical plate compared to fetuses from naïve uninfected dams and naïve ZIKV-infected dams (Fig. 3A). Quantification of cortical thickness from multiple litters showed that these reductions were significant and consistent for fetuses with both DENV2-immune and 4G2-injected dams compared to fetuses of naïve uninfected dams and naïve ZIKV-infected dams (Fig. 3B). The cortical thickness was also moderately reduced in the fetuses of naïve ZIKV-infected dams compared to fetuses of healthy controls (Fig. 3B), although the cortical layers were intact and discernable (Fig. 3A). Because we had observed that the fetal mass was smaller in all ZIKV-infected groups compared to controls (Fig. 2E), we also questioned whether the reduced cortical thickness was disproportionately small relative to the body mass or proportional to the body mass. Fetuses born to ZIKV-exposed pregnant mothers have been described as displaying both proportional and disproportional microcephaly in humans (2). The ratio of the cortical thickness compared to the body mass for all groups showed that, for ZIKV-infected mice, the cortex was disproportionately reduced compared to control animals (Fig. 3C and fig. S7), indicating a presentation consistent with disproportionate microcephaly.

The transcription factor Brain 1 (Brn1) is used as a marker of cortical development in mice (34); thus, we measured levels of Brn1 in the brains of E18 embryos from all groups. We observed that levels of Brn1 mRNA were reduced in fetuses from DENV2-immune or 4G2-injected dams (Fig. 3D), which was correlated with the impaired cortical thickness. We also measured levels of additional genes associated with early cortical neurogenesis, including forkhead box G1 (Foxg1), empty spiracles homologue 2 (Emx2), and paired box 6 (Pax6). Expression of these genes allows differentiation and proliferation of ventricular zone progenitors as well as expansion of the subventricular zone (35, 36). ZIKV infection resulted in reduced expression of both Pax6 and Foxg1, suggesting that early brain development was stunted because of ZIKV infection, although Emx2 was not significantly influenced (Fig. 3D). Brain 2 (Brn2) and orthodenticle homeobox protein family genes, Otx1 and Otx2, along with Brn1, aid the differentiation and migration of neurons (34, 37) as well as the development of neuronal layers in the cortex and cerebellum (38). ZIKV infection also suppressed the expression of both Brn2 and Otx family genes. For Pax6, Brn1, and Brn2, fetuses of DENV-immune dams showed greater deficits than those of naïve dams (Fig. 3D). Suppressed levels of selected cortical markers were verified at the protein level by immunohistochemistry (fig. S8). Together, these results show visually striking and quantifiable defects in the development of the cerebral cortex during ZIKV infection resulting in a phenotype consistent with disproportionate microcephaly, supported by evidence that cerebral cortex-associated transcription factors are reduced in the fetuses of DENV2-immune dams.

On the basis of these findings and the potential of the antibody 4G2, which is DENV directed but ZIKV cross-reactive, to cause an enhanced microcephaly-like phenotype in the fetuses of ZIKV-infected dams, we expected that antibodies might promote increased translocation of ZIKV into the fetus; therefore, we quantified ZIKV genome copies in the fetuses on E10, 3 days after the dams had been infected. This 3-day period was chosen to allow sufficient time for replication of ZIKV in the mother and for potential translocation of ZIKV into the fetus; it is also a time point at which placentation has already begun (24). We first confirmed that true replication of ZIKV occurs in this model by comparing infection levels in the dams and fetuses in mice injected with live ZIKV compared to ultraviolet light-inactivated ZIKV. This experiment demonstrated that the injected virus is detected only at very low levels in the dam’s spleen (Fig. 4A), and not detected at all in either the placenta (Fig. 4B) or the embryos (Fig. 4C). The viral burden did not differ in pregnant mice compared to nonpregnant mice (fig. S9). Furthermore, the virus negative strand, which is produced only during genome replication, was detectable by PCR in the dams’ spleens, the placentas, and the embryos (Fig. 4, D and E), confirming ZIKV replication. These findings support the fact that fetal ZIKV infection in this model relies on replication of ZIKV in vivo.

Our results examining the potential of maternal DENV immunity to influence fetal ZIKV infection showed that maternal antibodies to DENV or monoclonal antibody 4G2 each enhanced infection of the embryo compared to ZIKV infection of naïve dams (Fig. 4F). ZIKV RNA was detectable in approximately 50% of the embryos of naïve ZIKV-infected dams at E10 but in 100% of the embryos of DENV2-immune dams and in 80% of 4G2-injected dams (Fig. 4F). By E18, although brain development was impaired (Fig. 3), ZIKV RNA was undetectable in the brains of all fetuses by PCR; however, immunohistochemistry staining of E18 brain sections for ZIKV proteins NS2b and M showed positive staining, indicating that antigen persisted in the cortex (fig. S10). Enhanced detection of these antigens was observed in tissue sections from the fetuses of DENV-immune or 4G2-injected dams (fig. S10). DENV immunity also enhanced ZIKV RNA in the dam’s spleen, consistent with other reports that DENV antibodies can cause ADE, and we confirmed that this can occur in vivo in our model (Fig. 4G). In contrast to the mother’s spleen, previous DENV immunity did not significantly enhance the viral burden in the placenta at E10 (Fig. 4H). To confirm that this enhancement occurs in vivo, we performed flow cytometry staining for the ZIKV envelope (E) protein in single-cell suspensions of placentas at E10, after E7 infection of dams. We observed that ZIKV E protein could be detected intracellularly in fetal endothelial cells, defined by vimentin staining (Fig. 4, I and J), and in syncytiotrophoblast cells, defined by the marker cytokeratin, increased over baseline staining of cells from uninfected control placentas (Fig. 4, K and L). Furthermore, the percentage of placental fetal endothelial cells and syncytiotrophoblasts that contained ZIKV E protein was significantly increased in the placentas of DENV-immune compared to naïve dams (Fig. 4, I to L). These results indicated that heterologous immunity enhances ZIKV infection in both the pregnant dam and the fetus and enhances detection of the ZIKV structural protein, E, in placental cells.

Because antibodies are translocated into the fetus with a mechanism dependent on FcRN, and we observed enhanced staining of E protein in the placentas of DENV-immune dams in two cell types that are known to express FcRN, we examined whether FcRN contributes to fetal ZIKV infection in DENV-immune mice. However, because there are conflicting statements in the literature regarding the time of onset of FcRN expression in the fetus, we first verified that FcRN could be detected in fetal mice. Immunostaining of the mouse placenta at E10 (the time point when virus was detectable in mouse embryos by positive- and negative-strand PCR; Fig. 4, C, E, and F) revealed colocalization of FcRN with the fetal endothelial cell marker vimentin (Fig. 5A) and the syncytiotrophoblast marker cytokeratin (Fig. 5B). We next isolated mouse placentas from uninfected and
Fig. 4. Enhanced ZIKV infection of fetuses in DENV-immune dams. (A to C) ZIKV genome copies were quantified by PCR after injection of ultraviolet (UV) inactivation of the virus compared to injection of live virus in tissues including the (A) dams’ spleens (***P = 0.0047), (B) placentas, or (C) fetuses on E10, 3 days after infection. Input UV-ZIKV could be detected only in the dam’s spleen but at significantly lower genome copy numbers than live virus. PCR specific for the ZIKV negative strand was performed using tissue (D) from the mother’s spleen days 1 and 3 after infection and (E) from embryos or placentas on E10, 3 days after infection. For (D) and (E), uninfected and ZIKV-infected vero cell lysates were used as negative and positive controls, and uninfected tissues were used as negative controls. Expected band size is 188 base pairs (bp). The gels confirm active ZIKV replication in mother and fetal mice. (F) Real-time RT-PCR was used to quantify the ZIKV genome copies in the mouse fetuses of DENV-naïve uninfected dams (n = 10), DENV-naïve ZIKV-infected dams (n = 12), DENV2-immune ZIKV-infected dams (n = 17), and 4G2-injected ZIKV-infected dams (n = 10) derived from two to three independent experiments. Fetuses were harvested on E10 (3 days after maternal infection) or on E18 (11 days after maternal infection) for RNA isolation. Fetuses from DENV2-immune and 4G2-injected dams showed significant increases in ZIKV compared to those of naïve dams by one-way ANOVA with Holm-Sidak’s multiple comparison test (**P < 0.01, ***P < 0.001). (G) Quantification of ZIKV infection in the spleen of dams 3 days after infection when the fetuses were harvested on E10 (n = 3 per group). *P < 0.05. Error bars represent the SEM. (H) Quantification of ZIKV infection in the placentas at E10. ns, not significant. Representative flow cytometry plots showing intracellular staining for ZIKV E protein in vimentin+ fetal endothelial cells from the placenta at E10, following maternal ZIKV infection at E7. FSC, forward scatter. (J) ZIKV E protein was detectable in a significantly larger proportion of vimentin+ cells in the placentas from DENV-immune compared to naïve dams infected with ZIKV (n = 5 per group). (K) Representative flow cytometry plots showing intracellular staining for ZIKV E protein in cytokeratin+ syncytiotrophoblast cells from the placenta at E10, following maternal ZIKV infection at E7. E protein was detectable in a significantly larger proportion of cytokeratin+ cells in the placentas from DENV-immune compared to naïve dams infected with ZIKV (n = 5 per group). (J) and (L): ****P < 0.0001.
**Fig. 5. Maternal DENV immunity leads to FcRN-dependent enhancement of fetal ZIKV infection.** Placenta tissue sections from E10 were stained for FcRN, DNA (4′,6-diamidino-2-phenylindole), and either (A) the fetal endothelial cell marker vimentin or (B) the syncytiotrophoblast marker cytokeratin and were imaged by confocal microscopy. Both cytokeratin and vimentin colocalized with FcRN. Scale bars, 50 μm. Images are representative of three independent experiments. (C) FcRN mRNA expression in E8 and E10 mouse placentas. (D) Detection by flow cytometry of FcRN on cells from E8 placentas, gated on vimentin (left) or cytokeratin (right) from uninfected or ZIKV-infected dams, 24 hours after infection at E7. (E) Average mean fluorescence intensity (MFI) of FcRN on cells from E8 placentas, relative to levels on uninfected control endothelial cells (vimentin+); n = 5 per group. (F) Detection by flow cytometry of FcRN on cells from E10 placentas, gated on vimentin (left) or cytokeratin (right) from uninfected or ZIKV-infected dams, 72 hours after infection at E7. (G) Average MFI of FcRN on cells from E8 placentas, relative to levels on uninfected control endothelial cells (vimentin+); n = 5 per group. Flow cytometry gating strategy for (D) to (G) is provided as fig. S11. (H) Schematic showing experimental setup of transwells, where FcRN-expressing HULEC-5a cells were plated on transwell inserts to form a tight monolayer, and trophoblast placenta cells were plated on the bottom chamber. ZIKV or ZIKV + antibody 4G2 were added to the top chamber. (I) Permeability of the monolayers 24 hours after addition of ZIKV or ZIKV with various concentrations of antibodies was measured by quantitating fluorescein isothiocyanate (FITC)-dextran leakage. No differences in leakage were observed compared to uninfected controls. (J) Addition of antibody 4G2 significantly increased the amounts of infection in trophoblast target cells, in a dose-dependent manner, on the opposite side of the transwell insert 24 hours after exposure to ZIKV. (K) Addition of antibody 4G2 significantly increased the detection of ZIKV associated with trophoblast target cells 6 hours after exposure to ZIKV. (L) Addition of antibody 4G2 significantly increased the amounts of infection in trophoblast target cells, in a dose-dependent manner, on the opposite side of the transwell insert 24 hours after exposure to ZIKV. (M) No significant difference was observed in serum endpoint titers fourfold over naïve against DENV2 between WT and FcRN−/− female mice 21 days after infection. P = 0.32 by Student's unpaired t test; n = 5 per group. (N) Quantification of ZIKV in DENV2-immune (n = 17) or DENV2-naïve (n = 12) WT and DENV2-immune (n = 15) or DENV2-naïve (n = 21) embryos on E10 derived from three to four independent dams per group. (O) Proportions of PCR-positive embryos for each group in (N). Significance was determined by Fisher’s exact test (*P = 0.01, ns for P = 0.736). (P) Comparison of ZIKV titers for ZIKV+ fetuses alone shows enhanced viral burden in the embryos of DENV2-immune dams for both WT and FcRN−/− groups, determined by one-way ANOVA with Holm-Sidak’s multiple comparison test; *P = 0.019, n = 6 (DENV-naïve WT), n = 17 (DENV2-immune WT), n = 9 (DENV-naïve FcRN−/−), and n = 8 (DENV2-immune FcRN−/−), corresponding to the PCR-positive embryos from (N). For (N) to (P), all fetal measurements were derived from three to four independent dams. KO, knockout. (Q) and (R) Blocking antibody against FcRN (4C9) or IC antibodies were injected therapeutically into DENV-immune pregnant mice (n = 3 dams) before ZIKV infection on E7. Head circumference of the fetal mice was measured on E18. (Q) Head circumferences and (R) cortical thicknesses were significantly increased in 4C9-treated fetal mice compared to those given IC (*P < 0.0001, **P < 0.05, ***P < 0.01, ****P < 0.001, and *****P < 0.0001).
ZIKV-infected mice at E8 and E10 to represent early time points, 24 and 72 hours after maternal infection. We detected FcRN expression in infected and uninfected placentas at both E8 and E10 by PCR (Fig. 5C). Maternal ZIKV infection led to increased FcRN mRNA expression in E10 embryos compared to controls (Fig. 5C). This finding was confirmed by flow cytometry at E8 and E10, where nearly all fetal endothelial cells (vimentin+ and syncytiotrophoblasts (cytokeratin+) expressed FcRN (Fig. 5, D to G). Similar to PCR detection, a slight but significant increase in FcRN protein was observed on the vimentin+ cells of placentas from ZIKV-infected dams on E8 compared to uninfected controls (Fig. 5, D and E). This induction of FcRN was more dramatic by E10 and apparent on both fetal endothelial cells and syncytiotrophoblasts (Fig. 5, F and G). These results demonstrate that FcRN is present in the placenta during and preceding the E10 time point when antibody-dependent enhanced detection of ZIKV is observed in the fetus (Fig. 4F).

To determine whether ZIKV infection could be enhanced across a monolayer of FcRN-expressing endothelial cells in an antibody-dependent fashion, we used a transwell system. Human endothelial cells were cultured above a monolayer of human trophoblast cells, and supernatants containing ZIKV or ZIKV and various concentrations of 4G2 antibody were applied to the top chamber (Fig. 5H). After incubation for 24 hours, we assessed the permeability of the endothelial cell monolayers and observed no significant increase in permeability, measured by transendothelial resistance (Fig. 5I). However, we did observe an antibody dose-dependent increase in infection of the trophoblast target cells on the opposite side of the transwell insert (Fig. 5I). Increased ZIKV levels were detectable on the opposite side of the transwell insert as early as 6 hours of incubation, indicating that increased translocation occurs because this time point is insufficient for virus replication (Fig. 5K). In addition, DENV-immune human serum could enhance the translocation of ZIKV across the human FcRN-expressing endothelial cells, resulting in a higher titer of ZIKV on the opposite side of transwell inserts (Fig. 5L), supporting the role of antibodies in the process and the potential of human antibodies to induce this phenomenon. These results indicate that antibodies enhance translocation of ZIKV across FcRN-expressing endothelial monolayers and that ZIKV remains viable and able to infect target cells following translocation.

We next aimed to determine whether FcRN plays a functional role in enhancing ZIKV infection in fetal mice. First, we validated that FcRN−/− mice were able to induce anti-DENV antibodies at a similar titer to WT mice and observed no significant difference in the polyclonal anti-DENV response (Fig. 5M). We then compared viral titers in fetuses of both naïve and DENV2-immune FcRN−/− dams to WT controls. Overall, fetuses of DENV2-immune FcRN−/− mice showed reduced levels of ZIKV RNA at E10 compared to the fetuses of WT mice (Fig. 5N). Furthermore, ZIKV RNA levels were not significantly higher in the fetuses of DENV2-immune dams compared to naïve dams (Fig. 5N), in contrast to the significant enhancement of ZIKV infection observed in the context of DENV2 maternal immunity in WT animals (Fig. 5N). In FcRN−/− DENV2-immune dams, only 50% of the fetal mice showed ZIKV infection compared to 100% of the fetuses of WT DENV2-immune dams (Fig. 5O). This proportion was significantly lower than the proportion of infected fetuses in WT mice and not significantly different from ZIKV-infected naïve WT or naïve FcRN−/− mice (Fig. 5O) and suggests that FcRN-mediated translocation of immune complexes increases the likelihood of vertical transmission. Yet, antibodies may still have a role in enhancing titers in an FcRN-independent manner, because DENV2-immune FcRN−/− fetuses that were ZIKV+ showed higher viral titers than the DENV2-naïve FcRN−/− fetuses that were ZIKV+ (Fig. 5P). Similarly, the fetuses of dams that were DENV2 immune showed higher levels of ZIKV than naïve animals (Fig. 5P), suggesting that DENV2 antibodies also result in enhanced levels of ZIKV if the fetus becomes infected by a mechanism independent of FcRN. To confirm the contributions of FcRN by an alternate mechanism, we also injected dams with a blocking antibody against FcRN to neutralize its function in the translocation of immune complexes across the placenta. We observed that fetal head circumference was increased with FcRN blockade compared to IC injection (Fig. 5Q). Similarly, the cortical thickness, measured from brain sections, was also significantly increased after FcRN blocking compared to IC treatment (Fig. 5R). IC treatment was verified to have no influence on head circumference or cortical thickness in uninfected animals (fig. S12). These results support the conclusion that both a novel mechanism of FcRN-mediated immune complex translocation into the fetus and ADE can contribute to enhanced ZIKV infection in fetal mice.

DISCUSSION

Many viruses in the Flavivirus genus are neurotropic, but no others have been identified to cause microcephaly. Neurological complications occur only rarely with DENV (39), the most closely related known human pathogen to ZIKV, and have entirely different clinical presentation in the context of JEV and West Nile virus (1), involving encephalitis in adults and children. Some studies have reported neuronal tissue damage during ZIKV infection in humans and mice, involving infection of neural progenitor cells (7, 8). Immunocompromised mice develop severe fetal abnormalities (31), which are not necessarily consistent with the much lower rates of microcephaly that are observed in humans (40). Immunocompetent mice infected with high doses of ZIKV during slightly later stages of pregnancy than our model also have fetal abnormalities, including reduced fetal size that corresponded with a reduced cortical thickness (41). Here, using a model of maternal infection at a time point of mouse fetal development corresponding to the first trimester of pregnancy in humans and a moderate ZIKV inoculating titer of a strain epidemiologically associated with microcephaly, we report that vertical transmission of ZIKV occurs. Furthermore, maternal antibodies enhance transplacental infection of mouse fetuses and lead to an exacerbated phenotype consistent with microcephaly. This involves reduced cortical thickness, substantial loss of certain cortical layers, and impaired induction of transcriptional profiles key for brain development. Microcephaly in humans resulting from ZIKV infection has been described as both proportionate and disproportionate to the body mass (2). In our model, we observe a phenotype resembling disproportionate microcephaly relative to the reduction in overall fetal mass, which is enhanced by maternal DENV immunity. Infection was undetectable by PCR in E18 fetal brains, while the signs of damage to the cortex and ZIKV antigens remained.

Head size alone did not indicate the full severity of reduced cortical thickness in mice, nor could this effect be interpreted from the pregnant dam’s viral burden, although ADE in the dams was evidenced by a higher ZIKV burden in DENV-immune compared to naïve pregnant mice. Our results also indicate that FcRn mediates a significant amount of the antibody-enhanced effects of disease because FcRn−/− animals have a reduced percentage of embryonic mice
infected with ZIKV in utero. This finding is likely due to the essential role of FcRN in mediating transcytosis of immune complexes across the placenta from mother to fetus (42, 43) or across the syncytiotrophoblast cells before full placentation (24). FcRN mediates transcytosis within recycling endosomes that are only weakly acidic (pH ~6.6) (44), and release of the antibody from FcRN occurs at neutral pH (45, 46); therefore, transcytosis of ZIKV-immune complexes is not expected to inactivate ZIKV because Flaviviruses, including ZIKV, are not fully inactivated until a pH of <3.0 to 4.0 is achieved (47, 48). Consistent with this hypothesis, we observed enhanced infection of target trophoblasts in the presence of antibodies when ZIKV was exposed to the opposite side of an FcRN-expressing endothelial cell monolayer. Although it is possible that FcRN could act in a traditional ADE mechanism, promoting infection of FcRN-bearing target cells rather than merely translocation, the fact that enhanced levels of ZIKV can be detected crossing monolayers after short time periods that are insufficient for virus replication supports the view that translocation is a major component of the enhanced infection response. We also confirmed the contributions of FcRN to induction of reductions in cortical thickness in the fetuses of ZIKV-infected DENV-immune dams by using an alternate method where FcRN was targeted with a monoclonal neutralizing antibody. In addition to supporting the role of FcRN in the antibody-enhanced phenotype in fetal mice, this experiment suggests the possibility of therapeutic targeting of FcRN to limit severe pathologies in the subset of mothers that are Flavivirus immune. This possibility is consistent with a recently published study where targeting FcRN reduced ZIKV infection in human placental explants (49). However, FcRN cannot account for all of the antibody-enhanced effects because embryos of DENV-immune dams that are infected have higher levels of ZIKV than naive dams (with WT and FcRN−/− mice showing the same trends), indicating a contribution of ADE to enhanced fetal infection. In humans, maternal immunoglobulin G (IgG) is present in the fetus during the first trimester of human pregnancy (50), but fetal concentrations of IgG are lower in the early (compared to late) stages of pregnancy (50, 51). Thus, it will be important to determine whether certain stages of human pregnancy are susceptible to antibody-enhanced translocation of the virus and augmented microcephaly and whether the presence of Flavivirus cross-reactive antibody is a factor in extending the period of fetal susceptibility to ZIKV beyond the first trimester when most cases have been shown to occur (2). Additional studies are required to determine whether DENV immunity is a risk factor for microcephaly during maternal ZIKV infection in humans because of the inherent physiological and immune functional differences between humans and mice. A recent study examining this question found no association between the incidence of total abnormal pregnancy outcomes and positive tests for DENV-reactive IgG (52). However, because approximately 90% of the patients in that study were determined to be IgG positive, further studies are needed with a larger sample size of DENV IgG-negative patients to fully assess this question and to examine the effects of DENV immunity on specific manifestations of ZIKV congenital syndrome, such as microcephaly. Addressing whether DENV immunity enhances severity of human ZIKV congenital syndrome is further complicated by the fact that ZIKV- and DENV-specific antibodies in serum cross-react to a large degree (53), making it difficult to distinguish between immunity to ZIKV and DENV if a blood sample is not taken early enough after ZIKV infection to exclude the presence of DENV cross-reactive ZIKV antibodies. Genetic differences between Flaviviruses also play a strong role in the ability of antibodies raised against unique viruses to enhance or neutralize a heterologous infection, as do the concentrations of virus-specific or cross-reactive antibodies. Thus, future studies are also needed to examine the potential of multiple strains and serotypes of DENV to enhance ZIKV-induced microcephaly because the incidence of microcephaly in humans is not high enough to suggest that 100% of the fetuses of DENV-immune mothers develop microcephaly. This study provides important considerations for microcephaly screening, raises novel therapeutic targets to limit severe disease, informs our understanding of mechanisms that could influence the severity of ZIKV presentation, and establishes a novel mechanism of vertical transmission of infection.

METHODS

Female mice that were DENV naïve or DENV immune [3 weeks after infection with 1 × 10⁶ plaque-forming units (PFU) of Eden2 strain] were bred and infected with ZIKV (1 × 10⁶ PFU of H/PF/2013 strain) on E7. This route of infection was chosen to initiate uniform systemic infection in the dams to specifically study the process of vertical transmission. The SingHealth Institutional Animal Care and Use Committee approved all animal protocols. Detailed methods are provided in the Supplementary Materials.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/2/eaav3208/DC1

REFERENCES AND NOTES

Zika convalescent macaques display delayed induction of anamnestic cross-neutralizing antibodies.


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Maternal immunity and antibodies to dengue virus promote infection and Zika virus–induced microcephaly in fetuses
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