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Zika virus infection of first-trimester human placentas: utility of an explant model of replication to evaluate correlates of immune protection *ex vivo*

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Abstract

The emergence of congenital Zika virus (ZIKV) disease, with its devastating effects on the fetus, has prompted development of vaccines and examination of how ZIKV breaches the maternal-fetal barrier. Infection of placental and decidual tissue explants has demonstrated cell types at the uterine-placental interface susceptible to infection and suggests routes for transmission across the placenta and amniochorionic membrane. ZIKV replicates in proliferating Hofbauer cells within chorionic villi in placentas from severe congenital infection. Explants of anchoring villi recapitulate placental architecture and early-stage development and suggest infected Hofbauer cells disseminate virus to fetal blood vessels. ZIKV infection of explants represents a surrogate human model for evaluating protection against transmission by antibodies in vaccine recipients and passive immune formulations and novel therapeutics.

Introduction

The authors recall first hearing on National Public Radio of the sudden epidemic of microcephaly in northeastern Brazil that was tentatively attributed to an epidemic of an obscure flavivirus – Zika virus (ZIKV) – not theretofore associated with serious human disease or known to cross the placental barrier and infect the fetus. We were tempted to believe that the causative agent might be other viruses [1*], such as human cytomegalovirus (HCMV), long known to transmit infection across the human placenta, causing microcephaly and neurological defects [2,3]. In fact, we had modeled HCMV infection in explants of early gestation placentas, focusing on the very question of how maternal virus breaches the placental barrier, identifying specialized cells involved in dissemination [4–11]

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and antiviral antibodies as a correlate of protection [12–16]. But HCMV did not cause a rash or epidemics [17]. In early 2016, we came together as a team combining experience with HCMV models of transmission [18] and a longstanding knowledge of flavivirus biology and immunology [19–23] and began to apply the tools and knowledge gained in studying these disparate viruses to elucidating the mechanisms of transplacental ZIKV transmission.

Emergence of ZIKV as a serious public health problem

Zika virus (ZIKV) is an arbovirus of the *Flavivirus* genus, which includes several clinically important arboviruses, such as dengue virus, West Nile virus, and yellow fever virus, among others [24]. Originally isolated from a rhesus monkey in the Zika forest of Uganda in 1947, few cases had been documented in humans before 2014. Components of non-neonatal disease overlaps that of dengue and chikungunya, with maculopapular rash, conjunctivitis, low-grade fever, polyarthralgia, myalgia, and headache [25,26*] but this virus was not known to cause serious disease. This situation changed dramatically with outbreaks in Micronesia in 2007, French Polynesia in 2013–2014 and Brazil and the Americas beginning in 2015 [27]. ZIKV spread rapidly in these naïve populations; for example, in less than 10 months from the first reported case in northeastern Brazil, local transmission had been reported in 26 countries or territories in the Americas [28*]. The ZIKV epidemic was associated with an inordinate number of cases of microcephaly in the initial American epidemic in northeastern Brazil, which prompted frantic efforts by health officials and scientists to determine the relationship of ZIKV infection in pregnant women with congenital defects [28]. Several case reports confirmed the presence of ZIKV in babies with microcephaly and other brain abnormalities [29*,30*,31]. Consideration of the collective evidence, including timing of ZIKV infection during gestation relative to developmental defects observed and the specificity of the defects to ZIKV infection, has led to a consensus that congenital ZIKV infection, especially during early pregnancy, causes a variable syndrome of severe malformations in the fetus, termed congenital Zika syndrome (CZS), that can include microcephaly at delivery or postnatally, reduction in cerebral volume, ventriculomegaly, subcortical calcifications, ocular defects and neuromuscular abnormalities [25,32*,33*,34*,35]. A retrospective analysis of birth data later confirmed that the initial outbreak in Micronesia in 2007 – the first outside of Africa or Asia – was followed by an increase in microcephaly cases.

Epidemiology of ZIKV spread to the Americas

Studies have shown that export of viral lineages to the Americas occurred during a period of suitable climate conditions for vector transmission in recipient countries. In Central and South America, there was a 12-month interval between initial export and the date of ZIKV detection, suggesting a season of undetected transmission. In the Americas, ZIKV was first confirmed in May 2015 in northeast Brazil, a country with the highest number of reported cases worldwide (200,000 and over 2,366 cases of microcephaly). More than 45 countries in the Americas have reported local transmission, with severe disease reported in 24 countries. Analyses of viral genomes in conjunction with epidemiological data estimate that ZIKV was present in northeast Brazil by February, 2014, and disseminated before detection in the Americas. Since first being detected in Florida by sequencing of ZIKV genomes from

infected patients, studies have shown at least 4 introductions, but potentially as many as 40, contributed to the outbreak in Florida, and that local transmission is likely to have started in the spring of 2016 – several months before its initial detection [36]. By analyzing surveillance and genetic data, it was shown that ZIKV moved among transmission zones in Miami. Most introductions were linked to the Caribbean, a finding corroborated by the high incidence rates and traffic volumes from the region into the Miami area.

Studies to measure the prevalence of ZIKV in febrile patients in Senegal and Nigeria in samples collected from 1992 to 2016 indicated seroprevalence of 6.2% based on serological analysis [37]. Phylogenetic analysis showed that these isolates belonged to the African lineage, grouping with either the Nigerian or MR766 sublineages, evidence that ZIKV had been silently circulating in West Africa for two decades.

The potential for ZIKV spread into countries where *Aedes* spp. mosquitoes are endemic is high. Previously, cases tended to be sporadic and associated with mild, non-specific symptoms. Large-scale surveillance of ZIKV is challenging, since many cases are asymptomatic, and ZIKV co-circulates with other arthropod-borne viruses with overlapping symptoms. A system of continuous virus sequencing integrated with surveillance data could provide timely information for effective responses against ZIKV [38].

ZIKV infection of brain

How ZIKV impacts development of the fetal brain is poorly understood. Nonetheless, studies have begun to model specific interactions in vitro [39]. ZIKV preferentially infects neural stem cells, astrocytes, oligodendrocyte precursor cells, and microglia, whereas neurons were less susceptible to infection. These findings suggest mechanisms for microcephaly and other pathologic features of infants with congenital ZIKV infection that are not explained by neural stem cell infection alone, such as calcifications in the cortical plate. It may be revealing to compare mechanisms by which ZIKV crosses the blood-brain barrier and the placental barrier as these are elucidated.

Pregnancy and congenital ZIKV infection

Much attention has been focused on the question of how American ZIKV strains are able to cross from maternal tissue and blood into the protected fetal compartment. Although the mechanisms are ill-defined, they could include a possible role for vasoactive cytokines and preexisting cross-reactive antibodies, the latter potentially resulting in antibody-enhanced dissemination in dengue seropositive women [40,41**]. More recently, a secreted viral pathogenesis factor, the nonstructural protein-1 (NS1), whose counterpart in dengue can trigger endothelial permeability via degradation of the endothelial glycocalyx components [42**] and elicit secretion of vasoactive cytokines [43*] that contribute to severe disease [44], was reported to have distinct features in Zika, dengue and West Nile viruses [45, 46**, 47**,48]. Flavivirus cross-reactive antibodies have been shown to enhance ZIKV infection *in vitro* and in mouse models [43*,49]; however, the role of both host and viral secreted factors in ZIKV pathogenesis and transmission is yet to be determined.

Diagnosis of maternal ZIKV infection during gestation, especially as it relates to severe congenital outcomes, has been confounded by the fact that the clinical symptoms and serology of ZIKV infection overlap those of dengue and chikungunya viruses and by frequent coinfection [50*]. Of particular interest is the question of whether the timing of prior exposure to dengue virus could contribute to regional differences in the frequency of microcephaly associated with ZIKV infection [51,52]. Improved serological methods should help uncover these relationships: non-cross-reactive ZIKV NS1-specific monoclonal antibodies have recently been identified in a screen of human memory B cells and have been used to develop new ZIKV-specific serologic diagnostic tests [41**,53**].

Chorionic villi of the placenta and the fetal-derived avascular amniotic membrane are highly adapted to prevent inflammatory responses and the spread of ascending pathogens into the fetal compartment [54–56], and few viruses are known to cause congenital infection with significant placental and fetal pathology [57]. The special molecular adaptations of these viruses that enable them to cross the placental and membrane barriers and establish pathogenic infections in the fetus are therefore of great interest, as counteracting their functional effects could prevent transplacental transmission.

ZIKV infection of primary cells and tissues from human placentas: clues to mechanisms of transmission

Consistent with transplacental transmission, ZIKV antigens and RNA have been found in first and second trimester placentas from congenital infections [30*]. Of particular relevance is infection of Hofbauer cells, which have been found to support ZIKV replication *ex vivo* [58**]. Importantly, infected Hofbauer cells have been documented in cases of microcephaly in the second trimester and a study of placentas from congenital infections with spontaneous abortion [59,60**]. Surprisingly, ZIKV-infected placentas exhibit hyperplasia of Hofbauer cells, potentially amplifying virus production by these cells in the villus core, and lack classical signs of inflammation, suggesting a unique ability to evade innate immune responses [61].

We reported that infection of primary cells isolated from human placentas and fetal membranes has identified several cell types productively infected by both Nicaraguan ZIKV isolates and the African prototype strain MR766, including cytotrophoblasts, endothelial cells, trophoblast progenitor cells, placental fibroblasts, and amniotic epithelial cells [62**]. Explants from first-trimester human placentas cultured on an artificial extracellular matrix develop anchoring villi and produce invasive cytotrophoblasts, mimicking early development and remodeling of the decidualized uterus during pregnancy [63]. When villus explants were infected with either Nicaraguan ZIKV strains or MR766, Hofbauer cells became infected, consistent with placentas from cases of congenital ZIKV infection. An earlier report indicated that cytotrophoblasts isolated from term placentas were refractory to ZIKV infection, possibly due to high levels of secreted type III interferon [64]. However, using conditions in which mid-gestation primary cytotrophoblasts were allowed to differentiate, we showed positive immunostaining for ZIKV proteins and production of infectious progeny in MR766-infected cells suggesting gestational age and culture conditions influence

infection. Likewise, cytotrophoblasts in explants of anchoring villus were infected with both Nicaraguan strains and prototype MR766 *ex vivo*. All the cell types shown to be productively infected expressed T-cell immunoglobulin and mucin domain 1 protein (TIM1), a candidate cofactor for ZIKV infection that recognizes phosphatidyl ethanolamine in the viral membrane envelope, whereas AXL and Tyro3, TAM receptors, hypothesized to participate in ZIKV infection showed variable expression [39,62**,65–67]. Treatment of virions with the phosphatidyl ethanolamine-binding drug duramycin [68] potently blocked infection by in isolated placental cells, amniotic epithelial cells and explants of anchoring villi, suggesting that infection depended strongly on TIM1. Furthermore, TIM1 was shown to be widely expressed at the interfaces between uterine decidua and both placenta and extraplacental amniochorionic membranes.

Our recent detailed analysis of infection of chorionic villus explants from numerous first-trimester placentas has confirmed that ZIKV reproducibly infects specific sites and replicates in cell column and invasive cytotrophoblasts and Hofbauer cells in villus cores [69]. ZIKV-infected cytotrophoblasts and amniotic epithelial cells continued to proliferate and increased virus production. Notably, cell column cytotrophoblasts infected with the Nicaraguan ZIKV strains proceeded to differentiate and invade extracellular matrix, whereas those infected with the prototype African strain MR766 largely failed to do so, potentially suggesting functional differences between the American and African strains that could increase ZIKV dissemination. In this regard, invasion of basal decidua and uterine blood vessels by ZIKV-infected cytotrophoblasts could contribute to prolonged maternal viremia. ZIKV has been shown to replicate and produce infectious progeny in intact decidual explants and primary decidual macrophages [69–71*]. We showed that ZIKV replication in epithelial cells of endometrial glands in basal decidua is highly productive and could amplify the viral load in the maternal blood space where cell columns develop and increase the overall risk of transplacental transmission.

Vertical transmission of ZIKV by two potential routes

Based upon current understanding of susceptible cell types and patterns of infection at the interface of the decidualized uterus with the placenta proper and amniochorionic membranes, we present a model of ZIKV transmission to the fetal compartment (Figure 1). Cells highlighted in red in Figure 1 are susceptible cell types identified in explants of human placentas and basal decidua and among primary cells and reflect reproducible sites of infection in anchoring villus explants that could mediate ZIKV transmission to the fetus.

Transplacental transmission—The uterine-placental interface (upper panel) forms during the first trimester as cytotrophoblasts in contact with decidua establish an interface between maternal and placental cells that eventually develops into the basal plate [57]. The maternal blood space, in which the tree-like placental villi covered by the multinucleate syncytiotrophoblast are bathed in maternal blood, mediating exchange across the syncytiotrophoblast surface, increases gradually during the first trimester. ZIKV in the maternal blood space, potentially amplified by decidual infection, could transmit virus to proliferating cytotrophoblasts in cell columns and Hofbauer cells in villus cores. As our recent studies indicate that Hofbauer cell infection does not depend on nearby infection of

cytotrophoblasts, it remains unclear how virus is initially transmitted to Hofbauer cells in villus cores, but local proliferation of cytotrophoblasts facilitates access. Infected Hofbauer cells could proliferate and amplify virus titers in the villus core promoting spread to nearby fetal blood vessels and thus to the fetal circulation. As our recent study also shows that cytotrophoblasts infected with American ZIKV strains remain invasive, they could further spread infection as cells remodel uterine blood vessels, contributing to prolonged viremia and increasing risk of transplacental transmission. Recent studies in rhesus macaques provide support for local replication inasmuch as ZIKV infection persists in multiple tissues, including lymph nodes and the uterus, of infected rhesus macaques after viral clearance from circulation [72*,73*].

Transmembrane transmission—The interface between the extra-placental amniochorionic membrane and the parietal decidua increases in the second and third trimesters as the fetus grows and the amniotic sac expands and comes in contact with the parietal decidua (lower panel) [57]. Cytotrophoblasts originating from trophoblast progenitor cells in the chorionic membrane mediate contact with and undergo limited invasion of the parietal decidua [74]. Thus, infected cells in parietal decidua could transmit ZIKV to susceptible cytotrophoblasts, trophoblast progenitor cells in the chorion, and amniotic epithelial cells lining the amniotic sac. Proliferation of infected amniotic epithelial cells could increase virus titers in amniotic fluid and enable transmission to the fetus through susceptible cells in fetal skin, gastrointestinal tract, and lung. Additionally, infected amniotic epithelial cells could amplify the viral load in amniotic fluid, increasing fetal exposure, inflammation and disease [75–77].

Prospects for testing potential efficacy of a ZIKV vaccine to protect human placentas from ZIKV infection

Several groups have reported promising results from vaccine and passive antibody transfer studies in mice and non-human primates using multiple platforms [78**]. Five have reported DNA-based vaccines expressing pre-membrane and E proteins, which enable responses to quaternary epitopes known to elicit neutralizing activity [79–83]; all were shown to confer protection in animal models after virus challenge by various measures, including suppression of viremia and indicators of pathology. Similar results were obtained in rhesus macaques using purified inactivated virus and adenovirus-based expression of pre-membrane and E proteins [81]. Notably, depletion of $CD4^+$ and $CD8^+$ T lymphocytes from mice immunized with a DNA vaccine prior to challenge did not abrogate the protective effect of the vaccine [80], and passive transfer of immune serum from immunized animals showed similar protective effects, suggesting protection can be conferred by antibodies alone. Interestingly, no significant differences in ZIKV titers, neutralizing antibody levels, or immune cell kinetics were found following ZIKV infection of flavivirus-naïve and dengue-immune animals [84].

Although early reports are encouraging, the effectiveness of vaccines for the prevention of adult and fetal disease will be unclear in the near future, as efficacy and durability of immunity are evaluated. ZIKV infection will therefore remain a concern for pregnant

women even after immunization, as most ZIKV infections are asymptomatic in adults yet can cause severe fetal disease [85]. Importantly, interventions to prevent transplacental transmission after ZIKV exposure or seroconversion in pregnancy would be highly useful in this and other settings. However, demonstrating efficacy in preventing transmission before long-term studies can be completed is problematic. Moreover, correlates for vaccine and passive immune protection against transplacental transmission have not been defined.

Experiments to determine whether vaccine-induced antibodies or candidate therapeutic molecules could protect the human placenta from ZIKV infection and prevent transmission could be performed in the explant models of anchoring villi and basal decidua from first-trimester human placentas, which retain tissue architecture, cellular and innate immune functions and developmental potential. Furthermore, given the central role likely played by Hofbauer cells in transmission and the question of how virus reaches them initially, it would be important to specifically evaluate protection against Hofbauer cell infection, a capability that the explant model has already been shown to provide. We suggest that rigorous testing and proven effectiveness in a native human tissue model is critical to guide development and long-term investment in specific therapies.

Conclusion and future directions

The rapid emergence of congenital ZIKV disease caught the public health community by surprise and drew renewed attention to the problem of congenital viral disease, mostly absent since the days when birth defects from rubella virus were still in the news. In response, the research community quickly identified cell types susceptible to ZIKV infection and cell surface molecules that act as cofactors for infection that could plausibly mediate transplacental transmission, identifying Hofbauer cells as likely conduits for virus spread to fetal circulation. However, the role of maternal viremia and molecular mechanisms of transplacental transmission remain poorly understood. Multiple reports of success in vaccine and passive transfer studies in animal models suggest an effective ZIKV vaccine could soon be developed, but efficacy in preventing congenital disease may not be known for years to come. In the interim, sporadic outbreaks will likely occur in seronegative communities [51,52]. Immune sera from vaccine trials [78**] and passive immune therapies [41**,86], as well as candidate therapeutic compounds [87], could be tested in explant models of the human placenta and decidua for their ability to specifically prevent virus dissemination, as indicated by infection of Hofbauer cells and other surrogate markers of fetal infection [88*]. As previous work on HCMV infection of the human placenta greatly enabled our understanding of the early steps in ZIKV replication, ongoing testing in such models could enable responses to the emergence of new congenital viral diseases in the future.

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Highlights

- ZIKV crosses the uterine-placental interface and infects the developing fetus.
- ZIKV replicates in explants of human basal decidua and anchoring villi.
- Cytotrophoblasts and Hofbauer cells are ZIKV targets in first-trimester placentas.
- ZIKV-infected cells proliferate and titers increase *ex vivo*.
- Protective antibodies elicited by ZIKV vaccines can be evaluated in explants.

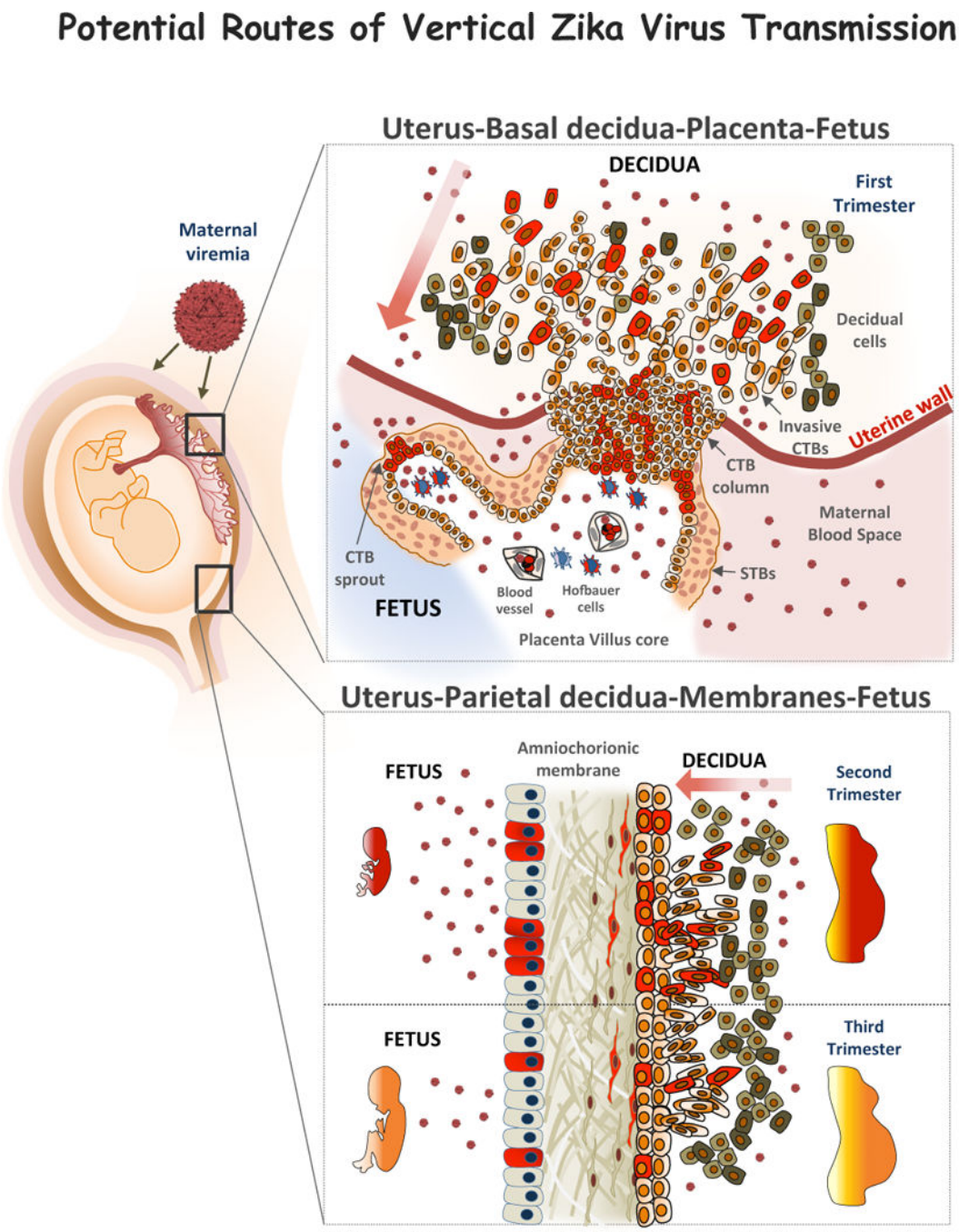

Figure 1. Potential routes of vertical Zika virus transmission during human pregnancy

Diagram of the maternal – fetal interface highlighting potential routes of ZIKV dissemination from the decidualized uterus to the developing human placenta. Drawing indicates sites of infection and cell types identified in studies of ZIKV replication in explants that model development in first-trimester human placentas and basal decidua and primary cells from the amniochorionic membranes [62**,71*]. Red virions and graduated arrows in basal and parietal decidua symbolize viremia (red) and viral dissemination from maternal blood stream, ZIKV-infected cells (red). Upper panel: At the basal decidua-placental

interface, virus released from infected cells in decidua contacts developing cell columns in first-trimester placentas. ZIKV infects proliferating cytotrophoblasts (CTBs) in cell columns and sprouts in branching villi, and nearby Hofbauer cells that proliferate and spread infection to fetal blood vessels in villus cores. Invasion of basal decidua and remodeling of uterine arterioles by infected CTBs could contribute to prolonged maternal viremia. Lower panel: At the interface between the parietal decidua and amniochorionic membranes established during the second and third trimesters, infected cells in decidua could spread ZIKV to nearby CTBs, proliferating trophoblast progenitor cells in the chorionic membrane, and amniotic epithelial cells (AmEpCs) lining the amniotic sac. ZIKV replication could transmit virus to the fetus or potentially amplify fetal infection. Virion modified with permission from Sirohi et al. [89]