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Zika Virus Targets Different Primary Human Placental Cells Suggesting Two Routes for Vertical Transmission

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Abstract

Zika virus (ZIKV) infection during pregnancy is linked to severe birth defects, but mother-to-fetus transmission routes are unknown. We infected different primary cell types from mid- and late-gestation placentas and explants from first-trimester chorionic villi with the prototype Ugandan and a recently-isolated Nicaraguan ZIKV strain. ZIKV infects primary human placental cells and explants – cytotrophoblasts, endothelial cells, fibroblasts and Hofbauer cells in chorionic villi and amniotic epithelial cells, and trophoblast progenitors in amniochorionic membranes expressing Axl, Tyro3 and/or TIM1 viral entry cofactors. ZIKV produced NS3 and E proteins and generated higher viral titers in amniotic epithelial cells from mid-gestation compared to late-gestation placentas. Duramycin, a peptide that binds phosphatidylethanolamine in enveloped virions and precludes TIM1 binding, reduced ZIKV infection in placental cells and explants. Our results suggest that ZIKV spreads from basal and parietal decidua to chorionic villi and amniochorionic membranes, and targeting TIM1 could suppress infection at the uterine-placental interface.

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SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, three tables and can be found with this article online at

AUTHOR CONTRIBUTIONS

Conceptualization, L.P., E.H., T.T.; Methodology and Investigation, T.T., H.P.G., M.P., D.M.; Writing, L.P., E.H., T.T., M.P., H.P.G., D.M.; Funding Acquisition and Supervision, L.P., E.H.

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INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne flavivirus that was relatively obscure until outbreaks in Yap and French Polynesia in 2007 and 2013, respectively, and the 2015–16 pandemic expanding rapidly from Brazil across the Americas brought it to worldwide attention. In the US, 691 imported cases and 11 cases of confirmed sexual transmission have been reported (CDC, 2016). ZIKV is linked to severe birth defects and Guillain-Barré Syndrome (Cao-Lormeau et al., 2016; Sarno et al., 2016), and in February of 2016, the World Health Organization declared the Zika pandemic a Public Health Emergency of International Concern (WHO, 2016). However, little is known about the causal mechanisms. Mounting evidence indicates that infection in early gestation can lead to miscarriage, stillbirth, intrauterine growth restriction (IUGR) and microcephaly, a malformation of the fetal brain (Brasil et al., 2016; Mlakar et al., 2016); however, infection of the mother in the second or third trimester and prolonged viremia could contribute to fetal abnormalities (Brasil et al., 2016; Driggers et al., 2016). ZIKV has been detected in brain glia and neurons, placenta and amniotic fluid of babies with microcephaly, further linking infection during pregnancy to congenital disease (Mlakar et al., 2016; Rasmussen et al., 2016). How ZIKV infects the placenta and reaches the fetal compartment is unknown.

In early gestation, trophoblasts from chorionic villi of the placenta develop into two major cell types, syncytiotrophoblasts (STB) that cover the villus surface and cytotrophoblasts (CTB). Villus CTB proliferate and switch from an epithelial to an endothelial phenotype, differentiate, invade the uterine wall, and remodel uterine arteries (Zhou et al., 1997). Chorionic villi anchor the placenta to the uterus and channel blood from circulation to the maternal blood space. To maintain immune tolerance to the hemiallogeneic placenta, natural killer cells, macrophages, and dendritic cells emigrate to the basal decidua, attracted by chemokine-receptor networks (Red-Horse et al., 2001). Opposite the basal decidua, where chorionic villi are anchored, a much larger portion of the uterine wall is lined by the parietal decidua. By 15 weeks gestation, the parietal decidua is in contact with the chorionic membrane, which is fused to the amniotic membrane lined on the fetal side by amniotic epithelial cells (AmEpC) (Benirschke and Kaufmann, 2000). Trophoblast progenitor cells (TBPC) in the chorion differentiate into invasive CTB that migrate into the parietal decidua and attach the amniochorionic membranes to the uterus (Genbacev et al., 2015). The parietal decidua contains maternal blood vessels and lymphatic vessels and functions as a paraplacental exchange organ that filters fluid from maternal circulation via the chorion and contributes to maintenance of equilibrium in the fetal compartment. As pregnancy advances and the fetus grows, the chorionic surface of the amniochorionic membrane adjoins the parietal decidua across almost the entire uterine surface.

Flaviviruses bind to a variety of surface molecules that serve as entry mediators or cofactors (Perera-Lecoin et al., 2014). Recently, dengue virus (DENV) was shown to bind the TAM family of tyrosine kinase receptors – Tyro3, Axl and Mertk – that clear apoptotic cells (Meertens et al., 2012) and regulate innate immune functions (Lemke and Rothlin, 2008; Rothlin et al., 2007). TAM is activated by ligands that bind phosphatidylserine (PS) in membranes of apoptotic cells and can form bridges between virions and TAM. DENV also binds TIM1, a member of the T cell immunoglobulin and mucin domain protein family that

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regulates innate and adaptive immune functions and cell survival (Freeman et al., 2010). Axl and Tyro3 and, to a lesser extent, TIM1 serve as entry cofactors for DENV (Meertens et al., 2012; Perera-Lecoin et al., 2014), and it was demonstrated that TIM1 directly binds PS and phosphatidylethanolamine (PE) in the virion envelope of dengue, West Nile and Ebola viruses (Jemielity et al., 2013; Richard et al., 2015). A recent ZIKV isolate was shown to infect human dermal fibroblasts, epidermal keratinocytes and immature DCs, with DC-SIGN, Axl, Tyro3 and TIM1 facilitating entry, and Axl playing a major role (Hamel et al., 2015).

In the present study, we took advantage of our previous work on human cytomegalovirus (HCMV) infection in the developing placenta and primary cells expressing viral receptors (Fisher et al., 2000; Maidji et al., 2007; Tabata et al., 2007; Tabata et al., 2015; Zydek et al., 2014) to understand how ZIKV infection and transmission from mother to fetus occur. We hypothesized that, like HCMV, hematogenous spread of ZIKV entails infection of cells at sites in the uterus perfused by maternal blood – decidua and placenta – and by amniotic fluid that bathes the amniotic membrane and fetus. To test this possibility, we isolated different primary cell types from mid- and late-gestation placentas and established explants from first-trimester chorionic villi, and we found that placental cells and explants were highly infectable with the prototype Uganda ZIKV strain and new 2016 ZIKV isolates from the current epidemic in Nicaragua. Cell types susceptible to ZIKV replication expressed Axl, Tyro3 and TIM1 cofactors to varying degrees, and immunohistological and immunoblot analyses of placental biopsy specimens and primary cells confirmed that expression varied by cell type, donor, differentiation state and gestational age. Notably, TIM1 was consistently and highly expressed throughout the basal decidua and parietal decidua, chorionic villi, and amniochorionic membranes surrounding the fetus, and in AmEpC that were productively infected and released high virus titers. Duramycin, a cyclic 19-amino acid peptide that specifically binds PE, potently blocked ZIKV infection in all primary placental cells and chorionic villus explants, indicating that TIM1 plays a critical role in infection and suggesting strategies for clinical management.

RESULTS

ZIKV infects primary human placental cells from mid and late gestation and chorionic villus explants from early gestation

To determine whether ZIKV infects primary cells from the human placenta, isolated AmEpC from mid- and late-gestation amniotic membranes, TBPC from chorion, and HPF and CTB from chorionic villi were isolated from donors (n=20). These cells, along with a primary HUVEC line from umbilical cord, were infected with prototype ZIKV strain MR766 and at 72 h post-infection, were immunostained with monoclonal antibodies (MAbs) to E glycoprotein (Figure 1, Panel I A–E) and nonstructural protein NS3 (F–J), which is not secreted and is widely used as a marker for flavivirus replication. AmEpC (Figure 1, Panel l A, B, F, G) were highly susceptible to ZIKV, as indicated by expression of E and NS3, while infection of TBPC (C, H), HPF (D, I), HUVEC (E, J) and CTB (Figure S1) occurred in single cells and/or discrete foci. Productivity of primary cell infection was indicated by release of infectious progeny quantified by plaque and focus-forming assays (Table S1).

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Most productive were AmEpC, which released high titers (10^5–10^7) at low MOI (0.001) as compared with TBPC, HPF, HUVEC and CTB, which were infected at higher MOI (1–2).

Having found that primary AmEpC from mid- and late-gestation placentas were susceptible to ZIKV infection, these cells were infected with clinical isolates Nica1–16 and Nica2–16 in parallel with MR766. Nica1–16 and Nica2–16 were isolated in Nicaragua in January 2016; full-length sequencing confirmed their close relationship to the Brazil 2015–16 strains. Like MR766, Nica1–16 readily infected AmEpC at low MOI, expressed E (Figure 1, Panel II A, C) and NS3 (B, D), and produced high titers at 3, 5 and 7 dpi (days postinfection) (Table S1). Interestingly, AmEpC from mid-gestation placentas produced 6- to 8-fold higher titers than those from late-gestation placentas at 5–7 dpi when infected with MR766 (Figure 2A), Nica1–16 (Figure 2B) or Nica2–16 (Figure 2C). Similarly, ZIKV release from Nica1–16-infected AmEpC titered on human ARPE-19 cells was ~4-fold greater in mid-gestation than in late-gestation cells at 5–7 dpi (Figure 2D).

We next cultured chorionic villus explants from first-trimester placentas (n=4), infected with MR766 or Nica1–16, and immunostained for ZIKV E and NS3 at 3 dpi (Figure 1, Panel III A, B). Low magnification (middle) showed E and NS3 proteins in a focus of proliferating villus CTB (cytokeratin (CK)- and Ki67-positive). Approximately 15 CTB near the villus core were infected, and both strong and weak staining was observed, suggesting cell-to-cell spread (Panel III A, B; Figure S2 H–S). ZIKV-infected CTB expressed CK but did not express Ki67, indicating they were no longer proliferating (Panel lll C; Figure S2 J, M, P, S). Invasive CTB were also infected, expressed E and NS3 proteins, and continued to express CK (Panel IV A, B, D, E) and MHC class I HLA-G, a differentiation antigen (Panel IV C, F). ZIKV proteins were also detected in villus CTB and numerous small foci with 2–4 infected cells (Figure S2 N–S). Detailed examination of the explants indicated that STB did not produce ZIKV NS3 protein and were not infected, although E glycoprotein was detected in small cytoplasmic vesicles (data not shown). In villus explants infected with Nica1–16, Hofbauer cells in the villus core were infected and expressed E and NS3 proteins (Panel V A, B, D, E) and CD68 (Panel C, F). Likewise, MR766 infected Hofbauer cells in villus explants (Figure S2 B–G). Titers of infectious progeny released from chorionic villus explants infected with MR766 or Nica1–16 were quantified in ARPE-19 cells and ranged from 2×10^3 PFU to 1×10^5 PFU.

Together, these results show that different types of primary cells from mid- and late-gestation placentas are permissive to infection with prototype and contemporary ZIKV strains. Further, in the tissue environment of early gestation chorionic villus explants, proliferating villus CTB in proximal cell columns were infected, after which proliferation was arrested. Differentiating/invasive CTB and Hofbauer cells in the villus core were also key targets of infection.

Primary placental cells express potential cofactors for ZIKV infection

We hypothesized that Axl, Tyro3 and TIM1 could be cofactors for ZIKV infection in the human placenta and that their expression could be modulated during cell isolation and culture *in vitro*. To this end, immunoblotting was used to measure expression of these

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cofactors in CTB, amniotic membranes, isolated AmEpC, and low-passage TBPC, HPF and HUVEC.

In CTB isolated from mid-gestation placentas, cofactor expression varied considerably during differentiation from 0 to 96 h (Figure 3A). Axl was either upregulated or not expressed, while Tyro3 levels were generally low. In contrast, TIM1 showed moderate or strong expression that declined over time. In late-gestation CTB, TIM1 expression was strong and detected throughout differentiation, in contrast to Axl and Tyro3 (A). Titers from MR766- and Nica1–16-infected CTB were about 100-fold lower than titers from mid-gestation CTB (Table S1). TIM1 was strongly produced in trophoblast cell lines (JEG-3 and HTR8) that are highly infected by ZIKV (Bayer et al., 2016; Miner et al., 2016), but Axl was not expressed by JEG-3 cells (B). In intact amniotic membranes from late gestation, little or no Axl was detected (C); however, in cells newly isolated from these membranes and subsequently at passage 0 (P0), faster-migrating forms of Axl were present, and a high-molecular weight form was strongly upregulated at P1. Tyro3 was initially weakly expressed but in some cases strongly upregulated after isolation (C). In contrast, TIM1 was consistently and strongly expressed in both intact amniotic membranes and isolated AmEpC from all donors (C). Next, we compared cofactor expression in AmEpC from mid- and late-gestation placentas (D). Axl was produced by AmEpC from seven of nine donors, but two mid-gestation donors made faster-migrating forms, and Tyro3 was not expressed in four donors (D). Uniformly robust TIM1 expression was found in AmEpC from all donors. In TBPC isolated from early- and mid-gestation chorionic membranes, Axl and Tyro3 were weakly expressed in early gestation and strongly in late gestation, whereas TIM1 expression was robust throughout (E). In HPF and HUVEC, Axl was strong, Tyro3 varied, and TIM1 expression was moderate (F).

We next used immunofluorescence to visualize the subcellular localization of Axl and TIM1. Axl was concentrated at plasma membranes and perinuclear sites in mid-gestation AmEpC; however, abundant perinuclear and vesicular staining was detected in late gestation (Figure 3G). TIM1 was concentrated in plasma membranes in mid-gestation AmEpC, but diffuse nuclear and cytoplasmic staining increased in late gestation (H). Immunostaining of first-trimester chorionic villus explants showed that TIM1 was expressed in villus CTB and Hofbauer cells (CD68-positive) (Figure S2 A). In summary, immunoblot and immunofluorescence studies showed that TIM1, a potential cofactor for ZIKV infection, was expressed in CTB and trophoblast cell lines and strongly in amniotic membranes and in primary AmEpC isolated from all mid- and late-gestation placentas and early-gestation chorionic villi. In contrast, Axl and Tyro3 expression differed by cell type and donor and was modulated in culture and as cells differentiated *in vitro*.

Potential cofactors for ZIKV infection are present at the uterine-placental interface

Axl was reported to play a major role in ZIKV infection in human keratinocytes (Hamel et al., 2015). To determine whether Axl was present in the decidua, placenta and fetal membranes, biopsy specimens from mid-gestation were co-stained for Axl and the cellular proteins vimentin (fibroblasts), cytokeratin (CTB), CD68 (Hofbauer cells/fetal macrophages (Mϕ)) and CD31 (endothelial cells in blood vessels). In basal decidua, Axl was expressed in

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decidual cells (Figure 4A) and a subset of deeply invasive CTB (B). In chorionic villi of the adjacent placenta, Axl was present in Hofbauer cells/Mϕ (C), endothelial cells in blood vessels (D), and HPF in the villus core (E). In amniochorionic membranes, Axl was expressed in AmEpC and TBPC (F). Interestingly, the intensity of Axl expression depended on location, especially in invasive CTB in decidua (A, B), Hofbauer cells and HPF (C, E), suggesting responses to the tissue environment. Modulation of cofactors was also shown by immunoblot analysis of differentiating CTB from various donors that upregulated Axl as they invaded extracellular matrix *in vitro* (Figure 3A).

To determine whether potential cofactors for ZIKV infection were expressed *in vivo*, we surveyed a library of 31 biopsy specimens from mid- and late-gestation placentas for Axl, Tyro3 and TIM1. Multiple tissue sections were screened from amniochorionic membranes, chorionic villi of the placenta, basal decidua, and parietal decidua. Detailed examination indicated that expression of specific cofactors depended on the donor, location of cells in tissues, and gestational age (Figure 5, Table S2). The strength of Axl signal varied and was absent from some mid-gestation and all late-gestation amniotic membranes (Figure 5A, D, G, P) and smaller chorionic villi (J); however, basal decidua and parietal decidua were positive (M and S, Table S2). Tyro3 was present in amniotic epithelium (B, E, H, Q) and in basal and parietal decidua (N, T), but not in chorionic villi (K). In contrast, TIM1 was strongly and consistently expressed in all tissues of mid- and late-gestation placentas – amniotic epithelium (C, F, I, R), STB, Hofbauer cells and HPF in chorionic villi (L), decidual cells and invasive CTB in basal decidua (O), TBPC in chorion, and was dramatically induced in the population of differentiating CTB that invade the parietal decidua (U).

Table S3 summarizes results of immunoblot and immunostaining experiments in isolated cells and biopsy specimens of placentas from mid and late gestation. TIM1 was consistently and strongly expressed on cells throughout the uterine-placental interface in basal and parietal decidua, chorionic villi, and amniochorionic membranes surrounding the fetus. In contrast, Axl and Tyro3 expression varied by donor, gestational age and tissue and was modulated in culture and differentiating cells.

TIM1 inhibitor blocks ZIKV infection across placental cell types and reduces infection in chorionic villus explants

Having found that ZIKV infects primary cells from chorionic villi and amniochorionic membranes that express Axl and TIM1, we considered they could function as cofactors. Although Axl was variably expressed and absent from amniotic membranes in late gestation (Figure 5A, D, G P, Table S3), we tested the effects of the Axl-specific tyrosine kinase inhibitor R428 on ZIKV infection. At the highest concentration tested (3.0 μM), R428 reduced infection of AmEpC by MR766 and Nica2–16 by ~40–50% (Figure 6A, B).

Since TIM1 was consistently made by all permissive cell types and was broadly expressed at the uterine-placental interface by all donors (Figure 5C, F, I, L, O, R, U, Table S3), we performed infection-blocking experiments using duramycin, a cyclic peptide that binds PE and inhibits infection by DENV, a close relative of ZIKV, in cells that express TIM1 (Richard et al., 2015). ZIKV MR766 was incubated with increasing concentrations of

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duramycin and added to AmEpC. Immunofluorescence assays revealed a striking reduction in ZIKV NS3-expressing cells in the range of 0.2 μM to 1 μM duramycin; few NS3-expressing cells were detected after treatment with ≥0.2 μM duramycin (Figure 6C). Quantification of NS3-expressing AmEpC showed that duramycin potently blocked ZIKV infection in cells from mid- (22.6 wks) and late-(40.2 wks) gestation placentas (Figure 6D (i)). Likewise, when duramycin-treated MR766 was added to placental TBPC, HPF and HUVEC, infection was reduced by >50% with 0.1 μM and >95% with 0.2 μM duramycin, even at 10-fold higher MOI (Figure 6D (ii)). Similar results were obtained when AmEpC were infected with Nica1–16 treated with duramycin (Figure 6D, (iii)). Titration of infectious progeny released from both mid- and late-gestation AmEpC showed that 0.2–1 μM duramycin significantly reduced virus titers (Figure 6E).

To determine whether duramycin affects ZIKV infection in the tissue environment, chorionic villus explants from first-trimester placentas were infected with untreated (n=2) or 1 μM duramycin-treated (n=2) MR766 (1×10^6 PFU) 2 h after plating, and infectious progeny were titered in ARPE-19 cells. Titers were reduced from 1×10^5 PFU/ml in the explants infected with untreated ZIKV to 4×10^3 and 0 PFU/ml in the explants infected with duramycin-treated virus. Together, these studies show that duramycin blocks ZIKV infection of numerous primary placental cell types from mid and late gestation, whereas the Axl inhibitor was far less effective in blocking infection. Moreover, duramycin reduces ZIKV infection in chorionic villus explants from early-gestation placentas.

DISCUSSION

In this study, we show that ZIKV prototype strain MR766 and recent ZIKV isolates from the current Zika pandemic infect numerous human primary cells – AmEpC, CTB, TBPC, HPF and HUVEC – isolated from mid- and late-gestation placentas and produce infectious progeny. In explants of chorionic villi from first-trimester placentas, ZIKV infected proliferating villus CTB, invasive CTB, and Hofbauer cells in the villus core and expressed E and NS3 proteins, indicating viral replication. Of note, ZIKV-infected CTB no longer proliferated. Virus titers were dependent on cell type and gestational age, as mid-gestation AmEpC produced significantly higher titers than late-gestation cells. Analysis of biopsy specimens revealed sites at the uterine-placental interface with strong and consistent TIM1 expression in cells where maternal blood perfuses the placenta. These include the basal decidua and adjacent chorionic villi (Figure 7, left) and the parietal decidua, lining most of the uterus and adjacent to amniochorionic membranes surrounding the fetus (Figure 7, right). Expression of other potential cofactors, Axl and Tyro3, varied by donor, location of susceptible cell types within biopsy specimens, and gestational age. Infection of isolated placental cells was potently inhibited by duramycin, a 19- amino acid cyclic peptide that specifically binds PE in the virion envelope and blocks infection of the closely related DENV (Richard et al., 2015). Duramycin blocked ZIKV infection of primary placental cells that contact maternal and fetal circulation and AmEpC in amniotic fluid and reduced infection in the tissue environment of early-gestation chorionic villus explants, potentially by precluding binding of ZIKV to TIM1, a cofactor we showed was universally expressed across the uterine-placental interface throughout gestation. In contrast, an inhibitor of Axl,

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reported to be a cofactor for ZIKV in dermal and epidermal cells (Hamel et al., 2015), modestly reduced infection, suggesting a secondary contribution in placental cells.

Figure 7 shows a model of the uterine-placental interface and identifies the type and location of primary cells infected *in vitro*, suggesting sites of viral replication and identifying placental and paraplacental routes of ZIKV transmission to the fetus. The basal decidua, the first zone of contact between the uterus and adjacent placenta, develops as placental CTB invade the uterine wall and remodel blood vessels. The opposed parietal decidua, the second zone of contact between the uterus and fetal membranes, constitutes a considerably larger surface where maternal and fetal zones remain in contact from mid-gestation to delivery (Genbacev et al., 2015). Our findings suggest that in the parietal decidua, ZIKV could disseminate from uterine blood vessels to invasive CTB to fetal membranes, infecting TBPC in the chorionic membrane and then AmEpC in the amniotic membrane. Progeny virions released into amniotic fluid could infect susceptible cells in fetal skin (Fujita et al., 1991; Kubo et al., 2009), analogous to DENV infection of the skin (Hamel et al., 2015; Wu et al., 2000). Recent reports support both placental and paraplacental routes of ZIKV transmission, including (i) persistence of ZIKV in maternal blood during pregnancy, (ii) high viral load in fetal membranes and viral RNA in amniotic fluid, (iii) limited placental damage with high viral titers in fetal brain, (iv) more cases of symptomatic congenital ZIKV infections with microcephaly in early gestation as compared with late gestation, and (v) a narrow window after primary infection until fetal anomalies develop, suggesting direct infection of the fetus (Brasil et al., 2016; Calvet et al., 2016; Driggers et al., 2016).

Regarding the first zone of contact between floating chorionic villi in the maternal blood space and anchoring villi in the basal decidua (Figure 7, left panel), it was recently reported that ZIKV fails to infect syncytialized CTB from late gestation due to secretion of Type III interferon (Bayer et al., 2016). Although the cofactor TIM1 is strongly expressed in chorionic villi, we observed that STB are spared from infection, suggesting innate immune responses protect the villus surface (Savidis et al., 2016). Nonetheless, we identified ZIKV infection with both NS3 and E expression in foci of proliferating CTB near the villus core and invasive CTB expressing HLA-G. In countries with endemic DENV infection, it is likely that pre-existing maternal antibodies cross-reactive with ZIKV but have low neutralizing activity could promote infection of immune cells bearing Fcγ receptors (FcγR) for IgG, as reported for DENV (Balsitis et al., 2010; de Alwis et al., 2014). These observations suggest a potential role for antibody-mediated transcytosis of IgG-ZIKV immune complexes by the neonatal Fc receptor expressed in STB and infection of underlying CTB as reported for HCMV (Maidji et al., 2006). ZIKV dissemination in the villus core could result in enhanced infection of FcγR-expressing Hofbauer cells, reported to be targets of ZIKV infection in the placenta (Quicke et al., 2016). Here, we found that fetal Hofbauer cells in the villus core of explants infected with ZIKV expressed E glycoprotein and NS3 protein, consistent with viral replication, suggesting that virion uptake can result in infection of fetal macrophages. Infection of cells bearing FcRs in basal and parietal decidua could potentially be enhanced by immune complexes of ZIKV with weakly neutralizing DENV-cross-reactive antibodies or antibodies that develop after primary maternal ZIKV infection.

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Our results have potential clinical relevance for congenital ZIKV infection and implications regarding disease outcome. We found that AmEpC from mid-gestation placentas produce higher virus titers than late-gestation cells, suggesting fetuses infected earlier in gestation are exposed to more virus for a longer time during pregnancy. Infection in early- and mid-gestation via basal decidua and chorionic villi could disseminate virus to fetal circulation at a critical stage in brain development, when high viral load increases the risk of malformations. We observed that ZIKV infected numerous clusters of proliferating CTB in intact villus explants, but ZIKV-infected cells stopped proliferating and virus replicated in invasive CTB. High viral titers were released that could potentially spread infection to Hofbauer cells, placental fibroblasts and endothelial cells in the villus core and fetal circulation. Moreover, the patterns of infection observed suggest downstream structural defects: calcification of the villus surface, truncation of developing cell columns that then fail to attach to the uterine wall, and a reduced population of invasive CTB, which would impair remodeling of uterine blood vessels, leading to IUGR, which is associated with congenital ZIKV infection.

Regarding potential cofactors of ZIKV infection, immunoblot and immunohistochemical analysis of isolated placental cells, amniotic membranes, and biopsies of placental and fetal membranes revealed that TIM1 was expressed consistently and uniformly, regardless of individual donor and gestational age, and was maintained during differentiation. In contrast, we found that Axl and Tyro3 cofactors varied substantially across donors, gestational age, and differentiation state. The consistent and strong expression of TIM1 on fetal membranes in mid- and late-gestation placentas and on numerous cell types at the uterine-placental interface and early-gestation chorionic villus explants, together with the susceptibility of proliferating and invasive CTB and Hofbauer cells to infection, suggest that TIM1 may be an important cofactor for ZIKV infection. This concept was supported by our results showing that duramycin – a cyclic peptide inhibitor that exclusively binds PE in the virion envelope, thus presumably precluding TIM1 binding of PE in the virion envelope – inhibits ZIKV infection of TIM1-expressing cells at the uterine-placental interface. In addition, a trophoblast cell line that does not express Axl is highly infected by ZIKV (Bayer et al., 2016; Miner et al., 2016), suggesting that Axl is not essential for infection. However, whether duramycin's effect on ZIKV infection is mediated solely by TIM1 remains to be shown and is the subject of ongoing experiments. Duramycin also blocks infection of dengue, Ebola and West Nile viruses (Richard et al., 2015) reported to bind TIM1 as a cofactor for virion entry (Kondratowicz et al., 2011; Moller-Tank et al., 2013), suggesting utility of duramycin as a broad-spectrum antiviral that could have clinical application for congenital ZIKV. Interestingly, a TIM1 polymorphism is associated with susceptibility to severe hepatitis A virus infection in certain populations (Kim et al., 2011), but other forms predispose toward inflammation associated with asthma and allergy (Gao et al., 2005). Whether genetic variants of TIM1 expressed by pregnant women infected with ZIKV are associated with severe congenital disease remains to be determined. Additionally, whether Axl, which we found varies in expression by donor, gestational age and tissue, plays a role in ZIKV infection during pregnancy is unclear given the limited effect of an inhibitor (Holland et al., 2010) against infection in primary placental cells.

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In summary, we have shown that ZIKV replicates in primary human placental cells from mid- and late-gestation and villus explants from first-trimester human placentas, suggesting placental and paraplacental routes of transmission, and that infection of these cells is inhibited by the cyclic peptide duramycin. Further, we suggest that these ex vivo model systems can be used to elucidate the molecular mechanisms of ZIKV infection and test antiviral drugs.

EXPERIMENTAL PROCEDURES

Human tissue samples

This study was approved by the Institutional Review Board of the University of California, San Francisco (UCSF). Tissue was collected from term deliveries and elective terminations at UCSF Medical Center. For tissue analysis, placenta membrane biopsies from 31 donors were fixed in 4% paraformaldehyde (PFA) and frozen in optimal cutting temperature compound (OCT; Tissue Tek) as described previously (Tabata et al., 2012) or fixed in 10% formalin, embedded in paraffin, and sectioned for immunohistochemistry and histology (McDonagh et al., 2006; Pereira et al., 2014).

Primary human cells, cell lines and cell culture

AmEpCs were isolated from early (16.5–23.6 weeks (wks); n=10) and late (38.6–41.0 wks; n=6) gestational age fetal membranes and cultured according to published methods (Miki et al., 2005) with minor modifications. AmEpCs were cultured on fibronectin (Sigma-Aldrich) in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco) with 20 ng/ml epidermal growth factor (EGF; R&D Systems), 10% fetal bovine serum (FBS; Invitrogen), nonessential amino acids, 55 μM 2-mercaptoethanol and Antibiotic-Antimycotic (Thermo Fisher). Only passage 1 cells 100% immunopositive for cytokeratin 19 were used. TBPC from human chorion (7.3 and 15.6 wks), provided by Susan Fisher (UCSF), were cultured on gelatin in DMEM/F12 (1:1) with 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems), 10 μM SB431542 (Tocris Bioscience) and 10% FBS (Genbacev et al., 2011). TBPC 100% immunopositive for cytokeratin (MAb 7D3) (Damsky et al., 1992) and GATA4 were used up to passage 15. CTB were isolated from second trimester (15.6–21.1 wks) (n=7) and third trimester placentas (37.2 wks) (n=1) as previously described (Fisher et al., 2000; Tabata et al., 2007) and cultured on Matrigel (BD Biosciences) in serum-free medium: DMEM/F12 (1:1) with 2% Nutridoma (Roche Diagnostics), 1% sodium pyruvate, 1% HEPES, penicillin/streptomycin (P/S) and gentamycin (UCSF Cell Culture Facility) (Fisher et al., 2000). Human placental fibroblasts (HPF; 8 wks) were grown in DMEM/M199 (4:1) with amino acids and 10% FBS. Trophoblast cell lines JEG-3 and HTR8, gift from Michael S. Diamond (Washington University, St. Louis) were grown in DMEM/F12 and RPMI 1640 with 10% FBS. Human umbilical vein endothelial cells (HUVEC) were grown in EBM-2 with EGM-2 SingleQuots (Lonza). Cells 98% immunopositive for von Willebrand factor (Novocastra Laboratories), passages 4–6, were used. Human adult retinal pigment epithelial cells (ARPE-19) (Dunn et al., 1996) were grown in DMEM (Gibco) with 10% FBS. BHK-21 clone 15 cells (gift from S. Kliks, San Francisco, CA) were grown in MEM with 5% FBS, nonessential amino acids, 2 mM L-glutamine, and P/S (Gibco). *Aedes albopictus*

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C6/36 cells (gift from Ralph Baric, University of North Carolina, Chapel Hill) were grown in MEM-α with 2% FBS and 1% P/S (Gibco) at 32°C with 5% CO_2.

ZIKV strains and infection *in vitro*

Stocks of prototype (Uganda 1947, MR766; resequenced, GenBank accession KX421193), a kind gift from Michael S. Diamond (Washington University, St. Louis), and Nica1–16 and Nica 2–16 strains at low passage were propagated in C6/36 cells. Cell-free supernatants were harvested 3 dpi, aliquotted and stored at −80°C. Ni ca1–16 and Nica 2–16 were isolated from ZIKV-infected patients by the National Virology Laboratory, Ministry of Health, Managua, Nicaragua, using C6/36 cells. Full-length sequencing by Mi-Seq confirmed them to be closely related to Brazilian 2015–16 strains (GenBank accession KX421195 and KX421194). Cells were infected with MR766 at multiplicities of infection (MOI) of 0.001–3 and maintained up to 10 d. AmEpC (21.2, 22.6, 38.6 and 40.2 wks) were infected with Nica1–16 or Nica 2–16 at MOI 0.001–0.009. CTB were infected with MR766 (MOI 1–2) 16 h after isolation and plating on Matrigel and maintained for 3 d (Yamamoto-Tabata et al., 2004).

Placental villus explants

Normal human placentas (7.5 to 8.4 wks, n=4) were obtained from elective termination of pregnancy (Advanced Bioscience Resources). Chorionic villus explants were isolated and cultured as anchoring villi described previously (Tabata et al., 2012). Explants were cultured on Millicell-CM inserts (0.4 μm pore size, Millipore) coated with Matrigel in DMEM/F12 (1:1) with 10% FBS, P/S, and amino acids. Two or 24 h after attachment to Matrigel, explants were infected with ZIKV MR766 (1×10^6 PFU/explant) or Nica1–16 (5×10^3 PFU/explant) and maintained for 2–3 d, before fixation and embedding (Tabata et al., 2012).

ZIKV titration

Infectious virions were titered by plaque assay (PA) as described previously (Diamond et al., 2000) with minor modifications or focus-forming assay (FFA) on BHK-21 cells. Focus forming units (FFU) were stained with anti-envelope (E) MAb 4G2 (Henchal et al., 1982), anti-mouse IgG-HRP (Jackson Laboratories) secondary antibody, and TrueBlue™ (KPL) substrate. Titers were also determined by rapid immunofluorescence-based infectivity assay in human ARPE-19 cells, as previously described for HCMV (Zydek et al., 2014), using anti-E antibody.

Antibodies and reagents

See the supplement information for details.

Immunofluorescence staining

See the supplement information for details.

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Immunohistochemical staining

Sections from 31 donors were stained with hematoxylin and eosin or immunostained (Pereira et al., 2014). Antigen retrieval was performed for all antibodies using 10 mM sodium citrate, pH 6.0 under high pressure and heat.

Immunoblotting

See the supplement information for details.

Inhibitor studies

For Axl kinase inhibition, confluent AmEpC grown in 24-well plates were incubated with R428 (0.03–3.0 μM; ApexBio) for 1 h at 37°C, 5% CO_2, then replaced with 250 μl of R428-containing medium with virus (MR766 or Nica2–16 at MOI 0.014). After 2 h at 37°C, 250 μL of medium was added to each well, and plates were incubated overnight. Medium was then replaced, and cells were incubated 1–2 d and fixed (4% PFA) for immunostaining with anti-NS3 antibody. Total and NS3-positive cells were counted, and the percentage of cells infected was determined. For duramycin inhibition, virus was incubated in 250 μL of medium containing duramycin (0.04–1.0 μM; Sigma-Aldrich) for 1 h, and was then added to confluent cells. After 2 h at 37°C, 5% CO_2, 250 μL of medium was added to each well, and cells were incubated, fixed and analyzed as above.

Statistical analysis

See the supplement information for details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- ZIKV infects various primary human placental cell types and chorionic villus explants
- Target cells from early, mid and late gestation suggest two routes of ZIKV transmission
- Placental villi and amniochorionic membranes strongly express the ZIKV cofactor TIM1
- A TIM1 inhibitor blocks ZIKV infection of placental cells and chorionic villus explants

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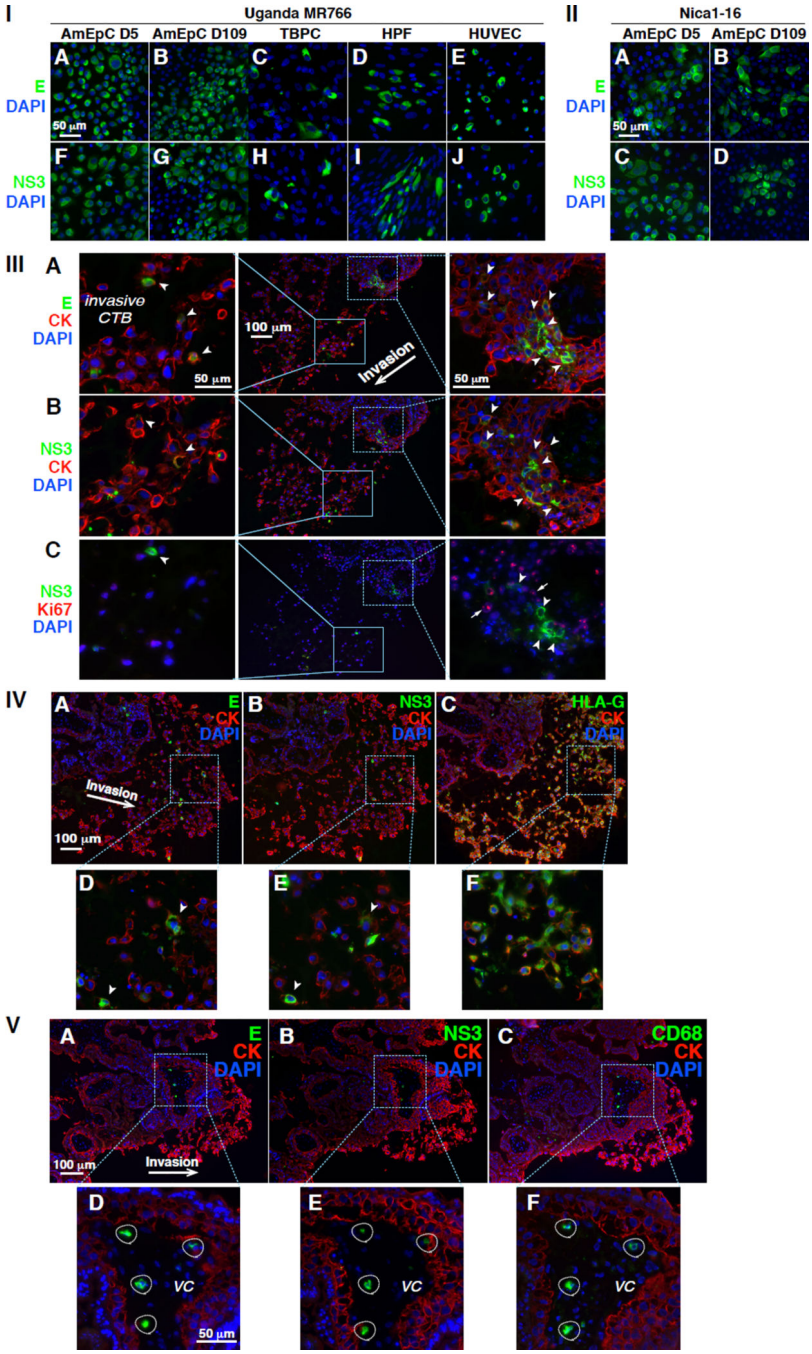

Figure 1. Prototype Uganda and Nicaraguan 2016 ZIKV strains infect primary cell types from human placentas

Panel I: Primary human placental cells were infected with ZIKV MR766 and immunostained for E (A–E) and NS3 (F–J) proteins at 3 dpi. (A, B, F, G) Primary amniotic epithelial cells (AmEpC) at mid and late gestation were infected at MOI 0.1. (C, H) Trophoblast progenitor cells (TBPC) (P15), (D, I) Human placental fibroblasts (HPF), and (E, J) Human umbilical vein endothelial cells (HUVEC) (P5) were infected at MOI 1. Panel II: Primary AmEpC from mid and late gestation were infected with ZIKV Nica1–16 at MOI 0.001 and immunostained for E glycoprotein (A, B) and NS3 protein (C, D) at 3 dpi. Panel

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III: (A–C) Chorionic villus explants (8.4 wks) infected with ZIKV MR766 (1×10^6 PFU) and immunostained for E and CK (A), NS3 and CK (B), and NS3 and Ki67 (C) in adjacent sections at 3 dpi. Left and right panels: higher magnification images. Arrowheads, cells expressing viral proteins; arrows, cells expressing Ki67. Images representative of n=4. Panel IV: Chorionic villus explants (8 wks) infected with ZIKV MR766 (1×10^6 PFU) and immunostained for E and CK (A), NS3 and CK (B), and MHC class I HLA-G (differentiation antigen) and CK (C). Panel V: (A–F) Chorionic villus explant infected with Nica1–16 (5×10^3 PFU) and immunostained for E and CK (A, D), NS3 and CK (B, E) and CD68 (Hofbauer cell/macrophage marker) and CK (C, F) in adjacent sections at 2 dpi. Villus core Hofbauer cells/Mϕ (circled) were positive for NS3 and E. Nuclei, blue (DAPI). VC, villus core. See also Figures S1 and S2 and Table S1.

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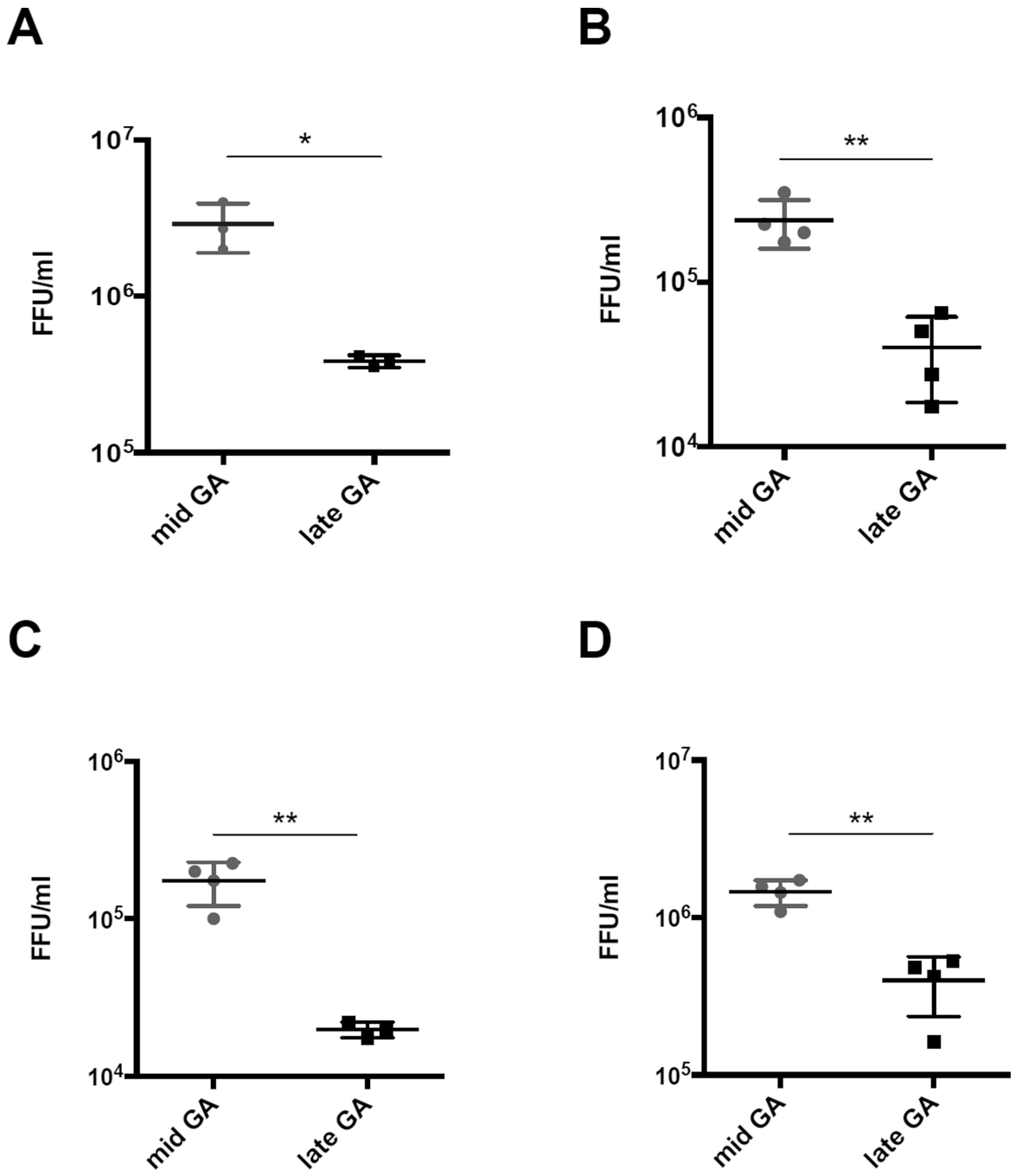

Figure 2. Viral titers from ZIKV-infected AmEpC from mid-gestation are higher than those from late gestation

AmEpC isolated from mid- (21.2 and 22.6 wks) and late- (38.6 and 40.2 wks) gestation were infected with (A) MR766 at MOI 0.001, (B, D) Nica1–16 at MOI 0.001, and (C) Nica2–16 at MOI 0.009. Viral titers were determined by FFA at 3 dpi (A) and 5 and 7 dpi (B, C). (D) Viral titers at 5 and 7 dpi were quantified in ARPE-19 cells, stained with mAb 4G2 at 28 hpi. GA, gestational age. n=4 from 2 independent experiments, *p<0.05, **p<0.01, unpaired t-test. See also Table S1.

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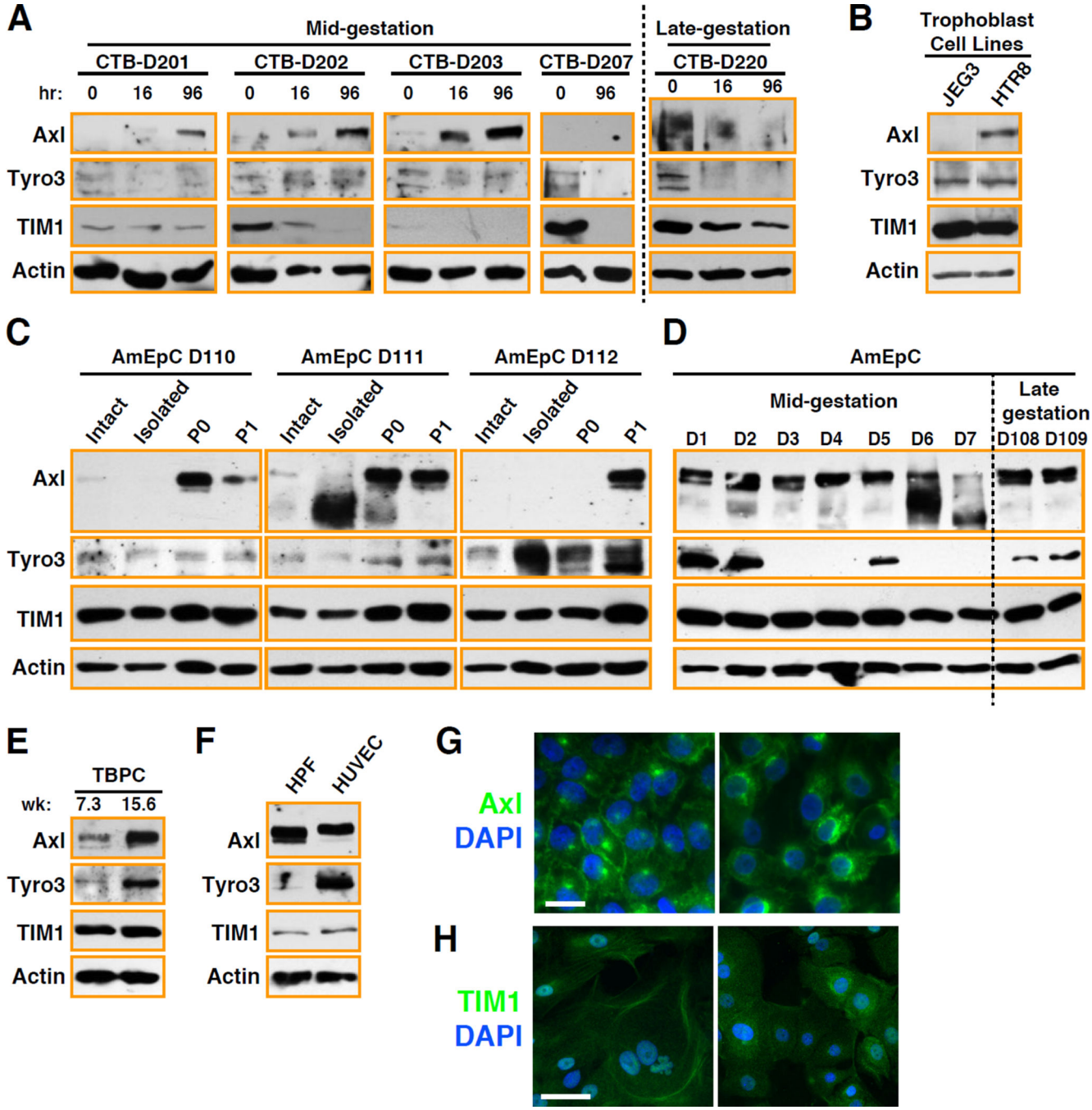

Figure 3. TIM1 is consistently expressed in primary placental cells, while Axl and Tyro3 expression varies according to cell type, differentiation state and individual donor

Expression of cofactors by immunoblotting in (A) differentiating CTB isolated from mid-gestation (n=4) and late-gestation placentas (n=1); (B) trophoblast cell lines JEG-3 and HTR8, (C) cells from 3 late-gestation amniotic membranes, including intact membranes, freshly isolated AmEpC before plating, and confluent P0 and P1 cells; (D) AmEpC (P1) from mid- and late-gestation placentas; (E) TBPC from early- and mid-gestation (P15) placentas; (F) HPF and HUVEC (P5). (G, H) Representative images of Axl and TIM1

staining in AmEpC from mid- (left) and late-gestation (right) placentas (n=6). See also Figure S2 and Table S3.

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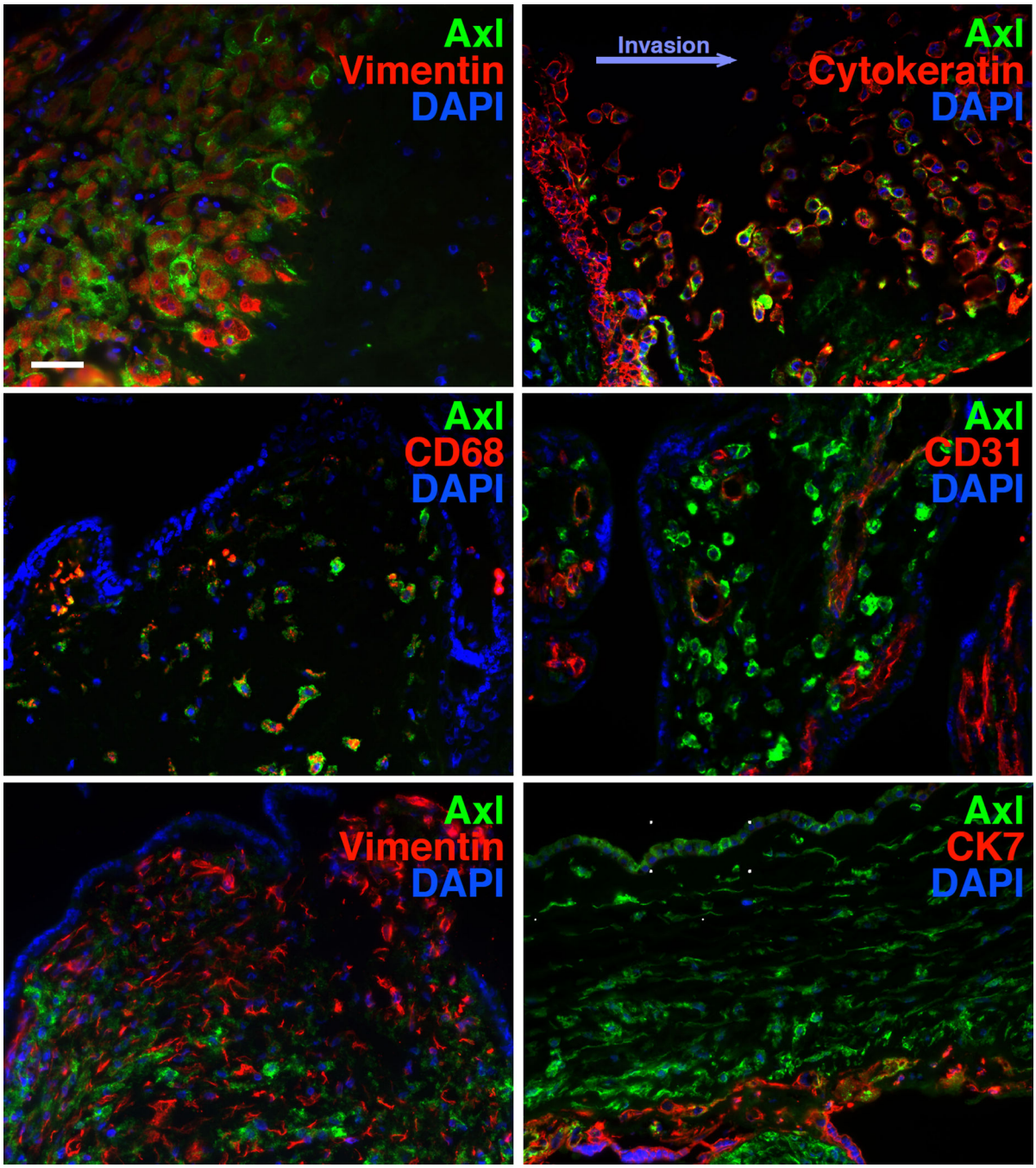

Figure 4. Axl is strongly expressed at the uterine-placental interface

Immunofluorescence staining of frozen sections from mid-gestation placentas for Axl (green) and cell type markers (red). (A) Axl and vimentin in cells of the basal decidua. (B) Axl and cytokeratin (mAb 7D3) in invasive CTB. (C) Axl colocalizes with CD68 (red), a marker of Hofbauer cells and macrophages (Mϕ). (D) Axl in CD31-positive vascular endothelial cells in a villus core. (E) Axl in villus core partially overlaps with vimentin, a marker of HPF. (F) Axl in the amniotic epithelium and cells of the mesenchymal portions of the amnion and chorion, where TBPC reside. Cytokeratin 7 (CK7) staining identifies CTB

lining the surface of the chorionic mesenchyme. DAPI, blue. Images representative of n=4. See also Figure S2 and Table S3.

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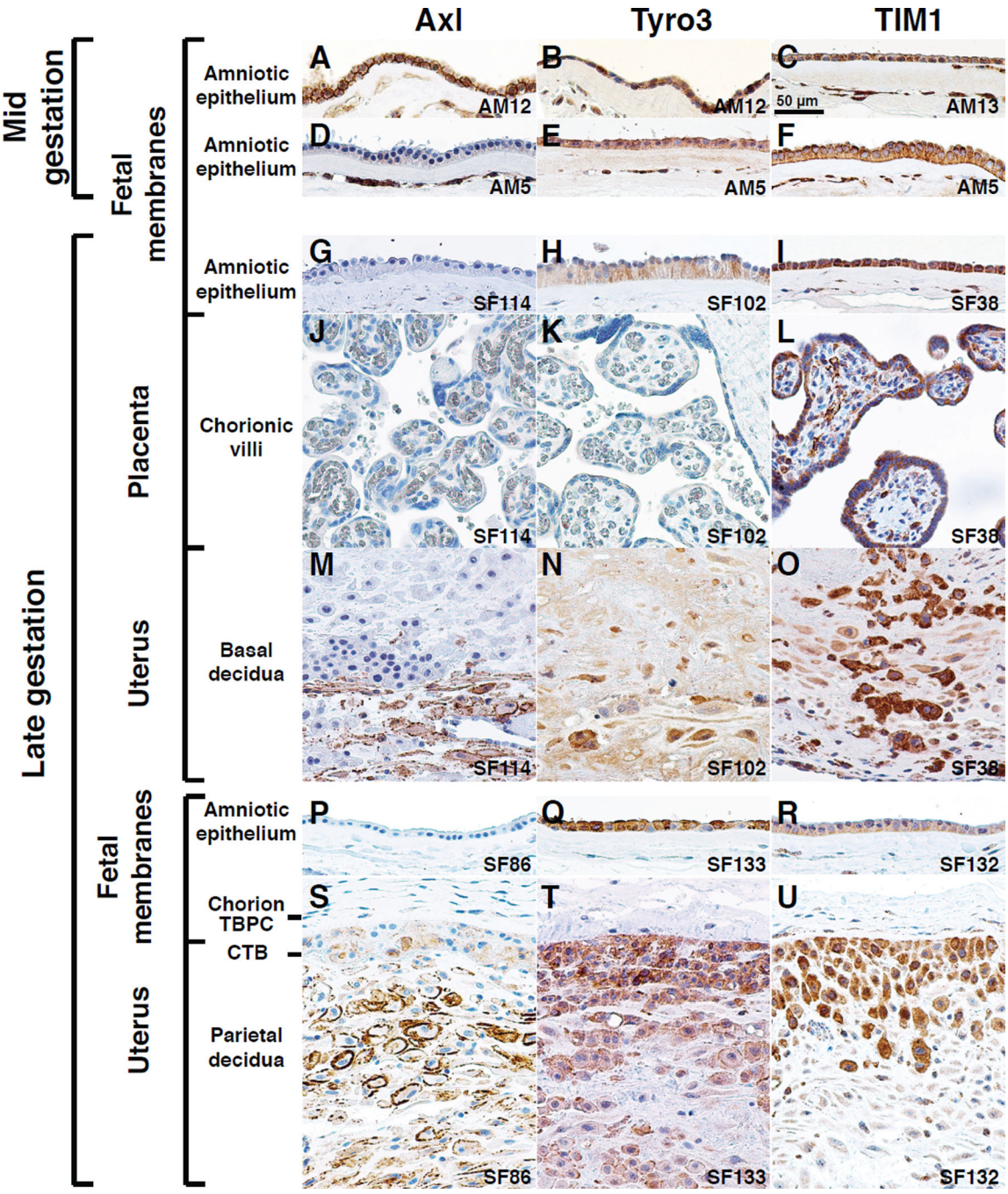

Figure 5. Axl and Tyro3 vary by site and gestational age, whereas TIM1 is consistently expressed

Axl, Tyro 3, and TIM1 expression in human placenta and fetal membranes was examined by immunohistochemical staining of fixed, paraffin-embedded placental biopsies from mid- and late-gestation (n=26). (A, D) Axl immunostaining in two mid-gestation amnions. (B, C, E, F) Mid-gestation AmEpC stain positively for Tyro 3 (B, E) and TIM1 (C, F). (G–O) Three zones of the maternal-placental interface from 3 placentas: amniotic epithelium on fetal side, bathed in amniotic fluid (G–I); chorionic villi of placenta with fetal blood vessels and villus core covered by STB in maternal blood (J–L); basal decidua containing invasive CTB from

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the placenta (M–O). (P–U) Interface of the amniochorion with parietal decidua from 3 placentas, including the amniotic epithelium (P–R) and the chorion, CTB layer and parietal decidua, containing invasive CTB (S–U). Images are representative of 26 donors (Table S2). Donor numbers are indicated in the lower right corner. See also Table S2.

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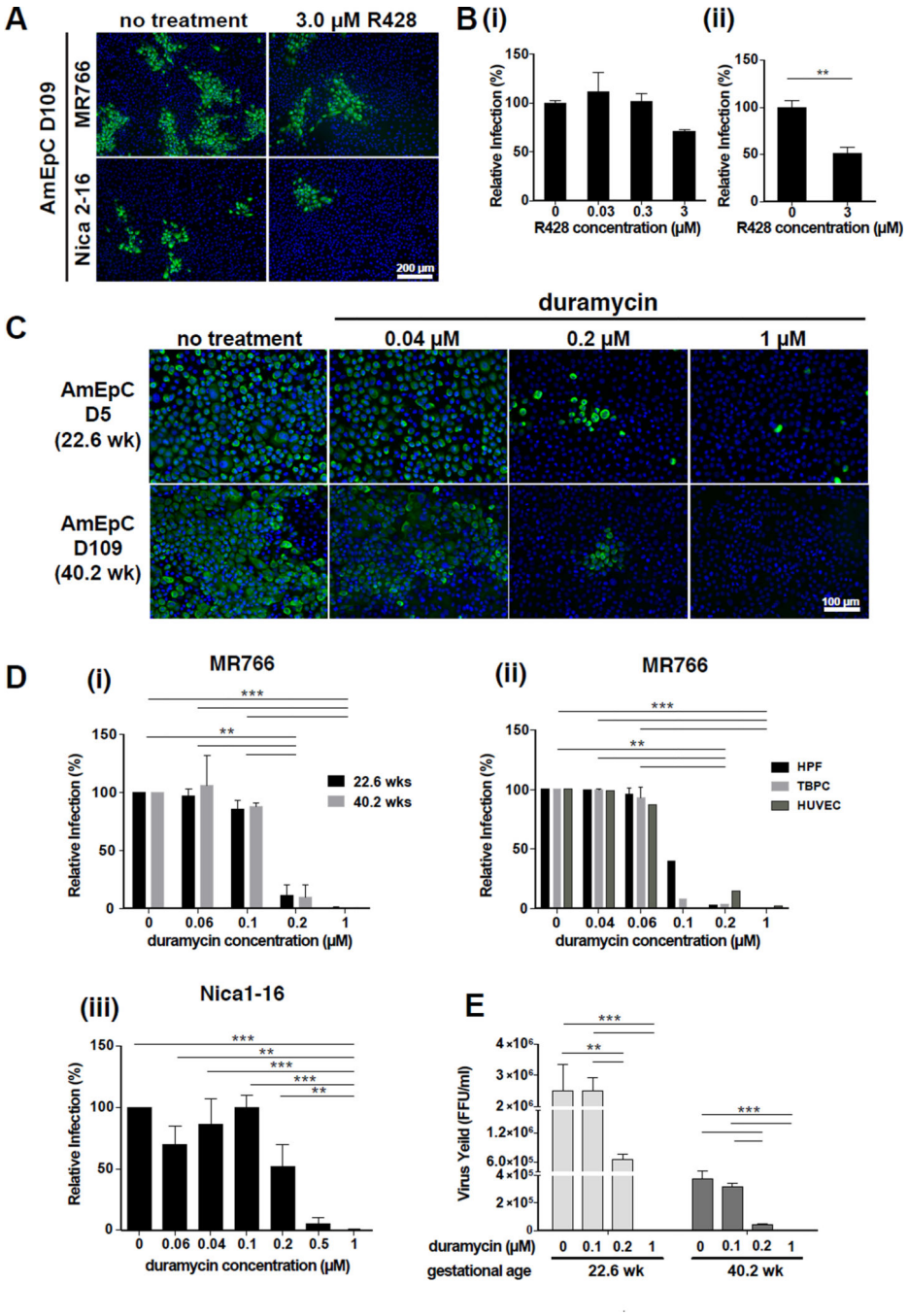

Figure 6. Axl and TIM1 inhibitors block ZIKV infection differentially in primary placental cells

(A and B) Axl kinase inhibitor reduces infection of (i) MR766 and (ii) Nica2–16 (both MOI 0.014) in AmEpC (40.2 wks). (A) Representative immunofluorescence images stained for NS3 and (B) quantification of relative infection (%) (mean±SD) of 2 replicates of each condition from 2 independent experiments. **p<0.01, unpaired t-test. (C and D) TIM1 inhibitor (duramycin) potently reduces ZIKV MR766 (MOI 0.3) and Nica1–16 (MOI 0.001) infection of various placental cell types. (C) Representative immunofluorescence images of ZIKV MR766 infection in AmEpC (22.6 and 40.2 wks). NS3 in green. (D) (i) Relative

infection of AmEpC with ZIKV MR766 (22.6 and 40.2 wks) and (ii) HPF, TBPC and HUVEC. n=2 for HPF and TBPC and n=1 for HUVEC. (iii) Relative infection of AmEpC with Nica1–16, n=3. (E) Virus titers from ZIKV MR766-infected AmEpC treated with duramycin at 3 dpi, quantified by FFA. n=2. D, E: **p<0.01, ***p<0.001, one-way ANOVA with Dunnett's post-test.

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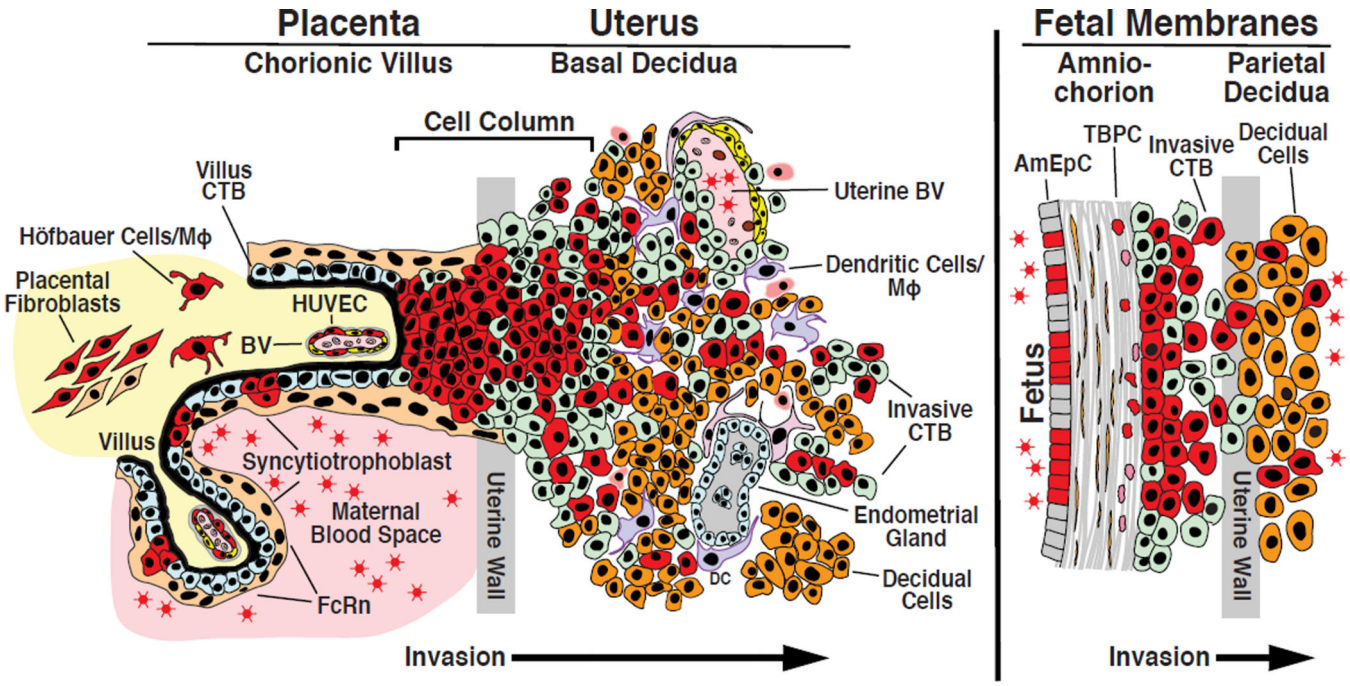

Figure 7. A model of uterine-placental interface indicating ZIKV infection of numerous cell types and possible routes of transmission

(Left). Cross-section of the pregnant uterus and anchoring villus with CTB that remodel blood vessels that channel maternal blood to the placenta surface. The basic structural unit is the chorionic villus, composed of a stromal fibroblast core with fetal capillaries and Hofbauer cells/macrophages (Mϕ). During early development, villus CTB detach from the basement membrane and either fuse to form STB, which covers floating villi, or proliferate and aggregate into a column of extravillus CTB that differentiate and invade the basal decidua and uterine wall (invasive CTB). These anchoring villi establish physical connections between mother and fetus through attachment of CTB cell columns. (Right) The amniochorionic membrane circumscribes the fetal compartment extending from the margins of the placenta to surround the fetus, with AmEpC in direct contact with amniotic fluid. At 15–20 wks gestation, as the amniotic sac enlarges, the amniochorionic membranes fuse and contact the parietal decidua, and a subset of CTB, derived from TBPC in the chorion and invade the parietal decidua lining the uterine wall (invasive CTB). ZIKV infects primary placental cells that strongly express TIM1 (red). These include proliferating villus CTB in proximal cell columns and invasive CTB, Hofbauer cells, HPF and HUVEC in chorionic villi and TBPC and AmEpC in fetal membranes, defining a continuum of susceptible cells that represent two routes of ZIKV transmission from the basal decidua to chorionic villi and fetal circulation (Left), and parietal decidua to amniochorionic membranes, amniotic fluid and the fetus (Right). Modified from Pereira, *et. al,* 2005.