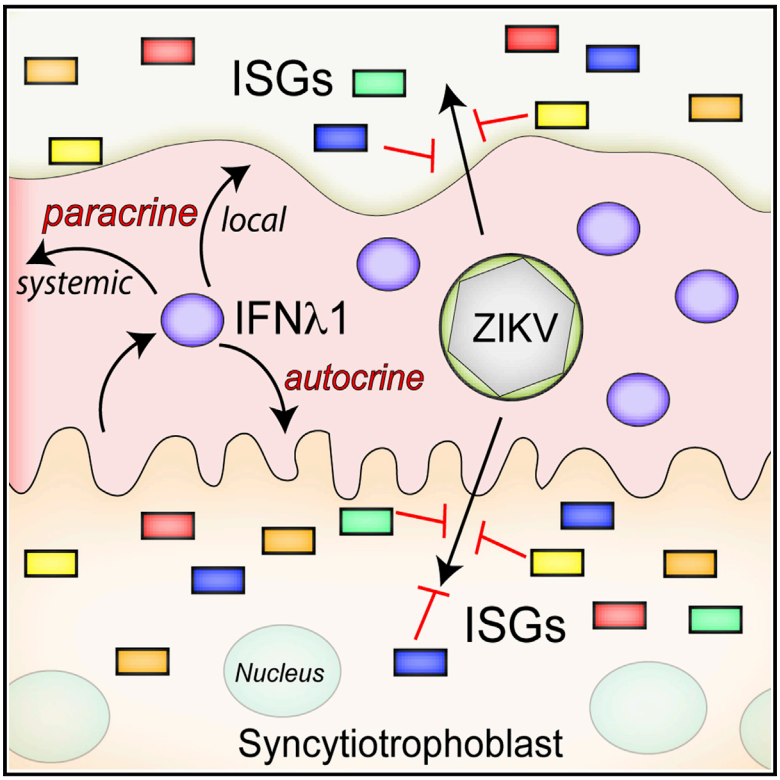
Short Article

Cell Host & Microbe

Type III Interferons Produced by Human Placental Trophoblasts Confer Protection against Zika Virus Infection

Graphical Abstract



Highlights

- Zika virus infects placental cell lines but not primary human trophoblast (PHT) cells
- PHT cells constitutively release the anti-viral type III interferon IFNλ1
- IFNλ1 acts in an autocrine and paracrine manner to protect cells from Zika virus

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In Brief

Bayer et al. find that primary human placental trophoblasts are refractory to ZIKV infection due to their constitutive release of antiviral type III interferons, which also serves to protect non-placental cells. These data suggest that rather than directly infecting the placenta, ZIKV likely uses alternative strategies to cross the placenta.

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Short Article

Type III Interferons Produced by Human Placental Trophoblasts Confer Protection against Zika Virus Infection

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SUMMARY

During mammalian pregnancy, the placenta acts as a barrier between the maternal and fetal compartments. The recently observed association between Zika virus (ZIKV) infection during human pregnancy and fetal microcephaly and other anomalies suggests that ZIKV may bypass the placenta to reach the fetus. This led us to investigate ZIKV infection of primary human trophoblasts (PHTs), which are the barrier cells of the placenta. We discovered that PHT cells from full-term placentas are refractory to ZIKV infection. In addition, medium from uninfected PHT cells protects non-placental cells from ZIKV infection. PHT cells constitutively release the type III interferon (IFN) IFNλ1, which functions in both a paracrine and autocrine manner to protect trophoblast and non-trophoblast cells from ZIKV infection. Our data suggest that for ZIKV to access the fetal compartment, it must evade restriction by trophoblast-derived IFNλ1 and other trophoblast-specific antiviral factors and/or use alternative strategies to cross the placental barrier.

INTRODUCTION

In eutherian organisms, the placenta acts as a physical and immunological barrier between the maternal and fetal compartments and protects the developing fetus from the vertical transmission of viruses. In the human hemochorial placenta, the frontline of fetal protection are the syncytiotrophoblasts, which cover the surfaces of the human placental villous tree and are directly bathed in maternal blood following the establishment of the maternal circulatory system during the later stages of the first trimester.

The mechanisms by which viruses can be transmitted vertically are multifaceted and can involve entry into the gestational sac via direct hematogenous spread, trophoblastic transcellular or paracellular pathways, transport within immune cells or infected sperm, pre-pregnancy uterine colonization, introduction during invasive procedures during pregnancy, and/or transvaginal ascending infection. The emerging Zika virus (ZIKV) pandemic poses a new threat to the developing fetus. While usually causing relatively mild symptoms in non-pregnant individuals, ZIKV infection in Brazil has been associated with increased incidence of microcephaly (Cauchemez et al., 2016; Oliveira Melo et al., 2016; Schuler-Faccini et al., 2016; Ventura et al., 2016a). In addition, ZIKV infections have also been associated with other disorders such as placental insufficiency and fetal growth restriction, ocular disorders, other CNS anomalies, and even fetal death (Brasil et al., 2016; Ventura et al., 2016b).

While direct evidence for a causal relationship between ZIKV infections and the development of abnormal pregnancy outcomes is still emerging, recent reports have directly identified the presence of viral RNA (vRNA) and infectious virus in the placentas, amniotic cavity, and brains of fetuses that had developed fetal anomalies (Calvet et al., 2016; Martines et al., 2016; Mlakar et al., 2016). Interestingly, other flaviviruses, such as dengue virus (DENV), which is endemic in the regions of Brazil most impacted by the recent ZIKV outbreak, have not been associated with microcephaly or other congenital disorders, suggesting that ZIKV may exhibit unique mechanism(s) to directly infect and/or bypass the placental barrier and to access the fetal compartment and cause organ-specific damage.

The innate immune system is a primary host defense strategy to suppress viral infections and converges on the induction of interferons (IFNs), which function in autocrine and paracrine manners to upregulate a cadre of other genes, known as IFN-stimulated genes (ISGs). The effects of IFNs and ISGs are potent and wide-ranging; they are pro-inflammatory, enhance adaptive immunity, and are directly antiviral (Schneider et al., 2014). In most cell types, type I IFNs, which include IFNα and IFNβ, are the primary IFNs that are generated in response to viral infections. In contrast, cells of epithelial origin mount antiviral responses primarily mediated by type III IFNs, which include IFNλ1–4 (also known as IL-29, IL-28A–C) (Lazear et al., 2015b). The role of IFN signaling in the protection of placental

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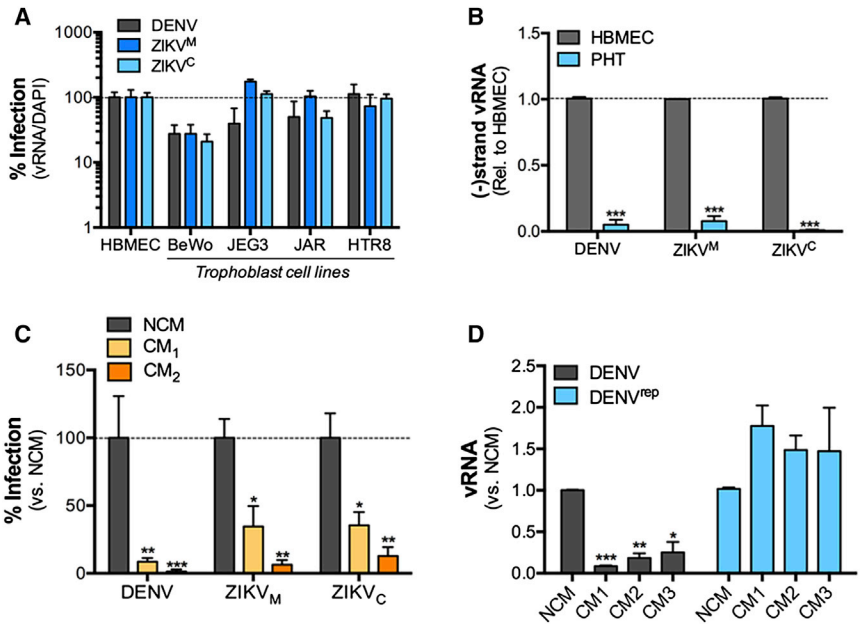

Figure 1. ZIKV Infects Placental Trophoblast Cell Lines, but Not PHT Cells

(A) The indicated cell lines were infected with DENV, ZIKV^M, or ZIKV^C for ~24 hr, fixed, and then stained with anti-dsRNA (J2) antibody. Data are shown as the percent of vRNA-positive cells relative to the total number of nuclei (as assessed by DAPI).

(B) Levels of DENV, ZIKV^M, or ZIKV^C negative-strand vRNA were assessed by RT-qPCR in HBMECs or PHT cells infected for ~48 hr.

(C) HBMECs were exposed to non-conditioned (NCM) PHT medium or conditioned PHT medium (CM, two independent preparations) for ~24 hr and then infected with DENV, ZIKV^M, or ZIKV^C. The level of infection was assessed by fluorescence microscopy for dsRNA. Data are shown as the percent of vRNA-positive cells relative to the total number of nuclei (as assessed by DAPI).

(D) Control HeLa cells or HeLa cells constitutively expressing a DENV replicon were exposed to NCM or three independent preparations of PHT CM, and then the levels of DENV vRNA were assessed by RT-qPCR ~24 hr after exposure. In all, data are shown as mean ± SD (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

trophoblasts from viral infections is unclear. Previous work has pointed to unidentified IFN(s) present in first-trimester human placentas (Lefèvre and Boulay, 1993). Ruminants express IFNτ at various stages of gestation (Bazer et al., 1996), and the mouse placenta can produce IFNλs in response to *Listeria monocytogenes* infection (Bierne et al., 2012).

Here we show that primary human trophoblast (PHT) cells, isolated from full-term placentas, are refractory to infection by two strains of ZIKV, one derived from an African lineage, and one derived from an Asian lineage that exhibits > 99% amino acid sequence similarity to strains currently circulating in Brazil (Haddow et al., 2012). We also found that conditioned medium (CM) isolated from PHT cells protected non-trophoblast cells from ZIKV infection through the constitutive release of the type III IFN IFNλ1. Our findings thus suggest that for ZIKV to infect syncytiotrophoblasts, it must overcome the restriction imparted by IFNλ1 and other syncytiotrophoblast-specific antiviral factors and/or gain access to the fetal compartment by a mechanism that does not involve syncytiotrophoblast infection, at least in the latter stages of pregnancy.

RESULTS

PHT Cells Resist ZIKV Infection

To assess the ability of ZIKV to replicate in human placental trophoblasts, we measured the replication of two strains of ZIKV, one of African lineage (Haddow et al., 2012) (MR766, termed ZIKV^M hereafter) and one of Asian lineage (Haddow et al., 2012) (FSS13025, termed ZIKV^C hereafter) in PHT cells and a panel of trophoblast-derived cell lines including BeWo, JEG-3, and JAR choriocarcinoma cells and the extravillous trophoblast cell line HTR8/SVneo (Graham et al., 1993). In addition, we compared the level of infection of these cell types by DENV. We also compared the infectivity of these cell types with that of human brain microvascular endothelial cells (HBMECs), a cell-based model of the blood-brain barrier (Stins et al., 2001) that is permissive to DENV and both strains of ZIKV (Figure 1A, Figure S1A). We found that BeWo, JEG-3, JAR, and HTR8/SVneo cells supported infection by both ZIKV^M and ZIKV^C, although BeWo cells were less susceptible to infection by both DENV and ZIKV than the other trophoblast-derived cells lines (Figure 1A, Figure S1A). In contrast, we were unable to detect any evidence of ZIKV or DENV replication in PHT cells by immunofluorescence microscopy (not shown). Consistent with this, we found that PHT cells resisted infection by ZIKV^M, ZIKV^C, and DENV, as evidenced by very low levels of total vRNA (Figures S1B and S1C) and the lack of production of the negative strand of vRNA, which is only produced during viral replication (Figure 1B). These results are consistent with our previous observations that PHT cells resist infection by diverse RNA and DNA viruses (Delorme-Axford et al., 2013) and show that ZIKV is unable to replicate efficiently in primary trophoblasts.

CM Isolated from PHT Cells Protects Non-placental Cells from ZIKV Infection

In addition to the resistance of PHT cells to ZIKV infection, we found that CM isolated from uninfected PHT cells protected non-placental recipient cells from infection by both isolates of ZIKV and DENV (Figure 1C). Interestingly, we found that this protection was lost when CM was added after the establishment of viral replication, as PHT CM exhibited no inhibitory effects on the production of vRNA in cells stably propagating a DENV subgenomic replicon (Figure 1D, Figures S1D and S1E).

Using microarrays, we found that exposure of human fibrosarcoma HT1080 (2fTGH) cells to PHT CM induced a subset of previously characterized ISGs (Schoggins et al., 2011), which did not occur in HT1080 cells with defective signal transducer and activator of transcription 1 (STAT1; 2fTGH-U3A cells) signaling (McKendry et al., 1991) (Figure 2A, Table S1). We obtained

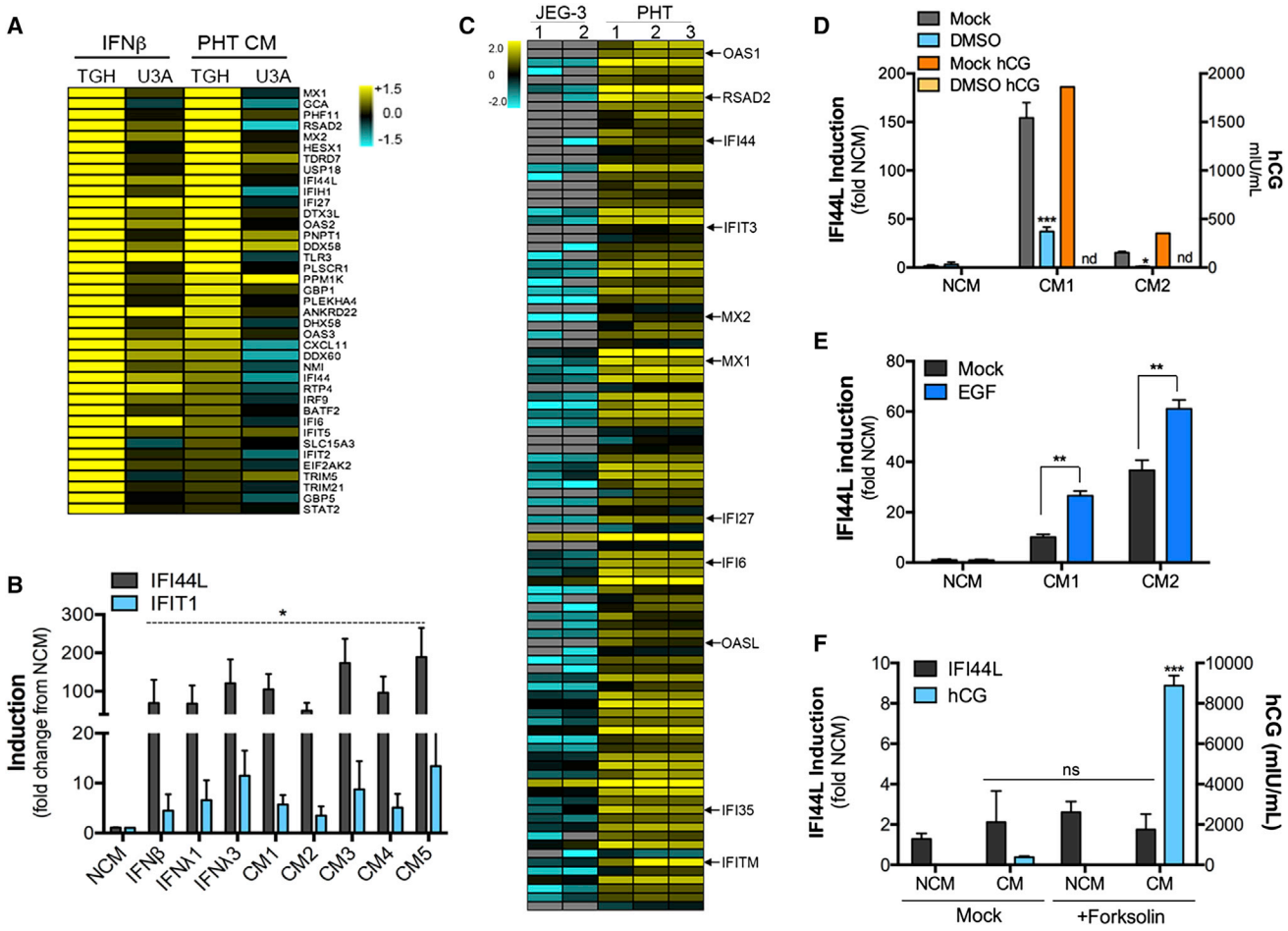

Figure 2. CM from PHT Cells Induces ISGs

(A) A heat map of IFN-stimulated genes (ISGs) differentially expressed between control (TGH) and STAT1 signaling-deficient (U3A) HT1080 cells exposed to purified IFNβ or PHT CM for 24 hr.

(B) RT-qPCR analysis for IFI44L or IFIT1 in U2OS cells exposed to control PHT non-conditioned medium (NCM) or five independent preparations of PHT CM. Data are shown as a fold change from NCM.

(C) Heat map of differentially expressed IFN-stimulated genes (ISGs) between two cultures of JEG-3 cells and two preparations of PHT cells (samples 2 and 3 are biological replicates of the same PHT preparation) as assessed by RNA-seq ($p < 0.05$).

(D) Two preparations of PHT cells were exposed to dimethyl sulfoxide (DMSO) to inhibit cell fusion, CM was collected, and then IFI44L induction was assessed by RT-qPCR (left y axis). In parallel, the levels of human chorionic gonadotropin (hCG) were determined by ELISA (right y axis).

(E) Two preparations of PHT cells were exposed to epidermal growth factor (EGF) to enhance cell fusion, CM was collected, and then IFI44L induction was assessed by RT-qPCR.

(F) BeWo cells were exposed to forskolin to induce fusion, CM was collected, and ISG induction in CM-exposed cells was assessed by RT-qPCR (for IFI44L, left y axis). In parallel, the levels of hCG were assessed by ELISA (right y axis).

In (B) and (D)–(F), data are shown as mean ± SD (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant; nd, not detected). The color intensity in (A) and (C) indicates the level of gene expression (yellow for upregulation and blue for downregulation), and gray indicates that no transcripts were detected in that sample.

similar results when cells were treated with IFNβ (Figure 2A) as previously described (Shu et al., 2015). We confirmed these results by RT-qPCR in human osteosarcoma U2OS cells that were exposed to PHT CM, which led to the robust induction of two known ISGs, IFN-induced protein 44-like (IFI44L) and IFN-induced protein with tetratricopeptide repeats 1 (IFIT1) (Figure 2B), and in human monocyte THP-1 cells as determined by an IFN regulatory factor (IRF)-inducible SEAP reporter assay (Figure S2A). In addition, RNA-seq revealed that PHT cells express high levels of ISGs (Figure 2C, Table S2). In contrast, the trophoblast cell line JEG-3 did not endogenously express ISGs (Figure 2C, Table S2), and CM isolated from these cells did not induce ISGs in non-placental recipient cells (Figure S2B).

During culturing in vitro, PHT cells undergo fusion to form syncytiotrophoblasts (Figure S2C) similar to their natural differentiation process in vivo, which can be inhibited by exposing the cultures to dimethyl sulfoxide (DMSO) (Thirkill and Douglas, 1997). We found that attenuation of PHT differentiation by DMSO reduced the ability of PHT CM to induce IFI44L in recipient cells (Figure 2D). Consistent with a role for syncytiotrophoblast fusion in the induction of ISGs, we found that exposure of PHT cells to epidermal growth factor (EGF), which promotes cell-cell fusion of

trophoblasts (Morrish et al., 1987), enhanced the ISG-inducing properties of PHT CM (Figure 2E). Importantly, ISG induction in recipient cells was specific for PHT CM and did not occur when cells were exposed to CM from the trophoblast-derived cell lines BeWo, JEG-3, JAR, or HTR8 cells, suggesting that this induction is specific for CM derived from primary trophoblasts (Figure 2E, Figure S2B). Furthermore, although BeWo cell fusion can be stimulated by forskolin treatment (Wice et al., 1990), this treatment did not confer ISG-inducing properties to BeWo CM (Figure 2F), suggesting that cell-cell fusion alone is not sufficient to confer ISG-inducing properties to trophoblasts. Lastly, we previously showed that PHT-derived exosomes released into PHT CM mediated some of the antiviral properties of PHT CM (Delorme-Axford et al., 2013). We found that CM depleted of vesicles was still capable of inducing ISGs in recipient cells (Figure S2D), indicating that an ISG-inducing pathway is present in PHT CM and bestows antiviral properties independently from PHT-derived exosomes.

PHT Cells Release the Type III IFN IFNλ1

We found by ELISAs that PHT CM contained negligible levels of IFNβ that were comparable to those in control non-CM, but contained IFNλ1 and, to a lesser extent, IFNλ2, which was detected in one PHT preparation (Figure 3A). In addition, PHT cells expressed high levels of IFNλ1 mRNA (Figure 3B), which were consistent with the levels induced in non-PHT cells (HBMECs) transfected with the synthetic ligand polyinosinic-polycytidylic acid (poly I:C) to induce IFN production (Figure 3B). In addition, we found that anti-IFNλ1/2 neutralizing antibodies partially inhibited the induction of the ISG IFI44L by PHT CM (Figure 3C). Furthermore, although CM isolated from uninfected trophoblast-derived cell lines did not contain detectable levels of IFNλ1 (Figure S3A), we found that these cells potently induced type III IFNs, primarily IFNλ1, in response to infection by Sendai virus (SeV, Figure 3D) and by both DENV and ZIKV (Figure 3E). In contrast, PHT cells did not induce IFNλ1 or the ISG 2′-5′-oligoadenylate synthetase 1 (OAS1) in response to ZIKV or DENV infection, yet were highly resistant to infection when compared to JEG-3 cells (Figure 3F, Figure S3B). However, PHT cells do induce both IFNλ1 and ISGs in response to Toll-like receptor 3 (TLR3) stimulation by poly I:C (Figure S3C). Finally, we found that RNAi-mediated silencing of a subunit of the type III IFN receptor (IL28RA) partially restored ZIKV infection in recipient cells exposed to PHT CM depleted of vesicles (Figure 3G). Collectively, these data point to a direct role for type III IFNs, particularly IFNλ1, in the antiviral signaling of placental syncytiotrophoblasts to restrict viral infections, including ZIKV.

DISCUSSION

The strong association between ZIKV infection in pregnant women with the development of fetal growth restriction and/or CNS and other fetal congenital abnormalities, in addition to the positive culture of ZIKV from feto-placental tissues of affected pregnancies, suggests that ZIKV is capable of gaining access into the intrauterine cavity to directly affect fetal development. Our work presented here suggests that ZIKV is unlikely to access the fetal compartment by its direct replication in placental syncytiotrophoblasts, at least in the later stages of pregnancy, unless ZIKV can bypass the antiviral properties of type III IFNs and other syncytiotrophoblast-derived antiviral pathways during in vivo infection of pregnant women. Because we observed potent protection from ZIKV infection by type III IFNs, specifically IFNλ1, which is constitutively produced by syncytiotrophoblasts, it is likely functioning in an autocrine manner to protect these cells from viral infections. In addition, we show that trophoblast-derived IFNλ1 protects non-placental cells from ZIKV infection in a paracrine manner. A schematic of the human placenta and the mechanisms by which IFNλ1 protects syncytiotrophoblasts from ZIKV infection is shown in Figure 4. Our work thus provides evidence that ZIKV may not directly infect placental villous syncytiotrophoblasts during later stages of pregnancy, suggesting instead that the virus must either evade the potent type III IFN antiviral signaling pathways generated by these cells and/or bypass these cells through an as-yet-unknown pathway to gain access to the fetal compartment.

Our previous studies implicated a role for trophoblast-specific miRNAs associated with the placental-specific chromosome 19 miRNA cluster (C19MC), contained within PHT-derived exosomes, as part of the antiviral arsenal secreted by PHT cells (Bayer et al., 2015; Delorme-Axford et al., 2013). Indeed, our work presented here demonstrates another facet of the antiviral mechanisms engaged by PHTs to protect the developing fetus. These potent antiviral pathways likely function in parallel to provide multiple mechanisms to protect syncytiotrophoblasts and other cell types at the maternal-fetal interface from ZIKV and other viruses. It is also possible that other as-yet-undiscovered pathways intrinsic to placental trophoblasts provide additional mechanisms to protect these cells from viral infections. While we have not been able to reliably measure IFNλ in the plasma of pregnant women, this may be because IFNλ is below the limits of detection in the expanded plasma volume of pregnant women and/or that the effects of IFNλ are local, affecting trophoblastic and non-trophoblastic placental cells (such as villous fibroblasts) in the immediate vicinity of the feto-placental unit.

Type III IFNs share significant structural homology with members of the IL-10 cytokine family (Gad et al., 2009), but induce ISGs similar to type I IFNs (Kotenko et al., 2003) through a distinct receptor (Sheppard et al., 2003). We found that PHT cells expressed high levels of IFNλ1. Remarkably, IFNλ1 was constitutively released from PHT cells and did not require the activation of antiviral innate immune signaling pathways to become induced. Thus, in addition to studies that implicate an important role for type III IFNs in antiviral signaling in the respiratory and gastrointestinal tracts and the blood-brain barrier (Lazear et al., 2015b), our work directly points to a role for type III IFNs, specifically IFNλ1, in antiviral signaling at the maternal-fetal interface. Although type I IFNs are conserved between mice and humans, there is significant divergence in the type III IFN pathway, where humans express IFNλ1–4 and mice express only IFNλ2 and IFNλ3. PHT cells expressed IFNλ2 at significantly lower levels than IFNλ1 and did not express mRNA for either IFNλ3 or IFNλ4. Thus, in addition to the morphological differences between the human and mouse placentas (Maltepe et al., 2010), these data suggest that the IFNλ1-mediated antiviral properties of placental syncytiotrophoblasts may be distinct

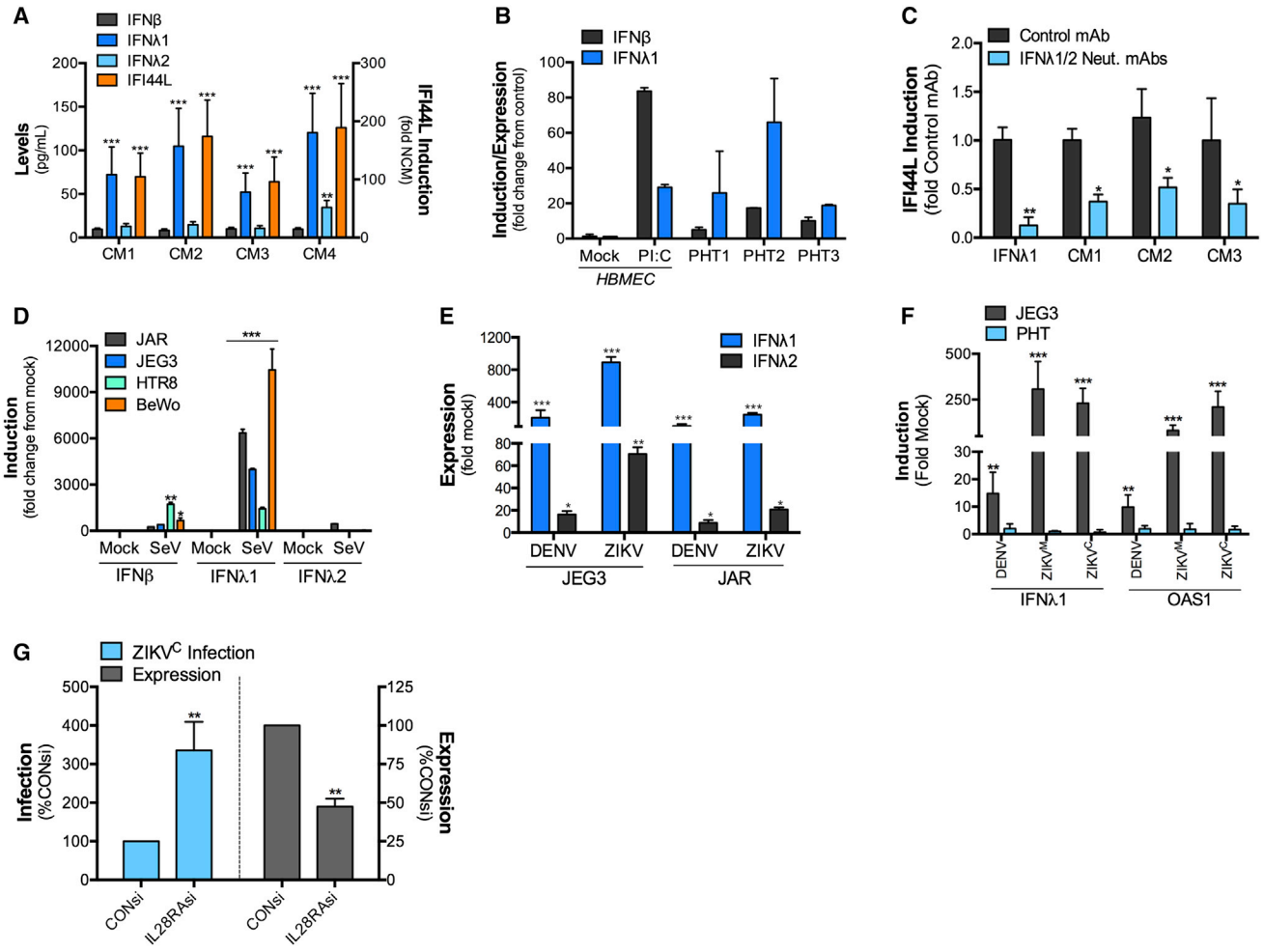

Figure 3. CM from PHT Cells Contains IFNλ1, which Is Required for ISG Induction

(A) ELISA for IFNβ, IFNλ1, and IFNλ2 in four independent PHT CM preparations (left y axis). In parallel, the extent of ISG induction in each sample was determined by RT-qPCR for the levels of IFI44L induced in U2OS cells exposed to the sample (right y axis).

(B) The levels of IFNβ and IFNλ1 mRNA in three preparations of PHT cells was assessed by RT-qPCR. In parallel, IFNβ and IFNλ1 mRNA levels were determined in mock-treated HBMECs or in HBMECs exposed to 10 μg poly I:C ("floated" in the medium) for ~24 hr. Data are shown as a fold change from mock-treated HBMECs.

(C) Level of ISG induction (as assessed by IFI44L RT-qPCR) in U2OS cells exposed to purified IFNλ1 or to three preparations of PHT CM incubated with a non-neutralizing monoclonal antibody (MOPC21) or anti-IFNλ1–3 neutralizing antibodies.

(D) RT-qPCR for IFNβ, IFNλ1, or IFNλ2 in indicated trophoblast cell lines infected with Sendai virus (SeV) for ~24 hr.

(E) RT-qPCR for IFNλ1 or IFNλ2 in the indicated trophoblast cell lines infected with DENV or $ZIKV^M$ for ~24 hr.

(F) RT-qPCR for IFNλ1 or OAS1 in JEG-3 or PHT cells infected with DENV, $ZIKV^M$, or $ZIKV^C$ for ~24 hr.

(G) $ZIKV^C$ infection in HBMECs transfected with control siRNA (CONsi) or IL28RA siRNAs and exposed to PHT CM depleted of vesicles for ~24 hr prior to infection.

In all panels, data are shown as mean ± SD (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant).

between humans and mice, which may complicate the use of the mouse placenta as a model for viral infections of the placenta during human pregnancy.

Another important implication of our work is that cells that do not express the type III IFN receptor or do not respond robustly to type III IFNs may be more susceptible to ZIKV infection, particularly at the maternal-fetal interface. In mice, the expression of the α subunit of the IFNλ receptor (IL-28RA) is restricted to epithelial-derived cells, which respond most robustly to type III IFNs (Sommereyns et al., 2008). Recent evidence also supports a role for type III IFNs in the microvascular endothelium comprising the blood-brain barrier (Lazear et al., 2015a). Because syncytiotrophoblasts and other trophoblasts that are epithelial are likely protected by the potent stimulation of ISGs in response to their constitutive production of IFNλ1, our data suggest that ZIKV may invade the intrauterine cavity by mechanisms that are independent of direct trophoblast infection. In addition to the trophoblast cell layers, the human placenta is also composed of mesenchymal cells, placental-specific macrophages (termed Hofbauer cells), and fibroblasts located within the villous core between trophoblasts and fetal vessels that may exhibit differences in their responsiveness to IFNλs. In addition, it is also

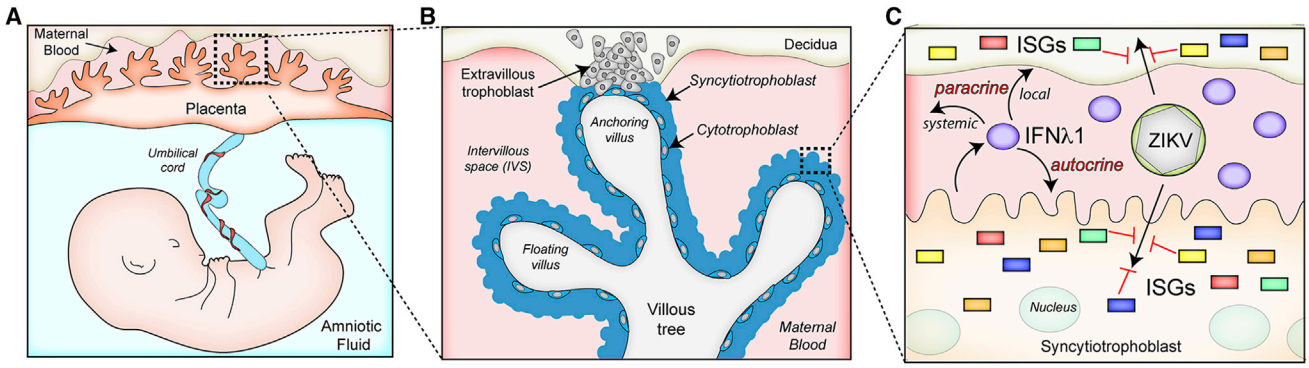

Figure 4. Schematic Depicting the Structure of the Human Placenta and the Role of IFNλ1 in Protecting against ZIKV Infection

(A) The intrauterine environment during human pregnancy. Embryonic structures include the villous tree of the human hemochorial placenta and the umbilical cord, which transfers blood between the placenta and the fetus.

(B) An overview of a single placental villus. Extravillous trophoblasts invade and anchor the placenta to the maternal decidua and to the inner third of the myometrium. The villous tree consists of both floating and anchoring villi. Multinucleated syncytiotrophoblasts overlie the surfaces of the villous tree and are in direct contact with maternal blood, which fills the intervillous space (IVS) once the placenta is fully formed. Mononuclear cytotrophoblasts are subjacent to the syncytiotrophoblasts and the basement membrane of the villous tree and serve to replenish the syncytiotrophoblast layer throughout pregnancy.

(C) In the work presented here, we show that syncytiotrophoblasts release IFNλ1 that can act in both autocrine and paracrine manners to induce ISGs, which protect against ZIKV and other viral infections. The paracrine function of IFNλ could work locally within the direct maternal-fetal compartment or might circulate more systemically to act on other maternal target cells.

possible that less differentiated, first-trimester trophoblasts as well as extravillous trophoblasts are more permissive than late-pregnancy villous trophoblasts to ZIKV infection and/or the antiviral effects of IFNλs. Finally, it is possible that the levels of IFNλ1 vary throughout pregnancy, or between individuals, which could markedly impact the ability of the virus to infect the syncytiotrophoblast cell layer at specific times during pregnancy or in specific individuals.

The rapidly emerging human health crisis associated with the ZIKV epidemic highlights the growing need to identify mechanisms by which ZIKV accesses the fetal compartment. These data will be instrumental in order to design therapeutic measures to limit ZIKV replication and/or spread. Our experimental cell system is directly relevant to the study of congenital ZIKV infections, by defining unique antiviral mechanisms at play in this specialized environment. We provide evidence that ZIKV is unlikely to access the fetal compartment by its direct infection of late-pregnancy villous syncytiotrophoblasts and potentially neighboring cells that express IL28RA, due to the role of type III IFNs in the antiviral defense produced by human trophoblasts, which suggests that the virus may circumnavigate these cells or overcome this restriction in vivo in order to bypass the placental barrier.

EXPERIMENTAL PROCEDURES

Culture of PHTs

PHT cells were isolated from healthy singleton term placentas using the trypsin-DNase-dispase/Percoll method as described (Kliman et al., 1986), with previously published modifications under an exempt protocol approved by the institutional review board at the University of Pittsburgh. Patients provided written consent for the use of de-identified and discarded tissues for research purposes upon admission to the hospital. Cells were maintained in DMEM (Sigma) containing 10% FBS (HyClone) and antibiotics at 37°C in a 5% CO_2 air atmosphere. Cells were then maintained for 72 hr after plating, with cell quality ensured by microscopy and production of human chorionic gonadotropin (hCG), determined by ELISA (DRG International). The cells exhibited a characteristic increase in medium hCG levels as the cytotrophoblasts differentiated into syncytiotrophoblasts.

Cells and Viruses

Human osteosarcoma U2OS cells, Vero cells, 2fTGH (STAT1 wild-type) cells, and U3A (STAT1 mutant) fibrosarcoma cells (previously described in McKendry et al., 1991) were cultured in DMEM supplemented with 10% FBS and antibiotics. BeWo cells were maintained in F12K Kaighn's modified medium supplemented with 10% FBS and antibiotics. JAR cells and immortalized, human, first-trimester, extravillous trophoblast cells (HTR8/SVneo) were maintained in RPMI 1640 medium supplemented with 10% FBS with antibiotics. Human choriocarcinoma JEG-3 cells were maintained in Eagle's Minimum Essential Medium (EMEM), supplemented with 10% FBS with antibiotics. HBMECs were maintained in RPMI 1640 medium supplemented with 10% FBS, 10% NuSerum, Minimum Essential Medium (MEM) vitamins, non-essential amino acids, sodium pyruvate, and antibiotics. HeLa CCL-2 cells were maintained in MEM supplemented with 10% FBS, non-essential amino acids, sodium pyruvate, and antibiotics. Development of HeLa cells stably propagating a DENV subgenomic replicon has been previously described (Ansarah-Sobrinho et al., 2008). Plasmids used to generate stable replicon cells were provided by Theodore Pierson (Viral Pathogenesis Section Laboratory of Viral Diseases, NIH/NIAID). *Aedes albopictus* midgut C6/36 cells were maintained in DMEM supplemented with 10% FBS and antibiotics at 28°C in a 5% CO_2 air atmosphere.

DENV2 16681 and ZIKV FSS13025 (Cambodian origin) were propagated in C6/36 cells, as previously described (Vasilakis et al., 2008). ZIKV MR766 (Ugandan origin) was propagated in Vero cells. Viral titers were determined by fluorescent focus assay, as previously described (Payne et al., 2006), using anti-DENV envelope protein monoclonal antibody 4G2 (provided by Margaret Kielian, Albert Einstein College of Medicine) for DENV and anti-double-stranded RNA monoclonal antibody J2 (provided by Saumendra Sarkar, University of Pittsburgh) for ZIKV (specificity of the J2 antibody is shown in Figure S1G). SeV was purchased from Charles River Laboratories. Experiments measuring productive DENV and ZIKV infection were performed with 1–3 focus-forming units/cell for 24 hr, unless otherwise stated, and SeV was used at 100 hemagglutination units/cell for 24 hr. Infection was determined by either RT-qPCR or immunofluorescence microscopy, as stated in the figure legends.

Preparation and Characterization of CM

CM samples from PHT cells or other cells were harvested at 72 hr after plating, followed by centrifugation at 800× *g* for 5 min. Non-conditioned medium

(NCM) was complete PHT medium (described above) that had not been exposed to PHT cells. Recipient cells were exposed to CM for ~24 hr before assays. Vesicle-depleted CM was generated by three centrifugation steps: 2,500× *g* for 5 min at room temperature, followed by 12,000× *g* for 20 min at room temperature, and 100,000× *g* for 2 hr at 4°C. Antiviral activity of CM preparations was determined in HBMECs exposed to CM for 24 hr prior to infection with DENV, ZIKV^{M}, or ZIKV^{C}.

RNA Extraction, cDNA Synthesis, and RT-qPCR

For cellular mRNA analysis, total RNA was extracted using TRI reagent (MRC) or GenElute total RNA miniprep kit (Sigma) according to the manufacturer's protocol. RNA samples were treated with RNase-free DNase (QIAGEN or Sigma). Total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) or iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Strand-specific cDNA was produced with primers targeting the negative RNA strand DENV or ZIKV using iScript Select cDNA Synthesis Kit (Bio-Rad). RT-qPCR was performed using SYBR Select or iQ SYBR Green Supermix (Bio-Rad) in a StepOnePlus real-time PCR system (Applied Biosystems), ViiA 7 System (Applied Biosystems), or CFX96 Real-Time System (Bio-Rad). Gene expression was calculated using the 2-delta delta CT method normalized to GAPDH or actin. Primer sequences are located in the Supplemental Experimental Procedures. The specificity of ZIKV and DENV primers were confirmed by RT-qPCR analysis (Figure S1F).

RNA-Seq and Microarray Analyses

RNA-seq from JEG-3 and PHT cells was performed as previously described (McConkey et al., 2016). Briefly, libraries were prepared with the NEB Ultra Library Preparation Kit, and library quality was determined using the Qubit Assay and the Agilent 2100 Bioanalyzer. Sequencing was performed with the Illumina HiSeq 2500 rapid-run mode on one flow cell (two lanes). CLC Genomics Workbench 8 (QIAGEN) was used to process, normalize, and map sequence data to the human reference genome (hg19). Differentially expressed genes were identified using DESeq2 (Love et al., 2014) with a significance cut-off of 0.05, and heat maps were generated using MeViewer software. Files associated with RNA-seq studies have been deposited into Sequence Read Archive under accession number SRA: SRP067137.

We used high-throughput microarray analysis as previously described (Shu et al., 2015) to screen for transcriptional changes in control (2fTGH) versus STAT1 signaling-deficient (U3A) HT1080 cells, both exposed to 100 U of purified IFNβ (PBL) or PHT CM for 24 hr. In parallel, mock-treated 2fTGH and U3A were also included and were used to identify differentially expressed genes in IFNβ- and CM-treated cells. Datasets related to these arrays have been deposited in GEO: GSE72342.

Statistics

Experiments were performed at least three times as indicated in the figure legends or as detailed. Data are presented as mean ± SD. Except where specified, a Student's t test was used to determine statistical significance for virus infection assays when two sets were compared, and one-way ANOVA with Bonferroni's correction used for post hoc multiple comparisons was used to determine statistical significance. Specific p values are detailed in the figure legends.

ACCESSION NUMBERS

Files associated with RNA-seq studies have been deposited into Sequence Read Archive under accession number SRA: SRP072501.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2016.03.008.

AUTHOR CONTRIBUTIONS

A.B., N.J.L., Y.O., J.C.B, S.M., and C.B.C. performed experiments; A.B., N.J.L., Y.S., and C.B.C. analyzed data; E.T.D.A.M. and S.C. contributed essential reagents; and A.B., N.J.L, S.C., Y.S., and C.B.C. wrote the manuscript.

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