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Type I interferons instigate fetal demise after Zika virus infection

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Abstract

Zika virus (ZIKV) infection during pregnancy is associated with adverse fetal outcomes including microcephaly, growth restriction, and fetal demise. Type I interferons (IFNs) are essential for host resistance against ZIKV, and IFN- α/β receptor (IFNAR)-deficient mice are highly susceptible to ZIKV infection. Severe fetal growth restriction with placental damage and fetal resorption is observed after ZIKV infection of type I IFN receptor knockout (*Ifnar1*^{-/-}) dams mated with wild-type sires, resulting in fetuses with functional type I IFN signaling. The role of type I IFNs in limiting or mediating ZIKV disease within this congenital infection model remains unknown. In this study, we challenged *Ifnar1*^{-/-} dams mated with *Ifnar1*^{+/-} sires with ZIKV. This breeding scheme enabled us to examine pregnant dams that carry a mixture of fetuses that express IFNAR (*Ifnar1*^{+/-}) or do not (*Ifnar1*^{-/-}) within the same uterus. Virus replicated to a higher titer in the

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placenta of *Ifnar1*^{-/-} than within the *Ifnar1*^{+/-} concepti. Yet, rather unexpectedly, we found only *Ifnar1*^{+/-} fetuses were resorbed after ZIKV infection during early pregnancy, whereas their *Ifnar1*^{-/-} littermates continue to develop. Analyses of the fetus and placenta revealed that, after ZIKV infection, IFNAR signaling in the conceptus inhibits development of the placental labyrinth, resulting in abnormal architecture of the maternal-fetal barrier. Exposure of mid-gestation human chorionic villous explants to type I IFN, but not type III, IFNs altered placental morphology and induced cytoskeletal rearrangements within the villous core. Our results implicate type I IFNs as a possible mediator of pregnancy complications, including spontaneous abortions and growth restriction, in the context of congenital viral infections.

Introduction

Zika virus (ZIKV), an emerging mosquito-borne flavivirus, infected over 500,000 individuals in 2015 and 2016 as it spread across the Americas and is now present in 62 countries across the world (1, 2). Symptoms in healthy individuals are mostly mild, including fever, rash, and conjunctivitis, with the majority of infections remaining asymptomatic. However, the recent outbreak has led to a worldwide concern over the ability of the virus to cause birth defects, including microcephaly, in infected pregnant women. In addition to microcephaly, ZIKV causes a range of other pregnancy complications including intrauterine growth restriction (IUGR), spontaneous abortion, and stillbirth (3). While ZIKV is primarily transmitted through the mosquito *Aedes aegypti*, increasing evidence supports sexual transmission as a route of infection: ZIKV RNA persists in semen for up to six months after infection, and there are a number of reports of ZIKV transmission amongst sexual partners in areas where mosquito transmission has not been reported (4, 5). However, it is unknown whether the mechanisms of ZIKV-induced fetal pathology after mosquito-borne and sexually-transmitted infection are similar.

The type I IFNs, including IFN- β and multiple subtypes of IFN- α , are key antiviral factors that mount a rapid and potent innate defense against viruses (6). Production of type I IFN is initiated through recognition of pathogen-associated molecular patterns (PAMPs), generated during viral infection (7, 8). Type I IFNs bind to their receptor, IFNAR, to induce an antiviral state through transcription of IFN-stimulated genes (ISGs), which restrict viral replication through a broad range of antiviral mechanisms (6, 9). In addition to inducing cell-intrinsic antiviral effects, IFNs have an extensive range of biological activities, including activating adaptive immune responses (10), blocking cell proliferation, and inducing apoptosis (11). Thus, in addition to restricting viral infection, IFNs also have the potential to contribute to pathogenesis.

Multiple mouse models of ZIKV infection have demonstrated adverse pregnancy outcomes (12–16). Because ZIKV is unable to suppress the mouse IFNAR signaling as successfully as it suppresses human IFNAR signaling (17, 18), many mouse models of ZIKV infection either require blockade of IFNAR using an antibody, use of *Ifnar1*-deficient mice, or the use of high levels of virus to induce pathology (12, 14, 19). Pregnancy studies of ZIKV have utilized a model in which *Ifnar1*^{-/-} females are crossed to wild-type males, creating *Ifnar1*^{+/-} fetuses with an intact IFN response (13, 14, 20). In these models, fetuses develop

severe growth restriction when pregnant dams are infected subcutaneously or intravaginally after embryonic day 7.5 (E7.5). If mice are infected earlier, between E4.5-E6.5 the majority of fetuses are resorbed. ZIKV infection in this model induces severe pathology of the placenta and abnormal placental architecture (13).

The placenta supports fetal development by facilitating exchange of nutrients and gases between the maternal and fetal blood. Additionally, it serves as a barrier by preventing transfer of pathogens from the mother to the fetus (3, 21). It is known that inflammation and infection can disrupt the function and development of the placenta, leading to IUGR, preeclampsia, preterm birth, and fetal demise, as demonstrated in humans and animal models (22, 23). Others have also suggested that the immune response to ZIKV at the maternal-fetal interface may be responsible for ZIKV-associated birth defects (24). Additionally, type I IFNs have been shown to mediate preterm birth in mouse models (25). On the other hand, IFNs are known to have important roles in supporting normal pregnancy and protecting the fetus from viral infections(26). For example, ISGs are upregulated during implantation in mice and humans (27), and human syncytiotrophoblasts constitutively express type III IFNs (IFN- λ s), making them resistant to infection by viruses (28, 29). In addition, type III IFNs play a role in restricting ZIKV vertical transmission in mice(20).

In this study, we interrogate the effect of type I IFN signaling on fetal development using a mouse model of ZIKV infection. Vaginal or subcutaneous ZIKV infection of *Ifnar1*^{-/-} dams crossed with *Ifnar1*^{+/-} sires enabled us to investigate the role of IFNAR signaling in antiviral protection and disease. Paradoxically, our results revealed a detrimental role of fetal IFNAR signaling in mediating IUGR and fetal resorption by causing abnormal placental development.

Results

Fetal IFNAR signaling instigates fetal demise despite controlling ZIKV replication

In our previous studies, we observed that vaginal infection of mice lacking the transcription factors upstream of type I IFN, *Irf3*^{-/-} *Irf7*^{-/-} dams crossed to *Irf3*^{-/-} *Irf7*^{-/-} sires, had higher levels of ZIKV in the placenta but had minimal growth restriction compared to fetuses from *Ifnar1*^{-/-} dams crossed to WT sires(14). While the *Irf3*^{-/-} *Irf7*^{-/-} mice are capable of responding to IFN, we hypothesize that the lack of pathology in this model, despite high levels of virus, is due to lack of IFN induction in the first place. Consistent with this, we observed that the ISGs were induced in *Ifnar1*^{+/-} fetuses and placenta, but they were absent in the *Irf3*^{-/-} *Irf7*^{-/-} matings, correlating with the level of pathology (Fig. S1A–F). These findings led us to hypothesize that IFN signaling, rather than the levels of virus, mediates fetal pathology after ZIKV infection.

In order to directly test the role of type I IFNs in antiviral defense and fetal development following ZIKV infection, we crossed *Ifnar1*^{-/-} females with *Ifnar1*^{+/-} males, producing a mixture of *Ifnar1*^{-/-} and *Ifnar1*^{+/-} fetuses in the same litter (Fig. 1A). Pregnant dams were infected intravaginally (ivag) with 1.5×10^5 PFU of Cambodian strain of ZIKV on either embryonic day 5.5 (E5.5) or E.8.5, corresponding to the mid and late first trimester in humans (30), respectively. Fetuses were harvested on E17.5, close to term. We observed

resorption of all fetuses of the *Ifnar1*^{+/-} genotype when dams were infected with ZIKV on E5.5 (Fig. 1B, C). Surprisingly, however, all *Ifnar1*^{-/-} littermates continued to develop after infection on E5.5, indicating that a functional copy of *Ifnar1* is required to mediate fetal demise following early ZIKV infection of pregnant dams (Fig. 1B, C). When dams were challenged with ZIKV on E8.5, *Ifnar1*^{+/-} fetuses were not resorbed but showed more severe growth restriction compared to their *Ifnar1*^{-/-} littermates (Fig. 1B, C). Analysis of viral RNA showed that, after E8.5 infection, there was more viral RNA detected in the placentas of the *Ifnar1*^{-/-} littermates compared to their *Ifnar1*^{+/-} littermates on E17.5 (9 days post infection (dpi)) (Fig. 1D). Plaque assays revealed a thousand-fold higher levels of infectious virus in the *Ifnar1*^{-/-} placentas compared to *Ifnar1*^{+/-} placentas (Fig S2A). After infection at E5.5, there were comparable levels of virus in the *Ifnar1*^{-/-} placenta and the resorbed *Ifnar1*^{+/-} conceptus, and these levels were lower than what was observed for the E8.5 *Ifnar1*^{-/-} placentas on E17.5 (12 dpi) (Fig. 1D). In the fetus, viral RNA was present but at low levels in both genotypes and at both time points of infection (Fig. 1E), and infectious virus was below the limit of detection for the majority of fetuses after infection at E8.5 (Fig. S2B). These results indicated that, IFNAR signaling in the conceptus, despite controlling ZIKV replication in the placenta, leads to fetal demise after congenital infection.

To understand how and when development is blocked in the *Ifnar1*^{+/-} conceptus, we harvested fetuses at various days after infection of dams on E5.5. Gross examination of the *Ifnar1*^{+/-} fetuses revealed no overt differences with *Ifnar1*^{-/-} fetuses or with uninfected counterparts on E9.5 (Fig. 2A, B). On E10.5, there was a modest reduction in size of the *Ifnar1*^{+/-} fetuses, and in 1 of 6 litters, all *Ifnar1*^{+/-} fetuses were resorbed (3/17), but fetuses otherwise appeared grossly normal (Fig. 2A, B). However, by E11.5, the majority of *Ifnar1*^{+/-} fetuses (7/11) were dead, and by E12.5, all *Ifnar1*^{+/-} fetuses were resorbed (Fig. 2A, B). Thus, IFNAR-dependent fetal demise after early ZIKV infection occurs between E10.5 and E12.5.

Placental labyrinth architecture is abnormal in IFNAR competent concepti

Post-implantation death between E5.5 and E12.5 is typically caused by defects in the fetal red blood cells (RBCs), vasculature, heart or the placenta (31). Thus, we focused on analysis of the placenta, which develops between E8.5 and E10.5 (32, 33). Global transcriptional analysis by RNA sequencing of placentas at E10.5 demonstrated evidence of active IFNAR signaling in the *Ifnar1*^{+/-} placenta, with robust induction of hundreds of IFN-stimulated genes (ISGs) (Fig. S3, Table S2). To determine which cells are infected by ZIKV, we stained the E10.5 placenta and decidua for ZIKV antigen. The decidua is the maternal-derived endometrial lining of the uterus, and the fetal-derived placenta is composed of the junctional zone and labyrinth zone, where nutrient exchange occurs between the maternal and fetal blood (Fig. 3A) (21, 32). We detected rare ZIKV-infected leukocytes (CD45⁺ cells) in both the *Ifnar1*^{+/-} and *Ifnar1*^{-/-} decidua but not in the underlying placenta (Fig. S4). These cells likely represent decidual macrophages or dendritic cells, and this is consistent with a report showing ZIKV RNA in leukocytes of maternal tissue surrounding the placenta in an infected patient (34, 35). These results suggest that ZIKV infects maternal cells in the decidua, which may be a source of type I IFNs that act on fetal cells of the underlying junctional zone or labyrinth layer to induce robust ISGs.

Next, we performed histological analysis of the developing placenta and decidua to determine the impact of ISG expression. At E9.5, the placenta and decidua were grossly normal in all groups: they showed normal decidualization, a layer of trophoblast giant cells, and a labyrinth with both maternal blood spaces, containing anucleated maternal RBCs, and fetal blood spaces, containing nucleated fetal RBCs (Fig. 3B, S5). By E10.5, we observed marked abnormality in the labyrinth of the ZIKV-infected *Ifnar1^{+/-}*, but not *Ifnar1^{-/-}*, placentas (Fig. 3B). Specifically, the labyrinth appeared to have denser cellularity, with decreased vascular spaces and minimal fetal blood cell content (Fig. 3B, arrow). In addition, there were abnormal spheroid structures (Fig. 3B, asterisks), likely composed of trophoblasts. The decidua and trophoblast giant cells were comparable between all groups. While the labyrinth of the infected *Ifnar1^{-/-}* placentas looked more disorganized with increased cellularity compared to the uninfected controls, there were still abundant fetal RBCs within the fetal blood space (Fig. 3B). By E11.5, when most ZIKV-infected *Ifnar1^{+/-}* fetuses were resorbed, the labyrinth appeared disorganized with reduced fetal blood vessels and abundant spheroid structures, and by E12.5, *Ifnar1^{+/-}* placentas show almost no maternal or fetal blood vessels (Fig. S5). In contrast, the labyrinths of *Ifnar1^{-/-}* placentas on E11.5 and E12.5 were indistinguishable from those of the uninfected controls with abundant adjacent fetal and maternal blood spaces (Fig. S5). These results suggested that type I IFN induced in response to ZIKV interferes with development of fetal vasculature in the placenta labyrinth.

To examine this possibility, tissue sections of E10.5 placentas from dams infected on E5.5 were stained with cytokeratin (CK) to label trophoblasts and CD31 to label blood vessels. We observed a network of closely associated CD31⁺ (endothelial cell demarcating fetal blood space) and CK⁺ (trophoblast demarcating maternal blood space) structures in both uninfected and ZIKV-infected *Ifnar1^{-/-}* placentas (Fig. 3C). In contrast, CD31 staining in the ZIKV-infected *Ifnar1^{+/-}* placentas was markedly reduced, wherein the limited CD31⁺ cells were found on the edge of the placental structure devoid of luminal spaces (Fig. 3C). Immunohistochemistry staining of the placentas with anti-CD31 antibody confirmed the collapsed vasculature and reduced number of the fetal endothelial cells (Fig. S6A). Anti-E-cadherin antibody, which stains the trophoblasts, revealed that many of the densely packed cells in the E10.5 placenta were Ecad⁺ trophoblasts (Fig. S6B). These results indicate that defective development of fetal vasculature and abnormal trophoblasts in the labyrinth immediately precede the death of the IFNAR-intact fetus following ZIKV infection of pregnant dams.

IFNAR signaling leads to increased apoptosis in the placental labyrinth, an abnormal maternal-fetal barrier, and fetal hypoxia

Based on the known functions of type I IFNs, we hypothesized that IFNAR signaling in the fetus inhibits placental development through three possible mechanisms: inducing immune cell recruitment and invasion of the labyrinth; blocking cellular proliferation; or inducing cell death (10, 11). At E10.5, CD45⁺ leukocytes were restricted to the decidua in all groups and did not infiltrate into the cytokeratin (CK) positive junctional zone or the labyrinth (Fig. S6C), ruling out the inflammatory leukocyte infiltration as the mechanism of fetal demise. Next, we examined cell proliferation in the labyrinth by Ki67 staining. All placentas showed

abundant Ki67⁺ cells at E10.5 (Fig. 4A), excluding the role of IFNAR signaling in blocking cell proliferation as the mechanism of fetal demise. To examine if IFNAR signaling is inducing cell death, we stained for activated (cleaved) caspase-3 (Casp3) as a marker of apoptotic cells. There was no Casp3 positive staining in the labyrinth of the uninfected placentas or infected *Ifnar1*^{-/-} placentas at E10.5 (Fig. 4B). In contrast, in the infected *Ifnar1*^{+/-} placenta, we detected Casp3 staining in the labyrinth in a pattern consistent with the endothelial cells, fetal blood cells, or adjacent trophoblasts surrounding the spheroid structure (Fig. 4B). These results are consistent with a previous report (13), and suggest a role for IFNAR in mediating apoptosis of fetal endothelial cells and trophoblasts as an underlying mechanism of placental dysfunction.

To examine the abnormal placenta architecture at a cellular level, we performed electron microscopy to analyze the maternal-fetal interface on E10.5. In the uninfected placentas and infected *Ifnar1*^{-/-} placentas, the expected trilaminar interhemal barrier was seen, with anucleated maternal blood (mrbc) and nucleated fetal blood (frbc) being separated by 4 layers of cells: the sinusoidal trophoblast giant cell (stgc) which directly contact the maternal blood, two continuous syncytial layers of trophoblast that are closely connected (ST-I and ST-II), and a layer of endothelial cells (ec) that directly contact the fetal blood (Fig. 4C) (32, 36). ST-I and ST-II were tightly adhered to one another (arrows, Fig. 4C). There was no mixing between maternal and fetal blood in the uninfected placenta. In the infected *Ifnar1*^{+/-} placentas, there were multiple instances of mixing between the maternal and fetal blood in the labyrinth (Fig. 4C). When separated maternal and fetal circulations were found, the barrier between the two was highly abnormal, with no evidence of the normal 4-cell layer barrier and breakdown between cells making the maternal-fetal barrier (Fig. 3C). Thus, IFNAR signaling in the placenta leads to an abnormal maternal-fetal blood barrier with local breakdown.

Based on the findings of an abnormal vasculature in the placental labyrinth and abnormal maternal-fetal barrier, we examined the transcriptional changes in the fetus to examine if lack of adequate gas exchange between mother and fetus may be contributing to fetal demise. We found that hypoxia response genes including *Vegfa*, *Adm*, *Bnip3*, *Glut1*, and *Pfkfb3* were all significantly upregulated in the ZIKV-infected *Ifnar1*^{+/-} fetuses relative to uninfected controls and to their infected *Ifnar1*^{-/-} littermates (Fig. 5A–E) (37, 38). Thus, the death of the *Ifnar1*^{+/-} fetuses following ZIKV infection is preceded by hypoxia.

IFNAR signaling mediates fetal death after subcutaneous ZIKV infection and Poly(I:C) treatment

To determine if IFNAR signaling mediates fetal resorption after other routes of ZIKV infection and with other strains of ZIKV, we challenged *Ifnar1*^{-/-} females crossed with *Ifnar1*^{+/-} males subcutaneously with Brazilian strain of ZIKV on E6.5 (13). After challenging with a high dose (3.4×10^5 PFU) of ZIKV, the majority of *Ifnar1*^{+/-} fetuses were resorbed by E12.5, but *Ifnar1*^{-/-} fetuses continued to develop (Fig. S7A, B). To analyze fetal development at later time points without maternal lethality, we challenged *Ifnar1*^{-/-} females mated with *Ifnar1*^{+/-} males with a sublethal dose of ZIKV (1×10^3 PFU) subcutaneously at E6.5 and harvested the fetuses at E17.5. Again, similar to the vaginal

ZIKV infection, all *Ifnar1*^{+/-} fetuses were resorbed, but *Ifnar1*^{-/-} fetuses were grossly normal (Fig. S7C, D). Thus, IFNAR-dependent fetal resorption occurs after subcutaneous and intravaginal ZIKV challenge, with both Brazilian and Cambodian ZIKV strains.

To examine if IFNAR signaling is sufficient to induce fetal resorption independent of ZIKV infection, we challenged mice with polyinosinic:polycytidylic acid (poly(I:C)), a double stranded RNA viral mimic capable of eliciting robust type I IFN responses (39). After intraperitoneal (IP) injection of 200 µg of poly(I:C) at E7.5, all fetuses of WT females mated with WT males were resorbed by E9.5, and we could not recover any fetal material in 5/6 injected females by E10.5, consistent with previous reports (Fig. 6A,C) (40). Notably, when we challenged *Ifnar1*^{-/-} females crossed with *Ifnar1*^{+/-} males with poly(I:C) on E7.5, the majority of both *Ifnar1*^{-/-} and *Ifnar1*^{+/-} fetuses continued to develop as examined on E10.5 and E12.5 (Fig. 6B,C). Thus, maternal IFNAR signaling was necessary for mediating poly(I:C)-induced fetal resorption.

Type I IFN, but not type III, treatment of human mid-gestation villous explants leads to deformation

To determine the impact of type I IFNs on the human placenta, we treated mid-gestation (19–23 weeks) human chorionic villous explants with a type I IFN, recombinant IFN-β, or type III IFN, recombinant IFN-λ3. Isolated villi were treated with recombinant IFN within hours following their isolation, when their structure and morphology remained completely intact as characterized by a continuous layer of cytokeratin-19 positive trophoblasts covering the surfaces of the isolated villi (Fig 7A, left panel, B, top panel). After ~16–20 hours of treatment, the architecture of IFN-β-treated villi became markedly abnormal, with areas of aggregated nuclei formed at the distal end of villi treated with IFN-β (either 100U or 1000U) (Fig. 7A, arrows, B, C). In contrast, treatment of the villous explants with IFN-λ3 showed no gross impact (Fig. 7A, C). The abnormal villous structures resembled syncytial knots, which are associated with pathological states of pregnancy; sprouts, which represent overproliferation of the syncytiotrophoblast layer; or apoptotic shedding (41, 42). In addition to the formation of syncytial knot- or sprout-like structures, we noted that IFNβ-treated villi also exhibited alterations in the actin cytoskeleton within the core of the villi as characterized by actin filament disassembly, suggesting widespread damage to the villi (Fig. 7D, bottom panel). To examine the global transcriptional changes in response to IFN-β and IFN-λ3 treatment, we performed whole-genome RNA sequencing on villi treated with IFN-β or IFN-λ isolated from three different placental preparations. We found that both IFN-β and IFN-λ treatment induced significant transcriptional changes (273 and 101 total genes, respectively, *p*<0.05) as illustrated by MA plots (Fig. 8A). However, interestingly, we found that there was little overlap between the genes induced by IFN-β and IFN-λ exposure—whereas IFN-β treatment induced the upregulation of the majority of transcripts (260 of 273), IFN-λ treatment correlated with the downregulation of the majority of transcripts (89 of 101) (Fig. 8B). Consistent with this differential expression pattern, only 12 transcripts were differentially expressed by both IFN-β and IFN-λ treatment (Fig. 8C). Surprisingly, we found that whereas IFN-β treatment induced many known ISGs, IFN-λ treatment had little impact on ISG expression (Fig. 8D–F). In addition, consistent with the significant morphologic alterations of villous architecture induced by IFN-β treatment, IFN-β-treated

villi exhibited suppression (by ~4-fold, $p=0.04$) in the expression of the β chain of human chorionic gonadotropin (hCG), which is exclusively produced by syncytiotrophoblasts and is associated with placental function. Collectively, these data suggest that type I IFN is sufficient to induce morphological alterations and also possibly adversely impact placental function in the human developing placental villi.

Discussion

Our findings highlight the detrimental impact of type I IFNs on the developing placenta and fetus by demonstrating that only the fetuses with a functional copy of IFNAR are resorbed after ZIKV infection. IFNAR signaling in the conceptus leads to abnormal placenta labyrinth development with apoptosis in the labyrinth, impaired fetal endothelial development and disrupted maternal-fetal blood barrier. IFNAR signaling clearly was important in controlling viral replication in the placenta. Despite this, IFNAR-mediated pathology outweighed the benefit of IFNAR-dependent control of viral replication. How exactly IFNAR signaling leads to the observed labyrinth pathology is unknown. The hypoxic state of the IFNAR-sufficient fetus, likely resulting from the fetal endothelial disruption, suggests an impaired delivery of oxygen and possibly nutrients being the underlying cause of fetal demise. Our results do not rule out a role for the direct action of ZIKV in mediating certain aspects of pathology such as microcephaly, ocular defects or other neurological abnormalities, which we do not address in this study. Despite having a less severe phenotype than their *Ifnar*^{+/-} littermates, *Ifnar*^{-/-} mice did exhibit growth restriction compared to their uninfected counterparts, consistent with previous reports (43). This growth restriction may be due to poor maternal health, but it could also be indicative of IFNAR-independent causes of birth defects, including direct pathogenic effects of the virus infection, or immune response unrelated to type I IFNs. One limitation to our study is that the host-pathogen interactions, including the suppression of host IFNAR signaling by ZIKV NS5 protein, are not preserved in mice. Thus, to what extent the mouse model recapitulates ZIKV infection and disease in humans is unknown. An additional limitation is that the structure and development of the mouse placenta is significantly different from that of the human placenta, making it difficult to directly compare the pathological changes seen in the mouse placenta with those of humans.

In order to extend our findings to humans, we examined the impact of recombinant IFN- β on second-trimester villous explants. This experimental system allowed us to examine the impact of type I IFNs in the pre-existing syncytium and in a model that fully retains the architecture and multicellular composition of the human placenta. IFN- β exposure induced morphological changes in the human placenta, which correlated with alterations in syncytial and actin cytoskeletal architecture. Altered syncytial morphology resembled syncytial knots, a feature commonly associated with placentas of adverse pregnancy outcomes including preeclampsia (42). Although common in full-term placentas, syncytial knots are rare in normal mid-gestation pregnancies (44). Given the shorter time-scale over which they developed, they could also be apoptotic shedding of damaged cells. Notably, our study required high levels (1000 U) of IFN- β to produce these effects, although we did note the appearance of syncytial knot-like structures at lower levels (100U). It is difficult to assess whether this level of IFN may be present locally in congenital infections and what effects

IFN- β may have over a longer time period and during earlier stages of development, which were not possible to assess in this model. Interestingly, we found that treatment of villi with recombinant type III IFN, IFN- λ 3, was not associated with the altered villous morphology or a strong ISG induction. These are consistent with previous reports that the syncytium constitutively produces IFN- λ s (28) and may suggest that type III IFNs primarily function in an autocrine and paracrine manner to defend the developing fetus against viruses, as has been shown to occur in mice (20). Interestingly, our data also showed that type I IFN, but not type III IFN, treatment led to robust ISG induction in the villous explants. The mechanistic basis for this differential signaling remains unknown, but may reflect the constitutive expression of type III IFN already secreted by the syncytium during mid-gestation, which may impact receptor binding by the recombinant protein or perhaps reflects some level of receptor desensitization (29). Alternatively, it could also reflect the more restricted tissue expression of IFN- λ -receptor which is limited to the epithelium (45). However, mid-gestation explants express high basal levels of many ISGs, suggesting that the tissue is likely responsive to type III IFNs (29). Consistent with this, even in the placentas of mice that lack IFNAR, and harbor high levels of virus in the placenta, the virus was mostly restricted from the fetus, indicating that other structures and pathways are capable of restricting ZIKV from the fetus.

How might type I IFNs trigger fetal death? We did not observe any differences in leukocyte infiltration into the placenta or block in proliferation of cells in the placenta. We did, however, observe apoptosis of cells in the labyrinth, which likely represented endothelial cells or adjacent trophoblasts. Consistent with this, human explants treated with IFN- β exhibited significant alterations in actin cytoskeletal structure, consistent with cellular damage. These results are consistent with a previous report showing similar placenta damage after ZIKV infection (13). In addition to these possibilities, type I IFN is also known to inhibit angiogenesis and blood vessel development (46) and explains the abnormal and reduced fetal blood vessels we observed in the labyrinth. Another possibility is that IFN may impair trophoblast fusion or differentiation. A previous report showed that IFN- β suppresses syncytin-1 expression (47). Consistent with this hypothesis, the placental histology and timing of death of the *Ifnar1*^{+/-} fetuses resembles that of mice lacking Syncytin-A, which have a defect in trophoblast fusion (36). We speculate that type I IFNs may serve as a quality control system to eliminate the developing embryo if coincident viral infection is detected and the levels of circulating IFNs reach a certain threshold. Many mammals carry their fetus for a prolonged time period, to upwards of ~650 days in elephants. Such costly investment by the mothers may justify high levels of scrutiny of the health of the fetus at every level but particularly early in pregnancy when the embryo is vulnerable to various stressors (48, 49). Type I IFNs may report on the viral infection status of the mother or the fetus within the local milieu, sending abortive signals to terminate pregnancy.

The effects of IFN on the developing placenta likely have implications for pregnancy complications beyond ZIKV, and it could be a mechanism for adverse pregnancy outcomes such as IUGR or early spontaneous abortions, which often occur without a known underlying etiology. Consistent with type I IFN being an underlying cause of pregnancy complications, many congenital infections or “TORCH” infections have a common presentation of microcephaly, cerebral calcifications, and IUGR (3). Beyond viral infections,

our study may be relevant to diseases in which type I IFNs are over-produced, collectively known as interferonopathies (50). Interferonopathies may be induced by monogenic mutations, such as those found in Aicardi-Goutières's syndrome, or polygenic diseases including systemic lupus erythematosus (SLE). Notably, fetuses with Aicardi-Goutières's syndrome can present with developmental defects similar to classic TORCH infection with fetal growth restriction, microcephaly, and intracerebral calcifications (50). SLE is well known for causing pregnancy complications, including fetal death *in utero*, preeclampsia, preterm birth, and elevated serum IFN is one of the key factors that closely correlates with poor pregnancy outcomes (51). Our study implicates type I IFNs as a possible common culprit for viral-associated pregnancy complications and suggests blockade of type I IFNs as a possible intervention to prevent pregnancy complications in the settings of non-viral interferonopathies.

Materials and Methods

Study Design

To test the effect that fetal IFNAR signaling has on development after ZIKV infection, *Ifnar1*^{-/-} females were crossed to *Ifnar1*^{+/-} males to allow for direct comparison between littermates of different genotypes. Fetuses and placentas were harvested at various timepoints after infection and appearance, histology, and transcriptional changes were analyzed. To test how human placentas were affected by IFN signaling, mid-gestation chorionic villous explants were treated with IFN- β or IFN- λ and villi were analyzed by immunofluorescent imaging or transcriptional changes were analyzed. For mouse studies, analysis was performed on litters containing both genotypes, and genotyping of litters was performed after initial analysis of fetal weight and appearance. No other formal randomization or blinding method was used. Subjects were assigned a litter and fetus number to allow unbiased selection for sample processing (RNA and imaging). A minimum of 3 infected litters were analyzed per timepoint. Exact N is indicated in figure legends. No formal statistical tool was used to determine power.

Mice

Ifnar1^{-/-}, *Ifnar1*^{+/-}, C57BL/6, and *Irf3*^{-/-} *Irf7*^{-/-} mice were bred and maintained at Yale University. All pregnant dams were between 9–20 weeks of age. Littermates were randomly assigned to infected or uninfected groups. *Ifnar1*^{+/-} male breeders are the F1 generation from cross between C57BL/6 (B6) and *Ifnar1*^{-/-} parents. Matings were timed by checking for the presence of a vaginal plug, indicating gestational age E0.5. Approximately 11% of infected analyzed between E9.5–12.5 and 17% of uninfected concepti developed into abnormal spheroid shapes (distinct from infection-induced resorbed fetuses) without presence of a fetus or yolk sac. These were excluded from analysis due to inability to obtain adequate fetal-derived tissue for genotyping analysis and because it was present in equal frequencies for infected and uninfected groups. All animal procedures were performed in compliance with Yale Institutional Animal Care and Use Committee protocols.

Mid-gestation Chorionic Villous Explants

Human fetal placental tissue from between 19–23 weeks gestation that resulted from elective terminations was obtained from the University of Pittsburgh Health Sciences Tissue Bank through an honest broker system after approval from the University of Pittsburgh Institutional Review Board and in accordance with the University of Pittsburgh anatomical tissue procurement guidelines. Chorionic villi (approximately 1cm x 1cm in size) were dissected and cultured in DMEM/F12 (1:1) supplemented with 10% FBS, penicillin/streptomycin, and amphotericin B. Immediately following isolation, isolated villi were treated with the indicated dose of recombinant IFN- β or IFN- λ for 24hr and then tissue was fixed and processed for imaging. For imaging studies, villi were fixed in 4% PFA followed by permeabilization in 0.25% Triton X-100 for 30min at roomed temperature with gentle agitation, washed in PBS, incubated with primary antibody, washed in PBS, and then incubated with Alexa Fluor conjugated secondary antibodies. Alexa Fluor- conjugated phalloidin was purchased from Invitrogen (A12379 or A12381). Rabbit anti- cytokeratin-19 (ab52625) was purchased from Abcam. Following staining, villi were mounted with VectaShield (Vector Laboratories) containing 4',6-diamidino-2-phenylindole (DAPI) and images captured using an Olympus FV1000 confocal or Zeiss LSM 710 confocal microscope. Images were adjusted for brightness/contrast using Adobe Photoshop (Adobe) and syncytial knot size calculated in Imaris.

Viruses and *in vivo* infections

ZIKV Cambodian FSS13025 strain, obtained from the World Reference Center for Emerging Viruses and Arboviruses at University of Texas Medical Branch, Galveston, was used for all studies. Stocks were propagated in Vero cells and titrated by plaque assay as previously described (14). ZIKV Brazilian PE243 was recovered from a 19-year old female in Brazil from 2015(52). Vero cells were obtained from ATCC. Cell lines were verified by morphology and were tested for mycoplasma contamination every 1–2 years.

Intravaginal infection was performed as previously described: at E5.5 or E8.5, a calginate swab (Fischer scientific) was used to remove mucous from the vaginal lumen, and 1.5×10^5 PFU of ZIKV was inoculated into the vagina using a pipette (14). Subcutaneous infection was performed by injecting 100 μ l of virus stock diluted in PBS (3.4×10^5 or 1×10^3 PFU) into the footpad. Pregnant mice were euthanized and tissues were harvested at indicated time points, and fetuses and placentas were either collected in Trizol (for RNA extraction), fixed in 4% PFA (for imaging), or collected in DMEM with 10% FBS and Penicillin/Streptomycin (for plaque assay). Fixed fetuses were imaged using a Zeiss Discovery.V8 stereomicroscope (Zeiss). Yolk sac for E9.5–12.5 or tail for E17.5 fetuses were collected for each fetus and genotyped using the primers F: ATTATTAAAAGAAAAGACGAGGCGAAGTGG R: AAGATGTGCTGTCCCTTCCTCTGCTCTGA, with a 150bp product indicating the presence of a WT copy of IFNAR.

Poly(I:C) challenge

200ug of HMW Vaccigrade PolyIC (Invivogen) was injected intraperitoneally into pregnant mice at E7.5. Mice were then harvested between E9.5 and E12.5 to examine fetuses.

Quantification of ZIKV genome, ISGs, and hypoxia response genes by qRT-PCR

Tissues were extracted using Trizol (Thermo Fischer Scientific) and purified using an RNeasy Mini Kit (Qiagen). iScript cDNA synthesis kit (Bio-Rad) was used to synthesize cDNA. Quantitative PCR was performed using SYBR green (Bio-Rad) was used to quantify ZIKV levels, ISGs, and hypoxia response genes and ran on a CFX Connect instrument (Bio-Rad). Primer sequences are provided in supplementary table 1. Virus and ISGs were normalized to *Hprt* (14).

Histology, immunofluorescence and immunohistochemistry staining of mouse placentas

Placentas were fixed in 4% PFA overnight at 4°C. For IHC and Hematoxylin and Eosin (H&E) staining, tissues were embedded in paraffin blocks and sectioned by the Yale Histology core. H&E was performed by the Yale Histology core. For IHC, paraffin sections were heated for 30 minutes at 55–60°. Antigen retrieval was performed by boiling in sodium citrate (J.T. Baker) pH 6.0 for 60 minutes. Blocking was performed using PBS (American Bio) 1% BSA (Sigma) and 0.5% Tween 20 (Sigma) adjusted to pH of 7.4. Slides were stained for CD31 (Goat, 1:1000, R&D AF3628), Casp3 (Rabbit, 1:1000, Cell Signaling Tech 9664T), Ecad (1:500, Thermo Fischer Scientific 13-1900), Ki67 (Rabbit 1:1000, Cell Signaling Tech 12202T) at 4°C overnight. Slide were blocked with Bloxall (Vector labs), stained with Rat (for Ecad), Rabbit (for Casp3, Ki67), or goat (for CD31) Impress antibodies (Vector lab) and DAB (Vector labs) per manufacturer's instructions. For immunofluorescence staining, slides were embedded in optimal cutting temperature (OCT) media (Tissue Tek). Five to seven µm frozen sections were cut using a cryostat, and sections were allowed to dry at room temperature. Sections were blocked with 2% normal Donkey serum (Jackson) in PBS-T and 1% BSA (Sigma) and stained with ZIKV-immune rat serum (53), CD45 (R&D Systems AF114), Cytokeratin (Dako Z0622) or CD31 (R&D Systems AF3628) overnight at room temperature. Sections were then stained with A488 anti-rat secondary (Thermo Fischer), A647 anti-rabbit (Thermo Fischer), Cy3 anti-rabbit (Jackson), A488 anti-goat (Thermo Fischer), or NL557 anti-goat (R&D Systems). Samples were stained with DAPI and mounted with ProLong Diamond Antifade Mountant (Molecular Probes). H&E and IHC images were captured using light microscopy (BX51; Olympus), and immunofluorescence images were captured using fluorescence microscopy (BX51; Olympus) or confocal microscopy (TCS SP2; Leica). Images were merged and brightness and contrast were adjusted using ImageJ.

Transmission electron microscopy of mouse placentas

Placentas were fixed in formaldehyde/glutaraldehyde 2.5% in phosphate buffer for at least 24 hours. Samples were washed and secondarily fixed in osmium tetroxide, negative stained with uranyl acetate, treated in ascending alcohols, and finally embedded in Durcupan ACM (EMS 14040). 70 nM ultrathin sections were cut on Leica ultra microtome, collected on Formvar-coated grids and analyzed on a Tecnai 12 FEI electron microscope.

RNAsequencing and qRT-PCR analysis of Mid-gestation Chorionic Villous Explants

RNA was isolated from the villous explants using GenElute RNA Total RNA Miniprep Kit (Sigma) and treated with DNase (Sigma). For RNAseq, as previously described (28, 54),

libraries were prepared using New England Biolabs Ultra Library Preparation kit. An Illumina HiSeq2500 was used for sequencing and CLC Genomics Workbench 9.0 (Qiagen) was used to process, normalize, and map sequence data to the human reference genome (hg19). DESeq2 in R (55) or CLC Genomics Workbench 9 were used to identify differentially expressed genes and to generate MA-plots.

RNAsequencing data is available through the GEO database, accession number GSE104349.

Statistical Analysis

In all analyses except for RNAseq, data analysis was performed using Microsoft Excel and Graphpad Prism. Exact statistical test and value of n are detailed in the figure legends. A Tukey's multiple comparison test or Dunnett's multiple comparison test was used to determine significance when determining significance between multiple groups (>3). A paired student t-test was used when comparing only 2 groups. T-tests assumed a normal distribution for all samples and t-tests assume an unequal standard deviation and variance between groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Fetal IFN signaling leads to severe intrauterine growth restriction and resorption after ZIKV infection

Ifnar1^{-/-} females mated to *Ifnar1*^{+/-} males were infected intravaginally with 1.5×10^5 PFU Cambodian ZIKV on E5.5 or E8.5 and harvested on E17.5. (A) Schematic showing mating strategy. Fetal weights were measured (B) and fetuses were visually inspected (C). RNA was isolated from the placenta or resorbed conceptus (D) or fetus (E) to determine ZIKV levels. Relative ZIKV levels were determined by normalization to *Hprt*. Individual data points with mean are shown. For fetal weights, ZIKV E5.5 *Ifnar1*^{-/-} n=15 fetuses, *Ifnar1*^{+/-} n=17 fetuses from 4 litters; ZIKV E8.5 *Ifnar1*^{-/-} n=21 fetuses, *Ifnar1*^{+/-} n=22 fetuses from 6 litters; Uninfected *Ifnar1*^{-/-} n=13 fetuses, *Ifnar1*^{+/-} n=21 from 4 litters. For ZIKV RNA, ZIKV E5.5 *Ifnar1*^{-/-} n=7, *Ifnar1*^{+/-} n=7 from 3 litters; ZIKV E8.5 *Ifnar1*^{-/-} n=9–10, *Ifnar1*^{+/-} n=11 from 5 litters; Uninfected n=11 from 3 litters with both genotypes pooled. Data are pooled from at least 2 independent experiments from each infection time point. Scale bar, 1cm. * P<0.05, ** P<0.01 by Tukey's multiple comparison test.

Fig. 2. ZIKV-infected fetuses with intact IFN signaling are resorbed between E10.5 and 12.5
Ifnar1^{-/-} females mated to *Ifnar1^{+/-}* males were infected intravaginally with 1.5×10^5 PFU Cambodian ZIKV at E5.5 and harvested at indicated time points. (A) Representative images of 3–5 litters for infected and 2–3 litters for uninfected are shown per time point. Scale bar, 1mm. (B) Crown rump length was measured by tracing distance from crown of head to end of tail using ImageJ. Mean with individual points are graphed. Data points shown represent: E9.5 uninfected *Ifnar1^{-/-}* n=7 and *Ifnar1^{+/-}* n=12 from 2 litters, infected *Ifnar1^{-/-}* n=9 and *Ifnar1^{+/-}* n=10 from 3 litters; E10.5 uninfected *Ifnar1^{-/-}* n=15 and *Ifnar1^{+/-}* n=5 from 3 litters, infected *Ifnar1^{-/-}* n=24 and *Ifnar1^{+/-}* n=17 from 6 litters; E11.5 uninfected *Ifnar1^{-/-}* n=5 and *Ifnar1^{+/-}* n=12 from 3 litters, infected *Ifnar1^{-/-}* n=12 and *Ifnar1^{+/-}* n=11 from 3 litters; E12.5 uninfected *Ifnar1^{-/-}* n=11 and *Ifnar1^{+/-}* n=11 from 3 litters, infected *Ifnar1^{-/-}* n=19 and *Ifnar1^{+/-}* n=19 from 5 litters. Data are pooled from at least 2 independent experiments from each infection time point. *P<0.05, **P<0.0001 compared to all other groups by Tukey's multiple comparison test. No significant differences were found between other groups.

Fig. 3. Placenta architecture of *Ifnar1*^{+/-} fetuses is disrupted at E10.5

Ifnar1^{-/-} females mated to *Ifnar1*^{+/-} males were infected intravaginally with 1.5×10^5 PFU Cambodian ZIKV at E5.5 and harvested at E10.5. (A) Schematic of decidua and placenta architecture and cell types. (B) Placentas were fixed in PFA and paraffin-embedded sections were stained by H&E. Whole placenta and decidua (top panel) or magnified labyrinth (lower panel) are shown. Representative images of 10 placentas/deciduas per genotype from 5 litters were analyzed for infected, and 4 placentas/deciduas per genotype per time point from 2 litters were analyzed for uninfected. Labyrinth, L, and decidua, D, are labeled with respective letters. The asterisk indicates abnormal spheroid structure. Arrows indicate fetal RBCs. Upper panel, scale bar, 100µm; lower panel, scale bar 50µm. (C) PFA-fixed frozen sections from infected littermates were costained for Cytokeratin (Red, CK, trophoblasts), CD31 (green, blood vessels), and 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, blue). Representative images from at least 3 placentas per genotype from at least 2 litters are shown. Scale bar, 75µm.

Fig 4. *Ifnar1*^{+/-} placentas show increased apoptosis and abnormal maternal-fetal blood barrier *Ifnar1*^{-/-} females mated to *Ifnar1*^{+/-} males were infected intravaginally with 1.5×10^5 PFU Cambodian ZIKV at E5.5 and harvested at E10.5. Paraffin embedded sections were stained for Ki67 (A, dividing cells) or cleaved caspase 3 (B, apoptotic cells) with 3,3'-diaminobenzidine (DAB). From A, B pictures from labyrinth are shown; scale bar, 50µm. Representative images from at least 3 placentas/deciduas per condition from at least 2 litters are shown. (C) Placentas were fixed at least 24 hours in formaldehyde with 2.5% glutaraldehyde at 4°C. The labyrinth was dissected and processed for electron microscopy (EM). Uninfected *Ifnar1*^{+/-} labyrinth shows four layers of cells between fetal RBC (frbc) and maternal RBC (mrbc): fetal endothelial cell (ec), two syncytiotrophoblast layers (ST-II, ST-I), and the sinusoidal trophoblast giant cell (stgc). Infected *Ifnar1*^{-/-} placenta is similar to the uninfected control. The barrier between maternal and fetal blood of ZIKV-infected *Ifnar1*^{+/-} placentas is highly abnormal with unfused cells (left panel). ZIKV-infected *Ifnar1*^{+/-} shows multiple examples of mixing between maternal and fetal blood (right panel). Scale bar, 2 or 10 µm as labeled on the image. Multiple sections and planes from one placenta per condition were analyzed.

Fig 5. ZIKV-infected *Ifnar1*^{+/-} fetuses show upregulated hypoxia response genes just before demise

Ifnar1^{-/-} females mated to *Ifnar1*^{+/-} males were infected intravaginally with 1.5×10^5 PFU Cambodian ZIKV at E5.5 and harvested at E10.5. RNA was extracted from fetuses, and expression of previously reported hypoxia- response genes Vegfa (A), Adm (B), Bnip3 (C), Pfkfb3 (D), and Glut1 (E) analyzed by RT-qPCR. Data represent n=9 fetuses per genotype from 3 litters from from ZIKV-infected litters, n=3 *Ifnar1*^{-/-} and n=5 *Ifnar1*^{+/-} fetuses from 2 uninfected litters. Data are pooled from at least 2 independent experiments per group.

*P<0.05, **P<0.01, ***P<0.0001 by Tukey's multiple comparison test. Data were normalized to *Hprt* and represented as fold-change over *Ifnar1*^{-/-} uninfected placentas.

Fig 6. Poly(I:C) injection of pregnant dams leads to fetal resorption in a maternal-IFNAR-dependent fashion

WT females mated to WT males or *Ifnar1*^{-/-} females mated to *Ifnar1*^{+/-} males were injected with 200ug HMW PolyIC at E7.5. (A) Representative images from mice harvested between E9.5 and E12.5 are shown. (B) Crown rump length was measured using ImageJ. Mean with SD and individual data points are shown. Data points represent: WTxWT litters untreated E9.5 n=17 from 2 litters, PIC E9.5 n= 33 from 4 litters, untreated E10.5 n=13 from 2 litters, PIC E10.5 n=8 from 1 litter; an additional 5 injected litters showed no fetal remnants at time of harvest at E10.5. For *Ifnar1*^{-/-} x *Ifnar1*^{+/-} litters, untreated E10.5 n=15 *Ifnar1*^{-/-} and n=5 *Ifnar1*^{+/-} from 3 litters; PIC E10.5 n=5 *Ifnar1*^{-/-} and n=11 *Ifnar1*^{+/-} from 2 litters; untreated E12.5 n=11 *Ifnar1*^{-/-} and n=11 *Ifnar1*^{+/-} from 3 litters; PIC E12.5 n=13 *Ifnar1*^{-/-} and n=7 *Ifnar1*^{+/-} from 2 litters. Uninfected measurements for *Ifnar1*^{-/-} x *Ifnar1*^{+/-} litters are the same as those shown in Fig 2B.

Fig. 7. Interferon treatment of human mid-gestation villous explants induces syncytial knot formation

(A), Human mid-gestation (19–23w) chorionic villi were isolated, placed in culture, and treated with 100 U or 1,000 U of recombinant human IFN β or 1,000 U of IFN λ 3 for ~16–20h. Villous explants were harvested, fixed in PFA, and stained for cytokeratin-19 (Cyt19, green, trophoblasts) and actin (red). DAPI-stained nuclei are shown in blue and differential interference contrast (DIC), lower panel. Scale bar, 100 μ m. Images are representative of villi isolated from four donors. Arrow indicates syncytial knot. (B), Three-dimensional image reconstruction of mock- or IFN- β (1000U)-treated explants stained for cytokeratin-19 (green) and actin (red). DAPI-stained nuclei are shown in blue. Scale bar, 20 μ m. (C) Quantification of syncytial knot size using Imaris in villi treated with 10U, 100U or 1000U of IFN β or 1000U of IFN λ 3 for ~24hrs. Each symbol represents an individual villous from a total of three donors, and the black line represents the mean. *** $P < 0.001$ by Dunnett's multiple comparison test to Mock. (D), Confocal micrographs of mock- or IFN- β (1000U)-treated villi stained for actin (red, at right). DIC is shown at left. White box denotes zoomed area shown at bottom left (mock) or right (IFN- β). Scale bar, 20 μ m.

Fig 8. Interferon stimulated genes are induced in IFN β -treated villous explants

Human mid-gestation (19–23w) chorionic villi were isolated, placed in culture and treated with 1,000 U of recombinant human IFN- β or 1,000 U of IFN- λ 3 for ~24h and RNA extracted. (A) MA plots generated in R following DeSeq2 analysis demonstrating the differential expression between IFN- β - (left) or IFN- λ (middle)-treated villi relative to mock-treated controls and between IFN- β - and IFN- λ -treated villi (right). Data are plotted as $\log_2$ fold changes (y-axis) and mean expression (x-axis). Red symbols denote transcripts whose expression was differentially expressed at $p < 0.05$. (B), Graph demonstrating the number of transcripts upregulated (in green) or down regulated (in red) following IFN- β or IFN- λ treatment of villi. (C), Venn diagram denoting the overlap of transcripts (12 total) between villi treated with IFN- β or IFN- λ (D), Heat map (based on log RPKM values) of known ISGs from mock-, IFN- β -, or IFN- λ -treated villi. (E, F), Volcano plots of villi treated with IFN- β (E) or IFN- λ (F) denoting specific ISGs differentially expressed by treatment. Red circles denote ISGs and purple circles denote non-ISG transcripts. For RNAseq experiments, data represent villi isolated from three independent placental preparations.