Innate immune signaling in trophoblast and decidua organoids defines differential antiviral defenses at the maternal-fetal interface

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Abstract

Infections at the maternal-fetal interface can directly harm the fetus and induce additional complications that adversely impact pregnancy outcomes. Innate immune signaling by both fetal-derived placental trophoblasts and the maternal decidua must provide antimicrobial defenses at this critical interface without compromising its integrity. Here, we developed matched trophoblast and decidua organoids from human placentas to define their relative contributions to innate immune antiviral defenses at the maternal-fetal interface. We show that trophoblast and decidua organoids recapitulate some, but not all, of the basal cytokine release observed in matched tissue explants. We further show that trophoblast organoids constitutively release antiviral type III interferons (IFNs) and that fetal and maternal organoids differentially respond to viral infections through the induction of organoid-specific cytokines. These findings define key differences in innate immune signaling generated by fetal-derived trophoblasts and the maternal decidua, which together must protect the fetus from viral infections.

Introduction

The maternal-fetal interface is comprised of both the maternal-derived decidua and the fetal-derived placenta, which together support fetal growth and provide immunologic defenses that protect the fetus from infections. The relative contributions of specific maternal- and fetal-derived cells to these critical functions remain largely undefined given the limitations of models that recapitulate the complex nature of this interface.

The placenta forms a key barrier to viral vertical transmission throughout pregnancy and is comprised in part by fetal-derived trophoblast cells. Trophoblast stem cells located in chorionic villi, the branch like projections that directly contact maternal blood, give rise to cytotrophoblasts (CTBs), the proliferative mononuclear cells of the placenta, extravillous trophoblasts (EVTs), and the syncytiotrophoblast (STB), a multinucleated contiguous cell layer that covers the villous surfaces. EVT directly remodel the maternal microvasculature to form spiral arteries, which ultimately deliver maternal blood to the surfaces of chorionic villi. Decidualization of the endometrium is required for implantation and appropriate placentation. This process involves the differentiation of fibroblast-like mesenchymal cells into secretory decidua cells. Prior to the formation of chorionic villi and the development of a hemochorial placenta, these secretory cells provide necessary nutrients to the developing embryo [1]. Together, the decidua and placenta thus provide the fetus with essential hormones and nutrients to support its growth.

While the placenta and decidua play complementary roles in fetal development, their contributions to antimicrobial defenses are less defined. Studies of tissue explants and histopathologic analysis of the placenta suggest that the decidua and chorionic villi have differing roles in the pathogenesis of relevant infections. The decidua may be a primary site of replication for several microorganisms associated with congenital disease, including cytomegalovirus (CMV) [2-4], Zika virus (ZIKV) [5-7], and *Listeria monocytogenes* [8], suggesting it may serve as a reservoir for these infections. By contrast, the placenta is largely resistant to infections and possesses several intrinsic mechanisms of innate defense. In the case of viruses, human trophoblasts constitutively

release antiviral interferons (IFNs) that restrict infection in both autocrine and paracrine manners [9, 10]. These IFNs are of the type III class, which include IFNs- λ 1-3 in humans [11, 12]. Constitutive release of IFNs is a unique feature of trophoblasts as IFNs are tightly regulated in most cell types and induced only after detection of the products of viral replication. In mice, type III IFNs also confer protection from Zika virus (ZIKV) vertical transmission and fetal disease, which occurs in a gestational-associated manner [13, 14], although it is not clear whether mice constitutively release these IFNs. Together, these findings suggest that maternal- and fetal-derived tissue differentially respond to and control viral infections. However, the lack of available models to fully interrogate the differences between maternal- and fetal-derived cells has limited a complete understanding of these processes, including how each tissue type responds to infection.

The recent development of trophoblast and decidua organoids from first trimester placental tissue has expanded the models available to study the maternal-fetal interface [15, 16]. Previous work has shown that isolation and propagation of trophoblast stem cells from first trimester placental chorionic villi leads to the development of three-dimensional organoids that differentiate to contain all lineages of trophoblast cells present in the human placenta and release well-known pregnancy hormones such as human chorionic gonadotropin (hCG) [15]. Similarly, decidua organoids isolated from uterine glands recapitulate the transcriptional profile of their tissue of origin and respond to hormonal cues [16]. These organoid models thus provide ideal systems to profile differences between maternal- and fetal-derived cells, however, these models have not yet been applied to pathogen infections.

To directly compare antiviral signaling pathways between the human placenta and decidua, we generated trophoblast and decidual organoids from matched human placentas and profiled the release of cytokines, chemokines, and other factors from these models under uninfected and virally infected conditions. In parallel, we performed similar profiling in matched tissue explants from which these organoids were developed and found that organoids produced distinct patterns of released cytokines. To determine whether there are differences in the responses of maternal- and fetal-

derived cells to viral infections, we also compared the immunological secretome from organoids treated with a ligand to stimulate toll-like receptor 3 (TLR3) signaling or after infection with ZIKV. These studies revealed that trophoblast organoids selectively secrete the type III IFN IFN- λ 2, which does not occur in matched decidua organoids. Moreover, trophoblast and decidua organoids differentially respond to TLR3 activation and ZIKV infection through the secretion of organoid-specific cytokines. Our studies highlight the differential responses of key cells comprising the maternal-fetal interface and provide new models to define the impact of pathogen exposure at this key site.

Results

Establishment and characterization of human trophoblast and decidual organoids

To generate matched human trophoblast organoids (TOs) and decidual organoids (DOs), we used human placental tissue isolated from the second and third trimesters of human pregnancy and cultured stem cells isolated from chorionic villi or isolated decidua in growth factor-containing medium like that described previously [15-20] (**Figure 1A**). Although the abundance of villous stem cells decreases after the first trimester [15], we successfully recovered organoids from all placental tissue harvested (five unique placentas). Isolated stem cells were embedded in Matrigel to allow for self-organization and propagated for ~3 weeks to generate organoid structures that were morphologically similar to those described previously that were isolated from the first trimester [15, 16] (**Figure 1B**). Also, like those isolated from the first trimester [15], cultured TOs developed projections that resembled villous structures (**Figure 1B**, arrows at bottom). Confocal microscopy confirmed the three-dimensional architecture of established organoids and verified that TOs expressed markers such as cytokeratin-19 (**Figure 1C**) and DOs expressed markers including cytokeratin-7 and -19 and E-cadherin (**Figure 1D**), consistent with previous reports [15, 16]. We also confirmed that TOs, but not DOs, secreted the trophoblast-specific hormone human chorionic gonadotropin (hCG) by both ELISA and rapid test strip assays (**Figure 1E**) and pregnancy-specific beta-1 glycoprotein 1 (PSG1) by ELISA, a major product released from the STB, similar to chorionic villous explants (**Figure 1F**).

TOs could be propagated for at least three months and passaged approximately every 2 weeks with stable hCG release (**Supplemental Figure 1A, 1B**).

Next, we performed transcriptional profiling of isolated TOs and DOs using RNASeq. We found that TOs and DOs were transcriptionally distinct based on volcano and principal component analysis (PCA) plots (**Figure 2A, Supplemental Figure 2A, 2B**). TOs highly expressed transcripts specifically enriched in the placenta, including HSD3B1, XAGE2 and 3, GATA3, and ERVW-1, which were absent or expressed at very low levels in DOs (**Figure 2B**). In addition, TOs highly expressed transcripts for PSGs and hCG (**Figure 2B**). The levels of expression of these transcripts were equivalent to those in primary human trophoblasts, confirming that TOs recapitulate the transcriptional signature of primary cells (**Figure 2B**). These markers were consistent with the presence of the syncytiotrophoblast (e.g., CGA, PSGs, CSH1, HSD3B1), cytotrophoblasts (e.g., TP63, ITGA6), and extravillous trophoblasts (e.g., ITGA5, HLA-G) (**Figure 2B**). In contrast, DOs expressed markers associated with the decidua and decidual stromal cells, including MUC1, MUC5A, MUC5B, and SOX17, which were absent or expressed at low levels in TOs and PHT cells (**Figure 2B**). To further confirm that TOs and DOs recapitulated their tissues of origin, we profiled the expression of three members of the chromosome 19 microRNA family (C19MC), which are specifically expressed in the human placenta [21]. We found that miR-516B-5p, miR-517A-3p, and miR-525-5p were expressed at high levels in TOs, comparable to levels observed in freshly isolated chorionic villi, but were not expressed in DOs (**Figure 2C**). Lastly, we found that TOs isolated from placentas obtained from male fetuses expressed Y-linked genes, which were absent in female-only derived DOs (**Supplemental Figure 2C**). Collectively, these data establish matched TOs and DOs as model systems of the maternal-fetal interface and that they can be isolated from tissue after the first trimester, including from mid-to-late in gestation.

Cytokine profiling of TOs and DOs compared to matched tissue explants

We have shown previously that human chorionic villous explants release a number of cytokines, chemokines, and growth factors that impact both maternal and fetal antimicrobial defenses [22]. Similarly, we have shown that primary human trophoblasts (PHTs) isolated from mid-gestation and full-term placentas secrete some of these same immunoactive factors [10, 22]. We therefore profiled the immunological secretome of TOs isolated from three independent placentas and compared these profiles to chorionic villous explants isolated from the same placentas. In addition, we compared these profiles to PHT cells to determine whether there were differences between stem cell-derived trophoblasts and primary trophoblasts isolated directly from full-term tissue. To do this, we used multianalyte Luminex-based profiling for ~80 cytokines, chemokines, growth and other factors from conditioned media (CM) isolated from these models under unstimulated conditions. Factors whose secretion was reliably measured over 50pg/mL were identified as present. We found that TOs secreted two cytokines (IL-6 and IL-28A/IFN- λ 2) and three immune-regulated secreted soluble receptors (sTNF-R1, gp130/sIL-6R β , sTNF-R2) (**Figure 2A**). By contrast, matched chorionic villous explants were immunologically more active and we reliably detected 51 factors (**Figure 2B**). Similarly, PHT cells also released a greater number of factors, with 31 detected (**Figure 2C**). The five factors released from TOs were also released from chorionic villous explants and PHT cells (**Figure 2D**). The most abundant cytokine present in CM isolated from TOs was IL-6, which was released at levels similar to those observed in both chorionic villi and PHT cells (**Figure 2E**). In contrast, whereas the most abundant factor in CM isolated from PHT cells was IL-8, which was also highly abundant in CM from chorionic villi, this cytokine was not detected in CM isolated from TOs, suggesting that there might be differential immune regulation between terminally differentiated PHT cells and those that are stem-cell derived (**Figure 2F**).

To define the immunological secretome of DOs, we performed similar Luminex-based profiling as the studies described above from CM isolated from DOs and decidua explants. Compared to TOs, DOs released a larger number of factors overall, with 21 detected in DO-derived CM (**Figure 3A**). Of these, 19 were also released from human decidua explants, which contained

50 total cytokines and other factors (**Figure 3B, 3C**). DOs and decidua explant tissue secreted similar levels of factors such as MMP-1 (**Figure 3D**). In contrast, we found that DOs secreted high levels of IL-10, which was not detected in CM from decidua explants (**Figure 3E**). Together, these data suggest that TOs and DOs partially recapitulate the secretion profile of chorionic villi and the decidua.

Trophoblast organoids constitutively secrete antiviral IFN- λ s

We next directly compared the secretion profiles from CM collected from matched TOs and DOs to identify factors enriched in either maternal- or fetal-derived cells. TOs selectively secreted two cytokines that were not present in CM isolated from matched DOs, IL-6 and IFN- λ 2 (**Figure 4A-B, D**). In contrast, DOs selectively released high levels of other factors such as IL-8 (**Figure 4C**). We showed previously that PHT cells isolated from full-term placentas and mid-gestation chorionic villi constitutively release type III IFNs, which protect from viral infections [9, 10]. We found that TOs recapitulated this phenotype and released high levels of IFN- λ 2, but not type I IFNs or IFN- λ 1 (**Figure 4E**). In contrast, DOs did not basally secrete any detectable IFNs (**Figure 2A, 4D**). Consistent with this, the expression of type III IFN transcripts was very high in TOs, but not DOs (**Figure 3F**). We monitored the release of IFN- λ 2 over the growth period of TOs and found that its release was detectable by ~10-days post-plating and reached high levels by >14-days in culture, which correlated with similar trends for IL-6 (**Supplemental Figure 3A**). We confirmed that CM isolated from TOs, but not DOs, induced interferon stimulated genes (ISGs) using a HEK293 based reporter assay (**Figure 4G**). Consistent with this, we found that CM isolated from TOs was antiviral and reduced ZIKV infections in non-placental cells treated with this CM (**Figure 4H**). PHT cells basally express high levels of ISGs, most likely from the autocrine effects of released IFN- λ s [10]. Therefore, we compared the basal expression of several ISGs in TOs and DOs and compared these levels to those in PHT cells, and in two choriocarcinoma cell lines, JEG-3 and JAR cells, which do not basally release type III IFNs [9]. We found that similar to PHT cells, TOs basally express high levels of ISGs,

which was not observed in either JEG-3 or JAR cells (**Supplemental Figure 3B**). However, despite the absence of released IFN- λ s, DOs also expressed high levels of these ISGs (**Supplemental Figure 3B**). These data show that TOs recapitulate the constitutive release of type III IFNs observed in primary trophoblast cells and tissue and that these IFNs function in paracrine to restrict viral infections.

Differential response to IFNs in TOs and DOs

Previous work has shown that chorionic villous and decidua explants respond to treatment with recombinant IFNs, which protect from ZIKV infection [13], although villous explants appear to be less responsive to type III compared to type I IFNs [23]. However, given that the tissue used in these studies contains many different cell types, we investigated the sensitivity of TOs and DOs to recombinant type I (IFN- β) or type III (IFN- λ) treatment. We found that DOs were highly responsive to both type I and III IFN treatment and robustly induced ISGs including IFI44L and OASL (**Figure 6A, 6B**). These ISGs were similarly induced by IFN treatment of decidua explants (**Figure 6C**). Although TOs did respond to both type I and III IFN treatment, the magnitude of these responses were lower than those in DOs (**Figure 6A, 6B**), which were consistent with the responses of IFN-treated chorionic villous explants (**Figure 6C, 6D**). These data suggest that there are differences in the sensitivity of maternal-and fetal-derived cells to type I and III IFNs.

Differential induction of antiviral signaling in TOs and DOs

Because we observed differences in the constitutive release of IFN- λ 2 from TOs and DOs, we next determined how these organoids responded to viral infections. To do this, we used poly (I:C), a synthetic ligand of TLR3 and measured differences in cytokine induction using Luminex-based profiling. We found that both TOs and DOs robustly responded to poly I:C treatment, which corresponded with the induction of ISGs in both models, although TOs induced higher transcript

levels of these ISGs (**Supplemental Figure 4A**). Cytokine profiling of CM isolated from poly I:C-treated TOs and DOs showed that TOs induced 15 factors (>5-fold change from mock) and DOs induced 14 factors (>5-fold change from mock) in response to TLR3 activation (**Figure 7A**). Of the factors induced, 9 were shared between both TOs and DOs (**Figure 7B**), which included IFN- λ 1, which was the most induced cytokine in both organoids (**Figure 7C**) and IFN- λ 2 (**Figure 7D**). Several cytokines were differentially induced in TOs versus DOs, including IFN- γ , which was induced in DOs but not TOs (**Supplemental Figure 4B**) and IL-8, which was induced in TOs but not DOs (**Supplemental Figure 4C**). In addition, we found that whereas TOs induced the type I IFNs IFN- α 2 and IFN- β in response to poly I:C, DOs induced no IFN- α 2 and low levels of IFN- β (**Figure 7E, 7F**).

We next profiled the differential response to viral infections in TOs and DOs using ZIKV. We found that both TOs and DOs supported low levels of ZIKV replication as assessed by focus-forming unit (FFU) assays at 72hrs post-infection (p.i.) (**Figure 8A**). However, levels of ZIKV infection were significantly lower than those observed in permissive JEG-3 cells (**Figure 8A**). To determine the impact of this infection on the transcriptional profiles of TOs and DOs, we performed RNASeq. Consistent with the low levels of infection observed by FFU assays, there were very low levels of viral RNA present in infected organoids, as determined by FPKM values (**Figure 8B**). Also consistent with low levels of viral replication, there were no significant changes in the transcriptional profile of ZIKV infected DOs (**Figure 8C**) and only changes in three transcripts in TOs (**Figure 8D**).

To determine if these transcriptional profiles were associated with changes in cytokine release, we performed luminex-based profiling of CM isolated from these cultures at 24hrs, 48hrs, and 72hrs p.i.. This profiling showed that few cytokines were induced by ZIKV infection and no cytokines or other factors were induced >5-fold in either TOs or DOs (**Figure 8E, 8F**). Consistent with poly I:C treatment, TOs induced very low levels of IFN- λ 1 and IFN- β at 72hrs p.i., which was not observed in DOs (**Figure 8G, 8H**). In contrast, DOs secreted low levels of the glycoposphoprotein osteopontin at 24hrs p.i., which decreased over the course of infection (**Figure**

8I). Taken together, these data show that TOs and DOs differentially respond to viral infections through the release of select cytokines and other factors.

Discussion

Our work presented here establishes fetal- and maternal-derived organoids as models of innate immune signaling at the maternal-fetal interface. We show that TOs constitutively secrete two cytokines at high levels, the pro-inflammatory cytokine IL-6 and the antiviral IFN- λ 2. In contrast, we show that DOs are immunologically more active under resting conditions and basally secrete several cytokines not observed in TOs, but do not release IFNs under unstimulated conditions. In addition, we show that TOs and DOs differentially respond to TLR3 activation and ZIKV infection and induce organoid-specific cytokines in response to activation of antiviral innate immune signaling. Collectively, our work suggests that cells that comprise the maternal-fetal interface defend from viral infections in a cell-type specific manner and highlight the unique contributions of fetal-derived trophoblasts in antiviral immunity.

The previous establishment of trophoblast and decidua-derived organoids has significantly expanded the *in vitro* systems that can model the complexity of the maternal-fetal interface [15, 16]. These previous studies utilized tissue isolated from the first trimester of pregnancy (6-9 weeks gestation for TOs and 8-12 weeks gestation for DOs) [15, 16], although another study successfully established DOs from full-term tissue [20]. Here, we show that TOs can also be isolated from later stages of gestation, including from full-term placental tissue. Moreover, we show that the growth medium and conditions used to grow organoids from first trimester tissue can be extrapolated to grow organoids from the second and third trimesters of pregnancy. Two-dimensional cultures of stem cell-derived primary trophoblasts are also restricted to tissue obtained in the first trimester [18]. Given that access to first trimester tissue can be complicated by access and/or regulatory restrictions, our studies provide an additional source for the development of placental organoids from tissue obtained

later in gestation. Moreover, the establishment of these organoids from tissues later in gestation may allow for elucidation of the role of maternal-fetal signaling in obstetric related pathologies.

Unlike many other barrier cell types such as the epithelium, human trophoblasts have the unique capacity to robustly secrete cytokines and other immunological factors without stimulation. Primary human trophoblasts from full-term placentas and mid-gestation chorionic villi secrete cytokines that mediate innate defenses from bacteria, parasites, and viruses [9, 22, 24]. Consistent with this, serum isolated from pregnant women often contains elevated levels of these cytokines, indicating that at least some have reached the systemic circulation [22, 25-27]. Similarly, the decidua releases specific cytokines and alterations in these profiles may be associated with pregnancy complications including miscarriage [28-33]. The decidua also responds to microbial infections via the release of additional cytokines that function in innate defense [6, 34, 35]. However, in many cases, these studies were performed on tissue explants that were multicellular in nature, thus preventing an assessment of the contributions of cytokine release from distinct cell types. Similarly, previous work utilizing tissue explants that are treated with recombinant cytokines, synthetic ligands of various innate immune pathways, or infected with pathogens are also unable to define the contributions of distinct cell types in these responses. The use of organoids therefore allows for analyses of cellular responses to infection or innate immune activation from trophoblasts and decidual glands in primary cell models that contain isolated cell types enriched at the maternal-fetal interface. An additional strength of the TO model is that trophoblast stem cells can differentiate into all three distinct trophoblast cell types comprising the human placenta [15], which is not possible in many other existing models including isolation of CTBs from full-term placentas.

The constitutive release of IFN- λ s from human trophoblasts is a highly unique phenomenon that runs counter to much of what is understood about antiviral IFN signaling, which is centered on a requirement for viral sensing to induce this pathway. Our findings from TOs support that IFN release occurs soon after trophoblast differentiation and is likely an early mechanism used by the placenta to defend from viral infections. Our study also indicates that TOs and DOs are largely

resistant to ZIKV infection and produced titers significantly lower than those observed in permissive cells. Previous work has shown that human CTBs and EVT_s are permissive to ZIKV whereas the STB is resistant [7, 36]. Given that TO_s contain EVT_s, CTBs, and that the STB faces inward in the organoid structure, it is possible that the low level of infection observed in TO_s results from these cells supporting ZIKV infection. It remains unknown whether EVT_s and CTBs exhibit differential responses to type III IFNs which might impact this differential susceptibility to ZIKV infection. The mechanism of resistance of DO_s is less clear, given that these organoids do not basally secrete the type I and III IFNs we measured. Of note, DO_s expressed high levels of many ISGs in the absence of stimulation, which might be linked to their relative resistance to ZIKV infection. Interestingly, our data also show that TO_s constitutively release IFN- λ 2 and that IFN- λ 1 is only induced upon activation of antiviral signaling pathways. IFN- λ s genes are located in tandem on chromosome 19 in humans and the mechanisms that might control the constitutive release of IFN- λ 2 specifically in trophoblasts is unclear. Our studies also show that type III IFNs are further induced by activation of antiviral innate immune signaling in both TO_s and DO_s, with IFN- λ 1 being the most induced cytokine detected in both organoid types. However, the type I IFN IFN- β is induced to greater levels in TO_s than DO_s, suggesting that at least some of the cells that comprise the maternal-fetal interface differentially rely on type I and III IFNs for antiviral defenses.

Previous work suggests that type I IFNs damage placental structure and function in mice and induce alterations in placental structure in *ex vivo* human chorionic villi [23]. In contrast, type III IFN signaling in mice is protective against viral infections and there are no deleterious effects of treatment of pregnant animals with recombinant IFN- λ s [13, 14]. While the mechanisms for the opposing effects of type I and III IFNs have yet to be fully elucidated, at least one ISG, interferon-induced transmembrane (IFITM), inhibits fusion of CTBs into the STB [37, 38], which may indicate that expression of select ISGs is tightly regulated to prevent IFN-mediated placental damage. Our data in TO_s and DO_s suggests that trophoblasts and decidual cells exhibit differential responsiveness to

recombinant IFN treatment, with treatment of TOs inducing lower levels of ISGs than those observed in DOs. While this might be attributed to reduced expression of the receptors associated with these IFNs in TOs, there might also be additional mechanisms employed by trophoblasts to protect from IFN- and ISG-mediated damage.

Our work presented here develops TOs and DOs from mid-to-late gestation placental tissue and demonstrates that these organoids recapitulate some of the immunologic secretory phenotypes of tissue explants isolated from matched placental tissue. We further show that TOs and DOs constitutively secrete unique cytokines and respond to viral infections through the release of organoid-specific cytokines. Our work also highlights the prominent role of type III IFNs in antiviral immunity at the maternal-fetal interface and demonstrates that TOs constitutively release IFN- λ 2. Collectively, these studies define the differential responses of key cells comprising the maternal-fetal interface and suggest that organoid-based models can be used to define innate immune signaling at this interface.

Materials and Methods

Human samples. All human tissue used in this study was obtained through the UPMC Magee-Womens Hospital Obstetric Maternal & Infant Database and Biobank after approval was received from the University of Pittsburgh Institutional Review Board (IRB) and in accordance with the guidelines of University of Pittsburgh human tissue procurement. Placental tissue used in this study was collected from the second (23rd and 27th) and third (35th and 38th) trimesters, including from dizygotic twins. Placental tissue was excluded for diagnosis of PPRM, labor, or maternal infection.

Derivation and culture of trophoblast organoid (TOs) cultures from human placental samples.

Villous trophoblast stem cells were isolated from fresh placental tissues as described previously [15]. Briefly, villi collected from fresh placental tissue were intensively washed, then were sequentially digested with 0.2% trypsin-250 (Alfa Aesar, J63993-09)/0.02% EDTA (Sigma-Aldrich, E9884-100G)

333 and 1.0 mg/mL collagenase V (Sigma-Aldrich, C9263-100MG). Pooled digests were washed with
334 Advanced DMEM/F12 medium (Gibco 12634-010) and pelleted by centrifugation, then resuspended
335 in approximate 10 times volume of ice-cold growth-factor-reduced Matrigel (Corning 356231).
336 Matrigel “domes” (40 µL/well) were plated into 24-well tissue culture plates (Corning 3526), placed in
337 a 37°C incubator to pre-polymerize for ~2 min, turned upside down to ensure equal distribution of
338 the isolated cells in domes for another 10 min, then carefully overlaid with 500 µL prewarmed
339 trophoblast organoid medium (TOM) consisting of Advanced DMEM/F12 (Life Technologies, 12634-
340 010) supplemented with 1× B27 (Life Technologies, 17504-044), 1× N2 (Life Technologies, 17502-
341 048), 2 mM GlutaMAXTM supplement (Life Technologies, 35050-061), 100 µg/mL Primocin
342 (InvivoGen, ant-pm-1), 1.25 mM N-Acetyl-L-cysteine (Sigma, A9165), 500 nM A83-01 (Tocris, 2939),
343 1.5 µM CHIR99021 (Tocris, 4423), 50 ng/mL recombinant human EGF (Gibco, PHG0314), 80 ng/mL
344 recombinant human R-spondin 1 (R & D systems, 4645-RS-100), 100 ng/mL recombinant human
345 FGF2 (Peprotech, 100-18C), 50 ng/mL recombinant human HGF (Peprotech, 100-39), 10mM
346 nicotinamide (Sigma, N0636-100G), 2 µM Y-27632 (Sigma, Y0503-1MG), and 2.5 µM prostaglandin
347 E2 (PGE2, R & D systems, 22-961-0). Cultures were maintained in a 37°C humidified incubator with
348 5% CO₂. Medium was renewed every 2-3 days. Small trophoblast organoid spheroids became visible
349 by approximately day 12 post-isolation. Derived TOs were passaged every 10 to 14 days depending
350 on their size and density. To passage, TOs were released from domes with ice-cold cell recovery
351 solution (Corning, 354253) and then were digested in prewarmed TrypLE Express (Gibco, 12605-
352 028) or Stem Pro Accutase (Gibco, A11105-01) at 37°C for 5-7 min. Further mechanical dissociation
353 was achieved with an Eppendorf Explorer Plus electronic pipettes at its “mix” function setting by
354 pipetting up and down around 400 times through small-bore pipette tips. Dissociated TOs fragment
355 were collected and washed by centrifuge, then resuspended in fresh ice-cold Matrigel and replated
356 as domes at the desired density for continuous culture. For freezing TOs, overlaid TOM was
357 aspirated, TOs with Matrigel were resuspended in CryoStor CS10 stem cell freezing medium
358 (STEMCell Technologies, 07930) frozen at -80 °C and then transferred to liquid nitrogen for long-

term storage. For thawing cryopreserved TOs, TOs were thawed as quickly as possible, diluted with 5 times volume of basic TOM containing Advanced DMEM/F12, 2 mM GlutaMAX supplement, 10 mM HEPES (Gibco, 15630-106), 1 × Penicillin/Streptomycin (Lonza, 17-602E) and centrifuged to pellet. Afterwards, TOs were resuspended in new ice-cold Matrigel and replated for recovery culture.

Derivation and culture of decidual organoids (DOs) cultures from human decidua samples.

Decidual gland-enriched cell suspension were acquired from fresh decidual tissue as described previously [16, 39]. Briefly, isolated decidual tissues were cut into small pieces then digested in 1.25 U/mL Dispase II (Sigma, D4693)/0.4 mg/mL collagenase V (Sigma, C-9263) solution. Glandular cells were filtered out from the digestion solution supernatant and resuspended in ice-cold Matrigel (Corning, 356231), plated as domes in 24-well plates (Corning 3526), and finally overlaid with 500 µL prewarmed organoid Expansion Medium (ExM) with the same composition as previously described[16]. The growth medium was renewed every 2-3 days. Mature organoids were passaged by mechanically disruption following TrypLE Express (Gibco, 12605) digestion every 7-10 days. Other maintenance were similar to those as previously described [39].

Generation of TO and DO conditioned media (CM). Overlaid TOM and ExM were collected every 2-4 days based on the growth status of TOs and DOs. Media from at least ten timepoints for each established organoid lines were collected as TO-CM or DO-CM. Non-conditioned medium (NCM) was TOM or ExM (described above) that had not been exposed to TOs or DOs, respectively.

Cells and Viruses. PHT cells were established and cultured as described previously [9] . Human osteosarcoma U2OS (ATCC HTB-96) and Vero (ATCC CCL-81) cells were grown in Dulbecco's minimal essential medium (DMEM) with 10% FBS and 1% antibiotic. JEG-3 and JAR were purchased from the ATCC and cultured as previously described [9, 40]. HEK-Blue IFN-λ reporter cells (hkb-ifnl) were purchased from InvivoGen and cultured according to the manufacturer's

instructions. ZIKV (Paraiba/2015, provided by David Watkins, University of Miami) was propagated in Vero cells. Viral titers were determined by fluorescent focus assay as previously described [41] using recombinant anti-double-stranded RNA monoclonal antibody J2 (provided by Abraham Brass, University of Massachusetts) in Vero cells. Infection was determined by qRT-PCR as described below. To assess antiviral activity, U2OS cells were exposed to TO or DO CM, or matched NCM, 24 hr prior to infection with ZIKV Paraiba/2015 for 72hrs. ZIKV infection of TOs and DOs was performed using 10^6 FFU virus per well for 24-72hrs, as indicated.

Immunofluorescence microscopy. TOs and DOs were released from Matrigel domes with 1 mL of cell recovery solution (Corning, 354253) per well without disrupting their 3D architecture, then were fixed in 4%PFA for 30 min at room temperature, followed by 0.5% Triton X-100/PBS to permeabilize for 30 min at 4°C. Organoids were washed and blocked in 5%(v/v) goat serum/0.1%(v/v) Tween-20 in PBS for 15 min at room temperature and then incubated with primary antibodies in the above-described blocking solution at 4°C overnight. Organoids were then washed with PBS and then incubated for 1-2 h at room temperature with Alexa Fluor-conjugated secondary antibodies (Invitrogen). Organoids were washed again with PBS and mounted in Vectashield (Vector Laboratories) containing 4',6-diamidino-2-phenylindole (DAPI) and were transferred onto microscope slides with large-orifice 200 μ L-tips (Fisher Scientific, 02707134). The following antibodies or reagents were used: cytokeratin-7 (Abcam, ab9021), cytokeratin-19 (Abcam, ab9221), E-cadherin (Invitrogen, PA5-85088), Abcam), Alexa Fluor 594-conjugated phalloidin (Invitrogen, A12381), Alexa Fluor Plus 488 Goat anti-Mouse IgG secondary antibody (Invitrogen, A32723), Alexa Fluor 488 Goat anti-Rabbit IgG secondary antibody (Invitrogen, A11034). Images were captured using a Zeiss LSM 710 inverted laser scanning confocal microscope and contrast-adjusted in Photoshop or Fiji.

RNA extraction, cDNA synthesis and quantitative PCR. Total RNA was extracted with the Sigma GenElute total mammalian RNA miniprep kit following the manufacturer's instruction and using the supplementary Sigma DNase digestion. RNA quality and concentration were determined using a Nanodrop ND-1000 Spectrophotometer. Total RNA was reverse-transcribed with the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions. Quantitative PCR was performed using the iQ SYBR Green Supermix (Bio-Rad, 1708882) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Gene expression was determined based on a ΔC_T method normalized to actin. The expression level of C19MC miRNAs hsa-miR-516b-5p, hsa-miR-517a-3p, hsa-miR-525-5p and reference gene RNU6 was quantified by using the MystiCq® MicroRNA® Quantitation System (Millipore Sigma) which includes miRNAs Poly(A) tailing, cDNA synthesis (MIRRT) and miRNA qPCR (MIRRM00). MicroRNAs were first polyadenylated in a poly(A) polymerase reaction, then reverse transcribed using an oligo-dT adapter primer according to the manufacturer's protocol (Sigma-Aldrich, MIRRT). Individual microRNAs (microRNA-516b-5p, microRNA-517a-3p and microRNA-525-5p) were quantified in real-time SYBR® Green RT-qPCR reactions with the specific MystiCq microRNA qPCR Assay Primer (MIRAP00475, MIRAP00477 and MIRAP00512) and the MystiCq Universal PCR Primer (MIRUP). Cq reading were normalized to the RNU6 internal reference by the $2^{-\Delta C_q}$ method. Primer sequences for actin, IFN- β , IL-28A/IFN- λ 2, IFI44L, OASL, GATA3, MUC20, and ZKIV vRNA have been previously described [22, 42]. All primer sequences used in this study can be found in Table S1.

RNA-seq analysis. For RNA-seq analysis, RNA was isolated from organoids as described above. Purified Total RNA was verified by Thermo scientific Nanodrop one. The libraries were prepared by the Duke Center for Genomic and Computational Biology (GCB) using the Tru-Seq stranded total RNA prep kit (Illumina). Sequencing was performed on the NovaSeq 6000 by using 150-bp paired-end sequencing. The reads were aligned to the human reference genome (GRCh38) using QIAGEN CLC Genomics (v20) or the ZIKV genome to calculate the FPKM values. DESeq2 [43] was used to

normalized count data and to do the differential gene expression analysis with a significance cutoff of 0.01 and a fold change cutoff of $\log_2(2)$. Heatmaps and hierarchical clustering was performed in R using pheatmap package in R and were based on RPKM (reads per kilobase million). Files associated with RNA-seq studies have been deposited into Sequence Read Archive under accession number SRA. Data from PHT cells (accession SRP067137 and SRP109039) and JAR (accession SRP095402) and JEG-3 cells (accession SRP109039) was based on published datasets [9, 10, 44]. Principal component analyses were performed using pcaExplorer in R [45]. Volcano plots were generated using the EnhancedVolcano package in R [46].

hCG and PSG1 ELISAs and Luminex assays. Human hCG (Sigma, RAB0092) and PSG1 (R&D Systems, DY6799) ELISAs were performed according to the manufacturer's instructions. Plates were read at 450 and 520 nm using a Synergy H1 microplate reader. All sample processing was performed in duplicates and each experiment was performed with at least three biological replicates. Luminex assays were performed using the following kits according to the manufacturer's instructions: Bio-Plex Pro Human Inflammation Panel 1, 37-Plex kit (171AL001M; Bio-Rad Laboratories) and Bio-Plex Pro Human Cytokine Screening Panel, 48-Plex kit (12007283; Bio-Rad Laboratories). The plates were read on a MAGPIX (EMD Millipore) Luminex machine and analyzed using xPONENT software (EMD Millipore).

IFN- λ activity reporter assay. For IFN- λ activity detection from CM, HEK-Blue IFN- λ reporter cells were used according to the manufacture's protocol. Briefly, IFN- λ reporter cells were plated into a 96-well plate, then CM was added and incubated at 37 °C for 24 hr. Supernatants (20 μ l) were collected and then transferred into a 96-well plate and activity measured using QUANTI-Blue Solution (InvivoGen, rep-qbs) after incubating at 37 °C for 2 hr using a spectrophotometer at 655 nm. Each CM preparation was run in triplicate.

Recombinant human IFN and poly(I:C) treatment. For Poly (I:C) treatment, TOs or DOs were incubated with 10 µg/mL Poly (I:C) diluted in organoid complete growth media for ~24 h, then supernatant was collected for Luminex-based multianalyte profiling or RNA isolated as described for qRT-PCR. For recombinant human IFN treatment, TOs or DOs were treated with IFN-β (8499-IF-010; R&D Systems) or IFN-λ3 (5259-IL-025, R&D Systems) for 24 hr, then total RNA was extracted as described above for following qRT-PCR analysis.

Statistics and reproducibility. All experiments reported in this study have been reproduced using independent samples (tissues and organoids) from multiple donors. All statistical analyses were performed using Prism software (GraphPad Software). Data are presented as mean ± SD, unless otherwise stated. Statistical significance was determined as described in the figure legends. Parametric tests were applied when data were distributed normally based on D'Agostino-Pearson analyses; otherwise, nonparametric tests were applied. P values <0.05 were considered statistically significant, with specific P values noted in the figure legends.

Author contributions LY designed and performed experiments and participated in manuscript review and writing, CM performed tissue dissections and participated in manuscript review and writing, and CC designed and performed experiments, participated in manuscript review and writing, and secured funding.

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Literature Cited

1. Burton GJ, Watson AL, Hempstock J, Skepper JN, Jauniaux E. Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy. *J Clin Endocrinol Metab.* 2002;87(6):2954-9. Epub 2002/06/07. doi: 10.1210/jcem.87.6.8563. PubMed PMID: 12050279.
2. McDonagh S, Maidji E, Chang HT, Pereira L. Patterns of human cytomegalovirus infection in term placentas: a preliminary analysis. *J Clin Virol.* 2006;35(2):210-5. Epub 2006/01/03. doi: 10.1016/j.jcv.2005.08.011. PubMed PMID: 16386950.
3. Weisblum Y, Panet A, Zakay-Rones Z, Haimov-Kochman R, Goldman-Wohl D, Ariel I, et al. Modeling of human cytomegalovirus maternal-fetal transmission in a novel decidual organ culture. *J Virol.* 2011;85(24):13204-13. Epub 2011/10/07. doi: 10.1128/JVI.05749-11. PubMed PMID: 21976654; PubMed Central PMCID: PMC3233115.
4. Pereira L, Maidji E, McDonagh S, Genbacev O, Fisher S. Human cytomegalovirus transmission from the uterus to the placenta correlates with the presence of pathogenic bacteria and maternal immunity. *J Virol.* 2003;77(24):13301-14. Epub 2003/12/04. doi: 10.1128/jvi.77.24.13301-13314.2003. PubMed PMID: 14645586; PubMed Central PMCID: PMC3296088.
5. Weisblum Y, Oiknine-Djian E, Vorontsov OM, Haimov-Kochman R, Zakay-Rones Z, Meir K, et al. Zika Virus Infects Early- and Midgestation Human Maternal Decidual Tissues, Inducing Distinct Innate Tissue Responses in the Maternal-Fetal Interface. *J Virol.* 2017;91(4). Epub 2016/12/16. doi: 10.1128/JVI.01905-16. PubMed PMID: 27974560; PubMed Central PMCID: PMC5286880.
6. Guzeloglu-Kayisli O, Guo X, Tang Z, Semerci N, Ozmen A, Larsen K, et al. Zika Virus-Infected Decidual Cells Elicit a Gestational Age-Dependent Innate Immune Response and Exaggerate Trophoblast Zika Permissiveness: Implication for Vertical Transmission. *J Immunol.* 2020;205(11):3083-94. Epub 2020/11/04. doi: 10.4049/jimmunol.2000713. PubMed PMID: 33139490.
7. Platt DJ, Smith AM, Arora N, Diamond MS, Coyne CB, Miner JJ. Zika virus-related neurotropic flaviviruses infect human placental explants and cause fetal demise in mice. *Sci Transl Med.* 2018;10(426). Epub 2018/02/02. doi: 10.1126/scitranslmed.aao7090. PubMed PMID: 29386359; PubMed Central PMCID: PMC6136894.
8. Rizzuto G, Tagliani E, Manandhar P, Erlebacher A, Bakardjiev AI. Limited Colonization Undermined by Inadequate Early Immune Responses Defines the Dynamics of Decidual Listeriosis. *Infect Immun.* 2017;85(8). Epub 2017/05/17. doi: 10.1128/IAI.00153-17. PubMed PMID: 28507070; PubMed Central PMCID: PMC5520438.
9. Bayer A, Lennemann NJ, Ouyang Y, Bramley JC, Morosky S, Marques ET, Jr., et al. Type III Interferons Produced by Human Placental Trophoblasts Confer Protection against Zika Virus Infection. *Cell Host Microbe.* 2016;19(5):705-12. Epub 2016/04/14. doi: 10.1016/j.chom.2016.03.008. PubMed PMID: 27066743; PubMed Central PMCID: PMC4866896.

- 552 10. Corry J, Arora N, Good CA, Sadovsky Y, Coyne CB. Organotypic models of type III interferon-
553 mediated protection from Zika virus infections at the maternal-fetal interface. *Proc Natl Acad Sci U*
554 *S A*. 2017;114(35):9433-8. Epub 2017/08/09. doi: 10.1073/pnas.1707513114. PubMed PMID:
555 28784796; PubMed Central PMCID: PMC5584447.
- 556 11. Lazear HM, Schoggins JW, Diamond MS. Shared and Distinct Functions of Type I and Type
557 III Interferons. *Immunity*. 2019;50(4):907-23. Epub 2019/04/18. doi: 10.1016/j.immuni.2019.03.025.
558 PubMed PMID: 30995506; PubMed Central PMCID: PMC6839410.
- 559 12. Wells AI, Coyne CB. Type III Interferons in Antiviral Defenses at Barrier Surfaces. *Trends*
560 *Immunol*. 2018;39(10):848-58. Epub 2018/09/17. doi: 10.1016/j.it.2018.08.008. PubMed PMID:
561 30219309; PubMed Central PMCID: PMC6179363.
- 562 13. Jagger BW, Miner JJ, Cao B, Arora N, Smith AM, Kovacs A, et al. Gestational Stage and
563 IFN-lambda Signaling Regulate ZIKV Infection In Utero. *Cell Host Microbe*. 2017;22(3):366-76 e3.
564 Epub 2017/09/15. doi: 10.1016/j.chom.2017.08.012. PubMed PMID: 28910635; PubMed Central
565 PMCID: PMC65647680.
- 566 14. Chen J, Liang Y, Yi P, Xu L, Hawkins HK, Rossi SL, et al. Outcomes of Congenital Zika
567 Disease Depend on Timing of Infection and Maternal-Fetal Interferon Action. *Cell Rep*.
568 2017;21(6):1588-99. Epub 2017/11/09. doi: 10.1016/j.celrep.2017.10.059. PubMed PMID:
569 29117563; PubMed Central PMCID: PMC5726784.
- 570 15. Turco MY, Gardner L, Kay RG, Hamilton RS, Prater M, Hollinshead MS, et al. Trophoblast
571 organoids as a model for maternal-fetal interactions during human placentation. *Nature*.
572 2018;564(7735):263-7. Epub 2018/11/30. doi: 10.1038/s41586-018-0753-3. PubMed PMID:
573 30487605; PubMed Central PMCID: PMC67220805.
- 574 16. Turco MY, Gardner L, Hughes J, Cindrova-Davies T, Gomez MJ, Farrell L, et al. Long-term,
575 hormone-responsive organoid cultures of human endometrium in a chemically defined medium. *Nat*
576 *Cell Biol*. 2017;19(5):568-77. Epub 2017/04/11. doi: 10.1038/ncb3516. PubMed PMID: 28394884;
577 PubMed Central PMCID: PMC5410172.
- 578 17. Sheridan MA, Fernando RC, Gardner L, Hollinshead MS, Burton GJ, Moffett A, et al.
579 Establishment and differentiation of long-term trophoblast organoid cultures from the human
580 placenta. *Nat Protoc*. 2020;15(10):3441-63. Epub 2020/09/11. doi: 10.1038/s41596-020-0381-x.
581 PubMed PMID: 32908314.
- 582 18. Okae H, Toh H, Sato T, Hiura H, Takahashi S, Shirane K, et al. Derivation of Human
583 Trophoblast Stem Cells. *Cell Stem Cell*. 2018;22(1):50-63 e6. Epub 2017/12/19. doi:
584 10.1016/j.stem.2017.11.004. PubMed PMID: 29249463.
- 585 19. Haider S, Meinhardt G, Saleh L, Kunihs V, Gamperl M, Kaindl U, et al. Self-Renewing
586 Trophoblast Organoids Recapitulate the Developmental Program of the Early Human Placenta.
587 *Stem Cell Reports*. 2018;11(2):537-51. Epub 2018/08/07. doi: 10.1016/j.stemcr.2018.07.004.
588 PubMed PMID: 30078556; PubMed Central PMCID: PMC6092984.

- 589 20. Marinic M, Rana S, Lynch VJ. Derivation of endometrial gland organoids from term placenta.
590 Placenta. 2020;101:75-9. Epub 2020/09/17. doi: 10.1016/j.placenta.2020.08.017. PubMed PMID:
591 32937244.
- 592 21. Mouillet JF, Ouyang Y, Coyne CB, Sadovsky Y. MicroRNAs in placental health and disease.
593 Am J Obstet Gynecol. 2015;213(4 Suppl):S163-72. Epub 2015/10/03. doi:
594 10.1016/j.ajog.2015.05.057. PubMed PMID: 26428496; PubMed Central PMCID:
595 PMCPMC4592520.
- 596 22. Megli C, Morosky S, Rajasundaram D, Coyne CB. Inflammasome signaling in human
597 placental trophoblasts regulates immune defense against *Listeria monocytogenes* infection. J Exp
598 Med. 2021;218(1). Epub 2020/09/26. doi: 10.1084/jem.20200649. PubMed PMID: 32976558.
- 599 23. Yockey LJ, Jurado KA, Arora N, Millet A, Rakib T, Milano KM, et al. Type I interferons instigate
600 fetal demise after Zika virus infection. Sci Immunol. 2018;3(19). Epub 2018/01/07. doi:
601 10.1126/sciimmunol.aao1680. PubMed PMID: 29305462; PubMed Central PMCID:
602 PMCPMC6049088.
- 603 24. Ander SE, Rudzki EN, Arora N, Sadovsky Y, Coyne CB, Boyle JP. Human Placental
604 Syncytiotrophoblasts Restrict *Toxoplasma gondii* Attachment and Replication and Respond to
605 Infection by Producing Immunomodulatory Chemokines. mBio. 2018;9(1). Epub 2018/01/11. doi:
606 10.1128/mBio.01678-17. PubMed PMID: 29317509; PubMed Central PMCID: PMCPMC5760739.
- 607 25. Liu J, Hao S, Chen X, Zhao H, Du L, Ren H, et al. Human placental trophoblast cells
608 contribute to maternal-fetal tolerance through expressing IL-35 and mediating iTR35 conversion. Nat
609 Commun. 2019;10(1):4601. Epub 2019/10/12. doi: 10.1038/s41467-019-12484-z. PubMed PMID:
610 31601798; PubMed Central PMCID: PMCPMC6787064.
- 611 26. Vassiliadis S, Ranella A, Papadimitriou L, Makrygiannakis A, Athanassakis I. Serum levels
612 of pro- and anti-inflammatory cytokines in non-pregnant women, during pregnancy, labour and
613 abortion. Mediators Inflamm. 1998;7(2):69-72. Epub 1998/12/04. doi: 10.1080/09629359891199.
614 PubMed PMID: 9836491; PubMed Central PMCID: PMCPMC1781827.
- 615 27. Benyo DF, Smarason A, Redman CW, Sims C, Conrad KP. Expression of inflammatory
616 cytokines in placentas from women with preeclampsia. J Clin Endocrinol Metab. 2001;86(6):2505-
617 12. Epub 2001/06/09. doi: 10.1210/jcem.86.6.7585. PubMed PMID: 11397847.
- 618 28. Lash GE, Ernerudh J. Decidual cytokines and pregnancy complications: focus on
619 spontaneous miscarriage. J Reprod Immunol. 2015;108:83-9. Epub 2015/03/17. doi:
620 10.1016/j.jri.2015.02.003. PubMed PMID: 25771398.
- 621 29. von Rango U, Classen-Linke I, Raven G, Bocken F, Beier HM. Cytokine microenvironments
622 in human first trimester decidua are dependent on trophoblast cells. Fertil Steril. 2003;79(5):1176-
623 86. Epub 2003/05/10. doi: 10.1016/s0015-0282(02)04829-x. PubMed PMID: 12738514.

- 624 30. Alam SM, Konno T, Sahgal N, Lu L, Soares MJ. Decidual cells produce a heparin-binding
625 prolactin family cytokine with putative intrauterine regulatory actions. *J Biol Chem.*
626 2008;283(27):18957-68. Epub 2008/05/10. doi: 10.1074/jbc.M801826200. PubMed PMID:
627 18467328; PubMed Central PMCID: PMCPMC2441551.
- 628 31. Denison FC, Kelly RW, Calder AA, Riley SC. Cytokine secretion by human fetal membranes,
629 decidua and placenta at term. *Hum Reprod.* 1998;13(12):3560-5. Epub 1999/01/14. doi:
630 10.1093/humrep/13.12.3560. PubMed PMID: 9886550.
- 631 32. Laham N, Brennecke SP, Rice GE. Interleukin-8 release from human gestational tissue
632 explants: the effects of lipopolysaccharide and cytokines. *Biol Reprod.* 1997;57(3):616-20. Epub
633 1997/09/01. doi: 10.1095/biolreprod57.3.616. PubMed PMID: 9282999.
- 634 33. Pitman H, Innes BA, Robson SC, Bulmer JN, Lash GE. Altered expression of interleukin-6,
635 interleukin-8 and their receptors in decidua of women with sporadic miscarriage. *Hum Reprod.*
636 2013;28(8):2075-86. Epub 2013/06/07. doi: 10.1093/humrep/det233. PubMed PMID: 23739222.
- 637 34. Qiu X, Zhu L, Pollard JW. Colony-stimulating factor-1-dependent macrophage functions
638 regulate the maternal decidua immune responses against *Listeria monocytogenes* infections during
639 early gestation in mice. *Infect Immun.* 2009;77(1):85-97. Epub 2008/10/15. doi: 10.1128/IAI.01022-
640 08. PubMed PMID: 18852237; PubMed Central PMCID: PMCPMC2612255.
- 641 35. Barber EM, Fazzari M, Pollard JW. Th1 cytokines are essential for placental immunity to
642 *Listeria monocytogenes*. *Infect Immun.* 2005;73(10):6322-31. Epub 2005/09/24. doi:
643 10.1128/IAI.73.10.6322-6331.2005. PubMed PMID: 16177303; PubMed Central PMCID:
644 PMCPMC1230899.
- 645 36. Tabata T, Petitt M, Puerta-Guardo H, Michlmayr D, Wang C, Fang-Hoover J, et al. Zika Virus
646 Targets Different Primary Human Placental Cells, Suggesting Two Routes for Vertical Transmission.
647 *Cell Host Microbe.* 2016;20(2):155-66. Epub 2016/07/23. doi: 10.1016/j.chom.2016.07.002. PubMed
648 PMID: 27443522; PubMed Central PMCID: PMCPMC5257282.
- 649 37. Buchrieser J, Degrelle SA, Couderc T, Nevers Q, Disson O, Manet C, et al. IFITM proteins
650 inhibit placental syncytiotrophoblast formation and promote fetal demise. *Science.*
651 2019;365(6449):176-80. Epub 2019/07/13. doi: 10.1126/science.aaw7733. PubMed PMID:
652 31296770.
- 653 38. Zani A, Zhang L, McMichael TM, Kenney AD, Chemudupati M, Kwiek JJ, et al. Interferon-
654 induced transmembrane proteins inhibit cell fusion mediated by trophoblast syncytins. *J Biol Chem.*
655 2019;294(52):19844-51. Epub 2019/11/19. doi: 10.1074/jbc.AC119.010611. PubMed PMID:
656 31735710; PubMed Central PMCID: PMCPMC6937555.
- 657 39. Turco M, Gardner L., Koo B., Moffett A., & Burton G. Derivation and long-term expansion
658 of human endometrial and decidual organoids. *Protocol Exchange.* 2017. doi:
659 10.1038/protex.2017.030.

40. Drummond CG, Nickerson CA, Coyne CB. A Three-Dimensional Cell Culture Model To Study Enterovirus Infection of Polarized Intestinal Epithelial Cells. *mSphere*. 2016;1(1). Epub 2016/06/16. doi: 10.1128/mSphere.00030-15. PubMed PMID: 27303677; PubMed Central PMCID: PMCPMC4863623.
41. Payne AF, Binduga-Gajewska I, Kauffman EB, Kramer LD. Quantitation of flaviviruses by fluorescent focus assay. *J Virol Methods*. 2006;134(1-2):183-9. Epub 2006/03/03. doi: 10.1016/j.jviromet.2006.01.003. PubMed PMID: 16510196.
42. Good C, Wells AI, Coyne CB. Type III interferon signaling restricts enterovirus 71 infection of goblet cells. *Sci Adv*. 2019;5(3):eaau4255. Epub 2019/03/12. doi: 10.1126/sciadv.aau4255. PubMed PMID: 30854425; PubMed Central PMCID: PMCPMC6402847.
43. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550. Epub 2014/12/18. doi: 10.1186/s13059-014-0550-8. PubMed PMID: 25516281; PubMed Central PMCID: PMCPMC4302049.
44. Van Twisk D, Murphy SP, Thakar J. Optimized logic rules reveal interferon-gamma-induced modes regulated by histone deacetylases and protein tyrosine phosphatases. *Immunology*. 2017;151(1):71-80. Epub 2017/01/06. doi: 10.1111/imm.12707. PubMed PMID: 28054346; PubMed Central PMCID: PMCPMC5382347.
45. Marini F, Binder H. Available from: <https://www.bioconductor.org/packages/release/bioc/vignettes/pcaExplorer/inst/doc/pcaExplorer.html>.
46. Blighe K, Rana S, Lewis M. EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. R package version 1.10.0 2021. Available from: <https://github.com/kevinblighe/EnhancedVolcano>.

Figure 1

Figure 1. Long-term three-dimensional organoids cultures can be established from human mid- and late-gestation trophoblasts and decidua. (A) Schematic representation of the derivation of trophoblast organoids (TOs) and decidual organoids (DOs) from placental tissue. **(B)** Representative bright-field images of TOs and DOs after passaging in complete growth medium TOM and ExM, respectively at 15 days (DO, top) or 32 days (bottom) post-passaging. Scale bar, 50 μ m. Black arrows denote placental-like villi extending from TOs (bottom). **(C)** Confocal microscopy for cytokeratin-19 (Cyt-19, green), and actin (red) in a TO. DAPI-stained nuclei are in blue. Scale bar, 50 μ m. White arrows denote placental-like villi. **(D)** Confocal microscopy for cytokeratin-7 (Cyt-7, green, left), cytokeratin-19 (Cyt-19, green, middle), or E-cadherin (E-cad, green, right) and actin (red in all) in DOs. DAPI-stained nuclei are in blue. Scale bar, 50 μ m. **(E)** Levels of hCG in conditioned medium (CM) isolated from TOs (blue) or DOs (orange) as determined by ELISA. Each symbol represents an individual CM preparation. Upper right shows the OTC pregnancy test stripe denoting an hCG positive line in TOs, but not DOs, after being placed into CM. **(F)** Levels of PSG-1 in conditioned medium (CM) isolated from TOs (light blue), chorionic villous explants (dark blue), or DOs (orange) as determined by ELISA. In (E) and (F) each symbol represents an individual CM

preparation and significance was determined by Mann-Whitney U test. ****, $P < 0.0001$, * $P < 0.05$, ns, not significant.

Figure 2

Figure 2. Whole genome transcriptional profiling of trophoblast and decidua organoids. (A), Volcano plot demonstrating differentially expressed transcripts in trophoblast organoids (TOs) or decidua organoids (DOs) as assessed by DeSeq2 analysis. Grey circles represent genes whose expression was not significantly changes and red denotes transcripts significantly enriched in TOs (right) or DOs (left) (significance was set at $p < 0.01$ and $\log_2$ fold-change of ± 2). **(B),** Heatmap (based on $\log_2$ RPKM values) of transcripts expressed in DOs, TOs, or primary human trophoblasts (PHT) cells that are associated with markers of trophoblasts (top, blue) or the decidua (bottom, orange). Key at left. Red indicates high levels of expression, blue indicates low levels of expression, and grey indicates no reads detected. Hierarchical clustering is shown at top. **(C),** Expression of three members of the C19MC family (miR-516-5p, 517A-3p, and 525-5p) in TOs (light blue) and DOs (orange), or in chorionic villi (dark blue) isolated from human placentas.

Figure 3

Figure 3. Cytokine secretion profiles from trophoblast organoids, matched placenta tissue, and primary cells. (A) Heatmap of twelve conditioned medium (CM) preparations generated from established TO lines analyzed by Luminex-based multianalyte profiling for the indicated cytokines, chemokines, and growth factors. Scale is shown at right. **(B)** Heatmap of CM generated from placental chorionic villi explants matched to TOs shown in A. Luminex assays were performed from three independent CM preparations. Scale is shown at right. **(C)** Heatmap of CM generated from primary human trophoblast cells cultured from full-term placentas. Luminex assays were performed from five independent CM preparations. Scale is at right. **(D)** Venn diagram showing the overlap of detectable factors in TOs, matched placenta chorionic villi, and primary cells from A-C. **(E, F)** Concentrations and comparison of the levels of IL-6 (E) and IL-8 (F) in CM from TOs (grey), matched chorionic villi (dark red), and primary cells (blue) shown in A-C. Each symbol represents a unique CM preparation. Significance was determined by Kruskal Wallance U test with multiple comparisons. **, P < 0.01; ns, not significant.

Figure 4

Figure 4. Cytokine secretion profiles from decidua organoids and decidua tissue. (A) Heatmap of twelve CM preparations generated from established DO lines analyzed by Luminex-based multianalyte profiling. Scale is at right. **(B)** Heatmap of CM generated from three independent decidua explants (two of these, at top and middle, are matched to DOs shown in A). Luminex assays were performed from three independent CM preparations. Scale is shown at right. **(C)** Venn diagram showing the overlap of detectable factors in CM isolated from DOs and decidua tissues. **(D, E)** Concentrations and comparison of the levels of MMP-1 (D) and IL-10 (E) in CM from DOs (orange) and decidua tissues (grey) shown in A and B. Each symbol represents a unique CM preparation. Significance was determined by Mann-Whitney U test. *, P < 0.05; ns, not significant.

Figure 5

Figure 5. Trophoblast organoids preferentially secrete high levels of antiviral IFN-λ2. (A) Venn diagram comparing the factors identified in CM isolated from TOs and DOs. (B, C) Concentrations and comparison of the levels of IL-6 (B) and IL-8 (C) released by TOs (blue) and DOs (orange) determined by Luminex-based profiling. Each symbol represents a unique CM preparation. (D) Comparison of the levels of IFN-λ2 in CM isolated from TOs (blue) and DOs (orange) as determined by Luminex. (E) Comparison of the levels of the type I IFNs IFN-α2 and IFN-β and type III IFNs IFN-λ1 and IFN-λ2 in CM isolated from TOs. (F), Heatmap (based on log2 RPKM values) of transcripts associated with type I (top) or III (bottom) IFNs in TOs or DOs. Key at right. Red indicates high levels of expression, blue indicates low levels of expression, and grey indicates no reads detected. (G) IFN-λ activity of CM isolated from TOs (blue) and DOs (orange) as assessed using an IFN-λ reporter 293T cells line. NCM (non-conditioned medium, grey) was as negative control, NCM supplemented with recombinant human IFN-λ2 (100ng) was used as a positive control (red). (H) Human osteosarcoma U2OS cells were treated with CM isolated from TOs (blue) or with recombinant IFN-λ2 (100ng) for 24hrs and then infected with ZIKV for 24hrs (in the presence of CM). Level of ZIKV replication was assessed by viral RNA as determined by qPCR. Data are shown as fold change from NCM and from at least three independent CM preparations. Significance was determined by Mann-

Whitney U test or by Kruskal Wallace U test with multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. In D-E,G–H, each symbol represents unique CM preparations.

Figure 6

Figure 6. Responses of TOs and DOs and placental tissue to recombinant IFNs. (A, B) RT-qPCR analyses for two ISGs, IFI44L (A) and OASL(B) in TOs (blue) and DOs (orange) treated with 100ng recombinant human IFN-β or IFN-λ3 treatment for ~24 hours. Data are shown as fold change from mock-treated controls two independent replicates performed in triplicate. **(C, D)**, RT-qPCR analyses for IFI44L (C) and OASL(D) in chorionic villous (blue) or decidua (orange) explants treated 100ng recombinant human IFN-β or IFN-λ3 treatment for ~24 hours. Significance was determined by Kruskal Wallce U test with multiple comparisons. *, P < 0.05; ***, ** P < 0.01; P < 0.001; ns, not significant.

Figure 7

Figure 7. Differential cytokine release in TOs and DOs treated with poly I:C. (A) Heatmaps demonstrating the induction of factors at left (shown as fold change from mock-treated controls) from TOs (left panel) and DOs (right panel) treated with 10 μ g poly (I:C) and analyzed by multiplex Luminex-based profiling. AVG denotes the average change in concentration of factors over CMs from individual preparations. Dark blue denotes significantly induced factors compared with untreated controls. Gray or white denotes little to no change (scale at top left). The red arrow demonstrates factors with induction greater than five-fold change observed in the average of separate experiments. Data are from four individual CM preparations from at least two unique matched organoid lines. (B) Venn diagram showing overlap of the factors released >5-fold by poly I:C treatment in TOs and DOs. (C-F) Levels of IFN- λ 1 (C), IFN- λ 2 (D), IFN- α 2 (E), and IFN- β (F) in CM isolated from poly I:C-treated TOs (blue) and DOs (orange) or in mock-treated controls (grey). In C-F, each symbol represents individual CM preparations. In C-F significance was determined using a Mann-Whitney U test. *, $P < 0.05$; ns, not significant.

(mock, grey). In G-I, each symbol represents individual CM preparation. Significance was determined with Mann-Whitney U test. ns, not significant.

Supplemental Figure 1

Supplemental Figure 1. (A,B) hCG levels as assessed by ELISA in conditioned medium isolated from two TOs lines isolated at the indicated weeks during culture.

Supplemental Figure 2

Supplemental Figure 2. RNASeq of TOs and DOs. . **(A)** Principal component analysis based on transcriptional profiles from DOs (orange) or TOs (blue). **(B)** Transcripts on the first principal component (PC1) with the greatest variance. Blue indicates enrichment in TOs and orange indicates enrichment in DOs. **(C)** Heatmap (based on log2 RPKM values) of representative Y-linked transcripts in TOs, DOs, or primary human trophoblasts (PHT). Red indicates high levels of expression, blue indicates low levels of expression, and grey indicates no reads detected. Hierarchical clustering is shown at top

955 **Supplemental Figure 3**

956

957 **Supplemental Figure 3. IFNs and ISGs in TOs and DOs. (A)** Concentrations of IL-6 (blue) and

958 IFN-λ2 (red) in CM generated from a single TO line throughout the first 25 days of culture. **(B)**

959 Heatmap (based on log2 RPKM values) of representative interferon stimulated genes (ISGs) in

960 TOs, DOs, primary human trophoblasts (PHT), or in JAR or JEG-3 cells. Red indicates high levels

961 of expression, blue indicates low levels of expression, and grey indicates no reads detected.

962 Hierarchical clustering is shown at top.

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Supplemental Figure 4

Supplemental Figure 4. (A) RT-qPCR for IFN- β , IFN- λ 2, IFI44L, and OASL in poly I:C treated TOs (blue) and DOs (orange). Data are shown as mean $\pm$ standard deviation as a fold change from mock-treated controls. **(B, C)** Levels of IFN- γ (B) or IL-8 (C) as assessed by Luminex in TOs (blue) and DOs (orange) treated with poly I:C or in mock-treated controls (grey). Data are shown as mean $\pm$ standard deviation. Symbols represent unique organoid preparations. **(D)** RT-qPCR for IFN- β , IFN- λ 2, IFI44L, and OASL in ZIKV-infected TOs (blue) and DOs (orange) 72hrs post-infection. Data are shown as mean $\pm$ standard deviation as a fold change from mock-infected controls. Significance was determined with Mann-Whitney U test. *, $p < 0.05$, ns, not significant.

Table S1. qPCR primers used in this study.

Target	Forward, 5'→3'	Reverse, 5'→3'
Actin	ACTGGGACGACATGGAGAAAA	GCCACACGCAGCTC
IFN-β	GAGCTACAACCTTGCTTGGATTC	CAAGCCTCCCATTCATTGC
IFN-λ2	ACATAGCCCAGTTCAAGTC	GACTCTTCTAAGGCATCTTTG
IFI44L	AACCTAGACGACATAAAGAGG	CTGAAACCAAGTCTGCATAG
OASL	GTACCAGCAGTATGTGAAAG	ATGGTTAGAAGTTCAAGAGC
GATA3	AAAATGAACGGACAGAACC	GGGGTCTGTTAATATTGTGAAG
MUC20	CTCTTCCTTCTGTCCCTC	CCATTTTTCACCTGCACTC
ZIKV	AGATGACTGCGTTGTGAAGC	GAGCAGAACGGGACTTCTTC