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Research article

Decidual production of interferon lambda in response to ZIKV persistence: Clinical evidence and *in vitro* modelling

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ABSTRACT

Zika virus (ZIKV) infections during pregnancy can result in Congenital Zika Syndrome (CZS), a range of severe neurological outcomes in fetuses that primarily occur during early gestational stages possibly due to placental damage. Although some placentas can maintain ZIKV persistence for weeks or months after the initial infection and diagnosis, the impact of this viral persistence is still unknown. Here, we aimed to investigate the immunological repercussion of ZIKV persistence in term placentas. As such, term placentas from 64 pregnant women diagnosed with Zika in different gestational periods were analyzed by ZIKV RT-qPCR, examination of decidua and placental villous histopathology, and expression of inflammation-related genes and *IFNL1-4*. Subsequently, we explored primary cultures of term decidual Extravillous Trophoblasts (EVTs) and Term Chorionic Villi (TCV) explants, as *in vitro* models to access the immunological consequences of placental ZIKV infection. Placenta from CZS cases presented low *IFNL1-4* expression, evidencing the critical protective role of theses cytokines in the clinical outcome. Term placentas cleared for ZIKV showed increased levels of *IFNL1*, *3*, and *4*, whether viral persistence was related with a proinflammatory profile. Conversely, upon ZIKV persistence placentas with decidual inflammation showed high *IFNL1-4* levels. *In vitro* experiments showed that term EVTs are more permissive, and secreted higher levels of IFN-α2 and IFN-λ1 compared to TCV explants. The results suggest that, upon ZIKV persistence, the maternal-skewed decidua contributes to placental inflammatory and antiviral signature, through chronic deciduitis and *IFNL* upregulation. Although further studies are needed to elucidate the mechanisms underlying the decidual

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responses against ZIKV. Hence, this study presents unique insights and valuable *in vitro* models for evaluating the immunological landscape of placentas upon ZIKV persistence.

1. Introduction

The Zika virus (ZIKV) belongs to the Flaviviridae family, genus Flavivirus, and is transmitted by *Aedes* sp. mosquitoes [1,2]. The ZIKV is the etiological agent of a myriad of congenital abnormalities, including microcephaly, cerebral anomalies, and ocular alterations named Congenital Zika Syndrome (CZS), more frequent in ZIKV infection during early stages of pregnancy [3–8]. In Brazil, between 2015 and 2019, there were 3563 confirmed cases of CZS, with only 27 cases reported in 2020 [9]. Although the number of cases has decreased, ZIKV continues to circulate in the Americas during the COVID-19 pandemic, despite an inherent underreporting of ZIKV and other arbovirus infections. This, combined with the high dispersion and abundance of mosquito vectors, means that CZS remains a constant threat [10].

The placenta is a mother-fetus mixed organ that plays a key role in TORCHZ infections (Toxoplasma, Others, Rubella, Cytomegalovirus, Herpes, and Zika) [11]. It has been shown that the ZIKV infection causes damage to the placenta, resulting in increased infiltration of $CD68^{+}$ cells, heightened vascular permeability, and overexpression of various cytokines, metalloproteinases, and chemokines [12–15]. Indeed, placental inflammation is a significant threat to fetal neurodevelopment and has been linked to neurodevelopmental impairment in children aged between four and seven years old, even considering third-trimester infections [16,17]. Noteworthy, it is currently unclear why some placentas exhibit ZIKV persistence at term many months after the symptomatic infection. Most importantly, what are the clinical and immunological consequences of ZIKV persistence?

A few studies have shown ZIKV persistence in the placental [18–21], and in other tissues as well [22,23]. This suggests that the fetus could be continuously exposed to the virus throughout pregnancy. However, no studies have been conducted to investigate the potential outcomes of such viral persistence. *In vitro* studies have demonstrated that ZIKV can infect the main regions of the maternal-fetal interface, the basal decidua, and the chorionic villi. In the basal decidua, first trimester extravillous cytotrophoblast cells (EVTs), dendritic cells, macrophages, lymphocytes, and decidual cells, whereas in the chorionic villi, cytotrophoblasts (CTBs) and other cell types, such as Hofbauer cells are thought to help viral titer amplification [24–27]. Fewer manuscripts focused on infections during third trimester, and the most used model was the term chorionic villi (TCV) explant culture [28–30]. The studies demonstrated that the syncytiotrophoblast (STB) is resistant to ZIKV infection throughout the entire pregnancy, presenting a protective IFN-λ production [26,31]. The IFN-λ1-4 are cytokines that act as an immunological barrier, mainly produced by cells with epithelial or monocytic origins [32]. Furthermore, so far studies including *in vitro ex vivo*, and murine models accessed critical aspects of placental IFN-λ upon ZIKV infection, focusing mostly on first trimester setups. Nevertheless, it remains elusive whether the placental damages caused by ZIKV in clinical samples reflect in production changes of this key local innate antiviral factor. Herein, we sought to clarify the immunological repercussion of ZIKV persistence in term placentas using clinical samples from 65 pregnant women diagnosed with Zika during pregnancy and primary culture models of term EVTs and TCV explants.

2. Materials and methods

2.1. Human subjects and sample collection

The samples were obtained from a retrospective study conducted at the Maternal and Child Hospital (IFF/Fiocruz) in Rio de Janeiro, Brazil. The study was approved by the Institutional Review Board (IRB/CAAE: 52675616.0.000.5269). This study analyzed placental samples from a clinical cohort of pregnant women with confirmed ZIKV infection according to the availability of samples, as well as healthy controls. Our study included pregnant adult women >18 years of age and their infants. The control group consisted of by women without any clinical symptoms or positive diagnosis of ZIKV infection. The ZIKV+ with confirmation of ZIKV infection during pregnancy by ZIKV PCR of maternal or neonatal urine, blood, or placental samples. Exclusion criteria included maternal HIV infection and pregnancies complicated by other congenital infections, known to cause infant neurologic damage (e.g., TORCH, CHIKV). All infants were evaluated for the following adverse neurologic outcomes: (a) microcephaly (head circumference z score of less than −2), (b) abnormal brain imaging by pre- or post-natal ultrasound (e.g., computed tomography and/or magnetic resonance imaging), and/or (c) abnormal eye examination by fundoscopic examination. For the present study the CZS cases within the ZIKV + cohort were classified by the presence of an abnormal diagnosis in at least one of these examinations. In addition, CZS classification was confirmed by a multidisciplinary team of neonatologists, neurologists, infectious disease specialists, geneticists, ophthalmologists, and physiotherapists. Clinical information regarding the trimester of pregnancy in which mothers first presented Zika symptoms (considered as the trimester of ZIKV infection) and CZS outcome was obtained from IFF/Fiocruz clinical staff records. The samples underwent processing and analysis for gene expression, placental ZIKV RT-qPCR, immunopathology, and immunofluorescence. The inclusion and exclusion criteria, as well as clinical and demographic details, were previously described [12]. The *in vitro* portion of this study utilized 12 term placentas obtained from healthy pregnant women who were being treated at Hospital Santo Antônio in Maceió, Alagoas, Brazil. The study was conducted with the permission of the local ethical committee (CAAE: 57828616.3.0000.5013) and followed the same inclusion and exclusion criteria.

2.2. Term placenta chorionic villi explants and extravillous trophoblast cell cultures

Two cotyledons from healthy placentas were dissected, conditioned in 25 mL Hanks balanced saline solution (HBSS) containing 2 % penicillin, and processed within 4 h. Each cotyledon was washed twice with HBSS 2 % penicillin for 5 min. The TCVs were dissected in Petri plates using a scalpel blade and incubated in plates of 48-wells plates in D-MEM/F12 culture medium (5 explants/well), for 2 h at 37 °C in an atmosphere of 5 % CO2. Primary EVT cells were isolated from three healthy placentas as previously described [33]. Briefly, basal plate fragments were coarsely minced, and ~5 g of wet tissue were incubated for 1 h with 20 mL of DMEM/F12 containing 4 % bovine serum albumin (Merck/Sigma-Aldrich, Germany), collagenase type II (125 U/mL, Merck/Sigma-Aldrich, Germany) and DNase type I (25 U/mL, Merck/Sigma-Aldrich, Germany), at 37 °C in a water bath, followed by inactivation with 20 % fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). The cell suspension was double filtered through a 100 μm mesh followed by a 70 μm mesh. The suspension was centrifuged at 400×*g* and the cells were washed and resuspended in DMEM/F12 (Merck/Sigma-Aldrich, Germany) supplemented with 1 % penicillin, 10 % FBS, 0.01 % insulin, 520 μg/mL calcium lactate, 56 μg/mL sodium pyruvate and 1 % nucleosides. Cells were isolated using a gradient of 30 and 60 % Percoll (GE Healthcare, Uppsala, Sweden) followed by centrifugation at 700×*g* for 30 min. EVT cells were collected from the top of the 30 % gradient. The cultures were incubated with 1.10^5 PFU of ZIKV for 4 h at 37 °C in a 5 % CO2 atmosphere, washed with HBSS 2 % penicillin, and incubated with D-MEM/F12 for 48 h at 37 °C in a 5 % CO2 atmosphere.

2.3. In vitro ZIKV incubation

The ZIKV PE243 strain (isolated in 2016 in Pernambuco, Brazil, third passage, stock titration of 1×10^8 FFU mL–1, GenBank accession MF352141.1) was kindly donated by Dr. Juliano Bordignon from the Instituto Carlos Chagas of Fundação Oswaldo Cruz (ICC/FIOCRUZ-PR). Viral propagation was performed with *Aedes albopictus* derived C6/36 cells were cultured with L-15 medium supplemented with 10 % (v/v) SBF, 10 % (v/v) tryptose, and 1 % PSA until 80 % confluence. Cell-free supernatants were harvested 5 days post-infection (dpi), aliquoted, and stored at −80 °C. Titration was performed after viral infection using green monkey kidney-derived Vero E6 cells cultured with supplemented DMEM for 1 h under gentle agitation. The media was changed, and cells were incubated with 1.5 % (w/v) carboxymethylcellulose (CMC) in DMEM with 2 % FBS at 1:1 (v/v). Plaque formation was observed at 5 dpi after 10 % (v/v) formalin fixation and 2 % (v/v) violet crystal staining. The viral titration of PE243 was 3×10^5 PFU mL–1. The EVT cells were plated at 5×10^5 cells per well in 24-well plates for 24 h, with DMEM/F12 medium supplemented with 2 % SBF. Cells were infected with 1 multiplicity of infection (MOI) of PE243 ZIKV strain for 1 h according to the literature [34]. The supernatant was removed, and the cells were washed thoroughly with PBS. A new culture medium was added, and the cells were cultured for 24 h. The ZIKV infection efficiency was accessed by flow cytometry and RT-qPCR. Chorionic villi explants were incubated with 1.5×10^5 PFU/mL of PE243 ZIKV strain for 24 h according to the literature [35]. Afterward, the explants were thoroughly PBS-washed and maintained with a new cultured media. The culture supernatants were used to quantify IFN-α-β-γ-λ1 and -λ2/3 using the LEGENDplex™ human type I, II, III IFN panel (BioLegend, San Diego, CA, USA) according to manufacturer instructions. The readings were performed employing a BD FACS Canto II (BD Biosciences, San Jose, CA, USA) and analyzed with Legendplex v. 8.0 (BioLegend, USA). The results were depicted in pg/mL.

2.4. ZIKV PCR detection

RT-qPCR was performed using the 2x QuantiTect Probe RT-PCR kit (Qiagen, Valencia, CA, USA) with the same primers and cycle times as previously described [36]. All the assays were performed in triplicate and fluorescence curves that crossed the threshold within or below 38 cycles were considered positive.

2.5. Gene expression

The placental gene expression data presented here are a re-analysis of raw data previously obtained [12]. Analysis of gene expression in placental tissue was performed using Fluidigm (Biomark platform) assays following a previously described workflow [37]. Briefly, the fluorescence accumulation data from each sample and each primer pair (Supplementary Table 1) were used to fit four-parameter sigmoid curves using the qPCR library from the R statistical package version 3.4.1. The cycle of quantification was determined according to the second derivative of the fit sigmoid curve and the gene efficiency. Endogenous controls *RPL13* and *18S*, selected by the geNorm method, were used to obtain the normalized expression of each sample [38]. For *IFNL1-4* gene expression, TaqMan™ probes (Thermo Fisher Scientific, Waltham, MA, USA) were used according to the manufacturer's instructions (respective assay IDs: Hs00601677_g1, Hs00820125_g1, Hs04193049_gH, and Hs04400217_g1) using *18S* (assay ID Hs03003631_g1) as endogenous control.

2.6. Histopathological analysis

The whole placentas were sent fresh to the Department of Pathological Anatomy and Cytopathology of the IFF and preserved at 4 °C for up to 48 h. They were weighed and photographed for macroscopic pathological analysis. Placentas were then fixed in buffered formalin for a maximum of 30 h and five or more fragments of the chorion plate, including the region of the umbilical cord insertion, were cut. The fragments were then embedded in paraffin, sectioned at 4 μm, stained with the Hematoxylin and Eosin method, and

analyzed by light microscopy.

2.7. Statistical analysis

For all datasets, the Shapiro-Wilk was applied for testing normality. For placental clinical data, pairwise comparisons were made using the Mann-Withey *U* test were applied. One-way ANOVA Kruskal-Wallis with Dunn's post-hoc test, correcting for multiple comparisons using statistical hypothesis testing, was used for comparisons between more than two groups or time points. For *in vitro*

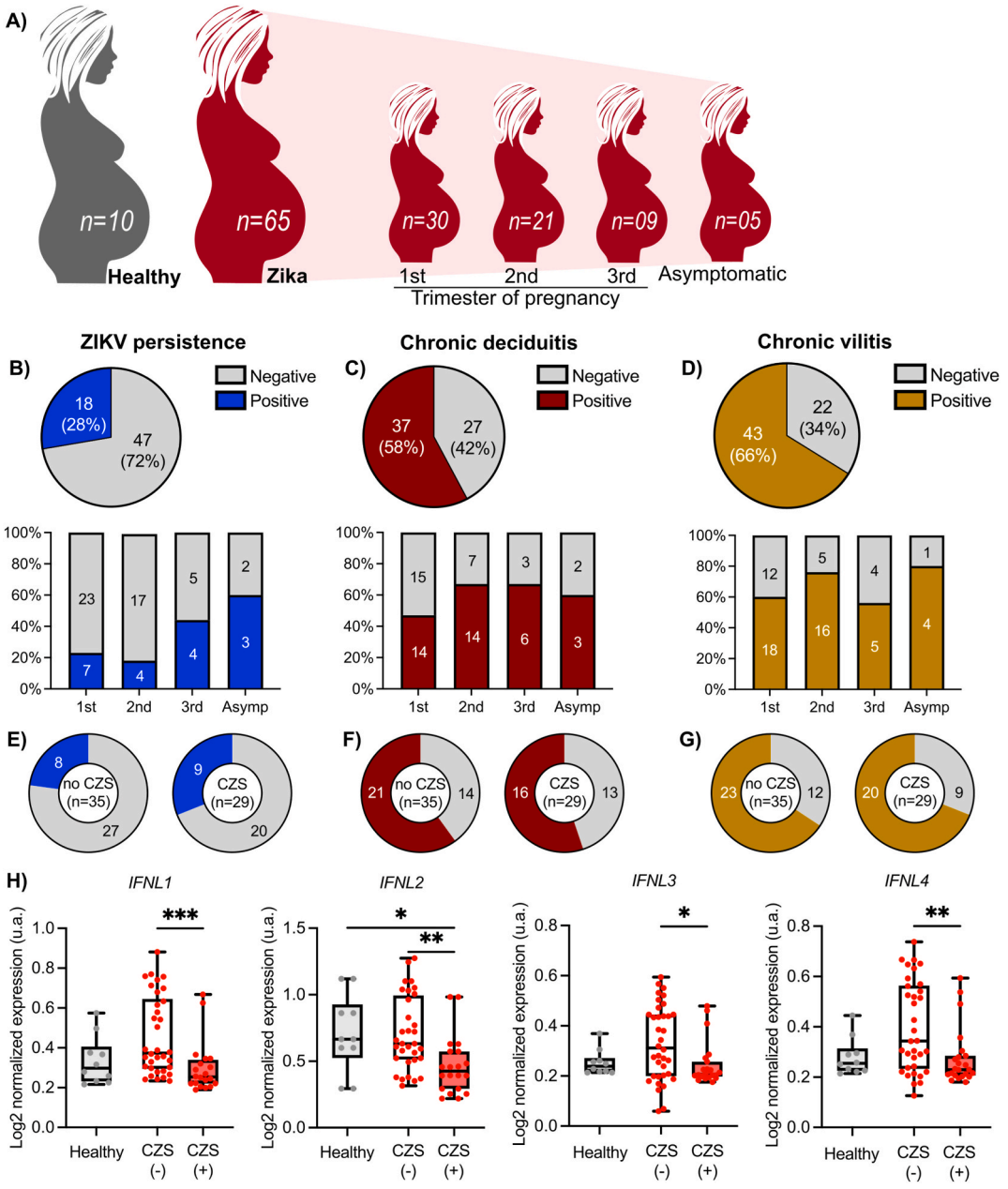

Fig. 1. Placental damage and IFN-λ response in relation to fetal CZS outcome. (A) Schematic representation of clinical cohort clustering, composed by not-infected healthy pregnant woman (n = 10) and pregnant woman diagnosed with Zika (n = 65). Mothers from Zika group presented first signs and symptoms of Zika during the first (n = 30), second (n = 21), and third trimester of pregnancy (n = 9), or asymptomatic Zika (n = 5). (B) Frequency of ZIKV persistence in at term placentas (C) chronic deciduitis, and (D) chronic villitis in the total Zika placenta and according to the trimester of Zika during pregnancy. (E) Frequency of ZIKV persistence in at term placentas (F) chronic deciduitis, and (G) chronic villitis in relation to CZS fetal outcome. (H) Detailed boxplots for log2 normalized expression of *IFNL1-4* in term placenta from healthy controls, and placentas derived from fetuses with or without CZS. Comparisons using Kruskal-Wallis with Dunn test. P-values * ≤0.05, ** ≤0.01, ***≤ 0.005.

data, comparisons between two paired conditions were made using the Wilcoxon test. Unpaired pairwise comparisons were performed using the unpaired *t*-test. Two-tailed levels of significance ≤0.01, 0.05, and 0.1 were considered as "highly significant", "significant", and "suggestive", respectively. All analyses and graphs were generated using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Chronic inflammation and ZIKV persistence in term placentas

Sixty-five pregnant women with RT-qPCR confirmed cases of Zika during pregnancy were grouped according to the trimester of pregnancy in which symptoms of ZIKV infection occurred: first trimester (n = 30, 46 %), second trimester (n = 21, 32 %), third trimester (n = 9, 14 %), and asymptomatic (n = 5, 8 %) (Fig. 1A). Of these 65 cases, 18 term placentas (28 %) were positive for ZIKV, indicating viral persistence. When divided according to the gestational period of ZIKV infection, the viral persistence is present in 7 samples (23 %) from ZIKV in the first trimester, 4 (19 %) in the second trimester, 4 (44 %) in the third trimester, and 3 (60 %) asymptomatic (Fig. 1B). The majority of Zika placentas had histopathological findings. Among them, 37 (58 %) placentas showed chronic deciduitis, including 14 (48 %) placentas from the first trimester, 14 (67 %) from the second trimester, 6 (67 %) from the third trimester, and 3 (60 %) asymptomatic (Fig. 1C). Chronic villitis was also found in 43 (66 %) placentas, including 18 (60 %) from the first trimester, 16 (76 %) from the second trimester, 5 (56 %) from the third trimester, and 4 (80 %) asymptomatic (Fig. 1D). The CZS outcome occurred in 29 cases (45 % of the total of Zika cases), coming mainly from in ZIKV infections during the first trimester (n = 22, 76 %), but also from second trimester (n = 2, 7 %), asymptomatic cases (n = 5, 17 %). No significant differences were observed in the frequency of CZS with ZIKV persistence in term placentas, with chronic deciduitis or with chronic villitis (Fig. 1E–G). Therefore, our data do not demonstrate a direct association between the placental parameters of ZIKV persistence, chronic deciduitis or villitis with the CZS outcome. However, the placental immunologic profile, known to be associated with CZS, is modulated by these parameters.

3.2. IFNL expression was decreased in term placentas from CZS pregnancies and with ZIKV persistence

The transcriptional profile of Zika placenta has already been investigated in a previous study, where ZIKV infection and CZS outcomes were associated with higher levels of genes related to type I IFNs and lower levels of the protective type III interferons [12].

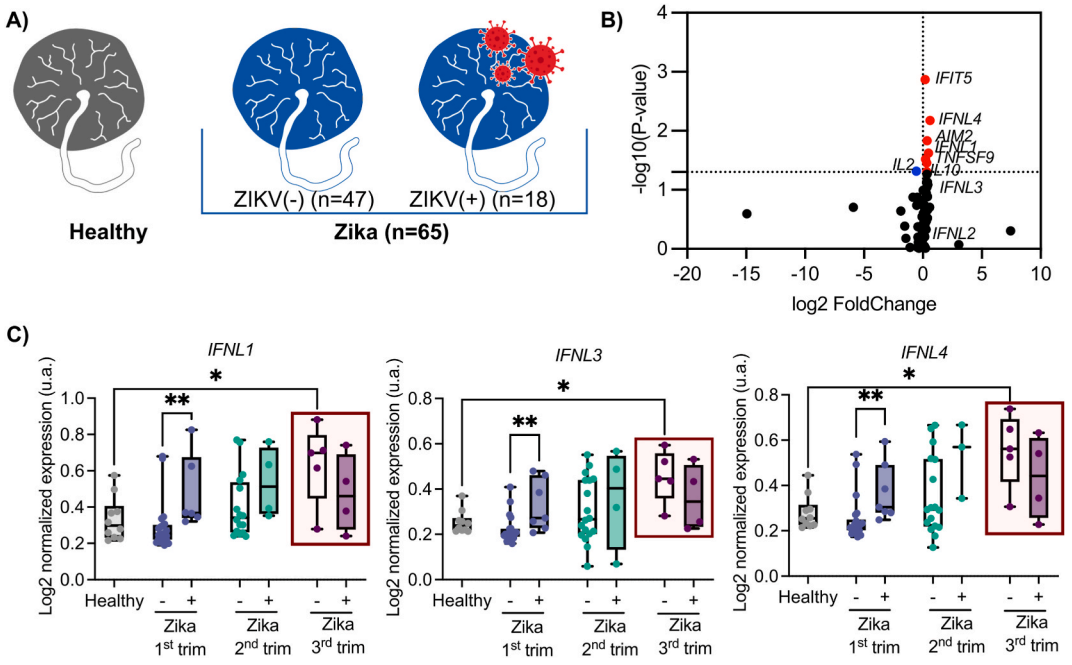

Fig. 2. *IFNL* expression in ZIKV persistent placentas. (A) Schematic representation of clinical cohort clustering. Term placentas were analyzed for ZIKV RT-qPCR and gene expression. The cohort was composed by healthy placentas (n = 10), and placentas from pregnancies with Zika (n = 65), further divided into ZIKV persistent (+) or absent (−) (n = 18 and 47, respectively). (B) Volcano plot representing genes differentially expressed in ZIKV persistent placentas and healthy placentas, using Mann-Whitney test, p-value <0.05. Dots representing genes with no significant gene expression regulation (black dots), upregulated (red dots), and downregulated (blue dots). All fold changes are relative to healthy placentas. (C) Detailed boxplots for log2 normalized expression of *IFNL1-4* dividing Zika placentas by which trimester they were diagnosed and subdivided in ZIKV persistence or absence as described. Each dot corresponds to one placenta analyzed. Comparisons using Kruskal-Wallis with Dunn test P-values ** ≤0.01, * ≤0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The current re-analysis of the previous data showed that the normalized expression of the placentas from neonates with CZS showed a reduction of all *IFNL* genes compared to Zika pregnancies without CZS (*IFNL1* - CZS (−) = median 0.37, quartiles Q1/Q3 0.29/0.64, CZS (+) = 0.25, 0.22/0.34, p < 0.001; *IFNL2* - CZS (−) = 0.63, 0.51/0.99, CZS (+) = 0.43, 0.29/0.57, p = 0.001; *IFNL3* - CZS (−) = 0.31, 0.20/0.45, CZS (+) = 0.21, 0.20/0.26, p = 0.014; *IFNL4* - CZS (−) = 0.34, 0.23/0.56, CZS (+) = 0.23, 0.21/0.28, p = 0.003). The placentas from CZS cases also showed a reduced expression of *IFNL2* compared to healthy control placentas (Healthy = 0.67, 0.52/0.93, CZS (+) = 0.43, 0.29/0.57, p = 0.010) (Fig. 1H).

Regarding persistence at term, the placentas positive for ZIKV persistence had increased expression of *IFIT5*, *IFNL1*, *IFNL4*, *AIM2*, *TNFSF9*, and *IL10*, with reduced expression of IL2 compared to healthy control placentas (Fig. 2A and B). Furthermore, the differential expression of *IFNL* genes was performed according to the semester in which Zika symptoms and diagnosis occurred. This allowed us to follow the IFNL expression dynamics after ZIKV infection. When the infection occurred in the first trimester, term placentas with viral persistence showed increased *IFNL1* (Zika 1st trimester (−) = 0.25, 0.23/0.41, Zika 1st trimester (+) = 0.37, 0.34/0.68, p = 0.004), *IFNL3* (Zika 1st trimester (−) = 0.20, 0.19/0.22, Zika 1st trimester (+) = 0.27, 0.23/0.46, p = 0.007), and *IFNL4* (Zika 1st trimester (−) = 0.22, 0.20/0.25, Zika 1st trimester (+) = 0.30, 0.28/0.49, p = 0.006) expression compared to placentas without the virus, which had similar expression to healthy control placentas (Fig. 2C). However, the opposite was true for third trimester infections, where ZIKV-negative placentas had higher IFNL expression in comparison to healthy placentas (*IFNL1*- Healthy = 0.30, 0.23/0.41, Zika 3rd trimester (−) = 0.70, 0.45/0.80, p = 0.023; *IFNL3* - Healthy = 0.24, 0.22/0.27, Zika 3rd trimester (−) = 0.34, 0.23/0.51, p = 0.029; and *IFNL4* - Healthy = 0.25, 0.23/0.31, Zika 3rd trimester (−) = 0.44, 0.26/0.61, p = 0.015) (Fig. 2C). Thus, the data suggest that the decrease in IFNL expression may result in the absence of a key placental protective response that is associated not only with CZS outcome, but also with viral persistence.

3.3. ZIKV persistence in term placentas correlates to inflammatory signature

A panel of genes were analyzed to verify whether viral persistence affected the transcriptional profile of term placentas. ZIKV-

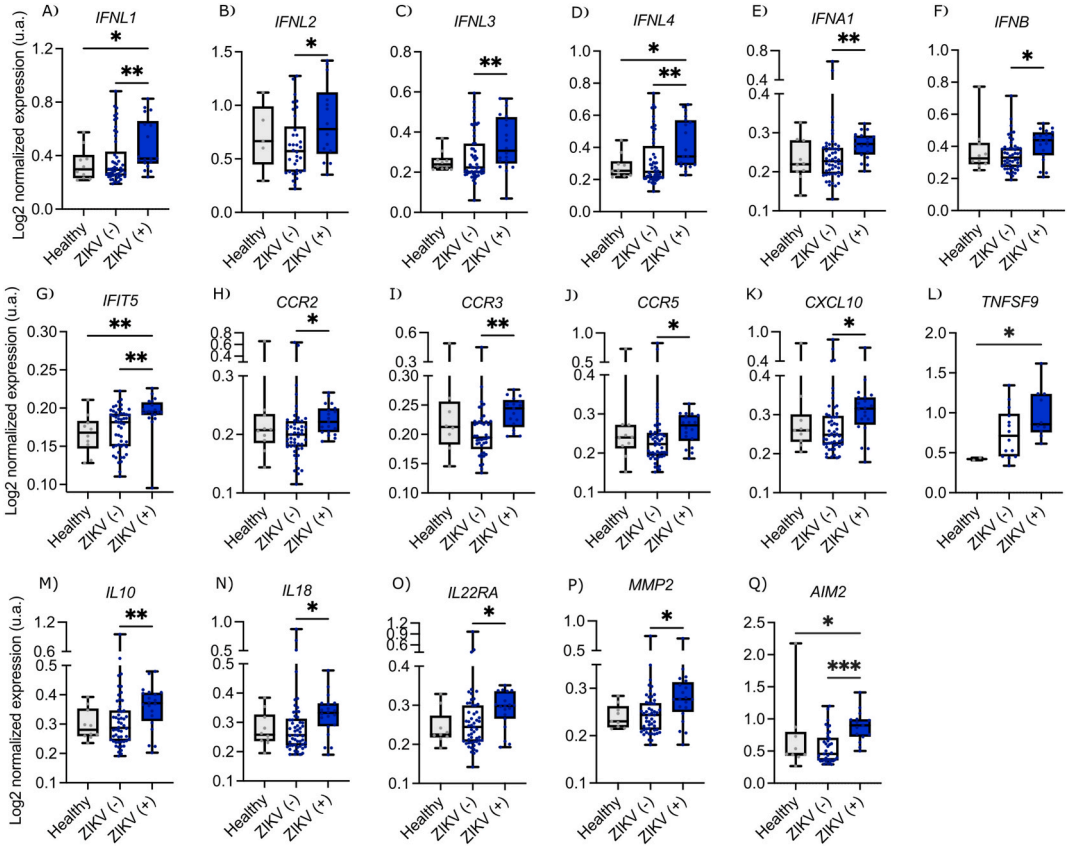

Fig. 3. ZIKV positivity in at-term placenta modulates transcriptional profile of IFN and inflammation-related genes. Detailed boxplots for log2 normalized expression comparing at-term placenta from healthy controls (n = 10), and placentas from pregnancies with Zika (n = 65), further divided into ZIKV persistent (+) or absent (−) (n = 18 and 47, respectively). Genes analyzed were related to IFN – (A) *IFNL1*, (B) *IFNL2*, (C) *IFNL3*, (D) *IFNL4*, (E) *IFNA1*, (F) *IFNB*, and (G) *IFIT5*, and inflammation – (H) *CCR2*, (I) *CCR3*, (J) *CCR5*, (K) *CXCL10,* (L) *TNFSF9*, (M) *IL10*, (N) *IL18*, (O) *IL22*RA, (P) *MMP2*, and (Q) *AIM2*. Each dot corresponds to one placenta analyzed. Comparisons using Kruskal-Wallis with Dunn test P-values ** ≤0.01, * ≤0.05.

persistent placentas showed an upregulation of: *IFNL1* (ZIKV (−) = 0.29, 0.25/0.43, ZIKV (+) = 0.38, 0.34/0.66, p = 0.005), *IFNL2* (ZIKV (−) = 0.57, 0.38/0.80, ZIKV (+) = 0.78, 0.55/1.10, p = 0.047), *IFNL3* (ZIKV (−) = 0.22, 0.20/0.34, ZIKV (+) = 0.31, 0.24/0.47, p = 0.005), *IFNL4* (ZIKV (−) = 0.25, 0.21/0.41, ZIKV (+) = 0.34, 0.29/0.57, p = 0.001), *IFNA1* (ZIKV (−) = 0.23, 0.19/0.26, ZIKV (+) = 0.27, 0.24/0.29, p = 0.005), *IFNB* (ZIKV (−) = 0.33, 0.27/0.39, ZIKV (+) = 0.44, 0.34/0.49, p = 0.026), *IFIT5* (ZIKV (−) = 0.18, 0.15/0.19, ZIKV (+) = 0.19, 0.19/0.21, p = 0.001), *CCR2* (ZIKV (−) = 0.20, 0.18/0.22, ZIKV (+) = 0.22, 0.20/0.24, p = 0.010), *CCR3* (ZIKV (−) = 0.19, 0.17/0.22, ZIKV (+) = 0.24, 0.21/0.26, p = 0.001), *CCR5* (ZIKV (−) = 0.22, 0.19/0.25, ZIKV (+) = 0.27, 0.23/0.30,

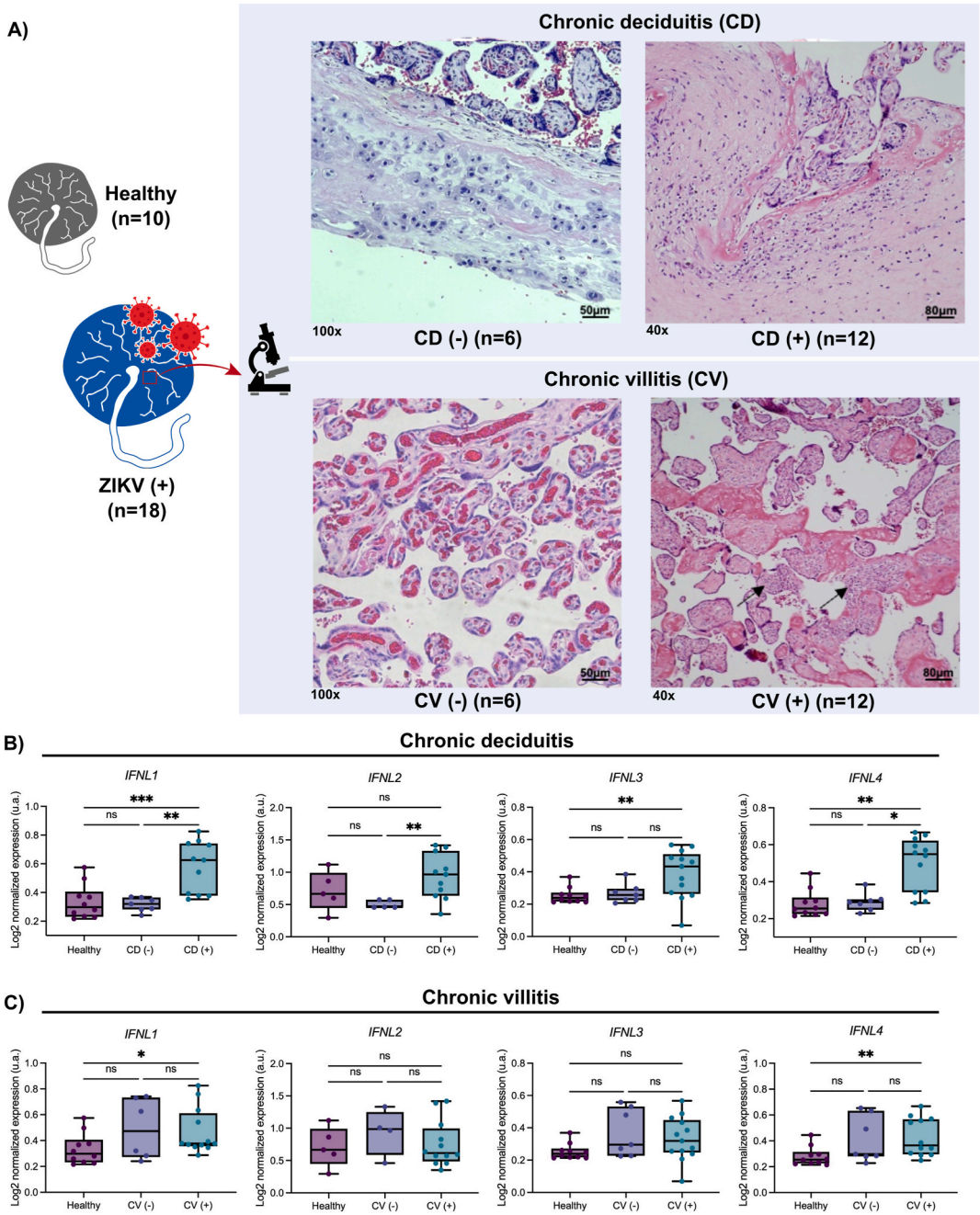

Fig. 4. ZIKV persistence is associated with placental inflammation and upregulation of *IFNL* expression. (A) Term healthy placentas (n = 10) and Zika placentas (ZIKV (+)) (n = 18) were analyzed according to absence (n = 6) or presence (n = 12) of chronic deciduitis (CD) as well as absence (n = 6) or presence (n = 12) of chronic villitis (CV). The Zika-induced CD is characterized by diffuse mononuclear inflammatory cells. CV is characterized by stromal fibrosis and a discrete infiltrate of mononuclear inflammatory (black arrows). (B) Detailed boxplots for log2 normalized expression of *IFNL1-4* in healthy and ZIKV persistence at term placenta CD (−) or CD (+), and (C) CV (−) or CV (+). Each dot corresponds to one placenta analyzed. Comparisons using Kruskal-Wallis with Dunn test. P-values ***≤ 0.005, ** ≤0.01, * ≤0.05, ns-non significant.

p = 0.010), *CXCL10* (ZIKV (−) = 0.25, 0.22/0.30, ZIKV (+) = 0.32, 0.27/0.34, p = 0.016), *IL10* (ZIKV (−) = 0.29, 0.24/0.35, ZIKV (+) = 0.37, 0.31/0.41, p = 0.008), *IL18* (ZIKV (−) = 0.26, 0.22/0.31, ZIKV (+) = 0.33, 0.29/0.37, p = 0.021), *IL22*RA (ZIKV (−) = 0.24, 0.21/0.30, ZIKV (+) = 0.30, 0.27/0.34, p = 0.014), *MMP2* (ZIKV (−) = 0.24, 0.21/0.27, ZIKV (+) = 0.28, 0.25/0.31, p = 0.012), and *AIM2* (ZIKV (−) = 0.46, 0.35/0.71, ZIKV (+) = 0.90, 0.72/1.00, p < 0.001) (Fig. 3). This scenario indicates a general inflammatory signature in these placentas compared to those without ZIKV persistence.

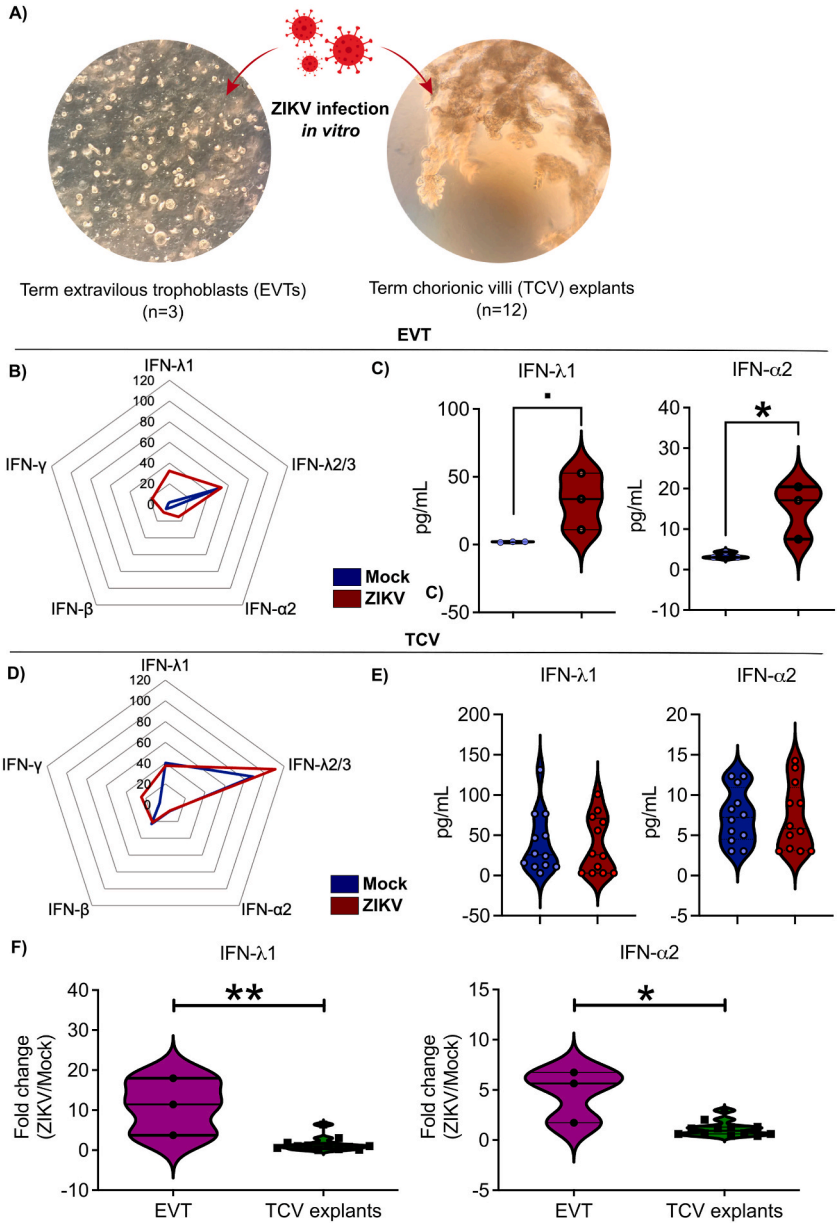

Fig. 5. Production of IFNs in term placental models upon *in vitro* ZIKV infection. (A) Schematic representation of placental cellular models used for functional interferon investigation. Primary EVT cells (n = 3) and terminal chorionic villi (TCV) explants (n = 12) were isolated from healthy pregnant women, following by ZIKV infection *in vitro* for 24 h. (B and D) Radar plot representing means of pg/mL values of IFN-λ1, IFN-λ2/3, IFN-α2, IFN-β, and IFN-γ under incubation of ZIKV (red line) or mock (blue line) in the supernatant of (B) EVT cells and (D) TCV explants. (C and E) Violin plot representing median and interquartile range of pg/mL values of IFN-λ1, and IFN-α2 in the supernatant of (C) EVT cells and (E) TCV explants under incubation of ZIKV (red) or mock (blue). (F) Fold change of IFN-λ1, and IFN-α2 calculated by the ratio of pg/mL values obtained in ZIKV and mock conditions in EVT cells and TCV explants. Considering *p* values. ≤ 0.01 * ≤0.05 and **≤ 0.01 by unpaired *t*-test. Statistical analysis by GraphPad Prism 5 program. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. IFNL expression correlates to decidual inflammation in ZIKV-persistent term placentas

Of the 18 ZIKV-persistent term placentas, most of them had chronic deciduitis or chronic vilitis (Fig. 4A). Notably, all *IFNL* genes were upregulated in ZIKV-persistent placentas with chronic deciduitis compared to ZIKV-persistent placentas without chronic deciduitis (*IFNL1* - CD (−) = 0.32, 0.28/0.37, CD (+) = 0.63, 0.38/0.74, p = 0.002; *IFNL2* - CD (−) = 0.46, 0.46/0.57, CD (+) = 0.97, 0.64/1.30, p = 0.007; and *IFNL4* - CD (−) = 0.29, 0.25/0.30, CD (+) = 0.55, 0.34/0.62, p = 0.033. Fig. 4B). The *IFNL1* (Healthy = 0.30, 0.23/0.41, CD (+) = 0.63, 0.38/0.74, p < 0.001), *IFNL3* (Healthy = 0.24, 0.22/0.27, CD (+) = 0.43, 0.26/0.51, p = 0.007) and *IFNL4* (Healthy = 0.25, 0.23/0.31, CD (+) = 0.55, 0.34/0.62, p = 0.002) expression was significantly higher in the ZIKV persistent placentas with chronic deciduitis compared to healthy control placentas (Fig. 4B). Furthermore, the presence of chronic vilitis in ZIKV-persistent placentas correlated with upregulation of *IFNL1* (Healthy = 0.30, 0.23/0.41, CV (+) = 0.38, 0.36/0.61, p = 0.024) and *IFNL4* (Healthy = 0.25, 0.23/0.31, CV (+) = 0.36, 0.30/0.57, p = 0.007) in comparison to healthy control placentas and, although no further changes were found compared to ZIKV persistent placentas without chronic vilitis (Fig. 4C). Therefore, in a scenario of viral persistence, decidua inflammation contributes to an increase in placental IFNL expression. Next, we hypothesized that a specific cell type in the decidua might be involved in this *IFNL* induction.

3.5. Term decidual EVT cells produce IFN-λ upon in vitro ZIKV infection

First trimester EVT cells are permissive for ZIKV replication [25]. However, no study up to date has evaluated ZIKV infection in third trimester/term EVT cells. As they still play an important immunological regulatory role on decidual leukocytes and chronic deciduitis has been clearly shown to correlate with ZIKV persistence and upregulation of *IFNL* genes, we observed the behaviour of these cells upon ZIKV infection *in vitro*. As a result, the EVT cell isolation was considered to be mostly pure, as they presented a typical morphology, and 75.25 % (±6.2 %) of the cells were HLA-G positive by flow cytometry (Supplementary Fig. 1A). Of the HLAG + EVT cells, an average of 58.2 % (±4.53 %) were positive for ZIKV after 24 h incubation (Supplementary Fig. 1B). RT-qPCR confirmed ZIKV replication in term EVT cells, as culture supernatants contained $5.06 \pm 1.4 \times 10^4$ PFU/μL after 24 h of infection (p = 0.036; Supplementary Fig. 1C). The EVT cells showed basal production in the mock condition of IFNLλ2-3, IFN-α2, and IFN-β in the supernatant, whereas IFN-λ1 production was below the detection limit (Fig. 5B). After ZIKV infection, EVT cells responded with high levels of IFN-λ1 (Mock = 2.00, 1.50/2.00, ZIKV = 33.60, 10.90/52.70, p = 0.064), together with increased levels of IFN-α2 (Mock = 3.03, 3.03/4.35, ZIKV = 17.10, 7.54/20.40, p = 0.042; Fig. 5C).

Regarding terminal villi explant, they were also permissive to the ZIKV strain PE243, with a viral load $5.78 \pm 1.34 \times 10^5$ PFU/μL (p = 0.023) after 24 h incubation (Supplementary Fig. 1D). In contrast to the EVT cells, the placental explants showed higher basal levels of the four cytokines analyzed (Fig. 5D). However, no changes in all the IFN were detected in the presence of the ZIKV (Fig. 5E). Finally, comparing those two different culture models, ZIKV-induced response was relatively higher in EVT cells than in chorionic villi explants for IFN-λ1 (EVT = 11.5, 3.72/18.00, TCV explants = 1.00, 0.34/1.70, p = 0.008) and IFN-α2 (EVT = 5.64, 1.73/6.74, TCV explants = 0.93, 0.60/1.30, p = 0.017) (Fig. 5F). Overall, considering the limitations of *in vitro* models, the data suggest the decidual EVT population as a responsive cell type contributing to the protective placental IFNL production.

4. Discussion

The ZIKV persistence has been demonstrated in term placentas [18–21] as well as in several other tissues [22,23]. However, the existing literature mainly reports the presence of ZIKV in term placentas without addressing the underlying consequences of this persistence. In particular, Bordoni et al. (2019) observed the persistence of ZIKV with replication capacity in mesenchymal stem cells derived from term placentas, infected during the second trimester of pregnancy [39]. Similarly, in a recent study by Barrozo et al. (2023), the persistence of ZIKV in term placentas was related with disruption of miRNA and RNAi pathways, using spatial transcriptomics [40]. However, these studies have not provided a comprehensive understanding of why only certain placentas exhibit such persistence, the dynamics of viral activity and the immune response during pregnancy, particularly in cases where no clinical symptoms are apparent following disease diagnosis, or whether infections or persistence in the third trimester correlates with fetal complications. While many questions remain unanswered, our current study aimed to investigate placental viral persistence and its potential correlation with CZS outcome, IFN-λ response and histopathological observations.

In this study, we assessed 18 term placentas (28 % of the total Zika diagnosed cases in our samples), that had persistent ZIKV infection. Notably, the data showed no association between viral persistence, chronic deciduitis, or villitis with CZS outcomes. However, as previously demonstrated, placentas derived from CZS-cases demonstrated a downregulation of all *IFNL* when compared to those from non-CZS cases, implicating a deficient antiviral response still occurring at term, despite the period of ZIKV infection [12]. Here, we confirmed this profile even comparing placenta from CZS cases with placentas from healthy controls. Thus, although there was no direct association between viral persistence and the development of CZS, the IFNL response was a common factor for these two outcomes.

It has already been showed that *IFNL* can exert a resolutive function in viral infections, both by acting as a local antiviral agent or by modulating immunological responses towards a regulatory profile [31,41,42]. These investigations have demonstrated the resistance of the STB to ZIKV infection throughout pregnancy, attributable to its protective IFN-λ production, effectively establishing a potential barrier [26,31]. Furthermore, the production of type III interferons by the placenta or decidual cells appears to be dependent on placental maturation, regulating ZIKV infection only after the mid-gestational stage [26,43], and inhibiting ZIKV replication in CTBs and placental organotypic models [31,44]. Here, focusing on placentas from cases of Zika diagnosed in the third trimester of

pregnancy, i.e. accessing recent infections, data demonstrate that induction of IFNL response was associated with resolution of viral infection at term. In contrast, cases of ZIKV infection occurring during the first trimester resulted in a lower expression of these *IFNL* in placentas that had subsequently tested negative for ZIKV, possibly due the timing of infection, reflecting the resolution phase that occurred sometime earlier [41].

Regarding the general immunological response in Zika placentas, the analysis of the transcriptional profile demonstrated sustained high expression of genes related to antiviral responses (*IFIT5*, *IFNL1*, *IFNL4*, and *AIM2)*, whereas the increase in *TNFSF9* and *IL10*, with a reduction of *IL2* could indicate an induction of T cell responses. Noteworthy, such alterations are expected during viral infections, but the ZIKV diagnostic was made during pregnancy, some of them several months before term. Other publications showed increased production of IL-1β, IL-10, TNF-α, TGF-β1, IL-6 and IL-8, MMPs, IFNs, and other immunological mediators, mostly associated with inflammation and vascular permeability in term placentas from ZIKV pregnancies [15,20,45,46]. The systemic inflammatory signature in newborns could also be associated with immunopathological responses that contribute to the CZS outcome [47]. Here, data suggests that ZIKV persistence can enhance this immunopathological profile, since the genes upregulated indicate an inflammatory signature (*CCR2*, *CCR3*, *CCR5*, *CXCL10*, *TNFS9*, *IL10*, *IL18*, *IL2*2RA, *MMP2*, and *AIM2*). Together with inflammation, ZIKV persistence led to upregulation of genes associated to antiviral response (*IFNL1*, *IFNL2*, *IFNL3*, *IFNL4*, *IFNA1*, *IFNB*, *IFIT5)*. This profile suggests that placental tissue could be trying continue fighting the long and continuous ZIKV infection, even months after Zika diagnosis.

As indicated by the upregulated gene signature suggestive of both inflammation and antiviral activity, we proceeded to investigate the presence of histopathological findings on the ZIKV persistent placentas. These persistent ZIKV-positive placentas with chronic vilitis and deciduitis exhibited heightened expression of *IFNL1* and *IFNL4*, and chronic deciduitis alone displayed upregulation of *IFNL2* and *IFNL3*. Taken together, data suggest that placentas harboring ZIKV but devoid of inflammatory modifications may not actively participate in combatting the virus throughout pregnancy. The association between histopathological alterations and ZIKV infection has been documented in prior studies [13,15,20,48]. These investigations have underscored variations in placentas stemming from pregnancies affected by Zika, including increased infiltration of neutrophils, macrophages, and lymphocytes within the chorionic villi [15,20]. Taken together, these observations suggest that maternal leukocyte infiltration may constitute a component of the resolution process aimed at eradicating ZIKV, possibly triggering IFNL response by trophoblasts, a major source of IFNL in the placenta [31].

Numerous studies have employed cell lines to elucidate the intricacies of ZIKV infection within the placenta [34,49–52]. Complementary experimental approaches, encompassing human placental explants and bi- and tri-dimensional culture models, have consistently reaffirmed the placenta's susceptibility to ZIKV infection and its ability to support viral replication [24,26]. Consequently, first trimester placentas are notably prone to ZIKV infection in a variety of cells [25–27,53]. Moreover, alternative 3D culture models employing primary cells from first trimester placentas have shown that decidual cells exhibit heightened responsiveness in terms of IFN-λ production when challenged by ZIKV, compared to cells derived from the chorionic villi. This heightened responsiveness is particularly noteworthy considering their relatively lower basal levels of IFN-λs [26].

To date, at term placentas studies conducted *in vitro* have centered around chorionic villi explants. These studies have consistently revealed that the STB and CTBs exhibit increased resistance to Zika virus (ZIKV) infection [31,53,54,55]. Additionally, the infected explants have been shown to secrete various cytokines, including IL-1β, IL-10, TNF-α, TGF-β1, IL-6, IL-8, matrix metalloproteinases (MMPs), and all types of IFNs [15,20,45]. In our explant experiments, we observed outcomes consistent with those described by Ribeiro and collaborators (2018) regarding IFN production [28]. All explants exhibited basal IFN production as previously reported. However, we did not observe an increase in any IFN production after 24 h of ZIKV infection. This discrepancy could potentially be attributed to the lack of further timepoints analyzed post ZIKV infection in both primary cell culture models, given the particular kinetics of IFN production in these specific cell types. Further, variations in the ZIKV strain used or genetic differences in IFNL expression, as single nucleotide polymorphisms (SNPs) in IFN genes have been shown to influence placental responsiveness [12]. Due to these limitations, further studies with large sample numbers and collection time points are needed to better characterize the effects of ZIKV infection on IFN-λ response in placental chorionic villi.

Furthermore, we opted to investigate infection in term isolated EVT cells, given their critical role in maternal immunomodulation and their location in the basal decidua [56]. Our findings demonstrate, for the first time, that term isolated EVT cells are permissive to ZIKV infection and support viral replication. Since these cells play a crucial role in the immune regulation of the basal decidua, we analyzed their IFN response pattern. Our results revealed that these cells exhibit basal production levels of all IFNs, with a prominent increase in IFN-α2 and IFN-λ1 production following ZIKV infection. This underscores their heightened responsiveness to viral presence compared to cells located in the chorionic villi. The elevated levels of the IFNs may upregulate chemokine genes, such as *CXCL10*, potentially increasing the recruitment of mononuclear leukocytes and contributing to the chronic deciduitis observed in these placentas [57]. It is worth noting that the *CXCL10* gene was indeed upregulated in these placentas with ZIKV persistence, as we have demonstrated.

Hence, our data suggest that EVT cells, along with maternal leukocytes significantly contribute to the increased expression of *IFNL* in placentas with chronic deciduitis resulting from ZIKV persistence. This also contributes to observed the inflammatory and antiviral signature in these placentas. This interpretation is supported by a previous study in the murine model, which inferred that the antiviral effect of IFN-λ resulted specifically from signaling in the maternal region of the maternal-fetal interface [41]. Indeed, a case-control study conducted with another Brazilian cohort demonstrated that the maternal *IFNL* polymorphism rs8099917 GG, characterized by low IFN-λ production, is associated with the CZS outcome [58,59]. While this interpretation may seem reasonable, there are still unanswered questions about the specific mechanisms that coordinate the resolution of ZIKV through inflammatory infiltration. Additionally, it is unclear why some placentas take longer to eliminate the virus compared to others that do not initiate the viral clearance process.

Due to the limited number of samples available, we were unable to perform cohort sub-stratifications, which would have been highly valuable for gaining a more nuanced understanding of the implications of viral and host variants on IFNL expression. Additionally, the unbalanced sample size among participant groups limits the statistical tests applied in this study. In summary, this study provides unique insights into the phenomenon of ZIKV persistence and its potential impact on the immune system. The data presented raises the question of why some placentas with ZIKV persistence do not show an increase in *IFNL* expression or chronic deciduitis. It is possible that the spatial distribution of ZIKV within the placenta may influence such a response.

Therefore, our findings suggest a correlation between deciduitis and increased *IFNL* expression, indicating the presence of chronic inflammation that could potentially extend for weeks or months. This chronic inflammatory state may have implications for fetal neurodevelopment, highlighting the need for further investigations to uncover the neonatal and infant consequences in these ZIKV placental persistence cases.

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Additional information

No additional information is available for this paper.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at https://zenodo.org/records/5567327/.

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at.https://10.1016/j.heliyon.2024.e30613.

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