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Insight into the expression of RIG-I-like receptors in human third trimester placentas following *ex vivo* cytomegalovirus or vesicular stomatitis virus infection

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ABSTRACT

A viral infection is detected through germline-encoded pattern-recognition receptors (PRRs) leading to the production of interferons (IFNs) and proinflammatory cytokines. The objective of this study was to investigate the expression of retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) in response to viral infection and the selected cytokine responses in the human term placenta. Placental villi and decidual explants were infected with human cytomegalovirus (CMV) or vesicular stomatitis virus (VSV) and cultured *ex vivo* to study viral infection. To evaluate *DDX58* (RIG-I), *IFIH1* (MDA5), and *DHX58* (LGP2) expression, quantitative real-time PCR (qRT-PCR) was used. The expression of RLRs was detected by Western blotting. Cytokine and chemokine production, as well as RLR protein levels, were quantified using ELISA. The increased expression of both RIG-I and MDA5 and the enhanced secretion of IFN-ß were observed in response to VSV infection compared to mock-infected tissues. CMV infection resulted in higher transcript levels of *DDX58* and *IFIH1*, while no changes in the cytokine production were observed. Our results indicate that RIG-I and MDA5 are specifically expressed in chorionic villi and deciduae in response to VSV infection. These findings suggest that RLRs may play a key role in pathogen recognition and the immune response against intrauterine viral transmission.

1. Introduction

Viral infections during pregnancy are a primary cause of maternal and fetal diseases. Several clinically relevant viruses, including cytomegalovirus (CMV), human immunodeficiency virus (HIV), rubella virus (RV), and varicella-zoster virus (VZV) (Fisher et al., 2000; Adams Waldorf & McAdams, 2013), can be vertically transmitted to the fetus subsequent to maternal infection. Most viruses are transmitted by the transplacental passage from the mother to the fetus during early pregnancy, although vertical infection may occur at any stage of gestation. CMV, a double-stranded DNA betaherpesvirus, is a leading cause of congenital infection, occurs in 0.64% of all live births in industrialized countries (Kenneson & Cannon, 2007). Mother-to-child transmission is mostly the result of primary maternal CMV infection, which carries a risk of transmission of 32.3% (ranging from 14.2% to 52.4%). The rate of transmission from pregnant women with recurrent infection to newborn infants is lower, at approximately 1.4% (1.1-1.7%) (Kenneson & Cannon, 2007). The majority of children with congenital CMV infection do not have clinical findings at birth. Symptomatic CMV infection can range from mild nonspecific findings to intrauterine growth restriction, newborn disease, long-term outcome, and stillbirth. Approximately 11.0% of newborns with congenital CMV infection have detectable manifestations, most common central nervous system damage, sensorineural hearing loss, visual impairment, and/or neurological dysfunction (Munro et al., 2005; Gabrielli et al., 2012; Paradowska et al., 2013). The overall risk of CMV infection increase with advancing gestational age and is greatest in the third trimester (75.0%), while most fetal and neonatal sequelae are associated with infection in the first or second trimester (Gindes et al., 2008; Adams Waldorf & McAdams, 2013; Lipitz et al., 2013).

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The innate immune system is triggered by pathogen-associated molecular patterns (PAMPs) and represents the first line of defense against invading microorganisms. The primary pattern recognition receptors (PRRs) that are involved in the sensing of viral nucleic acids include retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) expressed by most cells; Toll-like receptors (TLRs) expressed by many different immune and non-immune cell types, e.g., macrophages, dendritic cells, B cells, and placental cells (Patni et al., 2020; Kawai & Akira, 2011; Loo and Gale, 2011); members of the nucleotide oligomerization domain (NOD)-like receptors (NLRs) family (Kanneganti et al., 2006); and cytosolic DNA sensors (Takaoka et al., 2007). The RLRs have emerged as key cytosolic receptors for sensing various RNA viruses and consist of the following three DExD/H box RNA helicases: retinoic acid-inducible gene-I (RIG-I, encoded by *DDX58*), melanoma differentiated-associated gene 5 (MDA5, encoded by *IFIH1*), and laboratory of genetics and physiology 2 (LGP2, encoded by *DHX58*) (Yoneyama et al., 2004; Kato et al., 2005; Komuro & Horvath, 2006). RIG-I is activated by the binding of RNA substrate containing 5'-triphosphorylated, short, double-stranded RNA (dsRNA) produced during infection, whereas MDA5 responds to longer or more complex dsRNA networks (Hornung et al., 2006; Pichlmair et al., 2009; Schlee et al., 2009). RIG-I and MDA5 appear to differentially recognize different classes of RNA viruses. Genomes from vesicular stomatitis virus (VSV), Sendai virus (SV), respiratory syncytial virus (RSV), Newcastle disease virus (NDV), influenza A and B, and hepatitis C virus (HCV) all bind to RIG-I and serve as RIG-I agonists (Foy et al., 2005; Kato et al., 2005; Kato et al., 2006; Yoneyama et al., 2005; Rajsbaum et al., 2012), whereas MDA5 is able to recognize encephalomyocarditis virus (EMCV), Mengo virus, and Theiler's virus (Kato et al., 2005; Gitlin et al., 2006; Rajsbaum et al., 2012). Moreover, some DNA viruses, such as Epstein-Barr virus (EBV), herpes simplex type 1 (HSV-1), and adenovirus, encode short non-coding RNA transcripts, EBV-encoded small RNAs, and adenovirus virus-associated RNA I, which are transcribed by RNA polymerase III (Pol-III) and then trigger RIG-I or MDA5 activation (Ablasser et al., 2009; Melchjorsen et al., 2010; Minamitani et al., 2011).

RIG-I and MDA5 are characterized by the presence of two N-terminal caspase activation and recruitment domains (CARDs), which interact with interferon (IFN)-β promoter stimulator 1 (IPS-1, also called Cardif, MAVS, and VISA) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005) and are associated with inflammatory signaling pathways, the DExD/H box RNA helicase domain responsible for RNA-dependent adenosine triphosphate (ATP) hydrolysis, and the C-terminal regulatory domain (CTD), which is primarily involved in RNA recognition. Upon the recognition of viral RNAs through RIG-I or MDA5, receptors initiate downstream signaling, resulting in the activation of nuclear factor-κB (NF-κB), as well as interferon regulatory factors (IRFs) 3 and 7, which trigger the production of proinflammatory cytokines and IFN type I. In contrast to RIG-I and MDA5, LGP2 is postulated to act as a regulator of the other RLRs (Rothenfusser et al., 2005; Satoh et al., 2010).

The present study is the first to present data concerning the expression of *DDX58*, *IFIH1*, and *DHX58* in term chorionic villi and deciduae after CMV or VSV infection. RIG-I and MDA5 expression were induced during experimental VSV infection by the secretion of IFN-ß. Moreover, we found that CMV infection might influence RIG-I and MDA5 expression on the mRNA level in decidual tissues.

2. Materials and methods

2.1. Tissue collection

The study protocol was approved by the appropriate research ethics committee (Medical University of Lodz, Lodz, Poland; reference protocol RNN/120/09/KE) and written informed consent was obtained from each donor before clinical sampling. The study was conducted in accordance with the Declaration of Helsinki and all the experiments were performed in accordance with relevant guidelines and regulations of the concerned ethical committee. Twelve human placentas (38-42 weeks of gestation) were collected immediately from the cesarean section before the onset of labor. The CMV IgG antibodies were detected in all pregnant women. Women with immunological disorders or infectious diseases were excluded from the study.

2.2. Placental villous and decidual explants cultures

Third trimester human placental tissues were obtained immediately after cesarean section and were placed into sterile dishes in ice-cold phosphate-buffered saline (PBS; Sigma-Aldrich Co. Ltd., Ayrshire, UK). Placental explants were prepared and cultured as previously described (Jabłońska et al., 2018). Briefly, tissue biopsies were prepared from the chorionic villi on the fetal side and from the decidua basalis on the maternal side of the placenta. Both villous and decidual tissue biopsies were executed and tested separately in each organ. The explants were scraped from fetal membranes, washed several times with ice-cold PBS to remove contaminating blood, and then minced into small pieces (approximately 2 mm^3). Then, the tissue pieces (0.2 g) were transferred into 24-well tissue culture plates and cultured in 2 ml of Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 10% inactivated fetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine, and 100 µg streptomycin-100 U penicillin (Sigma-Aldrich). The plates were maintained at 37 °C in 5% CO_2 for 2 (VSV) or 5 (CMV) days. The explants and cell/tissue-free culture supernatants were collected by centrifugation and stored at −80 °C until use. The uninfected status of each placenta used in experiments was verified by quantitative real-time PCR (qRT-PCR) specific for CMV DNA.

2.3. Cell lines and virus strains

The MRC-5 (American Type Culture Collection (ATCC) CCL-171; Manassas, VA, USA) and L929 (ATCC CCL-1) cells were cultured in Eagle's Minimum Essential Medium (EMEM; Sigma-Aldrich) supplemented with 10% inactivated FBS, 2 mM L-glutamine, and 100 µg streptomycin-100 U penicillin at 37 °C in 5% CO_2 atmosphere until confluent. The CMV strain AD-169 (ATCC VR-538) was propagated in the MRC-5 cell line in EMEM supplemented with 2% inactivated FBS, 2 mM L-glutamine, and antibiotics. The VSV strain Indiana was cultured in the L929 cell line with EMEM supplemented with 2% FBS, 2 mM L-glutamine, and antibiotics. Briefly, supernatants from infected MRC-5 and L929 cells were harvested when at least 80% of cells showed cytopathic effect (CPE). A 13100 *g* centrifugation for 10 min at 4 °C was used to pellet cellular debris, and the supernatants were aliquoted and stored at −80 °C until use.

Virus stocks were prepared and titrated in MRC-5 (CMV) and L929 (VSV) cells before the experiment. The titer of the virus was expressed in plaque-forming units (PFUs) for CMV (Wentworth & French, 1970) and in the tissue culture infectious dose in 50% of cells ($TCID_{50}$) for VSV. The laboratory-adapted strains of CMV and VSV were used for the inoculation of explants.

2.4. Viral infection of placental villi and decidua

The chorionic villous and the decidual explants were infected with CMV (1×10^5 PFU/ml) or VSV (1×10^5 $TCID_{50}$/ml) (Paradowska et al., 1996a). Infection was performed in DMEM supplemented with 2% FBS, 2 mM L-glutamine, and antibiotics for 2 hr (CMV) or 1 h (VSV) at room temperature (RT). After adsorption, the virus inoculum was removed, organ cultures were washed 3 times with PBS, and supernatants were replaced with 2 ml of the fresh culture medium. Virus-infected explants that were maintained at 4 °C for 24 hours post-infection (hpi) served as controls of the efficacy of virus replication. The other explants were cultured for 48 hr (VSV) or 120 hr (CMV) at 37 °C. The supernatant samples were collected after 24 hpi, 48 hpi, and 120 hpi (CMV),

centrifuged at 13100 *g* for 10 min, and stored at −80 °C until analysis by enzyme-linked immunosorbent assay (ELISA) and viral load measurement.

2.5. Virus quantification; $TCID_{50}$ and plaque assays

The quantification of infectious viruses propagated in the susceptible cell cultures was measured using a plaque assay (CMV AD-169 strain) or $TCID_{50}$ (VSV). $TCID_{50}$ assay was also used to measure infectious virions of VSV in the villous and decidual tissue culture supernatants. The MRC-5 (2×10^5 cells per well) and L929 (2×10^4 cells per well) cells were seeded in 24-well plates or 96-well plates, respectively, and incubated for 24 hr. Then, MRC-5 and L929 cells were infected with serial dilutions of CMV and VSV, respectively. Collected supernatant fluids were diluted from 10° to 10^{-7} in EMEM supplemented with 2% FBS and aliquots were inoculated onto susceptible cell monolayers. After a suitable incubation period (48 hr for VSV and 120 hr for CMV), CPE induction was observed using an inverting microscope. The infected MRC-5 cells were fixed in methanol for 15 min and stained with 0.05% methylene blue for 15 min (Yu et al., 2010), then the number of plaques was counted under a microscope. The virus titer was calculated from the mean number of plaques for the 3 monolayers at a known dilution and was multiplied by the reciprocal of the dilution and the reciprocal of the volume added. Thus, the obtained value was the number of PFUs/ml. For VSV, the percentage of cell death (i.e., infected cells) was observed and evaluated using methyl thiazolyl tetrazolium (MTT) assay as described previously (Paradowska et al., 1996a). After overnight incubation at 37 °C, optical densities were measured at an absorbance wavelength of 550 nm and a reference wavelength of 670 nm using an ELISA reader (Benchmark Plus, Bio-Rad, Hercules, CA, USA).

2.6. DNA extraction

Genomic DNA isolation from villous and decidual specimens was performed using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of DNA were assessed using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.7. Real-time PCR quantification of CMV DNA in villous and decidual samples

A qRTPCR assay was used to quantify CMV DNA in DNA extracted from the tissues and supernatants of a villous and decidual cultures. Analyses of the CMV DNA copy number were performed using a qRT-PCR as previously described (Paradowska et al., 2006). Amplification was performed using a 7900 H T Fast Real-Time PCR system with TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA, USA).

2.8. RNA extraction and cDNA synthesis

The chorionic villous and the decidual biopsies were preserved in RNA*later* (Ambion, Austin, TX, USA), at −80 °C until use. Total RNA from tissue was isolated using Tri Reagent (Ambion), and first-strand complementary DNA (cDNA) was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's recommendations. The RT-PCR was performed in a Veriti® 96-Well Thermal Cycler (Applied Biosystems). The RNA and cDNA concentration and purity were evaluated using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific).

2.9. Analysis of gene expression by qRT-PCR

The qRT-PCR for *DDX58*, *IFIH1*, and *DHX58* genes, as well as for gene encoding tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*) as an endogenous housekeeping gene, was performed using a 7900 H T Fast Real-Time PCR System (Applied Biosystems). The *YWHAZ* gene was selected for normalization of RLR expression according to its expression stability and level in the human placenta (Meller et al., 2005; Jabłońska et al., 2018). For each qRT-PCR, 50 ng cDNA was added to a mix containing gene-specific primers (Komuro & Horvath, 2006), Power SYBR Green PCR Master Mix (Applied Biosystems), and nuclease-free water as previously described (Jabłońska et al., 2018). The primers efficiencies for each assay ranged from 96 to 102%. The qRT-PCR results were analyzed using the comparative C_t (ΔC_t) method, and the average C_t values were imported into Data Assist version 3.0 software (Life Technologies, Grand Islands, NY, USA) to calculate relative gene expression. All samples and controls were analyzed in triplicate.

2.10. Western blot analysis

The tissue homogenates from deciduae and chorionic villi were prepared using a Mammalian Cell Lysis Kit (Sigma-Aldrich) according to the manufacturer's instructions. A Western blot method was used for the evaluation of RLR expression in tissue homogenates. The samples (40 μg) were separated by electrophoresis using a 4-10% SDS-PAGE gel and transferred to nitrocellulose membranes. For the detection of proteins, the membranes were immunoblotted with rabbit polyclonal antibodies specific for DDX58 (1 μg/ml; Abcam, Cambridge, MA, USA), MDA5 (4 μg/ml; Abcam), DHX58 (1.5 μg/ml; Abcam) diluted in 5% non-fat milk in tris-buffered saline and Tween 20 (TBST). Protein loading was evaluated by examining ß-actin protein levels using the monoclonal β-actin antibody (Dako, Carpinteria, CA, USA). The primary antibody was used at 1:2000 dilutions in 5% non-fat milk in TBST overnight at +4 °C with gentle agitation. Then, the membranes were washed 3 times for 5 min in TBST and incubated for 2 hr at RT with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (diluted to 1:10000 in 5% non-fat milk in TBST; Abcam). Chemiluminescence detection was performed with Pierce ECL Western Blotting Substrate according to the manufacturer's instructions (Thermo Scientific, Rockford, IL, USA).

2.11. Protein quantification

The concentrations of RIG-I, MDA5, and LGP2 proteins in the fetal and the maternal tissues were determined using ELISA kits (EMELCA Bioscience, Breda, the Netherlands). The optical density of each sample was read at 450 nm using a Benchmark Plus microplate reader (Bio-Rad), and the selected protein concentrations were calculated using a standard curve. The protein levels of IFN-α and IFN-β (PBL Interferon Source, Piscataway, NJ, USA), interleukin (IL)-6, CXCL8/IL-8, and tumor necrosis factor (TNF)-α (R&D Systems, Minneapolis, MN, USA) were determined in supernatants from explants cultures using ELISA kits according to the manufacturer's instructions. After incubation, absorbance was measured using a Benchmark Plus microplate reader (Bio-Rad).

2.12. Statistical analysis

GraphPad Prism 5.00 software (GraphPad Software, San Diego, CA, USA) was used for all analyses. Statistical analyses of qRT-PCR data are presented as the mean ± standard error of the mean (SEM). The nonparametric Mann-Whitney U test was used to compare the levels of gene and protein expression between the two groups (virus-infected and mock-infected tissues). The Wilcoxon test was used to analyze two variables within the same group. One-way ANOVA followed by Tukey's post hoc testing was used for multiple comparisons. A *P*-value of less than 0.05 was considered statistically significant. The number of samples (*n*) and the *P*-values of significant differences are given in each Figure.

3. Results

3.1. Expression of DDX58, IFIH1, and DHX58 genes are found in both term chorionic villi and deciduae

First, we investigated the basal expression of *DDX58*, *IFIH1*, and *DHX58* in mock-infected (uninfected) cultures of third trimester chorionic villi and deciduae. We found that both chorionic villi and deciduae constitutively expressed mRNAs for all RIG-I-like receptors (Supplementary Fig. S1a). *DDX58* has been shown to exhibit higher constitutive expression than other RLR genes ($P \leq 0.001$ for both the deciduae and the chorionic villi; Supplementary Fig. S1a). The mRNA expression level of each RLR varied among individuals but no significant differences in the protein expression levels were found in both fetal and maternal tissues (Supplementary Fig. S1b). Notably, *DDX58* and *IFIH1* expression levels decreased until 48 hr of incubation in both the chorionic villi ($P = 0.016$ for both genes) and the deciduae ($P = 0.031$ and $P = 0.063$ for *DDX58* and *IFIH1*, respectively) as compared to mock-infected tissue at 24 hr of incubation (data not shown). The *DHX58* mRNA level increased during *ex vivo* culture until 24 hr of incubation ($P = 0.031$ for the chorionic villi and the deciduae) and then decreased by 48 hr of incubation (data not shown). Western blot analysis with specific antibodies confirmed the presence of the investigated proteins in maternal and fetal tissues. Immunostaining revealed a 107 kDa band corresponding to DDX58, a 117 kDa band corresponding to MDA5, and a 77 kDa band corresponding to DHX58. An ELISA provided quantitative information about RLR expression.

3.2. VSV infection activates RIG-I and MDA5 expression in the chorionic villi and the deciduae

We examined VSV-infected placental tissues using qRT-PCR to analyze the mRNA levels of all RLRs. VSV was selected as an indicatory virus because it does not cause natural infection in the human placenta. As shown in Figs. 1a and 1b, the expression of *DDX58* and *IFIH1* mRNAs was significantly higher, at 48 hpi in the chorionic villi ($P = 0.010$ and $P = 0.022$ for *DDX58* and *IFIH1*, respectively) and in the deciduae ($P = 0.038$ and $P = 0.010$ for *DDX58* and *IFIH1*, respectively) infected with VSV than that in mock-infected tissues at the equivalent time. Interestingly, the VSV-infected tissues showed significantly lower *DHX58* mRNA expression at 24 hpi than the mock-infected tissues ($P = 0.004$ for the deciduae, $P = 0.001$ for the chorionic villi) (Fig. 1c). These effects at the gene level were confirmed at the protein level by immunoblotting and ELISA (Fig. 1d–g). During VSV infection, enhanced RIG-I protein expression at 48 hpi was observed. The level of this protein was more than 10-fold higher than that in mock-infected explants ($P = 0.017$ and $P = 0.039$ for the deciduae and the chorionic villi, respectively; Fig. 1d). Moreover, a more than two-fold increase in MDA5 protein expression was found in response to VSV infection ($P = 0.020$ for the deciduae, $P = 0.049$ for the chorionic villi; Fig. 1e). These data suggest that RIG-I and, surprisingly, MDA5 are expressed most abundantly in both the fetal and maternal parts of the term placenta during VSV infection. No significant differences in LGP2 expression were detected in response to VSV infection (Fig. 1f). Results showed that an additional cleaved β-actin fragment was identified in some tissue. However, it is well known that β-actin is partially cleaved to two fragments by activated caspases during the development of apoptosis (Fig. 1g).

3.3. RLR expression during CMV infection in the chorionic villi and the deciduae

The expression of *DDX58*, *IFIH1*, and *DHX58* genes after CMV infection was studied in freshly isolated placental explants (Fig. 2a–c). The expression of *DDX58* and *IFIH1* increased in a time-dependent manner until 120 hpi in the CMV-infected chorionic villi (Figs. 2a and 2b). A comparison of mRNA expression in CMV-infected and mock-infected tissue samples revealed that *DDX58* expression was significantly higher in infected tissues than in mock-infected tissue at 48 hpi ($P = 0.003$ for both, deciduae and chorionic villi; Fig. 2a). A similar *IFIH1* expression profile was independently observed at 48 hpi for the infected and mock-infected explants ($P = 0.032$ and $P = 0.036$, respectively; Fig. 2b). In contrast, *DHX58* mRNA expression was significantly lower in the CMV-infected deciduae and chorionic villi than that in the mock-infected tissues ($P = 0.004$ and $P = 0.01$, respectively, at 24 hpi; Fig. 2c). These results were further evaluated at the protein level by immunoblotting and ELISA (Fig. 2d–g). The expression of RIG-I protein was slightly upregulated in the CMV-infected decidual explants compared to those of the mock-infected tissues ($P = 0.004$), whereas no specific effect on MDA5 expression was observed (Figs. 2c and 2d). No significant differences in the expression of LGP2 during virus infection were also noticed (Fig. 2f). Our results suggest that RIG-I expression increased in response to experimental CMV infection in term decidual explants compared to those of mock-infected tissues.

3.4. Viral replication

Transplacental CMV infection in the examined placental explants was excluded using qRT-PCR analysis, whereas naturally occurring human infection with VSV is rare. The kinetics of viral replication varies among individual tissues. Freshly isolated chorionic villi and decidua explants were relatively resistant to VSV infection. In most of the explants, the presence of low levels of VSV replication was observed; in others, VSV was replicated to a high titer at 48 hpi (mean: $10^{5.49}$ $TCID_{50}$/ml for both the deciduae and the chorionic villi). CMV multiplied in all examined chorionic villi and four of the five deciduae (Fig. 3a). The number of CMV DNA copies increased in the supernatants of infected decidual and chorionic villous cultures during the 120 hpi (2.34×10^4 genome equivalents (GE)/ml *vs.* 5.51×10^4 GE/ml in the deciduae and chorionic villi, respectively, $P > 0.05$; Fig. 3a). In contrast, the mean intracellular CMV DNA load decreased after 24 hpi in both the deciduae and the chorionic villi ($P > 0.05$; Fig. 3b). All mock-infected tissues remained uninfected during the culture periods.

3.5. Cytokine production

The concentrations of IFN-α, IFN-β, IL-6, IL-8, and TNF-α were measured in supernatants from mock-infected and virus-infected explants cultures. VSV induced IFN-β release in both fetal and maternal tissues ($P = 0.008$; Fig. 4a). In contrast, a significant decrease in the TNF-α level was observed in the VSV-infected explants compared to that in the mock-infected explants ($P = 0.001$ for the deciduae, $P = 0.008$ for the chorionic villi; Fig. 4b). No significant differences in IFN-α, IL-6, and IL-8 levels were observed between the mock-infected and VSV-infected explants ($P > 0.05$; data not shown). Infection with CMV did not affect the production of most cytokines, including IFN-α, IFN-β, IL-6, IL-8, and TNF-α. Finally, these data may suggest that VSV infection activates both RIG-I and MDA5 receptors in term chorionic villi and deciduae, which triggers IFN-ß production.

4. Discussion

The present study demonstrates that the expression of RIG-I-like receptors is induced during virus infection by the secretion of type I IFN in the third trimester human placenta. We previously described that both the deciduae and the chorionic villi constitutively express genes and proteins encoding two RLRs, RIG-I and MDA5, and that HSV-1 triggers enhanced RIG-I expression (Jabłońska et al., 2018). This study also found that *DDX58* encoding RIG-I displayed the highest expression compared to the expression of the other RLRs. The observed increase in *DDX58* and *IFIH1* mRNA levels after VSV infection compared to those in the mock-infected tissues were reflected in the increase in RIG-I and MDA5 protein levels. The specific expression of RIG-I and MDA5 in

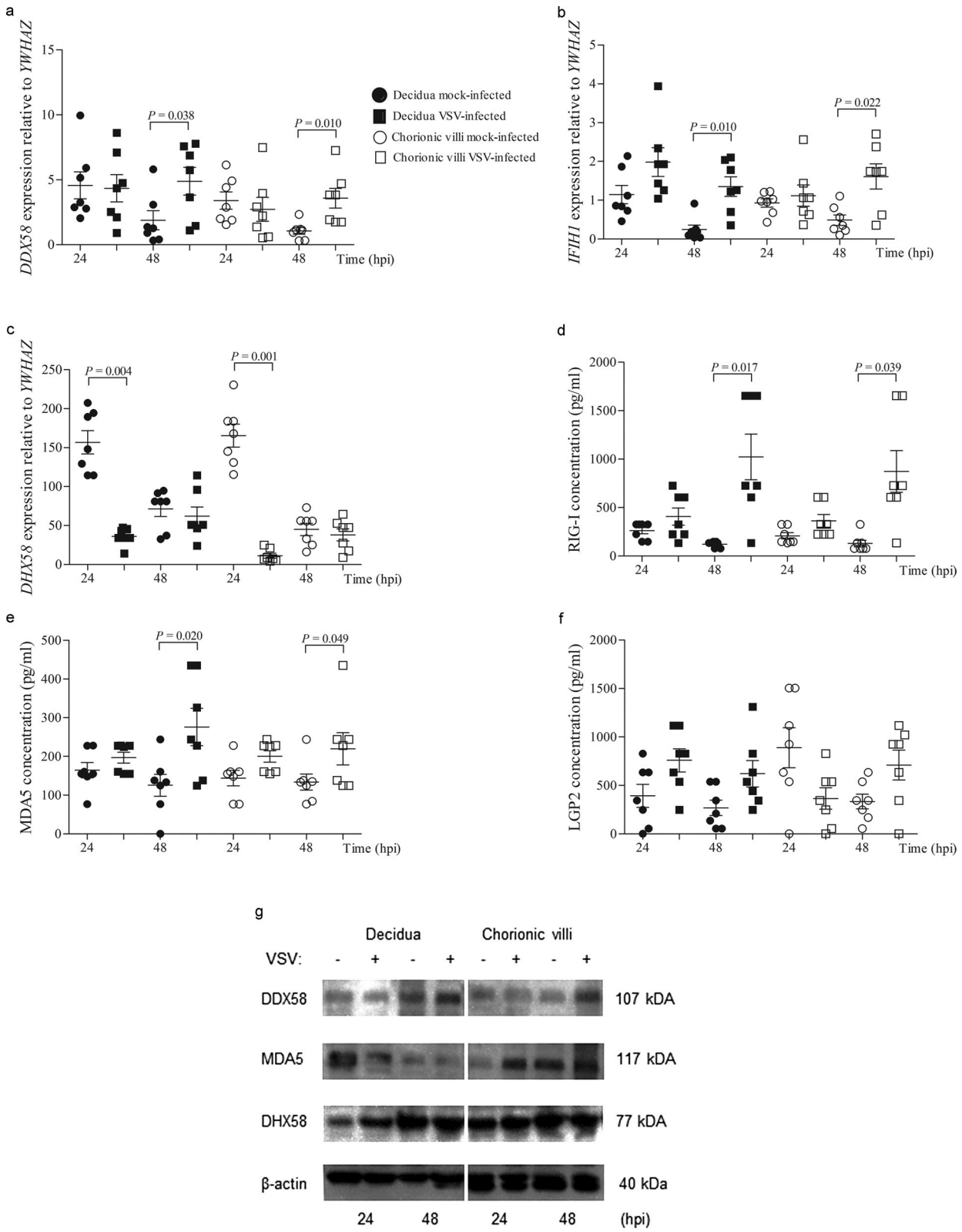

Fig. 1. Expression levels of (a) *DDX58*, (b) *IFIH1*, and (c) *DHX58* genes and (d) RIG-I, (e) MDA5, and (f) LGP2 proteins in third-trimester decidual and chorionic villi tissues. The explants were mock-infected (first rows) or infected (second rows) with VSV. Quantitative RT-PCR was performed using *YWHAZ* as a reference gene, while protein expression was determined by Western blot and ELISA. The samples derive from the same experiment and blots were processed in parallel. Data are reported as mean values ± standard error of the mean (SEM) ($n = 7$); Mann-Whitney U test. Statistical significant differences compared to mock-infected placental explant cultures are shown. In (g), total lysates of mock-infected (-) or VSV-infected explants (+) were analyzed by immunoblot using specific antibodies recognizing RLR proteins.

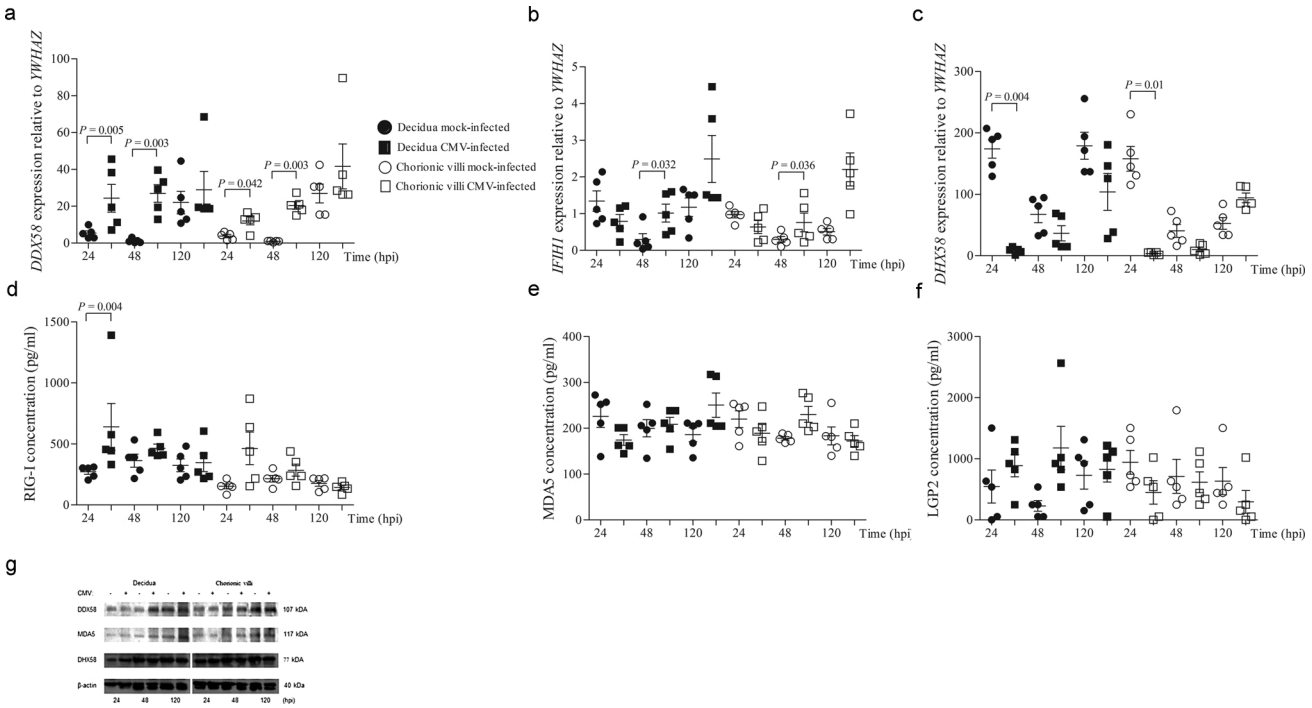

Fig. 2. Relative expression of (a) *DDX58*, (b) *IFIH1*, and (c) *DHX58* genes and (d) RIG-I, (e) MDA5, and (f) LGP2 proteins in third-trimester human tissues of deciduae and chorionic villi. The explants were mock-infected (first rows) or infected (second rows) with CMV. Data are reported as mean values ± SEM ($n = 5$); Mann-Whitney U test. For (d) $P = 0.049$, remained significant after removal of distal result. In (g), total tissue lysates were used for Western blot analysis using an anti-DDX58, anti-MDA5, anti-DHX58, and anti-ß-actin antibodies. Quantitative RT-PCR was performed using *YWHAZ* as a reference gene, while protein expression was determined by Western blot and ELISA. The samples derive from the same experiment and blots were processed in parallel.

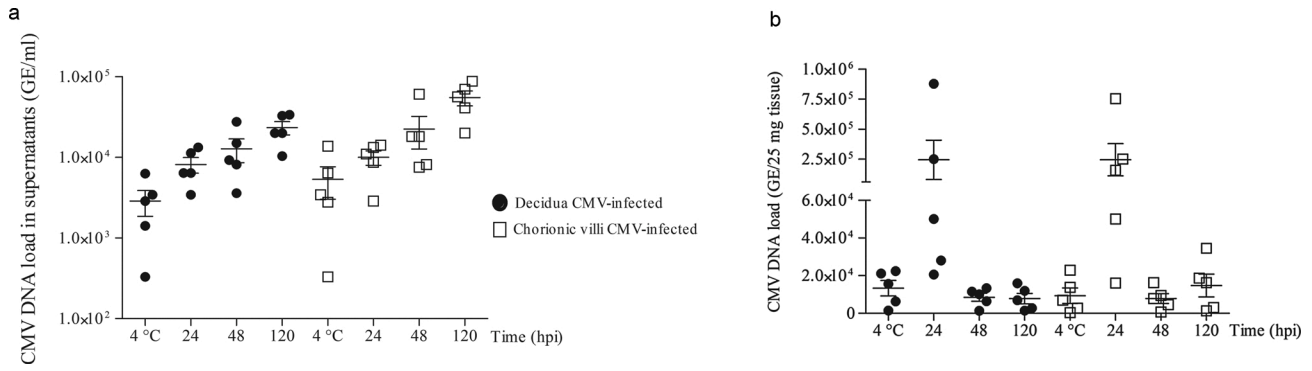

Fig. 3. Comparison of CMV DNA copies (a) supernatants and (b) tissues of decidual and chorionic villous cultures. Viral load quantification was performed by qRT-PCR. Data are expressed as the mean ± SEM ($n = 5$).

response to VSV infection resulted in enhanced IFN-ß secretion in both maternal and fetal tissues. CMV infection also upregulated the expression of *DDX58* and *IFIH1*, but the correlation between expression levels of mRNA and protein was poor. CMV infection upregulated the expression of RIG-I at a lower level than those induced by VSV, with much higher activities in the decidua.

The innate immunity activated by PRRs is a key element in the pathogen-induced nonspecific inflammatory response as well as specific adaptive immunity to invading microorganisms. Thus far, studies regarding PRR sensing of pathogen invasion have focused primarily on the role of TLRs and NLRs, primarily those in the trophoblast and placental explants from the first and third trimester of pregnancy (Krikun et al., 2007; Pontillo et al., 2013; Bryant et al., 2017; Stødle et al., 2018). It was demonstrated that responsiveness *via* TLR3, TLR7, TLR8, and RIG-I/MDA5 is a broad feature of human term gestation–associated tissues (Bryant et al., 2017). Immunohistochemistry revealed that RIG-I, MDA5, TLR3, TLR7, and TLR8 were expressed by the term placenta, choriodecidua, and amnion (Bryant et al., 2017). Both RIG-I and MDA5 were localized to the placental trophoblasts, chorionic trophoblasts, and decidual stromal cells, while MDA5 and LGP2 were also expressed in the villous stroma. Our results revealed constitutive expression of RIG-I and MDA5 in both the deciduae and the chorionic villi, whereas RIG-I showed a higher level of the mRNA expression than MDA5 and LGP2.

This is the first study to demonstrate RLR expression in the human term placenta during VSV and CMV infection. RIG-I is expressed at a low level in most cells of the body, and its level increases in response to infection with RNA viruses. VSV, a neurotropic ssRNA virus and a member of the *Rhabdoviridae* family, does not cause natural infection in the European human population, and no specific anti-VSV antibodies are present in human serum. However, VSV has the ability to replicate into almost all human cell types, including placental cells (Paradowska et al., 1996b; Jatczak et al., 2012). RIG-I is the primary receptor responsible

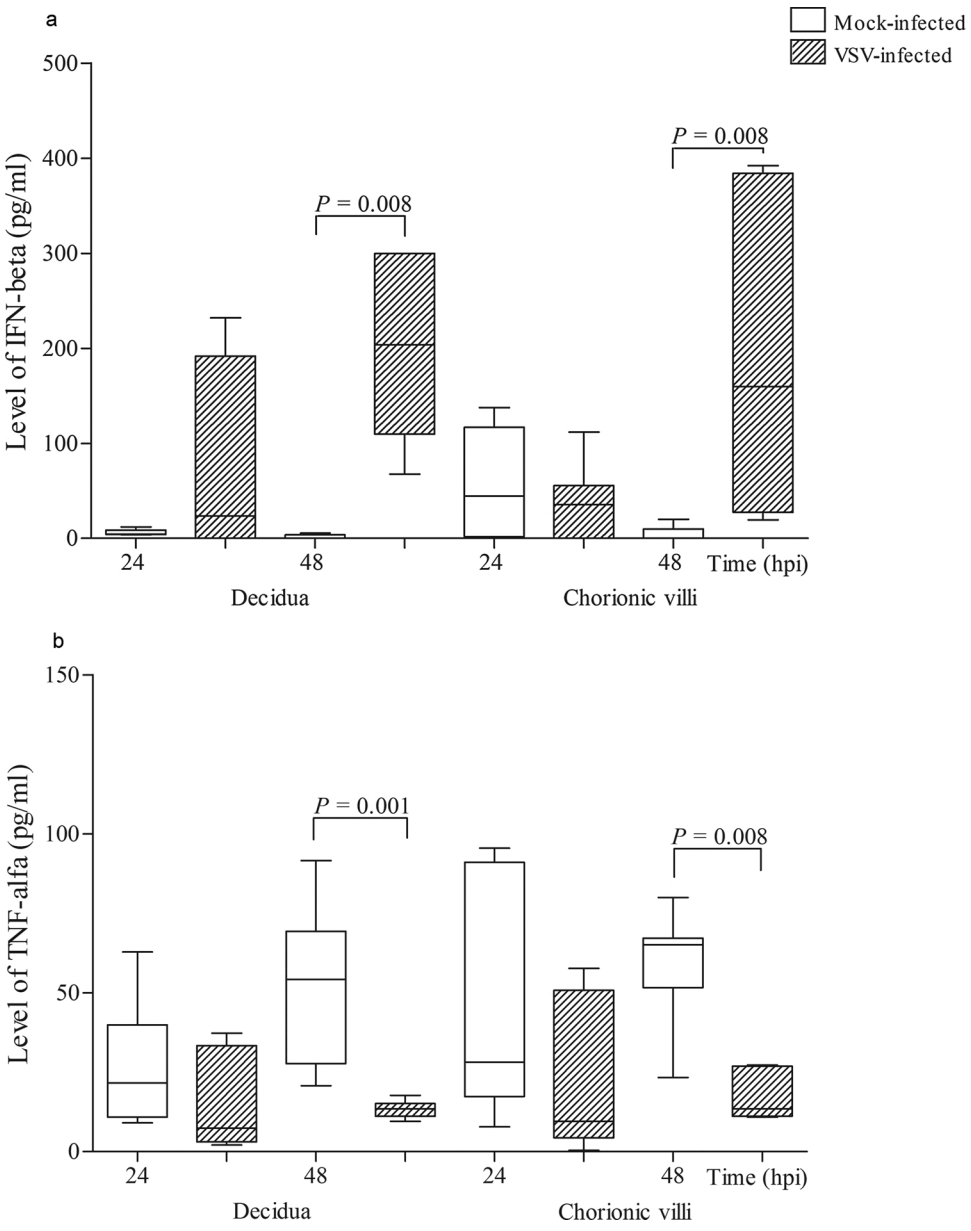

Fig. 4. (a) IFN-β and (b) TNF-α production in both decidual and chorionic villi after VSV infection. Human decidual and chorionic villi tissues were infected with 10^5 tissue culture infectious dose in 50% of cells ($TCID_{50}$)/ml of VSV ($n = 7$). The supernatants from explant cultures of mock-infected (first rows) or VSV-infected (second rows) deciduae and chorionic villi were shown. At 24 hpi and 48 hpi, the explants supernatants were collected and assayed for protein concentration by ELISA. Data are expressed as mean ± SEM; Mann-Whitney U test.

for the recognition of VSV infection in macrophages (Kandasamy et al., 2016), primary murine glial cells (Furr et al., 2008) and plasmacytoid dendritic cells (Waibler et al., 2007). In our studies, both RIG-I and MDA5 expression increased after VSV inoculation. We also observed that VSV infection was able to induce increased IFN-β secretion in placental cultures, indicating that RLR expression may result in antiviral cytokine production. RIG-I and MDA5 recognize distinct viral RNA species and activate RLR signaling through IPS-1 protein. Overexpression of IPS-1 induces the interaction of IKKε and TBK1 that leads to IRF3 and IRF7 phosphorylation and to activation of type I IFN production. Although the mechanism has not been confirmed in human placenta so far, it appears possible that it is activated during viral transmission. Notably, low levels of IFNs expression in the term placenta have been previously observed, although IFN production was dependent on the gestational age and type of inducer (Aboagye-Mathiesen et al., 1995; Paradowska et al., 1997). Consistent with the current findings, we speculate that low VSV replication in the present study may be associated with RLR expression and the production of IFN-β, which are important for the antiviral and autoimmune responses.

Some DNA viruses, including HSV-1 and EBV, induce IFN-β production through the RIG-I pathway after the conversion of DNA to RNA by the enzyme Pol-III (Samanta et al., 2006; Chiu et al., 2009). Viruses have evolved a variety of mechanisms to evade the RLR-mediated immune response. Many of these mechanisms antagonize host INF type I induction at the RIG-I level. CMV encodes proteins that bind to dsRNA and prevent their detection by RLRs in the cytoplasm (Child et al., 2004). Interestingly, we found a significant increase in RIG-I expression in response to CMV infection, which suggests that this pathogen may activate the RIG-I pathway, particularly in decidual tissue. Moreover, the elevated production of IL-8 and TNF-α during CMV infection indicates an overall shift toward NF-κB activation (Scott et al., 2012). IL-8 production was also significantly elevated in term placentas and choriodeciduae upon stimulation with nucleic acid ligands (Chatterjee et al., 2011). Because CMV is able to induce the production of IFN-λ (Brand et al., 2005; Egli et al., 2014), we suppose that it might induce both types I and III IFNs in the placenta. It is known that IFNs have been

generally considered to be antiproliferative proteins and IFN-β had greater growth inhibitory and proapoptotic effects than IFN-α (Chawla-Sarkar et al., 2001). Decreased *DDX58* and *IFIH1* mRNA expression in the fetal part of the placenta compared to the maternal part seems to be beneficial for the fetus, because it may prevent or decelerate proinflammatory cytokine activation and further fetal deformation and malformation. The role of RLRs in CMV infection and replication is not entirely understood. CMV detection by RIG-I and MDA5 in the cytoplasm is blocked by TRS1 and IRS1, which bind to dsRNA and prevent the downstream signaling activation of RLRs (Child et al., 2004). Scott (2009) observed a decrease in the expression of full-length RIG-I at 48 hpi and demonstrated for the first time that RIG-I and not MAVS is first upregulated and then degraded in a non-apoptotic manner during CMV infection (Scott, 2009).

Finally, we found high levels of LGP2 in freshly isolated placental tissue and differential expression of LGP2 in the deciduae and chorionic villi from the same organ after infection with both viruses. LGP2 shares considerable homology with RIG-I and MDA5 in the DExD/H-box helicase domain and C-terminal domain but lacks two N-terminal CARD domains that are required for signaling (Takahasi et al., 2009). LGP2, localized in the cytosol of different cell types, binds to dsRNA produced during viral replication. The increased expression of LGP2 may negatively regulate RIG-I/MDA5-mediated antiviral signaling (Yoneyama et al., 2005; Takahasi et al., 2009). Overexpressed LGP2 might act as a potent negative regulator because the sequestration of ssRNA and dsRNA through competitive binding could prevent the activation of RIG-I and MDA5. Another way in which LGP2 exerts an inhibitory role is by the presence of its repressor domain (RD). LGP2 inhibits RIG-I through RD interactions that block RIG-I dimerization, which is necessary for its activation (Saito et al., 2007). LGP2-deficient mice showed elevated type I IFN levels in response to VSV or poly(I:C) but decreased type I IFN levels following EMCV infection (Satoh et al., 2010). These studies indicate that LGP2 negatively or positively regulates RIG-I and MDA5 responses, depending on the type of RNA virus. Our results raise important questions as to why *ex vivo* viral infection did not increase the expression of *DHX58* and LGP2 compared to other receptors. In this regard, it is tempting to speculate that LGP2 in the placenta regulates RIG-I/MDA5 expression during intrauterine infection. It has been suggested that viruses have evolved a pleiotropic mechanism for limiting IFN induction because the interaction between specific viral proteins with LGP2 can inhibit the activation of both RIG-I and MDA5 (Childs et al., 2012; Deddouche et al., 2014). LGP2 may play an important role in modulating the innate recognition of viruses not only by serving as a pathogen sensor but also by activating signaling pathways. Hence, LGP2 may represent a mechanism to stimulate the innate and adaptive immune responses to viruses. Because the functional role of LGP2, as a regulatory molecule for RIG-I and MDA5, is still controversial, other studies are needed to determine its contribution to transplacental viral infection.

Some potential limitations of the study must be acknowledged. Because of the observational nature of our study, we were unable to understand the unique interaction between viruses, RLRs, and cells at the placental–decidual interface. Our results present RLR expression without controlling for other receptors known to bind dsRNA, e.g., TLR3. Moreover, it cannot be ruled out that the presence of the CMV IgG antibodies in women may affect CMV replication and RLRs expression. To determine whether viruses are specifically and functionally recognized by RIG-I and MDA5 in the human placenta, future in-depth and more advanced investigations using RNAi or CRISPR/Cas9 methodology are needed. Knocking down RLR expression before viral inoculation should be tested by future experiments using primary human placental cells e.g., cytotrophoblast cultures. Hence, the significance of these results must be handled with caution. This preliminary study was aimed at gaining novel insight into RLR expression and innate immune activation during placental infection, and it was not designed to determine the mechanism of the RLR signaling pathway. It highlights the potential utility of RIG-I-like receptors in the feto-maternal unit; however, it also drives further functional and mechanistic studies, including cellular localization by using immunohistochemistry. An extensive examination of IRF1/3 activation, nuclear translocation, and RLR-specific ISG expression would provide more detailed insights into the function of both RIG-I and MDA5 in the placenta.

In conclusion, our results reveal that RLRs are expressed in both human term chorionic villi and deciduae using an explant model. Enhanced RIG-I and/or MDA5 expression has been identified in response to VSV and CMV infection. Our findings raise the exciting possibility that both RIG-I and MDA5 might be involved in RNA and DNA viral recognition in fetal and maternal tissues and indicate that LGP2 is not activated during intrauterine viral infection. It is possible that this helicase functions as a regulator of RIG-I- and MDA5-mediated antiviral responses. However, further work is required to determine the functional activity of RLRs in human placenta. The discovery of an association between intrauterine viral infection, PRR expression levels, and certain adverse pregnancy outcomes might help in understanding the roles of components of the innate immune response at the feto-maternal interface and in developing efficient therapies.

CRediT authorship contribution statement

Agnieszka Jabłońska: Conceptualization, Funding acquisition, Investigation, Resources, Writing - original draft, Writing - review & editing. **Anna S. Świerzko:** Investigation, Writing - review & editing. **Mirosława Studzińska:** Investigation, Writing - review & editing. **Patrycja Suski:** Investigation, Writing - review & editing. **Jarosław Kalinka:** Resources, Writing - review & editing. **Zbigniew J. Leśnikowski:** Writing - review & editing. **Maciej Cedzyński:** Writing - review & editing. **Edyta Paradowska:** Conceptualization, Funding acquisition, Resources, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

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