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Human gestation-associated tissues express functional cytosolic nucleic acid sensing pattern recognition receptors

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Summary

The role of viral infections in adverse pregnancy outcomes has gained interest in recent years. Innate immune pattern recognition receptors (PRRs) and their signalling pathways, that yield a cytokine output in response to pathogenic stimuli, have been postulated to link infection at the maternal–fetal interface and adverse pregnancy outcomes. The objective of this study was to investigate the expression and functional response of nucleic acid ligand responsive Toll-like receptors (TLR-3, −7, −8 and −9), and retinoic acid-inducible gene 1 (RIG-I)-like receptors [RIG-I, melanoma differentiation-associated protein 5 (MDA5) and Laboratory of Genetics and Physiology 2(LGP2)] in human term gestation-associated tissues (placenta, choriodecidua and amnion) using an explant model. Immunohistochemistry revealed that these PRRs were expressed by the term placenta, choriodecidua and amnion. A statistically significant increase in interleukin (IL)-6 and/or IL-8 production in response to specific agonists for TLR-3 (Poly(I:C); low and high molecular weight), TLR-7 (imiquimod), TLR-8 (ssRNA40) and RIG-I/MDA5 (Poly(I:C)LyoVec) was observed; there was no response to a TLR-9 (ODN21798) agonist. A hierarchical clustering approach was used to compare the response of each tissue type to the ligands studied and revealed that the placenta and choriodecidua generate a more similar IL-8 response, while the choriodecidua and amnion generate a more similar IL-6 response to nucleic acid ligands. These findings demonstrate that responsiveness via TLR-3, TLR-7, TLR-8 and RIG-1/MDA5 is a broad feature of human term gestation-associated tissues with differential responses by tissue that might underpin adverse obstetric outcomes.

Keywords: hierarchical clustering, inflammation, pattern recognition receptors, reproductive immunology, viral

Introduction

Cytokine production at the materno–fetal interface is a part of normal pregnancy, and the changes that occur with adverse obstetric outcomes might offer therapeutic targets [1,2]. The relationship between bacterial infection and preterm rupture of the membranes (PROM) and preterm birth (PTB) has been given much attention [3], but recent years have seen the emergence of epidemiological evidence linking viral infection and adverse pregnancy [4,5]. These viral infections include human papillomavirus, hepatitis virus, herpes virus and cytomegalovirus, which have been identified in both gestational tissues and amniotic fluid, and are linked to increased risk of chorioamnionitis and spontaneous preterm birth [6]. Therefore, better understanding of the response of gestation-associated tissues to viruses is required.

Pattern recognition receptors (PRRs) are evolutionary conserved germline-encoded receptors which recognize pathogen-associated molecular patterns (PAMPs) from various microorganisms. Recognition of viral infections by the innate immune response occurs via nucleic acid-responsive Toll-like receptors (TLRs)-3, −7, −8 and −9 and retinoic

acid-inducible gene (RIG)-I-like receptors (RLRs) RIG-I, melanoma differentiation-associated protein 5 (MDA5) and Laboratory of Genetics and Physiology 2 (LGP2) [7–10]. These receptors are located intracellularly, where they are best positioned to encounter and respond to viruses [11]. Clearer understanding of the expression and function of viral responsive PRRs at the materno–fetal interface is required for our fundamental understanding of pregnancy immunology and for translating this to the development of new therapeutic approaches for adverse obstetric outcomes. There are no published studies of RLR expression in gestation-associated tissues. For TLRs-3, −7, −8 and −9, only the placenta has been investigated with transcripts and/or protein for all of these reported with functional responses observed to TLR-3, TLR-7 and TLR-7/8 but not TLR-9 agonists [12]. Therefore, we investigated the expression and activity of nucleic acid ligand-responsive TLRs (TLR-3, −7, −8 and −9) and RLRs (RIG-I, MDA5 and LGP2) in human term placenta, choriodecidua and amnion using an explant model to mimic more closely the cellular heterogeneity that occurs *in utero*. A hierarchical clustering approach, as described previously for cytokine data [13], was implemented to compare more clearly the response between different tissues and ligands. It is our hypothesis that nucleic acid ligands will induce an inflammatory response in gestation-associated tissues and that utilization of hierarchical clustering to examine cytokine production by the placenta, choriodecidua and amnion in response to these PAMPs will highlight distinct differences in their responsiveness not revealed in previous studies.

Materials and methods

Samples

Placenta and fetal membrane samples were collected from healthy term newborns (> 37 weeks of gestation) delivered by elective caesarean section (ECS) at Singleton Hospital, Swansea, UK. Written consent was obtained from all study participants following recruitment at the antenatal day assessment unit. Ethical approval for this study was given by Wales Research Ethics Committee 6 (REC no. 11/WA/0040).

Explant cultures

Placenta. Placental explant cultures were prepared as described previously [12]. The decidua basalis overlaying the maternal side of the placenta was removed and 1-cm^3 pieces of placental tissue were extracted from various sites across the placenta, avoiding the fetal membranes, and placed into sterile calcium and magnesium-free phosphate-buffered saline (PBS; Life Technologies, Paisley, UK). Tissue was washed repeatedly with PBS to remove contaminating blood. Tissue was then minced into smaller pieces and washed further. Pieces of tissue (1-mm^3 pieces to a total of 0·2 g) were transferred into the appropriate number of wells of a standard 12-well tissue culture plate (Greiner Bio-one, Frickenhausen, Germany) containing 1 ml Ultra-CULTURE™ medium (Lonza, Visp, Switzerland), supplemented with 2 mM GlutaMAX™ (Life Technologies) and 2 mM penicillin, streptomycin and fungisone (PSF; Life Technologies).

Membranes. Membranes were detached from the placenta. Choriodecidua and amnion were separated from each other by blunt dissection and placed individually and washed repeatedly in sterile Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (Life Technologies) to remove blood. Explants were cut with an 8-mm biopsy punch (Stiefel, Research Triangle Park, NC, USA): two discs of choriodecidua placed into 0·5ml advanced RPMI supplemented with 2 mM glutamax, 2 mM PSF, 5 mM 2-mercaptoethanol (2-ME; all Life Technologies), 2% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 5 mM 2-mercaptoethanol; and four discs of amnion placed into 0·5 ml advanced Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 2 mM glutamax, 2 mM PSF and 2% FBS.

Explant cultures were exposed to different stimuli; an unstimulated control was always included. Optimal levels of all agonists were determined by dose–course studies on gestation-associated tissues explants and the following final concentrations were used: Poly(I:C)LMW (TLR-3, 25 μg/ml), Poly(I:C)HMW (TLR-3, 25 μg/ml), imiquimod (TLR-7, 1 μg/ml) ssRNA40 (TLR-8, 1 μg/ml), Poly(I:C)LyoVec (RIG-I/MDA5, 1 μg/ml) (all from Invivogen, San Diego, CA, USA), ODN21798 control or ODN21798 (TLR-9, 1 μM; both Miltenyi Biotec, Bisley, UK). All treatments were performed in duplicate. Cultures were incubated for 24 h at 37°C in 5% CO_2. Tissue-free supernatants were collected by centrifugation for 7 min at 4°C, 515 ***g*** and stored at −20°C for analysis using cytokine-specific enzyme-linked immunosorbent assays (ELISAs).

Cytokine production

IL-6 and IL-8 in the tissue-free supernatants of placenta, choriodecidua and amnion explant cultures collected after 24 h were measured using commercially available ELISA kits (DuoSet, R&D Systems, Minneapolis, MN, USA) as per the manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections (4 μm) of placenta and fetal membranes using the Ventana ULTRA automated staining instrument (Ventana, Tucson, AZ, USA). The Optiview detection system (Ventana) was used without A/B blocker or amplification (except for anti-TLR-9). Antigen retrieval

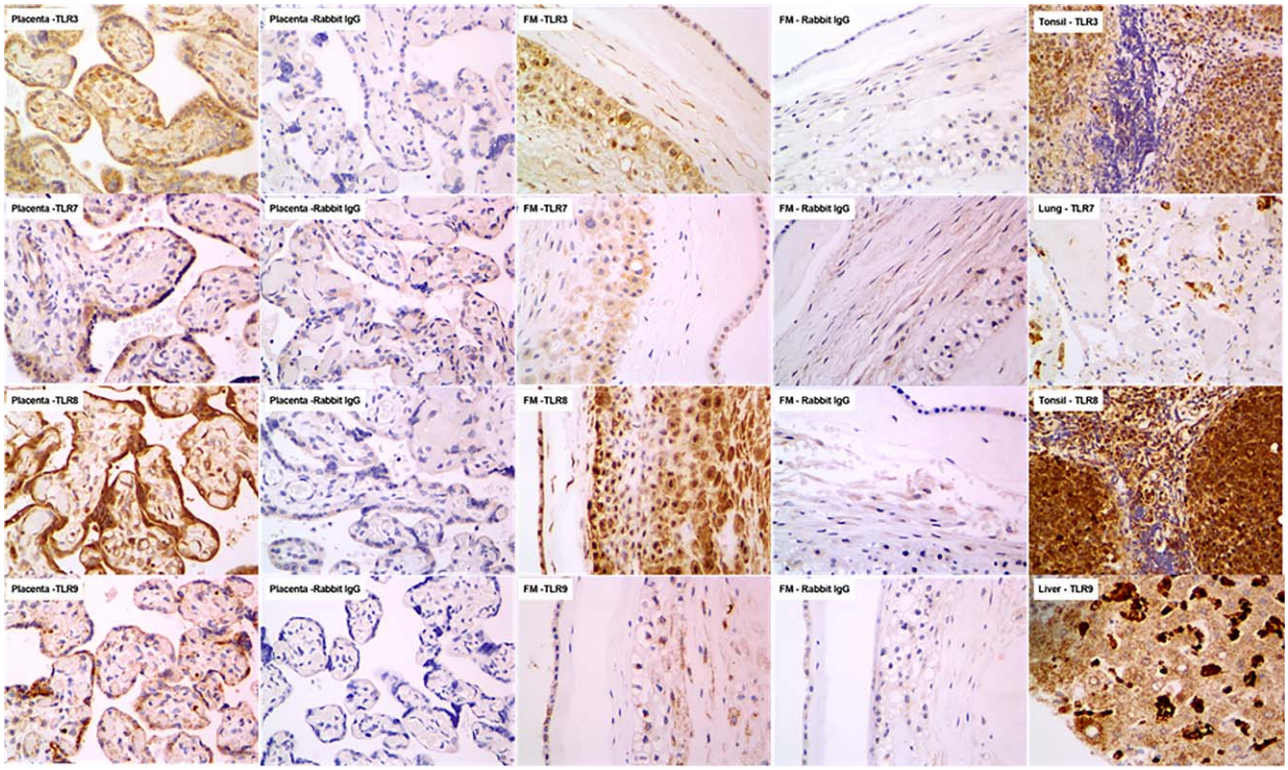

Fig. 1. Immunolocalization of nucleic acid sensing Toll-like receptors (TLRs) in human gestation-associated tissues. Negative (isotype match) and positive (TLR-3 and TLR-8, tonsil; TLR-7, lung; TLR-9, liver) controls are also shown. A representative example of seven is shown. Original magnification ×40.

was carried out in CC132 buffer for TLR-3, RIG-I and MDA5 and CC124 buffer for TLR-7, TLR-8 and LGP2. Primary antibodies, rabbit polyclonal anti-TLR-3 (5 μg/ml for 24 min), rabbit polyclonal anti-TLR-7 (10 μg/ml for 36 min), rabbit polyclonal anti-TLR-8 (5 μg/ml for 32 min), rabbit polyclonal anti-TLR-9 (10 μg/ml for 44 min), rabbit polyclonal anti-RIG-I (10 μg/ml for 32 min), rabbit polyclonal anti-MDA5 (5 μg/ml for 36 min) and rabbit polyclonal anti-LGP2 (5 μg/ml for 36 min) (all LifeSpan BioSciences, Inc., Seattle, WA, USA) were incubated at 36°C or, for anti-TLR-8, at room temperature. For control slides, primary antibody was replaced with polyclonal rabbit immunoglobulin (Ig)G (Biolegend, San Diego, CA, USA) at a corresponding concentration. A tissue reported to express the receptor of interest was used for optimization of staining and included as a positive control: tonsil (TLR-3, TLR-8, RIG-1, MDA5, LGP2), lung (TLR-7) and liver (TLR-9).

Data analysis

All experiments were performed a minimum of three times. Cytokine production in untreated *versus* treated tissue was evaluated by paired two-tailed Student's *t*-test or repeated-measures one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. A *P*-value of $\leq 0{\cdot}05$ was considered significant. Statistical significance was calculated using GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA, USA). Heatmaps were constructed for each cytokine using the mean values generated by ELISA, corrected for baseline constitutive levels, with the 'heatmap.2' function in the 'gplots' R package [14,15]. Hierarchical clustering was performed using a Euclidean distance method.

Results

Localization of TLR-3, TLR-7, TLR-8 and TLR-9 in human non-laboured gestation associated tissues

Immunohistochemistry was used to determine which cells within the placenta and fetal membranes expressed TLR-3, −7, −8 and −9 (Fig. 1). In the placenta, expression of all four TLRs by trophoblast was a common feature, with strong expression for both TLR-3 and TLR-8 in the trophoblast and villous stromal cells. In the fetal membranes, amnion epithelial cells, chorionic trophoblasts and decidual cells all exhibited strong expression of TLR-3 and TLR-8. Expression of TLR-7 was localized primarily to the chorion, with weak staining in the amnion intermittent expression of TLR-9 was also observed.

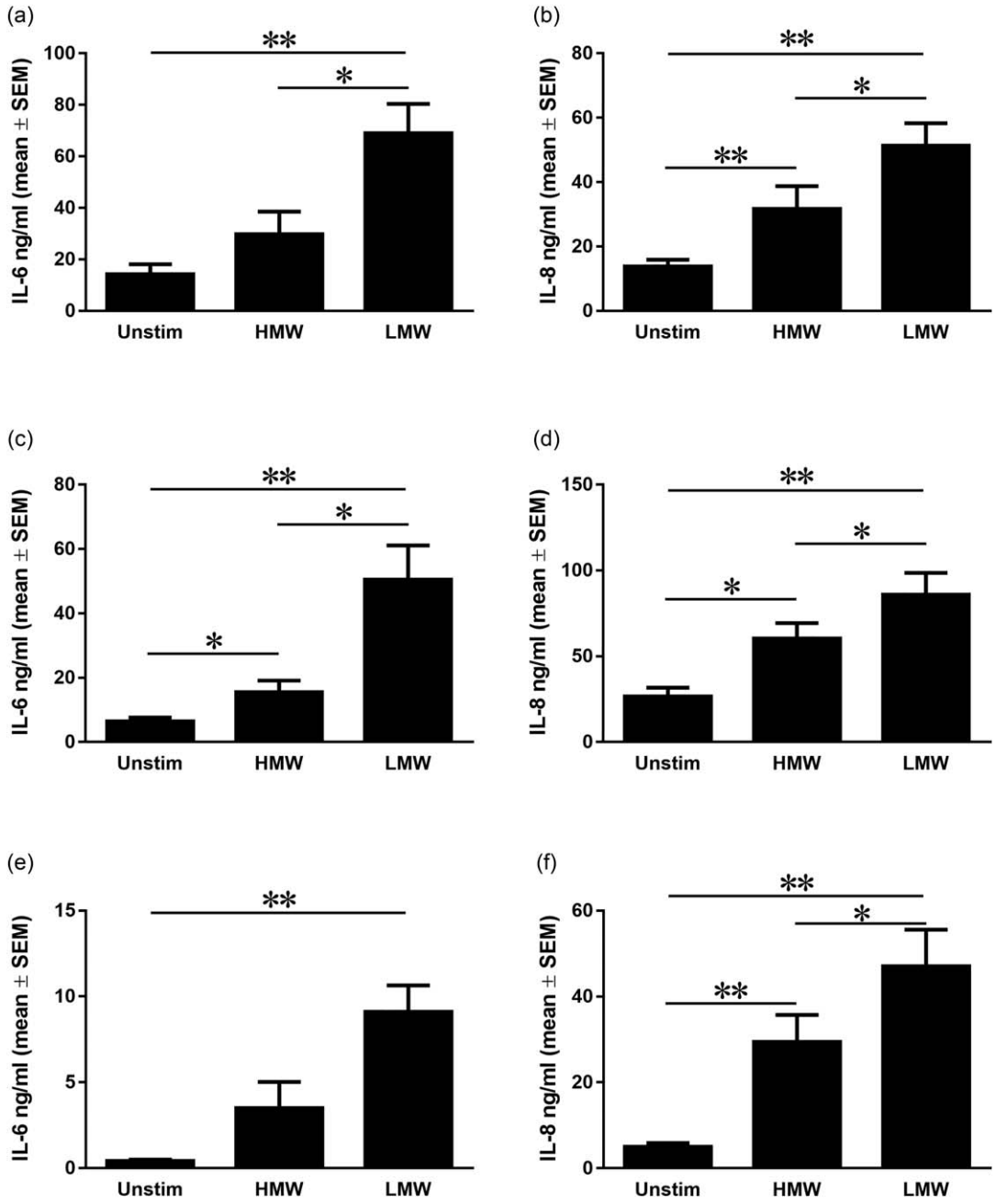

Fig. 2. Toll-like receptor (TLR)-3 agonist-induced cytokine response by the term non-laboured placenta, choriodecidua and amnion. Interleukin (IL)-6 and IL-8 production (ng/ml ± standard error of the mean) by the (a,b) placenta, (c,d) and choriodecidua (e,f) amnion following stimulation with polyinosine-polycytidylic acid [Poly(I:C)]LMW and Poly(I:C)HMW (both 25 μg/ml; $n = 9$). Statistically significant differences compared to unstimulated control are shown: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

Functional response of term non-laboured gestation associated tissue to specific TLR-3 TLR-7, TLR-8 and TLR-9 agonists

TLR-3. Functionality of TLR-3, a receptor involved in the recognition of dsRNA, was investigated using a synthetic dsRNA analogue Poly(I:C) (polyinosine-polycytidylic acid; both 25 μg/ml, $n = 9$). Both a high molecular weight (HMW, 1·5–8 kb) and a low molecular weight (LMW, 0·2–1 kb) version of the poly(I:C) were utilized based on reports of differences in activation efficacy determined by molecular weight [16]. For the placenta and amnion, IL-8 production was elevated significantly upon stimulation with both LMW and HMW Poly(I:C), whereas IL-6 was only elevated significantly upon stimulation with LWM Poly(I:C). For the choriodecidua, HMW and LMW Poly(I:C) resulted in a significant increase of both IL-6 and IL-8 (Fig. 2). HMW poly(I:C) gave a greater cytokine

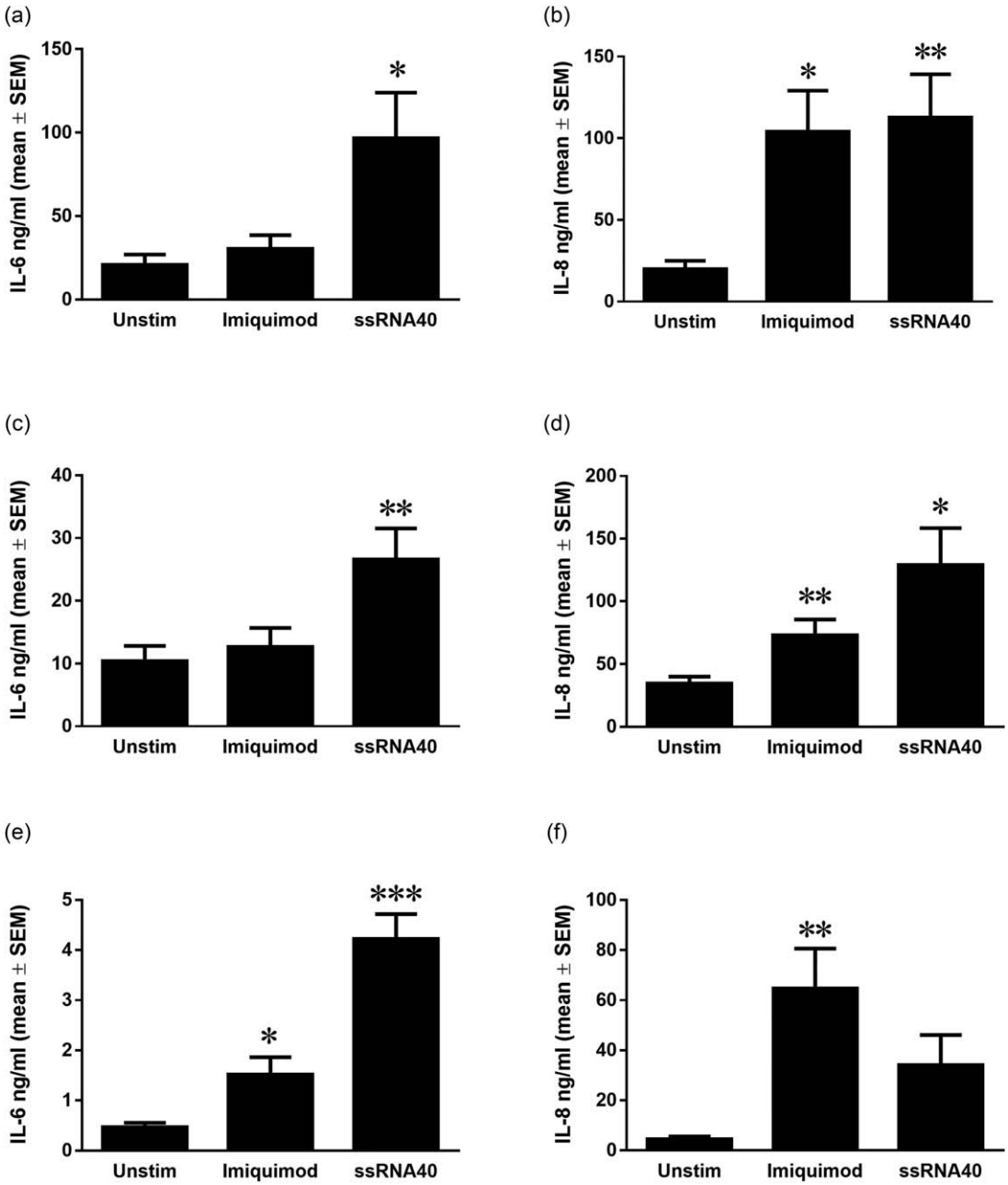

Fig. 3. Toll-like receptors (TLR)-7 and TLR-8 agonist-induced cytokine response by the term non-laboured placenta, choriodecidua and amnion. Interleukin (IL)-6 and IL-8 production (ng/ml ± standard error of the mean) by the (a,b) placenta, (c,d) choriodecidua and (e,f) amnion following stimulation with imiquimod (TLR-7) and ssRNA40/LyoVec (TLR-8) (both 1 μg/ml; $n = 9$). Statistically significant differences compared to unstimulated control are shown: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

response than LMW poly(I:C), which was significant in all cases except for IL-6 production in the amnion.

TLR-7 and TLR-8. TLR-7 and TLR-8 functionality was studied using imiquimod (R837) and ssRNA40/LyoVec, each at 1 μg/ml (Fig. 3; $n = 9$). Imiquimod is a small synthetic anti-viral molecule in the imidazoquinoline family specific to TLR-7, while ssRNA40/LyoVec is a single-stranded uridine-rich oligonucleotide derived from HIV-1 complexed with the transfection reagent LyoVec and is specific for TLR-8 [17,18]. The TLR-8 agonist ssRNA40 caused an increase in IL-6 and IL-8 in placenta, choriodecidua and amnion, which was significant in all cases except for IL-8 in the amnion (Fig. 3). In contrast, the TLR-7 agonist imiquimod induced a significant increase in IL-8 from all three tissues, but only from the amnion for IL-6 (Fig. 3).

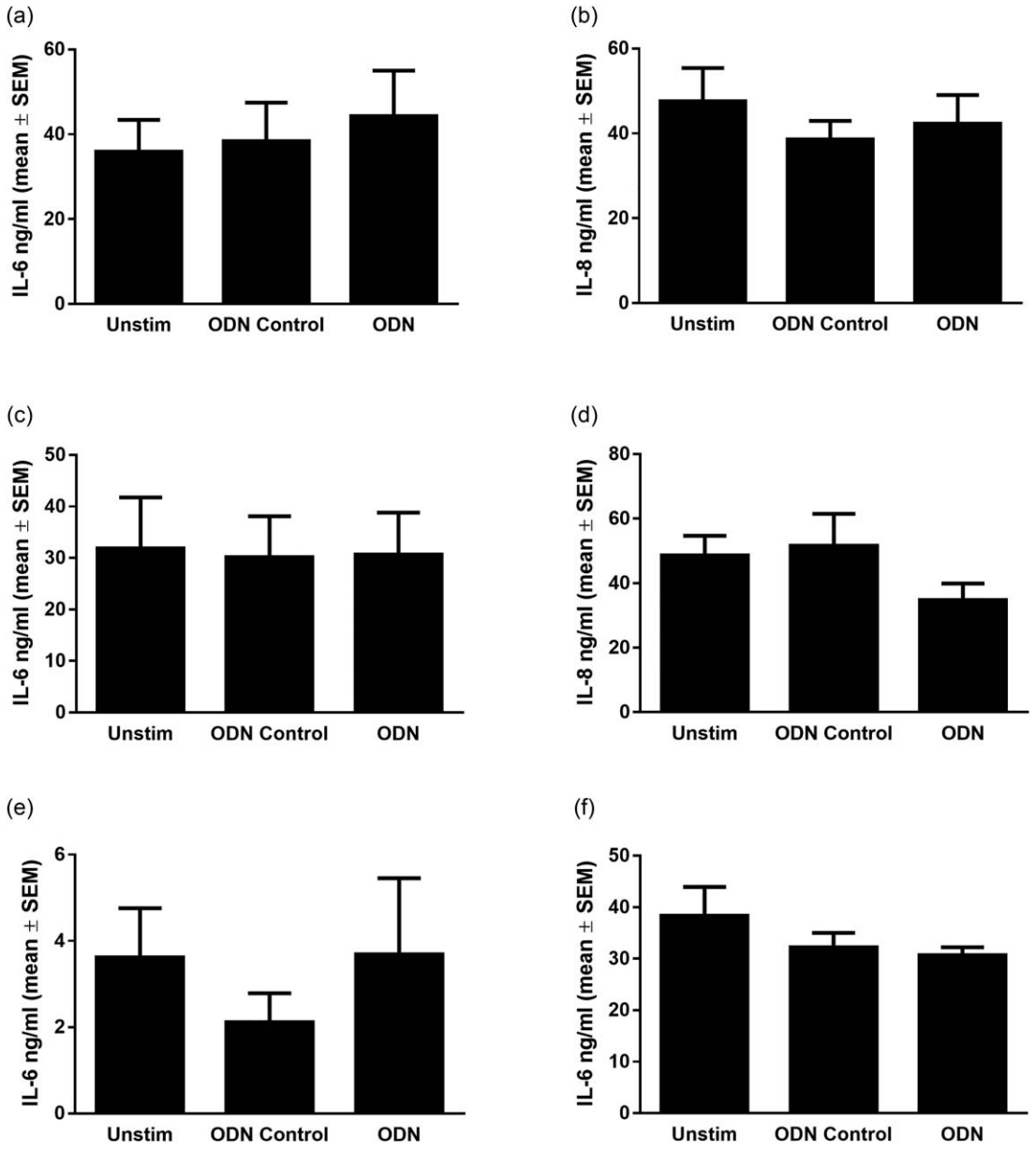

Fig. 4. Toll-like receptor (TLR)-9 agonist-induced cytokine response by the term non-laboured placenta, choriodecidua and amnion. Interleukin (IL)-6 and IL-8 production (ng/ml ± standard error of the mean) by the (a,b) placenta, (c,d) choriodecidua and (e,f) amnion following stimulation with ODN21798 control or ODN21798 (both 1 μM; $n = 7$). No statistically significant differences were observed.

TLR-9. The function of TLR-9 was investigated using a synthetic P-class oligodeoxyribonucleotide containing unmethylated cytosine–phosphate–guanosine (CpG) motifs (ODN 221798) at 1 μM (Fig. 4; $n = 7$). A sequence control was also included. There was no significant effect of ODN221798 on either cytokine in any tissue.

Localization of RIG-I, MDA5 and LGP2 in human non-laboured gestation associated tissues

Immunohistochemistry was used to determine which cells within the placenta and fetal membranes expressed RIG-I, MDA5 and LGP2 (Fig. 5). In the placenta, trophoblasts showed strong expression of all three RLRs, with expression of MDA5 and LGP2 also in the villous stroma. In the fetal membranes, expression of RIG-I, MDA5 and LGP2 was observed on chorionic trophoblasts, decidual cells and amnion epithelial cells.

Functional response of term non-laboured gestation associated tissue to a RIG-I/MDA5 agonist

RIG-I/MDA5 functionality was determined using Poly(I:C)/LyoVec, a complex between the transfection

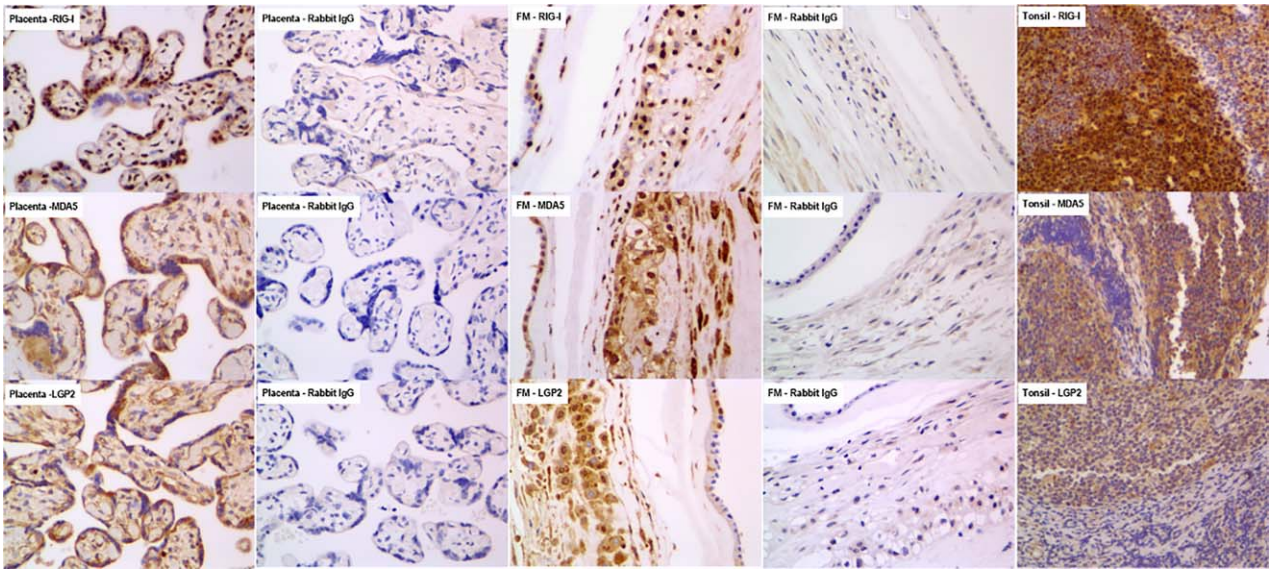

Fig. 5. Immunolocalization of RIG-I-like receptors (RLRs) in human gestation associated tissues. Negative (isotype match) and positive (all tonsil) controls are also displayed. A representative example of seven is shown. Original magnification ×40.

reagent LyoVec and HMW poly(I:C) at 1 μg/ml (Fig. 6; $n = 9$). Transfected Poly(I:C) is recognized by RIG-I/MDA5, unlike naked Poly(I:C), which is recognized by TLR-3 [19,20]. A significant increase in both IL-6 and IL-8 was observed in response to Poly(I:C)LyoVec in all three tissues.

Hierarchical clustering of the response to nucleic acid-sensing TLRs/RLRs

Heatmaps were drawn to visualize and enable a clearer comparison of the cytokine response by each of the tissues and each of the ligands (Fig. 7). Each row represents a tissue and each column represents a ligand, with light grey representing high production levels and dark grey/black low. Hierarchical clustering of the three tissues revealed that for IL-6 production the choriodecidua and amnion clustered together compared to the placenta, whereas for IL-8 production the placenta and choriodecidua clustered together compared to the amnion. When examining the response of the tissues to treatment with nucleic acid PAMPs, two primary clusters for each cytokine were evident. For IL-6, the response of TLR-3 activation by LMW poly(I:C) clustered with TLR-8, independently of the TLR-3/HMW poly(I:C), TLR-7 and RIG-I/MDA5. For IL-8, the response to TLR-7/8 activation clustered independently of TLR-3 (both LMW and HMW Poly(I:C)) and RIG-I/MDA5.

Discussion

This study reports for the first time, to our knowledge, expression and function of RLRs by the placenta, choriodecidua and amnion. Additionally, we note distinct differences in TLR-3, TLR-7 and TLR-8 function in these tissues compared to previous studies relating to the different agonists used. Furthermore, we provide a novel comparison of the cytokine producing capacity of the placenta, choriodecidua and amnion in response to nucleic acids.

Protein expression of TLRs-3, −7, −8 and −9 was observed within placental trophoblast, amnion epithelium, chorionic trophoblasts and the decidua. There was variability in expression: most notably, TLR-9 showed lowest levels of staining and TLR-7 expression was negligible within the decidua. Immunoreactivity of TLR-3, −7 and −8 has been reported previously in placental syncytiotrophoblasts and cytotrophoblasts of both normotensive and pre-eclampsia placentas [21]. Our findings confirm this and extend this observation to the fetal membranes, where immunoreactivity was also found. Broadly, patterns of immunoreactivity corresponded to the functional output, apart from TLR-9, where no functional output was observed.

While a functional response to the TLR-3 ligand poly(I:C) has been observed in both placental and fetal membrane explants, the efficiency of TLR-3 activation has been reported to be influenced by the size of the dsRNA used [16,22]. Here we note TLR-3 activation by LMW poly(I:C) induced a higher cytokine response than HMW poly(I:C), due possibly to more efficient penetration into the cell of smaller RNAs. This highlights the importance of considering the size of poly(I:C) used when examining TLR-3 function in gestational tissues, possibly offering an explanation for why no IL-8 production was observed in fetal membrane explants following poly(I:C) treatment [6].

While TLR-7 and TLR-8 are related phylogenetically and both recognize ssRNAs, the cytokine response resulting from activation of TLR-8 by both the placenta and fetal

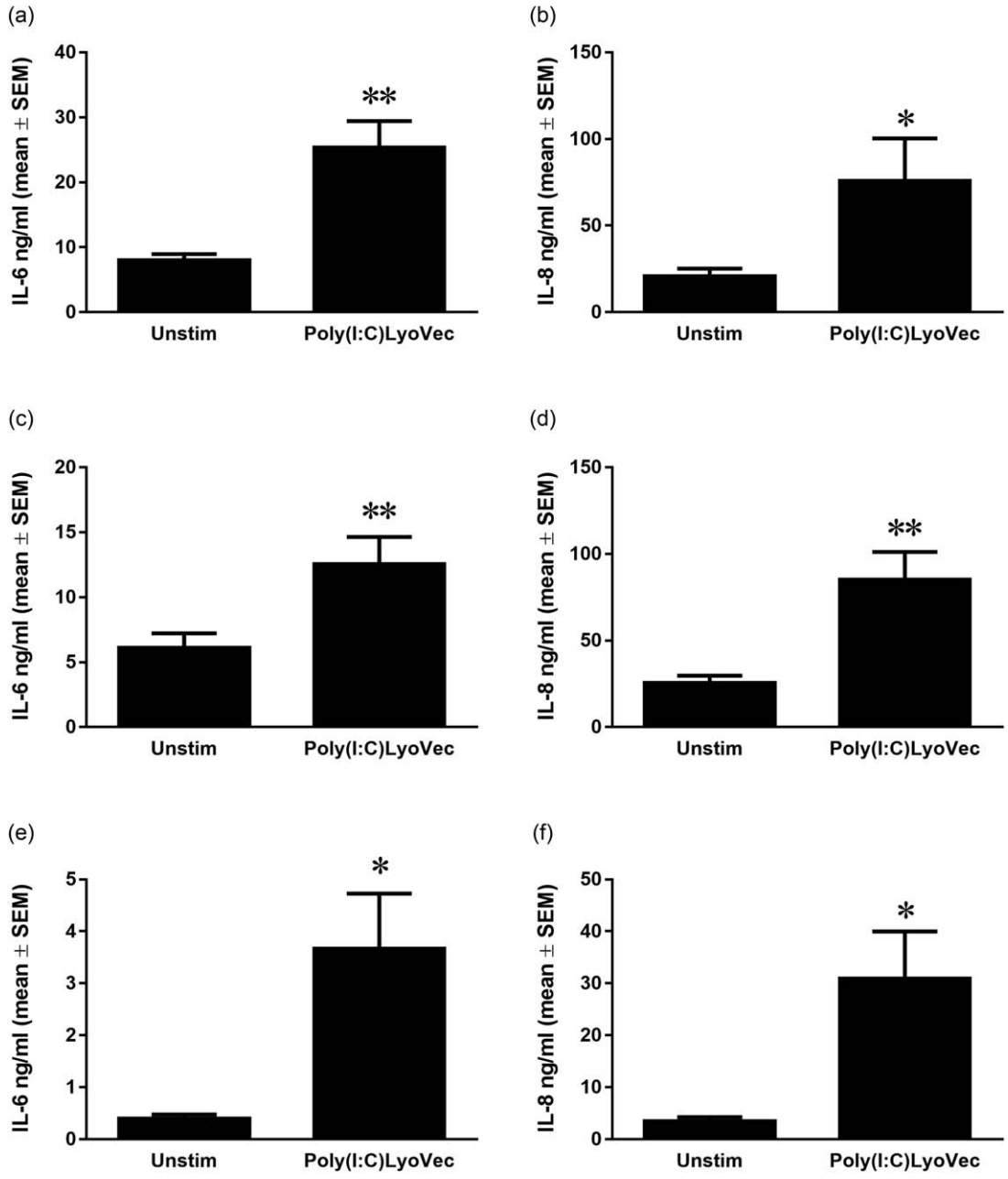

Fig. 6. Retinoic acid inducible gene I/melanoma differentiation-associated protein 5 (RIG-1/MDA-5) agonist-induced cytokine response by the term non-laboured placenta, choriodecidua and amnion. Interleukin (IL)-6 and IL-8 production (ng/ml ± standard error of the mean) by the (a,b) placenta, (c,d) choriodecidua and (e,f) amnion in response to polyinosine-polycytidylic acid [Poly(I:C)]LyoVec (1 μg/ml; $n = 9$). Statistically significant differences compared to unstimulated control are shown: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

membranes is more robust than that of TLR-7, apart from IL-8 production by the amnion. This might be related to the greater expression of TLR-8 than TLR7, as revealed by our immunohistochemical analysis. Previous functional investigations of TLR-7 and TLR-8 in gestational tissues have typically utilized dual synthetic agonists or ssRNAs [6,12], noting both IL-6 and IL-8 production by the placenta [12] and IL-8 production by the fetal membranes [6] in response to treatment. These discrepancies probably reflect the model of investigation used, i.e. total fetal membrane explants [6] *versus* explants of choriodecidua and amnion as here. Tissue processing for *ex-vivo* investigations can impact upon cytokine measurements with punch biopsies of amnion or choriodecidua, as used here, typically making greater amounts of cytokines than dual compartment Transwells [23]. Furthermore, the activation of TLR-7 and TLR-8 by synthetic agonists results in differences in target cell selectivity and cytokine profile [24].

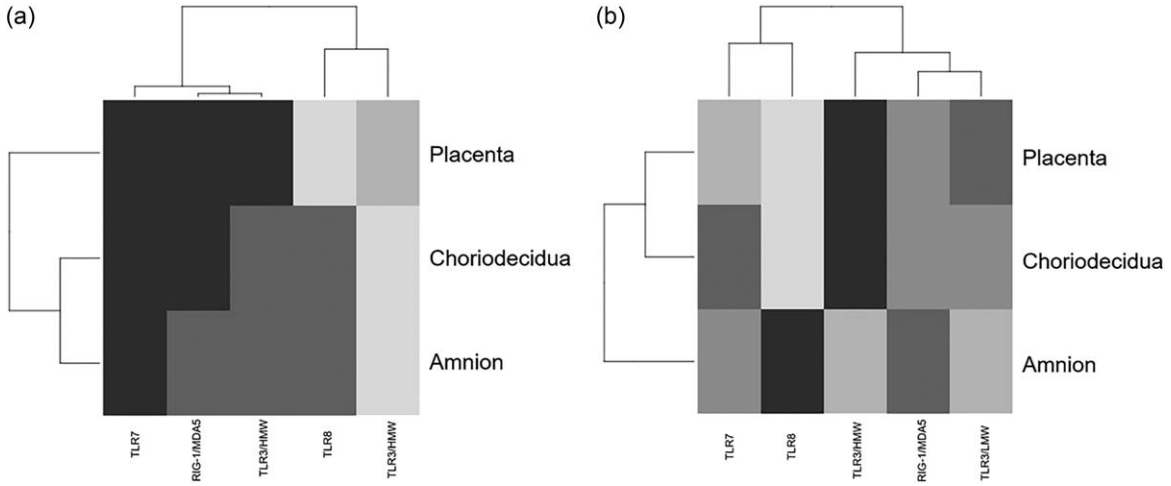

Fig. 7. Comparison of nucleic acid-induced cytokine production by the placenta, choriodecidua and amnion. Heatmap generated using the mean levels of (a) interleukin (IL)-6 and (b) IL-8 production as measured by enzyme-linked immunosorbent assay (ELISA); the production level was standardized by correction for background levels of cytokine from unstimulated tissues. Greyscale is used, with white signifying highest production and black lowest. Hierarchical clustering was performed on both rows (by tissue) and columns (by receptor).

We have reported previously that the human term placenta does not elicit either proinflammatory [IL-6, IL-8 and tumour necrosis factor (TNF)-α] or anti-inflammatory (IL-10) cytokines in response to an A-class CpG ODN [12]. However, several classes (A, B, C and P) of CpG ODN are available, with C-class combining features of A-class and B-class, while P-class activates at a higher efficiency than C-class [25,26]. Therefore, we chose to use a P-class ODN for this study. Again, we report no functional response by any tissues to P-class CpG ODN, despite TLR-9 immunoreactivity within placental trophoblasts and, more weakly, in various cells of the fetal membranes. To the best of our knowledge, this is the first report of TLR-9 protein localization in the placenta and fetal membranes. Limited expression of TLR-9 might offer an explanation as to why no functional response to CpG was observed corresponding to observations of expression *versus* function in monocytes and natural killer cells [27]. However, measurement of a wider array of cytokines/chemokines could reveal TLR-9 functionality. Increased MCP-1 and decreased granulocyte colony-stimulating factor (G-CSF), interferon (IFN)-γ, macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated upon activation, normal T cell expressed and secreted (RANTES) and vascular endothelial growth factor (VEGF) in response to class-A CpG ODN by human fetal membrane explants has been reported [28]. However, it is likely that TLR-9 activity at the materno–fetal interface is regulated tightly rather than non-functional to minimize placental inflammation from endogenous signals. Hypomethylated fetal DNA, found in the placenta and maternal circulation with circulating levels increased in pre-eclampsia [29], can activate TLR-9 signalling in human peripheral blood mononuclear cells (PBMCs) *in vitro* with elevated IL-6 production [30]. Placental-derived mitochondrial DNA is associated with TLR-9 activation and vascular dysfunction in pre-eclampsia [31] and TLR-9 expression is elevated on circulating plasmacytoid dendritic cells (pDCs) of pre-eclamptic *versus* healthy pregnant women, and is accompanied by a lesser cytokine output in response to TLR-9 activation [32].

The cytoplasmic RNA helicases of the RLR family play a major role in host anti-viral defence. They detect viral RNA ligands in the cytoplasm, triggering activation of transcription factors and leading to production of type I IFNs and expression of other anti-viral genes [33]. Our knowledge of RLR expression and function in human gestation associates tissues is relatively limited. Here we demonstrate, for the first time, immunoreactivity for RIG-I, MDA5 and LGP2 in the placenta and fetal membranes. Both RIG-I and MDA5 were localized to the placental trophoblasts, chorionic trophoblasts, decidual stromal cells and amnion epithelial cells. A corresponding functional output was observed with the dual agonist for these receptors. Further work is required to determine the relative contribution of RIG-I and MDA5 to this response. Recognition of dsRNAs by RIG-I and MDA5 is reported to be length-dependent [34]. As RIG-I selectively recognizes short dsRNA and MDA5 long, given that HMW dsRNA was used in our study the response is most probably MDA5-dependent, but further work is required to confirm this. LGP2 shared its expression pattern with RIG-I and MDA5. This is not surprising, given that LGP2 is a negative regulator of RLR signalling [7]. This negative feedback takes place on many levels, including competition for dsRNA, interaction with the adaptor molecule ISP-1, or direct binding of RIG-I [35]. LGP2 regulatory function of RLR within gestation-associated tissues remains to be determined.

Our investigation of the responsiveness of gestation-associated tissues to nucleic acid PRRs, namely TLR-3, −7, −8 and −9, and RIG-I and MDA5 provides further insight into inflammatory responses at the materno–fetal interface. Protein expression of these PRRs and LGP2 was a broad feature of human term gestation-associated tissues, yet there were subtle differences in functional responses. Hierarchical clustering enabled elucidation of these key differences between tissues and treatments to be highlighted. This approach revealed that the placenta and choriodecidua respond to nucleic acid ligands more similarly than the amnion regarding the production of IL-8. IL-8 is a key chemokine in the recruitment and activation of neutrophils [36], which have a role in anti-viral immunity [37]. Neutrophil infiltration of both the placenta and decidua occurs during bacterial infection [38,39], and this might also be the case during viral infection. In contrast, hierarchical clustering revealed that with regard to IL-6 production, the choriodecidua and amnion share a greater functional similarity.

The clustering approach utilized here has highlighted some key differences between tissues in relation to their responsiveness to nucleic acids PAMPs, but this has limitations. This is a study of heterogeneous tissue explants, so the relative numbers of responsive cells in each of the tissues will differ. This is evident by the constitutive production of both cytokines by each tissue, and we have corrected all comparisons for this. However, normalization of cytokine production to total protein content prior to baseline corrections could form the basis of future investigations. Furthermore, we have applied this approach only to the production of IL-6 and IL-8, based on the expectation that these would most probably yield responses to viral ligands [40,41] and have been implicated in anti-viral defence [42]. While these are key cytokines in the physiology and pathophysiology of labour, other cytokines including, IL-1β, IL-10 and TNF-α, have also been implicated [2]. Therefore, an extensive examination of other pro- and anti-inflammatory cytokines, perhaps after multiplex analysis, would provide a more detailed insight into the inflammatory response of gestational tissues.

While the focus of this study has been upon TLRs and RLRs, other nucleic acid sensing innate immune receptors have been described; namely, the cytosolic DNA sensors (CDS), known alternatively as the absent in melanoma 2 (AIM2)-like receptors (ALRs) [43]. Several CDS have been described, including DNA-dependent activator of IFN-regulatory factors (DAI), leucine-rich repeat Fli-I interacting protein (LRRFIPI), IFN-γ-inducible protein-16 (IFI16), DEAD-box helicase 41 (DDX41), cyclic-GMP-AMP synthase (cGAS) and AIM2. To date, little is known about either the expression or function of these cytosolic DNA sensors in gestational tissues apart from IFI16, which is associated with non-necroptotic programmed cell death of human trophoblasts following dsDNA exposure [44]. Furthermore, placenta expression of IFI16 is elevated significantly in women with pre-eclampsia and this can be mimicked *in vitro* by treating placental trophoblasts with poly(dA:dT) [45]. This implicates IFI16 and possibly other CDS and the DNA sensing receptors studied herein in pre-eclampsia, and highlights the importance of understanding the potential dysfunction of these receptors and their signalling pathways in pregnancy.

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Dislosure

None to declare.

References

1 Keelan JA, Blumenstein M, Helliwell RJ, Sato TA, Marvin KW, Mitchell MD. Cytokines, prostaglandins and parturition – a review. Placenta 2003; **24** (Suppl. A):S33–46.
2 Hamilton S, Oomomian Y, Stephen G *et al.* Macrophages infiltrate the human and rat decidua during term and preterm labor: evidence that decidual inflammation precedes labor. Biol Reprod 2012; **86**:39.
3 Nadeau HCG, Subramaniam A, Andrews WW. Infection and preterm birth. Semin Fetal Neonatal Med 2016; **21**:100–5.
4 Haun L, Kwan N, Hollier LM. Viral infections in pregnancy. Minerva Ginecol 2007; **59**:159–74.
5 Cardenas I, Means RE, Aldo P *et al.* Viral infection of the placenta leads to fetal inflammation and sensitization to bacterial products predisposing to preterm labor. J Immunol 2010; **185**: 1248–57.
6 Bakaysa SL, Potter JA, Hoang M *et al.* Single- and double-stranded viral RNA generate distinct cytokine and antiviral responses in human fetal membranes. Mol Hum Reprod 2014; **20**:701–8.
7 Loo YM, Gale M Jr. Immune signaling by RIG-I-like receptors. Immunity 2011; **34**:680–92.
8 Koyama S, Ishii KJ, Coban C, Akira S. Innate immune response to viral infection. Cytokine 2008; **43**:336–41.

9 Heil F, Hemmi H, Hochrein H *et al.* Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004; **303**:1526–9.

10 Guillot L, Le Goffic R, Bloch S *et al.* Involvement of Toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. J Biol Chem 2005; **280**:5571–80.

11 Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004; **5**:987–95.

12 Patni S, Wynen LP, Seager AL, Morgan G, White JO, Thornton CA. Expression and activity of Toll-like receptors 1–9 in the human term placenta and changes associated with labor at term. Biol Reprod 2009; **80**:243–8.

13 Martínez-Varea A, Pellicer B, Serra V *et al.* The maternal cytokine and chemokine profile of naturally conceived gestations is mainly preserved during in vitro fertilization and egg donation pregnancies. J Immunol Res 2015; **2015**:128616.

14 R Development Core Team. r: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2011.

15 Warnes GR, Bolker B, Bonebakker L *et al.* gplots: Various R Programming tools for Plotting Data. R package version 2009; 2. Vienna, Austria: R Foundation for Statistical Computing; 2009.

16 Zhou Y, Guo M, Wang X *et al.* TLR3 activation efficiency by high or low molecular mass poly I:C. Innate Immun 2013; **19**:184–92.

17 Ma F, Zhang J, Zhang J, Zhang C. The TLR7 agonists imiquimod and gardiquimod improve DC-based immunotherapy for melanoma in mice. Cell Mol Immunol 2010; **7**:381–8.

18 Alter G, Suscovich TJ, Teigen N *et al.* Single-stranded RNA derived from HIV-1 serves as a potent activator of NK cells. J Immunol 2007; **178**:7658–66.

19 Kato H, Sato S, Yoneyama M *et al.* Cell type-specific involvement of RIG-I in antiviral response. Immunity 2005; **23**:19–28.

20 Gitlin L, Barchet W, Gilfillan S *et al.* Essential role of MDA-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc Natl Acad Sci USA 2006; **103**:8459–64.

21 Chatterjee P, Weaver LE, Doersch KM *et al.* Placental Toll-like receptor 3 and Toll-like receptor 7/8 activation contributes to preeclampsia in humans and mice. PLOS ONE 2012; **7**:e41884.

22 Jiang M, Osterlund P, Sarin LP *et al.* Innate immune responses in human monocyte-derived dendritic cells are highly dependent on the size and the 5' phosphorylation of RNA molecules. J Immunol 2011; **187**:1713–21.

23 Miller MF, Loch-Caruso R. Comparison of LPS-stimulated release of cytokines in punch versus Transwell tissue culture systems of human gestational membranes. Reprod Biol Endocrinol 2010; **8**:121.

24 Gorden KB, Gorski KS, Gibson SJ *et al.* Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. J Immunol 2005; **174**:1259–68.

25 Vollmer J, Weeratna R, Payette P *et al.* Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. Eur J Immunol 2004; **34**:251–62.

26 Samulowitz U, Weber M, Weeratna R *et al.* A novel class of immune-stimulatory CpG oligodeoxynucleotides unifies high potency in type I interferon induction with preferred structural properties. Oligonucleotides 2010; **20**:93–101.

27 Hornung V, Rothenfusser S, Britsch S *et al.* Quantitative expression of Toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 2002; **168**:4531–7.

28 Hoang M, Potter JA, Gysler SM *et al.* Human fetal membranes generate distinct cytokine profiles in response to bacterial Toll-like receptor and nod-like receptor agonists. Biol Reprod 2014; **90**:39.

29 Martin A, Krishna I, Badell M, Samuel A. Can the quantity of cell-free fetal DNA predict preeclampsia: a systematic review. Prenat Diagn 2014; **34**:685–91.

30 Scharfe-Nugent A, Corr SC, Carpenter SB *et al.* TLR9 provokes inflammation in response to fetal DNA: mechanism for fetal loss in preterm birth and preeclampsia. J Immunol 2012; **188**:5706–12.

31 Goulopoulou S, Matsumoto T, Bomfim GF, Webb RC. Toll-like receptor 9 activation: a novel mechanism linking placenta-derived mitochondrial DNA and vascular dysfunction in pre-eclampsia. Clin Sci (Lond) 2012; **123**:429–35.

32 Panda B, Panda A, Ueda I *et al.* Dendritic cells in the circulation of women with preeclampsia demonstrate a pro-inflammatory bias secondary to dysregulation of TLR receptors. J Reprod Immunol 2012; **94**:210–5.

33 Yoneyama M, Fujita T. Function of RIG-I-like receptors in antiviral innate immunity. J Biol Chem 2007; **282**:15315–8.

34 Kato H, Takeuchi O, Mikamo-Satoh E *et al.* Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation–associated gene 5. J Exp Med 2008; **205**:1601–10.

35 Saito T, Hirai R, Loo YM *et al.* Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. Proc Natl Acad Sci USA 2007; **104**:582–7.

36 Hammond ME, Lapointe GR, Feucht PH *et al.* IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors. J Immunol 1995; **155**:1428–33.

37 Drescher B, Bai F. Neutrophil in viral infections, friend or foe? Virus Res 2013; **171**:1–7.

38 Rinaldi SF, Catalano RD, Wade J, Rossi AG, Norman JE. Decidual neutrophil infiltration is not required for preterm birth in a mouse model of infection-induced preterm labor. J Immunol 2014; **192**:2315–25.

39 Giaglis S, Stoikou M, Grimolizzi F *et al.* Neutrophil migration into the placenta: good, bad or deadly? Cell Adh Migr 2016; **10**:208–25.

40 Kumar A, Zhang J, Yu F-SX. Toll-like receptor 3 agonist poly(I:C)-induced antiviral response in human corneal epithelial cells. Immunology 2006; **117**:11–21.

41 Vanden Bush TJ, Bishop GA. TLR7 and CD40 cooperate in IL-6 production via enhanced JNK and AP-1 activation. Eur J Immunol 2008; **38**:400–9.

42 Mogensen TH, Paludan SR. Molecular pathways in virus-induced cytokine production. Microbiol Mol Biol Rev 2001; **65**: 131–50.

43 Shaw N, Liu Z-J. Role of the HIN domain in regulation of innate immune responses. Mol Cell Biol 2014; **34**:2–15.

44 Chu X, Chen W, Li N *et al.* Cytosolic double-stranded DNA induces nonnecroptotic programmed cell death in trophoblasts via IFI16. J Infect Dis 2014; **210**:1476–86.

45 Li N, Fu Y, Chen W *et al.* IFI16 mediates soluble Flt-1 and endoglin production by trophoblast cells. J Hypertens 2015; **33**: 1658–65.