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

Short Title: Viral TLRs and RLRs at the materno-fetal interface

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Key words

Inflammation; pattern recognition receptors; viral; hierarchical clustering, reproductive immunology

Abbreviations:

ECS, elective caesarean section; HMW, high molecular weight; IL-, interleukin; LMW, low molecular weight; PAMPs, pathogen associated molecular patterns; PRR, pattern recognition receptor; PTB, preterm birth; RIG-I, retinoic acid inducible gene I; RLR, RIG-I-like receptor; TLR, toll-like receptor.

Abstract

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 (TLR3, 7, 8 and 9), and retinoic acid-inducible gene 1 (RIG-I)-like receptors (RIG-I, MDA5 and 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 TLR3 (Poly(I:C); low and high molecular weight), TLR7 (Imiquimod), TLR8 (ssRNA40) and RIG-I/MDA5 (Poly(I:C)LyoVec) was observed; there was no response to a TLR9 (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 TLR3, TLR7, TLR8 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.

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 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 recognise 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)-1-like receptors (RLRs) RIG-I, MDA5 and LGP2 [7-10]. These receptors are located intracellularly where they are best positioned to encounter and respond to viruses [11]. Better 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 TLR3, TLR7 and TLR7/8 but not TLR9 agonists [12]. Therefore, we investigated the expression and activity of nucleic acid ligand responsive TLRs (TLR3, 7, 8 and 9) and RLRs (RIG-I, MDA5 and LGP2) in human term placenta, choriodecidua and amnion using an explant model to better mimic the cellular heterogeneity that occurs *in utero*. A hierarchical clustering approach, as previously described for cytokine data [13], was implemented to better compare the response between

different tissues and ligands. It is our hypothesis nucleic acid ligands will induce an inflammatory response in gestation associated tissues and that utilisation 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 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, UK). Tissue was washed repeatedly with PBS to remove contaminating blood. Tissue was then minced into smaller pieces and washed further. Pieces of tissue (1mm^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, Germany) containing 1ml UltraCULTURE™ medium (Lonza, Switzerland), supplemented with 2 mM GlutaMAX™ (Life Technologies, UK) and 2 mM penicillin, streptomycin, fungisone (PSF; Life Technologies, UK).

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 (PBS; Life Technologies) to remove blood. Explants were cut with an 8-mm biopsy punch (Stiefel): two discs of choriodecidua placed into 0.5ml Advanced RPMI supplemented with 2mM Glutamax, 2mM Penicillin streptomycin fungizone (PSF), 5mM 2-mercaptoethanol (2-ME; all Life Technologies) and 2% fetal bovine serum (FBS; Hyclone) and 5mM 2-mercaptoethanol; and four discs of

amnion placed into 0.5 ml Advanced DMEM (Life Technologies) supplemented with 2mM Glutamax, 2mM 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 (TLR3, 25 μg/ml), Poly(I:C)HMW (TLR3, 25 μg/ml), Imiquimod (TLR7, 1 μg/ml) ssRNA40 (TLR8, 1 μg/ml), Poly(I:C)LyoVec (RIG-I/MDA5, 1 μg/ml) (all from Invivogen, USA), ODN21798 control or ODN21798 (TLR9, 1 μM; both Miltenyi Biotec, UK). All treatments were performed in duplicate. Cultures were incubated for 24 hours at 37°C in 5% CO_2. Tissue free supernatants were collected by centrifugation for 7 minutes at 4°C, 515 x g and stored at -20°C for analysis using cytokine specific 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) as per 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. The Optiview detection system was used without A/B blocker or amplification (except for anti-TLR9). Antigen retrieval was carried out in CC132 buffer for TLR3, RIG-I and MDA5 and CC124 buffer for TLR7, TLR8 and LGP2. Primary antibodies, rabbit polyclonal anti-TLR3 (5 μg/ml for 24 minutes), rabbit polyclonal anti-TLR7 (10 μg/ml for 36 minutes), rabbit polyclonal anti-TLR8 (5 μg/ml for 32 minutes), rabbit polyclonal anti-TLR9 (10 μg/ml for 44 minutes), rabbit polyclonal anti-RIG-I (10 μg/ml for 32 minutes), rabbit polyclonal anti-MDA5 (5 μg/ml for 36 minutes), and rabbit polyclonal anti-LGP2 (5 μg/ml

for 36 minutes) (all LifeSpan BioSciences, Inc, USA) were incubated at 36°C or, for anti-TLR8, at room temperature. For control slides, primary antibody was replaced with polyclonal rabbit IgG (Biolegend) at a corresponding concentration. A tissue reported to express the receptor of interest was used for optimisation of staining and included as a positive control: tonsil (TLR3, TLR8, RIG-1, MDA5, LGP2), lung (TLR7), liver (TLR9).

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 *t*-test or repeated measures one-way ANOVA with Dunnett's multiple comparison test. A p-value of ≤ 0.05 was considered significant. Statistical significance was calculated using GraphPad Prism (Version 6, GraphPad Software Inc, 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 TLR3, TLR7, TLR8 and TLR9 in human non-laboured gestation associated tissues

Immunohistochemistry was used to determine which cells within the placenta and fetal membranes expressed TLR3, 7, 8 and 9 (Figure 1). In the placenta, expression of all four TLRs by trophoblast was a common feature with strong expression for both TLR3 and TLR8 in the trophoblast and villous stromal cells. In the fetal membranes, amnion epithelial cells, chorionic trophoblasts and decidual cells all exhibited strong expression of TLR3 and TLR8. Expression of TLR7 was primarily localised to the chorion with weak staining in the amnion Intermittent expression of TLR9 was also observed.

Functional response of term non-laboured gestation associated tissue to specific TLR3 TLR7, TLR8 and TLR9 agonists

TLR3. Functionality of TLR3, 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, 1.5-8kb) and a low molecular weight (LMW, 0.2-1kb) version of the poly(I:C) were utilised 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 (Figure 2). HMW poly(I:C) gave a greater cytokine response than LMW poly(I:C) which was significant in all cases except for IL-6 production in the amnion.

TLR7 and TLR8. TLR7 and TLR8 functionality were studied using imiquimod (R837) and ssRNA40/LyoVec each at 1 μg/ml (Figure 3; n=9). Imiquimod is small synthetic antiviral molecule in the imidazoquinoline family specific to TLR7, while ssRNA40/LyoVec, is a single stranded uridine-rich oligonucleotide derived from HIV-1 complexed with the transfection reagent LyoVec and is specific for TLR8 [17, 18]. The TLR8 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 (Figure 3). In contrast, the TLR7 agonist imiquimod induced a significant increase in IL-8 from all three tissues but only from the amnion for IL-6 (Figure 3).

TLR9. The function of TLR9 was investigated using a synthetic P-class oligodeoxyribonucleotide containing unmethylated CpG motifs (ODN 221798) at 1 μM (Figure 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 (Figure 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 reagent LyoVec and HMW poly(I:C) at 1 μg/ml (Figure 6; n =9). Transfected Poly(I:C) is recognised by RIG-I/MDA5 unlike naked Poly(I:C) which is recognised by

TLR3 [19, 20]. In all three tissues a significant increase in both IL-6 and IL-8 was observed in response to Poly(I:C)LyoVec.

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

Heatmaps were drawn to better visualise and enable a comparison of the cytokine response by each of the tissues and each of the ligands (Figure 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 TLR3 activation by LMW poly(I:C) clustered with TLR8, independent of the TLR3/HMW poly(I:C), TLR7 and RIG-I/MDA5. For IL-8, the response to TLR7/8 activation clustered independently of TLR3 (both LMW and HMW Poly(I:C)) and RIG-I/MDA5.

Discussion

This study reports for the first-time expression and function of RLRs by the placenta, choriodecidua and amnion. Additionally, we note distinct differences in TLR3, TLR7 and TLR8 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 TLR9 showed lowest levels of staining and TLR7 expression was negligible within the decidua. Immunoreactivity of TLR3, 7 and 8 has been reported previously in placental syncytiotrophoblasts and cytotrophoblasts of both normotensive and preeclampsia 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 TLR9 where no functional output was observed.

While a functional response to the TLR3 ligand poly(I:C) has been observed in both placental and fetal membrane explants, the efficiency of TLR3 activation has been reported to be influenced by the size of the dsRNA used [16, 22]. Here we note TLR3 activation by LMW poly(I:C) induced a higher cytokine response than HMW poly(I:C) possibly due to better penetration into the cell of smaller RNAs. This highlights the importance of considering the size of poly(I:C) used when examining TLR3 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 TLR7 and TLR8 are phylogenetically related and both recognise ssRNAs, the cytokine response resulting from activation of TLR8 by both the placenta and fetal membranes is more robust than that of TLR7 apart from IL-8 production by the amnion. This

might be related to the greater expression of TLR8 than TLR7 revealed by our immunohistochemical analysis. Previous functional investigations of TLR7 and TLR8 in gestational tissues have typically utilised 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 likely 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 on 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 TLR7 and TLR8 by synthetic agonists results in differences in target cell selectivity and cytokine profile [24].

We have previously reported that the human term placenta does not elicit either pro-inflammatory (IL-6, IL-8 and TNFα) 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 TLR9 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 TLR9 protein localisation in the placenta and fetal membranes. Limited expression of TLR9 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 TLR9 functionality. Increased MCP-1 and decreased G-CSF, IFNγ, MIP-1α, MIP-1β, RANTES and VEGF in response to Class-A CpG ODN by human fetal membrane explants has been reported [28]. However, it is likely that TLR9 activity at the materno-fetal interface is tightly regulated rather than non-functional to minimise placental inflammation from endogenous signals.

Hypomethylated fetal DNA, found in the placenta and maternal circulation with circulating levels increased in preeclampsia [29], can activate TLR9 signalling in human peripheral blood mononuclear cells (PBMCs) *in vitro* with elevated IL-6 production [30]. Placental derived mitochondrial DNA is associated with TLR9 activation and vascular dysfunction in preeclampsia [31] and TLR9 expression is elevated on circulating plasmacytoid dendritic cells (pDCs) of preeclamptic versus healthy pregnant women and is accompanied by a lesser cytokine output in response to TLR9 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 localised 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 recognises short dsRNA and MDA5 long, given HMW dsRNA was used in our study the response is most likely 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 TLR3, 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 in the regards to IL-6 production, the choriodecidua and amnion share a greater functional similarity.

The clustering approach utilised 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, normalisation of cytokine production to total protein content prior to baseline corrections would form the basis of future investigations. Furthermore, we have only applied this approach to the production of IL-6 and IL-8, based on the expectation that these would most likely 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 TNFα have also been implicated (2). Therefore, an extensive examination of other pro- and anti-inflammatory cytokine perhaps after multiplex analysis would provide a more detailed insight into the inflammatory response of gestational tissues.

While the focuses of this study has been TLRs and RLRs, other nucleic acid sensing innate immune receptors have been described, namely the cytosolic DNA sensors (CDS), alternatively known as the absent in melanoma 2 (AIM2)-like receptors (ALRs) [43]. Several

CDS have been described including DAI, LRRFIPI, IFI16, DDX41, 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 significantly elevated in women with preeclampsia 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 preeclampsia and highlights the importance of understanding the potential dysfunction of these receptors and their signalling pathways in pregnancy.

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Conflict of interests

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, Shynlova O, Tower CL, Garrod A, 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. Seminars in Fetal and Neonatal Medicine 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, Koga K, Lang SM, Booth C, 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, Han CS, Guller S, Norwitz ER, 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, Ampenberger F, Kirschning C, Akira S, 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, Escriou N, Akira S, Chignard M, 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, Hervás-Marín D, Martínez-Romero A, Bellver J, et al. The Maternal Cytokine and Chemokine Profile of Naturally Conceived Gestations Is Mainly Preserved during In Vitro Fertilization and Egg Donation Pregnancies. Journal of Immunology Research 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, Gentleman R, Huber W, Liaw A, et al. gplots: Various R programming tools for plotting data. R package version 2009; 2.
16. Zhou Y, Guo M, Wang X, Li J, Wang Y, Ye L, et al. TLR3 activation efficiency by high or low molecular mass poly I:C. Innate immunity 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, Meier A, Streeck H, Brander C, 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, Yamamoto M, Uematsu S, Matsui K, et al. Cell type-specific involvement of RIG-I in antiviral response. Immunity 2005; 23:19-28.
20. Gitlin L, Barchet W, Gilfillan S, Cella M, Beutler B, Flavell RA, et al. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc Natl Acad Sci U S A 2006; 103:8459-64.
21. Chatterjee P, Weaver LE, Doersch KM, Kopriva SE, Chiasson VL, Allen SJ, 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, Poranen MM, Bamford DH, Guo D, 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, Kedl RM, Kieper WC, Qiu X, 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, Jurk M, Schetter C, Laucht M, 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, Uhlmann E, Noll B, Krieg AM, 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, Krug A, Jahrsdörfer B, Giese T, 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. The Journal of Immunology 2002; 168:4531-7.
28. Hoang M, Potter JA, Gysler SM, Han CS, Guller S, Norwitz ER, 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, Keogh L, Doyle B, Martin C, 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, Abrahams VM, Norwitz ER, Stanic AK, et al. Dendritic cells in the circulation of women with preeclampsia demonstrate a pro-inflammatory bias secondary to dysregulation of TLR receptors. Journal of Reproductive Immunology 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, Hirai R, Kawai T, Matsushita K, et al. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid–

inducible gene-I and melanoma differentiation–associated gene 5. The Journal of Experimental Medicine 2008; 205:1601-10.
35. Saito T, Hirai R, Loo YM, Owen D, Johnson CL, Sinha SC, et al. Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. Proc Natl Acad Sci U S A 2007; 104:582-7.
36. Hammond ME, Lapointe GR, Feucht PH, Hilt S, Gallegos CA, Gordon CA, 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 research 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. The Journal of Immunology Author Choice 2014; 192:2315-25.
39. Giaglis S, Stoikou M, Grimolizzi F, Subramanian BY, van Breda SV, Hoesli I, 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. European journal of immunology 2008; 38:400-9.
42. Mogensen TH, Paludan SR. Molecular Pathways in Virus-Induced Cytokine Production. Microbiology and Molecular Biology Reviews 2001; 65:131-50.
43. Shaw N, Liu Z-J. Role of the HIN Domain in Regulation of Innate Immune Responses. Molecular and Cellular Biology 2014; 34:2-15.
44. Chu X, Chen W, Li N, Hu XZ, Du CT, Yu SX, 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, Hu GQ, Zhou M, Yu SX, et al. IFI16 mediates soluble Flt-1 and endoglin production by trophoblast cells. J Hypertens 2015; 33:1658-65.

Figure 1. Immunolocalisation of nucleic acid sensing TLRs in human gestation associated tissues. Negative (isotype match) and positive (TLR3 & TLR8 – tonsil; TLR7 – lung; TLR9 - liver) controls are also shown. A representative example of 7 is shown. Original magnification x40.

Figure 2. TLR3 agonist induced cytokine response by the term non-laboured placenta, choriodecidua and amnion. IL-6 and IL-8 production (ng/ml ± SEM) by the (A-B) placenta, (C-D) choriodecidua (E-F) amnion following stimulation with Poly(I:C)LMW and Poly(I:C)HMW (both 25 μg/ml; n=9). Statistically significant differences compared to unstimulated control are shown: * $p< 0.05$, ** $p<0.01$.

Figure 3. TLR7 and TLR8 agonist induced cytokine response by the term non-laboured placenta, choriodecidua and amnion. IL-6 and IL-8 production (ng/ml ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion following stimulation with imiquimod (TLR7) and ssRNA40/LyoVec (TLR8) (both 1 μg/ml; n=9). Statistically significant differences compared to unstimulated control are shown: * $p< 0.05$, ** $p<0.01$, *** $p< 0.001$.

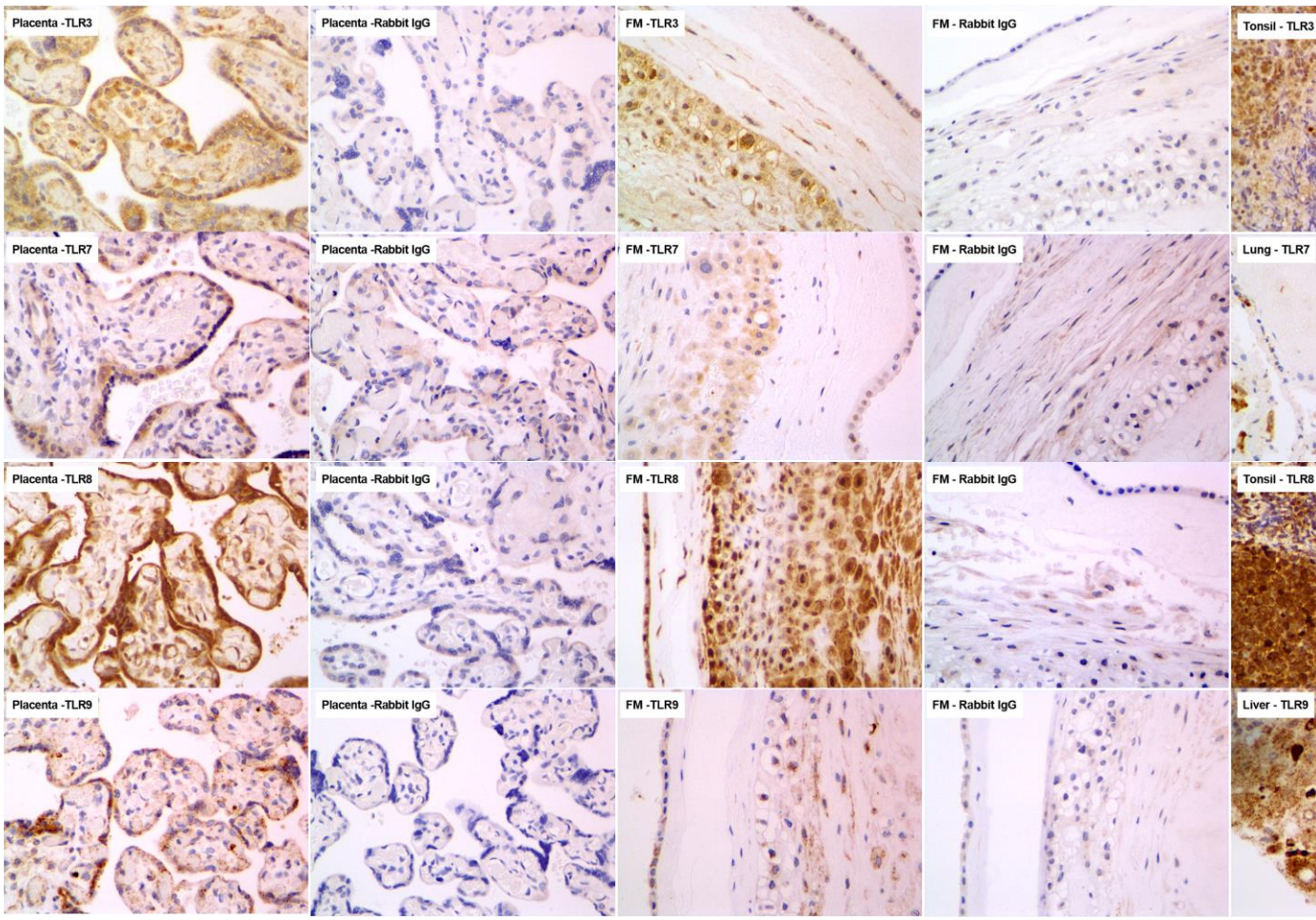
Figure 4. TLR9 agonist induced cytokine response by the term non-laboured placenta, choriodecidua and amnion. IL-6 and IL-8 production (ng/ml ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion following stimulation with ODN21798 control or ODN21798 (both 1 μM; n=7). No statistically significant differences were observed.

Figure 5. Immunolocalisation of RLRs in human gestation associated tissues. Negative (isotype match) and positive (all tonsil) controls are also displayed. A representative example of 7 is shown. Original magnification x40.

Figure 6. RIG-1/MDA-5 agonist induced cytokine response by the term non-laboured placenta, choriodecidua and amnion. IL-6 and IL-8 production (ng/ml ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion in response to Poly(I:C)LyoVec (1 μg/ml; n=9). Statistically significant differences compared to unstimulated control are shown: * $p < 0.05$, ** $p<0.01$.

Figure 7. Comparison of nucleic acid induced cytokine production by the placenta, choriodecidua and amnion. Heatmap generated using the mean levels of (A) IL-6 and (B) IL-8 production as measured by ELISA, the production level was standardised 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 the rows (by tissue) and columns (by receptor).

FIGURE 1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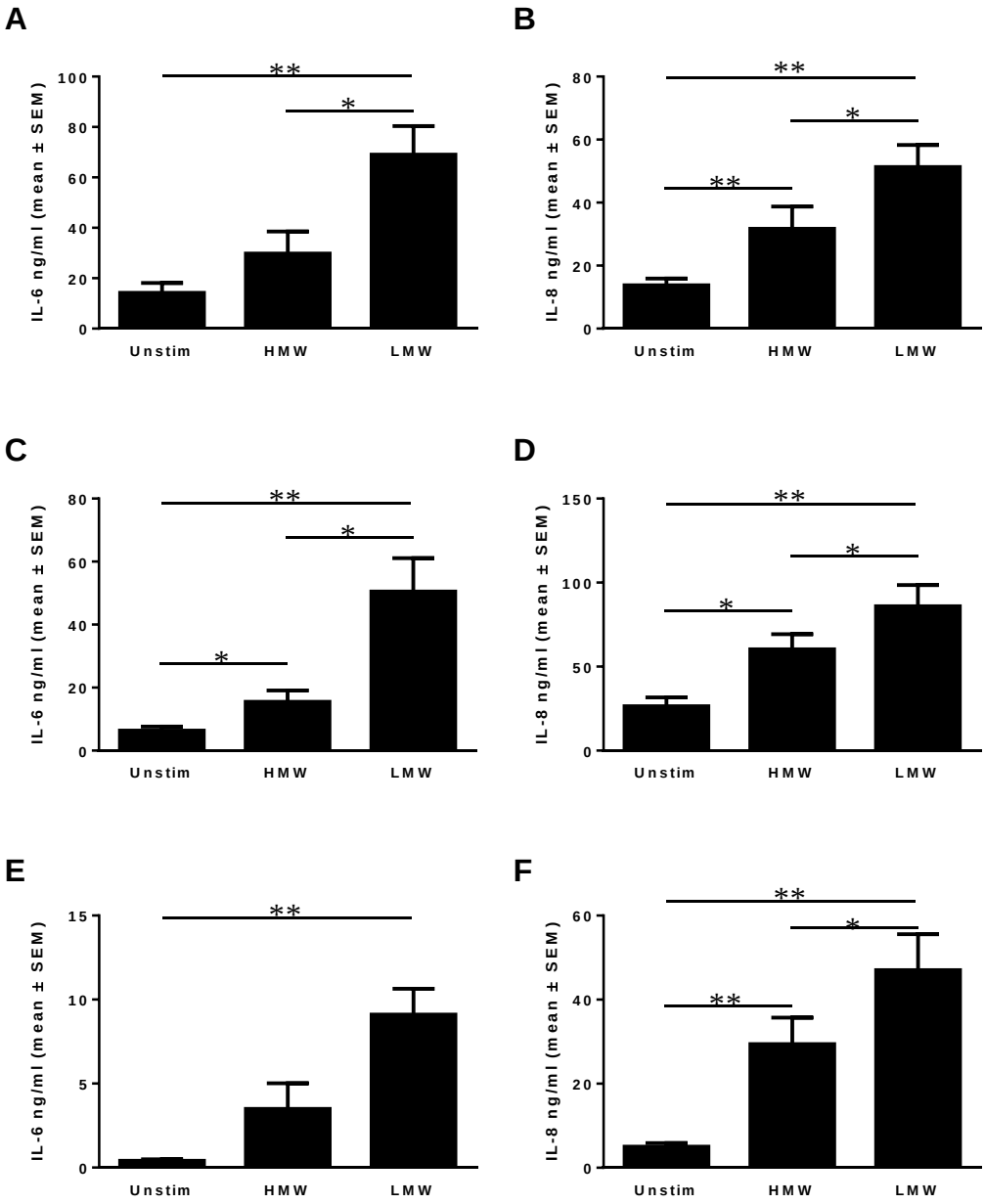
FM -TLR3
FM - Rabbit IgG
Tonsil - TLR3
Placenta -TLR7
Placenta -Rabbit IgG
FM -TLR7
FM - Rabbit IgG
Lung - TLR7
Placenta -TLR8
Placenta -Rabbit IgG
FM -TLR8
FM - Rabbit IgG
Tonsil - TLR8
Placenta -TLR9
Placenta -Rabbit IgG
FM -TLR9
FM - Rabbit IgG
Liver - TLR9

FIGURE 2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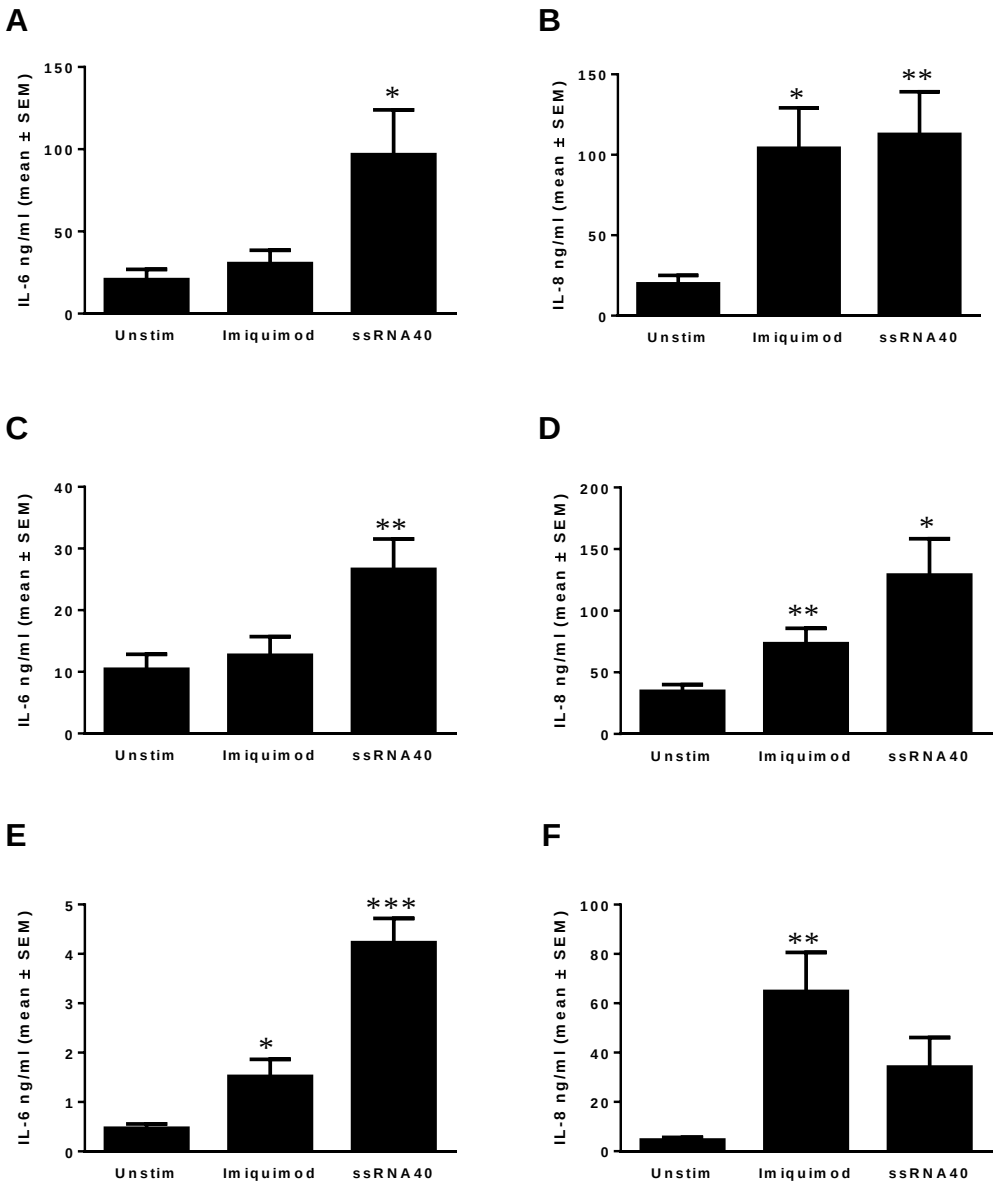
IL-6 ng/ml (mean ± SEM)
100
80
60
40
20
0
**
*
Unstim
HMW
LMW
B
IL-8 ng/ml (mean ± SEM)
80
60
40
20
0
**
**
*
Unstim
HMW
LMW
C
IL-6 ng/ml (mean ± SEM)
80
60
40
20
0
**
*
*
Unstim
HMW
LMW
D
IL-8 ng/ml (mean ± SEM)
150
100
50
0
**
*
*
Unstim
HMW
LMW
E
IL-6 ng/ml (mean ± SEM)
15
10
5
0
**
Unstim
HMW
LMW
F
IL-8 ng/ml (mean ± SEM)
60
40
20
0
**
**
*
Unstim
HMW
LMW

FIGURE 3


A
IL-6 ng/ml (mean ± SEM)
150
100
50
0
*
Unstim
Imiquimod
ssRNA40
B
IL-8 ng/ml (mean ± SEM)
150
100
50
0
*
**
Unstim
Imiquimod
ssRNA40
C
IL-6 ng/ml (mean ± SEM)
40
30
20
10
0
**
Unstim
Imiquimod
ssRNA40
D
IL-8 ng/ml (mean ± SEM)
200
150
100
50
0
**
*
Unstim
Imiquimod
ssRNA40
E
IL-6 ng/ml (mean ± SEM)
5
4
3
2
1
0
*
***
Unstim
Imiquimod
ssRNA40
F
IL-8 ng/ml (mean ± SEM)
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80
60
40
20
0
**
Unstim
Imiquimod
ssRNA40

FIGURE 4

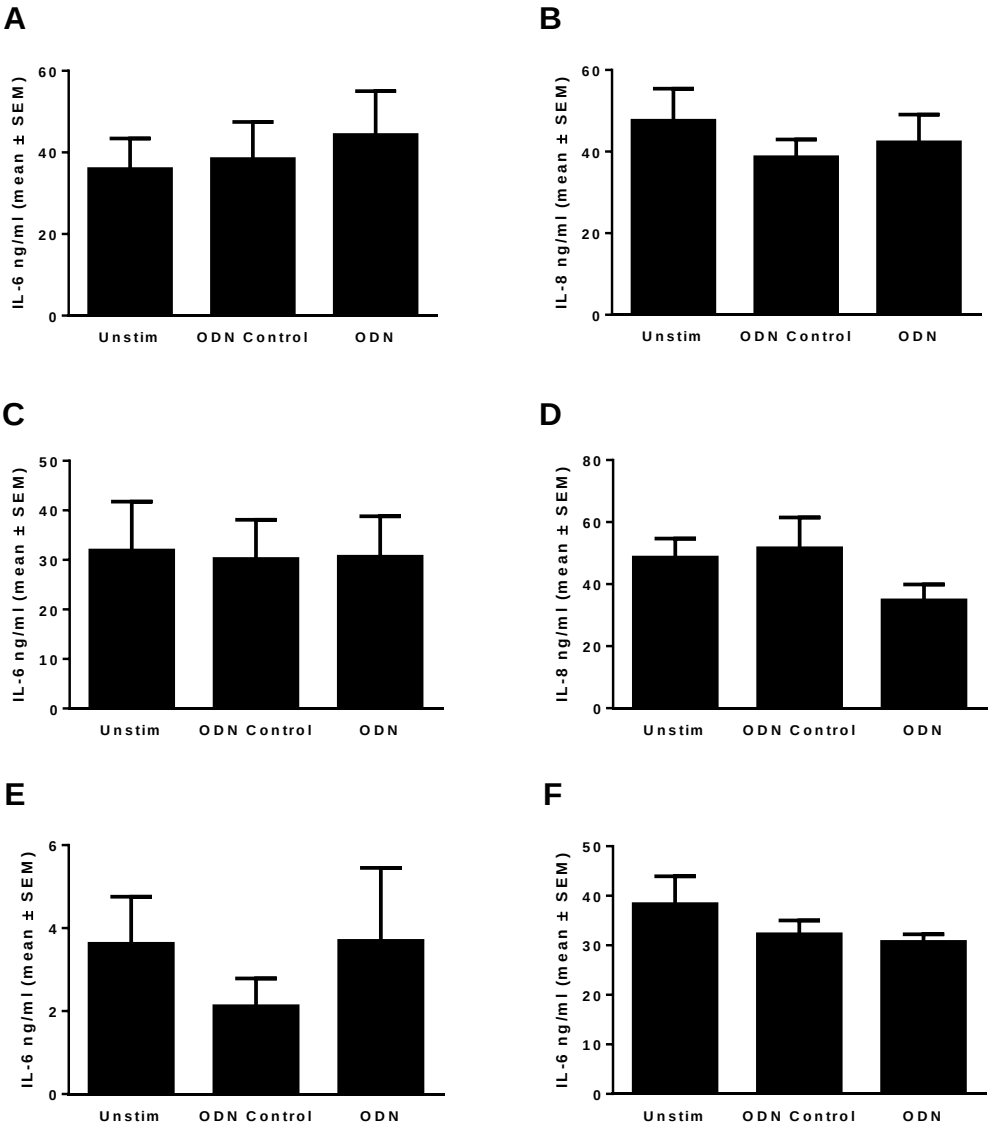

A
IL-6 ng/ml (mean ± SEM)
60
40
20
0
Unstim
ODN Control
ODN
B
IL-8 ng/ml (mean ± SEM)
60
40
20
0
Unstim
ODN Control
ODN
C
IL-6 ng/ml (mean ± SEM)
50
40
30
20
10
0
Unstim
ODN Control
ODN
D
IL-8 ng/ml (mean ± SEM)
80
60
40
20
0
Unstim
ODN Control
ODN
E
IL-6 ng/ml (mean ± SEM)
6
4
2
0
Unstim
ODN Control
ODN
F
IL-6 ng/ml (mean ± SEM)
50
40
30
20
10
0
Unstim
ODN Control
ODN

FIGURE 5

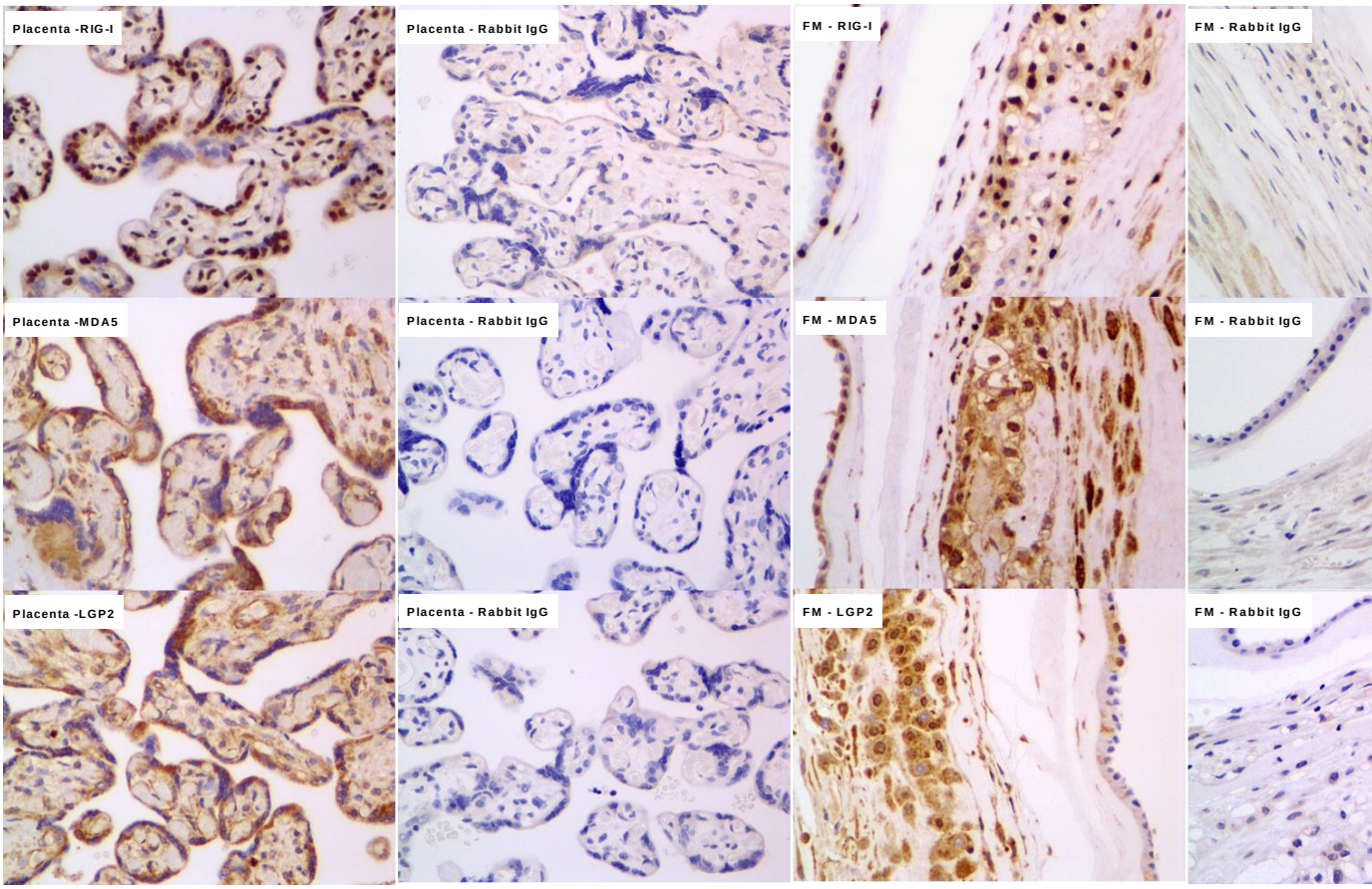

Placenta -RIG-I
Placenta - Rabbit IgG
FM - RIG-I
FM - Rabbit IgG
Placenta -MDA5
Placenta - Rabbit IgG
FM - MDA5
FM - Rabbit IgG
Placenta -LGP2
Placenta - Rabbit IgG
FM - LGP2
FM - Rabbit IgG

FIGURE 6

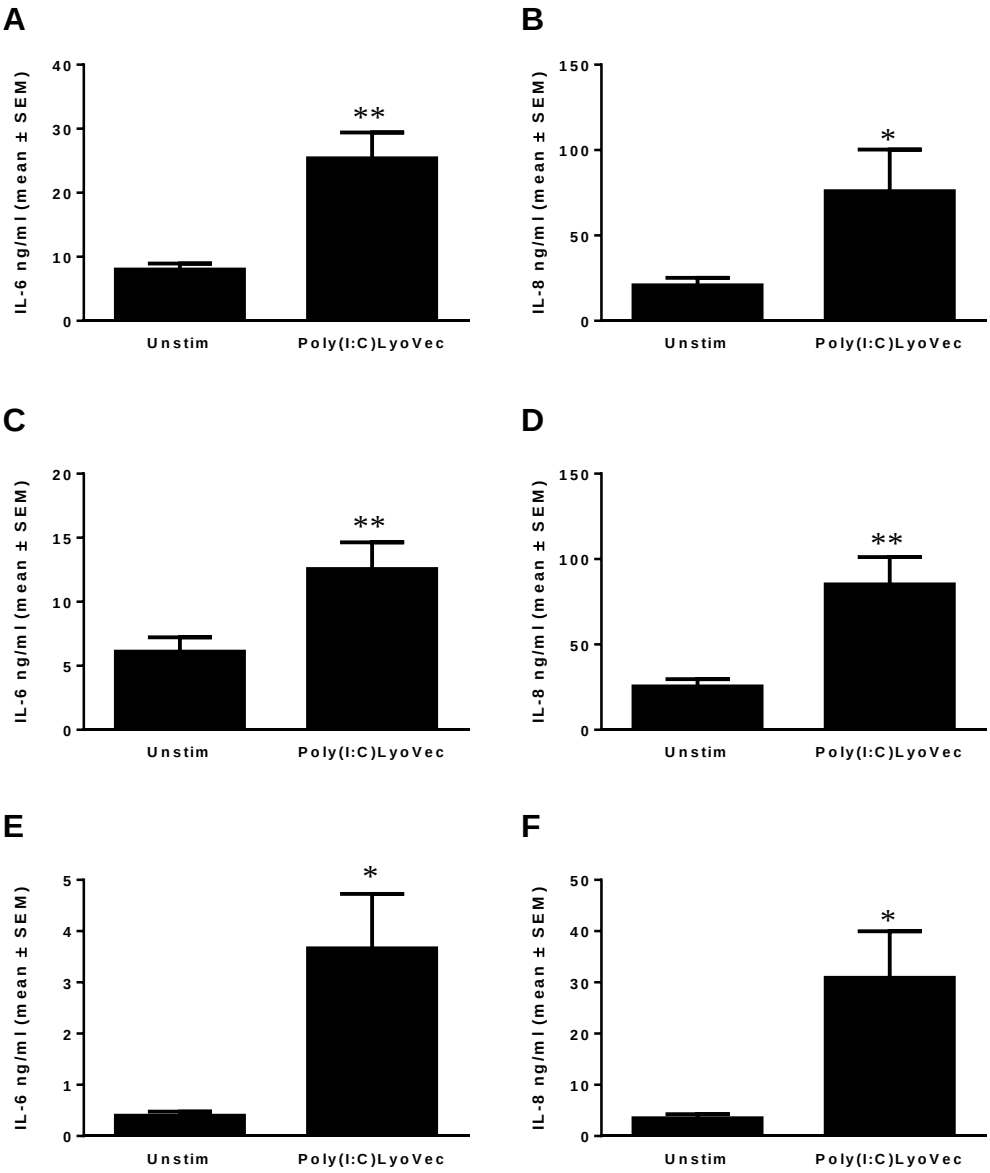
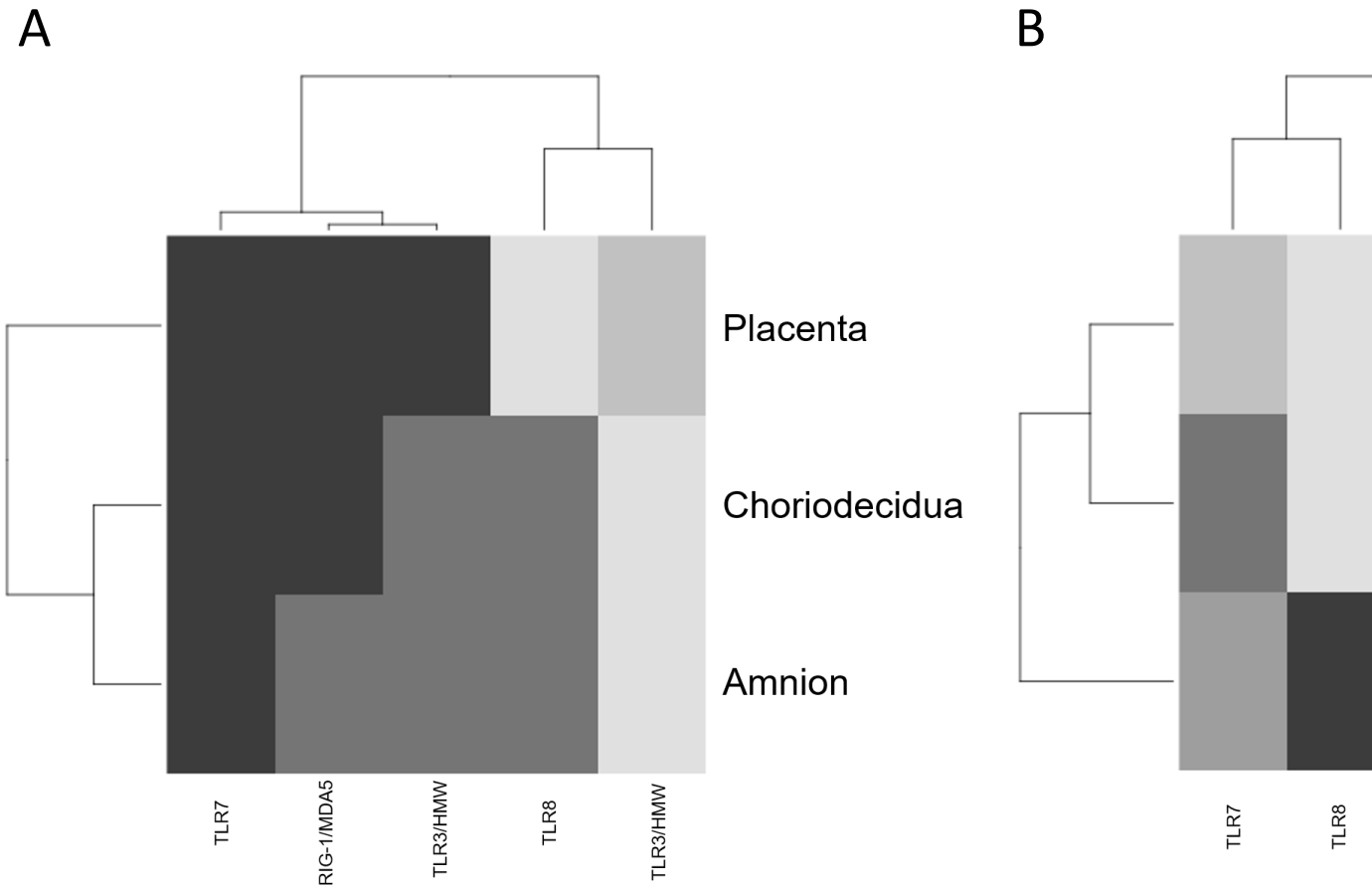

A
IL-6 ng/ml (mean ± SEM)
40
30
20
10
0
**
Unstim
Poly(I:C)LyoVec
B
IL-8 ng/ml (mean ± SEM)
150
100
50
0
*
Unstim
Poly(I:C)LyoVec
C
IL-6 ng/ml (mean ± SEM)
20
15
10
5
0
**
Unstim
Poly(I:C)LyoVec
D
IL-8 ng/ml (mean ± SEM)
150
100
50
0
**
Unstim
Poly(I:C)LyoVec
E
IL-6 ng/ml (mean ± SEM)
5
4
3
2
1
0
*
Unstim
Poly(I:C)LyoVec
F
IL-8 ng/ml (mean ± SEM)
50
40
30
20
10
0
*
Unstim
Poly(I:C)LyoVec

FIGURE 7


A
Placenta
Choriodecidua
Amnion
TLR7
RIG-1/MDA5
TLR3/HMW
TLR8
TLR3/HMW
B
TLR7
TLR8

1