Title: Expression and function of NOD-like receptors by human term gestation-associated tissues

Short title: NLRs at the materno-fetal interface

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Abstract

Introduction: Nucleotide-binding oligomerization domain (NOD)-like receptors or NOD-like receptors (NLRs) have been implicated in several disease pathologies associated with inflammation.

Since local and systemic inflammation is a hallmark of both term and preterm labour, a role for NLRs at the materno-fetal interface has been postulated.

Methods: Gene expression and immunolocalisation of NLR family members in human placenta, choriodecidua, and amnion were examined. Tissue explants were used to examine the response to activators of NOD1 (Tri-DAP), NOD2 (MDP) and NLRP3 (nigericin). Cell/tissue-free supernatants were examined for the production of interleukin (IL)-1β, IL-6, IL-8 and IL-10 using specific ELISAs.

Results: Expression of transcripts for *NOD1*, *NOD2*, *NLRP3*, *NLRC4*, *NLRX1*, *NLRP1* and *NAIP* and protein expression of NOD1, NOD2 and NLRP3 were a broad feature of all term gestation-associated tissues. Production of cytokines was increased significantly in response to all ligands in placenta and choriodecidua, except for MDP-induced IL-10. Similarly, there was a significant increase in cytokine production in the amnion except for MDP induced IL-1β and IL-10 response to either agonist. IL-1β production was dependent on caspase-1 regardless of agonist used or tissue examined.

Discussion: Term human gestation-associated tissues express functional NLRs which likely play a role in both sterile and pathogen-driven inflammatory responses at the materno-fetal interface.

Keywords: Inflammation; reproductive immunology; preterm labour; NOD-like receptors

Abbreviations: DAMP, damage associated molecular pattern; ECS, elective caesarean section; LPS, lipopolysaccharide; IL-, interleukin; MDP, muramyl dipeptide; NLR, NOD-like receptors; NOD, nucleotide-binding oligomerisation domain**;** PAMPs, pathogen associated molecular patterns; PRR, pattern recognition receptor; TLR, toll-like receptor; Tri-DAP, L-Ala-γ-D-Glu-mDAP.

Introduction

Nucleotide-binding oligomerization domain-like receptors, or NOD-like receptors (NLRs), are a family of intracellular pattern recognition receptors (PRRs). They are involved in the recognition of both pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) that have entered the cell [1]. Numerous NLRs have been described and they are divided into five subfamilies based on their N-terminal domain [1]. The first identified and most widely studied NLRs are NOD1 and NOD2 [2]. NOD1 and NOD2 recognise structures within bacterial peptidoglycan; NOD1 recognises iE-DAP and Tri-DAP specific to Gram-negative bacteria, whereas NOD recognises MDP, common to both Gram-negative and -positive bacteria [2]. Certain members of the NLR family, including NLRP1 and NLRP3, following the detection of PAMPs or DAMPs trigger the assembly of a large caspase 1 activating complex termed the inflammasome which enables the processing and secretion of pro-forms of IL-1β and IL-18 [3].

Preterm birth (PTB) is the leading cause of perinatal morbidity and mortality in the Western world. Intrauterine infection is a common mechanism of preterm labour but the role of damage in this pathology is becoming increasingly apparent [4]. As the common outcome of both PAMP and DAMP signals is local and systemic inflammation, a role for PRRs in the pathophysiology of preterm labour and birth has long been suggested [5]. It is increasingly apparent that NLRs play a role in several disease pathologies associated with inflammation [6,7] so a role for NLRs in preterm labour and other adverse pregnancy outcomes would not be unexpected.

NOD1 and NOD2 expression by term human decidua and only NOD1 expression in term amnion epithelial cells have been reported [8]. Functionally, NOD1 and NOD2 activity have been observed in total fetal membrane explants [9], but NLR activity by separated amnion and choriodecidua has not been examined to date. For the placenta, NOD1 but not NOD2 has been reported as expressed and functional in term trophoblast cells, whereas in first trimester placental trophoblast both NOD2 and

NOD1 are expressed with corresponding functional output [10]. However, several other cell populations are present within the placenta that would likely contribute to the inflammatory profile of this organ. Expression of transcripts for NLRP3 by first trimester placenta has been observed [11], and a functional response in the first trimester trophoblast cell line Sw.71 and term cytotrophoblasts to uric acid, a NLRP3 activator, has also been noted [12-14]. Term fetal membranes show expression of NLRP3 with a functional response observed in explants treated with the NLRP3 activators ATP and nigericin [15].

Our objective was to increase understanding of the possible role of NLRs in a reproductive setting by examining the expression and functional activity of the NLRs simultaneously in term placenta, choriodecidua and amnion. Where possible, functional activity was examined using an explant model to better mimic the cellular heterogeneity that occurs *in utero*.

Materials and Methods

Samples

Placenta and fetal membrane samples were collected from healthy term newborns (>37 weeks of gestation) delivered by elective caesarean section at Singleton Hospital, Swansea, UK. Informed, 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 [16,17]. Briefly, the overlaying decidua basalis of the maternal side of the placenta was removed and 1 cm^3 pieces of placenta tissue were cut from various sites 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 cultured in UltraCULTURE medium (Lonza, Switzerland), supplemented with 2 mM GlutaMAX (Life Technologies, UK) and 2 mM penicillin, streptomycin, Fungizone (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 into PBS. Tissue was washed repeatedly with PBS to remove any contaminating blood. Each membrane was cut with an 8mm biopsy punch (Stiefel, Medisave, UK). Two biopsies of choriodecidua were cultured in 0.5 ml of Advanced RPMI (Life Technologies, UK) supplemented with 2 mM GlutaMAX, 2% FBS (HyClone; Thermo Fisher Scientific, UK) 2 mM PSF and 5mM 2-mercaptoethanol (2-ME; Thermo Fisher Scientific). Three

biopsies of amnion were cultured in 0.5 ml of Advanced DMEM (Life Technologies, UK) supplemented with 2 mM GlutaMAX, 2% FBS and 2 mM PSF.

Once prepared, explant cultures were exposed to different stimuli with an unstimulated control always included. Optimal levels of all agonists were determined by dose course experiments on gestation-associated tissues explants and the following final concentrations were used; Tri-DAP (NOD1, 10 µg/ml), MDP (NOD2, 10 µg/ml), LPS (10 ng/ml) and nigericin (1µM) (all from Invivogen, USA). For inhibition experiments, cultures were treated with an inhibitor of caspase-1 (Z-WEHD-FMK, 5 µM; R&D systems) 30 min before the addition of Tri-DAP, MDP or nigericin. Cellular cytotoxicity was not observed with addition of inhibitors (data not shown) as determined by lactate dehydrogenase assay (Abcam). 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

Levels of IL-1β, IL-6, IL-8 and IL-10 in 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.

RNA Extraction from Gestation-Associated Tissue Samples

Biopsies of tissue were preserved in TRI reagent (Sigma-Aldrich, UK) at -20°C. DNA-free RNA was prepared from homogenised tissue (FastPrep FP120A Homogeniser; Qbiogene, The Netherlands) following the TRIzol method of extraction and DNA-free DNase kit (Ambion®, Thermo Fisher Scientific, UK) as per manufacturer's instructions. Purity and concentration of RNA were measured (NanoDrop 3300 flurospectrometer; NanoDrop Technologies, USA).

144

145 *Polymerase Chain Reaction (PCR)*

146 Reverse transcription was performed using the RETROscript kit (Ambion®, Thermo Fisher
147 Scientific, UK) as per manufacturer's instructions. Polymerase chain reaction was performed using
148 the Platinum® Taq DNA Polymerase kit and dNTP Mix (both Thermo Fisher Scientific, UK). All
149 primers were synthesised by Thermo Fisher Scientific, using sequences obtained using primer BLAST
150 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). *UBE2D2* housekeeping primers were based on
151 previously published sequences [18]. Primer sequences and specific conditions for each PCR are
152 listed in Table 1.

153

154 *Immunohistochemistry*

155 Immunohistochemical staining for NOD1, NOD2 and NLRP3 was performed on formalin fixed,
156 paraffin embedded sections (4 µm) of placenta and fetal membranes using the Ventana ULTRA
157 automated staining instrument as described previously (24). The following modifications were made:
158 Optiview detection system was used without A/B blocker or amplification, antigen retrieval was
159 carried out in CC1 buffer for 16 minutes for both NOD1 and NLRP3, and in protease 1 for 8 minutes
160 for NOD2. Mouse monoclonal anti-NOD1 (10 µg/ml; R&D Systems, USA), rabbit polyclonal anti-
161 NOD2 (3 µg/ml; LifeSpan BioSciences, Inc, USA) and mouse monoclonal anti-NLRP3 (10 µg/ml;
162 Abcam, USA) were incubated at 36°C for 40, 24 and 36 minutes, respectively. For control slides,
163 primary antibody was replaced with either rabbit IgG (3 µg/ml; Biolegend), mouse IgG1 (10 µg/ml;
164 eBioscience), or mouse IgG2b (10 µg/ml; eBioscience) isotype controls at the same concentration.

165

166 *Data analysis*

167 All experiments were performed a minimum of three times. Cytokine production by non-laboured
168 tissues was evaluated using 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).

Results

Expression of transcripts of NLRs by gestation-associated tissues

PCR analysis revealed expression of transcripts for *NOD1*, *NOD2*, *NLRP3*, *NLRC4*, *NLRX1*, *NLRP1* and *NAIP* in all gestation-associated tissues studied. PCR was performed using five individual samples of each tissue type (placenta, choriodecidua and amnion); three of these five samples are shown (Figure 1). Transcripts for each NLR examined were present in all five samples of each of the tissues.

Immunolocalisation and function of NOD1 and NOD2 by gestation-associated tissues

Expression of NOD1 and NOD2 was examined using immunohistochemistry (Figure 2A). In the placenta, expression of NOD1 was localised to the syncytiotrophoblast, while NOD2 expression was present in the syncytiotrophoblast and cells within the stroma. In the fetal membranes both NOD1 and NOD2 expression were present in the amnion epithelial cells, chorionic trophoblast and cells within the decidua. This expression pattern was similar for all 7 samples studied.

To investigate if NOD1 and NOD2 were functional, the agonists L-Ala-γ-D-Glu-mDAP (Tri-DAP; NOD1, 10 μg/ml) and muramyl dipeptide (MDP; NOD2, 10 μg/ml) were used. Both Tri-DAP and MDP induced a significant increase in IL-1β, IL-6, IL-8 and IL-10 by the placenta (Figure 3A). A significant increase in both IL-6 and IL-8 in response to NOD1 and NOD2 activation also was a common feature of both the choriodecidua and amnion (Figure 3B and C). Both Tri-DAP and MDP induced significant IL-1β by choriodecidua, however only Tri-DAP induced a significant IL-10 response (Figure 3C). With regards to the amnion, treatment with Tri-DAP induced significant IL-1β and IL-10, however MDP did not induce IL-1β above background and there was no significant effect on IL-10 production (Figure 3C).

Immunolocalisation and function of NLRP3 by gestation-associated tissues

NLRP3 expression was examined using immunohistochemistry (Figure 4A). In the placenta, expression of NLRP3 was localised primarily to syncytiotrophoblast and cells within the stroma. In the fetal membranes, NLRP3 was expressed by the chorionic trophoblast, cells within the decidua and amnion epithelial cells. For all tissues NLRP3 reactivity appeared to be localised to the nucleus. Tissue samples from seven different donors were studied and all were positive for NLRP3. However, while there was little variability in intensity of NLRP3 reactivity within the fetal membranes there were noticeable differences in the placenta and this seems to be inversely correlated to maternal BMI (Supplementary Figure 1).

To investigate if the NLRP3 inflammasome was functional, the potassium ionophore nigericin (1μM) derived from *Streptomyces hygroscopicus* was used. Prior to the addition of nigericin tissue explants were primed with LPS (10 ng/ml) for 3 hours. A significant increase in IL-1β, above both background and LPS treatment alone, was observed for each tissue following exposure to nigericin (Figure 4B-D).

NLR induced IL-1β production is caspase-1 dependent

As the production and secretion of IL-1β following NLRP3 activation is reliant on caspase-1, a caspase-1 inhibitor was utilized to confirm the involvement of the NLRP3 inflammasome. This approach was also applied to explants treated with both Tri-DAP and MDP due to the robust IL-1β response observed. In the presence of capase-1 inhibitor, both Tri-DAP- and MDP-induced IL-1β levels were inhibited in the placenta (Figure 5A), choriodecidua (Figure 5B) and amnion (Tri-DAP only; Figure 5C). A decrease in IL-1β in the presence of capase-1 inhibitor was also observed for treatment with both LPS alone and LPS and nigericin in all tissues examined (Figure 5D-F).

Discussion

Inflammation caused by infection or damage is a common feature of various pathologies [4] and NLRs have been implicated to have a role. Since a hallmark of both term and preterm labour is local and systemic inflammation a role for NLRs at the materno-fetal interface has been postulated [5]. Here we demonstrate that specific agonists for the NLRs - NOD1, NOD2 and NLRP3 - generate cytokine production by the placenta, choriodecidua and amnion, corresponding to expression at both the gene and protein level. Additionally, expression of transcripts of other NLRs, namely NLRC4, NLRX1, NLRP1 and NAIP by term gestation-associated tissues been demonstrated for the first time.

Previous investigations of NOD expression by the placenta have been limited to primary first and third trimester trophoblast. Transcripts and protein for both NOD1 and NOD2 were found in first trimester trophoblast but only for NOD1 in third trimester trophoblast with a corresponding functional response [19,20]. Here we demonstrate that transcripts and protein for both NOD1 and NOD2 are present in the term placenta. Furthermore, treatment of placental explants with Tri-DAP (NOD1) and MDP (NOD2) resulted in increased cytokine production (IL-1β, IL-6, IL-8 and IL-10) implying that both receptors are functional. Increases in pro-inflammatory cytokines are not unexpected as both Tri-DAP and MDP are known initiators of inflammation. The increase in the anti-inflammatory cytokine IL-10 might represent a compensatory mechanism: concentrations of anti-inflammatory cytokines are increased in the amniotic fluid from women who delivered preterm and had evidence of intra-amniotic infection [21] possibly representing a failed compensatory mechanism [22]. Discrepancies in NOD2 expression and function might be related to the model of investigation used, i.e. a dissociated cell culture model focussing on trophoblast only [19,20] in comparison to explants as here. The placenta is heterogeneous, composed of many cell types such as cytotrophoblast, syncytiotrophoblast, mesenchymal stem cells, endothelial cells and macrophages (Hofbauer cells). It is possible that other non-trophoblast cell types within the placenta are responsible for both NOD2 expression and activity as observed by us. For example, both monocyte derived macrophages (MDMs) [23] and tissue-resident macrophages [24,25] express NOD2 in humans. Immunohistochemistry of NOD1 and NOD2 expression revealed this to be the case. NOD1

expression within the placenta was localised to syncytiotrophoblast cells while NOD2 was expressed in both syncytiotrophoblast and other cells within the stroma. This observation of NOD2 expression by trophoblast cells of the term placenta clearly differs from previous reports where the MDP-stimulated IL-8 response in term cytotrophoblast lacking NOD2 expression was described as NOD2-independent [19].

MDP can also activate human NLRP1 [26] which is involved primarily in inflammasome activity typically measured via IL-1β output. Transcripts for NLRP1 have been reported in first trimester trophoblast in addition to decidual stroma and endothelial cells [11]. We found transcripts for both NLRP1 as well as other inflammasome-associated NLRs, namely NLRP3, NLRC4 and NAIP, in the term placenta. MDP was also able to induce IL-1β production in the placenta in a caspase-1 dependent manner. IL-1β production by MDP treated trophoblasts, either first or third trimester, has not been reported [10,19] and since transcripts for NLRP1 are present, it is possible that the NOD2-independent IL-8 observed [27,28] might relate to autocrine exposure to IL-1β.

NOD1 and NOD2 expression and responsiveness have been reported in the fetal membranes with NOD1 and NOD2 protein expressed by chorionic cytotrophoblasts and decidual cells but only NOD1 by amnion epithelial cells; both receptors were up-regulated following spontaneous labour [8]. In contrast, we found NOD1 and NOD2 protein expression using immunohistochemistry in cells of the amnion, chorion and decidua. Term choriodecidua and amnion both produced IL-6 and IL-8 in response to Tri-DAP and MDP treatment, corresponding to term fetal membrane explants treated with iE-DAP (NOD1) and MDP [8]. Additionally, we demonstrated that the choriodecidua produces IL-1β in response to both Tri-DAP and MDP, and IL-10 in response to Tri-DAP. The amnion produced IL-1β in response to Tri-DAP treatment, while neither Tri-DAP nor MDP treatment resulted in a significant IL-10 response. The caspase-1 dependence of Tri-DAP and/or MDP IL-1β production by the choriodecidua and amnion was confirmed. The balance of pro and anti-inflammatory cytokines

observed in the choriodecidua, mimics that of the placenta, suggesting a similar functional activity of NLRs in these tissues. However, while the amnion has both functional NOD2 and NLRP3 and active caspase-1, its unknown why activation with MDP does not result in an IL-1β response. MDP induced IL-1β might require the activity of other initiator caspases in the amnion.

NLRP3 expression in term placenta was localised primarily to the syncytiotrophoblast in keeping with the observation of NLRP3 gene expression by first trimester trophoblasts [11]. NLRP3 expression was noted in all samples studied but there was variability in this. NLRP3 expression and subsequent inflammasome activation can be affected by several different factors including smoking status [30], obesity [31] and age [32]. In our study, the expression of placental NLRP3 seemed to correlate negatively with BMI, i.e. decreasing NLRP3 expression with increasing maternal BMI. This contrasts with a previously published report of a positive correlation of trophoblast produced IL-1β with maternal BMI, though caspase-1 signalling was unaffected [33]. However, with only seven samples included in our study, caution should be taken until more samples can be examined (supplementary figure 1). Expression of NLRP3 was also observed by both the amnion and choriodecidua, however unlike the placenta, expression of NLRP3 was constant between donors. This observation of NLRP3 expression is in keeping previous reports [35] but we noted that NLRP3 expression was predominantly nuclear. While typically considered a cytoplasmic protein, NLRP3 has been shown to function as a nuclear transcription factor, positively regulating Th2 immunity, independent of inflammasome activity [36]. Nuclear translocation of NLRP3 might have a role in placental regulation of the Th2 predominance in pregnancy [37]. With a negative correlation between placental NLRP3 expression and maternal BMI, the loss of a NLRP3 driven Th2 environment might explain the increased inflammatory environment in obese pregnant women and the associated risk of adverse pregnancy outcomes [34].

Activation of the NLRP3 inflammasome by DAMPs, such as uric acid crystals or ATP, is associated with a "two-step" mode of activation [3]. Here functional placental NLRP3 is shown using an LPS prime (signal 1) and the potassium ionophore nigericin (signal 2) to give a significant increase in the production of IL-1β in a caspase-1 dependent manner. Previous studies have produced an IL-1β response in the first trimester trophoblast cell line Sw.71 [12,13] and term cytotrophoblasts [14] using uric acid crystals, although no priming signal was used in these studies. Caspase-1 dependent IL-1β production in response to nigericin was also observed by the choriodecidua and amnion, corresponding to IL-1β production by fetal membrane explants in response to ATP treatment [15,38].

In addition to NOD1, NOD2 and NLRP3, we have also shown that term placenta, choriodecidua and amnion express transcripts for NLRC4, NLRX1, NLRP1, and NAIP. Gene expression of NLRP1 and NAIP by the placenta [39] and gene and protein expression of NRLP1 and NLRC4 by term fetal membranes [35] has been reported by others. This is however the first report of placental NLRC4, fetal membranes NAIP expression, and NLRX1 expression by all three gestation-associated tissues. Furthermore while we have focussed on the casapase-1 dependence of IL-1β production, several other caspases (caspase-4, -5, -8 and -12) can either directly, by canonical and non-canonical inflammasomes, or indirectly modulate the production of IL-1β [40]. Functional activity of these NLRs and other caspases by gestation-associated tissues is still to be examined with our recent work showing a contribution of Caspases-1, -4 and -8 to IL-1β production in placenta, choriodecidua and amnion (Scott et al. Cytokine. Accepted).

The role of NLRs, especially through the production of IL-1β, highlights a potential role for this family of PRRs in the inflammation that occurs with preterm birth [41]. This inflammation can be driven by PAMPs or DAMPs but has common downstream outcomes related to chorioamnionitis, fetal membrane rupture, cervical ripening, and parturition [5,41]. Better understanding of the expression and activity of NLRs in adverse pregnancy outcomes is now required.

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461

462

463 **Table Legends**

464 **Table 1.** Sequences and optimum conditions for each pair of NOD-like receptor (NLR) primers used
465 for RT-PCR.

466

467 **Figure Legends**

468 **Figure 1. Gene Expression of NLRs by term non-laboured gestation-associated tissues.** RT-PCR
469 for NOD1, NOD2, NLRP3, NLRC4, NLRX1, NLRP1 and NAIP. Three representative samples of 5
470 are shown. Human spleen (S) was used as a positive control and UBE2D2 was used as a
471 housekeeping gene. L = 100 base pair ladder and the negative control (-ve) was with water replacing
472 cDNA in the reaction mix.

473 **Figure 2. Protein expression of NOD1 and NOD2 in human gestation-associated tissues.**

474 Immunolocalisation of NOD1 and NOD2 in placenta and fetal membranes. Negative (isotype match)
475 and positive (NOD1; tonsil and NOD2; colon) controls are also displayed. A representative example
476 of 7 is shown.

477

478 **Figure 3. Functional response of human gestation-associated tissues to NOD1 and NOD2**
479 **agonists.** NOD1/2 agonist induced cytokine response by term non-laboured placenta, choriodecidua
480 and amnion. IL-6, IL-8, IL-10 and IL-1β production (mean ± SEM) by the (A) placenta, (B)
481 choriodecidua, and (C) amnion following stimulation with 10 μg/ml Tri-DAP or MDP (n=6).
482 Statistically significant differences compared to unstimulated control are shown: * $p < 0.05$, **
483 $p<0.01$, *** $p< 0.001$.

484

485 **Figure 4. Expression and functional response of NLRP3 in human gestation-associated tissues.**
486 (A) Immunolocalisation of NLRP3 in placenta and fetal membranes. Negative (isotype match) and

positive (tonsil) controls are also displayed. Representative examples of 7 are shown; placenta 1 is from a woman of normal weight woman; placenta 2 is form a morbidly obese woman. (B-D) Nigericin-induced IL-1β (mean ± SEM) response by term non-laboured (B) placenta, (C) choriodecidua and (D) amnion. Statistically significant differences compared to unstimulated control are shown: * $p< 0.05$, ** $p<0.01$.

Figure 5. Caspase-1 is required for NLR-induced IL-1β production by gestation-associated tissues. IL-1β production from explants of (A & D) placenta, (B & E) choriodecidua and (C & F) amnion in response to Tri-DAP, MDP (A-C) or nigericin (D-F) pre-treated for 30 minutes with caspase-1 inhibitor (Z-WEHD-FMK; 5 μM) or vehicle control (n=3). Statistically significant differences compared to unstimulated control are shown: * $p< 0.05$, ** $p<0.01$, *** $p< 0.001$, **** $p< 0.0001$.

Supplementary Figure Legends

Figure S1. Immunolocalisation of NLRP3 in placenta is negatively correlated with body mass index.

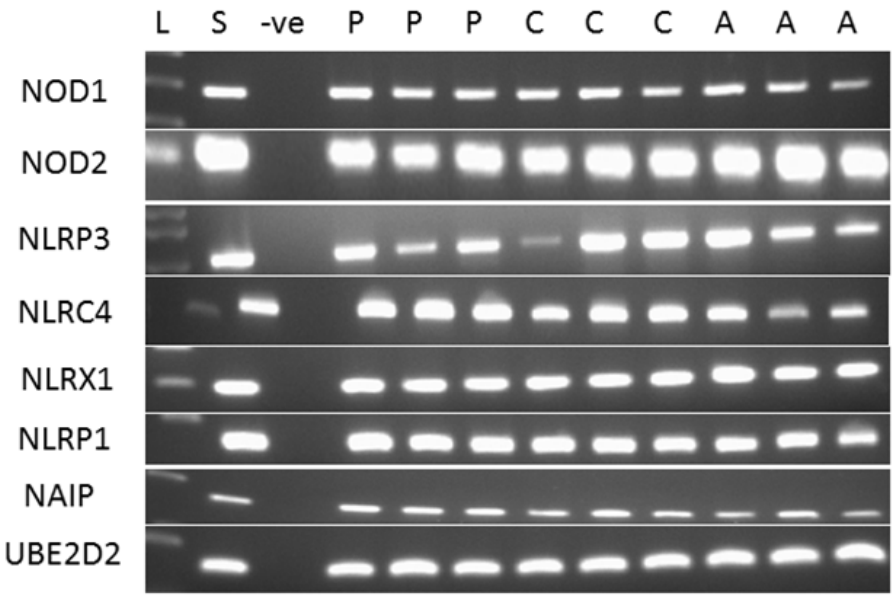
Immunohistochemical staining for NLRP3 performed on formalin fixed, paraffin embedded sections (4 μm) of placenta using mouse monoclonal anti-NLRP3 (10 μg/ml; Abcam, USA).

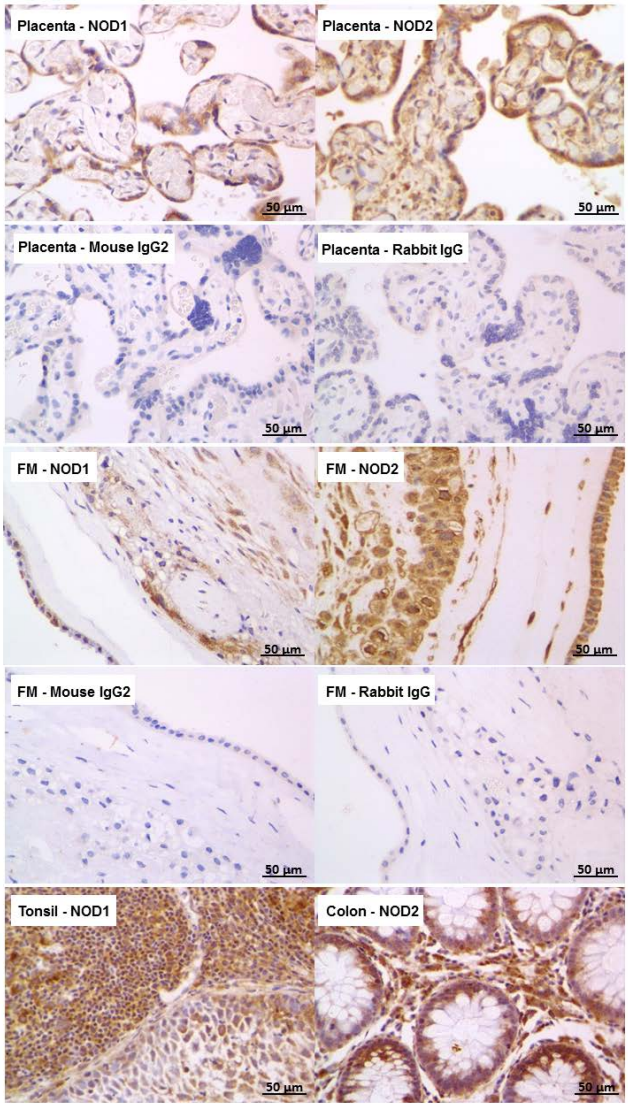
TABLE 1

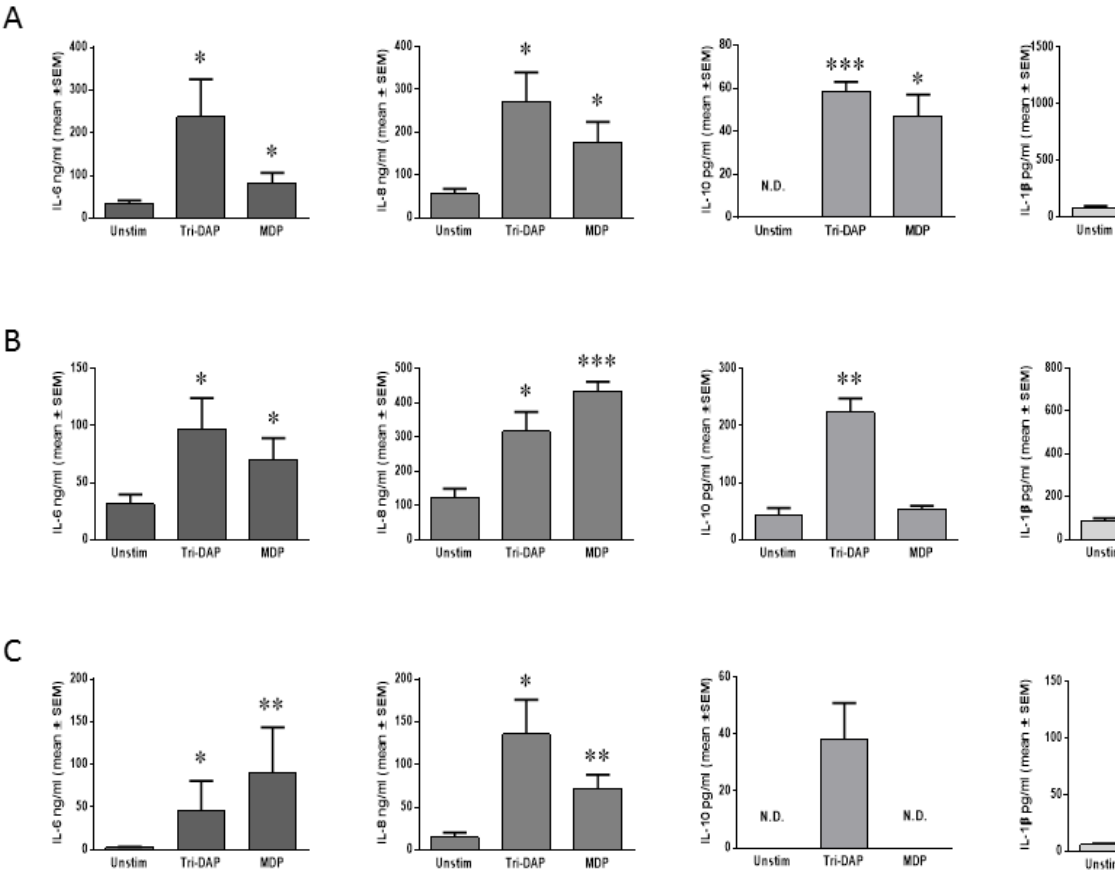
Gene		Primer	Mg^{2+} Conc (nM)	Annealing Temp (°C)	Fr Si
NOD1	***F***	5' AGGCTGAGTACCATGGGCTA	2	66	
	R	5' GCCCGTTTAGTCACCCTTCA			
NOD2	***F***	5' CAGGCAGCACAGGTCAGCCC	2	71	
	R	5' GTTGTGCGGCTCGGCCTTCT			
NLRP1	***F***	5' ATACGAAGCCTTTGGGGACT	3	65	
	R	5' CACCGCTTCTCTCATCACAA			
NLRP3	***F***	5' ACCGGAGCCAGCAGGAGAGG	1.5	71	
	R	5' GAAGGCTGCCCTGGCTTGGG			
NLRC4	***F***	5' GCCTCAGGCTGCAAATAAAG	2	68	
	R	5' CCAAGCTGTCAGTCAGACCA			
NLRX1	***F***	5' GCTCCATGGCTTAGAGCATC	1.5	62	
	R	5' ACGTACTTGCTGGGGATACG			
NAIP	***F***	5' TTCTTGCCCTGAAAACTGCT	3	66	

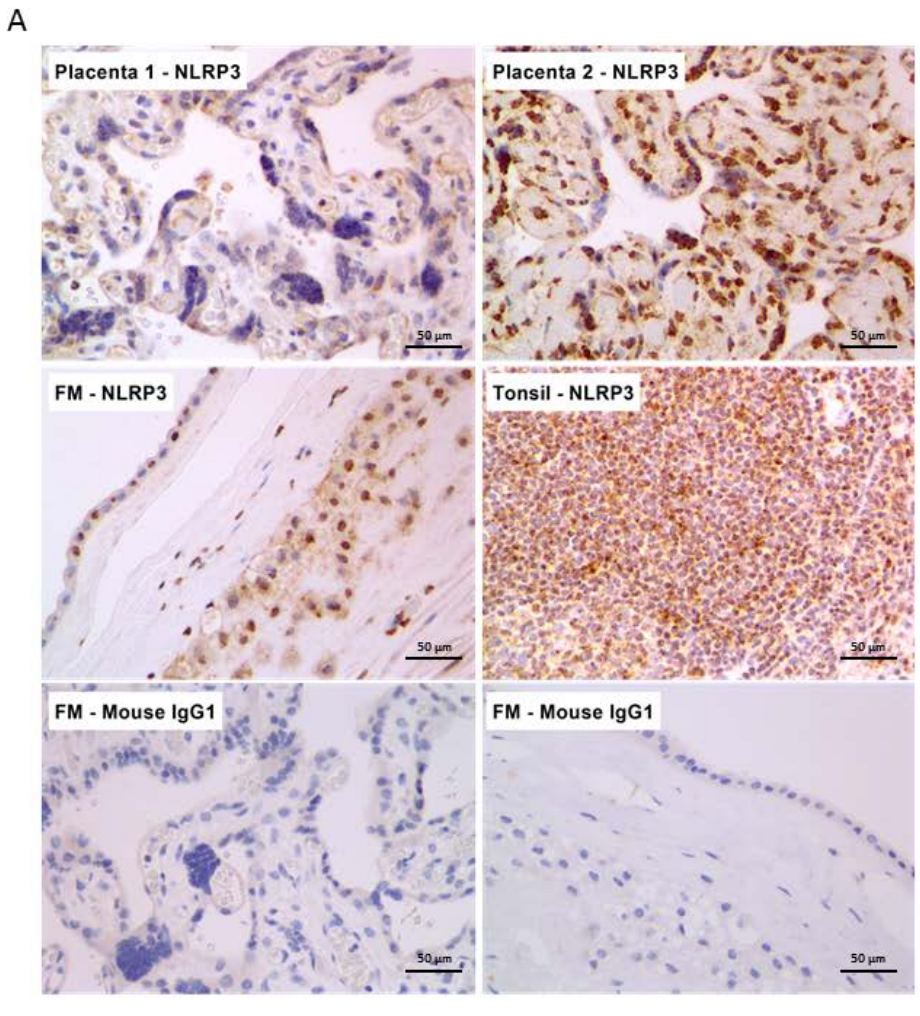
	R	5' CGTATTGGGAAGTGGATGCT		
UBE2D2	***F***	5' GATCACAGTGGTCTCCAGCA	3nM	65°C
	R	5' TCCATTCCCGAGCTATTCTG		

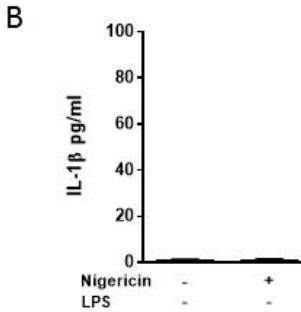
Figure 1

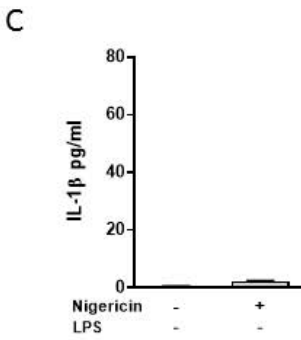

L
S
-ve
P
P
P
C
C
C
A
A
A
NOD1
NOD2
NLRP3
NLRC4
NLRX1
NLRP1
NAIP
UBE2D2

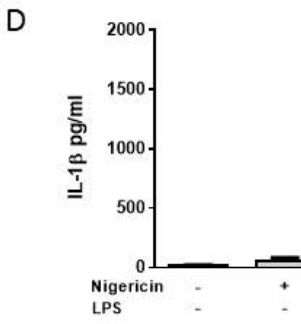

Placenta - NOD1
50 µm
Placenta - NOD2
50 µm
Placenta - Mouse IgG2
50 µm
Placenta - Rabbit IgG
50 µm
FM - NOD1
50 µm
FM - NOD2
50 µm
FM - Mouse IgG2
50 µm
FM - Rabbit IgG
50 µm
Tonsil - NOD1
50 µm
Colon - NOD2
50 µm

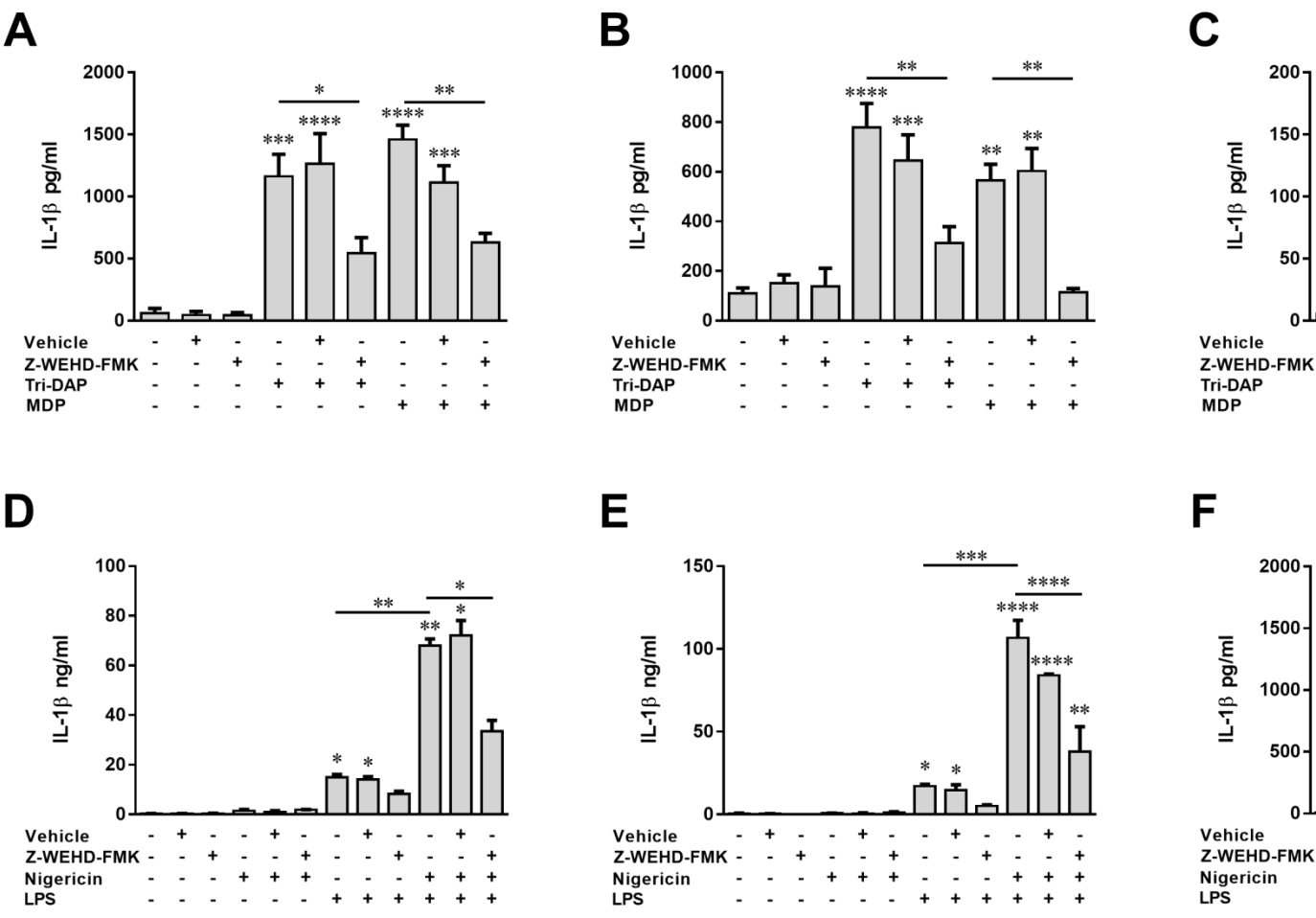

A
IL-6 ng/ml (mean ±SEM)
IL-8 ng/ml (mean ± SEM)
IL-10 pg/ml (mean ±SEM)
IL-1β pg/ml (mean ± SEM)
N.D.
Unstim
Tri-DAP
MDP
B
C


A
Placenta 1 - NLRP3
Placenta 2 - NLRP3
FM - NLRP3
Tonsil - NLRP3
FM - Mouse IgG1
FM - Mouse IgG1
50 µm


B
IL-1β pg/ml
100
80
60
40
20
0
Nigericin - +
LPS - -


C
IL-1β pg/ml
80
60
40
20
0
Nigericin - +
LPS - -


D
IL-1β pg/ml
2000
1500
1000
500
0
Nigericin - +
LPS - -


A
B
C
D
E
F
IL-1β pg/ml
IL-1β ng/ml
Vehicle
Z-WEHD-FMK
Tri-DAP
MDP
Nigericin
LPS

Supplementary Figure 1