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Functional activity but not gene expression of Toll-like receptors is decreased in the preterm versus term human placenta

Short title: Placental Toll-like receptors and preterm birth

Summary sentence: Pro-inflammatory cytokine output in response to multiple TLR ligands was decreased in the preterm compared to the term placenta but gene expression for each TLR tended to be similar.

Keywords: human, reproductive immunology, toll-like receptors, cytokines

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Abstract

INTRODUCTION: Toll-like receptor (TLR) activity within gestation-associated tissues might have a role in normal pregnancy progression as well as adverse obstetric outcomes such as preterm birth (PTB).

METHODS: The expression and activity of TLRs 1 – 9 in placentas collected following preterm vaginal delivery after infection-associated preterm labour (IA-PTL) at 25 – 36 weeks of gestation (preterm-svd, n = 10) were compared with those obtained after normal vaginal delivery at term (term-laboured; n=17). Placental explants were cultured in the presence of agonists for TLR2, 3, 4, 5, 7, 8 and 9 and cytokine production after 24 hours examined. Expression of TLR transcripts was determined using real time quantitative PCR.

RESULTS: Reactivity to all agonists except CpG oligonucleotides was observed indicating that other than TLR9 all of the receptors studied yielded functional responses both term and preterm. Significantly less TNFα and IL-6, but not IL-10, were produced by preterm than term samples in response to all TLR agonists. Changes in TLR mRNA expression did not underlie functional differences in the preterm and term groups; nor does a pre-exposure/tolerance model mimic this finding. While glucocorticoids suppressed cytokine production in an in vitro model using term tissue the association between lower gestational age and decreased cytokine outputs suggests a temporally regulated response.

DISCUSSION: Pro-inflammatory cytokine output in response to multiple TLR ligands was decreased in the preterm compared to the term placenta but gene expression for each TLR tended to be similar. Reduced cytokine production by the preterm placenta in response to stimulation of TLRs therefore must be regulated at the post-transcriptional level in a gestational age dependent manner.

Introduction

Cytokines and chemokines have a role in the normal physiological processes of pregnancy including parturition. IL-1β, IL-6, IL-8 and TNFα among others are produced by gestation-associated tissues both constitutively and/or in response to insult (1, 2). This has led to interest in the mechanisms of cytokine production in these tissues, with signalling pathways of the innate immune system that produce a defined cytokine output postulated as central to this (3). Toll-like receptors (TLRs), a family of pattern recognition receptors (PRRs), link microbial agonists to the production of inflammatory mediators. In humans, ten TLRs (TLRs 1 -10) have been identified (4). TLRs could provide a mechanism of cytokine production at the maternal-fetal interface in not only normal physiological aspects of pregnancy but also in various pathological states of pregnancy such as infection associated preterm labour (3, 5).

The placenta has been called a pregnancy-specific component of the innate immune system because it constitutes a physical and immunological barrier against invading infectious agents; the activity of TLRs and other PRRs within cells of the placenta would support such a role. Transcripts for TLRs 1-10 have been demonstrated in both the term and preterm human placenta and isolated cytotrophoblast and syncytiotrophobasts (6, 7). Trophoblastic choriocarcinoma cell lines express TLRs 1-10 and several co-receptors and accessory proteins (8). Functional activity of TLR2, TLR3 and TLR4 has been reported for first and third trimester trophoblast/placenta (9-12) and choriocarcinoma cell lines are responsive to ligands for TLRs 2, 3, 4 and 9 (8). Other cell types within the term placenta including Hofbauer cells express TLR2, TLR3 and TLR4 mRNA and/or protein and have functionally active TLR3 and TLR4 (13, 14). There is temporal variation in the expression of TLRs in the placenta: both first trimester primary trophoblast and trophoblast cell lines express TLRs 1-4 but not TLR6 which is expressed in third trimester trophoblast (9, 15), while TLR4 expression is higher at term than during the first trimester (16).

Globally around 10% or babies are born prematurely. There are three main antecedents of PTB: 30 – 35% maternal or fetal indications, 40 – 45% spontaneous preterm labour with intact membranes, and 20 – 25% preterm premature rupture of the membranes (PPROM): the latter two are grouped as spontaneous PTB (17). Pathophysiological mechanisms underlying spontaneous PTB are largely unknown but a wealth of evidence indicates that preterm labour is an inflammatory process (18, 19). The induced inflammatory milieu is likely heterogeneous depending on the underlying cause of PTB (18, 20), and both gestation-associated tissues themselves and infiltrating leukocytes contribute (21). However, there is little information about the possible role of PRRs and their ligands in spontaneous PTB especially that associated with infection. The probable role of TLRs in the pathogenesis of infection-associated preterm labour (IA-PTL) and other adverse pregnancy outcomes has been studied mostly with regards to TLR4: inflammatory cells infiltrating preterm placentas with chorioamnionitis express TLR4, and TLR4 expression on villous Hofbauer cells is increased in preterm placentas without chorioamnionitis and term placentas (14); functional TLR4 has been implicated in preterm labour triggered by administration of heat killed *E.coli* in mice (22); expression of TLRs 2, 4, 5 and 6 mRNA and TLR4 protein are also increased in the preeclamptic placenta (23, 24); the Asp299Gly TLR4 gene polymorphism associated with impaired TLR4 receptor function and an increased likelihood of Gram-negative sepsis (25) is carried more often by preterm than term infants or by mothers delivering preterm rather than at term (26). A role for functional TLR3 in preterm labour also has been described (27). Evidence for a role for TLRs in infection-associated preterm birth also comes from genetic studies.

Over the past few years there has been a dramatic increase in interest and information about activity mediated by microbial ligands/TLR combinations in various tissues and diseases. As we had previously described that multiple TLRs (with the exception of TLR9) were functional in the term placenta and stimulation with TLR agonists could lead to the production of relevant cytokines and chemokines (6) an investigation of the expression and activity of TLRs in preterm placentas delivered with evidence of intrauterine infection was undertaken.

Materials and Methods

Characteristics of preterm samples

Placentas were obtained following preterm labour at varying gestations, ranging from 25 weeks to 36 weeks (n=15; Table 1). The 10 cases which delivered vaginally after spontaneous onset of labour had no evidence of preeclampsia, intrauterine growth restriction or other obvious materno-fetal reasons but evidence of infection was found in all but one either in the form of histologic chorioamnionitis, positive swabs, urine examination or blood markers of infection (Table 1); all women had received steroid injections as part of their clinical care. None of these women were known to have any autoimmune or other immunological disorders. These cases (n=10) (IA-PTL) have been included in this study. Gestational age was calculated by ultrasound or by the first day of the last menstrual period. Women were approached either at admission in early labour or soon after delivery in full liaison with the midwives. Term samples were from women who delivered vaginally after spontaneous onset of labour and after 37 completed weeks of gestation. All women delivering preterm had received steroids prior to delivery as part of their clinical therapy for preterm labour; women delivering at term had not. All women gave informed written consent and Wales Research Ethics Committee 6 approved the study.

Placental explant culture

All placentas were weighed and then explant cultures were prepared as described [23]. Briefly, the overlaying decidua basalis on the maternal side of the placenta was removed and 1 cm^3 pieces of placental tissue were taken from different sites across the placenta and placed into sterile Ca^{++}/Mg^{++} free phosphate buffered saline (PBS; Life Technologies, UK). Care was taken to avoid contamination with chorioamnion. Tissue was washed repeatedly with PBS to remove contaminating blood and was then minced into smaller pieces (approximately 1-2 mm^3). Pieces of minced placental tissue (0.5 g in total) were transferred into each well of a 6-well tissue culture plate (Greiner Bio-one, Germany) containing 2.5 mls of Ultraculture medium (Cambrex, Belgium) supplemented with 2mM Glutamax (Life Technologies) and 100 U/ml Penicillin G, 100 µg/ml streptomycin sulphate and 0.25 µg/ml amphotericin B (PSF; Life Technologies). Care was taken to avoid any blood clots or fibrous tissue.

Optimal levels of all agonists were determined for the following final concentrations: peptidoglycan (PGN; TLR2, 3 µg/ml); poly I:C (TLR3, 25 µg/ml); LPS (TLR4, 100 ng/ml); flagellin

(TLR5, 100 ng/ml); R848 (TLR7/8, 100 ng/ml); loxoribine (TLR7/8, 100 µM); single stranded polyU/LyoVEC complexes (ssPoly, TLR7/8, 1 µg/ml); ODN2216 CpG (TLR9, 1 µM) and control ODN (all from Invivogen). An unstimulated control was always included. Plates were incubated at 37°C in 5% CO_2 for 24 hours. Cell/tissue free culture supernatants were collected by centrifugation and stored at -20°C until assayed.

Placental explant cultures prepared from placentas obtained following elective caesarean section at term were: (i) treated with the optimised concentration of each TLR ligand, then after incubation at 37°C for 24 hours, the tissue was washed by centrifugation and re-cultured for 24 hours in fresh media containing concentrations of TLR ligands as indicated in the results; (ii) treated with the optimised concentration of each TLR ligand alone and in the presence of 0.4, 4 or 40 ng/ml dexamethasone (Sigma, USA) for 20 - 24 hours. Supernatants were harvested and stored at -20°C until analysis of IL-6 levels by ELISA.

Extreme care was taken to limit endotoxin contamination during explant preparation. These precautions included the use of disposable plastic-ware and other consumables (e.g. scissors) whenever possible (28). All media/reagents were tested by the manufacturers and reported as endotoxin free.

Real time quantitative PCR (qPCR)

Placental biopsies were preserved in RNA*later*® (Sigma, Poole, UK) at -80°C. Preparation of DNA-free RNA from homogenised tissue and reverse transcription were performed as described in detail previously (6). Confirmation of genomic DNA-free status and successful reverse transcription was obtained by PCR amplification of the S15 ribosomal protein gene. Real-time PCR for all genes of interest (TLR 1-10) and 3 housekeeping genes was carried out using the iCycler IQ (ver.3.1 Bio-Rad). The house keeping genes, succinate dehydrogenase complex subunit A (SDHA), TATA box binding protein (TBP**)** and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide **(**YWHAZ) were selected as they are the most stably expressed in the human placenta (29). Primer sequences and the specific conditions particular to each PCR are listed in Table 2 with further details as described previously (6).

Enzyme linked immunosorbent assay (ELISA)

TNFα, IL-6, and IL-10 in supernatants from placental explant cultures were measured using commercial ELISA kits according to the manufacturer's instructions (OptEIA; BDBiosciences). The sensitivity of each of the ELISAs varied between 1 and 4pg/ml and the intra- and inter-assay coefficients of variation were <10%.

Statistical analysis

The Mann Whitney U test for two independent samples was used to compare TLR expression and activity; for the dexamethasone experiments the Friedmans test with Dunn multiple comparison posthoc test was used (GraphPad Prism Version 6, GraphPad Software Inc, USA).

Results

Comparison of TLR activity between the IA-PTL group and the term laboured group

Comparison of the cytokine outputs from the 10 placentae obtained following suspected IA-PTL was made with term-laboured group (Figure 1 a – g). The concentration of TNFα and/or IL-6 was statistically significantly reduced in preterm samples (n = 10) compared with term samples (n = 17) treated with PGN (TLR2) poly I:C (TLR3), LPS (TLR4), flagellin (TLR5), R848 (TLR7/8) but not zymosan or loxoribine (TLR7/8) (*p* values shown on graphs). None of the preterm or term samples responded to CpG for the cytokines measured in this study. Constitutive production of IL-6 was also significantly reduced in the preterm samples (p = 0.02; Figure 1 h). In contrast, irrespective of the TLR ligand used, IL-10 production did not differ significantly between term and preterm samples.

Comparison of mRNA for TLR1 - TLR10 between term laboured and preterm laboured groups.

Placental biopsies from all deliveries were placed in RNA*later*® and RNA extraction, cDNA synthesis and PCR were conducted as mixed batches (preterm and term). The expression of TLR transcripts by the two groups did not differ statistically for any of the TLRs (Figures 2 a – j; *p* values shown on graphs).

Effect of pre-exposure to TLR ligands on TLR ligand stimulated cytokine output of placental explants

As all but one of the 10 preterm-SVD placentas came from deliveries with evidence of intrauterine infection it was possible that they were exposed *in utero* to one or more of the TLR ligands used in this study. Pre-exposure to LPS is known to reduce the level of response upon secondary exposure to LPS, a process known as endotoxin tolerance (30). Therefore it was postulated that pre-exposure to any one ligand could explain the reduced cytokine outputs (at least for IL-6 and TNFα) in response to all ligands in the preterm group. Therefore, placental explants prepared from term non-laboured placenta were exposed to each TLR ligand and after 24 hours culture the first dose of ligand was removed by centrifugation and the tissue re-cultured in fresh medium in the presence of the same or another TLR ligand. After a further 24 hours culture, supernatants were harvested and analysed for IL-6. Data obtained for unstimulated, LPS-, PGN- and flagellin-treated samples are shown (Figure 3 a-d). There was no consistent trend in the cytokine output but reduced outputs were not an overarching feature.

Effect of dexamethasone and gestational age on cytokine output of preterm placental explants

As all the women in the IA-PTL group received steroids prior to delivery as part of their clinical therapy for preterm labour, the effect of dexamethasone (steroid) treatment on TLR-stimulated cytokine output by the placenta was explored. Placental explants prepared from non-laboured term deliveries (elective caesarean section) were treated with dexamethasone and IL-6 outputs in response to all TLR ligands determined. IL-6 was chosen as it can be induced by all ligands studied and is produced constitutively. Constitutive and TLR ligand-stimulated IL-6 was reduced in the presence of dexamethasone for all TLR ligands studied (only constitutive and the response to PGN, Poly (I:C), LPS and flagellin are shown; Figure 4a). The reduction in the cytokine output following treatment with 40 ng/ml dexamethasone was significant with $p < 0.05$ for ligand-stimulated explants (with the exception of flagellin). Given all women who delivered preterm had been administered corticosteroids prior to delivery and the in vitro steroid use model showed decreased responses via TLRs upon steroid exposure we also considered the impact of gestational age alone on the response. Figure 4b shows that decreasing gestational age is associated with decreasing LPS-stimulated IL-6, even when mothers have been administered coroticosteroids, suggesting that the reduced TLR ligand response by the preterm placenta is a consequence of gestational age and not steroid exposure.

Discussion

IL-6 and TNFα but not IL-10 outputs after stimulation of the placenta with TLR2, TLR3, TLR4, TLR5 and TLR7/8 ligands were significantly reduced in the preterm compared with the term laboured group. TLR9 activity was not detected in either group. Despite these functional differences on comparison with the term group there were no differences in the levels of mRNA for any of the TLRs studied. This is similar to our results obtained for term laboured placentae versus term non-laboured placentae, in which there was no apparent correlation between functional output and mRNA levels for any of the TLRs (6). These results suggest that TLR protein expression should be studied in greater detail and/or that negative regulators of TLR-dependent signalling might have a critical role. A number of inhibitors of TLR-mediated activity have now been described. Over-expression of Tollip (toll interacting protein) results in impaired NF-κB activation which can diminish the TLR response and thereby the associated cytokine output (31). Similarly, SIGGR (single immunoglobulin IL-1R-related molecule) acts as a negative regulator of interleukin (IL)-1 and lipopolysaccharide (LPS) signalling (32, 33). Also, the placenta is an abundant source of suppressor of cytokine signalling 1 (SOCS-1), with 3-fold greater gene expression than the spleen (34), and SOCS-1 protein has been identified as a negative regulator of TLR signalling (35). Investigation of the relative expression of these inhibitors in preterm versus term tissues might prove fruitful.

One of the challenges of undertaking studies such as this in humans is that samples tend to be only accessible once labour is completed (although this might not be the case in emergency caesarean sections). Consequently, results obtained using the samples for this study might not be representative of the response occurring *in utero* prior to or earlier in labour. All but one of the preterm deliveries had evidence of infection so the possibility that previous stimulation of a TLR (i.e. *in utero*) might down-regulate secondary responses (i.e. *in vitro*) was considered. This could be because of exhaustion or down regulation of the relevant TLR pathways *in vivo* as a result of response to the presence of infection. Pre-exposure to LPS is known to reduce the level of response upon secondary exposure to LPS, a process known as endotoxin tolerance (36, 37). Endotoxin tolerance is a well-known phenomenon, described both *in vivo* and *in vitro*, in which repeated exposure to endotoxin results in a diminished response, usually characterised as a reduction in pro-inflammatory cytokine release. With increasing understanding of the part played by TLR4 signalling in endotoxin release it has become clear that tolerance occurs to other TLR ligands in addition to endotoxin (36). To test this as an explanation for the lower IL-6 and TNFα responses in preterm samples we used term non-laboured placentas from elective caesarean sections and treated them *in vitro* with the study TLR ligands, washed the tissue, and then re-exposed it to the TLR ligands. There was no evidence of diminished response to any TLR ligand on secondary exposure. While a more sophisticated model might yield contrasting results our findings suggest that prior intrauterine exposure to the ligand does not explain the reduced cytokine output of preterm placental explants in comparison to term explants.

Women in preterm labour are treated with various pharmacotherapies to try and halt premature labour (tocolytics) but also to prepare the baby for premature delivery (corticosteroids). As corticosteroids are known to down-regulate inflammatory responses (38, 39) the possibility that corticosteroids might explain the findings was considered. Constitutive and TLR ligand-stimulated IL-6 was reduced in the presence of dexamethasone for all TLR ligands so prior exposure to steroids in the preterm group could contribute to reduced TLR function in these samples. However, as decreasing gestational age seems to associated with decreasing TLR-stimulated cytokine output, even with corticosteroid exposure, it is likely that gestational age is the overriding factor modulating the placental inflammatory response. This needs to better understood if we are to elucidate the role of the inflammatory response in preterm labour.

While TLRs were the focus here, other families of PRRs have been described including NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs) and cytosolic DNA sensors (CDS), that may contribute to the mechanisms of cytokine production at the maternal-fetal interface (5). However, to date only NLR activity has been examined in the placenta with both NOD1 and NOD2 expressed in the first trimester placenta where they are localised to the syncytiotrophoblast and cytotrophoblast; only NOD1 is expressed in term trophoblast cells (40, 41). This corresponds to the functional outputs of first versus third trimester trophoblast cells; first trimester cells respond to both iE-DAP (γ-D-Glu-mDAP; NOD1 ligand) and MDP (muramyl dipeptide; NOD2 ligand), while third trimester cells only respond to iE-DAP (42) which can induce preterm delivery in a murine model (43). Certain NLRs with ASC (apoptosis-associated speck-like protein containing a CARD) and caspase-1 can form the inflammasome for the processing and secretion of IL-1β and IL-18 (44). Several of these NLRs, including NLRP1, NLRP3 and NLRC4 are expressed in first trimester cytotrophoblasts with their expression enhanced and IL-1β induced in the presence of LPS (45).

This work clearly demonstrates that the human preterm placenta expresses functional TLR2, 3, 4, 5, and 7/8. However contradictory to the assumption that infection-associated preterm labour would be associated with enhanced cytokine levels secondary to its infection related etiology, we found reduced cytokine output in the preterm samples in comparison to term samples. This does not appear to result from a mechanism similar to endotoxin tolerance and whilst it could reflect exposure to steroids this reduced output by the preterm placenta might also be due to over expression of TLR inhibitors or post-transcriptional modification of TLR expression. Unfortunately, samples of placental membranes were not included in this study but the response by them might differ to that by the placenta. Further studies are required to consider spatial differences in TLR and other PRR expression and function with investigation of the expression of inhibitors of TLR activity particularly worthwhile pursuing.

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Figure Legends

Figure 1. Comparison of cytokine outputs (TNFα, IL-6 and IL-10) from placental explants of term-laboured (n=17) versus preterm-svd (n =10) in response to (a) PGN, (b) LPS, (c) flagellin, (d) zymosan, (e) R848 (f) poly I:C, and (g) loxoribine; IL-6 levels produced constitutively are also shown (h). Mann Whitney U test for two independent samples was employed for statistical analysis; $p < 0.05$ was considered significant. (^{o} denotes outliers)

Figure 2. TLR transcript expression in term and preterm placentas. Box and whisker plots depicting comparison between expression of mRNA for TLRs 1-10 in the term-laboured (T) (n=12) and preterm-SVD (P) (n = 10) groups. Mann Whitney U test for two independent samples was employed and the p values are shown on the graphs.

Figure 3. Effect of pre-exposure to TLR ligands on TLR ligand stimulated cytokine output of placental explants. Explants were prepared from term non-laboured placentas and treated with TLR ligands, as indicated on each figure for 24 hours. After washing the tissue it was re-cultured in the presence of the same or different ligand as indicated on the figure. Supernatants were harvested after a further 24 hours and IL-6 production in response to (a) constitutive (Cons)/no stimulation, (b) LPS, (c) PGN, (d) Flagellin for first 20 - 24 hours then no, flagellin, LPS or PGN stimulation of the second 20 – 24 hours determined by ELISA (n = 3; mean ± SEM).

Figure 4. Effect of dexamethasone and gestational age on IL-6 production by placenta. (a) Placental explants were prepared from term non-laboured placentas and treated with 0, 0.4, 4 and 40 ng/ml dexamethasone in the presence of various TLR ligands. Supernatants were harvested after 20 - 24 hours and IL-6 production in response to PGN, Poly (I:C), LPS or flagellin determined by ELISA (n = 3; mean ± SEM). Statistical significance as determined by Friedmans test with Dunn multiple comparison posthoc test; *<0.05. (b) Unstimulated and LPS stimulated IL-6 (pg/ml; mean ± SEM) by gestational age groups: < 32 weeks (n = 4), 32 to <37 weeks (n = 6), and >37 weeks (n = 12).

Case Number	Gestation in weeks	Mode of delivery	Reason for delivery	Evidence of infection
1	25	SVD	IAPTL	Funisitis
2	26	SVD	IAPTL	Urinary infection
3	27	SVD	IAPTL	Raised White cell count
4	31	SVD	IAPTL	None
5	33	SVD	IAPTL	GBS on swabs
6	33	SVD	IAPTL	Histologic Chorioamnionitis
7	34	SVD	IAPTL	Raised CRP
8	36	SVD	IAPTL	Coliforms on swabs
9	36	SVD	IAPTL	Raised White cell count
10	36	SVD	IAPTL	Urinary infection
11	35	SVD (induced)	Pre eclampsia	None
12	32	Caesarean	Pre eclampsia	None
13	31	Caesarean	IUGR	None
14	33	Caesarean	IUGR	None
15	36	Caesarean	Maternal Glaucoma	None

Table 1. Characteristics of preterm samples**.**

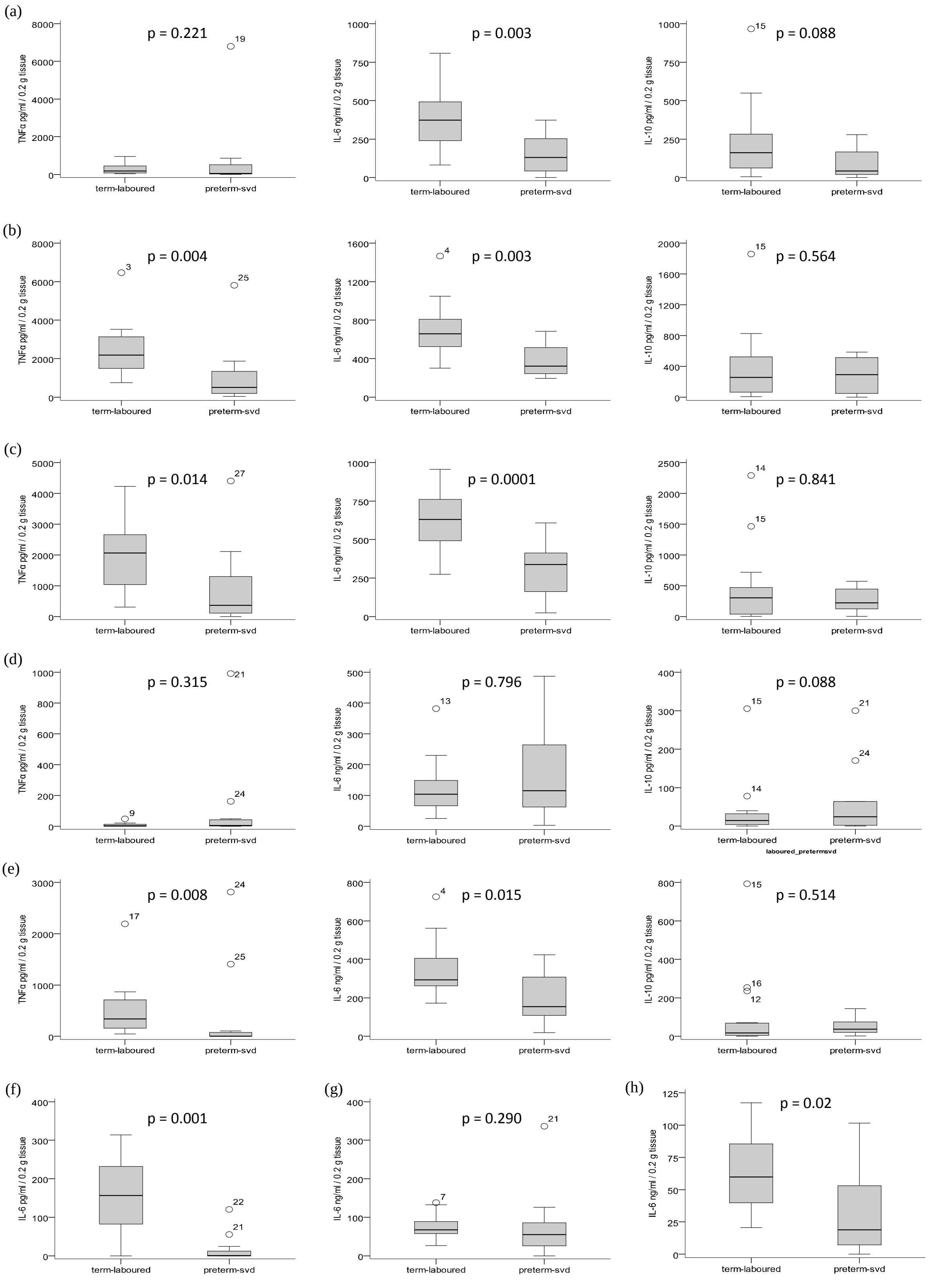
cDNA	Forward primer	Reverse primer	Fragment size	Pr conce
				F
TLR-1	5’-CAGTGTCTGGTACACGCATGGT-3’	5’-TTTCAAAAACCGTGTCTGTTAAGAGA-3’	104	5µM
TLR-2	5’-GGCCAGCAAATTACCTGTGTG-3’	5’-AGGCGGACATCCTGAACCT-3’	67	5µM
TLR-3	5’-CCTGGTTTGTTAATTGGATTAACGA-3’	5’-TGAGGTGGAGTGTTGCAAAGG-3’	82	5µM
TLR-4	5’-CAGAGTTTCCTGCAATGGATCA-3’	5’-GCTTATCTGAAGGTGTTGCACAT-3’	88	5µM
TLR-5	5’-TGCCTTGAAGCCTTCAGTTATG-3’	5’-CCAACCACCACCATGATGAG-3’	77	5µM
TLR-6	5’-GAAGAAGAACAACCCTTTAGGATAGC -3’	5’-AGG~~C~~CAAACAAAATGGAAGCTT-3’	88	5µM
TLR-7	5’-TTTACCTGGATGGAAACCAGCTA-3’	5’-TCAAGG~~C~~CTGAGAAGCTGTAAGCTA-3’	73	5µM
TLR-8	5’-TTATGTGTTCCAGGAACTCAGAGAA-3’	5’-TAATACCCAAGTTGATAGTCGATAAGTTTG-3’	83	5µM
TLR-9	5’-GGACCTCTGGTACTGCTTCCA-3’	5’-AAGCTCGTTGTACACCCAGTCT-3’	151	5µM
TLR10	5’-TGTTATGACAGCAGAGGGTGATG-3’	5’-GAGTTGAAAAAGGAGGTTATAGGATAAATC-3’	151	5µM
SDHA*	5’-TGGGAACAAGAGGGCATCTG-3’	5’-CCACCACTGCATCAAATTCATG-3’	86	5µM
TBP*	5’-TGCACAGGAGCCAAGAGTGAA-3’	5’-CACATCACAGCTCCCCACCA-3’	132	5µM
YWHAZ*	5’-ACTTTTGGTACATTGTGGCTTCAA-3’	5’-CCGCCAGGACAAACCAGTAT-3’	94	5µM

Table 2. Specifications for qPCR.

***SDHA**-Succinate dehydrogenase complex, subunit A;

TBP- TATA box binding protein;

YWHAZ- Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptid



(a)
p = 0.221
TNFα pg/ml / 0.2 g tissue
term-laboured
preterm-svd
p = 0.003
IL-6 ng/ml / 0.2 g tissue
p = 0.088
IL-10 pg/ml / 0.2 g tissue
(b)
p = 0.004
p = 0.003
p = 0.564
(c)
p = 0.014
p = 0.0001
p = 0.841
(d)
p = 0.315
p = 0.796
p = 0.088
laboured_pretermsvd
(e)
p = 0.008
p = 0.015
p = 0.514
(f)
p = 0.001
IL-6 pg/ml / 0.2 g tissue
(g)
p = 0.290
(h)
p = 0.02

Figure 1 (a-h)

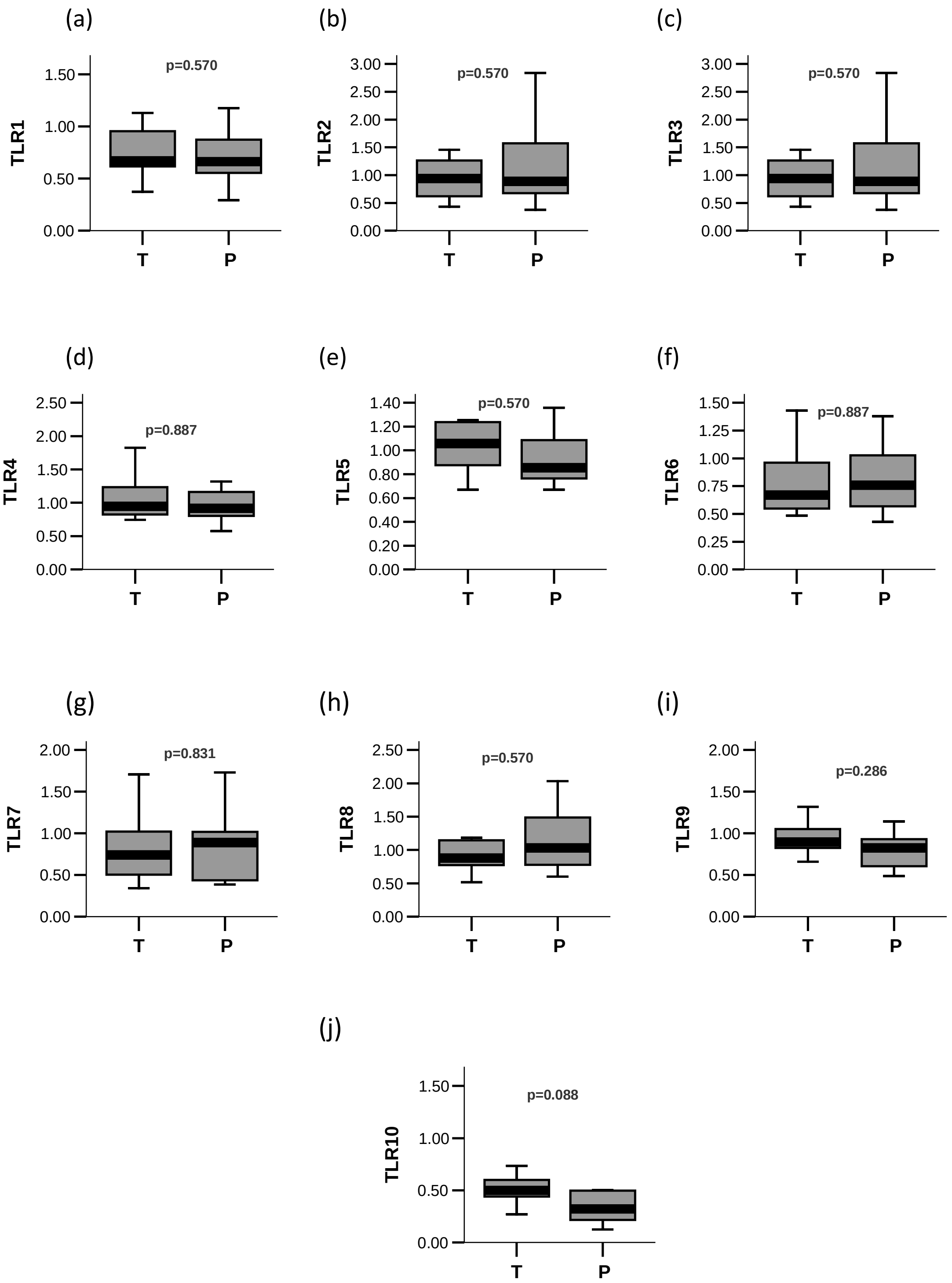

(a)
p=0.570
TLR1
T
P
(b)
p=0.570
TLR2
T
P
(c)
p=0.570
TLR3
T
P
(d)
p=0.887
TLR4
T
P
(e)
p=0.570
TLR5
T
P
(f)
p=0.887
TLR6
T
P
(g)
p=0.831
TLR7
T
P
(h)
p=0.570
TLR8
T
P
(i)
p=0.286
TLR9
T
P
(j)
p=0.088
TLR10
T
P

Figure 2 (a-j)

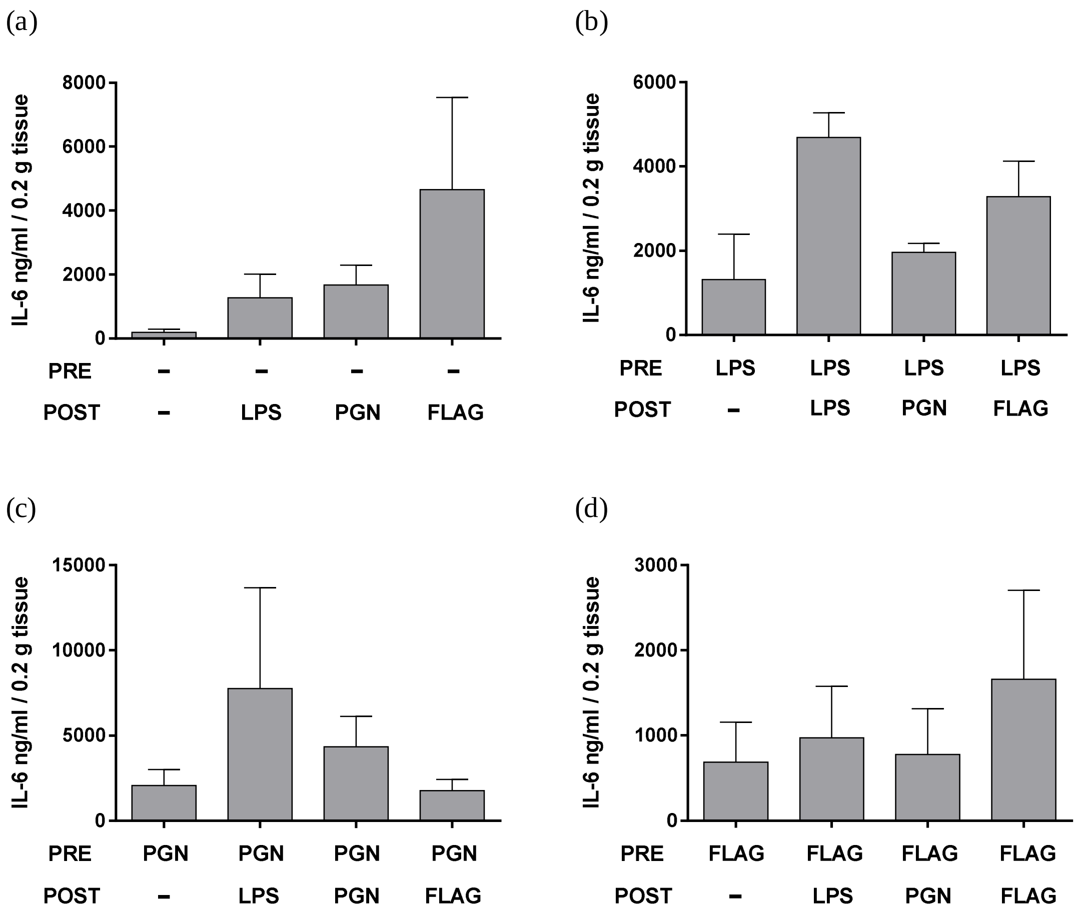
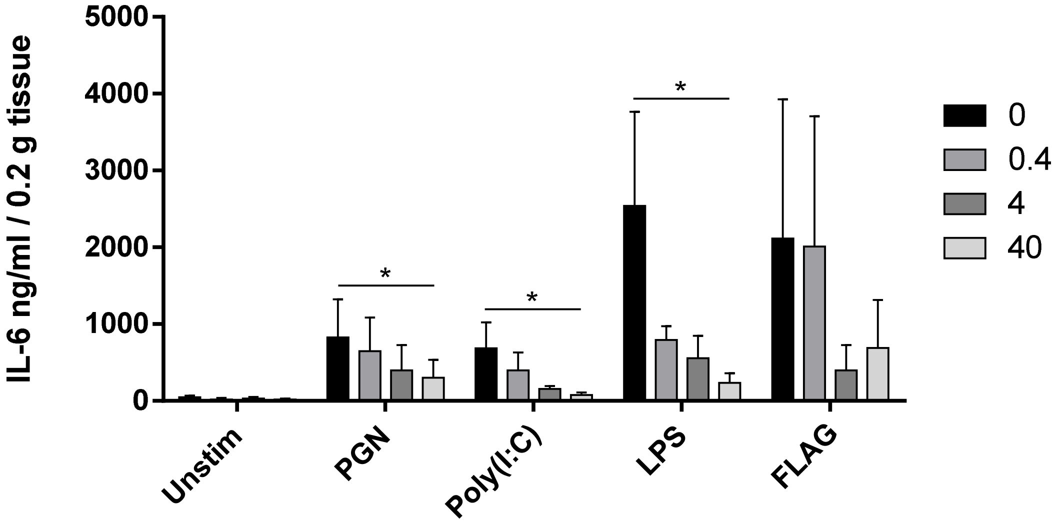

(a)
IL-6 ng/ml / 0.2 g tissue
8000
6000
4000
2000
0
PRE – – – –
POST – LPS PGN FLAG
(b)
IL-6 ng/ml / 0.2 g tissue
6000
4000
2000
0
PRE LPS LPS LPS LPS
POST – LPS PGN FLAG
(c)
IL-6 ng/ml / 0.2 g tissue
15000
10000
5000
0
PRE PGN PGN PGN PGN
POST – LPS PGN FLAG
(d)
IL-6 ng/ml / 0.2 g tissue
3000
2000
1000
0
PRE FLAG FLAG FLAG FLAG
POST – LPS PGN FLAG

Figure 3 (a-d)

(a)



(b)

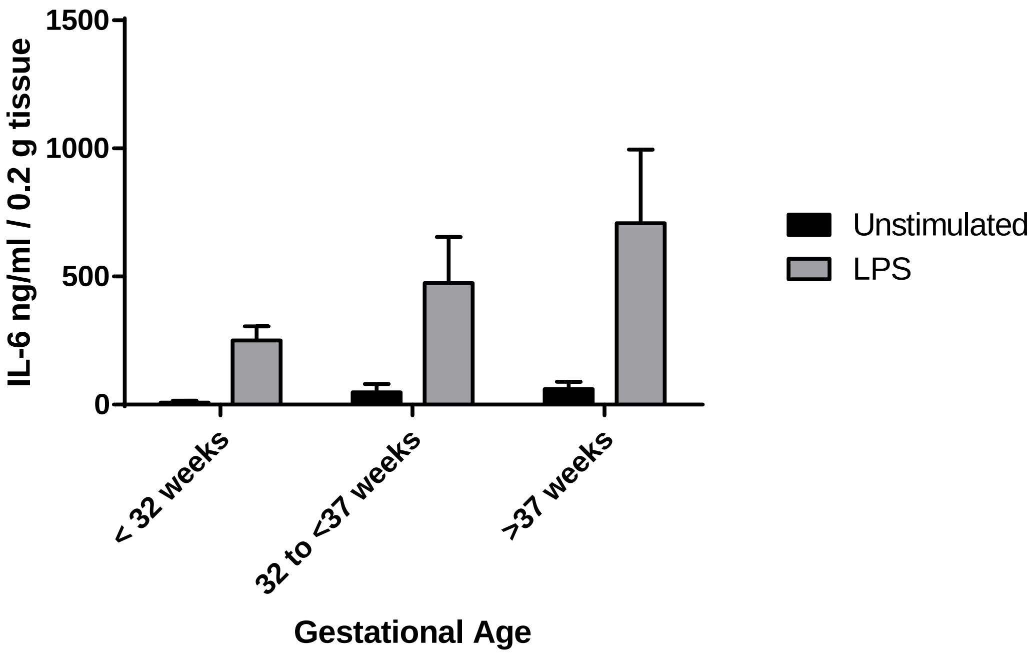


Figure 4 (a & b)