Toll-like receptors and mechanism of human parturition

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A thesis submitted for the Degree of Master of Science

February 2009

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Table of contents

List of figures

List of tables

List of abbreviations

mg	Milligram
µg	Microgram
ng	Nanogram
pg	Picogram
mL	Millilitre
µL	Microlitre
mM	Millimolar
cm	Centimetre
2-AP	2 Aminopurine
ANOVA	Analysis Of Variance
CD 14	Cluster Determinant 14
cDNA	Complementart Deoxy-Ribonucleic Acid
CI	Confidence Interval
COX-2	Cyclo-oxygenase-2
Cpn 10	Chaperonin 10 (Heat Shock protein 10)
CS	Caesarean Section
CRP	C reactive protein
C_T value	Threshold cycle value
DAB	Diaminobenzidine tetrahydrochloride
DD	Death Domain
DDW	Deionised distilled water
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
HCL	Hydrochloric acid
HRP	Horseradish peroxidase
HSP 70	Heat shock protein 70
ICC	Immuno-histochemistry
IDDM	Insulin dependent diabetes mellitus
IgG	Immunoglobulin G

IUGR	Intrauterine growth restriction
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IFN-γ	Interferon-gamma
iNOS	Inducible nitric oxide synthase
IQR	Interquartile range
IRAK	Interleukin 1 receptor associated kinase
KDa	Kilodalton
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LRRs	Leucine-rich repeats
mRNA	Messenger ribonucleic acid
MMP	Matrix metalloproteinase
MPA	Medroxyprogesterone acetate
MyD 88	Myeloid differentiation factor 88
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PG-E	Prostaglandin E
PG-F	Prostaglandin F
PIH	Pregnancy induced hypertension
PKR	Double stranded RNA-dependent protein
PNLs	Polymorphonuclear leukocyts
PRRs	Pattern recognition receptors
PTL	Pre-term labour
PPROM	Pre-term pre-labour rupture of membranes
RIG-I	Retinoic acid inducible gene I

rpm	Round per minute
RR	Relative risk
RT-PCR	Reverse transcriptase polymerase chain
SEM	Standard error of the mean
SP-A	Surfactant protein-A
TIR	Toll/Interleukin 1 receptor domain
TIRAP	Toll/Interleukin 1 receptor associated protein
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-alpha
TRAM	TRIF-related adaptor molecule
TREM-1	Triggering receptor in myeloid cells-1
TRIF	TIR domain-containing adaptor-inducing interferon-β

Acknowledgments

I am deeply indebted to Professor Jane E Norman for her constant encouragement, support and guidance during the course of this work. I am also grateful to Professor Scott M Nelson for his help and advice throughout this work.

My very special thanks are also due to Mrs Fiona Jordan for her guidance and assistance in RT-PCR techniques and myometrial tissue culture experiments. Thanks are also due to Mrs Anne Young for guidance in preparing myometrial tissue blocks for immunohistochemistry and western blotting and to Mrs Anne Brown for her guidance in ELISA techniques.

I also acknowledge the help and advice I received from Dr Dilys Freeman throughout the course of this work and Dr Alastair Gracie for his assistance in the Bio-Plex technique.

I am also grateful to Dr Shrikant Bollapragada for his assistance in patient recruitment, collection and preparation of myometrial samples. I would also like to thank the expectant mothers who agreed to participate in this research and staff at Glasgow royal maternity and Crosshouse Hospitals who assisted me in this study. Without their backing and co-operation this work would not have been possible.

I would like to express my deep gratitude to Tenovus Scotland and to Ayrshire and Arran NHS trust who provided financial support for the work presented in this thesis.

Finally, I wish to express my love and gratitude to my parents, my wife Mervat and to my sons George and Daniel, to whom this work is dedicated.

Declaration

I hereby declare that I am the author of this thesis and that I have consulted all the cited references. The work described here was done by my self unless otherwise stated and it has not been previously accepted for a higher degree.

I have read and approved the above listed statement and confirm that the conditions of the relevant Ordinance and Regulations have been fulfilled

Presentations to learned societies

1. Youssef RE, Norman JE. The role of Toll-like receptors in human parturition. The Blair Bell Society biennial meeting held in the Royal College of Obstetricians and Gynaecologists, London on November 17th 2005.
2. Youssef RE, Bollaprogada SS, Jordan F, Ferguson E, Norman JE. Toll-like receptors and mechanisms of labour. The 53rd annual meeting of the Society of Gynecologic Investigation (SGI), Westin Harbour Castle, Toronto, March 22 – 25, 2006.
3. Youssef RE, Bollapragada SS, Young A, Nelson SM, Norman JE. Toll-like receptor-2 protein expression is transcriptionally upregulated during labour at term. The 54th annual meeting of the Society of Gynecologic Investigation (SGI), Reno, Nevada, March 14 – 17, 2007.
4. Youssef RE, O'Gorman N, Jordan F, Bollapragada SS, Nelson SM, Norman JE. Effects of Medroxyprogesterone acetate and 2 Aminopurine on LPS induced cytokine production in human myometrium at term in vitro. The 55th annual meeting of the Society of Gynecologic Investigation (SGI), San Diego, California, March 26 – 29, 2008.

Summary

The process of parturition whether physiological at term or pathological in preterm labour is a complex one driven by fetal, placental and maternal signals. There is compelling evidence to suggest that inflammatory mediators, driven by endogenous factors or pathologically by infection, play a crucial role in stimulating the common parturition pathway leading into cervical ripening, uterine contractions and, finally, expulsion of the foetus and placenta.

Toll-like receptors (TLRs) are a relatively recently discovered family of pattern recognition molecules which have been shown to play a pivotal role in innate immune responses and inflammation. Products of micro-organisms, e.g. bacteria, viruses or fungi, can be considered to be primary ligands of Toll-like receptors. In addition, they are also activated by other endogenous ligands such as heat shock protein 60 and 70, extra domain A of fibronectin and surfactant protein A (SPA). Therefore, local activation of Toll-like receptors may be responsible for initiation of inflammatory cascade, in presence or absence of infection, leading to initiation of labouring mechanisms within the myometrium.

In this study, we hypothesized that Toll-like receptors (TLR 2 & 4) are expressed in labouring myometrium, and are involved in physiologic and pathophysiologic mechanisms of parturition. In order to test this hypothesis, we compared the pattern of expression of Toll-like receptors in myometrial samples obtained from consenting women before and after onset of term and preterm labour. We also aimed to investigate the activation, regulation of and the functional significance of TLR expression with respect to cytokine production in human myometrium at term, and to determine if progestogens could antagonise the effect of TLR ligands such as Lipopolysaccharide (LPS).

We identified the synthesis and presence of TLR2 & 4 in human myometrium as shown by expression of their mRNA and protein signals which appear to be increasing by gestation and

possibly by labour status. We also demonstrated a possible role for TLR2 as indicated by upregulation of its protein expression in labour compared with non-labouring myometrium. Although there was also a trend to an increase in TLR4 with labour, this did not reach statistical significance.

In our short-term tissue culture model, we demonstrated that Medroxyprogesterone acetate (MPA) significantly suppressed baseline and LPS induced production of IL-1β, IL-6 and IL-8 in pregnant human myometrium. Although LPS stimulated IL-10 production, there was no significant inhibitory effect with MPA. In contrast, we failed to demonstrate significant upregulation of either TNF-α or IFN-γ in response to LPS, and there was no effect of MPA. We also showed that the specific inhibitor for double stranded-RNA dependent protein kinase (PKR) 2-Aminopurine significantly inhibited both baseline and LPS stimulated myometrial cytokine production.

In conclusion, findings of this work have demonstrated a potential role for Toll-like receptors 2 & 4 in initiation of term and preterm labour and an anti-inflammatory role for the progestogen Medroxyprogestrone acetate in lipopolysaccahride stimulated myometrial tissue culture model in vitro. These findings highlight the need for further studies to examine the role of other progestogens / progesterone, benefits and risks to mothers and to their babies, in prevention of preterm labour.

1. INTRODUCTION

1.1 Premature labour

Spontaneous preterm labour and preterm birth are the leading cause of perinatal morbidity and mortality worldwide. Preterm birth, spontaneous and medically induced, complicated about 11.9% of all pregnancies in 2001 and the number of babies born preterm has been steadily increasing for the past two decades (1). Despite much effort during the past 40 years, nearly all of the improvements with treating this pregnancy complication have been due to advances in neonatology (2). Indeed, the probability of infant survival for 30 completed weeks of gestation is over 90 % and the limit of fetal survival has been extended to 24 weeks (3).

The 2% of babies born before 32 weeks, however, account for about 70 % of all infant mortality. A preterm born infant incurs a 40-fold increase in neonatal mortality. Long term neurological and developmental deficits such as cerebral palsy, chronic respiratory illness, blindness and deafness have been identified in as many as 70% of children with birth weights below 800 grams (4). These complications are inevitably associated with substantial healthcare costs (5) consuming approximately 10% of all paediatric health care costs in the United States, for example, in additional expenditures on health, education, and childcare (6).

Despite decades of investigation, the pathophysiology of premature labour is incompletely understood and consequently, therapies or preventive strategies tailored to each of the many potential causes do not exist. Spontaneous preterm labour appears to be a final common pathway for multiple aetiologies involving the cervix, fetus, fetal membranes, placenta and finally manifests in the responding organ; the myometrium (7). Known causes of preterm labour include poor nutrition, alcoholism, smoking, infection, prelabour rupture of membranes, multiple gestation and coagulation disorders (3). A common theme of many of these conditions is inflammation at the maternal-fetal interface, mediated by pro-

inflammatory cytokines (8). In turn, these will trigger a cascade of reactions ending the phase of myometrial quiescence and starting the process of labour.

1.2 Parturition as an inflammatory process

Parturition is the process of cervical ripening (softening) followed by contractions of the myometrium that leads into effacement and dilatation of the cervix and, finally, expulsion of the fetus. This process involves three independent processes:

- Remodelling of the cervix to allow it to dilate to the width of the reproductive tract
- Weakening and rupture of the membranes in the region overlying the cervix
- Finally, initiation of rhythmic uterine contractions

Increasing evidence indicates that inflammatory mediators play a crucial role in parturition (9, 10, 89). Young and colleagues (11) were among a group of investigators who showed a massive infiltration of leucocytes into the myometrium, cervix, fetal membranes and placenta during labour resulting in increased production of pro-inflammatory cytokines (e.g. IL-1β, IL-6, IL-8 and TNFα) (12). IL-1 and TNFα have been isolated from amniotic fluid prior to the onset of uterine contractions (13). Moreover, IL-1 can induce preterm labour in animal models (14).

Pro-inflammatory cytokines can induce ripening of the cervix in a number of ways. IL-1 β and TNFα increase the production of proteinases (matrix metalloproteinase MMP-1, MMP-3, MMP-9 and cathepsin S) that can digest the collagen and elastin fibres in the extracellular matrix of the cervix (15, 16). A similar effect occurring in the membranes can then weaken their tensile strength and lower their threshold for rupture (17). IL-1α and IL-1β have been shown experimentally to increase COX-2 and PGE2 production by rabbit cervical fibroblast cells (18). Beside the ability of PGE-2 to increase the production of proteinases, it also

increases the permeability of blood vessels for leukocytes trafficking. This effect is also shared by Nitric Oxide (NO), another inflammatory mediator that is increased at term (19).

Release of cytokines during inflammatory process results in upregulation of various inducible enzymes such as cyclo-oxygenase2 (COX2) and inducible nitric oxide synthase (iNOS). Cyclo-oxygenase converts free arachidonic acid into prostaglandin precursors and one of the isoforms, Cyclo-oxygease2, is rapidly inducible and can increase during labour (20). Prostaglandins have an important role in parturition and can stimulate uterine contractions. Additionally, Nitric Oxide, released by inducible Nitric Oxide Synthase (iNOS) stimulates processes important for labour such as cervical ripening (16). Both the iNOS and COX2 genes are activated by the transcription factor Nuclear Factor-κB (NF- κB). Upregulation of NF- κB activity is now known to be central to the process of human labour at term (21).

1.3 Role of infection

Infection is the most frequent cause of Preterm Pre-labour Rupture of Membranes (PPROM) and Preterm Labour (PTL) and is clearly identifiable in more than 30% of cases (22). The ability of systemic maternal infections to cause the onset of labour together with the frequent association of intra-uterine infection with pre-term labour and delivery has led to the current acceptance of intra-uterine infection as a major causal agent of pre-term birth. The route of intra-uterine colonization is most likely to be through ascending infection and commonly associated organisms include: Ureaplasma urealyticum, Mycoplasma hominis, Streptococcus agalactae and Escherichia coli. However, an obligate systemic component to the pathogenesis of bacterially induced labour is almost certain as mere microbial colonisation of the chorioamniotic membranes may not always elicit a fetal or maternal inflammatory response (23). In their interesting experiment on a murine model, Hirsch and Wang found that bacterial inoculation of a ligated uterine horn caused preterm delivery from the free horn in an

inoculation dose-dependent fashion. Further, the potency of these inocula to cause PTL and also of similar inocula administered in a distant organ (e.g. renal parenchyma) was in no way less than that injected into the intact bicornuate uterui (24).

Infection may cause preterm pre-labour rupture of membranes (PPROM) and preterm labour (PTL) by causing premature activation of cytokine cascades leading into activation of parturition mechanisms. A number of studies suggested that production of pro-inflammatory mediators such as IL-1 β, TNFα, IL-6, IL-8 and MMP-9 is increased during intra-amniotic infection, PPROM and PTL (14, 25, 26).

As previously discussed, pre-term labour was experimentally induced in rodents or primates through administration of bacteria or bacterial products in late pregnancy. This was preceded by increased production of pro-inflammatory cytokines including TNFα and IL-1 β. In fact, a causal role for IL-1 β was suggested, as its administration was able to mimic the effects of bacterial administration by causing PTL (26). The source of these cytokines is unclear but may originate from trophoblast cells as well as fetal or maternal macrophages. Cytokines could be experimentally produced in cultures of amnion, chorion and decidual cells in response to bacteria or bacterial products.

1.4 Human immune response

Vertebrate immunity can be broadly categorized into:

1.4.1 Adaptive immunity

Adaptive immune responses are mediated by clonally distributed B and T lymphocytes and are characterized by specificity and memory. Recognition relies on the generation of a random and highly diverse repertoire of antigen receptors, the T- and B-cell receptors, followed by clonal selection and expansion of receptors with relevant specificities (27). This

mechanism accounts for the generation of immunological memory, an important advantage, but has the main limitation that specific clones need to expand and differentiate into effector cells before they can participate in host defense. Therefore, adaptive immune responses are typically delayed for 4 to 7 days.

1.4.2 Innate immunity

To control the infection during the first days, our body relies on the evolutionarily ancient and more universal innate immune system. Its main functions include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of pro-inflammatory signalling cascades, and apoptosis (28). In contrast to adaptive immunity, it is non-specific, rapid and requires no previous exposure to antigens (immunologic memory).

The innate immune system also has an important function in activation and shaping of the adaptive immune response through the induction of co-stimulatory molecules and cytokines (29). In contrast to the clonotypic receptors, expressed by B and T lymphocytes, the innate immune system uses non-clonal sets of recognition molecules, called pattern recognition receptors. Pattern recognition receptors bind conserved molecular structures found in large groups of pathogens, termed pathogen-associated molecular patterns. There are various groups of pattern recognition receptors, which can be secreted, expressed on the cell surface, or resident in intracellular compartments (30).

Based on their sub-cellular localisation, PRRs can be classified into:

a) Soluble PRRs: as acute phase proteins, mannan-binding lectin and CRP
b) Trans-membrane PRRs: as scavenger receptors, Toll-like receptors (TLRs) 1, 2, 4, 5 and 11
c) Intracellular PRRs: as NOD1, NOD2, RIG-I family RNA helicases and TLRs 3, 7, 8 and 9 (28)

It seems that the innate rather than adaptive immunity plays the significant role in PTL. Although a previous preterm birth is the strongest predictor of a subsequent preterm birth, gestation length does not decrease in proportion to number of previous deliveries, as it would with a classical graft rejection mediated through the memory bearing adaptive (acquired) immunity. Conversely, many women are still able to have normal term pregnancies after a previous per-term birth (31, 32).

Based on existing literature, an inflammatory process is started by presence of either bacterial products or other endogenous stimulus (9, 12, 33). This leads to activation of the innate immune system through cell surface recognition molecules such as the Toll-like receptors family. In turn, they produce an intracellular signal transduction process that results in release of cytokines, chemokines, proteases and other factors. These are the substances responsible for cervical ripening and conversion of the normally quiet uterus into a contractile organ.

1.5 Toll-like receptors

The Toll-like receptors (TLRs) are one of the most important pattern recognition receptor families that can discriminate between chemically diverse classes of microbial products. Furthermore, activation of innate immunity is a critical step to the development of antigen-specific acquired immunity.

At the end of the 20th century, Toll was shown to be an essential receptor for host defence against fungal infection in *Drosophila*, a fruit fly that has innate immunity only (35). One year later, a mammalian homologue of the Toll receptor with similar sequence (now termed TLR4) was shown to induce expression of genes involved in inflammatory responses (36). In addition, a point mutation in the TLR4 gene has been identified in a mouse strain that is unresponsive to Lipopolysaccharide (LPS) (37).

After the characterization of the first mammalian TLR, TLR4, several proteins that are structurally related to TLR4 were identified and named Toll-like receptors. Mammalian TLRs comprise a large family consisting of at least 11 members. TLR1 – 9 are conserved between the human and mouse. However, although TLR10 is presumably functional in the human, the C-terminal half of the mouse TLR10 gene is substituted to an unrelated and non-productive sequence, indicating that mouse TLR10 is non-functional. Similarly, mouse TLR11 is functional, but there is a stop codon in the human TLR11 gene, which results in a lack of production of human TLR11 (27, 38). Gene mapping studies have revealed that TLR genes are dispersed throughout the mammalian genome (30).

Table 1: Types of Toll-like receptors, their chromosomal location and related ligands

Receptor type	**Encoding gene location**	**Microbial ligands**
TLR1	Chromosome 4	Triacyl lipopeptides
TLR2	Chromosome 4	Lipoproteins, glcolipids and peptidoglycans of Gram-positive organisms
TLR3	Chromosome 4	Double-stranded viral RNA
TLR4	Chromosome 9	LPS of Gram-negative organisms
TLR5	Chromosome 1	Bacterial flagellin
TLR6	Chromosome 4	Diacyl lipopeptides
TLR7	Chromosome X	Guanosine or uridine-rich single-stranded viral RNA
TLR8	Chromosome X	Guanosine or uridine-rich single-stranded viral RNA

TLR9	Chromosome 3	Unmethylatd bacterial CpG motifs
TLR10	Chromosome 4	Possibly similar to TLR1 & TLR6
TLR11	Chromosome 4	Non-functioning in humans

(From Armant MA,) Fenton MJ, 2002) (30)

1.5.1 Toll-like receptors and response to infection

Toll-like receptors appear to mediate almost all the changes in gene expression induced by pathogens, including bacteria, viruses and fungi. Expression of TLR receptors is highest in peripheral blood leukocytes, macrophages and monocytes. They are a family of type I transmembrane receptors (as with TLRs 1, 2, 4, 5 and 11) or intracellular (as with TLRs 3, 7, 8 and 9) (28). The transmembrane variety is characterized by an extracellular amino acid terminus. The cytoplasmic portion of TLRs shows high similarity to that of the IL-1 receptor family, and is termed a Toll/IL-1 receptor (TIR) domain. Despite this similarity, the extracellular portions of both types of receptors are structurally unrelated. The IL-1 receptors possess an immunoglobulin-like domain, whereas TLRs bear leucine-rich repeats (LRRs) in the extracellular domain (39). How leucine-rich repeats mediate ligand recognition is still puzzling, especially as it was demonstrated that 7 out of 10 leucine-rich repeat motifs of the CD14 receptor, a transmembrane protein implicated in LPS recognition, could be deleted without affecting LPS binding. Furthermore, each TLR can recognize the most diverse ligands, lacking any structural similarity, making it hard to conceive how one motif can interact with all these molecules (40).

1.5.2 TLRs ligands

All products of microorganisms including bacteria, viruses, fungi and even parasites (e.g. LPS) might be considered to be the primary ligands the TLRs family. Typically, members of the TLRs family may contribute with each other for discrimination between different ligands and eliciting a distinct immune response accordingly (41).

TLRs are also activated by other endogenous agents such as heat shock protein 60 and 70 and the extra domain A of fibronectin (42, 43). Therefore, local activation of TLRs may be responsible for the initiation of inflammation within the myometrium during labour in the presence or absence of infection (44). This mechanism has previously been shown in other inflammatory conditions such as distressed myocardium (45). Surfactant protein-A (SP-A) produced by alveolar type II cells has also been identified as a ligand for TLR4 however it can also act via TLR2. SP-A treatment of human peripheral blood mononuclear cells, lung alveolar macrophages and monocyte cell lines caused rapid activation of NF-κB and increased expression of proinflammatory cytokines (46). Similarly, pulmonary surfactant isolated from human amniotic fluid stimulated prostaglandin synthesis in human amnion discs. Condon et al, (47) proposed that augmented production of SP-A by the maturing fetal lung either physiologically at term or pathologically preterm (in presence of infection) operates via TLRs to provides a key hormonal stimulus for the cascade of inflammatory signalling pathways within the maternal uterus that culminate in enhanced myometrial activity leading to parturition. Yuan et al, (48) studied the characterization of peripheral blood leukocytes and concluded that both term and preterm labour were associated with increased monocyte and neutrophil subpopulation and activation as indicated by increased neutrophil migratory ability and cell surface marker expression. Although this model has been shown to operate in mice, data from human studies suggest that migration of fetal macrophages from

the amniotic cavity or the chorioamniotic membranes into myometrium does not occur during human labour which casts doubts on applicability of this model in human labour (49, 50).

1.5.3 TLRs signalling pathways

TLRs activate signal transduction pathways that induce expression of pro-inflammatory cytokines, chemokines and other immune response genes (Figure 1). Activation of specific TLRs leads to slightly different patterns of gene expression profiles. For example, activation of TLR3 and TLR4 signaling pathways results in induction of type I interferons (IFNs), but activation of TLR2- and TLR5-mediated pathways does not (51). TLR7, TLR8 and TLR9 signaling pathways also lead to induction of type I IFNs through mechanisms distinct from TLR3/4-mediated induction.

Toll-like receptors, with the exception of TLR3, share a common signalling pathway via the adaptor molecule Myeloid Differentiation factor (MyD88) which has a segment of the cytoplasmic Toll/IL-1R (TIR) domain in its C-terminal region and a death domain (DD) in its N-terminal region. Upon stimulation, TLRs recruit MyD88 through interaction of their respective TIR domain. The DD of MyD88 then binds the DD of IL-1R associated kinase (IRAK) and the signal is propagated via TNF receptor associated factor-6 leading to activation of NF-κB and mitogen-activated protein kinases and transcription of relevant genes (38). Recently, a second TIR-containing adaptor protein, TIR-associated protein (TIRAP) / MyD88-adaptor like was identified to be involved in the MyD88-dependent pathways of TLR1/2, TLR2/6 and TLR4 (52).

In addition to the common MyD88-mediated signalling pathway, a MyD88-independent pathway has been identified that involves a third TIR-containing adaptor molecule, TIR domain-containing adaptor-inducing interferon-β (IFN-β; TRIF), which is essential for TLR3 and TLR4 signalling, leading to induction of transcription factor IFN regulatory factor 3 and

the subsequent production of IFN-β and activation and maturation of dendritic cells (53). Recently, a fourth TIR-containing adaptor molecule, TRIF-related adaptor molecule (TRAM) has been shown to be involved specifically in TLR4 mediated MyD88-independent IFN-β production. Thus, these adaptor molecules provide specificities for TLR-mediated signalling (54).

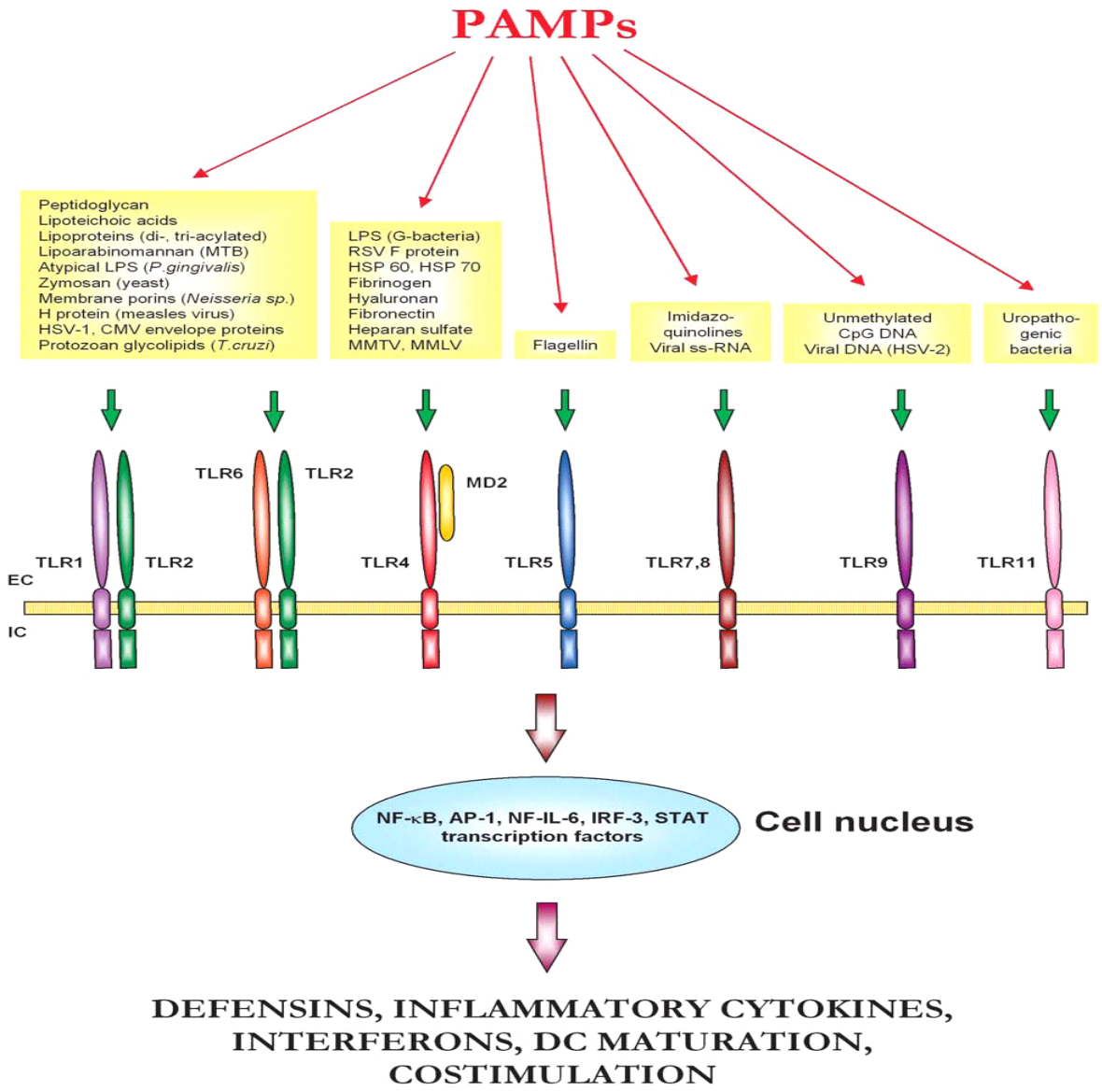


Figure 1: Schematic diagram of the trans-membrane group of mammalian Toll-like Receptors (TLRs) and their response upon stimulation by their potential ligands

(PAMP: pathogen associated molecular pattern, EC: extracellular, IC: intracellular)

(Sandor F & Buc M, 2005) (53)

1.5.4 Control of TLRs expression

Several reports suggest that Toll-like receptors expression is regulated in both a cell type and a stimulus-dependent fashion. According to their cellular expression pattern, TLRs can be categorised as either ubiquitous (as in TLR1) reflecting a possible role as a regulator of TLR signalling, restricted (as in TLR2, 4 and 5) or specific (as in TLR3) (41). Interestingly, TLRs expression declines with age (55) and also shows extreme variability among individuals; e.g., TLRs expression appears to be much higher in farmers' children, which might correlate with individual differences in pathogen susceptibility (56). TLRs participate in positive feedback loops, since the very pro-inflammatory cytokines, whose expression TLR activation stimulates, also stimulates TLR expression. For example, IL-1β, TNFα and IFNγ induce TLR4 transcription in human monocytes and polymorphonuclear leucocytes (57), and TLR4 activation consequently promotes further release of those cytokines. In human endothelial cells, TLR2 and TLR4 may be induced by LPS and IFNγ, leading to increased production of pro-inflammatory mediators (58). Since cytokines can themselves stimulate TLR expression, amplification loops promoting inflammatory responses through TLR likely exist.

On the other hand, several molecules are postulated to modulate TLR signalling pathways. Exposure to microbial components such as LPS results in a severely reduced response to a subsequent challenge by LPS. This phenomenon was first described over 50 years ago (59) and in now called endotoxin (or LPS) tolerance, however mechanism remains unclear (60). In addition, molecules that negatively regulate TLR signalling have been identified. IRAK-M, a member of the IRAK family of serine/threonine kinases, is induced by TLR stimulation in monocyte/macrophages (61). Combination of these negative regulators may finely coordinate the TLR signaling pathway to limit exaggerated innate responses causing harmful disorders.

1.5.5 Clinical implication

Compelling evidence exists for the crucial role of TLRs in inflammation, therefore, targeted inhibition of TLR activation is an attractive therapeutic option in inflammatory conditions. Aldara (Imiquimod) was the first TLR drug to reach the market which was developed by 3M Pharmaceuticals (Minnesota) and was approved in 1997 as a cancer therapy before the TLR mechanism of action was well understood. Many drug candidates have since emerged of which two are currently in phase 3 clinical trials. The first, PF-3512676, is being developed by Coley Pharmaceuticals for non-small cell lung cancer, and the second is a hepatitis B vaccine being developed by Dynavax (Hipsilav) (62). Toshchakov VY et al, (2007) identified cell penetrating peptides which have the ability to selectively affect different TLRs signalling pathways (63). Cpn10 (heat-shock protein 10) has been used clinically in small trials for treatment of rheumatoid arthritis, psoriasis and multiple sclerosis (64). As we learn more about TLRs signal transduction, it is expected that more option would be available for pharmacological targeting of infectious diseases, cancer and allergies (65).

1.6 Role of Progesterone in parturition

A role for progesterone in maintaining pregnancy has been suggested since the early 1960s (66). Although the exact mechanism of onset of labour in human remains incompletely understood, it appears that progesterone does have a role in prolongation of pregnancy. In animal models, rodents show a rapid fall in peripheral progesterone concentration before term and preterm labour (67, 68). However, administration of progesterone even in supra-physiological doses did not prevent infection or inflammation-induced preterm birth in the same animal model (69, 70). In early pregnancy, removal of the corpus luteum before establishment of placental progesterone production is associated with pregnancy loss (71). Pharmacological withdrawal of progesterone by administering 3-beta-hydroxysteroid

dehydrogenase inhibitors is associated with onset of labour and the anti-progesterone mifepristone has been used clinically in order to facilitate termination of pregnancy (72, 73). By contrast, women undergoing assisted reproductive techniques have been administered progesterone in order to maintain pregnancy (74). Progesterone is believed to contribute towards maintenance of pregnancy by maintaining uterine quiescence which is postulated to occur through suppression of the calcium-calmodulin-myosin light chain kinase system, reducing the calcium flux and altering the resting potential of uterine smooth muscles (71). Nevertheless, it is important to note that preterm birth is not a homogenous disease and as such, it is expected that progestational agents may work best only for a select group of patients. Vaginally administered progesterone did not prevent preterm birth in the double-blind placebo-controlled STOPPIT study involving 500 women with twin pregnancy (109).

While a fall in maternal progesterone concentrations has been noticed with connective tissue remodelling in the cervix in many animal species, these changes have not been documented to occur in women before labour. There are no apparent biochemical changes in circulating steroid hormones levels in human pregnancy (75). However, a functional withdrawal of progesterone has been suggested whereby the myometrial relaxing effects of progesterone are nullified (76). Several mechanisms have been proposed including: sequestration of free active progesterone, local cellular inactivation of progesterone bioactivity, production of natural progesterone antagonists and changes in progesterone receptor subtypes expression. Two types of progesterone receptors have been described: the A-form and the B-form. The A-form appears to inhibit signal transduction of the B-form. Myometrial cells that were transfected with both receptors failed to initiate gene expression, whereas those transfected with the B-form only did. The extent of progesterone responsiveness is inversely related to the progesterone-A/progesterone-B expression ratio. During labour, there is significant shift of progesterone receptor types in the myometrium from the B to the A-form causing a significant

increase in the ratio, and there was significantly less expression of both receptors in the amnion (77, 78). In their observation on pregnant human myometrial cell culture, Madsen at al (2004) demonstrated a functional progesterone withdrawal effect to prostaglandins by increasing the myometrial progesterone A/B expression ratio mediated via the protein kinase-A signalling pathway (79).

In the cervix, a shift from B to the A-form of the receptors has also been described with no total change in progesterone receptors. Progesterone receptors have been found to be important to suppression of the inflammatory properties of estradiol in mice. It has been suggested that blockage of the function of progesterone receptors could permit pro-inflammatory cascades to occur in utero at term in conjunction with rising estradiol levels.

In order to assess the benefits and harms, to mothers and to their infants, of its use in prevention of preterm birth, Dodd et al, (2008) identified eleven randomised controlled trials of progesterone supplementation for women at risk of preterm birth which met the inclusion criteria for their systematic review (85). These studies involved 2425 women and 3187 infants. Studies were subdivided by reason the women were considered to be at risk for preterm birth. Trials were excluded if progesterone was administered for the treatment of actual or threatened preterm labour or if progesterone was administered for prevention of miscarriage. Their primary outcomes were perinatal mortality, preterm birth before 34 weeks of gestation, developmental delay in childhood. In their subgroup analysis, they also assessed the effect of time of treatment commencing before or after 20 weeks of gestation, route of administration and different dosage regimens divided into a cumulative dose of less than 500 mg per week or a dose of equal to or greater than 500 mg per week. They showed that use of progesterone for women at risk of preterm birth is associated with a significant reduction in the risk of preterm birth before 34 weeks' gestation (RR 0.15, 95% CI 0.04 – 0.64),

significant reduction in the risk of infant birth weight less than 2500 g (RR 0.64, 95% CI 0.49 – 0.83) but no statistically significant differences for the outcome of perinatal death. Similarly, for women with a short cervix identified on ultrasound, progesterone was not associated with a significant reduction in perinatal death but there was a significant reduction in preterm birth before 34 weeks (RR 0.58, 95% CI 0.38 – 0.87) and a significant reduction in neonatal sepsis (RR 0.28, 95% CI 0.08 – 0.97). For women with a multiple pregnancy however, progesterone was not associated with any significant difference in perinatal birth or in preterm birth at less than 34 weeks of gestation. There was no differential effect on the outcomes when considering route of administration, time of commencement of supplementation or total weekly cummunlative dose of progesterone (80).

Similarly, Sanchez Ramos, et al, in their meta-analysis of 10 randomized controlled trials that compared progestational agents with placebo for patients at risk for preterm birth, concluded that the use of progestational agents and 17 α-hydroxyprogesterone caproate reduced the incidence of preterm birth and low birth weight newborns (81).

While promising, there remains a need for further large randomised controlled trials in order to assess more fully the benefits and potential risks, including those to the baby, and the optimal dose, route and timing of administration before its widespread supplementation for women considered to be at risk of preterm birth (82). The same was highlighted in the committee opinion of the American College of Obstetricians and Gynecologists relating to the use of progesterone supplementation for women at the risk of preterm birth (83). The results of current randomized trials (84, 85) will assist in further elucidation of the precise role, benefits and harms of progesterone therapy for women and for their unborn babies considered to be at risk of preterm birth.

1.7 Toll like receptors and mechanism of parturition

This study explores the hypothesis that Toll-like receptors (TLRs) are expressed in labouring myometrium, and are involved in physiologic and pathophysiologic mechanisms of parturition. Since the putative TLR ligands, HSP70 and the extra domain A of fibronectin are known to be expressed in the myometruim, activation of the TLR by these endogenous ligands could activate invading inflammatory leukocytes, leading to cytokine release, autocrine enhancement of TLR expression and thus amplification of myometrial inflammation. Additionally, TLR mediated upregulation of COX2 could promote prostaglandin release, thus stimulating uterine contractions. Under pathological conditions of infection-associated pre-term labour, microbial-derived products e.g. LPS could activate TLR leading to aberrant initiation of labouring mechanisms within the myometrium. Thus, if our hypothesis of myometrial Toll-like Receptors expression and regulation is correct, they could be a novel target through which inappropriate pre-term parturition can be inhibited.

1.8 Aims of this project

- To quantify TLR 2 & 4 mRNA and protein expression in human myometrial samples obtained from women before or after onset of spontaneous labour, both at term and preterm.

- To localise TLR 2 & 4 protein expression in human myometrium.

- To investigate the activation, regulation of and the functional significance of TLR 2 & 4 expression with respect to cytokine production in human myometrium at term, and to determine if progestogens could antagonise the effect of TLR ligands such as Lipopolysaccharide (LPS).

2. METHODS

2.1 Sample collection

Myometrial tissue biopsies were obtained from the following groups of pregnant women:

- at term (≥ 37 weeks gestation) undergoing elective caesarean section prior to onset of labour
- at term undergoing emergency caesarean section during spontaneous labour
- preterm pregnancy (24 -36 weeks gestation) undergoing elective caesarean section before labour onset
- preterm pregnancy undergoing emergency caesarean section during spontaneous labour

Women were excluded from the study if they had:

- a multiple pregnancy
- evidence of active infection: PROM for >24 hours, temperature >38o C
- induction of labour with prostaglandins or received oxytocic agents
- cervical dilatation < 4 cm or > 9 cm in labouring group

Women were recruited from both the Princess Royal Maternity and from Ayrshire Central Hospitals. The study was approved by the Research Ethics Committee at North Glasgow Hospitals University NHS Trust (05/S0705/18) and from the LREC in Ayrshire and Arran NHS Trust (R&D 272), and was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from each woman prior to recruitment.

Myometrial biopsies were obtained from the upper margin of the lower uterine segment incision following delivery of the placenta and membranes as described by Thomson et al (10). All tissue specimens were divided in three parts: 2 parts were flash frozen in liquid nitrogen for total RNA and protein extraction and stored in 2 separate refrigerators at -70^{o} C in pre-cooled metal pots until processed. The third part was fixed intact in 10% buffered formalin (BDH, Poole, UK) for 24 hours then embedded in paraffin blocks, subsequently cut in 5μm thick sections for immunohistochemistry

2.2 Real-time RT-PCR (TaqMan)

2.2.1 RNA isolation

The frozen tissue samples were ground using a mortar and pestle and the tissue transferred to a universal containing the appropriate amount of Trizol® (Invitrogen, 15596-018, Paisley, UK) [1 ml per 100mg of tissue]. Trizol® disrupts cells and dissolves the cell components freeing the RNA. The ground substance was homogenized in Trizol® using a homogenizer (Polytron®, Novara) for 45 seconds at speed of 5,000 rpm (1500g). The homogenate was aliquoted in 1 ml amounts into microcentrifuge tubes and incubated at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. 0.2ml of chloroform was then added per 1 ml of Trizol® homogenate to allow separation of the solution into an aqueous phase and an organic phase. The tubes were shaken vigorously for 15 seconds until the solution turned a cloudy red colour. The samples were then incubated at room temperature (RT) for 3 minutes followed by centrifugation at 13,000 rpm (10,000g) for 15 minutes at 4^{o}C. The upper aqueous phase of the sample was transferred to a fresh tube. The RNA was then precipitated from the aqueous phase by mixing with isopropyl alcohol (0.5 ml of isopropyl alcohol per 1 ml of Trizol® used in the initial homogenization). The samples were then incubated at RT for 10 minutes then centrifuged at 13,000 rpm (10,000g) for 15 minutes at

4°C. Isopropyl alcohol was then poured off and 1 ml of 75% alcohol was added to each RNA pellet sample. At this stage, samples were stored at -70°C until quantification.

2.2.2 Quantification of RNA

RNA samples were centrifuged at 10,000 rpm (6,000g) for 5 minutes at 4°C, the ethanol was poured off and the tubes gently tapped constantly observing the pellets at the bottom of the tube. The tubes were inverted and placed on filter paper and the pellets dried at RT for 20 minutes. An appropriate amount of Diethylpyrocarbonate- treated deionised water (DEPC DDW) was added to each pellet (to a large pellet, 40ul DEPC DDW and 15 ul to a small pellet). The samples were vortexed, spun down quickly and incubated at 65°C for 5 minutes in a Dri-Block (Techne). Samples were once more vortexed, spun down and incubated for a further 5 minutes at 65°C. All dissolved pellets from the same sample were combined in one tube and samples kept on ice at all times. The quantity of RNA present in the samples was calculated using a spectrophotometer (Ultrospec III, Amersham Pharmacia Biotech). Absorbance readings were taken at 260 nm and 280 nm. An aliquot of each sample was diluted in distilled water and placed in quartz cuvette. The spectrophotometer was first blanked with distilled water alone. The reading at 260 nm gives an indication of the amount of nucleic acid, in this case RNA, and the reading at 280 nm gives an estimation of the amount of protein present in each sample. The ratio of the 260 / 280 readings indicates the purity of the sample, for RNA a ratio between 1.8 – 2 being optimum. The 260 nm reading was used to calculate the amount of RNA present in each sample, 1OD unit at 260 nm is equivalent to 40 mg RNA. Samples were then DNase I treated to eliminate presence of genomic DNA contamination which can be amplified in the PCR process leading to a falsely high value for this sample.

2.2.3 DNase treatment of RNA samples

A DNA free kit (Ambion cat No.: 1906) was used to DNA decontaminate RNA samples. Briefly, to a 5 ul aliquot (upto5ug) of RNA, 16.5 ul of DEPC dH2O, 2.5ul of 10x DNase buffer and 1 ul of DNase I were gently mixed and incubated at 37^{o}C for 30 minutes in the OMN-E thermacycler. 2.5 ul of DNase inactivation reagent was then added and samples were incubated at room temperature for 2 minutes. Samples were centrifuged at 13,000 rpm (10,000g) for 1 minute to pellet the slurry DNase inactivation reagent and the supernatant solution containing the RNA was transferred to a fresh tube and stored at -70^{o}C.

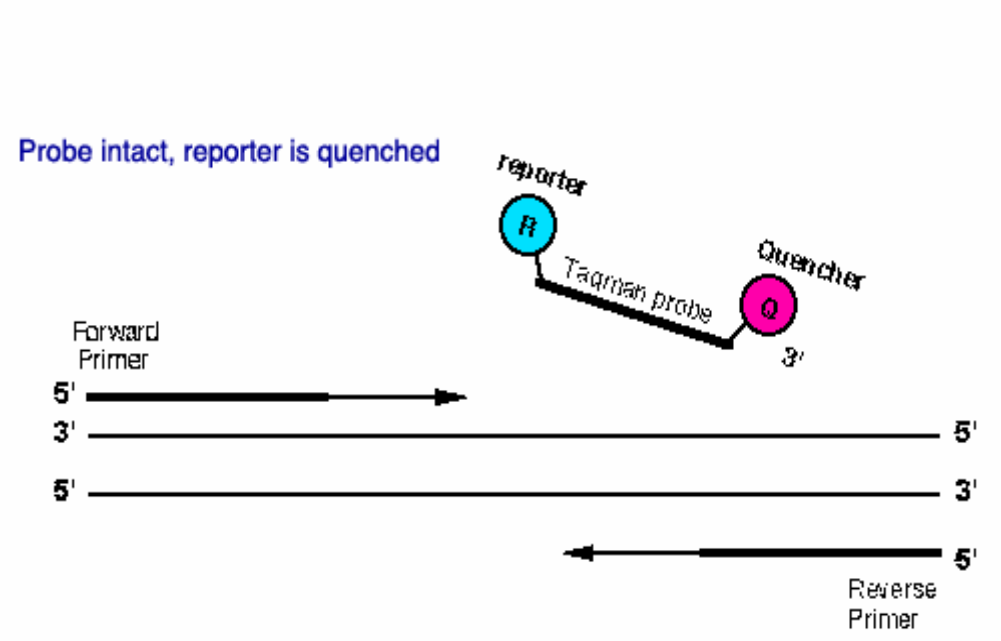
2.2.4 Converting RNA into cDNA

RNA cannot serve as a template for PCR and hence a reverse transcription step is required to convert RNA into cDNA. High Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK, 4322171) was used for this purpose according to the manufacturers instructions . 2x Reverse Transcriptase (RT) TaqMan Universal PCR MasterMix was prepared for the required number of samples. 2x RT MasterMix was prepared from 10x RT Buffer, 25x dNTP, 10x random primers, multiscribe Reverse Transcriptase, RNase inhibitor and nuclease-free water. Each time, a negative control sample was included omitting the mutiscribe Reverse Transcriptase (no RT control) which can detect presence of genomic DNA contamination, if present. 16 ul of 2x MasterMix was added to 4 ul of DNase treated RNA samples and mixed by pipetting. Samples were then briefly centrifuged and incubated at 25^{o}C for 10 minutes then 37^{o}C for 120 minutes (Omne programme 26). Serial dilutions from resulting cDNA (1/10 – 1/100,000) were prepared and samples were kept at -20^{o}C till used in TaqMan reaction.

2.2.5 Real-time reverse-transcriptase PCR (RT-PCR)

Real-time RT-PCR quantifies the initial amount of the template most sensitively and reproducibly and is a preferable alternative to other forms of quantitative RT-PCR that detect the amount of final amplified product at the end-point. Higuchi et al. (1993) pioneered the "real-time" analysis of PCR by constructing a system that detected PCR products as they accumulated (86). The system is based on detection and quantification of a fluorescent reporter, which increases in direct proportion to the amount of PCR product in the reaction. By recording the amount of fluorescence emission at each cycle, it is possible the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of nucleic acid target, the sooner a significant increase in florescence is observed. This method can be used for absolute or relative gene quantification and allelic discrimination assays that detects single base nucleotide mutations and polymorphisms.

Real-time PCR does not detect the size of the amplicon and thus does not allow the differentiation between DNA and cDNA amplification. However, unlike other quantitative PCR methods, real-time PCR does not require post-amplification processing, preventing potential PCR product carry-over contamination, thereby substantially increasing throughput (87).



(From ABI Prism® 7700 Sequence Detection System)

Figure 2: Schematic representation of Reverse Transcriptase Polymerase reaction (RT-PCR)

2.2.6 Setting up 96 well plate

It is important to set aside all reagents and equipments solely for RNA extraction and analysis. Contamination with RNase caused by poor sample handling techniques would degrade the RNA sample. The inadvertent introduction of a contaminating DNA or amplicon would lead to an erroneous result due to amplification of the contaminant. Therefore handling of equipments and reagents should always be carried out while wearing gloves. Accurate pipetting is also critical in order to ensure reproducible results.

All samples were carried out in duplicates. In addition to the control samples including “water only” and “no RT” controls, the housekeeping gene “18S” was used as an endogenous control to adjust for variations in the RNA quantity between samples. The housekeeping gene “18S” had already been previously established in our laboratory for myometrium after comparing it with GADP and β-actin. Results of a preliminary test-plate using cDNA standards in different

dilutions showed that the 1/100 diluted cDNA and the 18S (1/1000) produced a linear regression line and a Threshold Cycle (Ct values: defined as the fractional cycle number at which the fluorescence passes a fixed threshold) between 28 – 30. Therefore these concentrations were used in the rest of the 96 well plates. Sterile filter pipette tips were used at all times.

We used fluorogenic-labelled TLR2 (Hs00152932_m1) & TLR4 (Hs00152939_m1) and 18S probes (4310893E) from Applied Biosystems. The PCR reaction mixture (Applied Biosystems) was prepared as follows: 2x TaqMan mastermix (12.5 ul), 20x target assay mix (1.25 ul), RNase free water (10.25 ul) and 1 ul of cDNA (1/100 dilution) were prepared giving a final reaction mixture of 25 ul per well. PCR reaction mixture was added to every well followed by cDNA, using a fresh tip for every sample. All experiments were carried out in duplicate. The plate was then spun at 1400 rpm (120g) before being placed in the 7900HT TaqMan Machine (ABI), in order to reduce the risk of air bubbles interfering with the laser beam. Thermal cycle conditions are as follows: 50^{o}C for 2 minutes, 95^{o} C for 10 minutes (activation of the AmpliTaq-Gold Polymerase) then 40 cycles of 90^{o} C for 15 seconds (denaturation) followed by 60^{o} C for 60 seconds (annealing and extension). Fluorescence emission spectra are continuously monitored in real-time and analyzed by the 7900HT system using the SDS software. Once PCR run is complete, data collected during thermal cycling is saved and analysed using the Sequence detection software, which calculated the threshold cycle (C_T) values. The expression of the target assays were normalized by subtracting the C_T value of the 18S control from the C_T value of the relevant target assay. The fold increase relative to the control was obtained by using the formula $2^{-\Delta CT}$

2.3 Immunohistochemistry

Immunohistochemistry staining is the application of immunological methods to cells or tissue sections to enable protein visualization by demonstration of a marker conjugated to the final reactant (secondary antibody). Immunohistochemistry was used for studying pattern of expression and localization of TLR2 & 4 protein in sections from paraffin embedded myometrial tissue. Principally, sections were deparaffinized first and rehydrated before applying the primary antibody. Enzyme-conjugated secondary antibodies were then applied and the specific staining was then visualized after adding the enzyme specific substrate.

2.3.1 Preparation of Paraffin sections

Tissue samples were fixed in 10% buffered formalin (BDH, Poole, UK) for 24 hours, 50% ethanol for 8 hours then 70% ethanol for up to 34 hours. The tissue was then cut and placed in uni-cassettes. Each cassette was then placed in a histokinette 2000 and was taken through the following solutions: methylated spirits for 1 hour, methylated spirit phenol for 1 hour 30 minutes, industrial alcohol for 1 hour 30 minutes, then for 2 hours, 2 hours and 2 hours and 20 minutes, industrial alcohol / chloroform (50/50) for 1 hour, chloroform for 1hour 30 minutes, 2 hours, 2 hours and 2 wax treatments for 2 hours and 3 hours 30 minutes. The uni-cassettes were then transferred to metal uni-cassettes and embedded using a Tissue Tek 3. Sections 5 μm thick were cut from the paraffin embedded tissue using a microtome (Leica RM 2135) mounted on 2% silane (Sigma) in acetone (BDH, Poole, UK) coated slides, heated to 60^{o}C overnight and stored in slide boxes until used.

2.3.2 Dewaxing and antigen retrieval

Paraffin sections were heated to 60^{o}C for 35 minutes, deparaffinized in in Xylene (2x 10 minutes each), and rehydrated in 100% ethanol (2x 5 minutes each), 95% ethanol (2x 5minutes each), 70% ethanol (1x for minutes), 50% ethanol (1x for 5 minutes) then Phosphate

Buffered Saline (PBS, 1x for 5 minutes). Endogenous peroxidase activity was removed using 0.5% of freshly prepared hydrogen peroxide in methanol for 30 minutes at room temperature then washed in PBS. Antigen retrieval was performed by microwaving in a pressure cooker at full power for 5 minutes in citrate buffer (10mM, pH 6.0). Slides were then left to cool and washed in H2O for 5 minutes.

2.3.3 ABC method

Sections were then blocked with 20% goat serum / 20% human serum for 30 minutes at room temperature in a humidified box. Excess blocking serum was then tapped off and sections were incubated overnight at 4^{o}C with the primary antibody (TLR2: 1/5,000 in 2% rabbit serum, TLR4: 1/1000 in 2% normal goat serum) in a humidified box. Sections were washed with PBS (2x for 5 minutes each) and the secondary antibody was added (TLR2: biotinylated anti-goat IgG diluted in 2% rabbit serum with 5% of normal human serum, TLR4: biotinylated anti-rabbit IgG 1/200 in 2% normal goat serum and 5% of normal human serum) and slides were incubated at room temperature for 30 minutes in a humidifies box. Sections were washed in PBS 2x for 5 minutes each then incubated with avidin DH / biotinylated horseradish peroxidase H reagent (Vector) in PBS for 30 minutes before washing in PBS 2x 5 minutes each. The antigen was localized using 1 mg/ml Diaminobenzidine Tetrahydrochloride (DAB, Sigma, UK) 0.2% of hydrogen peroxide in 50mM Tris. Hcl, pH 7.6 for 10 minutes at room temperature. Sections were washed in PBS for 5 minutes and in running tap water for a further 5 minutes, staining in Harris haematoxylin (Sigma) for 15 – 20 seconds and finally washed in water. Sections were dehydrated through alcohol in a reverse way to the steps described in re-hydration step and mounted in DPX.

Negative controls included slides incubated without the primary antibody and sections from tonsillar tissue were used as positive control for the primary antibodies.

2.3.4 Image analysis

An Olympus BX50 microscope with x4, x10, x20, x44 lenses, connected to a 3-CCD colour camera (JVC) was used for digital image capture using the image analysis programme Image-Pro Plus 4. Images were imported to Adobe Photoshop 5.5 using the TWAIN 32 interface programme. Pictures were labelled and manually aligned using Adobe Photoshop software.

2.4 Western blotting

To determine protein expression of TLR2 & TLR4, western blot analyses were performed with tissue homogenates isolated using Trizol®.

2.4.1 Protein quantification (Bradford assay)

Protein content of each sample was estimated using the Bradford assay. The unknown protein is diluted to 1:100 in deionised distilled water. Protein standard containing a range of 0 – 10 ug / mL by diluting standard protein (Sigma P 0914) in DDW is prepared. Bradford reagent is then added (1 mL) to each tube in duplicate, mixed and incubated for 5 minutes. Absorbance reading at 595 nm is measured twice with the spectrophotometer with the deuterium lamp off and the mean of 2 readings is calculated. A standard curve of absorbance versus micrograms of protein is prepared and amount of protein is determined from the curve equation chart.

2.4.2 Western blotting

The XCell SureLock MiniCell and XCell blot module kit (Invitorgen) (Fig. 3) were used for protein electrophoresis and blotting. NuPage 4-12% Bis-Tris preset gel (NP0321, Invitrogen) was gently washed with running buffer solution to ensure that air bubbles are displaced from all wells. The gel is then placed in XCell SureLock tank and the upper buffer chamber is filled with 200 mL of running buffer solution (NP0002) containing 100 uL of the reducing agent DiThioThreitol (DTT) completely covering all the wells. Multimark Standard (LC5725,

Invitorgen) is then loaded in the first well (8 uL), positive control in the second well [TLR2: SW480 Cell Lysate (SC-2219), TLR4: HL-60 Whole Cell Lysate (SC-2209) (Santa Cruz)] and the rest of the lanes are then loaded with 5 ug of the protein samples and the lower chamber is the filled with 600 mL of running buffer. The unit is then covered and electrical leads are connected at 100 V for 1 hour.

Figure 3: Xcell SureLock MiniCell (Invitrogen)

Gel is then transferred nitrocellulose membrane (LC 2000, Invitrogen) pre-soaked in transfer buffer solution containing antioxidant. Briefly, gel is placed on transfer membrane ensuring that any air bubbles in between are removed and placed between pre-soaked filter papers and 2 pre-soaked blotting pads. The gel / membrane assembly is then fitted into the cassette with the gel closest to the cathode core (-) and returned to the tank. The inner buffer chamber is filled with the transfer buffer solution till the gel / membrane assembly is completely covered while the outer chamber is filled with deionised water and the lid is paced on the unit and electrical leads connected. Transfer is run at 30 V constant for 1 ½ hour with current between 150 – 110 mA.

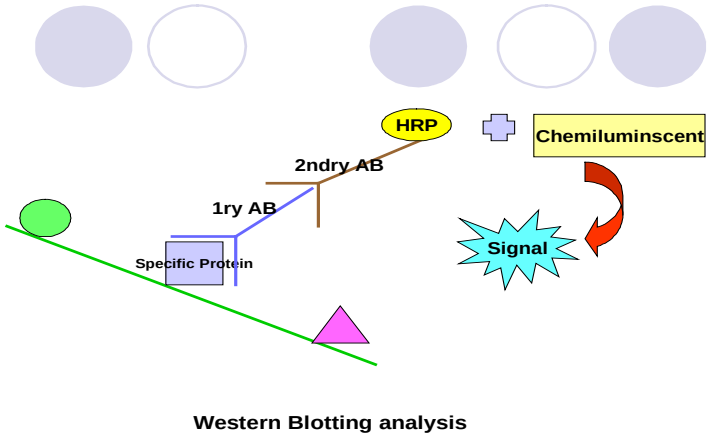


Figure 4: Schematic diagram illustrating principle of western blotting analysis

The membrane is then transferred to a Tupperware box with filtered Marvel solution (5%) in Phosphate Buffer Solution (PBS) with pH of 7.3 and kept on a platform shaker for agitation of membrane during incubation at 4^{o} C overnight. The membrane is washed 3 times with PBS and Tween 20 (0.1%) with each wash lasting 2 minutes followed by the primary antibody (TLR2 Santa Cruz SC-8689, TLR4 Santa Cruz SC-1074) at a concentration of 1:100 in filtered Marvel solution with PBS (pH 7.3) for 2 hours at room temperature. Such antibodies had been previously optimised using different concentrations and durations of exposure to the primary and secondary antibodies. Membrane is then washed 3 times as before with PBS and Tween 20 (0.1%) each lasting for 2 minutes followed by the secondary blocking antibody (for TLR2: rabbit anti-goat HRP, Santa Cruz SC-2922, for TLR4: goat anti-rabbit horseradish peroxidase (HRP), Santa Cruz SC-2054) at a concentration of 1:10.000 for 1 hour at room temperature. Membrane is washed as above and finally, the chemiluminescent substrate (SperSignal® West Pico, Pierce Biotechnology) is added as per manufacturer instructions for 5 minutes. Blot is covered with clear plastic wrap and exposed to X-ray film (Fujifilm, UK) for 1, 2, 5 and 15 minutes. Membranes were eventually re-probed with β-Actin (Santa Cruz

SC-1616) at a concentration of 1:500 overnight at 4^{o} C followed by secondary antibody anti-goat HRP at a concentration of 1:2000 for 1 hour at room temperature followed by washing and chemiluminescent substrate as above.

The density of expression bands on X-ray films were measured using Bio-Rad Multi-Analyst model GS-700 Image Densitometer. After adjustment of the contrast and background, different densities of the bands were measured in duplicates and averaged.

2.4.3 Stripping of western blot

Following immunoblotting, the blot could be reused by stripping and re-probing with anti-TLR4 antibody. Basically, the blot was incubated with the stripping buffer at 50^{o}C for 30 minutes with occasional shaking and was then washed 3 times (10 minutes each) with PBS and Tween 20 (0.1%). The blot was then re-blocked with Marvel 5% as above overnight before rewashing and probing with primary antibody.

2.5 Myometrial short-term tissue culture

This section of the study was performed to investigate the activation, regulation of and the functional significance of TLR expression with respect to cytokine production in human myometrium at term, and to determine if progestogens could antagonise the effect of TLR ligands such as Lipopolysaccharide (LPS). In preparation for this experiment, we have shown that LPS stimulates myometrial production of IL-6 in a dose dependent manner as measured by Enzyme-Linked ImmunoSorbent Assay (ELISA)

2.5.1 Enzyme-linked immunosorbent assay

This preparatory experiment was performed in order to select the LPS dose and time points for measuring myometrial cytokines *in vitro*. IL6 was used as an index cytokine for the inflammatory response.

2.5.1.1 Setting up 24 wells plate

Myometrial samples were obtained as described by Sehringer et al (88), transferred from maternity theatre to the laboratory in Hank's Buffered Salt Solution (HBSS). In tissue culture hood, sample was washed 3 times in Dulbecco's PBS to remove any excess blood. Weighed samples (100 mg) cut into 3 pieces were put in a 24 well plate containing 1 mL of Iscove's medium (Invitrogen) supplemented with Penicillin 100 U/mL, Streptomycin 100 ug /mL and Fungizone (Amphotricin) 250 ng/mL and pre-incubated at 37^{o} C for 60 minutes in a humidified chamber. Samples were then transferred to a new 24 well plate containing fresh culture medium with P.gingivalis lipopolysaccharide & ultra pure S.minnesota lipopolysaccharide (InvivoGen, CA, USA) at concentrations of 10, 100 and 1000 ng/mL. Control well was run without addition of LPS and all samples were carried out in duplicate. The plate is kept in a humidified chamber at 37^{o} C. Two Aliquots 100 uL each were obtained from each well and stored immediately at -20^{o} C for ELISA testing at a later date.

2.5.1.2 IL-6 immunoassay (ELISA)

This assay employs the quantitative sandwich enzyme immunoassay. A flat-bottomed 96-well microplate with monoclonal antibody specific for IL-6 was used (Quantikine®, R&D systems D6050).

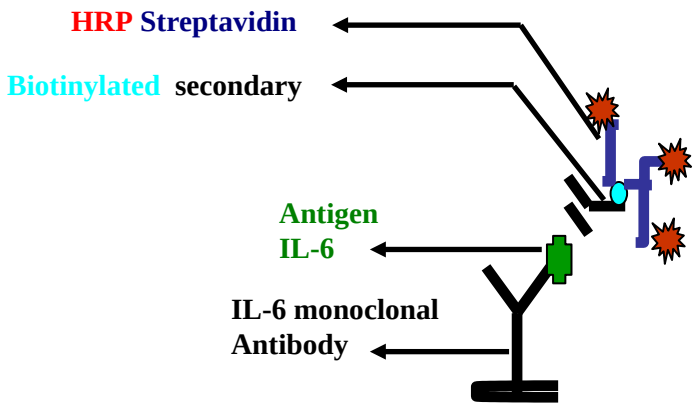


Figure 5: Schematic diagram illustrating the principles of enhanced-sensitivity ELISA using Biotin-Streptavidin system**.** IL-6 monoclonal antibody was used to coat the plate and capture the antigen. IL-6 was detected by a biotin-labeled secondary antibody which was in turn detected by an HRP-labeled streptavidin and the luminescent signal was detected by a plate reader.

Basically, 100uL of the assay diluent RD1W was added to each well followed by 100 uL of Standard, sample or control. The plate is then covered with adhesive strip and incubated for 2 hours at room temperature. This is followed by aspiration and washing of all well for a total of four times with the wash buffer and the plate is decanted by tapping and inverted against a clean paper towel. IL-6 conjugate is then added to each well and the plate is covered and incubated for two hours at room temperature and washed and decanted as before. Finally, 200 uL of Substrate Solution is added to each well and the plate is protected from light and incubated for 20 minutes at room temperature followed by addition of the Stop Solution (50 uL) to each well and the optical density of each well is determined within 30 minutes using a microplate reader set to 450 nm

2.5.1.3 Lactate Dehydrogenase activity assay

The Lactate Dehydrogenase (LDH) activity assay was performed in tissue culture medium obtained from all 24-wells plates in order to assess membrane integrity as an indicator of cytotoxicity by measuring the amount of cytoplasmic LDH released into the culture medium. The LDH assay is based on the reduction of NAD by the action of LDH. The resulting

reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye and the resulting coloured compound is measured spectrophotometrically. The Lacate Dehydrogenase based *in-vitro* toxicology assay kit (TOX-7, Sigma-Aldrich, St Louis, USA) was used for this purpose.

Cultures were removed from incubator into laminar flow hood and plates were centrifuged at 2500 rpm for 4 minutes to separate supernatant from intact cells. Aliquots were then transferred to clean flat-bottom plate and Lactate Dehydrogenase assay mixture is then prepared by adding 25 ml of DEPC water to bottle of lyophilized enzyme and added in amounts equal to half of the volume of culture medium. The plate is covered by an opaque material (Aluminium foil or a box) to protect it from light and incubated at room temperature for 20 – 30 minutes. The reaction is then terminated by addition of 1/10 volume of 1N HCl solution to each well. Finally, absorbance was measured spectrophotometrically (LKB Biochrom Ultrospec II, UK) at a wavelength of 490 nm in a plate reader. The background absorbance of multiwell plates was measured at 690 nm and was subtracted from the primary wavelength measurement.

2.5.2 Bio-Plex suspension array measurement

The aim of these detailed experiments is to measure the human myometrial expression of cytokines IL-1β, IL-6, IL-8, IL-10, TNF-α and IFN-γ in response to stimulation by P.gingivalis lipopolysaccharide (InvivoGen, CA) as a ligand for TLR2

2.5.2.1 Setting-up 24-well plate

Weighed myometrial samples (n=8) obtained from women undergoing elective CS were washed 3 times in Dulbeccos PBS to remove any excess blood, then cut into small pieces

using a sterile scalpel (100 mg weight) in tissue culture hood. Three small pieces of myometrium were put into each well of a 24 well plate containing 1ml of Iscoves medium supplemented with 100U/ml penicillin, 100ug/ml streptomycin and 250ng/ml fungizone (amphotericin). After a pre-incubation at 37^{o}C for 45 minutes in humidified chamber the explants were transferred to a new well containing fresh culture medium.

The explants were incubated for 24 hours with no additional treatment ("control"), 100ng/ml purified *P.gingivalis* LPS - a known TLR2 ligand ("LPS") InvivoGen, CA, USA), 2mg/ml Medroxyprogesterone acetate ("MPA") (Pharmacia & Upjohn), LPS with MPA or finally LPS with the PKR inhibitor 2-Aminopurine 10mM. Control wells were run without addition of any of these chemicals. All samples were tested in duplicate.

2.5.2.2 Cytokine assay

Expression of cytokines IL-1β, IL-6, IL-8, IL-10, TNFα, and IFNγ were measured in the cell culture supernatant using the Bio-Plex suspension array system (Bio-Rad) which is a multiplex bead-based assay designed to quantify multiple cytokines in diverse matrices using the X-MAP technology

Guided by IL-6 assay and performance characteristics of Bio-Plex assay, tissue culture supernatants used in this experiment as well as cytokine standards were 1/100 dilution. Bio-Plex standard is reconstituted in 500 uL of Iscove's medium (Invitrogen), gently vortexed and incubated for 30 minutes in ice. Bio-Plex beads working solution is then prepared, kept in ice in a foil wrap to protect it from light. 96-well filter plate is made wet with 100 uL of Bio-Plex assay buffer in each well followed by vacuum filtration to dry the filter plate. Bio-Plex beads working solution is then vortexed for 15 – 20 seconds at medium speed and added to wells, 50 uL each followed by vacuum filtration. Filter plate is then washed with 100 uL of Bio-Plex wash buffer into each well and removed again by vacuum filtration. Diluted standard to

samples are then added 50 uL into each well using a fresh pipette tip each time and the plate is covered with sealing tape and foil, shaken for 30 minutes at room temperature. Buffer solution is then removed by vacuum filtration and washed 3 times with wash buffer solution. Bio-Plex detection antibody solution is added 25 uL to each well, covered and shaken for further 30 minutes as before and buffer is removed by vacuum filtration. Plate is then washed 3 times with wash buffer followed by vacuum filtration after each wash. Streptavidin-PE is finally prepared adding 50 uL to each well, plate is then covered shaken for 10 minutes at room temperature and washed 3 times followed by vacuum filtration. Before the plate is read in Bio-Plex system, the beads are re-suspended with 125 uL of Bio=Plex assay buffer solution into each well and the plate is covered with sealing tape and briefly shaken at 1100 rpm for 30 seconds.

Results are expressed as pg/ml/mg of tissue except for IL-6 & IL-8 which are expressed as ng/ml/mg of tissue.

2.6 Statistical analysis

In order to study effect of a potential interaction between gestation and labour status on the dependent variables, a two-way between-group analysis of variance (ANOVA) was conducted. Two-way between-group analysis of variance is appropriate where there are two independent factors specifying the nature of the measurements (108). Where there was no significant interaction between gestation and labour, the main effect of these variables was reported as recommended. Where there was a significant interaction, the effects of labour and gestation were analysed and reported separately using either a two-sided t-test if the data were normally distributed or a Mann-Whitney *U* test if not normally distributed.

RT-PCR and Western blotting results were expressed as the Median ± IQR (data not normally distributed). To determine the differences between labouring and non-labouring samples, Mann-Whitney test was performed.

Cytokine concentration was compared between all treatment wells using the Kruskal-Wallis test. If significant, differences were explored between control and each of LPS and MPA, LPS and LPS/MPA and between LPS and LPS/2AP using the Mann Whitney test ($P < 0.05$).

3. RESULTS

3.1 Patients demographics

	Term		Pre-term	
	In labour	**Before labour**	**In labour**	**Before labour**
Number	23	37	5	12
Mean age	27.7 ± 6.5	30.7 ± 5.9	32 ± 6.1	30.9 ± 4.8
Gestation	39.6 ± 1.12	38.8 ± 0.6	34.4 ± 1.34	33.4 ± 1.5
Indication	FTP: 12 Brow: 1 Fetal distress: 6 Previous CS: 4	Previous CS: 19 Breech: 7 IUGR: 1 Maternal choice: 4 Other: 6	Previous 2 CS: 2 Breech: 1 Fetal distress: 2	IUGR: 5 Severe PIH: 5 IDDM: 2

Table 2: patients' characteristics and indications of delivery

CS: Caesarean section

IUGR: Intrauterine growth restriction

IDDM: Insulin dependent diabetes mellitus

PIH: Pregnancy-induced hypertension

3.2 mRNA expression of TLR2 and TLR4

In term myometrial samples, mean percentage values (± SEM) for TLR2 and TLR4 mRNA expression were [TLR2 105.53 (± 123.2) & TLR4 104.29 (± 118.6) (p = 0.06)], in labouring and [TLR2 30.9 (± 41.5) & TLR4 90.13 (± 140.8) (p = 0.88)], in non-labouring samples respectively. However, mean percentage values of TLR2 and TLR4 expression were significantly higher in myometrium at term [TLR2 56.7 (± 12) & TLR4 95 (± 18.4), p=0.009] compared with preterm myometrium [TLR2 8.8 (± 4.01), TLR4 14.85 (± 5.81), p= 0.03] respectively. (N = Term before labour: 37, Term in labour: 23, Pre-term before labour: 12, Pre-term in labour: 5)

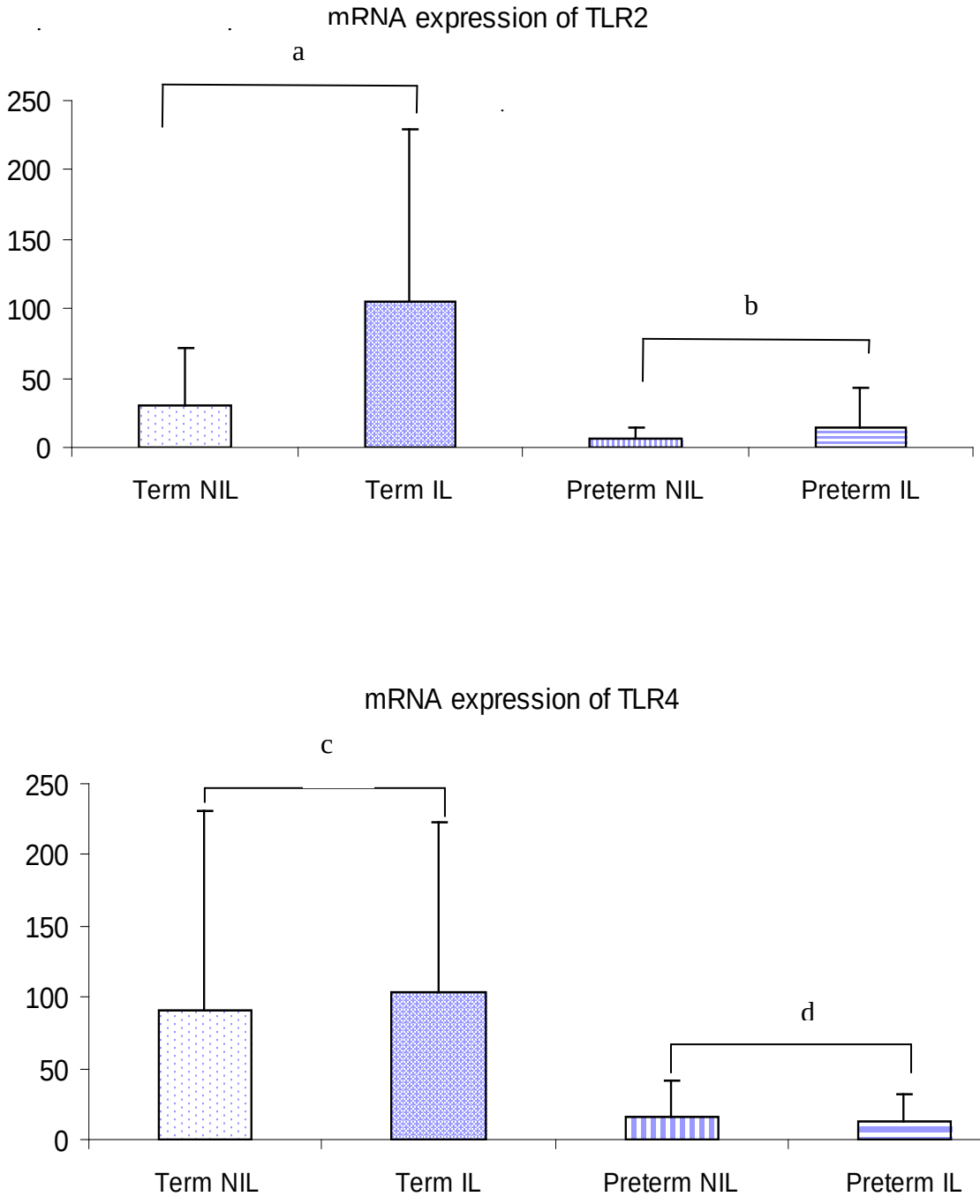
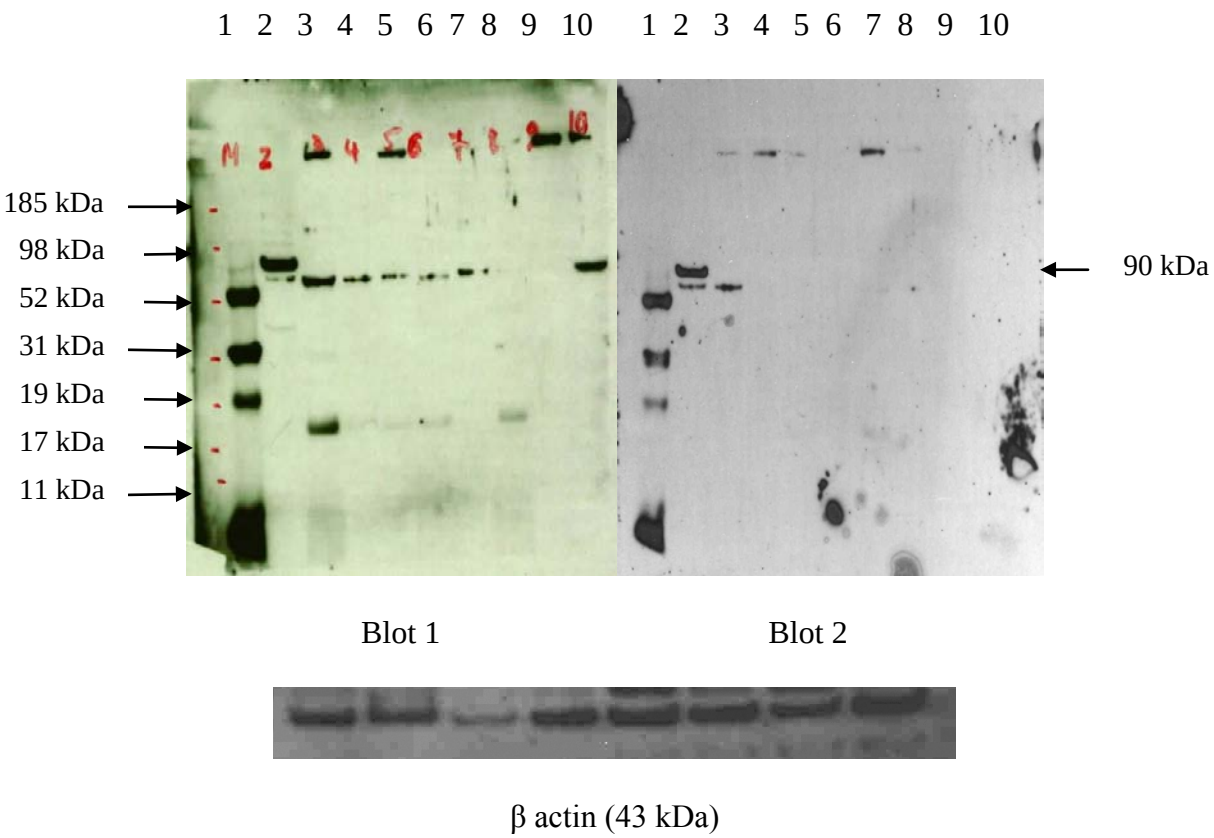


Figure 6: Expression of TLR2 & 4 mRNA in relation to gestation and labour status
a > b (P = 0.009)
c > d (P = 0.03)

3.3 TLR2 and TLR4 protein expression

On western blotting, a positive band corresponding to TLR2 protein was seen at 90kDA, to TLR4 at 89kDA and to β-actin at 43kDA. TLR2 protein expression was significantly increased in labouring myometrium (Mean = 23.3 ± 14.4,) as indicated by the density of the expression band on the blot in comparison with non-labouring myometrium at term (4.43 ± 3.8, *p=0.*004). TLR4 protein expression was similar in labouring and in non-labouring myometrium (5.47 ± 11.6 versus 6 ± 15.1, $p = 0.86$). However, in contrast to our previous mRNA data, we could not demonstrate statistically significant differences in TLR2 & 4 protein expression in term versus preterm myometrium (TLR2 = 14.99 ± 9.9 versus 12.03 ± 7.1, $p = 0.9$, TLR4 = 9.44 ± 11.1 versus 7.13 ± 14.1, $p = 0.62$) (N = Term before labour: 9, Term in labour: 9, Pre-term before labour: 9, Pre-term in labour: 5)



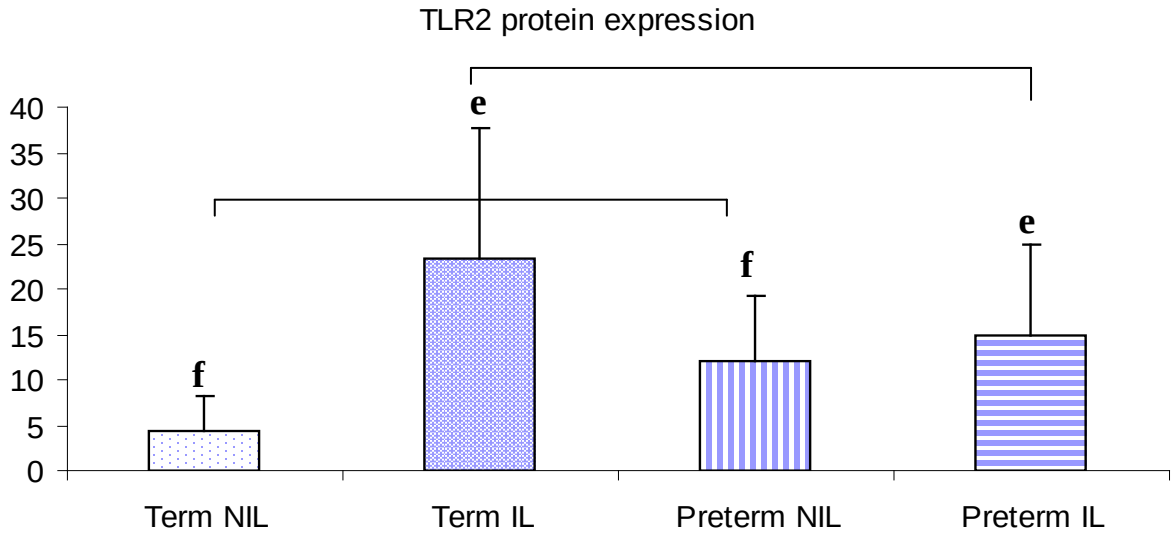
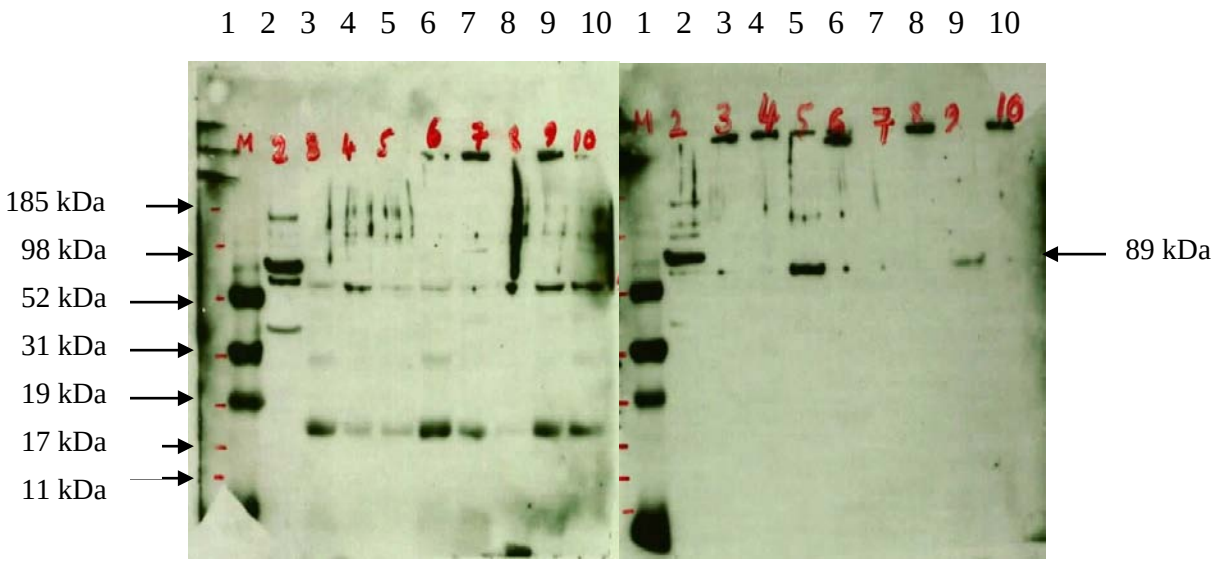


Figure 7**:** western blotting of TLR2 protein expression in relation to labour status and gestation. Lane 1 (M): MultiMark Standard (LC5725, Invitrogen), lane 2: positive control (SW cell lysate, SC2219), Lanes 3 – 6: myometrium at term in labour, lanes 7 – 10 myometrium at term before labour (Blot 1), Lanes 3 – 6 preterm myometrium in labour, Lanes 7 – 10: preterm myometrium not in labour (Blot 2)

e > f (P = 0.004)



β actin (43 kDa)

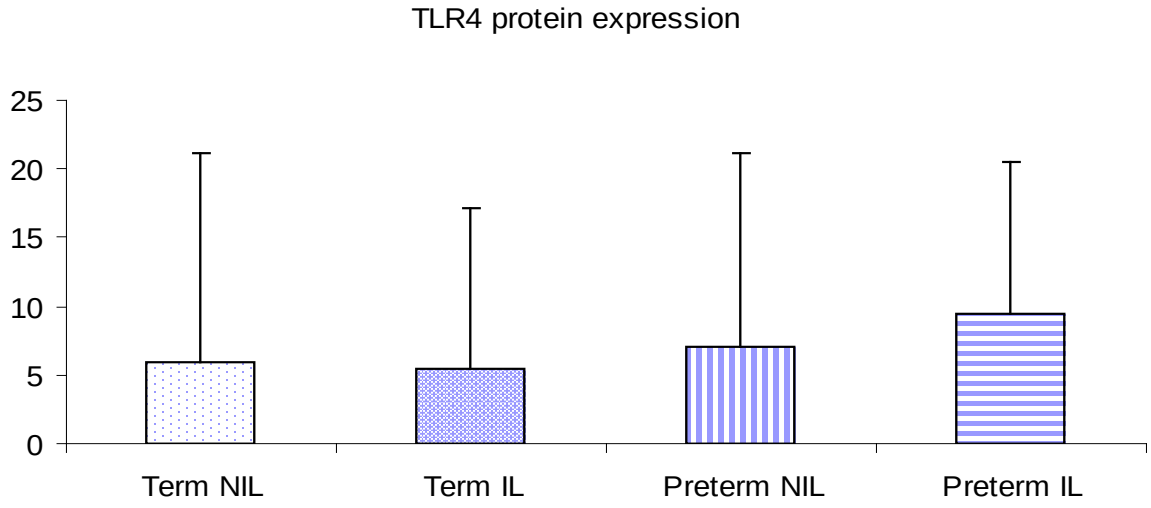


Figure 8: western blotting of TLR 4 protein expression in relation to labour status and gestation. Lane 1 (M): MultiMark Standard (LC5725, Invitrogen), lane 2: positive control (HL60 whole cell lysate, SC2209), Lanes 3 – 6: myometrium at term in labour, lanes 7 – 10 myometrium at term before labour (Blot 3), Lanes 3 – 6 preterm myometrium in labour, Lanes 7 – 10: preterm myometrium not in labour (Blot 4)

3.4 Immunohistochemical characterization of TLR2 & 4 expression

As previously noted in our laboratory and also by other investigators, labour is associated with widespread infiltration of myometrium with leukocytes. Expression of both TLR2 and TLR4 was readily noticed around blood vessels and in infiltrating leukocytes in myometrial sections in labour.

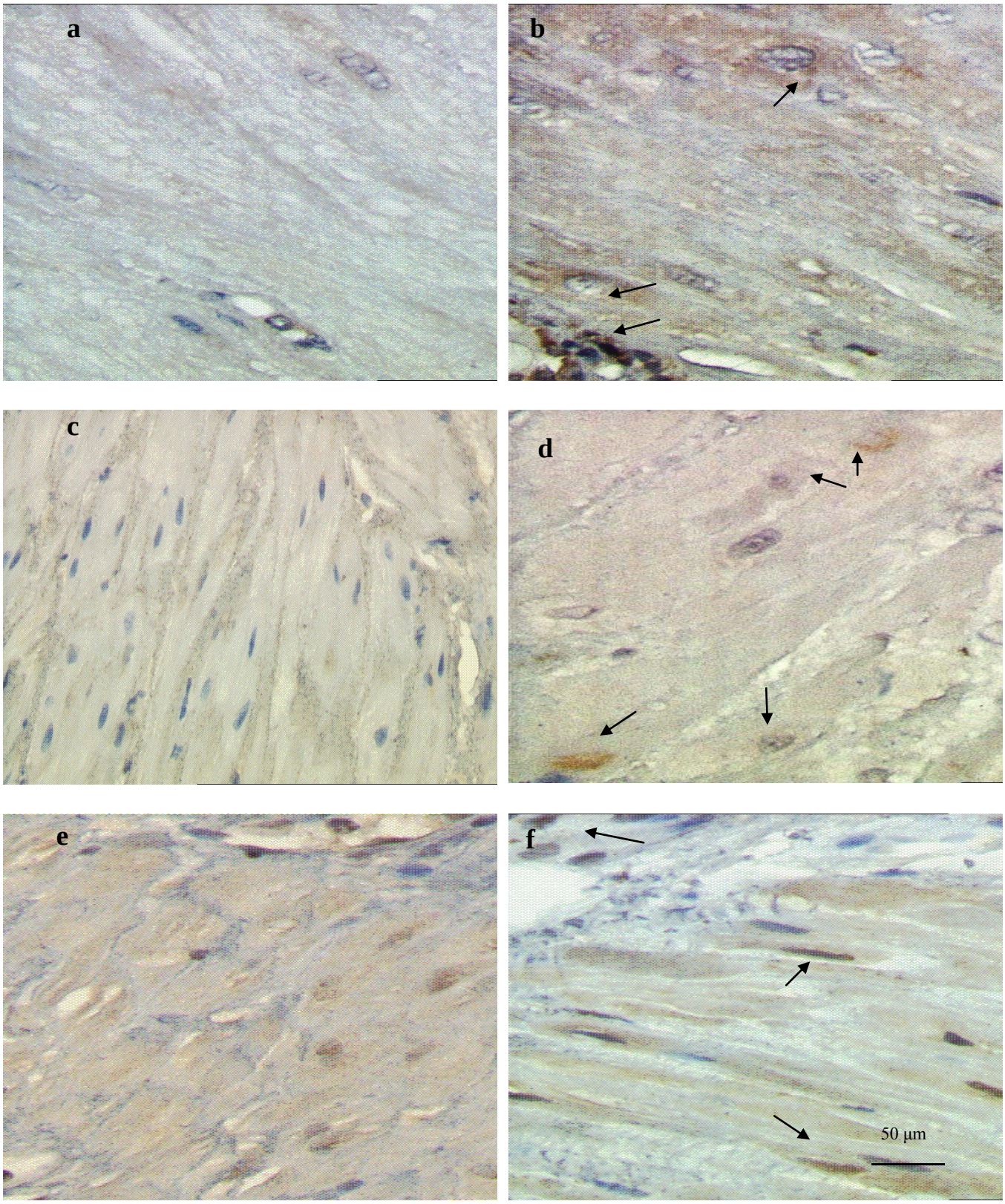


Figure 9: Immunolocalization of TLR2 & 4. a: TLR2 at term before labour, b: TLR2 at term in labour, c: TLR4 at term before labour, d: TLR4 at term in labour, e: TLR2 preterm before labour, f: TLR2 preterm in labour. (magnification x40). TLR2&4 protein expression visualized as a brown stain using diaminobenzidine is more pronounced around blood vessels and in infilterating leukocytes as indicated by the arrows.

3.5 Cytokine release by LPS, MPA and 2AP treated short-term myometrial tissue *in vitro*

In preparation for this experiment, we have shown that lipopolysaccharide (LPS) stimulates cytokine production in a dose and time dependent manner. (Figure 10) (N = 3)

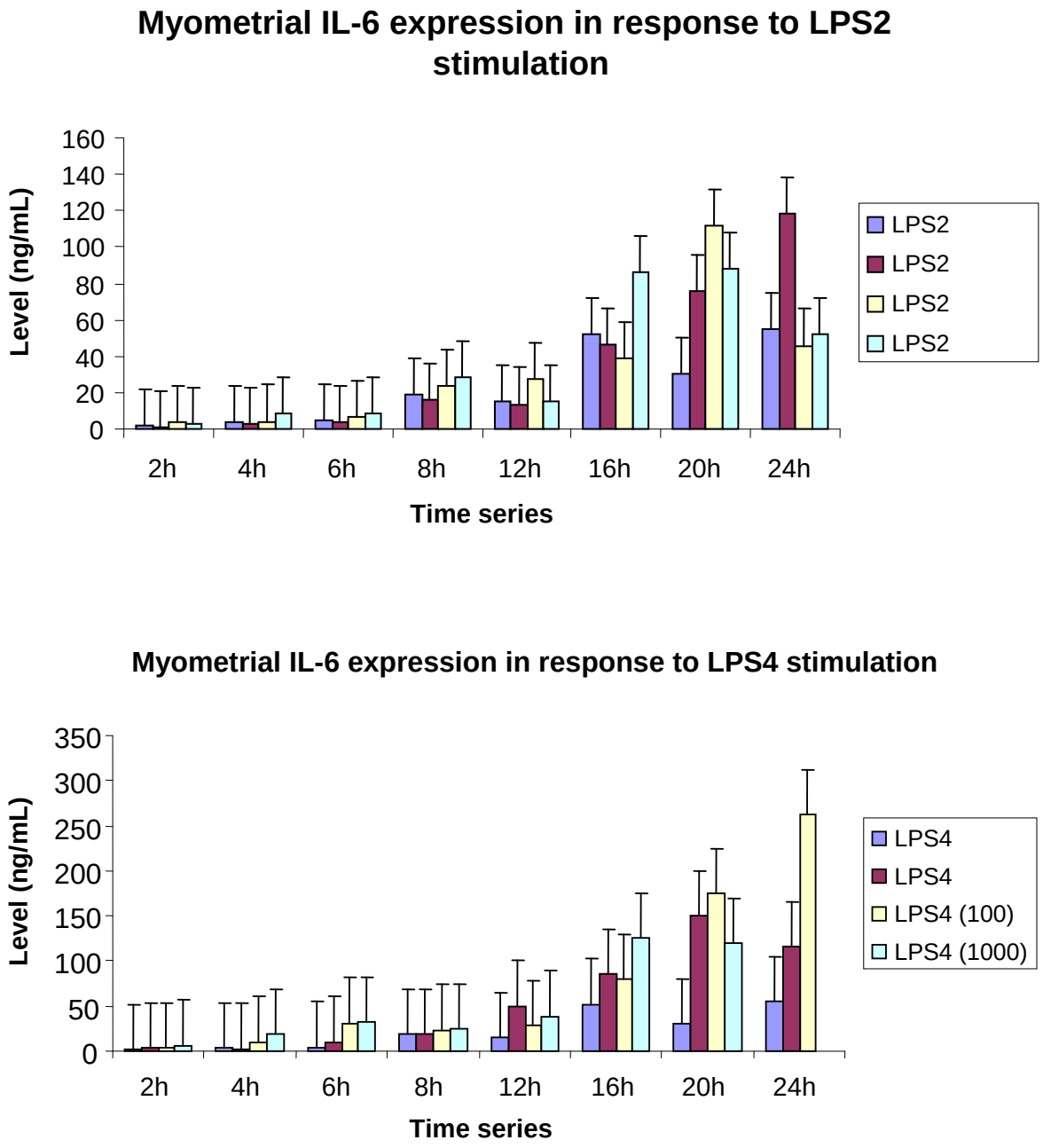


Figure 10: IL-6 production of human myometrium in response to stimulation by P.gingivalis LPS (LPS2) and S.minnesota LPS (LPS4)

MPA significantly suppressed baseline (un-stimulated samples) and LPS induced production of IL-1β, IL-6 and IL-8 in pregnant human myometrium. Although P.gingivalis LPS (LPS 2) stimulated IL-10 production, there was no significant inhibitory effect with MPA. In contrast, we failed to demonstrate upregulation of either TNF-α or IFN-γ in response to LPS, and there was no effect of MPA (Table 3)

2 Aminopurine which is a specific inhibitor for double stranded-RNA dependent protein kinase (PKR) significantly inhibited both baseline (un-stimulated) and LPS stimulated myometrial cytokine production. (Figure 11)

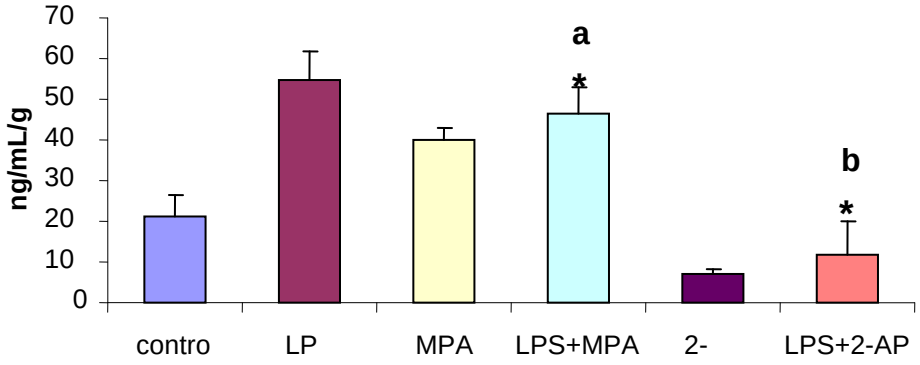
	Control	**LPS**	**MPA**	**LPS+MPA**	P^{*}	**2-AP**	**LPS+2AP**	P^{**}
IL-1β	214 (±53)	549 (±71)	402.6 (±26)	462 (±70)	0.04	68.5 (±16)	120 (±81)	0.008
IL-6	396.4 (±52.9)	771.3 (±157.5)	122.2 (±9.5)	222.7 (±45.2)	0.001	31.3 (±9.7)	6.4 (±0.2)	0.04
IL-8	488.7 (±105.9)	982.7 (±136.9)	193.2 (±46.5)	399.6 (±129)	0.007	13.1 (±6.2)	3.8 (±0.1)	0.008
IL-10	425 (±102)	1453 (±333)	625 (±46)	743 (±255)	0.08	110.5 (±30)	19.98 (±5.7)	0.03
TNF-α	2420 (±519)	3482 (±679)	2523 (±168)	2436 (±523)	0.3	528 (±201)	67.1 (±0.2)	0.04
IFN-γ	4207 (±461)	5522 (±583)	3367 (±168)	4639 (±1319)	0.2	611 (±217)	64.8 (±55)	0.03

Table 3: cytokine production by LPS, MPA and 2-AP short term myometrial tissue culture

P^{*}: compares cytokine expression by LPS stimulated samples with LPS + pre-treatment with Medroxyprogesterone acetate (MPA)

P^{**}: compares cytokine expression by LPS stimulated samples with LPS + pre-treatment with 2-Aminopurine (2-AP) (N = 6)

IL-1β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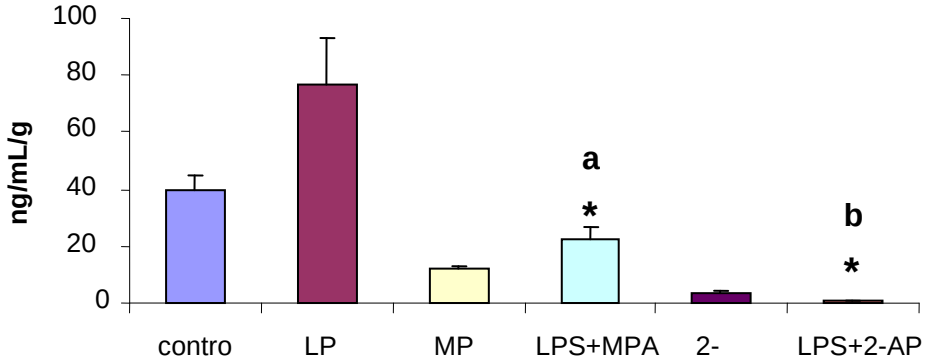
IL-6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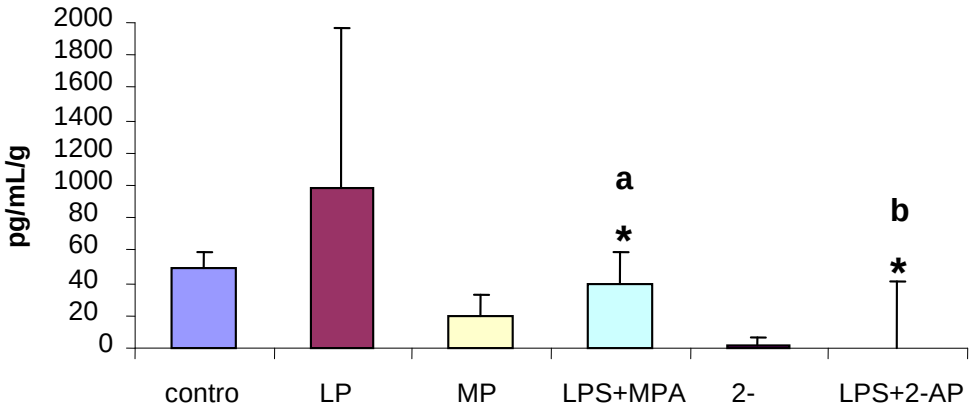
IL-8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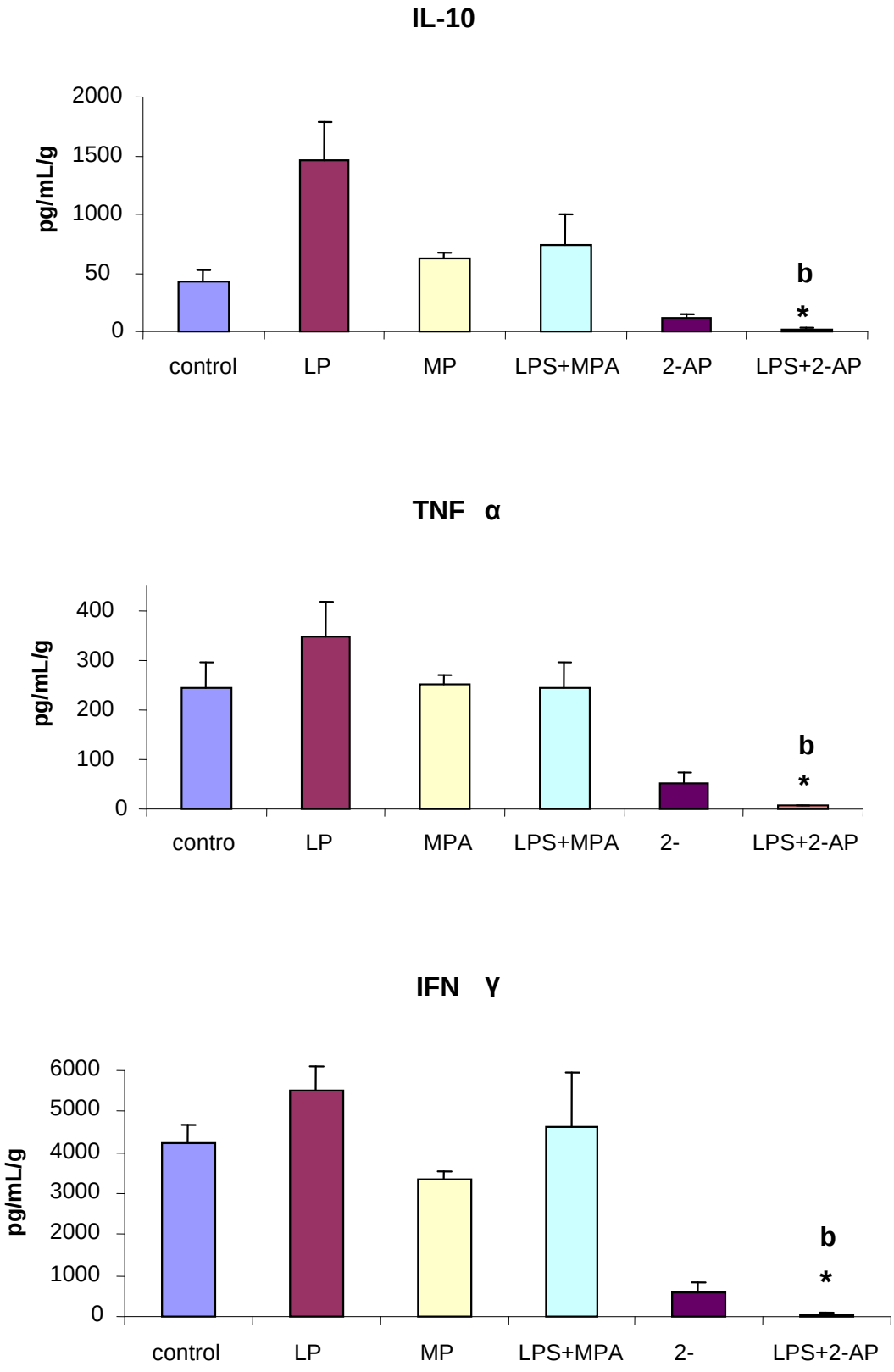

Figure 11: effect of P-gingivalis Lipopolysaccharide (LPS), Medroxyprogesterone acetate (MPA) and 2-Aminopurine (2-AP) on cytokine production by human myometrium at term

(*)= $p < 0.05$, a: compares LPS + MPA pre-treated myometrial samples to LPS stimulated samples, b: compares LPS + 2AP to LPS. Control: un-stimulated samples. (N = 6)

4. DISCUSSION

Discussion

In this study, we examined the hypothesis that Toll-like receptors are expressed in human myometrium and may play an important role in recognizing presence of the “danger signals” and initiation of the inflammatory cascade.

We identified the synthesis and presence of TLR2 & 4 in human myometrium as shown by expression of their mRNA and protein signals. mRNA expression of myometrial TLRs, as measured by RT-PCR showed significant upregulation at term compared with preterm gestation (TLR2 : $p = 0.009$, TLR4: $p = 0.03$).

Moreover, labour status also appears to modify the pattern of expression of TLRs. We demonstrated a possible role for TLR2 in labour as indicated by upregulation of its protein expression in labour compared with non-labouring myometrium ($p = 0.004$). Although there was a trend to an increase in TLR4 with labour, this did not reach statistical significance - observations similar to those noted by other investigators using the mouse model (88).

We also demonstrated that TLR2 protein expression was significantly increased in term labouring myometrium ($p = 0.004$) which lends further support to the previous notion of a possible role for TLR2 in human parturition.

It is possible that other members of the TLRs family may have similar role to play in human myometrium in labour which highlights the need for further studies in this field.

Although real-time RT-PCR technology has allowed quantification of mRNA targets with a degree of precision, biological validity of the quantitative data should be considered before concluding any results. There is no universal agreement yet on the inherent basic issues of this technique such as quality and quantity control of RNA, analysis and reporting of results and particularly, internal referencing. In this study, the internal reference (housekeeping) gene

"18S" had already been previously established in our laboratory for myometrium after comparing it with GADP and β-actin. It is also important noting that the preterm myometrial samples obtained before labour were collected from pregnant women with some disorders which are known to be associated with a degree of inflammation at the feto-maternal interface (e.g. IUGR, PIH and IDDM).

Accumulating evidence suggest that inflammation whether triggered by infection or by one or more of the endogenous stress signals, can start the unidirectional feed forward process of labour manifested by a progressive cascade of molecular events that lead to induction of a common pathway of parturition involving cervical effacement, rhythmic uterine contraction and finally expulsion of the fetus and placenta (7, 110). The inflammatory processes involved in human parturition appear to involve the complex co-ordination of various signalling pathways within the cervix and myometrium. Activation of such signalling pathways may result in up-regulation of transcription factors such as NFκB which in turn may contribute to cytokine, prostaglandin release, apoptosis and cell cycle regulation (89 - 91).

Since Toll-like receptors play a crucial role in recognition of a wide range of micro-organisms in addition to a host of possible endogenous ligands, it could be anticipated that defective signalling through TLRs pathway can have an effect of initiation of inflammatory cascade leading into myometrial activation associated with term or preterm labour. This is also supported by the finding of a strain of mice with a spontaneous mutation for TLR4 that was resistant to deliver preterm after intrauterine inoculation of heat-killed bacteria or lipopolysaccahride administration compared with wild type mice (24). Moreover, spontaneous labour at term or preterm was found to be associated with increased mRNA and protein expression of TLR2 and TLR4 in the chorioamniotic membranes (92). Our finding of

TLR2 and TLR4 expression in human myometrium would lend further evidence for the role of innate immune response in the mechanism of parturition.

As previously discussed, Toll-like receptors, with the exception of TLR3, share a common signalling pathway via the adaptor molecule MyD88 which has a Toll/IL-1R (TIR) domain in its C-terminal region and a death domain (DD) in its N-terminal region. Upon stimulation, TLRs recruit MyD88 through interaction of their respective TIR domain. The DD of MyD88 then binds the DD of IL-1R associated kinase (IRAK) and the signal is propagated via TNF receptor associated factor-6 leading to activation of NF-κB and mitogen-activated protein kinases and transcription of relevant genes (53, 93). Recently, a second TIR-containing adaptor protein, TIR-associated protein (TIRAP)/MyD88-adaptor like was identified to be involved in the MyD88-dependent pathways of TLR1/2, TLR2/6 and TLR4 (94).

In addition to the common MyD88-mediated signalling pathway, a MyD88-independent pathway has been identified that involves a third TIR-containing adaptor molecule, TIR domain-containing adaptor-inducing interferon-β (IFN-β; TRIF), which is essential for TLR3 and TLR4 signalling, leading to induction of transcription factor IFN regulatory factor 3 and the subsequent production of IFN-β and activation and maturation of dendritic cells (53). Recently, a fourth TIR-containing adaptor molecule, TRIF-related adaptor molecule (TRAM) has been shown to be involved specifically in TLR4 mediated MyD88-independent IFN-β production. Thus, these adaptor molecules provide specificities for TLR-mediated signalling.

In our short-term tissue culture model, we demonstrated that Medroxyprogesterone acetate (MPA) significantly suppressed baseline (cytokines produced in vivo proior to sample collection of in vitro during process of tissue culture) and LPS induced production of IL-1β, IL-6 and IL-8 in pregnant human myometrium. Although LPS stimulated IL-10 production, there was no significant inhibitory effect with MPA. In contrast, we failed to demonstrate

significant upregulation of either TNF-α or IFN-γ in response to LPS, and there was no effect of MPA. The pattern of expression of IL-10 in our myometrial tissue culture model is not consistent with its previously noted role in maintenance of pregnancy due to its anti-inflammatory effect. Previous studies have shown that IL-10 production is significantly reduced in the placenta at term without labour compared with that in first and second trimester tissue (95). Its expression was also noted to be reduced in the placental tissue of pregnancies complicated by preterm labour and chorioamnionitis when compared with placental tissues from normal controls. Administration of IL-10 was also noted to block interleukin-1β (IL-1β) induced uterine contractions in pregnant rhesus monkeys (96). Although IL-10 was also able to inhibit cyclooxygenase type 2 (COX-2) mRNA expression in cultured human placental explants from pregnancies complicated by preterm labour, it failed to do a similar effect in term labouring human myometrial explants (97). This may indicate that the mechanisms involved in regulation of inflammatory response during term labour may be different from preterm labour.

We also showed that the specific inhibitor for double stranded-RNA dependent protein kinase (PKR) 2 Aminopurine significantly inhibited both baseline and LPS stimulated myometrial cytokine production in a dose dependent manner. This effect is believed to be achieved mainly via prevention of Toll/IL-1 receptor domain (TRIF)-dependent rather than myeloid differentiation factor (MyD)88-dependent signalling (98). This finding lends further support to the significant role of TLRs in initiating inflammatory response as manifested by cytokine production in human myometrium at term.

Although animal studies have clearly demonstrated that interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) can induce labour (14, 26, 99), the question whether these cytokines do so during in vivo infections remains a difficult one to answer. Given the

complexity and redundancy of inflammatory response networks, it is likely that no single mediator will be identified as responsible for the induction of labour during infection. Preterm labour can still occur in knockout mice for the IL-1 type I receptor after exposure to bacteria, suggesting that IL-1 administration is sufficient but not necessary for the onset of labour in the context of infection (100). However, blockade of both IL-1 and TNF-α signalling in double knockout mice models has been associated with a decreased rate of preterm birth after bacterial inoculation. (101)

It is possible that TLRs may co-operate with other inflammatory receptors for induction or amplification of inflammatory response. TREM-1 (Triggering receptor in myeloid cells-1) is a newly discovered receptor, which is intimately involved in inflammatory processes. The specific ligands for TREM-1 remain unknown at present, but once activated TREM-1 acts as a critical amplifier of inflammatory signalling (100 - 102). TLR and TREM-1 may cooperate closely to induce an inflammatory response, as activation of TREM-1 causes a marked increment in the production of pro-inflammatory cytokines by macrophages when LPS is used as the co-stimulus. In contrast, TLRs directly recognise certain microbial products and components and using their adaptor molecules, they can potentially induce the production of proinflammatory cytokines via activation of NF-κB. If specific ligands for TREM-1 are located at the foci of microbe-induced inflammation, interaction then between TREM-1 and TLRs can synergistically induce inflammatory responses (103 - 105). Unfortunately, due to time and financial constraints, it was not possible to study the patterns of expression of TLR2 & TLR4 in the myometrial short term tissue culture samples to see if there is any correlation between cytokines production and TLRs profiles.

Although the concept of an important role for progesterone in maintaining pregnancy and prevention of preterm labour was put forth in the 1950s (65), it has recently gained

momentum with the findings of the Meis trial (2003) (81) and the recent support of the ACOG that 17P hydroxyprogesterone should be considered in high risk women to prevent preterm labour (82). The mechanisms by which progesterone administration prevent preterm birth remain speculative. Progesterone is known to promote myometrial quiescence, down regulate gap junction formation, inhibit cervical ripening and decrease the production of cytokines (e.g. IL-8) by chorioamniotic membranes (66, 67, 68, 70). Administration of progesterone receptor antagonist (i.e. Mifepristone or Onapristone) has been found to induce labour in pregnant women, non-human primates and in guinea pigs (71, 72). In many species, but not in humans, non-human primates and guinea pigs, a fall in maternal serum progesterone concentration occurs prior to spontaneous onset of parturition (74, 75). However, the concept of "functional progesterone withdrawal" has been suggested by some investigators (76 - 78) which may be invoked by:

- reduced availability of progesterone by binding to a high affinity protein
- competitive inhibition from increased cortisol concentration in late pregnancy on their common glucocorticoid receptor
- increased peripheral conversion of progesterone to an inactive form
- changes in progesterone receptor isoforms
- suppression of NF-κB by activated progesterone receptors

Our data from the short term myometrial tissue culture model lend further support to the hypothesis that progesterone may inhibit preterm labour via an anti-inflammatory effect. Further studies are needed to elucidate the specific roles and mechanisms of progesterone and progesterone-like agents in prevention of inflammation induced preterm birth and to identify the most effective preparation for this purpose.

In conclusion, findings of this work have demonstrated a potential role for Toll-like receptors 2 & 4 in initiation of term and preterm labour as manifested by their increased mRNA and protein expression with gestation and possibly with labour. We also demonstrated an anti-inflammatory role for the progestogen Medroxyprogestrone acetate in lipopolysaccahride stimulated myometrial tissue culture model in vitro which highlights the need for further studies to examine the role of other progestogens / progesterone in prevention of preterm labour. The results of current, long awaited, randomized trials (83, 84) will assist in further elucidation of the precise role, benefits and harms of progesterone therapy for women considered to be at risk of preterm birth.

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