TOLL-LIKE RECEPTOR 4 SIGNALLING IN INFLAMMATION PATHWAYS TO TERM AND PRETERM DELIVERY

A Thesis Submitted for the Degree of Doctor of Philosophy by

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Abstract

A pro-inflammatory signalling cascade initiates the events that bring about parturition, including uterine contractions, cervical ripening and the rupture of fetal membranes. One mechanism by which this inflammatory process can be initiated is via toll-like receptor (TLR)-mediated activation, elicited by danger associated molecular patterns (DAMPs) or other endogenous TLR4 regulators, such as platelet activating factor, surfactant protein A (SP-A), high mobility group box 1 (HMGB1), heat shock protein 70 (HSP70), uric acid and oxysterols which are released from gestational tissues after necrotic cell death as a result of tissue stretch, injury and remodelling in late gestation. It is postulated that preterm delivery results from a pathological process that involves an early increase in DAMPs and TLR4 regulators, premature activation of TLRs and the inflammatory pathways associated with on-time labour, leading to preterm delivery. This premature release of DAMPs and TLR4 regulators can be triggered by non-infectious events, leading to sterile inflammation and preterm delivery in the absence of infection. Many of the DAMPs and TLR4 regulators produced by the gestational tissues in late pregnancy and labour are known to trigger TLR4 signalling, raising the possibility that TLR4 acts as a point of convergence in the mechanistic pathways linking infection-driven and sterile preterm labour, and term labour, in mice and potentially humans. Prior to this study, the role of TLR4 and its association with DAMPs and TLR4 regulators in the timing of labour had not been evaluated in normal term parturition or sterile inflammation-driven preterm delivery. We hypothesised that TLR4 activation by DAMPs and TLR4 regulators acts upstream of the gene expression and leukocyte recruitment steps that mediate the physiological and sterile inflammation in normal term delivery and preterm delivery respectively.

In this thesis we describe experiments in mouse models, particularly mice with null mutation in the *Tlr4* gene, which demonstrate that TLR4 plays a critical role in the timing of labour. TLR4-deficient mice were found to have reduced expression of pro-inflammatory genes *Il1b*, *Il6*, *Il12b*, *Tnf* and *Il17a,* which was mirrored by immune cell populations with reduced percentages and numbers of inflammatory leukocytes in the gestational tissues, including placental neutrophils and uterine dendritic cells. The compromised pro-inflammatory environment likely impacted upon the downstream uterine activation genes *Ptgfr*, *Oxtr* and *Gja1* which were expressed at lower levels in TLR4-deficient mice compared to wildtype controls, in the gestational

tissues in late gestation. This led to an increased length of pregnancy and elevated pup mortality in the TLR4-deficient mice.

We then attempted to induce preterm birth using the endogenous TLR4 regulator carbamyl PAF (cPAF), which was administered intraperitoneally into pregnant mice. cPAF administration induced pro-inflammatory cytokine expression in the gestational tissues, leading to preterm delivery in wildtype mice and decreased fetal viability. TLR4-deficient females were resistant to cPAF induced preterm birth. Furthermore, cPAF-induced preterm delivery was blocked by administering the TLR4 antagonist (+)-naltrexone to mice, which prevented initiation of the pro-inflammatory cytokine cascade, strongly indicating that cPAF-mediated induction of inflammation within the gestational tissues and the resulting preterm delivery are TLR4 dependent. In summary, this thesis provides new evidence about the association between cPAF and TLR4 in inducing downstream inflammatory pathways, including cytokine expression and leukocyte recruitment that trigger uterine activation genes in both preterm and term delivery. This includes a crucial role of TLR4 as a key mediator of sterile inflammation in preterm and term delivery. The study provides fundamental insights on the inflammatory pathways underpinning the parturition cascade, and may inform future clinical studies to investigate prevention or delay of spontaneous preterm delivery in humans.

Declaration

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Abbreviations

ANOVA	Analysis of variance
ACTB	Beta actin
ASC	caspase recruitment domain
BSA	Bovine serum albumin
BMI	Body mass index
CCL2	Chemokine (C-C motif) ligand 2
CCL3	Chemokine (C-C motif) ligand 3
CCL4	Chemokine (C-C motif) ligand 4
CCL5	Chemokine (C-C motif) ligand 5
CCL8	Chemokine (C-C motif) ligand 8
CCL20	Chemokine (C-C motif) ligand 20
Cd24-/-	CD24 deficient
cDNA	Complementary DNA
cPAF	Carbamyl platelet activating factor
CX-43	Connexin-43
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL2	Chemokine (C-X-C motif) ligand 2
CXCL5	Chemokine (C-X-C motif) ligand 5
CXCL8	Chemokine (C-X-C motif) ligand 8
CXCR4	Chemokine (C-X-C motif) receptor type 4
DAMPs	Danger-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
EDA	Extra domain A
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay

FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FOXP3	Forkhead box P3
FSC-A	Forward scatter-area
FSC-H	Forward scatter-height
GD	Gestational day
GMCSF	Granulocyte macrophage stimulating factor
HA	Hyaluronic acid
HCl	Hydrochloric acid
HMGB1	High mobility group box 1
HRP	Horseradish peroxidase
HSPs	Heat shock proteins
HSP22	Heat shock protein 22
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
HSP110	Heat shock protein 110
IAI	Intra-amniotic infection
ICAM	Intercellular adhesion molecule
IFN	Interferon
IFNG	Interferon gamma
IKK	IKB kinase
IL	Interleukin
IL1	Interleukin 1
Il1r1/Tnfrsf1a-/-	Type 1 receptors for interleukin 1 and tumour necrosis factor deficient
IL1A	Interleukin 1 alpha
IL1B	Interleukin 1 beta
IL6	Interleukin 6
Il6-/-	Interleukin 6 deficient
IL10	Interleukin 10
Il10-/-	Interleukin 10 deficient

IL12B	Interleukin 12 beta
IL17A	Interleukin 17 alpha
IL17RA	Interleukin 17 receptor alpha
IL18	Interleukin 18
IRAK	IL1 receptor associated kinase
IRF	Interferon regulated factor
IUGR	Intrauterine growth restriction
Jα18-/-	Invariant Vα14-Jα18 receptor deficient
LAL	Limulus amebocyte lysate
LPCAT	lysophos-phatidylcholine acyltransferase-1
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Lyso-PAF	1-O-alkyl-*sn*-glycero-3-phosphocholine
Lyso-PAFAT	acetyl-CoA:lyso-PAF acetyltransferase
MAPK	Mitogen-activated kinase
MIAC	Microbial invasion of amniotic cavity
MLN	Mesenteric lymph nodes
MMPs	Matrix metalloproteinases
MMP1	Matrix metalloproteinase 1
MMP2	Matrix metalloproteinase 2
MMP3	Matrix metalloproteinase 3
MMP8	Matrix metalloproteinase 8
MMP9	Matrix metalloproteinase 9
mRNA	Messenger ribonucleic acid
MD2	Myeloid differentiation factor 2
MyD88	Myeloid differentiation factor 88
Myd88-/-	Myeloid differentiation factor 88 deficient
Myd88/Trif-/-	Myeloid differentiation factor 88 and TIR domain-containing adaptor deficient
NF-KB	Nuclear factor-kappa beta
NGP	Neutrophil granule protein

NLRP3	Nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain containing 3
NK cells	Natural killer cells
OxLDL	Oxidised low-density lipoprotein
OXTR	Oxytocin receptor
PAF	Platelet activating factor
PAF-AH	Platelet activating factor-acetylhydrolase
PAFR	Platelet activating factor receptor
Pafr-/-	Platelet activating factor receptor deficient
PALN	Para-aortic lymph nodes
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PECAM	Platelet-endothelial cell adhesion molecule
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGN	Peptidoglycan
Poly [I:C]	Polyinosinic:polycytidylic acid
pPROM	Preterm premature rupture of membrane
PTGER2	Prostaglandin E_2 receptor 2
PTGER4	Prostaglandin E_2 receptor 4
PTGFR	Prostaglandin $F_{2\alpha}$ receptor
PTGHS1	Prostaglandin H synthase 1
Ptghs1-/-	Prostaglandin H synthase 1 deficient
PTGHS2	Prostaglandin H synthase 2
Rag1-/-	Recombination activating gene 1 deficient
RAGE	Receptor for advanced glycation endproducts
RBC	Red blood cell
rHMGB1	Recombinant high mobility group box 1
RNA	Ribonucleic acid
RO	Reverse osmosis
RPMI	Roswell park memorial institute medium

rSP-D	Recombinant surfactant protein D
RT-PCR	Real-time polymerase chain reaction
SEM	Standard error mean
SP	Surfactant protein
SP-A	Surfactant protein A
SP-D	Surfactant protein D
Spa/d-/-	Surfactant protein A and surfactant protein D deficient
SRC1	Steroid receptor coactivator 1
SRC2	Steroid receptor coactivator 2
Src-1/-2 dhet	Steroid receptor coactivator 1 and steroid receptor coactivator 2 double heterozygous deficient
Th17 cells	T helper 17 cells
TNF	Tumour necrosis factor
TLR	Toll like receptor
TLR2	Toll like receptor 2
Tlr2-/-	Toll like receptor 2 deficient
TLR4	Toll like receptor 4
Tlr4-/-	Toll like receptor 4 deficient
Treg cells	Regulatory T cells
TRIF	TIR domain-containing adaptor
Trif-/-	TIR domain-containing adaptor deficient
TRAF6	TRF-associated factor 6
VECAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
ZAM	Zone of altered morphology

Chapter 1: Review of Literature

1.1 INTRODUCTION

Preterm delivery is defined as birth that occurs at less than 37 weeks of gestation (1). The obstetric precursors to preterm delivery are elective delivery for maternal or fetal indications, spontaneous preterm labour with intact membranes, and preterm premature rupture of the membranes (pPROM), where pPROM is defined as spontaneous rupture of the membranes at less than 37 weeks of gestation, at least one hour before the onset of contractions (2). The prevalence of preterm delivery is approximately 12%-13% in the USA, while in other developed countries including Australia the rate varies between 5%-9% (3). Globally, preterm birth affects 1 in 10 of babies born, resulting in an estimated 15 million preterm births every year (4). Preterm delivery is associated with over 1 million neonatal death and the preterm babies who survive often suffer serious life-long health problems, including cerebral palsy, brain injury, respiratory dysfunction and developmental delay (5). The risk factors for preterm delivery are summarised in Table 1.1 below:

Table 1.1 Epidemiological and clinical risk factors of preterm delivery

Risk factors	Comments
Infection	Over the past 20 years many studies have demonstrated that intrauterine infection affects 25% to 40% of preterm births (6-10). Intrauterine infection is considered as the main mechanism associated with preterm birth, by triggering pPROM and chorioamnionitis (9). In pPROM, inflammation-mediated membrane damage, instigate the loss of amniotic fluid and hence leads to preterm birth. Inflammation may affect the umbilical cord and chorionic villi blood vessels, initiating funisitis and chorionic vasculitis in chorioamnionitis (9,11). Extrauterine maternal infections, including pyelonephritis and malaria, are linked with preterm delivery (8).
Multiple pregnancy	An increase in the incidence of multiple births has been reported over the past 20 years, contributed by the use of assisted conception technologies (1). Preterm delivery occurrs in nearly 60% of all multiple pregnancy. One possible causative mechanism is uterine overdistention which results in premature uterine contractions (2,12). Premature cervical ripening is the predominant feature of cervical insufficiency (11), which may be caused by cervical shortening, congenital cervical weakness, surgery or trauma (11).
Cervical incompetence	Cervical insufficiency and intrauterine infection have been demonstrated to contribute to premature cervical ripening, leading to preterm birth (13). Cervical shortening, is identified as a risk factor and predicting factor for preterm delivery (14-16).
Psychosocial distress (including stress, anxiety and depression during pregnancy)	Greater poverty and poor quality of care in facilities cause women in low income countries to be exposed to more stress compared to women in high income countries (17). High prevalence of antenatal stress and depression have been reported in low income countries, and is comparable to the rates in the subpopulations of North American and European women with low socioeconomic status (18-20). Psychosocial distress is associated with preterm delivery (21-24).
Toxins	The use of multiple drugs, including heroin, methadone and marijuana are linked with preterm delivery (25,26).

	Cocaine use is associated with a higher rate of preterm delivery (27,28). Women who smoke during pregnancy have increased risk of preterm birth (29-31).
Short pregnancy interval	A link between short pregnancy interval of less than 1 year and preterm birth has been demonstrated (32-36).
Genetic history	An elevated risk for subsequent preterm delivery has been established if there is a history of previous preterm delivery, either if the mothers were born preterm or have sisters that have had preterm children (2,37,38). Delayed childbearing is reported to be increasingly common among women in developed countries including the United States and Canada (39-41). Increasing proportions of birth to women aged ≥ 35 years exposes greater risk for preterm birth (42-44).
Maternal age, weight and ethnicity	Systematic reviews and meta-analyses involving women from diverse international cohorts show that gestational weight gain below the Institute of Medicine guidliness is associated with increased risk of preterm delivery. Meanwhile, gestational weight gain above the guidelines is correlated with decreased risk of preterm delivery (45-47). Insufficient weight gain is positively correlated with high risk of preterm delivery, regardless of body mass index (BMI) (48). There is a link between maternal pregnancy BMI and higher risk of preterm delivery (47). Immigrant women with African ancestry and lifetime stress or pregnancy stress may be at increased risk of preterm delivery (49).

Currently, the management of women with threatened preterm labour involves the use of tocolytics including calcium channel blockers, beta-agonists and prostaglandin synthase inhibitors to delay or suppress uterine contraction. Tocolytics can provide additional time to administer antenatal corticosteroids such as betamethasone or dexamethasone in order to mature fetal tissues and reduce mortality and morbidity in the newborn (50). Meanwhile, in preterm birth arising from infectious aetiology, antibiotics are used to suppress bacterial growth with the aim to delay preterm birth (51). Neverheless, these attempts to prevent or arrest preterm delivery using currently available antibiotics are restricted by adverse side effects as well as poor efficacy (50-55).

Thus, there is an urgent need to better elucidate the complex underlying signalling mechanisms, especially the upstream, initiating events of preterm delivery. Defining the initial triggers and feed-forward events, and understanding how these relate to physiological events in normal term labour, is necessary to provide the detailed for developing future clinical solutions to reduce the prevalence of preterm delivery.

The parturition process can be represented as a pro-inflammatory signalling cascade, acting in harmony with hormonal cues (56). The mechanisms of parturition including uterine contractions, cervical ripening and the rupture of fetal membranes are all thought to be mediated in large part by inflammatory mediators. Inflammatory leukocytes (57,58), cytokines (59), and chemokines (59) then elicit specific responses, leading to the elevated synthesis of prostaglandins (60) and matrix metalloproteinases (MMPs) which in turn activate the uterine contractions and cervical dilatation of the parturition process. In humans, toll-like receptor 4 (TLR4) has been suggested to play a central role as a key upstream driver of the pathways underpinning preterm delivery with chorioamnionitis, as well as in normal term labour (61).

One biological agent which binds TLR4 to activate inflammation is bacterial lipopolysaccharide (LPS), which is a member of the family of agents known as exogenous pathogen-associated molecular patterns (PAMPs) (62). Previous studies have demonstrated the association between the presence of bacterial infection with preterm delivery in humans (8,63). Most importantly, TLR4 deficiency in mice has also been implicated in mediating the normal response to infection-induced preterm delivery (64-66).

Another class of biological agents which binds TLR4 to activate inflammation is the endogenous molecules known as danger-associated molecular patterns (DAMPs) (67). DAMPs are released during necrotic cell death which results from ischemia, toxic injury, stress or trauma

as well as from the degradation of extracellular matrix molecules following tissue injury (67). As well as these definitive DAMPs, other endogenous factors including platelet activating factor (PAF) and surfactant protein (SP) also found to contribute to TLR4 activation and can be released either under physiological or pathological conditions. In reproductive tissues, DAMPs and TLR4 regulators are released in late gestation and these are potential candidates for initiating term and preterm delivery. Key DAMPs and TLR4 regulators that are present in gestational tissues and increase as term approaches are platelet activating factor (PAF), surfactant protein (SP), heat shock protein 70 (HSP70), high mobility group box 1 (HMGB1), fetal fibronectin, uric acid, 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one.

Thus, DAMPs and TLR4 regulators which are synthesised in gestational tissues might play a crucial role in initiation of the physiological events of normal term labour. Their expression may also lead to an unscheduled and inappropriate inflammatory process, to initiate preterm delivery. These DAMPs and TLR4 regulators are proposed to induce inflammation by causing activation of TLR4. Therefore, we now seek to undertake research to address questions on the key roles of DAMPs, TLR4 regulators and TLR4 in term delivery and preterm delivery. This research will allow us to understand how TLR4 signalling might be crucial in determining the timing of labour and birth, and thus be a key step towards perinatal mortality and morbidity of premature birth.

1.2 MOUSE MODELS OF TERM AND PRETERM DELIVERY

The mouse has been found to be an informative and practical model to study the inflammatory pathways involved in preterm delivery. Its reproductive biology and immunology, especially the response to infectious stimuli has extensive similarities with that of humans (68). However, there are differences in various aspects of reproductive biology. For example, unlike humans, progesterone synthesis is limited to the ovary and does not occur in the placenta. In women, progesterone release from the corpus luteum is followed by synthesis in the placenta (68). In mice, systemic progesterone withdrawal is crucial for term parturition, in contrast to humans (68). Nevertheless treatment with antiprogestins including mifepristone results in labour at any stage of pregnancy in women, indicating that although progesterone synthesis is sustained, a functional progesterone withdrawal that interferes with progesterone signalling occurs in humans (69). As an animal model, mice are economical and readily manipulated in captivity, allowing sufficient numbers to be tested to demonstrate reproducibility and statistical significance in differences between treatments (68). They have a short gestational length and can reach sexual maturity rapidly to allow for time-efficient experimental development (68). This significantly shorter gestational length, which is approximately 20 days versus 266 days in humans, is a limitation in the study of pharmaceutical compound exposure or longitudinal analysis of effects of offspring (68). In addition, it makes it difficult for maternal and fetal instrumentation and surgical interventions due to the small anatomical size and larger litter size (68). Importantly mouse models offer the possibility of using genetically modified mice, in order to study the gene pathways involved during labour (68). We have summarized the different mice models that have been used to provide useful information on the role of inflammatory mediators in both term and preterm delivery (Table 1.2).

Table 1.2 Mouse models of infection-induced preterm delivery and term delivery

Model	Preterm phenotype	Term phenotype
TLR4 null mutant C3H/HeJ	Reduces susceptibility to heat killed *Escherichia coli* (*E.coli*)(64)	No phenotype reported
TLR2 null mutation	No phenotype (70)	Delay in labour by approximately 10 hours (71)
TRIF null mutation	No effect on response to killed *E.coli* (70)	No phenotype reported
MyD88 null mutation	Reduces susceptibility to killed *E.coli (70)*	No phenotype reported
MyD88 and TRIF double null mutation	Reduces susceptibility to killed *E.coli (70)*	No phenotype reported
IL1B null mutation	No effect on response to *E.coli* or LPS (72)	No phenotype reported
IL6 null mutation	Reduces susceptibility to LPS (73) No effect on response to killed *E.coli* (74)	Delay in labour by approximately 24 hours (73)
Type 1 receptors for IL1 and TNF double null mutation	Reduces susceptibility to killed *E.coli* (75)	No phenotype reported

IL10 null mutation	Increases susceptibility to LPS (76,77)	No phenotype reported
Jα18 null mutation (deficient in invariant NK T cells)	Reduces susceptibility to LPS (78)	No phenotype reported
Rag1 null mutation (deficient in T and B cells)	Increases susceptibility to LPS (79)	No phenotype reported
PTGHS1 null mutation	No phenotype reported	Delay in labour by approximately 12 hours (80)
SP-A and SP-D double null mutation	No phenotype reported	Delay in labour by approximately 17 hours in second pregnancies (71)
SRC1 and SRC2 double heterozygous null mutation (deficient in PAF and SP-A)	No phenotype reported	Delay in labour by approximately 35 hours (81)

1.3 PARTURITION AND INFLAMMATORY PATHWAYS IN TERM DELIVERY

Parturition involves complex, redundant mechanisms mediated in different organ systems and thus the elucidation of the initiators and effectors of normal and pathological pregnancies is challenging. Phase I represents the beginning of parturition and in humans may start several weeks prior to the onset of active labour. Nevertheless, there are very limited studies to elucidate the molecular mechanisms initiating the first steps towards activating inflammation in the gestational tissues of humans.

It is known that changes in the immune cell populations commence in the maternal and fetal compartments of the gestational tissues during Phase 1. Most notably, the cervix becomes infiltrated with neutrophils and macrophages prior to the onset of labour (82). Leukocyte extravasation into gestational tissues occurs via the increase in cell adhesion molecule expression, such as intercellular adhesion molecule 1 (ICAM-1), platelet-endothelial cell adhesion molecule (PECAM) and vascular cell adhesion molecule (VCAM) (83) which have been localised in vascular endothelium and leukocytes in the myometrium and cervix (84). *PECAM* and *VCAM* messenger ribonucleic acid RNA (mRNA) expression are increased as pregnancy advances in the myometrium and cervix respectively (84). In maternal circulation, an increase in the expression of CD11B on monocytes and granulocytes, which facilitate the adhesion of these leukocytes is observed towards the end of human gestation (85), suggesting that peripheral changes in immune cells accompany and may even precede those in the gestational tissues.

Inflammation occurs in the myometrium, decidua, placenta and cervix during this stage in mice as shown by increased infiltration and activation of macrophages and increased production of pro-inflammatory cytokines (86-90). A gradual increase in the myometrial contractility and softening of the cervix occurs as the results of changes in the gene expression in myometrial (87,91) and cervical cells respectively (92). Fetal membranes overlying the cervix are weaker and thinner when compared to other regions of the fetal membranes (93), implying that the remodelling process of fetal membranes occurs to develop a weakened area at the site of rupture of fetal membranes, in preparing for labour (56).

Phase II represents active labour and delivery which occurs as a direct consequences of phenotypic changes in the gestational tissues induced during phase I. Parturition mechanisms involve the onset of uterine contractions, cervical ripening and the rupture of human fetal

membranes. The infiltration of leukocytes and biochemical mediators of inflammation in the myometrium, decidua, placenta, fetal membranes and cervix initiate this inflammatory process to elicit specific responses, leading to activation of the reproductive tissues. The expression of *ICAM1* appears to become elevated during labour in these maternal tissues (84).

There are two isoforms of interleukin 1 (IL1), IL1A and IL1B, and in human tissues, IL1B is more abundant (59). IL1 is secreted by a variety of leukocytes as well as resident structural cells including smooth muscle cells, decidual stromal cells, placental Hofbauer cells (macrophages), syncytiotrophoblasts, endothelial cells within placental villi, and cervical fibroblasts (59,94,95). IL1 plays a role in inducing the expression of ICAM-1 and VCAM for adhesion of neutrophils, monocytes and lymphocytes (83). Tumour necrosis factor (TNF) which activates and causes the adhesion of neutrophils, is produced by a variety of leukocytes predominantly macrophages as well as mesenchymal cells, decidual stromal cells, placental stromal cells, extravillous trophoblast, cervical fibroblasts, smooth muscle cells and the glandular and surface epithelium of the cervix (59,83,95). Interleukin 6 (IL6) is known to be produced by activated leukocytes including macrophages and can also be secreted by mesenchymal cells, decidual stromal cells, extravillous trophoblasts, endothelial cells within placental villi and the glandular and surface epithelium of the cervix (59,83,95). Together IL1, TNF and IL6 cytokines are important for activation of lymphocytes during inflammation (83). nuclear factor-kappa beta (NF-KB) transcription factor activity is a key mediator and upstream regulator of expression the pro-inflammatory cytokines and chemokines within reproductive tissues (96,97). This is due to the presence of NF-KB recognition elements within the promoters of the pro-inflammatory cytokine genes (98).

Activation and infiltration of leukocytes from the intravascular space into the gestational tissues is also regulated by the secretion of chemokines, a family of low-molecular weight chemotactic cytokines (99), secreted by leukocytes and other types of cells. The most relevant chemokines associated with parturition are CCL2, CCL3, CCL5, CCL20, CXCL1 and CXCL2 and CXCL8. The function of CCL2 is to primarily attract and activate mainly monocytes (100,101); while CCL3 participates in attracting lymphocytes, monocytes, macrophages and eosinophils (102,103). CCL5 is identified as a potent chemotactic agent and an activator of monocytes, T lymphocytes and eosinophils (103,104). CCL20 plays a role in the chemotaxis of immature dendritic cells, as well as T and B cells (105). CXCL1 and CXCL2 are potent neutrophil

chemotactic and neutrophil-activating factors (106-108). CXCL8 attracts leukocytes and stimulates adhesion molecule expression and neutrophil activity (59). CXCL8 is also a major chemokine studied in normal term parturition and has been found to be produced by mesenchymal cells, decidual stromal cells, cervical stromal cells as well as the glandular and surface epithelium of the cervix (59).

It is thought that a positive feedback mechanism exists during labour, whereby the presence of pro-inflammatory cytokines contributes to invasion of pro-inflammatory leukocytes into the gestational tissues (59). These leukocytes then generate additional cytokines to further attract more leukocytes into the surrounding tissues (59).

1.3.1 Myometrium

The myometrium, which accounts for most of the uterine mass, is composed of specialised smooth muscle cells, arranged in randomly oriented interconnected bundles within an extracellular matrix that consist of collagen proteoglycans as well as adhesion molecules such as laminins and fibronectin (92). During labour, it has been shown that invasion of macrophages and neutrophils occurs in the upper segment of the myometrium, suggesting a crucial role in mediating uterine contraction directly via secretion of pro-inflammatory cytokines, including interleukins and TNF (57).

The lower segment of the myometrium, which interfaces with the cervix attracts elevated populations of macrophages, neutrophils and T cells during labour (57). These cells are implicated in tissue remodelling and thus facilitate cervical dilatation and the passage of the fetus (57). Furthermore, neutrophils are proposed to contribute to the increase in collagenase activity in the lower segment of the myometrium during labour (109). Neutrophils act as a major source of MMPs, which are a key family of endopeptidases found to cleave many constituents of the extracellular matrix including neutrophil elastase, neutrophil collagenase (MMP8), and MMP3 activated gelatinase B (MMP9) (110). This view is supported by a study demonstrating that MMP8, MMP9 as well as CXCL8, a key driver of neutrophil chemotaxis and activation, are elevated during labour and at the same time during cervical dilation in lower segment myometrium (111). Moreover, the release of MMP8 and MMP9 correlate strongly with CXCL8 concentration in this tissue (111). Other than neutrophils, resident structural cells can also secrete MMP, as inflammatory cytokines including IL1 and TNF induce MMP9 in smooth muscle

cells (112). In the myometrium the remodelling of this connective tissue might contribute to facilitate the uterine contraction by allowing the formation of gap junctions (113).

IL1B, IL6 and *CXCL8* mRNA expression is upregulated in the myometrium during labour (58). Using immunohistochemistry, leukocytes are found to be the predominant origin of IL1B and a low concentration of IL1B is also demonstrated within myocytes in the labouring myometrium (59). The localisation of IL6 is restricted to a sub-population of CD45+ cells, possibly neutrophils based on their morphology; however, it is not clear how this expression changes in the labouring versus non-labouring myometrium (59). CXCL8 is secreted by leukocytes only in the labouring myometrium (59). These results suggest that invading leukocytes are the main source of the increase in IL1B and CXCL8 observed in the myometrium, as opposed to the resident structural cells. Further experiments are required to determine which leukocytes are the major contributor to the elevation of these pro-inflammatory cytokines documented during labour. Other chemokines are likely to be crucial in attracting other leukocytes including macrophages as well as T cells into the myometrium and subsequently promoting their activation, and these are not well studied during human labour. The sequence of immune cell changes has not been documented in detail so the relationships between various populations is unclear. For example it could be that during labour, neutrophils infiltration and activation causes the increase in pro-inflammatory cytokines, which then recruit more inflammatory leukocytes via an increase in adhesion molecule and chemokine expression (83,114).

Pro-inflammatory cytokine mRNAs including *Tnf, Il1b, Il6* and *Il12b mRNA* are increased during labour in both the myometrium and decidua in mice (86,87). Importantly, it has been shown that parturition in *Il6*-/- mice is delayed by 24 hours when compared to wildtype mice and this delay in labour is restored when the *Il6*-/- mice are administered with exogenous IL6, suggesting an important role of this cytokine in mediating normal on-time labour (73). Overall leukocyte invasion and pro-inflammatory cytokine production may then be stimulated in the myometrium in a feed-forward loop which sustains and amplifies the process of parturition via prostaglandin production and uterine contractions.

Prostaglandins, including prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2) produced by the myometrium and intrauterine tissues of pregnancy, also play a vital role in the mechanisms of parturition (60). One of the mechanisms of parturition induced by prostaglandins is uterine muscular contraction, as shown by treatment of pregnant women with prostaglandins

which is demonstrated to induce labour (60). Furthermore, inhibition of prostaglandin activity is reported to result in delayed parturition in primates (115). The transition of the myometrium from an inactive state during pregnancy to a muscle that is spontaneously sensitive and responsive to contractile stimulants during labour is termed 'uterine activation'. It is proposed that the activation of the myometrium occurs from coordinated expression of uterine activation mediators including prostaglandin H synthase (PTGHS), prostaglandin $F_{2\alpha}$ receptor (PTGFR), prostaglandin E_2 receptor (PTGER), oxytocin receptor (OXTR) and connexion-43 (Cx-43) also known as GJA (91). Other than prostaglandins, oxytocin also contributes to myometrial contraction and GJA is important in increasing the formation of gap junctions to support an increase in the electrical coupling between myometrial cells to promote synchronised uterine contraction (92).

Two isoforms of PTGHS have been identified, PTGHS1 and PTGHS2, which catalyse the first step in prostaglandin synthesis from arachidonic acid (60). While in most cases PTGHS1 is expressed in many tissues for constitutive prostaglandins synthesis, PTGHS2 is generally induced by several agonists (60). *PTGHS2* mRNA expression is detected to be higher in both the upper and lower segments of the myometrium in women with term labour, when compared to women at term with no labour (116). In support of these results, a study in mice shows that *Ptghs2* mRNA expression in the uterus is upregulated in wildtype mice during parturition (91,117). On the other hand, uterine mRNA expression of *Ptghs1* is induced before parturition and its level remains the same during parturition in the uterine tissues (117). *Ptghs1*-/- mice have been shown to exhibit a delay in labour, due to a low level of prostaglandin $F_{2\alpha}$ production which inadequately initiates luteolysis (80). In turn, the expected fall in plasma progesterone fails to occur in *Ptghs1*-/- mice as expected on gd 19 (80). Thus, it stands to reason that that while PTGHS1-derived prostaglandins might play a crucial role the induction of luteolysis, PTGHS2-derived prostaglandins might facilitate the mechanisms of uterine contraction during parturition in mice (117).

PTGFR and GJA concentrations are shown to be elevated in the lower and upper segments of myometrium in women at term with labour, compared to term women with no labour (118,119). In addition, elevated expression of *OXTR* mRNA is reported with the onset of women term labour (120). Taken together, elevation in these uterine activation proteins by regulation at the transcriptional level appears to allow the uterus to respond to stimulants and associated

prostaglandins leading to uterine activation, allowing progression to the physiological events of delivery.

Studies in human myometrial cell cultures demonstrate that IL1B and TNF modulate arachidonic acid release and expression of PTGHS2, to promote prostaglandin synthesis (121-124). It is well documented by in vitro studies in humans and rats that pro-inflammatory cytokines act to stimulate expression of other uterine activation genes including *PTGFR* (125) and *Oxtr* (126). These observations in vitro are reinforced by an in vivo study in mice showing that the delay in labour in *Il6-/-* mice is accompanied by a delay in the expression of *Oxtr* and *Ptger4* mRNA in the uterus (73).

1.3.2 Decidua

The decidua comprises terminally differentiated endometrial stromal cells, decidual parenchymal cells which are derived from uterine stromal fibroblast-like cells in the endometrium and newly generated maternal vascular cells (127). An infiltration of inflammatory leukocytes and a higher concentration of IL1B are evident in the decidua during normal term labour (128,129). A greater density of macrophages and other leukocytes have been observed in the human decidua relative to amnion, amniotic connective tissue, and chorion (130). This explains why *IL1B* mRNA expression is amplified in the choriodecidua relative to the amnion (130).

1.3.3 Placenta

The placenta is composed of fibroblasts, endothelial cells, smooth muscle cells, cytotrophoblast cells, and syncytiotrophoblasts (92). A higher level of inflammatory cytokine production has been detected in human placental explant cultures, including IL1, IL6, and TNF when tissue is collected during labour (94,95,131,132). IL1B is shown to be produced at a higher level in comparison to IL1A in placental explants at term labour (94). Importantly the elevated level of TNF is demonstrated to result from secretion by placental macrophages (CD11B+ cells) (95). Placental stromal cells also contribute to the elevation in TNF production in the placenta during labour (95). On the other hand, the amplification in IL1B and IL6 cytokines during labour is contributed by the endothelial cells of the placental villi, and the activation of macrophages as late parturition approaches appears to also contribute (95). It seems possible that inflammation in the placenta contributes to amplifying the inflammatory process in the maternal and fetal tissues to initiate and/or accelerate the labour cascade.

1.3.4 Fetal membrane

The fetal membranes are composed of amnion and chorion epithelial cells. Together with the adjacent decidua of maternal origin, these tissues contribute to the regulation of both myometrial activity and fetal maturation (92). Together with the placenta, the fetal membrane acts as the interface between the fetus and the mother, to transmit the signals produced by the fetus to the maternal tissues including the myometrium and cervix (92). There is no observable difference in the density of leukocytes detected in the unseperated fetal membranes after the onset of labour, compared to before labour (58), although when the chemotaxic bioactivity is measured, the choriodecidua exhibits a higher chemotactic activity for granulocytes, T cells, monocytes and natural killer (NK) cells (133). There is a significantly higher density of leukocytes in the peri-placental region of the membranes when compared with the region of fetal membranes overlying the cervix, known as the zone of altered morphology (ZAM) (130). This leads to an interpretation that the inflammatory cytokines expressed in the placenta may contribute to the infiltration of leukocytes into peri-placental region of the fetal membranes.

Elevated *IL1B, IL6, TNF* and *CCXL8* mRNA expression occurs in the amnion and choriodecidua during human labour (58,134). NF-KB is reported to increase at the time of labour, compared to before labour in the fetal membranes (135) in order to initiate the transcription of these inflammatory cytokine genes. An increase in production of IL1B, IL6 and CXCL8 has also been implicated in the amnion and chorion during human labour (136,137). Using immunohistochemistry, the IL1B, IL6 and CXCL8 are localised to the leukocytes, and resident structural cells (59). Nevertheless, it is unclear whether the immune cells or resident structural cells cause the elevation in inflammatory cytokines observed in these tissues during labour (60). As the density of leukocytes are portrayed to be similar before labour and after the onset of labour (58), resident structural cells may contribute to the increase in these inflammatory cytokines in fetal membranes.

These inflammatory cytokines presumably stimulate the increased level of *PTGHS2* mRNA, which is positively correlated with PTGHS enzyme activity in the amnion of women delivered spontaneously at term when compared to women delivered by selective Caesarean section at term (138). This could result in an increase of PTGHS2 causing elevated synthesis of prostaglandin E_2 in the amnion, which is hypothesised to diffuse through the chorion and decidua to further induce myometrial prostaglandin production and hence contraction (139). In addition,

MMP8 and MMP9 are detected in the fetal membranes, and production of these is shown to be induced by TNF (140). The levels and activity of MMP9 has been shown to be elevated in the chorioamnion during labour (141). These enzymes are reported in degradation of the fibrillar collagen-rich extracellular matrix of the amnion and choriodecidua, which contribute to the increased structural integrity and tensile strength of the fetal membrane (111,141).

1.3.5 Amniotic fluid

The elevation of cytokines including IL6, TNF and ILB in the amniotic fluid during labour (136,137,142) correlates with the degree of leukocyte invasion into the placenta and fetal membranes (143). Several studies demonstrate that the concentrations of CCL2 (144), CCL3 (145,146), CCL5 (147), CCL20 (105) and CXCL1 (148) are upregulated in the amniotic fluid during human spontaneous labour at term suggesting that these chemokines play a role in leukocyte infiltration into the fetal tissues including the placenta, fetal membranes and the maternal tissues including myometrium, decidua and cervix. Nevertheless, there are limited studies to investigate the different populations and percentage of leukocytes in the amniotic fluid during labour and the specific sequence of the changes in gene expression and leukocyte infiltration.

1.3.6 Cervix

The cervix comprises the ectocervix which is lined by epithelial cells, projecting into the vagina; and the endocervix which forms the lining of the cervical canal; stromal fibroblasts which form the body of the cervix as well as the connective tissues with abundant collagen which is important for the rigidity of the cervix (92,149). The cervix is infiltrated with neutrophils and macrophages prior to the onset of labour; however, it has been reported that no further infiltration of these cells occurs once the final stage of labour commences (82). In contrast, another study demonstrated an infiltration of these leukocytes during labour (58). The primary difference in the study design is that Bokstrom et. al did not define active labour, whereas Osman et. al recruited only spontaneous labouring women with cervical dilation of 4-8 cm (58). The significance of labour stage is further emphasised by evidence showing a higher synthesis of CXCL8 in the cervix during parturition (150). During labour, ILB and IL6 levels are localised to leukocytes invading the cervix (59).

Cervical ripening is associated with the disorganization of these collagen bundles by MMPs (149). Most importantly, the infiltrating leukocytes have been suggested to release MMP8 and MMP9 to contribute to the cervical remodelling process (151). Thus, the elevated level of CXCL8 in the cervix can be associated with increasing activity of collagenase contributed by neutrophils found in the cervix during parturition (151). Other than infiltrating leukocytes, the local resident structural cells in the cervix also contribute to the production of these endopeptidases. IL1B and TNF participate in the production of MMP1, MMP3 and MMP9 from cervical fibroblasts (114) as well as cervical smooth muscle cells at term (152).

1.3.7 Peripheral blood

Higher populations of CD15+ neutrophils, CD14+ monocytes and CD16/56 + NK cells, with greater migratory activity of neutrophils has been reported during labour in the peripheral blood (153). These neutrophils and monocytes have elevated surface expression of CD62L, which mediates tethering and rolling of these leukocytes onto endothelium, prior to extravasation and migration into tissues (153). Elevated expression of CD11A and CD11B is also revealed on the surface of neutrophils and monocytes, facilitating the binding of these leukocytes to ICAM-1 (153) and thus may promote their adhesion to the myometrial and cervical endothelium (84). This evidence suggests that the leukocytes in the maternal circulation are primed for initiation of adherence and extravasation into the gestational tissues during parturition. Anti-inflammatory CD4+CD127 low+/-CD25+ regulatory T (Treg) cells are lower in the percentage and suppressive activity in the blood samples of women delivering spontaneously or by elective caesarean section at term when compared to the women in first-trimester of pregnancy (154,155). This evidence suggests there is a reduced availability to suppress inflammation during labour in the maternal circulation.

1.3.8 Summary

The mechanisms of inflammation during human labour in gestational tissues are summarised in Figure1.1 below:

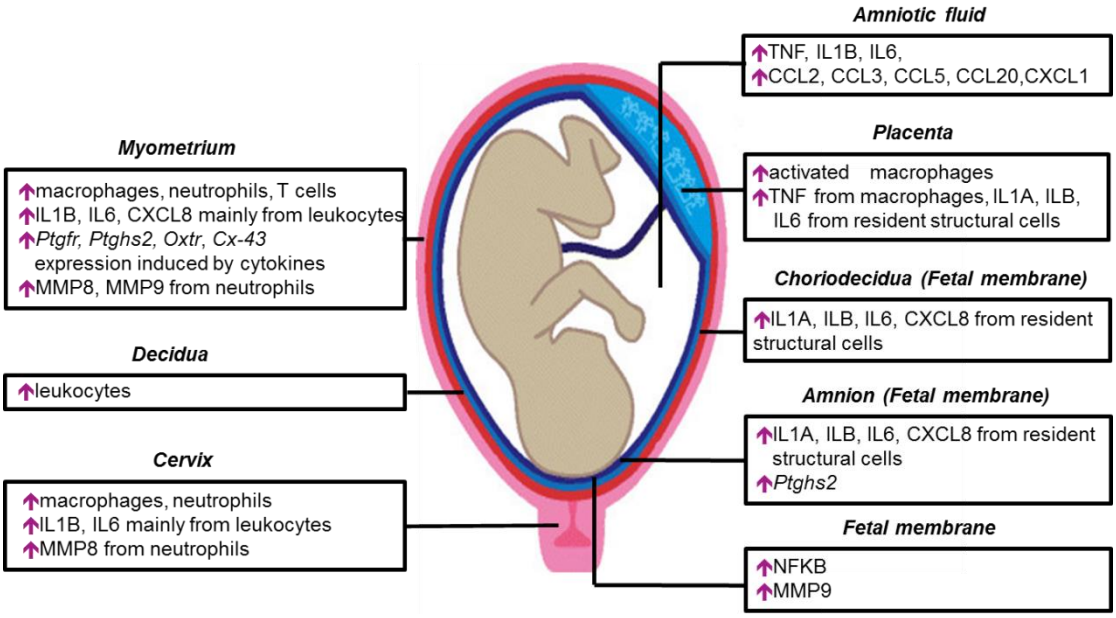


Figure 1.1 Schematic illustration of inflammation in the gestational tissues during labour

The invasion of pro-inflammatory leukocytes into maternal tissues including the myometrium, decidua and cervix is induced by elevated expression of pro-inflammatory cytokines. This in turn causes a further increase in inflammatory cytokine production, to upregulate uterine activation genes and increase the level of MMPs allowing uterine contraction and cervical dilation to be initiated. Similarly, an infiltration of macrophages is observed in the placenta and together with resident structural cells, contributes to elevated production of pro-inflammatory cytokines, to likely further amplify inflammation in both the maternal and fetal tissues. In the fetal membranes, the resident structural cells potentially contribute to increased production of inflammatory cytokines, leading to induction of uterine activation gene expression and amplified MMP production, instigating fetal membrane rupture. In the amniotic fluid elevated levels of both inflammatory cytokines and chemokines are demonstrated, and the inflammatory signals are suggested to be transmitted to the fetal and maternal tissues. Despite these observations, the sequence and causal relationships of the molecular mechanisms, particularly those that initiate the first steps towards activating inflammation and initiating the labour cascade, are not clear.

1.3.9 Role of TLR4 and its signalling pathways in normal term delivery

One possible mechanism via which the inflammatory process can be activated is via the toll-like receptors (TLRs). In humans, ten different TLRs have been identified according to the types of ligands they recognise, including LPS (also known as endotoxin) in the cell walls of Gram-negative bacteria, peptidoglycan (PGN) and lipoteichoic acid in Gram-positive bacteria, double stranded ribonucleic acid (RNA) viral motifs and flagellin.

Human reproductive and conceptus tissues including the uterus, endometrium (156-158), fetal membranes (159), placenta (160) and endocervix (156,161) express TLR4. Within the reproductive tract, TLR4 is also expressed by dendritic cells, monocytes, macrophages in the endometrium (157), NK cells in the uterus (162) as well as the macrophages and neutrophils in the placenta (163). Meanwhile in the mouse, TLR4 has been detected in the maternal reproductive tract including the uterine endometrium (164), epithelial cells derived from uterus (165) and cervix (166), as well as from fetal tissues including fetal membranes (167) and placenta (166). Uterine dendritic cells (168) and amniotic fluid macrophages (71) also express TLR4.

Interestingly, human spontaneous labour at term is correlated with increased chorioamniotic membrane expression of *TLR4* (61). Nevertheless, there are limited studies to elucidate the TLR4 expression in other gestational tissues during labour or to define whether TLR4 is differentially expressed or has any physiological role. Meanwhile in mice, TLR4 elevates in the cervix and uterus towards the end of gestation (166). This evidence in both human and mice suggests that TLR4 is potentially important in normal term parturition.

Montalbano et al. found that the surface expression of both TLR4 and TLR2 is increased in amniotic fluid macrophages towards the end of pregnancy (71). When these authors evaluated the role of TLR2 in normal on time parturition, they found that mice deficient in TLR2 had delayed labour compared to wildtype mice (71). This was accompanied by a decline in *Gja* and *F4/80* mRNA in the myometrium of TLR2 decient (*Tlr2-/-)* mice compared to wildtype mice on gd 18.5 (71). The amniotic fluid macrophages isolated from *Tlr2-/-* mice on gd 18.5 also displayed a decrease in *Il1b* and *Arg1* mRNA, which are activation markers for pro-inflammatory M1 and anti-inflammatory M2 macrophages respectively (71).

It is well established that TLR4 activates downstream inflammatory signalling via recruitment of adaptor molecules, including myeloid differentiation factor88 (MyD88) and MyD88 adaptor-like (Mal), as well as TIR domain- containing adaptor (TRIF) (169). The MyD88-dependent pathway is mediated by the MyD88 and Mal activation of IL1R associated kinases (IRAK), IRAK1 and IRAK4, TRF-associated factor 6 (TRAF6) and mitogen-activated kinases (MAPK) (169). These kinases then further activate the transcription factor NF-KB via the IKB kinase (IKK) complex (169). NF-KB acts to mediate the transcription of the pro-inflammatory cytokine genes (169). In addition, MyD88-independent pathway is initiated by TRIF, which results in activation of the interferon regulated factors (IRF) family of transcription factors, to mediate the

transcription of interferon (IFN) (169). These MyD88-dependent and independent pathways are demonstrated in Figure 1.2 below:

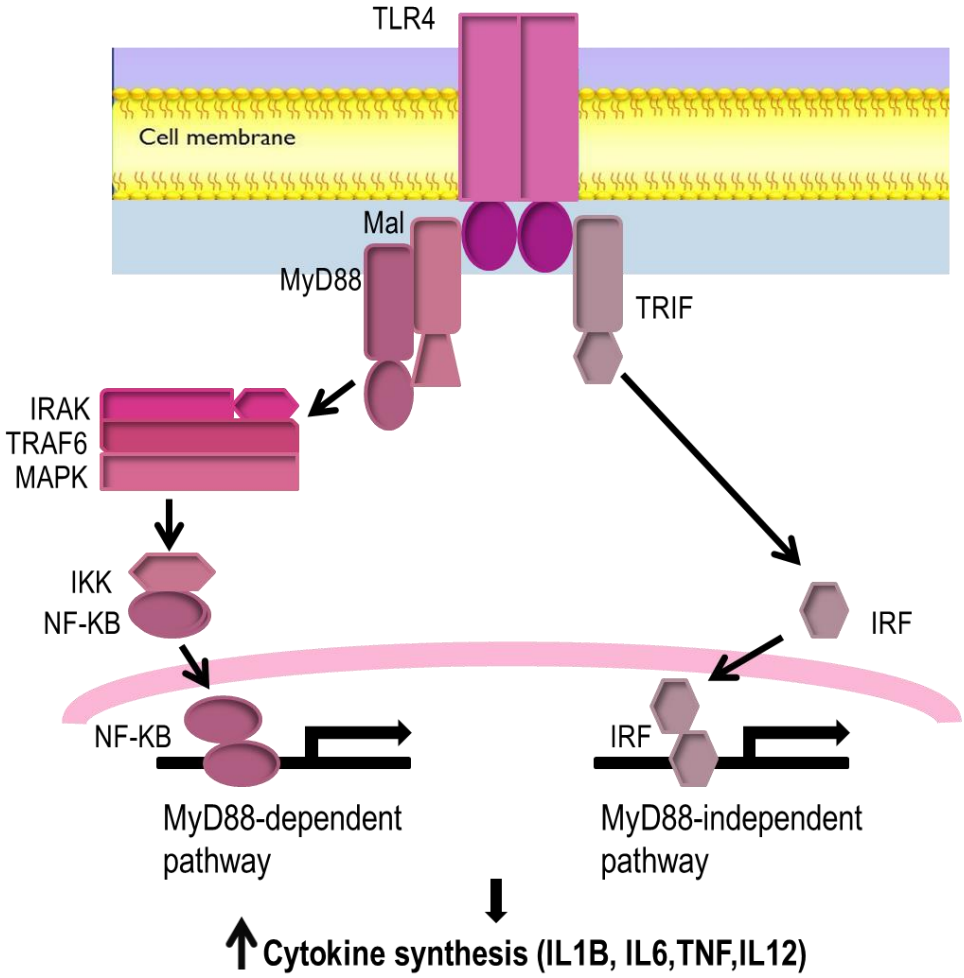


Figure 1.2 Schematic illustration of the MyD88-dependent and MyD88-independent pathways mediated by TLR4 ligation to induce cytokine gene expression

TLR4 activates the NF-KB transcription factor via the adaptor molecules including MyD88 and Mal, which act to activate the kinases including IRAK, TRAF6, MAPK and IKK in the MyD88-dependent pathway. The IRF transcription factor is activated by TLR4 via TRIF, as the adaptor molecule in the MyD88-independent pathway. Adapted from Diamond et. al (2007).

LPS as one of the TLR4 ligands has been demonstrated to induce the formation of the 'm' shaped TLR4, myeloid differentiation factor-2 (MD2) and LPS complex (170) as shown in Figure 1.3 below. Before binding to LPS, MD2 forms a stable heterodimer with the extracellular domain of TLR4. LPS binding forms an additional binding interface between TLR4 and MD2, leading to dimerization of TLR4-MD2 complex (170). This is proposed to allow the dimerization of intracellular TIR domain and hence recruitment of the adaptor molecules explained above such as MyD88 (170). The formation of TLR4, MD2 and LPS complex is induced by CD14, which

plays a role in loading the LPS onto cell surface TLR4-MD2 and CD14 coprecipitates with this complex (171).

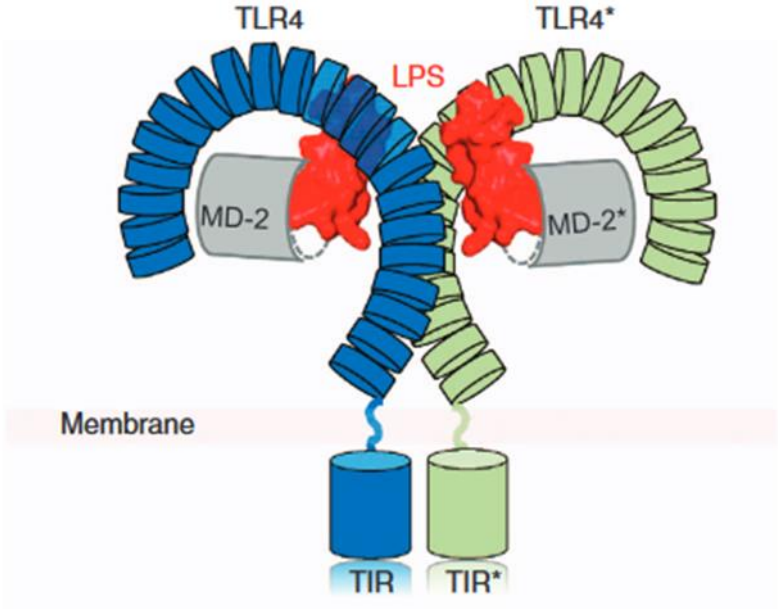


Figure 1.3 Schematic illustration of LPS recognition by TLR4-MD2

Dimerization of surface TLR4-MD2 is induced following binding to LPS, suggested to subsequently cause dimerization of the TIR domain for the recruitment of adaptor molecules. Adapted from Park et. al (2013).

1.4 INFLAMMATORY PATHWAYS IN INFECTION MEDIATED PRETERM DELIVERY

1.4.1 Intrauterine infection in human preterm delivery

Preterm delivery is proposed to result from a pathological process where in premature birth there is precocious activation of the inflammatory pathways common to term labour (11). Intrauterine infection can occur via microbial invasion of the amniotic cavity (MIAC), which is diagnosed by the isolation of microorganisms with standard culture techniques from amniotic fluid which is normally sterile. Amniotic fluid from women with preterm delivery is retrieved via transabdominal amniocentesis procedure, to allow amniotic fluid cultures to be performed and thus provide information about intra-amniotic infection (IAI) (8). From 33 studies, 13% (379/2963) of preterm delivery women with intact membranes were detected with positive amniotic fluid cultures and these women had a higher tendency to develop clinical chorioamnionitis, to be

refractory to tocolytic and to rupture their membranes spontaneously, compared to women with negative amniotic fluid cultures (8). Furthermore, from 18 studies, 32% (473/1462) of women with preterm premature rupture of membrane (PROM) were found to have a positive amniotic fluid cultures (8).

Polymerase chain reaction (PCR) has emerged as a more sensitive, more rapid and less expensive method than standard microbial culture (172). *Ureaplasma* species is detected in amniotic fluid from a higher percentage of women with preterm PROM using PCR techniques (28%) in comparison to culture techniques (16%) (172). In addition, amniotic fluid culture tested negative in 42% of women whom were detected as being positive for *Ureaplasma* species using PCR (172). Women with a PCR positive result but negative amniotic fluid cultures had a higher inflammatory response in the amniotic fluid and elevated neonatal morbidity, in comparison to women with both negative PCR and culture results (172,173). The more sensitive molecular techniques reveal a link between microbial invasion of the amniotic cavity with greater inflammation and adverse maternal and fetal outcomes (172,173).

Despite the evidence discussed above, the crucial question that remains to be addressed is why some women with preterm delivery exhibit microbial invasion of the amniotic cavity and others do not. Other risk factors, possibly non-infectious agents might also contribute to preterm delivery.

1.4.2 The role of TLR4 in preterm delivery

Interestingly, human preterm delivery with chorioamnionitis is associated with a higher percentage of TLR4 positive amniotic epithelial cells in fetal membranes when compared to preterm delivery without chorioamnionitis (61). This suggests expression of TLR4 by fetal membranes is upregulated during the pathological process of preterm delivery in the presence of infection (61). It has also been demonstrated that TLR4 is crucial in mediating normal response to inflammation-induced preterm delivery in mice (64-66).

1.4.3 Cytokines and chemokines in human preterm delivery

Infectious agents most frequently have access to the amniotic cavity and fetus via ascending transmission from the vagina and cervix (8). Infectious agents are proposed to reside in the decidua, chorion, and amnion prior to invading the amniotic cavity and fetus (8). The most common bacteria detected in preterm delivery are *Ureaplasma* species*, Mycoplasma hominis,*

Streptococus agalactiae, E.coli, Fusobacterieum species *and Gardnerella vaginalis* (174). Following infection, release of the bacterial membrane products including LPS and PGN occurs to trigger the pro-inflammatory processes leading to preterm delivery (174).

Women in preterm labour with IAI have higher IL1 bioactivity, most likely IL1B, TNF and IL6 concentrations in the amniotic fluid, when compared to women without infection (175-177). This suggests that the presence of infectious agents promotes the secretion of pro-inflammatory cytokines in the amniotic fluid of preterm labouring women.

Women with preterm labour and IAI exhibit higher concentrations of chemokines including CCL2 (178), CCL3 (145,146), CCL5 (147), CCL20 (105), and CXCL6 (179) in the amniotic fluid, compared to preterm labour without IAI. It is plausible that in response to pathogens, these inflammatory chemokines are released possibly by leukocytes within the amniotic fluid in late gestation. This might be an attempt to recruit more pro-inflammatory leukocytes from the maternal circulation to amplify the inflammatory pathways eliciting preterm labour.

1.4.4 Infection in mice preterm delivery

To mimic the conditions of human preterm delivery mediated by infection, various infectious agents have been utilised including killed *E.coli*, components of the cell wall of Gram-negative and Gram-positive bacteria, components of virus, and IL1. These infectious agents create an inflammatory state in the presence and absence of an overt infection. The dose, time interval, and route of infectious agents administration also contributed to the different rates of preterm delivery reported. Thus, efforts have been made to exploit the use of mouse models to provide insight into the inflammatory pathways involved in infection-induced preterm delivery, as one of the predominant causes of preterm delivery in humans. The different strains of mice and used in a range of studies, which show variations in the rate of preterm delivery are summarised in Table 1.3.

Table 1.3 Mouse models of infection-induced preterm delivery

Strain	Means of inducing preterm delivery	Rate of preterm delivery	Notes
CD-1 impregnated by CD-1	Intrauterine 2 to 10 $\times 10^3$ live *E.coli* on gd 14.5	91 % within 48 hours	Intrauterine administration of live *E.coli*, induces preterm delivery (180)
CD-1 impregnated by CD-1	Intrauterine killed *E.coli* on gd 15 at: • 1×10^8 • 1×10^9 • 1×10^{10}	• 9% • 80% • 100% *within 48 hours	The dose of gram negative bacterial inoculum influences the rate of preterm delivery (181). Mice given killed bacteria appeared mildly ill and developed piloerection as well as decreased mobility (181). The highest bacterial inoculum resulted in maternal death (181).
CD-1-timed pregnant mice	Intrauterine 250 µg LPS, on gd 15	• 0% at 0 hour • 0% after 2 hours • 8% after 6 hours • 71% after 12 hours • 92% after 18 hours 75% after 24 hours	Intrauterine administration of LPS, as a ligand from gram negative bacteria induces preterm delivery within 24 hours (182).
CD-1	On gd 15, • Intrauterine PGN at ➢ 0.15 mg/mouse ➢ 0.3 mg/mouse ➢ 0.6 mg/mouse ➢ 0.75 mg/mouse • LTA at ➢ 0.25 mg/mouse	 • 22% • 40% • 73% • 100% • 25%	Ligands from gram positive bacteria (PGN and LTA) are essential to induce preterm delivery in a dose dependent fashion. PGN is more efficient in inducing preterm delivery via intrauterine administration, compared to the intraperitoneal administration (182). Higher dose of poly [I:C], as a synthetic analogue of double-stranded RNA might be required to induce preterm delivery (182).

	➢ 0.5 mg/mouse ➢ 1.0 mg/mouse	• 100% • 100%
	• Intraperitoneal PGN at: ➢ 0.5 mg/mouse ➢ 1.0 mg/mouse Intrauterine: • Polyinosinic:polycytidylic acid (Poly [I:C]) on gd 15 at 0.5 mg/mouse • Poly [I:C] on gd 16 at 0.5 mg/mouse	 • 11% • 55% • 14% • 31%
C57BL/6	On gd 13, intracervical live Group B Streptococcus (GBS): • wildtype strain at 10^7 CFU/mL • βH/C-deficient strain at 10^7 CFU/mL	 • 54 % • 0% *within 72 hours	Ascending live GBS infection induces preterm delivery within 72 hours (183). βH/C toxin is important as one of the virulence factors to mediate GBS induction of preterm delivery following maternal vaginal colonization (183).
CD-1	Intraperitoneal 10^9 killed GBS on gd 14.5	100% within 36 hours	Systemic killed GBS infection causes preterm delivery (184). Intrauterine administration of killed GBS also induces preterm delivery (data not shown) (184).
C3H/HeN impregnated by C3H/HeN	Intraperitoneal: LPS on gd 12, at: • 50 µg/kg – twice every 3 hr LPS on gd 15, at: • 50 µg/kg -single • 100 µg/kg -single • 50 µg/kg – twice every 1 hr • 50 µg/kg – twice every 3 hr	 • 63% • 0% • 38% • 50% • 100%	Preterm delivery rate varies, depending on the strains of pregnant female mice and mated strains, the dose of LPS and the time intervals that the LPS is given (185).

	• 50 µg/kg – twice every 6 hr LPS on gd 17, at: • 50 µg/kg – twice every 3 hr 50 µg/kg of LPS twice every 3 hours on gd 15 for: • C3H/HeN impregnated by C3H/HeN • C3H/HeN impregnated by B6D2F1 BALB/c impregnated by B6D2F1	• 13% • 100% • 25% • 100% 0%	
C3H/HeN impregnated by B6D2F1	Intraperitoneal: • LTA on gd 15 at: ➢ 25mg/kg once ➢ 25mg/kg twice every 3 hr ➢ 50mg/kg once ➢ 50mg/kg twice every 3 hr ➢ 75mg/kg once ➢ 75mg/kg twice every 3 hr • LTA on gd 17 at: ➢ 12.5mg/kg once ➢ 12.5mg/kg twice every 3 hr ➢ 25mg/kg once ➢ 25mg/kg twice every 3 hr	 • 0% • 58% • 0% • 75% • 50% • 100% • 10% • 90% • 70% • 100%	Preterm delivery rate is influenced by the dose and the time interval that the LTA is administered (186).
C3H/ HeJ pregnant inbred mice	Subcutaneous recombinant human IL-1 at three doses within 6 hours	100% within 24 hours	The subcutaneous administration of IL-1, as one of the pro-inflammatory cytokines, is efficient in mediating preterm delivery within 24 hours (187).

1.4.5 PAMP activation of TLR-4 in mouse model of infection-induced preterm delivery

One of the advantages of using the mouse model is the feasibility of modifying the mouse genome through transgenic and gene knockout approaches, as well as the inhibition of the possible inflammatory mediators involved, in order to provide valuable insights into the possible inflammatory pathways participating in preterm delivery. TLR4 deficient (*Tlr4*-/-) C57BL/6 mice and C3H/HeJ mice (which carry null mutation in TLR4) exhibit a lower fetal death rate, accompanied by decreased placental necroinflammatory response following intravenous injection with *Fusobacterium nucleatum* on gd 16 or 17 when compared to wildtype mice (65). Inhibition of TLR4 using the synthetic TLR4 antagonist TLR4A significantly reduced the fetal death rate as well as placental necrosis in response to this Gram-negative bacterial infection on gd 16 (65). *Tlr4* null mutant C3H/HeJ mice showed no preterm delivery after intrauterine administration of an inoculum of 5 x 10^9 heat killed *E.coli* on gd 14.5 when compared to the *Tlr4* replete C3HeB/FeJ mice, with a 100% preterm delivery rate (64). In addition, the strategy of blocking TLR4 was efficient in reducing the rate of LPS-induced preterm delivery (66). This strong evidence suggests that TLR4 is a key signalling molecule at the apex of the inflammatory cascade which bind PAMPs that are introduced systemically. These observations suggest TLR4 inhibition maybe efficient in providing mice with protection against bacterial infection-induced preterm delivery. Given the common characteristics between these mouse models and human preterm delivery with infection, there is a prospect that TLR4 might also be a rate- limiting factor in human preterm birth.

1.4.6 TLR4 activation of inflammatory cytokines and chemokines in the mouse model of infection induced preterm delivery

MyD88, an adaptor protein downstream of TLR4 signalling, is required to activate the transcription factor NF-KB and its translocation from the cytoplasm into the nucleus (169). Following intrauterine administration of 1 x 10^{10} heat killed *E.coli* on gd 4.5, MYD88 deficient (*Myd88*-/-) and MYD88 and TRIF deficient (*Myd88/Trif*-/-) mice do not exhibit preterm delivery and have a significantly higher fetal viability in utero than their wildtype counterparts (70). In contrast, TRIF deficient (*Trif*-/-) mice administered *E.coli* have 100% preterm delivery and lower viability of fetuses retained in utero, a similar response to wildtype mice (70). These results emphasise that in response to Gram-negative bacterial infection, the downstream signalling of TLR4 is MyD88 dependent. Following bacterial exposure, *Myd88*-/- and *Myd88/Trif*-/- mice had reduced NF-KB proteins and in turn low levels of *Il1b* and *Tnf* mRNA in the uterus compared to *Trif*-/- and wildtype mice (70).

Expression of pro-inflammatory cytokine mRNAs, including *Il1b, Il1* and *Tnf* are detected using Northern blot in the uterus as early as 5 hours after intrauterine *E.coli*-administration on gd 14.5 (180). ELISA assays show that the IL1A, ILIB, IL6 and TNF cytokine levels are higher in the uterus and fetal membranes, as early as 3 hours after the inoculation of the uterus with 10^{10} killed *E.coli* compared to 10^{7} killed *E.coli* (181). Taken together, the inflammatory cytokines are upregulated in the gestational tissues in response to infection locally, in order to mimic human intrauterine infection. Interestingly, following inoculation of bacteria into the uterus, both the fetal and maternal tissues respond to the infection, which suggests that similar to humans, diffusion of bacteria into the fetal membrane occurs and probably infiltrates the amniotic fluid.

A genetic deficiency in IL6 or IL1B results in a similar rate of intrauterine heat killed *E.coli*-induced preterm delivery as in wildtype mice (72,74). The use of the IL6 antibody (MR16-1) is efficient in inducing a normal response towards intrauterine LPS-induced preterm birth, compared to the control IgG or phosphate buffer saline (PBS) (188). This is further supported by evidence that the use of doubly null mutant mice for type 1 receptors for IL1 and TNF (*Il1r1/Tnfrsf1a*-/-) significantly decreased the rate of heat-killed *E.coli*-induced preterm delivery when compared to wildtype mice, at a lower bacterial inoculum (75). These studies highlight the significance of complex, multisystem signalling involved between the cytokines in inflammation-induced preterm delivery.

To date, there has not been any research carried out to evaluate the role of TLR4 in activating the pro-inflammatory chemokines, which regulate leukocyte infiltration in the reproductive tissues and their activation, in response to infection-induced preterm delivery in mice. Nevertheless, a significant elevation in the *Ccl2* mRNA was detected in the uterus after 2 and 6 hours of injection with LPS intrauterine on gd 15 (182). Greater chemokine mRNA expression including *Cxcl1, Cxcl2* and *Cxcl5* were quantified in the uterus, placenta and fetal membranes 6 hours after intrauterine infusion of LPS on gd 17 (189). Similarly, elevated concentrations of CCL2 and CCL3 were measured using ELISA in the uterus 12 hours after intraperitoneal LPS administration on gd 16.5 (190). Taken together, it seems that infection that occurs locally or systemically induces significant chemokine production, possibly from the leukocytes which invade the gestational tissues. This would probably allow the invasion of more inflammatory leukocytes to further amplify the inflammation, culminating preterm delivery.

In humans NF-KB also regulates the IL1 mediated expression of PTGHS2 and prostaglandin in the uterus (123), which explains the observation of a trend towards a lower

expression level of *Ptghs2* in the myometrium and decidua of the *Tlr4-/-* mice, compared to the *Tlr4* replete mice (64). Thus, it is plausible that TLR4 is involved in infection-induced upregulation of *Ptghs2*. *Ptghs2* mRNA expression and prostaglandin levels were discovered to be increased in the uterus of mice in response to LPS-induced preterm delivery when compared to mice given PBS on gd 14.5 (191). Similarly, intrauterine infusion of LPS on gd 15.5 results in the upregulation of *Ptghs2* mRNA in the myometrium during LPS-induced preterm delivery when compared to control mice that were killed 24 hours after intrauterine PBS administration (87). Mice administered intraperitoneal LPS on gd 14.5 together with PTGHS2 inhibitor (SC-230) orally showed a lower rate of LPS-induced preterm delivery, compared to mice given LPS alone (191). The PTGHS1 inhibitor (SC-560) was also used in this experiment; however, it did not inhibit preterm delivery as efficiently as did PTGHS2 inhibitor (SC-230) (191). *Ptghs1-/-* mice were also demonstrated to have a similar rate of preterm delivery to the wildtype mice when induced with intraperitoneal LPS injection on gd 14.5 (191). These experiments demonstrate that in response to local as well as systemic infection, PTGHS2 is a more important uterine activation protein than is PTGHS1, that is induced for early onset synthesis of prostaglandin in the myometrium, to trigger preterm delivery in mice.

1.4.7 TLR4 activation of inflammatory leukocytes in mouse model of infection-induced preterm delivery

Blocking TLR4 reduces the percentage of activated T cells (in blood) and NK cells (in blood and placenta) in mice treated with LPS intraperitoneally on gd 15, when compared to mice with normal TLR4 function (66). This demonstrates a key role for TLR4 in the activation of pro-inflammatory leukocytes, including NK cells in the maternal circulation and gestational tissue in the infection-induced preterm delivery model. The importance of NK cells has been previously shown using mice deficient in invariant Vα14-Jα18 receptor (*Jα18-/-*), and consequently deficient in invariant NK cells. These mice have an attenuated response towards LPS induced preterm delivery (78). This is accompanied by a lower percentage of NK (CD45+CD49b+) cells and T cells (CD45+CD3+), quantified by flow cytometry in the decidua of *Jα18-/-* mice compared to *Jα18+/+* mice given LPS systemically on gd 15 (78). The percentage of activated NK cells (CD45+CD49b+CD69+), activated dendritic cells (CD45+CD11C+CD69+) and activated T cells (CD45+CD3+CD69+) was also reduced in the decidua of *Jα18-/-* mice when injected with LPS compared to *Jα18+/+* mice (78). These results underscore the crucial role of invariant NK cells in the activation of decidual NK cells, dendritic cells and T cells involved in preterm birth.

Mice with a null mutation in the gene encoding interleukin 10 (*Il10*-/- mice) were found to have a higher percentage and cytotoxicity of the uterine NK cells (CD45+CD3-NK1.1+) when compared to wildtype mice, when both groups of mice were infused with LPS intraperitoneally on gd 14 (77). Initially, *Il10*-/- mice were shown to have a marked increase in low dose LPS-induced preterm delivery when compared to wildtype mice (77). Using histochemistry, uterine NK cells were demonstrated to migrate into placental tissue and associate with placental cells undergoing apoptosis in *Il10*-/- mice, which was not observed in the wildtype mice 48 hours after treatement with LPS (77). Interestingly, the depletion of uterine NK cells by intraperitoneal injection anti-NK1.1 on gd 11 and 14, restored normal term labour in *Il10*-/- mice when compared to *Il10*-/- mice injected with IgG antibody (77).

There was a significant increase in the uterine NK cells that secrete TNF and to a lesser extent IFN, 48 hours after LPS administration of LPS to *Il10*-/- mice (77). Moreover, neutralisation of TNF which is important to reduce cytotoxicity as well as migration of NK cells, led to a significantly reduced infiltration of uterine NK cells into placental tissues of *Il10*-/- mice (77). Thus, the depletion of TNF and IFNG via intraperitoneal administration of anti-TNF monoclonal antibody and anti-IFN monoclonal antibody respectively protected the *Il10*-/- mice from preterm delivery when induced with LPS (77). Together, these results emphasize that both NK cells and TNF are crucial in mediating the susceptibility of *Il10*-/- mice to preterm birth mediated by systemic infection. This study also suggests the IL10 plays a role in regulating the function of uterine NK cells in the presence of infection. This is supported by a previous study demonstrating that IL10 reduces the activation of NKT cells in response to infection (192).

Neutrophils are also implicated in the response to LPS in mice. Using flow cytometry, neutrophils (CD11B+Ly6G+ cells) accumulate in both the myometrium and decidua 12 hours after intraperitoneal LPS administration at gd16.5 (232). Intrauterine infusion of LPS on gd 15.5 also resulted in neutrophil (CD45+Neu7/4+ Ly6G+) infiltration into both myometrium and decidua during the intrauterine LPS-induced preterm birth (86,87). Similarly, using immunohistochemistry an elevated number of Ly6G-positive neutrophils was also demonstrated in the decidua, but not myometrium after 6 hours of intrauterine LPS administration on gd 17 (189). These recruited cells displayed a higher expression of neutrophil granule protein (*Ngp)* mRNA, as a neutrophil marker in each of the uterus, placenta and fetal membrane tissues 6 hours after LPS was given (189). Nevertheless, the depletion of neutrophils using anti-Ly6G intraperitoneal injection on gd 16 did not delay intrauterine LPS induced preterm delivery on gd 17 (189). Although the pro-inflammatory responses observed in the uterus, placenta and fetal membrane were slightly

altered following neutrophil depletion, it was insufficient to alter the timing of preterm delivery (189). These observations suggest that neutrophils are not essential for LPS induced preterm birth.

Previously it has been reported that depletion of macrophages using F4/80 antibody injected intraperitoneally prevented LPS-induced preterm delivery (193). Following administration of LPS intravaginally on gd 15, antibody-mediated macrophage-depleted mice showed a decline in collagen degradation and MMP9 activity compared to isotype-antibody control mice (193). Meanwhile, using flow cytometry, the percentage of macrophages (CD45+F4/80+Neu7/4-) in mice given an intrauterine infusion of LPS was found to be similar to the mice administered PBS in both the myometrium and decidua (86,87). This is supported by a recent study revealing that the percentage of macrophages (CD11B+F4/80+) quantified using flow cytometry in maternal tissues, after intraperitoneal LPS administration was not significantly different to the control (190). Thus, when these mice have been challenged with infection there is a potential infiltration of macrophages into the cervix, but not maternal tissues to secrete MMP9 and hence mediate early onset of cervical remodelling.

Recombination activation gene null mutant (*Rag1-/-*) mice. which are deficient in T and B cells were more susceptible to preterm delivery compared to wildtype mice when induced by systemic LPS administration on gd 15 (79). The uterus of *Rag1-/-* mice expressed elevated *MyD88, Nfkb1, Il6* and *Ptghs2* mRNA 6 hours following LPS administration compared to PBS administration (79). Using flow cytometry, an elevated percentage of activated dendritic cells (CD11+MHCII+), but not macrophages (CD11B+CD14+) was observed in the uterus of *Rag1-/-* mice given LPS (79). This evidence suggests that infection-mediated preterm delivery in *Rag1-/-* mice results in the activation of dendritic cells to produce inflammatory cytokines, which affect the prostaglandin pathways in the uterus. It is plausible that the deficiency in T cell populations, especially Treg cells which suppress inflammation in the maternal tissues, causes these mice to be more susceptible to preterm delivery when induced with LPS.

The transfer of purified CD4+ T cells or whole lymphocytes intravenously on gd 10, before LPS injection, resulted in a lower proportion of *Rag1-/-* mice delivered prematurely compared to untreated Rag1-/- mice (79). 24 hours after LPS treatment, a population of CD4+TLR+FOXP3+ T cells were detected in the para-aortic lymph nodes (PALN) of *Rag1-/-* mice transferred with purified CD4+ T cells; however, these cells were fewer than in untreated wildtype

mice (79), suggesting that CD4+TLR+FOXP3+ T cells are important in mediating susceptibility of *Rag1-/-* mice to LPS-induced preterm delivery.

A recent study revealed that a decrease in the percentage and number of CD4+CD25+FOXP3+ uterine Treg cells occurrs 12 hours after intraperitoneal injection of LPS (190). This study suggests that a lack of regulatory T cell function, which acts to suppress the inflammatory response in the local microenvironment, might contribute to expulsion of the fetuses prematurely when induced with LPS. Despite this, there was an increase in both the percentage and number of CD4+CD25+FOXP3+ and CD8+CD25+FOXP3+ cells in the spleen of females 12 hours after injection with LPS intraperitoneally (190). Thus, a population of Treg cells is elevated in the secondary lymphoid organs to control the pro-inflammatory response induced by the systemic LPS administration.

Overall, there is limited information about regulation of the populations of leukocytes in the fetal tissues, such as felt membrane, placenta and amniotic fluid, especially the role of inflammatory cytokines and chemokines induced in these tissues in response to infection.

1.5 ROLE OF DAMPs IN TERM AND PRETERM DELIVERY

Other than pathogens, TLR can also be activated by endogenous ligands known as DAMPs. These host-derived non-microbial stimuli, are found to be released following necrotic cell death resulting from ischemia, toxic injury, stress or trauma. Following these events, the cells loose integrity, allowing the intracellular material to escape into the extracellular matrix (67). Chromatin-associated protein HMGB1 (194), heat shock proteins (HSPs) (195) and purine metabolites, including ATP (196) and uric acid (197) have been identified as DAMPs secreted from necrotic cells. Other than that, HMGB1, HSP70 and fetal fibronectin can also be secreted from pro-inflammatory cells especially macrophages in response to infection (198-204). DAMPs are also derived from the degradation of extracellular matrix molecules following injury or tissue damage, including fibronectin, hyaluronic acid (HA), heparin sulphate and biglycan (67).

Most importantly, there is emerging evidence showing that DAMPs might interact with several receptors, including TLRs, to induce downstream inflammatory responses. In vitro as well in vivo studies revealed that DAMPs, including HSP70 (205-207), HSP22 (208), HSP60 (209), HMGB1 (210-212), fetal fibronectin (204), biglycan (213), heparan sulfate (214) and HA fragments (215), can activate the inflammatory process by signalling through TLR4. Interestingly, HMGB1 (216), HSP70 (206), HSP60(217) and biglycan (213) has been demonstrated to bind the

surface TLR4-MD2 complex in a CD14-dependent mechanism, which is similar to LPS recognition by TLR4 (170,171). HA fragments require the MD2 accessory protein for interaction with TLR4 (218). DAMPs play a physiological role as the key danger signals which alert tissue stress, damage, and death and thereby initiate the tissue defence and repair mechanisms (169). Thus, low levels of DAMPs are likely to be important in mediating the physiological immune response to regain tissue homeostasis (169). For example HGMB1 in low amounts has a protective effect against liver damage induced by hepatic reperfusion injury via inhibition of TLR4 signalling (219,220).

In the events leading up to labour, the extensive tissue remodelling in the myometrium, fetal membrane and cervix is likely to involve the secretion of HSP70 and HMGB1 which are associated with tissue remodelling (221-223). The degradation of extracellular matrix components in fetal membrane including fetal fibronectin is hypothesised to cause the release of this DAMP during parturition (56). This raises the possibility that the inflammatory processes occurring during labour may be regulated by secretion of these DAMPs activating TLR4 activation.

Other than infection, the pathological states of sterile inflammation, including multiple gestations, cervical insufficiency, psychosocial distress and toxins also contribute to preterm delivery (Table 1.1). It is proposed that under these conditions, DAMPs are released by necrotic cell death or the degradation of extracellular matrix molecules to mediate sterile inflammation.

Previous studies have highlighted that DAMPs contribute to the pathogenesis of many chronic inflammatory and autoimmune diseases in humans, potentially via activation of TLR4 (169). HMGB1 and HSP70 have been detected in synovia of rheumatoid arthritis patients, but not the in synovia from normal joints (224,225). In support of these findings, in vivo experiments show that introduction of exogenous HGMB1 intra-articularly induced joint inflammation via the activation of macrophages and expression of IL1 via NF-KB activation in a mouse model of arthritis (226). Therapeutic blockade of HGMB1 using polyclonal antibody against DAMPs meanwhile suppresses the overproduction of IL1B in the joints and thus reduces collagen-induced arthritis in mice and rats models (227). Therefore, these experiments demonstrate that these endogenous TLR4 activators contribute to the disease and indicate the inhibition of TLR4 is potentially useful in ameliorating this pathological autoimmune disease by dampening inflammation.

Importantly, a recent study has revealed that intra-amniotic balloon inflation was efficient in eliciting preterm delivery in pigtail macaques when compared to saline infusion, which mimics human multiple pregnancy and uterine overdistention (228). Preterm delivery mediated by intra-amniotic balloon inflation was also correlated with a greater uterine wall stress and higher cytokines, chemokines and prostaglandin levels in the amniotic fluid in these nonhuman primates (228). This in vivo model was also consistent with in vitro observation that an increase in the expression of cytokines and chemokines mRNA was demonstrated when mechanical stretch was introduced in the human amniocytes (228).

In addition, women with twins and preterm labour exhibit elevated pro-inflammatory cytokine and chemokine levels in both the amniotic fluid and myometrium in comparison to multiple pregnancy without labour (228). Importantly, the genes that are differentially expressed in the nonhuman primate following the uterine overdistention stimulation and in women with twins were associated with HMGB1 signalling pathways (228). It is one of the pathways involved in tissue remodelling to possibly contribute to the extensive remodelling of myometrial smooth muscle following uterine overdistention (228). Thus, this evidence from humans and nonhuman primates suggests that in multiple pregnancy, uterine overdistention and extensive tissue remodelling occurs. This potentially causes secretion of DAMPs such as HMGB1 in gestational compartments to provoke inflammatory responses, eliciting early onset of labour.

Smoking is known to cause oxidative stress (229-232), which occurs when the production of reactive oxygen species is higher than intrinsic anti-oxidant defences (233). Reactive oxygen species is crucial in maintaining cell homeostasis; however, at higher levels it can result in damage of biological moleculs leading to loss of function and cell death (233). Most importantly, oxidative stress is associated with complications of pregnancy including preterm birth (233-235). Interestingly, a recent study showed that the oxidative stress marker, malondialdehyde and an anti-oxidative capacity marker, oxygen radical absorbance capacity were elevated and lowered, respectively, in women who delivered within 3 days of pPROM compared to women who delivered more than 3 days after pPROM (236). Furthermore, another study revealed that oxidative stress may be associated with fetal membrane rupture by inducing apoptosis and the degradation of extracellular matrix component (237). Meanwhile, antioxidants counteracted the effects of free radicals by protecting chorioamnion from damage caused by reactive oxidative species (238). Hence, it is possible that smoking during pregnancy results in oxidative stress at the end of gestation to cause elevated secretion HMGB1 to mediate pPROM.

Cigarette also contains an average 4% of carbon monoxide by volume. Carbon monoxide can readily cross the placenta to bind to fetal haemoglobin which has a higher affinity for carbon monoxide to form carboxyhaemoglobin (239). This elevated level of carboxyhaemoglobin and reduced unloading of blood oxygen resulted in fetal hypoxia, which is proposed to cause adverse effects on fetal growth and development (239). Nevertheless, the mechanisms in which the fetal hypoxia and oxidative stress can provoke inflammation to trigger preterm birth in women is unclear. Hence, further studies are required to understand the mechanisms of how multiple pregnancy, smoking and other risk factors can potentially cause secretions of higher levels of DAMPs to contribute to inflammation via TLR4 signalling to induce preterm birth. Thus, the candidate DAMPs that may be involve in mediating inflammation in preterm delivery are discussed below.

1.5.1 Heat Shock Protein 70 (HSP70)

Heat shock proteins (HSPs) are present in subcellular compartments of all cell types from prokaryotes and eukaryotes (240) and play an important role as molecular chaperones that regulate intracellular processes to maintain homeostasis during cell proliferation and differentiation (241,242). HSPs are categorised according to their approximate molecular weights, including HSP60, HSP70, HSP90 and HSP110. Amongst these, HSP70 is the best characterised HSP among HSP families (243).

Experiments using hepatocellular carcinoma cells show that inhibition of both TLR2 and TLR4 expression using an RNA interference method results in reduced cell after stimulating with HSP70 (244). Furthermore, interaction between HSP70 and the receptors including TLR2 or TLR4 expressed by this cell line is established using co-immunoprecipitation and pull-down assays (244). TLR2 and TLR expression at the mRNA and protein level is elevated especially in the tumour tissue of hepatocellular carcinoma patients (244). Overall, these results suggest that HSP70 plays a significant role in promoting the proliferation of hepatocellular carcinoma cells via activation of both TLR2 and TLR4 receptors.

Diabetic nephropathy patients have higher levels of HSP70 and TLR4 in the dilated tubules compared to non-diabetic controls (245). Similarly, diabetic mice also showed elevated HSP70 concentrations, protein expression of TLR2 and TLR4 as well as NF-KB activity in their kidneys. The blockade of HSP70 is reported to decrease inflammation induced by diabetes in mice (245). Most importantly, ex vivo experiments found that HSP70 induction of pro-inflammatory mediators mRNA including *Ccl2* and *Tnf* is inhibited in mouse proximal tubular cells

derived from *Tlr4*-/- mice, but not from *Tlr2*-/- mice (245). Hence, it seems that HSP70 activation of TLR4 is crucial in mediating inflammation-mediated diabetic nephropathy in both humans and mice.

The inhibition of CD40 receptor, which has been demonstrated to bind HSP70, suppresses the secretion of CCL5, following treatment of both activated CD34+ myelo-monocytic and monocyte cell lines with *Mycobacterium tuberculosis* derived HSP70 (246,247). Thus other than TLR2 and TLR4, CD40 is also an important receptor with which HSP70 interacts to promote inflammation in vitro.

HSP70 is detectable in amniotic fluid in mid-gestation and its concentration increases towards the end of gestation and during labour, supporting the postulate that HSP70 is released under the physiological conditions of normal term labour (248,249). Thus, it is plausible that during growth and development of the fetus, HSP70 plays a role as a chaperokine inside the amniotic cavity (248). HSP70 mediates the synthesis of PTGHS2 and PGE_2 in human umbilical vein endothelial cells (250), suggesting that HSP70 may have an effect on prostaglandin production to contribute to initiation of labour.

The mechanisms which induce HSP70 secretion in the amniotic fluid remain unclear. HSP70 has been shown to be secreted by human fetal membranes in response to infection (201,202) and clinical chorioamnionitis (251). Interferon gamma (IFNG) acts to trigger HSP70 synthesis in the K562 cell line, derived from a human erythroleukemia patient (203). Thus in late gestation, it is possible that elevated synthesis of cytokines in fetal membranes might increase the synthesis of HSP70. The extensive tissue remodelling which occurs prior to fetal membrane rupture might also contribute to inflammation via the release of HSP70 into amniotic fluid (223).

HSP70 has been localised in decidual stromal cells of pregnant woman in the first, second and third trimesters of pregnancy; however, the intensity of HSP70 staining declines as gestation advances. The decidua is not likely to be the source of this elevation of amniotic fluid HSP70 (252). Other than decidual stomal cells, HSP70 staining was detected in the cytotrophoblast, syncytiotrophoblast, intermediate trophoblast, Hofbauer and endothelial cells of the placenta; however, it is unclear whether placental HSP70 levels change as gestation advances (252). Therefore as well as the gestational compartments it seems likely that HSP70 also increases in the maternal circulation as gestation advances. There are no studies to demonstrate a specific role of HSP70 in the inflammatory processes that mediate normal on time parturition in either humans or mice.

Conditions that can increase the intracellular expression of heat shock protein include pathological events such as infection and inflammation, ischemia, oxidant injury and toxin exposure (253). HSP70 has been demonstrated to be released from stressed cells (254-257) as well as from necrotic cells (258,259). The concentration of HSP70 was observed to increase in umbilical cord serum, amniotic fluid, placenta and maternal serum of human preterm delivery with IAI in comparison to human preterm delivery without IAI or normal term delivery (248,260). Similarly, PROM women with IAI showed elevated HSP70 concentration in umbilical cord serum, amniotic fluid, placenta and maternal serum compared to PROM women without IAI or normal term labour women (248,260). Culture method was utilised to detect for the presence of the bacteria, which is a limitation of these studies (248,260). Hence, infection is implicated in elevated synthesis of HSP70 in gestational compartments and maternal circulation of human preterm delivery.

An association between the presence of *Mycoplasma hominis* in mid-trimester amniotic fluid with an increase in median intra-amniotic HSP70 levels was also revealed in another study (249). In vitro studies reinforce the above evidence where amniotic fluid cells produced a higher level of HSP70 when induced with PGN, compared to the control (249). Elevated expression of *HSP70* mRNA and HSP70 was also found in human fetal membrane following LPS or *E.coli* treatment respectively (201,202). This evidence postulates the role of bacterial components as well as the bacteria itself in inducing the secretion of HSP70 in amniotic fluid and fetal membranes.

There was also an elevation in HSP70 in the umbilical cord serum, placenta, and maternal serum of preterm delivery women without IAI, when compared to normal term delivery (260). From this study it can be assumed that sterile inflammation also induces elevated synthesis of HSP70 in gestational tissues and maternal circulation. Nevertheless, there are limited studies performed in mice to investigate the role of HSP70 in triggered by infectious agents or sterile inflammation as well as the inflammatory mechanisms that are involved.

1.5.2 High mobility group box 1 (HMGB1)

HMGB1 is a non-histone, chromatin-associated protein that is constitutively expressed in almost every cell type that has a nucleus (212) and plays a role in facilitating the deoxyribonucleic acid (DNA) transcription process (261).

In vitro experiments reveal that both anti-TLR2 and anti-TLR4 antibodies reduce the secretion of IL17, IL6 and IL23 in response to stimulation of macrophages from pulpitis patients

with HMGB1 (262). Increased secretion of HMGB1, IL6 and CCL2 as well as the protein expression of TLR2, C-X-C chemokine receptor type 4 (CXCR4), and nuclear NF-KB in renal tubular epithelial cells is reported when induced with contrast media, which is known to cause contrast-induced nephropathy (263). The contrast media-induced inflammation observed in renal tubular epithelial cells is abrogated following the inhibition of HMGB1, TLR2 and CXCR4 using Glycyrrhizin, anti-TLR2-IgG, and AMD3100 respectively. These results suggest that HMGB1 signals through TLR2, TLR4 and CXCR4 receptors to mediate inflammation in these inflammatory diseases.

In a murine model of autoimmune thyroiditis, the treatment of mice with 0.005% sodium iodine in drinking water for 16 weeks causes an elevation in serum HMGB1 and pro-inflammatory cytokines including TNF, IL6a and IL1B and in protein expression of HMGB1, TLR2, MYD88 and NF-KB in the thyroid gland (264). Direct inhibition of HMGB1 using Glycyrrhizin, which is a traditional medicine extracted from licorice root has been shown to ameliorate the severity of thyroiditis and the enhanced inflammation observed in the serum of mice treated with sodium iodine water (264). Most importantly, the thyroid gland of mice given sodium iodine water also show a decline in HMGB1, TLR2, MYD88 and NF-KB following treatment with Glycyrrhizin (264). Hence, it is plausible that HMGB1 interaction with TLR2, via MYD88 signalling pathway is important in increasing the severity autoimmune thyroiditis by enhancing inflammation locally and systemically.

In the mouse model of bladder pain, it has been shown that intravesical pre-treatment of mice with TLR4 antagonist, but not receptor for advanced glycation endproducts (RAGE) antagonist prevents intravesical-HMGB1 induced abdominal mechanical hypersensitivity. On the other hand, abdominal mechanical hypersensitivity triggered by bladder infusion of HMGB1 is reduced with the pre-treatment with both TLR4 and RAGE antagonist intraperitoneally. These results portray that HMGB1 interaction with TLR4 receptor locally in the bladder mediates the abdominal mechanical hypersensitivity and RAGE receptor modulate the effects of HMGB1 systemically, possibly in the central nervous system (265).

HMGB1 has been shown to mediate the intraperitoneal acetaminophen-induced lung injury as the blocking of HMGB1 using monoclonal antibody administered intravenously reduces the death rate and the concentration of IL6, TNF and CCL2 in the serum of wildtype mice (266). Interestingly, a higher fetal death rate, accompanied by elevation of serum pro-inflammatory cytokines and chemokine are observed in CD24 deficient (*Cd24-/-*) mice following

acetaminophen-stimulated liver injury (266). Thus CD24, which is demonstrated to co-immunoprecipitate with HMGB1 (266), can protect the mice from lung injury caused by acetaminophen potentially by suppressing the immune response induced by HMGB1.

Women at term in labour, with clinical chorioamnionitis, with or without evidence of IAI (n=46), were found to exhibit higher concentrations of amniotic fluid HMGB1 compared to women in term labour without clinical chorioamnionitis or IAI (n=48) (267). In this study, clinical chorioamnionitis was diagnosed by temperature elevation to 37.8°C or higher and two or more of the following criteria: uterine tenderness, vaginal discharge, fetal or maternal tachycardia and maternal leucocytosis (267). The presence of infection was determined using culture to detect for aerobic or anaerobic bacteria and genital mycoplasmas (267), which is a limitation of this study as the use of molecular techniques is known to reveal higher numbers of women with bacterial infection, compared with culture techniques. These results suggest that the presence of bacteria and cytokines during term labour induces the production of HMGB1 by the amniotic fluid possibly by macrophages.

In vitro studies utilising mouse macrophages demonstrated the capability of macrophages to secrete HMGB1 once stimulated with LPS and cytokines (198-200,268,269). HMGB1 has been immunolocalised to the cytoplasm and nucleus of amnion epithelium cells as well as the cytoplasm of myofibroblasts, macrophages and infiltrating neutrophils detected in the fetal membrane of women with histologic chorioamnionitis who delivered at term using immunofluorescence staining (270). Thus, it is plausible that following infection HMGB1 might be translocated from the nucleus into the cytoplasm of these cells, to then be released into amniotic fluid.

HMGB1 receptor soluble RAGE appears to decrease in the amniotic fluid of women at term in labour with clinical chorioamnionitis, inconsistent with the observed increase in HMGB1 (267). It could be that an alternate HMGB1 receptor, such as TLR4 is involved in engaging HMGB1. Apart from its presence in amniotic fluid, HMGB1 has also been detected at both protein and mRNA level in human term cervix (271) and placenta (272). The level of HMGB1 in the cervix did not alter during labour (271), thus it is unlikely that the cervix contributes to this increase level of HMGB1 observed in the amniotic fluid especially with the presence of infection.

HMGB1 can also be secreted passively by necrotic cell death due to stress, caused by ischemia or oxidative stress (212). Both preterm labour women and pPROM women with IAI were detected with higher concentrations of HMGB1, RAGE and IL6 in the amniotic fluid when

compared to preterm labour women and pPROM women without IAI, respectively (270). Therefore, it is suggested that the presence of infection induces the secretion of HMGB1, which may activate RAGE receptor, to induce elevated pro-inflammatory cytokines concentration in the amniotic fluid of preterm labour and pPROM women.

The women with preterm delivery without IAI who delivered within seven days of amniocentesis had a higher concentration of amniotic fluid HMGB1 (n=23) compared to women who delivered more than 7 days after amniocentesis (n=12) (273). Preterm delivery women with sterile inflammation and amniotic fluid HMGB1 concentration $\geq$ 8.55 ng/mL were observed with a higher concentration of IL6 in the amniotic fluid than women with sterile inflammation with amniotic fluid HMGB1 concentration $<$ 8.55 ng/mL (273). Most importantly, the cultivation technique as well as molecular technique including PCR accompanied by base composition analysis using electrospray ionisation mass spectrometry ESI-MS was used to detect the presence of microbial infection in this study (273). Thus, it can be interpreted that HMGB1 contributes in inducing pro-inflammatory cytokine in amniotic fluid to cause preterm delivery in the absence of infection.

A higher expression of *HMGB1* mRNA was also found in the fetal membranes of preterm delivery women and pPROM women when compared to normal term labour women (274). In this study, the preterm delivery women were not tested for the presence of infection in the amniotic fluid (274). An in vitro study demonstrated increased concentration of HMGB1 when the fetal membranes were treated with LPS or water soluble cigarette smoke extract (274). This suggests that infection and oxidative stress from smoking (229-232) induce secretion of the HMGB1 extracellularly by the resident cells in fetal membranes. The treatment of human fetal membranes with HMGB1 also induced the expression of both *TLR2* and *TLR4* mRNA but not *RAGE* mRNA and amplified the production of pro-inflammatory cytokines including IL1B, IL6 and TNF (274). Thus, HMGB1 induction of pro-inflammatory cytokines is potentially mediated by TLR2 and TLR4 in fetal membrane, attributing in preterm delivery or pPROM.

Other than in amniotic fluid and fetal membrane, higher *HMGB1* mRNA was detected in the cervix of women with preterm delivery when compared to the term delivery women (271). In this study, there was a mix of patients detected to have infection and without infection in both groups of preterm delivery women (271). Thus, both infectious and non-infectious agents may have induced the expression of *HMGB1* in the cervix, resulting in preterm delivery.

Intra-amniotic HMGB1 administration, at a dose of 9 ng induced preterm delivery in CD-1 mice, but not intraperitoneal administration (275). This intra-amniotic administration of HMGB1 also caused a 15% fetal death rate when they were born (275). A higher fetal death rate was recorded one week after the pups were born from the dams that were challenged with HMGB1 compared to PBS control (275). Similar results were observed with intraperitoneal injection of 50 µg HMGB1 on gd 14.5, where an increased percentage of fetal death one week post-birth was observed (275). Thus, it could also be that the dose used for the intraperitoneal administration is insufficient to trigger preterm delivery in mice. From this evidence it is clear that local HMGB1 administration is efficient in mediating preterm delivery and affecting the viability of the pups that were born; however, the inflammatory mechanisms in gestational tissues behind this HMGB1 induction of preterm delivery were not further explored.

1.5.3 Uric acid

Uric acid is a final product for purine degradation, produced by the liver from endogenous or exogenous precursor proteins and excreted from the system by the kidneys and intestines. It plays a physiological role as an antioxidant especially in the plasma (276). Higher levels of uric acid in the plasma can result in oxidative damage and inflammation (276). Interestingly, a large study in humans has found an association between maternal hyperuricemia during the third trimester of pregnancy and preterm delivery, in the absence of any hypertensive disorders or multiple pregnancies, history of gestational smoking, opiates or alcohol consumption, and infections or antibiotics use (276). In this study, hyperuricemia is defined as serum uric acid level one standard deviation greater than the appropriate for gestational age (276).

Maternal hyperuricemia was correlated with poor neonatal outcomes including NICU admission, neonatal hypoglycaemia and neonatal intraventricular haemorrhage (276). There was also a significant correlation between maternal and umbilical uric acid level (276), which can occur due to ability of uric acid to cross the placenta (277). Thus, a high level of uric acid in the neonatal sera can be a reflection of maternal sera, and is associated with poor neonatal outcomes. This is supported by a study showing that adolescents who were born prematurely with very low birth weight had higher uric acid level in their serum, compared to those who were born at term with normal birth weight (278). Taken together, high levels of uric acid in maternal as well as fetal circulation may contribute to sterile inflammation of preterm delivery, and affect neonatal outcomes.

Uric acid is known to activate inflammation via one of the subsets of inflammasome, nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain containing 3 (NLRP3) (279). Inflammasome forms when activated NLRP3 becomes associated with caspase-1 and adaptor molecule apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). Most importantly, the multi-protein complex induces caspase-1 activation, allowing caspase-1 to cleave both pro-IL1B and pro-IL18 to become biologically active (280-282). Uric acid stimulation of IL1B and TNF is inhibited in RNA-interference-based NLRP3-knockdown cell lines generated from the human monocytic cell line (279). Uric acid-mediated IL1B production by the human trophoblast cell line is both caspase-1 and ASC dependent (283). Ex vivo experiments show that macrophages derived from mice deficient in NLRP3, caspase-1 and ASC secrete lower concentration of IL1B when compared to macrophages derived from wildtype mice (284). Thus, uric acid activation of NLRP3 and other components of inflammasomes is crucial in inducing inflammation. So far there is no study demonstrating the interaction between uric acid and TLR4 to induce inflammation. Nevertheless, it has been shown that uric acid production in the human trophoblast cell line can be induced by anti-phospholipid antibodies, via TLR4 (285).

There are also limited studies in mice to unravel the potential role of uric acid in inducing preterm delivery especially when administered systemically as well as the inflammatory mechanisms stimulated by uric acid in gestational tissues.

1.5.4 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one

Both 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one are formed in vivo by enzymatic or non-enzymatic (catalytic) oxidation of cholesterol or can be obtained from various dietary sources including cholesterol rich food (286). Both 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one play a role in the regulation of cholesterol and steroid hormone biosynthesis as well as in lipid homeostasis (286). They also contribute to disease pathogenesis, by inducing inflammation in various cell types as well as cell apoptosis (287-291). There are limited studies to investigate the circulating of both 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one in normal term delivery women. Particles that carry both 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one, known as oxidised low-density lipoprotein (oxLDL) are increased in the serum of pregnant women compared to non-pregnant women (292). The level of oxidised oxLDL is elevated in the pathogenesis of pregnancies including pre-eclampsia (293,294) and intrauterine growth restriction (IUGR) (295). They were found to impair the differentiation and fusion (296) and cause cytotoxicity at a higher dose (297) of term primary trophoblast cells in

vitro. This might be explained by the in vitro experiments portraying that both 25-hydrocholesterol and 7-ketocholesterol promote the pro-inflammatory cytokines production including IL6, CCL4 and TNF in the primary trophoblast cells, via interaction with TLR4 (298). This is because the inhibition of TLR4 using OxPAPC and CLI095 causes a decrease in the secretion of these cytokines from primary trophoblast cells when stimulated with both 25-hydrocholesterol and 7-ketocholesterol (298).

These studies provide information about the potential role of both 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one in stimulating inflammatory cytokines production to affect placental function in humans as one of the triggering factors for preterm birth, especially mediated by oxidative stress. Thus, further studies in humans will be crucial to establish the level of both 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one in gestational tissues especially placenta or maternal circulation in human preterm delivery.

1.5.5 Fetal fibronectin

Fibronectin is a multidomain protein that binds to receptors, collagen, proteoglycans as well as other fibronectin molecule on the cell surface (204). Fetal fibronectin is diffusely distributed in fetal membranes, to contribute to structural support and adhesion of fetal membranes to the uterus layer (204). In humans, fetal fibronectin that is found in the cervicovaginal secretion obtained via vaginal swab, is one of the markers that is useful as a clinical predictive marker for preterm birth (299-304). This evidence supports the hypothesis that degradation of extracellular matrix components in fetal membrane including fetal fibronectin causes the release of this DAMP during normal parturition (56). Thus, presence of fetal fibronectin in cervicovaginal fluid is speculated to indicate the disruption of the fetal membrane, resulting in the secretion of fetal fibronectin into the vagina (204).

Intrauterine administration of full length fetal fibronectin purified from human amnion is able to induce preterm delivery and reduce fetal survival in mice, when compared to treatment with PBS (204). Further in vitro experiments showed that fetal fibronectin and its component, extra domain A (EDA) induced the expression of *MMP1*, *MMP2*, *MMP9* and *PTGHS2* mRNA in the human amnion mesenchymal, but not epithelial cells, via TLR4 signalling (204). TLR4 blockade using TLR4 antibody reduced the expression of *MMP1*, *MMP2*, *MMP9* and *PTGHS2* mRNA following treatement of human amnion mesenchymal cells with both fetal fibronectin or EDA (204). As a result, both fetal fibronectin and EDA also stimulated the production of PGE_2 in human amnion mesenchymal cells (204). It is clear that fetal fibronectin especially through its

component EDA is efficient in stimulating the synthesis of prostaglandin and MMPs in mesenchymal cells to possibly mediate preterm delivery. Other than sterile inflammation, infection may also trigger the release of fetal fibronectin in preterm delivery as both LPS and TNF can induce fetal fibronectin production from human epithelial cells but not mesenchymal cells in vitro (204).

1.6 ROLE OF OTHER ENDOGENOUS TLR4 REGULATORS IN TERM AND PRETERM DELIVERY

1.6.1 Platelet activating factor (PAF)

PAF; 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine is a potent phospholipid inflammatory mediator that can be secreted from various cells including immune cells (305). PAF can be synthesised via two pathways, one of them being a remodelling pathway. The initial step involves the action of phospholipase $A_{2,}$ synthesising the 1-O-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF) also known as PAF precursor from 1-O-alkyl-2- arachidonyl-*sn*-glycero-3-phosphocholine. Then, lyso-PAF is further converted to PAF by acetyl-CoA:lyso-PAF acetyltransferase (lyso-PAFAT). PAF can be degraded by PAF-acetylhydrolase (PAF-AH) (306-308). PAF elicits diverse effects through the G protein-coupled receptor, PAF-receptor (PAFR) (307,309), expressed mainly on human platelets, monocytes and neutrophils (310). PAFR, which is a G-protein coupled receptor, has 7 transmembrane domains. The binding of PAF to PAFR causes activation of several signalling mechanisms, including GTPase, protein kinase C and tyrosine kinases. Activation of GTPase leads to phospholipid turnover via phospholipase C,D and A2 pathway. The activation of these multiple signalling pathways produce second messengers, which is important in PAF induced expression of primary response genes leading to PAF responses (311).

PAF has been shown to play roles in various physiological processes including parturition where its level is elevated in the amniotic fluid and amnion during labour in humans (312). PAF in the amniotic fluid is thought to be released from the fetal lung and to play a role as one of the signals for parturition as well as fetal lung maturation (312). This hypothesis is supported by an in vitro experiment showing that there was an increase in PAF concentration and activity of lyso-PAFAT 6 days following the culture of human fetal lung (313). In mice, PAF levels are elevated towards the end of gestation in the amniotic fluid as well as fetal lung (81). PAF has been shown to increase in the human amnion during labour and treatment of amniotic disc stimulates production of prostaglandin (314), PAFR expression has also been demonstrated in amnion cells (315). The production of prostaglandin and expression of *PTGHS2* mRNA is also

increased in human fetal membrane disc following exposure to PAF (315). This evidence reflects that PAF, possibly via interactions with PAFR, is important during labour for the synthesis of prostaglandins via PTGHS2 induction in the fetal membrane.

An in vitro study shows that PAF stimulates human myometrium contraction, which is PAFR dependent as blocking of this receptor, using PAFR antagonist CV3988, reduces the contractions induced by PAF (316). This suggests that in addition to amniotic fluid accumulation, PAF can also be secreted to maternal tissues, possibly as an effect of diffusion of PAF from amniotic fluid into the uterus. One of the regulatory mechanisms that can influence the PAF accumulation in maternal tissues is via PAH-AH, which plays a role in degrading the PAF. This enzyme is secreted from human decidual macrophages isolated at term pregnancy, indicating these leukocytes could play an important role in PAF metabolism in normal term labour (317). cPAF as well as pro-inflammatory cytokines including TNF, IL1A, IL1B and CXCL8 reduce PAF-AH production by the decidual macrophages in vitro (318-320). This is further supported by a study in rats portraying a reduction in PAF-AH activity as gestation approaches its end, to cause elevation of the PAF level in the uterus of rats (321). Thus, PAF-AH plays an important role in regulating the elevated level of PAF in the maternal tissues towards the end of gestation and during labour.

Studies in mice reveal that steroid coactivator 1 (SRC1) and SRC2, which are crucial in activating the transcription of the lysophos-phatidylcholine acyltransferase-1 (LPCAT), which in turn regulates PAF and mediates normal term labour in mice (81). Female mice that are double heterozygous for *Src1* and *Src2*, referred as *Src-1/-2* dhet mice were mated to the same genotype females, and were found to have delayed labour when compared to wildtype matings (81). The PAF level was also observed to be lower on gd18.5 in the fetal lung and within the amniotic fluid of *Src-1/-2* double deficient fetuses (81). An important role of PAF is further indicated by experiments showing that the intra-amniotic administration of PAF on gd 17.5 initiated normal on time parturition in *Src-1/-2* dhet mice (81).

Most importantly, this local administration of cPAF on gd 17.5 also induced an increase in the contraction associated genes and proteins including *Gja* and Oxtr, and a decrease in the level PGF_2a synthase, known as AKR1B3 in the myometrium of these *Src-1/-2* dhet mice on gd18.5 when compared to PBS administration (81). As a result, an increase in $PGF_{2\alpha}$ measured in the myometrium was observed in *Src-1/-2* dhet mice on gd18.5 following administration of PAF in comparison to PBS on gd 17.5 (81). These experiments support the hypothesis that cPAF can

be transmitted from amniotic fluid into the myometrium in late gestation to activate uterine contractions via stimulation of the uterine activation proteins and prostaglandin synthesis (139).

There is a suspected inflammatory role for cPAF in the cervix, as shown by PAF inducing cytokines (322) and MMP1 (323) production in human uterine cervical fibroblasts. This inflammation is PAFR dependent, as inhibition of the receptor using WEB 2170 attenuates PAF-induced cytokine and MMP1 production (322,323). PAFR expression is also higher at term and after parturition in cervical stroma and glandular epithelium respectively (324). *Paf-ah* mRNA was also downregulated in the cervix of rats towards the end of pregnancy (325), which could lead to elevated PAF levels in the cervix. Intracervical administration of PAF also plays a physiological role in inducing cervical ripening and polymorphonuclear leukocytes infiltration into the cervix in rats (326). Collectively, this evidence suggests that PAF might diffuse into the cervix from the amniotic fluid during labour to trigger inflammation which mediates cervical ripening (139).

PAF concentration and *PAFR* mRNA are identified in placental trophoblast cells of normal healthy pregnancies after delivery (327); however, there is no work in humans which demonstrates that placental PAF contributes to labour. In the rat placenta, PAF-AH levels rise, leading to a decrease in placental PAF, as parturition approaches (321). This is postulated to alter blood pressure and consequently increase the fetoplacental circulation, which may be crucial for an adequate delivery of nutrients to the fetus during this rapid growth period.

PAF synthesis can also be triggered by hypoxia, infection and trauma (328) and has been shown to mediate the pathogenesis of inflammatory diseases such as asthma, endotoxin shock, diabetes, acute allergic reactions, thrombosis and ischemic bowel necrosis (305). An increase in PAF has been found in the amniotic fluid of women with preterm delivery (329,330). Studies in mice support this observation, where the intrauterine administration on gd 15 of 10-40 µg of cPAF caused preterm delivery in 4 out of 9 CD-1 mice (331). Thus, these studies in humans and mice reflect the crucial role of PAF in mediating preterm delivery.

Another study using C57BL/6 and C3HeB/FeJ showed that the intrauterine administration of 15 µg/mouse to 100 µg/mouse of cPAF caused death of the mice within few hours of administration (332). A lower dose of cPAF resulted in either death or no apparent effect (332). This gives the idea that different strains of mice used result in different outcomes when challenged with PAF locally. Meanwhile in rats, intravenous infusion of cPAF for 7 days from gd 14 to gd 21 resulted in a decrease in fetal and placental weights (333,334). Systemic administration of PAF also has an effect on the fetal outcomes causing intrauterine fetal growth

restriction on gd 21 (333,334). Nevertheless, the mechanisms of inflammation which trigger preterm delivery or poor fetal outcomes when these mice were challenged with PAF have not been studied.

A higher expression of pro-inflammatory cytokines and chemokines including *Tnf, Il1b* and *Ccl5* mRNA was detected in the uterus, decidua, and placenta of these *Paf/af*-/- mice which increases their susceptibility of cPAF when administered killed *E.coli* when compared to wildtype mice (332). A reduction in pro-inflammatory mediators including TNF and CCL5 was observed in peritoneal macrophages derived from PAF receptor deficient (*Pafr*-/-), *Tlr2-/-* and *Tlr4-/-*mice when compared to wildtype mice following stimulation with cPAF (332). Thus these results reflect that other than PAFR, PAF induction of inflammatory processes is also TLR2 and TLR4 dependent. Nevertheless, the mechanisms by which TLR2 and TLR4 mediates PAF stimulation of inflammatory response are not clear. There is no study to demonstrate direct binding of PAF to TLR2 or TLR4 so far; however, peritoneal macrophages derived from *Tlr4-/-* mice exhibit lower expression of *Pafr* mRNA, either with or without treatment with cPAF (332) and PAF has also been demonstrated to induce TLR4 expression in human and mouse intestinal epithelial cell lines, human embryonal intestinal cell line (335) as well as in an in vivo rat intestinal loop model at both protein and gene levels (328). Thus it seems likely that TLR4 has an effect on the expression of PAFR and PAF induction of PAFR is TLR4 dependent. It can be speculated that PAF acts to upregulate TLR4 synthesis to induce its sensitivity to ligation by both PAMPs and DAMPs.

Other than PAH-AF, an alternative method to regulate the level of endogenous PAF is by using PAF agonists. The administration of PAF antagonists CV-6209 intraperitoneally before intrauterine LPS administration elicited a lower percentage of preterm birth, accompanied by a higher percentage of viable pups amongst the undelivered preterm pups, compared to mice that were administered with LPS alone (331). Similarly in rats the administration of PAF antagonists, WEB-2170 intraperitoneally reduced LPS-induced preterm cervical ripening, assessed by the measurements of the cervical light-induced fluorescence and cervical resistance to stretch in timed pregnant rats (336).The results illustrate that the endogenous PAF production is crucial in enhancing infection-induced inflammation in maternal and fetal tissues, to cause early activation of cervical ripening and preterm delivery in mice. It is also possible that the infection stimulate higher level of endogenous PAF eliciting in preterm delivery.

1.6.2 Surfactant Protein (SP)

SP is a pulmonary glycerolphospholipid-rich lipoprotein produced by pulmonary alveolar type II cells (81). It is crucial for breathing as it reduces the surface tension within the lung and a deficiency in surfactants in infants that are born prematurely can lead to respiratory distress syndrome (81). In humans, SP-A synthesis by the fetal lung begins only after 80% of gestation is complete and the level of SP-A in the amniotic fluid peaks before term (337,338) before declining during spontaneous labour at term (339).

An vitro study has shown that SP isolated from human amniotic fluid stimulates the synthesis of PGE_2 and in human amniotic discs (340). SP-A also promotes an increase in PTGHS2 activity in human myometrial cells obtained from women delivered at term without labour, via elective caesarean section (341). This is consistent with results demonstrating that SP-A is detectable in myometrial cells along with SP-A binding protein expression (341). In contrast, a decrease in the expression of IL1B, CXCL2 and CXCL5 is reported when human amnion explants are treated with SP-A (338). The ability of the globular heads and collagen tails to mediate both anti-inflammatory and pro-inflammatory responses is speculated to occur by binding to signal-regulatory protein α and to calreticulin/ low-density lipoprotein-related protein 1 respectively (342). This demonstrates dual immunomodulatory roles for SP-A so, despite the elevation of SP-A towards the end of gestation in amniotic fluid, it is not clear whether and how this contributes to parturition in humans.

Meanwhile, a study in mice demonstrates detectable expression of *Sp-a* mRNA on gd 15 in fetal lung, which increases towards the end of gestation on gd 18 (343). In the amniotic fluid SP-A level is detected using immunoblotting on day 17 of gestation and is further elevated towards the end of gestation on gd 19 (344). The elevation of SP-A in amniotic fluid is also associated with an increase in IL1B in amniotic fluid macrophages detected using immunohistochemistry (344). These results provide an indication that in late gestation, elevated SP-A concentration may contribute to activating inflammatory leukocytes, allowing the release of inflammatory cytokines to amplify inflammation.

Experiments in mouse models that allow tracking of fetal macrophages show that amniotic fluid leukocytes activated by SP-A can infiltrate the uterus, to further amplify inflammation during late pregnancy. Heterozygous *Lac-Z* transgenic male mice, which express β-galactosidase in all cells have been mated to wildtype ICR females to generate pregnant ICR females with 50% heterozygous *Lac-Z* embryos and amniotic fluid macrophages that are positive

for β-galactosidase activity (344). A proportion of F4/80+ uterine macrophages in these pregnant females are detected to have β-galactosidase activity, and these cells are elevated towards the end of gestation. Most importantly, the intra-amniotic administration of SP-A in these pregnant females drives a higher β-galactosidase activity in the uterus (344). These results explain the increase in both IL1B and CD68 markers for activated macrophages observed in the uterus especially in response to the elevated intra-amniotic SP-A near term (344). In contrast, another study reports there is no elevation of CD68+ macrophages in the myometrium of C57BL/6 mice as gestation advances (338). It could be that CD68, as the only surface activation marker used in this study does not capture the total population of activated macrophages in the amniotic fluid.

SP-A and SP-D deficiency in *Spa/d-/-* mice causes delayed parturition in the second pregnancy, but not first pregnancy (71). F4/80+ macrophages isolated form the amniotic fluid on gd 18.5 of second pregnancies of *Spa/d-/-* mice display lower expression of the pro-inflammatory *Il1b* mRNA, and lower anti-inflammatory activation markers including *Arg1* and *Ym1* mRNA (71). A decline in the expression of inflammatory cytokines including *Il1b* and *Il6* mRNA and uterine activation genes, *Gja* and *Oxtr* mRNA was also exhibited in the myometrium of *Spa/d-/-* mice, when compared to the wildtype mouse on gd 18.5 of their second pregnancies (71). Nevertheless, it is not possible to conclude an essential role of SP-A and SP-D in the initiation of the physiological events of normal term labour as the delay is observed only in second pregnancies which suggests that other factors might also contribute to the delay of labour seen in *Spa/d-/-* mice.

Src-1/-2 dhet mice have a delay in labour in their first pregnancy (81) indicating SRC1 and SRC2 are also important in the upregulation of SP-A gene expression in fetal lung type II cell (81). Thus, the *Src-1/-2* double deficient fetuses also had reduced SP-A, but not SP-D and SP-B expression in the lungs, compared to the wildtype fetuses (81). Intra-amniotic administration of SP-A to these *Src-1/-2* dhet mice rescued the delayed-labour phenotype observed in these mice (81). SP-A also induce an elevation of contraction associated genes and proteins including GJA and OXTR as well as PGF_2a synthase, known as AKR1B3 in the myometrium of *Src-1/-2* dhet mice (81). As a result, an increase in $PGF_{2\alpha}$ measured in the myometrium and a decrease in maternal blood progesterone were observed in *Src-1/-2* dhet mice administered SP-A in comparison to PBS (81). These experiments highlight crucial role of SP-A which is regulated by SRC1 and SRC2 in mediating normal on-time parturition in mice. Importantly, this evidence supports the interpretation that there is transmission of SP-A into the myometrium (139), where SP-A has direct effects on production of pro-inflammatory cytokines

and uterine activation proteins, resulting in synthesis of prostaglandin, to possibly initiate myometrial contraction in mice. Overall, the evidence points to a difference in the role of SP-A during parturition between humans and mice, possibly due to the dual immunomodulatory roles for SP-A.

Intra-amniotic administration of SP-A on gd 15 was efficient in inducing preterm delivery within 24 hours in 14 out of 17 ICR mice, suggesting that exogenous SP-A can contribute to the pathogenesis of sterile inflammation during preterm delivery in mice (344). This is supported by increased nuclear localisation of NF-KB proteins from the cytoplasm of the uterine horn, 4.5 hours following the intra-amniotic administration of SP-A, when compared to the intra-amniotic injection of SP-A depleted preparation (344). In vitro experiments showed an increase in *Il1b* and *Nfkb* mRNA at each time point with the treatment of amniotic fluid macrophages using SP-A, implying that SP-A can activate macrophages, leading to secretion of IL1B (344). Taken together, these studies show that SP-A is efficient in activating NF-KB to potentially upregulate the pro-inflammatory cytokine expression contributing to preterm delivery in mice.

The administration of higher doses SP-A in the right uterine horns on gd 14.5 did not induce preterm delivery within 48 hours in CD1 mice (345). The intrauterine administration of SP-A alone had no effect on pro-inflammatory cytokines measured in gestational tissues including uterus, placenta and fetal bodies (345). This suggests that different routes of SP-A administration in different strains of mice result in different outcomes of preterm birth. In another study, mice over-expressing rat SP-A (rSP-A) or SP-D (rSP-D) had higher expression of SP-A and SP-D respectively in the fetal lung, amniotic fluid and uterus, compared to wildtype mice (346,347). The high endogenous level of SP-A and SP-D did not result in preterm birth (346,347). It could be that the endogenous level of SP-A or SP-D was not sufficient to induce preterm delivery in mice, compared to the exogenous level of SP-A which was administered in the Condon et al. study (344).

rSP-A and rSP-D mice also required only 12.5 μg of LPS in order to have preterm delivery with live born pups (346). Meanwhile, 25 μg of LPS was required to induce preterm delivery with live born pups for wildtype mice. At this dose, more than 50% of the pups were born dead for both rSP-A and rSP-D mice (346).Overall it seems like both rSP-A and rSP-D mice have higher susceptibility to poor neonatal outcomes resulting from preterm delivery when challenged with LPS. In contrast to this study, the administration of intrauterine SP-A together with the TLR ligands including LPS and PGN/ Poly [I:C] caused a significantly decreased rate of preterm

delivery and increased number of surviving pups 48 hours after surgery when compared to mice administered TLR ligands alone (345). The expression of pro-inflammatory cytokines including *Tnf, Il1b* and *Ccl5* mRNA in the placentas and fetal bodies were shown to decline after 8 hours of SP-A and PGN/ Poly [I:C] administration on gd 14.5, when compared to the administration of PGN/ Poly [I:C] alone (345). Thus in this study, SP-A causes suppression of preterm delivery mediated by intrauterine infection, via the inhibition of pro-inflammatory cytokines in the gestational tissues.

An anti-inflammatory action of SP-A is supported by an in vitro study showing that a decline in the percentage of both F4/80+ macrophages and decidual cells expressing TNF was observed following treatment with 10 µg rSP-A or rSP-D before incubation with LPS (348). Further in vitro studies provide evidence that SP-A suppressed the TLR ligands including LPS, PGN and PGN/ Poly [I:C]-induced expression of *Il1b* and *Tnf* mRNA in the mouse macrophage cell line, RAW 264.7(345). This anti-inflammatory effect of SP-A requires TLR2, but not TLR4 as the anti-inflammatory effect of SP-A was eliminated in macrophages lacking TLR2 and treated with LPS, but was present in macrophages lacking TLR4 and treated with PGN/ Poly [I:C] (345). This is supported by a study showing that SP-A downregulates the PGN stimulation of TNF in human lymphoblast lung cell line and rat alveolar macrophages by binding to TLR2 directly (349). SP-A also reduces PGN-induced NF-KB in human embryonic kidney 293 cells that are transiently infected with cDNA of TLR2 (349). There is also a possibility that SP-A excerts its anti-inflammatory effect via TLR4 as further ex vitro experiments reveal that SP-A reduces the secretion of IL10 from bone marrow derived macrophages from C3H/HeJ mice (350). SP-D interacts with both TLR2 and TLR4 and does not alter interaction with their ligands, phosphatidylinositol and LPS respectively (351).

Hence, in the presence of infection it appears that SP-A can act as both a pro-inflammatory and anti-inflammatory mediator in late gestation. Another possible explanation is that different methods used in synthesis and purification of SP-A or SP-D in different laboratories lead to these different effects of SP-A on inflammation. A human study has demonstrated that SP-A polymorphism is not associated with spontaneous preterm delivery (352).

1.6.3 Summary

The studies discussed above show that the signals of parturition are likely to be initiated by the fetus, where PAF and SP-A have been identified as signals secreted by the fetal lung and elevated in late gestation (81,312,313,337,340,343). On balance the evidence supports the view

that birth is induced at term by fetal signals, released when the fetus is physiologically prepared to maintain homeostasis as a neonate (353). PAF and SP-A are then secreted into amniotic fluid towards the end of gestation (81,344). HSP70 is hypothesised to be secreted from fetal membranes to contribute to the accumulation in the amniotic fluid as term gestation approaches (248,249). In late gestation, macrophages are activated by elevated SP-A level (344), possibly via signalling through TLR4 which is expressed by amniotic fluid macrophages (168). Activation of TLR4 by other DAMPs and TLR4 regulators might also help to activate inflammatory leukocytes in the amniotic fluid. PAF, SP-A, HSP70 and HMGB1 may all interact to provoke a controlled inflammation in the amniotic fluid during normal labour (81,248,249,267,312,343,344).

As TLR4 is expressed by resident structural cells in the fetal membranes of both humans and mice (159,167), elevated levels of PAF, SP-A, HSP70 and HMGB1 in amniotic fluid could also help to trigger TLR4-mediated inflammation as gestation approaches its end, instigating fetal membrane rupture during labour. Overall, this speculation supports the interpretation that fetal membrane senescence also contributes as an initiator of a coordinated, redundant signalling cascade leading to parturition (353). From the above studies, it seems that PAF and SP-A can be transmitted from amniotic fluid into maternal tissues of humans and rodents including the uterus and cervix, as pregnancy reaches its end (139). PAF and SP-A have an effect on the synthesis of cytokines (71,322), prostaglandins (71,81,341) and MMPs (323) in these maternal tissues (139). Inflammatory cells such as macrophages activated by SP-A have also been demonstrated to infiltrate into the maternal tissues from the amniotic fluid in late gestation (344). HSP70 and HMGB1 which are associated with tissue remodelling (221-223) could also be secreted following an increase in uterine stretch as well as cervical remodelling towards the end of pregnancy. In these maternal tissues DAMPs and TLR4 regulators could contribute to activating TLR4, expressed by the leukocytes (157,162) as well as resident structural cells in the uterus (156-158,165) and cervix (156,161,166), to induce inflammatory processes that progress the onset of uterine contractions and cervical dilatation during labour.

A better understanding of the inflammatory pathways which mediate the mechanisms of normal term labour, is crucial in order to address why preterm birth occurs in some women. If indeed inflammatory pathways can be initiated via activation of TLR4 by DAMPs and TLR4 regulators, this could comprise a mechanism to initiate parturition that could be common to term and preterm labour. Depending on the stage of gestation (term or preterm), accumulating DAMPs and TLR4 regulators might originate in different sites in the gestational tissues. One logical explaination is that the mechanisms of term parturition are initiated by fetal membrane remodelling following

accumulation of fetally-derived DAMPs and TLR4 regulators, which then emanate out to maternal tissues to be followed by uterine contraction and cervical ripening. However, it is important to note that these DAMPs and TLR4 regulators can also interact with other receptors including PAFR, TLR2, RAGE and inflammasome which are expressed in the amniotic fluid (71,267,270,354), fetal membrane (159,274,315,355-358), myometrium (316,355,356,359-362), and cervix (271,322,323,363-366). Thus, as well as TLR4, these other receptors might also play important roles in interacting with these endogenous ligands to promote inflammation in late gestation, leading to parturition.

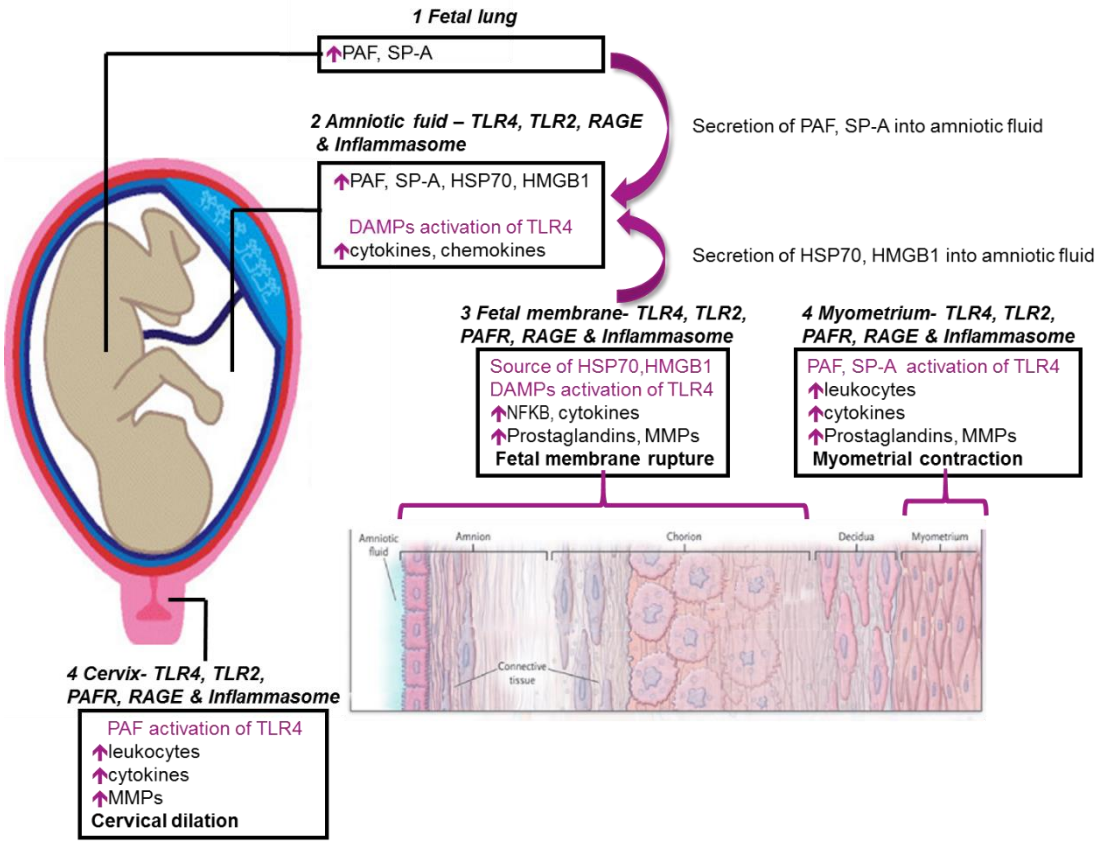


Figure 1.4 Schematic illustration of the inflammation mediated by DAMPs and TLR4 regulators in the gestational tissues during labour

Parturition is initiated by the signals produced by fetal lung including PAF and SP-A which are then released into amniotic fluid. PAF, SP-A, HSP70 and HMGB1 concentrations are elevated during labour in the amniotic fluid to potentially mediate inflammation in fetal membranes, initiating the process of fetal membrane rupture. PAF and SP-A are suggested to be transmitted from amniotic fluid into the maternal tissues including the myometrium and cervix as gestation approaches its end, to amplify inflammatory processes in these maternal tissues, ultimately causing uterine contraction, cervical dilation and delivery of the neonate. Adapted from Smith (2007).

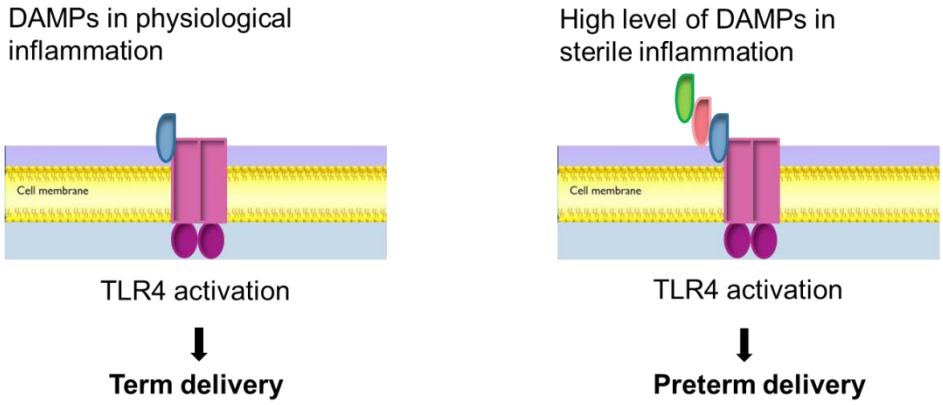
In the presence of infection, the levels of HSP70 (248) and HMGB1 (270) are increased in the amniotic fluid of human preterm delivery and pPROM. Higher levels of HMGB1, PAF and SP-A in the amniotic fluid are associated with preterm delivery without the presence of infection in humans and mice (273,329,330,344). It is speculated that smoking which results in fetal hypoxia (239) causes higher levels of PAF and SP-A to be synthesised from the fetal lungs to provoke early onset inflammation in amniotic fluid.

Oxidative stress from smoking (229-232) has been demonstrated to increase the synthesis of HMGB1 in the fetal membrane (274) to possibly contribute to elevated levels in the amniotic fluid (273). Multiple pregnancy, which causes extensive fetal membrane distention and remodelling (228) is hypothesised to contribute to elevated production of fetal fibronectin, HMGB1 and HSP70. In response to infection, HSP70 (201,202,249) and HMGB1 (274) are potentially secreted at higher levels in fetal membrane. Together, these DAMPs potentially stimulate greater inflammatory responses, to cause an increase in MMP production and thus mediate the rupture of membrane prematurely especially in pPROM women.

HMGB1 is increased in the cervix of preterm delivery women, both with or without infection (271). In the events of cervical insufficiency, occurring from cervical shortening, congenital cervical weakness, surgery or trauma, HMGB1 may be secreted in the cervix, to promote the inflammation causing early onset of cervical dilation and thus initiate preterm delivery. Multiple pregnancy which causes uterine overdistention and extensive tissue remodelling (228) is hypothesised to cause elevated release of the HMGB1 and HSP70 (221-223) to provoke the inflammation and thus trigger uterine contractions causing early onset of labour.

Other than in gestational compartments, DAMPs including HSP70 (260) and uric acid (276) are also increased in maternal circulation during preterm delivery, and this could occur due to maternal stress, exposure to toxins such as cigarette smoke and drugs, and maternal extrauterine infection. Overall, we hypothesise these infectious and non-infectious mediators stimulate activation of TLR4 by DAMPs and TLR4 regulators in gestational tissues to promote early onset of inflammatory process, eliciting preterm delivery.

A



B

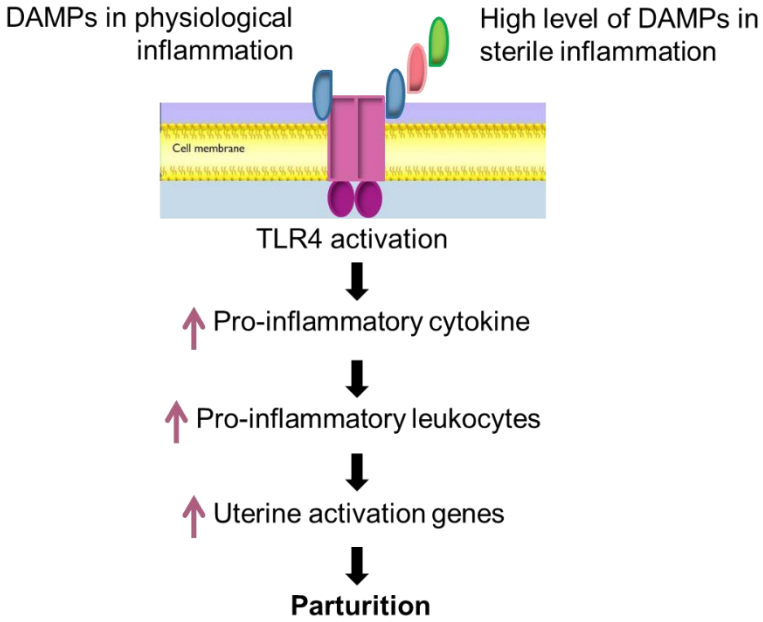


Figure 1.5 Working model of our hypotheses

(A) We predict that low levels of PAF, SP-A, HSP70, HMGB1 and fetal fibronectin produced from physiological inflammation in late gestation play a crucial role in activating TLR4, leading to the initiation of normal term labour. Meanwhile, we predict a high level of PAF, SP-A, HSP70, HMGB1, fetal fibronectin, uric acid and causes activation of TLR4 to mediate sterile inflammation, without the presence of infection. (B) We also propose that upon ligation with DAMPs and TLR4 regulators from physiological or sterile inflammation, the activated TLR4 acts to mediate the synthesis of pro-inflammatory cytokine genes including *Il6, Il1b, Tnf* and *Il12b*. These cytokines might mediate recruitment of pro-inflammatory leukocytes such as neutrophils, macrophages and T cells, to secrete more pro-inflammatory cytokines. These inflammatory mediators may participate in the upregulation of uterine activation genes, including *Ptghs, Ptgfr, Ptge, Oxtr* and *Gja*, leading to parturition.

1.6.4 Hypotheses

1. TLR4 activation by DAMPs and TLR4 regulators is crucial in mediating the physiological and sterile inflammation in normal term delivery and preterm delivery, respectively.

2. TLR4 activation by DAMPs and TLR4 regulators acts upstream of cytokine gene expression and leukocyte recruitment steps that mediate the mechanisms of parturition.

1.6.5 Aims : To utilize mouse models to evaluate:

1. The effect of TLR4 deficiency on activation of labour.

2. The effect of TLR4 deficiency on mechanisms of term parturition, including cytokine gene expression and leukocyte recruitment.

3. The efficiency of DAMPs and TLR4 regulators in inducing preterm delivery.

4. The effect of TLR4 deficiency in preventing preterm delivery induced by DAMPs and TLR4 regulators.

5. The effect of TLR4 deficiency on mechanisms of preterm parturition induced by DAMPs and TLR4 regulators, including cytokine gene expression.

Chapter 2: Materials and Methods

2.1 MICE AND MATING PROTOCOLS

BALB/c mice were obtained from the Animal Resource Centre (Perth, WA). Mice with a null mutation in the *Tlr4* gene (*Tlr4*-/- mice) backcrossed onto BALB/c for more than 10 generations were sourced from Professor Akira (Osaka University, Osaka, Japan) and supplied by Professor Paul Foster (University of Newcastle, New South Wales, Australia). The mice were housed and maintained in the specific pathogen-free University of Adelaide Medical School Animal House with a 12-hour light, 12-hour dark cycle. Breeder chow food and water were provided ad libitum, and animals were used according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes with approval from the University of Adelaide Animal Ethics Committee.

In our study, *Tlr4*-/- and BALB/c female mice were mated to males of the same genotype. One to 3 virgin female mice of 8-14 weeks of age were housed with a proven fertile male of the same genotype and checked daily between 8 and 10 am for vaginal plugs, as evidence of mating. The morning of vaginal plug detection was designated gestational day (gd) 0.5. Mated females were removed from the male and housed individually.

2.2 IN VIVO TREATMENTS

2.2.1 Danger associated molecular patters (DAMPs) and carbamyl PAF (cPAF)

At 10 am to 12 pm on gd 16.5, pregnant BALB/c female mice were injected intraperitoneally with the DAMPs or the same volume of vehicles in Table 2.1 below:

Table 2.1 Lists of DAMPs and cPAF utilised for in vivo studies in pregnant BALB/c mice

DAMPs/ cPAF	Dose	Source
cPAF	1 µg/mouse	Sigma-Aldrich (St Louis, MO, USA)
Recombinant HMGB1 (rHMGB1)	50 µg/mouse	Prospecbio (East Brunswick, NJ, USA)
Uric acid	3 mg/mouse	Invivo Gen (San Diego, CA, USA)
5-cholesten-3β, 25-diol	1.5-2.0 mg/mouse (50 mg/kg)	Steraloids (New Port, Rhode Island, USA)

2.2.2 Uric acid and cPAF

Pregnant *Tlr4*-/- and BALB/c female mice were given 6 mg/mouse uric acid and 2 µg/mouse of cPAF (1-O-palmitol-2-(N-methylcarbamyl)-*sn*-glycero-3-phosphocholine) intraperitoneally at 10 am to 12 pm on gd 16.5. The carbamyl group chemically incorporated in cPAF acts to confer resistance to the degradation by PAF-AH. Control treatment groups received the same volume of PBS. In other experiments pregnant BALB/c mice were administered intrauterine cPAF, 35 µg/mouse in PBS or vehicle at 10 am to 12 pm on gd 16.5 via surgical intervention. Isoflurane anaesthesia was used with an induction chamber, using a heat pad and a mask to deliver isoflurane/ oxygen anaesthesia. The mice were ensured to have reached deep anaesthesia with a toe pinch. A mini laparotomy was performed in the lower abdomen to expose the right uterine horn, until the two gestational sacs most proximal to the cervix were visualised. The cPAF was then injected slowly between the two gestational sacs, with care taken not to pierce the amniotic cavity and to avoid leaking of the cPAF. The incision was sutured using silk suture.

2.2.3 (+)-Naltrexone

Additional groups of pregnant BALB/c females were injected intraperitoneally with cPAF or vehicle on gd 16.5 together with TLR4 antagonist (+)-naltrexone intraperitoneally at the dose of 1.8-2.4 mg/mouse (60 mg/kg) on gd 16.5, 17.0, 17.5 and 18.0. Similarly females administered with intrauterine cPAF or vehicle on gd 16.5 also received TLR4 antagonist (+)-naltrexone intraperitoneally at the dose of 1.8-2.4 mg/mouse (60 mg/kg) on gd 16.5, 17.0, 17.5 and 18.0.

2.3 PREGNANCY OUTCOMES

For analysis of gestation length, pregnant females were monitored twice a day, in the morning as well as in the evening for the time of parturition and the number of pups born. The number of viable pups was recorded following delivery and at 7 and 21 days of age to calculate the percentage pup viability and postnatal mortality. To establish pup weights and neonatal growth, pups were weighed at 12-24 hours after delivery and at 7 and 21 days post-partum. Sex of surviving pups were recorded at 21 days post-partum.

Preterm delivery is defined as the delivery of at least one pup within 48 hours of treatment on gd 16.5. On gd 18.5, pregnant females that did not deliver preterm were killed by cervical dislocation, and the intact uterus of each female was removed. The total number of implantation sites was counted. Implantation sites were classified as viable (presence of live fetus and placenta); or not viable (anemic, malformed, severely growth retarded fetuses). When viable

fetuses were present, each viable fetus was dissected from the amniotic sac and umbilical cord. Then the fetuses and placentae were weighed, and the fetal: placental weight ratios calculated for each implant.

2.4 TISSUE COLLECTION FOR PCR

Pregnant *Tlr4*-/- and BALB/c females were killed by cervical dislocation on gd 16.5, 17.5, 18.5, or 19.5. In other experiments, pregnant *Tlr4*-/- and BALB/c females treated with PBS, cPAF, and/or (+)-naltrexone, they were killed 4 hours after treatment administration, by cervical dislocation on gd 16.5. The uterine myometrium (from implantation sites), and entire uterine decidua (at placental attachment site), placenta, fetal membranes and fetal head were dissected and snap-frozen in liquid N_2, then stored at −80°C. All tissues from pregnant mice were recovered prior to onset of labour. All the tissues harvested from two implantation sites per dam were pooled together and expression of cytokine genes as well as uterine activation was determined real time-PCR (RT-PCR).

2.5 RNA EXTRACTION

Uterine, placental, decidual, fetal membrane and fetal head tissues were disrupted and homogenised in 2 mL tough tubes (Mo Bio, Carlsbad, CA, USA) in the presence of 0.6 g of 1.4 mm ceramic beads (Mo Bio) and 1 mL of Trizol (Ambion RNA, Carlsbad CA, USA) using Powerlyzer 24 (Mo Bio) at 4°C to release RNA at 3500 rpm for 30 seconds in 1 cycle. The homogenised samples were then incubated at room temperature for 10 minutes to allow complete dissociation of the nucleoprotein complex, before 200 µL of chloroform (Biolab, Scoresby, Victoria, AUS) was added into each tube. The tubes were shaken vigorously by hand for 15 seconds and then incubated for 3 minutes at room temperature. The samples were then centrifuged at 12,000 x g for 15 minutes at 4°C which caused the mixture to separate into a lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase, which contains the RNA. This aqueous phase was removed, placed into a new tube with 1 mL of isopropanol (Sigma Life Science) incubated at room temperature for 10 minutes to precipitate RNA. Samples were then centrifuged at 12,000 x g for 10 minutes at 4°C to pellet the RNA. The supernatant from each sample was removed, leaving the pellet which then was washed with 2 mL of 75% ethanol (Sigma Life Science). The samples were centrifuged at 7,500 x g for 5 minutes at 4°C and the wash was discarded. The samples were then centrifuged again briefly and the remaining wash was discarded again to dry the RNA pellet. The RNA pellet was resuspended in

RNase free water (20 µL to 100 µL depending on the size of the pellet). The samples were incubated in a heat block set at 55°C for 10 minutes. RNA purity and concentration were determined by the A_{260} and A_{280} measurements using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and then stored at -80°C. The RNase free DNAse treatment was carried out on 10 µg of RNA in a volume of 50 µL using Turbo DNA-free Kit according to the manufacturer's instructions (Ambion RNA). The RNA integrity was verified by agarose gel electrophoresis to ensure that the 28S and18S bands were visible on the gel image captured using Gel Doc-EZ imager (Bio-Rad Laboratories).

2.6 RT-PCR

Total RNA (1000 ng) was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) in a volume of 20 µL using the protocols provided by the manufacturer. The complementary DNA (cDNA) obtained was then used for subsequent RT-PCR. Primer sequences for the uterine activation genes, pro-inflammatory cytokine genes, anti-inflammatory cytokine genes and the Treg cell transcription factor, forkhead box P3 (Foxp3) gene (Table 2.2) were designed, optimized and tested for their efficiency in house.

Table 2.2 Sequences, concentrations and Genbank accession numbers for RT-PCR primers

Gene (protein, synonym)	Primer seguences (5'-3')	Primer binding locations (nucleotide number)	Product length (bp)	Primer	GeneBank accession no.
Actb (ACTB)	Fwd: CGTGGGCCGCCCTAGGCACCA Rev: ACACGCAGCTCATTGTA	Fwd: 211 to 231 Rev: 396 to 380	186	1 µM	NM 007393.3
Ptgfr (PTGFR, FP)	Fwd: CTGGCCATAATGTGCGTCTC Rev: TGTCGTTTCACAGGTCACTGG	Fwd: 1053 to 1072 Rev: 1157 to 1137	105	0.1 µM	NM 008966
Ptgs1 (PTGS1,PGHS-1, COX-1)	Fwd: GTGAATGCCACCTTCATCCG Rev: CCAGCTGATGTAGTCATGCGC	Fwd: 450 to 469 Rev: 560 to 540	111	0.1 µM	NM 008969.3
Ptgs2 (PTGS2,PGHS-2, COX-2)	Fwd: GTTTGCATTCTTTGCCCAGC Rev: AGTCCACTCCATGGCCCAGT	Fwd: 742 to 761 Rev: 839 to 820	98	0.5 µM	NM 011198.3
Ptger4 (PTGER4, EP4)	Fwd: ATCGACTGGACCACCAACGTA Rev: AGAGCACGGTGGCAAGGAT	Fwd: 1141 to 1161 Rev: 1231 to 1213	91	0.25 µM	NM 001136079.1
Gja1 (GJA1, Cx43)	Fwd: TGGTGTCCTTGGTGTCTCTCG Rev: TCGCTTCTTCCCTTCACGC	Fwd: 876 to 866 Rev: 966 to 948	91	0.25 µM	NM 010288.3
Oxtr (OXTR,OTR)	Fwd: ATCACGCTCGCCGTCTACA Rev: TGAGTCGCAGATTCTGCCAGA	Fwd: 621 to 639 Rev: 711 to 691	91	0.5 µM	NM 001081147.1
Foxp3 (FOXP3)	Fwd: CCACCTGGAAGAATGCCATC Rev:AATTCATCTACGGTCCACACTGC	Fwd: 1478 to 1497 Rev: 1574 to 1552	97	0.25 µM	NM 001199347.1
IL17a (IL17A)	Fwd: TCAACCGTTCCACGTCACC Rev: TCCCTCCGCATTGACACAG	Fwd: 266 to 284 Rev: 378 to 360	113	0.5 µM	NM 010552.3

Tnf (TNF)	Fwd: GTAGCCCACGTCGTAGCAAAC Rev: CTGGCACCACTAGTTGGTTGTC	Fwd: 441 to 461 Rev: 558 to 537	118	0.1 µM	NM 013693.3
Il12b (IL12B)	Fwd: TGACACGCCTGAAGAAGA Rev: AGAGACGCCATTCCACAT	Fwd: 206 to 223 Rev: 571 to 554	366	0.5 µM	NM 001303244.1
Il6 (IL6)	Fwd: ACAACCACGGCCTTCCCTAC Rev: TCCACGATTTCCCAGAGAACA	Fwd: 139 to 158 Rev: 269 to 249	131	0.5 µM	NM 031168.1
Il1b (IL1B)	Fwd: CCAAAGCAATACCCAAAGAAA Rev: GCTTGTGCTCTGCTTGTGAG	Fwd: 700 to 716 Rev: 829 to 810	130	0.5 µM	NM 008361.3
Il1a (IL1A)	Fwd: CCGACCTCATTTTCTTCTGG Rev: GTGCACCCGACTTTGTTCTT	Fwd: 725 to 744 Rev: 828 to 809	104	0.5 µM	NM 010554.4
Il10 (IL10)	Fwd: AGGCGCTGTCATCGATTTCT Rev: TGGCCTTGTAGACACCTTGGT	Fwd: 437 to 456 Rev: 539 to 519	103	0.5 µM	NM 010548.2

The RT-PCR plate was set up using QIAgility (Qiagen, Valencia, CA, USA) a benchtop liquid handling system. Each reaction for RT-PCR contained 2 µL of cDNA (10 ng/uL) and 18 uL of master mix consisting of 1x Power SYBR® Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA), forward and reverse primer (refer to Table 2.2 for concentrations), and RNase free water. Non template control samples containing water substituted in place of cDNA were included in all assays to confirm the absence of non-specific amplification product. Real time RT-PCR was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 20 sec at 95°C and 1 min at 60°C. Melt curve analysis was included to ensure lack of amplification of non-specific products for all primer sets. Previous studies in our laboratory show that the expression of *Actb* is stable in gestational tissues including uterus, palcenta, fetal membrane and fetus of control mice or mice treated with infectious agents (76,367,368). Thus in this study, levels of each of gene of interest were expressed relative to *Actb* levels using the following formula (369):

mRNA level= Log_2-(Ct_{Bactin}-$Ct_{target\ gene}$)

2.7 FLOW CYTOMETRY

Pregnant *Tlr4*-/- and BALB/c mice were killed at gd 18.5 and myometrium, decidua, placenta and fetal membrane from all implantation sites as well as PALN were harvested. Mesenteric lymph node (MLN) was also harvested to be used in defining compensation controls for each antibody used. Myometrium, decidua, placenta and fetal membranes were placed in Roswell Park Memorial Institute medium (RPMI) 1640 containing 2% fetal calf serum (FCS) (RPMI-FCS, Life Technologies) plus 1 µg/mL collagenase from *Clostridium histolyticum* Type IA (Sigma-Aldrich) and 4 unit/ml of DNase I (Sigma-Aldrich), finely cut with scissors and incubated at 37°C with agitation for 1 hour. Myometrium, decidua, placenta and fetal membranes suspension were ground between the frosted ends of two glass slides to further extract cells out of the tissues. PALN and MLN were crushed between the frosted ends of two glass slides in 1 mL of RPMI each.

For each of the myometrium, decidua, placenta, fetal membranes and PALN tissues, the remaining tissue fragments were filtered from the cell suspension using a 70 µM cell strainer (In Vitro Technologies, Noble Park, Vic, AUS) into 50 mL falcon tubes. RPMI 1640 containing 2% FCS was then added to the cell suspension to make up to a total volume of 10 mL. Then 10% (1 mL) of the cell suspension was transferred into a 2 mL tube for count bead analysis. Placental

cell suspensions were washed first by centrifugation at 300 g for 5 minutes and the supernatant were aspirated before being incubated with 2 mL of 1x red blood cell (RBC) lysis buffer (10 × RBC lysis buffer- 1.55 M NH_4Cl, 100 mM $KHCO_3$, 992 µM EDTA disodium salt in RO water) for 2 min. The placental cell suspension with lysed red blood cells was diluted with RPMI culture medium to a total of 10 mL. To remove dead cells and debris, the cell suspension for each of the digested gestational tissues, 500 µL of FCS was added slowly to the bottom of tube slowly, before centrifugation at 300 g for 5 minutes without the brake. The PALN cell suspension was centrifuged without FCS.

The MLN cell suspension was diluted with 1 mL of ice-cold RPMI culture medium and then equally divided between the flow cytometry tubes for compensation controls for each of the antibody used. Cells in each of the compensation tubes were again washed in 3 mL of RPMI culture medium and centrifuged at 300 g for 5 minutes. After centrifugation the supernatant was removed and each of the cell pellets was incubated with Fc block (BD Biosciences, San Diego, CA, USA, clone 2.4G2, 10 µg/ml, 10 min on ice). The antibodies for cell surface markers from antibody panels #1, #2 and #3 (Table 2.3) were prepared in fluorescence activated cell sorter (FACS) buffer (0.1% bovine serum albumin (BSA) and 0.05% sodium azide in PBS) at 1 in 100 dilution for each of antibody. Each of the cell pellets was equally divided into three flow cytometer tubes for incubation with the three different panels (50 µL for each panel) of antibodies for 30 minutes on ice. Then the cells were washed by centrifugation at 300 g for 30 minutes.

For panels #1 and #2, cells were then incubated with 50 µL of biotinylated streptavidin-V500 as the secondary antibody for MHCII which was conjugated with biotin, at 1/100 dilution in FACS buffer for 30 minutes on ice. The cells were washed before addition of 2 µL 4',6-diamidino-2-phenylindole (DAPI) solution (200 µg/mL) (Sigma-Aldrich). For panel #3, cells were incubated with 300 µL fixable viability dye V450 (BD Biosciences) at 1/300 dilution with 1x PBS at room temperature. The cells were washed and then fixed, permeabilised using the FOXP3 staining kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions and labeled with anti-FOXP3 antibody.

A FACS Canto II (BD Biosciences, San Diego, CA, USA) was used to perform the multi-coloured flow cytometry analysis and the data were collected using FACS Diva software (version 6.0, BD Biosciences). Gates were firstly applied to the side scatter/ forward scatter density plot to exclude dead cells or tissue debris. Then another gate was applied on the forward scatter-area (FSC-A)/ forward scatter-height (FSC-H) density plot to discriminate the doublet cells, on the basis of the disproportion between the cell size and cell signal. DAPI positive staining was used to exclude the dead cells for the samples that did not require fixation (panel #1 and panel

#2). Additional gates were then applied to dot plot to show cells positive for staining with cell markers for macrophages (CD11B, F4/80, MHCII), neutrophils (CD11B, Ly6G), dendritic cells (CD11C, CD80, CD86, MHCII), Treg cells (CD3, CD4, CD25, FOXP3) and T helper 17 (Th17) cells (CD3, CD4, IL17RA) based on the unique fluorescence emission wavelengths of the fluorochrome conjugated to each antibody.

Samples for count bead analysis were labelled with antibodies against CD11C, F4/80, CD4 and CD45 (APC, eBioscience) at 1/100 dilution in FACS buffer, before addition of 10000 of CountBright™ Absolute Counting Beads (Molecular Probes, Invitrogen, Eugene, Oregon, USA) and DAPI. The ratio between beads and labelled cells in each sample was used to calculate the total number of cells in the whole myometrium scraped and PALN, and the total number of cells per implantation site in fetal membrane, placenta and decidua.

Table 2.3 Panels of antibodies, conjugates and dyes utilised in flow cytometry analysis of leukocyte populations

Panel	Antibody	Conjugate	Clone	Source
#1	CD11B	APC	M1/70	eBioscience
#1	F4/80	PE	BM8	eBioscience
#1	Ly6G/Gr-1	APCY-Cy7	1A8	BD Biosciences
#1	MHCII	Biotin	M5/114.15.2	eBioscience
#1	Streptavidin	V500	-	BD Biosciences
#1	DAPI	-	-	Sigma Life Science
#2	CD11C	Alexafluoro 488	N418	eBioscience
#2	CD80	PE-Cy 7	16-10A1	eBioscience
#2	CD86	APC	GL1	BD Biosciences
#2	MHCII	Biotin	M5/114.15.2	eBioscience
#2	Streptavidin	V500	-	BD Biosciences
#2	DAPI	-	-	Sigma Life Science
#3	CD3	FITC	17A2	eBioscience
#3	CD4	Per-CP-5.5	RM4-5	eBioscience

#3	CD25	PE-Cy7	PC61.5	eBioscience
#3	FOXP3	APC	FKJ-16s	eBioscience
#3	IL17A Receptor	PE	PAJ-17R	eBioscience
#3	Fixable viability dye	V450	-	BD Biosciences

2.8 BACTERIAL ENDOTOXIN LPS

The preparations of DAMPs and TLR4 regulators were tested for the presence of gram-negative bacterial endotoxin content (Table 2.4) using a QCL-100 limulus amebocyte lysate (LAL) assay (Lonza, Basel, Switzerland), according to the manufacturer's instructions, in which all reagents as well as the microtiter plate were required to be maintained at 37°C throughout the assay. Pyrogen-free plastic and glassware were throughout. Firstly, either LAL-free water control, standard, DAMPs or TLR4 regulators at different concentrations were added to the 96 well plate. 50 µL of limulus amebocyte lysate solution was then added to each well (T= 0 min) and incubated for 10 min (T= 10 min) before the addition of a chromogenic substrate for 6 min (T= 16 min) at 37°C. This was followed by acidification of the substrate product by the addition of 100 µL of 25% acetic acid (v/v) and measurement of absorbance at 405 nm. The concentration of endotoxin within the samples was calculated from a standard curve (4-parameter logistic curve) using known concentrations of *E.coli*-derived endotoxin. According to the manufacturer, the assay has a minimal detectable limit of 0.1 EU/ml, with intra-assay precision of <4% and inter-assay precision of <10%.

2.9 IN VITRO CULTURE OF J774 MACROPHAGES

2.9.1 General

All the experiments using cell lines were performed using aseptic technique, under sterile conditions and in a laminar flow hood to minimise the potential of microbial or endotoxin contamination.

2.9.2 Freezing J774 macrophages cells

J774 cells were cultured in medium consisting of RPMI 1640 medium + L-glutamine +10% (v/v) FCS + 1:50 of Penicillin-Streptomycin (10,000 U/mL) (Life Technologies). Cells from six 75 cm^2 flasks (In Vitro Technologies) were used per experiments when each of the flasks is about 90% confluent. 5 mL of culture medium was added to each of the flasks before the cells were scraped and pooled together in a 50 mL tube. The cell suspension was centrifuged at 1100 rpm for 6 min at room temperature and the supernatants were removed. The cells were resuspended in 6 mL of culture medium and then with 6 mL of the freezing medium consisting of 60% culture medium + 20% FCS + 20% dimethyl sulfoxide (DMSO) (Merck Millipore, Billerica, MA, USA). 1 mL of the cell suspension was added in each freezing tubes and then stored in

isopropanol in -80°C freezer overnight. The tubes were transferred into liquid nitrogen the next day.

2.9.3 Thawing J774 macrophages cell lines

1 mL of the cell suspension was transferred into 5 mL tube (In Vitro Technologies) before 1 mL of culture medium was added dropwise and shaken gently every few drops. The tube was left at room temperature in the hood for 10 minutes, then 2 mL of the culture medium was added dropwise as before, and left for 5 minutes. The cell suspension was centrifuged for 6 minutes at 1100 rpm at room temperature. The supernatant was discarded and the cell pellet was resuspended in 4 mL of the culture medium and then centrifuged again for 6 minutes at 1100 rpm at room temperature. The cell pellet was resuspended in 1 mL of culture medium and then transferred into a 75 cm2 flask. 25 mL of the culture medium was added into the flask and then cells were cultured in a humidified 5% CO_2 atmosphere in the incubator at 37°C. Using Nikon TMS inverted microscope (Nikon, Chiyoda-ku, Tokyo, Japan), cells were assessed daily to make sure that they were viable, adhering to the surface and proliferating. The culture medium was changed after 3 days to eliminate dead non-adherent cells. After 3 days cells achieved 90% confluence. In order to split the cells, the supernatant was removed and 10 mL of medium was added into the flask. The cells were then scraped using a sterile cell scraper (In Vitro Technologies) before 1.5 mL of the cell suspension was added to each of the flasks, in a total of six new flasks.

2.9.4 Seeding J774 macrophages cell lines

Once approximately 90% confluence was achieved, cells from the six flasks were scraped and pooled in a 50 mL tube and centrifuged at 1100 rpm at room temperature for 6 minutes. The supernatant was removed, the pellet was resuspended in 2 mL of culture medium and then added with culture medium to a total volume of 30 mL. The tube was vortexed briefly and 50 µL of the cell suspension was isolated and then diluted 1:2 with 50 µL of trypan blue (Sigma Life Science, St. Louis, MO, USA). Trypan blue selectively stains the dead cells blue, leaving live cells unstained. 10 µL of the cell suspension was loaded onto each chamber of the glass haemocytometer. The viable cells that were not blue in colour were counted under the Nikon TMS inverted microscope.

The total cell number was counted as:

$$\text{Total cells/mL} = \frac{\text{Number of cells counted} \times \text{dilution (2)}}{\text{\# of square (25)}} \times 10000$$

The cell suspension was diluted accordingly and counted again to achieve cell concentration of 4-5 x 10^4 cells/mL. The cell suspension was aliquoted in wells of a 48-well plate (In Vitro Technologies) at 20000 cells/ well. Culture medium was added to make up a total volume of 500 µL in each well, before pre-incubation for 24 hours to allow cell adherence in a humidified 5% CO_2 atmosphere in the incubator at 37°C. The cells were treated with DAMPs or TLR4 regulators (refer to Table 2.4) at a range of concentrations or control (culture medium alone) for 12 hours.

Table 2.4 Lists of DAMPs and TLR4 regulators utilised for in vitro study using J774 macrophages

DAMPs/ TLR4 regulators	Solutions used for reconstitution	Source	Endotoxin level (EU/µg)
cPAF	Endotoxin free water	Sigma-Aldrich	0.001
rHMGB1	Endotoxin free water	Prospecbio (East Brunswick, NJ, USA)	0.312
uric acid	Endotoxin free PBS	Invivo Gen (San Diego, CA, USA)	0.002
5-cholesten-3β, 25-diol	100% ethanol	Steraloids (Newport, Rhode Island, USA)	0.030
5-cholesten-3β-ol-7-one	100% ethanol	Steraloids (Newport, Rhode Island, USA)	0.002
Recombinant SP-D (rSP-D)	Endotoxin free PBS	R&D Systems (Minneapolis, MN USA)	0.020

After 12 hours the supernatant was harvested and placed in labelled tubes and stored at -20°C for later cytokine quantification using ELISA or bead array. The cells in each well were treated with 100 µL of Presto Blue (Life Technologies) at 1X (diluted from 10X with culture medium) per well and incubated in a humidified 5% CO_2 atmosphere in the incubator at 37°C for 20 minutes. Presto Blue is a membrane permeable solution, using the metabolic activity of the mitochondrial enzyme to reduce resazurin from the non-fluorescent to the fluorescent form of resorutin. The Presto Blue solution was transferred to a 96 well plate PS MaxiSorp non sterile clear U96 (Thermo Fisher Scientific) and then measured for absorbance at wavelength 570 nm

minus 595 nm. The optical density values of each well, with known approximate viable cell number/ well were used to generate a standard curve (4-parameter logistic curve) from which the approximate number of viable cells after treatment with DAMPs, TLR4 regulators and control medium was calculated.

2.9.5 J774 cell culture supernatant TNF ELISA

Mouse TNF Duoset ELISA (R&D Systems) was used to quantify the concentration of TNF in the J774 cell culture supernatants harvested, according to the manufacturer's instructions. Briefly 96 well plate PS MaxiSorp non sterile clear U96 was coated with goat anti-mouse TNF capture antibody overnight at room temperature. Then the plate was blocked with reagent diluent (1% BSA in PBS) for 1 hour at room temperature to inhibit the non-specific binding. The supernatants and standards were added and incubated for 2 hours, before addition with the biotinylated goat anti-mouse TNF detection antibody for 2 hours at room temperature. Bound antibody was detected by incubation with streptavidin conjugated horseradish peroxidase (HRP) for 20 minutes followed by incubation with substrate solution for 20 minutes, where both incubations occurred in the dark. The reaction was stopped using 50 µL of 1 M hydrochloric acid (HCl), and the optical density in each plate was measured at 450 nm (reference wavelength 570 nm) using a Benchmark microplate reader (Bio-Rad). The optical density values of the standards, with known concentrations of recombinant TNF were used to generate a standard curve (4-parameter logistic curve) from which the concentrations of TNF in the J774 cell culture supernatants were calculated.

2.10 BEAD ARRAY

DAMPs or cPAF were administered by intraperitoneal injection (Table 2.1) on gd 16.5 and 4 hours later the pregnant females were anaesthetised by the injection of 400 µL avertin intraperitoneally. An incision was made in the chest cavity and blood was collected via cardiac puncture before the females were humanely sacrificed. Blood was centrifuged at 2000 rpm for 10 minutes at room temperature before the aqueous layer of the serum was collected and stored at -80ºC. The level of inflammatory cytokines and chemokines was quantified using multiplex bead array in the serum of these mice as well as in the cell culture supernatants from J774 macrophages treated with 5-cholesten-3β, 25-diol (20 µg/mL), uric acid (200 µg/mL), rHMGB1 (20 µg/mL), cPAF (0.5 ug/mL) and medium controls.

A mouse cytokine/ chemokine magnetic bead panel (EMD Millipore) was performed, according to the manufacturer's instruction. Briefly wash buffer was added to each of the assay

wells and mixed on a plate shaker for 10 minutes at room temperature before the wash buffer was discarded. The standards or control (assay buffer) was then added to the plate in a vertical configuration and assay buffer was added to the sample wells. Appropriate matrix solution was then added to the standard, control and sample wells before the addition of serum samples (diluted 1:2 in assay buffer) or culture supernatants (neat). The plate was incubated with premixed beads overnight, with agitation at 4ºC. Following incubation, the plate was washed 2 times before the addition of detection antibody, followed by additional incubation for 1 hour. After 1 hour, streptavidin-phycoerythrin was added to each of the well and incubated for a further 30 minutes, with agitation at room temperature. After the washing step, sheath fluid was added to the plate, and then the beads were resuspended on a plate shaker for 5 minutes. The median fluorescent intensity (MFI) was analysed using Luminex 200 (Merck's Life Sciences, Darmstadt, Germany), with the following settings: 50 per bead of events, 100 µL sample size, 8000 to 15000 double discrimination gate settings and default (low PMT) reporter gain. The xPONENT software (Merck's Life Sciences) was used for analysis of data. The MFI of the standards, with known concentrations were used to generate a standard curve (4-parameter logistic curve) from which the concentrations of cytokines and chemokines in the serum or cell culture supernatants were calculated.

2.11 STATISTICAL ANALYSIS

All statistical analysis was conducted using SPSS for Windows, version 20.0 software (SPSS Inc, Chicago, IL). Data were tested for normality using a Shapiro–Wilk test. ANOVA and post hoc Sidak T-tests were used when data were normally distributed. Kruskal-Wallis and Mann-Whitney U-test were used when data were not normally distributed. Categorical data were compared by χ^2 analysis. The fetal weight, placental weight and fetal: placental weight ratio data were analysed using mixed model ANOVA and the data are given as the estimated marginal means ± SEM. The statistical test and number of samples/mice used in each experiment is shown in the corresponding table of the figure legend. Differences between groups were considered significant when $p < 0.05$.

Chapter 3: Effect of Genetic Deficiency in TLR4 on Term Labour and Gene Expression in Gestational Tissues

3.1 INTRODUCTION

The parturition process can be thought of as a pro-inflammatory signalling cascade which drives the mechanisms of parturition to result in uterine contractions, cervical ripening and the rupture of fetal membranes. During human labour, elevated expression of pro-inflammatory cytokine genes including *IL1B, IL6* and *TNF* has been documented in the uterus, cervix and fetal membranes (58,59,128,136,137). In the reproductive tissues, these pro-inflammatory cytokines have been suggested to originate in the invading leukocytes as well as non-leukocyte structural cells during parturition (59). Studies in mice also demonstrated that pro-inflammatory cytokine mRNAs expression are elevated during labour in both the myometrium and decidua (86,87). This is supported by an observation showing an increase in decidual cytokine genes as gestation approaches its end (86). The important role of pro-inflammatory cytokines in mediating normal on-time labour is further demonstrated by the finding that parturition is delayed by 24 hours in IL6 deficient mice, which can be corrected by administration of exogenous IL6 (73). NF-KB recognition elements are detected within the promoters of each of these pro-inflammatory cytokine genes (98). Thus, it is well established that the activity of this transcription factor is a key upstream regulator of the pro-inflammatory cytokine expression in the reproductive tissues (96,97). Anti-inflammatory CD4+CD127 low+/-CD25+ Treg cells are reduced in the percentage and suppressive activity during labour (154,155).

The transition of myometrium from an inactive state during pregnancy to a muscle that is spontaneously sensitive and responsive to contractile stimulants during labour is termed ‘uterine activation’. The activation of myometrium is suggested to be mediated by coordinated expression of PTGHS, PTGFR, PTGER, OXTR and GJA (91). In vitro experiments and genetic disruption of cytokines in vivo in mice also imply that the induction of uterine activation genes is downstream of pro-inflammatory cytokine synthesis in the birth cascade (73,121-126,370). Previous studies also reported that the physiological events of delivery are facilitated by the elevation in these uterine activation proteins by regulation at the transcriptional level, to potentially allow the uterus to respond to contractile stimulants such as prostaglandins and oxytocin, leading to uterine activation (80,87,91,117-120,138,371). Other than prostaglandins, oxytocin also contributes to myometrial contraction and GJA is important in increasing the formation of gap junctions to support an increase in the electrical coupling between myometrial cells to promote synchronised uterine contraction (92).

Human spontaneous labour at term is associated with an increase in *TLR4* expression in the chorioamniotic membrane (61).TLR4 activation occurs after association with PAMPs or endogenous ligands, including DAMPs and TLR4 regulators. These may include PAF and SP-A secreted from fetal lung, and HSP70 is thought to be secreted by the fetal membranes. Levels of these endogenous ligands are elevated in the amniotic fluid of humans and mice towards the end of gestation suggesting they may play a crucial role for on-time parturition in mice (81,248,249,312,313,337,340,343,344).

PAF and SP-A are implicated in normal term parturition as *Src-1/-2* dhet mice that are deficient in these TLR4 regulators have delayed labour when compared to wildtype mice. (81). Human studies have documented an increase of PAF (312) and HSP70 (248) in the amniotic fluid during labour. Human studies by Romero have demonstrated that HMGB1 concentration is also elevated in the amniotic fluid in human term labour associated with infection (267). This evidence suggests that under the physiological conditions of term labour, these DAMPs and TLR4 regulators are secreted in the amniotic fluid to trigger labour with or without the presence of infection. Most importantly, PAF (332), SP-A (350,351) HSP70 (205,206) and HMGB1 (210,211) can contribute to TLR4 activation to induce inflammation, and thereby these factors may play a role in initiating the events culminating in parturition.

We hypothesise that TLR4 is an important driver of physiological inflammation of normal term labour. If so, it seems likely that TLR4 activation acts upstream of the upregulated cytokine gene expression which mediates the mechanisms of parturition in normal term labour. We have utilised TLR4 deficient mice (*Tlr4*-/- mice) to study the activation of labour and mechanisms of term parturition in mice, including induction of key genes involved in labour. We find that activation of TLR4 is indeed required for on-time labour, and that the activation of TLR4 in late gestation leads to upregulation of pro-inflammatory cytokines and induction of uterine activation genes, leading to on-time parturition.

3.2 THE EFFECT OF TLR4 DEFICIENCY ON TIMING OF NORMAL TERM LABOUR AND PERINATAL VIABILITY

In order to investigate whether TLR4 deficiency impacts the timing of normal time labour and perinatal outcomes, *Tlr4*-/- BALB/c females and wildtype BALB/c females were mated with males of the same genotype. The duration of gestation was extended by 13.2 hours in the *Tlr4*-/- females as compared to control wildtype BALB/c females (with parturition at 20.05 ± 0.09 days *versus* 19.50 ± 0.08 days respectively, mean ± SEM, $p < 0.001$, Fig. 3.1A). This was accompanied by a 26% decrease in the mean number of viable pups born in *Tlr4*-/- mice compared with control mice ($p = 0.017$, Fig. 3.1B). The pups born from *Tlr4*-/- pregnancies also showed a 8% increase in weight at 12-24 hours after birth compared to wildtype pups ($p = 0.002$, Fig. 1C). Later these *Tlr4*-/- pups exhibited a 3.1-fold higher rate of postnatal death within 24 hours of birth ($p < 0.001$, Fig. 3.1D). This then leads to a 35% decrease in viability of the *Tlr4*-/- pups at weaning age, compared to control mice ($p = 0.018$, Fig. 3.1E). These observations demonstrate that TLR4 is essential for normal on-time birth in mice, and in the absence of TLR4, litter size at birth and pup viability in the postnatal period are reduced.

3.3 THE EFFECT OF TLR4 DEFICIENCY ON THE PRO-INFLAMMATORY AND ANTI-INFLAMMATORY CYTOKINE GENES IN PLACENTA, FETAL MEMBRANE AND FETAL HEAD

To examine the effect of TLR4 deficiency on expression of pro-inflammatory and anti-inflammatory cytokines known to be induced in the parturition cascade in mice, *Tlr4*-/- BALB/c females and wildtype control females were mated to males of the same genotype, then placenta, fetal membranes and fetal head were recovered at time points over the course of late gestation on gd 16.5, 17.5, 18.5 or 19.5. The fetal head was harvested to determine if TLR4 also affects the inflammation in the fetal brain. Using RT-PCR, the expression of pro-inflammatory cytokine genes including *Il1b*, *Il6*, *Il12b, Tnfa* and *Il17a* as well anti-inflammatory cytokine gene *Il10* and the Treg cell transcription factor *Foxp3* was quantified. The *Il17a* and *Foxp3* genes were not quantified in the fetal head, as T cells were not expected to be abundant in the tissues.

In the placenta from the wildtype BALB/c group, pro-inflammatory *Il1b, Il6, Tnf* and *Il17a* mRNA expression were all increased from gd 16.5 to gd 19.5 (all $p < 0.05$, Fig. 3.2A-3.2E). The placenta collected from *Tlr4*-/- mice showed a difference in mean expression levels of several cytokines, including 50% lower *Tnf* at gd 16.5 (Fig. 2D), 31% lower *Il1b* at gd 18.5 (Fig. 3.2A), and 46% lower *Il6* at gd 19.5 (Fig. 3.2B) compared to wildtype (all $p < 0.05$). Although *Il12b* did

not increase in either group towards the end of gestation, expression declined in *Tlr4*-/- mice at gd 16.5 and 18.5 (both $p < 0.05$, Fig. 3.2C).

In fetal membranes from control mice, expression of *Il1b* and *Il6* increased dramatically on gd 19.5 ($p < 0.05$, Fig. 3.2F, 3.2G). Induction of *Il1b*, *Il6* and *Tnf* failed to occur in *Tlr4*-/- mice, with expression at gd 19.5 reduced by 60-85% compared with wildtype control mice ($p < 0.05$, Fig. 3.2F, 3.2 G, 3.2I). In contrast, there was a reduction in *Il12b* and *Il17a* from gd 16.5 to gd 19.5 in wildtype females ($p < 0.05$, Fig. 3.2H, 3.2J). Nevertheless, at gd 16.5 *Il17a* expression was elevated in this control group compared with the *Tlr4*-/- group ($p < 0.05$, Fig. 3.2C). In the fetal head of *Tlr4*-/- females, the expression of *Il1b*, *Il6*, *Il12b* and *Tnf* were lower than the controls at gd 18.5, which were not different to gd 16.5 (all $p < 0.05$, Fig. 3.2K-3.2N).

For the anti-inflammatory cytokine *Il10*, levels in wildtype control mice increased from gd 16.5 to gd 18.5 in placenta and fetal head ($p < 0.05$, Fig. 3.3A, 3.3E), with a similar trend in fetal membrane, from gd 16.5 to 19.5 ($p < 0.1$, Fig. 3.3C). The induction of *Il10* was perturbed in *Tlr4*-/- mice at gd 18.5 in placenta and fetal head and at gd 17.5 and 19.5 in fetal membrane ($p < 0.05$, Fig. 3.3A-E*).* Despite a reduction in *Foxp3* from gd 16.5 to gd 18.5 in fetal membrane of wildtype mice ($p < 0.05$, Fig. 3.3B, 3.3D), elevated expression was observed on gd 16.5 in the control fetal membrane as well as placenta compared to *Tlr4*-/- mice ($p < 0.05$, Fig. 3.3B, 3.3D).

3.4 THE EFFECT OF TLR4 DEFICIENCY ON THE PRO-INFLAMMATORY AND ANTI-INFLAMMATORY CYTOKINE GENES IN DECIDUA AND MYOMETRIUM

To examine the effect of TLR4 deficiency on pro-inflammatory and anti-inflammatory cytokine expression in the maternal compartment of the gestational tissues, uterine decidua and uterine myometrium were also analysed by RT-PCR. In the decidua of wildtype control mice there was an increase in *Il1b* expression at gd 19.5 compared to gd 16.5 ($p < 0.05$, Fig. 3.4A) and a trend towards increase in expression of *Il6* and *Il12b* ($p < 0.1$, Fig. 3.4B, 3.4C*).* In *Tlr4*-/- mice there was a significant decrease in *Il17a* expression on gd 19.5 ($p<0.05$, Fig. 3.4E) relative to control mice. Only a trend towards a similar decrease in *Il1b* and *Il12b* was observed on gd 19.5 in decidua of *Tlr4*-/- mice, compared to wildtype mice ($p < 0.1$, Fig. 3.4A, 3.4C).

In the myometrium of wildtype control mice, *Il1b* and *Il6* mRNA expression increased from gd 16.5 to gd 19.5 ($p<0.05$, Fig. 3.4F, 3.4G), but no differences were evident between control and *Tlr4*-/- females at any time points over the course of late gestation (Fig. 3.4F, 3.4G). *Il12b, Tnf* and *Il17a* were not differentially expressed in the myometrium across the later stages of gestation, irrespective of genotype (Fig. 3.4H-I).

In decidua collected from wildtype control mice, anti-inflammatory *Il10* mRNA expression progressively increased before reaching a peak at gd 19.5 ($p < 0.05$, Fig. 3.5A). A trend towards a similar elevation in *Foxp3* was also observed as gestation progressed in the control decidua ($p < 0.1$, Fig. 3.5B). In *Tlr4*-/- mice there was significantly lower *Il10* at gd 19.5 in decidua ($p = 0.009$, Fig. 3.5A) compared with control mice. Conversely, *Foxp3* expression was clearly elevated in *Tlr4*-/- mice at gd 16.5 in both decidua and myometrium relative to wildtype mice ($p < 0.05$, Fig. 3.5B, 3.5D*)*.

3.5 THE EFFECT OF TLR4 DEFICIENCY ON THE UTERINE ACTIVATION AND PROSTAGLANDIN PATHWAY GENES IN DECIDUA AND MYOMETRIUM

To examine the effect of TLR4 deficiency on the uterine activation cascade, decidua and myometrium were analysed in control wildtype and *Tlr4*-/- mice by RT-PCR for expression of uterine activation genes and prostaglandin pathway genes. In decidua of control and Tlr4-/- mice, *Ptgfr* mRNA expression progressively increased over late gestation, peaking at gd 19.5. Mean expression in *Tlr4*-/- mice was 71% and 39% less at gd 17.5 and 18.5 respectively ($p < 0.05$, Fig. 3.6A), before comparable expression at gd 19.5, suggesting delayed induction. In the myometrium *Ptgfr* surged at gd 19.5 in both control and *Tlr4*-/- mice, with a trend to reduced expression compared to controls ($p = 0.096$, Fig. 3.6E).

Expression of *Oxtr* progressively increased in both the decidua and myometrium, reaching a maximum at gd 19.5 ($p < 0.05$, Fig. *3.6C*). Although *Oxtr* reached comparable levels in the decidua and myometrium of control and *Tlr4*-/- mice at gd 19.5, it was reduced by 40%-83% in both tissues in *Tlr4*-/- mice, indicating delayed induction (all $p < 0.05$, Fig. 3.6C, 3.6D). Myometrial *Gja1* was reduced by 40% in *Tlr4*-/- mice at day 18.5 ($p = 0.011$), while decidual *Gja1* was not different between control and *Tlr4*-/- mice (Fig. 3.6D, 3.6H). The delay in labour in *Il6-/-* mice is accompanied by a delay in the expression of only *Ptger4* mRNA, but not *Ptger2* or *Ptger3* mRNA in the uterus on gd 18 (73). TLR4 activation mediates the synthesis of inflammatory cytokines including IL6 (169) which upregulates *Ptger4* as one of the uterine activation genes (73). Thus, it is possible that TLR4 deficiency would also reduce *Ptger4* expression. In our study, *Ptger4* expression was investigated as in the previous study; however, it was not differentially expressed between these two groups of mice across the later stages of gestation (Fig. 3.6B, 3.6F).

In wildtype control mice the expected increase in uterine prostaglandin synthase *Ptghs1* expression was seen in late gestation in the myometrium ($p < 0.05$, Fig. 3.7C) only but not decidua as the expression did not change throughout late gestation (Fig. 3.7A). Similarly, *Ptghs2* expression in decidua (Fig. 3.7B) and myometrium (Fig. 3.7D) of control females also did not alter as gestation reached term. *Ptghs1* was not differentially expressed between the two genotypes at any time point (Fig. 3.7A, 3.7 C) in either maternal tissues. In the decidua there was a trend towards lower *Ptghs2* expression in *Tlr4*-/- females at 17.5 and gd 19.5 relative to control females ($p < 0.1$, Fig. 3.7B), while in myometrium *Ptghs2* expression was comparable between genotypes at time points other than at gd 16.5, when expression was 32% higher in *Tlr4-/-* mice ($p < 0.05$, Fig. 3.7D).

Figure 3.1 The effect of TLR4 deficiency on activation of normal term labour and perinatal viability

Tlr4-/- BALB/c or wildtype BALB/c mice were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5 and from gd 18.0 the females were monitored to record the gestational length (A) and the number of viable pups born (B). 12-24 hours after birth the pups were weighed (C) and the survival of the pups were recorded to determine the percentage of pups lost in the first 7 days (D) as well as viable pups per litter at weaning (E). Data are shown as mean± SEM, with the number of dams or pups per group is given in parentheses. (A-C,E) were analysed by ANOVA and SIdak t test to determine the effect of genotype. Categorical data (D) were compared by χ^2 analysis. Different letters (a,b) indicate statistical significance ($p \leq 0.05$) between genotypes.

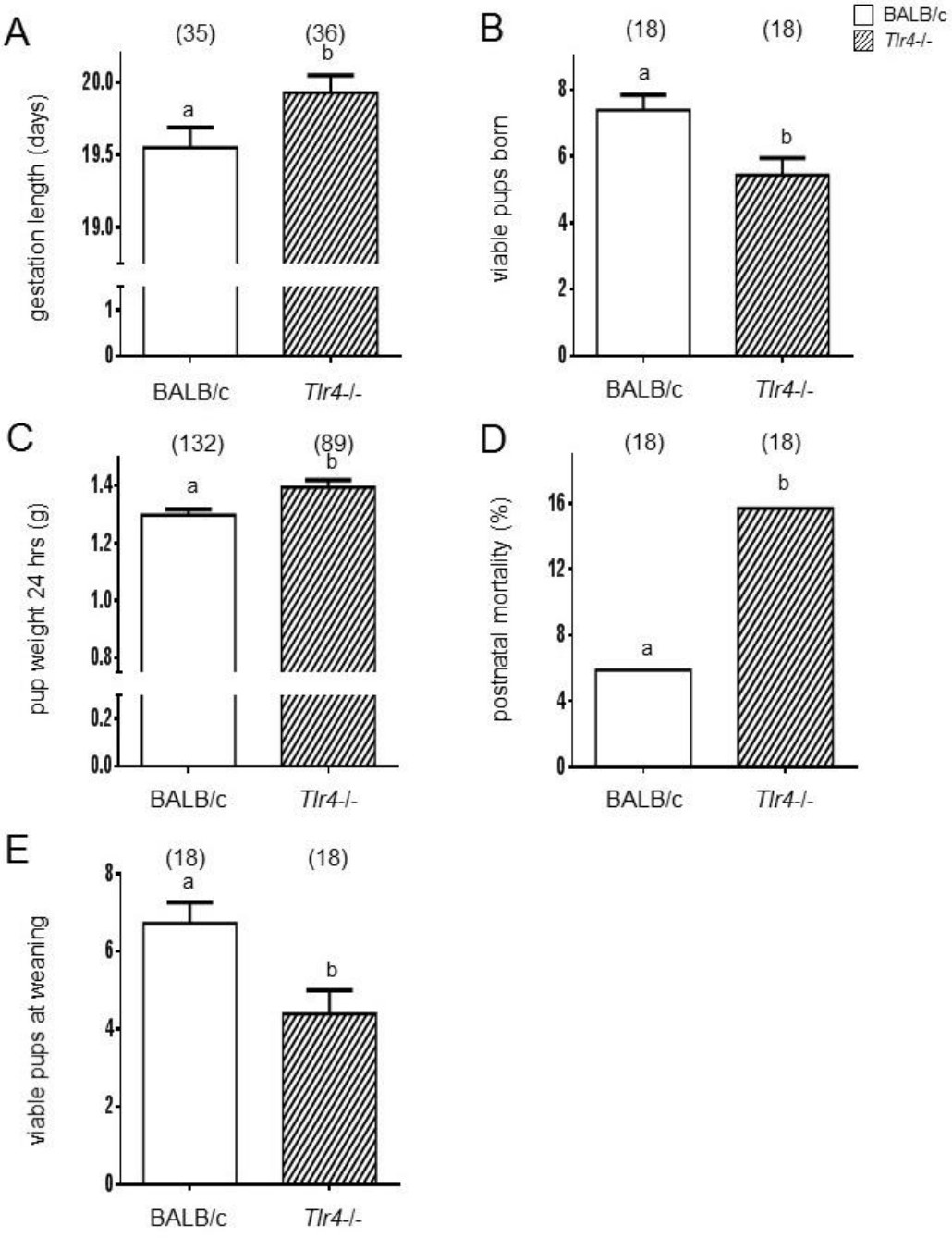
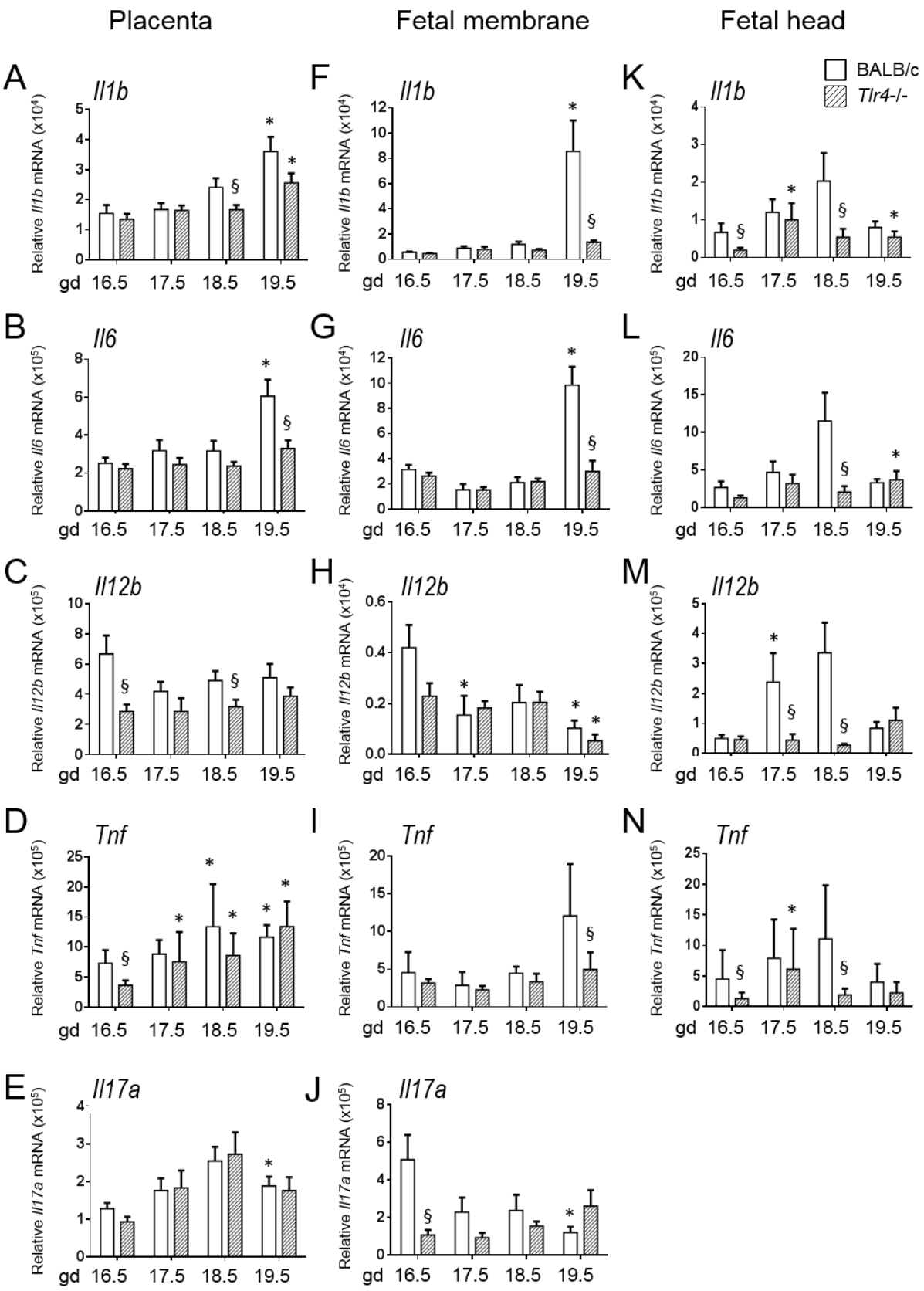

A
(35)
(36)
b
a
20.0
19.5
19.0
1
0
gestation length (days)
BALB/c
Tlr4-/-
B
(18)
(18)
BALB/c
Tlr4-/-
a
b
8
6
4
2
0
viable pups born
BALB/c
Tlr4-/-
C
(132)
(89)
b
a
1.4
1.2
1.0
0.8
0.2
0.0
pup weight 24 hrs (g)
BALB/c
Tlr4-/-
D
(18)
(18)
b
16
12
8
4
0
a
postnatal mortality (%)
BALB/c
Tlr4-/-
E
(18)
(18)
a
b
8
6
4
2
0
viable pups at weaning
BALB/c
Tlr4-/-

Figure 3.2 The effect of TLR4 deficiency on late gestation induction of pro-inflammatory cytokine gene expression in fetal tissues including placenta, fetal membrane and fetal head

Tlr4-/- BALB/c (hatched bars ▨) or wildtype BALB/c (white bars □) mice were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5, females were sacrificed at gd 16.5, gd17.5, gd 18.5 or gd 19.5 and placenta, fetal membrane as well as fetal head were harvested. Using RT-PCR, relative expression of *Il1b, Il6, Il12b, Tnf* and *Il17a* mRNA were quantified in each tissue and normalised to *Actb*. The relative expression of *Il17a* was not determined in the fetal head. Data are shown as mean± SEM relative gene expression in tissue pooled from two implantation sites, with n= 6-10 dams/group. Data were analysed using Kruskal-Wallis and the non-parametric Mann-Whitney U-test to determine the effect of genotype and the time of gestation. § indicates statistical significance ($p \leq 0.05$) in relative to the wildtype at equivalent time point. * indicates statistical significance ($p \leq 0.05$) compared with gd 16.5 for the same genotype.


Placenta
Fetal membrane
Fetal head
BALB/c
Tlr4-/-
A
Il1b
Relative Il1b mRNA (x10⁴)
F
Il1b
Relative Il1b mRNA (x10⁴)
K
Il1b
Relative Il1b mRNA (x10⁴)
B
Il6
Relative Il6 mRNA (x10⁵)
G
Il6
Relative Il6 mRNA (x10⁴)
L
Il6
Relative Il6 mRNA (x10⁵)
C
Il12b
Relative Il12b mRNA (x10⁵)
H
Il12b
Relative Il12b mRNA (x10⁴)
M
Il12b
Relative Il12b mRNA (x10⁵)
D
Tnf
Relative Tnf mRNA (x10⁵)
I
Tnf
Relative Tnf mRNA (x10⁵)
N
Tnf
Relative Tnf mRNA (x10⁵)
E
Il17a
Relative Il17a mRNA (x10⁵)
J
Il17a
Relative Il17a mRNA (x10⁵)
gd 16.5 17.5 18.5 19.5
§
*

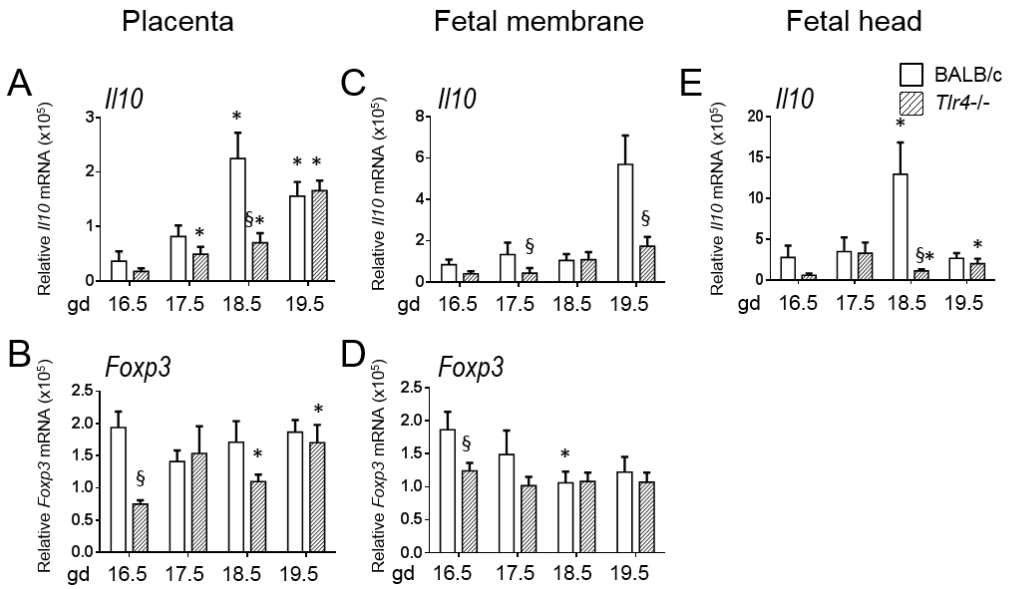
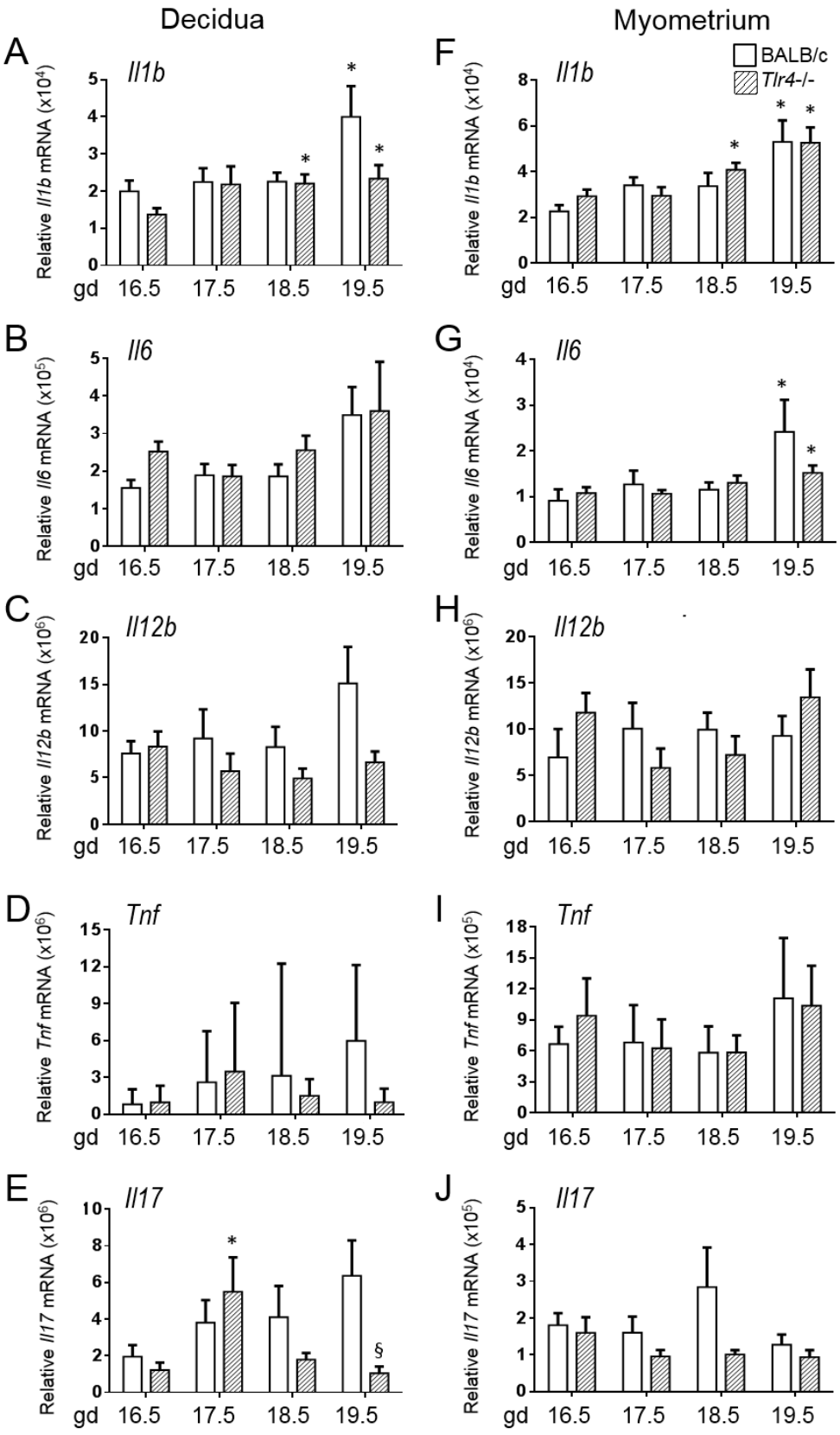


Figure 3.3 The effect of TLR4 deficiency on late gestation induction of anti-inflammatory cytokine gene expression in fetal tissues including placenta, fetal membrane and fetal head

Tlr4-/- BALB/c (hatched bars ▨) or wildtype BALB/c (white bars □) mice were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5, females were sacrificed at gd 16.5, gd17.5, gd 18.5 or gd 19.5 and placenta, fetal membrane as well as fetal head were harvested. Using RT-PCR, relative expression of *Il10* and *Foxp3* mRNA were quantified in each tissue and normalised to *Actb*. The relative expression of *Foxp3* was not determined in the fetal head. Data are shown as mean± SEM relative gene expression in tissue pooled from two implantation sites, with n= 6-10 dams/group. Data were analysed using Kruskal-Wallis and the non-parametric Mann-Whitney U-test to determine the effect of genotype and the time of gestation. § indicates statistical significance ($p \leq 0.05$) in relative to the wildtype at equivalent time point. * indicates statistical significance ($p \leq 0.05$) compared with gd 16.5 for the same genotype.

Figure 3.4 The effect of TLR4 deficiency on late gestation induction of pro-inflammatory cytokine gene expression in uterine decidua and uterine myometrium

Tlr4-/- BALB/c (hatched bars ▨) or wildtype BALB/c (white bars □) mice were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5, females were sacrificed at gd 16.5, gd17.5, gd 18.5 or gd 19.5 and decidua as well as myometrium were harvested. Using RT-PCR, relative expression of *Il1b, Il6, Il12b, Tnf* and *Il17a* mRNA were quantified in each tissue and normalised to *Actb*. Data are shown as mean± SEM relative gene expression in tissues pooled from two implantation sites with n= 6-10 dams/group. Data were analysed using Kruskal-Wallis and the non-parametric Mann-Whitney U-test to determine the effect of genotype and the time of gestation. § indicates statistical significance ($p \leq 0.05$) in relative to the wildtype at equivalent time point. * indicates statistical significance ($p \leq 0.05$) compared with gd 16.5 for the same genotype.


Decidua
Myometrium
BALB/c
Tlr4-/-
A
Il1b
Relative Il1b mRNA (x10⁴)
F
Il1b
Relative Il1b mRNA (x10⁴)
B
Il6
Relative Il6 mRNA (x10⁵)
G
Il6
Relative Il6 mRNA (x10⁴)
C
Il12b
Relative Il12b mRNA (x10⁶)
H
Il12b
Relative Il12b mRNA (x10⁶)
D
Tnf
Relative Tnf mRNA (x10⁶)
I
Tnf
Relative Tnf mRNA (x10⁵)
E
Il17
Relative Il17 mRNA (x10⁶)
J
Il17
Relative Il17 mRNA (x10⁵)
gd 16.5 17.5 18.5 19.5
*
§

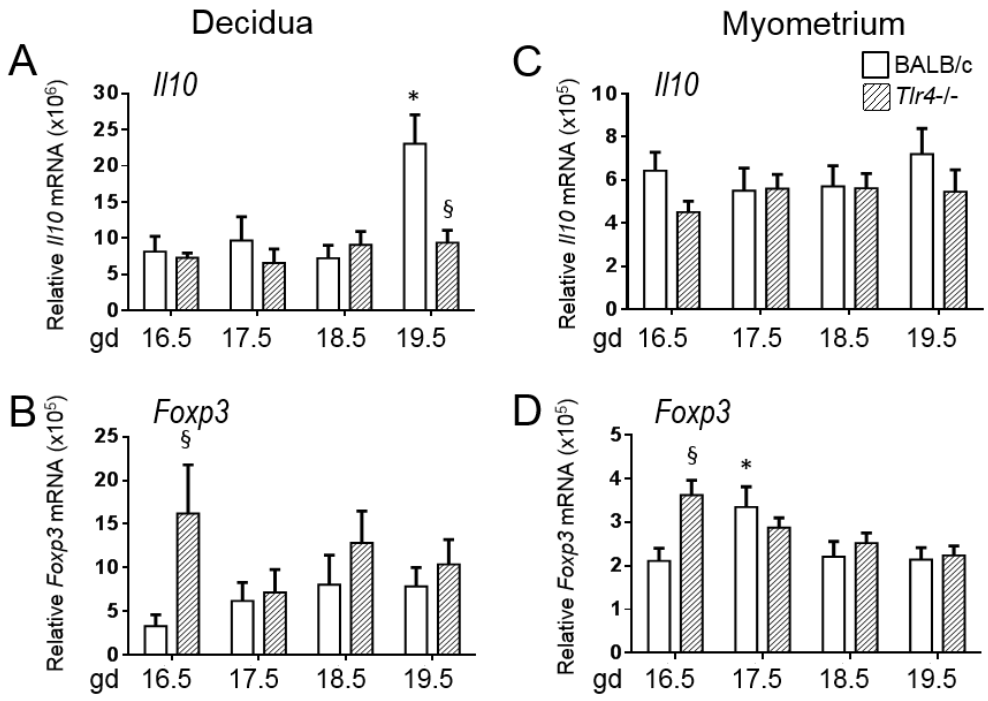
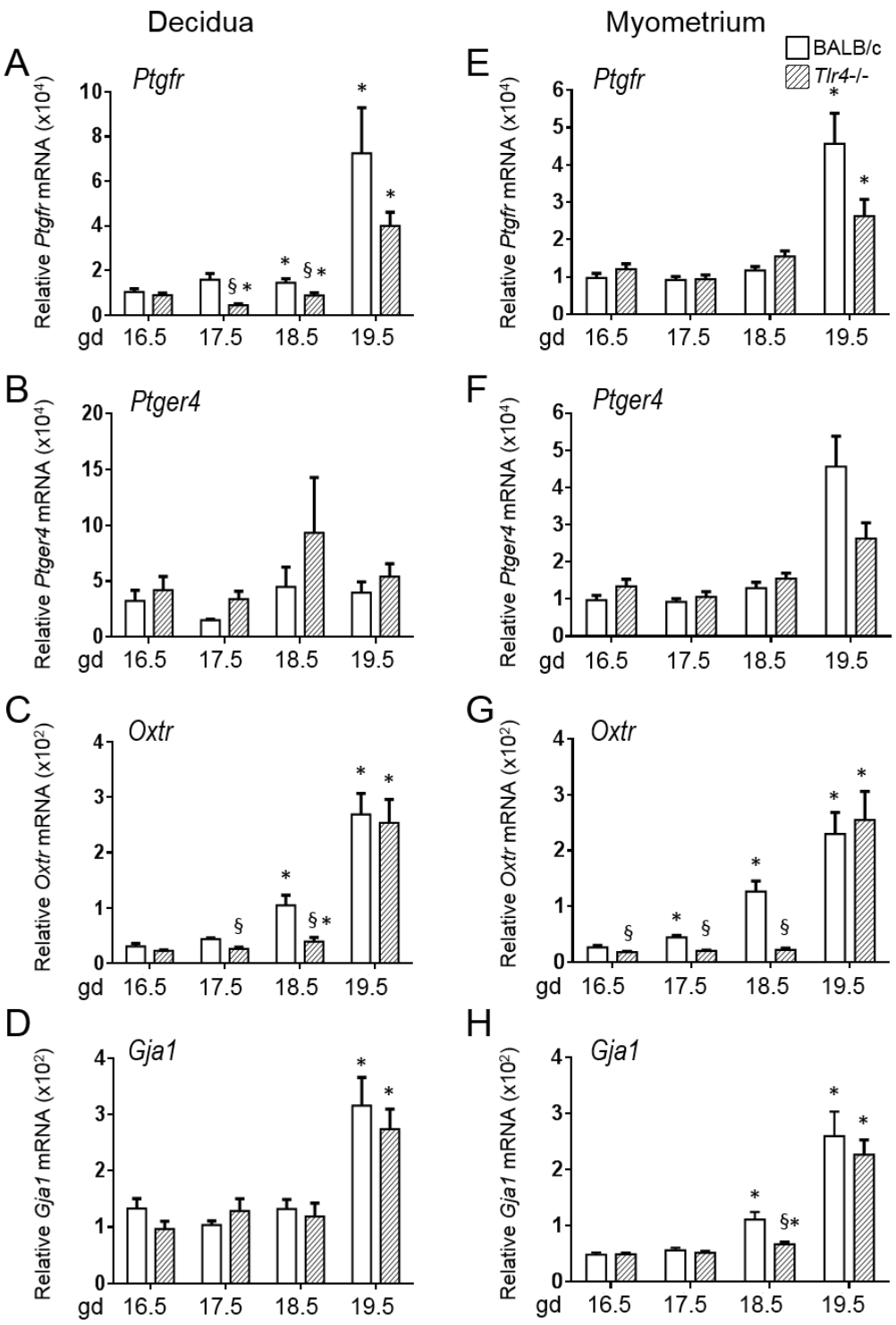


Figure 3.5 The effect of TLR4 deficiency on late gestation induction of anti-inflammatory cytokine gene expression in uterine decidua and uterine myometrium

Tlr4-/- BALB/c (hatched bars ▨) or wildtype BALB/c (white bars ☐) mice were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5, females were sacrificed at gd 16.5, gd17.5, gd 18.5 or gd 19.5 and decidua as well as myometrium were harvested. Using RT-PCR, relative expression of *Il10* and *Foxp3* mRNA were quantified in each tissue and normalised to *Actb*. Data are shown as mean± SEM relative gene expression in tissue pooled from two implantation sites, with n= 6-10 dams/group. Data were analysed using Kruskal-Wallis and the non-parametric Mann-Whitney U-test to determine the effect of genotype and the time of gestation. § indicates statistical significance ($p \leq 0.05$) in relative to the wildtype at equivalent time point. * indicates statistical significance ($p \leq 0.05$) compared with gd 16.5 for the same genotype.

Figure 3.6 The effect of TLR4 deficiency on late gestation induction of uterine activation gene expression in uterine decidua and uterine myometrium

Tlr4-/- BALB/c (hatched bars ▨) or wildtype BALB/c (white bars ☐) mice were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5, females were sacrificed at gd 16.5, gd17.5, gd 18.5 or gd 19.5 and decidua as well as myometrium were harvested. Using RT-PCR, relative expression of *Ptgfr, Ptger4, Oxtr* and *Gja1* mRNA were quantified in each tissue and normalised to *Actb*. Data are shown as mean± SEM relative gene expression in tissue pooled from two implantation sites, with n= 6-10 dams/group. Data were analysed using Kruskal-Wallis and the non-parametric Mann-Whitney U-test to determine the effect of genotype and the time of gestation. § indicates statistical significance ($p \leq 0.05$) in relative to the wildtype at equivalent time point. * indicates statistical significance ($p \leq 0.05$) compared with gd 16.5 for the same genotype.


Decidua
Myometrium
BALB/c
Tlr4-/-
A
Ptgfr
Relative Ptgfr mRNA (x10⁴)
E
Ptgfr
Relative Ptgfr mRNA (x10⁴)
B
Ptger4
Relative Ptger4 mRNA (x10⁴)
F
Ptger4
Relative Ptger4 mRNA (x10⁴)
C
Oxtr
Relative Oxtr mRNA (x10²)
G
Oxtr
Relative Oxtr mRNA (x10²)
D
Gja1
Relative Gja1 mRNA (x10²)
H
Gja1
Relative Gja1 mRNA (x10²)
gd 16.5 17.5 18.5 19.5

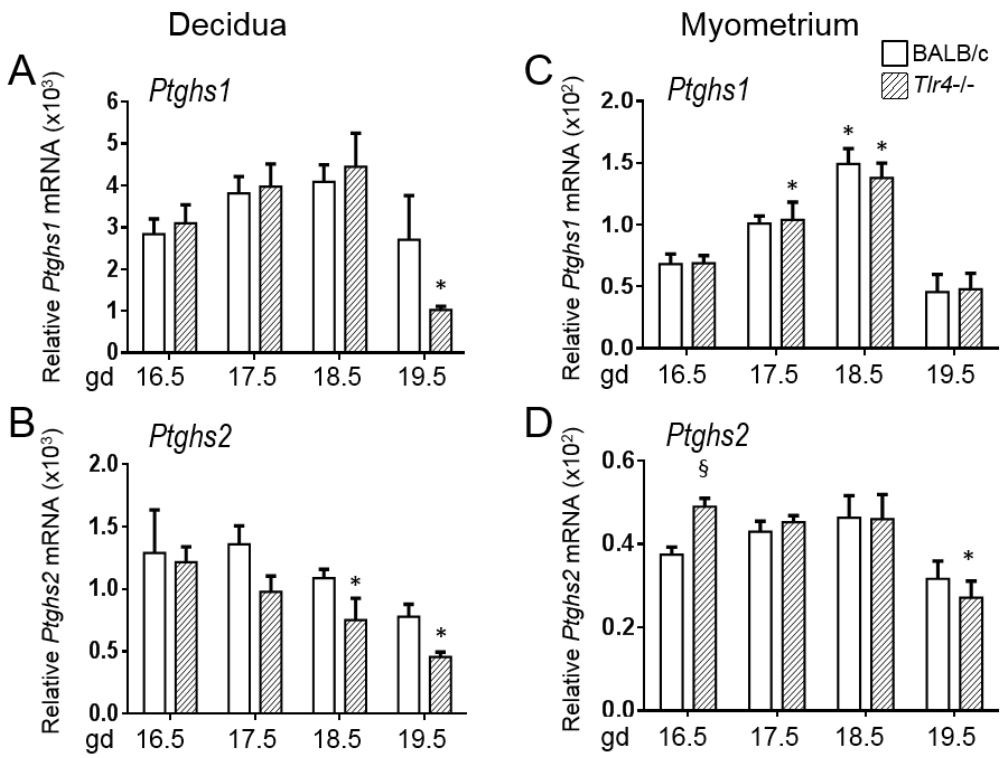


Figure 3.7 The effect of TLR4 deficiency on late gestation induction of prostaglandin H synthase gene expression in uterine decidua and uterine myometrium

Tlr4-/- BALB/c (hatched bars ▨) or wildtype BALB/c (white bars □) mice were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5, females were sacrificed at gd 16.5, gd17.5, gd 18.5 or gd 19.5 and decidua as well as myometrium were harvested. Using RT-PCR, relative expression of *Ptghs1* and *Ptghs2* mRNA were quantified in each tissue and normalised to *Actb*. Data are shown as mean± SEM relative gene expression in tissue pooled from two implantation sites, with n= 6-10 dams/group. Data were analysed using Kruskal-Wallis and the non-parametric Mann-Whitney U-test to determine the effect of genotype and the time of gestation. § indicates statistical significance ($p \leq 0.05$) in relative to the wildtype at equivalent time point. * indicates statistical significance ($p \leq 0.05$) compared with gd 16.5 for the same genotype.

3.6 DISCUSSION

The mechanisms of parturition require activation to be induced by inflammatory mediators, including inflammatory cytokines which elicit specific response in the reproductive and gestational tissues (59). TLR4 is expressed by immune cells and structural resident cells in the reproductive compartments including the cervix, uterus, fetal membrane, placenta and amniotic fluid of humans and mice (156-167,372). Most importantly, expression of *Tlr4* mRNA and abundance of TLR4 protein has been demonstrated to be elevated during labour in human fetal membrane as well as towards the end of gestation in the uterus and cervix of mice (61,166).

Here we found that in the sterile inflammation associated with term labour, genetic TLR4 deficiency leads to a 13-hour delay in labour and affects the survival of the pups born, indicating that TLR4 impacts the timing of labour as well as perinatal outcomes. The expression of inflammatory cytokine genes including *Il1b*, *Il6*, *Il12b* and *Tnf*, *Il17a, Il10* and the transcription factor *Foxp3* in the gestational tissues harvested from TLR4-deficient pregnancies is reduced, accompanied by a delay in the expected upregulation of expression of uterine activation genes including *Ptgfr*, *Oxtr* and *Gja1.* These results emphasize that in mice, TLR4 influences the inflammatory response that regulates the timing of birth in a healthy, infection-free pregnancy.

The delay in *Tlr4*-/- mice is also accompanied by an increase in pup weight 24 hours after they were born suggesting that the fetuses continue to grow in the uterus along the normal trajectory. It is also possible that this observation is due to an impact of TLR4 signalling on growth of the fetus in utero, leading to greater size at birth. Previously, pups that were delivered by *Tlr2*-/- mice which also manifest a delay in labour are also heavier at birth (71). Unfortunately other perinatal outcomes were not further investigated in the *Tlr2*-/- study (71). In our study, the reduced viability of the pups from *Tlr4*-/- mice could be attributed to loss of pups in late gestation and at the time of birth as opposed to earlier in gestation. This is because there was no significant difference in overall implantation rate or viable fetuses at gd 18.5 between *Tlr4*-/- mice and the wildtype females. When the viability of the pups delivered by *Tlr4*-/- females were further tracked, it was observed that they continue to have higher postnatal mortality. This suggests that as well as significantly extending the timing of normal term labour, TLR4 activation at the end of gestation has physiological roles in fetal development in late gestation and at the time of birth that promote postnatal viability. We have reported for the first time that there is a downregulation in both pro-inflammatory and anti-inflammatory cytokines in fetal brain of *Tlr4-/-* females on gd 18.5. Overall, TLR4 may have influenced the maturation of fetal brain; however, further study is required to

investigate this potential physiological role of TLR4 in late gestation, which could in turn affect the development and survival of the fetuses.

A previous study in CD-1 wildtype mice showed elevation of *Il1b* and *Tnf* towards the end of gestation in decidua (86). We showed a similar increase in *Il1b*, *Il6,* and *Il10* expression from gd 16.5 to gd 19.5 in the myometrium and decidua harvested from wildtype BALB/c females. Most importantly, in the fetal tissues including placenta and fetal membrane, we demonstrated for the first time that there was an elevation in inflammatory cytokine genes including *Il1b, Il6, Tnf, Il17a* and *Il10* as gestation reaches the end. Thus as well as in maternal tissues, inflammation genes are also induced in the fetal tissues to mediate the inflammation that accompanies the onset of labour. It also indicates that there is an upregulation of anti-inflammatory cytokines to control the inflammation to form a balance in inflammatory regulation in these gestational tissues of wildtype females,

This induction in both pro-inflammatory and anti-inflammatory cytokine genes observed in fetal tissues was found to depend on activation by TLR4, as a lower degree of induction of *Il1b*, *Il6, Il12b, Il17a* and *Tnf* was observed in *Tlr4-/-* placenta and fetal membranes in the later stages of gestation. A failure in the induction of anti-inflammatory cytokine *Il10* and Treg cell transcription factor *Foxp3* were also demonstrated throughout the course of late gestation in these fetal tissues of *Tlr4-/-* females. Thus, the normal induction of anti-inflammatory mediators in these fetal tissues of wildtype females doesn't occur to the same extent in the *Tlr4-/-* females.

In decidua TLR4 deficiency resulted in low *Il17a* and an increase in *Foxp3* which suggests that TLR4 deficiency may alter the Th17/ Treg balance. Expression levels of pro-inflammatory cytokine genes including *Il1b*, *Il6, Il12b* and *Tnf* were not as substantially affected by TLR4 deficiency in maternal tissues as compared to the fetal tissues in late gestation. Similar results were described in *Tlr2-/-* females where there was no change in the level of *Il1b* and *Il6* mRNA level in relative to the wildtype females in the myometrium at gd 18.5 (71). They observed a significant decrease in *Il1b* expressed by amniotic fluid macrophages isolated from *Tlr2-/-* females at gd 18.5 (71). In addition, elevation of *Tnf, Il1b, Il6* and *Il12b* mRNA occurred during labour in both myometrium and decidua in mice (86,87). During postpartum, there is an increase in IL1B, both at gene and protein level in decidua (86). This evidence gives an understanding that perhaps these inflammatory cytokines are physiologically more important in the labouring and post-partum phases.

Overall, this can be interpreted as evidence that the first phase of TLR4 signalling required to initiate the parturition cascade occurs in the fetal membranes and placenta, as opposed to the maternal compartment. We therefore postulate that TLR4 activation and stimulation of inflammatory cytokines in the fetal tissues can have a role in responding to the elevated concentrations of PAF, SP-A and HSP70 in amniotic fluid that is observed towards the end of gestation (81,248,249,267,312,343,344). This might be an early step in activating inflammation to mediate fetal membrane rupture, supporting the hypothesis that fetal membrane senescence is the initiator of coordinated, redundant signal cascade leading to parturition (353).

Previous studies done have demonstrated that pro-inflammatory cytokines can stimulate the expression of uterine activation genes (73,121-126,370). Firstly, we have shown there is a significant elevation in *Ptgfr, Oxtr* and *Gja* from gd 16.5 to gd 19.5, in both the decidua and myometrial tissues of the wildtype and *Tlr4*-/- females. Similarly, these *Ptgfr, Oxtr* and *Gja* mRNA also increase in the uterus of mice, accompanied by a higher level of $PGF_{2\alpha}$ on gd 19.3 when compared to gd 16 (87,91). Human studies are consistent with these observations with PTGFR and GJA levels increased in the lower and upper segment myometrium of women at term with labour, compared to term women with no labour (118,119). In addition, elevated expression of *OXTR* mRNA was reported with the onset of term labour in women (120).

Importantly, we have shown that TLR4 deficiency causes a transient delay in expression of *Ptgfr* and *Oxtr* in the decidua as well as *Oxtr* and *Gja1* in the myometrium. Interestingly by gd 19.5 the expression levels of *Ptgfr, Oxtr* and *Gja1* in the *Tlr4*-/- females were comparable to wildtype females in these maternal tissues demonstrating that the delay in uterine activation gene expression is caught up in the 24 hours prior to labour. It is documented by in vitro studies that Il1B and IL6 act to stimulate the expression of *PTGFR* in humans (125) and *Oxtr* in rats (126) respectively. These observations in vitro are supported by an in vivo study in mice showing that the delay in labour in *Il6-/-* mice is accompanied by a pronounced delay in *Oxtr* expression, but little change in *Gja1* in the uterus (73). It could be that the delayed myometrial uterine activation gene expression is the consequence of reduced pro-inflammatory cytokine genes in the absence of TLR4 activation in the fetal tissues.

The extended gestational length observed in *Src-1/-2* dhet mice as well as *Spa/d*-/- mice is accompanied by a lower synthesis of contraction associated genes and proteins including GJA and OXTR in the myometrium at gd 18.5 (81). Intra-amniotic administration of PAF and SP-A in these *Src-1/-2* dhet mice on gd 17.5 also promoted an increase in myometrial uterine activation

protein on gd18.5 (81). Thus, it possible that TLR4 deficiency results in a decreased response to the secretion of PAF and SP-A from amniotic fluid in the maternal tissues as pregnancy reaches its conclusion (139), in turn causing a delay in the expression of *Ptgfr*, *Oxtr* and *Gja1.*

In this study, the level of $PGF_{2\alpha}$ was not measured; however, the expression of *Ptghs1* and *Ptghs2*, which catalyse the first step in the prostaglandin synthesis from arachidonic acid (60) was quantified in both the myometrium and decidua. The expression of *Ptghs1* increased progressively between gd 16.5 and gd 18.5, peaking at gd 18.5 in the myometrium of wildtype females. By gd 19.5, the expression of *Ptghs1* decreased significantly in the myometrium. Again, this observations are similar to a published study portraying that uterine mRNA expression of *Ptghs1* is induced from gd 15 to gd 17 and then declines by gd 20 (117). These findings stand to reason that PTGHS1-derived prostaglandins might play a crucial role in the induction of luteolysis (117). The expression of *Ptghs1* was not diminished with the absence of TLR4 in both the myometrium and decidua.

On the other hand, the expression of *Ptghs2* remained relatively constant throughout late gestation, in both the myometrium and decidua of wildtype females. This is consistent with the observations that *Ptghs2* mRNA expression in the uterus was not different from gd 15 to gd 17 and was only upregulated on gd 19.3 or during parturition of wildtype females (91,117). PTGHS2-derived prostaglandins might facilitate the mechanisms of uterine contraction during parturition in mice. It is noticeable that there is a trend towards a decline in *Ptghs2* in the decidua, (but not myometrium) of *Tlr4*-/-mice. The data support the interpretation that the synthesis of prostaglandin is unlikely to be affected or to contribute to the delay in labour in *Tlr4*-/- mice.

It would add strength to this study if the expression of the inflammatory cytokines and uterine activation genes in late gestation could be confirmed at the protein level using ELISA, bead array or western blot assays. In this study the cellular source of the inflammatory cytokines which are upregulated in late gestation was not identified. Using immunohistochemistry these inflammatory cytokines could be localised, either to leukocytes or different resident structural cells in the gestational tissues.

Overall, in this study we have demonstrated for the first time that activation of TLR4 in late gestation leads to upregulation of inflammatory cytokine and induction of uterine activation genes in the maternal and fetal tissues to regulate to on-time parturition. TLR4 deficiency causes a 13-hour delay in labour and affects the viability of the pups born. The expression of both pro-

inflammatory and anti-inflammatory genes including *Il1b*, *Il6*, *Il12b* and *Tnf*, *Il17a, Il10* and *Foxp3* mainly in the fetal tissues harvested from TLR4-deficient pregnancies are decreased when TLR4 is absent. This is also accompanied by a transient delay in the expression of uterine activation genes in the maternal tissues, as the mediators downstream of TLR4 activation including *Ptgfr*, *Oxtr* and *Gja1.*

Chapter 4: Effect of Genetic Deficiency in TLR4 on Leukocyte Infiltration into Gestational Tissues

4.1 INTRODUCTION

One of the mechanisms that contributes to activation of labour is the invasion of leukocytes into the gestational tissues, to promote the inflammatory process. The infiltration of pro-inflammatory leukocytes, predominantly macrophages and neutrophils into both the upper and lower segments of myometrium during (57), the decidua (129) and the cervix (58) have been demonstrated during labour in women. These leukocytes appear to infiltrate from the maternal circulation; as larger populations of neutrophils, monocytes and NK cells with higher potentials for adherence and extravasation as well as greater migratory activity are present in the peripheral blood during labour (153). Anti-inflammatory Treg cells have also been studied in humans where the percentage and suppressive activity of CD4+CD127 low+/-CD25+ Treg cells were shown to decrease during labour (154,155) potentially indicating reduced availability to suppress inflammation in gestational tissues.

Neutrophils and macrophages infiltrate human cervix as gestation approaches its end (82). In maternal circulation, elevated expression of CD11B on monocytes and granulocytes, which facilitate the adhesion of these leukocytes is observed at the end of human gestation (85). In the myometrium, decidua and cervix of mice higher percentages of macrophages have been quantified, accompanied by an increase in decidual *Il1b* and *Tnf* at the end of gestation, prior to labour (86-89).

There is also induction in *Cxcl1* and *Cxcl2* mRNA in the myometrium and decidua of mice during labour (86,87). In humans, there are limited studies to examine the expression of inflammatory chemokines in maternal tissues prior to labour.The infiltration of these leukocytes from the intravascular space into the gestational tissues is proposed to be regulated by the secretion of chemokines including CCL2 (144), CCL3 (145,146), CCL5 (147), CCL20 (105) and CXCL1 (148) which increase in human amniotic fluid during spontaneous labour at term. CCL2 is crucial to attract and activate mainly monocytes (100,101) while CCL3 is identified as a potent chemotactic agent of lymphocytes, monocytes, macrophages and eosinophils (102,103). CCL5 is involved in attracting and activating monocytes, T lymphocytes and eosinophils (103,104). CCL20 attracts immature dendritic cells, T and B cells (105), meanwhile CXCL1 is a potent neutrophil chemotactic and neutrophil-activating factors (106-108).

CXCL8, as a major chemokine studied in normal term parturition, is found to be secreted by mesenchymal cells, decidual stromal cells, cervical stromal cells as well as the glandular and surface epithelium of the cervix (59). CXCL8 palys a role in chemotaxis of leukocytes and

stimulates adhesion molecule expression and neutrophil activity (59) and is elevated in the lower segment of the myometrium, as well as the amnion, chorion and cervix during labour in women (58,59,111).

During parturition the invading leukocytes have been found to be a key source of pro-inflammatory cytokines and chemokines particularly IL1, IL6, TNF and CXCL8 which presumably amplify and accelerate feed-forward mechanisms in the myometrium and cervix (59). Resident structural cells may contribute to the majority of pro-inflammatory cytokines and chemokine secretion in the fetal membrane (59) during labour. Additionally neutrophils produce a range of MMPs thought to assist in cervical ripening (110,149) in the active phase of human delivery.

Importantly pro-inflammatory leukocytes found in the gestational tissues including dendritic cells (92), monocytes (92), macrophages (157,168), NK cells (162) and neutrophils (163) are also known to express TLR4. Montalbano et. al have revealed that the surface expression of *Tlr4* increases in amniotic fluid macrophages towards the end of pregnancy in mice (71). We hypothesise that TLR4 is crucial in activating the leukocyte recruitment that mediates parturition associated with inflammation in normal term labour. By utilising the TLR4 deficient mice we have shown that the TLR4 has an essential role in late gestation in regulating leukocyte recruitment into the fetal and maternal tissues. Given the delayed labour in TLR4 deficient mice, this implies that these leukocytes have a role in normal term parturition.

4.2 THE EFFECT OF TLR4 DEFICIENCY ON LEUKOCYTE RECRUITMENT AND ACTIVATION BEFORE TERM LABOUR

To determine whether leukocyte populations are affected by TLR4 deficiency, *Tlr4*-/- and wildtype control mice were mated to males of the same genotype. Using flow cytometry the pro-inflammatory and anti-inflammatory leukocyte populations were analysed in the decidua, myometrium, placenta, fetal membranes and PALN on gd 18.5. The percentage of macrophages, neutrophils, dendritic cells and T cells were identified as CD11B+F4/80+, CD11B+Ly6G+, CD11C+ and CD3+CD4+ cells respectively. It was a concern that changes in the proportions of these leukocytes could be masked by expansion of the total viable cells in response to TLR4 deficiency. Therefore, using counting beads, the absolute numbers of these CD11B+F4/80+, CD11B+Ly6G+, CD11C+ and CD4+ cells were also quantified. The phenotype of these leukocytes were further analysed, including of expression of MHCII by CD11B+F4/80+ macrophages, expression of CD80, CD86 and MHCII by CD11C+ dendritic cells and expression of FOXP3 and IL17RA by CD3+CD4+ T cells.

The percentage of total viable cells comprised by $F4/80^{+}CD11B^{+}$ macrophages in the fetal membranes and placenta of *Tlr4*-/- mice was reduced by 46% and 53% compared to wildtype, respectively ($p < 0.005$, Fig. 4.1A). The absolute number of macrophages however was not different between genotypes in any of the gestational tissues collected (Fig. 4.5A). In addition, the placental macrophages had a less activated phenotype, with 18% fewer macrophages expressing MHCII in the placenta of *Tlr4*-/- mice ($p <0.001$, Fig. 4.1B).

Neutrophils comprised a 33% and 39% smaller proportion of total viable cells in the placenta and myometrium, respectively, of *Tlr4*-/- mice compared with controls (both $p < 0.04$, Fig. 4.2A). Similarly the number of neutrophils was reduced in the placenta ($p < 0.05$, Fig. 4.5B), but not the myometrium (Fig. 4.5B).

The percentage of $CD11C^{+}$ dendritic cells were reduced by 63% in TLR4 deficient myometrium ($p < 0.001$) and a similar trend was seen in the decidua ($p = 0.076$, Fig. 4.3A). This was accompanied by fewer numbers of CD11C+ dendritic cells in the myometrium ($p = 0.05$, Fig. 4.5C). There was no change in the activation status of dendritic cells, as assessed by CD80, CD86 and MHCII expression, between wildtype and *Tlr4*-/- mice in all gestational tissues harvested (Fig. 4.3B).

The percentage of $CD3^{+}CD4^{+}$ T cells were 38% fewer in the placenta ($p = 0.025$) and were higher in the PALN ($p < 0.05$) of *Tlr4*-/- mice compared to control (Fig. 4.4A). The absolute number of CD3+CD4+ cells was not different between wildtype and *Tlr4*-/- females in all of the tissues collected ($p > 0.05$, Fig. 4.5D) The proportion of myometrial $CD3+CD4^{+}$ T cells

expressing the Treg cell marker CD25 and FOXP3 increased from 2.9% in control mice to 6% in *Tlr4*-/- mice (p = 0.039, Fig. 4.4B). A 1% increase in Th17 cells, as indicated by CD3+CD4+ T cells that express IL17RA, occurred in the myometrium of *Tlr4*-/- mice (p = 0.044, Fig. 4.4C).

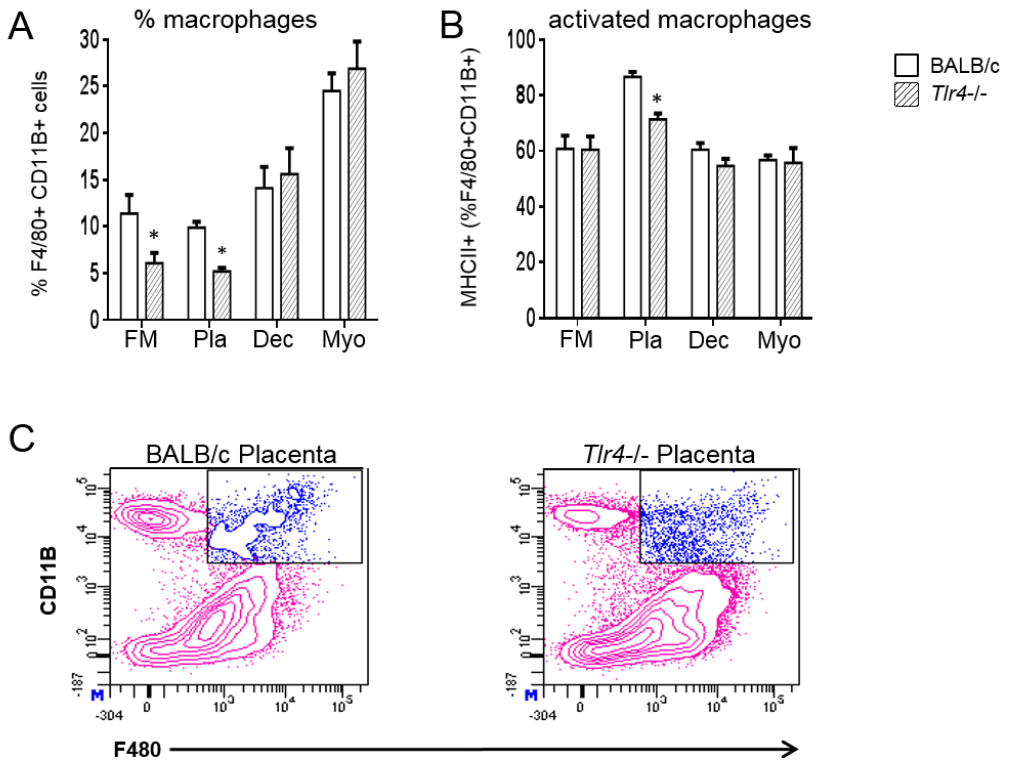


Figure 4.1 The effect of TLR4 deficiency on macrophage recruitment and activation

Tlr4-/- BALB/c (hatched bars ▨) or wildtype BALB/c (white bars □) mice were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5, females were sacrificed at gd 18.5 and gestational tissues including fetal membrane (FM), placenta (Pla), decidua (Dec) and myometrium (Myo) were harvested. Using flow cytometry, the percentage of $F4/80^{+}CD11B^{+}$ macrophages out of viable cells (A) and the expression of MHCII by $F4/80^{+}CD11B^{+}$ macrophages (activated macrophages, B) were determined. Representative dot plots were included to show the gating strategy used to identify macrophages in the placenta of both wildtype and *Tlr4-/-* BALB/c (C). Data are shown as mean± SEM cell number per implantation sites for fetal membrane, placenta and decidua, or for entire uterine myometrium, n= 6-10 dams/group. Data were analysed using Kruskal-Wallis and the non-parametric Mann-Whitney U-test to determine the difference between genotype. * indicates statistical significance ($p \leq 0.05$) in relative to wildtype for the same tissue.

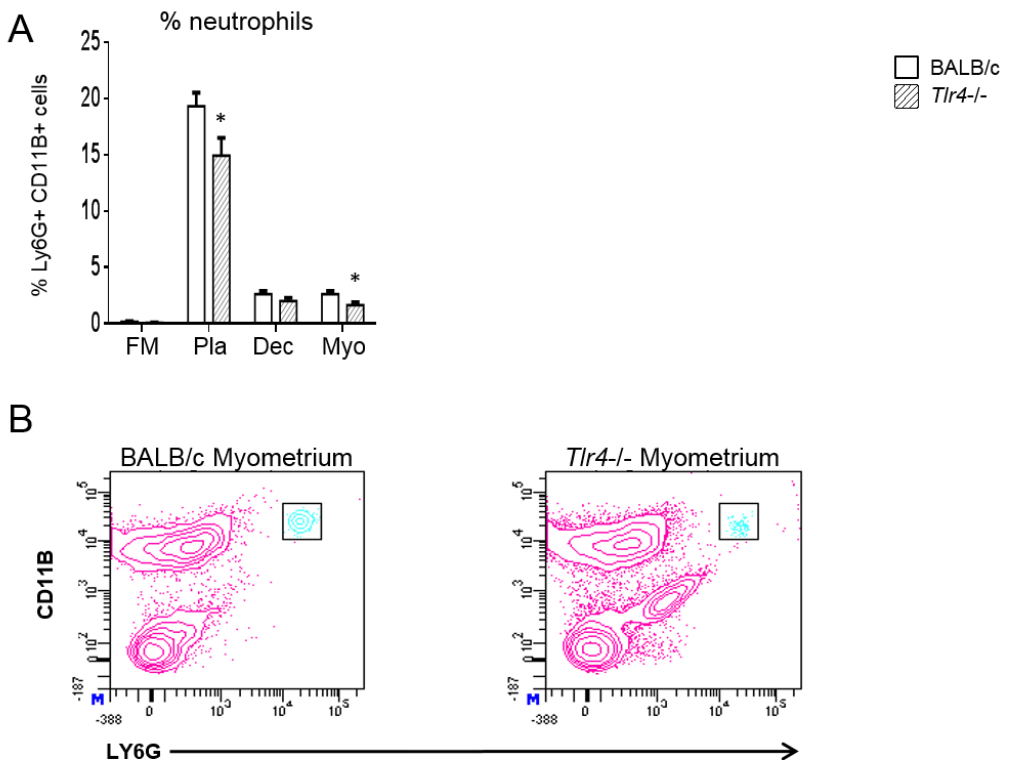


Figure 4.2 The effect of TLR4 deficiency on neutrophil recruitment

Tlr4-/- BALB/c (hatched bars ▨) or wildtype BALB/c (white bars ☐) mice were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5, females were sacrificed at gd 18.5 and gestational tissues including fetal membrane (FM), placenta (Pla), decidua (Dec) and myometrium (Myo) were harvested. Using flow cytometry, the percentage of $Ly6G^{+}CD11B^{+}$ neutrophils out of viable cells (A) was determined. Representative dot plots were included to show the gating strategy used to identify neutrophils in the myometrium of both wildtype and *Tlr4-/-* BALB/c (B). Data are shown as mean± SEM cell number per implantation sites for fetal membrane, placenta and decidua, or for entire uterine, n= 6-10 dams/group. Data were analysed using Kruskal-Wallis and the non-parametric Mann-Whitney U-test to determine the difference between genotype. * indicates statistical significance ($p \leq 0.05$) in relative to wildtype for the same tissue.

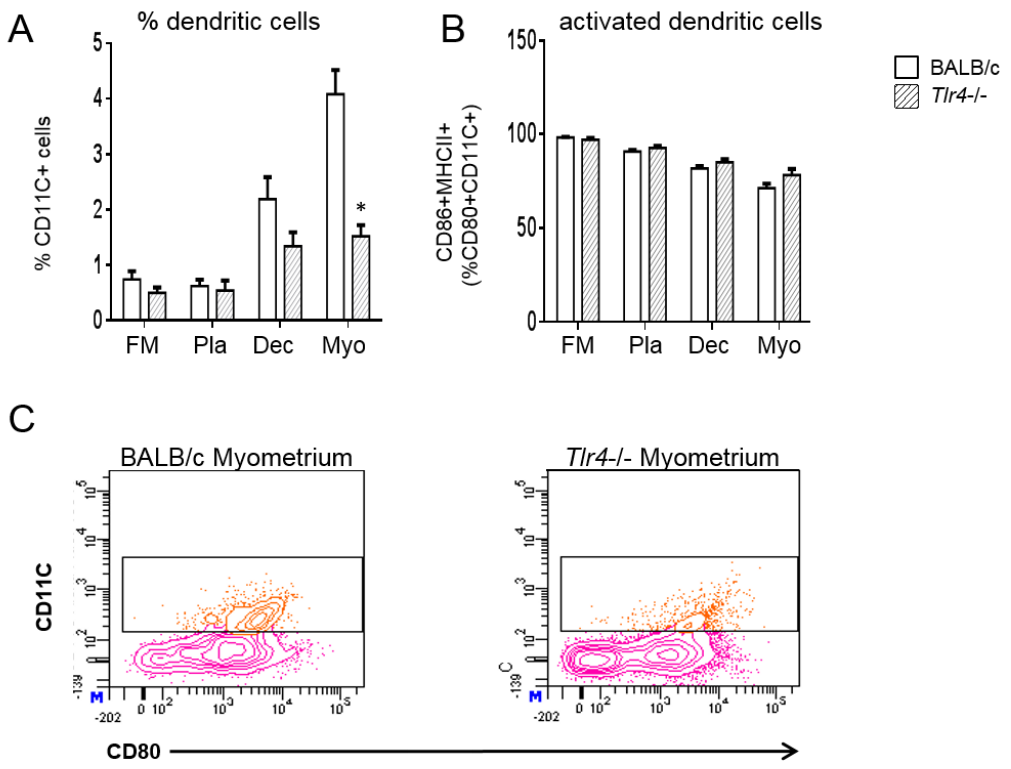


Figure 4.3 The effect of TLR4 deficiency on dendritic cell recruitment and activation

Tlr4-/- BALB/c (hatched bars ▨) or wildtype BALB/c (white bars ☐) mice were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5, females were sacrificed at gd 18.5 and gestational tissues including fetal membrane (FM), placenta (Pla), decidua (Dec) and myometrium (Myo) were harvested. Using flow cytometry, the percentage of $CD11C^+$ dendritic cells out of viable cells (A) and expression of CD80, CD86 and MHCII by $CD11C^+$ dendritic cells (activated dendritic cells, B) were determined. Representative dot plots were included to show the gating strategy used to identify dendritic cells in the myometrium of both wildtype and *Tlr4-/-* BALB/c (C). Data are shown as mean± SEM cell number per implantation sites for fetal membrane, placenta and decidua, or for entire uterine myometrium, n= 6-10 dams/group. Data were analysed using Kruskal-Wallis and the non-parametric Mann-Whitney U-test to determine the difference between genotype. * indicates statistical significance ($p \leq 0.05$) in relative to wildtype for the same tissue.

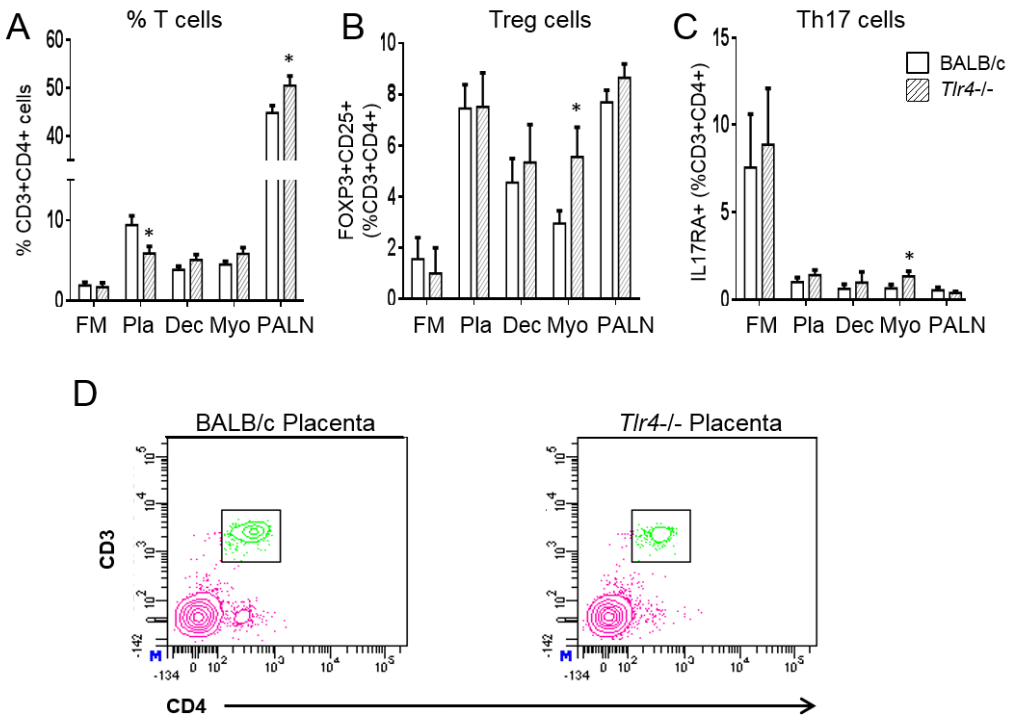


Figure 4.4 The effect of TLR4 deficiency on T cell recruitment and activation

Tlr4-/- BALB/c (hatched bars ▨) or wildtype BALB/c (white bars □) mice were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5, females were sacrificed at gd 18.5 and gestational tissues including fetal membrane (FM), placenta (Pla), decidua (Dec), myometrium (Myo) and PALN were harvested. Using flow cytometry the percentage of CD3+CD4+ T cells out of viable cells (A), expression of FOXP3 by CD3+CD4+ T cells (Treg cells, B) and expression of IL17RA by CD3+CD4+ T cells (Th17 cells, C) were determined. Representative dot plots were included to show the gating strategy used to identify T cells in the placenta of both wildtype and *Tlr4-/-* BALB/c (D). Data are shown as mean± SEM cell number per implantation sites for fetal membrane, placenta and decidua, or for entire uterine myometrium and PALN, n= 6-10 dams/group. Data were analysed using Kruskal-Wallis and the non-parametric Mann-Whitney U-test to determine the difference between genotype. * indicates statistical significance ($p \leq 0.05$) in relative to wildtype for the same tissue.

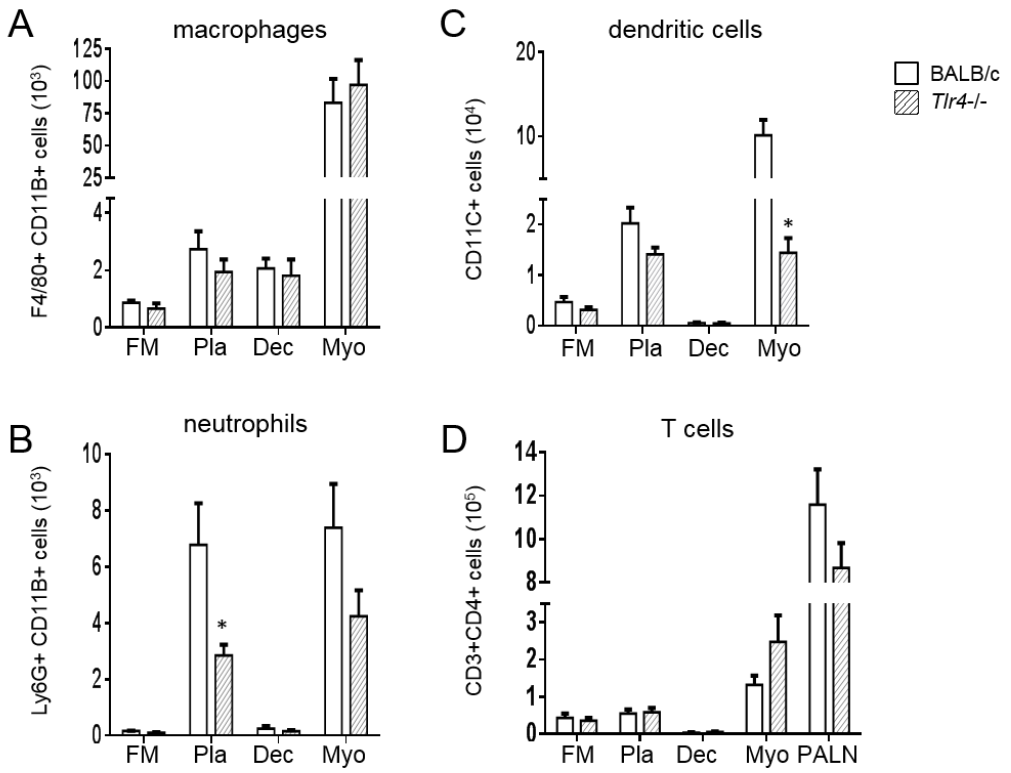


Figure 4.5 The effect of TLR4 deficiency on absolute number of leukocytes

Tlr4-/- BALB/c (hatched bars ▨) or wildtype BALB/c (white bars □) mice were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5, females were sacrificed at gd 18.5 and gestational tissues including fetal membrane (FM), placenta (Pla), decidua (Dec), myometrium (Myo) and PALN were harvested. Count beads were used in order to determine the absolute number of $F4/80^{+}CD11B^{+}$ macrophages (A), $Ly6G^{+}CD11B^{+}$ neutrophils (B), $CD11C^{+}$ dendritic cells (C) and $CD4^{+}$ T cells (D). Data are shown as mean± SEM cell number per implantation sites for fetal membrane, placenta and decidua, or for entire uterine myometrium and PALN, n= 6-10 dams/group. Data were analysed using Kruskal-Wallis and the non-parametric Mann-Whitney U-test to determine the difference between genotype. * indicates statistical significance ($p \leq 0.05$) in relative to wildtype for the same tissue.

4.3 DISCUSSION

It is well established from both human and mouse studies that as gestation approaches its end, there is increased invasion of pro-inflammatory leukocytes including macrophages and neutrophils (57,58,86-89) into gestational tissues to facilitate the mechanisms of parturition. Most importantl, TLR4 has been documented to be expressed by the pro-inflammatory leukocytes such as dendritic cells (92), monocytes (92), macrophages (157,168), NK cells (162) and neutrophils (163) that were found in gestational tissues of both human and mouse. At the end of pregnancy in mice, the surface expression of TLR4 also increases in amniotic fluid macrophages (71). We have shown in this study that in mice, TLR4 deficiency is associated with a decline in the population of neutrophils and dendritic cells in the gestational tissues harvested on gd 18.5. This evidence indicates that in mice, TLR4 influences one of the key mechanisms of inflammation-leukocyte infiltration into gestational tissues.

Genetic deficiency in TLR4 causes a reduced leukocyte response to infectious and non-infectious inflammatory stimuli in a variety of pathogenic settings (169,373). Similarly, the blockade of TLR4 using antibody also cause an abrogation in LPS induced leukocytes in the blood as well as placenta (66). In our study, the percentage and number of CD11B+Ly6G+ neutrophils were observed to decline in the placenta on gd 18.5 of *Tlr4-/-* females. As demonstrated in Chapter 3, TLR4 deficient females displayed a decrease in the expression of placental *Il1b*, which plays important role in the adhesion and migration of neutrophils (83). During inflammation neutrophils either spontaneously or following stimulation play a role in secreting the chemokines that in turn cause infiltration of the macrophages, dendritic cells and T cells to the inflammation site (374). The percentage, but not the number of CD11B+F4/80+ macrophages and CD3+CD4+ T cells was declined in the placenta of *Tlr4-/-* females, suggesting that the total viable cells were increased, but not the absolute number of macrophages and T cells. Likewise TLR4 deficiency did not affect the percentage and number of CD11C+ dendritic cells in the placenta of *Tlr4-/-* mice.

Thus, TLR4 deficiency resulted in reduced neutrophils, to in turn potentially reduce the migration of other leukocytes into the placenta on gd 19 or gd 19.5 instead of gd 18.5. Furthermore, an elevation in macrophages is demonstrated in the human placenta at labour (95). These observations support our results in Chapter 3 showing that TLR4 deficiency causes decreased *Il1b*, *Il6*, and *Tnf* mRNA expression, potentially from a reduced invasion and activation of macrophages and dendritic cells on gd 19.5. It is likely that these cytokines also play roles in

adhesion and migration of more leukocytes into the placenta. The results also suggest that TLR4 signalling potentially mediates the resident structural cell induction of *Il1b* and *Il12b* mRNA in the placenta on gd 18.5 that we observed in Chapter 3.

The percentage and number of neutrophils were not affected by TLR4 deficiency which may explain why the percentage but not the number of macrophages was reduced in the fetal membrane. Similarly it implies that the total number of macrophages was not different; however, they were lower in density as the total viable non-leukocytic cells were increased in TLR4 deficient mice. The populations of dendritic cells and T cells were also not reduced in the fetal membrane in the absence of TLR4. These observations are consistent with a study demonstrating that there is no increase in invasion of leukocytes into fetal membrane during human labour (58). It is possible that these results may suggest that resident structural cells play a crucial role in the TLR4 mediated induction of pro-inflammatory cytokine expression including *Il1b*, *Il6*, and *Tnf* on gd 19.5 in fetal membrane as shown in Chapter 3, to initiate the mechanism of labour.

Previous studies in mice have reported increased infiltration of Neu7/4+Ly6G+ neutrophils into the myometrium in the postpartum period, but not before (87) In the decidua there was an increase in the percentage of Neu7/4+Ly6G+ neutrophils during labour as well as the postpartum period (86). A recent human study showed that decidual neutrophils are increased in women with infection-driven preterm delivery but not on-time labour (375). Most importantly, the deletion of neutrophils using antibody against Ly6G in mice neither changed gestation length (376) nor protects against LPS-triggered preterm delivery (189). Based on this evidence, it seems that neutrophils may have an important role during postpartum repair, rather than in facilitating delivery. Therefore, it seems unlikely that neutrophil-dependent mechanisms account for the delayed birth in *Tlr4-/-* mice.

In our study, it was evident that the percentage of neutrophils was reduced, accompanied by only a trend towards a decline in the total number of neutrophils in the myometrium of the *Tlr4*-/- mice on gd 18.5. Likewise in the decidua, the percentage and number of neutrophils were not seen to decline in *Tlr4*-/- mice. Thus, TLR4 deficiency has no effect on the infiltration of macrophages in the myometrium and decidua either on gd 18.5. This is consistent with the observation that TLR4 deficiency did not reduce the expression of pro-inflammatory cytokines *Il1b*, *Il6*, and *Tnf* as portrayed in Chapter 3, given that these cytokines can be induced by activated macrophages in the myometrium.

Increased migration of macrophages into maternal tissues is implicated towards the end of gestation and during labour-associated inflammation in the maternal tissues, instead of during the postpartum period. Using immunohistochemistry a higher number of F4/80+ macrophages was observed in the uterus of mice on gd 15 compared to non-pregnant controls, and then decreased during the postpartum period (88). Other studies in mice showed that an elevated number of F4/80+ Neu7/4- macrophages amongst total CD45+ cells were detected using flow cytometry on gd 18.5 when compared to gd 15, which then decreased in the postpartum period in the myometrium and decidua (86,87). Macrophages were also upregulated in human decidua during labour (375). Thus, macrophages are implicated in labour-associated inflammation in the maternal tissues, instead of during the postpartum period. At the same time the delay in labour in *Tlr2-/-* mice was accompanied by a decline in *F4/80* mRNA in the myometrium when compared to wildtype mice on gd 18.5 (71). This would also possibly explain why we do not see any effect of TLR4 deficiency on the population macrophages in the myometrium and decidua. It could be that instead of TLR4, mediates the infiltration of macrophages into these maternal tissues.

In the myometrial compartment the impact of TLR4 deficiency was to substantially reduce both the percentage and number of dendritic cells. Previous in vitro experiments have demonstrated that HSP70 activation of TLR4 causes secretion of chemokines from tumour cells for chemoattraction of dendritic cells (377). Towards the end of gestation, a higher level of HSP70 and HMGB1 may be secreted from the myometrium as a result of cell stress following the increase in uterine stretch (221-223), to potentially cause TLR4-mediated induction of chemokines for the increased invasion of dendritic cells into the myometrium. HMGB1 and HSP70 engagement of TLR4 induces maturation and activation of dendritic cells (373,377-379), although we did not find evidence of altered CD11C+CD80+CD86+MHCII+ dendritic cells. Other co-stimulatory molecules including MHCI, CD40 and CD83 were not examined in this study. These markers are also shown to be important markers indicating the maturation of dendritic cells (378).

A pathway by which matured dendritic cells may progress inflammation to accelerate labour is through reversing T cell-mediated immune suppression, to reinforce the direct effects of TLR4 on CD4+ T cells (380). Ligation of TLR4 on the surface of dendritic cells can abrogate the anti-inflammatory and pro-tolerance functions of Treg cells (381). Treg cells have been reported to lose potency in their suppressive function in late gestation prior to labour (382). Failure of dendritic cells to remove and silence Treg cells in the absence of TLR4 signalling may explain the elevated percentage of Treg cells we observed in the myometrium of *Tlr4-/-* mice.

This is consistent with the elevation in *Foxp3* mRNA in the myometrium of *Tlr4-/-* females, observed in Chapter 3. Thus, excessive Treg cells would be expected to quell progression of inflammation and so may contribute to delayed delivery. TLR4 signalling can influence the production of IL17 and other cytokines in CD3+CD4+ T cells (383), consistent with the increased expression of IL17RA we observed in myometrial CD3+CD4+T cells. *Tlr4-/-* mice also displayed an increase in the percentage, but not the number of T cells in the PALN, suggesting that T cells trafficking from uterine draining lymph nodes into the uterus is not affected by the TLR4 deficiency at the end of gestation.

Towards the end of gestation the surface expression of TLR4 increased on the amniotic fluid macrophages (71), and the secretion of inflammatory chemokines increased in human amniotic fluid during labour (105,144-148). Therefore, it would be beneficial to investigate the role of TLR4 in activating the leukocyte recruitment into the amniotic fluid. Also there is evidence in mice that the blockade of TLR4 reduces the percentage of LPS-induced NK cells in blood and placenta (66). In the *Jα18-/-* mouse model which are deficient in invariant NK cells, mice were resistant to LPS-induced preterm delivery (78). This evidence demonstrates the role of TLR4 in recruiting NK cells, which have been shown to be important in the infection-induced preterm delivery in mice. Hence, it would be interesting to include NK cells in this study, to determine if NK cells contribute to the delayed birth in *Tlr4-/-* mice. This is especially relevant because neutrophils also play a role in secreting the chemokines for the infiltration of NK cells to the inflammation sites (374).

Neutrophils and cytokines contribute to the increased level of MMPs presumed to assist in the mechanisms of labour, including uterine contraction, cervical ripening and fetal membrane rupture (110,111,113,141,149). Thus, it would be relevant to examine the effect of TLR4 deficiency on the expression of MMP genes especially in the myometrium and fetal membrane. Furthermore, in both humans and mice, chemokines are upregulated to mediate the increased infiltration of inflammatory leukocytes into the gestational tissues during labour (86,87,105,144-148). It would also be reasonable to study the effect of TLR4 on the expression of pro-inflammatory chemokines in the gestational tissues across the later stages of pregnancy.

Overall, we have demonstrated that TLR4 deficiency in late gestation decreases the percentage of placental neutrophils and myometrial dendritic cells that are part of the normal inflammatory cascade leading to onset of labour. The absolute number of placental neutrophils and myometrial dendritic cells are also reduced due to the TLR4 deficiency in mice. These

changes in leukocytes may in part be a consequence of altered cytokine expression from non-leukocytic cell lineages in the gestational tissues, and conversely may also contribute to the altered cytokine production within these tissues.

Chapter 5: Effect of DAMPs and TLR4 regulators on TLR4-induced inflammation to elicit preterm delivery in mice

5.1 INTRODUCTION

In addition to pathogen-derived molecular patterns, toll-like receptors (TLRs) can also be activated by endogenous host-derived non-pathogen stimuli, known as damage-associated molecular patterns (DAMPs). As the name suggests, these molecules are released from necrotic cells as a result of tissue damage from ischemia, toxic injury, stress or trauma. Following these events, the cells loose integrity, and cause intracellular material to escape into the extracellular matrix (67). Chromatin-associated protein HMGB1 (194), uric acid (197) have all been identified as DAMPs released from necrotic cells. In addition, HMGB1 can be actively secreted from pro-inflammatory cells especially macrophages in response to infection (198-200). Most importantly, both in vivo and in vitro experiments have shown TLR4 to be one of the TLRs that HMGB1 interacts with to induce downstream pro-inflammatory responses (210,211). PAF (328,332,335) has been shown to be secreted from necroctic cells (328) as well as from pro-inflammatory cells following infection (384,385).

Several DAMPs have significant physiological roles and maintain homeostasis. For example HGMB1 has been revealed to have a protective effect against liver damage induced by hepatic reperfusion injury via inhibition of TLR4 signalling (219,220). Furthermore, both 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one play crucial physiological roles in regulation of cholesterol and steroid hormone biosynthesis (286), and uric acid acts as an antioxidant, especially in the plasma (276). SP as one of the TLR4 regulators (345,348-351) is also important for breathing as it reduces surface tension within the lung (81). It is also critical for fetal lung maturation when towards the end of a normal pregnancy, higher levels of SP and PAF are found in the fetal lung and amniotic fluid in both human and mice (81,312,343,344). In contrast high level of HMGB1, 5-cholesten-3β, 25-diol, 5-cholesten-3β-ol-7-one, uric acid, PAF and SP contribute to the pathogenesis of many chronic inflammatory and autoimmune diseases such as rheumatoid arthritis, asthma, endotoxin shock, diabetes, acute allergic reactions, thrombosis, ischemic bowel necrosis and inflammatory airway disease (224,287-289,305,386-390). In all these conditions, their effects may be mediated via activation of TLR4 (169).

An elevation in HMGB1 and PAF levels in amniotic fluid and serum of women has been associated with preterm delivery (273,329,330). In pregnancy inflammation mediated by rHMGB1 (275), rSP-A (344) and cPAF (331) elicits preterm birth in mice. Nevertheless, the efficiency of other DAMPs including 5-cholesten-3β, 25-diol, 5-cholesten-3β-ol-7-one, uric acid

and rSP-D in inducing preterm delivery as well as the role of TLR4 in preterm birth mediated by DAMPs and TLR4 regulators in mice has not been investigated.

To assist in selection of DAMPs and TLR4 regulators to further study in in vivo models of preterm delivery, firstly examined whether rHMGB1, 5-cholesten-3β, 25-diol, 5-cholesten-3β-ol-7-one, uric acid, cPAF and rSP-D could stimulate inflammation in the murine J774 macrophage cell line. We followed with in vivo experiments to determine the effect of DAMPs and TLR4 regulators in inducing inflammatory cytokines and chemokines in the serum of mice. From these results, we then proceeded to investigate if these DAMP and TLR4 regulators were efficient in inducing inflammation in vivo sufficient to trigger preterm birth in the BALB/c strain of mice. Finally, we have utilised TLR4-deficient mice as well as the small molecule TLR4 antagonist (+)-naltrexone, to study the role of TLR4 in preterm delivery induce by DAMPs and TLR4 regulators, as well as the mechanism of any early onset of parturition including inflammatory cytokine and uterine activation gene expression.

5.2 THE EFFECT OF DAMPs AND TLR4 REGULATORS ON INFLAMMATORY CYTOKINE AND CHEMOKINE SECRETION FROM THE J774 MACROPHAGE CELL LINE

From previous studies and our observations, macrophages are shown to present in the gestational tissues including myometrium, decidua, placenta, fetal membrane and amniotic fluid (16, 36-37). This is why the J774 macrophage cell line was used to study the inflammatory effects of these DAMPs and TLR4 regulators in vitro before proceeding to establish if these endogenous ligands also have similar inflammatory effects in vivo in BALB/c mice. TNF is the main cytokine produced by macrophages especially in response to inflammatory stimuli. In vitro studies have shown that TNF is produced by freshly isolated macrophages, as well as macrophage cell lines or primary cells derived from gestational tissues following stimulation with 5-cholesten-3β, 25-diol, 5-cholesten-3β-ol-7-one, uric acid, rHMGB1 or cPAF (274,298,391-395). Therefore, we aimed to investigate whether exposure of J774 macrophages to the various DAMPs and TLR4 regulators could alter TNF secretion by these cells. Before starting these studies, it was critical to establish that any inflammatory effects of these DAMPs and TLR4 regulators were not due to endotoxin contamination. This was achieved using the LAL assay. Each DAMPs and TLR4 regulators preparation was demonstrated to contain a very low level of endotoxin, insufficient to induce inflammation in vitro or in vivo as shown in Table 2.4 in Chapter 2, Materials and Methods.

Macrophages were incubated for 12 hours with increasing concentrations of 5-cholesten-3β, 25-diol, uric acid, 5-cholesten-3β-ol-7-one, rSP-D, rHMGB1 and cPAF or a media alone control. The preliminary in vitro data demonstrated that TNF was induced in J774 macrophages following treatment with 1 μg/mL of LPS. Both 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one were revealed to promote inflammatory cytokines and chemokines after 12 hours of incubation in primary trophoblast cells (298). Whilst many of the DAMPs and TLR4 regulators showed the ability to induce inflammatory changes much earlier than 12 hours post-treatment in immune cells (332,391,392), the 12 hour time point was selected as the best time to observe activity across all DAMPs and TLR4 regulators tested in this study. The dose range has been used as previously (283,298,332,348,391,394,395). In all in vitro experiments, LPS treatment was included as positive control to confirm the J774 macrophages responded to exogenous TLR4 ligand as expected.

Following treatment, the supernatants were harvested and the approximate number of viable cells remaining were quantified using Presto Blue (as described in Chapter 2, 3.9), in order to investigate whether treatment with individual DAMPs, TLR4 regulators or LPS affected the proliferation and/or viability of the cells, compared to the medium control. This important step was performed so that we could confirm that any change in production of TNF (or other cytokines) was a result of the macrophages responding to the DAMPs or TLR4 regulators and not due to reduced cell number because of toxicity caused by DAMPs and TLR4 regulators or increased cell number due to proliferative effects. TNF concentrations in the supernatants were then quantified using TNF-specific ELISA, with concentrations expressed in pg/mL, normalised to the approximate number of cells.

LPS induced a significant TNF response in the J774 macrophages in all of the independent experiments that were carried out (all $p = 0.05$, Fig 5.1A-D). For 5-cholesten-3β, 25-diol, uric acid, 5-cholesten-3β-ol-7-one and rSP-D three independent experiments were performed, and each experiment was carried out in triplicate. As 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one were both dissolved in 100% ethanol, a 0.04% ethanol (final concentration) control was included in the appropriate experiments to ensure that ethanol did not affect the viability or the cytokine response of J774 macrophages. The ethanol control was found not to alter TNF production by macrophages, nor was it found to alter the number of viable cells after the treatments were collected, compared to the medium control (data not shown). 5-cholesten-3β, 25-diol was observed to stimulate the production of TNF in a dose-dependent manner, as a higher concentration of TNF was observed when treated with 20 μg/mL ($p = 0.05$, Fig. 5.1A)

when compared to 4 µg/mL or medium control with 0.04% ethanol (all p = 0.05, Fig. 5.1A). The 5-cholesten-3β, 25-diol at a concentration of 4 µg/mL also induced a greater increase in TNF compared to the 0.8 µg/mL or medium control (all p = 0.05, Fig. 5.1A). In contrast, only the highest concentration of uric acid tested (200 µg/mL) was found to induce TNF production, with a 2.61-fold increase observed in J774 macrophages (all p = 0.05, Fig. 5.1B), relative to control medium alone. For 5-cholesten-3β-ol-7-one and rSP-D, both failed to alter TNF production by J774 macrophages at any of the concentrations tested (all p > 0.05, Fig. 5.1C,D).

For rHMGB1 and cPAF, two independent experiments were carried out and each experiment was performed in triplicate. It appeared that rHMGB1 stimulated production of TNF in J774 cells at the highest concentration of 20 µg/mL (Fig. 5.1E). cPAF did not induce secretion of TNF from J774 macrophages at any of the concentrations tested (Fig. 5.1F). It is important to note that the same pattern of results was observed irrespective of whether TNF concentrations were examined as pg/mL or normalised to viable cell number (data not shown), reflecting that the treatments with DAMPs or TLR4 regulators did not affect the proliferation or viability of the cells. These results were also consistent across each of the individual replicate experiments, for each treatment.

To determine if any of the DAMPs induced pro-inflammatory cytokines other than TNF, the supernatants from the highest concentrations of 5-cholesten-3β, 25-diol, uric acid and rHMGB1 treatments of J774 macrophages were analysed using multiplex bead array (Luminex Assay). This allowed a broad range of inflammatory cytokines and chemokines including IL1B, IL6, IL10, IL12B, TNF, GMCSF, CXCL1, CXCL2, CCL2 and VEGF to be examined in a very small supernatant sample volume. Pooled supernatants from two independent experiments were used, with analysis revealing that in addition to TNF, incubation of J774 macrophages with 20 µg/mL 5-cholesten-3β, 25-diol seemed to also stimulate an increase in CCL2 and CXCL2, compared to the medium control (Fig 5.2A). It also appeared as an increase in the secretion of CXCL2 and TNF was evident following 12 hours of incubation with 200 µg/mL uric acid compared to the medium control (Fig 5.2B). In comparison to medium control, 20 µg/mL rHMGB1 seemed to induce synthesis of GMCSF, CXCL2 and TNF (Fig 5.2C).

Previously it has been demonstrated that intrauterine administration of cPAF is efficient in inducing preterm birth in mice (331). Given that the supernatants collected from macrophages exposed to 0.5 ug/mL of cPAF showed induction of TNF (332) in peritoneal macrophages, this concentration of cPAF was used for further cytokine and chemokine analysis using Luminex

assay. It appeared that none of the cytokines and chemokines measured showed elevated secretion from J774 cells following exposure to cPAF for 12 hours (Fig 5.2D).

5.3 THE EFFECT OF INTRAPERITONEAL DAMPs AND cPAF's INJECTION ON CYTOKINE AND CHEMOKINE LEVEL IN THE MOUSE SERUM

To investigate the effect of 5-cholesten-3β, 25-diol, uric acid, rHMGB1 and cPAF in stimulating cytokines and chemokines in vivo, wildtype BALB/c female mice were mated to wildtype male mice. On gd 16.5, pregnant females were injected intraperitoneally with 1.5-2.0 mg/mouse (50 mg/kg) 5-cholesten-3β, 25-diol, 3 mg/mouse uric acid, 50 μg rHMGB1/mouse, 1 μg/mouse cPAF, or the appropriate vehicle control, at the similar dose that had been shown to induce inflammatory cytokines in mice in vivo (391,396,397). Previous studies observed a maximal stimulation in pro-inflammatory cytokines in peritoneal lavage fluid (396) as well as serum (391) after 6 hours following intraperitoneal injections of uric acid or rHMGB1 respectively in mice. Intraperitoneal injection of cPAF was found to induce a significant increase in the production of TNF within the serum of mice as early as 1 hour after administration (397). Since uric acid and rHMGB1 were found to be more potent in inducing inflammation in J774 macrophages, 6 hours was chosen as the time point for blood collection after administration of DAMPs or cPAF. Serum was prepared (as described previously) and the level of inflammatory cytokines and chemokines was quantified by Luminex assay.

Stimulation of IL6 ($p = 0.04$, Fig. 5.3B) and CXCL1 ($p = 0.02$, Fig. 5.3B) was observed in the serum of mice, 6 hours following injection of uric acid, compared to vehicle control. Interestingly, administration of cPAF was shown to induce a significant increase in CCL2 ($p = 0.01$, Fig. 5.3D) and IL10 ($p = 0.03$, Fig. 5.3D) in the serum of mice, compared to vehicle control. No cytokine or chemokine stimulation was seen in the serum of mice following the intraperitoneal injection of rHMGB1 or 5-cholesten-3β, 25-diol, when compared to the vehicle controls (all $p > 0.05$, Fig. 5.3A,C).

5.4 THE EFFICIENCY OF INTRAPERITONEAL URIC ACID AND cPAF IN INDUCING PRETERM DELIVERY

Given the effects of cPAF and uric acid on cytokines in vivo, these ligands were studied for their effects in pregnant mice. To investigate the effect of intraperitoneal administration of uric acid and cPAF in inducing preterm delivery, BALB/c females were mated with BALB/c males and were administered 6 mg/mouse uric acid or 2 μg cPAF/mouse on gd 16.5. Mice were observed for preterm delivery, and in the absence of preterm birth mice were killed on gd 18.5 and

implantation sites, fetal viability and fetal and placental weights were determined. The fetal: placental weight ratio, which is an index of placental efficiency (367) was calculated. Intraperitoneal uric acid administration did not induce any preterm delivery and also did not affect the fetal weight, placental weight or fetal: placental weight ratio ($p > 0.05$, Table 5.1).

cPAF administration in BALB/c females elicited preterm birth in 9 out of 14 pregnant mice (64%) ($p < 0.05$, Fig. 5.4A) of pregnant mice, accompanied by a 36% reduction in percentage of viable pregnancies ($p < 0.05$, Fig. 5.4B), compared to 100% in vehicle control. There was a 27-36% decline in the number ($p < 0.001$, Fig. 5.4D) and percentage ($p < 0.001$, Fig. 5.4E) of viable fetuses per dam compared to PBS control groups. The total implantation sites per pregnant dam was not different across the treatment groups ($p > 0.05$, Fig. 5.4C), thus the differences observed in fetal number were due to late gestation fetal loss as opposed to reduced implantations. The fetal weight (Fig. 5.5A), placental weight (Fig. 5.5B) and fetal: placental weight ratio, (Fig. 5.5C) recorded from the viable fetuses undelivered on gd 18.5 were not affected by the administration of cPAF (all $p > 0.05$).Taken together, systemic administration of cPAF but uric acid was efficient in inducing preterm birth as well as causing fetal death in BALB/c mice.

5.5 THE EFFECT OF BLOCKADE OF cPAF-INDUCED PRETERM DELIVERY BY (+)-NALTREXONE OR TLR4 ABLATION IN PREVENTING INTRAPERITONEAL cPAF INDUCED PRETERM DELIVERY

To determine whether disruption in TLR4 signalling prevents cPAF induction of preterm birth, *Tlr4*-/- and BALB/c mice were mated to males of the same genotype and injected intraperitoneally with 2 µg/mouse of cPAF or vehicle on gd 16.5. *Tlr4*-/- mice were protected from preterm delivery with only 2 out of 12 mice delivering preterm, compared to 9 of 14 in wildtype BALB/c mice ($p < 0.05$, Fig. 5.4A). despite the lower preterm birth, cPAF treatment acted in *Tlr4*-/- females to cause a 58% reduction in percentage of viable pregnancies ($p < 0.05$, Fig. 5.4B) and 58% fewer viable fetuses per dam ($p = 0.02$ Fig. 5.5E) in comparison to PBS-treated *Tlr4*-/- females. This mirrored the significant fetal loss observed in pregnant wildtype BALB/c females given cPAF. *Tlr4*-/- females administered with cPAF exhibited lower fetal weight ($p = 0.031$, Fig. 5.5A), compared to the *Tlr4*-/- females given vehicle, while placental weight was not affected ($p > 0.05$, Fig. 5.5A). The administration of cPAF resulted in a lower fetal: placental weight ratio, which is an index of placental efficiency ($p = 0.013$, Fig. 5.5C) in *Tlr4*-/- females in comparison to wildtype BALB/c females. The fetal: placental weight ratio ($p < 0.001$, Fig. 5.5C) also declined significantly in *Tlr4*-/- females in comparison to BALB/c females given PBS, suggesting these mice had a reduced placental efficiency regardless of cPAF treatment.

The role of TLR4 ligation in driving cPAF-induced preterm birth was further studied by utilising the TLR4 antagonist (+)-naltrexone. Pregnant BALB/c females were administered 2 μg/mouse of cPAF or vehicle intraperitoneally on gd 16.5 together with four doses of (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg) on gd 16.5, 17.0, 17.5 and 18.0. Administration of (+)-naltrexone rescued mice from cPAF-induced preterm delivery, with none of 14 mice experiencing preterm birth ($p < 0.05$, Fig. 5.4A). The percentage of viable pregnancies (Fig. 5.4 B) as well as both the number (Fig. 5.4D) and percentage (Fig. 5.4E) of viable fetuses per dam were also comparable to PBS-treated or (+)-naltrexone-only treated control groups (all $p > 0.05$), indicating (+)-naltrexone treatment prevented cPAF-induced fetal loss. Placental weight (Fig. 5.5B) and fetal: placental weight ratio (Fig. 5.5C) from the wildtype females injected with both cPAF and (+)-naltrexone were not significantly different to either naltrexone-treated or cPAF-treated wildtype control females (all $p > 0.05$).

Overall TLR4 deficiency partially protected mice from cPAF driven preterm delivery although it did not prevent fetal loss or growth restriction in surviving fetuses. (+)-naltrexone protected mice from having preterm delivery as well as fetal death when challenged with cPAF systemically.

5.6 THE EFFECT OF BLOCKADE OF cPAF-INDUCED PRETERM DELIVERY BY (+)-NALTREXONE IN PREVENTING INTRAUTERINE cPAF INDUCED PRETERM DELIVERY

The efficiency of intrauterine administration of cPAF in inducing preterm birth was then investigated, whereby BALB/c females were mated with BALB/c males and injected with 35 μg of cPAF or vehicle intrauterine on gd 16.5 before the perinatal outcomes were recorded on gd 18.5. Preterm birth occurred in 4 out of 13 of pregnant wildtype mice (31%) ($p < 0.05$, Fig. 5.6A) when administered with intrauterine cPAF, accompanied by a 62% reduction in percentage of viable pregnancies ($p < 0.05$, Fig. 5.6B), compared to vehicle control. The number ($p < 0.001$, Fig. 5.6D) and percentage ($p < 0.001$, Fig.5.6E) of viable fetuses per dam also declined by 36-40% compared to PBS control. The total implantation sites per dam was not significantly different across the groups, thus the difference observed was attributed to the late gestation fetal loss instead of reduced implantations ($p > 0.05$, Fig. 5.6C). The fetal weight ($p = 0.004$, Fig. 5.7A) and fetal: placental weight ratio ($p = 0.01$, Fig. 5.7C) were lower in the BALB/c female group administered with cPAF compared to vehicle control.

Using the TLR4 antagonist (+)-naltrexone, the role of TLR4 ligation on the responsiveness of mice to intrauterine cPAF-induced preterm birth was further investigated. BALB/c mice were mated to BALB/c males and injected intrauterine with 35 µg of cPAF or vehicle control on gd 16.5 together with (+)-naltrexone at 60 mg/kg on gd 16.5, 17.0, 17.5 and 18.0. Female mice treated with both cPAF and (+)-naltrexone showed a comparable percentage of viable pregnancies (Fig. 5.6B) as well as both the number (Fig. 5.6D) and percentage (Fig. 5.6E) of viable fetuses per dam relative to PBS-treated or (+)-naltrexone-treated control groups (all $p > 0.05$). The fetuses from BALB/c females administered both cPAF and (+)-naltrexone were significantly smaller ($p= 0.001$, Fig. 5.7A), compared to the (+)-naltrexone-only treated control group, and were similar to the cPAF treated females ($p > 0.05$, Fig. 5.7A). The fetal: placental weight ratio (Fig. 5.7C) was similar to cPAF-treated and (+)-naltrexone-treated control groups (all $p > 0.05$).

Overall, the local administration of cPAF directly into uterine tissues also induced preterm delivery and fetal death, as well as growth restriction and poor placental function in surviving fetuses. The administration of (+)-naltrexone rescued cPAF- induced fetal loss, but not the cPAF induced growth restriction of the surviving fetuses.

5.7 THE EFFECT OF BLOCKADE OF cPAF-INDUCED PRETERM DELIVERY BY (+)-NALTREXONE OR TLR4 ABLATION ON cPAF INDUCTION OF THE INFLAMMATORY CYTOKINE GENES IN DECIDUA AND MYOMETRIUM

To define the cytokine expression response to intraperitoneal administration of cPAF, pregnant BALB/c females were given 2 µg/mouse of cPAF or vehicle control on gd 16.5. After 4 hours, the decidua and myometrium were harvested for mRNA expression analysis.

In response to cPAF injection there was a 1.5 to 11.8-fold elevation in *Il1b* ($p = 0.001$, Fig. 5.8A), *Il6* ($p < 0.001$, Fig. 5.8B) and *Il10* ($p < 0.001$, Fig. 5.9A) mRNA in the decidua of wildtype females compared to PBS control. Similarly in the myometrium, a 3.0 to 9.4-fold induction in *Il1b* ($p = 0.002$, Fig. 5.8F), *Il6* ($p = 0.007$, Fig. 5.8G) and *Il10* ($p = 0.02$, Fig. 5.9A) mRNA was observed 4 hours after cPAF administration to the BALB/c females in comparison to vehicle control. Other pro-inflammatory cytokines including *Il12b, Tnf* and *Il1a* failed to be upregulated in response to cPAF administration in decidua (Fig. 5.8C-E) and myometrium (Fig. 5.8H-J), when compared to controls (all $p > 0.05$).

To determine the TLR4-dependent cytokine response to cPAF in preterm birth, pregnant BALB/c or *Tlr4*-/- females were administered 2 µg/mouse of cPAF intraperitoneally, with an additional group of BALB/c mice treated with (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg).

After 4 hours, the decidua and myometrium were harvested for mRNA expression analysis. The expression of *Il1b* was suppressed by 68% in the decidua of BALB/c females treated with cPAF and (+)-naltrexone ($p = 0.012$, Fig. 5.8A), compared to cPAF alone. Similarly, in response to cPAF and (+)-naltrexone treatment in wildtype females, a lack of induction in *Il6* ($p = 0.024$, Fig. 5.8B) and *Il10* mRNA ($p = 0.03$, Fig. 5.9A) was evident in the decidua relative to administration of cPAF alone in the BALB/c females and expression levels were similar to those observed in the (+)-naltrexone-treated control group.

In addition, *Tlr4*-/- females treated with cPAF ($p = 0.005$, Fig. 5.8A) had 49% reduced decidual *Il1b* expression relative to wildtype females administered cPAF alone, similar to that observed in *Tlr4*-/- females that received vehicle control ($p > 0.05$, Fig. 5.8A). The upregulation of *Il6* (Fig. 5.8B) and *Il10* (Fig. 5.9A) was not suppressed in the decidua of *Tlr4*-/- females 4 hours after treatment, as the level of expression was similar to cPAF treated BALB/c females (both $p > 0.05$). In the decidua of BALB/c females treated with cPAF and (+)-naltrexone as well as *Tlr4*-/- females which received cPAF, the expression of *Il12b, Tnf* and *Il1a* was not differentially expressed in comparison to BALB/c females challenged with cPAF (all $p > 0.05$, Fig. 5.8C-E).

In myometrium collected from the BALB/c females administered with cPAF and (+)-naltrexone, there was no induction of *Il10* ($p < 0.02$, Fig. 5.9B) compared to wildtype females treated with cPAF alone, similar to the (+)-naltrexone treated control ($p > 0.05$, Fig. 5.9B). The induction of *Il1b* and *Il6* (Fig. 5.8F,G) in the myometrium of females administered with cPAF was not inhibited by (+)-naltrexone with similar expression of these genes compared to cPAF treated females (all $p > 0.05$). Similarly, in *Tlr4*-/- females treated with cPAF, the expression of *Il1b* (Fig. 5.8F), *Il6* (Fig. 5.8G) and *Il10* (Fig. 5.9B) failed to be suppressed as the expression of these genes was similar to wildtype females administered cPAF alone (all $p > 0.05$).

Wildtype females administered with cPAF and (+)-naltrexone and *Tlr4*-/- females treated with cPAF had similar levels of myometrial *Tnf* and *Il1a* to wildtype females challenged with cPAF or their respective vehicle control groups (all $p > 0.05$, Fig. 5.8I,J). The *Il12b* synthesis in the myometrium of BALB/c females treated with cPAF and (+)-naltrexone ($p < 0.003$, Fig. 5.8H) as well as *Tlr4*-/- females treated with cPAF ($p < 0.034$, Fig. 5.8H) was suppressed when compared to the group of BALB/c females treated with cPAF alone. cPAF alone did not induce *Il12b* expression , thus these observations are difficult to interpret.

In summary, the inflammatory cytokines *Il1b, Il6 and Il10* were induced in the maternal tissues of wildtype females when challenged with cPAF. The administration of (+)-naltrexone suppressed the cPAF stimulation of *Il1b, Il6* and *Il10* in the decidua and *Il10* in the myometrium.

Genetic TLR4 deficiency did not affect the expression of any cytokine induced by cPAF, other than *Il1b* in the decidua.

5.8 THE EFFECT OF BLOCKADE OF cPAF-INDUCED PRETERM DELIVERY BY (+)-NALTREXONE OR TLR4 ABLATION ON cPAF INDUCTION OF THE UTERINE ACTIVATION AND PROSTAGLANDIN PATHWAY GENES IN DECIDUA AND MYOMETRIUM

To investigate uterine activation gene expression in response to intraperitoneal administration of cPAF, pregnant BALB/c females were given 2 µg/mouse of cPAF or vehicle control on gd 16.5. After 4 hours, the decidua and myometrium were harvested for mRNA expression analysis. In the decidua ($p = 0.017$, Fig. 5.10A) and myometrium ($p = 0.054$, Fig. 5.10F) of wildtype females, cPAF injection resulted in a 1.9-fold elevation in *Ptghs2* mRNA compared to PBS control. The expression of other uterine activation genes such as *Oxtr, Gja, Ptghs1* and *Ptgfr* were not stimulated 4 hours after cPAF challenge in either decidua ($p > 0.05$, Fig. 5.10B-E) or myometrium ($p > 0.05$, Fig. 5.10G-J) in comparison to the vehicle control group.

To determine the TLR4 dependent cytokine response to cPAF in preterm birth, pregnant BALB/c or *Tlr4*-/- females were administered 2 µg/mouse of cPAF intraperitoneally, and an additional group of BALB/c mice was treated with (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg). After 4 hours, the decidua and myometrium were harvested for mRNA expression analysis. The expression of *Ptghs2* in the decidua and myometrium was not suppressed in BALB/c females by (+)-naltrexone following injection with cPAF as *Ptghs2* levels in these maternal tissues were not significantly different to wildtype females treated with cPAF alone (all $p > 0.05$, Fig. 5.10A,F). Similarly, *Tlr4*-/- females did not show any abrogation of response of *Ptghs2* when challenged with cPAF when compared to wildtype females treated with cPAF alone in both decidua and myometrium (all $p > 0.05$, Fig. 5.10A,F).

Both groups of wildtype females administered with cPAF and (+)-naltrexone and *Tlr4*-/- females treated with cPAF had similar expression of decidual *Oxtr* and *Gja* to wildtype females challenged with cPAF or their respective vehicle control groups (all $p > 0.05$, Fig. 5.10B,C). Similar results were observed for *Oxtr* and *Gja* mRNA in myometrium, in which no significant difference in the expression of these genes was observed in *Tlr4*-/- females treated with cPAF when compared to either wildtype females challenged with cPAF (all $p > 0.05$, Fig. 5.10G,H). Unexpectedly, myometrial *Oxtr* mRNA was significantly induced in wildtype females that received both cPAF and (+)-naltrexone when compared to wildtype females challenged with cPAF alone ($p = 0.001$, Fig. 5.10G) or (+)-naltrexone control ($p = 0.003$, Fig. 5.10G). Myometrial *Gja* mRNA

was stimulated in females which received both cPAF and (+)-naltrexone when compared to (+)-naltrexone control ($p = 0.038$, Fig. 5.10H); however, the level of expression was similar to wildtype females challenged with cPAF alone ($p > 0.05$, Fig. 5.10H). These results are difficult to interpret given cPAF alone did not induce either gene.

In summary, only the expression of *Ptghs2* was stimulated in the decidua and uterus following intraperitoneal challenge with cPAF, and this was not affected either via blockade of TLR4 using (+)-naltrexone or TLR4 deficiency.

5.9 THE EFFECT OF BLOCKADE OF cPAF-INDUCED PRETERM DELIVERY BY (+)-NALTREXONE OR TLR4 ABLATION ON cPAF INDUCTION OF THE INFLAMMATORY CYTOKINE GENES IN PLACENTA, FETAL MEMBRANE AND FETAL HEAD.

To define the cytokine expression in response to intraperitoneal administration of cPAF, pregnant BALB/c females were given 2 μg/mouse of cPAF or vehicle on gd 16.5. After 4 hours, the placenta and fetal membrane were harvested for mRNA expression analysis. Analysis of placental gene expression from BALB/c females demonstrated a 2.0 to 2.4-fold upregulation of pro-inflammatory cytokines *Il1b* ($p < 0.001$, Fig. 5.11A) and *Il6* ($p < 0.001$, Fig. 5.11B) in response to cPAF administration. The expression of the pro-inflammatory cytokines *Il12b*, *Tnf* and *Il1a* (all $p > 0.05$, Fig. 5.11B-E) and anti-inflammatory cytokine *Il10* ($p > 0.05$, Fig. 5.12A) were not increased in the placental tissues of females challenged with cPAF. In fetal membrane, cPAF treated wildtype females resulted in a 5.3-fold induction of *Il10* ($p = 0.015$, Fig. 5.12B) mRNA in comparison to vehicle control. cPAF administration did not affect expression of *Il1b, Il6, Il12b*, *Tnf* and *Il1a* in fetal membranes of wildtype females (all $p > 0.05$, Fig. 5.11F-J).

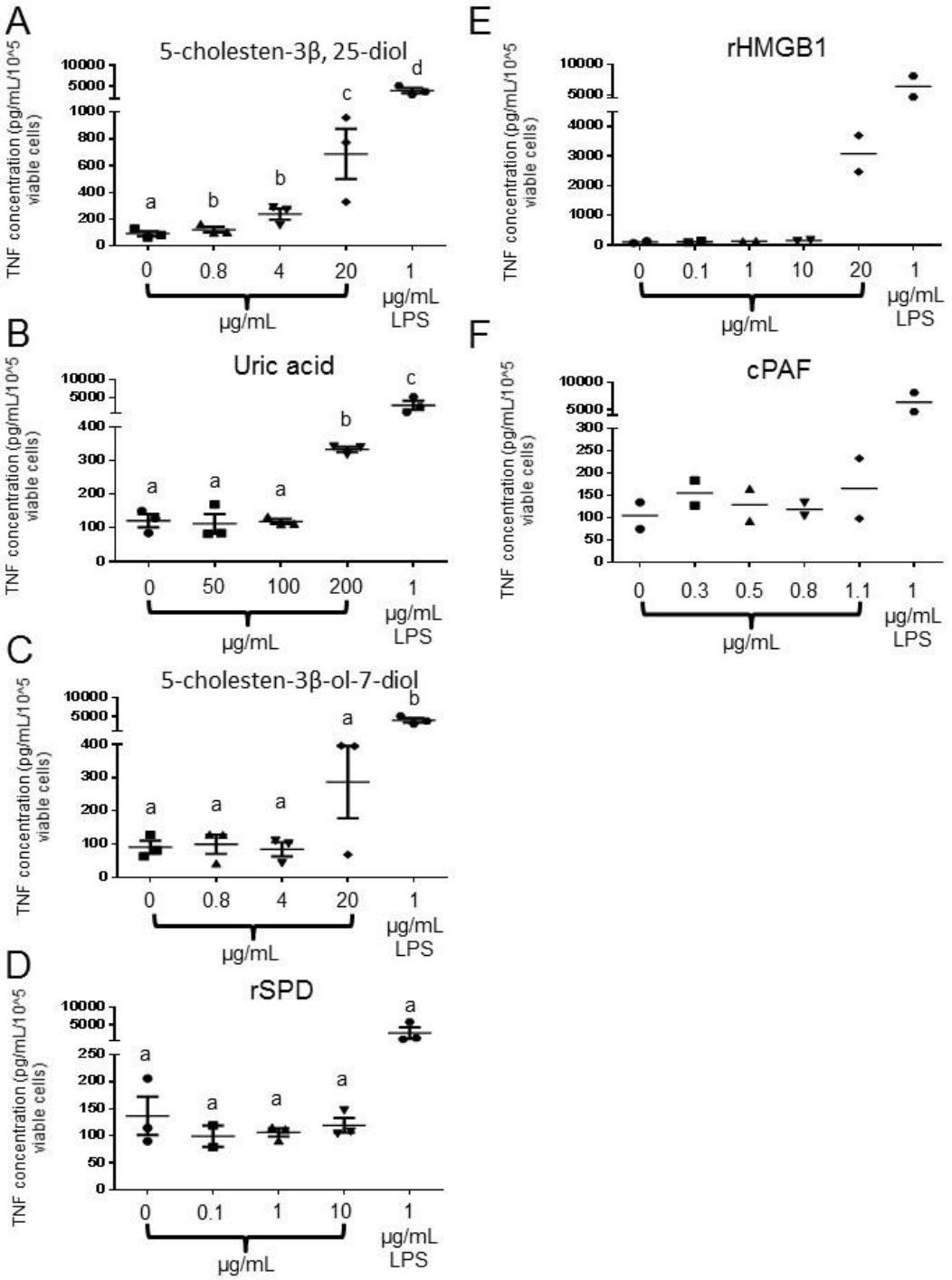
To determine the TLR4 dependent cytokine response to cPAF in preterm birth, pregnant BALB/c or *Tlr4*-/- females were administered 2 μg/mouse of cPAF intraperitoneally, with an additional group of BALB/c mice treated with (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg). After 4 hours, the placenta and fetal membranes were harvested for mRNA expression analysis. In placenta, BALB/c females treated with cPAF and (+)-naltrexone (all $p < 0.05$) displayed nearly 50% suppression in *Il1b* and *Il6* (Fig. 5.11A,B) expression relative to wildtype females treated with cPAF alone, similar to the (+)-naltrexone control group (all $p > 0.05$). In placenta, *Tlr4*-/- females treated with cPAF ($p = 0.004$, Fig. 5.11A) displayed a 58% inhibition of *Il1b* when compared to wildtype females treated with cPAF alone, similar to the *Tlr4*-/- females receiving vehicle control. The expression of *Il6* in the placenta of *Tlr4*-/- females was significantly upregulated ($p = 0.022$, Fig. 5.11B) when injected with cPAF compared to vehicle, at a level similar to the cPAF treated BALB/c females ($p > 0.05$, Fig. 6.1.1B).

In addition, expression of placental *Il12b, Tnf, Il1a* and *Il10* in both BALB/c females administered with cPAF and (+)-naltrexone and *Tlr4*-/- females challenged with cPAF were not significantly different to BALB/c females challenged with cPAF or their control groups (all $p > 0.05$, Fig. 5.11C-E, 5.12A).

In fetal membrane, the expression of placental *Il1b, Il6, Il112b, Tnf, Il1a* and *Il10* in both BALB/c females administered cPAF and (+)-naltrexone and *Tlr4*-/- females challenged with cPAF were not significantly different to BALB/c females receiving cPAF or their control groups (all $p > 0.05$, Fig. 5.11F-J, 5.12A).

Figure 5.1 The effect of DAMPs and TLR4 regulators on TNF secretion from J774 macrophages cell line

J774 macrophages were treated for 12 hours with increasing concentrations of 5-cholesten-3β, 25-diol (A), uric acid (B), rHMGB1 (C), cPAF (D), rSP-D (E) and 5-cholesten-3β-ol-7-one (F) and TNF concentration was measured using ELISA and normalised to the approximate number of viable cells. The TNF concentration was also normalised to the approximate cell number determined after the treatment of J774 macrophages with rHMGB1 (D), 5-cholesten-3β, 25-diol (E) and uric acid (F). Data are shown as mean± SEM, from 2-3 experiments on different days, with each experiment performed in triplicate. The data were analysed Kruskal-Wallis and Mann-Whitney U-test. Different letters (a,b,c,d) indicate statistical significance ($p < 0.05$) between treatment groups.


A
5-cholesten-3β, 25-diol
TNF concentration (pg/mL/10^5 viable cells)
10000
5000
1000
800
600
400
200
0
a
b
b
c
d
0
0.8
4
20
1
µg/mL
LPS
µg/mL
B
Uric acid
TNF concentration (pg/mL/10^5 viable cells)
10000
5000
400
300
200
100
0
a
a
a
b
c
0
50
100
200
1
µg/mL
LPS
µg/mL
C
5-cholesten-3β-ol-7-diol
TNF concentration (pg/mL/10^5 viable cells)
10000
5000
400
300
200
100
0
a
a
a
a
b
0
0.8
4
20
1
µg/mL
LPS
µg/mL
D
rSPD
TNF concentration (pg/mL/10^5 viable cells)
10000
5000
250
200
150
100
50
0
a
a
a
a
a
0
0.1
1
10
1
µg/mL
LPS
µg/mL
E
rHMGB1
TNF concentration (pg/mL/10^5 viable cells)
10000
5000
4000
3000
2000
1000
0
0
0.1
1
10
20
1
µg/mL
LPS
µg/mL
F
cPAF
TNF concentration (pg/mL/10^5 viable cells)
10000
5000
300
250
200
150
100
50
0
0
0.3
0.5
0.8
1.1
1
µg/mL
LPS
µg/mL

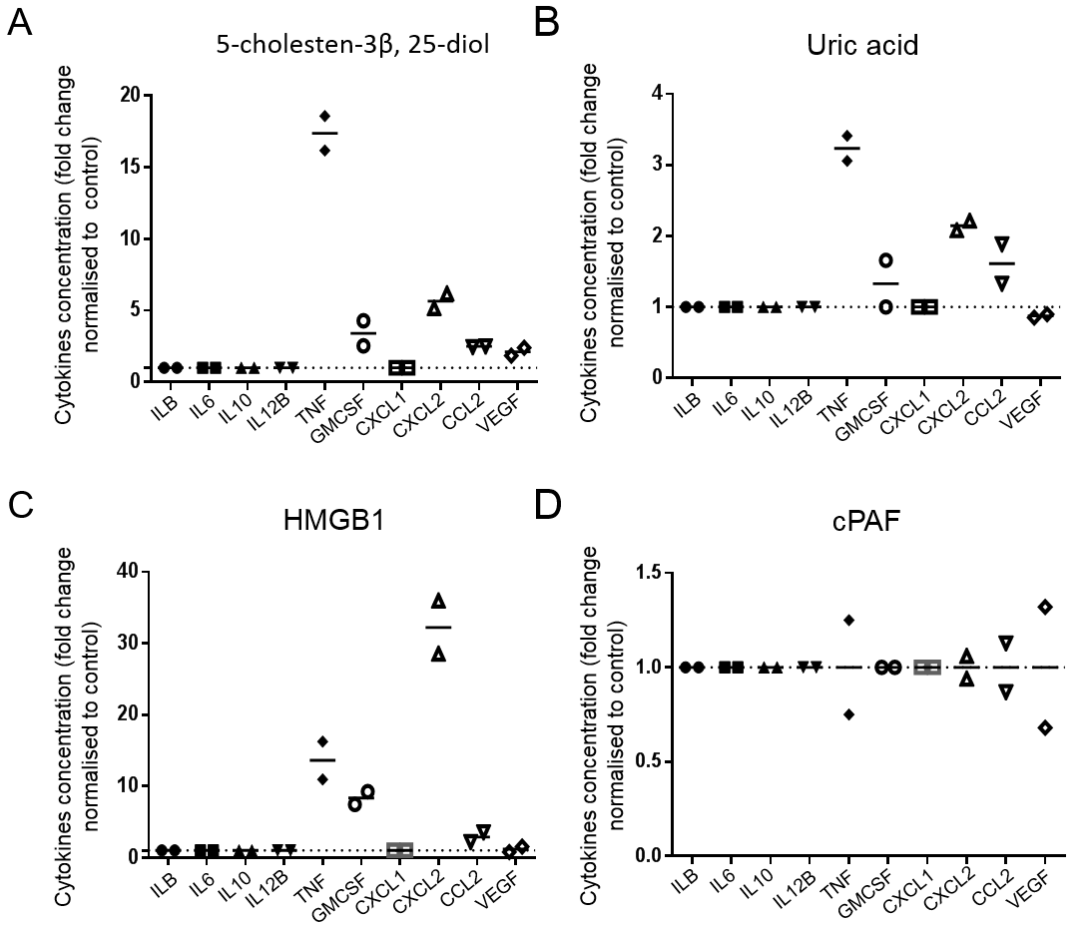


Figure 5.2 The effect of DAMPs and cPAF on inflammatory cytokines and chemokines from J774 macrophages cell line

J774 macrophages were cultured with either 20 µg/mL 5-cholesten-3β, 25-diol (A), 200 µg/mL uric acid (B), 20 µg/mL rHMGB1 (C) or 0.5 ug/mL cPAF for 12 hours before media were collected with cytokine and chemokine expression analysed by bead array (D). Data represent fold change normalised to medium control and are shown as mean from 2 experiments done in triplicate on different days. The dotted line on each graph represents the level of medium control for each of the cytokine or chemokine measured.

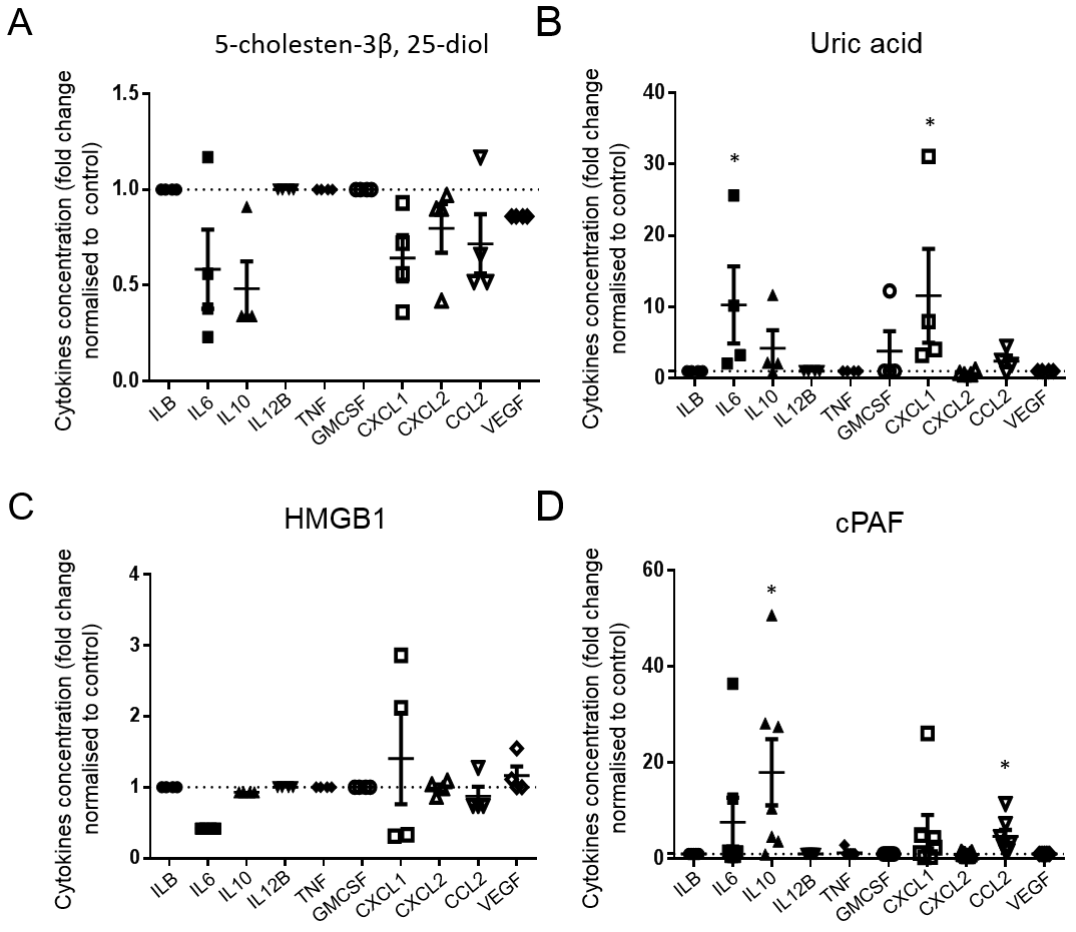


Figure 5.3 The effect of DAMPs and cPAF on inflammatory cytokines and chemokines in the serum of mice

On gd 16.5, pregnant BALB/c females were administered intraperitoneally with 1.5-2.0 mg/mouse (50 mg/kg) 5-cholesten-3β, 25-diol (A), 3 mg/mouse uric acid (B), 50 µg/mouse rHMGB1 (C) and 1 µg/mouse cPAF (D) and the blood was collected 6 hours later. The cytokine and chemokine expression in the serum was analysed by bead array. Data represent fold change normalised to medium control and are shown as mean ± SEM. The dotted line on each graph represents the level of medium control for each of the cytokine or chemokine measured. Data were analysed using Kruskal-Wallis and Mann-Whitney U-test, with n= 4-7 dams/group. *indicate statistical significance ($p < 0.05$) compared to vehicle control group.

Table 5.1 The effect of uric acid on preterm delivery outcomes

	PBS (n=7)	Uric acid (n=9)
% preterm delivery *	0	0
% viable pregnancies *	100	100
Total implantation sites *	5.71 ± 0.20	6.56 ± 0.17
Viable fetuses/dam *	5.71 ± 0.20	6.00± 0.15
% viable fetuses/dam *	100 ± 0	92.66 ± 1.310
Fetal weight ^	968.90 ± 18.068	940.45 ± 16.74
Placental weight ^	111.04 ± 2.55	104.29 ± 2.33
Fetal:placental weight ratio^	8.93 ± 0.265	9.17 ± 0.24

* Data are shown as mean ± SEM

^Data were analysed using mixed model and given as the estimated marginal mean ± SEM

There are no significant differences between treatment and control groups

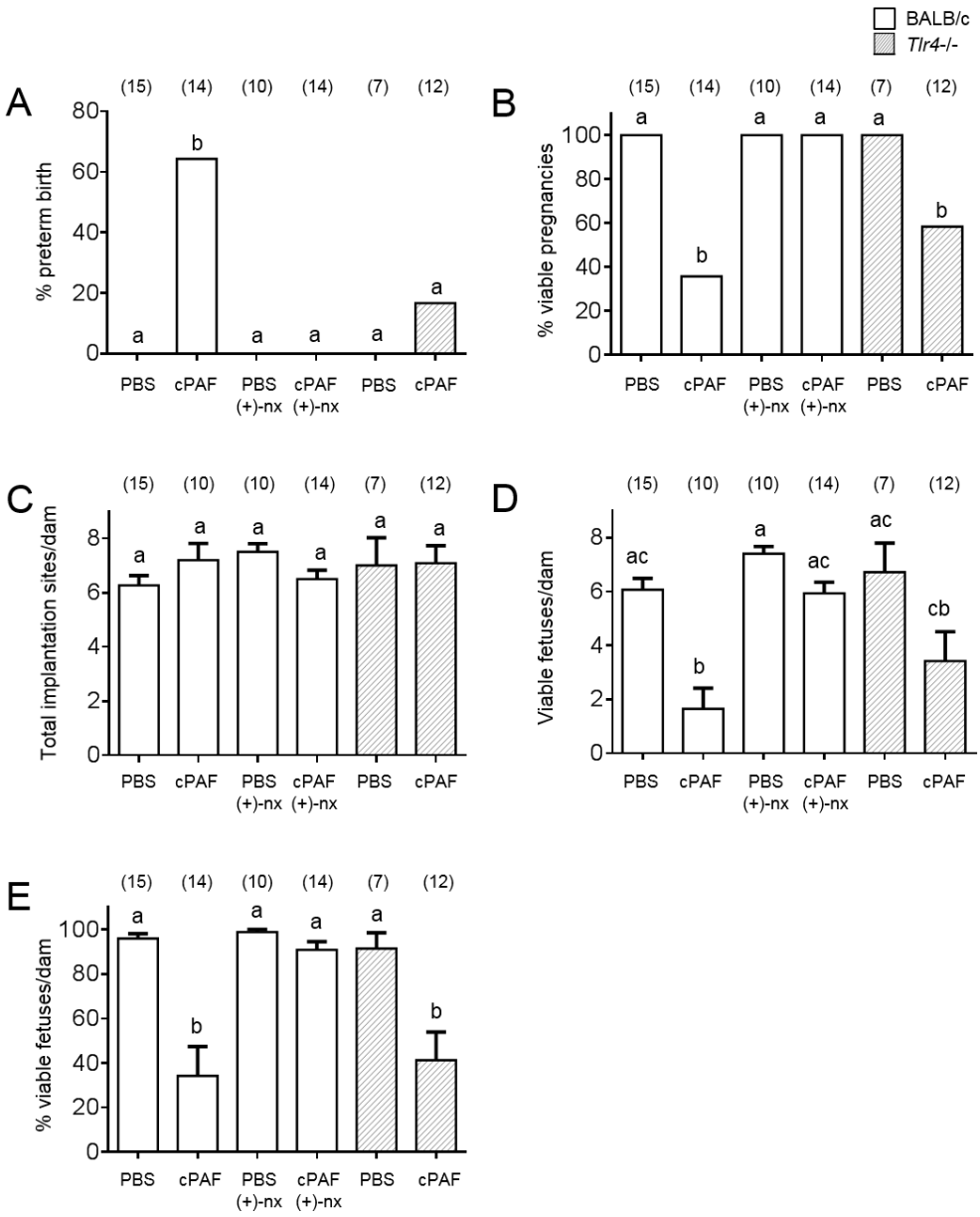


Figure 5.4 The effect of TLR4 antagonist (+)-naltrexone or TLR4 deficiency in preventing intraperitoneal cPAF induced preterm birth

Wildtype BALB/c females (white bars ☐) or *Tlr4-/-* females (hatched bars ▨) were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5 and on gd 16.5, pregnant BALB/c females were administered 2 µg/mouse of cPAF or PBS vehicle control intraperitoneally, followed by intraperitoneal administration of (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg) on gd 16.5, 17.0, 17.5 and 18.0 or PBS control. Mice were observed for preterm delivery within 48 hours of cPAF administration to record percentage of preterm birth (A). In the absence of preterm birth, mice were killed on gd 18.5 and pregnancy outcomes were measured to record percentage viable pregnancy (B), total implantation sites per dam (C), number of viable fetuses per dam (D) and percentage of viable fetuses per dam. Data are shown as mean± SEM, with the number of dams given in parentheses. Categorical data (A,B) were compared by χ^2 analysis (C-E), and were analysed by ANOVA and Sidak t test to determine the effect of genotype. Different letters (a,b,c) indicate statistical significance ($p < 0.05$) between treatments in different genotypes.

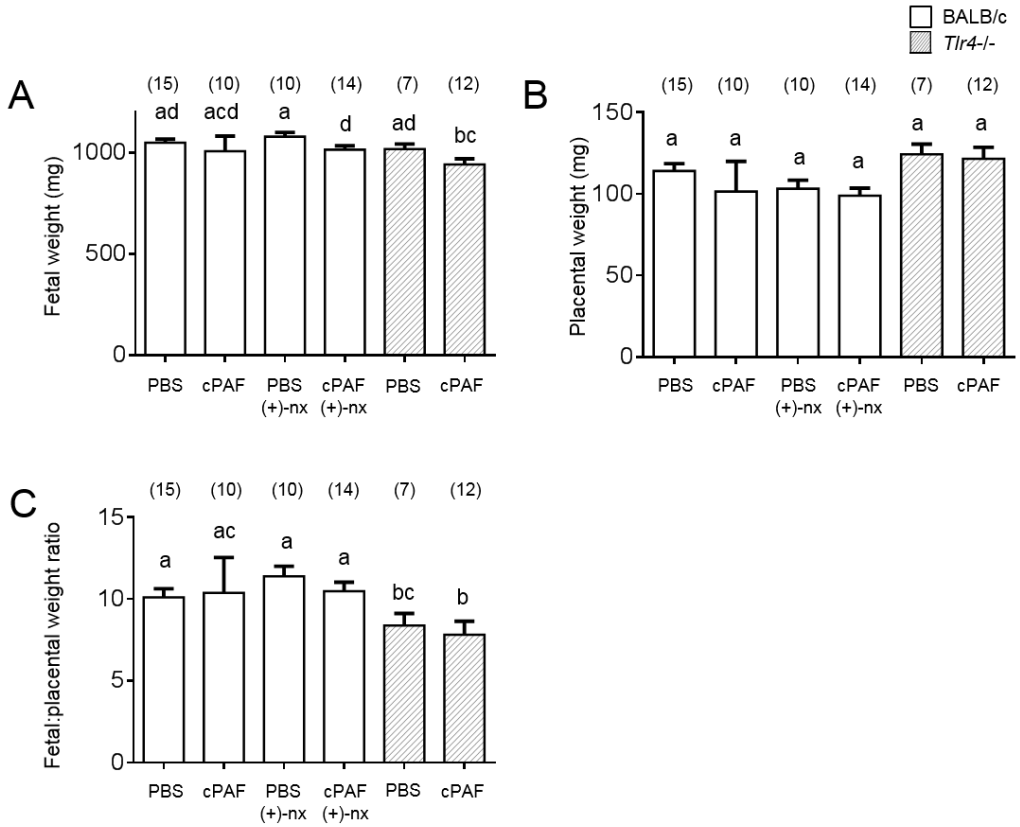


Figure 5.5 The effect of TLR4 antagonist (+)-naltrexone or TLR4 deficiency in preventing intraperitoneal cPAF induced preterm birth

Wildtype BALB/c females (white bars ☐) or *Tlr4-/-* females (hatched bars ▨) were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5 and on gd 16.5, pregnant BALB/c females were administered 2 µg/mouse of cPAF or PBS vehicle control intraperitoneally, followed by intraperitoneal administration of (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg) on gd 16.5, 17.0, 17.5 and 18.0 or PBS control. Mice were observed for preterm delivery within 48 hours of cPAF administration to record percentage of preterm birth (A). In the absence of preterm birth, mice were killed on gd 18.5 and pregnancy outcomes were measured to record fetal weight (A), placental weight (B) and the fetal: placental weight ratio was calculated (C). Data were analysed using mixed model and given as the estimated marginal means ± SEM, with the number fetuses per group is given in parentheses. Different letters (a,b,c,d) indicate statistical significance ($p < 0.05$) between treatments in different genotypes.

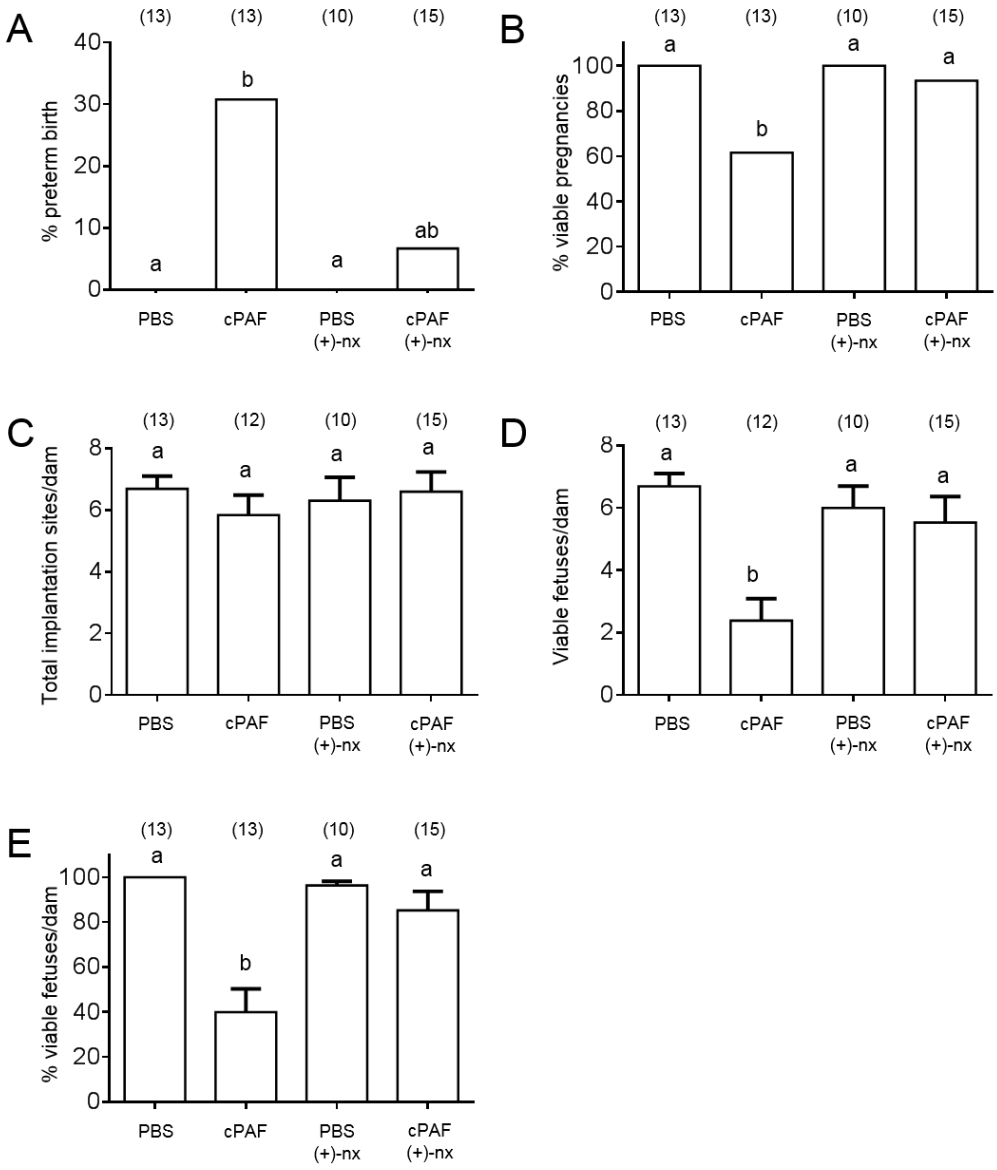


Figure 5.6 The effect of TLR4 antagonist (+)-naltrexone in preventing intrauterine cPAF induced preterm birth

Wildtype BALB/c females were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5 and on gd 16.5, pregnant BALB/c females were administered 35 µg/mouse of cPAF or PBS vehicle control intrauterine, followed by intraperitoneal administration of (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg) on gd 16.5, 17.0, 17.5 and 18.0 or PBS control. Mice were observed for preterm delivery within 48 hours of cPAF administration to record percentage of preterm birth (A). In the absence of preterm birth, mice were killed on gd 18.5 and pregnancy outcomes were measured to record percentage viable pregnancy (B), total implantation sites per dam (C), number of viable fetuses per dam (D) and percentage of viable fetuses per dam. Data are shown as mean± SEM, with the number of dams given in parentheses. Categorical data (A,B) were compared by χ^2 analysis (C-E), and were analysed by ANOVA and Sidak t test to determine the effect of genotype. Different letters (a,b) indicate statistical significance ($p < 0.05$) between treatments.

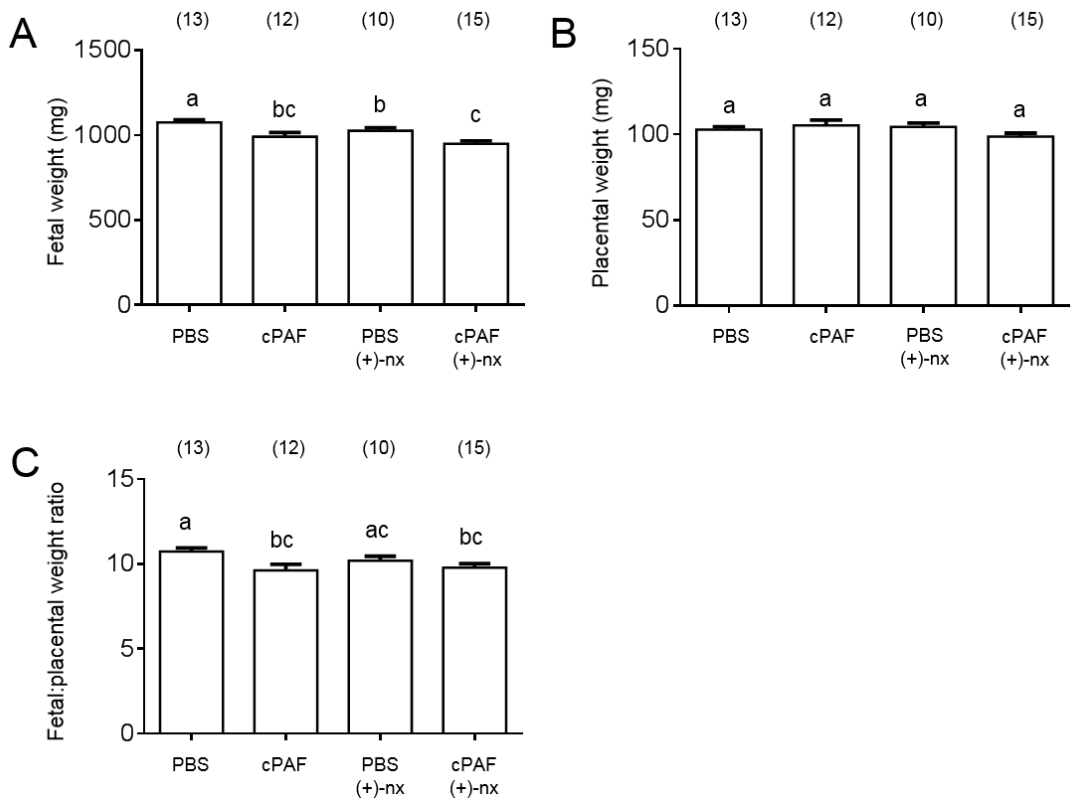
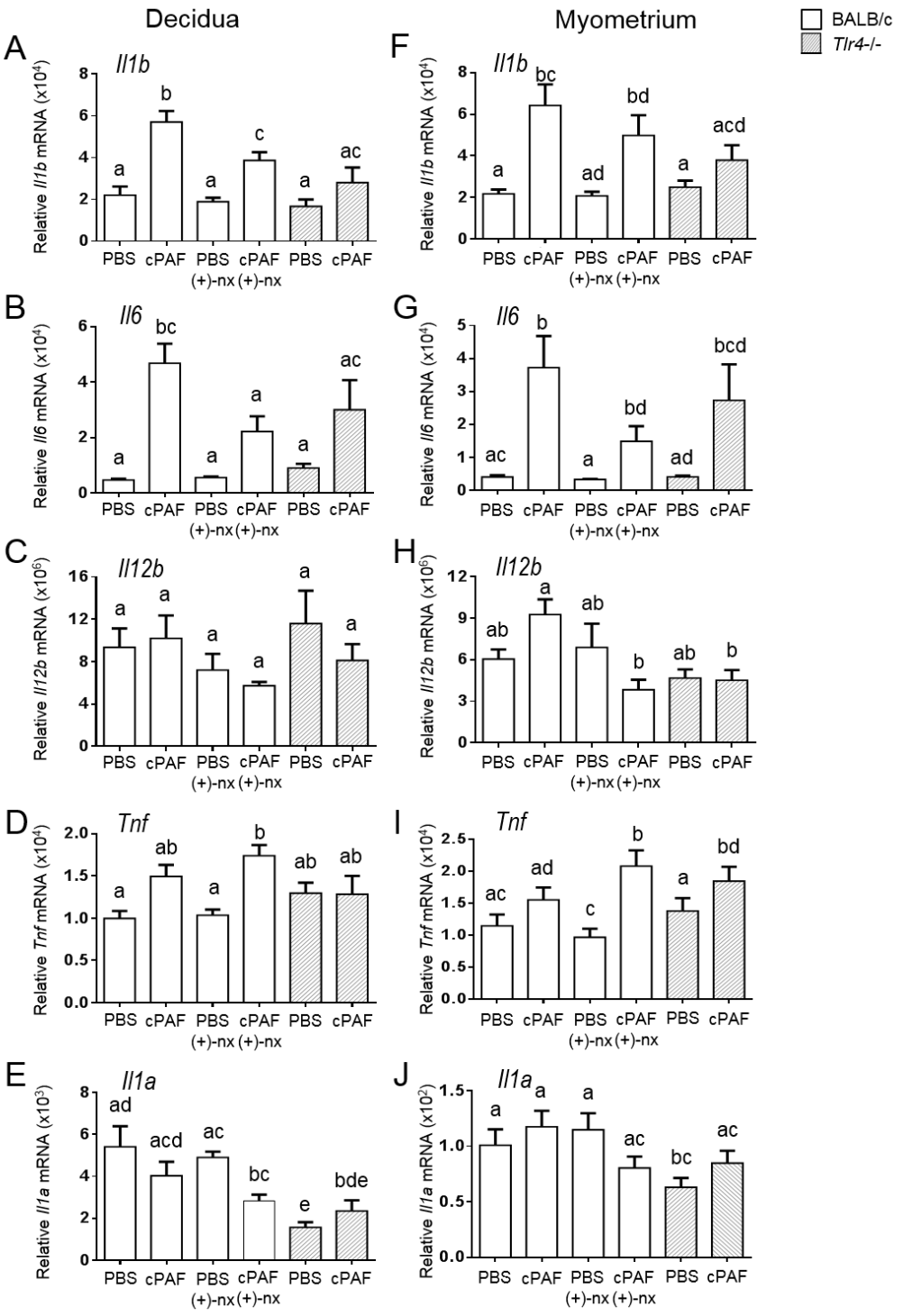


Figure 5.7 The effect of TLR4 antagonist (+)-naltrexone in preventing intrauterine cPAF induced preterm birth

Wildtype BALB/c females were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5 and on gd 16.5, pregnant BALB/c females were administered 35 µg/mouse of cPAF or PBS vehicle control intrauterine, followed by intraperitoneal administration of (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg) on gd 16.5, 17.0, 17.5 and 18.0 or PBS control. Mice were observed for preterm delivery within 48 hours of cPAF administration to record percentage of preterm birth (A). In the absence of preterm birth, mice were killed on gd 18.5 and pregnancy outcomes were measured to record fetal weight (A), placental weight (B) and the fetal: placental weight ratio was calculated (C). Data were analysed using mixed model and given as the estimated marginal means ± SEM, with the number fetuses per group is given in parentheses. Different letters (a,b,c) indicate statistical significance ($p < 0.05$) between treatments.

Figure 5.8 The effect of TLR4 antagonist (+)-naltrexone or TLR4 deficiency on intraperitoneal cPAF induction of the pro-inflammatory cytokine genes in the decidua and myometrium

Wildtype BALB/c females (white bars ☐) or *Tlr4-/-* females (hatched bars ▨) were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5 and on gd 16.5, pregnant BALB/c females were administered 2 µg/mouse of cPAF or PBS vehicle control intraperitoneally, followed by intraperitoneal administration of 1 dose of (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg) or PBS control. 4 hours after treatment, decidua (A-E) and myometrium (F-J) were harvested and relative expression of the *Il1b*, *Il6*, *Il12b*, *Tnf* and *Il1a* mRNA in each tissue were quantified using RT-PCR, normalised to *Actb*. Data are shown as mean± SEM relative gene expression in tissue pooled from two implantation sites per pregnancy, with n= 6-10 dams/group. Data were tested for normality using a Shapiro–Wilk test. ANOVA and post hoc Sidak T-tests were used when data were normally distributed. Kruskal-Wallis and Mann-Whitney U-test were used when data were not normally distributed. Different letters (a,b,c,d) indicate statistical significance ($p < 0.05$) between treatments in different genotypes.


Decidua
Myometrium
BALB/c
Tlr4-/-
A
Il1b
Relative Il1b mRNA (x10⁴)
F
Il1b
Relative Il1b mRNA (x10⁴)
B
Il6
Relative Il6 mRNA (x10⁴)
G
Il6
Relative Il6 mRNA (x10⁴)
C
Il12b
Relative Il12b mRNA (x10⁵)
H
Il12b
Relative Il12b mRNA (x10⁵)
D
Tnf
Relative Tnf mRNA (x10⁴)
I
Tnf
Relative Tnf mRNA (x10⁴)
E
Il1a
Relative Il1a mRNA (x10³)
J
Il1a
Relative Il1a mRNA (x10²)
PBS cPAF PBS cPAF PBS cPAF
(+)-nx (+)-nx

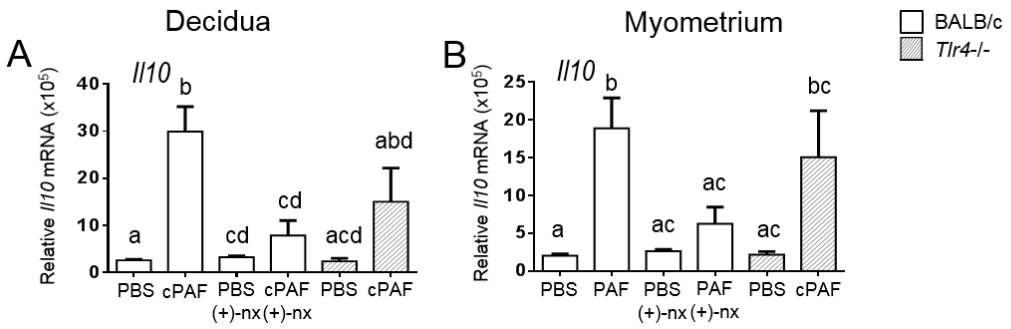


Figure 5.9 The effect of TLR4 antagonist (+)-naltrexone or TLR4 deficiency on intraperitoneal cPAF induction of the anti-inflammatory cytokine gene *Il10* in the decidua and myometrium

Wildtype BALB/c females (white bars □) or *Tlr4-/-* females (hatched bars ▨) were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5 and on gd 16.5, pregnant BALB/c females were administered 2 µg/mouse of cPAF or PBS vehicle control intraperitoneally, followed by intraperitoneal administration of 1 dose of (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg) or PBS control. 4 hours after treatment, decidua (A) and myometrium (B) were harvested and relative expression of the *Il10* mRNA in each tissue were quantified using RT-PCR, normalised to *Actb*. Data are shown as mean± SEM relative gene expression in tissue pooled from two implantation sites per pregnancy, with n= 6-10 dams/group. Data were tested for normality using a Shapiro–Wilk test. ANOVA and post hoc Sidak T-tests were used when data were normally distributed. Kruskal-Wallis and Mann-Whitney U-test were used when data were not normally distributed. Different letters (a,b,c,d) indicate statistical significance ($p < 0.05$) between treatments in different genotypes.

Figure 5.10 The effect of TLR4 antagonist (+)-naltrexone or TLR4 deficiency on intraperitoneal cPAF induction of the uterine activation genes in the decidua and myometrium

Wildtype BALB/c females (white bars ☐) or *Tlr4-/-* females (hatched bars ▨) were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5 and on gd 16.5, pregnant BALB/c females were administered 2 µg/mouse of cPAF or PBS vehicle control intraperitoneally, followed by intraperitoneal administration of 1 dose of (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg) or PBS control. 4 hours after treatment, decidua (A-E) and myometrium (F-J) were harvested and relative expression of the *Ptghs2*, *Oxtr*, *Gja*, *Ptghs1* and *Ptgfr* mRNA in each tissue were quantified using RT-PCR, normalised to *Actb*. Data are shown as mean± SEM relative gene expression in tissue pooled from two implantation sites per pregnancy, with n= 6-10 dams/group. Data were tested for normality using a Shapiro–Wilk test. ANOVA and post hoc Sidak T-tests were used when data were normally distributed. Kruskal-Wallis and Mann-Whitney U-test were used when data were not normally distributed. Different letters (a,b,c,d,e) indicate statistical significance ($p < 0.05$) between treatments in different genotypes.

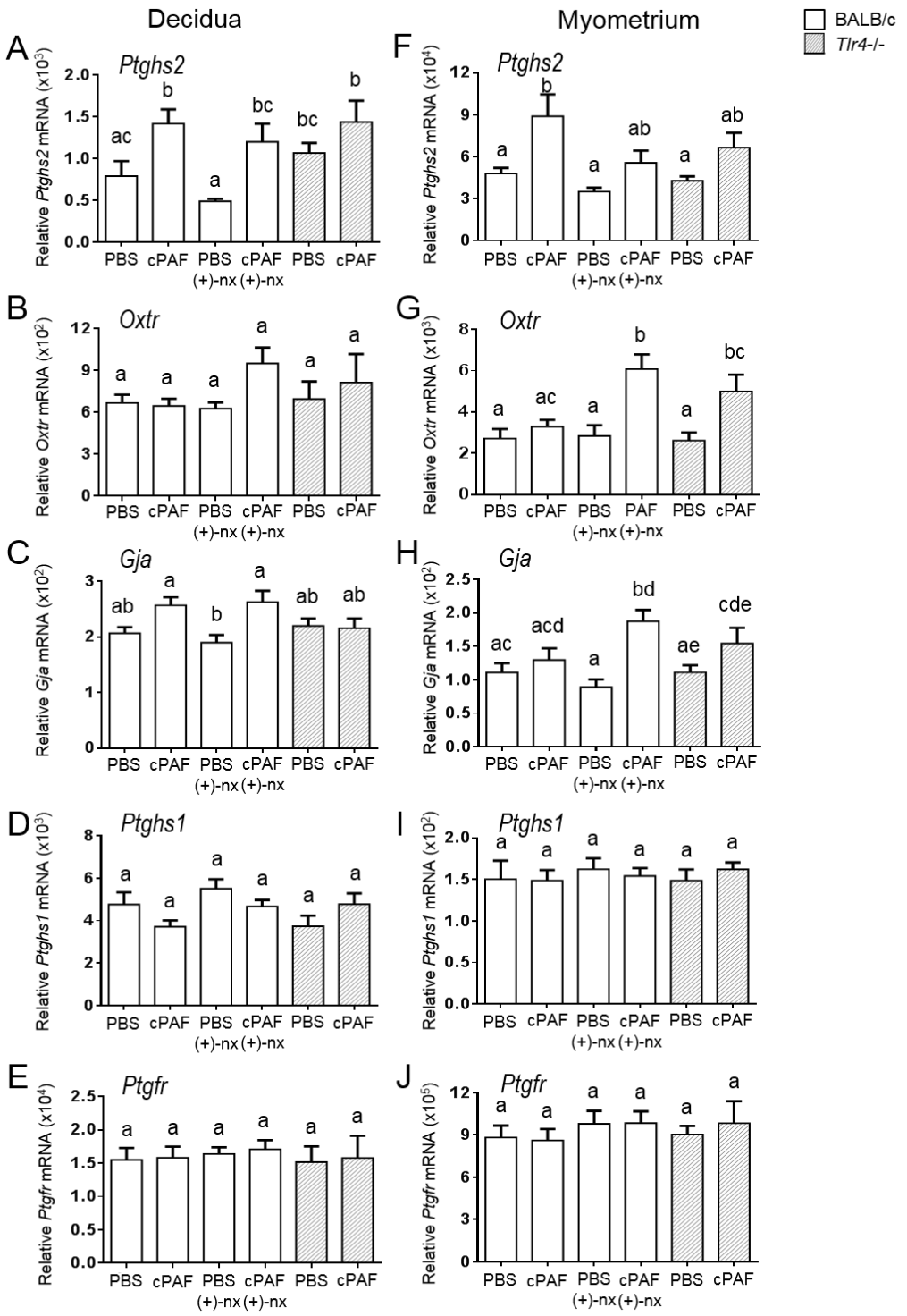
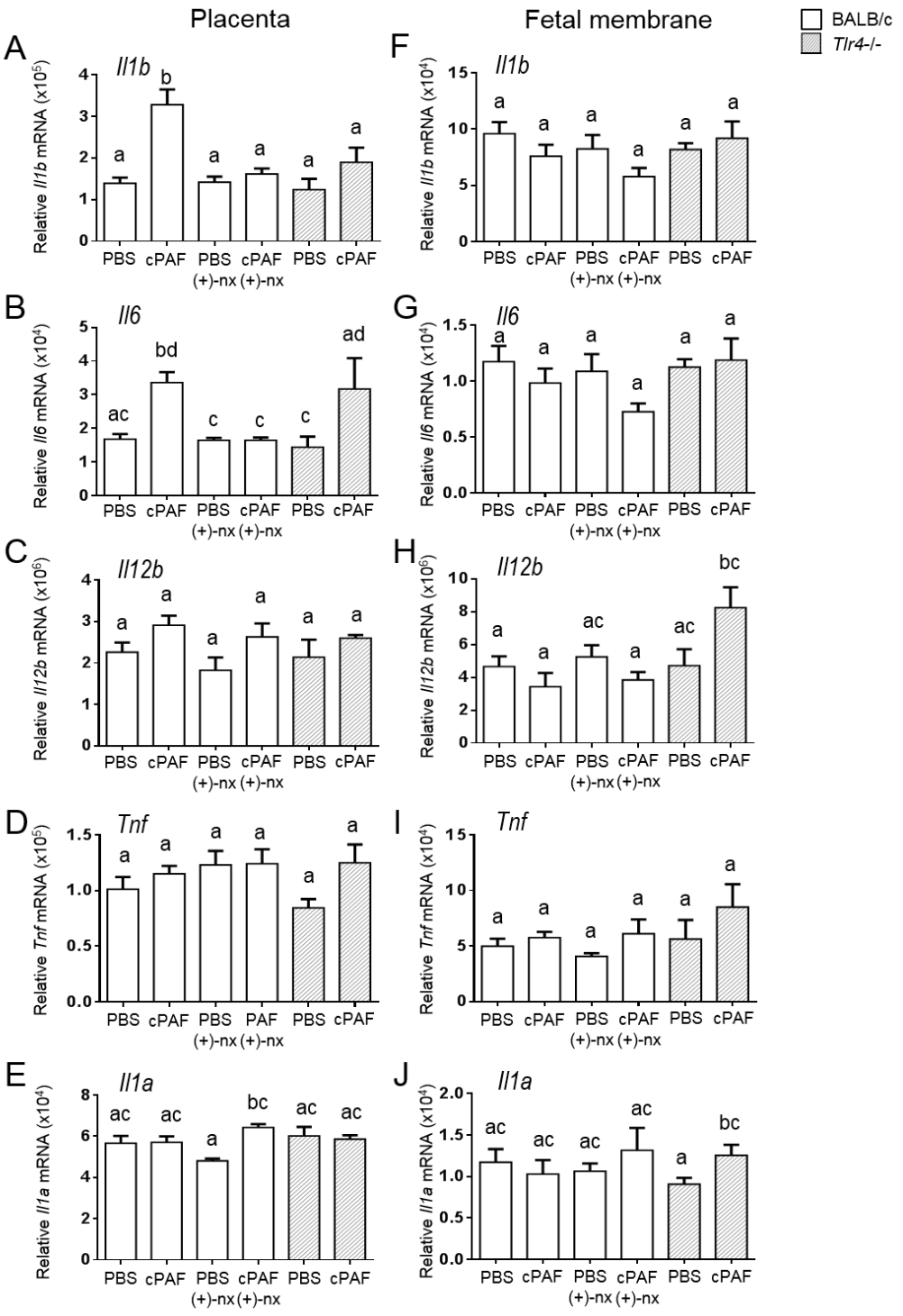

Decidua
Myometrium
BALB/c
Tlr4-/-
A
Ptghs2
Relative Ptghs2 mRNA (x10³)
ac b a bc bc b
F
Ptghs2
Relative Ptghs2 mRNA (x10⁴)
a b a ab a ab
B
Oxtr
Relative Oxtr mRNA (x10²)
a a a a a a
G
Oxtr
Relative Oxtr mRNA (x10³)
a ac a b a bc
C
Gja
Relative Gja mRNA (x10²)
ab a b a ab ab
H
Gja
Relative Gja mRNA (x10²)
ac acd a bd ae cde
D
Ptghs1
Relative Ptghs1 mRNA (x10³)
a a a a a a
I
Ptghs1
Relative Ptghs1 mRNA (x10²)
a a a a a a
E
Ptgfr
Relative Ptgfr mRNA (x10⁴)
a a a a a a
J
Ptgfr
Relative Ptgfr mRNA (x10⁵)
a a a a a a
PBS cPAF PBS cPAF PBS cPAF
(+)-nx (+)-nx
PAF

Figure 5.11 The effect of TLR4 antagonist (+)-naltrexone or TLR4 deficiency on intraperitoneal cPAF induction of the pro-inflammatory cytokine genes in the placenta and fetal membrane

Wildtype BALB/c females (white bars ☐) or *Tlr4-/-* females (hatched bars ▨) were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5 and on gd 16.5, pregnant BALB/c females were administered 2 µg/mouse of cPAF or PBS vehicle control intraperitoneally, followed by intraperitoneal administration of 1 dose of (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg) or PBS control. 4 hours after treatment, placenta (A-E) and fetal membrane (F-J) were harvested and relative expression of the *Il1b*, *Il6*, *Il12b*, *Tnf* and *Il1a* mRNA in each tissue were quantified using RT-PCR, normalised to *Actb*. Data are shown as mean± SEM relative gene expression in tissue pooled from two implantation sites per pregnancy, with n= 6-10 dams/group. Data were tested for normality using a Shapiro–Wilk test. ANOVA and post hoc Sidak T-tests were used when data were normally distributed. Kruskal-Wallis and Mann-Whitney U-test were used when data were not normally distributed. Different letters (a,b,c) indicate statistical significance ($p < 0.05$) between treatments in different genotypes.


Placenta
Fetal membrane
BALB/c
Tlr4-/-
A
Il1b
Relative Il1b mRNA (x10⁵)
B
Il6
Relative Il6 mRNA (x10⁴)
C
Il12b
Relative Il12b mRNA (x10⁶)
D
Tnf
Relative Tnf mRNA (x10⁵)
E
Il1a
Relative Il1a mRNA (x10⁴)
F
Il1b
Relative Il1b mRNA (x10⁴)
G
Il6
Relative Il6 mRNA (x10⁴)
H
Il12b
Relative Il12b mRNA (x10⁶)
I
Tnf
Relative Tnf mRNA (x10⁴)
J
Il1a
Relative Il1a mRNA (x10⁴)
PBS cPAF PBS cPAF PBS cPAF
(+)-nx (+)-nx

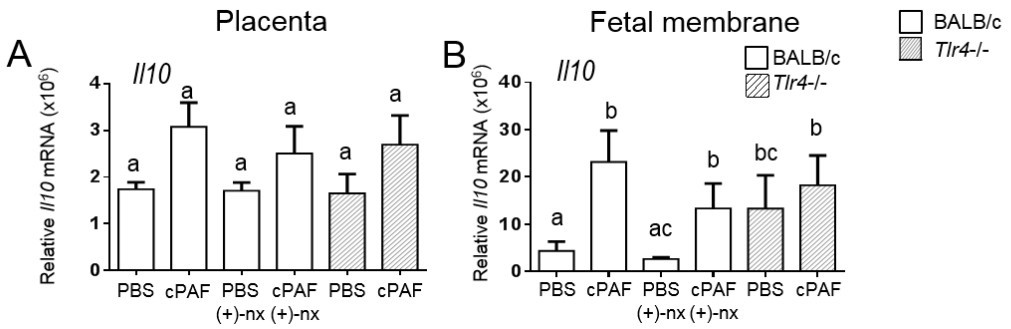


Figure 5.12 The effect of TLR4 antagonist (+)-naltrexone or TLR4 deficiency on intraperitoneal cPAF induction of the anti-inflammatory cytokine gene *Il10* in the placenta and fetal membrane

Wildtype BALB/c females (white bars ☐) or *Tlr4-/-* females (hatched bars ▨) were mated to the males of the same genotype. The vaginal plug detection was designated gd 0.5 and on gd 16.5, pregnant BALB/c females were administered 2 µg/mouse of cPAF or PBS vehicle control intraperitoneally, followed by intraperitoneal administration of 1 dose of (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg) or PBS control. 4 hours after treatment, placenta (A) and fetal membrane (B) were harvested and relative expression of the *Il10* mRNA in each tissue were quantified using RT-PCR, normalised to *Actb*. Data are shown as mean± SEM relative gene expression in tissue pooled from two implantation sites per pregnancy, with n= 6-10 dams/group. Data were tested for normality using a Shapiro–Wilk test. ANOVA and post hoc Sidak T-tests were used when data were normally distributed. Kruskal-Wallis and Mann-Whitney U-test were used when data were not normally distributed. Different letters (a,b,c) indicate statistical significance ($p < 0.05$) between treatments in different genotypes.

5.10 DISCUSSION

DAMPs including HMGB1 and 5-cholesten-3β, 25-diol, 5-cholesten-3β-ol-7-one and TLR4 regulators such as PAF and SP are endogenous ligands that have been shown to contribute to TLR4 activation to induce downstream pro-inflammatory processes (210,211,298,328,332,335,349-351). Following ischemia, toxic injury, stress or trauma HMGB1 (194), uric acid (197) and PAF (328) are released from necrotic cells. HMGB1 and PAF can also be actively secreted by pro-inflammatory macrophages in response to infection (198-200,384,385). High levels of these DAMPs and TLR4 regulator are known to mediate the pathogenesis of many chronic inflammatory and autoimmune diseases (224,287-289,305,386-390), with HMGB1 and PAF concentrations increased in the amniotic fluid of women who deliver preterm (273,329,330). Others have found that rHMGB1 (275), cPAF (331) and rSP-A (344) are efficient in inducing preterm delivery in mice when administered locally, either intra-amniotic or intrauterine.

Initially, we aimed to investigate whether DAMPs and TLR4 regulators might act via the TLR4 in gestational tissues to promote inflammation. Initially we evaluated whether candidate DAMPs and TLR4 regulators could stimulate inflammation in the mouse J774 macrophage cell line in vitro. Cultures incubated with 5-cholesten-3β, 25-diol, uric acid and rHMGB1 acted to promote inflammatory cytokine and chemokine production in J774 macrophages. We then proceeded with in vivo experiments to determine if DAMPs and TLR4 regulators could induce pro-inflammatory cytokines and chemokines in the serum of mice. It was found that 6 hours after intraperitoneal administration of uric acid and cPAF in day 16.5 pregnant BALB/c females, there was an increase in several cytokines and chemokines in the serum.

The effect of systemic route of administration of these ligands was firstly studied, in which cPAF but not uric acid was efficient in triggering preterm delivery in BALB/c mice as well as causing fetal loss. cPAF also promoted an increase in the expression of inflammatory cytokines and uterine activation gene in the gestational tissues. TLR4 deficiency rescued cPAF induction of preterm delivery, but not fetal loss and did not efficiently suppress cPAF induction of inflammatory cytokines. The use of TLR4 antagonist (+)-naltrexone was efficient in preventing the induction of inflammatory cytokine genes to inhibit both preterm delivery and fetal death triggered by cPAF.

There is limited knowledge on the role of both 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one in inducing pro-inflammatory cytokines in human or murine immune cells. Primary

trophoblast cells isolated from placenta at term showed the doses of 6.7 µg/mL and 20 µg/mL of the 5-cholesten-3β, 25-diol caused induction of pro-inflammatory cytokines secretion including IL6, CCL4 and TNF after 12 hours of incubation in dose dependent manner (298). This result is consistent with our study in that 5-cholesten-3β, 25-diol caused macrophages to produce TNF in a dose dependent manner, and perhaps other chemokines such as CCL2 and CXCL2. Conversly, 5-cholesten-3β-ol-7-one did not stimulate a TNF response, which contradicts the above study in primary trophoblast cells, in which the 5-cholesten-3β-ol-7-one at the doses of 6.7 µg/mL and 20 µg/mL triggered IL6, CCL4 and TNF production. This may be attributed to the different responses between the macrophage cell line and the primary cells derived from human gestational tissues, which may express different receptor profiles and therefore exhibit different scales of responsiveness.

An in vitro study revealed that 1000 µg/mL of uric acid induced ILB and TNF secretion in a mouse macrophage cell line RAW 264.7 after 36 hours (394). A dose of 100 µg/mL triggered IL1B in a human first trimester trophoblast cell line after 72 hours (283). We decided to use uric acid between 50 to 200 µg/mL and found that uric acid at the highest dose of 200 µg/mL promoted TNF and probably CXCL2 secretion in J774 macrophages. There are no studies on the role of TLR4 in mediating uric acid activation of pro-inflammatory cytokines although there is evidence that TLR4 plays a role in mediating the anti-phospholipid antibody induction of uric acid secretion from human first trimester trophoblast cells (285).

Previously, rHMGB1 at doses between 0.01-1 µg/mL caused the secretion of TNF from human peripheral blood mononuclear cells (PBMCs) after 4 hours, in a dose dependent manner (391). We have used similar doses of rHMGB1 between 0.01 to 1 µg/mL and found that these doses did not induce TNF production in J774 macrophages. Therefore, when higher rHMGB1 dose of 20 µg/mL was used, TNF production was triggered in J774 macrophages. In pregnancy, the treatment of human fetal membranes with rHMGB1 induces the expression of both *Tlr2* and *Tlr4* mRNA but not *Rage* mRNA (274). rHMGB1 also amplified the production of pro-inflammatory cytokines including IL1B, IL6 and TNF in the human fetal membranes (274) reflecting that HMGB1 induction of pro-inflammatory cytokines could be mediated by TLR2 or TLR4.

cPAF at the doses between 0.1 to 10 nM has been shown to stimulate *Tnf* mRNA expression after 3 hours of treatment in mouse bone marrow-derived macrophages (395). Ex vivo experiments demonstrated that 0.5 ug/mL of cPAF induced TNF, CCL3 and NO secretion after 5 hours of treatment in peritoneal macrophages via TLR4 signalling (332). In our in vitro

experiments, treatment of J774 macrophages with cPAF at concentrations of 0.3 to 1.1 μg/mL for 12 hours did not cause stimulation of TNF as measured by ELISA. The concentration of 0.5 ug/mL, as used in previous study also failed to trigger the secretion of any other inflammatory cytokines and chemokines analysed using bead array. In order to examine if the incubation time could influence these results, we also incubated J774 macrophages with cPAF at concentrations of 0.3 to 1.1 μg/mL for 5 hours; however, this did not induce any of the inflammatory cytokines and chemokines assessed (data not shown). Again, the different responses between cell lines and primary cells may contribute to the differences in the results observed between this study and previous studies. It is possible that a higher dose is required to induce inflammatory cytokines in J774 cells; however, higher doses than those used in this study would likely be supraphysiological and elicit non-specific, toxic effects in vitro.

The J774 macrophages did not produce TNF when treated with 0.1 to 10 μg/mL rSP-D for 12 hours, which is consistent with a previous study demonstrating that 10 μg of rSP-D resulted in a reduced percentage of F4/80+ macrophages and decidual cells expressing TNF compared to the controls (348). The percentage of both F4/80+ macrophages and decidual cells that expressed TNF in response to LPS declined following treatment with 10 μg rSP-D (348). Further in vitro studies demonstrating LPS, PGN and PGN/ Poly [I:C] induced expression of *Il1b* and *Tnf* mRNA could be inhibited by SP-A in the mouse macrophage cell line, RAW 264.7 (345). From this evidence, it is unsurprising that we did not see any cytokine or chemokine response following the treatment with SP-D in vitro.

From the above evidence, it is obvious from in vitro experiments using J774 macrophages, other macrophage cell lines or other immune cells that 5-cholesten-3β, 25-diol, uric acid, rHMGB1 and cPAF can stimulate pro-inflammatory cytokines and chemokines. Previously it has been reported that human fetal membranes (274), term primary trophoblast cells (298) and first trimester trophoblast cell line (283) also respond to most of these DAMPs and TLR4 regulators. Therefore, as well as immune cells, DAMPs and TLR4 regulators are likely to induce cytokines in placental trophoblasts in mice. The pro-inflammatory effects of these DAMPs and TLR4 regulators on different cell lineages isolated from mouse gestational tissues including trophoblast cells need to be elucidated in the future. These experiments would be useful in understanding the local effects of DAMPs and TLR4 regulators on tissues involved in triggering preterm birth in mice. Whilst in this study, we did not examine the role of TLR4 in mediating the induction of inflammatory cytokines and chemokines by DAMPs and TLR4 regulators in J774 cells in vitro, it would be possible to use TLR4 antagonists (+)-naltrexone to test this.

Therefore, the potential role of these DAMPs and cPAF in stimulating cytokines and chemokines in the serum of the 16.5 pregnant BALB/c mice were further investigated. 3 mg of uric acid was seen to promote IL6 and CXCL1 after 6 hours of administration in the serum of pregnant BALB/c mice. In addition, 1 μg/mouse of cPAF increased CCL8 and IL10 in the serum of pregnant females. These results are consistent with previous studies revealing that the level of cytokines in the serum and peritoneal lavage fluid of mice were induced following intraperitoneal administration of 1 μg/mouse of cPAF and 3 mg/mouse of uric acid, respectively (396,397). None of the inflammatory cytokines or chemokines examined here were upregulated in the serum of mice after injection with rHMGB1. This is consistent with a previous study demonstrating that intraperitoneal injection of the same dose of rHMGB1 was not efficient in inducing preterm delivery in C57BL/6 mice, but was sufficient to affect the viability of the pups one week after they were born (275). Furthermore, intraperitoneal injection of a higher dose (100 μg) rHMGB1 in non-pregnant BALB/c mice induced elevated production of TNF in the serum after 6 hours (391). Therefore, the dose used in these studies may have been insufficient to promote an inflammatory response in vivo to elicit preterm delivery.

There are limited studies to examine the inflammatory effects of 5-cholesten-3β, 25-diol in vivo. The concentration of 5-cholesten-3β, 25-diol in this study was based on a study showing that the administration of 50 mg/kg 5-cholesten-3β, 25-diol intraperitoneally 12 hours before intravenous HIV infection led to an increase in the percentage of CD3+CD4+T cells in the peripheral blood 10 days after infection (398). This suggests that the dose of 5-cholesten-3β, 25-diol used in this study was inadequate or the time point after administration at which blood was collected did not represent the peak of inflammatory response, potentially explaining why induction of the inflammatory cytokines and chemokines in the serum of mice was not observed.

Overall these results provided justification for the administration of uric acid and cPAF in wildtype BALB/c females, to examine if these ligands which triggered inflammation in vivo could elicit preterm delivery. In order to amplify the inflammation and thus increase the likelihood of inducing preterm birth in mice, we decided to double the dose of uric acid and cPAF utilised in the in vivo experiments. Uric acid at the dose of 6 mg/ mouse was not efficient in inducing preterm delivery or affecting fetal outcomes; however, we were successful in showing for the first time that intraperitoneal administration of cPAF at the dose of 2 μg/mouse was efficient in triggering preterm delivery. cPAF administration also caused significant fetal loss; however, the surviving fetuses did not show any reduction in fetal weight, placental weight or the fetal: placental weight ratio. In contrast, systemic administration of cPAF in rats induced intrauterine fetal growth

restriction. Intravenous infusion of cPAF at the dose of 0.5-5 μg/kg per hour for 7 days from gd 14 to 21 led to in a decrease in the fetal and placental weights (333,334). It is possible that the small number of surviving fetuses that could be analysed in our study, and the large variation and low statistical power, masked a potential effect that might become evident with a larger study.

Previously a higher dose of LPS is required to induce preterm delivery in mice when administered intrauterine when compared to intraperitoneal (182,368). This is because intrauterine administration of LPS only induces localised inflammation in the gestational tissues when compared to systemic inflammation caused by intraperitoneal injection. Therefore, in this study a higher cPAF dose is required to elicit preterm birth when given directly to the uterus. This is consistent with a previous study showing a preterm delivery rate of 44% (4/9) as well as fetal death in response to intrauterine administration of 10-40 μg of cPAF on gd 15 (331). In our study, cPAF dose of 40 μg/mouse intrauterine caused maternal death (data not shown). Meanwhile, 4 out of 13 (31%) of pregnant BALB/c mice delivered preterm, and remaining mice showed a significant fetal loss after administration 35 μg of cPAF directly into the uterus. At the same time intrauterine cPAF elicited poor fetal outcomes in surviving fetuses, evident as growth restriction and poor placenta efficiency. These parameters in surviving fetuses were not investigated in previous studies (331). These effects of intrauterine cPAF on preterm delivery and fetal viability resemble to some extent human preterm delivery mediated by sterile inflammation, in which the level of PAF is elevated in the amniotic fluid during preterm delivery (329,330). In humans, PAF may be transmitted into the uterus, to further amplify inflammation (139). Another possibility is that PAF may be secreted by the resident structural cells or immune cells in the uterus following stress (328) mediated by multiple pregnancy which introduces further myometrial stress (1,2,11,399-401). Interestingly, preterm labour in women with twins is associated with increased concentration of IL6, CXCL8 and CCL2 in the myometrium, in comparison to women with twins who are not in labour (228). This suggests that in the absence of infection, uterine overdistention causes upregulation of inflammatory cytokines and implicating non-infectious DAMPs and TLR4 regulators as eliciting agents.

A higher percentage of TLR4 positive amniotic epithelial cells is documented in fetal membranes of human preterm delivery with chorioamnionitis when compared to preterm delivery without chorioamnionitis (61). This observation in humans is supported in mice, as TLR4 is required to mediate normal responses towards inflammation-induced preterm delivery in mice (64-66). These studies emphasize the important role of TLR4 in the pathological process of preterm delivery mediated by infection in both humans and mice. In this study we investigated

the role of TLR4 in mediating the sterile inflammation in cPAF induction of preterm delivery, as cPAF stimulation of inflammation in vitro has been demonstrated to be TLR4 dependent (332). *Tlr4* expression in human and mouse intestinal epithelial cell lines as well as in vivo rat intestinal loop model is also induced by PAF (328); however, there is no study to show that cPAF directly binds to TLR4 to induce inflammation.

Interestingly, absence of TLR4 prevented intraperitoneal cPAF induction of preterm delivery, but failed to prevent fetal loss or the poor fetal outcomes in surviving fetuses, including fetal growth restriction. Thus, while activation of the early onset of labour in the setting of systemic inflammation is TLR4 dependent, the mechanisms of cPAF induction of fetal death and poor fetal outcomes appear to occur independently of TLR4. Another possible explanation is that given *Tlr4-/-* females had significantly lower fetal: placental weight ratio compared to BALB/c females independently of cPAF treatment, a poorly functioning placenta may increase susceptibility to alternate pathways of cPAF-induced fetal loss, such as via PAFR and TLR2. PAFR has been shown to be expressed in the myometrium, cervix and fetal membrane, to potentially interact with elevated cPAF to induce inflammation leading to preterm birth. PAF stimulation of human myometrium contraction in vitro and cPAF-induced secretion of pro-inflammatory cytokines from human uterine cervical fibroblasts are demonstrated to be PAFR dependent (316) (322). Furthermore, cPAF stimulation of pro-inflammatory mediators is reduced in peritoneal macrophages derived from *Pafr-/-* and *Tlr2-/-* mice when compared to wildtype mice (332). This evidence reflects that PAFR and TLR2 also play important role in mediating cPAF-induced inflammatory processes in vitro and may contribute to mediating the cPAF-induced fetal death observed in *Tlr4-/-* females.

Another strategy to study the role of TLR4 was to use the small molecule TLR4 antagonist, (+)-naltrexone, which is the (+)-isomer of the opiod receptor antagonist (-)-naltrexone. As the two most common opiod receptor antagonists, both (-)-naltrexone and (-)-naloxone have been reported to block the biological effects of LPS (402) and are used clinically for the treatment of drug and alcohol abuse. Both these isomers are orally active and readily cross the blood brain barrier and presumably placenta (403). Most importantly, (+)-naltrexone and (+)-naloxone are found to be inactive opiod receptors and thus do not antagonize the anaelgesic effects of opiods, unlike the opid active isomers, (-)-naltrexone and (-)-naloxone. Importantly, a recent study found that both (+)-naltrexone and (+)-naloxone bind to the LPS binding pocket of MD2 to inhibit the TLR4-TRIF-IRF3 signalling pathway, but not LPS-induced MAPK and NF-κB activation in vitro (403,404). It is demonstrated that (+)-naltrexone and (+)-naloxone have potential for the

treatment of neuropathic pain and drug abuse, proposed to be mediated by TLR4 activation of inflammatory processes (403,405-409).

Intraperitoneal administration of 60 mg/kg dose of (+)-naloxone 10 minutes before injection of 2.5 mg/ kg of morphine intraperitoneally has been demonstrated to cause an enhanced morphine analgesia in wildtype BALB/c mice (407). In this study, morphine analgesia is demonstrated by hotplate latencies measured prior to and 20 minutes after (+)-naloxone and morphine administration (407). Meanwhile in humans, repeated doses of (-)-naloxone are required to treat narcotic analgesic overdose as it has a relatively short half life (410). This evidence suggests that repeated doses are necessary within the period of 48 hours after cPAF administration, until the pregnancy is considered as term. Nevertheless, preliminary studies in our laboratory have shown that (+)-naltrexone is more efficient that (+)-naloxone in preventing LPS-induced preterm birth in BALB/c mice (unpublished).Therefore in our study, four doses of (+)-naltrexone at 1.8-2.4 mg/mouse (60 mg/kg) on gd 16.5, 17.0, 17.5 and 18.0 have been administered intraperitoneally together with either intraperitoneal or intrauterine administration of cPAF on gd 16.5 in BALB/c females.

Administration of of (+)-naltrexone suppressed both intraperitoneal and intrauterine cPAF-induced preterm delivery as well as fetal death. These results imply existence of both local and systemic mechanisms of cPAF induction of preterm delivery, and show fetal loss is at least partly TLR4 dependent. It is possible that the blocking of TLR4 results in a downregulation of PAFR expression, to interact with cPAF in mediating the downstream inflammation leading to preterm delivery and fetal loss. Furthermore, it also seems that PAF induction of PAFR is also TLR dependent. It is because, peritoneal macrophages derived from *Tlr4-/-* mice have lower expression of *Pafr* mRNA, either with or without treatment with cPAF (332). These results also suggest that further studies are required to demonstrate direct binding of cPAF to TLR4, this could be achieved by co-immunoprecipitation and pull-down assays.

(+)-Naltrexone prevented the reduction in placental efficiency but not growth restriction induced by intrauterine cPAF administration, suggesting that a TLR4-independent mechanism is involved in perpetuating the local cPAF induction of fetal growth restriction. It seemed evident that systemic administration of cPAF is more efficient in inducing preterm delivery, in comparison to a local route of administration, and thus this route of delivery was used to further study the mechanisms of cPAF induction of preterm delivery in mice. A study investigating the role of cPAF in mediating tumour growth and metastasis has shown that *Tnf* mRNA is induced in the lung of

mice after 4 hours of cPAF intraperitoneal administration, at a dose of 0.1 mg/kg (411). This was supported by an in vitro study demonstrating the stimulation of IL6 and CXCL8 at both the gene and protein level following 4 hours of the treatment of human uterine cervical fibroblast with 10^{-8} M cPAF (322).

Here, after 4 hours of intraperitoneal cPAF injection on gd 16.5, there was a significant elevation in *Il1b* and *Il6* mRNA in the decidua, myometrium and placenta. There was also an upregulation of *Il10* expression presumably in order to control the inflammation induced by cPAF in these gestational tissues. This is consistent with previous studies demonstrating that *Il10*-/- have higher susceptibility to LPS induced preterm delivery (76,77) in mice. The other pro-inflammatory cytokine genes including *Il12b, Tnf* and *Il1a* were not upregulated after 4 hours of cPAF administration in maternal or fetal tissues of wildtype females. This might reflect the timing at which the tissues were harvested for analysis, as a previous ex vivo study showed significant production of TNF cytokine 5 hours after treatment of peritoneal macrophages with cPAF (332).

Whilst there are limited studies investigating the role of TLR4 in mediating infection-induced inflammatory cytokines in gestational tissues, one study has shown that uterine inflammatory cytokine gene expression including *Il1b* and *Tnf* mRNA was reduced in *Myd88/Trif*-/- mice 3 hours after intrauterine heat killed *E.coli* administration on gd 14.5 (70). Both MYD88 and TRIF are the adaptor molecules for TLR4 which are recruited and activated following TLR4 ligation, to induce the downstream inflammatory cytokines, thus deficiency in either of these adaptor molecules would result in abrogation of the inflammatory response. Both TRIF and MYD88 were shown to be important in cPAF stimulation of pro-inflammatory cytokines in ex vivo experiments (332). Hence, it is important to investigate the role of TLR4 in mediating the induction of inflammatory cytokine gene expression in gestational tissues, as the underlying mechanism eliciting preterm delivery in mice.

Mice deficient in TLR4 only exhibited downregulation of *Il1b* in the decidua and placenta 4 hours following cPAF injection when compared to wildtype females administered with cPAF. This likely contributed to the observed fetal deaths as well as poor fetal outcomes in the surviving fetuses from the *Tlr4-/-* females treated with cPAF, as most of the pro-inflammatory cytokines were not altered by TLR4 deficiency in these gestational tissues. These results also reflect a possible TLR4-independent pathway, which may be mediated by PAFR and TLR2 (323,332) to induce upregulation of pro-inflammatory and anti-inflammatory cytokines in response to cPAF administration in *Tlr4-/-* mice.

Interestingly, the cPAF induction of *Il1b*, *Il6* and *Il10* was reduced in both decidua and placenta by the administration of one dose of (+)-naltrexone. The administration of (+)-naltrexone inhibited cPAF induction of *Il10*, but not other cytokines in the myometrium. This is presumably because the myometrium is in contact with the peritoneal cavity, with high levels of inflammatory mediators stimulated by intraperitoneal cPAF administration. One dose of (+)-naltrexone might not be sufficient in suppressing the inflammation in this tissue. Thus following TLR4 blockade, suppression of cytokine expression in the fetal and maternal tissues appears to contribute to inhibiting cPAF effects and to protect mice from preterm delivery as well as fetal death.

cPAF injection resulted in an elevation in *Ptghs2* mRNA to vehicle control in both the decidua and myometrium of wildtype mice. These results are supported by an in vitro study revealing that cPAF treatment induced the upregulation of *Ptghs2*, resulting in the synthesis of prostaglandin E in human choriodecidua (315). Moreover, mice administered PTGHS2 inhibitor (SC-230) orally were also resistant to LPS-induced preterm delivery on gd14.5, when compared to mice given LPS alone (191), suggesting that PTGHS2 production is also crucial in infection-mediated preterm delivery. In vitro studies from both human and mice revealed that pro-inflammatory cytokines play a role in inducing the expression of uterine activation genes such as *Ptghs2, Ptgfr and Oxtr* (121-126). Thus, it is also possible that a time point beyond 4 hours is required to capture the induction of other uterine activation genes such as *Ptghs1, Oxtr, Gja* and *Ptgfr* expression triggered by cPAF injection.

Expression of *Ptghs2* mRNA was not suppressed in these maternal tissues in response to both cPAF and (+)-naltrexone administration in BALB/c females as well as cPAF injection in *Tlr4-/-* females, compared to cPAF administered wildtype females. This evidence suggests that other signalling pathways (323,332) contribute to the cPAF upregulation of this uterine activation gene. In the myometrium, an induction in *Oxtr* mRNA was observed in cPAF and (+)-naltrexone treated wildtype females relative to (+)-naltrexone control wildtype females or the cPAF treated wildtype females, which suggests that the drug might have an effect on stimulation of *Oxtr* gene expression especially in the presence of cPAF, and should be further studied.

In the future, it would be interesting if the expression of the inflammatory cytokines and uterine activation genes in the gestational tissues can be confirmed at the protein level using ELISA, bead array or western blot assays. In response to systemic administration of cPAF or cPAF together with (+)-naltrexone, the concentrations of inflammatory cytokines could be measured using bead array in the serum as well as peritoneal cavity lavage of mice, other than in the gestational tissues. In vitro studies portrayed that cPAF treatment of human uterine cervical

fibroblasts resulted in the secretion of MMP1 (323) as well as chemokines such as CCL2 and CCL5 production in human uterine cervical fibroblasts (322). Thus, the effect of cPAF administration on the expression of MMP genes especially in the fetal membrane as well as pro-inflammatory chemokines in the gestational tissues could be further examined. The role of TLR4 blockade as well as TLR4 deficiency in mediating cPAF induction of MMP and inflammatory chemokine expression could be investigated to understand how gene expression is linked to leukocyte recruitment.

Overall, we have demonstrated for the first time that intraperitoneal cPAF administration induces preterm delivery in mice and affects the viability of the fetuses, and that these effects can be abrogated by treatment with the TLR4 antagonists, (+)-naltrexone. *Tlr4-/-* females are also resistant to cPAF induction of preterm delivery indicating a contribution of TLR4 in the underlying mechanism. The fetal loss as well as poor fetal outcomes were not prevented by TLR4 deficiency, showing cPAF operates through pathways in addition to TLR4, such as PAFR and TLR2. cPAF induction of pro-inflammatory and anti-inflammatory cytokines including *Il1b, Il6* and *Il10* in gestational tissues was suppressed via this TLR4 blockade using (+)-naltrexone, but not efficiently byTLR4 deficiency. cPAF upregulated *Ptghs2* mRNA, and this was not downregulated by TLR4 blockade or TLR4 deficiency, again implying TLR4 interacts with other pathways to mediate cPAF-induced effects in the gestational tissues.

Chapter 6: General Discussion and Conclusion

6.1 DISCUSSION AND CONCLUSION

Prior to and during parturition, increased synthesis of inflammatory cytokines and infiltration of leukocytes into the gestational tissues initiates a progressively accelerating inflammatory cascade that eventually upregulates prostaglandin pathways and MMP synthesis, leading to activation of uterine contractions, cervical ripening and the rupture of fetal membranes. Previous studies demonstrate that levels PAF, SP-A, HMGB1 and HSP70 are all elevated and can have inflammatory effects in the gestational tissues during labour (81,248,249,267,312,322,323,341,343,344). PAF, SP-A, HMGB1, HSP70, uric acid, 5-cholesten-3β, 25-diol and 5-cholesten-3β-ol-7-one are able to mediate the sterile inflammation in preterm delivery that occurs without infection in the event of multiple gestations, cervical insufficiency, or exposures to other stressors or toxins (260,271,273,276,329,330,344). This is consistent with studies in mice, where local administration of rHMGB1 (275), cPAF (331), rSP-A (344) and fetal fibronectin (204) can trigger preterm delivery, without the presence of infection. Interestingly, the induction of pro-inflammatory processes by many of these DAMPs and TLR4 regulators is associated with TLR4 (204-207,210,211,298,332,350,351,372), which is expressed by immune cells and structural resident cells in the reproductive compartments of humans and mice (156-167,372). TLR4 has been indicated to potentially play a key role in the mechanism underlying preterm delivery mediated by infection. There is elevated expression of *TLR4* in human fetal membranes at normal term delivery (61) and in the cervix as well as uterus of mice as pregnancy approaches its end (166). Prior to this study, it was not known whether TLR4 is essential for on-time term parturition and in particular, a contribution to sterile inflammation of preterm delivery mediated by DAMPs and TLR4 regulators in mice had not been explored.

In this study, we provide new evidence that TLR4 activation in late gestation plays an important role in inducing an inflammatory cascade in the gestational tissues leading to on-time labour in mice. Our experiments suggest that elevated level of cPAF in the fetal lung and amniotic fluid (81) may contribute to TLR4 activation of inflammation, to initiate the process of parturition. In addition, we have shown that administration of cPAF causes activation of TLR4 and is efficient to provoke greater inflammatory responses than in normal term delivery in the gestational tissues, leading to early onset of parturition and preterm delivery in mice.

In chapter 3, we describe experiments showing that in the sterile inflammation of normal term labour, genetic TLR4 deficiency caused a 13-hour delay in labour accompanied by an increase in pup weight at birth. This adds to existing knowledge that TLR2 is required for normal

on time birth and birth weight (71). In *Tlr4*-/- mice a downregulation in inflammatory cytokines in the brain of the fetuses on gd 18.5 and a reduced viability of the pups after birth suggests that TLR4 activation at the end of gestation is not only crucial for on-time parturition, but also is likely to have physiological roles in fetal development, which affects the viability of the pups. Thus, the role of TLR4 in mediating the induction of inflammatory cytokines and uterine activation gene expression which regulates on-time labour in healthy, infection-free pregnancy was explored.

The expression of pro-inflammatory cytokine genes including *Il1b*, *Il6*, *Il12b, Tnf* and *Il10* in the placenta and fetal membranes in TLR4-deficient pregnancies was reduced near term compared to wildtype mice. The expression levels of pro-inflammatory cytokines in the maternal tissues were not as substantially affected by TLR4 deficiency. It is notable that myometrial *Il1b* and *Il6* mRNA expression were also not affected in late gestation by TLR2 genetic deficiency (71). Overall, it can be interpreted that in late gestation, the first phase of TLR4 activation occurs in the fetal membranes and placenta as opposed to the maternal compartment, possibly in response to the elevated concentrations of PAF, SP-A and HSP70 in amniotic fluid, to initiate the progress of parturition (81,248,249,267,312,343,344). The transient delay in the upregulation of expression of uterine activation genes including *Ptgfr*, *Oxtr* and *Gja1* in maternal tissues of TLR4 deficient mice is likely to be a consequence of reduced pro-inflammatory cytokines in the absence of TLR4 activation in the fetal tissues, as these pro-inflammatory cytokines are known to induce the uterine activation genes and proteins (73,121-126,370).

In chapter 4 we further elucidate the role of TLR4 in mediating the mechanism of inflammatory leukocyte infiltration, which contributes to parturition. We showed that TLR4 deficiency resulted in reduced neutrophils on gd 18.5, in turn affecting immigration of other leukocytes including macrophages, dendritic cells and T cells into the placenta immediately prior to delivery on gd 19 and gd 19.5. This might then explain the reduced pro-inflammatory cytokine genes that were observed in the placenta of *Tlr4*-/- mice on gd 19.5 as demonstrated in chapter 3, since at least a proportion of cytokine expression reflects production within infiltrating leukocytes. In the fetal membranes, the populations of these inflammatory leukocytes were not altered in the absence of TLR4 in late gestation. It is possible that leukocytes in this tissue fluctuate less in late gestation than other sites, since there is no increase in invasion of leukocytes into fetal membrane during human labour (58). It is plausible that in late gestation TLR4-mediated activation of resident structural cells occurs to induce pro-inflammatory cytokine expression in the fetal membranes, as shown in Chapter 3. TLR4 deficiency also did not result in decreased infiltration of neutrophils and macrophages in the myometrium and decidual tissues consistent

with our data in chapter 3 where the expression of pro-inflammatory cytokines were not decreased in these maternal tissues of *Tlr4*-/- mice. Signalling pathways other than TLR4 might compensate for TLR4 deficiency, and be involved in mediating inflammatory cytokine expression and infiltration of neutrophils and macrophages in the maternal tissues.

In the myometrial compartment dendritic cells were reduced in *Tlr4*-/- mice possibly because TLR4 activation by DAMPs and TLR4 regulators causes secretion of chemokines to regulate dendritic cell recruitment (377) as well as to induce their maturation and activation (373,377-379). Nevertheless, we did not find evidence of altered activation state within dendritic cells due to TLR4 deficiency. Failure of dendritic cells to remove and silence Treg cells (381,382) in the absence of TLR4 signalling may explain the elevated Treg cell population we observed in the myometrium of *Tlr4*-/- mice.

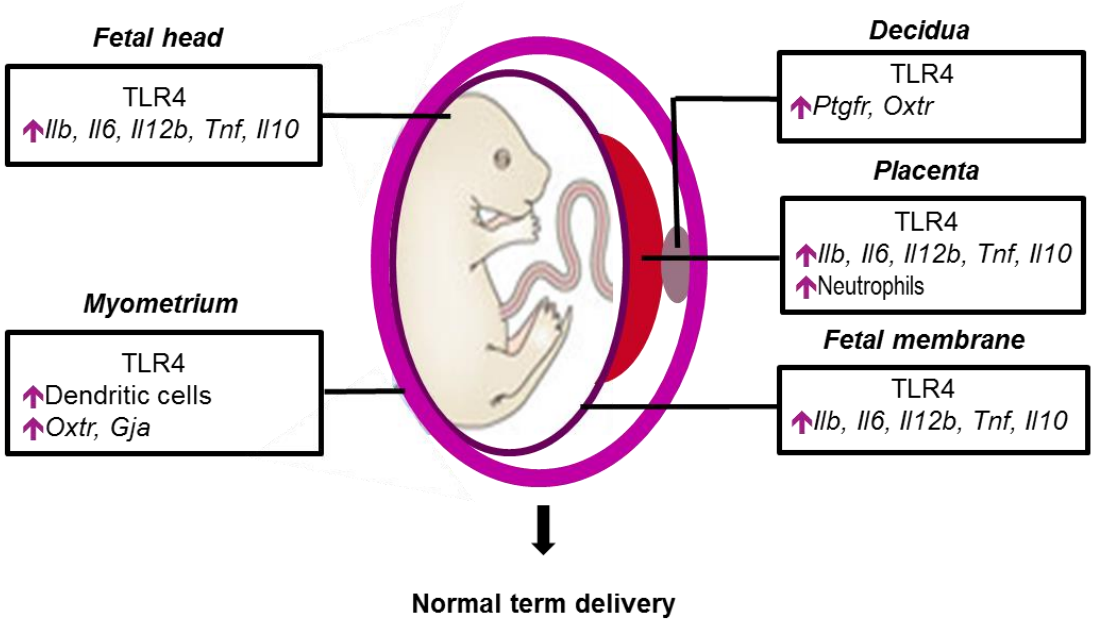


Figure 6.1: Schematic illustration of the mechanisms of TLR4 activation of normal term delivery

Our results suggest that TLR4 activation in late gestation induces the synthesis of pro-inflammatory cytokine genes including *Il1b*, *Il6, Il12b, Tnf* and *Il10* in the fetal tissues such as fetal head, fetal membrane and placenta. TLR4 activation in late gestation also mediates the recruitment of leukocytes such as neutrophils and dendritic cells into both placenta and myometrium respectively. These inflammatory cytokines and cells may participate in the upregulation of uterine activation genes, including *Ptgfr, Oxtr* and *Gja* in the maternal myometrium and decidua. Together, these mechanisms appear to contribute to normal on-time delivery.

In chapter 5, we sought to investigate how TLR4 may be involved in preterm birth mediated by DAMPs and TLR4 regulators. Initially, in vitro experiments were carried out to determine the pro-inflammatory activity of DAMPs and TLR4 regulators including 5-cholesten-3β, 25-diol, 5-cholesten-3β-ol-7-one uric acid, rHMGB1, cPAF and SP-D, using the J774 macrophage cell line. 5-cholesten-3β, 25-diol, uric acid, rHMGB1 were demonstrated to promote inflammatory cytokines and possibly chemokines in these macrophages. Even though cPAF did not have inflammatory effects in the macrophage cell line, it has been demonstrated to promote inflammatory cytokine and chemokine release (332,395) in primary macrophages. Thus, we proceeded with in vivo experiments to determine the inflammatory effects of 5-cholesten-3β, 25-diol, uric acid, rHMGB1 and cPAF in the serum of mice. It was found that intraperitoneal administration of uric acid or cPAF to day-16.5 pregnant females resulted in an increase in cytokine and chemokine levels in the serum.

Overall this data provided justification for the intraperitoneal administration of uric acid and cPAF in wildtype females to examine if these ligands were capable of inducing preterm delivery. We were unable to elicit preterm delivery or affect fetal viability with uric acid. Interestingly, intraperitoneal administration of cPAF triggered preterm delivery accompanied by significant fetal loss. This is consistent with a previous study revealing that systemic administration of cPAF induced intrauterine fetal growth restriction in rats (333,334). This model of systemic cPAF induction of preterm delivery mimics some aspects of human preterm delivery mediated by sterile inflammation. Maternal exposure to toxins such as drugs, cigarette smoke or maternal stress (21,25,27,28,31) can be a source of PAF secretion systemically, following cell stress.

Interestingly, both TLR4 deficiency and blocking of TLR4 using (+)-naltrexone were efficient in inhibiting intraperitoneal cPAF induction of preterm delivery, indicating that the activation of the early onset of labour in the setting of systemic inflammation is TLR4-dependent. Possibly, the downregulation of PAFR expression caused by TLR4 deficiency and blocking of TLR4 affects cPAF interaction with PAFR to mediate inflammation, causing preterm birth. It is also probable that PAF induction of PAFR is also TLR4 dependent. Additionally, the blockade of TLR4 using (+)-naltrexone, but not genetic TLR4 deficiency, was shown to prevent cPAF-induced fetal loss, showing some aspects of cPAF activity that cause fetal demise are mediated independently of TLR4. We speculate that the altered fetal: weight ratio in *Tlr4*-/- mice compared to wild-type control mice indicates a placental defect which might increase their susceptibility to TLR4-independent induction of fetal death.

Then the mechanism of inflammatory cytokines and uterine activation gene expression behind cPAF induction of preterm delivery via TLR4 signalling was further studied. An in vitro study revealed that cPAF stimulated pro-inflammatory cytokine and chemokine in human uterine cervical fibroblasts (322). In our study, exogenous cPAF promoted an elevation in pro-inflammatory cytokine genes including *Il1b*, *Il6* and *Il10* mRNA in the decidua, myometrium and placenta of wildtype mice. This implies that cPAF provokes a pro-inflammatory response in the fetal and maternal tissues to mediate the early onset of parturition in mice. (+)-Naltrexone prevented the upregulation of these inflammatory cytokines especially in the decidua and placenta to inhibit preterm delivery and fetal death induced by cPAF. The only suppression evident in mice deficient in TLR4 was in *Il1b* mRNA in the decidua and placenta following cPAF injection. This indicates IL1B must be critical in protection from preterm delivery in the *Tlr4*-/- females.

cPAF injection also resulted in an elevation in *Ptghs2* mRNA in both decidua and myometrium of wildtype mice, which is consistent with an in vitro study revealing that cPAF induced upregulation of *Ptghs2* in human choriodecidua (315). *Ptghs2* mRNA was not suppressed in these maternal tissues by either TLR4 deficiency or TLR4 blockade, suggesting that as well as TLR4, TLR4-independent pathways are involved in cPAF stimulation of uterine activation genes.

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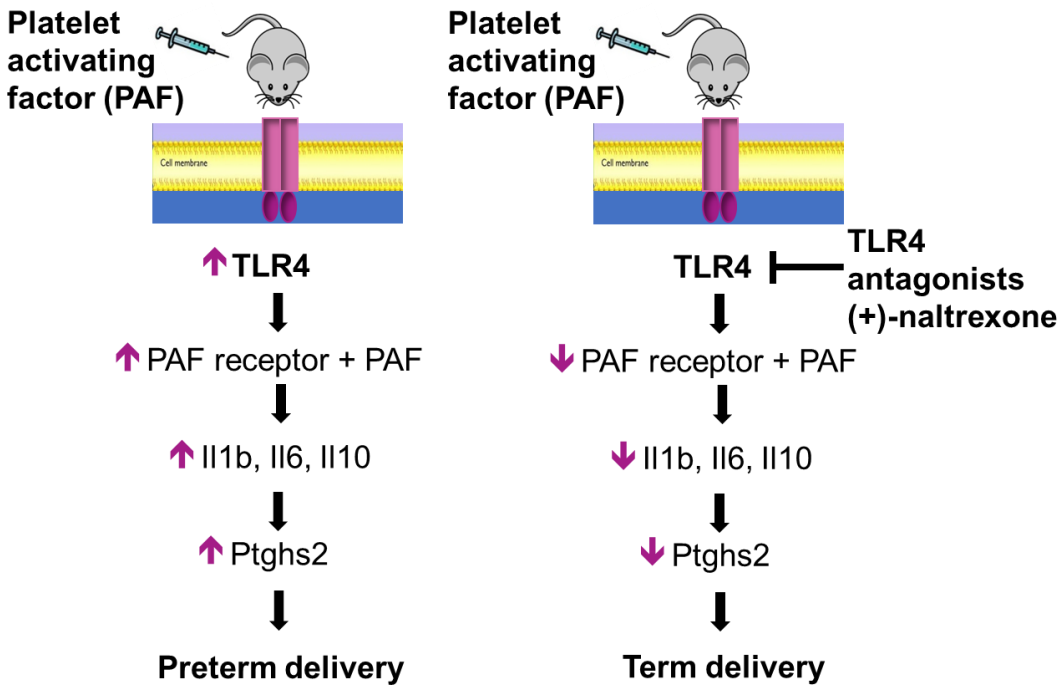

Figure 6.2 Schematic illustration of the mechanisms of TLR4 activation of preterm delivery induced by cPAF

Intraperitoneal cPAF administration may cause elevation of TLR4, which can induce PAFR expression to to promote an inflammatory response, including elevated pro-inflammatory cytokines and uterine activation gene expression, to trigger preterm delivery and reduce the viability of fetuses in mice. Administration of TLR4 antagonist (+)-naltrexone inhibits the inflammatory response towards cPAF and prevents ensuing induction of preterm delivery. The results also suggest that premature elevation of PAF may be an endogenous sterile trigger of preterm delivery.

Table 6.1 Expression of inflammatory cytokines and uterine activation genes in gestational tissues in response to administration of cPAF alone or cPAF together with (+)-naltrexone

	*Intraperitoneal cPAF	^Intraperitoneal Cpaf + (+)-Naltrexone
Cytokines:		
Fetal membrane	↑*Il10*	_
Placenta	↑*Il1b, Il6*	↓*Il1b, Il6*
Decidua	↑*Il1b, Il6, Il10*	↓*Il1b, Il6, Il10*
Myometrium	↑*Il1b, Il6, Il10*	↓*Il10*
Uterine activation genes (decidua and myometrium)	↑Ptghs2	_
Preterm delivery	↑↑	↓↓

* change with cPAF treatment, relative to PBS control

^ change with cPAF + (+)-naltrexone, relative to cPAF treatment

A limitation of this study is that we are not able to delineate the relative contribution of TLR4 in fetal and maternal tissues. In future, the effect of matings between *Tlr4*-/- BALB/c females and wildtype BALB/c males, to give rise to *Tlr4*+/- heterozygous (TLR4 replete) fetuses in TLR4 deficient mothers, on the timing of labour and the inflammatory mechanisms in the gestational tissues could be examined to determine whether fetal or maternal TLR4 is more important in the inflammation required for normal term labour. Importantly, TLR4 is not the only toll-like receptor implicated in term labour as previous experiments show that TLR2 deficiency leads to a similar increase in gestation length as does TLR4 deficiency (71). TLR2 and TLR4 share endogenous ligands that are produced in the later stages of gestation, including HSP70 (206), SP (71) and HMGB1 (210,211,412). Thus, it would be interesting to examine the effect of both TLR2 and TLR4 deficiency in double deficient *Tlr2/Tlr4*-/- mice on the timing of parturition and inflammatory mechanisms that involved.

It would be valuable to investigate the role of other TLR4 ligands particularly HSP70 and HMGB1 on the gestational length, perinatal outcomes as well as the mechanisms of inflammation in the gestational tissues in late gestation. This could be achieved by utilising HSP70 deficient mice or by inhibition of these DAMPs using specific antagonists or antibodies. This is because

HSP70 mice are viable, but not the HMGB1 ablated mice (413,414). It will be of interest to also investigate the effect of TLR4 deficiency on the release of DAMPs and TLR4 regulators including PAF, HSP70 and HMGB1, at time points over the course of late gestation on gd 16.5, 17.5, 18.5 or 19.5. This is because inflammatory cytokines including TNF, IL1A, IL1B and IFN, released after TLR4 activation, can stimulate the secretion of PAF, HSP70 and HMGB1 in in vitro experiments (203,268,269,318), showing a feed forward loop in cytokine and DAMP release may exist.

Investigation of different time points after cPAF administration would be required to capture the upregulation of other inflammatory cytokines including *Il12b, Tnf* and *Il1a* and other uterine activation genes *Ptghs1, Oxtr, Gja* and *Ptgfr*. It seems likely that the latter uterine activation genes would require more than 4h to be induced after cPAF administration, since previous studies show these genes are induced secondary to cytokine synthesis. At a longer time point, such as 12h after cPAF treatment, it seems likely an effect of TLR4 in mediating the induction of additional inflammatory cytokines and uterine activation genes could be observed. It would also be interesting to investigate higher doses of cPAF to see whether a higher rate of adverse effect will be elicited. There are only limited studies to explore the mechanism of leukocyte invasion including macrophages, neutrophils, dendritic cells, T cells and NK cells in the gestational tissues in response to cPAF or other DAMPs and TLR4 regulators in mouse models of preterm delivery. It would be reasonable to determine if TLR4 is required to elicit recruitment of these leukocytes into the gestational tissues, in response to exogenous cPAF.

In humans, smoking which results in fetal hypoxia (239), may cause increased synthesis of PAF in the fetal lung to contribute to its elevated level in the amniotic fluid (329,330), and thus provoke inflammation leading to preterm delivery. Therefore, a human population-based study to investigate the association between smoking and elevated level of PAF in human preterm delivery would be of value. If tissue samples could be collected, such a study could also determine the association between genetic polymorphisms in TLR4 and PAF-signalling genes with human preterm delivery, and their interaction with smoking and other clinical and environmental factors linked with sterile spontaneous preterm labour, such as multiple pregnancy, exposure to air pollution and environmental toxins, and psychosocial stress.

In order to mimic the intra-amniotic elevation of PAF in preterm birth (329,330), it would be interesting to investigate the effect of cPAF intra-amniotic administration in mediating preterm birth in mice. Additional experiments should investigate the mechanisms involved, including

effects on inflammatory cytokines and uterine activation gene expression as well leukocyte recruitment into gestational tissues. If these are demonstrated to be induced by cPAF, an elevated level of PAF in the amniotic fluid could potentially be a predictive marker for threatened preterm delivery in humans.

One of the limitations of this study is the use of exogenous cPAF to model elevated endogenous levels of PAF. cPAF is modified to prevent degradation by PAF-AH; however, this form of PAF does not naturally exist either systemically or locally in the gestational tissues. It is possible that in humans, deficiency in PAH-AH systemically or locally could potentially contribute to the elevation of endogenous PAF to trigger preterm delivery. The effects of PAF-AH deficiency or PAF agonists in abrogating sterile inflammation of cPAF-induced preterm delivery, and the inflammatory mechanisms involved could be investigated in mice. It would then be worthwhile to measure PAH-AH activity in the serum or plasma of women at risk of preterm delivery using colorimetric assay (415,416), and to link this with genomic tests to investigate the association between genetic polymorphisms in PAF-AF and preterm delivery.

In summary, our study provides new evidence on the association between PAF and TLR4 in induction of the downstream inflammatory pathways in preterm delivery. Our study also suggests a possible role of PAF as a ligand of TLR4 in mediating inflammation generally, which needs to be addressed in future studies. The work implicates a crucial role for TLR4 as a key agent at the apex of the inflammatory cascade in both sterile inflammation of preterm and term delivery, adding to prior knowledge of TLR4 as central in infection-associated preterm labour. This study provides new pathways for investigation in potential clinical studies especially to investigate prevention or delay of preterm delivery in humans. The significance of TLR4 is especially exciting because genetic polymorphisms in TLR4 are associated with preterm delivery risk in humans (417). In our study, as well as preventing preterm delivery, the TLR4 antagonist (+)-naltrexone also rescued fetal loss via the inhibition of inflammation in gestational tissues upon challenge with cPAF. This work suggests that (+)-naltrexone may have potential as a therapeutic invention in human spontaneous preterm delivery associated with PAF-inducing conditions such as smoking, drug use, stress and multiple pregnancy. Nevertheless, extensive studies will be required to explore how PAF-induced activation of inflammation is involved in human tissues, as we have demonstrated in mice. Next it will be necessary to elucidate the relevant signalling pathways by which PAF signals in human tissues including the contribution of TLR4, PAFR and TLR2 pathways. Only then it can be determined whether blocking TLR4 is likely to be effective and sufficient to achieve suppression of PAF induction and delay of preterm delivery in humans.

But it is important to also note that in our study, inhibition of TLR4 in late pregnancy interferes with fetal growth in late gestation and at the time of birth and thus it is potential that TLR4 plays an important physiological role in fetal development. This should be taken into consideration in any development of pharmacological approaches targeting TLR4, as it may have significant implications for the safety and acceptability of of drugs that suppress TLR4 signalling. In addition, PAF levels have also been demonstrated to be induced by infection, via the reduction of PAH-AH in vitro (318,384,385,418). In vivo experiments revealed that the endogenous PAF production is crucial in enhancing infection-induced inflammation in maternal and fetal tissues, to cause early activation of cervical ripening and preterm delivery in mice (331,332,336). From this evidence it seems likely that in human preterm delivery associated with intra-amniotic fluid infection, higher levels of PAF accumulate in the amniotic fluid and amplify the infection-induced inflammation. New insight on the contribution of PAF, other TLR4 regulators and DAMPs to infection-associated preterm birth may explain why attempts to prevent or arrest preterm delivery using antibiotics often have poor efficacy. It may well be that in infection-associated preterm birth, inhibition of TLR4 activation, could suppress amplification of inflammation driven by PAMPs, DAMPs and TLR4 regulators and may be a useful strategy in preventing preterm delivery that could be employed as an adjunct therapy with antibiotics.

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