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EXPRESSION AND ACTIVITY OF THE TOLL-LIKE RECEPTOR FAMILY IN THE TERM AND PRETERM HUMAN PLACENTA.

by

Shalini Patni

Submitted to the University of Wales in fulfilment of the requirements for the Degree of Doctor of Medicine (MD)

Swansea University

2007

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Summary

Toll like receptors (TLR) have emerged as key upstream mediators of inflammation at many tissue sites in humans. Parturition is considered an inflammatory process so it was hypothesised that TLR activity within gestation-associated tissues, such as the placenta, might have an important role in the initiation and/or maintenance of normal term labour and in various pathological states of pregnancy such as infection associated preterm labour.

Expression of transcripts for TLR1–10 was confirmed in term (> 37 weeks of gestation) human placentas collected in the absence of labour (elective caesarean sections, *term non-laboured*) and after the completion of labour at term (normal vaginal delivery, *term laboured*,) and preterm (<37 weeks gestation, preterm delivery). Explants of placental tissue were cultured *in vitro* in the presence of ligands for TLR 1-9 (an agonist of TLR10 has not yet been identified). Cytokine (TNFα, IL-6, IL-8 & IL-10) production into the culture supernatants was then measured using ELISA.

Reactivity to all agonists except CpG oligonucleotides was observed in all the three groups studied indicating that, other than TLR9, all of the receptors studied yielded functional responses. Placentas collected after the completion of labour (*term laboured*; n=17) had significantly more LPS (TLR4 agonist) and R848 (TLR7/8) agonist induced TNFα than those obtained in the absence of labour (*term non-laboured*; n=17). In contrast, gene expression analysis revealed that transcripts for TLR2 and TLR5 only were significantly elevated in association with labour. These findings indicate that there is no relationship between changes in mRNA expression and function of these receptors within the placenta.

The study of placentas collected following preterm spontaneous vaginal delivery (*preterm svd*; n=10) compared with those obtained after *term laboured* (n=17) revealed that significantly less TNFα and IL-6 were produced by placentas following preterm delivery in response to all TLR agonists. In contrast, the expression of TLR transcripts was found not to be statistically different for any of the TLRs1-10 in these two groups.

Thus the human term placenta expresses a variety of functional TLRs and whilst LPS and R848 mediated TNFα increase at term labour, more detailed analysis of contributing cell types and signalling molecules is required to elucidate the role of this family of receptors in parturition.

Declaration

This work has not been accepted previously in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed...Dr. S Patni

Date......26.08.07..

Statement 1

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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Dedicated to

To my husband Anjum and my son Abhinav for their unabated patience and support

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ABBREVIATIONS

BSA	Bovine Serum Albumin
COX2	Cyclo-oxygenase
DD	Death domain
ECS	Elective caesarean section
ELISA	Enzyme linked immunosorbent assay
GCSF	Granulocyte colony-stimulating factor
IA-PTL	Infection associated preterm labour
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN- γ	Interferon gamma
IL-1β	Interleukin 1 beta
IL-1ra	Interleukin 1 receptor antagonist protein
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-18	Interleukin 18
IKK	IkappaB kinase kinase
IUGR	Intra-uterine Growth restriction
IRFs	Interferon regulatory factors
IRAK	Interleukin-1receptor associated kinase
JNK	c-Jun N-terminal kinase

LPS	Lipopolysaccharide
LBP	Lipopolysaccharide binding protein
MAP	Mitogen activated protein kinase
MAL	MyD88-adaptor-like
MCP-1	Monocyte chemotactic peptide-1
MIP-1α	Macrophage inflammatory protein-1alpha
MMP	Matrix metalloproteinase
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor-kappa B
NEMO	NF-κB essential modifier
NO	Nitric oxide
NVD	Normal vaginal delivery at term
PAMP	Pathogen associated molecular patterns
PBS	Phosphate Buffered Saline
PG	Prostaglandins
PGN	Peptidoglycan
PolyI:C	Polyinosine-polycytidilic acid
PPROM	Preterm premature rupture of membranes
PRR	Pattern recognition receptors
PTD	Preterm delivery
qPCR	Quantitative real-time polymerase chain reaction
RANTES	Regulated upon Activation, Normal T cell Expressed and presumably Secreted

RT-PCR	Reverse transcriptase-polymerase chain reaction
SVD	spontaneous vaginal delivery
TAK-1	Transforming growth factor-β activated kinase-1
TANK	TRAF-associated NFκB activator
TBK-1	TANK-binding kinase-1
$TGF\beta_2$	Transforming growth factor-β2
TIMP	Tissue inhibitor of metalloproteinase
TIR	Toll Interleukin 1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TLRs	Toll-like receptors
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor-alpha
Tollip	Toll-interacting protein
TRAF-6	TNF-receptor associated factor-6
TRIF	TIR domain-containing adaptor inducing IFN-β
TIRP/ TRAM	TRIF-related adaptor molecule

CHAPTER 1

INTRODUCTION

1.1 PRETERM LABOUR

Preterm birth, defined as delivery before 37 weeks of pregnancy (WHO 1993) has an estimated worldwide incidence of 13 million per annum. It complicated about 11.9% of all pregnancies in 2001 (Martin, Hamilton et al. 2002). The number of babies born preterm has been increasing steadily for the past two decades reaching up to 25% in some developed and developing countries (Steer 2005). Prematurity alone accounts for 75% of perinatal morbidity and mortality in the developed world (Besinger 1993). Of the 600,000 live births in the UK every year, about 8,000 will be very premature and weigh less than 1500 grams: of these around 1,600 die, 600 develop cerebral palsy, and many others will have a variety of disabilities such as bronchopulmonary dysplasia, intra-ventricular haemorrhage, patent ductus arteriosus, retinopathy of prematurity and poor school performance in later years (Hole and Tressler 2001). Thus attempts to reduce the present wastage of foetal life are essential.

The second and third trimesters of pregnancy are vital for maturation of the fetal lungs and other organs in preparation for extrauterine life. Interruption of this development by very early delivery markedly diminishes the chances of survival of the newborn even with the best neonatal care. Preterm birth thus also imposes a significant economic burden, from both immediate neonatal intensive care unit costs (estimated at approximately 10% of all paediatric health care costs in the United States and amounting to approximately $5.5–6 billion/year) together with the long-term costs for the residential care or support of infants with residual disabilities (Lewitt, Baker et al. 1995).

If the problem of prematurity is to be tackled successfully, there are three facets to consider – firstly, and ideally, the prevention of preterm labour, or failing that successful recognition and implementation of treatment directed at inhibiting myometrial contractions, and lastly the care of the preterm newborn. Any or all of these would considerably affect perinatal morbidity and mortality statistics and therefore deserve to be the number one priority of contemporary obstetrics.

Despite much effort during the past 40 years, nearly all of the improvements with treating this pregnancy complication are due to advances in neonatology (Lumley 2003). Indeed, the probability of infant survival for 30 completed weeks of gestation is now 90% compared to only 45% in 1960 (Reichman 2005) and the limit of fetal survival has been extended to 24 weeks (Creasy and Iams 1999). The 2% of babies born before 32 weeks, however, account for about 70% of all infant mortality (Lopez-Bernal and Tamby 2000; Martin, Hamilton et al. 2002).

Whilst delaying delivery by using tocolysis would decrease the rate of long-term morbidity there is as yet no effective treatment available for this. Alternatively, accurate prediction of preterm birth would at least allow the administration of corticosteroids known to significantly reduce the associated mortality and morbidity. However, there is currently no accurate method of predicting preterm birth and, moreover, half of all preterm births occur in women with no known clinical risk factors (Iams, Goldenberg et al. 2001).

Thus, the prevention of preterm labour seems to be the most viable step in dealing with the problem of prematurity. Certainly this requires a better understanding of the basic aetiology of preterm labour. However the onset and progress of labour, both at term and preterm are only beginning to be understood (Farina and Winkelman 2005).

1.1.1 Aetiology of Preterm labour

In his report on 'Epidemiology of preterm birth', Main (Main 1988) classified factors inherent in causation of preterm birth into proximate causes and risk factors.

Proximate causes were categorised into four definite obstetric diagnoses directly responsible for preterm delivery namely, 1) idiopathic preterm labour, 2) preterm premature rupture of membranes (PPROM), 3) maternal medical and obstetrical complications, 4) fetal distress or demise.

Romero (Romero and Mazor 1988) later reported the individual contribution of these categories to be approximately equal, i.e. one third each for idiopathic preterm labour, PPROM and for the combination of maternal and fetal factors. Idiopathic preterm labour comprised of those cases in which no obvious maternal or fetal cause could either be discerned or attributed to the occurrence of spontaneous onset of labour remote from term. However, the main contributory factor to this preterm labour group is now considered to be infection as discussed in detail below (Lamont 2003) and the 'term' infection associated preterm labour (IA-PTL) will be used here on in. The PPROM group includes the pregnancies in which rupture of membranes occurs prior to 37 weeks of gestation. Finally, the maternal and fetal factors that contribute to preterm delivery include preeclampsia, abruption placentae and intra-uterine growth restriction (IUGR) that demand an early delivery to avoid any major complications to mother and/or fetus.

The known *risk factors* of prematurity include extremes of age and parity (nulliparity and grand multiparity), rapid succession of pregnancy, poor socio-economic status, race, maternal smoking, alcoholism and other substance misuse, poor nutrition, poor education, maternal anxiety, physical and sexual high risk activity, poor antenatal care and last, but not least, previous preterm births (Patni 1991).

1.1.2 Infection associated preterm labour

As prematurity secondary to maternal and fetal causes is inevitable, the main group to focus on in the fight against prematurity seems to be the infection associated preterm labour (IA-PTL) group, which to some extent merges into the PPROM group as membrane rupture could be consequential to the factors which initiated the preterm labour. Indeed, infections have been reported as responsible for up to 40% of all preterm labour cases (Lamont 2003). Furthermore, 80% of preterm deliveries occurring at less than 30 weeks of gestation have evidence of infection (Goldenberg, Hauth et al. 2000). Bacterial infection can occur in the choriodecidual space, fetal membranes, or within the amniotic fluid, placenta, umbilical cord or fetus. Ascending infection from the urinary or genital tract is considered to be the most common route of infection (Goldenberg and

Rouse 1998), however haematogenous spread of organisms from the other sites, such as opportunistic pathogens like *Fusobacterium nucleatum* from the oral cavity, is of increasing interest (Bearfield, Davenport et al. 2002). In this regard, amniotic fluid samples from 14% women were polymerase chain reaction (PCR) positive for *F. nucleatum.* A significant association was found between detection of microbial DNA (*F. nucleatum*) and complications in previous pregnancies including miscarriage, intrauterine death, neonatal death, preterm delivery and premature rupture of membranes. However, generally in most cases it is the cervicovaginal infection (ascending) that progresses to intra-uterine infection and inflammation.

Several investigators have studied the role of infection in preterm labour by performing amniocentesis and isolating bacteria from amniotic fluid. Bobbitt and Ledger were the first to suggest that unrecognised amnionitis, i.e. subclinical amniotic fluid infection, might be causally related to preterm labour (Bobitt and Ledger 1977). The amniotic cavity is normally sterile and therefore the isolation of any microorganism from the amniotic fluid constitutes evidence of microbial invasion (Romero, Gomez et al. 2001). Both Gram positive and Gram negative species have been isolated and the most common isolates from the amniotic cavity of women with IA-PTL are *Ureaplasma urealyticum*, *Fusobacterium* species, *Mycoplasma hominis, Escherichia coli,* Group B *Streptococcus* and *Gardnerella vaginalis* (Hillier, Martius et al. 1988; Hillier, Krohn et al. 1991) However, other species are also implicated: *Lactobacillus* species, coagulase negative *Staphylococcus*, *Enterococcus*, *Trichomanas vaginalis*, *Chlamydia trachomatis*, *Bacteroides* and *Peptococcus* species (Gravett, Nelson et al. 1986; Hitti, Hillier et al. 2001; Gojnic, Pervulov et al. 2005).

Similarly, unsuspected chorioamnionitis has been held responsible for a significant number of preterm births. Severe chorioamnionitis is found in 71% of all premature deliveries compared with 10% of term spontaneous deliveries (Sebire, Goldin et al. 2001). Chorioamnionitis arises from the infiltration of leukocytes, recently shown to be of maternal origin (Steel, O'Donoghue et al. 2005), into the chorion and amnion in response to intrauterine infection. The precise molecular mechanisms by which

infection so dramatically affects pregnancy remain undefined (Abrahams and Mor 2005b), however, the inflammatory mediators (cytokines and chemokines) themselves are postulated to play a prominent role in the pathogenesis of premature labour and birth (Gomez, Ghezzi et al. 1995; Goldenberg and Rouse 1998). Therefore in order to reduce the incidence of IA-PTL, an understanding of the molecular processes which occur in the gestational tissues (placenta, amnion and choriodecidua) following an infection and how the two interact to initiate labour is required.

1.1.3 Inflammation/Cytokines and term and preterm labour

Inflammation has long been recognised as a key feature of both preterm and term labour, with an influx of inflammatory cells into the uterus and elevated levels of pro-inflammatory cytokines observed during parturition (Tamsin M Lindstrom 2005). Elevated concentrations of inflammatory mediators, like cytokines and prostaglandins, in the amniotic fluid and extraembryonic tissues occurs in pregnancies with demonstrable microbial invasion of the materno-fetal interface. However, pregnancies without overt infection can also have elevated levels of amniotic fluid cytokines and prostaglandins, and it is unclear to what extent this reflects a secondary response in the fetal membranes to an underlying decidual infection or a physiological response as a normal part of labour. The elaboration of cytokines, chemokines and immunomodulatory proteins that support an inflammatory response in the placenta and gestational membranes has been investigated extensively in the context of both normal and abnormal pregnancy and delivery. While a robust inflammatory response accompanies premature labour and delivery, particularly in the presence of intrauterine infection, a more modest inflammatory response typifies normal term labour and labour has been called 'physiological inflammation' (Peltier 2003). Thus increased expression of cytokines such as interleukin-8 (IL-8) and interleukin-1beta (IL-1β) occurs in healthy amnion and choriodecidua in the third trimester (compared to earlier in pregnancy) and after labour, supporting a role for these cytokines in the establishment and/or maintenance of labour irrespective of gestation (Elliott, Loudon et al. 2001).

A link between the presence of bacterial infection at the materno-fetal interface and the initiation of labour is provided by studies showing that microbial products can stimulate cytokine production by extraembryonic tissues (Keelan 2004) as discussed below. A consistent picture of how intrauterine infection and perhaps other conditions lead to preterm delivery is emerging. Bacterial products activate cells within the placenta, decidua, chorion and amnion leading to the production of pro-inflammatory cytokines such as interleukin-6 (IL-6), tumour necrosis factor-alpha (TNFα) and IL-1β; chemokines such as IL-8 and macrophage inflammatory protein-1alpha (MIP-1α) ; and immunosuppressive or anti-inflammatory cytokines such as interleukin-10 (IL-10) and interleukin-4 (IL-4) (Erroi, Fantuzzi et al. 1993; Deetz, Jagielo et al. 1997) . Some of these mediators are also postulated to contribute to the normal maintenance of pregnancy and disruption of the delicate balance of these cytokines at the materno-fetal interface by bacteria or other factors is postulated to trigger a cascade of events leading to cyclo-oxygenase (COX2) activity and the production of prostaglandins, that cause uterine contractions thereby activating the parturition mechanism prematurely (Ville 2001). Elevated levels of proinflammatory mediators like IL-6, IL-8 and TNFα and IL-1β in patients with chorioamnionitis (+/- infection) are reported by many groups to occur in the amniotic fluid, maternal serum, cervical secretions, placenta, fetal membranes and other compartments of the placento-maternal unit (Denison, Kelly et al. 1998; Laham, Brennecke et al. 1999; Dowd, Laham et al. 2001).

IL-6: IL-6 is a proinflammatory cytokine which, during the early phase of inflammation, acts to activate and mature neutrophils and macrophages, and to promote differentiation of natural killer cells. These are the leukocytes that invade cervical and endometrial tissue prior to labour and contribute to labour progression (Osman, Young et al. 2003). IL-6 also acts as a growth factor and can stimulate the expression of oxytocin receptors on myometrial cells to increase responsiveness to oxytocin (Rauk, Friebe-Hoffmann et al. 2001). Plasma IL-6 is significantly elevated in women with premature labour compared to gestationaly matched, non-labouring controls (Laham, Rice et al. 1993).

Vaginal levels of IL-6 are also significantly elevated among women with threatened preterm labour and cervico-vaginal concentration of IL-6 has been found to be a good predictor of preterm delivery (Torbe and Czajka 2004). Stimulation of explants of amnion, placenta or choriodecidua with lipopolysaccharide (LPS) from Gram negative bacteria leads to elevated IL-6 levels in culture supernatants (Laham, Brennecke et al. 1996) thereby linking IL-6 production with a microbial stimulus.

IL-6 is also a physiological constituent of amniotic fluid and its production by gestational tissues has been described to not only be regulated by exposure to LPS but by mode and time of delivery. IL-6 was released from all choriodecidual and placental explants both prior to labour and after term labour and delivery with levels significantly higher in the laboured group in both choriodecidual and placental explants. In contrast, IL-6 was detected in only 33% of amniotic explant cultures prior to onset of labour at term but from 100% amniotic explants after term labour and delivery, and again mean IL-6 levels were higher in the latter group (Laham, Brennecke et al. 1996).

Thus, microbial product mediated production of IL-6 in intrauterine tissues has been proposed as an important etiological factor contributing to preterm labour (Casey, Cox et al. 1990), but IL-6 may also play a role in term labour onset and/or maintenance (Cox, King et al. 1993). It has been proposed that, following cervical ripening and dilatation, the decidua lining the fetal membranes is exposed to vaginal-cervical fluid and activated by bacterial toxins present in the fluid. This activation of the decidua results in the release of cytokines, such as IL-6. If this model is correct, IL-6 release may be an epi-phenomenon of the onset of human labour and delivery both at term and preterm gestations.

IL-8: Like IL-6, the α chemokine IL-8, is postulated to have a role in preterm labour as well as in normal labour at term. Enhanced expression of chemokines such as IL-8 presumably recruits infiltrating leukocytes into the fetal membranes thereby amplifying the inflammatory process and hastening membrane rupture and delivery. IL-8 is notable for its selective chemotaxis, degranulation and activation of neutrophils into tissue stroma. It is present within the uterus, cervix, placenta, myometrium, amnion and

choriodecidua: explants of amnion, placenta and choriodecidua all release IL-8 constitutively with choriodecidual explants producing significantly greater levels of IL-8 than amnion or placenta (Laham, Brennecke et al. 1997). The concentration of IL-8 tends to increase in the third trimester, particularly in the amniotic fluid, choriodecidua and uterus (Laham, Rice et al. 1993; Laham, Brennecke et al. 1997; Laham, Brennecke et al. 1999).

A statistically significant increase in IL-8 protein and mRNA expression has been shown to occur in the amnion in association with parturition at term (Elliott, Allport et al. 2001). However, in another study, spontaneous onset of labour, irrespective of the gestation and eventual mode of delivery, was not associated with any significant changes in IL-8 release from human gestational tissues, including amnion, compared to non-laboured tissues. Similarly, chorioamnionitis did not impact significantly on IL-8 release (Laham, Brennecke et al. 1997; Laham, Brennecke et al. 1999). However upon stimulation with lipopolysaccharide (LPS) IL-8 release is significantly increased from both choriodecidual and placental explants but not from amniotic explants (Laham, Brennecke et al. 1999).

Despite the general lack of significant increase from gestation-associated tissues, IL-8 concentrations are increased in the amniotic fluid in association with labour at term with respect to the gestational-age matched controls. However, although the concentration of IL-8 in amniotic fluid obtained from women in preterm labour was higher it was not statistically different from that in amniotic fluid obtained from gestational aged-matched non-labouring controls. Thus whilst microbial stimulation can induce production of a relevant mediator by appropriate tissues most of the data relating to IL-8 production is consistent with the suggestion that IL-8 release is an early event in chorioamnionitis that precedes the appearance of clinically overt symptoms. Moreover, IL-8 might be particularly relevant to events surrounding cervical ripening. The IL-8 concentration in cervical secretions at 28 weeks of gestation was significantly higher in women who went on to deliver preterm compared to women who continued until term and there was a greater than five-fold increase from 18 to 28 weeks in this subset of women (Dowd, Laham et al. 2001).

Cervical ripening is a result of an extensive remodelling process in the cervical tissue during pregnancy and labour. It is recognized as softening and dilatation of the cervical canal, and starts as a slow process during pregnancy, becoming rapid, as labour approaches. Cytokines have been postulated as possible mediators of the cervical ripening. Levels of mRNA for IL-8, IL-6 and granulocyte colony-stimulating factor (G-CSF) are up-regulated in ripe postpartum cervical tissue compared to unripe tissue (Sennstrom, Ekman et al. 2000). Likewise, the release of these cytokines by cervical tissue increases from the non-pregnant to the term-pregnant state with a further increase at delivery. For example, IL-8 concentrations increased 4-fold from the non-pregnant to the term-pregnant and a further 10-fold to the postpartum state. IL-8 immunoreactivity was identified in the epithelia of cervical tissue from pregnant women and was most pronounced in the epithelia and stroma of tissue collected postpartum (Sennstrom, Ekman et al. 2000). Thus cervical ripening is an inflammatory process with a central role mediated by IL-8.

TNFα: TNFα is a 17-kD pleiotropic peptide implicated in mediating the cellular events that occur at labour, both term and preterm. This potent proinflammatory cytokine stimulates the increased synthesis of prostaglandins by gestation-associated tissues both at term and preterm (Mitchell, Trautman et al. 1993) and is postulated to play an etiological role in the onset and/or maintenance of labour (Rice 1990). LPS-treated explants of choriodecidual and placental tissue release significantly greater levels of TNFα than unstimulated samples.

IL-1: IL-1 is also implicated in the onset and/or maintenance of term and preterm labour. Its biological activity has been attributed to two polypeptides, IL-1α and IL-1β, as well as the opposing effect of the IL-1 receptor antagonist protein (IL-1ra). It is known that whereas IL-1β is present in the placenta and stimulates prostaglandin formation (Mitchell, Romero et al. 1991), there is a predominance of IL-1α and IL-1ra in the human amniotic fluid and decidua (Romero, Brody et al. 1989). Similar to IL-6, IL-8 and TNFα, IL-1α concentrations did not change significantly in maternal plasma and

amniotic fluid with spontaneous term labour and delivery (Laham, Brennecke et al. 1996). However IL-1α was released in detectable amounts from human placental and choriodecidual explants in association with term labour and delivery compared to non-labour tissues. LPS stimulated a significant increase in IL-1α from human placental explants from both term non-laboured and term laboured tissues. Levels of IL-1α and IL-1β are significantly elevated in cervicovaginal fluid from women with bacterial vaginosis (Kalinka, Wasiela et al. 2003) suggesting that like cytokines already discussed above, they also might be linked with preterm labour. Indeed preterm parturition can be triggered in rabbits by intra-amniotic injections of IL-1α or TNFα (Bry and Hallman 1993).

Evidence for a role for inflammatory cytokines in IA-PTL is also provided by genetic studies. It has been proposed that genetic predisposition to immune hyper-responsiveness to an infectious insult might result in overproduction of cytokines such as TNFα and IL-1 (Dizon-Townson 2001). This genetic predisposition to cytokine over-expression might manifest on either the maternal, fetal, or both sides of the maternal-fetal interface. A polymorphism in intron 2 of the IL-1ra gene (IL1RN * 2), was associated with a blunted proinflammatory IL-1β response to abnormal vaginal flora and the carriers of this polymorphism had a decreased susceptibility to infection-associated preterm birth (Genc, Onderdonk et al. 2004). This highlights the role of IL-1β in the aetiology of preterm labour.

The apparent association of proinflammatory cytokines and chemokines with labour has led to an interest in anti-inflammatory cytokines as potential *in vivo* regulators of cytokine activity and consequently, as possible therapeutic agents. Anti-inflammatory cytokines, like IL-10, might be involved in protecting the fetal allograft throughout pregnancy and at parturition (Denison, Kelly et al. 1998). Anti-inflammatory effects for IL-10 were found on choriodecidual explants, but not in the adjacent amnion where, in contrast, it had pro-inflammatory actions (Mitchell, Simpson et al. 2004). IL-10 was expressed by explants of placenta from all trimesters of normal pregnancies with its expression significantly diminished in term placental tissues collected before the onset

of labour and term labour had no significant effect on placental IL-10 levels. The down-regulation of IL-10 near term might be an important mechanism underlying the subtle changes associated with the initiation of normal parturition (Hanna, Hanna et al. 2000). Furthermore in animal models (rhesus monkeys) IL-10 inhibited IL-1β induced uterine activity, possibly by decreased prostaglandin production (Sadowsky, Novy et al. 2003). IL-4, although considered an anti-inflammatory agent, was found to be elevated in women with preterm labour and delivery, particularly in association with chorioamnionitis in one study (Dudley, Hunter et al. 1996). This could indicate a proinflammatory role for IL-4 or mark a failed attempt to control proinflammatory cytokines by upregulating this anti-inflammatory cytokine. Transforming growth factor-beta 2 (TGFβ2), also known to have anti-inflammatory properties, has been shown to prevent the preterm delivery induced by IL-1α and TNFα in rabbits (Bry and Hallman 1993) and can downregulate TNFα release from amniochorion in *invitro* models (Fortunato, Menon et al. 1997).

In addition to those discussed in detail above, other cytokines are postulated to play pivotal roles in immune recognition, acceptance of the fetal allograft and maintenance of pregnancy alongside parturition. Interleukin 18 (IL-18), for example, is a proinflammatory pleiotropic cytokine that has been implicated in the host defence against infection. IL-18 is increased in amniotic fluid in cases of microbial invasion of the amniotic cavity in both term and preterm labour and is detectable in the amniotic, maternal, and fetal compartments (Pacora, Romero et al. 2000). Other mediators such as monocyte chemotactic peptide-1 (MCP-1) and RANTES (Regulated upon Activation, Normal T cell Expressed and presumably Secreted) also appear to have a role in inflammatory responses within gestational tissues, but their secretion and regulation within the third trimester uterus is less well defined (Denison, Kelly et al. 1998).

A critical role for pro-inflammatory cytokines in labour could be the induction of prostaglandins (PG). PG released by the membranes in response to stretch and the actions of pro-inflammatory cytokines act not only upon the myometrium and cervix, but might also exert paracrine/autocrine effects on cell viability and matrix protein

integrity. The localisation and regulation of enzymes, responsible for converting PGH_2 to bioactive prostanoids such as PGE_2 and $PGF_{2\alpha}$, are being studied in the myometrium and cervix, particularly in the context of cytokine interactions. Furthermore gestational tissues are sources of PGD_2 and PGJ_2 and their derivatives, but the regulation of production of these prostaglandins has yet to be studied in any detail and their actions, which could include apoptosis and anti-inflammatory activity, remain poorly defined (Keelan 2004).

Whilst the published literature suggests that inflammatory cytokines are elevated in preterm labour and that cytokines can account for the histological changes that characterise chorioamnionitis, it is essential to establish a causal link between the bacteria known to infect the gestation associated tissues and the consequent cascade of events leading to labour. An *ex vivo* system was designed to model fetal membrane biology and immunology, i.e. linking cytokine response and infection (Keelan 2004). Studying membranes from term delivery (non-laboured, caesarean section) exposed to LPS on either the maternal or fetal side revealed that accumulation of inflammatory mediators in the amniotic cavity can arise in the absence of intra-amniotic infection as a result of activation of the fetal membranes secondary to decidual activation. Elevated IL-6 levels in amniotic fluid were predicted by this model to be a good marker of intrauterine infection, as is observed clinically. TNFα can cross the membrane barrier, albeit with low efficiency, explaining its poorer diagnostic profile.

From the above it is clear that many of the processes necessary for term or preterm labour (e.g. remodelling of the cervix, rupturing of the membranes and myometrial contractions) are initiated/mediated by inflammatory cytokines. Many of the cell types that are sources of these cytokines and mediate these remodelling events are also involved in the early and crucial events triggering host defences against invading pathogens and so are implicated in infection associated preterm labour. In this regard, Nuclear factor kappa B (NF-κB) has been implicated by a number of groups in the regulation of pro-inflammatory cytokine and prostaglandin production by gestation-

associated tissues. NF-κB is a transcription factor family classically associated with inflammation. For example, NF-κB is essential for basal and IL-1β stimulated IL-8 production by primary amnion cells and NF-κB activity can be detected in amnion only after labour has occurred (Elliott, 2001, Allport-2001). Thus NF-κB might have a critical role in signalling pathway(s) that increase inflammatory cytokine production and antimicrobial peptides in gestation-associated tissues (Ulevitch 2004). Similarly studies by Tamsin and Bennett (Allport, Pieber et al. 2001; Tamsin M Lindstrom 2005) suggest a role for NF-κB in the physiology and pathophysiology of labour. NF-κB activity increases with labour onset and is central to multiple pro-labour pathways. Premature or aberrant activation of NF-κB may thus contribute to preterm labour

How bacterial pathogens interact with cells of the reproductive tract to initiate inflammatory processes and cytokine production at the materno-fetal interface in term and preterm labour are poorly understood. Recent advances in developmental biology and functional genomics have seen dramatic advances in our understanding of signalling pathways involved in innate immune defences and have led to the discovery of a family of receptors, the Toll-like receptors (TLRs), which are now of great interest to those who study the immunology of gestation-associated tissues.

1.2 TOLL LIKE RECEPTORS

Toll-like receptors (TLR) have emerged as key upstream mediators of inflammation at many tissue sites in humans and other organisms. TLRs are a family of proteins, consisting of ten type 1 transmembrane receptor proteins (Beutler and Poltorak 2000) in humans and thirteen in mice. (Tabeta, Georgel et al. 2004). It is not known if a recently identified 11th member of this family is functional in humans (Zhang, Zhang et al. 2004).

'***Toll***', derived from the German word for "amazing" or "mad", was first discovered in the fruit-fly *Drosophila melanogaster* as a plasma membrane protein with a role in development of dorsoventral polarity during embryogenesis (Hashimoto, Hudson et al.

1988). Later, Toll was also found to have a role in the fly's immunity to fungal infections (Lemaitre, Nicolas et al. 1996). Upon infection of *Drosophila* the Toll ligand Spatzle is processed into a biologically active form which binds to Toll and induces a signalling cascade resulting in the production of the antimicrobial peptide drosomycin (Lemaitre, Nicolas et al. 1996; Levashina, Langley et al. 1999).

The first mammalian TLR, so called because of its sequence homology to the *Drosophila melanogaster* gene Toll (Grohol's 2005), was identified in 1997 (Medzhitov, Preston-Hurlburt et al. 1997). Given the apparent conservation of this receptor type through evolution, it is perhaps not surprising that all animals studied, including goldfish and chickens, have TLRs (Grohol's 2005). The presence of homologous receptors in plants indicates that they are of ancient evolutionary origin and, after the defensins, might be the oldest components in the immune system (Goldstein 2004).

The TLR family is the best-characterised class of mammalian pattern recognition receptors (PRRs). The pattern recognition strategy of these receptors is based upon the detection of a limited set of conserved molecular patterns that are unique to the microbial world. The targets of pattern recognition, sometimes called pathogen-associated molecular patterns (PAMPs) signal to the host the presence of infection (Janeway and Medzhitov 2002). TLRs are expressed by cells involved in innate immunity and enable recognition of invading microbes and initiation of anti-microbial activity. Whilst TLRs are now recognised to play a crucial role in the regulation of immune responses, the factors that regulate TLR expression and function are poorly understood.

All members of mammalian TLRs described to date share a unique structure comprising of three parts: a highly homologous cytoplasmic domain, similar to the IL-1 receptor with which it shares a conserved homophilic domain of approximately 200 amino acids known as the Toll/IL-1Receptor domain (TIR) (Cook, Pisetsky et al. 2004); a very short transmembrane domain; and an extracellular portion consisting of leucine-rich repeats (Wright 1999) and one or two cysteine-rich regions (Cario 2003) (Figure 1.1).

Expression of TLRs occurs predominantly at the plasma membrane but these receptors are also expressed within intracellular phagolysosomes.

TLRs1, 2, 4, 5 and 6 tend to specialise in the recognition of bacterial products that are present on the outer surfaces of the microorganisms whereas TLRs3, 7, 8 and 9, specialise in recognising nucleic acids, especially those of viral origin, and are localised intracellularly (Ulevitch 2004). Self/non-self discrimination of nucleic acids is mediated not so much by the molecular nature of the ligands as by their accessibility to the TLRs (Iwasaki and Medzhitov 2004). These TLRs (3, 7, 8 and 9) are typically localised in intracellular compartments, especially late endosomes-lysosomes, which are not normally accessible to host nucleic acids (Lund, Sato et al. 2003; Diebold, Kaisho et al. 2004; Lund, Alexopoulou et al. 2004).

The extracellular domains of TLRs, usually composed of 24-29 amino-acid repeats, function as sentinels for 'molecular patterns' on different classes of pathogens. Ligands for TLRs include exogenous/microbial (e.g. bacterial/viral RNA/DNA, lipopolysaccharide and lipopeptides) as well as endogenous/host-derived (human heat shock proteins 60, 70 and host RNA, β-defensins and oxidised lipids) stimuli (Vabulas, Ahmad-Nejad et al. 2002; Wan, Zhou et al. 2004). With the exception of TLR10, microbial ligands for each of the human TLR family members have been described, as shown in Table 1.1. The presence of endogenous ligand, i.e. various host-derived products released during tissue damage or "danger signals" (Matzinger 2002), indicates a possible role for TLR signalling pathways in normal physiological phenomena and are postulated to cause sterile inflammation (Ulevitch 2004).

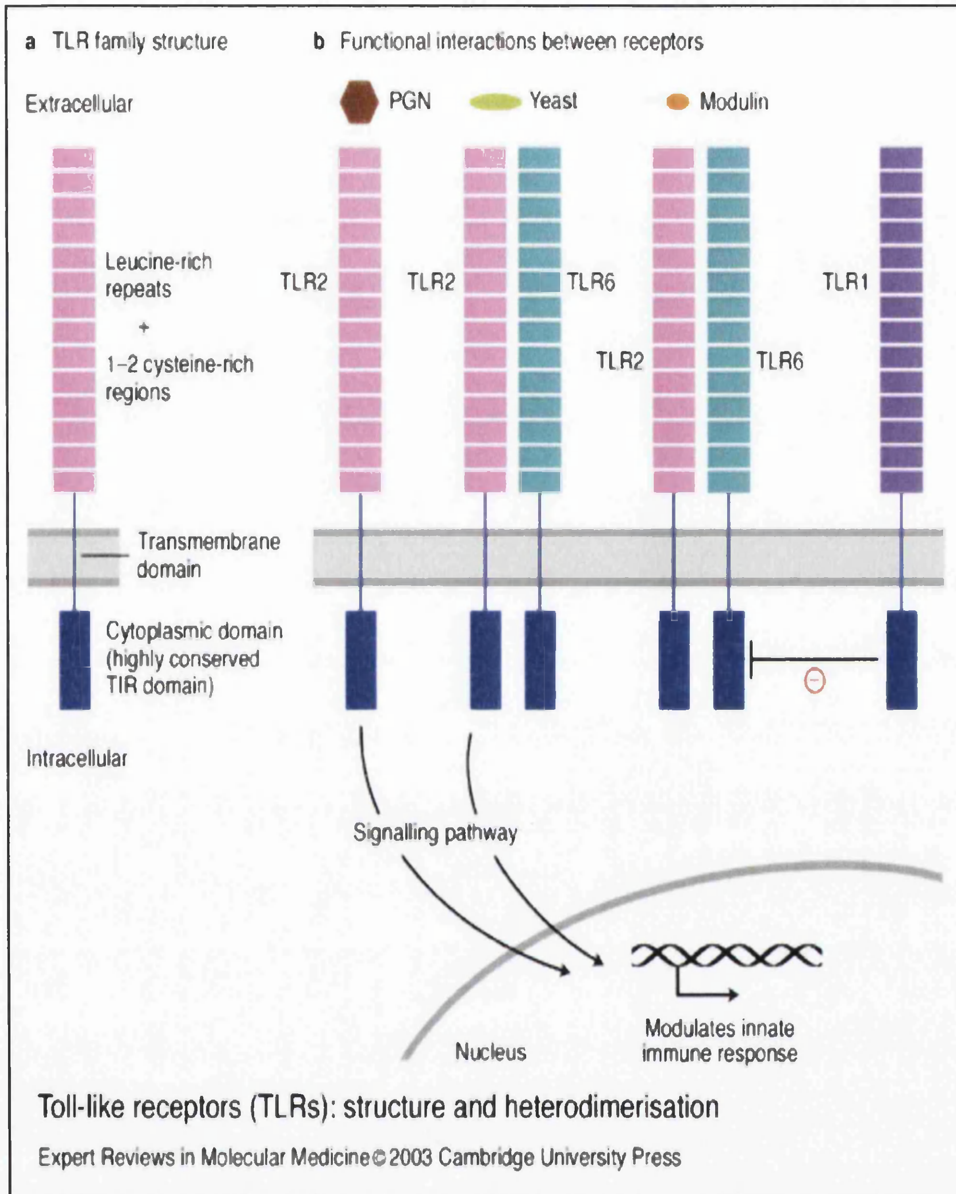


Figure 1.1 *Toll-like receptors: structure and heterodimerisation. (a) The structure of mammalian TLR family - type I transmembrane protein; b) TLRs function in activating innate immunity by recognising conserved molecular patterns carried by microorganisms. Pattern recognition can be achieved by individual TLRs. (Elke Cario, 2003)*

TLR	Gene location	Microbial Ligands (natural and synthetic)	Natural Ligands	Known Activation Cascades
TLR1 (TLR 2)	Chromosome-4	Triacetylated lipoproteins		Unknown
TLR 2	Chromosome-4	Gram positive Peptidoglycan, Lipopeptides (di and tri acyl), Lipoarabinomannan , Lipotechoic acids (Mycoplasma), Zymosan (fungal hyphae and conidia)	Heat shock protein 70	MyD88 dependent TIRAP
TLR 3	Chromosome-4	Double stranded viral RNA Polyinosine-polycytidilic acid (poly I:C)	Host RNA	MyD88 independent TRIF/TICAM
TLR 4	Chromosome-9	Gram negative lipopolysaccharide Taxol	Neutrophiles Elastase, Heat shock protein 60,Fibronectin A	MyD88 dependent TIRAP; MyD88 independent TRIF/TICAM/TRAM
TLR 5	Chromosome-1	Bacterial flagellin		MyD88 dependent IRAK
TLR6 (TLR 2)	Chromosome-4	Zymosan (fungal hyphae and conidia), Diacetylated Lipoproteins		Unknown
TLR 7	Chromosome-X	Single stranded RNA-Small synthetic compounds- R848, Loxaribine, Bropirimine		MyD88 dependent IRAK
TLR 8	Chromosome-X	Single stranded RNA-Small synthetic compounds- R848, Bropirimine		MyD88 dependent IRAK
TLR 9	Chromosome-3	Unmethylated CpG DNA		MyD88 dependent IRAK
TLR 10	Chromosome-4	Unknown		Unknown
TLR 11	Chromosome-unknown	Profilin (*Toxoplasma gondii*)		MyD88 dependent IRAK

Table 1.1 *Chromosome location and ligands for TLRs and their known signalling pathways.*

TLR4, the first human Toll-like receptor to be well characterised, is a receptor for LPS of Gram-negative bacteria (Medzhitov, Preston-Hurlburt et al. 1997) (Poltorak, He et al. 1998) (Hoshino, Takeuchi et al. 1999; Qureshi, Lariviere et al. 1999). It was found to be in the target region of chromosome 4 in mice and this might explain the origin of its name. Recently, a TLR4 polymorphism (Asp299Gly) was shown to be associated with impaired TLR4 receptor function and an increased likelihood of Gram-negative sepsis (Agnese, Calvano et al. 2002). TLR4 mediated responses generally require additional molecules, such as CD14, lipopolysaccharide binding protein (LBP) and MD2 to initiate the signalling cascade. The latter is an apoprotein with a secreted leucine-rich repeat domain that is required for surface expression and LPS-regulated activation of TLR4. MD2 exists both in soluble form and as a glysosylphosphatidylinisitol anchored (GPI-anchored) protein. TLR4 forms a complex with MD2 leading to translocation of nuclear factor-kappaB (NF-κB) to the nucleus and cytokine production in response to LPS stimulation (Shimazu, Akashi et al. 1999).

In addition to sensing LPS, TLR4 mediates signals initiated by other exogenous compounds like respiratory syncytial virus protein F, and several endogenous proteins that may be present during inflammation such as heat shock protein 60 and the proinflammatory extra domain A of fibronectin (Wan, Zhou et al. 2004). A TLR-related protein, called RP105, was identified in B cells and acts as both a LPS sensor and a regulator of B cell proliferation (Yazawa, Fujimoto et al. 2003). However, more extensive expression of RP105 has been described recently and RP105 might be a negative regulator of TLR4 activity. Like TLR4, RP105 requires an MD2-related protein, MD1, for its surface expression. (Divanovic, Trompette et al. 2005).

TLR2 has the broadest specificity of the TLRs since it can recognise Gram-positive, Gram-negative and mycobacterial associated lipoproteins, Gram-positive peptidoglycan and lipoteichoic acid, and fungal zymosan (Yoshimura, Lien et al. 1999). A Arg753Gln TLR2 polymorphism in humans has been associated with increased susceptibility to infection with *Staphylococcus aureus* (Lorenz, Mira et al. 2000). TLR2 knockout mice are similarly susceptible and this demonstrates the role of TLR2 in responses to bacterial

lipoproteins. TLR2 recognition of some microbial products appears to be dependent upon the formation of heterodimers with either TLR1 or TLR6 (Takeuchi, Kawai et al. 2001; Krutzik, Ochoa et al. 2003). TLR1 recognise bacterial triacylated lipoproteins like peptidoglycan (Takeuchi, Sato et al. 2002), while TLR2/TLR6 complexes recognise mycoplasmal diacylated lipoproteins like lipotechoic acid, as well as fungal zymosan (Takeuchi, Kawai et al. 2001; Morr, Takeuchi et al. 2002).

TLR3 binds viral double stranded RNA (dsRNA). On stimulation with its synthetic ligand, polyinosine-polycytidilic acid (poly I:C) proinflammatory cytokines and chemokines, particularly type I interferons (IFNα/β) are released (Schaefer, Fahey et al. 2005). This is the only TLR which utilises MyD88 independent TRIF/TICAM signalling pathway. This contrasts with all other TLRs which have MyD88 dependent cascades.

TLR5 is a signalling mediator of bacterial flagellin, a principal component of bacterial flagella, and may play a role in resistance to *Salmonella* infection. Mammalian TLR5 recognizes bacterial flagellin from both Gram-positive and Gram-negative bacteria and upon its activation the receptor mobilizes NF-κB and stimulates TNFα production (Hayashi, Smith et al. 2001). A common stop codon polymorphism in the ligand-binding domain of TLR5 (Arg392STOP) is associated with susceptibility to pneumonia caused by *Legionella pneumophila*, a flagellated bacterium, due to lack of flagellin-mediated signalling (Hawn, Verbon et al. 2003).

TLR7 and **8** recognise small antiviral molecules and detect single stranded viral RNA like that of influenza (Lund, Alexopoulou et al. 2004). There are several synthetic ligands for the stimulation of TLR7/8 such as R848 (antiviral synthetic compound), loxoribine, bropirimine, and single stranded poly-oligonucleotides. Both TLR7 and 8 independently confer responsiveness to R848, which is an imidazoquinoline compound with antiviral activity. Although TLR7 and TLR8 are phylogenetically (Figure 1.2) and structurally related, their relative functions are largely unknown. The role of TLR7 has been established using TLR7-deficient mice and small molecule TLR7 agonists. TLR agonists selective for TLR7 or TLR8 were used to determine the repertoire of human

innate immune cells that are activated through these TLRs: TLR7 agonists directly activated purified plasmacytoid dendritic cells and, to a lesser extent, monocytes, whereas TLR8 agonists directly activated purified myeloid dendritic cells, monocytes, and monocyte-derived dendritic cells. Accordingly, TLR7 selective agonists were more effective than TLR8 selective agonists at inducing IFN-α and IFN-regulated chemokines. In contrast, TLR8 agonists were more effective at inducing proinflammatory cytokines and chemokines, such as TNFα, IL-12, and MIP-1α (Gorden, Gorski et al. 2005). Thus TLR7 and TLR8 agonists differ in their target cell selectivity and cytokine induction profile.

TLR9 detects unmethylated CpG (deoxycytidine-deoxyguanosine dinucleotide) motifs that occur in microbial DNA (Krieg 2006). TLR9 is expressed predominantly by plasmacytoid dendritic cells and B cells. TLR9 detects a pattern that is present in the DNA of invading intracellular pathogens but is not present in mammalian DNA to trigger a proinflammatory response.

TLR10 is an orphan member of the human TLR family (Hasan U, 2005). Genomic studies indicate that TLR10 is in a locus that also contains TLR1 and TLR6, both known to function as co-receptors for TLR2 and to which TLR10 is phylogenetically most closely related (Figure 1.2). Interestingly TLR10 not only forms homodimers but also heterodimerises with TLR1 and TLR2. Unlike widely expressed TLR1 and TLR6, TLR10 expression is more restricted and has been detected in B cells and weakly expressed in plasmacytoid dendritic cells (Hornung, Rothenfusser et al. 2002). Although the specific ligand for TLR10 is not yet identified, functional activity of TLR10 has been demonstrated using a recombinant CD4-TLR10 molecule (Hasan, Chaffois et al. 2005).

TLR11 existence and/or function in humans remain unclear. Transcripts for TLR11 are highly expressed in the urinary tract of mice, thus contrasting with the expression patterns of other TLRs (Zhang, Zhang et al. 2004) and suggesting that TLR11 might be involved in responses to uropathogenic bacteria. Recently, a *Toxoplasma gondii*-derived profilin-like protein has been identified as a ligand for murine TLR11 (Yarovinsky, Zhang et al. 2005). However, the human TLR11 gene contains several stop codons

suggesting that TLR11 might not be expressed and/or functional in humans (Zhang, Zhang et al. 2004).

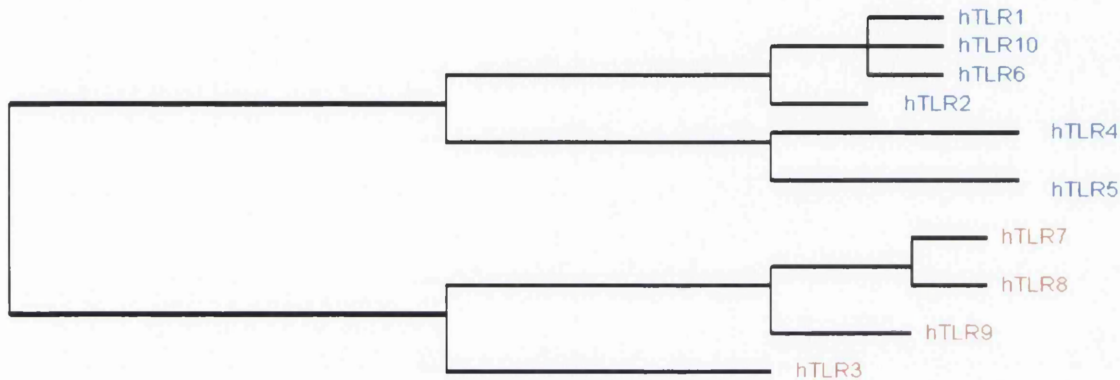


Figure 1.2 Phylogenetic tree of the human toll-like receptor family (Chuang and Ulevitch 2000).

1.2.1 Mechanism of action of TLR

The innate immune system represents the immunological first line of defence against invading pathogens through the ability to distinguish between what is non-infectious self and infectious non-self (Medzhitov and Janeway 2002). Each TLR forms either a homodimer or heterodimer in the recognition of a specific or set of specific molecular determinants present on microorganisms. The specificity of TLRs and other innate immune receptors cannot be changed rapidly, so these receptors must recognise patterns that are constantly present on threats, not subject to mutation, and highly specific to threats (i.e. not normally found in the host). Patterns that meet this requirement are usually critical to the pathogen's function and not readily eliminated or changed through mutation; and thus are said to be evolutionary conserved (Medzhitov R 2000).

Ligation of a TLR by microbial or endogenous products results in an inflammatory immune response characterised by the production of cytokines and anti-microbial factors (Medzhitov and Janeway 1997). Furthermore, through the regulation of co-stimulatory

molecules, TLRs might also facilitate the development of adaptive immune responses (Medzhitov and Janeway 1997). Thus while individual TLRs respond to limited ligands, collectively the family of TLRs can respond to a wide range of products associated with bacteria, viruses, fungi and parasites as shown in Figure 1.3. It is not clear how a restricted family of receptors has the capacity to recognise the wide spectrum of microbial stimuli known to exist. Co-transfection of different TLRs might lead to either enhancement or inhibition of recognition of particular PAMPs (Ozinsky, Underhill et al. 2000), suggesting that cellular responses to PAMPs are dependent on the total repertoire of TLRs displayed on a cell, necessary cofactors, and the levels of each protein present.

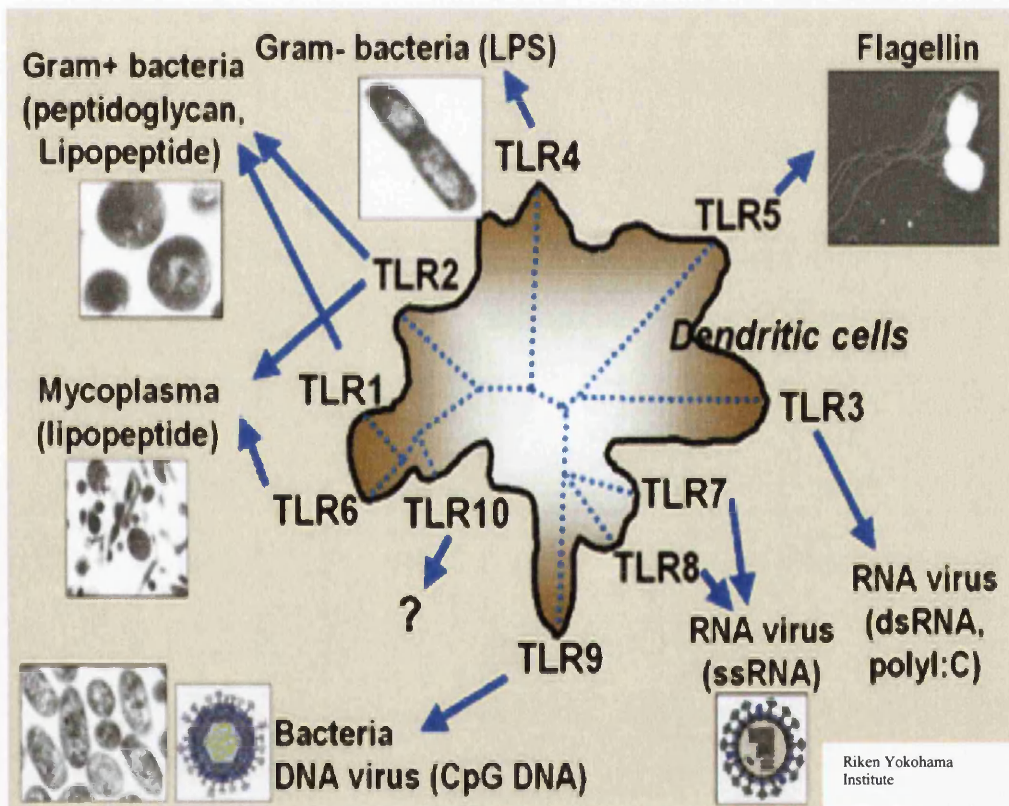


Figure 1.3 Ligands and phylogenetic tree of human Toll-like receptors as shown on a dendritic cell (Kaisho 2004).

The requirement for TLR cooperation is supported by the finding that TLR2 needs a partner to activate TNFα production in macrophages. Dimerisation of the cytoplasmic

domain of TLR2 does not induce TNFα production in macrophages, whereas similar dimerisation of the TLR4 cytoplasmic domain does. Thus, the cytoplasmic tails of TLRs are not functionally equivalent, with certain TLRs requiring assembly into heteromeric complexes, whereas others are active as homomeric complexes. The best example of this is elicited by the TLR2 as discussed earlier (Takeuchi, Kawai et al. 2001; Krutzik, Ochoa et al. 2003). Thus TLRs can establish a combinatorial repertoire to discriminate among the large number of pathogen-associated molecular patterns found in nature.

1.2.2 TLR signalling pathways

Each TLR has distinct extracellular specificity but all receptors signal through shared pathways. Consequently, following ligation with their specific ligands, TLRs elicit different but often overlapping immune responses leading to transcriptional activation of inflammatory genes. The common cytoplasmic TIR signalling domain mediates the interaction between TLRs and a family of TIR-containing adaptors that recruit receptor-associated kinases as shown in Figure 1.4a&b and Table 1.1. Currently four intracellular proteins function as adaptors for TLRs: myeloid differentiation factor 88 (MyD88, the most commonly used and initially thought to be the crucial mediator of TLR signalling) (Takeuchi, Takeda et al. 2000), TIR domain containing adaptor inducing IFN-β (TRIF), TRIF related adaptor molecule (TIRP/TRAM) and MAL (MyD88-adapter-like). These adaptors link the receptors via a series of kinases (e.g. mitogen activated protein kinase (MAPK) and IκB kinases) (Henneke and Golenbock 2004) to the transcriptional machinery typically via NF-κB and AP-1 (activating protein-1) which in turn leads to the production of several different cytokines.

As an example, MyD88 contains a TIR domain within its C-terminal and a death domain (DD) within its N-terminal (Fitzgerald, Palsson-McDermott et al. 2001) (Hardiman, Rock et al. 1996). Following ligation of a TLR by its ligand, MyD88 becomes associated with the intracellular domain of the receptor through a TIR-TIR interaction (Sato, Sugiyama et al. 2003). In turn MyD88, through its DD, recruits and activates the DD-containing serine/threonine kinase IL-1-receptor associated kinase (IRAK) (Wesche, Henzel et al. 1997). IRAK then dissociates from the receptor complex and becomes

associated with TNF-receptor associated factor (TRAF-6) (Cao, Xiong et al. 1996). TRAF-6 then dissociates from this complex and associates with another complex containing TGFβ activated kinase (TAK-1). TAK-1 is thus activated, which in turn activates the MAPK signalling pathways including the IκB kinase kinase (IKK) complex. The kinase activity of this complex is modulated by its IKKγ subunit, the transcription factor NF-κB essential modulator (NEMO). IKK-mediated phosphorylation of IκB unmasks the nuclear localisation domain of NF-κB (Cook, Pisetsky et al. 2004). After its translocation into the nucleus, NF-κB activates multiple cytokine genes (Takaesu, Kishida et al. 2000) (Takaesu, Ninomiya-Tsuji et al. 2001) resulting in the production of cytokines, chemokines and antimicrobial peptides.

Experiments using MyD88 deficient cells revealed that NF-κB and JNK (c-Jun N-terminal kinase) activation induced by TLR3 and TLR4, unlike other TLRs, was not completely abolished, but was instead delayed (Adachi, Kawai et al. 1998). Thus some TLRs signal via MyD88 independent pathways (Figure 1.4a). In addition to MyD88, TLR4 can associate with TRIF, which via its N-terminal end can directly bind TRAF-6 and subsequently activate NF-κB (Sato S, 2003). The production of type-1 interferons (IFNα and IFNβ) via stimulation of TLR4 and TLR3 with LPS and dsRNA respectively utilises MyD88 independent pathways and expression of IFN-inducible genes (Akira and Takeda 2004), as a result of TRIF, through TBK-1 (TANK-binding kinase-1), and activation of the transcription factor, IFN regulatory factor (IRF3/7) (Han, Su et al. 2004). Further analysis of other TLR pathways has revealed additional MyD88-independent mechanisms. TLR2 interacts with Rac1GTPase initiating a signalling cascade that also results in NF-κB translocation and possibly other non-transcriptional responses (Arbibe, Mira et al. 2000). Thus, while TLRs share many features, some unique properties of individual receptors can have a significant impact on immunological and functional outcomes.

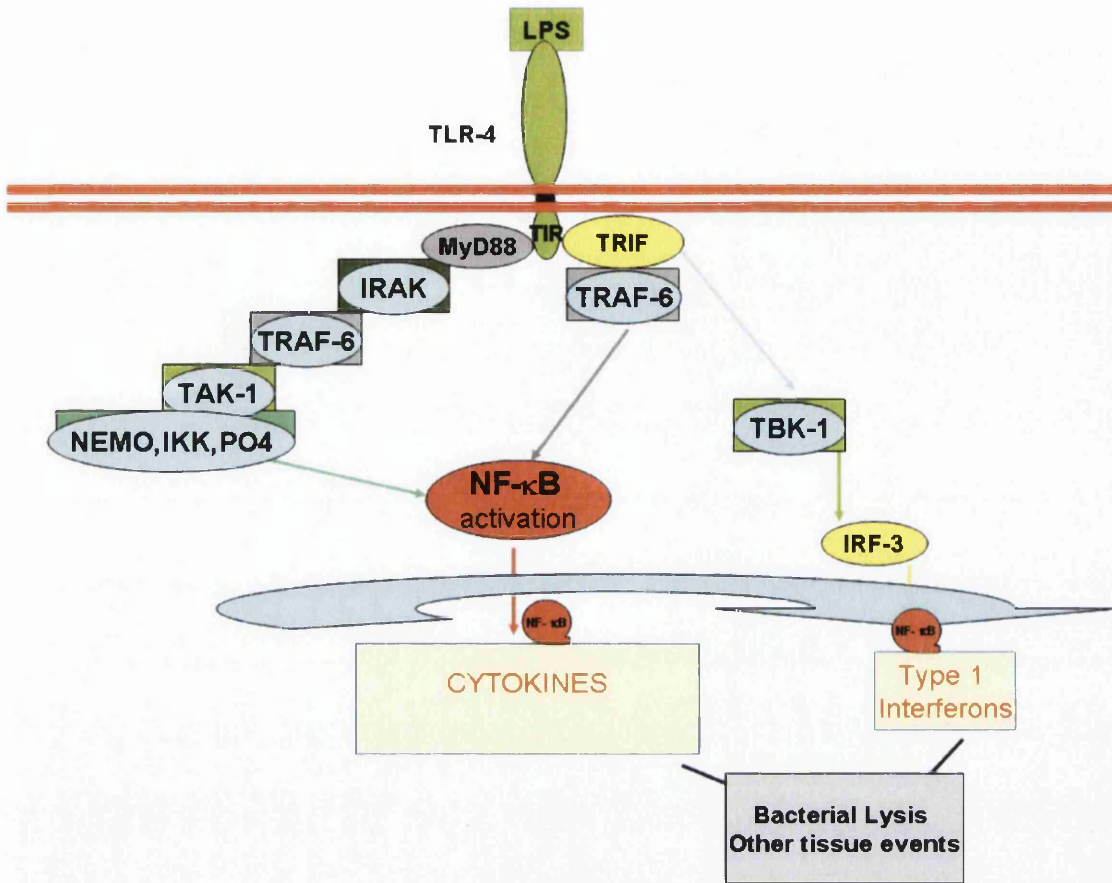


Figure 1.4a *MyD88-dependent and -independent signalling of TLR4. Activation of NF-κB by TLR-4 can occur through the classical MyD88 signalling pathway, or in a MyD88-independent manner through the recruitment of TRIF.*

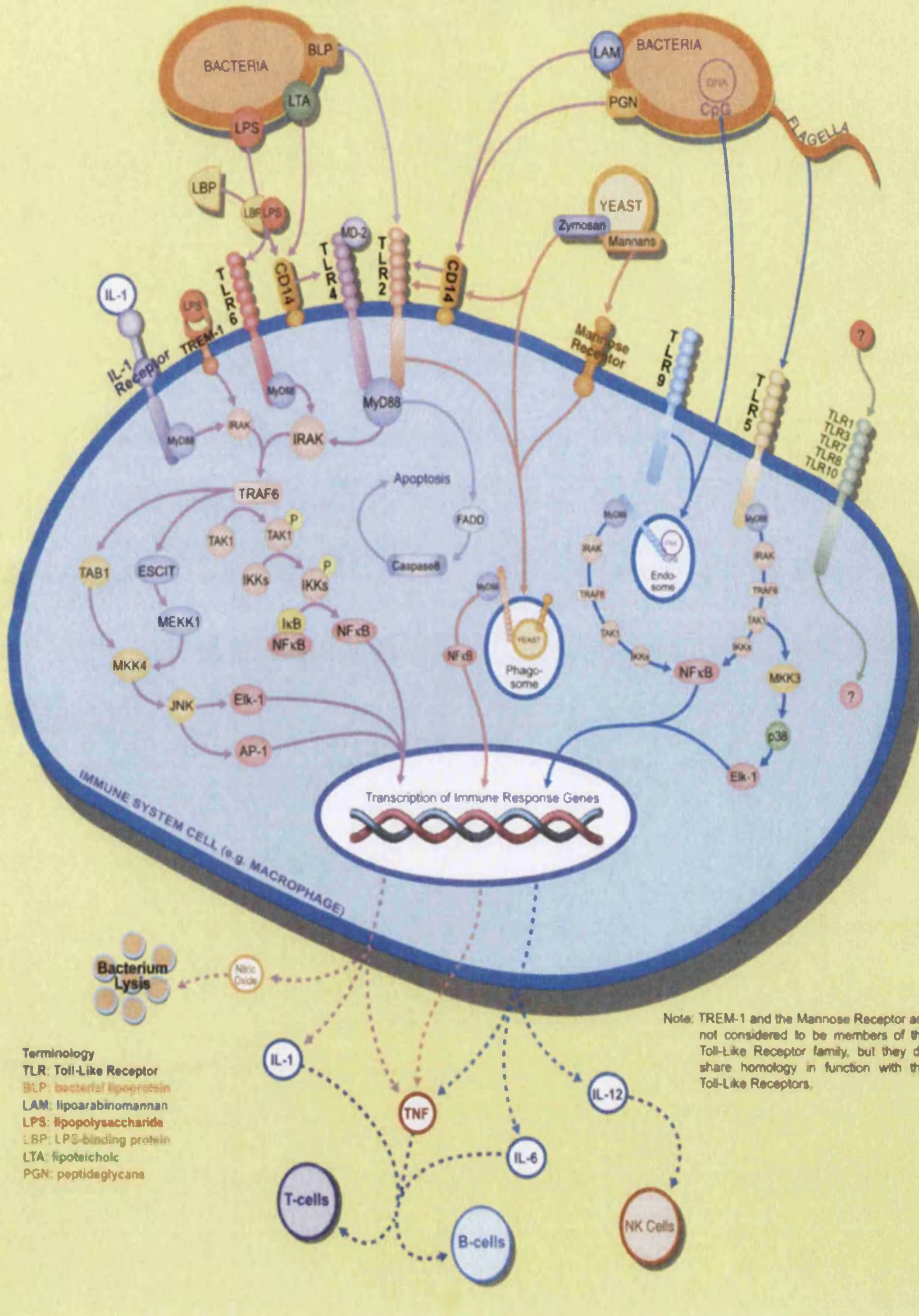


Figure 1.4b *Toll like receptor signalling pathways.*
(http://www.komabiotech.co.kr/technical/review/toll_like_receptor.gif)

'Ligand specificity' primarily determines which TLR is activated in a given cell but the presence of other TLRs and the secondary signalling pathways engaged provide a further level of control of the tissue response to a particular PAMP. Additional molecules such as CD14, MD2 and LBP have also been found to affect the activity and response of TLRs (especially TLR4).

The importance of intact TLR signalling pathways is highlighted by genetic studies of human primary immunodeficiencies caused by germ line mutations in genes encoding molecules involved in cell signalling downstream from TLRs (Ku, Yang et al. 2005). The cells of subjects with anhidrotic ectodermal dysplasia with immunodeficiency (have developmental anomalies of skin appendages and a broad spectrum of infectious diseases) show a broad defect in NF-κB activation, with an impaired, but not abolished response to a large variety of stimuli including TLR agonists. However patients with autosomal recessive amorphic mutations in IRAK4 present a purely immunological syndrome with more restricted defects. In these subjects, the NF-κB and MAPK mediated induction of inflammatory cytokines in response to TLR agonists is impaired. The patients present with a narrow range of pyogenic bacterial infections that become increasingly rare with age.

1.3 DISTRIBUTION OF TLRs IN HUMAN TISSUES

Whilst leukocytes within the peripheral blood are relatively well studied there is much interest in the contribution of these receptors to normal and pathological processes in various tissues. Typically, TLR expression is monitored by investigation of mRNA and/or protein expression with functional responses to TLR agonists much less frequently studied. TLR distribution in a broad range of tissues from adult humans has been examined using quantitative PCR (Zarember and Godowski 2002; Nishimura and Naito 2005). To facilitate comparison between tissues in Zarember's study, all samples were normalised to the expression of the particular TLR in spleen (spleen = 1). The

tissues involved in immune function (e.g., spleen, peripheral blood leukocytes) displayed the most diverse repertoire of TLR transcripts, as did those expected to encounter microbes (e.g., lung, small intestine and colon). TLR3 and TLR5 were expressed ubiquitously, whereas RP105 and TLR10 were more restricted. Of particular interest the placenta expressed an abundance of TLR3 (*4.99x more than spleen*), TLR5 (*1.75x*) and TLR7 (*1.78x*). TLR1, TLR2, TLR4 and TLR8 were also expressed in placenta but in lesser amounts than in the spleen. As TLR expression by mucosal tissues is important for host defence against pathogens (Gewirtz 2003; Yuan and Walker 2004), most studies have therefore focused on the intestinal and respiratory tracts, however there is growing evidence that the mucosal epithelium of the female reproductive tract is also an important site of TLR activity (Quayle 2002; Wira and Fahey 2004).

1.4 TLR EXPRESSION AND FUNCTION IN HUMAN REPRODUCTION

In recent years there has been much interest in the role of various cytokines in human reproduction. As TLRs have emerged vital to cytokine production, the existence of various TLRs and their intracellular pathways have begun to be explored in human reproductive tissues. Whilst the studies of TLRs in the male reproductive tract is still in its infancy (Robertson 2005), more progress regarding the female reproductive tract has been made.

1.4.1 The female reproductive tract

Tissues of the lower reproductive tract are populated by a rich commensal microflora, which limit the growth of more virulent microorganisms. The major commensals at this site include *Lactobacillus*, *Gardnerella vaginalis* and *Escherichia coli*. In addition, common pathogens that can populate the lower genital tract include *Neisseria gonorrhoeae, Chlamydia trachomatis* and *Candida albicans* (Larsen and Monif 2001; Quayle 2002). The mucus produced by the cervix provides somewhat of a barrier, however, this can be breached and the upper tract is, on occasion, exposed to both commensal bacteria and pathogenic microbes through either peristaltic contractions or intercourse (Profet 1993; Crane-Godreau and Wira 2004). Infection within the upper

tract can have serious consequences, such as chronic pelvic inflammation, infertility and pregnancy complications (Witkin 2002). Therefore, such infectious agents must be quickly removed, whilst sustaining the resident microbial population of the lower tract. However, commensal and pathogenic microorganisms share PAMPs, and should thus have the same ability to trigger innate immune response pathways such as those mediated by TLRs. This creates the possibility of either hyper-responsiveness to resident microorganisms or the depletion of commensal populations. Yet mucosal systems, including the female reproductive tract, are colonized by commensal microflora, and these systems are not subject to constant inflammation under normal conditions. However, in the presence of a pathogen, a robust immune response can be generated and the infection is resolved. This then raises the question of how the mucosal immune system, such as that of the female reproductive tract, can distinguish between what is pathogenic and what is not.

TLRs are differentially expressed in the epithelia of different regions of the female reproductive tract and this has been postulated to prevent inappropriate reactivity to commensal populations. For example, TLR4 protein was detected immunhistochemically in the endocervix, endometrium and uterine tubes but not in the vagina and ectocervix (Fazeli, Bruce et al. 2005). TLR4 could thus play an important role in host defence against ascending infection. Similarly, endometrial epithelial cells were found to express TLRs 1-9 (Schaefer, Desouza et al. 2004).

Alternative hypotheses for maintenance of commensal flora and anti-pathogen activity have been forwarded (Strober 2004). Rather than avoiding a response towards commensals by minimal expression of TLRs at relevant sites, it has been suggested that these microbes may in fact be essential for protecting mucosal epithelia from direct tissue injury by inducing the production of cytoprotective factors such as TNFα, IL-6 and heat shock proteins in response to commensal microbes (Hooper and Gordon 2001; Rakoff-Nahoum, Paglino et al. 2004). These findings shed new light on the function of TLRs in host defence and the role of commensal microorganisms in mucosal immunity,

especially in the female reproductive tract. This in turn raises the question of whether TLRs also play a role in innate immune responses during pregnancy (Abrahams 2005a).

1.4.2 The function of Toll-like receptors in pregnancy

There is a wealth of literature supporting the production of cytokines by gestation-associated tissues in response to both intra-uterine infection or *ex vivo* stimulation with microbial products such as LPS. As TLRs have been found to link microbial ligands and the production of various inflammatory mediators, it has been postulated that TLRs provide a mechanism of cytokine production at the materno-fetal interface.

1.4.2.1 Placenta

The placenta constitutes a physical and immunological barrier against invading infectious agents and has been called a pregnancy-specific component of the innate immune system (Holmlund, Cebers et al. 2002). Most TLR research in the context of pregnancy has focused on the placenta, although even these studies remain limited. Transcripts for TLRs 1-10 have been detected in the term human placenta (Zarember and Godowski 2002; Klaffenbach D 2005; Nishimura and Naito 2005; Mitsunari M 2006). Various cell types in the preterm and term placenta, including Hofbauer cells, villous, extravillous and intermediate trophoblasts, express TLR2 and TLR4 protein (Holmlund, Cebers et al. 2002; Kumazaki, Nakayama et al. 2004; Beijar EC 2006) as revealed by immunohistochemical studies. Holmlund *et al* demonstrated that term syncytiotrophoblast and intermediate trophoblast cells express TLR2 and TLR4 (Holmlund, Cebers et al. 2002). In contrast, Kumazaki *et al* found the TLR4 positive placental cells to be term extravillous and intermediate trophoblasts. Such contradictory findings might result from the type of antibodies used or the methodology employed. Kim *et al* (Kim, Romero et al. 2004), using real-time quantitative reverse transcriptase polymerase chain reaction (q-PCR), found TLR2 and TLR4 mRNA levels to be significantly higher in chorioamnion from women at term with spontaneous labour than women not in labour. This is the only study to date suggesting that changes in TLR expression are associated with labour. However, these three studies do clearly indicate the expression of at least TLR2 and TLR4 by cells of the placenta. Similarly, in preterm

placentae with chorioamnionitis the inflammatory cells infiltrating the placenta expressed TLR4, and TLR4 expression on the villous Hofbauer cells was increased in comparison to preterm placenta without chorioamnionitis as well as term placentae (Kumazaki, Nakayama et al. 2004). These results suggest an important role of the villous Hofbauer cells in the activation of innate immune system in response to infectious pathogens in preterm placentas.

Thus, cells of the placenta including trophoblasts could indeed interact with microorganisms and initiate an immune response, as was proposed by Gulieria and Pollard (Guleria and Pollard 2000). Functional activity of TLR2, TLR3 and TLR4 have been reported for first and third trimester trophoblast/placenta (Holmlund, Cebers et al. 2002; Abrahams, Bole-Aldo et al. 2004; Abrahams VM 2005; Abrahams VM 2006). Explants of term placenta do respond to ligands for TLR2 and TLR4 (zymosan and LPS, respectively) by producing IL-6 and IL-8 into culture supernatants (Holmlund, Cebers et al. 2002). However, this is the only work published so far in which the functional activity of TLRs at the materno-fetal interface has been considered. Moreover, definitive data that these responses occur via TLR 2 and/or 4 have not been provided yet.

Since an intrauterine infection might precede implantation, recent work has focused on the function of TLRs in the placenta during the first trimester of pregnancy. Shorter duration of pregnancies following *in vitro* fertilization treatment and repeated implantation failure is postulated to reflect infection and/or LPS contamination of the gestation-associated tissues as the consequence of the extensive invasive procedures involved in the treatment (Romero, Espinoza et al. 2004). Ligation of TLR4 by LPS triggers first trimester villous cytotrophoblasts and extravillous trophoblast cells to generate a classical inflammatory response, characterized by the increased production of both pro- and anti-inflammatory cytokines like IL-6, IL-8, TNF-α, IL-1, IL-10 and IL-4 (Abrahams, 2004). In contrast, the ligation of TLR2 by PGN fails to upregulate cytokine production by trophoblast cells but instead induces apoptosis of first trimester trophoblast cells (Abrahams, Bole-Aldo et al. 2004). Elevated trophoblast apoptosis occurs in pregnancies complicated with IUGR, pre-eclampsia and in preterm births (Ishihara, Matsuo et al. 2002). These complications have also been associated with

intrauterine infections (Romero, 2003). As mediators of both a proinflammatory response and apoptosis, TLRs expressed at the maternal-fetal interface could play an important role in the pathogenesis of all of these disorders. In this regard, a very recent study by Kim *et al* revealed significantly higher percentage of TLR4 positive interstitial trophoblasts on the placental bed biopsies from patients with preeclampsia (Kim, Romero et al. 2005).

Intrauterine infections during pregnancy could have either a direct or indirect effect upon trophoblast cell survival, depending upon which TLR is activated. A pathogen might directly promote trophoblast cell death through TLR2. Alternatively, triggering TLR4 would lead to production of high levels of cytokines, including TNFα and IFNγ, which in turn could promote trophoblast cell apoptosis (Crocker, Barratt et al. 2001). Together, these studies suggest that trophoblast cells can indeed function similarly to cells of the innate immune system, by recognising and responding to components of microorganisms and in cases of chronic ligand exposure can promote tissue dysfunction leading to pregnancy complications.

1.4.2.2 Decidua

The decidua contains many cell types able to produce a variety of inflammatory mediators and with the potential to mount an immune response against a pathogen (Mor and VM 2002). TLR2 and TLR4 protein have been detected in infiltrating cells, most likely macrophages and neutrophils, during inflammation of term decidua (Kim, Romero et al. 2004). Term decidual stromal cells also express these innate immune receptors, suggesting these cells might also contribute to the resolution of an invasive infection (Canavan , Shaival et al. 2004). Cells from first trimester decidua also express TLR1, 2 and 4 and studies to further characterise the expression and function of decidual TLRs are currently underway by this group (Abrahams and Mor. 2004). Recently TLRs have been studied in human laboured and non-laboured myometrium, and the authors found TLR expression to be upregulated with increasing gestation and not by onset of labour (R Youssef 2006).

1.4.2.3 Amnion

Amniotic epithelial cells clearly possess the mechanisms necessary to specifically recognise and respond to bacteria. Both TLR2 and TLR4 protein is expressed by amniotic epithelial cells and TLR2 expression was significantly higher in patients with chorioamnionitis (Kim, Romero et al. 2004). Interestingly, TLR2 expression was confined to the basal surface of amniotic epithelial cells in cases without chorioamnionitis, but this polarisation was lost in the presence of inflammation. (Kim, Romero et al. 2004). This expression pattern is analogous to TLR5 expression in gut epithelium, supporting the hypothesis that a pathogen must breach certain barriers before a response can be mounted (Gewirtz, Navas et al. 2001). Interestingly in cases of inflammation, such as in chorioamnionitis, this polarised distribution of TLR2 is lost and both TLR2 and TLR4 expression is upregulated (Kim, Romero et al. 2004). Similar upregulation of TLR2 and 4 occurs in membranes from women with spontaneous labour at term compared to women who were not in labour (Kim, Romero et al. 2005).

1.4.2.4 Cervix

As discussed above the cervix undergoes dramatic remodelling during parturition with numerous inflammatory mediators implicated in this. To date, only one study has been published in which the gene expression of TLRs in the cervix after vaginal delivery or elective caesarean section was determined. The microarrays used in this analysis covered approximately 47,000 probe sets including ones for TLR2, TLR3, TLR4 and TLR5 and revealed a significant decrease in expression of transcripts for TLR3 and TLR5 that could be confirmed by conventional quantitative PCR (the apparent up-regulation of TLR2 and TLR4 in the microarray study could not be confirmed) (Hassan, Romero et al. 2006; Hassan SS 2006). The biological significance of this remains to be determined but could reflect a direct role for TLR3 and TLR5 in labour via perhaps as yet unidentified endogenous ligands or down-regulation of these two receptors by any of the inflammatory mediators up-regulated in the cervix at this time.

In conclusion, elucidation of biological functions of TLRs in placenta and other gestation-associated tissues under physiological conditions requires further investigation.

1.5 TLRs AND LABOUR

1.5.1 TLRs and Term labour

A comparison has been made between the cellular and autocoid events that occur at the time of human labour and delivery and those that occur in association with inflammation and infection (Liggins, Fairclough et al. 1973). This analogy is highlighted in cases of bacterial infection associated preterm labour (Romero, Gomez et al. 2001) and also in term labour (Elliott, Loudon et al. 2001). The process of labour involves three physiologically interdependent processes: remodelling of the cervix to allow it to stretch open to the width of the reproductive tract, weakening and rupture of the membranes in the region that overlies the cervix and the initiation of rhythmic contractions of increasing amplitude and frequency that ultimately forces the fetus and placenta from the uterus.

Some or most of these processes seem to be mediated by pro-inflammatory cytokines, suggesting that the immune privileges that the fetal-placental unit has enjoyed during pregnancy are revoked at the time of labour (Elliott, Allport et al. 2001; Peltier 2003). TLR signalling could have a role in initiating these responses (Figure 1.5). An inflammatory response during labour might also help to remove placental fragments and prepare the uterus for the pathogens that it will undoubtedly encounter during the immediate postpartum period.

So far we know that progesterone withdrawal plays a role in initiation of labour. In rodents and ruminants labour is preceded by a rapid decline in peripheral progesterone concentrations (Liggins, Fairclough et al. 1973). However, in primates and guinea pigs progesterone concentrations remain elevated until the delivery of the placenta. Recent studies have suggested that there may be a "functional withdrawal" of progesterone in primates by modulation of progesterone receptors and that this might be mediated via

TLRs. In the decidua there is a significant loss in progesterone receptor binding to its response element in samples collected after labour (Henderson and Wilson 2001). During labour, there is a significant shift of progesterone receptor types in the myometrium from the B-form to the A-form and expression of both receptors on the amnion is significantly reduced (Haluska, Wells et al. 2002). The A-form of the progesterone receptor appears to inhibit signal transduction of the B-form because myometrial cells transfected with both receptors failed to initiate gene expression, whereas those transfected with the B-form only did (Pieber, Allport et al. 2001). In human cervical stroma, a shift from B-form to A-form of the progesterone receptor with no total change in progesterone receptors has also been described (Deng, P et al. 2003). The progesterone receptor is important in suppressing the inflammatory properties of estradiol in mice (Tibbetts, Conneely et al. 1999) and abrogating the function of progesterone receptors could permit proinflammatory cascades to occur *in utero* at term in conjunction with rising estradiol levels.

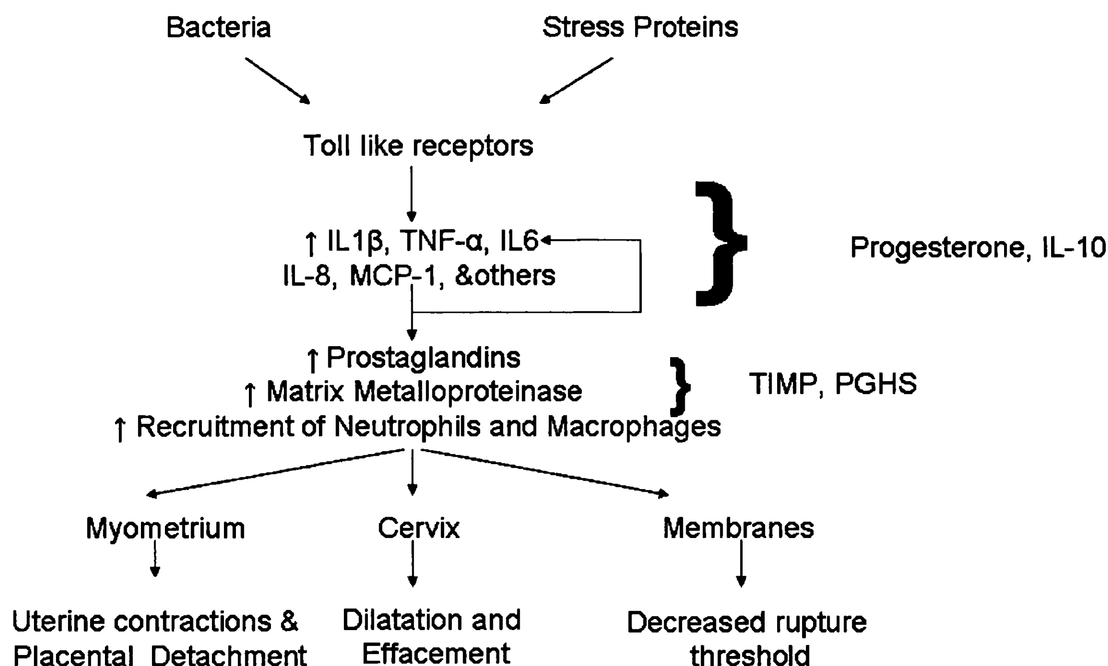


Figure 1.5 Model for biochemical cascades involved in labour.

With the influence of progesterone abrogated, IL-8, IL-1β, IL-6 and TNF-α production increases in the human cervix, perhaps via a TLR mediated pathway, during cervical ripening and labour. Proinflammatory cytokines can induce the ripening of the cervix in a number of ways. IL-1β and TNF-α increase the production of matrix metalloproteinase (MMP)-1, MMP-3, MMP-9, and cathepsins (Watari, Watari et al. 1999) and IL-1β down-regulates the expression of tissue inhibitor of metalloproteinase (TIMP)-2, an endogenous inhibitor of MMP-2. A shift in the balance towards proteinase activity favours digestion of collagen and elastin fibres in the extracellular matrix of the cervix to further increase cervical compliance. IL-1β can also act on a number of cell types to increase the production of cyclooxygenase (COX)-2 and thereby PGE_2, the most effective chemical for inducing cervical dilation in women. IL-1α, which uses the same receptor as IL-1β, has been shown experimentally to increase COX-2 and PGE_2 production by rabbit cervical smooth muscle cells (Sato, Michizu et al. 2001). PGE_2 may then further stimulate labour by increasing the production of proteinases or may play an indirect role by increasing the permeability of blood vessels for leukocyte trafficking (Kelly 2002). Nitric oxide (NO), another proinflammatory mediator that is increased at term, may also contribute to vasodilatation in order to facilitate leukocyte trafficking.

A similar proinflammatory process takes place in the membranes and the myometrium. During labour, the production of IL-8, TNF-α, IL-6 and IL-1β increases in the membranes (Young, Thomson et al. 2002) and these mediators increase the production of prostaglandins. Prostaglandins, in turn, stimulate myometrial contractility and cervical ripening. Increased levels of protein and/or mRNA for IL-1β, TNF-α and IL-6 in the myometrium are also associated with labour (Young, Thomson et al. 2002) (Osman, Young et al. 2003). These pro-inflammatory cytokines have been immunolocalised to the leukocytes in the myometrium, numbers of which are increased during labour. IL-1β and TNF-α stimulate arachidonic acid release, activate phospholipid metabolism and increase the production of prostaglandins by the myometrium (Pollard, 1991). IL-1β activates a signal transduction system involving NF-κB to increase the expression of COX-2 (Belt, 1999), which is increased in the myometrium during labour (Slater, 1999), and stimulates the production of PGE_2 by

myometrial cells (Todd, 1996). These effects of IL-1β on myometrial cells are similar to the effects of oxytocin which also upregulates COX-2 and PGE_2 production by myometrial cells. Oxytocin and PGE_2 both increase the intracellular calcium concentration in myometrial cells, which is required for uterine contractions (Thornton, Gillespie et al. 1992).

Although IL-6 has no effect on prostaglandin production by myometrial cells (Todd, Dundoo et al. 1996) and is unable to stimulate myometrial contractions (Dajani, 1994), this cytokine might play a role in labour by increasing the expression of oxytocin receptors on myometrial cells (Rauk, Friebe-Hoffmann et al. 2001) thereby increasing their responsiveness to oxytocin. Like IL-1β, IL-6 can also increase oxytocin secretion by myometrial cells (Friebe-Hoffmann, Chiao et al. 2001). IL-1β and TNF-α can also increase the production of MMP-9 by myometrial cells, which may be important for detachment of the placenta (Roh, Oh et al. 2000).

Thus, undoubtedly, cytokine production increases several folds at the materno-fetal interface at the onset (none of the cytokines or related molecules however can be measured at the onset of labour in humans) of labour whether at term or preterm. It is still not clear how this happens. However TLR-mediated signalling pathways leading to a defined cytokine output from the stimulated cell might be one of the mechanisms instrumental in this process.

1.5.2 Preterm labour

As discussed previously, bacterial infections, if present within the uterus during pregnancy, can represent a significant threat to the well being of the fetus. Indeed, animal models of pregnancy complications have been generated by the administration of Gram-negative bacterial LPS (Bennett, Terrone et al. 2000; Wang and Hirsch 2003). Since clinical studies have shown an association between intrauterine infections and preterm labour (Elovitz, Wang et al. 2003; Wang and Hirsch 2003), it can be hypothesised that certain cell types within gestation-associated tissues recognise and respond to bacterial components through TLRs. LPS from Gram-negative bacteria has

been implicated in IA-PTL and there have been a number of studies exploring the role of TLR4 in preterm labour. Functional TLR4 has been implicated in heat killed *E.coli* mediated preterm labour in mice (Wang and Hirsch 2003). Thus, TLRs expressed at the maternal-fetal interface could play an important role in the pathogenesis of infection-associated prematurity (Abrahams 2005a). Besides the pathophysiological evidence, a role for TLRs in IA-PTL also comes from the genetic studies. A polymorphism (Asp299Gly) in TLR4 associated with impaired TLR4 function and an increased likelihood of Gram-negative sepsis was carried more often by preterm infants than term infants or by mothers delivering preterm than at term (Lorenz, Hallman et al. 2002). Unpublished data referred to by this same group highlight that studies should not be restricted to TLR4 as in the same Finnish population there was a trend towards higher frequency of TLR2 mutations in preterm compared with term infants.

Thus one can hypothesise that once present within the uterus; an infectious agent might take one of two, not mutually exclusive, main routes. Firstly, an infectious agent might cross the fetal membranes and if successful, proceed to infect the fetus. Alternatively, it might gain access to the maternal-fetal interface by infecting the placenta or maternal deciduas (Goncalves, Chaiworapongsa et al. 2002). As shown in Figure 1.4b TLRs recognise conserved motifs of microbial origin or that are indicative of cellular damage and mediate the production of various proinflammatory mediators that differ depending on the TLR(s) stimulated (Erroi, Fantuzzi et al. 1993; Deetz, Jagielo et al. 1997). These mediators (such as TNFα, IL-6, IL-8) interact to augment their own production, recruit neutrophils and macrophages to the maternal-fetal interface, and increase the production of downstream mediators of inflammation - the most important of which seem to be prostaglandins and matrix metalloproteinases. As discussed above for term labour (Figure 1.5) these effects include cervical ripening and dilatation and uterine contractions thereby activating the parturition mechanism prematurely (Ville 2001). During much of pregnancy, immunosuppressive substances such as progesterone and IL-10 control these proinflammatory cascades and the current literature suggests that excessive levels of stress proteins or infectious organisms override the protective effects of such molecules to cause preterm labor and/or PPROM (Peltier 2003).

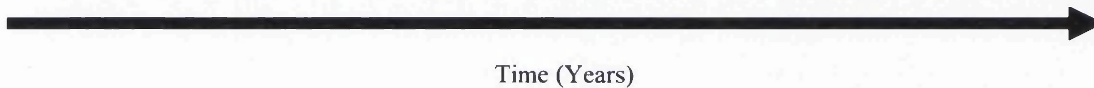
Recently it has been suggested that infection might trigger apoptosis and that disturbances in the regulation of apoptosis within the placenta might be associated with abnormal pregnancy outcomes (Jerzak and Bischof 2002). Elevated trophoblast apoptosis is seen during the first trimester of pregnancies complicated with IUGR or preeclampsia (Ishihara N, 2002; Smith SC et al, 1997) and in preterm births (Kakinuma, Kuwayama et al. 1997; Balkundi, Ziegler et al. 2003). It has been suggested that certain intrauterine infections during pregnancy have either a direct or indirect effect upon trophoblast cell survival, depending upon which TLR is activated (Abrahams VM, 2004b).

The study of TLR mediated inflammation at the maternal-fetal interface is in its infancy. A better understanding of these proteins and the signal transduction cascades that they mediate might explain why some pregnancies are complicated by PTL and PPROM whereas others are only affected by PPROM. More investigations into the endogenous activators of the TLRs might also explain how PTL and PPROM can occur in the absence of infection (e.g. preeclampsia, multiple gestation, teenage pregnancy, or with excessive tobacco and alcohol consumption). These molecules might offer excellent targets for therapeutic strategies because they are upstream mediators of the proinflammatory cascade that ultimately results in PTL.

1.6 CLINICAL PERSPECTIVE

Despite the recognition over the past two decades that intrauterine infection and inflammation are causal factors for a significant number of preterm deliveries, attempts to reduce the number of preterm births using various antibiotic treatment regimes have proved ineffective. Again none of the tocolytic drugs known and used so far have been successful in stopping preterm labour (Sanchez-Ramos, Kaunitz et al. 1999) (Figure-1.6).

Silent infections and PTL	Infection associated PTL	Proinflammatory cytokines and labour	Tocolytics	Cortico-steroids	Oral antibiotics	TLRs and labour
1987 Iams and co-workers	1988 Romero Casey	1990 Casey MI, Macdonald PC	1960's - 2002 Sanchez-Ramos et al	1994 Kari MA et al	2002 Kenyon S et al	2004- Holmund; Kumazaki; Kim



Time (Years)

Figure 1.6 *Time-line for investigations of causes and prevention of preterm labour.*

Histological and *in vitro* studies suggest that the solution might be the rapid suppression of inflammatory reactions triggered by microbial products before the irreversible initiation and propagation of positive feed-forward cascades that precipitate preterm labour. The NF-κB transcription factor serves as a focal point for inflammatory mediators such as TNFα, LPS and the TLR family. A number of pharmacological inhibitors of the NF-κB activation pathway have been evaluated, using gestational membrane explants and perfusion methodologies, in an attempt to determine if this might be a valuable approach for the treatment and prevention of IA-PTL (Keelan 2004). Four NF-κB inhibitors were initially evaluated across a range of doses: Sulfasalazine (SSZ), BAY-11-7085, Helanalin, and Pyrrolidine dithiocarbamate. SSZ treatment of LPS stimulated fetal membranes with attached decidua resulted in significantly reduced production of TNF-α in both the maternal and fetal compartments. Similarly, IL-6 levels after treatment were abrogated in the maternal compartment and significantly reduced in the fetal compartment.

These studies suggest that the NF-κB inhibitor SSZ is capable of suppressing LPS-stimulated pro-inflammatory cytokine production in gestational membranes with a rapid onset of action (2 hours or less) and at least 20 hours duration. The administration of

SSZ does not appear to be associated with onset of cell death, arguing against the possibility that NF-kB inhibition might be pro-apoptotic in these tissues. These data support the further evaluation of SSZ, a drug used in the treatment of inflammatory bowel disease and rheumatoid arthritis, as a treatment modality for the prevention of IA-PTL.

The identification of TLRs as upstream mediators of inflammation offers alternative therapeutic targets for preventing common pregnancy complications such as preterm labour, and hopefully others such as IUGR and preeclampsia. Alternatively, these receptors might serve as clinical biomarkers of disease. There are at least three strategies for interfering with TLR signalling (Opal and Huber 2002), with the specific goal of reducing the consequences of their biological effects.

1. The generation of specific soluble TLRs to bind and neutralise their respective microbial ligands. Recently, soluble TLR2 was identified in human plasma and breast milk and found to be produced by peripheral blood monocytes (LeBouder, Rey-Nores et al. 2003). Soluble TLRs might function by modulating specific TLR mediated responses or alternatively, might bind to microorganisms and flag them for destruction by the complement system or by phagocytosis (Medzhitov and Janeway 2002). Soluble TLRs might, therefore, provide new markers of pregnancy complications as well as a potential target for therapeutic interventions.

2. The development of small antagonistic molecules or antibodies that interfere with the extracellular domain of TLRs and act as TLR antagonists.

3. The generation of small molecules that interfere with the intracellular domain of TLRs and prevent the interaction with distal intracellular signalling molecules (e.g., MyD88, IKK) (Ulevitch 2004).

A possible fourth strategy is the use of an inhibitor of NF-κB, like sulfasalazine, as suggested by Keelan et al, 2004.

Similarly a genetic approach might provide important information regarding an individual's susceptibility to developing infection-based fertility problems or pregnancy complications. Polymorphisms in TLR2 and 4 have been identified and have been linked with altered responsiveness to TLR ligands and these genetic mutations might have an impact clinically (Lorenz, Hallman et al. 2002; von Aulock, Schroder et al. 2004).

It is very much possible that therapeutic use of any of the above could neutralise beneficial components of the host defence. However, if the genetic basis of susceptibility to infection can be determined, tools will become available that might help identify patients at high risk for pregnancy complications like preterm labour and appropriate interventions could then be individually tailored (pharmacogenetics).

1.7 HYPOTHESIS

The contribution of TLRs to inflammation at the materno-fetal interface is only beginning to be understood. It is clear that a better understanding of TLRs and their signalling cascades is of relevance to labour, be it at term or preterm, as the downstream effects of activating any particular TLR will be similar irrespective of whether an endogenous or exogenous ligand is the stimulus. Spontaneous term labour and infection-associated preterm labour can therefore feasibly be mediated by the same TLR-mediated pathways leading to the common outcome of elevated pro-inflammatory cytokine production and the effects on gestation-associated tissues for the initiation and maintenance of labour.

Therefore the hypothesis to be tested is: "that activation of toll-like receptors by endogenous and exogenous ligands contributes to term and preterm labour, respectively".

1.8 OBJECTIVES

1. *To determine TLR expression and activity in the term human placenta*: Placentas will be collected after delivery at term in the absence of labour. Explants of placental tissue will be cultured *in vitro* in the presence of optimised concentrations of various TLR ligands (for TLR1–9) and pro- and anti-inflammatory cytokine production (TNFα, IL-6, IL-8 & IL-10) into the culture supernatants will be measured using ELISA. Expression of each TLR will be further investigated using RT-PCR and q-PCR for mRNA analysis.

2. *To investigate changes in TLR response and expression in term labour:* The methodologies used in (1) above will be applied to placentas collected after the completion of spontaneous labour at term.

3. *To determine TLR expression and response in preterm labour:* As in (1) above biopsies will be taken from placentas delivered after preterm labour for explant culture, mRNA analysis and quantification of TLR expression by RT-PCR and q-PCR.

1.9 OUTCOMES:

The proposed outcomes are:

- (i) Basal expression and activity of TLR1-9 in the human term placenta.
- (ii) Ontogeny of TLR1- 9 expression and/or activity in the human placenta.
- (iii) Changes in TLR expression and/or activity in spontaneous term labour.
- (iv) TLR1-9 expression and/or activity in infection-associated preterm labour compared with term labour.

CHAPTER 2

MATERIALS AND METHODS

2.1 PATIENT RECRUITMENT AND SAMPLES

All participants gave informed written consent to have the placenta collected after delivery. Approval for the study was given by the Local Research Ethics Committee covering Bridgend, Neath Port Talbot and Swansea (LREC Project Registration No: 04/WMW02/18). Relevant documentation (Consent form) is shown in Appendix 1.

2.1.1 Patient Groups

Otherwise healthy pregnant women with a singleton fetus were recruited to give the following three groups (Appendix 2):

1. **Term non-laboured group** (gestational age >37 weeks): Women booked for elective caesarean section at term due to varied obstetric reasons such as cephalopelvic disproportion, breech presentation and/or previous caesarean section were approached when attending the antenatal day assessment unit at Singleton Hospital, Swansea for a pre-operative assessment. The placenta was collected at the time of the caesarean section by liaising with the delivery suite team.
2. **Term laboured group (**gestational age >37 weeks): Women were approached in the delivery suite either during early labour or soon after a spontaneous vaginal delivery as deemed suitable by the midwife responsible for the case.
3. **Preterm group (**gestational age >24 weeks to <37 weeks): Women were approached either at their admission in early labour or soon after delivery again in full liaison with the midwives. Whilst the primary aim was to include preterm deliveries with infectious etiology, 'all cases' were accepted.

Exclusion criteria: Women with autoimmune or other immunological disorders were excluded from the study as were the women with diabetes mellitus including gestational diabetes and multiple pregnancies and any other medical infections and illnesses.

Demographic and obstetric details: Gestational age was calculated either by an early ultrasound or by the first day of the last menstrual period (when women were sure of their dates). The necessary demographic and obstetric details were obtained from the hospital notes of both mother and baby and were recorded on a set proforma (Appendix 3). The mothers were aged 17-37 years old (median 28.5 years) and had up to 5 previous children. The term babies (18 boys, 16 girls) weighed 2610-4760 grams (median 3685 grams). The preterm babies (9 boys, 6 girls) weighed 860-3230 grams (median 2045 grams). The weight of the term placentae ranged from 438-831 grams (median 634.5 grams) and the preterm placentae ranged from 271-835 grams (median 553.0 grams). The details are shown in Table 2.1.

Variables	**Non-Laboured (ECS)**	**Laboured (SVD)**	**Preterm** (25-36 weeks)
Maternal age (years)	20 - 36 (median 30.5) p=0.002	17 - 35(median 26)	15-37 (median 26) p=0.949
Parity	1 – 3 (median 1) p=0.887	1 – 5 (median 1)	1-2 (median 1) p=0.119
Placental weight (grams)	438 - 818 (median 683) p=0.964	478 - 831 (median 648)	271 - 515 (median 309)
Sex	7 boys; 10 girls p=0.506	10 boys; 7 girls	9 boys; 6 girls p=0.908
Birth weight (grams)	2610 – 4450 (median 3550). p=0.977	2950 - 4760 (median 3440).	860-3260 (median 2175).

Table 2.1 *Demographic and obstetric details of the two experimental groups. p values in column two are between the term nonlaboured and term laboured while p values in column four are between term svd and preterm groups.*

2.1.2 Sample Size

Power calculation analysis was based on previous studies by the group in which placental cytokine production was the primary outcome and was performed using PS-Power and Sample Size Calculation software (Version 2.1.31). This analysis revealed that 17 placentae were required per group for the comparison of labour versus non-labour at term. Due to time limitations as many preterm samples as possible were collected (n = 15 at varying gestational ages).

2.1.3 Collection of Samples

Immediately following delivery, placentae were collected into a clean bag and placed in a clean bucket and taken to the laboratory for processing.

2.2 PROCESSING OF PLACENTA

2.2.1 Placental Explant Culture

Care was taken in handling the placenta to limit contamination. The placenta was weighed and then transferred to a Class II tissue culture cabinet wherein all procedures were performed. Placental explant cultures (0.2 g of wet tissue/ml) were prepared as described (Hanna, Hanna et al. 2000). The overlaying decidua basalis on the maternal side of the placenta was removed and 1cm^3 pieces of placental tissue were taken from different sites across the placenta and placed into sterile phosphate buffered saline (PBS; Invitrogen, UK). Disturbance of the chorioamnion was avoided. Pieces of tissue were washed repeatedly with PBS to remove contaminating blood. The tissue was then minced into smaller pieces (approximately 1-2 mm^3) and washed further with PBS. Pieces of placental tissue (0.5g) were transferred into each well of a standard tissue culture 6-well plate (Grenier Bio-one, Germany) containing 2.5mls of Ultraculture medium (Cambrex, Belgium) supplemented with 2mM Glutamax (Invitrogen) and 100U/ml penicillin G, 100µg/ml streptomycin sulphate and 0.25µg/ml amphotericin B (PSF; Invtirogen). Care was taken to avoid any blood clots or fibrous tissue.

Once the explant cultures were prepared, different stimuli were added to each well as shown in Table 2.2 (details in *Chapter 3).* An unstimulated control was always included. Immediately after adding ligands, the plates were incubated at 37°C in 5% CO_2 for 24 hours. All stimuli were purchased from InvivoGen, SanDiego, CA, USA. Cell/tissue free culture supernatants were collected by centrifugation and stored at -20°C until assayed.

Maintenance of LPS-free conditions

Extreme care was taken to limit LPS/endotoxin contamination during explant preparation. These precautions included the use of disposable plastic-ware and other consumables (e.g. scissors) whenever possible (Jones, Finlay-Jones et al. 1997). All media/reagents were tested by the manufacturers and found to be endotoxin free.

2.2.2 Biopsies for RNA analysis

Additional placental tissue was removed and washed several times in cold PBS to remove contaminating blood. A fixed amount of the tissue (0.2-0.26g) was transferred into 1.3ml of RNA*later*® (Sigma, USA). Biopsies in RNA*later*® were stored in the fridge for 48 hours before storing at -80°C until analysis.

2.3 TREATMENT OF PLACENTAL EXPLANTS

2.3.1 TLR Ligands

The placental explants from each donor were treated with each of the ligands as shown in the Table 2.2. Response curves for each ligand were conducted to determine optimal concentration to use.

TLR Ligands	TLR stimulated
Peptidoglycan (*S. aureus*)	TLR2
Polyinosine-polycytidilic acid (Poly (I:C))- ds RNA	TLR3
Ultra Pure *E.coli* (0111:B4) LPS	TLR4
Flagellin (*S. typhimurium*)	TLR5
Zymosan	TLR2/6
R848 (anti viral synthetic compound)	TLR7/8
Loxoribine	TLR7/8
ssPoly-single stranded polyU oligonucleotide	TLR7/8
CpG - unmethylated deoxycytidine-deoxyguanosine dinucleotide	TLR9
ODNC-control oligonucleotide 2216	Control (for CpG TLR9)

***Table 2.2** TLR ligands added to placental explant cultures.*

2.3.2 Dexamethsone

Placental explants from term non-laboured placentas were treated with the optimised concentration of each TLR ligand in the presence of 0.4, 4 or 40 ng/ml dexamethasone (Sigma, USA) for 20 - 24 hours. Supernatants were harvested and stored at -20°C until analysis of IL-6 levels by ELISA.

2.3.3 Pretreatment with Ligands

Placental explants from term non-laboured placentas were treated with the optimised concentration of each TLR ligand. After incubation at 37 °C for 20-24 hours, the tissue was washed by centrifugation and recultured in fresh media containing concentrations of TLR ligands as indicated in the results (*Chapter 5)*. Supernatants were harvested after further 20 - 24 hours incubation and stored at -20 °C until analysis of IL-6 levels by ELISA.

2.4 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Cytokine concentrations in supernatants from placental explant cultures were determined using commercial ELISA kits according to the manufacturers' instructions. The different cytokines measured and their sensitivities as specified by the manufacturers are shown in Table 2.3.

Cytokine	Sensitivity	Company
TNFα	4pg/ml	OptEIA ELISA BD Biosciences, USA
IL-10	4pg/ml	OptEIA ELISA
IL-6	1pg/ml	OptEIA ELISA
IL-8	1-3pg/ml	Pelkine ELISA Sanquin Reagents, The Netherlands.
IFN-α	8pg/ml	Module Set ELISA Med Systems Diagnostics, Austria.
IFN-γ	2pg/ml	OptEIA ELISA

***Table 2.3** Details of ELISA used for this study.*

The methodology for all assays was essentially the same. Briefly, half area 96 well plates (Costar, Corning, USA) were coated with 50µl of capture antibody prepared in 0.05M carbonate/bicarbonate buffer pH 9.6 (prepared from carbonate-bicarbonate buffer capsules; Sigma, USA) or PBS as appropriate and incubated overnight in the fridge. After discarding excess coating antibody, nonspecific reactivity was blocked by incubation for 1 hour at room temperature with 1% bovine serum albumin (BSA; Sigma) in PBS (150µl/well). The plates were then washed three times with wash buffer (1% BSA in PBS with 0.05% Tween-20 (Sigma, USA); 200µl/well) before the sample or standard was applied in duplicates (50µl/well). After 2 hr incubation at room temperature, the plates were washed four times with the wash buffer as before and then,

for OptEIA assays 50µl/well of biotinylated detecting antibody and avidin-horseradish peroxidase mix was added. For non-OptEIA assays, the biotinylated detecting antibody and avidin-horseradish peroxidase (both 50µl/well) were added separately and required, respectively, 1 or 2hrs and 30 or 60 minute incubation at room temperature (time of incubation was assay dependent). Plates were washed four times between detecting antibody as the next step. Upon completion of the incubation with avidin-horseradish peroxidase the contents of the wells were discarded and the plate was washed six times with wash buffer as before. Substrate chromogen (tetramethylbenzidine, BD Biosciences; 5̃0µl/well; prepared according the manufacturer's instructions) was then added and the blue colour allowed to develop (variable times for each cytokine assay). The reaction was stopped with 1M H_2SO_4 and the subsequent colour intensity was recorded as the optical density at 450nm using an ELISA plate reader (Multiskan Ascent, model no. 354, Thermo Lab systems).

Cytokine concentrations in the samples were calculated in pg/ml from the standard curve (EXCEL; Microsoft office 2003).

2.5 GENE EXPRESSION

2.5.1 RNA Extraction

RNA was extracted using a modified method based on Reno et al (1997) known as the 'TRIspin method'. This method involves a homogenisation step with TRIzol™ (Invitrogen) followed by purification using the RNeasy® Mini Kit (Qiagen, UK).

Approximately 50mg of placental tissue was removed from the RNA*later*® and homogenised in a FastPrep FP120A (Bio101 Savant, Qbiogene; The Netherlands) using 2ml Lysing Matrix D tubes (Bio101 Systems; Qbiogene) containing 600µl TRIzol™. The homogenised sample was centrifuged at 20,000rpm for 5 minutes at 4ºC. The supernatant was removed and incubated for 5 minutes at room temperature to allow for complete dissociation of nucleoprotein complexes. Chloroform (150µl) was then added to the supernatant and the sample mixed by repeated inversion prior to centrifugation at

20000 *x g* for 15 minutes at 4°C. The upper aqueous phase was removed, and one volume of 70% ethanol was added to the aqueous phase and mixed well. This was transferred to a Qiagen assay minicolumn and RNA was isolated according to the manufacturer's protocol. This kit isolates all RNA molecules longer than 200 nucleotides, while low molecular weight RNAs, including 5S rRNA and tRNAs, are excluded. A total of 60µl RNA extract was obtained from each sample.

Any contaminating genomic DNA in the RNA extract was removed using the DNA-free DNase Kit (Ambion, UK) according to the manufacturer's instructions. The yield of DNA free RNA was then quantified spectrophotometrically using NanoDrop (ND-3300 flurospectrometer, NanoDrop Technologies; USA) and optical density ratios (OD_{260}/OD_{280}) were determined. A ratio ≥ 1.7 was taken as satisfactory. RNA quality was also checked using the Agilent system (RNA 6000 Nano LabChip run on Agilent 2100 Bioanalyzer).

2.5.2 Complementary DNA (cDNA) Synthesis (reverse transcription)

Reverse transcription was completed using Ambion's RETROscript kit following the manufacturer's instructions. RNA (1µg) was reversed transcribed into cDNA using murine leukaemia virus reverse transcriptase enzyme and random decamers in a 20µl volume. To ensure that all of the reagents used for reverse transcription were DNA free, a no template control (NTC) which contained all of the reagents used for the reverse transcription and water in place of RNA was always included. To confirm that the reverse transcription worked a positive control (mouse liver RNA) provided in the kit was always included. The reaction mixture was incubated in a Polymearse Chain Reaction (PCR) thermal cycler (Flexigene; Techne, Cambridge, UK) at 44°C for 60 minutes, and then heated to 92°C for 10 minutes to inactivate the enzyme.

To confirm that cDNA was synthesised a PCR amplifying the human S15 (ribosomal protein) gene was performed using 1.0µl of the cDNA mixture (the rest was aliquoted for storage at -80°C). For each PCR, the following samples were included: (1) cDNA obtained from the experimental samples; (2) the corresponding RNA from each sample

to test for genomic DNA within the extracts; (3) a negative control which had all the PCR reagents but water in place of cDNA; (4) a NTC brought forward from the reverse transcription step; (5) a positive control which had cDNA from the mouse liver RNA as described above.

The inclusion of the controls assured the reliability of the PCR and the reverse transcription reactions. A total volume of 20μl containing cDNA, primers, dNTP, 10xPCR buffer from the kit along with thermostable Taq DNA polymerase (5U/μl, ABgene, UK) and nuclease free water was prepared for each sample according to the manufacturer's instructions and was placed in the thermal cycler. The primer sequences and cycling parameters are shown in Table 2.4.

Primer sequences- forward	5'- TTCCGCAAGTTCACCTACC
Primer sequences- reverse	5'- CGGGCCGGCCATGCTTTACG
Fragment length	361bp
PCR conditions	94°C for 1 minute, 30 cycles of (94°C for 30 seconds; 59°C for 30 seconds; 72°C for 30 seconds), 72°C for 4 minutes

***Table 2.4** Primer sequences and cycling specifications for the S15 PCR used to confirm that cDNA synthesis was successful.*

PCR products were analysed by electrophoresis through 1% agarose gel in 1X Tris-borate-EDTA (TBE; Sigma-Aldrich, St. Louis, USA) containing ethidium bromide (18μl/100ml of agarose; Fluka, Biochemica, Sigma) at 100 volts for at least 30 minutes and subsequently visualised using UV light. If all stages of RNA extraction and cDNA synthesis were successful, bands of the appropriate size should be visible for all of the experimental samples and for the positive control but not for the RNA from the corresponding sample, NTC or the negative control. If bands were visible for the RNA extracts from the experimental samples, thereby indicating the presence of genomic DNA, these extracts were discarded, and fresh extracts prepared from the tissue stocks. Only upon ensuring that, after S15 PCR, there were no bands visible from the fresh

RNA extracts but that there were bands visible from the new cDNA samples was the cDNA used for real time quantitative PCR.

2.5.3 Real Time Quantitative PCR (qPCR)

Quantitative PCR was carried out using an iCycler PCR machine (iCyler IQ version 3.1, Bio-Rad, UK) for all of the genes of interest (TLR 1-10) and three house keeping genes; succinate dehydrogenase complex, subunit A **(SDHA)**; TATA box binding protein **(TBP)**; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide **(YWHAZ)**, (Meller, Vadachkoria et al. 2005). The primers and PCR conditions used are listed in Table 2.5. All of the primers used have been published previously [(Vandesompele, De Preter et al. 2002; Zarember and Godowski 2002); http://medgen.ugent.be/rtprimerdb,primer ID2629)] and were synthesised by MWG Biotech AG (Ebersberg, Germany). The housekeeping genes were chosen based on a study that demonstrated that these three genes are the most stably expressed house keeping genes in the human placenta (Meller, Vadachkoria et al. 2005). This ensures that the gene expression profile comparisons have more sensitivity and stability.

Broadly for each qPCR, 1µl of the cDNA (prepared as in *section 2.5.2*) was added to a 20µl mix containing 1µl each of appropriate forward and reverse primers (5µM), 7µl IQ SYBR-green supermix (Bio-Rad, UK) and 10µl nuclease free water. Optimisation was performed for each qPCR for primer concentration, annealing temperature and, if required, magnesium chloride concentration (Table2.5). Apart from TLR7 (15µM of reverse primer) and TLR9 (3.5mM of magnesium chloride), all of the qPCRs had the same proportions of different components in the PCR mix.

The qPCR conditions for all PCRs only differed for the annealing temperatures. Each plate was treated at 95°C for 210 seconds and then 40 cycles of: 95°C for 30 seconds, appropriate annealing temperature for 30 seconds and 72°C for 10 seconds. The programme (iCyler IQ version 3.1) also enabled a melt curve for each qPCR to be undertaken to assist in the identification of any non-specific PCR products: the PCR product was heated at 95°C for 60 seconds, then 55°C for 60 seconds and then the

cDNA	Forward primer	Reverse primer	Fragment size	Primer concentration		Mg^{++}	Annealing temperature
				Forward	Reverse		
TLR-1	5'-CAGTGTCTGGTACACGCATGGT-3'	5'-TTTCAAAAACCGTGTCTGTTAAGAGA-3'	104	5µM	5µM	3mM	60
TLR-2	5'-GGCCAGCAAATTACCTGTGTG-3'	5'-AGGCGGACATCCTGAACCT-3'	67	5µM	5µM	3mM	62
TLR-3	5'-CCTGGTTTGTTAATTGGATTAACGA-3'	5'-TGAGGTGGAGTGTTGCAAAGG-3'	82	5µM	5µM	3mM	57
TLR-4	5'-CAGAGTTTCCTGCAATGGATCA-3'	5'-GCTTATCTGAAGGTGTTGCACAT-3'	88	5µM	5µM	3mM	56
TLR-5	5'-TGCCTTGAAGCCTTCAGTTATG-3'	5'-CCAACCACCACCATGATGAG-3'	77	5µM	5µM	3mM	56
TLR-6	5'-GAAGAAGAACAACCCTTTAGGATAGC -3'	5'-AGGCCAAACAAAATGGAAGCTT-3'	88	5µM	5µM	3mM	56
TLR-7	5'-TTTACCTGGATGGAAACCAGCTA-3'	5'-TCAAGGCCTGAGAAGCTGTAAGCTA-3'	73	5µM	15µM	3mM	59
TLR-8	5'-TTATGTGTTCCAGGAACTCAGAGAA-3'	5'-TAATACCCAAGTTGATAGTCGATAAGTTTG-3'	83	5µM	5µM	3mM	56
TLR-9	5'-GGACCTCTGGTACTGCTTCCA-3'	5'-AAGCTCGTTGTACACCCAGTCT-3'	151	5µM	5µM	3.5mM	61.2
TLR10	5'-TGTTATGACAGCAGAGGGTGATG-3'	5'-GAGTTGAAAAAGGAGGTTATAGGATAAATC-3'	151	5µM	5µM	3mM	56
SDHA*	5'-TGGGAACAAGAGGGCATCTG-3'	5'-CCACCACTGCATCAAATTCATG-3'	86	5µM	5µM	3mM	60
TBP*	5'-TGCACAGGAGCCAAGAGTGAA-3'	5'-CACATCACAGCTCCCCACCA-3'	132	5µM	5µM	3mM	63
YWHAZ*	5'-ACTTTTGGTACATTGTGGCTTCAA-3'	5'-CCGCCAGGACAAACCAGTAT-3'	94	5µM	5µM	3mM	63

***Table* 2.5** *Specifications for qPCR*

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

TBP- TATA box binding protein

YWHAZ- tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

temperature was increased by 0.5°C for 10 seconds each time for a total of 80 cycles. A single peak at the correct melting temperature indicated a reliable and desired PCR product.

As all samples and controls were to be analysed in triplicate, the total number of wells required meant that each qPCR had to be run over two plates. To ensure that the results were comparable, samples from the experimental groups were spread equally over both plates. The RNA extractions were performed in three batches as well, and care was taken to ensure that samples from each batch also were spread equally over both plates. Furthermore, to address plate to plate variation four 'calibrator samples': placental cDNA (1000ng/ml and 300ng/ml) and spleen cDNA (1000ng/ml and 300ng/ml) were included. These were run on both plates (Appendix 4). Therefore, in summary, the samples included on each plate were: (1) cDNA obtained from the experimental samples; (2) a no template control (NTC) brought forward from the reverse transcription step; (3) a negative control containing all PCR reagents but water in place of cDNA; (4) the four calibrator samples as detailed above. (See Appendix 5 for generalised layouts of Plates 1 and 2 for all qPCRs.)

PCR efficiency:

The efficiency of each PCR was determined for all TLR genes and the three house keeping genes. This was performed by generating two sets of standards using six different concentrations of RNA from two separate sources:

1. Placental tissue (elective caesarean section; RNA extracted using same protocol as outlined in *section 2.5.1*) - 3000ng/ml, 1000ng/ml, 300ng/ml, 100ng/ml, 30ng/ml and 10ng/ml.

2. Spleen RNA (commercially produced RNA from Clontech, CA; USA) - 3000ng/ml, 1000ng/ml, 300ng/ml, 100ng/ml, 30ng/ml and 10ng/ml.

These sets of standards were derived as follows: RNA concentration was determined using the Nanodrop (as described previously) and dilutions made with nuclease-free water to give the six concentrations required. These were individually reverse

transcribed and the success of cDNA synthesis checked by S15 PCR (all as described above). The resulting cDNA were run in triplicates on the iCycler along with the NTC and negative controls as before. Efficiencies were derived from both sources from three or four independent PCR runs.

Threshold cycle (Ct) values were obtained for each replicate of each standard and a standard curve (logarithm of the RNA concentration plotted against Ct) was drawn using the iCycler software. The standard curve was accepted if the R-squared value was ≥ 0.985. PCR efficiency was derived from the slope of the line, expressed as follows:

Efficiency = $10^{(-1/slope)} - 1$

Standard curves for all TLRs were run four times and the average of the three best efficiencies considered. Similarly standard curves for the three house keeping genes were run three times and the resulting average was taken for final calculation. These are presented in Table 2.6.

GENE	EFFICIENCY-PLACENTAL TISSUE	EFFICIENCY-SPLEEN TISSUE
TLR1	1.89	2.07
TLR2	1.92	2.14
TLR3	1.94	2.08
TLR4	1.85	2.03
TLR5	1.91	2.02
TLR6	1.89	2.11
TLR7	1.91	2.02
TLR8	1.83	1.93
TLR9	1.99	2.16
TLR10	1.91	2.00
SDHA	1.92	2.06
TBP	1.82	1.91
YWHAZ	1.86	2.00

Table 2.6 *PCR efficiency for experimental and house-keeping genes from placental and spleen RNA.*

Ideally the efficiency of a PCR reaction should be 2. As shown in Table 2.6 values for the placenta were less than 2. The efficiencies for spleen were higher than those of placenta but this is not unexpected as the spleen RNA is a commercially produced product, and probably much purer than the placental samples prepared specifically for this study. Therefore, for all subsequent analyses the efficiency results obtained from the placental tissue were used for calculating the expression of genes of interest and housekeeping genes as this was deemed more appropriate. Spleen was included as it provided a positive control as it is known to express TLRs 1- 10 (Zarember and Godowski 2002).

Analysis of data:

Data were obtained using iCycler software and Ct values obtained from all samples were imported into qbase (version: 1.2.2). This software (http://medgen.ugent.be/qbase/) enabled a normalisation factor allowing for plate-to-plate variation/efficiencies of all genes but especially the three house keeping genes to be incorporated into any calculations. Thereby relative gene expression of the genes of interest could be calculated (Vandesompele, De Preter et al. 2002). All results were normalised against the 1000ng/ml placental sample.

2.6 STATISTICAL ANALYSIS

(i) Cytokine responses by non-laboured tissue to TLR ligands:
2-tailed Student's t-test assuming samples to have unequal variance was applied. The statistical analysis was done using SPSS version 13 (SPSS Inc Chicago, USA). The differences were considered to be statistically significant when the p value was < 0.05.

(ii) Cytokine responses by laboured versus non-laboured placental explants: Statistical comparison was made between the activities of each TLR in the two groups. As the data were not normally distributed the logarithmic values

of the raw data were employed in the *t-test* for 2 independent samples. The statistical analysis was done using SPSS version 13. The differences were considered to be statistically significant when the *p* value was < 0.05.

(iii) Gene expression analysis between laboured versus non-laboured placental explants: The comparison was made between the expression of each TLR in the two groups using the *Mann Whitney U test* for two independent samples The statistical analysis was done using SPSS version 13. The differences were considered to be statistically significant when the *p* value was < 0.05.

(iv) Cytokine responses by preterm laboured versus term laboured placental explants: Statistical comparison was made between the activities of each TLR in the 2 groups using the *Mann Whitney U* test for two independent samples. The statistical analysis was done using SPSS version 13. The differences were considered to be statistically significant when the *p* value was < 0.05.

(v) Gene expression analysis between preterm laboured versus term laboured placental explants: The comparison between the expression of each TLR in the 2 groups was made using the *Mann Whitney U test* for two independent samples The statistical analysis was done using SPSS version 13. The differences were considered to be statistically significant when the *p* value was < 0.05.

CHAPTER 3

BASAL EXPRESSION AND ACTIVITY OF TLRs IN THE TERM HUMAN PLACENTA

3.1 INTRODUCTION

The placenta has many roles during pregnancy including being a physical and immunological barrier against invading infectious agents and, consequently, has been called a pregnancy-specific component of the innate immune system. The trophoblast in particular is postulated to play an important role in innate immune function (Holmlund, Cebers et al. 2002). As discussed in *Chapter 1*, TLRs have been identified as a family of pathogen recognition receptors with a central role in innate immunity. There is much interest in the contribution of TLRs to immunological functions at many tissue sites including those at the materno-fetal interface. Although most pregnancy related TLR research has focused on the placenta, currently there are only few such studies.

Gene expression studies using human tissue cDNA libraries revealed that the placenta expressed transcripts for TLRs 1-10 (Zarember and Godowski 2002; Klaffenbach D 2005; Nishimura and Naito 2005) with TLR3, TLR5, TLR7 and TLR8 being the most abundant. Detailed studies of TLR protein expression and function are more limited and generally focus on TLR2 and TLR4. Various cell types in the preterm and term placenta, including Hofbauer cells and villous, extravillous and intermediate trophoblasts, express TLR2 and TLR4 protein (Holmlund, Cebers et al. 2002; Kumazaki, Nakayama et al. 2004; Beijar EC 2006) with preferential expression of TLR4 on the trophoblast plasma membrane facing the maternal circulation. Similarly, choriocarcinoma cell lines are positive by flow cytometry for cell surface TLR2 and TLR4, and intracellular TLR9 (Klaffenbach D 2005).

Functional activity of TLR2, TLR3 and TLR4 have been reported for first and third trimester trophoblast/placenta (Holmlund, Cebers et al. 2002; Abrahams, Bole-Aldo et al. 2004; Abrahams VM 2005; Abrahams VM 2006); and choriocarcinoma cell lines are responsive to TLR2, TLR3, TLR4 and TLR9 agonists (Klaffenbach D 2005). Holmlund et al (2002) found that stimulation of term placental explants with either zymosan (a TLR2 ligand) or LPS (a TLR4 ligand) induced IL-6 and IL-8 cytokine production into culture supernatants.

The functionality of TLRs other than TLR2, 3 and 4 within the human placenta and/or primary trophoblast remains unknown. Therefore, we undertook an investigation of the expression and activity of TLRs 1 - 10 in the term human placenta in the absence of labour and following completion of spontaneous onset term labour (*chapter 4*) to explore whether TLRs expressed in the term human placenta are functional, and whether changes in the expression and activity of these receptors is associated with spontaneous term labour (*chapter 4*).

The primary objectives of the work presented in this chapter were:

(i) To evaluate the functional activity of TLRs 1-9 in the human term placenta (as no ligand for TLR10 was identified at the time of the study, only TLRs 1-9 were studied).

(ii) To confirm the presence of transcripts for each TLR.

3.2 APPROACH

Placental explants from the '***term non-laboured group***' were stimulated with TLR specific ligands. Cytokine output in response to stimulation was then an index of the capacity of the placenta to signal its response to that pathogen via a specific TLR. Several different cytokines, namely TNF-α, IL-6, IL-8 and IL-10, were measured using ELISA. Initial experiments determined the optimum concentration for each TLR ligand. On identifying optimal doses for each ligand, a further series of term non-laboured placental explant cultures *(n=17)* were exposed to TLR ligands and cytokine production determined. The expression of TLRs in the term non-laboured human placenta was also determined by polymerase chain reaction *(n=12)*.

Statistical comparison was done by applying *two-tailed student's t-test* assuming samples to have unequal variance using SPSS Version 13 (SPSS Inc Chicago, USA). The differences were considered to be statistically significant when p value was < 0.05.

3.3 RESULTS

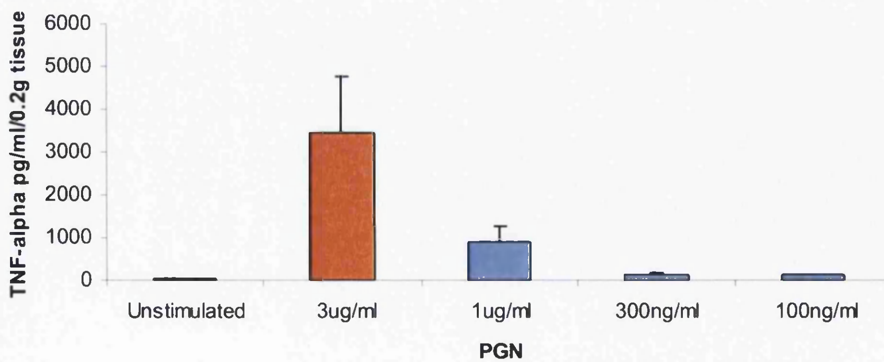
3.3.1 Dose response curves for each TLR ligand

Dose response curves based on published information and laboratory experience with stimulating peripheral blood mononuclear cells via TLR were conducted for all ligands of interest. These results (Figure 3.1a-f; *n=3*) were used to determine optimum concentration for use in all subsequent experiments (Table 3.1).

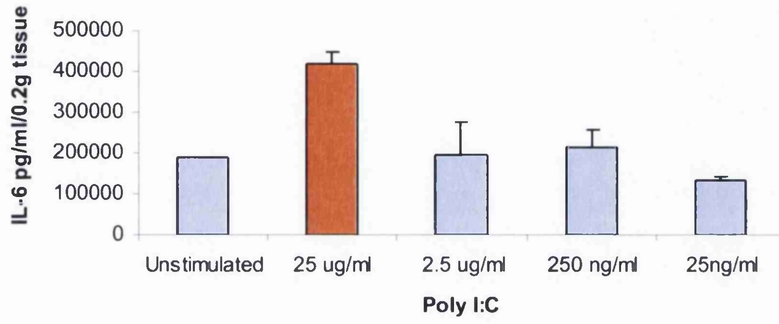
TLR Ligand	TLR stimulated	Treatment Dose
Peptidoglycan (*S. aureus)*	TLR2	3µg/ml
Zymosan	TLR2/6	3µg/ml
Polyinosine-polycytidilic acid (Poly (I:C))- dsRNA	TLR3	25µg/ml
Ultra Pure *E.coli* (0111:B4) LPS	TLR4	100 ng/ml
Flagellin (*S. typhimurium*)	TLR5	100ng/ml
R848 (anti viral synthetic compound)	TLR7/8	100ng/ml
Loxoribine	TLR7/8	100µM
ssPoly - single-stranded polyU oligonucleotide	TLR7/8	1µg/ml
CpG - unmethylated deoxycytidine-deoxyguanosine dinucleotide	TLR9	1µM
ODNC-control oligonucleotide 2216	Control	1µM

Table 3.1 The treatment doses used in the experimental work.

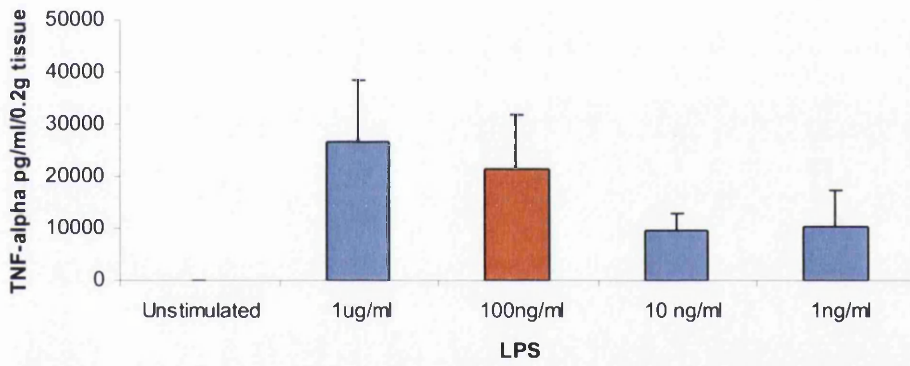
(a)



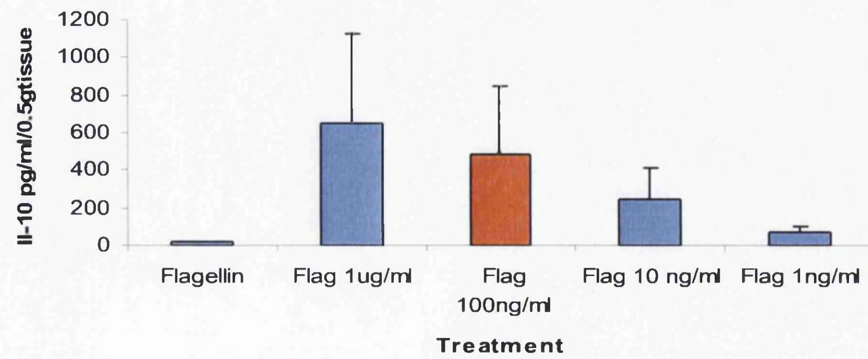
(b)



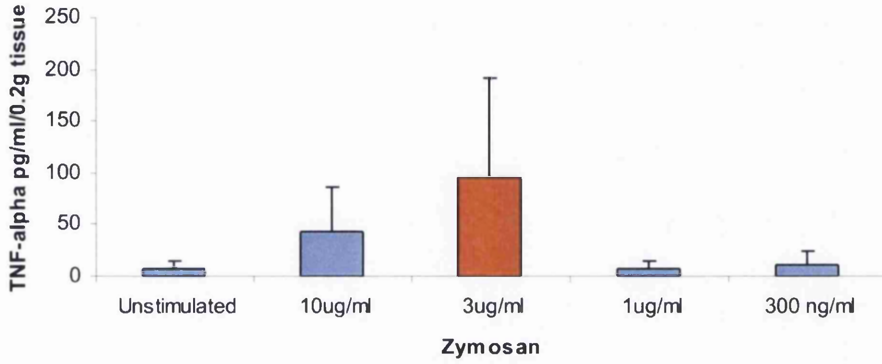
(c)



(d)



(e)



(f)

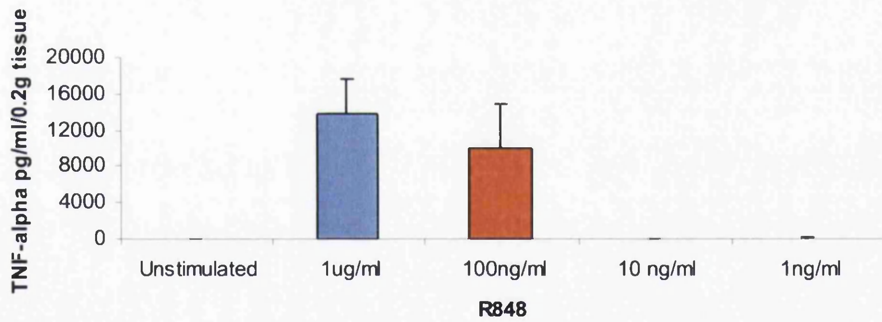


Figure 3.1 *Dose response curves for different TLR ligands. Explants of non-laboured placentas (n=3) were cultured for 24 hours with doses of a) PGN, a TLR2 ligand; b) Poly I:C, a TLR3 ligand; c) LPS, a TLR4 ligand; d) Flagellin, a TLR5 ligand; e) Zymosan, a TLR2 ligand; f) R848 a TLR7/8 ligand. Mean ± SEM for the most appropriate cytokine is shown. The dose chosen for subsequent experiments is shown in red.*

3.3.2 Response of Placental explants to various TLR ligands

Placental explants from term non-laboured placentas (*n=17*) were stimulated with optimal doses of ligands and production of TNF-α, IL-6, IL-8 and IL-10 was examined for all agonists studied (Table 3.2).

Ligand	Cytokines			
	TNF-α	**IL-10**	**IL-6**	**IL-8**
PGN	√	√	√	√
Poly I: C	x	x	√	√
LPS	√	√	√	√
Flagellin	√	√	√	√
Zymosan	√	√	√	√
R848	√	√	√	√
LOX	x	x	√	√
ssPoly	x	x	√	√
CpG	x	x	√	√

Table 3.2 *The cytokines measured for each of the ligands (Examined-√; not examined-x).*

Results for cytokine production in response to all ligands are presented in Figures 3.2-3.9. PGN, LPS, flagellin, R848 all induced significant increase in TNF-α, IL-6, IL-8 and IL-10 production from placental explant cultures (*n=17; p values shown within figures).* Notably, IL-6 and IL-8 were produced constitutively with production significantly enhanced by the ligands. Whilst zymosan stimulated an increase in IL-6 and IL-8 this was only statistically significant for IL-8 (*p=0.030,* Figure 3.3). As preliminary investigations (n=9) indicated that poly I:C, loxoribine, ssPoly and CpG did not induce TNF-α and IL-10 production, only IL-6 and IL-8 continued to be analysed. Whilst polyI:C, ssPoly and loxoribine did not stimulate TNFα or IL-10 production they did induce significant increases in IL-6 alone (Figure 3.5), both IL-6 and IL-8 (Figures 3.9),

and IL-8 alone (Figure 3.8), respectively. CpG (or the oligonucleotide control) did not stimulate production of any of the cytokines studied. Additional analysis indicated that R848, loxoribine, ssPoly and CpG, did not induce the production of INF-α or IFN-γ from the placental explants (data not shown).

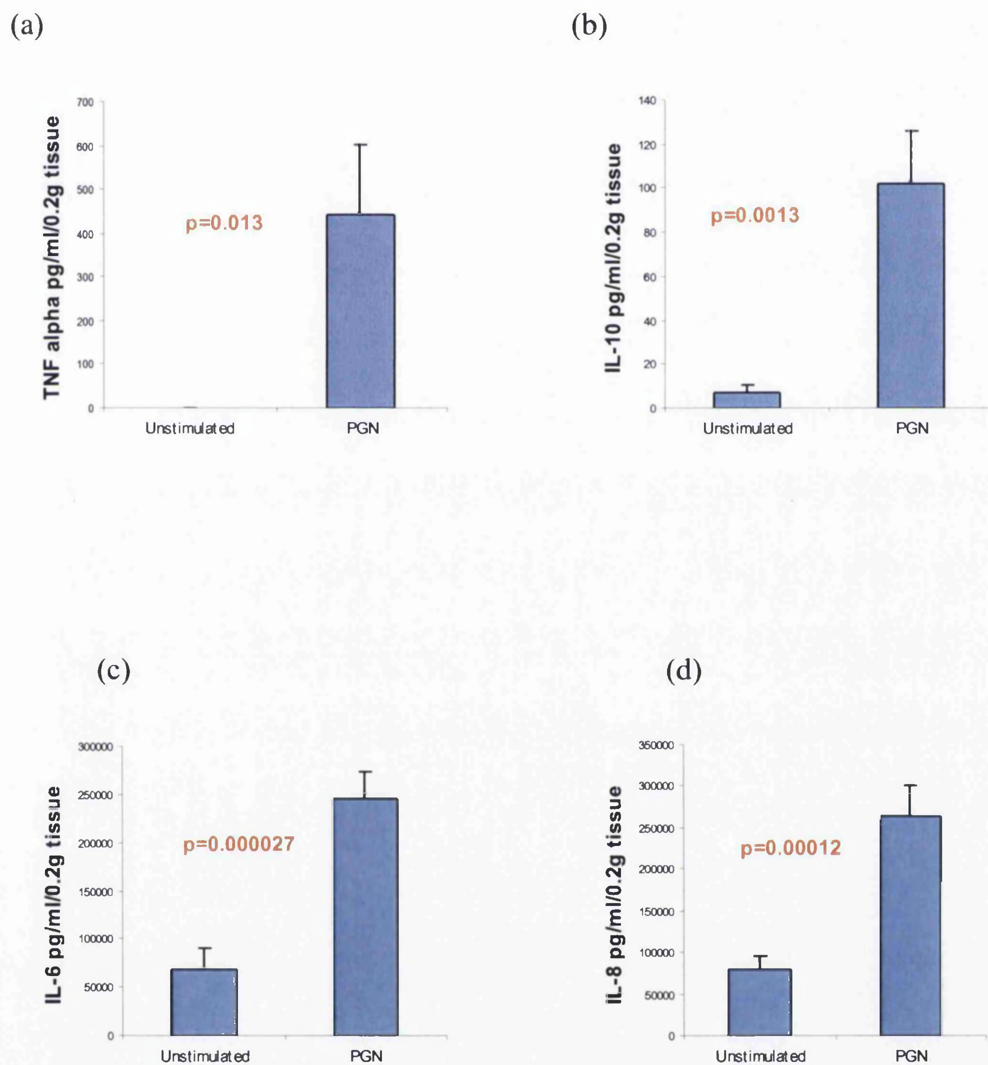


Figure 3.2 *Cytokine outputs of placental explants in response to TLR2 ligand PGN (mean ± SEM; n=17). Explants were left unstimulated or stimulated with PGN for 24 hours. Levels of (a) TNFα, (b) IL-10, (c) IL-6 ,and IL-8 were measured using a specific ELISA and statistically significant differences determined using two-tailed student's t-test. p values are as shown in red. p<0.05 was considered significant.*

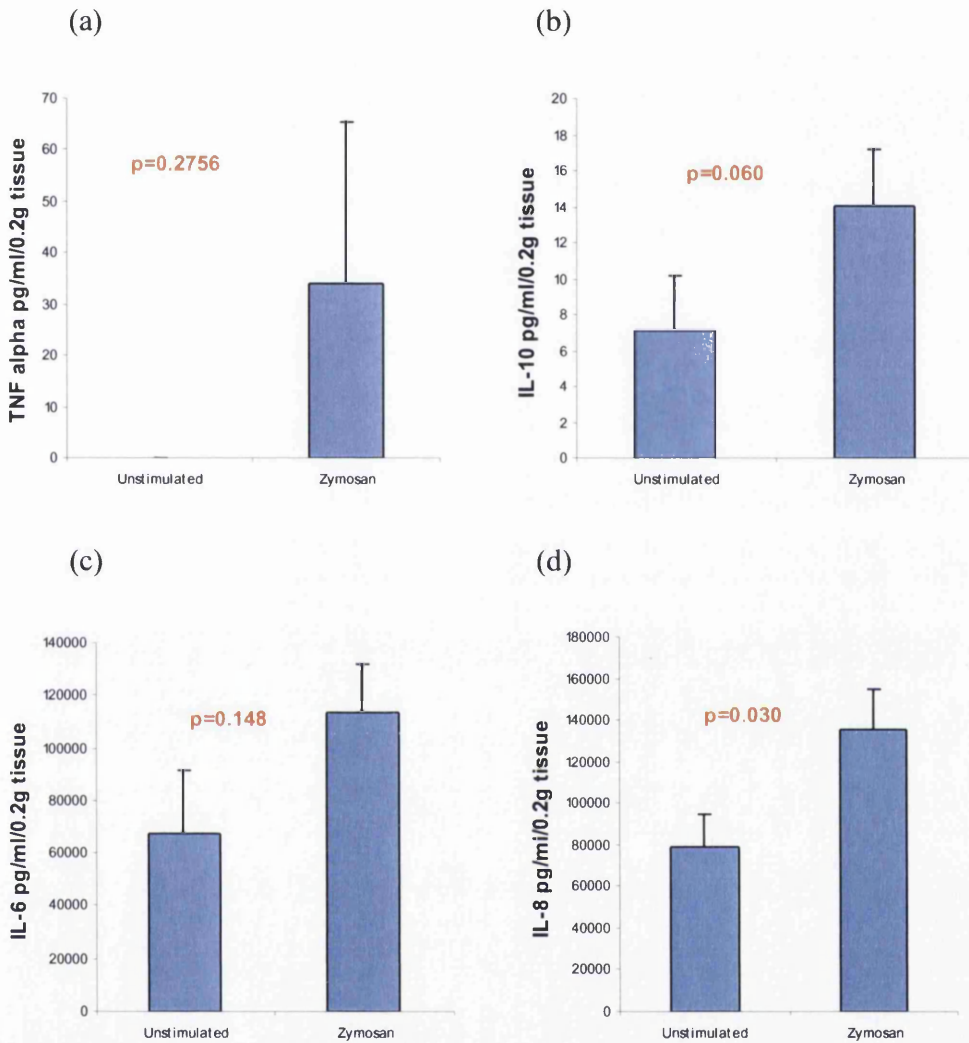


Figure 3.3 *Cytokine outputs of placental explants in response to TLR2 ligand zymosan (mean ± SEM; n=17). Explants were left unstimulated or stimulated with zymosan for 24 hours. Levels of (a) TNFα, (b) IL-10, (c) IL-6 and (d) IL-8 were measured using a specific ELISA and statistically significant differences determined using two-tailed student's t-test. p values are as shown in red.* $p<0.05$ *was considered significant.*

(a) (b)

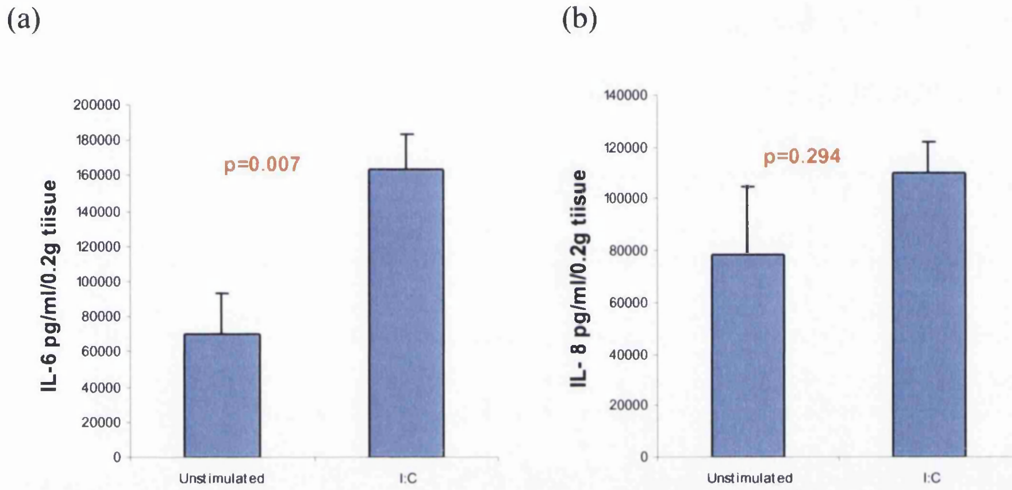


Figure 3. 4 *Cytokine outputs of placental explants in response to TLR3 ligand poly I:C (mean ± SEM; n=17) . Explants were left unstimulated or stimulated with poly I:C for 24 hours. Levels of (a) IL-6 and (b) IL-8 were measured using a specific ELISA and statistically significant differences determined using two-tailed student's t-test. p values are as shown in red. p<0.05 was considered significant.*

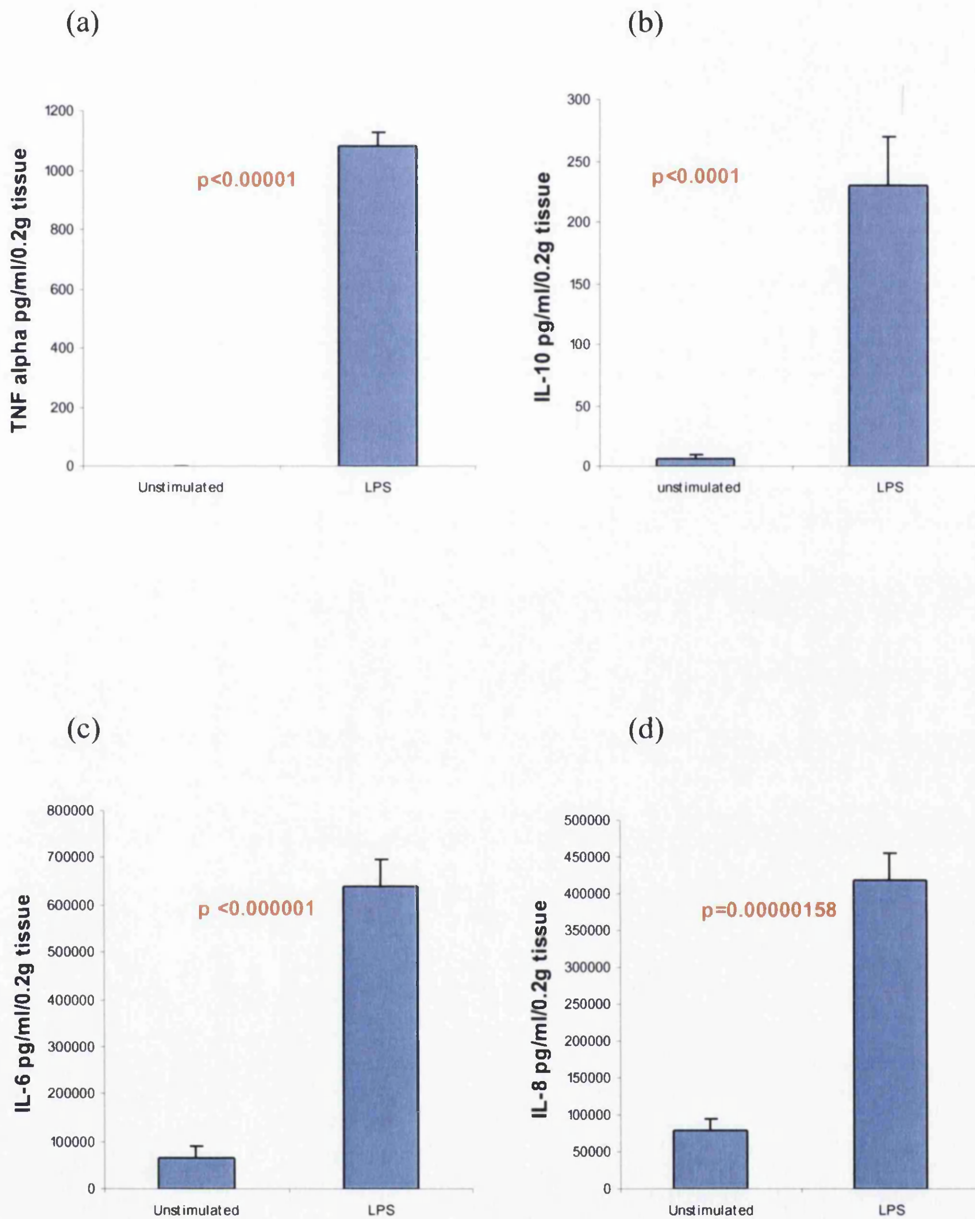


Figure 3.5 *Cytokine outputs of placental explants in response to TLR4 ligand LPS (mean ± SEM; n=17). Explants were left unstimulated or stimulated with LPS for 24 hours. Levels of (a) TNFα, (b) IL-10, (c) IL-6 and (d) IL-8 were measured using a specific ELISA and statistically significant differences determined using two-tailed student's t-test. p values are as shown in red. p<0.05 was considered significant.*

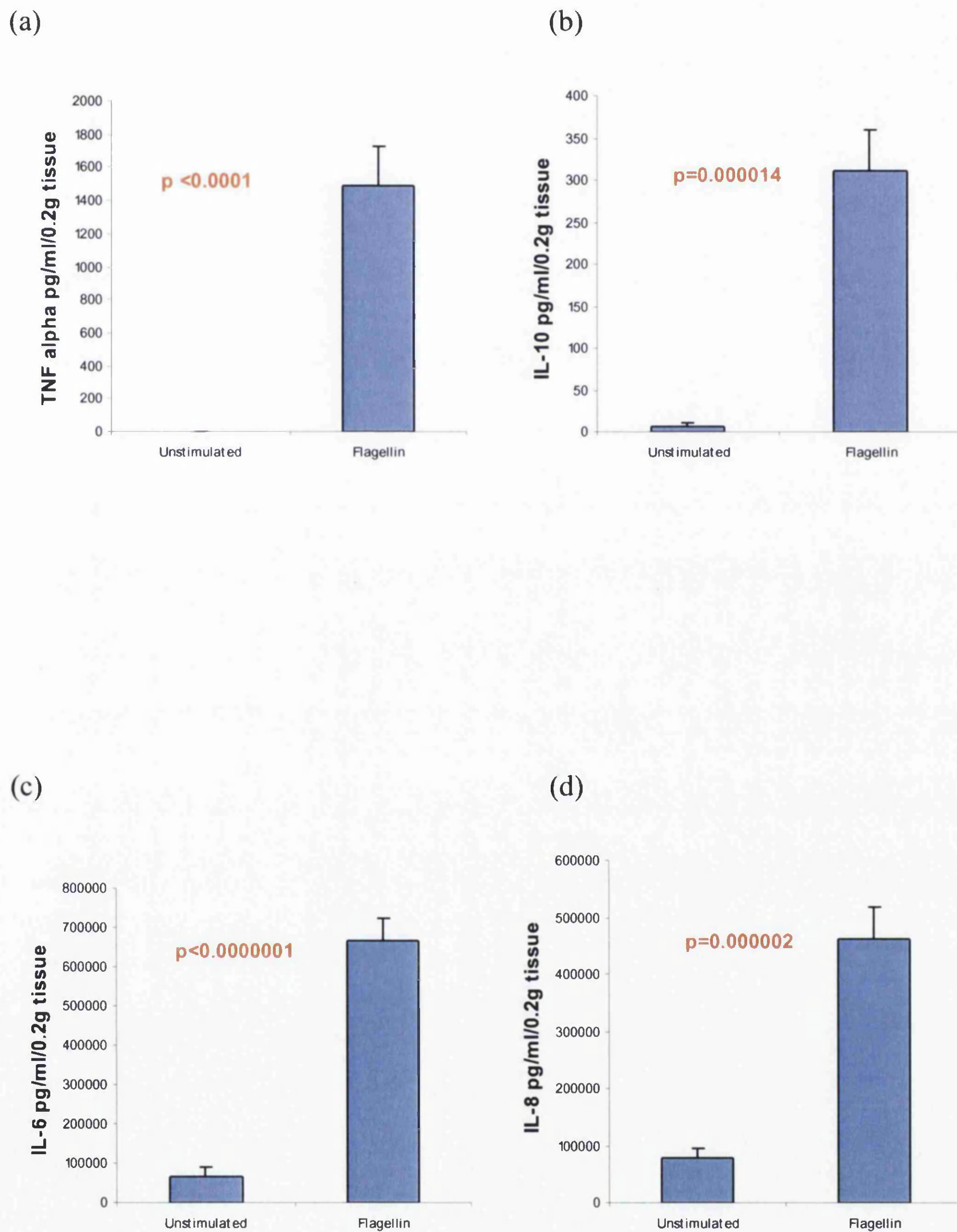


Figure 3.6 *Cytokine outputs of placental explants in response to TLR5 ligand flagellin (mean ± SEM; n=17) . Explants were left unstimulated or stimulated with flagellin for 24 hours. Levels of (a) TNFα, (b) IL-10, (c) IL-6 and (d) IL-8 were measured using a specific ELISA and statistically*

significant differences determined using two-tailed student's t-test. p values are as shown in red. p<0.05 was considered significant.

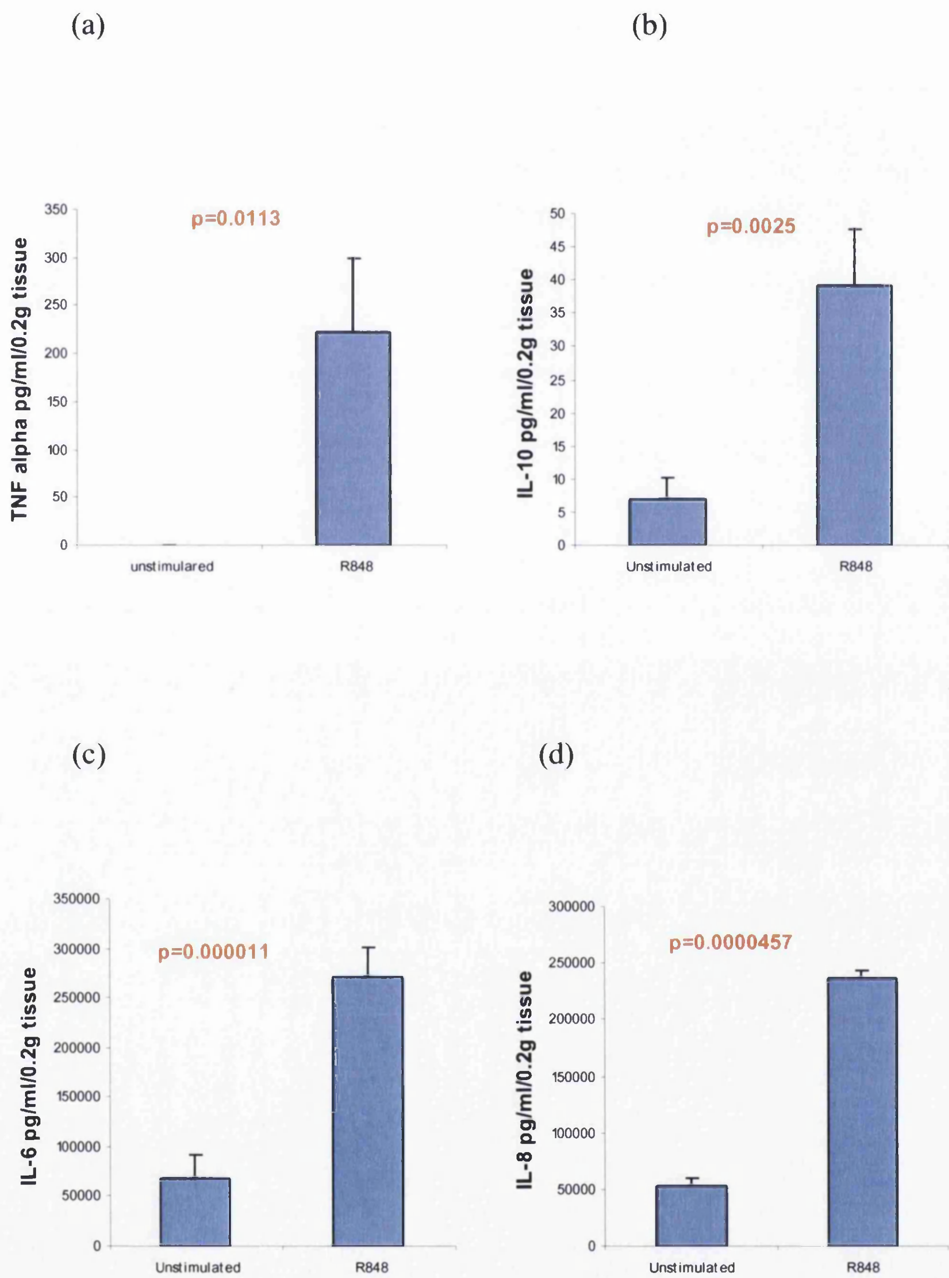


***Figure** 3.7 Cytokine outputs of placental explants in response to TLR7/8 ligand R848(mean ± SEM; n=17). Explants were left unstimulated or stimulated with R848 for 24 hours. Levels of (a) TNFα, (b) IL-10, (c) IL-6 and (d) IL-8 were measured using a specific ELISA and statistically*

significant differences determined using two-tailed student's t-test. p values are as shown in red. p<0.05 was considered significant.

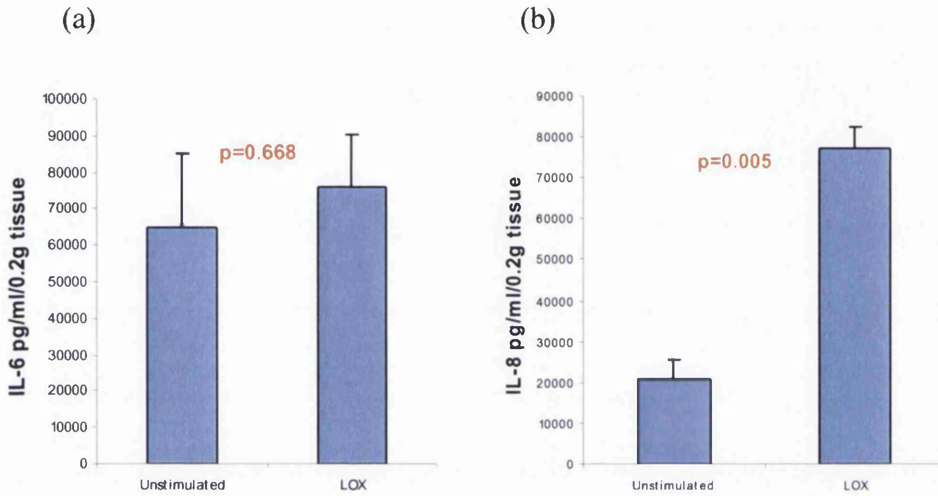


Figure 3.8 *Cytokine outputs of placental explants in response to TLR7/8 ligand loxoribine (mean ± SEM; n=17). Explants were left unstimulated or stimulated with loxoribine for 24 hours. Levels of (a) IL-6 and (b) IL-8 were measured using a specific ELISA and statistically significant differences determined using two-tailed student's t-test. p values are as shown in red. p<0.05 was considered significant.*

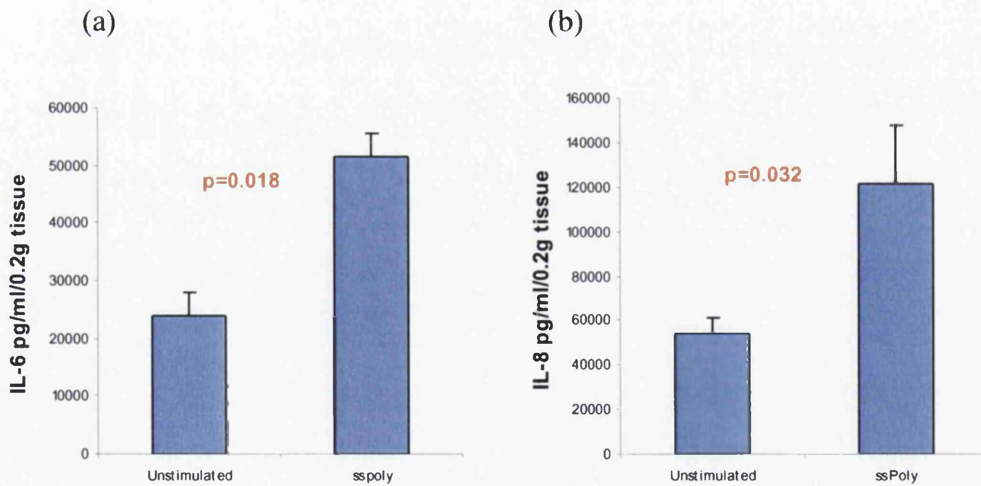


Figure 3.9 *Cytokine outputs of placental explants in response to TLR7/8 ligand (ssPoly) (mean ± SEM; n=17). Explants were left unstimulated or stimulated with sspoly for 24 hours. Levels of (a) IL-6 and (b) IL-8 were measured using a specific ELISA and statistically significant differences*

determined using two-tailed student's t-test. p values are as shown in red p<0.05 was considered significant.

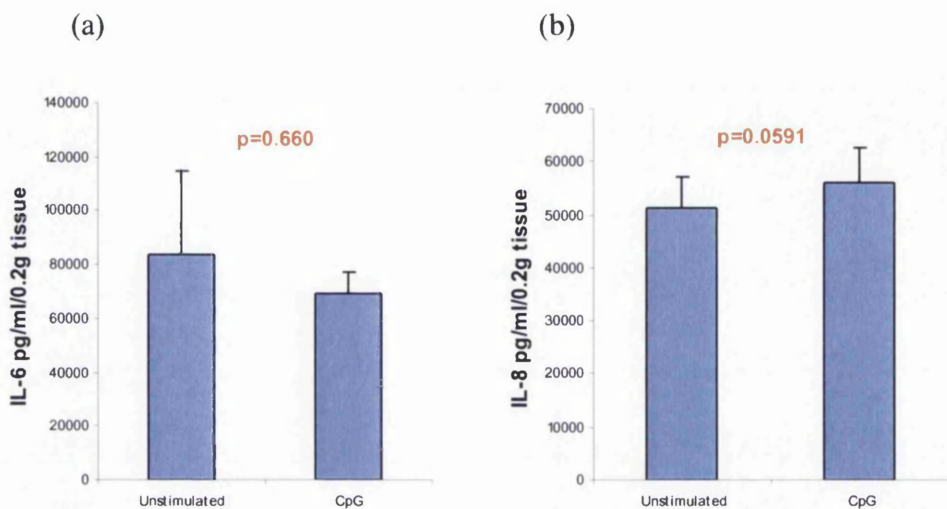


Figure 3.10 *Cytokine outputs of placental explants in response to TLR9 ligand CpG (mean ± SEM; n=17). Explants were left unstimulated or stimulated with CpG for 24 hours. Levels of (a) IL-6 and (b) IL-8 were measured using a specific ELISA and statistically significant differences determined using two-tailed student's t-test. p values are as shown in red p<0.05 was considered significant.*

3.3.3. Gene Expression

Transcripts for TLRs 1 – 10 were expressed in all of the term non-laboured placentas studied (n = 11). All samples were analysed using the iCycler and a representative example was visualised on 2% agarose gel as shown in Figure 3.11. A negative control (water) and a positive control (human spleen) were also included.

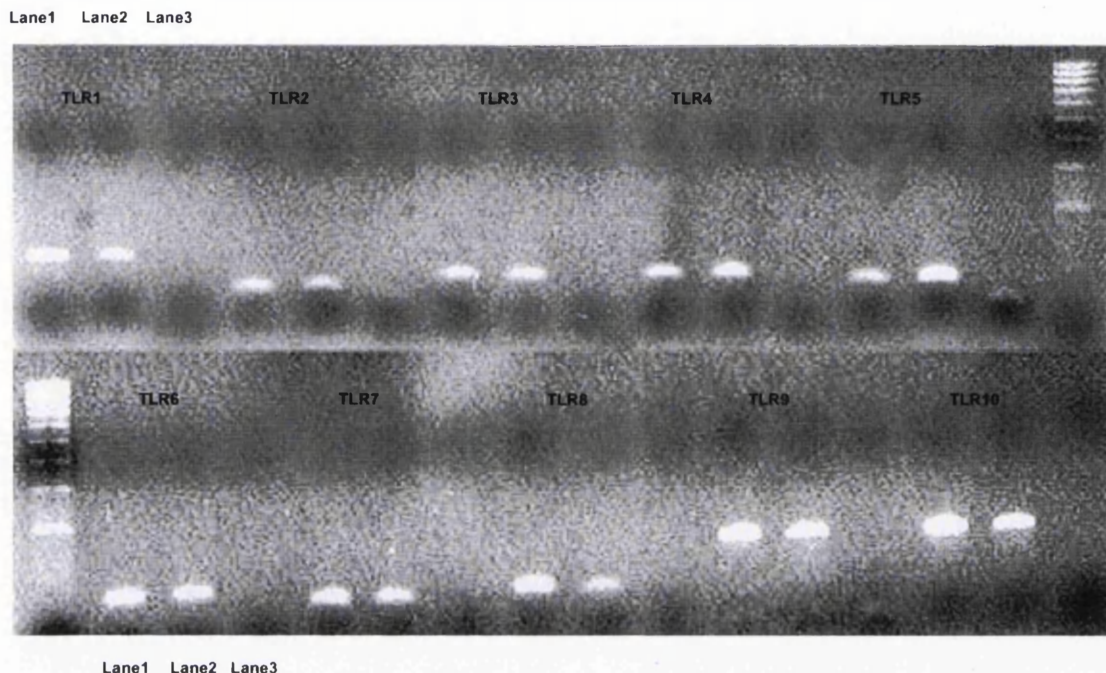
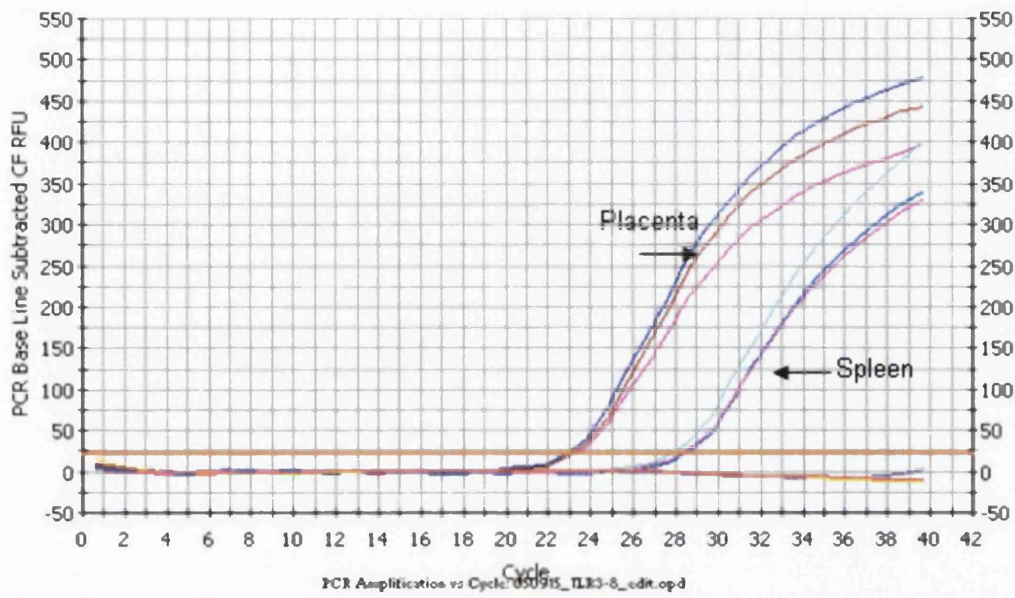


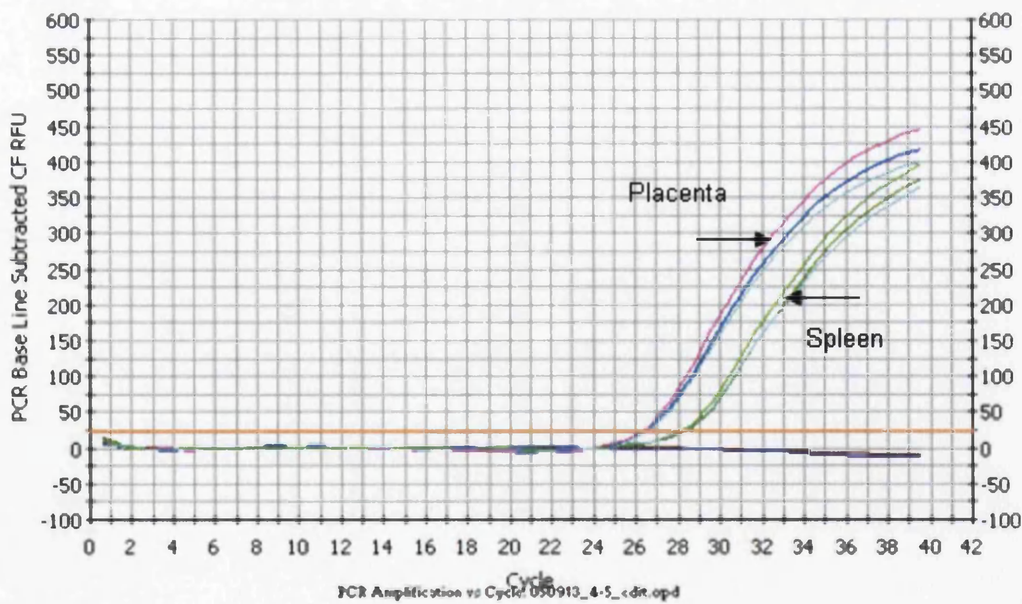
Figure 3.11 *PCR products obtained after gene expression analysis of TLRS 1-10. PCR was performed on the iCycler and a representative example was visualised on a 2% agarose gel; for all TLRs- Lane 1: Positive control (Spleen); Lane 2: Placenta; Lane 3: Negative control (water). Base pair ladder is shown at top right and bottom left corners of gel (range: 100-1000 base pairs with 100 base pair increments).*

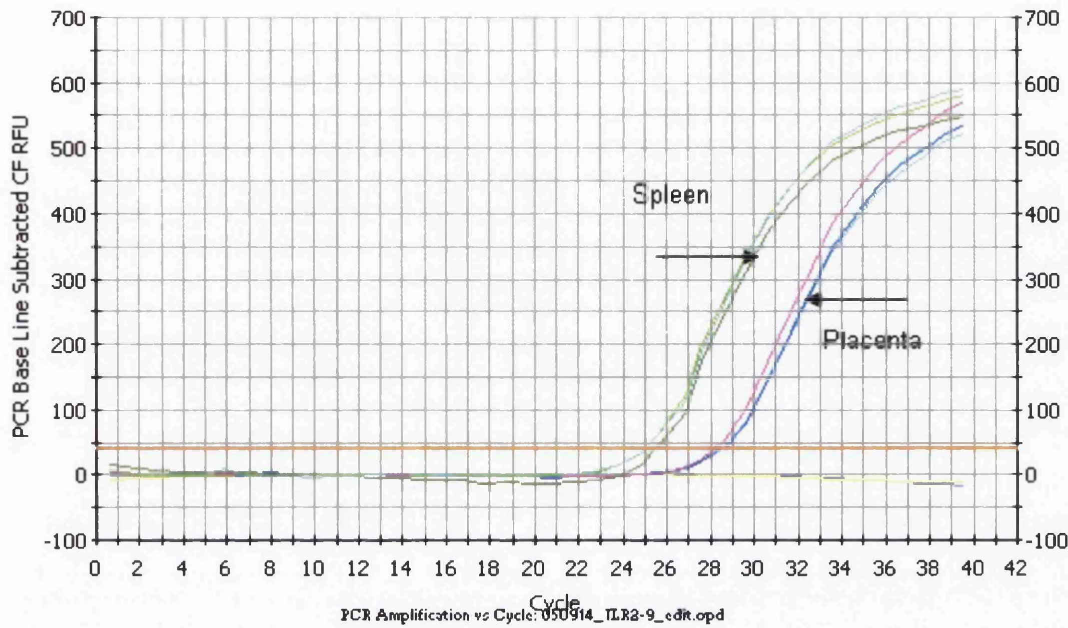
Analysis using q-PCR also provided anecdotal evidence of relative expression by the placenta compared to the spleen. Ct values as shown in Figure 3.12 (a-d) indicate relatively increased expression of TLR 3 and 5 by the placenta compared to spleen but relatively decreased expression of TLR 9 and 10. The plots for the other TLRs are in Appendix 6. Ct is the point at which fluorescence crosses threshold and is the average of each sample in triplicate. These results were confirmed in a larger sample size of non-laboured placentas (n=11, details, *chapter4*).

(a)



(b)





(d)

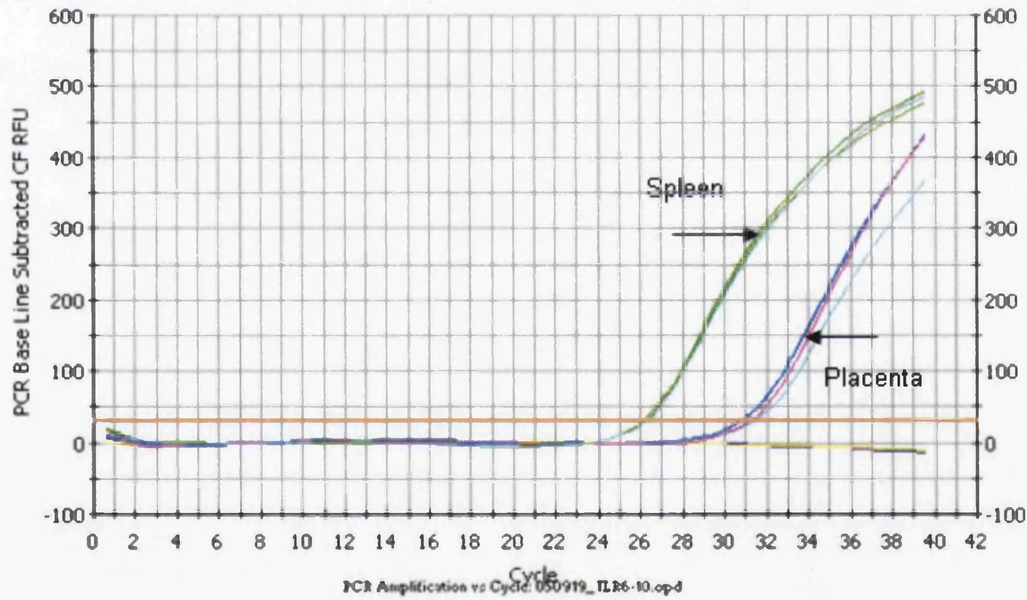


Figure 3.12 *Ct values for placenta and spleen obtained using the iCycler for (a) TLR3 [placenta Ct = 23.03, spleen Ct = 28.13], (b) TLR5 [placenta Ct = 26.33, spleen Ct = 28.10], (c) TLR9 [Ct = 28.37, spleen Ct = 25.17], and (d) TLR10 [placenta Ct = 31.13, spleen Ct = 26.23].*

3.3 DISCUSSION

This study shows for the first time that the human term placenta can respond to a variety of TLR ligands additional to TLR2 and TLR4 as already described (Holmlund, Cebers et al. 2002; Kumazaki, Nakayama et al. 2004; Beijar, Mallard et al. 2006). Statistically significant increased production of at least one of the various cytokines studied (TNF-α, IL-6, IL-10 and IL-8) was found to occur in response to ligands for TLR2, 3, 4, 5, 6 and 7/8. Notably, a response to stimulation via TLR9 could not be detected - CpG did not induce any changes in cytokine outputs even though functional activity of the ligand in use was confirmed by its ability to induce IFNα from peripheral blood mononuclear cells (data from other work in the laboratory being conducted at the same time as the placental work). Whilst expression of transcripts for TLR10 by the placenta was confirmed, however as no agonist for this receptor was available at the time of this study functionality could not be determined.

PGN, LPS, flagellin and R848 were able to significantly stimulate production of all four cytokines studied including significantly increased IL-6 and IL-8 in comparison to high constitutive levels. Even though the remaining agonists (poly I:C, a TLR3 agonist, loxoribine and ssPolyU, TLR7/8 agonists) variably stimulated the four cytokines studied these TLRs are clearly functional. Notably, the ability to stimulate cytokine production via TLR3 indicates that MyD88-dependent and MyD88-independent (presumably TRIF-dependent) intracellular signalling pathways are functional in the placenta (Adachi, Kawai et al. 1998; Akira and Takeda 2004). The discrepancies in responsiveness to the various TLR7/8 agonists used (loxoribine, ssPolyU and R848) could be explained by differences in the intracellular signalling pathways initiated by each of these ligands. In particular, it is now becoming clear that in contrast to R848 which stimulates both TLR7 and 8, loxoribine specifically stimulates TLR7 (Heil, PAhmad-Nejad et al. 2003; Levy, Eugénie et al. 2006) and ssPoly stimulates TLR8 .

The inability to detect a response to CpG using the four cytokines noted above prompted consideration of the ability of this ligand to induce other cytokines, namely IFN-α and IFNγ. However, neither IFNα or IFNγ were detectable from untreated or CpG treated explants. Similarly, IFNα and IFNγ could not be detected after treatment with R848, loxoribine or ssPoly. This is in keeping with other studies that show that IFNγ is generally not detectable in placental tissue throughout much of pregnancy (Hanna, Bonifacio et al. 2004).

In accordance with published data, transcripts for TLRs 1-10 were detectable in all non-laboured term human placentas studied (Zarember and Godowski 2002; Nishimura and Naito 2005). Intriguingly, transcripts for TLR9 could be detected from all placentas despite the inability to detect a functional response. Further investigation of the regulation of expression of TLR9 protein is warranted by this finding. Also, whilst anecdotal, our study confirms that transcripts for TLR3 , TLR5 and TLR7 are more abundant in the human placenta than they are in the human spleen which is in accordance with reports elsewhere (Zarember and Godowski 2002).

The cytoplasmic tails of TLRs are not functionally equivalent, with certain TLRs requiring assembly into heteromeric complexes, whereas others are active as homomeric complexes. TLR2 provides a good model for this for understanding the complexity of TLR biology. TLR1 and TLR6 act as co receptors for TLR2 to promote or inhibit cellular responsiveness to activating ligands (Ozinsky, Underhill et al. 2000; Hajjar, O'Mahony et al. 2001). This could explain the results obtained for the TLR2 ligands PGN and zymosan in this study. Whilst a robust response was seen for TLR2 when stimulated with PGN, another TLR2 ligand, zymosan, stimulated production of all cytokines to a much lesser extent and this increase was only significant for IL-8. TLR2/TLR1 recognises bacterial triacylated lipoproteins like peptidoglycan (Takeuchi, Sato et al. 2002) while TLR2/TLR6 complexes recognise mycoplasmal diacylated lipoproteins like lipotechoic acid, as well as fungal zymosan (Takeuchi, Kawai et al. 2001; Morr, Takeuchi et al. 2002). Studies using macrophages showed that TLR2 needs a partner to activate TNF-α production in macrophages. Dimerisation of the cytoplasmic

domain of TLR2 does not induce TNF-α production in macrophages, whereas similar dimerisation of the TLR4 cytoplasmic domain does. However TLR1/TLR2 heterodimerisation overcomes the inability of TLR2 alone to produce TNF-α. Thus, the cytoplasmic tails of TLRs are not functionally equivalent, with certain TLRs requiring assembly into heterodimeric complexes, whereas others are active as homodimeric complexes. Therefore, more detailed analysis of TLR2 and its partner TLRs is required and is currently underway.

Results from this chapter clearly demonstrate that the human term placenta expresses functional TLR1, 2, 3, 4, 5, and 7/8 but highlights the need to undertake more detailed functional analysis of each of the TLR-mediated pathways in placental and other extraembryonic tissues. Such an analysis, of even non-functional pathways such as that for TLR9, would delineate the exact signalling pathways operational for each TLR thereby enhancing knowledge of the intrinsic differences in the activity of a particular receptor on stimulation with a specific ligand in a particular tissue. It may well be possible that in placenta, the ratio of different TLRs within a cell could modify the response to a given ligand (Hajjar, O'Mahony et al. 2001) and that manipulation of TLR activity could yield numerous therapeutic benefits.

CHAPTER 4

CHANGES IN TLR ACTIVITY AND EXPRESSION IN THE PLACENTA WITH TERM LABOUR

4.1 INTRODUCTION

Labour is considered an inflammatory process and TLRs have a central role in the initiation of the inflammatory response by mediating cytokine/chemokine production (as discussed in Chapter 1). Therefore, TLRs might have a role in the onset and/or maintenance of labour. Having established that TLRs 1, 2, 3, 4, 5, 6, 7 and 8 but not TLR9 are expressed and functional in the healthy term non-laboured placenta (Chapter 3) the possibility that changes in the function and expression of these receptors are associated with spontaneous labour at term was explored. The review of the published scientific literature to date does not reveal any studies on the role of TLRs expressed by cells of the placenta in term labour. Studies of other gestation-associated tissues identified that transcripts for TLR2 and TLR4 (other TLRs were not studied) could be detected in amnion at term. Notably, TLR2 and TLR4 mRNA levels were significantly higher in choriodecidua and amnion collected after spontaneous delivery at term than those collected at term in the absence of labour (Kim, Romero *et al.* 2004).

The primary objectives of the work presented in this chapter were:

(i) To compare the functional activity of TLRs 1-9 in non-laboured and laboured term human placenta.

(ii) To compare the level of expression of transcripts for each TLR in the same two groups.

4.2 APPROACH

Placental explants from the '***term non-laboured group***' *(n=17)* and the '***term laboured group***' *(n=17)* collected after spontaneous vaginal delivery, were stimulated with receptor specific ligands and cytokine output measured as per *Chapter 3*. Differences in the expression of TLRs at the mRNA level in term non-laboured *(n=11)* and laboured *(n=12)* human placenta was determined using **real time quantitative PCR**.

Statistical comparison was made between the activities of each TLR in the two groups. As the data were not normally distributed the logarithmic values of the raw data were employed in the *t-test for 2 independent samples* and confidence intervals were calculated. SPSS version 13 (SPSS Inc Chicago, USA) was used for statistical analysis. Similarly comparison was made between the expressions of each TLR in the 2 groups by using the *Mann Whitney U test for two independent samples* and SPSS version 13.

4.3 RESULTS

4.3.1 Comparison of activity of TLR 1-10 between non-laboured and laboured groups

As this component of the study generated a large volume of data, preliminary analysis was undertaken using forest plots for each of the cytokines assayed (Figure 4.1a-d). Each forest plot shows the results for each of the different ligands used to induce the cytokine in question. The midpoint on each horizontal bar is the mean difference between the laboured and non-laboured samples and the horizontal cross bars represent the upper and the lower confidence intervals of the mean difference. A statistically significant result is identified when the confidence intervals remain entirely on one side of the mid line. In this case if the confidence intervals remain entirely on the right hand side, cytokine outputs from the laboured samples were greater than non-laboured samples. These plots revealed that the TLR-mediated cytokine outputs that differed significantly between the laboured and non-laboured groups were TNFα production in response to LPS (TLR4 ligand; $p=0.01$) and R848 (TLR7/8 ligand; $p=0.01$) (Figure 4.2 a & b). The plots for the other TLRs are in Appendix 7. Table 4.1 shows the results of the statistical analysis for all the cytokine/ligand combinations studied.

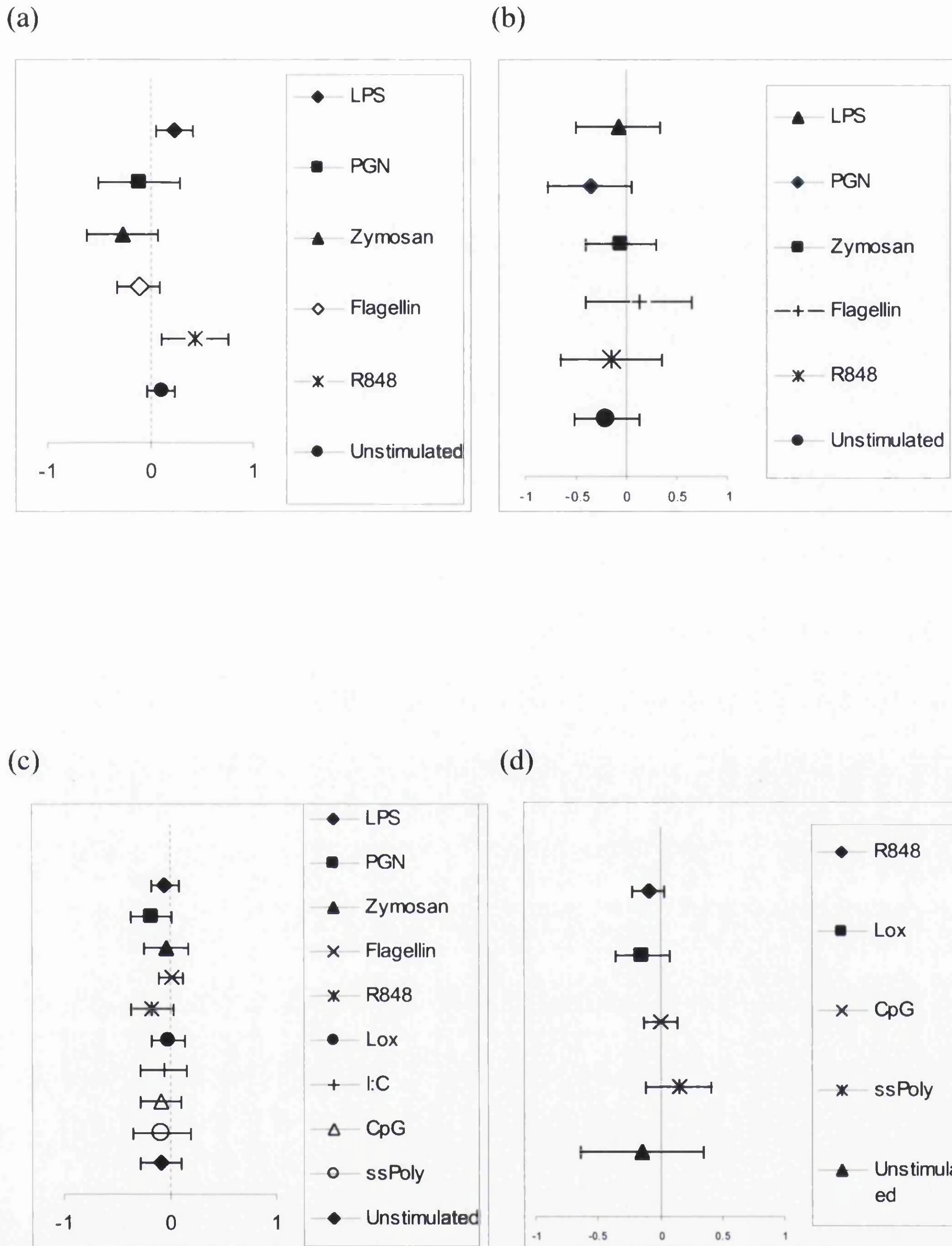


Figure 4.1 *Horizontal confidence interval plots for the effects of labour on the response to each TLR ligand as measured by cytokine output. Each forest plot shows the results for each of the different ligands used to induce (a) TNFα, (b) IL-10, (c) IL-6, and (d) IL-8. The midpoint on each horizontal bar is the mean difference between the laboured and non-laboured samples and the horizontal bars represent the upper and the lower confidence intervals of the mean difference.*

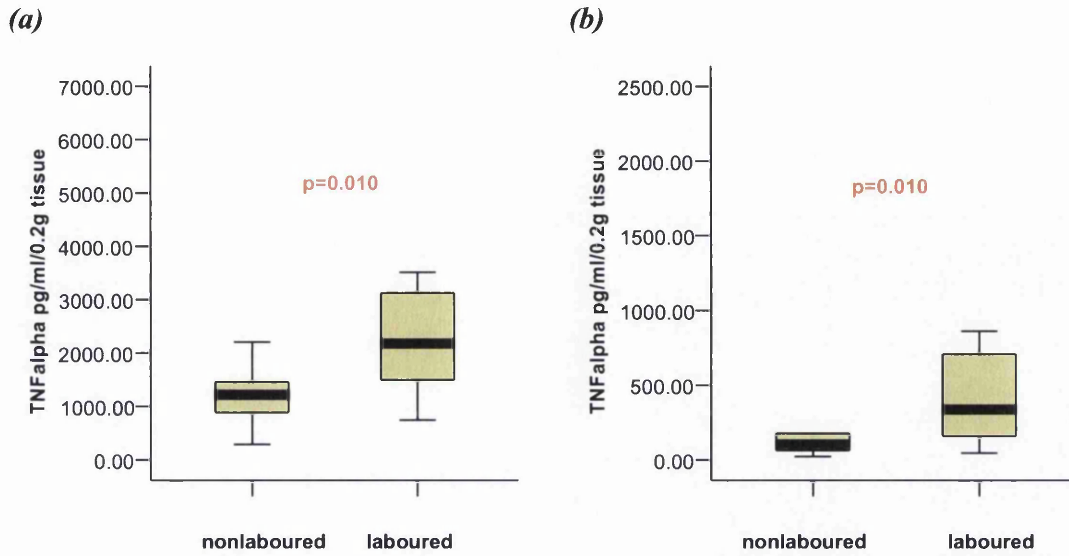


Figure 4.2 *TNFα production in response to (a) LPS and (b) R848 stimulation of non-laboured (n=17) and laboured (n=17) placental explants. Cytokine levels after 24 hours incubation were compared using Mann Whitney U test for two independent samples (p values are shown on the graphs).*

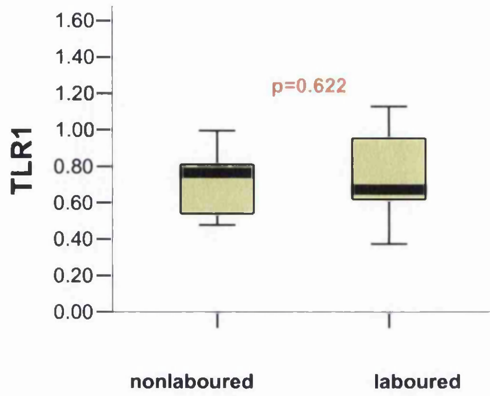
TLRs	**TNFα**	**IL-6**	**IL-8**	**IL-10**
TLR2 (PGN)	0.543	0.069	ND	0.091
TLR3 (Ploy I:C)	ND	0.617	ND	ND
TLR4 (LPS)	0.010	0.482	ND	0.683
TLR5 (Flagellin)	0.389	0.863	ND	0.636
TLR2/6 (Zymosan)	0.109	0.702	ND	0.735
TLR7/8 (R848)	0.010	0.095	0.180	0.547
TLR7/8 (sspoly)	ND	0.789	0.202	ND
TLR7/8(loxoribine)	ND	0.523	0.232	ND
TLR9 (CpG)	ND	0.341	0.950	ND
Unstimulated	0.125	0.356	0.547	0.223

Table 4.1 *Summary of the p values obtained after statistical analysis of the cytokine outputs obtained for each cytokine in response to each TLR ligand in the non-laboured (n = 17) and laboured (n = 17) groups. ND – not done*

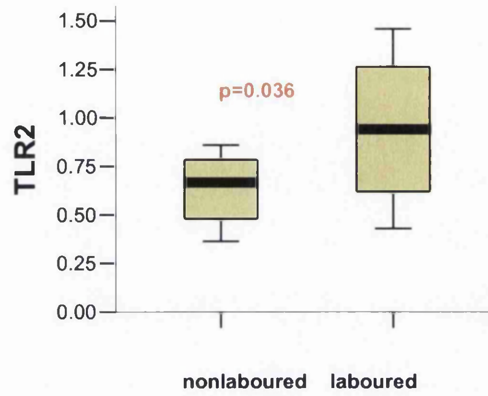
4.3.2 Comparison of the expression of mRNA for TLR 1-10 between the non-laboured and laboured groups

Data were obtained using iCycler software (Details in *Chapter 2*) and Ct values obtained from all samples were imported into qbase (Version 1.2.2). This software (http://medgen.ugent.be/qbase/) enabled a normalisation factor allowing for plate-to-plate variation/efficiencies of all genes but especially the three house keeping genes to be incorporated into any calculations. Thereby relative gene expression of the genes of interest could be calculated (Vandesompele, De Preter et al. 2002). All results were normalised against the 1000ng/ml placental sample. Testing of commonly used endogenous control genes such as GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β-actin show that their level of expression is affected by a number of conditions including different endocrine and immune system changes which happen normally in pregnancy. It is for this reason that succinate dehydrogenase complex, subunit A (**SDHA**); TATA box binding protein (**TBP**); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide **(YWHAZ)** were used as the housekeeping genes as these are relatively stable (Meller, Vadachkoria et al. 2005). Results for expression of mRNA for TLR1-10 in the 2 experimental groups, non-laboured ($n=11$) and laboured ($n=12$), are shown in Figure 4.3 a-j. The p values are shown within the figures. Generally the expression of all TLRs was greater in the laboured than the non-laboured group however a statistically significant increase in association with labour was seen only for TLR2 and TLR5.

(a)



(b)



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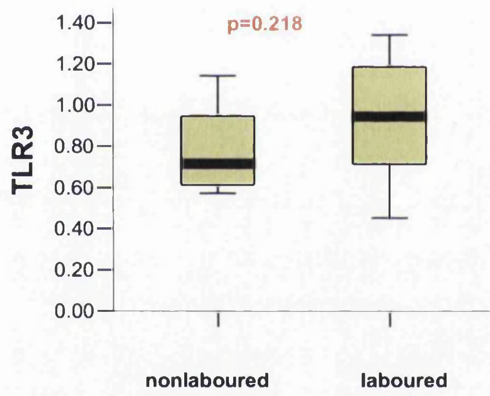
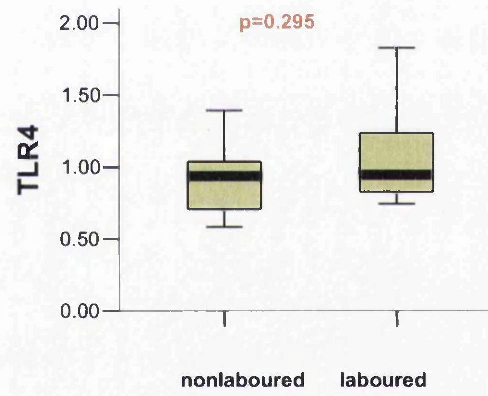
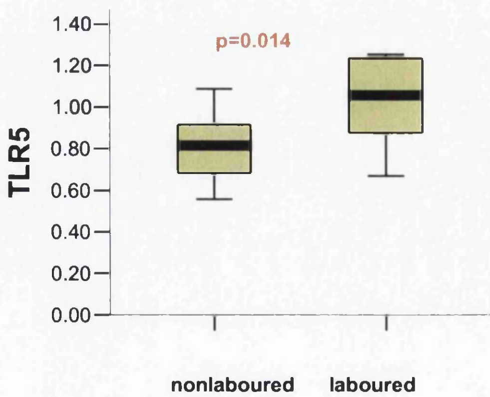


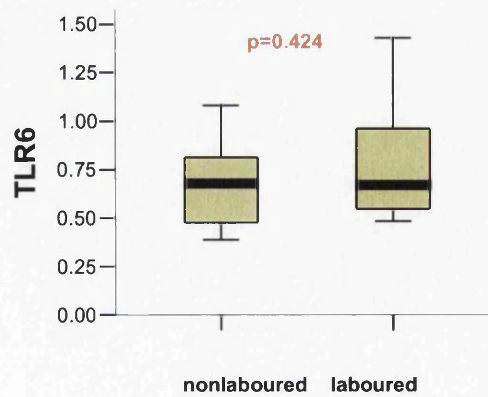
(d)



(e)



(f)



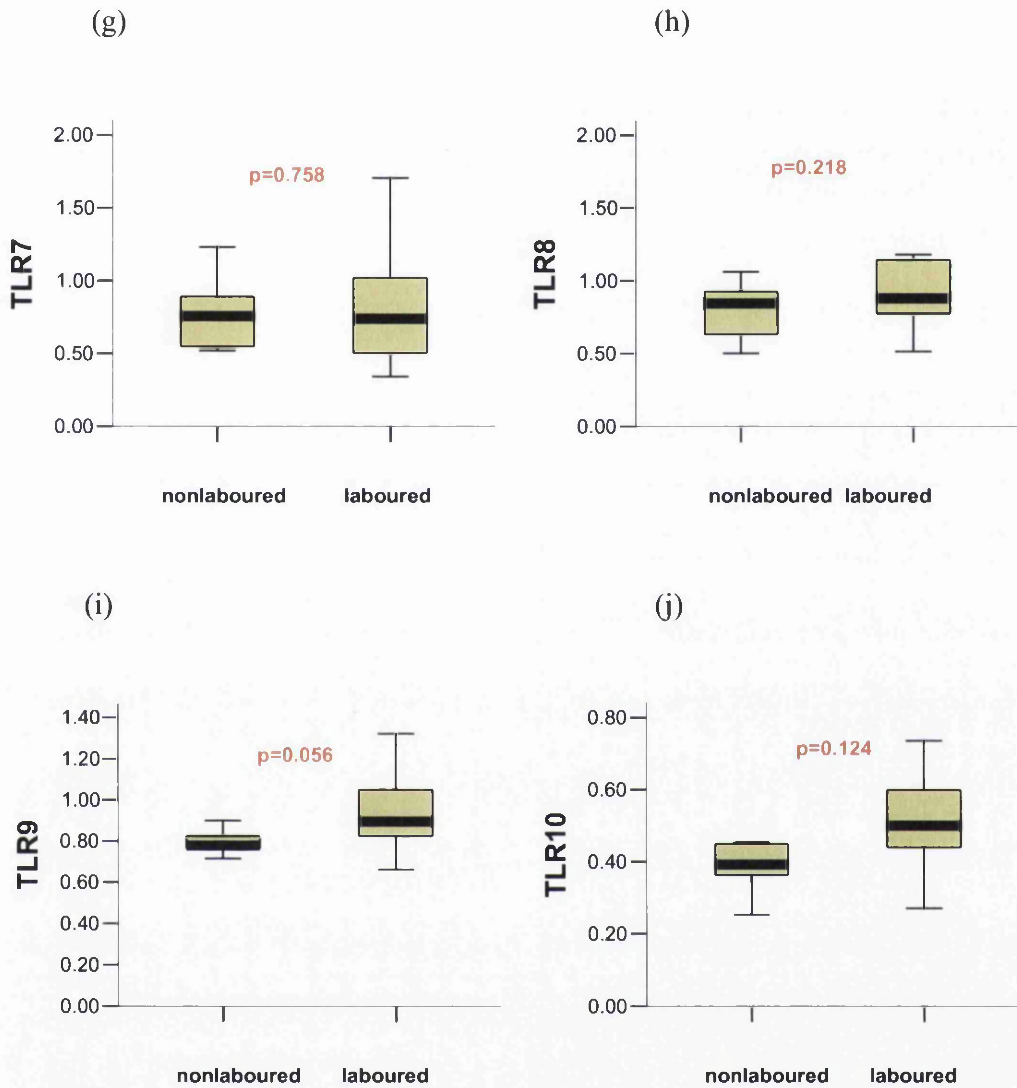


Figure 4.3 *Box and whisker plots depicting comparison between expression of mRNA for TLRs 1-10 in the term-non-laboured (n=11) and term laboured (n=12) groups. Mann Whitney U test for two independent samples* was employed and the *p* values are shown on the graphs.

4.4 DISCUSSION

The extensive analysis of differences in the functional response of laboured and non-laboured term placentas to various TLR ligands revealed statistically significantly increased levels of TNFα in response to a TLR4 ligand (LPS) and a TLR7/8 ligand (R848) in the laboured group. However, this change in function was not explained by an increase in the level of transcripts for these two TLRs in the laboured group. Whilst, placental levels of transcripts for TLR4, TLR7 and TLR8 did not change with labour, TLR2 mRNA and TLR5 mRNA were significantly higher in the laboured group despite no functional changes in the cytokine output in response to stimulation via these receptors being detected.

Although a wide range of TLRs were found to be expressed in the term human placenta it seems that the function of only TLR4 and TLR7/8 might differ with labour. Whilst there is ample evidence in the literature regarding amplified cytokine responses with and without stimulation by, typically, LPS in gestational tissues following labour (Laham, Brennecke et al. 1994; Laham, Brennecke et al. 1996; Laham, Brennecke et al. 1996; Laham, Brennecke et al. 1999; Elliott, Loudon et al. 2001) these generally relate to the study of the amnion and/or choriodecidua. Although these tissues are being studied by the group, the placenta formed the focus of this project. Studies by other groups of the effect of labour on cytokine production by the term placenta have yielded similar results to those presented herein. Although TNFα as well as IL-1α and IL-1ß were all significantly up-regulated in placentas from normal term pregnancies in response to labour-associated conditions, the constitutive and/or LPS-stimulated production of IL-6, IL-8 and IL-10 by the placenta does not differ "before" and "after" labour (Laham, Brennecke et al. 1996; Laham, Brennecke et al. 1999; Hanna, Hanna et al. 2000). Furthermore whilst the expression of IL-10 (measured using quantitative PCR) by the placenta declined over gestation it did not differ "before" and "after" labour at term. These results suggest that IL-10 expression remains at lower levels during labour to allow parturition-associated pro-inflammatory cytokines to be over-expressed. Notably,

IL-6 levels were significantly higher in amniotic and choriodecidual but not placental cultures obtained after the completion of labour at term compared to samples taken at the time of elective caesarean section at term (Laham, Brennecke et al. 1996). Also similar to our findings spontaneous onset of labour, irrespective of the eventual mode of delivery, was not found to be associated with any significant changes in IL-8 release from the amnion, choriodecidua, and placenta tissues compared to not in labour tissues both at term and preterm (Laham, Brennecke et al. 1999).

The explanation for the significant increase in TNFα in this and other studies and not other proinflammatory mediators might relate to the fact that IL-6 and IL-8 are produced constitutively by the placenta. It could also be that the levels of immunoreactive IL-6 and IL-8 as measured by ELISA, remain the same and rather it is their bioavailability that changes favourably. For example, IL-8 binds macromolecules that affect its activity such that the total amount of IL-8 can remain the same but its functional activity is altered by the release of the chemokine from macromolecules that otherwise inhibits its activity (Mire-Sluis AR 1997). This possibility is now being explored. Another possible explanation is that measurements can only be made before the onset (elective caesarean sections) and after the completion (vaginal deliveries). Although inclusion of samples from women who have been in labour but have had to undergo emergency caesarean section before the natural completion of labour might resolve this issue, the variable reasons for emergency delivery of the baby might confound any results.

In contrast to the lack of differences between TLR2 and TLR4 mRNA in the laboured placenta, increases in both TLR2 and TLR4 mRNA have been described in chorioamnion collected after the completion of spontaneous labour at term compared to that collected in the absence of labour (Kim, Romero et al. 2004). Changes in TLR2 and TLR4 expression associated with labour might therefore be tissue specific as no difference in transcripts were seen in either the placenta (this study) or in the myometrium before and after the completion of labour in humans (R Youssef 2006).

Data about protein expression of the receptors themselves or the intracellular signalling pathways being utilised should enhance our understanding of the contribution of TLR-dependent processes to parturition. Changes in receptor expression have been identified in the placenta and thus TLRs might have a role in pathological inflammatory conditions within the gestation associated tissues, particularly infection-associated preterm labour and preeclampsia, as discussed in *Chapter 5*. A study of preterm placentae with chorioamnionitis revealed that the inflammatory cells infiltrating the placenta expressed TLR4, and that TLR4 expression on the villous Hofbauer cells was increased in comparison to preterm placenta without chorioamnionitis as well as term placentae (Kumazaki, Nakayama et al. 2004). TLR4 protein expression by interstitial trophoblast is also increased in preeclampsia (Kim, Romero et al. 2004; Kim, Romero et al. 2005).

These observations reflect the complexity and interplay between TLR signalling pathways and support a lack of relationship between protein and mRNA levels for TLRs, at least in the placenta, and/or a role for various regulatory mechanisms including those that are TLR-independent to explain the changes in LPS and R848 mediated TNFα production. In summary, whilst the human term placenta expresses a variety of functional TLRs and labour-associated changes do occur, a more detailed analysis of contributing cell types and signalling molecules is required to elucidate the role of this family of receptors in parturition.

CHAPTER 5

TLR EXPRESSION AND RESPONSE BY THE PLACENTA IN PRETERM LABOUR

5.1 INTRODUCTION

An extensive literature highlights the probable contribution of cytokines and chemokines to infection-associated preterm labour (IA-PTL) (Gomez, Ghezzi et al. 1995; Goldenberg and Rouse 1998; Lamont 2003). In particular, elevated levels of IL-6, IL-8 and TNFα are measurable in amniotic fluid, choriodecidual tissue and maternal plasma from deliveries characterised by infection-associated preterm labour (Mitchell, Trautman et al. 1993; Laham, Brennecke et al. 1996; Laham, Brennecke et al. 1999). These effects can be replicated *in vitro* by treatment of the placenta, choriodecidua or amnion with the microbial product LPS. The Asp299Gly TLR4 gene polymorphism which is associated with impaired TLR4 receptor function and an increased likelihood of Gram-negative sepsis (Agnese, Calvano et al. 2002) also has been shown to be carried more often by preterm infants than term infants or by mothers delivering preterm rather than at term (Lorenz, Hallman et al. 2002). Unpublished data referred to by this same group highlight that studies should not be restricted to TLR4 as in the same Finnish population there was a trend towards higher frequency of TLR2 mutations in preterm compared with term infants.

Thus TLRs within the gestation associated tissues might have a role in pathological inflammatory conditions, particularly infection-associated preterm labour and preeclampsia. To date, a study of preterm placentae with chorioamnionitis revealed that the inflammatory cells infiltrating the placenta expressed TLR4, and that TLR4 expression on the villous Hofbauer cells was increased in comparison to preterm placenta without chorioamnionitis as well as term placentae (Kumazaki, Nakayama et al. 2004). TLR4 protein expression by interstitial trophoblast is also increased in preeclampsia (Kim, Romero et al. 2004; Kim, Romero et al. 2005).

Over the past few years there has been a dramatic increase in interest and information about activity mediated by microbial ligands/TLR combinations in various tissues and

diseases. However, there is little information about the possible role of these ligands and receptors in infection associated preterm labour. As TLRs (with the exception of TLR9) were found to be functional in the term placenta and stimulation via TLRs could lead to the production of relevant cytokines and chemokines an investigation of the activity of TLRs in preterm placentas was undertaken.

The primary objectives of the work presented in this chapter were:

(i) To evaluate the functional activity of TLRs 1-9 in the human preterm placenta.

(ii) To confirm the presence of transcripts for each TLR in the human preterm placenta.

(iii) To evaluate the expression and/or activity of TLRs in infection-associated preterm labour in comparison with term labour.

5.2 APPROACH

Placentae (n=15) were obtained following preterm labour at varying gestations for various reasons. Explant cultures were prepared and treated with TLR ligands as before (*Chapter 3 and 4*). Cytokine output in response to stimulation was then an index of the capacity of the placenta to signal its response via that specific TLR. The expression of transcripts of TLRs in the preterm human placenta was also determined by qPCR (n=12). The TLR activity and expression in the preterm group was compared with the term laboured group. Statistical comparison between the activities and expression of each TLR in the 2 groups was performed using the *Mann Whitney U test for two independent samples* and SPSS Version 13 (SPSS Inc Chicago, USA).

5.3 RESULTS

5.3.1 Characteristics of preterm samples

The placentae in the study were obtained following preterm labour at varying gestations, ranging from 25 weeks to 36 weeks. Eleven of the 15 cases delivered vaginally and the other 4 underwent elective caesarean section. All 4 cases delivered by caesarean section were for maternal (preeclampsia) or fetal reasons (IUGR). One case from the vaginal delivery group was induced prematurely at 35 weeks due to preeclampsia and the remaining 10 cases which delivered vaginally after spontaneous onset of labour had no obvious materno-fetal reasons to explain the premature delivery. On checking the medical records of these 10 cases, evidence of infection was found in most cases either in the form of histologic chorioamnionitis, positive swabs, urine examination or blood markers of infection as shown in Table 5.1.

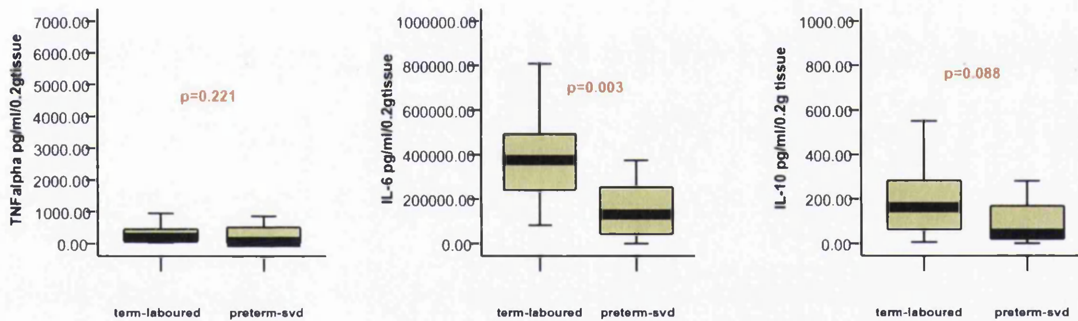
Case Number	Gestation in weeks	Mode of delivery	Reason for delivery	Evidence of infection
1	25	SVD	IAPTL	Funisitis
2	26	SVD	IAPTL	Urinary infection
3	27	SVD	IAPTL	Raised White cell count
4	31	SVD	IAPTL	None
5	33	SVD	IAPTL	GBS on swabs
6	33	SVD	IAPTL	Histologic Chorioamnionitis
7	34	SVD	IAPTL	Raised CRP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

Table 5.1 *Characteristics of preterm samples.*

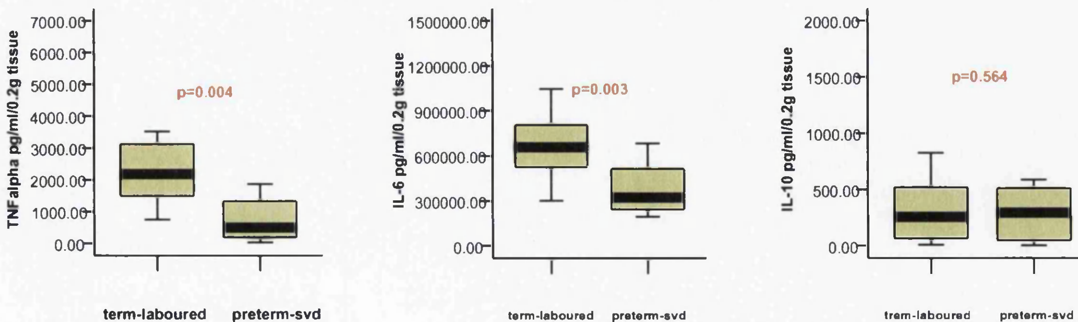
5.3.2 Comparison of TLR activity between the IA-PTL group and the term laboured group

As the preterm samples were a heterogeneous group and of varying gestations, an initial comparison of the cytokine outputs from the 10 placentae obtained following suspected IA-PTL (preterm-SVD) was made with term laboured group (term laboured) (Figures 5.1 a – h). The concentration of TNFα and/or IL-6 was statistically significantly reduced in preterm samples (n = 10) compared with term samples (n = 17) treated with PGN (TLR2) poly I:C (TLR3), LPS (TLR4), flagellin (TLR5), R848 (TLR7/8) but not zymosan or loxoribine (TLR7/8) (*p* values shown on graphs). None of the preterm samples responded to CpG. Constitutive production of IL-6 was also significantly reduced in the preterm samples (p = 0.023; Figure 5.2). In contrast, irrespective of the TLR ligand used, IL-10 production did not differ significantly between term and preterm samples.

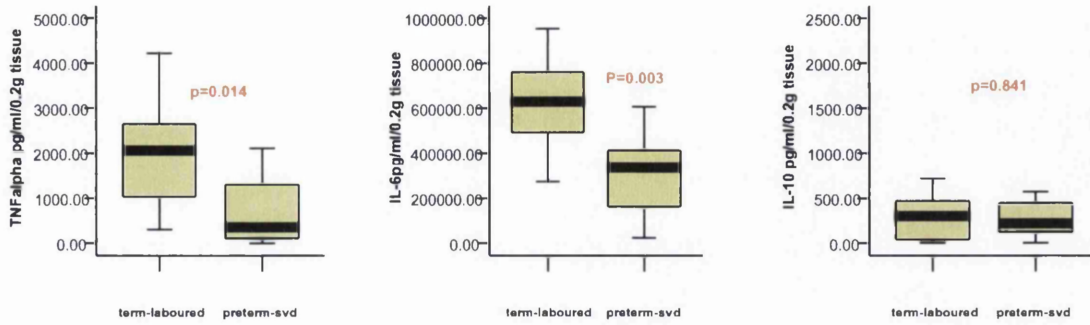
(a)



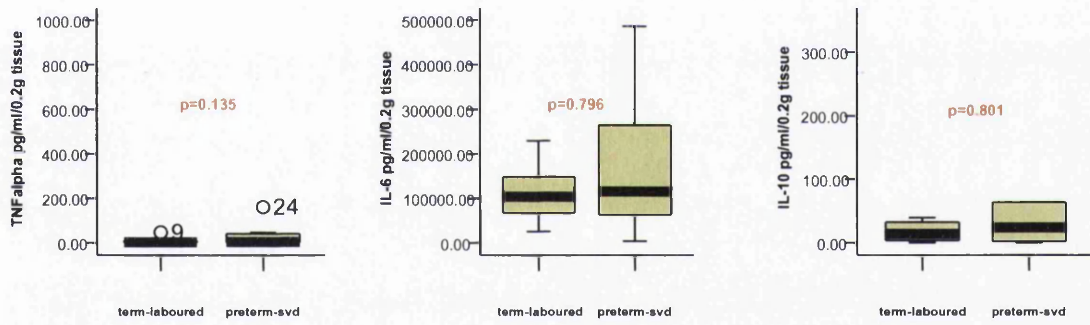
(b)



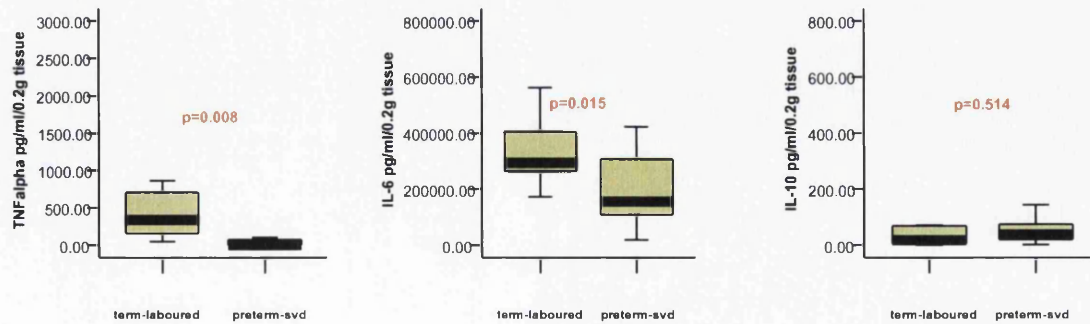
(c)



(d)



(e)



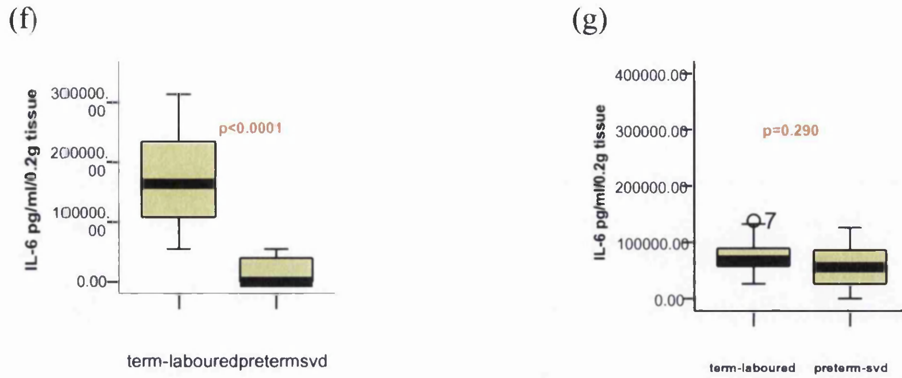


Figure 5.1 *Comparison of cytokine outputs (TNFα, IL-6 and IL-10) from placental explants of term-laboured (n=17) versus preterm-SVD (n =10) in response to (a) PGN, (b) LPS, (c) flagellin, (d) zymosan, (e) R848 (f) poly I:C, and (g) loxoribine. Mann Whitney U test for two independent samples was employed for statistical analysis.* $p < 0.05$ *was considered to be significant.*

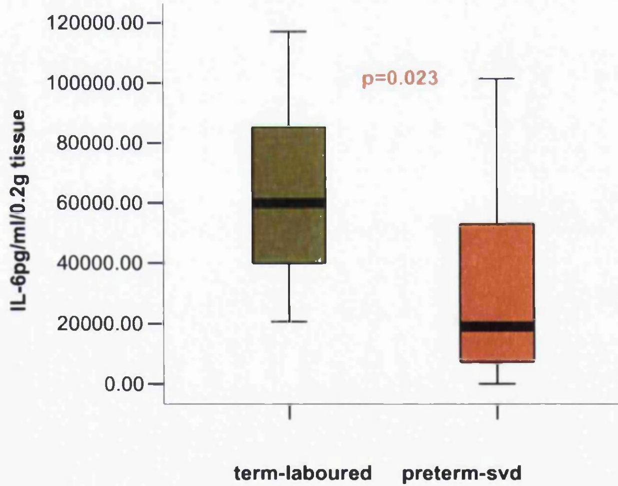


Figure 5.2 *Comparison of IL-6 levels produced constitutively from placental explants of term laboured (n = 17) versus preterm-SVD (n = 10). Mann Whitney U test for two independent samples was employed for statistical analysis.* $p < 0.05$ *was considered to be significant.*

5.3.3 Effect of Dexamethasone on cytokine output of pretrm placental explants

As all the subjects in the IA-PTL group received steroids as part of the clinical therapy for preterm labour, the effect of dexamethasone (steroid) treatment on TLR-stimulated cytokine output by the placenta was explored. Placental explants prepared from non-laboured term deliveries (elective caesarean section) were treated with dexamethasone and IL-6 outputs in response to all TLR ligands determined. IL-6 was chosen as it can be induced by all ligands studied and is produced constitutively. Constitutive and TLR ligand-stimulated IL-6 was reduced in the presence of dexamethasone for all TLR ligands (only LPS, flagellin and Poly I:C are shown) (Figure 5.3 a-d). The reduction in the cytokine output following treatment with dexamethasone was significant with p values of <0.0001 for both the constitutive and ligand-stimulated explants (with exception of I/0.4 versus I/0 and F/0.4 versus F/0).

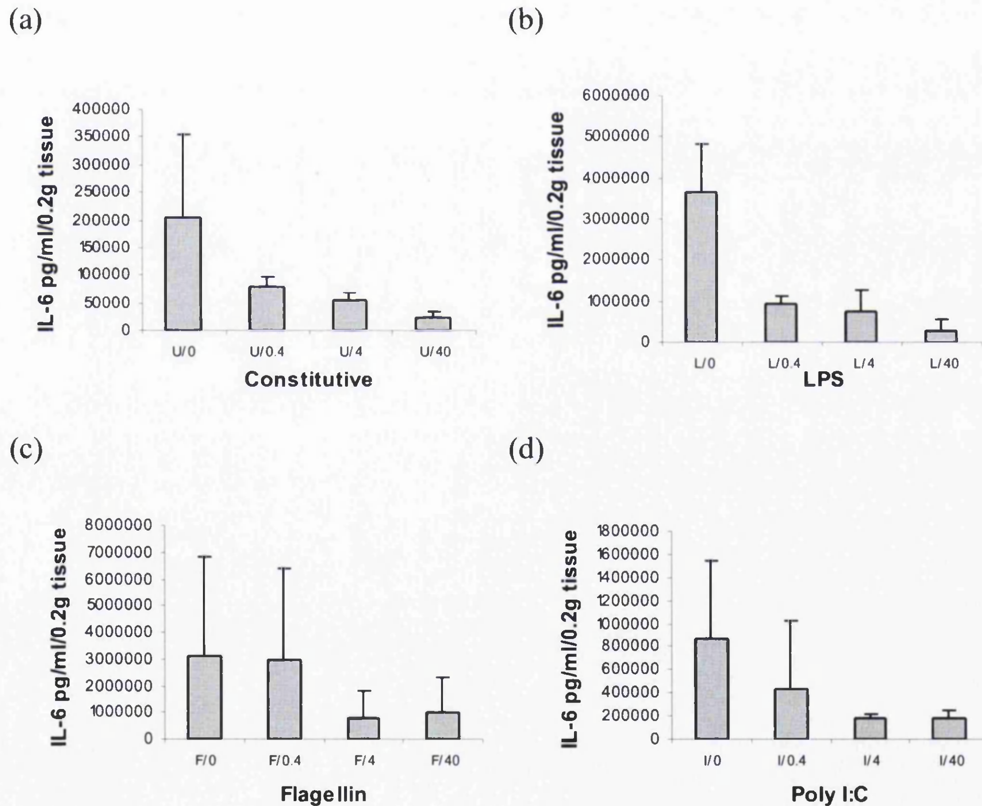


Figure 5.3 *Effect of dexamethasone on IL-6 production by placental explants. Explants were prepared from term non-laboured placentas and treated with 0.4, 4 and 40 ng/ml dexamethasone in the presence of various TLR ligands. Supernatants were harvested after 24 hours and IL-6 (mean ± SEM) production in response to (a) no stimulation/constitutive, (b) LPS, (c) flagellin, (d) Poly I:C determined by ELISA.*

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

As the 10 preterm-SVD placentas came from deliveries associated with intrauterine infection it was possible that they were exposed *in utero* to one or more of the TLR ligands used in this study. Pre-exposure to LPS is known to reduce the level of response upon secondary exposure to LPS, a process known as endotoxin tolerance (Broad A 2006). Therefore it was postulated that pre-exposure to any one ligand could explain the reduced cytokine outputs (at least for IL-6 and TNFα) in response to all ligands in the preterm group. Therefore, placental explants prepared from term non-laboured placenta were exposed to each TLR ligand and after 24 hours culture the first dose of ligand was removed by centrifugation and the tissue recultured in fresh medium in the presence of the same or another TLR ligand. After a further 24 hours culture, supernatants were harvested and analysed for IL-6. Data obtained for unstimulated, LPS, PGN and Flagellin are shown in Figure 5.4 a-d. This approach did not reveal any consistent trend in the cytokine output although reduced outputs were not an overarching feature. Thus prior intrauterine exposure to the ligand does not explain the reduced cytokine output of preterm placental explants in comparison to term explants.

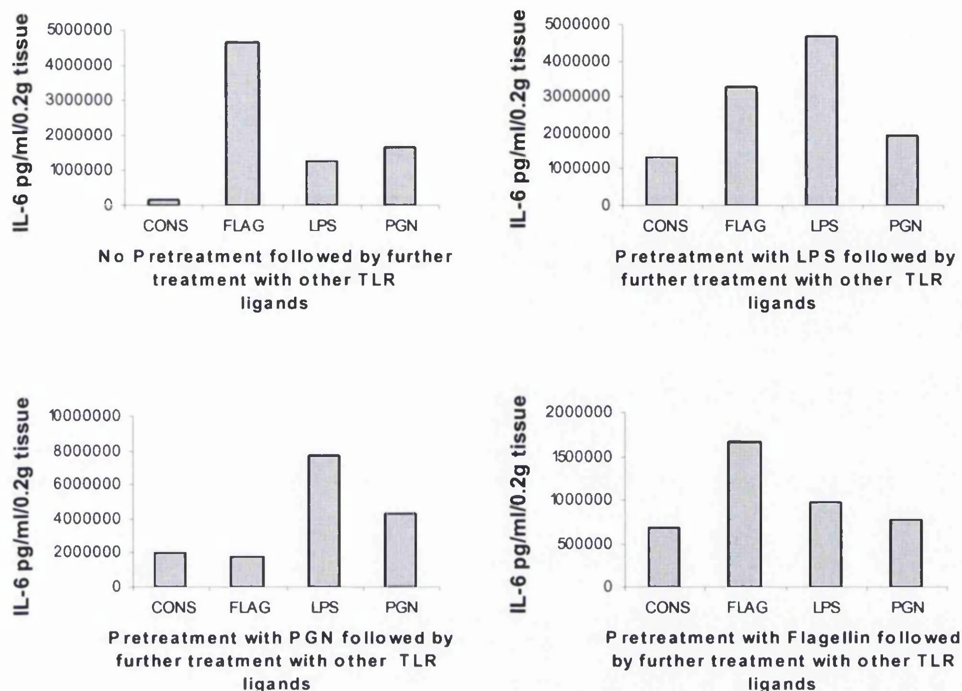


Figure 5.4 *Effect of pre-exposure to TLR ligands on TLR ligand stimulated cytokine output of placental explants. Explants were prepared from term non-laboured placentas and treated with TLR ligands.. Supernatants were harvested after further 24 hours and IL-6 (mean ± SEM) production in response to (a) constitutive (Cons)/no stimulation, (b) LPS, (c) PGN, (d) Flagellin determined by ELISA (where standard error bars are missing the errors are too small to show)*

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

As preterm samples were of varying gestations and for various reasons, initial comparison of the TLR transcripts was made between the preterm spontaneously laboured group (obtained following IA-PTL, preterm-svd, $n=9$) and the term laboured group (term-laboured, $n=12$; Figures 5.6 a – j). Placental biopsies from all deliveries were placed in RNA*later*® so although RNA extraction, cDNA synthesis and PCR could not be conducted simultaneously they were spread over three mixed batches for extraction and cDNA synthesis and 2 plates PCR as described in detail in *Chapter 2, section 2.5.3*). The expression of TLR transcripts by the two groups did not differ statistically for any of the TLRs (p values shown on graphs).

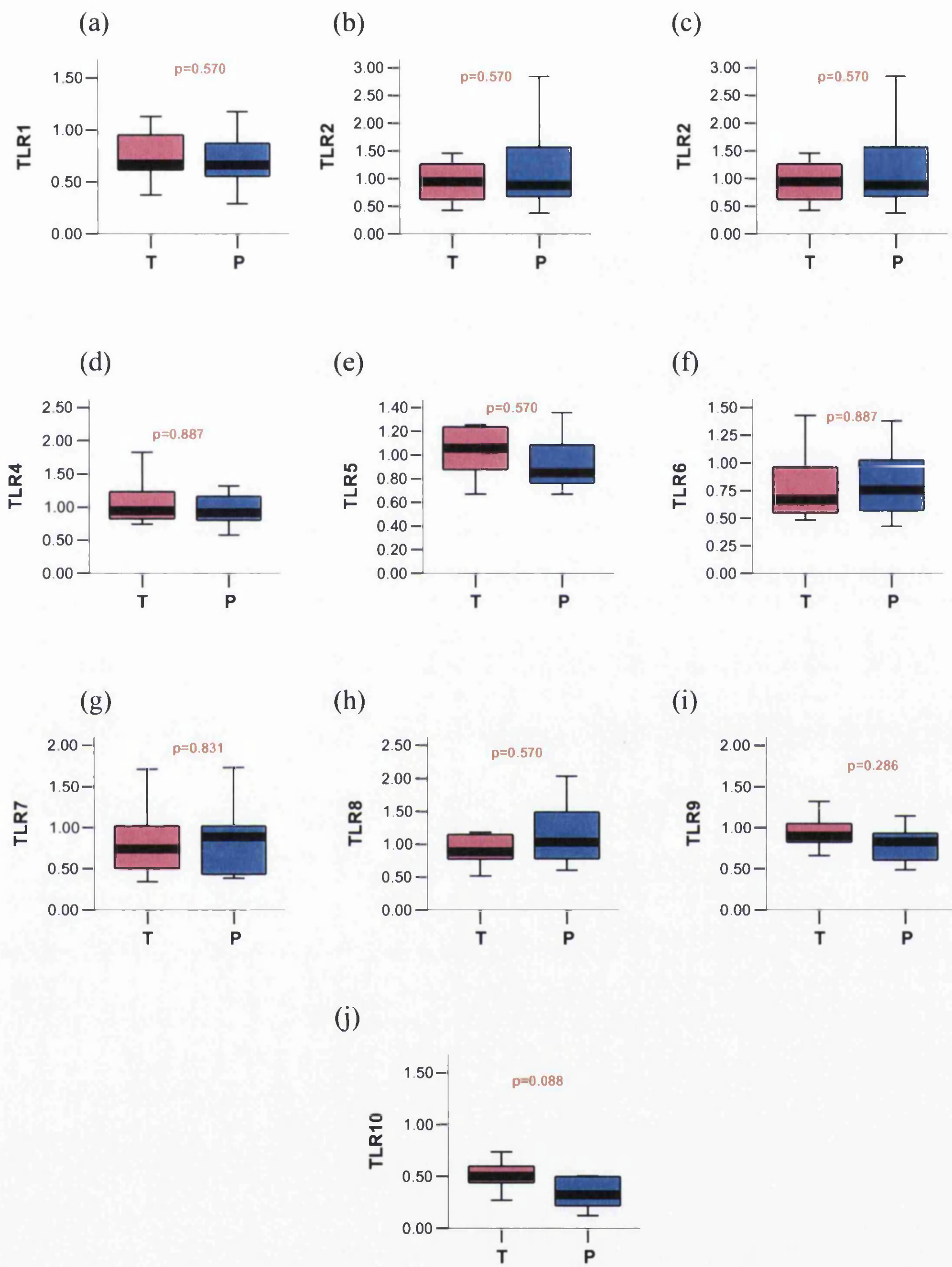


Figure 5.6 *Box and whisker plots depicting comparison between expression of mRNA for TLRs 1-10 in the term-laboured (T) (n=12) and preterm-SVD (P) (n = 9) groups. Mann Whitney U test for two independent samples was employed and the p values are shown on the graphs.*

5.4 DISCUSSION

IL-6 and TNFα outputs after stimulation of the placenta via TLR2, TLR3, TLR4, TLR5 and TLR7/8 were significantly reduced in the preterm compared with term laboured group. As for the term group, TLR9 activity was not detected. Despite these functional differences on comparison with the term group there were no differences in the levels of mRNA for any of the TLRs studied. This is similar to the results obtained in Chapter 4 in which there was no apparent correlation between functional output and mRNA levels for any of the TLRs (in the term laboured and non-laboured groups). The comparable levels of placental TLR transcripts in the preterm and term laboured groups is also similar to findings in human myometrium where both TLR2 and TLR4 mRNA expression was comparable in labouring versus nonlabouring myometrium both at term and preterm gestations (R Youssef 2006). These results suggest that TLR protein expression should be studied in greater detail and/or that changes in inhibitors of TLR-dependent signalling might have a critical role.

A number of inhibitors of TLR-mediated activity have now been described. Over-expression of Tollip (toll interacting protein) results in impaired NF-kappaB activation (Burns K 2000) which can thus diminish the TLR response and thus the cytokine output. Similarly SIGGR (single immunoglobulin IL-1R-related molecule), acts as a negative regulator of interleukin (IL)-1 and lipopolysaccharide (LPS) signaling (Wald D 2003; Qin J 2005; Huang X 2006).Thus investigation of the relative expression of these inhibitors in preterm versus term tissues might prove fruitful.

One of the challenges of undertaking studies such as this in humans is that it is impossible to obtain samples during labour and samples can only be obtained once the delivery is completed. Consequently, results obtained at this time might not be representative of the response occurring *in utero* earlier in labour. Additionally, women in preterm labour are treated with various pharmacotherapies to (i) prevent premature delivery (tocolytics) and (ii) prepare the baby for premature delivery (corticosteroids).

Therefore to explore the possibility that these treatments might explain the results obtained in this chapter, a number of additional studies were undertaken. Firstly, the effect of dexamethasone on TLR-mediated responses was explored. The constitutive and TLR ligand-stimulated IL-6 was reduced in the presence of dexamethasone for all TLR ligands studied and the reduction in the cytokine output following treatment was statistically significant with doses of 4 and 40ng/ml of dexamethasone.

Secondly, the possibility that previous stimulation of a TLR (i.e. *in utero*) might down-regulate secondary responses (i.e. *in vitro*) was considered. This could be because of exhaustion or downregulation of the relevant TLR pathways *in vivo* as a result of response to the presence of infection. Pre-exposure to LPS is known to reduce the level of response upon secondary exposure to LPS, a process known as endotoxin tolerance (Fan and Cook 2004; Broad, Jones et al. 2006). Endotoxin tolerance is a well known phenomenon, described both *in vivo* and *in vitro*, in which repeated exposure to endotoxin results in a diminished response, usually characterised as a reduction in pro-inflammatory cytokine release. With increasing understanding of the part played by TLR-4 signalling in endotoxin/LPS release it has become clear that response tolerance occurs to other TLR ligands in addition to LPS/endotoxin. However, pre-treatment of explants from non-laboured term placentas with any TLR ligand *in vitro* was not associated with diminished responses to any TLR ligand. Thus prior intrauterine exposure to the ligand does not explain the reduced cytokine output of preterm placental explants in comparison to term explants.

In keeping with findings from the term placenta as discussed in chapter 3 and 4, results from this chapter clearly demonstrate that the human preterm placenta also expresses functional TLR1, 2, 3, 4, 5, and 7/8. However contradictory to the assumption that preterm labour would be associated with enhanced cytokine levels secondary to its infection related etiology, we found reduced cytokine output in the preterm samples in comparison to term samples. This does not appear to result from a mechanism similar to endotoxin tolerance and whilst it could reflect exposure to steroids this reduced output by the preterm placenta might also be due to overexpression of TLR inhibitors. Further

studies to determine expression of inhibitors of TLR activity would be worthwhile pursuing.

CHAPTER 6

CONCLUSIONS

6.1 INTRODUCTION

Toll like receptors have emerged as key upstream mediators of inflammation at many tissue sites in humans. Parturition is considered an inflammatory process so it was hypothesised that TLR activity within gestation-associated tissues, such as the placenta, might have an important role in the initiation and/or maintenance of labour and in various pathological states of pregnancy such as infection associated preterm labour.

The study reported in this thesis examined the expression and the activity of the TLRs in the preterm and term laboured and non-laboured human placenta. The underlying aim of the study was to achieve a better understanding of the mechanisms which underlie the onset of labour whether at term or preterm. Understanding TLR-mediated inflammation at the materno-fetal interface is of relevance to labour and could reveal novel targets for either the prevention of labour when preterm or induction of labour at term. The former is a priority of contemporary obstetrics.

Despite much effort during the past 40 years in tackling preterm labour, this remains the most important cause of perinatal mortality and morbidity. Prematurity alone accounts for 75% of perinatal morbidity and mortality in the developed world (Besinger 1993). If the problem of prematurity is to be tackled successfully, there are three facets to consider – firstly, and ideally, the prevention of preterm labour, or failing that successful recognition and implementation of treatment directed at inhibiting myometrial contractions, and lastly the care of the preterm newborn. With the advances made so far, the prevention of preterm labour seems to be the most viable step in dealing with the problem of prematurity. Certainly this requires a better understanding of the basic etiology of preterm labour. With this background, this study was planned and was commenced with the determination of baseline characteristics at the maternofetal interface in the non-laboured state. This then progressed to evaluation of the changes with onset of labour at term and finally to the changes in association with the preterm labour.

6.2 MAIN CONCLUSIONS OF THIS STUDY

6.2.1 TLR expression and activity in the human placenta

This study describes the expression of transcripts for TLRs 1-10 in the term human placenta in keeping with published studies (Zarember and Godowski 2002; Nishimura and Naito 2005). With the exception of TLR9 (and TLR10 which could not be studied) all TLRs were found to be functional. However further study is required to determine the activity of TLR1 and TLR2; and TLR2 and TLR6 heterodimers. Our study indicated differential response on stimulating TLR2 with different ligands (PGN and zymosan). Thus, the cytoplasmic tails of TLRs are not functionally equivalent, with certain TLRs requiring assembly into heterodimeric complexes, whereas others are active as homodimeric complexes. Therefore, more detailed analysis of TLR2 and its partner TLRs is required and is currently underway. Also the recent development of TLR7 and TLR8 specific ligands in contrast to R848 used in this study which stimulates both TLR7 and TLR8 means more detailed study of these pathways should now be possible. Ideally these studies should be complemented with analysis of protein expression (e.g. immunohistochemistry and/or immunoblotting). However the lack of antibodies suitable for anything other than TLR2 and TLR4 at the time of study precluded such analysis but is an ongoing aim of the group.

6.2.2 Changes in TLR response and expression in term labour

The extensive analysis of differences in the functional response of laboured and non-laboured term placentas to various TLR ligands made in this study revealed statistically significant increased levels of TNFα in response to a TLR4 ligand (LPS) and a TLR7/8 ligand (R848) and of transcripts for TLR2 and TLR5 in the laboured group. Changes in TLR4 mediated and TLR7/8 mediated function was not accounted for by an increase in the level of transcripts for these two TLRs. This discrepancy could be due to changes in

the protein expression of the TLRs themselves of any of the accessory molecules known to modify their activity, e.g. CD14 for TLR4 mediated responses. As noted above, the recent availability of TLR7 or TLR8 specific ligands means that further studies should reveal if labour associated changes are peculiar to just one of these receptors.

6.2.3 TLR expression and response in preterm labour

The primary objective of this component of the study was to elicit the possible etiology of preterm labour at the cellular level. Cytokine levels (TNFα and IL-6 but not IL-10) in response to stimulation via TLR2, 3, 4, 5 and 7/8 were significantly reduced in placentae obtained after preterm labour compared with those obtained after term labour. However the expression of transcripts for these TLRs did not differ between the two groups. Reduced concentration in the absence of changes in levels of transcripts might be explained by steroid exposure as most patients with preterm labour received steroid injections as part of the treatment for preterm labour. In support of this, dexamethasone treatment reduced IL-6 output via all ligands as well as constitutively. However the physiological significance of this remains unknown. Alternatively, pre-exposure to any TLR stimuli *in utero* might lead to the downregulation of the receptors leading to reduced output of cytokines on their subsequent stimulation. However the data generated do not support this. Another possible explanation is that TLR pathway inhibitory molecules, such as Tollip, SIGGR and other similar inhibitory molecules are relatively over expressed in the preterm placenta and this possibility is worthwhile investigating.

6.3 SUMMARY

This study clearly demonstrates that the human term placenta expresses functional TLR2, 3, 4, 5, 7 and 8 and, because of the observed response to PGN, TLR1 must also be active (Takeuchi, Sato et al. 2002). This study also highlights the need to undertake more detailed functional analysis of each of the TLR-mediated pathways in placental and other extraembryonic tissues. Such an analysis, of even non-functional pathways such as that for TLR9, would delineate the exact signalling pathways operational for each TLR thereby enhancing knowledge of the intrinsic differences in the activity of a particular receptor on stimulation with a specific agonist in a particular tissue. It might well be possible that in placenta, the ratio of different TLRs active within different cell populations could modify the response to a given agonist (Hajjar, O'Mahony et al. 2001) and that manipulation of TLR activity could indeed yield numerous therapeutic benefits.

CHAPTER 7

FUTURE DIRECTIONS

FUTURE WORK

The study in this thesis has concentrated on the function of TLRs in the placenta by measuring the biological activity of these receptors on stimulation with their agonists using cytokine outputs measured by ELISA. At the same time gene expression studies were also performed to illustrate the expression of TLRs at the mRNA level.

This work has demonstrated that these receptors are both present and functional in the human placenta. This is a novel finding and an extension to previously reported studies (Holmlund, Cebers *et al.* 2002; Zarember and Godowski 2002; Kim, Romero *et al.* 2004; Kumazaki, Nakayama *et al.* 2004). Changes in TLR activity and expression in response to term and preterm labour have also been shown. In general, these findings indicate that there is no relationship between mRNA expression and function of these receptors within the placenta indicating that specific inhibitory mechanisms and/or TLR-independent mechanisms might supplement TLR-dependent activity. Thus the human term placenta expresses a variety of functional TLRs but more detailed analysis of contributing cell types and signalling molecules is required to elucidate the role of this family of receptors in parturition.

Future work should, in particular, aim to determine the protein expression of TLR1/2/6, TLR4 and TLR7/8 receptors by using immunohistochemistry and/or immunomodulatory studies (of course, once a TLR10 ligand is identified, functional activity of this receptor pathway should be determined). More detailed analyses of the TLR4 and TLR7/8 signalling pathway could explain why increased TLR activity occurs in absence of any change in the expression of the mRNA for these TLRs with the onset of term labour. Moreover, elucidating the complexity in the TLR2 singalling pathway might explain why increased TLR2 mRNA expression is increased with labour in the apparent absence of any functional changes in TLR2-meidated cytokine outputs. There may also be changes in the expression of accessory molecules like CD14 which could explain increased activity in response to LPS. Notably, expression of soluble CD14 and soluble TLR2 was demonstrated using immunoblotting during the course of this study.

Furthermore it would be worthwhile to look at other gestational tissues such as amnion as altered activity of this tissue is likely to contribute to changes in the cytokine profile of amniotic fluid. Of note, this work revealed a reduced cytokine output following stimulation of TLRs after preterm labour and, as already underway, the study of both protein expression levels and inhibitory pathways is warranted.

Several lines of evidence support a genetic predisposition to spontaneous preterm labour and preterm birth. A leading risk factor for spontaneous preterm labour and preterm birth is a personal or family history. If a woman previously delivered preterm, her subsequent babies are also more likely to be born preterm. Women who experienced an early preterm birth (<32 completed weeks) in their first pregnancy have the highest rate of recurrent preterm birth in subsequent pregnancies. Spontaneous preterm labour and preterm birth in subsequent pregnancies tend to recur at equivalent gestational ages. If a woman herself was born preterm, she is also at an increased risk of spontaneous preterm labour and preterm birth, with the risks being highest for those women who themselves were born most preterm. This predisposition does not apply if the father was born preterm. Thus a genetic approach might provide important information regarding an individual's susceptibility towards preterm labour. Polymorphisms in TLR2 and 4 in both mother and fetus have been identified and have been linked with altered responsiveness to TLR ligands and these genetic mutations might have an impact clinically (Lorenz, Hallman *et al.* 2002; von Aulock, Schroder *et al.* 2004).

Furthermore, it is well established that upper genital tract infection and/or inflammation is seen in association with spontaneous preterm labour and preterm birth. Previous investigations have focused primarily on an infectious aetiology for this finding. In keeping with our hypothesis that preterm labour represents an abnormal inflammatory response (histological evidence of infection/inflammation and elevated body fluid concentrations of inflammatory cytokines) investigations have been performed on single gene polymorphisms of relevant cytokines in both mother and fetus. The polymorphisms TNFα-308, IL-1β+3953/3954 and IL-6-174 have been most consistently associated with spontaneous preterm labour and preterm birth (Varner MW 2005). These findings

confirm a clear genetic predisposition to spontaneous preterm labour and preterm birth and raise hopes that patient-specific therapies might be developed in the future. Focusing on functional responses of gestation-associated tissues from women of known genotype might be of interest. Similarly polymorphisms in other TLRs like TLR5 and TLR7/8 could be of interest.

Toll-like receptors could also play a role in the pathogenesis of certain pregnancy complications in addition to preterm labor such as preeclampsia or IUGR. If this is the case then these receptors might be used as clinical biomarkers of disease. Recently, soluble TLR2 has been identified (LeBouder, Rey-Nores *et al.* 2003) and has been postulated to modulate specific TLR mediated responses. Alternatively, soluble forms of TLRs might bind to microorganisms and flag them for destruction by the complement system or by phagocytosis (Medzhitov and Janeway 2002). Soluble Toll-like receptors might, therefore, provide new markers of pregnancy complications as well as a potential target for therapeutic interventions.

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Appendices

Appendix1

CONSENT FORM

VERSION NO: 2 (12/08/04)

LREC number 04/WMW02/18
Patient identification number for this trial..............................
***N.B.* Three copies should be made for (1) patient, (2) researcher, (3) hospital notes**

Title of project : Maternal allergy and smoking and the immune response of the newborn.
Name of researcher : Catherine Thornton, PhD
Contact telephone number: 01792 602122

Please initial Box

1. **I confirm that I have read and understood the information sheet for the above study and have had the opportunity to ask questions.** ☐

2. **I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.** ☐

3. **I understand that sections of any of my medical notes may be looked at by responsible individuals from University of Wales Swansea, Swansea NHS Trust or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.** ☐

4. **I agree to take part in the above study.** ☐

____

Name of Patient **Date** **Signature**

____

Name of Person taking consent **Date** **Signature**

____

Researcher **Date** **Signature**

Appendix2

Details of experimental Groups

Numbers	Group	Maternal Age (years)	Parity	Gestation(weeks)	Birth weight(grams)	Gender
1	EL CS	30	1+0	39	3890	Female
2	EL CS	29	3+0	39	2890	Male
3	EL CS	31	0+0	42+	3460	Female
4	EL CS	31	1+0	40+1	4450	Male
5	EL CS	21	3+0	38+	3280	Male
6	EL CS	36	0+1	38	2610	Female
7	EL CS	29	0+0	39	3800	Male
8	EL CS	34	P0+2	40	3180	Female
9	EL CS	23	1+1	38+3	3640	Female
10	EL CS	32	1+0	41	4170	Female
11	EL CS	35	1+0	39+	3970	Female
12	EL CS	20	1+0	40+4	3800	Male
13	EL CS	36	1+0	39	3380	Male
14	EL CS	35	2+0	38+5	3180	Female
15	EL CS	29	1+0	39+1	3800	Male
16	EL CS	22	1+0	39	3940	Male
17	EL CS	22	1+0	39	3120	Female
1	SVD	22	0+1	39+4	2950	Male
2	SVD	28	1+0	41+	3550	Female
3	SVD	20	1+2	39	3400	Male
4	SVD	24	P3+0	39+1	4370	Male
5	SVD	26	1+0	39+6	3400	Male
6	SVD	35	0+0	40	3090	Male
7	SVD	19	0+0	40+3	3940	Male
8	SVD	26	0+0	38+5	3090	Female
9	SVD	17	0+0	39+6	4200	Female
10	SVD	31	1+0	40+1	4760	Male
11	SVD	22	1+0	41+2	3490	Female
12	SVD	34	1+0	40+4	3830	Female
13	SVD	31	1+0	41+1	3580	Male
14	SVD	35	0+0	40+3	3280	Female
15	SVD	29	5+0	40+	3400	Male
16	SVD	18	0+0	39	3250	Male
17	SVD	24	2+0	40+2	3060	Female
1	Pre term-CS	31	P0+0	36+1	3060	Male
2	Pre term -CS	25	0+0	32+6	1805	Female
3	Pre term-SVD	27	0+0	35+4	2670	Male
4	Pre term-SVD	37	2+3	34	2175	Female
5	Pre term-SVD	19	0+0	36+2	2870	Male
6	Pre term-SVD	28	1+0	36+4	3230	Male
7	Pre term-SVD	18	0+0	27+6	1075	Female
8	Pre term-SVD	22	1+0	33+4	2280	Male
9	Pre term-SVD	31	2+3	26	870	Female
10	Pre term-SVD	26	0+0	31+6	1715	Female
11	Pre term-SVD	18	0+0	25	860	Male
12	Pre term-SVD	34	0+0	36+4	3110	Male
13	Pre term -CS	31	0+1	31	1270	Male
14	Pre term -CS	25	0+0	33	2020	Female
15	Pre term-SVD	15	0+0	33+6	2610	Male

Appendix3

Proforma for recording maternal and fetal details

Addressograph/Label- **MUM**

Age **Gravida /Parity**

Obstetric History

EDD **Date of Delivery** **Time of delivery**

Medical Condition

Complications in Pregnancy

Smoking/Alcohol

Medications/Drugs

Gestation at delivery

Mode of delivery **Indication (If relevant)**

Placental weight **Time arrived in lab** **Time of PEC** **Time of Harvest**

BABY-

Hospital No.

Birth Weight (Kgs/gms)

Length(cms)

Head Circumference (cms)

Apgar Scores

Gender

Any Medical condition

Record of Fetal Distress

Appendix4*

Calibration qPCR

1	2	3	4	5	6	7	8	9	10	11	12
←	—	NOT	—	—	IN	—	—	USE	—	—	→
Placenta ←	3000	→	Placenta ←	1000	→	Placenta ←	300	→	Placenta ←	100	→
Placenta ←	30	→	Placenta ←	10	→	Spleen ←	3000	→	Spleen ←	1000	→
Spleen ←	300	→	Spleen ←	100	→	Spleen ←	30	→	Spleen ←	10	→
Placenta ←	NTC	→	NEG					NEG	Spleen ←	NTC	→
Placenta ←	3000	→	Placenta ←	1000	→	Placenta ←	300	→	Placenta ←	100	→
Placenta ←	30	→	Placenta ←	10	→	Spleen ←	3000	→	Spleen ←	1000	→
Spleen ←	300	→	Spleen ←	100	→	Spleen ←	30	→	Spleen ←	10	→

*

- **Placenta and spleen samples at different strengths in triplicates.**
- **NEG- Negative control in triplicate**
- **NTC- No Template Control in triplicate**

Appendix5
Plate Plan per PCR *

	1	2	3	4	5	6	7	8	9	10	11	12
A	←	—	**NOT**	—	—	**IN**	—	—	**USE**	—	—	→
B	←	064	→	←	080	→	←	118	→	←	123	→
C	←	069	→	←	082	→	←	083	→	←	086	→
D	←	133	→	←	139	→	←	093	→	←	125	→
E	←	194	→	←	224	→	←	124	→	←	128	→
F	←	137	→	←	172	→	←	NEG	→	←	NTC	→
G	Placenta ←	3000	→	Placenta ←	1000	→	Placenta ←	300	→	Placenta ←	100	→
H	Placenta ←	30	→	Placenta ←	10	→	Spleen ←	1000	→	Spleen ←	300	→

Plate 1

Plate-2 *

	1	2	3	4	5	6	7	8	9	10	11	12
A	←	—	**NOT**	—	—	**IN**	—	—	**USE**	—	—	→
B	←	117	→	←	149	→	←	134	→	←	135	→
C	←	143	→	←	148	→	←	091	→	←	196	→
D	←	142	→	←	197	→	←	150	→	←	173	→
E	←	234	→	←	239	→	←	136	→	←	138	→
F	←	175	→	←	176	→	←	NEG	→	←	NTC	→
G	Spleen ←	3000	→	Spleen ←	1000	→	Spleen ←	300	→	Spleen ←	100	→
H	Spleen ←	30	→	Spleen ←	10	→	Placenta ←	1000	→	Placenta ←	300	→

*

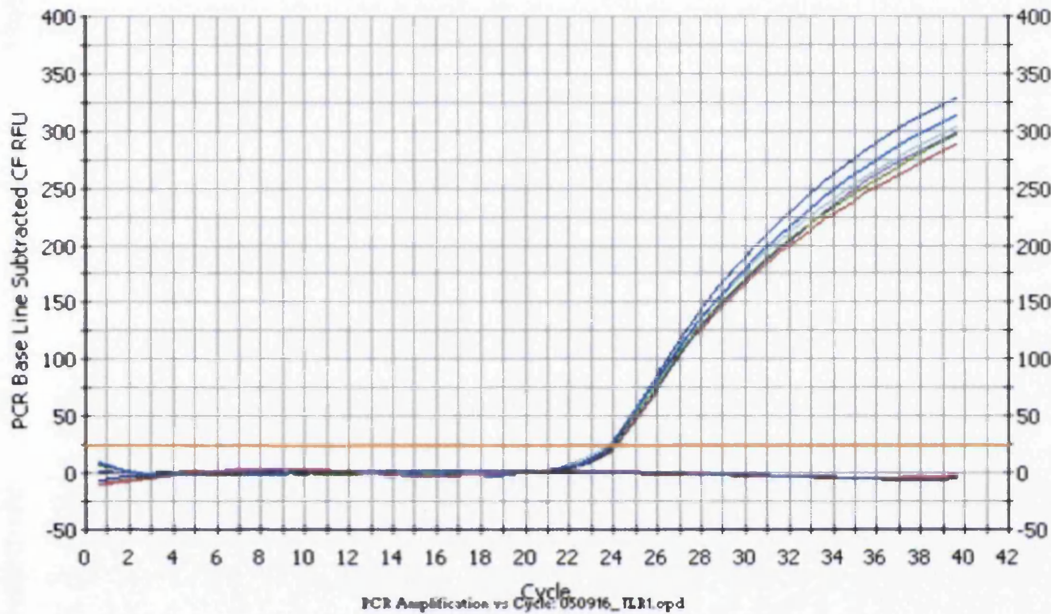
- The numbers on the plate (rows-A-F) are the sample numbers employed in the study (Appendix-2) and include samples from the 3 experimental groups. Each sample is in triplicate.
- NEG- Negative control in triplicate
- NTC- No Template Control in triplicate
- Placenta and spleen samples (rows-G-H) served both as standards and calibrators in triplicate.

Appendix6
(qPCR Results from Chapter3)

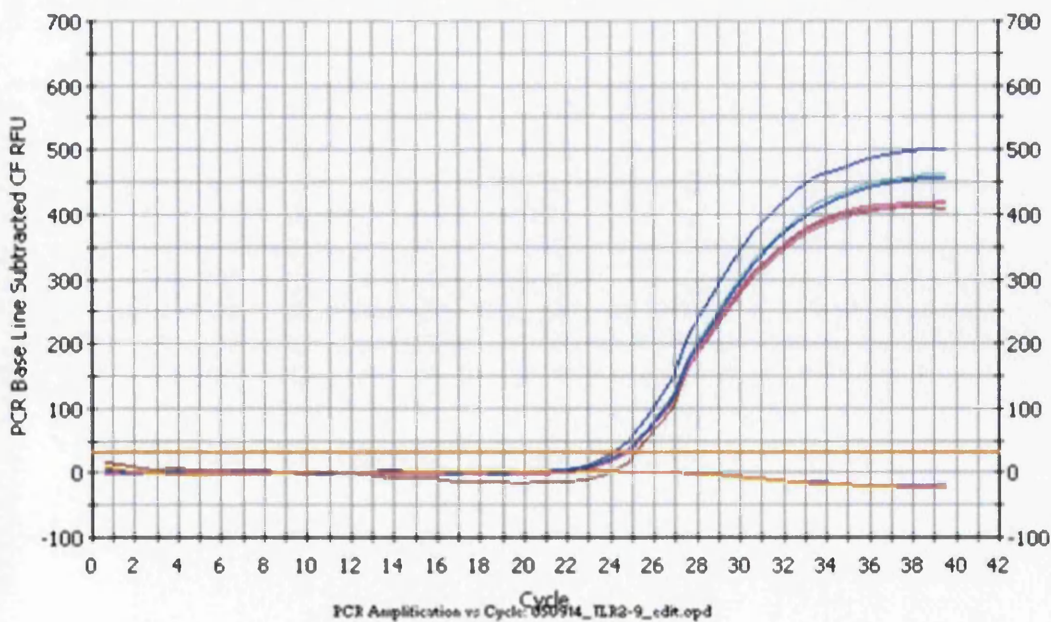
Comparison of qPCR results for TLRs 1-10 from placenta and spleen along with negative control.

Ct is the point at which fluorescence crosses threshold and the values shown are the average of triplicate.

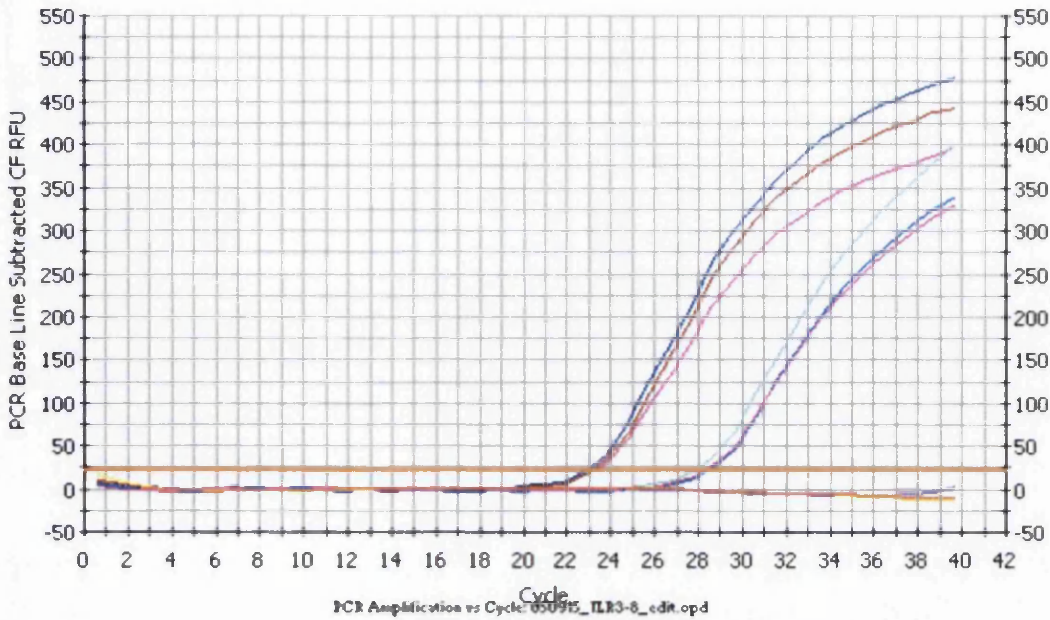
TLR1: placenta Ct = 24.0, spleen Ct = 23.9



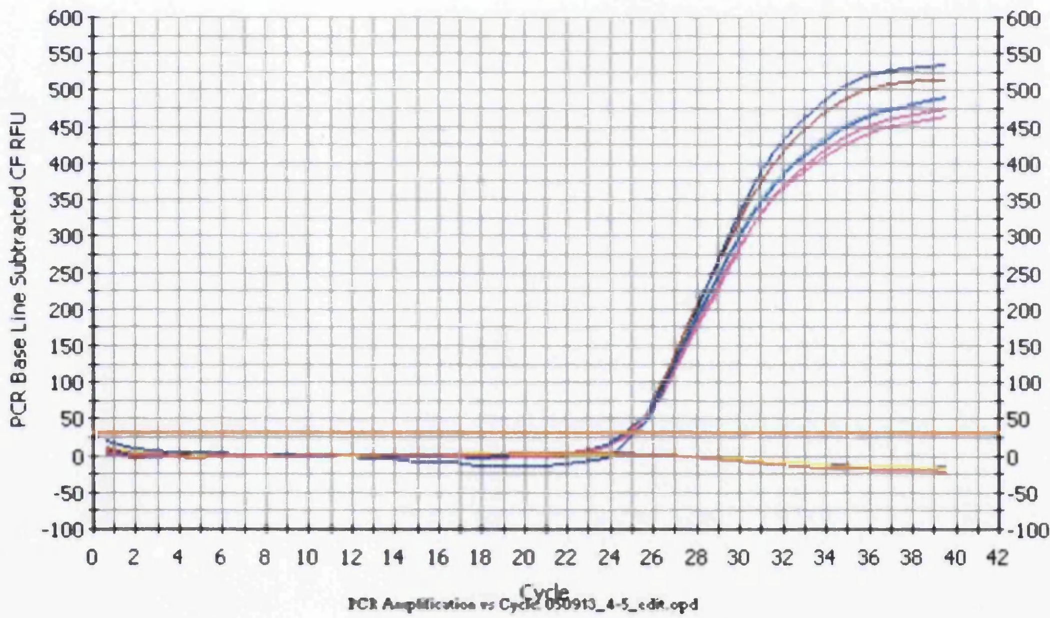
TLR2: placenta Ct = 24.57, spleen Ct = 24.67



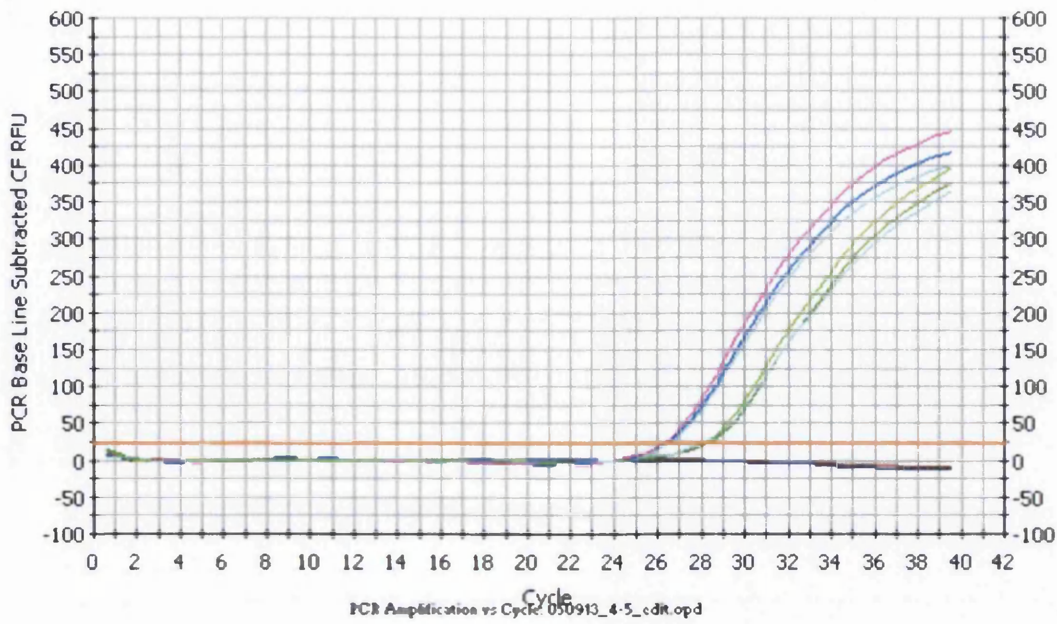
TLR3: placenta Ct = 23.03, spleen Ct = 28.13



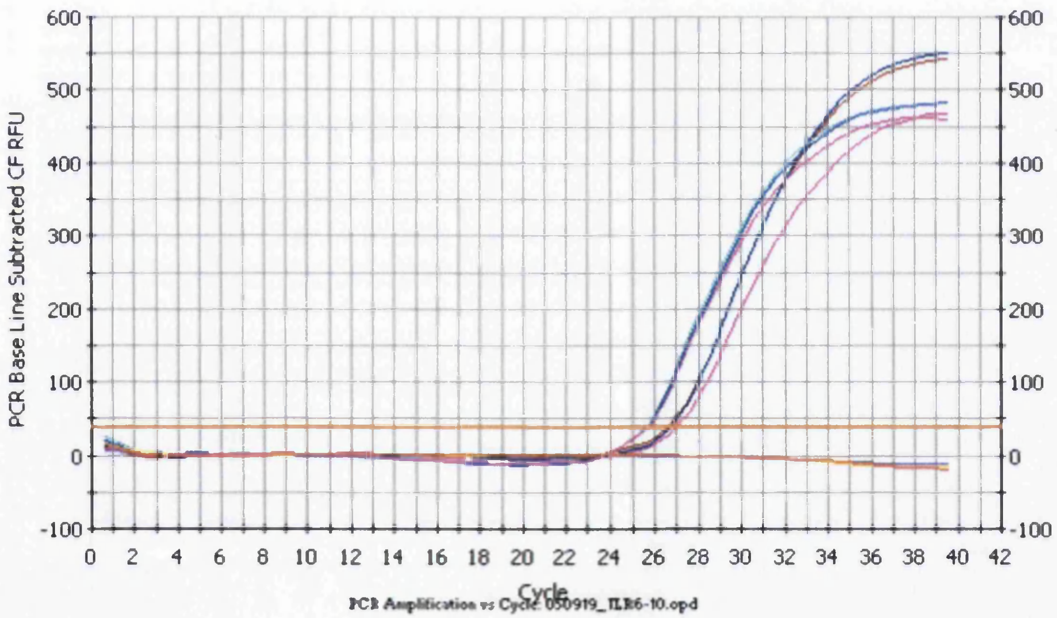
TLR4: placenta Ct = 24.83, spleen Ct = 24.67



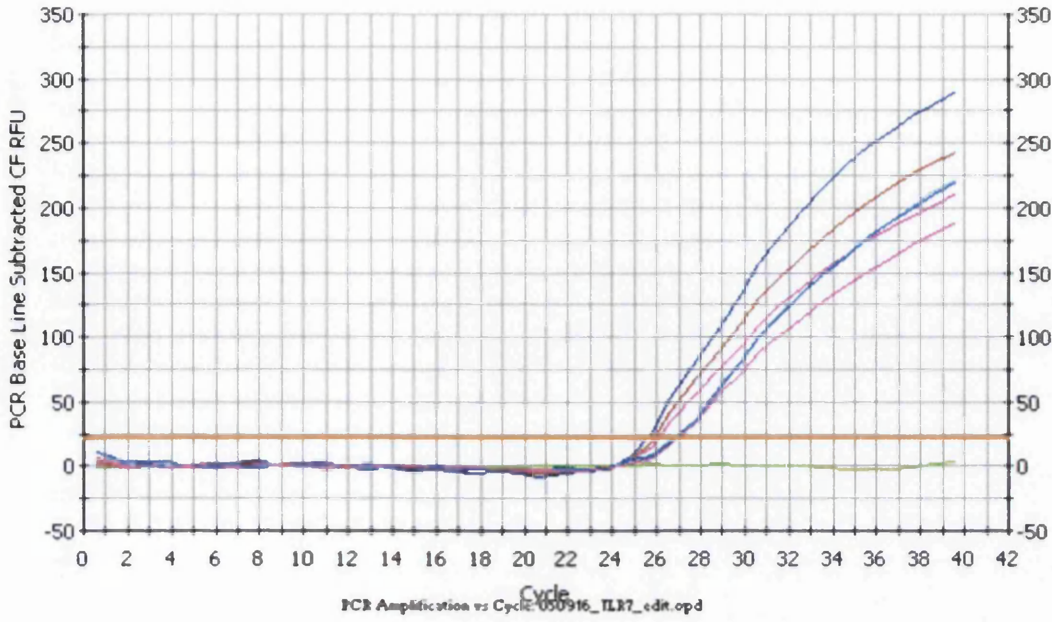
TLR5: placenta Ct = 26.33, spleen Ct = 28.10



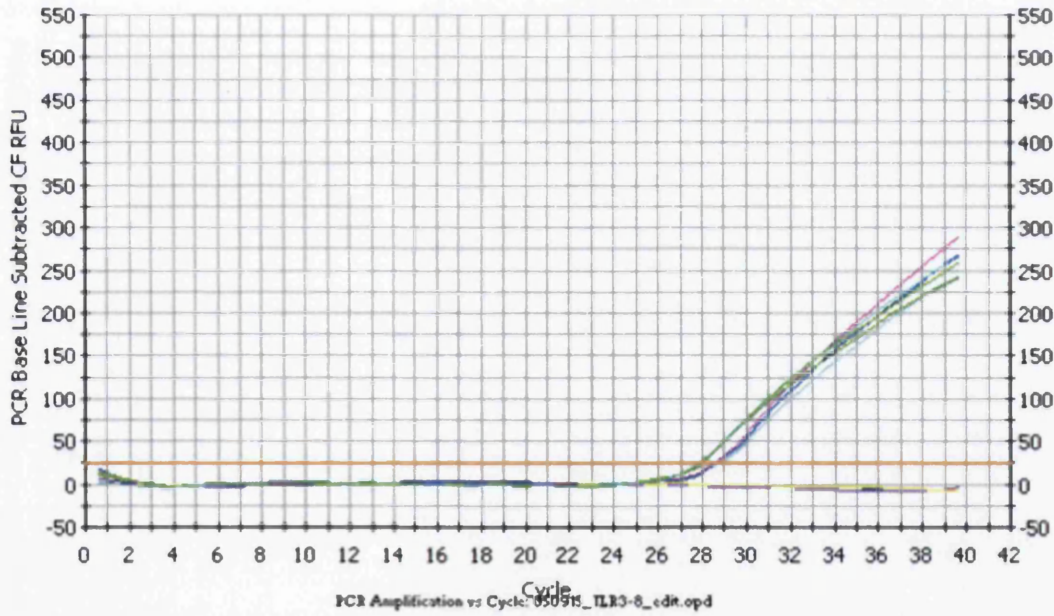
TLR6: placenta Ct = 26.70, spleen Ct = 25.63



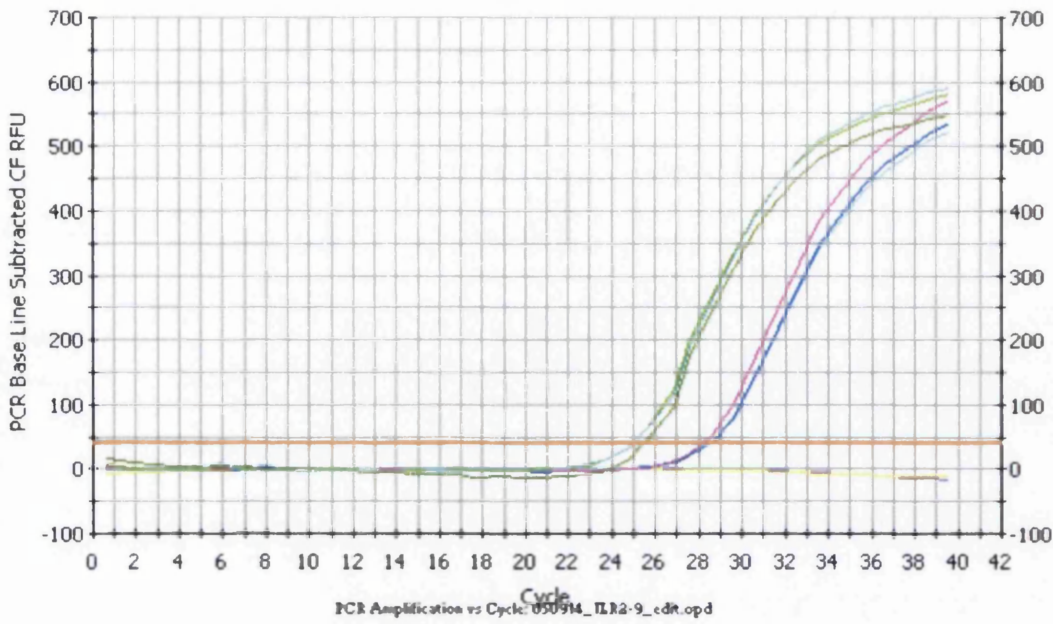
TLR7: placenta Ct = 25.87, spleen Ct = 26.80



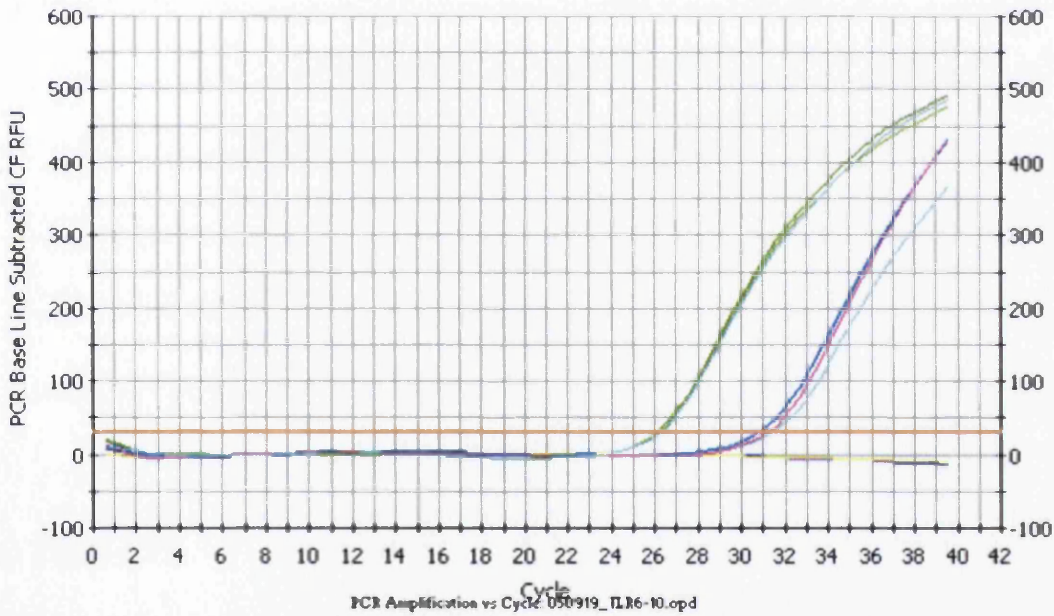
TLR8: placenta Ct = 28.53, spleen Ct = 27.90



TLR9: placenta Ct = 28.37, spleen Ct = 25.17



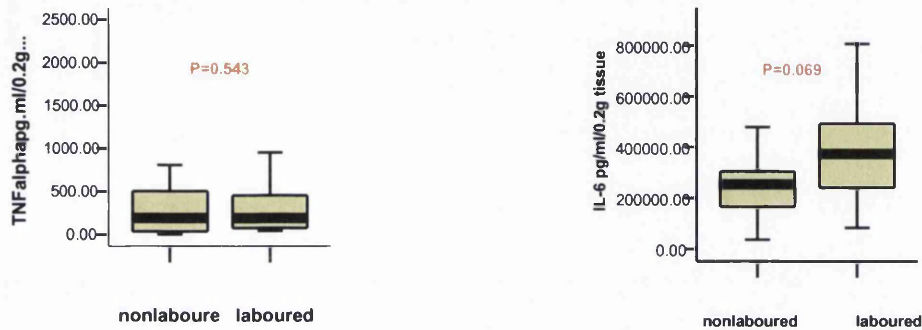
TLR10: placenta Ct = 31.13, spleen Ct = 26.23



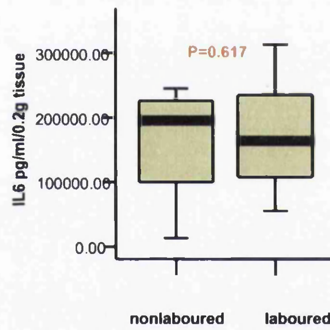
Appendix7
Results from Chapter 4

Comparison of cytokine outputs of non-laboured versus laboured placental explants in response to TLR ligands

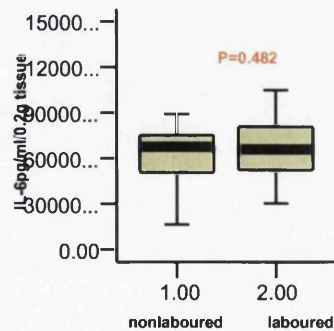
1(a) TLR2-PGN



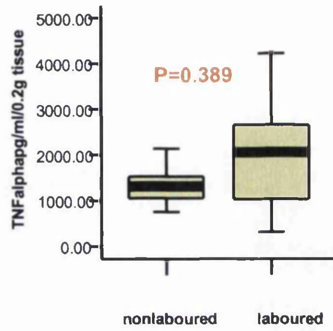
1(b) TLR3-PolyI: C

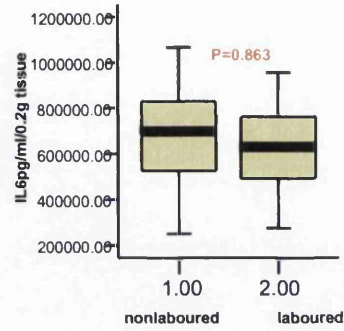


1(c) TLR4-LPS

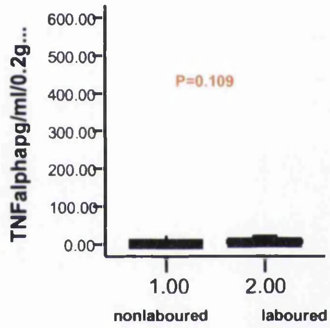


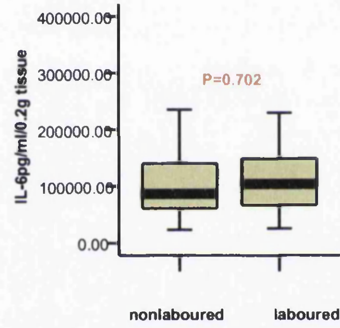
1(d) TLR5-Flagellin



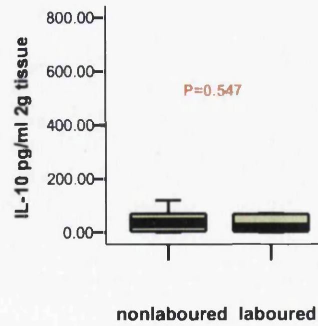


1(e) TLR2/6-Zymosan

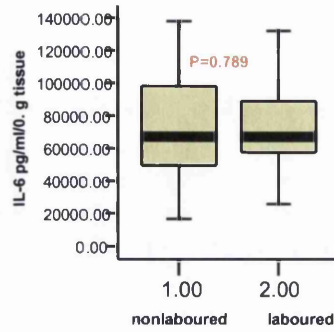




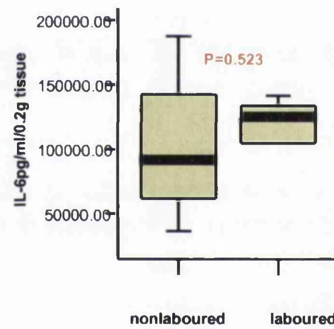
1(f) TLR7/8-R848



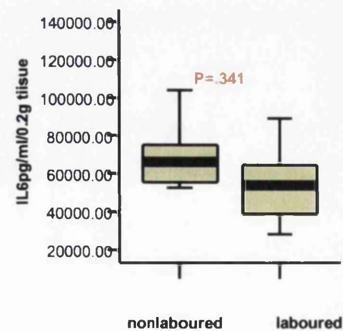
1(g) TLR7/8-Lox



1(h) TLR7/8-ssPoly



1(i) TLR9-CpG



PRESENTATIONS AND PUBLICATIONS IN PEER REVIEWED INTERNATIONAL AND NATIONAL SCIENTIFIC MEETINGS

PRESENTATIONS AT INTERNATIONAL MEETINGS

- Expression and activity of Toll like receptors in infection-associated preterm labour in comparison with term labour. **S Patni**, L Wynen, P, Flynn, JO White, CA Thornton.**Oral presentation** in the **19th European Congress of Obstetrics and Gynaecology, Turin, Italy,** 5th-8th **April 2006.**

- Expression and activity of Toll like receptors in term human placenta. Patni S, L Wynen, P, Flynn, JO White, CA Thornton. **Poster presentation** at **IFFPA and European Placenta conference** at **Glasgow,** September 2005.

PRESENTATIONS AT NATIONAL MEETINGS

- "Expression and activity of Toll like receptors in infection-associated preterm labour". Patni S, L Wynen, P, Flynn, JO White, CA Thornton. **Poster** presentation in *11th British Maternal and Fetal medicine society annual conference*, Cardiff, UK, 6th -7th April 2006.

- Activity of toll-like receptors in term human placenta and changes following labour. **Oral** presentation at ***Blair Bell Research society*** meeting at Liverpool -2-3rd June 2005.

PUBLISHED ABSTRACTS

- **S Patni**; LP Wynen; P Flynn; JO White; CA Thornton. Expression and activity of toll like receptors in infection-associated preterm labour. *J Obstet Gynaecol.* Volume 26, Supplement 1 April 2006.

- **S Patni**; LP Wynen; P Flynn; JO White; CA Thornton. Toll like receptor (1–9) activity in the human placenta and changes associated with labour. *BJOG: An International Journal of Obstetrics and Gynaecology.* Vol. 112 Issue 10, 1446 October, 2005.

- **S Patni,** L Wynen, P, Flynn, JO White, CA Thornton. Expression and activity of Toll like receptors in term human placenta. *Placenta*, vol 26, issue 8-9, Sept-Oct 2005.

Submitted articles

- ***Review article to BJOG***

Shalini Patni, Paul Flynn, Louise P Wynen, Anna L Seager, Gareth Morgan, John O White, Catherine A Thornton. An introduction to Toll-like receptors (TLRs) and their possible role in the initiation of labour. Accepted for publication in BJOG *(Reference no: 2007-RV-3716R,*. BJOG 2007; DOI: 10.1111/j.1471-0528.2007.01488.x)

- **Peer reviewed article for submission to Journal of Immunology**

Shalini Patni, Louise P Wynen, Anna L Seager, Ruth H Jones, Gareth Morgan, Paul Flynn, John O White, Catherine A Thornton.. Expression and activity of Toll Like Receptors 1 – 9 in the human term placenta and changes associated with labour at term.