Pattern recognition receptor activity at the maternal fetal interface: Implications for preterm labour.

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Pattern recognition receptor activity at the maternal-fetal interface: Implications for preterm labour

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Summary

Interest in the innate immune response at the maternal-fetal interface has developed due to the association between intrauterine infection, inflammation and adverse pregnancy outcomes. Pattern recognition receptors (PRRs) offer a link between microbial derived agonists and the production of inflammatory mediators by gestation-associated tissues (placenta, choriodecidua and amnion). An improved understanding of these receptors and the signal transduction cascades they initiate in these tissues might explain why some pregnancies are complicated by preterm labour (PTL) and preterm premature rupture of the membranes (PPROM) whereas others are only affected by PPROM. However while this may be the eventual aim of this field of study; a greater understanding of PRR expression and activity in term non-laboured tissues is required to provide a baseline comparison for these receptors, in order to determine any potential role they may play in normal term labour but also in preterm labour and other adverse pregnancy outcomes.

Examination of PRRs in term non-laboured gestation-associated tissues demonstrated the expression of transcripts for Toll-like Receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like Receptors (RLRs) and C-type lectin Receptors (CLRs). A functional role for TLRs 1-7, NOD1, NOD2, RIG-I/MDA5 and Dectin-1 can be inferred by an increase in the production of IL-6 and IL-8 following stimulation with receptor specific agonists. IL-1β production and activation of the caspase-1 and/or caspase-8 inflammasome was observed in the placenta and choriodecidua in response to fungal β-glucan and bacterial flagellin. The anti-inflammatory cytokines IL-4, IL-10 and IL-13 are able to down-regulate the lipopolysaccharide-stimulated cytokine responses by the placenta, choriodecidua and amnion. This highlights the potential utility of these cytokines in preventing preterm birth.

Declaration and Statements

DECLARATION

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

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STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

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I dedicate this thesis in memory of my Mam.
Diolch am eich cefnogaeth ac am gredu ynddo fi.

Abbreviations

ACA	Acute chorioamnionitis
AMP	Adenosine monophosphate
AMPK	AMP-activated kinase
AP1	Activator Protein 1
APC	Antigen presenting cell
ART	Assisted reproductive technologies
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BIR	Baculovirus inhibitor of apoptosis protein repeat
BMI	Body mass index
BSA	Bovine serum albumin
CARD	Caspase activation and recruitment domain
CCA	Chronic chorioamnionitis
CD	Cluster of differentiation
cDNA	Complementary DNA
CDS	Cytosolic DNA sensor
cIAP	Cellular inhibitors of apoptosis
CLR	C-type lectin receptor
COX-2	Cyclooxygenase-2
CRD	Carbohydrate recognition domain
CRP	C-reactive protein
CSF	Colony stimulating factor
CTD	C-terminal domain
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Damage associated molecular pattern
DED	Death effector domain
DZYM	Depleted Zymosan
ESC	Elective caesarean section
ELISA	Enzyme-linked immunoabsorbant assay
FBS	Fetal Bovine Serum
FcRγ	Fc receptor common gamma chain
FLAG	Flagellin
dsRNA	Double stranded RNA
HIV	Human immunodeficiency virus

HSV	Herpes simplex virus
iE-DAP	D-γ-glutamyl-meso-DAP dipeptide
IFN	Interferon
IHC	Immunohistochemistry
IKK	IκB Kinase
IL-	Interleukin-
IP-10	Interferon inducible protein-10
IRAK	IL-1 receptor associated kinase
IRF-	Interferon regulatory factor-
ISRE	Interferon stimulated gene factor
I-TAC	Interferon-inducible T-cell alpha chemoattractant
ITAM	Immunoreceptor tyrosine-based activation motif
IUGR	Intrauterine growth restriction
LBP	LPS-binding protein
LDH	Lactate dehydrogenase
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipoteichic acid
MAP	Mitogen-activated protein
MBL	Mannose binding lectin
MCM	Murine cytomegalovirus
MCP-1	Monocyte chemotactic protein-1
MD2	Lymphocyte antigen 96
MDA5	Melanoma differentiation associated factor 5
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MIG	Monokine induced by interferon gama
MINCLE	Macrophage inducible C-type lectin
MIP-1α	Macrophage inflammatory protein 1 alpha
MMP	Matrix metalloptoteinase
MNC	Mononuclear cell
MR	Mannose receptor
MSU	Monosodium urate
mtDNA	Mitochondrial DNA
MyD88	Myeloid differentiation primary response gene 88

NAIP	NLR family apoptosis inhibitory protein
NBD	Nucleotide binding domain
NEMO	NF-κB essential modulator
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-kappa B
NLR	NOD-like receptor
NLRA	NOD-like receptor containing a acidic transactivating domain
NLRB	NOD-like receptor containing a BIR domain
NLRC	NOD-like receptor containing a CARD domain
NLRP	NOD-like receptor containing a PYD domain
NOD	Nucleotide binding oligomerisation domain
NTC	No template control
OD	Optical density
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PG	Prostaglandin
PGN	Peptidoglycan
Poly(I:C)	Polyinosinic-polyctidylic acid
PPROM	Preterm premature rupture of the membranes
PRR	Pattern recognition receptor
PTB	Preterm Birth
PYD	Pyrin domain
RAMP	Resolution associated molecular pattern
RD	Repressor domain
RIG-I	Retinoic acid-inducible gene I
RIP	Receptor interacting protein
RLR	RIG-1-like receptor
ROS	Reactive oxygen species
SAP	Splicesome associated protein
SDS	Sodium dodecyl sulfate
SOCS	Suppressor of cytokine synthesis
sPTL	Spontaneous preterm labour
SR	Scavenger receptor
ssRNA	Single stranded RNA

STING	Stimulator of interferon genes
Syk	Spleen tyrosine kinase
TBE	Tris-borate-Ethylenediaminetetraacetic acid
TBK	TANK-binding kinase
TBS	Tris-buffered saline
TIMP	Tissue inhibitor of metalloproteinsase
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain-containing adapter protein
TNFα	Tumor necrosis factor alpha
TRAF	TNF receptor associated factor
TRAM	TRIF related adapter molecule
TRIF	TIR domain-containing adapter proteins inducing IFNβ
Tri-DAP	L-Ala-gamma-D-Glu-mDAP
TLR	Toll-like receptor
UBE2D2	Ubiquitin-conjugating enzyme E2 D2
WHO	World Health Organisation
WST	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate sodium salt

Chapter 1

Introduction

1 Introduction

1.1 Overview

Labour is the climax of pregnancy resulting in the expulsion of the fetus from the uterus. It is a complex process and the mechanisms involved in the initiation of labour are poorly understood despite decades of investigation. Generally, labour is not a sudden occurrence but one for which the body prepares: numerous physiological, endocrinological, biochemical and immunological events take place at the maternal-fetal interface and in both mother and fetus in the lead up to parturition [1-5]. These same processes might be accelerated or other pathways brought into play when labour occurs prematurely. The bulk of perinatal morbidity and mortality is associated with premature labour and delivery of a preterm infant [6, 7]. Understanding the mechanisms of labour in healthy and in adverse obstetric outcomes should provide insight into the pathogenesis of preterm birth (PTB).

1.2 Human labour

Human labour and delivery have been compared to an inflammatory response [8-10] of at least three physiologically interdependent processes (Figure 1.1): 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 force the fetus and placenta from the uterus. Pro-inflammatory cytokines produced primarily by gestation associated tissues (placenta and fetal membranes; Figure 1.2) have a role in most of these processes, suggesting that the immune privileges that the fetal-placental unit has enjoyed during pregnancy might be revoked at the time of labour [9]. Beneficial effects of strong pro-inflammatory activity during labour could include removal of placental fragments and a heightened innate immune response in the postpartum uterus to combat the pathogens undoubtedly encountered at this time.

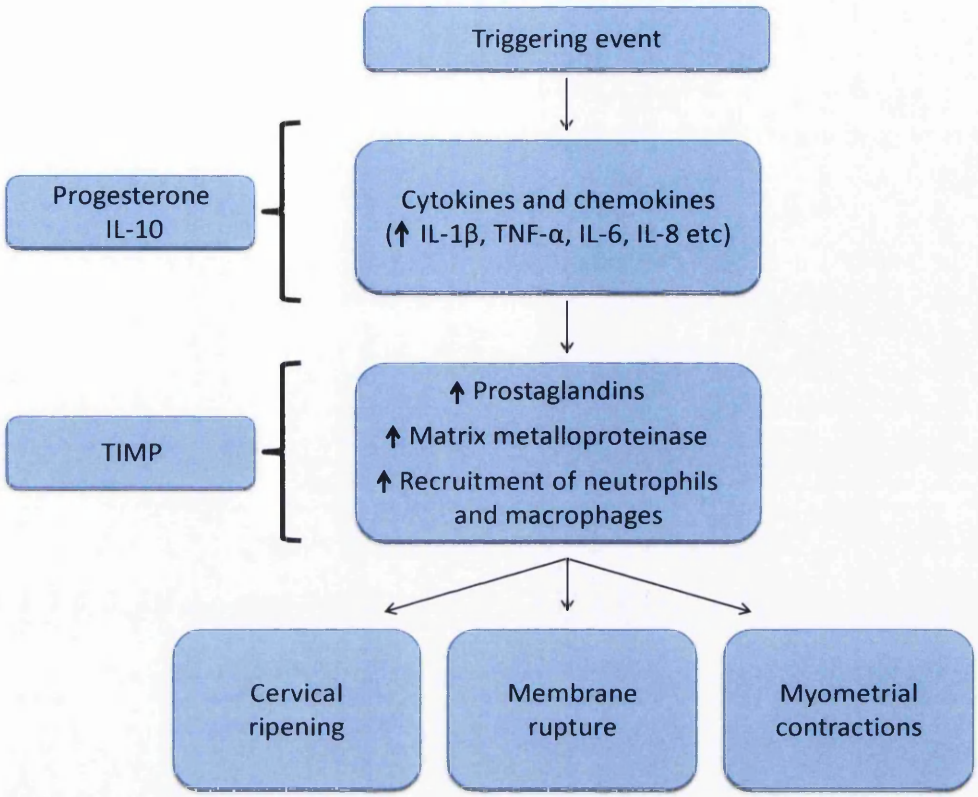

Figure 1.1 Overview of human labour.

Increased production and release of inflammatory cytokines is caused by a triggering event (e.g. microbial stimuli). This leads to the recruitment of inflammatory cells and the increased production of other inflammatory mediators. These events facilitate the three physiological process of human labour; cervical ripening, membrane rupture and myometrial contractions. These events can also be hindered by several inhibitors including IL-10, progesterone and tissue inhibitor of metalloproteinsase (TIMP). Adapted from Patni et al [11].

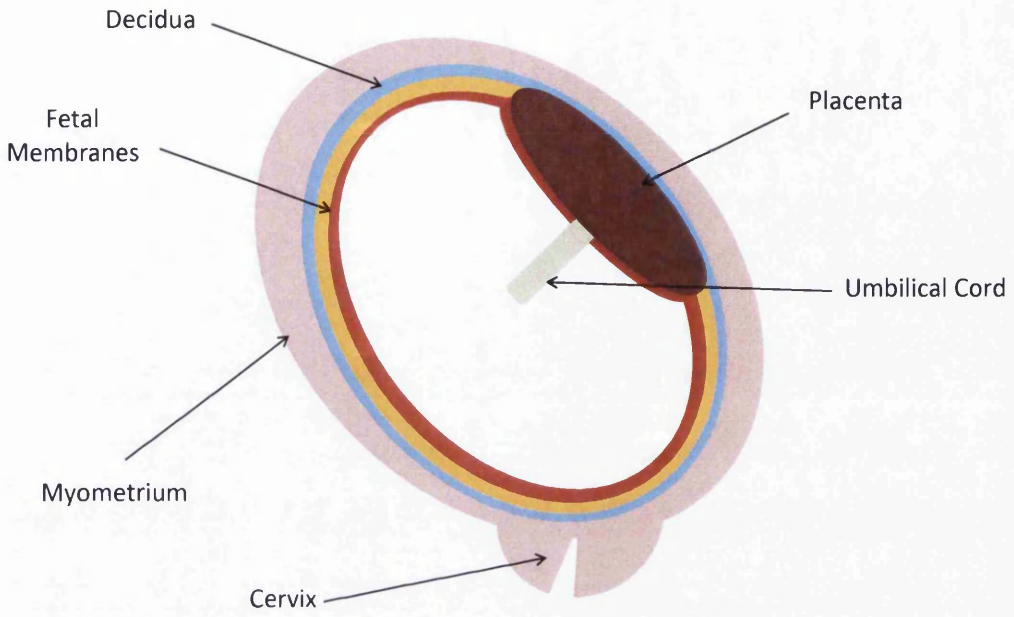

Figure 1.2 Gestation associated tissues.

During pregnancy the fetal environment includes the gestation associated tissues - placenta and the fetal membranes (amnion and choriodecidua).

1.2.1 Cervical ripening

Re-modelling of the extracellular matrix, including decreased collagen concentration and the dispersion of collagen fibrils, is a feature of cervical ripening [12, 13]. This is facilitated by increased local production of many pro-inflammatory cytokines and chemokines, such as interleukin-1β (IL-1β), IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1; CCL2) and tumour necrosis factor α (TNFα) [14, 15]. There is also an influx of neutrophils and macrophages that also secrete cytokine and chemokines and amplify the inflammatory response via the recruitment of other inflammatory cells to the cervix [16-18]. Cytokines such as TNFα and IL-1β activate the nuclear factor (NF)-κB pathway, leading to increased production of proteases, cathepsins and matrix metalloproteinase (MMPs),that allow for the digestion of collagen [19]. Inhibitors of MMP are down-regulated by IL-1β which also increases production of cyclooxygenase (COX)-2 and prostaglandin E_2 (PGE_2) and this further increases production of proteinases and modulates leukocyte trafficking [20]. Murine

models show that mechanisms regulating cervical ripening might differ in preterm and term birth, and with underlying cause of labour: preterm birth subsequent to progesterone withdrawal in the absence of infection was comparable to term cervical ripening but preterm ripening in response to infection (using lipopolysaccharide (LPS)) was associated with a robust pro-inflammatory response including neutrophil influx and activation of the prostaglandin synthesis cascade [21].

1.2.2 Membrane rupture

Membrane rupture is characterised by extracellular matrix remodeling: fibronectin is degraded by MMPs and other proteases [22, 23] facilitating separation of the previously fused chorioamniontic membranes and deciduas. Fetal fibronectin present in vaginal secretions during early pregnancy can be used as a marker for PTB [24]. Similar to the cervix, production of MMPs is increased in response to the augmented production of pro-inflammatory cytokines such as IL-8, IL-6, TNFα and IL-1β [25-27]. Collagen, particularly collagen I and collagen IV are responsible for the strength of the fetal membranes. Specific MMPs modulated by tissue inhibitors of matrix metalloproteinases (TIMPs) control collagen degradations, thus the ratio of MMPs to TIMPs can be used as an indicator of collagen degradation [28]. It has also been noted that inflammatory cytokines can weaken the fetal membranes. This is achieved by production of reactive oxygen species (ROS), which has been associated with the induction of prostaglandins and MMP-9 [29]. TIMP-1, which modulates the activity of MMP-9, is decreased with labour and premature rupture of the membranes (PROM) [30, 31].

1.2.3 Myometrial contractions

A similar pattern of cytokine activity is observed in the myometrium: increased levels of IL-6, IL-1β, TNFα and IL-8 result in increased production of COX-2 and PGE_2 [32, 33]. IL-6 in particular promotes increased production of oxytocin and expression of the oxytocin receptor by myometrial cells [34]. Contractions of the uterine smooth muscle are primarily facilitated by intracellular calcium and phosphorylation of myosin light chains [35]. Oxytocin and prostaglandins are key in this process, whereby activation of uterine contractility either directly or indirectly by oxytocin via PGF_2 results in the activation of phospholipase C, and subsequently the activation of calcium channels, resulting in increased intracellular calcium levels [36, 37]. Additionally oxytocin facilitates gap junction formation between cells by increasing

the expression of connexion-43, a gap junction protein, resulting in enhanced cell-cell coupling, facilitating the synchronisation of the uterine contractions [38].

1.3 Preterm birth

Preterm Birth (PTB) as defined by the World Health Organisation (WHO) is "all births prior to 37 completed weeks of gestation or fewer than 259 days since the first day of a woman's last menstrual period" and can be further sub-divided based on gestational age [39, 40] (Table 1-1).

Table 1-1 Preterm deliveries subdivided by gestation.

PTB Sub-Category	Gestation (Weeks)	Occurrence (%)
Extremely Preterm	< 28	5%
Very Preterm	28 – < 32	12%
Moderate to late Preterm	32 – < 37	83%

Adapted from March of Dimes [39], Hamilton & Tower [40] and Goldenberg et al [41]. PTB, preterm birth.

PTB is the leading cause of perinatal morbidity and mortality in the Western world; 75% of perinatal mortality and nearly 50% of long-term neurological morbidity are associated with PTB [41]. Preterm infants are prone to higher rates of complications of the gastrointestinal, renal and respiratory systems [42]. Increasing PTB rates over the past 30 years in industrialised countries reflects increased indicated preterm births for reasons such as preeclampsia and intrauterine growth restriction (IUGR), and the fallout of multiple gestations associated with assisted reproductive technologies (ART). Other risk factors for preterm birth include ethnicity, previous preterm birth, extremes of maternal body mass index (BMI), genetic variation, infection including periodontal disease, and adverse behaviours [41] (Figure 1.3). Annual estimates currently suggest that 12.9 million infants worldwide (about 10%) are born prematurely [43]. The prevention and management of PTB is one of the major challenges of contemporary obstetrics and gynaecology. PTB also has a large economic impact. The cost of preterm birth to the UK is £939 million per year, with

the average the cost of a preterm baby is more than one and half times that of a full term neonate [44]. Recent decades have seen improvements in the survival rates of preterm infants due to advances in neonatal care and the increased use of antenatal steroids but little change in the ability to prevent PTB [45].

There are three obstetric precursors leading to PTB. The primary precursor is spontaneous preterm labour (sPTL) with intact membranes, defined as uterine contractions and cervical changes before 37 weeks gestation, which accounts for 45% of preterm births. Accounting for 25% of preterm births is preterm premature rupture of the membranes (PPROM), characterised by spontaneous membrane rupture before 37 weeks gestation [41]. Both sPTL and PPROM are the outcome of maternal and/or fetal inflammation that can have a systemic component or remain localised to the reproductive tract. The final precursor, accounting for 30% of preterm births, is when the infant is delivery by caesarean section before 37 weeks for reasons including preeclampsia, placental abruption and growth restrictions. This is referred to as delivered for maternal or fetal indications [40].

Intrauterine infection is a common mechanism of preterm labour accounting for 25 – 40% of all sPTL cases, although limitations in microbiological culture techniques might make this a conservative estimate [46]. Several routes have been suggested for microbial invasion into the intrauterine cavity. The most common route is the ascension of microorganisms from the vagina through the cervix and into the uterus. The infection can ultimately gain access to the amniotic fluid thereby exposing the fetus to infection [47]. Other routes of infection include hematogenous spread through the placenta of non-genital tract infections such as those from the oral cavity [48]. Irrespective of the route, microbial invasion results in infection at various sites including the placenta, the fetal membranes, the amniotic fluid, the umbilical cord, and the fetus itself. Many microorganisms have been associated with PROM, sPTL and PTB. Microorganisms resulting in intrauterine infection include *Escherichia coli* [49], *Ureaplasma urealyticum* [50], *Streptococcus agalactiae* [51], *Chlamydia trachomatis* [51] and *Candida albicans* [52] among many others. For example, genital mycoplasmas have been associated with a higher maternal white blood count and C-reactive protein (CRP), and more leukocytes in the amniotic fluid [53].

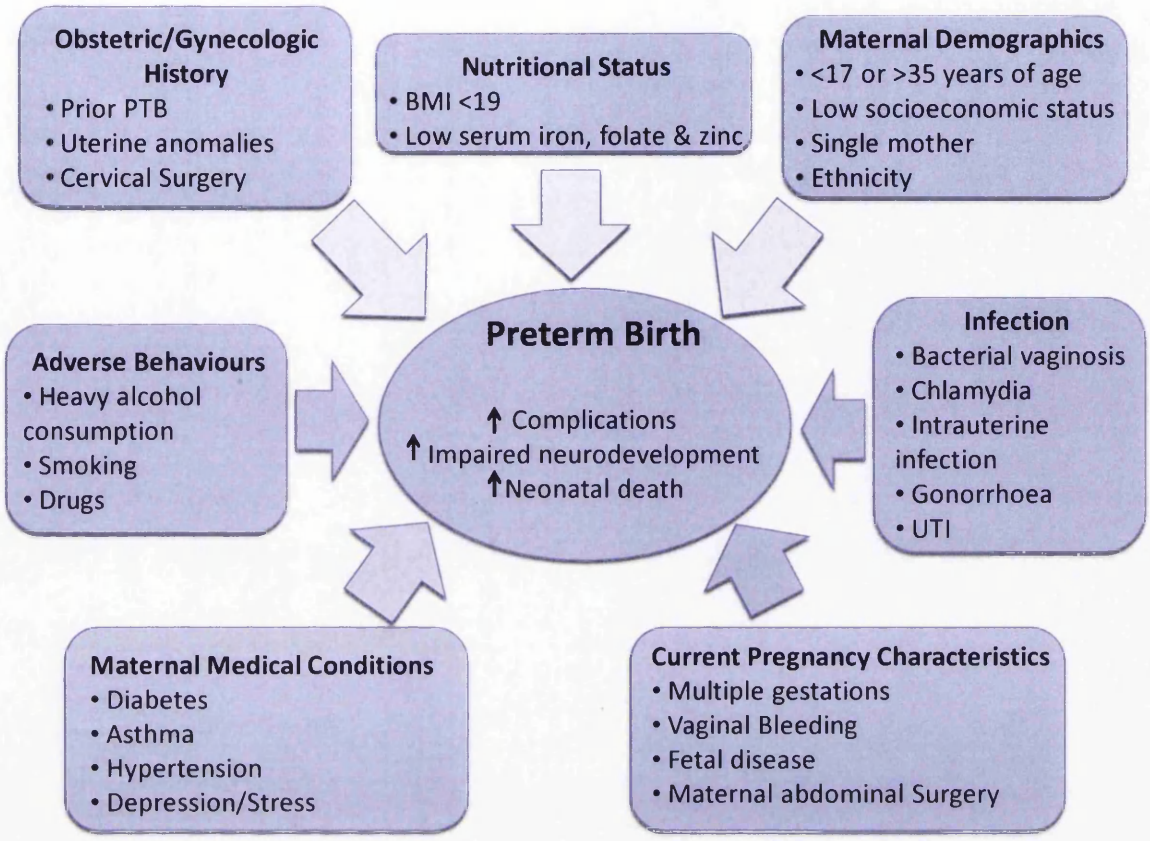

Figure 1.3 Risk factors for preterm birth.

Adapted from Goldenberg et al [41]. BMI, body mass index; PTB, preterm birth; UTI, urinary tract infection.

1.4 Inflammation and cytokines in healthy and adverse pregnancy outcomes

A wealth of evidence indicates that labour is an inflammatory process [10]. Inflammation is a primary response mechanism resulting from the biological activity of cytokines and other mediators produced in response to harmful stimuli of both infectious and non-infectious origins. Cytokines are small (~ 5 – 20 kDa) proteins secreted by many cells types that function as extracellular signalling molecules to facilitate communication between various cells of the body, a response induced by binding to specific receptors expressed by target cells. Cytokines including IL-6, IL-8 and TNFα are key immunological and inflammatory mediators which can act in an autocrine, paracrine or endocrine manner [54, 55].

The pathophysiological mechanisms underlying preterm birth are largely unknown but might relate to premature activation of the normal labour process or the response to an insult. Proposed triggers of preterm birth include: uterine over-distension [56], stress [3, 57, 58], infection and inflammation [53, 59-61], and other immunologically-mediated processes e.g allergy/hypersensitivity [62-64]. Irrespective of the triggering event, local and systemic inflammation tends to be a feature of preterm labour and delivery. Understanding the inflammatory pathways that contribute to the initiation and maintenance of preterm (and term) labour could be used to develop strategies to: (i) identify those women most at risk of preterm labour and birth, and (ii) prevent preterm birth. Whilst infection-associated PTB is the focus of this thesis, clinical studies have revealed an association between infection and other pregnancy complications such as preeclampsia and IUGR [65, 66].

A burgeoning body of literature implicates numerous cytokines in the normal physiological processes of pregnancy (e.g. implantation, placental function, parturition) and in the inflammatory response during infection associated preterm labour [10, 67-72]. The analysis of changes in candidate cytokines has proved worthwhile in identifying potential underlying mechanisms of PTB. IL-1, the first cytokine implicated is up-regulated in the human decidua in response to microbial products (e.g. lipopolysaccharide (LPS)), resulting in the production of prostaglandins by the amnion and decidua [73-75]. Mid-trimester amniotic fluid levels of IL-1β are associated positively with preterm delivery [76], and IL-6 concentrations in amniotic fluid are considered a marker for infection [77, 78]. Other cytokines including IL-10 [79], TNFα [80], granulocyte colony stimulating factor (G-CSF) [81] and IL-18 [82] among others have been linked to infection associated preterm labour. There are now a number of groups developing strategies to identify cytokine and other protein signatures that might rapidly identify those women most at risk of delivering prematurely especially in the setting of intrauterine infection. Mass spectrometry-based proteomic profiling of amniotic fluid from women with PTL or PROM found an inverse relationship between time to delivery and severity of intra-amniotic inflammation as determined by measurement of 4 biomarkers (neutrophil defensins-1 and −2 and calgranulins A and C). While even minimal inflammation was also associated with preterm birth, the extent of intra-amniotic inflammation correlated with negative outcomes for the neonate [83]. The potential of proteomic profiling of cervico-vaginal fluid also has been evaluated in a non-

human primate model of intra-amniotic infection. Differential expression of proteins was observed in control versus infected samples and this might offer a relatively non-invasive strategy for detection of infection via signatures created by specific biomarkers [84]. Numerous novel predictors of intra-amniotic infection; including immune modulators and acute-phase reactants, have been identified using a comprehensive proteome analysis of vaginal fluid from a cohort of pregnant women in spontaneous preterm labour. In the intraamniotic infection group, an increase in amniotic fluid proteins (e.g. vitamin D binding proteins and insulin-like growth factor binding protein-1) and a decrease of extracellular matrix-signalling proteins (e.g. fatty acid binding protein) were noted when compared to women in preterm labour who lacked evidence of infection. This suggests that the mechanisms of preterm birth may differ in the presence of infection [85].

The cytokines of interest are produced by cells normally present in the gestation-associated tissues such as trophoblast cells (syncytiotrophoblast and cytotrophoblast) and macrophages, and by leukocytes that infiltrate these tissues in response to inflammatory stimuli. An accumulation of leukocytes evident upon histological analysis of the placental membranes, so-called chorioamnionitis, occurs in around one-third of preterm deliveries. Chorioamnionitis was once considered a hallmark of infection but it was soon recognised to occur in the absence of any detectable signs of infection. Now, two types of chorioamnionitis have been classified: acute chorioamnionitis (ACA) associated with infection, and chronic chorioamnionitis (CCA) of immunologic origin related to maternal anti-fetal allograft rejection and graft-versus-host disease in the placenta [86]. The local cytokine profile differs with the type of chorioamnionitis: IL-6 is the prototypic cytokine elevated in amniotic fluid in ACA whereas for CCA amniotic fluid levels of CXCL10 (IP-10 – interferon-inducible protein-10) are increased and there is elevated gene expression of not only CCL10 but also CXCL9 (MIG – monokine induced by interferon gamma) and CXCL11 (I-TAC - interferon-inducible T-cell alpha chemoattractant) [87].

While chorioamnionitis is generally associated with adverse pregnancy outcomes, around 9 - 20% of term deliveries have evidence of histologic chorioamnionitis and the duration of labour might impact on the occurrence of this [86, 88]. Notably chorioamnionitis is also increased in spontaneous versus induced preterm birth [87]. Preterm chorioamnionitis is accompanied by villitis in around 40% of cases [87].

Placental villous macrophages (Hofbauer cells) in particular increase when there is evidence of chorioamnionitis. It has been suggested that fibroblast production of MCP-1 in response to bacterial products such as LPS or inflammatory cytokines such as IL-1β or TNFα might drive the accumulation of macrophages within placental villi in this setting [89].

1.5 The innate immune response and inflammation: pattern recognition receptors and cytokine production

Changes in cytokine production at the maternal-fetal interface are a feature of both term and preterm labour. This has generated much interest in the mechanisms of cytokine production in the placenta and attached membranes; and whether such changes precede labour or are simply a consequence of it. Signalling pathways of the innate immune system which produce a defined cytokine output in response to microbial stimuli have been postulated as central to this. Several studies have found a link between the treatment of various gestation-associated tissues with microbial stimuli and cytokine outputs [45, 59, 90-92]. These studies have shown that microbial products such as LPS can trigger the production of key molecular events ultimately leading to the production of relevant cytokines such as IL-1β, IL-6 and TNF-α, the roles of which have been noted above.

The innate immune system uses evolutionary conserved germline encoded receptors, termed pattern recognition receptors (PRRs), to recognise and respond to a variety of pathogenic and non-pathogenic microorganisms. Identification of PRRs was demonstrated initially by work on the *Drosophila* protein Toll, a protein involved in the development of dorsoventral polarity during embryonic growth. However, Hoffman and colleagues demonstrated that Toll was also required for an effective immune response to *Aspergillus fumigates* in the fly [93]. This realisation inspired a search for mammalian homologues of Toll, which lead to the discovery initially of Toll-like receptor 4 (TLR4), and consequently the remainder of the Toll-like receptor family (TLRs) [94]. As increasing research attention has been placed on the mechanisms of innate immune recognition and signalling, other pattern recognition families have been discovered. These include; RIG-I (retinoic acid-inducible gene I)-like receptors (RLRs) [95], NOD (nucleotide oligomerisation domain)-like receptors (NLRs) [96], C-type lectin receptors (CLRs) [97] and Cytosolic DNA sensors (CDS) [98]. Each family of PRRs has numerous members (Table 1-2).

PRRs detect conserved molecular patterns called pathogen associated molecular patterns (PAMPs) on a wide range of pathogens. Detection of a PAMP by its specific PRR, activates intracellular signalling, leading to cytokine gene expression and eventual activation of inflammatory and antimicrobial responses [99]. Examples of PAMPs include LPS, a cell wall component of Gram-negative bacteria, and peptidoglycan (PGN), a component of Gram-positive bacteria cell walls [100]. PRRs have also been implicated in the recognition of endogenous danger signals by the host, referred to as both damage associated molecular patterns (DAMPs) and alaramins [101]. However, no consistent terminology has been adopted, with some using these terms interchangeably, while others consider DAMPs as an umbrella term for both exogenous PAMPs and endogenous alarmins [102].

Table 1-2 Pattern recognition receptors and their ligands.

PRR Name	Group	Ligand / PAMP	Localization
TLR1	TLR	Triacyl Lipopeptides	Plasma Membrane
TLR2	TLR	Peptidoglycan (PGN), Lipopeptides, Glycolipids, B-glucan	Plasma Membrane
TLR3	TLR	Viral double-stranded RNA	Endosome
TLR4	TLR	Bacterial lipopolysaccharide (LPS)	Plasma Membrane
TLR5	TLR	Flagellin	Plasma Membrane
TLR6	TLR	Diacyl Lipopeptides	Plasma Membrane
TLR7	TLR	Single-stranded RNA of viral and bacterial origin	Endosome
TLR8	TLR	Single-stranded RNA of viral and bacterial origin	Endosome
TLR9	TLR	CpG motifs common in bacterial and viral DNA	Endosome
TLR10	TLR	Unknown	Plasma Membrane
RIG-1	RLR	Short dsRNA	Cytoplasm
MDA5	RLR	Long dsRNA	Cytoplasm
NOD1	NLR	Distinct PGN Motifs e.g. iE-DAP	Cytoplasm
NOD2	NLR	Distinct PGN Motifs e.g. MDP	Cytoplasm
NLRP3	NLR	Adenosine-5'-triphosphate (ATP), Monosodium Urate (MSU), Nigericin	Cyoplasm
Dectin-1	CLR	B-glucan	Plasma Membrane
Dectin-2	CLR	High-mannose structures	Plasma Membrane
MINCLE	CLR	α-mannose, trehalose-6'6'-dimycolate (TDM), SAP130	Plasma Membrane
DAI	CDS	cytosolic dsDNA	Cytoplasm
AIM2	CDS	cytosolic dsDNA	Cytoplasm

Adapted and modified from Takaoka & Akira [103]. ATP, adenosine-5'-triphosphate; CDS, cytosolic DNA sensor; CLR, C-type lectin receptor; CpG, "—C—phosphate—G—"; dsDNA, double stranded deoxyribonucleic acid; dsRNA, double stranded ribonucleic acid; iE-DAP, γ-D-Glu-mDAP; LPS, lipopolysaccharide; MDA5, melanoma differentiation-associated protein 5; MSU, monosodium urate; NOD, nucleotide-binding oligomerisation domain; NLR, NOD receptor; NLRP, NOD-like receptor containing a PYD domain; PAMP, pathogen associated molecular pattern; PGN, peptidoglycan; PRR, pattern recognition receptor; RIG-I, retinoic acid-inducible gene 1; RLR, RIG-I like receptor; SAP, splicesome associated protein; TDM, trehalose-6'6'-dimycolate; TLR, toll-like receptor.

1.5.1 Toll-like receptors

The first characterised mammalian TLR was TLR4, and a further 12 mammalian TLRs have since been identified [104]. Only TLRs 1 – 10 are expressed and functional in humans. TLRs 1-9 are functional in both human and mice, however TLR10 is likely non-functional in mice due to substitution of the C-terminal half of the mouse TLR10 gene with a non-productive sequence. In contrast, a stop codon in the human TLR11 gene results in the gene not being expressed, while mouse TLR11 is functional and has a role in urogenital tract infections in particular [105]. Stimulation of TLRs by their specific PAMPs initiates an intracellular signalling cascade involving numerous proteins, most notably MyD88. Activation of these signalling molecules ultimately leads to the activation of NF-κB and other transcription factors to induce the production of inflammatory cytokines.

TLRs are type I transmembrane glycoproteins. The extracellular N-terminal end of all TLRs is composed of leucine-rich repeats (LRRs), which mediate PAMP binding and receptor dimerisation [104]. The LRR domain is composed of 19-25 tandem LRR motifs, of 24-29 amino acids in length [106]. The mechanisms by which TLRs can differentiate between PAMPs or how any one TLR can respond to multiple PAMPs are only now being revealed. It has been suggested that specific ligand binding sites are created in each TLR by specific insertions of the PAMP into the LRR [107]. The conserved cytoplasmic region of each TLR is termed the Toll/IL-1 receptor (TIR) domain due its similarity to the cytoplasmic domains of the interleukin-1 receptor family. The TIR domain varies between 135 and 160 amino acids in length and functions as a binding site for downstream adapter molecules [108].

TLRs can be characterised into two groups based on their cellular location and ligand specificity: plasma membrane localised TLRs - 1, 2, 4, 5, 6 and 10 – that generally recognise lipid based PAMPs; however no ligand has yet been identified for TLR10. TLRs localised to intracellular endosomes - 3, 7, 8 and 9 – that recognise nucleic acid based PAMPs.

The most extensively studied member of the TLR family is TLR4. TLR4 is expressed on various haematopoietic cells including monocytes, macrophages, polymorphonuclear (PMN) cells, dendritic cells, and B cells. It is also expressed by non-haematopoietic cells including epithelial cells and fibroblasts [109]. TLR4

predominantly recognises LPS, a cell wall component of Gram-negative bacteria, composed of O-antigen, lipid A (endotoxin) and an oligosaccharide core [110]. TLR4 recognition of LPS requires formation of a complex with CD14 and MD2 [111]. MD2 binds to the extracellular region of TLR4, enabling it to bind to the lipid A component of LPS. CD14, a glycosyl phosphatidylinositol (GPI)-anchored, high affinity membrane protein, binds LPS in the presence of LPS-binding protein (LBP). LBP exchanges monomers of LPS for other lipids bound in its lipid binding site prior to transferring the LPS monomers to CD14. This enables CD14 to concentrate the LPS which is released from the bacterium in small amounts prior to presenting the LPS to the TLR4-MD2 complex [112]. TLR4 also has been implicated in the detection of fungal PAMPs including glucuronoxylomannan from *Cryptococcus neoformans,* and mannan derived from *Saccharomyces cerevisiae* and *Candida albicans* [113].

TLR2, which has a similar expression profile to TLR4, recognises a variety of PAMPs from both Gram-positive and Gram-negative bacteria including lipoproteins/lipopeptides and peptidoglycan, glycolipids, lipoteichoic acid and non-endobacterial LPS [114]. TLR2 also recognises fungal PAMPs including cell surface phospholipomannan of *Candida albicans,* and *Saccharomyces cerevisiae* derived zymosan [113, 115]. TLR2 forms a heterodimer with its structural relatives, TLR1 or TLR6 [116, 117]. These heterodimers - TLR2/TLR1 and TLR2/TLR6 - can detect subtle variations in the lipid component of lipoproteins: tri-acetylated lipopeptides by TLR2/TLR1 and di-acetylated lipopeptides by TLR2/TLR6 [117, 118]. Recognition of di-acetylated lipopeptides by TLR2/TLR6, is facilitated by the co-receptor CD36, a class II scavenger protein [119]. Expression patterns of TLR1 and TLR6 are similar to that of TLR2, however both are highly expressed on B cells; while TLR2 is not [109]. The regulation of TLR2 expression differs between various cell types depending on their specific function and this is not the case for TLR1 and TLR6 [109].

TLR5 recognises bacterial flagellin, a structural protein which is the major component of flagella of Gram-negative bacteria [120]. Recognition of flagellin by TLR5 is possibly via highly conserved regions in the flagellin protein [121]. A common stop codon in TLR5 is associated with loss of signalling to flagellin and increased susceptibility to pneumonia caused by *Legionella pneumophila* [122]. In addition to the conventional expression of TLR5 by haematopoietic cells, TLR5 is expressed on the basolateral surface of healthy human intestinal epithelium,

suggesting that bacterial recognition by TLR5 only occurs as the bacteria invade across the epithelium [123].

TLR3 is involved in the recognition of double-stranded RNA (dsRNA) from double-stranded viruses, such as reovirus, or that generated during viral replication of single stranded viruses [124, 125]. Multiple haematopoietic, such as dendritic cells, and non-haematopoietic cells, such as epithelial cells, express TLR3 which is located within endosomes. Recognition of dsRNA by TLR3 was first demonstrated in TLR3 deficient mice, which showed susceptibility to mouse cytomegalovirus [126]. The type I interferon (IFN) inducing dsRNA synthetic analog, polyinosine-deoxycytidylic acid (poly I:C) is often used *in vitro* to induce TLR3 activity [124].

TLR7 and TLR8 are structurally highly conserved and recognise uridine or guanosine-rich single stranded RNAs from a variety of viruses, including the influenza virus and human immunodeficiency virus (HIV) [127-129]. Additionally, they can recognise several synthetic imidazoquinoline-like molecules, such as resiquimod, which have potent antiviral activities due to their structural similarity to ribonucleic acids. While TLR7 and TLR8 recognise viral nucleic acid structures [129, 130] their expression within endosomes prohibits, under normal circumstances, interaction with host derived ssRNA.

TLR9 recognises the unmethylated CpG motifs common in single-stranded DNA present in the genomes of many viruses and bacteria [131-133]. DNA viruses shown to induce inflammatory cytokine and type I IFN production via TLR9, include herpes simplex virus-1 (HSV-1), HSV-2 and murine cytomegalovirus (MCM). Two structurally different forms of CpG exist: A-type CpG oligodeoxynucleotides (ODNs) which stimulate plasmacytoid DCs (pDCs) to produce IL-12 and IFNα and B-type CpG ODNs, which induce IL-6, IL-12 and TNFα production by pDCs. B-type CpG ODNs also up-regulate expression of MHC Class II and the costimulatory molecules CD80 and CD86 on B cells and pDCs [131, 134]. TLR9 might also recognise host derived CpGs but these are weak inducers of activation due to the presence of highly methylated cytosine bases.

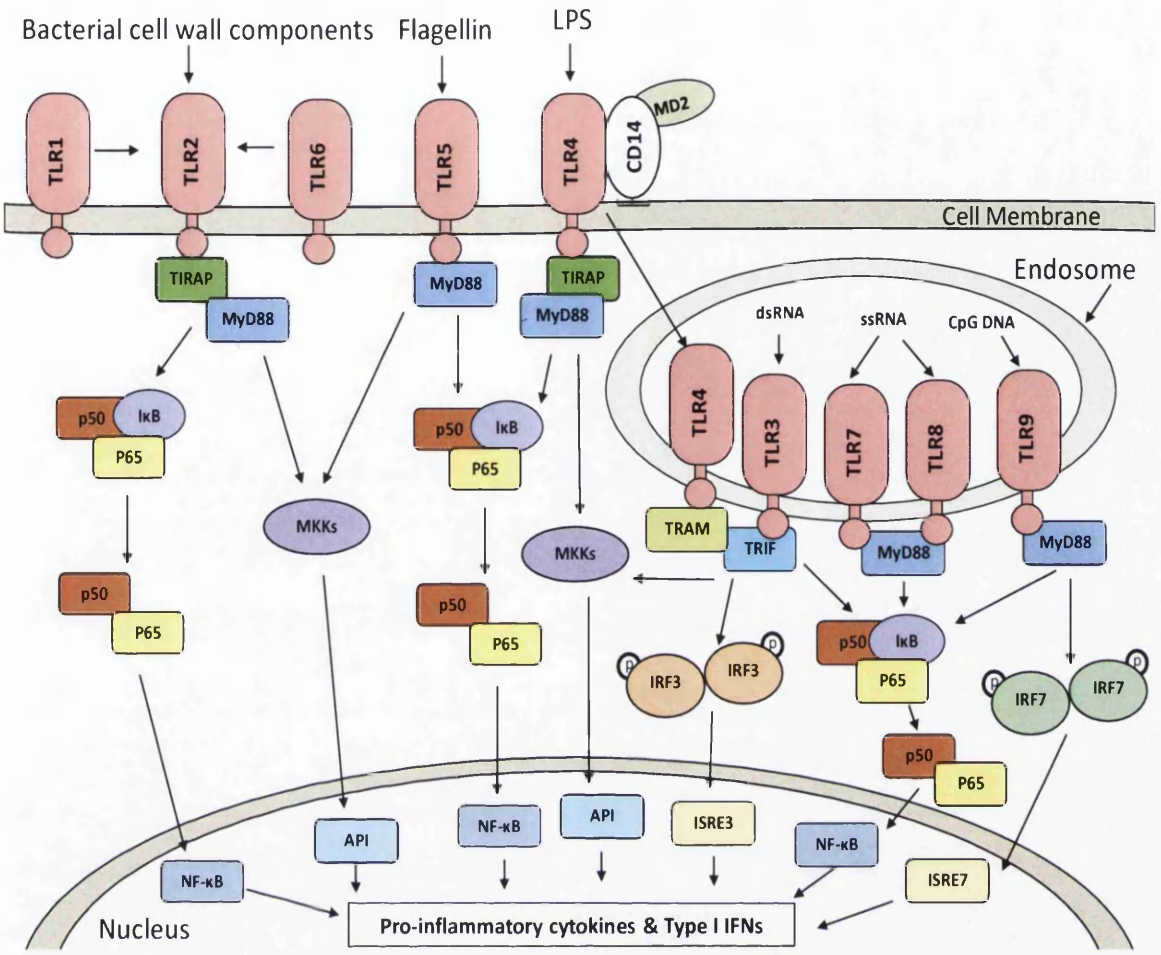

Figure 1.4 Overview of Toll-like receptor (TLR) signalling.

Activation of TLRs by their specific PAMPs (pathogen associated molecular patterns) initiates an intracellular signalling cascade involving numerous adapter proteins (MyD88, TRIF, TRAM, TIRAP), ultimately leading to the activation of several transcription factors (NF-κB, ISRE3, ISRE7, AP-1) to induce the production of inflammatory cytokines. Adapted from O'Neill et al [135], Kawai & Akira [136] and Kopp and Medzhitov [137]. AP1, activator protein 1; CD, cluster of differentiation; CpG, "—C—phosphate—G—"; dsRNA, double stranded ribonucleic acid; IFN, interferon; IκB, inhibitor of kappa B; IRF, interferon regulatory factor; ISRE, interferon stimulated gene factor; LPS, lipopolysaccharide; MD2, lymphocyte antigen 96; MKK, Mitogen-activated protein kinase kinase; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor-kappa B; PGN, peptidoglycan; ssRNA, single stranded ribonucleic acid; TIR, toll/interleukin-1 receptor; TIRAP, TIR domain-containing adapter protein; TLR, toll-like receptor; TRAM, TRIF related adapter molecule; TRIF, TIR-domain-containing adapter-inducing interferon-β.

1.5.1.1 TLRs: recognition and response

Binding of PAMPs leads to the dimerisation of TLRs, which triggers the activation of the TLR mediated signalling pathways and the expression of various genes involved in the immune response. As previously described TLR2 forms a heterodimer with both TLR1 and TLR6, the remaining TLRs form homodimers [138]. TLR signalling originates from the TIR domain of the receptor, which associates with an adapter containing a TIR domain. Various adapters have been characterised including MyD88 (Myeloid differentiation primary response gene 88), TRIF (TIR domain-containing adapter proteins inducing IFNβ), TRAM (TRIF related adapter molecule) and TIRAP (TIR domain-containing adapter protein); each are involved in slightly different signalling pathways, dependent on which TLR is activated [139]. Two main TLR signalling pathways have been described: the MyD88-dependent pathway and the MyD88-independent pathway also known as the TRIF-dependent pathway.

The adapter MyD88, which has a C-terminal TIR domain in addition to an N-terminal death domain, associates with the TIR domain of the TLR. Upon activation of a TLR, MyD88 recruits IRAK-4, a member of the IL-1 receptor associated kinase (IRAK) family, via interactions between the death domains of both molecules. This enables activation of IRAK-1 by IRAK-4 mediated phosphorylation. IRAK-1 then associates with TRAF6 (TNF receptor associated factor 6), resulting in the eventual activation of the IκB kinase (IKK) complex, which consists of IKKα, IKKβ and NEMO/IKKγ. The IKK complex induces the phosphorylation of IκB, resulting in translocation of the nuclear transcription factor NF-κB, from the cytosol to the nucleus. Once in the nucleus NF-κB can induce expression of multiple inflammatory cytokines [140-142]. Alternatively, activation of TRAF6 can lead to the activation of MAP kinases, resulting in AP-1 transcription factor activation. As shown in MyD88-deficient mice, MyD88 is vital for signalling via this pathway. A second adapter, TIRAP/Mal (MyD88-adapter-like) has been shown to be associated with the MyD88-dependent pathway for signalling via TLR2 and TLR4 [143].

Evidence for a My88-independent pathway was first shown in MyD88-deficient macrophages, in which NF-κB activation was not observed but production of inflammatory cytokines was [144]. Further investigation noted that TLR4 stimulation results in IRF3 (interferon regulatory factor) activation, a transcription factor involved in the production of IFNα [145]. Additionally IRF3 was activated in response to

dsRNA or viral infection, via TLR3, suggesting that both TLR3 and TLR4 can utilise a common MyD88-independent pathway [146].

Two main adapters have been identified as involved in the MyD88-independent pathway: TRIF also known as TICAM-1 (TIR domain containing molecule), and TRAM or TICAM-2. TRIF has been associated with MyD88-independent signalling via TLR3, while TRIF and TRAM are associated with MyD88-independent signalling via TLR4 [147-149]. The TIR domain of TRIF is located in the centre of the molecule and the flanking N-terminal and C-terminal regions can both mediate the activation of NF-κB but by two different actions: the N-terminal region associates with TRAF6, while the C-terminal region associates with RIP1 (receptor interacting protein) [150, 151]. Activation of the IFNα promoter is exclusively via the N-terminal region in association with the non-canonical IKKs, TBK1 (TANK-binding kinase) and IKKi/IKKε, which mediate the phosphorylation and nuclear translocation of IRF3, resulting in the induction of IRF-3 dependent IFNβ production.

1.5.1.2 TLRs: negative regulation

Excessive production of inflammatory cytokines in response to PAMPs by TLRs can lead to detrimental outcomes including sepsis and autoimmune disease. Thus negative regulatory mechanisms have developed in response to TLR-mediated signalling. Once such mechanism is the phenomenon known as tolerance, in which a subsequent challenge by a PAMP results in a reduced response. This was first document in relation to LPS; however the exact mechanisms are not yet fully understood. A number of inhibitors of TLR signalling have also been described. One such molecule is IRAK-M. IRAK-M lacks kinase activity and prevents the dissociation of IRAK-1/IRAK4 from MyD88 which in turn prevents the IRAK-1/TRAF6 complex forming [152, 153]. Another inhibitor SOCS1 (suppressor of cytokine synthesis 1) has been shown to directly modulate TLR signalling [154].

1.5.2 Nod-like receptors (NLRs)

NLRs (also known as CATERPILLERs) consist of a large family of 23 human intracellular (cytosolic) PRRs, which recognise both PAMPs and/or DAMPs. Activation via NLRs leads to cytokine production via NF-κB, or the inflammasome (see section 1.4.3.1) [155-157]. NLRs have also been implicated in autophagy, a lysosomal degradation and cell death pathway that follows infection [158]. Structurally, NLRs are characterised by the presence of a trimodular structure: a

central nucleotide binding domain (NBD) flanked by leucine rich repeats (LRRs) at the C-terminal and a protein binding domain - caspase activation and recruitment domain (CARD), baculovirus inhibitor of apoptosis protein repeat (BIR), death effector domain (DED) or pyrin domain (PYD) - at the N-terminal. These N-terminal domains, also termed the effector region, are responsible for the protein-protein interactions needed to activate downstream signal transduction. NLRs can be categorised into subfamilies based on this effector domain including: NLRC (Nod-like receptor containing a CARD domain) and CIITA or NLRA (class II, major histocompatibility complex, transactivator) all contain CARD effector domains, while NLRPs contain a pyrin effector domain, and NAIPs or NLRBs contain three BIR domains [155, 159].

The first identified and most widely studied NLRs are NOD1 and NOD2. These belong to the NLRC subfamily and are highly expressed in monocytes, macrophages and dendritic cells, in addition to other hematopoietic cells and epithelial cells [160]. Both NOD1 and NOD2 recognise peptidoglycan (PGN) an essential building block of Gram-positive bacteria cell walls and to lesser extent Gram-negative bacteria. PGN consists of glycan chains cross-linked via short peptides [161], NOD1 and NOD2 recognise different motifs in this structure: NOD2 recognises the conserved muramyl dipeptide (MDP) motif found in all PGNs [162], whereas NOD1 recognises D-γ-glutamyl-meso-DAP dipeptide (iE-DAP), which is present in all Gram-negative, but only some Gram-positive PGNs [163, 164]. NOD2 can also recognise viral ssRNA and mycobacterial N-glycolylmuramyl dipeptides [165, 166]. Thus both NOD1 and NOD2 are involved in the recognition of a variety of pathogenic bacteria including: *E. coli*, *Chlamydia spp*, *Haemophilus influenza* by NOD1, and *M. tuberculosis* and *Streptococcus pneumonia* by NOD2 [159]. Since the majority of these bacteria replicate outside of the cytoplasm where NOD1 and NOD2 are located, a mechanism by which PGN can cross the cell membrane to activate them is required. Although numerous transport proteins including PepT1, PepT2, and pannexin have been identified to facilitate MDP passage into the cytoplasm, an exact mechanism is not fully understood [167-169].

In general, activation of NOD1 and NOD2 by their respective ligands results in a conformational change allowing interaction with CARD domain containing receptor–interacting serine-threonine kinase 2 (RIP2) in a homophilic CARD-CARD manner. Cellular inhibitors of apoptosis 1 and 2 (cIAP1 and 2) are also involved [170]. RIP2

can then mediate the ubiquitination of NF-κB essential modulator (NEMO)/IKK, subsequently leading to the activation of NF-κB and the production of pro-inflammatory cytokines and antimicrobial peptides [171]. Activation of NOD2 by MDP also can result in the activation of the MAP kinase pathways via the adapter CARD9 [172].

Other NLRs including NLRX1 and NLRC5 have gained interest as regulators of PRR signalling pathways. The mitochondrial protein NLRX1 has the ability to inhibit RIG-I signalling by interacting with mitochondrial antiviral-signalling protein (MAVS) a mitochondrial adapter [173]. NLRX1 can trigger the generation of reactive oxygen species (ROS) thereby amplifying NF-κB and JNK signalling [85]. NLRC5 inhibits NF-κB and IRF mediated signalling by interacting with the NF-κB signalling intermediates IKKα and IKKβ, NLRC5 blocks the phosphorylation of these proteins and thus inhibits NF-κB signalling. Inhibition of IRF driven type I interferon response via RLRs occurs by interaction of NLRC5 with RIG-I and MDA5 [3].

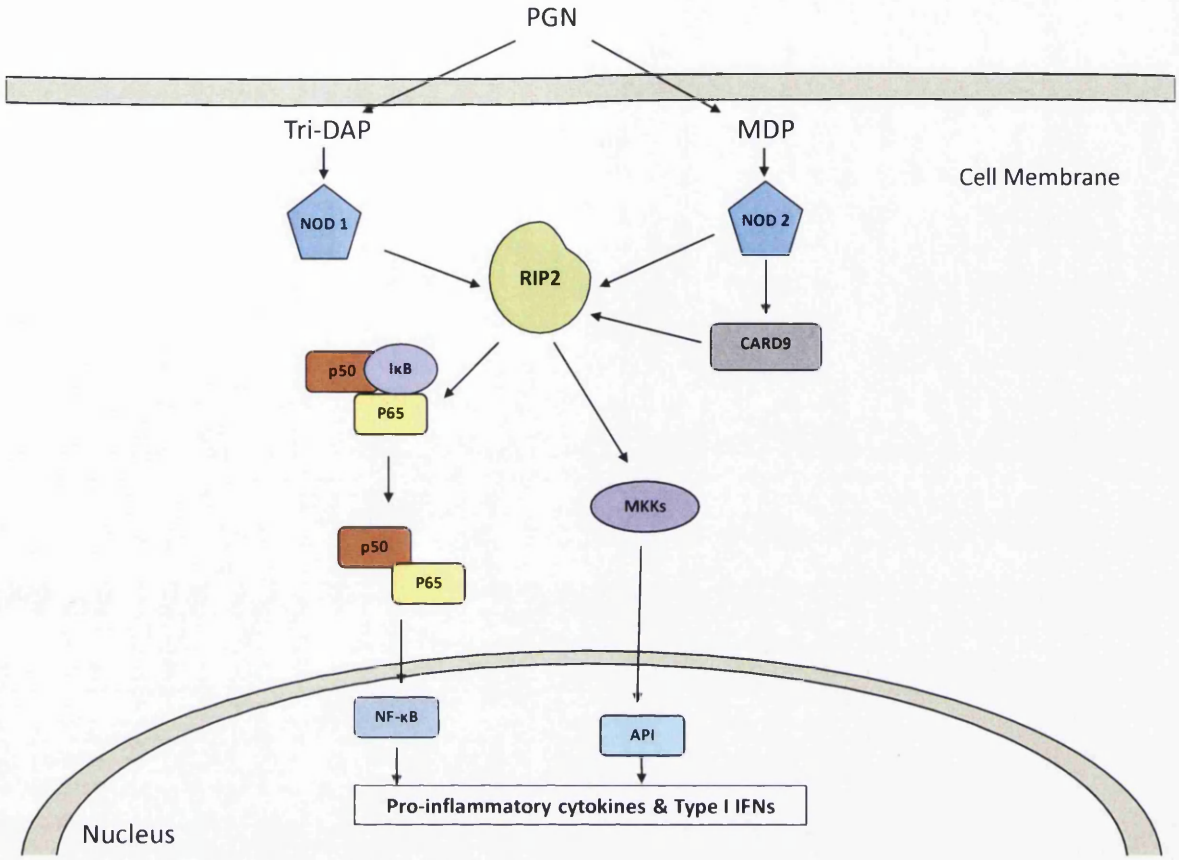

Figure 1.5 Overview of NOD signalling.

Activation of NOD1/NOD2 by their ligands facilitates the interaction with the adapter protein RIP2 and the subsequent activation of transcription factors to induce the production of pro-inflammatory cytokines. Adapted from Shaw et al [157] and Kanneganti et al [155]. AP1, activator protein 1; CARD9, caspase activation and recruitment domain 9; IFN, interferon; IκB, inhibitor of kappa B; MDP, muramyl dipeptide; MKK, mitogen-activated protein kinase kinase; NF-κB, nuclear factor-kappa B; NOD, nucleotide binding oligomerisation domain; PGN, peptidoglycan; RIP2, Receptor-interacting serine/threonine-protein kinase 2; Tri-DAP, L-Ala-gamma-D-Glu-mDAP.

1.5.2.1 The inflammasome

Certain members of the NLR family detect microbial components in the cytosol and trigger the assembly of a multiprotein oligomer termed the inflammasome. This complex supports the autocatalytic cleavage of caspase-1 which enables the processing and secretion of proforms of IL-1β and IL-18. NLRP1, NLRP3, NLRC4 and the adapter apoptosis-associated speck-like protein containing a CARD (ASC) are critical components of the inflammasome but components of emerging interest include NLRP6 [174-176]. While much of the focus has been on the caspase-1

inflammasome, caspase-8 and caspase-11 (in mice) are also associated with an inflammasome-triggered response in a caspase-1-independent manner [177, 178].

The NLRP3 inflammasome (Figure 1.6) is the most extensively studied: it comprises NLRP3, ASC, and caspase-1 [179]. Like other NLRP family members, NLRP3 is composed of a C-terminal LRR domain, a central NOD domain and an N-terminal PYD domain. NLRP3 is expressed in many types of hematopoietic cells, in addition to osteoblasts, skin keratinocytes and transitional epithelium of the urinary tract [180]. A wide variety of pathogens of bacterial, fungal and viral origin can initiate NLRP3 inflammasome formation: *Listeria monocytogenes*, *Staphylococcus aureus*, *Candida albicans, Saccharomyces cerevisiae*, and adenovirus and influenza viruses. A number of host-derived DAMPs indicative of cellular injury, including extracellular ATP and uric acid among others, have been shown to activate the inflammasome [181, 182]. The mechanisms involved in the recognition of these stimuli by NLRP3 are currently not fully understood, but may involve cellular mediators e.g. reactive oxygen species (ROS) relaying signals to NLRP3 [180]. However physical interaction between NLRP3 and the PAMPs/DAMPs has not been ruled out [183]. Following activation, NLRP3 oligomerisation leads to the clustering of PYD domains which can then recruit the CARD containing adapter ASC which by a CARD-CARD interaction can then recruit pro-caspase-1. The clustering of pro-capase-1 results in its autocleavage to the active caspase-1 p10/p20 tetramer enabling the processing of cytokine proforms to yield mature molecules for secretion. Production of these proforms depends on NF-κB driven transcriptional activity resulting from signalling from other PRRs such as NOD2 and TLRs [184].

Less is known about the NLRP1 inflammasome, which is comprised of ASC, caspase-1, caspase-5, and NLRP1 and induced by the NOD2 agonist MDP [179]. NLRP1 is expressed in various haematopoietic cells including: T and B cells, monocytes, dendritic cells and granulocytes, as well as on neurons and non-haematopoietic cells within the testes [180]. The structure of NLRP1 is different to that of NLRP3, as NLRP1 contains a C-terminal CARD domain, allowing NLRP1 to interact directly with pro-caspase-1. However ASC forms part of the inflammasome complex, allowing for the recruitment of caspase-5 providing additional inflammasome activity [179].

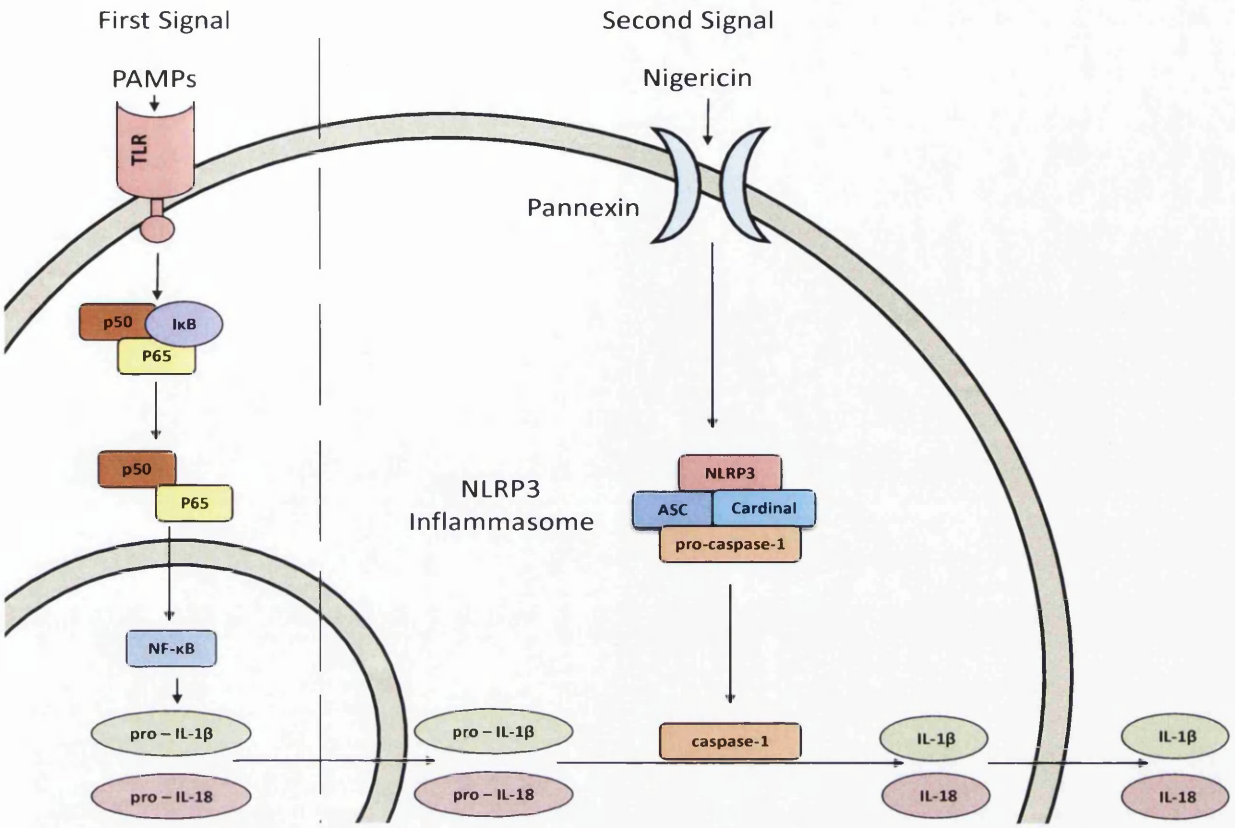

Figure 1.6 Overview of the NLRP3 inflammasome.

Upon TLR activation an inactive pro-IL-1β precursor is produced that must ultimately be cleaved into the bioactive mature IL-1β, a process that is regulated by the inflammasome. A second signal for example the microbial toxin nigericin activates NLRP3 resulting in the autocatalytic cleavage of pro-caspase-1 to active caspase-1, which in turn enables the processing of pro-IL-1β (and pro-IL-18) and secretion of mature IL-1β (and IL-18). Adapted from Schroder et al [184] and Franchi et al [185]. ASC, apoptosis-associated speck-like protein containing a CARD (caspase activation and recruitment domain); IκB, inhibitor of kappa B; IL-1β, interleukin-1β; NF-κB, nuclear factor-kappa B; NLRP, NOD-like receptor containing a PYD domain; PAMP, pathogen associated molecular pattern; TLR, toll-like receptor.

NLRC4 (also known as IPAF) is expressed primarily in lymphoid tissue. Like NOD1 and NOD2 it has a C-terminal LRR domain, a central NOD domain, and an N-terminal CARD domain [186]. Activation of the NLRC4 by microbial flagellin leads to the activation of caspase-1, IL-1β secretion and pyrotosis, a rapid form of cell death [187, 188]. Similar to NLRP1, NLRC4 can interact directly with pro-caspase-1 via its CARD domain. The role of ASC in the NLRC4 inflammasome remains inconclusive: ASC cannot interact with NLRC4 which lacks a PYD domain but a role for ASC in regulation of this inflammasome has been suggested [184].

1.5.3 C-type Lectin receptors (CLRs)

CLRs are a family of receptors that recognise and bind carbohydrates in a calcium-dependent manner. Binding is mediated via a conserved carbohydrate recognition domain (CRD) first identified on circulating mannose binding lectin (MBL). Other than MBL, CLRs are primarily expressed on antigen presenting cells (APCs) such as macrophages and dendritic cells, and are involved particularly in fungal recognition and modulation of the anti-fungal innate immune response. CLR intracellular signalling is activated via immunoreceptor tyrosine-based activation motif (ITAM) present in the cytoplasmic tail of the receptor or on ITAM containing adapters. Examples of such CLRs include;- Dectin-1, Dectin-2, macrophage-inducible C-type lectin (MINCLE), DC-SIGN and the mannose receptor [97].

Mannose binding lectin has two to six clusters of CRDs which facilitate the identification of an attachment to repetitive mannose and fucose residues found on various microorganisms. The fixed orientation of the CRDs requires not only the presence of these residues but their specific spatial arrangement to initiate interaction between MBL and the target micro-organism. Recognition of mannose and fucose by MBL leads to the activation of the lectin complement pathway and enhanced polymorphonuclear cell uptake [189-191].

The mannose receptor (MR) has similar recognition strategies to MBL. Primarily expressed by macrophages and dendritic cells, the mannose receptor is a type-1 membrane protein with eight tandemly arranged CRD domains. These allow the recognition of various carbohydrates terminating in L-fucose, D-mannose and N-acetyl glucosamine [192]. In addition to CRDs at the extracellular region, MR has two additional domains, an N-terminal cysteine-rich domain and a fibronectin II domain; these are involved in calcium-independent binding to sulphated sugars and collagen, respectively. Unlike the CRDs, these additional domains are only involved in the recognition of endogenous ligands and not those of microbial origin [192, 193]. The role of the MR in host defence remains a mystery: animal knockout models do not demonstrate an increased susceptibility to pathogens such as *Candida albicans* and mycobacteria which are known to contain MR ligands [194, 195].

The most widely studied CLR is Dectin-1. Dectin-1, also known as C-type lectin domain family 7 member A (CLEC7A), is a small (33kDa) type II glycosylated

transmembrane receptor with an extracellular CRD connected to a cytoplasmic ITAM-like motif by a stalk domain. Two functional isoforms exist - Dectin-1A, and Dectin-1B which lacks the stalk domain, due to alternative splicing [196]. Dectin-1 expression in humans can be found on myeloid cells, including neutrophils, monocytes/macrophages and dendritic cells, however limited expression has been noted on other cell types [196]. Dectin-1 recognises specifically the glucose polymers β-1-3 and/or -1-6-glucans primarily found in the cell walls of fungi, including *Candida albicans* and *Saccharomyces cerevisiae,* but which can also be found in the cell walls of some plants [197]. Upon binding of the appropriate PAMP, phosphorylation of Dectin-1 occurs by a non-receptor tyrosine kinase, Src, via interaction with its ITAM motif. This leads to the activation of another kinase, Syk (spleen tyrosine kinase), which induces the activation of the CARD9-Bcl10-Malt1 adapter complex leading ultimately to the activation of NF-κB and the production of pro-inflammatory cytokines [198]. In addition, activation of Dectin-1 mediates the production of reactive oxygen species (ROS), which can trigger the NLRP3 inflammasome and further modulation of cytokine expression occurs via the NFAT (nuclear factor of activated T-cells) pathway [199, 200]. Attenuation of the Dectin-1 mediated pro-inflammatory response has been linked to internalisation of the receptor [201]. It has been shown that signalling via Dectin-1 and TLR2/TLR6 combined enhances the response triggered by each receptor alone [202].

Similarly to Dectin-1, Dectin-2 which can be found on myeloid cells, has a role in antifungal immunity, particularly in hyphal recognition by detecting high mannose structures [97]. However unlike Dectin-1, Dectin-2 must associate with an Fc receptor γ-chain (FcRγ), leading to of the CARD9 complex and NF-κB activation [203]. In response to *Candida albicans*, Dectin-2 induces the production of TNFα, and has been implicated in Th17 inducing activity [204].

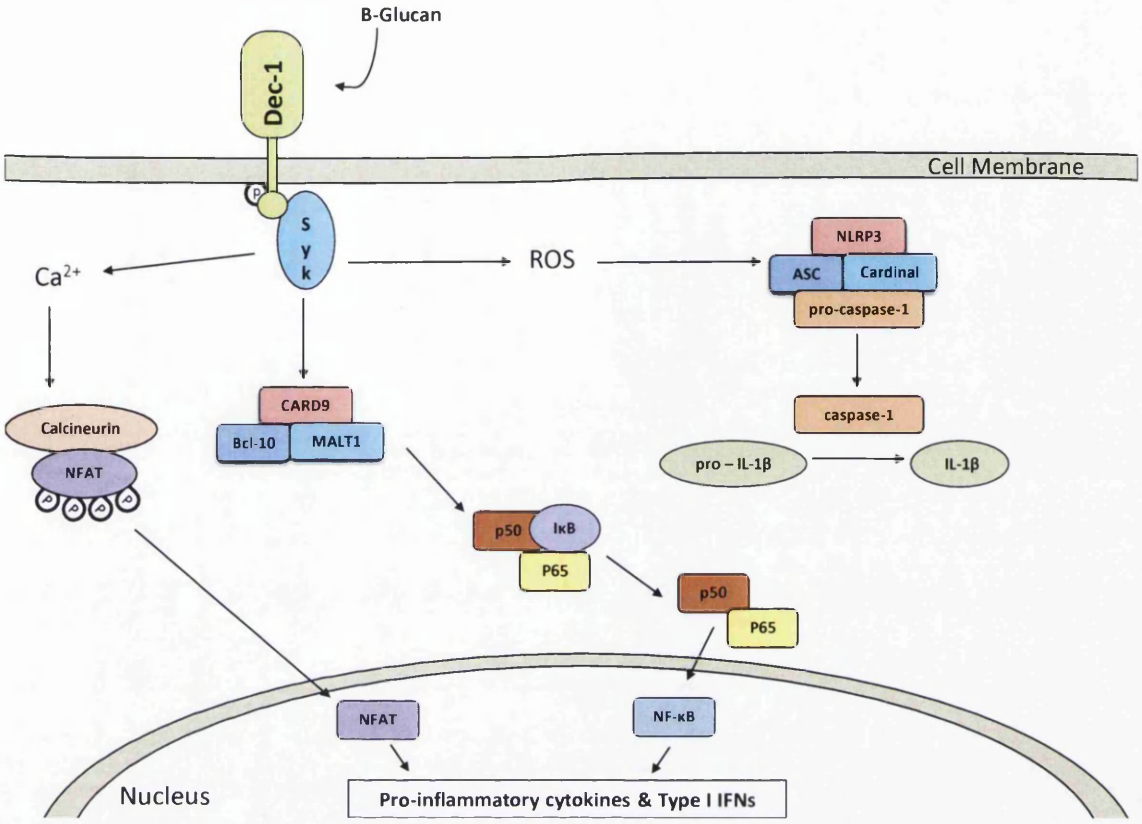

Figure 1.7 Overview of Dectin-1 signalling.

Activation of Dectin-1 by β-glucans initiates several intercellular signalling pathways via the adapter protein syk to induce cytokine production. Similar pathways are activated by Dectin-2 and MINCLE. Adapted from Taylor et al [205], Brown et al [206] and Reid et al [207]. ASC, apoptosis-associated speck-like protein containing a CARD; Bcl10, B cell lymphoma/leukemia 10; CARD9, caspase activation and recruitment domain 9; IκB, inhibitor of kappa B; IL-1β, interleukin-1β; MALT1, mucosa associated lymphoid tissue lymphoma translocation gene 1; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor-kappa B; NLRP, NOD-like receptor containing a PYD domain; ROS, reactive oxygen species; Syk, spleen tyrosine kinase.

MINCLE which is structurally similar to Dectin-2 recognises numerous exogenous PAMPs and endogenous DAMPs, including those from mycobacteria, *C. albicans* and necrotic cells [208, 209]. The identification of necrotic and damaged cells is mediated via recognition of splicesome-associated protein 130 (SAP130), secreted by these cells. MINCLE was the first known example of a CLR that can interact with PAMPs and DAMPs. MINCLE and another CLR, Galectin-3, have a role in anti-*Candida* defence, by recognition of α-mannose [209]. MINCLE also recognises the immunostimulatory component of *Mycobacterium tuberculosis* - trehalose-6'6'-dimycolate better known as cord factor [210].

DC-SIGN expressed primarily on monocyte-derived dendritic cells, is a type II transmembrane receptor with only one C-type lectin domain and has been implicated in the recognition and uptake of *Candida albicans* by detecting high mannose structures [211]. DC-SIGN, also interacts with *Leishmania* but has gained increasing interest because of its involvement in the recognition of several viruses including human immunodeficiency virus (HIV) [212-214].

1.5.4 RIG-I like Receptors (RLRs)

The cytoplasmic RNA helicases that comprise the RIG-I like family of receptors play a major role in host anti-viral defence. RLRs include the well characterised RIG-1 (retinoic acid-inducible gene I), MDA5 (melanoma differentiation associated factor 5) and LGP2 (laboratory of genetics and physiology 2). These are able to detect a variety of viral RNA ligands present in the cytoplasm and triggering the activation of transcription factors resulting in the production of type I IFNs in addition to expression of other anti-viral genes [215]. Viral recognition also occurs via TLR3. It seems that RIG-1 and MDA5 play a greater role in viral recognition by fibroblasts, macrophages and myeloid dendritic cells whereas TLR3 plays a more important role in viral recognition by plasmacytoid dendritic cells (pDCs) [216].

Both RIG-1 and MDA5 share a number of structural similarities. They contain a central DExD/H box RNA helicase domain flanked by an N-terminal of tandem CARD domains and a C-terminal domain (CTD). In the case of RIG-1 the CTD also contains a repressor domain (RD), involved in autoregulation, which is not present in the CTD of MDA5. Despite their similar structures, RIG-1 and MDA5 are able to detect distinct viral species: RIG-1 is involved in the recognition of Paramyxoviridae, Filoviridae and Rhabdoviridae among others, whereas MDA5 is important in the recognition of Picornaviruses [217]. More is known about RLR signalling in regard to RIG-1 however it's believed that both RIG-1 and MDA5 share a common signalling pathway, involving the adapter ISP-1/MAVS (mitochondrial antiviral signalling protein) [218]. Prior to recognition of RNA by the RD or CTD region, RIG-1 is inactive in a "closed" conformation, where the CARD domain is bound to the RD. Activation of RIG-1 results in a conformational change whereby CARD is released from the RD, allowing the CARD domain to interact with the adapter ISP-1 [217]. ISP-1 can then initiate two distinct signalling routes resulting in the activation of various transcription factors including NF-κB which induces the production of pro-inflammatory cytokines, and IRF3 and IRF7 which are responsible for the

expression of type 1 IFNs [218]. Unlike RIG-1, MDA5 does not contain a RD to regulate its activation and when expressed ectopically signalling occurs in the absence of RNA recognition [219]. Unlike RIG-I and MDA5, LGP2 lacks the N-terminal CARD domain, consisting only of the RNA helicase domain and the C-terminal domain containing an RD. It has been suggested that LGP2 is involved in the negative regulation of RIG-1 and possibly MDA5 [217]. This negative feedback is thought to take place on many levels including acting as a competitor for dsRNA, interaction with ISP-1, and maybe direct binding to RIG-1 via RD interactions [219].

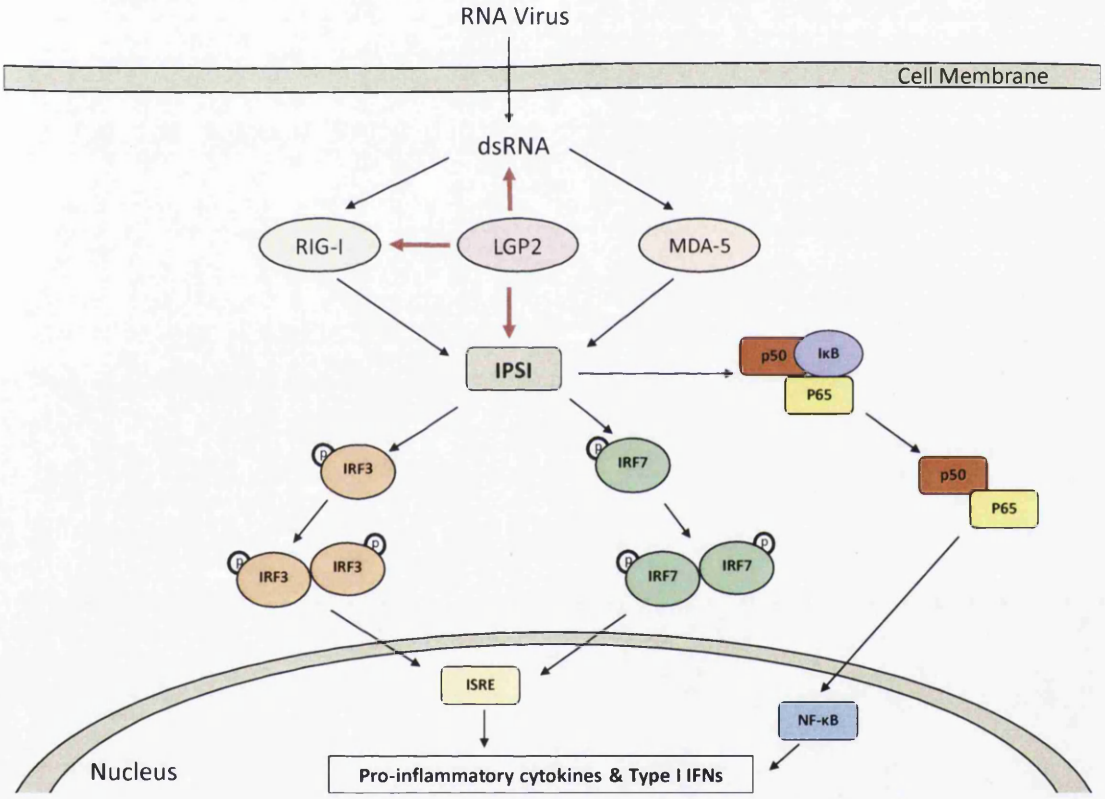

Figure 1.8 Overview of RLR signalling.

Activation of RIG-I/MDA-5 by dsRNA triggers a signal cascade via the adapter ISP-I resulting in the activation of several transcription factors including ISRE and NF-κB to induce the production of pro-inflammatory cytokines and type I interferons. A third RLR, LGP2 is thought to act as a negative regulator of this system. Adapted from Creagh et al [220], Yoneyama et al [95] and Loo et al [217]. dsRNA, double stranded ribonucleic acid; IFN, interferon; IκB, inhibitor of kappa B; IPS-I, mitochondrial antiviral signalling protein (MAVS); IRF, interferon regulatory factor; ISRE, interferon stimulated gene factor; LGP2, laboratory of genetics and physiology 2; MDA5, melanoma differentiation-associated protein 5; NF-κB, nuclear factor-kappa B; RIG-I, retinoic acid-inducible gene 1; RNA, ribonucleic acid; RLR, RIG-I like receptor.

1.5.5 Other pattern recognition receptors

Other groups of pattern recognition receptors have also been described, including cytosolic DNA sensors (CDS) and scavenger receptors (SR).

DNA-dependent activator of IFN-regulatory factors (DAI, also known as DLM-1 and ZBP1) was the first CDS to be described. It was first identified in murine macrophages and murine tumour stromal cells. DAI is up-regulated by exposure to cytosolic DNA or IFNγ suggesting that DAI functions as a DNA sensor [221, 222]. A human homologue containing two N-terminal Zα domains and a C-terminal domain has since been described but it is of unknown function [223]. Activation of DAI by either microbial or host derived DNA, results in the activation of both the NF-κB pathway and the IRF pathway [224]. Additional CDS including LRRFIPI, IFI16, DDX41, cGAS and AIM2 have now been described [225]. RIG-1, a RLR family member has also been characterised unexpectedly as a CDS. However RIG-1, unlike other CDSs does not recognise cytosolic DNA directly but rather a dsRNA intermediate produced by RNA polymerase III mediated transcription [226].

Two pathways that mediate the response to cytosolic DNA have been described; activation of STING dependent signalling and activation of caspase-1 [225]. The primary pathway induced by cytosolic DNA is the production of type I IFNs and IFN stimulated genes via the transcription factor IRF3. A key upstream molecule of this is STING (stimulator of IFN genes). STING which is located on the endoplasmic reticulum, acts as a platform for the phosphorylation of IRF3 via TBK1 [227]. As described above (see 1.4.3.1) activation of caspase-1 occurs through a multi protein complex called an inflammasome. AIM2 (absent in melanoma 2), in response to cyctosolic DNA, actively promotes the formation of an inflammasome (AIM2 inflammasome), interacting with the adapter ASC, leading to the cleavage of pro-caspase-1 to activate the caspase and formation and secretion of mature forms of IL-1β and IL-18 [228].

Scavenger receptors (SRs) are a group of cell surface transmembrane receptors that are important in the clearance of several pathogens, host modified molecules, and apoptotic cells by endocytotic internalisation. These receptors also play a role in lipid metabolism. Expressed primarily on myeloid cells and some endothelial cells, both their expression and structure are regulated by various cytokines including IL-6 and TNFα [229]. Numerous SRs have been shown to play a role in innate immunity,

including SR-A I, SR-A II, CD36, LOX-1, MARCO, SR-CL I, SR-CL II, SCARF1 and DSR-C1 [230, 231].

1.6 Pattern recognition receptors at the maternal-fetal interface

The discovery of an association between intrauterine infection, inflammation and certain adverse pregnancy outcomes has led to increased interest in the innate immune response at the maternal-fetal interface [232]. The production by gestation-associated tissues of cytokines in response to microbial products has been well documented. Since pattern recognition receptors are a key component of the innate immune response, linking infection by various microorganisms to the production of inflammatory mediators, a role for PRRs at the maternal–fetal interface has been postulated. To date studies have principally focused on the role of TLRs and most recently NLRs, primarily in the placenta and the trophoblast.

1.6.1 Placenta

Expression of transcripts for TLR 1-10 in the term placenta has been demonstrated with all but TLR9 shown to be functional (note that TLR10 function has not been studied due to the lack of an identified ligand) [233]. Changes were observed in both mRNA expression and functional cytokine outputs in response to labour at term. An increase in TNFα in response to LPS and R848 (TLR7/8 ligand) were associated with labour, however a significant difference in expression of transcripts was noted only with TLR2 and TLR5 [233]. TLR2 and TLR4 protein has been localised to term syncytiotrophoblast and intermediate cytotrophoblast cells and both receptors are highly expressed in first trimester placental tissue [90, 234]. First trimester trophoblast cells also express TLR6. TLR6 blocks apoptosis induced by PGN via TLR1 and TLR2 and mediates NF-κB activation and secretion of IL-6 and IL-8 leading to the postulate that TLR6 might regulate the balance of apoptosis and inflammation in response to Gram-positive infection [235].

The NLRs, NOD1 and NOD2 are both expressed in the first trimester placenta where they are localised to the syncytiotrophoblast and cytotrophoblast. In contrast only NOD1 is expressed in term trophoblast cells. This corresponds to the functional outputs of first versus third trimester trophoblast cells; first trimester cells respond to both MDP and iE-DAP, and third trimester cells only respond to iE-DAP [60, 236]. The NOD1 ligand iE-DAP can induce preterm delivery in a murine model. When lower doses that did not induce preterm delivery were used there was heightened

inflammation at the maternal-fetal interface and in the fetus itself [237]. Inflammasome components including caspase-1, the adapter PYCARD and NLR members, NLRP1, NLRP3 and NLRC4 are expressed in first trimester cytotrophoblasts. Moreover in the presence of LPS, expression of these was enhanced and IL-1β secretion induced. When compared to the other NLRs, a 2-fold up-regulation of NLRP3 expression was observed [238].

Immunohistochemical studies have shown that the CLR DC-SIGN is expressed by fetal macrophages (Hofbauer cells) within the chorionic villi of the term placenta [214]. Viral ssRNA also activates cytokine, chemokine, and type I IFN production by primary first trimester trophoblast cells. This ligand also induces apoptosis in trophoblast cells in an IFNγ-dependent fashion [239].

1.6.2 Amnion

Amniotic epithelial cells represent the first line of defence against intra-amniotic infection. Earlier studies were restricted to TLR2 and TLR4 and found that both of these receptors are up-regulated in the amnion from women with chorioamnionitis compared to those without [59], although not all studies support this finding [240]. Transcripts for TLRs 1-10 have been detected in human amniotic epithelial cells. However only TLR2/6, TLR4 and TLR5 have been reported to be functional: activation of TLR2/6 and TLR5 resulted in increased production of IL-6 and IL-8, while activation of TLR4 reduced cell viability via apoptosis [241]. Immunohistochemical studies of human fetal membranes have shown that the expression of TLR4 is greater in the chorion than the amnion, that expression decreases with gestational age but that expression does not differ by anatomic location within the uterus [240].

1.6.3 Decidua

Despite its juxtaposition to the myometrium the expression and function of PRRs within the maternally derived decidua is not studied extensively. Initially transcripts for TLRs 1-6 were detected in term decidual cells but only TLR1, TLR2, TLR4 and TLR6 were shown to be functional via production of IL-8 in response to stimulation with LPS or PGN [242]. Transcripts for TLRs 1-10 have since been detected in both first trimester and term decidual cells [243]. Despite no significant change in mRNA levels between first and third trimester cells, it was observed that expression levels of all TLRs other than TLR3, 7 and 9 were lower at term. In contrast to previous

studies no functional response via production of IL-8 was observed resulting from TLR2 and TLR4 in response to PGN or LPS. However LPS induced IL-6 was observed in addition functional TLR3 by poly(I:C) induced IL-6 and IL-8 [243].

Immunohistochemical studies have demonstrated the expression of NOD1 and NOD2 in first trimester decidualised stroma [244]. Similarly to first trimester trophoblasts, the NLRP1, NLRP3 and NLRC4 inflammasomes are expressed in first trimester decidual stromal cells and, in the presence of LPS, their expression is enhanced and IL-1β secretion induced [238]. DC-SIGN has also been detected on decidual macrophages [245].

1.7 PRRs and adverse pregnancy outcomes

PRRs expressed at the maternal-fetal interface could play an important role in the pathogenesis of infection-associated preterm birth and other adverse pregnancy outcomes [246]. This possibility has been studied mostly with regards to TLRs, especially TLR4. LPS from Gram-negative bacteria has been implicated in infection associated preterm birth and there have been a number of studies exploring the possible role of TLR4 in preterm labour. Functional TLR4 has been implicated in preterm labour triggered by administration of heat killed *E.coli* in mice [92]. Evidence for a role for TLRs in infection-associated preterm birth also comes from genetic studies. A polymorphism (Asp299Gly) known to be associated with impaired TLR4 function and an increased likelihood of Gram-negative sepsis [247] was carried more often by preterm infants than term infants or by mothers delivering preterm than at term [248]. Genetic variation in TLRs is also associated with other adverse obstetric outcomes: *TLR4*, *TLR9* and *TLR1* but not *TLR2* variants are associated with placental malaria (low birth weight and maternal anaemia; no difference in parasite densities) [249]. Other genetic variants are also associated with risk of PTB: maternal genetic variants in extracellular matrix metabolism with risk of PPROM and fetal genetic variation (e.g. IL-6R1) are associated with risk of PTB [250].

While LPS has been used as the model for infection associated PTB for many years it is worth noting that LPS from different species of bacteria might differentially regulate inflammatory responses from gestation-associated tissues such as the amniochorion [251]. Also tissue processing for ex vivo investigations can impact on cytokine measurements. For example, in a comparison of punch biopsies of amnion or choriodecidua versus dual compartment transwells, the punch biopsies typically

made greater amounts of cytokines [252]. Such observations highlight the need to take these factors into account when designing studies and when comparing data from different studies.

In addition to the direct pro-inflammatory effects initiated by exposure to infection, disturbances in the regulation of apoptosis might also be associated with sub-optimal pregnancy outcome [253]. Increased trophoblast apoptosis is seen during the first trimester of pregnancies complicated with IUGR or preeclampsia [81, 254], and elevated trophoblast apoptosis has been observed in preterm births [255, 256]. It has been suggested that the direct or indirect effects of infectious microorganisms upon trophoblast cell survival might depend upon which TLRs are activated with TLR2-mediated events apparently favoring apoptosis [235].

Recently there has been growing interest in how a viral infection might itself cause preterm birth but also how it might increase the risk of pregnancy failure during subsequent or concurrent bacterial infection. In a murine model, intraperitoneal injection of a synthetic TLR3 ligand, poly I:C, caused preterm delivery within 24 hours. This was associated with inflammation in multiple gestation-associated tissues (polymorphonuclear cell infiltrate, necrosis and haemorrhage), infiltration of NK cells and macrophages into the placenta, and placental cytokine (e.g. IL-6) and chemokine (e.g. MCP-1) production that could also be detected systemically. This did not occur in TLR3 knock out animals. The cytokine response could be replicated *in vitro* by polyI:C treatment of primary murine trophoblast and a human trophoblast cell line and involved activation of NF-κB [257]. In contrast, intra-peritoneal injection of murine herpes virus was associated with evidence of inflammation in the placenta and spleen but no adverse pregnancy outcomes. Evidence of viral infection in the placenta and decidua but not the fetus led the investigators to postulate that these tissues act as a barrier to capture virus and prevent infection of the fetus. However, this might not prevent developmental impacts on the fetus. Human primary first trimester trophoblast also can be infected with herpes virus *in vitro* but unlike the response to poly I:C treatment, cytokine and chemokine production tended to be down-regulated. Viral infection but not polyI:C induced increased expression of TLR2 and TLR4 in human trophoblast cells and in the accompanying mouse model, viral infection sensitised for a response – preterm delivery in less than 24hrs in all mice accompanied by 100% fetal death - to intraperitoneal infection of LPS [258,

259]. These observations have highlighted a need for better understanding of the expression and activity of viral detecting PRRs at the materno-fetal interface.

Preeclampsia, a pregnancy specific hypertensive disorder, also is characterised by inflammation. Pathways related to stress, inflammation (including TLR signalling pathways), growth, tissue remodelling, and metabolism are all altered during preeclampsia [260]. The differences might reflect acute inflammation secondary to microbial infection versus chronic inflammation secondary to oxidative stress. Possible involvement of the inflammasome in preeclampsia has been suggested. Uric acid is known to activate the NLRP3 inflammasome: circulating uric acid levels increase prior to clinical manifestations of preeclampsia and levels relate to disease severity. Components of the inflammasome, including ASC, are expressed in first and third trimester trophoblasts and monosodium urate (MSU) up-regulates IL-1β production in an inflammasome-dependent manner [261]. However it must be noted that despite an observed up-regulation of IL-1β in response to MSU this was only slight and experimental design was sub optimal as a priming first signal, such as LPS, prior to MSU stimulation was not used. There is also interest in the potential role of viral PAMPs or related DAMPs from necrotic cells in preeclampsia. Activation via TLR3 or the RLRs RIG-1 and MDA-5 leading to downstream inflammation, anti-angiogenesis and oxidative stress converging on endothelial dysfunction has been postulated [262]. A proposed role for TLR9 has also been implicated in preeclampsia and preterm birth. In complicated pregnancies significantly elevated levels of circulating free fetal DNA is found in the maternal plasma [263]. Since TLR9 recognises hypomethylated microbial DNA, it has been suggested that it may also be able to recognise fetal DNA which itself is hypomethylated [264]. Additionally higher circulating levels of mitochondrial DNA (mtDNA) are observed during pregnancies with preeclampsia. An evolutionary conserved relationship between bacteria and mitochondria may facilitate recognition of mtDNA via TLR9 [265].

1.8 Regulating the inflammatory response at the materno-fetal interface

The resolution of inflammation is essential for immune homeostasis. It has become apparent that there are intracellular stress proteins that have extracellular properties related to the regulation of the innate immune response and inflammation. These so-called RAMPs (resolution-associated molecular patterns [266]) have anti-inflammatory activity or the ability to resolve inflammation and counterbalance the

activity of PAMPs and DAMPs. HSP10, HSP27, αB-crystallin and BiP have been suggested as founding members of this family of molecules [266]. There is keen interest in the potential therapeutic use of these [267].

There are also a number of cytokines well recognised for their anti-inflammatory activity. These include IL-4, IL-10 and IL-13. IL-10 can down-regulate LPS- and LTA (lipoteichi acid)-induced cytokine/chemokine responses by the healthy term placenta [268]. Paradoxically, IL-10 is increased in amniotic fluid from women in term labour and women with intra-amniotic infection at term and preterm, and is also increased in those without infection who delivered preterm rather than term [269]. Elevated IL-10 in these circumstances might represent a compensatory mechanism that has failed. Similarly, an anti-inflammatory cytokine (IL-4, IL-10 and IL-13) bias within the cervix prior to 16 weeks of gestation might identify those women most likely to suffer microbial invasion of the utero-placental unit and then spontaneous preterm labour and delivery [270]. Whether the greater anti-inflammatory milieu permits ascending infection or is a compensatory response to a pro-inflammatory response to existing infection that when no longer controlled tips in favour of pro-inflammatory response and the initiation of labour remains to be determined.

A key component of PRR signalling pathways, essential for the expression of cytokines and other immune response gene is the transcription factor NF-κB. NF-κB pathway intermediates including IKK-α, IKK-β, IKK-γ and IκB-α have been identified in first trimester decidua, while binding activity has been reported in a variety of intrauterine cells and tissues, including first trimester placenta, amnion epithelial WISH cells and nuclear extracts prepared from term amnion, choriodecidua and placenta [271]. The functionality of NF-κB is determined by several heterodimer combinations of various subunits including p50, p52, Rel-A (p65), Rel-B and c-Rel [272]. Several of these subunits have been reported in nuclear extracts from first trimester trophoblast, term cytotrophoblast and term amnion, choriodecidua and placenta [271].

With labour characterised by an increase in pro-inflammatory cytokine production, it might be expected that during pregnancy, NF-κB activity would be supressed. The expression of several pathway intermediates responsible for NF-κB inactivation has been shown to increase in the decidua in response to increased progesterone production [273]. More so in term cytotropholasts the DNA binding activity of NF-κB

is suppressed by the production of glucocorticoids [274]. An important role for NF-κB during labour has also been shown. Following term labour, increased activity of p65 in the amnion, but not choriodecidua has been noted; with binding of p65 to the IκBα gene promoter also increased in response to labour in the amnion [275, 276], with varying degrees of p65 expression in preterm samples [272]. Additionally, activation of NF-κB is critical for the activation of numerous genes associated with labour, including MMPs and COX-2 [277]. In the myometrium, increased expression and DNA binding activity of p65 is observed during labour. However other investigators have been unable to record any changes in p65 activity in amnion or choriodecidua, but this might relate to differences in experimental approaches especially explants models versus primary cultures of isolated cells [278]. Considering its central role in inflammation and labour, directed targeting may have the potential as an effective treatment for preterm labour. However considering the broad role of NF-κB the identification of labour specific components of this pathway is key, in order to minimise toxicity [277].

1.9 Aims and Objectives

The study of PRR-mediated inflammation at the maternal-fetal interface has only just begun. Ultimately, a better understanding of these receptors and the signal transduction cascades they initiate might explain why some pregnancies are complicated by PTL and PROM whereas others are only affected by PROM. Moreover, investigations into the endogenous activators of PRRs might explain how PTL and PROM can occur in the absence of infection (e.g. preeclampsia, multiple gestation, teenage pregnancy, or excessive tobacco and alcohol consumption). These molecules (either the receptors or their signalling molecules) might therefore be excellent targets for therapeutic strategies because they are upstream mediators of the pro-inflammatory cascade that ultimately results in premature labour and preterm birth.

However while this may the eventual aim of this field of study; a greater understanding of PRR expression and activity in term non-laboured tissues is required to provide a baseline comparison for these receptors, in order to determine any potential role they may play in normal term labour but also in preterm labour and other adverse pregnancy outcomes. With this in mind the primary aim of this thesis is to determine the expression and activity of PRRs in term non-laboured gestation-associated tissues. Secondary to this, the work presented here will start to examine

the signalling pathways involved upon PRR activation and to try and identify molecules that may reduce PRR mediated responses in these tissues. With this in mind the objectives are as follows:-

(i) To investigate PRR expression and activity in the term non-laboured placenta, choriodecidua and amnion.(Chapter 3 & 4)

(ii) To examine inflammasome activity in the term non-laboured placenta, choriodecidua and amnion. (Chapter 4 & 5)

(iii) To examine the impact of anti-inflammatory cytokines on PRR mediated responses in the term non-laboured placenta, choriodecidua and amnion. (Chapter 6)

Chapter 2

Materials and Methods

2 Materials and methods

2.1 Samples and recruitment

Informed written consent was obtained from all participants for the collection of peripheral blood and the placenta following delivery in the case of pregnant women. Ethical approval for this study was given by the South West Wales Research Ethics Committee (REC 11/WA/0060, 11/WA/0040 and 04/WMW02/68). Documents (participant information sheet, questionnaires and consent forms) are shown in Chapter 8.1.

2.1.1 Participant groups

The following participant groups were recruited:

1. Term non-laboured women (gestational age > 37 weeks) – During pre-operative assessment at the antenatal day assessment unit at Singleton Hospital, Swansea, healthy pregnant women scheduled for elective caesarean section (ESC) at term were approached. Women undergoing elective section for fetal or maternal anomalies were not recruited therefore samples were typically from women scheduled for section because of breach presentation, cephalo-pelvic disproportion or emergency section at previous delivery. Maternal blood was collected into sodium heparin containing tubes during the pre-operative assessment at the same time as samples for routine clinical care. Following liaison with midwives on the delivery suite, the placenta was collected after the ESC.
2. Healthy Adults – Peripheral blood was collected from healthy adult donors into sodium heparin containing tubes (Vacutainer, Greiner Bio-One) at the Institute of Life Science, Swansea University.

Exclusion Criteria: Women with documented evidence of infection such as HIV or hepatitis C, autoimmune diseases, severe medical conditions or stillborn births. Women with poor English language skills were also excluded.

2.1.2 Sample collection

The placenta was collected into a sealed clean plastic bag and placed into a clean plastic bucket immediately following delivery. Samples were transported to the laboratory at ambient temperature in specialised transport bag and processed within

1.5 hours of delivery. Blood was collected into sodium heparin containing tubes and processed within 30 minutes of collection.

2.2 Processing of gestation-associated tissues

A. When handling the placenta and the attached membranes, care was taken to minimise contamination by LPS/endotoxin. All procedures are performed in a class II tissue culture cabinet using disposable sterile consumables; including surgical grade sterile equipment for dissection. Reagents used were determined by manufactures to be endotoxin free.
B. Total weight (placenta and attached membranes) was recorded prior to preparation of explant cultures.

2.2.1 Placental explant cultures

The deciduas basalis, overlaying the maternal side of the placenta was removed with scissors. Pieces of placenta tissue (1 cm^3) were then cut from various sites across the placenta and placed into sterile calcium and magnesium free PBS (phosphate buffered saline; Life Technologies, UK). Tissue was washed repeatedly with PBS to remove any contaminating blood. Tissue was then minced into smaller pieces and washed further. Pieces of tissue (1 mm^3 pieces to a total of 0.2 g) were transferred into the required number of wells of a standard 12-well tissue culture plate (Greiner Bio-one, Germany; Figure 2.1) containing 1 ml Ultraculture medium (Lonza, UK), supplemented with 2 mM Glutamax (Life Technologies, UK) and 100 U/ml penicillin, 100 µg/ml streptomycin sulphate and 0.25 µg/ml amphotericin B (PSF; Life Technologies, UK).

Explant cultures were exposed to different stimuli as detailed in individual chapters; an unstimulated control was always included. All treatments were performed in duplicate. Cultures were incubated for 24 hours at 37°C in 5% CO_2. Tissue free supernatants were collected by centrifugation for 7 minutes at 4°C, 515 x g and stored at -20°C until analysis.

2.2.2 Fetal membrane explant cultures

Pieces of membranes were cut away from the placenta. Choriodecidua and amnion were separated from each other by blunt dissection and placed separately into PBS. Tissue was washed repeatedly with PBS to remove any contaminating blood. Each membrane was cut with an 8 mm biopsy punch (Steifel; Medisave, UK). Biopsies of

amnion, six pieces, were transferred into each individual well of a 12-well tissue culture plate containing 1ml Advanced DMEM (Life Technologies, UK) supplemented with 2 mM Glutamax and 2% FBS (Fetal Bovine Serum, Hyclone; Fisher-Scientific, UK), or three pieces into each individual well of a 24-well tissue culture plate containing 0.5 ml of the same medium. Biopsies of choriodecidua, four pieces, were transferred into each individual well of a 12-well tissue culture plate containing 1 ml Advanced RPMI supplemented with 2-Mercaptoethanol (2-ME), 2 mM Glutamax and 2% FBS, or two pieces into each individual well of a 24-well tissue culture plate containing 0.5 ml of the same medium (Figure 2.1).

Once prepared, explants cultures were exposed to different stimuli as detailed in individual chapters; an unstimulated control was always included. All treatments were performed in duplicate. Cultures were incubated for 24 hours at 37°C in 5% CO_2. Tissue free supernatants were collected and stored at -20°C until analysis.

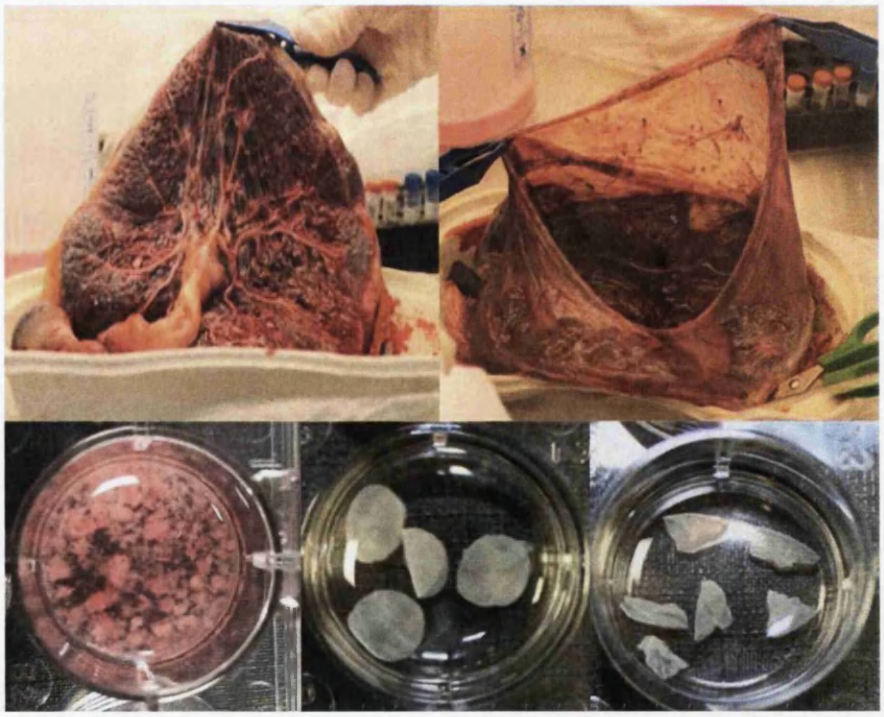

Figure 2.1 Gestation associated tissues – Placenta and fetal membranes.
(A) Fetal side of the placenta showing umbilical cord. (B) Maternal side with attached fetal membranes (choriodecidua & amnion). (C) Placental explant culture. (D) Choriodecidua explant. (E) Amnion explant.

2.2.3 Storage of gestation-associated tissues for RNA extraction

PBS washed tissue (0.2 g) was placed into a 2ml tube containing Lysing Matrix D (Fisher Scientific Ltd, UK) with 600 µl TRI reagent (Sigma-Aldrich, USA) within 30 minutes of collection. Specimens were stored at -20°C until extraction.

2.2.4 Lactate dehydrogenase (LDH)-cytotoxicity assay

Cytotoxicity was evaluated using the LDH-Cytotoxicity Assay Kit II (Abcam, UK), utilising a WST (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)- 2*H*-5-tetrazolio]-1,3-benzene disulfonate sodium salt)reagent, a tetrazolium salt substrate, for detection of LDH released from damaged cells (Figure 2.2). In a 96 well plate, 100 µl of LDH reaction mix working solution (49 parts LDH assay buffer, 1 part WST substrate mix) was added to either, 10 µl of supernatant or 10 µl of the appropriate control (background control; cell culture medium, negative control; dH_20, positive control; LDH). All tests were performed in triplicate. The reaction was then incubated for 30 minutes at room temperature. Following this, the absorbance of all samples and controls at 450 nm was read using a plate reader (POLARStar; BMG) and the levels of LDH calculated as a comparison to the unstimulated.

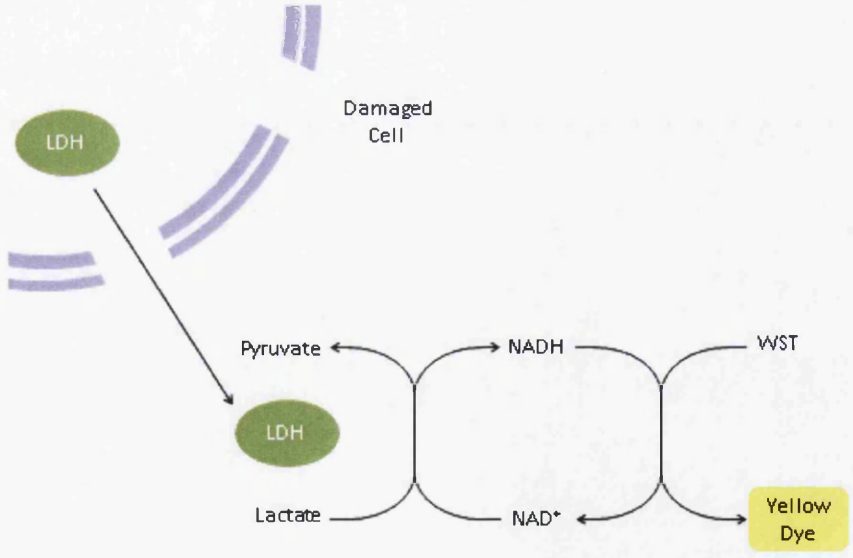

Figure 2.2 Lactate dehydrogenase cytotoxicity assay.

During cell death the plasma membrane is damaged resulting in the release of LDH, an enzyme that oxidises lactate, generating NADH. The cytotoxicity assay takes advantage of this process by utilising a WST substrate mix, which interacts with NADH generating a yellow colour. LDH, lactate dehydrogenase; NAD/NADH, Nicotinamide adenine dinucleotide; WST, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)- 2*H*-5-tetrazolio]-1,3-benzene disulfonate sodium salt. Adapted from Sarker et al [279].

2.3 Processing of adult peripheral blood

To limit contamination, handing of adult peripheral blood took place in a class II tissue culture cabinet. Manufacture endotoxin tested reagents and disposable sterile consumables were used.

2.3.1 Isolation of mononuclear cells (MNCs) by density gradient centrifugation

Whole anti-coagulated blood (10 ml) was layered onto 10 ml Histopaque 1077 (Sigma-Aldrich, Poole, UK) in a 50 ml falcon tube (Greiner, Bio-One, Germany) and centrifuged at 800 x g for 20 minutes with no brake (Figure 2.3). The plasma was removed and discarded. The mononuclear cell layer was removed and placed in a 30 ml Universal (Greiner, Bio-One, Germany), topped up with RPMI 1640/Glutamax (Life Technologies, UK) and centrifuged at 500 x g for 10 minutes at room temerature. The supernatant was discarded and the cell pellet resuspended in RPMI 1640/Glutamax and centrifuged at 500 x g for 7 minutes. The supernatant was discarded and the cell pellet resuspended in RPMI 1640/Glutamax supplemented with 5% FBS and 2-ME – the volume of this was dependent on the stating volume of blood. MNCs were then counted using the Countess® automated cell counter (Life Technologies, UK).

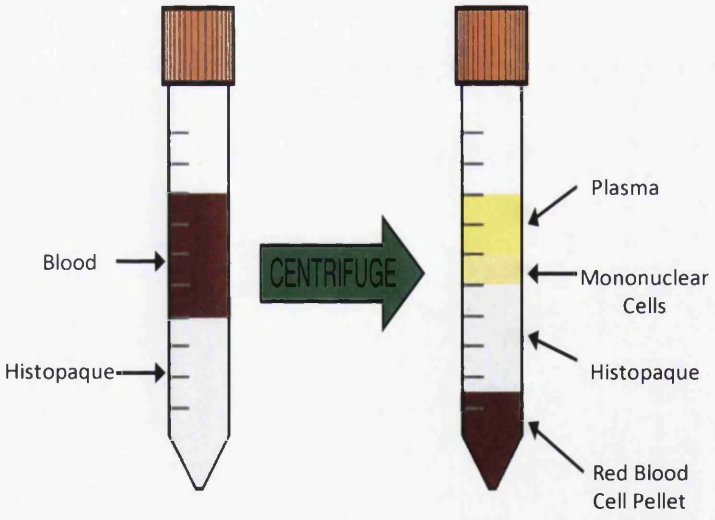

Figure 2.3 Density gradient centrifugation of whole blood.

Diagram illustrating the isolation of mononuclear cells by density gradient centrifugation.

2.3.2 Mononuclear cell culture

Isolated MNCs were cultured at 0.5 x 10^6 cells in 500 µl of RPMI 1640/Glutamax/5% FBS/2-ME in a 4.5 ml culture tube (Greiner Bio-One, Germany; Figure 2.4). Optimal levels of all agonists were then added as detailed in individual chapters. An untreated sample was always included. All treatments were performed in duplicate. Cultures were then incubated for 24 hours. Following incubation, cultures were centrifuged for 7 minutes at 4°C, 515 x g. Cell free supernatants were removed and stored at -20°C until analysis.

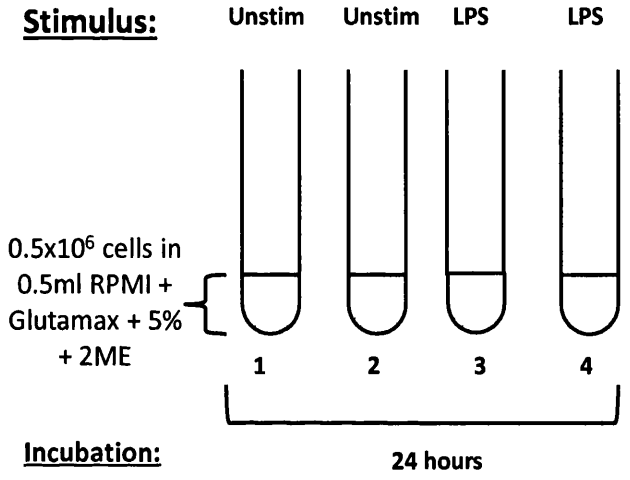

Figure 2.4 Mononuclear cell cultures.

Diagram illustrating the set up and principle of MNC cultures. Unstimulated/LPS example.

2.4 Gene expression

2.4.1 RNA extraction from gestation-associated tissue samples

The Trizol method of RNA extraction was used. Previously archived samples (section 2.2.3) were thawed and placed into a FastPrep FP120A Homogeniser (Bio101 Savant, Qbiogene, The Netherlands) and homogenised for 45 seconds at speed setting 5. Lysates were then centrifuged at 20,000 x g for 5 minutes at 4°C. The supernatant were then transferred to a 1.5 ml tube (Greiner, Bio-One, Germany), prior to incubation for 5 minutes at room temperature. Chloroform (150 µl:- Sigma-Aldrich, Poole, UK) was added, mixed vigorously and incubated at room temperature for a further 15 minutes, prior to centrifugation at 18,000 x g at 4°C for 15 minutes. Following centrifugation, 3 phases are present (Figure 2.5). The upper aqueous phase which contains RNA was removed and mixed with 250 µl

isopropanol (Sigma-Aldrich, Poole, UK) prior to incubation at room temperature for 10 minutes. This was then centrifuged for 5 minutes at 4°C, 18, 000 x g. An RNA pellet forms on the bottom of the tube. Supernatant was removed and the RNA pellet washed with 500 µl 70% ethanol, vortexed and centrifuged for 5 minutes at 4°C, 11,000 x g. On removing ethanol the pellet was allowed to dry for 5 – 10 minutes at room temperature, with care taken to ensure that the pellet doses not dry out completely. The pellet was then reconstituted in 50 µl RNase-free water (Ambion, UK).

To ensure that the RNA was free of genomic DNA, the RNA extract was processed using the DNA free DNase Kit (Ambion, UK) as per manufacturer's instructions. Spectrophotometric quantification of DNA free RNA was performed using a NanoDrop (ND-3300 fluorospectrometer, NanoDrop Technologies, USA). Optical density ratios (OD260/OD280) were determined with a ratio of ≥ 1.8 accepted as satisfactory.

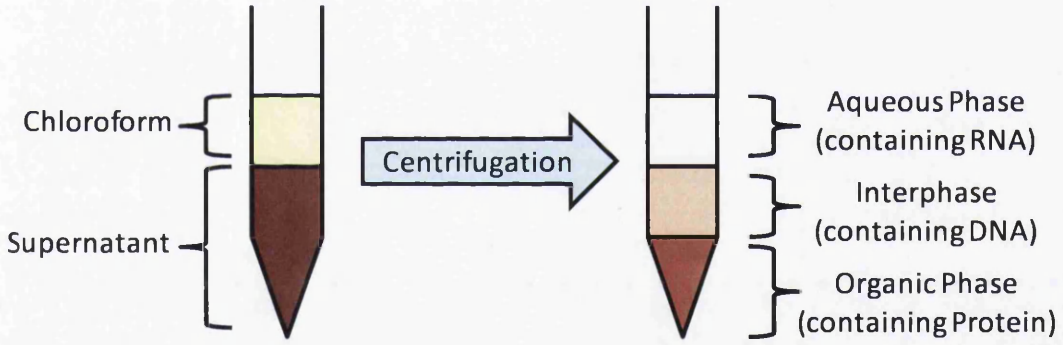

Figure 2.5 RNA separation.

After centrifugation, three phases are present: a lower red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA).

2.4.2 Reverse transcription polymerase chain reaction (RT-PCR) – Complementary cDNA synthesis

Reverse transcription was performed using the RETROscript kit (Ambion, UK) as per manufacturer's instructions. Briefly, using random decamers and murine leukaemia virus reverse transcriptase, 1 µg of RNA was transcribed into cDNA. The reaction was incubated at 44°C for 60 minutes then 92°C for 10 minutes in a polymerase chain reaction (PCR) thermal cycler (DNAEngine, Bio-Rad, UK). A

positive control of mouse liver RNA provided in the kit was used as a positive control. A no template control (NTC) of water and reverse transcriptase was performed to ensure that all reagents were DNA free.

For confirmation of successful cDNA synthesis a "check" PCR was performed with products analysed by gel electrophoresis (see 2.4.3.2).

2.4.3 Polymerase chain reaction (PCR)

Polymerase chain reaction was performed using the Platinum® Taq DNA Polymerase kit and 10 mM dNTP Mix (both Life Technologies, UK). cDNA (1 µl) was added to a master mix of 10X PCR buffer, dNTP, specific forward and reverse primers for the gene of interest, optimised concentration of $MgCl_2$, nuclease free water and a thermostable Taq DNA polymerase for a total reaction mix of 20 µl. A no template control (NTC) of water and PCR master mix was performed to ensure that all reagents were DNA free. Human spleen cDNA (prepared from total human spleen RNA as in section 2.4.2; Clontech, UK) was used as a positive control. PCR reaction was performed in a DNAEngine thermal cycler. Cycling parameters were 95°C for 5 minutes, 40 cycles of (94°C for 30 seconds, reaction specific annealing temperature for 30 seconds, 72°C for 30 seconds), 72°C for 4 minutes. Primer sequences and optimised conditions are detailed in the appropriate chapters.

2.4.3.1 Optimisation of PCR primers

To ensure a successful PCR reaction, each primer pair was optimised for a specific annealing temperature and magnesium concentration by performing a temperature/$MgCl_2$ gradient using human spleen cDNA (Figure 2.6). Primer information is detailed in individual chapters.

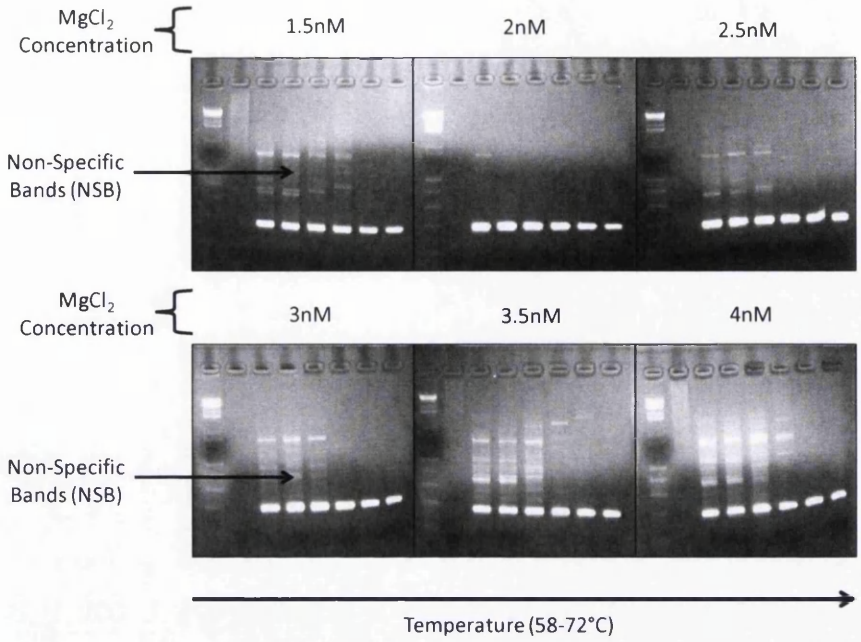

Figure 2.6 Example temperature/$MgCl_2$ gradients.

PCR reactions of different $MgCl_2$ were placed on a temperature gradient in thermal cycler. Under optimum conditions non-specific bands (NSB) are no longer present.

2.4.3.2 "Check" PCR

For confirmation of successful cDNA synthesis a "check" PCR was performed. For each sample a reaction mix of 20 µl containing 10X PCR buffer, dNTP and control primers from the RETROscript kit, a thermostable Taq DNA polymerase, nuclease free water and cDNA as per manufacturer's instructions. A "check" PCR was performed on the experimental samples cDNA produced during the RT, in addition to the corresponding RNA from the experimental samples to ensure that each sample was free of genomic DNA. The NTC and positive control from the RT step and a negative control containing all PCR reagents with water substituted for the cDNA were also included.Cycling parameters were 95°C for 1 minutes, 30 cycles of (94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds), 72°C for 4 minutes.

If each stage of the RNA extraction and RT-PCR were successful, upon analysis by gel electrophoresis, bands of the appropriate size should be present in the positive control and for all experimental samples, but not in the NTC or negative control or the corresponding RNA from the experimental samples. If bands were present in the RNA samples, RNA was subjected to further DNase treatment until RNA was DNA free.

2.4.4 Agarose gel electrophoresis

Agarose gels (2%) were prepared (agarose tablets; Bioline, UK; Tris-Borate-EDTA (TBE); Sigma-Aldrich, Poole, UK) and allowed to set for 30 minutes at room temperature. PCR products were loaded alongside DNA standard (TrackIt; Life Technologies, UK) and run for a minimum of 30 minutes at 120 V. Gels were subsequently visualised under UV light (Gel Doc XR, Bio-Rad).

2.5 Protein expression

2.5.1 Protein extraction from gestation-associated tissue samples

PBS washed tissue (0.2 g) was place into a 2ml tube containing Lysing Matrix D with 500 µl RIPA Buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with Protease Inhibitor Cocktail (104 mM AEBSF, 80 µM Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin, 1.5 mM Pepstatin A) and Phosphatase Inhibitor Cocktail (all Sigma-Aldrich, Poole, UK).

Specimens were placed into a FastPrep homogeniser and homogenised for 45 seconds at speed setting 5. Lysates were then centrifuged for 20 minutes at 4°C, 12,000 x g. Supernatants were transferred to a clean 1.5ml tube with the remaining cellular debris discarded. A small volume of supernatant was aliquoted for protein estimation. Lysates were stored at -20°C until needed.

2.5.2 Protein estimation

Protein content of each sample was estimated using the Bicinchoninic acid (BCA) Assay. In a 96 well plate (Greiner, Bio-One, Germany), 80 µl of a working solution (50 parts bicinchoninic acid solution, 1 part copper (II) sulphate pentahydrate 4% solution, all Sigma-Aldrich, Poole, UK) was added to either, 10ul of protein sample or 10 µl protein standard (Bovine serum albumin [BSA], 0.2 – 1 mg/ml; Sigma-Aldrich, Poole, UK, blank of distilled water also included). The reaction was then incubated for 30 minutes at 37°C. Following this, the absorbance at 562 nm of all samples and standards was measured using a plate reader (POLARstar Omega; BMG LABTECH, Germany).

2.5.3 Western blotting

2.5.3.1 Preparation of protein samples

Previously quantified protein (50 µg) was added to 5ul LDS samples buffer (Life Technologies, UK) and sufficient deionised water to make a total volume of 25 µl. All samples were then incubated for 10 minutes at 70°C.

2.5.3.2 SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

Polyacrylamide running gels (7.5-15%) were prepared (see Table 2-1) and placed into a 1.5 mm gel mould (Bio-Rad, UK). Water saturated butanol was added to ensure a clean top edge and the gel left to polymerise for 30 minutes at room temperature. Once set, the butanol was washed off, stacking gel (1.75 ml H_2O, 0.75 ml Tris + SDS, 0.5 ml 30% acrylamide, 100 µl 10% APS [0.1 g APS in 1 ml dH_2O], 3 µl TEMED) was added and comb inserted before leaving gel for 20 minutes at room temperature. Un-polymerised staking gel was removed by washing with dH_20 and gels placed into a mini tank (Mini-PROTEAN Tetra Cell, Bio-Rad, UK) filled with 1 X SDS-PAGE running buffer (900 ml deionised water, 100 ml 10 X SDS-PAGE running buffer; 500 ml Tris-Glycine, 5 g SDS, both Sigma-Aldrich, Poole, UK). Protein samples were loaded alongside a protein standard (All Blue; Bio-Rad, UK) and run for 50 minutes at 200 V.

2.5.3.3 Semi-dry membrane transfer

Once the gel has run, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, UK) using a transfer dock (Bio-Rad TransBlot SD) for 75 minutes at 15V. PVDF membrane was pre-soaked (methanol for 30 seconds, water for 2 minutes, transfer buffer for at least 5 minutes) then placed upon three pre-soaked pieces of filter paper on the transfer dock. The SDS-PAGE gel was then placed on top of the PVDF membrane followed by three further pieces of filter paper. Care was taken to ensure no air bubbles. Protein was then transferred using the Trans-Blot SD Semi-Dry Electrophoretic Transfer cell (Bio-Rad, UK). Following transfer each membrane was treated with Ponceau S stain (Sigma-Aldrich, Poole, UK) to visualise the protein to ensure that the transfer was successful. The membrane was then washed in 1 X TBS-Tween-20 (Both Sigma-Aldrich, Poole, UK) to remove the stain before continuing.

Table 2-1 Polyacrylamide running gels.

Reagent	7.50%	10%	12%	15%
Water	5 ml	4.2 ml	3.5 ml	2.5 ml
4 X Tris and SDS	2.5 ml	2.5 ml	2.5 ml	2.5 ml
30% acrylamide	2.5 ml	3.3 ml	4 ml	5 ml
APS	100 µl of 10% solution	100 µl of 10% solution	100 µl of 10% solution	100 µl of 10% solution
TEMED	10 µl	10 µl	10 µl	10 µl

Different percentage 1.5 mm polyacrrylamide gels (7.5-15%) were prepared by varying the concentration of acrylamide in the mixture. The polymerising agents APS and TEMED were not added until ready to cast to minimise the amount of oxidation the mixture undergoes, as this will impair polymerisation. 10% APS – 0.1 g APS in 1 ml dH_2O. APS, ammonium persulfate; TEMED. Tetramethylethylenediamine.

2.5.3.4 Membrane blocking and antibody staining

PVDF membrane was blocked for 1 hour at room temperature in 5% non-fat milk in TBS. Blocking buffer was then removed and 2ml of primary antibody (diluted in either 5% BSA, 0.1% Tween-20 in TBS or 5% non-fat milk in TBS to the appropriate concentration as noted in each chapter) was added to the PVDF membrane which was then sealed in a plastic bag and incubated over-night at 4°C. The following morning, the primary antibody was removed and the membrane washed in TBS-Tween-20 three times for 7 minutes each. Secondary antibody as indicated in each chapter was prepared and the membrane incubated with 2 ml of this preparation for 1-2 hours at room temperature in a sealed plastic bag. The membrane was then washed in TBS-Tween-20 three times for 7 minutes as before and TBS for a minimum of 5 minutes before visualisation.

2.5.3.5 Enhanced chemiluminesence (ECL)

PVDF membrane was incubated face down in ECL for 1-5 minutes at room temperature – time varied depending on the ECL reagent used; 1 minute for Amersham ECL Select Western blotting detection reagent (GE Healthcare, UK) and 5 minutes for West PICO Chemiluminescent substrate (Fisher Scientific, UK). The

membrane was then visualised using a ChemiDoc XRS (Bio-rad, UK) at several different time points (1, 10, 30, 60, 90, 270 seconds).

2.5.3.6 Stripping and re-probing of PVDF membrane

The membrane was placed in room temperature TBS-Tween-20 for 10 minutes, prior to being sealed in a plastic bag with 2 ml stripping buffer (Fisher-Scientific, UK) for 15-20 minutes. Following incubation the membrane was washed in TBS-Tween-20. The membrane was then ready to be reprocessed as described above (see 2.5.3.4 – 2.5.3.5).

2.6 Enzyme linked immunoabsorbant assay (ELISA)

Production of cytokines/chemokines were examined in the supernatants of placenta, choriodecidua and amnion explant cultures and whole blood culture supernatants collected after 24 h using commercially available ELISA kits as per manufactures instructions. The different cytokines analysed are listed in Table 2-2.

An overview of a general ELISA protocol can be seen in Figure 2.7. Briefly, a half-area 96 well plate (Greiner Bio-one, Germany) is coated with a capture antibody and incubated overnight at 4°C. The coating antibody is discarded and the plate incubated for 1 hour at room temperature with 1% BSA in PBS (150 µl per well). The plate is subsequently washed 3 times with wash buffer (1% BSA in PBS 0.005% Tween-20) using an ELx50 Microplate Strip Washer (BioTek, UK) and supernatants of interest in addition to known standards are added for 2 hours at room temperature. Following incubation the plate is washed 4 times before the addition of detection antibody at room temperature for 2 hours. The plate is once again washed 4 times, before the addition of strepdavidin horseradish peroxidase (HRP) linked secondary antibody for 20 minutes' at room temperature and once again washed 6 times. A substrate solution is prepared according to manufactures instructions (TMB, BD Bioscience, USA) and added allowing for the development of a blue colour. A yellow is then produced followed the addition of 1M H_2SO_4 to stop the reaction. The intensity of the colour is measured at 450 nm using a plate reader (POLARstar Omega; BMG LABTECH, Germany), with the concentration of the cytokine calculated based on the standard curve.

Table 2-2 ELISA kits used in this body of work.

Cytokine/Chemokine	Manufacturer	Sensitivity (pg/ml)
IL-1β	R&D Systems	3.91 pg/ml
IL-6	R&D Systems	9.38 pg/ml
IL-8 (CXCL8)	R&D Systems	31.2 pg/ml
MIP-1α (CCL3)	R&D Systems	7.81 pg/ml

Sensitivity is listed as provided by manufacturers.

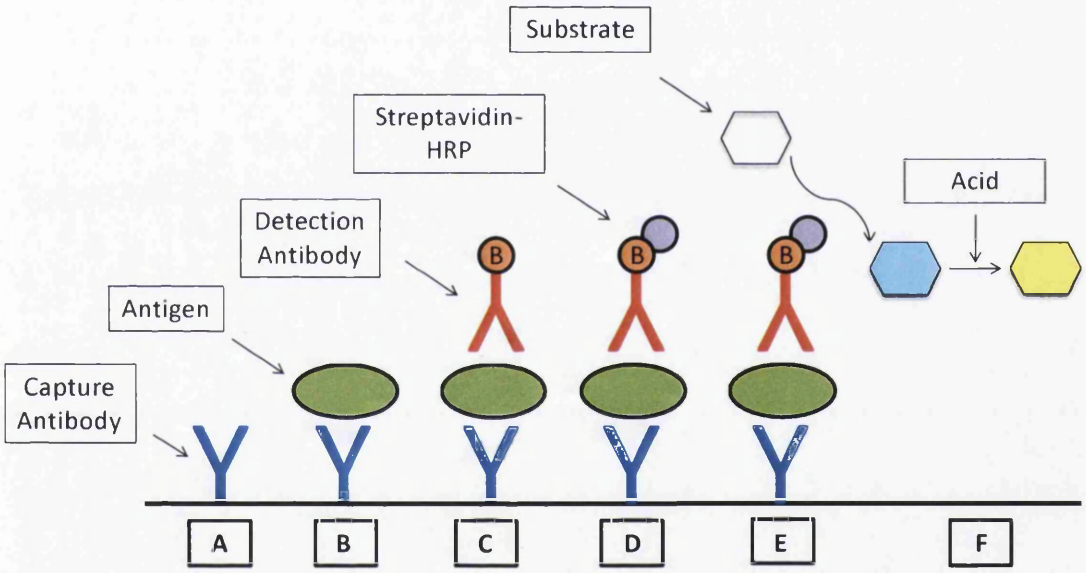

Figure 2.7 Overview of ELISA protocol.

(A) Capture antibody is immobilised on plastic. (B) Antigen is added and binds to the capture antibody. (C) Biotin-conjugated detection antibody is added and binds the antigen. (D) HRP linked streptavidin binds to biotin on the detection antibody. (E) TMB is added and catalysed by the HRP producing a blue colour. (F) Acid is added, stopping the reaction producing a yellow colour.

2.7 Caspase activity assay

Caspase activity was evaluated using the Caspse-1 Colorimetic Assay Kit and Caspase-8 Colorimetic Assay Kit (both Abcam, UK), utilising a caspase specific substrate, which in the presence of active caspase is cleaved releasing a chromophore. Spectrophotometric detection of light emitted by the chromophore is relative to the amount of active caspase. In a 96 well half-area plate 100 µg of protein in 25 µl was added with 25 µl of 2X reaction buffer (containing 10 mM DTT) and 5 µl substrate (200 µM; YVAD-*p*-NA, Caspase-1 substrate; IETD-*p*-NA, Caspase-8 substrate). All tests were performed in duplicate. Commercially available active Caspase-1 and Caspase-8 (both Abcam) were used as positive controls. The reaction was incubated at 37°C for a minimum of 2 hours. Following this, the absorbance of all samples and controls at 405nm were read using a plate reader (POLARstar Omega; BMG LABTECH, Germany). Protein and buffer without substrate were included permitting background correction.

2.8 Statistical analysis

All experiments were performed a minimum of three times (i.e. a minimum of three biological replicates) in duplicate (two technical replicates) with data presented as mean ± SEM.

Since the data sets contained small sample numbers it was important to consider which statistical test would be most appropriate. To choose a parametric test, the data must be normally distributed i.e. following a Gaussian distribution; if the data is not normality distributed type II error will occur. However, tests used to determine normality do not have the power to determine if a data set comes from a Gaussian population, when the sample number is small. Since normality cannot be determined, the alternative is to perform non-parametic testing. However, it should be noted that applying non-parametric tests to a data set without knowing if it is normally disturbed could increase the chances of type I errors occurring. As normality could not be determined, all data was therefore treated as non-parametric. The Wilcoxon matched pairs signed rank test was applied when comparing to groups (e.g treated and untreated), and the Freidman's test with Dunn's posthoc test when comparing three or more groups (e.g. untreated, treatment 1 and treatment 2). A p vaule of ≤ 0.05 was determined significant. Statistical significance was calculated using GraphPad Prism (Version 6, GraphPad Software Inc, USA).

Chapter 3

Expression and activity of TLRs, NLRs and RLRs in human gestation-associated tissues

3 Expression and activity of TLRs, NLRs and RLRs in human gestation-associated tissues

3.1 Introduction

It is now well established that a number of cytokines and chemokines play a role in the normal physiological process of pregnancy including parturition. In particular the cytokines IL-1β, IL-6, IL-8 and TNFα among others are produced by gestation-associated tissues (placenta and attached membranes) both constitutively and/or in response to an insult [10, 67-72]. Pattern recognition receptors (PRRs) have been identified as a link between both exogenous microbial agonists (pathogen associated molecular patterns, PAMPs) and endogenous host derived agonists (damage associated molecular patterns, DAMPs) [103, 280]. However, the study of PRR expression and activity by gestation-associated tissues is still in its infancy.

The study of PRRs expression and activity by gestation-associated tissues has principally focused on Toll-like receptors (TLRs) and more recently Nod-like receptors (NLRs). Transcripts for TLRs 1-10 have been demonstrated in the term placenta, decidua and amnion [233, 241-243]. Functional activity of TLRs 1-9 have been reported for the term placenta, with only functional activity for TLRs 2/6, 4 and 5 in amniotic epithelial cells and TLRs 1-4 and 6 in the decidua [233, 241-243]. Expression of the NLRs, NOD1 and NOD2 has been demonstrated by the term decidua, while only NOD1 is expressed by the term placenta [60, 244]. However, NOD2 in addition to NOD1 has been shown to be expressed in the first trimester placenta with a corresponding functional output; functional NOD1 and 2 in first vs. functional NOD1 at term [281]. NLR activity by the amnion has not been examined to date.

Other families of PRRs have also been described namely RIG-I-like receptors which are associated with recognition of viral RNAs and C-type lectin receptors, which are associated with recognition of fungal derived carbohydrates [196, 217]. However the expression and activity of these PRRs by gestation associated tissues remains unknown. It is therefore clear that a great deal remains to be understood regarding PRR activity in gestation-associated tissues.

3.2 Aims

The aim of this chapter is to examine the expression and activity of Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) in the term non-laboured human placenta, choriodecidua and amnion. The activity of oC-type lectin receptors (CLRs) will be examined in the following chapter.

3.3 Methods

3.3.1 Samples

Healthy pregnant women scheduled for elective caesarean section (ESC) were approached in the antenatal day assessment unit at Singleton Hospital, Swansea, during their pre-anaesthetic assessment. Informed written consent was obtained following explanation of the study. After delivery, the placenta and attached fetal membranes (n = 3-11) from these women were collected and processed within 1.5 hours of delivery (see Chapter 2.1 for details).

3.3.2 PCR

Gestation associated tissue samples (placenta, choriodecidua and amnion) were stored in TRI reagent and frozen within 30 minutes of collection of the tissue. RNA was extracted using the Trizol method and was cDNA subsequently produced following DNase treatment (see Chapter 2.4 for method). PCR was performed for TLRs, NLRs and RLRs using the primers tested in Table 3-1 and 3-2.

3.3.3 Gestation-associated tissues explants

Placenta, choriodecidua and amnion tissue explants were cultured as described in chapter 2.2. Cultures were initially stimulated with several PRR specific agonists (all Invivogen) at a range of concentrations (Table 3-3) to identify an optimum concentration of each agonist. Once identified the optimised concentration was used in subsequent *in vitro* culture experiments.

3.3.4 Cytokine production

IL-6 and IL-8 in the tissue free supernatants of placenta, choriodecidua and amnion explant cultures collected after 24 h were measured using commercially available ELISA kits (DuoSet, R&D Systems) as per manufacturer's instructions.

3.3.5 Statistical analysis

Agonist mediated cytokine production by non-laboured tissues was evaluated by Wilcoxon matched pairs signed rank test or Freidman's test with Dunn's posthoc test for multiple comparisons. A p-value of ≤ 0.05 was considered significant.

Table 3-1 TLR and Housekeeping primer sequences.

Gene		Primer	Mg^{2+} Conc (nM)	Annealing Temp (°C)	Fragment Size (bp)	Ref
TLR1	*F*	5' CAGTGTCTGGTACACGCATGGT	3	63	104	[233]
	R	5' TTTCAAAAACCGTGTCTGTTAGAGA				
TLR2	*F*	5' GGCCAGCAAATTACCTGTGTG	2	69	67	[233]
	R	5' AGGCGGACATCCTGAACCT				
TLR3	*F*	5' CCTGGTTTGTTAATTGGATTAACGA	1.5	63	82	[233]
	R	5' TGAGGTGGAGTGTTGCAAAGG				
TLR4	*F*	5' CAGAGTTTCCTGCAATGGATCA	3.5	60	88	[233]
	R	5' GCTTATCTGAAGGTGTTGCACAT				
TLR5	*F*	5' TGCCTTGAAGCCTTCAGTTATG	1.5	59	77	[233]
	R	5' CCAACCACCACCATGATGAG				
TLR6	*F*	5' GAAGAAGAACAACCCTTTAGGATAGC	2.5	68	88	[233]
	R	5' AGGCCAAACAAAATGGAAGCTT				
TLR7	*F*	5' TTTACCTGGATGGAAACCAGCTA	2	66	73	[233]
	R	5' TCAAGGCCTGAGAAGCTGTAAGCTA				
TLR8	*F*	5' TTATGTGTTCCAGGAACTCAGAGAA	3	66	83	[233]
	R	5' TAATACCCAAGTTGATAGTCGATAAGTTTG				
TLR9	*F*	5' GGACCTCTGGTACTGCTTCCA	2	66	151	[233]
	R	5' AAGCTCGTTGTACACCCAGTCT				
TLR10	*F*	5' TGTTATGACAGCAGAGGGTGATG	1.5	63	151	[233]
	R	5' GAGTTGAAAAAGGAGGTTATAGGATAAATC				
UBE2D2	*F*	5' GATCACAGTGGTCTCCAGCA	3	65	156	[282]
	R	5' TCCATTCCCGAGCTATTCTG				

Sequences and optimum conditions for each pair of Toll-like receptor (TLR) and housekeeping primers used for PCR. TLR primers sequences obtained from Patni et al 2009 [233] and UBE2D2 sequences from Popov et al 2010 [282]. Bp, base pairs; F, forward primer sequence; nM, nanomolar; R, reverse primer sequence; TLR, Toll-like receptor; UBE2D2, ubiquitin-conjugating enzyme E2 D2.

Table 3-2 NLR and RLR primer sequences.

Gene		Primer	Mg^{2+} Conc (nM)	Anneling Temp (°C)	Fragment Size (Bp)	Accession Number
NOD1	*F*	5' AGGCTGAGTACCATGGGCTA	2	66	184	NM_006092
	R	5' GCCCGTTTAGTCACCCTTCA				
NOD2	*F*	5' CAGGCAGCACAGGTCAGCCC	2	71	300	NM_001293557
	R	5' GTTGTGCGGCTCGGCCTTCT				
NLRP1	*F*	5' ATACGAAGCCTTTGGGGACT	3	65	164	NM_014922
	R	5' CACCGCTTCTCTCATCACAA				
NLRP3	*F*	5' ACCGGAGCCAGCAGGAGAGG	1.5	71	659	NM_001127461
	R	5' GAAGGCTGCCCTGGCTTGGG				
NLRC4	*F*	5' GCCTCAGGCTGCAAATAAAG	2	68	213	NM_001199139
	R	5' CCAAGCTGTCAGTCAGACCA				
NLRX1	*F*	5' GCTCCATGGCTTAGAGCATC	1.5	62	189	NM_001282358
	R	5' ACGTACTTGCTGGGGATACG				
NAIP	*F*	5' TTCTTGCCCTGAAAACTGCT	3	66	169	NM_004536
	R	5' CGTATTGGGAAGTGGATGCT				
RIG-I	*F*	5' TGTTTCCAGGGATCCCAGCAATGA	1.5	70	839	NM_014314
	R	5' ACTTCACATGGATCCCCCAGTCATGGC				
MDA5	*F*	5' GCTCACAGTGGATCCGGAGTTATCGAA	2	59	420	NM_022168
	R	5' CACCATCATGGATCCCCAAGCCTGGCC				
LGP2	*F*	5' GTGGTGGTGCAGGCGAGACC	3	70	752	NM_024119
	R	5' GCTGGGGGCAGCAGTTCTGG				

Sequences and optimum conditions for each pair of NOD-like receptor (NLR) and RIG-I like receptor (RLR) primers used for PCR. Bp, base pairs; F, forward primer sequence; LGP2, laboratory of genetics and physiology 2; MDA5, melanoma differentiation-associated protein 5; NAIP, NLR family apoptosis inhibitory protein; nM, nanomolar; NOD, nucleotide-binding oligomerisation domain; NLRC, NOD-like receptor containing a CARD domain; NLRP, NOD-like receptor containing a PYD domain; R, reverse primer sequence; RIG-I, retinoic acid-inducible gene 1.

Table 3-3 TLR, NLR and RLR ligands.

Ligand	Receptor Targeted	Concentrations Tested
Pam3CSK4	TLR2/1	100 ng – 1 ug/ml
HKLM	TLR2	10^6 - 10^8 cells/ml
FSL1	TLR2/6	1 ng – 1 ug/ml
Poly(I:C)HMW	TLR3	5 – 25 µg/ml
Poly(I:C)LMW	TLR3	5 - 25 µg/ml
LPS	TLR4	1 ng – 10 µg/ml
Flagellin	TLR5	1 ng – 100 ng/ml
Imiquimod	TLR7	10 ng – 1 µg/ml
ssRNA40	TLR8	10 ng – 1 µg/ml
Tri-DAP	NOD1	100 ng – 10 µg/ml
MDP	NOD2	100 ng - 10 µg/ml
Poly(I:C)LyoVec	RIG-1 & MDA-5	10 ng -1 µg/ml

Ligands at a range of concentrations were tested on placenta, choriodecidua and amnion to identify an optimum concentration of each agonist. HKLM, heat killed *Listeria monocytogenes*; HMW, high molecular weight; LMW, low molecular weight; LPS, lipopolysaccharide; MDA5, melanoma differentiation-associated protein 5; MDP, Muramyl dipeptide; NOD, nucleotide-binding oligomerisation domain; NLR, NOD receptor; Poly(I:C), polyinosine-polycytidylic acid; RIG-I, retinoic acid-inducible gene 1; RLR, RIG-I like receptor; ssRNA, single stranded RNA; TLR, toll-like receptor; Tri-DAP, L-Ala-γ-D-Glu-mDAP.

3.4 Results

3.4.1 Expression of transcripts for TLRs, NLRs and RLRs by gestation-associated tissues

To investigate whether transcripts for a variety of pattern recognition receptors, including TLRs, NLRs and RLRs are expressed in gestation associated tissues, PCR was performed using five individual samples of each tissue type (placenta, choriodecidua and amnion). Three of these five samples are shown in Figure 3.2 – Figure 3.4. Transcripts for each PRR examined were present in all five samples of each of the tissues.

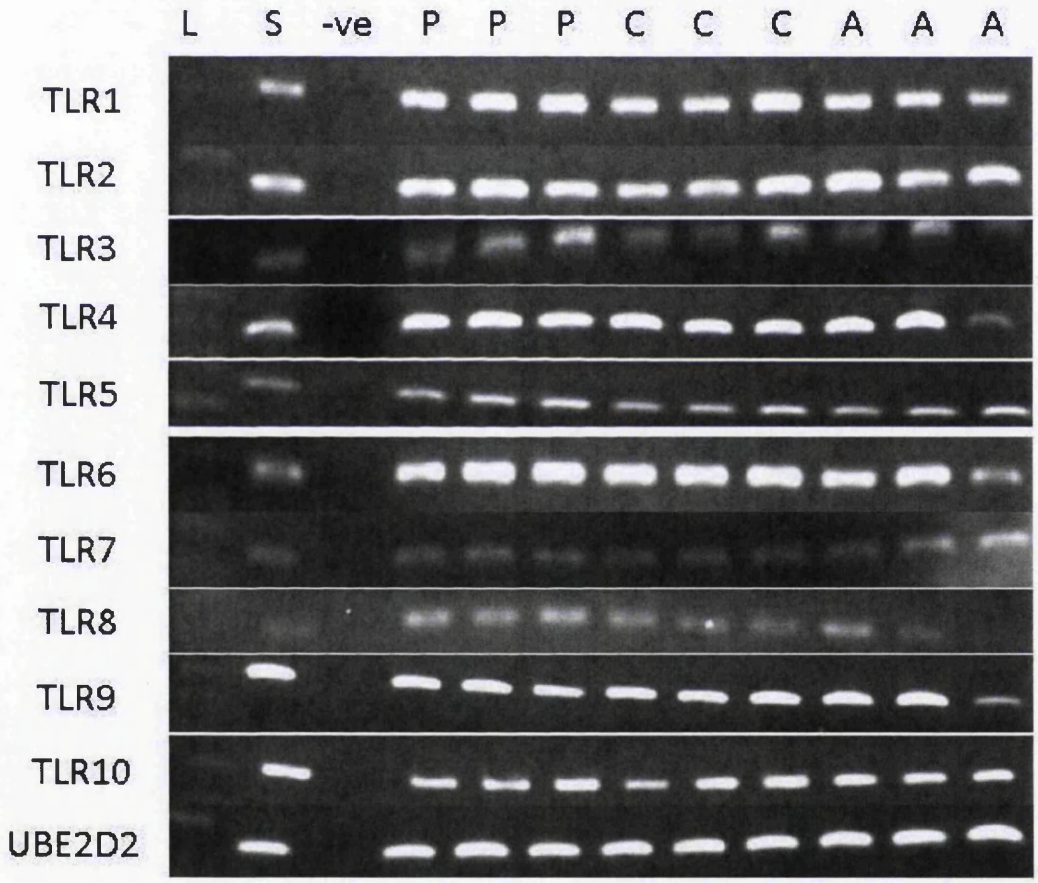

Figure 3.1 Transcripts for TLR1-10 in gestation-associated tissues.

Each TLR was present in all five of the samples tested from each of the tissues examined, three representative samples are shown; placenta (P), choriodecidua (C) and amnion (A). Human spleen (S) was used as a positive control and UBE2D2 was used as a housekeeping gene. L = 100 base pair ladder and the negative control (-ve) was with water replacing cDNA in the reaction mix.

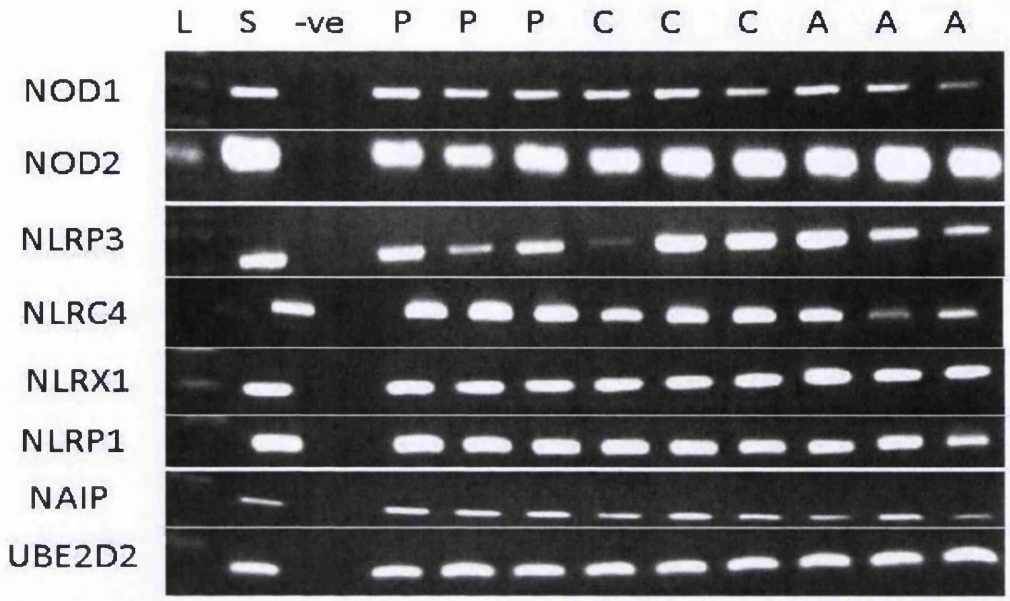

Figure 3.2 Transcripts for NLR family members in gestation associated tissues.

Each NLR family member was present in all five of the samples tested from each of the tissues examined, three representative samples are shown; placenta (P), choriodecidua (C) and amnion (A). Human spleen (S) was used as a positive control and UBE2D2 was used as a housekeeping gene. L = 100 base pair ladder and the negative control (-ve) was with water replacing cDNA in the reaction mix.

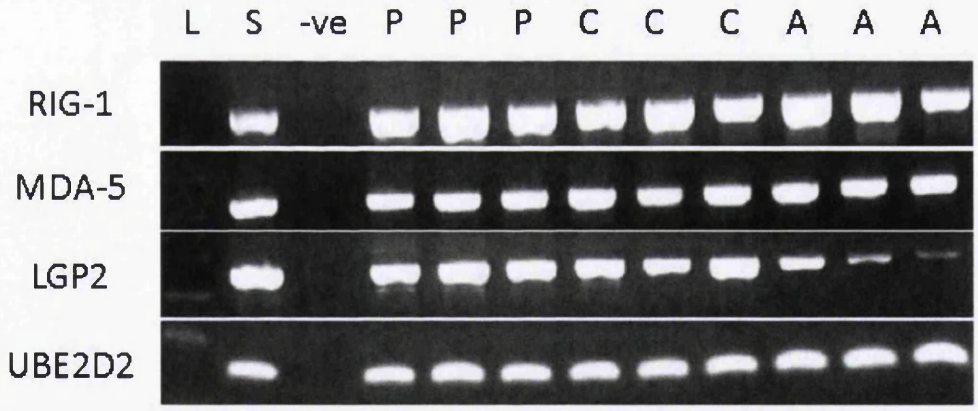

Figure 3.3 Transcripts for RLR family members in gestation associated tissues.

Each RLR family member was present in all five of the samples tested from each of the tissues examined, three representative samples are shown; placenta (P), choriodecidua (C) and amnion (A). Human spleen (S) was used as a positive control and UBE2D2 was used as a housekeeping gene. L = 100 base pair ladder and the negative control (-ve) was with water replacing cDNA in the reaction mix.

3.4.2 Optimisation of PRR agonists

In order to investigate the functional activity of PRRs in the placenta, choriodecidua and amnion, preliminary investigations (n=3) were performed in order to determine an optimised concentration of each agonist for subsequent experiments. In doing so a range of concentrations, based on the manufacturer's recommendations was tested for each agonist (Table 3-3) on all three tissues. If no significant outcome was observed at any concentration, a concentration for further experiments was chosen based on its ability to provide the greatest increase for both IL-6 and IL-8 for all three tissues. Optimised concentrations of each agonist are summarised in Table 3-4 with each dose response shown in the appendix (Chapter 8.3, Figure 8.1 – 8.14).

Table 3-4 Optimised concentrations of TLR agonists.

Ligand	Receptor Targeted	Optimised Concentration
Pam3CSK4	TLR2/1	100 ng/ml
HKLM	TLR2	10^7 cells/ml
FSL1	TLR2/6	10 ng/ml
Poly(I:C)HMW	TLR3	25 µg/ml
Poly(I:C)LMW	TLR3	25 µg/ml
LPS	TLR4	10 ng/ml
Flagellin	TLR5	100 ng/ml
Imiquimod	TLR7	1 µg/ml
ssRNA40	TLR8	1 µg/ml
Tri-DAP	NOD1	10 µg/ml
MDP	NOD2	10 µg/ml
Poly(I:C)LyoVec	RIG-1 & MDA-5	1 µg/ml

Optimised concentrations of PRR agonists as determined following preliminary investigations. HKLM, heat killed *Listeria monocytogenes*; HMW, high molecular weight; LMW, low molecular weight; LPS, lipopolysaccharide; MDA5, melanoma differentiation-associated protein 5; MDP, Muramyl dipeptide; NOD, nucleotide-binding oligomerisation domain; NLR, NOD receptor; Poly(I:C), polyinosine-polycytidylic acid; RIG-I, retinoic acid-inducible gene 1; RLR, RIG-I like receptor; ssRNA, single stranded RNA; TLR, toll-like receptor; Tri-DAP, L-Ala-γ-D-Glu-mDAP.

3.4.3 Response of term non-laboured gestation associated-tissues explants to TLR agonists

3.4.3.1 TLR1/2/6

Since TLR2 can form both a homodimer and heterodimer complex with TLR1 and TLR6, distinct agonists were used to determine if these receptors are functional in the placenta, choriodecidua and amnion. The agonists Pam3CSK4 (100 ng/ml), a synthetic triacylated lipopeptide, and FSL-1 (10 ng/ml), a synthetic lipoprotein, were used to examine the TLR2/1 and TLR2/6 heterodimers respectively. A freeze-dried heat-killed preparation of *Listeria monocytogenes* (HKLM; 10^7 cells/ml) was used as a general activator of TLR2. As shown in Figure 3.4, a significant increase in IL-6 and IL-8 production was observed by both the placenta and choriodecidua in response to all three agonists. A significant increase in cytokine production was also observed by amnion to all three agonists with the exception of HKLM induced IL-8.

3.4.3.2 TLR3

To investigate if TLR3, a receptor involved in the recognition of dsRNA is functional in the placenta, choriodecidua and amnion both a high molecular weight (HMW, 1.5-8 kb) and a low molecular weight (LMW, 0.2-1 kb) version of the synthetic dsRNA analogue Poly(I:C) (Polyinosine-polycytidylic acid) was used at a optimised concentration of 25 µg/ml. As shown in Figure 3.5, a increase in both IL-6 am IL-8 production was observed in response to both HMW and LMW Poly(I:C) in the placenta and choriodecidua, however a significant increase in cytokine production was only observed by these tissues in response to treatment with LMW Poly(I:C). Similarly increased IL-6 and IL-8 was observed by the amnion following treatment with both agonists, with only LMW induced IL-8 significant.

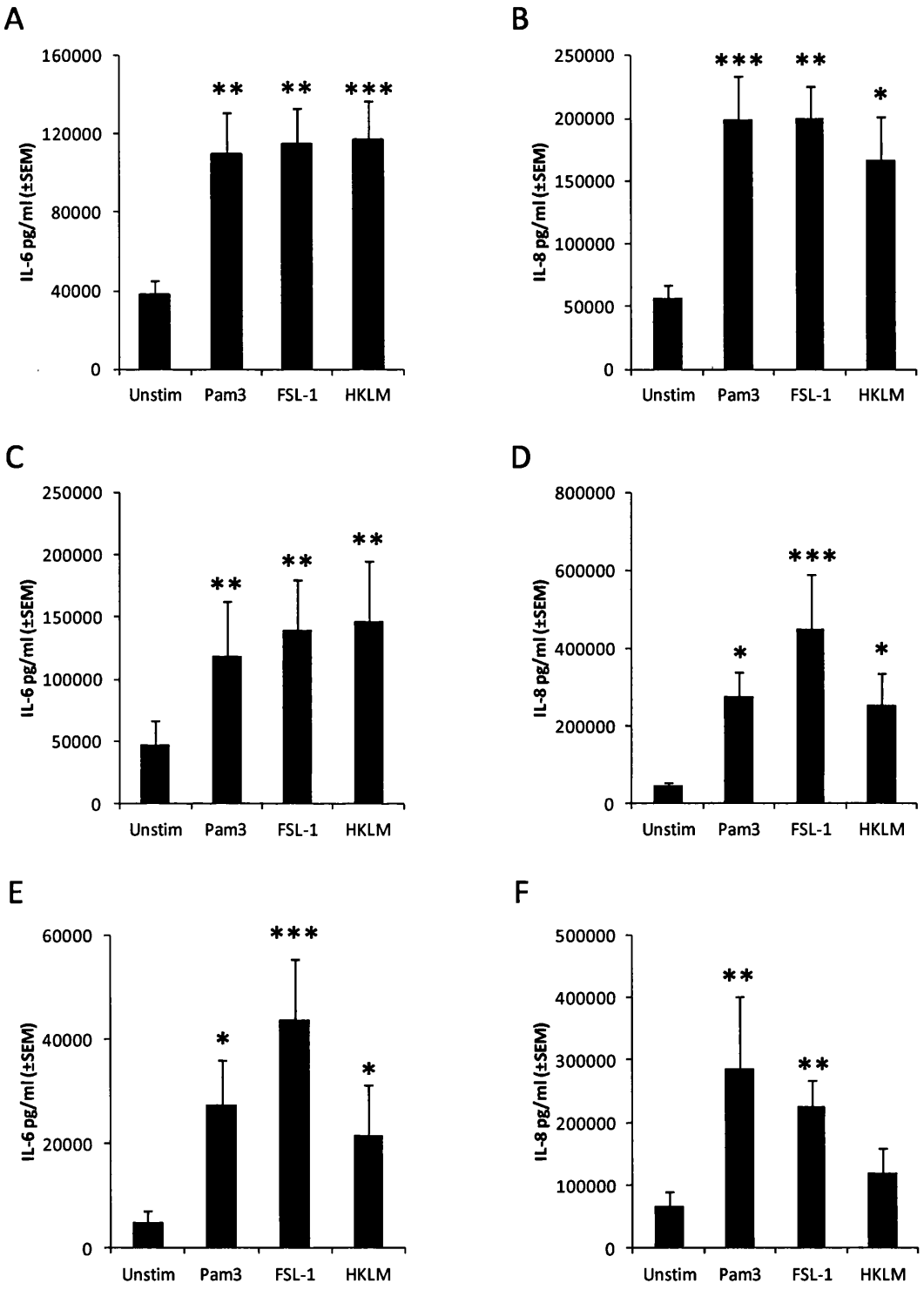

Figure 3.4 TLR2 agonist induced cytokine response by the term non-laboured placenta, choriodecidua and amnion.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua (E-F) amnion following stimulation with Pam3CSK4 (100 ng/ml), HKLM (10^7 cells/ml) and FSL-1 (10 ng/ml) (n=10). Statistical significance compared to unstimulated control as determined by Freidman's test with Dunn's posthoc test are shown: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

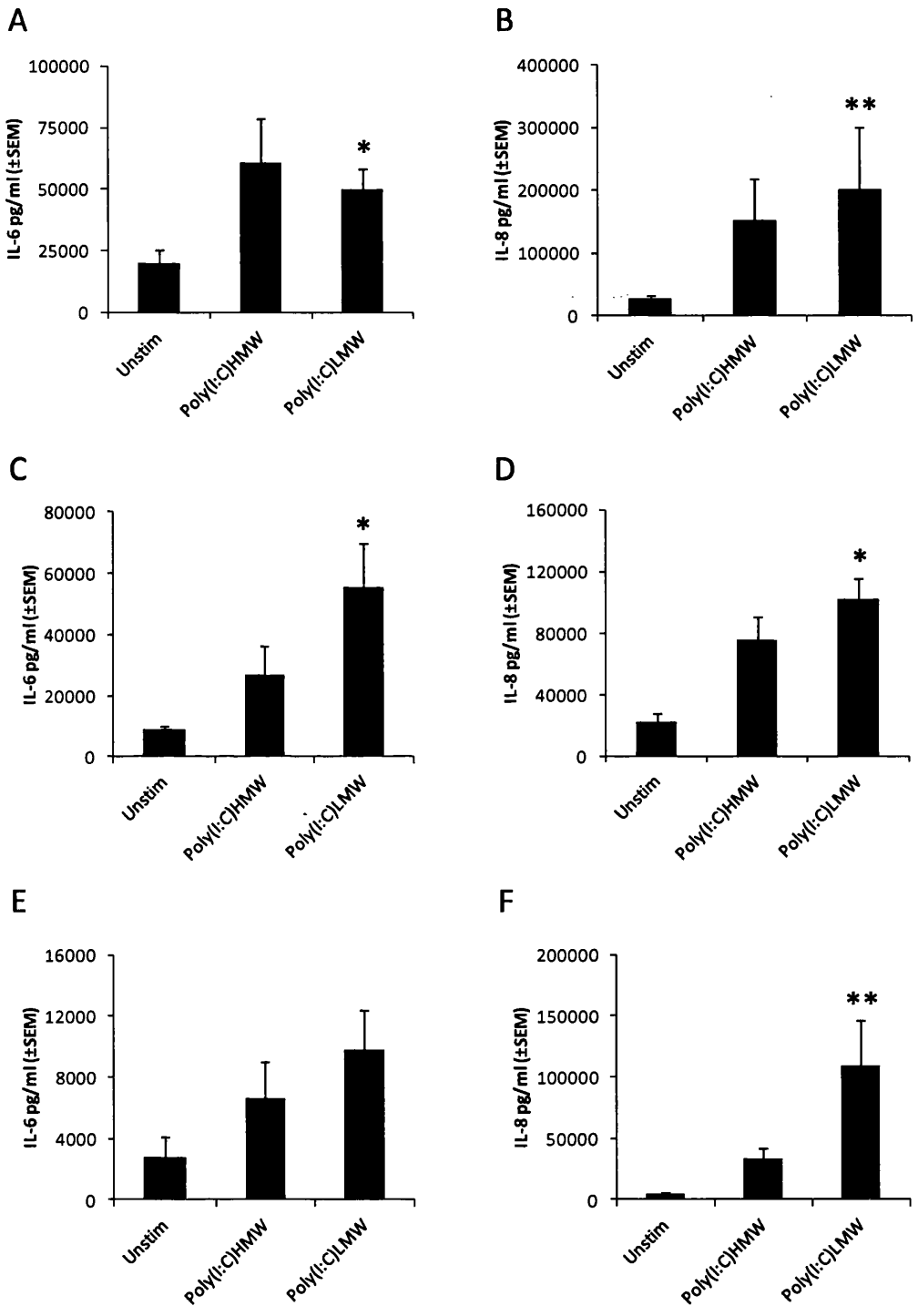

Figure 3.5 TLR3 agonist induced cytokine response by the term non-laboured placenta, choriodecidua and amnion.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua (E-F) amnion following stimulation with Poly(I:C)HMW and Poly(I:C)LWM (both 25 μg/ml; n=5). Statistical significance compared to unstimulated control as determined by Freidman's test with Dunn's posthoc test are shown: * $p \leq 0.05$, ** $p \leq 0.01$.

3.4.3.3 TLR4

To investigate if TLR4 is functional in the placenta, choriodecidua and amnion an ultrapure form of LPS isolated from *E. coli 0111:B4* was used. Since LPS is routinely used within the group a concentration of 10 ng/ml has been determined previously [233]. A significant increase in both IL-6 and IL-8 production in response to LPS is observed in all three tissues (Figure 3.6).

3.4.3.4 TLR5

To investigate if TLR5 is functional in the placenta, choriodecidua and amnion an ultrapure form of flagellin isolated from *Salmonella typhimurium* was used at a concentration of 100 ng/ml.A significant increase in both IL-6 and IL-8 production in response to flagellin is observed in all three tissues (Figure 3.7).

3.4.3.5 TLR7/8

To investigate if TLR7 and TLR8 were functional in the placenta, choriodecidua and amnion the agonists imiquimod (R837) and ssRNA40/LyoVec were used at an optimised concentration of 1 µg/ml (n=5). Imiquimod is a small synthetic antiviral molecule specific to TLR7 composed of an imidazoquinoline amine analogue to guanosine, while ssRNA40/LyoVec is a single stranded GU-rich oligonucleotide complexed with the transfection reagent LyoVec, specific for TLR8. In response to both Imiquimod and ssRNA40 a statistically significant increase in IL-8, but not IL-6 was observed in the placenta. In the choriodecidua only a statistically significant increase was observed for ssRNA40 induced IL-6, there was no significant increase in IL-8 in response to either agonist. Similarly to the placenta, the amnion produced a statistically significant increase in IL-8 in response to both agonists. Additionally, a significant increase in IL-6 production in response to imiquimod, but not ssRNA40 was observed in the amnion.

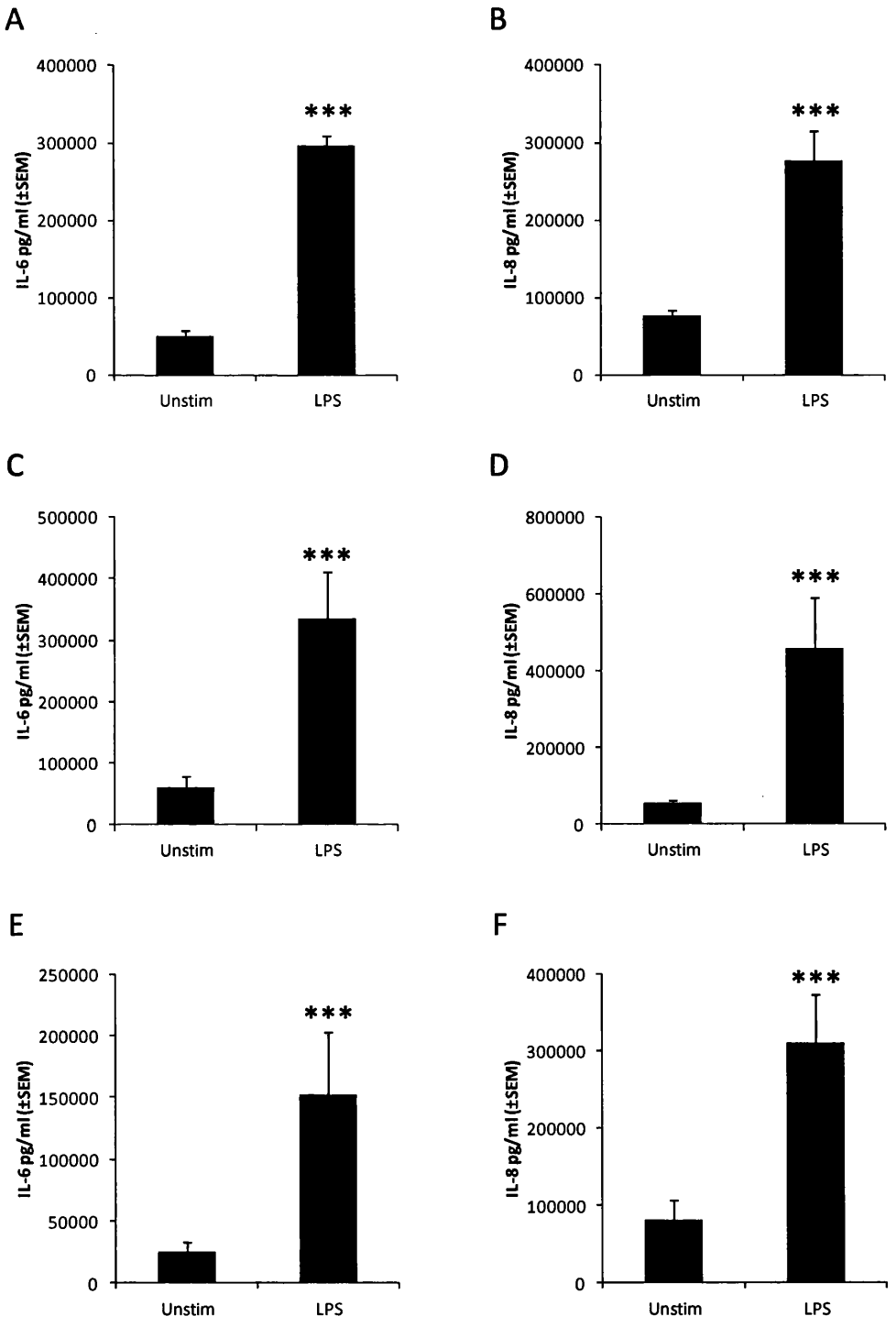

Figure 3.6 LPS induced cytokine response by the term non-laboured placenta, choriodecidua and amnion.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion following stimulation with 10 ng/ml of LPS (n=11). Statistical significance compared to unstimulated control as determined by Wilcoxon matched pairs signed rank test are shown: *** $p \leq 0.001$.

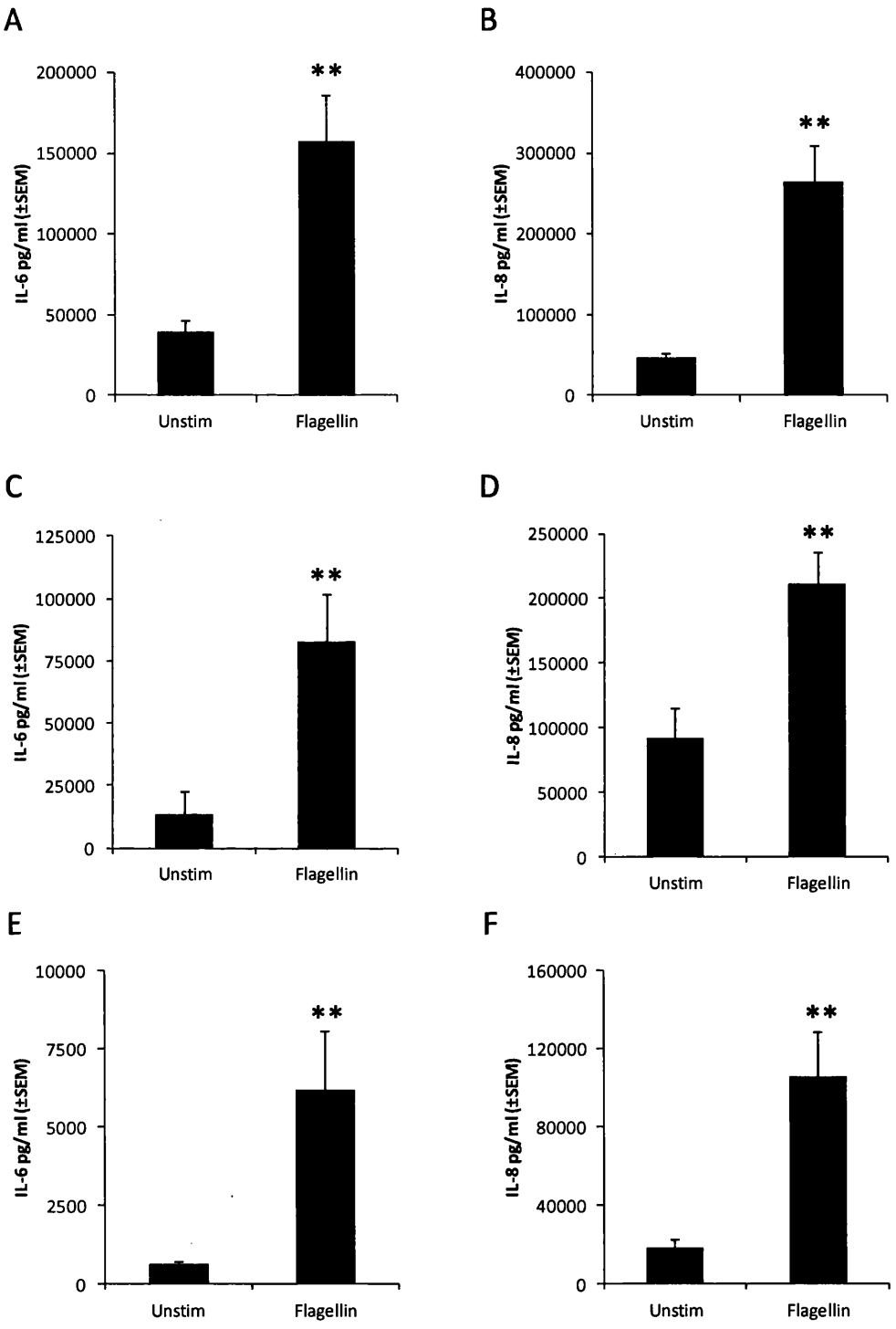

Figure 3.7 Flagellin induced cytokine response by the term non-laboured placenta, choriodecidua and amnion.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion following Flagellin (100 ng/ml; n=9). Statistical significance compared to unstimulated control as determined by Wilcoxon matched pairs test are shown: ** $p \leq 0.01$.

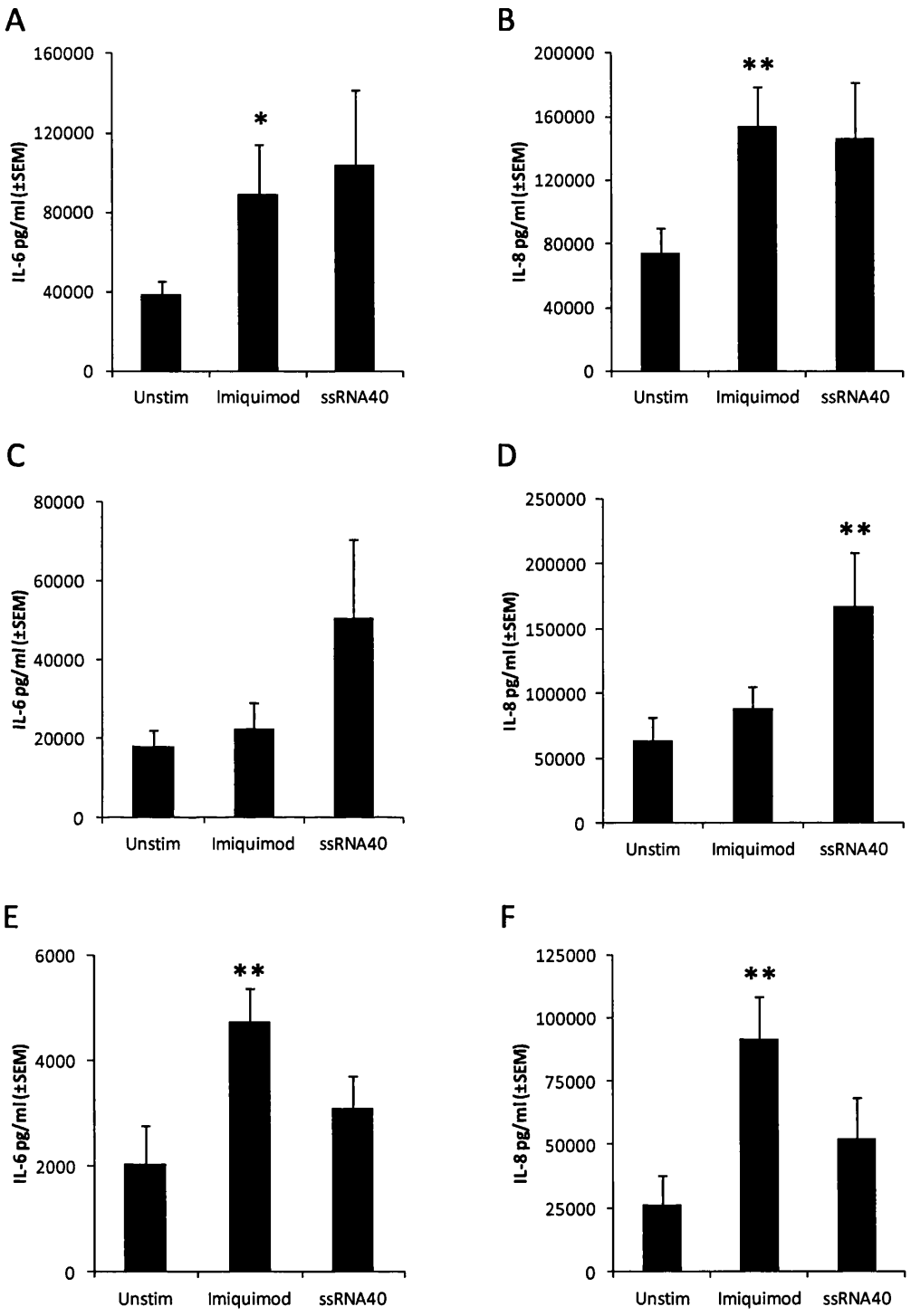

Figure 3.8 TLR7/8 agonist induced cytokine response by the term non-laboured placenta, choriodecidua and amnion.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua (E-F) amnion following stimulation with either imiquimod or ssRNA40 (both 1 μg/ml; n=5). Statistical significance compared to unstimulated control as determined by Freidman's test with Dunn's posthoc test are shown: * $p \leq 0.05$, ** $p \leq 0.01$.

3.4.4 Response of term non-laboured gestation-associated tissues explants to NLR agonists

To investigate if the NLRs, NOD1 and NOD2 were functional in the placenta, choriodecidua and amnion, the agonists L-Ala-γ-D-Glu-mDAP (Tri-DAP) and muramyl dipeptide (MDP) which are specific for NOD1 and NOD2 respectively were used. As shown in Figure 3.9, a significant increase in IL-6 and IL-8 production by the placenta is observed in response to Tri-DAP treatment. An increase in MDP induced IL-8 is also observed, however this was not significant. An increase in cytokine production is observed in response to both agonists by the choriodecidua, with a significant increase in Tri-DAP induced IL-6 and MDP induced IL-8. In the amnion, a significant increase in MDP induced IL-6 and Tri-DAP induced IL-8. Elevated IL-8 induced by MDP treated amnion is also observed.

3.4.5 Response of term non-laboured gestation-associated tissue explants to RLR agonists

To investigate if the RLRs, RIG-I and MDA5 were functional in the placenta, choriodecidua and amnion the agonist Poly(I:C)/LyoVec, a complex between the transfection reagent LyoVec and poly(I:C) was used. Transfected Poly(I:C) has been shown previously to be recognised by RIG-I/MDA5, unlike naked Poly(I:C) which is recognised by TLR3 [216, 283]. The results are shown in Figure 3.10. Elevated IL-8 production was observed in all three tissues in response to treatment with Poly(I:C)LyoVec, however this was not significant. In contrast, no difference was observed in relation to IL-6 production, by the placenta and amnion. Elevated IL-6 production was observed by the choriodecidua, but this was not significant.

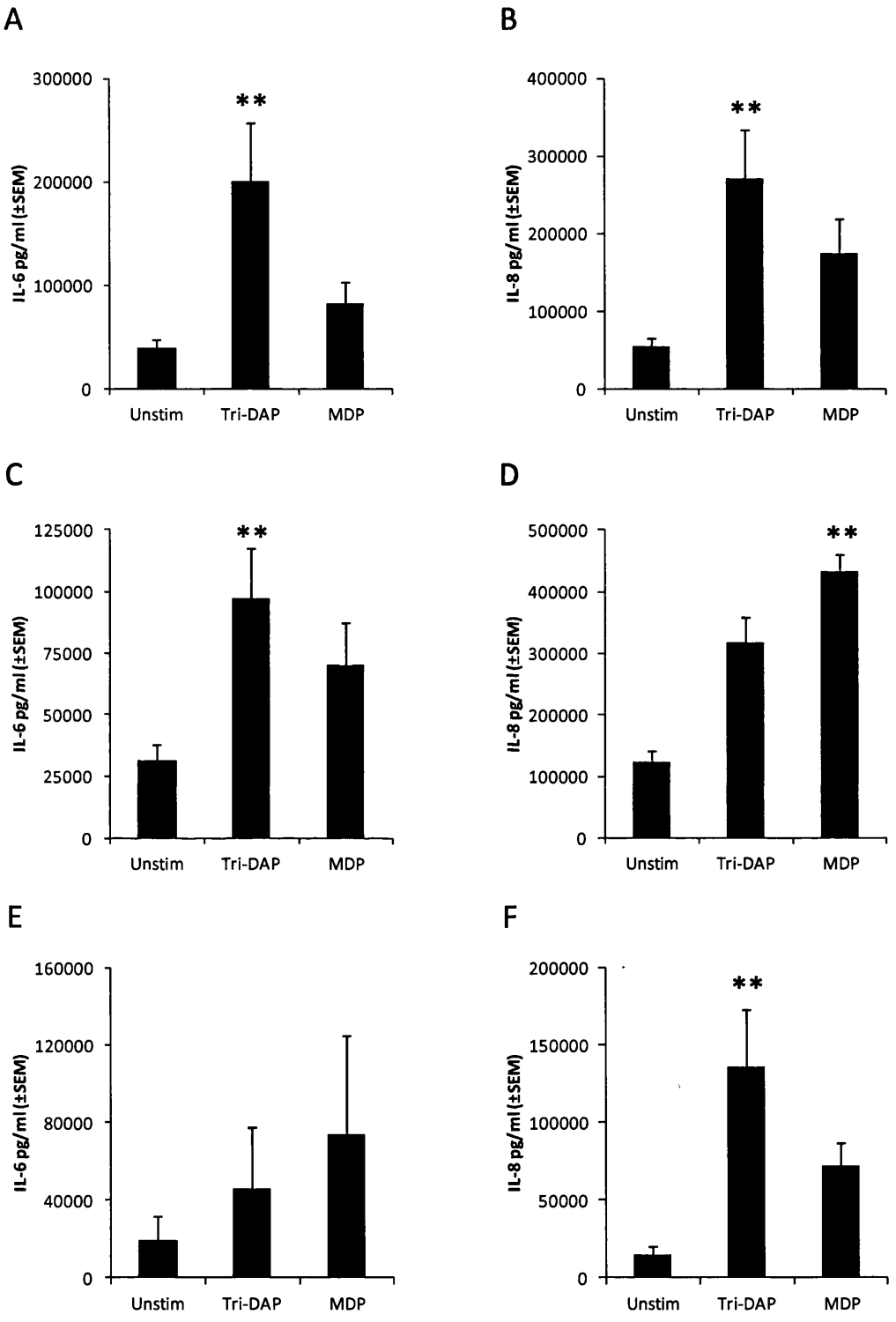

Figure 3.9 NOD1/2 agonist induced cytokine response by the term non-laboured placenta, choriodecidua and amnion.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua (E-F) amnion following stimulation with either Tri-DAP or MDP (both 10 µg/ml; n=6). Statistical significance compared to unstimulated control as determined by Freidman's test with Dunn's posthoc test are shown: * $p \leq 0.05$, ** $p \leq 0.01$.

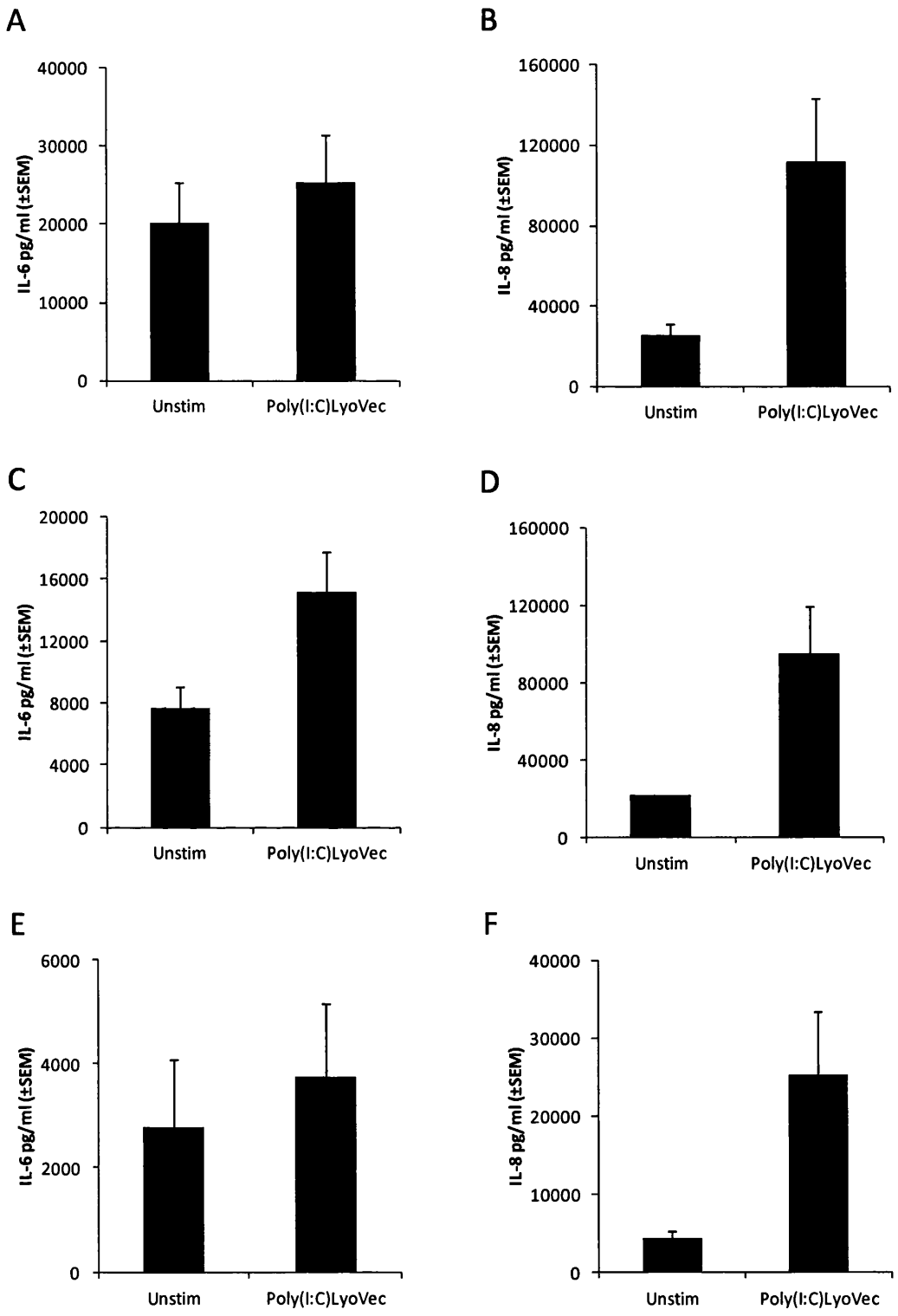

Figure 3.10 RIG-I/MDA5 agonist induced cytokine response by the term non-laboured placenta, choriodecidua and amnion

IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion following treatment with Poly(I:C)LyoVec (1 μg/ml; n=5). Statistical significance was determined by Wilcoxon matched pairs test. No significant difference was observed.

3.5 Discussion

Since changes in cytokine production at the maternal-fetal interface are a feature of both term and preterm labour, the mechanisms involved in their production by the placenta and attached membranes have gathered much interest. PRRs play a key role in the production of inflammatory mediators in response to microbial stimuli, it has therefore been suggested that PRRs have a central role in cytokine production by these tissues. With this in mind, the aim of this chapter was to examine whether several families of PRRs, including Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) were expressed and were able to elicit a cytokine response (IL-6 and IL-8) following stimulation with known agonists. The cytokines IL-6 and IL-8 were chosen due to their documented role in both term and preterm labour [11, 15, 25, 27] and as key cytokine produced following PRR activation [233, 284-286].

Work previously reported by the group had demonstrated the expression of TLR 1 – 10 in the term placenta with TLR 2-5 and 7/8 shown to be functional based on a significant increase in one or more of the cytokines analysed (IL-6, IL-8, IL-10 and TNFα) [233]. The data generated here mainly corresponds to this in regards to expression and function of TLRs by the placenta. In relation the functional response of the placenta to TLR agonists, the data shown here, demonstrates a significant increase in the production of either IL-6, IL-8 or both in response to Pam3CSK4 (TLR2/1), FSL-1 (TLR2/6), HKLM (TLR2) Poly(I:C)LWM (TLR3), LPS (TLR4), Flagellin (TLR5) and Imiquimod (TLR7), thus implying functional TLR 1-7 in the placenta. In comparison to previous studies where PGN is used as an activator of TLR2, this work utilised the specific agonists for the TLR2 heterodimers TLR2/1 and TLR2/6, thus implying for the first time that both are functional in the term placenta. However, neutralisation experiments are required to confirm this. While functional TLR7 and 8 has been previously reported utilising a dual agonist for both receptors, the work presented here would suggests that TLR8 is not functional as significant increase in cytokine production was not observed to the TLR8 agonist ssRNA40. However, this is likelya result of heterogeneity within the small sample population examined. Similarly, low sample number may offer an explanation as to why no significant cytokine production was observed to the TLR3 agonist Poly(I:C)HMW in comparison to Poly(I:C)LMW. However, it is not entirely surprising that a difference was observed between the two agonists, as these synthetic dsRNA molecules differ in size, therefore it's possible that the smaller LMW (0.2 – 1 kb) poly(I:C) gains

access to the endosomose located TLR3 with greater ease that the larger HMW (1.5 – 8 kb) poly(I:C).

Similarly to the term placenta, the work in this chapter demonstrates expression of transcripts for TLR 1 – 10 by the term choriodecidua and amnion, corresponding to that previously reported in decidual cells and amnion epithelial cells [240, 243, 287]. Functional TLR 1-6 and 8 are implied in the term choriodecidua as suggested by the significant increase in the production of either IL-6, IL-8 or both in response to Pam3CSK4 (TLR2/1), FSL-1 (TLR2/6), HKLM (TLR2) Poly(I:C)LWM (TLR3), LPS (TLR4), Flagellin (TLR5) and ssRNA40 (TLR8). In response to ssRNA40 only a significant increase in IL-8 was observed, possibly due to heterogeneity within low sample number examined. This is the first time functional response to ligands for TLR5 and TLR8 within these tissues have been described. Previous investigation showed only functional TLR1-4 and 6 in term decidual cells, while functional TLR5 and TLR8 have been shown in fetal membranes explant [242, 243, 288].

The difference in the response of isolated decidual cells in comparison to explants of choriodecidua or whole fetal membrane to TLR5 and TLR8 agonists highlights the importance of the model of investigation used. While a dissociated cell model has the advantage of greater efficiency and reproducibility, resulting from the generation of millions of cells from a single source, the activity of these cells in isolation is artificial and does not factor in the interactions between various cells found in any given tissue or how utilisation of signalling pathways may differ. The response to TLR5/TLR8 agonists might be the result of resident leukocytes (macrophages and neutrophils) within the choriodecidua. Leukocyte recruitment from the maternal circulation into the choriodecidua accomplished by selective chemotaxis has been proposed as a key step in the preparation for labour [14, 289, 290]. These resident leukocytes have been shown to secrete various pro-inflammatory cytokines including IL-1β, IL-8 and TNFα at both term and preterm, contribution to the inflammatory environment during labour [289, 291]. Since the expression of TLRs by macrophages and neutrophils has been well documented [292], it's more than likely that these cells contribute to the TLR agonist induced cytokine production by the choriodecidua. Alternatively, this might suggest differential expression of these receptor between the chorion and decidua at the protein level. Immunohistochemical studies would be needed to confirm protein expression and the cell populations expressing these receptors.

To date, TLR2/6, TLR4 and TLR5 have been reported to be functional in amnion epithelial cells, based on increased IL-6 an IL-8 in addition to the translocation of the NF-κB, p65 subunit. No induction of IL-6 or IL-8 was observed in response to agonist for TLR1/2, 7 and 9, while no TLR8 agonist was used [287]. The data presented here implies functional TLR2/1, TLR2/6, TLR3, TLR4, TLR5 and TLR7 as suggested by the significant increase in the production of IL-6, IL-8 or both in response to specific agonists. This data therefore verifies a functional response to TLR2/6, TLR4 and TLR5 agonists in the term amnion, in addition to showing a functional response TLR2/1, and TLR7 agonists.

While a functional response to TLR9 agonists (CpG ODN) was not examined during initial investigations, preliminary data recently generated (Chapter 8.3 – Figure 8.15) implies that TLR9 is not functional in the term non-laboured placenta, corresponding to previously published work by the research group [233]. While no impact was observed on IL-6 production in response to CpG ODN by the choriodecidua and amnion, a down-regulation of constitutive IL-8 was observed. A recent report noted and increase in MCP-1 and a inhibitory effect on the constitutive production of G-CSF, IFNγ, MIP-1α, MIP-1β, RANTES and VEGF in response to CpG ODN by human fetal membrane explants [288]. Therefore, activation of TLR9 may play a regulatory role within human fetal membranes.

Previous investigations of NLR expression by the placenta have been limited to primary first and third trimester trophoblast noting expression of transcripts and protein for both NOD1 and NOD2 in first trimester trophoblast, but only NOD1 in third trimester trophoblast. A corresponding functional response was observed [60, 236]. The data generated here show transcripts for both NOD1 and NOD2 in the term placenta. In response to treatment with Tri-DAP (NOD1) and MDP (NOD2) a significant increase in both IL-6 and IL-8 production was observed in response to Tri-DAP but not MDP. This data implies that in the term placenta that only NOD1 is functional, while NOD2 is not, corresponding to response of the third trimester trophoblast. It should be noted however that an increase in IL-8 production was noted in response to the NOD2 agonist MDP in the term cytotrophoblast, in a NOD2 independent manner [60]. Elevated levels of IL-8 in response to MDP were also observed here. In addition to NOD2, MDP has been shown to activate human NLRP1 and NLRP3; however these NLRs are primarily involved in inflammasome activation [293, 294]. While the data herein shows transcripts for both NLRP1 and

NLRP3 in the term placenta and others have demonstrated transcripts in both the first and third trimester trophoblast [238, 295], no significant induction of IL-1β was observed (See Chapter 8.3 – Figure 8.16) in response MDP stimulation of the term placenta, likely resulting from low sample. Additionally the term placenta expresses transcripts for other NLRs including NLRC4, NAIP and NLRX1 (Figure 3.3). Transcripts for NLRC4 have been reported previously reported in first trimester trophoblasts [238].

Recently, expression of both NOD1 and NOD2 has been reported in the fetal membrane, with expression of both up-regulated following spontaneous labour [286]. Further examination at the protein level noted expression of NOD1 and NOD2 by chorionic cytotrophoblasts and decidual cells. However, only protein for NOD1 was shown in amnion epithelial cells. Additionally fetal membrane explants in response to NOD agonist resulted in production of IL-6 and IL-8 [286]. The data generated here shows that both the term choriodecidua and amnion express transcripts for NOD1 and NOD2. Significantly elevated IL-6 production is observed in response to Tri-DAP by the choriodecidua, while elevated IL-8 is observed in response to MDP, implying both receptors are functional in the choriodecidua. Only Tri-DAP induced IL-8 is observed by the amnion, implying functional NOD1. Heterogeneity within the small sample population examined likely is the cause of the discrepancy between cytokines observed.

Transcripts for the inflammasome associated NLRs, NLRP1, NLRP3 and NLRC4 have been reported in the first trimester decidual stromal and endothelial cells [238]. The data reported here demonstrate the expression of these NLRs in the term choriodecidua and amnion in addition to the NLRs, NAIP and NLRX1.

To date, the examination of the expression and function of RLRs in human gestation associates tissues is relatively limited. It has previously been reported that third trimester placenta and decidua express RIG-I, MDA5 and LGP2 [296]. This work demonstrates the expression of transcripts for the three described RLRs by the placenta, choriodecidua and amnion. Immunohistochemical studies are needed to confirm protein expression and the cell populations expressing these receptors. Tissues stimulated with the agonist Poly(I:C)LyoVec (RIG-I/MDA5) did not elicit a significant production of their IL-6 or IL-8, implying that the RLRs are not functional is gestational tissues. However elevated levels of IL-8 were observed by all three

tissues in response to treatment, and with increased sample number may be significant. RLR induced IL-8 production may play a role in neutrophil recruitment to gestational tissues. IL-8 is a key chemokine involved neutrophil chemotaxis [297]. During infection neutrophils are one of the first leukocytes to be recruited from the circulation into tissues, to mediate bacterial clearance. Neutrophil recruitment is also a feature of viral infection, were they have been implicated in providing protection from viral infection by the release of neutrophil extracellular traps and the production of type I interferons [298, 299]. Therefore IL-8 production by RLRs and other viral sensing PRRs (TLR3/7/8) which also upon activation induce IL-8 production, maybe a key step in promoting viral clearance in gestational tissues. Since Poly(I:C)LyoVec is a dual agonist neutralisation experiments would be required to determine if either RIG-I, MDA5 or both are potentially functional. Additionally quantitative PCR would be required to examine the role of LGP2, as there is no agonist for this receptor due to regulatory function in RIG-I and MDA5 signalling [217].

In summary, this work clearly demonstrates that human term gestation associated tissues mount a functional response to known ligands of a variety of PRRs. Further work is required to reveal the PRR expressing cells within these tissues. In addition, work is required to examine in detail PRR-mediated signalling pathways in these tissues in response to each PRR agonist and the cross-talk resulting from the activation of multiple receptors. Subsequent chapters will start examining these, focusing on C-type lectin receptors (CLRs) and the signalling pathways involved in flagellin recognition in addition to examining modulators of cytokine production induced by PRRs.

Chapter 4

Expression and activity of CLRs, in human gestation-associated tissues and the innate immune response to *Candida albicans*

4 Expression and activity of CLRs in human gestation associated tissues and the innate immune response to *Candida albicans*

4.1 Introduction

Vulvovvaginal candidiasis (VVC) or vaginal thrush is a common reproductive infection and only second to bacterial vaginosis as a cause of vaginal inflammation [300]. More than 75% of women of reproductive age will be diagnosed with VVC at least once during their lifetime, with recurrence occurring in 40-50% of women, while 5-8% will suffer with recurrent (R) VVC (three or more episodes) [301, 302]. *Candida albicans* are a frequent cause of VVC [303]. Several risk factors have been described for VVC, including steroid and immunosuppressive therapy, diabetes and pregnancy [302, 304]. The higher occurrence of VVC and RVVC in pregnant women compared to non-pregnant is associated with changes in reproductive hormone levels e.g. estrogens. As the concentrations of estrogens increase, glycogen production in vaginal tissues increases, providing *Candida* species a source of carbon for growth [305, 306]. Additionally estrogens enhance the adherence of *Candida* to the muscosal surface of the vagina [301]. While colonisation of the amniotic cavity by C*andida* is rare, the consequences of intra-amniotic *Candida* infections include preterm labour (PTL) and preterm rupture of membranes (PROM), neonatal infection and fetal death [52, 307].

Candida albicans and other *Candida* species are dimorphic fungal organisms that at any given time asymptomatically colonises 30-50% of healthy individuals; however under certain conditions they can cause a broad spectrum of muscosal and systemic infections [308]. While several virulence factors are important in the pathogenesis of *Candida*, the role of the cell wall is critical, as the first point of contact with the host [309]. The *Candida* cell wall is composed of numerous polysaccharide structures such as mannoproteins (N-linked and O-linked), chitin and β-glucans (β-1,3 glucan and β-1,6 glucan) that function as pathogen associated molecular patterns (PAMPs) recognised by pattern recognition receptors (PRRs) of the innate immunes system [202, 207, 310]. Several groups of PRRs play role in anti-candida defence; including Toll-like receptors (TLRs), NOD-like receptors (NLRs) and Scavenger receptors, C-type lectin receptors (CLRs) are the primary contributors (Table 4-1). Despite the prevalence of *Candida albicans* infection during pregnancy and the possible associated pregnancy complications of intra amniotic colonisation, very little is in known about CLRs at the maternal-fetal interface.

Table 4-1 Pattern recognition receptors involved in anti-*Candida* immunity.

PRR	Group	Fungal PAMP
TLR2	TLR	Phospholipomannan
TLR4	TLR	Mannan
TLR9	TLR	CpG Oligodecoxynucleotides
Dectin-1	CLR	β-Glucan
Dectin-2	CLR	α-mannans, O-linked mannobiose rich glycoproteins
MINCLE	CLR	α-mannos, glyceroglycolipids
DC-SIGN	CLR	High Mannose Structures
Mannose Receptor	CLR	Mannan, N-linked Mannose Residues
Galectin-3	CLR	β-Mannosides
SCARF-1	SR	β-Glucan
CD36	SR	β-Glucan
NLRP3	NLR	Unknown - activation via Dectin-1 induced ROS

Several pattern recognition receptors from the Toll-like receptor, C-type lectin receptor, NOD-like receptor and scavenger receptor families have been implicated in the innate immune response to *candida albicans*. Adapted from [97] and updated with information from [311-314]. CLR, C-type lectin receptor; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; F, forward primer sequence; MINCLE, macrophage-inducible C-type lectin; NLR, NOD-like receptor; NLRP, Nod-like receptor containing a PYD domain; ROS, reactive oxygen species; SCARF-1, scavenger receptor class F, member 1, SR, scavenger receptor; TLR, Toll-like receptor.

4.2 Aims

The aim of this chapter was to examine the innate immune response to fungal-derived PAMPs and to *Candida albicans*, particularly the expression and function of CLRs in the term non-laboured human placenta, choriodecidua and amnion.

4.3 Methods

4.3.1 Samples

Healthy pregnant women scheduled for elective caesarean section (ESC) were approached in the antenatal day assessment unit at Singleton Hospital, Swansea, during their pre-anaesthetic assessment. Informed written consent was obtained upon explanation of the study. Following delivery, placenta and attached fetal membranes (n = 3-12) from these women were collected and processed within 1.5 hours of delivery. Peripheral blood (n = 3) was also collected from healthy adults at the Institute of Life Science, Swansea University who were approached and consented as controls.

4.3.2 PCR

Gestation associated tissue samples (placenta, choriodecidua and amnion) were stored in TRI reagent and frozen within 30 minutes of collection. RNA was extracted using the Trizol method and cDNA was subsequently produced following DNase treatment (see Chapter 2.4 for method). PCR was performed for CLRs and scavenger receptors (see Chapter 2.4 for method and Table 4-2 for primer details).

4.3.3 Gestation-associated tissue explants

Placenta, choriodecidua and amnion tissue explants were cultured as described in Chapter 2.2. Cultures were initially stimulated with several ligands at various concentrations (Table 4-3) to identify an optimum concentration of each agonist. Once identified the optimised concentration was used in subsequent in vitro culture experiments. Cultures were incubated for 24 hours. For inhibition/neutralisation experiments cultures were incubated for 30 minutes prior to the addition of agonist with the following blocking antibodies or inhibitors; anti-hDectin-1 IgG1 (5 µg/ml, Invivogen), Mouse IgG1 isotype (5 µg/ml, Invivogen), piceatannol (Syk inhibitor, 100 µM, Tocris), BAY11-7082 (IκB-a inhibitor, 50 µM, Merck Chemicals), butylated hydroxyanisole (BHA, ROS inhibitor, Sigma), Z-WEHD-FMK (Caspase-1 inhibitor, 5uM, R&D Systems), Z-IETD-FMK (Caspase-8 inhibitor, 5µM, R&D Systems).

Table 4-2 CLR primer sequences.

Gene		Primer	Mg^{2+} Conc (nM)	Anneling Temp (°C)	Fragment Size (Bp)	Accession Number
Dectin-1 Variant a	*F*	5' CTGTGGTCCTGGGTACCATGGCT	1.5	69	388	NM_197947.2
	R	5' ACCTCAGTCTGGGGCCGAGAAAG				
Dectin-1 Variant b	*F*	5' TCCTGGGTACCATGGGGGTTCT	2	60	234	NM_022570.4
	R	5' GGGGCCGAGAAAGGCCTATCCAA				
Dectin-2	*F*	5' ATGGTGAAACTGGCAAAAGG	2.5	67	205	NM_001007033
	R	5' ACCAAATGTGCTCCCATCTC				
MINCLE	*F*	5' TGGGTGGACGGCACACCTTTG	1.5	70	88	NM_014358
	R	5' TGGCACAGTCCTCCAGGGTAGC				
DC-SIGN	*F*	5' GACTGCAGCAGCTGGGCCTC	1.5	60	147	NM_021155
	R	5' GGAGCCCAGCCAAGAGCGTG				
Galectin-3	*F*	5' GAGCCAGCCAACGAGCGGAA	2	70	658	NM_002306
	R	5' CTGCAACCTTGAAGTGGTCAGGTTC				
Mannose Receptor	*F*	5' GCCCACGACGACTCCCGAAC	3.5	70	580	NM_002438
	R	5' CCTCTTGCAAAACGCTCGCGC				
SCARF-1	*F*	5' CCTCAGCTCCCACAAGCTAC	1.5	59	152	NM_003693
	R	5' GAACCTCATCTGCCTCTCCA				
CD36	*F*	5' AGGCCCTGCATTCCTGTCATCCT	1.5	70	992	NM_001001548
	R	5' TGGCGGCTATCATAAGGAGGAGGC				

Sequences and optimum conditions for each pair of C-type lectin receptors (CLRs) primers used for PCR. Bp, base pairs; CD, cluster of differentiation; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; F, forward primer sequence; MINCLE, macrophage-inducible C-type lectin; R, reverse primer sequence; SCARF-1, scavenger receptor class F, member 1.

Table 4-3 CLR ligands.

Ligand	Receptor Targeted	Concentrations Tested
Curdlan	Dectin-1	1-100 µg/ml
Depleted Zymosan	Dectin-1	1-100 µg/ml
TDB	MINCLE	1-100 µg/ml

Ligands at various concentrations were tested on placenta, choriodecidua and amnion to identify an optimum concentration of each agonist. MINCLE, macrophage-inducible C-type lectin; TDB, Trehalose-6, 6-dibehenate.

4.3.4 Tissue lysates

Total protein was extracted from placenta and choriodecidua explants treated with curdlan after 3h, 6h and 18h. An unstimulated control at each time point and T0 tissue was also included (see chapter 2.5.1 for method). Total protein was estimated using the BCA assay (see chapter 2.5.2 for method).

4.3.5 Peripheral blood mononuclear cell cultures

Mononuclear cells (n=3) were isolated as described in chapter 2.3. Cultures were stimulated with LPS (10 ng/ml) and a range of Trehalose-6,6-dibehenate (TDB) concentrations (1-100 µg/ml).

4.3.6 Cytokine production

IL-1β, IL-6 and IL-8 in the tissue free supernatants of placenta, choriodecidua and amnion explant cultures collected after 24 h were measured using commercially available ELISA kits (DuoSet, R&D Systems) as per manufacturer's instructions. IL-1β in tissue lysates and corresponding tissue free supernatants of placenta and choriodecidua collected after 3h, 6h and 18h were measured.

4.3.7 Cytotoxicity assay

As outlined in chapter 2.2.4, cytotoxicity of all inhibitors was evaluated utilising a WST reagent for detection of LDH in tissue free supernatants (LDH-Cytotoxicity Assay Kit II, Abcam).

4.3.8 Caspase-1/8 activity

The activity of caspase-1 and caspase-8 was measured in tissue lysates at T0 and after 3h, 6h and 18h of culture in the presence of absence of with curdlan using commercially available colorimetric kits (Caspase-1 Assay Kit and Caspase-8 Assay Kit, Both Abcam) as per manufacturer's instructions (see chapter 2.7 for method). Active caspase-1 and active caspase-8 (Both Abcam) were used as positive controls.

4.3.9 Statistical analysis

Agonist mediated cytokine production by non-laboured tissues was evaluated by Wilcoxon matched pairs signed rank test or Freidman's test with Dunn's posthoc test for multiple comparisons. A p-value of ≤ 0.05 was considered significant.

4.4 Results

4.4.1 Expression of CLRs and scavenger receptors by gestation-associated tissues

To investigate whether transcripts for a variety of CLRs and scavenger receptors, including Dectin-1 (variant a & b), Dectin-2, MINCLE, DC-SIGN, Galactin-3, Mannose Receptor, SCARF-1 and CD36 are expressed in gestation associated tissues, a PCR was performed using 5 individual samples of each tissues type (placenta, choriodecidua and amnion); 3 examples of each tissue is shown in Figure 4.1. Transcripts for each PRR examined were present in all samples in each of the tissues.

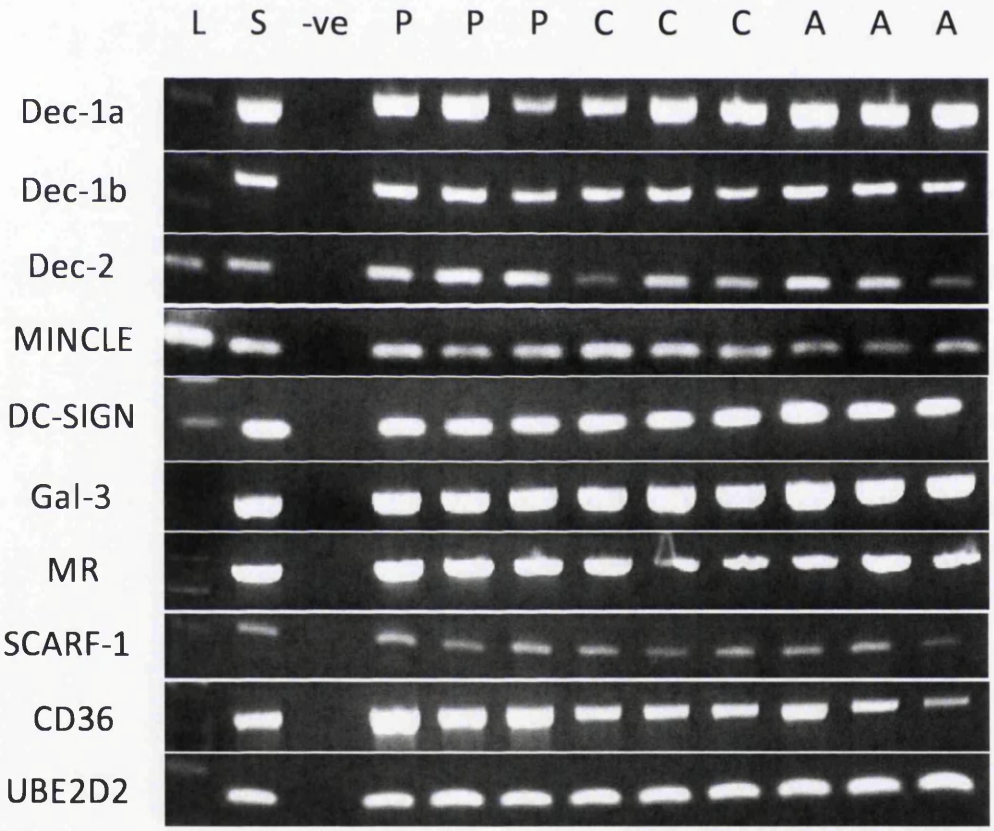

Figure 4.1 Expression of transcripts for CLR and scavenger receptors by gestation Associated tissues.

RNA was extracted from placenta (P), choriodecidua (C) and amnion (A), reverse transcribed and then used for PCR of CLR and scavenger receptor family member. Three representative examples of a total of 5 are shown. Total RNA from human spleen (S) was used as a positive control and UBE2D2 was used as a housekeeping gene. L = 100 base pair ladder and the negative control (-ve) was with water replacing cDNA in the reaction mix.

4.4.2 Response of term non-laboured gestation-associated tissue explants to CLR agonists

4.4.2.1 Optimisation of CLR agonists

In order to investigate the functional activity of CLRs in the placenta, choriodecidua and amnion, preliminary investigations (n=3) were performed using distinct CLR agonists, to determine an optimised concentration of each agonist for subsequent experiments. In doing so a range of concentrations, based on the manufacturer's recommendations was tested for each agonist (Table 4-4) on all three tissues. If no significant outcome was observed at any concentration, a concentration for further experiments was chosen based on its ability to provide the greatest increase for both IL-6 and IL-8 for all three tissues. Optimised concentrations of each agonist are summarised in Table 4-4 with each dose response shown in the appendix (Chapter 8.3, Figure 8.17 – 8.20).

Table 4-4 Optimised concentrations of Dectin-1 and MINCLE agonists.

Ligand	Receptor Targeted	Optimised Concentration
Curdlan	Dectin-1	100 µg/ml
Depleted Zymosan	Dectin-1	100 µg/ml
TDB	MINCLE	100 µg/ml

Concentrations of agonists for Dectin-1, as determined following preliminary investigations. MINCLE, macrophage-inducible C-type lectin; TDB, Trehalose-6,6-dibehenate.

4.4.2.2 Dectin-1

To investigate if Dectin-1, a receptor involved in the recognition of β-glucans is functional in the placenta, choriodecidua and amnion, the agonists depleted zymosan, a cell wall preparation from *Saccharomyces cerevisiae* treated with hot alkali to remove TLR2 stimulating properties and curdlan, a β-1,3-glucan from *Alcaligenes faecalis* and were used. The results are shown in Figures 4.2. Elevated IL-6 and IL-8 was observed in response to both depleted zymosan and curdlan in the placenta, however a significant increase was not observed for depleted zymosan induced IL-8. Similarly, in response to both agonists elevated cytokine levels were observed in response to both agonists by the choriodecidua. A difference in the responsiveness of the amnion to both agonists was observed, with elevated cytokine production only occurring following treatment with curdlan.

4.4.2.3 MINCLE

To investigate if MINCLE, a receptor involved in the recognition of trehalose-6,6-dimycolate (TDM), better known as cord factor, the agonist trehalose-6,6-dibehenate (TDB), a synthetic analogue of TDM, which has been shown to bind MINCLE was used [315]. The results are shown in Figures 4.5. In response to TDB treatment no effect was observed in any of the three tissues. To confirm the bioactivity of TDB, peripheral blood mononuclear cells (PBMCs) isolated from healthy adult donors (n=3) were treated with varying concentrations of TDB. An LPS positive control was also included (Figure 4.4). A significant increase in both IL-6 and IL-8 production from PBMCs was observed in response to LPS only and not any concentration of TDB tested.

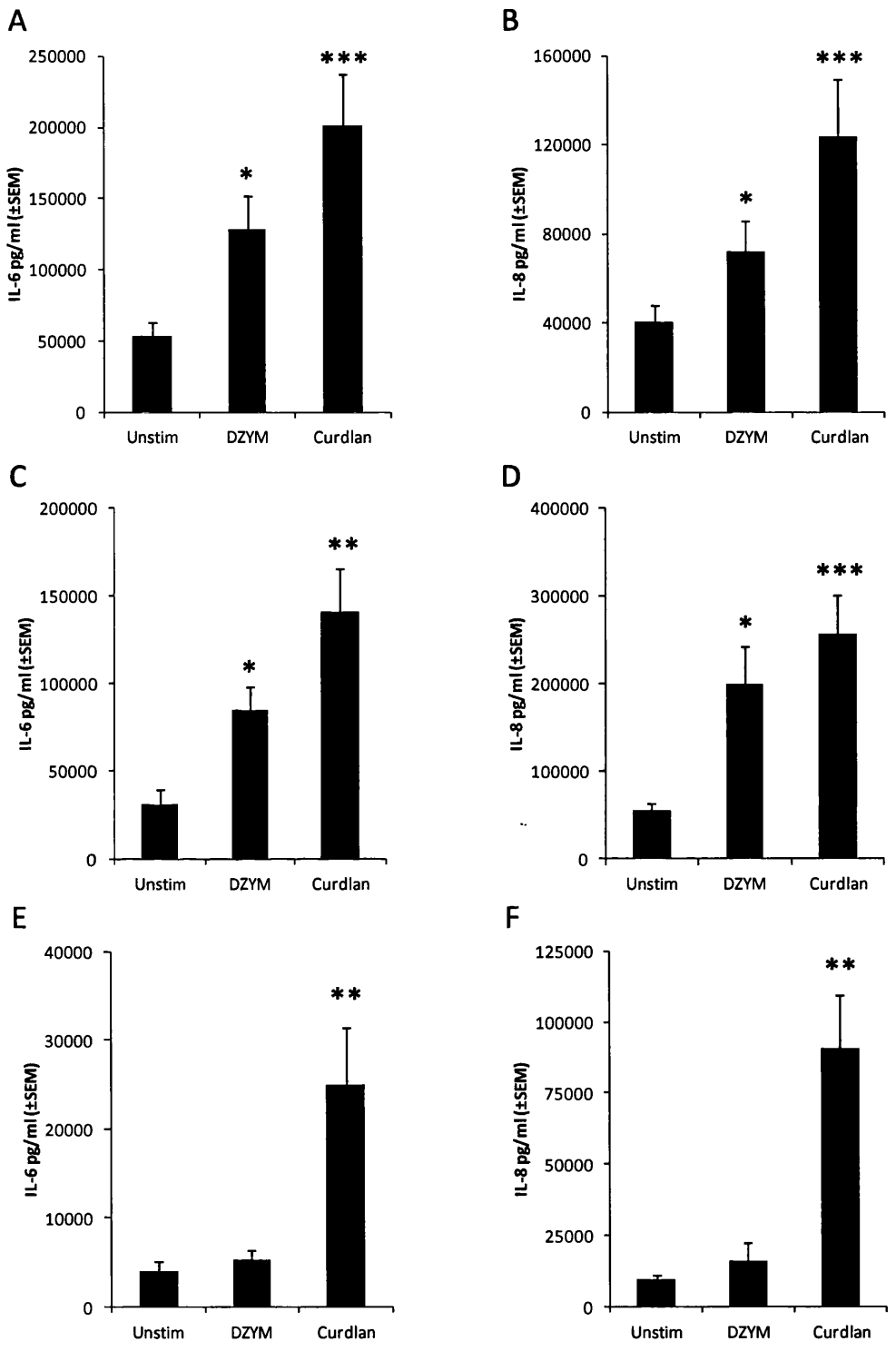

Figure 4.2 Dectin-1 agonist induced cytokine response by the term non-laboured placenta, choriodecidua and amnion.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion following stimulation with depleted zymosan (DZYM) and curdlan (both 100 µg/ml; n=9). Statistical significance compared to unstimulated control as determined by Freidman's test with Dunn's posthoc test is shown: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

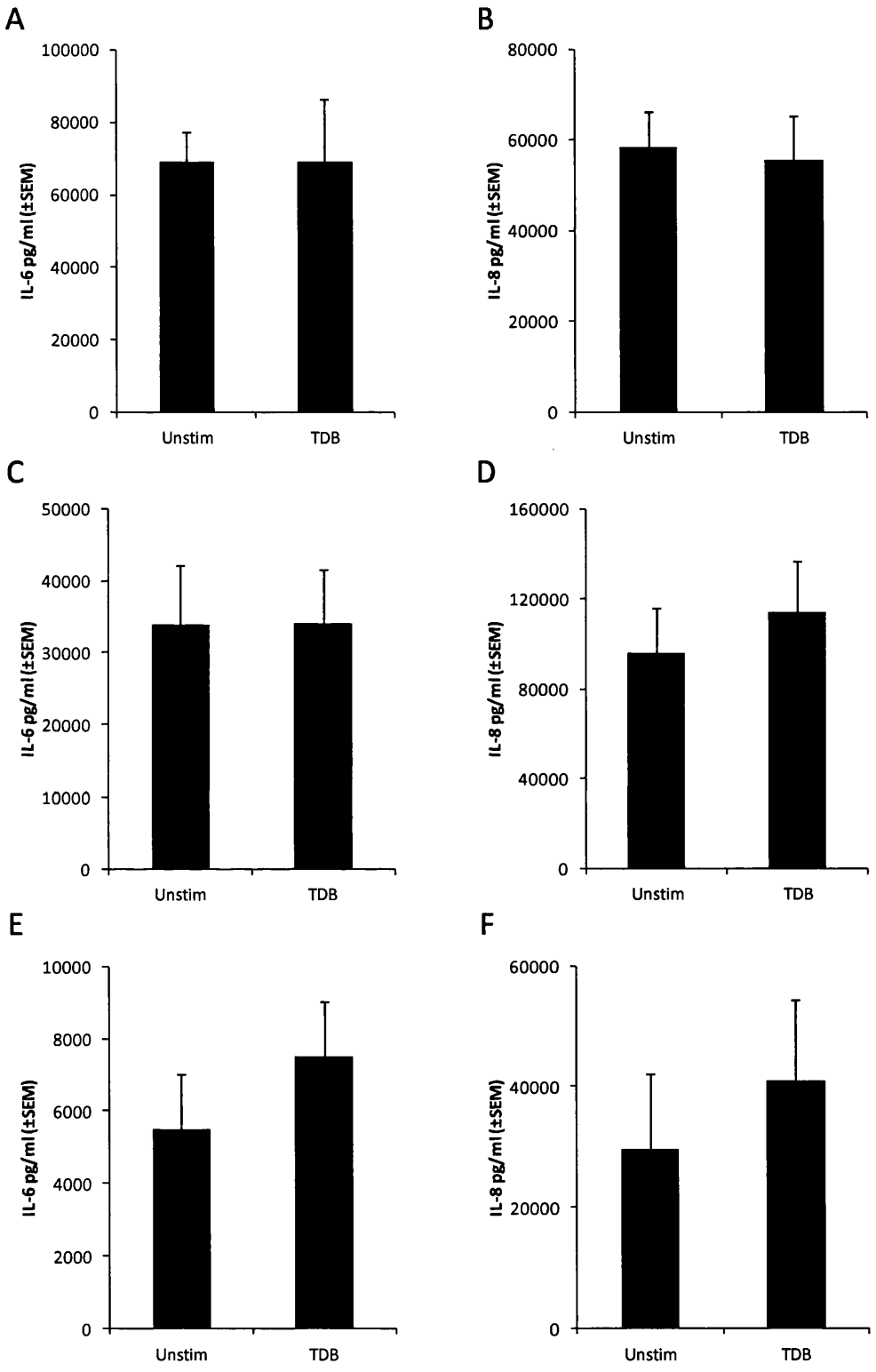

Figure 4.3 MINCLE induced cytokine response by the term non-laboured placenta, choriodecidua and amnion.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion following stimulation with 100 μg/ml of trehalose-6,6-dibehenate (TDB; n=11). Statistical significance was determined by Wilcoxon matched pairs test. No significant difference was observed.

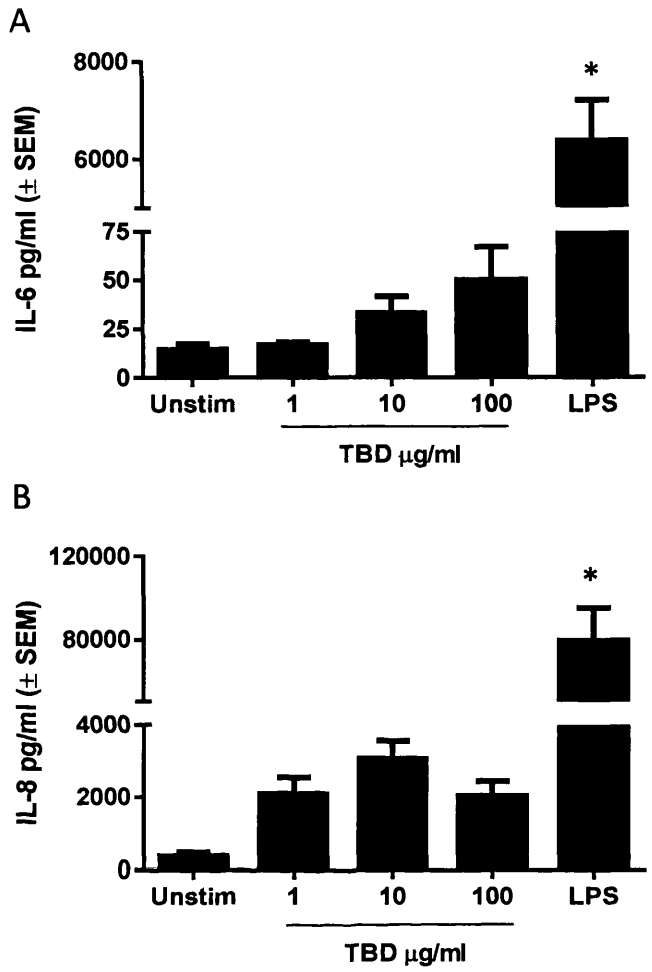

Figure 4.4 Cytokine response by peripheral blood mononuclear cells to LPS and varying doses of TDB.

IL-6 and IL-8 production (pg/ml mean ± SEM) by peripheral blood mononuclear cells (n=3) in response to varying concentrations of TDB (1 – 100 µg/ml) and LPS (10 ng/ml). Statistical significance compared to unstimulated control as determined by Freidman's test with Dunn's posthoc test are shown: * $p \leq 0.05$.

4.4.3 The Dectin-1 signalling pathway

Activation of Dectin-1 by the appropriate agonist leads to a series of signalling events involving notably spleen tyrosine kinase (Syk) and the transcription factor NF-κB [198, 199, 206].

4.4.3.1 Dectin-1 activation

To confirm the role of Dectin-1 in the pro-inflammatory response triggered by curdlan and depleted zymosan, term non laboured tissues were treated with both Dectin-1 agonists and LPS in the presence and absence of a neutralising monoclonal anti-human Dectin-1 mouse IgG1 antibody (Figure 4.5). Mouse IgG1 isotype was used as a control. In this preliminary investigation, no inhibition of cytokine production was observed in response to either curdlan or depleted zymosan treatment in the presence of the neutralising antibody.

4.4.3.2 Spleen tyrosine kinase (Syk) activation

To examine the role of Syk in the curdlan and depleted zymosan induced pro-inflammatory response in the placenta, choriodecidua and amnion, tissues were treated with both agonists in the presence or absence of the Syk inhibitor, piceatannol (PIC) for 24 hours. The optimum concentration of the inhibitor was determined following initial dose course (see Chapter 8.3 – Figure 8.21), corresponding to previously published use of the inhibitor [316]. As shown in Figure 4.6, treatment with piceatannol resulted in decreased agonist induced cytokine production by the placenta. However this was not significant. A similar trend is observed following piceatannol treatment of both the choriodecidua (Figure 4.7) and amnion (Figure 4.8). Treatment with piceatannol did not impact tissue viability as observed by LDH assay.

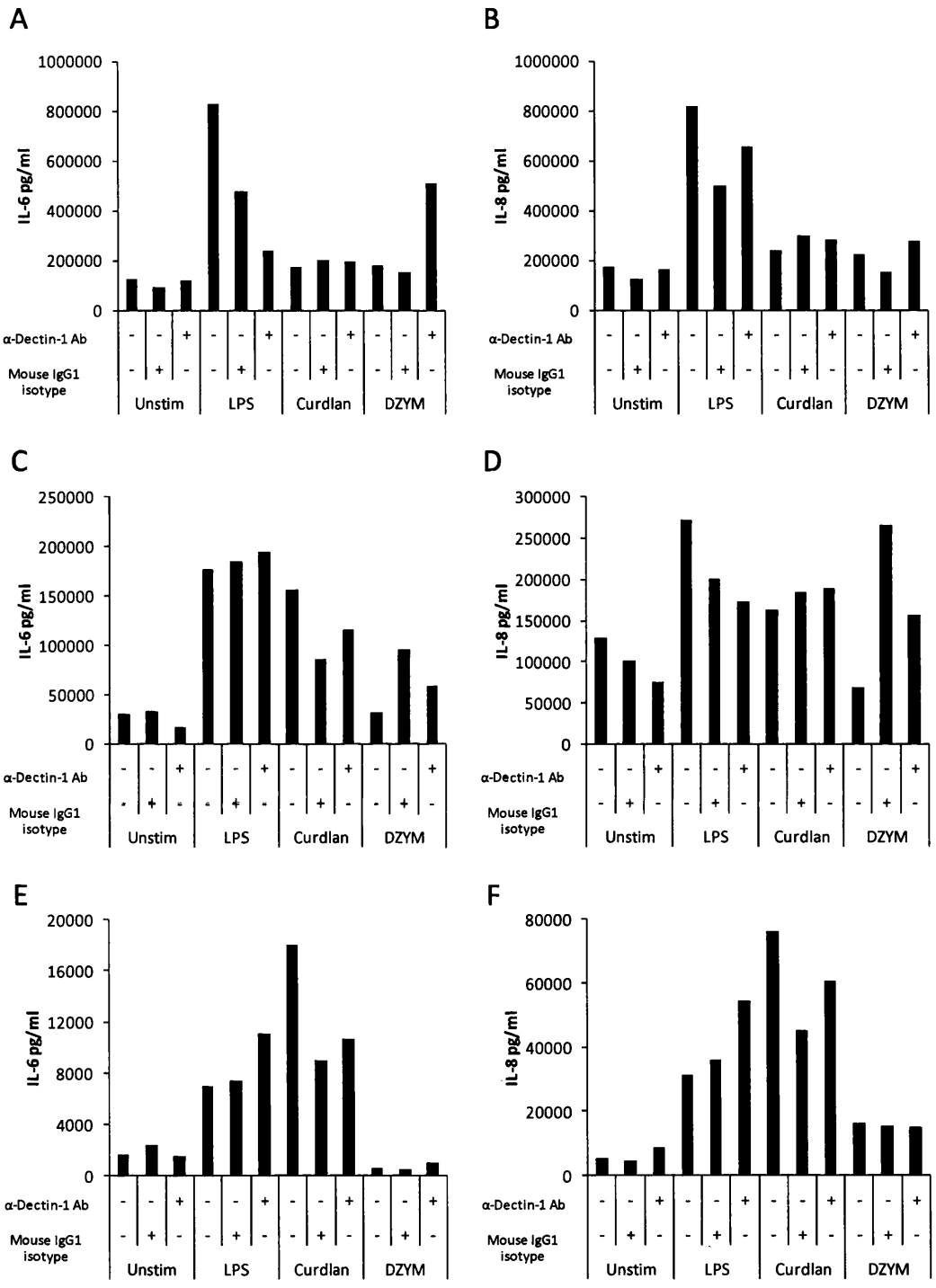

Figure 4.5 Dectin-1 neutralisation in term non-laboured gestation associated tissues.

Explants of (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion were stimulated with LPS (10 ng/ml), curdlan (100 µg/ml) and depleted zymosan (DZYM, 100 µg/ml) in the presence (5 µg/ml) of anti-human Dectin-1 mouse IgG1 antibody or mouse IgG1 isotype (n=1) and IL-6 and IL-8 levels (pg/ml) in tissue free supernatants measured.

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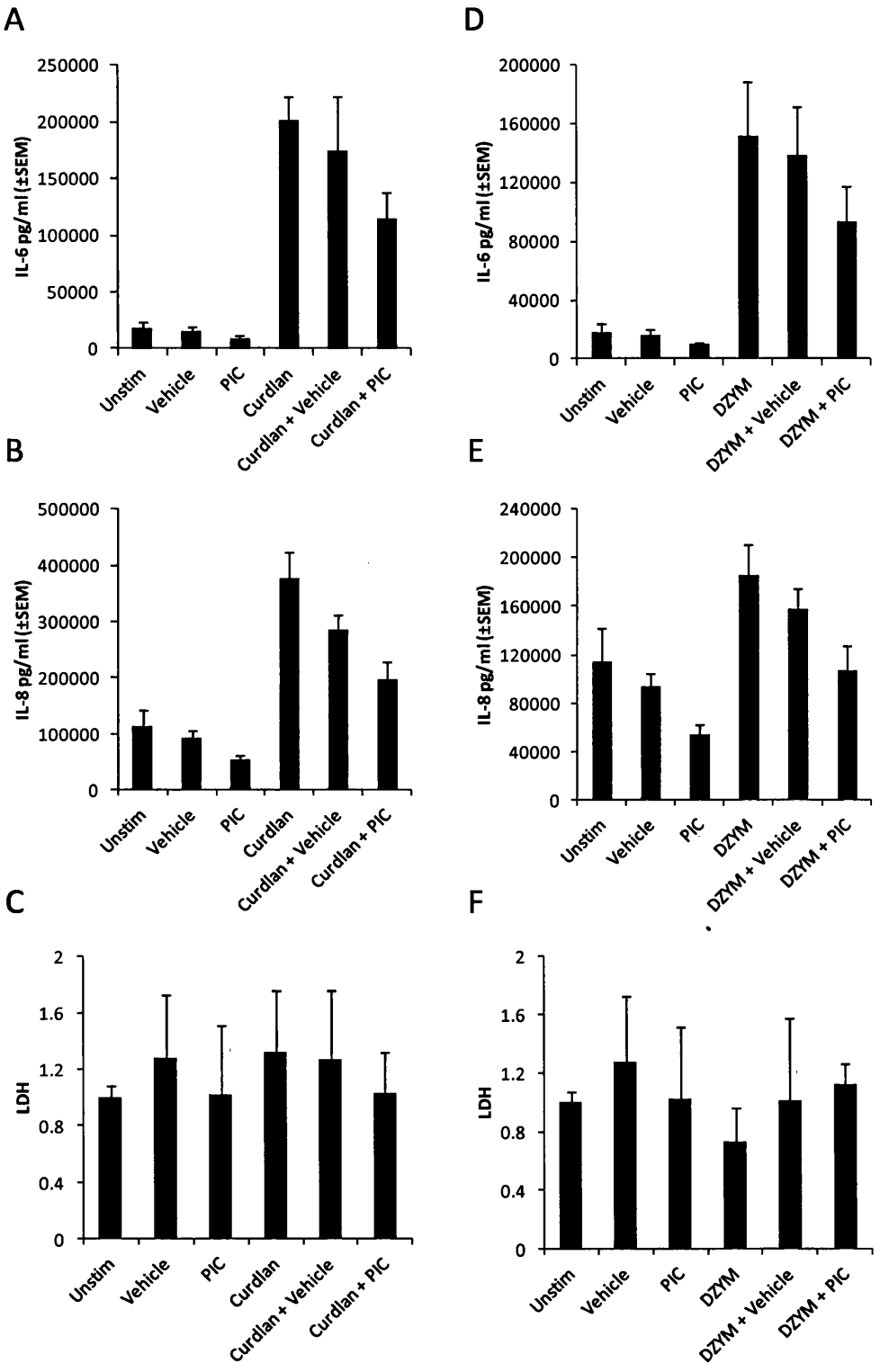

Figure 4.6 Syk inhibition in the term non-laboured placenta.

IL-6 & IL-8 production (pg/ml mean ±SEM) by the placenta, following stimulation with (A-B) curdlan or (D-E) depleted zymosan (both 100 μg/ml, n=3) in the presence or absence of 100 μM piceatannol. (C &F) Relative lactate dehydrogenase (LDH) levels compared to the unstimulated. Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

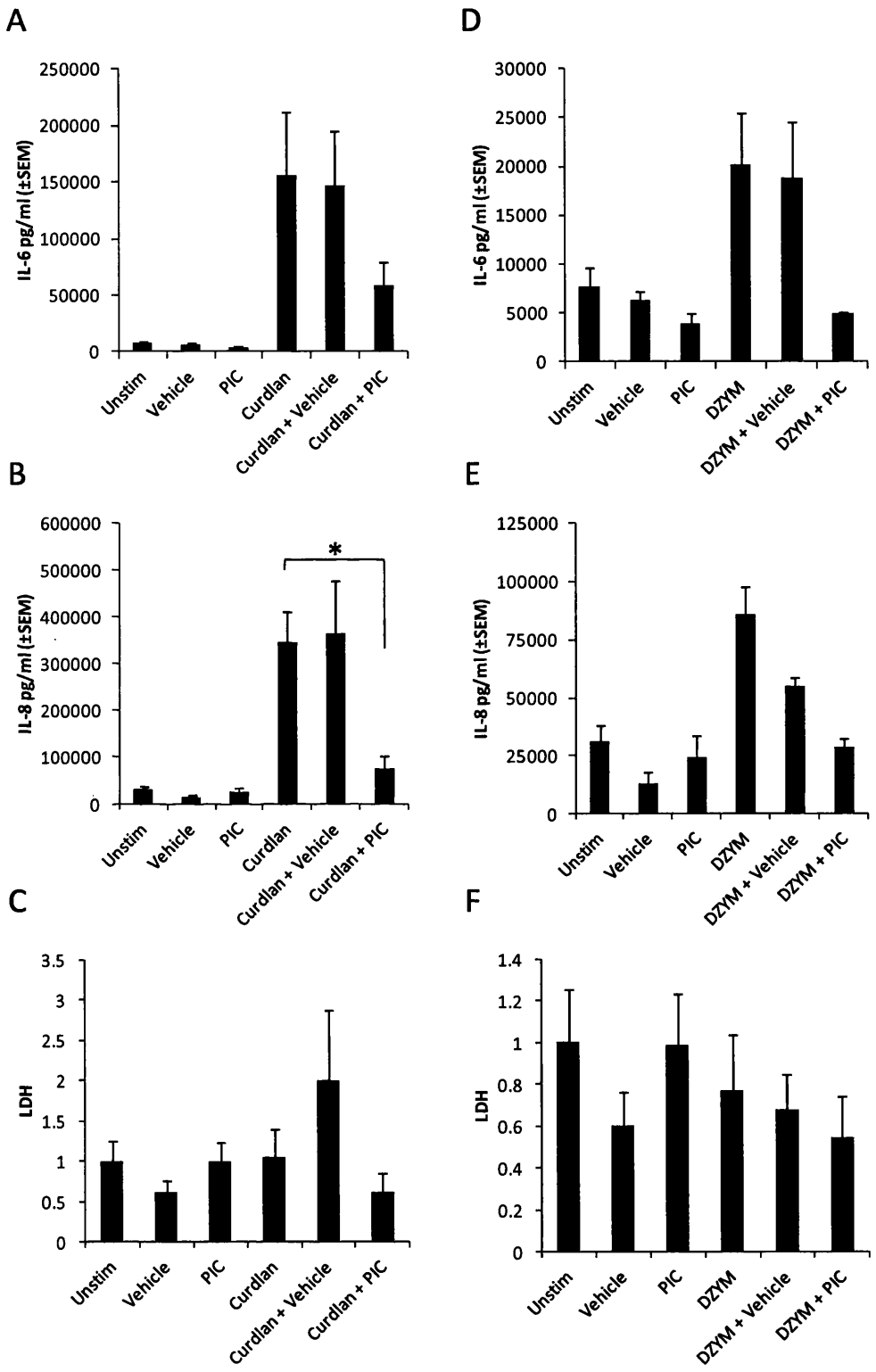

Figure 4.7 Syk inhibition in the term non-laboured choriodecidua.

IL-6 & IL-8 production (pg/ml mean ±SEM) by the choriodecidua, following stimulation with (A-B) curdlan or (D-E) depleted zymosan (both 100 μg/ml, n=3) in the presence or absence 100 μM of piceatannol. (C & F) Relative lactate dehydrogenase (LDH) levels compared to the unstimulated. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to curdlan is shown: * $p \leq 0.05$.

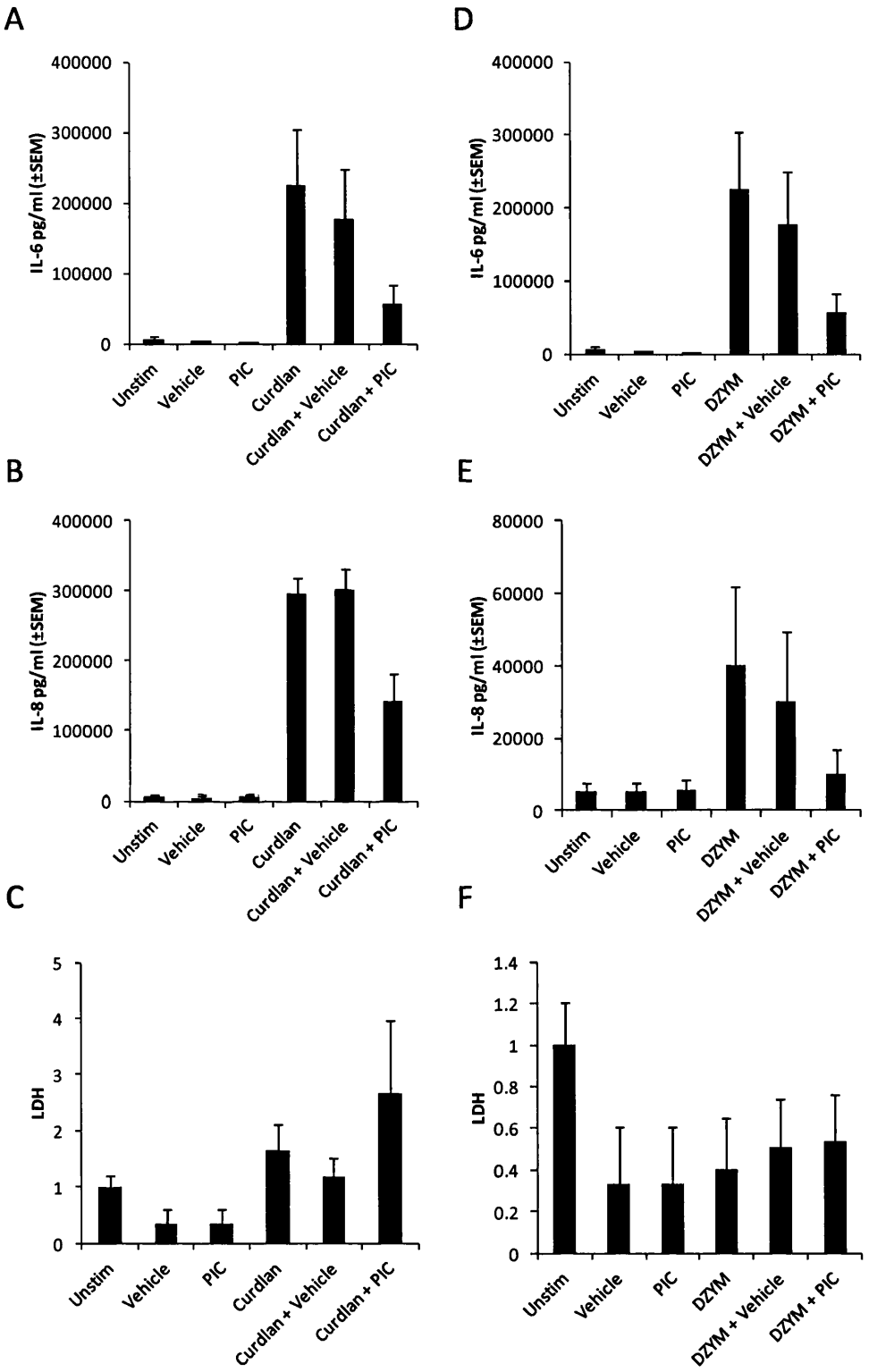

Figure 4.8 Syk inhibition in the term non-laboured amnion.

IL-6 & IL-8 production (pg/ml mean ±SEM) by the amnion, following stimulation with (A-B) curdlan or (D-E) depleted zymosan (both 100 µg/ml, n=3) in the presence or absence of 100 µM piceatannol. (C &F) Relative lactate dehydrogenase (LDH) levels compared to the unstimulated. Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

4.4.3.3 NF-κB activation

To investigate the role of NF-κB in the curdlan induced pro-inflammatory response in the placenta, choriodecidua and amnion, tissues were treated in the presence or absence of the irreversible IκB-a inhibitor, BAY11-7082, which inhibits IκB-a phosphroylation causing the inactivation of NF-κB [317]. To determine an optimum concentration of the inhibitor an initial dose course was performed based on manufacturer's recommended working concentrations, utilising LPS as a known NF-κB activator. No reduction in LPS induced cytokine production was observed at any concentration tested in the placenta or choriodeicuda. However a reduction was observed in the amnion at 10 μM inhibitor. Following reports in the literature of the inhibitor on placental tissue [318, 319], a concentration of 50 μM was tested (see Chapter 8.3 - Figure 8.22). A reduction in LPS induced cytokine production was observed in all three tissues in the presence of the inhibitor. As a result this concentration of BAY11-7082 was used in subsequent experiments, to examine curdlan induced NF-κB activation. As seen in Figures 4.9 – 4.11, a reduction curdlan induced IL-6 and IL-8 is observed in all three tissues in the presence of the inhibitor. However this was not significant. Treatment with BAY11-7082 did not impact tissue viability as observed by LDH assay.

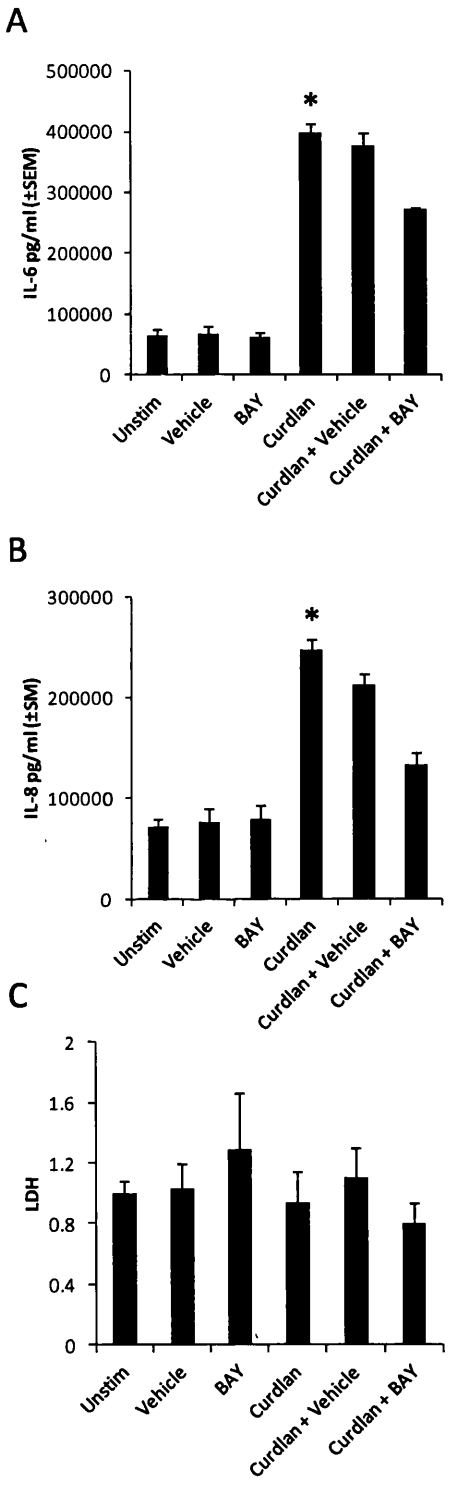

Figure 4.9 NF-κB inhibition in the term non-laboured placenta.

Placental explants were stimulated with curdlan (100 μg/ml, n=3) in the presence or absence of 50 μM of BAY11-7082 and (A) IL-6 and (B) IL-8 (both pg/ml mean ± SEM). (C) Relative lactate dehydrogenase (LDH) levels compared to the unstimulated. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

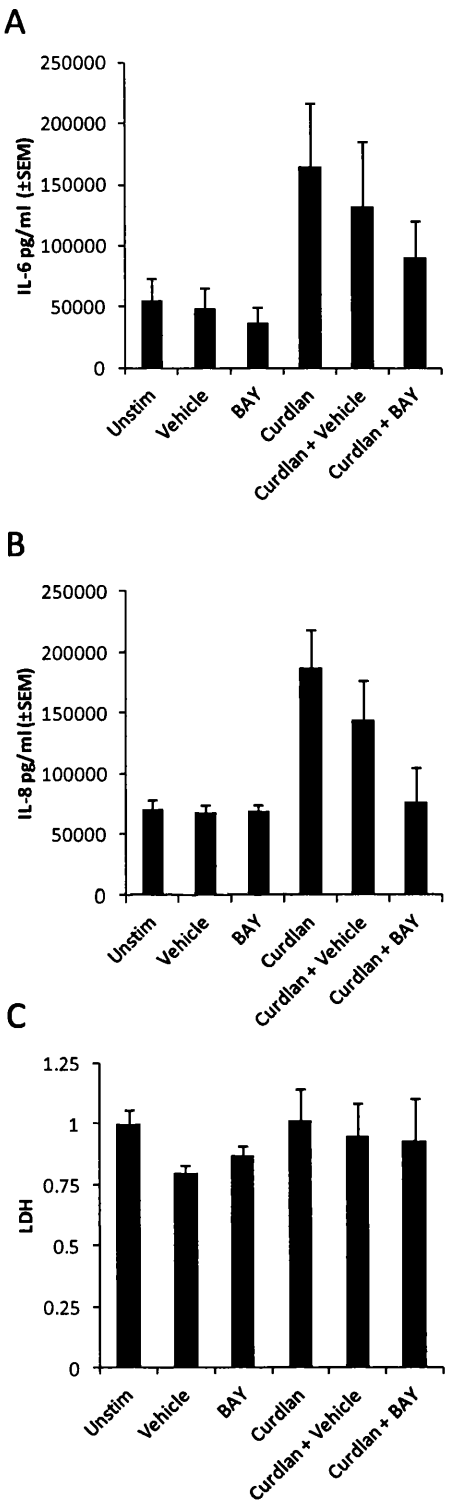

Figure 4.10 NF-κB inhibition in the term non-laboured choriodecidua.

Choriodecidual explants were stimulated with curdlan (100 μg/ml, n=3) in the presence or absence of 50 μM of BAY11-7082 and (A) IL-6 and (B) IL-8 (both pg/ml mean ± SEM). (C) Relative lactate dehydrogenase (LDH) levels compared to the unstimulated. Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

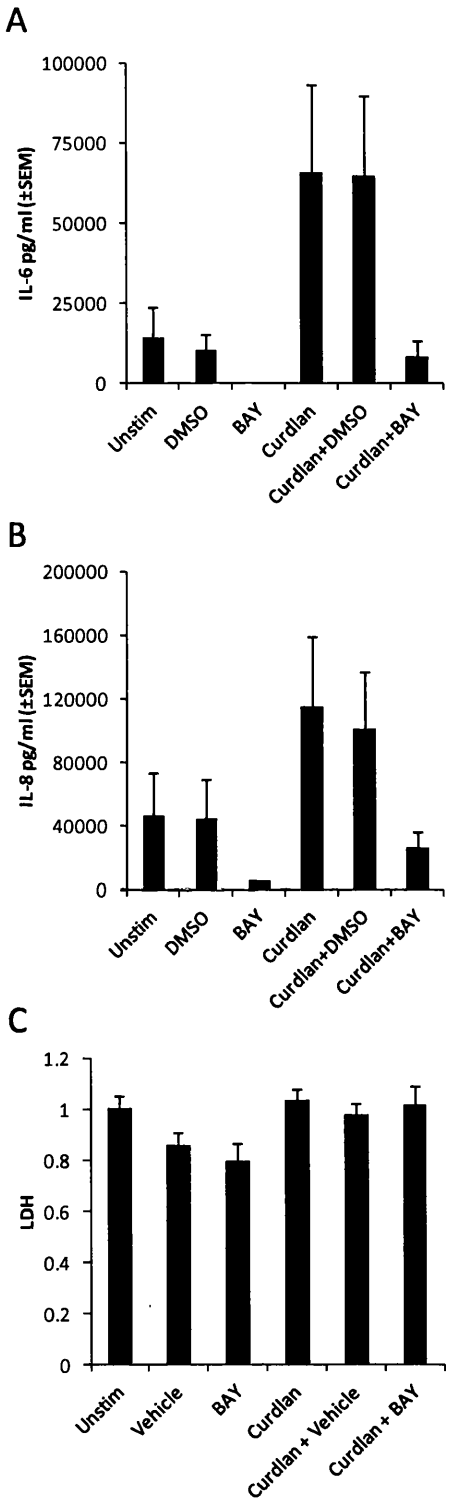

Figure 4.11 NF-κB inhibition in the term non-laboured amnion.

Amnion explants were stimulated with curdlan (100 μg/ml, n=3) in the presence or absence of 50 μM of BAY11-7082 and (A) IL-6 and (B) IL-8 (both pg/ml mean ± SEM). (C) Relative lactate dehydrogenase (LDH) levels compared to the unstimulated. Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

4.4.4 β-glucan induced inflammasome activation by term non-laboured gestation-associated tissue explants

4.4.4.1 Curdlan induced IL-1β production

Elevated levels of IL-1β present in amniotic fluid are a hallmark of intra-amniotic infection during pregnancy [320]. As a result the mechanisms of IL-1β production by the gestation associated tissues have gained interest. To date, β-glucan induced activation of dectin-1 and production of IL-1β in macrophages and dendritic cells, have yielded evidence of NLRP3/capase-1 and a non-canonical capase-8 inflammasome respectively [177, 314]. IL-1β production by DC's was independent of NLRP3 and caspase-1 [177]. To investigate if β-glucans can induce IL-1β production in the placenta, choriodecidua and amnion, tissue was treated with curdlan for 24 hours (n=12). A significant increase in IL-1β production was observed in all three tissues (Figure 4.12).

4.4.4.2 Reactive oxygen species (ROS) Production

Generation of reactive oxygen species is associated with activation of the NLRP3 inflammasome [321]. Furthermore, in macrophages β-glucan triggered IL-1β production is dependent on ROS production in a Syk dependent manner [199, 314]. To investigate the role of ROS in β-glucan triggered IL-1β in the placenta, choriodecidua and amnion, the known ROS inhibitor butylated hydroxyanisole (BHA) was used [314, 322, 323]. BHA functions as ROS scavenger [324]. Tissues were treated with curdlan either in the presence or absence of BHA at various concentrations (n=3). As shown in Figure 4.13, no inhibition of IL-1β production was observed in response any of the concentrations of BHA tested in any of the tissue.

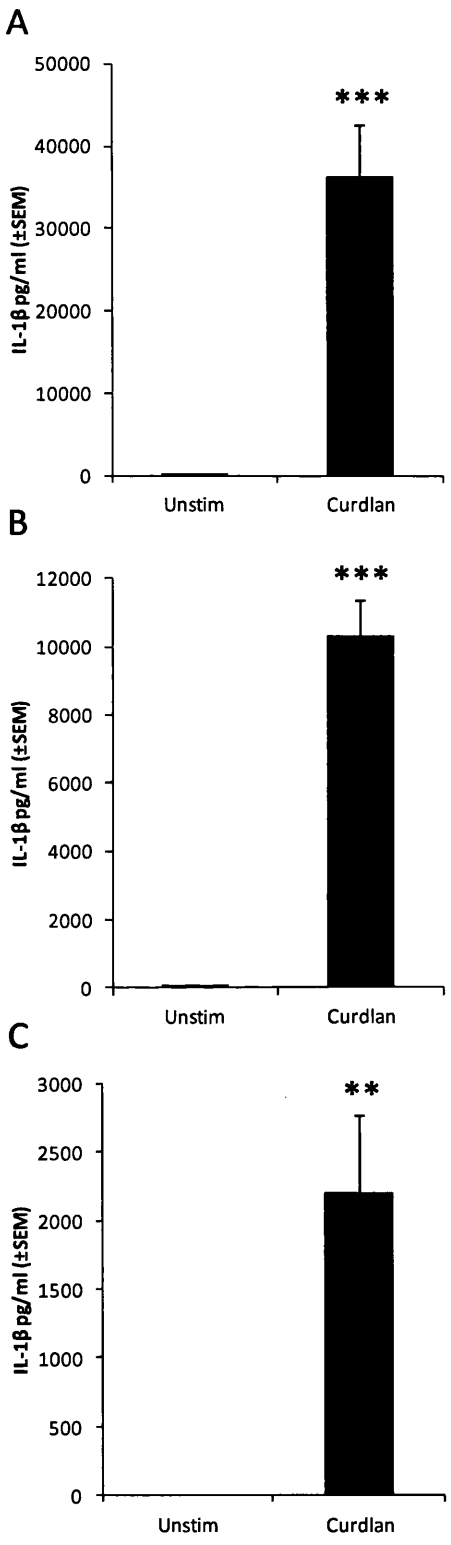

Figure 4.12 Curdlan induced IL-1β production by term non-laboured gestation associated tissues.

Explants of (A) placenta, (B) choriodecidua and (C) amnion were treatment with curdlan (100 μg/ml; n=12) and IL-1β production (pg/ml mean ± SEM) measured in tissue free culture supernatants measured. Statistical significance compared to unstimulated control as determined by Wilcoxon matched pairs signed rank test are shown: ** $p \leq 0.01$, *** $p \leq 0.001$.

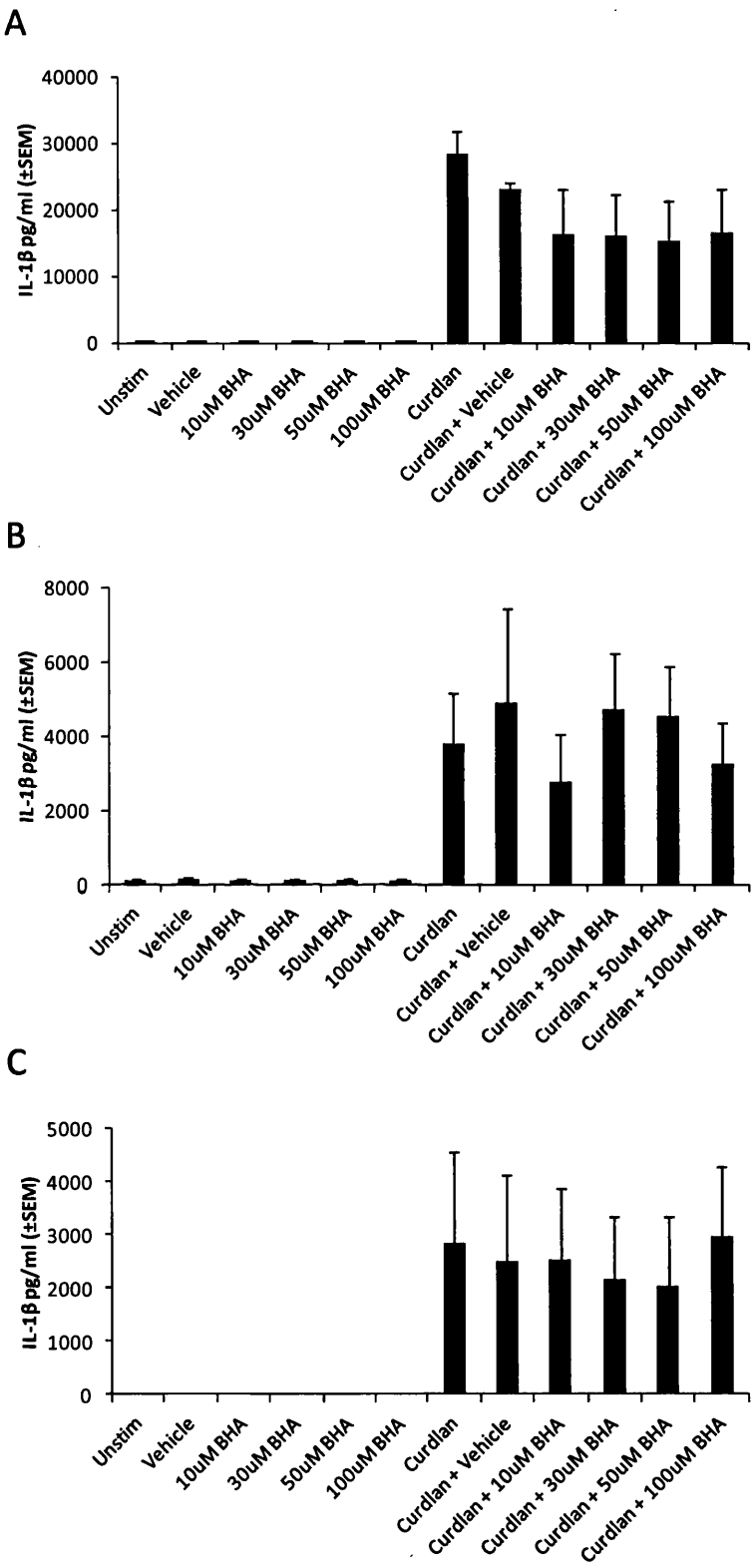

Figure 4.13 Inhibition of reactive oxygen species (ROS) production.

Explants of (A) placenta, (B) choriodecidua and (C) amnion were treated with curdlan (100 μg/ml) in the presence or absence of the ROS inhibitor, BHA at varying concentrations (n=3) and IL-1β levels (pg/ml mean ± SEM) in tissue free supernatants measured. Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

4.4.4.3 Caspase inhibition & activity

Since β-glucan induced IL-1β production has been associated with both caspase-1 and caspase-8, the role of both enzymes in the processing of pro-IL-1β was examined utilising specific inhibitors. Tissues were treated with curdlan either in the presence or absence of the caspase-1 inhibitor, Z-WEHD-FMK or caspase-8 inhibitor, Z-IETD-FMK for 24 hours (n=3). The optimum concentration of both inhibitors was determined following initial dose course (see Chapter 8.3 Figure 8.23 - 8.24). A reduction in curdlan induced IL-1β production in all three tissues was observed in the presence of both inhibitors; however this was not significant (Figure 4.14 and 4.15). Treatment with either inhibitor did not impact tissue viability as observed by LDH assay.

To examine specific caspase activity in these tissues, lysates of placenta and choriodecidua were prepared at 3, 6 and 18 hours following curdlan treatment (Figure 4.16 and 4.17). Caspase activity at T0 was also examined. No discernible difference was observed in caspase-1 or caspase-8 activity regardless of time or curdlan stimulation. To determine the functionality of the assay, background reagents and T0 placental lysates were spiked with 1 unit of either active caspase-1 or caspase-8 (Figure 4.18). When compared with un-spiked controls, both spiked reagents and lysates demonstrated increased caspase activity as expected. Additionally in order to determine that the recommended incubation time of 2 hours was sufficient to observed caspase activity, subsequent time points of 24, 48 and 72 hours were included. While increasing the incubation time did not impact the caspase activity within the treated placental and choriodecidual lysates (Figure 4.16 and 4.17), increased activity was observed in both the spiked reagents and lysates (Figure 4.18). This would confirm that both assays are functional and that there is nothing present in the lysate that would interfere with the assay.

In addition to caspase activity, lysates were assayed for IL-1β, representing putative pro-IL-1β. IL-1β production was also examined in corresponding tissue free supernatants. Lysates showed an increase in putative pro-IL-1β production in both a curdlan and time dependent manner, which was also observed in corresponding supernatants (Figure 4.19 - 4.20).. In the placenta, an increase in both putative pro-IL-1β and IL-1β were observed from 3 hours and continued to increase over time. In the choriodecidua, however an increase both putative pro-IL-1β and IL-1β were not observed until 6 hours and continued to increase over time.

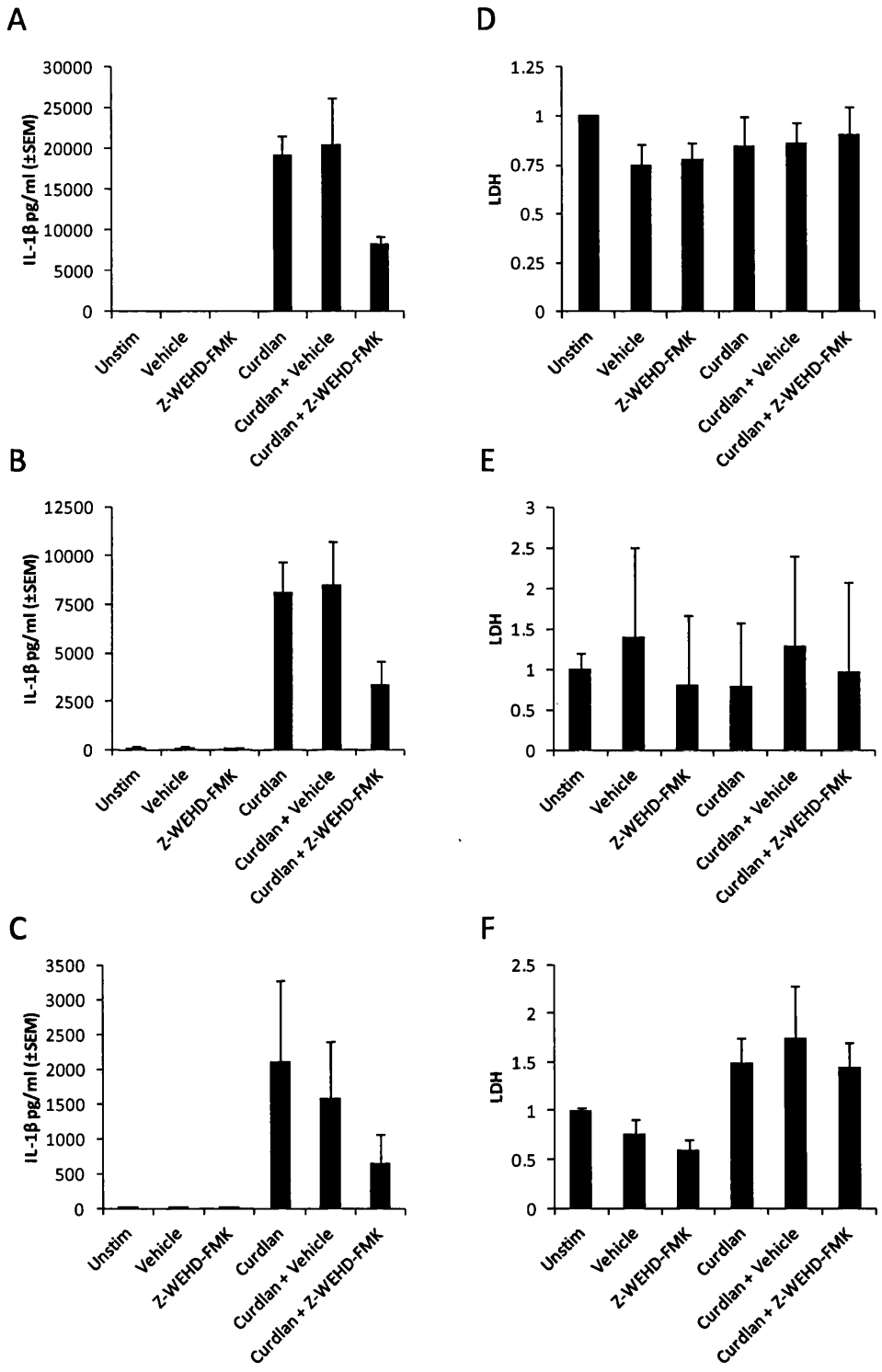

Figure 4.14 Inhibition of capase-1.

Explants of (A) placenta, (B) choriodecidua and (C) amnion were treated with curdlan (100 μg/ml) in the presence or absence of the caspase-1 inhibitor Z-WEHD-FMK (5 μM; n=3) and IL-1β levels (pg/ml mean ± SEM) in tissue free supernatants were measured. Relative lactate dehydrogenase (LDH) levels in the (D) placenta, (E) choriodecidua and (E) amnion compared to the unstimulated control. Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

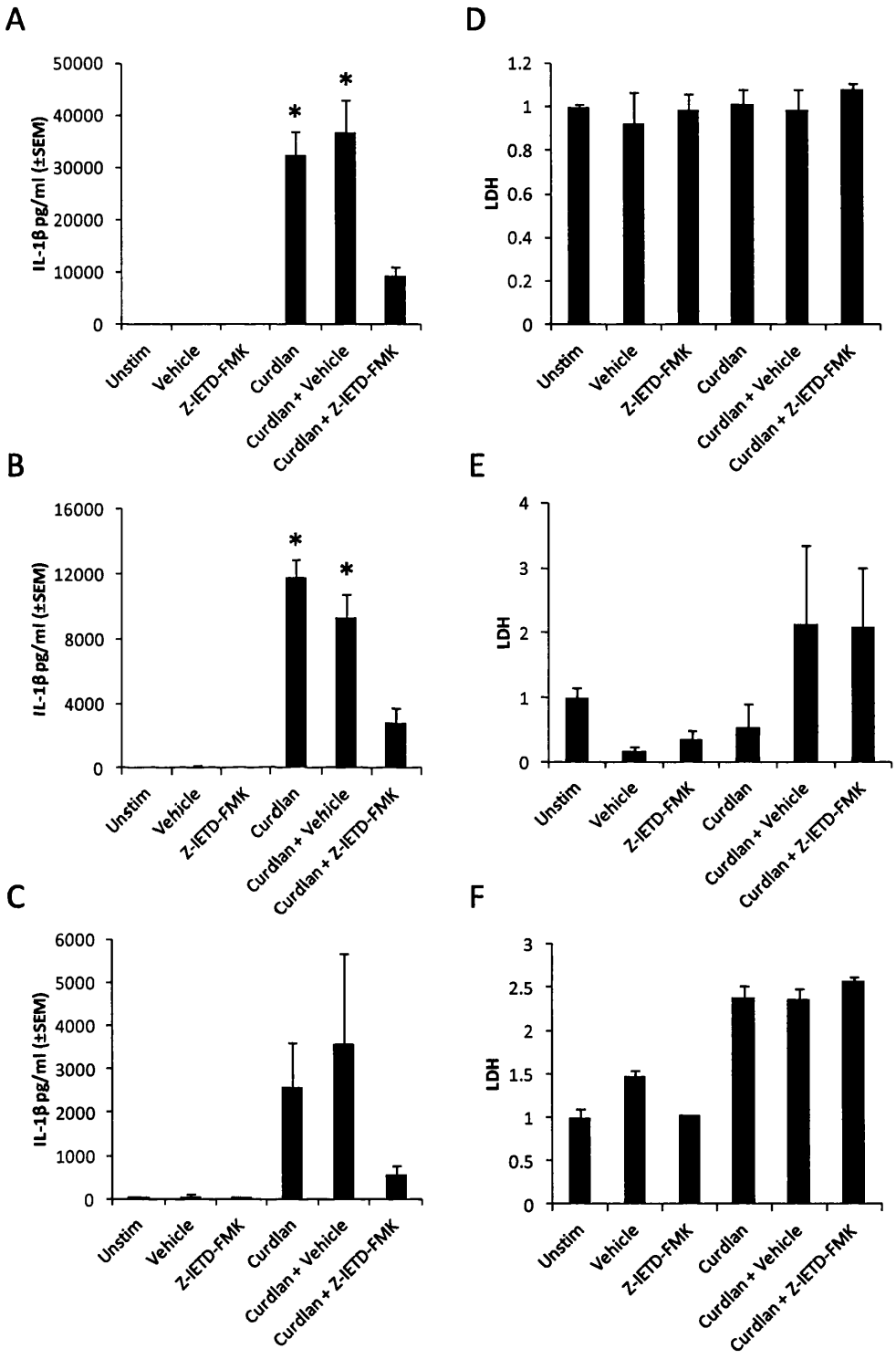

Figure 4.15 Inhibition of caspase-8.

Explants of (A) placenta, (B) choriodecidua and (C) amnion were treated with curdlan (100 µg/ml) in the presence or absence of the caspase-8 inhibitor Z-IETD-FMK (5 µM; n=3) and IL-1β levels (pg/ml mean ± SEM) in tissue free supernatants were measured. Relative lactate dehydrogenase (LDH) levels in the (D) placenta, (E) choriodecidua and (E) amnion compared to the unstimulated control. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated are shown: * $p \leq 0.05$.

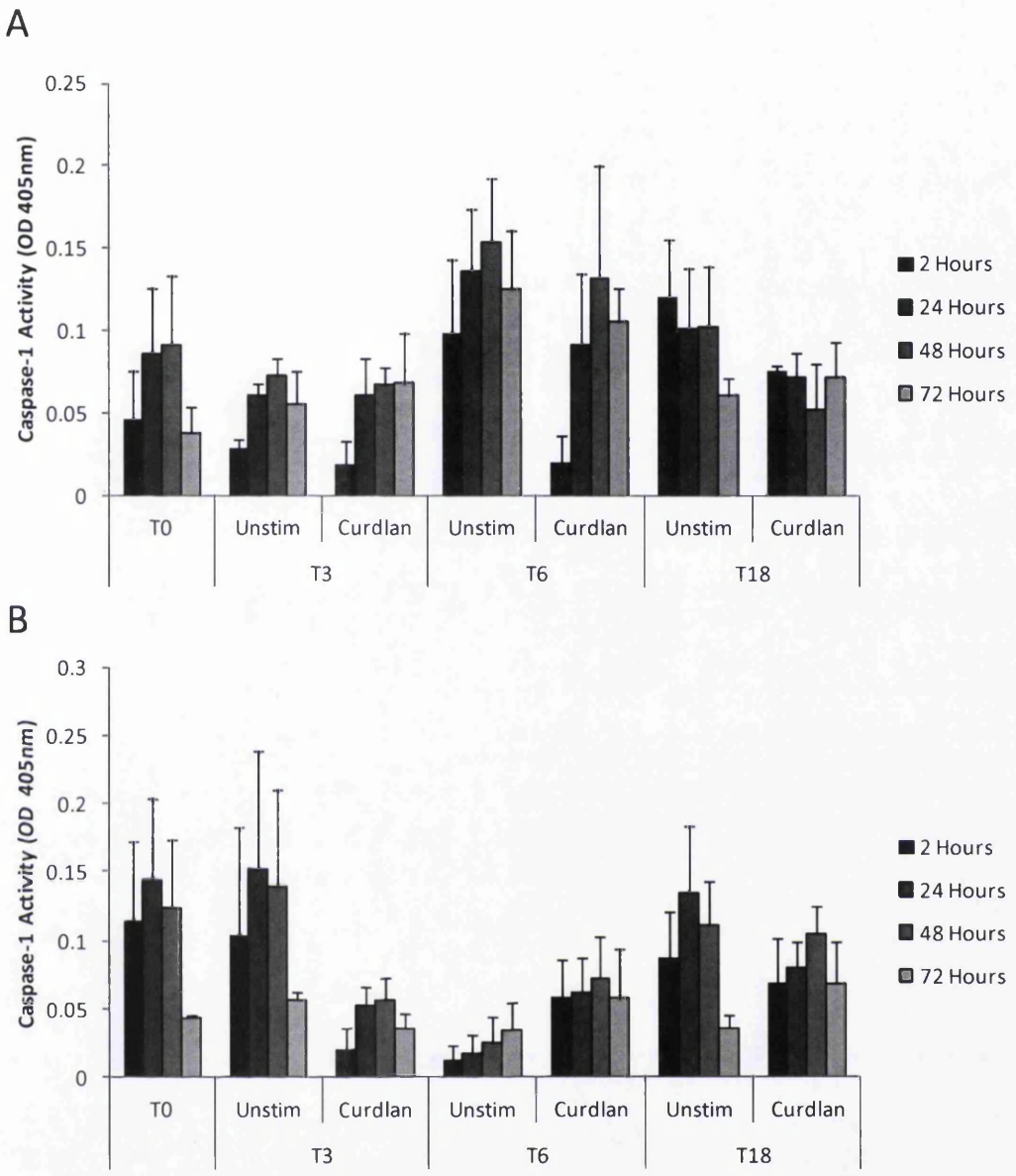

Figure 4.16 Caspase-1 activity in the placenta and choriodecidua.

Tissue lysates prepared from (A) placenta and (B) choriodecidua with and without treatment with curdlan (100 µg/ml; n=3) at 3, 6 and 18 hours were assayed for Caspase-1 activity (OD 405 nm at 2, 24, 48 and 72 hours; mean ± SEM). Caspase activity at T0 was also examined.

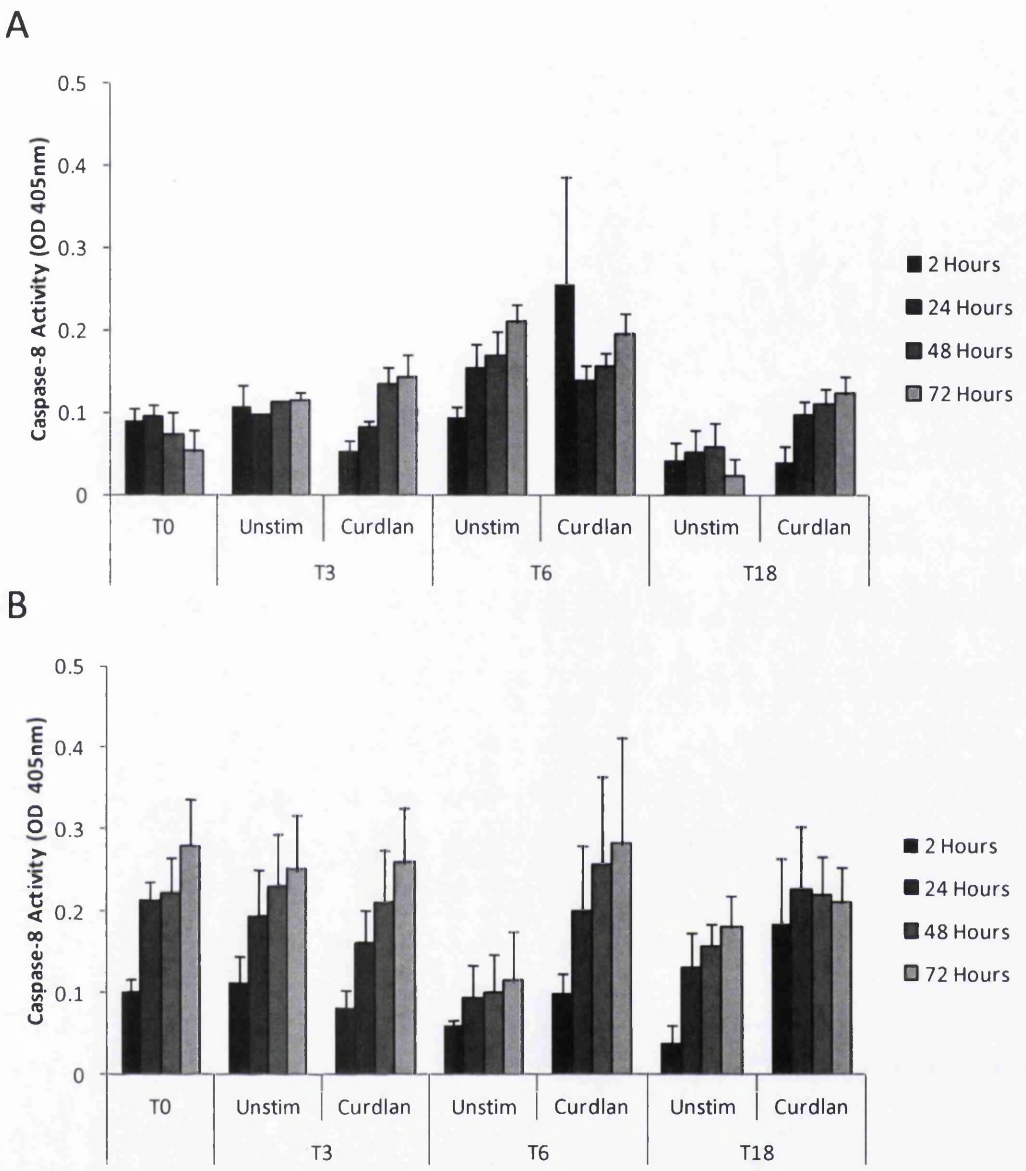

Figure 4.17 Caspase-8 activity in the placenta and choriodecidua.

Tissue lysates prepared from (A) placenta and (B) choriodecidua with and without treatment with curdlan (100 µg/ml; n=3) at 3, 6 and 18 hours were assayed for Caspase-8 activity (OD 405 nm at 2, 24, 48 and 72 hours; mean ± SEM). Caspase activity at T0 was also examined.

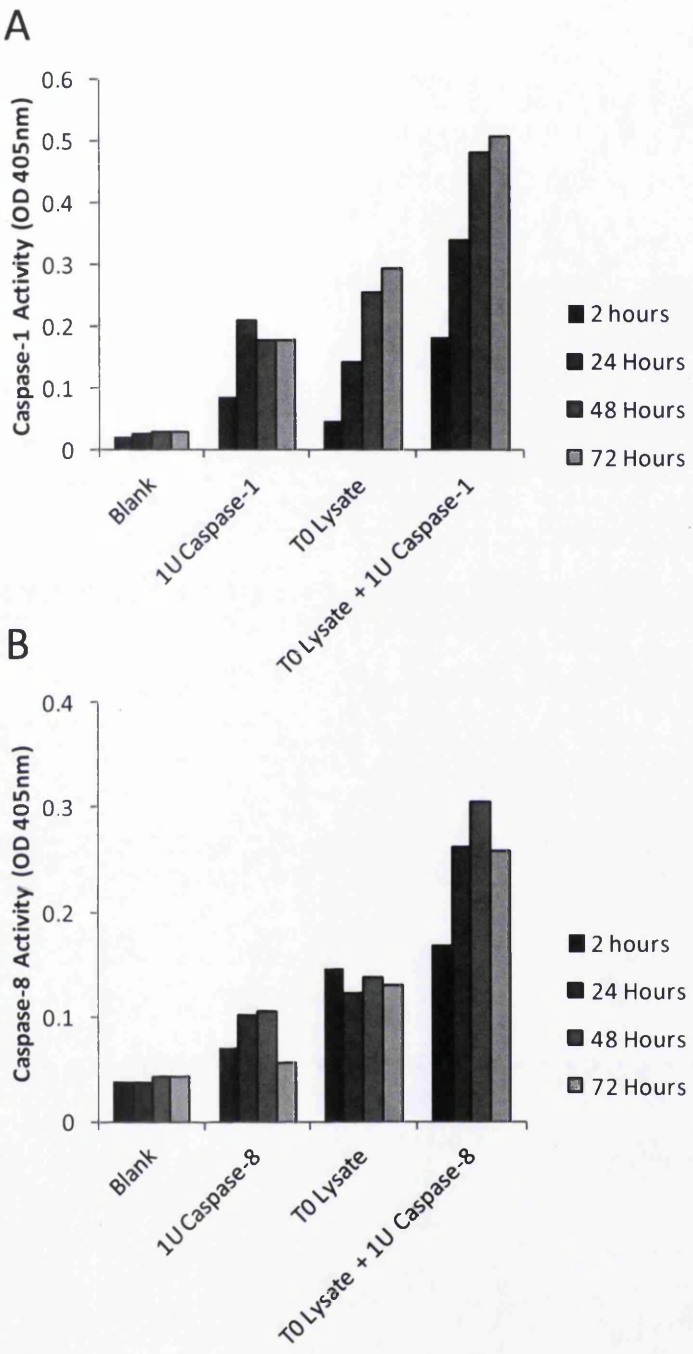

Figure 4.18 Caspase activity assay test.

Assay reagents (blank) and T0 placental lysate were spiked with 1 unit (1U) of active (A) Caspase-1 or (B) Caspase-8 to determine validity of both activity assays (n=1). Caspase activity (OD 405 nm at 2, 24, 48 and 72 hours).

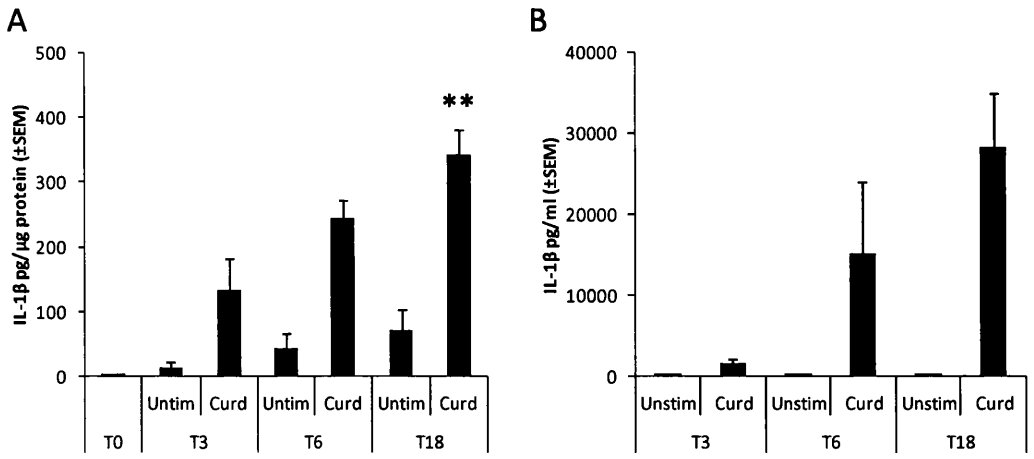

Figure 4.19 IL-1β production in placental tissue lysates and tissue free supernatants.

IL-1β levels (pg/ml mean ± SEM) in placental (A) tissue lysates (total protein) and in (B) tissue free supernatants in response to treatment with curdlan at 3, 6 and 18 hours (100 μg/ml; n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to T0 lysate is shown: ** $p \leq 0.01$.

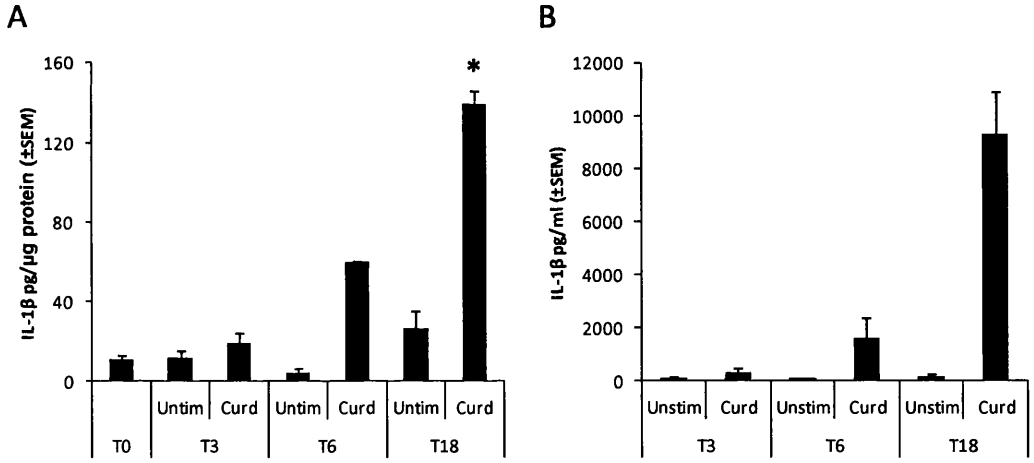

Figure 4.20 IL-1β production in choriodecidual tissue lysates and tissue free supernatants.

IL-1β levels (pg/ml mean ± SEM) in choriodecidual (A) tissue lysates (total protein) and in (B) tissue free supernatants in response to treatment with curdlan at 3, 6 and 18 hours (100 μg/ml; n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to T0 lysate is shown: * $p \leq 0.05$.

4.4.5 Response of term non-laboured gestation-associated tissue explants to *Candida albicans*

While the work to date has utilised β-glucan agonists such as curdlan to examine the functional activity of Dectin-1, a whole array for other PRRs are involved in the recognition of *Candida albicans* (Table 4.1). As noted in Figure 4.1, transcripts for CLRs and scavenger receptors involved in *Candida* recognition are expressed in the placenta, choriodecidua and amnion, and work presented in chapter 3, and implies functionally active TLRs in these tissues also. Based on this, these tissues likely would be able to mount an inflammatory response to *Candida albicans*.

To investigate the functional response to *Candida albicans*, in the placenta, choriodecidua and amnion, tissues were treated with both live and UV inactived *Candida albicans* strain SC5314 (Figure 4.21). The results are shown in Figures 4.22 – 4.24. A general trend is observed that as the concentration of *Candida* increases, the amount of cytokine produced also increases. Additionally these a trend that the cytokine response to live *Candida albicans* is higher when compared to inactived C*andida albicans*, however this was only significant for IL-8 in the placenta at a concentration from 10^6 cells/ml and 10^7 cells/ml respectively and IL-1β in the placenta and amnion at 10^7 cells/ml.

In the placenta (Figure 4.22) a significant increase in both IL-1β and IL-8 production is observed in response to live C*andida albicans* at a concentration of 10^6 cells/ml and higher, however a significant increase in IL-6 production is observed at a concentration of 10^7 cell/ml. A similar response is observed by the choriodecidua (Figure 4.23), In the amnion a significant increase in both IL-6 and IL-8 in response to live *Candida albicans* was observed a concentration 10^6 cells/ml, with a significant increase in IL-1β at a concentration of 10^7 cell/ml (Figure 4.24).

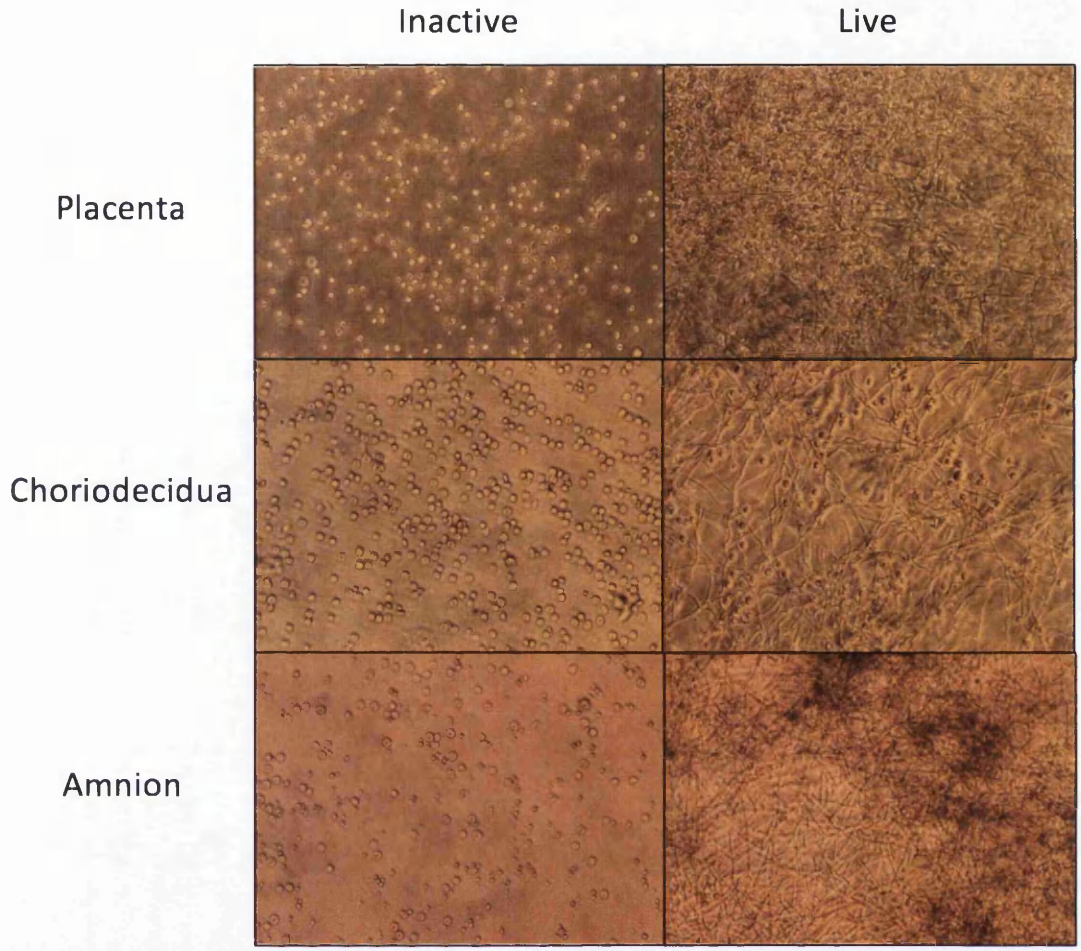

Figure 4.21 Inactive and live *Candida albicans* present in gestation associated tissue cultures.

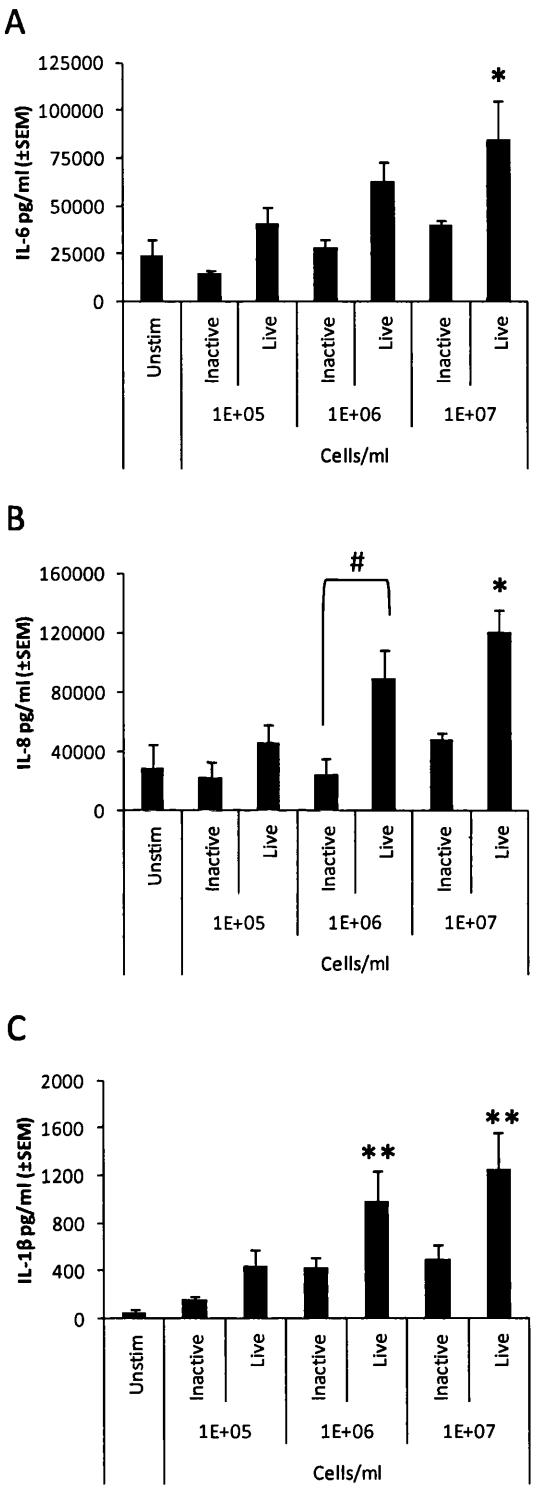

Figure 4.22 *Candida albican* induced cytokine response in the term non-laboured placenta.
Cytokine production; (A) IL-6, (B) IL-8 and (C) IL-1β (pg/ml; mean ±SEM) by the placenta in response to live and inactive *Candida albicans* (n=4). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$, ** $p \leq 0.01$. Statistical significance inactive vs life: # $p \leq 0.05$.

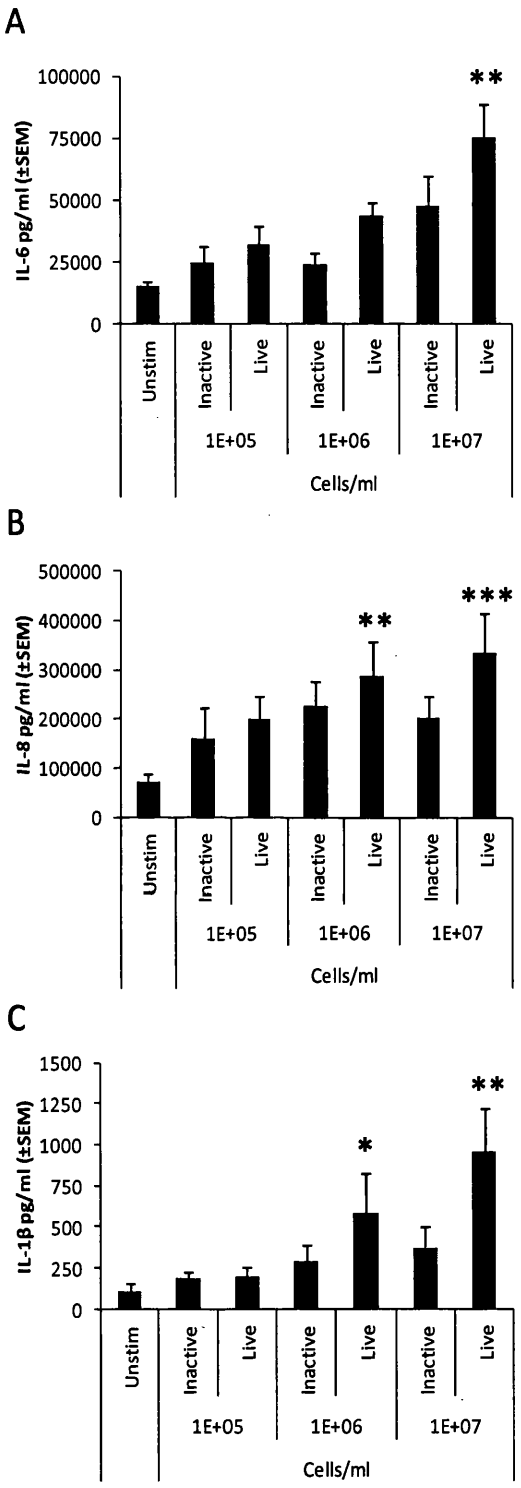

Figure 4.23 *Candida albican* induced cytokine response in the term non-laboured choriodecidua.

Cytokine production; (A) IL-6, (B) IL-8 and (C) IL-1β (pg/ml; mean ±SEM) by the choriodecidua in response to live and inactive *Candida albicans* (n=4). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

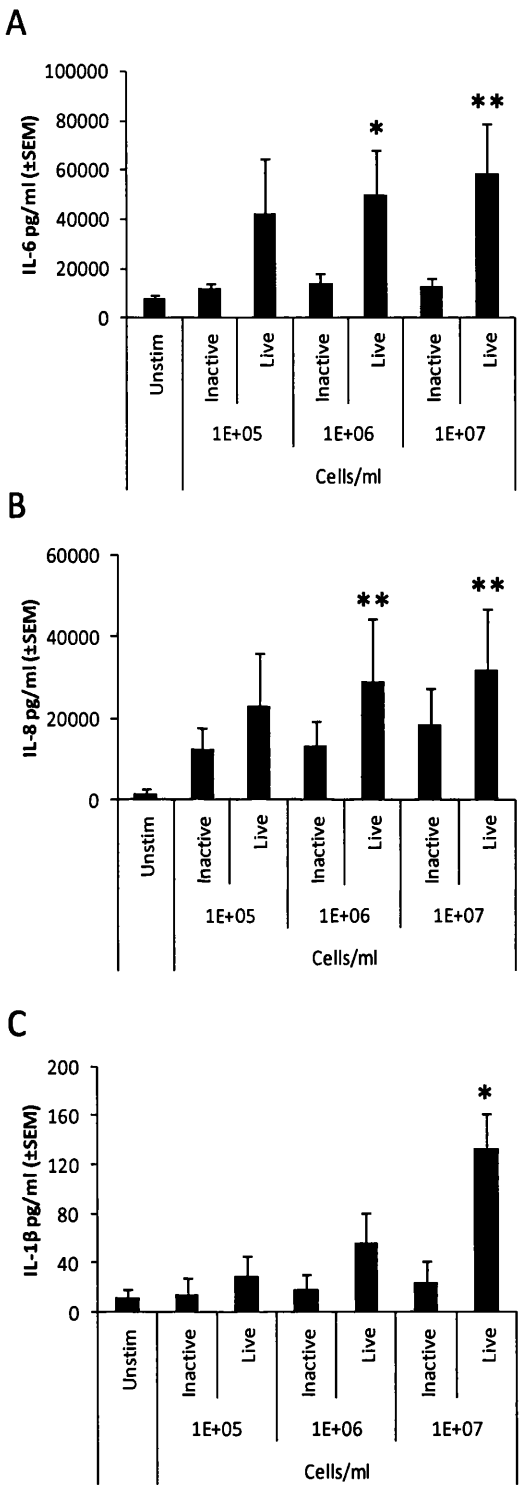

Figure 4.24 *Candida albican* induced cytokine response in the term non-laboured amnion.
Cytokine production; (A) IL-6, (B) IL-8 and (C) IL-1β (pg/ml; mean ± SEM) by the amnion in response to live and inactive *Candida albicans* (n=4). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$, ** $p \leq 0.01$.

4.5 Discussion

One of the aims of this chapter was to examine the expression and function of C-type lectin receptors (CLRs) in the placenta, choriodecidua and amnion. Analysis of these tissues by PCR showed that transcripts of the CLRs - Dectin-1, Dectin-2, MINCLE, DC-SIGN, Galectin-3 and the Mannose Receptor - are expressed in these tissues. This includes the two described functional variants of Dectin-1 [325]; Dectin-1a and Dectin-1b respectively. Additionally, transcripts for the scavenger receptors SCARF-1 and CD36 are also expressed. However protein expression of these receptors and their cellular source within these tissues is yet to been determined and is currently underway. To date only the expression of DC-SIGN has been reported in the placenta where it is expressed on Hofbauer cells (fetal macrophages) within the chorionic villi [214].

One of the most extensively studied CLR is the β-glucan receptor Dectin-1 [197, 205, 206]. The data shown here illustrate that stimulation of the placenta, choriodecidua and amnion with Dectin-1 agonists; curdlan and depleted zymosan is sufficient to elicit a significant increase in pro-inflammatory cytokine production in these tissues, implying functional dectin-1. While both agonists were used at the same concentration, on the whole a greater response in cytokine production was observed following curdlan treatment. This was most evident in the amnion, where no significant increase in cytokine production was observed following treatment with depleted zymosan compared with a 6-fold and 9-fold increase in IL-6 and IL-8 respectively following curdlan treatment. While all β-glucans are linear β-glycosidic 1 → 3 linked glucose polymers, they differ in branching structure and length [326]. These characteristic differences in addition to the organism of origin have been shown in porcine leukocyte studies to have different immunomodulating activity [327, 328]. This indeed might explain the difference between depleted zymosan derived from *Saccharomyces cerevisiae* versus the *Alcaligenes faecalis* derived curdlan. The two isoforms of Dectin-1 are functionally different, which is suggested to play a role in the regulation of the cellular response to β-glucans, likely via the N-linked glycosylation of Dectin-1 which has been shown to effect ligand binding and expression [329, 330]. While transcripts for both isoforms are expressed in the placenta, choriodecidua and amnion, the relative abundance of each is yet to be determined.

To further investigate the role of β-glucan mediated Dectin-1 activation by the gestation associated tissues, neutralisation/inhibition experiments utilising specific antibodies/inhibitors were performed to examine key components of the dectin-1 signalling pathway; including Dectin-1, Syk and the transcription factor NF-κB. No consistent inhibition of Dectin-1 with a specific antibody was observed. This could be due to lack of access for the antibody to target cells within the explants, however similar experiment in subsequent chapter utilising neutralising antibodies to block other cell surface receptors have been successful. Alternatively the antibody might not be a neutralising antibody, and although it is supplied as such a lack of a positive control leaves this unknown in our hands.

Following recognition of β-glucans by Dectin-1, a Syk dependent pathway of cytokine production via the transcription factor NF-κB is activated. The data presented here, implies that both Syk and NF-κB are required for β-glucans induced cytokine production in gestational tissues. However it should be noted that only partial inhibition was observed. This might relate to pre-incubation time of the inhibitor. While 30 minute pre-incubation was used here, based on previous experience within the research group, in comparison a 60 minute pre-incubation is used by others in explant models [286]. It's also important to consider other downstream signalling events of Dectin-1 activation. While the Syk dependent pathway is the primary pathway activated, a Syk independent pathway signalling through Raf-1 has also been described. Raf-1 signalling ultimately feeds back into the Syk pathway for synergistic NF-κB activation [331]. In addition to NF-κB, other downstream events including the activation of MAPKs and NFAT play a role in Dectin-1 induced cytokine production [198, 200, 332].

In macrophages and dendritic cells, β-glucan, specifically curdlan, induced activation of dectin-1 was associated with the production of IL-1β [177, 314]. The mechanisms of IL-1β production via the inflammasome recently have gained interest especially at the maternal fetal interface due to the association of intra-amniotic infection during pregnancy and elevated levels of IL-1β present in the amniotic fluid [320]. To date little is known regarding inflammasome activation in the gestation associated tissues, with only limited examination of production of IL-1β induced by uric acid crystals and the up-regulation of inflammasome gene expression by LPS in the trophoblast [238, 295, 333]. The data presented here illustrates that curdlan

treatment is able to induce a significant increase in IL-1β in the placenta, choriodecidua and amnion.

Dectin-1 mediated IL-1β production in macrophages is associated with the generation of reactive oxygen species (ROS) and the activation of the NLRP3/caspase-1 inflammasome [199, 314]. More recently activation of a non-canonical capase-8 inflammasome has been described independent of NLRP3 and caspase-1 [177]. Since a role of ROS in IL-1β production was not confirmed using the known ROS scavenger BHA, it would be prudent to measure ROS levels in the future. Examination of caspase-1 and caspase-8 using specific inhibitors implied both caspases contribute to IL-1β production by gestation associated tissues. When the activity of both caspases was further examined using total protein lysates of the placenta and choriodecidua treated with curdlan, no discernible difference was observed, despite the proven validity of the assay. This would likely suggest that a minor cell population within these tissues, most likely a macrophage population, is responsible for the generation of IL-1β induced by curdlan and that in a total protein colorimetric assay the signal would likely be below the limit of detection. While a more sensitive fluorescence assay might be appropriate, it might initially be more prudent to use an immunohistochemical approach to determine the specific cell type involved and purify out that cell population for further investigation. While these data demonstrates that both capase-1 and caspase-8 are involved in the production IL-1β induced by curdlan, it cannot conclusively confirm the involvement of the NLRP3 inflammasome or the non-canonical caspase-8 inflammasome. Examination of putative pro-IL-1β in tissue lysates, show an increase as early as 3 hours post treatment with curdlan in the placenta, with a corresponding increase observed IL-1β in matched supernatants. The choriodecidua however, appear to require longer, with no increase in either putative pro-IL-1β or IL-1β until 6 hours post curdlan treatment. This warrants further investigation especially gene expression by quantitative PCR.

The role of MINCLE, a CLR primarily involved in the recognition mycobacterial cord factor but also in the antifungal response to *Candida albicans* was also examined [334, 335]. Stimulation of the placenta, choriodecidua and amnion with a synthetic analogue TDB, did not induce the production of either IL-6 or IL-8. In peripheral blood mononuclear cells, elevated levels of IL-8 was observed in response to TDB treatment. While IL-6 and IL-8 are induced by numerous agonists in these tissues,

TDB may not stimulate the production of these cytokines in these tissues. In murine bone marrow derived dendritic cells (BMDCs), TDB was able to induce IL-1β production via the NLRP3 inflammasome [336]. Examination of TDB stimulated placenta, choriodecidua and amnion showed that TDB was able to elicit significant IL-1β production in the placenta and choriodecidua but not the amnion requiring further investigation (see Chapter 8.3 – Figure 8.25).

While examination of single agonists provides insight into specifics of receptor activity and signalling, in response to a pathogen, *in vivo*, single PRR activation does not occur. This makes it important to investigate the immune response to whole organisms. *Candida albicans* are able to grow as both budding yeast and hyphae [337]. The yeast form is able to colonise mucosal surfaces, while the primary role of the hyphae is host invasion [338]. Therefore the response to both live, which will be able to transition from yeast to hyphae, and inactivated *Candida albicans* were examined. The data shown here illustrates that culturing the placenta, choriodecidua and amnion in the presence of *Candida albicans* induces an inflammatory response in these tissue. Furthermore live *Candida albicans* were able induced a greater inflammatory response compared to UV inactivated *Candida albicans*. As *Candida albicans* switches between yeast and hyphael forms, different components of the cell wall are exposed. For example during budding, glucans and chitin are exposed, in contrast to being shielded by mannans and mannoproteins in the hyphae. As a result, different PRRs will be activated as the *Candida* changes forms [309, 339]. Additionally the switch from bud to hyphae formation trigger the activation of the NLRP3 inflammasome [340]. Therefore it's not surprising that live *Candida albicans* would stimulate a greater inflammatory response. However this contrasts previously reported data in human PBMCs where both heat killed and UV irradiated *Candida albicans* induced a significantly higher inflammatory response compared to live *Candida albicans*, with the authors citing an increased exposure of β-glucan in the "killed" *Candida* as the reason [341]. However it should be noted that a different stain of Candida was used.

In summary the data contained in this chapter demonstrate the expression of transcripts in the placenta, choriodecidua and amnion for several CLRs and scavenger receptors involved in the innate immune response to *Candida albicans* and other fungi. Furthermore these tissues produce an inflammatory response to *Candida albicans.* Detailed investigation of Dectin-1, demonstrates that the

activation of this CLR in response to β-glucans, induces an inflammatory response in gestation-associated tissues including activation of the inflammasome.

Chapter 5

Flagellin induced inflammasome activation and IL-1β at the maternal-fetal interface

5 Flagellin induced inflammasome activation and IL-1β at the maternal-fetal interface

5.1 Introduction

Interleukin-1 beta (IL-1β) is a pro-inflammatory cytokine that in response to pathogenic invasion can induce a local and systemic inflammatory response. Elevated levels of IL-1β in amniotic fluid are a hallmark of intra-amniotic infection during pregnancy [320]. IL-1β is considered a major factor in the initiation of infection associated preterm labour by up-regulation of matrix metalloproteinase activity, prostaglandin production resulting in myometrial contractions, and membrane rupture and cervical ripening [19, 26, 75, 342]. Despite this little is known about the sources of IL-1β in these circumstances.

Production of IL-1β is tightly regulated due to its potency as a pro-inflammatory mediator. Unlike many cytokines it is not constitutively expressed, with expression dependent on both exogenous and endogenous stimuli, resulting in the production of an inactive pro-IL-1β precursor that must ultimately be cleaved into the bioactive mature IL-1β, a process that is regulated by the inflammasome [343-345]. Inflammasomes are a cytosolic multi-protein complex typically including a member of the NLR family, that support the autocatalytic cleavage of pro-caspase-1 to active caspase-1, which in turn enables the processing of pro-IL-1β (and pro-IL-18) and secretion of mature IL-1β (and IL-18) [185, 346, 347].

In the previous chapter (Chapter 4), it was shown that the placenta, choriodecidua and amnion are capable of producing IL-1β in response to fungal stimuli. Parallel work within the laboratory has demonstrated that these tissues can produce IL-1β in response to a wide variety of activators including the microbial toxin nigericin, hemozoin produced by the parasite *Plasmodium* and monosodium urate (MSU) crystals. The majority of these activators are associated with IL-1β processing via caspase-1 and the NLRP3 inflammasome, while the β-glucan curdlan activates the recently described non-canonical caspase-8 inflammasome ([177, 181, 348, 349] and Chapter 4). Several other inflammasomes have been described including NLRP1 and NLRC4, which are activated by MDP and flagellin, respectively. Both of these inducers are components of bacteria; MDP is bioactive motif of peptidoglycan, while flagellin is the major protein components of bacterial flagella [294, 350]. Several bacterial species associated with preterm labour are flagellated including

Escherichia coli [49]. While transcripts for NLRC4 are expressed in the placenta, choriodecidua and amnion (Chapter 3), a functional role of NLRC4 is yet to be determined.

5.2 Aims

The aim of this chapter is to examine if flagellin can induce IL-1β production by the term non-laboured human placenta, choriodecidua and amnion and to examine the signalling pathways that are involved in this process.

5.3 Methods

5.3.1 Samples

Healthy pregnant women scheduled for elective caesarean section (ESC) were approached in the antenatal day assessment unit at Singleton Hospital, Swansea, during their pre-anaesthetic assessment. Informed written consent was obtained upon explanation of the study. Following delivery, placenta and attached fetal membranes (n = 3–12) from these women were collected and processed within 1.5 hours of delivery.

5.3.2 Gestation-associated tissue explants

Placenta, choriodecidua and amnion tissue explants were cultured as described in Chapter 2.2. Cultures were stimulated with ultrapure flagellin isolated from *Salmonella typhimurium* (100 ng/ml, endotoxin level: <0.05 EU/μg, Invivogen) for in vitro culture experiments. For inhibition/neutralisation experiments cultures were incubated for 30 minutes prior to the addition of flagellin with blocking antibodies or inhibitors as follows; anti-hTLR5 IgA2 (10 μg/ml, clone Q2G4; Invivogen), human IgA2 isotype control (10 μg/ml, clone T9C6; Invivogen), Z-WEHD-FMK (Caspase-1 inhibitor, 5 μM; R&D Systems), Z-IETD-FMK (Caspase-8 inhibitor; 5 μM, R&D Systems). The anti-human TLR5 and isotype control antibodies were generated by combining the constant domains of human IgA2 with the variable domains of mouse IgG from mice immunised with either human TLR5 or, for the control, *E. coli* β-galactosidase (Invivogen).

5.3.3 Tissue lysates

Total protein was extracted from placenta and choriodecidua explants treated with flagellin after 3h, 6h and 18h. An unstimulated control at each time point and T0 tissue was also included (see Chapter 2.5.1 for method). Total protein was estimated using the BCA assay (see Chapter 2.5.2 for method).

5.3.4 Cytokine production

IL-1β, IL-6 and IL-8 in the tissue free supernatants of placenta, choriodecidua and amnion explant cultures collected after 24 h were measured using commercially available ELISA kits (DuoSet, R&D Systems) as per manufacturer's instructions. IL-1β in tissue lysates and corresponding tissue free supernatants of placenta and choriodecidua collected after 3h, 6h and 18h were measured.

5.3.5 Cytotoxicity assay

As outlined in Chapter 2.2.4, cytotoxicity of all inhibitors was evaluated utilising a WST reagent for detection of LDH in tissue free supernatants (LDH-Cytotoxicity Assay Kit II, Abcam).

5.3.6 Caspase-1/8 activity

The activity of caspase-1 and caspase-8 was measured in tissue lysates at T0 and after 3h, 6h and 18h of culture in the presence of absence of with flagellin using commercially available colorimetric kits (Caspase-1 Assay Kit and Caspase-8 Assay Kit, Both Abcam) as per manufacturer's instructions (see Chapter 2.7 for method).

5.3.7 Statistical analysis

Agonist mediated cytokine production by non-laboured tissues was evaluated by Wilcoxon matched pairs signed rank test or Freidman's test with Dunn's posthoc test for multiple comparisons. A p-value of ≤ 0.05 was considered significant.

5.4 Results

5.4.1 IL-1β production by term non-laboured gestation-associated tissue explants in response to flagellin

To investigate if flagellin induced IL-1β production in the placenta, choriodecidua and amnion an ultrapure form of flagellin isolated from *Salmonella typhimurium* was used at a pre-optimised concentration (Chapter 3) of 100 ng/ml (n=12). A significant increase in IL-1β was observed in both the placenta and choriodecidua (Figure 5.1). IL-1β was not detectable in supernatants from amnion explant cultures.

5.4.2 The role of TLR5 in the production of IL-1β by term non-laboured gestation-associated tissue explants in response to flagellin

To determine if TLR5 played a role in the production of IL-1β by the placenta and choriodecidua, neutralising anti-human TLR5 monoclonal antibody (10 μg/ml) was used; an appropriate isotype control was included (n=3). A decrease in flagellin- but not LPS-induced IL-1β, IL-6 and IL-8 production was observed in the placenta choriodecidua and amnion in the presence of anti-human TLR5 antibody but not isotype control (Figure 5.2 – 5.4). However this was not significant.

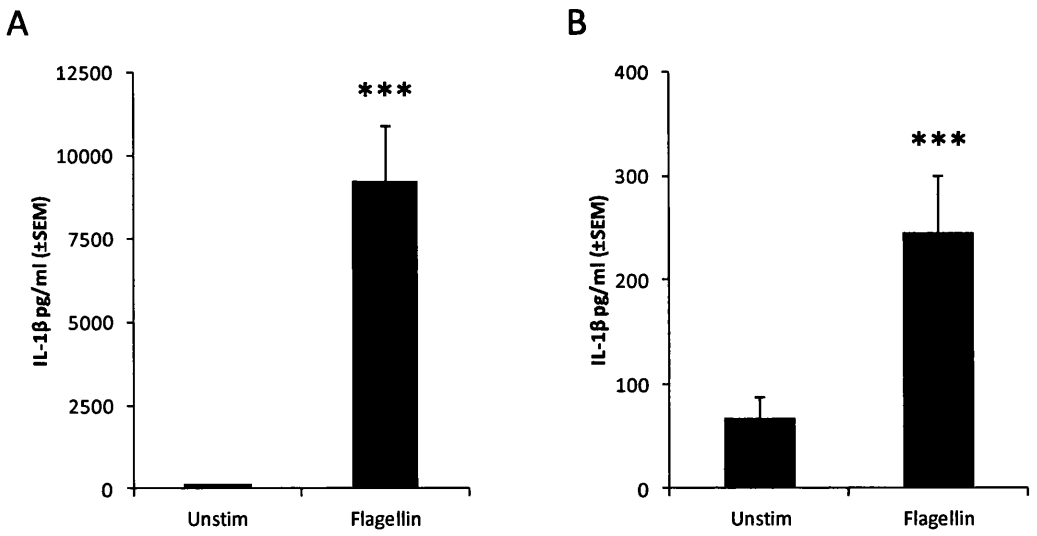

Figure 5.1 Flagellin induced IL-1β production.

Explants of (A) placenta and (B) choriodecidua were treated with ultrapure flagellin (100ng/ml) and levels of IL-1β production (pg/ml mean ± SEM) in tissue free culture supernatants measured (n=12). Statistical significance compared to unstimulated control as determined by Wilcoxon matched pairs signed rank test are shown: *** $p \leq 0.001$.

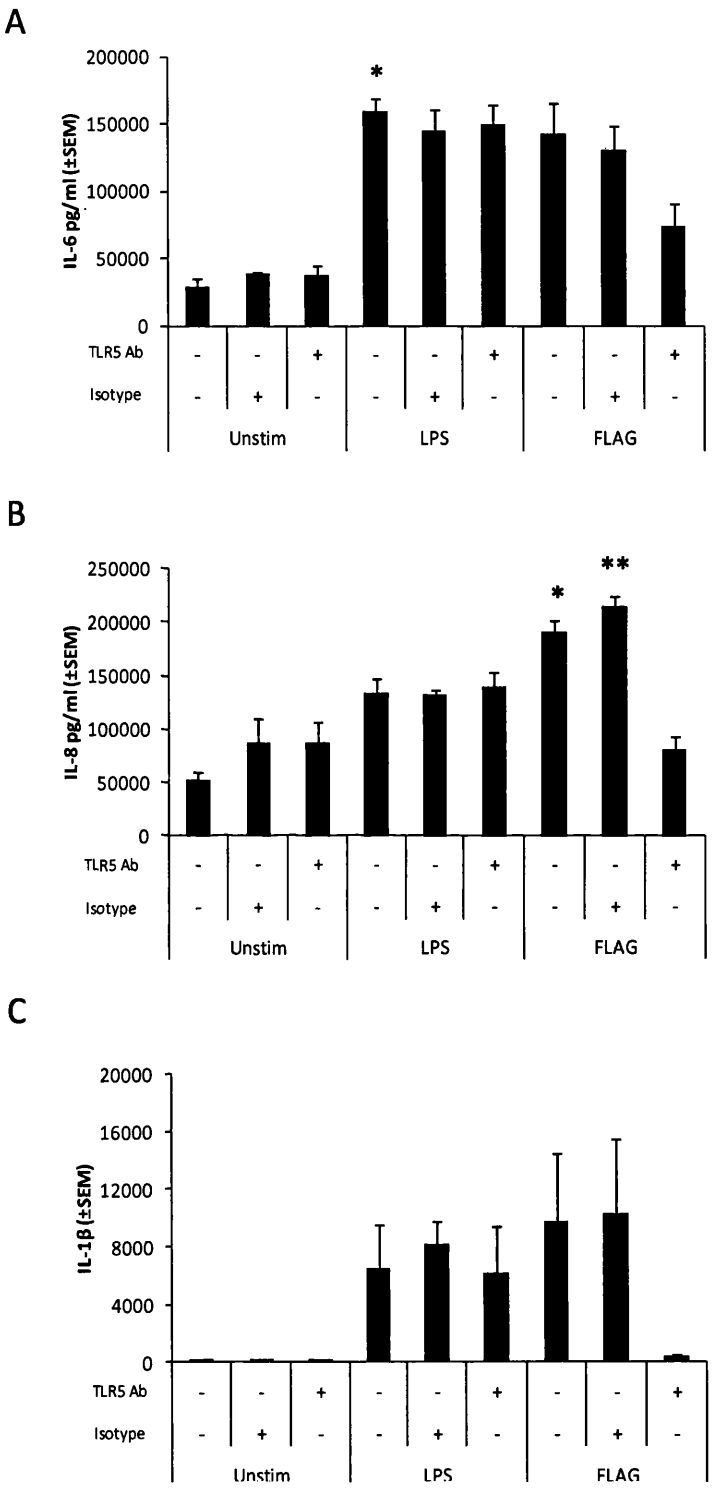

Figure 5.2 Neutralisation of TLR5 in the placenta.

Levels of (A) IL-6, (B) IL-8 and (C) IL-1β (pg/ml mean ± SEM) from explants of term non-laboured placenta (n=3) treated with flagellin (FLAG, 100 ng/ml) or LPS (10 ng/ml) alone or following 30 minutes pre-treatment with either anti-hTLR5-IgA or IgA2 isotype control (both 10 μg/ml). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$, ** $p \leq 0.01$.

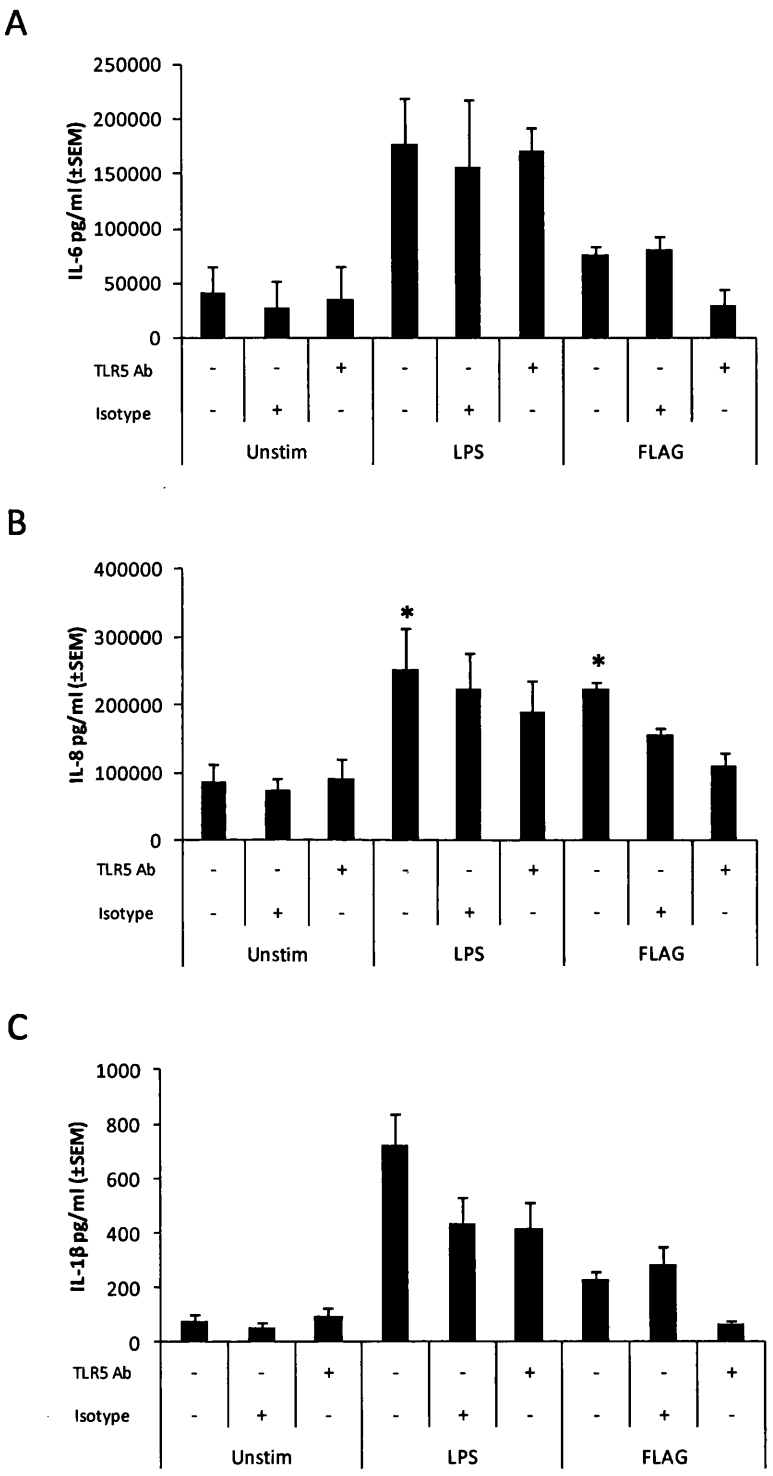

Figure 5.3 Neutralisation of TLR5 in the choriodecidua.

Levels of (A) IL-6, (B) IL-8 and (C) IL-1β (pg/ml mean ± SEM) from explants of term non-laboured choriodecidua (n=3) treated with flagellin (FLAG , 100 ng/ml) or LPS (10 ng/ml) alone or following 30 minutes pre-treatment with either anti-hTLR5-IgA or IgA2 isotype control (both 10 μg/ml). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

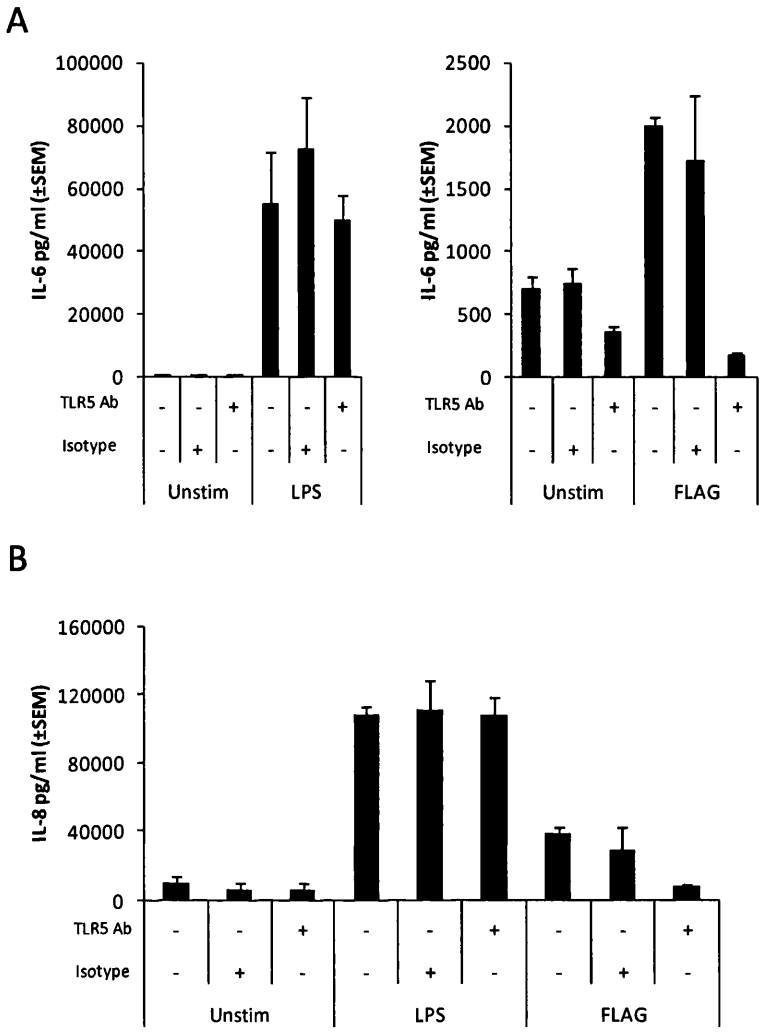

Figure 5.4 Neutralisation of TLR5 in the amnion.

Levels of (A) IL-6 and (B) IL-8 (pg/ml mean ± SEM) from explants of term non-laboured amnion (n=3) treated with flagellin (FLAG, 100 ng/ml) or LPS (10 ng/ml) alone or following 30 minutes pre-treatment with either anti-hTLR5-IgA or IgA2 isotype control (both 10 µg/ml). Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

5.4.3 The role of caspase-1 and caspase-8 in the production of IL-1β by term non-laboured gestation-associated tissue explants in response to flagellin

Since flagellin-induced IL-1β production is associated with caspase-1 and NLRC4 inflammasome activation [350], the role of capase-1 in pro-IL-1β processing was examined using the caspase-1 inhibitor, Z-WEHD-FMK (5μM). A reduction in flagellin-induced IL-1β levels in both the placenta and choriodecidua (n=3) was observed, however this was not significant (Figure 5.5). Since caspase-8 has a role in IL-1β production in response to curdlan, the role of capase-8 in flagellin induced IL-1β was examined using the caspase-8 inhibitor, Z-IETD-FMK (5μM). A reduction in IL-1β was observed in the placenta, however this was not significant. Inhibiting caspase-8 had no effect on IL-1β production in the choriodecidua (Figure 5.6). Monitoring LDH in the culture supernatants showed that there was no effect of the inhibitors on cell viability.

To examine specific caspase activity in these tissues, caspase-1 and caspsae-8 activity was examined in tissue lysates prepared at 3, 6 and 18 hours following flagellin treatment (Figures 5.7 - 5.8). Caspase activity at T0 was also examined. No discernible difference was observed in caspase-1 or caspase-8 activity regardless of time or flagellin stimulation. Examining activity at subsequent time points of 24, 48 and 72 hours did not alter this. As shown in Chapter 4, both assays are indeed functional. When assayed for IL-1β, lysates showed an increase in putative pro-IL-1β production in both a flagellin and time dependent manner, which was also observed in corresponding supernatants (Figures 5.9 - 5.10). In the placenta, an increase in both putative pro-IL-1β and IL-1β was observed from 3 hours and this continued to increase over time, with a significant increase in putative pro-IL-1β from 6 hours. In the choriodecidua, an increase in both putative pro-IL-1β and IL-1β was not observed until 6 hours and this continued to increase over time, with a significant increase of putative pro-IL-1β at 18 hours.

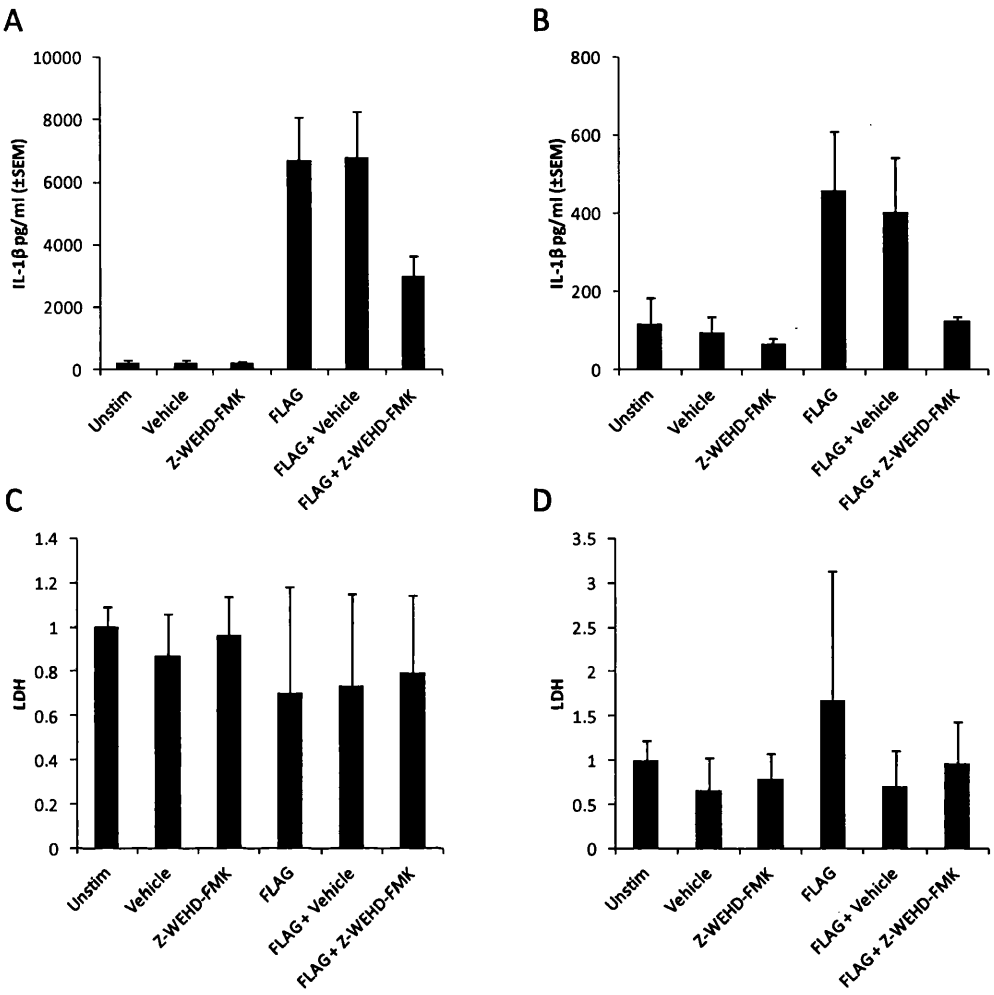

Figure 5.5 Inhibition of capase-1.

Explants of (A) placenta and (B) choriodecidua were treated with flagellin (100 ng/ml) in the presence or absence of the caspase-1 inhibitor Z-WEHD-FMK (5 μM; n=3) and levels of IL-1β (pg/ml mean ± SEM) in tissue free supernatants measured. Relative LDH levels in the (C) placenta and (D) choriodecidua compared to the unstimulated control. Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

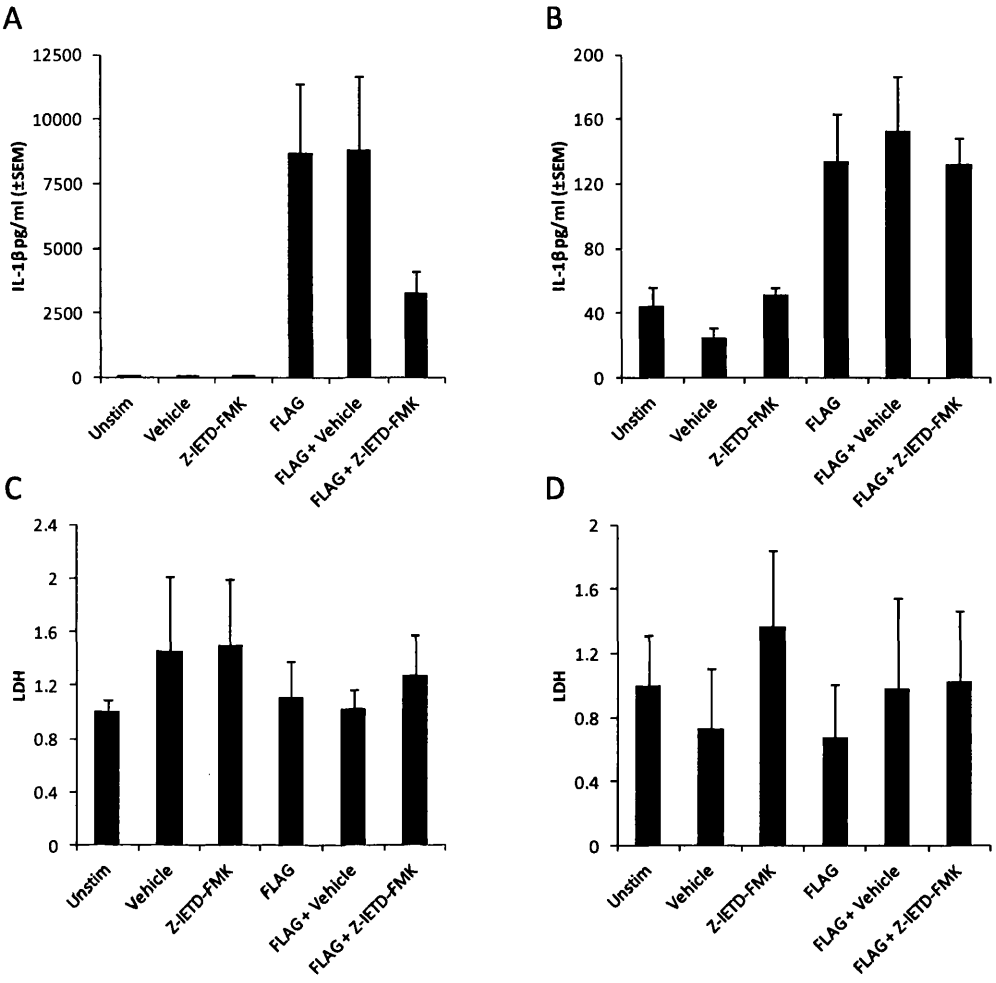

Figure 5.6 Inhibition of caspase-8.

Explants of (A) placenta and (B) choriodecidua were treated with flagellin (100 ng/ml) in the presence or absence of the caspase-8 inhibitor Z-IETD-FMK (5 μM; n=3) and levels of IL-1β (pg/ml mean ± SEM) in tissue free supernatants measured. Relative LDH levels in the (C) placenta and (D) choriodecidua compared to the unstimulated control. Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

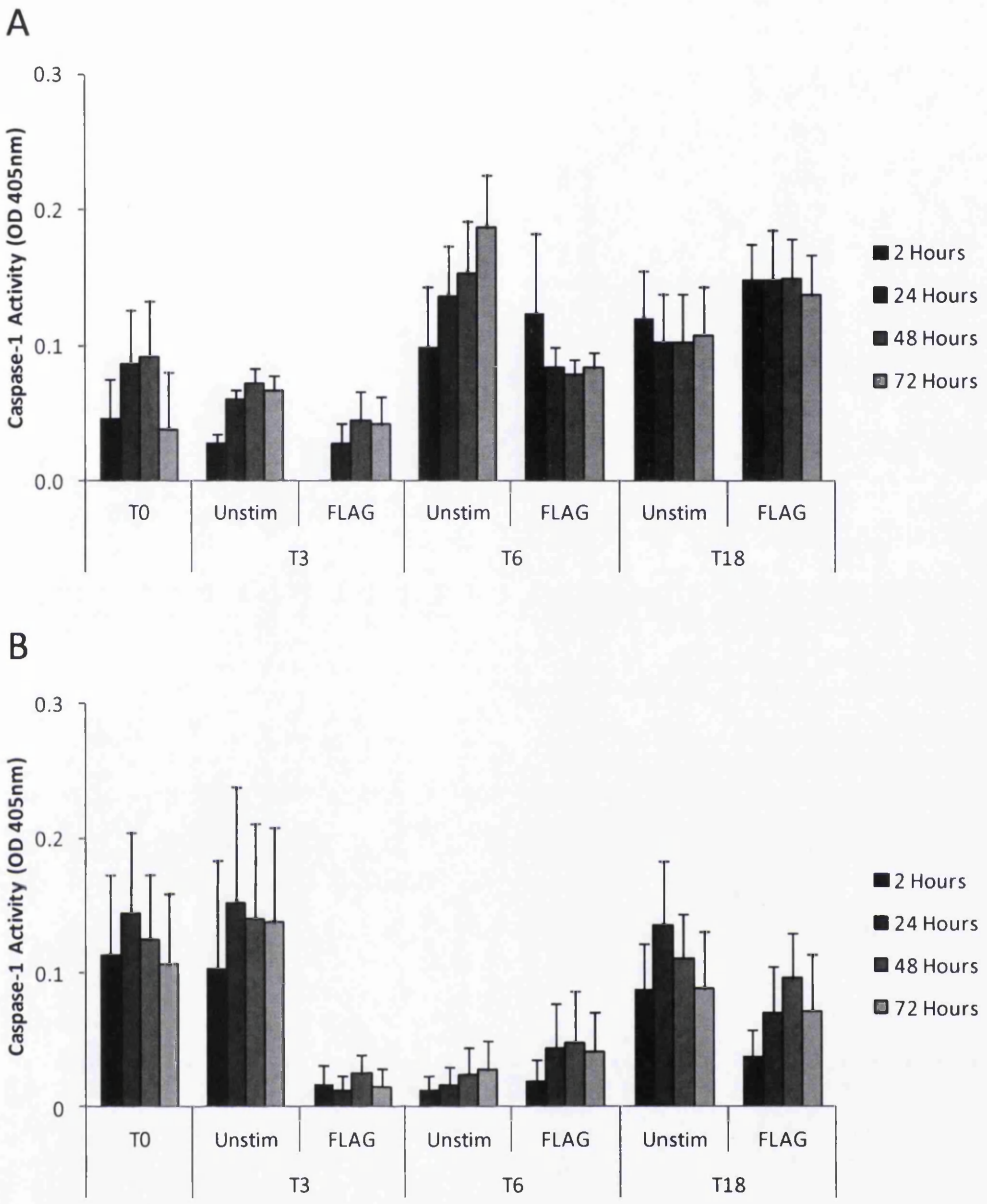

Figure 5.7 Caspase-1 activity in the placenta and choriodecidua.

Tissue lysates were prepared by homogenisation of explants of (A) placenta and (B) choriodecidua with and without treatment with flagellin (100 ng/ml; n=3) for 3, 6 an 18 hours. After determining total protein content, lysates were assayed for caspase-1 activity (OD 405 nm at 2, 24, 48 and 72 hours; mean ± SEM). Caspase-1 activity at T0 was also examined.

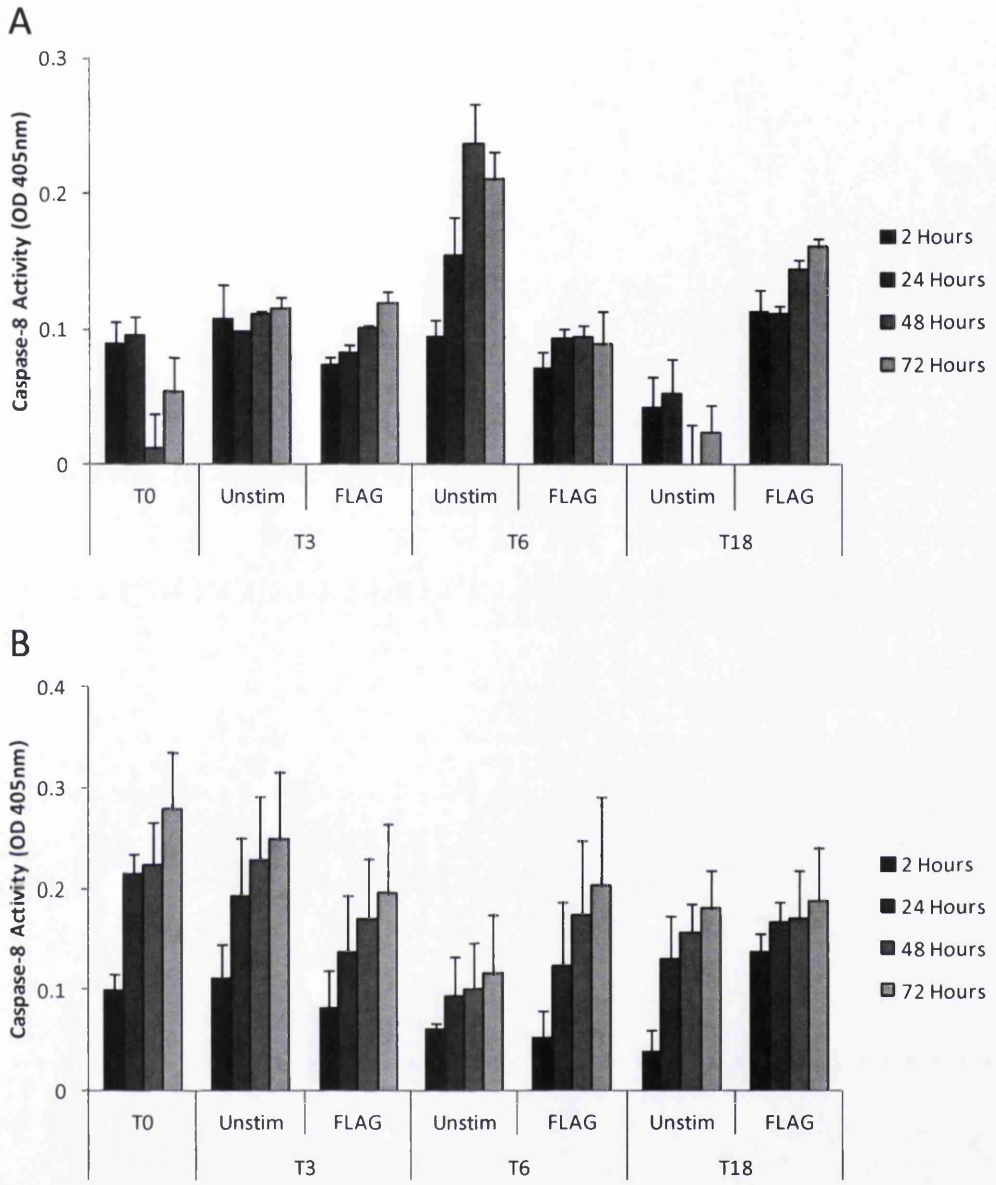

Figure 5.8 Caspase-8 activity in the placenta and choriodecidua.

Tissue lysates were prepared by homogenisation of explants of (A) placenta and (B) choriodecidua with and without treatment with flagellin (100 ng/ml; n=3) for 3, 6 an 18 hours. After determining total protein content, lysates were assayed for caspase-8 activity (OD 405 nm at 2, 24, 48 and 72 hours; mean ± SEM). Caspase-8 activity at T0 was also examined.

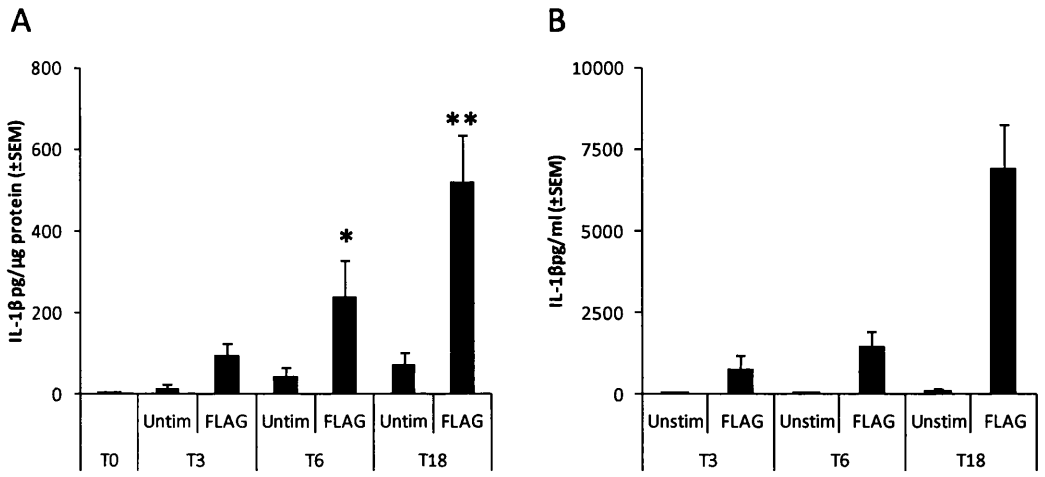

Figure 5.9 IL-1β production in placental tissue lysates versus issue free supernatants.
Explants of placenta were treated with flagellin (100 ng/ml; n=3) and then levels of IL-1β (pg/ml mean ± SEM) measured in (A) tissue lysates (total protein) and (B) tissue free supernatants. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to T0 lysate is shown: * $p \leq 0.05$, ** $p \leq 0.01$.

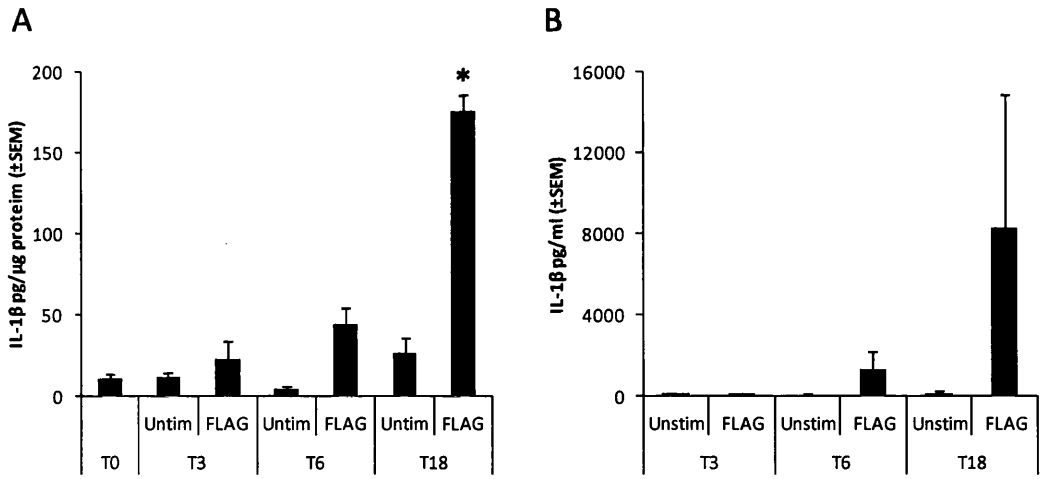

Figure 5.10 IL-1β production in choriodecidual tissue lysates versus tissue free supernatants.
Explants of choriodecidua were treated with flagellin (100 ng/ml; n=3) and then levels of IL-1β (pg/ml mean ± SEM) measured in (A) tissue lysates (total protein) and (B) tissue free supernatants. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to T0 lysate is shown: * $p \leq 0.05$.

5.5 Discussion

Since IL-1β is a key cytokine associated with intra-amniotic infection during pregnancy and a major factor in the initiation of infection associated preterm labour, identifying the key tissues involved in IL-1β production at the maternal-fetal interface and the initiators of this process is of much interest. Chapter 4 demonstrated that the gestation-associated tissues are capable of producing IL-1β in response to fungal β-glucans, while work running in parallel in the research group demonstrated IL-1β production in response to several other described agonists of the NLRP3 inflammasome. In addition to NLRP3, transcripts for several other NLRs that are associated with the formation of an inflammasome are expressed by these tissues (Chapter 3). Therefore the aim of this chapter was to determine if flagellin, which is associated with the activation of the NLRC4 inflammasome [350], could induce IL-1β production in the term non-laboured placenta, choriodecidua and amnion.

The data generated shows that flagellin is able to induce the production of IL-1β in both the placenta and choriodecidua, but not the amnion. The innate immune response to flagellin is traditionally associated with TLR5, while flagellin-induced IL-1β is associated with the activation of the NLRC4 inflammasome. The expression of transcripts for TLR5 and NLCR4 within placenta, choriodecidua and amnion was shown in Chapter 3. However, protein expression of these receptors and their cellular source within these tissues has yet to been determined and is currently underway. It would be expected that TLR5 protein would be observed in all three tissue, as flagellin treatment induced elevated levels of IL-6 and IL-8 (Chapter 3). In contrast, since only the placenta and choriodecidua induced IL-1β production, it would suggest that NLRC4 protein is not expressed by the amnion. However it's entirely possible that NLRC4 protein might be expressed in the amnion, and the lack of IL-1β results for defect in other inflammasome components.

To examine if TLR5 had a functional role in IL-1β production, a TLR5 neutralising antibody was used. In the presence of anti-hTLR5 Ab, a reduction in IL-1β, IL-6 and IL-8 was observed in both the placenta and choriodecidua, suggesting that TLR5 is required for the production of IL-1β. It is probable that the flagellin-TLR5 interaction is the priming signal required for the initial production of pro-IL-1β, while simultaneous activation of the inflammasome occurs to process the pro-IL-1β to IL-1β, but this needs to be investigated. Examination of placental tissue lysates

demonstrate that by 3 hours there was a trend for increased level of putative pro-IL-1β occurs, with a corresponding increase of IL-1β in the supernatant, suggesting a rapid and simultaneous response. The choriodecidua however, appear to require a longer prime, with no increase in either pro-IL-1β or IL-1β until 6 hours post flagellin treatment. Regardless of the length of the priming event, replication of this experiment utilising the anti-hTLR5 Ab, might demonstrate no pro-IL-1β in the tissue lysate or IL-1β in the supernatant, further implying a role for TLR5. Downstream of TLR5, production of pro-inflammatory cytokines is dependent on the MyD88 dependent pathway and the activation of NF-κB and MAPKs [120]. While not examined here, it would be expected they signalling would be required for the production of pro-IL-1β.

Recognition of cytoplasmic flagellin by the NLRC4 inflammasome is associated with caspase-1 dependent cleavage of pro-IL-1β to active IL-1β. Inhibition of caspase-1 using a caspase-1 specific inhibitor, resulted in lower levels of IL-1β from flagellin treated placenta and choriodecidua. Recent work in a murine model has identified that NAIP5 and NAIP6 act as the direct sensors for flagellin and type three secretion systems signalling though NLRC4 [350, 351]. In humans only one NAIP protein is expressed and as shown in chapter 3 transcripts for NAIP are expressed in all three gestation-associated tissues. The human NLRC4-NAIP complex has not yet been extensively studied, however it has been shown to recognise a type three secretion system in human U937-derived macrophages in a NAIP dependent manner [352]. Therefore it is possible that human NAIP functions in a similar manner to murine NAIP5 and NAIP6, acting as a direct sensor for cytoplasmic flagellin, signalling through NLRC4 to activate caspase-1. In contract to NLRP3 which requires the adapter protein ASC, NLRC4 has been shown to directly interact with pro-caspase-1 due the presence of CARD domain within its structure. However NLRC4-ASC dependent processes do occur [353, 354], therefore the exact role of for ASC within the NLRC4 inflammasome remains inconclusive.

A role for caspase-8 by macrophages in the processing of IL-1β has been described [177]. Furthermore recent reports have shown that in a murine model a caspase-8 dependent pathway is activated via NLRC4 in response to *Salmonella* infection inducing an ASC-caspase-8-caspase-1 complex [354]. Inhibition of caspase-8 resulted in the reduction of IL-1β production by the placenta and not the choriodecidua. As shown in chapter 4, caspase-8 is present in the choriodecidua

and can be activating under certain conditions. It is therefore possible that gestation-associated tissue can utilise caspase-1 and caspase-8 in an agonist and tissue dependent manner. However at this time the mechanisms relating to this are unknown. Gestation-associated tissues might be utilising a caspase-1 dependent inflammasome e.g. NLCR4 or NLRP3 and the non-canonical caspase-8 inflammasome independently or alternatively a caspase-8-caspsae-1 complex could be formed. Furthermore a role for caspase-8 in the processing of caspase-1 has been described [355]. A co-immunoprecipitation approach would likely assist in confirming what type inflammasome/caspase complexes are involved.

In summary the data contained in this chapter show that flagellin stimulates the production of IL-1β by the placenta and choriodecidua, but not the amnion. There is a role for caspase-1 in IL-1β production by both tissues but only placental IL-1β production in response to flagellin seems to utilise caspase-8. This contrasts with the response to curdlan shown in Chapter 4 where caspase-1 and caspase-8 were both involved in the curdlan stimulated IL-1β.

Chapter 6

Use of anti-inflammatory cytokines to modulate the inflammatory response of gestation-associated tissues

6 Use of anti-inflammatory cytokines to modulate the inflammatory response of gestation-associated tissues

6.1 Introduction

Human labour and delivery has been well characterised as an inflammatory process with several pro-inflammatory cytokines produced by the gestation associated tissues involved in this process [10, 11, 14]. Work in this thesis and by others has shown that the gestation associated tissues are capable of producing an inflammatory response to an array of PAMPs via the activation of PRRs [233, 236, 238, 242, 286, 287, 296]. While this response to microbial derived stimuli might offer an explanation of the mechanisms of infection associated preterm labour, it is possible that PRRs in response to endogenous stimuli play a role in term labour also. The ability to regulate the inflammatory response via PRRs in the gestation associated tissue might yield novel new approaches to the prevention of preterm labour and other adverse pregnancy outcomes.

To date treatment strategies for PTL have focused on inhibiting myometrial contractions utilising an array of compounds including cyclooxygenase inhibitors, oxytocin antagonists, magnesium sulphate and β-mimetics [356]. The potential benefits of progesterone treatments for PTL have also been examined [357]. While no significant change in the levels of progesterone are observed preceding labour, a functional withdrawal of progesterone activity in the uterus is associated with term and preterm labour [358, 359]. Several studies utilising progesterone in women have cautiously positive results [357, 360, 361], but indications from animal studies have shown an increased susceptibility to bacterial infection in the uterus [362, 363]. Regardless, progesterone supplementations was approved to prevent recurrent preterm birth in the United States in 2011 [357]. NF-κB has been suggested as another therapeutic target because of its role in both inflammation and labour. However considering its broad role, the identification of labour specific components is key in order to minimise toxicity [277].

A number of cytokines are well recognised for their anti-inflammatory activity, including IL-4, IL-10 and IL-13 [364]. IL-4 and IL-13 are shown to signal though a signal transducer and activator of transcription 6 (STAT6) dependent pathway (Figure 6.1), while IL-10 utilises STAT3 (Figure 6.2). During pregnancy anti-inflammatory cytokines have been long associated with a protective role including

maintaining the Th1:Th2 cytokine balance by decreasing the production of pro-inflammatory cytokines [365, 366]. The trophoblast, decidua and amnion have all been reported to produce IL-4, IL-10 and IL-13 contributing to the reported Th2 cytokine bias of pregnancy [366]. Interestingly, IL-10 is increased in amniotic fluid from women in term labour and women with intra-amniotic infection at term and preterm, and is also increased in those without infection who delivered preterm rather than term [269]. A similar observation for IL-4 has been described [367]. Elevated anti-inflammatory cytokines in these circumstances might represent a compensatory mechanism that has failed.

Investigations into the use of anti-inflammatory cytokines therapeutically to prevent PTL have focused primarily on IL-10. Animal studies have shown that co-administration of IL-10 and LPS decreased placental nitric oxide, TNF-α and apoptosis compared to LPS treatment alone [368]. Furthermore, intravenous IL-10 administration was able to prevent PTB resulting from intrauterine LPS infusion in rats [369]. IL-10 has been shown to down-regulate LPS- and LTA (lipoteichoic acid)-induced cytokine and chemokine responses by the healthy term placenta [268]. In addition IL-10 can inhibit the production of IL-1β and prostaglandin E_2 production and inhibit COX-2 expression in intact term fetal membranes [370]. The anti-inflammatory effects of IL-4 and IL-13 have also been noted. In human monocytes and human umbilical vein endothelial cells, IL-4 has been shown to inhibit LPS induced IL-1β and TNFα [371]. Furthermore IL-4 can down-regulate formyl methionyl leucyl phenylalanine (fMLP) induced IL-8 and TNFα in human polymorphonuclear leukocytes (PMNs) [372]. In monocytes and macrophages, IL-13 has been shown to down-regulate the production of IL-1β, IL-8, MIP-1α and TNF-α [373-376]. It is therefore possible that other anti-inflammatory cytokines have effects similar to IL-10 in gestation associated tissues.

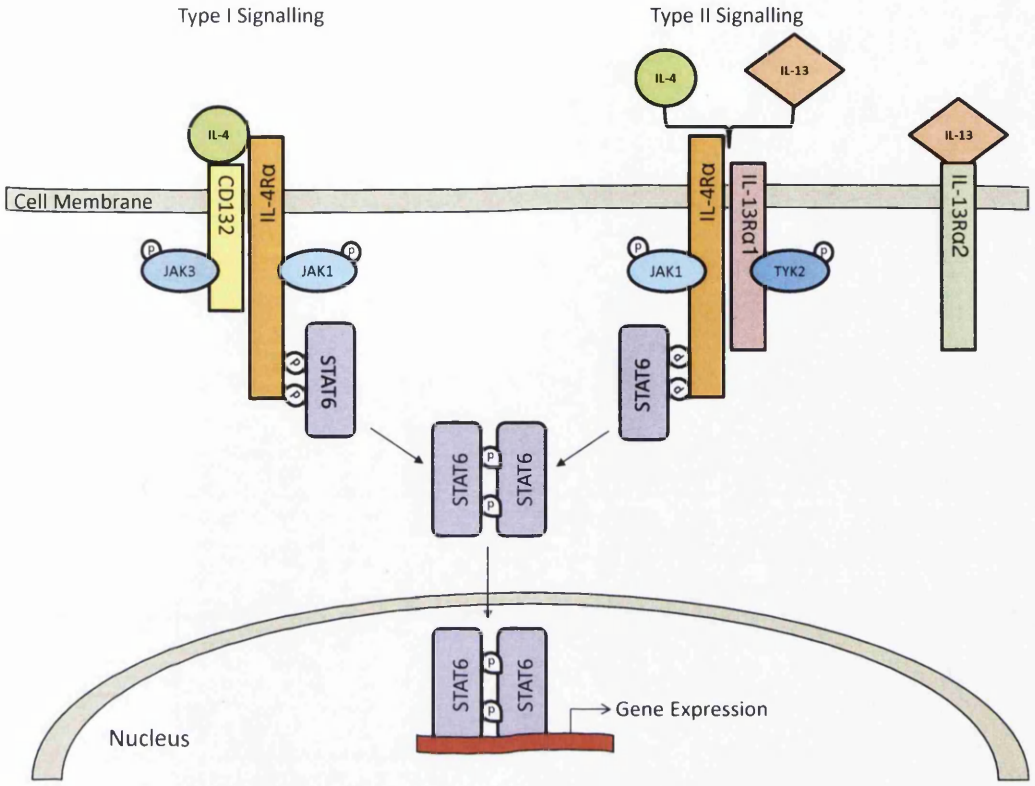

Figure 6.1 Overview of IL-4/IL-13 signalling pathway.

There are two types of signalling receptors – type I consisting of the common gamma chain (g_c; CD132) and IL-4Rα; and type II consisting of IL-4Rα and IL-13Ra1. IL-4 first binds to IL-4Rα and can signal via both type I and type II, whereas IL-13 first binds to IL-13Ra1 and only signals via type II. IL-13Rα2 has been suggested to be a decoy. Activation of their receptors by IL-4 and IL-13 leads to transphosphorylation of non-receptor protein tyrosine kinases (JAKs) and STAT6 phosphorylation and dimerization, and translocation to the nucleus [377].

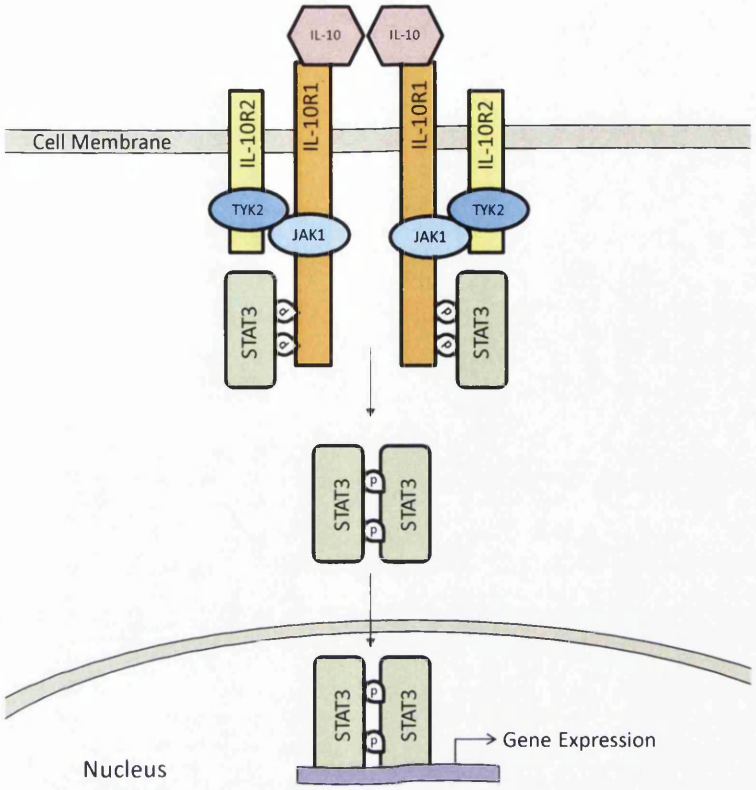

Figure 6.2 Overview of the IL-10 signalling pathway.

Two copies of IL-10 receptor 1 (IL-10R) and IL-10 receptor 2 (IL-10R2), form a receptor complex. Association of JAK1 and Tyk2 occurs upon IL-10 binding to its receptor, resulting in the phosphorylation of the cytoplasmic tail of IL-10R1 facilitating the recruitment of STAT3. Homondimerisation of STAT3 occurs facilitating its release from IL-10R1 and translocation into the nuclease [378, 379].

6.2 Aims

The aim of this chapter is to examine if anti-inflammatory cytokines, notably IL-4 and IL-13, can regulate the LPS induced inflammatory response in the term non-laboured human placenta, choriodecidua and amnion.

6.3 Methods

6.3.1 Samples

Healthy pregnant women scheduled for elective caesarean section (ESC) were approached in the antenatal day assessment unit at Singleton Hospital, Swansea, during their pre-anaesthetic assessment. Informed written consent was obtained upon explanation of the study. Following delivery, placenta and attached fetal membranes (n=3-9) from these women were collected and processed within 1.5 hours of delivery.

6.3.2 PCR

RNA was extracted and cDNA produced from gestation associated tissue samples (placenta, choriodecidua and amnion) stored in TRI reagent (see Chapter 2.4 for method). PCR was performed for IL-4/13 receptor components; IL-4Rα, IL-13α1, IL-13α2 and CD132 (common gamma chain), the transcription factor STAT6 (see Chapter 2.4 for method and Table 6-1 for primer details).

Table 6-1 IL-4/13 receptor and STAT6 primer sequences.

Gene		Primer	Mg^{2+} Conc (nM)	Anneling Temp (°C)	Fragment Size (Bp)	Accession Number
IL-4R	*F*	5' GACCTGGAGCAACCCGTATC	2.5	70	335	NM_000418
	R	5' CATAGCACAACAGGCAGACG				
CD132	*F*	5' ACGGGAACCCAGGAGACAGG	2	70	275	NM_000206
	R	5' AGCGGCTCCGAACACGAAAC				
IL-13Rα1	*F*	5' GAGCTGACCAAAGTGAAGGA	3	69	518	NM_001560
	R	5' ATTGCACCTGCGACGATGACTG				
IL-13Rα2	*F*	5' GGCATAGGTGATCTTCTTGA	2	60	559	NM_000640
	R	5' GCCAGAAACGATGCAAAGTTT				
STAT6	*F*	5' AGAGGGGTTGCCGAGGTGA	4	70	755	NM_001178078
	R	5' TGTCCACCAGGCTTTCACAC				

Sequences and optimum conditions for each pair of primers used for PCR.

6.3.3 Gestation-associated tissue explants

Placenta, choriodecidua and amnion tissue explants were cultured as described in Chapter 2.2. Cultures were stimulated with previously optimised concentrations of cytokines and agonists as follows; ultrapure LPS (10 ng/ml; Invivogen), IL-4 (10 ng/ml), IL-10 (10 ng/ml) and IL-13 (10 ng/ml). An unstimulated control was also included. Cytokines were added either pre- or post-LPS stimulation as detailed in the appropriate figure legends. Cultures were incubated for 24 hours. For inhibition/neutralisation experiments cultures were incubated for 30 minutes prior to the addition of cytokines/agonists with the following blocking antibodies: anti-hIL-4Rα IgG2A (1 µg/ml; R&D Systems), Mouse IgG2A isotype (1 µg/ml; R&D Systems).

6.3.4 Cytokine production

IL-1β and MIP-1α in the tissue free supernatants of placenta, choriodecidua and amnion explant cultures collected after 24 h were measured using commercially available ELISA kits (DuoSet, R&D Systems) as per manufacturer's instructions.

6.3.5 Western blotting

Total protein was extracted and quantified from gestation associated tissue samples (placenta, choriodecidua and amnion) treated with IL-4 for 0.5, 2 and 24 hours (see Chapter 2.5 for method). Immunoblotting was performed using 10 µg of protein for total and phosphorylated STAT6. Antibodies were used at the following dilutions; total STAT6 (rabbit polyclonal IgG; 1:1000; R&D systems), phospho-STAT6 (Y641; rabbit polyclonal IgG; 1:1000; R&D systems), GAPDH (rabbit polyclonal IgG; 1:1000; santa cruz biotechnology); goat anti-rabbit IgG (secondary antibody; 1:2000; santa cruz biotechnology).

6.3.6 Statistical analysis

Agonist mediated cytokine production by non-laboured tissues was evaluated by Wilcoxon matched pairs signed rank test or Freidman's test with Dunn's posthoc test for multiple comparisons. A p-value of ≤ 0.05 was considered significant.

6.4 Results

6.4.1 Effect of IL-4 and IL-13 on the inflammatory response of term non-laboured gestation-associated tissues

To investigate the effect of IL-4 and IL-13 on the inflammatory response in the term non-laboured placenta, choriodecidua and amnion, tissues were pre-treated for 90 minutes with previously optimised concentrations (10 ng/ml) of IL-4 or IL-13 prior to the addition of LPS (10 ng/ml). Results are represented as percentage decrease in cytokine production (IL-1β and MIP-1α) compared to LPS set at 100%. These cytokines were chosen because of their low constitutive production by gestation associated tissues (see Chapter 8.3, Figure 8.26), because of this only the data from LPS treated samples are shown. As shown in Figure 6.3, pre-treatment with IL-4 decreases LPS induced IL-1β and MIP-1α production in all three tissues. IL-13 pre-treatment had the same effect (Figure 6.4). However, a varied effect is seen across the three tissues: the greatest impact of IL-4 /13 pre-treatments was on the placenta with the least impact on the amnion. Additionally a greater impact on IL-1β than MIP-1α was observed.

While pre-treatment with either IL-4 or IL-13 was able to reduce LPS induced cytokine levels, this does not mimic the typical clinical setting. Therefore the ability of both IL-4 and IL-13 to modulate cytokine production post-LPS exposure was examined. To investigate this term non-laboured placenta, choriodecidua and amnion were treated with LPS and IL-4 or IL-13 either concurrently, 90 minute or 240 minute post-LPS treatment. Pre-treatment (90 minutes) was also performed to provide a base line response for IL-4/IL-13. Results are represented as percentage decrease in cytokine production compared to LPS set at 100%. As shown in Figure 6.4, the inhibitory effect of IL-4 diminishes over time post LPS treatment. A similar effect is observed for IL-13 (Figure 6.5).

Since the effect of both IL-4 and IL-13 diminishes post exposure to LPS, it was decided to examine whether co-treatment with both cytokines would enhance their anti-inflammatory effect. To investigate this term non-laboured placenta, choriodecidua and amnion, tissues were treated with IL-4 alone, IL-13 alone or a co-treatment of IL-4 and IL-13 at 90 and 240 minutes post-LPS exposure. The results are shown in Figure 6.6. IL-4 and IL-13 co-treatment did not enhance the ability of these cytokines to down regulate the LPS response.

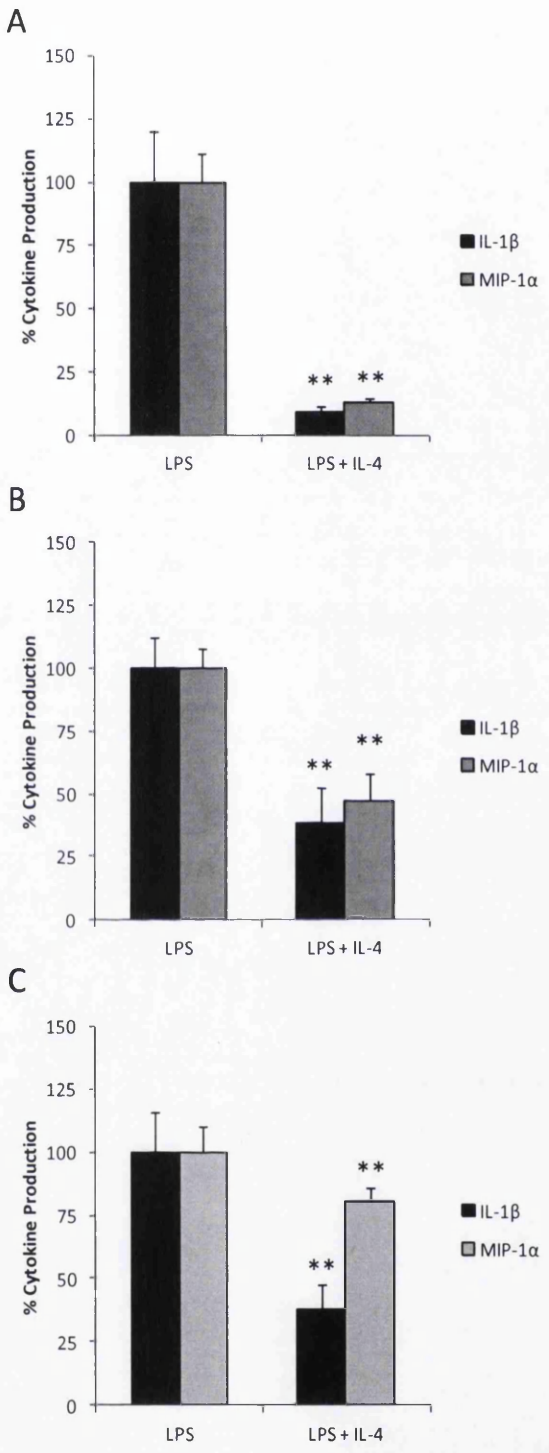

Figure 6.3 Effect of IL-4 on LPS induced cytokine production by term non-laboured gestation associated tissues.

Cytokine levels (IL-1β & MIP-1α) from explants of (A) placenta (B) choriodecidua and (C) amnion in response to treatment with IL-4 (10 ng/ml) 90 minutes prior to treatment with LPS (10 ng/ml). Data shown as percentage of response to LPS alone (n=9); error bars represent SEM. Statistical significance compared to unstimulated LPS as determined by Wilcoxon matched pairs signed rank test are shown: ** $p \leq 0.01$.

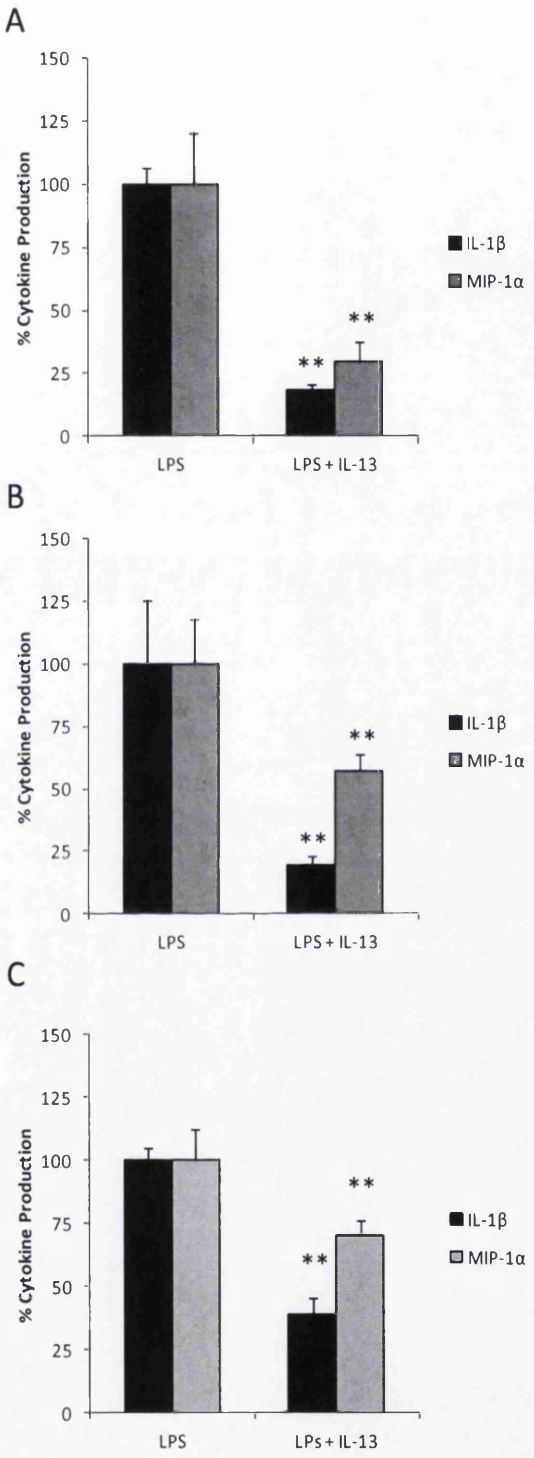

Figure 6.4 Effect of IL-13 on LPS induced cytokine production by term non-laboured gestation associated tissues.

Cytokine levels (IL-1β & MIP-1α) from explants of (A) placenta (B) choriodecidua and (C) amnion in response to treatment with IL-13 (10 ng/ml) 90 minutes prior to treatment with LPS (10 ng/ml). Data shown as percentage of response to LPS alone (n=9); error bars represent SEM. Statistical significance compared to LPS as determined by Wilcoxon matched pairs signed rank test are shown: ** $p \leq 0.01$.

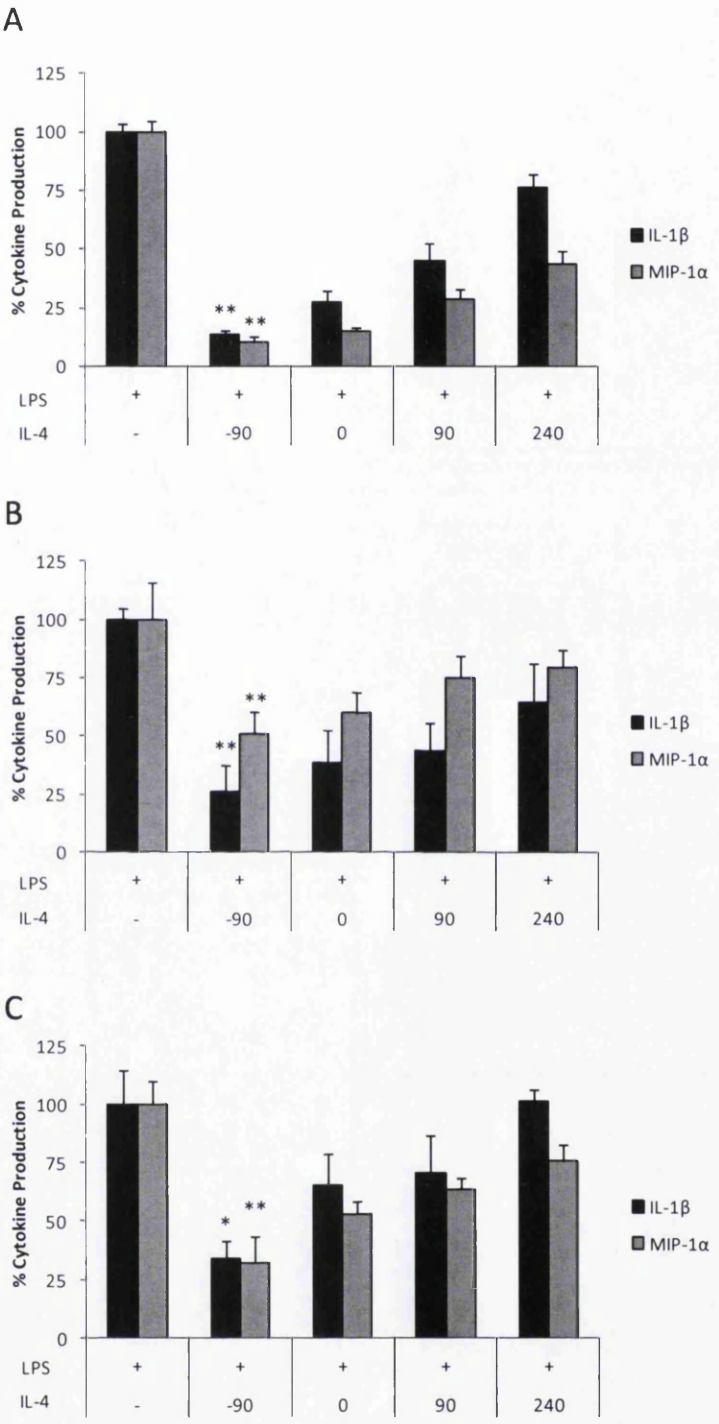

Figure 6.5 Effect of IL-4 treatment over time on LPS induced cytokine production by term non-laboured gestation associated tissues.

Cytokine levels (IL-1β & MIP-1α) from explants of (A) placenta (B) choriodecidua and (C) amnion in response to LPS (10 ng/ml) in the presence of IL-4 (10 ng/ml) added 90 minutes prior to LPS (T-90), at the same time as LPS (T0), or 90 minutes (T+90) or 4 hours (T+240) after LPS (n=3). Data are shown as percentage of response to LPS alone; error bars represent SEM. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to LPS is shown: * $p \leq 0.05$, ** $p \leq 0.01$.

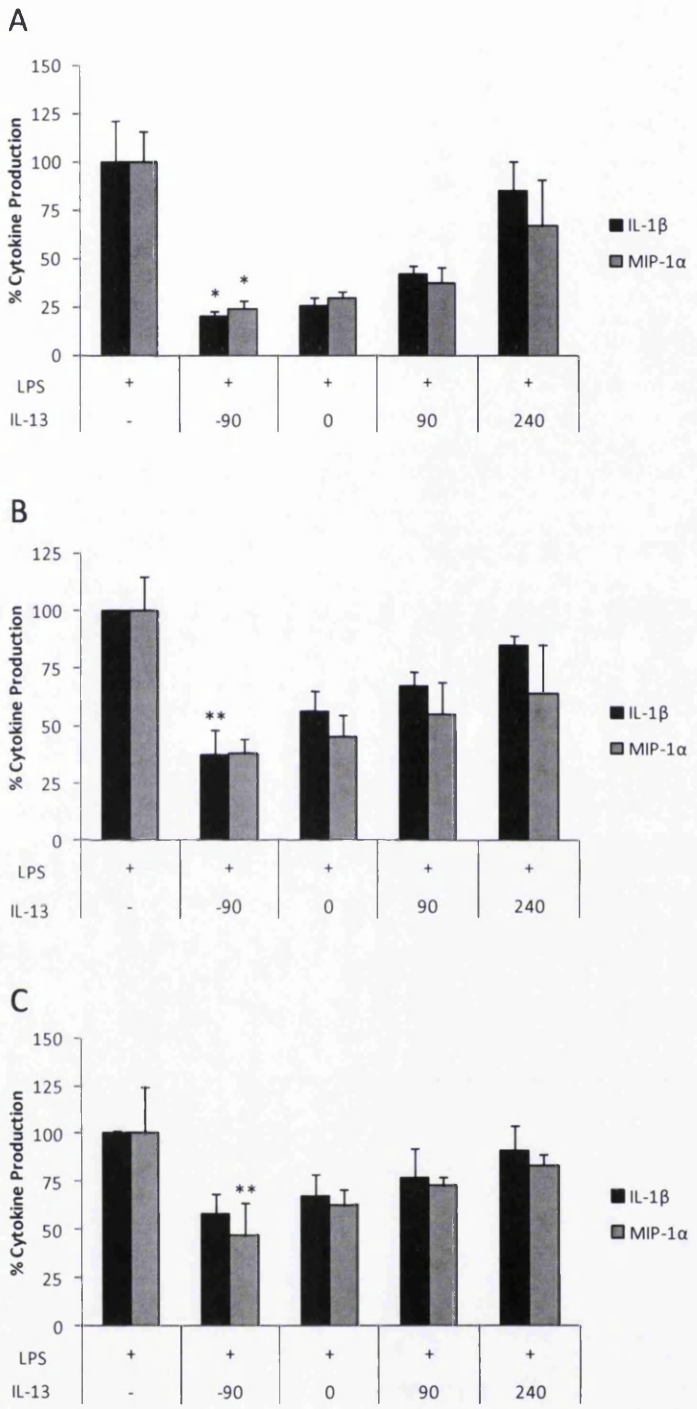

Figure 6.6 Effect of IL-13 treatment over time on LPS induced cytokine production by term non-laboured gestation associated tissues.

Cytokine levels (IL-1β & MIP-1α) from explants of (A) placenta (B) choriodecidua and (C) amnion in response to LPS (10 ng/ml) in the presence of IL-13 (10 ng/ml) added 90 minutes prior to LPS (T-90), at the same time as LPS (T0), or 90 minutes (T+90) or 4 hours (T+240) after LPS (n=3). Data are shown as percentage of response to LPS alone; error bars represent SEM. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to LPS is shown: * $p \leq 0.05$, ** $p \leq 0.01$.

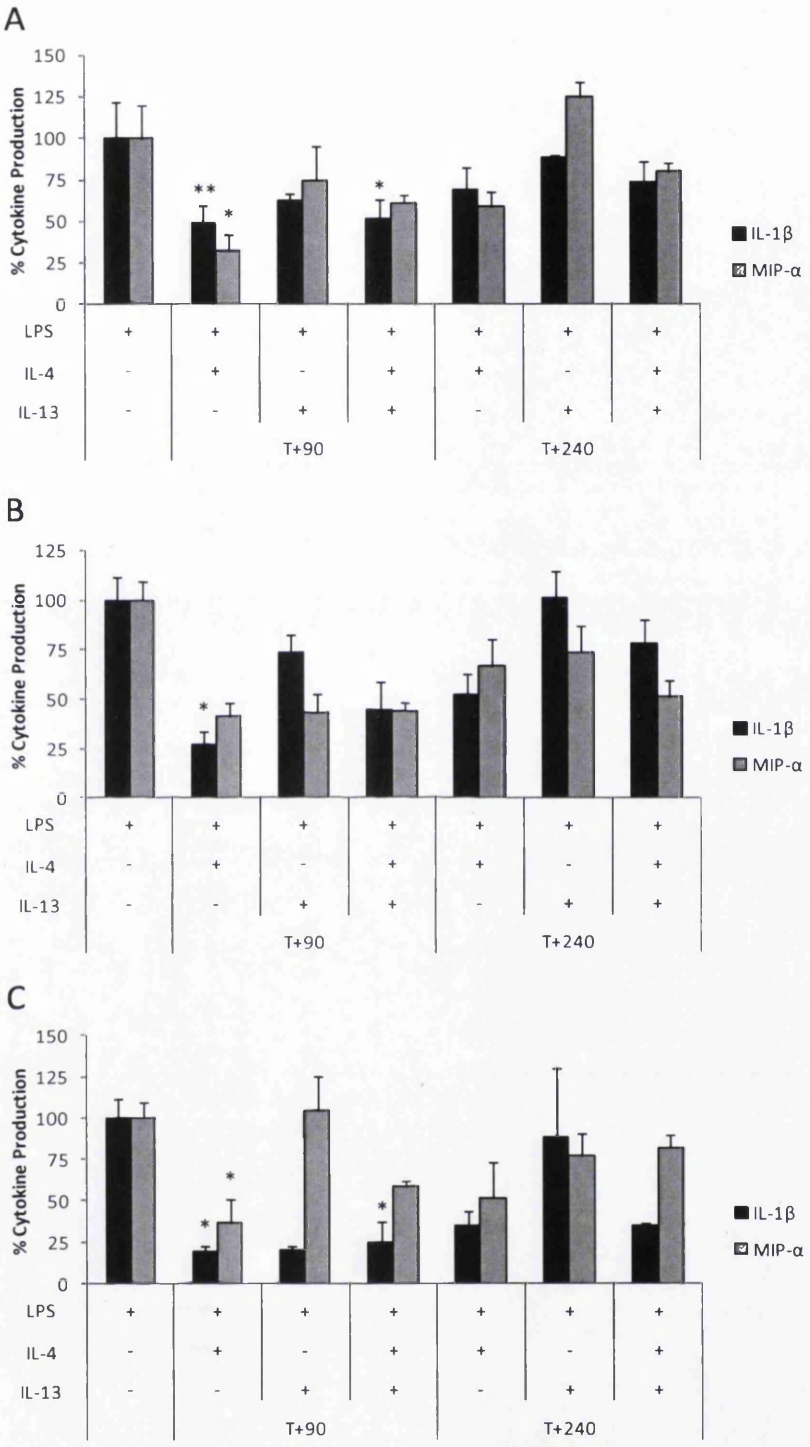

Figure 6.7 Combined effect of IL-4 and IL-13 treatment on LPS induced cytokine production by term non-laboured gestation associated tissues.

Cytokine levels (IL-1β & MIP-1α) from explants of (A) placenta (B) choriodecidua and (C) amnion in response to LPS (10 ng/ml) in the presence of IL-4, IL-13 or IL-4 + IL-13 (both10 ng/ml) added 90 minutes (T+90) or 4 hours (T+240) after LPS (n=3). Data are shown as percentage of response to LPS alone; error bars represent SEM. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to LPS is shown: * $p \leq 0.05$, ** $p \leq 0.01$.

6.4.2 The IL-4/IL-13 signalling pathway

IL-4 and IL-13 share a number of functional characteristics. Both IL-4 and IL-13 utilise the receptor component IL-4Rα and activate a common signalling pathway; the JAK/STAT pathway to activate signal transducer and activator of transcription 6 (STAT6). An overview of IL-4 and IL-13 signalling is shown in Figure 6.1.

6.4.2.1 Expression of transcripts for IL-4/IL-13 signalling pathway by gestation-associated tissues

To investigate whether transcripts for components of the IL-4/IL-13 signalling pathways, including IL-4 receptor (IL-4Rα), the common gamma chain (CD132), IL-13 receptor alpha 1 (IL-13Rα1), IL-13 receptor alpha 2 (IL-13Rα2) and STAT6 are expressed in gestation associated tissues, PCR was performed using five individual samples of each tissue type (placenta, choriodecidua and amnion). Three of these five samples are shown in Figure 6.8. Transcripts for each component examined were present in all five samples of each of the tissues, with the exception of IL-13Rα2, which showed varied expression in the amnion across all 5 samples, from no expression to strong expression.

6.4.2.2 IL-4Rα neutralisation

To confirm that both IL-4 and IL-13 utilise their classically described signalling pathway in the gestation associated tissues, IL-4Rα, as a common receptor component to both IL-4 and IL-13 signalling was examined. Term non-laboured tissues were pre-treated for 90 minutes with either IL-4 or IL-13 prior to the addition of LPS in the presence and absence of a neutralising Mouse IgG2A anti-hIL-4R (1 μg/ml; n=3). Mouse IgG2A isotype (1 μg/ml) was used as a control. The results are shown in Figures 6.9 – 6.11. In the presence of the anti-IL-4Rα antibody, the effect of IL-4/IL-13 on LPS-induced IL-1β and MIP-1α is abrogated, with no effect of isotype control.

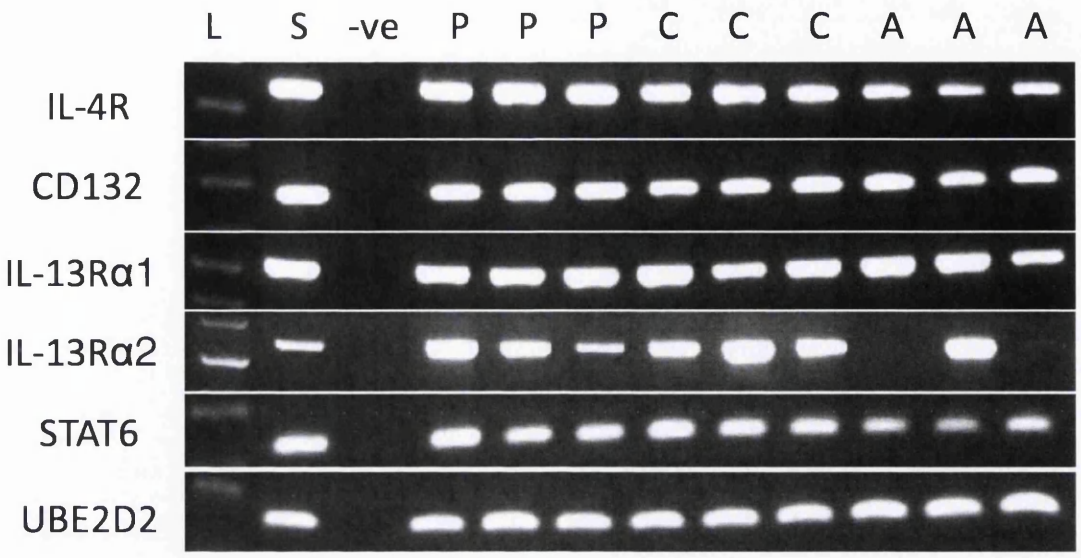

Figure 6.8 Expression of transcripts of the IL-4/13 signalling pathway by gestation-associated issues.

PCR for each of the receptor chains involved in IL-4 and IL-13 signalling as well as the main transcription factor STAT6. Three representative examples of a total of 5 are shown; placenta (P), choriodecidua (C) and amnion (A). Human spleen (S) was used as a positive control and UBE2D2 was used as a housekeeping gene. L = 100 base pair ladder and the negative control (-ve) was with water replacing cDNA in the reaction mix.

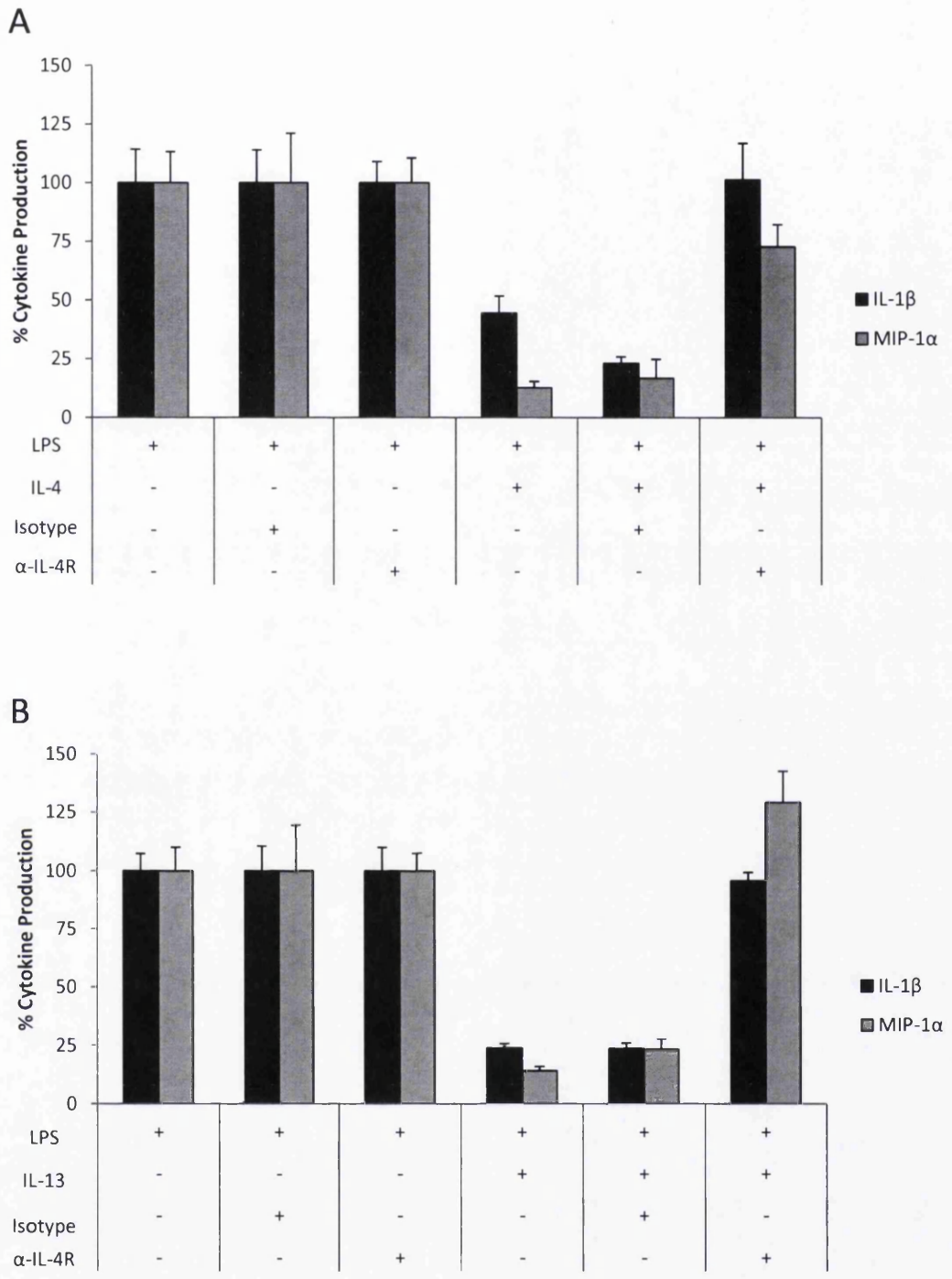

Figure 6.9 Neutralisation of IL-4 receptor in the placenta abrogates the anti-inflammatory effects of IL-4 and IL-13.

Cytokine levels (IL-1β & MIP-1α) from explants of placenta in response to LPS (10 ng/ml) pre-treated for 90 minutes with (A) IL-4 and (B) IL-13 (both 10 ng/ml) in the presence or absence of mouse IgG2a anti-human IL-4R antibody or mouse IgG2a isotype. Data are shown as % of LPS alone (n = 3); error bars represent SEM. Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

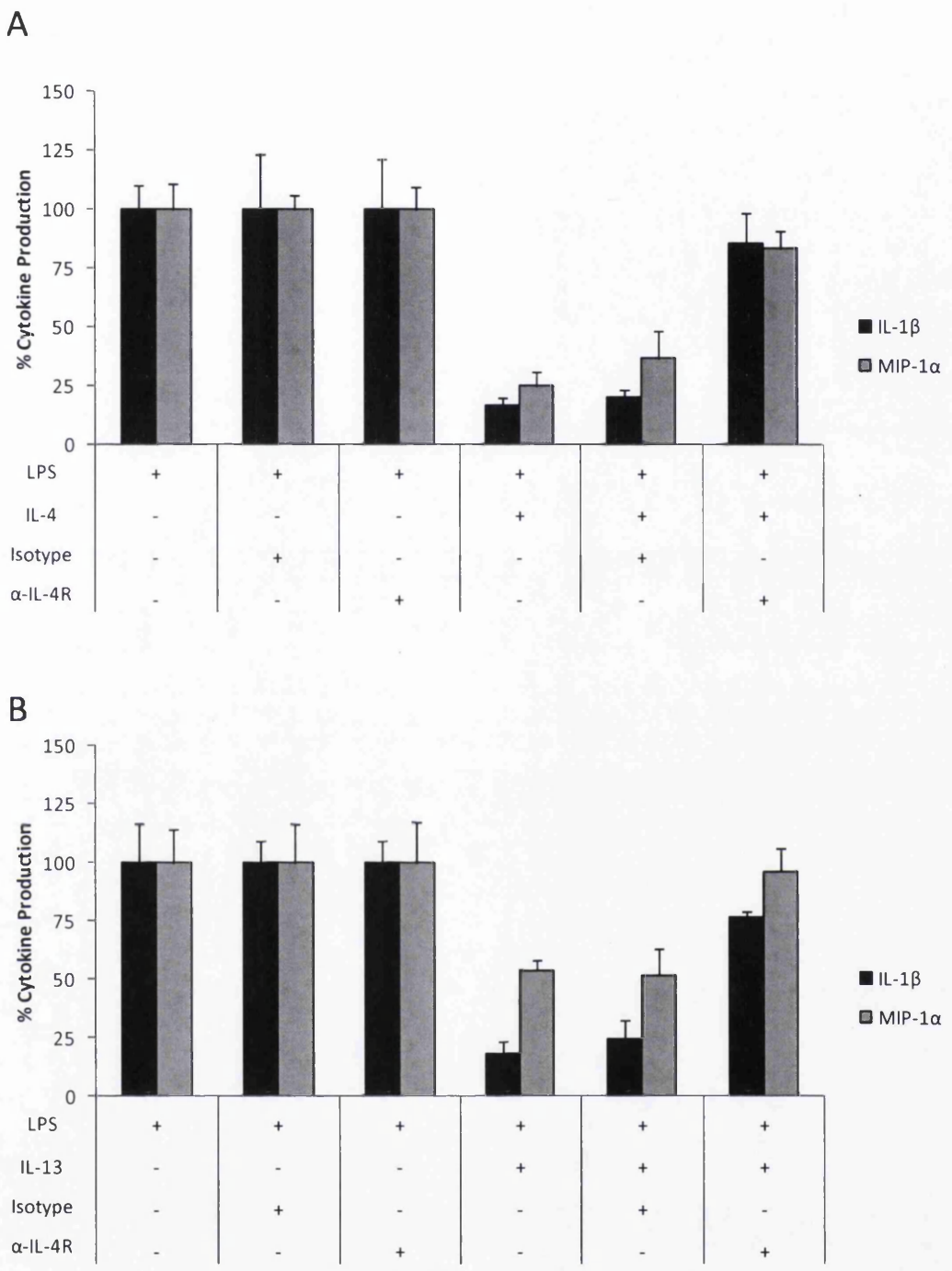

Figure 6.10 Neutralisation of IL-4 receptor in the choriodecidua abrogates the anti-inflammatory effects of IL-4 and IL-13.

Cytokine levels (IL-1β & MIP-1α) from explants of choriodecidua in response to LPS (10 ng/ml) pre-treated for 90 minutes with (A) IL-4 and (B) IL-13 (both 10 ng/ml) in the presence or absence of mouse IgG2a anti-human IL-4R antibody or mouse IgG2a isotype. Data are shown as % of LPS alone (n = 3); error bars represent SEM. Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

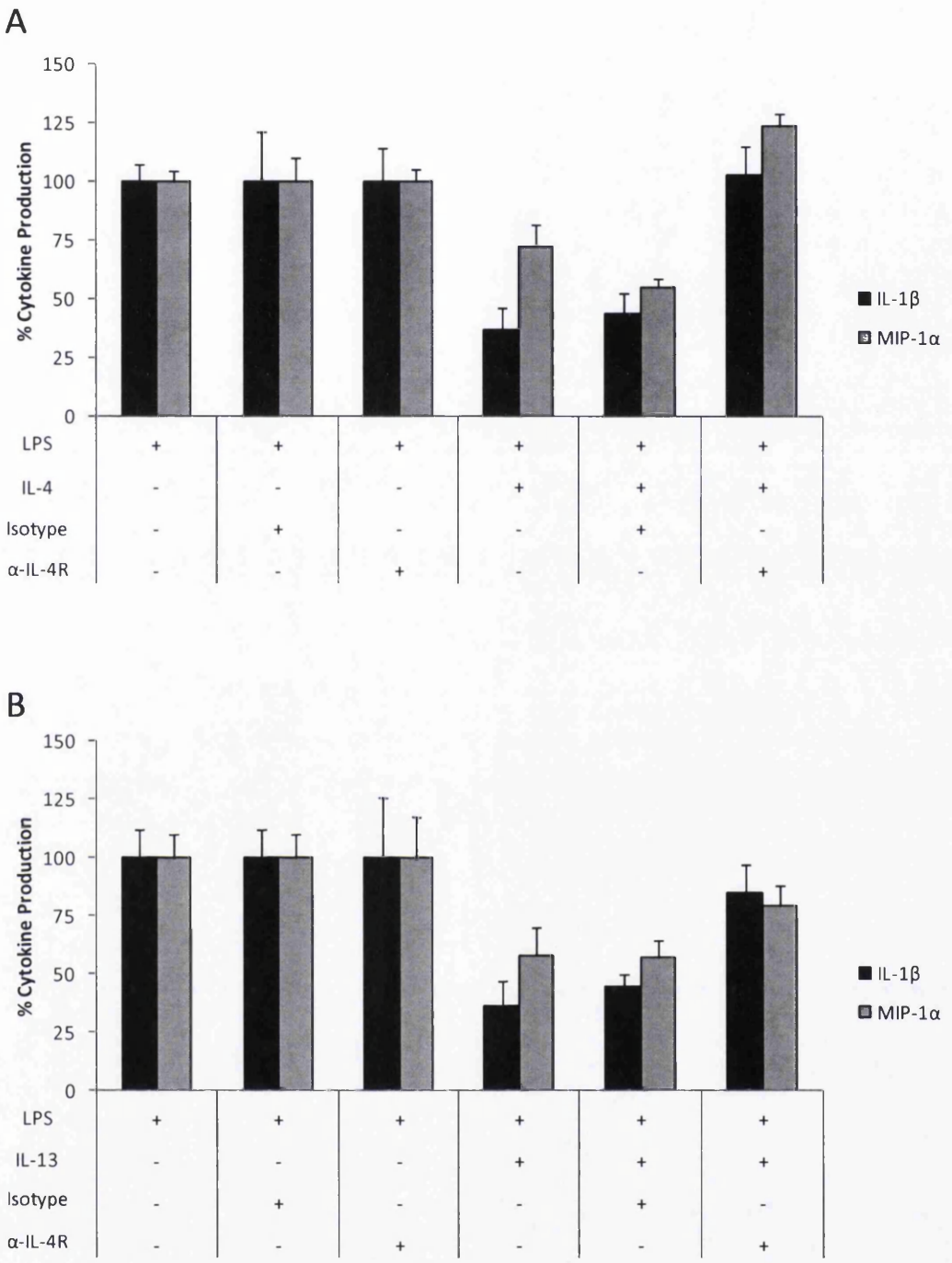

Figure 6.11 Neutralisation of IL-4 receptor in the amnion abrogates the anti-inflammatory effects of IL-4 and IL-13

Cytokine levels (IL-1β & MIP-1α) from explants of amnion in response to LPS (10 ng/ml) pre-treated for 90 minutes with (A) IL-4 and (B) IL-13 (both 10 ng/ml) in the presence or absence of mouse IgG2a anti-human IL-4R antibody or mouse IgG2a isotype. Data are shown as % of LPS alone (n = 3); error bars represent SEM. Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

6.4.2.3 STAT6 phosphorylation

To confirm that both IL-4 and IL-13 induce STAT6 phosphorylation in these tissues, lysates of placenta were prepared at 0.5, 2 and 24 hours in the presence or absence of cytokine treatment. Phosphorylation at T0 was also examined. Figure 6.12 shows the effect of IL-4 (10 ng/ml and 100 ng/ml) on STAT6 phosphorylation compared to total STAT6 in the placenta. It would appear IL-4 treatment does result in phosphorylation of STAT6; however this result was not reproducible. Therefore the validity is questionable.

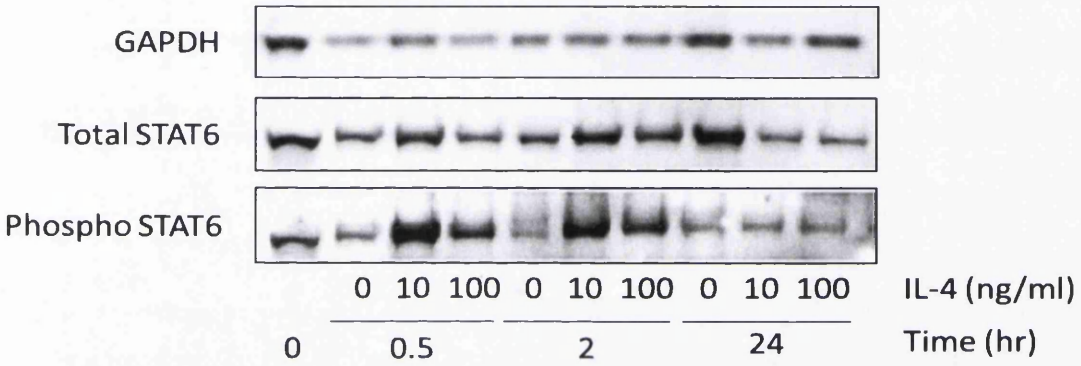

Figure 6.12 STAT6 phosphorylation in the placenta

Tissue lysates were prepared fromplacental explants treated with IL-4 (10 ng/ml and 100 ng/ml) for 0.5, 2 and 24 h and STAT6 phosphorylation examined. IL-4 treatment appeared to result in phosphorylation of STAT6, however this result was not reproducible.

6.4.3 Can simultaneous treatment with IL-10 augment the anti-inflammatory effect of IL-4?

Since IL-10 has been shown previously to inhibit cytokine production in the term placenta and fetal membranes, it was decided to examine whether co-treatment with IL-10 could enhance the inhibitory effect of IL-4 post-LPS stimulation. Initial investigations were performed to confirm the inhibitory effect of IL-10 on LPS induced cytokine production. Term non-laboured placenta, choriodecidua and amnion, tissues were pre-treated for 90 minutes with previously optimised concentration (10 ng/ml) of IL-10 prior to the addition of LPS. Results are represented as percentage decrease in cytokine production (IL-1β and MIP-1α) compared to LPS set at 100%. As shown in Figure 6.13, treatment with IL-10 was able to decrease LPS-induced IL-1β and MIP-1α production in all three tissues.

To investigate the effects of co-treatment with IL-10 and IL-4 on term non-laboured placenta, choriodecidua and amnion, tissues were treated with IL-4 alone, IL-10 alone or a combination of IL-4 and IL-10 at 90 and 240 minutes post-LPS exposure. The results are shown in Figure 6.13. In the placenta, while a reduction in both IL-1β and MIP-1α was observed at 90 minutes post-LPS in response to the combination of IL-4 and IL-10 compared to IL-4 or IL-10 alone this was not significant. In the choriodecidua, there was a significant reduction in MIP-1α at 90 minutes post-LPS in the presence of both IL-4 and IL-10 compared to IL-4 alone. Additionally at 240 minutes post-LPS, a significant reduction in IL-1β and MIP-1α was observed compared to IL-10 and IL-4 alone, respectively. A significant reduction in MIP-1α at 240 minutes was observed compared to IL-4 and IL-10 alone in the amnion. Additionally a reduction in IL-1β at 240 minutes post-LPS was observed compared to IL-4 alone. These data would imply that co-treatment of IL-4 and IL-10 may have a greater effect than either cytokine individually.

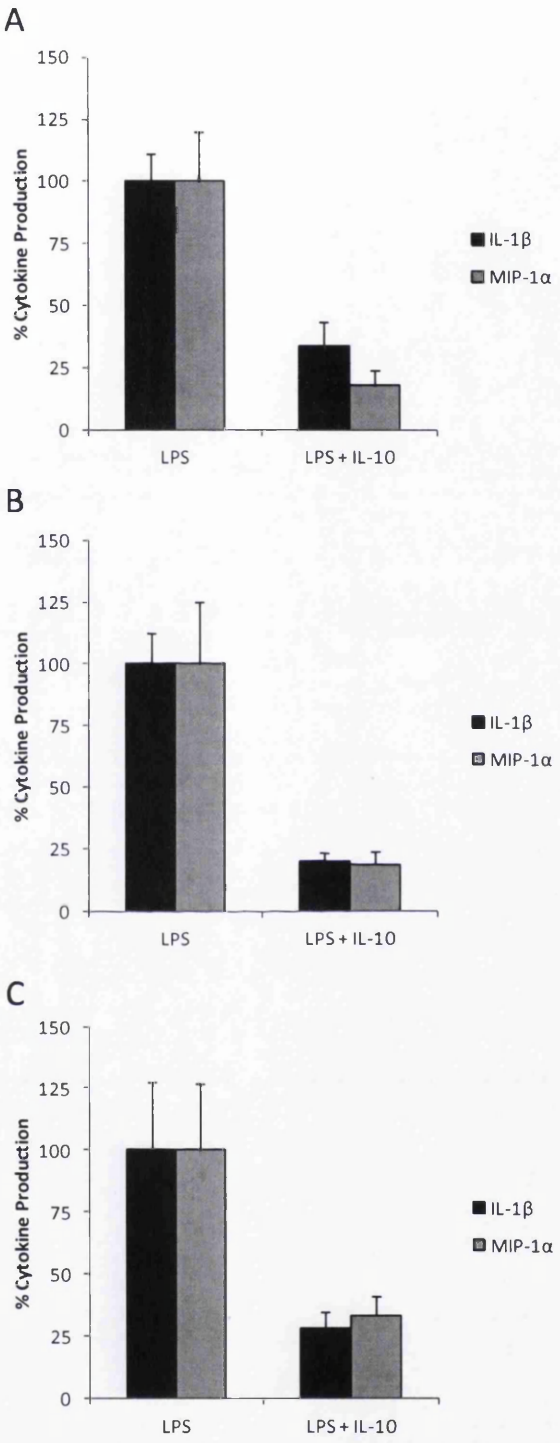

Figure 6.13 Effect of IL-10 on LPS induced cytokine production by term non-laboured gestation associated tissues

Cytokine levels (IL-1β & MIP-1α) from explants of (A) placenta (B) choriodecidua and (C) amnion in response to treatment with IL-10 (10 ng/ml) 90 minutes prior to treatment with LPS (10 ng/ml). Data are shown as % of response to LPS alone (n=6); error bars represent SEM. Statistical significance compared to LPS as determined by Wilcoxon matched pairs signed rank test. No significant difference was observed.

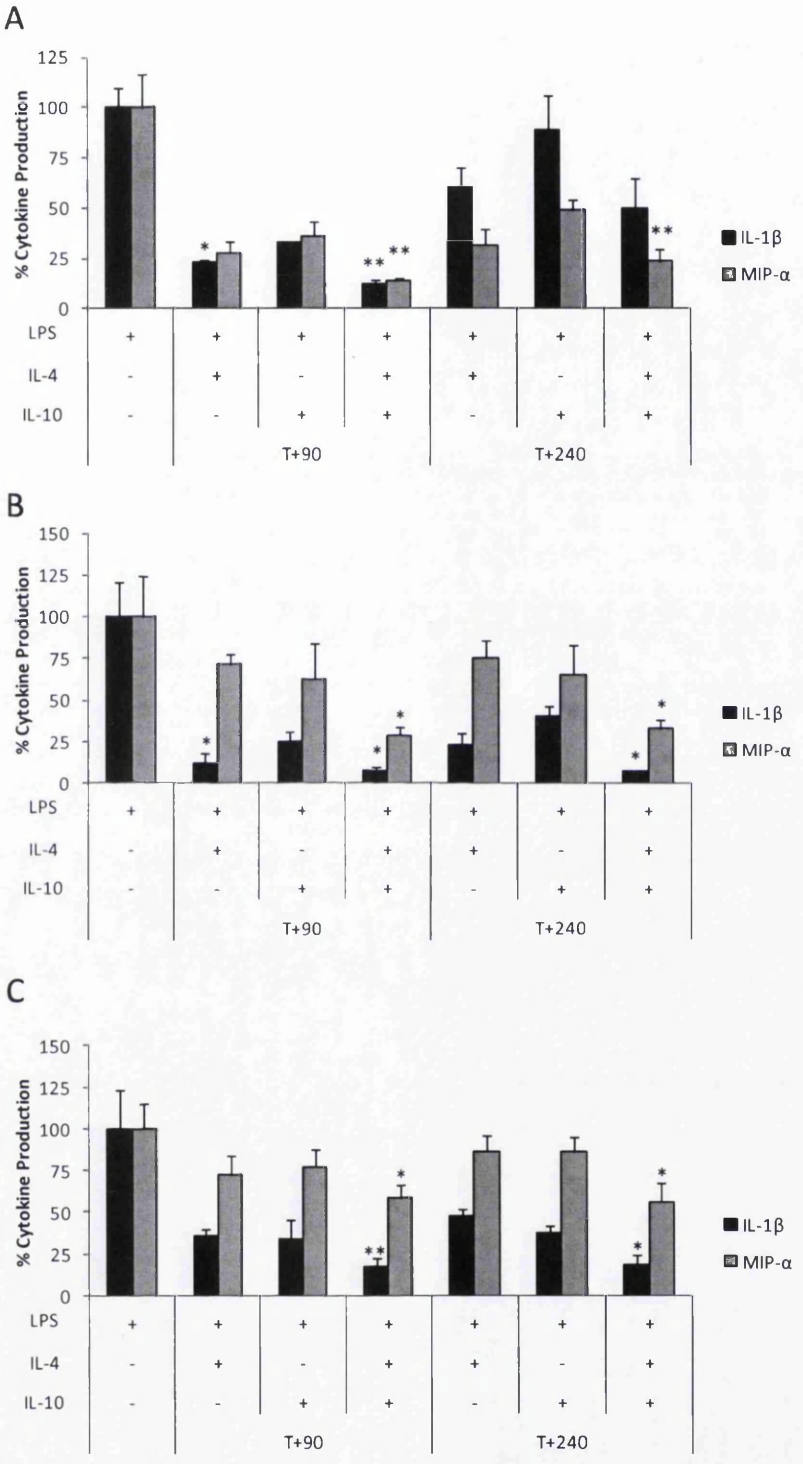

Figure 6.14 Effect of simultaneous treatment with IL-4 and L-10 on LPS-induced cytokine production by term non-laboured gestation associated tissues.

Cytokine levels (IL-1β & MIP-1α) from explants of (A) placenta (B) choriodecidua and (C) amnion in response to LPS (10 ng/ml) in the presence of IL-4, IL-10 or IL-4 + IL-10 (both10 ng/ml) added 90 minutes (T+90) and 4 hrs (T+240) after LPS treatment. Data are shown as % of LPS alone (n=3); error bars represent SEM. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to LPS is shown: * $p \leq 0.05$, ** $p \leq 0.01$.

6.5 Discussion

The cytokines IL-4, IL-10 and IL-13 have anti-inflammatory activity in a number of experimental settings [372-376]. Anti-inflammatory cytokines are also associated with a protective role during pregnancy [365, 366]. Studies to date have demonstrated that IL-10 can down-regulate LPS- and LTA-induced cytokine/chemokine and prostaglandin production, in addition to COX-2 expression by the healthy term placenta and intact term fetal membranes [268, 370]. However, it is unknown if other anti-inflammatory cytokines such as IL-4 and IL-13 have similar effects. The data presented here show that IL-4 and IL-13 pre-treatment of the placenta, choriodecidua and amnion reduced the production of the pro-inflammatory cytokines IL-1β and MIP-1α in response to LPS. The effect of IL-4 and IL-13 treatment after LPS stimulation was also considered as this was postulated to better mimic the clinical scenario. The anti-inflammatory effect of these cytokines was diminished when the cytokines were treated post-LPS exposure. Co-treatment with both IL-4 and IL-13 did not enhance the anti-inflammatory effects of either cytokine alone post-LPS exposure.

Two types of IL-4 signalling pathways have been described; type I signalling and type II signalling [377]. The type I signalling consists of a receptor complex between the common gamma chain (g_c; CD132) and IL-4Rα; and type II consisting of IL-4Rα and IL-13Rα1. IL-4 can signal via both type I and type II, whereas IL-13 first binds to IL-13Rα1 and can only signal via type II. Analysis of the placenta, choriodecidua and amnion by PCR showed transcripts for both type I and type II receptor components (IL-4Rα, CD132 and IL-13Rα1), the IL-13 decoy receptor IL-13Rα2 and the transcription factor STAT6. However the expression of IL-13Rα2 varied in the amnion. Furthermore neutralisation of IL-4Rα, which is required for both type I and type II, was able to block the effects of both IL-4 and IL-13 in these tissues. IL-4 induced phosphorylation of STAT6 was also examined, but was not reproducible i.e. no phospho-STAT6 or total STAT6 was observed under the same conditions or with alternative antibodies. Examination of STAT6 phosphorylation utilising a plate based assay may overcome this, however similar issues to the capase-1/8 activity assays in Chapter 4 and 5 may occur. Immunoprecipitation of STAT6 prior to blotting might also overcome the detection problems.

When comparing the anti-inflammatory effect of IL-4 and IL-13, it was generally observed that IL-4 had a great effect in reducing the LPS induced cytokine production. The ability of IL-4 to utilise both type I and type II might account for these differences as there are more target receptors for it to bind. Similarly this would explain why there was no enhancement of the anti-inflammatory effect of these cytokine with co-treatment as in this situation both IL-4 and IL-13 would be competing for the type II receptor complex. This highlight the need to further examine the function of other receptor chains notably IL-13Rα1.

As IL-10 has already been reported to have anti-inflammatory effects on gestation associated tissues, investigation into whether co-treatment of IL-10 could enhance the anti-inflammatory effect of IL-4, which had a greater effect than IL-13, was undertaken. Simultaneous treatment with IL-4 and IL-10 was able to enhance the down-regulation of LPS-induced cytokine response by IL-4 or IL-10 alone. IL-4 has been shown to both up-regulate and inhibit IL-10 production [380, 381] and as LPS can also induce IL-10 production in gestation-associated tissues [233, 382], it would be worthwhile measuring IL-10 in supernatants from LPS/IL-4 treated tissues explants. It is likely that the effect observed by addition of IL-10, results from the activation of a second distinct signalling pathway or enhancing its effect.

These results suggest that further investigation of the therapeutic potential of IL-4 and IL-10 is warranted. Targeting the receptor with small peptides would be worth pursuing. Understanding the signalling mechanisms might also reveal therapeutic targets. The mechanisms of how IL-4 and IL-13 inhibit LPS induced pro-inflammatory cytokine production might relate to the down-regulation of TLR4 expression and function [383]. However, this may result in an undesired impaired innate immune response [384]. Activation of TLR4 by LPS has been shown to directly up-regulated suppressor cytokine signalling 1 (SOCS1) expression, a negative regulator of both TLR and JAK/STAT signalling pathways [377, 385]. SOCS1 is also up-regulated by IL-4, but it has been suggested that SOCS1 does not mediate the anti-inflammatory effects of IL-4 [386]. Therefore it's possible that the diminished effect of IL-4 and IL-13 post LPS treatment is mediated by SOCS1 induced by LPS.

An overview of the IL-10 signalling pathway is shown in Figure 6.2. Activation of STAT3 is key in mediating the anti-inflammatory effects of IL-10 [387], however the

exact mechanisms of how this occurs remain unclear but might result from the induction of SOCS3 [388]. The potential of IL-10 to inhibit NF-κB activation has been examined, with several, contradictory reports in the literature. Some have reported the inhibition of NF-κB activation by IL-10 [389, 390], while negligible effects have been suggested by others [391, 392]. However, IL-10 has been shown to induce the expression of Bcl-3 in macrophages [393], a suggested negative regulator of NF-κB [394, 395]. Both IL-10 and IL-4 have been shown to enhance the expression of IL-1 receptor antagonist (IL-1Ra) [396], a potent anti-inflammatory cytokines involved in the inhibition of IL-1α and IL-1β [364].

While LPS is a classically used agonist to induce inflammation and to initiate infection associated preterm labour in animal models, the work in this thesis has demonstrated that gestation associated tissues can response to a wide variety of agonists of bacterial, viral and fungal origin. Whether the effects of IL-4, IL-10 and IL-13 shown in this chapter, apply to other agonist or indeed other cytokines is yet to be determined. However based on the ability of IL-10 to down-regulate LTA (TLR2 agonist)-induced cytokine production in healthy term placenta [268], it is possible this maybe also be the case for cytokine production induced by other agonists.

In summary the data contained in this chapter demonstrate that both IL-4 and IL-13 are able to down-regulate LPS induced cytokine production in the placenta, choriodecidua and amnion likely functioning via their classically described pathways. Furthermore co-treatment of IL-4 and IL-10 results in a greater down-regulation of LPS induced cytokine production.

Chapter 7

General discussion and directions for further work

7 General discussion and directions for further work

7.1 Overview

Increased interest in the innate immune response and its receptors at the maternal-fetal interface has arisen because of the association between intrauterine infection, inflammation and certain adverse pregnancy outcomes. However, the study of PRR expression and activity by gestation-associated tissues is still in its infancy. While ultimately an improved understanding of these receptors and the signal transduction cascades they initiate might offer an explanation as to why some pregnancies are complicated by PTL and PPROM whereas others are only affected by PPROM, this is beyond the scope of this current study. Here the focus was to examine PRR expression and activity in term non-laboured tissues, providing a baseline comparison and greater understanding of these receptors in gestational tissues, to aid in examining any potential role they may play in normal term labour but also in preterm labour and other adverse pregnancy outcomes in the future.

7.2 Expression and activity of PRRs by gestation associated tissues.

At the outset of this work, investigation into Toll-like receptors (TLRs) in the gestation associated tissues was the primary focus. However during the early months of research , it became clear as the literature surrounding PRRs evolved that the scope of the work should be expanded to include other PRR families, including Nod-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs). Initial investigations were performed to determine the expression profile of PRRs by the placenta, choriodecidua and amnion. Transcripts for all PRRs examined were shown to be expressed in these tissues, corresponding to some previously published reports (including TLRs 1-10 [233] and the NLRs, NOD1 and NOD2 [60, 288]), while demonstrating the expression of others for this first time notably Dectin-1, Dectin-2 and MINCLE.

Following this, the placenta, choriodecidua and amnion were treated with a wide variety of agonists shown to bind and activate specific PRRs to determine if these receptors were functional in these tissues. An increase in IL-6 and/or IL-8 in response to stimulation of all three tissues with receptor specific agonists, implied a functional role for TLRs 1-8, NOD1, NOD2, RIG-I/MDA5 and Dectin-1. Detailed investigation of the Dectin-1 signalling pathway, demonstrated that β-glucans, a primary component of the *Candida albicans* cell wall was able to elicit a robust

inflammatory response in a spleen tyrosine kinase and NF-κB dependent manner. Furthermore exposure of these tissues to *Candida albicans* resulted in an increase in IL-6 and IL-8 production. Of all PRRs examined, TLR9 and MINCLE agonists appeared not to elicit a response suggesting that either that these receptors are not functional in these tissues or that a broader screen of cytokines is required. It has been previously reported that fetal membranes treated with a TLR9 agonist significantly increased MCP-1 production, while significantly inhibiting the constitutive production of G-CSF, IFNγ, MIP-1α, MIP-1β, RANTES and VEGF [288]. Additionally, further examination of placenta and choriodecidua treated with the MINCLE agonist TDB, based on reports that in may activate the NLRP3 inflammasome [336], resulted in an increase in IL-1β production (Chapter 8 – Figure 8.25).

An increase in the local production of pro-inflammatory cytokines at the maternal-fetal interface has been long associated with initiating the physiological processes of labour; remodelling of the cervix, weakening and rupture of the membranes and the initiation of uterine contractions [11]. These processes are facilitated by the activity of matrix metalloproteinases (MMPs), the expression of which are augmented by pro-inflammatory cytokines [11, 356]. Based on the knowledge that PRRs are expressed and are functional in the placenta, choriodecidua and amnion it is likely that the activation of PRRs might lead to the production of MMPs. Indeed activation of TLRs in various cell types including monocytes, keratinocytes and fibroblasts has been shown to induce the expression of several MMPs [397-399]. Examinations of PRR induced MMP expression in gestation associated tissues is rather limited and has notably focused on the amnion. Murine studies have shown activation of MMP-1 and MMP-9 in a TLR4 dependent manner [400]. Examination of human amniotic epithelial cells and amnion explants has noted the induction of MMP-9 via TLR5 and TLR2/6 and in response to LPS [287, 401]. Additionally induction of MMP-9 has been reported in fetal membranes in response to a mixed bacterial population and in response to NOD1 agonist iE-DAP and NOD2 agonist MDP [286, 402]. Furthermore LPS induced secretion of MMP-9 by human chorion trophoblasts and placental syncytiotrophoblast has been reported, however no significant effect was observed on TIMP-1, an inhibitor of MMPs [403].

Since PRRs utilise a number of microbial derived agonists, it's easy to understand how PRRs may play a role in the pathogenesis of infection associated preterm

labour and birth. However PTB has multiple etiologies, with several risk factors identified including extremes of maternal body mass index (BMI), hypertension, multiple gestations, uterine anomalies and adverse behaviours (smoking and alcohol consumption) among others [41]. PRRs have been implicated in the recognition of endogenous danger signals released by the host; referred to as both DAMPs or alarmins. These include HMGB1, fibronectin and heat shock proteins (HSPs) [101, 404]. HMGB1 mediates its activity though multiple TLRs including TLR2, TLR4 and TLR9 and the receptor for advanced glycation end products (RAGE) [405-407]. HMGB1 localised to amnion epithelial cells is associated with membrane rupture with elevated levels present in the amniotic fluid of women in PTL with PPROM compared to women in PTL with intact membranes [408]. Fetal fibronectin (fFN) present in vaginal secretions has long been suggested as a marker for PTL [22, 409]. Murine studies have shown the ability of fFN to activate NF-κB and ERK1/2 in a TLR4 dependent manner in amnion mesenchymal cells, resulting in several downstream effects including increased COX-2 mRNA, PGE_2 biosynthesis, and enzymatic activity of MMP-1 and MMP-9 [400] Elevated levels of alarmins (HMGB1 and HSP70) in the amniotic fluid are also associated with intra-amniotic infection [408, 410]. Therefore it is possible that alarmins at the maternal-fetal interface play a role in preterm labour with and without the presence of infection.

7.3 IL-1β production and inflammasome activation by gestation associated tissues

IL-1β is tightly regulated due to its potency as a pro-inflammatory mediator. Initially expressed as an inactive pro-IL-1β precursor it requires caspase activity mediated by several described inflammasomes to be processed into mature IL-1β [185, 343-347].

The data presented in this thesis, demonstrates production of IL-1β in response to the β-glucan curdlan and bacterial flagellin in both the placenta and choriodecidua. Parallel work within the laboratory has demonstrated that these tissues can produce IL-1β in response to a wide variety of inflammasome activators including nigericin, hemozoin and monosodium urate (MSU) crystals. Studies of β-glucan induced IL-1β in macrophages and dendritic cells have yielded evidence of activation of both NLRP3/capase-1 and a non-canonical capase-8 inflammasome respectively [177, 314]; flagellin induced IL-1β is associated with the activation of caspase-1 and the

NLRC4 inflammasome [350]. Investigation into these pathways using specific inhibitors, yielded evidence that both caspase-1 and caspase-8 are required for curdlan and flagellin induced IL-1β production in the placenta. A similar observation is seen the choriodecidua for curdlan induced IL-1β; however flagellin induced IL-1β in this tissue was dependent on caspase-1 only. Despite showing a role for caspase-1 and capase-8 in IL-1β production in these tissues, the results cannot confirm conclusively the involvement of the NLRP3 inflammasome, the non-canonical caspase-8 inflammasome or the NLRC4 inflammasome and these should be the focus of further investigation utilising a co-immunoprecipitation approach.

It has been well documented that IL-1β is a major factor in the initiation of both term and preterm labour via its ability to up-regulate MMP activity and prostaglandin production which facilitate the physiological processes of labour [19, 26, 75, 342]. Additionally, elevated levels of IL-1β in amniotic fluid is a hallmark of intra-amniotic infection during pregnancy [320]. Although gestation associated tissues produce IL-1β, it's not known whether IL-1β acts in either an autocrine or paracrine manner or both at the maternal-fetal interface. IL-1β signals through the IL-1 receptor (IL-1R) [411]. Examination of IL-1R expression by gestational tissues has not been investigated; however it would be highly unlikely that IL-1R is not expressed by some component of the maternal-fetal interface due to the highlighted role of IL-1β in term and preterm labour. Furthermore the IL-1 signalling pathway utilises several signalling molecules associated with TLR signalling [412], which are functional in gestation-associated tissue, further suggesting a functional IL-1 signalling pathway. Activation of the IL-1 pathway by IL-1β is associated with the production of a number of pro-inflammatory cytokines including IL-6 [413, 414]. Furthermore, IL-1β has been shown to induce the expression of several DAMPs including HMGB1 and HSP70, by cortical astrocytes and islet beta cells respectively [415, 416]. Therefore, IL-1β production by gestation-associated tissues might contribute to sustained inflammation at the maternal-fetal interface.

Activation of the inflammasome and the production of IL-1β have gained extensive interest as a link between inflammation and metabolic disorders including obesity [417]. The inflammatory state during obesity is linked to the activation of adipose tissue macrophages by lipids including ceramide and palmitate, which have been shown drive the activation of NLRP3 and caspase-1 and the production of IL-1β, mediating insulin resistance [417-419]. This occurs due to the down-regulation of

AMP-activated kinase (AMPK) activity, a regulator of cellular energy status, resulting in enhanced reactive oxygen species (ROS) production from accumulated dysfunctional mitochondria, promoting the activation of NLRP3 [418].

Because of the obesity epidemic there are an increased number of obese pregnant women, and maternal obesity is associated with increased risk of PTL [420-425]. Since the placenta and attached membranes are the interface between the maternal and fetal environment, it is likely to have a central role in the impact of maternal obesity on adverse pregnancy outcomes and the short and long term health of the offspring [426]. Examinations of the impact of maternal obesity on the placenta have demonstrated elevated oxidative and nitrative stress, pro-inflammatory cytokine gene expression and placental lipotoxicity [426-428]. An association of increased risk of PTL with low BMI also has been reported [429]. Since caloric restriction is associated with AMPK activation [430], adverse pregnancy outcomes such as PTL at the lower extremes of maternal BMI, may be associated with increased oxidative phosphorylation.

While the work here examined the production of IL-1β by gestation-associated tissues, inflammasome activation is also associated with processing of pro-IL-18 to mature IL-18. To date, IL-18 production in response to inflammasome stimuli by gestation associated tissue has not been examined; evidence however of IL-18 at the maternal-fetal interface has been described. IL-18 is constitutively expressed in human chorion and decidua [431]. Furthermore significant amniotic fluid concentrations of IL-18 are observed following microbial invasion of the amniotic cavity in patients with intact membranes and PPROM at term and preterm [431, 432]. Elevated placental IL-18 is associated with preeclampsia [433]. Similar to IL-1β, IL-18 mediates its pro-inflammatory effect by stimulating the production of a variety of cytokine including IL-1β and TNFα [434, 435], both of which facilitate the physiological processes of labour. Therefore is likely that IL-18 may play a role in the onset of labour and in adverse pregnancy outcomes.

7.4 Modulating the inflammatory response in gestation associated tissues

With preterm labour, regardless of infection status, characterised by an increase of pro-inflammatory cytokines at the maternal-fetal interface, resolution of this inflammatory response might be beneficial in prolonging the pregnancy and providing a better outcome for the fetus [1, 15, 436]. For immune homeostasis, several endogenous resolving and anti-inflammatory mediators have been described, including; anti-inflammatory cytokines (IL-4, IL-10, IL-13, TGF-β), bioactive lipids (lipoxins, protectins), glucocorticoids, neuropeptides (ghrelin) and resolution associated molecular patterns (RAMPs; HSP10, HSP27) [266].

At the maternal-fetal interface anti-inflammatory cytokines, have been long associated with a protective role, maintaining the Th1:Th2 cytokine balance by decreasing the production of pro-inflammatory cytokines [365, 366]. The potential benefits of anti-inflammatory cytokine treatment on PRR induced inflammation by the placenta, choriodecidua and amnion was considered. Firstly IL-4 and IL-13 were used. While pre-treatment with both cytokines was able to reduce LPS induced cytokine production, utilising classically described signalling receptors; this effect was diminished post-treatment. Further investigation with IL-10 demonstrated that simultaneous treatment with IL-4 and IL-10 was able to enhance the down-regulation of LPS-induced IL-1β and MIP-1α by IL-4 or IL-10 alone. The mechanisms of how these cytokines might have their effect are yet to be determined but might relate to the down regulation of PRRs themselves, induction of suppressor of cytokine signalling (SOCS) proteins or negative regulation of NF-κB [383, 388, 395]. While these anti-inflammatory cytokines were able to down-regulate IL-1β and MIP-1α, it would be worthwhile examining whether this effect applies to other pathogenic stimuli, whole organisms or indeed other pro-inflammatory cytokines. While anti-inflammatory cytokine treatments *in vitro* may have a positive effect in relation to reducing PAMP induced cytokine response, in order to determine any potential benefit for the treatment of PTL *in vivo* studies would be required.

Maternal nutrition has been shown to influence fetal development and pregnancy outcomes, with poor maternal nutrition linked to adverse pregnancy outcomes [437-441]. Some reports have implicated that women following a Mediterranean-type diet during pregnancy may reduce the risk of PTB [442, 443]. However no benefit was observed by others [444]. Regardless a Mediterranean-type diet contains high levels

of phytonutreints. Plant derived phytonutrients or phytochemicals, have gained clinical interest due to their anti-oxidant and anti-inflammatory properties [445-447]. Evidence now exists that phytonutrients can inhibit PRR mediated inflammation by a variety of mechanisms including inhibition of receptor dimerisation, MyD88-independent signalling and NF-κB activation [448]. Additionally several phytonutrients including curcumin, capsaicin and quercetin have been shown to activate AMPK in 3T3-L1 adipocytes and MCF-7 breast cancer cells [449-452]. It is therefore possible that phytonutreints might impact the PRR mediated inflammatory response by gestation-associated tissues at the maternal-fetal interface. To date, there are only a limited number of conflicting reports examining the impact of phytonutreints on adverse pregnancy outcomes. High plasma concentrations of several carotenoids, including β-carotene and lycopene were associated with reduced risk of preterm birth [453]. Low carotenoid levels in placental tissue are associated with preeclampsia [454]. However, others have reported that lycopene was associated with increased risk of preterm birth and low birth weight [455]. Therefore further investigations into the impact of phytonutreints on pregnancy outcomes are required.

7.5 Limitations

Many of the experiments performed in this study had a limited sample size, resulting in no significant outcomes for many of the experiments. This was notably evident with inhibition experiments, where a reduction in cytokine output was observed however the results were typically not significant. This is even more problematic with primary human material, due to the variation in response between donors. It would have been beneficial initially for a power calculation to be performed to determine sample numbers that would be required. However, when working with human samples it's often difficult to achieve high sample numbers, which can be complicated further depending on the nature of the experiments to be performed.

Hetergeneity within the small sample population is likely to be another contributing factor to the lack of significance observed. While all the tissue used in this study was from healthy pregnant women and therefore the data derived is not impacted by the presence of infection, severe medical conditions or adverse pregnancy outcomes; there are other factors that may impact the results. For example it's now been established that BMI can impact immune function [456], however this was not accounted for during this study, and therefore the maternal BMI of the pregnant

women recruited would likely add to the heterogeneity observed. Other demographic factors including age and ethnicity may also play a role.

7.6 Future Directions

The work presented here was all performed on term non-laboured gestation associated tissues; however it's important to consider how PRR expression and function might differ in term laboured tissue and preterm tissues in order to asses any potential therapeutic targets for PTL. Murine studies have implicated functional TLRs associated with PTB. Functional TLR4 has been implicated in preterm labour triggered by administration of heat killed *E.coli* [92], while intraperitoneal injection of poly I:C, caused preterm delivery within 24 hours [257]. In humans, it has been previously shown that labour can impact the expression and function of TLRs in the placenta, with labour associated with increased TLR2 and TLR5 expression, while increase TNFα production was observed in response to TLR4 and TLR7/8 agonists [233]. Elevated TLR2 and TLR4 expression in chorioamniotic membranes following preterm birth compared to membranes without chorioamnionitis has been documented [59]. While the knowledge and understanding in this area is ever expanding, much of relates to TLRs and not the wider array PRRs described here, highlighting a knowledge grab in this area.

There is also an increasing interest in the role of sterile inflammation as a cause of PTL. Recently it has been reported that sterile intra-amniotic inflammation is a more frequent cause of PTL than infection associated intra-amniotic inflammation [457]. While the exact mechanisms for this are currently unknown, DAMPs/alarmins including HMGB1 and IL-1 receptor antagonist (IL-1Ra) have been suggested as key to this process, with elevated levels observed in the amniotic fluid and circulation of women with high risk pregnancies [408, 410, 458]. HMGB1 is one of the most extensively studied DAMPs and has been shown to signal via TLR2, TLR4 and RAGE (see chapter 7.2), while IL-1Ra functions as a negative regulator of IL-1β, by binding to the IL-1R [343]. However the mechanism of action for many DAMPs has not been determined. Furthermore, how gestation-associated tissue respond to an array of DAMP has yet to be determined and may occur via PRR activation. However, if they act in a different manner to PAMPs, it might highlight the possible need for different treatment/prevention strategies for intra-amniotic inflammation in the presence or absence of infection.

A link between inflammation and metabolism is becoming increasingly apparent. Glucose and fatty acids are the main energy substrates used by cells through oxidative metabolism and glycolysis to generate the ATP needed to power cellular activity [430]. Studies have shown evidence of a metabolic shift in activated macrophages, from oxidative phosphorylation to aerobic glycolysis, the so called Warburg effect, to provide the energy needed during host defence [418, 430]. Considering that gestation-associated tissues have a highly inflammatory nature both in term and preterm labour little is known about the energy substrate requirement of these tissues. Understanding this might offer an explanation why women at the extremes of BMI are at greatest risk of preterm birth: women with low BMI of spontaneous preterm labour and women in obese classes II of PPROM [429].

7.7 Final Summation

In summary the work detailed here clearly demonstrates that human term gestation-associated tissues express transcripts for a variety of PRRs, including TLRs, NLRs, RLRs and CLRs. Furthermore an implied functional role for TLRs 1-7, NOD1, NOD2, RIG-I/MDA5 and Dectin-1 was determined by an increase in the production of IL-6 and IL-8 following stimulation with receptor specific agonists. Inflammasome activity, noted by an increase in IL-1β levels was observed for the placenta and choriodecidua in response to β-glucan and flagellin. Evidence that both caspase-1 and caspase-8 are involved in this was found. Modulation of LPS-induced cytokine production in the placenta, choriodecidua and amnion was observed with treatment of these tissues with the anti-inflammatory cytokines IL-4 and IL-13, likely functioning via their classically described pathways. Furthermore co-treatment of IL-4 and IL-10 resulted in a greater down-regulation of LPS-induced cytokine production.

Chapter 8

Appendices

8 Appendices

8.1 Study Information and Consent Forms

Swansea University
Prifysgol Abertawe

Preterm Birth Study
Participant Information Sheet

REC No: 11/WA/0060 **Version 2, 23/03/12**

You are being invited to take part in a research study. We appreciate that this is a distressing time for you but we are doing research to determine why preterm birth occurs. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, discuss it with your friends and relatives if you wish and decide whether or not you want to take part.

What is the purpose of the study?

Around 10% of pregnant women will deliver their baby before full term. We are trying to understand why preterm birth occurs. We particularly want to study the inflammatory response in the mother, baby and placenta to see if this might contribute to preterm labour and/or birth.

Why have I been chosen?

We are approaching pregnant women who are at risk of delivering their baby prematurely and inviting them to be part of this study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you or your baby receives.

What will happen to me if I take part?

If you agree to help with this research, we will take some blood (up to two tablespoonfuls) from you at the same time as blood is taken for the tests that are part of your normal care. If you do not need a blood test for other reasons, we will

ask to take a sample specifically for our research. When your baby is born, we will collect a sample of blood from the discarded placenta after the delivery. This will not harm you or your baby. We will ask you questions about you general health, any medicine you have taken, smoking and diabetes. Your hospital notes will be read to gain information about you and your newborn baby, such as birth weight and whether you had a boy or a girl.

The samples of blood and placenta will be studied in the laboratory to find out how many and what type of immune cells they contain and how these react in various tests. Samples will be kept for ten years and may be used for other tests in the future.

What are the side-effects of taking part?

Taking the blood sample from you may cause some minor discomfort. There is no discomfort for your baby.

What are the possible benefits of taking part?

There are no immediate benefits of taking part in this study.

What if something goes wrong?

We hope that any problems can be sorted out easily and quickly, at the time they arise and with the person concerned. If the problem cannot be sorted out in this way and you wish to make a complaint the normal National Health Service and Swansea University complaints mechanisms are available to you. You may have grounds for a legal action but you may have to pay for it.

Will my taking part in this study be kept confidential?

All information which is collected about you and your baby during the course of the research will be kept strictly confidential. You will be given a unique identifying number and this will be used for labelling of samples and all analyses undertaken using the samples you provide.

What will happen to the results of the research study?

Results will be presented at scientific meetings and published in scientific journals. You will not be identified in any report/publication. Some of the samples might be

used by research students who will present results in their dissertation. Each year we will prepare a summary of the progress/results of the study which we can send to you if requested.

Who has reviewed the study and who is funding the research?

This study has been reviewed by and given a favourable opinion by South West Wales Research Ethics Committee and is organised by the College of Medicine, Swansea University.

Further Information

If you want more information about this study please contact Cathy Thornton (College of Medicine, Swansea University, Swansea, SA2 8PP or 01792 602122).

Thank you for taking the time to read this, and please feel free to ask any questions.

Swansea University
Prifysgol Abertawe

PARTICIPANT CONSENT FORM (Version 1, 28/11/11; REC No: 11/WA/0060)

Participant identification number for this study.....

***N.B.* Two copies should be made for (1) participant, (2) researcher**

Title of project : Preterm birth study

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 or my baby's medical notes may be looked at by responsible individuals from Swansea University 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 Participant	**Date**	**Signature**
Name of Person taking consent	**Date**	**Signature**
Researcher	**Date**	**Signature**

Swansea University
Prifysgol Abertawe

Preterm Birth Study

Maternal Questionnaire (Version 1, 30/11/11; REC No: 11/WA/0060)

Study ID	ID number

Date of questionnaire

dd	m	vear

Date of birth

dd	mm	vear

Age

.....years

Gestation

Estimated due date

dd	m

Reason for preterm birth if known:

.....

.....

Additional information:

.....

.....

Number of pregnancies (including this one): ☐

How many older siblings will your newborn have? ☐

Have you had a previous preterm delivery?
Yes/No

Reason for previous preterm birth (if known):

Additional information:

.....
.....

Are you taking any medication? Yes/No

If yes,
what?.....
.....

Do you smoke cigarettes at the moment?
Yes/No

If yes, **How many cigarettes per day?** ☐

☐

If no, Did you smoke before you were pregnant?

Did you smoke at any time during this pregnancy? ☐

If yes, When did you smoke? .....

How many cigarettes per day? ☐

Does anyone else in your household smoke? ☐

If yes, How many cigarettes per day? ☐

Have you ever been diagnosed with diabetes?
Yes/No

If yes, what type of diabetes? Type 1/ Type 2/Gestational

Were you tested for gestational diabetes in

(i) this pregnancy?
Yes/No

(ii) a previous pregnancy?

Yes/No

Height .....cm

Weight .....kg

BMI .....

The Immune Response at Birth

Participant Information Sheet **REC No: 11/WA/0040**

Version 2, 12/07/11

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, discuss it with your friends and relatives if you wish and decide whether or not you want to take part.

What is the purpose of the study?

The immune response made by healthy pregnant women and babies is very different to that of non-pregnant women or men. Differences in the immune response of babies might relate to the later health of the child. We want to study why the immune response of pregnant women and babies is different. We also want to study if common health issues in pregnant women such as allergy, smoking, diabetes or obesity affect the immune response of mother or baby.

Why have I been chosen?

We are approaching pregnant women and inviting them to be part of this study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you or your baby receives.

What will happen to me if I take part?

If you agree to help with this research, we will take some blood (up to two tablespoonfuls) from you at the same time as blood is taken for the tests that are part of your normal care in pregnancy. If you do not need a blood test for other

reasons, we will ask to take a sample specifically for our research. When your baby is born, we will collect a sample of blood from the umbilical cord and will take the placenta. This will not harm you or your baby. The cord and placenta would otherwise be disposed of by the hospital.

We will ask you questions about any allergies you have, medicine you have taken, smoking and diabetes. Your hospital notes will be read to gain information about you and your newborn baby, such as birth weight and whether you had a boy or a girl. The samples of blood and placenta will be studied in the laboratory to find out how many and what type of immune cells they contain and how these react in various tests. Samples will be kept for ten years and may be used for other tests in the future.

What are the side-effects of taking part?

Taking the blood sample from you may cause some minor discomfort. There is no discomfort for your baby.

What are the possible benefits of taking part?

There are no immediate benefits of taking part in this study.

What if something goes wrong?

We hope that any problems can be sorted out easily and quickly, at the time they arise and with the person concerned. If the problem cannot be sorted out in this way and you wish to make a complaint the normal National Health Service and Swansea University complaints mechanisms are available to you. You may have grounds for a legal action but you may have to pay for it.

Will my taking part in this study be kept confidential?

All information which is collected about you and your baby during the course of the research will be kept strictly confidential. You will be given a unique identifying number and this will be used for labelling of samples and all analyses undertaken using the samples you provide.

What will happen to the results of the research study?

Results will be presented at scientific meetings and published in scientific journals. You will not be identified in any report/publication. Some of the samples might be used by research students who will present results in their dissertation. Each year we will prepare a summary of the progress/results of the study which we can send to you if requested.

Who has reviewed the study and who is funding the research?

This study has been reviewed by the Local Research Ethics Committee and is organised by the College of Medicine, Swansea University.

Further Information

If you want more information about this study please contact Cathy Thornton (College of Medicine, Swansea University, Swansea, SA2 8PP or 01792 602122).

Thank you for taking the time to read this, and please feel free to ask any questions.

PARTICIPANT CONSENT FORM (Version 1, 23/02/11; REC No: 11/WA/0040)

Participant identification number for this study.....

***N.B.* Two copies should be made for (1) participant, (2) researcher**

Title of project : The immune response at birth
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 or my baby's medical notes may be looked at by responsible individuals from Swansea University 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 Participant	**Date**	**Signature**
Name of Person taking consent	**Date**	**Signature**
Researcher	**Date**	**Signature**

The immune response at birth

Maternal Questionnaire **(Version 1, 23/02/11; REC No: 11/WA/0040)**

Study ID	ID number

Date of questionnaire

dd	m	vear

Date of birth

dd	mm	vear

Age

.....years

Gestation [ ]

Estimated due date

dd	m

Number of pregnancies (including this one) [ ]

How many older siblings will your newborn have? [ ]

Do you suffer from	**Eczema?**	**Yes/No**
	Asthma?	**Yes/No**
	Hayfever/Rhinitis?	**Yes/No**

Have you ever had allergy/asthma diagnosed by your doctor?

Yes/No

If yes to any of the above, what are you allergic to?

.....

.....

Are you taking any medication? **Yes/No**

If yes,

what?.....

Do you smoke cigarettes at the moment?

Yes/No

If yes, **How many cigarettes per day?** ☐

If no, **Did you smoke before you were pregnant?** ☐

Did you smoke at any time during this pregnancy? ☐

If yes, **When did you smoke?** .....

How many cigarettes per day? ☐

Does anyone else in your household smoke? ☐

If yes, How many cigarettes per day? ☐

Have you ever been diagnosed with diabetes?

Yes/No

If yes, what type of diabetes? Type 1/ Type 2/Gestational

Were you tested for gestational diabetes in

(i) this pregnancy?

Yes/No

(ii) a previous pregnancy Yes/No

Height .....cm

Weight .....kg

The Immune Response at Birth - Mother and Baby Data

Mother:

Study number assigned: Date consent taken:

Hospital number: DOB:

Name:

Age: BMI:

Smoking whilst pregnant: Yes / No

Gravidity/Parity: G /P

Any other information:

Baby:

DOB: Gender: Male/Female

Birth Weight: (grams) Mode of delivery:

Gestation: Apgar@1: Apgar@5:

Time of delivery:

Other:

Placental weight: (grams)

Peanut, tree nut and sesame seed allergy

LREC No: 04/WMW02/68 **Version 1**
(20/09/04)

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, discuss it with your friends and relatives if you wish and decide whether or not you want to take part. Ask us if anything is not clear or if you want more information. Consumers for Ethics in Research (CERES) publish a leaflet entitled "Medical Research and You". The researchers have copies of this leaflet that gives you more information about medical research.

What is the purpose of the study?

Allergy to peanuts, other nuts such as cashews and almonds, and sesame seeds occurs in some children and adults. These allergies make some people very ill and we are trying to work out what parts of the nuts and seeds cause this illness.

Why I have been chosen?

We are approaching healthy adults with and without allergies and inviting them to be part of this study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

What will happen to me if I take part?

If you agree to participate we will ask you some questions about your general health and whether or not you suffer from any allergies. We will also take some blood (1 – 2 tablespoonfuls) from you. The blood sample we collect will be used to study the effect of extracts of various nuts and seeds on immunological responses by your white blood cells. We plan to store any unused samples for up to 10 years for further studies that

arise from our initial research but any additional studies would still relate only to nut and seed allergies.

What are the side-effects of taking part?

Taking the blood samples from you may cause some minor discomfort.

What are the possible benefits of taking part?

There are no immediate benefits of taking part in this study. In the unlikely event that your blood sample or the questions we ask you indicate that something else is wrong with you, we will discuss this with you and make sure the right treatment is made available to you.

What if something goes wrong?

If you are harmed by a mistake made by the researchers or by chance you can seek compensation but you may have to pay the legal costs. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. You will be given a unique identifying number and any information about you will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?

Results will be presented at scientific meetings and published in scientific journals. You will not be identified in any report/publications. Each year we will prepare a summary of the progress/results of the study which can send to you if requested.

Who has reviewed the study and who is organising the research?

This study has been reviewed by the Local Research Ethics Committee.

Further Information

If you want more information about this study please contact Cathy Thornton (Institute of Life Science, Swansea University, SA2 8PP, Tel: 01792 602122).

Thank you for taking time to read this, and please feel free to ask any questions.

Peanut, tree nut and sesame seed allergy

QUESTIONNAIRE (Version 1, 20/09/04, LREC No. 04/WMW02/68)

Patient identification for this trial ☐

Date of questionnaire ☐ day ☐ month ☐ year

Date of birth ☐ day ☐ month ☐ year

Age ☐

Gender ☐

Do you suffer from

Eczema? ☐

Asthma? ☐

Hayfever/ Rhinitis? ☐

Have you ever had allergy/asthma diagnosed by your doctor? ☐

If yes to any of the above, what are you allergic to?

.....

Are you taking any medication?

If yes, what? .....

Do you smoke cigarettes at the moment?

If yes, **How many cigarettes per day?**

If no, **Have you ever been a smoker?**

If you used to be a smoker:

How long ago? .....

How many cigarettes per day?

Would you be happy to be invited back for follow-up study?

CONSENT FORM (Version 2, 17/03/05, LREC No. 04/WMW02/68)

Patient identification for this trial.....

N.B. Two copies should be made for (1) patient and (2) researcher.

Title of Project: Peanut, tree nut and sesame seed allergy.

Person in charge of the study: Catherine Thornton, PhD

Contact telephone numbers: 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 reasons, 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 of Abertawe Bro Morgannwg University 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**

8.2 Supplementary data

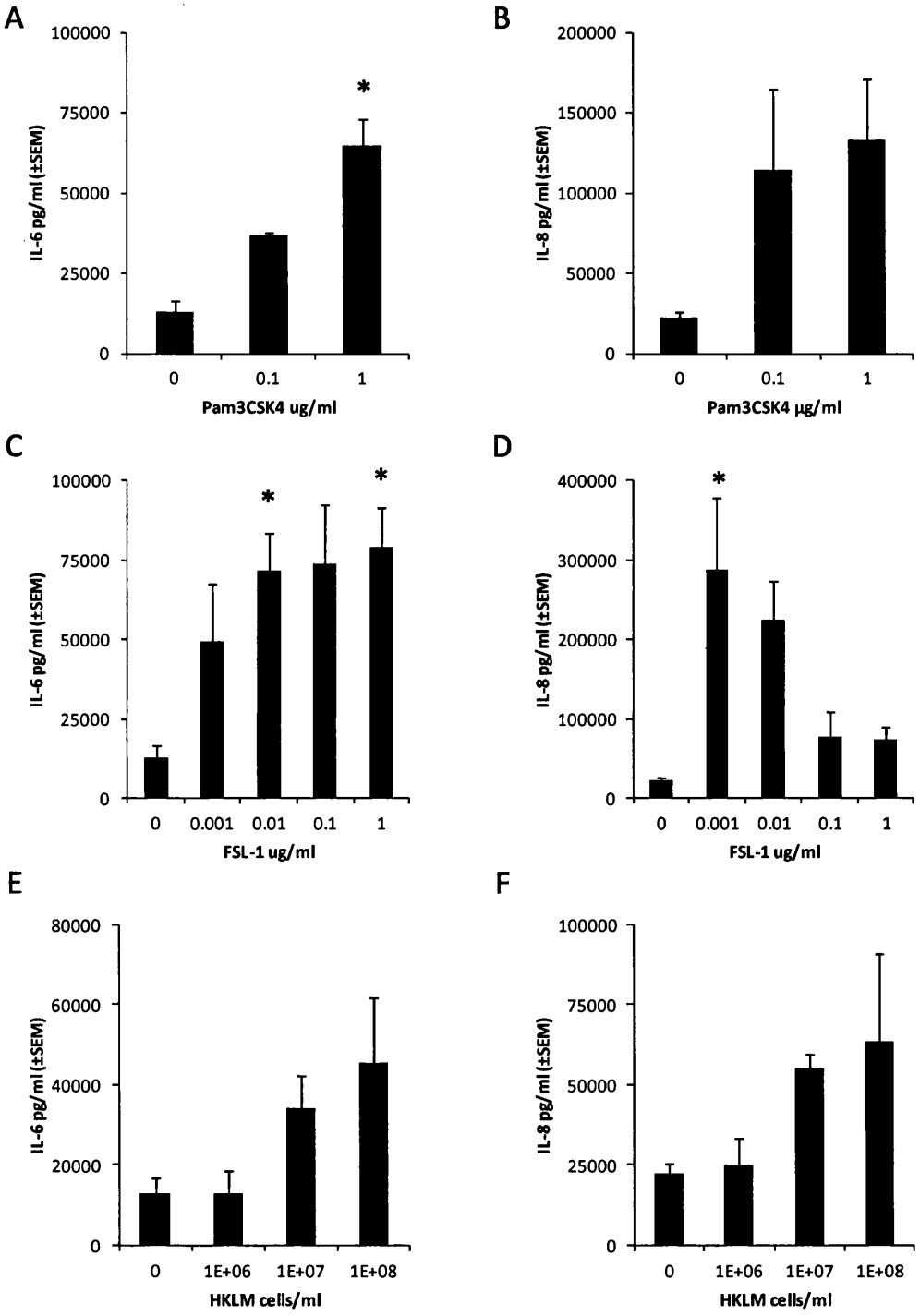

Figure 8.1 TLR2 agonist induced cytokine response by the term non-laboured placenta.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the placenta (n=3) in response to varying concentrations of (A-B) Pam3CSK4, (C-D) FSL-1 and (E-F) HKLM. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

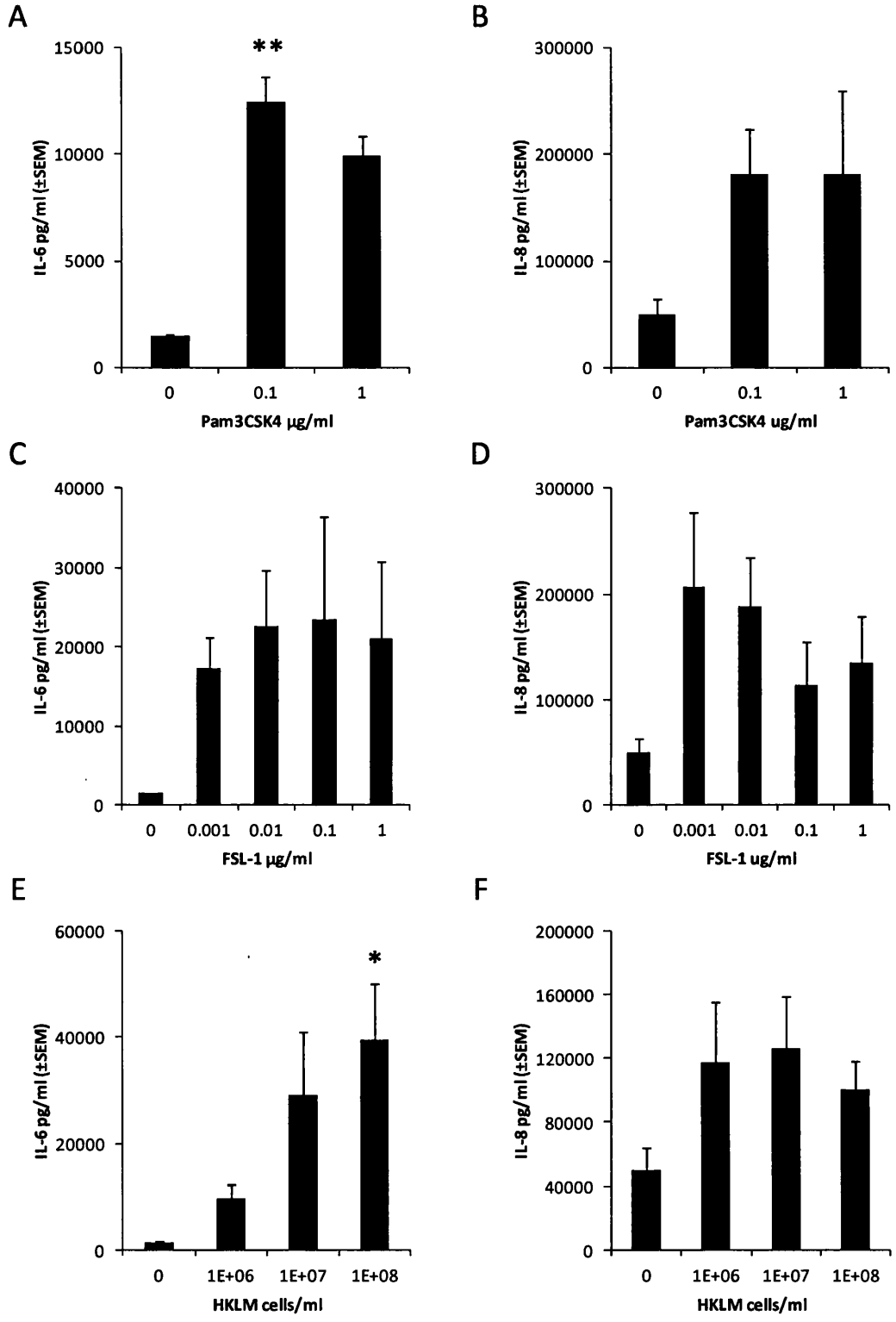

Figure 8.2 TLR2 agonist induced cytokine response by the term non-laboured choriodecidua.
IL-6 and IL-8 production (pg/ml mean ± SEM) by the choriodecidua (n=3) in response to varying concentrations of (A-B) Pam3CSK4, (C-D) FSL-1 and (E-F) HKLM. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

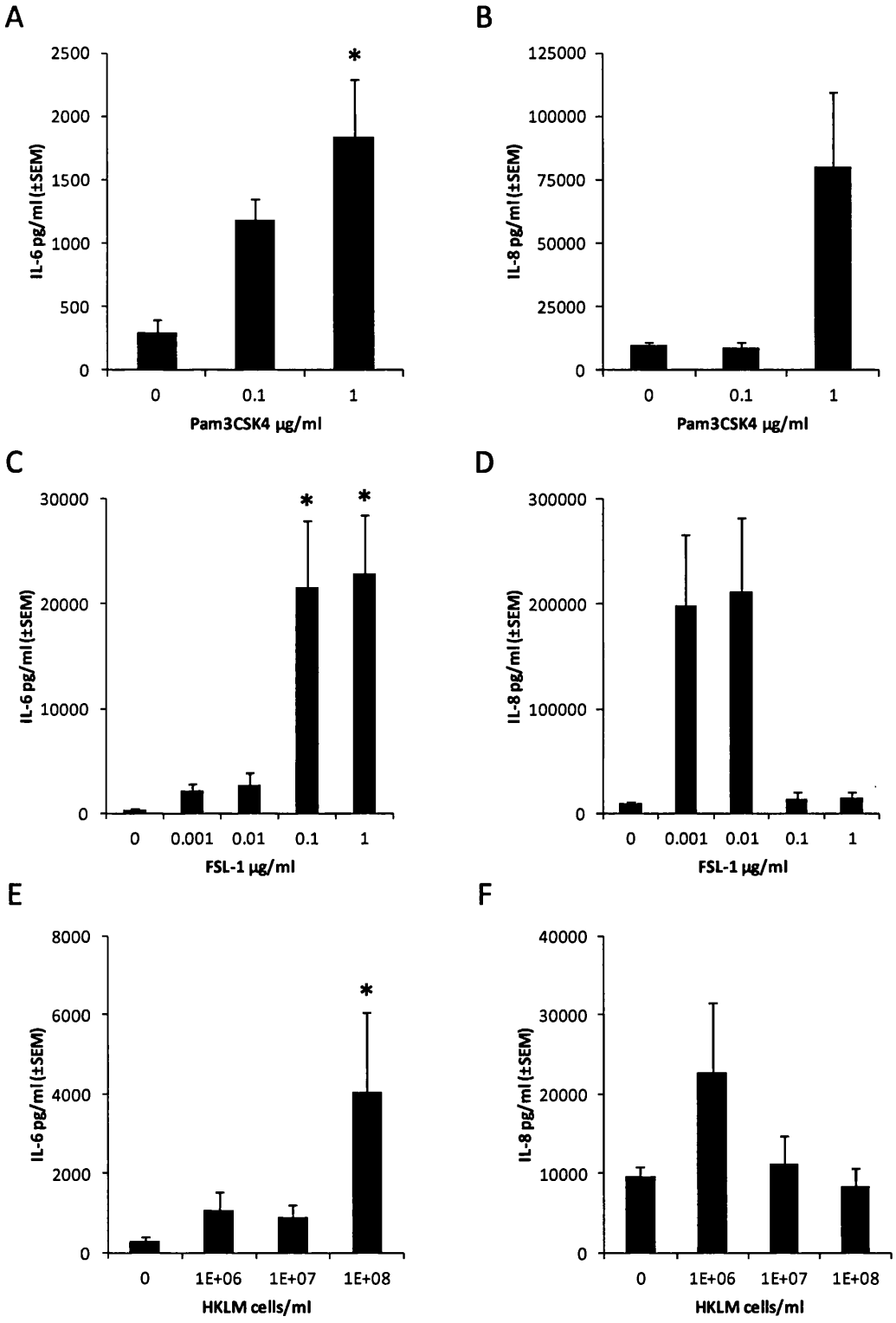

Figure 8.3 TLR2 agonist induced cytokine response by the term non-laboured amnion.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the amnion (n=3) in response to varying concentrations of (A-B) Pam3CSK4, (C-D) FSL-1 and (E-F) HKLM. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

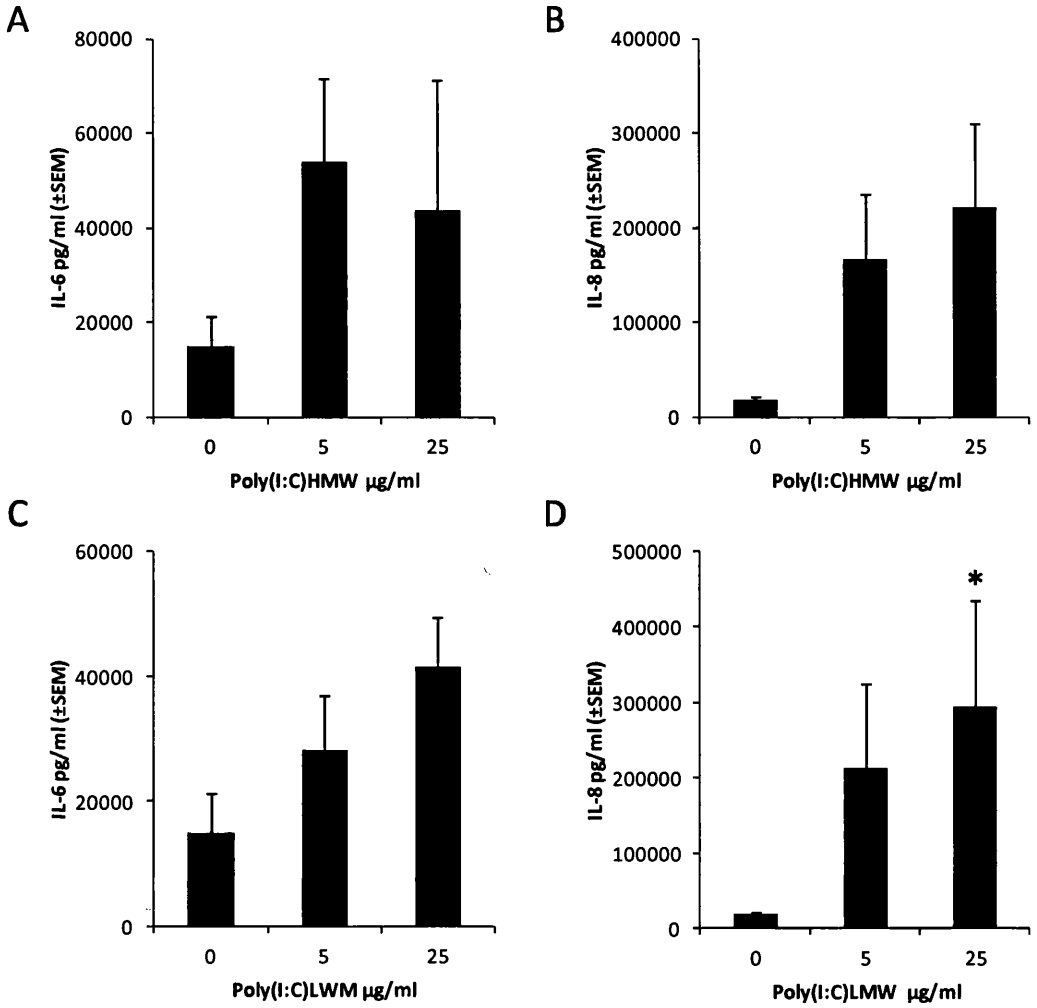

Figure 8.4 TLR3 agonist induced cytokine response by the term non-laboured placenta.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the placenta in response to varying concentrations of (A-B) Poly(I:C)HMW and (C-D) Poly(I:C)LWM (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

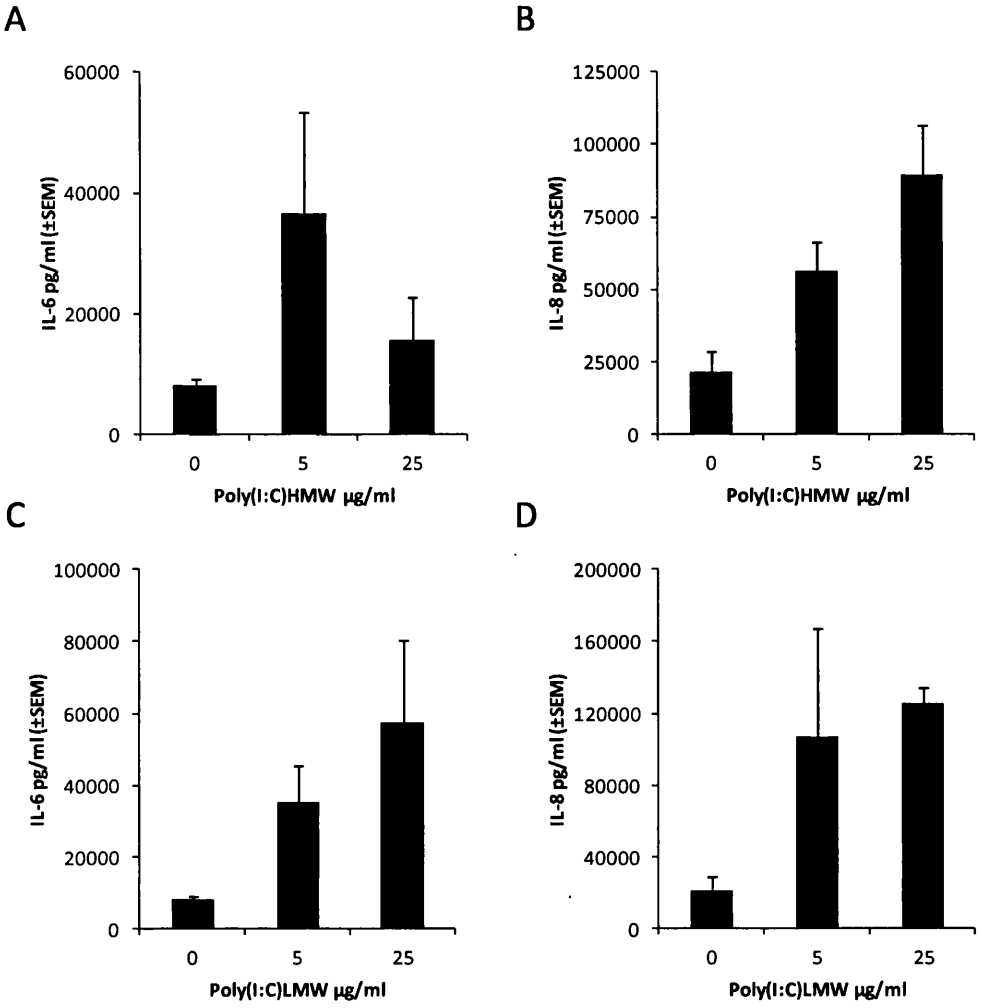

Figure 8.5 TLR3 agonist induced cytokine response by the term non-laboured choriodecidua.
IL-6 and IL-8 production (pg/ml mean ± SEM) by the placenta in response to varying concentrations of (A-B) Poly(I:C)HMW and (C-D) Poly(I:C)LWM (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

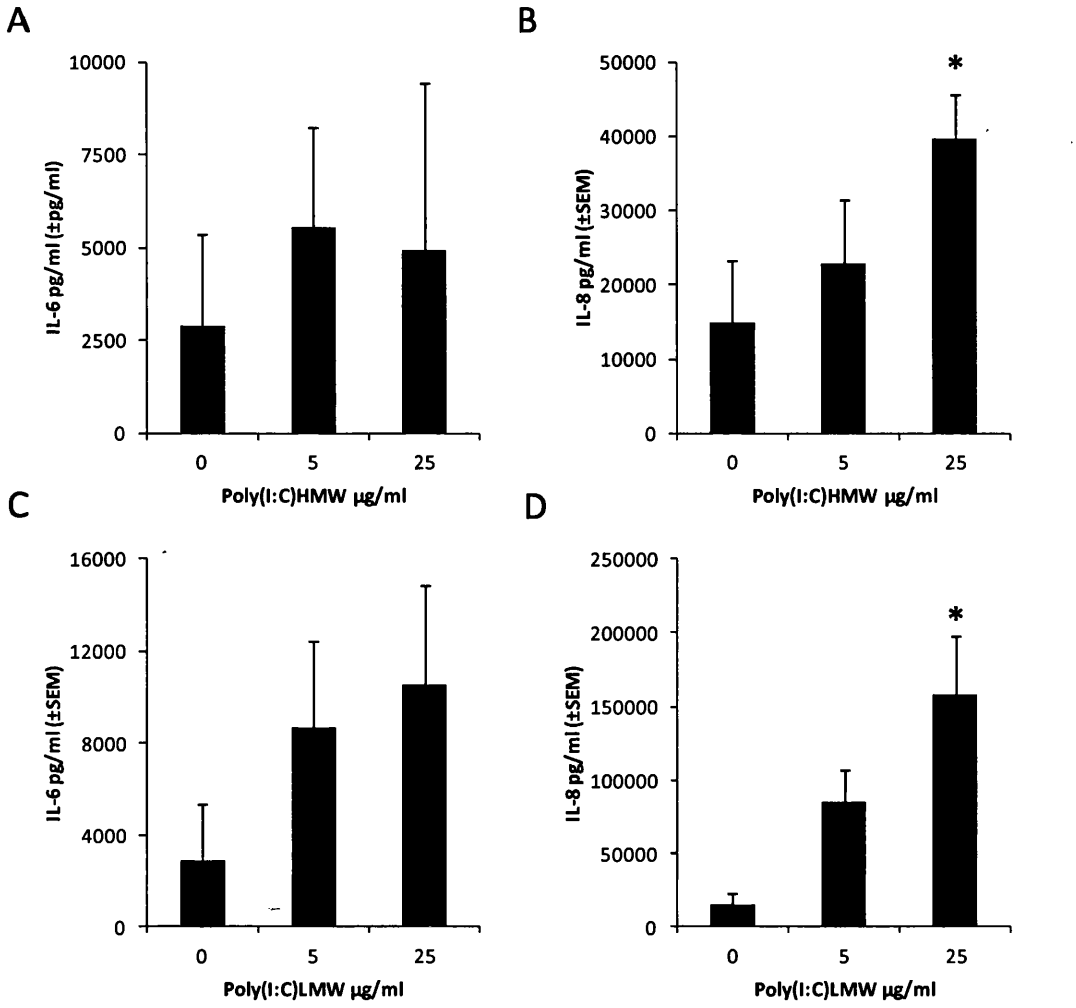

Figure 8.6 TLR3 agonist induced cytokine response by the term non-laboured amnion.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the amnion in response to varying concentrations of (A-B) Poly(I:C)HMW and (C-D) Poly(I:C)LWM (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

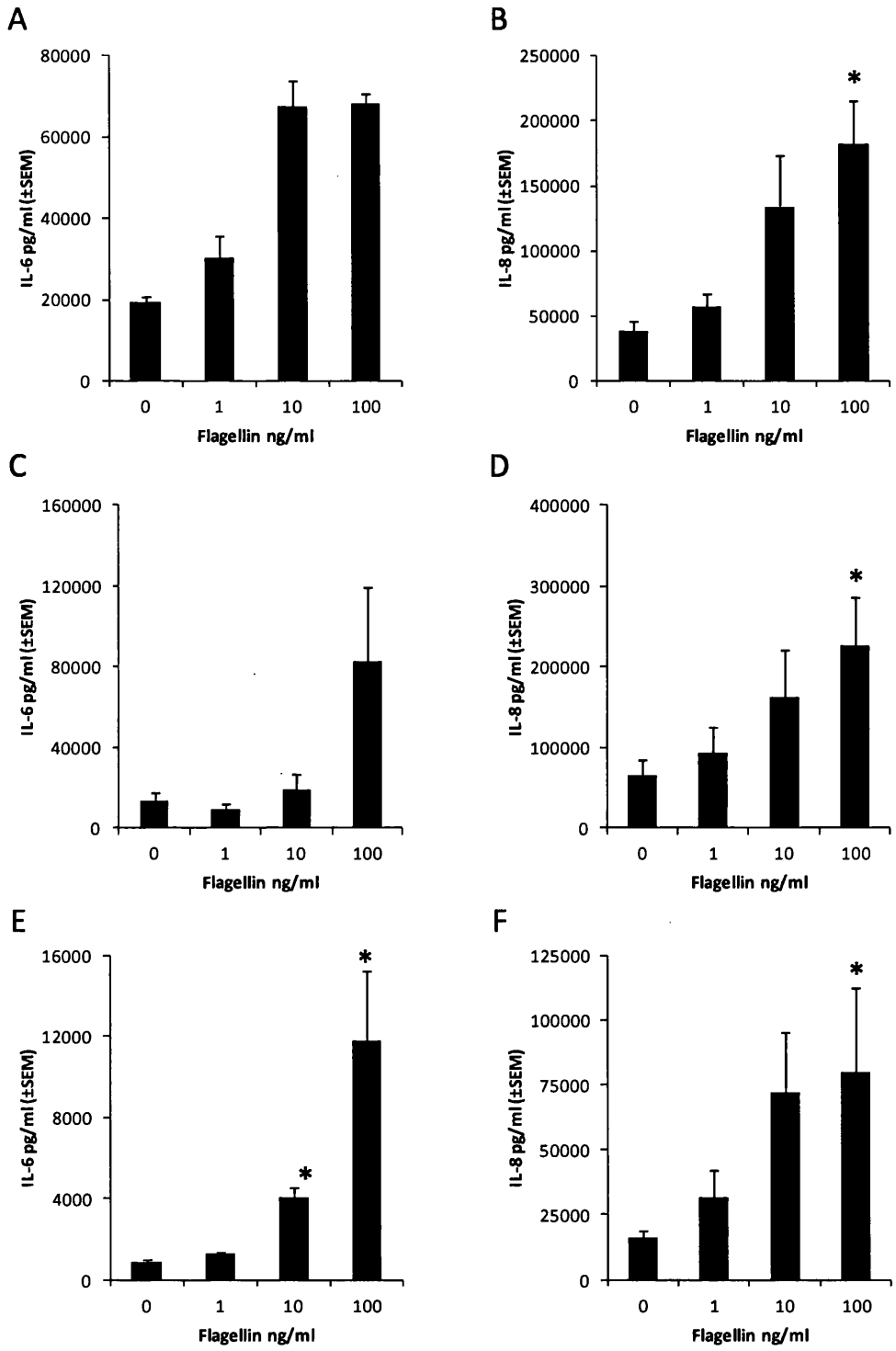

Figure 8.7 TLR5 induced cytokine response by the term non-laboured placenta, choriodecidua and amnion.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion in response to varying concentrations of flagellin (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

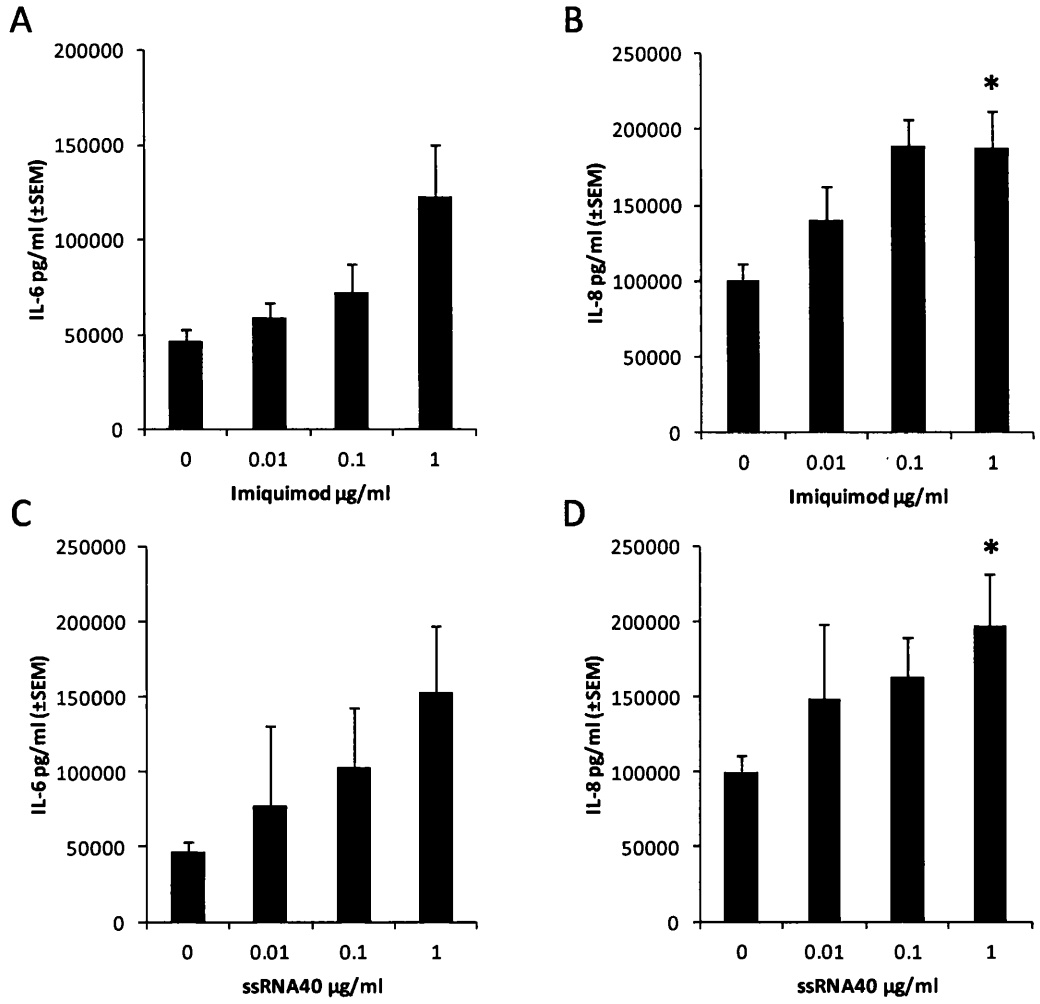

Figure 8.8 TLR7/8 agonist induced cytokine response by the term non-laboured placenta.
IL-6 and IL-8 production (pg/ml mean ± SEM) by the placenta in response to varying concentrations of (A-B) imiquimod and (C-D) ssRNA40 (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

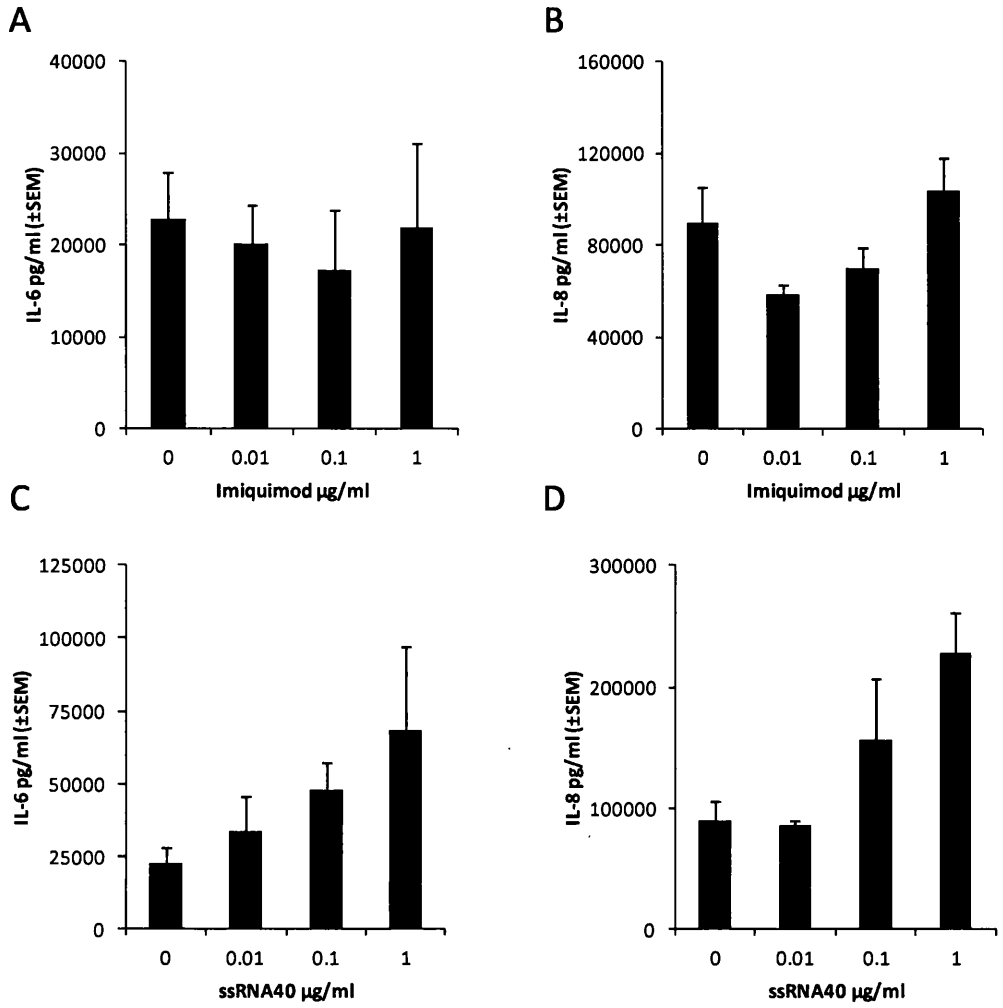

Figure 8.9 TLR7/8 agonist induced cytokine response by the term non-laboured choriodecidua.
IL-6 and IL-8 production (pg/ml mean ± SEM) by the choriodecidua in response to varying concentrations of (A-B) imiquimod and (C-D) ssRNA40 (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

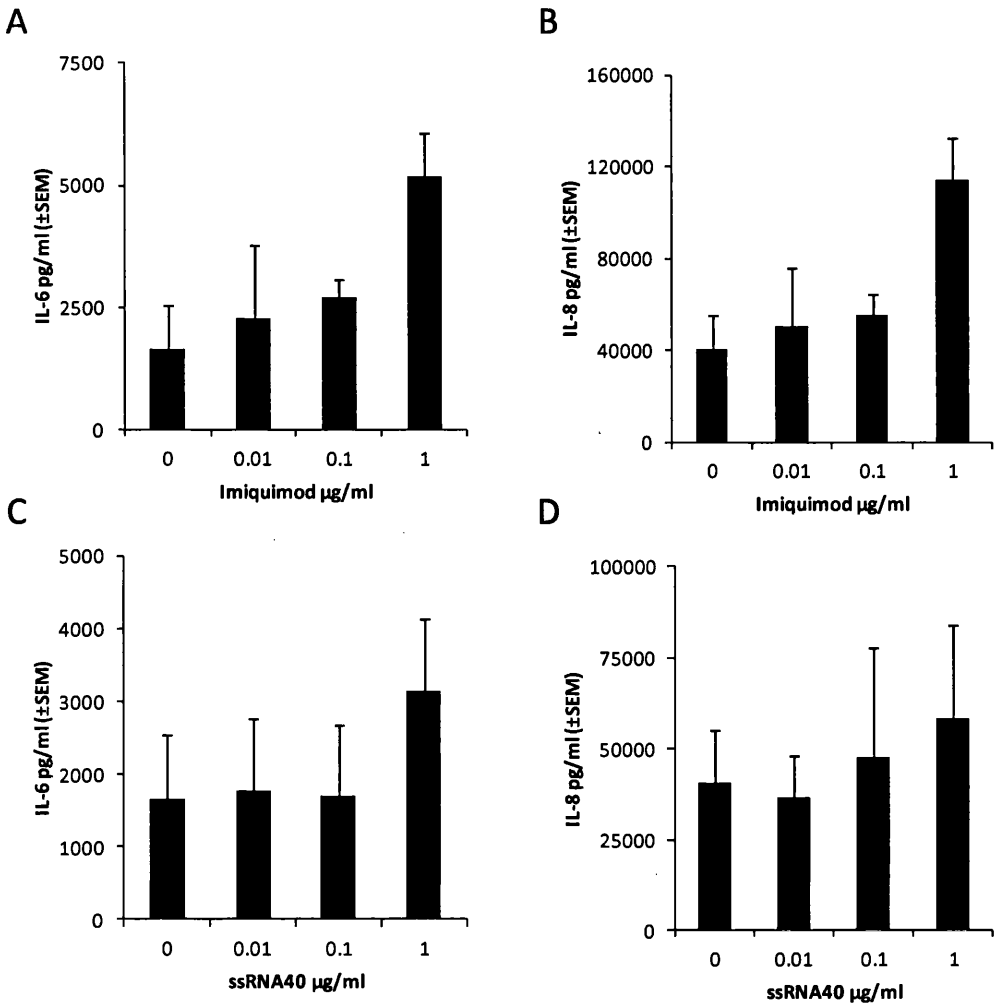

Figure 8.10 TLR7/8 agonist induced cytokine response by the term non-laboured amnion.
IL-6 and IL-8 production (pg/ml mean ± SEM) by the amnion in response to varying concentrations of (A-B) imiquimod and (C-D) ssRNA40 (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

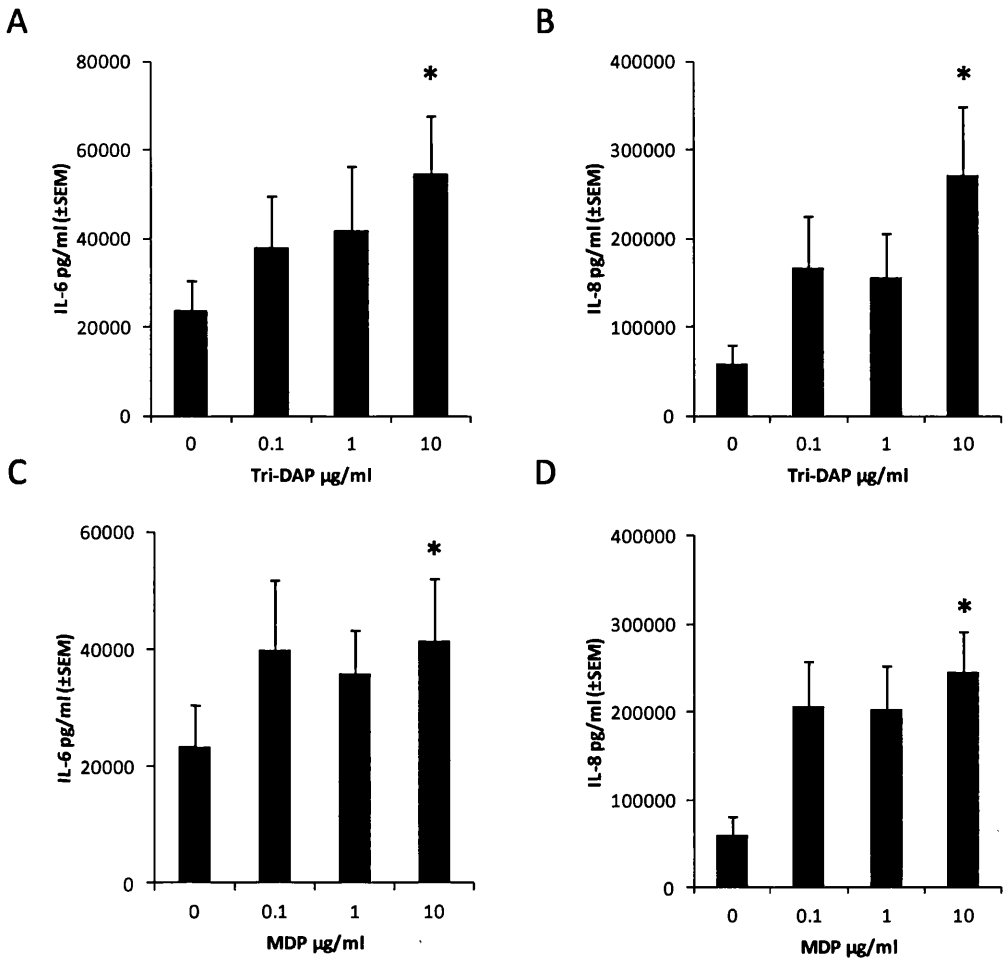

Figure 8.11 NOD1/2 agonist induced cytokine response by the term non-laboured placenta.
IL-6 and IL-8 production (pg/ml ± SEM) by the placenta in response to varying concentrations of (A-B) Tri-DAP and (C-D) MDP (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$, ** $p \leq 0.01$.

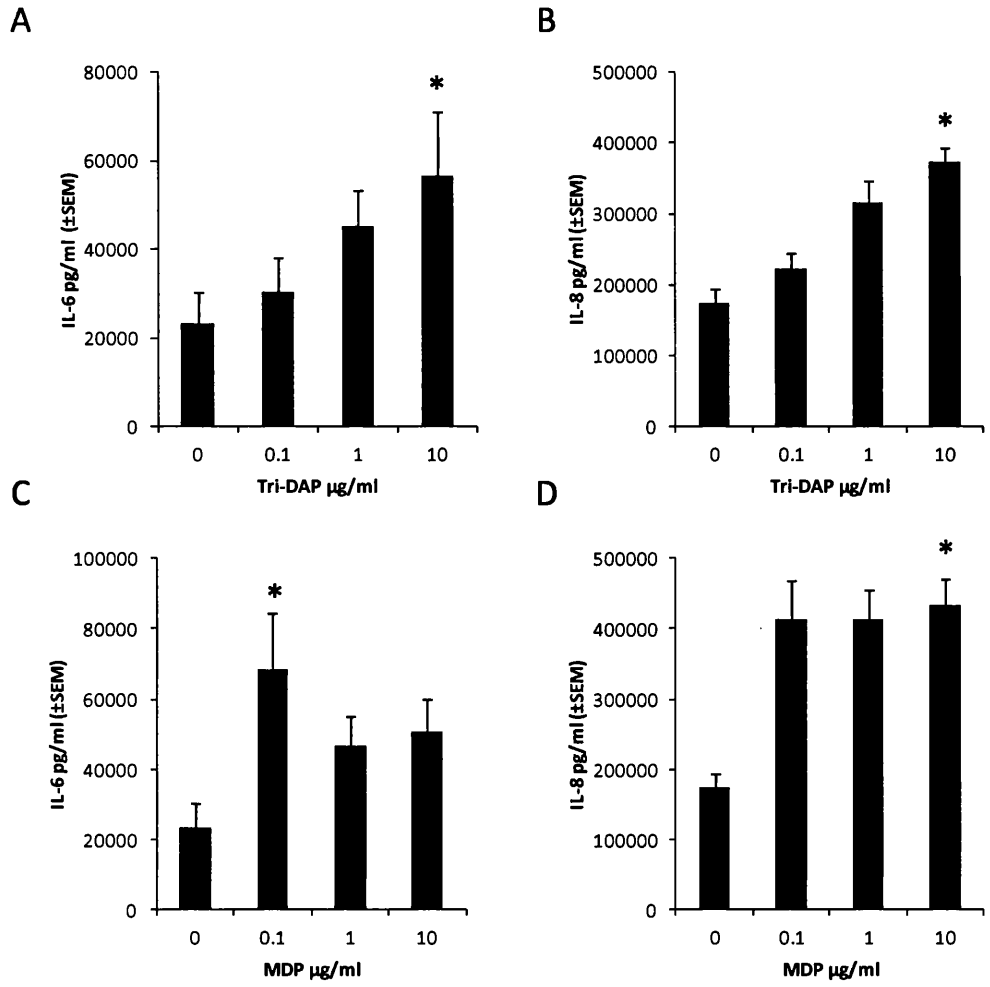

Figure 8.12 NOD1/2 agonist induced cytokine response by the term non-laboured choriodecidua.

IL-6 and IL-8 production (pg/ml ± SEM) by the choriodecidua in response to varying concentrations of (A-B) Tri-DAP and (C-D) MDP (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

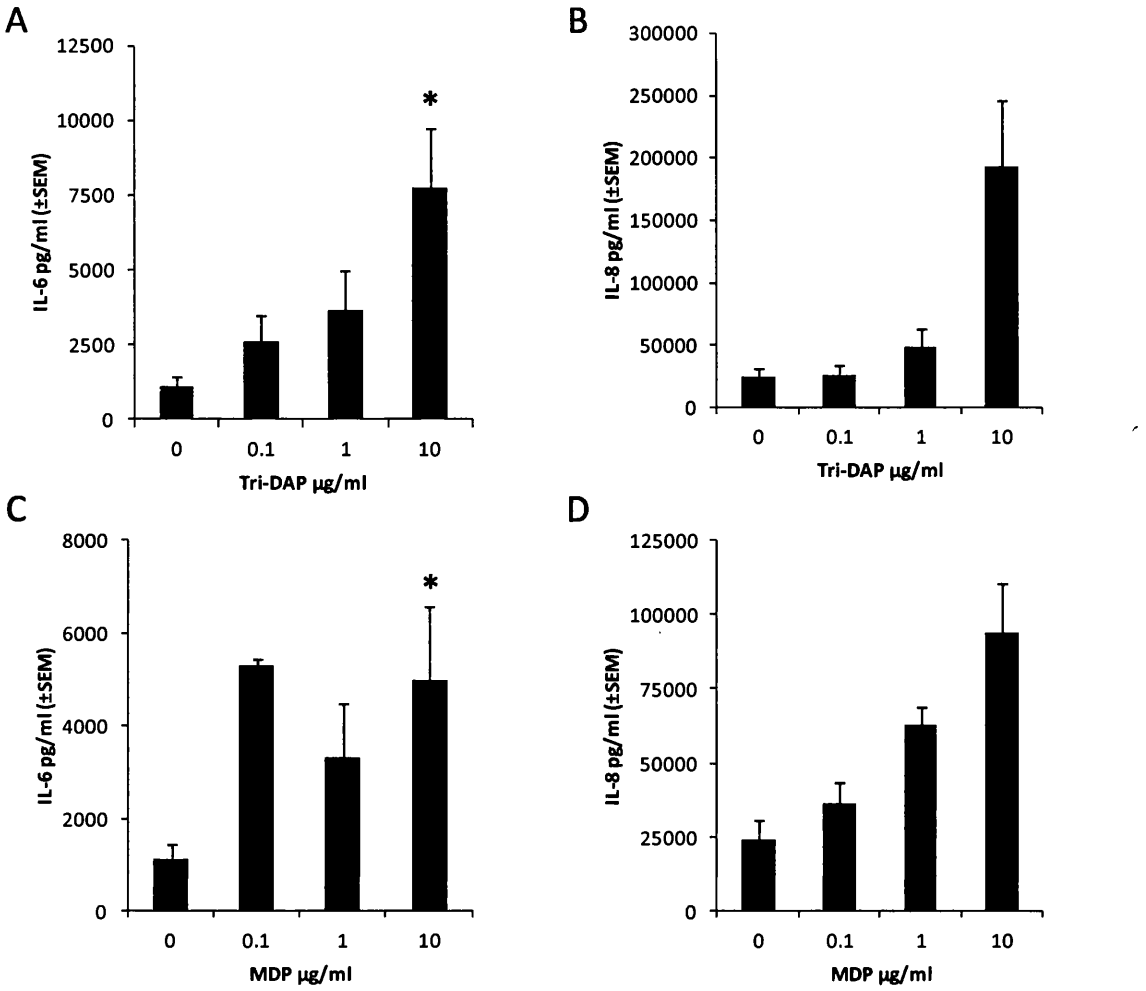

Figure 8.13 NOD1/2 agonist induced cytokine response by the term non-laboured amnion.

IL-6 and IL-8 production (pg/ml ± SEM) by the amnion in response to varying concentrations of (A-B) Tri-DAP and (C-D) MDP (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

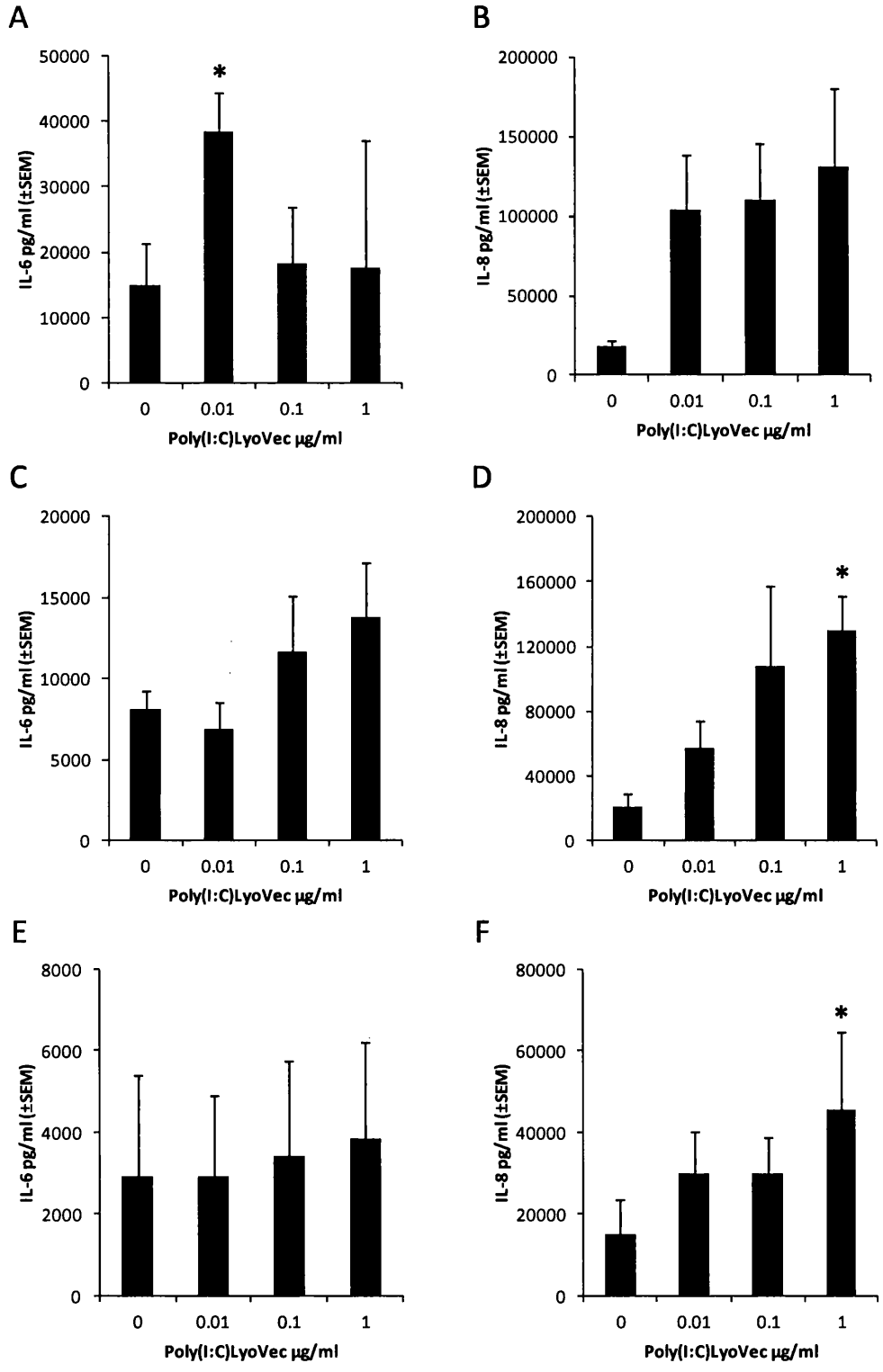

Figure 8.14 RIG-I/MDA5 agonist induced cytokine response by the term non-laboured placenta. IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion in response to varying concentrations of Poly(I:C)LyoVec (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

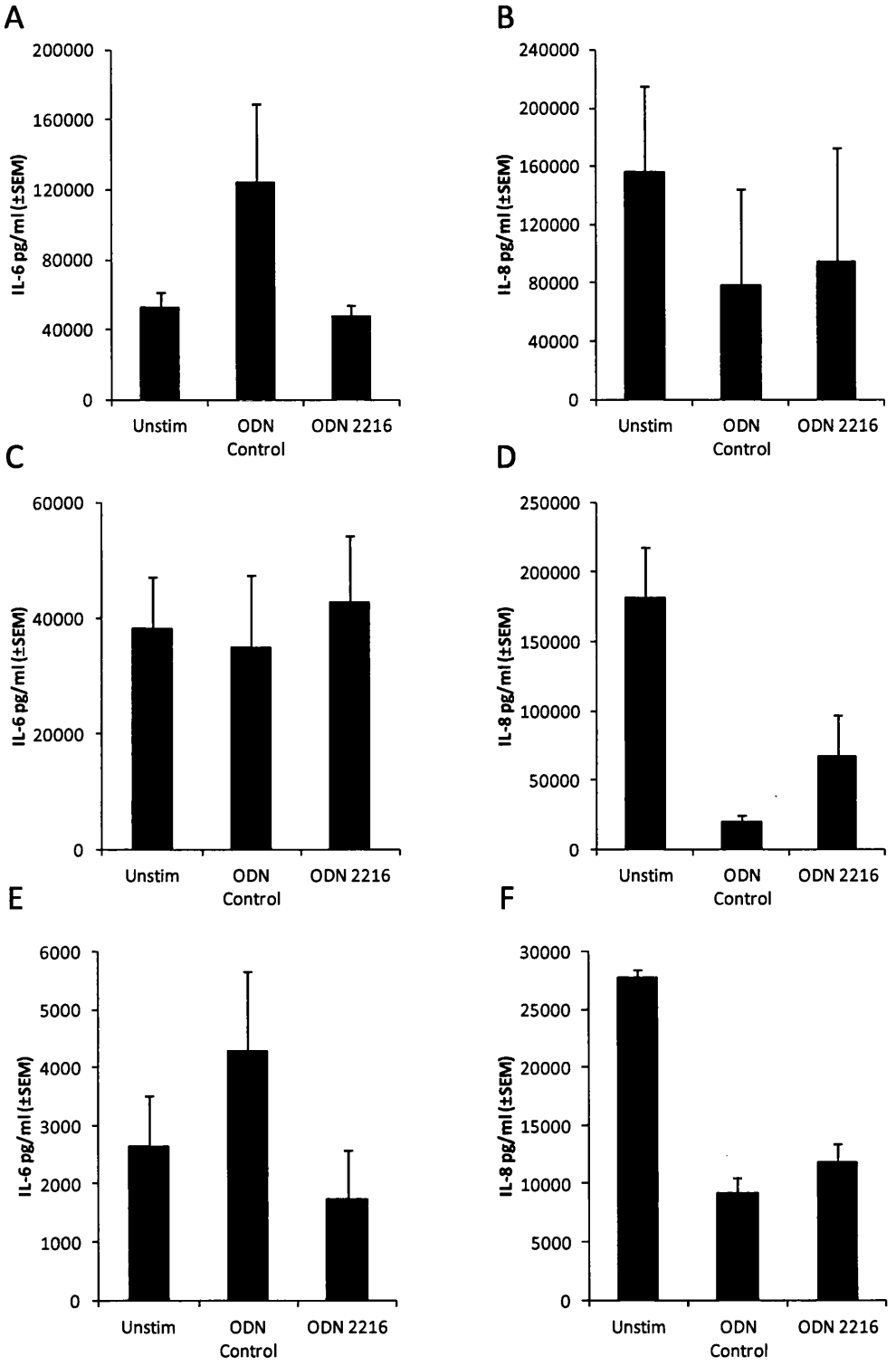

Figure 8.15 CpG ODN induced cytokine response by the term non-laboured placenta, choriodecidua and amnion.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion following stimulation with 5 μM of CpG ODN (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

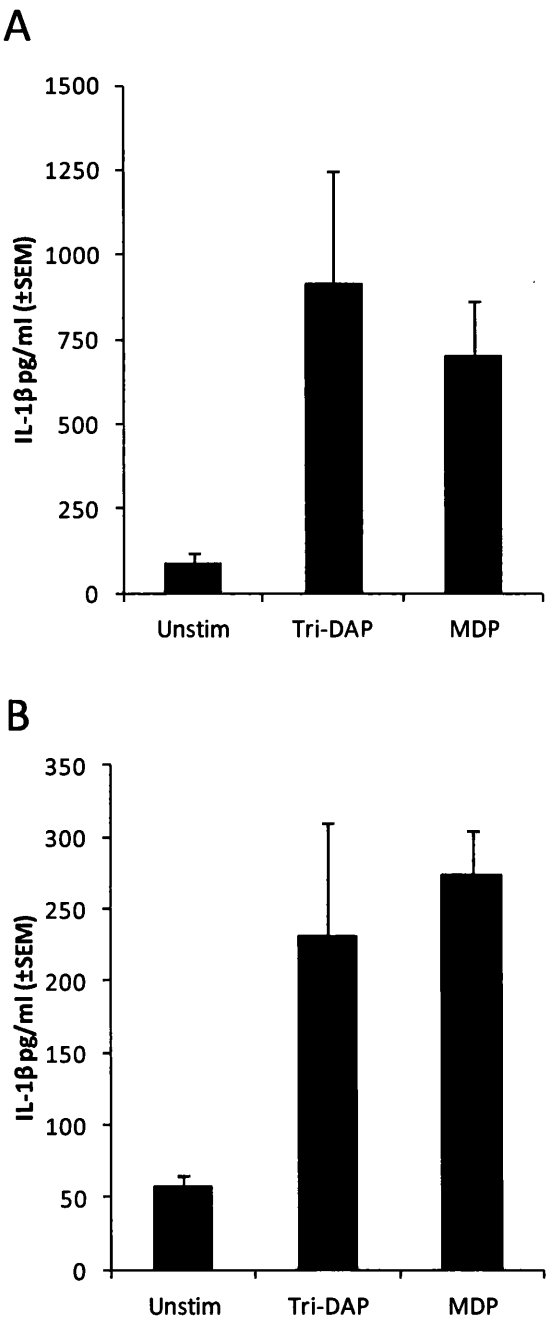

Figure 8.16 NOD1/2 agonist induced IL-1β by the term non-laboured placenta and choriodecidua
Explants of (A) placenta and (B) choriodecidua were treated with either Tri-DAP or MDP (both 10 μg/ml) and levels of IL-1β production (pg/ml mean ± SEM) in tissue free culture supernatants measured (n=3). Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

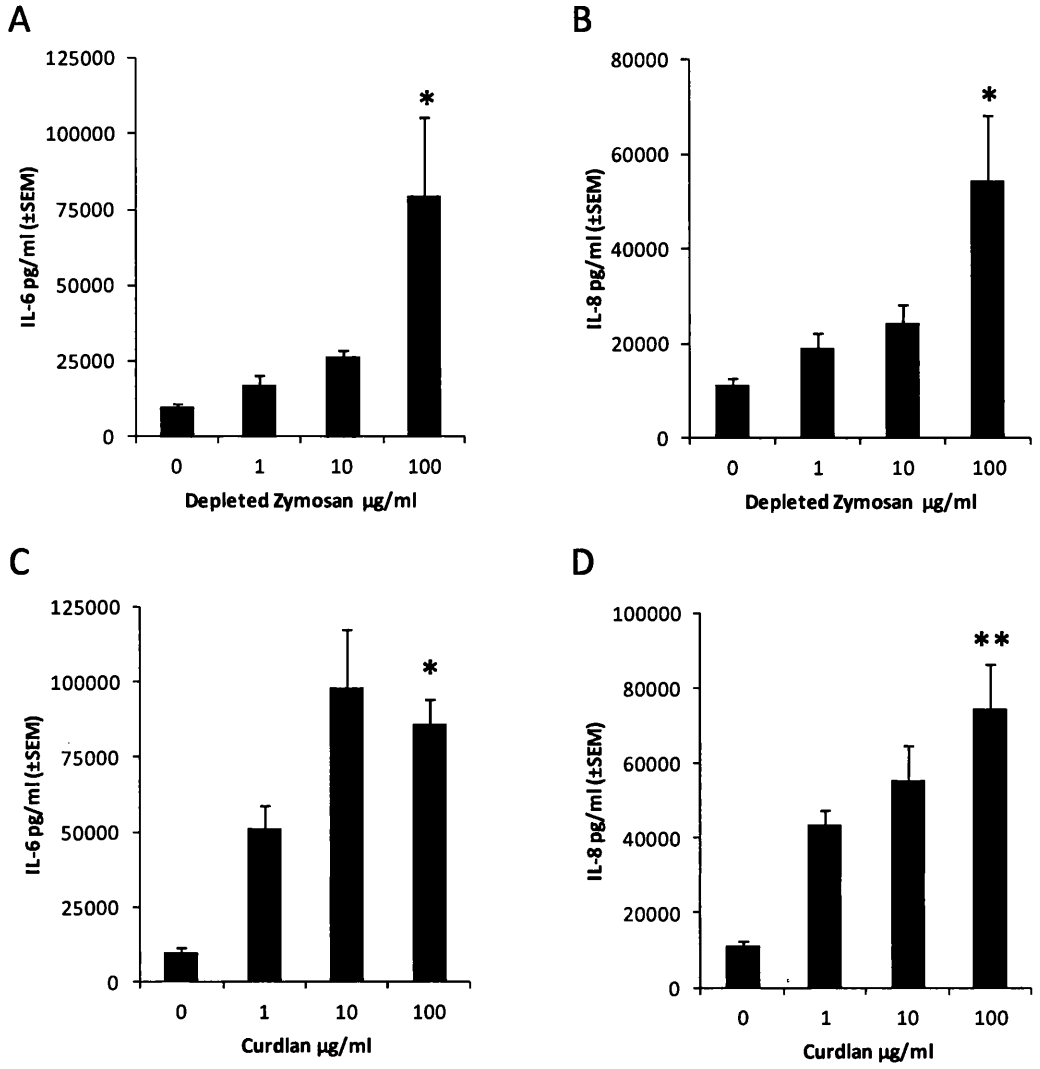

Figure 8.17 Dectin-1 agonist induced cytokine response by the term non-laboured placenta.
IL-6 and IL-8 production (pg/ml ± SEM) by the placenta in response to varying concentrations (n=3) of (A-B) depleted zymosan (DZYM) and (C-D) curdlan. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$, ** $p \leq 0.01$.

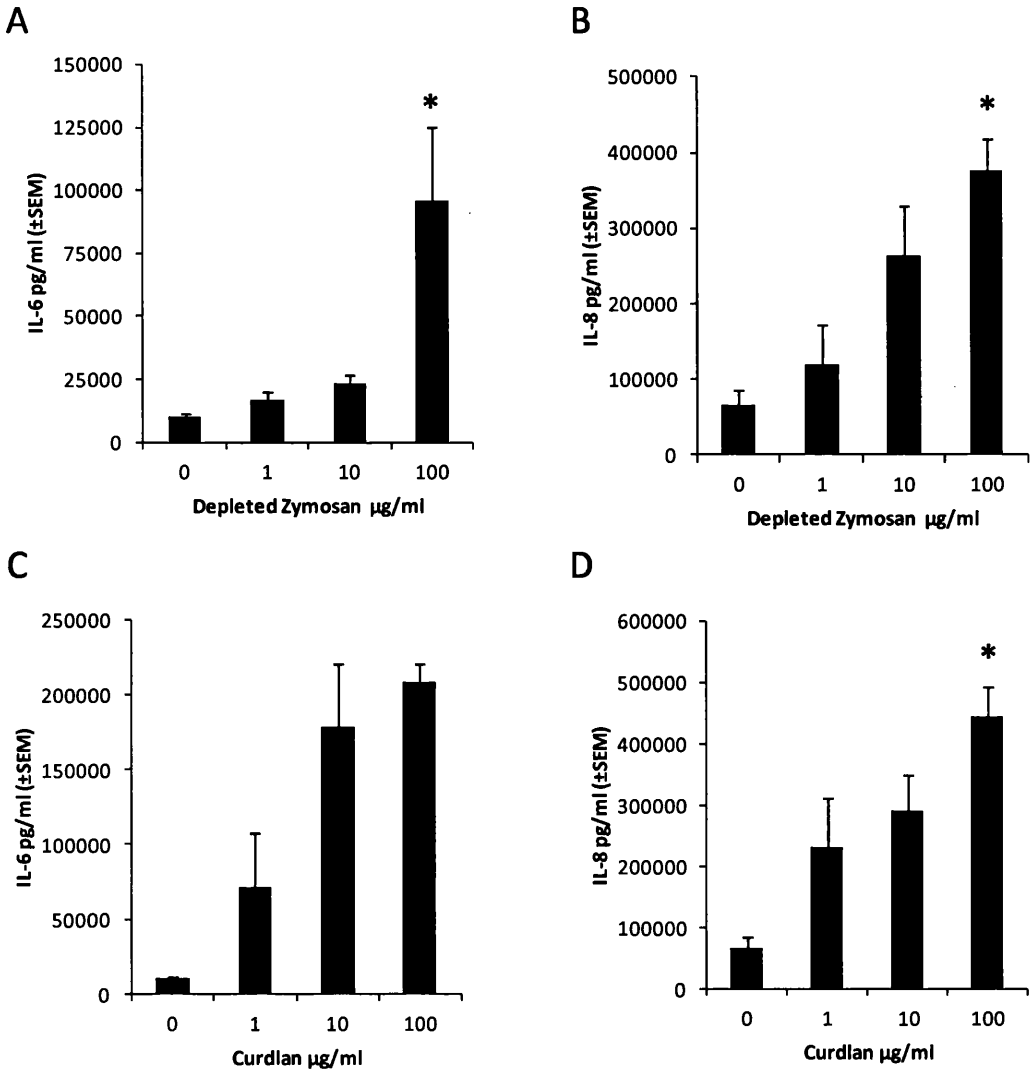

Figure 8.18 Dectin-1 agonist induced cytokine response by the term non-laboured choriodecidua.

IL-6 and IL-8 production (pg/ml ± SEM) by the choriodecidua in response to varying concentrations (n=3) of (A-B) depleted zymosan (DZYM) and (C-D) curdlan. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

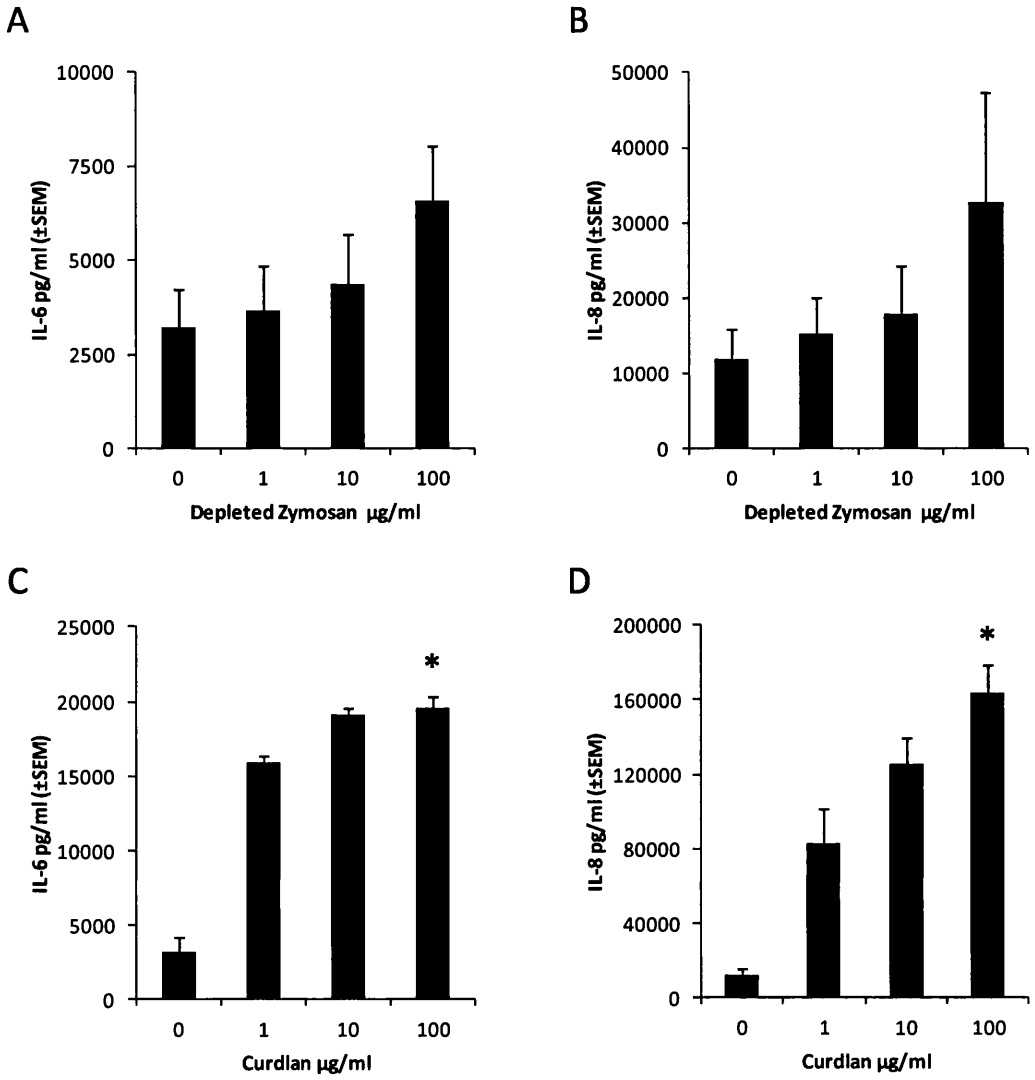

Figure 8.19 Dectin-1 agonist induced cytokine response by the term non-laboured amnion.
IL-6 and IL-8 production (pg/ml ± SEM) by the amnion in response to varying concentrations (n=3) of (A-B) depleted zymosan (DZYM) and (C-D) curdlan. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown: * $p \leq 0.05$.

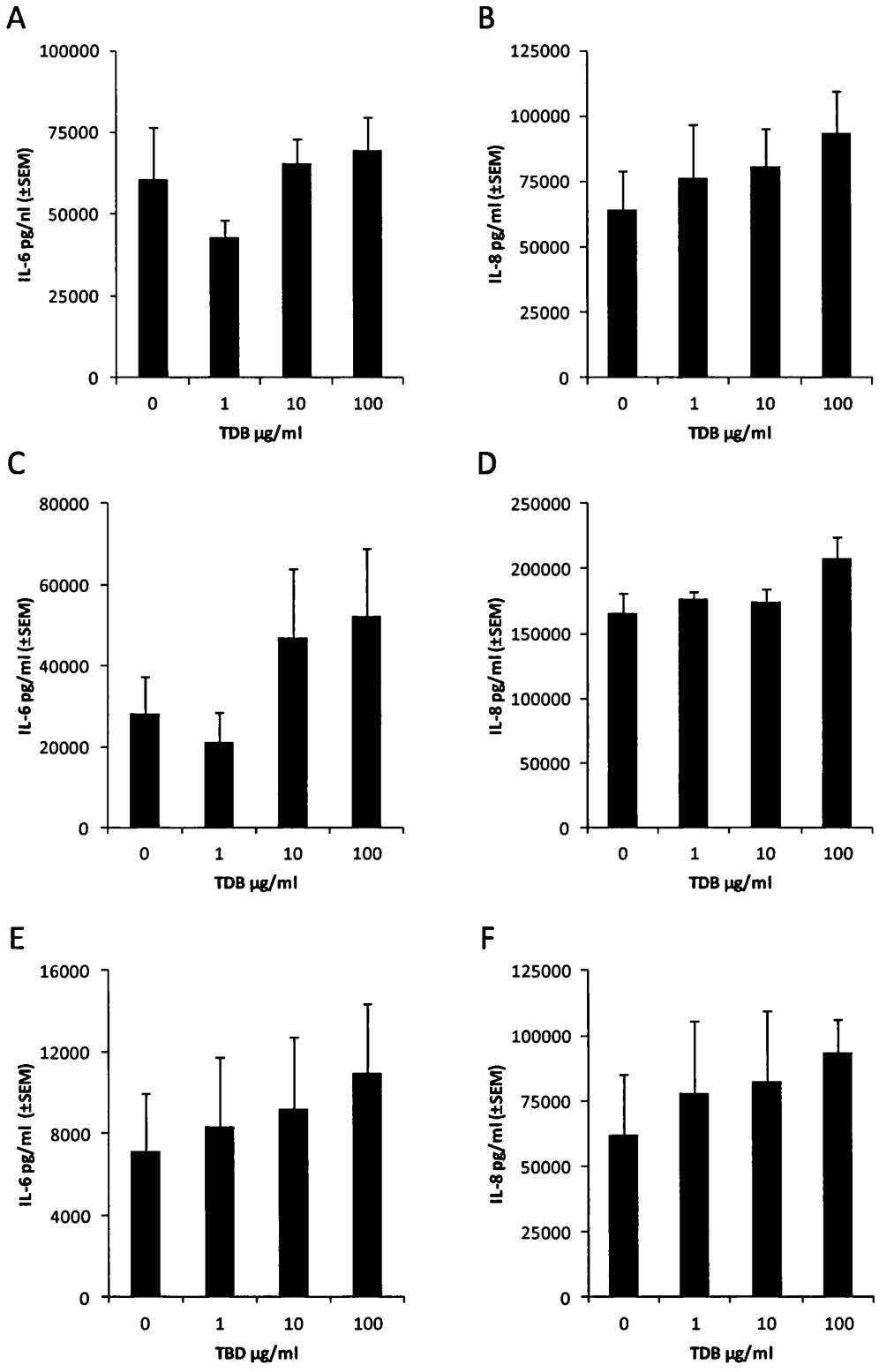

Figure 8.20 MINLCE agonist induced cytokine response by the term non-laboured placenta.

IL-6 and IL-8 production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion in response to varying concentrations of TDB (n=4). Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

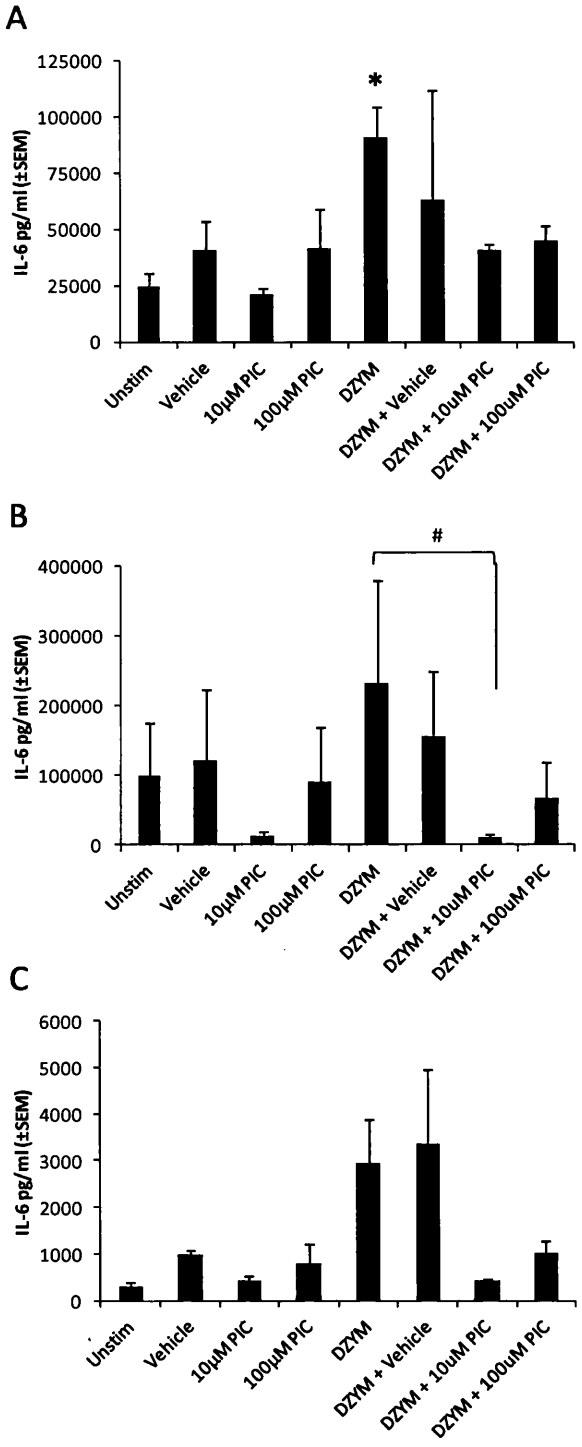

Figure 8.21 Optimisation of the Syk Inhibitor Piceatannol.

Explants of (A) placenta, (B) choriodecidua, and (C) amnion were stimulated with depleted zymosan (100 μg/ml) in the presence or absence of varying concentrations of the Syk inhibitor piceatannol (n=3) and IL-6 (pg/ml; mean ± SEM) in tissue free supernatants measured. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown as: * $p \leq 0.05$. Statistical significance compared to curdlan is shown as: # $p \leq 0.05$.

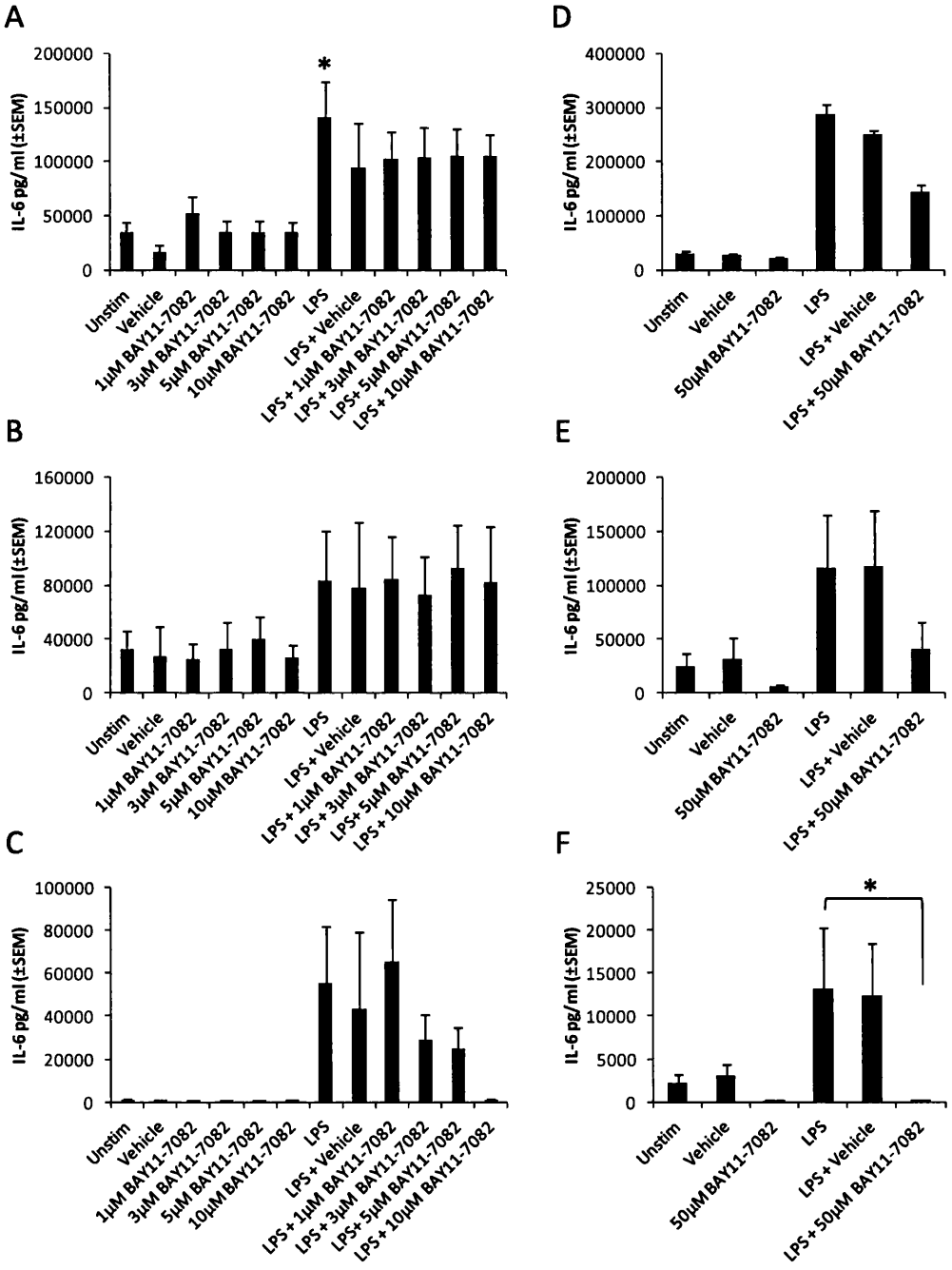

Figure 8.22 Optimisation of the NF-κB Inhibitor BAY11-7082

Explants of (A) placenta, (B) choriodecidua, and (C) amnion were stimulated with LPS (10 ng/ml) in the presence or absence of varying concentrations of the NF-κB inhibitor BAY11-7082 (n=3) and IL-6 levels (pg/ml mean ± SEM) in tissue free supernatants measured. Explants of (D) placenta, (E) choriodecidua, and (F) amnion following stimulation with LPS (10 ng/ml) in the presence or absence of 50 μM BAY11-7082 (n=3) and IL-6 levels (pg/ml mean ± SEM) in tissue free supernatants measured. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown as: * $p \leq 0.05$. Statistical significance compared to LPS is shown as: # $p \leq 0.05$.

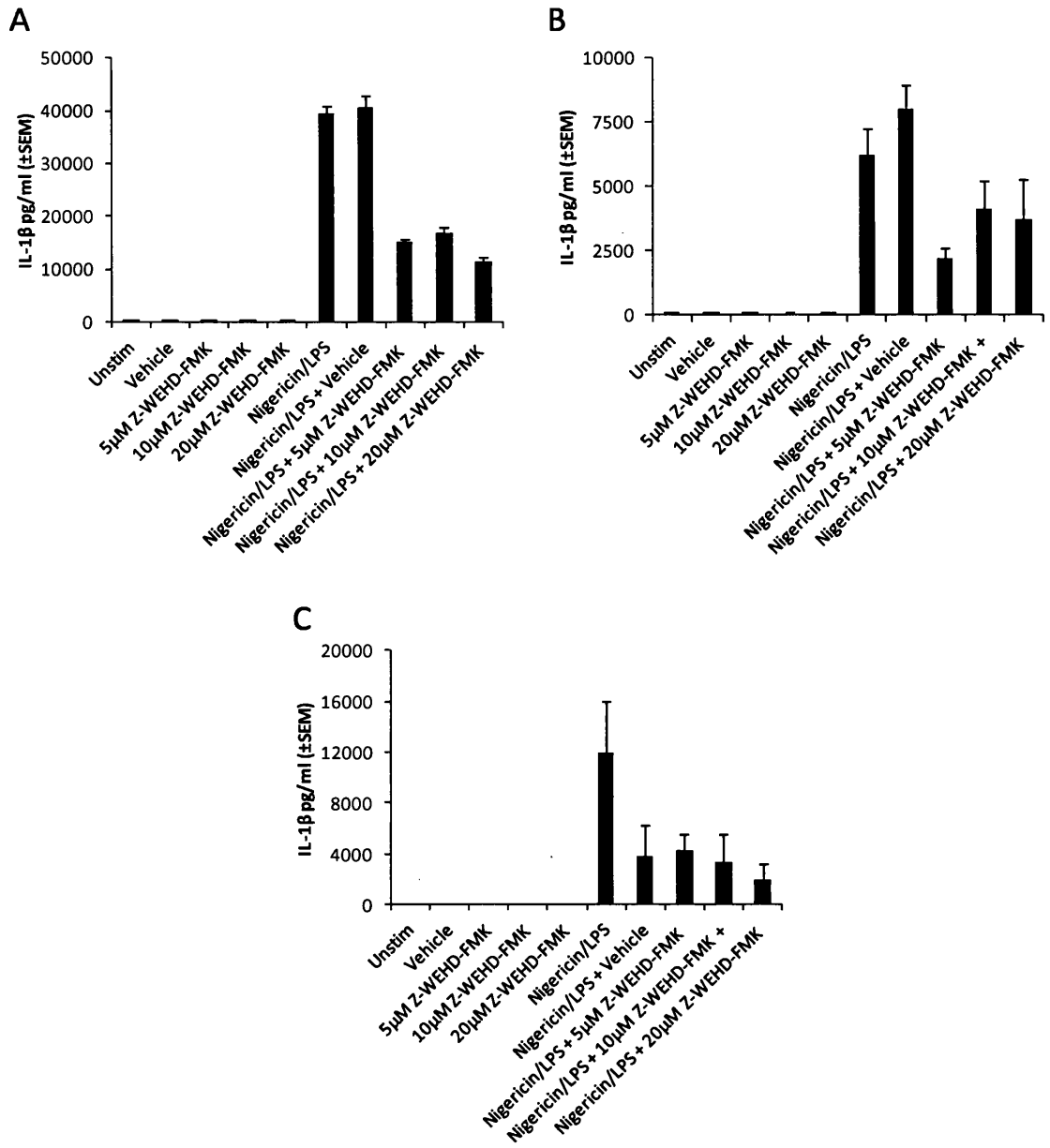

Figure 8.23 Caspase-1 Inhibitor optimisation

Explants of (A) placenta, (B) choriodeicuda and (C) amnion were treated with LPS (10 ng/ml) + Nigericin (1 μM) in the presence or absence of the caspase-1 inhibitor Z-WEHD-FMK (n=3) and IL-1β production (pg/ml mean ± SEM) in tissue free supernatants measured. Statistical significance was determined by Freidman's test with Dunn's posthoc test. No significant difference was observed.

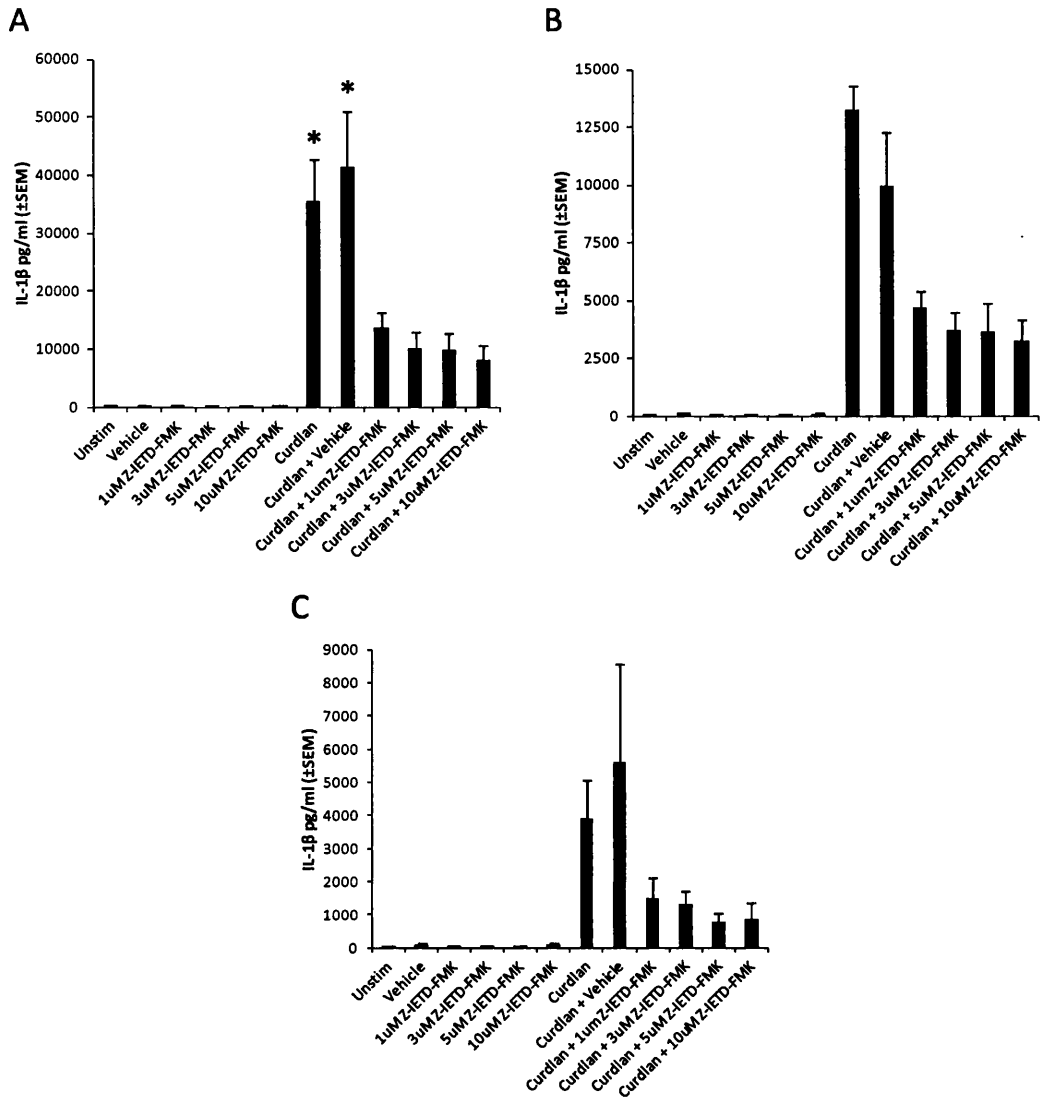

Figure 8.24 Caspase-8 Inhibitor Optimisation

Explants of (A) placenta, (B) choriodeicuda and (C) amnion were treated with curdlan (100 µg/ml) in the presence or absence of the caspase-8 inhibitor Z-IETD-FMK (n=3) and IL-1β production (pg/ml mean ± SEM) in tissue free supernatants measured. Statistically significant differences compared to curdlan are shown: * $p \leq 0.05$, ** $p \leq 0.01$. Statistical significance was determined by Freidman's test with Dunn's posthoc test. Statistical significance compared to unstimulated is shown as: * $p \leq 0.05$.

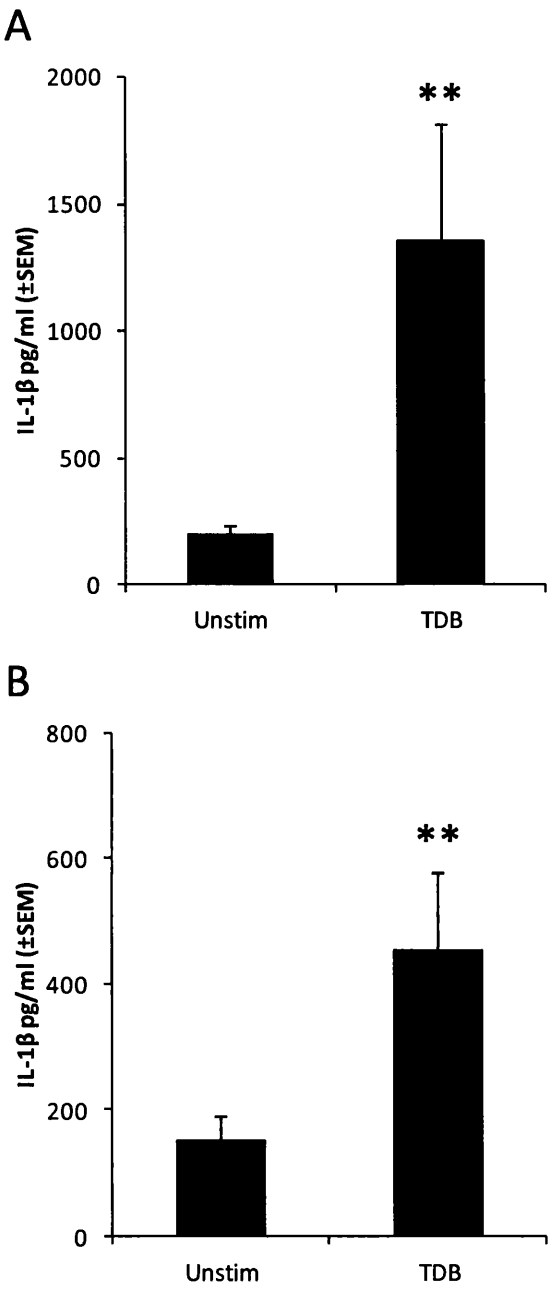

Figure 8.25 TDB induced IL-1β by the term non-laboured placenta and choriodecidua

Explants of (A) placenta and (B) choriodecidua were treated with either TDB (both 100 μg/ml) and levels of IL-1β production (pg/ml mean ± SEM) in tissue free culture supernatants measured (n=9). Statistical significance compared to unstimulated control as determined by Wilcoxon matched pairs signed rank test are shown: ** $p \leq 0.01$.

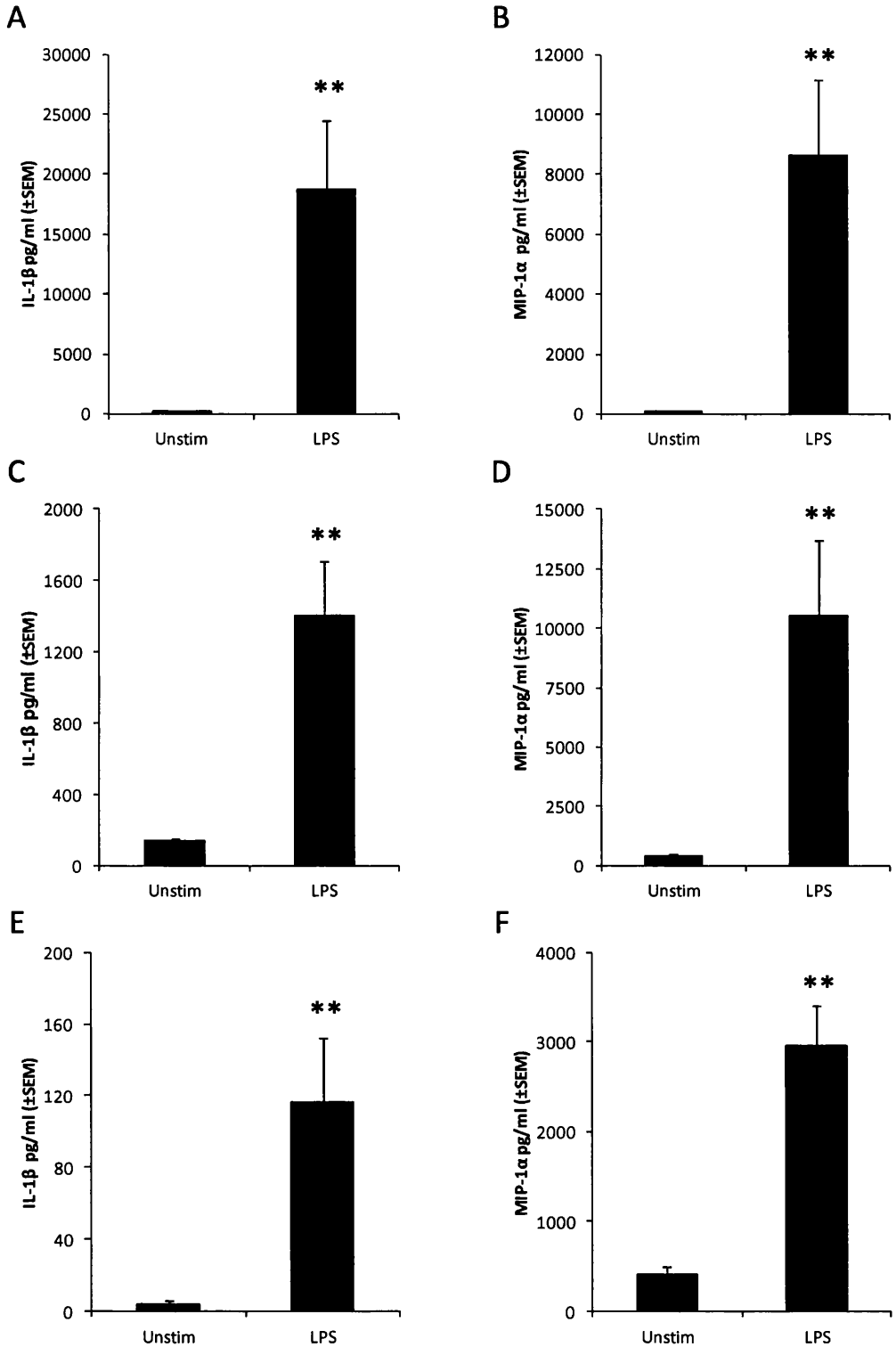

Figure 8.26 LPS induced cytokine response by the term non-laboured placenta, choriodecidua and amnion.

IL-1β and MIP-1α production (pg/ml mean ± SEM) by the (A-B) placenta, (C-D) choriodecidua, and (E-F) amnion following stimulation with 10 ng/ml of LPS (n=9). Statistical significance compared to unstimulated control as determined by Wilcoxon matched pairs signed rank test are shown: ** $p \leq 0.01$.

8.3 Conference presentations and publications

8.3.1 Conference posters

1. European Congress of Immunology (2012) – Poster titled: Modulation of the inflammatory response in gestation associated tissues by IL-4.

2. International Congress of Immunology (2013) – Poster titled: Fungal stimulated inflammatory response of gestation associated tissues.

8.3.2 Publications

1. Aled H. Bryant and Catherine A. Thornton (2012). Cytokines and the Innate Immune Response at the Materno-Fetal Interface, Recent Advances in Research on the Human Placenta, Dr. Jing Zheng (Ed.), ISBN: 978-953-51-0194-9, InTech, DOI: 10.5772/33219. Available from: http://www.intechopen.com/books/recent-advances-in-research-on-the-human-placenta/immunological-responsiveness-of-the-human-placenta

Chapter 9

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