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Exploring the potential role of CRTH2 agonists in the prevention of inflammation induced preterm labour

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Declaration

Statement of originality

All work presented in this thesis was performed by myself unless otherwise stated.

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Publications

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The 15dPGJ2 reduces NF-κB in peripheral blood mononuclear cells and reduces the production of the pro inflammatory cytokines IFN-γ and TNF-α in T helper cells
Sykes L, Ponnampalam S, Yap XJ, Teoh TG, Bennett PR.
The Society of Gynecological Investigation, San Diego, USA. March 2012.

The CRTH2 agonist Pyl A alters the Th1 and Th2 interleukin profile in the myometrium of LPS treated mice.
Sykes L, Herbert B, Lee Y, Johnson M, Teoh TG, Bennett PR.
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The CRTH2 agonist Pyl A prevents LPS induced fetal death but induces preterm labour.
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CRTH2 is not present on amniocytes and myocytes.
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The Th1:Th2 Dichotomy of Pregnancy and Preterm Labour
Sykes L; MacIntyre DA; Yap XJ; Teoh TG; Bennett PR.
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Changes in the Th1: Th2 Cytokine Bias in Pregnancy and the Effects of the Anti-Inflammatory Cyclopentenone Prostaglandin 15-Deoxy-Δ12,14-Prostaglandin J2
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MacIntyre DA*; Sykes L*; Teoh TG; Bennett PR
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List of Abbreviations

AP-1	Activator protein 1
APC	Allophycocyanin
AUC	Area under the curve
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCL	Chemokine (c-c motif) ligand
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD11b	Cluster of differentiation molecule 11B
COX	Cyclo-oxygenase
cPLA2	Cytosolic phospholipase A2 isoenzymes
CRTH2	Chemoattractant receptor homologous to the T helper 2 cell
CXCL	Chemokine (c-x-c motif) ligand
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphates
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinases
FACS	Fluorescent activated cell sorter
FBS	Fetal bovine serum
FIRS	Fetal inflammatory response syndrome
FITC	Fluorescein Isothiocyanate
GFP	Green Fluorescent Protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSP	Heat shock protein
HRP	Horse radish peroxidase
Ig	Immunoglobulin
IKB	Inhibitory Kappa Beta
IKK	IKB kinase
IL-1β	Interleukin 1 Beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IFN-γ	Interferon- Gamma
iNOS	Inducible nitric oxide synthase
JAK	Janus Kinase
JNK	C-Jun N terminal Kinase
LETZ	Loop exision of the transformation zone
LPS	Lipopolysaccharide
MAP kinase	Mitogen activated protein kinase

MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMP	Matrix metalloproteinase
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear Factor –Kappa B
mRNA	Messenger Ribonucleic acid
OCT-1	Organic Cation transporter-1
OPPTIMUM	Does progesterone prophylaxis to prevent preterm labour improve outcome?
OTR	Oxytocin receptor
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PCR	Polymerase chain reaction
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin F2-alpha
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
$15dPGJ_2$	15 deoxy-delta- Prostaglandin J_2
PHA	Phytohaemagglutinin
PI3K	Phsophoinositol-3-kinase
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PPAR-γ	Peroxisome proliferators receptor gamma
PROM	Prelabour rupture of membranes
PRR	Pattern recognition receptors
PTL	Preterm labour
PVDF	Polyvinylidene fluoride membrane
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide
STAT	Signal transducers and activators of transcription
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TBST	Tris –buffered saline-Tween
TEMED	N, N,N, N-tetramethyl-1,2-diaminoethane
Th1 and Th2	T helper cell 1 and T helper cell 2
TNF-α	Tumour Necrosis Factor
TLR	Toll like receptor

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Abstract

Introduction: Preterm labour occurs in 76,000 women in England and Wales each year and accounts for 75% of neonatal mortality. Infection and inflammation are key triggers of preterm labour. The transcription factor NF-κB plays a key regulatory role in the expression of the labour associated genes such as COX-2, prostaglandins and MMPs, as well as pro-inflammatory cytokines such as IL-8, IL-1β, IFN-γ and TNF-α. The anti-inflammatory prostaglandin 15-deoxyΔ-^{12,14} Prostaglandin J_2, a CRTH2 agonist can inhibit NF-κB and delay preterm labour in a murine model of inflammation induced preterm labour. It is well recognised that the immunology of pregnancy is biased towards a Th2 rather than Th1 response. CRTH2 agonists induce the production of the anti-inflammatory cytokines IL-4 and IL-10 in T-helper 2 cells *in vitro*, thus have potential to further alter the Th1:Th2 ratio in favour of the Th2 bias. **Aims:** This thesis examines the role of the CRTH2 receptor and its agonists in pregnancy and parturition, and its potential as a therapeutic target for the prevention of preterm labour. **Methods:** PCR, western analysis and flow cytometry were used for CRTH2 expression studies. To examine the effect of CRTH2 agonists on NF-κB activation, western analysis of p65 and phospho-p65 was performed. The effect of the agonists on interleukin profiles was demonstrated by flow cytometry. Myometrial contractility response to agonists was measured using a myograph. To determine the *in vivo* effect of a small molecule CRTH2 agonist on inflammation induced preterm labour, CD1 mice were given an intrauterine injection of LPS and the CRTH2 agonist. Delivery outcome and the effect on labour associated genes and interleukins were determined by PCR, western analysis and ELISA. **Results:** Amniocytes and myocytes show low CRTH2 mRNA levels however do not express the protein at detectable levels. The small molecule CRTH2 agonist Pyl A does not inhibit NF-κB in amniocytes and myocytes, indicating that the inhibition of NF-κB by $15dPGJ_2$ is independent of CRTH2. Although $15dPGJ_2$ was able to inhibit the production of pro-inflammatory cytokines in peripheral blood mononuclear cells, Pyl A was unable to alter the Th2 cytokine profile. Pyl A inhibits myometrial contractility, although in a mechanism independent of CRTH2. In the murine model of inflammation induced preterm labour fetal viability was significantly increased by Pyl A, however, preterm labour was augmented rather than prevented. **Conclusion:** $15dPGJ_2$ inhibits NF-κB and inflammation induced preterm labour in the mouse in a mechanism independent of CRTH2. Contrary to anticipated, Pyl A does not modulate the Th1:Th2 bias or delay preterm labour *in vivo*. However, $15dPGJ_2$ suppresses the Th1 response *in vitro*, thus represents a potential anti-inflammatory therapeutic target for both the prevention of preterm labour and inflammation induced brain injury.

CHAPTER 1

Introduction

1.1 Preterm labour

Incidence

Preterm labour is defined as labour occurring between the 24th and the 37th week of pregnancy. It can be further classified as extremely preterm (24-27 completed weeks), very preterm (28-31 completed weeks) and late preterm (32-36 completed weeks) [1]. Its incidence in the western world remains between 6-15% depending on the geography and demographics of the population [2]. The singleton preterm delivery rate in England over the last decade has been between 5.7 and 6.7%, with a transient increase to 7.5% between 2005 and 2006 (Figure 1.1)[3]. The incidence of extreme prematurity is around 3%, which equates to roughly 2600 of the 76,000 premature births per year in England and Wales [4].

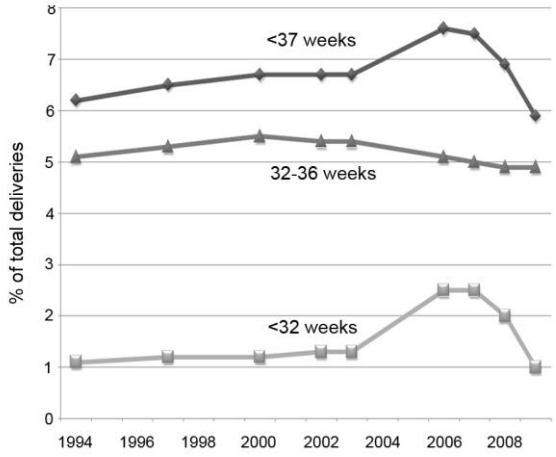


Figure 1.1 Singleton preterm delivery rates in the UK (Sykes et al 2011) [3]
Examination of singleton preterm delivery rates in the UK for the last 15 years reveal that while increasing efforts have been placed into understanding preterm labour, little, if any clinical impact has been made. Recent reviews have described an increasing rate of PTL particularly < 32 weeks. However, analysis of the latest data suggests a transient increase in the past 6 years, the cause of which is unclear.
(Data available online from NHS, http://www.ic.nhs.uk/statistics-and-data-collections, http://www.dh.gov.uk/en/Publicationsandstatistics/Statistics/index.htm).

Preterm labour is a heterogeneous condition [5], which contributes to the challenge of finding a universal preventative strategy. Despite increased research in preterm labour

(Figure 1.2), the incidence has remained unchanged over the last 2 decades, reflecting the lack of any major advances in its prevention.

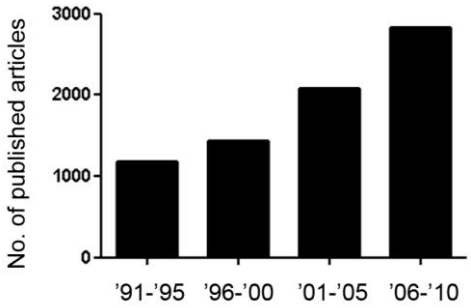


Figure 1.2 PubMed literature search for causes of preterm labour over the last 2 decades
A blast search using "preterm labour" or "preterm labor" and "causes" or "mechanisms" highlights the increasing efforts in the field to better understand the mechanisms underlying preterm labour. A search was performed from 1991-2010 in five-year intervals.

Neonatal morbidity and mortality

Around 75% of neonatal deaths can be accounted for by preterm birth [6]. 77% of neonatal deaths reported in the UK in 2006 were of babies that were born preterm [7]. Survival rates sharply decrease from 99% at 32 weeks to 1% at 23 weeks, and this is associated with a reciprocal increase in the risk of handicap [8]. 70% of long term health problems, such as neurodevelopmental impairment and respiratory disease [6] can be accounted for by preterm birth. Short-term complications of prematurity include necrotising enterocolitis, sepsis, respiratory distress, periventricular haemorrhage, and long-term sequale include chronic lung disease and neurodevelopmental impairment. As reported in the EPICure study, of those babies born before 25 weeks who survive to discharge, 23% developed severe disability by 30 months of corrected age [9]. Improvements in neonatal survival over the last few decades can be attributed to improvements in the provision of neonatal care and antenatal corticosteroids, rather than any major advances in the prevention of preterm labour.

Economic cost

The financial cost to the NHS is increased the earlier the gestation of delivery. The estimated cost of hospitalisation for a term infant is £584 compared with £317,166 for an extremely preterm neonate. The projected financial burden for all babies born preterm per year is estimated at £2.946 billion for the subsequent 18 years [10].

1.2 Physiology of term labour

During pregnancy the myometrium remains quiescent and the cervix remains long and closed. The mechanisms controlling the final signal for the onset of labour have been extensively researched; however the single final trigger is still debatable. In preparation for and during labour the myometrium, fetal membranes and cervix undergo significant biochemical and physiological changes which lead to uterine contractility, membrane rupture and cervical dilation.

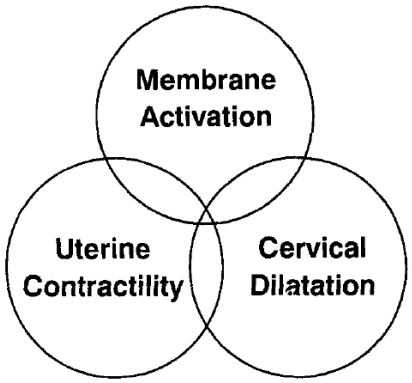


Figure 1.3 Components of the common terminal pathway of human parturition (Romero et al 1994) [11]
Labour involves many physiological changes to the myometrium, fetal membranes and the cervix which lead to uterine contractility, membrane activation and rupture, and cervical dilatation.

Membrane activation

Human amnion consists of a single layer of epithelial cells and a sub-epithelial layer of mesenchymal cells which is the major site of prostaglandin production. The final trigger for membrane rupture is likely to be controlled by different proteins to uterine contractility [12-14], since contractions do not always ensue following either natural or artificial membrane rupture. However, there is likely to be significant overlap in the biochemical events leading to membrane activation and rupture; for example, the expression of COX-2 and the production of PGE_2 both increase in the amnion at term [15-19]. Prior to the rupture of amnion, activation and weakening of the membranes occur. Collagen type I is the predominant collagen providing strength to the amnion, along with collagen IV which is a component of the basement membrane and the connection between the epithelial and mesenchymal layers [20]. Matrix metalloproteinases (MMP's) degrade collagen, with MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9 readily detected in amniochorion [21]. There is significant evidence that particularly MMP-9 is involved in remodelling of the amnion leading to weakness and susceptibility to membrane rupture [22-24]. Inflammatory cytokines also contribute to membrane activation even in term labour not complicated by chorioamnionitis. The pro-inflammatory cytokines IL-1β and IL-8 are up-regulated during labour in the amnion as are IL-6 and IL-8 in the choriodecidua [25]. Pro-inflammatory cytokines including IL-1β and IL-6 drive the production of COX-2 and PGE_2 in amnion [26-29].

Cervical ripening and dilatation

Similar to the remodelling that occurs during membrane activation prior to membrane rupture; the cervix undergoes significant remodelling in order to efface and dilate. As with the biochemical changes seen in the amnion, the cervix also remodels with the combination of both structural and inflammatory components. A reduction in collagen synthesis, along with an increase in collagenase and MMP activity contributes to cervical ripening at term [30-32]. The predominant collagen components are type I and type II, and as the cervix ripens, the concentration of collagen decreases by as much as 30-50% [33-35]. The cervix becomes infiltrated with leukocytes as part of a

physiological inflammatory reaction, which contributes to the production of pro-inflammatory cytokines [25, 36]. Cervical prostaglandins also play a key role in effacement and dilation of the cervix through several mechanisms. PGE_2 decreases the concentration of collagen [37], increases the synthesis of proteoglycans [38], enhances migration of leukocytes towards the cervix the [35] and augments the potency of pro-inflammatory chemokines [39].

Uterine contractility

The myometrium is the muscular portion of the uterus which is responsible for uterine contractility. Human myometrium is composed of smooth muscle cells arranged into bundles which are delineated by connective tissue and interspersed with microvasculature [40]. Interaction between actin and myosin in the smooth muscle cell leads to myocyte contractility [41]. This interaction is regulated by the enzyme myosin light chain kinase (MLCK) [42], which phosphorylates myosin, and can be inhibited by myosin phosphatase (MLCP). MLCK is activated by intracellular calcium and calmodulin [43]. The sources of calcium include extracellular calcium which enters through calcium channels after depolarisation of the myocyte, and the intracellular calcium stores [44]. The control of contractility is proportional to the activity ratio of the MLCK and MLCP which determine the phosphorylation status of myosin regulatory light chain [40]. Oxytocin and prostaglandin F2 alpha ($PGF_{2\alpha}$) alter the activity ratio of MLCK and MLCP which leads to the increase in sensitivity of the contractile unit to calcium [45-47]. The connexins, which are a family of gap junction proteins, also regulate contractility by controlling the interaction between cells for the propagation of action potentials. The main connexins present in term myometrium are connexin-43, connexin-40 and connexin-45 [48, 49].

Oxytocin

Oxytocin is a physiological agonist of uterine contractility. It is a nonapeptide released from the posterior pituitary and the choriodecidua [50, 51]. Oxytocin exerts its action

through the G protein coupled oxytocin receptor (OTR). Upon stimulation of the receptor, $G_{\alpha q/11}$ and $G_{\beta\gamma}$ (from $G_{\alpha i}$) G-protein subunits are released leading to activation of the phospholipase C (PLC) isoforms β1, β3 and β2, β3 respectively [52]. In turn, this leads to release of calcium stores, which leads to depolarisation of the myocyte and ultimately to myometrial contractility [40].

Prostaglandins; Prostaglandin E2 (PGE2) and Prostaglandin F2α (PGF2α)

Prostaglandins play a key role in parturition by aiding cervical ripening and myometrial contractility. Although the majority of prostaglandins are derived from the amnion, the source of prostaglandins involved in myometrial contractility is likely to be the decidua and myometrium [53]. Prostaglandins are derived from arachidonic acid from cell membrane phospholipids by the action of the phospholipase enzymes PLA_2, PLC and PLD [54]. The cyclo-oxygenase enzymes lead to the production of the intermediate PGH2, which can be converted into PGE_2, $PGF_{2\alpha}$ and also PGI_2 and PGD_2. COX-2 is the rate limiting step for prostaglandin synthesis and is the primary regulator of prostaglandin synthesis in labour [55]. Figure 1.4 summarises the pathway of prostaglandin synthesis [56].

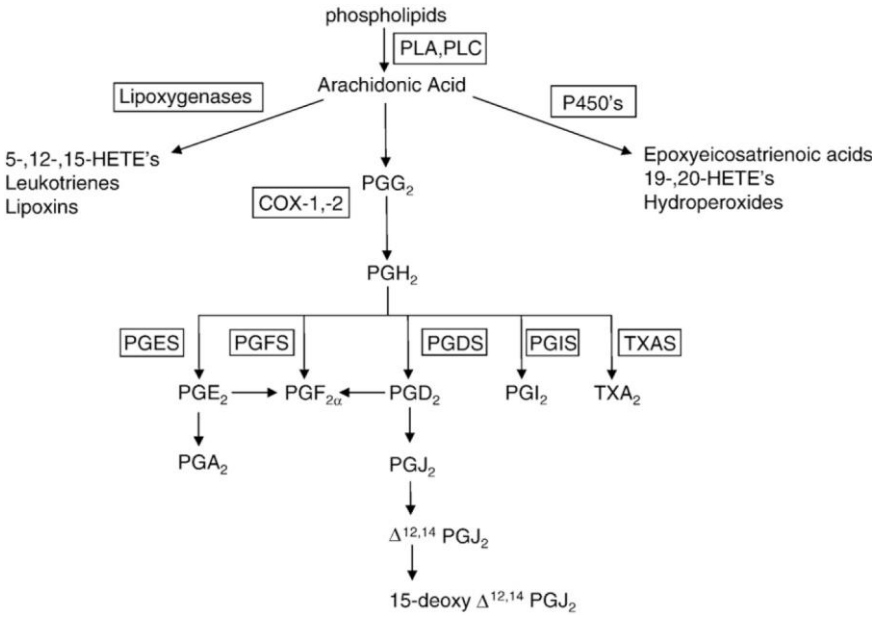


Figure 1.4 The biosynthesis of prostaglandins (Black et al 2008) [56]
This diagram shows the pathway of prostaglandin production from arachidonic acid.

The main prostaglandins contributing to uterine contractility are PGE_2 and $PGF_{2\alpha}$. PGE_2 has 4 receptors with the EP_1 ($G_{\alpha q}$) and EP_3 ($G_{\alpha i}$) being pro- contractile and EP_2 and EP_4 (both $G_{\alpha s}$) being considered as relaxatory [57-60]. Upon stimulation of the EP_1 and EP_3 receptors, PGE_2 leads to calcium release via inositol triphosphate (InsP3) and inhibition of adenylate cyclase and cAMP respectively [40]. Recent data suggests however, that spontaneous and PGE_2-induced myometrial contractility is via the EP_3 rather than the EP_1 receptor [61]. $PGF_{2\alpha}$ stimulates myometrial contractility via the FP receptor, which is likely to be via an increase in intracellular calcium mobilisation [62].

1.3 Pathophysiology of preterm labour

Approximately 30% of preterm births are due to elective deliveries for reasons such as pre-eclampsia and intrauterine growth retardation [63]. The remainder are spontaneous labours. Preterm labour is a heterogeneous condition, which results from many contributing factors and is often referred to as the "preterm labour syndrome". The main aetiological factors involved in the pathophysiology of preterm labour are vascular disorders, structural factors such as stretch or cervical or uterine disorders, but most commonly infection and inflammation. However, a clinical spectrum exists that covers all, and women can present with all or one of the following manifestations as shown in Figure 1.5.

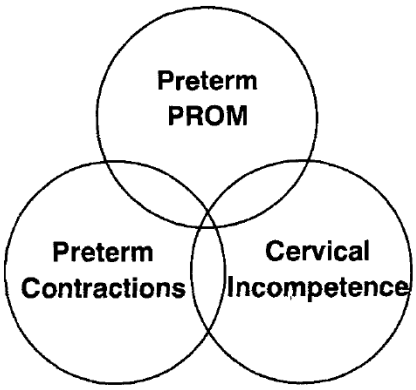


Figure 1.5 Components of the clinical manifestations of premature activation of the terminal pathways involved in human parturition (Romero et al 1994) [11]
Premature activation of membranes leads to preterm rupture of membranes, whereas early and asymptomatic dilatation of the cervix is seen in women with cervical insufficiency. Premature uterine contractility is present in many women who present in threatened preterm, labour.

Vascular disorders

Uteroplacental ischaemia and placental abruption

The most common pathological features in the placenta of women who show no signs of inflammation who deliver preterm are maternal and fetal vascular lesions [5]. Vascular lesions in the decidual vessels attached to the placenta are associated with a mean odds ratio of 3.8 for delivering preterm with intact membranes, and an odds ratio of 4 with premature rupture of membranes [64]. Placental abruption is also more commonly seen in women delivering preterm than at term [65-67]. The rennin-angiotensin system is a possible mechanism of ischemia induced preterm labour [68, 69] since angiotensin II can induce myometrial contractility both directly [70] and indirectly via the production of prostaglandins [71]. The presence of release of thrombin with decidual necrosis and haemorrhage can also activate labour, with *in vitro* studies showing a thrombin induced dose-dependent increase in myometrial contractility [72]. Thrombin is also capable of inducing an inflammatory response by activating neutrophils, chemoattraction of monocytes and by aiding lymphocyte proliferation [73].

Structural factors

Cervical insufficiency

Cervical insufficiency is defined by the inability to retain a pregnancy as a result of painless dilatation of the cervix. The incidence of true cervical insufficiency is 1% [74]. The cause of an inherently insufficient cervix is unknown, and is usually diagnosed retrospectively after a mid trimester loss or early preterm delivery in the absence of painful contractions. However, a weakened cervix can also be a result of direct cervical trauma from multiple dilations following several surgical terminations or cone biopsies/LETZs [75, 76]. Exposure to diethylstilbestrol in utero is also thought to result in a weakened cervix [77]. Inflammation and infection are also associated with cervical

insufficiency since a shortened cervix fails to effectively maintain the barrier to ascending infection [74].

Uterine anomalies

The presence of a uterine anomaly has an incidence of 0.5% [78] and is a recognized risk factor for preterm birth. Uterine didelphys [79], a unicornuate uterus[80] and a septate uterus [81] can lead to an increased rate of preterm labour, although the mechanism for this is not fully understood.

Uterine stretch

Women with multiple pregnancies or polyhydramnios have a higher rate of preterm delivery. This has directed many studies to investigate a role of uterine over distension and stretch in preterm labour. *In vitro* studies have confirmed that stretch of both amniocytes and myocytes can lead to the expression of labour associated genes such as prostaglandin E2, COX-2, and the oxytocin receptor [82-84].

Infection and Inflammation

A long recognised correlation between intrauterine infection and preterm birth exists. Goldenburg et al discovered that 80% of women delivering before 30 weeks had evidence of infection in the amniotic fluid and/or fetal membranes compared with only 30% of women delivering after 37 weeks [63]. Specifically in the amniotic fluid microbial invasion occurs in only 1% of women not in labour at term [85]. In comparison, 12.8% of women with intact membranes in labour have evidence of microbial invasion [86], and as many as 75% of women in labour with preterm premature rupture of membranes [87]. The most common microorganisms found in amniotic fluid are the genital mycoplasmas including *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Streptococcus agalactiae, Escherichia coli*, and *Gardnerella*

vaginalis [88, 89]. It is likely that ascending infection from the vaginal and cervix is the most common source of intrauterine infection. Other routes include haematogenous spread via the placenta, retrograde seeding from the peritoneal cavity via the fallopian tubes and introduction via invasive procedures such as amniocentesis, as shown in Figure 1.6.

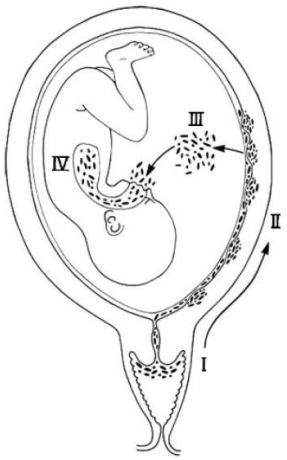


Figure 1.6 The proposed routes of transmission of microbes causing intrauterine infection. Adapted from Romero et al 1988 [90]
I: ascending from the vagina and cervix, II: transplacental, III: retrograde seeding from the peritoneum, IV: invasive procedures.

The presence of microorganisms results in the recruitment of leukocytes leading to an inflammatory response. Gram negative bacteria contain endotoxins, whereas gram positive bacteria contain peptidoglycans and lipoteichoic acid, and mycoplasmas contain lipoglycans [73]. These components are recognised by pattern recognition receptors (PRRs) which further enhance the inflammatory response upon activation. As part of the inflammatory response, pro-inflammatory cytokines are produced and this leads to activation of a biochemical cascade; including the production of pro-labour proteins such as prostaglandins [91] required for uterine contractility and cervical ripening and MMP's [92, 93] which are important in membrane rupture.

As revealed by an interrogation of the Pubmed literature, increasing research efforts are being expended on deducing the role that inflammation plays in preterm birth (see Figure 1.7) [3]. Evidence has emerged that inflammation in the absence of infection can be sufficient to cause preterm labour [94]. There is also significant evidence that

inflammation leads to an increase in neonatal morbidity regardless of an absence of intrauterine infection [95, 96].

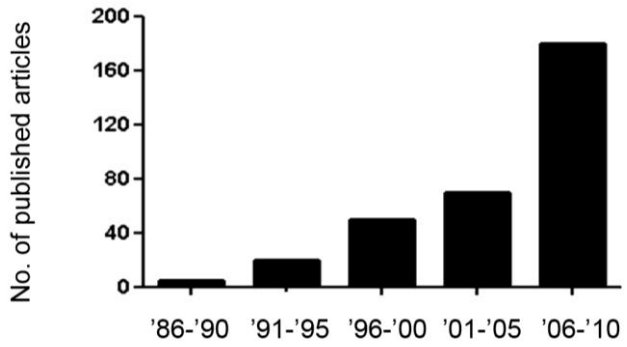


Figure 1.7 PubMed literature search for causes of preterm labour over the last 2 decades (Sykes et al 2011) [3]
A blast search using "preterm labour" or "preterm labor" and "inflammation" highlights the increasing increased awareness and efforts to better understand the role of inflammation in preterm labour. The search was performed from 1991-2010 in five-year intervals.

The fetal inflammatory response syndrome refers to the fetal response to the presence of maternal inflammation/infection [97]. The fetus mounts an inflammatory response, which was first classified as an elevated plasma IL-6 and CRP [98, 99]. Subsequently it has also been found to be associated with activation of neutrophils and monocytes [100], and a polarised Th1 response characterised by an increase in IFN-γ producing cells [101]. The fetal inflammatory response syndrome is an independent risk factor for severe neonatal morbidity due to the potential involvement of several target organs [97], as summarised by Figure 1.8.

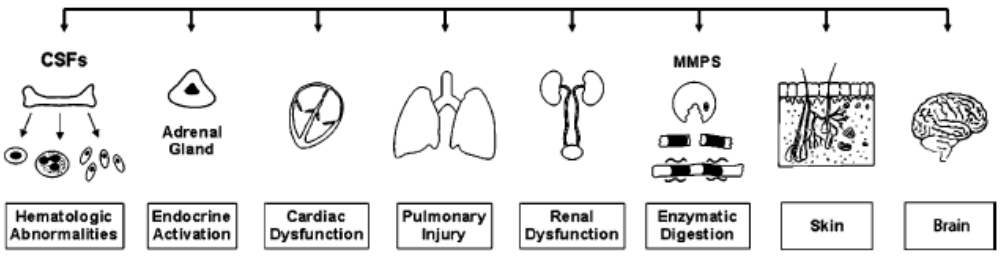


Figure 1.8 Target organs of the fetal inflammatory response syndrome (Gotsch et al 2007) [97]

1.4 The management of preterm labour

Presentation and risk factors

Preterm labour can present with preterm rupture of membranes, cervical dilation and/or uterine contractions. Symptoms of threatened preterm labour increases the risk of early delivery, yet in 70% of cases the pregnancy continues until term [102]. Women in established preterm labour present with cervical dilation in the presence of regular contractions, (with the exception of women with cervical incompetence, where cervical dilation is painless). Known risk factors for preterm labour include a history of prior mid trimester loss or preterm labour [103], women with multiple pregnancies [104], previous cervical treatment [105] and uterine anomalies [80].

Screening

Cervical length screening can be adopted in women at high risk of preterm labour. The shorter the cervix, the higher the risk is of preterm labour. A cervical length of <25mm in asymptomatic high risk women has a positive predictive value of 70% for delivery <35 weeks if detected at 14-18 weeks, and 40% if detected at 18-22 weeks [106]. In women presenting with threatened preterm labour a recent meta analysis concluded a cut off of 15mm gives a positive predictive value 60% and negative predictive value of 96% of delivery within 1 week if presenting under 34 weeks of gestation [107].

Collection of fetal fibronectin from the posterior fornix is also commonly used as a screening tool both in asymptomatic and symptomatic women. Fetal fibronectin is an extracellular matrix glycoprotein secreted by the chorion. If any biochemical disruption to the fetal membranes occurs, this glycoprotein will be detected in the high vagina. A negative result places both asymptomatic and symptomatic women at low risk of preterm labour. A systematic review of 26,876 cases revealed a likelihood ratio for

positive results of 4.01 (95% CI 2.39-5.49) for predicting birth before 34 weeks in asymptomatic women, with a corresponding LR for negative results of 0.78 (0.72 to 0.84). In symptomatic women the LR for a positive result was 5.42 (95% CI 4.36-6.74) for delivery within 7-10 days, and 0.25 (95% CI 0.2-0.31) for a negative result [108]. Cost savings are seen with the introduction of fetal fibronectin testing into clinical practice as a result of a reduction in hospitalisation, administration of corticosteroids and the reduction of *in utero* transfers to tertiary referral centres in women with a negative result [109].

Prevention

Cerclage

Cervical cerclage for cervical insufficiency was first described by Shirodkar in 1955 [110], and later by McDonald in 1957 [111]. Both techniques provide structural support for a weakened cervix, but may also improve the cervical immunological barrier by improving retention of the mucous plug, and preventing ascending infection by maintaining cervical length. Cervical cerclage can be placed electively in women with a strong history suggestive of cervical insufficiency, or can be placed if the cervix becomes short as detected by ultrasound. A multicentre randomized trial of history-indicated cervical cerclage in women with 3 or more mid-trimester losses of preterm births showed a reduction in the risk of PTB before 33 weeks from 32 to 15% [112]. A meta-analysis of 208 women at high risk of preterm labour with a short cervix (<25mm), concluded a statistically significant reduction in rate of preterm delivery <35 weeks with cerclage placement (relative risk 0.61; 95 % CI 0.4-0.92) [113]. There is also some evidence that cervical cerclage in women at high risk results in a statistically significant reduction of a composite of perinatal mortality and morbidity [114].

Progesterone

There have been several large trials looking at the potential benefits of vaginal progesterone to reduce the rates of preterm delivery using gestational age of delivery

as a primary outcome [115-118]. A large multicentre randomised controlled trial including mainly women without a prior history of preterm birth has shown a 45% reduction in preterm labour at <33 weeks using 90mg of vaginal progesterone in women with a short cervix of 10-20mm [118]. A composite of neonatal indices were also reduced, including a 60% reduction in respiratory distress syndrome. The multicentre trial OPPTIMUM, a randomised controlled trial of women at high risk or preterm labour is specifically powered for long-term neonatal outcome, and therefore should provide robust evidence of the effect of progesterone on neonatal morbidity and mortality.

Tocolytics

Tocolytics are aimed at suppressing uterine contractility. They include NSAIDS, beta agonists, calcium channel blockers and oxytocin receptor antagonists. Although tocolytics have been shown to increase the delay to delivery at 48 hours and 7 days [119], there is no evidence of a clear reduction in perinatal mortality or neonatal morbidity [120]. However, a short course of tocolysis for women with threatened preterm labour is given to allow time for the administration of corticosteroids to stimulate fetal maturation. It is likely that the inability of tocolytics to prevent preterm labour is partly because most women in threatened preterm labour are not destined to labour; and conversely for those women that really did need tocolysis, once uterine contractility is established it is likely that the multiple biochemical pathways involved are too advanced to be overcome by transient inhibition of the contractions. Nifedipine, (a calcium channel blocker) and Atosiban (an oxytocin antagonist) are the most commonly used tocolytics in the UK. Although there have been no direct randomised controlled trials comparing Atosiban with Nifedipine, using adjusted indirect comparisons there is a non-significant trend to an increased delay in delivery by 48 hours with Nifedipine [121], with a trend also towards an improvement in perinatal morbidity, lower side effect profile and a lower cost [122]. A recent meta-analysis reviewed 95 randomised controlled trials of tocolytic therapies including prostaglandin inhibitors, calcium channel blockers, oxytocin receptor antagonists, magnesium sulphate and beta mimetics on [123]. Maternal outcomes included a 48 hr

delay in delivery and side effects, whereas neonatal outcomes of respiratory distress syndrome and mortality were reported on. The meta-analysis concluded that concluded that prostaglandin inhibitors and calcium channel blockers had the highest probability of delaying delivery and improving neonatal and maternal outcomes.

1.5 The immunology of pregnancy, term and preterm labour

The immunology of pregnancy

The immunology of pregnancy is a complex phenomenon that involves a balance between maternal immune tolerance and suppression, often referred to as the "immune paradox of pregnancy". The fetus has been described as a semi-allogeneic graft, being a product of two histoincompatable individuals [124, 125]. This poses a challenge to the mother; to both tolerate the expression of paternal antigens, whilst maintaining the ability to reject in the case of overwhelming infection [126]. This challenge is undertaken in part by the immune system. It was first proposed by Medawar that the fetus essentially acts as an allograft that avoids rejection due to a physical separation from the mother, its own antigenic immaturity and the immunological inertness of the mother (i.e. maternal immune suppression) [127]. Highly regulated responses from both the innate and adaptive immune systems facilitate immune tolerance and immune activation when needed.

The immune system has two main defence systems which coordinate the immune response: the innate and the adaptive system. The innate immune response is a non-specific reaction towards foreign antigens, whereas the adaptive response forms an antigen specific reaction [128]. Although different immune components are involved in these systems, much overlap and cross talk exist between the two. Figure 1.9 is a pictorial representation of the key components of these systems during pregnancy.

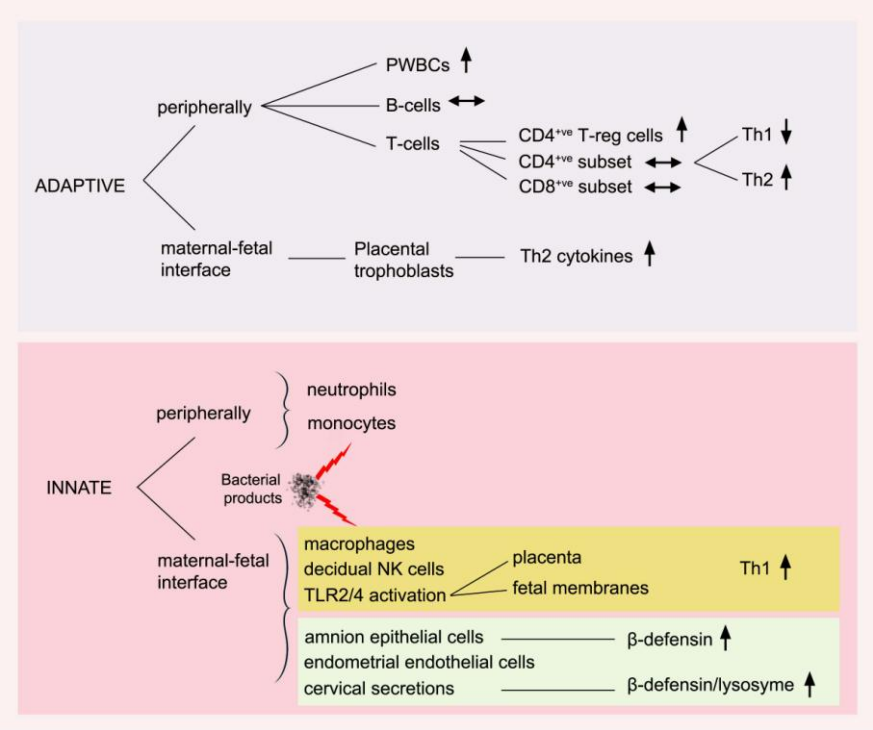


Figure 1.9 The key components of the adaptive and innate immune system in pregnancy (Sykes et al 2012) [129] Mediators of the adaptive and innate immune system work in parallel to facilitate a balance between immune tolerance of the fetus whilst maintaining the ability to mount a response against invading pathogens. PWBC: Peripheral white blood cells.

Innate immune system in pregnancy

The innate immune system the most primitive form and is the first line in the host's ability to defend against pathogens. Although it is non-specific, it is critical for the prevention of microbial invasion. The innate immune system includes physical and biochemical barriers to non-self. For example, the amnion acts as a membrane barrier to the mother throughout gestation. Also, immunologically active secretions such as lysozymes and defensins serve as biochemical barriers to invasive pathogens. Cellular components of the innate immune system include neutrophils, monocytes and macrophages, which protect against pathogens by phagocytosis. The innate immune system has also evolved to recognize non-self utilising pattern recognition receptors (PRRs) [85] that recognise molecular signatures of pathogens [130]. PRRs are also capable of recognising non-infections endogenous proteins , such as Hsp70 [131].

Toll like receptors (TLRs) are a major component of pattern recognition receptors. To date 11 members have been discovered in mammals [132]. The Toll like receptors (TLRs) TLR 2 and TLR 4 are pattern recognition receptors stimulated by gram positive and gram negative bacteria respectively [133]. TLR 2 and TLR 4 are of particular interest in relation to pregnancy and preterm labour. Both receptors are expressed on gestational tissues including trophoblasts [134], amnion epithelial cells, [135] and the myometrium [136]. The expression of these receptors is increased in term labour in amnion and in myometrium [135, 136]. An important activator downstream of TLRs is NF-κB which leads to the production of immune response proteins such as cytokines and chemokines [137].

Adaptive immune system in pregnancy

In contrast to the innate immune response, the adaptive immune response is a more specific response. The adaptive immune system relies upon B and T lymphocytes, which are responsible for the humoral and cellular responses respectively [138]. B cells differentiate into plasma cells which then secrete antibodies. Antibodies work in concert with the rest of the immune system by activating complement and immune cells including macrophages and neutrophils to target pathogens [139]. T cells ($CD3^+$) can be subdivided into T helper cells (Th cells) ($CD4^+$) and cytotoxic T cells (Tc cells) ($CD8^+$), with Th cells being subdivided further into Th1 and Th2 cells depending on the cytokines they produce. The cytokines produced by T helper cells play a central role in the protection against pathogens, but also play a role in allergy and autoimmune conditions. The cytotoxic T cells generally target virally infected cells resulting in cell death [139].

The hypothesis of immune suppression during pregnancy, proposed originally by Medawar [140] has been superseded by a more complex hypothesis where a change in the ratio and function of maternal leukocytes occurs rather than complete suppression. For example there is an increase in the total peripheral white cell count from the early stages of pregnancy with no change in the CD4 and CD8 counts (T

helper and cytotoxic T cells respectively) [141]. However, within the T helper CD4 positive population an increase in T regulatory cells is seen in pregnancy [142].

An important component of the adaptation of the immune system in pregnancy is the regulation of specific cytokine production [143]. Pregnancy is largely represented by a shift towards Th2 activity [143] [128] [144, 145]. During pregnancy, peripheral blood lymphocytes show an increase in mRNA of the prototypical Th2 interleukin IL-4 and lower mRNA levels of the prototypical Th1 interleukin interferon gamma (IFN-γ) [146]. Also, *in vitro* stimulation of peripheral lymphocytes in pregnant women display increased production of IL-4 and reduced production of IFN-γ compared with non pregnant controls [147]. The Th2 cytokine bias is likely to be more pronounced at the maternal fetal interface [148]. Decidual leukocytes produce IL-4 and IL-10 [149], with Th2 cytokines also being produced by non-immune cells including trophoblasts and decidual cells to contribute to the Th2 cytokine predominance [150] [151]. Th2 interleukins are capable of suppressing the Th1 interleukins as a form of regulating the Th1:Th2 ratio [152], and can also suppress the production of IL-8 and prostaglandins [153]. In turn this offers a mechanism by which the Th1:Th2 ratio can contribute to pregnancy maintenance along with immune tolerance of the fetus as an allograft.

The immunology of term labour

Term labour is seen as an inflammatory event with physiological activation of the immune system. Local inflammation in the fetal membranes, cervix and uterus plays a key role in the cascades leading to both term and preterm birth [73].

Immune activation in the decidua and fetal membranes at the maternal fetal interface

In preparation for delivery, the fetal membranes and decidua undergo complex biochemical events leading to the physical separation of the membranes from the decidua and their rupture. The decidua forms the interface between the maternal and fetal tissues, and it was proposed by Casey and MacDonald that it may also regulate maternal-fetal immuno-tolerance during pregnancy [154]. In the amnion, the pro-inflammatory cytokines IL-1β and IL-8 are up regulated during labour as are IL-6 and IL-8 in the choriodecidua [25]. IL-1β stimulates COX-2 production, which in turn leads to the production of PGE_2 in the amnion. The amnion is a major source of prostaglandins which are required for uterine contractility and cervical ripening [26].

Immune activation in the cervix

At the beginning of parturition, the cervix undergoes a major remodelling process referred to as “ripening”. This occurs independently, yet concurrently with the activation of the fetal membranes and myometrium. Cervical ripening involves activation of an inflammatory cascade that involves the release of pro-inflammatory cytokines and the infiltration of leukocytes [25, 155]. IL-8 is a chemokine produced by many immune cells but primarily macrophages and monocytes [156] and is increased in the cervix with the onset of labour [157]. The invading inflammatory cells contribute to the release matrix metalloproteinases (MMPs) that, along with activated collagenases, degrade the structural connective tissue and collagen fibres of the cervix [32].

Immune activation in the uterus

During term labour, there is a significant influx of inflammatory cells including lymphocytes, macrophages and neutrophils [155]. These infiltrating leukocytes contribute to the source of pro-inflammatory interleukins and prostaglandins which are involved in term labour. Immunohistochemistry demonstrates that the immune cells are the likely origin of the majority of pro-inflammatory cytokines within the myometrium during labour [158]. From this study IL-1β was predominantly located in leukocytes, with weak staining in myocytes, whereas IL-6 and TNF-α were restricted to leukocytes. A number of studies have also identified increases of IL-8 in the myometrium with the onset of labour [157-159].

There has been some debate as to whether the activation of inflammatory pathways in the myometrium is a cause or consequence of labour. Recent work by Bisits and colleagues used mathematical modelling to distinguish which pathway of myometrial activation was most likely to occur first [160]. Of the three pathways investigated; inflammatory stimulation, functional progesterone withdrawal or oxytocin receptor activation, data provided from quantitative RT-PCR on key genes of each pathway strongly supported inflammation as the primary driver for activation of the myometrium.

The immunology of preterm labour

Although the initial triggers of preterm and term labour may well differ, with term labour seen as physiological and preterm labour seen as a pathological condition, the terminal events leading to delivery of the fetus are common to both [161]. The inflammatory response present in term labour also exists in preterm labour, except with a greater rate of association with infection, with approximately 30% of preterm deliveries being associated with an infective process [162]. The earlier the gestation of preterm labour, the higher the association with infection is. 80-85% of women in preterm labour < 28 weeks show evidence of intrauterine infection, with >90% around 24 weeks, compared with 15% of women delivering late preterm at 34-36 weeks [163].

The mechanism by which infection is able to trigger the parturition cascade is undoubtedly linked to an integrated network of interactions between the pro inflammatory cytokines and prostaglandins. In response to intrauterine infection, infiltrating leukocytes and decidual cells are capable of secreting the proinflammatory cytokines IL-1, IL-6, IL-8 and TNF-α [90, 154, 164] These act in concert to stimulate uterine contractions, and remodel both the cervix and the fetal membranes leading to cervical dilation and rupture of membranes respectively [162, 163].

Although microbial infection is a common cause of inflammation, the presence of infection is not essential for inflammation driven preterm labour. There is a causal role for cytokines in labour even in the absence of infection [165]. We have previously shown that pregnancies without clinical overt signs of infection still show increased levels of IL-8 in the myometrium [166]. Moreover, a recent genomic study of amnion tissues from women in spontaneous labor without histological chorioamnionitis revealed that these tissues present an acute inflammation gene expression signature [167].

Immune activation in the decidua and fetal membranes at the maternal fetal interface

In response to intrauterine infection, infiltrating leukocytes and decidual cells are capable of secreting the pro-inflammatory cytokines IL-1, IL-6, IL-8 and TNF-α [90, 154, 164]. These cytokines induce prostaglandin production in the fetal membranes and decidua leading to initiation of parturition [168, 169]. IL-6 is a pro-inflammatory cytokine and a major mediator of host response to inflammation and infection [170]. IL-6 is significantly increased in amnion and choriodecidua in women with preterm delivery compared with term delivery [171] and is the most sensitive and specific indicator of infection-associated preterm labour [11, 172]. Detection of increased in IL-6 in amniotic fluid and cervicovaginal fluid is an independent risk factor for preterm labour and neonatal morbidity [173], including cerebral palsy [174] and bronchopulmonary dysplasia [175]. IL-8 mRNA expression is also increased in preterm labour by more than 50-fold and more than 1000-fold in preterm labour with evidence of chorioamnionitis [176].

Immune activation in the cervix

Premature ripening of the cervix usually occurs in conjunction with uterine contraction, but in the case of cervical insufficiency is classically painless. The cervix plays not only a mechanical role but also a biochemical role in the prevention of ascending infection as demonstrated by the higher incidence of microbial invasion of the amniotic fluid in women with a shorter cervix [177, 178]. Consistent with the protective role of the immune response within the cervix, women who are destined to deliver preterm have a lower number of macrophages antenatally compared with women delivering at term [179]. In contrast, an exaggerated immune response in the cervix is also associated with preterm labour. Both IL-6 and IL-8 levels are significantly higher in cervical secretions of women destined to deliver preterm [180]. Although the specific role of IL-6 in inducing preterm birth is not fully understood, the function of IL-8 is by chemoattracting leukocytes such as neutrophils that contribute to cervical ripening by producing MMPs [181].

Immune activation in the uterus

Preterm labour as well as term labour is characterised by marked leukocyte invasion into the myometrium [155]. Pro-inflammatory cytokines such as IL-8 and IL-1β are increased in the myometrium in women in preterm labour compared to women not in labour [182]. As well as pro-inflammatory cytokines there is also increased expression of chemokines including CCL5, CXCL5, and CCL20 [183]. As with term labour, pro-inflammatory cytokines lead to the increased production of prostaglandins in the myometrium. IL-1β and TNF-α stimulate arachidonic acid release thereby activating phospholipid metabolism [184] and increasing the production of prostaglandins [185, 186]. IL-1β up regulates COX-2 expression [187] stimulating the production of PGE_2 [188] and increases oxytocin secretion both of which contribute to uterine contractility [189].

1.6 The Th1 and Th2 interleukins in pregnancy, term and preterm labour

The Th1/Th2 cytokines

Th1 and Th2 cells originate from undifferentiated Th0 cells under the influence of their prototypical cytokines IFN-γ and IL-4 respectively. Th1 and Th2 cells differ in the cytokines that they produce and their function. Th1 cells produce pro-inflammatory cytokines; IFN-γ [190], IL-2 [191], and TNF-α [192], and are the major effectors of phagocyte-mediated host defence which protect against intracellular pathogens [191, 193, 194]. Th2 cells produce a mixture of pro- and anti-inflammatory cytokines; IL-4, IL-5, IL-13, IL-10 [190] and IL-6. IL-4 and IL-10 are considered to be anti-inflammatory cytokines [195] and IL-6 is considered to be pro-inflammatory [196]. Although IL-10 and IL-6 are typically classed as Th2 cytokines [144, 197-201], IL-10 is also produced by Th1 cells, macrophages and B cells [202, 203], whereas IL-6 is also produced by macrophages, fibroblasts and B cells [204].

The Th2 cytokines have several functions and are commonly associated with facilitating antibody responses [205]. IL-4 stimulates IgE and IgG_1 antibody production [206]. IL-5 promotes the growth and differentiation of eosinophils whereas IL-13 and IL-10 inhibit the activity of macrophages [206]. Transcriptional regulation of the Th1 cell cytokine production is mainly by T-bet and STAT-4 [148], whereas the predominant Th2 cytokine IL-4 is regulated by STAT-6, c-maf, GATA-3 and NFAT [207]. Although the Th1 and Th2 responses may be described as discrete responses, there is considerable cross talk and overlap between the functions of the T helper cells. For example the Th1 cytokines can promote the production of complement-fixing antibodies involved in antibody dependent cell cytotoxicity [208]. A balance between Th1 and Th2 exists rather than complete suppression of one over the other. Figure 1.10 (from O'Garra et al) summarises the cytokines secreted by the Th1 and Th2 cells, their function, and the internal regulation that exists to maintain the Th1/Th2 interleukin profile [194].

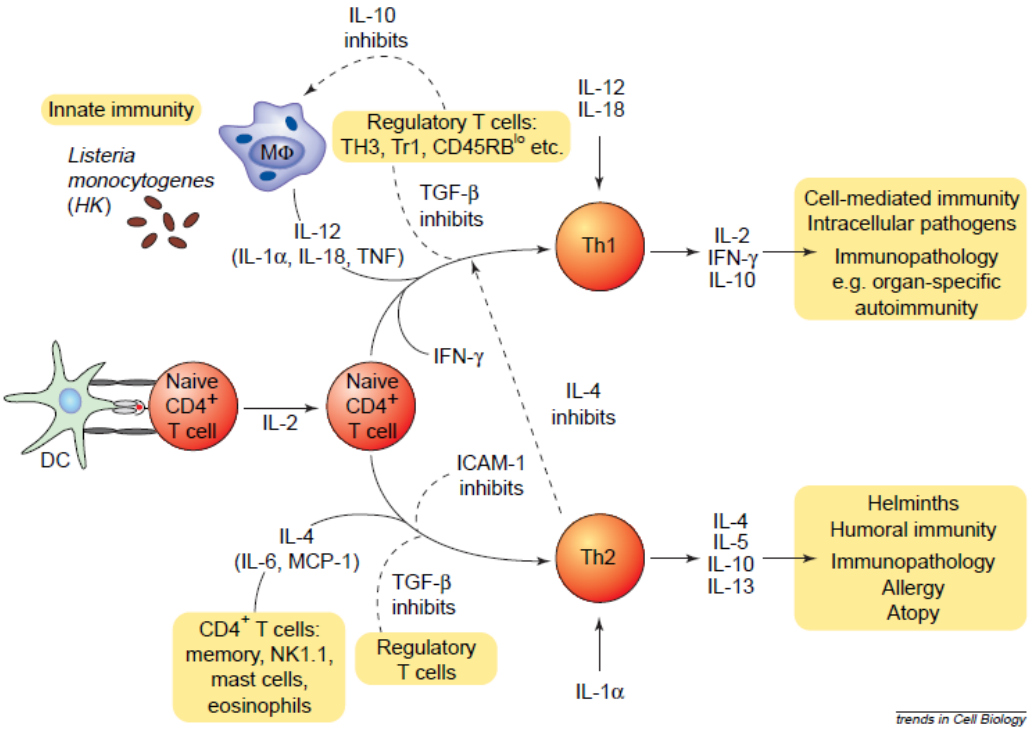


Figure 1.10 The regulation of T helper cell responses (O'Garra et al 2000)
Th1 development is dependent on IL-12 and IFN-γ and is required mainly for cell-mediated immunity of the adaptive immune system. Th1 development is antagonised directly by IL-4 and indirectly by IL-10. Th1 development is dependent on IL-4 and is important for immune tolerance, allergy and atopy.

The Th1/Th2 cytokines and pregnancy maintenance

Recognition of a bias towards the Th2 cytokine profile during pregnancy has been well documented. Wegmann and colleagues were the first group to hypothesise that Th2 predominance together with downregulation of the Th1 response is important for pregnancy maintenance [209]. This has subsequently been supported by both murine studies and the clinical course of Th2 and Th1 based conditions in pregnancy. For example, IL-2, IFN-γ and TNF-α induce miscarriage in mice, which can be reversed by inhibitors of the Th1 cytokines, or by administering the anti-inflammatory Th2 cytokine IL-10 [210, 211]. Th1 mediated autoimmune conditions like rheumatoid arthritis generally improve in pregnancy [212], whereas the Th2 autoimmune spectrum tend to worsen, for example systemic lupus erythematosus [213]. The Th2:Th1 bias and thus

diminished cell-mediated immunity may be responsible for the increased susceptibility in pregnancy of conditions caused by intracellular pathogenesis such as influenza, and *listeria monocytogenes* [145]. Pregnancy hormones such as progesterone [214], leukaemic inhibitory factor [215], estradiol [216] and prostaglandin D_2 (PGD_2) [217] promote the Th2 cell profile, and are likely to be in part responsible for the Th2 bias associated with pregnancy.

Peripheral blood

Several studies have reported on a Th2 bias in peripheral blood leukocytes in pregnancy. Marzi and colleagues stimulated peripheral blood mononuclear cells (PBMCs) with phytohaemagglutinin (PHA), and measured interleukin secretion by ELISA showing an increase in IL-4 and IL-10 with a reduction in IFN-γ and IL-2 in pregnancy compared with non-pregnant controls [147]. These results were reproduced by using flow cytometry by Reinhard et al in phorbol myristate acetate (PMA) stimulated cells, demonstrating an increase in intracellular IL-4 production and a reduction in intracellular IFN-γ and IL-2 in pregnancy compared with non-pregnant controls [218]. *In vivo* confirmation of this bias has since been demonstrated by PCR showing decreasing mRNA of IFN-γ through pregnancy along with a concurrent increase in IL-4 mRNA which peaks in the 7^{th} month compared with non-pregnant controls [146]. Whilst the majority of studies support this bias, there are some studies that do not consistently reproduce this bias. Shimoaka *et al.,* reported a reduction in PMA-stimulated IL-4 during pregnancy [219] while Matthiesen and colleagues presented data suggesting an increase in both IL-4 and IFN-γ secreting cells in pregnancy compared with non-pregnant controls [220, 221]. Such discrepancies may be due to characterisation of cytokine profiles in either isolated cell populations or whole blood, the gestational stage at time of blood collection, or the undiagnosed presence of infection or inflammation, confounding results.

Maternal fetal interface and non-immune cells

Local cytokine production at the maternal interface may be of greater significance than measurements obtained in the peripheral blood [193], however, access to tissue makes researching this challenging. Despite this, a number of studies have successfully shown a bias towards the Th2 cytokine profile during pregnancy. IL-4 and IL-10 production by T cells at the maternal fetal interface is associated with successful pregnancy [193]. Decidual $CD4^+$ cells from women undergoing unexplained recurrent pregnancy loss typically exhibit reduced IL-4 and IL-10 production [149]. Trophoblast, decidua and amnion all contribute to the Th2 cytokine environment by production of IL-13 [222], IL-10 [223], IL-4 and IL-6 [224, 225].

Local regulation of the Th1/Th2 to promote the Th2 bias at the maternal fetal interface is likely to occur in a number of ways. Local production of IL-4 and IL-10 inhibits the function of both Th1 cells and macrophages [226]. Co–culture of trophoblasts and T cells results in an increase in the transcription factors GATA-3 and STAT-6 (which regulate Th2 cytokine production), and a reduction in the Th1 transcription factor STAT-4 and subsequently decreased production of IFN-γ and TNF-α [227]. *In vitro* studies also show Th2 interleukins can modulate labour associated genes by inhibiting the production of the Th1 cytokine TNF-α [152], and TNF-α-induced COX-2 and/or PGE_2 synthesis in amnion-derived wish cells. Similar effects are also observed in decidual and placental cells [153, 228-230].

The Th1/Th2 cytokines and term labour

Peripheral blood

Labour is often seen as a pro-inflammatory state marking the end of the pregnancy and thus it would be anticipated that a Th1 bias exists during labour, however, there are limited studies looking at the Th1:Th2 ratio in peripheral blood cells in labour. One study has reported a reduction in serum IL-4 in the later part of labour with a peak in the cytokine TNF-α in early labour consistent with a Th1 bias over Th2 [231].

Consistent with this, an increase in IFN-γ and IL-1β in women in active labour has also been reported [232].

Maternal fetal interface and non-immune cells

There is substantial evidence that the Th1 cytokines play a role in the initiation of labour at term [192]. The importance of local rather than peripheral production of the cytokines is highlighted by their direct input into the biochemical pathways involved in parturition. TNF-α and IL-1β are both increased in amnion, amniotic fluid and decidua at term [233] and can induce PGE_2 production in amniocytes and decidual cells in *vitro* [234, 235]. Therefore, despite the pro-inflammatory nature of the Th1 cytokines they are required for successful pregnancy contributing to the physiology of term labour.

The Th1/Th2 cytokines and preterm labour

Immune and non-immune cells contribute to a cytokine-rich environment in the presence of infection and inflammation. Since preterm labour is associated with both infection and inflammation, the increase in the Th1 pro-inflammatory cytokines seen in preterm labour is not surprising. Pro-inflammatory cytokines, (typically of Th1 type), ultimately result in the production of prostaglandins and MMPs [236]. This triggers a cascade of pro-labour events including uterine contractility and fetal membrane rupture. If this cascade is activated too early in pregnancy then preterm labour can ensue.

Peripheral blood

Several studies support the hypothesis of an altered bias towards the pro-inflammatory Th1 cytokines in peripheral blood during preterm labour. Serum taken from women with preterm pre-labour rupture of membranes (pPROM) compared to women who deliver at term exhibit a higher concentration of IFN-γ, with undetectable

levels of IL-4 and IL-5 in both groups [237]. In a study of 30 women in preterm labour, mitogen and antigen stimulated PBMCs show a higher production of the pro-inflammatory cytokines IFN-γ and IL-2 favouring a Th1 response compared with controls who delivered at term [238]. Activation of the Th1 cytokines also occurs as a specific response to infection caused by intracellular bacteria, parasites and viruses [239], and thus a prominent Th1 response would be expected in infection mediated preterm labour. While the Th1 cytokine response may be suppressed by both the maternal and fetal immune system during pregnancy [240], it still maintains the capacity to mount a defensive response in the context of infection. Activated T cells drive both local and systemic cytokine production [239]. This is evident for example in neonates exposed to intrauterine infection in that there is an increased percentage of IFN-γ-producing peripheral leukocytes [241].

Maternal fetal interface and non-immune cells

The inflammatory response at the maternal fetal interface is likely to reflect the most pronounced aberrant Th1 biased profile in preterm labour. A recent meta-analysis concluded that pro-inflammatory cytokines at the maternal fetal interface play a role in the events leading to spontaneous preterm labour, while systemic inflammation does not appear to be present in asymptomatic women early on in pregnancy who then go on to deliver preterm [242].

Placentas from women following preterm delivery compared with term delivery show a bias towards the Th1 profile with significantly higher levels of IFN-γ and IL-2 as well as the Th1 inducing cytokine IL-12 [243]. Also, placentas from women delivering preterm have lower levels of the Th2 cytokines IL-4 and IL-10 than placentas from term deliveries. The Th1 cytokine TNF-α is increased in choriodecidual tissues [171] and amniotic fluid [244] in preterm labour. TNF-α is known to stimulate prostaglandin production through the TNF receptor 2, leading to uterine contractions, and is also likely to contribute to MMP-9 production leading to PROM via activation of its receptor TNF Receptor 1 (TNFR1) [92].

The source of pro-inflammatory cytokines at the maternal fetal interface in preterm labour may be from both non-immune cells and infiltrating leukocytes. Cytokine levels in placentas and fetal membranes from preterm deliveries (with and without intrauterine infection) correlate with the extent of leukocyte infiltration in fetal membranes [233]. This supports the suggestion that in the presence of infection, the primary cellular source of cytokine production in fetal membranes is likely to be infiltrating leukocytes rather than amniocytes or choriodecidual cells [233].

1.7 The role of Nuclear Factor-κB in pregnancy, term and preterm labour

Nuclear factor Kappa B (NF-κB) is a transcription factor that plays a central role in controlling the inflammatory response [161]. The NF-κB signaling pathway also plays an important role in the process of human labour [245]. Therefore targeting the inhibition of this transcription factor is an attractive therapeutic strategy for the prevention of both preterm labour and inflammation induced neurological damage associated with inflammation induced preterm labour.

The NF-κB family consists of 5 members; NF-κB1 (p105/p50), NF-κB2 (p100/52), Rel A (p65), Rel B and c-Rel [245]. They play a central role in controlling the inflammatory response, apoptosis, tissue remodelling and cell proliferation [161]. Pro-inflammatory cytokines such as IL-1β and TNF-α are both regulated by and can activate NF-κB giving rise to a positive feed forward loop and thus further activating the NF-κB regulated genes [246]. The following labour associated genes; phospholipase A2 isoenzymes (cPLA2) [247], COX-2, IL-8, IL-6, and MMPs [245] are also transcriptionally regulated by NF-κB.

NF-κB signalling

NF-κB transcription factor activity is inducible by the release of its inactive subunits in the cytoplasm for nuclear translocation. The inactive subunits of the NF-κB family are localised as homo or hetero dimers in the cytoplasm together with the NF-κB inhibitor

IκB. The IκB family has several members; IκB α, IκBβ, IκBε, IκB-γ, IkB-z, p105, p100, and Bcl3. The IκB proteins contain multiple ankyrin repeat motifs important for the maintenance of NF-κB in the cytoplasm [248], by mediating binding to the NF-κB rel homology domain (RHD) [245]. IκB degradation and the subsequent release of the NF-κB subunits is regulated by the IκB kinases (IKK). The first IKK complex identified consists of two subunits IKK-α (IKK-1) and IKK-β (IKK-2), and the regulatory component IKK-γ (also known as NEMO) [247]. IKK-β phosphorylates IκB in response to proinflammatory stimuli and stress [249]. Recently another IKK complex was been identified containing IKK- ε, which mediates activation of NF-κB by Phorbol ester [250]. Upstream kinases mediate the phosphorylation of the IKK complexes including; MAP Kinase, NF-κB-inducing kinase (NIK), NF-κB-activating kinase, (NAK), receptor activator for NF-κB and its ligand (RANK and RANKL), mitogen activated protein kinase/ERK kinase kinase 1, 2 and 3 (MEKK1,2,3) [251, 252]. Once activated the IKK proteins phosphorylate IκB at specific serine residues [253] which result in the attachment of ubiquitin residues [254]. Ubiquitated IκB is then cleaved by the 26S prosteosome [255] which allows the NF-κB subunit to translocate into the nucleus to bind specifically to DNA response elements.

Three signalling pathways are involved in NF-κB activation; the atypical, non-canonical and the canonical pathways. DNA damage activates the atypical pathway and results in phosphorylation of IκBα by p38 induced casein kinase-2 (CK-2). The non-canonical pathway is activated by lymphotoxin β or B-cell activating factor (BAFF) and upon activation IKKα phosphorylates p100 generating p52, releasing RelB/p52 dimers for translocation into the nucleus. More relevant to the work in this thesis is the canonical pathway, which is activated by lipopolysaccharide, TNF-α and IL-1β. IκBα is phosphorylated by IκB kinase (IKK) and is degraded to release p50/p65 dimers for translocation into the nucleus where it functions as a transcriptional activator of labour associated genes and pro-inflammatory cytokines. Figure 1.11 summarises the pathways involved in NF-κB activation from Lindstrom et al [245].

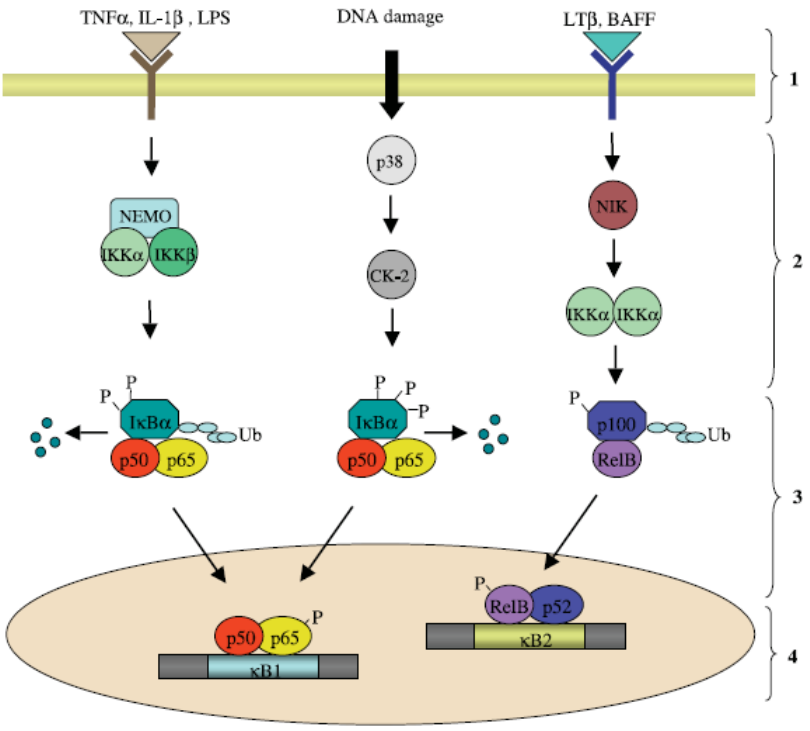


Figure 1.11 Schematic illustration of the three signalling pathways of NF-κB activation. (Lindstrom et al 2005) [245]
The canonical pathway is activated by pro-inflammatory cytokines, the atypical pathway by DNA damage, and the non-canonical pathway by lymphotoxin β and BAFF. Inactive subunits reside in the cytoplasm attached to the IKB inhibitory proteins. Once phosphorylated the active subunits translocate into the nucleus.

Peripheral blood

NF-κB is known to play a key regulatory role in controlling the Th1 immune response by modulating the expression of Th1 cytokines [256]. NF-κB activity in PBMCs has been shown to be suppressed in pregnancy compared with non-pregnant controls [257]. The reduction in Th1 cytokine production in PBMCs and specifically T cells during pregnancy is likely to be as a result of this down regulation of the NF-κB/IκB signalling [240, 258]. It is not known whether NF-κB activity is increased in PBMCs in term or preterm labour.

Amnion

NF-κB plays a key role in the regulation of prostaglandin synthesis in labour through more than one mechanism. NF-κB regulates the production of cytosolic phospholipases A2 isoenzymes (cPLA2) in human gestational tissues. These enzymes generate arachidonic acid from glycerophospholipids [247]. Secondly NF-κB is a transcriptional regulator of COX-2 in amnion epithelial cells. COX-2 contains two NF-κB response elements in its promoter region, and site directed mutagenesis of these regions represses the activity of the COX-2 promoter reporter construct in amnion epithelial cells [259].

NF-κB activity is increased in amnion during labour [259], with term pre-labour samples showing varying degrees of p65 expression at different time points, which is likely to reflect the physiological transition that precedes parturition [260]. Pro-inflammatory cytokines such as IL-1β, IL-8 and IL-6 are elevated in the amnion and choriodecidua of women in term and preterm labour choriodecidua [233]. These interleukins are regulated by NF-κB [161]. Inhibition NF-κB can result in the inhibition of LPS induced pro-inflammatory cytokines including IL-8 and TNF-α in amnion and choriodecidua [261].

Myometrium

Chapman and colleagues have previously reported the presence of all subunits except Rel B and p52 in the myometrium. Although total p65 was found to be reduced in lower segment myometrium in labouring women, DNA binding activity was actually increased [262]. In contrast expression of the p65 subunit shows a marked increase in the fundal myometrium during labour [263]. This is consistent with the concept that the fundal myometrium is more important than the lower segment for the propagation of uterine contractility. These findings are consistent in animal studies

with NF-κB activity increased in the pregnant mouse uterus [264] and the fundal myometrium in baboons at term [265].

NF-κB plays a key role in the transcriptional regulation of contractile associated genes in the myometrium at the time of labour. The oxytocin receptor (OTR) [266] and COX-2 [259] genes contain NF-κB response elements in their promoter regions. OTR mediates oxytocin induced myometrial contractions through activation of phospholipase C and downstream calcium release from intracellular stores [267]. The COX-2 enzyme is the rate-limiting step for prostaglandin synthesis, which is responsible for uterine contractions. COX-2 is elevated in lower segment myometrium towards term [268], and in labour verses non labour samples [269], as is IL-1β at the onset of labour [270]. NF-κB is required for IL-1β induced expression of COX-2 in myocytes [271], representing another mechanism of NF-κB mediated inhibition of COX-2.

NF-κB also regulates the pro-inflammatory cytokines IL-8 and IL-6 in the myometrium, both of which contain NF-κB recognition elements within their promoter regions [245]. IL-8 is dramatically increased in labouring myometrium [272], and is detected in the supernatant of non-stimulated and IL-1β stimulated myocytes *in vitro* [273]. Microarray analysis on myocytes overexpressing p65 shows that NF-κB regulates genes principally related to immunity and inflammation in these “non-immune” cells [274]. This provides evidence that the myometrium is capable of functioning as a distinct immune organ and that inflammatory pathways are critical components during parturition [159].

Cervix

NF-κB is also involved in transcriptional regulation of matrix metalloproteinases (MMPs), including MMP-9, which are required for remodelling of the extracellular matrix [269], leading to both cervical ripening and fetal membrane rupture.

NF-κB activity in preterm labour

As already highlighted, the terminal biochemical pathways that are activated in term labour are also activated in preterm labour. Premature enhanced activation of NF-κB can be triggered by the presence of infection and inflammation. For example, a positive feed forward loop also exists from the activation of NF-κB by the pro-inflammatory cytokines such as TNF-α and IL-1β and subsequently leading to their own transcriptional activation [187, 245, 275].

NF-κB inhibition is therefore an attractive target for the prevention of preterm labour since, as well as inhibiting labor-associated genes involved in uterine contractility, cervical ripening and fetal membrane rupture, it would also inhibit the pro-inflammatory cytokine production. The outcome of which would be to both inhibit preterm labour and the neurological damage associated with inflammation induced preterm labour.

1.8 15-deoxy Δ ^{12,14} Prostaglandin J2 (15dPGJ$_2$)

15-deoxy-Δ 12,14-Prostaglandin J2 (15dPGJ$_2$) is an anti-inflammatory cyclopentenone prostaglandin. It exerts anti-inflammatory effects through inhibiting the activation of NF-κB [276]. 15dPGJ$_2$ inhibits NF-κB and COX-2 in both in human myocytes and amniocytes *in vitro* [271]. It is also capable of delaying inflammation induced preterm labour in the mouse *in vivo* [277]. In addition it reduces pup mortality from 75% to 5% and inhibits pup brain NF-κB and JNK activation. 15dPGJ$_2$ is an endogenous prostaglandin, and is a product of PGD$_2$ via a series of dehydration reactions, [278] as summarised in Figure 1.4. It can accumulate intracellularly, and is detected in amniotic fluid [279], placenta [280] and urine [281].

$15dPGJ_2$ has multiple cellular targets. It is a ligand with high affinity to the peroxisome proliferator receptors for example PPAR-γ [247, 282]. $15dPGJ_2$ can also act independently of the PPARγ through inhibition of different transcription factors such as NF-κB [283] AP-1 [284] and NF-AT [285, 286]. Additionally, $15dPGJ_2$ is a ligand for chemoattractant receptor-homologous molecule 2 (CRTH2) that is present on Th2 cells, eosinophils and basophils [287]. The mechanism by which $15dPGJ_2$ inhibits NF-κB in amniocytes and myocytes is not yet fully understood. It is plausible that CRTH2 may play a role in the inhibition, which warrants further investigation.

1.9 Chemoattractant receptor-homologous molecule expressed on T helper 2 cells

Chemoattractant receptor-homologous molecule expressed on T helper 2 cells (CRTH2) was first discovered by Nagata et al [287] in 1999 as a second receptor to Prostaglandin D_2 (PGD_2). Other names for CRTH2 include DP2, GPR44 and CD294. The human CRTH2 gene is located at chromosome 11q12, its transcript length is 2.9kb and it has two exons and one intron [288]. As a typical G Protein coupled receptor it has seven putative transmembrane domains [289], with two glycosylation sites at its N terminus, and a long cytoplasmic tail with multiple phosphorylation sites [287]. CRTH2 is coupled to the G_i type G proteins enabling it to inhibit adenylate cyclase and thus decrease cAMP production.

Distribution of CRTH2 expression

CRTH2 is expressed on Th2 cells, eosinophils, basophils, monocytes and dendritic cells [289, 290]. The T cells that express CRTH2 secrete interleukins typical of Th2 cells; IL-4, IL-5, IL-13 [291]. These T cells include Th2 cells and Th2-type $CD8^+$ T cells (Tc2). In cultured $CD4^+$ T cells CRTH2 expression is increased by IL-4, and inhibited by the Th1

cytokine IL-12 [291, 292]. CRTH2 is not expressed on human Th1 cells, and this has led to CRTH2 becoming a cell differentiation marker of Th2 cells [291].

Studies of the expression of CRTH2 on non-immune cells are limited, probably due to the lack of antibodies available for detection using techniques other than FACS. Human bronchial epithelial cells express CRTH2 as demonstrated by FACS analysis by Chiba et al [293]. Other cell types reported to express CRTH2 include chondrocytes [294], osteoblasts [295] and keratinocytes [296]. Nagata et al have demonstrated CRTH2 mRNA in many human tissues; with high expression in heart, brain, stomach, small intestine, liver, thymus, and placenta and moderate expression in the colon, uterus, pancreas, prostate and peripheral blood leukocytes [287]. Although, it is possible that the expression demonstrated in these tissues is through the presence of infiltrating leukocytes.

Function of CRTH2

As alluded to by its name, upon stimulation CRTH2 leads to chemotaxis of leukocytes including Th2 cells, basophils and eosinophils [291, 297, 298] . Up regulation of cell adhesion molecules such as CD11b in eosinophils and basophils are likely to aid migration of the cells to target locations [298]. CRTH2 stimulation also leads to up regulation of CD40 in Th2 cells which is needed for T cell to B cell interaction, thus implying a functional role in aiding leukocyte effector response [287]. It is likely that CRTH2 plays a functional role in maintaining a Th2 cytokine bias since CRTH2 agonists result in a dose dependent increase in the production of IL-4, IL-5, IL-13 and IL-10 in cultured Th2 cells [217, 299]. $CRTH2^+$ T cells are increased at the maternal fetal interface during pregnancy in women undergoing elective termination compared to women with recurrent miscarriage, suggesting a protective role of CRTH2 in the maintenance of pregnancy [300]. PGD_2 production from the placenta is likely to be responsible for chemoattracting the CRTH2 positive cells and maintaining a pro-pregnancy Th2 cytokine predominance at the maternal fetal interface [301].

CRTH2 agonists and their downstream effects

Endogenous CRTH2 agonists include PGD_2 and $15dPGJ_2$. In response to PGD_2, CRTH2 induces intracellular calcium mobilization and chemotaxis in Th2 cells, eosinophils and basophils in a $G_{\alpha/i}$ dependent manner [297]. PGD_2 induces cytokine production via the calcineurin and PI3K pathways as demonstrated by inhibition with calcineurin inhibitors and the PI3K inhibitor Wartmannin [302]. $15dPGJ_2$ induces calcium mobilisation, actin polymerisation and up regulation of CD11b eosinophils via CRTH2 [303]. Up regulation of CD11b via CRTH2 may be mediated via Gq class proteins leading to activation of p38 MAP kinase and phosphoinositol-3-kinase (PI3K), as these responses are resistant to the pertussin toxin [304]. The non-steroidal anti-inflammatory agent Indomethacin is also an agonist of CRTH2. Indomethacin induces Ca^{2+} mobilization and cell migration via CRTH2 in Th2 cells, which is sensitive to the pertussin toxin indicating a $G_{\alpha/i}$ dependent mechanism [305]. Indomethacin also induces Th2 cytokines *in vitro* [299]. The potential role of CRTH2 and CRTH2 agonists for the immuno-modulation during pregnancy has not previously been examined.

1.10 Summary and Aims

Preterm labour and long-term neonatal morbidity is associated with infection and inflammation. NF-κB is a transcription factor that regulates both inflammatory and labour associated gene expression. Preterm labour is associated with a switch from a Th2 bias to a proinflammatory Th1 bias. Our group have previously shown that the CRTH2 agonist 15dPGJ2 inhibits NF-κB in cultured human amniocytes and myocytes, and delays inflammation induced preterm labour in the mouse.

This study aims establish if a small molecule CRTH2 agonist can replicate the effects of 15dPGJ2 with the potential of delaying preterm labour and reducing the adverse effects on the neonate which are associated with inflammation induced preterm labour.

In this thesis the following questions will be answered:

1. Is CRTH2 expressed in human amniocytes and myocytes?

2. Does a small molecule CRTH2 agonist inhibit NF-κB?

3. Do CRTH2 agonists modulate the Th1/Th2 cytokine profile in peripheral blood mononuclear cells in pregnancy and labour?

4. Is uterine contractility inhibited by CRTH2 agonists?

5. Can a small molecule CRTH2 agonist delay inflammation induced preterm labour?

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals, Reagents and Solvents

Acrylamide (Biorad)

Acetic Acid (VWR)

Agarose (VWR)

Ampicillin (Sigma)

Bovine Serum Albumin (Sigma)

Brefeldin (Sigma)

Ca Cl_2 (Sigma)

Chloroform (Sigma)

Coomassie Brilliant Blue (Biorad)

15-deoxy-Δ 12,14-Prostaglandin J2 (15dPGJ_2) (Cayman Chemicals)

DEPC treated water (Ambion)

Dextran (Fluka)

13, 14 dihydro 15 keto Prostaglandin D2 (Cayman Chemicals)

Dimethyl Sulfoxide (DMSO) (Sigma)

Ethanol (VWR)

Ethylenediaminetetraacetic acid (EDTA)

Ficoll Paque Plus (GE healthcare)

Fugene Transfection reagent (Promega)

GeneJuice Transfection reagent (Novagem)

Glucose (Sigma)

Glycerol (BDH Chemicals)

Indomethacin (Sigma)

Interleukin-1β (R&D systems)

Ionomycin (Sigma)

Isopropanol (Sigma)

LPS 0111:B4 (Sigma)

LPS 055:B5 (Sigma)

LPS 0128:B12 (Sigma)

LPS 0127:B8 (Sigma)

LPS Salmonella Smooth Form (Alexis)

Methanol (VWR)

$MgCl_2$ (Sigma)

$NaCl_2$ (Sigma

NP-Alternative (Calbiochem)

Optilyse (Beckman Coulter)

Paraformaldehyde (VWR)

Phorbol 12 myristate 13 acetate (PMA) (Sigma)

Prostaglandin D_2 (Cayman Chemicals)

Protein ladder (Promega)

Pyl A and Pyl B (Oxygen Healthcare)

Rat Serum (Sigma)

Robosep (Stem Cell Technologies)

Saponin (Sigma)

SDS 20% (National Diagnostics)

Skimmed Milk (Sigma)

S.O.C Media (Invitrogen)

Sodium Azide (BDH Chemicals)

Sybr green (Applied Biosystems)

Sybr safe DNA gel stain (Invitrogen)

TEMED (Sigma)

Tetraethyl ammonium chloride (Sigma)

Tris (Sigma)

Trypan Blue (Sigma)

Tween 20 (BDH)

Versalyse (Beckman Coulter)

X481 (Glaxo Smith Kline)

2.1.2 Antibodies

Primary Antibodies

β-actin Cat no. A 2228, Sigma

CD4-APC Cat no. 551980, BD Pharminogen

CD11b- PE Cat no. 555388, BD Pharminogen

COX-2 (C-20) Cat no. 1745, Santa Cruz

CRTH2-PE Cat no. A07413, Beckman Coulter

CRTH2 Cat no. PR- 4029, ProScience

CRTH2 Cat no. PR- 4027, ProScience

CRTH2 Cat no. 23092, Santa Cruz

CRTH2 Cat no. 2178, Santa Cruz

p-65 Cat no 8008, Santa Cruz

Phospho-p65 (Ser 536) Cat 3033s, Cell signalling

CD49d-APC cat no 130-093-281, Miltenyi Biotec

IFN-γ-FITC Cat No. 554700, BD Pharminogen

IL-4- PECy7 Cat no. 500824, BioLegend

TNF-α–FITC

Secondary antibodies

Anti Rabbit IgG HRP Cat no. 7074, Cell Signalling

Anti Rat HRP Cat 61-9520, Invitrogen

Donkey anti Goat-HRP Cat no. SC 2020 Santa Cruz

Goat anti Mouse-HRP Cat no. SC 2005 Santa Cruz

Isotype controls

APC Mouse IgG1 κ isotype Control Cat no. 550854, BD Pharminogen

APC mouse IgG2b isotype control Cat no. 130-092-217, Miltenyi Biotec

FITC Mouse IgG1 κ isotype control Cat no. 554679, BD Pharminogen

PE Rat IgG2a isotype control (matched to CRTH2-PE) Cat no. 731666 Beckman Coulter

PE/Cy7 Rat IgG1 κ isotype control antibody Cat no. 400416, BioLegend

PE Mouse IgG1 κ isotype control Cat 555749, BD Pharminogen

2.1.3 Cell culture materials

Collagenase XI or IA (Sigma)

Dispase (Gibco)

Dulbecco's Modified Eagles Medium (Sigma)

Fetal calf serum (Sigma)

L-glutamine (Sigma)

Penicillin Streptomycin (Sigma)

RPMI medium (Invitrogen)

Trypsin/EDTA (Sigma)

Plastic ware (from Appleton woods): T25 cell culture flask, 24 well plates cell strainer, filter unit, 50 and 15 ml falcons, conical flasks.

2.1.4 Buffers

Buffer A (Cytosolic)

10 mM HEPES

10 mM KCL

0.1 mM EDTA

0.1 mM EGTA

2 mM DTT

1% v/v NP-40 and Complete protease inhibitor (Roche)

Buffer B (Nuclear)

10 mM HEPES

10 mM KCL

0.1 mM EDTA

0.1 mM EGTA

2 mM DTT

400 mM $NaCl_2$

1% v/v NP- 40 and Complete protease inhibitor (Roche)

Whole cell lysis buffer 10X (Cell Signalling)

2.1.5 RNA and DNA synthesis

DNAse Buffer 10x (Invitrogen)

DNAse Grade 1 (Invitrogen)

DNA ladder (Invitrogen and Bioline)

DNA loading dye (Invitrogen)

dNTP 10 mM (Invitrogen/Novogem)

DTT 0.1mM (Invitrogen)

EDTA 25 mM

Oligo DT (Invitrogen)

Primers (Thermo Scientific)

Random Hexamer (Invitrogen)

RNA later (Sigma)

RNAse Out (Invitrogen)

Solution Q 5x (Qiagen)

Superscript III (Invitrogen)

Taq Pol (Qiagen)

Trizol (Invitrogen)

Qiagen PCR buffer 10x (Qiagen)

2.1.6 Cloning, sequencing, transfection and *in vitro* transcription translation

AMAXA Basic Nucleofector Smooth Muscle Cell Kit (Lonza)

AMAXA Basic Nucleofector Primary Mammalian Epithelial Cell kit (Lonza)

DNA Maxiprep/Miniprep kit (Qiagen)

Endonuclease (New England Biolabs)

Escherichia coli TOP 10 (Invitrogen)

Geneclean Column kit (Qiagen)

Geneclean Spin Kit (Qbiogene)

Gen Elute Gel Extraction Kit (Qiagen)

Methionine 1mM/^{35}S (Perkin Elmer),

pSG5 Vector (Agilent Technologies)

pCR® -BluntII-TOPO® (Invitrogen)

T4 DNA ligase (Invitrogen)

T7 TNT Coupled Reticulocyte Lysate System (Promega)

TOPO cloning kit (Invitrogen)

2.1.7 Western and ELISA

High Performance chemiluminescence film (GE healthcare)

Laemelli buffer x4

0.2 M Tris pH 6.8

8% SDS

40% Glycerol

0.4M DTT

0.4% Bromophenol Blue

Polyacrylamide 10% Resolving gel

10% Acrylamide
Tris HCl pH 8.8
0.1% SDS
0.1% Ammonium Persulfate
0.04% TEMED

Polyacrylamide 5% Stacking gel

5% Acrylamide
Tris HCl pH 6.8
1% SDS
1% Ammonium Persulfate
0.1% TEMED

Phosphate Buffered Saline (PBS) (In house)

PVDF membrane (Millipore)

Running Buffer *1x SDS* (In house)

1x TBS (In house)

1x TBS-T (In house)

Transfer Buffer

100 mls TB buffer 10x

200 mls Methanol

700 mls Distilled Water

3.75 mls 10% SDS

TH1/Th2 Multiplex ELISA kit, Meso scale Discovery

2.1.8 Contractility

Gas 95% 0_2 5% CO_2, BOC

Krebs henseleit buffer pH 7.4

Glucose 2.0g/L

$MgSO_4$ 0.141g/L

KH_2PO_4 0.16g/L

KCl 0.35 g/L

$NaCl_2$ 6.9g/L

$CaCl_2$ 0.373g/L

$NaHO_3$ 2.1g/L

Muscle Strip Myograph 800MS, DMT

Powerlab software, ADInstruments

2.1.9 Murine study

Morphine

Isofluorane

CD1 Outbred Mice (Charles River)

2.2 Primary Cell culture

2.2.1 Amnion epithelial cells (Human)

Amnion was collected from women on the Labour ward at Queen Charlotte's hospital in accordance with the Ethical approval Ref 2002/6283. Pre-labour amnion was taken from women undergoing elective caesarean section at term.

Amnion was separated from the choriodecidua, cut into strips and washed in phosphate buffered saline (PBS). It was then incubated in 0.5 mM of EDTA-PBS for 15 minutes at room temperature, followed by a PBS rinse. The intracellular matrix was digested in pre-warmed dispase at 2g/L in PBS for 45 minutes at 37°C. The epithelial cells were then isolated by shaking the amnion strips vigorously in Dulbecco's modified Eagle's medium, DMEM for 3 minutes, then pelleted by centrifugation for 10 minutes at 2000rpm. Cells were resuspended in DMEM containing 10% fetal calf serum, 2 mM/L L-glutamine, 100U/ml Penicillin and 100 µg/ml of Streptomycin and grown to confluence in T25 flasks at 5% CO_2.

2.2.2 Myometrial Smooth Muscle cells (Human)

Myometrial biopsies were collected from women on the Labour ward at Queen Charlotte's hospital in accordance with Ethical approval (Ref 3358). Pre-labour myometrium was collected at time of elective caesarean section.

Myometrial biopsies were taken from the upper margin of lower segment incisions during elective caesarean sections. Tissue was washed in PBS, and mechanically dissected with two sterile blades to form a paste-like texture. Cells were isolated by incubating with 15 mg of Collagenase 1A, 15 mg of Collagenase X and 50 mg of BSA in 30 mls of PBS for 45 mins at 37°C. The suspension was filtered through a cell strainer and centrifuged at 400 g for 5 minutes. The cells were resuspended and cultured in DMEM as described in above.

2.2.3 Peripheral Blood Mononuclear cells (Human)

Blood from pregnant and non-pregnant women was collected in accordance with the South East London Ethics approval Ref: 10/H0805/54. 4mls of blood was collected in sodium citrate vacutainers. Two methods were used for leukocyte isolation, depending on the leukocyte fraction of interest. For isolating granulocytes, dextran was used, and for isolating lymphocytes Ficoll was used.

a) Dextran

The white cell fraction was isolated by incubating 4 mls of blood with 4mls of 4.5% Dextran (Fluka Analytical) in PBS for 45 minutes at 4°C. The leukocyte fraction was centrifuged at 500g for 10minutes, and the pellet was resuspended in PBS.

b) Ficoll

For lymphocyte isolation, 4 mls of blood was mixed with 4 mls of PBS. 4 mls of blood/PBS was layered over 3 mls of Ficoll (GE healthcare), and centrifuged at RT for 40mins at 400g. The supernatant was discarded and the Lymphocyte halo was washed in PBS and resuspended in RPMI 1604 medium with 10% fetal calf serum and 2 mM/L L-glutamine, 100U/ml Penicillin and 100 µg/ml of Streptomycin and plated in 24-well plates.

2.3 Protein extraction

2.3.1 Whole cell lysate preparation

Adherent cultured cells (amniocytes and myocytes) were collected using a cell scraper and placed in whole cell lysis buffer (Cell Signalling) with 5 µl/ml of protease inhibitor (Sigma). Cells were incubated on ice for 20 minutes and centrifuged at 13,000rpm for 10 minutes at 4°C. Whole cell lysates from tissue were prepared by homogenising in lysis buffer, centrifugation and collection of supernatant. Leukocytes were collected using a 1ml pipette and incubated on ice in lysis buffer for 5 minutes and centrifuged for 20 minutes at 13,000rpm at 4°C. The supernatant was stored at -80°C until use.

2.3.2 Nuclear and Cytosolic protein extraction

Cells were collected as described in 2.2.1 and lysed in Buffer A (10 mM HEPES, 10 mM KCL, 0.1 mM EGTA, 0.1 mM EDTA, 1mM DTT, 1%(v) NP-40 with protease inhibitor), with the cytosolic fraction collected following centrifugation at 13,000rpm for 60s at 4°C. The pellet was then resuspended in Buffer B (10 mM HEPES, 10 mM KCL, 0.1 mM EGTA, 0.1 mM EDTA, 2MM DTT, 400 mM $NaCl_2$ and 1% v/v NP-40 and protease inhibitors), incubated on a shaker for 15 minutes at 4°C, and then centrifuged at 13,000 rpm for 5 minutes at 4°C . The supernatants were stored at -80°C until analysis.

2.3.3 MSD protein extraction buffer for Multispot Elisa

Murine brain and myometrium was collected and snap frozen on dry ice and stored at -80 until use. For protein extraction, tissue was grinded with a pestle and mortar in liquid nitrogen and homogenised in MSD lysis buffer (150mM $NaCl_2$, 20mM Tris pH 7.5, 1mM EDTA, 1mM EGTA, 1% Triton X-100, with Phosphatase Inhibitor (Sigma) and Protease inhibitor (Roche)).

2.3.4 Protein quantification

Protein quantification was performed using the BIORAD assay, measuring absorbance at 655 nm.

2.4 RNA extraction

Total RNA was isolated from tissue, primary cell cultures and Peripheral blood mononuclear cells using Trizol© according to the manufacturer's instructions. Briefly, tissue was washed in PBS and homogenised using the power homogeniser in 1ml of Trizol© per 100 mg of tissue. For primary cell cultures 1 ml of Trizol© was added to each T25 flask or 200ul per well of a 6 well plate and cells were collected using a cell scraper. Prior to RNA extraction from white blood cells, the white cell fraction was prepared from blood by incubation with 4.5% Dextran in PBS for 45 minutes at 4°C. This fraction was then centrifuged for 10 minutes at 200g and the resulting pellet was resuspended in 1 ml of Trizol© per 1 ml of white cell fraction.

2.5 cDNA synthesis

RNA was treated with DNase to remove contaminating DNA. 1 µg of RNA was incubated with 1 µl DNase and 1 µl of DNase buffer made up to 10 µl volume with DEPC treated water for 15 minutes at room temperature. The reaction was terminated by the addition of 1 µl of 25mM EDTA for 10 minutes at 65°C. 8 µl of the DNAse treated mix was incubated with 1 µl of 10 mM dNTP and 1 µl of Oligo DT (12-18 0.5ug/ml) for 5 minutes at 65°C. To this mix 2 µl of 10x RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1m DTT, 1 µl of RNAse Out and 1µl of Superscript III was added. (In the reverse transcriptase controls no Superscript III was added). The mix was incubated at 42°C for 10 minutes and the reaction was terminated at 70°C for 15 minutes. 0.5 µl of RNAse H was added and mix was incubated at 37°C for 20 minutes. Samples were stored at -20°c until further use.

2.6 Polymerase Chain Reaction (PCR)

PCR is used to amplify specific DNA sequences from a small amount of template and provides information on gene expression as it amplifies cDNA reverse transcribed from messenger RNA. Paired oligonucleotide primers for amplification of genes of interest were either designed by Primer 3 software or were based on publications. Primers were checked against sequences of the genes of interest using GenBank sequences. The primer sets produced amplicons where the intron/exon boundary was crossed wherever possible. Non-template reverse transcriptase controls were used.

2.6.1 Reverse Transcriptase Polymerase Chain Reaction

The PCR programmes varied depending on the primers and the gene of interest. All reactions included a denaturing step to denature the primers and DNA template. Cooling then allowed for annealing of the primers to the target sequence. The DNA polymerase (Taq Po or PFU Turbo) extends the primers at the 3 prime end. Repeated cycling results in exponential production of the target sequence.

For detection of human CRTH2 in Amniocytes, Amnion, Myocytes, Myometrial, Choriodecidual and Placental tissue a 309bp CRTH2 transcript was amplified using PCR mix A, PCR program A, and Primers A, Tables 1-3. In preparation for cloning human CRTH2 white blood cell cDNA was used and a 1.188kb transcript was amplified using PCR mix C, Program C, and Primers C, Tables 1-3.

A 344bp CRTH2 transcript was amplified from murine myometrial tissue cDNA using PCR mix A, Primers E, with the following programme: 94°C for 3 minutes, and 34 cycles of 94°C for 1 minute to denature, 63°C for 1 minute to anneal, and 72°C for 3 minutes to extend, followed by a 10 minute holding step at 72 °C. (See Tables 1-4)

2.6.2 Quantitative Polymerase Chain Reaction

Quantitative PCR was performed with SYBR Green PCR mix. The cycle at which the fluorescence reaches a preset cycle threshold (non-template control threshold) was used for quantification. The $\Delta\Delta C_T$ method was used for comparative analysis, with data normalised relative to two endogenous housekeeping genes, B actin and L19 or GAPDH to serve as endogenous controls.

For quantification of human amniocyte and myocyte CRTH2 a 114bp transcript was amplified using PCR mix B, TAQMAN program B, and Primers B, Tables 1-3. Myometrial murine tissue was used to quantify the Th1 and Th2 cytokines using PCR mix B, TAQMAN program B and the relevant primers in Table 4.

2.6.3 Agarose gel electrophoresis

PCR products were detected using 1-1.5% Agarose gels to confirm product sizes. Agarose was microwaved in 1x TBE buffer until dissolved. On cooling 1:10000 Sybr Safe gel stain was added, and the gel was allowed to set. Once set the PCR product was diluted with 6x loading buffer and loaded onto the gel. The gel electrophoresed at 100 V for 20-40 minutes. The gel was transilluminated and photographed under UV light.

Table 1 PCR Mix

PCR Mix	A	B	C
Enzyme	Taq Po 0.5 µl	Sybergreen 12.5ul	PFU turbo 0.5 µl
CRTH2 Forward Primer	0.5 µl (3.2 pmol stock)	0.06 µl (20um stock)	0.5 µl (3.2 pmol stock)
CRTH2 Reverse Primer	0.5 µl (3.2 pmol stock)	0.06 µl (20um stock)	0.5 µl (3.2 pmol stock)
cDNA	2 µl (600ng/ µl)	1 µl	1 µl
Water	17 µl	11.38 µl	11 µl
Buffer	2.5 µl X10 Qiagen buffer	n/a	2.5 µl 10% buffer PFU
dNTP mix (2mM Novagem)	2.5 µl	n/a	2.5 µl
5x Solution Q	5 µl	n/a	5 µl
25mM $MgCl_2$	1.5 µl	n/a	1.5 µl
Volume of mix	25 µl	25 µl	25 µl

Table 2 PCR Program

Program	A	B	C
Polymerase activation	95°C 3 min	50°C 2 min	95°C 3min
Denature	94°C 1 min	95°C 10 min	94°C 1 min
Anneal	67°C for 1 min	95°C 15s	75°C 1 min
Extend	72°C for 2 mins	60°C 1min	72°C 2 min
Hold	72°C for 10mins	Hold	72°C 10min
Cycles	35	40	36

Table 3 Primers (Human)

		Forward	Reverse (Anti sense)	Size
A	CRTH2	CCTCTGTGCCCAGAGCCCCACGATGTCGGC	CACGGCCAAGAAGTAGGTGAAGAAG	309bp
B	CRTH2	CCTCTGTGCCCAGAGCCCCACGATGTCGGC	ATGTAGCGGATGCTGGTGTTG	114bp
C	CRTH2	GGC ACTCACACGCGAAAGTAT	ACCAGGGTGCCGCGGTTC	209bp
D	CRTH2	CTGCCCTTCTTCACCTACTTCTTGGC	GTGTCCCGGAACACGAAATAGGGCAC	265bp
E	CRTH2	**GGATCC**ATGTCGGCCAACGCCACACTGAAGCC **BamH I site**	**AGATCT**CTAACTCGAGGTGCTGCTCAGCGCCC **Bgl II site**	1188bp
F	CRTH2	T7 TAATACGACTCACTATAGG For CRTH2 insert TCACCAGCCTGGCCTTCTTC	SG5 GAAATTTGTGATGCTATTGC CRTH2 insert TGCAGTTTGCAGAAGGTGGTG	n/a
G	GAPDH	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTAGGCCAT	240bp

Table 4 Primers (Murine)

		Forward	Reverse	Size
H	M CRTH2	CGTAGCCGTGAGCCTGCGACTG	GGCGATTGCGGAGCCCACCACT	344bp
I	M IFN γ	AGCGGCTGACTGAACTCAGATTGTAG	GTGTAGTCAGTTTTCAGCTGTATAGG	252bp
J	M TNF α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC	175bp
K	M IL-10	ACCTGGTAGAAGTGATGCCCCAGGCA	ACCTGGTAGAAGTGATGCCCCAGGCA	237bp
L	M IL-4	CGAAGAACACCACAGAGAGTGAGCT	GACTCATTCATGGTGCAGCTTATCG	181 bp
M	M B Actin	AGGCTGTGCTGTCCCTGTAT	GCTGTGGTGGTGAAGCTGT	201 bp
N	M GAPDH	CTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA	170bp
O	M L-19	GAAAAAGAAGGTCTGGTTGGA	TGATCTGCTGACGGGAGTTG	72 bp

2.7 Cloning and sequencing of CRTH2

In order to achieve a good positive control for determining the presence of CRTH2 at the protein level I cloned CRTH2. Human peripheral leukocytes were used to extract mRNA and to reverse transcribe to cDNA in order to amplify the whole length of CRTH2 for cloning.

2.7.1 cDNA synthesis

Peripheral blood leukocytes were isolated using Dextran 4.5% and mRNA was extracted with Trizol© as described in section 2.4. 1µg of DNAse treated RNA was incubated with 1 µl of 10 mM dNTP and 3 µl of Random hexamer for 5 minutes at 65°C. To this mix 1µl of 10x RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1mDTT, 1 µl of RNAse Out and 1 µl of Superscript III was added. The mix was incubated at 25°C for 10 minutes 50°C for 50 minutes and 85°C for 5 minutes. The mix was chilled on ice and 1 µl of RNAse H was added and incubated at 37°C for 20 minutes.

2.7.2 CRTH2 amplification

A 1.18kb transcript was amplified from white cell cDNA with primers from Table 3 (C). The reaction mix was as described in Table 1 (C). The reaction protocol was as described in Table 2 (C). The primers introduced a BamH 1 digest sequence upstream of the CRTH2 coding sequence and a Bgl II restriction digest sequence downstream. The fragment was then separated on a 1% Agarose DNA gel.

2.7.3 Purification of DNA from agarose gel

The whole length of CRTH2 was cut out of the gel under UV light vision and the Gene Clean Spin Kit was used according the manufacturer's instructions. The gel was melted

in 400 µl of GC SPIN GLASSMILK in the Geneclean spin filter at 55°C for 5 minutes. The liquid was spun at 12,000g into a catch tube. The filter was washed with 500 µl of GC SPIN NEW Wash, and spun for 30 seconds, a repeat wash step was performed and then a repeat spin for 1 minute was performed to dry the DNA pellet. The DNA was eluted with 50 µl of GC SPIN Elution Solution, and the spin filter was transferred to a fresh catch tube and spun for 30 seconds at 12,000 x g to collect the eluted DNA.

2.7.4 Cloning into intermediate vector; pCR® -BluntII-TOPO®

The PCR product (gel elute) was cloned into a TOPO Vector according to manufacturer's instructions. Briefly, ligation was performed with 1 µl of PCR product, 1 µl of TOPO salt solution, 3 µl of Nuclease free water H_20 and 1 µl of TOPO–II vector for 30 minutes at room temperature. Top10 E.coli cells were transformed with 1 µl of cloning reaction by heat shock at 42°C for 45 seconds and cooled on ice for 2 minutes. 150 µl of S.O.C medium was added to the transformed cells and incubated at 37 °C for 1 hour, and then plated onto ampicillin plates and incubated overnight at 37°C. Colonies were then selected the next day and 3mls of L-Medium was inoculated and incubated overnight at 37°C on a shaker set at 200rpm. The next day the medium was centrifuged at 3800rpm at 4°C and the DNA pellet was isolated using the QIAprep Miniprep Spin Kit. Briefly the pellet was resuspended in 250 µl of Buffer P1, 250 µl of Buffer P2, 350 µl of Buffer N3 and centrifuged at 13000 rpm for 10 minutes. The supernatant was transferred to the Miniprep spin column and centrifuged for 15 seconds. The column was washed with Buffer PE and centrifuged dry. DNA was eluted with 50 µl of dH_20 and quantified by Nanodrop.

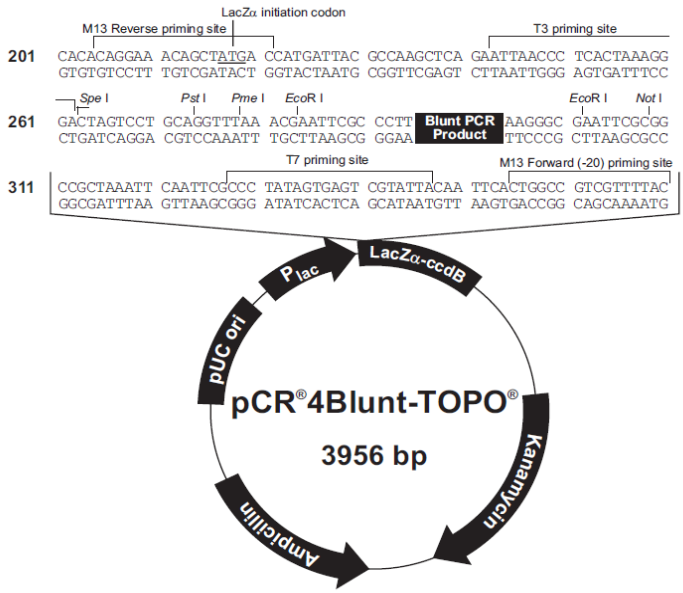


Figure 2.1 Map of the pCR Blunt Topo Vector
This map shows insertion and ligation sites from the Invitrogen data sheet.

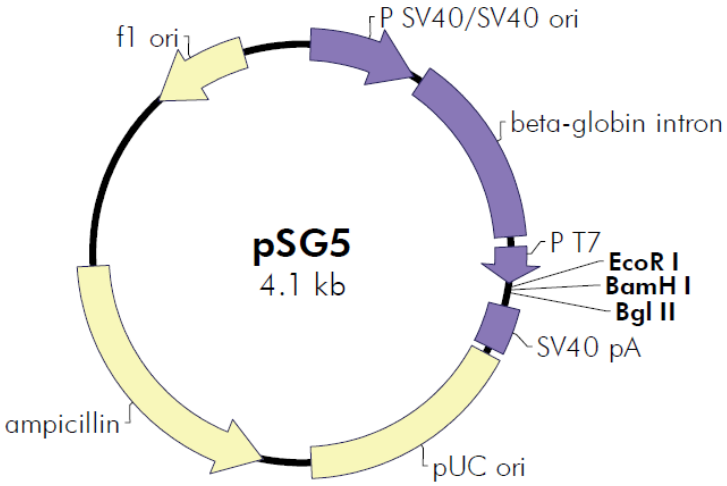
The plasmid was digested at the BamH 1 and Bgl II sites using 2 µl of Buffer 3 (New England Biolabs), 0.5 µl BSA, BamH 1 and Bgl II digestion enzymes and 2 µg of Plasmid DNA for 90 minutes at 37 °C. The products were then run on a 1% DNA Agarose gel, cut and purified using the Geneclean kit for insertion into the pSG5 vector. The map of the pCR Blunt TOPO is shown in Figure 2.1 showing the insertion point of the PCR product.

2.7.5 Cloning into the pSG5 Vector

Ligation was performed with 1 µl of T4 ligation enzyme, 2 µl of T4 buffer, 1 µl of pSG5 vector, 1.5 µl of insert, and 4.5 µl of water overnight at 16°C. E.coli Top 10 competent cells were transformed as above. Colonies were grown overnight on ampicillin plates at 37°C, all colonies were picked and L medium was inoculated and incubated overnight at 37°C. All colonies were purified using the QuickLyse Miniprep kit. Plasmid DNA for each colony was digested at the BamH 1 and Bgl II sites, as shown in Figure 2.2, and run out on a 1% Agarose gel to confirm product insertion and the correct size of product. 8 colonies of 24 were selected based on correct size of product insertion to transform and place into the pSG5 vector, plated out, and isolated again with the QIAprep Miniprep spin kit for good quality DNA. Plates were stored at 4°C. Plasmids were digested the insert purified for sequencing.

Figure 2.2 Map of the pSG5 Vector
This map shows insertion and ligation sites. From the Agilent Technologies data sheet

The pSG5 Vector



2.7.6 DNA sequencing

Sequencing of the plasmid DNA was performed by the MRC Clinical Sciences Centre at Imperial College. The sequencing reaction contained the 1 µl of T7 Forward or pSG5 Reverse primer with 600ng of DNA in a 10 µl volume. See Table 3 for primers sequence.

2.7.7 Plasmid DNA preparation for transfection

Once sequences were confirmed to be correct and inserted in the correct orientation, the stored plates with the colonies of transformed cells were re-picked, and 5mls of LB-Broth was inoculated and incubated at 37°C on the shaker at 200rpm for 8 hours. 1ml of the LB- Broth starter culture was placed into 250mls of LB-Broth on the shaker overnight ay 37°C. The following day the medium was transferred into a conical centrifuge tube and centrifuged for 20 minutes at 3600 rpm at 4°C. The supernatant was discarded and the bacterial pellet was purified with the QIAprep Maxiprep kit. 10mls of Buffer P1 and 10mls of Buffer P2 were added and incubated for 5 minutes at

room temperature. 10mls of Buffer P3 was added and incubated for 10minutes in a filter cartridge. The lysate passed through the cartridge into a HiSpeed Maxi tip and entered a resin and was then washed with 60mls of Buffer QC. 15mls of Buffer QC was used to collect the DNA elutant which was then precipitated with Isopropanol. The elutant was passed though the QIAprecipitator and was dried with flushing of air. 1.5mls of Buffer TE was passed through the precipitator to collect the DNA into a microcentrifuge tube.

2.8 Transfection of CRTH2

Myocytes

Myometrial smooth muscle cells were cultured in six well plates until they reached 90-95% confluence. The AMAXA Basic Nucleofector Smooth Muscle Cell Kit was used to transfect the cells. GFP was used as a positive control for the transfection efficiency. On the day of transfection cells were Trypsinised and centrifuged at 100 x g for 10 mins at room temperature. Approximately $1x10^{6}$ cells were then resuspended in 100 µl of the Nucleofector solution with either 2µg of CRTH2 expression vector or 2 µg of GFP. The cell suspension was transferred into the cuvette and placed into the Nucleofector Program and electroporated on Program A-033. 500 µl of prewarmed medium was added and cells were gently plated into fresh six well plates. Cells were incubated at 37 °C in humidified 5% CO_2. Medium was changed 18 hours post transfection. Gene expression of GFP was determined by fluorescent microscope on day 1, day 2 and day 3-post transfection. Gene expression of CRTH2 was determined by Flow cytometry on day 1, day 2 and day 3.

Amniocytes

For transfection of CRTH2 into amniocytes; primary amnion epithelial cells were cultured until 80% confluence. GeneJuice® (Novogem) or Fugene (Promega) transfection reagent was used according to manufacturer's guidance with 1 µg of DNA

per well. Cells were grown in 6 well plates and at the time of confluence the transfection reagent was added to the wells together with 1µg of CRTH2 expression vector. Cells were harvested for analysis after 24 hours. GFP was used as a positive control for the transfection efficiency.

2.9 In vitro transcription translation

CRTH2 expression in a cell free system was examined with the T7 TNT Coupled Reticulocyte Lysate System. This was in order to produce high quantities of CRTH2, and to demonstrate CRTH2 at the protein level without the need for antibodies. The TNT mix and cold Methionine 1mM were incubated with 0.2 µg of the CRTH2 expression vector 30°C for 90 minutes. The product underwent gel electrophoresis and western blotting as described in section 2.10. The membrane was probed with antibodies 1, 2 and 3 from Table 5 and detection was with chemilluminescent substrate of horseradish peroxidase. In order to demonstrate CRTH2 without the use of antibodies cold Methionine was substituted with radioactive ^{35}S Methionine. The product underwent gel electrophoresis and was transferred onto a membrane. Detection was by x-ray film.

2.10 Sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis and Western blotting

2.10.1 SDS polyacrylamide gel electrophoresis

The SDS polyacrylamide gel was prepared as the stacking gel and then the resolving gel. TEMED and Ammonium persulphate polymerise both stacking and resolving gels. Once the stacking gel was set, the resolving gel was layered on top, and the 10 well comb added. As the gel was setting, protein was denatured at 100°C with 4x loading dye for 5 minutes. Once set, the gels were mounted in the BIORAD tanks and combs

were removed and wells were rinsed with running buffer prior to loading protein. Protein ladder and protein of interest were loaded and gels electrophoresed in 1x SDS Running buffer at 100V until passage into the stacking gel, and then 140V until the dye front had migrated to the bottom of the gel.

2.10.2 Western blotting

PVDF membrane was activated with methanol for 1 minute and distilled water for 3 minutes prior to transfer. Resolved gels were allowed to equilibrate in Transfer buffer prior to transfer. The BIORAD wet transfer technique was used. The layers consisted of (from bottom to top): sponge; two layers of Whatman paper; the gel; the membrane; two layers of Whatman paper and sponge.

The sandwiched unit was then enclosed in the BIORAD transfer tank with the membrane towards the anode and the gel towards the cathode. Transfer was for 90 minutes at 100V at 4°C. Following transfer, the membrane was re-activated in methanol for 1 minute and distilled water for 3 minutes. The membrane was then blocked in 5% milk in 1x TBS-T for 1 hour. Primary antibodies were used at varying concentrations and incubated at varying times. (See Table 5) After primary and secondary antibody incubations membranes were washed in 1x TBS-T for 10, 10 and 10 minutes. Detection was achieved using chemilluminescent substrate of horseradish peroxidase.

Table 5 Antibodies

Primary Antibody	Catalogue No	Dilution	Incubation	Secondary Antibody 1 hour at room temp
1 CRTH2 goat	Sc- 23092	1:200 TBS 1% milk	Overnight, 4°C	1:2000 TBS 1% milk donkey anti goat
2 CRTH2 rat	Sc-21798	1:500 TBS 1% milk	Overnight, 4°C	1:3000 TBS 1% milk goat anti rat
3 CRTH2 rabbit	PR-4029	1:500 TBS 1% milk	Overnight, 4°C	1:2000 TBS 1% milk mouse anti rabbit
4 CRTH2 rabbit	PR-4027	1:500 TBS 1% milk	Overnight, 4°C	1:2000 TBS 1% milk mouse anti rabbit
5 P65 mouse	SC8008	1:1000 TBS 1% milk	1 hour Room temp	1:2000 TBS 1% milk goat anti mouse
6 Phospho-p65 rabbit	3031S	1:1000 in TBS	Overnight, 4°C	1:2000 TBS 1% milk anti rabbit
7 Progesterone mouse	N/a	1:1000 TBS 1% milk	Overnight, 4°C	1: 2000 TBS 1% milk goat anti mouse

2.10.3 Protein Sequencing

Whole cell lysates were prepared from positive controls (human heart and white cells), amnion and myometrial samples, and underwent electrophoresis. Membranes were stained with Coomassie blue (BIORAD) and bands cut at relevant sizes according to ladder and corresponding western blots. Membranes and bands were sent to Cambridge University Department of Biochemistry for N terminal sequencing using the Edman technique, carried out by Cambridge University Department of Biochemistry.

2.11 Flow cytometry

2.11.1 Cell surface staining (CD11b and CD49D)

Blood was collected from non-pregnant patients in vacutainers containing the anticoagulant citrate. The white cell fraction was isolated by incubating with 4.5% Dextran for 45 minutes at 4°C. Following centrifugation of the fraction the cells were resuspended with PBS containing $CaCl_2$ (0.9mM) and $MgCl_2$ (0.5mM) and counted. Cells were then pre-incubated at 37°C, followed by treatment with the CRTH2 agonists at 0.1 µm, 1 µm and 10 µm for 15 minutes. The reaction was terminated by the addition of 1 ml of ice cold FACSFlow. In experiments with the CRTH2 antagonist, pre incubation with x481 was performed for 10 minutes at 37°C. The cells were then centrifuged at 400g for 5 minutes at 4°C and resuspended in Robosep for labelling with PE anti-CD11b and APC anti-CD49D for 10 minutes at 4°C in the dark. The red cells were then lysed by the addition of Optilyse-C for 10 minutes in the dark at room temperature. Cells were then washed and resuspended in PBS and 1% FBS for analysis. Eosinophils and basophils were identified as CD49D positive and by high side and forward scatter, as shown in Figure 2.3. Flow cytometry settings were as follows: Forward scatter E0 Voltage, 1.00 Amp gain Lin, and Side scatter of 329 Voltage, 1.00 Amp gain Lin.

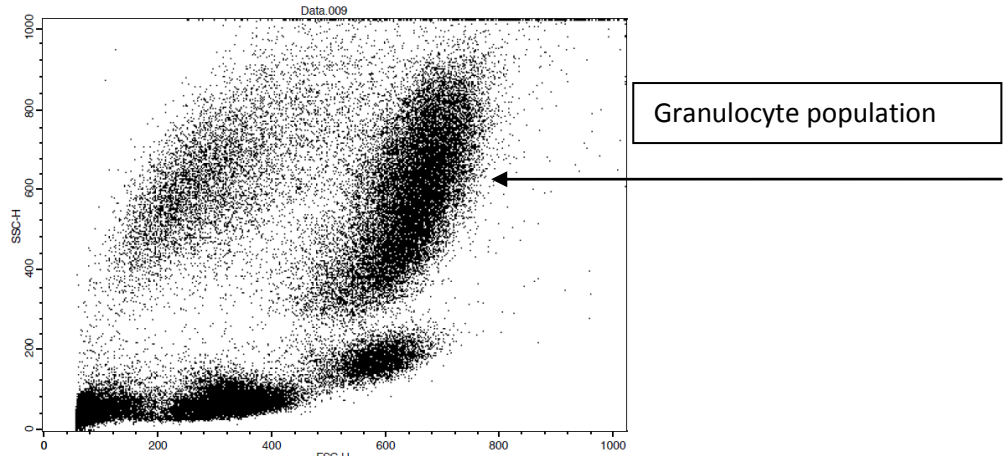


Figure 2.3 Forward and Side scatter profile of Peripheral Blood cells isolated using Dextran
Granulocytes have the highest forward and side scatter. Lymphocytes have a lower forward and side scatter than granulocytes and monocytes. Monocytes have a high forward scatter but low side scatter

2.11.2 Cell surface staining (CRTH2 and CD4)

Blood was collected in vacutainers containing the anticoagulant citrate and diluted 1:1 in PBS. 4mls of blood/PBS was carefully layered over 4 mls of Ficoll-Paque^{TM} PLUS and centrifuged at 400 x g for 40 minutes at room temperature. The peripheral blood mononuclear cells formed a halo and was collected gently and washed twice in PBS. During the second wash, 10ul of the PBMC suspension was added to 10ul of Trypan Blue for a cell count with the Neubauer haemocytometer.

The PBMC pellet was resuspended to obtain roughly 3.5 x 10^6 per eppendorph in 30ul of staining buffer (1% Fetal Calf Serum, 0.09% Sodium Azide in PBS). 20ul of CRTH2-PE and 3ul of CD4-APC was added and cells were incubated for 1 hour at 37°C. 3ul of Mouse IgG1 κ –APC and 1ul of Rat IgG2a- PE Isotype controls were added to relevant tubes. Cells were washed and resuspended in PBS for analysis. Flow cytometry settings were as follows: Forward scatter E0 Voltage, 1.00 Amp gain Lin, and Side scatter of 329 Voltage, 1.00 Amp gain Lin. 50,000 cells were counted and gating was on lymphocytes based on the forward and side scatter. Lymphocytes were identified according to forward and side scatter, as shown in Figure 2.4.

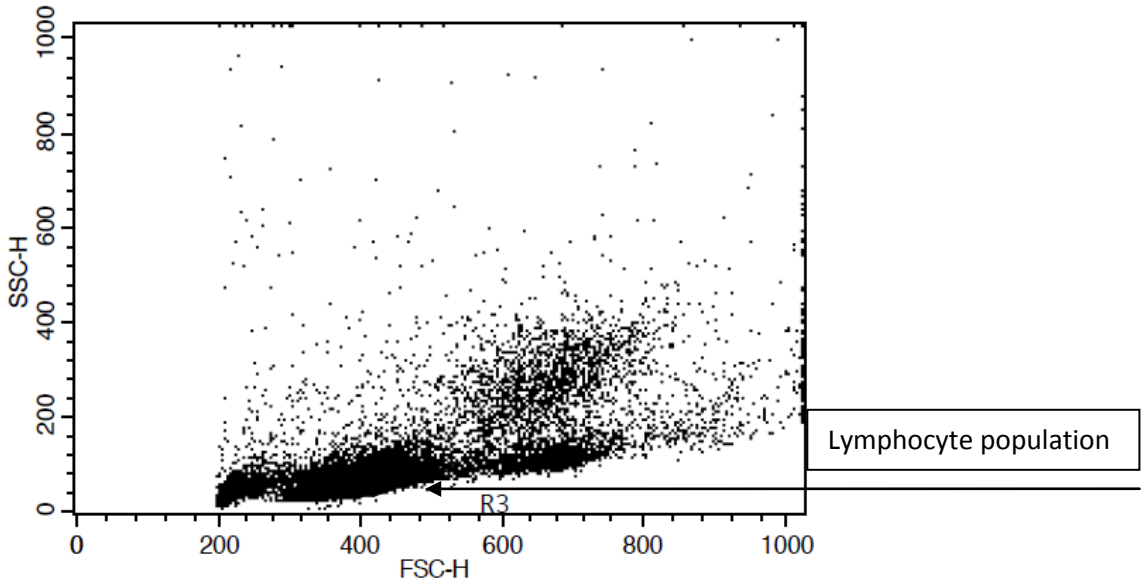


Figure 2.4 Forward and Side scatter profile of Peripheral Blood cells isolated using Ficoll Paque. Lymphocytes are the most abundant leukocytes isolated with this technique.

2.11.3 Cell surface staining of Amniocytes and Myocytes

Amniocytes

Amnion epithelial cells were prepared as described in section 2.2.1. Cell surface staining with the CRTH2-PE antibody was performed on cells before culturing and on day 1 of culture. Cultured cells were trypsinised and washed and resuspended in Robosep for staining. Cells were incubated with either 20 µl of CRTH2-PE or Isotype control 1 hour at 37 ^{o}C. The following settings were used for analysis: Forward Scatter E1; Voltage 4.77; Amp gain Lin; Side scatter of 320; Voltage 1.00; Amp gain Lin; FL2 347 log. A discrete population of amniocytes is shown in Figure 2.5.

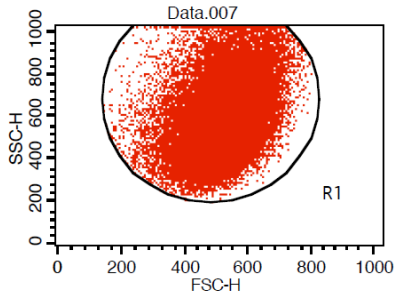


Figure 2.5 Forward and Side scatter profile of amniocytes
A discrete population of amniocytes are seen.

Myocytes

Myometrial smooth muscle cells were prepared as described in section 2.2.2. Cells were grown to 100% confluence and harvested by trypsinisation. Cells were washed and resuspended in PBS with 2% FCS for staining. Cells were incubated with 20 µl of CRTH2-PE or isotype control for 1 hour at 37^{o}C. Cells were washed with PBS and resuspended in 250 µl of PBS for analysis. The following settings were used for analysis: Forward Scatter E1; Voltage 4.37; Amp gain Lin; Side scatter of 197; Voltage log; FL2 350 log. Two discrete populations of myocytes are shown in Figure 2.6, which is likely to reflect the different spindle and more rounded cells, or the different responses seen to trypsinisation.

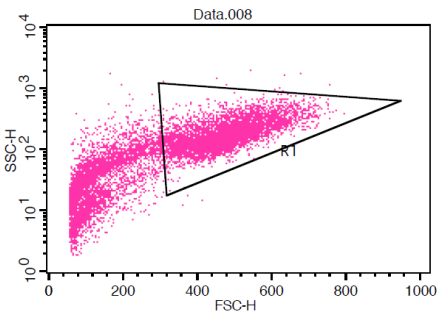


Figure 2.6 Forward and side scatter profile of myocytes The discrete population of myocytes are gated in Region 1. Myocytes appear as two populations, one is likely to reflect the spindle shaped myocytes and the other is likely to represent the more rounded myocytes.
It is also possible that these populations may reflect the recovery of the shape of the myocytes post trypsinisation.

2.11.4 Intracellular interleukin staining of PBMCs (IL-4, IFN-γ, TNF-α)

The PBMCs were isolated as described earlier. The pellet was then resuspended in 11ml of pre-warmed at 37^{o}C RPMI-1640 medium with 10% FCS and Penicillin/Streptomycin. 1ml was plated per well of a 24 well plate and incubated overnight at 37^{o}C in 5% CO_2 and 21 % O_2. 18 hours after collection 10 µg of Brefeldin A was added to each well to plug the interleukins in the Golgi Apparatus. For Interleukin 4 analysis 0.5 ul of Phorbol myristate acetate (PMA) to make (50ng/ml) and 1ul of Ionomycin to make (0.5µg/ml) were added to the appropriate wells for 5 hours of stimulation. For IFN-γ and TNF-α analysis 4 hours of PMA and Ionomycin stimulation was performed at the same concentrations as for Interleukin 4 analysis. PMA is a protein kinase C activator and Ionomycin is a Ca^{2+} ionophore and are standard *in vitro* stimuli of T cell cytokines and are used to reflect the responsiveness of T cells *in vivo*. Pre incubation with CRTH2 agonists or vehicle prior to stimulation was at 6 hours prior to harvest at the time of addition of Brefeldin A.

Prior to intracellular staining, cell surface staining was performed as described above. Directly after the wash step post cell surface staining cells were resuspended in 250µl of 2% paraformaldehyde (PFA) and incubated at 37 ^{o}C for 15minutes in the dark to fix the cell surface antibodies to their receptors. Cells were centrifuged at 1000xg at room temperature for 10 minutes and resuspended in 500 µl of 0.5% Saponin and incubated for 30 minutes on ice in the dark to permeabilize the cells. After incubation, cells were centrifuged at 1000 x g for 10 minutes and resuspended in 0.5% Saponin to make up a volume of 50 µl with the relevant antibody (20ul of IL-4 PE/Cy7, 1 µl of IFN-γ -FITC and 0.5µl of TNF-α-FITC). Relevant isotype controls were used; 0.25 µl of PE/Cy7 Rat IgG1, κ or FITC Mouse IgG1 κ. Cells were incubated for 1 hour at room temperature for IL-4 or 20 minutes on ice for IFN-γ and TNF-α in the dark. The cell suspensions were then washed with 500 µl of 0.5% Saponin and centrifuged at 1000 x g for 10 mins at 4^{o}C. The pellets were then resuspended in 250 µl of PBS for FACS analysis. Flow cytometry settings were as follows: Forward scatter E0; Voltage 1.00; Amp gain Lin; and Side scatter of 329; Voltage 1.00; Amp gain Lin.

2.11.5 Compensation adjustments for FACS analysis

Fluorochromes do not emit light at just a single discrete wavelength, but over a range of wavelengths. As a result, there may be overlap of spectral emission into the specified laser channel selected for analysis creating false positive emissions. This can be overcome through compensation. Single fluorophores for positive and negative controls were run to set the compensation percentages between channels prior to double and triple colours.

The populations of singly labeled cells should not appear in other channels and discrete populations should be clearly seen. Under-compensation occurs when signals from the singly labeled cells are being detected in the other channels. Over-compensation can also occur, resulting in the singly labeled cells off-scale on the low end in a correlated dot plot. Examples of each are shown below in Figure 2.7.

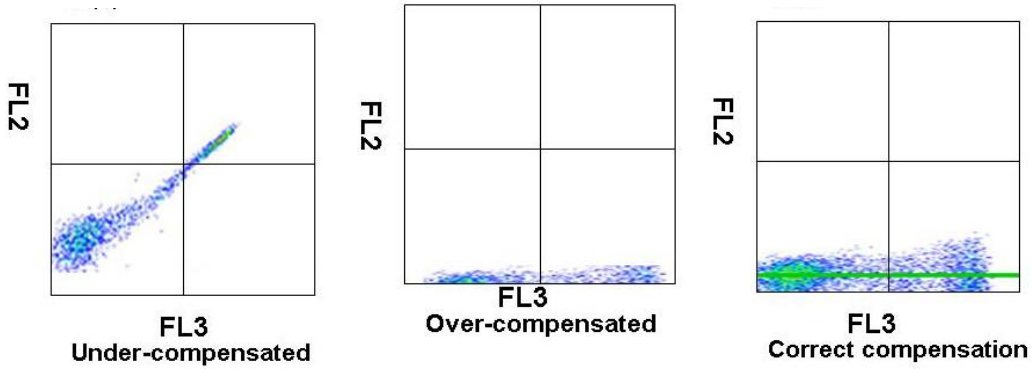


Figure 2.7 Examples of different spectral compensation
(a) Under compensation occurs when the signals detected in other channels, e.g. FL2.
(b) Over compensation occurs when the signals are off the scale.
(c) Correct compensation occurs when positive cells occur in the correct channel only.

Compensation was required for IL-4 (FL3) and CRTH2 (FL2) and CD4 (FL4) experiments. Settings required were as follows: “FL2- 20% - FL3”, “FL3 - 2.5% - FL2”, FL1= 430, FL2= 460, FL3= 465 and FL4 = 716.

2.12 Multi-spot Enzyme linked immunosorbant assay

2.12.1 Tissue preparation

For analysis of Th1:Th2 interleukins in LPS and CRTH2 agonist treated mice, maternal myometrium and pup brain was collected at 4 hours post injection and snap frozen in dry ice until processing. Whole cell lysate was prepared with the whole cell extraction buffer recommended by meso scale discovery. 100mg of tissue per 1 ml of extraction buffer was used. Following grinding of tissue with liquid nitrogen tissue was homogenised in ice-cold extraction buffer, and centrifuged for 10 minutes at 13,000rpm. Protein concentration was determined by Bradford assay (BIORAD).

2.12.2 Meso scale discovery protocol

The mesoscale discovery kit contained standards, detection antibodies, and read buffer. Standards were serially diluted and a range was pre determined with a test run of sample tissue from the tissue which was anticipated to have the lowest and highest concentration of interleukins. The 8-point standard curve ranged from 0-2500 pg/ml. While standards were being prepared the 96 well plate was blocked in 1% BSA in PBS for 1 hour on the shaker. The plate was washed in PBS-T three times, and then 40 µg of murine myometrium or 100µg of pup brain protein lysate was added in a volume of 25 µl. The plate was gently rocked for 2 hours. The plate was then washed three times with 150 µl of PBS-T and a 25 µl mix of detection antibody, Blocker B and Diluent 100 was added and gently rocked for 2 hours at room temperature. Following this the plate was washed three times in PBS-T and 150 µl of read buffer was added. The plate was read with the Sanger analyser.

2.12.3 Analysis

The multi spot Elisa plate is read by the Sanger 2400 imager using electrochemiluminescence detection techniques. The quantities of interleukins were determined against the standard curve and transferred into an excel spreadsheet.

2.13 Myometrial Contractility

2.13.1 Tissue preparation and mounting

Human tissue

Myometrium was dissected on a petri dish on ice into 8 strips of approximately 5mm x 10mm and mounted onto the DMT myograph shown below in Figure 2.8. The tissue was stretched to 4g and was allowed to equilibrate for 90 minutes in 4 mls of modified Krebs solution (Glucose 2.0g/L, Magnesium sulphate 0.141g/L, Potassium phosphate 0.16g/L, Potassium Chloride 0.35 g/L, Sodium Chloride 6.9g/L, Calcium Chloride dehydrate 0.373g/L, Sodium bicarbonate 2.1g/L, pH 7.4) and gassed with 95% O_2 and 5% CO_2.

Murine tissue

Mice where sacrificed by cervical dislocation at E16 of gestation under general anaesthetic. A midline laparotomy was performed to obtain the uterus. The uterus was transported from the animal house to the laboratory in PBS on ice and was used within 5 hours of harvesting. Myometrial strips (5mm x 10mm) were mounted on the DMT myograph along the circular or longitudinal muscle orientation, and stretched to 3g of tension. Each organ bath contained 4 mls of Krebs solution (Glucose 2.0g/L, Magnesium sulphate 0.141g/L, Potassium phosphate 0.16g/L, Potassium Chloride 0.35 g/L, Sodium Chloride 6.9g/L, Calcium Chloride dehydrate 0.373g/L, Sodium bicarbonate 2.1g/L, pH 7.4) and was gassed with 95% O_2 and 5% CO_2. Tissue was allowed to equilibrate for 30 minutes.



Figure 2.8 Image of contractility machine (800MS DMT)
8 channel organ bath contractility machine.

2.13.2 Dose response to CRTH2 agonists

Cumulative dose responses were performed after 30 minutes of spontaneous contractions were recorded to serve as baseline contractility. At the end of the experiment 10^{-7} M of Oxytocin was added to demonstrate strip viability. 0.1-100 um concentrations were added every 20-30minutes at the time of organ bath wash out. In some experiments a preincubation step with the CRTH2 antagonist x481 was performed.

2.13.3 Analysis of contractility

Contractility was analysed using the Powerlab software V 5.5.6 (ADI instruments) using the Peak parameters extension. Parameters analysed were area under the curve (work done by the tissue), peak amplitude (peak tissue tension), duration of contractions and rate of contractility as shown in Figure 2.9. Data were transferred from the datapad of the Powerlab software into an excel spreadsheet and statistical analysis was performed with Graph-Pad Prism (v5.0; GraphPad Software, San Diego, CA). Response to treatment was measured by normalising to baseline spontaneous contractility and divided by the relevant timepoint for the vehicle control.

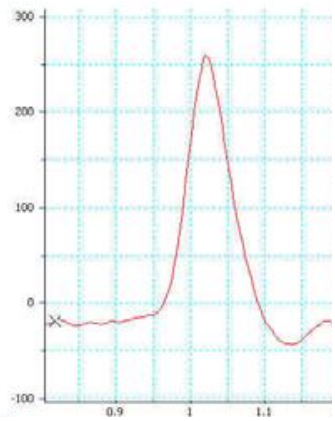


Figure 2.9 Image of a myometrial contraction
Area under the curve represents the work done by the tissue, the peak of the contraction represents the peak tension and the width of the base of the contraction represents the duration of the contraction.

2.14 Murine model of preterm labour

2.14.1 Husbandry

CD1 mice (Charles River) were housed in conditions compliant with Home Office regulations. All of the animal experiments were performed under the Home Office Project Licence (Ref 70/6906). A personal licence was obtained from the Home Office for my work, and was adhered to at all times (Ref 70/22094).

2.14.2 Surgery

Mice were time mated such that plug day was day 0. On day 16 mice were anaesthetised with Isofluorane briefly and given an intramuscular injection of Morphine 2.5mg/kg. Mice were allowed to recover, and the morphine was given 15 minutes to work. The mice were re-anaesthetised and a midline mini laparotomy was performed under aseptic conditions, the uterus was exteriorised, and an intrauterine injection of Lipopolysaccharide in a total volume of 25 µl was delivered to the right

horn between the two most distal pups. The peritoneum was closed with continuous non-locking sutures with 2/0 vicryl, and the skin was sutured with interrupted 2/0 vicryl. The mice were recovered in a warm incubator until active and were then housed separately. Mice were either sacrificed at 4.5 hours post injection for harvesting myometrium and pup brain, or allowed to deliver spontaneously. Continuous monitoring was achieved via infrared CCTV system that was viewed remotely.



Figure 2.10 CD1 mouse anaesthetised at time of intrauterine injection at laparotomy
The uterus is exteriorised briefly and the LPS is administered between the two most distal pups of the right horn by Hamilton syringe.

2.14.3 Dose response

A dose response for the Lipopolysaccharide was first performed to obtain the lowest dose at which preterm delivery was consistently obtained. Control mice were given PBS alone. A dose of 20 µg was chosen for the model of infection induced preterm labour.

2.14.4 CRTH2 agonist administration

In experiments where the CRTH2 agonist Pyl A was used, a separate Hamilton syringe was used first to administer the 250 µg of Pyl A between the second and third most distal pups. In those mice which received LPS also, another syringe was used to administer the LPS between the two most distal pups of the right horn.

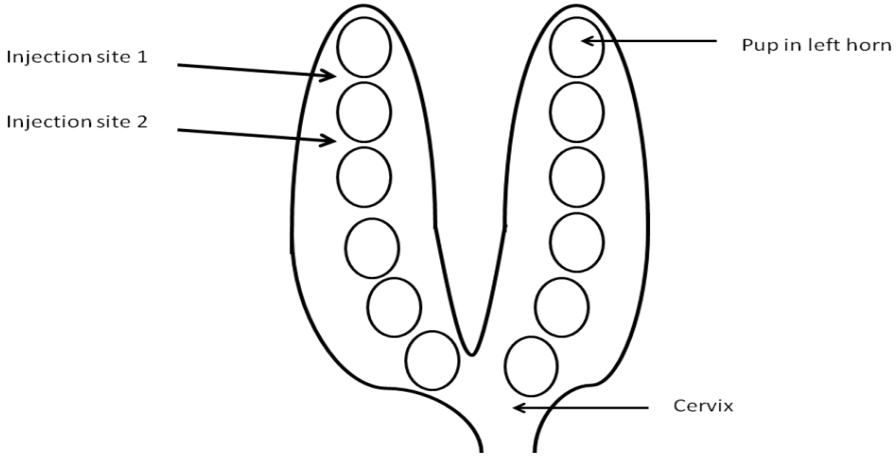


Figure 2.11 Murine bicornuate uterus demonstrating site of intrauterine injection
LPS is administered between the two most distal pups of the right horn at site 1. Site 2 is used for administration of the CRTH2 agonist.

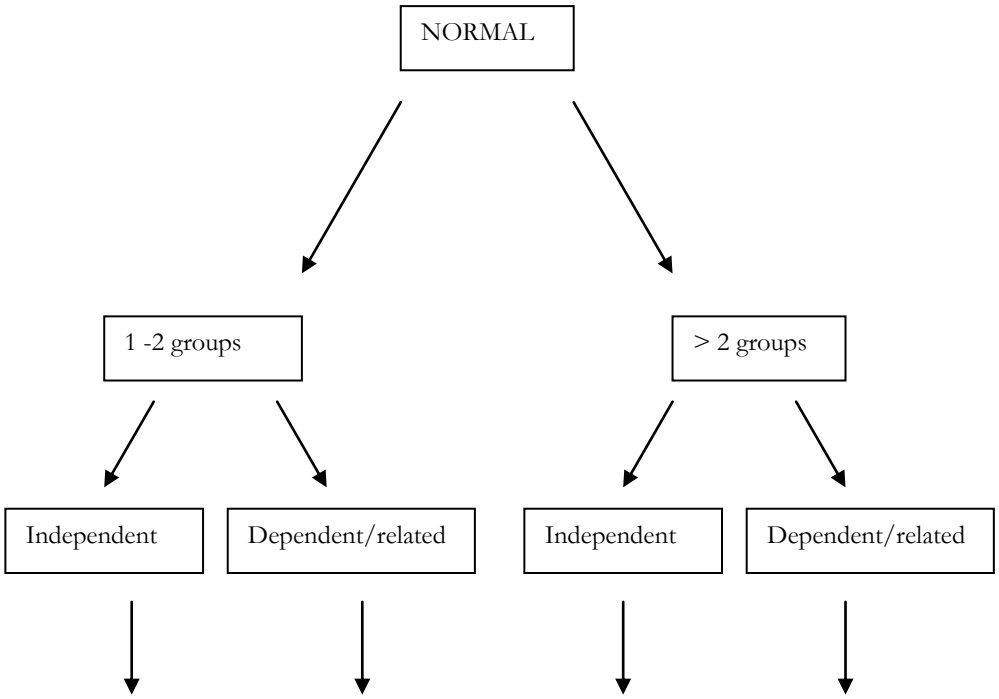
2.14.5 Tissue harvesting

For tissue harvesting, mice were anaesthetised and sacrificed by cervical dislocation. A laparotomy was performed immediately and pups were sacrificed by decapitation in accordance with Schedule 1. Myometrium and pup brain were snap frozen and stored at -80 ^{o}C until processing.

2.15 Statistical analysis

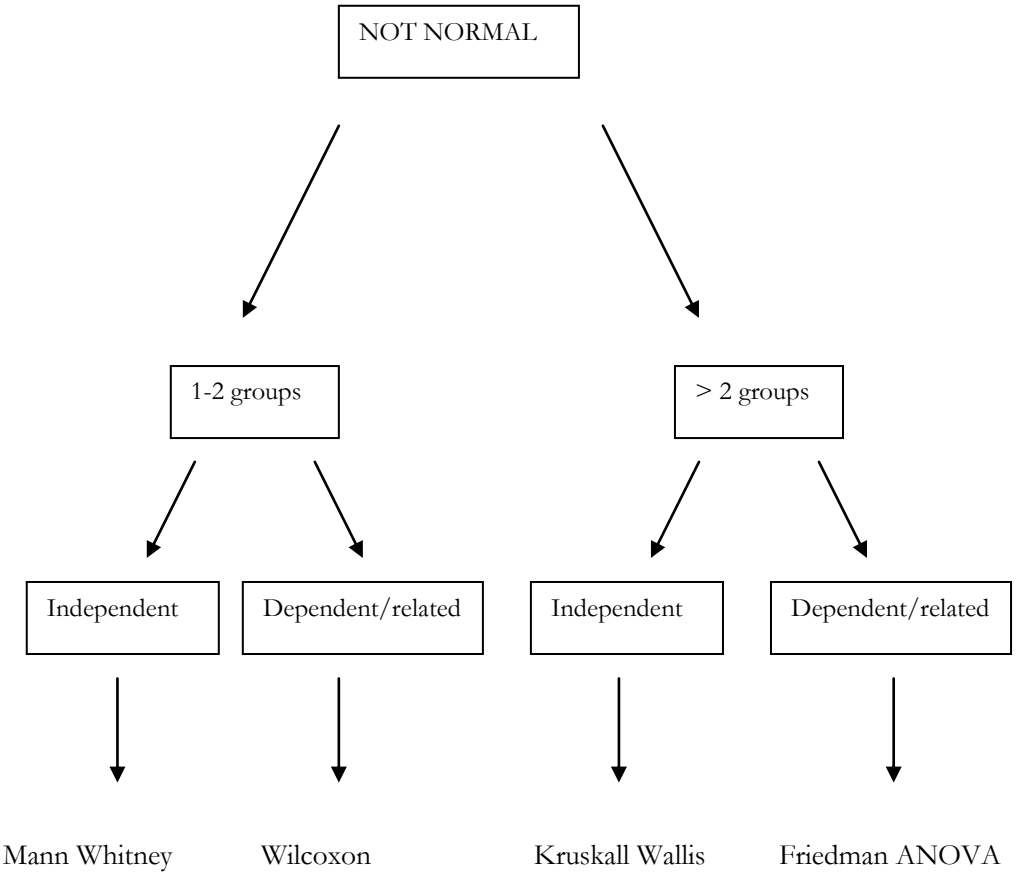
All data was processed firstly with Microsoft Excel 2007. Statistical analysis was performed with Graph-Pad Prism v5 (GraphPad Software, San Diego, CA). The correct statistical test was carried out depending on normality and the number of groups analysed. The flow diagram below was used as an aid to determining the correct statistical test. More detail on statistical analysis will be given in each results section.

A. Flow diagram to determine statistical test for data with normal distribution



* For Catagorical dependant variable and continuous outcome variable the difference in means between groups needs to be normal rather than the sample distribution itself

B. Flow diagram to determine statistical test for data with non-parametric distribution



Figures 2.12 A and B. Flow diagrams to aid in determining the correct statistical test to be used The flow diagram in A was used for normally distributed data, and the flow diagram in B was used if the data was not normally distributed.

CHAPTER 3

CRTH2 expression

Chapter summary

Hypothesis:

$15dPGJ_2$ is a CRTH2 agonist. Our group has previously shown that it inhibits NF-κB in human amniocytes and myocytes, and also delays preterm labour in the murine model of inflammation induced preterm labour. It is possible that CRTH2 plays a role in these effects seen with $15dPGJ_2$.

Aims:

Firstly, data presented in this chapter aims to determine if the CRTH2 receptor is expressed in gestational tissues including amnion, choriodecidua, placenta and myometrium, and at the cellular level in amniocytes and myocytes. Secondly, the expression profile of CRTH2 in T helper cells was examined at different stages of pregnancy and in labour, to determine if there is a potential up regulation of CRTH2 in pregnancy, consistent with the Th2 bias that exists in pregnancy.

Methods:

mRNA expression was determined by PCR. Protein expression was explored using western analysis and flow cytometry.

Results:

CRTH2 is not present at the protein level in amniocytes and myocytes. There is no statistically significant increase in CRTH2 expression in lymphocytes during pregnancy, and no difference in expression prior to the onset of labour compared with during labour.

Conclusion:

It is unlikely that CRTH2 plays a role in $15dPGJ_2$-mediated inhibition of NF-κB since CRTH2 is not expressed in amniocytes and myocytes, although it may still play a role in the delay in preterm labour and increased pup survival in the murine model. Despite not seeing a significant increase in CRTH2 expression on Th2 cells during pregnancy, an increase in the functional activity of the receptor during pregnancy is still possible.

Introduction

$15dPGJ_2$ inhibits NF-κB in cultured human amniocytes and myocytes in a mechanism that remains to be fully explained [271]. $15dPGJ_2$ also delays preterm labour and increases pup survival in the murine model of inflammation induced preterm labour [277]. $15dPGJ_2$ is an agonist of the CRTH2 receptor and therefore this receptor could play a role in NF-κB inhibition. CRTH2 is the second prostaglandin D_2 receptor, first discovered by Nagata et al in a search to find a specific marker expressed in Th2 cells but not Th1 cells [291]. It has been shown to be expressed on Th2 cells, eosinophils, basophils and monocytes at the protein level [289]. However, there are limited data on the expression profile of CRTH2 non-immune cells, gestational tissues and myometrium.

The function of CRTH2 appears to be important in maintaining a Th2 type interleukin rich environment, with PGD_2 inducing IL-4, IL-5 and IL-10 production *in vitro* [217]. Since endogenous PGD_2 and $15dPGJ_2$ are found in the amniotic fluid, it is plausible that CRTH2 is expressed in the amnion. Tsuda et al have shown a higher percentage of CRTH2 positive decidual T cells in women undergoing elective termination compared to women with spontaneous miscarriage [300]. They suggested a role for CRTH2 in the maintenance of pregnancy, by providing a Th2 rich cytokine environment at the maternal-fetal interface [198]. It is possible that CRTH2 expression in peripheral blood also aids in the maintenance of a bias in the Th1:Th2 cytokine ratio in favour of Th2 profile.

In this chapter, the expression profile of CRTH2 in fetal membranes, placental and myometrial tissue along with expression at the cellular level in amniocytes and myocytes was explored. The trend in expression of CRTH2 in Th2 cells through gestational time points was examined, with a comparison of the expression between pre-labour and in labour samples, and between term labour and preterm labour samples.

Results

3.1 *CRTH2* mRNA

CRTH2 mRNA expression pattern was analysed by PCR. Standard PCR was run with 35 cycle replications and RT-PCR was run at up to 40 cycles. mRNA from peripheral blood mononuclear cells (PBMCs) isolated from non-pregnant human blood was used as a positive control, and non-template water controls or non reverse transcriptase samples used as negative controls. Four primer sets were used (see Table 3).

Initial experiments revealed detectable levels of CRTH2 mRNA in amniocytes, choriodecidua, myocytes and placenta with two primer sets (Figure 3.1.1A and B). Water control samples were introduced along with a third primer set (Figure 3.1.2A and B). Experiments were then tailored to specific tissue/cell types using primer set A and B.

mRNA from fetal membranes and myometrium was examined using primer set A (n=5). Amnion, myometrium, choriodecidua and placenta were collected from women at the time of pre-labour caesarean section. CRTH2 mRNA was detected in all five samples of amnion (A), myometrium (B), choriodecidua (C) and placenta (D) using PBMCs as a positive control and water non-template water negative control (Figure 3.1.3 A-D). Although the aim of this was qualitative rather than quantitative, very faint bands were seen in both choriodecidual and amnion samples, suggesting low expression.

CRTH2 mRNA levels at the cellular level were next investigated. CRTH2 was amplified by standard PCR with primer set A and by RT-PCR which primer set B, and products were subjected to agarose gel electrophoresis. Detectable levels where seen in 4 of 6 amniocyte samples (Figure 3.1.4A) and all 6 myocyte samples (Figure 3.1.4B). PBMCs were used as a positive control, with reverse transcriptase negative controls.

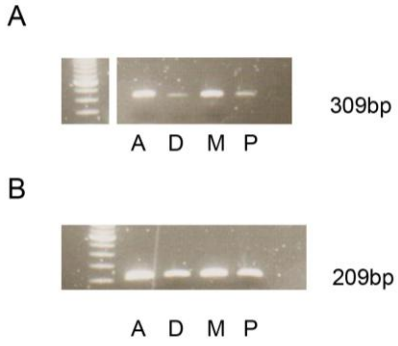


Figure 3.1.1 CRTH2 mRNA initial detection
mRNA was isolated from cultured amniocytes, myocytes, choriodecidual and placental biopsies and converted to cDNA. Qualitative PCR was used with two primer sets to amplify CRTH2 showing product sizes of 309bp (A) and 209bp (B). A&B: amniocytes (lane 1), choriodecidua (lane 2), myocytes (lane 3), placenta (lane 4). (A= amniocytes, D= choriodecidua, M= myocytes, P= placenta)

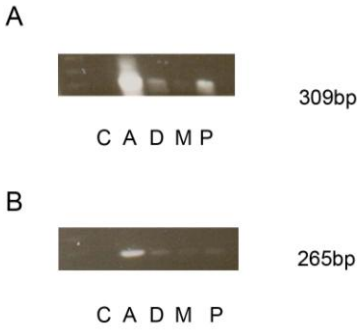


Figure 3.1.2 CRTH2 mRNA with controls
The same cDNA samples from figure 3.1.1 were used, along with a negative control (non-template) and amplified by PCR using two primer sets showing correct product sizes of 309bp (A) and 265bp (B). A&B: non-template control (lane 1), amniocytes (lane 2), choriodecidua (lane 3), myocytes (lane 4), placenta (lane 5). (C= control, A= amniocytes, D= choriodecidua, M= myocytes, P= placenta)

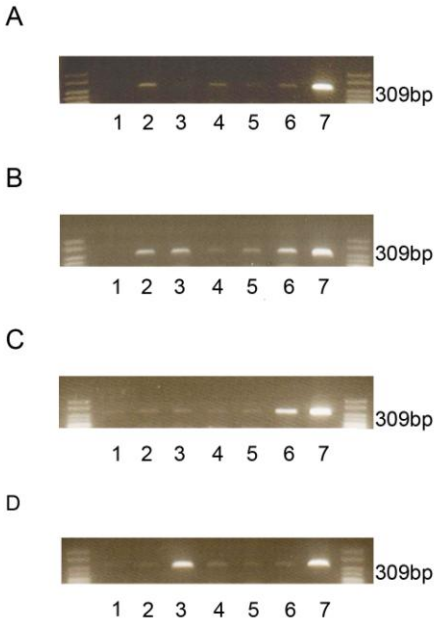


Figure 3.1.3 CRTH2 mRNA in tissue of fetal membranes, myometrium and placenta
mRNA was isolated from tissue biopsies of fetal membranes, placenta and myometrium and converted to cDNA (n=5). Qualitative PCR was used to amplify CRTH2 showing the correct product size of 309bp in amnion (A), myometrium (B), choriodecidua (C) and placenta (D). Non-template negative controls were used and peripheral blood mononuclear cells (PBMCs) were used as a positive control. A-D: Non-template control (lane 1), tissue samples (lanes 2-6), PBMCs (lane 7).

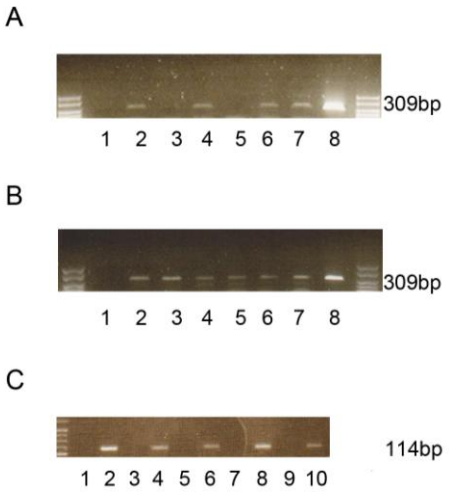


Figure 3.1.4 CRTH2 mRNA in amniocytes and myocytes
mRNA was isolated from cultured amniocytes and myocytes and converted to cDNA (n=6). Qualitative PCR was used to amplify CRTH2 showing the correct product size of 309bp in amniocytes (A) and myocytes (B) (n=6 samples each) using non-template negative controls and PBMCs as a positive control. Products from RT-PCR using another primer set were run out on a gel showing the correct size of 114bp for amniocytes and myocytes (n=2 samples each) using reverse transcriptase negative controls and PBMCs as a positive control. A&B: Non-template control (lane 1), cell samples (lanes 2-7), PBMCs (lane 8). C: Reverse transcriptase controls (lanes 1,3,5,7,9), PBMCs (lane 2), amniocytes (lanes 4,6), myocytes (lanes 8,10).

3.2 Identification of endogenous CRTH2 protein by western analysis

Western analysis was used for the detection of CRTH2 in myometrium and fetal membranes, performed as described in section 2.10. The Sc 23092 antibody was used for detection and its data sheet demonstrates CRTH2 at a molecular weight of 34kDA. Human heart (kindly donated by Dr Grisha Pirianov) and peripheral blood mononuclear cell protein lysates were used as positive controls. Amnion, choriodecidua, placenta and myometrium gave strong bands at 55 correlating with the positive controls, with no bands seen at 34kDa, Figure 3.2.1 A, B, C and D respectively. A second antibody was also used (ProSci 4027) as shown in Figure 3.2.1 E, F, G, H. However, a non-specific band was detected at 70kDA which did not show up in the human heart and peripheral blood mononuclear cell control samples.

CRTH2 protein expression was also investigated at the cellular level in amniocytes and myocytes. A faint band at 55 was seen in amniocyte cell lysate, however this band was as strong as many other bands seen on the blot which is suggestive of being non-specific (Figure 3.2.2A). No band was detected with myocyte protein lysate (Figure 3.2.2B).

The presence of the band at 55 could represent the glycosylated from of CRTH2. Since no band was detected at the cellular level, (and if this in the correct band), it is possible that infiltrating leukocytes account for the expression seen in the tissue.

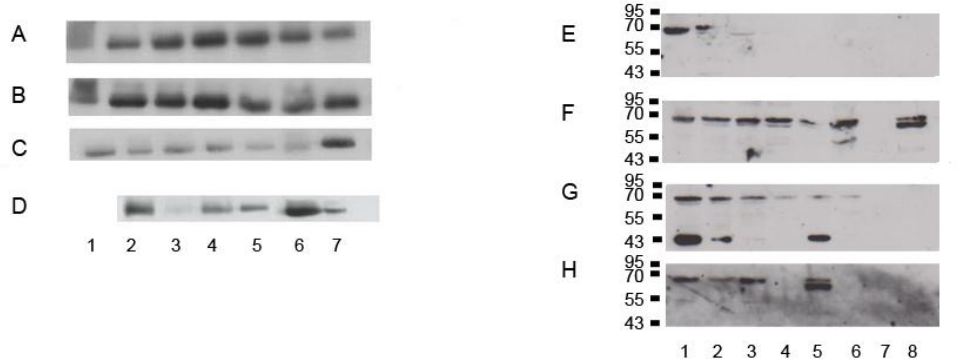


Figure 3.2.1 Detection of CRTH2 in gestational tissues and myometrium
Protein lysate from gestational tissues and myometrium were subjected to western analysis. **A-D**; Immunoblots were probed with the SC-23092 CRTH2 antibody. Blot A represents amnion, blot B choriodecidua, blot C placenta, blot D myometrium. Lanes 1-3 of blots A-C represent labour minus samples, and lanes 4-6 of blots A-C represent labour plus samples. Lane 7 of blots A-C contains protein lysate from human heart as a positive control. Lanes 2-4 of blot D represent labour minus myometrium, lanes 5,6 and 7 are positive controls; human heart, PBMCs sample 1 and PBMCs sample 2 respectively. A single band appeared on the immunoblot at 55kDa. No band was seen at 34kDa. E-H; Immunoblots were probed with the ProScience 4027 antibody. Blot E represents amnion in lanes 1-6, lane 7 human heart, lane 8 white blood cells. Blot F represents choriodecidua in lanes 1-6, lane 7 human heart, lane 8 mouse heart. Blot G represents placenta in lanes 1-6, lane 7 human heart, lane 8 white blood cells. Blot H represents myometrium in lanes 1-3, lane 4 human heart, lane 5 mouse heart, lanes 6-8 white blood cells.

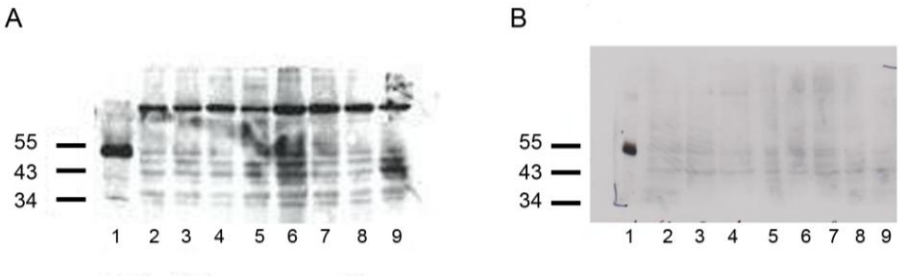


Figure 3.2.2 Detection of CRTH2 in amniocyte and myocyte whole cell lysate
Protein lysate from cultured amniocytes and myocytes were subjected to western analysis. Immunoblots were probed with the SC-23092 CRTH2 antibody. Blot A represents amniocytes (n=8) and blot B represents myocytes (n=8). Human heart was used as a positive control in blot A and B in lane 1. A strong single band appeared on the immunoblot at 55kDa with the positive control only. No significant band was seen at 34kDa in either amniocytes, myocytes or positive control.

3.3 Sequencing the band

In an attempt to confirm that the band persistently seen at 55 was CRTH2, N-terminal protein sequencing of the band on these immunoblots was carried out by the Biochemistry department at Cambridge University. Figure 3.3A shows an example of the protein bands that were sequenced, from PBMCs, myometrium, human heart and amnion protein lysate. The bands were cut off from the immunoblot shown in Figure 3.3B for sequencing. CRTH2 was not identified from these bands at 55kDa. Keratin and Immunoglobulin heavy chain proteins were seen, possibly through contamination which is a recognised limitation of this technique. If CRTH2 was present, it was masked by other proteins of far greater concentration.

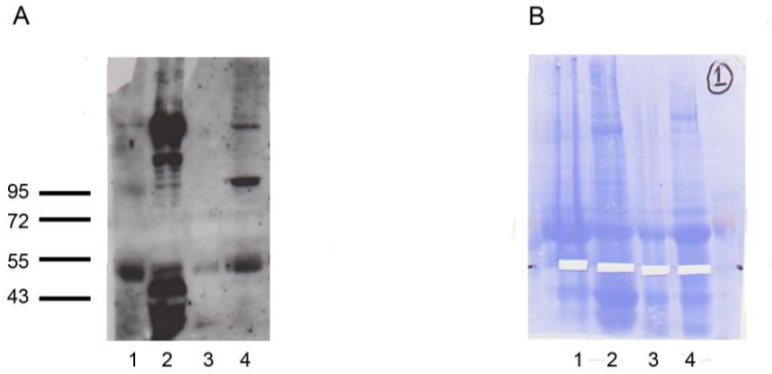


Figure 3.3 Sequencing of the band at 55kDa by N-terminal sequencing
Protein lysate from PBMCs (lane 1), myometrium (lane 2), human heart (lane 3), and amnion (lane 4), were subjected to western analysis. Blot A was probed with primary and secondary antibodies to detect CRTH2. Blot B was stained with methylene blue and the bands at 55kDa were cut out and underwent N-terminal sequencing.

3.4 Cloning CRTH2

In order to provide a highly concentrated protein lysate of CRTH2 for a positive control CRTH2 was cloned. The 1.18kb CRTH2 transcript was amplified from white cell cDNA as described in section 2.7. The product was run out on a gel to confirm amplification and the correct product size (Figure 3.4A). The PCR was repeated in order to cut the band containing the cDNA under the UV light to purify ready for insertion into the intermediate vector pCR®-BluntII-TOPO®, figure 3.4B. The presence of CRTH2 was confirmed by digesting at the BAMH I and BGL II insertion site to produce two products: the sPG5 expression vector and the 1.18kb CRTH2 insert (Figure 3.4C). The transcript was also sequenced to confirm the presence of CRTH2 and to confirm that it was correctly orientated in the expression vector.

The cell free *in vitro* transcription translation kit (TNT kit) was used with 35^S Methionine to confirm the correct size of the protein product. Detection of the 34kDa CRTH2 product was by x-ray film (Figure 3.4D). The progesterone expression vector (a kind donation by Dr Yun Lee) was used as a positive control confirming the effectiveness of the TNT kit. The CRTH2 product roughly correlated with the Kozak rule [306], and was consistent with the CRTH2 transfect produced by Nagata et al [291]. The product size also matched the expected size of CRTH2 detected by the Santa Cruz antibody SC-23092 as published in the data sheet.

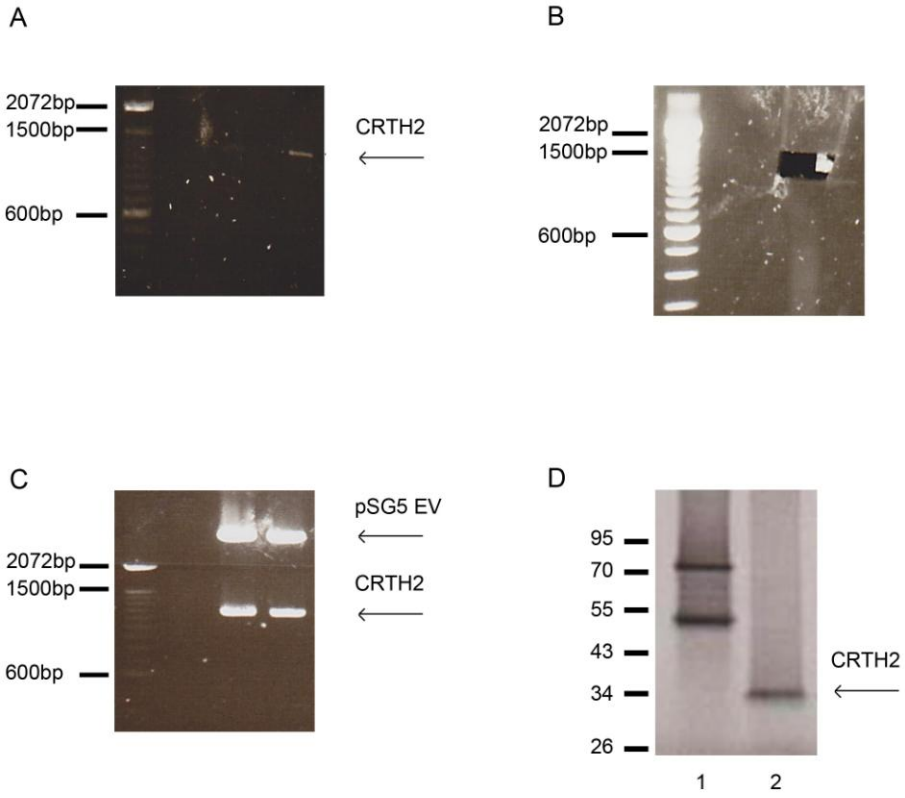


Figure 3.4 Cloning CRTH2

A 1.18kb transcript was amplified from white cell cDNA (A). This transcript was purified for insertion into an intermediate vector (B). The expression vector containing CRTH2 was digested to confirm the presence of CRTH2 (n=2) (C). The *in vitro* transcription translation kit was used to produce CRTH2 with 35^S Methionine and the product was detected by x-ray film, lane 2 (D). The progesterone (PRC 301) expression vector was used as a positive control, lane 1 (D).

3.5 Western of cloned CRTH2

Knowing that the pSG5 expression vector was functional I then sought to determine if the commercially available antibodies were able to detect CRTH2. Firstly, I used the protein lysate from the cell free system; both the exact lysate seen in Figure 3.4D as well as a non-radiolabelled lysate and performed western analysis with these products. As negative controls, the same volume from the kit and the progesterone lysate were used. The immunoblots were probed with three commercially available antibodies designed for western blotting. No band was detected at 34kDa, with non-specific bands appearing also in the negative control of the TNT kit without plasmid DNA (Figure 3.5.1).

Secondly, amniocytes were transfected with CRTH2 using GeneJuice and Fugene transfection reagents. Whole cell protein lysate was subjected to western analysis, using PBMC lysate as a positive control (Figure 3.5.2). No band was seen at the expected 34kDa size. A band was seen at 55 in blot A with the SC-23092 antibody, however, this corresponds with the band that was sequenced and found not to be CRTH2. A secondary antibody control blot was also included, which also showed a band at 55, consistent with this band being non-specific. Upon probing with the ProSci antibody, non-specific bands were also seen at 70 and 43kDA (Blot B). These bands were not detected in the positive control samples, and no difference in intensity was seen between non-transfected and transfected lysate.

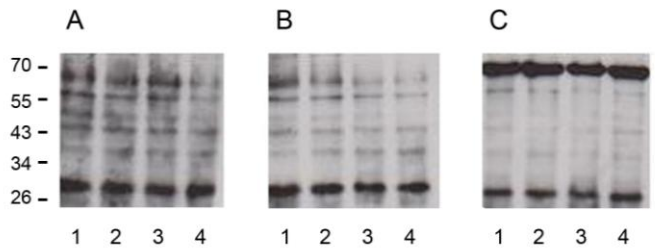


Figure 3.5.1 Detection of CRTH2 produced in the cell free system by immunoblot
The protein lysate from the TNT kit was subjected to western analysis; negative control: TNT kit with no plasmid DNA (lane 1), negative control: progesterone PSG5 expression vector (lane 2), positive control: 35^{S} Methionine labelled CRTH2 (Lane 3), cold Methionine CRTH2 protein product (lane 4). Membranes were probed with 3 commercially available antibodies; SC-23092 (A), SC-21798 (B), ProSci 4027 (C). None of the antibodies detected CRTH2.

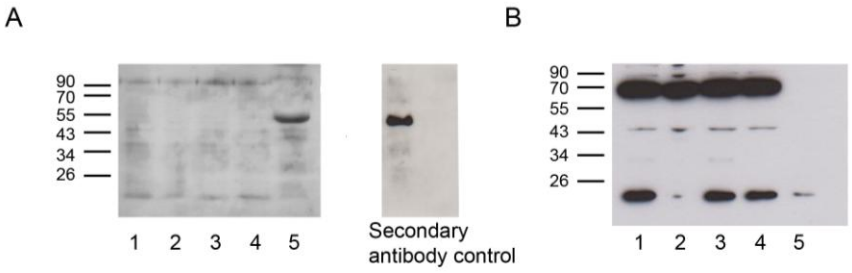


Figure 3.5.2 Detection of CRTH2-transfected amniocytes by immunoblot
The protein lysate from non transfected and transfected amniocytes was subjected to western analysis. Blot A was probed with the SC-23092 antibody and blot B was probed with the ProSci 4027 antibody. Human peripheral blood cell protein lysate was used as a positive control. Protein lysates from myocytes (lane 1), amniocytes (lane 2), Genejuice-transfected amniocytes (Lane 3), Fugene-transfected amniocytes (lane 4), and PBMCs (lane 5) were examined. Blot A failed to show a band at the expected 34kDa. However, it did show the consistently seen band at 55. This band was confirmed to be non-specific with the secondary antibody control. The Proscience antibody failed to detect a band in the positive control.

3.6 FACS of endogenous CRTH2

Flow cytometry is the most commonly reported technique used for the identification of CRTH2. Many papers have successfully demonstrated expression mainly in immune cells, but also in some non-immune cells. Having failed to demonstrate sensitivity of commercially available antibodies suitable for western analysis I used a flow cytometry antibody, as used by Chiba et al, firstly for demonstrating CRTH2 expression on peripheral blood lymphocytes, and secondly to examine endogenous and transfected expression of CRTH2 in amniocytes and myocytes.

3.6.1 Detecting CRTH2 in human lymphocytes by flow cytometry

Peripheral blood mononuclear cells were isolated as described in section 2.2.3 by Ficoll separation. Subjects were non-pregnant women without asthma or active infection, (n=6). Lymphocytes were determined on the basis of forward and side scatter profile as shown in Figure 3.6.1.1A. CRTH2 cell surface staining was performed as described in section 2.11. Example histograms are shown for cells with no staining, isotype control and CRTH2-PE (Figure 3.6.1.1B). Graphs are shown in Figure 3.6.1.2 summarising the percentage of lymphocytes labelled with the fluorophore phycoerythrin (PE), and the mean, geometric mean and median. A mean of 2.15% of lymphocytes express CRTH2, with a background staining of 2.54% in the isotype control indicating non-specific background staining and a low level of expression. However, CRTH2 expression is clearly demonstrated by the high mean and median fluorescence intensity seen with CRTH2-PE compared with isotype control. The mean, geo mean and median fluorescence intensity of CRTH2 positive cells were 483.32, 218.09, and 342.13 respectively compared with 61.60, 23.63 and 18.15 for isotype control ($P< 0.0001$ for each parameter), (Figures 3.6.1.2 A-D).

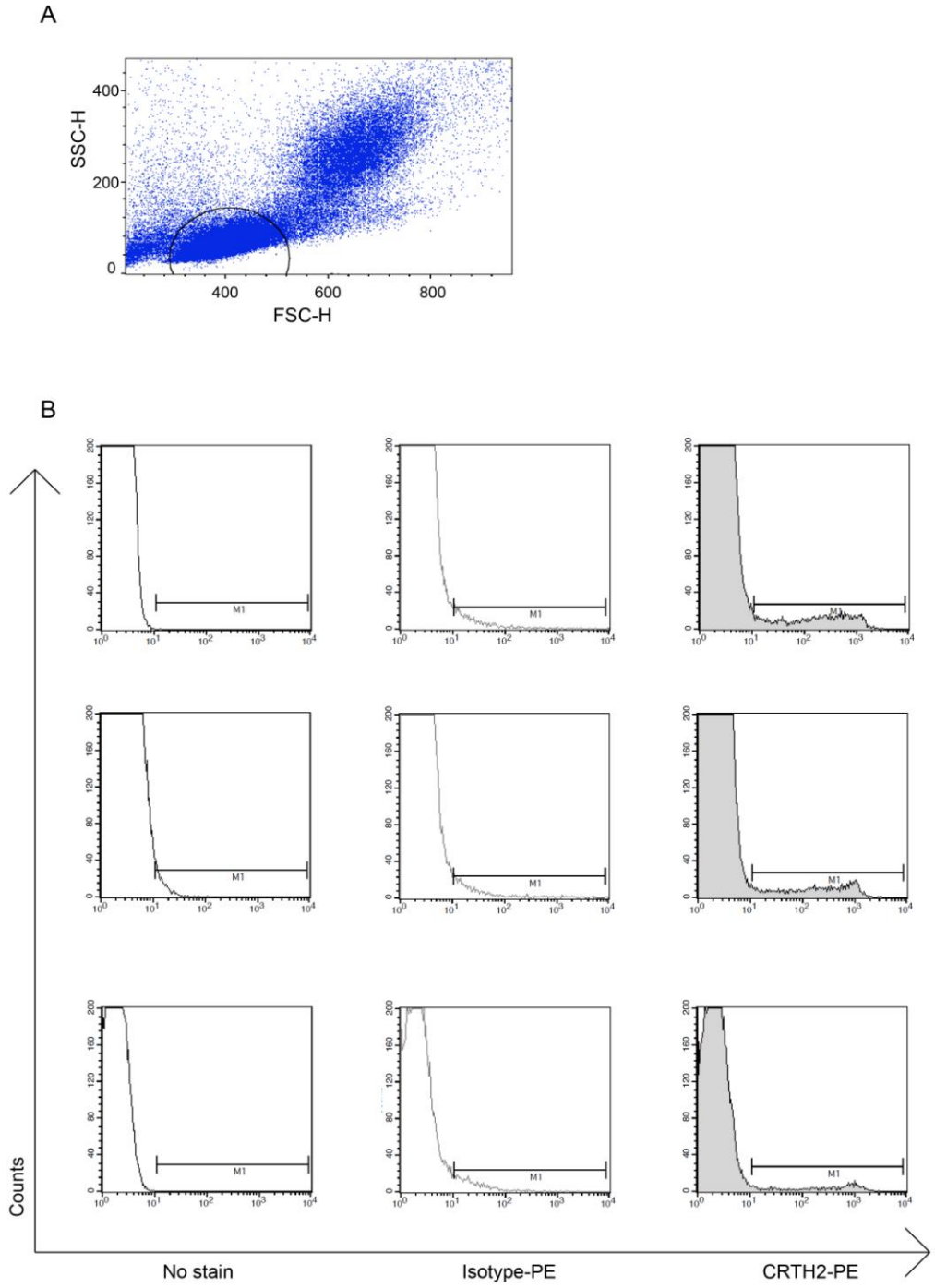


Figure 3.6.1.1 CRTH2 expression in human lymphocytes
Lymphocytes were gated according to forward scatter and side scatter (n=6) A representative dot plot shows the lymphocyte population (A). Representative histograms of three subjects are shown for no staining, isotype controls and CRTH2 stained cells (B). Cells expressing CRTH2 are shown towards the right of the histogram.

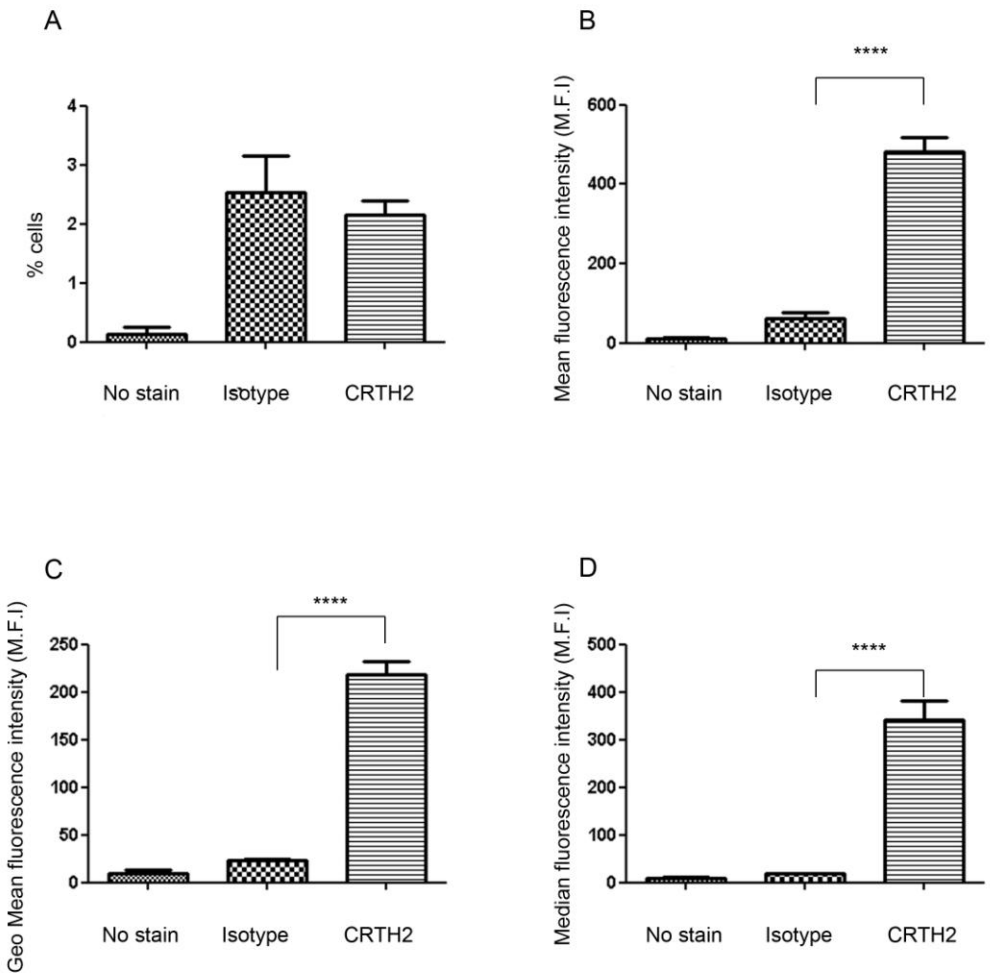


Figure 3.6.1.2 Graphs showing CRTH2 expression in human lymphocytes
The mean percentage of CRTH2 positive lymphocytes is 2.15%. High background staining with the isotype is evident with 2.54% percent of cells binding to the isotype control (A). CRTH2 expression is clearly seen as reflected by a higher mean (B), geo mean (C) and median (D) fluorescence compared with isotype control. For statistical analysis One way ANOVA was used with Bonferroni's *post hoc* analysis; **** $P<0.0001$.

3.6.2 Exploring CRTH2 expression in amniocytes by flow cytometry

Cultured primary amniocytes from amnion of pre-labour caesarean sections were prepared as described in section 2.2, (n=6). Cells were harvested on day 3 and labelled with CRTH2-PE. A representative dot blot shows the amniocyte population in Figure 3.6.2.1A. The histograms of three representative subjects are shown in Figure 3.6.2.1B, showing cells with no stain, isotype control and CRTH2-PE. There was no significant difference between isotype controls and CRTH2 labelled samples with all parameters (percentage of labelled cells, mean and median fluorescence intensity), indicating no endogenous expression of CRTH2 on amniocytes (Figures 3.6.2.2A-D).

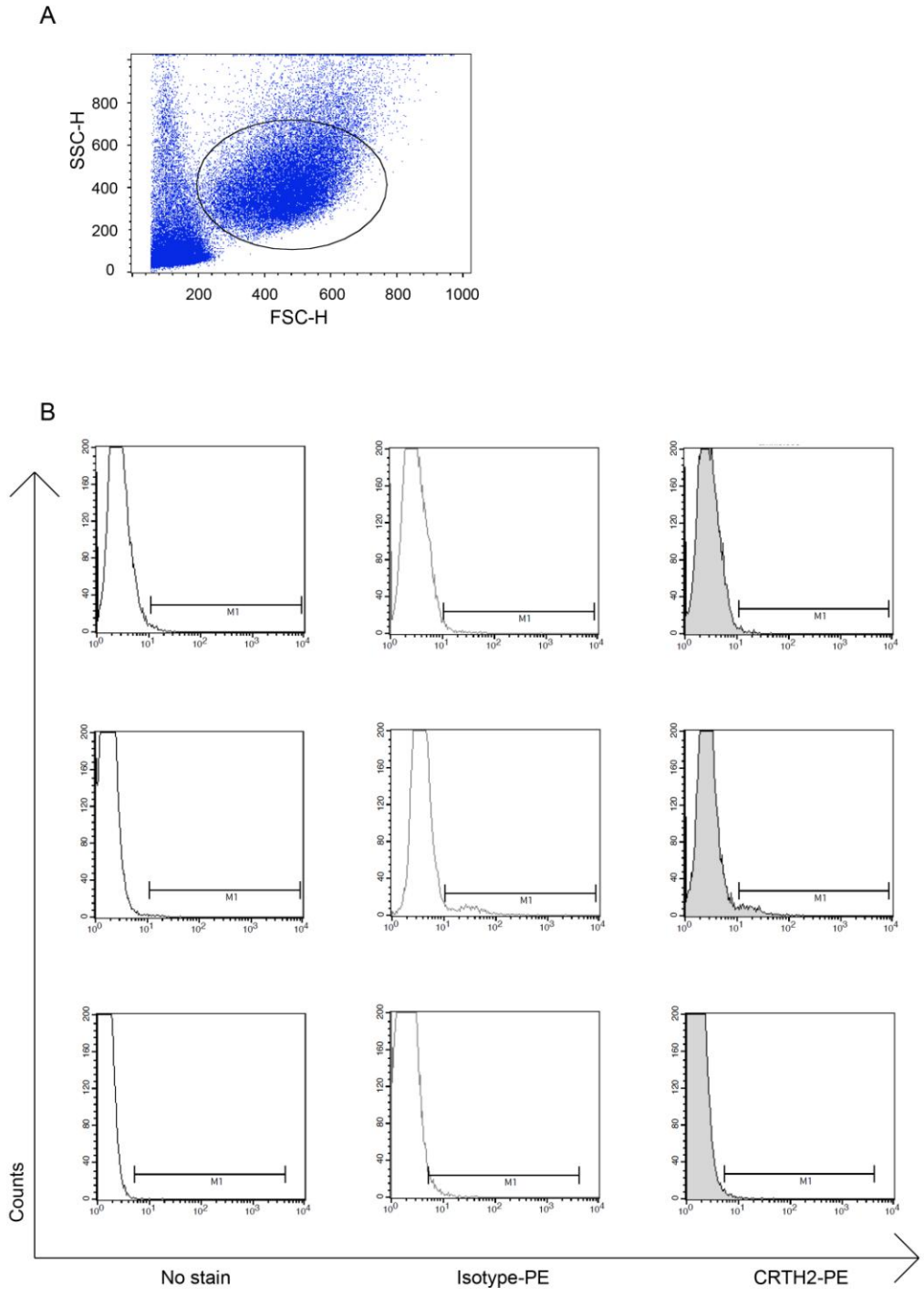


Figure 3.6.2.1 CRTH2 is not present on human amniocytes
CRTH2 expression on amniocytes was examined by flow cytometry (n=6). A representative dot plot shows the amniocyte population (A). Representative histograms of three subjects are shown for no staining, isotype controls and CRTH2 stained cells (B). No difference is seen between the histograms of the isotype controls and CRTH2 stained samples reflecting no expression of CRTH2 on amniocytes.

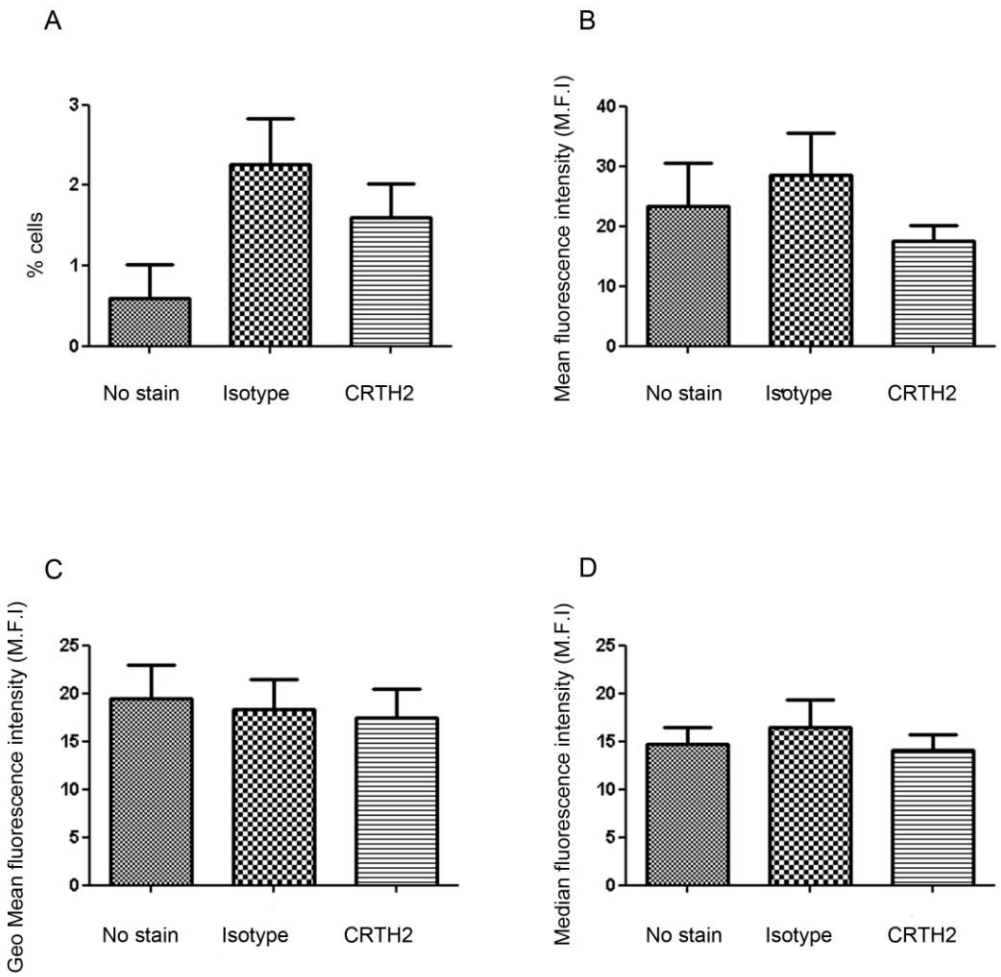


Figure 3.6.2.2 Graphs showing no expression of CRTH2 expression in amniocytes
CRTH2 expression on amniocytes was examined by flow cytometry (n=6). There was no significant difference in mean (B), geo mean (C) or median fluorescence intensity (D) between isotype control and CRTH2 labelled samples indicating no expression of CRTH2. There was a higher percentage of cells which stained with the isotype control compared with CRTH2-PE (A). For statistical analysis ANOVA of repeated measured was used with Bonferroni's *post hoc* analysis.

3.6.3 Exploring CRTH2 expression in myocytes by flow cytometry

Cultured myocytes from myometrium of pre-labour caesarean sections were prepared as described in section 2.2 (n=6). Cells were harvested at passage 1-3 and labelled with CRTH2-PE. A representative dot blot shows the myocyte population in Figure 3.6.3.1A. The histograms of three representative subjects are shown in Figure 3.6.3.1B, showing cells with no stain, isotype control and CRTH2-PE. There was no significant difference between cells with no staining and CRTH2 labelled samples with all parameters of (percentage of labelled cells, mean and median fluorescence intensity), indicating no expression of CRTH2 on myocytes, (Figures 3.6.3.2A-D). There was a higher mean, geo mean and median fluorescence intensity in the isotype control samples compared with the CRTH2-PE samples indicating a degree of background non-specific staining.

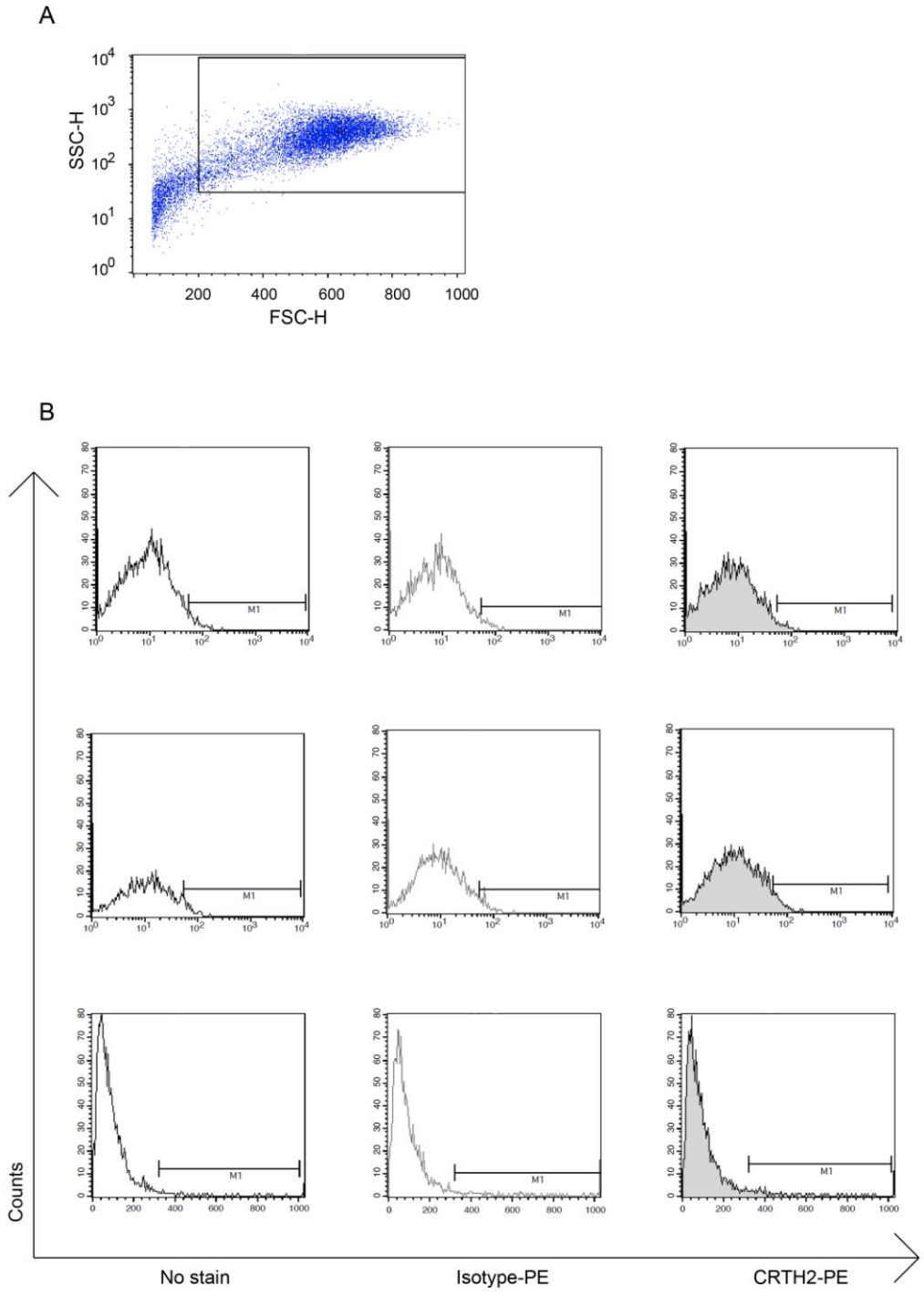


Figure 3.6.3.1 CRTH2 is not present on human myocytes
CRTH2 expression on myocytes was examined by flow cytometry (n=6). A representative dot plot shows the myocyte population (A). Representative histograms of three subjects are shown for no staining, isotype controls and CRTH2 stained cells (B). No difference is seen between the histograms of the isotype controls and CRTH2 stained samples reflecting no expression of CRTH2 on myocytes.

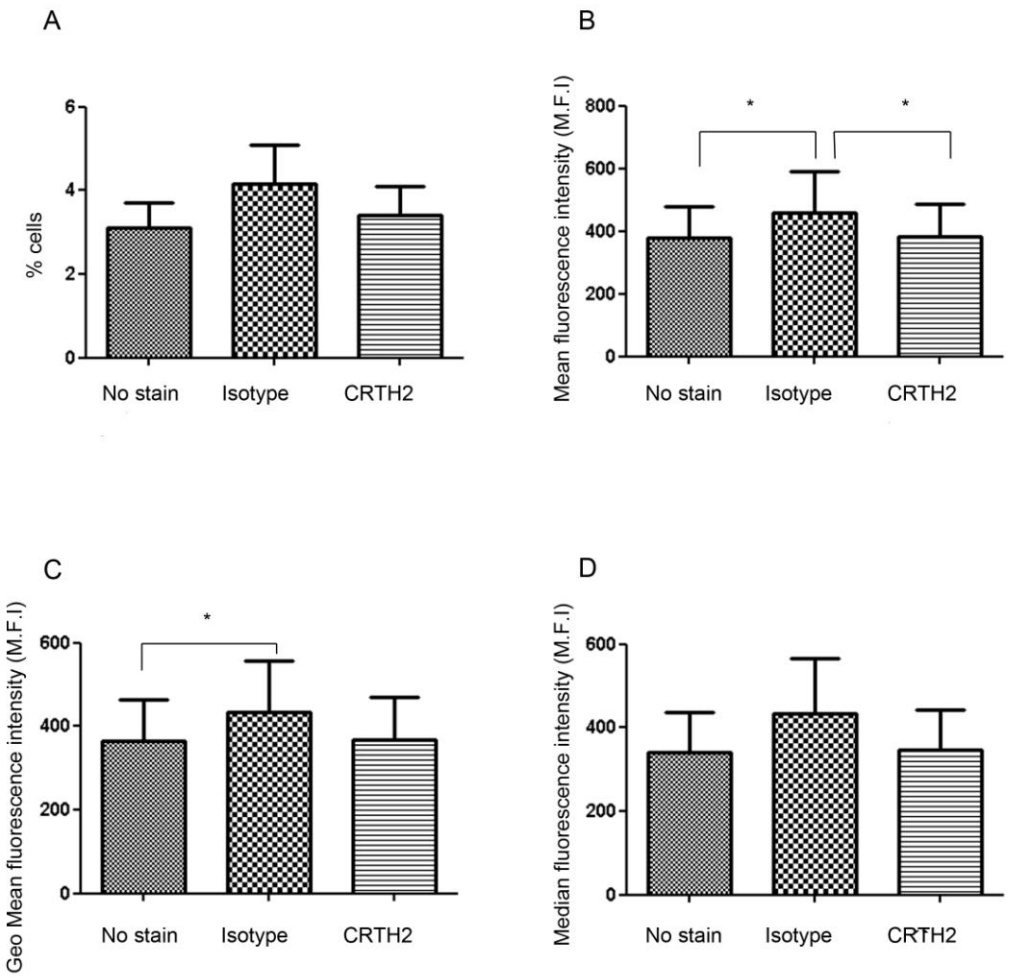


Figure 3.6.3.2 Graphs showing no expression of CRTH2 expression in myocytes
These graphs summarise the results of cultured myocytes (n=6). There was no significant difference in the percentage of cells labelled with CRTH2-PE (A), mean (B), geo mean (C) or median fluorescence intensity (D) and cells with no stain, or isotype control. There was a higher mean fluorescence intensity of cells which were stained with the isotype control compared with CRTH2-PE (B-D), indicating some background staining. For statistical analysis ANOVA of repeated measured was used with Bonferroni's *post hoc* analysis. * $P<0.05$.

3.7 Detection of transfected CRTH2 by flow cytometry

Having found an antibody that detects CRTH2, amniocytes and myocytes were transfected with the CRTH2 pSG5 expression vector and analysed by flow cytometry.

GeneJuice was used to transfect amniocytes which were harvested on day 1 post transfection as described in section 2.8. GFP was used as a control for transfection efficiency. Figure 3.7.1 shows the histograms for the cells with no staining, isotype-PE and CRTH2-PE.

Myocytes were transfected with the CRTH2 pSG5 expression vector by electroporation as described in section 2.8. Cells were harvested on day 2 post transfection and analysed by flow cytometry. CRTH2 expression was seen, again at a low level. Histograms are shown in Figure 3.7.2. A representative image of GFP transfected cells is shown to demonstrate adequate transfection efficiency, Figure 3.7.3.

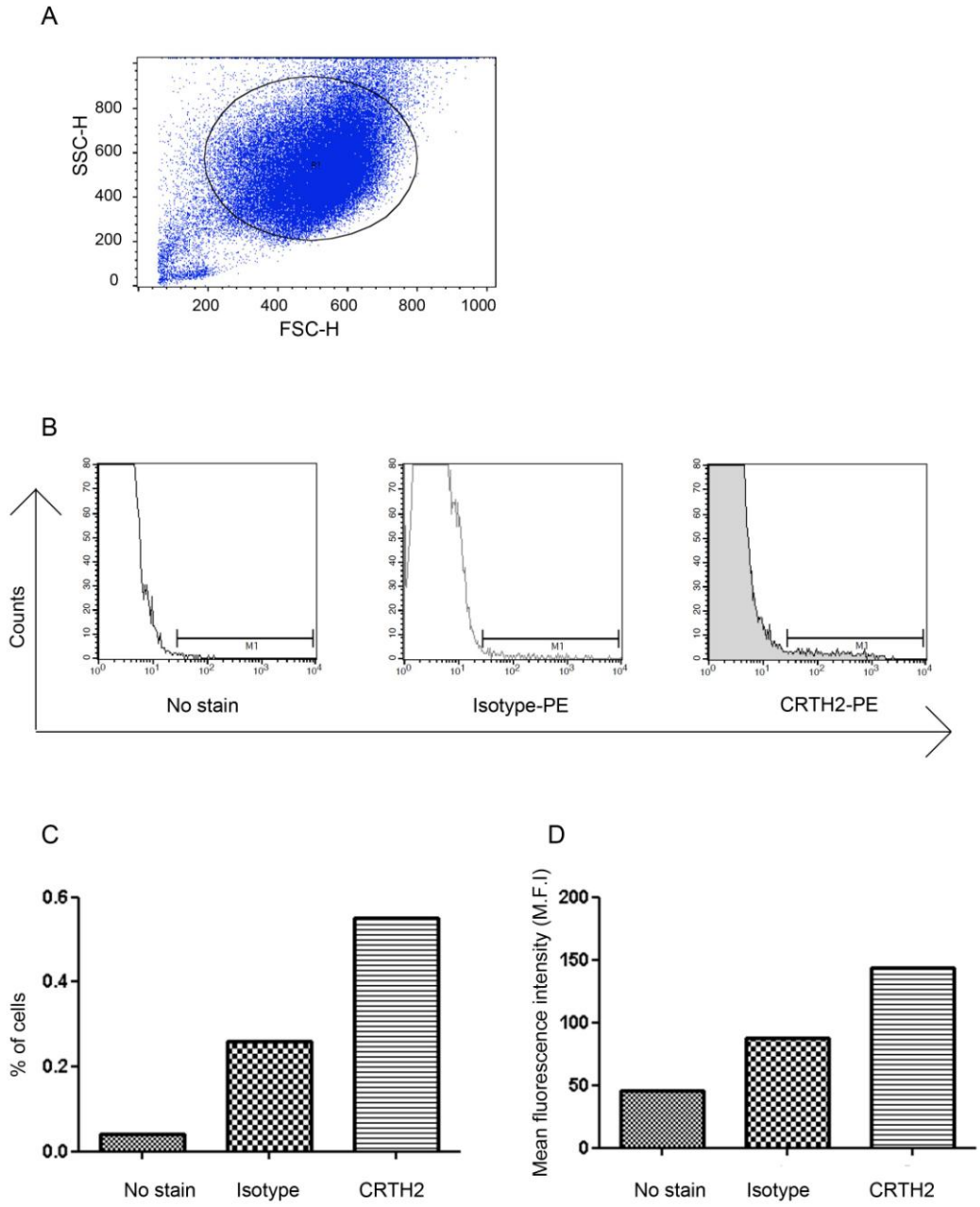


Figure 3.7.1 CRTH2 expression in transfected amniocytes
A representative dot plot shows the transfected amniocyte population (A). Histograms of the transfected cells are shown for no staining, isotype controls and CRTH2 stained cells (B). The percentage of CRTH2 transfected cells is shown in (C), along with the mean fluorescence intensity (D). CRTH2 expression was very low, which is consistent with the cells not being able to express an endogenous stable CRTH2 protein.

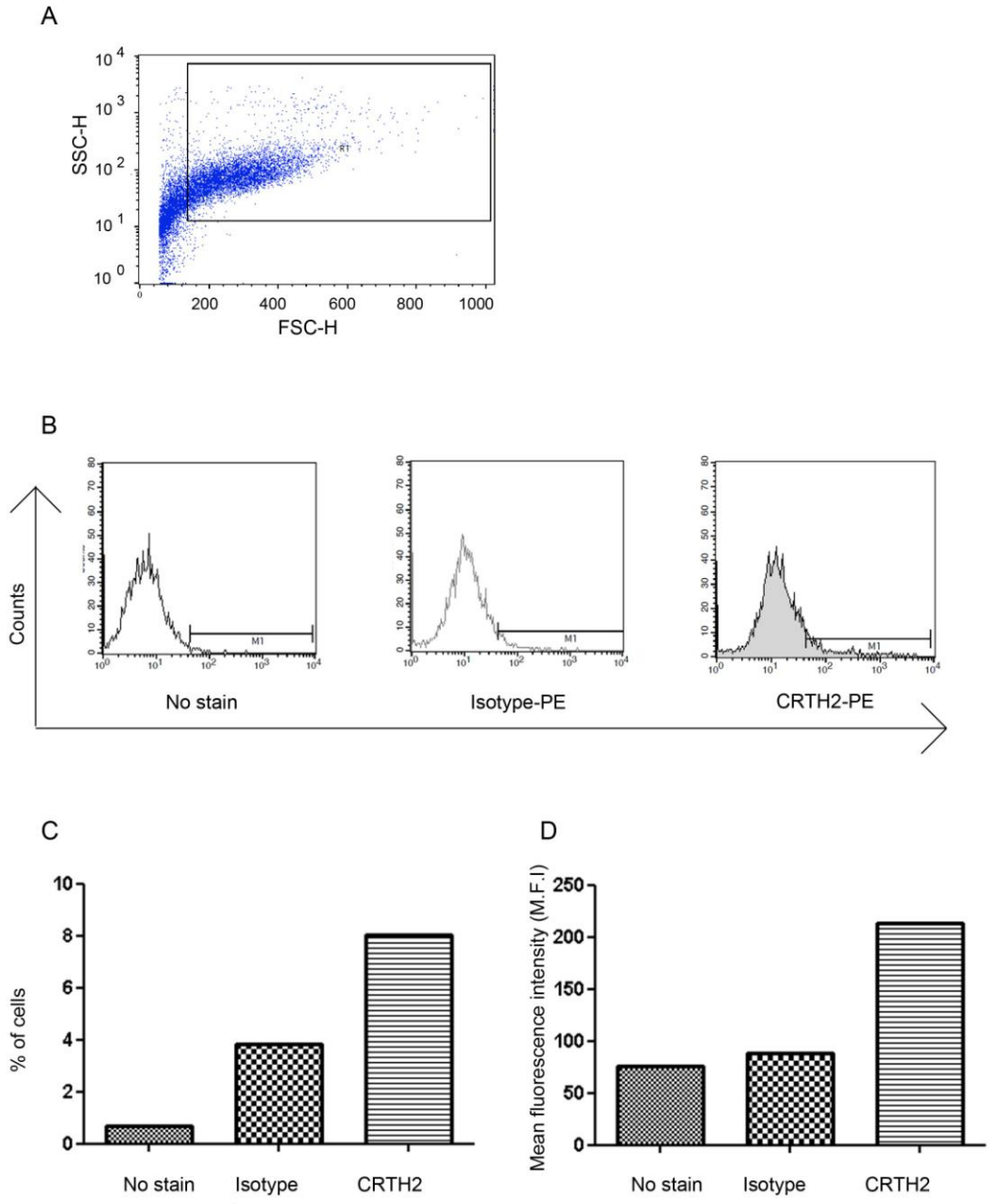


Figure 3.7.2 CRTH2 expression in transfected myocytes
A representative dot plot shows the transfected myocyte population (A). Histograms of the transfected cells are shown for no staining, isotype controls and CRTH2 stained cells (B). The percentage of CRTH2 transfected cells is shown in (C), along with the mean fluorescence intensity (D). CRTH2 expression was low, which is consistent with the cells not being able to express an endogenous stable CRTH2 protein.

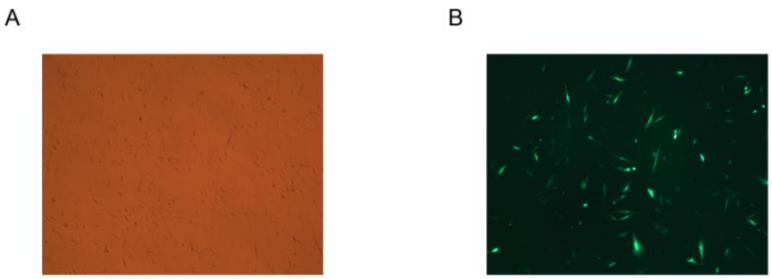


Figure 3.7.3 GFP transfected cells
An example of the GFP transfected myocytes is shown. Myocytes were viewed under the microscope (A). The GFP (green fluorescent protein) in the myocytes was detected with the fluorescent microscope (B).

3.8 CRTH2 expression in peripheral blood mononuclear cells during pregnancy

The expression of CRTH2 in peripheral blood T helper cells in pregnancy was next explored to determine if CRTH2 could be used as a marker of the hypothesised Th2 bias of pregnancy. Blood was collected from women at 12 and 28 weeks of pregnancy, and at term pre-labour and in labour, with non-pregnant women serving as controls (n=8-10). T helper cells were identified firstly by gating lymphocytes based on forward and side scatter and secondly with CD4 cell surface staining. Cells were also stained with CRTH2-PE. Flow cytometry was used to detect CD4-APC and CRTH-2-PE. An example cytogram is shown in Figure 3.8A, with the upper right quadrant representing the CRTH2 positive CD4 cells. The percentage of CD4 positive cells expressing CRTH2 was calculated (Figure 3.8B) and the geometric mean of the double positives was evaluated (Figure 3.8C). A trend was seen of an increase in the percentage of CRTH2 expressing cells in the 3rd trimester, however this did not reach statistical significance. No difference in the mean fluorescence intensity was seen, implying no increase in CRTH2 receptor expression. The percentage of cells expressing CRTH2 in labour was not statistically different from non-labouring women. Although a decrease in mean fluorescence was seen in women in preterm labour, the error bar was large and this did not reach statistical significance.

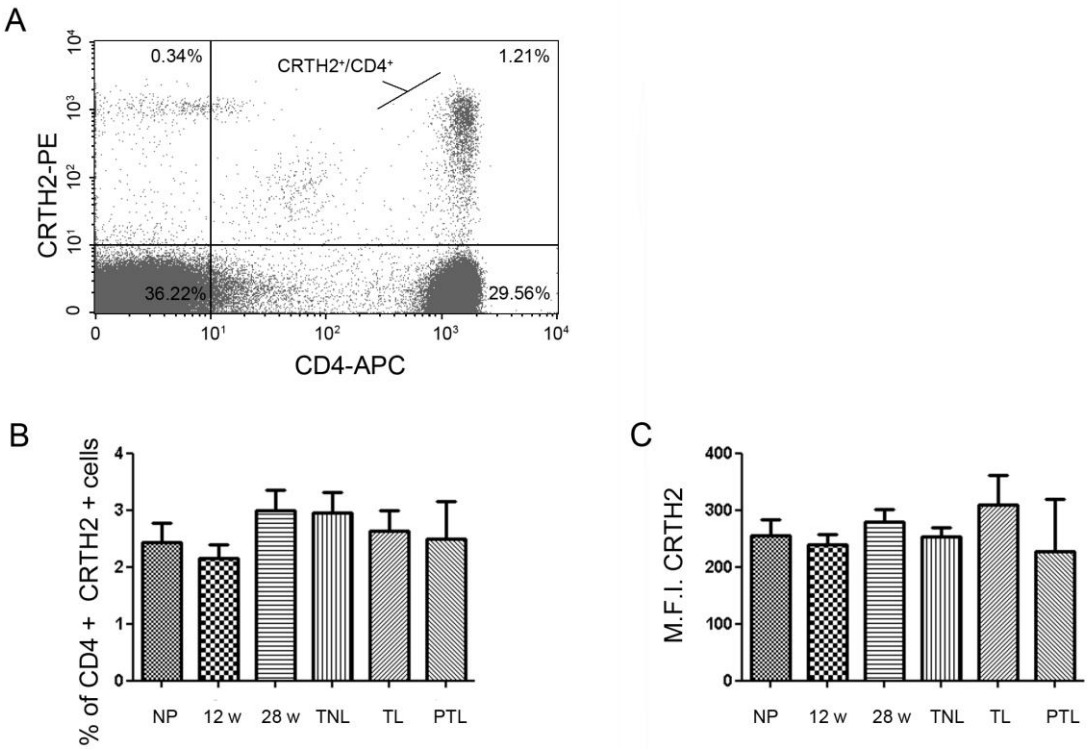


Figure 3.8 CRTH2 expression in CD4 positive lymphocytes during pregnancy
Lymphocytes were gated according to forward scatter and side scatter and T helper cells were identified using CD4 as a cell surface marker (n=8-10 for each group). A representative cytogram from lymphocytes taken at 28 weeks gestation is presented with the right upper quadrant showing $CRTH2^+/CD4^+$ lymphocytes (A). The percentage of CRTH2 positive T helper cells (presented as a percentage of CRTH2 positive CD4 positive cells) increased slightly in the third trimester although statistical significance was not seen (B). No significant difference was seen in the mean fluorescence intensity between non-pregnant controls and women at each gestational timepoint (C). For statistical analysis ANOVA with Bonferroni's multiple comparison test was used. NP = non-pregnant, 12 w= 12 weeks, 28 w = 28 weeks, TNL = term not in labour, TL = term in labour, PTL=preterm labour.

Summary and discussion

CRTH2 is a ligand for $15dPGJ_2$, a cyclopentenone prostaglandin that inhibits NF-κB in human amniocytes and myocytes [271], and delays preterm labour in inflammation induced preterm labour in the mouse [277]. CRTH2 is a receptor typically associated with immune cells, particularly Th2 cells. The function of CRTH2 on T helper cells appears to be important for the maintenance of the Th2 interleukin profile, whereas on eosinophils it appears to be important for chemotaxis in allergic conditions such as asthma [307]. Few studies have examined its expression in non-immune cells. In this chapter I explored the expression of CRTH2 in gestational tissues and myometrium as a basis for exploring the possibility that a) CRTH2 could be involved in $15dPGJ_2$-mediated inhibition of NF-κB, and b) CRTH2 could play a functional role in contributing to a balance in the Th1:Th2 interleukin profile at the maternal fetal interface.

CRTH2 is the second receptor to Prostaglandin D_2, discovered over a decade after the DP1 receptor. The amnion, choriodecidua and placenta all show basal production of PGD_2 in cultured explants at a concentration of 2.6, 2.5 and 95.8 pg/mg of tissue over 24 hours respectively [279]. Low or undetectable mRNA levels of the DP1 receptor are seen in human myometrium [308, 309] and amnion [279]. It is plausible that the target of endogenous PGD_2 is the second prostaglandin D_2 receptor, CRTH2. There is limited data available on the mRNA expression and no data available on the protein expression of CRTH2 in these tissues. Nagata and colleagues have shown relatively high levels of mRNA expression in placenta and moderate levels in non-pregnant myometrium [287]. Helliwell and colleagues demonstrated mRNA expression present in 24/24 placentas, 7/12 choriodecidua samples and none of the 24 amnion samples examined [279].

<u>Detection of CRTH2 mRNA by PCR</u>

In this study all five pre-labour myometrial, amnion, choriodecidual and placental samples showed detectable levels of CRTH2 mRNA (Figure 3.1.3). Although standard

PCR was used for detection rather than quantitative PCR it was still possible to conclude that expression was weak in these tissue types, particularly in amnion and choriodecidua, since a cycle number of 35 was used to determine expression. In order to establish if the expression seen in amnion and myometrium was due to leukocyte infiltration, mRNA from cultured amniocytes and myocytes was determined. CRTH2 mRNA was detected in 4/6 samples of amniocytes and 6/6 myocyte samples (figure 3.1.4), again at a high cycle number of 35. This is consistent with the high cycle numbers required to demonstrate expression in monocytes and dendritic cells [290]. Increasing the cycle number is useful in order to analyse low copy number targets [310]. Rameckers et al reviewed the determinants of cycle numbers and demonstrated the requirement of cycles up to 45 for the detection of low target sequences [311]. However, with cycles above 40 there is an increased risk of introducing non-specific products. The consistent absence of a product in the non-template and reverse transcriptase negative controls in this study supports that neither random nor systematic contaminants such as genomic DNA are the sources of the products seen, but rather low target numbers of CRTH2.

Detection of CRTH2 by western analysis

Since mRNA expression was low in the cells and tissues examined, examination of CRTH2 at the protein level was explored to determine if the mRNA is translated. Western analysis was employed using two commercially available antibodies to detect CRTH2 in tissue samples (Figure 3.2.1). A single band at 55kDa was detected with the Sc-23092 antibody and a single band at 70kDa was detected with the Proscience 4027 antibody in all tissue samples. The band seen at 70kDa was deemed to be non-specific since it did not appear in the human heart and PBMC positive controls. The band seen at 55 was consistently seen in the positive controls human heart and PBMCs. Nagata et al have previously reported detection of CRTH2 in its glycosylated form at 55dKa and at 34 with oligosaccharide cleavage [291]. No band was detected at 34kDa or 55kDa at the cellular level in amniocytes or myocytes (Figure 3.2.2), meaning that either CRTH2 is not expressed at the cellular level, or the bands detected in the

positive controls were non-specific. This led to sequencing of the band at 55kDa and cloning CRTH2 to provide a highly concentrated positive control.

N-terminal sequencing of the band detected in amnion, myometrium, human heart and PBMCs failed to detect CRTH2. Immunoglobulin and keratin were detected; both are contaminants and a recognised limitation of the technique. This led to inconclusive results since proteins with low expression may be masked by the more highly expressed proteins of this size. By cloning CRTH2, a highly concentrated protein lysate was produced both in the cell free system and endogenously by transfecting amniocytes and myocytes. However, none of the three commercially available antibodies used for western analysis detected CRTH2 produced by the *in vitro* transcription translation kit (Figure 3.5.1). It is likely that the explanation for this is due to the reliance of the antibodies on the folded G-protein structure and/or the post-translational modifications such as glycosylation. CRTH2 is known to have the classical 7 transmembrane domains of a G-protein coupled receptor, and also has several potential sites for N-glycosylation and protein kinase C phosphorylation [291].

To overcome the potential requirement for post-translational modifications for CRTH2 detection, protein lysate from CRTH2-transfected amniocytes was probed with the Sc-23092 and Proscience 4027 antibodies (Figure 3.5.2). The Santa cruz antibody again detected a band at 55 but no band in the transfected cells. With the persistence of the band at 55 in the positive control yet with no band seen in the transfected cells a secondary antibody control blot was examined. This band was shown to be non-specific since it was still present in the absence of the primary antibody. The Proscience antibody detected the non-specific band at 70 again and a weak band at 43. The band at 43 was considered non-specific since it was not present in the PBMC positive control and there was no increase in band intensity in the transfected cells compared with the non-transfected cells.

Studies of the expression of CRTH2 on non-immune cells are limited, probably due to the lack of specific antibodies available for detection using techniques other than flow cytometry. There are only three publications using western analysis for the detection

of human CRTH2. Zayed et al detected bands at 41 and 55 for chondrocytes, using osteoblasts as a positive control [294]. However, they did not include blocking or secondary antibody controls to confirm specificity. Colombe et al detected a band for CRTH2 in keratinocytes and melanocytes at 45kDa with a Cayman Chemicals antibody (also suitable for immunohistochemistry) which has subsequently been taken of the market for lack of specificity [296]. The third group to publish on detection of CRTH2 by western analysis is Nagata's group who raised several antibodies to CRTH2, however, these antibodies are not available commercially. Together with data from these publications, the data presented in this chapter suggest there are no commercially available antibodies suitable for detecting CRTH2 by western analysis.

Detection of CRTH2 by flow cytometry

Flow cytometry is used successfully for the detection of CRTH2 on immune cells [291, 312-316]. Flow cytometry detection of CRTH2 on non-immune cells has only been reported in human bronchial epithelial cells [317]. The antibody from this study was used to detect CRTH2 firstly in peripheral blood lymphocytes as a positive control, and then for the detection of CRTH2 in amniocytes and myocytes. CRTH2 was detected on a mean of 2.15% lymphocytes and 2.43% of $CD4^+$ cells (Figures 3.6.1 and 3.8), consistent with published reports of between 0.4 and 6.5% of peripheral blood $CD4^+$ cells expressing the receptor [291].

Having established a way of demonstrating CRTH2 expression, flow cytometry was used to explore endogenous and transfected CRTH2 expression in amniocytes and myocytes. No CRTH2 was detected in any amniocyte and myocyte samples (Figures 3.6.2 and 3.6.3), and low expression was seen in the transfected samples (Figure 3.7). Good transfection efficiency was seen with GFP, and thus the low expression of CRTH2 in the transfected samples is potentially explained by the inability of amniocytes and myocytes to express a stable CRTH2 protein. This is consistent with the absence of endogenous CRTH2 protein expression in these cells.

The expression of CRTH2 in peripheral blood lymphocytes during pregnancy at different gestational time points was next examined. Since the immunology of pregnancy is characterised by a bias towards Th2, and that CRTH2 is a specific marker of T helper 2 cells [287], an increase in CRTH2 expression in $CD4^+$ cells was expected during pregnancy. Although a small increase was seen in expression at 28 weeks and at term compared with non-pregnant controls, this did not reach statistical significance. Tsuda *et al* have previously reported an increase in CRTH2 expression in $CD4^+$ cells of women at 7 weeks gestation (3.43%, P=0.02) compared with non-pregnant women (2.08%) [312] but it is possible that this effect is diminished with advancing gestation.

Expression levels of CRTH2 were also compared between women not in labour and in term and preterm labour. Since early preterm labour is associated with infection and inflammation, a Th1 bias is plausible leading to a diminished Th2 bias. For this reason, CRTH2 expression in women in preterm labour was anticipated to be reduced compared with women in term labour. No statistically significant reduction was seen in either the percentage of cells expressing CRTH2 or the mean expression levels of CRTH2 as determined by the mean fluorescence intensity. Despite no significant increase being seen in CRTH2 expression in peripheral lymphocytes in pregnancy, this does not rule out a functional role for the receptor in the immunology of pregnancy.

In summary, CRTH2 is not expressed at the protein level in amniocytes and myocytes, and can be used as a negative control for future CRTH2 expression studies. The most reliable technique for protein expression studies of CRTH2 is flow cytometry. It is likely that any detectable CRTH2 seen in gestational tissues and myometrium is from leukocyte infiltration. Although no significant increase is seen in CRTH2 expression in T helper cells in pregnancy, this does not rule out a functional role for the receptor in the maintenance of the balanced immunology of pregnancy. Since CRTH2 is not expressed on amniocytes and myocytes, it is not likely to contribute to $15dPGJ_2$ mediated inhibition of NF-κB, however, it may still play a role in $15dPGJ_2$ mediated delay in inflammation induced preterm labour in the mouse.

CHAPTER 4

The effect of CRTH2 agonists on NF-κB

Chapter summary

Hypothesis:

NF-κB controls the expression of many labour associated genes and its activity is increased in the amnion and myometrium at term and in labour. It may therefore play a key role in initiating preterm labour. The anti-inflammatory prostaglandin $15dPGJ_2$ inhibits NF-κB in human amniocytes and myocytes [271]. The mechanism of $15dPGJ_2$ mediated NF-κB inhibition remains to be elucidated. $15dPGJ_2$ is a CRTH2 agonist; however, since CRTH2 is not expressed on human myocytes and amniocytes it is unlikely that this receptor plays a role in $15dPGJ_2$-mediated inhibition of NF-κB. Nuclear phospho-p65 is suppressed in T helper cells during pregnancy [240]; but it is not known if NF-κB becomes activated in peripheral leukocytes during labour. If basal NF-κB activity is increased in peripheral leukocytes during labour and $15dPGJ_2$ has the same inhibitory effect on NF-κB in these cells, then $15dPGJ_2$ could provide a beneficial systemic anti-inflammatory effect, as well as the local anti-inflammatory effect seen on amniocytes and myocytes.

Aims:

The aims of the work described in this chapter are; first of all, to confirm that CRTH2 plays no role in $15dPGJ_2$-mediated inhibition of NF-κB, secondly to determine if NF-κB activity is altered during pregnancy and in labour in peripheral blood mononuclear cells, and thirdly to determine if $15dPGJ_2$-mediated inhibition of NF-κB can be replicated in peripheral blood mononuclear cells (PBMCs).

Methods:

Prior to commencing experiments with the CRTH2 agonist Pyl A, its activity at the CRTH2 receptor was demonstrated by up regulation of CD11b expression. The effect of $15dPGJ_2$ and Pyl A on IL-1β induced NF-κB activity in amniocytes and myocytes was determined by western analysis of nuclear p65 and phospho-p65. NF-κB activity in peripheral blood mononuclear cells from women at 28 weeks of pregnancy, term pre-labour and in labour, and non-pregnant controls was determined by western analysis

of whole cell phospho-p65. To confirm that CRTH2 plays no role in $15dPGJ_2$-mediated inhibition of NF-κB, PBMCs were incubated with $15dPGJ_2$ with or without a CRTH2 antagonist and analysed for phospho-p65 by western analysis.

Results:

$15dPGJ_2$ inhibited NF-κB in amniocytes and myocytes. This effect was not reproduced by the CRTH2 agonist Pyl A. No change in PBMC phospho-p65 was seen during pregnancy compared to non-pregnant controls, or in labour compared with pre-labour. $15dPGJ_2$ inhibited NF-κB activity in PBMCs, but this effect was not attenuated by the CRTH2 agonist.

Conclusion:

CRTH2 plays no role in $15dPGJ_2$-mediated inhibition of NF-κB. $15dPGJ_2$ was able to inhibit NF-κB in peripheral blood mononuclear cells. This inhibition is likely to lead to further anti-inflammatory effects such as the inhibition of pro-inflammatory cytokines, which may be of beneficial therapeutic effect in inflammation associated preterm labour.

Introduction

$15dPGJ_2$ inhibits NF-κB in cultured human amniocytes and myocytes in a mechanism that remains to be fully explained [271]. $15dPGJ_2$ is an agonist of the CRTH2 receptor and therefore the work in this thesis aims to explore the potential for the use of small molecule CRTH2 agonists to induce NF-κB inhibition. However, from the CRTH2 expression studies of CRTH2 in amniocytes and myocytes, (presented in chapter three), it is evident that CRTH2 is not expressed in these cell types. This implies that CRTH2 is not involved in $15dPGJ_2$-mediated NF-κB inhibition. Data presented in this chapter aims to prove definitively that CRTH2 plays no role in $15dPGJ_2$-mediated inhibition of NF-κB.

NF-κB activity is increased in both amnion [259] and myometrium at term [262], consistent with labour being considered a pro-inflammatory process. There is an increased production of pro-inflammatory cytokines in the amnion, amniotic fluid, choriodecidua, myometrium and cervix during labour, including IL-1β, TNF-α, IL-6 and IL-8 [25, 157, 158, 233]. Preterm labour is associated with infection and inflammation, and any trigger of NF-κB can lead to switching on a cascade of pro-inflammatory and labour associated genes such as IL-8, IL-1β, COX-2 and the MMPs [245]. The presence of inflammation is also associated with neonatal brain injury [318]. Systemic infection and inflammation even in term labour is associated with a poorer neonatal outcome [319]. Therefore, NF-κB is an attractive therapeutic target for both preventing preterm labour and neonatal brain injury.

NF-κB activity is suppressed in T cells during pregnancy [240]. This suppression is thought to contribute to the suppression of Th1 interleukins which ultimately alter the Th1:Th2 ratio to favour the Th2 bias seen in pregnancy. It is not known whether this suppression in PBMCs is reversed towards term and during labour. The effect of $15dPGJ_2$ on NF-κB in PBMCS has also not yet been reported on.

Data presented in this chapter examines the effect of the CRTH2 agonists Pyl A on phospho-p65 amniocytes and myocytes to see if the effects of $15dPGJ_2$ can be

replicated. Secondly, the effect of these CRTH2 agonists on NF-κB activity in peripheral blood mononuclear cells was determined.

Results

Prior to commencing experiments with the small molecule CRTH2 agonist Pyl A, its activity at the CRTH2 receptor was determined by demonstrating its ability to up-regulate the expression of CD11b on eosinophils.

4.1 Pyl A increases CD11b expression in eosinophils via CRTH2

The small molecule CRTH2 agonist Pyl A was synthesised by Oxygen Healthcare (Cambridge, UK) based on the L-888,607 compound from Merck Frosst Centre for Therapeutic Research (Quebec, Canada) [320]. To prove the CRTH2 agonist activity of the compound, and based on the knowledge that the CRTH2 agonists PGD_2 and $15dPGJ_2$ increase CD11b expression on eosinophils and basophils via CRTH2, the effect of Pyl A on CD11b expression was examined [303, 321]. Granulocytes were isolated from non-pregnant women as described in section 2.2.3 and were treated with Pyl A. Flow cytometry was used to detect CD11b expression on eosinophils which were identified by high intensity CD49d expression and forward and side scatter characteristics (Figure 4.1). Up regulation of CD11b expression with Pyl A treatment was demonstrated by an increase in mean fluorescence intensity of CD11b-PE *(P<0.5)*. This effect was attenuated with prior incubation of cells with the CRTH2 antagonist GSKCRTH2X, obtained from Glaxo Smith Kline, (London, UK) *(P<0.5)*.

An example dot blot is shown Figure 4.1A, with CD49d expression along the x-axis and CD11b along the y-axis. The double positive population is seen in the right upper quadrant. The cell population shows a subtle shift in the direction of the y-axis showing increased CD11b expression, which is reversed with pre-incubation with the CRTH2 antagonist GSKCRTH2. The histogram in Figure 4.1B shows the shift to the right with Pyl A treatment (grey filled histogram), reflecting increased CD11b expression, which is reversed with the CRTH2 antagonist (black outlined histogram). The graph in C summarises the results of three biological replicates.

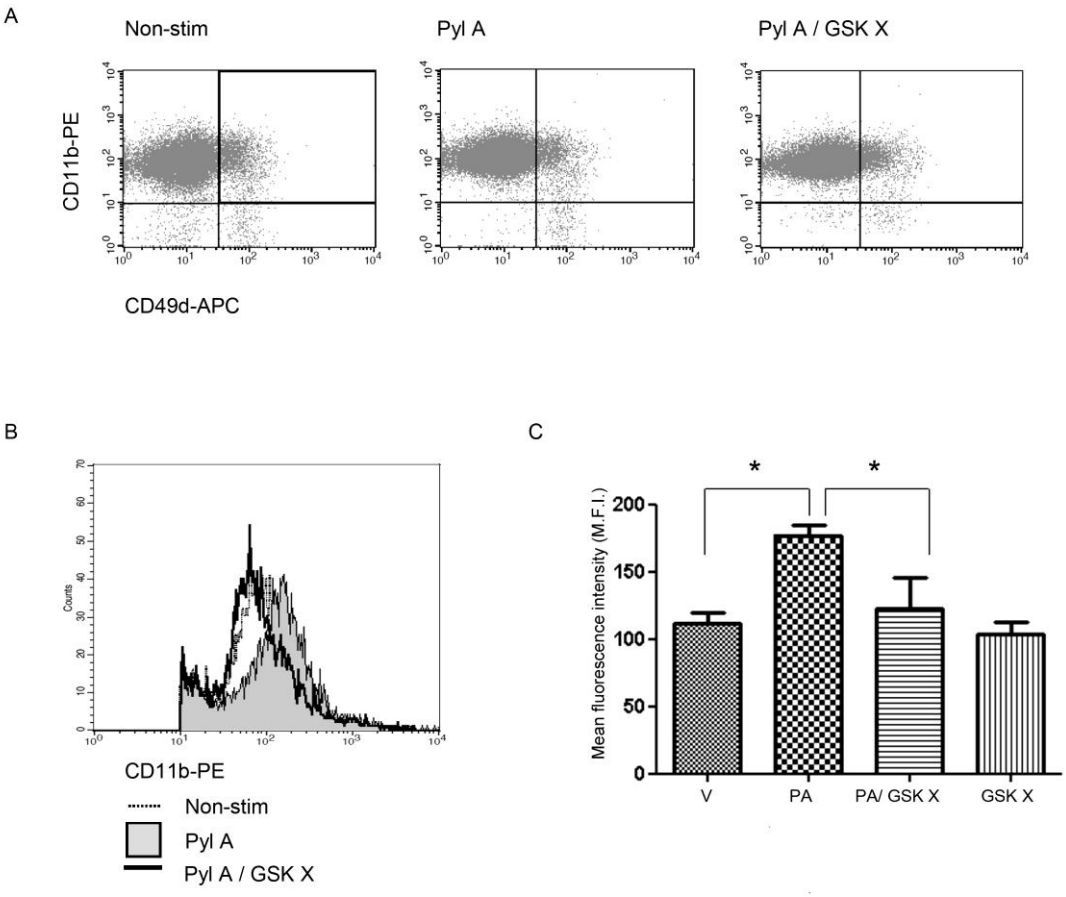


Figure 4.1 Pyl A increases CD11b expression on eosinophils

32 µm of PYL A was used to increase CD11b expression on eosinophils. Pre-treatment with the CRTH2 antagonist GSKCRTH2X (100 µm) was used to confirm this effect was via CRTH2. Eosinophils were identified by labelling with anti-CD49d and on the basis of forward and side scatter. A representative cytogram is presented with the right upper quadrant showing $CD11b^{+}/CD49d^{+}$ cells (A). A representative histogram reveals a clear shift to the right with Pyl A treatment indicative of an increase in CD11b expression. This effect was attenuated with CRTH2 antagonist pre-treatment (B). A summary of CD11b expressed in eosinophils with each treatment is shown in the graph (n=3) (C). V= vehicle, PA =Pyl A, GSK X= GSKCRTH2X. For statistical analysis ANOVA of repeated measures with Bonferroni's multiple comparison test was used; * P<0.05.

4.2 15dPGJ$_2$ inhibits NF-κB in amniocytes and myocytes

It has previously been shown that 15dPGJ$_2$ inhibits IL-1β induced NF-κB activity in human amniocytes and myocytes in a mechanism independent of PPAR-γ [271]. For the purpose of this thesis these results were replicated showing a significant reduction in IL-1β induced NF-κB, as determined by nuclear p65, to below basal levels in amniocytes at 32 µm and in myocytes from 16 µm (Figure 4.2A and B).

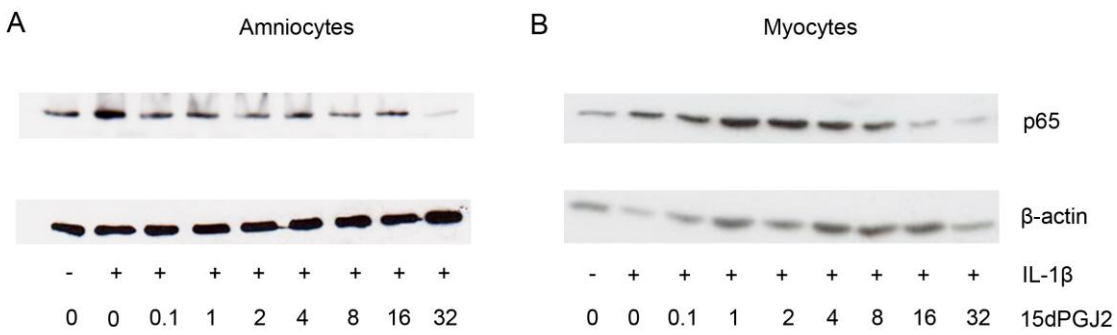


Figure 4.2 15dPGJ$_2$ reduces NF-κB activity in amniocytes and myocytes
Protein was extracted from IL-1β stimulated and 15dPGJ$_2$ treated cells and levels of nuclear p65 were examined using immunoblotting. A dose response of 0.1-32 µm of 15dPGJ$_2$ was performed (n=3). Representative immunoblots are shown for amniocytes, (A), myocytes (B). Immunoblots were re-probed for β-actin as an internal loading control.

4.3 Pyl A has no effect on NF-κB activity in amniocytes and myocytes

The precise mechanism of $15dPGJ_2$-mediated inhibition of NF-κB is yet to be fully understood. In chapter three data was presented showing that CRTH2 is not expressed in amniocytes and myocytes. Therefore, this suggests that CRTH2 does not play a role in NF-κB inhibition. As anticipated, the small molecule CRTH2 agonist Pyl A did not reproduce the effects of $15dPGJ_2$; having no effect IL-1β induced NF-κB in amniocytes and myocytes. NF-κB activity was assessed by examining nuclear p65 and phospho-p65 by immunoblot. Representative immunoblots for amniocytes and myocytes are shown in Figures 4.3A and B respectively. Densitometric analysis, taking into account β-actin loading controls, was performed and a summary of n=3 experiments is shown in figures 4.3C-F. Figures 4.3C and D represent p65 in amniocytes and myocytes respectively, and figures 4.3E and D represent phopsho-65 in amniocytes and myocytes respectively.

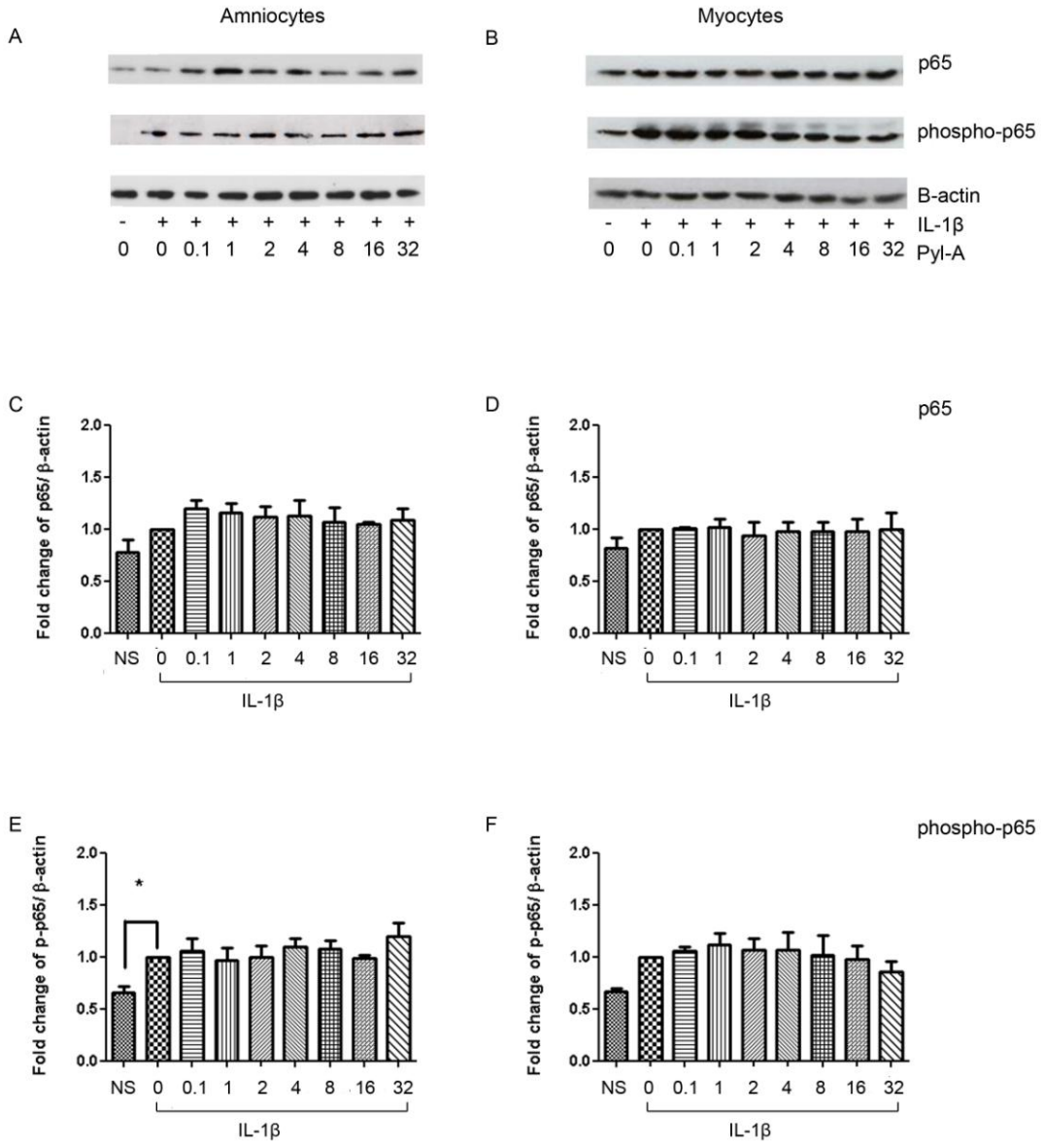


Figure 4.3 Pyl A has no effect on NF-κB activity in amniocytes and myocytes
Protein was extracted from IL-1β stimulated and Pyl A treated cells and levels of nuclear p65 and phosphorylated p65 (p-p65) were examined using immunoblotting. A dose response of 0.1-32 μm of Pyl A was used (n=3). Representative immunoblots are shown for amniocytes, (A), myocytes (B). Immunoblots were re-probed for β-actin as an internal loading control. Densitometric analysis of the immunoblots was conducted revealing no effect of Pyl A on p65 or p-p65 in amniocytes (C, E) or myocytes (D, F). Effect of treatment was examined for statistical significance using ANOVA of repeated measures with Bonferroni's multiple comparison test; *P<0.05.

4.4 15dPGJ$_2$ reduces NF-κB in peripheral blood mononuclear cells

To determine if the inhibitory effect of 15dPGJ$_2$ could be replicated in peripheral blood mononuclear cells, blood was collected and prepared as described in section 2.2.3. Cells were incubated with 1-32 µm of 15dPGJ$_2$ or vehicle for 2 hours and whole cell lysate was analysed for phopsho-p65, the transcriptionally active form of NF-κB. A significant reduction in NF-κB activity was seen with 32 µm of 15dPGJ$_2$ as shown by a representative blot (Figure 4.4A), and in the graph summarising results of three biological replicates (Figure 4.4B).

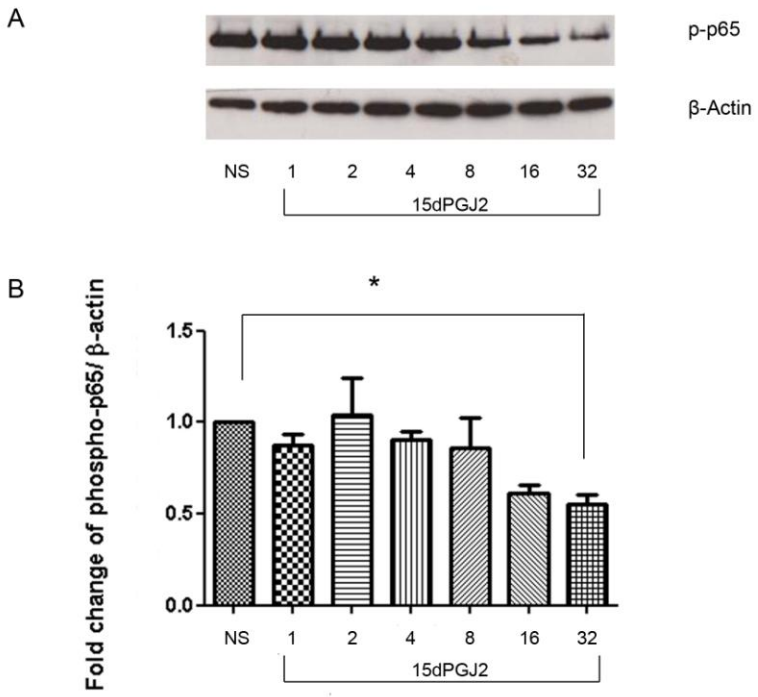


Figure 4.4 15dPGJ$_2$ reduces NF-κB activity in peripheral blood mononuclear cells
Protein was extracted from peripheral blood mononuclear cells after a 2 hr incubation with 15dPGJ$_2$ and levels of whole cell phsopho-p65 were examined using immunoblotting. A dose response of 1-32 µm of 15dPGJ$_2$ was used (n=3). Representative immunoblots are shown for phospho-p65 and β-actin (A). Immunoblots were re-probed for β-actin as an internal loading control and densitometric analysis of the immunoblots for all experiments was conducted and summarised in the graph (B). A reduction in NF-κB activity was seen from 16 µm concentration. Effect of treatment was examined for statistical significance using One-way ANOVA with Dunnett's post-hoc analysis; *P<0.05.

4.5 Pyl A has no effect on NF-κB in peripheral blood mononuclear cells

The CRTH2 agonist Pyl A was used to determine if the inhibitory effect of $15dPGJ_2$ could be replicated. Cells were incubated with 0.1-32 µm of Pyl A or vehicle for 2 hrs and whole cell lysate was analysed for phopsho-p65. Four biological replicates were performed, with an example blot in Figure 4.5 A and a summary of all replicates in Figure 4.5 B.

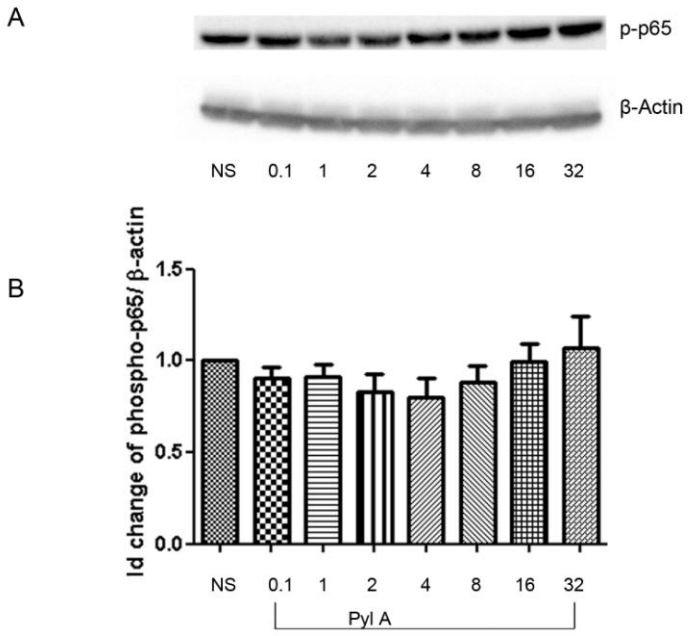


Figure 4.5 Pyl A has no effect on NF-κB activity in peripheral blood mononuclear cells
Protein was extracted from peripheral blood mononuclear cells after a 2 hr incubation with Pyl A and levels of whole cell phsopho-p65 were examined using immunoblotting. A dose response of 0.1-32 µm of Pyl A was used (n=4). Representative immunoblots are shown for phospho-p65 and β-actin (A). Immunoblots were re-probed for β-actin as an internal loading control and densitometric analysis of the immunoblots for all experiments is summarised in the graph (B). No effect was seen on NF-κB activity at any concentration of Pyl A. Effect of treatment was examined for statistical significance using One-way ANOVA with Dunnett's post-hoc analysis.

4.6 DK- PGD_2 has no effect on NF-κB in peripheral blood mononuclear cells

A second specific CRTH2 agonist was used to confirm that CRTH2 agonists have no effect on NF-κB activity. A dose response from 0.1-32 μm showed no effect on whole cell phospho-65 in peripheral blood mononuclear cells. A representative blot is shown in Figure 4.6A, and a graph summarising the absence of effect with three replicates is shown in Figure 4.6B.

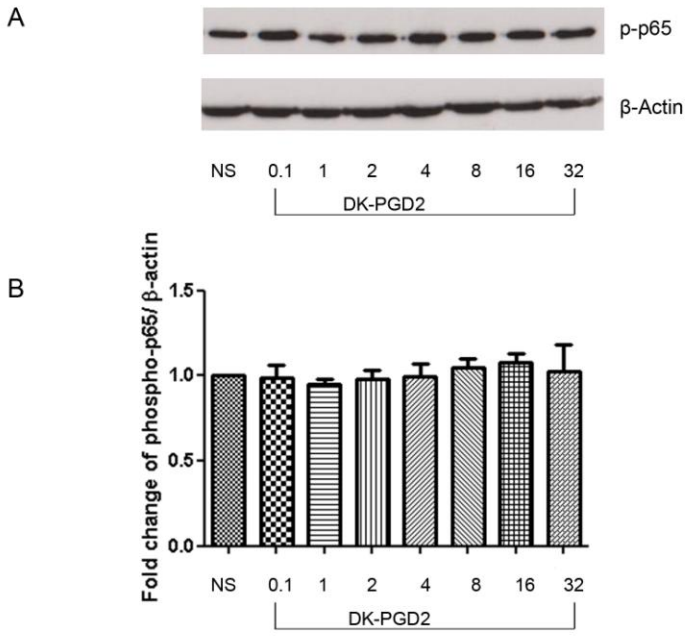


Figure 4.6 DK- PGD_2 has no effect on NF-κB activity in peripheral blood mononuclear cells
Protein was extracted from peripheral blood mononuclear cells after a 2 hr incubation with DK- PGD_2 and levels of whole cell phsopho-p65 were examined using immunoblotting. A dose response of 0.1-32 μm of DK- PGD_2 was used (n=3). Representative immunoblots are shown for phospho-p65 and β-actin (A). Immunoblots were re-probed for β-actin as an internal loading control and densitometric analysis of the immunoblots for all experiments is summarised in the graph (B). No effect was seen on NF-κB activity at any concentration of DK-PGD_2. Effect of treatment was examined for statistical significance using One-way ANOVA with Dunnett's post-hoc analysis.

4.7 $15dPGJ_2$ inhibits basal on NF-κB in a mechanism independent of CRTH2

Data presented so far supporting the hypothesis that CRTH2 is not involved in $15dPGJ_2$-mediated inhibition of NF-κB includes the inability of Pyl A to replicate NF-κB inhibition in amniocytes, myocytes and peripheral blood mononuclear cells. The second CRTH2 agonist used (DK- PGD_2) also did not replicate the effects of $15dPGJ_2$ in PBMCs. As a final confirmation that CRTH2 plays no role in $15dPGJ_2$ -mediated NF-κB inhibition, prior to treatment of PBMCs with $15dPGJ_2$ cells were pre-incubated with the CRTH2 antagonist (GSKCRTH2X). The CRTH2 antagonist could not attenuate the effects of $15dPGJ_2$, Figure 4.7.

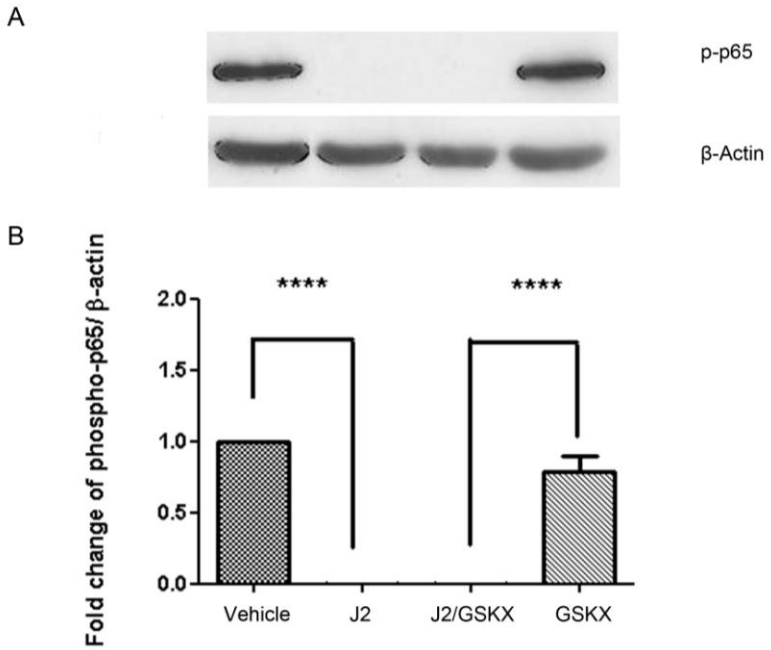


Figure 4.7 The effect of $15dPGJ_2$ on basal NF-κB p65 phosphorylation in peripheral blood mononuclear cells PBMCs were treated with 32 μm of $15dPGJ_2$ for 2 hrs with or without preincubation with 100 μm of GSKCRTH2X for 45 mins. Whole cell protein lysate was examined for levels of phosphorylated p65 (p-p65) using immunoblotting. A representative immunoblot from n=6 samples are shown (A). Immunoblots were reprobed for β-actin as an internal loading control. Densitometric analysis of the immunoblots was conducted revealing a complete inhibition of p-p65 levels with $15dPGJ_2$. Pre-incubation with the CRTH2 antagonist GSKCRTH2X had no effect on inhibition (B). Effect of treatment was examined for statistical significance using ANOVA of repeated measures with Bonferroni's multiple comparison test; **** $P<0.0001$.

4.8 15dPGJ$_2$ reduces PMA stimulated NF-κB in peripheral blood mononuclear cells

PMA and ionomycin are stimulants used *in vitro* to represent *in vivo* T cell activation. To determine if 15dPGJ$_2$ was able to inhibit PMA/ionomycin activated T cells, peripheral blood mononuclear cells were incubated with vehicle control or 32 μM of 15dPGJ$_2$ and stimulated with PMA/ionomycin for 10 mins. Whole cell protein lysate was extracted and assessed for levels of phosphorylated p65, the transcriptionally active subunit of NF-κB. PMA/ionomycin treatment stimulated NF-κB phosphorylation in all samples. Pre-treatment of cells with 15dPGJ$_2$ reduced levels of p-p65 in all samples but narrowly missed out on statistical significance (n=4). A representative immunoblot is shown in Figure 4.8A and a summary of all experiments is expressed in the graph in Figure 4.8B.

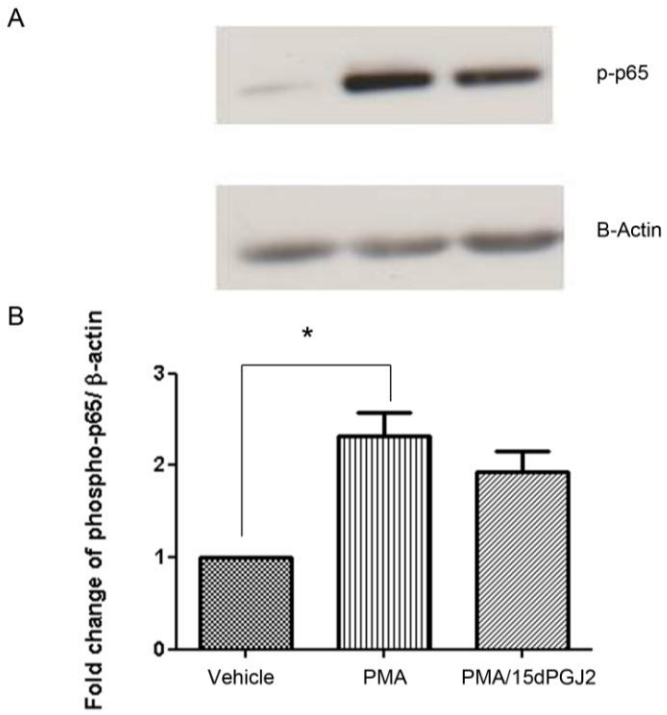


Figure 4.8 15dPGJ$_2$ reduces PMA stimulated NF-κB activity in peripheral blood mononuclear cells

PBMCs were treated with 15dPGJ$_2$ for 2 hrs then stimulated for 10 mins with PMA/ionomycin. Whole cell protein lysate was extracted and levels of phosphorylated p65 examined using immunoblotting. A representative immunoblots is shown (A). Immunoblots were reprobed for β-actin as an internal loading control. Densitometric analysis of the immunoblots was conducted revealing a significant increase in p-p65 levels upon stimulation and a decrease with the addition of 15dPGJ$_2$ (B). Effect of treatment was examined for statistical significance at each gestational timepoint using ANOVA of repeated measures with Bonferroni's multiple comparison test; *P<0.05.

4.9 NF-κB activity is unchanged during pregnancy and labour

Previous studies have shown that NF-κB activity is suppressed in T cells in pregnancy. However, it is not known if this suppression is reversed at term and in labour. Peripheral blood mononuclear cell whole cell lysate from non-pregnant women, women at 28 weeks gestation and at term pre-labour and in labour was analysed for phospho-65 by western analysis. In this leukocyte population there was no difference in phosopho-p65 between non-pregnant women and pregnant women, or between labouring and non-labouring women. Figure 4.9A shows a representative immunoblot and Figure 4.9B shows a summary of three to four replicates in the form of a graph.

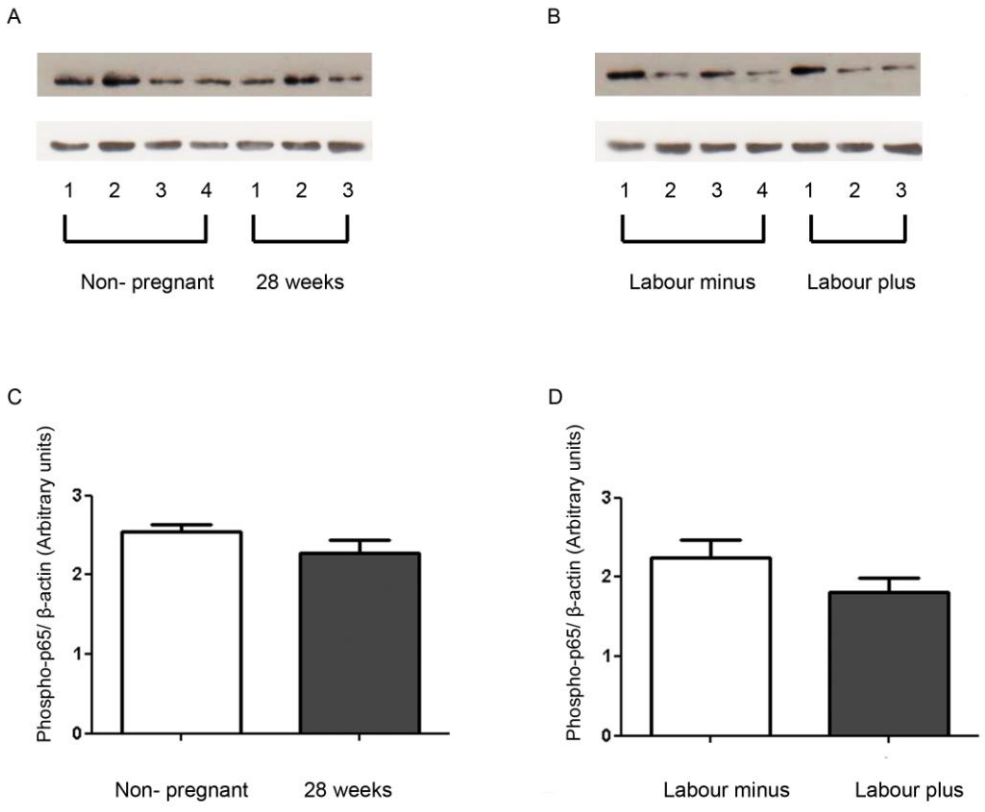


Figure 4.9 No difference in Phospho-p65 in peripheral blood mononuclear cells during pregnancy and in labour

Whole cell lysate was extracted from peripheral blood mononuclear cells from non-pregnant women, women at 28 weeks and at term not in labour or in labour. Levels of phosphorylated p65 (p-p65) were examined using immunoblotting. Representative immunoblots are shown for each gestational time point (A), n=3 or 4. Immunoblots were reprobed for β-actin as an internal loading control. Densitometric analysis of the immunoblots showed no change in p-p65 between gestational timepoints (B). Effect of treatment was examined for statistical significance at each gestational timepoint using the Students t-test.

4.10 $15dPGJ_2$ reduces PMA stimulated phospho p65 at all gestational time points

Since $15dPGJ_2$ was shown to reduce PMA stimulated NF-κB in non-pregnant women, the effect on NF-κB during pregnancy and in labour was also determined. A reduction was seen at all gestational time points, with the most significant effect seen at 28 weeks, (*P*<0.0001) and at term in labour (*P*<0.05), Figure 4.10.

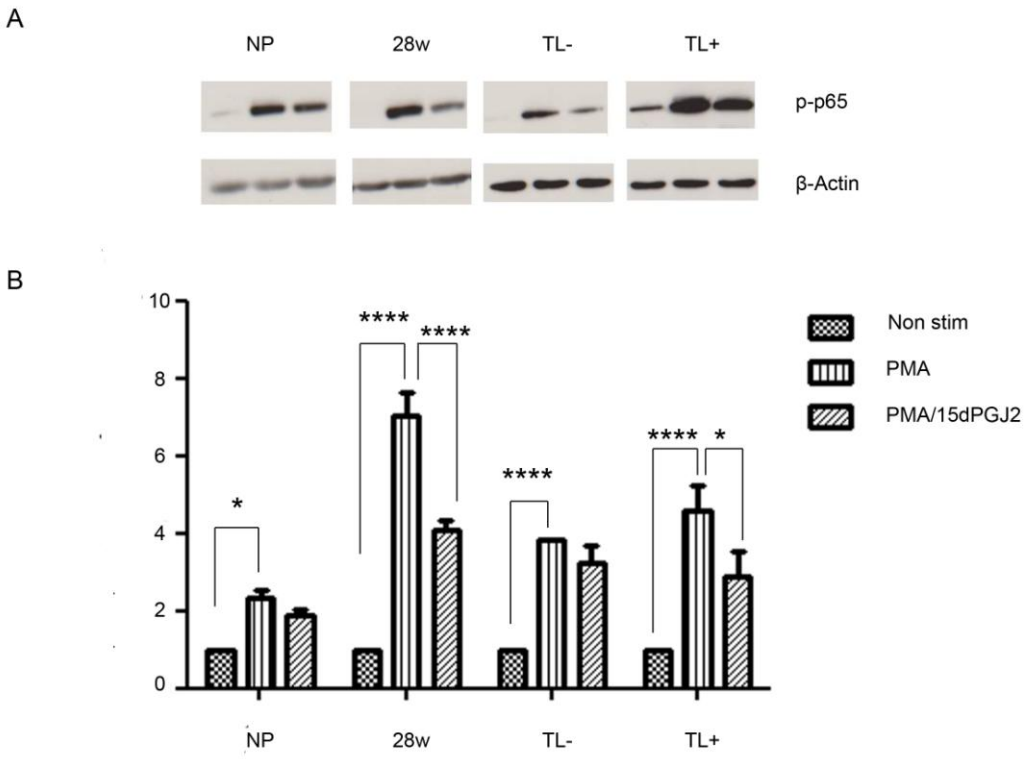


Figure 4.10 $15dPGJ_2$ reduces PMA stimulated NF-κB p65 phosphorylation in peripheral blood mononuclear cells during pregnancy and in labour
PBMCs were treated as previously described and whole cell lysate was extracted. Levels of phosphorylated p65 (p-p65) were examined using immunoblotting. Representative immunoblots are shown for each gestational time point (A) (n=3). Immunoblots were reprobed for β-actin as an internal loading control. Densitometric analysis of the immunoblots was conducted revealing a significant increase in p-p65 levels upon stimulation with PMA/ionomycin in all samples (B). A significant decrease in PMA/ionomycin stimulated p-p65 was observed following $15dPGJ_2$ treatment in 28 week and term in labour samples. Effect of treatment was examined for statistical significance at each gestational timepoint using 2 way ANOVA with Bonferroni's multiple comparison test; **** *P*<0.0001*** *P*<0.001, ***P*<0.01, **P*<0.05.

Summary and discussion

The discovery that the anti-inflammatory prostaglandin $15dPGJ_2$ inhibits NF-κB in human amniocytes and myocytes [271], and delays inflammation induced preterm labour in the mouse [277] by an unknown mechanism led us to investigate a potential role for the CRTH2 receptor and small molecule CRTH2 agonists. Data presented in chapter 3 concludes that CRTH2 is not expressed on human amniocytes and myocytes, thus implying there is no role for CRTH2 in $15dPGJ_2$-mediated inhibition of NF-κB in these cells. It is not known what effect CRTH2 agonists have on NF-κB signalling, and the effect of $15dPGJ_2$ on NF-κB in human peripheral mononuclear cells has not been examined before. Data presented in this chapter conclusively rules out any role for CRTH2 in $15dPGJ_2$-mediated inhibition of NF-κB, however it shows that $15dPGJ_2$ does have inhibitory effects on NF-κB in PBMCs, which is maximal in activated cells at 28 weeks of gestation and in labour.

<u>The CRTH2 agonist Pyl A</u>

Pyl A is a stable synthetic, potent, and selective CRTH2 agonist which was made by Oxygen Healthcare based on the published compound L-888,607 synthesised by Merck [320]. The compound was shown to bind to DP1 and CRTH2 with affinity (*K*i) of 2331 and 0.8 nM (2.333 μM and 0.0008 μm) respectively. The structures of CRTH2 agonists are shown in Figure 4.11 (adapted from Gervais et al 2005). The CRTH2 agonist Pyl A (L-888,607) was developed from L-883,595 which generated two stereoisomers; L-888,291 and L-888,607, with the latter being the most potent. The arrow (1) shows the point which reflects the stereoisomer structure. The cyclopentenone ring (2) contributes to the potency as does the presence of the sulphur molecule (3). Substituting a heavier molecule at the site of the fluoride molecule was found to reduce the potency (4). At higher concentrations, the compound is able to bind to other prostanoid receptors with the rank of order of affinity as follows: CRTH2> TP> EP3> DP> EP4> EP2> FP> IP> EP1.

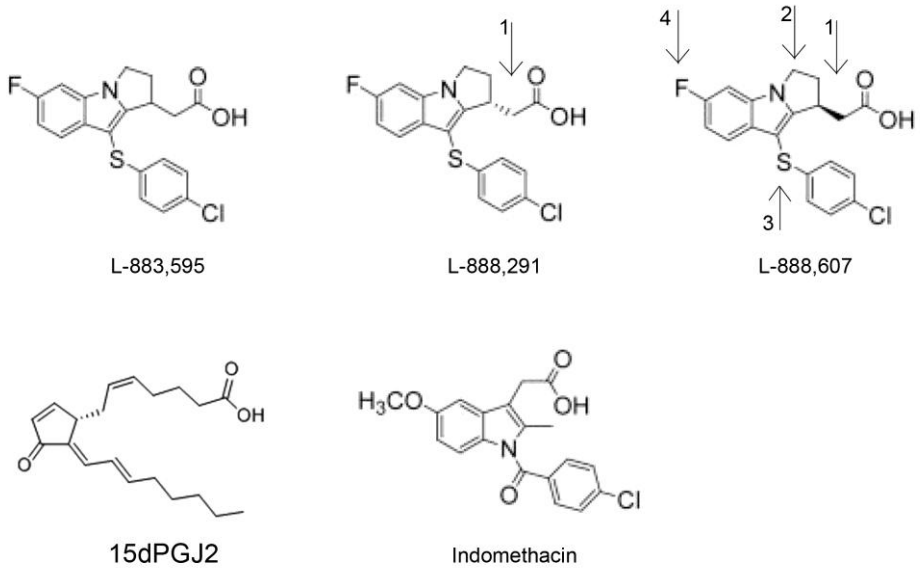


Figure 4.11 The structure of CRTH2 agonists. Adapted from Gervais et al, 2005.
The potent stereoisomer L-888,607 was developed from the L-883,595 compound by Gervais et al. The structure is shown alongside its stereoisomer L-888,291 with arrow 1 at the point which reflects the stereoisomeric structure. Arrow 2 points to the presence of the cyclopentenone ring which can be seen in all structures including $15dPGJ_2$. The presence of the sulphur molecule appears to be important for the affinity of the compound as shown by arrow 3. By substituting the fluoride molecule (arrow 4) the potency is reduced.

The activity of L-888,607 at the CRTH2 receptor was demonstrated by its ability to cause eosinophil morphological shape change and maximal eosinophil chemotaxis at 100 nM concentration [320]. For the purpose of this thesis, the activity at the CRTH2 receptor was demonstrated by its ability to increase the expression of CD11b in eosinophils (Figure 4.1). PGD_2, $15dPGJ_2$ and Indomethacin increase its expression via CRTH2, which is thought to contribute to eosinophil migration [303, 304, 321]. As added confirmation that Pyl A was acting through CRTH2, the CRTH2 antagonist GSKCRTH2X inhibited this response.

The CRTH2 agonist Pyl A has no effect on NF-κB in amniocytes, myocytes and PBMCs

The agonist Pyl A had no effect on phospho-p65 in human amniocytes and myocytes (Figure 4.3). This was expected in view of the absence of detectable levels of the CRTH2 protein in amniocytes and myocytes. The effect of the CRTH2 agonists Pyl A and DK- PGD_2 on NF-κB activity was explored in peripheral blood mononuclear cells, with known expression of CRTH2. However, no effect on phospho-65 was seen in PBMCs either, (Figures 4.5 and 4.6). The only other study to explore the effect of CRTH2 agonists on NF-κB showed that an IKB inhibitor had no effect PGD_2 induced cytokine production. Despite rocaglamide (an NF-κB inhibitor) causing a reduction in PGD_2-induced cytokine production by 30%, PGD_2 did not lead to an increase phospho-IκB, suggesting a non-pathway specific effect of rocaglamide [302]. Together with this data reporting no direct effect on NF-κB signalling upon CRTH2 activation with PGD_2, our data also supports no direct effect on NF-κB signalling via CRTH2. The main pathway activated with CRTH2 agonists appears to be the phosphatidylinositol 3-kinase (PI3K) pathway which is involved in indomethacin induced eosinophil morphological change, CD11b expression [304] and PGD_2 induced production of IL-4, IL-5 and IL-13, and chemotaxis in Th2 cells [302].

The CRTH2 agonist $15dPGJ_2$ inhibits NF-κB in amniocytes, myocytes and PBMCs independently of CRTH2

As previously described, $15dPGJ_2$ is an endogenous prostaglandin which accumulates intracellularly [278], and can be measured endogenously in picomolar concentrations in body fluids [322]. As well as being a ligand for PPAR-γ, it is also an agonist of the CRTH2 receptor [287]. $15dPGJ_2$ has previously been shown to inhibit NF-κB in amniocytes and myocytes *in vitro* [271], the effect of which was replicated here as seen in Figure 4.2A and B. Since NF-κB controls the expression of many labour associated genes and pro-inflammatory cytokines; targeting its inhibition seems

attractive to prevent both inflammation induced preterm labour and the associated risk of brain injury associated with inflammation. As well as the potentially beneficial effect of $15dPGJ_2$ locally at the maternal fetal interface, a peripheral anti-inflammatory effect in women with systemic signs of chorioamnionitis could also be beneficial in preventing neonatal adverse outcome.

The inhibitory effect of $15dPGJ_2$ on NF-κB activity was replicated in peripheral blood mononuclear cells at similar concentrations used with amniocytes and myocytes, (Figure 4.4). A reduction was also seen in PBMCs stimulated with the classical activators of T cells, PMA and ionomycin (Figure 4.8). To finally rule out a role of CRTH2 in $15dPGJ_2$ mediated inhibition of NF-κB activity, PBMCs were pre-incubated with the CRTH2 antagonist GSKCRTH2X (Figure 4.7). No attenuation of NF-κB inhibition was seen with the CRTH2 antagonist. As demonstrated in chapter 3 CRTH2 is only present on a small subset of T cells, and the PBMCs population examined here will contain a significant proportion of non-CRTH2 expressing T and B cells. Since there was complete inhibition of NF-κB (Figure 4.7), whatever the mechanism of action, it must be a mechanism which is occurs in all subsets of leukocytes in the PBMC population examined.

Although $15dPGJ_2$ is also a high affinity ligand to the nuclear PPAR-γ receptor, this is not the mechanism of NF-κB inhibition in amniocytes and myocytes [271]. $15dPGJ_2$ contains an electrophilic cyclopenteneone ring, a property which permits it to ligate nuclear receptors and modify intracellular signalling molecules. The highly reactive ring of $15dPGJ_2$ can form a covalent interaction with components of the NF-κB signalling pathway (IkB kinase complex B, p50, and p65 subunits) via the Michael reaction, resulting in impaired nuclear entry and DNA binding activity [323]. PGA_1, which contains an electrophilic cyclopentenone ring mimics the effect of $15dPGJ_2$ in amniocytes and myocytes, whereas 9, 10-dihydro-$15dPGJ_2$ (in which the ring is disrupted) does not [271]. Although Pyl A contains a cyclopentenone ring, it is not highly electrophilic like $15dPGJ_2$. It is highly likely that the electrophilic cyclopentenone ring of $15dPGJ_2$ is also essential for the inhibition of NF-κB seen in PBMCs. Although prostaglandin J2 has been shown to enter the cell by active transport [324], it is not

yet clear if this is the mechanism of entry into amniocytes, myocytes and PBMCs. Data presented in this chapter excludes a CRTH2-dependent receptor-mediated entry into the cell as a requirement for NF-κB inhibition, and excludes a CRTH2 dependent downstream signalling effect for the inhibition of NF-κB.

NF-κB activity in PBMCs during pregnancy and in labour

The immune system undergoes significant adaptation during pregnancy to accommodate the "foreign" fetus with a degree of immune-suppression whilst maintaining adequate immune function to ward off infection. The precise immuno-regulatory mechanisms remain to be determined. There is significant evidence supporting the bias towards a favourable Th2 interleukin profile away from the pro-inflammatory Th1 profile for successful pregnancy maintenance, which is discussed in more detail in chapter five. One proposed immuno-regulatory mechanism is that of suppressed NF-κB activity in T cells during pregnancy compared to non-pregnant controls which is thought to explain the suppressed Th1 response in pregnancy [240]. Peripheral blood mononuclear cells from women in the third trimester stimulated with pooled non-pregnant serum and LPS show reduced NF-κB DNA binding activity, reduced nuclear p65 and reduced cytosolic Iκ-Bα and Iκ-Bβ [257]. Isolated T cells of pregnant women in the third trimester also show a reduced response to PMA/ionomycin stimulation with absent nuclear and whole cell p65 and reduced whole cell and cytosolic Iκ-Bα and Iκ-Bβ [240].This group demonstrated that levels of PMA stimulated nuclear p65 returned to non-pregnant values by 72 hours post partum [256].

Contrary to what was anticipated, basal NF-κB activity as determined by whole cell phospho-p65 was unchanged throughout pregnancy and in labour in peripheral blood mononuclear cells (Figure 4.9). PMA/ionomycin stimulation led to a significantly increased activity of NF-κB at all time points in pregnancy with no apparent difference compared to non-pregnant controls (Figure 4.10). Two of the studies above isolated T cells, and therefore the results from the PBMCs, which will include B cells are not

representative of the cell population in McCracken's studies [240, 257]. In the one study in which they also used PBMCs, cells were stimulated with pooled serum and LPS, which again is not a comparable method to the one which was used in this thesis. Whilst all studies are valid and provide useful insights into potential *in vivo* leukocyte NF-κB activity, isolation of T cells provides more information on the potential immuno-regulation specifically in these cells which may then alter the Th1/Th2 balance. However, the presence of B cells *in vivo* also serve a potentially important role, and the effect on peripheral blood cells as a whole with cell-cell interaction may be more relevant in reflecting the overall immune response which occurs *in vivo*.

$15dPGJ_2$ was able to reduce basal and PMA/ionomycin activated NF-κB, with the most significant effect seen at 28 weeks and at term in labour. The women at these time points would be the target populations of any proposed administration of $15dPGJ_2$ as an anti-inflammatory and anti-labour therapeutic agent.

In summary, from the data presented in this chapter, a conclusion can be drawn that $15dPGJ_2$ inhibits NF-κB in a mechanism independent of CRTH2. The discovery that $15dPGJ_2$ also suppresses NF-κB activity in peripheral blood mononuclear cells raises the possibility that it may also be used to suppress systemic inflammation and reduce production of pro-inflammatory cytokines in circulating peripheral leukocytes.

CHAPTER 5

CRTH2 agonist effect on the Th1/Th2 interleukins

Chapter summary

Hypothesis:

The CRTH2 agonist PGD_2 induces the production of the Th2 interleukins *in vitro*. $15dPGJ_2$ inhibits NF-κB in peripheral blood mononuclear cells (PBMCs). It is possible that the CRTH2 agonists $15dPGJ_2$ and Pyl A can modulate the systemic production of Th1/Th2 interleukins.

Aims:

The work presented in this chapter aims to determine if the Th1 interleukins are suppressed and if the Th2 interleukin IL-4 is increased in PBMCs during pregnancy. The Th1/Th2 interleukin profile during labour was also examined to determine if there is a shift towards the pro-inflammatory Th1 response, consistent with the pro-inflammatory profile seen locally in amnion and myometrium. The potential immunomodulatory effect of the CRTH2 agonists $15dPGJ_2$ and Pyl A on the Th1/Th2 profile in PBMCs was examined at different stages of pregnancy and in labour.

Methods:

Blood was collected from women at different gestational time points and during labour. PBMCs were treated with CRTH2 agonists or vehicle and stimulated with PMA/ionomycin. Expression of intracellular interleukins in CD4 or CRTH2 positive cells was determined by flow cytometry.

Results:

The Th1 interleukins IFN-γ and TNF-α were suppressed in pregnancy, with no increase in labour. There was no change in IL-4 production during pregnancy or in labour. $15dPGJ_2$ suppressed PMA/ionomycin stimulated Th1 interleukin production; however neither $15dPGJ_2$ nor Pyl A increased IL-4 production.

Conclusion:

Unlike Pyl A, $15dPGJ_2$ is able to modulate the Th1:Th2 interleukin ratio, by suppressing the Th1 cytokines. This effect is not via CRTH2 and is likely to be via inhibition of NF-κB.

Introduction

The hypothesis of Th2 predominance and downregulation of the Th1 response for the maintenance of successful pregnancy originated from Wegmann and colleagues [209] and was reinforced by evidence from both murine studies and the clinical course of Th2 and Th1 based conditions in pregnancy. Th1 and Th2 cell subsets originate from undifferentiated Th0 cells under the influence of IFN-γ and IL-4 respectively. Type 1 $CD4^+$ T cells produce an array of inflammatory cytokines including IFN-γ [190], IL-2 [191], and Tumor necrosis factor-alpha (TNF-α) [192], whereas the type 2 $CD4^+$ T cells (Th2) produce IL-4, IL-5, IL-13, IL-10 [190].

Term labour is often seen as a pro-inflammatory state and therefore it is plausible that labour is associated with a reversal in the bias back towards Th1 rather than Th2 in the peripheral blood cells. There is substantial evidence that the Th1 cytokines play a role in the initiation of labour at term locally at the maternal fetal interface [192]. Fetal membranes [166, 171] and myometrium [325] produce IL-1β at term, and TNF-α and IL-1β are both increased in amnion, amniotic fluid and decidua at term [233]. TNF-α and IL-1β are potent inducers of the transcription factor NF-κB [271]. NF-κB regulates the expression of several labour-associated genes including COX-2, the oxytocin receptor, IL-8 and matrix metalloproteinase-9 (MMP-9) [245].

Preterm labour is often associated with infection and inflammation, particularly early preterm labour [63]. Activation of the Th1 cytokines occurs as a specific response to infection caused by intracellular bacteria, parasites and viruses [239]. During infection IL-12 is produced by macrophages and dendritic cells [326, 327], which leads to polarisation from Th0 to Th1 type cells [194]. The Th1:Th2 ratio is further altered by IFN-γ up-regulating the IL-12 receptor leading to further Th1 development, and by the inhibition of Th2 cells [328]. As part of the fetal response to infection and PROM, there is also evidence of a fetal Th1 polarised response with an increase IFN-γ production in cord blood [101]. Pro-inflammatory cytokines, such as TNF-α and IL-1β can ultimately result in the production of prostaglandins and MMPs [236], via NF-κB. The production

of pro-inflammatory Th1 interleukins therefore has the potential for triggering a cascade of pro-labour events including uterine contractility and fetal membrane rupture leading to preterm labour. The presence of infection and inflammation is now considered an independent risk factor for the neonatal brain injury in neonates born preterm [319], and at term [329]. For example, amniotic fluid and cord blood pro-inflammatory cytokines IL-1β, IL-6, and TNF-α are associated with radiological evidence of cerebral damage [330, 331].

PGD_2 promotes the production of the Th2 interleukins IL-4, IL-13, IL-5 and IL-10 in T helper 2 cells *in vitro* via the second PGD_2 receptor, CRTH2 [217]. PGD_2 is produced by the placenta and may play a role in the chemo-attraction of Th2 cells, via the CRTH2 receptor, to the maternal fetal interface to produce a localised Th2 bias [198]. The non-immune cells of the maternal fetal interface; trophoblast, decidua and amnion can contribute to the Th2 cytokine environment by production of IL-13 [222], IL-10 [223], IL-4 and IL-6 [224, 225]. There is substantial evidence for the importance of a Th2 bias during pregnancy, mainly at the maternal fetal interface [192, 208, 332]. Consistent with this, decidual $CD4^+$ cells from women undergoing unexplained recurrent pregnancy loss typically exhibit reduced IL-4 and IL-10 production [149].

In this chapter, the potential for CRTH2 agonists as immunomodulatory therapeutic agents was explored. The expression profile of the Th1 interleukins IFN-γ and TNF-α, and the Th2 interleukin IL-4 in T helper cells were examined to identify if; firstly a Th2 bias exists during pregnancy, and secondly if this is altered during term labour. The effect of $15dPGJ_2$ on the Th1 and Th2 interleukins was examined to determine if a Th1 response could be suppressed via its ability to inhibit NF-κB in PBMCs, and if a Th2 response could be induced via its agonist effect on the CRTH2 receptor. The small molecule agonist Pyl A was also used to determine if a Th2 response could be induced, and if a Th1 response could be indirectly suppressed by the production of Th2 interleukins.

Results

5.1 The Th1/Th2 interleukin profile during pregnancy and labour

Many groups have reported on a bias in the Th1:Th2 interleukin ratio in favour of Th2 interleukins during pregnancy at a local level, however there is limited data on the Th1:Th2 profile in peripheral blood mononuclear cells in labour. This chapter reports on the Th1:Th2 profile in T helper cells during pregnancy and in labour, and the effects of the CRTH2 agonists $15dPGJ_2$ and Pyl A on the interleukins. PBMCs were examined from women at 28 weeks gestation, at term pre labour and in labour, and from non-pregnant women serving as controls. Cells were stimulated with PMA/ionomycin (or vehicle control). For examining the effects of the CRTH2 agonists cells were incubated for 2 hrs with the agonist prior to PMA/ionomycin stimulation. Intracellular production of the Th1 interleukins IFN-γ and TNF-α, and the Th2 interleukin IL-4 were examined by flow cytometry. The percentage of cells producing the interleukins, as well as the mean fluorescence intensity of expression was analysed. Four biological replicates were used in all experiments presented in this chapter.

5.1.1 IFN-γ production in $CD4^+$ (T helper cells) at different gestational time points

PMA /ionomycin stimulated cells from women at 28 weeks, term pre labour, term in labour and non-pregnant women were examined for IFN-γ production. Cells were labelled with CD4-APC to identify T helper cells. The percentage of IFN-γ^+/$CD4^+$ cells and the geometric mean fluorescence intensity of IFN-γ in these cells were determined. Figure 5.1.1A shows a representative dot blot of non-stimulated cells, non-pregnant and 28 week PMA/ionomycin treated lymphocytes. The right upper quadrant shows the IFN-γ^+/$CD4^+$ cells. A clear increase in the percentage of cells producing IFN-γ is seen with PMA/Ionomycin treatment. A lower percentage of cells produced IFN-γ during pregnancy, as shown by the representative non-pregnant and 28 week dot blots.

Panel B summarises the results for n=4. The mean percentage of IFN-γ-producing $CD4^+$ lymphocytes in response to stimulation reduced from 10.74% in non pregnant women to 6.57% at 28weeks, 5.065% at term pre-labour and 5.55% at term in labour respectively ($P < 0.05$, $P < 0.001$ and $P < 0.01$ respectively). There was also a reduction in the percentage of cells secreting IFN-γ in the non-stimulated state, however this did not reach statistical significance. PMA/ionomycin significantly increased the production of IFN-γ at all time points, however the mean fluorescence intensity of production was not significantly different between gestational time points in stimulated cells; with a mean fluorescence intensity of 101.92, 115.7, 110.14 and 88.28 in non pregnant, 28 weeks, term pre-labour and term in labour samples respectively (Figure 5.1.1C). No significant increase in either the percentage of cells producing IFN-γ or the expression of IFN-γ was seen in labour compared to the pre-labour samples in stimulated and non-stimulated cells (Figure 5.1.1B). There was nevertheless a small but clear increasing trend in the basal percentage of cells producing IFN-γ from 28 weeks to term, with a further increase in labour (Figure 5.1.1B – seen more clearly with different axis range later in Figure 5.2.1A panel B).

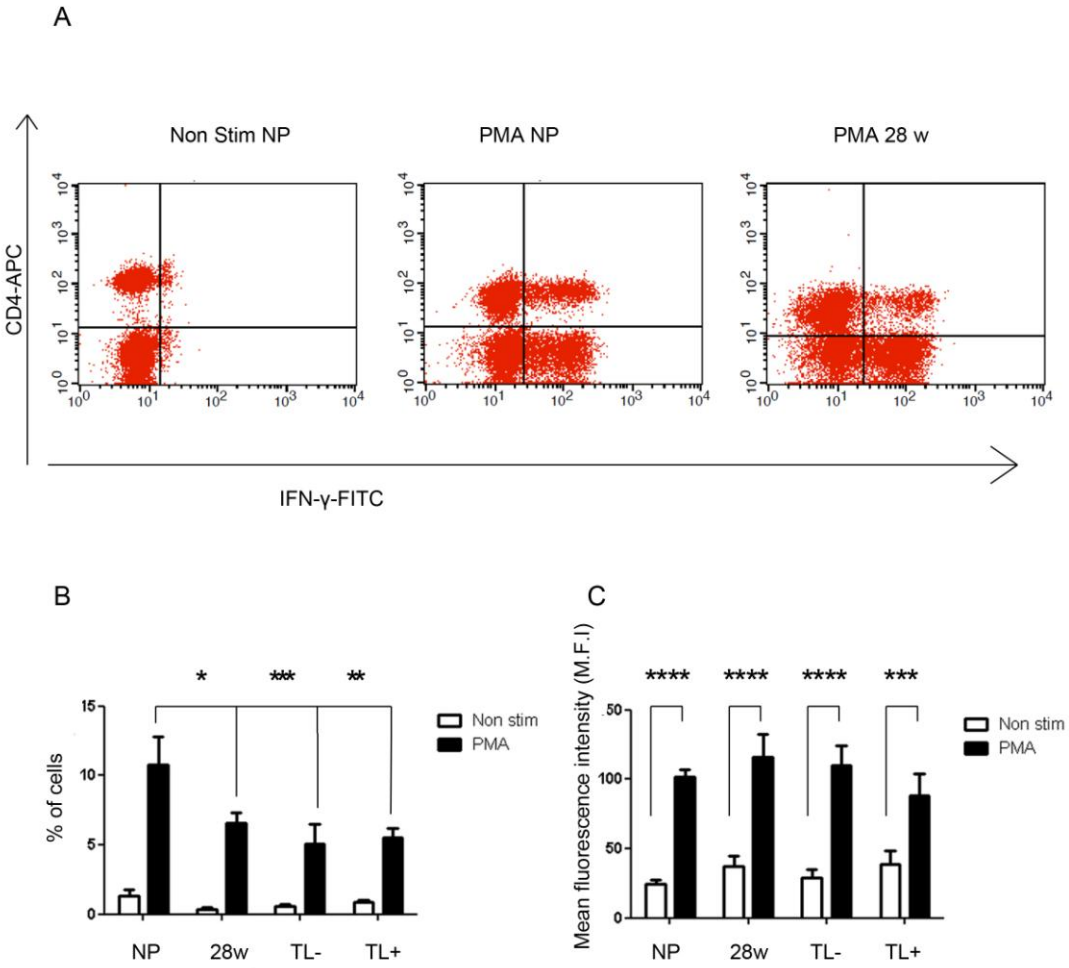


Figure 5.1.1 IFN-γ production in $CD4^+$ T helper cells at different gestational time points
Peripheral blood mononuclear cells were isolated and stimulated with PMA/ionomycin. The percentage of IFN-γ^+/$CD4^+$ positive cells and the geometric mean fluorescence intensity of IFN-γ^+ were detected by flow cytometry in samples derived from non-pregnant (NP), 28 week pregnant, term pre-labour (TL-) and term in labour (TL+) samples. A representative dot blot shows the effect of stimulation on IFN-γ production in CD4 positive cells in non-pregnant and 28 week lymphocyte samples, (seen in the right upper quadrants in panel A). A significant reduction in the percentage IFN-γ secreting $CD4^+$ lymphocytes was seen at all gestational time points compared to non-pregnant controls (B). Geometric mean fluorescence intensity (M.F.I.) was also assessed as a measure of total cytokine production (C). PMA/ionomycin stimulation significantly increased IFN-γ production in all sample groups, however no differences in gestation-dependent responses were detected. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test using NP or non-stimulated samples as a control was used; ****P<0.0001, ***P< 0.001, **P<0.01, *P<0.05.

5.1.2 TNF-α production in $CD4^+$ (T helper cells) at different gestational time points

The second Th1 interleukin to be examined in PMA /ionomycin stimulated cells at the different gestational time points was TNF-α. The percentage of TNF-α^+/$CD4^+$ cells and the geometric mean fluorescence intensity of TNF-α in these cells were determined. Figure 5.1.2A shows a representative dot blot of non-stimulated cells, non-pregnant and 28 week PMA/ionomycin treated lymphocytes. The right upper quadrant shows the TNF-α^+/$CD4^+$ cells. An increase in the percentage of cells producing TNF-α is seen with PMA/ionomycin treatment. A lower percentage of cells produced TNF-α during pregnancy, as shown by the representative non-pregnant and 28 week dot blots, although the effect was more subtle than in the IFN-γ dot blots.

Panel B of figure 5.1.2 summarises the results for n=4. The mean percentage of TNF-α -producing $CD4^+$ lymphocytes in response to stimulation reduced from 20.6% in non-pregnant women to 14.53% at 28 weeks, 15.78% at term pre-labour and 13.26% at term in labour respectively, however this effect did not reach statistical significance. There was also a non-significant reduction in the percentage of cells secreting TNF-α in the non-stimulated cells. PMA/ionomycin significantly increased the percentage of cells producing TNF-α and the expression within the positive cells at all time points, however the mean fluorescence intensity of production was not significantly different between gestational time points in stimulated cells; with a mean fluorescence intensity of 63.28, 72.62, 64.44 and 55.96 in non pregnant, 28 weeks, term pre-labour and term in labour samples respectively (Figure 5.1.2C). No significant increase in either the percentage of cells producing TNF-α or the expression of TNF-α was seen in labour compared to the pre-labour samples in PMA/ionomycin or non-stimulated cells (Figures 5.1.2B). There was nevertheless a small but clear increasing trend in the basal percentage of cells producing TNF-α from 28 weeks to term, with a further increase in labour (Figure 5.1.2B – seen more clearly with different axis scale later in Figure 5.2.2A panel B).

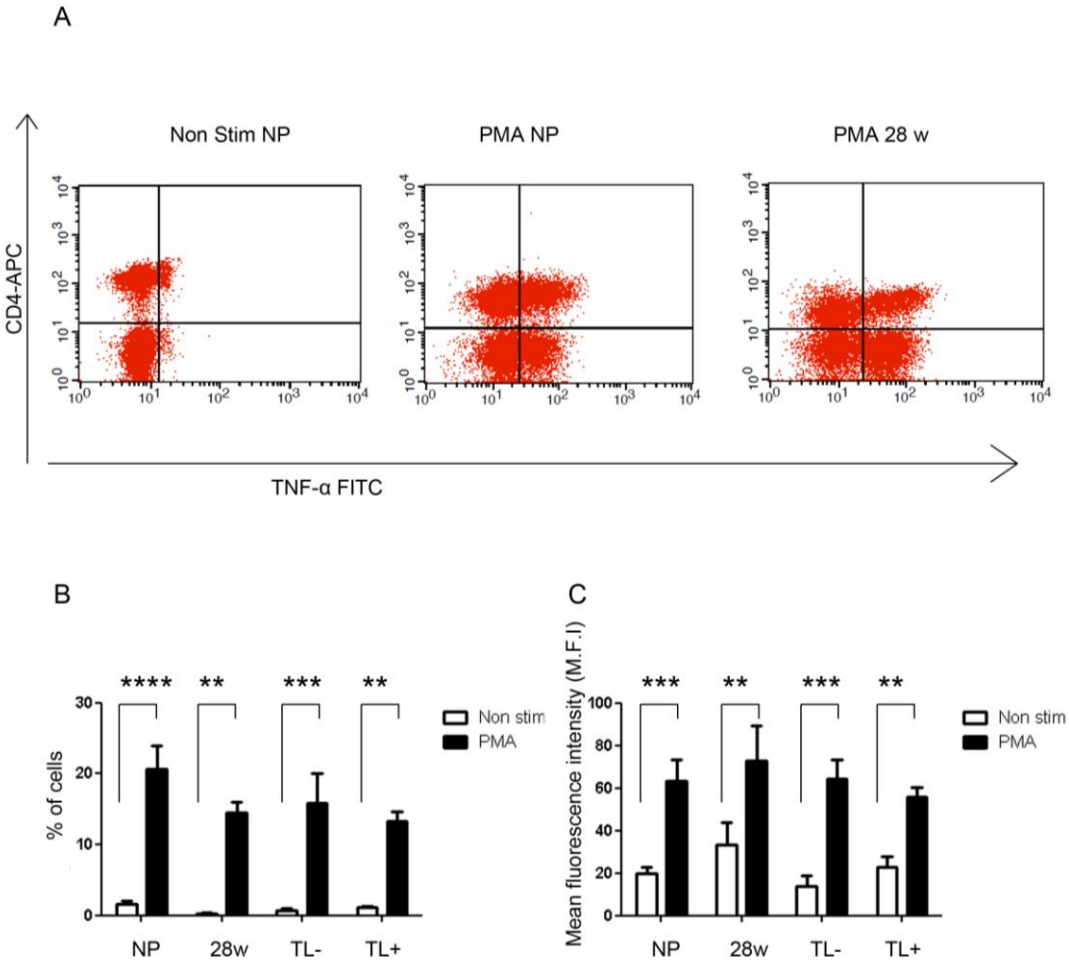


Figure 5.1.2 TNF-α production in CD4$^+$ T helper cells at different gestational time points
Peripheral blood mononuclear cells were isolated and stimulated with PMA/ionomycin. The percentage of TNF-α$^+$/CD4$^+$ positive cells and the geometric mean fluorescence intensity of TNF-α were determined by flow cytometry in samples derived from non-pregnant (NP), 28 week pregnant, term pre-labour (TL-) and term in labour (TL+) samples. A representative dot blot shows the effect of stimulation on TNF-α production in CD4 positive cells in non-pregnant and 28 week lymphocyte samples, (seen in the right upper quadrants in panel A). PMA/ionomycin significantly increased the percentage of cells producing TNF-α and mean expression (B). Although a reduction was seen in the percentage of TNF-α/CD4$^+$ cells during pregnancy in both non-stimulated and stimulated cells, this narrowly missed out on statistical significance. Geometric mean fluorescence intensity (M.F.I.) was also assessed as a measure of total cytokine production (C). PMA/ionomycin stimulation significantly increased TNF-α production in all sample groups, however no differences in gestation-dependent responses were detected. For statistical analysis Two-way ANOVA with Bonferroni's multiple comparison test using NP or non-stimulated samples as a control was used; ****P<0.0001, ***P< 0.001, **P<0.01.

5.1.3 IL-4 production in $CRTH2^+$ cells at different gestational time points

The classic Th2 interleukin is IL-4. CRTH2 cells produce IL-4 upon stimulation. The effect of PMA/ionomycin stimulation at the different gestational time points was examined. The percentage of $IL\text{-}4^+/CRTH2^+$ cells and the geometric mean fluorescence intensity of IL-4 in these cells were determined. Figure 5.1.3A shows a representative dot blot of non-stimulated cells, and non-pregnant and 28 week PMA/ionomycin stimulated lymphocytes. The right upper quadrant shows the $IL\text{-}4^+/CRTH2^+$ cells.

A very small increase in the percentage of CRTH2 positive cells producing IL-4 is seen with PMA/ionomycin treatment during pregnancy, although this does not reach statistical significance; see Figure 5.1.3 panel B. PMA/ionomycin significantly increased the mean production of IL-4 in PMA/ionomycin stimulated cells at 28 weeks and at term pre-labour from 33.07 to 39.35 and 31.5 to 39.4 respectively. However the mean fluorescence intensity of production was not significantly different between gestational time points in stimulated cells. Despite there being an increase in the percentage of cells producing IL-4 at 28 weeks compared to non –pregnant samples, with a subsequent reduction pre labour at term, this narrowly missed out on statistical significance with the use of the Bonferroni's multiple comparisons test. No significant difference in either the percentage of cells producing IL-4 or the expression of IL-4 was seen in labour compared to the pre-labour samples.

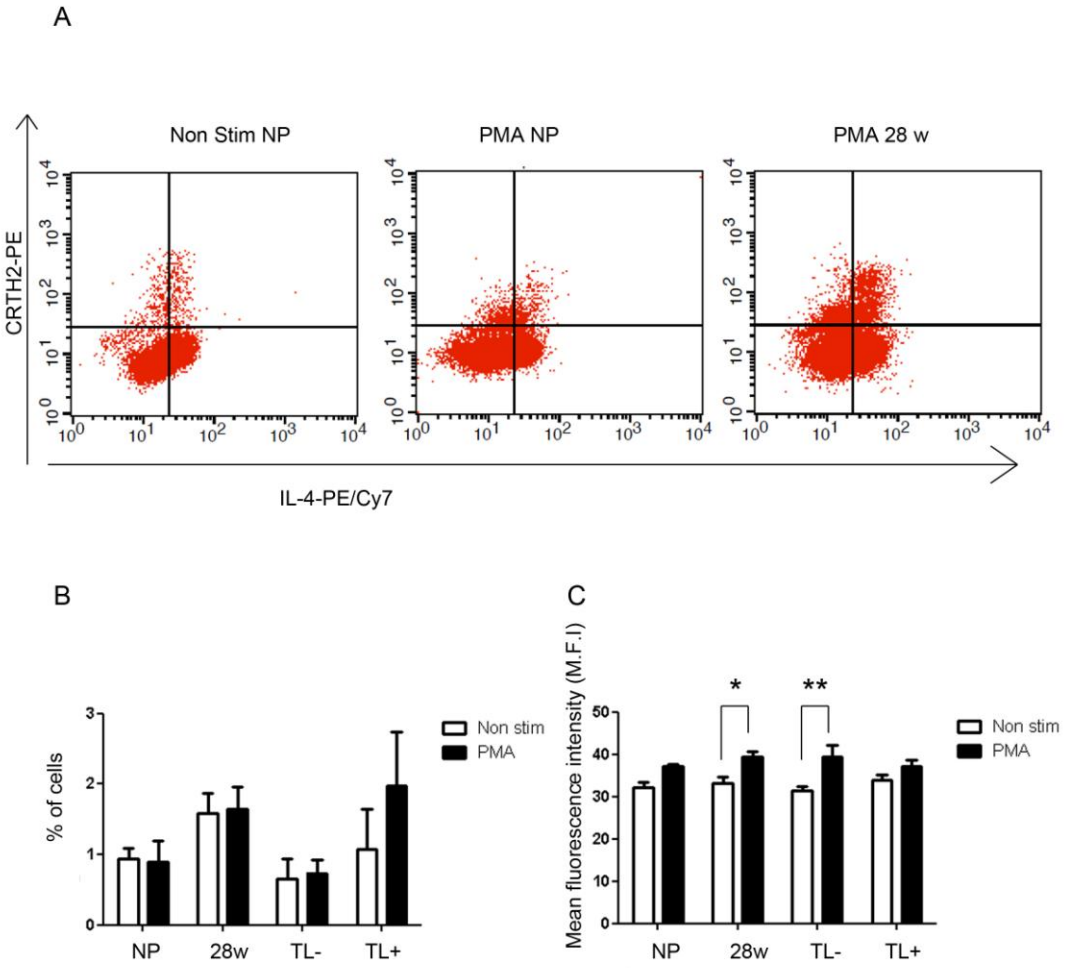


Figure 5.1.3 IL-4 production in CRTH2$^+$ T helper cells at different gestational time points
Peripheral blood mononuclear cells were isolated and stimulated with PMA/ionomycin. The percentage of CRTH2$^+$/ IL-4 positive cells and the geometric mean fluorescence intensity of IL-4 were determined by flow cytometry in samples derived from non-pregnant (NP), 28 week pregnant, term pre-labour (TL-) and term in labour (TL+) samples. A representative dot blot shows the effect of stimulation on IL-4 production in CRTH2 positive cells in non-pregnant and 28 week lymphocyte samples, (seen in the right upper quadrants in panel A). PMA/ionomycin increased the percentage of cells producing IL-4 (B) and mean expression during pregnancy (C). No statistically significant difference was seen in the percentage of IL-4/CRTH2$^+$ cells or IL-4 production during pregnancy compared to non-pregnant controls in both non-stimulated and stimulated cells. For statistical analysis two-way ANOVA with Bonferroni's multiple comparison test using NP or non-stimulated samples as a control was used; $^{**}P<0.01$, $^{*}P<0.05$.

5.2 The effect of the CRTH2 agonist $15dPGJ_2$ on the Th1 and Th2 interleukins

$15dPGJ_2$ is an anti-inflammatory prostaglandin and a ligand for CRTH2. We therefore explored the effect of $15dPGJ_2$ on the Th1 cytokines; IFN-γ and TNF-α. To do this, cells were pre incubated with 32 μM of $15dPGJ_2$ or vehicle control prior to the addition of PMA/ionomycin or vehicle before intracellular cytokines were detected by flow cytometry.

5.2.1 Effect of $15dPGJ_2$ on IFN-γ production in $CD4^+$ (T helper cells)

5.2.1A Non PMA/Ionomycin stimulated cells

The effect of $15dPGJ_2$ on basal IFN-γ production was examined. $15dPGJ_2$ appeared to create a $CD4^+/IFN\text{-}\gamma^+$ population as demonstrated in the representative dot plot in panel A of figure 5.2.1A. This led to an increase in the percentage of $IFN\text{-}\gamma^+/CD4^+$ cells (panel B) and an increase in IFN-γ production, (panel C). The percentage of $IFN\text{-}\gamma^+/CD4^+$ lymphocytes increased from 1.3% to 2.2% in non-pregnant women (non-significant), 0.35% to 2.36% at 28 weeks (P<0.001), 0.57% to 1.84% at term (P<0.05), and 0.87% to 1.78% (non- significant) at term in labour. $15dPGJ_2$ also led to a small increase in the production of IFN-γ (measured as the geometric mean fluorescence intensity) in T helper cells from 24.36 to 28.08 (non-significant) in non-pregnant controls, 37.29-45.59 (P<0.05) in 28 week samples and from 29.26 to 34.35 in term pre-labour (non-significant), and 38.85 to 45.34 in term in labour (non significant) samples. An example histogram shows the mean intensity of $IFN\text{-}\gamma^+/CD4^+$ cells under the different conditions, panel D.

5.2.1B PMA/Ionomycin stimulated cells

The effect of $15dPGJ_2$ on PMA/ionomycin IFN-γ production was examined, with a representative dot plot shown in panel A of figure 5.2.1B. $15dPGJ_2$ reduced the percentage of PMA/ionomycin stimulated IFN-γ^+/$CD4^+$ cells and the mean production of IFN-γ at all gestational time points, see panel B and C Figure 5.2.1B. The percentage of IFN-γ^+/$CD4^+$ lymphocytes reduced from 10.7% to 5.13% in non-pregnant women (P<0.001), 6.6% to 3.4% at 28 weeks (P<0.05), 5.1% to 2.5% at term L- and 5.6 % to 2.9% at term L+. The reduction in PMA/ionomycin stimulated IFN-γ (measured as the geometric mean fluorescence intensity) was from 101.9 to 52.96 (P<0.01) in non-pregnant controls, 115.7 to 52.35 (P<0.001) in 28 week samples and from 110.1 to 60.73 in term pre-labour (P<0.01), and 88.28 to 66.30 in term in labour (P>0.05) samples. An example histogram shows the mean intensity of IFN-γ^+/$CD4^+$ cells under the different conditions, panel D.

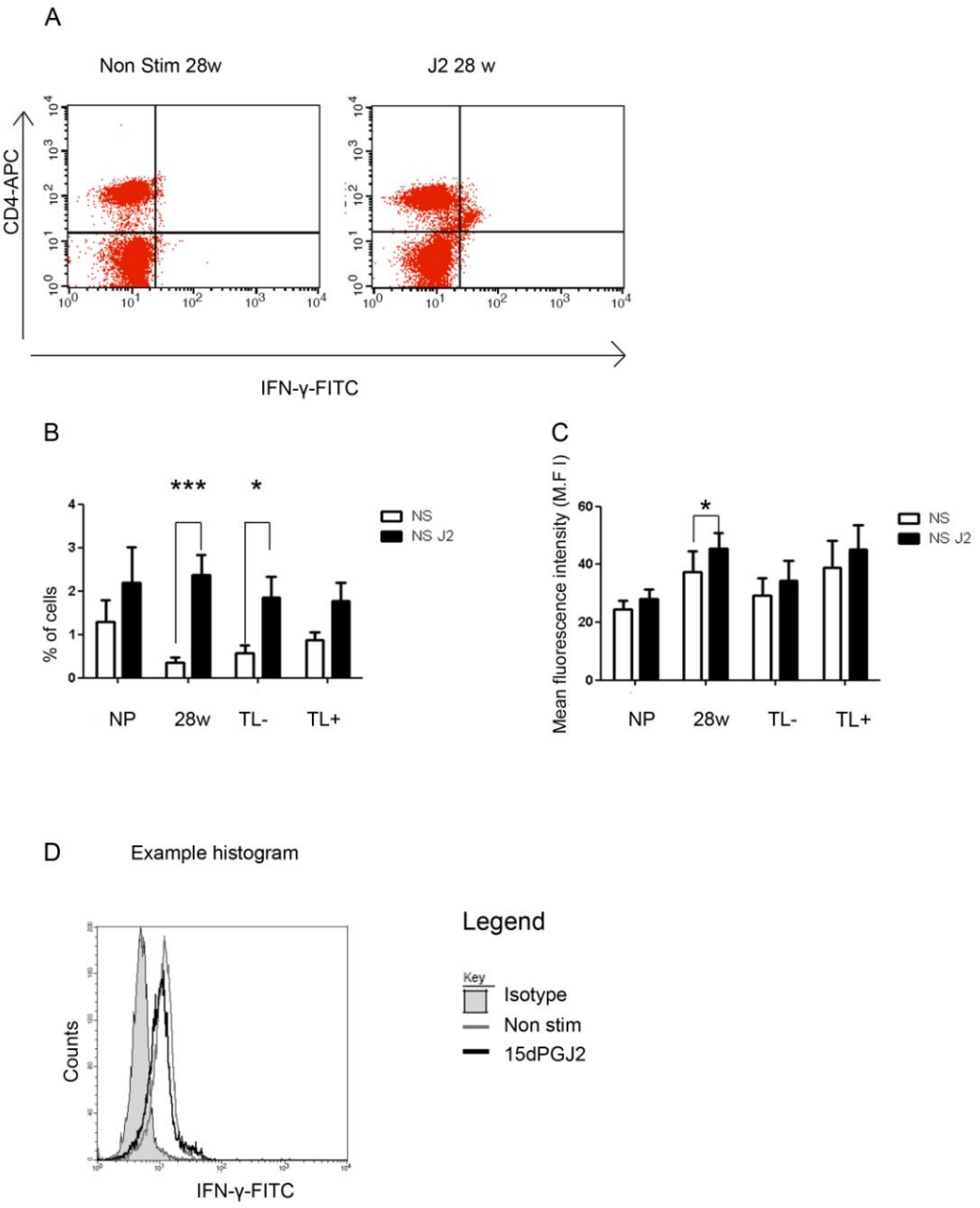


Figure 5.2.1A Effect of $15dPGJ_2$ on IFN-γ production in non-stimulated $CD4^+$ (T helper cells)
Peripheral blood mononuclear cells were incubated with vehicle control or 32 µM of $15dPGJ_2$ before being gated on the lymphocyte population according to forward and side scatter. $15dPGJ_2$ induced a population of IFN-γ^+/$CD4^+$ cells as shown in a representative 28 week patient sample, with IFN-γ^+/$CD4^+$ cells shown in the right upper quadrant (A). The percentage of IFN-γ^+/$CD4^+$ cells increased with $15dPGJ_2$ treatment (B), as did the mean production of IFN-γ^+ as summarised in the graphs (C). A representative histogram reveals a small shift to the right upon treatment indicative of an increase in IFN-γ (D). For statistical analysis a two-way ANOVA with Bonferroni's multiple comparison test was used with non-treated cells as controls; ***P<0.001, *P<0.05.

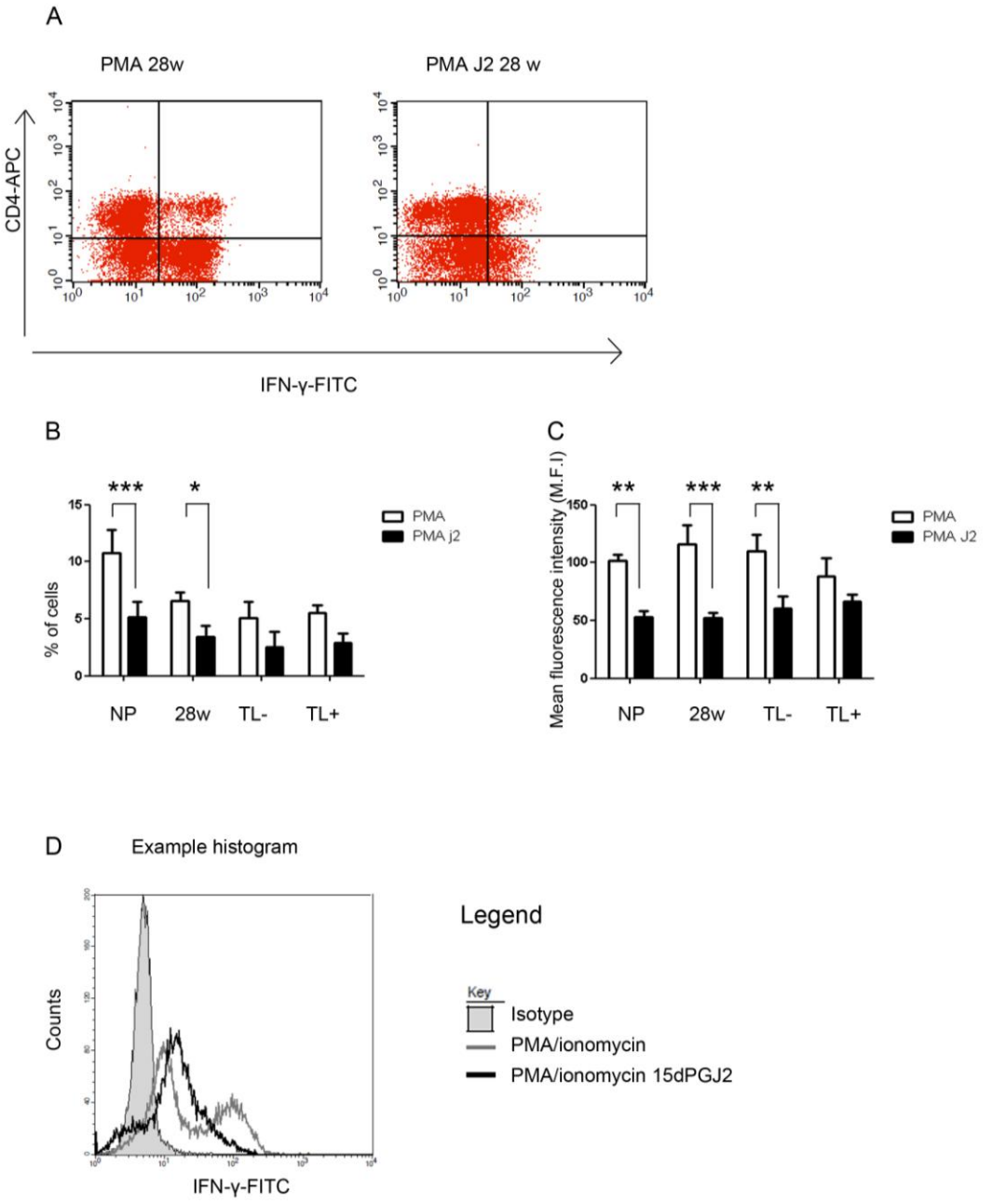


Figure 5.2.1B Effect of 15dPGJ$_2$ on IFN-γ production in PMA stimulated CD4$^+$ (T helper cells)
Peripheral blood mononuclear cells were incubated with vehicle control or 32 µM of 15dPGJ$_2$ and stimulated with PMA/ionomycin before being gated on the lymphocyte population according to forward and side scatter. 15dPGJ$_2$ reduced the percentage of IFN-γ$^+$/CD4$^+$ cells as shown in a representative 28 week patient sample, with IFN-γ$^+$/CD4$^+$ cells shown in the right upper quadrant (A). The percentage of IFN-γ$^+$/CD4$^+$ cells reduced with 15dPGJ$_2$ treatment at all gestational time points (B), as did the mean production of IFN-γ$^+$ as summarised in the graphs (C). A representative histogram reveals a clear shift to the left upon treatment with 15dPGJ$_2$ indicative of a decrease in IFN-γ (D). For statistical analysis a two-way ANOVA with Bonferroni's multiple comparison test was used with non-treated cells as controls; ***P<0.001, **P<0.01, *P<0.05.

5.2.2 Effect of 15dPGJ2 on TNF-α production in CD4+ (T helper cells)

5.2.2A Non PMA/Ionomycin stimulated cells

The effect of $15dPGJ_2$ on basal TNF-α production was examined to determine if the same effect was seen as with IFN-γ. $15dPGJ_2$ also appeared to create a TNF-$\alpha^+/CD4^+$population as demonstrated in the representative dot plot in panel A of Figure 5.2.2A. This led to an increase in the percentage of TNF-$\alpha^+/CD4^+$ cells (panel B) and a small increase in TNF-α production, (panel C). The percentage of TNF-$\alpha^+/CD4^+$lymphocytes increased from 1.57% to 3.28% in non-pregnant women (non-significant), 0.31% to 4.36% at 28 weeks ($P<0.001$), 0.72% to 3.08% at term ($P<0.05$), and 1.12% to 3.13% (non-significant) at term in labour. $15dPGJ_2$ also led to a small increase in the production of TNF-α (measured as the geometric mean fluorescence intensity) in T helper cells from 19.85 to 24.75 (non-significant) in non-pregnant controls, 33.4-34.54 (non-significant) in 28 week samples and from 13.73 to 23.96 in term pre-labour ($P<0.05$), and 22.65 to 26.67 in term in labour (non-significant) samples. An example histogram shows the mean intensity of TNF-$\alpha^+/CD4^+$ cells under the different conditions, panel D.

5.2.2B PMA/Ionomycin stimulated cells

The effect of $15dPGJ_2$ on PMA/ionomycin TNF-α production was also examined, with a representative dot plot shown in panel A of figure 5.2.2B. $15dPGJ_2$ reduced the percentage of PMA/ionomycin stimulated TNF-$\alpha^+/CD4^+$ cells (panel B) and the mean production of TNF-α at all gestational time points, see panel C Figure 5.2.2B. The percentage of TNF-$\alpha^+/CD4^+$ lymphocytes reduced from 20.6% to 16.75% in non-pregnant women ($P<0.05$), 14.53% to 8.99% at 28 weeks ($P<0.001$), 15.79% to 12.92% at term (non-significant) and 13.96% to 8.93% ($P<0.05$). $15dPGJ_2$ also reduced the production of PMA/ionomycin stimulated TNF-α (measured as the geometric mean fluorescence intensity) in T helper cells from 63.29 to 45.65 ($P<0.05$) in non-pregnant controls, 72.62 to 53.14 ($P<0.01$) in 28 week samples and from 64.44 to 46.05 in term

pre-labour (P<0.01), and 55.96 to 45.97 in term in labour (non-significant) samples. An example histogram shows the mean intensity of TNF-α^+/CD4$^+$ cells under the different conditions, panel D.

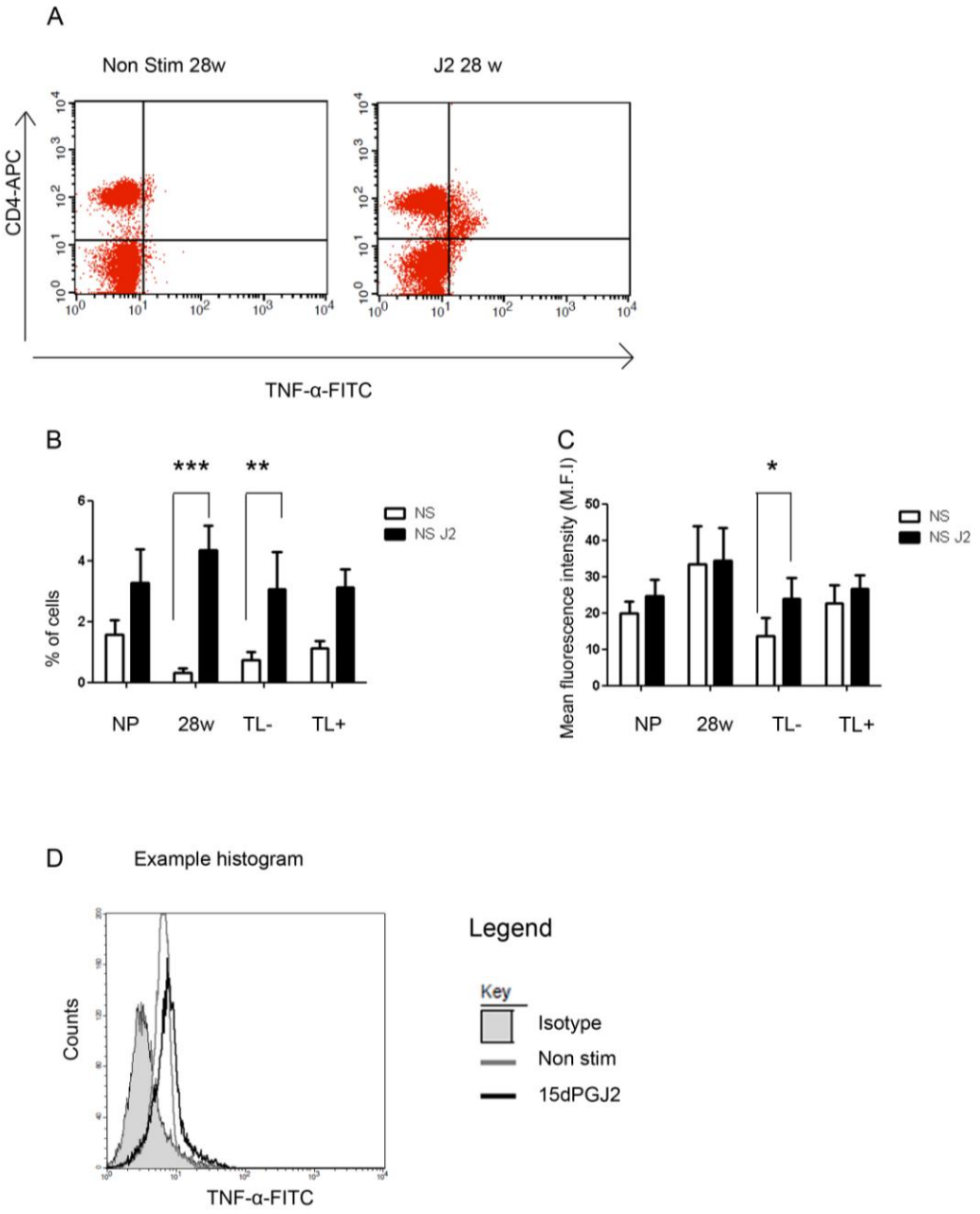


Figure 5.2.2A Effect of 15dPGJ$_2$ on TNF-α^+ production in non-stimulated CD4$^+$ (T helper cells)
Peripheral blood mononuclear cells were incubated with vehicle control or 32 µM of 15dPGJ$_2$ before being gated on the lymphocyte population according to forward and side scatter. 15dPGJ$_2$ induced a population of TNF-α^+/CD4$^+$ cells as shown in a representative 28 week patient sample, with TNF-α^+/CD4$^+$ cells shown in the right upper quadrant (A). The percentage of TNF-α^+/CD4$^+$ cells increased with 15dPGJ$_2$ treatment (B), as did the mean production of TNF-α as summarised in the graphs (C). A representative histogram reveals a small shift to the right upon treatment indicative of an increase in TNF-α (D). For statistical analysis a two-way ANOVA with Bonferroni's multiple comparison test was used with non-treated cells as controls; ***P<0.001, **P<0.01 *P<0.05.

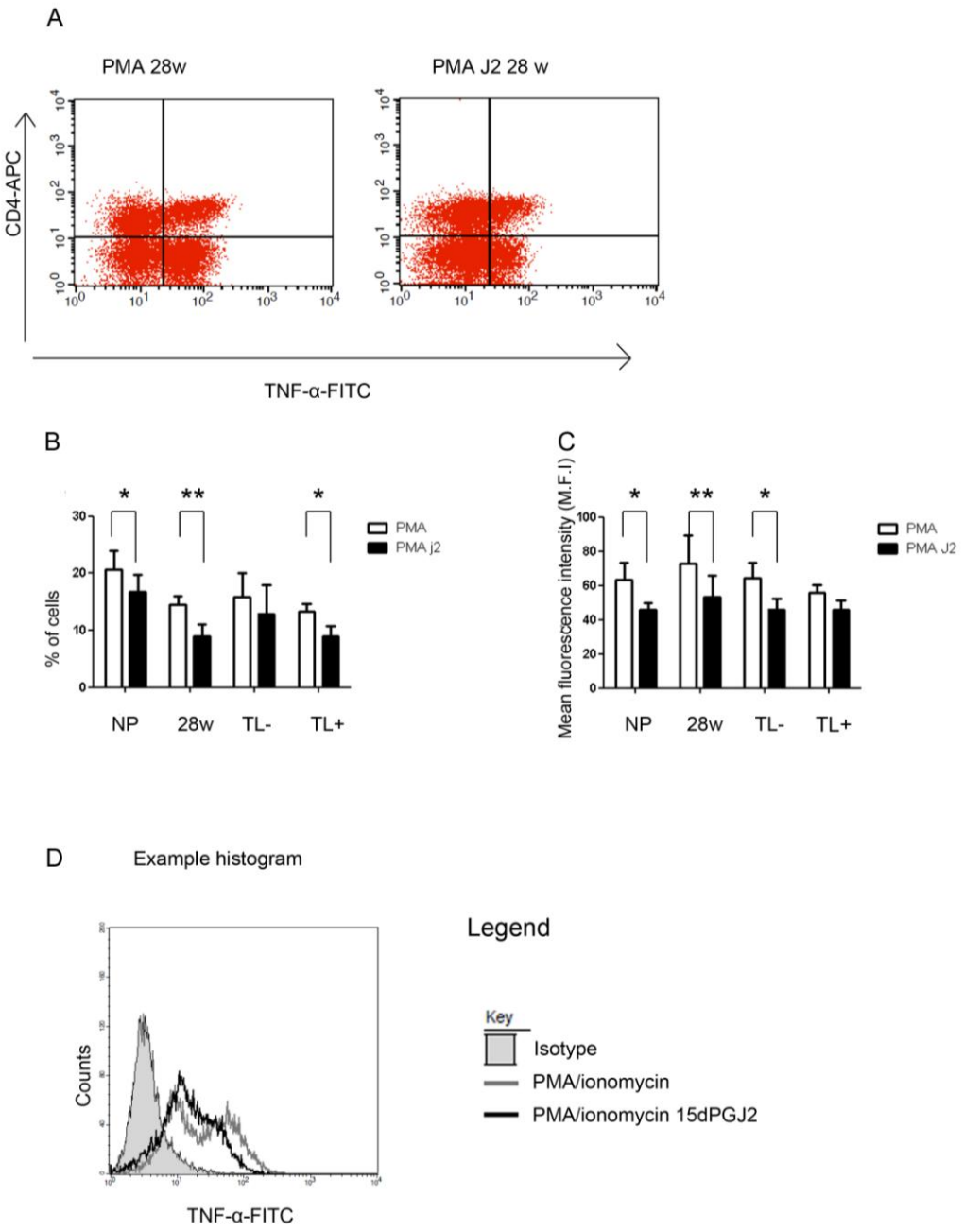


Figure 5.2.2B Effect of 15dPGJ$_2$ on TNF-α production in PMA stimulated CD4$^+$ (T helper cells)
Peripheral blood mononuclear cells were incubated with vehicle control or 32 µM of 15dPGJ$_2$ and stimulated with PMA/ionomycin before being gated on the lymphocyte population according to forward and side scatter. 15dPGJ$_2$ reduced the percentage of TNF-α$^+$/CD4$^+$ cells as shown in a representative 28 week patient sample, with TNF-α$^+$/CD4$^+$ cells shown in the right upper quadrant (A). The percentage of TNF-α$^+$/CD4$^+$ cells reduced with 15dPGJ$_2$ treatment at all gestational time points (B), as did the mean production of TNF-α as summarised in the graphs (C). A representative histogram reveals a clear shift to the left upon treatment with 15dPGJ$_2$ indicative of a decrease in TNF-α (D). For statistical analysis a two-way ANOVA with Bonferroni's multiple comparison test was used with non-treated cells as controls; ***P*<0.01, **P*<0.05.

5.2.3 Effect of $15dPGJ_2$ on IL-4 production in $CRTH2^+$ cells

Since the CRTH2 agonists indomethacin and PGD_2 induce IL-4 production in T helper cells via CRTH2, it was hypothesized that $15dPGJ_2$ would induce an increase in IL-4 production via its action on the CRTH2 receptor.

5.2.3A Non PMA/Ionomycin stimulated cells

The effect of $15dPGJ_2$ on basal IL-4 production was examined. $15dPGJ_2$ had no effect on the percentage of IL-4 producing CRTH2 cells and did not increase the mean production of IL-4, (Figure 5.2.3A, panels A and B).

5.2.3B PMA/Ionomycin stimulated cells

PMA/ionomycin stimulation induced a population of IL-4 producing $CRTH2^+$ cells. These cells were pre-treated with 32 µm of $15dPGJ_2$ for 2 hours. As with non-stimulated cells, pre-treatment with $15dPGJ_2$ in the PMA/ionomycin stimulated cells had no effect on the percentage CRTH2 cells producing IL-4, neither did it increase the mean production of IL-4 in these cells, (Figure 5.2.3B, panels A and B)

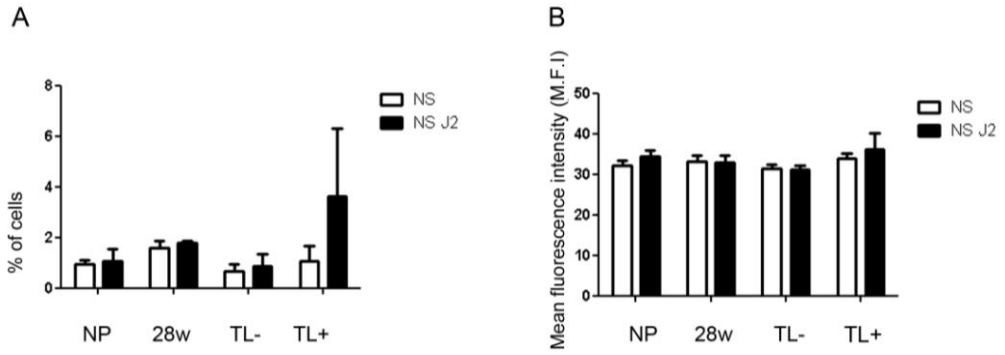


Figure 5.2.3A Effect of $15dPGJ_2$ on IL-4 production in non-stimulated $CRTH2^+$ cells
Peripheral blood mononuclear cells were incubated with vehicle control or 32 μM of $15dPGJ_2$ before being gated on the lymphocyte population according to forward and side scatter. No significant change in the percentage of IL-4 producing $CRTH2^+$ cells was detected (A). Treatment with $15dPGJ_2$ also had no effect in the mean production of IL-4 production (B). For statistical analysis a two-way ANOVA with Bonferroni's multiple comparison test was used with non-treated cells as controls.

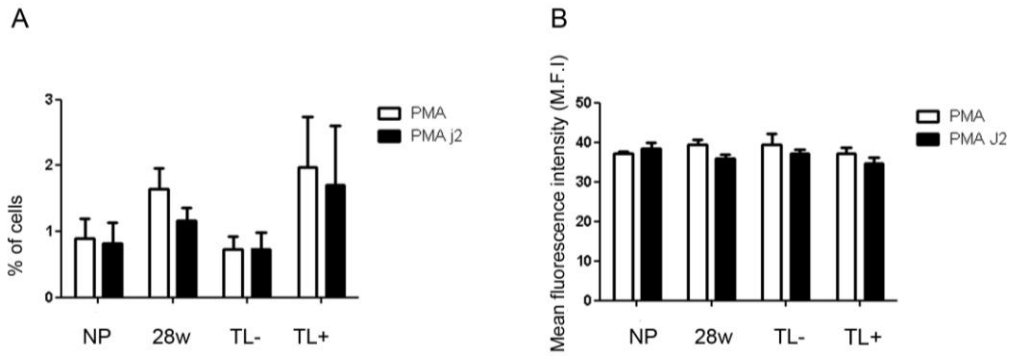


Figure 5.2.3B Effect of $15dPGJ_2$ on IL-4 production in PMA-stimulated $CRTH2^+$ cells
Peripheral blood mononuclear cells were incubated with vehicle control or 32 μM of $15dPGJ_2$ and stimulated with PMA/ionomycin before being gated on the lymphocyte population according to forward and side scatter. No significant change in the percentage of IL-4 producing $CRTH2^+$ cells was detected (A). Treatment with $15dPGJ_2$ also had no effect in the mean production of IL-4 production (B). For statistical analysis a Two-way ANOVA with Bonferroni's multiple comparison test was used with non-treated cells as controls.

5.3 The effect of the CRTH2 agonist Pyl A on the Th1/Th2 interleukins

The effect of the in-house CRTH2 agonist Pyl A on the Th1/Th2 interleukins was next examined. No direct effect on the Th1 response should be seen, since CRTH2 is not expressed on the Th1 cells. However, an indirect reduction in the Th1 interleukins is possible secondary to a possible CRTH2 mediated induction of IL-4 or IL-10 production in Th2 cells. The effect of Pyl A on the Th1 interleukins IFN-γ and TNF-α, along with the Th2 interleukin IL-4 was examined by flow cytometry.

5.3.1 Effect of Pyl A on IFN-γ production in $CD4^+$ (T helper cells)

5.3.1A Non PMA/Ionomycin stimulated cells

The effect of 32 μm of Pyl A on basal IFN-γ production was examined. Unlike $15dPGJ_2$, Pyl A did not create a $CD4^+$/IFN-γ^+ population as demonstrated in the representative dot plot in figure 5.3.1A (A). Pyl A had no effect on the percentage of IFN-γ^+/$CD4^+$ cells at all gestational time points examined (Figure 5.3.1A, panel B). There was also no effect seen on the production of IFN-γ (measured as the geometric mean fluorescence intensity) (Figure 5.3.1A, panel C). An example histogram shows the mean intensity of IFN-γ^+/$CD4^+$ cells under the different conditions, with no shift in either direction with Pyl A treatment (Figure 5.3.1A, panel D).

5.3.1B PMA/Ionomycin stimulated cells

The effect of 32 μm of Pyl A on PMA/ionomycin induced IFN-γ production was examined, with a representative dot plot shown in Figure 5.3.1B (A). Pyl A had no effect on the percentage of PMA/ionomycin stimulated IFN-γ^+/$CD4^+$ cells or the mean production of IFN-γ at all gestational time points, (Figure 5.3.1B, panel B and C). An example histogram shows the mean intensity of IFN-γ^+/$CD4^+$ cells under the different conditions, with no shift in either direction with Pyl A treatment (Figure 5.3.1B, panel D).

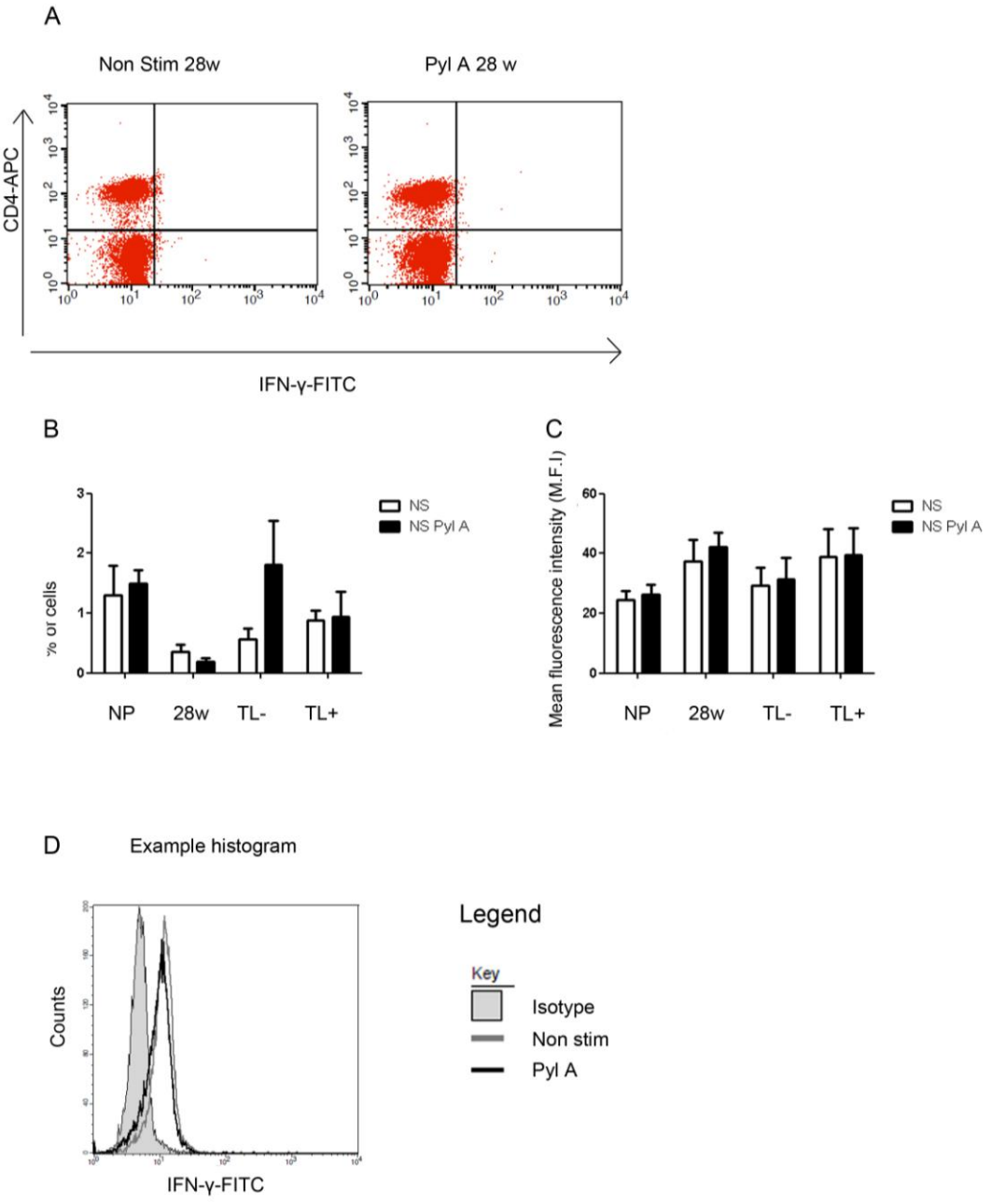


Figure 5.3.1A Effect of Pyl A on IFN-γ production in non-stimulated $CD4^+$ (T helper cells)
Peripheral blood mononuclear cells were incubated with vehicle control or 32 µM of Pyl A before being gated on the lymphocyte population according to forward and side scatter. Pyl A did not have any effect on IFN-γ production in $CD4^+$ cells. A representative dot blot shows the absence of IFN-γ^+/$CD4^+$ cells in the right upper quadrant in this Pyl A treated 28 week sample (A). The percentage of IFN-γ^+/$CD4^+$ cells and the mean production of IFN-γ^+ are summarised in the graphs, showing no difference between non-treated and Pyl A treated samples at all gestational time points (B and C). A representative histogram reveals no shift indicating no effect on IFN-γ (D). For statistical analysis a two-way ANOVA with Bonferroni's multiple comparison test was used with non-treated cells as controls.

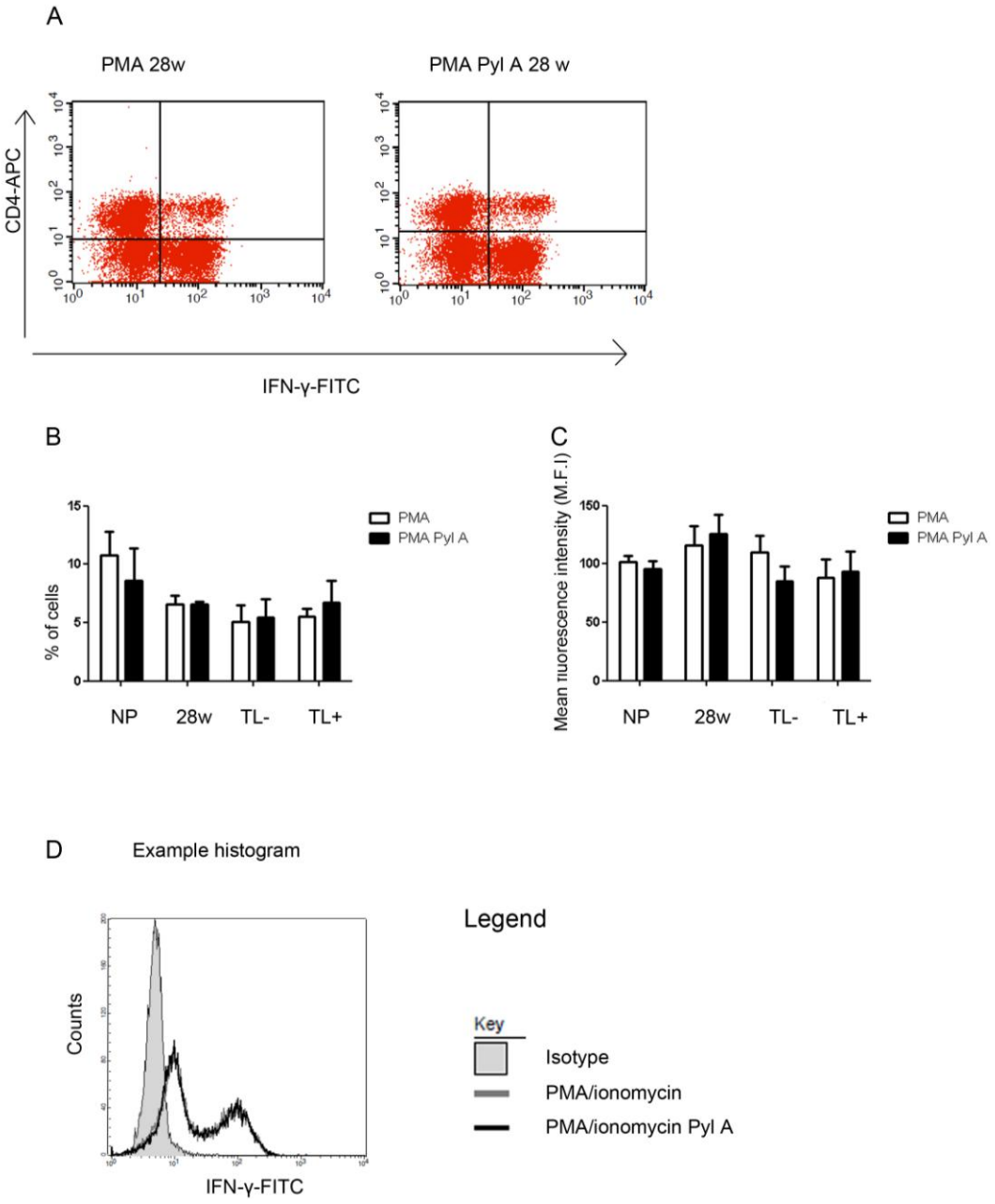


Figure 5.3.1B Effect of Pyl A on IFN-γ production in PMA-stimulated CD4$^+$ (T helper cells)
Peripheral blood mononuclear cells were incubated with vehicle control or 32 µM of Pyl A, then stimulated with PMA/ionomycin before being gated on the lymphocyte population according to forward and side scatter. Pyl A did not have any effect on IFN-γ production in PMA stimulated CD4$^+$ cells. A representative dot blot shows no difference in the percentage of IFN-γ$^+$/CD4$^+$ cells in the right upper quadrant in this 28 week sample with Pyl A treatment (A). The percentage of IFN-γ$^+$/CD4$^+$ cells and the mean production of IFN-γ$^+$ are summarised in the graphs, showing no effect with Pyl A (B and C). A representative histogram reveals no shift indicating no effect on IFN-γ (D). For statistical analysis a two-way ANOVA with Bonferroni's multiple comparison test was used with non-treated cells as controls.

5.3.2 Effect of Pyl A on TNF-α production in $CD4^+$ (T helper cells)

5.3.2A Non PMA/Ionomycin stimulated cells

The effect of 32 μm of Pyl A on basal TNF-α production was examined. Unlike $15dPGJ_2$, Pyl A did not create a TNF-α^+/$CD4^+$ population as demonstrated in the representative dot plot in figure 5.3.2A, panel A. Pyl A had no effect on the percentage of TNF-α^+/$CD4^+$ cells at all gestational time points examined (Figure 5.3.2A, panel B). There was also no effect seen in the production of TNF-α (measured as the geometric mean fluorescence intensity) (Figure 5.3.2A, panel C). An example histogram shows the mean intensity of TNF-α $^+$/$CD4^+$ cells under the different conditions, with no shift in either direction with Pyl A treatment (Figure 5.3.2A, panel D).

5.3.2B PMA/Ionomycin stimulated cells

The effect of 32 μm of Pyl A on PMA/ionomycin induced TNF-α production was examined, with a representative dot plot shown in figure 5.3.2B, panel A. Pyl A had no effect on the percentage of PMA/ionomycin stimulated TNF-α $^+$/$CD4^+$ cells or the mean production of TNF-α at all gestational time points, figure 5.3.2B, panel B and C. An example histogram shows the mean intensity of TNF-α $^+$/$CD4^+$ cells under the different conditions, with no shift in either direction with Pyl A treatment (Figure 5.3.2B, panel D).

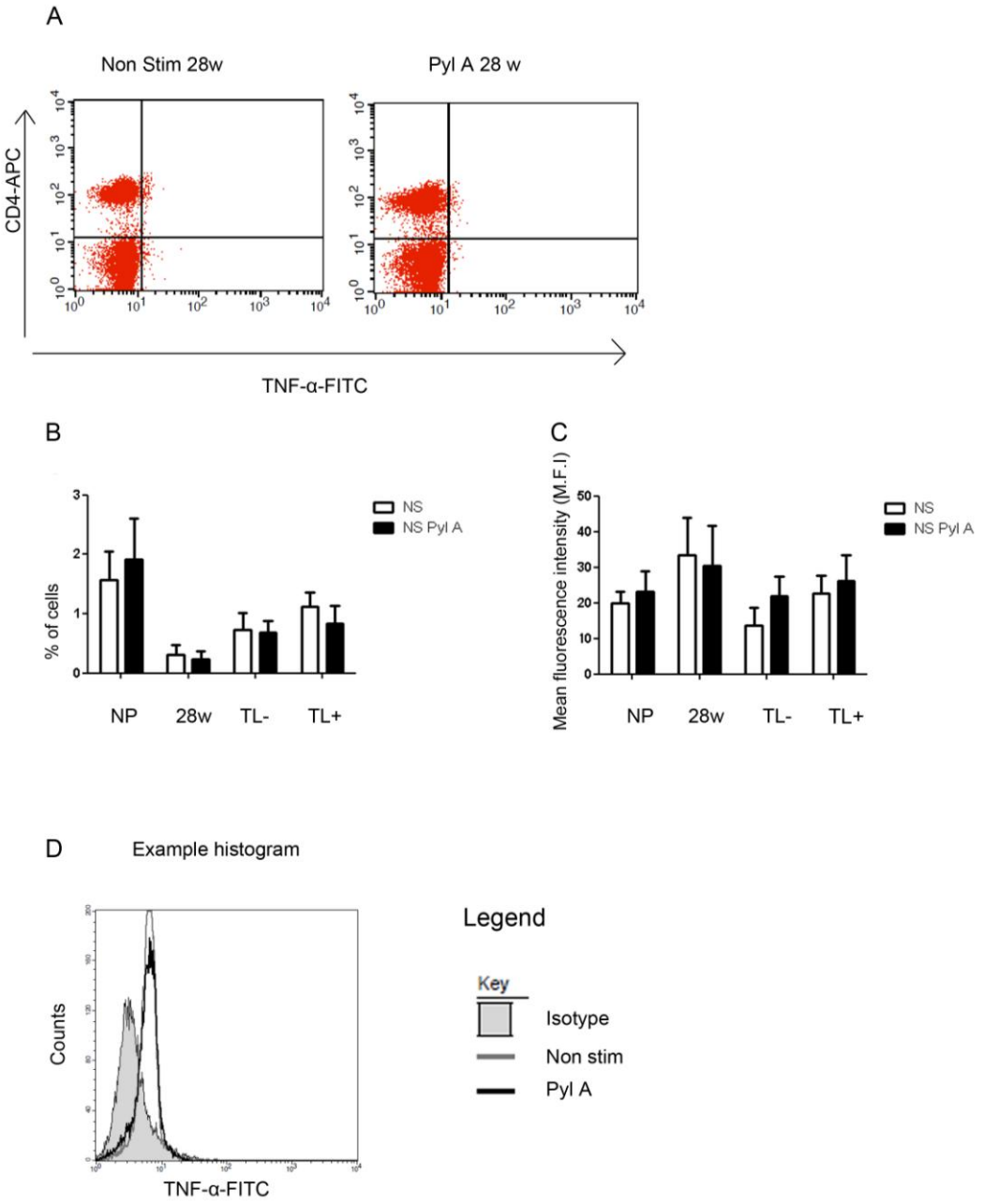


Figure 5.3.2A Effect of Pyl A on TNF-α production in non-stimulated CD4$^+$ (T helper cells)
Peripheral blood mononuclear cells were incubated with vehicle control or 32 µM of Pyl A before being gated on the lymphocyte population according to forward and side scatter. Pyl A did not have any effect on TNF-α production in CD4$^+$ cells. A representative dot blot shows the absence of TNF-α$^+$/CD4$^+$ cells in the right upper quadrant in this Pyl A treated 28 week sample (A). The percentage of TNF-α$^+$/CD4$^+$ cells and the mean production of TNF-α$^+$ are summarised in the graphs, showing no difference between non-treated and Pyl A treated samples at all gestational time points (B and C). A representative histogram reveals no shift indicating no effect on TNF-α (D). For statistical analysis a two-way ANOVA with Bonferroni's multiple comparison test was used with non-treated cells as controls.

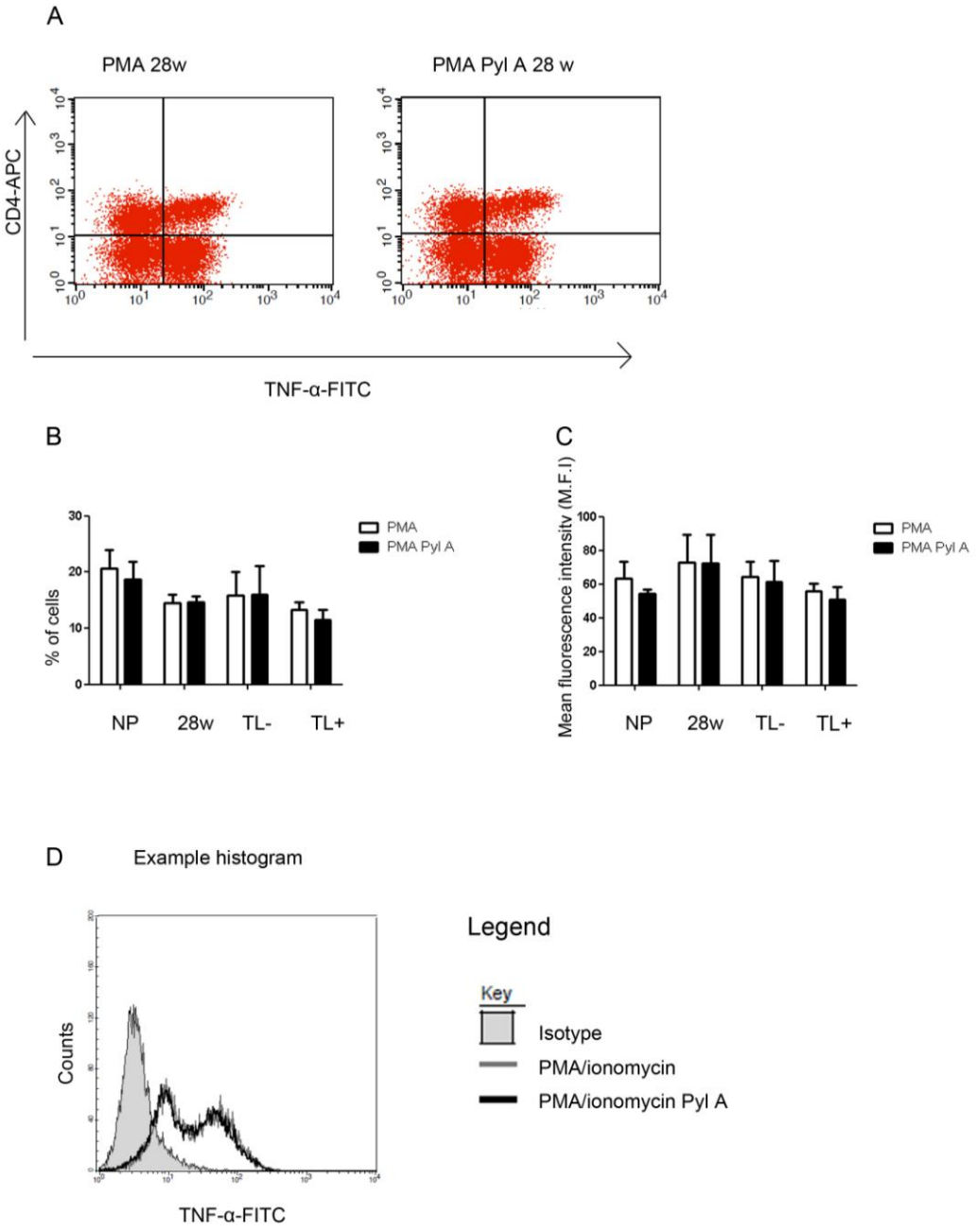


Figure 5.3.2B Effect of Pyl A on TNF-α production in PMA-stimulated $CD4^+$ (T helper cells)
Peripheral blood mononuclear cells were incubated with vehicle control or 32 µM of Pyl A, then stimulated with PMA/ionomycin before being gated on the lymphocyte population according to forward and side scatter. Pyl A did not have any effect on TNF-α production in PMA stimulated $CD4^+$ cells. A representative dot blot shows no difference in the percentage of TNF-α^+/$CD4^+$ cells in the right upper quadrant in this 28 week sample with Pyl A treatment (A). The percentage of TNF-α^+/$CD4^+$ cells and the mean production of TNF-α^+ are summarised in the graphs, showing no difference effect of Pyl A (B and C). A representative histogram reveals no shift indicating no effect on TNF-α (D). For statistical analysis a two-way ANOVA with Bonferroni's multiple comparison test was used with non-treated cells as controls.

5.3.3 Effect of Pyl A on IL-4 production in $CRTH2^+$ cells

The effect of the specific small molecule agonist Pyl A on IL-4 production was also examined. Two concentrations were used; 32 μm and 1 μm. 32 μm was used to serve as a direct comparison with $15dPGJ_2$. 1 μm was also used since activity at the CRTH2 receptor should be seen at this concentration [320] with minimal non-specific effects that may be seen with a higher concentration of 32 μm.

5.3.3A Non PMA/Ionomycin stimulated cells

Firstly the effect of Pyl A on basal IL-4 production was examined. A representative dot plot is shown of cells labelled with CRTH2-PE and IL-4-PECy7 in a 28 week sample (Figure 5.3.3A, panel A). Pyl A had no significant effect on the percentage of IL-4 producing CRTH2 cells or the mean production of IL-4 (Figure 5.3.3A, panel B). The example histogram shows no effect on IL-4 production with Pyl A in non-PMA treated cells from a 28 week sample (Figure 5.3.3A, panel CD.

5.3.3B PMA/Ionomycin stimulated cells

PMA/ionomycin stimulation induced a population of IL-4 producing $CRTH2^+$ cells. Cells were pre-treated with 1 or 32 μm of Pyl A for 2 hrs. A representative dot plot is shown of cells labelled with CRTH2-PE and IL-4-PECy7 in a 28 week sample (Figure 5.3.3B, panel A). Pre-treatment with Pyl A in the PMA/Ionomycin stimulated cells had no significant effect on the percentage CRTH2 cells producing IL-4, or mean production of IL-4 during pregnancy (panel B and C). There was a statistically significant increase in the mean production of IL-4 from 37.08 to 43.6 in non-pregnant samples ($P<0.01$), with no significant increase in the percentage of cells producing of IL-4 (Figure 5.3.3B, panel B and C). An example histogram shows the effect of Pyl A on IL-4 production in a 28 week sample (Figure 5.3.3B, panel D).

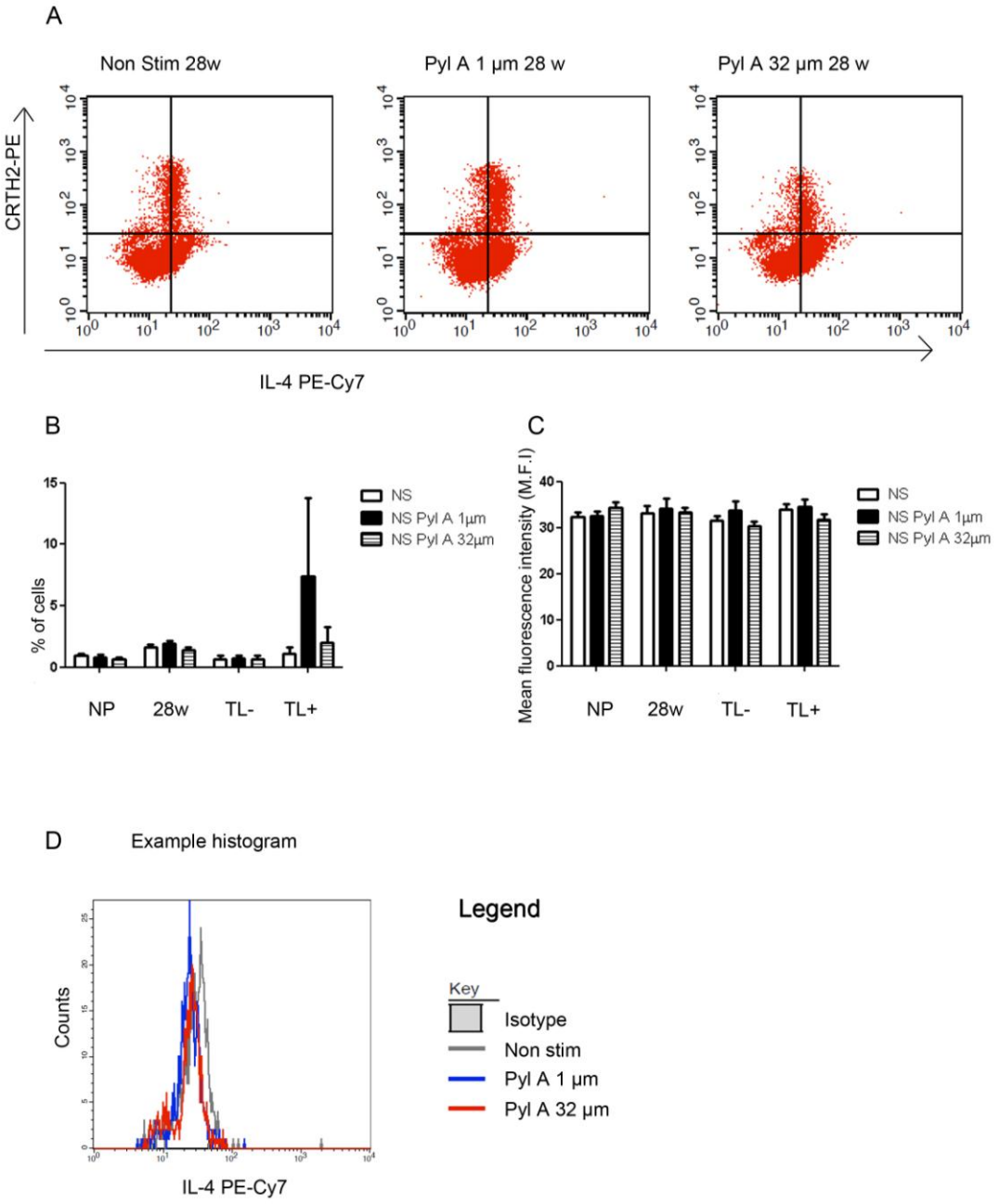


Figure 5.3.3A Effect of Pyl A on IL-4 production in non-stimulated CRTH2$^+$ cells
Peripheral blood mononuclear cells were incubated with vehicle control, 1 µM or 32 µM of Pyl A before being gated on the lymphocyte population according to forward and side scatter. Pyl A did not have any effect on IL-4 production in CRTH2$^+$ cells. A representative dot blot shows the absence of IL-4$^+$/CRTH2$^+$ cells in the right upper quadrant in this Pyl A treated 28 week sample (A). The percentage of IL-4 $^+$/CRTH2$^+$ cells and the mean production of IL-4 are summarised in the graphs, showing no difference with Pyl A (B and C). A representative histogram reveals no shift indicating no effect on IL-4 (D). For statistical analysis a two-way ANOVA with Bonferroni's multiple comparison test was used with non-treated cells as controls.

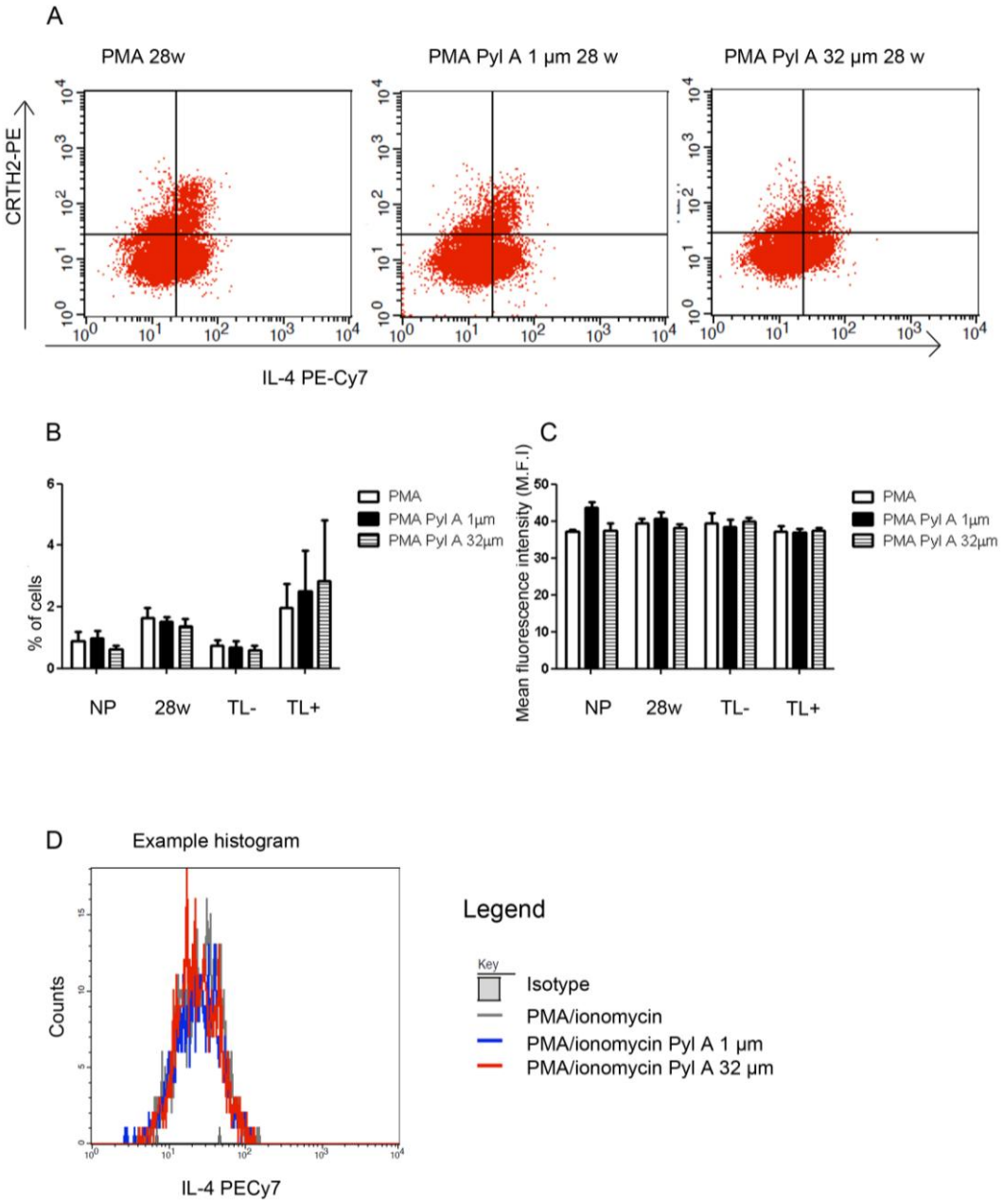


Figure 5.3.3B Effect of Pyl A on IL-4 production in PMA stimulated CRTH2$^+$ cells
Peripheral blood mononuclear cells were incubated with vehicle, 1 µM or 32 µM of Pyl A and then stimulated with PMA/ionomycin before being gated on the lymphocyte population according to forward and side scatter. Pyl A did not have a significant effect on IL-4 production in CRTH2$^+$ cells. A representative dot blot shows the IL-4$^+$/CRTH2$^+$ cells in the right upper quadrant with no difference between treatment groups (A). The percentage of IL-4$^+$/CRTH2$^+$ cells and the mean production of IL-4 are summarised in the graphs, showing no difference with Pyl A (B and C). A small increase in the mean was seen in non-pregnant women (B). A representative histogram reveals no shift indicating no effect on IL-4 (D). For statistical analysis a two-way ANOVA with Bonferroni's multiple comparison test was used with non-treated cells as controls.

Summary and discussion

A balance of immune suppression and immune tolerance during pregnancy to facilitate the growing fetus was first hypothesised by Medawar and colleagues in the 1950's through his work on transplant rejection [140]. Wegmann and colleagues further developed this hypothesis and specifically introduced the notion of a shift from the Th1 response towards a Th2 bias during pregnancy [209]. Compelling evidence particularly from murine studies suggests an important role in this altered ratio for the maintenance of successful pregnancy [210, 333, 334]. Although many studies have explored the Th1:Th2 interleukin profile at the maternal fetal interface [149, 223, 335] and in peripheral blood during human pregnancy [147, 218, 336], data is lacking on the profile in peripheral blood during labour. Endogenous pro-pregnancy hormones such as progesterone and leukaemia inhibitory factor have the ability to modulate the Th1:Th2 profile [149, 337], and therefore developing immunomodulating therapeutic agents could form and adjunct to maintaining the Th1:Th2 profile. Here I examine the production of the Th1 cytokines IFN-γ and TNF-α, and the Th2 cytokine IL-4 throughout pregnancy and in labour, and the potential of the CRTH2 agonists $15dPGJ_2$ and Pyl A, to act as therapeutic agents to modulate the Th1:Th2 response.

<u>The Th1/ Th2 interleukins in pregnancy and in labour</u>

Consistent with the notion of suppressed Th1 cytokine response throughout gestation [146, 147, 218, 338], the results presented here revealed a reduction in the percentage of IFN-γ and TNF-α-secreting T helper cells during pregnancy in response to PMA/ionomycin stimulation (Figure 5.1.1B and 5.1.2B respectively). Interestingly, the mean production of IFN-γ and TNF-α remained constant within the $CD4^+$/IFN-γ^+ and $CD4^+$/TNF-α^+ cell populations (Figure 5.1.1C and 5.1.2C respectively). This indicates that while fewer cells maintain the capacity to initiate a Th1 response to inflammatory stimuli with advancing gestation, the cells which maintain the ability to produce these cytokines do no show reduced production.

Since normal human labour reflects a pro-inflammatory state, an increase in the percentage of IFN-γ and TNF-α producing T helper cells with the onset of labour was anticipated. However, no statistically significant difference in the percentage of T helper cells producing IFN-γ and TNF-α, or the mean production of interleukins was seen between term non-labouring and term labouring samples in non-stimulated or PMA/ionomycin stimulated cells. However, a trend of a small increase in the basal percentage of cells producing IFN-γ and TNF-α was seen from 28 weeks to term with a further increase in labour. It is likely that the extent of localized pro-inflammatory response in the uterus and fetal membranes seen in labour does not occur systemically, which indeed would lead to adverse neonatal outcome by the transfer of maternal cytokines across the placenta. However, the evidence presented here of a degree of inflammation may suggest a biological requirement for some systemic inflammation. Examination of these cytokines in women with suspected chorioamnionitis and who are pyrexial during labour may however have shown a more exaggerated inflammatory response.

Several studies have shown an increase in intrinsic [146, 221] and mitogen stimulated IL-4 production in PBMCs during pregnancy [147, 218, 220]. An increase in basal and PMA/ionomycin induced IL-4 production in CRTH2 positive cells during pregnancy was anticipated, with a reduction in the labouring samples. CRTH2 is a classic marker of Th2 cells and thus should reflect their functional role in pregnancy. However, no significant difference in the percentage of $CRTH2^{+}/IL\text{-}4^{+}$ secreting cells, or the mean production of IL-4 was seen during pregnancy and in labour compared to non-pregnant controls, (figure 5.1.3B and 5.1.3C respectively). Although an increase in the percentage of IL-4 secreting cells was observed in both non-stimulated and stimulated 28 week samples, which returned to non-pregnant levels at term, this narrowly missed out on statistical significance when the Bonferroni's multiple comparisons test was applied. The fact that the percentage of CRTH2 expressing $CD4^{+}$ cells is low in the peripheral blood, and the numbers in this experiment were small, it is not possible to rule out a modest yet biologically significant change in IL-4 production in these cells.

The effect of $15dPGJ_2$ on the Th1/Th2 interleukins

To examine the potential of further modulating the Th1:Th2 bias in pregnancy the effect of the CRTH2 agonist and anti-inflammatory cyclopentenone prostaglandin on the Th1 and Th2 interleukins was determined by flow cytometry. $15dPGJ_2$ is an endogenous product of PGD_2. PGD_2 is readily converted to $15dPGJ_2$ by a series of dehydration reactions in the presence of albumin and is found in amniotic fluid [279] and the placenta [280]. PGD_2 has been shown to promote the Th2 bias through IL-4, IL-10 and IL-5 production in Th2 cells *in vitro* via CRTH2 [217]. Therefore, the combined properties of being both an anti-inflammatory prostaglandin and a CRTH2 agonist led to the hypothesis that it could both suppress the Th1 interleukins whilst promoting the Th2 response.

$15dPGJ_2$ pre-treatment significantly reduced the PMA-stimulated production of both Th1 cytokines; IFN-γ and TNF-α, in non-pregnant and all pregnant samples (Figure 5.2.1B and 5.2.2B respectively). The anti-inflammatory properties of $15dPGJ_2$ are thought to be at least partly via the inhibition of the transcription factor NF-κB [339]. NF-κB is known to play a key regulatory role in controlling the Th1 immune response by modulating the expression of Th1 cytokines [256]. Data is presented in chapter four showing the ability of $15dPGJ_2$ to inhibit basal NF-κB (Figure 4.4) and significantly reduce PMA stimulated NF-κB in PBMCS of women at 28 weeks of pregnancy and at term in labour, as determined by western analysis of the transcriptionally active NF-κB subunit p-p65 (Figure 4.10). A non-significant reduction was also observed in non-pregnant controls and samples acquired at term pre-labour. This data provides evidence that the Th1 inhibitory properties of $15dPGJ_2$ are likely to be regulated through NF-κB.

The effect of $15dPGJ_2$ on basal production of IFN-γ and TNF-α was surprising. A small population of $CD4^+$/IFN-γ^+ and $CD4^+$/TNF-α^+ appeared upon treatment with 32μm of $15dPGJ_2$ (Figure 5.2.1A and 5.2.2A). This led to a statistically significant increase in the percentage of cells producing IFN-γ and TNF-α at 28 weeks and at term pre-labour. However, the percentage of IFN-γ and TNF-α producing cells was still low, 2.3% and

4.3% at 28 weeks and 1.7% and 3.1% at term in labour respectively, and therefore the potential *in vivo* biological relevance of this is uncertain. $15dPGJ_2$ significantly improves pup survival with intrauterine injection in LPS treated mice [277], and therefore this suggests an overall *in vivo* neuroprotective effect rather than causing adverse effects. In other cell types $15dPGJ_2$ has been shown to suppress both IFN-γ [340] and LPS and PMA induced TNF-α [341].

The relevance of $15dPGJ_2$-mediated inhibition of IFN-γ and TNF-α seen in the PMA/ionomycin treated cells is nevertheless still significant, since PMA and ionomycin are standard *in vitro* stimulants used to most closely mimic the degree potential of *in vivo* activation of T cells in an *in vitro* setting. T cell activation involves several steps including activation of protein tyrosine kinases, plasma membrane inositol phospholipid hydrolysis and an increase in intracellular Ca^{2+} to activate calcineurin [342]. PMA activates protein kinase C and ionomycin artificially increases intracellular Ca^{2+} [343], and therefore mimics the *in vivo* mechanism of T cell activation.

Interestingly, no increase was seen on the production of the Th2 cytokine, IL-4, in response to $15dPGJ_2$ treatment. An increase may have been counteracted by an overriding effect of a non-CRTH2 mediated mechanism such as the inhibition of other signalling proteins involved the transcriptional regulation of IL-4 expression. Evidence for this has been previously reported in a PMA/ionomycin activated T cell line where $15dPGJ_2$ inhibited IL-4 production in a dose dependent manner through the down-regulation of Nuclear factor of activated T-cells (NF-AT) activation [286].

<u>The effect of the CRTH2 agonist Pyl A on the Th1/Th2 interleukins</u>

Pyl A had no effect on the Th1 interleukins. Th1 cells do not express the CRTH2 receptor, however, an indirect inhibition of the Th1 cytokines could have been possible if CRTH2 mediated IL-4 and IL-10 production occurred in the T helper cells. Pyl A alone did not induce IL-4 production (Figure 5.3.3A). With the addition of PMA/ionomycin, Pyl A led to a statistically significant increase in IL-4 production in

non-pregnant samples at 1 µm concentration (Figure 5.3.3B). No significant response was seen during pregnancy and in labour. Two studies have shown a CRTH2-mediated increase in the Th2 interleukins in T helper cells *in vitro*, including IL-4. Tanaka et al used treated purified $CRTH2^+$ cells with PGD_2, Indomethacin and DK- PGD_2 for 6 hours which resulted in an increase in the percentage of cells producing IL-4, IL-5 and IL-13 [299]. Xue et al also treated purified $CRTH2^+$ cells with PGD_2 and demonstrated a dose dependent increase in the production of IL-4, IL-5, IL-13 and IL-10 [217]. Both of these studies differ in design to this study in that they used a purified CRTH2 population for determining the effects of the CRTH2 agonists. Only approximately 13% of the $CRTH2^+$ cells in Tanaka's study produced IL-4, and therefore, with only roughly 2% of lymphocytes expressing CRTH2 in our study any CRTH2 agonist effect could be difficult to detect without using purified $CRTH2^+$ cells.

Summary

While $15dPGJ_2$ appears to have no effect on the peripheral Th2 response, the demonstrated inhibition of the Th1 response represents a collective shift towards a Th2 bias, or in other words, an increase in the Th2:Th1 ratio. The lack of a demonstrable effect on the Th2 profile by Pyl A could be explained by the design of the experiment by not isolating a purified $CRTH2^+$ population. Nevertheless, this data presents evidence that the Th1:Th2 balance is naturally altered during pregnancy through Th1 suppression rather than Th2 promotion. It also provides further evidence of the potential anti-inflammatory effects of $15dPGJ_2$; through the suppression of NF-κB, and the Th1 pro-inflammatory cytokines IFN-γ and TNF-α. These properties may be of therapeutic benefit for the prevention of inflammation associated preterm labour and the reduction of inflammation induced neonatal morbidity where an aberrant profile favouring the Th1 response is seen.

CHAPTER 6

The effect of CRTH2 agonists on myometrial contractility

Chapter summary

Hypothesis:

$15dPGJ_2$ inhibits NF-κB in human myocytes [271]. NF-κB controls the expression of many genes required for uterine contractility. It is possible the $15dPGJ_2$ could inhibit myometrial contractility via NF-κB. Although CRTH2 is not expressed on human myocytes, the CRTH2 agonist indomethacin inhibits myometrial contractility in a mechanism independent of CRTH2. Indomethacin has a similar structure to Pyl A and therefore it is possible that Pyl A could replicate the inhibitory effects of indomethacin on myometrial contractility.

Aims:

To determine if the CRTH2 agonists $15dPGJ_2$ and Pyl A inhibit human myometrial contractility.

Methods:

Myometrial biopsies were collected from women undergoing pre-labour caesarean section, dissected into strips and mounted on the DMT myograph. A cumulative dose response was performed with 1-100 μm of $15dPGJ_2$, Pyl A, PGD_2 and indomethacin. Vehicle control strips were also used. The total and average area under the curve, peak amplitude and rate of contractions were analysed as markers of myometrial contractility.

Results:

$15dPGJ_2$ and PGD_2 had no effect on myometrial contractility. Pyl A mimicked the effects of indomethacin and inhibited myometrial contractility.

Conclusion:

Although $15dPGJ_2$ did not inhibit myometrial contractility, the CRTH2 agonist Pyl A did so in a mechanism independent of CRTH2.

Introduction

Throughout pregnancy and parturition the uterus undergoes changes which can be separated into four phases, as described by Challis et al [53]. During pregnancy the uterus remains quiescent, but as labour approaches an activation phase occurs with up-regulation of contraction-associated proteins, and is followed by a stimulation phase when the uterus becomes responsive to prostaglandins and oxytocin. Finally, post-partum the uterus involutes. Throughout pregnancy, uterine quiescence is thought to be attributed in part to the effects of progesterone. It is likely that progesterone represses NF-κB activity [344] until term, when NF-κB activity is increased in the myometrium in association with an increase in PR-A:PR-B ratio and possibly the expression of truncated forms of the inhibitory progesterone receptors PR-C [263] .

NF-κB controls the expression of several genes required for uterine contractions. Prostaglandins are essential for stimulating myometrial contractions [55]. Prostaglandins are synthesised in a 2-step reaction, firstly from the generation of arachidonic acid from glycerophospholipids via PLA2 isozymes, and then from the conversion of arachidonic acid via COX-2 which is the rate limiting step [345]. The cPLA2 and sPLA2 isozymes are expressed in gestational tissues and appear to play significant roles in parturition [346]. cPLA2 and sPLA2 have response elements for NF-κB in their promoter regions [247], suggesting a regulatory role for NF-κB in their activity. In support of this, NF-κB inhibition with sulphalazine [275] and NAC [347] results in the inhibition of LPS induced production of these isozymes in placenta amnion and choriodecidua. The COX-2 gene also contains two NF-κB response elements, and site directed mutation of either of these elements leads to suppression of COX-2 promoter activity [259]. The promoter region of the oxytocin receptor contains three NF-κB binding sites, suggesting a role for NF-κB in the regulation of the oxytocin receptor [54]. The oxytocin receptor can also be activated by a synergistic interaction between NF-κB and the C/EBP transcription factor, forming a complex which binds with the receptor [266].

NF-κB appears to play a key role in regulating proteins that are central to the mechanism of uterine contractility. Since inhibition of NF-κB is seen in myocytes treated with $15dPGJ_2$, it is plausible that $15dPGJ_2$ can also lead to the inhibition of myometrial contractility.

Indomethacin can be used as a tocolytic [348]; however, due to the potential adverse effects on the fetus and the advent of other safer tocolytics, it is now less commonly used. Indomethacin is a non-selective COX inhibitor and an agonist of the CRTH2 receptor. Many of the adverse effects of indomethacin are thought to be due to COX-1 inhibition since it is mainly COX-1 that contributes to prostaglandin synthesis in the fetus [348, 349]. Myometrial contractility is inhibited *ex vivo* by indomethacin in a relatively rapid response [349, 350]. It is likely that inhibition is due to inhibition of calcium channels rather than the inhibition of COX-2 and prostaglandin synthesis. Since CRTH2 is not expressed in myocytes, the inhibitory mechanism is also independent of CRTH2. The structure of Pyl A is similar to indomethacin; therefore it is plausible that Pyl A may reproduce the effects of indomethacin and inhibit myometrial contractility. This chapter explores the effects of the CRTH2 agonists $15dPGJ_2$, indomethacin, Pyl A and PGD_2 on human myometrial contractility.

Results

To determine if the CRTH2 agonists $15dPGJ_2$, Pyl A and PGD_2 have a direct effect on uterine contractility, myometrial biopsies were collected from women undergoing pre-labour caesarean section. Strips were dissected and mounted on a myograph. A cumulative dose response from 1 µm to 100 µm was performed. Effect of treatment was compared to spontaneous contractions at the beginning of the experiment and divided by the equivalent timepoint for vehicle control. The total area under the curve per time point of the experiment, the average area under the curve, average peak amplitude and the rate of contractions were analysed.

6.1 $15dPGJ_2$ does not inhibit human myometrial contractility

No inhibition of myometrial contractility was seen with cumulative doses of $15dPGJ_2$, (Figure 6.1). There was a small non-significant decrease in the area under the curve, peak amplitude and rate of contractility, but this did not follow a dose response. There was also variability within subjects and between subjects which is reflected in the large error bars seen at some time points. Figure 6.1A shows a representative experiment with $15dPGJ_2$ and vehicle control strips. Overall, no effect on myometrial contractility was seen with $15dPGJ_2$.

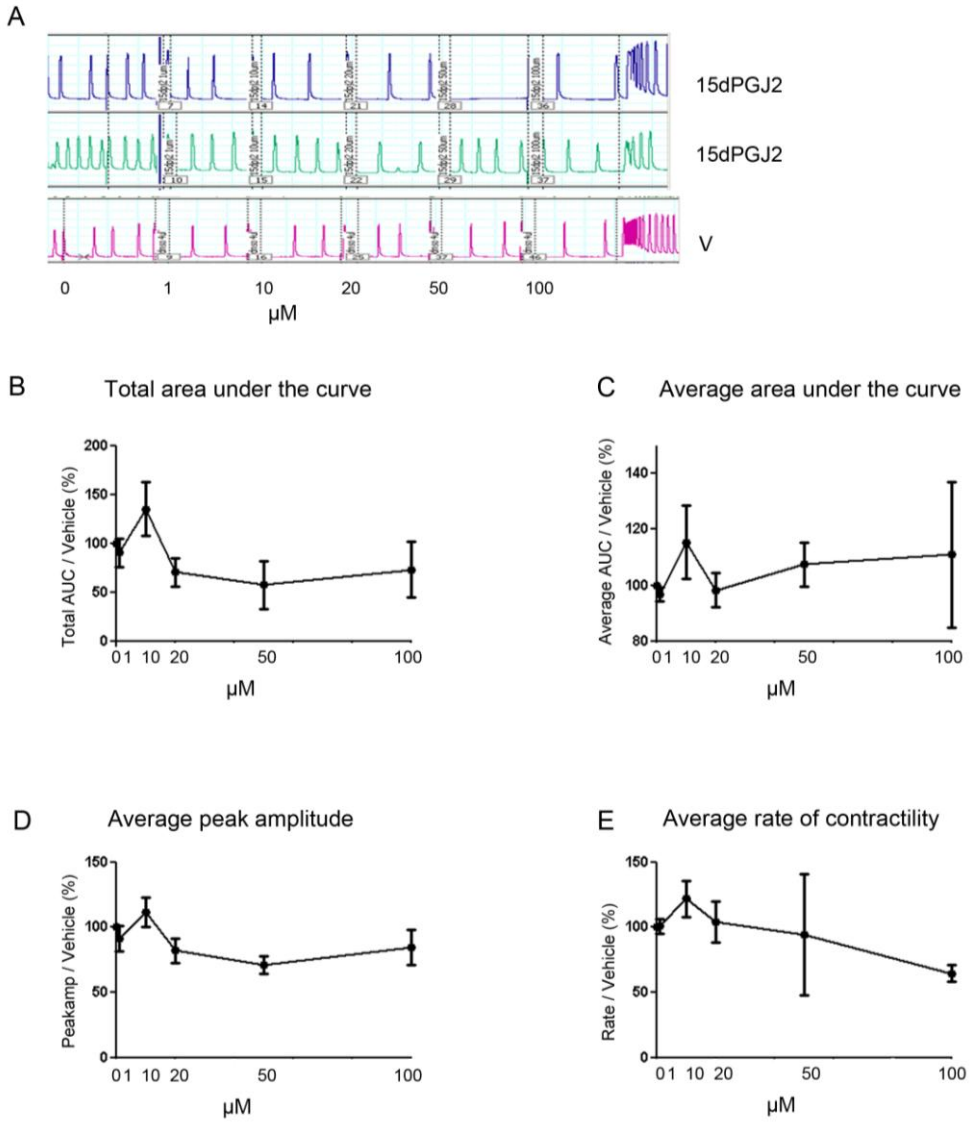


Figure 6.1 The effect of 15dPGJ$_2$ on myometrial contractility

Non-labouring myometrial biopsies were dissected and mounted on the myograph (n=3 subjects with 2-3 strips each). A representative trace is shown for 15dPGJ$_2$ treated strips and vehicle control (A). A cumulative dose response with 15dPGJ$_2$ or vehicle was performed and the effect on total and average area under the curve (B and C), peak amplitude (D) and rate of contractility (E) was determined. 15dPGJ$_2$ had no significant effect on myometrial contractility. V=vehicle. For statistical analysis ANOVA of repeated measures was used with Dunnett's multiple comparison test.

6.2 Indomethacin inhibits human myometrial contractility

Indomethacin has previously been shown to inhibit myometrial contractility *ex vivo*. Indomethacin has several known actions. For example, it is a CRTH2 agonist, a COX-2 inhibitor, and is likely to inhibit contractility via modulation of intracellular calcium. A cumulative dose response from 1 µm to 100 µm was performed. Figure 6.2A shows contracting strips from a representative experiment. Total area under the curve was significantly reduced from 10 µm (Figure 6.2B) and the peak amplitude and rate of contractions was significantly reduced from 100 µm $p<0.01$ (Figure 6.2 D and E).

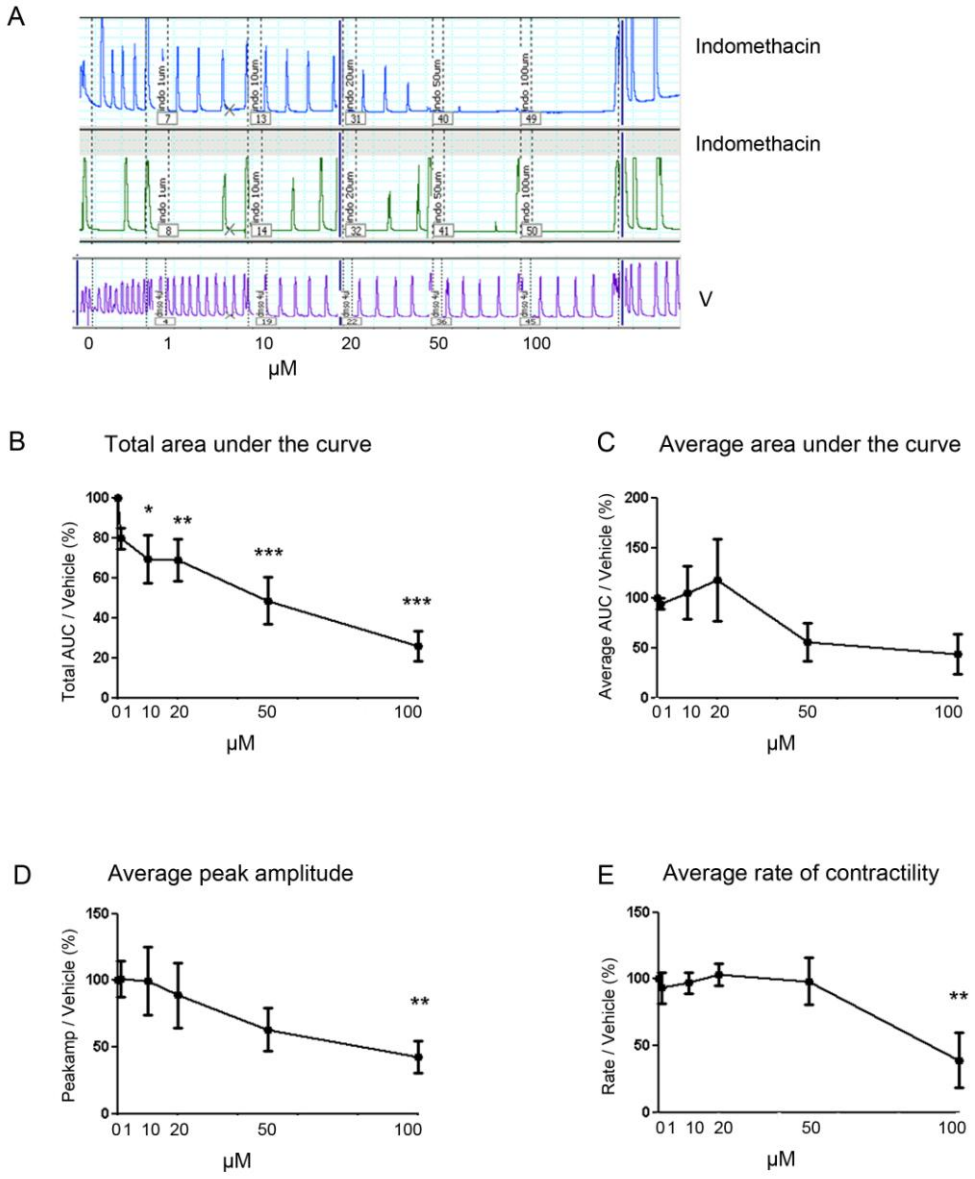


Figure 6.2 The effect of Indomethacin on myometrial contractility

Non-labouring myometrial biopsies were dissected and mounted on the myograph (n=3 subjects with 2-3 strips each). A representative trace is shown for indomethacin treated strips and vehicle control (A). A cumulative dose response with indomethacin or vehicle was performed and the effect on total and average area under the curve (B and C), peak amplitude (D) and rate of contractility (E) was determined. Indomethacin significantly reduced myometrial contractility with maximal response seen at 100 μm. V=vehicle. For statistical analysis ANOVA of repeated measures was used with Dunnett's multiple comparison test; (* *P<0.05,* ** *P<0.01*, *** *P<0.001*).

6.3 Pyl A inhibits human myometrial contractility

Although CRTH2 is not expressed on human myometrium, Pyl A has a similar chemical structure to indomethacin and therefore its effect on myometrial contractility was examined. A cumulative dose response from 1 µm to 100 µm was performed. Figure 6.3A shows contracting strips from a representative experiment. Total area under the curve was significantly reduced from 1 µm (Figure 6.3B) and the rate of contractions was significantly reduced from 20 µm ($p<0.001$) (Figure 6.3 D and E). Complete inhibition was seen with 100 µm.

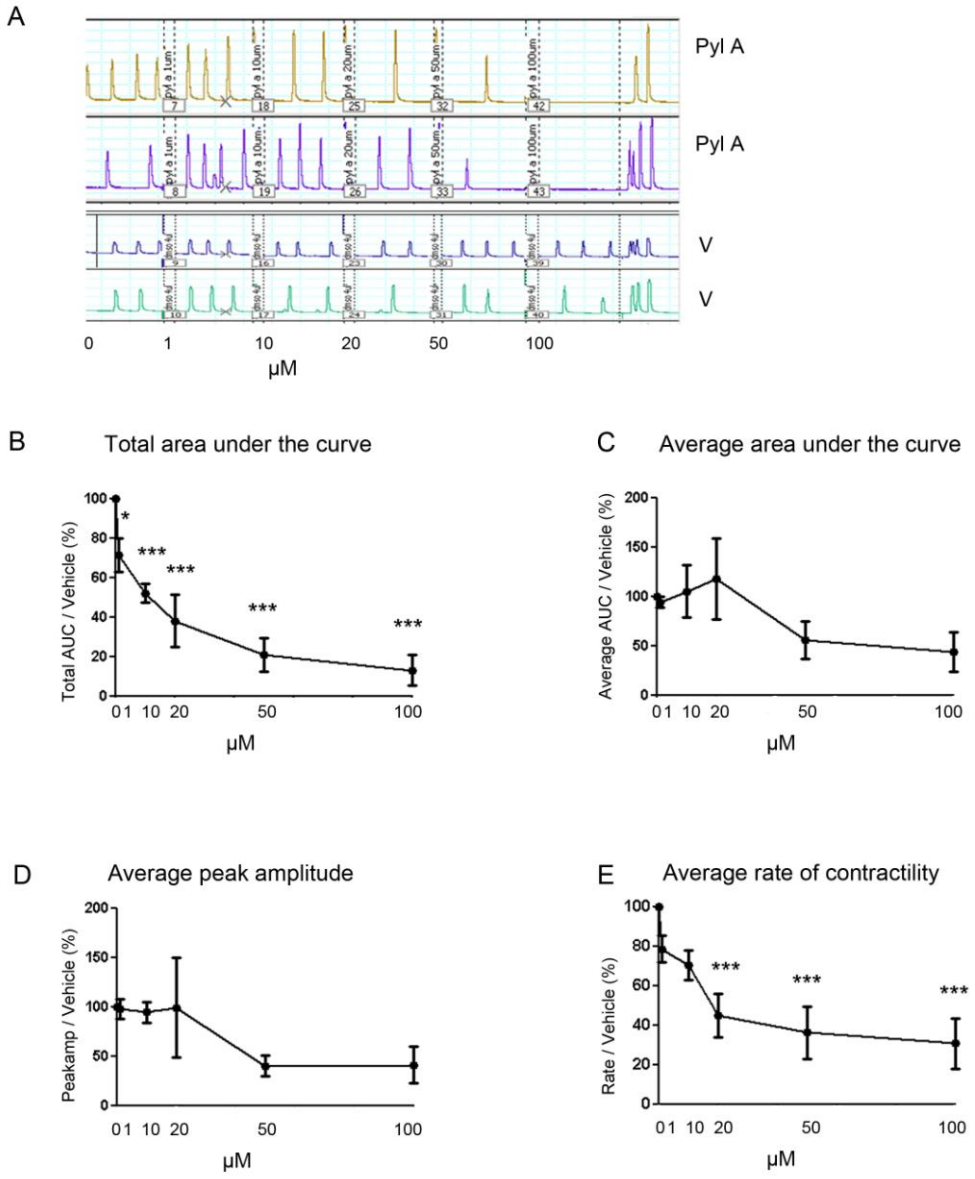


Figure 6.3 The effect of Pyl A on myometrial contractility

Non-labouring myometrial biopsies were dissected and mounted on the myograph (n=4 subjects with 2-3 strips each). A representative trace is shown for Pyl A treated strips and vehicle control (A). A cumulative dose response with Pyl A or vehicle was performed and the effect on total and average area under the curve (B and C), peak amplitude (D) and rate of contractility (E) was determined. Pyl A significantly reduced myometrial contractility with maximal response seen at 100 μm. V=vehicle. For statistical analysis ANOVA of repeated measures was used with Dunnett's multiple comparison test; (* *P<0.05,* ** *P<0.01*, *** *P<0.001*).

6.4 PGD_2 does not inhibit human myometrial contractility

PGD_2 has two receptors: DP_1 and DP_2. The effect of PGD_2 on myometrial contractility was determined in order to establish if Pyl A was acting in a non-CRTH2 mediated mechanism by inhibiting the DP_1 receptor at the high concentrations used. However, no inhibition of myometrial contractility was seen with the cumulative dose response (Figure 6.4). There was a non-statistically significant trend in increased myometrial contractility as seen in all parameters examined. There was significant variability within subjects and between subjects which is reflected in the large error bars seen at some most time points. Overall, no inhibition of myometrial contractility was seen with PGD_2, which excludes the DP_1 receptor as a target for Pyl A mediated inhibition of contractility.

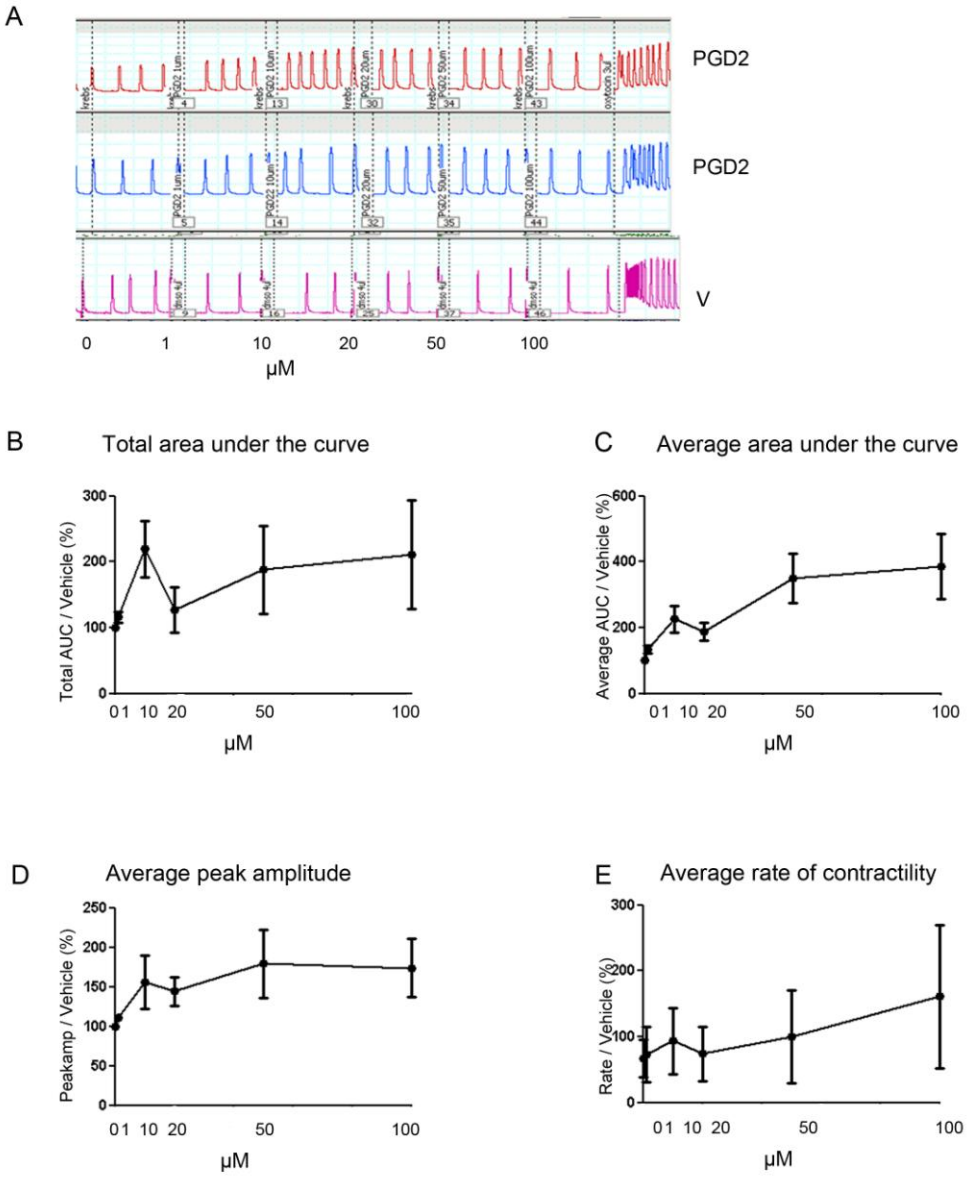


Figure 6.4 The effect of PGD_2 on myometrial contractility

Non-labouring myometrial biopsies were dissected and mounted on the myograph (n=3 subjects with 2 strips each). A representative trace is shown for PGD_2 treated strips and vehicle control (A). A cumulative dose response with PGD_2 or vehicle was performed and the effect on total and average area under the curve (B and C), peak amplitude (D) and rate of contractility (E) was determined. PGD_2 had no significant effect on myometrial contractility. V=vehicle. For statistical analysis ANOVA of repeated measures was used with Dunnett's multiple comparison test.

Summary and Discussion

For the uterus to become activated for labour to ensue, there is an increase in the expression of contraction-associated proteins. These proteins enhance the interaction between actin and myosin, increase the excitability of myometrial cells and promote intercellular connectivity which aids the development of synchronous contractions [41]. The contractile force of the myometrium is due to cyclical attachments and detachments of cross bridges between myosin and actin microfilaments which is triggered by the activation of the regulatory 20 kDa chain of myosin molecules [351]. Myosin light chain activation occurs by phosphorylation by myosin light chain kinase (MLK), which is in turn activated by calmodulin [40]. An increase in intracellular calcium and calmodulin activate MLK [43]. After depolarization of the myocyte there is an influx of extracellular calcium and a release of intracellular calcium stores [44], which then promotes myosin actin interaction and further promoting contractions.

The major contractile pathways activate phospholipase C (PLC), the release of intracellular calcium and the stimulation of calcium entry [54]. PGE_2 and $PGF_{2\alpha}$ activate ligand-regulated channels leading to the release of calcium from intracellular stores [352, 353]. Oxytocin activates the $G\alpha_q$ OTR, which is linked to PLC, leading to the release of inositol triphosphate and in turn the release of calcium from intracellular stores [43, 354, 355]. Relaxant pathways often target G protein coupled receptors which upon activation increase cAMP or cGMP [54], such as the B adrenergics, or target the inhibition of calcium entry such as nifedipine and oxytocin antagonists [356].

The major disadvantage with the currently available tocolytics is their lack of anti-inflammatory effects. Therefore, delaying preterm labour with tocolytics to provide steroid treatment for lung maturation, may lead to a detrimental increase in duration of exposure of the fetus to a hostile inflammatory environment. For this reason, the effects of the anti-inflammatory prostaglandin $15dPGJ_2$ were explored to determine if, by inhibition of NF-κB and thus COX-2 and prostaglandins, inhibition of myometrial

contractility could be elicited. Acute administration of $15dPGJ_2$ in a cumulative dose response failed to inhibit myometrial contractility. It is possible that the duration of the experiment was not long enough to assess for inhibition of protein expression, via NF-κB. A reduction in NF-κB and cPLA2 can be demonstrated *in vivo* in the murine model by 1 hr after administration of $15dPGJ_2$, with COX-2 inhibition at the protein level seen at 6 hrs [277]. Unfortunately the control strips failed to contract for long enough to demonstrate meaningful results at time points long enough to reliably assess for inhibition of prostaglandin synthesis. However, pre-incubation with $15dPGJ_2$ for 2 hours prior to mounting strips on the contractility machine led to a reduction in contractility [357].

Mechanical stretch activates NF-κB in cultured myocytes [183], increases the expression of the oxytocin receptor [84] and the gap junction proteins: Connexin-43 and Connexin-26 in the rat [358], and thus stretch may contribute to triggering uterine contractility and labour. In support of this, women with multiple pregnancies and polyhydramnios are at a higher risk of delivering preterm [359, 360]. The stretch on the uterus at term is estimated to correlate with a resting tone of 0.5g per 1mm diameter of myometrial strips *ex vivo* on a myograph [361]. The stretch of 3-4g which is applied to the individual 5-10 mm strips in this study correlates with the resting tone of the uterus. It was anticipated that $15dPGJ_2$ would inhibit contractility via NF-κB inhibition in the stretched myometrium. The assumption was made however, that $15dPGJ_2$ was able to penetrate the myometrial tissue to act at the cellular level on NF-κB. To allow for this, higher concentrations of up to 100 µm was used compared to 32 µm used in the *in vitro* studies, yet despite this, no inhibition of contractility was seen, (Figure 6.1).

Indomethacin did however cause inhibition of myometrial contractility (Figure 6.2) consistent with previous experiments performed by this research group [349, 350]. Although 10 µm of indomethacin reduces PGE_2 production by 90% in fetal membranes [362], the rapid inhibitory effect on myometrial contractility seen with indomethacin suggests that its mechanism is not via the inhibition of COX-2 and prostaglandin synthesis. The effect of indomethacin on calcium influx has therefore been explored in myocytes, which revealed an inhibition in the Ca^{2+} channel current, although with a

smaller inhibitory effect than the effect seen on actual contractility [349]. This could be explained by an alternative and as yet unidentified mechanism for indomethacin-induced inhibition of contractility. By demonstrating the absence of CRTH2 on human myocytes, the role of CRTH2 in indomethacin-induced inhibition of myometrial contractility has been excluded in this study.

Despite this, the effect of the CRTH2 agonist Pyl A on human myometrial contractility was examined, since it shares a similar chemical structure to indomethacin. Pyl A also inhibited myometrial contractility (figure 6.3), but more potently than indomethacin. The mechanism of inhibition, which is independent of CRTH2, may be via the alteration of the Ca^{2+} channel current, as with indomethacin. However, at higher concentrations, Pyl A also reacts with other prostanoid receptors at a potency of TP> EP_3> DP> EP_4> EP_2> FP> IP> EP1 [320]. The prostaglandin receptors can be grouped into two categories; EP_1, EP_3 FP, and TP which stimulate contraction, and DP_1, EP_2, EP_4, and IP which are considered to be relaxatory receptors [363]. It is likely that the effect of Pyl A is due to non-specific binding with either the EP_2 or EP_4 receptors, or the DP_1 receptor.

Upon activation of the stimulatory receptors EP_1, EP_3 and FP, an increase in intracellular calcium leads to increased uterine contractility [352, 364]. The relaxatory receptors EP_2 and EP_4 are expressed in the myometrium, but the quiescence associated with pregnancy is more likely to be due to EP2 rather than the EP_4 receptor, [308] by increasing cAMP thereby inducing smooth muscle relaxation [352]. Our group have shown inhibition of myometrial contractility with EP_2 receptor but not with EP_4 agonists [365]. Typically the DP1 receptor is considered to be relaxatory. Expression studies of the prostanoid receptors in the human myometrium reveal higher mRNA expression of DP_1 than EP_2, with no difference in either between non-pregnant or pre-labour and in-labour myometrial samples [366]. The effect of PGD_2 on human myometrial contractility was therefore explored to see if myometrial contractility could be inhibited via the DP_1 receptor. However no inhibition was seen. Previous studies of human non-pregnant and pregnant myometrium have shown initial excitatory responses [308, 367], consistent with the slight increase in

contractility seen in Figure 6.4. However both studies then showed the excitatory response was followed by a subsequent inhibitory effect with PGD_2. The excitatory effects of PGD_2 are likely to be due to cross-reactivity with the EP_3, FP or TP receptor.

In conclusion, Pyl A inhibited myometrial contractility in a mechanism independent of CRTH2. It is likely that the inhibitory effect of Pyl A is through non-specific binding with the EP_2 receptor or via alteration of calcium channel current. Contrary to anticipated $15dPGJ_2$ failed to inhibit human myometrial contractility. The use of Pyl A as a tocolytic may therefore be considered.

CHAPTER 7

CRTH2 agonist effect on murine myometrial contractility and inflammation induced preterm labour

Chapter summary

Hypothesis:

$15dPGJ_2$ delays inflammation induced preterm labour and significantly reduces pup mortality. The mechanism behind this effect is not fully understood. $15dPGJ_2$ is a ligand for the CRTH2 receptor; therefore it is plausible that a small molecule agonist could replicate some of the effects of $15dPGJ_2$.

Aims:

CRTH2 expression in murine myometrium was determined, and the direct effects of the CRTH2 agonists $15dPGJ_2$ and Pyl A on murine myometrial contractility was investigated. The effects of Pyl A on both LPS induced preterm labour and fetal viability were explored, together with the effects on the inflammatory proteins; NF-κB, COX-2 and the Th1:Th2 interleukins in the myometrium.

Methods:

CRTH2 mRNA expression was determined by PCR, and the direct effect of the CRTH2 agonists on myometrial contractility was examined *ex-vivo* using a myograph. Intra-uterine LPS was administered on E16 to CD1 mice with vehicle or Pyl A. Dams were sacrificed at 4.5 hours post injection and myometrium and pup brain was harvested for mRNA and protein analysis by PCR, western analysis or ELISA. Fetal viability was also assessed. In a subsequent experiment mice were allowed to deliver spontaneously, and delivery time and pup survival was recorded.

Results:

CRTH2 mRNA was detected by PCR. Pyl A inhibited circular myometrial contractility in a mechanism independent of CRTH2. Pyl A augmented an inflammatory response in murine myometrium leading to an increase in NF-κB, TNF-α, IFN-γ and IL-1β. Despite this an increase in fetal viability was seen which was associated with an increase in myometrial IL-10. In contrast to the anticipated delay in preterm labour, but in

keeping with the pro-inflammatory effect seen with Pyl A, preterm labour was augmented rather than delayed.

Conclusion:

$15dPGJ_2$-mediated delay in inflammation induced preterm labour is not mediated via CRTH2. CRTH2 agonist activity is associated with augmentation of inflammation induced preterm birth, therefore CRTH2 should not be considered as potential therapeutic target for the prevention of preterm labour and neonatal morbidity.

Introduction

In a murine model of inflammation induced preterm labour, $15dPGJ_2$ delays preterm labour. More importantly $15dPGJ_2$ improves pup survival from 30% with LPS, to 95% with co-injection of $15dPGJ_2$ [277]. The mechanisms by which $15dPGJ_2$ exert these effects are not fully understood, but it is likely that the inhibition of NF-κB in the myometrium and the brain is important. Alternative mechanisms such as the direct inhibition of uterine contractility or an alteration Th1:Th2 interleukin profile via CRTH2, are also plausible.

Murine CRTH2 expression at the protein level is hampered by the lack of specific antibodies [368]. Prior to this thesis, mRNA expression of CRTH2 in murine myometrium had not been explored. Although CRTH2 was not detected at the protein level in human myometrium, the distribution of CRTH2 differs in the mouse compared to the human. For example, CRTH2 is detected on both Th1 and Th2 cells in the mouse [369].

Murine CRTH2 shares 77% homology with the human CRTH2 receptor amnio acid sequence [369], and shares 90% homology within the transmembrane domains which explains the virtually identical pharmacology between receptors [370]. The CRTH2 agonists Pyl A, $15dPGJ_2$, DK- PGD_2, PGD_2, and indomethacin all show activity at the mouse CRTH2 receptor [290, 320, 371-373]. Human contractility studies with the CRTH2 agonists (presented in chapter six) revealed an inhibitory effect with Pyl A which was not mediated via the CRTH2 receptor. It is plausible that this inhibitory effect could be demonstrated in murine myometrium.

$15dPGJ_2$ inhibits NF-κB in human amniocytes, myocytes and PBMCS, as shown in chapter four [271, 374]. Upon intrauterine injection in the mouse it also reduces basal and LPS induced NF-κB in both the myometrium and the pup brain [277]. Pyl A failed to replicate this effect in human amniocytes, myocytes and PBMCs (see chapter four) and therefore is also unlikely to replicate the effects of $15dPGJ_2$ in the murine myometrium and pup brain. The effects of Pyl A on both NF-κB and COX-2 in the

murine myometrium were nevertheless explored in this chapter to confirm that $15dPGJ_2$ inhibits NF-κB in the mouse independently of CRTH2.

The importance of the balance between the Th1:Th2 profile at the maternal fetal interface in the mouse for successful pregnancy maintenance is well established. In the deciduas/placental compartment IL-4, IL-5 and IL-10 are present in all three trimesters with IFN-γ present at low levels only in first trimester. Interestingly, all are undetectable in the spleen indicating local production of the interleukin [334]. IL-10 protects the fetus by reducing fetal loss as a result of pro-inflammatory cytokines and LPS administration [210, 375]. In the human, PGD_2 stimulates the production of the Th2 cytokines IL-4, IL-5, IL-13 and IL-10 in cultured Th2 cells *in vitro* [217]. IL-4 is able to inhibit the Th1 response directly, with IL-10 inhibiting the production of inflammatory mediators indirectly [194]. Although $15dPGJ_2$ was able to modulate the Th1:Th2 bias in human PBMCs *in vitro* by suppressing the Th1 response , Pyl A could not mimic the effects of $15dPGJ_2$ or the increase in Th2 cytokine profile seen with PGD_2 (data shown in chapter five). It is possible that the lack of effect seen may represent a limitation of *in vitro* models.

The data presented in this chapter focuses on the *in vivo* effects of the CRTH2 agonist Pyl A in the mouse. CRTH2 expression at the mRNA level in murine myometrium was examined. The direct effects of the CRTH2 agonists $15dPGJ_2$, Pyl A and DK- PGD_2 on murine myometrial contractility were examined to determine if this is an additional mechanism of action of $15dPGJ_2$-mediated delay in murine preterm labour. Finally, the *in vivo* effects of Pyl A on NF-κB and the Th1:Th2 interleukin profiles were examined to conclude if there is any potential for *in vivo* administration of CRTH2 agonists in the prevention of preterm labour.

Results

7.1 CRTH2 mRNA expression in murine myometrium

Murine myometrium was harvested from mice treated with intrauterine administration of vehicle, LPS +/- Pyl A. mRNA was extracted and a *CRTH2* transcript was amplified using RT-PCR, along with the housekeeping gene L-19, (Figure 7.1). Amplification of CRTH2 was seen by cycle 33 (four-fold higher than the human) and L-19 by cycle 19.

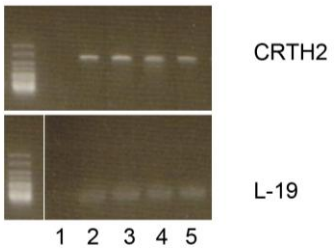


Figure 7.1 Murine myometrial *CRTH2* mRNA
mRNA was isolated from murine uterus and converted to cDNA (n=3 per treatment group). A *CRTH2* transcript of product size of 344bp and L-19 transcript of product size 72bp were amplified by RT-PCR. No difference in *CRTH2* expression was seen between the treatment groups. Non-template control (lane 1), vehicle treated mouse (lane 2), Pyl A treated (lane 3), LPS treated (lane 4), LPS/Pyl A treated (lane 5).

7.2 The effects of the CRTH2 agonists on murine myometrial contractility

To determine if the CRTH2 agonists 15dPGJ$_2$, Pyl A and DK- PGD$_2$ have a direct effect on uterine contractility, uteri were harvested from mice on E15-16, dissected, and mounted on the myograph in the circular orientation. A cumulative dose response from 1 μm to 100 μm was performed, with vehicle control strips.

7.2.1 The effect of 15dPGJ$_2$ on murine myometrial contractility

No inhibition of myometrial contractility was seen with the cumulative dose response of 15dPGJ$_2$, (Figure 7.2.1). This indicates that the delay in inflammation induced preterm labour seen with 15dPGJ$_2$ is not due to a direct effect on myometrial contractility.

7.2.2 The effect of Pyl A on murine myometrial contractility

Pyl A reduced myometrial contractility from 10 μm concentration *(P<0.01)*, with complete inhibition seen with 100 μm *(P<0.001)* (Figure 7.2.2). The effect of Pyl A on longitudinal muscle was also examined by mounting the strips along the longitudinal orientation. Contractility was not maintained in the longitudinal orientation for the whole duration of the experiment in control strips to robustly examine the effect of Pyl A on longitudinal muscle contractility. Despite this, the clear inhibition seen in the circular muscle was not evident in the longitudinal strips.

7.2.3 The effect of DK- PGD$_2$ on murine myometrial contractility

No inhibition of myometrial contractility was seen with the cumulative dose response of DK- PGD$_2$ (Figure 7.2.3), indicating no role for CRTH2 in Pyl A mediated inhibition of circular myometrial contractility.

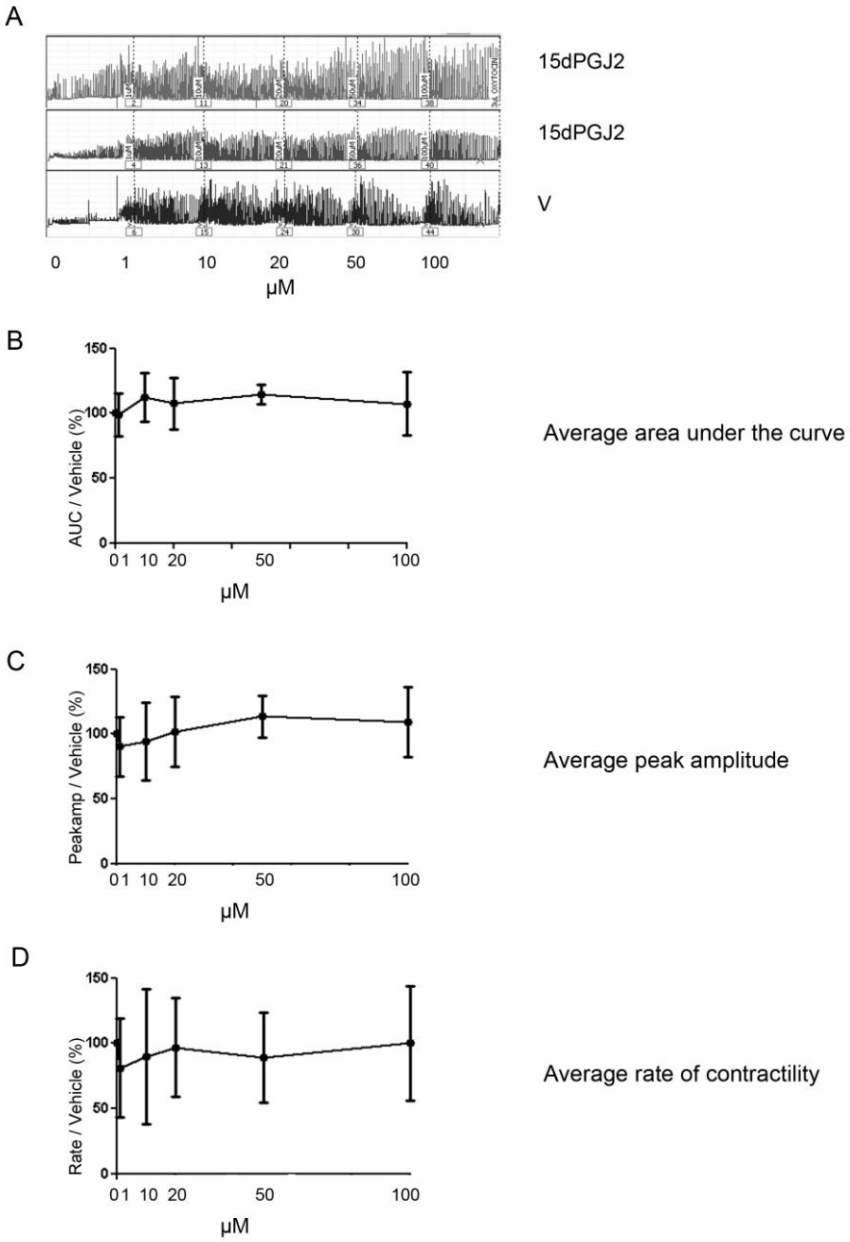


Figure 7.2.1 The effect of 15dPGJ$_2$ on circular muscle contractility
CD1 mice were sacrificed on E15-16 and myometrial tissue was harvested and mounted on the myograph in the circular orientation (n=3). A cumulative dose response with 15dPGJ$_2$ or vehicle was performed and the effect on average area under the curve, peak amplitude and rate of contractility was determined. 15dPGJ$_2$ had no effect on myometrial contractility. A representative trace is shown in (A), with graphs summarising parameters of n=3 experiments (B,C,D). V=vehicle. For statistical analysis ANOVA of repeated measures was used with Dunnett's multiple comparison test.

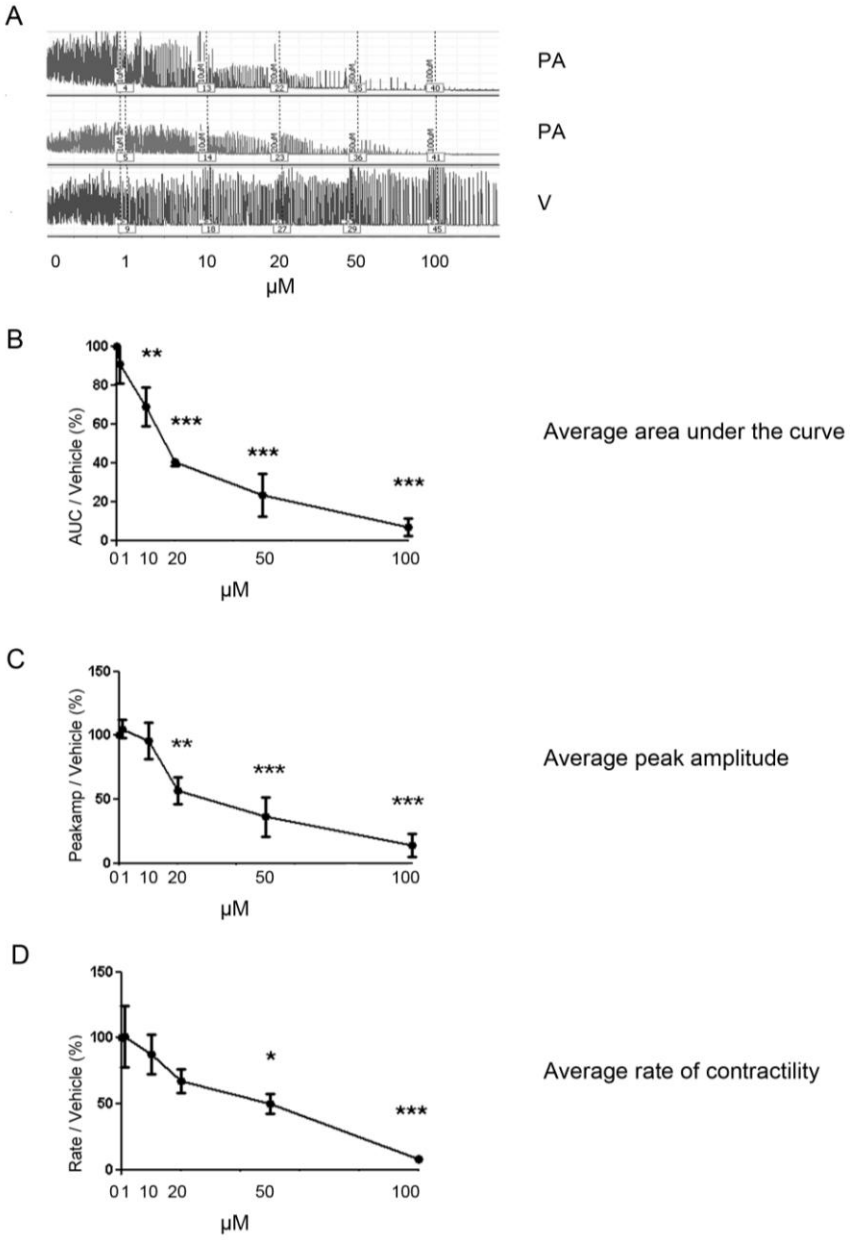


Figure 7.2.2 The effect of Pyl A on circular muscle contractility
CD1 mice were sacrificed on E15-16 and myometrial tissue was harvested and mounted on the myograph in the circular orientation (n=3). A cumulative dose response with Pyl A or vehicle was performed and the effect on average area under the curve, peak amplitude and rate of contractility was determined. Pyl A inhibited myometrial contractility. A representative trace is shown in (A), with graphs summarising parameters of n=3 experiments (B,C,D). V=vehicle, PA = Pyl A. For statistical analysis ANOVA of repeated measures was used with Dunnett's multiple comparison test; (* *P<0.05,* ** *P<0.01*, *** *P<0.001*)

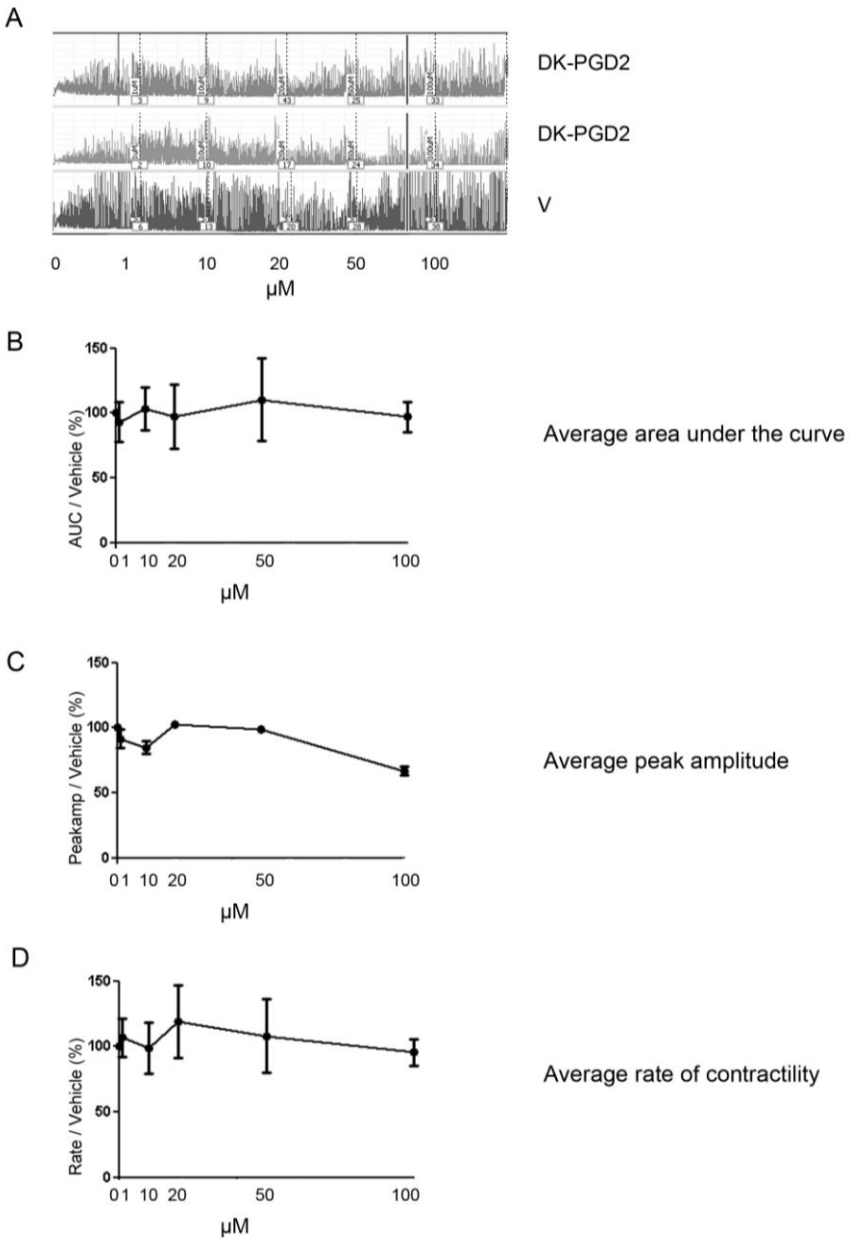


Figure 7.2.3 The effect of DK- PGD_2 on circular muscle contractility

CD1 mice were sacrificed on E15-16 and myometrial tissue was harvested and mounted on the myograph in the circular orientation (n=3). A cumulative dose response with DK- PGD_2 or vehicle was performed and the effect on average area under the curve, peak amplitude and rate of contractility was determined. DK- PGD_2 had no effect on myometrial contractility. A representative trace is shown in (A), with graphs summarising parameters of n=3 experiments (B,C,D). V=vehicle. For statistical analysis ANOVA of repeated measures was used with Dunnett's multiple comparison test.

7.3 LPS induced murine preterm labour

Prior to commencing experiments exploring the effect of the CRTH2 agonist on inflammation induced preterm labour, a dose response with the LPS serotype 0111:B4 was performed to find the optimal concentration for reliably inducing preterm labour. CD1 mice were time-mated and an intrauterine infection of LPS or vehicle was performed by mini laparotomy and mice were allowed to delivery spontaneously. Mice were monitored by infrared CCTV, allowing continuous monitoring without physical intrusion, in order not to disrupt labour. The optimal concentration with the minimal variation was found to be 20 µg which induced preterm labour at a mean 12.45 hrs (SEM +/- 1.33). No pups survived labour with >10 µg of LPS.

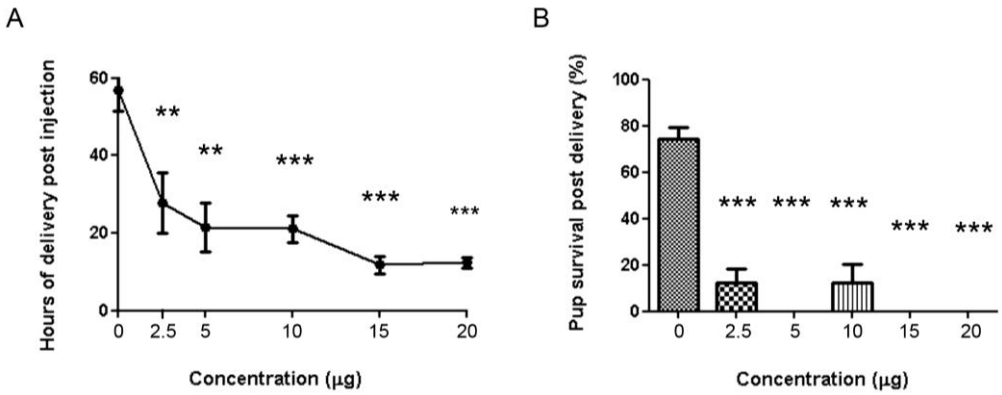


Figure 7.3 Dose response of LPS induced preterm labour
CD1 mice received an intrauterine injection of vehicle or LPS at E16 and were allowed to deliver, (n=3-10 per treatment group). No vehicle control mice delivered preterm and LPS induced preterm labour in a dose response effect, (A). No surviving pups were seen at an LPS dose of >10 µg (B). For statistical analysis one-way ANOVA with Dunnett's post hoc test comparing all groups to the vehicle control; ***P<0.01, *** P<0.001.*

7.4 The effect of the CRTH2 agonist Pyl A on LPS induced murine preterm labour

We sought to determine if the CRTH2 agonist Pyl A had the same feto-protective effect as $15dPGJ_2$ in delaying preterm labour in LPS treated mice. 20 μg of LPS with vehicle or 250 μg of Pyl A was introduced by intrauterine injection and mice were allowed to labour spontaneously. Vehicle control mice delivered 64.5 hrs post injection and LPS treated mice delivered 7.7 hrs post injection *(P<0.001).* Co-injection of LPS and Pyl A augmented delivery to 5.8 hrs (mean) post injection (Figure 7.4A). This effect was more pronounced with a higher dose of Pyl A of 500 μg and lower dose of LPS of 10 μg, shortening delivery time from 14.7 to 8.7 hrs post injection (P<0.01) (Figure 7.4B). Although at 250 μg Pyl A alone did not induce labour earlier than control mice, at 500 μg labour was induced earlier with a mean of 44.8 hrs post injection compared to 64.6 in the vehicle control group (Figure 7.4C). None of the vehicle control treated mice delivered preterm.

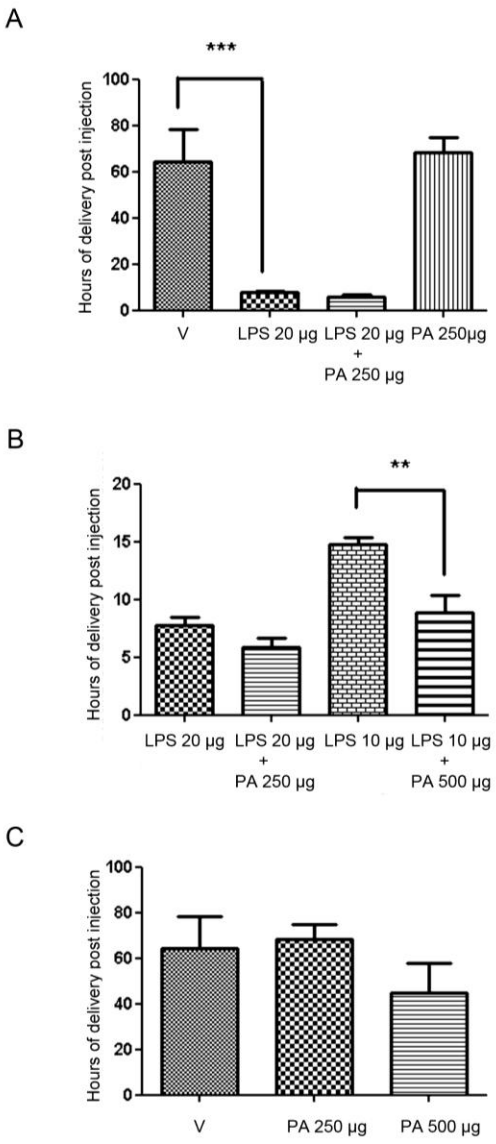


Figure 7.4 The effect of Pyl A on LPS induced preterm labour
CD1 mice received an intrauterine injection of vehicle, LPS or Pyl A at E16 and were allowed to deliver, (n=3 per treatment group). No vehicle control mice delivered preterm, and at 250 µg Pyl A alone did not induce preterm labour (A). LPS induced preterm labour in a dose response effect, and this effect was augmented with the addition of Pyl A (B). 250 µg of Pyl A alone did not induce preterm labour, however at 500 µg Pyl A augmented labour (C). V= vehicle, PA= Pyl A. For statistical analysis one-way ANOVA with Bonferroni's multiple comparison's test or Dunnett's post hoc test was used; ***P<0.01, *** P<0.001.*

7.5 The effect of the CRTH2 agonist Pyl A on the inflammatory cascade

7.5.1 Effect of Pyl A on NF-κB and COX-2 in the myometrium and pup brain

To explore the mechanisms behind Pyl A-augmented LPS-induced preterm labour, key mediators of inflammation in the myometrium were investigated. Myometrium and pup brain were harvested at 4.5 hrs post intrauterine injection and western analysis was used to detect whole cell phospho-p65 and COX-2. LPS did not lead to an increase in NF-κB in the myometrium; however an increase was seen with co-administration of LPS and Pyl A ($P<0.05$) (Figure 7.5.1 A). A reduction was seen in NF-κB in pup brain with LPS compared to vehicle control, with no increase with co-administration with Pyl A (Figure 7.5.1 B). No difference in COX-2 expression was seen between treatment groups in the myometrium or pup brain at this timepoint (Figure 7.5.1 C and D).

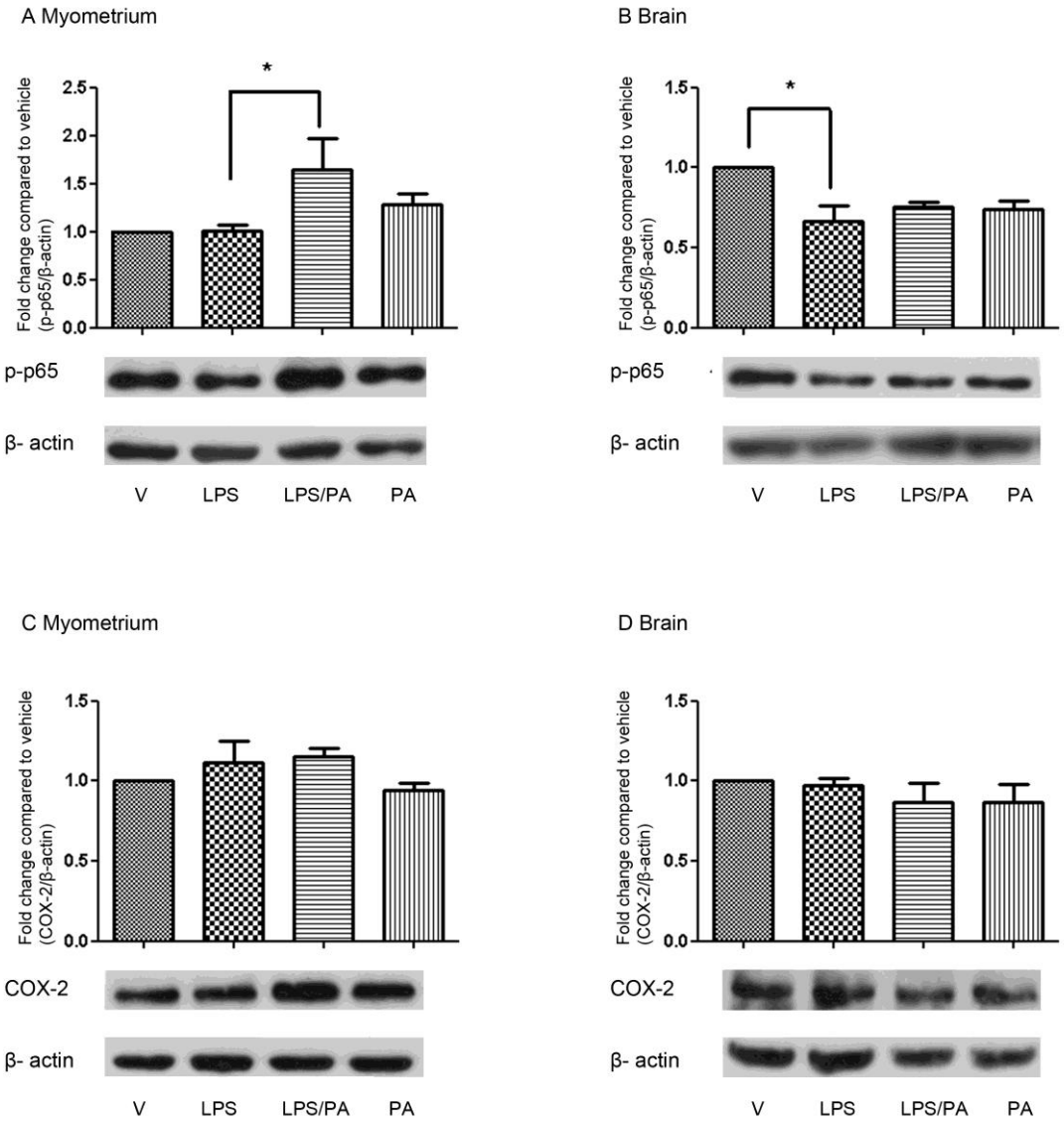


Figure 7.5.1 The effect of LPS and Pyl A on myometrial and pup brain NF-κB and COX 2
Dams were sacrificed at 4.5 hrs post intrauterine injection and tissue was harvested for protein for detection of phospho-p65 and COX-2 by western analysis (n=3). Co-injection of LPS and Pyl A increased myometrial phospho-p65 (A), with no effect on pup brain phospho-p65. Phospho-p65 was decreased in pup brain of dams treated with LPS alone (B). Representative phospho-p65 immunoblots are shown for each treatment group, and β-actin was used as a loading control. No significant changes in COX-2 were seen in any treatment group in either the myometrium or pup brain (C,D). For statistical analysis one-way ANOVA and Bonferroni's multiple comparison test was used; * *P< 0.05.*

7.5.2 Effect of Pyl A on inflammatory cytokines in the myometrium and pup brain

With the activation of NF-κB upon co-administration of Pyl A and LPS, the effect on downstream activation of pro-inflammatory cytokines was next examined. Since the CRTH2 agonist PGD_2 induces the production of the Th2 cytokines IL-10 and IL-4 in human T cells [217], the anticipated effect of Pyl A was to see an increase in these anti-inflammatory cytokines and an inhibition of the pro-inflammatory cytokines. However, contrary to anticipated but yet consistent with NF-κB activation, Pyl A and LPS led to an increase in the pro-inflammatory cytokines (Figure 7.5.2). mRNA of the Th1 interleukins IFN-γ and TNF-α was significantly increased at 4.5 hrs post injection (*P <0.05* and *P< 0.01* respectively) (Figure 7.5.2 A), but the increase in protein expression missed out on statistical significance. A significant increase in protein expression of other pro-inflammatory cytokines was seen including IL-1β, KC/GRO (the murine chemokine equivalent of human IL-8 [376]), and IL-12 *(P<0.05)* (Figure 7.5.2 B).

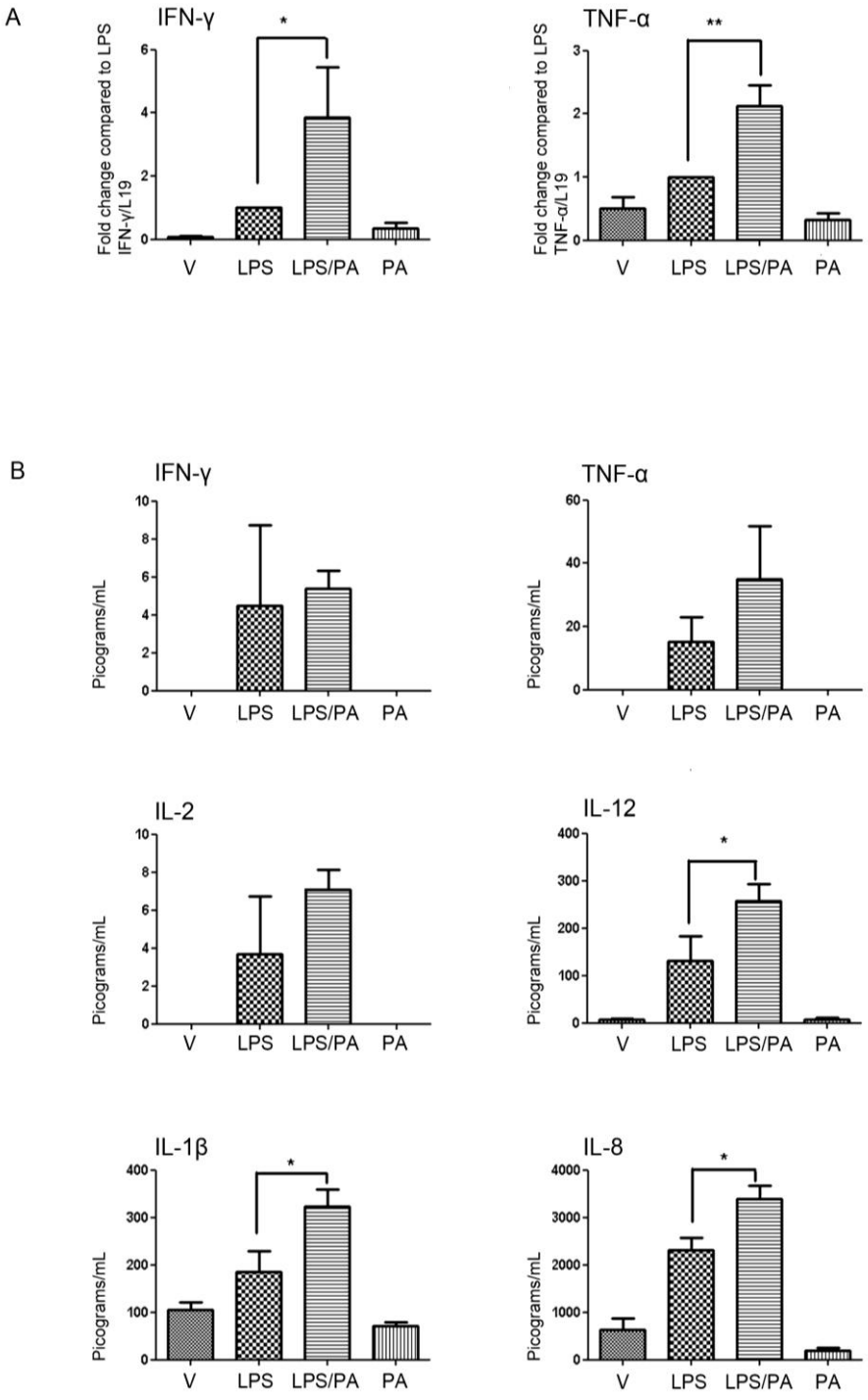


Figure 7.5.2 Effect of LPS and Pyl A on the pro-inflammatory cytokines in the myometrium
Dams were sacrificed at 4.5 hrs post intrauterine injection of 20 µg of LPS/250 µg of Pyl A for harvesting myometrium. mRNA and protein was extracted and analysed by PCR and ELISA respectively (n=3). With co-injection of LPS and Pyl A, IFN-γ and TNF-α mRNA were significantly increased (A) as well as protein levels of IL-12, IL-1β and KC-GRO (IL-8) (B). V= vehicle, PA = Pyl A. For statistical analysis one-way ANOVA with Bonferroni's multiple comparison test was used; * *P<0.05*, ** *P<0.01.*

7.5.3 Effect of Pyl A on inflammatory cytokines in the pup brain

The effect of Pyl A on the pro-inflammatory cytokines in the pup brain was examined by ELISA. Only KC-GRO (the murine equivalent of IL-8) was found at detectable levels. KC-GRO was slightly elevated in LPS treated mice with a further increase with co-administration of Pyl A, although this was not statistically significant (Figure 7.5.3). Overall, no statistically significant difference was seen between treatment groups.

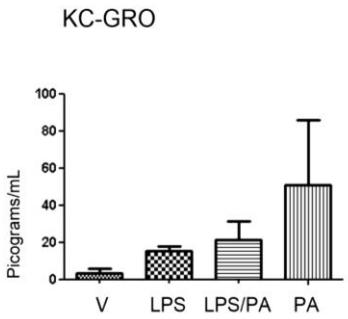


Figure 7.5.3 Effect of LPS and Pyl A on the pro-inflammatory cytokines in the brain
Dams were sacrificed at 4.5 hrs post intrauterine injection of 20 μg of LPS/250 μg of Pyl A and protein was extracted from the pup brain (n=3). The Th1:Th2 profile was determined by ELISA, with only KC-GRO (IL-8) expressed at detectable levels. No statistically significant difference was seen between groups. V= vehicle, PA = Pyl A. For statistical analysis one-way ANOVA with Bonferroni's multiple comparison test was used.

7.6 The effect of the CRTH2 agonists on *in utero* viability in LPS treated mice

To determine if the CRTH2 agonist Pyl A maintained the same feto-protective effect as $15dPGJ_2$, fetal wellbeing was examined at 4.5 hrs post intrauterine injection of LPS with vehicle or Pyl A. Mice were anaesthetised and a mini laparotomy was performed, and uteri were incised. Fetuses were assessed for viability by assessment of movement with or without physical stimulus. Contrary to expected, in view of the findings of the effect of Pyl A and LPS on the pro-inflammatory cascade, a significant improvement in fetal viability was observed when LPS treated mice were injected with Pyl A compared to LPS and vehicle control. Fetal survival was increased from 20% in LPS treated mice to 100% in LPS/Pyl A treated mice, (*P<0.0001*) (Figure 7.6A). However, this benefit was not seen post spontaneous labour (Figure 7.6B), due to the adverse effects of preterm labour.

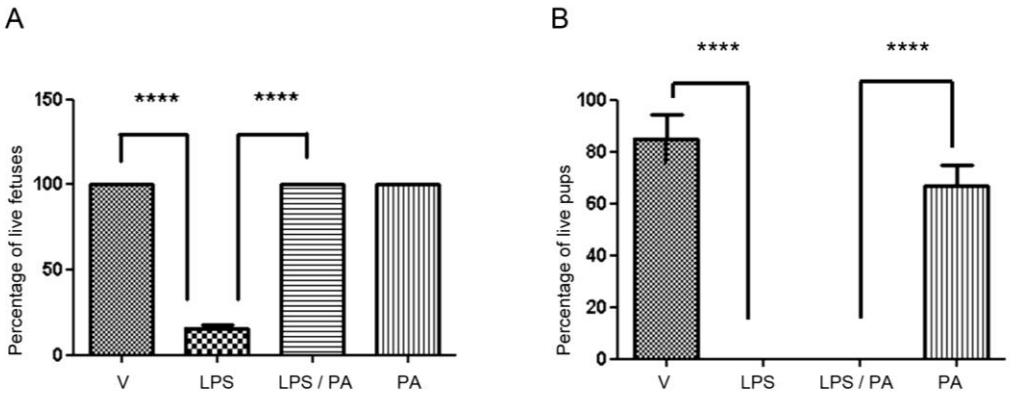


Figure 7.6 Effect of Pyl A on intrauterine fetal viability at 4.5 hours post intrauterine injection and post spontaneous labour
Dams were sacrificed at 4.5 hrs post intrauterine injection of 20 µg of LPS/ 250 µg of Pyl A and pup viability was assessed (n=3 dams). There was an average of 11-14 pups per dam in all treatment groups. Pyl A significantly reduced fetal death at 4.5 hrs post injection from 100% with LPS alone to 20% with Pyl A (A). However, post labour, no surviving pups were seen, which is likely to be attributed to the effects of prematurity (B). V= vehicle, PA = Pyl A. For statistical analysis one-way ANOVA with Bonferroni's multiple comparison test was used; ****$p<0.0001$.

7.7 The effect of the CRTH2 agonist Pyl A on the anti-inflammatory Th2 interleukins

The mechanism of increased *in utero* fetal survival seen with Pyl A was explored by analysing the mRNA and protein expression of Th2 anti-inflammatory cytokines in the myometrium and pup brains. There was no difference in IL-4 mRNA between treatment groups, and protein concentrations were below the detection level of the assay. There was a slight increase in the production of IL-5, and an increase in both mRNA (Figure 7.7A) and protein expression (Figure 7.7B) of IL-10, which also did not achieve statistical significance. These interleukins were not detectable in fetal brain samples.

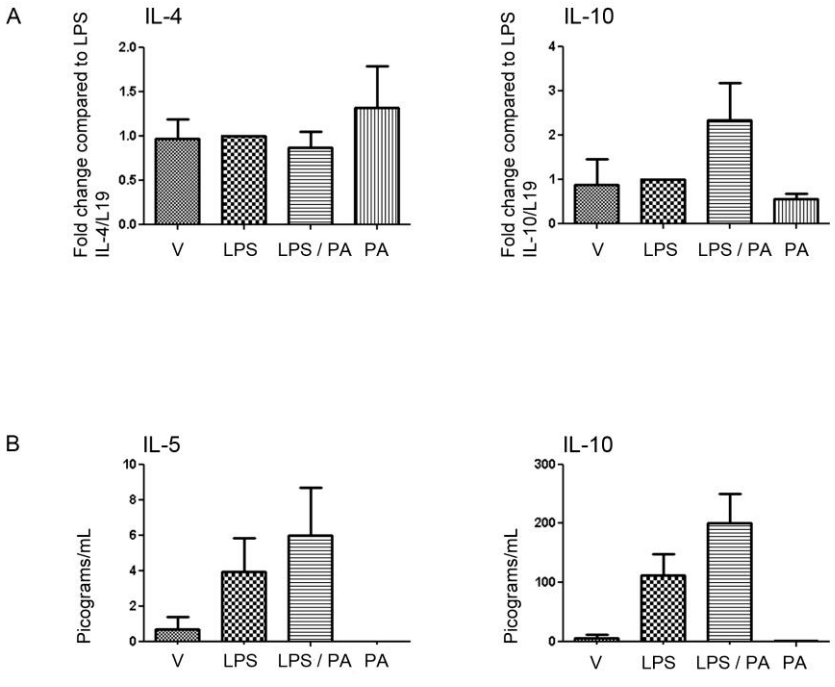


Figure 7.7 Effect of LPS and Pyl A on the anti-inflammatory cytokines in the myometrium
Dams were sacrificed at 4.5 hrs post intrauterine injection of 20 µg of LPS/ 250 µg of Pyl A. mRNA and protein was extracted from the myometrium and analysed by PCR or ELISA respectively. No change in IL-4 mRNA expression was seen, and a non-significant increase in IL-10 was seen with co-administration of Pyl A and LPS (A). Although there was an increase in protein levels of IL-10 and IL-5 with co-injection of LPS and Pyl A, this did not reach statistical significance (B). V=Vehicle, PA= Pyl A.

Summary and Discussion

The search for preventative therapies for both preterm birth and related neurological damage has recently focused upon anti-inflammatory in addition to tocolytic strategies. It is generally accepted that parturition is a pro-inflammatory event, with preterm labour being associated with an exaggerated inflammatory response and/or infection. With the discovery that the CRTH2 agonist $15dPGJ_2$ delays inflammation induced preterm labour in the mouse and increases pup survival [277], this led to the hypothesis that this effect could be replicated with stable small molecule CRTH2 agonists. This chapter explored the potential for the small molecule agonist Pyl A to both delay preterm labour and improve fetal outcomes in a model of inflammation induced preterm labour in the mice.

CRTH2 expression in murine myometrium

Murine CRTH2 mRNA was detected in the mouse myometrium using the primers as described by Abe et al [369], (Figure 7.1). Detection at the protein level was hampered by the lack of available antibodies [368]. However, mRNA expression of CRTH2 has been shown in murine Th1 and Th2 cells, and in various tissues including the lung, kidney, brain heart thymus and spleen [369]. It is possible that detection of CRTH2 in tissue samples is through contamination of leukocytes. However, it is unlikely that the CRTH2 detected in the murine myometrium in this study can be explained by this since there was no difference in mRNA expression between vehicle, Pyl A, LPS or LPS/Pyl A treated mice, and one would anticipate leukocyte infiltration particularly in the LPS treated group. Although we cannot definitively conclude that CRTH2 is expressed at the protein level in murine myometrium on the basis of mRNA expression alone, amplification at a cycle number of 33, (four fold higher than the human), is suggestive that the mRNA is expressed and thus is likely to be translated. This may imply a biological relevance for CRTH2 in the myometrium of the mouse.

The effects of the CRTH2 agonists on murine myometrial contractility

$15dPGJ_2$ inhibits the pro-labour transcription factor NF-κB at both the basal level and in LPS treated mice [277]. NF-κB controls the expression of labour associated genes such as prostaglandins, connexin 43 and the oxytocin receptor [245] and therefore $15dPGJ_2$ may indirectly inhibit myometrial contractility via NF-κB. The effect of $15dPGJ_2$ on murine myometrial contractility was examined *ex vivo* with a myograph. As with human myometrial contractility, no effect was seen with acute administration of $15dPGJ_2$ in a cumulative dose response of 1-100 μm over a two and a half hour time frame (Figure 7.2.1). Arguably this may not be long enough to assess a downstream inhibitory effect at the level of control of gene expression.

A non-CRTH2 mediated inhibition of human contractility was seen with Pyl A (see chapter six) which indeed was replicated in murine circular muscle (Figure 7.2.2). Again this was shown to be independent of CRTH2 since neither $15dPGJ_2$ nor DK- PGD_2 had the same effects (Figures 7.2.1 and 7.2.3). The robust inhibitory effect was clearly demonstrated in circular muscle, however, was not demonstrated in longitudinal muscle in part since the control strips did not contract for long enough to carry out the cumulative dose response. At high doses Pyl A cross-reacts with other prostanoid receptors including the EP_2 and EP_4 receptors [320]. Previously we have shown that the EP_2 agonist, but not EP_4 agonist inhibits human myometrial contractility [365]. Stimulation of the EP_2 and EP_4 receptors leads to cAMP production via the G protein G_s leading to smooth muscle relaxation [377]. Thus the effect seen with Pyl A study could be due to non-specific binding with the EP_2 receptor.

The effect of the CRTH2 agonist Pyl A on LPS induced murine preterm labour

Pyl A does not replicate the inhibitory effects of $15dPGJ_2$ on NF-κB in human cultured amniocytes and myocytes (chapter four), and therefore it is unlikely to inhibit NF-κB in the murine myometrium. However, in view of the discovery that Pyl A inhibits murine

circular myometrial contractility, it is still possible that a delay in LPS induced preterm labour could be demonstrated. Contrary to anticipated, co-injection of LPS treated mice with Pyl A was found to augment the time to preterm labour in a dose dependant response (Figure 7.4B). At a high dose of Pyl A alone, labour was augmented to a mean of 44 hrs post delivery compared to 65 hrs in the vehicle control (Figure 7.4C). Several mechanisms were explored in order to explain these findings.

Firstly, current understanding of the functional role of the individual muscle layers of the mouse uterus in pregnancy and parturition suggests that inhibition of the circular myometrial muscle is unlikely to prevent labour. Although there is limited knowledge of the role of the individual muscle layers in the mouse, it has been suggested that the function of the longitudinal muscle myometrium in other species such as the pig and rat is to move luminal contents by contraction [378], and that tonic contraction of the circular muscle may be required for spacing and retention of the embryos/fetuses [379]. Circular muscle cells have a higher spontaneous electrical activity than longitudinal muscle cells during rat pregnancy [380], supporting its potential role in the maintenance of pregnancy. If circular muscle contraction is necessary for retention of uterine contents, this could explain how inhibition of circular muscle contraction by Pyl A leads to preterm expulsion of the fetuses seen in this study. In the human a relatively new, although controversial, concept has emerged relating to the importance of relaxation of uterine tone during human labour [381]. It is proposed that relaxation of the lower segment of the uterus, in conjunction with contractions of the fundal region, is required for the passage of the fetus through the birth canal. Secondly, the effect of Pyl A on the pro-labour transcription factor NF-κB in the myometrium was examined, and thirdly its effect on the anti and pro-inflammatory cytokines was determined.

<u>The effect of the CRTH2 agonist Pyl A on the inflammatory cascade</u>

Treatment of animals with Pyl A alone increased NF-κB activity in the myometrium which was enhanced with co-administration of LPS (Figure 7.5.1 A). Surprisingly,

myometrial COX-2 levels remained unchanged 4.5 hrs post treatment in all groups (Figure 7.5.1 C). As preterm labour was induced following LPS/Pyl A treatment at 5.8 hrs (SEM $\pm$ 0.7) it was expected that any COX-2 up-regulation in the myometrium should have already been apparent by 4.5 hrs post treatment. It is possible that COX-2 was already up-regulated prior to intrauterine injection in preparation for term labour. In support of this COX-2 is suppressed in pregnancy and by E16 it is already up-regulated in preparation for term labour and cannot be further increased [382]. Analysis of pup brain extracts collected from LPS treated dams revealed a decrease in levels of phospho-p65. This may reflect protein degradation induced by the pre-terminal state of the live pups (Figure 7.5.1 B).

Co-injection of Pyl A and LPS also led to an increase in the production of the pro-inflammatory cytokines TNF-α, IFN-γ, IL-12, IL-1β and KC-GRO (Figure 7.5.2). Pro-inflammatory stimuli such as LPS [383] and the cytokines TNF-α [384] and IL-1 [385] have been shown to activate NF-κB via the canonical pathway by phosphorylating serines leading to IKB degradation and translocation of the NF-κB complex into the nucleus, activating gene expression of pro-inflammatory cytokines which further augments the pro-inflammatory response in a positive feed-forward loop [245]. The assessed cytokines were also increased (but to a lesser degree) in the absence of NF-κB activation with LPS treatment alone (Figure 7.5.2 A and B). It is therefore plausible that LPS and Pyl A co-administration activates a strong cytokine response which then further induces NF-κB activation via a feed forward mechanism.

The pro-labour and pro-inflammatory effect seen with the CRTH2 agonist Pyl A was disappointing. However, consistent with the effect seen here, several murine studies with CRTH2 agonists/antagonists and/or the use of CRTH2 knock-out mice have shown a pro-inflammatory role for the CRTH2 receptor [371, 372, 386-390]. The CRTH2 agonist DK- PGD_2 causes eosinophilia in mouse lung [390] and intra-peritoneal administration of DK- PGD_2 causes a 2-fold induction of MCP-1 and a 25-fold induction of MIP-2 (the functional equivalent of the IL-8 receptor in the mouse) [371]. Furthermore, in a murine study of FITC induced inflammation of the skin (a model of contact hypersensitivity), a CRTH2 antagonist was found to significantly reduce the

production of the pro- inflammatory cytokines TNF-α, IL-1β and the chemokines MIP-2 and GRO-α [391]. Similarly reduced levels of lung IFN-γ, IL-4 and IL-5 have been observed in a mouse model of airways inflammation upon administration of a CRTH2 antagonist [389].

The Pyl A and LPS induced pro-inflammatory effects on both NF-κB and the Th1 type cytokines, along with the inhibition of circular muscle contractility are the likely mechanisms explaining augmented preterm labour.

The effect of Pyl A on *in-utero* viability in LPS treated mice and the anti-inflammatory Th2 interleukins

With the overwhelming pro-inflammatory response seen in the myometrium, it was surprising to discover the significant increase in fetal viability seen with co-injection of Pyl A compared to LPS alone (Figure 7.6). The mechanism behind the increase in fetal viability seen with administration of Pyl A and LPS at 4.5 hours post injection was explored by analysing the effect on the anti-inflammatory Th2 type cytokines. While no changes in the levels of IL-4 were detected, an increase (non-significant) in IL-5 was observed (Figure 7.7). Moreover, a non-significant increase in IL-10 mRNA and protein with LPS and Pyl A treatment was also detected. Although the increase did not reach statistical significance with an n=3, the biological relevance of a small increase is not always necessarily reflected by statistical analyses. IL-10 is a potent protective interleukin against fetal loss in the mouse, including loss associated with LPS administration [375].

In summary, this chapter reveals that the CRTH2 agonist Pyl A augments a pro-inflammatory response in LPS induced preterm labour in the mouse. Pyl A shortened the time interval from intrauterine injection to preterm delivery via increased NF-κB activity and increased production of pro-inflammatory cytokines. Pyl A also led to an inhibition of circular myometrial contractility *ex vivo* which was likely to contribute to

the rapid expulsion of the fetuses. Despite increased fetal viability seen with Pyl A in LPS treated dams, which was likely to be secondary to an increase in IL-10, an overwhelming pro-inflammatory response was seen with the CRTH2 agonist in the mouse. This may be secondary to a functional CRTH2 receptor on murine Th1 cells, unlike in humans. Therefore, in conclusion, $15dPGJ_2$-mediated delay in murine preterm labour is not mediated via CRTH2. Finally, since the CRTH2 agonist seems to augment inflammation induced preterm birth, CRTH2 should not be considered as a potential therapeutic target for the prevention of preterm labour and neonatal morbidity.

CHAPTER 8

Summary discussion

8.1 Summary of hypotheses

Prior to the work presented in this thesis, $15dPGJ_2$ was found to be a promising agent for the prevention of inflammation associated preterm labour and the neurological sequale associated with inflammation. $15dPGJ_2$ was shown to inhibit the pro-labour and pro-inflammatory transcription factor NF-κB in human amniocytes and myocytes [271] and delay inflammation induced preterm labour in the mouse where it offered neuroprotection and increased pup survival [277]. However, the mechanism of action by which $15dPGJ_2$ operates in these systems was not fully understood.

Since $15dPGJ_2$ is a CRTH2 agonist, the key hypotheses of this work were that CRTH2 plays a role in the following effects of $15dPGJ_2$;

a) NF-κB inhibition
b) Delay in inflammation induced preterm labour
c) Increased pup survival

Secondly, it was hypothesised that the CRTH2 agonist effects could be exploited, to create a Th2 cytokine bias and promote positive pregnancy outcomes.

Thirdly, it was hypothesised that if CRTH2 was involved in the mechanism of action of $15dPGJ_2$, then a stable small molecule agonist could be used as a future therapeutic agent for both the prevention of inflammation associated preterm labour and the reduction of inflammation induced brain injury.

8.2 Summary of results and discussion

CRTH2 expression

The potential expression of CRTH2 on amniocytes and myocytes was first explored as part of the investigations to determine if CRTH2 plays a role in $15dPGJ_2$ mediated inhibition of NF-κB. There are limited studies examining CRTH2 expression in fetal membranes and the uterus. One study failed to demonstrate mRNA expression in amnion [279], and another study examining non-pregnant uterus detected moderate levels [287]. As presented in chapter three, CRTH2 mRNA was detected at low levels in both amniocytes and myocytes. However, this mRNA was not translated since CRTH2 could not be detected at the protein level using a variety of approaches. The work presented in this thesis demonstrates the lack of sensitive and specific commercially available antibodies hampering the use of western blotting for detecting CRTH2. By using peripheral blood leukocytes and CRTH2-transfected amniocytes and myocytes as positive controls, flow cytometry was successful in detecting both endogenous PBMC CRTH2 and transfected amniocyte and myocyte CRTH2. Flow cytometry failed to detect endogenous CRTH2 on amniocytes and myocytes, thus providing conclusive evidence that these cells do not express CRTH2.

CRTH2 and NF-κB

In view of the absence of the CRTH2 receptor on amniocytes and myocytes, it seemed unlikely that CRTH2 had a functional role in $15dPGJ_2$-mediated inhibition of NF-κB. However, to fully conclude this, a series of experiments were performed with $15dPGJ_2$, a specific CRTH2 agonist (Pyl A), and the CRTH2 antagonist GSKCRTH2 on amniocytes, myocytes and peripheral blood mononuclear cells (PBMCs), results of which are presented in chapter four. Pyl A failed to replicate $15dPGJ_2$-mediated inhibition of IL-1β-stimulated NF-κB in amniocytes and myocytes. Although $15dPGJ_2$ was found to

inhibit NF-κB in peripheral blood mononuclear cells, this effect was not replicated by the specific CRTH2 agonists DK-PGD_2 or Pyl A, and its effect was not attenuated by pre-incubation with the CRTH2 antagonist. These experiments provide compelling evidence that CRTH2 is not involved in 15dPGJ_2-mediated NF-κB inhibition.

Although 15dPGJ_2 is also a ligand for PPAR-γ, inhibition of NF-κB in amniocytes and myocytes is independent of this receptor [271]. The electrophilic cyclopentenone ring of 15dPGJ_2 appears to contribute to its actions. The cyclopentenone prostaglandin PGA1 (which contains one reactive electrophilic carbon as opposed to two) also inhibits NF-κB activity, although at markedly higher concentrations. 15dPGJ_2 is able to inhibit multiple steps in the IKK/NF-κB signalling pathway. It inhibits nuclear translocation by preventing the IL-1β induced phosphorylation of IKKα and IKKβ, and also by preventing IKBα degradation and thus the release of the NF-κB subunits [271]. Endogenous 15dPGJ_2 can accumulate intracellularly and can bind to components of the NF-κB signalling pathway directly. For example, direct modification of cysteine 179 of IKKβ by covalent interaction with 15dPGJ_2 has been demonstrated in HeLa cells [392]. Furthermore, the formation of covalent adducts between 15dPGJ_2 and the p50 subunit has been demonstrated [323]. Despite these mechanisms having been described, it is still not known how 15dPGJ_2 enters the cell, or if a receptor dependent factor is required for the inhibition of NF-κB. However, work presented in this thesis has at least excluded the possibility that the effects of 15dPGJ_2 are via the G protein coupled receptor CRTH2.

<u>Effect of pregnancy, labour and CRTH2 agonists on the Th1/Th2 profile</u>

A bias exists in pregnancy to favour the Th2 interleukin profile, especially at the maternal fetal interface [193]. The suppression of Th1 interleukins peripherally is thought to be at least in part due to suppression of NF-κB in T helper cells in pregnancy [240]. Several groups have demonstrated this bias in PBMCs during pregnancy which returns to the non-pregnant state post-partum [146, 147, 218]. However, during labour it is unclear if cells are activated or if the bias has already reversed consistent

with the post-partum state [393, 394] . PBMCs of women in labour were examined to see if a systemic pro-inflammatory state was reflected, consistent with local activation of NF-κB and pro-inflammatory cytokines seen at the maternal fetal interface and in the uterus at term and in labour.

Confirmation of the reduction in both IFN-γ and TNF-α production in PMA/ionomycin and non-stimulated cells during pregnancy is reported in chapter five. Although the mean production per cell of IFN-γ and TNF-α was generally unchanged, the percentage of cells secreting the interleukins was reduced. This potentially reflects regulation at the levels of differentiation or development of the cells rather than at an individual cellular level. However, no significant increase in the production of Th1 interleukins was seen in PMA/ionomycin treated PBMCs of women in labour. A moderate increase in the basal percentage of cells producing IFN-γ and TNF-α was seen from 28 weeks to term with a further increase with the onset of labour. However, this increase was not statistically significant. The biological relevance of this increase is not known and indeed the physiological implications of a systemic inflammatory response could potentially be detrimental to both mother and fetus. Studies to explore both NF-κB and the Th1 profile in women with clinical evidence of chorioamnionitis are required to determine if this basal inflammatory response is more pronounced, and therefore amenable to immuno-modulation.

Several groups have demonstrated an increase in IL-4 production systemically during pregnancy [146, 147, 218], however the contrary has been reported by one group where a reduction in IL-4 during pregnancy was observed [219]. Since CRTH2 is a marker of Th2 cells, the production of IL-4 in this population was examined during pregnancy and in labour. An increase in the percentage of cells secreting IL-4 was seen at 28 weeks compared to non-pregnant women, with return to non-pregnant values at term, however this did not reach statistical significance when the multiple comparisons test was applied. Had more subjects have been examined, or if the $CRTH2^{+}$ cells producing IL-4 had been purified, a more marked response may have been demonstrated. The increase in IL-4 producing cells, together with the absence of any change in the mean production is consistent with a potential regulation at the

levels of differentiation or development of the CRTH2 positive cells rather than at an individual cellular level, as seen with the Th1 interleukin profile.

The CRTH2 agonists PGD_2 and Indomethacin both induce the production of the Th2 interleukins IL-4, IL-5 and IL-10 in Th2 cells *in vitro* [217, 299]. As part of a negative feedback regulatory system, IL-4 can directly inhibit the development of Th1 cells, whereas IL-10 achieves this indirectly [194]. This leads to the inhibition of the production of pro-inflammatory cytokines. Having demonstrated a bias towards Th2 with suppression of the percentage of cells producing Th1 interleukins as well as a moderate increase in the CRTH2 positive cells secreting IL-4 during pregnancy, the immunomodulatory effects of CRTH2 agonists were examined. It was anticipated that the agonists would be able to further modulate interleukin production at the cellular level by modifying mean interleukin production.

Only 1 μM of Pyl A had any effect on CRTH2 positive cells. A small, yet non-significant, increase was seen in the percentage and mean production of IL-4 at 28 weeks in non-PMA/ionomycin treated cells. A statistically significant increase in the mean production in PMA/ionomycin treated cells in non-pregnant samples was also found. The subtle response may be due to the experimental design in view of the small amount of non-purified $CRTH2^+$ cells being analysed, but also the PMA/ionomycin may be maximally stimulating IL-4 preventing further stimulation with Pyl A. No change was seen in IFN-γ or TNF-α production with either concentration used, probably due to only a minor effect on IL-4 production. Overall, no significant immuno-modulation could be demonstrated with Pyl A.

In contrast, significant modulation of the Th1 cytokines was seen with $15dPGJ_2$, with substantial downregulation of PMA/ionomycin stimulated IFN-γ and TNF-α at all gestational time points. Data presented in chapter four reveals the inhibitory effect of $15dPGJ_2$ on NF-κB in peripheral blood mononuclear cells. Since NF-κB is known to play a key regulatory role in controlling the Th1 immune response by modulating the expression of Th1 cytokines [256], it is likely that the Th1 inhibitory properties of $15dPGJ_2$ are regulated through NF-κB. Conversely, no effect was seen on IL-4

production. This may be due to an overriding effect of a non-CRTH2 mediated mechanism such as the inhibition of other signalling proteins involved the transcriptional regulation of IL-4 expression. For example, $15dPGJ_2$ inhibits nuclear factor of activated T-cells (NF-AT), which transcriptionally regulates IL-4 production, in a PMA/ionomycin activated T cell line in a dose dependent manner [286]. Overall, Th1 suppression is seen with $15dPGJ_2$, thereby leading to a Th2 bias indirectly. Preterm labour, particularly in the presence of chorioamnionitis, is associated with a shift towards the Th1 cytokine response [192]. Specifically PMA stimulated PBMCs of women in threatened preterm labour who deliver preterm have significantly higher IFN-γ compared to women who go on to deliver at term [238]. Data presented in this thesis demonstrates that the anti-inflammatory properties of $15dPGJ_2$, previously demonstrated in the amnion and myometrium [271], can also be applied systemically resulting in the inhibition of NF-κB and the Th1 interleukins in PBMCs.

Effect of CRTH2 agonists on myometrial contractility

An ideal therapeutic agent for the prevention of preterm labour would have both anti-inflammatory and tocolytic properties. The inhibitory effect of $15dPGJ_2$ on NF-κB and COX-2 in myocytes [271], together with stretch mediating an increase in NF-κB activity [183], led to the hypothesis that $15dPGJ_2$ could also have tocolytic properties. This was investigated *ex vivo* by mounting myometrial strips collected from women at term (pre-labour) and treating them with $15dPGJ_2$ in a cumulative dose response, as presented in chapter six. No effect on contractility was seen with acute administration. This may be because activation of NF-κB may not be required for uterine contractions induced *ex vivo* by the myograph. Surprisingly, treatment of myometrial strips with Pyl A led to a dose dependent inhibition of myometrial contractility, although in a CRTH2 independent mechanism. Possible mechanisms of inhibition include wither via non-specific interaction with the EP_2 receptor, or via its structural similarity to Indomethacin.

Effect of the CRTH2 agonist Pyl A on a murine model of inflammation induced preterm labour

Despite not being able to replicate the anti-inflammatory effect of $15dPGJ_2$ on NF-κB, or showing immunomodulatory effects on the human Th1/Th2 profile *in vitro*, the considerable tocolytic effect of Pyl A led us to investigate if the same effect could be achieved *in vivo*. Whilst it is appreciated that tocolysis in the presence of clinically apparent inflammation is not advisable, it was also important to establish if CRTH2 plays a role in $15dPGJ_2$-mediated delay in inflammation induced preterm labour and increased pup survival. Results of the effect of Pyl A on murine myometrial contractility and inflammation induced preterm labour were presented in chapter seven.

Pyl A showed significant tocolytic effects on murine circular myometrium *ex vivo*, consistent with the effects seen in the human, and again in a CRTH2 independent mechanism. However, *in vivo* administration of Pyl A led to augmentation of labour, which was amplified with co-injection of LPS. Controversy remains with regards to the function of the circular muscle in the mouse. Tonic contraction of the circular muscle in the rat may be required for spacing and retention of embryos/fetuses [379], and weak high frequency contractions in the circular muscle layer are thought to prevent movement of fetuses towards the cervix during pregnancy [395], supporting the potential role of tonic contraction in the maintenance of pregnancy. Also, in support of this, uterine relaxation of the lower segment during human labour in the human is thought to aid passage of the fetus through the birth canal [381].

Alternatively the tocolytic effect of Pyl A may not have contributed to the augmentation of labour. This may have been purely due to the overwhelming inflammatory response induced by the co-administration of Pyl A and LPS. Activation of NF-κB was demonstrated which also lead to downstream activation of several pro-inflammatory cytokines including IL-1β, IFN-γ, TNF-α and KC-Gro (the murine equivalent of IL-8). Although this pro-inflammatory effect was not induced by Pyl A

alone, and only a small effect with LPS alone, there is a clear synergistic effect with both LPS and Pyl A together. It is plausible that a subtle trigger in NF-κB activation with LPS together with a subtle trigger from Pyl A is enough to lead to an amplified response and a continued feed forward loop. Despite the significant pro-inflammatory response caused by Pyl A, a surprising increase in fetal viability was seen at the 4.5 hr sacrificial timepoint, likely to be due to the increase in IL-10 production. This protective effect however was not maintained post delivery, with zero pup survival since all delivered at a pre-viable gestation. In conclusion, data presented here provides conclusive evidence that CRTH2 does not play a role in 15dPGJ$_2$ mediated delay in preterm labour or increased pup survival.

The differences in effect seen with Pyl A between the human and the mouse highlight the limitations of *in vivo* work in species other than humans. The mouse is commonly used for models of preterm labour, due to its relatively short gestation and low cost of experiments and maintenance, however, there are clear differences in pregnancy and parturition compared to that of the human. For example, the human myometrium is not composed of such a discrete longitudinal/circular muscle arrangement, nor is the uterus bicornuate. A biochemical withdrawal of progesterone occurs in the mouse at term [396] rather than a functional progesterone withdrawal that is seen in the human [397]. Specifically with regards to CRTH2, unlike in the humans, its expression is detected on murine Th1 cells [369]. Although it is not known if the CRTH2 receptor is functional in murine Th1 cells, this could account for the significant production of Th1 cytokines induced by Pyl A. Finally, although protein expression of CRTH2 in murine myocytes was hampered by the lack of commercially available antibodies, the comparable mRNA expression in the myometrium of vehicle vs LPS vs LPS Pyl A suggests that CRTH2 expression in the myometrium is not solely due to infiltrating leukocytes.

8.3 Conclusion

In conclusion, the CRTH2 agonist Pyl A does not replicate the anti-inflammatory effects of $15dPGJ_2$ in the human or the mouse. It can be deduced that CRTH2 does not play a functional role in NF-κB inhibition; delay in inflammation induced murine preterm labour or increased pup survival. Finally, Pyl A and $15dPGJ_2$ do not modulate the Th2 interleukin profile in peripheral blood mononuclear cells via CRTH2. However, it was found that the anti-inflammatory effects of $15dPGJ_2$ can be extended to activated peripheral blood mononuclear cells resulting in both the inhibition of NF-κB and Th1 interleukins, thereby indirectly modulating the peripheral Th1/Th2 interleukin profile to favour the Th2 response.

8.4 Clinical relevance

Preterm labour remains a common challenge to obstetrics. The incidence has not reduced, despite increased efforts in understanding the mechanisms controlling its onset. The management of women with preterm rupture of membranes, particularly pre-viability or at the margin of viability, remains variable amongst obstetricians. The use of tocolytics also remains variable worldwide in terms of the type of tocolytic used, the indication and the duration. Economically, the neonatal sequale of preterm labour and indeed the long-term health issues that follow account for a significant portion of NHS resources. It is therefore imperative that preventive strategies are developed to target those at risk of preterm labour.

Controversy still remains with regards to aiming to prevent preterm labour in those presenting with threatened preterm labour. This is mainly due to the risk of delaying preterm labour in the presence of subclinical infection/inflammation, thereby potentially increasing neonatal morbidity and mortality. The focus of this thesis was on the potential use of $15dPGJ_2$ or a specific CRTH2 agonist with a view to targeting the inhibition of the biochemical pathways associated with both inflammation and labour.

The ultimate aim is to prophylactically administer such an agent to prevent the initial biochemical triggers of both inflammation and labour that precede the onset of clinical symptoms. Although specific small molecule CRTH2 agonists should not be considered as future therapeutic agents, the anti-inflammatory prostaglandin $15dPGJ_2$ shows considerable promise for the future.

8.5 Future work

Future work with $15dPGJ_2$ will include exploring its role in the inhibition of NF-κB and pro-inflammatory cytokines both locally at the maternal fetal interface and systemically in maternal peripheral blood mononuclear cells in women in preterm labour and term labour associated with clinically evident chorioamnionitis. Effects of the anti-inflammatory compound will also be explored in cord blood obtained from matched samples to ascertain if it has potential for preventing the fetal inflammatory response syndrome. If these results are consistent with the anti-inflammatory effects seen so far, as presented in this thesis, then it will be proposed that $15dPGJ_2$ should be used in a primate model of inflammation induced preterm labour.

Published manuscripts

Pre-term Labour and Delivery

Targeting Immune Activation in the Prevention of Pre-term Labour

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Abstract

Pregnancy involves a continuing interplay between inflammatory mediators that facilitate implantation, gestation and the onset of labour. The latter in particular involves a reduction in the influence of hormonal and regulatory factors that promote myometrial quiescence and the activation of proinflammatory cascades that precede myometrial activation and contractions. In the majority of early pre-term labour cases (at less than 32 weeks of gestation), there is evidence of inflammation, often induced by infection. There is increasing evidence that nuclear factor kappa B is a key modulator of the major inflammatory signalling pathways involved in both term and pre-term labour. There is also increasing evidence of the anti-inflammatory role of current therapies such as progesterone, cerclage and non-steroidal anti-inflammatory drugs. Selectively targeting immune activation and key mediators of inflammation may therefore prove to be useful strategies for preventing pre-term labour and improving neonatal outcome by inhibiting immune-mediated damage to the foetus in utero. This article discusses the immunology of pregnancy, immune activation in term and pre-term labour and the neonatal sequale of infection-induced pre-term labour. Current and future strategies targeting immune activation in the prevention of pre-term labour are also explored.

Keywords

Pre-term labour, immune activation, nuclear factor kappa B, inflammation, cytokines

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Following implantation, which is largely an inflammatory event, the maternal unit tightly regulates inflammatory mediators to enable the development of a semi-allergenic foetus, while still providing protection against infection. Towards the end of normal pregnancy, inflammatory pathways are again activated, culminating in myometrial contractility and birth. If this inflammatory response is triggered early, for example, in the case of ascending infection, myometrial activation and contractions ensue, resulting in premature labour and birth.

Pre-term labour is defined as a labour occurring between the 24th and the 37th week of pregnancy. It can be further classified as extremely pre-term (24–27 completed weeks), very pre-term (28–31 completed weeks) and late pre-term (32–36 completed weeks).[1] While increasing research efforts have attempted to address the understanding and prevention of pre-term birth, its incidence has remained largely unchanged over the last two decades (see *Figure 1*). Our failure to improve premature birth rates has proved socially and economically costly. Around 75 % of neonatal deaths and 70 % of long-term health problems, such as neurodevelopmental impairment and respiratory disease,[2] can be accounted for by pre-term birth. Neonatal morbidity is estimated to cost £2.9 billion per year.[3] A long-recognised correlation between intrauterine infection and pre-term birth exists; however, as revealed by an interrogation of the PubMed literature, increasing research efforts are being expended on deducing the role that inflammation plays in pre-term birth (see *Figure 1*). Despite the increase in awareness of inflammation and infection in pre-term labour, rates of pre-term labour have not reduced.

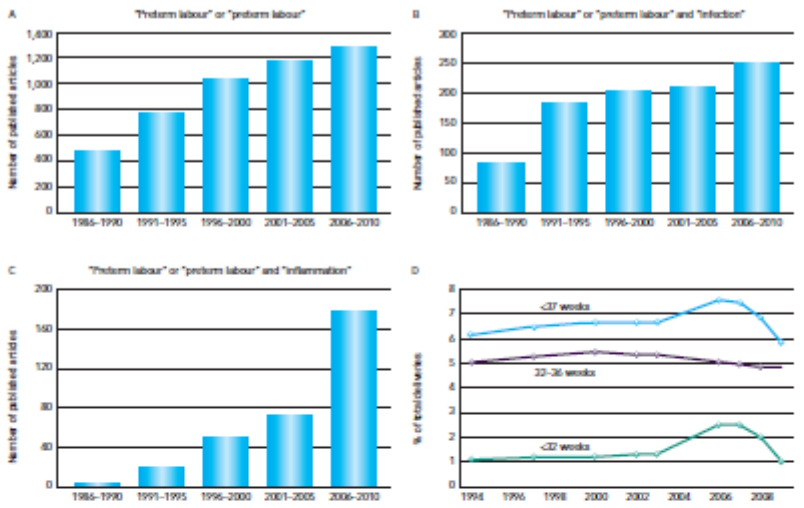
Approximately 30 % of pre-term births are a result of elective deliveries for reasons such as pre-eclampsia and foetal growth restriction. The remainder are spontaneous labours and are mostly the result of an inflammatory and/or infective process.[4] Since inflammation and infection are the most common pathogenic processes in extreme premature labour, targeting immune activation in the prevention and treatment of pre-term labour is an attractive strategy.

Pre-term Labour, Inflammation and Neonatal Sequelae

The neonatal sequelae of pre-term delivery are mainly dependent on gestational age at delivery. Survival rates improve from 39% at 25 weeks to 98 % at 32 weeks.[5] Pre-term delivery results in an increased risk of necrotising enterocolitis, chronic lung disease, periventricular leukomalacia (PVL) and intraventricular haemorrhage (IVH).[6] Cerebral palsy, for which PVL is a predictor, is 60 times more frequent in neonates born before 28 weeks of gestation compared with those born at term.[7] The additional insult of inflammation and infection further increases the risk of chronic lung disease,[8] PVL[9] and IVH by three- to four-fold.[10]

Intrauterine inflammation is probably mediated by both the maternal and foetal response. Foetal inflammatory response syndrome (FIRS) was proposed by Romero and colleagues and is characterised by the activation of the foetal innate immune system.[11] This condition was initially defined as elevated interleukin-6 (IL-6) concentrations in foetal plasma in women with pre-term labour and prelabour premature rupture of membranes (PPROM).[12] However, other indicators of foetal

Figure 1: PubMed Literature Search for Relevant Terms Over the Last 25 Years



A: A blast search using 'preterm labour' highlights the continued, growing efforts in the field to better understand the mechanisms underlying untimely onset of labour. While infection has long been associated with pre-term labour (B), research is increasingly being directed towards understanding the role of inflammation in driving the onset of parturition and labour, particularly in the last five years (C). Examination of singleton pre-term delivery rates in the UK for the last 15 years reveals that, while increasing efforts have been placed into understanding pre-term labour, little if any clinical impact has been made (D). Recent reviews have described an increasing rate of pre-term labour, particularly at less than 32 weeks of gestation. However, analysis of the latest data suggests a transient increase over the past six years, the cause of which is unclear.
Data available online from NHS: www.ic.nhs.uk/statistics-and-data-collections and www.ch.gov.uk/en/Publicationsandstatistics/Statistics/index.htm

inflammation include umbilical cord C-reactive protein,[72] amniotic fluid white cell counts[74] and other proinflammatory cytokines. It is likely that inflammatory mediators released in FIRS play a functional role in triggering the main signalling pathways that result in labour, to shorten the exposure of the foetus to a hostile environment. However, these inflammatory mediators may be partly responsible for the damaging effects of inflammation on the neonatal central nervous system.

As part of the foetal response to infection and PROM, there is evidence of a foetal Th1 polarised response with increased interferon (IFN) gamma production in cord blood.[75] Amniotic fluid and cord blood proinflammatory cytokines IL-1β, IL-6 and tumour necrosis factor-alpha (TNF-α) are associated with radiological evidence of cerebral damage.[76,77] Intra-amniotic proinflammatory cytokines including IL-8 are associated with the development of cerebral palsy.[78] There is mounting evidence from *in vitro* and *in vivo* animal studies that the same signalling pathways activated in pre-term labour, involving Toll-like receptors (TLRs) and nuclear factor kappa B (NF-κB), also play a role in inflammation-induced brain damage.[79] Thus, targeting inflammatory pathways may also prove useful for the prevention of neonatal morbidity.

Immunology of Pregnancy

The immunology of pregnancy is a complex phenomenon that balances immune tolerance and suppression. The immune paradox of pregnancy exists as the mother does not reject the foetus, contrary to an anticipated rejection of paternally inherited foetal antigens. As first proposed by Medawar, the foetus essentially acts as an allograft that avoids rejection owing to a physical separation from the mother, its own antigenic immaturity and the immunological inertness of the mother (i.e. maternal immune suppression).[80] Highly regulated responses from both the innate and adaptive immune systems facilitate this process.

The Innate Immune System in Pregnancy

The innate immune system is the first line in the host's ability to defend against pathogens. In its most primitive form, it includes physical barriers that protect against entry of foreign material. In the case of the foetus, the amnion acts as a membrane barrier to the mother throughout gestation. Immunologically active secretions such as lysozymes and defensins serve as biochemical barriers to invasive pathogens. The innate immune system has evolved to recognise non-self-utilising pattern recognition receptors (PRRs)[81] that recognise molecular signatures of pathogens.[82] PRRs are also capable of recognising non-infectious endogenous proteins such as Hsp70.[83]

TLRs are a major component of PRRs, and to date 11 members have been discovered in mammals.[84] Evidence increasingly points towards

TLR-mediated inflammatory mechanisms as a trigger to infection-induced pre-term birth, with TLR receptors 2 and 4 particularly implicated. TLR 2 recognises the products of Gram-positive bacteria and mycoplasma, whereas TLR 4 recognises the products of Gram-negative bacteria.[35] TLR 2 and 4 are expressed in immune cells within the placenta but also in the trophoblasts at different stages of pregnancy.[36] Amnion epithelial cells also express TLR 2 and 4, with an increase in messenger RNA (mRNA) in both term and pre-term labour.[37] Similarly, both receptors are expressed in myometrium, with protein levels of TLR2 increased in labour versus non-labour.[38] An important activator downstream of TLRs is NF-κB, which can lead to the production of immune-response proteins such as cytokines and chemokines.[39]

The Acquired Immune System in Pregnancy

In contrast to the innate immune system, the adaptive immune system relies on B and T cells, which are responsible for the humoral and cellular responses.[9] Activation of the innate immune system can be seen as a prerequisite for triggering the acquired immune response, particularly aiding T-cell polarisation towards T helper 1 effector cells.[12] An important component of the adaptation of the immune system in pregnancy is the regulation of specific cytokine production.[13] Cytokines participate in either T helper 1-mediated responses (cell-mediated immunity) or T helper 2 responses (humoral immunity). Pregnancy is largely represented by a shift towards Th2 activity,[12,13] while the presence of infection and inflammation triggers an opposite shift towards the Th1 response. Consistent with this, increases in the Th1-specific cytokine TNF-α are highly correlated with infection-related pre-term birth.[14,17] Furthermore, the double-knockout TNF-α and IL-1β murine model does not suffer pre-term labour when administered lipopolysaccharide, a major component of Gram-negative bacteria cell walls.

A shift in the bias from Th1 to Th2 is evident both systemically and locally at the maternal–foetal interface. During pregnancy, peripheral blood lymphocytes show an increase in mRNA of the prototypical Th2 interleukin IL-4 and lower mRNA levels of the prototypical Th1 interleukin IFN-γ.[18] Also, *in vitro* stimulation of peripheral lymphocytes in pregnant women displays increased production of IL-4 and reduced production of IFN-γ compared with non-pregnant controls.[19] The reduction in T helper 1 cytokine production is likely a result of downregulation of the NF-κB/IκB signalling pathway observed in T cells throughout pregnancy.[20]

In addition to decidual leukocyte production of the Th2 cytokines IL-4 and IL-10,[21] non-lymphoid cells in the placenta and decidua also contribute to the Th2 cytokine predominance.[22] These Th2 interleukins are capable of suppressing the Th1 interleukins,[23] IL-8 and prostaglandin production,[24] which are likely to contribute to pregnancy maintenance and uterine quiescence while also contributing to immune tolerance of the foetus as an allograft.

Immune Activation in Term Labour

Term labour occurs at between 38 and 42 weeks of gestation, at which time the cervix undergoes remodelling and ripening to facilitate the expulsion of the foetus from the contractile uterus. Despite extensive research, the exact triggers and network coordination of biochemical and endocrinological components of these processes are poorly understood. This has created the biggest challenge for developing therapies for the prevention of pre-term labour. Although the initial triggers of pre-term and term labour may well differ, with term labour seen as physiological and pre-term labour seen as a pathological condition, the terminal events leading to delivery of the foetus are common to both conditions.[25] We now have increasing evidence that inflammation in the foetal membranes, cervix and uterus is a major player in the cascades leading to both term and pre-term birth.[26]

Immune Activation in the Decidua and Foetal Membranes at the Maternal–Foetal Interface

Activation of the foetal membranes and decidua involves a complex set of biochemical events culminating in the separation of the membranes from the decidua in the lower uterine segment and, ultimately, their rupture. As the decidua acts as an interface between the maternal and foetal tissues, it was proposed by Casey and MacDonald that it may also regulate maternal–foetal immunotolerance during pregnancy.[27] In the amnion, proinflammatory cytokines IL-1β and IL-8 are upregulated during labour, as are IL-6 and IL8 in the choriodecidua.[28] IL-1β is known to stimulate cyclo-oxygenase 2 (COX-2) production, which in turn leads to the production of prostaglandin E2 in the amnion, a major source of prostaglandins that acts on the uterus to stimulate contractions and on the cervix to aid cervical ripening.[29]

Immune Activation in the Cervix

Throughout pregnancy, the cervix is rigid and tightly closed. At the beginning of parturition, the cervix undergoes a major remodelling process referred to as 'ripening'. This occurs independently yet concurrently with the activation of the myometrium and involves an inflammatory cascade that induces the release of proinflammatory cytokines and the infiltration of leukocytes.[30,31] These release matrix metalloproteinases (MMPs), which, along with activated collagenases, degrade the structural connective tissue and collagen fibres of the cervix.[32]

Immune Activation in the Uterus

The myometrium is the muscle layer responsible for uterine contractility. It consists of smooth-muscle cells, myocytes, surrounded by connective tissue, vasculature and infiltrating leukocytes capable of secreting cytokines.[33] During term labour, there is an massive influx of inflammatory cells into the myometrium, mainly the lower segment, including neutrophils, macrophages and T lymphocytes.[34] These infiltrating leukocytes are a source of proinflammatory interleukins and prostaglandins, which are likely to contribute to normal parturition.

A comprehensive study by Young et al. used immunohistochemistry to localise these cytokines to their cellular origin, comparing pre-labour term myometrium and in-labour myometrium.[33] IL-1β was predominantly located in leukocytes, whose presence increased during term labour. Weak staining was also demonstrated in myocytes. IL-6 and TNF-α were restricted to leukocytes; from the morphological appearance of these leukocytes, they are likely to be neutrophils and macrophages respectively. Interestingly, IL-8 was observed infiltrating leukocytes in the labouring myometrium and not in the myocytes themselves; however, as discussed below, others have attributed increased levels of IL-8 in the labouring myometrium to the myocytes themselves.[33]

Whether the activation of inflammatory pathways in the myometrium is a cause or consequence of labour is debatable. Recent work by Bisits and colleagues has gone some way to addressing this.[26] Using a novel application of structural equations modelling, the mathematical plausibility of three commonly proposed pathways of myometrial activation was tested. These pathways were inflammatory stimulation,

functional progesterone withdrawal and oxytocin receptor activation. Data provided from quantitative reverse transcriptase polymerase chain reaction performed on key genes involved in these pathways were used to generate alternative causal pathways using mathematical formulae known as directed graphs. The data analysed from 12 non-labouring and 12 labouring women strongly supported inflammation as a primary driver of human myometrial activation for labour.

Nuclear Factor Kappa B as a Mediator of Labour Onset

NF-κB is a family of transcription factors classically associated with inflammation that control expression of cytokines and chemokines, for example, TNF-α and IL-1β.[10] The NF-κB family comprises five members; NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-rel.[11] Interactions between these members and their intracellular location dictate their function and ability to interact with DNA to promote the expression of inflammatory genes. Chapman and colleagues have previously reported the presence of all subunits except RelB and p52 in the myometrium. Although total RelA was found to be reduced in lower-segment myometrium in labouring women, DNA-binding activity was actually increased.[12]

NF-κB also appears to play a pivotal role in the transcriptional regulation of contractile associated genes in the myometrium and foetal membranes at the time of labour. COX-2, the rate-limiting step for prostaglandin synthesis, is elevated in lower-segment myometrium towards term[13] and in labour versus non-labour samples.[14] Work by our own group has confirmed *in vitro* that NF-κB is required for IL-1β-induced expression of COX-2 in myocytes[15] and, consistent with this, that IL-1β concentration increases in myometrium with the onset of labour.[12] NF-κB also regulates the proinflammatory cytokines IL-8 and IL-6, which both contain NF-κB recognition elements within their promoter regions.[16] IL-8 is dramatically increased in labouring myometrium[12] and is detected in the supernatant of non-stimulated and IL-1β-stimulated myocytes *in vitro*.[15] Transcriptomic studies comparing myometrial biopsies from non-labouring versus labouring term women revealed that the largest differences were in IL-8 (47-fold increase with labour), IL-6 (14-fold increase) and IL-1β (11-fold increase).[16] This study highlights the importance of proinflammatory genes in lower-segment myometrium in labour. We have also used microarray analysis on myocytes overexpressing p65 and showed that NF-κB regulates genes principally related to immunity and inflammation in these cells.[12] These studies, along with earlier work by Chan et al.,[14] provide evidence that the myometrium is capable of functioning as a distinct immune organ and that inflammatory pathways are critical components during parturition. Consistent with a proposed role in regulating labour onset, the administration of SN-50, an NF-κB inhibitor, in the amniotic fluid of mice was shown to delay pre-term birth.[17]

Immune Activation in Pre-term Labour

Apart from gestational age, the outcome of pre-term labour and term labour is phenotypically similar.[18] Both share a final pathway of cervical ripening, uterine contractility and rupture of membranes. Intuitively, this suggests that there is likely to be considerable overlap in the biochemical pathways leading to the final trigger of labour and expulsion of the foetus. Infection and/or inflammation are the only well-defined pathological processes at the molecular level in which a firm causal link with pre-term labour has been established.

As discussed above, immune activation and inflammation are physiological components of normal, term labour. We propose that, in events leading to pre-term labour, there is a pathological presence of inflammation, with or without infection, which precipitates cervical ripening, uterine contractility and premature rupture of membranes. This may present as PPROM only, isolated uterine irritability or cervical dilation in the absence of contractions or PPROM, depending on the location of inflammation and/or individual patient characteristics.

Infection and/or Inflammation and Pre-term Labour

One of the most common causes of inflammation is infection. In the context of pregnancy, approximately 30 % of pre-term deliveries are associated with an infective process.[19] Upon review of the literature, Goldenburg et al. summarised that 80–85 % of spontaneous pre-term labours at less than 28 weeks of gestation showed evidence of intrauterine infection, rising to over 90 % around 24 weeks, in contrast to around 15 % of women who delivered late pre-term at 34–36 weeks of gestation.[20] The most commonly identified pathogens associated with pre-term labour prior to rupture of membranes are Ureaplasma urealyticum and Mycoplasma hominis.[21] Interestingly, the latter is a Gram-negative bacteria that can instigate inflammation through TLR4 signalling.[22] Routes of transmission of infection into the uterus and subsequently the foetus may be via haematogenous spread via the placenta, iatrogenic procedures such as amniocentesis or, as most commonly described, by an ascending route from the vagina through the cervix.[23]

The mechanism by which infection is able to trigger the parturition cascade is undoubtedly linked to an integrated network of interactions between the proinflammatory cytokines and prostaglandins. These act in concert to stimulate uterine contractions and remodel both the cervix and the foetal membranes, leading to cervical dilation and rupture of membranes, respectively.[24,25] In response to intrauterine infection, infiltrating leukocytes and decidual cells are capable of secreting the proinflammatory cytokines IL-1, IL-6, IL-8 and TNF-α.[26,27] These cytokines then induce prostaglandin production in the foetal membranes and decidua, leading to initiation of parturition.[25,27]

Although microbial infection is a common cause of inflammation, the presence of infection is not essential for inflammation-driven pre-term labour. There is a causal role for cytokines in labour even in the absence of infection.[28] We have previously shown that pregnancies without clinically overt signs of infection still show increased levels of IL-1β and IL-8 in the myometrium, amnion and choriodecidua.[29] Moreover, a recent genomic study of amnion tissues from women in spontaneous labour without histological chorioamnionitis revealed that these tissues present an acute inflammation gene expression signature.[30] Recently published data suggest that relaxin, a hormone secreted by the corpus luteum, can also modulate cytokine production by decidual macrophages at the maternal–foetal interface and contribute to a localised 'sterile' inflammatory response.[31] Together, these data lend support to the notion that inflammation, but not necessary infection-induced inflammation, is a common pathway in the activation of labour.

Therapies for the Prevention of Pre-term Labour – An Anti-Inflammatory Perspective

Progesterone

Several large randomised controlled trials have now shown that progesterone is able to delay pre-term labour in high-risk pregnancies.[32–35] The ongoing multicentre OPPTIMUM (ISRCTN: 14568373) trial aims to determine if this prolongation of pregnancy will confer benefit to the

infant in the long term. The precise mechanism by which progesterone is able to delay pre-term labour is still not known, yet it is likely that the anti-inflammatory role of endogenous progesterone contributes to a favourable immunological environment for the foetus.

The traditionally accepted primary role of progesterone in pregnancy maintenance is its contribution to myometrial quiescence. Several proposed mechanisms exist for this, including downregulation of labour-associated genes such as connexin[61,62], MMP-9[63] and COX-2.[64] Non-genomic mechanisms include modulation of the contractile apparatus by a surpassing intracellular free calcium[65] and increasing cyclic adenosine monophosphate, which results in relaxation.[66] The immunomodulatory effects of progesterone have been demonstrated both locally in the uterus and foetal membranes as well as systemically in peripheral blood leukocytes.

At the maternal–foetal interface, the placenta is a major source of progesterone. Decidual leukocytes produce progesterone-induced blocking factor in response to progesterone, which regulates the shift in Th2 cytokine bias, and a reduced cytotoxic natural killer activity.[67] *In vitro* studies have shown that progesterone negatively interacts with and suppresses NF-κB activity in myocytes,[67] amniocytes[68] and lower uterine segment fibroblasts,[69] leading to downregulation of COX-2. The suppressive effect of progesterone on NF-κB also results in the suppression of proinflammatory cytokines such as TNF-α.[70] Progesterone has also been shown to induce a Th2 phenotype in resting peripheral blood mononuclear cells in cell culture and induces IL-4 expression,[71] consistent with the Th2 dominance seen *in vivo*. Despite the immunomodulatory effects of progesterone in the maintenance of pregnancy, it appears that administration of progesterone for the prevention of pre-term labour may not translate these effects. In a murine model of pre-term labour, progesterone delayed labour onset yet inflammatory markers were unchanged and no increase in pup survival was observed.[72] Similarly, a recent study examining the effects of vaginal progesterone in women with a shortened cervix showed no reduction in cytokine production in the cervicovaginal fluid.[17] Another study of progesterone administration compared interleukin levels in myometrial tissue and peripheral blood leukocytes at the time of pre-labour caesarean section and found no statistically significant differences.[73]

Cervical Cerclage

Cervical shortening is associated with an increased risk of pre-term delivery: a cervical length of less than 15 mm leads to a 50 % risk of pre-term labour before 32 weeks of gestation.[74] Placement of a cervical cerclage reduces the risk of delivery before 35 weeks of gestation by 30 %, with a 36 % reduction in perinatal mortality and morbidity.[75] Cervical cerclage provides mechanical support to a weakened cervix and is typically placed in women with suspected cervical insufficiency. However, as well as contributing to mechanical support, preservation of cervical length and the mucous plug is likely to contribute to protection against ascending infection.[76]

There is a correlation between shortening cervical length and positive amniotic fluid culture.[77,78] Intrauterine inflammation (using MMP-8 as a marker) is also increased with shortening cervix length, appearing in 22 % of women with a cervical length of less than 25 mm compared with 73 % of women with a cervical length of less than 5 mm.[79] In a study of women with a shortened cervix undergoing cerclage, a reduction in pre-term labour before 37 weeks with cerclage was more likely in those women with low cervicovaginal IL-8 levels.[80] In women who already have established inflammation or infection, the triggers of parturition are not likely to be reversed by cerclage. If cerclage does play a role in targeting immune activation, its effectiveness is likely to be in the prevention of inflammation and ascending infection and not the reversal of inflammation. Thus, cerclage combined with anti-inflammatory/antimicrobial treatment regimens may enhance and improve positive patient outcomes by physically inhibiting cervical shortening and managing underlying biochemical inflammation.

Non-steroidal Anti-inflammatory Drugs

The role of non-steroidal anti-inflammatory drugs (NSAIDs) in the prevention of pre-term labour has been evaluated mainly in the context of tocolysis. COX-2 is the rate-limiting step for the production of prostaglandins. Since prostaglandins are integral to the mechanism of cervical ripening and uterine contractility, this class of drug initially seems attractive as a preventative strategy in asymptomatic women. Indometacin is a non-selective COX inhibitor and is effective in reducing pre-term labour.[81] Originally, the mechanism of action was thought to be solely via inhibition of prostaglandin synthesis.[82] However, indometacin is also able to inhibit calcium channel currents in myocytes, leading to complete inhibition of myometrial contractility *in vitro*.[83] Aspirin and salicylate have been shown to inhibit NF-κB activity by direct inhibition of IκB kinase.[84] As previously mentioned, NF-κB is fundamental to the biochemical pathways involved in both term and pre-term labour, thus the anti-inflammatory effects of NSAIDs are also likely to contribute to their efficacy in reducing pre-term labour. Unfortunately, the adverse effect profile of NSAIDS, particularly that of indometacin (increased risk of premature closure of the ductus arteriosus, increased risk of necrotising enterocolitis and IVH), has prevented its more frequent use in the prevention of pre-term labour in high-risk women.[82] Specific inhibition of COX-2 in women at high risk of pre-term labour appears to further increase their risk, which suggests that COX-2 may play dual roles in both pregnancy maintenance and parturition.[72]

Antibiotics

As discussed above, there exists a clear association between infection and pre-term labour. However, there is a paucity of evidence demonstrating a benefit in the administration of antibiotics for the prevention of pre-term labour. Antibiotics appear ineffective when abnormal vaginal flora[77] or foetal fibronectin are used as indicators of high-risk pregnancies;[75] in fact, the use of antibiotics in high-risk asymptomatic women increases the risk of pre-term delivery.[77] In symptomatic women with intact membranes, antibiotics have been shown to increase long-term functional impairment, including cerebral palsy.[77] As the administration of antibiotics in these cases follows overt clinical indicators, it is tempting to speculate that inflammatory pathways have long been activated and the cascades of parturition have already been initiated. Thus, the efficacy of antibiotic treatment may be improved with earlier diagnosis of infection.

Conclusion

This article has highlighted the integral role of the inflammatory response in the activation of both premature and term labour and its neonatal consequence. Current treatments, despite having potential immunomodulating effects, have proved disappointing in terms of delaying pre-term labour and improving neonatal outcome. While it is feasible that treatment doses, population targets or timing of treatment have yet to be correctly optimised, we feel that it is more

likely that inflammation- or infection-induced pre-term labour involves multiple components that contribute to a final common pathway. Targeting a key modulator of this common pathway, such as NF-κB, may prove particularly beneficial in the rational design of therapeutic treatments for pre-term birth.

Specifically targeting NF-κB would lead to inhibition of COX-2 and prostaglandin production and, subsequently, uterine contractility and cervical ripening. Additionally, it might also reduce responsiveness to the TLR-activated pathways, resulting in the reduction of proinflammatory cytokines such as IL-1β and IL-8. We have demonstrated in an animal model of lipopolysaccharide-induced pre-term labour that the anti-inflammatory prostaglandin 15-deoxy Δ prostaglandin J2 (15dPGJ2) not only delays pre-term labour but also provides neuroprotection and increases pup survival.[74] 15dPGJ2 inhibits NF-κB and COX2 in both human myocytes and amniocytes *in vitro*.[42] Additionally, 15dPGJ2 is a ligand for chemoattractant receptor-homologous molecule 2 , which is present on TH2 cells and which may augment beneficial Th2 cytokine bias.

We conclude with the vision of a potential immunomodulating agent that will not only delay pre-term labour but also suppress the foetal inflammatory response, leading to neuroprotection and, ultimately, improved neonatal outcome. ■

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REVIEW ARTICLE

Prevention of preterm labour via the modulation of inflammatory pathways

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Pregnancy is characterized by a complex interplay of inflammatory events regulated by both the innate and acquired immune systems. Similarly, parturition can be viewed as the activation of "pro-labour" inflammatory pathways, which drive cervical ripening and myometrial activation. Premature activation of these pathways, for example, by infection, can lead to preterm labour and birth. Nuclear factor κβ is a key modulator of these pathways and functions by regulating the expression of prostaglandins, chemokines and pro-inflammatory cytokines involved in both term and preterm labour. Future design of therapeutics that target key mediators of inflammation and immune activation would therefore be a rational approach for preventing preterm labour and immune-mediated neonatal brain damage.

Keywords: Inflammation, nuclear factor κβ, pregnancy

Inflammation and preterm labour

Preterm birth is the major cause of infant death and handicap in the developed world, accounting for 65% neonatal deaths and 50% neurological disabilities. Survival rates sharply decrease from 99% at 32 weeks to 1% at 23 weeks, and this is associated with a reciprocal increase in the risk of handicap [1]. As reported in the EPICure study, of those babies born before 25 weeks who survive to discharge, 25% developed severe disability and less than 50% are normal at 30 months of age.

Inflammatory mediators play important roles in all aspects of successful pregnancy. After implantation, which itself involves localized activation of inflammatory mediators, there is the need for strict maternal regulation of immune and inflammatory mediators to prevent rejection of the semi-allogeneic fetus but to still provide protection against infection. Term labour results from a physiological reduction in the influence of endocrine signals and other factors that act to inhibit myometrial contractility, in conjunction with the activation of pro-inflammatory biochemical pathways that precede myometrial activation and contraction. Premature activation of inflammatory mediators is a major feature of the pathophysiology of preterm labour and in particularly very preterm labour where it is often induced by infection [2].

The transcription factor nuclear factor κβ (NF-κβ) is an important modulator of the inflammatory signalling pathways involved in both term and preterm labour and directly regulates the expression of various labour associated genes such as the oxytocin receptor, which are not typically considered inflammatory. There is growing evidence that activation of inflammation within the uterus not only leads to the onset of preterm labour but can independently be damaging to the fetal brain [3]. Data from our laboratory and others suggest that this is mediated by toll-like receptors (TLRs) and NF-κβ [4]. The targeting key mediators of inflammation within the uterus may therefore prove to be useful strategies for both preventing preterm labour and improving neonatal outcome.

The immune system during pregnancy

The complex balance between immune tolerance and suppression is often referred to as the "immune paradox of pregnancy" and is critical in ensuring that the mother does not reject the semi-allogeneic fetus. This is largely achieved by the physical separation of the fetus from the mother by the maternal-fetal interface in combination with its own antigenic immaturity and through maternal adaption of the innate and acquired immune systems [5].

The amnion acts as a membrane barrier between fetus and mother and is the most elemental feature of the innate immune system and is complemented by other nonspecific biochemical barriers including lysosymes and defensins. The innate immune system also incorporates non-self pattern recognition receptors, TLRs which play a major role in activation of inflammatory mechanisms in the context of inflammation or infection induced preterm birth. In particular, expression of TLR2 and TLR4 in the amnion increases with both term and preterm labour and expression of TLR2 increases in the myometrium with the onset of labour [6]. Activation of TLR2 or TLR4 leads to downstream upregulation of pro-inflammatory transcription factors including NF-κβ and Activator Protein-1 (AP1) leading to the production of pro-inflammatory cytokines and chemokines.

The adaptive immune system is based upon B and T lymphocytes which mediate humoral and cellular responses [7] in which cytokine production is an important component of adaption in pregnancy. Cytokines play roles in either T helper 1 (Th1; cell mediated immunity) or T helper 2 (Th2) mediated responses (humoral immunity). A critical step in the activation of the acquired immune response is the triggering of the innate immune response, aiding the polarization of T cells towards Th1 effector cells [8]. Normal pregnancy is associated with a change in the

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Th1/Th2 cytokine response towards Th2 activity and that infection within the uterus triggers an opposite shift towards the Th1 response [9]. In support of this concept, increased synthesis of the Th1 specific cytokine, tumour necrosis factor α (TNF-α), is highly correlated with infection associated preterm birth [10]. Preterm labour can be induced in the mouse by exposure to lipopolysacharide, a major component of gram negative bacterial cell walls, either through intra or extra amniotic or intraperitoneal injection. However, the TNF-α/interleukin-1β (IL-1β) double knock out mouse is protected from preterm labour under these circumstances.

The bias towards Th2 cytokines during pregnancy can be seen both systemically and locally at the maternal-fetal interface. Pregnant maternal peripheral blood lymphocytes show increased expression of IL-4, the archetypal Th2 cytokine, and reduced expression of the interferon γ (IFN-γ), the classical Th1 cytokine [11]. Stimulation of peripheral lymphocytes from pregnant women leads to a greater increase in IL-4 and a greater decrease in IFN-γ production when compared with lymphocytes from nonpregnant women [12]. The NF-κβ/Iκβ signalling pathway has also been shown to be downregulated in maternal T cells throughout pregnancy and this is probably a major component of the mechanism leading to the reduction in Th1 cytokines in pregnancy 13. At the maternal-fetal interface, there is decidual leukocyte production of Th2 cytokines, IL-4 and IL-10 [14]. There is also a contribution from nonlymphoid cells in the placenta and the decidua. These Th2 cytokines suppress the synthesis of Th1 interleukins, IL-8, cyclo-oxygenase-2 (COX-2), prostaglandins and therefore contribute to pregnancy maintenance both through affects on immune tolerance of the fetus as an allograft and an inhibition of cervical ripening and myometrial contractions.

Immune regulation at the onset of term labour

Term labour involves an initial phase of cervical remodelling leading to dilation and uterine contractions leading to expulsion of the fetus. The exact triggers and networked co-ordination of these processes are yet to be elucidated impeding advancements in developments of therapies to prevent preterm birth. Regardless of the initial triggers of preterm and term labour, a common junction in the mechanisms leading to contractions is likely to exist. Inflammation in fetal membranes, cervix and uterus is a common prelude to both term and preterm labour.

The decidua acts as an interface between maternal and fetal compartments and regulates maternal/fetal immune tolerance during pregnancy. With the onset of labour there is upregulation of the pro-inflammatory cytokines, IL-1β, IL-6 and IL-8 in the amnion and chorion deciduas [15]. IL-1β upregulates COX-2 synthesis and this in turn drives the synthesis of prostaglandin E2, particularly in the amnion, which then plays important roles in membrane remodelling, cervical ripening and myometrial contractions.

For most of pregnancy the cervix remains rigid and closed but prior to the onset of established labour, it undergoes major remodelling associated with the production of pro-inflammatory cytokines, chemokines and leukocyte infiltration [15]. This leads to increased matrix metalloproteinases and collagenase expression which degrades the connective tissue and collagen fibres of the cervix.

During labour, there is a large influx of inflammatory cells including neutrophils, macrophages and T lymphocytes into the myometrium which are an important source of pro-inflammatory interleukins and prostaglandins and are presumed to play an important role in normal parturition. The concept that activation of inflammation within the myometrium is a cause rather than a consequence of labour has been supported by mathematical modelling undertaken by Bisets and colleagues [16] who modelled three possible pathways leading to labour, inflammatory stimulation, functional progesterone withdrawal or oxytocin receptor activation. These studies strongly supported the hypothesis that the primary event is activation of inflammation with less evidence for functional progesterone withdrawal and oxytocin receptor activation as the initiating events.

Immune regulation at the onset of preterm labour

The final common pathways of cervical ripening and uterine contractility occur both in term and preterm labour suggesting considerable overlap in the terminal biochemical pathways. A firm causal link has been established between infection and/or inflammation with the uterus and preterm labour. This is particularly important in preterm labour at early gestational ages with evidence of infection in 80–85% cases of preterm labour before 28 weeks and over 90% cases before 24 weeks. This contrasts markedly with only 15% of women delivering late preterm, beyond 34 weeks, showing clear evidence of infection. The most commonly identified pathogens are Ureaplasma urealyticum and Mycoplasma hominis, the latter being a gram negative bacterium that can activate TLR4 signalling. It is thought that these pathogens enter the uterus by haematogenous spread via the placenta through iatrogenic mechanisms such as amniocentesis but most commonly by ascending from the vagina through the cervix. Although infection is commonly associated with inflammation and preterm birth, infection is not essential for inflammation to occur and there is a causal role for inflammatory cytokines in preterm labour in the absence of infection. There are reports of increased levels of IL-1β and IL-8 in the myometrium, amnion and chorion decidua in preterm labour without clinically overt signs of infection [17]. A recent transcriptomics study of amnion from women in spontaneous labour without histological chorioamnionitis showed that there was nevertheless evidence of an acute inflammatory gene expression [18]. The correlation between activation of inflammation and the presence of bacteria within the uterus is not strong suggesting that inflammation, but not necessarily infection induced inflammation, is a common pathway in the activation of preterm labour and that some cases of preterm labour may be due to an excessive inflammatory response to relatively trivial number of bacteria. Not surprisingly, studies associating genetic polymorphisms with a risk of preterm birth have found links between a risk of preterm birth and polymorphisms in or near a range of genes known to participate in the inflammatory response.

NF-κβ as a mediator of labour onset

NF-κβ is a transcription factor important in the regulation of a range of cytokines and chemokines and other inflammatory mediators such as COX-2. NF-κβ also plays a key role in the regulation of other labour associated proteins at the time of labour. For example, the expression of COX-2, the central enzyme for prostaglandin synthesis, increases in the lower segment of the uterus towards term and again with the onset of labour [19]. NF-κβ also modulates the expression of the pro-inflammatory cytokines, IL-6 and IL-8. Production of IL-8 is increased in

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labouring myometrium and in the supernatant of myocytes stimulated with IL-1β *in vitro* [20]. Studies of the transcriptome of myometrial biopsies comparing myometrium before and after the onset of labour showed large differences in IL-8, IL-6 and IL-1β expression. Activation of NF-κβ in myocytes in culture shows that NF-κβ regulates genes principally related to immunity and inflammation in these cells [21]. Myocytes therefore appear to be capable of functioning as immune cells as well as contractile cells and it is probable that activation of inflammatory pathways in myocytes are critical components during parturition consistent with a proposed role for NF-κβ in the regulation of the onset of labour.

Anti-inflammatory therapies for the prevention of preterm birth

A number of well-conducted large randomized controlled trials have demonstrated that progesterone can reduce the risk of preterm birth in women at high risk of preterm labour [22]. Although there is now evidence of short-term neonatal benefit, there is still no evidence of long term infant benefit, which is the goal of the currently ongoing multicentre trial, OPPTIMUM (https://www.opptimum.org.uk/: ISRCTN 14568373). The generally accepted primary role of progesterone in maintaining pregnancy is through repression of factors leading to myometrial contractility including downregulation of labour associated proteins, connexin 43, matrix metalloproteinase-9 and COX-2. Progesterone may also modulate contractility by suppressing intracellular free calcium and increasing cyclic AMP concentrations. However it is rational to suggest that progesterone also plays immune-modulatory roles, both at the maternal/fetal interface and systemically.

The placenta is a major source of progesterone. Progesterone stimulates progesterone-induced blocking factor expression in decidual leukocytes inducing a shift towards Th2 cytokine bias and a reduction in the cytotoxic natural killer cell activity [23]. Several studies have shown that progesterone suppresses NF-κβ activity in myometrium, amnion and fibroblast from the lower segment of the uterus leading to suppression of COX-2 expression and downregulation of prostaglandin synthesis [24]. The repression of NF-κβ by progesterone also leads to suppression of pro-inflammatory cytokine synthesis, such as TNF-α [25]. While progesterone appears to play a variety of immune-modulatory roles, its therapeutic use in the treatment of preterm labour does not appear to transfer these characteristics. In a mouse model of preterm labour, although progesterone delayed the onset of labour, inflammatory markers were not changed and there was no increase in pup's survival. Moreover, the CLIC (cervical length inflammation cerclage) study showed that administration of vaginal progesterone to women with a short cervix did not lead to a reduction in cytokine production in cervico-vaginal fluid.

Progressive cervical shortening during pregnancy is associated with an increased risk of preterm delivery. Placement of a cervical cerclage in a woman with a short cervix reduces the risk of preterm delivery before 35 weeks by 30% [26]. In addition to providing mechanical support, cervical cerclage probably also contributes to protection against ascending infection by preserving cervical length and the cervical mucus plug. Evidence for this includes the fact that there is a positive correlation between amniotic fluid cultures and shortened cervix. There is also an association between evidence of intrauterine inflammation and a short cervix with one study showing that amniotic fluid MMP-8 concentrations, were present in 73% women whose cervical length was less than 5 mm compared to 22% women with a cervical length greater than 25 mm [27]. Additionally, a study of women with a short cervix who underwent cervical cerclage showed that the probability of reducing the risk of preterm birth before 36 weeks was higher in those with low cervico-vaginal IL-8 concentrations [28]. It seems likely that women who already have an established inflammation or infection are unlikely to have this reversed by cervical cerclage.

Nonsteroidal, anti-inflammatory drugs have largely been evaluated in the role of acute tocolysis. Indomethacin, a nonselective inhibitor of cyclo-oxygenase, is effective in short-term suppression of preterm labour [29]. Originally, the mechanism of action was thought to be via inhibition of prostaglandin synthesis; however, indomethacin is also a calcium channel blocker. Indomethacin, aspirin and other salicilates have also been shown to inhibit NF-κβ activity, and thus contribute to its anti-inflammatory properties [30]. Due to the fetal side effect profile of most nonsteroidal, anti-inflammatory drugs, particularly that of indomethacin, the use in high risk women is usually limited to short courses in women who are extremely high risk. The finding that the principle enzyme responsible for prostaglandin synthesis at the time of labour is COX-2 has lead to the hope that specific inhibition of COX-2 might reduce the risk of preterm birth without the associated fetal side effects. However, the TOCOX (type-2 cycylo-oxygenase inhibition to prevent preterm birth) study study showed that although the fetal side effect profile is improved, prophylactic use of specific COX-2 inhibitors actually increases the risk of preterm labour suggesting that COX-2 plays dual roles in both pregnancy maintenance and the onset of labour.

The future

It is clear that inflammatory responses play key roles in both preterm and term labour and in the adverse neonatal outcomes associated with preterm labour. Targeting key modulators of this pathway, such as NF-κβ, may prove to be beneficial both in reducing the risk of preterm birth and in improving the outcome for the preterm neonate. Specifically, targeting of NF-κβ would inhibit COX-2 and prostaglandin production and reduce responsiveness to TLR activated pathways resulting in a reduction in the synthesis of pro-inflammatory cytokines such as IL-1β and IL-8. As an example of this possible approach we have demonstrated that the anti-inflammatory prostaglandin 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 (15dPGJ_2) can inhibit NF-κβ, COX-2 and prostaglandin synthesis in human amnion and myometrium, and in an animal model of inflammation induced preterm labour not only delays labour but also increases pup survival with some evidence of neuro-protection. 15dPGJ_2 is also a ligand for the chemo-attractant receptor homologous molecule expressed on Th2 cells (CRTh2) and may therefore augment a beneficial Th2 cytokine bias. Other anti-inflammatory approaches to the prevention of preterm labour are being studied or developed and much more is yet to be learned about the mechanisms and potentials of established treatments such as progesterone. It is essential however that future study of drugs intended to prevent or delay preterm labour have, as their outcome, long term benefit to the preterm neonate not just short-term obstetric outcomes such as gestation of delivery.

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Review Article

The Th1:Th2 Dichotomy of Pregnancy and Preterm Labour

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Pregnancy is a unique immunological state in which a balance of immune tolerance and suppression is needed to protect the fetus without compromising the mother. It has long been established that a bias from the T helper 1 cytokine profile towards the T helper 2 profile contributes towards successful pregnancy maintenance. The majority of publications that report on aberrant Th1:Th2 balance focus on early pregnancy loss and preeclampsia. Over the last few decades, there has been an increased awareness of the role of infection and inflammation in preterm labour, and the search for new biomarkers to predict preterm labour continues. In this paper, we explore the evidence for an aberrant Th1:Th2 profile associated with preterm labour. We also consider the potential for its use in screening women at high risk of preterm labour and for prophylactic therapeutic measures for the prevention of preterm labour and associated neonatal adverse outcomes.

1. Introduction

Preterm labour occurs in some 10% of pregnancies [1]. In many developed countries, the rates are rising. Birth before 37 weeks of gestation is thought to account for up to 70% of neonatal deaths, and the extremely high neonatal intensive care costs required to support those who do survive make preterm birth both a social and economic burden. It is now widely acknowledged that the aetiology of preterm labour is multifactorial, and, as such, the underlying cause of preterm labour is often unknown. There is a strong association between preterm labour and infection and inflammation, and research in this field has dramatically increased over the last few decades [2]. However, we still have made little significant progress in the prevention of preterm labour. Evidence of the detrimental direct impact of maternal infection/inflammation on neonatal outcome is emerging, yet we do not fully understand if anti-inflammatory therapeutic agents would provide benefit or harm to the neonate born under conditions of infection/inflammation-induced preterm labour.

The immunology of pregnancy is complex, in that the mother must tolerate the "foreign" fetus, and thus requires a degree of immunosuppression whilst on the other hand needs to maintain immune function to fight off infection. One mechanism which is involved in successful pregnancy maintenance is the proposed switch from the T helper 1 (Th1) cytokine profile to the T helper 2 (Th2) profile. This paper explores the evidence for an imbalance in the Th1:Th2 profile in women at risk of and who are in established preterm labour.

2. The Immunology of Pregnancy

The fetus can be described as a semiallogeneic graft, being a product of two histoincompatible individuals [3, 4]. This poses a challenge to the mother, to both tolerate and accommodate the fetus, which will express paternal antigens, and maintain an ability to reject in case of overwhelming infection [5]. This challenge is undertaken in part by the immune system. The immune system has two main defence systems: the innate and the adaptive. The innate immune response is a nonspecific reaction towards foreign antigens, whereas the adaptive response forms a very specific reaction towards antigens [6]. Although different immune components are involved in these systems, much overlap and

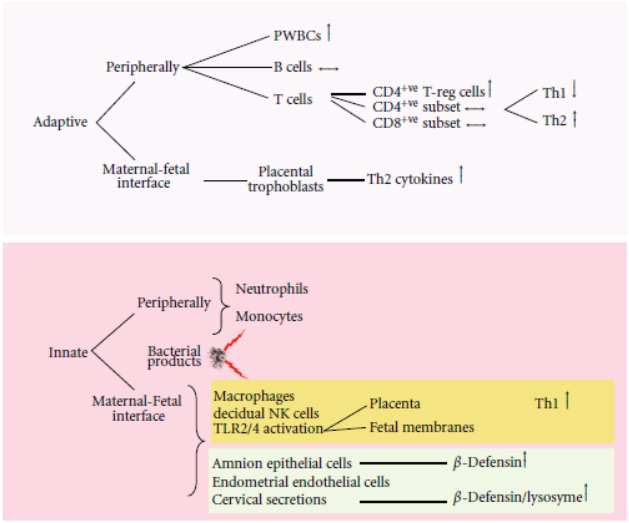


FIGURE 1: Summary of the adaptive and innate immune system in pregnancy. Mediators of the adaptive and innate immune system work in parallel to facilitate a balance between immune tolerance of the fetus whilst maintaining the ability to mount a response against invading pathogens. PWBC: peripheral white blood cells.

cross-talk exist between the two. Figure 1 summarises the key elements of these systems during pregnancy.

The immune cells that make up the adaptive immune response include B and T lymphocytes. Activation by antigen presenting cells and cytokines leads to cytokine release by T cells in a cell-mediated response, or antibody release by B cells in a humoral response [7]. Although Medawar originally hypothesised that pregnancy represents a time of immune suppression [8], a more complex picture has recently emerged where a change in the ratio and function—rather than a complete suppression—of the maternal leukocytes occurs during pregnancy. For example, there is an increase in the total peripheral white cell count from the early stages of pregnancy with no change in the CD4 and CD8 counts [9]. Within the CD4 positive population, an increase in T regulatory cells is seen in pregnancy [10]. The function of the T cells adapts in pregnancy to favour the T helper 2 cytokine profile, which is more pronounced at the maternal fetal interface [11]. Nonimmune cells, for example, placental trophoblasts also contribute to the Th2 cytokine predominance in pregnancy [12].

The innate immune system provides a less specific response nevertheless is critical for the prevention of microbial invasion. Cellular components include neutrophils, monocytes, and macrophages, which protect against pathogens by phagocytosis. The Toll-like receptors (TLRs) TLR2 and TLR4 are pattern recognition receptors stimulated by Gram-positive and Gram-negative bacteria, respectively [1]. TLRs are expressed on nonimmune cells in the placenta and fetal membranes, which mediate part of the innate immune system at the maternal fetal interface [13]. TLR2 and 4 mutations are associated with an increased risk of preterm birth [14, 15]. During pregnancy, there is tight regulation and considerable cross-talk between the adaptive and the innate adaptive immune system that is responsible for preventing or activating rejection of the conceptus.

3. Th1:Th2 Cytokines

T helper 1 and 2 cell subsets originate from undifferentiated Th0 cells under the influence of interferon-gamma (IFN-γ) and interleukin-4 (IL-4), respectively. Pregnancy hormones such as progesterone [16], leukaemic inhibitory factor [17], estradiol [18], and prostaglandin D_2 (PGD_2) [19] promote the T helper 2 cell profile and are likely to be in part responsible for the Th2 bias associated with pregnancy.

Type 1 $CD4^+$ T cells (Th1) produce an array of inflammatory cytokines including IFN-γ [20], IL-2 [21], and Tumor necrosis factor-alpha (TNF-α) [22] and are the major effectors of phagocyte-mediated host defence, protective against intracellular pathogens [21, 23, 24]. Type 2 $CD4^+$ T cells (Th2) produce IL-4, IL-5, IL-13, IL-10 [20], and IL-6. Whilst IL-4 and IL-10 are considered to be anti-inflammatory cytokines [25], IL-6 has proinflammatory properties [26]. Although IL-10 and IL-6 are frequently referred to as Th2 cytokines [27–32], they are both produced by other cell types including Th1 cells, macrophages, and B cells for IL-10 [33, 34], and macrophages, fibroblasts, and B

cells for IL-6 [35]. The T helper 2 cytokines are commonly associated with strong antibody responses [36], for example, IL-4 stimulates IgE and IgG_1 antibody production [37]. However, the Th2 cytokines also serve other functions, for example; IL-5 promotes the growth and differentiation of eosinophils, whereas IL-13 and IL-10 inhibit the activity of macrophages [37]. T helper 2 cell responses are also associated with protection against parasites, since IL-4 mediates IgE production, and IL-5 mediates an eosinophilia, both of which are hallmarks of parasitic infection [38]. It is important to note that, although the Th1 and Th2 responses can be seen as discrete responses, there is considerable cross-talk and overlap between the functions of the T helper cells. For example, the Th1 cytokines can promote the production of complement-fixing antibodies involved in antibody-dependent cell cytotoxicity [39], and thus the dichotomy described may be an oversimplified representation of the complex immune system. Transcriptional regulation of the predominant Th2 cytokine IL-4 is by STAT-6, c-maf, GATA-3, and NFAT [40], whereas Th1 cell cytokine production is transcriptionally regulated by T-bet and STAT-4 [11].

4. Th1:Th2 and Pregnancy Maintenance

The hypothesis of Th2 predominance and downregulation of the Th1 response originated from Wegmann and colleagues [41] and was reinforced by evidence from both murine studies and the clinical course of Th2 and Th1 based conditions in pregnancy. IL-2, IFN-γ, and TNF-α induce miscarriage in mice, which can be reversed by inhibitors of the Th1 cytokines or by administering the anti-inflammatory Th2 cytokine IL-10 [42, 43]. Autoimmune conditions where Th1 is involved in the pathophysiology generally improve in pregnancy (e.g., rheumatoid arthritis [44]), whereas the Th2 autoimmune spectrum tends to worsen (e.g., systemic lupus erythematosus [45]). With a Th2:Th1 bias, the diminished cell-mediated immunity may be responsible for the increased susceptibility in pregnancy of conditions caused by intracellular pathogenesis (e.g., influenza, leprosy, and *Listeria monocytogenes* [46]).

4.1. Peripheral Blood. Several techniques are available to establish the function of Th1 and Th2 cells in pregnancy; enzyme-linked immunosorbent assay (ELISA) can be used to measure maternal serum interleukins; peripheral T cells can be isolated and stimulated with a mitogen such as phorbol myristate acetate (PMA) or phytohaemagglutinin (PHA) to measure the cytokine production either by ELISA or flow cytometry during pregnancy compared with nonpregnant controls.

Marzi and colleagues isolated PBMCs, stimulated them with PHA, and measured interleukin secretion by ELISA showing a reduction in IFN-γ and IL-2 and an increase in IL-4 and IL-10 in pregnancy compared with nonpregnant controls [47]. In support of this study, Reinhard et al. stimulated cells with PMA and demonstrated by flow cytometry a reduction in intracellular IFN-γ and IL-2, and an increase in intracellular IL-4 production in pregnancy compared with nonpregnant controls [48]. *In vivo* confirmation of this bias has since been demonstrated by polymerase chain reaction (PCR) reflecting decreasing messenger ribonucleic acid (mRNA) of IFN-γ through pregnancy and a concurrent increase in IL-4 mRNA which peaks in the 7th month compared with nonpregnant controls [49]. However, not all studies support the Th1 to Th2 bias. Shimaoka et al. reported a reduction in PMA-stimulated IL-4 during pregnancy [50], while Matthiesen and colleagues presented data suggesting an increase in both IL-4 and IFN-γ secreting cells in pregnancy compared with nonpregnant controls [51, 52]. Such discrepancies may be due to characterisation of cytokine profiles in either isolated cell populations or whole blood, the latter arguably being a more biologically relevant system.

4.2. Maternal Fetal Interface and Nonimmune Cells. While much research has been dedicated toward circulating cytokines in pregnancy, local cytokine production at the maternal interface may be of greater significance than measurements obtained in the peripheral blood [23]. IL-4, IL-10, and macrophage colony-stimulating factor (m-CSF) production by T cells at the maternal fetal interface is associated with successful pregnancy [23]. Trophoblast, decidua, and amnion all contribute to the Th2 cytokine environment by production of IL-13 [53], IL-10 [54], IL-4 and IL-6 [55, 56]. Coculture of trophoblasts and T cells results in an increase in the transcription factors GATA-3 and STAT-6 (which regulate Th2 cytokine production), and a reduction in the Th1 transcription factor STAT-4 and subsequently decreased production of IFN-γ and TNF-α [57]. The placenta also synthesises PGD_2, which may act as a chemoattractant of Th2 cells to the maternal fetal interface via the classic Th2 receptor CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) [28]. $CRTH2^+$ cells are reduced at the maternal fetal interface of women suffering from recurrent loss compared with women undergoing elective termination [58].

Local production of IL-4 and IL-10 inhibits the function of both Th1 cells and macrophages, which serves to prevent fetal allograft rejection [59]. Other anti-inflammatory effects of these interleukins result in inhibition of the Th1 cytokine TNF-α [60], and TNF-α-induced cyclo-oxygenase-2 (COX-2), and/or PGE_2 synthesis in amnion-derived wish cells. Similar effects are observed in decidual and placental cells *in vitro* [61–64], which is thought to inhibit the onset of labour. Consistent with such a role, decidual CD4 positive cells from women undergoing unexplained recurrent pregnancy loss typically exhibit reduced IL-4 and IL-10 production [65].

5. Th1:Th2 Cytokines in Labour

5.1. Peripheral Blood. The Th2 cytokine predominance which exists during pregnancy has been shown to return to nonpregnant Th1:Th2 ratios by 4 weeks postpartum [66]. Labour is often seen as a proinflammatory state marking the end of the pregnancy, and thus it is plausible that labour is associated with a reversal in the bias back towards Th1 rather than Th2. Rather, Kuwajima and colleagues

have shown that the Th2:Th1 ratio remained constant in favour of Th2 through pregnancy and labour, with a reversal back to nonpregnant parameters at 7 days postpartum [67]. However, this finding is somewhat contrary to an earlier report indicating that serum IL-4 levels measured by ELISA in women through pregnancy and at different stages of labour were reduced in the later part of labour and by day 1 postpartum in both normotensive and preeclamptic women [68]. In this study, the Th1 proinflammatory cytokine TNF-α peaked in early labour consistent with labour being a proinflammatory state. Consistent with this, an increase in IFN-γ and IL-1β in women in active labour has also been reported [69].

5.2. Maternal Fetal Interface and Nonimmune Cells. There is substantial evidence that the Th1 cytokines play a role in the initiation of labour at term [22]. The importance of local rather than peripheral production of the cytokines is highlighted by their direct input into the biochemical pathways involved in parturition. Fetal membranes [70, 71] and myometrium [72] produce IL-1β at term, a potent inducer of NF-κB [73]. This transcription factor regulates the expression of numerous labour-associated genes including COX-2, the oxytocin receptor, IL-8, and matrix metalloproteinase-9 (MMP-9) [74]. TNF-α and IL-1β are both increased in amnion, amniotic fluid, and decidua at term [75] and can induce PGE_2 production in amniocytes and decidual cells *in vitro* [76, 77]. Despite the proinflammatory nature of the Th1 cytokines they are required for successful pregnancy contributing to the physiology of term labour.

6. Th1:Th2 Cytokines in Infection

Activation of the Th1 cytokines occurs as a specific response to infection caused by intracellular bacteria, parasites, and viruses [78]. The necessary proinflammatory type 1 response elicited by infection, along with the action of the activated T cells, drives local and systemic cytokine production that, if left unchecked, can be harmful to the host [78]. In some situations, the Th1 response is balanced by the production of Th2 cytokines, particularly IL-4 and IL-10 [79–82]. In the early stages of infection, IL-12 is produced by macrophages and dendritic cells [83, 84], which lead to polarisation from Th0 to Th1 type cells [24]. IFN-γ enhances Th1 development by upregulating the IL-12 receptor and inhibiting the growth of Th2 cells [85]. IFN-γ also primes macrophages to begin phagocytosis and to stimulate the release of interleukin-1 [86].

While the Th1 cytokine response may be suppressed by both the maternal and fetal immune system during pregnancy [87], it still maintains the capacity to mount a defensive response in the context of infection. For example, cord blood mononuclear cells cultured with lipopolysaccharide (LPS) *in vitro* show an increased production of IFN-γ concurrent with reduced IL-4 secretion [88]. Similarly, neonates exposed to intrauterine infection have an increased percentage of IFN-γ-producing cells, with some neonates also showing an increase in IL-4-producing cells [89]. In response to LPS amnion, chorion, deciduas, and placenta also release proinflammatory cytokines [64, 90, 91].

7. Th1:Th2 Cytokines in Preterm Labour

Approximately, 30% of preterm births are associated with infection [92], with a higher rate of 80–85% in early preterm birth (<28 weeks) [93]. Immune and nonimmune cells contribute to a cytokine-rich environment in the presence of infection and inflammation. Proinflammatory cytokines such as TNF-α and IL-1β ultimately result in the production of prostaglandins and MMPs [86], via NF-κB. This triggers a cascade of prolabour events including uterine contractility and fetal membrane rupture, and if this cascade is activated early in pregnancy, preterm labour can ensue.

7.1. Peripheral Blood. As discussed above, the peripheral response may not be as potent as the local Th1:Th2 response and may instead reflect a more significant inflammatory response at the fetal placental compartment. A large case control study of 101,042 Danish women showed that an elevated mid pregnancy IFN-γ plasma level was associated with moderate and late spontaneous preterm delivery, whereas no increased risk was seen with elevated TNF-α or IL-2 [94]. However, a study comparing women in active preterm labour and no labour looked at mitogen-stimulated production of IFN-γ and the Th2 cytokines IL-4, IL-10, and IL-13 and showed no difference in median cytokine production in the supernatant *in vitro* [86]. The differing results between these studies could be explained by the fact that the *in vitro* cells lack the presence of other cells of the immune system and thus lack the ability to reflect the complexity of the immune system as a whole. This same study did however show a higher IL-12 and lower IL-4 in cervical secretions of women in preterm labour, reflecting the localised Th1:Th2 dichotomy. Bahar and colleagues did not demonstrate any difference in serum TNF-α or IFN-γ in women with preterm labour compared to term labour or matched controls not in labour [95]. However, those women in the preterm labour group received indomethacin, an anti-inflammatory COX-2 inhibitor, which could have dampened a typical proinflammatory response. Serum taken from women with preterm prelabour rupture of membranes (pPROM) compared to women who delivered at term exhibit a higher concentration of IFN-γ. Levels of IL-4 and IL-5 were undetectable in both groups [96]. In a study of 30 women in preterm labour, mitogen- and antigen-stimulated PBMCs showed a higher production of the proinflammatory cytokines IFN-γ and IL-2, along with an altered Th1:Th2 ratio favouring a Th1 response compared with controls who delivered at term [97]. Taken together, these results suggest that, rather than a decrease in the Th2 response, preterm labour most likely represents an activation of the Th1 response. Thus, future development of therapeutic targets would likely be more effective if directed towards the modulation of the Th1 cytokines.

The Th1:Th2 dichotomy likely represents an oversimplification of the complexity of the cross-talk between the

Th1 and Th2 cytokines. The ratio of Th1:Th2 is likely to be of more physiological importance than the actual concentrations produced. In support of such a notion, women in threatened preterm labour with high serum levels of IL-12 (which induces a Th1 cytokine response) and no change in serum IL-18 (which can induce both Th1 and Th2 response) do not show significant associations with preterm labour. However, women with high IL-12 levels and low IL-18 and thus a high IL-12 : IL-18 ratio increasing the Th1 predominance are associated with a twofold risk of preterm labour when presenting with threatened preterm labour [98].

7.2. Maternal Fetal Interface and Nonimmune Cells. The inflammatory response at the maternal fetal interface more likely reflects the true importance of the Th1:Th2 dichotomy and the aberrant profile in preterm labour. A recent meta-analysis concluded that proinflammatory cytokines at the maternal fetal interface play a role in the events leading to spontaneous preterm labour, while systemic inflammation does not appear to be present in asymptomatic women early on in pregnancy who then go on to deliver preterm [99]. This is consistent with a more local intrauterine inflammatory response syndrome, where no organisms are identified. Understanding the pathophysiology at the maternal interface is essential for developing new therapies for the prevention of inflammation-induced preterm labour, although using such local changes for the prediction is challenging because of lack of access to the maternal fetal interface.

Placentas from women with pPROM and preterm delivery have higher Th1, inducing cytokines [100], and placentas from women following preterm delivery compared with term delivery show a bias towards the Th1 profile with significantly higher levels of IFN-γ and IL-2 as well as the Th1-inducing cytokine IL-12 [100]. Moreover, term placentas exhibit comparatively higher levels of the Th2 cytokines, IL-4, and IL-10, compared with the preterm placentas.

TNF-α is increased in choriodecidual tissues [71] and amniotic fluid [101] in preterm labour. TNF-α is known to stimulate PG production through the TNF receptor 2, leading to uterine contractions likely via activation of NF-κB, but is also likely to contribute to MMP-9 production leading to PROM via activation of its receptor TNF Receptor 1 (TNFR1) [102]. Interestingly, samples of myometrium collected women in preterm labour and samples collected preterm before labour express comparable mRNA levels of TNF-α. However, mRNA levels of the receptors, TNF R1 A and B, are increased in preterm labour and term labour compared with nonlabour controls [103] suggesting a receptor-mediated increase in sensitivity to TNF-α.

Although placental, amnion, and choriodecidual cells secrete proinflammatory cytokines, cytokine levels in tissues from preterm deliveries (with and without intrauterine infection) correlate with the extent of leukocyte infiltration in fetal membranes [75]. In the presence of infection, the primary cellular source of cytokine production in fetal membranes is likely to be infiltrating leukocytes rather than amniocytes or choriodecidual cells. [75].

7.3. Polymorphisms of the Th1 and Th2 Cytokines. Studying genetic polymorphisms of the Th1 and Th2 cytokines could provide a novel screening method for determining women at high risk of preterm labour. Polymorphisms giving rise to functional alterations can also provide information on the importance of the interleukins in preterm labour. There has yet to be any promising genetic polymorphisms identified in the Th1:Th2 cytokines for the prediction of preterm labour, the work conducted warrants consideration (see Table 1).

8. Non-Th1:Th2 Interleukins

8.1. IL-8. Interleukin 8 is a chemokine produced by many immune cells but primarily macrophages and monocytes [116]. Its production is stimulated by LPS, TNF, and IL-1 [117] and, in the context of pregnancy, is thought to attract leukocytes to the gestational tissues and the cervix at the onset of term and preterm labour. IL-8 mRNA expression has been reported to be increased more than 50-fold in preterm labour and more than 1000-fold in preterm labour with evidence of chorioamnionitis in amnion and choriodecidua [118]. A number of studies have also identified increases of IL-8 in the myometrium and cervix with the onset of labour [119, 120]. Placental IL-8 is also higher in preterm deliveries compared with term deliveries [71].

8.2. IL-6. Although IL-6 is produced by Th2 cells, it is a proinflammatory cytokine and a major mediator of host response to inflammation and infection [121]. IL-6 levels are moderately increased in placenta, significantly increased in amnion and choriodecidua in women with preterm delivery compared with term delivery [71]. IL-6 appears to be among the most sensitive and specific indicators of infection-associated preterm labour [122, 123]. The presence of an increase in IL-6 in amniotic fluid and cervicovaginal fluid is an independent risk factor for preterm labour and neonatal morbidity [124] including cerebral palsy [125] and bronchopulmonary dysplasia [126].

9. Therapeutic Modulation of Th1 and Th2 Profile

Various therapeutic strategies have been proposed to prevent preterm labour, with the primary objectives of (1) delaying delivery to increase gestation at delivery and (2) to improve neonatal condition at birth [127]. Currently, many of the strategies adopted for the prevention of preterm labour involve targeting the proposed pathways and events that result in uterine contractions and cervical shortening and dilation rather than targeting immune activation. As described here, an aberrant proinflammatory profile exists in both term and preterm labour, which is associated with neonatal morbidity. The limitation of tocolytics is the inability to counteract the exposure of the fetus to proinflammatory cytokines, which lead to the fetal inflammatory response syndrome. This may in fact worsen neonatal outcome by prolonging the exposure of the fetus to a hostile

Table 1: Cytokine polymorphism associations with preterm labour (PTL).

Gene	Polymorphism	Th1/Th2	Function	Reference
IFN-γ	+874A>T	Th1	Classic Th1 cytokine. Proinflammatory. No clear association between IFN-γ polymorphisms and PTL	[104, 105]
TNF-α	−308G>A	Th1	Regulatory role in PG synthesis elevated at maternal fetal interface controversial link between PTL and TNF-α polymorphisms	Refute association [104, 106, 107] Support association [108, 109]
IL-4	−590	Th2	Classic Th2 cytokine. The IL-4 590 C/C genotype is associated with preterm birth but unclear. IL-4-590 SNP has been associated with both low and high IL-4 expression. Link also exists between IL-4 promoter polymorphisms and preterm birth in multiple pregnancies; however, polymorphism actually associated with increased IL-4	[110, 111]
IL-10	−1082G>A −819C>T −592C>A	Th2	Anti-inflammatory Th2 cytokine inhibits production of cytokines, chemokines, and prostaglandins in LPS stimulated amnion, choriodecidual, and placental explants [112–114]. However, no clear association between IL-10 polymorphisms and PTL or adverse neonatal outcome	[104–106, 115].

environment. There is mounting evidence that periventricular leukomalacia and cerebral palsy are associated with fetal exposure to intra-amniotic inflammation and the development of fetal inflammatory response syndrome [128]. Thus, a strategy for targeting immune activation through the modulation of the Th1:Th2 bias may be beneficial for both the prevention of preterm labour as well as the reduction of neurological insult to the fetus.

9.1. Progesterone. There have been several studies indicating a positive response to progesterone treatment for the prevention of preterm labour in specific patient populations [129–131]. The strongest evidence for improvement in neonatal outcomes comes from the most recent multicentre randomised controlled trial which showed a 45% reduction in preterm labour (<33 weeks) and a 60% reduction in respiratory distress syndrome at <33 weeks using 90 mg of vaginal progesterone in women with a short cervix of 10–20 mm [132]. The mechanism by which progesterone contributes to pregnancy maintenance has traditionally been attributed to maintenance of uterine quiescence by increasing cyclic AMP (cAMP) and a reduction in intracellular calcium thus reducing contractility [133]. Moreover, progesterone appears to inhibit the phosphorylation of myosin, a critical step in the activation of the myometrial contractile machinery required for labour onset [134, 135].

Progesterone also has immunomodulatory effects on the Th1:Th2 bias. Progesterone is able to suppress Th1 differentiation and enhance Th2 differentiation in peripheral blood mononuclear cells *in vitro* [136]. A more potent and orally bioavailable progestogen, dydrogesterone (6-dehydro-9β,10α-progesterone) upregulates IL-4 and downregulates IFN-γ in PHA-stimulated PBMCs more significantly than progesterone *in vitro* [137]. There is also *in vivo* evidence of an anti-inflammatory effect of prolonged administration of vaginal progesterone. In a study of pregnant women receiving either progesterone or placebo from 24 to 34 weeks, peripheral blood leukocytes were collected before and after treatment [138]. mRNAs of the proinflammatory cytokines IL-1β and IL-8 were reduced with progesterone treatment, whereas the anti-inflammatory IL-10 was increased. A multicentre placebo controlled trial (OPPTIMUM, https://www.opptimum.org.uk/: ISRCTN 14568373) powered on neonatal outcome will provide us with evidence of any potential beneficial effect of vaginal progesterone on neonates born preterm.

9.2. NF-κB Inhibitors. Inhibition of NF-κB activation is another attractive strategy to prevent preterm labour as NF-κB activation is central to the activation of labour-associated genes in labour [139]. NF-κB activation also leads to a proinflammatory response in various cytokines including IFN-γ [140], IL-1β [74], TNF-α, and IL-8 [141]. *Ex vivo* studies with the anti-inflammatory sulfasalazine suppress LPS-induced IL-6 and TNF-α production in fetal membranes via inhibition of translocation of p65 to the nucleus [142]. The reported clinical safety profile of sulfasalazine has been variable [143–145], however, if used in pregnancy is often supplemented with folate. The anti-inflammatory characteristics of the cyclopentenone PG, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15dPGJ$_2$) appears to be derived from its ability to inhibit NF-κB activation in human amnion and myometrial cell culture [146]. We have also shown that 15dPGJ$_2$ inhibits activation of NF-κB in human peripheral blood mononuclear cells and reduces the percentage of cells producing the proinflammatory cytokines, IFN-γ and TNF-α, [147]. Work conducted in our laboratory has also shown that 15dPGJ$_2$ is able to delay labour and provide

neuroprotection by reducing pup mortality from 75% to 5% in a murine model of inflammation induced preterm labour [148].

10. Conclusion

There has been extensive interest in the Th1:Th2 dichotomy for the maintenance of successful pregnancy. A trend towards the Th2 cytokine profile and a suppression of the Th1 cytokine profile appears to exist both in the peripheral blood but more significantly at the maternal fetal interface. Activation of the proinflammatory Th1 profile—rather than suppression of the Th2 profile—is apparent in preterm labour and thus should be considered as the logical target for immunomodulating therapies for the prevention of preterm labour and improving neonatal outcome.

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Research Article

Changes in the Th1 : Th2 Cytokine Bias in Pregnancy and the Effects of the Anti-Inflammatory Cyclopentenone Prostaglandin 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2

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Pregnancy is a complex immunological state in which a bias towards T helper 2 (Th2) protects the fetus. Evidence suggests that proinflammatory cytokines increase the risk of poor neonatal outcome, independently of the direct effect of preterm labour. The anti-inflammatory prostaglandin 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 (15dPGJ$_2$) inhibits nuclear factor Kappa B (NF-κB) in amniocytes and myocytes *in vitro* and is a ligand for the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) receptor. Here we examine the Th1:Th2 cytokine bias in pregnancy and whether 15dPGJ$_2$ could be used to inhibit the production of the proinflammatory cytokines through inhibition of NF-κB while simultaneously promoting Th2 interleukin 4 (IL-4) synthesis via CRTH2 in T helper cells. Peripheral blood mononuclear cells (PBMCs) from women at 28 weeks, term pre-labour, term labour as well as non-pregnant female controls were cultured with 15dPGJ$_2$ or vehicle control and stimulated with phorbol myristyl acetate (PMA)/ionomycin. The percentage of CD4$^+$ cells producing interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) in response to PMA/ionomycin was significantly reduced in pregnancy. 15dPGJ$_2$ reduced IFN-γ and TNF-α production in stimulated T helper cells, but did not alter IL-4 production in CRTH2^{+ve} cells. 15dPGJ$_2$ also reduced phospho-p65 in stimulated PBMCs. In summary, 15dPGJ$_2$ suppresses the Th1 response of PBMCs during pregnancy and active labour whilst maintaining the Th2 response suggesting a therapeutic benefit in reducing neonatal morbidity in inflammation-induced PTL.

1. Introduction

Preterm labour <34 weeks occurs in about 4% of pregnancies [1]. In 80–85% of cases of spontaneous preterm labour (PTL) <28 weeks, there is evidence of intrauterine infection [2]. Despite increased awareness of the association between inflammation and preterm labour [3], there have been no major advances in the prevention of preterm labour which have been shown to improve long-term neonatal outcomes. During normal term labour, the uterus and cervix become infiltrated with leukocytes and undergo changes in response to local secretion of proinflammatory cytokines. A similar pattern of biochemical events occur during PTL; however, the triggers for this premature activation are still not fully understood. Regardless, the presence of increased proinflammatory cytokines during pregnancy is clearly associated with a poor prognosis for babies born preterm [4].

The immune system can generally be divided into the innate and adaptive immune system. The former is a nonspecific system providing immediate defence against pathogens, while the latter is more targeted, characterised by T and B lymphocytes. Although cross-talk between these lymphocytes exist, B cells and their antibodies mainly give rise to humoral immunity, whereas T cells primarily provide cell mediated immunity [5]. T helper cells (CD4$^+$) form a subset of T cells and can be further subdivided into T helper 1 cells (Th1) and T helper 2 cells (Th2) depending on their pattern of cytokine production. Th1 cells secrete

pro inflammatory cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), whereas the Th2 cells secrete anti-inflammatory cytokines such as interleukin 4 (IL-4), IL-10, and IL-13 [6]. A mutually exclusive interaction exists between the Th1 interleukin, IFN-γ, and the Th2 interleukin, IL-4. IL-4 is the dominant factor for promoting growth and differentiation from the Th0 to the Th2 subtype, and directly inhibits the development of the Th1 cells [7]. IFN-γ indirectly promotes Th1 differentiation by upregulating the IL-12 receptor whilst inhibiting the growth of Th2 cells [8, 9].

Wegmann and colleagues first developed the concept that during pregnancy there is a shift from a T helper 1 (Th1) response to a T helper 2 (Th2) bias during pregnancy that functionally induces maternal tolerance and suppression [10]. Consistent with this notion, administration of the Th1 interleukins IFN-γ [11] and IL-2 [12] leads to fetal loss and preterm labour in the mouse. Similarly, CBA × DBA/2 mice that have placentas deficient in IL-4 and IL-10 are prone to fetal resorption. Treatment of these mice via intraperitoneal injection of IL-10 protects the fetuses from resorption [13]. Several human studies have shown a Th2 bias in the ratio of circulating T helper cytokine profile in normal pregnancy; and an increase in the Th1 ratio in cases of recurrent miscarriage [14] and in preeclampsia [15]. Peripheral blood lymphocytes taken from women in the first trimester show an increased production of IL-4 and IL-10 and less IFN-γ compared to nonpregnant controls *in vitro* [16]. There is *in vivo* evidence in pregnant women of increased expression of IL-4 messenger ribonucleic acid (mRNA) and lower expression of the IFN-γ mRNA [17].

Not all studies support the requirement of the shift from Th1 to Th2 for successful pregnancy outcome [18, 19]. Despite showing a suppression of IFN-γ and an increase in IL-10 during pregnancy compared to nonpregnant controls, Bates et al. showed no difference in IFN-γ, IL-10, or IL-4 secretion in women who subsequently miscarried compared with those who went on to complete their pregnancy [18]. However, contrary to this study, women with recurrent miscarriage have been shown to have increased IFN-γ and TNF-α levels compared with women that go on to have successful pregnancies [20]. While the mechanism regulating the Th1 : Th2 ratio is yet to be fully elucidated the importance of maternal immune tolerance during pregnancy is unquestionable. Several pregnancy-related proteins are known to promote Th2 bias such as leukemia inhibitory factor [21], progesterone, progesterone-induced blocking factor [22], and estradiol [23]. Prostaglandin D_2 (PGD_2) promotes IL-4, IL-13, IL-5, and IL-10 production in T helper 2 cells *in vitro* via the second PGD_2 receptor; chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) [24]. PGD_2 is produced by the placenta and may play a role in the chemoattraction of Th2 cells via the CRTH2 receptor to the maternal fetal interface to produce a localised Th2 bias [25].

15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 ($15dPGJ_2$), an endogenous prostaglandin (PG), is a product of PGD_2 via a series of dehydration reactions [26]. As well as being a ligand for Peroxisome proliferator-activated receptor gamma (PPARγ), it is also an agonist of the CRTH2 receptor [27]. We have previously demonstrated that this anti-inflammatory PG inhibits nuclear factor Kappa B (NF-κB) in amniocytes and myocytes *in vitro* [28] and delays infection induced preterm labour and increases pup survival in the mouse via NF-κB inhibition [29]. Moreover, recent work has shown that the first trimester of pregnancy is associated with the suppression of available T cell NF-κB and reduced levels of IFN-γ compared to nonpregnant controls [30, 31]. Collectively these data suggest that the Th1 : Th2 ratio is modulated through the regulatory interplay of both Th1 suppression and Th2 promotion.

In this study, we examined the expression of the dominant Th1 interleukins IFN-γ and TNF-α, and the Th2 interleukin IL-4 in T helper cells obtained from nonpregnant women, women in the early and late third trimester, and during labour. We examined the effect of the CRTH2 agonist, $15dPGJ_2$, on these interleukins and on NF-κB activation at different gestational time points to determine its potential role in suppressing the Th1 response via NF-κB whilst simultaneously promoting the Th2 response via CRTH2.

2. Materials and Methods

2.1. Subjects. Pregnant women at 28 weeks and term (prelabour and in labour) and nonpregnant women of child bearing age were included in the study in accordance with ethical approval from the South East London Ethical Committee Ref. 10/H0805/54. Exclusion criteria included women with Asthma and HIV, women not of childbearing age, and women with a fever. After obtaining informed consent, 5 mL of peripheral venous blood was collected in sodium citrate tubes, and processed within 30 mins of collection. Unless otherwise stated, a minimum of 4 subjects were included for each experimental sample group.

2.2. Isolation of Peripheral Blood Mononuclear Cells (PBMCs). Blood was diluted 1 : 1 with phosphate-buffered saline (PBS) and carefully layered onto Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) before centrifuging at 400 ×g for 40 mins at room temperature. After centrifugation, the halo containing PBMCs was carefully transferred into a clean centrifuge tube and washed twice with 7 mL of PBS. After centrifugation (400 ×g for 10 mins), the cell pellet was resuspended in either PBS or RPMI 1640 (Invitrogen Life Technologies, Grand Island, NY, USA) culture medium.

2.3. CRTH2 Expression Studies. The PBMC pellet was resuspended PBS, and cells were counted with a Neubauer haemocytometer and then resuspended in staining buffer (1% fetal calf serum; 0.09% sodium azide in PBS) to obtain roughly 3.5×10^6 cells per sample. Preparations were incubated in the dark for 1 h at 37°C with 20 μL of CRTH2-phycoerythrin (CRTH2-PE) (Beckman Coulter, High Wycombe, UK) and 3 μL of CD4-Allophycocyanin (CD4-APC) (BD Pharminogen, Oxford, UK) or the relevant isotype controls; Rat Immunoglobulin (Ig)G_{2a}-PE (Beckman Coulter) and Mouse $IgG_{1\kappa}$-APC (BD Pharminogen). After incubation, the PBMC suspension was washed twice in

1 mL of PBS and then resuspended in PBS for analysis. Flow cytometry settings were as follows: forward scatter *E0* voltage, 1.00 Amp gain Lin, and side scatter of 329 voltage, 1.00 Amp gain Lin. A total of 50,000 cells were counted and gating was on lymphocytes based on the forward and side scatter.

2.4. Intracellular Cytokine Studies (IL-4, IFN-γ, and TNF-α). For intracellular cytokine studies, cells were resuspended in RPMI 1640 (Invitrogen) media supplemented with 10% fetal calf serum, 2 mM/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL of streptomycin (Sigma, St. Louis, MO, USA) before being plated in 24-well plates and incubated for 18 h in 5% CO_2/humidified air at 37°C. Following this, 10 μg of brefeldin A (Sigma) was added to each well to immobilize the interleukins in the golgi apparatus. Cells were pretreated with 32 μM of 15dPGJ$_2$ (Cayman Chemicals, Ann Arbor, MI, USA) or dimethyl sulfoxide (DMSO) (Sigma) vehicle control for 2 h in the case of IFN-γ and TNF-α experiments or 1 h for IL-4. Following this 50 ng/mL of phorbol myristate acetate (PMA) and 0.5 μg/mL of ionomycin were added for 4 h in the case of IFN-γ and TNF-α experiments or 5 h for IL-4. Prior to intracellular staining, cell surface staining with either CRTH2 and CD4, or CD4 antibodies alone, was performed as described in Section 2.3. Cells were then fixed with 2% paraformaldehyde (PFA) and incubated at 37°C for 15 mins in the dark. Cells were washed and resuspended in 0.5% saponin and incubated for 30 mins on ice in the dark to permeabilize the cells. After incubation, cells were washed and resuspended in 0.5% saponin for intracellular staining with the relevant antibody; IL-4 PE/Cy7 (BioLegend, San Diego, CA, USA), IFN-γ-(fluorescein isothiocyanate (FITC) or TNF-α-FITC (BD Biosciences, Franklin Lakes, NJ, USA). Appropriate isotype controls were used; PE/Cy7 Rat IgG$_{1\kappa}$ (Biolegend), or FITC Mouse IgG$_{1\kappa}$ (BD Biosciences, Oxford, UK). Cells were incubated in the dark for 1 h at room temperature for IL-4 staining or 20 mins on ice for IFN-γ and TNF-α staining. Finally, cell suspensions were washed with 0.5% saponin and resuspended in PBS for flow cytometry analysis as follows: Forward scatter *E0* voltage; 1.00 Amp gain Lin; side scatter of 329 Voltage; 1.00 Amp gain Lin.

2.5. Flow Cytometry. Flow cytometry of lymphocytes was carried out using FACSCalibur flow cytometer (BD Biosciences) equipped with FACSCalibur software for analysis. The lymphocyte population was gated on the scatter plot as determined by the characteristic forward scatter (FS) and side scatter (SC) which indicates the cell size and shape. The analysis was of CD4$^+$, CRTH2$^+$, and CRTH2$^+$/CD4$^+$ in the CRTH2 expression studies and IL-4$^+$, IL-4$^+$/CRTH2$^+$, TNF-α^+/CD4$^+$, and IFN-γ^+/CD4$^+$ for the intracellular cytokines. The expression levels of CRTH2, CD4, and intracellular cytokines were evaluated by the percentage of cells expressing the protein of interest or by mean fluorescence intensity (MFI).

2.6. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting. Following culture and treatments, cells were collected using a 1 mL pipette and incubated on ice in whole cell lysis buffer (Cell Signalling, Beverly, MA, USA) with 5 μL/mL of protease inhibitor (Sigma) lysis buffer for 5 mins and centrifuged for 20 mins at 13,000 rpm at 4°C. The supernatant was stored at −80°C until use. Prior to SDS-PAGE, protein concentrations were determined using the BIORAD quantification assay measuring absorbance at 655 nm. Approximately 15 μg of extracted protein per sample was resolved by SDS-PAGE and subsequently transferred onto polyvinyl difluoride (PVDF) membranes (GE Healthcare) 100 V constant at 4°C. Following transfer, the membrane was then blocked in 5% (wt/vol) milk in tris-buffered saline (TBS) supplemented with 0.1% Tween 20 (TBST) for 1 h. The membrane was then probed with phospho-p65 (Ser 536) (Cell signalling) primary antibody (1 : 1000 in tris-buffered saline) overnight at 4°C followed by the appropriate secondary antibody (1 : 2000 in 1% milk/TBS) for 1 h at room temperature. Chemiluminescence detection was then carried out with ECL Plus (GE Healthcare), and the membranes developed using a high-performance chemiluminescence film (GE Healthcare). Blots were scanned, and densitometry was performed with ImageJ (v1.44p).

2.7. Statistical Analysis. Experimental sample groups consisted of at least 4 biological replicates unless otherwise stated. Statistical analysis was performed with Graph-Pad Prism (v4.0; GraphPad Software, San Diego, CA, USA). Paired Student's *t*-test or repeated measures ANOVA was conducted where appropriate. Samples with $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Gestational Effect on Th1 and Th2 Cytokines. A change in the production of the Th1 and Th2 cytokines in pregnancy has previously been described. In this study, we employed flow cytometry to examine the effect of stimulation by the mitogen PMA/ionomycin on cytokine production at different gestational stages of pregnancy and during labour (see Figure 1). Th1 cytokine profiles of CD4 positive cells were assessed for intracellular IFN-γ and TNF-α (Figures 1(a)–1(d)) and compared to levels of nonpregnant controls. The percentage of peripheral T cells producing IFN-γ in response to stimulation reduced in pregnancy from 10.7% in nonpregnant women to 6.7% at 28 weeks, 5.1% at term ($P < 0.05$), and 5.6% at term in labour ($P < 0.05$). Similarly, the proportion of TNF-α producing cells was reduced, although not significantly, from 20.6% in nonpregnant women to 14.5% at 28 weeks, 15.8% at term and 13.3% at term in labour. Overall levels of Th1 cytokine production (expressed as mean fluorescence intensity), in the CD4$^+$/IFN-γ^+ or CD4$^+$/TNF-α^+ cells remained consistent throughout gestation and unchanged compared to nonpregnant controls. The Th2 cytokine, IL-4, was similarly assessed in CRTH2 positive cells (Figures 1(e) and 1(f)). While PMA/ionomycin stimulation did not increase the percentage of IL-4 expressing cells, the mean fluorescence intensity of

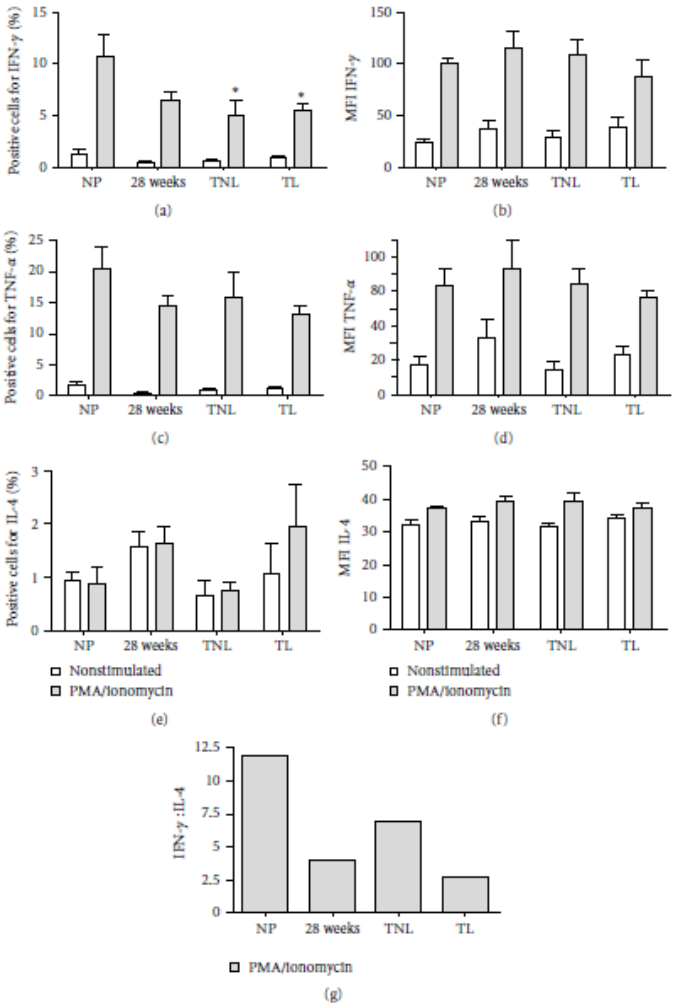


FIGURE 1: Th1 and Th2 cytokine production in peripheral T cells from nonpregnant and pregnant women. Peripheral blood mononuclear cells were isolated and stimulated with PMA/ionomycin. The percentage of $CD4^+/IFN\text{-}\gamma^+$, $CD4^+/TNF\text{-}\alpha^+$, and $CRTH2^+/IL\text{-}4$ positive cells were detected by flow cytometry in samples derived from nonpregnant (NP), 28-week pregnant, term no labour (TNL), and term labour (TL) samples (a), (c), and (e). A reduction in the percentage of peripheral CD4 positive cells secreting the Th1 cytokines IFN-γ and TNF-α was observed, whereas the percentage of cells secreting the Th2 cytokine, IL-4, remained consistent throughout gestation. Mean fluorescence intensity (MFI) was also assessed as a measure of total cytokine production (b), (d), and (f). PMA/ionomycin stimulation induced both Th1 and Th2 cytokine production in all sample groups compared to NL controls. No differences in gestation-dependent responses were detected. (g) a ratio of IFN-γ : IL-4 revealed a decrease in the Th1 : Th2 ratio during pregnancy, which was increased prior to the onset of labour. For statistical analysis ANOVA with Dunnett's multiple comparison test with NP as a control was used; $^{**}P < 0.01$ and $^{*}P < 0.05$.

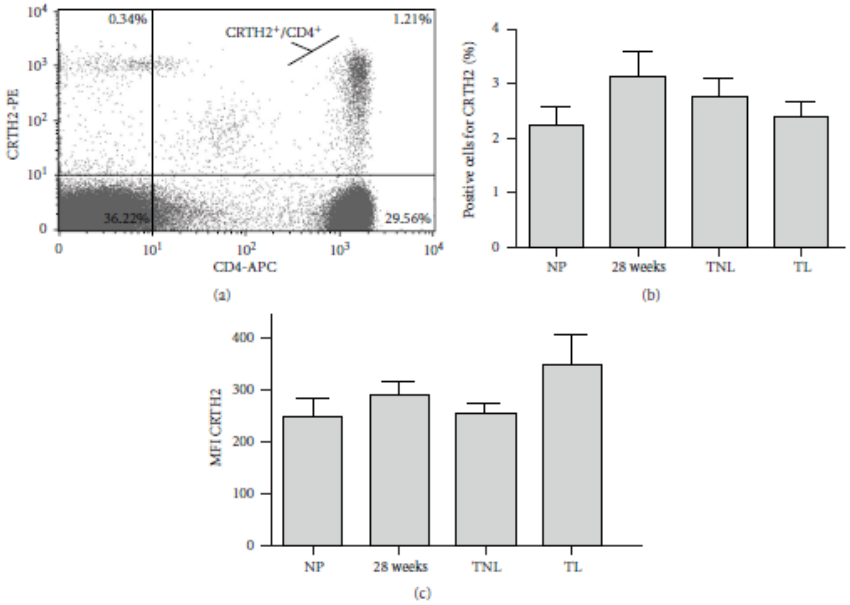


FIGURE 2: CRTH2 expression in lymphocytes derived from nonpregnant and pregnant subjects. Lymphocytes were gated according to forward scatter and side scatter and T helper cells were identified using CD4 as a cell surface marker ($n = 8$ for each group). A representative cytogram from a woman of 28-week gestation is presented with the right upper quadrant showing CRTH2$^+$/CD4$^+$ lymphocytes (a). The percentage of CRTH2 positive T helper cells (presented as a percentage of positive CD4 positive cells) increased slightly in the third trimester, although statistical significance was not reached (b). No change in mean fluorescence intensity was detected in either the nonpregnant or pregnant samples (c). For statistical analysis ANOVA with Dunnett's multiple comparison test with NP as a control was used.

IL-4 was significantly increased in samples collected from women at 28 weeks (39.3, $P < 0.01$) and at term (39.4, $P < 0.05$) compared to levels of nonpregnant controls (37.1). Levels of IL-4 in term labouring samples were consistent with nonlabouring samples (37.1). The ratio of the IFN-γ : IL-4 producing cells reduces during pregnancy, due to the suppression of the Th1 rather than the promotion of the Th2 cytokine production (Figure 1(g)).

3.2. CRTH2 Expression. To determine whether the Th2 response in pregnancy is reflected by an increase in the percentage of CRTH2 positive cells, we used CD4 as a marker of T helper cells and calculated the percentage these cells that express CRTH2 (Figure 2(a)). The percentage of CRTH2 positive cells in the CD4 positive population was observed to increase in the second trimester of pregnancy from 2.24% in nonpregnant women to 3.12% at 28 weeks before reducing to 2.75% at term and 2.4% in term labour, although statistical significance was not reached (Figure 2(b)). There was no significant increase in the mean fluorescence intensity of CRTH2 in T helper cells between nonpregnant and pregnant subjects (Figure 2(c)), although a slight increase in CRTH2 production was noted in term labouring samples ($P - 0.15$).

3.3. The Effect of 15dPGJ$_2$ on the Th1 and Th2 Cytokines. 15dPGJ$_2$ is an anti-inflammatory PG and a ligand for CRTH2. We therefore explored the effect of 15dPGJ$_2$ on the Th1 cytokines, IFN-γ and TNF-α. To do this, cells were preincubated with 32 μM of 15dPGJ$_2$ or vehicle control prior to the addition of PMA/ionomycin before intracellular cytokines were detected by flow cytometry (Figures 3(a) and 3(b)). 15dPGJ$_2$ reduced the production of PMA/ionomycin stimulated IFN-γ production (measured as mean fluorescence intensity) in T helper cells from 101.9 to 53 ($P < 0.01$) in nonpregnant controls, 115.7 to 52.4 ($P < 0.01$) in 28 week samples and from 110 to 60.7 in term ($P < 0.01$) samples. There was a nonsignificant decrease observed in term labouring samples also (88.3 to 66.3, Figure 3(c)). Similarly, a significant decrease in PMA/ionomycin-induced TNF-α production following treatment with 15dPGJ$_2$ was detected in all pregnant samples assessed (Figure 3(d)).

The percentage of T helper cells producing IFN-γ was also reduced from 10.7% to 5.13% in nonpregnant women

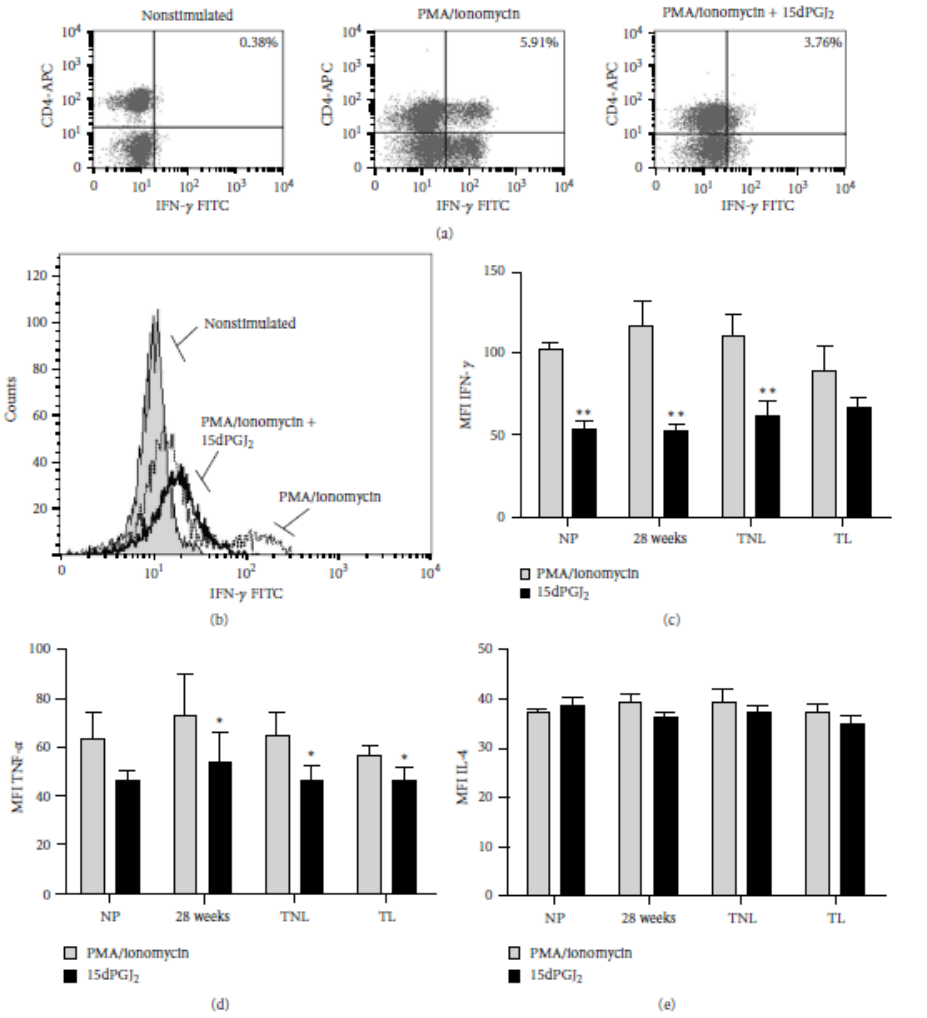


FIGURE 3: Effect of 15dPGJ2 on Th1 and Th2 cytokines. Peripheral blood mononuclear cells were incubated with vehicle control or 32 μM of $15dPGJ_2$ and stimulated with PMA/ionomycin before being gated on the lymphocyte population according to forward and side scatter. A representative 28-week patient sample is presented with $CD4^+/IFN\text{-}\gamma^+$ cells shown in the right upper quadrant (a). A representative histogram reveals a clear shift to the right upon stimulation indicative of increased IFN-γ producing cells (b). This effect was attenuated with $15dPGJ_2$ pre-treatment. PMA/ionomycin induced IFN-γ and TNF-α production was decreased with $15dPGJ_2$ treatment (c) and (d); however, levels of IL-4 remained unchanged. No change in IL-4 producing cells was detected (e). For statistical analysis a Student's t-test was used to compare means between paired treated and nontreated samples; $^{**}P < 0.01$ and $^{*}P < 0.05$.

($P < 0.01$), 6.6% to 3.4% at 28 weeks ($P < 0.01$), 5.1% to 2.5% at term ($P < 0.05$), and 5.6% to 2.9% ($P < 0.01$). A reduction in the percentage of T cells producing TNF-α was also seen with $15dPGJ_2$ from 20.6% to 16.7% in nonpregnant women, 14.5% to 9.0% at 28 weeks ($P < 0.05$), 15.8% to 12.9% at term ($P < 0.01$), and 13.2% to 8.93% at term in labour ($P = 0.05$) (data not shown).

Considering its anti-inflammatory properties, it was hypothesized that $15dPGJ_2$ would induce an increase in IL-4 production via its action on the CRTH2 receptor. Contrary to what was anticipated, $15dPGJ_2$ had no effect on the production of IL-4 (Figure 3(e)). Consistent with this, the percentage of CRTH2+/IL-4+ lymphocytes remained constant following $15dPGJ_2$ treatment except for samples collected at 28 weeks gestation (1.65% to 1.16%, $P < 0.05$; data not shown).

3.4. $15dPGJ_2$ Inhibits PMA/Ionomycin-Stimulated NF-κB Activation in Peripheral Blood Mononuclear Cells. Peripheral blood mononuclear cells were incubated with vehicle control or 32 μM $15dPGJ_2$ and stimulated with PMA/ionomycin for 10 min before being extracted and assessed for levels of phosphorylated p65, the transcriptionally active subunit of NF-κB. PMA/ionomycin treatment stimulated NF-κB phosphorylation in all samples and at all gestational time points (Figure 4). Pretreatment of cells with $15dPGJ_2$ significantly reduced levels of p-p65 in samples collected from women at 28-week gestation. A clear trend of decreased p-p65 following $15dPGJ_2$ was observed in the remaining samples yet they did not return to basal levels of p-p65.

4. Discussion

The immunological paradox of pregnancy relies on a careful balance of both immune tolerance and immune suppression. Work by Medawar, and later Wegmann and colleagues, has led to the proposal that this balance is determined by a functional immune suppression via a shift from a Th1 response to a Th2 bias during pregnancy [10, 20, 32]. Although not all human and murine studies support the importance or the simplification of the Th1 : Th2 paradigm [18, 19, 33], further understanding of this hypothesis to aid the development of new therapeutic strategies is still required. In this study, we have explored the Th1 cytokines IFN-γ and TNF-α, and the Th2 cytokine IL-4 throughout pregnancy, and examined the potential of the cyclopentenone PG CRTH2 agonist, $15dPGJ_2$, to act as a therapeutic agent to modulate the Th1 : Th2 response.

Consistent with the notion of suppressed Th1 cytokine response throughout gestation [16, 17, 34, 35], our results revealed a reduction in the percentage of IFN-γ and TNF-α-secreting T helper cells during pregnancy in response to PMA/ionomycin stimulation (Figures 1(a) and 1(c)). However, mean production of IFN-γ and TNF-α remained constant within the $CD4^+$/IFN-γ^+ and $CD4^+$/TNF-α^+ cell populations (Figures 1(b) and 1(d)). This indicates that while fewer cells maintain the capacity to initiate a Th1 response to inflammatory stimuli with advancing gestation, a compensatory mechanism facilitates increased IFN-γ and TNF-α production. These results suggest that Th1 response during gestation is likely to be regulated at both an individual cellular level as well as at the level of the cell population.

As normal human labour reflects a proinflammatory state, we anticipated that the percentage of IFN-γ and TNF-α producing T helper cells would increase with the onset of labour. However, no difference between term nonlabouring and term labouring samples was detected. This may be due to the proinflammatory state of labour being a localized response in the uterus and fetal membranes, rather than an extending to peripheral inflammation. This would be consistent with the localised increase in the DNA binding activity of NF-κB in amnion cells postlabour compared to prelabour [36] and in myometrium during labour compared to pre-labour [37] as well as the reported increase in labour of IFN-γ in amnion, choriodecidua, and placenta [38], and TNF-α in the amniotic fluid, myometrium, and cervix [39, 40] rather than in the peripheral blood.

A number of studies have shown that mitogen stimulation can induce IL-4 production in PBMCs of the second and third trimesters of pregnancy *in vitro* [16, 35, 41]. Intrinsic IL-4 production by PBMCs also shows an increase in pregnancy compared with nonpregnant controls [17, 42]. We therefore hypothesized that PMA/ionomycin stimulation of PMBCs would drive IL-4 production during pregnancy, and that this phenomenon would subside at term concurrent with the activation of inflammatory pathways involved in normal labour pathways. To examine any evidence of a shift towards the Th2 profile in peripheral blood during pregnancy, PBMCs were gated for CRTH2 (a marker for Th2 cells) and the level of IL-4 assessed (Figures 1(e) and 1(f)). The percentage of $CRTH2^+$/$IL\text{-}4^+$ secreting cells, as well as mean production levels of IL-4 remained consistent throughout pregnancy and advancing gestation (Figures 1(f) and 1(e)) although a slight increase in the percentage of IL-4 secreting cells was observed in both nonstimulated and stimulated 28-week samples. Between 0.4–6.5% of peripheral blood $CD4^+$ cells of nonpregnant women express CRTH2 and secrete IL-4, but not IFN-γ, in response to PMA and ionomycin and are thus typical of Th2 cells [43]. Consistent with this, we found that between 2–4% of $CD4^+$ PMBCs express CRTH2 (Figure 2(a)). This percentage was maintained throughout gestation with a minor, statistically nonsignificant, increase observed at 28 weeks (Figure 2(b)). Tsuda et al. have previously reported an increase in CRTH2 expression in $CD4^+$ cells between nonpregnant (2.08%) and 7-week gestation (3.43%, $P = 0.02$) [44], but it is possible that this effect is diminished with advancing gestation. Mean levels of CRTH2 expressed in $CD4^+$ cells were also unchanged through gestation and labour (Figure 2(c)). Taken together with the fact that we were unable to detect any difference in IL-4 secretion levels in these cells upon mitogen stimulation, our results collectively suggest that the Th2 response in PMBCs is maximised during pregnancy and cannot be further stimulated. This would imply that any beneficial effects of therapeutic agents intended to push the Th2 : Th1 ratio towards Th2 would need to do so through inhibition of Th1, rather than upregulation of Th2. Although

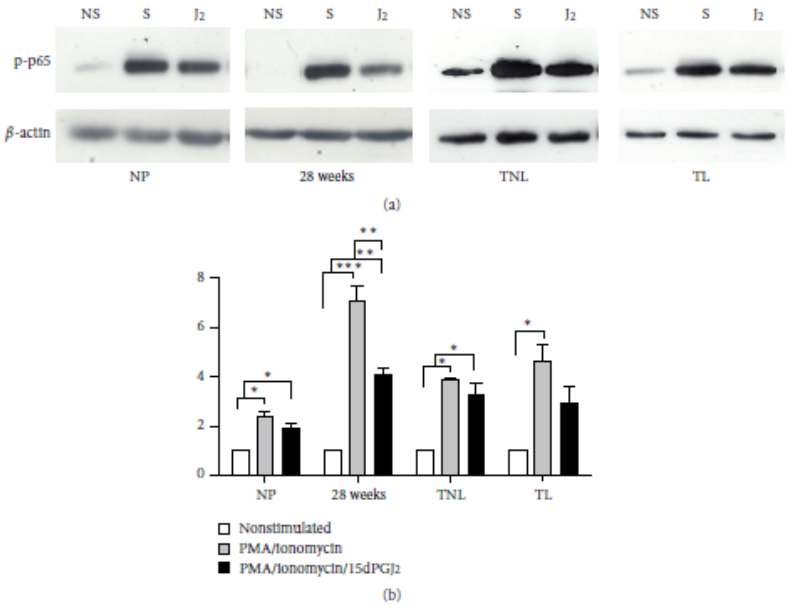


FIGURE 4: The effect of 15dPGJ2 on NF-κB p65 phosphorylation in peripheral blood mononuclear cells. PBMCs were treated as previously described before being extracted and levels of phosphorylated p65 (p-p65) examined using immunoblotting. Representative immunoblots are shown for each gestational time point (a). Immunoblots were reprobed for β-actin as an internal loading control. Densitometric analysis of the immunoblots was conducted revealing a significant increase in p-p65 levels upon stimulation with PMA/ionomycin in all samples (b). A significant decrease in PMA/ionomycin stimulated p-p65 was observed following $15dPGJ_2$ treatment in 28-week samples. The capacity of $15dPGJ_2$ to inhibit PMA/ionomycin-induced p-p65 was lost in those samples collected at term. NS: nonstimulated, S: PMA/ionomycin stimulated, and J_2: $15dPGJ_2$ pretreatment. Effect of treatment was examined for statistical significance at each gestational timepoint using ANOVA with Bonferroni's multiple comparison test; $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$.

the percentage of CRTH2 expressing $CD4^+$ cells is low in the peripheral blood, we are unable to rule out any modest yet biologically significant changes in IL-4 production from these cells at a tissue-specific level.

To further investigate the potential of Th2 promotion versus Th1 suppression, we examined the response of PBMCs to $15dPGJ_2$, an endogenous product of Prostaglandin D_2 (PGD_2) (and potential therapeutic agent) that has been shown to promote Th2 bias through IL-4 production in T helper 2 cells *in vitro* via CRTH2 [24]. PGD_2 is readily converted to $15dPGJ_2$ by a series of dehydration reactions in the presence of albumin and is found in the amniotic fluid [45], placenta [46], and urine [47]. $15dPGJ_2$ pretreatment significantly inhibited the PMA-stimulated production of both Th1 cytokines: IFN-γ and TNF-α, in nonpregnant and all pregnant samples (Figures 3(a)–3(d)). However, no effect on the production of the Th2 cytokine, IL-4, could be detected in response to $15dPGJ_2$ treatment. This may be due to an overriding effect of a non-CRTH2-mediated mechanism such as the inhibition of other signalling proteins involved the transcriptional regulation of IL-4 expression. Evidence for this has been previously reported in a PMA/Ionomycin-activated T-cell line, where $15dPGJ_2$ inhibited IL-4 production in a dose-dependent manner through the downregulation of nuclear factor of activated T-cells (NF-AT) activation [48].

The anti-inflammatory properties of $15dPGJ_2$ are thought to be at least partly via the inhibition of the transcription factor NF-κB [49]. NF-κB is known to play a key regulatory role in controlling the Th1 immune response by modulating the expression of Th1 cytokines [31]. NF-κB activity in PBMCs during pregnancy is reduced compared to nonpregnant controls [50], and it has therefore been suggested that this is responsible for suppressed Th1 response in pregnancy [30]. To determine whether the observed inhibitory effects of $15dPGJ_2$ on Th1 cytokine production may be via NF-κB, treated cells were extracted, and levels of the transcriptionally active NF-κB subunit p-p65 were examined by immunoblotting. Our results indicate that $15dPGJ_2$ significantly inhibits p-p65 in PMA/ionomycin stimulated T helper at 28 weeks of gestation. A nonsignificant reduction was also observed in nonpregnant controls and

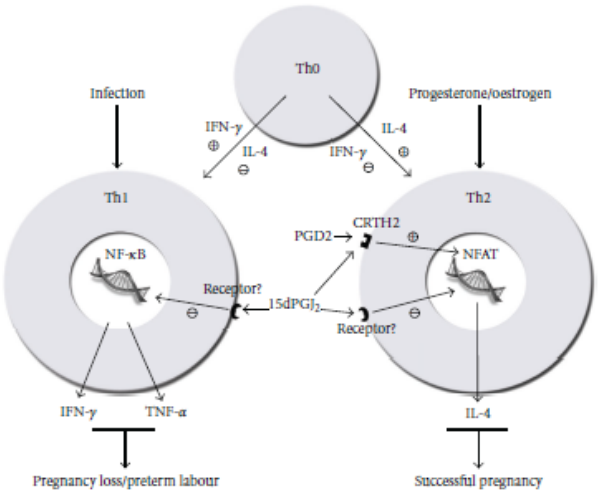


FIGURE 5: Schematic proposal of the Th1 and Th2 balance and the role of CRTH2 and NF-κB. T-helper cell precursors are directed toward committed immunophenotype by the typical Th1 and Th2 interleukins, IFN-γ and IL-4, respectively. Infection or propregnancy hormones such as progesterone can further modulate the Th1/Th2 bias. Based upon our findings and those of others, we propose that $15dPGJ_2$ maintains a Th2 bias principally through the suppression Th1 interleukins through the inhibition of NF-κB.

samples acquired at term. This data provides evidence that the Th1 inhibitory properties of $15dPGJ_2$ are likely regulated through NF-κB.

$15dPGJ_2$ is a cyclopentenone PG which possesses an α,β-unsaturated carbonyl group in the cyclopentenone ring [51]. This group can form Michael adducts with I Kappa B kinase (IKK) [52], and the p65 [53] and p50 subunits leading to modification of these proteins, thus inhibition of NF-κB activity [51]. We have shown that in amnion and myometrial cells, inhibition of NF-κB by $15dPGJ_2$ is not via PPAR-γ [28]. However, $15dPGJ_2$-mediated suppression of Th1 interleukin expression in lymphocytes via PPAR-γ receptor inhibition or activator protein 1 (AP-1) has not been ruled out in this study.

While $15dPGJ_2$ appears to have no effect on the peripheral Th2 response, the demonstrated inhibition of the Th1 response represents a collective shift toward a Th2 bias, or in other words, an increase in the Th2:Th1 ratio. This has important ramifications for the future design of therapeutic strategies to prevent preterm labour and pregnancy loss (Figure 5). The percentage of CRTH2 positive decidual lymphocytes is far higher, accounting for 10.5% of T lymphocytes and 18% of CD4+ lymphocytes [44]. Exploring the effect of $15dPGJ_2$ on purified decidual T cells may give rise both Th1 suppression and Th2 cytokine promotion.

Immunomodulating therapies such as progesterone (for women at high risk of preterm labour) and intravenous Immunoglobulins (for women with recurrent miscarriage) are currently being used in a clinical context. Progesterone suppresses IFN-γ production in peripheral blood mononuclear blood cells cultured with trophoblasts [54] and dydrogesterone suppresses both IFN-γ and TNF-α whilst increasing IL-4 in phytohaemagglutinin (PHA) stimulated PBMCs leading to a substantial Th1 to Th2 shift [55]. Immunoglobulins are capable of altering the Th1 : Th2 balance leading to a reduction in the $CD4^+IFN\text{-}\gamma^+/CD4^+IL\text{-}4^+$ lymphocyte ratio [56]. The endogenous protein, progesterone-induced blocking factor (PIBF), is capable of increasing the production of IL-4 and IL-10 in PBMC, but has no effect on the Th1 cytokines IFN-γ and TNF-α [57].

$15dPGJ_2$ may show more promising immunomodulating effects for the prevention of preterm labour exploiting its ability to both suppress the Th1 cytokines with the added benefit of inhibiting NF-κB activity. Preterm labour, particularly in the presence of chorioamnionitis, is associated with a shift towards the Th1 cytokine response [58]. PBMCs of women in preterm labour when stimulated with PMA produce significantly higher IFN-γ compared to women who go on to deliver at term [59]. The presence of IFN-γ in cervicovaginal fluid in the late second and early third trimesters has also been shown to be a risk factor for preterm labour in asymptomatic women [60]. Similarly, TNF-α is thought to be key cytokine involved in the initial trigger of biochemical events leading to infection-mediated preterm birth, for example, by causing prostaglandin E_2 (PGE_2) synthesis by intrauterine tissues which drive pathways leading to uterine contractility and cervical ripening [61–63]. While global changes in the PBMCs likely reflect important systemic inflammatory events, their sub-components (e.g., T cells, B cells, and monocytes) may play functionally independent

roles at the maternal fetal interface. Future work using an *in vivo* model examining effects of $15dPGJ_2$ and CRTH2 agonists on Th1 and Th2 interleukins at local sites (e.g., decidua and myometrium) would allow us to extrapolate how immune cells influence nonimmune cell function.

Prophylactic administration of $15dPGJ_2$ to women at high risk of preterm birth may lead to the inability of infection to activate NF-κB and thus preventing transcription of labour associated genes, and the inability to alter the Th1 : Th2 bias to favour Th1, thus preventing the pro inflammatory detrimental environment of the fetus. Consideration for the route of administration should be supported by further studies of the potential local effect of $15dPGJ_2$ on interleukin production from the cervix, myometrium, and fetal membranes. The anti-inflammatory effect on NF-κB and the Th1 interleukins may not be replicated in the cervix, for example, which would serve as the least invasive route of administration of $15dPGJ_2$ in the form of a pessary. Absorption at the local level is also unlikely to provide systemic levels high enough to effect peripheral cytokine production, likewise, the effect of peripheral administration on the local maternal fetal interface is not guaranteed to result in a physiological effect. Nevertheless, this study shows promising potential for the future application of $15dPGJ_2$ as an immunomodulating therapy for infection-mediated preterm labour, and thus further studies examining its effect on gestational tissues should be pursued.

5. Conclusion

This study presents evidence that the Th1 : Th2 balance is changed in pregnancy through Th1 suppression rather than Th2 promotion. We have also demonstrated the potential anti-inflammatory effects of $15dPGJ_2$ through the suppression of NF-κB and the Th1 proinflammatory cytokines IFN-γ and TNF-α. These properties may be of therapeutic benefit for the prevention of infection-mediated preterm labour and the reduction of inflammation induced neonatal morbidity, where an aberrant profile favouring the Th1 response is seen. Thus, future work in this area may be best directed toward designing therapeutic strategies aimed to manipulate the Th1 response during pregnancy as a rational strategy for the prevention of preterm labour and pregnancy loss/miscarriage.

Conflict of Interests

The authors report that no conflict of interests exist.

Acknowledgment

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Chemoattractant Receptor Homologous to the T Helper 2 Cell (CRTH2) Is Not Expressed in Human Amniocytes and Myocytes

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Abstract

Background: 15-deoxy-Δ 12,14- Prostaglandin J2 (15dPGJ2) inhibits Nuclear factor kappa B (NF-κB) in human myocytes and amniocytes and delays inflammation induced preterm labour in the mouse. 15dPGJ2 is a ligand for the Chemoattractant Receptor Homologous to the T helper 2 cell (CRTH2), a G protein-coupled receptor, present on a subset of T helper 2 (Th2) cells, eosinophils and basophils. It is the second receptor for Prostaglandin D2, whose activation leads to chemotaxis and the production of Th2-type interleukins. The cellular distribution of CRTH2 in non-immune cells has not been extensively researched, and its identification at the protein level has been limited by the lack of specific antibodies. In this study we explored the possibility that CRTH2 plays a role in 15dPGJ2-mediated inhibition of NF-κB and would therefore represent a novel small molecule therapeutic target for the prevention of inflammation induced preterm labour.

Methods: The effect of a small molecule CRTH2 agonist on NF-κB activity in human cultured amniocytes and myocytes was assessed by detection of p65 and phospho-p65 by immunoblot. Endogenous CRTH2 expression in amniocytes, myocytes and peripheral blood mononuclear cells (PBMCs) was examined by PCR, western analysis and flow cytometry, with amniocytes and myocytes transfected with CRTH2 acting as a positive control in flow cytometry studies.

Results: The CRTH2 agonist had no effect on NF-κB activity in amniocytes and myocytes. Although CRTH2 mRNA was detected in amniocytes and myocytes, CRTH2 was not detectable at the protein level, as demonstrated by western analysis and flow cytometry. 15dPGJ2 inhibited phospho-65 in PBMC'S, however the CRTH2 antagonist was not able to attenuate this effect. In conclusion, CRTH2 is not expressed on human amniocytes or myocytes and plays no role in the mechanism of 15dPGJ2-mediated inhibition of NF-κB.

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Introduction

Preterm delivery complicates 6–11% of pregnancies in Europe and North America [1]. Preterm labour is a heterogenous condition [2], however there is a strong association between infection and inflammation, particularly in early preterm deliveries [3]. Despite our increased awareness of this association [4], limited progress has been made in drug development targeted towards anti-inflammatory pathways involved in infection/inflammation induced preterm labour.

Nuclear factor Kappa B (NF-κB) is a transcription factor that plays a central role in controlling the inflammatory response [5]. Human T cell NF-κB activity is suppressed during pregnancy, which likely explains the altered bias from the Th1 to Th2 cytokine ratio [6]. The NF-κB signaling pathway also plays an important role in the process of human labour [7]. Expression of the p65 subunit shows a marked increase in the fundal myometrium during labour [8], associated with an increase in DNA binding activity [9]. NF-κB activity is also increased in amnion during labour [10], with pre-labour samples showing varying degrees of p65 expression, which is likely to reflect the physiological transition that precedes parturition [11]. Many labour associated genes, such as the phospholipases A2 isoenzymes (cPLA2) [12], cyclooxygenase-2 (COX-2), interleukin-8 (IL-8), IL-6, and matrix metalloproteinases (MMPs) [7] are transcriptionally regulated by NF-κB. Pro-inflammatory cytokines such as IL-1β and TNF-α are both regulated by and can activate NF-κB giving rise to a positive feed forward loop and thus further activating the NF-κB regulated genes [13].

Given the importance of NF-κB in the regulation of inflammation and labour, it represents an important potential therapeutic target for the prevention of preterm labour and the neonatal sequale as a direct result of both inflammation and prematurity. We have previously investigated the potential of 15-deoxy $\Delta^{12,14}$

Prostaglandin J2 (15dPGJ2), an anti-inflammatory cyclopenentone prostaglandin, in the prevention of inflammation/infection induced preterm labour. 15dPGJ2 inhibits IL-1β stimulated NF-κB in human amniocytes and myocytes, independently of PPAR-γ [14], and reduces the percentage of peripheral blood mononuclear cells producing IFN-γ and TNF-α during pregnancy [15]. Moreover, in a mouse model of lipopolysaccharide-induced preterm labour, 15dPGJ2 delays preterm labour and increases pup survival from 30% to 95% [16]. The precise mechanism of NF-κB inhibition is still not fully understood. 15dPGJ2 is also a ligand for the second Prostaglandin D2 (PGD2) receptor, Chemoattractant receptor-homologous molecule expressed on T helper 2 cells (CRTH2), and binds to it with equivalent affinity to PGD2 as the specific CRTH2 agonist DK-PGD2 [17].

The human CRTH2 gene is located at chromosome 11q12, its transcript length is 2.9 kb consisting of two exons and one intron [18]. It was first named and described by Nagata and colleagues as being expressed on Th2 but not Th1 clones following a gene expression search method [19]. Subsequently it was entitled 'receptor D2', a second novel receptor of Prostaglandin D2 (PGD2) [20] as well as 'GPR44' following a search for novel G protein coupled receptors [21]. It is now apparent that these are the same receptor, which is also referred to as the cluster differentiation protein CD294 [22]. As a typical GPCR it has seven putative transmembrane domains, with two potential glycosylation sites at its N terminus, and a long cytoplasmic tail with multiple phosphorylation sites [19]. Despite PGD2 acting as a ligand to both the DP1 and CRTH2 receptor, there is a lack of homology between their sequences and their downstream effects differ substantially. CRTH2 is expressed on T helper 2 cells, eosinophils and basophils [23]. The $CD4^+$ T cells that express CRTH2 secrete interleukins typical of Th2 cells; IL-4, IL-5, IL-13, and little if any INF-γ [19]. CRTH2 is not expressed on human Th1 cells, and this has led to CRTH2 becoming a cell differentiation marker of Th2 cells [19].

CRTH2 mRNA expression has been described in a number of tissue types, with high expression in heart, brain, stomach, small intestine, liver, thymus, and placenta and moderate expression in the colon, uterus, pancreas, prostate and peripheral blood leukocytes [24]. Other groups have demonstrated CRTH2 mRNA expression at the cellular level in non-immune cells including osteoblasts [25], chondrocytes [26] bronchial epithelial cells [27] and keratinocytes [28]. However, studies of CRTH2 expression at the protein level in non-immune cells are limited, probably due to the lack of specific commercially available antibodies available for detection of CRTH2 using techniques other than flow cytometry. The protein expression profile of CRTH2 in human amniocytes and myocytes has not been investigated to date.

In this study we explored the potential role of CRTH2 in 15dPGJ2 mediated NF-κB inhibition. We examined the effect of a small molecule CRTH2 agonist (Pyl A) on NF-κB activity in human cultured amniocytes and myocytes and examined both the mRNA and protein CRTH2 expression profile in these cell types.

Materials and Methods

Ethics Statement

Ethical approval was obtained for placenta (Ref 2002/6283) and myometrium (Ref 3358) from the ethics committees of the Imperial College Healthcare NHS Trust/Imperial College or from the South East London ethical committee for peripheral blood (Ref 10/H0805/54), and in accordance with Imperial College NHS Healthcare Trust Research and Development. Written consent was obtained from all subjects. All clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki.

Reagents

15dPGJ2 was purchased from Cayman Chemicals (Ann Arbor, MI). The small molecule CRTH2 agonist, Pyl A, was synthesised commercially by Oxygen Healthcare, (Cambridge, London) based on the L-888,607 compound from Merck Frosst Centre for Therapeutic Research (Quebec, Canada) [29]. The CRTH2 antagonist GSKCRTH2X was obtained from Glaxo Smith Kline, (London, UK). IL-1β was from R&D Systems (Abingdon, UK). Antibodies against phospho-p65 (Ser 536), p65 and CRTH2 for western analysis were purchased from Cell Signaling Technology (Beverly, MA), Santa Cruz Biotechnology (Santa Cruz, CA) or Santa Cruz and ProSci Incorporated (Poway, CA) respectively. The β-actin antibody was purchased from Sigma (Gillingham, UK). The flow cytometry antibodies and their isotype controls for CRTH2 were purchased from were from Beckman Coulter (High Wycombe, UK), and CD4 from BD Pharminogen (Oxford, UK). All primers were from Thermo-Scientific (Waltham, MA). For cloning and transfection experiments the sources of reagents were as follows: the intermediate vector pCR® -BluntII-TOPO®, Invitrogen Life Technologies (Grand Island NY); pSG5 expression vector, Agilent Technologies (Wokingham, UK); T7 TNT Coupled Reticulocyte Lysate System, Promega (Madison, WI); GeneJuice® transfection reagent, Novagem (Hertford UK); and AMAXA Basic Nucleofector Smooth Muscle Cell Kit, Lonza (Basel, Switzerland).

Cell Culture

Placentas and myometrial biopsies were collected at the time of pre-labour caesarean section, and blood was taken from non pregnant women.

Preparation of amnion epithelial cells. Amnion was separated from the choriodecidua, cut into strips and washed in phosphate buffered saline (PBS). It was then incubated 0.5 mM of EDTA-PBS for 15 mins at room temperature, and washed in PBS. The intracellular matrix was digested in pre-warmed Dispase at 2 g/L in PBS for 45 mins at 37°C. The epithelial cells were then isolated by shaking the amnion strips vigorously in Dulbecco's modified Eagle's medium, DMEM for 3 mins, then pelleted by centrifugation for 10 mins at 2000 rpm. Cells were resuspended in DMEM containing 10% fetal calf serum, 2 mM/L L-glutamine, 100 U/ml Penicillin and 100 µg/ml of Streptomycin and grown to confluence in T25 flasks at 5% CO_2. Cells were cultured for 24 hours until confluent.

Preparation of myocytes. Myometrial biopsies were taken from the upper margin of lower segment incisions during elective caesarean sections. Tissue was washed in PBS and mechanically dissected with two sterile blades to form a paste-like texture. Cells were isolated by incubating with 15 mg of Collagenase 1A, 15 mg of Collagenase X and 50 mg of BSA in 30 mls of PBS for 45 mins at 37°C. The suspension was filtered through a cell strainer and centrifuged at 400 *g* for 5 mins. The cells were resuspended and cultured in DMEM as described above. Myocytes were cultured until confluent. Passage one myocytes were used for transfection studies and passage 0–4 for studies of endogenous CRTH2.

Preparation of placenta and choriodecidua. A biopsy of placenta and choriodecidua were snap frozen and ground in liquid nitrogen in preparation for mRNA extraction.

Preparation of peripheral blood mononuclear cells. Blood was diluted 1:1 with phosphate buffered saline (PBS) and carefully layered onto Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden) before centrifuging at 400 *g* for

40 mins at room temperature. After centrifugation, the halo containing PBMCs was carefully transferred into a clean centrifuge tube and washed twice with 7 ml of PBS. After centrifugation (400 *g* for 10 mins), the cell pellet was resuspended in extraction buffer or RPMI 1640 culture medium (Invitrogen Life Technologies, Grand Island, NY) culture medium.

Cell Treatment

A dose response with the CRTH2 agonists 15dPGJ2 and Pyl A from 0.1 μM-32 μM for 2 hrs was used to determine the effect on NF-κB activity in IL-1β treated amniocytes and myocytes. Stimulation was required with 1 ng/ml of IL-1β, since basal activity in pre-labour amnion and myometrium is low [14]. Based on previous work with 15dPGJ2 on amniocytes, myocytes [14] and PBMC's [15], 32 μM of 15dPGJ2 was used for determining the effect of NF-κB activity in PBMC's. Prior to treatment with 15dPGJ2, cells were pre-treated with 2 μM of GSKCRTH2X (a CRTH2 antagonist) or vehicle for 45 mins.

PCR

Total RNA was extracted using TRIzol® (Invitrogen) and reverse transcribed by Superscript III (Invitrogen). Taq Po was used for qualitative PCR. CRTH2 was detected using qualitative PCR with the Primer sets A and B (Table 1) under cycling conditions of; 95°C for 3 min, amplification by 35 cycles of PCR (94°C, 1 min; 67°C, 1 min; 72°C, 2 min) for set A and 95°C for 3 min, amplification by 40 cycles of PCR (94°C, 1 min; 63°C, 1 min; 72°C, 2 min) for set B with ABI StepOne. Products of quantitative PCR were also qualitatively assessed on an agarose gel having been amplified with primer set C (Table 1) with SYBRGreen PCR Master mix (Applied Biosystems) under the following cycling conditions of activation at 50°C for 2 min with 40 cycles of (95°C, 1 min; 95°C, 15 s; 60°C, 1 min). Peripheral mononuclear blood cell cDNA was used to amplify a 1.188 kb CRTH2 transcript using primer set D with activation at 95°C for 3 min and 36 cycles of (94°C, 1 min; 75°C, 1 min; 72°C, 2 min). The primer sets produced amplicons where the intron/exon boundary was crossed wherever possible. Non-template controls and reverse transcriptase negative controls were used. Products were subjected to gel electrophoresis and detected by staining with Sybersafe (Invitrogen) to assess for correct product size.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Protein was extracted from cells with whole cell lysis buffer (Cell Signalling, Beverly, MA) and 5 μl/ml of protease inhibitor (Sigma). Cells were incubated with ice cold buffer for 5 mins and centrifuged for 20 mins at 13,000 rpm at 4°C. Protein extraction from tissue was performed by homogenising in whole cell lysis buffer prior to the above incubation steps. For nuclear protein extraction cells were lysed in Buffer A (10 mM HEPES, 10 mM KCL, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 1%(v) NP-40 with protease inhibitor), with the cytosolic fraction collected following centrifugation at 13,000 rpm for 60s at 4°C. The pellet was then resuspended in Buffer B (10 mM HEPES, 10 mM KCL, 0.1 mM EGTA, 0.1 mM EDTA, 2MM DTT, 400 mM NaCl and 1% v/v NP-40 and protease inhibitors), incubated on a shaker for 15 mins at 4°C, and then centrifuged at 13,000 rpm for 5 mins at 4°C. Prior to SDS-PAGE, protein concentrations were determined using the BIORAD quantification assay measuring absorbance at 655 nm. Between 15 and 50 μg of extracted protein per sample, was resolved by SDS-PAGE and subsequently transferred onto polyvinyl difluoride (PVDF) membranes (GE Healthcare) at 100 V constant at 4°C. Following transfer, the membrane was then blocked in 5% (wt/vol) milk in tris-buffered saline (TBS) supplemented with 0.1% Tween 20 (TBST) for 1 h. The membrane was then probed with the relevant primary antibody followed by the appropriate secondary antibody under conditions specified in (Table 2). Chemiluminescence detection was then carried out with ECL Plus (GE Healthcare) and the membranes developed using a high Performance chemiluminescence film (GE Healthcare). Blots were scanned and densitometry was performed with ImageJ (v1.44p).

Cloning

Human peripheral leukocytes were used to extract mRNA and to reverse transcribe to cDNA with random hexamers with Superscript III according to manufacturer's instructions (Invitrogen Life Technologies). A 1.18 kB CRTH2 transcript of the coding region was amplified with primers which introduced BamH I digest sequence upstream of the CRTH2 coding sequence and a BGL II restriction digest sequence downstream (Table 2). The transcript was cloned into an intermediate TOPO Vector (pCR®-BluntII-TOPO®) according to manufacturer's instructions (Invitrogen Life Technologies). The plasmid was digested at the BamH1 and BglII sites, run on a 1% DNA Agarose gel, cut and purified using the Geneclean kit for insertion into the pSG5 expression vector. Plasmids were digested and the insert was purified for sequencing to confirm that CRTH2 was present and in frame. The pSG5 expression vector was selected since this has previously been shown to lead to high and stable expression of multiple proteins in myocytes [30].

In vitro Transcription Translation and Transfection

CRTH2 expression in a cell free system was examined with the T7 TNT Coupled Reticulocyte Lysate System. The TNT kit was used as per manufacturer's instructions (Promega) to produce CRTH2 with both cold Methionine and radioactive ^{35}S Methionine for detection by both western analysis and x-ray film respectively. The progesterone pSG5 expression vector, a

Table 1. The primer sequences used for amplification of CRTH2.

	Forward 5′	Anti-sense 5′	bp
Set A	CCTCTGTGCCCAGAGCCCCACGATGTCGGC	CACGGCCAAGAAGTAGGTGAAGAAG	309
Set B	CTGCCCTTCTTCACCTACTTCTTGGC	GTGTCCCGGAACACGAAATAGGGCAC	265
Set C	CCTCTGTGCCCAGAGCCCCACGATGTCGGC	ATGTAGCGGATGCTGGTGTTG	114
Set D*	5′GGATCCATGTCGGCCAACGCCACACTGAAGCC (*Includes BAMH I site)	AGATCTCTAACTCGAGGTGCTGCTCAGCGCCC (*Includes BGL II site)	1188

doi:10.1371/journal.pone.0050734.t001

Table 2. The incubation conditions and the catalogue numbers for all antibodies used.

	Primary antibody	Secondary antibody	Cat number
P-p65 (Ser536)	1:1000 Overnight at 4°C	Anti-rabbit; 1:2000 1 hr RT	Cat 3033s
p65	1:1000 Overnight at 4°C	Anti-mouse; 1:200 1 hr RT	Cat no 8008
CRTH2	1:200 Overnight at 4°C	Anti-goat: 1:2000 1 hr RT	Cat no. 23092
CRTH2	1:500 Overnight at 4°C	Anti-rat; 1:3000 1 hr RT	Cat no. 2178
CRTH2	1:500 Overnight at 4°C	Anti-rabbit; 1:2000 1 hr RT	Cat no. PR-4029
β-actin	160:000 30 minutes at RT	Anti-mouse; 20:1000 15 mins RT	Cat no. A 2228

doi:10.1371/journal.pone.0050734.t002

kind donation of Dr Yun Lee, served as a positive control for the efficiency of the transcription-translation reaction. For transfection of CRTH2 into amniocytes; primary amnion epithelial cells were cultured until 80% confluence. GeneJuice® transfection reagent was used according to manufacturer's guidance (Novagem) with 1 µg of DNA per well. For transfection of CRTH2 into myocytes; myometrial smooth muscle cells were cultured until 100% confluence. The AMAXA Basic Nucleofector Smooth Muscle Cell Kit was used as per manufacturer's instructions (Lonza). Electroporation was performed with the Nucleofector Program using Program A-033. GFP was used as a positive control for the transfection efficiency. Gene expression of Green Fluorescent Protein (GFP) was determined by fluorescent microscope on days 1 and 2 post transfection. Gene expression of CRTH2 was determined by Flow cytometry on days 1 and 2 post transfection.

Flow Cytometry for Detection of CRTH2

PBMC, amniocyte and myocyte pellets were resuspended in staining buffer (1% Fetal Calf Serum, 0.09% Sodium Azide in PBS). Cells were incubated in the dark for 1 h at 37°C with 20–40 µl of CRTH2-PE. PBMCs were also incubated with 3 ul of CD4-APC. Mouse IgG1 κ –APC and Rat IgG_{2a}-PE were used as isotype controls. After incubation, the PBMC suspension was washed twice in 1 ml of PBS and then resuspended in PBS for analysis. The FACSCalibr flow cytometer was used for CRTH2 detection and settings were as follows: PBMCs; Forward scatter E0 Voltage, 1.00 Amp gain Lin, and Side scatter of 329 Voltage, 1.00 Amp gain Lin, amniocytes; Forward Scatter E1; Voltage 4.77; Amp gain Lin; Side scatter of 320; Voltage 1.00; Amp gain Lin; FL2 347 log, Myocytes; Forward Scatter E1; Voltage 4.37; Amp gain Lin; Side scatter of 197; Voltage log; FL2 350 log. Results are expressed as percentage of cells expressing the receptor and mean fluorescence intensity of receptor expression. The geographical mean was used since data was collected on a logarithmic scale and is a better indicator of the central tendency of the population.

Statistical Analysis

Experimental sample groups consisted of 3–6 biological replicates. Statistical analysis was performed with Graph-Pad Prism (v5.0; GraphPad Software, San Diego, CA). ANOVA of repeated measures was conducted where appropriate, with Bonferroni's multiple comparison's test for *post-hoc* analysis. Samples with $P<0.05$ was considered to be statistically significant.

Results

The CRTH2 Agonist 15dPGJ2 Inhibits IL-1β Induced NF-κB Activity in Human Amniocytes and Myocytes but the CRTH2 Agonist Pyl A does not Replicate this Effect

We have previously shown that 15dPGJ2 inhibits IL-1β induced NF-κB activity in human amniocytes and myocytes in a mechanism independent of PPAR-γ [14]. Here we initially replicated these results showing a significant reduction in IL-1β induced NF-κB, as determined by nuclear p65, to below basal levels in amniocytes at 32 µM and in myocytes from 16 µM (Figure 1A and 1B). If CRTH2 is expressed in amniocytes and myocytes and is the mechanism of action of 15dPGJ2, we would expect a small molecule CRTH2 agonist to replicate the effect of

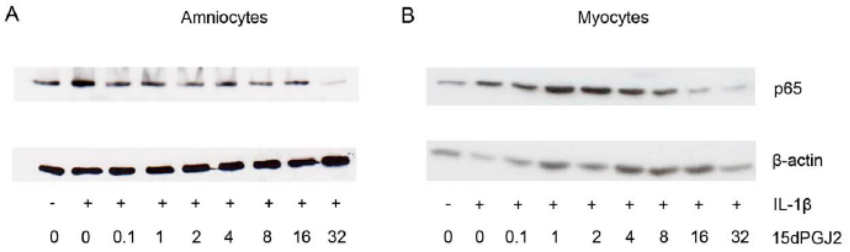


Figure 1. 15dPGJ2 reduced NF-κB p65 activity in amniocytes and myocytes. Protein was extracted from IL-1β stimulated and 15dPGJ2 treated cells and levels of nuclear p65 were examined using immunoblotting. A dose response of 0.1–32 µM of 15dPGJ2 was used (n = 3). Representative immunoblots are shown for amniocytes, (**A**), myocytes (**B**). Immunoblots were re-probed for β-actin as an internal loading control.
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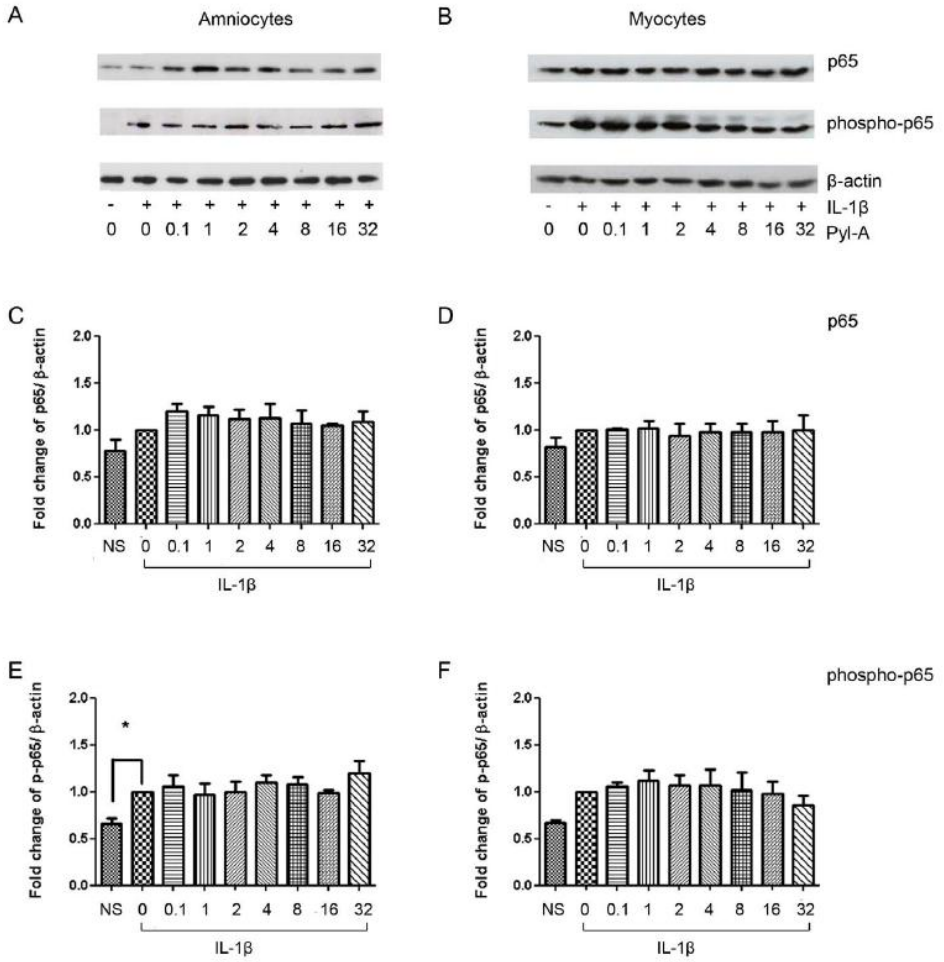


Figure 2. Pyl A has no effect on NF-κB p65 activity in amniocytes and myocytes. Protein was extracted from IL-1β stimulated and Pyl treated cells and levels of nuclear p65 and phosphorylated p65 (p-p65) were examined using immunoblotting. A dose response of 0.1–32 μM of Pyl A was used. Representative immunoblots are shown for amniocytes, (**A**) and myocytes (**B**). Immunoblots were re-probed for β-actin as an internal loading control. Densitometric analysis of the immunoblots was conducted revealing no effect of Pyl A on p65 or p-p65 in amniocytes (**C**, **E**) or myocytes (**D**, **F**). NS = non-stimulated (non-IL-1β treated cells). Effect of treatment was examined for statistical significance using ANOVA of repeated measures with Bonferroni's multiple comparison test; $^{*}P<0.05$.
doi:10.1371/journal.pone.0050734.g002

15dPGJ2 We therefore examined the effect of the CRTH2 agonist Pyl A on IL-1β induced NF-κB in amniocytes and myocytes to determine if the small molecule agonist could replicate the effects of 15dPGJ2. NF-κB activity was assessed by examining nuclear p65 and phospho-p65 by immunoblot. There was no difference in IL-1β stimulated NF-κB activity seen with Pyl A treatment in either cell type. Representative immunoblots for amniocytes and myocytes are shown in Figures 2A and 2B respectively, with densitometric analysis taking into account β-actin loading controls seen in Figures 2C–F.

CRTH2 mRNA is Expressed in Amniocytes and Myocytes

The absence of Pyl A mediated inhibition of NF-κB in amniocytes and myocytes led us to question if this was due to a

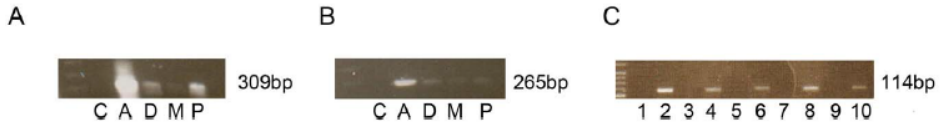


Figure 3. CRTH2 mRNA is expressed in amniocytes and myocytes. mRNA was isolated from cultured amniocytes, myocytes, PBMCs, choriodecidual and placental extracts and converted to cDNA (n = 6). Qualitative PCR was used with three primer sets to amplify CRTH2 showing product sizes of 309 bp, 265 bp, and 114 bp. Non-template and reverse transcriptase negative controls were used and mRNA from placenta, choriodecidua and peripheral blood mononuclear cells were used as a positive controls. (**A,B**): Non-template control (lane 1), amniocytes (lane 2), choriodecidua (lane 3), myocytes (lane 4), placenta (lane 5); (**C**): Reverse transcriptase controls (lanes 1,3,5,7,9), PBMCs (lane 2), amniocytes (lanes 4,6), myocytes (lanes 8,10).
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lack of expression of CRTH2 in these cells, or, alternatively that there is no role for CRTH2 in 15dPGJ2 mediated inhibition of NF-κB. Accordingly, CRTH2 mRNA and protein expression of in amniocytes and myocytes was examined by PCR and western analysis. CRTH2 mRNA was detected in amniocytes and myocytes as well as choriodecidual tissue and placental tissue with two primer sets. Placenta served as a positive control [24] and non-template negative controls were included (Figure 3A and 3B). A third primer set was used as confirmation of consistent expression in amniocytes and myocytes with reverse transcriptase negative controls and PBMCs as a positive control (Figure 3C). N = 6 biological replicates were examined.

CRTH2 is not Expressed at the Protein Level in Amniocytes and Myocytes

Western analysis of endogenous CRTH2. CRTH2 expression at the protein level was then examined by immunoblot using the CRTH2 antibody Sc-23092 (Figure 4). 50 µg of total protein derived from PBMC whole cell lysate, amniocytes, myocytes was used. Multiple bands are seen in both the positive control (PBMCs) and in the amniocyte and myocyte lanes. A band appears at Mr~34 000 in amniocytes, faintly in the myocyte lane, but is absent in the positive control PBMCs lane. However, the strongest bands appear at M_r~15 000 and at just above M_r~43 000 in all lanes. No culture dependent effect was seen as demonstrated by myocytes from passage 0–4 in panel C, Figure 4. With the absence of CRTH2 detection in the positive control, we cannot determine the presence or absence of endogenous CRTH2 in amniocytes and myocytes from this immunoblot alone.

Detection of CRTH2 in the pSG5 expression vector. With the possibility of very low endogenous protein expression of CRTH2 explaining the reason for the antibody failing to detect CRTH2 in the positive control of the immunoblot in Figure 4, we cloned and synthesised CRTH2 using an *in-vitro* transcription translation kit to form a highly concentrated CRTH2 protein lysate to serve as a positive control. A band at M_r~34 000, consistent with the reported expected size could be clearly observed following detection of radioactive ^{35}S Methionine by x-ray film (Figure 5A). The protein lysate was then loaded and transferred onto immunoblots and probed with three commercially available antibodies designed for western blotting. No band was detected at M_r~34 000, with non-specific bands appearing also in the negative control of the TNT kit without plasmid DNA (Figure 5B–D).

Detection of endogenous CRTH2 by flow cytometry. Several studies have used commercially available antibodies for flow cytometry successfully for the detection of endogenous CRTH2 [27,31,32]. Similarly, we were able to detect endogenous CRTH2 by flow cytometry on human lymphocytes. Lymphocytes were gated based on forward and side scatter profile. A representative cytogram is shown with $CRTH2^+/CD4^+$ cells seen in the right upper quadrant (Figure 6A). 1.6% of lymphocytes were $CRTH2^+/CD4^+$, and 0.57% were $CRTH2^+/CD4^-$. A representative histogram of cells with no staining, isotype control and CRTH2 staining is shown, with the mean fluorescence intensity of 157.80 compared to 22.8 in the isotype control sample (Figure 6B). No CRTH2 expression was seen in amniocytes or myocytes, with a mean fluorescence intensity of 14.41 with labelling compared to 15.71 for the isotype in amniocytes; and 69.69 with labelling compared to 69.30 in the isotype control labelled myocytes (Figure 6C and 6D), n = 6. Table 3 summarises the mean fluorescence intensity and percentage of cells expressing CRTH2 in the gated populations.

Detection of transfected CRTH2 in amniocytes and myocytes by flow cytometry. Since we could not detect CRTH2 in amniocytes or amniocytes by flow cytometry we transfected in a CRTH2 expression vector to confirm that endogenous CRTH2 could have been detected if present. CRTH2 was detected in amniocytes and myocytes transfected with CRTH2, as seen by an increase in the mean fluorescence intensity. Representative histograms are shown for transfected samples, with isotype controls for amniocytes (Figure 7A) and myocytes (Figure 7B). The mean fluorescence intensity increased from 87.75 in the isotype control to 143.49 in amniocytes, and from 68.84 to 96.97 in myocytes. A GFP transfection control is shown to confirm adequate transfection efficiency (Figure 7C). Table 4 summarises the mean fluorescence intensity and percentage of cells expressing CRTH2 in the gated populations.

15dPGJ2 Inhibits Basal NF-κB Activity in PBMC's in a Mechanism Independent of CRTH2

Thus far we have demonstrated that CRTH2 is not expressed at detectable levels in amniocytes and myocytes. We therefore proposed that CRTH2 does not play a role in 15dPGJ2-mediated inhibition of NF-κB. To confirm this we treated PBMCs with 15dPGJ2 with or without the CRTH2 antagonist, GSKCRTH2X (n = 6). 15dPGJ2 showed complete inhibition of phospho-65 ($P<0.0001$) which was not attenuated by pre-incubation with the CRTH2 antagonist (Figure 8).

Discussion

Preterm birth accounts for 70–75% of neonatal morbidity and mortality [33]. In 80–85% of cases of spontaneous preterm labour <28 weeks there is evidence of intrauterine infection [34]. The presence of inflammation can increase the risk of neonatal morbidity, independently of prematurity [35]. Inflammatory

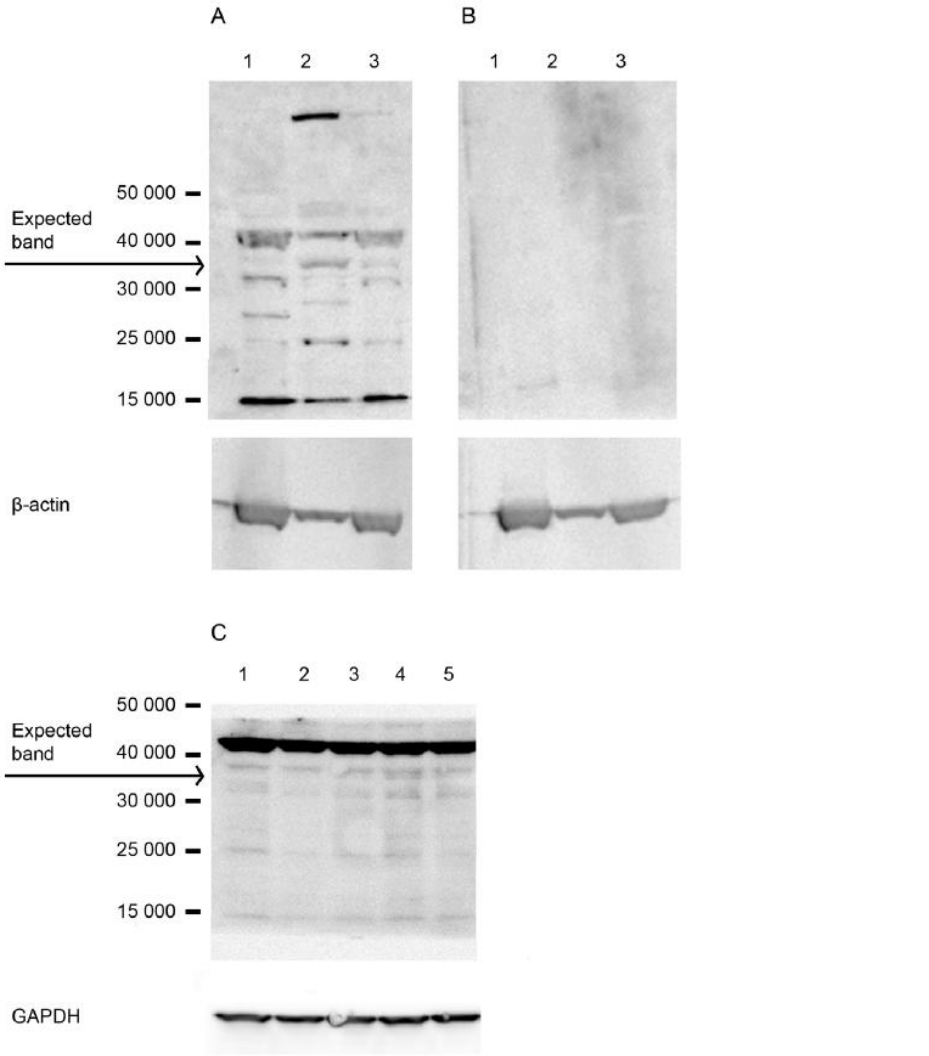


Figure 4. Western blot of protein lysate from PBMCs, amniocytes, myocytes. 50 μg of whole cell lysate from PBMCs, amniocytes, myocytes were subject to western analysis. Membranes were probed with the primary antibody -SC-23092 to detect a Mr~34 000 product (**A**; Lanes 1–3). The secondary antibody control blot is also shown (**B**; Lanes 1–3). β-actin was used as a loading control. Lane 1 = PBMCs, lane 2 = amniocytes, lane 3 = myocytes. Multiple bands are seen on the blot. A band appears at Mr~34 000 in amniocytes, faintly in the myocyte lane, but is absent in the positive control PBMCs lane. However, the strongest bands appear at Mr~15 000 and at just above Mr~43 000 in all lanes. Myocytes from passage zero, one, two, three and four (lanes 1–5 respectively) were also examined for CRTH2 expression showing a very faint band at Mr~34 000, with no effect of passage number (**C**).
doi:10.1371/journal.pone.0050734.g004

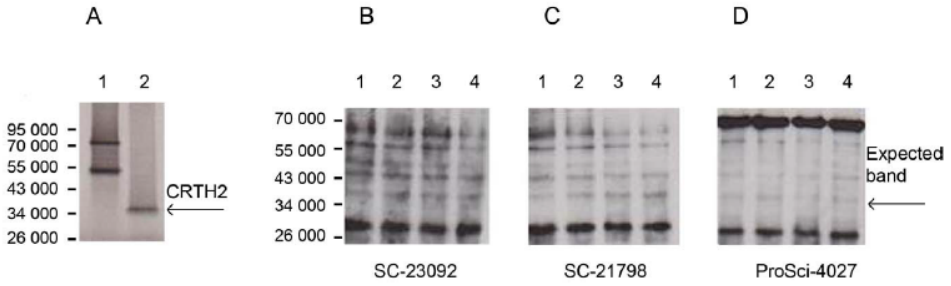


Figure 5. Detection of radiolabelled CRTH2 in pSG5 expression vector by x-ray and immunoblot. The in vitro transcription translation kit was used with 35^S Methionine to demonstrate the expression of CRTH2 and to provide a positive control for detection of CRTH2. A Mr~34 000 product was detected by x-ray (Lane 2). The progesterone expression vector was used as a control for the TNT kit (**A**). The protein lysate from the TNT kit was subjected to western analysis; Negative Control: TNT kit with no plasmid DNA (lane 1), Negative control: Progesterone PSG5 Expression vector (lane 2), Positive control: 35^S Methionine labelled CRTH2 (Lane 3), Cold Methionine CRTH2 protein product (lane 4). Membranes were probed with 3 commercial antibodies; SC-23092 (**B**), SC-21798 (**C**), ProSci 4027 (**D**). None of the antibodies detected CRTH2.
doi:10.1371/journal.pone.0050734.g005

cytokines, produced in association with infection, can activate the transcription factor NF-κB. Since NF-κB controls the expression of many labour associated genes and pro-inflammatory cytokines, targeting the inhibition of NF-κB seems attractive to prevent both infection induced preterm labour and the associated risk of brain injury associated with inflammation. In this study we explored the possibility that CRTH2 plays a role in 15dPGJ2-mediated inhibition of NF-κB both at a local level in cells of the maternal fetal interface; amniocytes and myocytes, and systemically in peripheral blood mononuclear cells. If this were true, then small molecule agonists of CRTH2 could potentially be used in the management of preterm labour.

We have previously shown that 15dPGJ2 inhibits IL-1β induced NF-κB activity in human amniocytes and myocytes through a mechanism independent of the PPAR-γ receptor [14]. 15dPGJ2 is a CRTH2 agonist, and upregulates CD11b expression in eosinophils via CRTH2 [36]. This led us to examine the effect of a small molecule CRTH2 agonist, Pyl A, on NF-κB activity in human amniocytes and myocytes. We saw no effect of Pyl A on IL-1β stimulated p65 or phospho-p65 in either cell type (Figure 2). We have also previously seen differing effects between 15dPGJ2 and Pyl A on NF-κB activity in the murine myometrium. Intrauterine administration of 15dPGJ2 inhibits LPS induced NF-κB in the murine myometrium and is associated with a delay in preterm labour [16]. In contrast, Pyl A did not inhibit NF-κB but instead augmented LPS stimulated NF-κB, which led to earlier preterm delivery of the pups (unpublished data). Despite both 15dPGJ2 and Pyl A activating the CRTH2 receptor, cross talk may exist between their other downstream targets of varying cell types, which could explain their contrasting effects when given *in vivo*.

Few studies have sought to characterise CRTH2 expression in human gestational tissues. Helliwell et al examined mRNA expression in amnion, choriodecidua and placental tissue [37]. They failed to detect CRTH2 in all 24 amnion samples, both preterm and term. 3/12 preterm choriodecidual samples expressed CRTH2, all three had histological evidence of chorioamnionitis, whereas 7/12 term samples expressed CRTH2. All placental samples had detectable but variable levels of CRTH2 mRNA, with no difference between preterm and term samples. Nagata et al have reported detectable CRTH2 mRNA in many human tissue types including the placenta and non-pregnant myometrium [24]. Since CRTH2 is classically expressed on immune cells, it is plausible that the mRNA detected in these tissue samples is from infiltrating leukocytes, which would explain why CRTH2 can be detected choriodecidual samples associated with chorioamnionitis.

Several studies have examined the presence of CRTH2 positive T cells in the decidua in early pregnancy in association with the potential CRTH2 mediated bias towards the anti-inflammatory Th2 cytokine profile at the maternal fetal interface [38,39,40]. The percentage of $CRTH2^+$ cells in $CD4^+/CD3^+$ cells are higher in the decidua than the peripheral blood in early pregnancy [38]. It has been proposed that decidual Th2 cells are recruited to the maternal fetal interface by PGD2 mediated chemoattraction via the CRTH2 receptor [39]. In this study we examined CRTH2 at the cellular level and found detectable levels of CRTH2 mRNA in amniocytes and myocytes with three primer sets (Figure 3), using choriodecidua and placental tissue and PBMCs as positive controls. Expression levels were low with cycle numbers of between 35 and 40 used for detection. This is consistent with the low expression levels seen in other cell types such as monocytes and dendritic cells, being amplified with up to 40 cycles [41].

To determine if the mRNA was translated into protein in amniocytes and myocytes we used a polyclonal antibody against the extracellular domain of CRTH2 intended for detection of human CRTH2 at Mr~34 000 by immunoblot. This antibody failed to convincingly detect CRTH2 in amniocyte and myocyte protein lysate (Figure 4). Although a faint band was detected in amniocytes and myocytes, no band was detected in the positive control (PBMCs). Since no band was detected in the positive control, we cannot conclude with this immunoblot if CRTH2 is endogenously expressed or not. There are relatively few studies that have used western analysis for the detection of human CRTH2 by western analysis. Zayed et al detected bands at Mr~41 000 and Mr~55 000 for chondrocytes, using osteoblasts as a positive control [26]. However, they did not include blocking or secondary antibody controls to confirm specificity. CRTH2 has previously been demonstrated in osteoclasts by immunohistochemistry with appropriate secondary antibody controls [42]. However, sale of this antibody has since been discontinued because of non-specificity. Colombe et al detected a band for CRTH2 in keratinocytes and melanocytes at Mr~45 000 with the

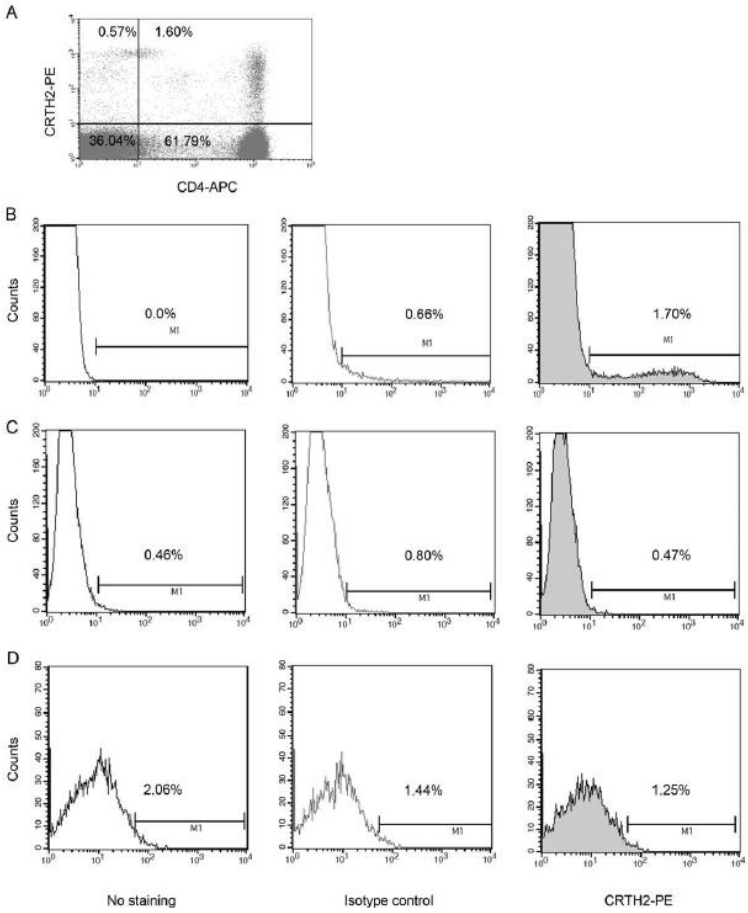


Figure 6. Flow cytometry for the detection of endogenous CRTH2. Lymphocytes were gated according to forward scatter and side scatter and T helper cells were identified using CD4 as a cell surface marker (n = 6). A representative cytogram is presented with the right upper quadrant showing $CRTH2^+/CD4^+$ lymphocytes (**A**). Histograms showing no staining, isotype control and $CRTH2^+$ labelled lymphocytes (**B**), amniocytes (**C**) and myocytes (**D**), (n = 5).
doi:10.1371/journal.pone.0050734.g006

Table 3. An overview of the mean fluorescence intensities and percentages in the gated cells during detection of endogenous CRTH2 in lymphocytes, amniocytes and myocytes.

Endogenous expression	No stain	Isotype-PE	CRTH2-PE
Lymphocytes	12.98 (MFI) 0.0 (%)	22.8 (MFI) 0.66 (%)	157.80 (MFI) 1.70 (%)
Amniocytes	13.91 (MFI) 0.46 (%)	15.71 (MFI) 0.80 (%)	14.41 (MFI) 0.47 (%)
Myocytes	71.38 (MFI) 2.06 (%)	69.30 (MFI) 1.44 (%)	69.69 (MFI) 1.25 (%)

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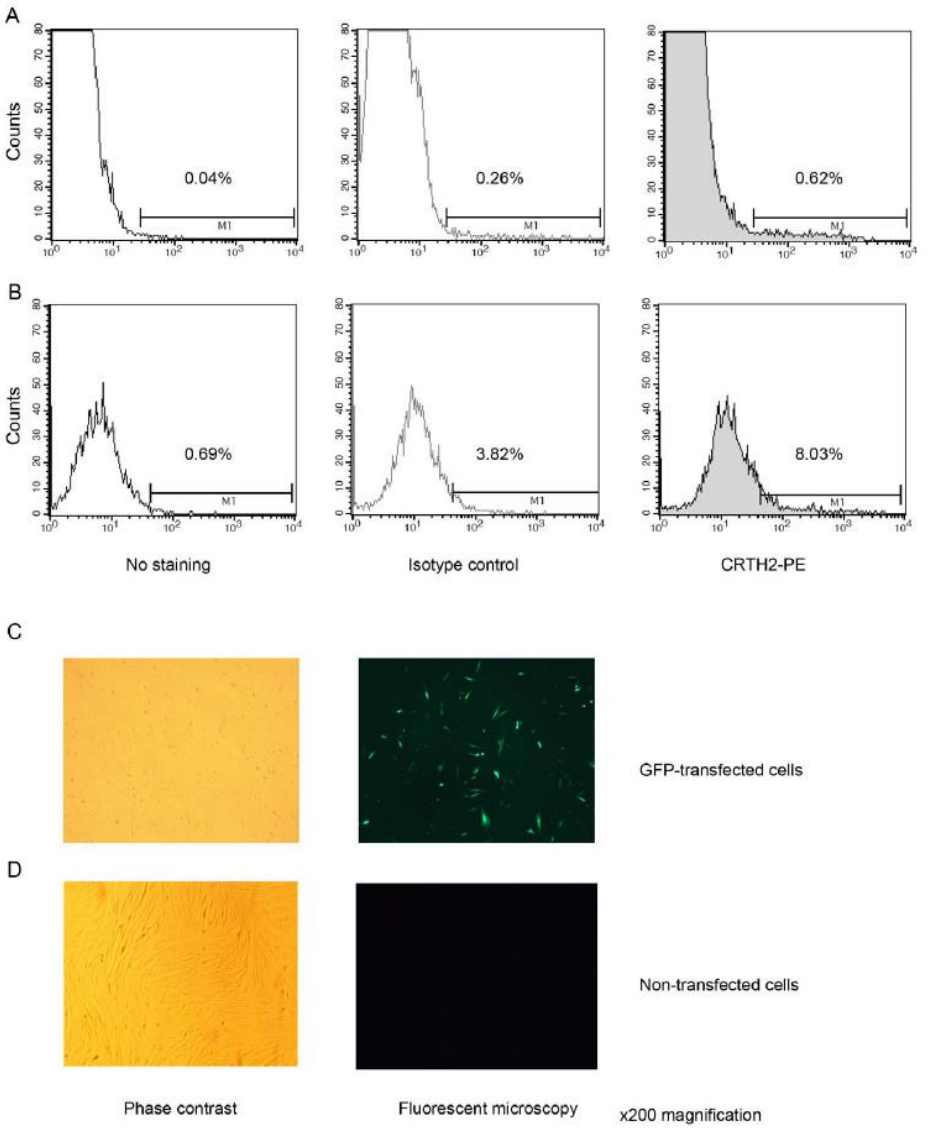


Figure 7. Flow cytometry for the detection of transfected CRTH2. Amniocytes (**A**) and myocytes (**B**) were transfected using gene juice and electroporation respectively with CRTH2 PSG5 expression vector and labeled with anti-CRTH2-PE. A small shift to the right is seen in the histogram showing expression of CRTH2 in transfected cells with an increase in the mean fluorescence intensity from 87.75 in the isotype control to 143.49 in amniocytes, and from 68.84 to 96.97 in myocytes. An example of a GFP control is given for the myocyte transfection, with cells imaged with phase contrast and with the fluorescent microscope to detect GFP transfected cells (**C**). A direct comparison is also shown with non-GFP transfected cells (**D**).

doi:10.1371/journal.pone.0050734.g007

Table 4. An overview of the mean fluorescence intensities and percentages in the gated cells during detection of transfected CRTH2 in amniocytes and myocytes.

Transfected expression	No stain	Isotype-PE	CRTH2-PE
Amniocytes	45.20 (MFI) 0.04 (%)	87.75 (MFI) 0.26 (%)	143.49 (MFI) 0.62 (%)
Myocytes	63.88 (MFI) 0.69 (%)	68.84 (MFI) 3.82 (%)	96.97 (MFI) 8.03(%)

doi:10.1371/journal.pone.0050734.t004

same antibody, again without negative or secondary antibody controls [28].

We cloned CRTH2 to provide a highly concentrated positive control for detection by western analysis and flow cytometry. CRTH2 was synthesized at the correct size of Mr~34 000 (Figure 5A), conforming roughly with the Kozak rule, and consistent with the size determined by Nagata et al [19]. Nagata et al raised three antibodies against CRTH2 which detected the presumed glycosylated form of CRTH2 at Mr~55 000 and the Mr~34 000 CRTH2 product following oligosaccharide cleavage with Endo F, consistent with CRTH2 carrying N-glycosylation sites as predicted by its amnio acid sequence.

Three commercially available antibodies failed to detect in vitro translated CRTH2 by immunoblot (Figure 5B–D). CRTH2 has 7 transmembrane domains which is typical of the G protein coupled seven transmembrane receptor superfamily. There are two potential sites for N-glycosylation in the first extracellular domain, and four consensus sites for protein kinase c phosphorylation on a long cytoplasmic tail [19]. It is very likely that the antibodies require the correct folding structure and/or post translational modifications of the CRTH2 protein to bind to their epitopes. The limitation of the cell free system is the inability to construct the protein to its correctly folded structure or to reproduce the post-translational modifications that exist in the cell, which may explain why CRTH2 was not detected.

To overcome the limitation of the cell free system, we transfected CRTH2 into amniocytes and myocytes. There are a number of studies in which flow cytometry successfully detected human CRTH2 on immune cells [19,31,32,38,43,44]. Since no conclusion could be made on CRTH2 expression in amniocytes and myocytes by immunoblot, we used flow cytometry to examine CRTH2 expression on amniocytes and myocytes, using PBMCs as a positive control. We used a monoclonal antibody raised against the BM16 clone which has also successfully detected CRTH2 on

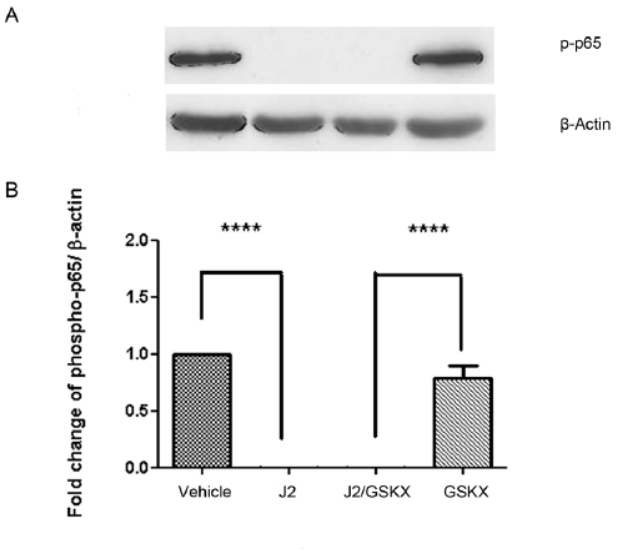


Figure 8. The effect of 15dPGJ2 on basal NF-κB p65 phosphorylation in peripheral blood mononuclear cells. PBMCs were treated with 32 μM of 15dPGJ2 for 2 hours with or without preincubation with 2 μM of GSKCRTH2X for 45 mins. Whole cell protein lysate was examined for levels of phosphorylated p65 (p-p65) using immunoblotting. A representative immunoblot from n = 6 samples are shown (**A**). Immunoblots were reprobed for β-actin as an internal loading control. Densitometric analysis of the immunoblots was conducted revealing a complete inhibition of p-p65 levels with 15dPGJ2. Pre-incubation with the CRTH2 antagonist GSKCRTH2X had no effect on inhibition (**B**). J2 = 15dPGJ2, GSKX = GSKCRTH2X. Effect of treatment was examined for statistical significance using ANOVA of repeated measures with Bonferroni's multiple comparison test; **** $P<0.0001$.
doi:10.1371/journal.pone.0050734.g008

non immune cells; human bronchial epithelial cells [27]. The expression of CRTH2 is low in known cellular populations, with between 0.4–6.5% of peripheral blood $CD4^+$ cells expressing the receptor [19]. Our data is consistent with this, with the representative sample showing 2.5% of $CD4^+$ cells expressing CRTH2, (Figure 6B). This antibody did not detect endogenous CRTH2 in amniocytes and myocytes (Figure 6C and 6D). However, CRTH2 was detected at low levels in transfected cells (Figure 7A and 7B). The pSG5 expression vector leads to high expression of multiple proteins in myocytes [30], and good transfection efficiency was achieved as demonstrated by the GFP control (Figure 7C). Therefore, the inefficiency of amniocytes and myocytes to express a stable CRTH2 protein supports our conclusion that CRTH2 is not endogenously expressed in amniocytes and myocytes.

The absence of CRTH2 on human amniocytes and myocytes imply that the mechanism of 15dPGJ2 mediated inhibition of NF-κB is independent of CRTH2. Moreover, the CRTH2 antagonist failed to attenuate the effects of 15dPGJ2 in peripheral blood mononuclear cells confirming that CRTH2 plays no role in NF-κB inhibition (Figure 8). There are several signalling pathways known to be involved upon CRTH2 activation and control the varying effects of CRTH2 agonists. Indomethacin induces Ca^{2+} mobilization via CRTH2 in Th2 cells, which is inhibited by pertussin toxin indicating a $G_{\alpha/i}$ dependent mechanism [45]. Indomethacin induced eosinophil morphological change and upregulation of CD11b via CRTH2 can be attenuated with inhibitors of MAP Kinase and phosphatidylinositol 3-kinase (PI3K), suggesting an important role for these signalling pathways in CRTH2 mediated effects [46]. PGD2 induces IL-4, IL-5 and IL-13 production and cell migration in Th2 cells via CRTH2, which is also attenuated by inhibiting the PI3K pathway [47]. The only other study to explore the effect of CRTH2 agonists on NF-κB showed that an IKB inhibitor had no effect PGD2 induced cytokine production, however the NF-κB inhibitor rocaglamide reduced PGD2 induced cytokine production by 30% [47]. The authors of this study concluded that since PGD2 did not lead to an increase phospho-IκB, a marker of NF-κB activation, that this effect was likely to be non-pathway specific. Together with their data showing no direct effect on NF-κB signalling upon CRTH2 activation with PGD2, our data also supports no direct effect on NF-κB signalling with CRTH2 activation or inhibition (Figure 8).

The CRTH2 agonist 15dPGJ2 is an endogenous anti-inflammatory prostaglandin, formed from a non-enzymatic dehydration of PGD2 [48]. It can accumulate intracellularly [49], and can be measured endogenously in picomolar concentrations in body fluids [50]. Although it is a high affinity ligand to the nuclear PPAR-γ receptor, 15dPGJ2 inhibits NF-κB independently of this receptor in amniocytes and myocytes [14]. We have previously shown that 15dPGJ2 is able to inhibit multiple steps in the IKK/NF-κB pathway. It inhibits IL-1β induced phosphorylation of IKKα and IKKβ, prevents IKBα degradation and thus prevents the release of the NF-κB subunits for nuclear translocation [14]. Direct modification of cysteine 179 of IKKβ by covalent interaction by 15dPGJ2 has been demonstrated in HeLa cells [51].

15dPGJ2 contains an electrophilic cyclopenteneone ring, a property which permits it to ligate nuclear receptors and modify intracellular signalling molecules. The highly reactive ring of 15dPGJ2 can form a covalent interaction with several components of the NF-κB signalling pathway (IkB kinase complex B, p50, and p65 subunits) via the Michael reaction, resulting in impaired nuclear entry, as described above, and impaired DNA binding activity [52]. It is highly likely that the cyclopentenone ring is essential for the inhibition of NF-κB seen in PBMCs in this study, since we have previously shown the ability of PGA_1, but not 9,10-dihydro-15dPGJ2 (in which the ring is disrupted) to mimic the effect of 15dPGJ2 in amniocytes and myocytes.

Despite the above mechanisms having been described, it is not yet clear how 15dPGJ2 enters the cell to reach its targets, or if there is an alternative receptor mediated mechanism. Although Prostaglandin J2 has been shown to enter the cell by active transport [53], it is not yet clear if this is the mechanism of entry of 15dPGJ2 into amniocytes, myocytes and PBMCs. In this study we have excluded a CRTH2 dependent receptor mediated entry into the cell as a requirement for NF-κB inhibition, and CRTH2 dependent downstream signalling effect on the inhibition of NF-κB. Further studies should be carried out to investigate other potential mechanisms of 15dPGJ2 mediated NF-κB inhibition, including mechanism of entry into the cell, and to explore its effect on the production of NF-κB regulated interleukins in amniocytes and myocytes.

Conclusions

We have demonstrated that despite being able to detect low levels of mRNA in amniocytes and myocytes, neither cell type express CRTH2 at a detectable protein level. We conclude that the mechanism of 15dPGJ2 mediated inhibition of NF-κB does not involve CRTH2. Small molecule CRTH2 agonists are therefore unlikely to be of value in the inhibition of inflammation associated preterm birth.

Author Contributions

Conceived and designed the experiments: LS YL SK DM XY SP TT PB. Performed the experiments: LS YS SK XY SP. Analyzed the data: LS YL DM SK XY SP. Contributed reagents/materials/analysis tools: LS YL SK TGT PB. Wrote the paper: LS YL DM TGT PB.

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IMMUNOLOGY ORIGINAL ARTICLE

The CRTH2 agonist Pyl A prevents lipopolysaccharide-induced fetal death but induces preterm labour

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Summary

We have previously demonstrated that the anti-inflammatory prostaglandin 15-deoxy-Δ 12,14-prostaglandin J_2 ($15dPGJ_2$) delays inflammation-induced preterm labour in the mouse and improves pup survival through the inhibition of nuclear factor-κB (NF-κB) by a mechanism yet to be elucidated. The $15dPGJ_2$ is an agonist of the second prostaglandin D_2 receptor, chemoattractant receptor homologous to the T helper 2 cell (CRTH2). In human T helper cells CRTH2 agonists induce the production of the anti-inflammatory interleukins IL-10 and IL-4. We hypothesized that CRTH2 is involved in the protective effect of $15dPGJ_2$ in inflammation-induced preterm labour in the murine model. We therefore studied the effects of a specific small molecule CRTH2 agonist on preterm labour and pup survival. An intrauterine injection of lipopolysaccharide (LPS) was administered to CD1 mice at embryonic day 16, ± CRTH2 agonist/vehicle controls. Mice were killed at 4.5 hr to assess fetal wellbeing and to harvest myometrium and pup brain for analysis of NF-κB, and T helper type 1/2 interleukins. To examine the effects of the CRTH2 agonist on LPS-induced preterm labour, mice were allowed to labour spontaneously. Direct effects of the CRTH2 agonist on uterine contractility were examined *ex vivo* on contracting myometrial strips. The CRTH2 agonist increased fetal survival from 20 to 100% in LPS-treated mice, and inhibited circular muscle contractility *ex vivo*. However, it augmented LPS-induced labour and significantly increased myometrial NF-κB, IL-1β, KC-GRO, interferon-γ and tumour necrosis factor-α. This suggests that the action of $15dPGJ_2$ is not via CRTH2 and therefore small molecule CRTH2 agonists are not likely to be beneficial for the prevention of inflammation-induced preterm labour.

Keywords: 15-deoxy-Δ12,14-prostaglandin J_2; chemoattractant receptor homologous to the T helper 2 cell; lipopolysaccharide; murine preterm labour; nuclear factor-κB.

Introduction

Preterm labour is one of the most challenging complications of human pregnancy. Its incidence in the western world remains between 6 and 15% depending on the geography and demographics of the population.[1] It is a heterogeneous condition,[2] with the only firm causal link being that of infection.[3] Despite the increased awareness of the association between infection and inflammation and preterm labour,[4] there have been limited advances in the treatment and prevention of preterm labour. Currently, there is a drive to develop anti-inflammatory therapies to not only delay preterm labour, but to prevent the long-term neurological damage thought to be a result of the impact of pro-inflammatory factors on fetal inflammatory response syndrome.

The transcription factor nuclear factor-κB (NF-κB), which is classically associated with inflammation, is central to regulating the biochemical pathways involved in both term labour and preterm labour.[5] The oxytocin receptor and cyclo-oxygenase-2 (COX-2) genes contain NF-κB response elements in their promoter regions.[6,7]

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Oxytocin receptor mediates oxytocin-induced myometrial contractions through activation of phospholipase C and downstream calcium release from intracellular stores.[8] The COX-2 enzyme is the rate-limiting step for prostaglandin synthesis, which is responsible for uterine contractions and cervical dilatation. The NF-κB is also involved in the transcriptional regulation of matrix metalloproteinases, including matrix metalloproteinase-9, which are required for remodelling of the extracellular matrix,[9] leading to cervical ripening and fetal membrane rupture. A positive feed-forward loop also exists from activation of NF-κB by the pro-inflammatory cytokines and subsequently their transcriptional activation, including tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β).[5,10,11] Hence, premature activation of NF-κB by pro-inflammatory cytokines, or activation of the Toll-like receptors as a result of infection and inflammation, can lead to the amplification of the pro-inflammatory response and the transcription of the labour-associated genes via NF-κB activation, resulting in preterm labour.

Inhibition of NF-κB is an attractive therapeutic target because apart from inhibiting labour-associated genes involved in uterine contractility, cervical ripening and fetal membrane rupture, it would also target pro-inflammatory cytokine production, which may contribute to the neurological damage seen independently of the effect of prematurity. We have previously shown that 15-deoxy-Δ 12,14-prostaglandin J_2 ($15dPGJ_2$), an anti-inflammatory cyclopentenone prostaglandin, inhibits NF-κB activity and COX-2 *in vitro* in both human cultured myocytes and amniocytes.[12] In a murine model of inflammation-induced preterm labour, $15dPGJ_2$ delays preterm labour from 20 hr post lipopolysaccharide (LPS) injection to 30 hr post LPS plus $15dPGJ_2$ injection. More importantly $15dPGJ_2$ improved pup survival from 30% with LPS, to 95% with co-injection of LPS and $15dPGJ_2$.[13] The mechanism by which $15dPGJ_2$ inhibits NF-κB is not entirely understood. The $15dPGJ_2$ has more than one ligand, including peroxisome proliferator-activated receptor-γ[14] and the second prostaglandin D_2 (PGD_2) receptor chemoattractant receptor homologous to the T helper 2 cell (CRTH2).[15] We have shown that $15dPGJ_2$ does not inhibit NF-κB via the peroxisome proliferator-activated receptor-γ receptor.[12] Whether CRTH2 plays a role in the mechanism of NF-κB and COX-2 inhibition by $15dPGJ_2$ is currently unknown.

CRTH2 is a G protein-coupled receptor linked to the $G_{\alpha i/o}$ subunit.[16] It is the classical receptor of the T helper type 2 (Th2) cell,[17] and has also been identified on eosinophils[18] and basophils.[19] CRTH2 mRNA has been detected in non-pregnant human uterine tissue,[20] placenta and choriodecidua.[21] Prostaglandin D_2 stimulates the production of the Th2 cytokines IL-4, IL-5, IL-13 and IL-10 in cultured Th2 cells *in vitro*.[22] Interleukin-4 is a classic Th2 cytokine that is able to inhibit the Th1 response directly, with IL-10 inhibiting the production of inflammatory mediators indirectly.[23] Interleukin-10 has also been shown in the mouse to protect the fetus by reducing fetal loss as a result of pro-inflammatory cytokines.[24] The function of CRTH2 in non-immune cells remains unclear.

We sought to determine if a small molecule CRTH2 agonist was able to mimic the effects of $15dPGJ_2$ by exerting anti-inflammatory effects and subsequently delaying preterm labour and providing neuroprotection for the fetus and increased pup survival. The effect of CRTH2 agonists on murine uterine contractility was examined *ex vivo* using a myograph.

Materials and methods

Reagents

The small molecule agonist CRTH2, referred to from now on as Pyl A, was synthesized commercially by Oxygen Healthcare, (Cambridge, UK) and is chemically identical to the L-888 607 compound from the Merck Frosst Centre for Therapeutic Research (Quebec, QC, Canada).[25] The compound has an indole core with an acetic acid side chain and a phenyl sulphide group, which is para-substituted by a chlorine atom. Based on the pharmacokinetics of 5 mg/kg described by Gervais *et al.*,[25] 250 μg of Pyl A was used for intrauterine injection. The CRTH2 antagonist GSKCRTH2X was obtained from Glaxo Smith Kline, (London, UK) and $15dPGJ_2$ from Cayman Chemicals (Ann Arbor, MI). *Escherichia coli* LPS serotype 0111: B4 (Sigma, St Louis, MO) was used in the murine model of inflammation-induced preterm labour.

Ethics statement

Human blood from non-pregnant women of childbearing age was collected in accordance with the South East London Ethics Committee approval Ref: 10/H0805/54, and in accordance with Imperial College NHS Healthcare Trust Research and Development department where recruitment took place. All blood was collected with written informed consent. Animal studies were performed under UK Home Office Licence 70/6906 and in accordance with the UK Animals (Scientific Procedures) Act of 1986, and the Imperial College Ethics Review Board.

Flow cytometry of granulocytes for detection of CR3 (CD11b) expression

A protocol based on previous studies on CR3 (CD11b) expression was followed.[15] Four millilitres of human blood was collected in sodium citrate vacutainers and the granulocyte fraction was isolated by incubating 1 : 1 blood : 4·5% Dextran (Fluka Analytical) in PBS for

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45 min at 4°. The leucocyte fraction was centrifuged at 500 ***g*** for 10 min, and the pellet was resuspended in PBS containing $CaCl_2$ (0·9 mM) and $MgCl_2$ (0·5 mM) and counted. Cells were then pre-incubated at 37°, followed by treatment with the CRTH2 agonists Pyl A or $15dPGJ_2$ for 15 min. The reaction was terminated by the addition of 1 ml ice-cold FACSFlow. In experiments with the CRTH2 antagonist, pre-incubation with GSKCRTH2X was performed for 10 min at 37°. The cells were then centrifuged at 400 ***g*** for 5 min at 4° and resuspended in PBS with 2% fetal calf serum for labelling with phycoerythrin-conjugated anti-CD11b and allophycocyanin-conjugated anti-CD49d for 10 min at 4° in the dark. The red cells were then lysed by the addition of Optilyse-C for 10 min in the dark at room temperature. Cells were then washed and resuspended in PBS and 1% fetal bovine serum for analysis. Eosinophils were identified as CD49d positive and by high side and forward scatter. Flow cytometry settings were as follows: Forward scatter E0 Voltage, 1·00 Amp gain Lin, and Side scatter of 329 Voltage, 1·00 Amp gain Lin.

Murine model of infection-induced preterm labour

CD1 outbred virgin female and stud male mice (Charles River, Margate, UK) were purchased at 6–8 weeks of age. All mice were housed in open cages at 21 ± 1°, on a 12 : 12 light : dark cycle regimen, with *ad libitum* access to standard chow and water. Timed mating was performed, with the presence of a copulatory plug being classed as E0 (day 0) of gestation. A mini-laparotomy was performed on embryonic day 16 (E16) of gestation correlating with human gestation of between 33 and 34 weeks. Morphine analgesia (2·5 mg/kg) was administered subcutaneously 20 min before surgery. Both uterine horns were exteriorized and the number of live fetuses per horn was determined. Twenty micrograms (25 μl total volume) *Escherichia coli* LPS serotype 0111:B4 (Sigma) or sterile PBS was injected into the upper right uterine horn between the first and second sacs taking care not to enter the amniotic cavity. Two-hundred and fifty micrograms Pyl A or vehicle control was then injected between the second and third sacs. Treatment groups consisted of (i) vehicle, (ii) LPS, (iii) LPS and Pyl A and (iv) Pyl A alone.

Animals were allowed to recover before fetal wellbeing assessment and tissue collection (myometrium and pup brain) at 4·5 hr post injection. A qualitative assessment of fetal viability was made in accordance with Pinto-Machado[26] Fetuses were deemed viable if they were pink and moved spontaneously or in response to stimulus. In subsequent experiments dams were allowed to deliver spontaneously. Continuous monitoring was achieved via a remote infrared CCTV system. A dose—response for the LPS was first performed to obtain the lowest dose at which preterm delivery was consistently obtained. For tissue harvesting, mice were anaesthetized and killed by cervical dislocation. A laparotomy was performed immediately and pups were killed by decapitation in accordance with the project licence. Before processing tissue, uteri were incised in the longitudinal direction and pups were expelled. Right and left horns of the uterus were snap frozen separately with placentas and vasculature removed. Myometrium from the frozen left uterine horns were used for analysis. Pup brains were also extracted and snap frozen. Tissue was stored at −80° until processing.

Protein extraction

Tissue was ground with a pestle and mortar in liquid nitrogen and homogenized in whole cell lysis buffer (150 mM NaCl, 20 mM Tris–HCl pH 7·5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, with phosphatase Inhibitor (Sigma) and protease inhibitor (Roche)). The homogenate was incubated on ice for 5 min and centrifuged for 20 min at 13 000 rpm at 4°. The supernatant was stored at −80° until use. Protein quantification was performed using the Bio-Rad assay, measuring absorbance at 655 nm (Bio-Rad, Hercules, CA).

SDS–PAGE and Western blotting

Approximately 15 μg extracted protein per sample was resolved by SDS–PAGE and subsequently transferred onto PVDF membranes (GE Healthcare) at 100 constant V at 4°. Following transfer, the membrane was then blocked in 5% (weight/volume) milk in TBST × 1 for 1 hr. The membrane was then probed with phospho-p65 (Ser 536) (Cell Signalling) primary antibody (1 : 1000 in TBS) overnight at 4° or COX-2 (Santa Cruz) primary antibody (1 : 2000 in 1% milk in TBS) for 2·5 hr at room temperature, followed by secondary antibody (1 : 2000 in 1% milk/TBS) for 1 hr at room temperature. Chemiluminescence detection was then carried out with ECL Plus (GE Healthcare). The membranes were developed using a high-performance chemiluminescence film (GE Healthcare). Blots were scanned and densitometry was performed with ImageJ (v1.44p).

Detection of CRTH2 and interleukin mRNA

Total RNA was isolated from tissue with Trizol© according to the manufacturer's instructions. Tissue was washed in PBS and homogenized using the power homogenizer in 1 ml Trizol© per 100 mg of tissue. 1 μg RNA was incubated with 1 μl DNase and 1 μl DNase buffer made up to 10 μl volume with diethylpyrocarbonate-treated water for 15 min at room temperature for removal of contaminating DNA. Eight microlitres of the DNAse-treated mix was incubated with 1 μl 10 mM dNTP and 1 μl oligo-dT(12–18) (0·5 μg/ml) for 5 min at 65°. To

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this mix, 2 μl 10X RT buffer, 4 μl 25 mM $MgCl_2$, 2 μl 0·1 mM dithiothreitol, 1 μl RNAse Out and 1 μl Superscript III was added. (In the reverse transcriptase controls no Superscript III was added.) The mix was incubated at 42° for 10 min and the reaction was terminated at 70° for 15 min. Then 0·5 μl RNAse H was added and the mix was incubated at 37° for 20 min. Samples were stored at −20° until further use. PCR was used to amplify the cDNA. Paired oligonucleotide primers for amplification of the genes of interest were designed to produce amplicons where the intron/exon boundary was crossed wherever possible. Non-template reverse transcriptase controls were used. Table 1 provides the primers for CRTH2, L-19, COX-2 and the cytokines IL-4, IL-10, interferon-γ (IFN-γ) and TNF-α.

Multi-spot enzyme-linked immunosorbent assay

The mesoscale discovery multi-spot ultrasensitive mouse Th1/Th2 9-plex assay was used as per the manufacturer's protocol for the detection of the following cytokines: IL-12, IFN-γ, TNF-α, IL-1β, KC/GRO, IL-4, IL-5, IL-10 and IL-2. Cytokines were quantified against an eight-point calibration curve from 0 to 2500 pg/ml, constructed from serially diluted standards provided by the kit. The 96-well multi-spot plate was blocked in 1% BSA in PBS for 1 hr before the addition of 40 μg of murine myometrium or 100 μg of pup brain protein lysate and incubated for 2 hr at room temperature. The multi-spot ELISA plate was read using a Sanger 2400 imager. The quantities of cytokines were determined against the standard curve and transferred into an EXCEL spreadsheet for further analysis.

Myometrial contractility

Mice were killed by cervical dislocation at E15–16 of gestation; the uterus was harvested, kept in PBS on ice and was used within 5 hr of harvesting. The uterus was dissected either in the longitudinal or horizontal direction to expel the fetuses and the placentas. Vasculature and decidua were removed macroscopically, and 5 × 10 mm strips were mounted on the DMT myograph (DMT, Aarhus, Denmark) in the orientation dependent on the muscle type being examined; longitudinal direction for longitudinal muscle and horizontally for the circular muscle orientation. Strips were stretched to 3 g of tension in the organ baths containing 4 ml Krebs solution (glucose 2·0 g/l, magnesium sulphate 0·141 g/l, potassium phosphate 0·16 g/l, potassium chloride 0·35 g/l, sodium chloride 6·9 g/l, calcium chloride dehydrate 0·373 g/l, sodium bicarbonate 2·1 g/l, pH 7·4) and was gassed with 95% O_2 and 5% CO_2. Tissue was allowed to equilibrate for 30 min. Cumulative dose responses were performed after 30 min of spontaneous contractions were recorded to serve as baseline contractility. At the end of the experiment 10^{-7} M oxytocin was added to demonstrate strip viability. Concentrations from 0·1 to 100 μm were added every 20 min at the time of organ bath wash out. Contractility was analysed using the POWERLAB software V 5.5.6 (ADI instruments) using the peak parameters extension. Data were transferred from the datapad of the POWERLAB software onto an EXCEL spreadsheet for analysis. Response to treatment was measured by normalizing to baseline spontaneous contractility and divided by the relevant time-point for the vehicle control.

Statistical analysis

Experimental groups consisted of at least three replicates unless otherwise stated. Statistical analysis was performed with GRAPH-PAD PRISM v5 (GraphPad Software, San Diego, CA). One-way analysis of variance or analysis of variance of repeated measures was conducted, with either Dunnett's or Bonferroni's multiple comparisons tests. Samples with $P < 0·05$ were considered to be statistically significant.

Results

CRTH2 mRNA

CRTH2 mRNA was detected in murine myometrium by RT-PCR, using L-19 as a housekeeping gene. No significant difference in CRTH2 expression was seen between

Table 1. Primer sequences used for amplification of CRTH2, cyclo-oxygenase 2 (COX-2), L-19 and the cytokines: interleukin-4 (IL-4), IL-10, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α)

	Forward 5′–3′	Reverse 5′–3′	bp
CRTH2	CGTAGCCGTGAGCCTGCGACTG	GGCGATTGCGGAGCCCACCACT	344
COX-2	AGCCAGGCAGCAAATCCTTGCTGTT	TCAAATCCTGTGCTCATACATTCC	71
IFN-γ	AGCGGCTGACTGAACTCAGATTGTAG	CGGGTGTAGTCACAGTTTTCAGCTGTATAGG	252
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC	175
IL-4	CGAAGAACACCACAGAGAGTGAGCT	GACTCATTCATGGTGCAGCTTATCG	237
IL-10	ACCTGGTAGAAGTGATGCCCCAGGCA	CTATGCAGTTGATGAAGATGTCAAA	181
L-19	GAAAAAGAAGGTCTGGTTGGA	TGATCTGCTGACGGGAGTTG	72

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the treatment groups (Fig. 1). Amplification of CRTH2 was seen by cycle 33 and L-19 by cycle 19.

Pyl A up-regulates CR3 (CD11b) via CRTH2

The CRTH2 agonists PGD_2 and $15dPGJ_2$ increase the expression of CR3 (CD11b) on eosinophils and basophils via CRTH2.[15,27] Before experiments with the CRTH2 agonist Pyl A, activity at the CRTH2 receptor was confirmed by demonstrating up-regulation of CR3 (CD11b) in human eosinophils. We used flow cytometry to detect CR3 (CD11b) expression on eosinophils, identified by high intensity CD49d expression and forward and side scatter characteristics (Fig. 2). Up-regulation of CR3 (CD11b) expression with Pyl A treatment was demonstrated by an increase in mean fluorescence intensity of CD11b-PE ($P < 0{\cdot}01$). This effect was attenuated with previous incubation of cells with the CRTH2 antagonist GSKCRTH2X (Fig. 2a,b). The effect of Pyl A was identical to the effect of $15dPGJ_2$ in causing increased expression of CR3 (Fig. 2c).

Pyl A induces preterm labour in the mouse

We sought to determine if the CRTH2 agonist Pyl A had the same tocolytic and feto-protective effect as $15dPGJ_2$ in delaying preterm labour in LPS-treated mice. A dose–response effect was demonstrated with LPS (serotype 0111:B4) since varying potencies can be seen between serotypes and within batches.[28] Administration of 20 μg LPS led to reliable preterm delivery with the least variation between mice (Fig. 3a). No surviving pups at the time of delivery were seen with concentrations above 10 μg (Fig. 3b). Subsequent experiments were performed with

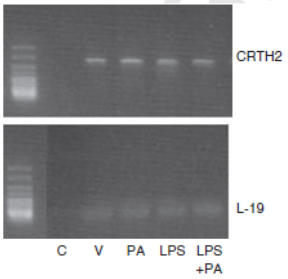


Figure 1. Murine myometrial CRTH2 mRNA. The mRNA was isolated from murine uterus and converted to cDNA ($n = 3$ per treatment group). RT-PCR was used to amplify CRTH2 showing a product size of 344 bp. No difference in CRTH2 expression was seen between treatment groups. CRTH2 mRNA expression was comparable between mice. C = Non-template control, V = vehicle, PA = Pyl A, LPS = lipopolysaccharide.

20 μg LPS. Twenty micrograms LPS with vehicle or 250 μg Pyl A was given by intrauterine injection under general anaesthetic and mice were allowed to labour spontaneously. Vehicle control mice delivered 64·5 hr post injection and LPS-treated mice delivered 7·7 hr post injection ($P < 0{\cdot}001$) (Fig. 4a). Co-injection of LPS and Pyl A augmented delivery to 5·8 hr (mean) post injection (Fig. 4a). This effect was more pronounced with a higher dose of Pyl A (500 μg) and lower dose of LPS (10 μg), shortening delivery time from 14·7 to 8·7 hr post injection ($P < 0{\cdot}01$) (Fig. 4b). Although at 250 μg Pyl A alone did not induce labour, at 500 μg labour was induced at 44·8 hr post injection from 64·6 hr in the vehicle control group. None of the vehicle control-treated mice delivered preterm.

Pyl A prevents LPS-induced intrauterine death

We then determined if the CRTH2 agonist Pyl A maintained the same feto-protective effect as $15dPGJ_2$ by examining fetal wellbeing at 4·5 hr post intrauterine injection of LPS with vehicle or Pyl A. Mice were anaesthetized and underwent a caesarean section. Fetuses were assessed for viability by assessment of colour and movement with or without mechanical stimulus. A significant improvement in fetal viability was observed when LPS-treated mice were co-injected with Pyl A compared with LPS and vehicle control. There was a clear difference in the appearance between both groups, in that the LPS-treated mice were clearly dead with no respiratory effort, whereas the LPS/Pyl A-treated mice were pink, moved spontaneously or with stimulus, and had respiratory effort. Fetal survival was increased from 20% in LPS-treated mice to 100% in LPS/Pyl A-treated mice, ($P < 0{\cdot}0001$) (Fig. 5a). However, following spontaneous labour no pups were viable in the LPS-treated and LPS/Pyl A-treated groups (Fig. 5b).

The effect of Pyl A on the inflammatory cascade in the myometrium and fetal brain

To explore the mechanisms behind Pyl A-augmented LPS-induced preterm labour, key mediators of inflammation in the myometrium were investigated. Myometrium and pup brain were harvested at 4·5 hr post intrauterine injection and Western blotting was used to detect whole cell phospho-p65 and COX-2. Administration of LPS did not lead to an increase in NF-κB in the myometrium; however, an increase was seen with co-administration of LPS and Pyl A ($P < 0{\cdot}05$) (Fig. 6a). A reduction was seen in NF-κB in pup brain with LPS compared with vehicle control, with no increase with co-administration with Pyl A (Fig. 6b). No significant difference in COX-2 protein expression was seen between treatment groups in the myometrium or pup

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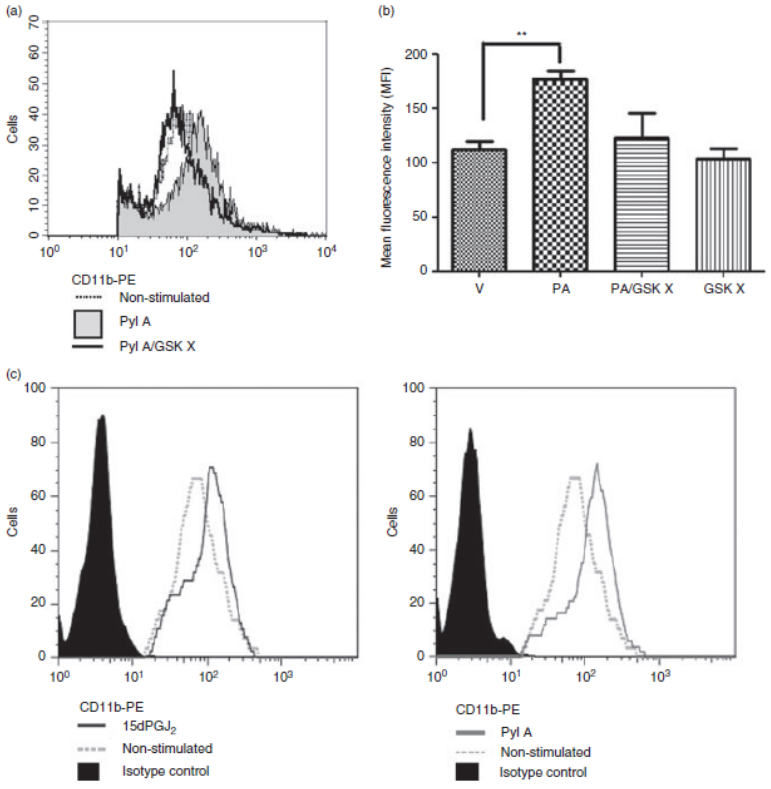


Figure 2. The effect of Pyl A and GSKCRTH2X on CR3 (CD11b) expression on eosinophils. Pyl A (32 μm) was used to increase CR3 (CD11b) [30] expression on eosinophils. Pre-treatment with the CRTH2 antagonist GSKCRTH2X (100 μm) was used to confirm this effect was via CRTH2. Eosinophils were identified by labelling with anti-CD49d and on the basis of forward and side scatter. A representative histogram reveals a clear shift to the right with Pyl A treatment indicative of an increase in CR3 (CD11b) expression. This effect was attenuated with CRTH2 antagonist pre-treatment (a). A summary of CR3 (CD11b) expressed in eosinophils with each treatment is shown in the graph ($n = 3$) (b). The effect of Pyl A on CR3 expression is identical to that of $15dPGJ_2$. A representative histogram is shown for the effect of both $15dPGJ_2$ and Pyl A on CR3 expression (c). V = vehicle, PA = Pyl A, GSK X = GSKCRTH2X. For statistical analysis, analysis of variance of repeated measures with Dunnett's post hoc test was used; $^{**}P < 0.01$.

brain at this time-point (Fig. 6c,d). However, the messenger RNA of COX-2 was increased in the myometrium of dams treated with Pyl A and LPS compared with other treatment groups (Fig. 6e).

The effect of Pyl A on inflammatory cytokines

We next sought to determine whether activation of NF-κB resulted in downstream activation of pro-inflammatory cytokines. As the CRTH2 agonist PGD_2 induces the production of the Th2 cytokines IL-10 and IL-4 in human T cells,[22] we anticipated that Pyl A would lead to an increase in these anti-inflammatory cytokines and an inhibition of the pro-inflammatory cytokines. However, consistent with NF-κB activation, Pyl A and LPS led to an increase in the pro-inflammatory cytokines (Fig. 7). Messenger RNA of the Th1 cytokines IFN-γ and TNF-α [24] was significantly increased at 4·5 hr post injection ($P < 0·05$ and $P < 0·01$, respectively); however, the increase in protein expression did not reach statistical significance. Protein expression levels of other pro-inflammatory cytokines were significantly elevated including IL-1β, KC/GRO (the murine chemokine equivalent of human IL-8[29]), and IL-12 ($P < 0·05$).

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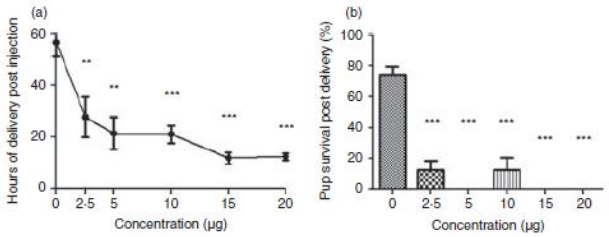


Figure 3. Dose–response of lipopolysaccharide (LPS) induced preterm labour. CD1 mice received an intrauterine injection of vehicle or LPS at E16 and were allowed to deliver, (n = 3–10 per treatment group). No vehicle control mice delivered preterm and LPS induced preterm labour in a dose–response effect, (a). No surviving pups were seen at an LPS dose of >10 μg (b). For statistical analysis, one-way analysis of variance with Dunnett's post hoc test comparing all groups to the vehicle control was used; $^{**}P < 0.01$, $^{***}P < 0.001$.

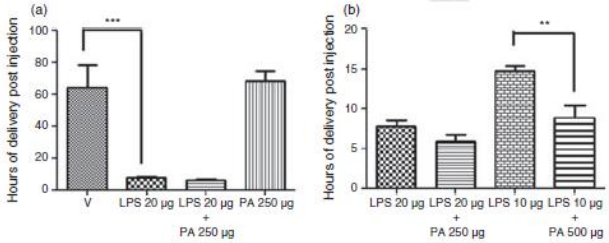


Figure 4. The effect of Pyl A on lipopolysaccharide (LPS) -induced preterm labour. CD1 mice received an intrauterine injection of vehicle, LPS or Pyl A at E16 and were allowed to deliver, (n = 4 per treatment group). No vehicle control mice delivered preterm, and at 250 μg Pyl A alone did not induce preterm labour (a). LPS induced preterm labour in a dose–response manner, and this effect was augmented with the addition of Pyl A (b). V = vehicle, PA = Pyl A. For statistical analysis, one-way analysis of variance with Bonferroni's multiple comparison's test was used; $^{**}P < 0.01$, $^{***}P < 0.001$.

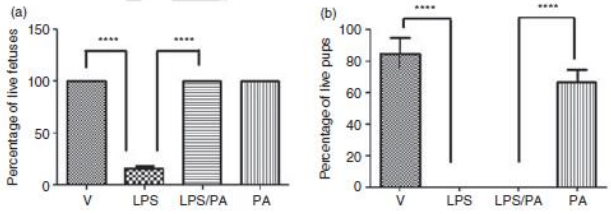


Figure 5. Effect of Pyl A on intrauterine fetal viability at 4.5 hr post intrauterine injection. Dams were killed at 4.5 hr post intrauterine injection of 20 μg of lipopolysaccharide (LPS), 250 μg of Pyl A and pup viability was assessed (n = 3 dams). An average of 11–14 pups per dam was seen in each treatment condition. Pyl A significantly increased viability at 4.5 hr post injection from 20% survival to 100% (a). In a subsequent experiment mice were allowed to deliver spontaneously. No pups in the LPS-treated or LPS/Pyl A-treated groups survived premature delivery (b). V = vehicle, PA = Pyl A. For statistical analysis, one-way analysis of variance with Bonferroni's multiple comparison test was used; $^{****}P < 0.0001$.

The effect of Pyl A on the anti-inflammatory cytokines

The mechanism of increased *in utero* fetal survival seen with Pyl A was explored by analysing the mRNA and protein expression of Th2 anti-inflammatory cytokines in the myometrium and pup brains. There was no difference in IL-4 mRNA between treatment groups, and protein concentrations were below the detection level of the assay. There was a slight increase in the production of IL-5, and

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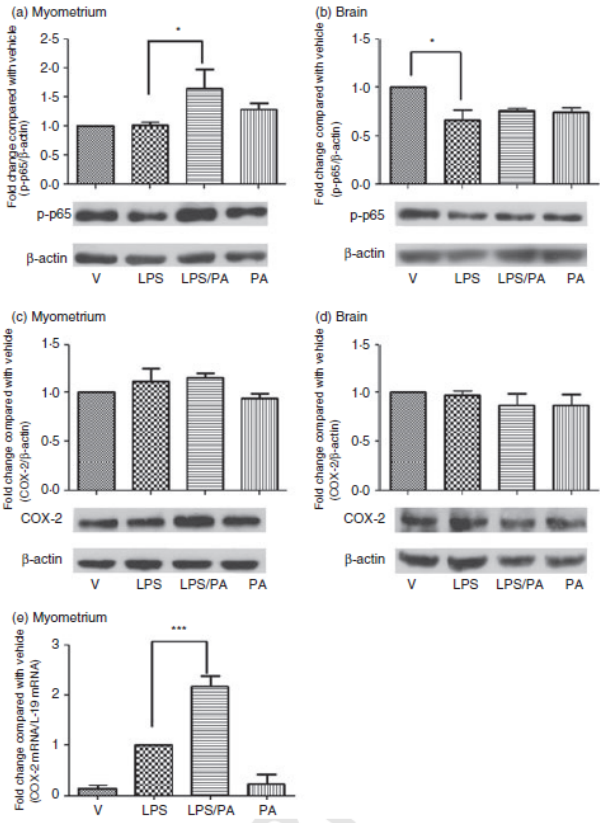


Figure 6. The effect of lipopolysaccharide (LPS) and Pyl A on myometrial and pup brain nuclear factor-κB (NF-κB) and cyclo-oxygenase 2 (COX 2). Dams were killed at 4.5 hr post intrauterine injection and tissue was harvested for protein analysis of phospho-p65 and COX-2 ($n = 3$). Co-injection of LPS and Pyl A increased myometrial phospho p65 (a), with no effect on pup brain phospho-p65. Phospho-p65 was decreased in brains of pups from dams treated with LPS alone (b). Representative phospho-p65 immunoblots are shown for each treatment group with B actin used as a loading control. No significant changes in COX-2 protein expression were seen in any treatment group in both myometrium and pup brain (c,d). However, an increase in messenger RNA of COX-2 was seen in LPS-treated and LPS/Pyl A-treated mice (e). For statistical analysis, one-way analysis of variance with Bonferroni's multiple comparison test was used; $^{*}P < 0.05$, $^{***}P < 0.001$.

an increase in both mRNA and protein expression of IL-10, which did not achieve statistical significance (Fig. 8). These interleukins were not detectable in fetal brain samples (data not shown).

The effect of Pyl A on uterine contractility

To determine if Pyl A had a direct effect on uterine contractility, uteri were harvested from mice on E15–16, dissected and mounted on the myograph in the circular orientation. Pyl A inhibited myometrial contractility from a concentration of 10 μm ($P < 0{\cdot}01$), with complete inhibition seen with 100 μm ($P < 0{\cdot}001$) (Fig. 9a,b). The effect of Pyl A on longitudinal muscle was also examined by mounting the strips along the longitudinal orientation. Contractility was not maintained in the longitudinal orientation for the whole duration of the experiment in control strips to robustly examine the effect of Pyl A on longitudinal muscle contractility. Despite this, the clear inhibition seen in the circular muscle was not evident in the longitudinal strips (data not shown). The inhibition of contractility in circular muscle was probably not CRTH2-mediated because other agonists, $15dPGJ_2$ and 13,14-dihydro-15-keto-prostaglandin D_2 (DK-PGD_2), did not have the same effect (Fig. 9c–f).

Discussion

The search for preventative therapies for both preterm birth and related neurological injury has largely focused upon anti-inflammatory strategies. It is generally accepted that parturition is a pro-inflammatory event, with preterm labour being associated with an exaggerated inflammatory response and infection. When women present in preterm labour, it is likely that inflammation precedes any clinical symptoms. We have previously reported that

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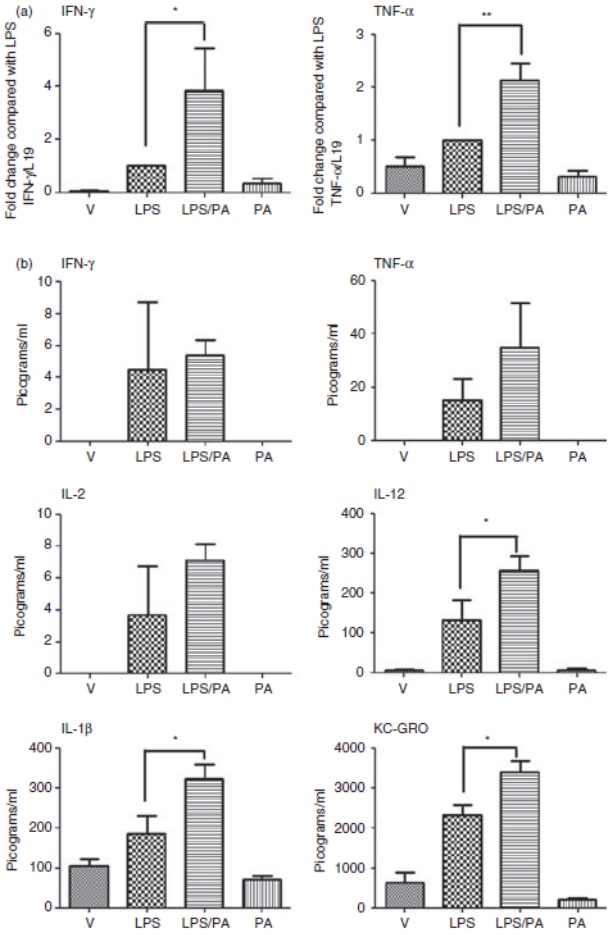


Figure 7. Effect of lipopolysaccharide (LPS) and Pyl A on the pro-inflammatory cytokines in the myometrium. Dams were killed at 4.5 hr post intrauterine injection of 20 μg LPS/250 μg Pyl A and mRNA and protein was extracted from the myometrium and analysed by PCR or ELISA ($n = 3$). With co-injection of LPS and Pyl A, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) mRNA were significantly increased (a) as well as protein levels of interleukin-12 (IL-12), IL-1β and KC-GRO (b). V = vehicle, PA = Pyl A. For statistical analysis, one-way analysis of variance with Bonferroni's multiple comparison's test was used; $^{*}P < 0.05$, $^{**}P < 0.01$.

the anti-inflammatory cyclopentenone prostaglandin and CRTH2 agonist $15dPGJ_2$ delay inflammation-induced preterm labour in the mouse and increases pup survival.[13] In this study we have examined the potential for acute administration of a small molecule CRTH2 agonist to improve both maternal and fetal outcomes in LPS-induced murine preterm labour.

The CRTH2 agonist Pyl A was manufactured based on the L-888 607 compound from the Merck Frosst Centre for Therapeutic Research in which radioligand binding assays were used to demonstrate the affinity of the compound to the CRTH2 receptor.[25] The CRTH2 agonist activity of Pyl A was confirmed with a gold standard experiment based on the work of Cossette, Monneret and Nagata, in which the CRTH2 agonists PGD_2, $DK\text{-}PGD_2$, indomethacin and $15dPGJ_2$ cause up-regulation of CR3 (CD11b) in granulocytes.[15,27,30–32] Pyl A caused a significant increase in the expression of CR3 (CD11b) in human eosinophils, which could be attenuated by pre-incubation with the CRTH2 antagonist GSKCRTH2X (Fig. 2), further confirming activity at the CRTH2 receptor. CR3 (CD11b) up-regulation via CRTH2 is believed to aid cell adhesion to the vascular wall for migration of cells from the blood into tissue at sites of inflammation.[33] The murine *CRTH2* gene was first cloned and characterized by Abe *et al.*[34] and shares 77% homology with the

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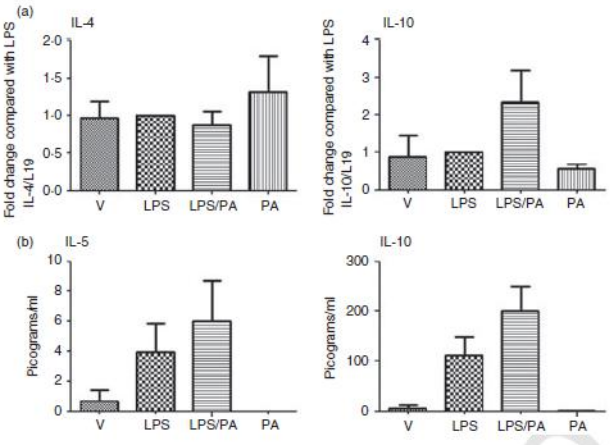


Figure 8. Effect of lipopolysaccharide (LPS) and Pyl A on the anti-inflammatory cytokines in the myometrium. Dams were killed at 4.5 hr post intrauterine injection of 20 μg LPS/250 μg Pyl A and mRNA and protein was extracted from the myometrium and analysed by PCR or ELISA. No change in interleukin-4 (IL-4) mRNA expression was seen, and a non-significant increase in IL-10 was seen with co-administration of Pyl A and LPS (a). Although there was an increase in protein levels of IL-10 and IL-5 with co-injection of LPS and Pyl A, this did not reach statistical significance (b). V = Vehicle, PA = Pyl A.

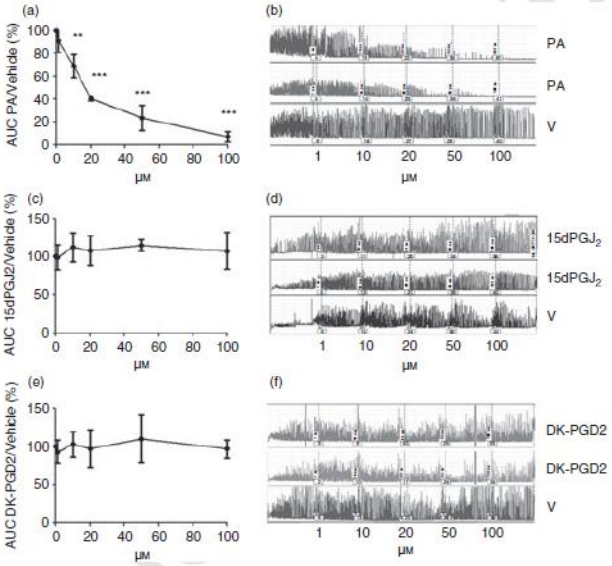


Figure 9. The effect of CRTH2 agonists on circular muscle contractility. CD1 mice were killed on E15–16 and myometrial tissue was harvested to mount on the myograph in the circular orientation ($n = 3$). A cumulative dose–response of CRTH2 agonists or vehicle was performed and the average area under the curve was determined. An inhibition in myometrial contractility was seen with Pyl A, but no effect with $15dPGJ_2$ or $DK\text{-}PGD_2$. Graphs showing the effects of the CRTH2 agonists are shown (a,c,e), with representative traces of myometrial contractility (b,d,f). V = vehicle, PA = Pyl A. For statistical analysis, analysis of variance of repeated measures was used with Dunnett's multiple comparison test; $^{**}P < 0.01$, $^{***}P < 0.001$.

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human CRTH2 receptor gene. The pharmacologies of the human and mouse CRTH2 receptors are virtually identical, and the receptors share 90% homology within the transmembrane domains.[35] The CRTH2 agonists PGD_2, $DK\text{-}PGD_2$, $15dPGJ_2$ and indomethacin all show activity to the mouse CRTH2 receptor.[36–39] $15dPGJ_2$ binds to the mouse CRTH2 receptor with an affinity several orders of magnitude greater than that seen for peroxisome proliferator-activated receptor-γ.[39,40] We detected CRTH2 mRNA in the mouse myometrium using the primers used by Abe *et al.*,[34] (Fig. 1). There was no difference in mRNA expression between vehicle and Pyl A-treated or LPS-treated mice and LPS/Pyl A-treated mice. However, the degree of expression seen at the mRNA level suggests that CRTH2 is expressed in the myometrium. Determining if expression is seen on both myocytes and infiltrating leucocytes or leucocytes alone has not been possible because of the lack of available specific antibodies to murine

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CRTH2. Human studies have demonstrated mRNA expression in the myometrium, but flow cytometry confirms the absence of the expressed protein in cultured myocytes.[41] CRTH2 positive leucocytes are also detected in the endometrium and are likely to be recruited to decidua via PGD_2.[42,43]

We have previously reported that the CRTH2 agonist $15dPGJ_2$ delays LPS-induced preterm labour in the mouse, which is thought to be via NF-κB inhibition in the myometrium.[13] $15dPGJ_2$ also inhibits NF-κB in human cultured amniocytes and myocytes;[12] however, the mechanism by which NF-κB inhibition is achieved is unclear. It was therefore hypothesized that Pyl A could reproduce the effects of $15dPGJ_2$ of delaying preterm labour by diminishing the pro-inflammatory effect of LPS via NF-κB inhibition. However, co-injection of LPS-treated mice with Pyl A was found to exacerbate time to preterm labour in a dose-dependent response (Fig. 4b). To establish the mechanism of Pyl A-augmented labour onset we first examined the effect of Pyl A and LPS on the pro-inflammatory and pro-labour transcription factor NF-κB and its downstream targets. Treatment of animals with Pyl A alone increased NF-κB activity in the myometrium, which was enhanced with co-administration of LPS (Fig. 6a). The inability of Pyl A to inhibit NF-κB implies that CRTH2 is not involved in the mechanism of $15dPGJ_2$-mediated inhibition. In support of this, we demonstrated that CRTH2 is not required for $15dPGJ_2$-mediated inhibition of NF-κB in human amniocytes, myocytes and lymphocytes.[41]

Surprisingly, myometrial COX-2 protein levels remained unchanged 4·5 hr post treatment in all groups. As preterm labour was typically induced following LPS/Pyl A treatment at 5·8 hr (SEM ± 0·7) it was expected that any COX-2 up-regulation in the myometrium should have already been apparent by 4·5 hr post treatment. It is possible that COX-2 was already up-regulated before intrauterine injection in preparation for term labour, which is one limitation of using a model at E16. Progesterone withdrawal in the mouse occurs late E16 and so downstream activation of pro-labour genes is not likely to have been initiated in our model.[44] Consistent with this the majority of labour-associated proteins such as PGE_2, $PGF_{2\alpha}$, the oxytocin receptor and Connexin-43 are not significantly up-regulated until E18.[45,46] We have shown, however, that COX-2 is suppressed in pregnancy and is up-regulated from E16, which was not increased further in term labour.[47] We further explored the possibility that, despite seeing no change at the protein level, COX-2 was still activated by LPS and LPS plus Pyl A. Messenger RNA was indeed increased in LPS-treated mice, and was further increased with co-injection of Pyl A (Fig. 6e). COX-2 requires peroxidases for activation and the endogenous peroxide tone of smooth muscle cells can be mimicked by nitration.[48] Previous studies have shown that peroxynitrite increases the activity of COX-2 with no alteration of COX-2 protein expression.[49,50] Consistent with our results, Aisemberg *et al.*[51] demonstrated an increase in LPS-induced mRNA COX-2 with no effect at the protein level. It is plausible that this is a result of LPS-induced NO leading to the formation of peroxynitrite, which in turn, activates COX-2 without alteration of protein expression. Alternatively, it is also plausible that the nitrated form of COX-2 is not recognized by the COX-2 antibody.

Analysis of pup brain extracts collected from LPS-treated dams revealed a decrease in levels of phosphorylated p65 (ser 536). It is thought that this may reflect protein degradation induced by the pre-terminal state of the live pups (Fig. 6b). A significant increase in *in utero* fetal viability was achieved with Pyl A treatment (Fig. 5a) but this was not associated with altered NF-κB activity. This also highlights the contrasting effects of Pyl A compared with the $15dPGJ_2$ because we have previously shown that $15dPGJ_2$ inhibits NF-κB in the pup brain of dams treated with LPS.[13] Co-injection of Pyl A and LPS led to an increase in the production of the pro-inflammatory cytokines TNF-α, IFN-γ, IL-12, IL-1β and IL-8 (Fig. 7b). Pro-inflammatory stimuli such as LPS[52] and the cytokines TNF-α[53] and IL-1[54] have been shown to activate NF-κB via the canonical pathway by phosphorylating serines, leading to IκB degradation and translocation of the NF-κB complex into the nucleus, thereby activating gene expression of pro-inflammatory cytokines, which augments the pro-inflammatory response in a positive feed-forward loop.[5] The assessed cytokines were also increased but to a lesser degree in the absence of NF-κB activation with LPS treatment alone (Fig. 7a,b). It is therefore plausible that LPS and Pyl A co-administration activates a strong cytokine response, which then further induces NF-κB activation via a feed-forward mechanism. Lipopolysaccharide induces a strong inflammatory response and leads to the recruitment of leucocytes.[55,56] CRTH2 agonists also chemoattract CRTH2-positive leucocytes,[19,57] including Th2 cells,[19] eosinophils[58] and dendritic cells.[37] The increase in inflammatory cytokines seen with combined injection of both LPS and the CRTH2 agonist Pyl A may be as a result of the increase in infiltrating leucocytes rather than a direct effect on myocytes. Importantly, CRTH2 is also expressed on Th1 cells in the mouse, unlike the human, which is likely to have contributed to the unexpected pro-inflammatory response seen in the mouse.

Several murine studies with CRTH2 agonists/antagonists and the use of CRTH2 knock-out mice have shown a pro-inflammatory role for the CRTH2 receptor.[36,38,59–63] The CRTH2 agonist DK-PGD_2 causes eosinophilia in mouse lung[63] and intra-peritoneal administration of DK-PGD_2 causes a two-fold induction of monocyte chemoattractant protein-1 and a 25-fold induction of macrophage

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26 inflammatory protein-2 (the functional equivalent of the IL-8 receptor in the mouse).[36] Furthermore, in a murine study of FITC-induced inflammation of the skin (a model of contact hypersensitivity), a CRTH2 antagonist was found to significantly reduce the production of the pro-inflammatory cytokines TNF-α, IL-1β and the chemokines macrophage inflammatory protein-2 and GRO-α.[64] However, no distinction between the Th1 or Th2 type cytokines being modulated was made. Similarly reduced levels of lung IFN-γ, IL-4 and IL-5 have been observed in a mouse model of airway inflammation upon administration of a CRTH2 antagonist.[62]

Our finding of increased fetal viability with Pyl A in LPS-treated mice was surprising in view of the shortened time interval from injection to delivery. Although following spontaneous labour there were no surviving pups in the LPS and LPS/Pyl A treatment groups (Fig. 5b). We attribute this to the pups delivering preterm, based on unpublished data showing non-viability at E16 even in the absence of inflammation-induced preterm labour. The CRTH2 agonists indomethacin and PGD_2 lead to an increase in the production of the Th2 cytokines in human T cells *in vitro*,[22,30] which in turn can antagonize the Th1 response. *In vitro* stimulation of Th2 cells by PGD_2 requires much higher concentrations to stimulate IL-10 production compared with IL-4, IL-5 and IL-13.[1,22] We therefore examined the effect of Pyl A on the Th2-type anti-inflammatory cytokines in the myometrium (Fig. 8). Although no changes in levels of IL-4 were detected, an increase (non-significant) in IL-5 was observed (Fig. 8). Moreover, a non-significant increase in IL-10 mRNA and protein with LPS and Pyl A treatment was detected consistent with improved protection against LPS-induced fetal loss in mice[65] as well as the reduced rate of naturally occurring fetal loss in IL-10-deficient mice.[24]

Although Pyl A led to a small increase in the pro-labour transcription factor NF-κB and the pro-inflammatory cytokines, we did not see an increase in COX-2 protein expression. We therefore examined the direct effect of Pyl A on myometrial contractility *ex vivo*. Contrary to the expected uterotonic effect, Pyl A administration resulted in complete inhibition of circular muscle contractility (Fig. 9), but had no effect on longitudinal muscle. There is limited knowledge on the functional role of the individual muscle layers of the mouse uterus, the inner circular and outer longitudinal muscle, in pregnancy and parturition. In the myometrium of other species such as the pig and rat, it has been suggested that the function of the longitudinal muscle is to move luminal contents by contraction[66] and that tonic contraction of the circular muscle may be required for spacing and retention of embryos/fetuses.[67] Circular muscle cells have a higher spontaneous electrical activity than longitudinal muscle cells during rat pregnancy,[68] and weak high-frequency contractions in the circular muscle layer prevent movement of fetuses towards the cervix during pregnancy,[69] supporting its potential role in the maintenance of pregnancy. If circular muscle contraction is necessary for retention of uterine contents, this would explain how inhibition of circular muscle contraction by Pyl A leads to preterm expulsion of the fetuses, as seen in this study. Consistent with this, relaxation of uterine tone is also believed to be important during human labour.[70] It is proposed that relaxation of the lower segment of the uterus, in conjunction with contractions of the fundal region, is required for the passage of the fetus through the birth canal.

Alternatively, relaxation of circular muscle may not be important in murine labour. Many rodent studies suggest that by term, the function of circular muscle becomes more similar to the longitudinal layer, and that contractility of both the circular and longitudinal muscle is required for labour.[71–74] It is possible that despite the inhibitory effect on contractions seen with Pyl A *ex vivo*, that the overwhelming *in vivo* inflammatory effect was enough to overcome the tocolytic effect resulting in preterm labour. It is also plausible that the *in vivo* uterotonic effect of Pyl A occurs through indirect stimulation of immune cells and activation of the myometrium via an inflammatory response, rather than a direct action of Pyl A on myocytes. This is comparable to the indirect effect of LPS-induced labour, because addition of LPS to myometrial strips *ex vivo* does not lead to increased myometrial contractility.

The observed inhibition in myometrial contractility seen with Pyl A is likely to be through a CRTH2-independent mechanism as the other CRTH2 agonists $15dPGJ_2$ and $DK\text{-}PGD_2$ did not show the same effect. At higher concentrations, Pyl A is able to bind to other prostanoid receptors with the rank of order of affinity as follows: CRTH2> TP> EP3> DP> EP4> EP2> FP> IP> EP1.[25] Since the TP/IP/EP3/EP1 receptors are considered to be excitatory and the EP2/EP4 and DP1 receptors relaxatory, we hypothesize that Pyl A may be having off-target effects on one of the latter mentioned receptors. The effect of DP1 agonists on murine contractility has been investigated previously by several groups. We have shown that the EP2 agonist, but not EP4 agonist, inhibits human myometrial contractility.[75] Stimulation of the EP2 and EP4 receptors leads to cAMP production via the G protein Gs leading to smooth muscle relaxation.[76] Hence the effect seen in our study is potentially a result of non-specific binding with the EP2 receptor.

Conclusion

This study presents evidence that the CRTH2 agonist Pyl A augments a pro-inflammatory response in LPS-induced preterm labour in the mouse. Pyl A shortened the time interval from intrauterine injection to preterm delivery

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via increased NF-κB activity and increased production of pro-inflammatory cytokines. We also demonstrated a non-CRTH2-mediated inhibition of circular myometrial contractility *ex vivo*, which was likely to contribute to rapid expulsion of the fetus. Despite increased fetal viability seen with Pyl A in LPS-treated dams, an overwhelming pro-inflammatory response was seen with the CRTH2 agonist in the mouse. This may be secondary to a functional CRTH2 receptor on murine Th1 cells, unlike in humans. We conclude that $15dPGJ_2$-mediated inhibition of NF-κB is not mediated via CRTH2. The CRTH2 agonist seems to augment inflammation-induced preterm birth, so CRTH2 is unlikely to be a suitable therapeutic target for the prevention of preterm labour and neonatal morbidity.

Disclosures

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