The potential role of viral priming in bacterial induced preterm labour, and 15dPGJ2 in its prevention

Zahirrah Begam Mohamed Rasheed

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Department of Surgery and Cancer, Imperial College London

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Abstract

Introduction: Preterm labour occurs in 10% of pregnancies and is a major contributor of neonatal morbidity and mortality. Bacterial-induced preterm labour is thought to contribute to up to 40% of preterm births. However, evidence is mounting for the role of viruses in preterm labour with a 'double-hit' of both a viral and bacterial insult during pregnancy increasing susceptibility to bacterial-induced preterm labour. The transcription factors NF-κB and AP-1 are important in regulating key mediators for labour and are activated by the recognition of the toll-like receptors (TLRs) to invading microorganisms. The use of an anti-inflammatory 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15dPGJ2) has been shown to successfully inhibit IL-1β-induced NF-κB activation and delay preterm labour in a murine model of inflammation-induced preterm labour. **Aims:** The effect of 'double-hit' in human gestational tissue using *in vitro* models of ascending and haematogenous spread of infection was studied. 15dPGJ2 as an anti-inflammatory agent in a solid lipid nanoparticle form is investigated. **Methods:** Vaginal epithelial cells (VEC), amniocytes and myocytes were used as an *in vitro* model of ascending infection and placental explants and PBMC were used as an *in vitro* model of systemic infection. PCR, RT-QPCR were used for examining TLR expression. Western immunoblotting was used to detect phospho-p65, phospho-c-Jun and phospho-IRF3. ELISAs were used to examine cytokine, chemokine and PGE2 production. **Results:** Gestational tissues expressed all TLRs with the exception of vaginal epithelial cells which lacked TLR4 mRNA. TLR3 protein was detected and further induced with poly I:C (TLR3 agonist) stimulation in vaginal epithelial cells (VEC), amniocytes, myocytes, PBMC and placental explants. In the ascending infection model, local TLR3 priming prior to TLR4 and TLR2/6 stimulation augmented cytokine, chemokine and PGE2 production in amniocytes and myocytes compared to treatment with the individual ligand alone. In VECs, only TLR2/6 (following TLR3 priming) showed augmented pro-inflammatory cytokine and PGE2 production. In the haematogenous infection model, local TLR3 priming prior to TLR2 and 2/6 stimulation augmented pro- and anti-inflammatory, chemokines, and PGE2 production in PBMCs. In placental explants, only chemokines and IL-10 production were augmented. However, culturing fresh placental explants with TLR3-primed and TLR2-treated media derived from the PBMCs priming experiment led to augmented IL-6 and IL-8 production. Poly I:C stimulation alone increased the expression of TLR3 and TLR2 and phospho-IRF3 protein. The 15dPGJ2-loaded SLN failed to inhibit IL-1β induced NF-κB and AP-1. **Conclusion:** A 'double-hit' of viral and bacterial stimuli leads to an augmented pro-inflammatory response in human gestational tissue, with the greatest and most consistent effect seen with TLR3 priming prior to TLR2/6 agonist stimulation. It is possible that this is due to augmented activation of phospho-IRF3 and TLR3 mediated increased expression of the TLR2 receptor. These findings should alert us to the potential additive risk of preterm labour in women exposed to viruses in pregnancy. Whilst no anti-inflammatory effect was demonstrated with the 15dPGJ2-loaded SLN.

Statement of originality

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

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List of Conferences presentations

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1. **1st Prize for Scientific Undergraduate Academic Award**
   The effect of TLR3 activation on pro-labour & pro-inflammatory mediators in human placenta.
   Eberechi Anucha, Zahirrah Begam, Phillip Bennett, Lynne Sykes.
   Royal College of Obstetrician and Gynaecologist (RCOG), London, UK. March 2017.

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   The effect of 15-deoxy-Δ12,14-prostaglandin J2 and 15-deoxy-Δ12,14-prostaglandin J2 nanoparticles/nanocapsules on IL-1β induced NFκB activation in amnion and vaginal epithelial cells.
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   TLR3 Priming Increases TLR4- and TLR6-mediated Pro-inflammatory Cytokine Production in Amniocytes, Myocytes and Vaginal Epithelial Cells.
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3. The effect of TLR3 receptor priming on TLR2, 4 and 6 agonist-induced inflammation in placental explants.
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LIST OF ABBREVIATIONS

15dPGJ2	15deoxy$\Delta^{12,14}$-PGJ2
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of Variance
AP-1	Activator Protein 1
β-actin	Beta actin
bp	Base pairs
BPE	Bovine Pituitary Extract
BSA	Bovine serum albumin
CAPs	Contraction associated proteins
CCL5	chemokine ligand 5
CD14	cluster of differentiation 14
cDNA	Complementary deoxyribonucleic acid
CEBP/β	CCAAT-enhancer-binding protein beta
COX-2	Cyclooxygenase-2
cPLA2-α	Cytosolic phospholipase A2 alpha
CRH	Cortisol releasing hormone
Ct	cycle threshold value
DEPC	Diethylpyrocarbonate
DIC	Differential interference contrast
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
E.coli	Escherichia coli
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
F-12 HAM	Nutrient Mixture (F-12)
FBS	Fetal Bovine Serum
FIRS	Fetal inflammatory response syndrome
FRAP	Fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
FSL-1	Fibroblast-stimulating lipopeptide-1
HCL	Hydrocloric acid
HKLM	Heat Killed *Listeria Monocytogenes*
IFN	Interferon
IFN-α	Interferon Alpha
IFN-β	Interferon Beta

IFN-γ	Interferon Gamma
IgG	Immunoglobulin G
IL-10	Interleukin 10
IL-12p40	Interleukin-12 Subunit p40
IL-12p70	Interleukin-12 Subunit p70
IL-18	Interleukin 18
IL-1RA	Interleukin-1 Receptor Antagonist
IL-1α	Interleukin 1 Alpha
IL-1β	Interleukin 1 Beta
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IRF	Interferon regulatory factor
IRF5	Interferon regulatory factor 5
JNK	c-Jun NH2-terminal kinase
KCl	Potassium chloride
kDa	Kilo Dalton
KH_2PO_4	Monopotassium phosphate
KSFM	Keratinocytes Serum Free Media
L19	Ribosomal protein L19
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
MAPK	Mitogen activated protein kinase
Mg	Magnesium
$MgCl_2$	Magnesium chloride
MIAC	Microbial invasion of amniotic cavity
MIP-1α	Macrophage Inflammatory Protein 1-alpha
MIP-1β	Macrophage Inflammatory Protein 1-beta
MMP	matrix metallopeptidase
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response 88
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NF-κB	Nuclear factor-kappa B
NO	nitric oxide
OTR	Oxytocin receptor
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween
PCR	Polymerase Chain Reaction
PVDF	Polyvinyl difluoride
PGE_2	Prostaglandin E_2
p-IRF3	Phosphorylated interferon regulatory factor 3

PMSF	Phenylmethylsulfonyl Fluoride
Poly I:C	polyinosinic:polycytidylic acid
PPROM	Preterm premature rupture of the membranes
PR	Progesterone receptor
PRRs	Pattern recognition receptors
PTL	Preterm labour
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SDS	Sodium dodecyl sulphate
SLN	Solid lipid nanoparticle
TBE	Tris-borate-EDTA
TBST-T	Tris Buffered Saline with Tween® 20
TEMED	N, N, N, N-tetramethyl-1,2-diaminoethane
TGF-β	Transforming growth factor beta
TIR	Toll/interleukin-1 receptor
TIRAP	TIR Domain Containing Adaptor Protein
TLR	Toll-like receptor
TLR1	Toll like receptor 1
TLR10	Toll like receptor 10
TLR2	Toll like receptor 2
TLR3	Toll like receptor 3
TLR3/2	TLR3 agonist stimulation pre TLR2 agonist stimulation
TLR3/4	TLR3 agonist stimulation pre TLR4 agonist stimulation
TLR3/6	TLR3 agonist stimulation pre TLR6 agonist stimulation
TLR4	Toll like receptor 4
TLR6	Toll like receptor 6
TNF-α	Tumour Necrosis Factor Alpha
TNF-β	Tumour Necrosis Factor-beta
TRAF3	TNF receptor-associated factor 3
TRAF6	TNF receptor-associated factor 6
UV	Ultraviolet
VEC	Vaginal epithelial cells

CHAPTER 1

Introduction

1.1 Immunology

1.1.1. The immune system

Living organisms are exposed to a variety of potentially infectious organisms that can cause pathology and disease. The immune system has evolved to provide a carefully mediated response involving recognition, inflammatory activation and clearance of pathogens. The immune system can be broadly divided into the innate and adaptive immune system.

The innate immune response

The innate immune system is the first line of defence in protecting against harmful pathogens and includes physical (e.g. mucosal epithelia), chemical (e.g. soluble molecules such as cytokines and chemokines) and cellular barriers (e.g. epithelial and immune cells). The cells of innate immunity comprise of immune cells such as dendritic cells, neutrophils, natural killer cells, monocytes, eosinophils, basophils and macrophages [1]. These cells secrete a plethora of mediators such as TNF, interleukins (IL-1β, IL-4, IL-6, IL-10, IL-12, IL-18, IFN-γ), RANTES, TGF-β and others when evoked by an array of cellular receptors [1, 2].

When an organism is infected with a potentially harmful microbe, survival of the host organism depends on a rapidly mounted defence response. Such protective mechanisms are evolutionarily conserved in all multicellular organisms and are collectively referred to as innate immunity. Pattern recognition receptors (PRRs) recognize conserved molecular patterns called pathogen-associated molecular patterns (PAMPs), which distinguish foreign organisms such as viruses, bacteria, fungi and parasites from host cells. PAMPs include viral nucleic acids, components of bacterial and fungi cell walls and flagellar proteins. The PRRs family include NOD-like receptors (NLRs), RIG-like helicases (RLHs) and Toll-like receptors (TLR), which are the most extensively studied [3, 4].

The adaptive immune response

The adaptive immune system functions by recognizes specific antigens through T lymphocytes and B lymphocytes, however, it takes days or weeks to develop.

Adaptive immunity is also capable of forming memory cells that lead to a more vigorous and rapid response when exposed to the same antigens in the event of subsequent exposure. Invading pathogens can be destroyed by the adaptive immune response either directly or by facilitating cells of the innate immune response. These actions are carried out by the lymphocytes and include B cells for antibody responses, and T cells for cell-mediated immune responses. Antibodies, also called immunoglobulins, are secreted into the bloodstream or into mucosal secretions. They bind to pathogens and inactivate them by blocking their ability to bind to host cell receptors and by labelling them for destruction by the phagocytic cells of the innate immune system. Activated T cells, on the other hand, act by eliminating the infected cells based on the antigens that are presented on the cell surface of the infected host cell. Thus, stopping invasion of the viruses to the adjacent cells. T cells also are responsible for producing cytokines to activate macrophages to destroy microbes that they have phagocytosed [5].

1.1.2. Immunology of pregnancy

During pregnancy, the immune system is required to adapt a highly regulated balance between immune tolerance and activation. This balance is required in order to prevent rejection of the semi-allogenic fetus whilst protecting the mother and fetus from pathogens. In support of this, there is evidence to suggest weakening of cell mediated immunity during pregnancy in conjunction with a strengthening of humoral immunity [6].

The bias shifting from Th1 to Th2 during pregnancy is coordinated by both the innate and adaptive immune system. The innate immune system, although very primitive and non-specific, is very important in microbial infection during pregnancy. It compasses the physical barriers of the mucosal epithelia and other membranes, but, also includes neutrophils, macrophages and monocytes that protect the host by phagocytosing microbes or infected cells. For example, while the amniotic membrane acts as a physical barrier, neutrophils are able to cross into the amniotic cavity and protect the fetus from infectious organisms such as S*treptococcus agalactiae*, *Gardnerella vaginalis*, and *Ureaplasma urealyticum* [7]. These bacteria are recognised by TLRs, as part of the innate immune system at the maternal-fetal interface [8-10]. T cells at

this interface also favour a Th2 cytokine profile to adapt with pregnancy [11] in conjunction with other nonimmune cells such as trophoblast cells [12].

Immune adaptations during pregnancy also appear to be dependent on gestational age, both at the maternal-fetal interface and systemically [6, 13]. The first trimester is predominantly described as a pro-inflammatory state in order for implantation to be successful, whereby an anti-inflammatory state is described during the second trimester to aid fetal growth. During the first trimester, there is an accumulation of uterine-specific natural killer cells (uNK), macrophages (Mø) and dendritic cells (DCs) in the decidua [14]. These immune cells are vital since absence of either of these has a deleterious effect as proven in mice [15] and humans [16-18]. Depletion of uNK cells has been shown to impede trophoblast invasion in the uterus leading to miscarriage [19], while the absence of DCs impede decidualisation and angiogenesis [20]. Towards the third trimester there is a shift back to a pro-inflammatory Th1:Th2 dominance, which is thought to allow for the biochemical changes required for labour [21, 22].

Just prior to labour, a pro-inflammatory state is observed peripherally [23] and locally with fetal membranes [24, 25] and myometrium [26] by producing IL-1β, a potent inducer of Nuclear factor-kappa B (NF-κB) that can induce an array of pro-labour and pro-inflammatory genes [27].

1.2 Physiology and mechanisms of term labour

Throughout pregnancy, the myometrium remains quiescent while the cervix remains long and closed. In preparation for labour, three significant biochemical and physiological changes occur. First, the myometrium becomes activated and coordinated and regular contractions are initiated. Secondly, the amniotic membrane undergoes pro-inflammatory changes leading to membrane rupture, and finally the cervix undergoes remodelling to allow for shortening and dilation (Figure 1.1) [28]. These changes are collectively characterised by a transition towards a pro-inflammatory state.

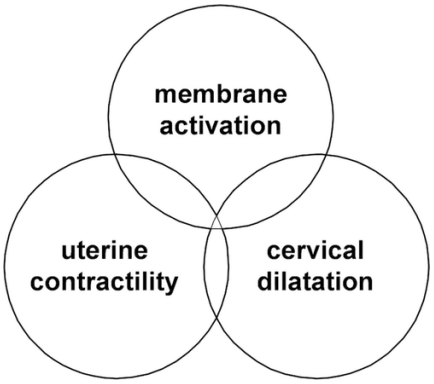


Figure 1.1 Common terminal pathway of human parturition process [28]. Physiological changes approaching labour leads to uterine contractility, membrane activation and rupture, and cervical dilatation.

1.2.1. Hormonal regulation of labour

Various hormones play a crucial role in the initiation of labouring process. Estrogen, which is primarily produced by the placenta increases throughout gestational [29] with its metabolite, Estriol, then rapidly increases the production of cortisol releasing hormone (CRH) by a positive feedback mechanism [30]. This leads to inhibition of placental production of progesterone (P4) by placental cells [31]. Estrogen will in turn lead to uterine activation and cervical ripening by up-regulating contraction associated proteins (CAPs) required for contraction, such as connexin 43, prostaglandins and the oxytocin receptor (OTR) [32-37].

Role of hormones in myometrial contractility:

Progesterone and CRH are key hormones involved in the transformation of the quiescent myometrium to the highly contractile and excitable state required to expel the fetus from the uterus. Unlike mice and many other animals, levels of progesterone in human pregnancy steadily increases throughout pregnancy [38]. The responsiveness of progesterone is tightly regulated by the ratio of the genomic receptors PR:A and PR:B that eventually leads to the concept of a functional progesterone withdrawal [39]. This refers to increased PR:A expression at term, which leads to a coordinated increased in estrogen expression on that is initially inhibited by PR:B during pregnancy. Estrogen thus increases the expression of contraction associated proteins (CAPs) that augment myometrial contractility and excitability [39]. The CAPs include oxytocin, prostaglandin, COX-2 and gap-junction protein connexin 43 (Con43) [40,

41]. At the time of labour, the CRH concentration increases in the circulation, which was in an inactive state during pregnancy by binding to the CRH binding protein (CRHBP) [42, 43]. The increase of CRH enhances Con43 [44] and the myometrial response towards prostaglandin F2α [45] thus promotes myometrial contraction.

Role of hormones in membrane rupture:

Fetal membranes express CRH receptors [46] and the ability of CRH to produce PGE2 from the fetal membrane [47] drives membrane rupture. CRH has also been found to enhance fetal adrenocorticotropic hormone (ACTH) and adrenal cortisol production to stimulate fetal lung maturation and the production of surfactant proteins [48]. The surfactant provides the source of arachidonic acid as a precursor for prostaglandin synthesis by the amnion [49]. Cortisol also has been shown to be able to increase COX-2 expression [50].

Role of hormones in cervical remodelling:

Cervical changes preceding the onset of labour are associated with a significant elevation of maternal dehydroepiandrosterone sulphate levels, an upstream of estrogen biosynthesis important for cervical softening [51]. The altered expression of PR:A and PR:B isoforms also could contribute to the cervical changes during labour [52, 53]. Up regulation of COX-2 by cortisol increases the production of prostaglandin, thereby promoting cervical ripening [29].

1.2.2. Inflammatory regulation of labour

Inflammation is widely accepted as playing a key role in labour onset. NF-κB, a classical inflammatory transcription factor regulates many pro-inflammatory and labour-associated genes such as TNF-α, IL-8, IL-6, IL-1β (reviewed in [27]), OTR [54], MMP-1, MMP-3, MMP-9 [55] and also COX-2 [56]. In addition to NF-κB, other transcription factors are also associated with term and preterm labour in which the involvement of these proteins is detailed in section 1.4.

Role of inflammation in myometrial contractility:

During labour an influx of macrophages, neutrophils and lymphocytes occurs in the myometrium [57, 58] and these activated leukocytes increase myometrial levels of IL-

1β and IL-8 [59-61]. The former stimulates prostaglandin production that is important for uterine contractility and the latter promotes further neutrophil infiltration and activation to enhance the cascade [39, 62]. Examination of the myometrium at the onset of labour demonstrates increased NF-κB p65 nuclear localisation in this tissue [63] which is an important transcription factor regulating COX-2, IL-8 and chemokines [27]. The former is an enzyme for prostaglandin synthesis [56, 64]. Term labouring myometrial tissue also produces increased IL-8, IL-1β, IL-6 and COX-2 mRNA level compared to non-labouring samples [65]. This provides further evidence that myometrial tissue is capable of producing inflammatory cytokines, and supports the suggestion that it is an inflammatory organ as well as a contractile organ.

Role of inflammation in membrane rupture:

Activation of NF-κB and increased downstream inflammatory cytokines is evident [66], in the myometrium prior to labour [27]. This contributes to fetal membrane rupture by promoting the production of metalloproteinases [67]. While IL-1β elicits a feed-forward response by increasing IL-8 and prostaglandin synthesis via COX-2, this is at least in part regulated by NF-κB [68].

Role of inflammation in cervical remodelling:

Infiltration of leukocytes into the cervix occurs with the onset of labour at term [69], and increases IL-8, IL-1β, IL-6 cytokine production are seen, as well as MMPs [61, 70] which aids collagen degradation, thus aiding cervical remodelling.

1.3 Preterm labour

Preterm labour is commonly defined as birth before 37 weeks of gestation. It can be further divided according to the gestational age at delivery; (i) extremely preterm, occurring before 28 weeks (5% of PTD), (ii) very preterm, occurring between 28^{+0}-31^{+6} weeks (15% of PTD), (iii) moderately preterm, occurring between 32^{+0} and 33^{+6} (20% of PTD) and (iv) late preterm, occurring between 34^{+0}-36^{+6}, (60% of PTD) (Figure 1.2) [71-73]. Although 30% of preterm deliveries are iatrogenic, 45% are due to the spontaneous onset of labour, and 25-40% follow preterm premature rupture of the membranes (PPROM) [74].

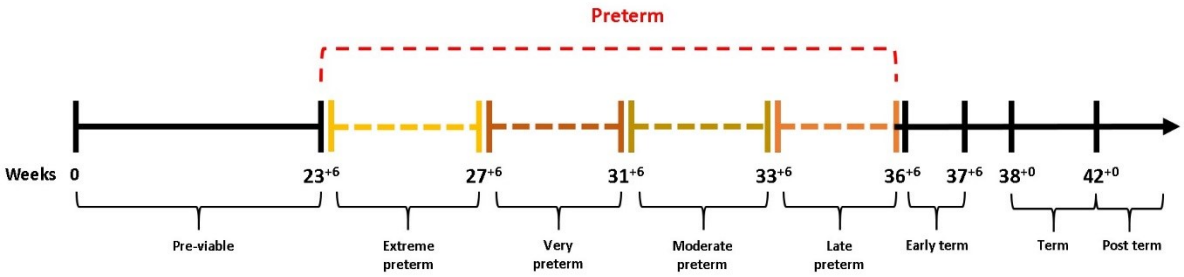


Figure 1.2 Definition of preterm, term and post term (adapted from [72]).

Approximately 75% of neonatal deaths and neonatal morbidities are attributed to prematurity [75], with the severity of adverse effects being inversely proportional to the gestational age of delivery [72, 73]. This causes a huge economic burden to parents and the NHS. The estimated cost per child surviving up to 18 years old is £61,781 if born very preterm, and £94,740 if born extremely preterm [76]. The severity of neonatal outcome is also worsened by the presence of infection and inflammation. This represents a major challenge for the obstetrician in deciding whether to delay delivery to gain fetal maturity without detriment to the fetus by keeping them in a potentially hostile inflammatory environment, which may actually worsen outcome.

1.3.1. Preterm labour treatment

Tocolytics, which inhibit uterine contractility, are used in women who present in threatened preterm labour. The aim of this treatment is to delay delivery until lung maturation is enhanced with administration of corticosteroids to the mother, and to allow for safe *in utero* transfer to a unit with appropriate neonatal care facilities. The most common tocolytics used are calcium channel blockers (Nifedipine) and oxytocin receptor antagonists (Atosiban). Although tocolytics appear to reduce the proportion of births within 7 days, this does not translate to a clear beneficial effect on overall perinatal mortality or serious neonatal morbidity rates [77]. In view of this, preventative strategies should be adopted.

1.3.2. Preterm labour prevention

While several preventative strategies exist, none are widely effective in preventing preterm birth. One of the widely used strategies for the prevention of preterm birth is progesterone treatment, which is thought to be effective at reducing rates of preterm

labour, neonatal morbidity and mortality in women with cervical insufficiency or a short cervix [78-81]. However, a large trial recently refuted this finding by demonstrating that vaginal progesterone is not associated with reduced risk of preterm labour or neonatal adverse outcome [82]. The differences in results between research studies is likely to reflect the heterogeneity of preterm labour aetiology, and the variation in dosage and timing of progesterone administration [83]. Cervical cerclage is also employed in women with cervical shortening [84] and has been reported to reduce PTB in 20% of women with a history of cervical incompetence [85, 86]. Research indicates that the suture material used for cerclage may influence effectiveness of this approach, with the nylon suture offering most benefit [87, 88].

Despite preventative and acute treatments being widely available, they are not effective in preventing all cases of preterm birth. The most likely explanation for this is the multifactorial aetiology and/or our lack of understanding of the mechanisms underpinning the pathophysiology of preterm labour, and lack of ability in stratifying individuals to tailor the most appropriate treatment. There are many plausible explanations for this, including the complex and multifactorial aetiology of preterm birth, which is yet to be fully understood as well as the difficulty in identifying women at risk of preterm birth.

1.4 Pathophysiology of preterm labour

Preterm labour is associated with multiple aetiologies such as infection or inflammation, uterine over-distention, uteroplacental ischemia and vascular disorders (Figure 1.3) [89, 90].

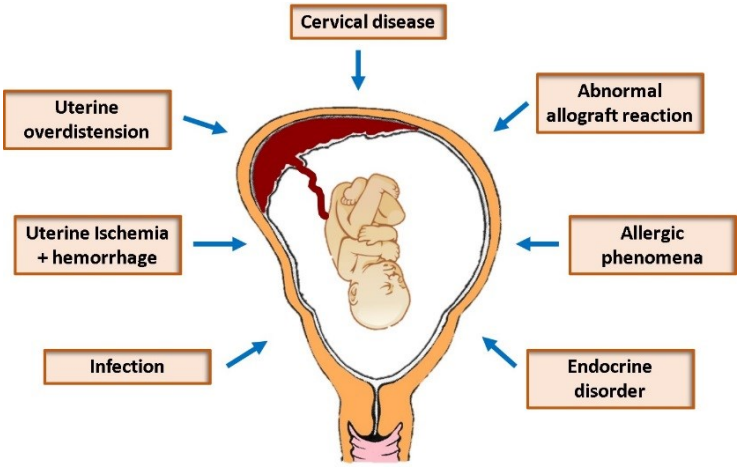


Figure 1.3 The pathological processes associated with induction of preterm labour. Leading cause of preterm parturition syndrome is intrauterine infection. However, vascular lesions have been documented as the second cause of preterm birth which constitutes uterine ischemia, cervical insufficiency and uterine overdistension (adapted from [90]).

A long established relationship exists between infection and inflammation with preterm labour. Infection is the only mechanism by which a direct causal link has been shown. There are multiple routes by which microorganisms can gain entry into the uterine cavity. The most common is by ascending route from the vagina and cervix (Figure 1.4). Evidence for the role of ascending infection is supported by the increased presence of microbial invasion of the amniotic cavity (MIAC) in women with a short cervix and ruptured membranes compared with women who have intact membranes as well as the presence of common vaginal bacterial species in the amniotic cavity [91]. However, other routes have been reported such as trans-placental haematogenous dissemination, retrograde spread through the fallopian tubes and accidental introduction during invasive procedures (e.g. amniocentesis) [92, 93]. Adverse outcomes including cerebral palsy, chronic lung disease and periventricular leucomalacia can be further worsened by the presence of infection and the fetal inflammatory response syndrome (FIRS) [94, 95]. Maternal extra-uterine infections such as malaria, pyelonephritis, pneumonia and appendicitis have also shown to contribute to preterm labour [92]. Animal models of preterm labour induced by systemic and intraperitoneal administration of pathogens also provide support for the role of haematogenous spread of infection in preterm labour [90].

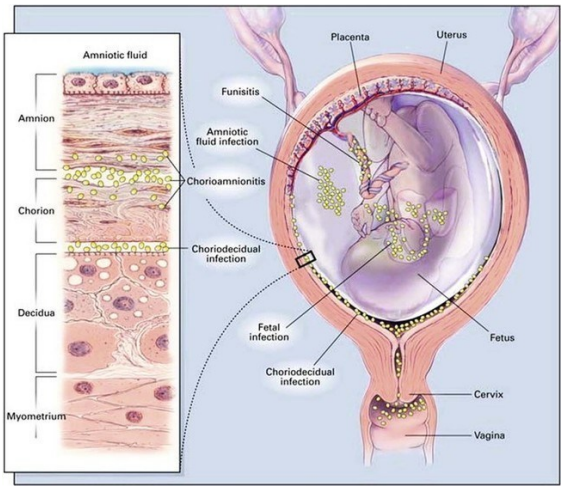


Figure 1.4 Ascending intrauterine infection route. Microorganisms found in the vagina ascend through the cervix to the choriodecidual space, and eventually invade the intrauterine cavity and infect the fetus [96].

1.4.1 NF-κB

Recognition of the pathogen associated molecules by PRRs such as TLRs including TLR2/4/6 and 3 all have the ability to activate the transcription factor NF-κB which is a key regulator of pro-labour and pro-inflammatory genes. The NF-κB family consists of 5 members; NF-κB1 (p105/p50), NF-κB2 (p100/p52), p65 (RelA), c-rel and RelB that share the same conserved N-terminal region called Rel homology domain (RHD). This domain controls subunit dimerization, DNA binding, nuclear localisation and binding to its inhibitor. The most dominant activated form of NF-κB is p50/p65 heterodimer [27].

Three distinct NF-κB signalling pathways have been outlined; canonical, atypical and non-canonical pathways as in Figure 1.5 [27].NF-κB is maintained in an inactive state in the cytoplasm in association with its inhibitory protein, IκB in the form of NF-κB:IκB complex which prevents it from being translocated into the nucleus [97, 98].

DNA damage triggers the atypical pathway, which is IKK-independent and depends on phosphorylation of IkappaB-alpha (IκBα) on Tyr42 or Ser residue. The non-canonical pathway is activated by the specific TNF cytokine family; B-cell activating factor (BAFF), lymphotoxin-β, or CD40 and the ligation induces the phosphorylation of p100 by IKKα which leads to the cleavage of the protein into p52 [27, 99, 100].

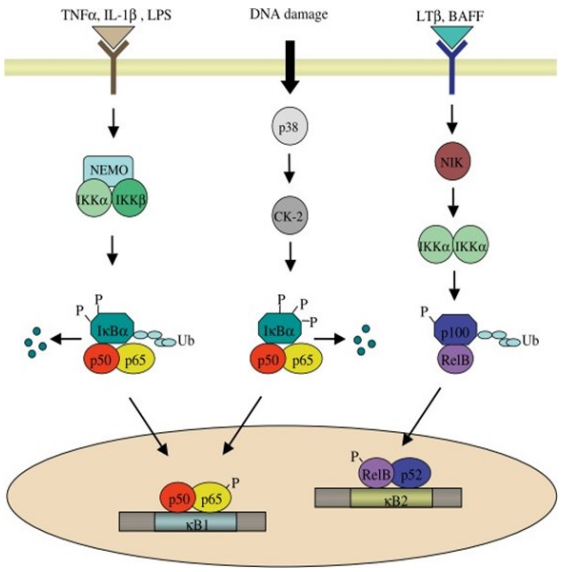


Figure 1.5 Three NF-κB signalling pathways. The *c*anonical pathway is induced by stimulation with inflammatory cytokines such as TNF-α, IL-1β and LPS, mostly associated with preterm labour. The effect elicits an array of pro-labour genes are being up-regulated. The atypical pathway is activated by DNA damage results in the phosphorylation of p100 and release of p50/p65 subunits. The non-canonical pathway on the other hand is stimulated by BAFF, lymphotoxin-β, or CD40 and results in the production of p52/RelB, which activates a different subset of genes [27].

The canonical (classical) pathway is stimulated by LPS, TNF-α and IL-1β (Figure 1.5) and results in the phosphorylation of IκB when the IKK complex is activated. The IKK complex consists of, IKKα, NEMO (or IKKγ) and the most predominant, IKKβ, which phosphorylates IκBα on Ser32 and Ser36 later leads to ubiquitination of IκBα. This releases the NF-κB subunits from its complex to make its way into the nucleus and switches on an array of pro-labour genes [98].

NF-κB role during pregnancy

During pregnancy, NF-κB activity is suppressed. Isolated T cells from non-pregnant and pregnant women show down-regulation of NF-κB/IκB signalling during pregnancy, and suppression of NF-κB in the decidua has also been reported [101, 102]. This suppression may be due to the mutual antagonism between NF-κB transcriptional activity and the progesterone receptor as seen in amnion epithelial cells [56, 103]. Progesterone also suppresses OTR signalling by directly binding to the receptor thus maintaining the quiescent state of the uterus throughout gestation [104].

NF-κB role during labour

In association with labour, the fundal myometrium [63] and cervix [105] have increased NF-κB activation. Post-labour amnion samples also show increased NF-κB activation compared to the pre-labour samples [56]. The promoter region of NF-κB contains the binding site for pro-inflammatory cytokines TNF-α, IL-8, IL-6, IL-1β, OTR [27] , COX-2 and also MMP-1 and MMP-9 [55]. These are required for uterine contractility, membrane rupture and cervical modelling (Figure 1.6).

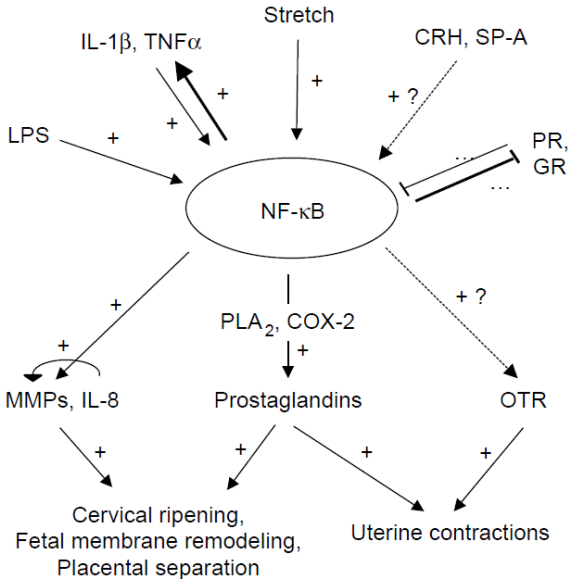


Figure 1.6 NF-κB central role in preterm labour. NF-κB is stimulated by stretch, LPS and CRH. The stimulated NF-κB regulates the expression of pro-inflammatory cytokines including ECM remodelling enzymes precedes labour [27].

1.4.2 AP-1

Activator of protein-1 (AP-1) is another key pro-inflammatory transcription factor that has been implicated in the physiology of labour and pathophysiology of preterm labour. AP-1 is composed of dimeric transcription factors belonging to the Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1, Fra-1) subfamilies [106] and it binds to promoter regions for genes encoded for cell differentiation, proliferation [107] and inflammation [108]. The mitogen-activated protein kinases (MAPKs) constitute of 3 groups namely extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 MAPK that are crucial in activating AP-1 [109, 110] (Figure 1.7). They can be activated by cytokines, stress, and bacterial infection, thus implying

the role of MAPKs in inducing inflammation and infection-induced preterm labour [107, 109].

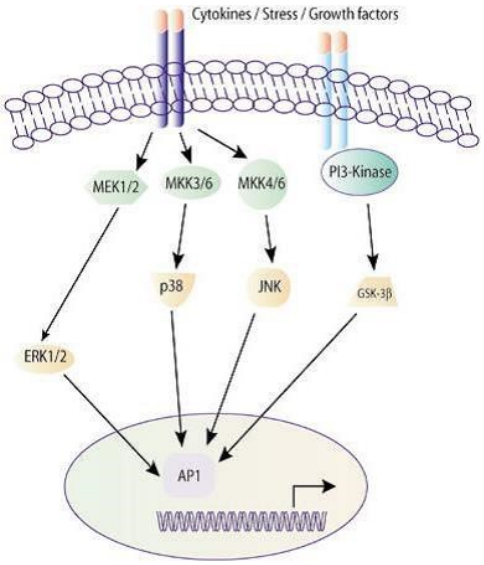


Figure 1.7 Activation pathway of AP-1. AP-1 is stimulated by stress, growth factor and cytokines. Stimulated cell induces the MAPK cascade activation and ERK1/2, p38 and JNK activation. Activated ERK1/2 and JNK stimulate AP-1 subunit c-Jun for gene expression [111].

High expression of phosphorylated MAPKs proteins of ERK, JNK and p38 have been demonstrated in term pre-labour supracervical fetal membranes [109]. The activation of AP-1 upregulates an array of pro-inflammatory and pro-labour genes as the promoter regions for the following genes IL-8 [112], COX-2 [113, 114], OTR [115, 116], Con43 and MMP-9 contains a binding site [109, 115].

1.5 The role of bacteria in preterm labour

Parturition and preterm labour has been proposed to be driven by sterile 'intra-amniotic inflammation', which refers to an inflammatory process in the absence of microorganisms [117, 118]. Sterile inflammation can be caused by triggering of receptors recognising foreign markers, such as alarmin. These alarmins are produced independent of infection and can be caused by tissue injury, hypoxia/ischemia, cellular senescence or another stressor [119]. Although sterile inflammation is prevalent in many preterm labour cases, infection is considered to be the highest contributor. Numerous studies have shown an inversely proportional relationship between the presence of inflammation and infection with the gestational age of preterm delivery.

1.5.1. Evidence for the influence of bacteria in ascending infection

Histological chorioamnionitis is seen in up to 85% of women delivering before 28 weeks compared to only 10% between 33 and 36 weeks [92, 120]. Examples of microbial species detected in MIAC include *Mycoplasma* sp. and *Ureaplasma urealyticum* [120-123], *Streptococcus agalactiae*, *Escherichia coli*, *Fusobacterium* sp., *Gardnerella vaginalis* [90], bacteroides (30%), as well as anaerobes including *Group B Streptococcus* (GBS, 15%) [124]. These bacteria are common constituents of the cervicovaginal microbiome [125]. Some of these bacteria produce protease enzymes that could potentially affect the integrity of the fetal membranes or maternal decidua [126, 127]. In addition, these bacteria also may play a role in uterine contractility by producing the prostaglandin precursor, phospholipase A_2 [128]. Infection by these bacteria could potentially cause an inflammatory cascade by producing pro-inflammatory cytokines such as IL-1β [129], which is increased in the gestational tissue that has inflammation [24, 61].

The host microbiota in the vagina during pregnancy may also play an important role in influencing the risk of preterm labour. In healthy pregnancy, a shift towards *Lactobacillus spp*. dominance occurs [130-133]. These *Lactobacillus* produces lactic acid to maintain an acidic environment (pH<4.5), which is thought to have a protective effect against infections [134, 135] and acts as a barrier to inhibit pathogen growth [136]. Significant evidence exists reporting the protective effect of the presence of *L. crispatus* [137-139]. On the contrary, the absence of *Lactobacillus spp,* which is seen in vaginal dysbiosis, [88, 140-142] or colonisation by the *L. iners* have been associated with preterm birth and a short cervix [137, 143].

1.5.2. Evidence for the influence of bacteria in haematogenous spread

Contamination from oral cavity microbiota such as *Fusobacterium* or *Streptococcus* [144-150] and *Listeria monocytogenes* [151, 152] from the digestive system into the amniotic cavity provides support for haematogenous dissemination. *Fusobacterium nucleatum* have been found in both cord blood [153] and fetal membranes [154]. Fluorescence *in situ* hybridisation (FISH) study on preterm chorion and amnion samples with chorioamnionitis related the presence of maternal leukocytes leading to a local inflammatory response to intrauterine infection [155].

A recent study however, failed to detect any microbiome in both term and preterm placenta tissues [156]. The lack of detection in the study could be due to the small placental sample numbers, with only 20 samples per category. Furthermore, although 20 preterm placenta samples were included, only a total of 6 preterm placentas had histological evidence of chorioamnionitis. Additionally, only 4 women were <28 weeks and of those 4 only 1 woman had clinical signs of chorioamnionitis. Therefore, these numbers may have been too small to extrapolate any conclusions on the role of haematogenous bacterial spread via the placenta.

The discovery of pathogenic microbiota in the vagina and amniotic fluid suggests that gestational tissue is a suitable environment for the microorganisms to survive within the host. The mechanism by which some women are susceptible to this and others are not is still not fully understood. However, if the immune system detects these pathogens the response may lead to labour as a protective mechanism for maternal survival. This complex interaction between the host immune response and the microbes appears to be vital, yet not fully understood.

1.6 The role of viruses in preterm labour

Since the advent of next generation sequencing, there is growing interest in investigating the role of bacteria in preterm birth. Although the potential role of the viruses is dramatically under-researched in comparison, there are research groups starting to look at the role of the virome in healthy pregnancy [157] and preterm labour [158]. The importance of understanding the role of viral infection on maternal and fetal morbidity and mortality has become evident from reports of pandemics and epidemiological studies [159] [160, 161]. Among all, human immunodeficiency virus (HIV) [162, 163], herpes simplex virus (HSV) [164, 165], respiratory syncytial virus (RSV) [166], West Nile Virus (WNV) [167] and Hepatitis B [168] are viruses that have been associated with preterm birth.

Histological studies using preterm and term placenta unveil a significant increase of chorioamnionitis in preterm adenovirus-positive placentas compared to both preterm adenovirus-negative placentas and term adenovirus-positive placentas [169] suggesting a potential role for viruses in preterm labour. However, research at the molecular level looking at the role and the mechanisms of viral infections and preterm birth are still under-researched.

1.6.1 Evidence for the influence of viruses in ascending infection

The cervix is one of the most vulnerable parts in the reproductive tract. Being exposed to the external environment, it becomes the most accessible route for pathogens including viruses to enter the reproductive tract. Some of the viruses commonly infecting the cervix include human herpes virus 6 (HHV6), HHV7 [170], cytomegalovirus (CMV) [171, 172], Epstein-Barr virus (EBV) [173], HSV [174] with the most common being human papillomavirus (HPV) [175]. The latter two viruses have been linked with preterm birth in humans [174, 175]. The depletion of *Lactobacillus* species is also associated with viral infection which subsequently increasing the polymicrobial flora (bacterial vaginosis, BV) in the vagina [176, 177] that is associated with preterm births [141, 178] and ascending colonisation of bacteria in the placenta during pregnancy [179-181]. Studies also suggest that BV is also associated with the acquisition of HPV in the cervix, upper region of vagina and development and progression of cervical intraepithelial neoplasia (CIN, a form of cervical cancer) [182-185]. In addition, BV-positive women have increased levels of pro-inflammatory cytokines [186, 187].

A murine study of HSV infection revealed histological changes in the cervix mimicking cervical ripening and significant collagen remodelling due to the upregulation of hyaluronic acid (HA) synthesis [188]. The importance of HA in the cervix is vital as an important mediator and regulator for cervical ripening [189].

1.6.2 Evidence for the influence of viruses in haematogenous spread

Several systemic viruses can colonise the placenta and this has been found to be associated with preterm labour [165, 169]. Adeno-associated virus (AAV) [190], HIV [191], Hepatitis B [192], CMV and HPV [165] are associated with preterm labour but the underlying mechanism is still not fully understood. In one study, adenoviral DNA was detected in as many as 40.8% of preterm placentas compared with 20.5% of term placentas [169]. Besides infecting the cervix, a case-control study identified extravillous trophoblast HPV-infection in women with spontaneous preterm birth compared to term labour [193]. Another study also found an increased rate of

thrombosis and villitis with a strong association with a high preterm birth rate in women who are HPV DNA positive [175].

1.7 Toll like receptors

Toll-like receptors (TLR) are pattern recognition receptors, which recognise a variety of pathogenic molecular signatures. Upon binding, this leads to the activation of the innate immune response by stimulating the inflammatory cascade producing chemokines, cytokines and other effectors as summarised in Figure 1.8 [89]. To date, 10 TLR receptors have been characterised in the human and 12 in the mouse [194]. The ligands for each of the receptors are listed in Table 1.1.

Table 1.1 Toll-like receptors and their respective ligands

Bacterial TLRs		
Receptor	**Exogenous ligand**	**Dimeric activity**
TLR2/1	Lipoproteins	Heterodimer [195]
TLR2/6	Mycoplasma lipoproteins and peptidoglycan	Heterodimer [196]
TLR4	Gram-negative bacteria	Forms homodimers [197] [198]
TLR5	Flagellin	Forms homodimers and heterodimers with TLR4 [197] [198]
TLR10	Lipopeptides	Forms homodimer [199] and heterodimers with TLR1 [199] and TLR2 [200]
Viral TLRs		
TLR3	dsRNA	Dimerises on activation [198]
TLR7/8	ssRNA	Preformed dimer [198]
TLR9	Unmethylated DNA fragments	Preformed dimer [198]

With reference to infection induced preterm labour, the toll-like receptors 2 and 4 have been most extensively studied. Upon ligand binding of these receptors by gram negative and gram positive bacteria respectively, the transcription factor NF-κB is activated [201]. NF-κB is central to the biochemical process of inflammation/ infection induced preterm labour since it regulates the expression of labour associated genes (cPLA2α, COX-2, Prostaglandin E2) and pro-inflammatory cytokines (IL-1β, TNF-α, IL-6 and IL-8) [68]. NF-κB also interacts with progesterone and downregulates its pro-pregnancy quiescent effect.

TLRs are also responsible in detecting nucleic acid PAMPs products in addition to bacterial products displayed on the invading organisms. With respect to viruses, four classes of PAMPs have been characterised [202] and detected by four members of the TLRs. TLR3 recognises double-stranded RNA (dsRNA constitutes the genome of one of the viruses, but is also generated during the life cycle of many viruses), TLR7 and TLR8 on the other hand recognise single-stranded RNA (ssRNA) and the last member in the group, TLR9 recognises non-methylated viral CpG motifs in the DNA (CpG DNA) [203]. Envelope glycoproteins on the other hand have been shown to be detected by TLR2 and TLR4. These are not classified as viral TLRs and sense pathogen components on the cell surface as opposed to TLR3,7,8 and 9 that are found in the endosomal compartments [203-205].

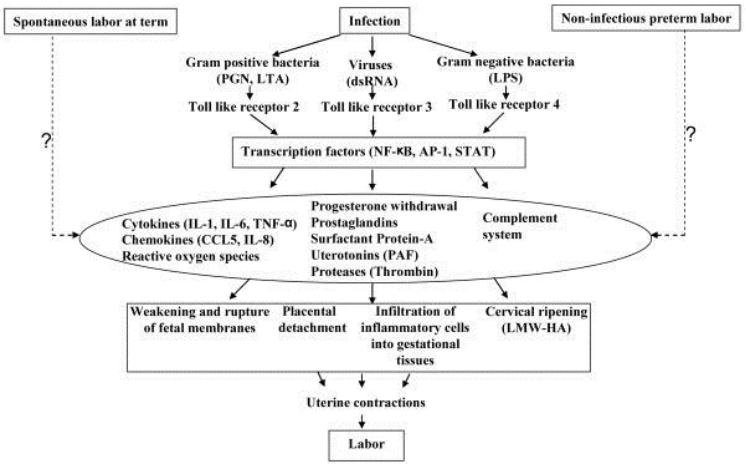


Figure 1.8 Overview of the role of TLRs in infection mediated preterm labour. Upon binding with the TLRs, pathogens activate the inflammatory cascade via pro-inflammatory transcription factors. This in turn up-regulates the expression of chemokines, cytokines and prostaglandins which are key proteins involved in preterm labour [89].

This study focused on the role of TLR2/4/6 and 3 in infection-induced preterm labour. For this reason, a more detailed description of these TLRs is presented below.

1.7.1 TLR2 and 6

Structure

Toll-like receptor receptors are type I integral transmembrane glycoproteins composed of a conserved intracellular toll–interleukin-1 receptor (TIR) homology domain, a single transmembrane helix domain and a horseshoe-like solenoid extracellular

ectodomain (ECD). The ECD is responsible for pathogen recognition and is composed of 16-28 leucine-rich-repeat (LRR) [3]. With this diversity of LRR repeats, TLRs can be divided into six major families according to the LRR repeat numbers and the motifs of two cysteine clusters flanking the LRRs. Therefore, TLR2, TLR1, TLR6 and TLR10 are members of the TLR1 subfamily [206].

The structural differences between TLR1, TLR2, and TLR6 and other TLRs are the three distinctive subdomains in the ECD: N terminal, central and C terminal. The central domain is the unique characteristic that is distinguishable among TLR1, 2 and 6 due to the irregular LRR sequence. The central domain lack the asparagine networks that is important in stabilising the overall shape of the horseshoe-like structures. This allows for structural distortions and is important in fulfilling the function of these TLRs because the ligand-binding pocket of these TLRs is situated within the structural changes of C-terminal and the central domains [207, 208].

Therefore, due to the similar structure and the need for TLR6 to heterodimerise with TLR2 for activation and signalling propagation, TLR6 description is incorporated in the same section as TLR2.

Expression

TLR2 and TLR6 are expressed on cell membranes and reports have shown that the dimerization of TLR2/TLR6 pre-exist on the cell membrane without any active signalling [209]. The expression of TLR2 has been detected on immune cells, endothelial and epithelial cells [210, 211]. With regards to gestational tissue, the expression of TLR2 has been recorded in the amnion and chorion [212], decidua [213], myometrium [214] and vagina [215]. TLR6 is also expressed on the decidua [213], cervix and vagina [215], amnion and chorion [216, 217].

As pregnancy progresses, the placenta exhibits changes in TLR2 expression between pre-labour and in-labour [218]. A temporal and spatial pattern of TLR2 and TLR6 expression were also observed in the placenta between first trimester and third trimester. A study has recorded the lack of first trimester trophoblast TLR6 expression [219] whereas expression appears in the third trimester trophoblast [220], suggesting a differential function for TLR6 which is dependent on gestational age. Similarly, although the first trimester villous cytotrophoblast and extravillous trophoblast

express TLR2, the syncytiotrophoblasts do not [219]. In contrast, term placenta express TLR2 and TLR4 at the syncytiotrophoblasts [221]. This implies that the placenta can only respond to bacteria that has breached the syncytiotrophoblast, the outer layer of trophoblast.

Function

TLR2 is an important response modulator for the innate immune system by detecting a wide range of gram-positive bacteria cell wall components mainly lipoproteins to mediate a protective response of the host. The triacyl lipoproteins exhibited by most gram positive bacteria is recognised by TLR2/1 and diacyl lipoproteins as those found in mycobacteria is recognised by TLR2/6 [222]. These heterodimerisations confer its different function. The unique response of the different ligation of TLR2 is observed in the placenta with an apoptotic pathway in TLR2/1 heterodimerisation whereas cell death is prevented and a cytokine response ensues through NF-κB activation via TLR2/6 ligation [223].

Signalling

Upon ligand binding, TLR2 heterodimers initiate a MyD88-dependent pathway, an intracellular signalling that is common to all membrane bound TLRs except for TLR4. Once initiated, this pathway induces the translocation of NF-κB into the nucleus to modulate inflammatory gene transcription and consequently cytokines and chemokines production. In addition to NF-κB, the MAPKs pathway is also triggered activating the AP-1 transcription factors thus influencing the transcription of inflammatory genes and production of cytokines and chemokines [224].

Human evidence for the role of TLR2 agonists in PTL

TLR2 heterodimerises with TLR1 and TLR6 in the cellular membrane upon activation [194]. Gram-positive bacterial products are the main ligand, however, *ureaplasma urealyticum*, group B *streptococcus* (GBS) and CMV (cytomegalovirus) are among the TLR2-specific human ligands [225, 226], with GBS being associated with bacterial vaginosis and high loads being associated with prematurity [227]. *Ureaplasma* spp is a common bacteria found in intrauterine infections. It is isolated from women with preterm labour with intact membrane [228, 229], PPROM [230],

MIAC with short cervix [231] and infected placentas [230]. The expression of TLR2 has been found to be up-regulated in fetal membranes from women who deliver preterm with histological chorioamnionitis compared to without chorioamnionitis [212].

Murine evidence for the role of TLR2 agonists in PTL

Murine studies also support the role of TLR2 in bacterial induced preterm labour. Mice injected with the TLR2 agonist lipoteichoic acid (LTA) systemically or intrauterine deliver preterm [232]. The dimerization of TLR2 with TLR1 has been shown to lead to apoptotic pathway by using first trimester trophoblast lacking TLR6 expression. This effect is however, reversed in the presence of TLR6 but with the induction of NF-κB activation and production of pro-inflammatory cytokines [223]. GBS infection has also been studied in mice. Intraperitoneal and intrauterine injection of heat-killed GBS in pregnant mice at 14.5 days of pregnancy showed a high percentage of preterm delivery (86%). Furthermore, GBS colonisation in the vagina day 16 of pregnancy caused 75% of the mice to deliver preterm, and this could be prevented with administration of a GBS vaccination prior to mating [233].

1.7.2 TLR4

Structure

TLR4 has the same horseshoe-like solenoid structure with the rest of the other TLR1, 2 and 6 with the same unique central domain, lacking asparagine network for stabilisation. One characteristic that is unique to the TLR4 structure is the binding site for the co-receptor protein MD-2, located between the central domain and N-terminal [234].

Expression

TLR4 differs to other TLRs since it is expressed in both the cell membrane and the endosome [235]. TLR4 is highly expressed in immune cells [236-238], however, plasmacytoid dendritic cells (pDCs) do not express TLR4 [239]. In non-immune cells, TLR4 is expressed in a variety of tissues but is not detected in the skeletal muscle or the skin [236]. In gestational tissues, placenta [218, 219] amnion and chorion [216, 217], myometrium [240] have been shown to express TLR4 but not in the vagina or

the cervix [241], although the expression is debatable in the literature [242-244]. A change in TLR4 expression was observed in the myometrium between pre-labour and in-labour [245].

Function

TLR4 is responsible in detecting lipopolysaccharides (LPS), a major component of gram-negative bacteria [194]. In addition to exogenous pathogens recognition, TLR4 also recognises endogenous PAMPs such as heat shock protein [246].

Signalling

TLR4 signalling is unique since it can initiate two different intracellular pathways: (i) MyD88-dependent and (ii) TRIF pathway (MyD88-independent) upon ligand binding. These signalling pathways produce a cascade of different proteins and molecules such as pro-inflammatory mediators [194].

Human evidence for the role of TLR4 agonists in PTL

TLR4 recognises bacterial lipopolysaccharides (LPS) of gram-negative bacteria such as *Escherichia coli, Mycoplasma hominis* and *Trachtomonas vaginalis,* all of which are associated with preterm birth [247]. Increased expression of TLR4 is also found in the fetal membranes [212, 248] and placenta villi [249] of patients with chorioamnionitis. An association of *TLR4* single nucleotide polymorphisms (SNP) and preterm birth has also been recorded in human studies. *TLR4* SNP at Asp299Gly and its co-segregation allele Thr399Ile increases the risk of premature birth [250, 251].

Murine evidence for the role of TLR4 agonists in PTL

Several mouse models using TLR4 agonists have been used to examine the effects of infection-induced preterm labour. Many studies have demonstrated that LPS is a potent inducer of preterm delivery in mice via intrauterine or systemic injection [252-255] and vaginal administration [256]. An intrauterine LPS injection in mice showed preterm delivery and high percentage of fetal demise compared to PBS control group [257]. Injection of live heat-killed *E. coli* also shows the same potent effect as LPS with preterm delivery leading to delivery within 7-8 hours post injection at 14.5-15.5 days of pregnancy. However, the numerous LPS serotypes have differential effects upon the activation of pro-inflammatory responses, delivery time post-injection and

varying levels of offspring survival [253]. Studies using TLR4 mutant mice also strengthen the role of TLR4 in LPS [252, 258] and heat-killed [258] bacterial induced preterm labour. Inoculation of the TLR4 mutant mice with these ligands are less likely to deliver preterm than wild type mice.

1.7.3 TLR3

Structure

TLR3 is a Type I transmembrane receptor with an N-terminal ECD, a single transmembrane helix, and a C-terminal cytoplasmic signalling domain of the TIR (Toll/IL-1 receptor) family. The ECD of TLR3 is located in the interior of endosomes of where it encounters and binds dsRNA to propagate the signals that initiate inflammatory and adaptive antiviral responses [259].

TLR3 is the most heavily glycosylated TLR. Structural analyses of human TLR3 ectodomain reveals that it consists of a solenoid with 23 leucine rich repeats that bends into a horse-shoe shape and is capped with a specialised structure known as leucine-rich repeats (LRR) at the end of the N and C terminals [260, 261]. One face of the horse-shoe shape is largely devoid of the glycan and is free to interact with molecules while the other face is largely masked by carbohydrates.

TLR3 molecules exist as monomeric in solution but the crystal dimers form a homodimer interface at the C-terminal highly-conserved surface residues and a TLR3-specific LRR insertion at LRR20 [260, 262]. The homodimer binds to dsRNA and requires a dsRNA length of at least 40-50 bp to induce signalling [263]. A site-directed mutagenesis revealed an essential role for the residue His-539 (H^{539}) and Asn-541 (N^{541}) in the C terminus for ligand binding and signalling and also residues His-39 (H^{39}), His-60 (H^{60}) and His-108 (H^{108}) in the N terminus for the binding of the dsRNA [260, 262] [264].

Cleaved and non-cleaved

Endogenously, TLR3 has been shown to have different molecular weights at different locations in the cell compartments. In the study conducted by Toscano et al [265], using HEK293, mDC (mouse dendritic cells) and other cell lines, at the basal conditions, cells primarily expressed the C-terminal fragment 72kDa and N-terminal

fragment 60kDa TLR3 forms. Treatments with IFN-α allowed the detection of the higher molecular mass form 135kDa.

Both de-glycosylated TLR3 (95kDa and 50kDa) and glycosylated TLR3 (135kDa, 130kDa and 72kDa) have been found in the endoplasmic reticulum (ER), Golgi apparatus (GA) and endosomes. The higher molecular weight TLR3, 130kDa undergoes 2 glycosylation steps. TLR3 is newly synthesized (neo-synthesized) and N-glycosylated in the ER producing 130kDa and 72kDa TLR3 forms. The 130kDa TLR3 (called as $TLR3_{FL}$) is then fully glycosylated in the GA to become EndoH resistant (EndoH is used to cleaves N-glycans) (called $TLR3_{FL+}$) and gives rise to a 135kDa form. Using PNGase treatment (removes all N-glycans, the glycosylation), $TLR3_{FL}$ (130kDa) and $TLR3_{FL+}$ (135kDa) shifts to a 95kDa respectively and the C-terminal (72kDa) form shifts to 50kDa. This indicates that both cleaved and uncleaved TLR3 are glycosylated and are rapidly being cleaved when it leaves Golgi apparatus to enter the endosome membranes [265] (Figure 1.9).

The full-length TLR3 protein that is synthesized in the ER exits from ER in an UNC93B1-dependent manner (UNC93B1 is an endoplasmic reticulum membrane protein). It is trafficked rapidly through the GA to reach endolysosomes where TLR3 is cleaved by cathepsins and accumulates ready for a signal. The cleavage and signalling of TLR3 is dampened by treatment with a cathepsins inhibitor (z-FA-fmk) or leupeptin. The cleavage of TLR3 is mediated by cathepsins B and H [266].

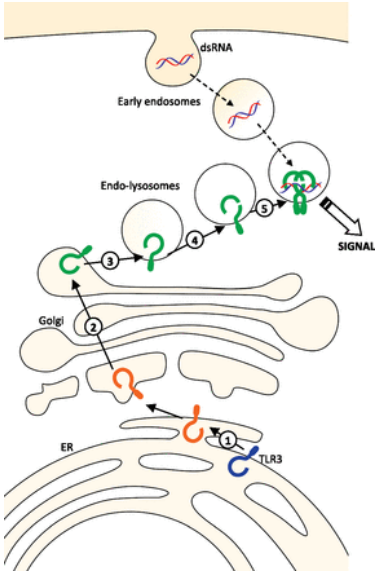


Figure 1.9 TLR3 processing. (1) In a resting state, TLR3 is neosynthesized and N-glycosylated in the ER continuously. (2) Glycosylated TLR3 crosses the Golgi apparatus and

is further glycosylated to become EndoH resistant, giving rise to $TLR3_{FL+}$ where it (3) exits and enters the endosomal membrane. (4) It is then cleaved by cathepsins and (5) the cleaved fragments dimerize and accumulate readily to signal (5) [265].

Expression

TLR3 expression is broadly expressed and conserved in vertebrates. TLR3 mRNA expression has been detected in the placenta [218], vaginal epithelial cells [215], amnion epithelial cells [216], pancreas, lung, liver, heart, brain [267], intestinal epithelial cells [268] and fibroblasts [269]. Immune cell expression has been demonstrated on PBMCs [270, 271] such as mast cells (MC) [272], dendritic cells [273], NK cells [274], monocytes [273], T and B cells [238]. In addition, the plasmacytoid pre-DCs (dendritic cells) do not express TLR3 but express TLR7 and TLR9 suggesting that these cells have different roles in anti-viral immune responses [239].

TLR3 is commonly found in the endosomes, however, human fibroblasts and epithelial cells have been shown to express TLR3 both intracellularly and on the cell surface while dendritic cells only express TLR3 intracellularly [269]. However, TLR3 signalling arises in the intracellular compartment in both fibroblasts and dendritic cells suggesting that it requires endosomal maturation [275]. In addition to primary cells, several cell lines such as human corneal epithelial cells (HCEC), lymphatic endothelial cells (LEC) and human bronchial epithelial cell (BEAS-2B) have been shown to express TLR3 on the cell surface [276-278]. In murine, unpublished data from our group has demonstrated increased TLR3 mRNA expression in the uterine tissue throughout gestation [279].

Function

TLR3 recognises both virus–derived double-stranded RNA (dsRNA) [280], a replication intermediate of most viruses [281] and its synthetic analogue polyriboinosinic:polyribocytidylic acid (poly (I:C)).

Interestingly, dsRNA from non-viral origin particularly, mRNA and RNA released from necrotic cells have been shown to trigger the TLR3 pathway as well [282, 283]. Since TLR3 responds to poly (I:C) and viral dsRNA and induces IFN-β gene transcription, it is thought that TLR3 may play a role in the anti-viral immune response

to most of the clinically relevant viral infections in the human such as flaviviruses, hepatitis B and C viruses, herpesvirus, rotavirus, retroviruses, orthomyxoviruses, and poxviruses [284].

The anti-viral immune response by TLR3 is still controversial [284, 285], however, Wang *et al* demonstrated the important role of TLR3 in infections caused by West Nile Virus (WNV) in the CNS. In the absence of TLR3, WNV-infected mice had impaired cytokine production and enhanced viral load in the periphery whereas a reduction of viral load, inflammatory responses and neuropathology were seen in the brain as compared to the wild-type mice [286]. TLR3, in general, facilitates the entry of the WNV in the brain causing lethal encephalitis.

Several reports have demonstrated the dual role of TLR3 in immune response contributing in the elimination of specific viruses or the benefits of TLR3 receptor on specific type of viruses as shown in Table 1.2. The function of TLR3 in general is probably dependent on the type of the virus, the viral load, the site of TLR3 activation either endoplasmic or cytoplasmic, the type of cells and the stage of infection.

Table 1.2 The role of TLR3 in virus infections (adapted from [287])

Type of virus	Target system, organ, tissue, and/or cells expressing TLR3	Role of TLR3	Reference(s)
Encephalomyocarditis virus	Heart	protective	[288]
Hepatitis C virus	Liver	protective / detrimental?	[289, 290]
	Kidney	detrimental?	[291]
Herpes simplex virus 1	CNS (neuronal cells)	detrimental?	[292, 293]
Herpes simplex virus 2 PTL	Female genital tract	protective?	[294-296]
Human immunodeficiency virus	Skeletal muscle	detrimental?	[297]
Influenza A virus	Respiratory tract	detrimental	[298]
	CNS	protective	[299]
Punta Toro virus	Liver	detrimental	[300]
Rabies virus	CNS (neuronal cells)	detrimental?	[292]
Respiratory syncytial virus	Respiratory tract	protective	[301]
Theiler's murine encephalomyelitis virus	CNS (astrocytes)	protective?	[302]
West Nile virus	CNS	detrimental	[286]
Zika virus	CNS	detrimental	[303]

Signalling

TLR3 recognises dsRNA and its synthetic analogue, poly (I:C) and mediates downstream signalling producing cytokines and chemokines. Additionally, several studies report that poly (I:C) is also detected by two cytosolic dsRNA receptors; RIG-I and MDA5 [304-306].

The TLR3 proteolytic processing is poorly understood even though it regulates the stability and endosomal localisation [266]. However, tyrosine phosphorylation of TLR3 in the endosome is vital for recruiting the adaptor protein toll-interleukin-1 (IL-1) receptor (TIR) domain-containing adaptor protein interferon-β (IFN-β) (TRIF) to the TIR domain of TLR3. This is in contrast to other endosomal TLRs (TLR7-9) that do not require tyrosine phosphorylation due to the MyD88-dependent signalling.

Unlike other TLR family members, TLR3 does not use myeloid differentiation factor 8 (MyD88) as the signalling adaptor protein [3]. TLR3 mediates signal via the adaptor protein TICAM-1/TRIF [307, 308] (Figure 1.10) activating transcription factors NF-κB,AP-1 and IRF3, leading to the induction of type 1 interferon, cytokine and chemokine production and DC maturation [280]. A study on TRIF knockout mice shows no response to poly (I:C), indicating that TRIF is crucial for TLR3 signalling pathway [308]. NF-κB activation in TLR3 signalling is mediated by two molecules which are receptor-interacting protein 1 (RIP-1) and TRAF6. RIP-1 deficient murine embryonic fibroblasts show complete loss of poly (I:C)-induced NF-κB activation, demonstrating that RIP-1 is an essential mediator in NF-κB activation in the TRIF pathway [309]. TRAF6 then recruits RIP-1, interacts with and activates the TAK1 complex leading to activation of NF-κB and MAPK family members such as ERK1/2, p38 and JNK. These MAPKs proteins than mediates the activation of the AP-1 family of transcription factors [3, 4], thus suggesting that TRAF6 is responsible in AP-1 activation in TLR3 signalling. Transfection of human myometrial primary cells with siRNA against TRIF, TRAF6, RIP-1 and TAK1 shows a significant reduction of poly I:C-induced NF-κB, cytokines and chemokines [310].

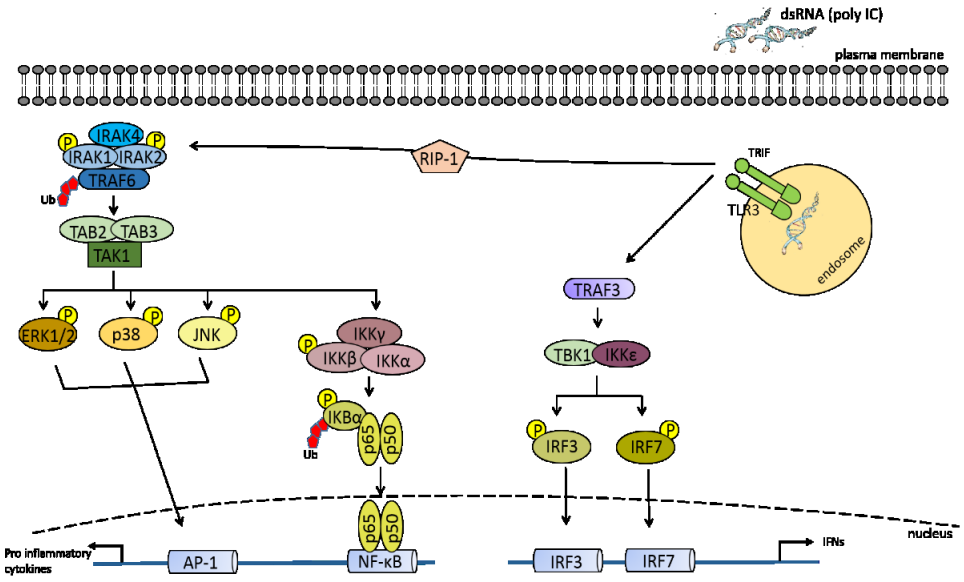


Figure 1.10 TLR3/TRIF signalling. TLR3 recruits TRIF to the TIR domain that interacts with TRAF6 and TRAF3. This lead to the phosphorylation of MAPKs, NF-κB, IRF3 and IRF7 transcription factors thus mediated the downstream signalling and produces pro-inflammatory cytokines and IFNs (modified from [311]).

Entry of synthetic poly I:C into the cells

Poly I:C has been reported to enter cells via directly binding to the CD14 co-receptor at the cell surface leading to endocytosis [312]. However, monocytes-derived iDCs and HEK293 cells do not express CD14 [313] suggesting that other cell surface molecules are involved in the cellular uptake of poly I:C in both of these cells. Other studies have also suggested that extracellular dsRNA is being captured by various cell surface receptors including MSR [314], SR-A [315], CD14 [312] and Raftlin [316] before being taken up using the clathrin-mediated endocytosis [317] into the cells.

One study confirms that extracellular poly I:C enters the cells by sensing the SR-A or raftlin (cytoplasmic lipid raft protein) on the cell surface and enters via endocytosis. SR-A communicates a negative signal to endosomal TLR3 while raftlin communicates a positive signal to endosomal TLR3. The dsRNA escapes the endosome and is detected in the cytoplasm by MDA5 and/or RIG-I, which induces IFN-β production via IPS-1 without TLR3 signalling [318]. This hypothesis has been proved by Nguyen et al when they showed that SIDT2 is responsible in escaping the endocytosed poly I:C (dsRNA) to the cytoplasm to be signalled via the RLR pathway [319] (Figure 1.11).

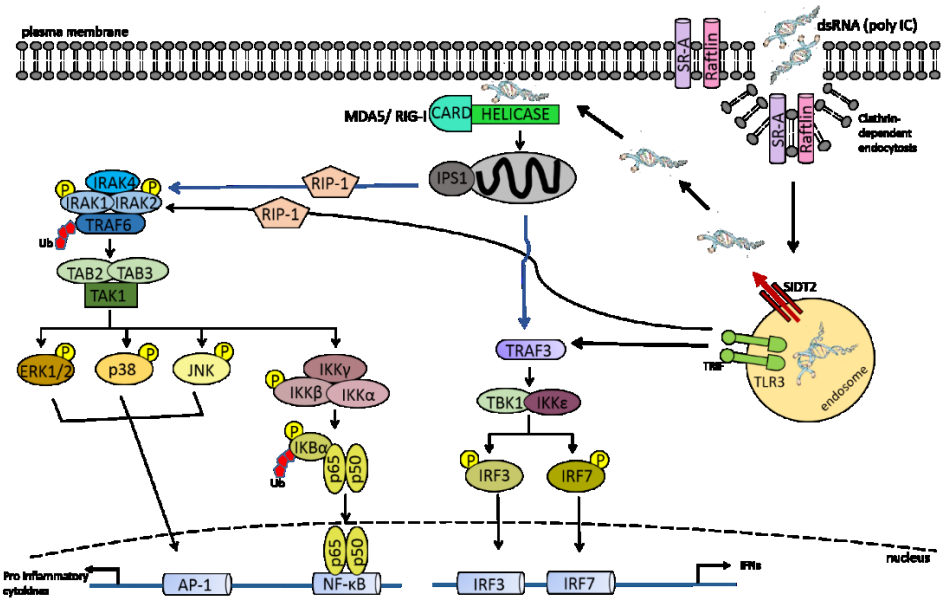


Figure 1.11 Uptake of the exogenous poly I:C. Poly I:C is captures by the lipid raft protein and is endocytose via clathrin-dependent process. Some poly I:C escape the endosome via the transmembrane protein, SIDT2, and were sense by the RLR in the cytoplasm. Thus, poly I:C can act on both TLR3, situated in the endosome and RLR in the cytoplasm to activate the downstream pathway NF-κB, AP-1, IRF3 and IRF7 to produce cytokines, chemokines and type 1 IFN (modified from [318, 319]).

Human evidence for the role of TLR3 agonists in PTL

The TLR3 receptor has been demonstrated to be a ligand for a few viruses that are associated with adverse pregnancy outcome such as HPV, HSV and Hepatitis B. Some of these viruses can cross the blood-brain barrier (BBB) thus infecting the growing fetus resulting in multiple neurological disorders such as the Zika virus. Evidence also suggests that viral invasion of the amniotic cavity can cause intrauterine inflammation, and cytomegalovirus, parvovirus and adenovirus have all been isolated from amniotic fluid [320, 321].

HIV is the most studied virus in relation to PTL. The link between HIV and TLR3 has been proved when TLR3 mRNA and protein level increased with stimulation of HIV on primary human brain microvascular endothelial cells (HBMEC) [322]. Human herpesvirus 8 (also known as Kaposi's sarcoma-associated herpesvirus) has been detected in PBMC's from pregnant-infected HIV women [323] and this virus signals via TLR3 by upregulating TLR3 expression and TLR3-specific cytokines and chemokines in KSHV-infected human monocytes [324].

Hepatitis B is also associated with preterm labour [167, 168, 192]. The presence of the hepatitis B e antigen compared to the s antigen increased the risk of preterm labour in the chronic carriers of HBV. This is postulated to reflect a higher viral load as e antigen serves as a marker for active replication in chronic hepatitis [168].

HSV-1 and HSV-2 are part of a large family of DNA viruses and HSV-1 is sensed by TLR3 among other PRRs [325]. An epidemiological study on women with subclinical primary genital HSV infection showed the association between preterm delivery and genital shedding of HSV at the onset of labour [164].

Epidemiological studies have showed that pregnant women are at a higher risk of developing severe complications of influenza virus during seasonal influenza periods [326] and pandemics compared to non-pregnant women [162, 327, 328]. Furthermore, increased preterm birth risk has been reported during the influenza pandemic of 1918 [327], the Asian influenza pandemic in 1957 [162] and the 2009 pandemic [329]. Influenza virus has been shown to signal via TLR3 demonstrated using lung epithelial cells *in vitro* [330, 331].

Despite the evidence above suggesting that TLR3 stimulating viruses appear to be associated with a higher risk of PTL, the recent ZIKA outbreak does not appear to have impacted on rates of preterm birth [332].

Murine evidence for the role of TLR3 agonists in PTL

The most compelling evidence for the potential role of TLR3 in preterm labour comes from murine studies. Poly I:C treated mice deliver preterm, with a dose dependent increase in delivery rate with higher concentrations of poly I:C. Furthermore, This effect is dampened using TLR3 knockout mice emphasising the critical role of TLR3 in poly I:C-induced preterm delivery [333].

1.8 The double hit hypothesis

There is growing interest in the potential synergistic effect of a 'polymicrobial' infection, also referred to as a 'double hit' in the aetiology of preterm labour. It refers to the ability of a viral infection to modulate the immune response to subsequent bacterial infection leading to an exaggerated pro-inflammatory response and increased susceptibility to preterm labour.

Murine evidence

Several murine studies support the synergistic effect of exposure to both viral and bacterial TLR agonists in inducing preterm labour. In a study using MHV-68 and LPS models, MHV-68 injection at 8.5 days of pregnancy showed 0% preterm delivery as compared to 29% of preterm delivery with LPS injection at day 15.5 of pregnancy. The preterm delivery rate however, increases to 100% when the mice receive MHV-68 injection prior to LPS. In addition, a 100% rate of fetal death is also seen with MHV-68 priming prior to LPS injection [334, 335]. In another study, LPS or poly I:C injection alone at low doses did not elicit any preterm delivery, however, a 100% preterm delivery rate was recorded when the mice were primed with poly I:C for 4 hours prior to LPS stimulation [336]. Although no preterm delivery rate was recorded, a study using MHV-68 injected at day 8.5 of pregnancy and a subsequent LPS injection at day 15.5 of pregnancy increased IL-1β mRNA levels in the fetal membranes [337]. In contrast to systemic injection, vaginal inoculation of MHV-68 at day 8.5 prior to live *U. urealyticum* at day 15.5, led to an accumulation of the bacteria

in the decidua and lymph node compared to no accumulation with the bacteria alone [338]. Despite no PTD rate being reported, this study implies that local viral priming causes increased susceptibility to bacterial ascending infection relevant to human PTL.

Besides looking at the viral priming effect before a subsequent bacterial infection in a mouse model, the double-hit hypothesis has also been examined by administering both viral and bacterial components simultaneously. One study used poly I:C as the viral agonist and PGN as the bacterial TLR2/6 agonist to show the synergistic effect of intrauterine administration of both agonists. Poly I:C injection alone gave rise to a preterm delivery rate of 22%, and PGN injection alone showed a 14% preterm delivery rate. However, the rate increased to 100% with a combined injection [339].

Human evidence

Following on from these murine studies, there have been limited human *in vitro* studies examining the role of a double hit on inflammation in the context of preterm labour.

Fetal membranes

In one study, various combinations of viral stimuli including poly I:C, HSV-2 and MHV-68 have been used to examine the double-hit effect. Fetal membrane explants were primed with these viruses for 24 hours before a subsequent LPS 0111:B4 stimulation. This resulted in increased IL-1β protein production in all combinations in addition to increase in the other cytokines and chemokines. Pre-treatment with MHV-68 increased IL-6 and IL-8; poly I:C increased IL-6, MIP-1α and RANTES; HSV-2 increased MIP-1α before subsequent LPS 0111:B4 stimulation [337]. However, no studies have been done to look at the effects on amnion epithelial cells in isolation, which are the cells at the interface of the amniotic cavity.

Placenta

Human primary trophoblast and trophoblast cell lines treated with MHV-68 infection for 24 hours prior to LPS stimulation show an increase in the production of pro-inflammatory cytokines GRO-α, IL-6 and IL-8 in both types of cells compared with either treatment alone [335]. However, no study has looked at the priming effect of Poly I:C, or with a broader panel of bacterial TLR agonists.

The local effect of a double-hit on vaginal epithelial cells, amniocytes and myocytes has not been reported on.

1.9 The potential role of anti-inflammatories for the prevention of infection-induced PTL

Many strategies have been devised to combat and reduce the high risk pregnant mothers for preterm labour. Current preventative measures currently used include progesterone and cervical cerclage. Controversy still remains with regards to the efficacy of progesterone, which is likely to be due to the inclusion of women with multiple aetiologies in research trials. In support of its potential benefit, significant evidence exists for its anti-inflammatory actions via inhibition of TLR-induced cytokine production [340], suppression of the innate immune responses in macrophages, NK cells, suppression of NF-κB signalling [341-343]. During pregnancy, progesterone influences the Th1 and Th2 balance by suppressing the development of Th1 immune responses and enhancing the development of Th2 cells [344, 345]. Despite cerclage providing mechanical support to the cervix to prevent of preterm labour, it is also considered to contribute to forming an immunological barrier by preventing ascending infection [346].

Treatment of women who present in threatened preterm labour already contracting involves the use of tocolytics such as a calcium channel blocker or oxytocin receptor antagonist [347] [348]. However, tocolytics have only been shown to maximally delay delivery by 48 hours to 7 days [349]. Furthermore, there is limited evidence for significant improvement in neonatal outcome which may be as a result of there being no anti-inflammatory effects from these treatments, and the inability to completely reverse the process of parturition once the process has advanced. A more attractive agent would have both anti-inflammatory properties in targeting the transcription factors that regulate both the inflammatory and labour associated genes, and the ability to self-administer prophylactically is desirable.

With the evidence that infection and inflammation increase the risk of preterm delivery, and that a ‘double-hit’ effect is seen to increase the risk of preterm labour in the mouse, the advancement on anti-inflammatories that could target the main transcription factors involved in labour is an exciting and a novel strategy to prevent preterm labour.

1.9.1 15dPGJ2

Non-enzymatic dehydration of PGD2 gives rise to PGJ2 and its metabolite cyclopentenone prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15dPGJ2) (Figure 1.12) [350, 351]. The highly reactive ring of 15dPGJ2 can form a covalent interaction with multiple components of the NF-κB signalling pathway which inhibits the translocation of p65 into the nucleus [352, 353]. 15dPGJ2 has also been reported to bind directly to c-Jun (component of AP-1) and subsequently inhibits DNA binding activity in rat mesangial cells [354].

Studies on cultured human amniocytes and myocytes stimulated with IL-1β show that 15dPGJ2 is an inhibitor of IKBα degradation, IKK phosphorylation and targets directly the p65 subunit [64]. The compound also reduces NF-κB activation in PBMCs from preterm samples, and suppresses the Th1 cytokines profile while maintaining Th2 cytokines profile during pregnancy and active labour [355]. Furthermore, 15dPGJ2 delays preterm labour and increases pup survival in LPS treated mice [356]. Using poly I:C as a stimulus, 15dPGJ2 inhibits stimulated p-p38 and p-ERK in human dendritic cells, but no effect on NF-κB was reported [357]. However, what is not known is whether this translates into inhibition of pro-labour and pro-inflammatory mediators in human gestational tissue. If 15dPGJ2 proves to have beneficial inhibitory effects on pro-labour and pro-inflammatory mediators, the progression of this compound from bench to bedside could be accelerated by its availability in the form of a nanoparticle.

Nanoparticle technology has been utilised in many areas including medicine for drug therapy and drug delivery. Advances have been made in drug delivery utilising polymer- and liposome-based systems for tocolytics for the prevention of preterm birth in rat and murine models of preterm labour, and in *ex vivo* experiments on uterine contractility and also preterm birth [358]. The benefit of nanoparticle use is that drugs can be delivered in far lower doses than conventional drug preparations, and can be used for targeting specific cells/tissue. Furthermore, it increases the stability of drugs [359], allowing the medications to be stored at room temperature, therefore of important practical benefit in the third world countries where suitable refrigeration may not be possible.

A group in Brazil has successfully synthesized solid lipid nanoparticle (SLN) embedded with 15dPGJ2 in the core [360]. SLN has been shown to have several advantages over other types of nanoparticles. The low toxicity, good stability, wide range of administration routes [361, 362], safe and sustained drug delivery in *in vivo* experiments [363-366], stable at room temperature and for storage and drug delivery, the ability to modify the drug release profile and its ability to maintain a good cell viability as a vehicle [367] leads to SLN being an attractive way to deliver unstable drugs. The stability of SLN at room temperature is a great opportunity to form a pessary, as the drug alone is unstable at room temperature, to be used in prevention of preterm labour on a global scale, including in third world settings.

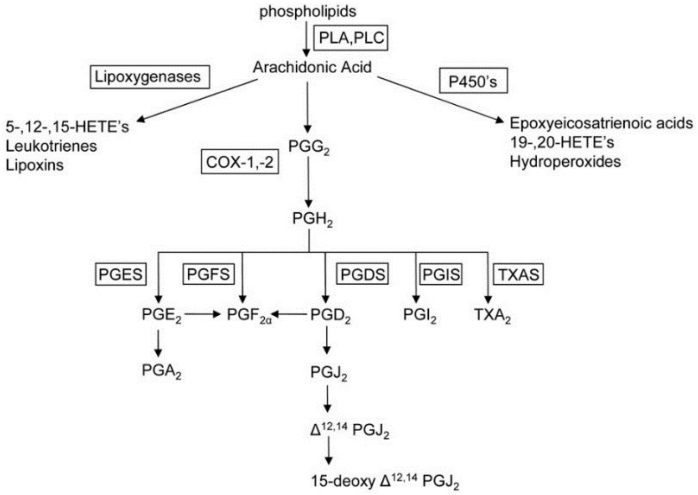


Figure 1.12 PGD2 metabolism pathway. Arachidonic acid is metabolized by cyclooxygenase for prostaglandin metabolism to form an intermediate PGH_2 product. PGH_2 is further metabolized to PGD_2, PGJ_2, $\Delta^{12,14}$-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 [368].

1.10 Hypothesis and aims

Hypothesis:

Viral infections stimulating the TLR3 receptor during pregnancy sensitise gestational tissues to subsequent bacterial infection and increases susceptibility to preterm labour.

Immunomodulation by 15dPGJ2 with the use of nanoparticle technology offers a novel therapeutic strategy and feasible drug delivery system for the prevention of infection/inflammation induced preterm labour (Figure 1.13).

Aims:

1. To determine the expression profile of TLR2, 3, 4, and 6 mRNA in pre-labour and in-labour gestational tissue and cells.

2. To determine TLR3 protein expression in pre-labour and in-labour gestational tissue and cells.

3. To determine the effect of the TLR3 agonist (poly I:C) treatment on TLR3 expression in VEC, amniocytes, myocytes, PBMC and placental explants.

4. To examine the effects of TLR3 agonist (poly I:C) priming prior to bacterial agonist TLR2, TLR4 and TLR2/6 treatment on pro inflammatory and pro-labour mediators in a model of ascending infection.

5. To examine the effects of TLR3 agonist (poly I:C) priming prior to bacterial agonist TLR2, TLR4 and TLR2/6 treatment on pro inflammatory and pro-labour mediators in a model of haematogenous spread of infection.

6. To determine the potential anti-inflammatory effect of solid lipid nanoparticle-loaded with 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15dPGJ2) to inhibit IL-1β-induced NF-κB and AP-1 in VECs, amniocytes and myocytes.

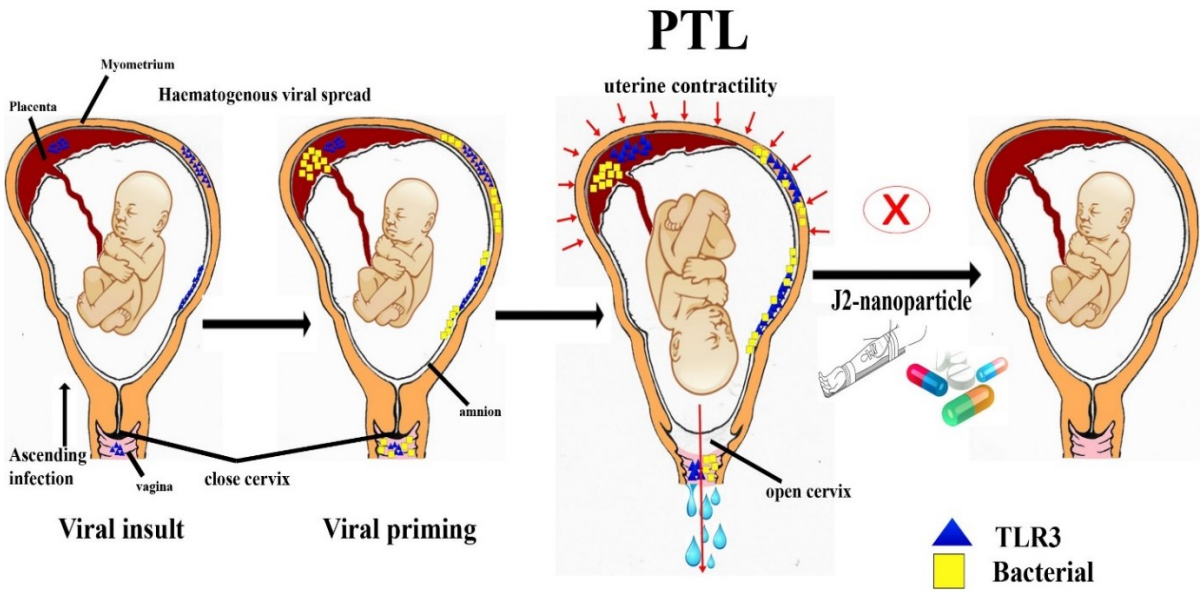


Figure 1.13 Schematic diagram of study hypothesis: TLR3 viral priming of the gestational tissues sensitizes for subsequent bacterial insult and leads to preterm labour. Viral infection during pregnancy via haematogenous spread or ascending infection may stimulate the TLR3 receptor in placenta, myometrium, amnion and vaginal cells. This viral insult may sensitize this tissue to subsequent bacterial infection and increase the risk of PTL. 15dPGJ2 nanoparticles could be delivered as a pessary or intravenously to help prevent preterm labour.

CHAPTER 2

Materials and methods

2.1 Materials

2.1.1 Chemicals, reagents and solvents

Chemical	Supplier
Absolute Ethanol	VWR
Acrylamide	Sigma-Aldrich
Agarose	VWR
Ammonium Persulphate (APS)	VWR
Ampicillin	Sigma
Bovine serum albumin (BSA)	Sigma-Aldrich
Chloroform	Sigma
DEPC-treated water	Ambion
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich
Dithiothreitol (DTT)	Sigma
Ficoll Paque Plus	GE healthcare
Isopropanol	Sigma
Methanol	VWR
Non-fat dried milk powder	VWR
NuPage sample buffer 4x Dye	Invitrogen
SYBR safe DNA gel stain	Invitrogen
TEMED	Sigma-Aldrich
Trizol	Invitrogen
Tween 20	Sigma-Aldrich
Calcium Chloride ($CaCl_2$)	Sigma

2.1.2 Antibodies

Antibody Epitope	Dilution used	Catalogue number	Supplier
Primary Antibody			
β-actin	1:50000	A5441	Sigma-Aldrich
COX-2	1:2000	sc-1745	Santa Cruz
Phospho-c-Jun (Ser73)	1:1000	9164	Cell Signaling
Phospho-ERK (Thr202/Tyr204)	1:1000	9101	Cell Signaling
Phospho-JNK (Thr183/Tyr185)	1:2000	9252	Cell Signaling
Phospho-p38 (Thr180/Tyr182)	1:1000	9211	Cell Signaling
Phospho-p65 (Ser536)	1:1000	3031	Cell Signaling
TLR3	1:1000	6961	Cell Signaling
Phospho-IRF3 (Ser396)	1:1000	29047	Cell Signaling
Secondary Antibody			
Anti-goat IgG	1:2000	sc-2020	Santa Cruz
Anti-mouse IgG	1:25000	7076	Cell Signaling
Anti-rabbit IgG	1:2000	7074	Cell Signaling

2.1.3 Treatments and inhibitors

Compound	**Supplier**
E.Coli LPS serotype 055:B5	Sigma-Aldrich
E.Coli LPS serotype 055:B5 Ultrapure	Invivogen
FSL-1 (Pam2CGDPKHPKSF)	Invivogen
HKLM (Heat-Killed *Listeria Monocytogenes*)	Invivogen
IL-1β	R&D system
Phosphatase inhibitor cocktail	Sigma-Aldrich
Polyinosinic-polycytidylic acid (poly (I:C))	P9582-5MG, Sigma-Aldrich
Protease inhibitor cocktail	Sigma-Aldrich
15deoxy$\Delta^{12,14}$PGJ2 (15dPGJ2)	Cayman Chemicals
Solid lipid nanoparticle	Centro de Pesquisas em Odontologia Sao Leopoldo Mandic, Brazil

2.1.4 Reagents and buffers

General buffers	
Phosphate Buffered Saline (PBS)	140mM NaCl 2.5mM KCl 1.5mM KH_2PO_4 10mM Na_2HPO_4 pH 7.2
Tris Buffered Saline (TBS)	50mM Tris-HCL 150mM NaCl pH 7.5

Cell Culture materials and media

Reagents	Supplier
Calcium Chloride	Sigma
Cell strainer (70μm)	Falcon
Collagenase XI or IA	Sigma-Aldrich
Dispase	Gibco
DMEM/Nutrient Mixture F-12 HAM	Sigma-Aldrich
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich
Fetal calf serum	Sigma-Aldrich
FluoroBrite DMEM media	Thermo Fisher
Keratinocyte-SFM Medium (Kit)	Fisher Scientific
L-glutamine	Sigma-Aldrich
Penicillin/Streptomycin	Sigma-Aldrich
RPMI 1604 medium	Life Technologies
Tissue culture plastic-ware	Corning,
Trypsin/EDTA	Sigma-Aldrich

Protein extraction buffer

Whole cell protein extraction buffer	Cell lysis buffer (10X), Cell Signaling #9803 1mM PMSF Protease and phosphatase inhibitor cocktail

Western Blotting reagents and buffers

30% Acrylamide/Bis	Sigma-Aldrich
Clarity ECL Western blotting reagent	Bio-Rad
ECL plus hyper film	Amersham Biosciences
Luminata Forte Western reagent	Millipore
PVDF membrane	Millipore
Pre-stained protein markers	Fisher Scientific
Whatman 3MM paper	Bio-Rad
Tris-Glycine running buffer	25mM Tris-base 250mM glycine 0.1% (w/v) SDS pH 8.3
Transfer buffer (TB)	39mM glycine 48mM Tris-base 20% (v/v) methanol
Blocking Buffer	0.01% (v/v) Tween-20 5% (w/v) non-fat milk in TBS-T 5% (w/v) Bovine Serum Albumin in TBS-T
Washing Buffer (TBS-T)	0.01% (v/v) Tween-20 in TBS

RNA, cDNA synthesis and PCR reagents and buffer

DNAse Buffer 10x	Invitrogen
DNAse Grade 1	Invitrogen
dNTP 10mM	Invitrogen/Novogen
Trizol	Invitrogen
Oligo DT	Invitrogen
Primers	Thermo scientific
DNA ladder	Invitrogen and Bioline
DNA loading dye	Invitrogen

2.1.5 Kits

Kits	**Supplier**	**Catalogue number**
U-PLEX Biomarker Group 1 (hu) Assays, SECTOR (5 PL)	Meso Scale Discovery (MSD)	K15067L-2
U-PLEX Human IL-1β Assay, SECTOR (1 PL)	Meso Scale Discovery (MSD)	K151TUK-1
U-PLEX Human IL-8 Assay, SECTOR (1 PL)	Meso Scale Discovery (MSD)	K151TYK-1
U-PLEX Human IL-10 Assay, SECTOR (1 PL)	Meso Scale Discovery (MSD)	K151TZK-1
U-PLEX Human IL-6 Assay, SECTOR (1 PL)	Meso Scale Discovery (MSD)	K151TXK-1
Human RANTES Tissue Culture Kit	Meso Scale Discovery (MSD)	K151BFB-2
Prostaglandin E2 Assay	R&D Systems	KGE004B
15-deoxy-Delta12,14-PGJ2 ELISA kit	Enzo Life Sciences	ADI-900-023
RNeasy Micro Kit	Qiagen	74004
SYBR Green master mix	Sigma	S4438-500RXN

2.2 Methods

2.2.1 Primary cell culture

2.2.1.1. Human peripheral blood mononuclear cell (PBMC) culture

Blood was collected from non-pregnant and pregnant women in accordance with the South-East London Ethics approval Ref: 10/H0805/54 in sodium heparin vacutainers. The Ficoll density centrifugation method was used for PBMC isolation as previously described by our group [369]. Blood was diluted 1:1 with PBS (4mls blood: 4mls PBS). 4mls of the diluted blood was then layered on top of 3mls of Ficoll (GE healthcare) and centrifuged at 400 x g for 40 minutes at room temperature. The supernatant was discarded, and the lymphocyte halo, yielding approximately $4x10^6$ PBMC was collected in a fresh tube and washed with PBS twice. The pellet was then either used for cell treatments with TLR agonists or processed immediately for RNA and protein extraction.

a) Cell culture and TLR agonist treatment

The pellet was resuspended in RPMI 1604 medium with 10% fetal calf serum and 2mM/L L-glutamine, 100U/ml Penicillin and 100µg/ml of Streptomycin and plated in a 24-wells plate for treatment. Each treatment contained approximately $1.7x10^5$ PBMC per well.

2.2.1.2. Primary human amnion epithelial cells (amniocytes) culture

Amnion was provided with consent from women on the labour ward at Queen Charlotte's and Chelsea Hospital in accordance with ethical committee approval (Ref 2002/6283). Pre-labour and post-labour placentas were taken from women undergoing elective caesarean section at term or following spontaneous vaginal delivery respectively.

Amniocytes were extracted from amnion as described previously by our group [369-371]. Firstly, amnion was separated from the choriodecidua and washed 3 times with phosphate buffer saline (PBS) to remove the blood. The amnion was cut into 5cm strips and incubated in 0.5mM EDTA-PBS for 15 minutes at room temperature, and subsequently rinsed in PBS. The intracellular matrix was then digested in 60mls of

pre-warmed dispase (Gibco) (2g/L in PBS) for 50 minutes at 37°C. The amnion strips were then shaken vigorously in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) (DMEM 10% FCS), 2mM/L L-glutamine, 100U/ml Penicillin and 100µg/ml Streptomycin for 4 minutes to isolate the epithelial cells from the intracellular matrix. The floating strips were removed, and the suspension was centrifuged for 10 minutes at 2000rpm to pellet the epithelial cells. Cells were either used immediately for protein and RNA extraction or resuspended in 50mls DMEM and grown to confluence at 37°C, 5% CO_2 for treatment with TLR agonists.

2.2.1.3. Primary myometrial smooth muscle cell (myocytes) culture

Myometrial biopsies were provided with consent from women on labour ward at Queen Charlotte's and Chelsea Hospital under the granted ethical approval (Ref 3358, Riverside Research Ethics Committee). Pre-labour and in-labour samples were taken from women undergoing elective caesarean section at term either prior to the onset of labour, or at the time of emergency caesarean following the onset of labour.

Myometrial biopsies were taken from the upper margin of lower segment incisions, placed in PBS, stored at 4˚C and used within 2 hours of collection. Myocytes were extracted from myometrial biopsies as previously described by our group [116, 369, 372]. The tissue was washed with PBS to rinse off excess blood before being dissected mechanically using two sterile blades to form a fine paste-like texture. Cells were isolated by incubating the homogenate for 45 minutes at 37°C with a sterile enzyme mix of 10mg of Collagenase 1A, 10mg of Collagenase XI and 200mg of BSA in 30mls of 1:1 of DMEM and F-12 HAM. DMEM (10% FCS) was added to inactivate the enzymes before the suspension was filtered through a 100µm cell strainer and centrifuged at 3000rpm for 5 minutes. The pellet was resuspended in DMEM (10% FCS) and cultured in a flask at 37°C, 5% CO_2 to confluence. Cells were passaged and used for experiments between passage three and six.

2.2.1.4. Vaginal epithelial cell line (VK2/E6E7) (VEC) culture

VK2/E6E7 (ATCC® CRL2616™) cell lines were purchased from American Type Culture Collection (ATCC) company. The cells were kept in liquid nitrogen vapour

phase until use, to avoid loss of viability. At the time of culturing, cells were thawed at 37°C in a water bath for approximately 2-3 minutes and transferred into pre-warmed DMEM (10% FCS). Cells were centrifuged at 125g for 5 minutes at room temperature, the supernatant was discarded, and the cell pellet was resuspended in 7mls of keratinocyte serum free medium (KSFM) supplemented with bovine pituitary extract (BPE), epidermal growth factor (EGF) and calcium chloride ($CaCl_2$). Cells were grown at 37°C, 5% CO_2 until confluent. Cell treatments were performed between passage numbers three and six.

2.2.2 Placental explant culture

Placentas were obtained from labour ward inpatients at Queen Charlotte's and Chelsea Hospital. Written consent was obtained from all patients under the granted ethical approval by Hammersmith, Queen Charlotte's & Chelsea Hospitals' Research Ethics Committee (Ref 2002/6283). Tissue samples were collected and thoroughly washed in PBS. Tissue was either prepared immediately for explant culture or was snap frozen on dry ice and promptly stored at -80˚C for future RNA and protein extraction.

For placental explant studies a 2cm portion of placental tissue was dissected from the maternal side and washed in PBS to remove excess blood. The tissue was then dissected in Dulbecco's Modified Eagle's Medium F12 (DMEM-F12) (Sigma-Aldrich) on ice with sterile forceps and a blade, yielding explants each weighing between 30mg. DMEM was supplemented with 10% Fetal Bovine Serum (FBS), Penicillin and Streptomycin (100µl/ml). Culture conditions were based on previously published protocols [373-376]. Explants were cultured in 12-well plates in 8% oxygen, 5% CO_2. Explants were left to equilibrate in culture for 5 days prior to stimulation to allow the regeneration of the syncytiotrophoblast after injury during dissection [377, 378]. DMEM-F12 was changed every 48 hours leading up to treatments, and samples were serum starved on day 4 prior to treatments.

2.2.3 Cells and explant stimulation and drug treatment

2.2.3.1 Toll-like receptor stimulation

Human cultured PBMCs, amniocytes, myocytes and VECs were stimulated with TLR2, 3, 4 and 2/6 agonists at various time points and concentrations (concentrations

and time points are described in the chapter summary of each Chapter and in Appendix A). For priming studies, cells/tissue were stimulated first with the TLR3 agonist, and then stimulated with either TLR2, TLR3, TLR4 or TLR2/6 agonists. The stimulated cells/tissue were subjected to RNA and protein extraction for NF-κB/AP-1 activation or TLR expression, whereas the supernatant was used to measure the cytokines, chemokines and PGE2 quantification as described in section 2.2.11.

2.2.3.2 15-deoxy-Δ12,14-PGJ2 (15dPGJ2) and nanoparticle treatment

Human amniocytes, myocytes and VEC were incubated with 15dPGJ2 for 2 hours prior to IL-1β stimulation. Solid lipid nanoparticle (SLN)-loaded 15dPGJ2 was also used to treat myocytes. Myocytes were incubated for 2.5 hours prior to IL-1β stimulation. Cultured cells were used for RNA and protein extraction for MAPKs, NF-κB, AP-1 activation and supernatant was used to quantify cytokines, PGE2 and 15dPGJ2 production.

2.2.4 RNA extraction

Total RNA was isolated from tissues, primary cell cultures, cell lines and peripheral blood mononuclear cells using Trizol© according to the manufacturer's instructions. The tissue was ground in liquid nitrogen using a pestle and mortar and homogenised using the power homogeniser in 1ml of Trizol© per 50-100mg of tissue. For primary cell culture, 600µl of Trizol© was added and the cells were collected using cell scraper. Ficoll separated PBMCs pellets were resuspended in 1ml of Trizol© per 1 million cells.

RNA was extracted by adding 0.2 volume of chloroform to 1 volume of the homogenate and precipitated using 0.5 volume of isopropanol. The solution was incubated for 30s and pelleted at 12,000 g for 10 minutes at 4ºC. The pellet was washed in 75% ethanol, air-dried and re-suspended in 20µl of RNase-free water. The concentration and purity of the RNA was measured using NanoDrop Nd-1000 spectrometer. RNA was stored at -80ºC until further analysis.

For cultured PBMCs, total RNA extraction was performed using RNeasy Micro Kit according to the manufacturer's instruction. Briefly, the pelleted PBMCs after treatment were digested in RLT buffer supplemented with β-mercaptoethanol. The

homogenised lysate was then supplemented with the same volume of 70% ethanol. The mixture was spun in the spin column at 10,000 x g for 15s. The flow through was discarded and the spin column was carried on with DNase digestion using a mixture of buffer RDD and DNAse (1:8) at a total of 80µl after washing step with buffer RW1. The spin column was incubated for 15 minutes at room temperature and spun at 10,000 x g for 15s. The spin column was washed with 500µl of buffer RPE at 10,000 x g for 15s and a second wash with 500µl of 80% ethanol at 10,000 x g for 2 minutes. 10µl of RNase-free water was added into the spin column and spun at 16,000 x g for 1 minute.

2.2.5 DNase treatment and first-strand cDNA synthesis

Prior to generation of cDNA, the total RNA was treated with DNase to remove any contaminating DNA. 2µg of RNA (300ng of RNA from plated PBMC) was added to the mixture of 1µl of DNAse 10x buffer and 1µl of DNAse. DEPC-treated water was used to make a total volume of 10µl. The solution was incubated for 15 minutes at room temperature and the reaction was stopped using 1µl of 25mM EDTA for 10 minutes at 70ºC. 8µl of the DNAse treated samples were then added to the mixture of 1µl 10mM dNTP and 1µl of Oligo DT (0.5µg/ml) and incubated at 70°C for 10 minutes. Next, 2µl 10x M-MLV RT buffer, 1µl M-MLV RT, 0.5µl RNase Inhibitor and 6.5µl DEPC-treated water were added and the solution was incubated at room temperature for 15 minutes, 37°C for 50 minutes and 90°C for 10 minutes.

2.2.6 Polymerase chain reaction

Polymerase chain reactions (PCR) were performed using Taq DNA Polymerase. A PCR reaction mix was prepared following the manufacturer's protocol (Sigma-Aldrich) (Table 2.1). Briefly, 4µl of cDNA was mixed with 10µl of 10x PCR buffer, 2µl of dNTP mix, 5µl of both forward and reverse primers (Table 2.3), 0.5µl of Taq enzyme and 70.5µl of H_2O. Polymerase activation was set at 94°C for 3 minutes and the reaction was subjected to 35 thermo-cycles of DNA denatured for 45 seconds at 94°C, primer annealing at 60°C for 30 seconds and extension at 72°C for 1 minute 30 seconds. This was followed by the final extension step of 72°C for 10 minutes (Table 2.2). The PCR final product was analysed by agarose gel electrophoresis.

Table 2.1 PCR mix

PCR mix	Volume (µl)
Buffer	10µl 10x PCR buffer (minus Mg)
10mM dNTP mix	2µl
10mM forward primers	5µl
10mM reverse primers	5µl
cDNA	4µl
Taq polymerase enzyme	0.5µl
50mM MgCl2	3µl
H_2O	70.5µl
Volume of mix	100µl

Table 2.2 PCR program

Process	Temperature, time
Polymerase activation	94°C for 3 minutes
Denature	94°C for 45s
Anneal	60°C for 30s
Extend	72°C for 1-minute 30s
Hold	72°C for 10 minutes
Cycles	35

Table 2.3 Primers

	Forward	Reverse	Size (bp)
β-actin	AGGCATCCTCACCCTGAAGTA	CACACGCAGCTCATTGTAGA	105
CCL2	TCTGTGCCTGCTGCTCATAG	AGATCTCCTTGGCCACAATG	203
Con 43	AGGAGTTCAATCACTTGGCG	GCAGGATTCGGAAAATGAAA	174
COX-2	TGTGCAACACTTGAGTGGCT	ACTTTCTGTACTGCGGGTGG	77
cPLA2-α	TGCATTCTGCACGTGATGTG	CACCATGGCTCGGAAACC	68
IL-6	CCTTCCAAAGATGGCTGAAA	AGCTCTGGCTTGTTCCTCAC	153
IL-8	GCCTTCCTGATTTCTGCAGC	CGCAGTGTGGTCCACTCTCA	151

L19	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAA	77
OTR	GATGGGAAGGGTGGT	CAAGGACCCCAGCATTTGTT	77
TLR1	ACACCAAGTTGTCAGCGATGTG	ACCATGCGTGTACCAGACACTG	105
TLR2	TGCTGCCATTCTCATTCTTCTG	AGGTCTTGGTGTTCATTATCTTCC	92
TLR3	CCTGGTTTGTTAATTGGATTAACG A	TGAGGTGGAGTGTTGCAAAGG	82
TLR4	AATCCCCTGAGGCATTTAGG	CCCCATCTTCAATTGTCTGG	100
TLR6	GCCACCATGCTGGTGTTGGCT	CGCCGAGTCTGGGTCCACTG	101
TNF-α	TCCTTCAGACACCCTCAACC	CAGGGATCAAAGCTGTAGGC	208

2.2.7 Agarose gel electrophoresis

PCR products were detected using a 2% agarose gel to confirm the correct product size, a single product, and absence of product in the water negative control. Agarose was made up in 1x TBE buffer and heated until completely dissolved. The gel was then cooled under the tap, and on cooling, 1:10 000 SYBR Safe was added before allowing the gel to set. The PCR product was diluted in 6x loading buffer and loaded in the gel. The gel was electrophoresed at 100V for 30-40 minutes, transilluminated and photographed immediately under the UV light.

2.2.8 Real-time polymerase chain reaction – Quantitative PCR

The target gene was quantified using SYBR Green PCR mix (Applied Biosystem) (Table 2.4) using the program listed in Table 2.5 and carried out using ABI StepOne Real Time PCR system (Applied Biosystems). At each cycle, the SYBR Green 1 fluorescent dye was incorporated into the double-stranded DNA and the emitted fluorescent dye is detected by the Real Time PCR system throughout each cycle. The accumulation of the products led to a high SYBR Green fluorescence. Amplification was carried out using specific primers listed in Table 2.3. The cycle number where the fluorescence reached a preset cycle threshold, defined as Ct value, was used as a quantitative measurement of the input target. A low Ct value referred to high target cDNA. Data was normalised to either 1 or 2 housekeeping genes of β-actin and L19.

Table 2.4 RT-QPCR mix

Reagent	Volume
SYBRGreen	10µl
Rox Dye	0.2µl
Forward primer (20µM)	0.3µl
Reverse primer (20µM)	0.3µl
Water DEPC	5.2µl
cDNA	4µl
Total volume	20µl

Table 2.5 RT-QPCR program

Process	Temperature, time
Polymerase activation	95°C 2 minute
Denature	95°C 15 sec
Anneal	60°C 30 sec
Extend	72°C 30 sec
Hold	95°C 15 sec
Cycles	40

2.2.9 Protein extraction

2.2.9.1 Whole cell protein extraction

Suspension cells (PBMCs) and adherent cultured cells (amniocytes, myocytes and VEC) which were collected with a cell scraper, were lysed with whole cell lysis buffer (Cell Signaling) containing 5µl/mL of protease inhibitor cocktail (Sigma), 5µl/mL of phosphatase inhibitor cocktail (Sigma), 1mM of PMSF. Cells were incubated on ice for 5 minutes in the lysis buffer and centrifuged at 14,000 x g for 10 minutes at 4°C. The supernatant was collected and stored at -80°C until used.

Whole cell lysates from amnion, myometrial and placental tissue were prepared by homogenising in lysis buffer, followed by centrifugation. Supernatants were collected and stored at -80°C until use.

2.2.9.2 Protein quantification

Protein quantification was performed using the BIORAD assay. Standards of 0.25, 0.5, 1.0 and 2.0 mg/ml were created using 4 mg/ml stock from Bovine Serum Albumin (BSA) and lysis buffer. 5µl of sample was added to 96 well plates in duplicates and then 25µl of Reagent A & S (1:20) was added to each well followed by 200µl of Reagent B. The plate was incubated for 20 minutes before being analysed using a microplate spectrophotometer measuring the absorbance at 650 nm.

2.2.10 Western blot and immunodetection

2.2.10.1 SDS Page gel electrophoresis

10% and 8% resolving gels and 5% stacking gels were prepared from the reagents listed in Table 2.6. Gels were then mounted in BIORAD tanks and filled with 1x sodium dodecyl sulphate (SDS) running buffer. Protein along with 4x loading dye was denatured at 80°C for 10 minutes before being loaded into wells adjacent to a protein ladder. Gels were electrophoresed at 128V until the dye front reached the bottom of the gel.

Table 2.6 Solutions for preparing resolving and stacking gel

Reagents	10% Resolving gel	8% Resolving gel	5% Stacking gel
H2O	7.9ml	9.3ml	5.5ml
30% Acrylamide mix	6.7ml	5.3ml	1.3ml
1.5M Tris (pH 8.8)	5.0ml	5.0ml	0ml
1M Tris (pH 6.8)	0ml	0ml	1.0ml
10% SDS	0.2ml	0.2ml	0.08ml
10% ammonium persulfate (APS)	0.2ml	0.2ml	0.08ml
TEMED	0.008ml	0.012ml	0.008ml

2.2.10.2 Western Blotting

The BIORAD wet transfer was used to transfer the protein from the gel to the membrane. The polyvinyl difluoride (PVDF) membranes were activated in 100% methanol (for 30 seconds) and washed in distilled water for a few seconds prior to transfer. The gels were then removed from the tank and layered on Whatman paper and sponge. The unit was transferred in a BIORAD transfer tank filled with transfer buffer and left for 90 minutes at 300mA. The membrane was then re-activated in 100% methanol and blocked in 5% milk in 1x TBS-T for 1 hour. The membranes were hybridised with primary antibody overnight at 4°C. Secondary antibody incubation was carried out the next day. Following antibody incubation, the membranes were washed in 1x TBS-T for 10 minutes and three times for 5 minutes and proteins were detected using Clarity ECL Western (BioRad) or Luminata Forte (Millipore) blotting substrate of horseradish peroxidase.

2.2.11 Enzyme linked immunosorbent assay (ELISA)

2.2.11.1 Cytokines and chemokines

The cytokines and chemokines were analysed from cultured VEC, amniocytes, myocytes, PBMCs and placental explant stimulated with various TLRs and 15dPGJ2 treatments. The supernatants from these cells were collected and subjected to cytokine measurement using the U-Plex protocol from MSD (Meso Scale Diagnostics, Rockville, Maryland). 96-well plates were set up to analyse 16 different cytokines (a total of 8 cytokines per plate). The individual wells were coated with linker and antibody and were left on the shaker for 1 hour. A visual representation of the principles behind how each cytokine was detected can be seen in Figure 2.1. A summary of the cytokines that were analysed in each plate is listed in Table 2.7. A titration test of supernatants from each treatment/cell type was used to ensure readings were within the standard curve and assay range. Samples were plated alongside a panel of calibration curve standards. All samples and standards were plated in duplicates. Following incubation for 1 hour, plates were washed in 0.02% PBS with Tween (PBS-T) before incubation with detection antibody solution for a further 1 hour. Plates were

washed with 0.02% PBS-T and 2 x Read Buffer was added to each well. Plates were read by the QuickPlex SQ 120 (Meso Scale Diagnostics).

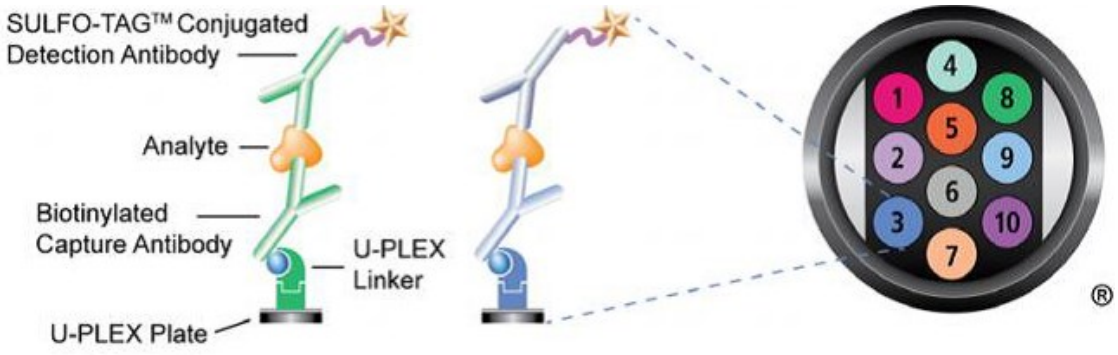


Figure 2.1 A schematic representation of how the 96-well U-PLEX plates are able to detect the specific cytokines of interest. Each well has 10 different spots to which each of the well has been assigned to one specific U-Plex linker. The biotinylated capture antibody is prepared and bound to each linker and this antibody-linker conjugate is left to incubate on shaker allowing the binding of the linker to its specific spot on the U-Plex plate. Derived from the MSD protocol for U-Plex plate.

Table 2.7 A summary of the different cytokines analysed on each of the two 96 well U-Plex plates. The first column shows the 8 cytokines that each well on plate 1 was designed to detect. The second column shows the 8 cytokines that each well on plate 2 was designed to detect.

First Plate	Second Plate
TNF-α	MIP-1α
IL-8	MIP-1β
IFN-γ	IFN-β
IL-6	IL-12p40
IL-12p70	TNF-β
IL-1β	IFN-α
IL-1α	IL-4
IL-1RA	IL-10

2.2.11.2 PGE2

The supernatants collected after treatment with 15dPGJ2 and TLR stimulation were used for PGE2 measurements according to the manufacturer's guidance (KGE004B, R&D Systems). In brief, the supernatant was diluted in a 1:3 dilution with the assay diluent. Samples were plated alongside a panel of calibration curve standards. All samples and standards were plated in duplicates. Following a 1 hour incubation period on a shaker at 500 rpm, the PGE2 conjugate was added and the plate was incubated further for 2 hours. The plate was washed 4 times with the wash buffer and a substrate solution was added and incubated in the dark for 30 minutes at room temperature. Following this, a stop solution was added and the absorbance was measured at 450 nm, 540 nm and 570 nm at 30 minutes. The reading at 450 nm was subtracted from the 540 nm and 570 nm readings and this value was used for analysis.

2.2.11.3 15-deoxy-Δ12,14-PGJ2

The supernatant from cultured myocytes treated with SLN alone and 15dPGJ2-loaded SLN were used to measure the release of 15dPGJ2 from the nanoparticles. The supernatant was diluted 1:200 with the relevant matched culture medium. Samples and standards were plated in duplicates. Following a 2 hour incubation on a shaker at 500 rpm at room temperature, the plate was washed 3 times. The substrate solution was added into each well and incubated for 3 hours at 37°C. A stop solution was added after 3 hours and the absorbance was measured at 405 nm, 570 nm and 590 nm. The reading at 405 nm was subtracted from the 570 nm and 590 nm readings and this value was used for analysis.

2.2.12 Imaging

2.2.12.1 Standard light microscopy

This imaging technique was used to assess the confluency of the cells and the morphology pre and post treatment as a measure the general health of the cells.

2.2.12.2 Differential interference contrast (DIC)

Solid lipid nanoparticle (SLN) (Figure 2.2) is a submicron particle ranging from 50-100 nm and has been widely utilised as a drug carrier for therapeutic purposes [379]. The entry point of this nanoparticle in the myocytes was determined before looking at the effect on NF-κB and AP-1 with the nanoparticle incubation prior to IL-1β stimulation. Term non-labouring myocytes were grown in a bottom glass chamber and incubated overnight at 37°C. Cells were washed with PBS twice. Following washing, serum free fluorobrite DMEM media with 0.32μM of RHB-tagged 15dPGJ2 was added to the cells. Water and vehicle controls were used. The bright-field optical images were captured using differential interferences contrast (DIC) modes at the intervals of 0, 15 minutes, 30 minutes, 45 minutes and 1 hour. The fluorescence images were acquired using UV-diode laser at the excitation wavelength of 405nm and He/Ne laser at excitation wavelength of 543/633nm. The images were captured using Leica TCS SP5 (Leica Microsystems)

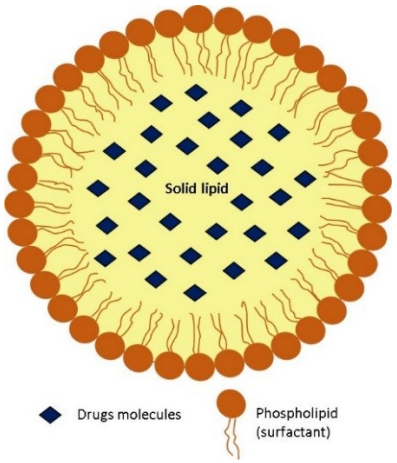


Figure 2.2 A representative structure of solid lipid nanoparticle (SLN). A submicron particles made from lipid and remain solid at room temperature (adapted from [380]).

2.2.13 Statistical analysis

All data were analysed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Statistical tests were selected depending on the number of groups analysed and the distribution of the data. When comparing two groups, a Student's T-test was used for if data was normally distributed, and a Mann-Whitney (for unpaired) or Wilcoxon test (for paired) was used if the data were not normally distributed. One-way ANOVA was

used when comparing more than two groups of one variable if data were normally distributed, or by using the Kruskal-Wallis for non-parametric distributions. A Two-way ANOVA was used when comparing data with more than one variable for both data found to be normally distributed, and for experiments whereby experimental replicates were less than 5, due to the robustness of the Two-way ANOVA [381, 382]. The *Bonferroni* or *Dunnett's* post hoc tests were used when required. A $p<0.05$ was considered as statistically significant.

CHAPTER 3

Toll-like receptor expression

Introduction

Background:

Successful pregnancy requires a highly regulated immune system. The innate immune system, although primitive, acts as the first line of defence and subsequently contributes to activation of the adaptive immune response. The innate immune response consists of several cells and receptors that are vital for the recognition of the invading pathogens. One of the most researched type of pattern recognition receptors are the family of the toll-like receptors (TLRs). Gestational tissues express TLRs which serve to recognise invading microorganisms and commensals. The expression of these TLRs has been shown to change throughout gestational age to aid implantation and labour. Some of the TLRs such as TLR2, TLR4 and TLR6 can also change at term, preterm and with infection in the gestational tissues. These TLRs respond to the gram negative and gram positive bacteria that are associated with chorioamnionitis. Much of the focus on TLR expression in pregnancy has been on the bacterial ligands TLR2, 4 and 6 in the context of bacterial-induced preterm labour.

There are limited human studies examining the expression of viral TLRs in gestational tissues, and the role of viruses in preterm labour, however several murine studies support the role of TLR3 in virally-induced preterm labour.

Hypothesis:

1. Toll-like receptors (TLR) 3, 2, 4 and 6 are expressed in all gestational tissue and the expression increases with the onset of labour.
2. The TLR3 agonist poly I:C increases TLR3 expression.

Aims:

The aims of the first part of this study are to confirm the expression of TLR2, 4 and 6 in amniocytes, myocytes, vaginal epithelial cells, placental explants and PBMCs. I will also determine if expression is increased with the onset of labour.

Finally, I will examine if the TLR3 agonist (poly I:C) alters TLR3 protein expression in amniocytes, myocytes, vaginal epithelial cells, placental explants and PBMCs.

Results

3.1 Endogenous TLR3, 2, 4 and 6 mRNA expression

TLR2, 3, 4 and 6 mRNA expression were compared between pre-labour and in-labour samples of matched amnion and amniocytes, matched myometrium and myocytes, and in VECs, placental explants and PBMCs. Total RNA was extracted from all tissues and cells and real time QPCR (RT-QPCR) was performed to quantify mRNA expression. TLRs were detected using the primers described in Table 2.3 (Chapter 2, section 2.2.6).

For vaginal epithelial cells, a cell line was used to detect the presence of TLR mRNA due to the difficulty in accessing primary cells during pregnancy and labour. Expression of TLR2, 3 and 6 was confirmed, however no TLR4 mRNA expression was seen (Figure 3.1).

RT-QPCR was performed on amnion and amniocytes on both pre-labour and in-labour samples to detect the expression of TLR2, 3, 4 and 6. TLR2 (**p<0.01) and TLR3 (*p<0.05) showed increased expression in labour in amnion tissue. However, no differences were detected in TLR2, 3, 4 and 6 expression in amniocytes between pre-labour and in-labour samples (Figure 3.2).

In myometrium, TLR2, 4 and 6 (*p<0.05) expression increased significantly in labour. However, at the cellular level only TLR6 (**p<0.01) increased significantly in labour (Figure 3.3).

In PBMCs, no significant difference in expression was detected for any of the TLRs between pre-labour and in-labour samples (Figure 3.4).

Placental explants showed only a significant increase in TLR2 (*p<0.05) and TLR3 (*p<0.05) mRNA in labour compared to pre-labour samples (Figure 3.5).

3.1.1 Vaginal epithelial cells (VEC)

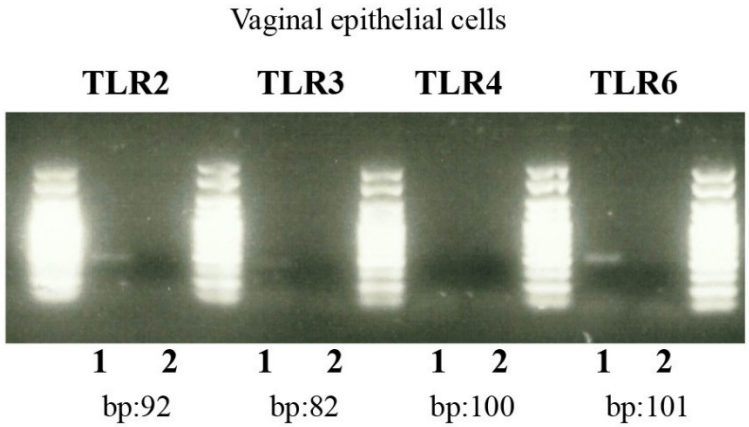


Figure 3.1 TLR2, 3, 4, 6 mRNA expression in VEC. Expression of TLR2, 3, 4 and 6 mRNA was examined in VEC using RT-PCR. The PCR products were run on a 2% agarose gel and a single band was observed in TLR2 (lane 1), 3 (lane 2) and 6 (lane 4). No band was observed in TLR4 (lane 3). A water control was run on the adjacent lane as a negative control. bp = base pairs. 1 = vaginal cells, 2 = water control.

3.1.2 Amnion and amniocytes

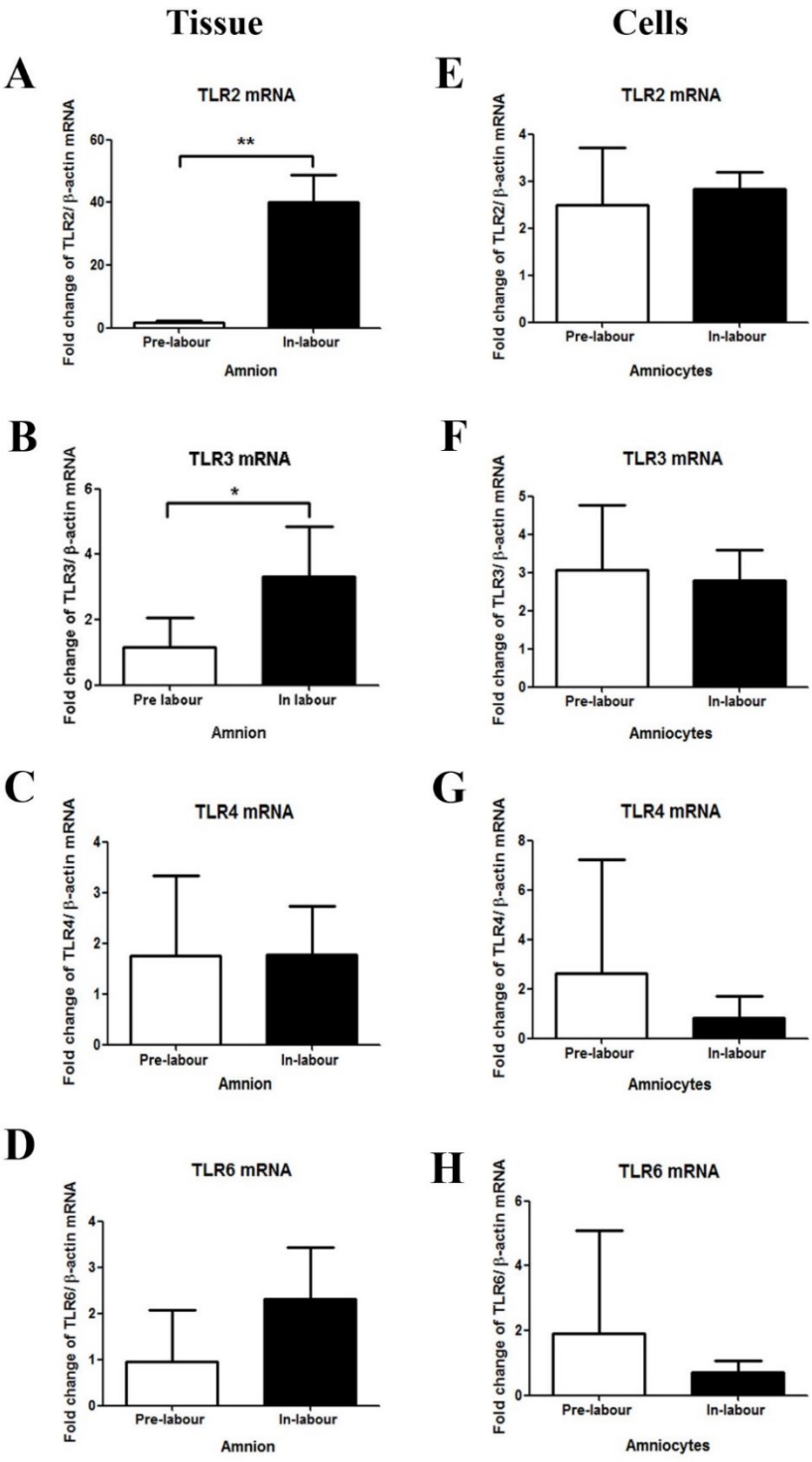


Figure 3.2 TLR2, 3, 4 and 6 mRNA expression in pre-labour and in-labour of matched amnion and amniocytes. Expression of TLR2, 3, 4 and 6 mRNA was quantified using RT-QPCR with β-actin as the housekeeping gene. In amnion tissue, only **(A)** TLR2 (**p<0.01) mRNA expression is significantly increased as compared to the pre-labour amnion while no significant changes were seen with **(B)** TLR3, **(C)** TLR4 and **(D)** TLR6 mRNA expression. No difference in expression was observed in the amnion cells of **(E)** TLR2, **(F)** TLR3, **(G)** TLR4 and **(H)** TLR6 between pre-labour and in-labour. Statistical analysis was performed using unpaired t-test for normally distributed data and Mann-Whitney U test for data not normally distributed, n=6 biological replicates.

3.1.3 Myometrium and myocytes

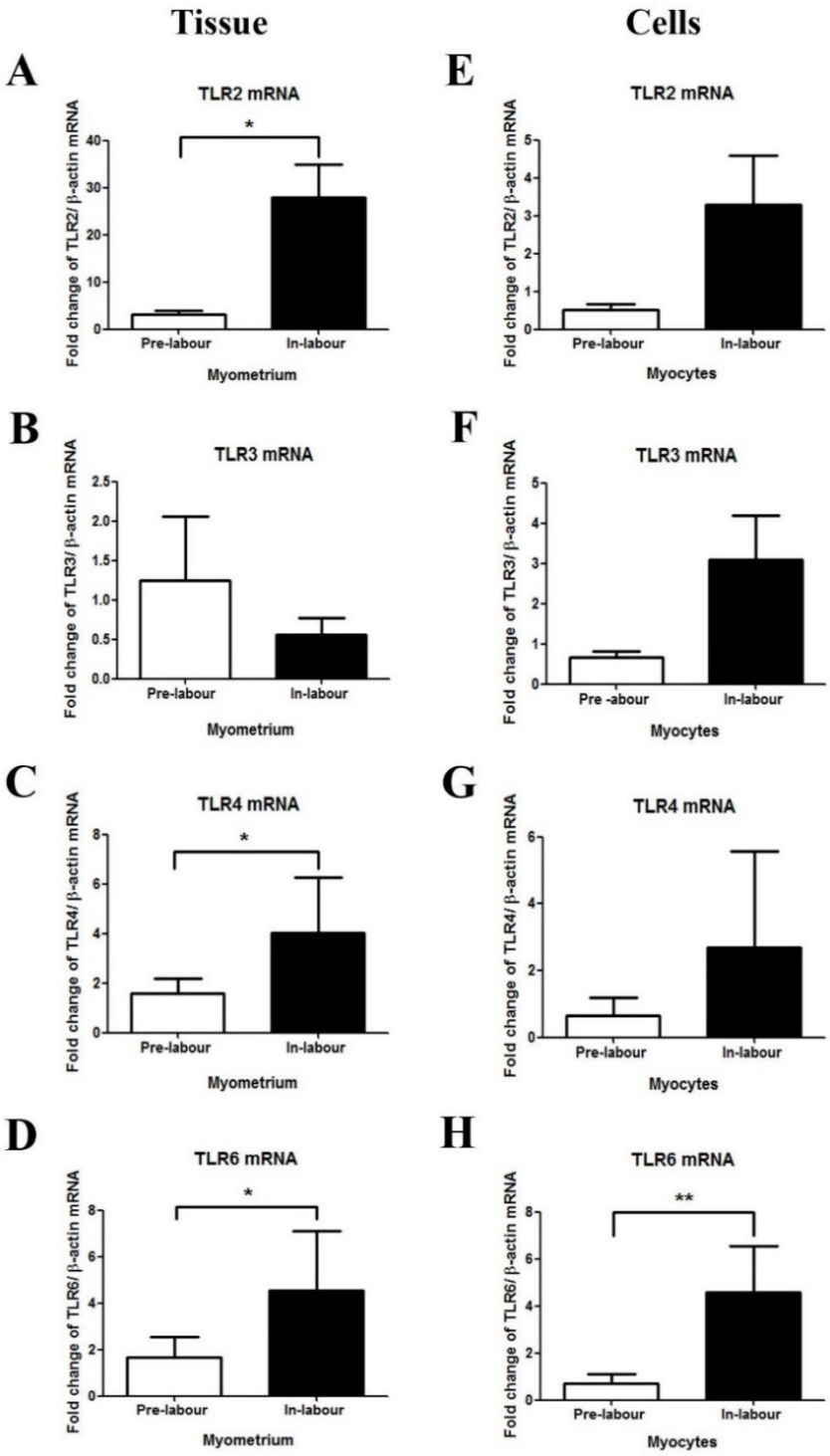


Figure 3.3 TLR2, 3, 4 and 6 mRNA expression in pre-labour and in-labour matched myometrium and myocytes. Expression of TLR2, 3, 4 and 6 mRNA was quantified using RT-QPCR with β-actin as the housekeeping gene. In myometrium tissue, a significant difference in expression was observed in **(A)** TLR2, **(C)** TLR4 and **(D)** TLR6 (*p<0.05) between pre-labour compared to in-labour but not in **(B)** TLR3 mRNA expression. No difference in expression was observed in the cells for **(E)** TLR2, **(F)** TLR3, **(G)** TLR4 except **(H)** TLR6 (**p<0.01) mRNA expression. Statistical analysis was performed using unpaired t-test, n=6 biological replicates.

3.1.4 PBMCs

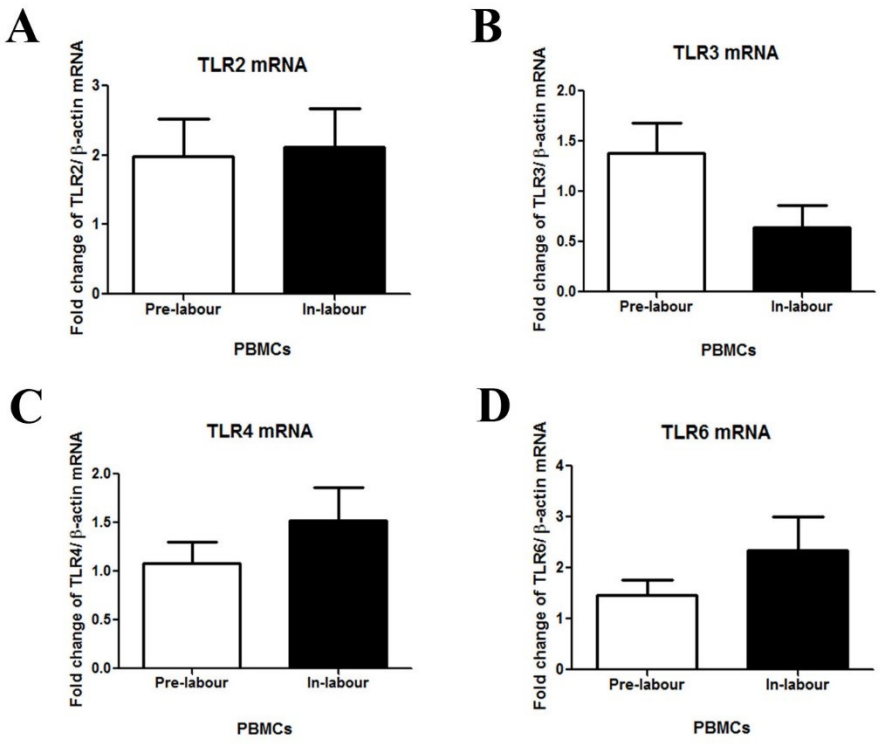


Figure 3.4 TLR2, 3, 4 and 6 mRNA expression in pre-labour and in-labour PBMCs. Expression of TLR2, 3, 4 and 6 mRNA was quantified using RT-QPCR with β-actin as the housekeeping gene. No difference of mRNA expression was observed in **(A)** TLR2, **(B)** TLR3, **(C)** TLR4 and **(D)** TLR6 between pre-labour compared to in-labour samples. Statistical analysis was performed using Mann-Whitney U test, n=4 biological replicates.

3.1.5 Placental explants

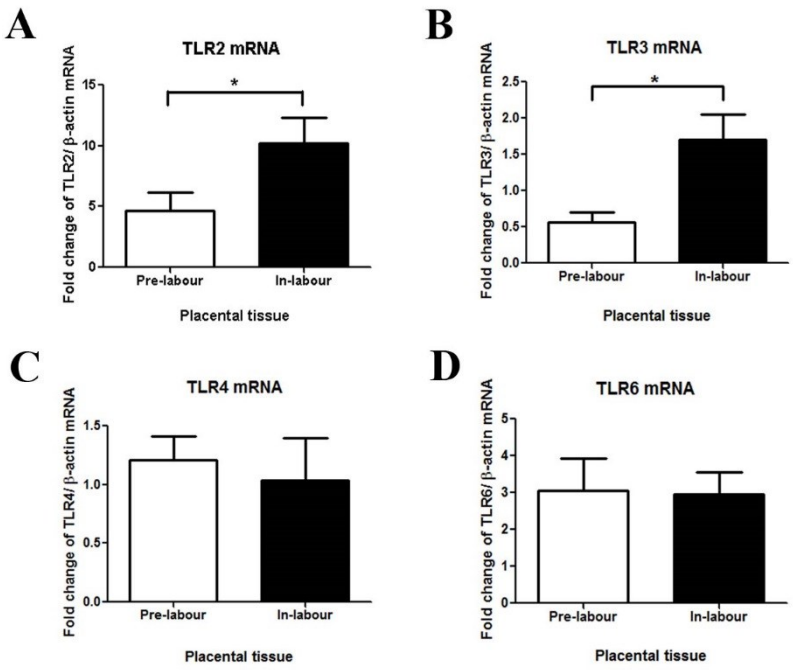


Figure 3.5 TLR2, 3, 4 and 6 mRNA expression in pre-labour and in-labour placental explants. Expression of TLR2, 3, 4 and 6 mRNA was quantified using RT-QPCR with β-actin as the housekeeping gene. A significant increase in **(A)** TLR2 (**p*<0.05) and **(B)** TLR3 (**p*<0.05) mRNA expression was observed in in-labour compared to pre-labour samples. **(C)** TLR4 and **(D)** TLR6 mRNA expression showed no difference between pre-labour and in-labour placentas. Statistical analysis was performed using Mann-Whitney U test, n=4 biological replicates.

3.2 Endogenous TLR3 protein expression

With the confirmation of TLR3 mRNA expression in the tissues and cells, expression at the protein level was examined. The matched tissue and cell samples were subjected to whole cell protein extraction and western immunoblotting was used to detect TLR3 protein. Protein expression of TLR2, 4 and 6 was not performed, as the novel work in this study is TLR3.

The basal level of TLR3 protein in VECs was detected in the poly I:C stimulation blot labelled as non-stim (non-stimulated) (Figure 3.10).

TLR3 protein was demonstrated at the basal level in amniocytes and no difference was detected comparing pre-labour and in-labour samples, consistent with there being no difference in mRNA expression (Figure 3.6). Despite seeing an increase in mRNA in amnion from laboured placentas, no protein expression was demonstrated in the amnion from both pre labour and in labour amnion (data not shown).

In myometrium and myocytes, consistent with the mRNA expression data, there was no difference in TLR3 protein expression in the pre-labour and in-labour samples, (Figure 3.7).

Pre-labour PBMC TLR3 protein was expressed faintly in one pre labour sample. In the in-labour samples, a variation in expression was seen between patients with two patients having clear expression of the TLR3 protein and the other two patients showing no expression (Figure 3.8).

Placental explants showed a significant increase in TLR3 protein expression between pre-labour and in-labour samples (Figure 3.9) (*p<0.05).

3.2.1 Vaginal epithelial cells (VEC)

The expression of TLR3 protein in VEC is shown in section 3.3.1 at the basal level before stimulation with poly I:C.

3.2.2 Amniocytes

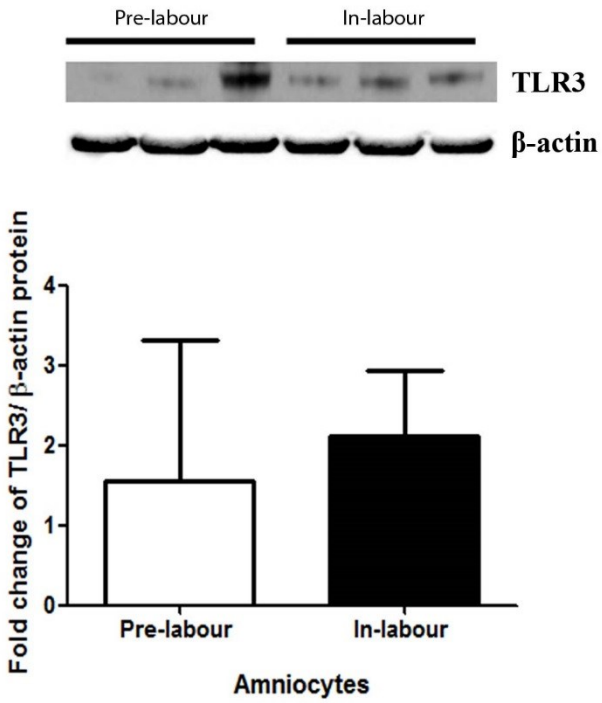


Figure 3.6 TLR3 protein expression in pre-labour and in-labour matched amniocytes. Confluent monolayer amniocytes were lysed in the lysis buffer and Western immunoblotting was performed to detect the presence of TLR3 protein. A representative blot was shown above the graph with β-actin as a loading control. No difference of TLR3 protein was observed between pre-labour and in-labour samples. Statistical analysis was performed using Mann-Whitney U test, n=6 biological replicates.

3.2.3 Myometrium and myocytes

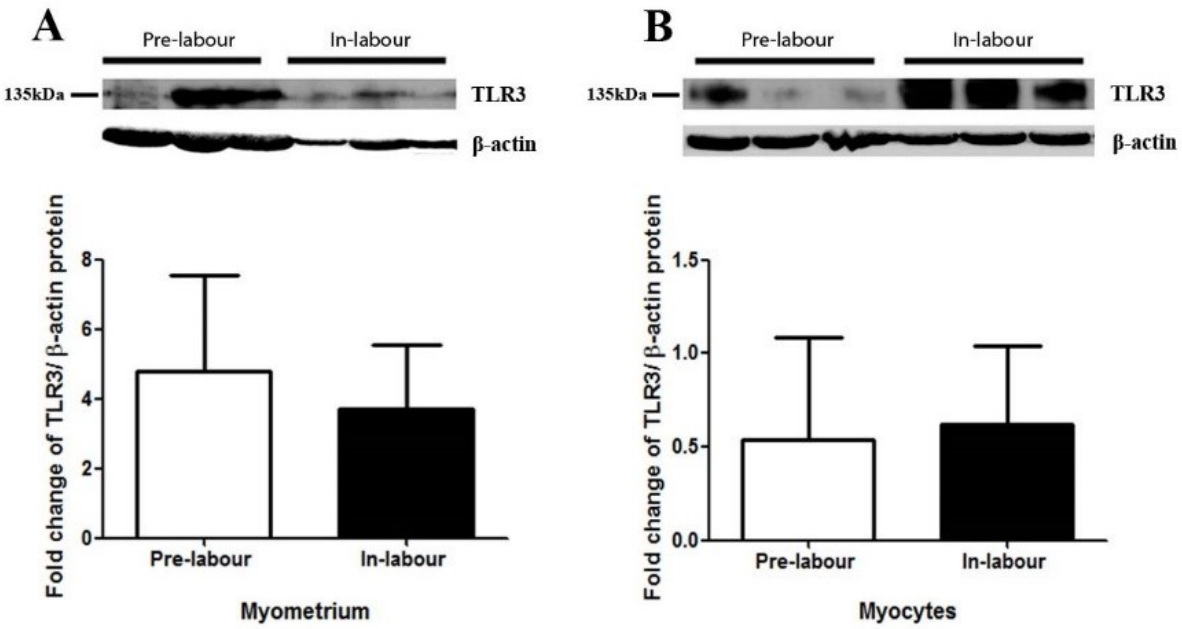


Figure 3.7 TLR3 protein expression in pre-labour and in-labour matched myometrium and myocytes. Myometrium tissue was homogenised, and confluent monolayer myocytes were lysed in the lysis buffer and Western immunoblotting was performed to detect the presence of TLR3 protein. A representative blot was shown above each graph with β-actin as a loading control. No difference in TLR3 protein was observed between pre-labour and in-labour samples in **(A)** myometrium and **(B)** myocytes. Statistical analysis was performed using unpaired t-test for normally distributed data and Mann-Whitney U test for data not normally distributed , n=6 biological replicates.

3.2.4 PBMCs

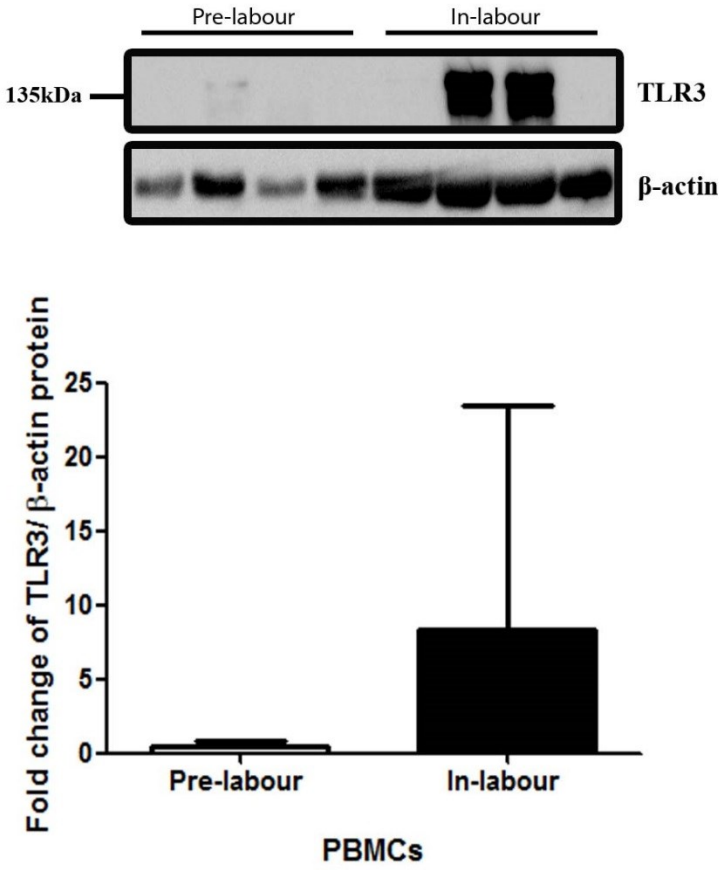


Figure 3.8 TLR3 protein expression in pre-labour and in-labour PBMCs. PBMCs were lysed in the lysis buffer and Western immunoblotting was performed to detect the presence of TLR3 protein. A representative blot was shown above each graph with β-actin as a loading control. A significant variation in expression was seen between patients, either no expression was seen or high expression. Statistical analysis was performed using Mann-Whitney U test, n=4 biological replicates.

3.2.5 Placental explants

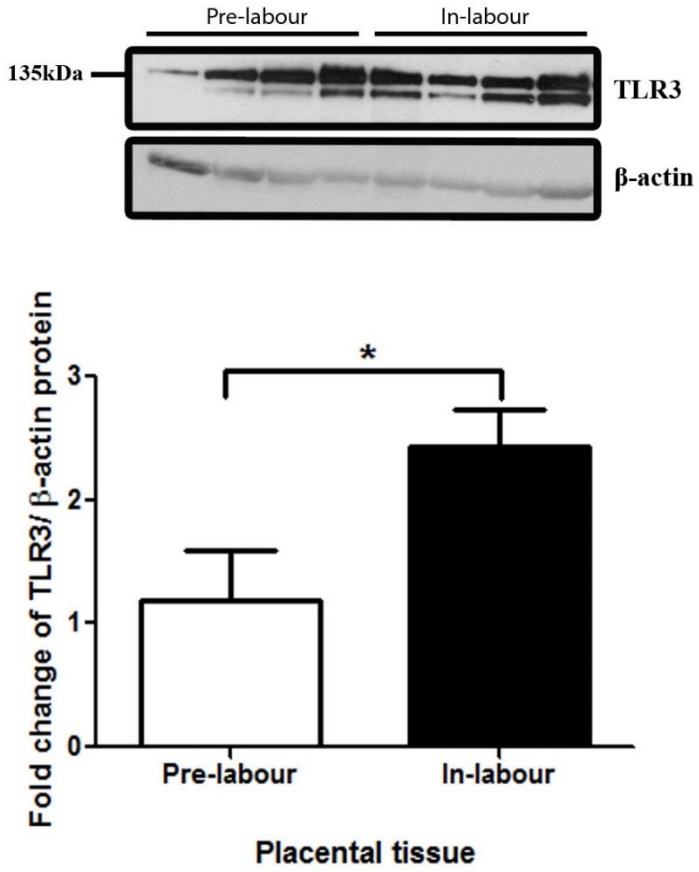


Figure 3.9 TLR3 protein expression in pre-labour and in-labour placental explants. Placental explants were homogenised in the lysis buffer and Western immunoblotting was performed to detect the presence of TLR3 protein. A representative blot was shown above each graph with β-actin as a loading control. A significant increase of TLR3 (*p<0.05) protein was observed in in-labour samples as compared to the pre-labour samples. Statistical analysis was performed using unpaired t-test, n=8 biological replicates.

3.3 TLR3 protein expression is induced with the TLR3 agonist poly I:C

Having demonstrated the presence of TLR3 protein at basal levels in gestational tissues, the expression of TLR3 protein was further evaluated following stimulation with its own agonist, poly I:C. Increased TLR3 protein with poly I:C stimulation has been successfully demonstrated in other cell types including bone marrow-derived cultured mast cells (BMMC) with 10μg/ml over 30 minutes [383] and primary human lung microvascular endothelial cells (HLEC) with 0.1, 1 and 10μg/ml for 3, 6, 12 and 24 hours [384]. Pre-labour samples of amniocytes, myocytes, PBMCs and placental explants, and VECs were stimulated with poly I:C over a time course of up to 24 hours and at different concentrations of 5μg/ml, 10μg/ml, 25μg/ml, 50μg/ml and 100μg/ml. This was to examine the effect of a positive feedforward loop in a TLR3 viral infection setting in the gestational tissues. Western immunoblotting was performed to detect TLR3 protein expression.

In VECs, TLR3 protein expression increased significantly by 12 hours (**p<0.001) with 5μg/ml of poly I:C, (Figure 3.10 A). In the dose response experiment, performed at 6 hours, there was an increase in TLR3 expression with 25μg/ml, 50μg/ml and 100μg/ml (*p<0.05) of poly I:C (Figure 3.10 B). Based on this result, 25μg/ml of poly I:C was used in a 6 hour stimulation for future priming experiments in VECs.

In amniocytes, TLR3 protein increased significantly at 12 (**p<0.01) and 24 (**p<0.01) hours (Figure 3.11 A) with 5μg/ml poly I:C. In the dose response experiment, performed at 12 hours, an increase in TLR3 expression was seen with 5μg/ml, however statistical significance was achieved only with 25μg/ml (*p<0.05), 50μg/ml and 100μg/ml (**p<0.01) (Figure 3.11 B). Based on this result, future priming experiments in amniocytes used 25μg/ml of poly I:C in a 12 hour stimulation.

Myocytes also showed increased TLR3 expression with 5μg/ml of poly I:C at 12 hours (**p<0.01) and 24 (**p<0.01) hours (Figure 3.12 A). In the poly I:C dose response stimulation, at 12 hours, an increase in TLR3 expression was observed with 10μg/ml, 25μg/ml, 50μg/ml and 100μg/ml (*p<0.05) at 12 hours (Figure 3.12 B). Despite a significant increase with 10μg/ml this was not consistent with the other samples, therefore future priming experiments were carried out with 25μg/ml of poly I:C to also be consistent with the concentrations used in VECs and myocytes.

There was no statistically significant increase in TLR3 expression with 5μg/ml poly I:C stimulation in PBMCs in the time course leading up to 24 hours (Figure 3.13). There was no statistically significant increase in TLR3 expression with 25μg/ml poly I:C stimulation in placental explants in the time course leading up to 24 hours (Figure 3.14). In both PBMC [385, 386] and placental explants [218, 333], since other publications have conducted poly I:C stimulation on these cell types, the same concentrations were used according to the reported data.

In conclusion, TLR3 expression is inducible with the TLR3 agonist, poly I:C, and shows a clear dose response in vaginal epithelial cells, amniocytes and myocytes. Furthermore, an increase in expression was demonstrated after 12 hours of incubation. However, at 5μg/ml and 25μg/ml there was no increased expression in PBMCs and placental explants respectively by 12 or 24 hours of stimulation.

3.3.1 Vaginal epithelial cells (VEC)

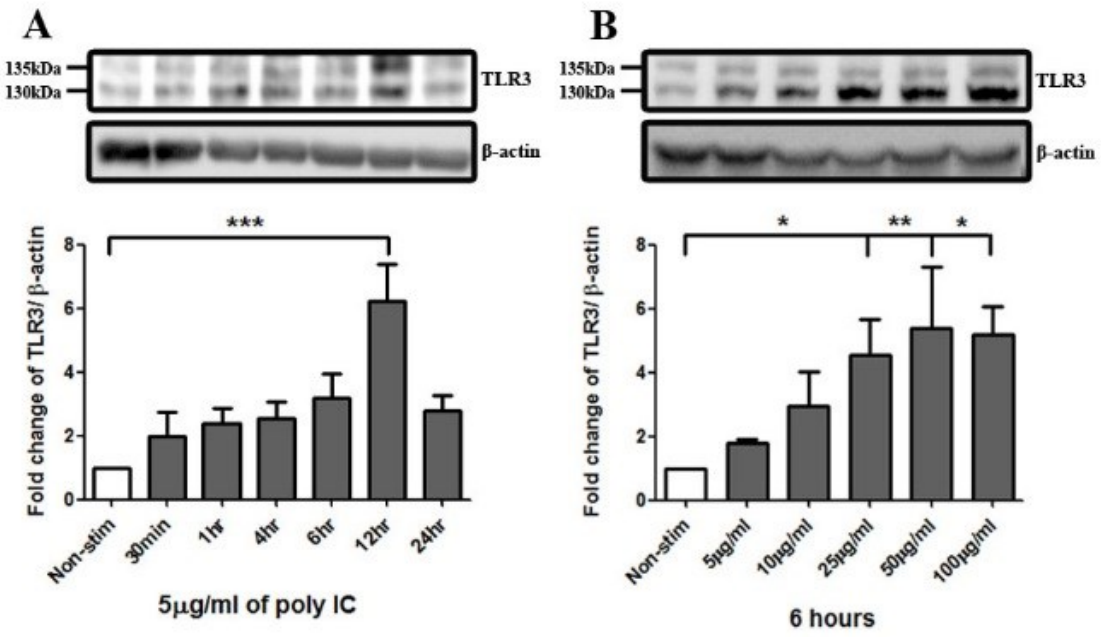


Figure 3.10 TLR3 protein expression increases with poly I:C agonist stimulation in VECs. A representative blot was shown above each graph with β-actin as a loading control. TLR3 protein significantly increased with 5μg/ml of poly I:C stimulation at **(A)** 12 hours (****p*<0.001). At 6 hours, a dose response of poly I:C stimulation showed a significant increase of TLR3 protein at **(B)** 25μg/ml (**p*<0.05), 50μg/ml (***p*<0.01) and 100μg/ml (**p*<0.05). For statistical analysis One-way ANOVA with *Dunnett's* multiple comparison test was used. n=3.

3.3.2 Amniocytes

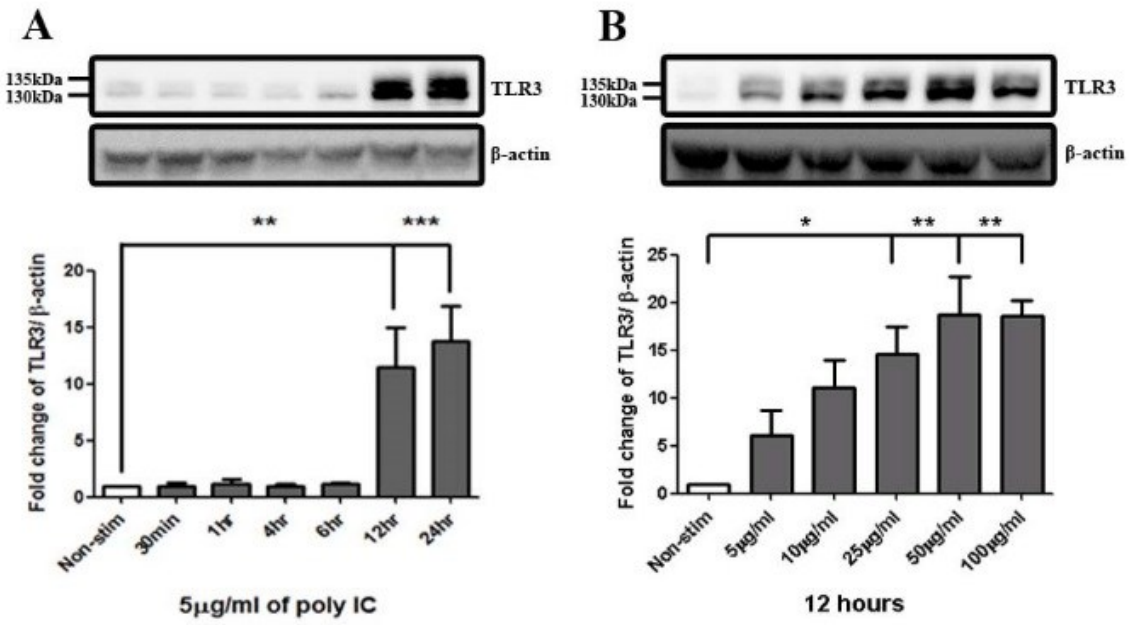


Figure 3.11 TLR3 protein expression increases with poly I:C agonist stimulation in amniocytes. A representative blot was shown above each graph with β-actin as a loading control. TLR3 protein significantly increased with 5μg/ml of poly I:C stimulation at **(A)** 12 (**p<0.01) and 24 hours (***p<0.001). At 12 hours, a dose response of poly I:C stimulation showed a significant increase of TLR3 protein at **(B)** 25μg/ml (*p<0.05), 50μg/ml and 100μg/ml (**p<0.01). For statistical analysis, One-way ANOVA was used for normally distributed data and Kruskal-Wallis was used for data not normally distributed with *Dunnett's* multiple comparison test. n=3-4.

3.3.3 Myocytes

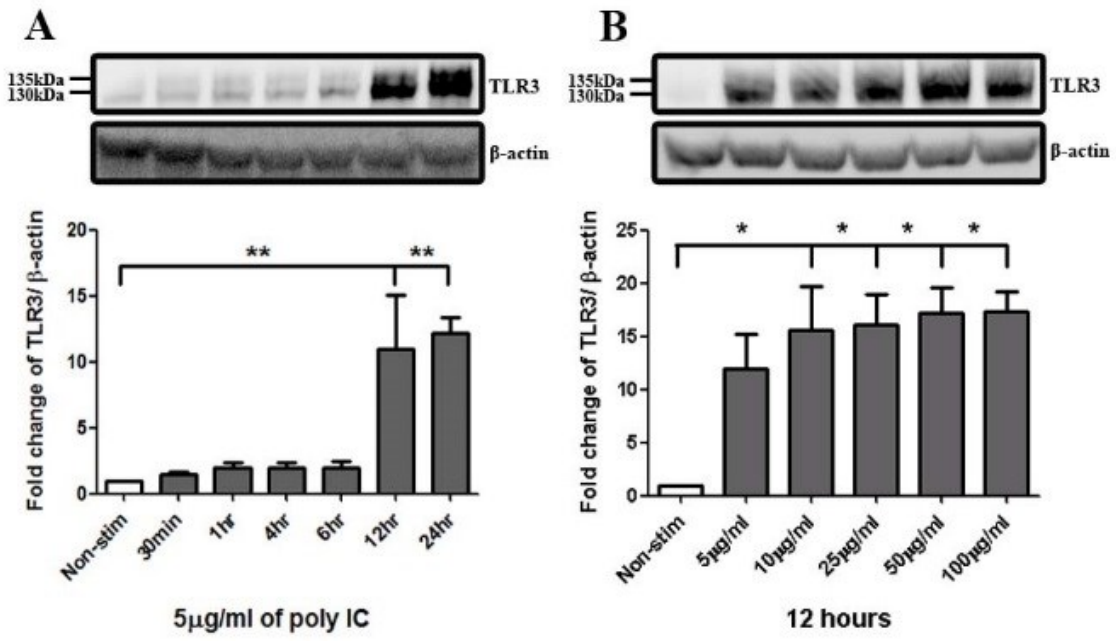


Figure 3.12 TLR3 protein expression increases with poly I:C agonist stimulation in myocytes. A representative blot was shown above each graph with β-actin as a loading control. TLR3 protein significantly increased with 5μg/ml of poly I:C stimulation at **(A)** 12 and 24 hours (**p<0.01). At 12 hours, a dose response of poly I:C stimulation showed a significant increase of TLR3 protein at **(B)** 10μg/ml, 25μg/ml, 50μg/ml and 100μg/ml (*p<0.05). For statistical analysis Kruskal-Wallis with *Dunnett's* multiple comparison test was used. n=3.

3.3.4 PBMCs

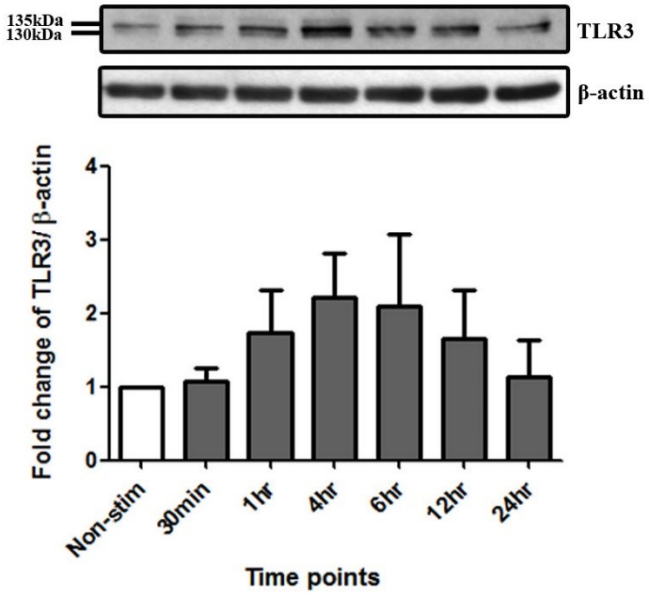


Figure 3.13 TLR3 protein expression with poly I:C agonist stimulation in PBMCs. PBMCs were cultured and stimulated with 5μg/ml of poly I:C over 24 hours. A representative blot was shown above each graph with β-actin as a loading control. TLR3 protein showed an increasing trend from 1 to 6 hours but no statistically significant was achieved. For statistical analysis Kruskal-Wallis with *Dunnett's* multiple comparison test was used. n=4.

3.3.5 Placental explants

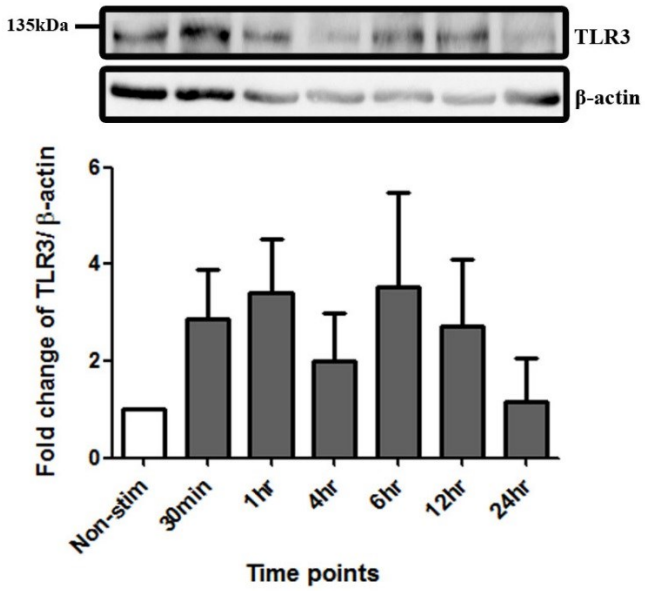


Figure 3.14 TLR3 protein expression with poly I:C agonist stimulation in placental explants. Placental explants were cultured and stimulated with 25μg/ml of poly I:C over 24 hours. A representative blot was shown above each graph with β-actin as a loading control. No significant increase was seen in TLR3 expression upon treatment with poly I:C. For statistical analysis Kruskal-Wallis with *Dunnett's* multiple comparison test was used. n=4.

Discussion

VEC, amnion and amniocytes, myometrium and myocytes, PBMCs and placental explants expressed TLRs and are inducible when stimulated with poly I:C. Amnion and amniocytes expressed all TLR mRNAs, but no difference was observed in relation to labour. In myometrium, TLR2, 4 and 6 mRNA increased in labour, with no differences seen in myocytes except for TLR6. In contrast, only mRNA for TLR2, 3 and 6 were detected in VECs. Only placental explants showed increased endogenous TLR3 protein with labour. Poly I:C stimulation induced TLR3 protein expression in amniocytes, VECs and myocytes, but not in PBMCs or placental explants.

The first part of the study confirmed the expression of bacterial TLR2, 4 and 6 in the gestational tissues and the increased TLR2, 4 and 6 mRNA expression in labour compared to pre-labour as shown in the literature [214, 218, 387]**.** The regulation of the expression of TLRs are likely to be finely tuned to maintain a balanced innate immune response for successful pregnancy. Infiltration of leukocytes bearing TLRs into gestational tissue occurs during the physiological changes of labour [57, 69, 388]. To our knowledge, this is the first study reporting expression of TLR3 in myometrium and myocytes and showing an increase in expression with the onset of labour in amnion and placental explants. My study has also revealed that these gestational tissues are capable of responding to TLR3 recognising viruses during pregnancy. The agonist used to activate TLR3, poly I:C, is a synthetic stimulus mimicking dsRNA [389] which is the intermediate of viral replication, including ssRNA viruses [286, 390]. Due to the limitation in the difficulty of obtaining preterm samples without pathology, the changes in TLR3 expression were assessed using term samples of healthy controls.

Conclusion:

This study confirmed the findings of other studies on the expression of TLR2, 4 and 6 in gestational tissue. The novel findings presented are of TLR3 protein expression in amniocytes and myocytes. Furthermore, in amniocytes, VEC and myocytes, TLR3 expression is increased when cells are treated with poly I:C. The relevance of this finding is unclear in the context of healthy pregnancy, labour and preterm labour and will be explored in subsequent chapters.

CHAPTER 4

Effect of TLR3 priming in a model of ascending infection induced sPTL

Introduction

Background:

Bacterial infection is commonly cited as one of the causes of preterm labour and the ascending pathway is considered to be the main route of entry for the microorganisms. Recently, murine models have identified the role of viral infection in preterm labour. The synergistic effect when combining the viral TLR3 agonist poly I:C and a TLR2 agonist results in a 100% preterm delivery in mice when compared to 14% and 22% with TLR3 and TLR2 agonists alone, respectively. This double-hit hypothesis has been previously explored *in vivo* using mice and locally using human fetal membrane explants and trophoblast cells. In this chapter, I will explore the local response to TLR3 viral agonist priming prior to bacterial TLR2, 4 and 6 agonist stimulation in 3 different cell types exposed during ascending infection, namely VECs, amniocytes and myocytes.

Hypothesis:

1. NF-κB and AP-1 increase with TLR3 agonist (poly I:C), TLR2 agonist (Heat-killed *Listeria Monocytogenes*, HKLM), TLR4 agonist (Lipopolysaccharide 055:B5, LPS 055) and TLR2/6 agonist (fibroblast–stimulating lipopeptide, FSL-1) stimulation in VEC, amniocytes and myocytes.
2. Local TLR3 priming prior to TLR2, 4 and 2/6 agonist stimulation augments the production of pro-labour and pro-inflammatory mediators in VEC, amniocytes and myocytes.

Aims:

The aims of the first part of this chapter are to determine the time points and concentrations of the TLR3, 2, 4 and 2/6 agonists needed to activate the transcription factors NF-κB and AP-1 to be used for the priming experiment. The second part involves priming the cells with the TLR3 agonist poly I:C before subsequent bacterial TLR2, 4 and 2/6 agonist stimulation. The priming effect will be assessed by determining the activation status of NF-κB and AP-1 and the downstream endpoint of these transcription factors by quantifying the production of pro-inflammatory and anti-inflammatory cytokines, chemokines and PGE2. The expression of TLR3, 2, 4 and 6 mRNA and p-IRF3 are also evaluated after poly I:C stimulation.

Results

4.1 Vaginal epithelial cells (VECs)

4.1.1 Poly I:C (viral TLR3 agonist) stimulation increases NF-κB and AP-1

Before carrying out the viral priming experiments, a time course and dose response experiments were performed with poly I:C (viral TLR3 agonist) to examine its effect on the transcription factors NF-κB and AP-1. Western immunoblotting was performed and phosphorylated p65 (p-p65) and phosphorylated c-Jun (p-c-Jun) were used as markers for NF-κB and AP-1 activation status.

Cells were cultured, and confluent monolayer cells were stimulated with 5µg/ml and 10µg/ml of poly I:C in a time course experiment for up to 24 hours. NF-κB increased significantly at 4, 6 and 24 hours (**p<0.01) with 5µg/ml (Figure 4.1 A) and at 1 (***p<0.001), 4 (**p<0.01), 6 and 12 (*p<0.05) hours with 10µg/ml (Figure 4.1 B). A higher fold-change was observed in AP-1 compared to NF-κB with significant increase at 1, 4 (**p<0.01) and 6 (*p<0.05) hours with 5µg/ml (Figure 4.1 C) only at 1 hour (*p<0.05) with 10µg/ml (Figure 4.1 D) of poly I:C.

The cells were also cultured and stimulated with increasing doses of poly I:C starting with 5, 10, 25, 50 and 100µg/ml for 6 hours. This time point was chosen to be consistent with the poly I:C stimulation dose response test that shows increasing TLR3 protein expression at 6 hours (see Chapter 3, section 3.3.1). NF-κB (Figure 4.2 A) and AP-1 (Figure 4.2 B) showed a trend of an increase starting with the lowest poly I:C dose at 5µg/ml.

For priming purposes, a dose of 25ug/ml poly I:C was selected for further priming experiments as this dose leads to significant increase of TLR3 protein levels at 6 hours (see Chapter 3, section 3.3.1).

4.1.1.1 Poly I:C time course

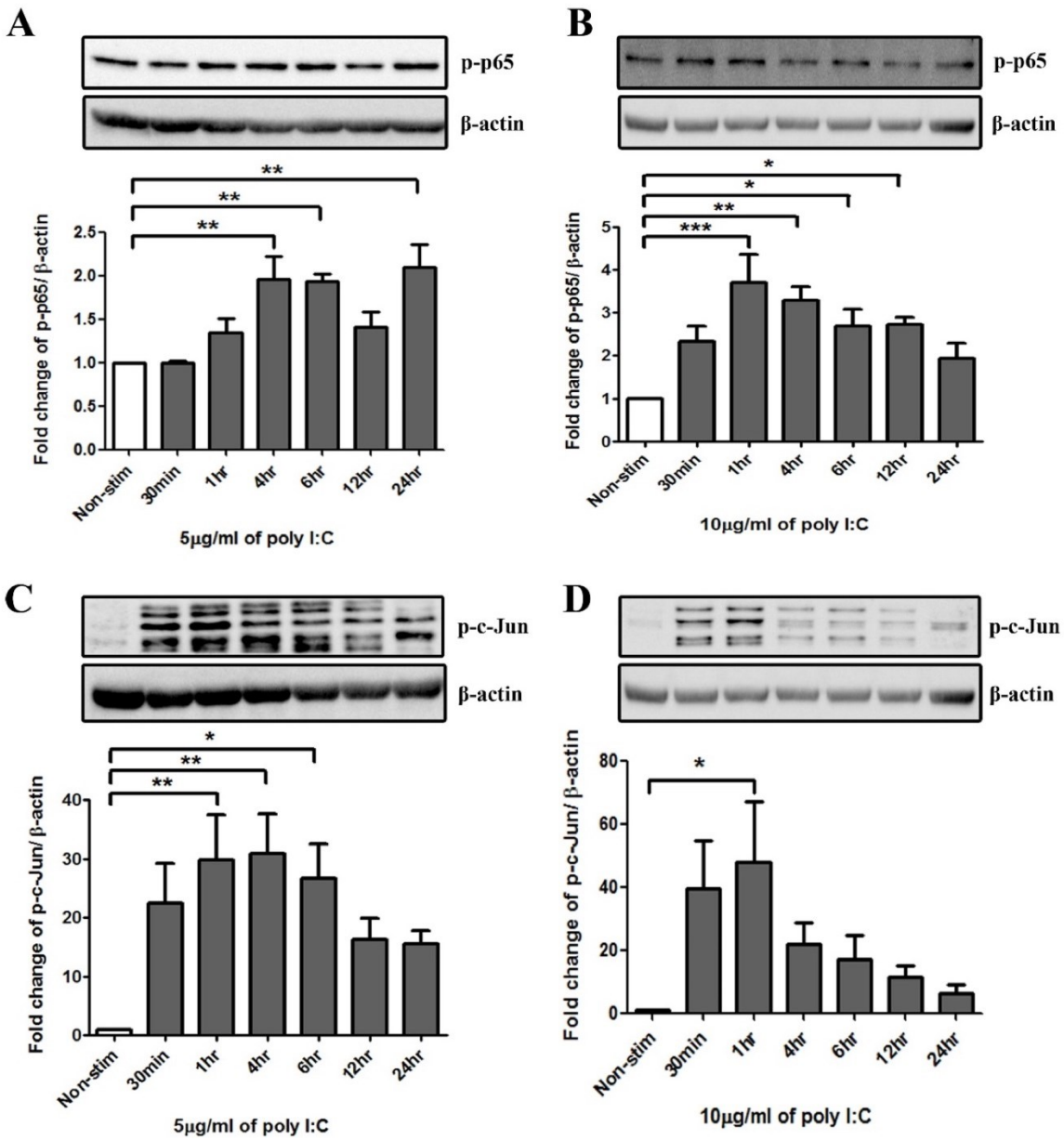


Figure 4.1 The effect of poly I:C, a viral TLR3 agonist stimulation on NF-κB and AP-1 activation in VEC. VECs were cultured and stimulated with 5μg/ml and 10μg/ml of poly I:C for a duration of 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. NF-κB increased significantly with **(A)** 5μg/ml of poly I:C at 4, 6 and 24 (**p<0.01) hours and **(B)** 10μg/ml of poly I:C at 1 (***p<0.001), 4 (**p<0.01), 6 and 12 (*p<0.05) hours. AP-1 also increased significantly with **(C)** 5μg/ml of poly I:C at 1, 4 (**p<0.01) and 6 (*p<0.05) hours and **(D)** 10μg/ml of poly I:C at 1 hour (*p<0.05). For statistical analysis One-way ANOVA with *Dunnett's* multiple comparison test was used. n=3-4.

4.1.1.2 Poly I:C dose response at 6 hours

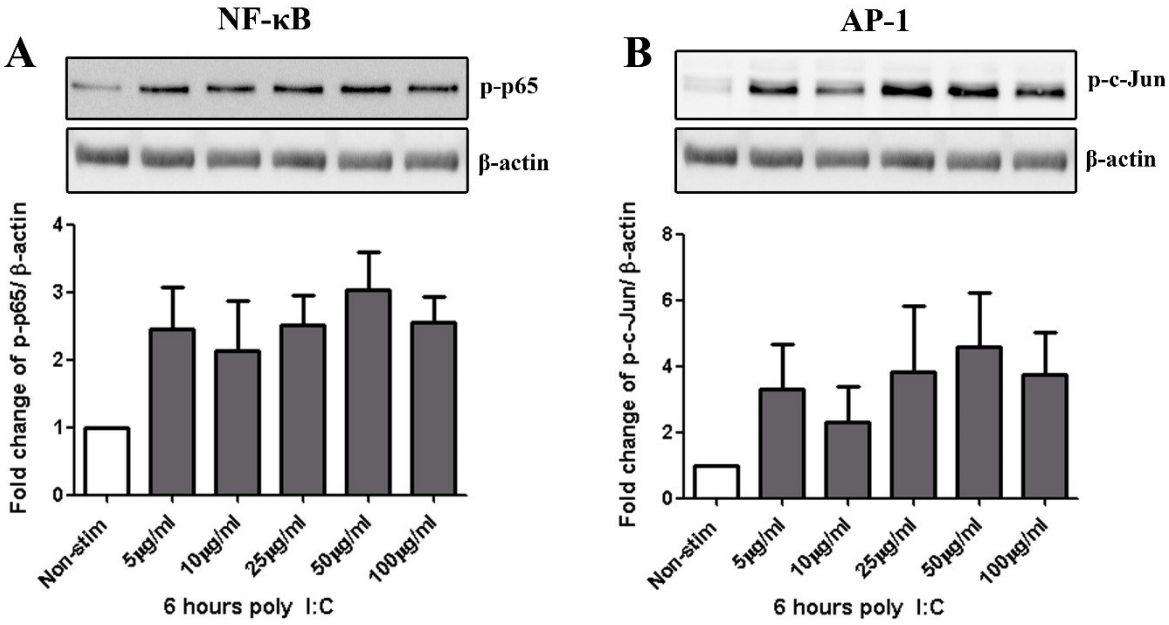


Figure 4.2 The effect of different dose of poly I:C, a viral TLR3 agonist stimulation on NF-κB and AP-1 activation at 6 hours in VEC. VECs were cultured and stimulated with 5μg/ml, 10μg/ml, 25μg/ml, 50μg/ml and 100μg/ml of poly I:C for 6 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. **(A)** NF-κB and **(B)** AP-1 showed no statistically significant increase but a trend of increase was observed in all poly I:C doses. For statistical analysis One-way ANOVA with *Dunnett's* multiple comparison test was used. n=3.

4.2.2 Heat-killed *Listeria Monocytogenes* (HKLM) (TLR2 agonist) stimulation increases NF-κB and AP-1

The same set of experiments as in section 4.1.1 were carried out with HKLM to find the optimal concentration and time points before conducting the priming experiment. Cells were cultured, and confluent monolayer cells were stimulated for up to 24 hours in a time course experiment with either 10^5 or 10^6 cells/ml concentrations of the TLR2 agonist. These concentrations were picked based on the lowest concentration used in the literature [391]. Western immunoblotting was performed and p-p65 and p-c-Jun were used as markers of NF-κB and AP-1 activation status.

NF-κB increased significantly at 6, 12 (*p<0.05) and 24 hours (**p<0.01) with 10^5 cells/ml (Figure 4.3 A). No statistical significance was achieved with 10^6 cells/ml (Figure 4.3 B).

At both concentrations, AP-1 increased significantly at 30 minutes and 1 hour (***p<0.001) (Figure 4.3 C and D).

For the priming experiment, 10^5 cells/ml at 1 hour was selected. This concentration is lower than the concentration suggested in the data sheet (10^8 cell/ml), but it clearly shows a reliable effect on NF-κB and AP-1. Although NF-κB increased significantly at 6 hours, 1 hour was chosen to capture both activation of NF-κB and AP-1 since NF-κB starts to increase from 1 hour.

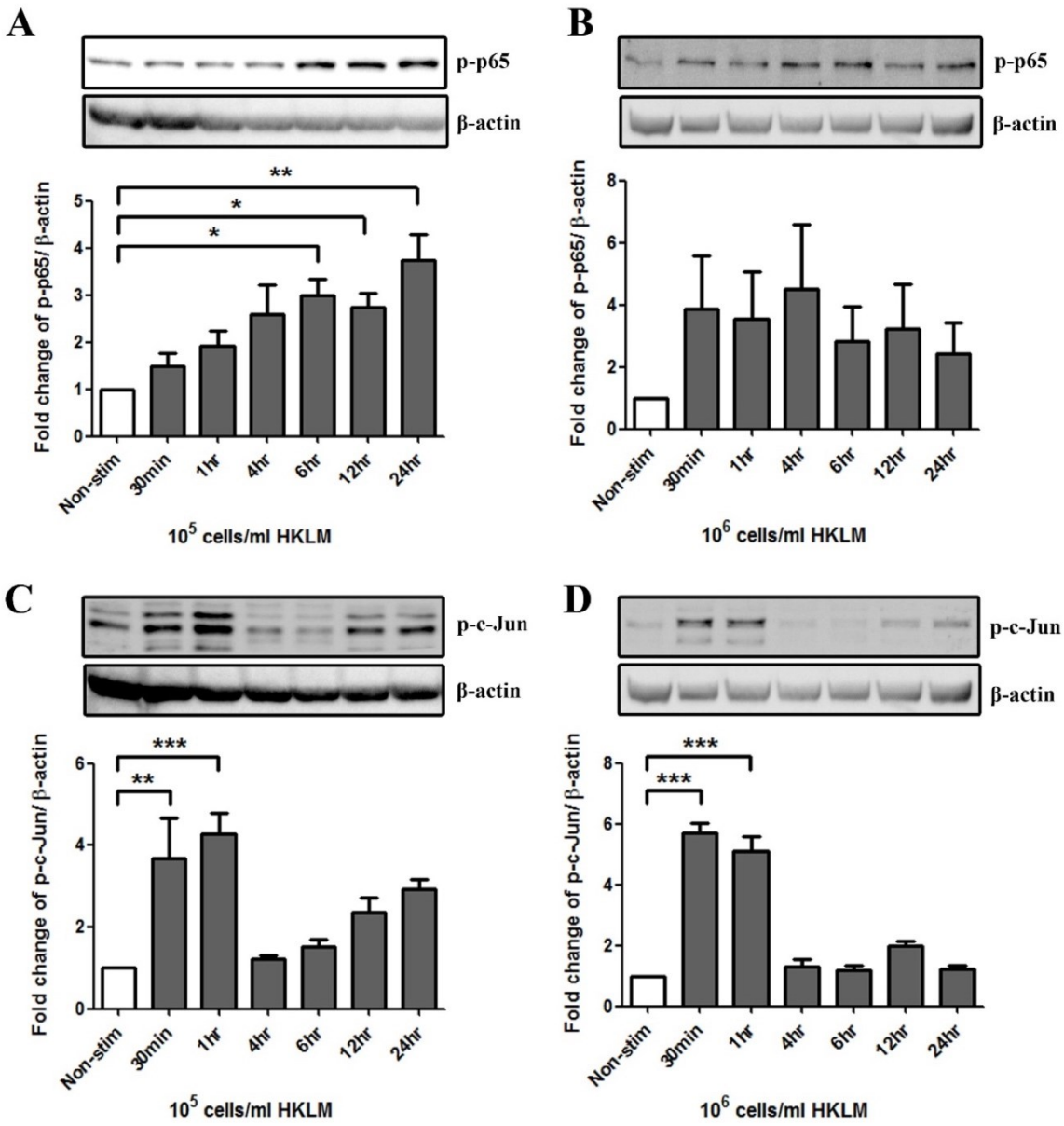


Figure 4.3 The effect of Heat-killed *Listeria Monocytogenes* (HKLM), a TLR2 agonist stimulation on NF-κB and AP-1 activation in VEC. VECs were cultured and stimulated with 10^5 or 10^6 cells/ml of HKLM for a duration of 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. Representative blots are shown above each graph with β-actin as a loading control. NF-κB increased significantly with **(A)** 10^5 cells/ml of HKLM at 6, 12 and 24 (*p<0.05) hours while an increase was observed with **(B)** 10^6 cells/ml, no statistical significance was achieved. AP-1 also increased significantly with **(C)** 10^5 cells/ml and **(D)** 10^6 cells/ml of HKLM at 30 minutes (**p<0.01, ***p<0.001) and 1 hour (***p<0.001). For statistical analysis One-way ANOVA with *Dunnett's* multiple comparison test was used. n=3-4.

4.2.3 TLR3/TLR2 priming in VECs

Based on the results from the dose and time course experiments in section 4.1.1 and 4.2.2, 25μg/ml of poly I:C and 10^5 cell/ml of HKLM were chosen as the optimal concentration for priming experiments. Monolayer VK2 cells were primed with poly I:C for 6 hours before a subsequent HKLM stimulation for 1 hour. Protein was extracted and analysed using Western immunoblotting to examine the activation status of NF-κB and AP-1.

For cytokines, chemokine and PGE2 studies, cells were primed for 6 hours with poly I:C prior to a 24 hour incubation with the TLR2 agonist HKLM. Supernatant was stored at -80°C until use and analytes were detected using an ELISA.

4.1.3.1 NF-κB or AP-1 activation is not augmented with TLR3/2 priming

Poly I:C stimulation at 6 hours yielded a small non-significant increase in NF-κB activation. Similarly, a non-significant increase was observed with HKLM stimulation at 1 hour, but with the same fold change as seen in section 4.2. Poly I:C priming prior to HKLM stimulation showed no augmented NF-κB response compared with the individual agonist stimulation alone (Figure 4.4 A).

For AP-1, poly I:C stimulation at 6 hours showed no statistically significant increase, which could be due to the earlier activation of AP-1 prior to the 1 hour time point (section 4.1.1). No statistical significance was observed with HKLM stimulation at 1 hours. This could be due to the increased AP-1 activation at the basal level at 1 hour suggesting that the basal level of AP-1 changes over time. TLR3/2 priming resulted in a significant increase in AP-1 activation compared to poly I:C stimulation alone (**p<0.01). However, no priming effect was seen when compared to treatment with HKLM (TLR2 agonist) alone (Figure 4.4 B). Thus, overall there is an additive effect with TLR3/2 priming compared to TLR3 treatment alone, but not with TLR2 alone.

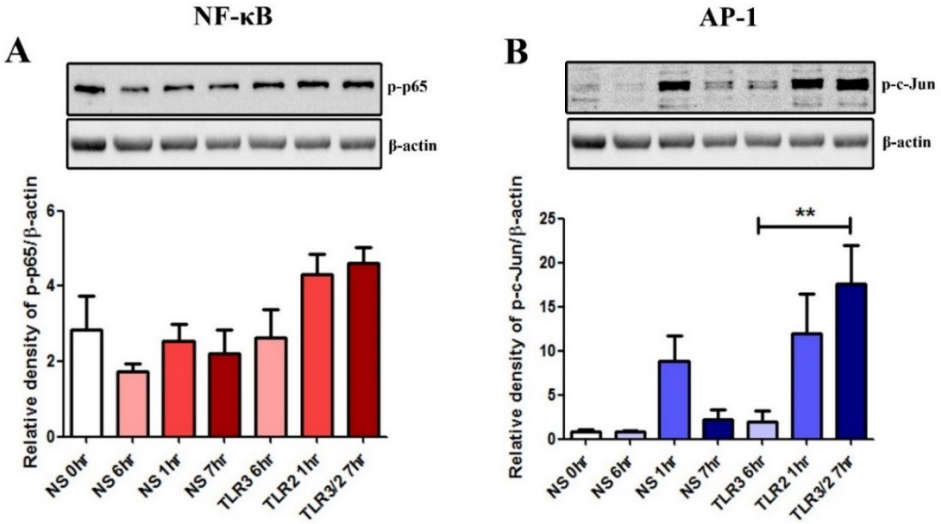


Figure 4.4 Effect of TLR3 priming before TLR2 agonist stimulation on NF-kB and AP-1 in VEC. Confluent monolayer VECs were plated and were primed with 25μg/ml of poly I:C (TLR3 agonist) for 6 hours prior to 10^5 cells/ml of HKLM (TLR2 agonist) stimulation for 1 hour. Protein was extracted, and Western immunoblotting was performed to determine the activation status of serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1, respectively. A representative blot is shown above each graph with β-actin as a loading control. TLR3 priming prior to subsequent TLR2 agonist stimulation showed no augmentation in **(A)** NF-κB activation. In **(B)** AP-1, although an augmentation was observed comparing TLR3/2 to TLR3 agonist stimulation alone (**p<0.01), no further augmentation was observed compared to TLR2 agonist stimulation alone. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3.

4.1.3.2 Pro-inflammatory cytokine production is not augmented with TLR3/2 priming

Although the NF-κB or AP-1 levels were not significantly affected by priming, the possibility of other transcription factor/s being activated with priming could lead to the production of the pro-inflammatory and pro-labour mediators via alternative pathways. Therefore, the following pro-inflammatory mediators were examined including IL-1α, IL-1β, IL-6, IL-8, IFN-γ, IL-12p40, IL-12p70, TNF-α and TNF-β. However, TNF-β, IFN-γ, IL-12p40 and IL-12p70 were not detected in all samples hence these data were not presented.

The vehicle control showed a trend of increase with the detection level of all cytokines over time. A significant increase above baseline was seen with poly I:C stimulation alone of IL-1α (***p<0.001 at 24 hours) (Figure 4.5 A), IL-1β (**p<0.01 at 24 hours) (Figure 4.5 B), IL-6 (***p<0.001 at 6 and 24 hours) (Figure 4.5 C), IL-8 (***p<0.001 at 6 and 24 hours) (Figure 4.5 D), and TNF-α (***p<0.001 at 6 and 24 hours) (Figure 4.5 E). No increase was seen with HKLM stimulation alone. Furthermore, no augmentation of any of the cytokines was seen with TLR3/2 priming.

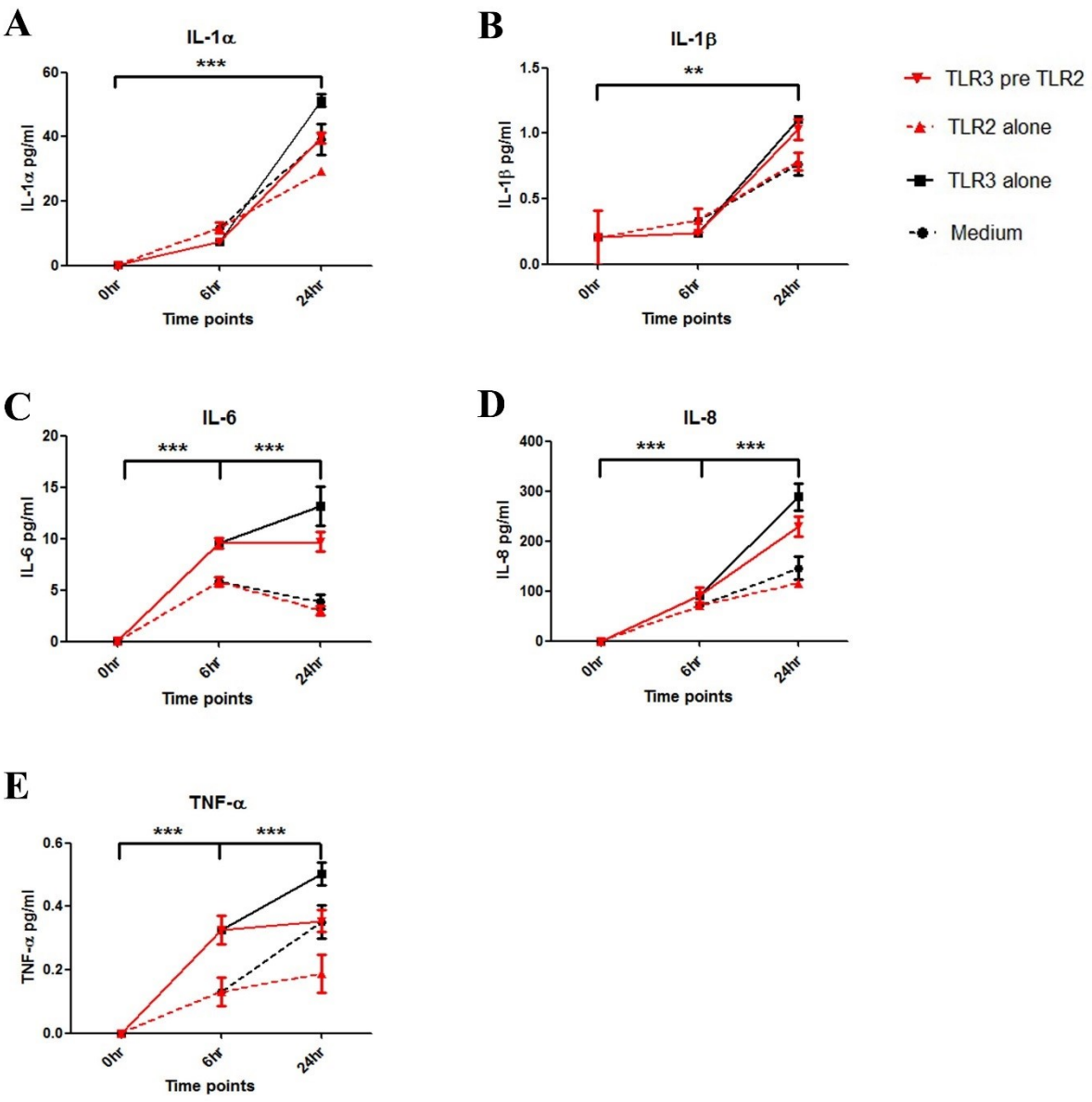


Figure 4.5 The effect of TLR3 priming prior to TLR2 agonist stimulation on pro-inflammatory cytokines production in VEC. VECs were plated and grown in monolayer until confluency was reached. Cells were primed with 25μg/ml of poly I:C for 6 hours prior to 10^5 cells/ml of HKLM stimulation for 24 hours. Supernatant was collected, and neat samples were used to run an ELISA to determine the level of the cytokine production. TLR3 stimulation significantly increased the production of IL-1α, IL-1β, IL-6, IL-8 and TNF-α. However, TLR3 priming prior to TLR2 agonist stimulation did not result in a further increase in **(A)** IL-1α, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-8 or **(E)** TNF-α. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. ***p*<0.01 **=** effect of TLR3 compared to 0hr.

4.1.3.3 Chemokine production is not augmented with TLR3/2 priming

Three chemokines, MIP-1α, MIP-1β and RANTES, were analysed since they are typically responsive towards viral stimuli *in vivo*. Neat samples were run for MIP-1α and MIP-1β whilst a 1 in 10 dilution was used for RANTES quantification.

There was a non significant increase in chemokine production with vehicle control over time. Poly I:C stimulation significantly increased MIP-1α (***p<0.001 at 24 hours) (Figure 4.6 A), MIP-1β (***p<0.001 at 6 and 24 hours) (Figure 4.6 B) and RANTES (***p<0.001 at 24 hours) (Figure 4.6 C) production compared to 0hr. HKLM stimulation showed no significant increase in chemokine production. Furthermore, no chemokine augmentation was observed with TLR3/2 priming.

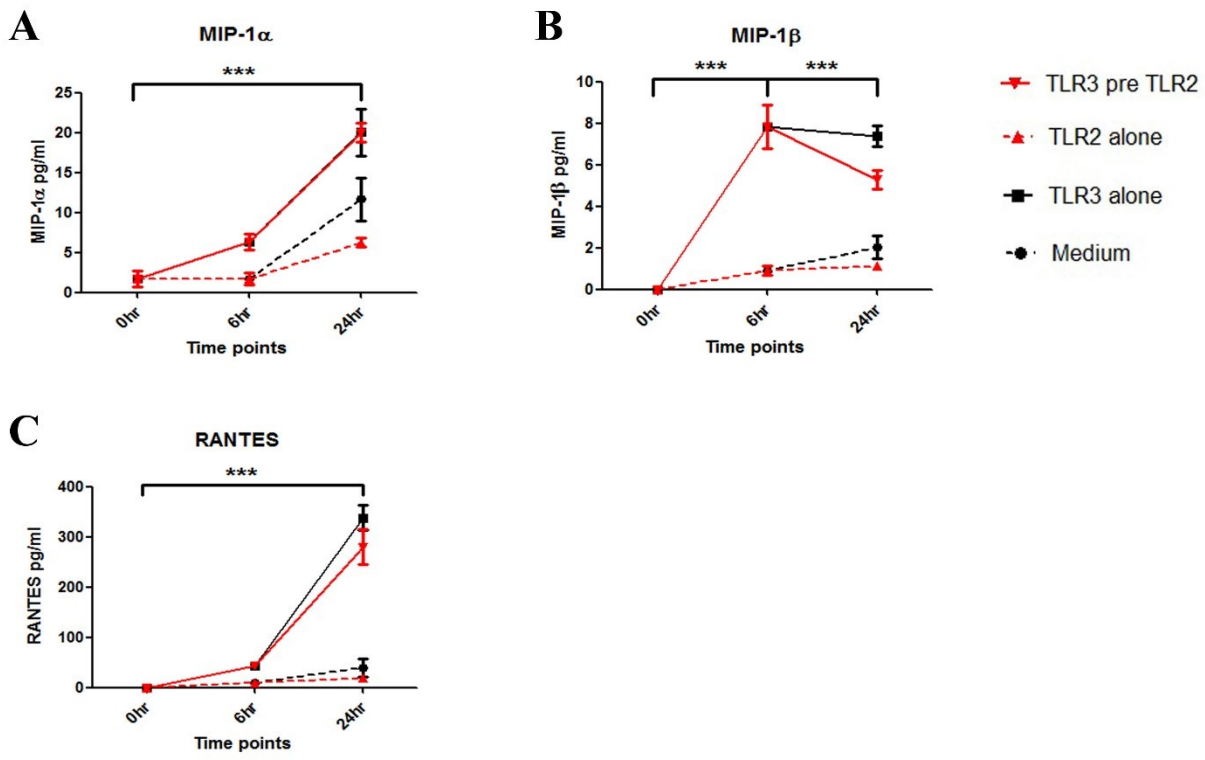


Figure 4.6 The effect of TLR3 priming prior to TLR2 agonist stimulation on chemokines production in VEC. Confluent monolayer VECs were plated and were primed with 25μg/ml of poly I:C for 6 hours prior to 10^5 cells/ml of HKLM stimulation for 24 hours. Supernatant was collected and was used to run ELISA to determine the levels of the chemokines production. TLR3 stimulation significantly increased the production of MIP-1α, MIP-1β and RANTES. No augmentation was observed with TLR3 priming before TLR2 agonist stimulation in **(A)** MIP-1α, **(B)** MIP-1β or **(C)** RANTES. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. ***p<0.001 **=** effect of TLR3 compared to 0hr.

4.1.3.4 Anti-inflammatory cytokine production is not augmented with TLR3/2 priming

The production of the following anti-inflammatory cytokines was analysed: IL-4, IL-10, IL-1RA, IFN-α and IFN-β. Neat samples were used to quantify these cytokines, however, only IL-1RA was detectable in all samples.

There was no difference in the IL-1RA (Figure 4.7) levels in samples treated with poly I:C alone, HKLM alone or primed samples compared to the basal levels in the vehicle control samples.

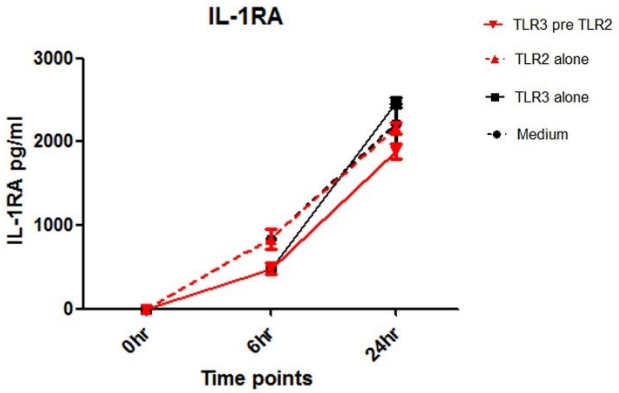


Figure 4.7 The effect of TLR3 priming prior to TLR2 agonist stimulation on anti-inflammatory IL-1RA production in VEC. Confluent monolayer VECs were plated and were primed with 25µg/ml of poly I:C for 6 hours prior to 10^5 cells/ml stimulation for 24 hours. Supernatant was collected and neat samples were used to run ELISA to determine the level of IL-1RA production. Neither the TLR agonists alone nor TLR3 priming prior to TLR2 agonist stimulation increased the production of IL-1RA. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3.

4.1.3.5 PGE2 production is not augmented with TLR3/2 priming

PGE2 production was analysed using an ELISA. PGE2 (Figure 4.8) production with poly I:C or HKLM stimulation or priming showed no difference compared to the vehicle control samples.

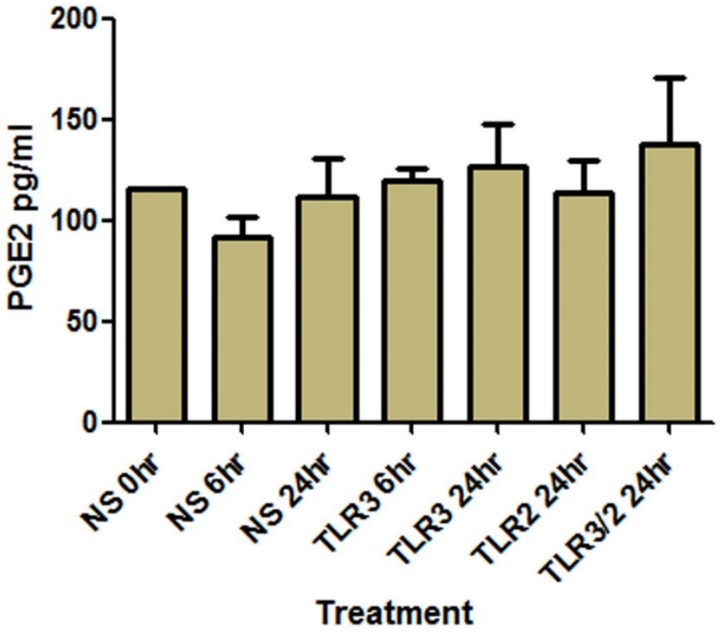


Figure 4.8 The effect of TLR3 priming prior to TLR2 agonist stimulation on PGE2 production in VEC. Confluent monolayer VECs were plated and were primed with 25µg/ml of poly I:C for 6 hours prior to 10^5 cells/ml of HKLM for 24 hours. TLR3 priming prior to TLR2 agonist stimulation showed no augmentation of PGE2 production compared to the individual TLR agonist stimulation alone. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3.

4.2.4 Lipopolysaccharides from Escherichia coli 055:B5 (LPS 055) ultrapure (TLR4 agonist) stimulation increases NF-κB and AP-1

There is some controversy in literature regarding the expression of TLR4 in the vagina. Nonetheless, we decided to explore any potential priming effect of poly I:C with a subsequent TLR4 agonist stimulation. In this experiment, an ultrapure lipopolysaccharides from Escherichia coli 055:B5 (LPS 055) was used to minimise the possibility of cross reactivity with other TLR receptors, especially TLR2, which is expressed in the vagina (section 3.1.1).

Cells were stimulated for up to 24 hours with 0.1μg/ml and 1μg/ml of LPS 055 ultrapure. These concentrations were chosen based on the suggested concentration in the datasheet (100pg - 1μg/ml) and published experiments in the same cell type [392]. Western immunoblotting was performed and p-p65 and p-c-Jun were used as markers of NF-κB and AP-1 activation.

LPS stimulation resulted in a significant increase in NF-κB activation at 6 hours (*p<0.05) (Figure 4.9 A) with 1-fold difference compared to non-stimulated control. This increase however is not consistent in all 3 samples (Figure 4.10) suggesting that this increase could be an erroneous and spurious result. 1μg/ml of LPS did not have an effect on NF-κB activation (Figure 4.9 B).

Although TLR4 mRNA has been shown not to be expressed in the vagina in this and other studies, an unsuspected significant increase in AP-1 activation was seen at 30 minutes (*p<0.05) and 1 hour (***p<0.001) with 0.1μg/ml (Figure 4.9 C), while no statistically significant change was achieved with 1μg/ml (Figure 4.9 D) of LPS. Although a 100-fold change increase was observed with 1μg/ml, the value was not significant due to variability in kinetic responses between replicates. The responses seen with LPS on AP-1 activation could be due to a non-specific reactivity of the agonist, despite attempts to avoid this by using low concentrations.

For the purpose of the priming experiment, 0.1μg/ml at 6 hours was chosen to be consistent with other cell types in this chapter.

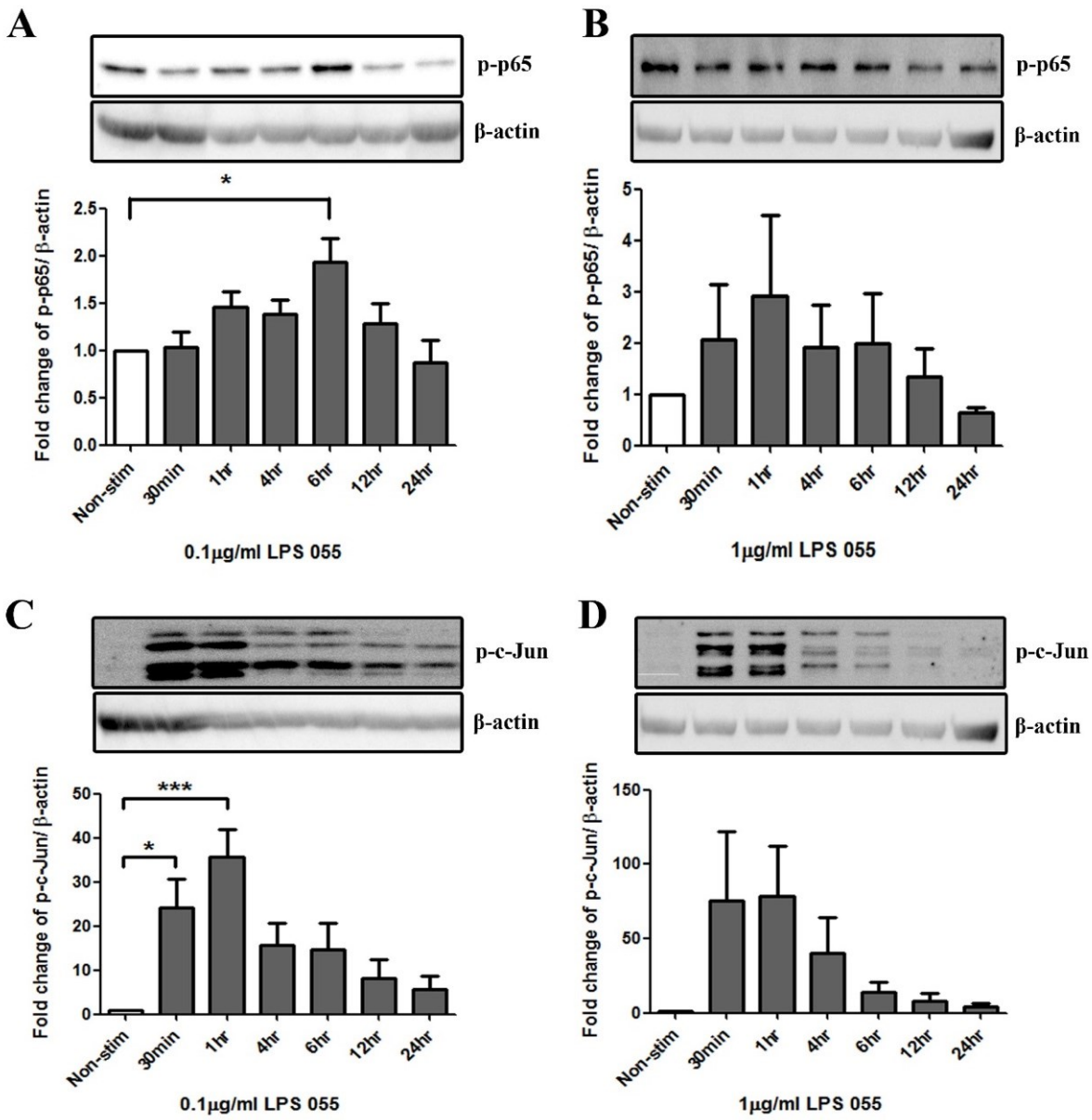


Figure 4.9 The effect of lipopolysaccharides from Escherichia coli 055:B5 (LPS 055) ultrapure stimulation on NF-κB and AP-1 activation in VEC. VECs were cultured and stimulated with 0.1µg/ml and 1µg/ml of LPS 055 ultrapure for a duration of 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. NF-κB increased significantly with **(A)** 0.1µg/ml of poly I:C at 6 (**p*<0.05) hours and **(B)** no increase was seen with 1µg/ml. AP-1 also increased significantly with **(C)** 0.1µg/ml of LPS 055 at 30 minutes (**p*<0.05) and 1 hour (****p*<0.001) and **(D)** 1µg/ml of LPS 055 at 30 minutes and 1 hour although no statistical significance was achieved. For statistical analysis One-way ANOVA with *Dunnett's* multiple comparison test was used. n=3-4.

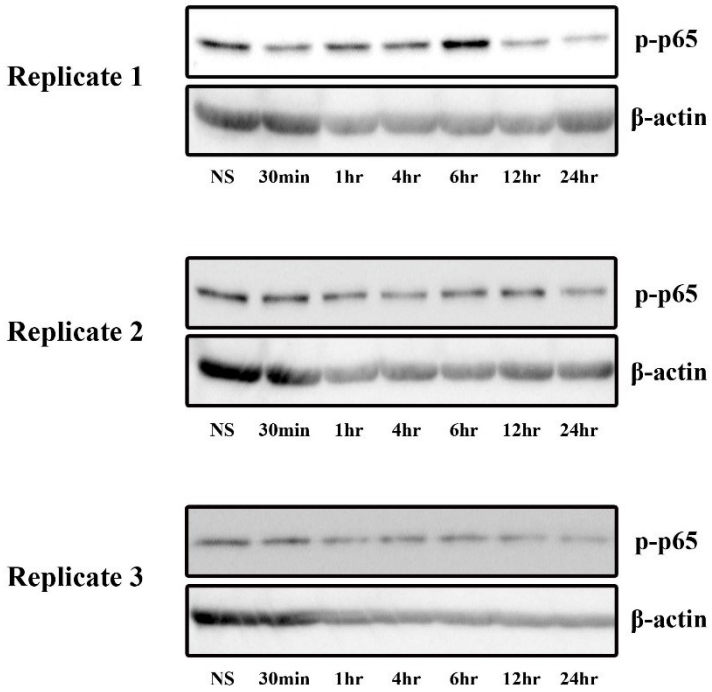


Figure 4.10 The differential effect of 0.1µg/ml of lipopolysaccharides from Escherichia coli 055:B5 (LPS 055) ultrapure stimulation (TLR4 agonist) on NF-κB activation in VEC. VECs were cultured and stimulated with 0.1µg/ml of LPS 055 ultrapure for a duration of up to 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) a marker of NF-κB activation. All three biological replicates are shown above with β-actin as a loading control. Replicate 1 showed clear increase NF-κB at 6 hours but this is not consistent in other replicates which did not show an increase in NF-κB activation.

4.2.5 TLR3/TLR4 priming in VECs

25μg/ml of poly I:C for 6 hours and 0.1μg/ml of LPS 055 ultrapure were used for the priming experiment. Monolayer VK2 cells were primed with poly I:C for 6 hours before a subsequent LPS 055 ultrapure stimulation for 6 hours. Protein was extracted and analysed using Western immunoblotting to examine the activation status of NF-κB and AP-1.

For cytokines, chemokine and PGE2 studies, cells were primed with poly I:C for 6 hours and then stimulated with LPS 055 for 24 hours. An ELISA was used to quantify the analytes.

4.1.5.1 NF-κB or AP-1 activation is not augmented with TLR3/4 priming

There was no increase in NF-κB activation (Figure 4.11 A) or AP-1 activation (Figure 4.11 B) with poly I:C, or LPS or primed stimulation compared with the basal level at 0hr.

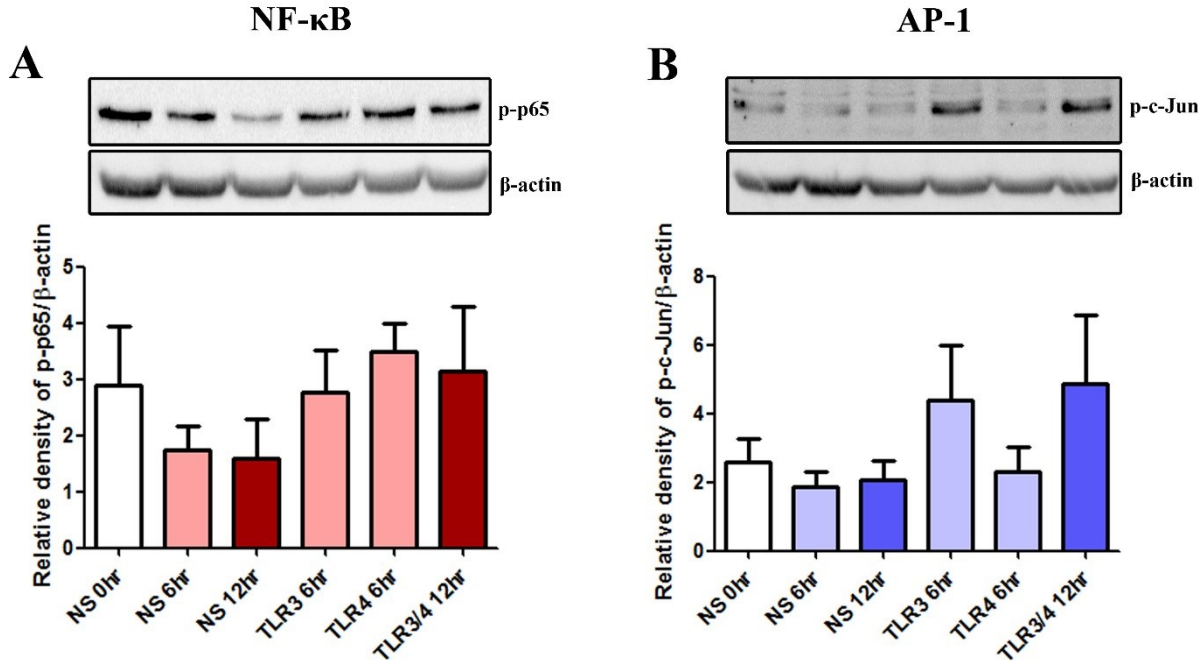


Figure 4.11 The effect of TLR3 priming before TLR4 agonist stimulation on NF-κB and AP-1 in VEC. Confluent monolayer VECs were plated and were primed with 25μg/ml of poly I:C for 6 hours prior to 0.1μg/ml of LPS 055 ultrapure stimulation for 6 hours. Protein was extracted, and Western immunoblotting was performed to determine the activation status of serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. TLR3 priming prior to subsequent TLR4 stimulation showed no augmentation of **(A)** NF-κB or **(B)** AP-1 activation. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3.

4.1.5.2 Pro-inflammatory cytokine production is not augmented with TLR3/4 priming

Although NF-κB and AP-1 were not augmented in TLR3/4 priming, which could be due to lack of TLR4 expression in VEC, the same downstream pro-inflammatory cytokines listed in section 4.3.2 were quantified. TNF-β, IFN-γ, IL-12p40 and IL-12p70 were not detected in any of the samples.

The vehicle control showed a trend of increased cytokines production over time. Poly I:C stimulation alone significantly increased IL-1α (***p<0.001 at 24 hours) (Figure 4.12 A), IL-6 (***p<0.001 at 6 and 24 hours) (Figure 4.12 C), IL-8 (***p<0.001 at 6 and 24 hours) (Figure 4.12 D) and TNF-α (***p<0.001 at 6 and 24 hours) (Figure 4.12 E) production compared to 0hr. There was no effect of LPS on cytokine production, consistent with TLR4 not being expressed in these cells. Furthermore, TLR3/4 priming showed no augmentation on any of the cytokines.

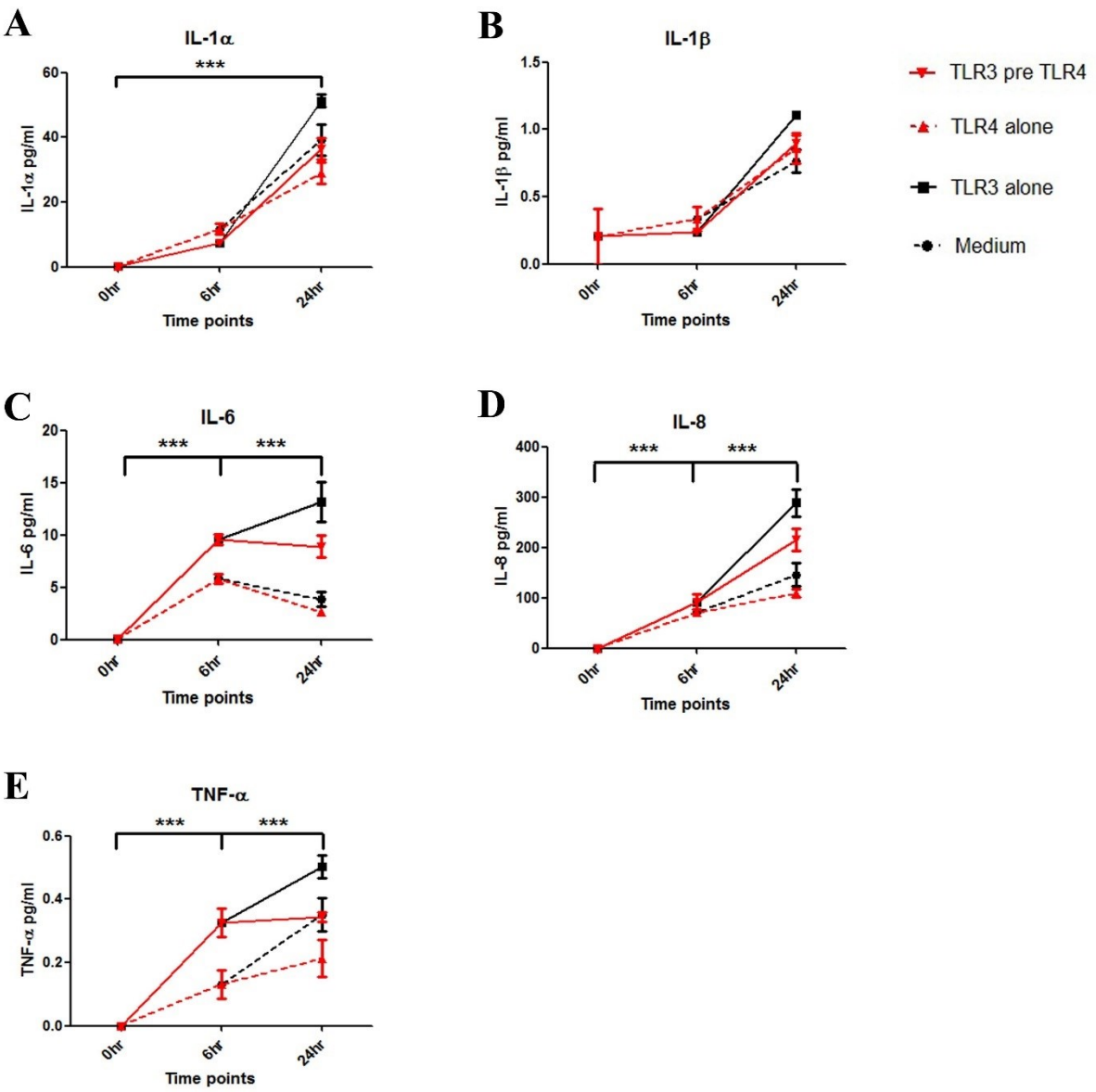


Figure 4.12 The effect of TLR3 priming prior to TLR4 agonist stimulation on pro-inflammatory cytokines production in VEC. Confluent monolayer VEC were plated and were primed with 25μg/ml of poly I:C for 6 hours prior to 0.1μg/ml of LPS 055 ultrapure stimulation for 24 hours. Supernatant was collected and neat samples were used to run ELISA to determine the level of the cytokines production. TLR3 stimulation significantly increased the production of IL-1α, IL-6, IL-8 and TNF-α. However, no further augmentation was observed with TLR3 priming prior to TLR4 agonist stimulation on **(A)** IL-1α, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-8 or **(E)** TNF-α. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. ****p*<0.001 = effect of TLR3 compared to 0hr.

4.1.5.3 Chemokine production is not augmented with TLR3/4 priming

The same chemokines in section 4.3.3 were quantified using neat samples for MIP-1α and MIP-1β and a 1 in 10 dilution for RANTES.

The vehicle control showed an increased chemokines production trend over time. Poly I:C stimulation alone significantly increased MIP-1α (***p<0.001 at 24 hours) (Figure 4.13 A), MIP-1β (***p<0.001 at 6 and 24 hours) (Figure 4.13 B) and RANTES (***p<0.001 at 24 hours) (Figure 4.13 C) production compared to 0hr. There was no effect of LPS on chemokine production, consistent with TLR4 not being expressed in these cells. Furthermore, there was no augmentation in the TLR3/4 treated cells.

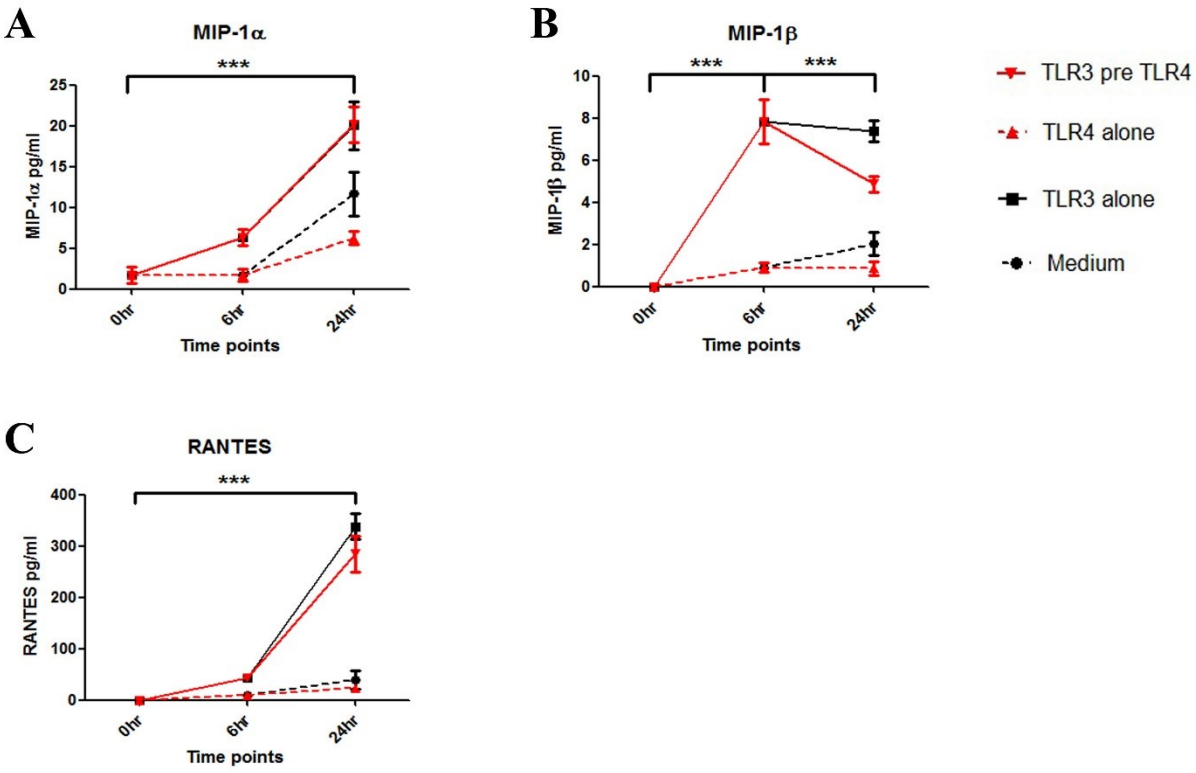


Figure 4.13 The effect of TLR3 priming prior to TLR4 agonist stimulation on chemokines production in VEC. Confluent monolayer VECs were plated and were primed with 25μg/ml of poly I:C for 6 hours prior to 0.1μg/ml of LPS 055 ultrapure stimulation for 24 hours. Supernatant was collected and were used to run ELISA to determine the level of the chemokines production. TLR3 stimulation significantly increased the production of MIP-1α, MIP-1β and RANTES. Priming with TLR3 before TLR4 agonist stimulation however do not augment further **(A)** MIP-1α, **(B)** MIP-1β or **(C)** RANTES. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. ***p<0.001 **=** effect of TLR3 compared to 0hr.

4.1.5.4 Anti-inflammatory cytokine production is not augmented with TLR3/4 priming

The same samples above were used to detect the same anti-inflammatory cytokines listed in section 4.3.4 using neat samples. However, only IL-1RA was detectable in all samples.

There was no difference in the IL-1RA (Figure 4.14) in cells treated with poly I:C or HKLM alone or in the primed samples compared to the basal levels in the vehicle control.

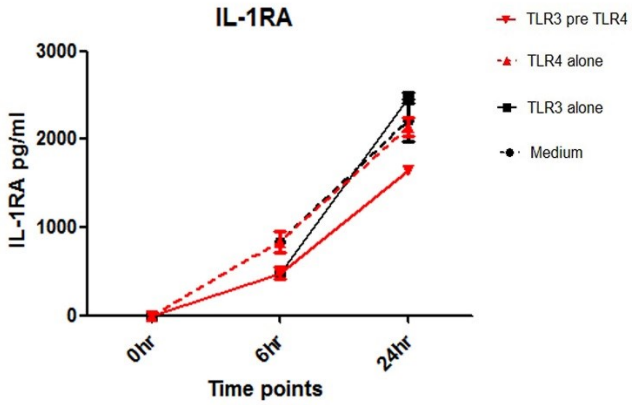


Figure 4.14 The effect of TLR3 priming prior to TLR4 agonist stimulation on anti-inflammatory IL-1RA production in VEC. Confluent monolayer VECs were plated and were primed with 25µg/ml of poly I:C for 6 hours prior to 0.1µg/ml of LPS 055 ultrapure stimulation for 24 hours. Supernatant was collected and neat samples were used to run ELISA to determine the level of the IL-1RA production. Neither the TLR agonists alone nor TLR3 priming prior to TLR4 agonist stimulation increased the production of IL-1RA above the basal level. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3.

4.1.5.5 PGE2 production is not augmented with TLR3/4 priming

PGE2 production was analysed. PGE2 production after poly I:C stimulation remained at the basal level. LPS stimulation alone significantly increased PGE2 production at 24 hours (*p<0.05). This could be due to the off-target effect of the agonist used. No augmentation was observed with TLR3/4 priming (Figure 4.15).

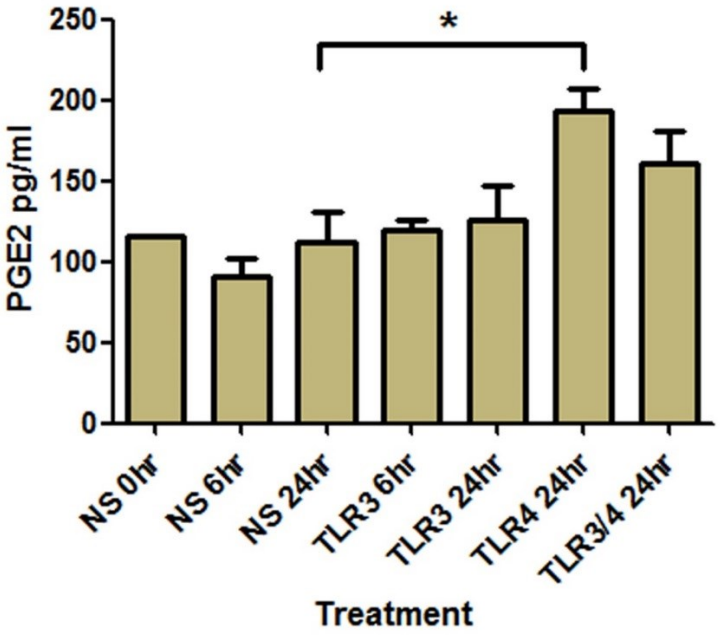


Figure 4.15 The effect of TLR3 priming prior to TLR4 agonist stimulation on PGE2 production in VEC. Confluent monolayer VECs were plated and were primed with 25µg/ml of poly I:C for 6 hours prior to 0.1µg/ml of LPS 055 ultrapure stimulation for 24 hours. TLR4 agonist stimulation increased the production of PGE2. However, TLR3 priming prior to TLR4 agonist stimulation showed no augmentation of PGE2 production compared to the individual TLR agonist stimulation alone. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3. *p<0.05 = effect of TLR4 agonist stimulation compared to non-stimulated control (NS).

4.2.6 Fibroblast–stimulating lipopeptide FSL-1 (TLR2/6 agonist) stimulation increases NF-κB and AP-1

FSL-1 is a TLR2/6 agonist and relies onTLR2 and TLR6 dimerization for its effect. Therefore, this agonist was used to compare and contrast the effects of the TLR2 agonist HKLM. In this section, the same set of experiments was carried out as in section 4.2.2 and 4.2.4 above with fibroblast–stimulating lipopeptide (FSL-1), a TLR2/6 agonist to find the optimal concentration and time points before conducting priming experiment.

Cells were cultured, and confluent monolayer cells were stimulated for 24 hours with 0.001µg/ml and 0.01µg/ml concentrations of the agonist based on the lowest concentration suggested in the datasheet (1ng/ml - 1µg/ml). Western immunoblotting was performed and p-p65 and p-c-Jun were used as marker for NF-κB and AP-1 activation status.

The lowest concentration of FSL-1, 0.001µg/ml showed no difference in NF-κB activation (Figure 4.16 A) in comparison to basal level. 10-fold concentration increase of the agonist showed a significant increase in NF-κB activation at 30 minutes and 1 hour (***p<0.001) with 0.01µg/ml (Figure 4.16 B).

In contrast to NF-κB, 0.001µg/ml FSL-1 significantly increased AP-1 activation at 30 minutes, 1, 4 and 6 hours(*p<0.05) (Figure 4.16 C). A much higher increase in AP-1 activation was seen with 0.01µg/ml FSL-1 at 1 hour (***p<0.001) (Figure 4.16 D).

Since NF-κB activation was not observed with 0.001µg/ml, the concentration of 0.01µg/ml of FSL-1 at 1 hour was chosen for priming to see an effect on both NF-κB and AP-1.

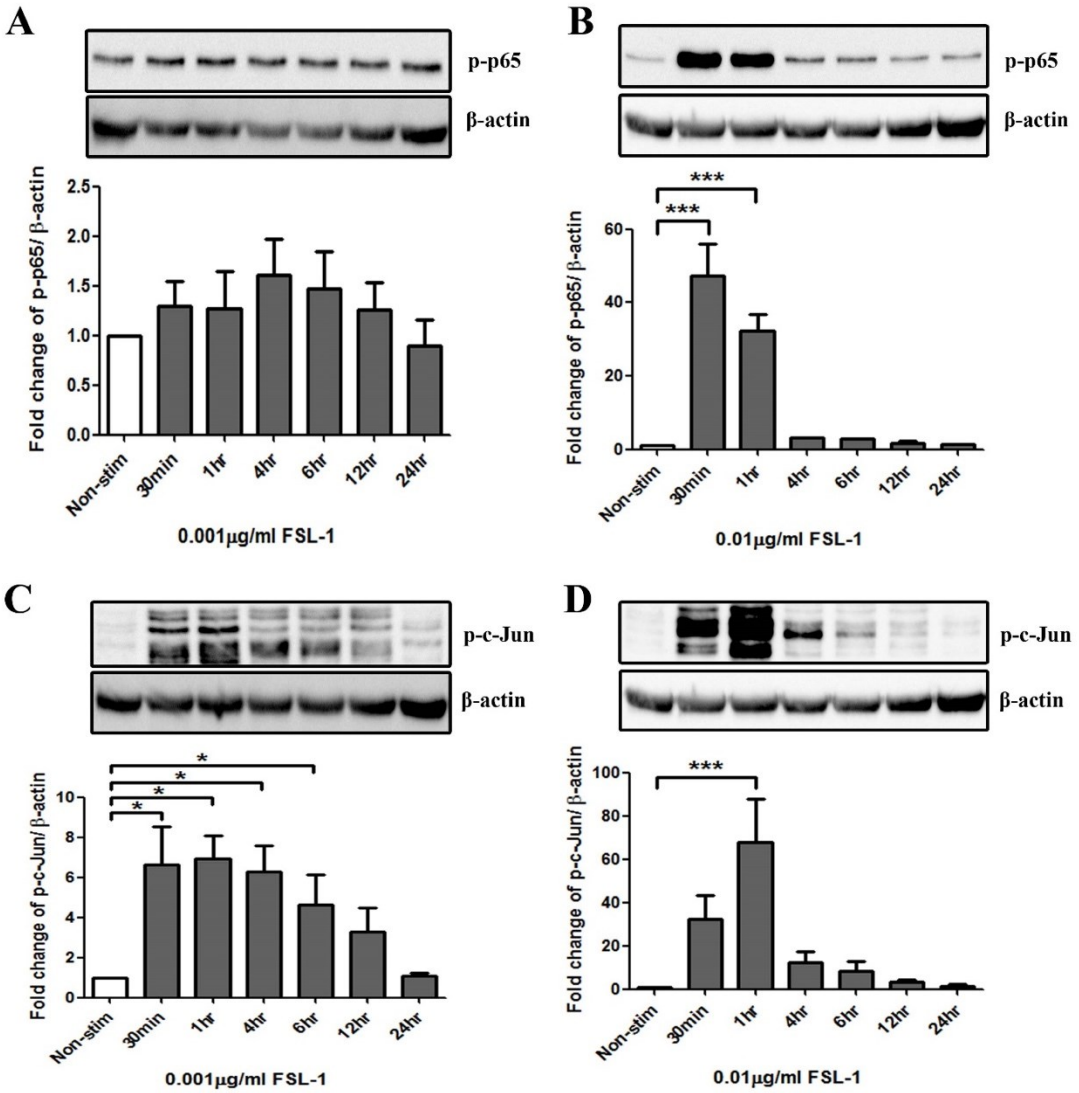


Figure 4.16 The effect of FSL-1, a TLR2/6 agonist stimulation on NF-κB and AP-1 activation in VEC. VECs were cultured and stimulated with 0.001µg/ml and 0.01µg/ml of FSL-1 for a duration of 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. NF-κB showed no increased with **(A)** 0.001µg/ml of FSL-1 but **(B)** a significant increase was observed with 0.01µg/ml of FSL-1 at 30 minutes and 1 hour (***p<0.001). AP-1 significantly increased with **(C)** 0.001µg/ml of FSL-1 at 30 minutes, 1, 4 and 6 (*p<0.05) hours and **(D)** 0.01µg/ml of FSL-1 at 1 (***p<0.001) hour. For statistical analysis One-way ANOVA with *Dunnett's* multiple comparison test was used. n=3.

4.2.7 TLR3/TLR2/6 priming in VECs

Based on the time course experiment, 25μg/ml of poly I:C and 0.01μg/ml of FSL-1 were chosen as the optimal concentration for priming experiment. Monolayer VK2 cells were primed with poly I:C for 6 hours before a subsequent FSL-1 stimulation for 1 hour. Protein was extracted and analysed using Western immunoblotting to examine the activation status of NF-κB and AP-1.

For cytokines, chemokine and PGE2 studies, cells were primed with poly I:C for 6 hours and then stimulated with FSL-1 for 24 hours. An ELISA was used to quantify the analytes.

4.1.7.1 NF-κB or AP-1 activation is not augmented with TLR3/TLR2/6 priming

Poly I:C stimulation yielded the same fold change difference in NF-κB activation as reported in section 4.1.2, although this did not reach statistical significance. FSL-1 stimulation increased NF-κB activation at 6 hours, however this did not reach statistical significance. No further augmentation of NF-κB was observed with priming (Figure 4.17 A).

For AP-1, poly I:C stimulation alone showed no difference at 6 hours compared to the basal level, which could be due to the earlier activation of AP-1 at 1 hour (section 4.1.1). Priming showed a significant increase compared to poly I:C stimulation alone (**p<0.01) but not when compared with FSL-1 alone (Figure 4.17 B).

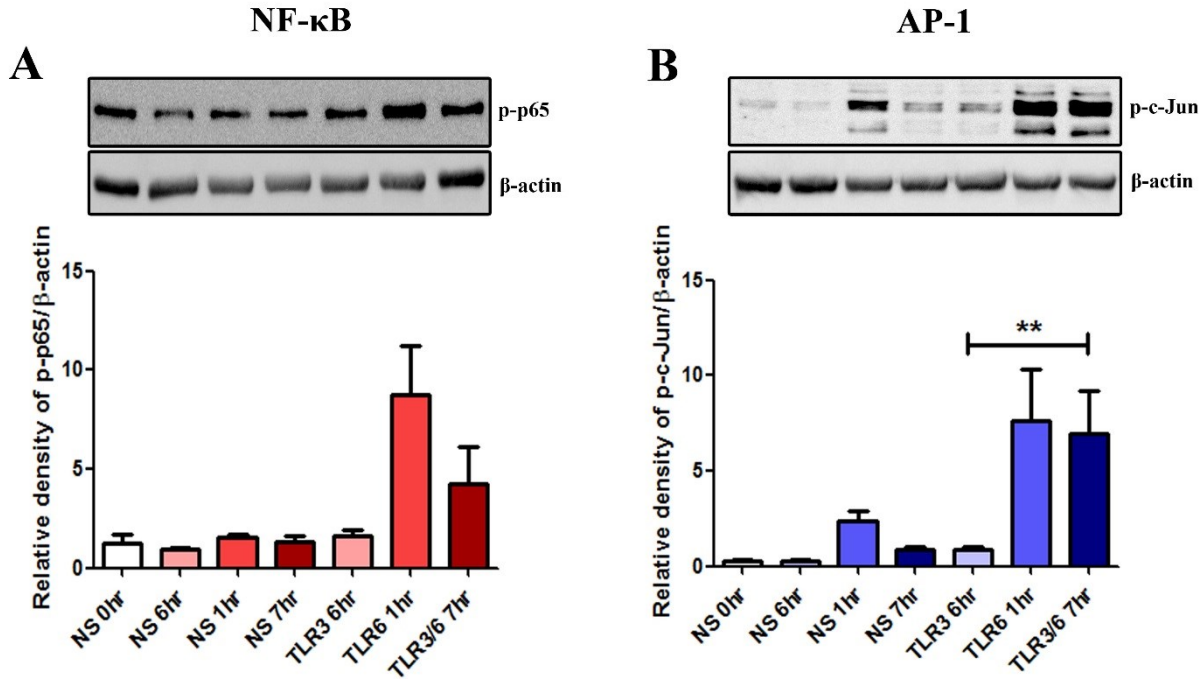


Figure 4.17 The effect of TLR3 priming before TLR2/6 agonist stimulation on NF-κB and AP-1 in VEC. Confluent monolayer VECs were plated and were primed with 25μg/ml of poly I:C for 6 hours prior to 0.01μg/ml of FSL-1 stimulation for 1 hour. Protein was extracted, and Western immunoblotting was performed to determine the activation status of serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1, respectively. A representative blot is shown above each graph with β-actin as a loading control. TLR3 priming prior to subsequent TLR2/6 stimulation showed no augmentation of **(A)** NF-κB. In **(B)** AP-1, although an augmentation was observed when comparing with TLR3 agonist stimulation alone (**p<0.01), no further augmentation was observed with TLR2/6 agonist stimulation. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3.

4.1.7.2 IL-6, IL-8 and TNF-α are augmented with TLR3/TLR2/6 priming

Although neither NF-κB nor AP-1 showed significant augmentation with priming, activation of other transcription factor/s could lead to the production of pro-inflammatory and pro-labour mediators. The same pro-inflammatory cytokines listed in section 4.3.2 were examined in this section. TNF-β, IL-12p40 and IL-12p70 were not detected in any of the samples.

Consistent with the previous section, the vehicle control showed a trend of increasing cytokine production over time. IL-1α (***p<0.001 at 24 hours), IL-1β (***p<0.001 at 24 hours), IL-6 (***p<0.001 at 6 and 24 hours), IL-8 (***p<0.001 at 24 hours) and TNF-α (*p<0.05 at 6 hours and ***p<0.001 at 24 hours) production increased significantly with poly I:C stimulation alone compared to 0hr.

FSL-1 stimulation significantly increased the production of IL-1β ($^{\dagger\dagger}p$<0.01 at 24 hours), IFN-γ ($^{\dagger\dagger\dagger}p$<0.001 at 24 hours), IL-6 ($^{\dagger}p$<0.05 at 6 hours and $^{\dagger\dagger\dagger}p$<0.001 at 24 hours), IL-8 ($^{\dagger\dagger\dagger}p$<0.001 at 24 hours) and TNF-α ($^{\dagger\dagger\dagger}p$<0.001 at 24 hours) compared to 0hr.

Priming with poly I:C before FSL-1 stimulation augmented IL-6 ($^{\S\S\S}p$<0.001) (Figure 4.18 D), IL-8 ($^{\S\S}p$<0.01) (Figure 4.18 E) and TNF-α ($^{\S\S}p$<0.01) (Figure 4.18 F) compared to FSL-1 stimulation alone. No effect on the other pro-inflammatory cytokines was observed.

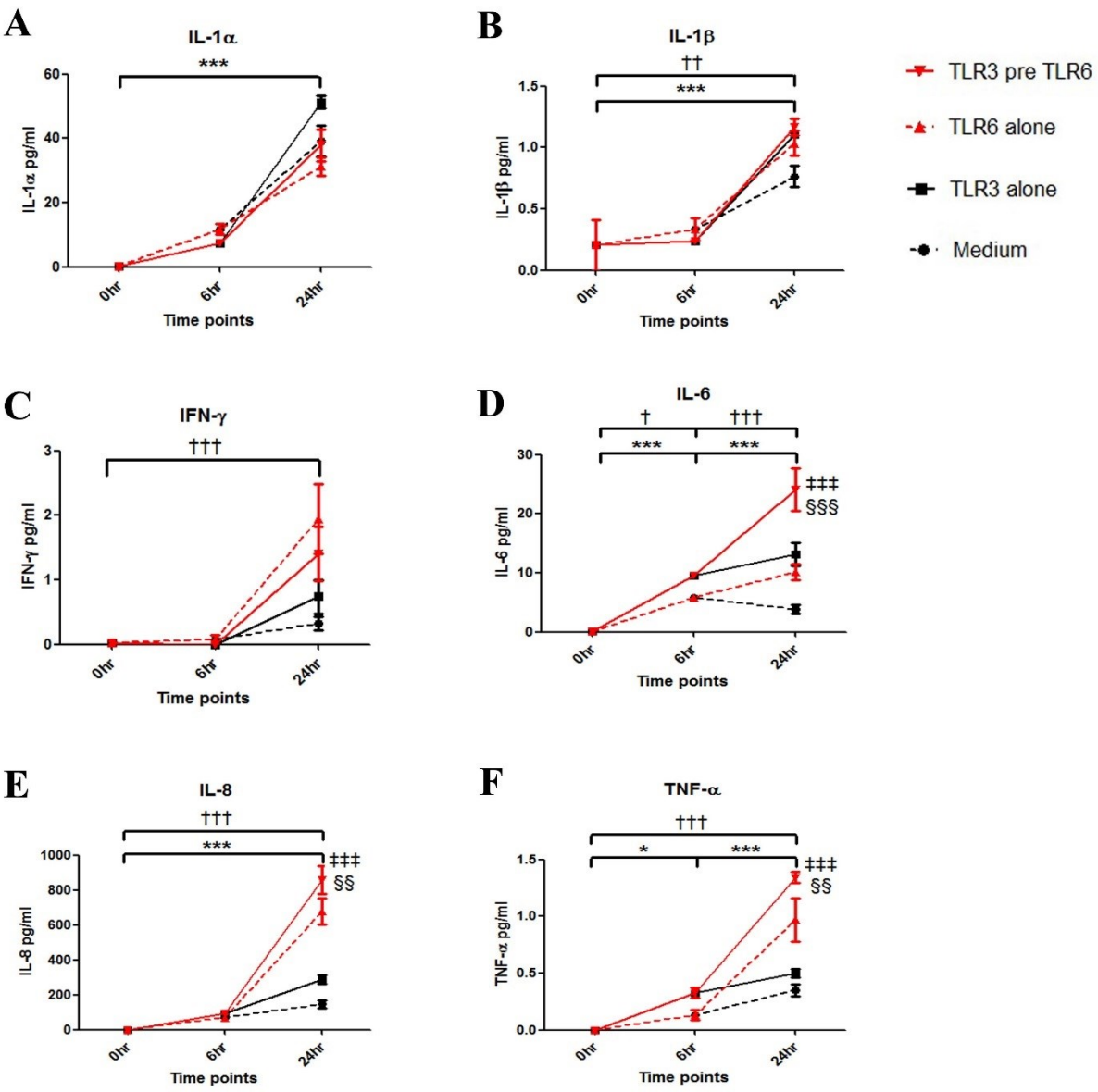


Figure 4.18 The effect of TLR3 priming prior to TLR2/6 agonist stimulation on pro-inflammatory cytokines production in VEC. Confluent monolayer VECs were plated and were primed with 25µg/ml of poly I:C for 6 hours prior to 0.01µg/ml of FSL-1 stimulation for 24 hours. Supernatant was collected and neat samples were used to run ELISA to determine the level of the cytokines production. TLR3 and TLR2/6 agonist stimulation significantly increased the production of the cytokines. Priming with TLR3 before TLR2/6 agonist stimulation did not augment **(A)** IL-1α, **(B)** IL-1β, **(C)** IFN-γ, however, a significant increase was observed with **(D)** IL-6, **(E)** IL-8 and **(F)** TNF-α cytokines production. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3. **p*<0.05 **=** effect of TLR3 compared to 0hr. †*p*<0.05 **=** effect of TLR2/6 compared to 0hr. ‡‡‡*p*<0.001 = effect of TLR3 pre TLR2/6 compared to TLR3 alone. §§*p*<0.01 = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone.

4.1.7.3 MIP-1α and MIP-1β are augmented with TLR3/TLR2/6 priming

Neat samples were run for MIP-1α and MIP-1β while a 1 in 10 dilution was used for RANTES quantification.

An increased trend of chemokine production was observed in the vehicle control samples. Poly I:C stimulation alone significantly increased the production of MIP-1α (***p<0.001 at 24 hours) (Figure 4.19 A), MIP-1β (***p<0.001 at 6 and 24 hours) (Figure 4.19 B) and RANTES (***p<0.001 at 24 hours) (Figure 4.19 C) compared to 0hr. FSL-1 stimulation increased the production of MIP-1α ($^{\dagger\dagger\dagger}p$<0.001 at 24 hours) and MIP-1β ($^{\dagger\dagger\dagger}p$<0.001 at 24 hours) compared to 0hr. Poly I:C priming prior to FSL-1 stimulation augmented MIP-1α ($^{\S\S\S}p$<0.001) and MIP-1β ($^{\S\S\S}p$<0.001) production compared to FSL-1 stimulation alone while no effect on RANTES was seen.

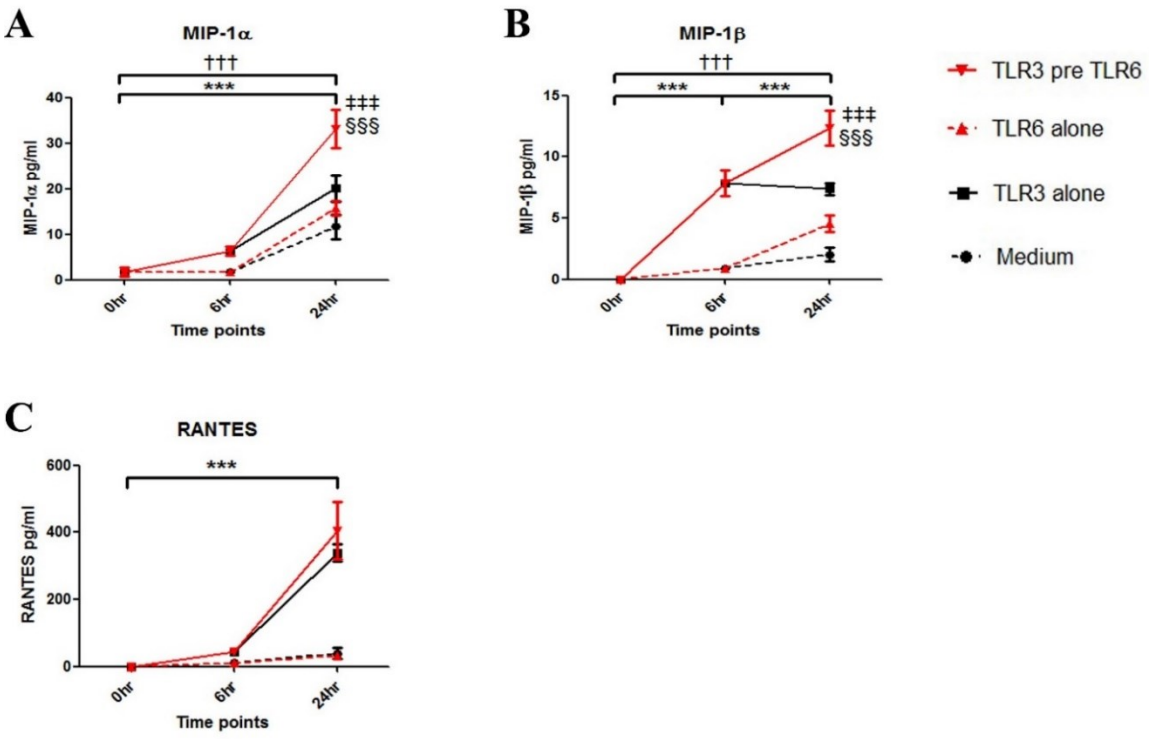


Figure 4.19 The effect of TLR3 priming prior to TLR2/6 agonist stimulation on chemokines production in VEC. Confluent monolayer VECs were plated and were primed with 25μg/ml of poly I:C for 6 hours prior to 0.01μg/ml of FSL-1 stimulation for 24 hours. Supernatants were collected and were used to run ELISA to determine the level of the chemokines production. TLR3 and TLR2/6 agonist stimulation significantly increased the production of MIP-1α, MIP-1β and RANTES. Further augmentation of **(A)** MIP-1α or **(B)** MIP-1β production was observed with TLR3 priming before TLR2/6 agonist stimulation, however, no further production was observed in **(C)** RANTES. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. ***p<0.001 **=** effect of TLR3 compared to 0hr. $^{\dagger\dagger\dagger}p$<0.001 **=** effect of TLR2/6 compared to 0hr. $^{\ddagger\ddagger\ddagger}p$<0.001 = effect of TLR3 pre TLR2/6 compared to TLR3 alone. $^{\S\S\S}p$<0.001 = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone.

4.1.7.4 Anti-inflammatory cytokine production is not augmented with TLR3/TLR2/6 priming

The same anti-inflammatory cytokines listed in section 4.3.4 were quantified using neat samples. However, only IL-1RA was detectable in all the samples.

There was no difference in IL-1RA (Figure 4.20) production in cells treated with poly I:C or FSL-1 alone, or with priming compared to the basal levels in the vehicle control.

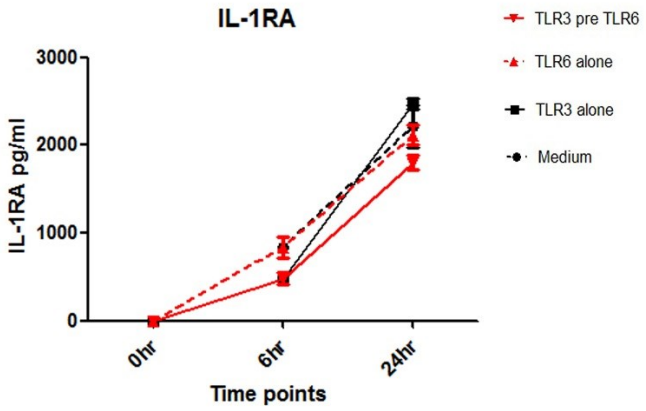


Figure 4.20 The effect of TLR3 priming prior to TLR2/6 agonist stimulation on anti-inflammatory IL-1RA production in VEC. Confluent monolayer VEC were plated and were primed with 25µg/ml of poly I:C for 6 hours prior to 0.01µg/ml of FSL-1 stimulation for 24 hours. Supernatant was collected and neat samples were used to run ELISA to determine the level of the IL-1RA production. Neither the TLR agonists alone nor TLR3 priming prior to TLR2/6 agonist stimulation increased the production of IL-1RA above basal level. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3.

4.1.7.5 PGE2 production is augmented with TLR3/TLR2/6 priming

PGE2 production was analysed. The PGE2 production remained at basal level with poly I:C or FSL-1 stimulation. Priming with poly I:C prior to FSL-1 stimulation augmented PGE2 production compared to stimulation with individual agonists alone (***p<0.001) (Figure 4.21).

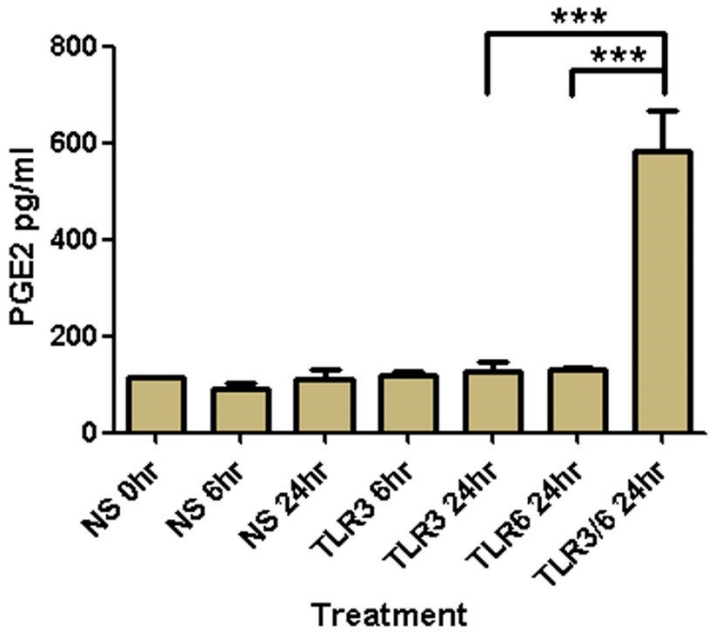


Figure 4.21 The effect of TLR3 priming prior to TLR2/6 agonist stimulation on PGE2 production in VEC. Confluent monolayer VECs were primed with 25µg/ml of poly I:C for 6 hours prior to 0.01µg/ml of FSL-1 stimulation for 24 hours. TLR3 priming prior to TLR2/6 agonist stimulation augmented PGE2 production compared to the individual TLR agonist stimulation alone. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3. ***p<0.001 = effect of TLR3 pre TLR2/6 compared to TLR3 and TLR2/6 alone.

4.2 Amniocytes

4.3.1 Poly I:C (viral TLR3 agonist) stimulation increases NF-κB and AP-1

Before carrying out the viral priming experiment in amniocytes, the experiments to determine the optimal concentration and time points for poly I:C were performed to examine its effect on the transcription factors NF-κB and AP-1. Western immunoblotting was performed and phosphorylated p-65 (p-p65) and phosphorylated c-Jun (p-c-Jun) were used as markers for NF-κB and AP-1 activation status.

Cells were cultured, and confluent monolayer cells were stimulated with 5µg/ml and 10µg/ml of poly I:C in a time course experiment for up to 24 hours. Poly I:C stimulation at 5µg/ml increased NF-κB activation at 12 hours (**p<0.01) (Figure 4.**Figure 4.**22 A) and 10µg/ml showed an increase at 12 and 24 hours (*p<0.05) (Figure 4.22 B). A higher fold change was recorded with AP-1 with a significant increase with 5µg/ml at 12 hours (*p<0.05) (Figure 4.22 C). Although a 10-fold change increase was observed with 10µg/ml in AP-1 at 12 and 24 hours, no statistical significance was observed due to the large variation in response seen between biological replicates (Figure 4.22 D).

With the consistent increase of TLR3 protein expression and transcription factors NF-κB and AP-1 with poly I:C stimulation at 12 hours, a dose response was performed at 12 hours starting with 5, 10, 25, 50 and 100µg/ml of poly I:C. Both NF-κB (Figure 4.23 A) and AP-1 (Figure 4.23 B) showed a significant increase at 25µg/ml (*p<0.05), 50µg/ml (**p<0.01) and 100µg/ml (**p<0.01, ***p<0.001) with poly I:C stimulation.

To be consistent with experiments in vaginal epithelial cells, 25µg/ml of poly I:C was chosen for priming. 12 hours was chosen for the duration of treatment in amniocytes based on the time course response experiment.

4.2.1.1 Poly I:C time course

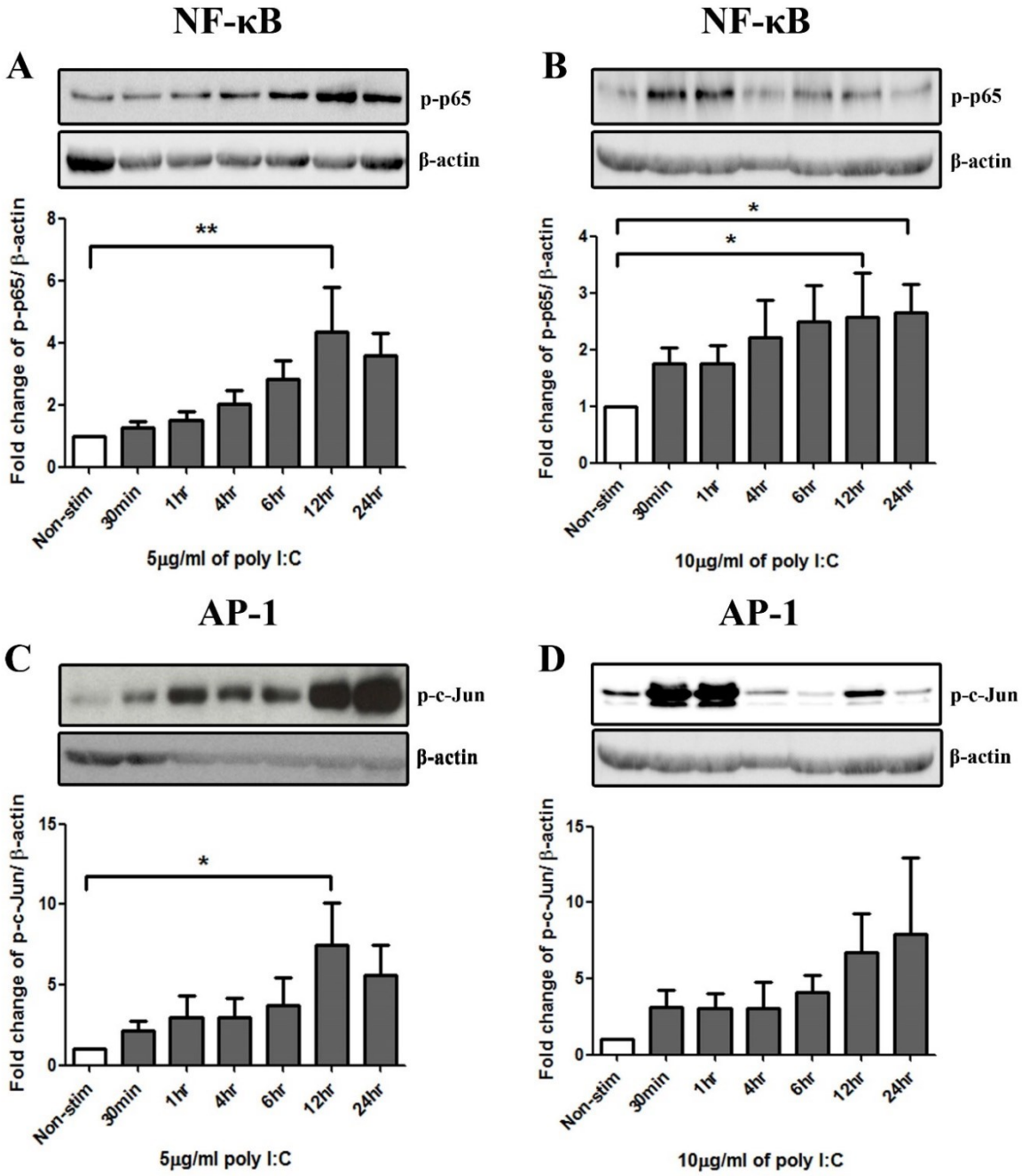


Figure 4.22 The effect of poly I:C, a viral TLR3 agonist stimulation on NF-κB and AP-1 activation in amniocytes. Amniocytes were cultured and stimulated with 5μg/ml and 10μg/ml of poly I:C for a duration of 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. NF-κB increased significantly with **(A)** 5μg/ml of poly I:C at 12 (**p<0.01) hours and **(B)** 10μg/ml of poly I:C at 12 (*p<0.05), 24 (*p<0.05) hours. AP-1 also increased significantly with **(C)** 5μg/ml of poly I:C at 12 (*p<0.05) hours. An increase in AP-1 was observed with **(D)** 10μg/ml of poly I:C at 12 and 24 hours, but no statistical significance was achieved at this concentration. For statistical analysis One-way ANOVA with *Dunnett's* multiple comparison test was used. n=4-5.

4.2.1.2 Poly I:C dose response at 12 hours

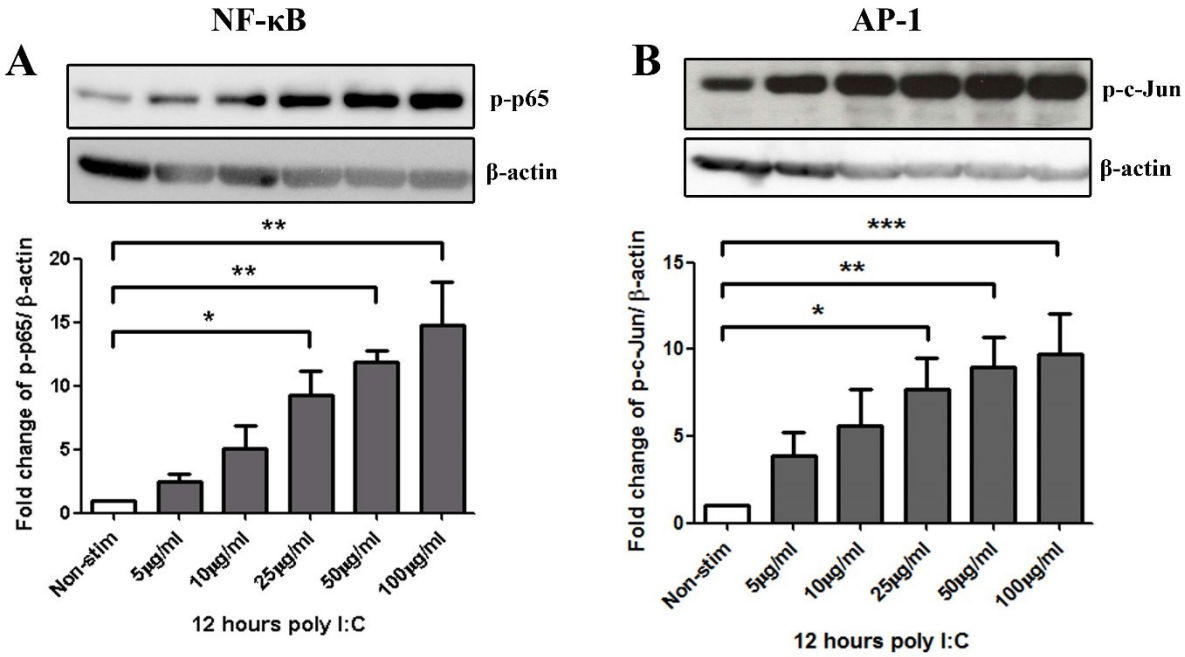


Figure 4.23 The effect of different doses of poly I:C, a viral TLR3 agonist stimulation on NF-κB and AP-1 activation at 12 hours in amniocytes. Amniocytes were cultured and stimulated with 5μg/ml, 10μg/ml, 25μg/ml, 50μg/ml and 100μg/ml of poly I:C for 12 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot was shown above each graph with β-actin as a loading control. **(A)** NF-κB and **(B)** AP-1 increased significantly with 25μg/ml (*p<0.05), 50μg/ml (**p<0.01) and 100μg/ml (**p<0.01, ***p<0.001) of poly I:C at 12 hours. Statistical analysis was performed using One-way ANOVA for normally distributed data and Kruskal-Wallis for data not normally distributed with *Dunnett's* multiple comparison test. n=4-5.

4.3.2 Heat-killed *Listeria monocytogenes* (HKLM) (TLR2 agonist) stimulation increases NF-κB and AP-1

The same time course experiment using10^5 cells/ml and 10^6 cells/ml experiments as in section 4.2.2 was repeated in amniocytes to find the optimal concentration and time point for the priming experiment.

10^5 cells/ml of HKLM increased NF-κB activation significantly by 30 minutes (***p<0.001), 1 and 4 hours (**p<0.01) (Figure 4.24 A) while no significant increase was observed with 10^6 cells/ml (Figure 4.24 B). At both concentrations, AP-1 increased significantly at 30 minutes and 1 hour (***p<0.001, *p<0.05) (Figure 4.24 C and D).

For consistency with the experiments in VECs, 10^5 cells/ml at 1 hour was used for the priming experiment. Although no statistical significance was seen with 10^5 cells/ml at 1 hour, a mean of 10-fold increase of NF-κB activation was seen, and there was a consistent increase in all samples tested. Therefore, this suggests that this concentration and time point is reliable indicator for NF-κB activation and was therefore chosen for the priming experiment.

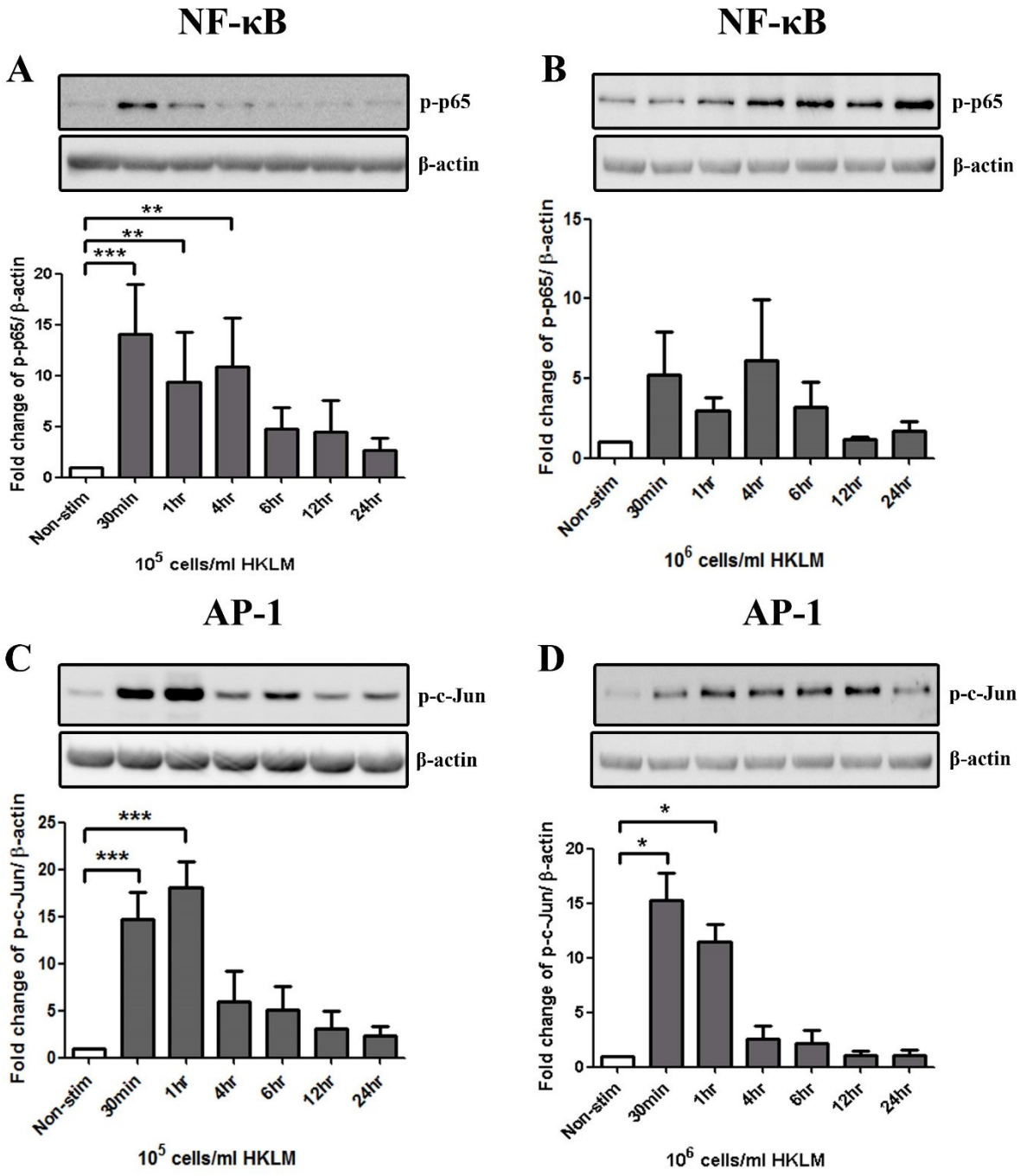


Figure 4.24 The effect of Heat-killed *Listeria monocytogenes* (HKLM), a TLR2 agonist stimulation on NF-κB and AP-1 activation in amniocytes. Amniocytes were cultured and stimulated with 10^5 and 10^6 cells/ml of HKLM for a duration of 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. NF-κB increased significantly with **(A)** 10^5 cells/ml of HKLM at 30 minutes (***p<0.001), 1 and 4 hours (**p<0.01) while although an increase was observed with **(B)** 10^6 cells/ml, no statistically significance was achieved. AP-1 also increased significantly with **(C)** 10^5 cells/ml and **(D)** 10^6 cells/ml of HKLM at 30 minutes and 1 hour (***p<0.001, *p<0.05). Statistical analysis was performed using One-way ANOVA for normally distributed data and Kruskal-Wallis for data not normally distributed with *Dunnett's* multiple comparison test. n=4-5.

4.3.3 TLR3/TLR2 priming in amniocytes

Based on the time course and dose response experiment with poly I:C and HKLM in amniocytes, 25μg/ml of poly I:C and 10^5 cell/ml of HKLM were chosen as the optimal concentrations for the priming experiment. Cells were primed with poly I:C for 12 hours before subsequent HKLM stimulation for 1 hour. Protein was extracted and analysed using Western immunoblotting to examine the activation status of NF-κB and AP-1.

For cytokines, chemokine and PGE2 studies, cells were primed with poly I:C for 12 hours and then stimulated with HKLM for 24 hours. An ELISA was used to quantify the analytes.

4.2.3.1 AP-1 activation is augmented with TLR3/2 priming but not NF-κB

Poly I:C stimulation and HKLM stimulation alone had no significant effect on NF-κB activation. TLR3/2 priming demonstrated augmented NF-κB activation compared to HKLM stimulation alone (*p<0.05) but not compared with poly I:C (Figure 4.25 A).

TLR3/2 priming showed augmented AP-1 activation (**p<0.01) (Figure 4.25 B) compared to poly I:C and HKLM alone.

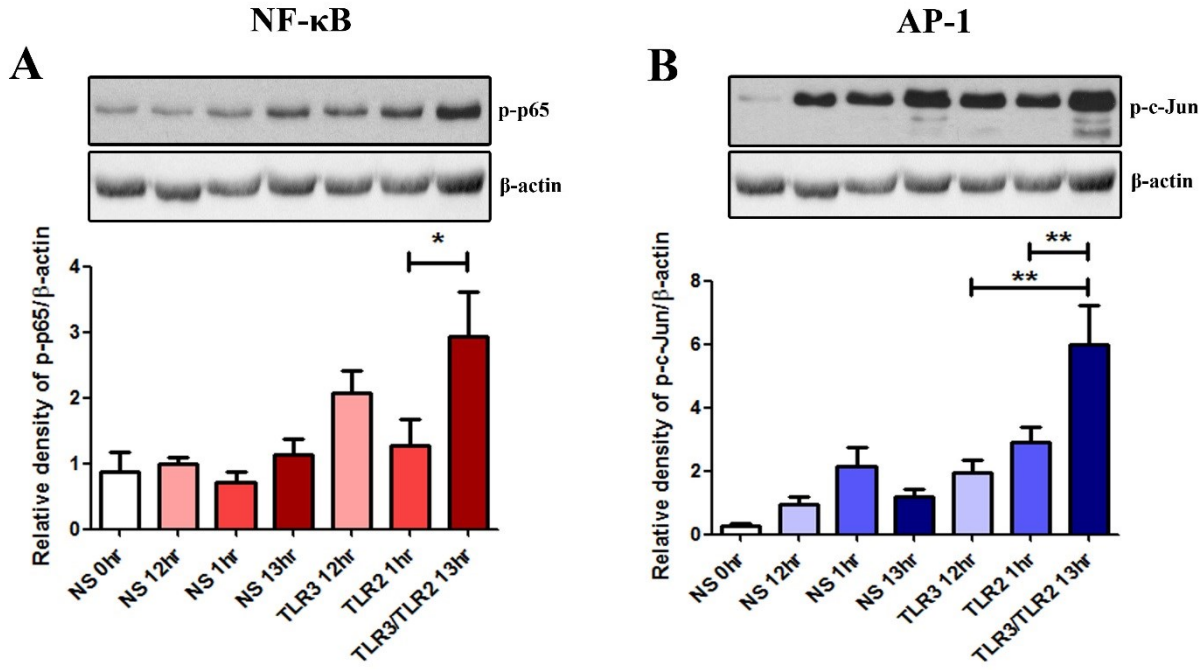


Figure 4.25 The effect of TLR3 priming before TLR2 agonist stimulation on NF-κB and AP-1 in amniocytes. Confluent monolayer amniocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 10^5 cells/ml of HKLM stimulation for 1 hour. Protein was extracted, and Western immunoblotting was performed to determine the activation status of serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1, respectively. A representative blot is shown above each graph with β-actin as a loading control. In **(A)** NF-κB, TLR3 priming prior to subsequent TLR2 agonist stimulation showed no augmentation compared to TLR3 agonist stimulation but there is an augmentation compared to TLR2 agonist stimulation (*p<0.05). **(B)** AP-1 however, increased significantly compared to both of the individual agonist alone (**p<0.01). For statistical analysis One-way ANOVA with *Bonferroni*'s multiple comparison test was used. n=5-6.

4.2.3.2 Pro-inflammatory cytokine production is not augmented with TLR3/2 priming

Since the TLR3/2 priming increased AP-1 activation, the effect on pro-inflammatory and pro-labour mediators listed in section 4.3.2 was quantified. Only 5 pro-inflammatory cytokines were detected in the samples with IFN-γ, TNF-β, IL-12p40 and IL-12p70 being below the detection level.

IL-1α (Figure 4.26 A) and IL-1β (Figure 4.26 B) production showed no difference with poly I:C or HKLM stimulation alone or with priming compared to the basal level.

No change was observed in the vehicle control over time. Poly I:C stimulation alone significantly increased IL-6 (***p<0.001 at 12 and 24 hours) (Figure 4.26 C), IL-8 (**p<0.01 at 12 and 24 hours) (Figure 4.26 D) and TNF-α (*p<0.05 at 12 hours and **p<0.01 at 24 hours) (Figure 4.26 E) production compared to 0hr. No increase was observed with HKLM compared to vehicle control and no augmentation was observed with TLR3/2 priming.

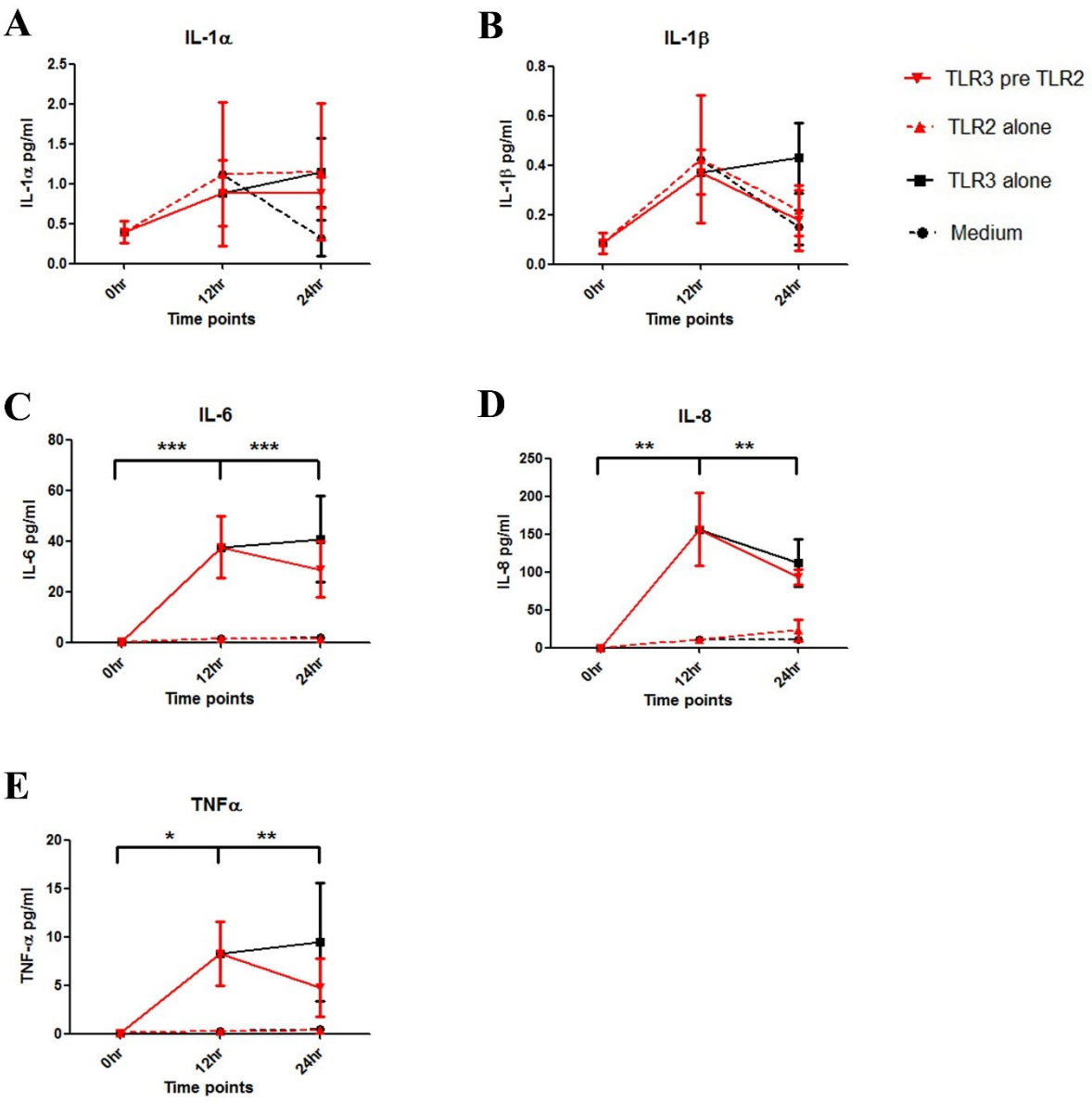


Figure 4.26 The effect of TLR3 priming prior to TLR2 agonist stimulation on pro-inflammatory cytokines production in amniocytes. Confluent monolayer amniocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 10^5 cells/ml of HKLM stimulation for 24 hours. Supernatant was collected and neat samples were used to run ELISA to determine the level of the cytokines production. TLR3 stimulation significantly increased the production of IL-6, IL-8 and TNF-α. However, no further augmentation was observed with TLR3 priming prior to TLR2 agonist stimulation on **(A)** IL-1α, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-8 or **(E)** TNF-α. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. **p*<0.05 **=** effect of TLR3 compared to 0hr.

4.2.3.3 Chemokine production is not augmented with TLR3/2 priming

Three different chemokines were quantified; MIP-1α, MIP-1β using neat samples and RANTES using 1 in 10 dilution.

No change was observed in chemokine production compared to basal production with HKLM stimulation alone. Poly I:C stimulation alone significantly increased MIP-1α (**p<0.01 at 12 and 24 hours) (Figure 4.27 A), MIP-1β (*p<0.05 at 12 and **p<0.01 at 24 hours) (Figure 4.27 B) and RANTES (**p<0.01 at 24 hours) (Figure 4.27 C) production compared to 0hr. No augmentation of the chemokines was observed with TLR3/2 priming.

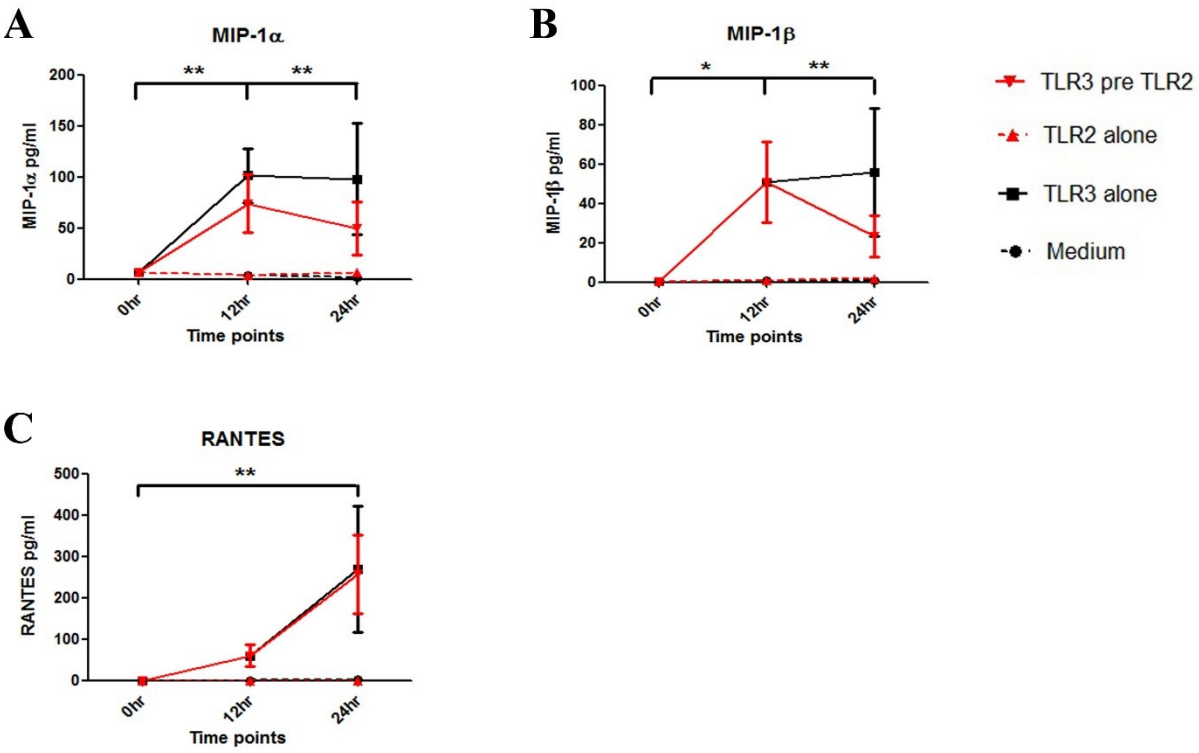


Figure 4.27 The effect of TLR3 priming prior to TLR2 agonist stimulation on chemokines production in amniocytes. Confluent monolayer amniocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 10^5 cells/ml of HKLM stimulation for 24 hours. Supernatants were collected and were used to run ELISA to determine the level of the chemokines production. TLR3 stimulation significantly increased the production of MIP-1α, MIP-1β and RANTES. No augmentation was observed with TLR3 priming before TLR2 agonist stimulation in **(A)** MIP-1α, **(B)** MIP-1β or **(C)** RANTES. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. *p<0.05 **=** effect of TLR3 compared to 0hr.

4.2.3.4 Anti-inflammatory cytokine production is not augmented with TLR3/2 priming

Five different anti-inflammatory cytokines, namely IL-4, IL-10, IL-1RA, IFN-α and IFN-β, were analysed. Neat samples were used to quantify these cytokines, however, only IL-4 and IL-1RA were detectable in all the samples.

HKLM stimulation did not increase the IL-4 production which stayed at the vehicle control level at all time points. Poly I:C stimulation alone significantly increased IL-4 production compared to 0hr however, no augmentation was seen with TLR3/2 priming (Figure 4.28 A).

No change in IL-1RA production was seen with poly I:C or HKLM stimulation alone, or with priming (Figure 4.28 B).

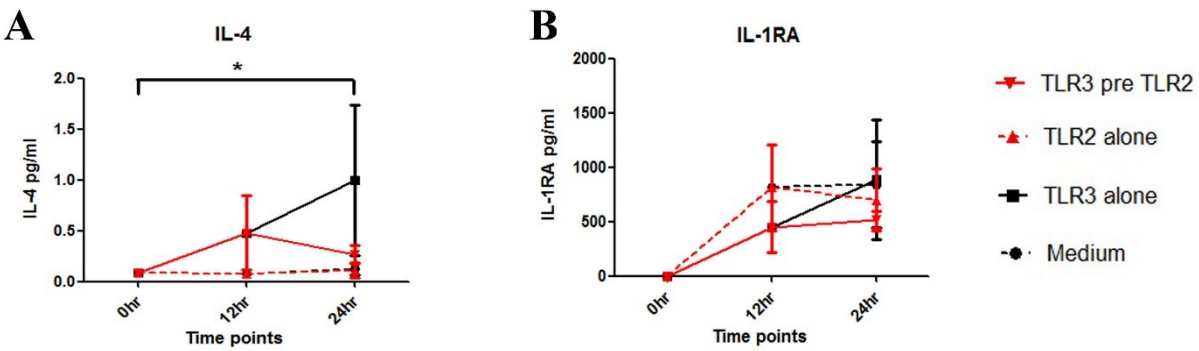


Figure 4.28 The effect of TLR3 priming prior to TLR2 agonist stimulation on anti-inflammatory IL-4 and IL-1RA production in amniocytes. Confluent monolayer amniocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 10^5 cells/ml stimulation for 24 hours. Supernatant was collected and neat samples were used to run ELISA to determine the level of IL-4 and IL-1RA production. TLR3 agonist stimulation increased the production of IL-4. TLR3 priming prior to TLR2 agonist stimulation however showed no further **(A)** IL-4 or **(B)** IL-1RA production. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. *p<0.05 **=** effect of TLR3 compared to 0hr.

4.2.3.5 PGE2 production is not augmented with TLR3/2 priming

PGE2 production was quantified and poly I:C, HKLM stimulation alone or priming did not increase the PGE2 production above the basal level (Figure 4.29).

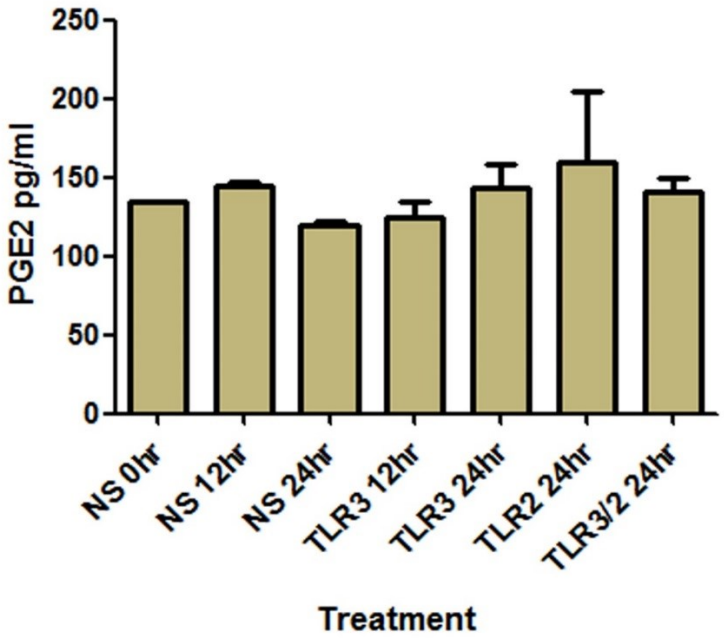


Figure 4.29 The effect of TLR3 priming prior to TLR2 agonist stimulation on PGE2 production in amniocytes. Confluent monolayer amniocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 10^5 cells/ml of HKLM stimulation for 24 hours. TLR3 priming prior to TLR2 agonist stimulation showed no augmentation of PGE2 production. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3.

4.3.4 Lipopolysaccharides from Escherichia coli 055:B5 (LPS 055) (TLR4 agonist) stimulation increases NF-κB and AP-1

To find the optimal concentration and time point for the priming experiment, the same concentrations of LPS were used as in previous experiments with VECs (section 4.2.4).

NF-κB activation increased significantly at 6 hours (*p<0.05) with the lowest concentration 0.1µg/ml (Figure 4.30 A). In contrast, earlier activation was observed with 1µg/ml at 30 minutes (*p<0.05) (Figure 4.30 B)

LPS stimulation at 0.1µg/ml significantly increased AP-1 at 1 hour (**p<0.01) (Figure 4.30 C). 1µg/ml of LPS also significantly increased AP-1 activation at 1 hour (**p<0.01) with higher fold change compared to 0.1µg/ml.

For priming, 0.1µg/ml of LPS at 6 hours was chosen to capture the peak activation of NF-κB.

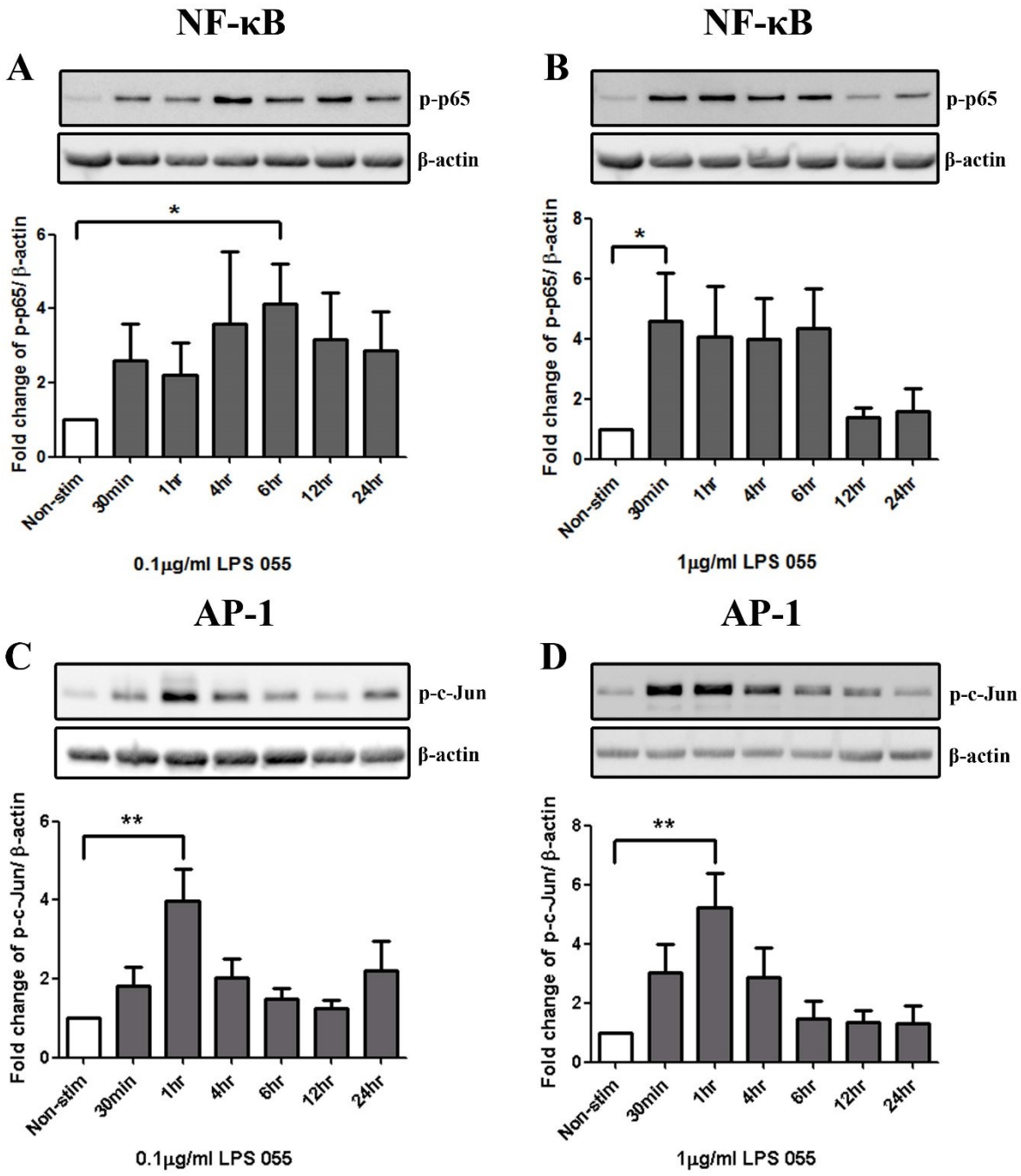


Figure 4.30 The effect of lipopolysaccharides from Escherichia coli 055:B5 (LPS 055) (TLR4 agonist) stimulation on NF-κB and AP-1 activation in amniocytes. Amniocytes were cultured and stimulated with 0.1μg/ml and 1μg/ml of LPS 055 for a duration of 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. NF-κB increased significantly with **(A)** 0.1μg/ml of LPS 055 at 6 (**p*<0.05) hours and with **(B)** 1μg/ml at 30 minutes (**p*<0.05). AP-1 also increased significantly with **(C)** 0.1μg/ml of LPS 055 at 1hour (***p*<0.01) and **(D)** 1μg/ml of LPS 055 at 1 hour (***p*<0.01). Statistical analysis was performed using One-way ANOVA for normally distributed data and Kruskal-Wallis for data not normally distributed with *Dunnett's* multiple comparison test. n=4-6.

4.3.5 TLR3/TLR4 priming in amniocytes

Based on the time course and dose response experiment in sections 4.3.1 and 4.3.4, 25μg/ml of poly I:C and 0.1μg/ml of LPS were chosen for the priming experiment. The same concentration as in VECs was used in order to be able to compare and contrast the outcome seen in amniocytes with other cell types. Cells were primed with poly I:C for 12 hours before a subsequent LPS 055 stimulation for 6 hours. Protein was extracted and analysed using Western immunoblotting to examine the activation status of NF-κB and AP-1.

For cytokine, chemokine and PGE2 studies, cells were primed with poly I:C for 12 hours and then stimulated with LPS 055 for 24 hours. An ELISA was used to quantify the analytes.

4.2.5.1 NF-κB and AP-1 activation are not augmented with TLR3/4 priming

Poly I:C stimulation alone did not result in significant NF-κB activation compared to the basal level. A decreased fold change was also noted compared to the NF-κB activation demonstrated in section 4.8.2 possibly due to differences shown by the biological replicates. A high basal level (5-fold change) at 6 hours could be the reason no statistical significance was observed with LPS stimulation. TLR3/4 priming only showed augmentation compared to poly I:C stimulation alone but not compared with LPS, suggesting in fact that poly I:C did not have an additive effect on TLR4 mediated NF-κB activation. (Figure 4.31 A).

No significant difference was observed with poly I:C and LPS stimulation on AP-1 activation which could be due to the variation in response and high fold change in the basal AP-1 activation level at 6 and 12 hours compared with 0hr. TLR3/4 priming showed no further augmentation of AP-1 activation (Figure 4.31 B).

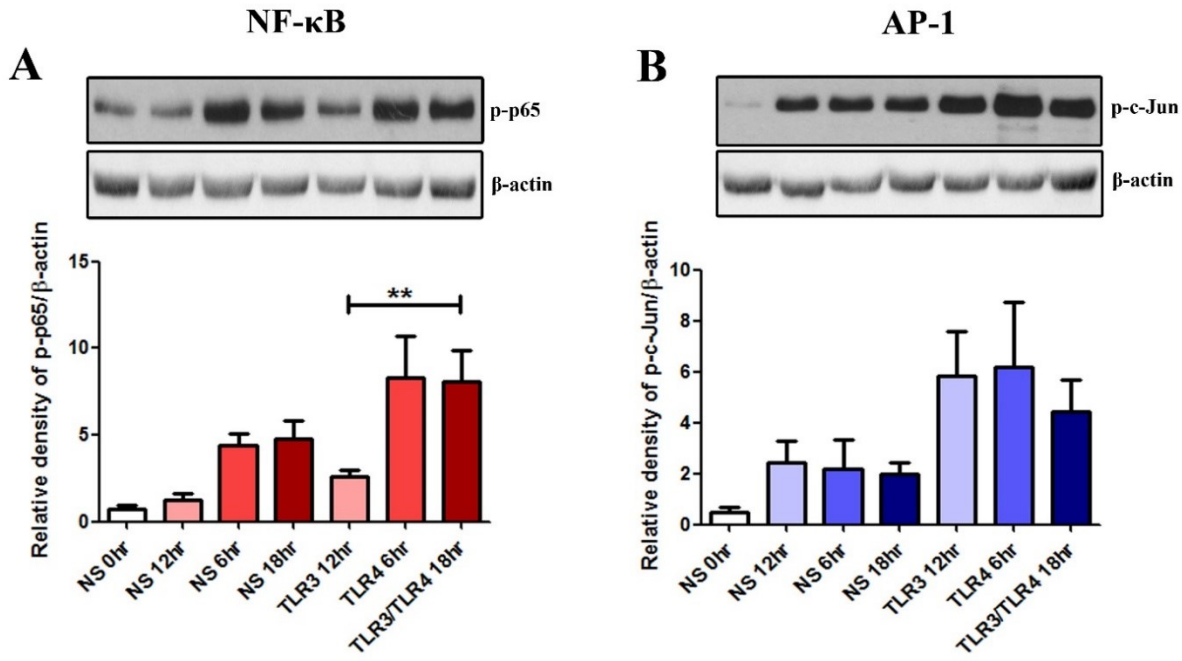


Figure 4.31 The effect of TLR3 priming before TLR2 agonist stimulation on NF-kB and AP-1 in amniocytes. Confluent monolayer amniocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 0.1μg/ml of LPS 055 stimulation for 6 hours. Protein was extracted, and Western immunoblotting was performed to determine the activation status of serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1, respectively. A representative blot is shown above each graph with β-actin as a loading control. In **(A)** NF-κB, although a significant increase was observed when compared to TLR3 agonist stimulation alone (**p<0.01), no further augmentation was observed with TLR4 agonist stimulation and no further augmentation was observed in **(B)** AP-1 with TLR3 priming prior to subsequent TLR4 stimulation. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3.

4.2.5.2 IL-6 and TNF-α are augmented with TLR3/4 priming

Although no significant augmentation in NF-κB and AP1 was seen with TLR3/4 priming, it is plausible that other transcription factors could be activated. Therefore, the same pro-inflammatory cytokines as listed in section 4.3.2 were examined in this section. IFN-γ, TNF-β, IL-12p40 and IL-12p70 were below the detection level, therefore these data were not shown.

IL-1α (Figure 4.32 A) and IL-1β (Figure 4.32 B) production showed no difference with poly I:C or LPS stimulation alone, or with priming compared to the basal level.

Poly I:C stimulation alone increased the production of IL-6 (***p<0.001 at 12 and 24 hours) and TNF-α (**p<0.01 at 6 hours) compared to 0hr. IL-1β, IL-8 ($^{\dagger\dagger}p$<0.01 at 24 hours) (Figure 4.32 C) and TNF-α ($^{\dagger}p$<0.05 at 24 hours) production increased significantly with LPS stimulation alone compared to 0hr. Priming with poly I:C prior to LPS stimulation augmented IL-6 ($^{\S\S\S}p$<0.001) (Figure 4.32 D) and TNF-α ($^{\S\S}p$<0.01) (Figure 4.32 E) when compared to LPS stimulation alone.

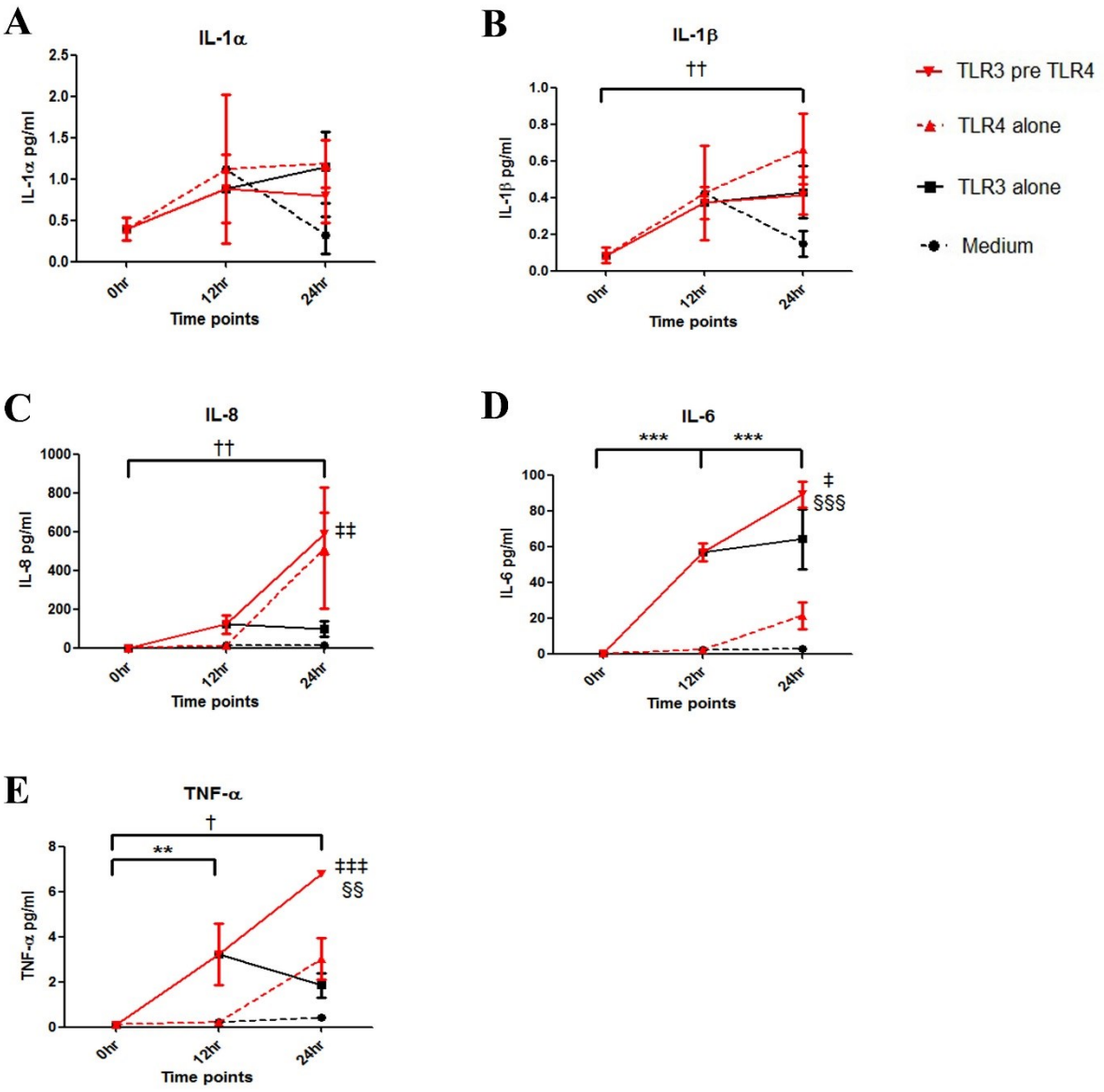


Figure 4.32 The effect of TLR3 priming prior to TLR4 agonist stimulation on pro-inflammatory cytokines production in amniocytes. Confluent monolayer amniocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 0.1μg/ml of LPS 055 stimulation for 24 hours. Supernatant was collected and neat samples were used to run ELISA to determine the level of the cytokines production. TLR3 and TLR4 agonist stimulation alone significantly increased the production of the cytokines. TLR3 priming prior to TLR4 agonist stimulation showed no augmentation of **(A)** IL-1α, **(B)** IL-1β and **(C)** IL-8. However, further augmentation was observed with **(D)** IL-6 and **(E)** TNF-α. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. $^{**}p<0.01$ **=** effect of TLR3 compared to 0hr. $^{\dagger}p<0.05$ **=** effect of TLR4 compared to 0hr. $^{\ddagger}p<0.05$ = effect of TLR3 pre TLR4 compared to TLR3 alone. $^{\S\S}p<0.01$ = effect of TLR3 pre TLR4 compared to TLR4 alone.

4.2.5.3 Chemokine production is not augmented with TLR3/4 priming

The chemokines MIP-1α, MIP-1β were also quantified neat and RANTES with a 1 in 10 dilution.

A significant increase in the production of MIP-1α ($^{\dagger\dagger}p<0.01$ at 24 hours) (Figure 4.33 A) was seen with LPS stimulation alone, and MIP-1β (Figure 4.33 B) and RANTES (Figure 4.33 C) ($^{*}p<0.05$) with poly I:C stimulation alone compared to 0hr. TLR3/4 priming showed no augmented production of these chemokines.

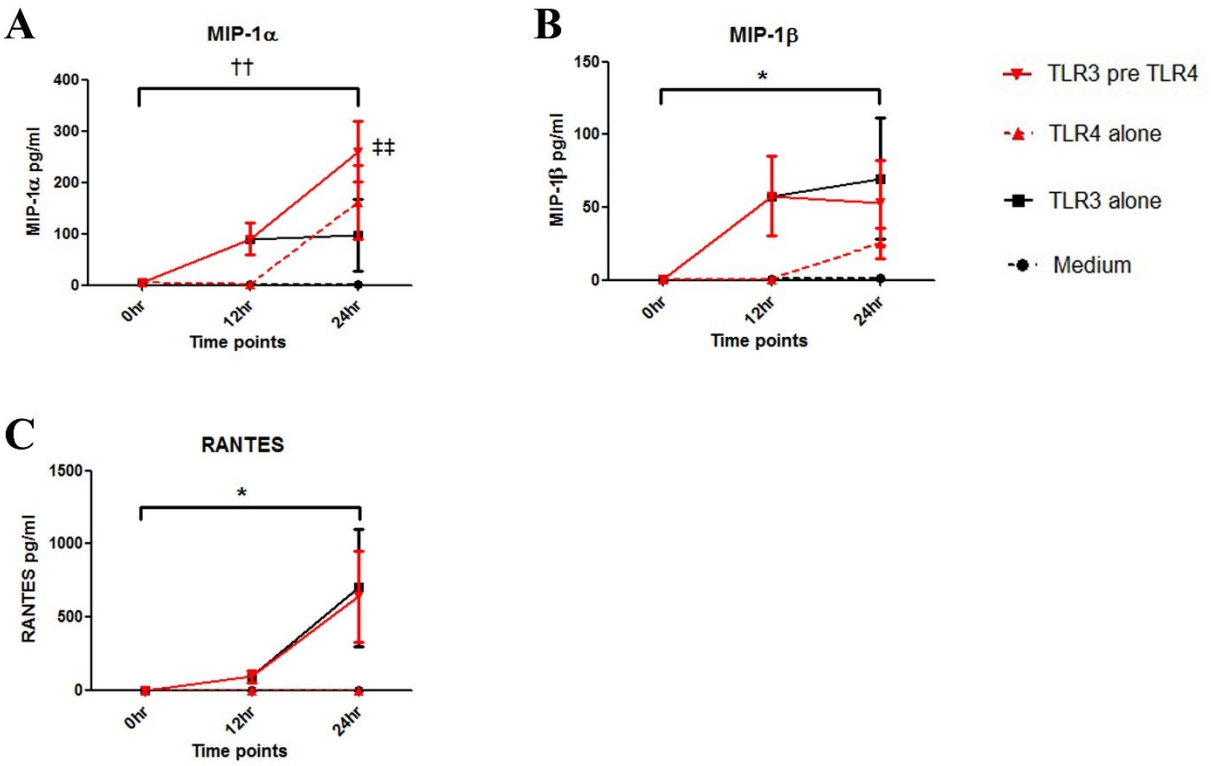


Figure 4.33 The effect of TLR3 priming prior to TLR4 agonist stimulation on chemokines production in amniocytes. Confluent monolayer amniocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 0.1μg/ml of LPS 055 stimulation for 24 hours. Supernatants were collected and were used to run ELISA to determine the level of the chemokines production. TLR3 and TLR4 agonist stimulation increased the production of MIP-1α, MIP-1β and RANTES. Priming with TLR3 before TLR4 agonist stimulation however did not further increase the levels of **(A)** MIP-1α, **(B)** MIP-1β or **(C)** RANTES. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. $^{*}p<0.05$ **=** effect of TLR3 compared to 0hr. $^{\dagger\dagger}p<0.01$ **=** effect of TLR4 compared to 0hr. $^{\ddagger\ddagger}p<0.01$ = effect of TLR3 pre TLR4 compared to TLR3 alone.

4.2.5.4 Anti-inflammatory cytokine production is not augmented with TLR3/4 priming

Neat samples were used to quantify anti-inflammatory cytokines IL-4, IL-10, IL-1RA, IFN-α and IFN-β, however, only IL-4 and IL-1RA were detectable in all the samples.

Only poly I:C stimulation significantly increased IL-4 (*p<0.05 at 24 hours) (Figure 4.34 A) production compared to 0hr. No further augmentation of IL-4 production was seen with TLR3/4 priming.

IL-1RA (Figure 4.34 B) production in the vehicle control showed a trend of increase over time. However, no difference was observed with poly I:C and LPS stimulation alone or with priming compared to the basal level.

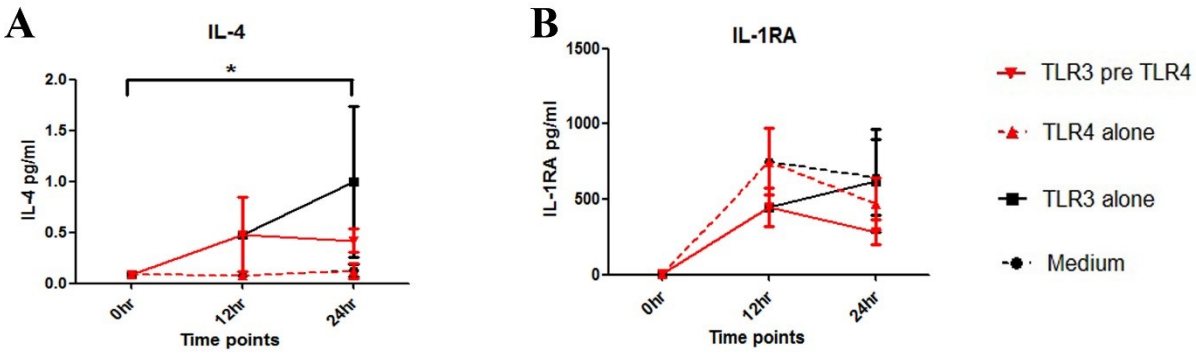


Figure 4.34 The effect of TLR3 priming prior to TLR4 stimulation on anti-inflammatory IL-4 and IL-1RA production in amniocytes. Confluent monolayer amniocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 0.1μg/ml of LPS 055 stimulation for 24 hours. Supernatant was collected and neat samples were used to run ELISA to determine the level of IL-4 and IL-1RA production. TLR3 agonist stimulation increased the production of IL-4. TLR3 priming prior to TLR4 agonist stimulation however, showed no further **(A)** IL-4 or **(B)** IL-1RA production. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. *p<0.05 **=** effect of TLR3 compared to 0hr.

4.2.5.5 PGE2 production is not augmented with TLR3/4 priming

Only LPS stimulation alone increased the production of PGE2 (**p<0.01) with no augmented response observed with TLR3/4 priming (Figure 4.35).

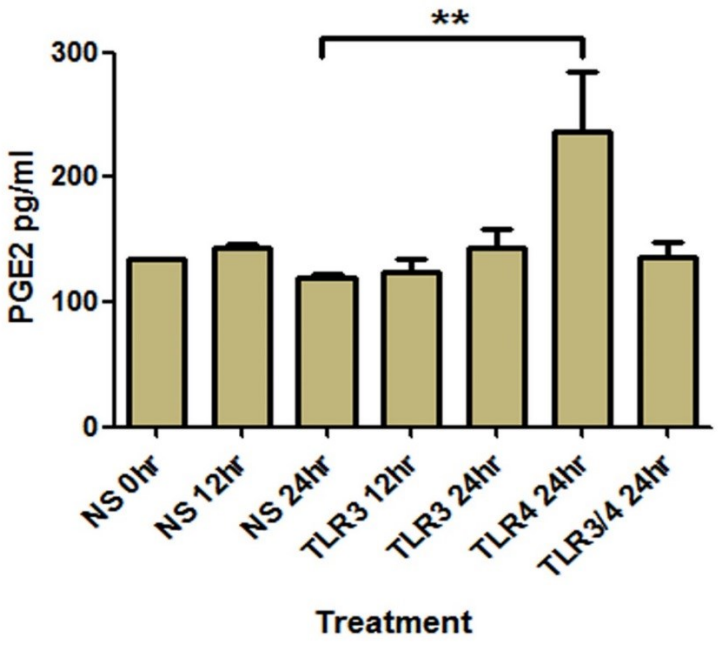


Figure 4.35 The effect of TLR3 priming prior to TLR4 agonist stimulation on PGE2 production in amniocytes. Confluent monolayer amniocytes were primed with 25µg/ml of poly I:C for 12 hours prior to 0.1µg/ml of LPS stimulation for 24 hours. TLR3 priming prior to TLR4 agonist stimulation showed no augmentation of PGE2 production. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3. **p<0.05 = effect of TLR4 compared to non-stimulated control.

4.3.6 Fibroblast–stimulating lipopeptide FSL-1 (TLR2/6 agonist) increases NF-κB and AP-1

A dose response and time course with 0.01µg/ml and 0.1µg/ml of FSL-1 were performed in amniocytes similarly as in the experiments performed in VECs (section 4.2.6).

FSL-1 stimulation at 0.01µg/ml increased NF-κB activation significantly at 30 minutes and 1 hour (*p<0.05) (Figure 4.36 A). A higher increase was observed at 1 hour with 0.1µg/ml (**p<0.01) (Figure 4.36 B).

For AP-1, 0.01µg/ml significantly increased the activation at 30 minutes (*p<0.05), 1 (**p<0.01), 4 and 6 hours (*p<0.05). A significant increase at 30 minutes (**p<0.01) and 1 hour (***p<0.001) was observed with 0.1µg/ml, the strength of response was greater than with 0.01µg/ml.

In order to achieve both NF-κB and AP-1 activation and to be consistent with the experiments in VECs, 0.01µg/ml was chosen to stimulate amniocytes at 1 hour in the priming experiments.

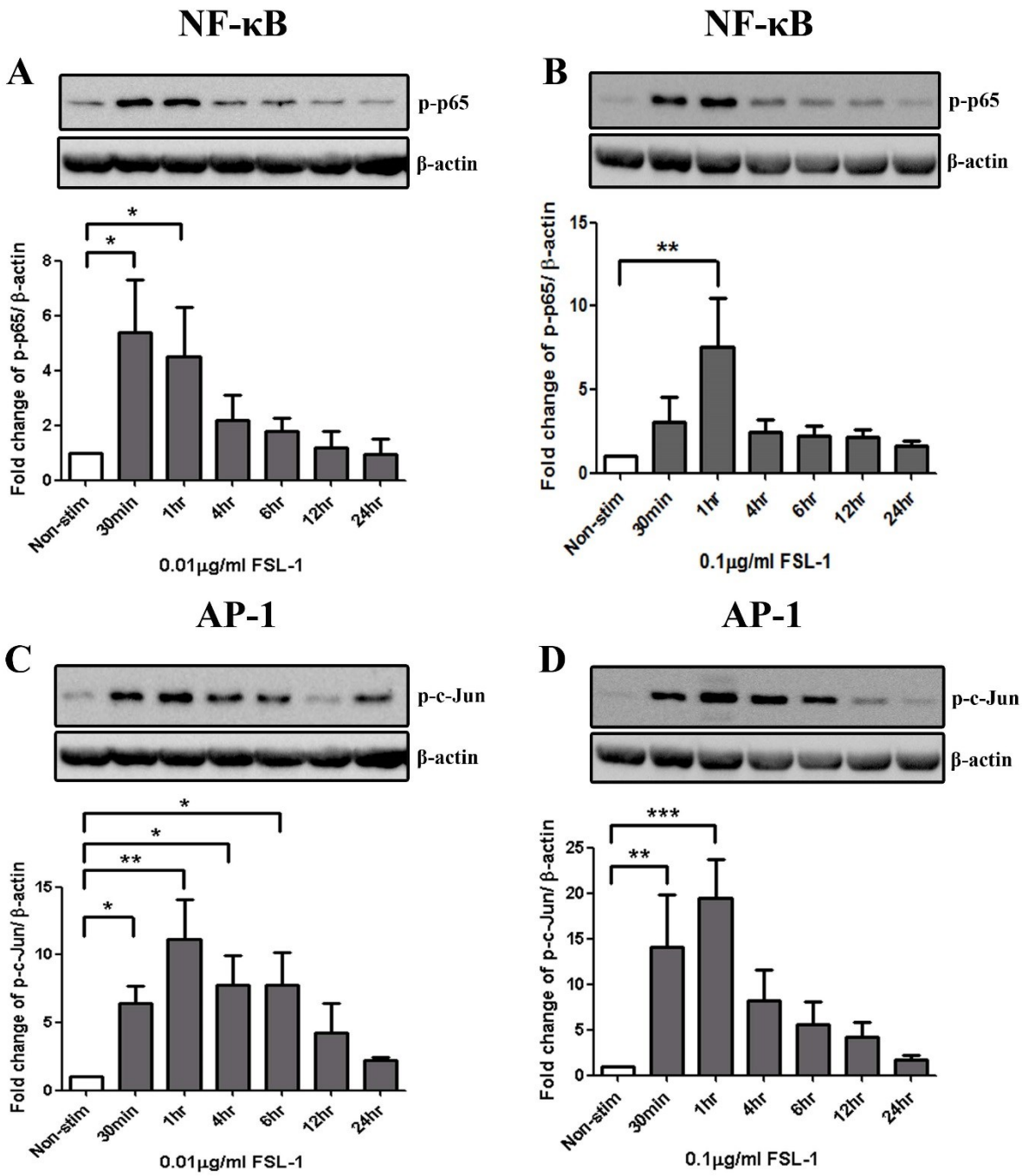


Figure 4.36 The effect of FSL-1, (TLR2/6 agonist) stimulation on NF-κB and AP-1 activation in amniocytes. Amniocytes were cultured and stimulated with 0.01μg/ml and 0.1μg/ml of FSL-1 for a duration of 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. NF-κB increased significantly with **(A)** 0.01μg/ml of FSL-1 at 30 minutes and 1 hour (*p<0.05) and **(B)** 0.1μg/ml of FSL-1 at 1 hour (**p<0.01). AP-1 also significantly increased with **(C)** 0.01μg/ml of FSL-1 at 30 minutes (*p<0.05), 1 (**p<0.01), 4 and 6 hours (*p<0.05) and **(D)** 0.1μg/ml of FSL-1 at 30 minutes (**p<0.01) and 1 hour (***p<0.001). For statistical analysis Kruskal-Wallis with *Dunnett's* multiple comparison test was used. n=3-4.

4.3.7 TLR3/TLR2/6 priming in amniocytes

Cells were primed with poly I:C for 12 hours before a subsequent 0.01μg/ml FSL-1 stimulation for 1 hour. Protein was extracted and analysed using Western immunoblotting to examine the activation status of NF-κB and AP-1.

For cytokine, chemokine and PGE2 studies, cells were primed with poly I:C for 12 hours and then stimulated with FSL-1 for 24 hours. An ELISA was used to quantify the analytes.

4.2.7.1 NF-κB and AP-1 activation are not augmented with TLR3/TLR2/6 priming

No difference in NF-κB activation was seen with poly I:C stimulation or FSL-1 stimulation. TLR3 priming prior to TLR2/6 stimulation only showed augmentation compared to TLR3 stimulation alone (*p<0.05) but no statistically significant difference compared to FSL-1 stimulation alone (Figure 4.37 A).

No significant difference was observed with poly I:C and FSL-1 stimulation alone on AP-1 activation. Priming showed no augmented AP-1 activation (Figure 4.37 B).

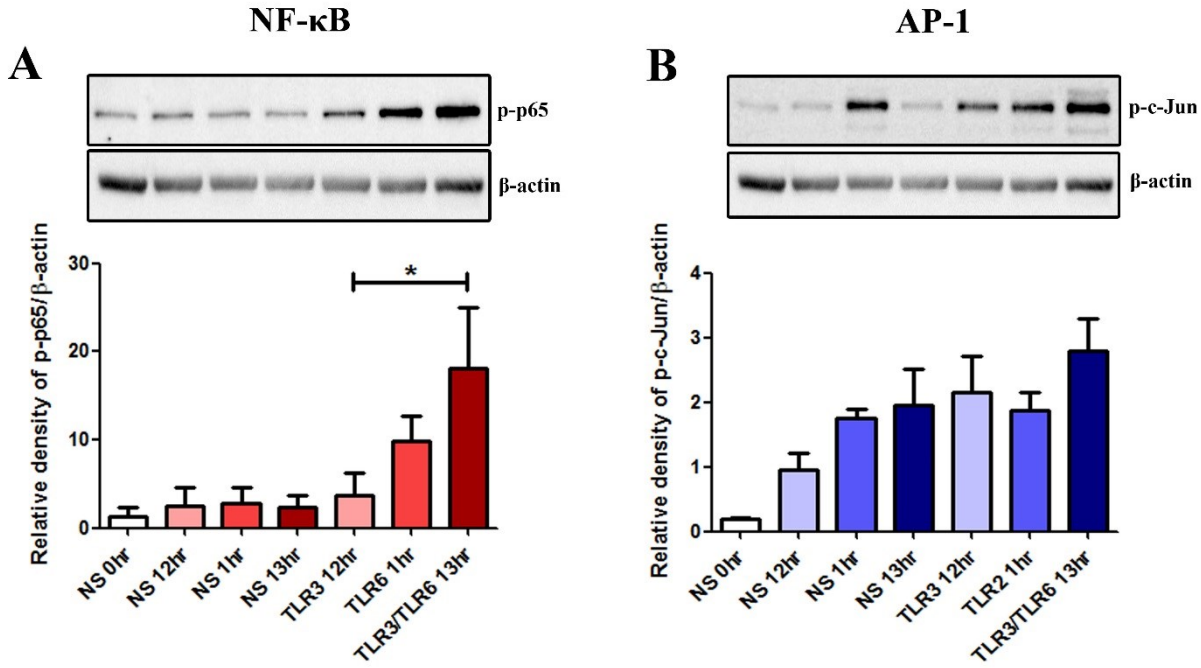


Figure 4.37 The effect of TLR3 priming before TLR2/6 agonist stimulation on NF-κB and AP-1 in amniocytes. Confluent monolayer amniocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 0.01μg/ml of FSL-1 stimulation for 1 hour. Protein was extracted, and Western immunoblotting was performed to determine the activation status of serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1, respectively. A representative blot is shown above each graph with β-actin as a loading control. In **(A)** NF-κB, although a significant increase was observed in TLR3 priming prior to TLR2/6 stimulation comparing to TLR3 stimulation alone (*p<0.05), no further augmentation was observed with TLR2/6 agonist stimulation and no further augmentation was observed in **(B)** AP-1 with TLR3 priming prior to subsequent TLR2/6 stimulation. Statistical analysis was performed using One-way ANOVA for normally distributed data and Kruskal-Wallis for data not normally distributed with *Dunnett's* multiple comparison test. n=3.

4.2.7.2 IL-6 is augmented with TLR3/TLR2/6 priming

The effect of TLR3 priming prior to TLR2/6 stimulation on pro-inflammatory and pro-labour mediators was determined. The same pro-inflammatory cytokines listed in section 4.3.2 were examined in this section. IFN-γ, TNF-β, IL-12p40 and IL-12p70 were below the detection level, therefore these data were not shown.

IL-1α (Figure 4.38 A) and IL-1β (Figure 4.38 B) production showed no difference with poly I:C or HKLM stimulation alone, or with priming compared to the basal level.

Poly I:C stimulation alone increased the production of IL-6 (***p<0.001 at 12 and 24 hours) (Figure 4.38 C) and TNF-α (**p<0.01 at 12 hours) (Figure 4.38 E) while FSL-1 only increased the production of IL-8 ($^{\dagger}p$<0.05 at 24 hours) (Figure 4.38 D) compared to 0hr. Priming with poly I:C before FSL-1 stimulation only augmented IL-6 ($^{\S\S\S}p$<0.001) production compared to FSL-1 stimulation alone.

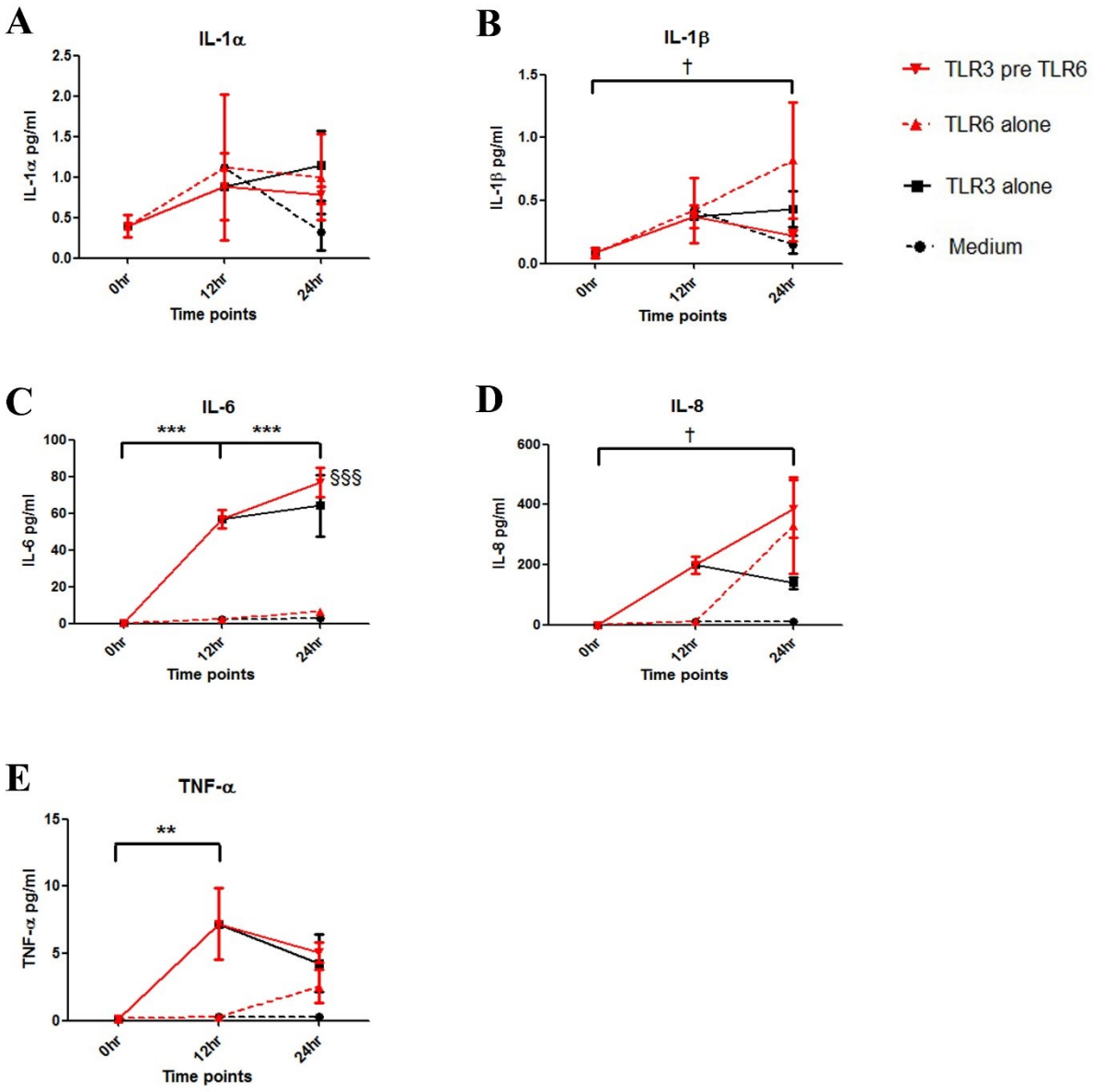


Figure 4.38 The effect of TLR3 priming prior to TLR2/6 agonist stimulation on pro-inflammatory cytokines production in amniocytes. Confluent monolayer amniocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 0.01μg/ml of FSL-1 stimulation for 24 hours. Supernatant was collected and neat samples were used to run ELISA to determine the level of the cytokines production. TLR3 and TLR2/6 agonist stimulation alone significantly increased the production of the cytokines. TLR3 priming prior to TLR2/6 agonist stimulation showed no augmentation of **(A)** IL-1α, **(B)** IL-1β and **(D)** IL-8 or **(E)** TNF-α. However, further augmentation was observed in **(C)** IL-6. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. $^{**}p<0.01$**=** effect of TLR3 compared to 0hr. $^{\dagger}p<0.05$ **=** effect of TLR2/6 compared to 0hr. $^{§§§}p<0.001$ = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone.

4.2.7.3 MIP-1α, MIP-1β and RANTES are augmented with TLR3/TLR2/6 priming

MIP-1α, MIP-1β using neat samples and RANTES with 1 in 10 dilutions were quantified after priming.

Poly I:C stimulation alone increased MIP-1α (*p<0.05 at 12 and 24 hours) and RANTES (*p<0.05 at 24 hours) production compared to 0hr. While the FSL-1 alone did not increase the chemokine production, priming with poly I:C prior to FSL-1 augmented MIP1-1α (Figure 4.39 A), MIP-1β ($^{§§}p$<0.01) (Figure 4.39 B) and RANTES ($^{§§§}p$<0.001) (Figure 4.39 C) compared to FSL-1 stimulation alone.

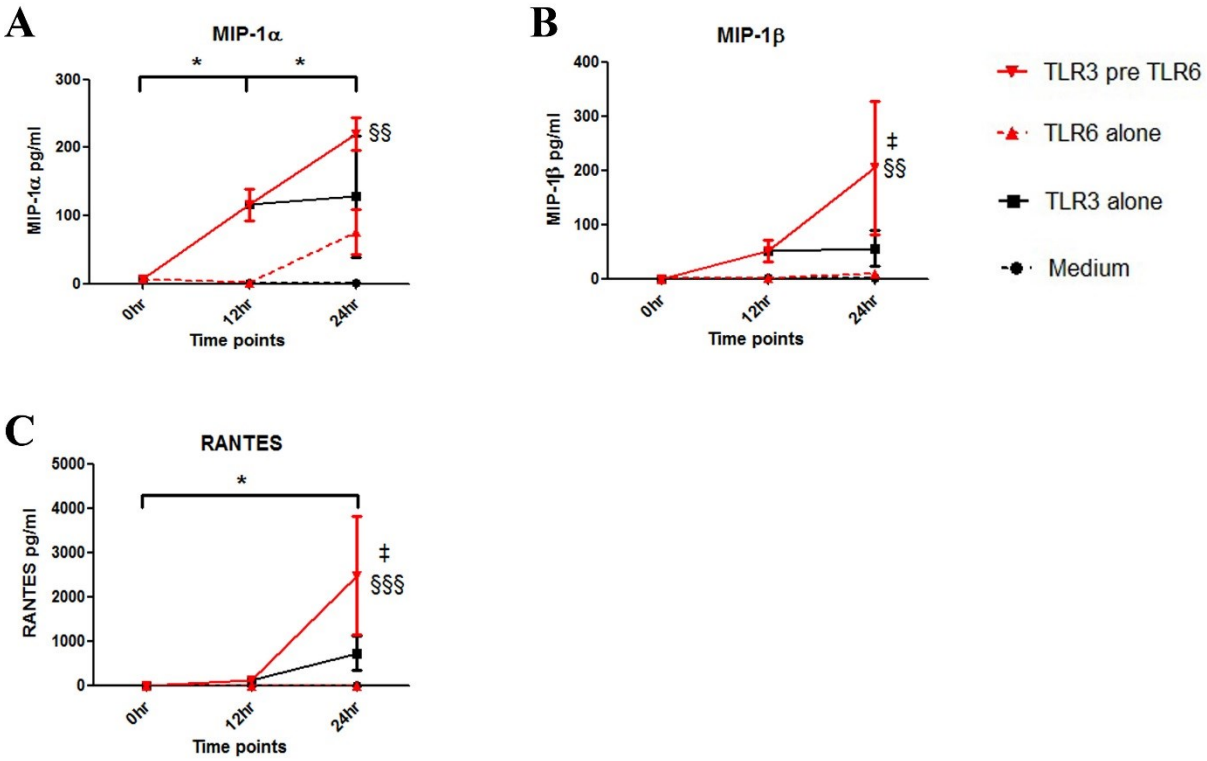


Figure 4.39 The effect of TLR3 priming prior to TLR2/6 agonist stimulation on chemokines production in amniocytes. Confluent monolayer amniocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 0.01μg/ml of FSL-1 stimulation for 24 hours. Supernatants were collected and were used to run ELISA to determine the level of the chemokines production. TLR3 stimulation significantly increased the production of MIP-1α and RANTES. Further augmentation of **(A)** MIP-1α, **(B)** MIP-1β and **(C)** RANTES was observed with TLR3 priming before TLR2/6 agonist stimulation. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. *p<0.05 **=** effect of TLR3 compared to 0hr. ‡p<0.05 = effect of TLR3 pre TLR2/6 compared to TLR3 alone. $^{§§}p$<0.01= effect of TLR3 pre TLR2/6 compared to TLR2/6 alone.

4.2.7.4 Anti-inflammatory cytokine production is not augmented with TLR3/TLR2/6 priming

Despite neat samples being used to quantify the cytokines only IL-4 and IL-1RA were detectable in all the samples.

Poly I:C stimulation significantly increased IL-4 (*p<0.05 at 24 hours) (Figure 4.40 A) production compared to 0hr. No further augmentation of IL-4 production with TLR3/TLR2/6 priming.

No difference in IL-1RA (Figure 4.40 B) was observed with poly I:C or FSL-1 stimulation alone or with priming compared to the basal level.

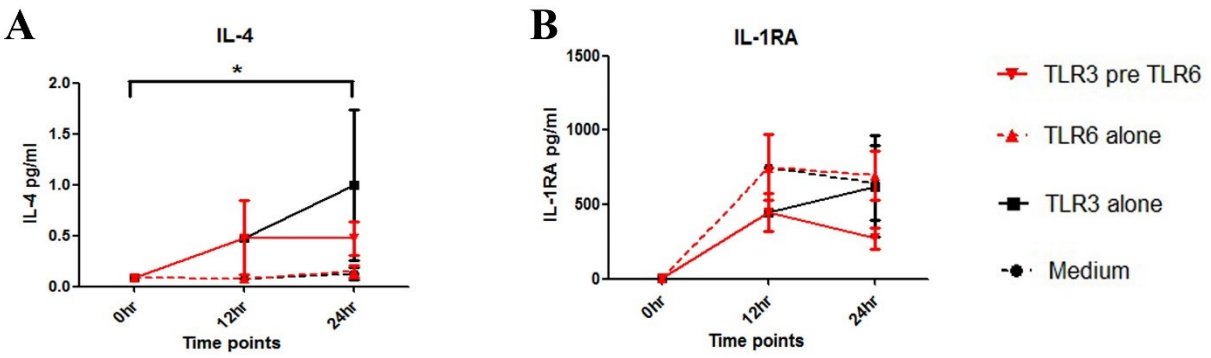


Figure 4.40 The effect of TLR3 priming prior to TLR2/6 stimulation on anti-inflammatory IL-4 and IL-1RA production in amniocytes. Confluent monolayer amniocytes were primed with 25µg/ml of poly I:C for 12 hours prior to 0.01µg/ml of FSL-1 stimulation for 24 hours. Supernatant was collected and neat samples were used to run ELISA to determine the level of IL-4 and IL-1RA production. TLR3 agonist stimulation increased the production of IL-4. TLR3 priming prior to TLR2/6 agonist stimulation however showed no further **(A)** IL-4 or **(B)** IL-1RA production. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. *p<0.05 **=** effect of TLR3 compared to 0hr.

4.2.7.5 PGE2 production is augmented with TLR3/TLR2/6 priming

Poly I:C and FSL-1 stimulation alone showed no increase in PGE2 production compared to the basal level. Priming with poly I:C prior to FSL-1 stimulation augmented PGE2 production compared to the individual agonist stimulations alone (***p<0.001) (Figure 4.41).

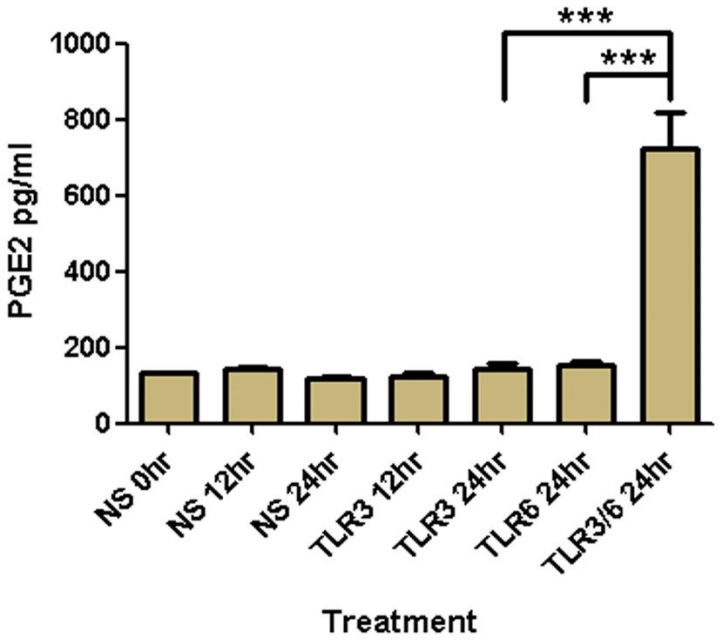


Figure 4.41 The effect of TLR3 priming prior to TLR2/6 agonist stimulation on PGE2 production in amniocytes. Confluent monolayer amniocytes were primed with 25µg/ml of poly I:C for 12 hours prior to 0.01µg/ml of FSL-1 stimulation for 24 hours. TLR3 priming prior to TLR2/6 agonist stimulation augmented PGE2 production compared to the individual TLR agonist stimulation alone. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3. ***p<0.001 = effect of TLR3 pre TLR2/6 compared to TLR3 and TLR2/6 alone.

4.3 Myocytes

4.3.1 Poly I:C (viral TLR3 agonist) stimulation increases NF-κB and AP-1

Priming experiments were also conducted in myocytes as a model of ascending infection model in preterm labour. Before carrying out the priming experiments, the optimal concentration and time points of poly I:C (viral TLR3 agonist) stimulation on NF-κB and AP-1 activation were determined.

Cells were cultured, and confluent monolayer cells were stimulated with 5μg/ml and 10μg/ml of poly I:C in a time course experiment for up to 24 hours. Poly I:C stimulation at 5μg/ml increased NF-κB activation at 24 hours (*p<0.05) (Figure 4.**Figure 4.**42 A) and 10μg/ml showed an increased at 12 and 24 hours (**p<0.01) (Figure 4.42 B). For AP-1, a significant increase was observed at the earlier time point of 30 minutes (**p<0.01) and a constant increase at 1, 4, 6, 12 until 24 hours (*p<0.05) with 5μg/ml (Figure 4.42 C). However, no statistical significance was achieved with 10μg/ml (Figure 4.42 D).

Since poly I:C stimulation increased TLR3 protein expression at 12 hours, a dose response experiment with poly I:C stimulation looking at the effect of NF-κB and AP-1 was performed at 12 hours. This time point was also chosen based on the increased NF-κB and AP-1 with the time course experiment. NF-κB increased significantly with 10μg/ml (*p<0.05), 25μg/ml (**p<0.01), 50μg/ml (***p<0.001) and 100μg/ml (***p<0.001) (Figure 4.43 A). Poly I:C also showed a dose dependent increase in AP-1 activation but it only reached statistical significance with 100μg/ml (*p<0.05) (Figure 4.43 B).

25μg/ml of poly I:C at 12 hours was chosen for priming experiment to obtain an increase on TLR3 expression (Chapter 3, section 3.3.3), NF-κB and AP-1 activation.

4.3.1.1 Poly I:C time course

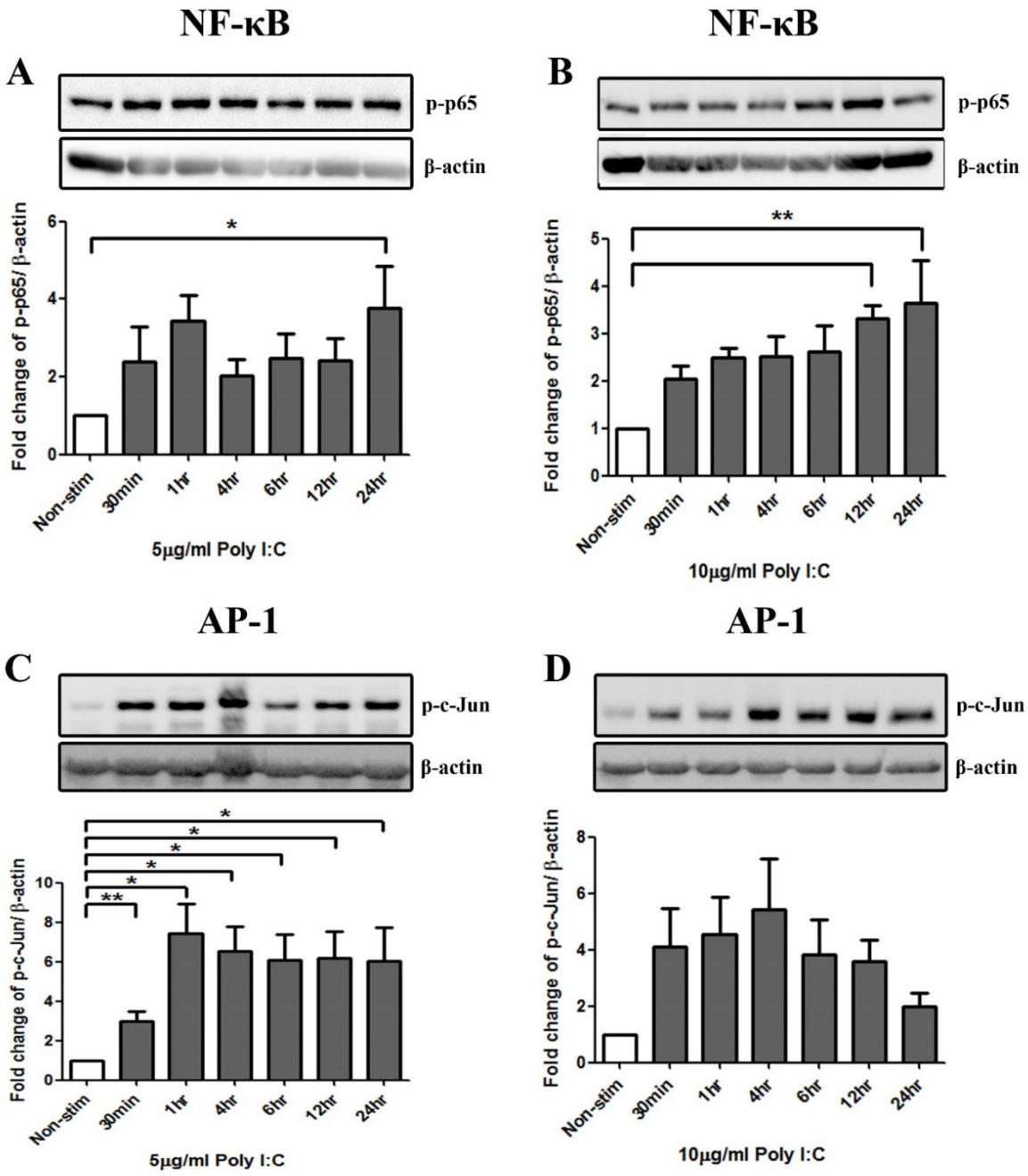


Figure 4.42 The effect of poly I:C, a viral TLR3 agonist stimulation on NF-κB and AP-1 activation in myocytes. Myocytes were cultured and stimulated with 5μg/ml and 10μg/ml of poly I:C for a duration of 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. NF-κB increased significantly with **(A)** 5μg/ml of poly I:C at 24 (**p*<0.05) hours and **(B)** 10μg/ml of poly I:C at 1, 4 and 6 (**p*<0.05) hours. AP-1 also increased significantly with **(C)** 5μg/ml of poly I:C at 30minutes (***p*<0.01), 1, 4, 6, 12 and 24 (**p*<0.05). Although an increase was observed with **(D)** 10μg/ml of poly I:C, no statistical significance was achieved. For statistical analysis One-way ANOVA with *Dunnett's* multiple comparison test was used. n=6-8.

4.3.1.2 Poly I:C dose response at 12 hours

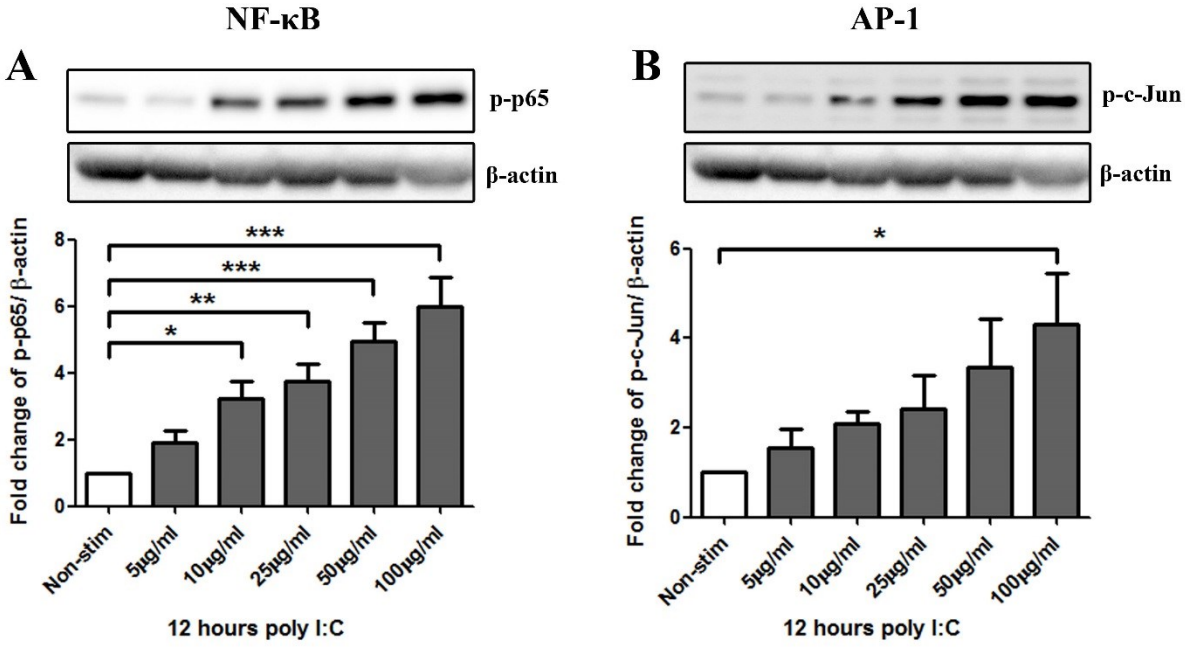


Figure 4.43 The effect of different doses of poly I:C, a viral TLR3 agonist stimulation on NF-κB and AP-1 activation at 12 hours in myocytes. Myocytes were cultured and stimulated with 5μg/ml, 10μg/ml, 25μg/ml, 50μg/ml and 100μg/ml of poly I:C for 12 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. **(A)** NF-κB increased significantly with 10μg/ml (*p<0.05), 25μg/ml (**p<0.01), 50μg/ml and 100μg/ml (***p<0.001) of poly I:C stimulation. **(B)** AP-1 on the other hand showed an increasing trend but only reached significance at 100μg/ml (*p<0.05) of poly I:C stimulation at 12 hours. For statistical analysis One-way ANOVA with *Dunnett's* multiple comparison test was used. n=5-6.

4.3.2 Heat-killed *Listeria monocytogenes* (HKLM) (TLR2 agonist) stimulation increases NF-κB and AP-1

Before performing TLR3/2 priming in myocytes, the effect of HKLM stimulation on NF-κB and AP-1 transcription factors was examined. The same concentrations of HKLM were used as with VECs and amniocytes. Western immunoblotting was performed and phosphorylated p-65 (p-p65) and phosphorylated c-Jun (p-c-Jun) were used as markers of NF-κB and AP-1 activation.

NF-κB increased significantly at 1 hour with 10^5 cells/ml (*p<0.05) (Figure 4.44 A) HKLM while no significant difference was observed with 10^6 cells/ml (Figure 4.44 B). For AP-1, 10^5 cells/ml increased the activation of this protein at 1 and 4 hours (*p<0.05) (Figure 4.44 C) and at 30 minutes, 1 and 4 hours with 10^6 cells/ml (*p<0.05) (Figure 4.44 D).

For the priming experiment, 10^5 cells/ml of HKLM at 1 hour was chosen to obtain an increase in NF-κB and AP-1 activation, as well as being consistent with the dosing of VECs and amniocytes.

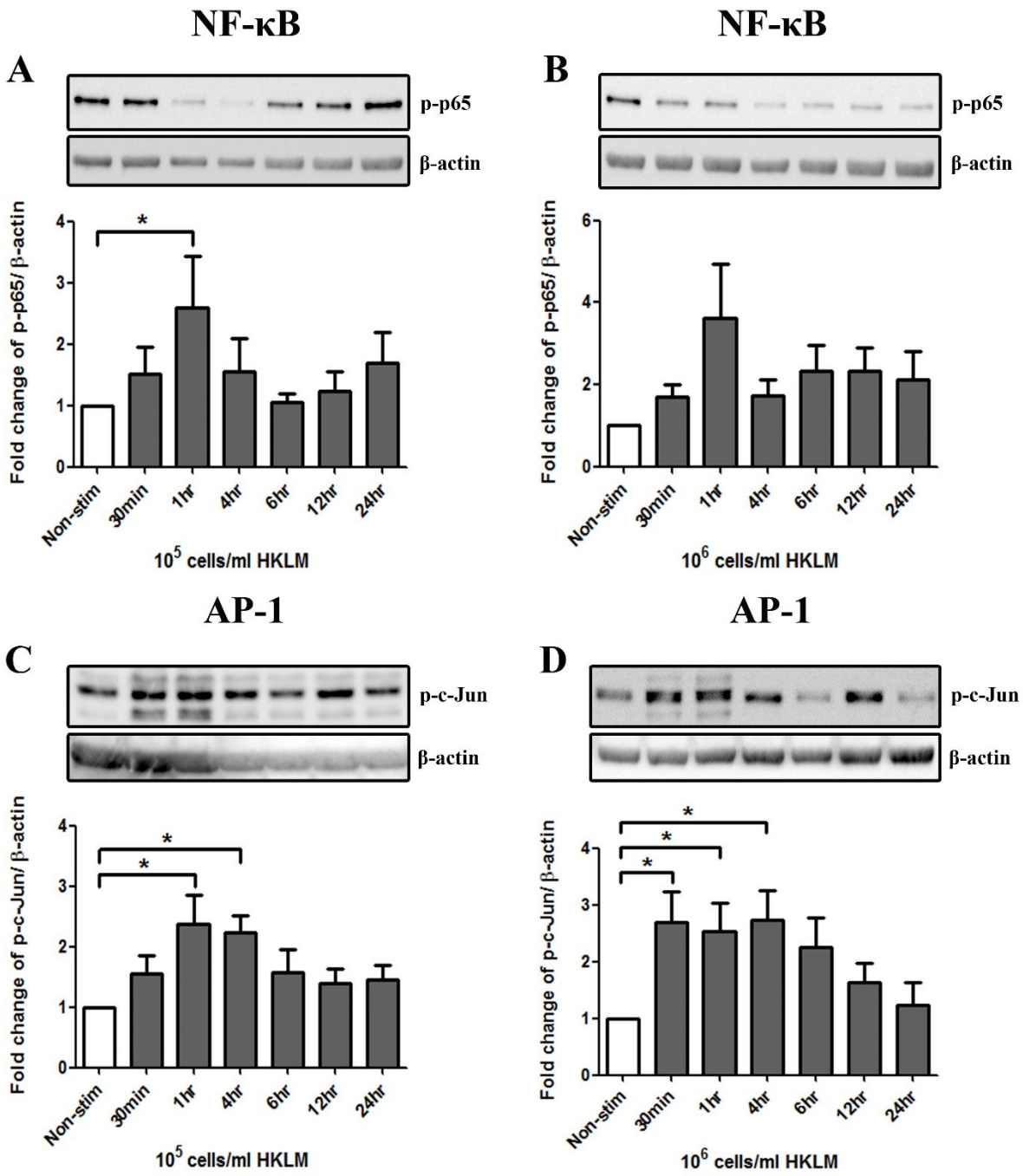


Figure 4.44 The effect of Heat-killed *Listeria monocytogenes* (HKLM), a TLR2 agonist stimulation on NF-κB and AP-1 activation in myocytes. Myocytes were cultured and stimulated with 10^5 and 10^6 cells/ml of HKLM for a duration of 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. NF-κB increased significantly with **(A)** 10^5 cells/ml of HKLM at 1 hour (*p<0.05) while although increased was observed with **(B)** 10^6 cells/ml, no statistically significant was achieved. AP-1 also increased significantly with **(C)** 10^5 cells/ml at 1 and 4 hours (*p<0.05) and **(D)** 10^6 cells/ml at 30 minutes, 1 and 4 hours (*p<0.05). For statistical analysis One-way ANOVA with *Dunnett's* multiple comparison test was used. n=6-8.

4.3.3 TLR3/TLR2 priming in myocytes

The same concentration of poly I:C (25μg/ml) and HKLM (10^5 cells/ml) was used as in section 4.3.3 to treat myocytes. Myocytes were primed with poly I:C for 12 hours before subsequent HKLM stimulation for 1 hour to examine the activation status of NF-κB and AP-1 using Western immunoblotting.

For cytokine, chemokine and PGE2 studies, cells were primed with poly I:C for 12 hours and then stimulated with HKLM for 24 hours. An ELISA was used to quantify the analytes.

4.3.3.1 NF-κB and AP-1 activation are not augmented with TLR3/2 priming

Poly I:C and HKLM stimulation alone showed no statistically significant increase in NF-κB activation. Priming with poly I:C prior to HKLM stimulation did not augment the NF-κB response compared to the stimulation with individual agonists (Figure 4.45 A).

No significant increase was seen in AP-1 activation (Figure 4.45 B) with poly I:C or HKLM treatment alone or with priming.

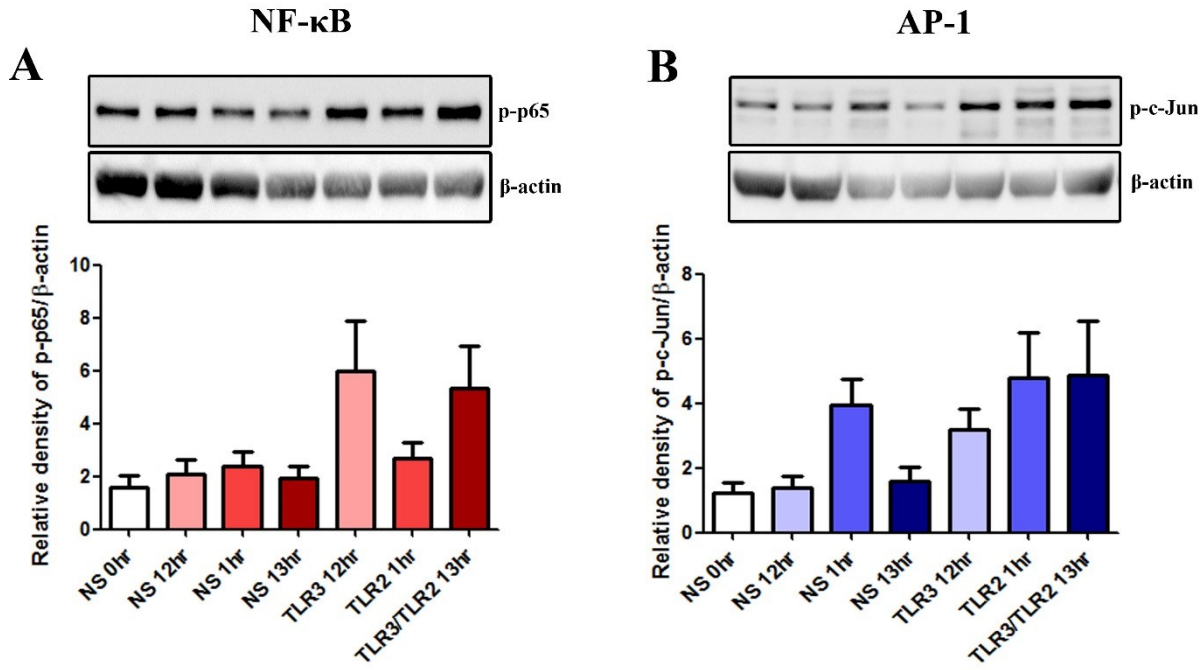


Figure 4.45 The effect of TLR3 priming before TLR2 agonist stimulation on NF-kB and AP-1 in myocytes. Confluent monolayer myocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 10^5 cells/ml of HKLM stimulation for 1 hour. Protein was extracted, and Western immunoblotting was performed to determine the activation status of serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1, respectively. A representative blot is shown above each graph with β-actin as a loading control. TLR3 priming prior to subsequent TLR2 stimulation showed no augmentation of **(A)** NF-κB or **(B)** AP-1. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used. n=4.

4.3.3.2 Pro-inflammatory cytokine production is not augmented with TLR3/2 priming

The effect of TLR3 priming on TLR2 stimulated cytokine production was examined. The cytokines listed in section 4.3.2 were quantified. Cytokines were measured in a 1:5 dilution. IL-1α, IFN-γ, TNF-β, IL-12p40 and IL-12p70 were below the detection level.

Poly I:C stimulation alone significantly increased the production of IL-1β (**p<0.01 at 12 hours and ***p<0.001 at 24 hours) (Figure 4.46 A), IL-6 (Figure 4.46 B), IL-8 (Figure 4.46 C) and TNF-α (Figure 4.46 D) (***p<0.001 at 12 and 24 hours) compared to 0hr. HKLM stimulation showed no increase in cytokine production compared to the vehicle control. TLR3/2 priming showed no augmentation of any of the cytokines.

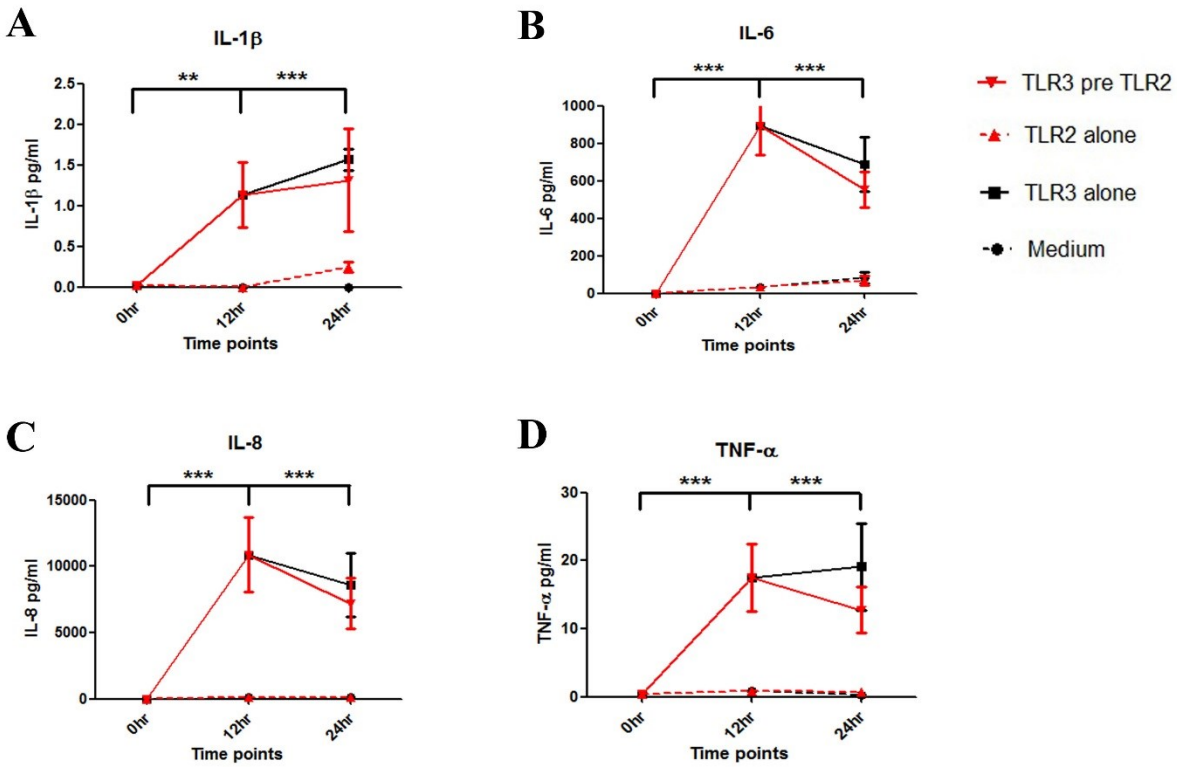


Figure 4.46 The effect of TLR3 priming prior to TLR2 agonist stimulation on pro-inflammatory cytokines production in myocytes. Confluent monolayer myocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 10^5 cells/ml of HKLM stimulation for 24 hours. Supernatant was collected and 1 in 5 dilutions were used to run ELISA to determine the level of the cytokine production. TLR3 stimulation significantly increased the production of IL-1β, IL-6, IL-8 and TNF-α. However, no further augmentation was observed with TLR3 priming prior to TLR2 agonist stimulation on **(A)** IL-1β, **(B)** IL-6, **(C)** IL-8 or **(D)** TNF-α. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=4. ***p*<0.01**=** effect of TLR3 compared to 0hr.

4.3.3.3 Chemokine production is not augmented with TLR3/2 priming

The same supernatant above (1 in 5 dilution) was used to quantify MIP-1α, MIP-1β as well as RANTES (1 in 10 dilution). MIP-1α, MIP-1β value were below the detection limit, but RANTES was within the detection range.

RANTES production in cells treated with HKLM was no different to vehicle control samples. Poly I:C stimulation alone increased the production of RANTES significantly at 12 and 24 hours (***p<0.001) compared to 0hr. No augmented production was observed with TLR3/2 priming (Figure 4.47).

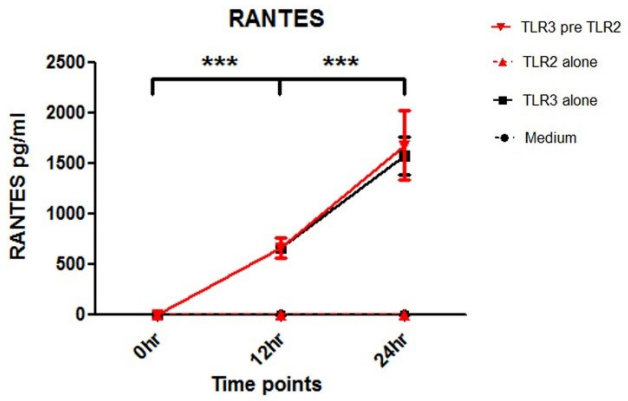


Figure 4.47 The effect of TLR3 priming prior to TLR2 agonist stimulation on chemokines production in myocytes. Confluent monolayer myocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 10^5 cells/ml of HKLM stimulation for 24 hours. Supernatants were collected and were used to run ELISA to determine the level of the chemokines production. TLR3 stimulation significantly increased the production of RANTES. However, no further augmentation of RANTES was observed with TLR3 priming before TLR2 agonist stimulation. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=4. ***p<0.001 **=** effect of TLR3 compared to 0hr.

4.3.3.4 Anti-inflammatory cytokines were not detected with TLR3/2 priming

The anti-inflammatory cytokines IL-4, IL-10, IL-1RA, IFN-α and IFN-β were undetectable in all the samples.

4.3.3.5 PGE2 production is not augmented with TLR3/2 priming

PGE2 level was quantified using ELISA. Poly I:C and HKLM stimulation alone and with priming had no effect on PGE2 production compared to basal levels (Figure 4.48).

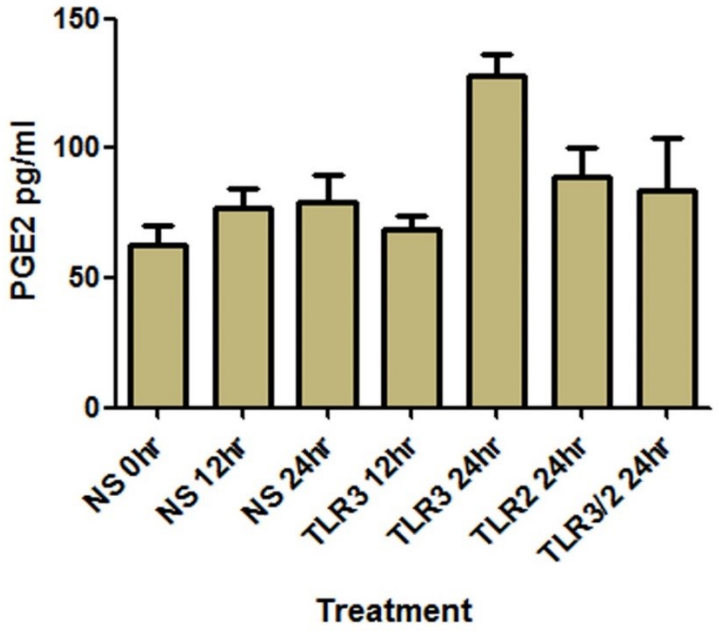


Figure 4.48 The effect of TLR3 priming prior to TLR2 agonist stimulation on PGE2 production in myocytes. Confluent myocytes were primed with 25µg/ml of poly I:C for 12 hours prior to 10^5 cells/ml of HKLM stimulation for 24 hours. TLR3 priming prior to TLR2 agonist stimulation showed no augmentation on PGE2 production. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

4.3.4 Lipopolysaccharides from Escherichia coli 055:B5 (LPS 055) (TLR4 agonist) stimulation increases NF-κB and AP-1

With the evidence of the presence of TLR4 mRNA in myocytes, the cells were stimulated with the TLR4 agonist LPS 055 to find the optimal concentration and time point before conducting the priming experiment. The same concentrations of LPS 055 (0.1μg/ml and 1μg/ml) used in VECs and amniocytes were used in myocytes. Western immunoblotting was performed and phosphorylated p-65 (p-p65) and phosphorylated c-Jun (p-c-Jun) were used as markers for NF-κB and AP-1 activation.

An increased was observed in NF-κB with 0.1μg/ml (Figure 4.49 A) of LPS while no increase in NF-κB at 1μg/ml (Figure 4.49 B) and 0.1μg/ml of AP-1 (Figure 4.49 C) activation was observed. 1μg/ml of LPS significantly increased AP-1 activation at 30 minutes (*p<0.05) and 4 hours (**p<0.01) (Figure 4.49 D).

For priming purposes, 0.1μg/ml at 6 hours was chosen to keep it consistent with the dosing in VECs and amniocytes.

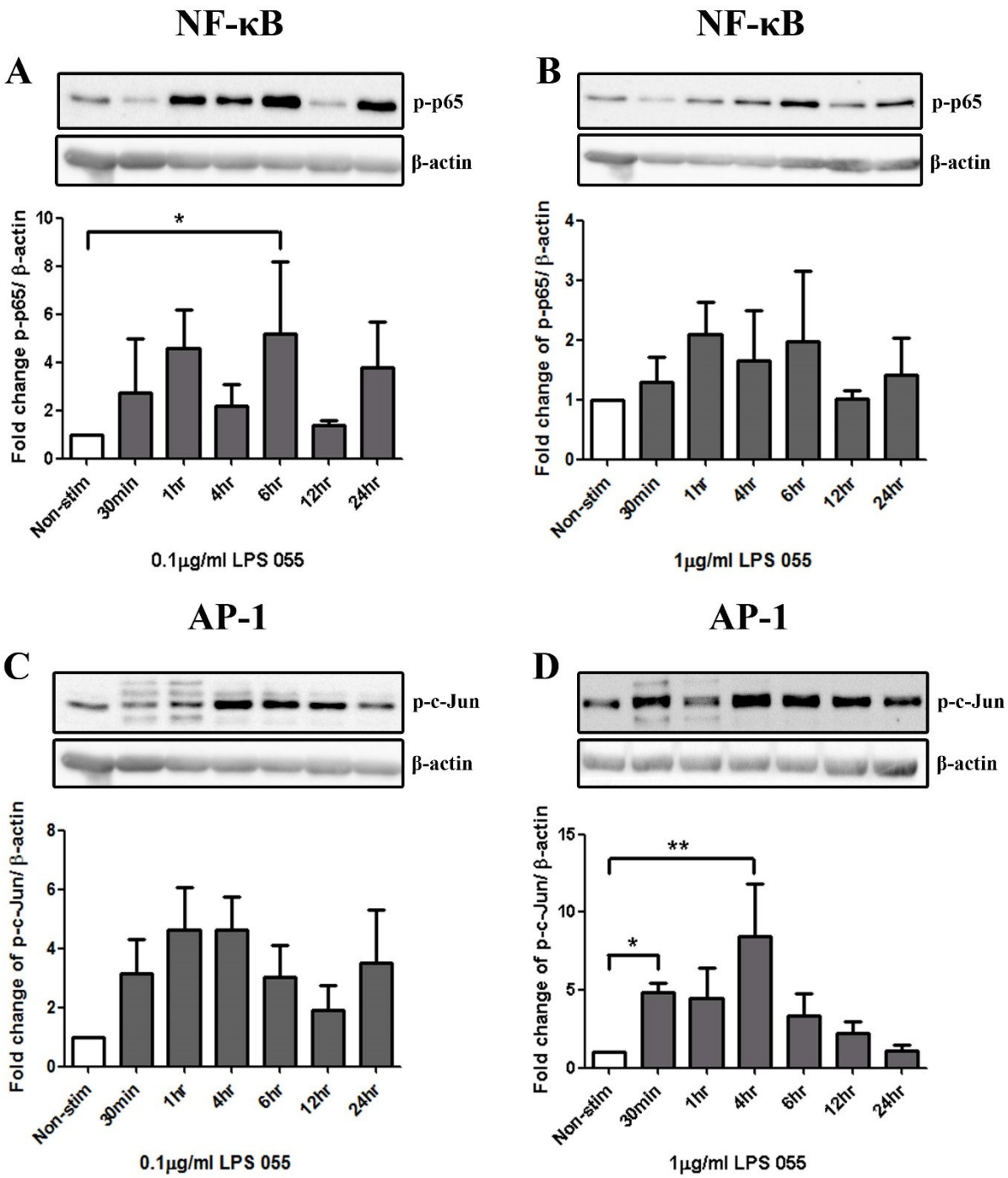


Figure 4.49 The effect of lipopolysaccharides from Escherichia coli 055:B5 (LPS 055) a TLR4 agonist stimulation on NF-κB and AP-1 activation in myocytes. Myocytes were cultured and stimulated with 0.1μg/ml and 1μg/ml of LPS 055 for a duration of 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. A statistical significant was observed in NF-κB with **(A)** 0.1μg/ml at 6 hours (*p<0.05) while an increased trend was observed with **(B)** 1μg/ml of LSP 055 but statistical significance was not achieved. The same increasing trend was observed with AP-1 in **(C)** 0.1μg/ml while a statistical significance increased was observed with **(D)** 1μg/ml of LPS 055 at 30 minutes (*p<0.05) and 4 hours (**p<0.01). For statistical analysis Kruskal-Wallis with *Dunnett's* multiple comparison test was used. n=5-6.

4.3.5 TLR3/TLR4 priming in myocytes

The same poly I:C (25μg/ml) and LPS (0.1μg/ml) concentrations used in VECs and amniocytes were also used to stimulate myocytes. To examine the activation status of NF-κB and AP-1 using Western immunoblotting, confluent monolayer cultured myocytes were primed with poly I:C for 12 hours before a subsequent LPS stimulation for 6 hours.

For cytokine, chemokine and PGE2 studies, cells were primed with poly I:C for 12 hours and then stimulated with LPS 055 for 24 hours. An ELISA was used to quantify the analytes.

4.3.5.1 NF-κB or AP-1 activation is not augmented with TLR3/4 priming

A significant increase was observed with poly I:C stimulation in NF-κB (*p<0.05). However, due to increased NF-κB at the basal level (6h, 18h), no statistical significance was observed with LPS stimulation alone or with TLR3/4 priming (Figure 4.50 A).

The significant increase in AP-1 activation was also observed with poly I:C stimulation (**p<0.01) but not with LPS stimulation alone. It is possible that AP-1 activation could occur at an earlier time point and therefore, was not captured in this experiment. No augmented response was observed with TLR3/4 agonist treatments (Figure 4.50 B).

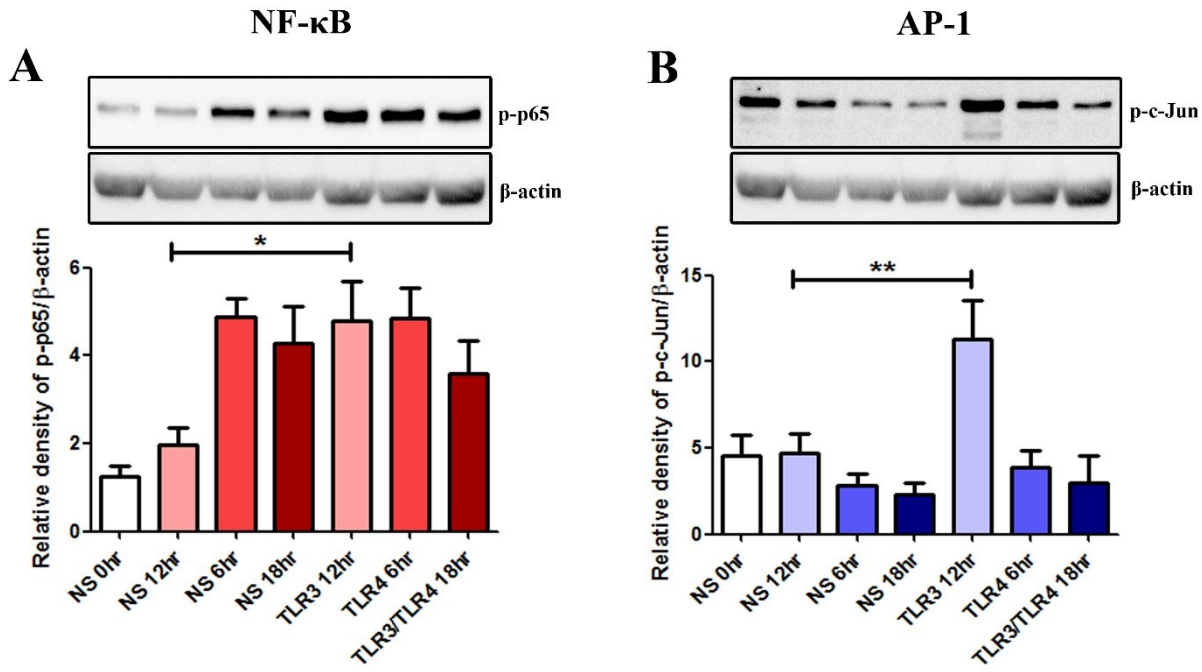


Figure 4.50 The effect of TLR3 priming before TLR4 agonist stimulation on NF-kB and AP-1 in myocytes. Confluent myocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 0.1μg/ml of LPS 055 stimulation for 6 hours. Protein was extracted, and Western immunoblotting was performed to determine the activation status of serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1, respectively. A representative blot is shown above each graph with β-actin as a loading control. TLR3 agonist stimulation alone significantly increased NF-κB and AP-1 activation. TLR3 priming prior to subsequent TLR4 stimulation showed no augmentation of **(A)** NF-κB or **(B)** AP-1. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. *p<0.05 = effect of TLR3 agonist compared to basal level.

4.3.5.2 IL-6 and IL-8 are augmented with TLR3/4 priming

Nine pro-inflammatory cytokines listed in section 4.3.2 were analysed. A 1 in 5 dilution was chosen to quantify all the cytokines based on the dilution test that was conducted. IL-1α, IFN-γ, TNF-β, IL-12p40 and IL-12p70 were below the detection level.

Poly I:C stimulation alone increased IL-1β (**p<0.01 at 12 hours and ***p<0.001 at 24 hours) (Figure 4.51 A), IL-6 (***p<0.001 at 12 and 24 hours) (Figure 4.51 B), IL-8 (***p<0.001 at 12 hours and **p<0.01 at 24 hours) (Figure 4.51 C) and TNF-α (***p<0.001 at 12 and 24 hours) (Figure 4.51 D) production compared to 0hr. LPS stimulation only significantly increased IL-6 ($^{\dagger}p$<0.05 at 24 hours) production compared to 0hr. TLR3/4 priming augmented IL-6 and IL-8 ($^{\S\S}p$<0.01) compared to LPS stimulation alone.

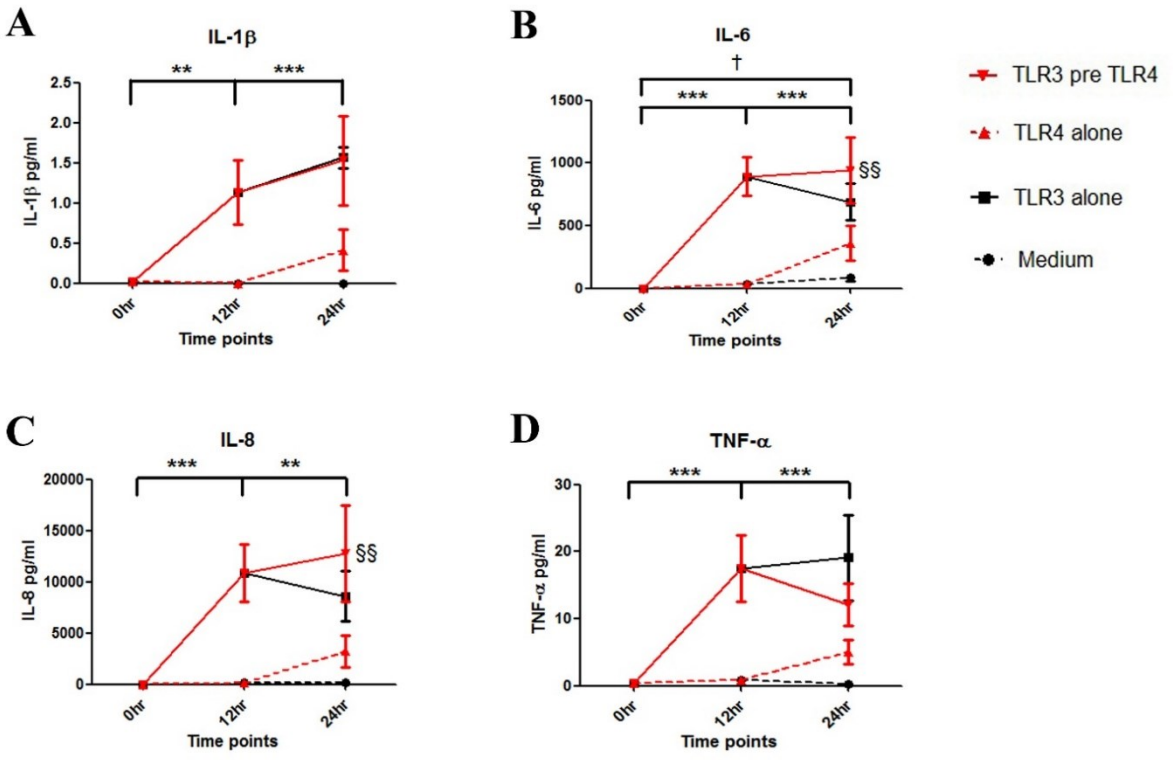


Figure 4.51 The effect of TLR3 priming prior to TLR4 stimulation on the pro-inflammatory cytokines production in myocytes. Confluent monolayer myocytes were plated and were primed with 25μg/ml of poly I:C for 12 hours prior to 0.1μg/ml of LPS 055 stimulation for 24 hours. Supernatants were collected and 1 in 5 dilutions were used to run ELISA to determine the level of the cytokine production. TLR3 agonist stimulation alone significantly increased the production of all cytokines and IL-6 increased with TLR4 agonist stimulation. TLR3 priming prior to TLR4 agonist stimulation showed no augmentation on **(A)** IL-1β but significantly augmented in **(B)** IL-6 and **(C)** IL-8. No augmentation was observed in **(D)** TNF-α. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=4. §§p<0.01 = effect of TLR3 pre TLR4 compared to TLR4 alone.

4.3.5.3 Chemokine production is not augmented with TLR3/4 priming

The same supernatant above (1 in 5 dilution) was used to quantify MIP-1α, MIP-1β as well as RANTES (1 in 10 dilution). MIP-1α, MIP-1β values were below the detection limit, but RANTES was within the detection range.

RANTES levels were no different between vehicle control and LPS treated samples. Poly I:C stimulation significantly increased the production of RANTES (***p<0.001 at 12 and 24 hours) compared to 0hr while no augmented response was seen with TLR3/4 priming (Figure 4.52).

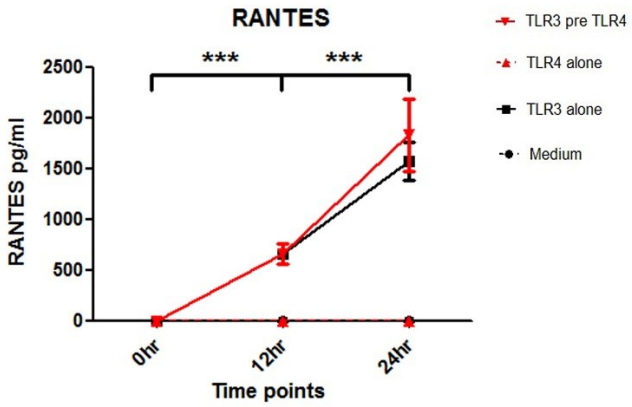


Figure 4.52 The effect of TLR3 priming prior to TLR4 stimulation on chemokines production in myocytes. Confluent monolayer myocytes were primed with 25μg/ml of poly I:C for 12 hours prior to 0.1μg/ml of LPS 055 stimulation for 24 hours. Supernatants were collected and were used to run ELISA to determine the level of the chemokine production. TLR3 stimulation significantly increased the production of RANTES. However, no further augmentation of RANTES was observed with TLR3 priming before TLR4 stimulation. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=4.

4.3.5.4 Anti-inflammatory cytokines were not detected with TLR3/4 priming

The anti-inflammatory cytokines IL-4, IL-10, IL-1RA, IFN-α and IFN-β were not detectable in any of the samples.

4.3.5.5 PGE2 production is not augmented with TLR3/4 priming

PGE2 production was quantified and no increase was seen with poly I:C or LPS stimulation alone or with TLR3/4 priming (Figure 4.53).

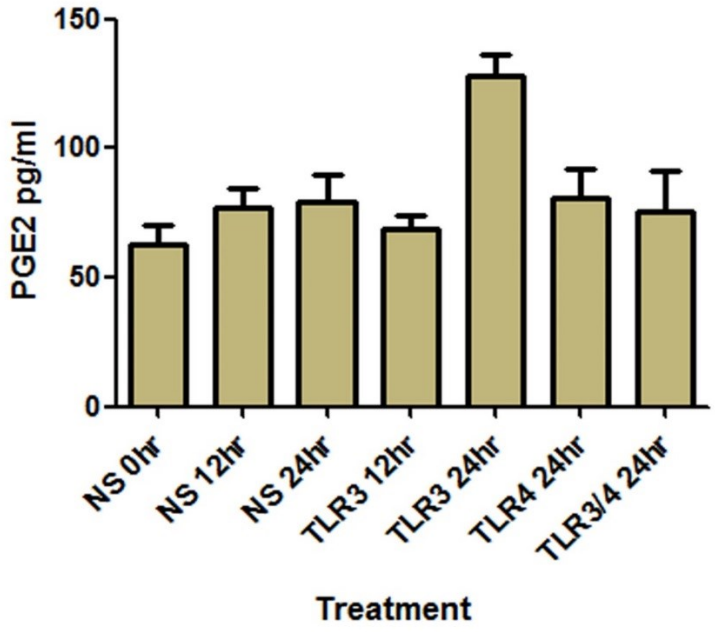


Figure 4.53 The effect of TLR3 priming prior to TLR2 agonist stimulation on PGE2 production in myocytes. Confluent myocytes were plated and were primed with 25µg/ml of poly I:C for 12 hours prior to 0.1µg/ml of LPS 055 stimulation for 24 hours. TLR3 priming prior to TLR4 agonist stimulation showed no augmentation of PGE2 production. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3.

4.3.6 Fibroblast–stimulating lipopeptide FSL-1 (TLR2/6 agonist) increases NF-κB and AP-1

FSL-1 stimulation in myocytes with 0.01μg/ml and 0.1μg/ml was used as in VEC and amniocyte treatments. Phosphorylated p-65 (p-p65) and phosphorylated c-Jun (p-c-Jun) were detected using Western immunoblotting as markers of NF-κB and AP-1.

No statistically significant increase in NF-κB was observed with both 0.01μg/ml (Figure 4.54 A) and 0.1μg/ml (Figure 4.54 B) of FSL-1. No significant increase was observed in AP-1 activation with 0.01μg/ml (Figure 4.54 C) but a statistically significant increase was seen at 4 hours with 0.1μg/ml (Figure 4.54 D).

Since no clear increase was observed at 1 hour in myocytes unlike in VECs and amniocytes, and only 0.1μg/ml showed statistical significance in AP-1 activation, this concentration and time point were chosen for priming experiments.

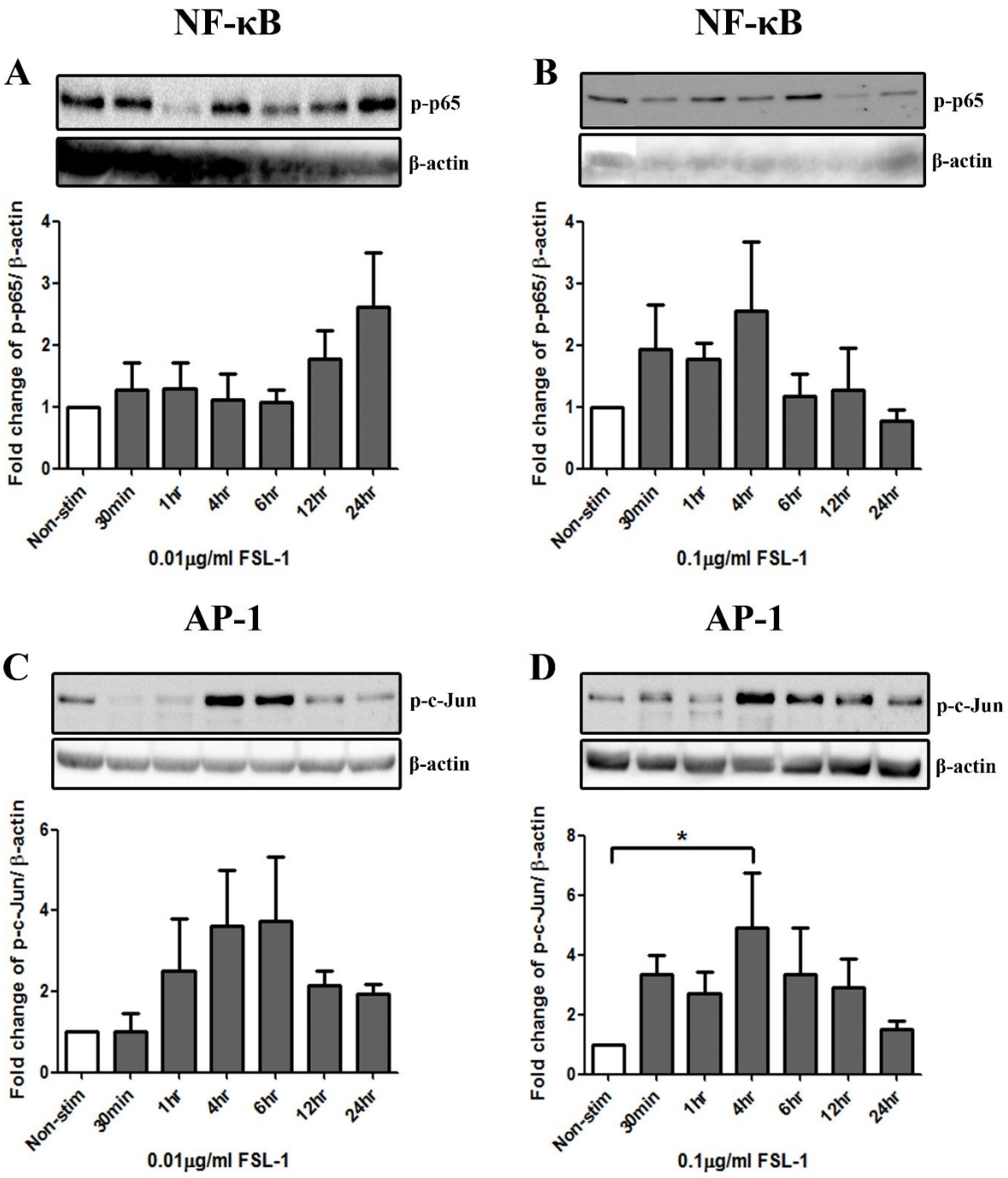


Figure 4.54 The effect of FSL-1 a TLR2/6 agonist stimulation on NF-κB and AP-1 activation in myocytes. Myocytes were cultured and stimulated with 0.01μg/ml and 0.1μg/ml of FSL-1 for a duration of 24 hours. Protein was extracted and whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. A representative blot is shown above each graph with β-actin as a loading control. NF-κB showed no increase with **(A)** 0.01μg/ml of FSL-1 but **(B)** a trend of increase was observed with 0.1μg/ml of FSL-1 at 4 hours but no statistical significance was achieved. AP-1 at **(C)** 0.01μg/ml of FSL-1 showed an increase trend from 1 hour to 6 hours but statistical significance was not achieved. At **(D)** 0.1μg/ml of FSL-1, AP-1 significantly increased at 4 (**p*<0.05) hours. For statistical analysis One-way ANOVA was used for normally distributed data and Kruskal-Wallis was used for data not normally distributed with *Dunnett's* multiple comparison test. n=4.

4.3.7 TLR3/TLR2/6 priming in myocytes

Myocytes were primed with the same poly I:C concentration as in VECs and amniocytes (25μg/ml), but 0.1μg/ml of FSL-1 was used to stimulate cells. Cells were primed with poly I:C for 12 hours before a subsequent FSL-1 stimulation for 4 hours. Protein was extracted and analysed using Western immunoblotting to examine the activation status of NF-κB and AP-1.

For cytokines, chemokine and PGE2 studies, cells were primed with poly I:C for 12 hours and then stimulated with FSL-1 for 24 hours. An ELISA was used to quantify the analytes.

4.3.7.1 NF-κB or AP-1 activation is not augmented with TLR3/TLR2/6 priming

No statistically significant increase in NF-κB activation was observed with poly I:C or FSL-1 stimulation and no difference was seen with priming (Figure 4.55 A).

Poly I:C stimulation alone increased AP-1 activation (**p<0.01) but no difference was noted with FLS-1 stimulation alone or with priming (Figure 4.55 B).

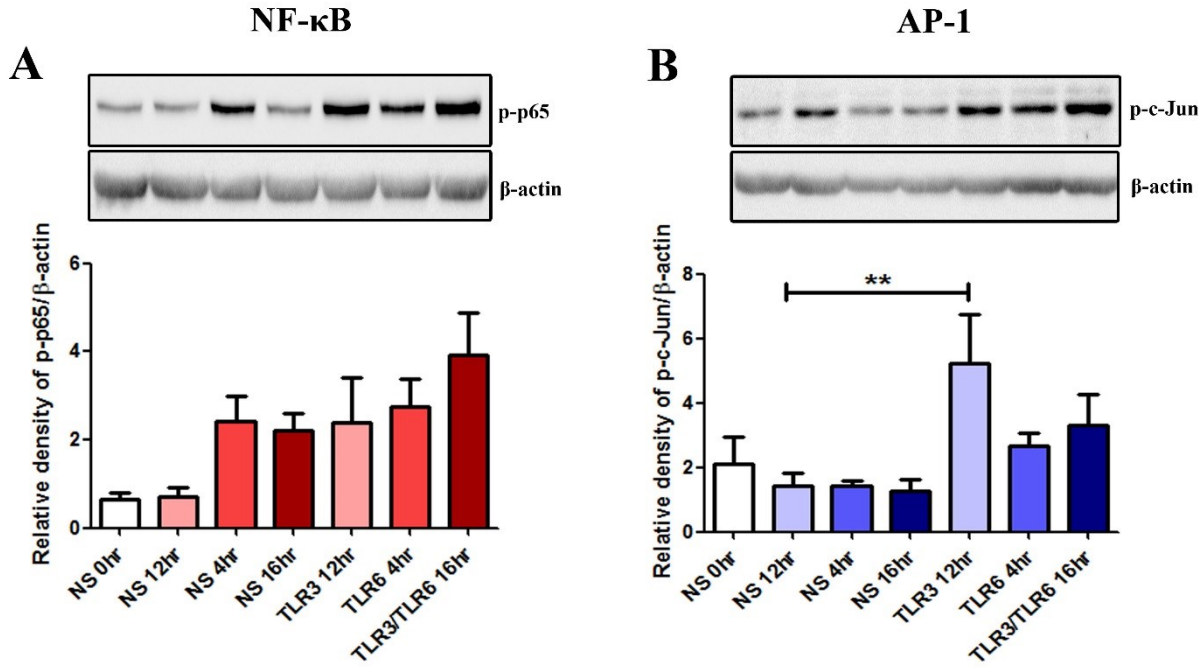


Figure 4.55 The effect of TLR3 priming before TLR2/6 agonist stimulation on NF-kB and AP-1 in myocytes. Confluent monolayer myocytes were plated and were primed with 25μg/ml of poly I:C for 12 hours prior to 0.1μg/ml of FSL-1 stimulation for 4 hours. Protein was extracted, and Western immunoblotting was performed to determine the activation status of serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1, respectively. A representative blot is shown above each graph with β-actin as a loading control. TLR3 agonist stimulation alone significantly increased AP-1 activation. TLR3 priming prior to subsequent TLR2/6 stimulation showed no augmentation of **(A)** NF-κB or **(B)** AP-1. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3. **p<0.01 = effect of TLR3 agonist compared to basal level.

4.3.7.2 IL-1α, IL-1β, IL-6, IL-8 and TNF-α cytokine production are augmented with TLR3/TLR2/6 priming

A 1 in 5 dilution was chosen to quantify all the cytokines. Only 5 pro-inflammatory cytokines were detected in the samples with IFN-γ, TNF-β, IL-12p40 and IL-12p70 being below the detection level.

Vehicle control showed no difference in the production of the cytokines over time. Poly I:C stimulation alone increased IL-8 (***p<0.001 at 12 hours and **p<0.01 at 24 hours) and TNF-α (*p<0.05 at 12 hours and **p<0.01 at 24 hours) production compared to 0hr. IL-1β ($^{\dagger\dagger}p$<0.01 at 24 hours), IL-6, IL-8 and TNF-α ($^{\dagger\dagger\dagger}p$<0.001 at 24 hours) production increased with FSL-1 stimulation alone compared to 0hr. Poly I:C priming prior to FSL-1 stimulation augmented the production of IL-1α ($^{\S}p$<0.05) (Figure 4.56 A), IL-1β ($^{\S\S\S}p$<0.001) (Figure 4.56 B), IL-6 ($^{\S\S\S}p$<0.001) (Figure 4.56 C), IL-8 ($^{\S\S}p$<0.01) (Figure 4.56 D) and TNF-α ($^{\S\S\S}p$<0.001) (Figure 4.56 E) compared to the FSL-1 stimulation alone.

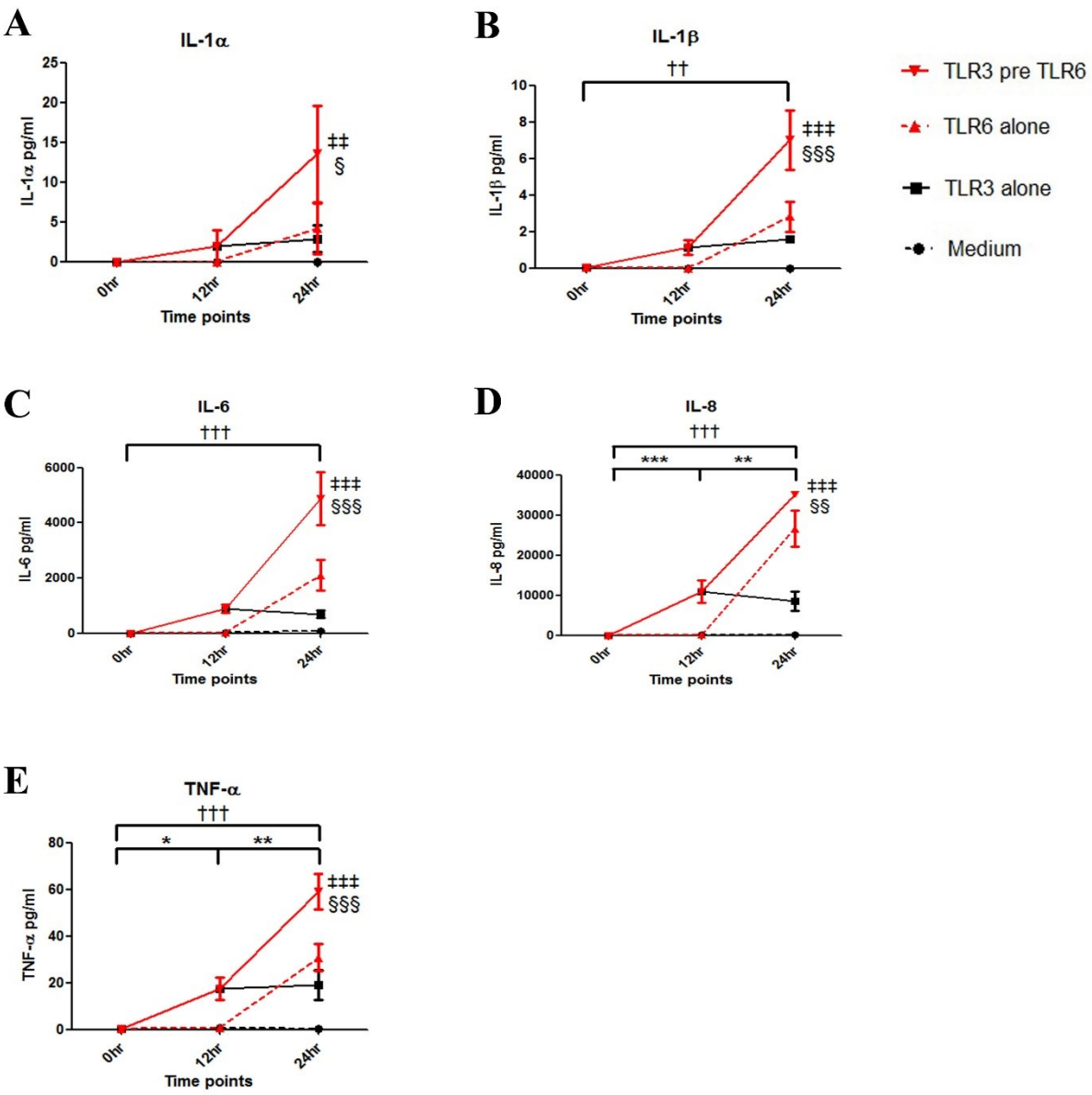


Figure 4.56 The effect of TLR3 priming prior to TLR2/6 stimulation on the pro-inflammatory cytokines production in myocytes. Confluent monolayer myocytes were plated and were primed with 25μg/ml of poly I:C for 12 hours prior to 0.1μg/ml of FSL-1 stimulation for 24 hours. Supernatants were collected and 1 in 5 dilutions were used to run ELISA to determine the level of the cytokines production. TLR3 and TLR2/6 agonist stimulation alone significantly increased the production of the cytokines. TLR3 priming prior to TLR2/6 agonist stimulation showed a significant augmentation of **(A)** IL-1α, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-8 and **(E)** TNF-α. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=4. ‡‡p<0.01 = effect of TLR3 pre TLR2/6 compared to TLR3 alone. §§p<0.01 = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone.

4.3.7.3 RANTES chemokine production is augmented with TLR3/TLR2/6 priming

Three different chemokines were analysed; MIP-1α, MIP-1β using 1 in 5 dilution and RANTES with a 1 in 10 dilution. Only RANTES production was within the detection range.

FSL-1 stimulation did not alter RANTES production. Poly I:C stimulation increased RANTES production (***p<0.001) compared to 0hr and priming augmented the production compared to FSL-1 stimulation alone ($^{§§§}p$<0.001) (Figure 4.57).

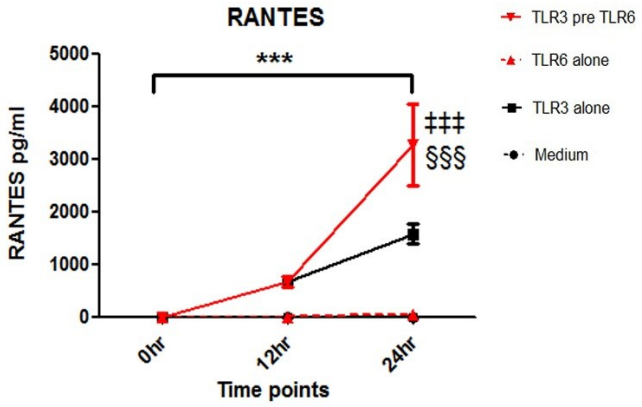


Figure 4.57 The effect of TLR3 priming prior to TLR2/6 stimulation on chemokines production in myocytes. Confluent monolayer myocytes were plated and were primed with 25μg/ml of poly I:C for 12 hours prior to 0.1μg/ml of FSL-1 stimulation for 24 hours. Supernatants weres collected and were used to run ELISA to determine the level of the chemokines production. TLR3 stimulation significantly increased the production of RANTES. A further augmentation of RANTES was observed with TLR3 priming before TLR2/6 stimulation. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=4. $^{‡‡‡}p$<0.001 = effect of TLR3 pre TLR2/6 compared to TLR3 alone. $^{§§§}p$<0.001 = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone.

4.3.7.4 Anti-inflammatory cytokines were not detected with TLR3/TLR2/6 priming

The anti-inflammatory cytokines IL-4, IL-10, IL-1RA, IFN-α and IFN-β were undetectable in all of the samples.

4.3.7.5 PGE2 production is not augmented with TLR3/TLR2/6 priming

The production of PGE2 was quantified and no difference was observed with poly I:C or FSL-1 stimulation alone or with priming (Figure 4.58).

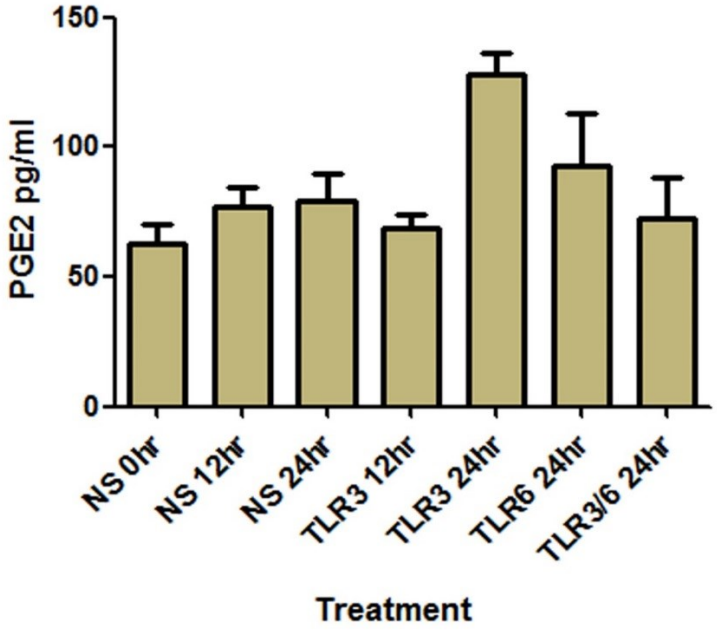


Figure 4.58 The effect of TLR3 priming prior to TLR2 agonist stimulation on PGE2 production in myocytes. Confluent monolayer myocytes were plated and were primed with 25µg/ml of poly I:C for 12 hours prior to 0.1µg/ml of FSL-1 stimulation for 24 hours. TLR3 priming prior to TLR2/6 agonist stimulation showed no augmentation of PGE2 production. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

4.4 Phosphorylated IRF3 (p-IRF3) transcription factor increases with poly I:C stimulation

Poly I:C acts through TLR3 and mediates its signalling via the TRAF3 and TRAF6 proteins [311]. TRAF3 signalling regulates the transcription factor IRF3 and the downstream genes of type I IFN production whereas TRAF6 signalling regulates NF-κB and MAPKs transcription factors. The TLR3 priming experiments above showed no consistent augmentation of the responses in NF-κB and AP-1 transcription factors in all cell types. The augmented effects on cytokine production could have been via alternative transcription factors. Therefore, the effect of poly I:C stimulation on the transcription factor IRF3 was examined. Cells were stimulated with 25μg/ml of poly I:C for up to 24 hours. Western immunoblotting was performed to detect the phosphorylated IRF3 as an activation marker of IRF3.

In VEC, poly I:C stimulation significantly increased p-IRF3 at 12 hours (*p<0.05) (Figure 4.59).

Meanwhile in amniocytes, p-IRF3 protein increased significantly at 30 minutes (*p<0.05), 1 (**p<0.01), 4 (**p<0.01), 12 (*p<0.05) and 24 hours (**p<0.01) (Figure 4.60).

In myocytes, p-IRF3 also increased significantly at 4 (*p<0.05) and 6 (**p<0.01) hours after poly I:C stimulation (Figure 4.61).

This confirms that IRF3 is activated by poly I:C in VECs, amniocytes and myocytes.

4.4.1 VEC

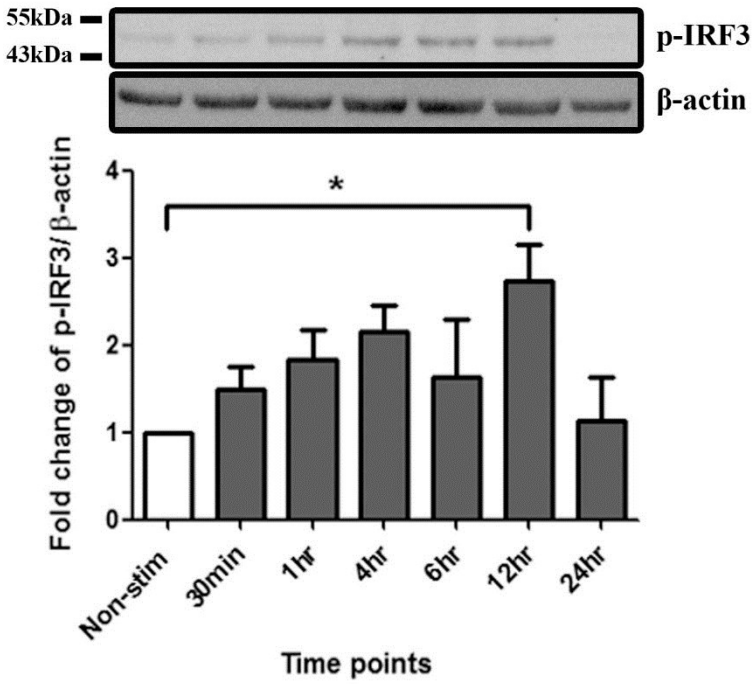


Figure 4.59 The effect of poly I:C stimulation on phospho-IRF3 in VEC. Confluent monolayer VECs were stimulated with 25μg/ml of poly I:C for a duration of 24 hours. Protein was extracted, and Western immunoblotting was performed to determine the activation status of serine 396-phosphorylated IRF3 (Ser396-p-IRF3). A representative blot is shown above each graph with β-actin as a loading control. Poly I:C stimulation significantly increased p-IRF3 at 12 (*p<0.05) hours. For statistical analysis One-way ANOVA with *Dunnett's* multiple comparison test was used. n=6.

4.4.2 Amniocytes

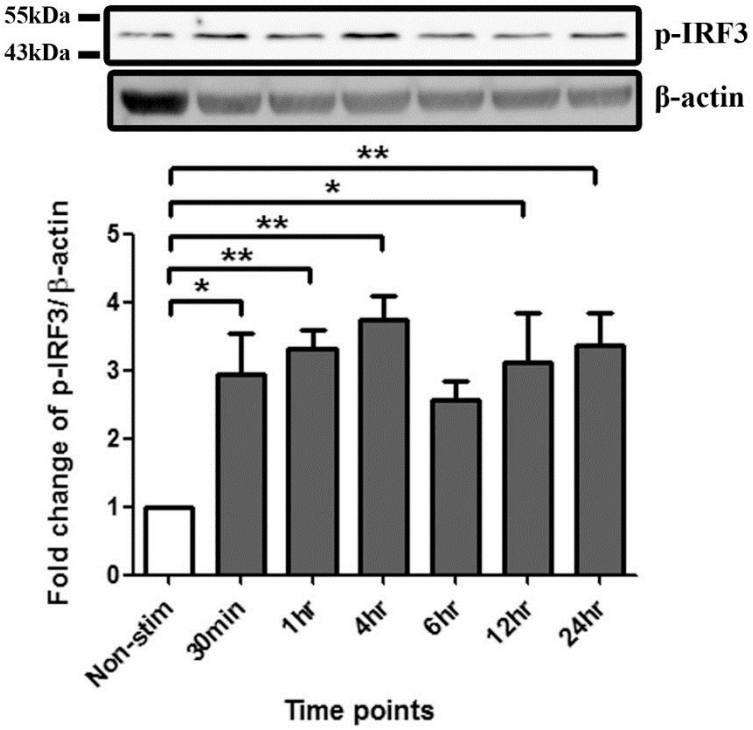


Figure 4.60 The effect of poly I:C stimulation on phospho-IRF3 in amniocytes. Confluent monolayer amniocytes were stimulated with 25μg/ml of poly I:C for a duration of 24 hours. Protein was extracted, and Western immunoblotting was performed to determine the activation status of serine 396-phosphorylated IRF3 (Ser396-p-IRF3). A representative blot is shown above each graph with β-actin as a loading control. Poly I:C stimulation significantly increased p-IRF3 at 30 minutes (*p<0.05), 1, 4 (**p<0.01), 12 (*p<0.05), and 24 (**p<0.01) hours. For statistical analysis One-way ANOVA with *Dunnett's* multiple comparison test was used. n=6.

4.4.3 Myocytes

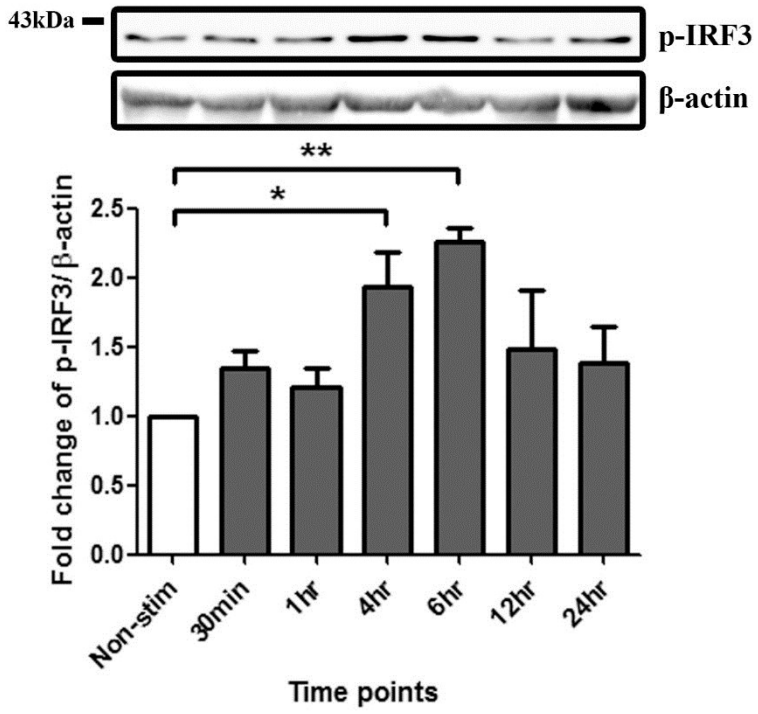


Figure 4.61 The effect of poly I:C stimulation on phospho-IRF3 in myocytes. Confluent monolayer myocytes were stimulated with 25μg/ml of poly I:C for a duration of 24 hours. Protein was extracted, and Western immunoblotting was performed to determine the activation status of serine 396-phosphorylated IRF3 (Ser396-p-IRF3). A representative blot is shown above each graph with β-actin as a loading control. Poly I:C stimulation significantly increased p-IRF3 at 4 (*p<0.05) and 6 (**p<0.01) hours. For statistical analysis Kruskal-Wallis with *Dunnett's* multiple comparison test was used. n=3.

4.5 Poly I:C stimulation increases TLR3 and TLR2 mRNA in amnion, VEC and myocytes

The augmented response seen in TLR2, 4 and 6 agonist stimulation after TLR3 agonist priming leads to the question of whether poly I:C stimulation increases the expression of these TLRs. Therefore, VECs, amniocytes and myocytes were stimulated with 25μg/ml of poly I:C for 2, 4, 6 and 12 hours. Total RNA was extracted, and RT-qPCR was performed to quantify the levels of TLR3, 2, 4 and 6 mRNA by using β-actin as the loading control.

Poly I:C stimulation significantly increased TLR3 and TLR2 mRNA (***p<0.001) expression at 6 and 12 hours in VECs (Figure 4.62).

TLR3 and TLR2 mRNA (***p<0.001) also increased significantly at 12 hours in amniocytes when treated with poly I:C (Figure 4.63).

The same response was also observed in myocytes with a significant increase of TLR3 (**p<0.01) and TLR2 (***p<0.001) mRNA at 12 hours when the cells were stimulated with poly I:C (Figure 4.64).

4.5.1 VEC

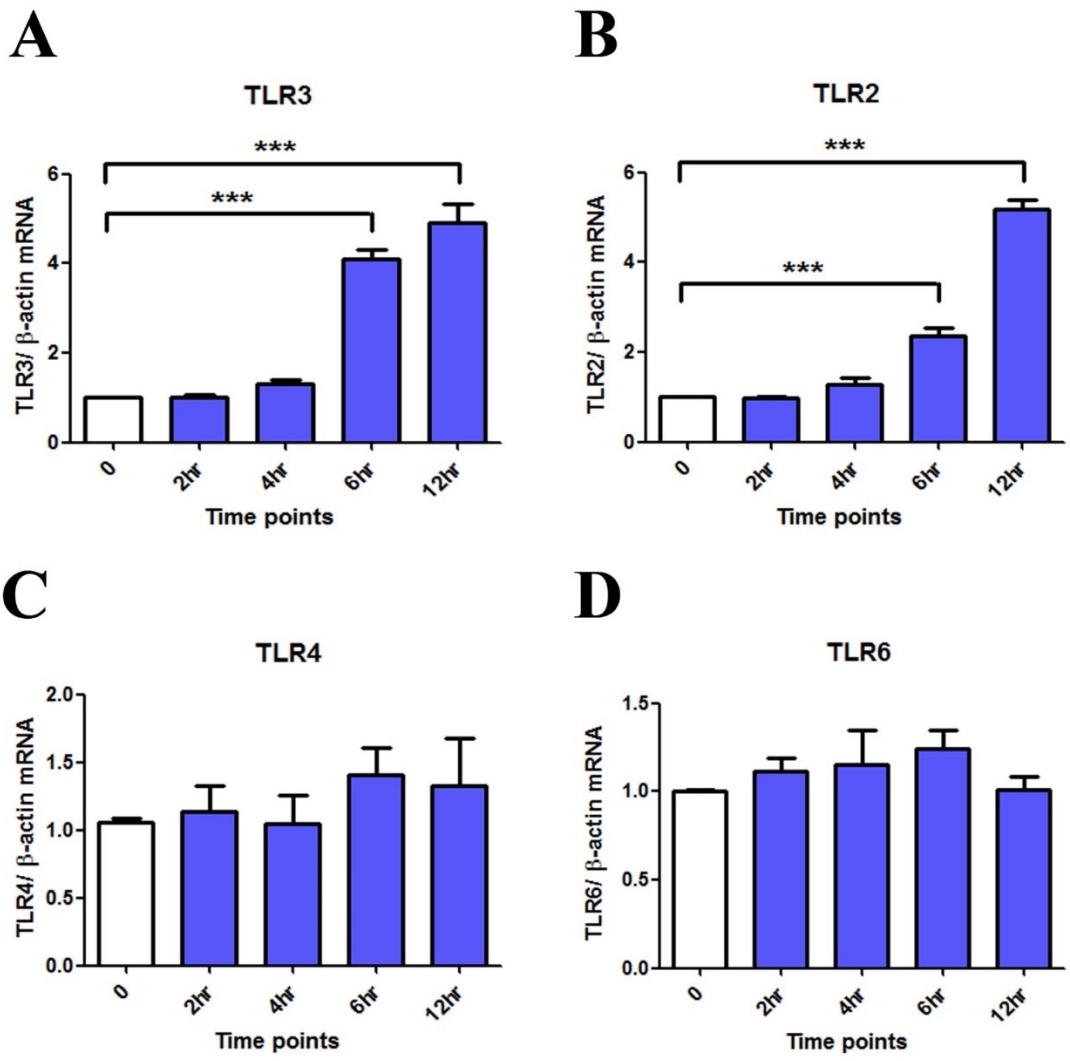


Figure 4.62 The effect of 25µg/ml of poly I:C stimulation on TLR3, 2, 4 and 6 mRNA expression in VEC VECs were stimulated with 25µg/ml of poly I:C at 0, 2, 4, 6 and 12 hours. Quantitative RT-PCR was used to determine the effect of poly I:C on TLR2, 3, 4 and 6 mRNA. Poly I:C significantly increased the mRNA expression of **(A)** TLR3 and **(B)** TLR2 at 6 and 12 hours (***p<0.001). However, no change in expression was observed in **(C)** TLR4 and **(D)** TLR6 mRNA. For statistical analysis One-way ANOVA was used with *Dunnett's* multiple comparison test. The results of 4 biological replicates were shown as above with β-actin was used as a loading control. n=4.

4.5.2 Amniocytes

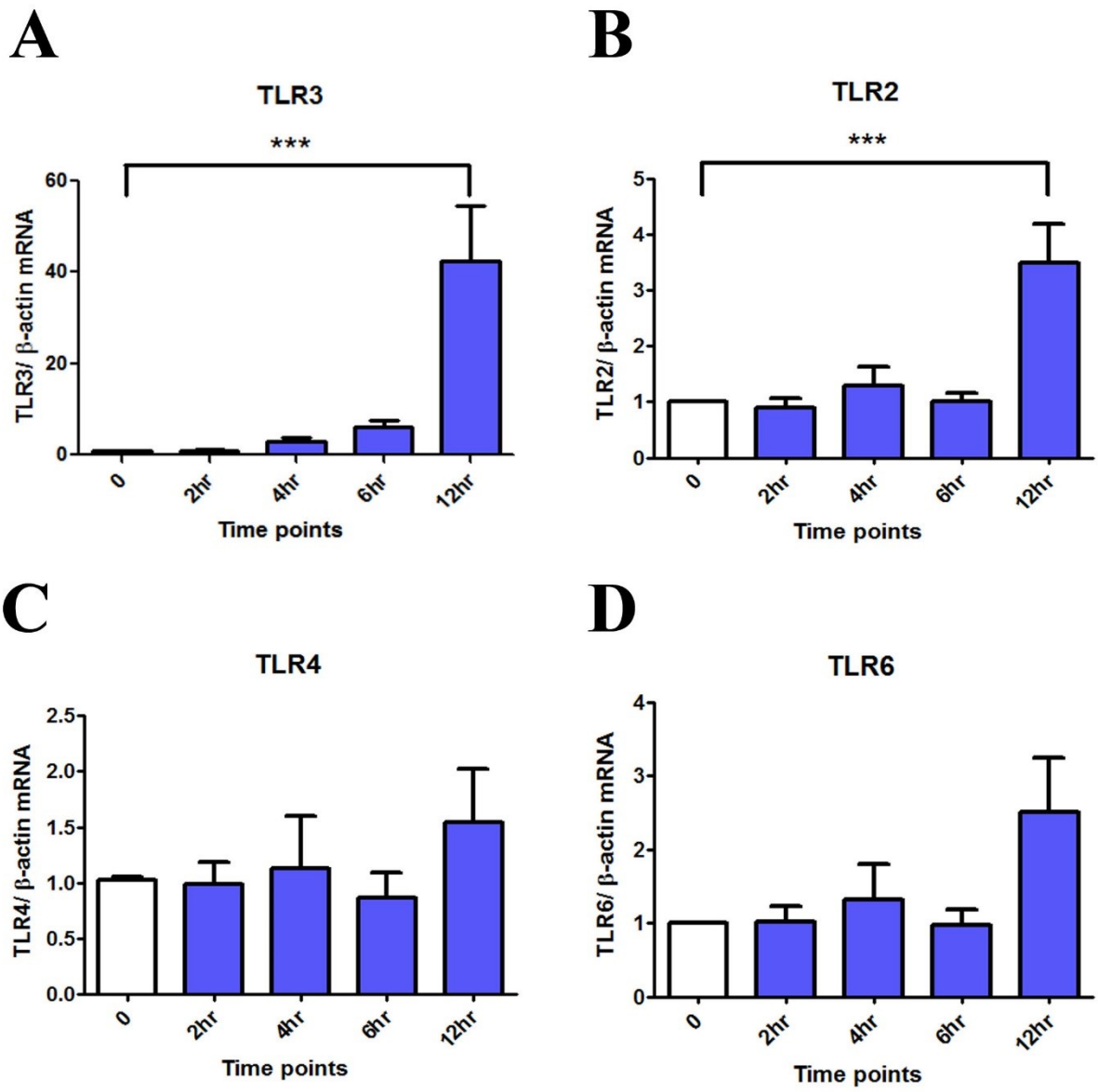


Figure 4.63 The effect of 25µg/ml of poly I:C stimulation on TLR3, 2, 4 and 6 mRNA expression in amniocytes. Amniocytes were stimulated with 25µg/ml of poly I:C at 0, 2, 4, 6 and 12 hours. Quantitative RT-PCR was used to determine the effect of poly I:C on TLR2, 3, 4 and 6 mRNA. Poly I:C significantly increased the mRNA expression of **(A)** TLR3 and **(B)** TLR2 at 12 hours (***p<0.001). A slight increase was observed in **(C)** TLR4 and **(D)** TLR6 mRNA at 4 and 12 hours but no statistical significance was achieved. For statistical analysis One-way ANOVA was used with *Dunnett's* multiple comparison test. The results of 6 biological replicates were shown as above with β-actin was used as a loading control. n=6.

4.5.3 Myocytes

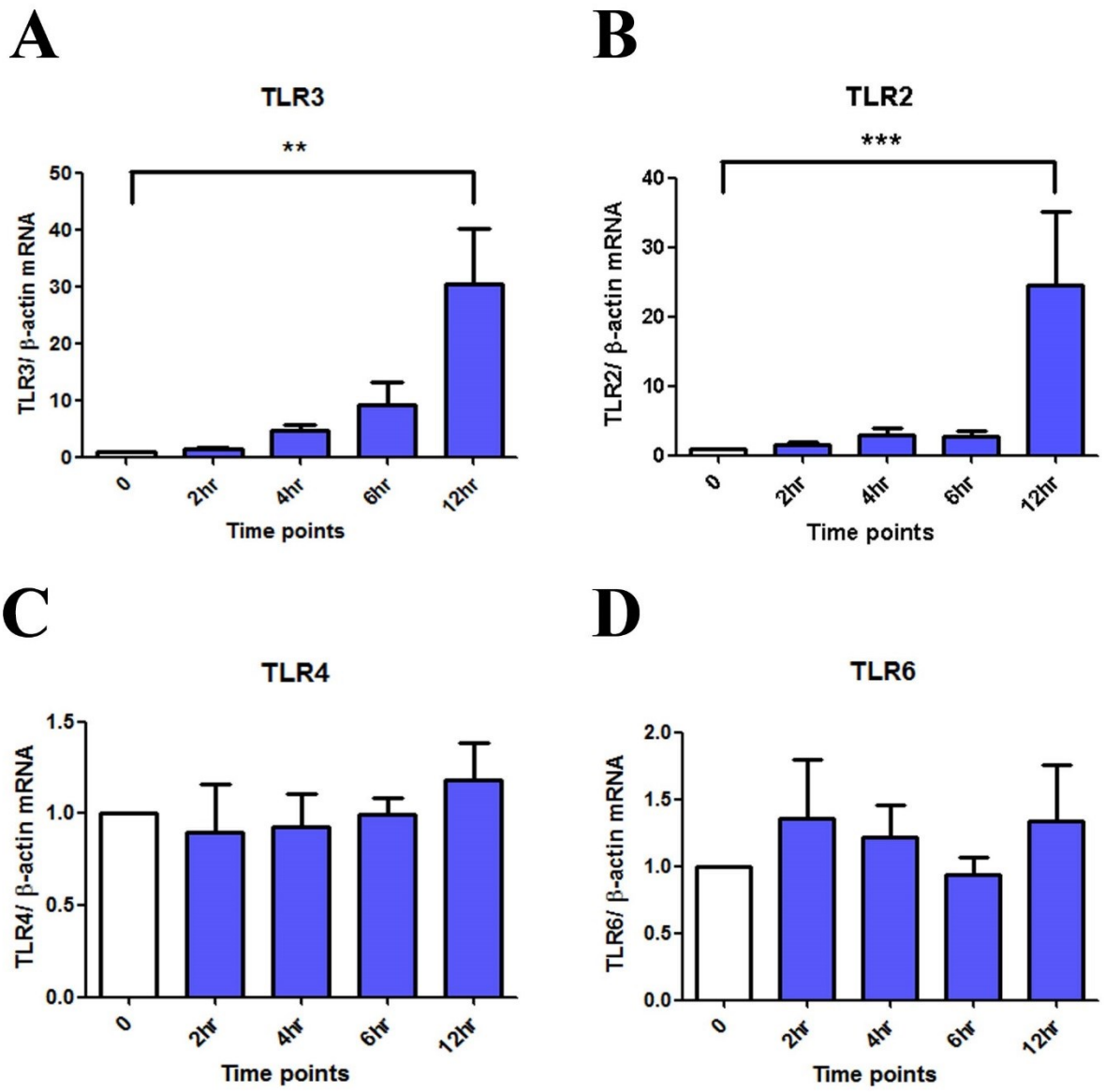


Figure 4.64 The effect of 25µg/ml of poly I:C stimulation on TLR3, 2, 4 and 6 mRNA expression in myocytes. Myocytes were stimulated with 25µg/ml of poly I:C at 0, 2, 4, 6 and 12 hours. Quantitative RT-PCR was used to determine the effect of poly I:C on TLR2, 3, 4 and 6 mRNA. Poly I:C significantly increased the mRNA levels of **(A)** TLR3 (**p<0.01) and **(B)** TLR2 (***p<0.001) at 12 hours. However, no change in expression was observed in **(C)** TLR4 and **(D)** TLR6 mRNA. For statistical analysis Kruskal-Wallis was used with *Dunnett's* multiple comparison test. The results of 6 biological replicates were shown as above with β-actin was used as a loading control. n=4.

Table 4.1 Concentrations and time point of TLRs agonist used for priming experiment in VEC, amniocytes and myocytes

Cell type	Poly I:C (TLR3)			HKLM (TLR2)			LPS (TLR4)			FSL-1 (TLR2/6)		
	Concentration	Time point		Concentration	Time point		Concentration	Time point		Concentration	Time point	
		NF-κB, AP-1	Cytokine, chemokine, PGE2		NF-κB, AP-1	Cytokine, chemokine, PGE2		NF-κB, AP-1	Cytokine, chemokine, PGE2		NF-κB, AP-1	Cytokine, chemokine, PGE2
VEC	25μg/ml	6hours	24 hours	10^5 cells/ml	1hour	24 hours	0.1μg/ml	6hours	24 hours	0.01μg/ml	1hour	24 hours
Amniocytes	25μg/ml	12hours	24 hours	10^5 cells/ml	1hour	24 hours	0.1μg/ml	6hours	24 hours	0.01μg/ml	1hour	24 hours
Myocytes	25μg/ml	12hours	24 hours	10^5 cells/ml	1hour	24 hours	0.1μg/ml	6hours	24 hours	0.1μg/ml	4hours	24 hours

Table 4.2 Effects on transcription factor, cytokine, chemokines and PGE2 with priming experiment in VEC, amniocytes and myocytes

Cell type	TLR3/2					TLR3/4					TLR3/TLR2/6				
	Transcription factors		Cytokine	Chemokine	PGE2	Transcription factors		Cytokine	Chemokine	PGE2	Transcription factors		Cytokine	Chemokine	PGE2
	NF-κB	AP-1				NF-κB	AP-1				NF-κB	AP-1			
VEC	X	X	X	X	X	X	X	X	X	X	X	X	IL-6, IL-8, TNF-α	MIP-1α, MIP-1β	✓
Amniocytes	X	✓	X	X	X	X	X	IL-6, TNF-α	X	X	X	X	IL-6	MIP-1α, MIP-1β, RANTES	✓
Myocytes	X	X	X	X	X	X	X	IL-6, IL-8	X	X	X	X	IL-1α, IL-1β, IL-6, IL-8, TNF-α	RANTES	X

X = no priming effect

Discussion

Priming with the TLR3 viral agonist prior to bacterial TLR2, 4 and 2/6 agonists augments pro-inflammatory mediators responses. However, priming does not augment the activation of the transcription factors NF-κB or AP-1 in any of the cell types. The production of pro-inflammatory mediators, chemokines and PGE2 is augmented in VECs with TLR2/6 stimulation after TLR3 viral agonist priming but no effect was seen with TLR2 or TLR4 agonist stimulation. Amniocytes showed augmented pro-inflammatory mediator and chemokine production with TLR4 and TLR2/6 agonist stimulation after TLR3 viral agonist priming. PGE2 was also augmented with TLR2/6 agonist stimulation prior to priming. In myocytes, TLR4 and TLR2/6 agonist stimulation after TLR3 priming increased pro-inflammatory mediators and chemokines production. Poly I:C stimulation leads to activation of the transcription factor IRF3 and increases TLR3 and TLR2 mRNA in all cell types.

In preterm labour, ascending infection is the most common route of infection [96] and the most widely researched area in the literature. Microorganisms detected in the amniotic fluid in the presence of chorioamnionitis often originate from the microbiota residing in the cervicovaginal area, supporting the concept of ascending infection [120-123, 125]. In addition to the presence of bacteria, the lower reproductive tract region is also a home for a local TLR3-virus human papillomavirus (HPV) that can be acquired as early as the first sexual experience [393, 394]. Furthermore, the presence of other viruses has also been discovered in the vaginal area and are associated with preterm birth [158]. In this study, priming VECs, amniocytes and myocytes with TLR3 agonist led to an augmented response to bacterial TLRs in the production of pro-inflammatory cytokines (especially IL-6, IL-8 and TNF-α) and pro-labour mediator PGE2. These pro-inflammatory and pro-labour mediators are vital for the physiological changes during labour such as myometrial contraction, membrane rupture and cervical dilation.

Conclusions:

Tissues exposed to ascending infection are highly susceptible to secondary bacterial infection following viral priming since they demonstrate enhanced pro-inflammatory responses compared to exposure to single pathogens alone. This implies that women

who have viruses that stimulate the TLR3 receptor in pregnancy could be at an additional risk of bacterial-induced preterm labour.

CHAPTER 5

Effect of TLR3 priming in a model of haematogenous infection induced sPTL

Introduction

Background:

Having demonstrated susceptibility of gestational tissue to a double viral/bacterial hit in the context of ascending infection, my next aim was to determine if PBMCs and the placenta are also susceptible in an *in vitro* model of haematogenous spread of infection induced preterm labour. In this chapter, I explored the effect of TLR3 agonist viral priming and subsequent bacterial TLR2, 4 and 6 agonist stimulation on PBMCs and placental explants. Following this, my aim was to elucidate whether the placenta is responsive to systemic multi-pathogen exposure by treating placental explants with PBMC-primed and PBMC-TLRs agonist treated supernatant.

Hypothesis:

1. NF-κB and AP-1 increases with TLR3 agonist (poly I:C), TLR2 agonist (Heat-killed *Listeria Monocytogenes*), TLR4 agonist (Lipopolysaccharide 055:B5) and TLR2/6 agonist (FSL-1) stimulation in both PBMCs and placental explants.
2. Local TLR3 priming prior to TLR2, 4 and 2/6 agonists stimulation augments the production of pro-labour and pro-inflammatory mediators in PBMC and in placental explants.

Aims:

The aims of the first part of this chapter was to prime PBMC and placental explants before subsequent bacterial TLR2, 4 and 6 agonist stimulation. The activation status of NF-κB and AP-1, and production of pro- inflammatory and pro-labour mediators were examined. Following this, placental explants were cultured with supernatant collected from treated PBMCs, and the effect on NF-κB and AP-1, pro-inflammatory and pro-labour mediators were examined. Finally, the effect of poly I:C treatment on TLR3, 2, 4 and 6 mRNA expression and p-IRF3 protein expression was examined.

Results

5.1 Peripheral Blood Mononuclear Cells (PBMC)

5.1.1 TLRs agonist stimulation increases NF-κB activation

PBMC stimulation with poly I:C was conducted at 5μg/ml, the lowest concentration reported in previous literature using PBMCs [385] and dendritic cells [386]. The concentration of poly I:C used in VECs, amniocytes and myocytes was 5-fold higher than PBMCs. As PBMCs are innate immune surveillance cells, high sensitivity to even a low dose of infection is needed to fight off the infection. Thus, this dose of 5μg/ml deems suitable as to avoid overloading the system. The concentration used for the TLR agonist HKLM was 10^8 cells/ml in keeping with the suggested working concentration in the datasheet (Invivogen) and with what has been used in the literature in PBMCs [395]. 0.1μg/ml of LPS was used based on the same concentration of LPS used in the PBMCs in the literature [396]. For the TLR2/6 agonist, 0.1μg/ml FSL-1 was used based on previously reported increase in cytokines production by PBMCs at this dose [397]. Western immunoblotting was performed and phosphorylated p-65 (p-p65) and phosphorylated c-Jun (p-c-Jun) were used as markers for NF-κB and AP-1 activation status.

Cells were cultured and stimulated with the agonists in a time course experiment for up to 24 hours. NF-κB activation peaked at 30 minutes and 1 hour with 5μg/ml of poly I:C (*p<0.05) (Figure 5.1 A).

Stimulation with 10^8 cells/ml of HKLM showed a significant increase at 24 hours post stimulation (*p<0.05) although a non-significant 2- fold increase was observed as early as 30 minutes (Figure 5.1 B).

LPS stimulation also showed early NF-κB activation at 30 minutes and 1 hour (*p<0.05 for both) (Figure 5.1 C), while FSL-1 stimulation showed no significant difference (Figure 5.1 D).

The transcription factor AP-1 was undetectable with all TLR stimulations. Therefore, only the transcription factor NF-κB was examined in the following priming studies.

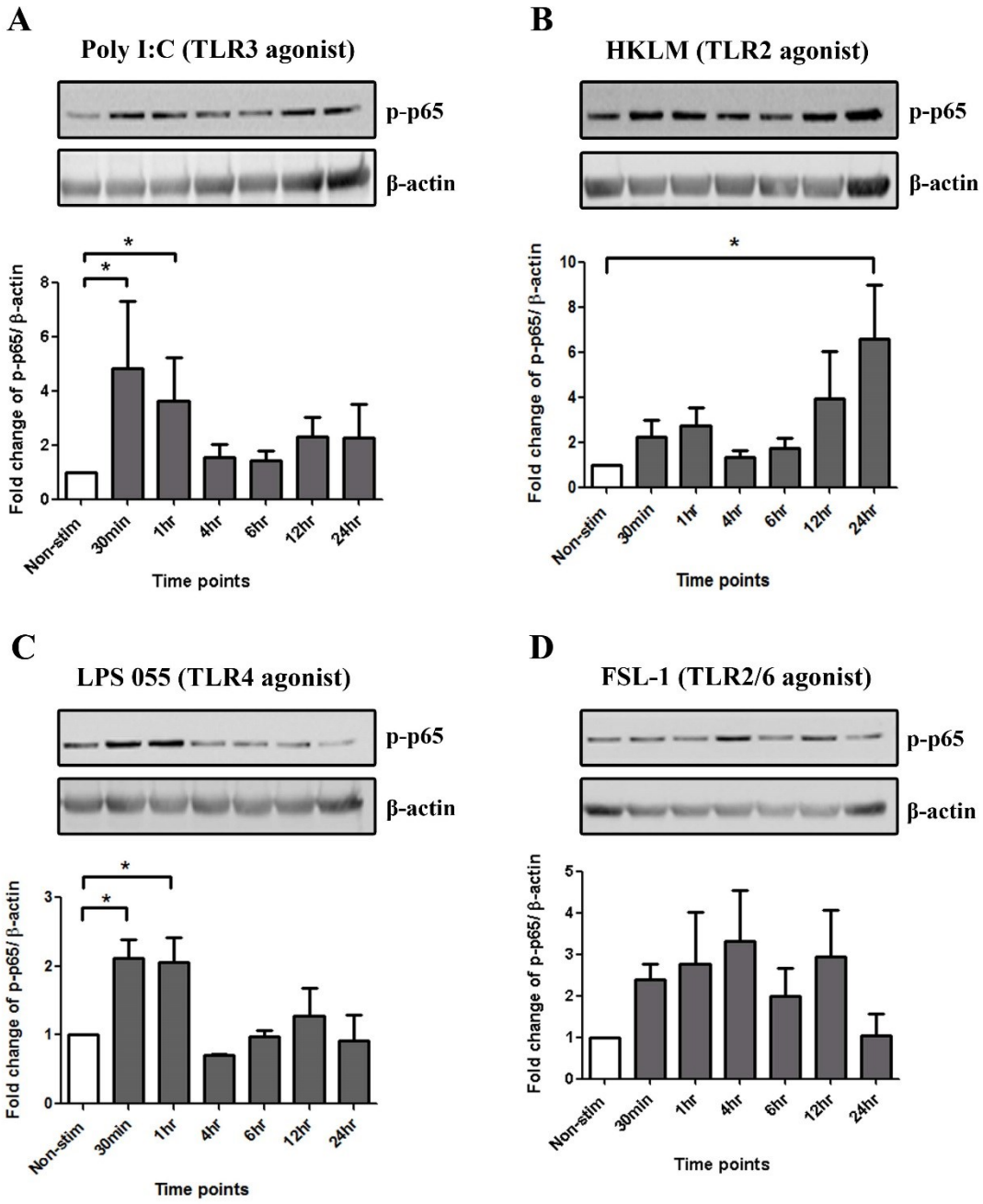


Figure 5.1 Effect of TLR agonists on NF-κB activation in PBMC. PBMCs were extracted using Ficoll and plated on 12 well plates. PBMCs were stimulated with 5μg/ml of poly I:C, 10^8 cells/ml of HKLM, 0.1μg/ml of LPS 055 and 0.1μg/ml of FSL-1 over 24 hours. Whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) as a marker of NF-κB activation. A representative blot was shown above each graph with β-actin as a loading control. NF-κB activation significantly increased at **(A)** 30 minutes and 1 hour (*p<0.05) with poly I:C, **(B)** 24 hours (*p<0.05) with HKLM, **(C)** 30 minutes and 1 hour (*p<0.05) with LPS 055 while **(D)** no statistical significance difference was observed with FSL-1. For statistical analysis a One-way ANOVA was used for normally distributed data and a Kruskal-Wallis test was used for data not normally distributed with *Dunnett's* multiple comparison test; *p<0.05. n=3-6.

5.1.2 TLR3/TLR2 priming in PBMCs

Based on the results from section 5.1.1, 30 minutes of 5μg/ml poly I:C and 1 hour of 10^8 cells/ml HKLM were chosen for the priming experiments. Even though 1 hour HKLM stimulation did not reach statistical significance, the trend shown was a 4-fold increase which was consistent in all replicates. It probably did not reach significance due to the subtle increase (Figure 5.1 B). This is also important not to overload the system and to achieve the minimum effect. Prior to stimulation, the cells were left to rest overnight after plating. Cells were centrifuged and fresh medium was used to re-suspend the pellet before 30 minutes of priming with poly I:C or vehicle control. After 30 minutes, the cells were centrifuged again, and the pellet was re-suspended in a fresh medium to wash out the poly I:C (or vehicle control) before subsequent TLR agonist simulation with either poly I:C (30 minutes) or HKLM (1 hour). Protein was extracted and analysed using western immunoblotting to examine the activation status of NF-κB.

The most cited time points for cytokine production following agonist stimulation in PBMCs are between 12 to 24 hours [398, 399] and also 18 hours [400]. Therefore, 18 hours was chosen as the optimal time point to capture cytokine production in PBMCs. For cytokine, chemokine and PGE2 studies, cells were primed for 30 minutes with poly I:C (or vehicle control) prior to an 18 hour incubation with the TLR2 agonist, HKLM. Supernatant was stored at -80°C until use and analytes were detected using ELISA.

5.1.2.1 NF-κB activation is not augmented with TLR3/2 priming and AP-1 is undetected

In this section, Figure 5.2 is repeated in sections 5.1.3.1 and 5.1.4.1. The focus of priming in each section will be highlighted using different colours on the histograms. TLR3/2 priming effect was shown in red, with the necessary controls.

The basal level of NF-κB was not affected after 2 spinning processes that are required for this experiment. However, this is not applicable when the cells were incubated for longer than 1 hour after 2 spinning processes, although this is not statistically significant (Figure 5.2, 6th bar from the left). AP-1, however was not detected on all blots.

A trend for an increase of NF-κB activation was observed with TLR3 (see # sign) and TLR2 (see solid black line) agonist alone compared to controls, but no statistical significance was achieved. This could be due to the approximately 2-fold increase of NF-κB basal activation after 30 minutes and 1 hour of culturing. Priming with poly I:C before subsequent TLR2 agonist stimuli showed no NF-κB augmentation (see bold black line) (Figure 5.2).

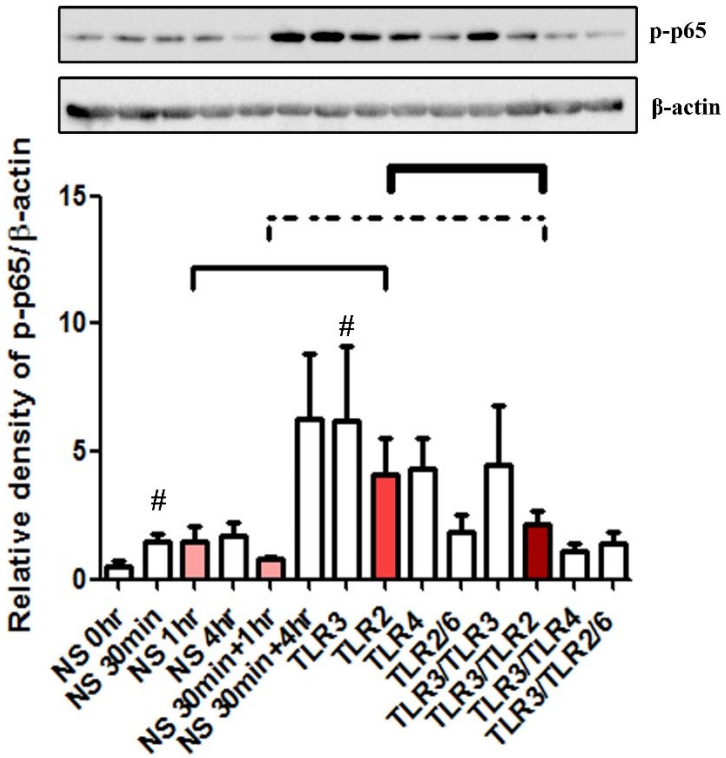


Figure 5.2 Effect of TLR3 priming before TLR2 agonist stimulation on NF-κB activation in PBMC. PBMCs were extracted using Ficoll and plated on 12 well plates. Primed and non-primed samples were assigned. Priming with 5μg/ml of poly I:C (TLR3 agonist) was for 30 minutes. After 30 minutes, the cells were stimulated with either poly I:C or 10^8 cells/ml of HKLM (TLR2 agonist) for 4 hours in both primed and non-primed samples. Whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) as a marker of NF-κB activation. A representative blot is shown with β-actin as a loading control. **Solid black line and # sign** showing comparison between TLR stimulated compared to non-stimulated at the same time point. **Dashed black line** showing priming compared to non-stimulated at the same time point and **bold black line** showing priming compared to non-primed. An increase in NF-κB activation was observed with TLR2 agonist stimulation but no statistical significance was achieved **(*solid black line*)**. Poly I:C priming did not augment any further NF-κB increase with subsequent HKLM agonist stimulation **(*bold black line*)**. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used. n=8.

5.1.2.2 IL-1α, IL-1β, IL-6 and IL-8 are augmented with TLR3/2 priming

Although NF-κB was not augmented with priming, there is a possibility that other transcription factors could be activated and lead to increased production of pro-inflammatory and pro-labour mediators. Therefore, IL-1α, IL-1β, IL-6, IL-8, IFN-γ, IL-12p40, IL-12p70, TNF-α and TNF-β pro-inflammatory mediators were examined. TNF-β was not detected in all treatments and in all biological replicates, hence these data were not presented.

Prior to performing the ELISA, a serial dilution test was conducted using the samples. The chosen dilution was based on the dilution that showed detection of the cytokines within the range as specified by the manufacturer. Therefore, the samples were diluted 1:5 for TNF-β and IL12-p40, 1:10 for IFN-γ, IL-6, TNF-α, IL-12p70, IL-1β, IL-1α while 1:50 dilutions for IL-8.

The vehicle control showed no difference in cytokine production over time and poly I:C stimulation alone showed no statistically significant increase in any of the cytokines.

However, IL-1α ($^{\dagger\dagger\dagger}p<0.001$), IL-1β ($^{\dagger\dagger}p<0.01$), IL-6, IL-8, TNF-α and IL-12p70 ($^{\dagger\dagger\dagger}p<0.001$) production were increased following HKLM stimulation alone (Figure 5.3).

Poly I:C priming prior to HKLM stimulation significantly augmented IL-1α ($^{\S\S\S}p<0.001$) (Figure 5.3 A), IL-1β ($^{\S\S}p<0.01$) (Figure 5.3 B), IL-6 ($^{\S\S\S}p<0.001$) (Figure 5.3 C) and IL-8 ($^{\S}p<0.05$) (Figure 5.3 D) compared to HKLM stimulation alone. No augmented response was observed in TNF-α (Figure 5.3 E), IL-12p40 (Figure 5.3 F), IFN-γ (Figure 5.3 G) or IL-12p70 (Figure 5.3 H).

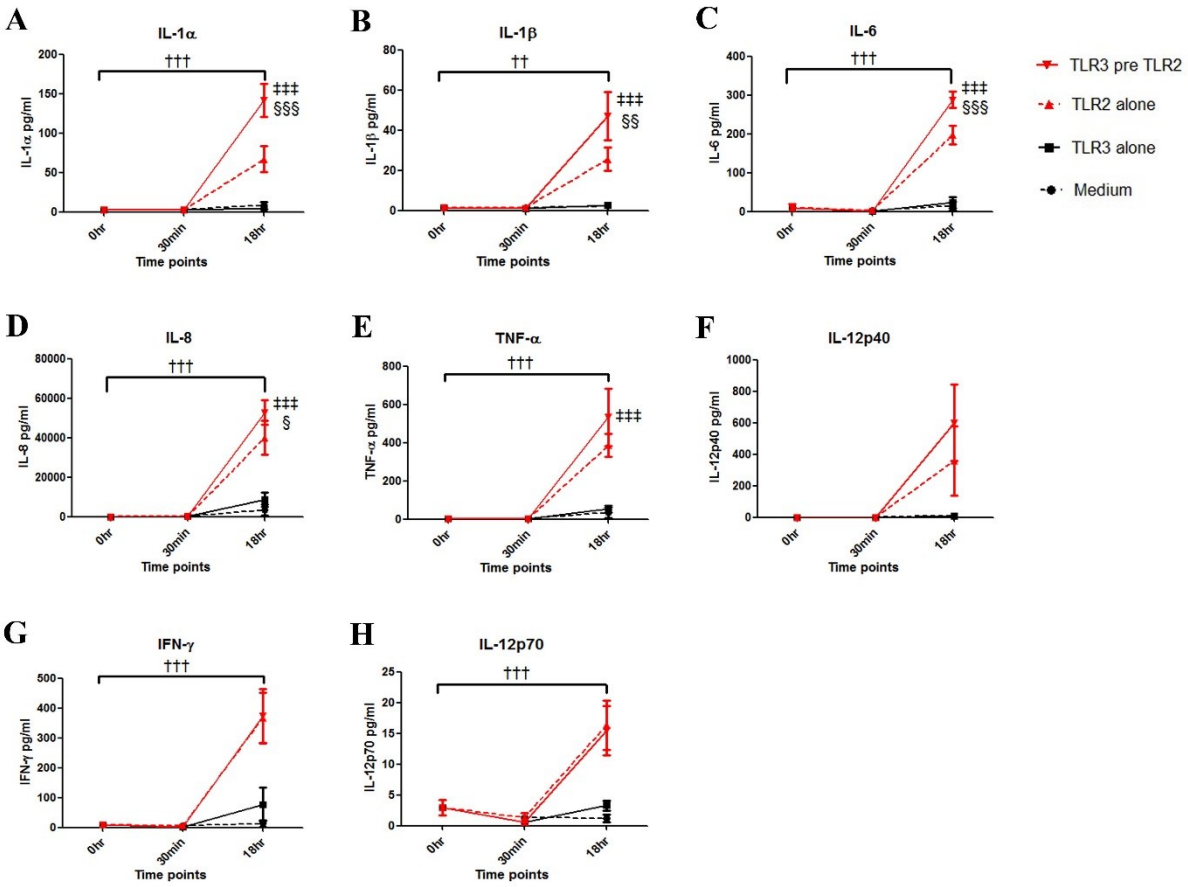


Figure 5.3 The effect of TLR3/2 priming on pro-inflammatory cytokines. PBMCs were primed with 5μg/ml of poly I:C for 30 minutes. After 30 minutes, cells were stimulated with 10^8 cells/ml of HKLM for 18 hours. Supernatant was collected and an ELISA was performed to determine the level of each cytokines. TLR2 agonist stimulation alone increased cytokine production ($^{\dagger\dagger\dagger}p<0.001$). Priming the cells with poly I:C before HKLM stimulation significantly augmented **(A)** IL-1α, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-8 and **(E)** TNF-α production. No significant increase was observed in **(F)** IL-12p40. No further augmentation was observed in **(G)** IFN-γ and **(H)** IL-12p70. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3-4. $^{\ddagger\ddagger\ddagger}p<0.001$ = effect of TLR3 pre TLR2 compared to TLR3 alone and $^{\S}p<0.05$ = effect of TLR3 pre TLR2 compared to TLR2 alone.

5.1.2.3 MIP-1α and MIP-1β are augmented with TLR3/2 priming

The chemokines MIP-1α, MIP-1β (1:5 dilution) and RANTES (1 in 10 dilution) were analysed.

A non-significant increase in RANTES production was observed with the vehicle control over time, however, a further increase was seen with poly I:C stimulation alone (**p<0.01 at 30 minutes) compared to 0hr. No significant increase in MIP-1α and MIP-1β was seen with poly I:C treatment alone.

HKLM stimulation alone significantly increased MIP-1α ($^{\dagger\dagger\dagger}p$<0.001), MIP-1β ($^{\dagger\dagger}p$<0.01) and RANTES ($^{\dagger}p$<0.05) production over the time course (Figure 5.4).

Poly I:C priming prior to HKLM stimulation significantly augmented the production of MIP-1α ($^{\S\S}p$<0.01) (Figure 5.4 A) and MIP-1β ($^{\S}p$<0.05) (Figure 5.4 B) but not RANTES (Figure 5.4 C).

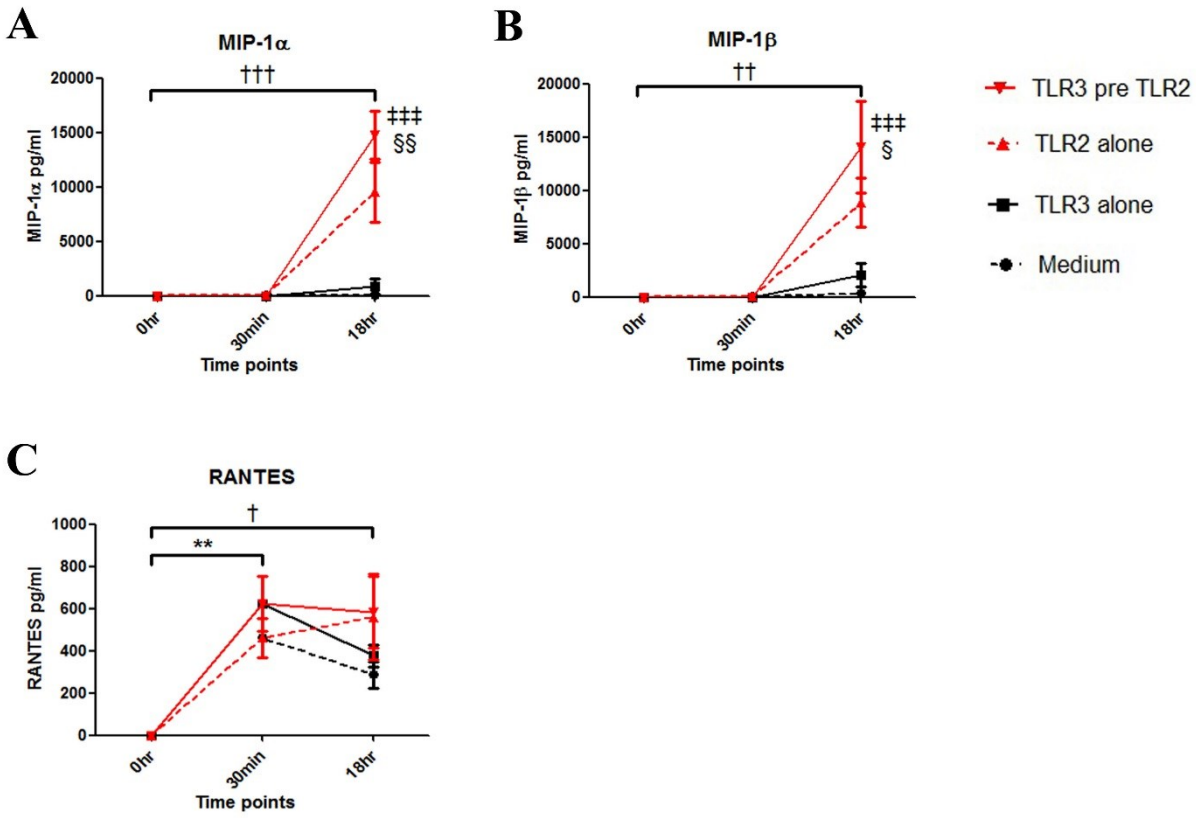


Figure 5.4 The effect of TLR3/2 priming on macrophage inflammatory proteins (MIP) and RANTES. PBMCs were primed with 5μg/ml of poly I:C for 30 minutes. After 30 minutes, cells were stimulated with 10^8 cells/ml HKLM for 18 hours. Supernatant was collected, and an ELISA was performed to determine the level of MIPs and RANTES chemokines. TLR2 agonist stimulation alone increased chemokine production and TLR3 agonist stimulation increased RANTES production. Priming with poly I:C significantly augmented further **(A)** MIP-1α and **(B)** MIP-1β production with subsequent TLR2 stimulation. However, no further augmented **(C)** RANTES production was observed. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3-4. $^{‡‡‡}p<0.001$ = effect of TLR3 pre TLR2 compared to TLR3 alone and $^{§}p<0.05$ = effect of TLR3 pre TLR2 compared to TLR2 alone.

5.1.2.4 IL-10 is augmented with TLR3/2 priming

Anti-inflammatory cytokines IL-1RA, IL-4, IL-10, IFN-α and IFN-β are key players in providing a balanced protective response to both viral and bacterial pathogens. A dilution test was conducted, and the chosen dilution was based on the dilution that showed detection of the cytokines within the range as specified by the manufacturer. Therefore, concentrations were determined from supernatant diluted to 1:5. IFN-α, IFN-β and IL-4 production were undetectable in all experimental conditions and in all biological replicates.

No significant differences in the production of the cytokines were seen in either the vehicle control or with poly I:C treatment.

Increased IL-10 ($^{\dagger\dagger\dagger}p<0.001$) and IL-1RA ($^{\dagger\dagger\dagger}p<0.001$) productions were seen with HKLM stimulation alone at 18 hrs (Figure 5.5).

Priming with poly I:C before HKLM stimulation only augmented IL-10 ($^{\S\S\S}p<0.001$) (Figure 5.5 A) production but not IL-1RA (Figure 5.5 B) compared to HKLM stimulation alone.

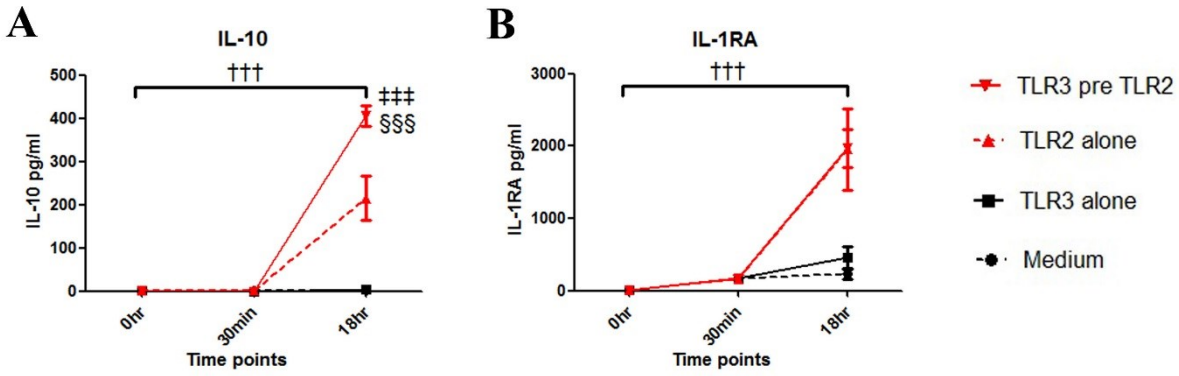


Figure 5.5 The effect of TLR3/2 priming on anti-inflammatory cytokines. PBMCs were primed with 5μg/ml of poly I:C for 30 minutes. After 30 minutes, cells were stimulated with 10^8 cells/ml HKLM for 18 hours. Supernatant was collected, and an ELISA was performed to determine the level of each cytokine. TLR2 agonist stimulation alone increased the cytokines ($^{\dagger\dagger\dagger}p<0.001$). Priming with poly I:C significantly augmented further **(A)** IL-10 production but no further increase of **(B)** IL-1RA production was observed with subsequent TLR2 stimulation. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3-4. $^{\ddagger\ddagger\ddagger}p<0.001$ = effect of TLR3 pre TLR2 compared to TLR3 alone and $^{\S\S\S}p<0.001$ = effect of TLR3 pre TLR2 compared to TLR2 alone.

5.1.2.5 PGE2 production is augmented with TLR3/2 priming

PGE2 production with priming was also evaluated using an ELISA. No significant increase in PGE2 production was seen with poly I:C and HKLM stimulation alone. However, priming PBMCs with poly I:C prior to HKLM stimulation significantly augmented PGE2 production (***p<0.001) (Figure 5.6) compared to individual stimuli.

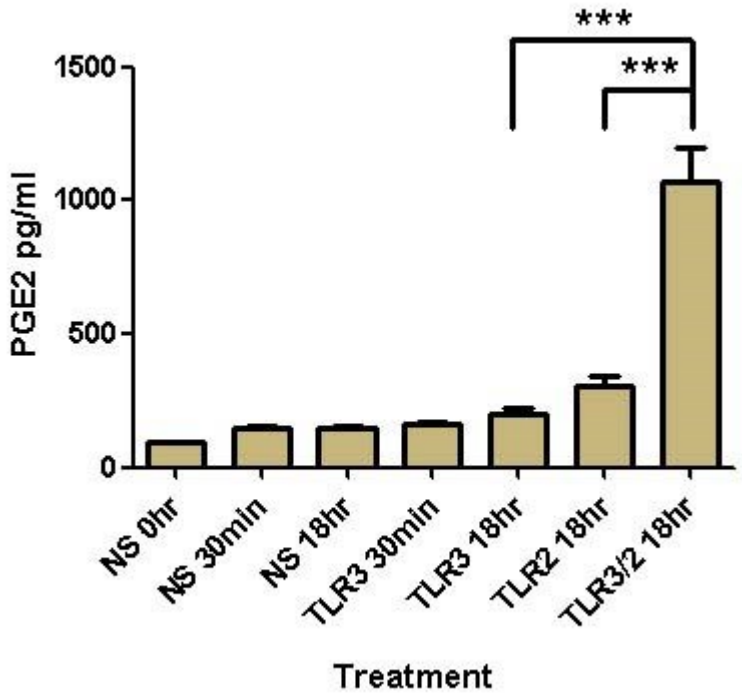


Figure 5.6 The effect of TLR3 priming prior to TLR2 agonist stimulation on PGE2 production in PBMCs. PBMCs were primed with 5µg/ml of poly I:C for 30 minutes. After 30 minutes, cells were stimulated with either poly I:C or 10^8 cells/ml of HKLM for 18 hours. Supernatant was collected, and an ELISA was performed to determine the level of PGE2. TLR3 priming significantly increased PGE2 production after TLR2 (***p<0.001) agonist stimulation compared to the individual TLR agonist stimulation alone. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3. ***p<0.001 = effect of TLR3 pre TLR2 compared to TLR3 and TLR2 alone.

5.1.3 TLR3/TLR4 priming in PBMCs

Since TLR3/4 priming augmented cytokine production in other cell types in Chapter 4, the same concentration of LPS, 0.1μg/ml was used to examine if the same priming effect could be seen in PBMCs. Hence, the same priming experiment was conducted as in section 5.2 above using 0.1μg/ml LPS 055 for 1 hour looking at NF-κB and AP-1 activation.

For cytokine, chemokine and PGE2 studies, cells were primed with poly I:C for 30 minutes and then stimulated with LPS 055 for 18 hours. ELISA was used to quantify the analytes.

5.1.3.1 NF-κB activation is not augmented with TLR3/4 priming and AP-1 is undetected

The same figure from section 5.1.2.1 (Figure 5.2) was repeated in this section but with blue-coloured histograms to highlight the effect of TLR3/4 priming with the necessary controls.

No significant changes were observed in NF-κB activation at the basal level over time in the control samples. LPS stimulation alone showed a 5-fold yet not statistically significant increase (see solid black line). No significant increase in NF-κB was seen with poly I:C treatment alone (see # sign). Priming with poly I:C before subsequent TLR4 agonist stimuli showed no NF-κB augmentation (see bold black line) (Figure 5.7). AP-1 was not detected in these samples.

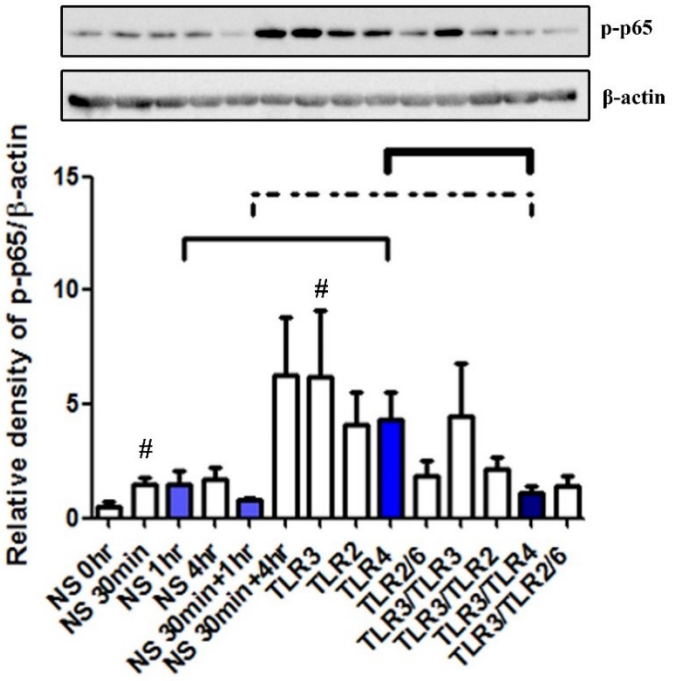


Figure 5.7 Effect of TLR3 priming before TLR4 agonist stimulation on NF-κB activation in PBMCs. PBMCs were isolated using Ficoll and plated on 12 well plates. Primed and non-primed samples were assigned. 5μg/ml of poly I:C (TLR3 agonist) was used to stimulate cell for 30 minutes. After 30 minutes, the cells were stimulated with either poly I:C or 0.1μg/ml of LPS 055 (TLR4 agonist) for 1 hour in both primed and non-primed samples. Whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) as a marker of NF-κB activation. A representative blot is shown with β-actin as a loading control. **Solid black line and # sign** showing comparison between TLR stimulated compared to non-stimulated at the same time point. **Dashed black line** showing priming compared to non-stimulated at the same time point and black bold line showing priming compared to non-primed. A non-significant increase in NF-κB activation was observed with individual TLR4 agonist stimulation (***solid black line***). Poly I:C priming does not augment any further NF-κB increase with subsequent LPS 055 agonist stimulation (***bold black line***). For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used. n=8.

5.1.3.2 Pro-inflammatory cytokine production is not augmented with TLR3/4 priming

Although no augmentation of NF-κB activation was observed with TLR3/4 priming, other transcription factors could be activated, leading to augmented pro-inflammatory and pro-labour mediator production. The same pro-inflammatory cytokines were analysed with the same sample dilution as mentioned in section 5.2.2.

No difference in the production of the cytokines was seen with the vehicle control and poly I:C stimulation alone. LPS stimulation alone significantly increased the production of IL-1α ($^{\dagger}p<0.05$) (Figure 5.8 A), IL-6 (Figure 5.8 C), IL-8 ($^{\dagger\dagger\dagger}p<0.001$) (Figure 5.8 D), TNF-α ($^{\dagger}p<0.05$) (Figure 5.8 E), IFN-γ ($^{\dagger\dagger\dagger}p<0.001$) (Figure 5.8 F), IL-12p40 ($^{\dagger}p<0.05$) (Figure 5.8 G) and IL-12p70 ($^{\dagger\dagger}p<0.01$) (Figure 5.8 H) compared to basal levels at 0hr (Figure 5.8 B).

However, poly I:C stimulation prior to LPS stimulation showed no further augmentation of any of the cytokines compared to LPS stimulation alone. TNF-β cytokine production was undetectable in all samples.

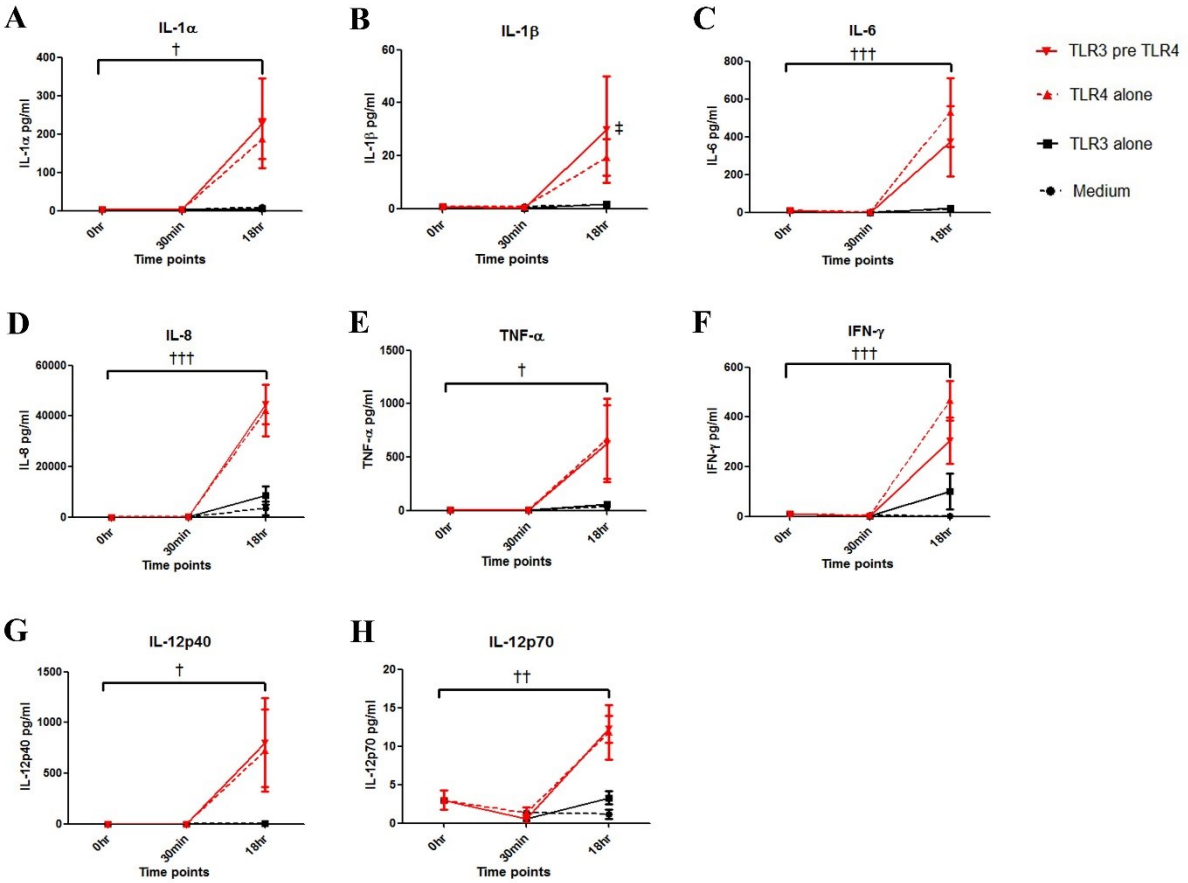


Figure 5.8 The effect of TLR3/4 priming on the pro-inflammatory cytokines production in PBMCs. PBMCs were primed with 5μg/ml of poly I:C for 30 minutes. After 30 minutes, cells were stimulated with 0.1μg/ml of LPS 055 for 18 hours. Supernatant was collected, and an ELISA was performed to determine the level of each cytokine. TLR4 agonist stimulation alone increased the cytokine level ($^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$). Priming the cells with poly I:C before LPS stimulation had no augmented response on either **(A)** IL-1α, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-8 and **(E)** TNF-α, **(F)** IFN-γ, **(G)** IL-12p40 or **(H)** IL-12p70. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3-4. $^{\ddagger}p<0.05$ = effect of TLR3 pre TLR4 compared to TLR3 alone.

5.1.3.3 Chemokine production is not augmented with TLR3/4 priming

MIP-1α, MIP-1β (1:5 dilution) and RANTES (1 in 10 dilution) were also analysed.

No significant increase in MIP-1α, MIP-1β or RANTES was seen with vehicle control or poly I:C stimulation alone (Figure 5.9).

LPS stimulation alone significantly increased the production of MIP-1α (Figure 5.9 A), MIP-1β (Figure 5.9 B) ($^{\dagger\dagger\dagger}p<0.001$) and RANTES ($^{\dagger}p<0.05$) (Figure 5.9 C) production compared to basal levels at 0hr.

Poly I:C priming before LPS stimulation showed no further augmentation in any of the chemokines production compared to LPS stimulation alone.

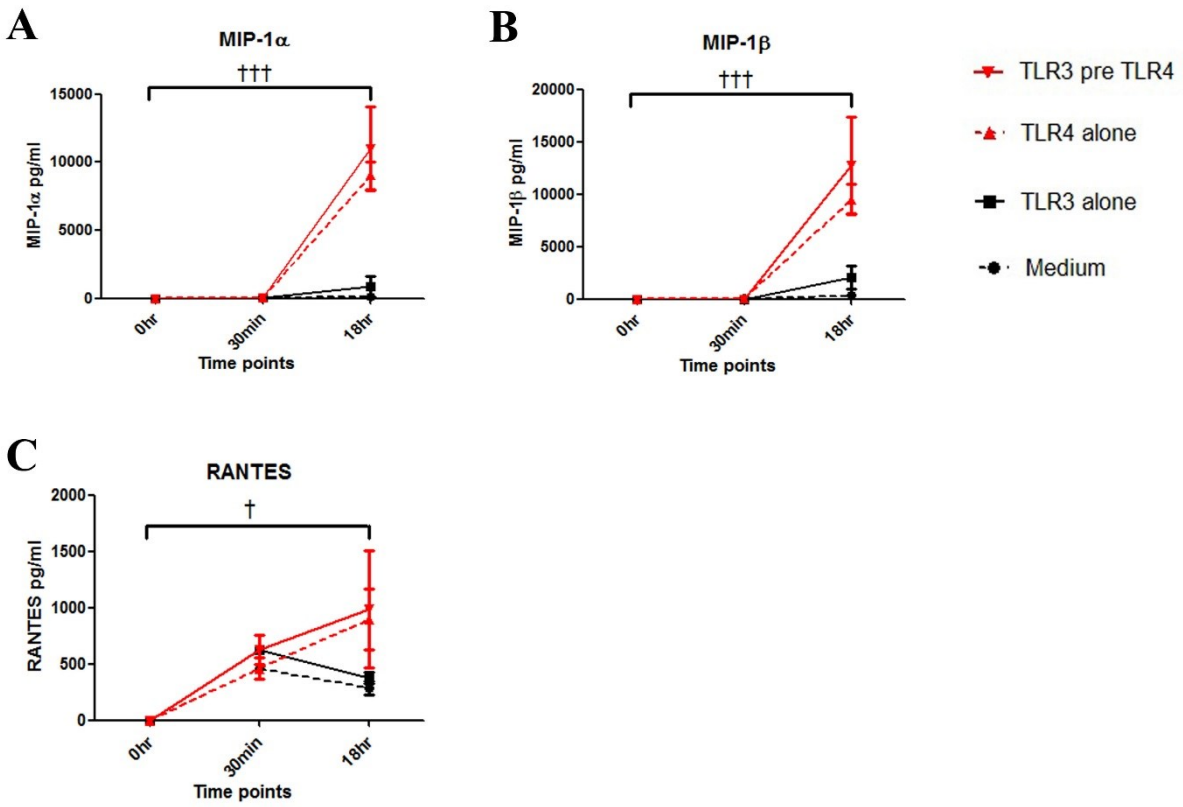


Figure 5.9 The effect of TLR3/4 priming on macrophage inflammatory proteins (MIP) and RANTES in PBMCs. PBMCs were primed with 5μg/ml of poly I:C for 30 minutes. After 30 minutes, cells were stimulated with 0.1μg/ml of LPS for 18 hours. Supernatant was collected, and an ELISA was performed to determine the level of MIP and RANTES chemokines. TLR4 agonist stimulation alone increased the chemokine concentrations ($^{\dagger}p<0.05$, $^{\dagger\dagger\dagger}p<0.001$). Priming with poly I:C did not augmented further either **(A)** MIP-1α, **(B)** MIP-1β or **(C)** RANTES production with subsequent TLR4 stimulation. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3-4.

5.1.3.4 Anti-inflammatory cytokine production is not augmented with TLR3/4 priming

The same anti-inflammatory cytokines listed in section 5.1.2.4 were analysed (1:5 dilution). However, only IL-10 and IL-1RA were detectable in all experimental conditions and in all biological replicates.

No differences were detected in the cytokines following either vehicle or poly I:C treatment alone (Figure 5.10).

IL-10 ($^{\dagger\dagger\dagger}p<0.001$) (Figure 5.10 A) and IL-1RA ($^{\dagger}p<0.05$) (Figure 5.10 B) significantly increased with LPS stimulation alone compared to 0hr. However, no augmentation was observed with TLR3/4 priming.

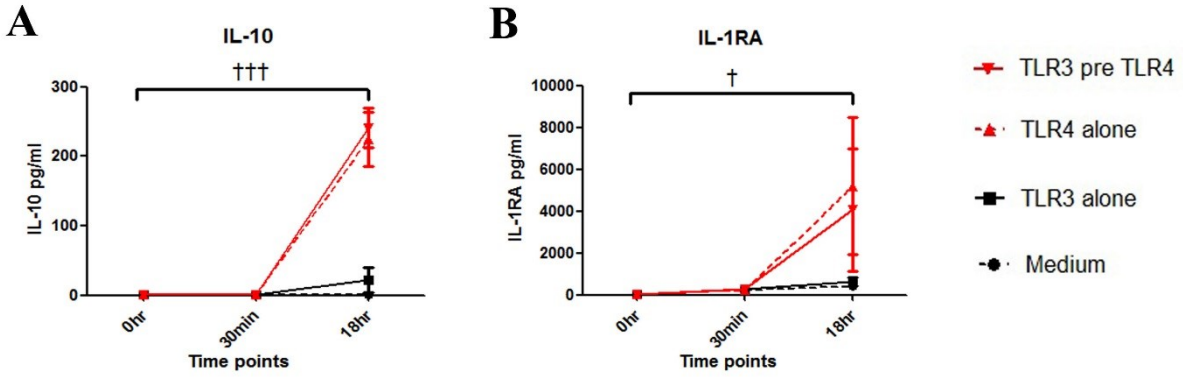


Figure 5.10 The effect of TLR3/4 priming on anti-inflammatory cytokines. PBMCs were primed with 5µg/ml of poly I:C for 30 minutes. After 30 minutes, cells were stimulated with 0.1µg/ml of LPS for 18 hours. Supernatant was collected, and an ELISA was performed to determine the level of each cytokine. TLR4 agonist stimulation alone increased cytokines levels ($^{\dagger}p<0.05$, $^{\dagger\dagger\dagger}p<0.001$). Priming with poly I:C did not augment further **(A)** IL-10 or **(B)** IL-1RA production with subsequent TLR4 stimulation. For statistical analysis One-way ANOVA with *Bonferroni*'s multiple comparison test was used; n=3-4.

5.1.3.5 PGE2 production is augmented with TLR3/4 priming

PGE2 production was measured using ELISA. LPS stimulation alone increased the production of PGE2 (*p<0.05) whilst no difference was observed in poly I:C stimulation alone compared to the basal level. Priming PBMCs with poly I:C prior to LPS stimulation significantly augmented PGE2 production compared to LPS (**p<0.01) and poly I:C (***p<0.001) stimulation alone (Figure 5.11).

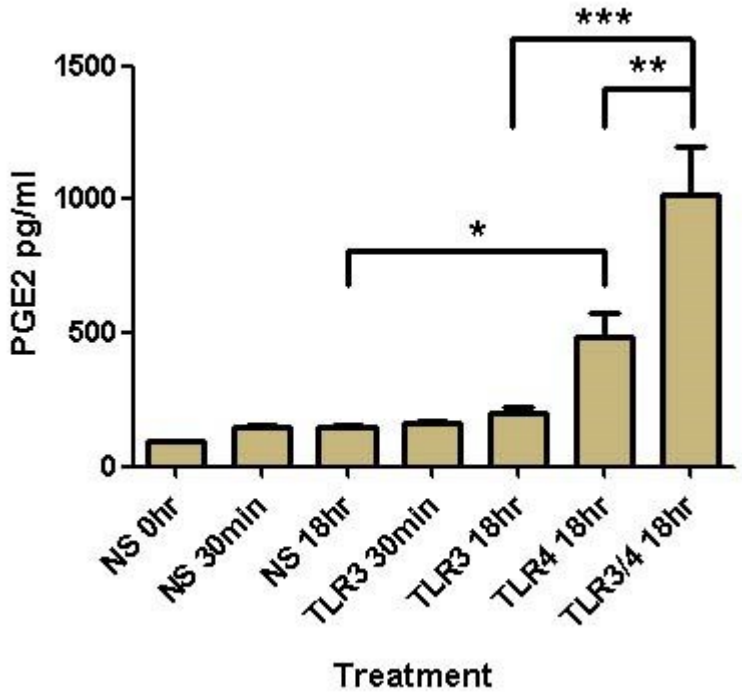


Figure 5.11 The effect of TLR3 priming prior to TLR4 agonist stimulation on PGE2 production in PBMCs. PBMCs were primed with 5µg/ml of poly I:C for 30 minutes. After 30 minutes, cells were stimulated with either poly I:C or 0.1µg/ml of LPS 055 for 18 hours. Supernatant was collected, and an ELISA was performed to determine the level of PGE2. TLR4 agonist stimulation increased PGE2 production (*p<0.05). TLR3 priming significantly increased PGE2 production after TLR4 (***p<0.001) agonist stimulation compared to the individual TLR agonist stimulation alone. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3. ***p<0.001 = effect of TLR3 pre TLR4 compared to TLR3 alone, **p<0.01 = effect of TLR3 pre TLR4 compared to TLR4 alone.

5.1.4 TLR3/TLR2/6 priming in PBMCs

Unlike in other cell types (Chapter 4), TLR3/2 priming significantly augmented pro-inflammatory, pro-labour and anti-inflammatory production in PBMCs. This demonstrates that there are differences in the responses to different types of gram positive bacterial components in this cell type. It is therefore intriguing to see the responses to TLR2/6 agonist (FSL-1) stimulation in PBMCs. Poly I:C priming prior to FSL-1 stimulation was performed in PBMCs using 0.1μg/ml of FSL-1 for 4 hours to examine the effect on NF-κB and AP-1 activation. Although no statistical significance was achieved at this time point, 2 replicates out of 3 showed a robust response maximally at 4 hours.

For cytokine, chemokine and PGE2 studies, cells were primed with poly I:C for 30 minutes and then stimulated with FSL-1 for 18 hours. ELISA was used to quantify the analytes.

5.1.4.1 NF-κB activation is not augmented with TLR3/TLR2/6 priming and AP-1 is undetected

Figure 5.2 from section 5.1.2.1 was repeated in this section but with purple-coloured histograms to highlight the effect of TLR3 priming prior to TLR2/6 stimulation with the necessary controls.

No significant changes were observed in NF-κB activation at the basal level over time in the control samples. No significant changes increase in FSL-1 stimulation alone (see solid black line). No significant increase in NF-κB was seen with poly I:C treatment alone (see # sign). Priming with poly I:C before subsequent TLR2/6 agonist stimuli showed no NF-κB augmentation (see bold black line) (Figure 5.12). AP-1 was undetected in these samples.

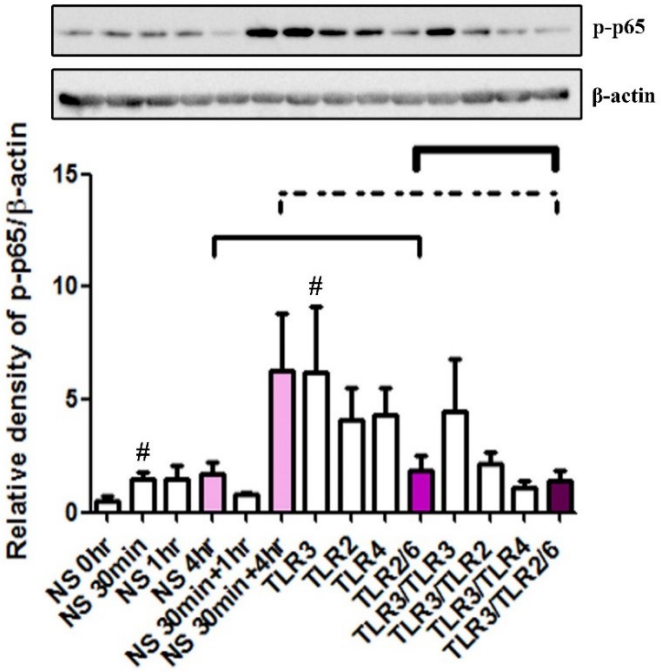


Figure 5.12 Effect of TLR3 priming before TLR2/6 agonist stimulation on NF-κB activation in PBMCs. PBMCs were isolated using Ficoll and plated on 12 well plates. Primed and non-primed samples were assigned. 5μg/ml of poly I:C (TLR3 agonist) was used to prime samples for 30 minutes. After 30 minutes, the cells were stimulated with either poly I:C or 0.1μg/ml of FSL-1 (TLR2/6 agonist) for 4 hours in both primed and non-primed samples. Whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) as a marker of NF-κB activation. A representative blot is shown with β-actin as a loading control. **Solid black line and # sign** shows the comparison between TLR stimulated and non-stimulated cells at the same time point. **Dashed black line** shows priming compared to non-stimulated at the same time point and **black bold line** shows priming compared to non-primed cells. No increase in NF-κB activation was observed with individual TLR2/6 agonist stimulation (***solid black line***). Poly I:C priming did not augment any further NF-κB increase with subsequent FSL-1 agonists stimulation (***bold black line***). For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used. n=8.

5.1.4.2 IL-1β, IL-6, IL-8 and TNF-α are augmented with TLR3/TLR2/6 priming

The same pro-inflammatory cytokines were analysed as in section 5.1.2.2 with the same dilutions.

Vehicle control, poly I:C and FSL-1 stimulation alone showed no difference in cytokine production at all time points (Figure 5.13).

Poly I:C priming prior to FSL-1 stimulation augmented IL-1β ($^{\S}p<0.05$) (Figure 5.13 B), IL-6 ($^{\S}p<0.05$) (Figure 5.13 C), IL-8 ($^{\S\S}p<0.01$) (Figure 5.13 D) and TNF-α ($^{\S}p<0.05$) (Figure 5.13 E). No augmentation was seen in IL-1α (Figure 5.13 A), IFN-γ (Figure 5.13 F), IL-12p40 (Figure 5.13 G) or IL-12p70 (Figure 5.13 H) production. TNF-β was undetectable in all experimental conditions and in all biological replicates.

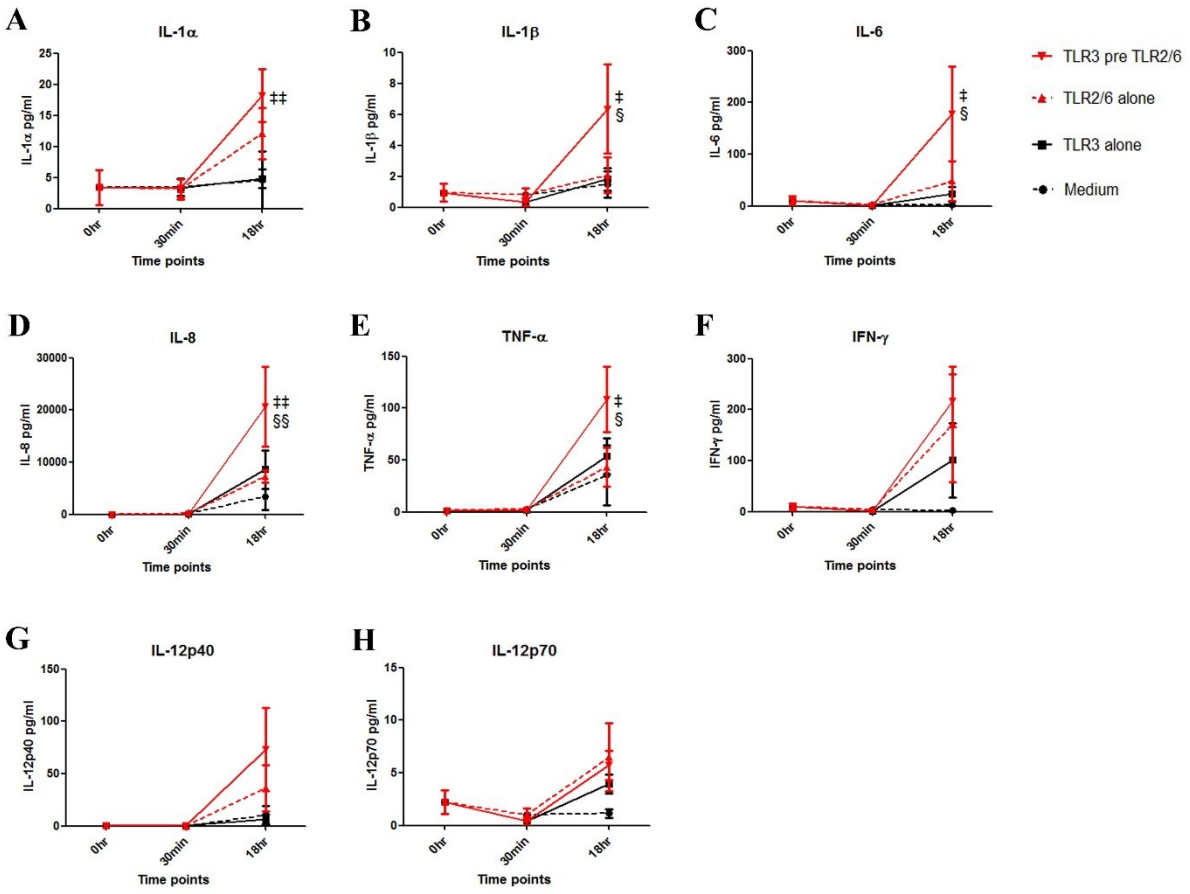


Figure 5.13 The effect of TLR3/6 priming on the pro-inflammatory cytokines. PBMCs were primed with 5μg/ml of poly I:C for 30 minutes. After 30 minutes, cells were stimulated with 0.1μg/ml of FSL-1 for 18 hours. Supernatant was collected, and an ELISA was performed to determine the level of each cytokines. Priming the cells with poly I:C before FSL-1 stimulation augmented **(A)** IL-1α, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-8 and **(E)** TNF-α production. However, no augmented response was observed with (**F)** IFN-γ, **(G)** IL-12p40 or **(H)** IL-12p70. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3-4. ‡p<0.05 = effect of TLR3 pre TLR2/6 compared to TLR3 alone. §p<0.05 = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone.

5.1.4.3 MIP-1α and MIP-1β are augmented with TLR3/TLR2/6 priming

Three chemokines; MIP-1α, MIP-1β (1:5 dilution) and RANTES (1 in 10 dilution) were also analysed.

No difference in the levels of these chemokines were seen with the vehicle control. RANTES production increased significantly with poly I:C (***p<0.001 at 30 minutes and *p<0.05 at 18 hours) and FSL-1 ($^{\dagger\dagger}p$<0.01) stimulation alone compared to basal levels at 0hr (Figure 5.14).

Priming with poly I:C prior to FSL-1 stimulation significantly augmented the production of MIP-1α (Figure 5.14 A) and MIP-1β ($^{\S\S}p$<0.01) (Figure 5.14 B) but not RANTES (Figure 5.14 C).

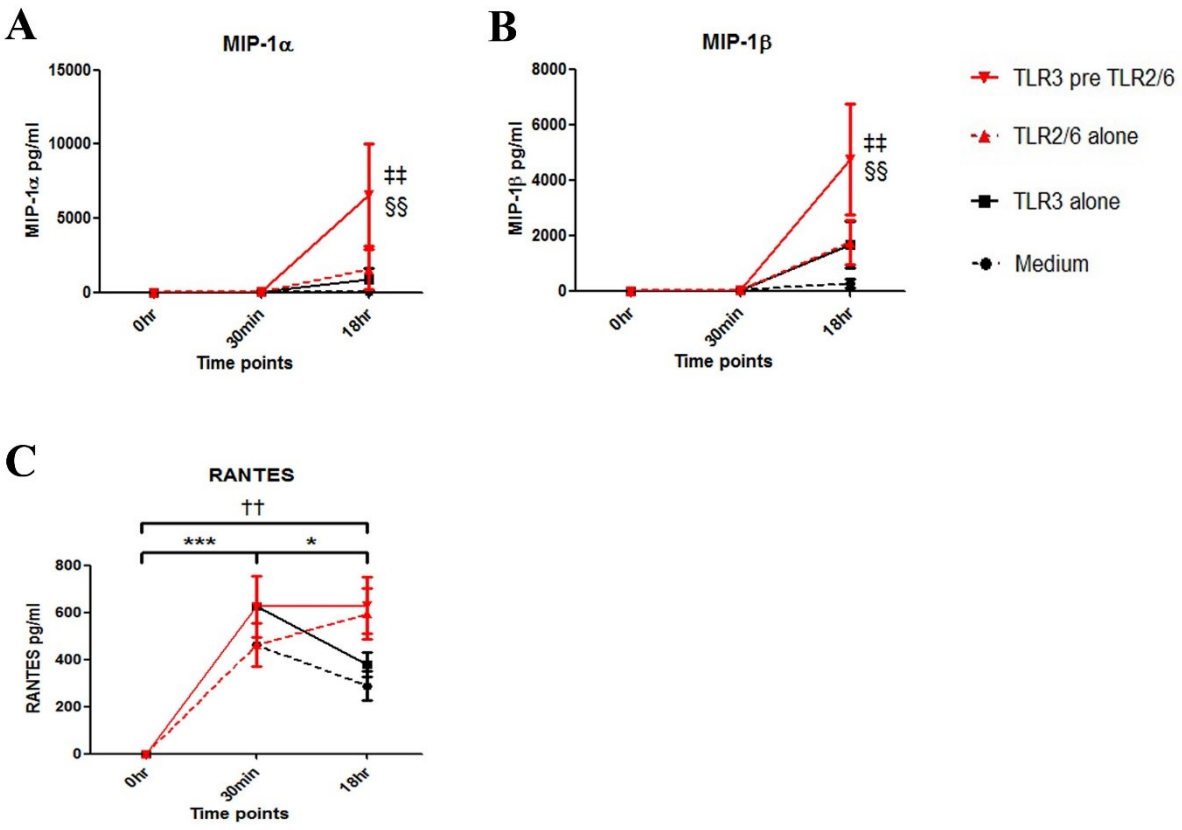


Figure 5.14 The effect of TLR3/6 priming on macrophage inflammatory proteins (MIP) and RANTES. PBMCs were primed with 5μg/ml of poly I:C for 30 minutes. After 30 minutes, cells were stimulated with 0.1μg/ml of FSL-1 for 18 hours. Supernatant was collected, and an ELISA was performed to determine the level of MIP and RANTES. TLR2/6 and TLR3 agonist stimulation alone increased RANTES production. Priming with poly I:C augmented **(A)** MIP-1α and **(B)** MIP-1β production but no further augmentation was seen with **(C)** RANTES. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3-4. ‡‡p<0.01 = effect of TLR3 pre TLR2/6 compared to TLR3 alone. §§p<0.01 = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone.

5.1.4.4 IL-10 is augmented with TLR3/TLR2/6 priming

Anti-inflammatory cytokines listed in section 5.1.2.4 were analysed using the same dilution. However, only IL-10 and IL-1RA were detectable in all experimental conditions and in all biological replicates.

No statistically significant increase in IL-1RA production was seen in the vehicle control. FSL-1 alone only increased IL-1RA production ($^{\dagger}p<0.05$). Poly I:C alone only increased IL-10 production (Figure 5.15).

Poly I:C priming prior to FSL-1 stimulation only augmented IL-10 production ($^{\S\S\S}p<0.001$) (Figure 5.15 A) and not IL-1RA (Figure 5.15 B).

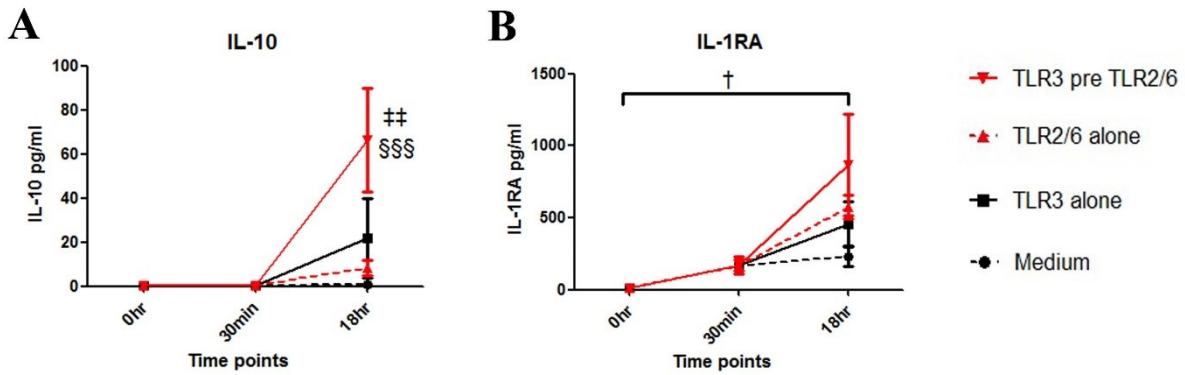


Figure 5.15 The effect of TLR3/6 priming on anti-inflammatory cytokines. PBMCs primed with 5µg/ml of poly I:C for 30 minutes. After 30 minutes, cells were stimulated with 0.1µg/ml of FSL-1 for 18 hours. Supernatant was collected, and an ELISA was performed to determine the level of each cytokine. TLR2/6 agonist stimulation alone increased the IL-1RA level ($^{\dagger}p<0.05$). Priming with poly I:C augmented further **(A)** IL-10 but not **(B)** IL-1RA production with subsequent TLR2/6 stimulation. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3-4. $^{‡‡}p<0.01$ = effect of TLR3 pre TLR2/6 compared to TLR3 alone. $^{\S\S\S}p<0.001$ = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone.

5.1.4.5 PGE2 production is augmented with TLR3/TLR2/6 priming

FSL-1 stimulation alone increased PGE2 production (*p<0.05) compared to the control. Priming PBMCs with poly I:C prior to FSL-1 stimulation significantly augmented PGE2 production compared to poly I:C and FSL-1 stimulation alone (***p<0.001) (Figure 5.16).

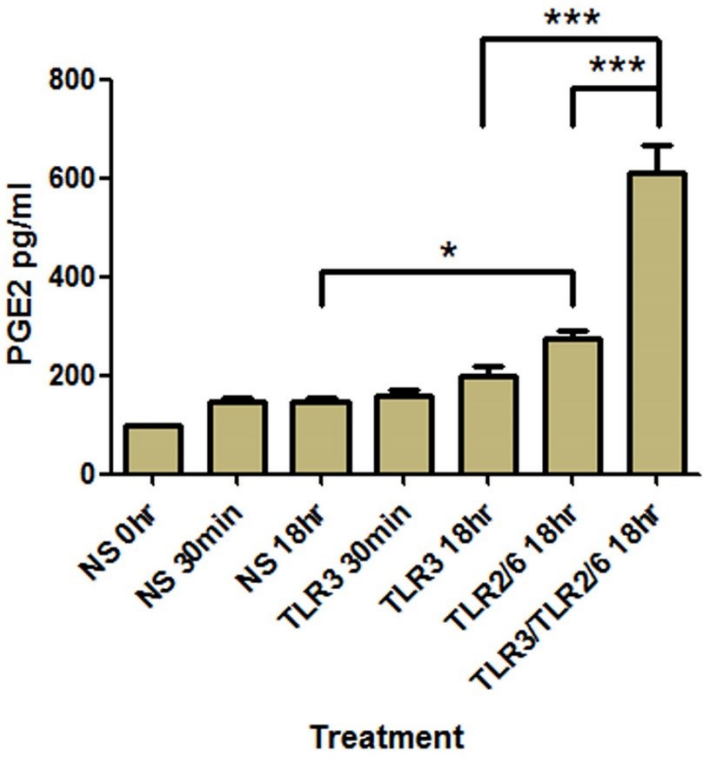


Figure 5.16 The effect of TLR3 priming prior to TLR2/6 agonist stimulation on PGE2 production in PBMCs. PBMCs were extracted and primed with 5µg/ml of poly I:C for 30 minutes. After 30 minutes, cells were stimulated with either poly I:C or 0.1µg/ml of FSL-1 for 18 hours. Supernatant was collected, and an ELISA was performed to determine the level of PGE2. TLR2/6 agonist stimulation increased PGE2 production (*p<0.05). TLR3 priming significantly increased PGE2 production after TLR2/6 (***p<0.001) agonist stimulation compared to the individual TLR agonist stimulation alone. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3. ***p<0.001 = effect of TLR3 pre TLR2/6 compared to TLR3 and TLR2/6 alone.

5.2 Placental explants

5.2.1 TLR3/TLR2 priming in placental explants

Besides the effects of priming in PBMCs, the effect of priming in placental tissue was also evaluated in order to examine the effect of haematogenous spread of multi-pathogen infection. 25μg/ml of poly I:C concentration was chosen based on previous studies [218, 333] whereas 10^8 cells/ml of HKLM was used in line with the recommendation in the datasheet and published concentration used in the choriocarcinoma cell line (BeWo cells) [401]. Three pieces of explants weighing 20-30mg were cultured in each well of 12 well plates and assigned to being primed or non-primed. Explants were cultured until day 4 and then primed on day 5 with poly I:C for 4 hours. After 4 hours, medium was changed and stimulated with HKLM for another 4 hours. Explants were homogenised and extracted protein was subjected to western immunoblotting to determine the activation status of NF-κB and AP-1.

For cytokine, chemokine and PGE2 studies, cells were primed with poly I:C for 24 hours following previously published 24 hours poly I:C stimulation on the placental explant looking at the production of cytokines [218]. The explants were then stimulated with HKLM for 24 hours. ELISA was used to quantify the analytes.

5.2.1.1 NF-κB or AP-1 activation is not augmented with TLR3/2 priming

Figure 5.17 was duplicated in the priming experiments in this section using coloured-histograms to differentiate each priming experiment. In this section, red-coloured histograms represent TLR3/TLR2 priming with necessary controls. Poly I:C stimulation alone showed a significant increase in NF-κB (**p<0.01) activation whilst no statistically significant increase was seen with HKLM stimulation in either NF-κB or AP-1 activation (see solid black line). Poly I:C priming showed no augmentation on NF-κB (Figure 5.17 A) or AP-1 (Figure 5.17 B) activation with subsequent HKLM stimulation (see bold black line).

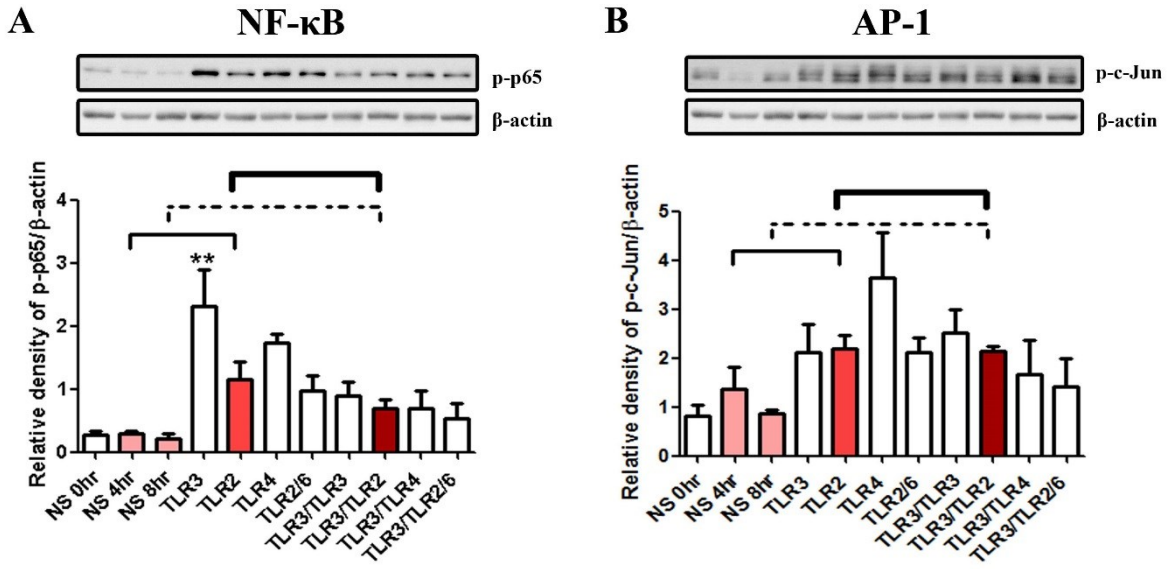


Figure 5.17 Effect of TLR3/2 priming on NF-κB and AP-1 activation in placental explants. Placenta tissue was dissected into small pieces and 3 small pieces weighing 20-30mg and were cultured on 12 well plates per well. 25μg/ml of poly I:C (TLR3 agonist) was used to stimulate primed samples for 4 hours. After 4 hours, explants were stimulated for another 4 hours with either poly I:C or 10^8 cells/ml of HKLM (TLR2 agonist) in both primed and non-primed samples. Whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) as a marker of NF-κB activation and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as a marker of AP-1 activation. A representative blot is shown with β-actin as a loading control. **Solid black line** shows the comparison between TLR stimulated compared to non-stimulated at the same time point. **Dashed black line** shows priming compared to non-stimulated at the same time point and the **bold black line** shows priming compared to non-primed. A significant increase in NF-κB activation was seen with poly I:C stimulation alone (**p<0.01). No significant increase in NF-κB and AP-1 activation was observed with TLR2 (***solid black line***). Poly I:C priming does not augment **(A)** NF-κB or **(B)** AP-1 with HKLM agonist stimulation (***bold black line***). For statistical analysis Kruskal-Wallis with *Bonferroni's* multiple comparison test was used. n=4.

5.2.1.2 Pro-inflammatory cytokine production is not augmented with TLR3/2 priming

Although neither NF-κB nor AP-1 activation were augmented with TLR3 priming, the level of 9 pro-inflammatory cytokines: IL-1α, IL-1β, IL-6, IL-8, IFN-γ, IL-12p40, IL-12p70, TNF-α and TNF-β were analysed after priming. Supernatants were used for ELISA to quantify the production of pro-inflammatory cytokines. 1:50 dilution was used for IL-6, 1:500 for IL-8 and 1:10 dilution was used for IL-1α, IL-1β, IFN-γ, IL-12p70, and TNF-α, whereas a 1:5 dilution was used for IL-12p40 and TNF-β.

Vehicle control showed a trend of increased cytokine production in IL-1α, IL-1β and IFN-γ over time. A significantly increased cytokine production was observed with poly I:C and HKLM stimulation alone on IL-1α (***p<0.001 at day 6, **p<0.01 at day 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.18 A), IL-1β (***p<0.001 at day 6 and 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.18 B), IL-6 (***p<0.001 at day 6, $^{\dagger\dagger}p$<0.01 at day 7) (Figure 5.18 C), IL-8 (***p<0.001 at day 6, **p<0.01 at day 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.18 D), IL-12p40 (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.18 E), IL-12p70 (***p<0.001 at day 6 and 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.18 F), TNF-α (Figure 5.18 G), TNF-β (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.18 H) and IFN-γ (**p<0.01 at day 6, *p<0.05 at day 7, $^{\dagger}p$<0.05 at day 7) (Figure 5.18 I) compared to basal levels at 0hr.

However, no augmented cytokine production was observed with TLR3 priming prior to TLR2 agonist stimulation.

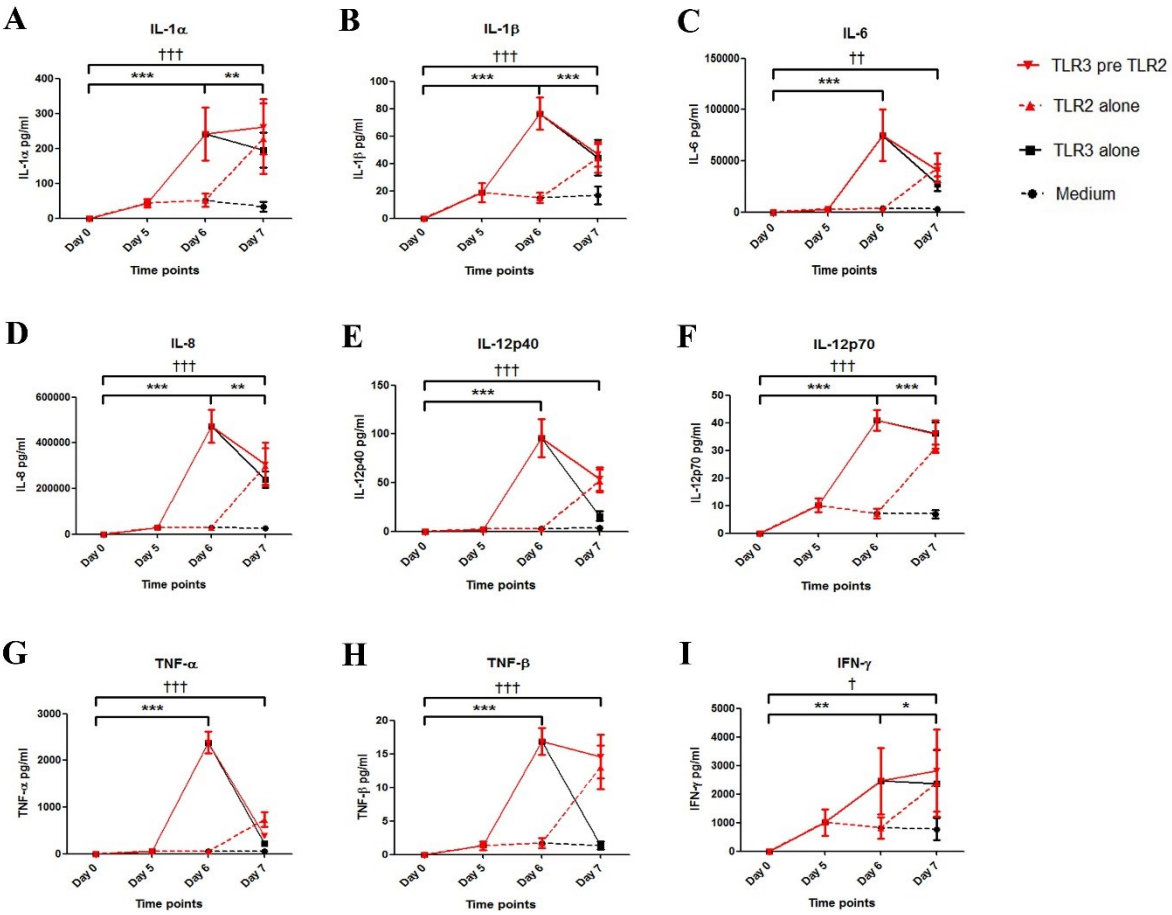


Figure 5.18 The effect of TLR3/2 priming on the pro-inflammatory cytokines. Placental explants were cultured and primed with 25µg/ml of poly I:C for 24 hours. After 24 hours, cells were stimulated with 10^8 cells/ml of HKLM for 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of each cytokine. TLR3 (**p<0.01, ***p<0.001) and TLR2 ($^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01, $^{\dagger\dagger\dagger}p$<0.001) agonist stimulation alone increased the cytokine levels. Priming the explants with poly I:C before HKLM stimulation had no augmented response on either **(A)** IL-1α, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-8, **(E)** IL-12p40, **(F)** IL-12p70, **(G)** TNF-α, **(H)** TNF-β or **(I)** IFN-γ. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

5.2.1.3 MIP-1α and RANTES are augmented with TLR3/2 priming

Three chemokines namely MIP-1α, MIP-1β and RANTES were also analysed. 1:50 dilution were used for MIP-1α and MIP-1β whereas a 1:10 dilution was used for RANTES.

No changes in cytokine productions were seen with the vehicle control. Poly I:C and HKLM stimulation alone increased the production of MIP-1α (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.19 A), MIP-1β (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.19 B) and RANTES (Figure 5.19 C) (**p<0.01 at day 6) compared to 0hr.

Poly I:C priming only augmented MIP-1α ($^{\S\S}p$<0.01) and RANTES ($^{\S\S\S}p$<0.001) compared to HKLM stimulation alone.

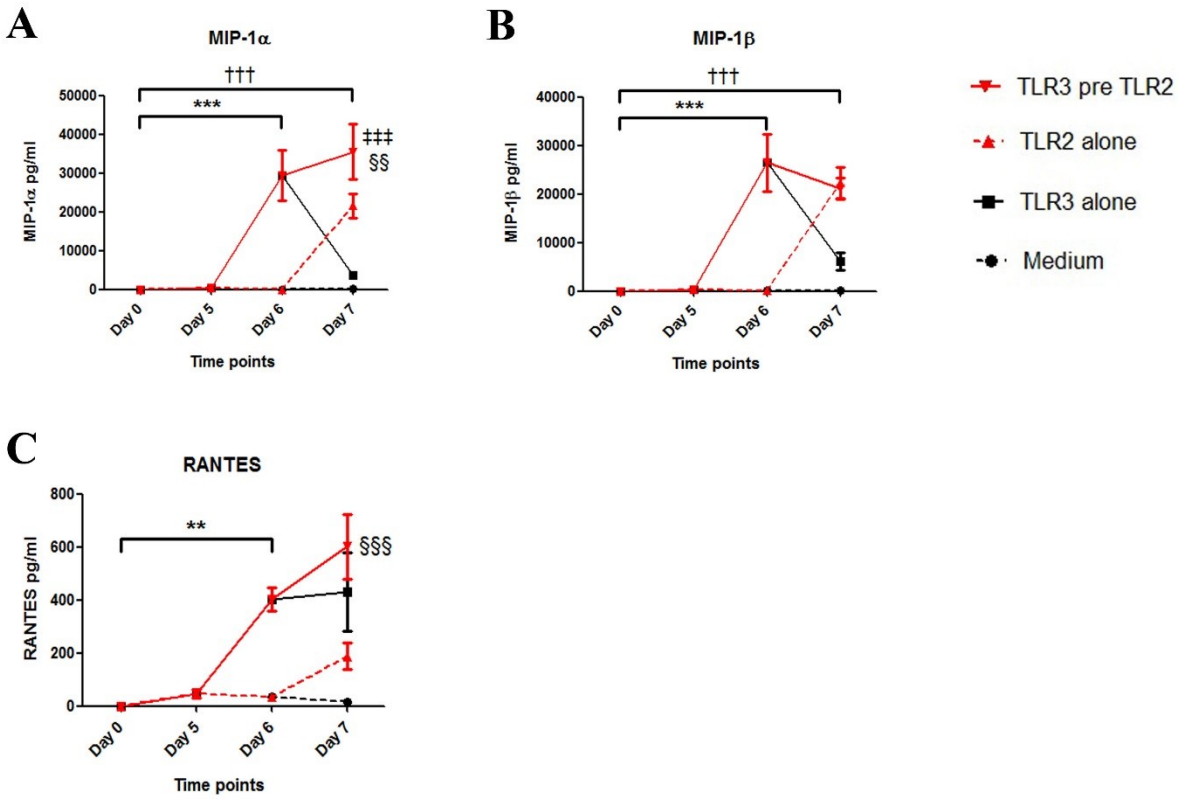


Figure 5.19 The effect of TLR3/2 priming on macrophage inflammatory proteins (MIP) and RANTES. Placental explants were cultured and primed with 25μg/ml of poly I:C for 24 hours. After 24 hours, cells were stimulated with 10^8 of HKLM for 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of each chemokines. The agonist stimulation alone increased chemokine production. Priming with poly I:C significantly augmented further **(A)** MIP-1α but not **(B)** MIP-1β production with subsequent TLR2 stimulation. **(C)** RANTES was also augmented with TLR3 priming before TLR2 agonist stimulation. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4. $^{\ddagger\ddagger\ddagger}p$<0.001 = effect of TLR3 pre TLR2 compared to TLR3 alone and $^{\S\S}p$<0.01= effect of TLR3 pre TLR2 compared to TLR2 alone.

5.2.1.4 IL-10 is augmented with TLR3/2 priming

The anti-inflammatory cytokines IL-4, IL-10, IL-1RA, IFN-α and IFN-β were also analysed. A 1:5 dilution was used to detect these cytokines.

IL-10 (Figure 5.20 A), IL-4 (Figure 5.20 B), IFN-α (Figure 5.20 C) (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7), IFN-β (Figure 5.20 D) (***p<0.001 at day 6, $^{\dagger\dagger}p$<0.01 at day 7) and IL-1RA (Figure 5.20 E) (***p<0.001 at day 6 and 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) production increased with poly I:C and HKLM stimulation alone compared to 0hr.

Only IL-10 ($^{\S\S}p$<0.01) production was augmented with TLR3 priming compared to HKLM stimulation alone.

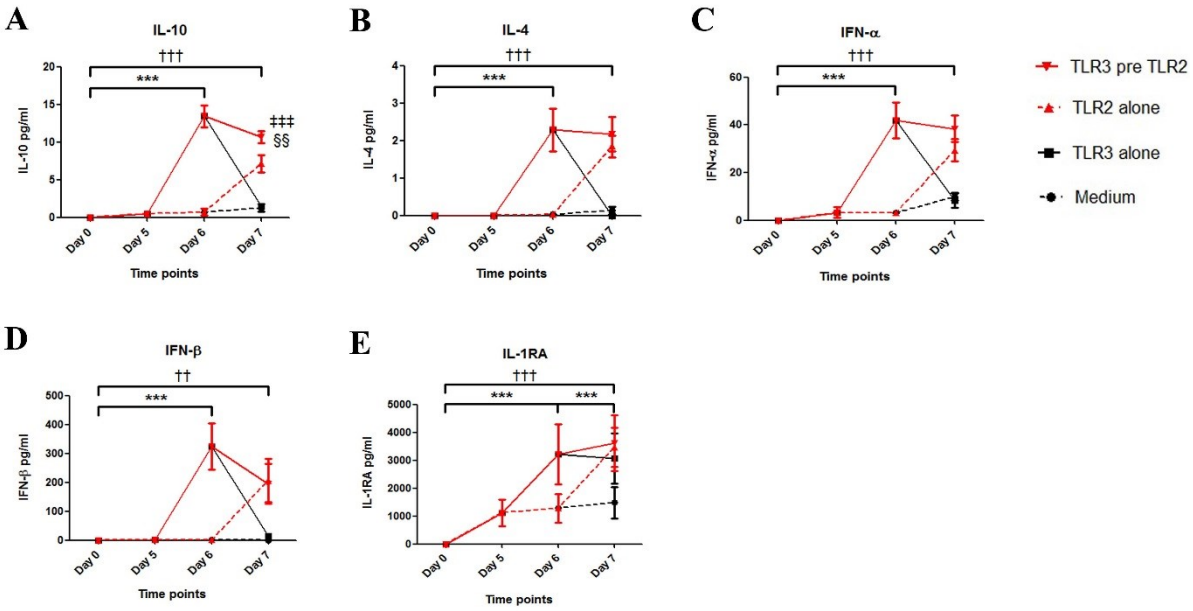


Figure 5.20 The effect of TLR3/2 priming on anti-inflammatory cytokines. Placental explants were cultured and primed with 25μg/ml of poly I:C for 24 hours. After 24 hours, cells were stimulated with 10^8 cells/ml of HKLM for 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of each cytokine. TLR3 (***p<0.001) and TLR2 ($^{\dagger\dagger}p$<0.01, $^{\dagger\dagger\dagger}p$<0.001) agonist stimulation alone increased the cytokine level. Priming with poly I:C significantly augmented further **(A)** IL-10 production. No further increase was seen in **(B)** IL-4, **(C)** IFN-α, **(D)** IFN-β or **(E)** IL-1RA. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4. $^{\ddagger\ddagger\ddagger}p$<0.001 = effect of TLR3 pre TLR2 compared to TLR3 alone and $^{\S\S}p$<0.01 = effect of TLR3 pre TLR2 compared to TLR2 alone.

5.2.1.5 PGE2 production is not augmented with TLR3/2 priming

PGE2 production was analysed using ELISA. Poly I:C (**p<0.01) and HKLM (***p<0.001) stimulation alone increased the production of PGE2 compared to the basal level, but no augmented effect was observed with TLR3/TLR2 priming (Figure 5.21).

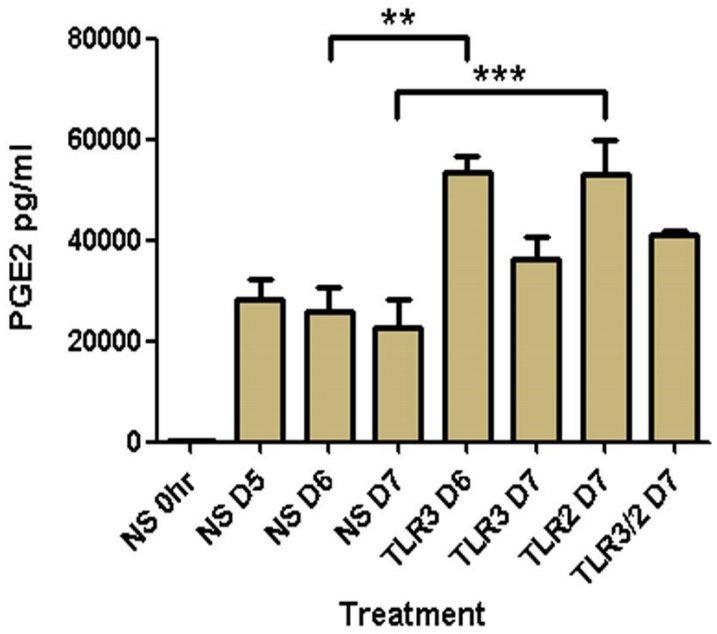


Figure 5.21 The effect of TLR3 priming prior to TLR2 agonist stimulation on PGE2 production in placental explants. Placental explants were cultured and primed with 25µg/ml of poly I:C for 24 hours. After 24 hours, explants were stimulated with either poly I:C or 10^8 cells/ml of HKLM for 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of PGE2. TLR3 (**p<0.01) and TLR2 (***p<0.001) agonist alone significantly increased PGE2 production. However, TLR3 priming prior to TLR2 agonist stimulation did not augment any further PGE2 production. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

5.2.2 TLR3/TLR4 priming in placental explants

Placental explants were cultured and primed with poly I:C before subsequent treatment with 0.1μg/ml of LPS 055 (TLR4 agonist) for 4 hours. Activation of NF-κB and AP-1 were examined.

For cytokine, chemokine and PGE2 analysis, placental explants were primed with poly I:C for 24 hours and then stimulated with LPS for 24 hours which is parallel with the most cited time points in the literature [402, 403]. ELISA was used to quantify the analytes.

5.2.2.1 NF-κB or AP-1 activation is not augmented with TLR3/4 priming

The blue-coloured histogram represents TLR3/TLR4 placental explant priming. Poly I:C (**p<0.01) and LPS (*p<0.05) stimulation alone showed significantly increased NF-κB activation but not AP-1. However, no augmented NF-κB (Figure 5.22 A) or AP-1 (Figure 5.22 B) activation was seen with priming and subsequent LPS stimulation (see bold black line).

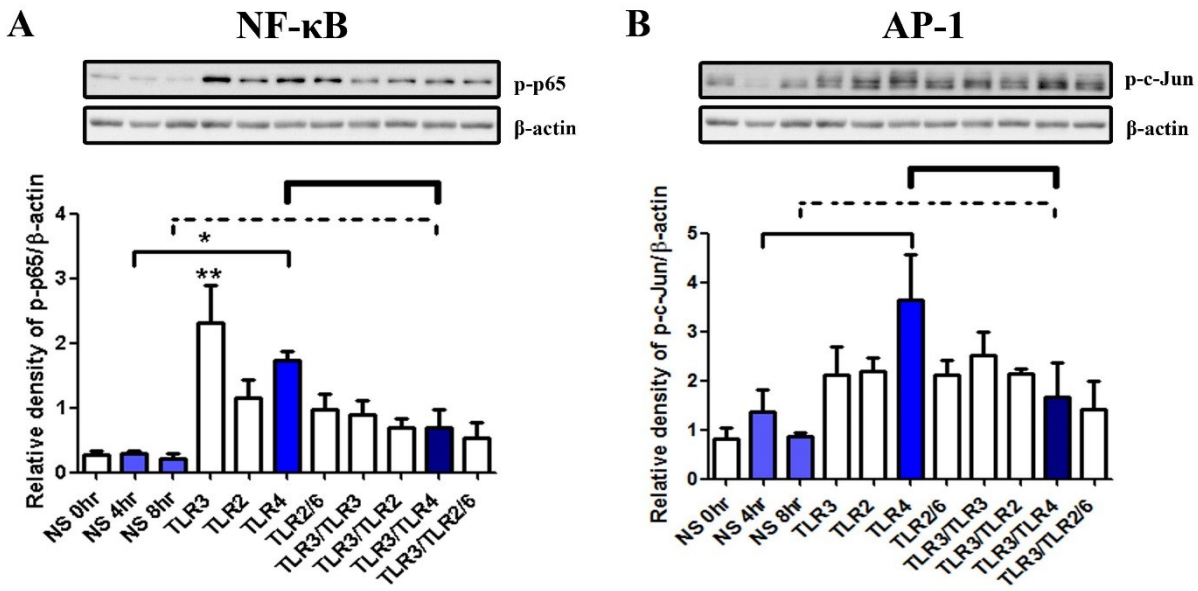


Figure 5.22 Effect of TLR3/4 priming on NF-κB and AP-1 activation in placental explants. Placental tissue was dissected into small pieces and 3 small pieces weighing 20-30mg were cultured per well in in 12 well plates. Primed and non-primed samples were assigned. 25μg/ml of poly I:C (TLR3 agonist) was used to stimulate samples for 4 hours. After 4 hours, explants were stimulated for another 4 hours with either poly I:C or 0.1μg/ml of LPS 055 (TLR4 agonist) in both primed and non-primed samples. Whole cells protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) as a marker of NF-κB activation and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as a marker of AP-1 activation. A representative blot is shown with β-actin as a loading control. **Solid black line** shows the comparison between TLR stimulated compared to non-stimulated explants at the same time point. **Dashed black line** shows primed compared to non-stimulated at the same time point and the **bold black line** shows primed compared to non-primed explants. A significant increase in NF-κB was observed with TLR3 agonist (**p<0.01) and TLR4 agonist alone (*p<0.05) but not in AP-1 (***solid black line***). Poly I:C priming did not augment **(A)** NF-κB or **(B)** AP-1 with LPS 055 agonist stimulation (***bold black line***). For statistical analysis Kruskal-Wallis with *Bonferroni's* multiple comparison test was used. n=4.

5.2.2.2 Pro-inflammatory cytokine production is not augmented with TLR3/4 priming

The 9 pro-inflammatory cytokines listed in section 5.2.1.2 were quantified to determine whether priming leads to an augmented cytokine response in placental explants.

Poly I:C and LPS stimulation alone significantly increased the production of IL-1α (**p<0.01 at day 6 and 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.23 A), IL-1β (***p<0.001 at day 6, *p<0.05 at day 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.23 B), IL-6 (**p<0.01 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.23 C), IL-8 (***p<0.001 at day 6, *p<0.05 at day 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.23 D), IL-12p40 (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.23 E), IL-12p70 (***p<0.001 at day 6 and 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.23 F), TNF-α (Figure 5.23 G), TNF-β (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.23 H) and IFN-γ (**p<0.01 at day 6 and 7, $^{\dagger\dagger}p$<0.01 at day 7) (Figure 5.23 I) compared to 0hr

However, no augmented cytokine production was observed with the priming.

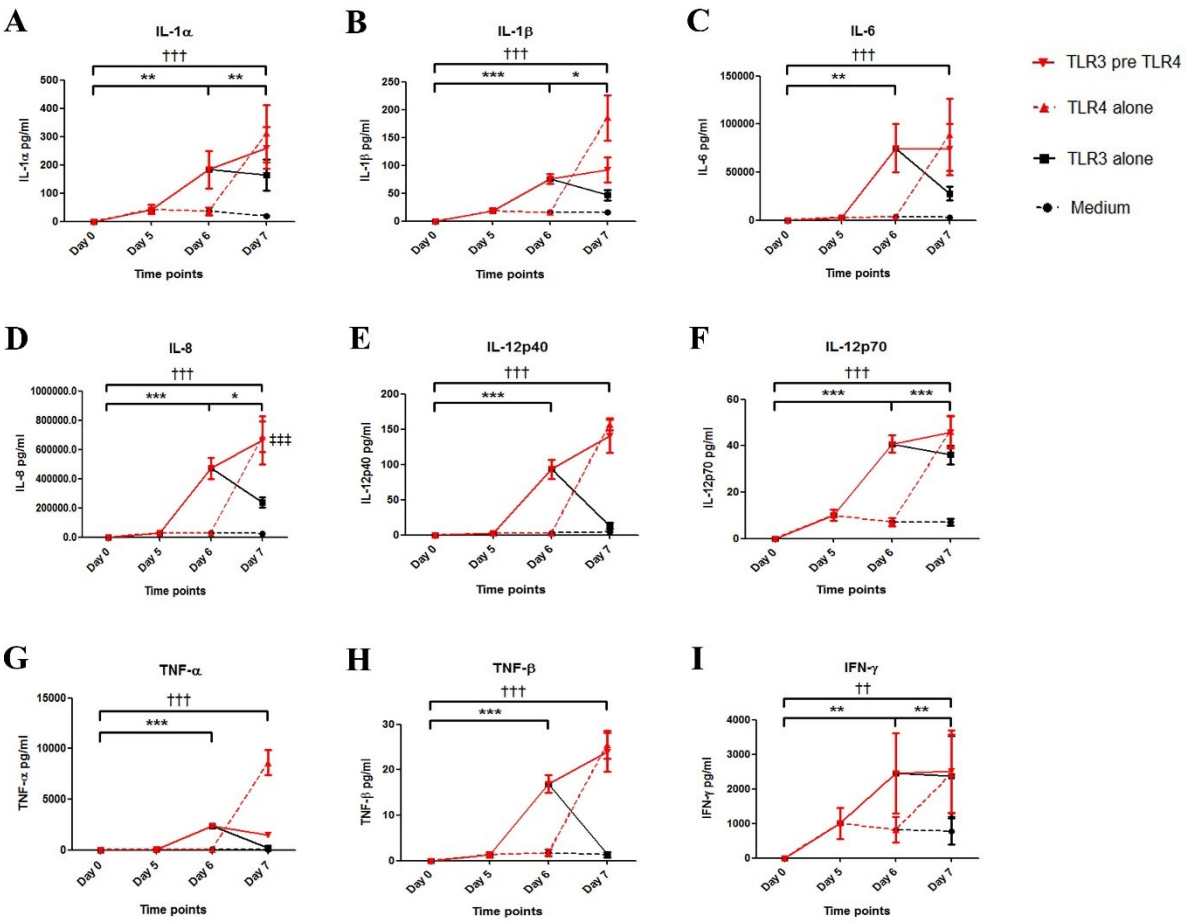


Figure 5.23 The effect of TLR3/4 priming on the pro-inflammatory cytokines. Placental explants were cultured and primed with 25μg/ml of poly I:C for 24 hours. After 24 hours, cells were stimulated with 0.1μg/ml of LPS 055 for 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of each cytokines. TLR3 (**p<0.01, ***p<0.001) and TLR2 ($^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01, $^{\dagger\dagger\dagger}p$<0.001) agonist stimulation alone increased the cytokine production. Priming the explants with poly I:C before LPS 055 stimulation had no augmented response on either **(A)** IL-1α, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-8, **(E)** IL-12p40, **(F)** IL-12p70, **(G)** TNF-α, **(H)** TNF-β or **(I)** IFN-γ. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

5.2.2.3 RANTES is augmented with TLR3/4 priming

Three chemokines MIP-1α, MIP-1β and RANTES were analysed Poly I:C stimulation significantly increased MIP-1α (Figure 5.24 A), MIP-1β (Figure 5.24 B) (***p<0.001) and RANTES (Figure 5.24 C) (**p<0.01), while LPS increased only MIP-1α, MIP-1β ($^{\dagger\dagger\dagger}p$<0.001) production compared to 0hr.

With poly I:C priming prior to LPS stimulation, only RANTES ($^{\S\S\S}p$<0.001) production was augmented compared to LPS stimulation alone.

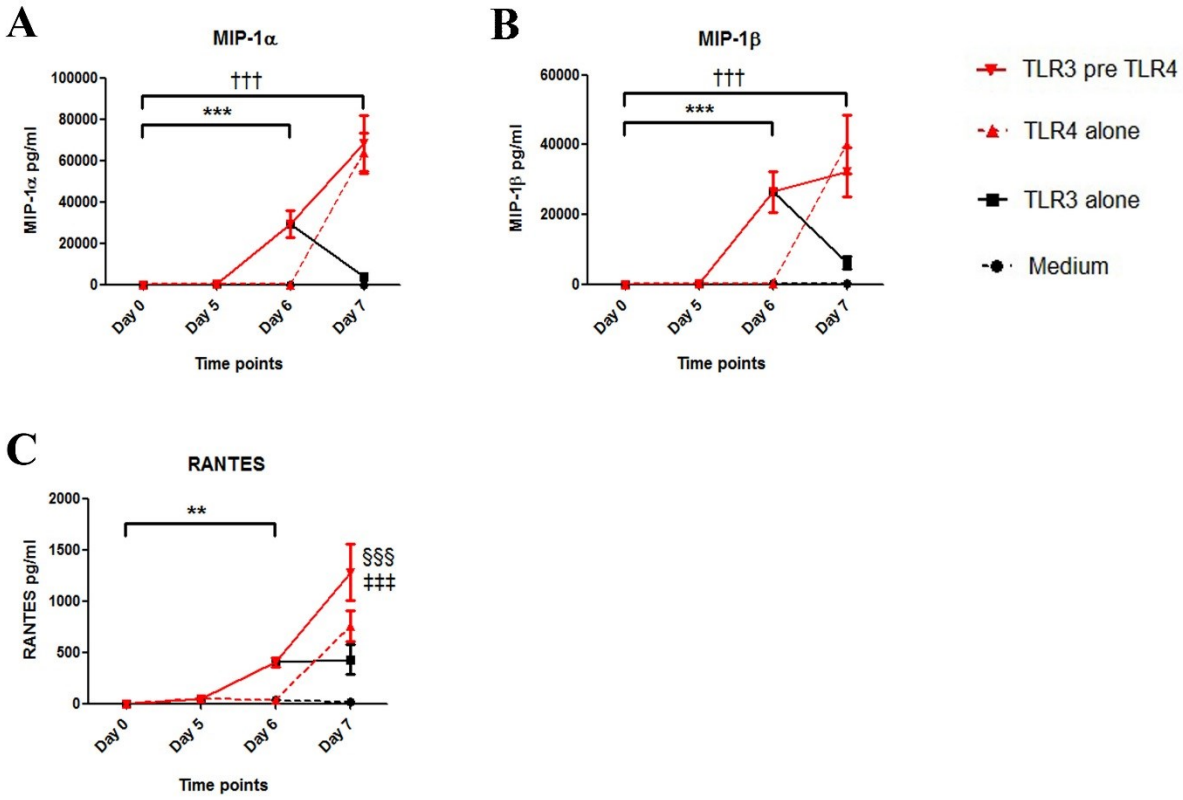


Figure 5.24 The effect of TLR3/4 priming on macrophage inflammatory proteins (MIP) and RANTES. Placental explants were cultured and primed with 25μg/ml of poly I:C for 24 hours. After 24 hours, cells were stimulated with 0.1μg/ml of LPS 055 for 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of each chemokine. Agonist stimulation alone increased chemokine production. Priming with poly I:C did not augment **(A)** MIP-1α or **(B)** MIP-1β but **(C)** RANTES production was significantly increased with subsequent TLR4 stimulation. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4. $^{‡‡‡}p$<0.001 = effect of TLR3 pre TLR4 compared to TLR3 alone and $^{\S\S\S}p$<0.001 = effect of TLR3 pre TLR4 compared to TLR4 alone.

5.2.2.4 Anti-inflammatory cytokine production is not augmented with TLR3/4 priming

The anti-inflammatory cytokines listed in section 5.2.1.4 were quantified.

Poly I:C and LPS stimulation alone significantly increased IL-10 (Figure 5.25 A), IL-4 (Figure 5.25 B), IFN-α (Figure 5.25 C), IFN-β (Figure 5.25 D) (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) and IL-1RA (Figure 5.25 E) (***p<0.001 at day 6 and 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) production compared to 0hr. However, poly I:C priming prior to LPS stimulation showed no augmented anti-inflammatory cytokine production.

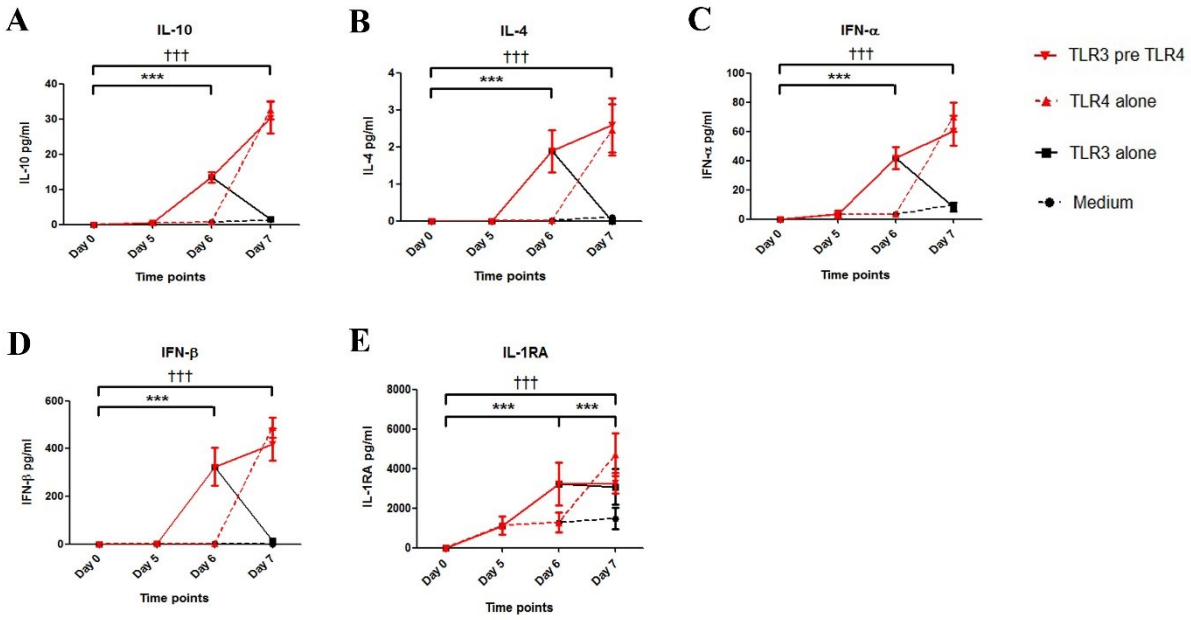


Figure 5.25 The effect of TLR3/4 priming on anti-inflammatory cytokines. Placental explants were cultured and primed with 25μg/ml of poly I:C for 24 hours. After 24 hours, cells were stimulated with 0.1μg/ml of LPS 055 for 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of each cytokine. TLR3 (***p<0.001) and TLR4 ($^{\dagger\dagger\dagger}p$<0.001) agonist stimulation alone increased cytokine production. Priming with poly I:C did not augment **(A)** IL-10, **(B)** IL-4, **(C)** IFN-α, **(D)** IFN-β or **(E)** IL-1RA with subsequent TLR4 stimulation. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

5.2.2.5 PGE2 production is not augmented with TLR3/4 priming

PGE2 increased with individual poly I:C and LPS (***p<0.001) stimulation but no augmented effect was observed with TLR3 priming prior to TLR4 agonist stimulation (Figure 5.26).

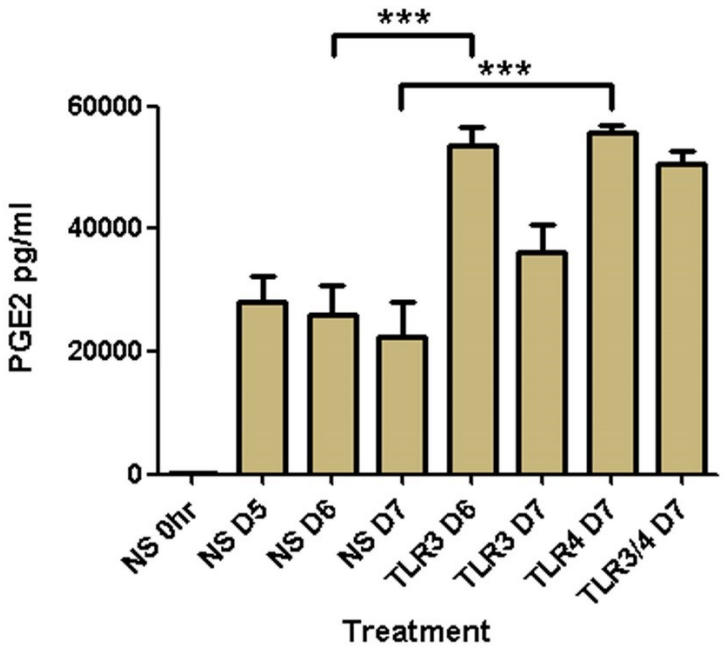


Figure 5.26 The effect of TLR3 priming prior to TLR4 agonist stimulation on PGE2 production in placental explants. Placental explants were cultured and primed with 25µg/ml of poly I:C for 24 hours. After 24 hours, explants were stimulated with either poly I:C or 0.1µg/ml of LPS 055 for 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of PGE2. TLR3 and TLR4 (***p<0.001) agonist alone significantly increased PGE2 production. However, TLR3 priming prior to TLR4 agonist stimulation did not augment further PGE2 production. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

5.2.3 TLR3/TLR2/6 priming in placental explants

Since placental explants were robust in preventing augmentation of pro-inflammatory and pro-labour mediators with poly I:C priming prior to HKLM and LPS stimulation, stimulation with FSL-1 was also examined. FSL-1 consistently led to an augmented response following poly I:C priming in myocytes, VECs and amniocytes (Chapter 4), as well as in PBMCs. Placental explants were cultured and primed with 25μg/ml of poly I:C prior to 0.1μg/ml of FSL-1 (TLR2/6 agonist) for 4 hours, and changes in NF-κB and AP-1 activation were examined.

For cytokine, chemokine and PGE2 studies, cells were primed with poly I:C for 24 hours and then stimulated with LPS 055 for 24 hours. ELISA was used to quantify the analytes.

5.2.3.1 NF-κB and AP-1 activation is not augmented with TLR3/TLR2/6 priming

Poly I:C alone significantly increased NF-κB activation (**p<0.01) whilst FSL-1 stimulation alone showed no statistically significant increase in NF-κB and AP-1 activation (see solid black line). Poly I:C priming prior to FSL-1 stimulation showed no augmentation in NF-κB (Figure 5.27 A) or AP-1 (Figure 5.27 B) activation (see bold black line).

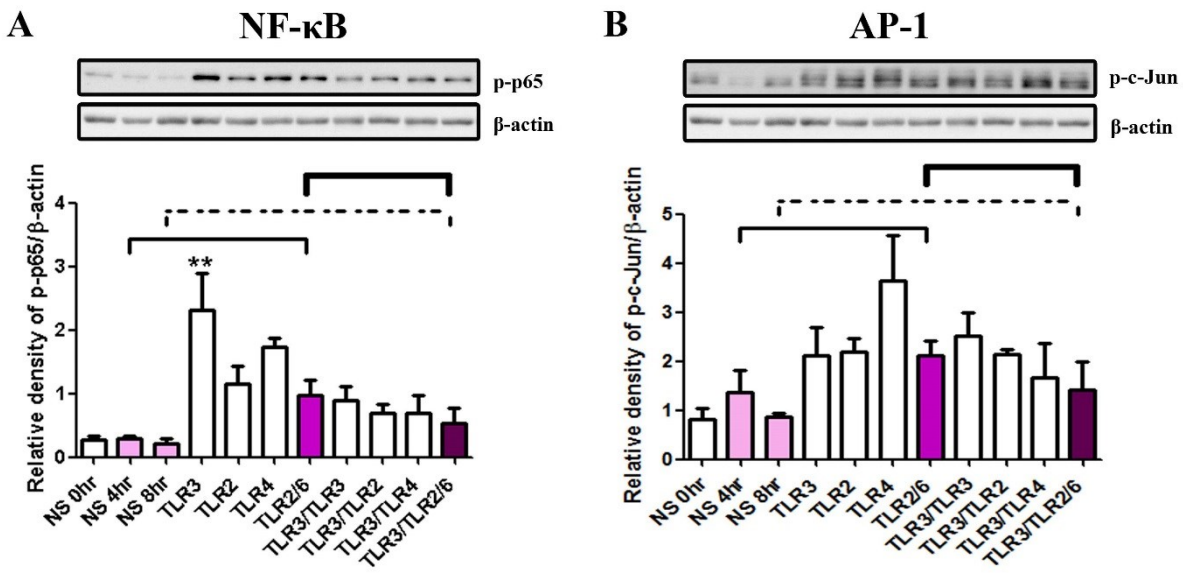


Figure 5.27 Effect of TLR3 priming before TLR2/6 agonist stimulation on NF-κB and AP-1 activation in placental explants. Placental tissue was dissected into small pieces and 3 small pieces weighing 20-30mg per well were cultured in 12 well plates. Primed and non-primed samples were assigned. 25μg/ml of poly I:C (TLR3 agonist) was used to primed samples for 4 hours. After 4 hours, explants were stimulated for another 4 hours with either poly I:C or 0.1μg/ml of FSL-1 (TLR6 agonist) in both primed and non-primed samples. Whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) as a marker of NF-κB activation and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as a marker of AP-1 activation. A representative blot is shown with β-actin as a loading control. **Solid black line** shows a comparison between TLR stimulated compared to non-stimulated explants at the same time point. **Dashed black line** shows primed compared to non-stimulated at the same time point and **bold black line** shows primed compared to non-primed explants. Significant NF-κB increased seen with TLR3 agonist stimulation (**p<0.01). No significant increase in NF-κB and AP-1 activation was observed with TLR2/6 agonist stimulation (***solid black line***). Poly I:C priming did not augment **(A)** NF-κB or **(B)** AP-1 with FSL-1 agonist stimulation (***bold black line***). For statistical analysis Kruskal-Wallis with *Bonferroni's* multiple comparison test was used. n=4.

5.2.3.2 Pro-inflammatory cytokine production is not augmented with TLR3/TLR2/6 priming

The same pro-inflammatory cytokines listed in section 5.2.1.2 were examined following TLR3/TLR2/6 priming.

Poly I:C and FSL-1 stimulation alone significantly increased the production of IL-1α (**p<0.01 at day 6, *p<0.05 at day 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.28 A), IL-1β (***p<0.001 at day 6 and 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.28 B), IL-6 (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.28 C), IL-8 (***p<0.001 at day 6, *p<0.05 at day 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.28 D), IL-12p40 (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.28 E), IL-12p70 (***p<0.001 at day 6 and 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.28 F), TNF-α (Figure 5.28 G), TNF-β (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) (Figure 5.28 H) and IFN-γ (**p<0.01 at day 6 and 7, $^{\dagger\dagger}p$<0.01 at day 7) (Figure 5.28 I) compared to 0hr.

However, no further augmentation in cytokine production was seen with poly I:C priming and FSL-1 stimulation.

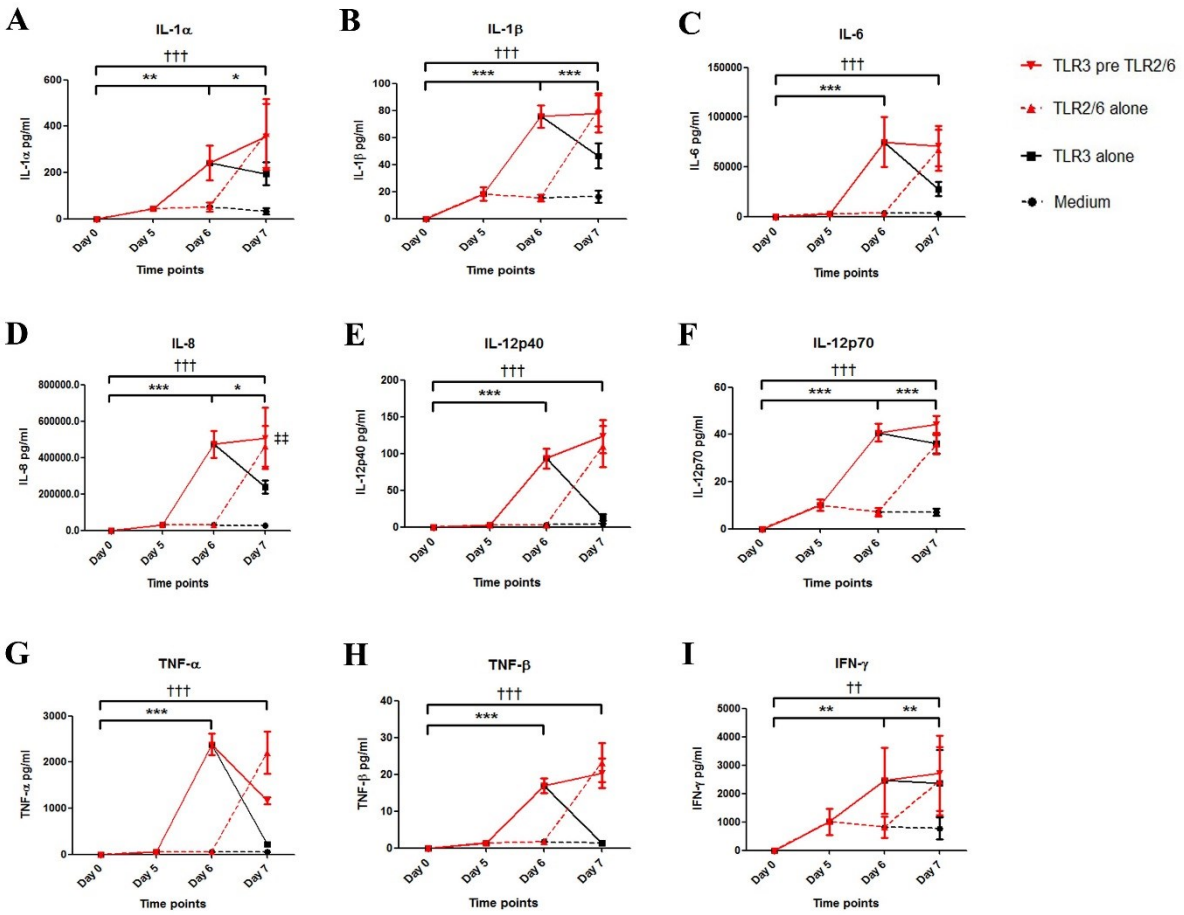


Figure 5.28 The effect TLR3 priming before TLR2/6 agonist stimulation on the pro-inflammatory cytokines. Placental explants were cultured and primed with 25μg/ml of poly I:C for 24 hours. After 24 hours, cells were stimulated with 0.1μg/ml of FSL-1 for 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of each cytokine. TLR3 (**p*<0.05, ***p*<0.01, ****p*<0.001) and TLR2 (†*p*<0.05, ††*p*<0.01, †††*p*<0.001) agonist stimulation alone increased cytokine production. Priming the explants with poly I:C before FSL-1 stimulation had no augmented response on either **(A)** IL-1α, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-8, **(E)** IL-12p40, **(F)** IL-12p70, **(G)** TNF-α, **(H)** TNF-β or **(I)** IFN-γ. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

5.2.3.3 RANTES is augmented with TLR3/TLR2/6 priming

MIP-1α, MIP-β and RANTES were analysed using ELISA.

There were no changes in the chemokines production in the vehicle control over time. Poly I:C and FSL-1 stimulation alone significantly increased MIP-1α (Figure 5.29 A), MIP-1β (Figure 5.29 B) (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) and RANTES (**p<0.01) (Figure 5.29 C) production compared to 0hr.

Priming with poly I:C prior to FSL-1 stimulation led to augmentation of RANTES ($^{\S\S}p$<0.01), but not MIP-1α or MIP-β.

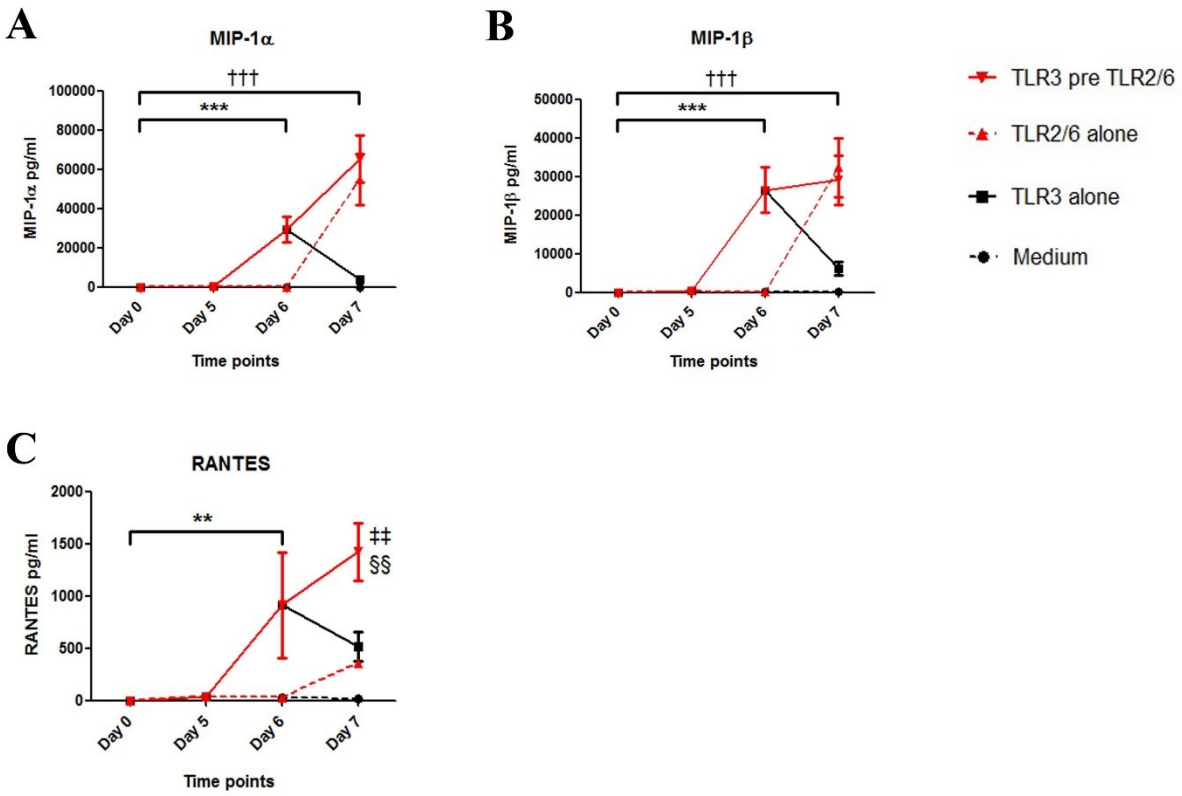


Figure 5.29 The effect of TLR3 priming before TLR2/6 agonist stimulation on macrophage inflammatory proteins (MIP) and RANTES. Placental explants were cultured and primed with 25μg/ml of poly I:C for 24 hours. After 24 hours, cells were stimulated with 0.1μg/ml of FSL-1 for 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of each cytokine. Agonist stimulation alone increased chemokine production. Priming with poly I:C did not augment**(A)** MIP-1α or **(B)** MIP-1β production. However, **(C)** RANTES production augmented further with subsequent TLR2/6 stimulation. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4. $^{\ddagger\ddagger}p$<0.01 = effect of TLR3 pre TLR2/6 compared to TLR3 alone and $^{\S\S}p$<0.01 = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone.

5.2.3.4 IL-10 is augmented with TLR3/TLR2/6 priming

The anti-inflammatory cytokines as listed in section 5.2.1.4 were also analysed.

Poly I:C and FSL-1 stimulation alone significantly increased IL-10 (Figure 5.30 A), IL-4 (Figure 5.30 B), IFN-α (Figure 5.30 C), IFN-β (Figure 5.30 D) (***p<0.001 at day 6, $^{\dagger\dagger\dagger}p$<0.001 at day 7) and IL-1RA (Figure 5.30 E) (***p<0.001 at day 6 and 7, $^{\dagger\dagger\dagger}p$<0.001 at day 7) production compared to 0hr.

Only IL-10 ($^{\S\S\S}p$<0.001) production was augmented in with priming compared to FSL-1 stimulation alone.

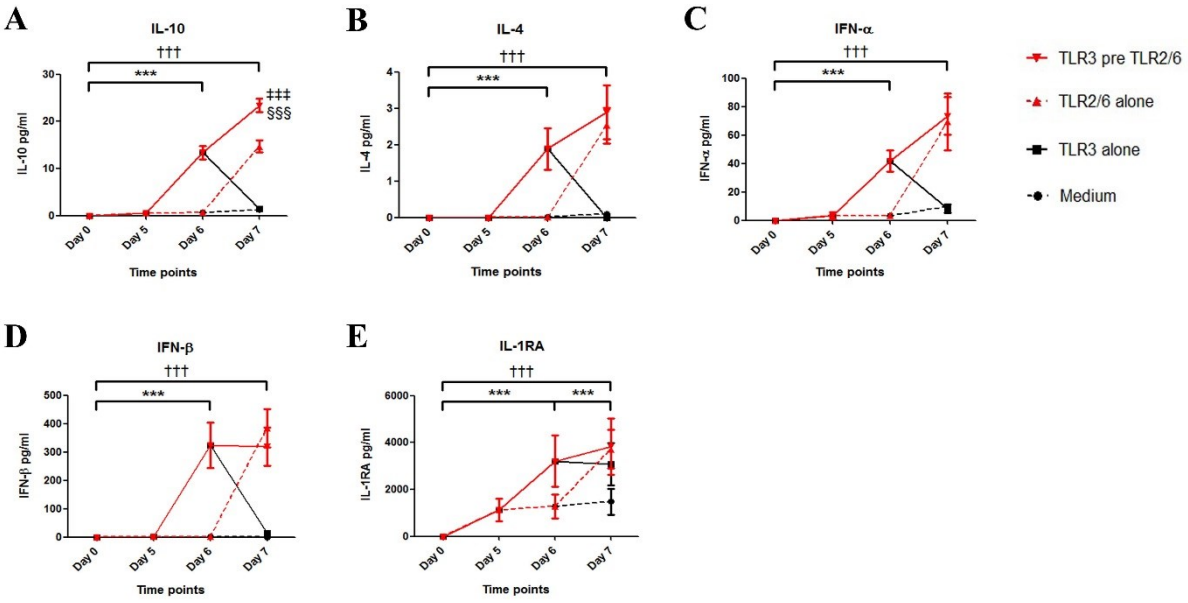


Figure 5.30 The effect of TLR3 priming before TLR2/6 agonist stimulation on anti-inflammatory cytokines. Placental explants were cultured and primed with 25μg/ml of poly I:C for 24 hours. After 24 hours, cells were stimulated with 0.1μg/ml of FSL-1 for 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of each cytokines. TLR3 (***p<0.001) and TLR2/6 ($^{\dagger\dagger\dagger}p$<0.001) agonist stimulation alone increased cytokine production. Priming with poly I:C significantly augmented **(A)** IL-10 production but not **(B)** IL-4, **(C)** IFN-α, **(D)** IFN-β or **(E)** IL-1RA. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4. $^{\ddagger\ddagger\ddagger}p$<0.001 = effect of TLR3 pre TLR2 compared to TLR3 alone and $^{\S\S\S}p$<0.001 = effect of TLR3 pre TLR2/6 compared to TLR2/6 alone.

5.2.3.5 PGE2 production is not augmented with TLR3/TLR2/6 priming

The same supernatants as those used in cytokine/chemokine analyses were used to detect PGE2. PGE2 production increased with poly I:C (**p<0.01) and FSL-1 (***p<0.001) stimulation alone compared to the basal level. However, no augmented effect was observed with TLR3 priming prior to TLR2/6 agonist stimulation (Figure 5.31).

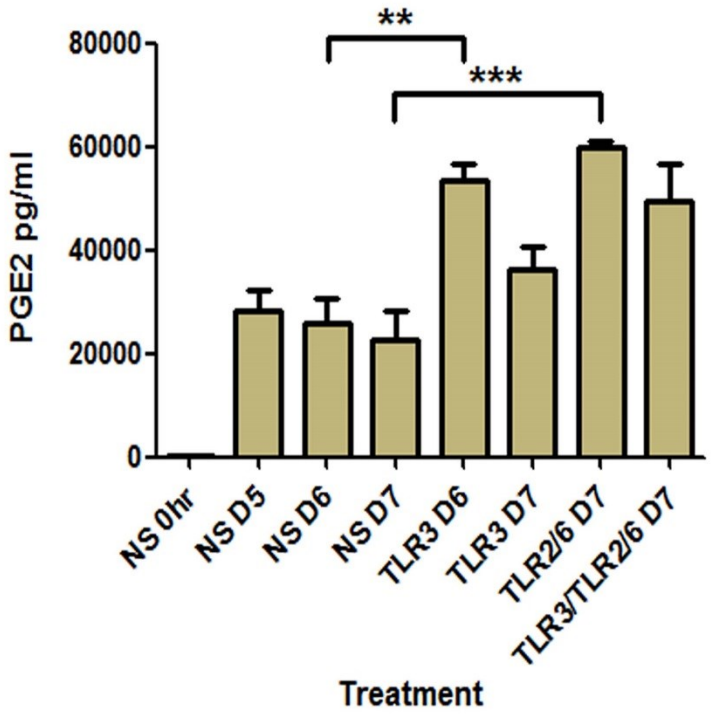


Figure 5.31 The effect of TLR3 priming prior to TLR2/6 agonist stimulation on PGE2 production in placental explants. Placental explants were cultured and primed with 25µg/ml of poly I:C for 24 hours. After 24 hours, explants were stimulated with either poly I:C or 0.1µg/ml of FSL-1 for 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of PGE2. TLR3 (**p<0.01) and TLR2/6 (***p<0.001) agonist alone significantly increased PGE2 production. However, TLR3 priming prior to TLR2/6 agonist stimulation did not augment kPGE2 production. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

5.3 Co-culture

The results presented in this chapter so far suggest that priming with the TLR3 agonist leads to augmented pro-inflammatory and pro-labour responses which are more pronounced in PBMCs compared to placental explants. This suggests that a multi pathogen infection that has reached the placenta via haematogenous spread is less able to cause an inflammatory response, implying that the placenta holds a robust protective mechanism. In support of this, the main cytokine that increases with priming in placenta is in fact the anti-inflammatory cytokine, IL-10. Despite the controversy over the presence of a placental microbiome, my results suggest that the placenta is equipped with a protective mechanism against exposure to multi-pathogen infection.

In view of the evidence supporting an increased risk of preterm labour in women with systemic immune activation [404], another model was utilised to examine the potential effect of circulating pro-inflammatory mediators produced during systemic infection on the placenta. Placental explants were cultured in the supernatant taken from the priming experiments on PBMCs described in section 5.1.2, 5.1.3 and 5.1.4. The explants were cultured for 4 hours to evaluate the activation status of NF-κB/AP-1 and for further 24 hours to assess the effect on pro-inflammatory mediators, IL-1β, IL-6, IL-8 and TNF-α, and the anti-inflammatory mediator, IL-10.

5.3.1 The effect of culturing placental explants in supernatant from TLR-treated PBMCs on NF-κB and AP-1 activation

Although a trend was seen for increased of NF-κB and AP-1 activation in placental explants treated with supernatant from TLR-treated PBMCs, this did not reach statistical significance. Furthermore, no augmentation of NF-κB (Figure 5.32 A) and AP-1 (Figure 5.32 B) was seen when incubated with the primed media.

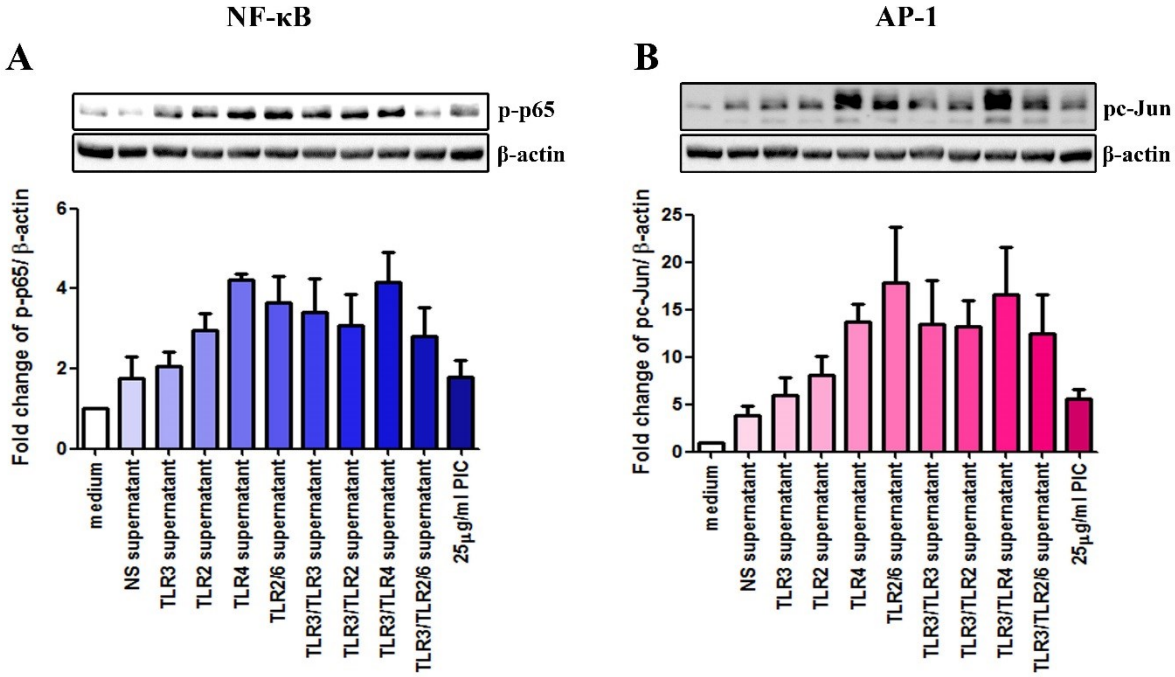


Figure 5.32 The effect of co-culturing placental explants with media derived from TLR agonist treated PBMCs. Placental explants were cultured and treated with media from PBMCs treated with either TLR agonists alone or following priming for 4 hours. Whole cell protein lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as markers of NF-κB and AP-1 activation, respectively. Representative blots with β-actin as a loading control are shown. No augmentation of **(A)** NF-κB or **(B)** AP-1 activation was observed comparing the PBMC-primed supernatant to PBMC-TLR agonist treated alone supernatant on placental explants. For statistical analysis Kruskal-Wallis with *Bonferroni's* multiple comparison test was used; n=4.

5.3.2 The effect of culturing placental explants in supernatant from TLR-treated PBMCs on pro-inflammatory cytokines

In this section, the results were laid out according to the TLR agonist treatment and the cytokines that was analysed. Section 5.3.2.1 represents TLR3/2, section 5.3.2.2 represents TLR3/4 and section 5.3.2.3 represents TLR3/TLR2/6 PBMC-derived media treated placental explants. IL-1β, IL-6, IL-8 and TNF-α production were measured using ELISA.

In each of the graphs, there were 3 independent clustered histograms representing different experimental conditions which are highlighted with different colours.

Pink-coloured histograms represent placental explant priming results (the same experiment conducted in section 5.2.1, 5.2.2 and 5.2.3).

Light blue-coloured histograms represent PBMC priming experiment results (the same experiment conducted in section 5.1.2, 5.1.3 and 5.1.4).

The dark blue-coloured histograms represent results obtained when fresh placental explants were cultured with the media derived from the light blue-coloured histograms (PBMC priming experiment).

5.3.2.1 TLR3/2

Although TLR stimulation alone increased IL-1β, TNF-α, IL-6 and IL-8 production in placental explants (shown in pink), there was no augmentation with the priming.

The PBMC priming results (shown in light blue) shows augmentation for IL-1β, TNF-α, IL-6 and IL-8 (Figure 5.33).

Cytokine production from placental explants cultured in media collected from TLR treated PBMCs are shown in dark blue-coloured histograms. IL-6 (Figure 5.33 C) and IL-8 (Figure 5.33 D) production were augmented when cultured in primed media compared to media from PBMCs treated with TLR3 (***$p<0.001$) or TLR2 (*$p<0.05$) agonists alone. In contrast, no augmentation was seen in the production of IL-1β (Figure 5.33 A) or TNF-α (Figure 5.33 B).

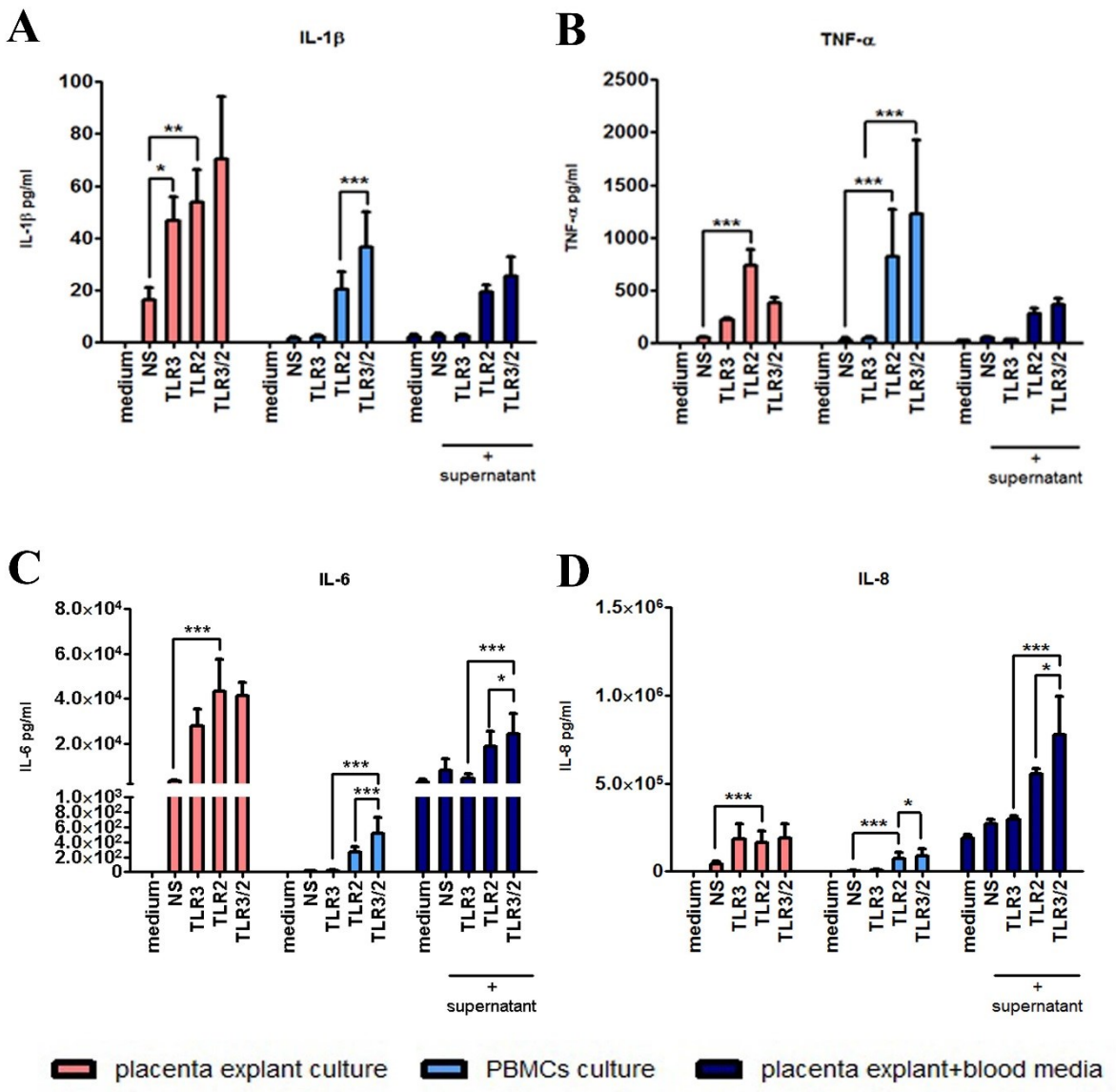


Figure 5.33 The effect of co-culturing placental explants with media derived from TLR3-primed PBMC and PBMCs-treated with the TLR2 agonist alone on IL-1β, TNF-α, IL-6 and IL-8. Placental explants were cultured and treated with TLR3-primed or TLR2 or TLR3 agonist treated PBMC media 24 hours. Supernatant was collected, and an ELISA was performed to determine the level of IL-1β, TNF-α, IL-6 and IL-8 pro-inflammatory cytokines. Pink-coloured histograms demonstrated placental explants priming experiment that showed no augmentation on the cytokines production. Light blue-coloured histograms demonstrated PBMC priming experiment that showed augmentation on IL-1β, TNF-α, IL-6 and IL-8 production. In the co-culture (dark blue-coloured histograms), no further augmentation of **(A)** IL-1β or **(B)** TNF-α on the placental explants. However, **(C)** IL-6 and **(D)** IL-8 were significantly augmented compared to placental explants cultured in PBMC-treated with agonist alone media. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

5.3.2.2 TLR3/4

As previously demonstrated in section 5.2.2.2, TLR stimulation alone increased IL-1β, TNF-α, IL-6 and IL-8 production in placental explants (shown in pink) with no augmentation with the priming.

The light blue-coloured histograms are the PBMC priming results (as shown in section 5.1.3.2) which demonstrate increases in IL-1β, TNF-α and IL-8 production in presence of the individual ligands but no augmentation for IL-1β, TNF-α, IL-6 and IL-8 when primed.

Cytokine production from placental explants cultured in media collected from TLR treated PBMCs are shown in dark blue-coloured histograms. Media derived from TLR4-treated PBMCs significantly increased IL-1β (**$p<0.01$) (Figure 5.34 A), TNF-α (***$p<0.001$) (Figure 5.34 B), IL-6 (**$p<0.01$) (Figure 5.34 C) and IL-8 (***$p<0.001$) (Figure 5.34 D) production in placental explants compared to the basal level derived media from PBMCs. There were no augmented responses in any of the cytokines when stimulated with PBMC-derived media with TLR3/TLR4 priming compared to that treated with TLR3 or TLR4 agonists alone.

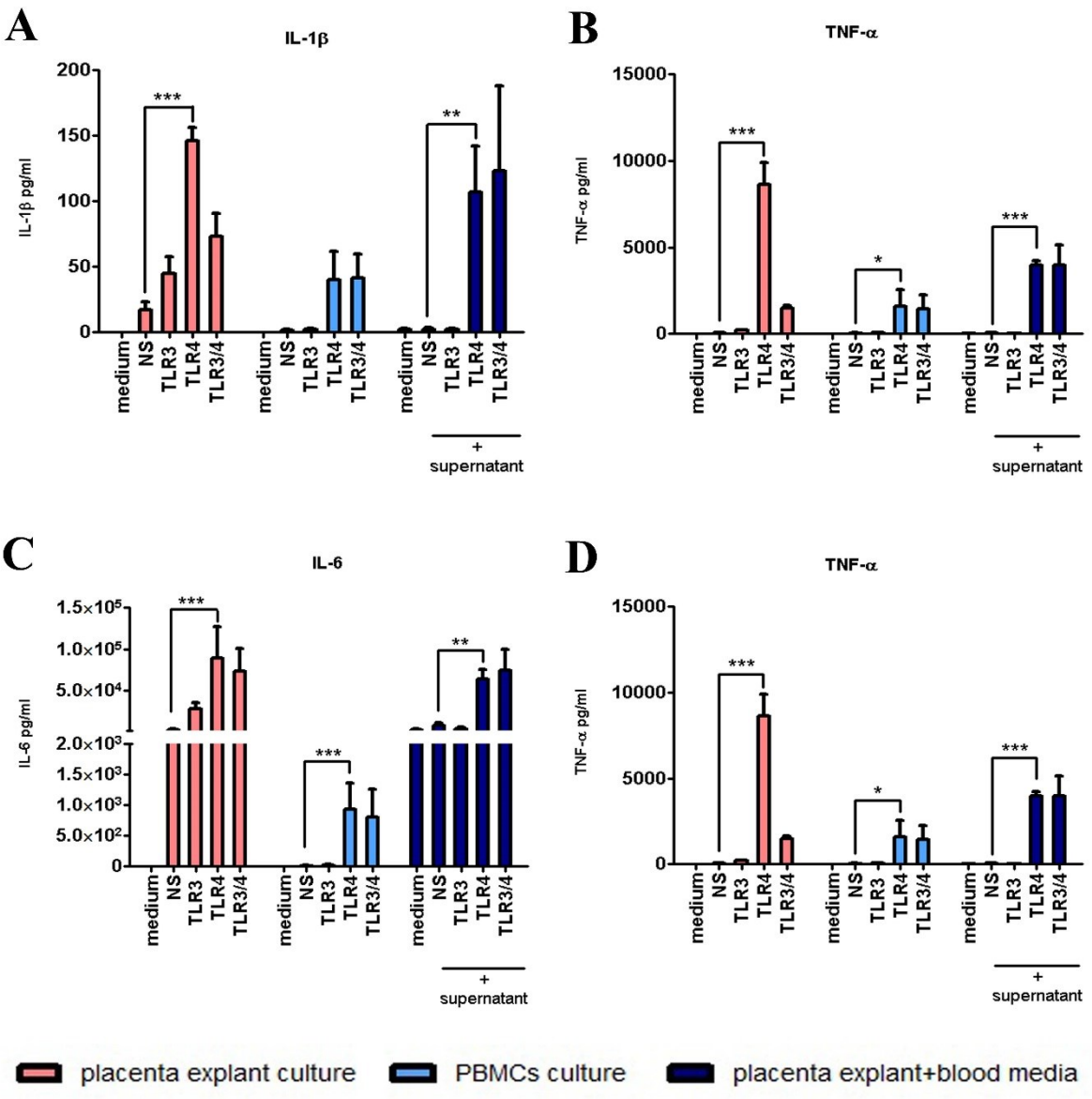


Figure 5.34 The effect of co-culturing placental explants with media derived from TLR3-primed PBMC and PBMCs-treated with the TLR4 agonist alone on IL-1β, TNF-α, IL-6 and IL-8. Placental explants were cultured and treated with TLR3-primed and TLR4 agonist treated alone media derived from PBMC for 24 hours. Supernatant was collected and ELISA was performed to determine the level of IL-1β, TNF-α, IL-6 and IL-8 pro-inflammatory cytokines. Pink-coloured and light blue-coloured histograms demonstrated placental explants and PBMC priming experiment, respectively that showed no augmentation on the cytokines production. In the co-culture (dark blue-coloured histograms), no further augmentation of **(A)** IL-1β, **(B)** TNF-α, **(C)** IL-6 or **(D)** IL-8 on the placental explants compared to the PBMC-treated with agonist alone media. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

5.3.2.3 TLR3/TLR2/6

No augmentation with the priming was observed in the placental explants although TLR stimulation alone increased IL-1β, TNF-α, IL-6 and IL-8 production (shown in pink).

Augmentation on the IL-1β, TNF-α, IL-6 and IL-8 was observed in the PBMC priming (shown in light blue).

Cytokine production from placental explants cultured in media collected from TLR treated PBMCs are shown in dark blue-coloured histograms. Media derived from TLR2/6-treated PBMC also increased the production of IL-1β (Figure 5.35 A), TNF-α (Figure 5.35 B), IL-6 (Figure 5.35 C) and IL-8 (***p<0.001) (Figure 5.35 D) compared to the basal level derived media from PBMC. No augmented response in any of the cytokines in PBMC-treated media with TLR3-primed prior to TLR2/6 agonist stimulation compared to TLR3 and TLR2/6 derived media.

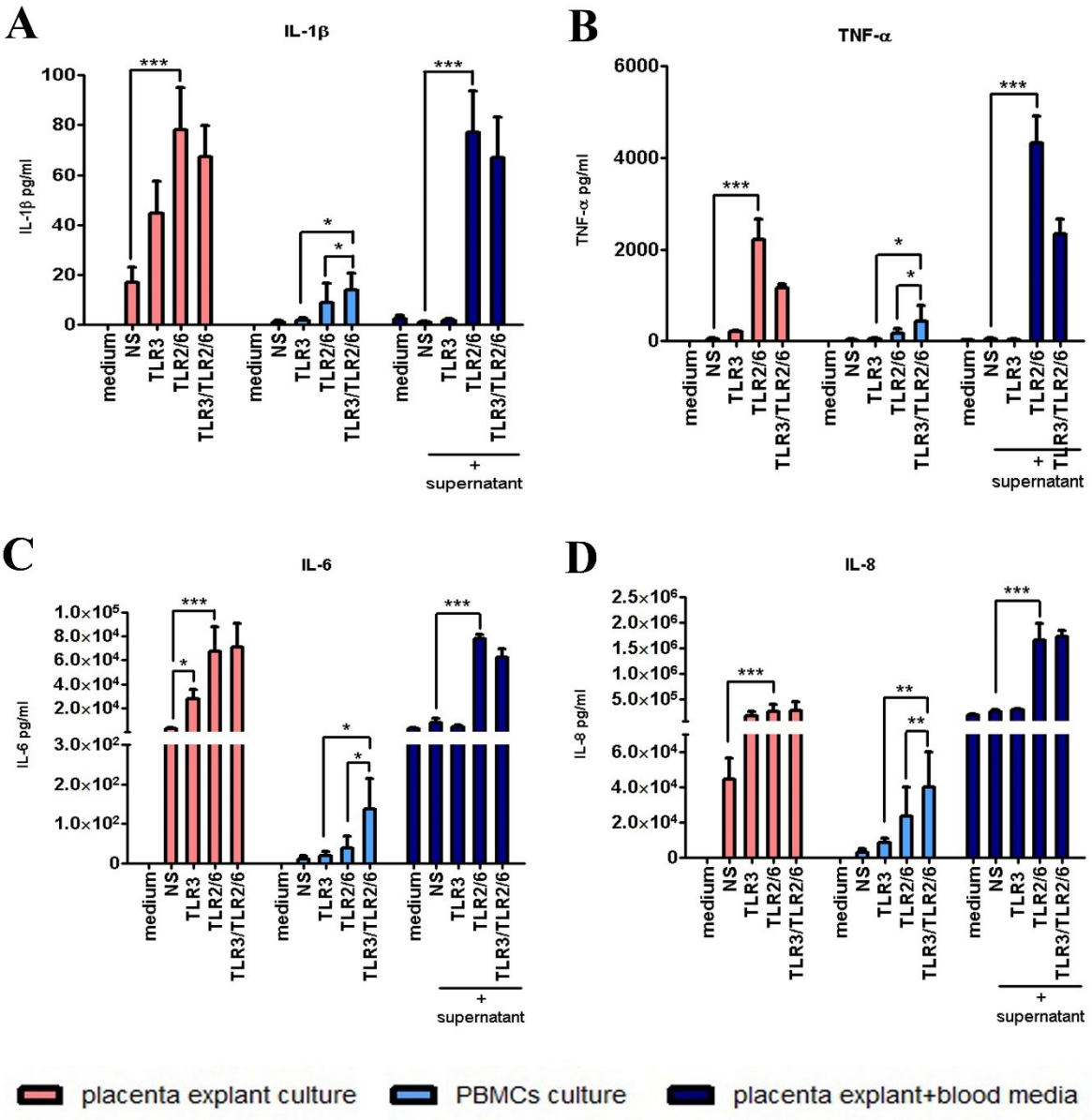


Figure 5.35 The effect of co-culturing placental explants with media derived from TLR3-primed PBMC and PBMCs-treated with the TLR2/6 agonist alone on IL-1β, TNF-α, IL-6 and IL-8. Placental explants were cultured and treated with TLR3-primed and TLR2/6 agonist treated alone media derived from PBMC for 24 hours. Supernatant was collected and ELISA was performed to determine the level of IL-1β, TNF-α, IL-6 and IL-8 pro-inflammatory cytokines. Pink-coloured histograms demonstrated placental explants priming experiment that showed no augmentation on the cytokines production. Light blue-coloured histograms demonstrated PBMC priming experiment that showed augmentation on IL-1β, TNF-α, IL-6 and IL-8 production. In the co-culture (dark blue-coloured histograms), no further augmentation of **(A)** IL-1β, **(B)** TNF-α, **(C)** IL-6 or **(D)** IL-8 on the placental explants compared to the PBMC-treated with agonist alone media. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

5.3.3 The effect of culturing placental explants in supernatant from TLR-treated PBMCs on the anti-inflammatory cytokine

In this section, IL-10 results from fresh placental explants were presented in Figure 5.36 where Figure 5.36 A represents treatment with TLR3/2, Figure 5.36 B with TLR3/4 and Figure 5.36 C with TLR3/TLR2/6 PBMC-derived media.

In each of the graphs, there were 3 independent clustered histograms representing different experimental conditions which are highlighted with different colours. Pink-coloured histograms represent placental explant priming results (the same experiment conducted in section 5.2.1.4, 5.2.2.4 and 5.2.3.4). Light blue-coloured histograms represent PBMC priming experiment results (the same experiment conducted in section 5.1.2.4, 5.1.3.4 and 5.1.4.4). The dark blue-coloured histograms represent results obtained when fresh placental explants were cultured with the media derived from the light blue-coloured histograms (PBMC priming experiment). Although increased IL-10 production was observed in media derived from TLR2- (Figure 5.36 A), TLR4- (Figure 5.36 B) and TLR6-treated PBMCs (Figure 5.36 C), no statistical significance was achieved. Moreover, there were no augmented responses observed when comparing TLR3-primed PBMC derived media to individual TLR agonist-treated PBMC derived media.

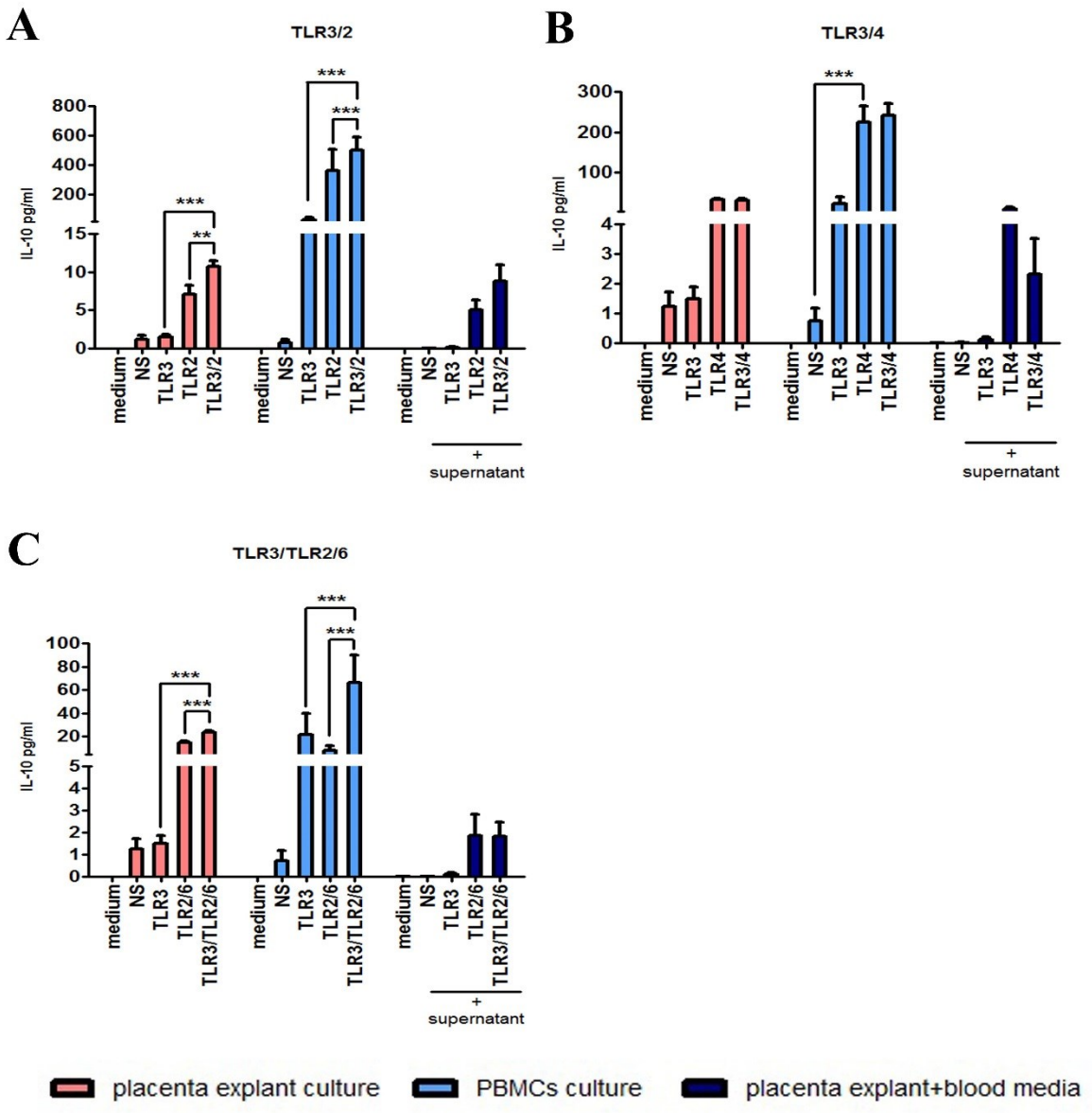


Figure 5.36 The effect of co-culturing placental explants with media derived from TLR3-primed PBMC and PBMCs-treated with TLR2 or TLR4 or TLR2/6 agonist alone on IL-10. Placental explants were cultured and treated with TLR3-primed and TLR agonist treated alone media derived from PBMC for 24 hours. Supernatant was collected and ELISA was performed to determine the level of IL-10 anti-inflammatory cytokine. Pink-coloured and light blue-coloured histograms demonstrated placental explants and PBMC IL-10 production, respectively that showed IL-10 augmentation with TLR3/2 and TLR3/TLR2/6 priming. With the co-culture (dark blue-coloured histograms), no augmentation of IL-10 was observed on the placental explants treated with media derived from TLR3-primed compared to the PBMC-treated with **(A)** TLR2, **(B)** TLR4 or **(C)** TLR2/6 agonist alone media. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4.

5.3.4 Phosphorylated IRF3 increases with poly I:C stimulation in placental explants

Since NF-κB and AP-1 were not activated with TLR3 priming in PBMCs or placental explants, the effect on another transcription factor known to be important for TLR3 signalling, IRF3, was examined. PBMCs and placental explants were stimulated with poly I:C (25µg/ml and 5µg/ml respectively) in a time course experiment, and western immunoblotting of p-IRF3 was used to examine its activation.

Blots from PBMCs probed with p-IRF3 antibody showed multiple bands (Figure 5.37 A) and no clear trend with poly I:C stimulation was observed at the intended molecular weight. Since myocytes and VECs showed different p-IRF3 molecular weight than amniocytes, no deduction was made to pinpoint the right band of p-IRF3 in the PBMCs. In placental explants, a clear band around 40kDa was observed with a significant increase in activation at 30 minutes (***p<0.001) (Figure 5.37 B).

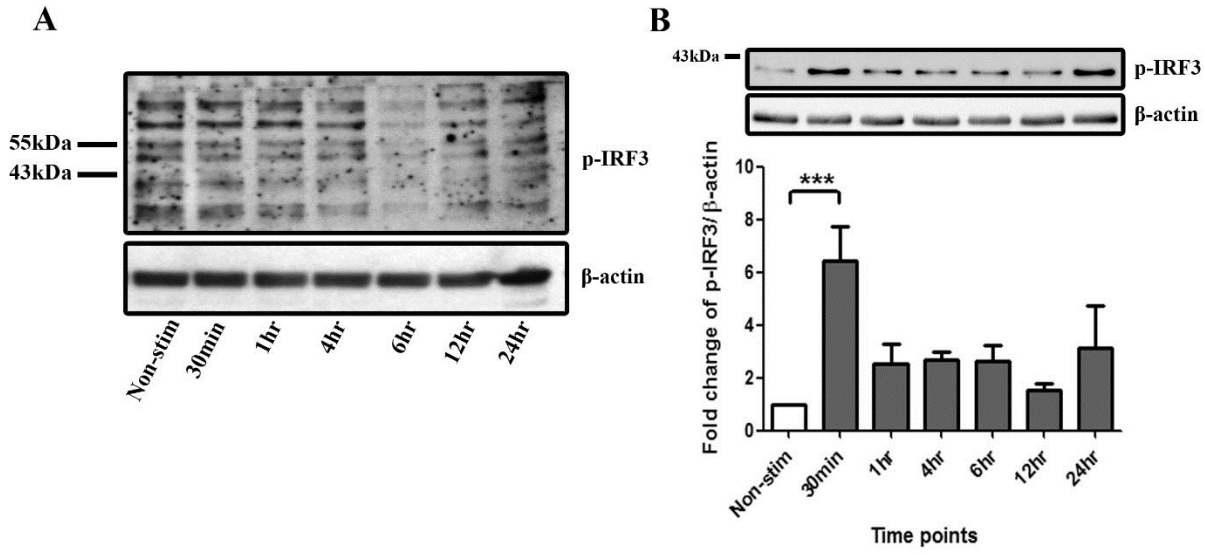


Figure 5.37 Effect of poly I:C stimulation on phosphorylated-IRF3 in PBMC and placental explants. PBMC and placental explants were cultured and stimulated with 5µg/ml and 25µg/ml of poly I:C, respectively for a duration of 24 hours. Protein was extracted and Western immunoblotting was performed to determine the activation status of serine 396-phosphorylated IRF3 (Ser396-p-IRF3). A representative blot was shown above each graph with β-actin as a loading control. In **(A)** PBMC, a representative blot of 3 independent experiments was shown demonstrated multiple bands of p-IRF3 with poly I:C stimulation. Poly I:C stimulation in the **(B)** placental explants significantly increased p-IRF3 at 30 minutes (*p<0.05) with a 2-fold decreased after 30 minutes and peaking at 24 hours but no statistical significant was achieved. For statistical analysis Kruskal-Wallis with *Dunnett's* multiple comparison test was used. n=3-4.

5.3.5 Poly I:C stimulation increases TLR3 mRNA expression in PBMCs and TLR2 mRNA expression in placental explants

TLR3 agonist priming was shown to increase pro-labour and pro-inflammatory cytokine production with TLR2 and TLR2/6 agonists in PBMCs and anti-inflammatory IL-10 in placental explants. A further production of pro-inflammatory cytokine was augmented when PBMC-derived media was cultured together with fresh placental explants.

These results imply that poly I:C enhances the responsiveness of PBMCs and placental explants to bacterial TLR agonists. Therefore, the mRNA expression of TLR2, 3, 4 and 6 were determined after poly I:C stimulation in a time course experiment up to 12 hours in both PBMCs and placental explants.

PBMCs and placental explants were stimulated with 5μg/ml and 25μg/ml of poly I:C, respectively at 1, 4, 6 and 12 hours. TLR3, 2, 4 and 6 mRNAs were quantified using quantitative PCR and β-actin was used as a house-keeping gene. Poly I:C stimulation in PBMCs showed a significant increase of TLR3 mRNA expression at 6 hours (**p<0.01) whereas no effect was seen on TLR2,4 or 6 mRNA levels (Figure 5.38). In contrast, a statistical significance was observed in TLR2 mRNA at 4 and 6 hours (*p<0.05) but no change in TLR3, 4 and 6 following TLR3 agonist stimulation in placental explants (Figure 5.39).

5.3.5.1 PBMCs

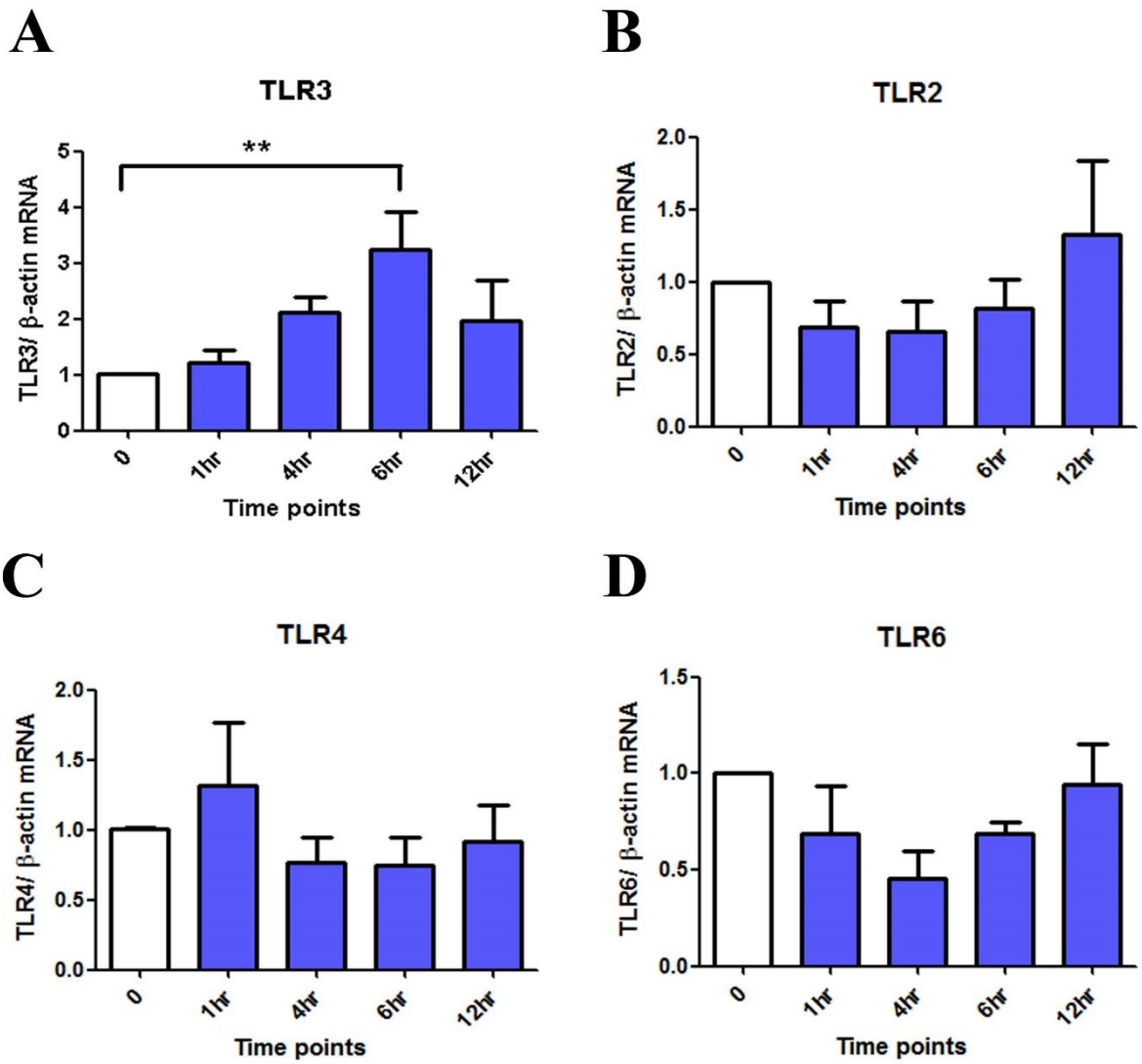


Figure 5.38 The effect of TLR mRNA expression with poly I:C stimulation in PBMCs. PBMCs were cultured and stimulated with 5μg/ml of poly I:C for 1, 4, 6 and 12 hours. Total RNA was extracted and RT-QPCR was performed to quantify the level of TLR3, 2, 4 and 6 mRNA expression using β-actin as the loading control. Poly I:C stimulation significantly increased **(A)** TLR3 (**p<0.01) mRNA expression at 6 hours. No change was seen with **(B)** TLR2 **(C)** TLR4 or **(D)** TLR6 mRNA expression. For statistical analysis Kruskal-Wallis with *Dunnett's* multiple comparison test was used; n=4.

5.3.5.2 Placental explants

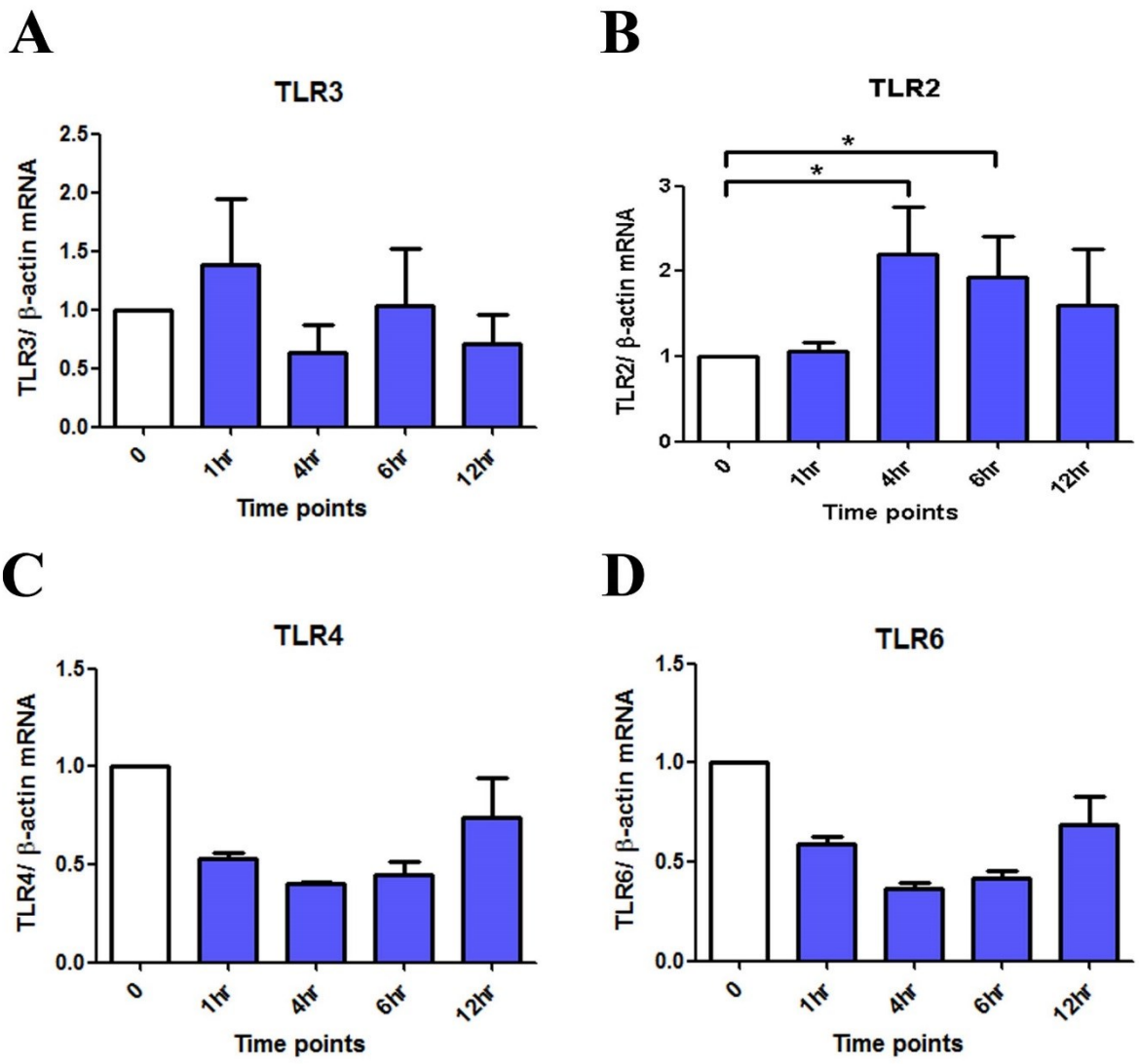


Figure 5.39 The effect of TLR mRNA expression with poly I:C stimulation in placental explants. Placental explants were cultured and stimulated with 25µg/ml of poly I:C for 1, 4, 6 and 12 hours. Total RNA was extracted and RT-QPCR was performed to quantify the level of TLR3, 2, 4 and 6 mRNA expression using β-actin as the loading control. Poly I:C stimulation showed a trend of increase on **(A)** TLR3 and achieved **(B)** statistically significant in TLR2 at 4 and 6 hours (**p*<0.05) but not in **(C)** TLR4 and **(D)** TLR6 mRNA expression. For statistical analysis Kruskal-Wallis with *Dunnett's* multiple comparison test was used; n=4.

Table 5.1 Concentrations and time point of TLRs agonist used for priming experiment in PBMCs and placental explants

Cell type	Poly I:C (TLR3)			HKLM (TLR2)			LPS (TLR4)			FSL-1 (TLR2/6)		
	Concentration	Time point		Concentration	Time point		Concentration	Time point		Concentration	Time point	
		NF-κB, AP-1	Cytokine, chemokine, PGE2		NF-κB, AP-1	Cytokine, chemokine, PGE2		NF-κB, AP-1	Cytokine, chemokine, PGE2		NF-κB, AP-1	Cytokine, chemokine, PGE2
PBMC	5µg/ml	30mins	18 hours	10^8 cells/ml	1hour	18 hours	0.1µg/ml	1hour	18 hours	0.1µg/ml	4hours	18 hours
Placental explants	25µg/ml	4hours	24 hours	10^8 cells/ml	4hours	24 hours	0.1µg/ml	4hours	24 hours	0.1µg/ml	4hours	24 hours

Table 5.2 Effects on transcription factor, cytokine, chemokines and PGE2 with priming experiment in PBMCs and placental explants

Cell type	TLR3/2					TLR3/4					TLR3/TLR2/6				
	Transcription factors		Cytokine	Chemokine	PGE2	Transcription factors		Cytokine	Chemokine	PGE2	Transcription factors		Cytokine	Chemokine	PGE2
	NF-κB	AP-1				NF-κB	AP-1				NF-κB	AP-1			
PBMC	X	-	IL-1α, IL-1β, IL-6, IL-8, IL-10	MIP-1α, MIP-1β	✓	X	-	X	X	✓	X	-	IL-1β, IL-6, IL-8, TNF-α, IL-10	MIP-1α, MIP-1β	✓
Placental explants	X	X	IL-10	MIP-1α, RANTES	X	X	X	X	RANTES	X	X	X	IL-10	RANTES	X
PBMC-media cultured on placental explants															
Placental explants	X	X	IL-6, IL-8	-	-	X	X	X	-	-	X	X	X	-	-

X = no priming effect

Discussion

Pro-inflammatory and anti-inflammatory cytokines and chemokines were augmented with TLR3 priming prior to TLR2 and TLR2/6 agonist stimulation. PGE2 was also augmented in TLR3/4 and TLR3/ 2/6 treatments. However, NF-κB activation was not augmented with priming whilst AP-1 was undetectable in the PBMCs. No augmentation on the mediators and transcription factors NF-κB and AP-1 in the placental explants.

In contrast to the effects of priming with the TLR3 viral agonist prior to TLR2, 4 and 6 agonist stimulation observed in PBMCs, placental explants did not show augmented NF-κB or AP-1 activation, pro-inflammatory cytokine or PGE2 production with priming. IL-10 on the other hand increased with TLR3 priming prior to TLR2 and TLR6 agonist stimulation. Stimulation of fresh placental explants with media from PBMC-primed supernatant did not augment NF-κB or AP-1 when compared to the supernatant from the TLR-agonist treated samples alone. However, supernatant from TLR3-primed and TLR2 agonist treated PBMCs caused augmented production of placental IL-6 and IL-8 compared to supernatant collected from PBMCs treated with the TLR agonists alone. An increase in TLR3 mRNAs with poly I:C stimulation was observed only in the PBMCs whilst no increase in the mRNA levels of TLR3, TLR2, TLR4 or TLR6 was seen in the placental explants. Although no conclusive result was obtained for the activation of transcription factor, IRF3, in the PBMCs, an increase in p-IRF3 was observed in the placental explants. Taken together, the placenta appeared to behave as a robust barrier to local multipathogen exposure by failing to stimulate a pro-inflammatory response but are susceptible to systemic multipathogen mediators. This likely to reflect the possible danger signal from the maternal-fetal interface in augmenting an inflammatory response which could lead to adverse effects to the fetus, such as preterm labour.

Conclusion:

Both PBMCs and placentas respond to individual TLR agonists, however, only PBMCs demonstrate enhanced pro-inflammatory responses with viral priming. Collectively, the results from this chapter indicate that the placenta has selective tolerance to haematogenous viral priming effects of multi-pathogen infection.

CHAPTER 6

The potential use of 15dPGJ2 and 15dPGJ2 loaded solid lipid nanoparticles as novel therapeutic agents in the prevention of inflammation/infection induced preterm labour

Introduction

Background:

Inflammation and infection are the leading causes for early preterm labour. There have been no significant advances in the development and implementation of new preventative therapeutic strategies for many decades. In this chapter, I present data exploring the potential role of the anti-inflammatory prostaglandin 15deoxy$\Delta^{12,14}$PGJ2 (15dPGJ2) for inhibiting both labour associated and pro-inflammatory mediators, along with the potential for utilising nanoparticle technology for drug delivery.

Hypothesis:

1. 15dPGJ2 inhibits the transcription factors NF-κB and AP-1, the pro-labour and pro-inflammatory mediators in human gestational tissue and could therefore be developed as a novel therapeutic strategy for inflammation and/or infection induced preterm labour.
2. The use of nanoparticle technology can be applied to provide a stable method for drug delivery of 15dPGJ2.

Aims:

1. To determine if 15dPGJ2 can inhibit IL-1β-induced NF-κB and AP-1 in an *in vitro* model of local and ascending infection/inflammation.
2. To determine if 15dPGJ2 can inhibit IL-1β-induced pro-contractile gene and protein expression, and inhibit myometrial contractility
3. To determine if 15dPGJ2 can inhibit IL-1β-induced pro-inflammatory cytokine production
4. To determine if 15dPGJ2- loaded solid lipid nanoparticles exhibit the same effect as 15dPGJ2 alone

Results

The effect of 15dPGJ2 and 15dPGJ2-SLN on myocytes

6.1 15dPGJ2 inhibits IL-1β-stimulated NF-κB and AP-1 in myocytes

32μM of 15dPGJ2 has been shown by our group to inhibit IL-1β-stimulated NF-κB in myocytes [64, 369]. We confirmed these results by demonstrating that 32μM of 15dPGJ2 inhibits IL-1β stimulated phosphorylation of p65. Myocytes were pre-treated with 32μM of 15dPGJ2 for 2 hours and the cells were stimulated with IL-1β for 15 minutes. Whole cell protein extraction was performed and phospho-p65 (p-p65) was detected by immunoblotting as a marker of NF-κB activation. IL-1β stimulation significantly increased p-p65 expression (*p<0.01) and this effect was attenuated by 15dPGJ2 (*p<0.05) (Figure 6.1 A).

AP-1 has been implicated as a transcription factor that also plays a significant role in both human and murine labour [108, 113]. The effect of 15dPGJ2 on IL-1β induced AP-1 activation was therefore examined using phosphorylated c-Jun as a marker of activation. 15dPGJ2 inhibited IL-1β induced AP-1 activation (*p<0.05) (Figure 6.1 B).

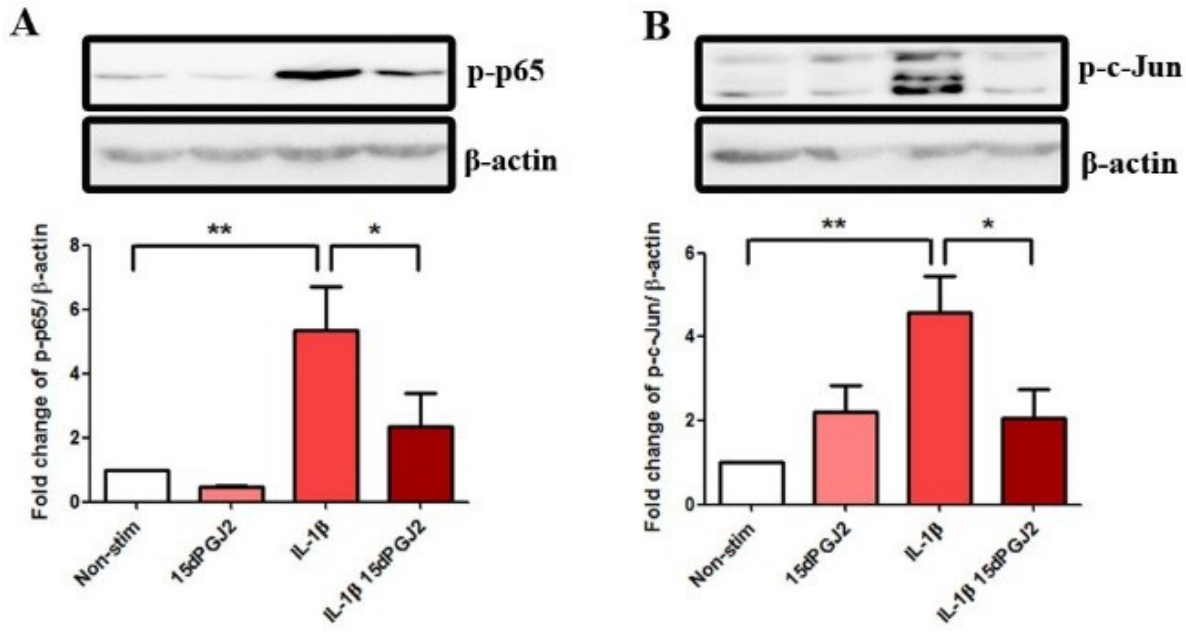


Figure 6.1 Effect of 15dPGJ2 on NF-κB and AP-1 in myocytes. Myocytes were pre-incubated with 32μM of 15dPGJ2 for 120 minutes then stimulated with 1ng/ml of IL-1β for 15 minutes. Whole cell lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) as a marker of NF-κB activation and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as a marker of AP-1 activation via Western immunoblotting. A representative blot was shown above each graph with β-actin as a loading control. Pre-incubation with 15dPGJ2 inhibited IL-1β stimulated **(A)** NF-κB (*p<0.05) and **(B)** AP-1 (*p<0.05). For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; *p<0.05, **p<0.01. n=4-6.

6.2 15dPGJ2 inhibits IL-1β induced contraction associated genes and proteins

NF-κB and AP-1 regulate the expression of *Connexin 43* [113, 405], *OTR* [115] and the *COX-2* [114] gene. COX-2 is the rate limiting enzyme that leads to PGE2 production (refer Figure 1.12 in Chapter 1) and prior to the onset of labour, contractility genes Con 43 and OTR are up-regulated in myocytes [406, 407]. The effect of 15dPGJ2 on endogenous and IL-1β stimulated Con 43, OTR, cPLA2-α and COX-2 mRNA was examined. Confirmation of COX-2 protein expression was determined using western blotting. For PGE2 production, myocytes were pre-incubated for 2 hours before stimulation with 1ng/ml and 10ng/ml of IL-1β for 18 hours.

cPLA2-α mRNA increased significantly with IL-1β stimulation at 8 and 12 hours and this effect is significantly inhibited (***p<0.001; $^{\dagger\dagger\dagger}p$<0.001) (Figure 6.2 A) by 15dPGJ2. Similarly, 15dPGJ2 also inhibited IL-1β stimulated COX-2 mRNA (Figure 6.2 B) and protein (*p<0.05) (Figure 6.2 C) at 4 hours and IL-1β stimulated PGE2 production (*p<0.05) (Figure 6.2 D).

Despite the reduction in PGE2 production, no inhibition was seen on myometrial contractility in an *ex-vivo* model (see Appendix A for unpublished data produced from the Sykes lab). In this experiment, human myometrial strips were dissected from myometrial biopsies taken from pre-labour caesarean section. Strips were incubated with either 15dPGJ2 (Figure S1 in Appendix A) or Indomethacin as a positive control (Figure S2 in Appendix A), or vehicle control at increasing concentrations at 20 minute intervals for up to 1 hour. No inhibition was seen with 15dPGJ2.

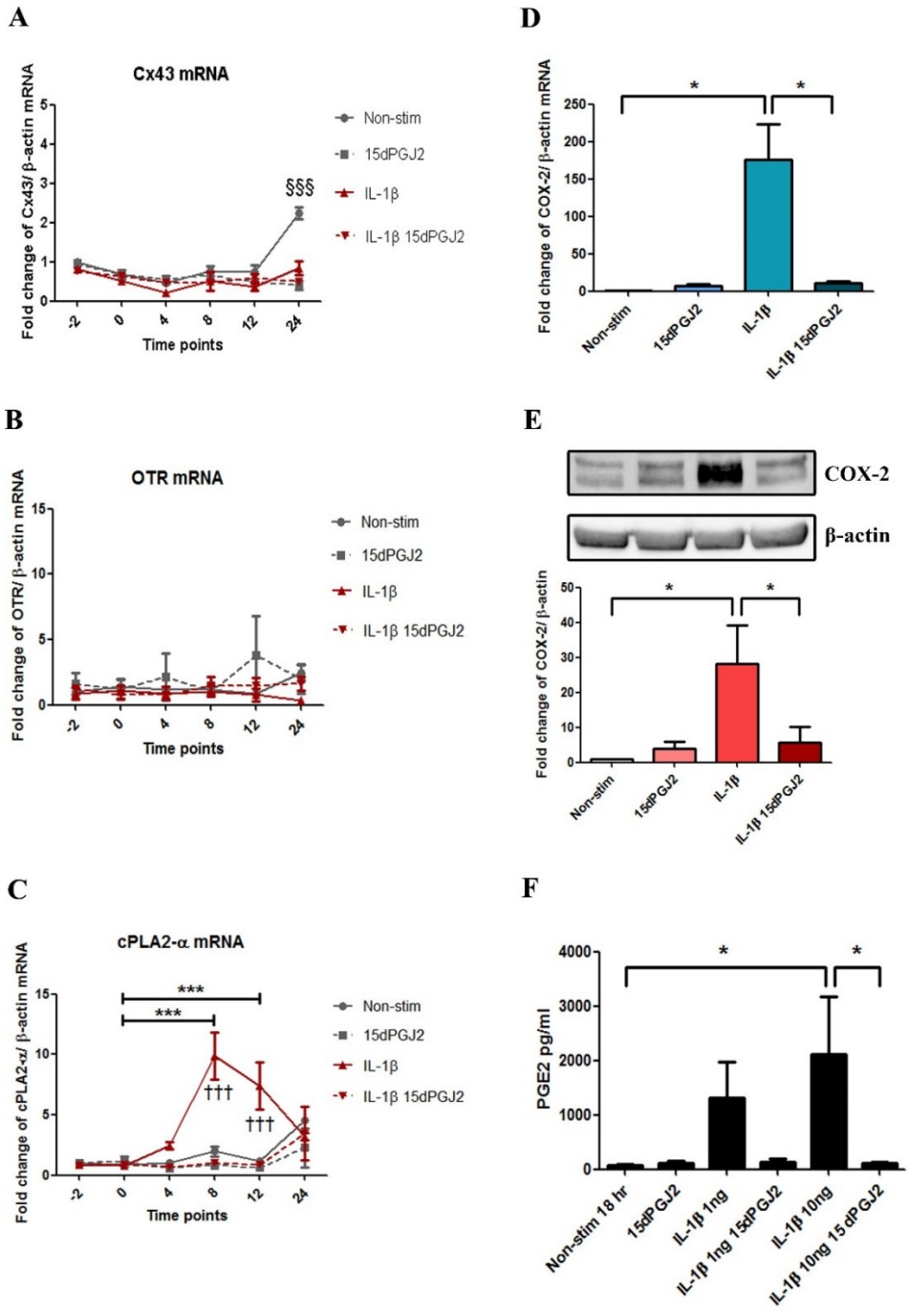


Figure 6.2 Effect of 15dPGJ2 on contraction associated genes and proteins. Myocytes were pre-incubated with 32μM of 15dPGJ2 for 120 minutes then stimulated with 1ng/ml of IL-1β for up to 24 hours. mRNA expression of Con 43, OTR and cPLA2-α was examined. Protein and mRNA level of COX-2 was determined after 4 hours of pre-incubation. 1ng/ml and 10ng/ml of IL-1β were used to stimulate PGE2 production from myocytes. Whole cell lysate was used to determine COX-2 protein expression, quantitative PCR was used to quantify mRNA expression while ELISA was used to determine the PGE2 production. Pre-incubation with 15dPGJ2 had no effect on **(A)** Con 43 and **(B)** OTR mRNA expression but **(C)** cPLA2-α expression was inhibited at 8 and 12 hours (†††p<0.001) after IL-1β stimulation. **(D)** mRNA and **(E)** protein expression of COX-2 was also inhibited after 4 hours (*p<0.05) of IL-1β stimulation. 10μg/ml of IL-1β stimulation of **(F)** PGE2 was also inhibited by 15dPGJ2 after 18 hours (*p<0.05). For statistical analysis Two-way ANOVA was used for Cx43, OTR and cPLA2-α and One-way ANOVA was used for COX-2 and PGE2 with *Bonferroni's* multiple comparison test was used. n=4-6.

6.3 15dPGJ2 inhibits IL-1β-induced pro-inflammatory mediator production in myocytes

NF-κB and AP-1 have been found to regulate a cassette of pro-inflammatory genes [66, 408]. Having shown that 15dPGJ2 inhibits these transcription factors, the expression of pro-inflammatory cytokines was assessed. Myocytes were pre-incubated for 2 hours with 15dPGJ2 before addition of 1ng/ml of IL-1β. Cells were harvested at either baseline (-2 time point), after 2 hours of 15dPGJ2 incubation (0 time point) or at 4,8,12, and 24 hours following IL-1β stimulation, (4, 8, 12 and 24 time points). Quantitative PCR was performed do determine IL-6, IL-8, TNF-α and CCL2 mRNA expression.

IL-1β stimulation increased IL-6 (Figure 6.3 A) and IL-8 (Figure 6.3 B) peaking at 8 hours (**p<0.01), and this increase was inhibited by 15dPGJ2 ($^{\dagger}p$<0.05). TNF-α expression (Figure 6.3 C) peaked at 4 hours with IL-1β (***p<0.001) and 15dPGJ2 inhibited this effect ($^{\dagger\dagger\dagger}p$<0.001). The same effect was observed with CCL2 at 12 hours (***p<0.001; $^{\dagger}p$<0.05) (Figure 6.3 D).

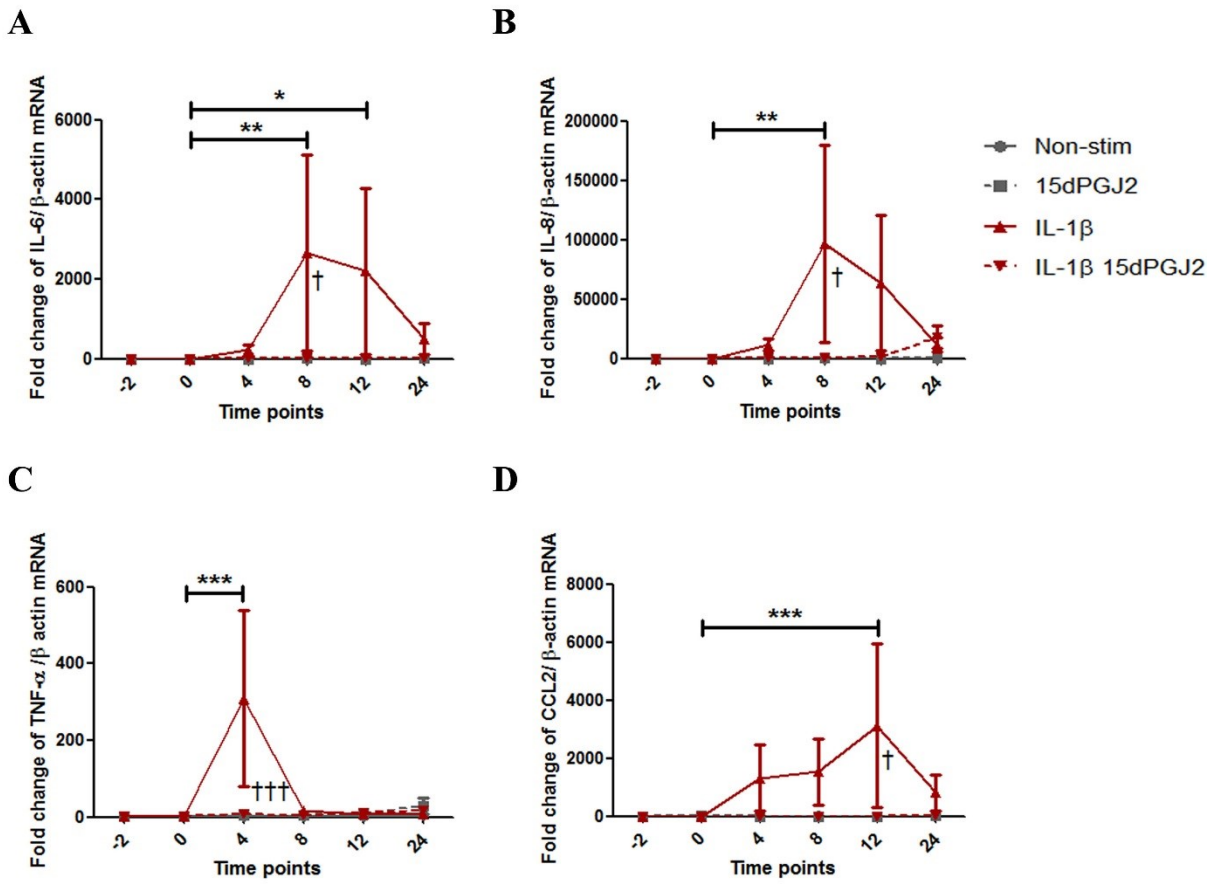


Figure 6.3 Effect of 15dPGJ2 on pro-inflammatory cytokines IL-6, IL-8 and TNF-α. Myocytes were pre-incubated with 32μM of 15dPGJ2 for 120 minutes then stimulated with 1ng/ml of IL-1β. Total RNA was extracted at -2, 0, 4, 8, 12 and 24 hours and quantitative PCR was performed looking at IL-6, IL-8, TNF-α and CCL2 mRNA. IL-1β significantly increased **(A)** IL-6 expression at 8 (**p<0.01) and 12 hours (*p<0.05) and 15dPGJ2 significantly inhibited this effect at 8 hours ($^{\dagger}p$<0.05). IL-1β also significantly increased **(B)** IL-8 expression at 8 hours (**p<0.01) in which 15dPGJ2 inhibited this effect ($^{\dagger}p$<0.05). The same effect was observed in **(C)** TNF-α at an earlier time point, 4 hours (***p<0.001 with IL-1β and $^{\dagger\dagger\dagger}p$<0.001 with IL-1β pre 15dPGJ2). **(D)** CCL2 expression on the other hand increased with IL-1β stimulation at 12 hours (***p<0.001) and this increase was inhibited by 15dPGJ2 ($^{\dagger}p$<0.05). For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=4-6.

6.4 15dPGJ2-loaded solid lipid nanoparticle (SLN) accumulated around the cells at 30 minutes

Our collaborators in Brazil supplied us with 15dPGJ2-loaded solid lipid nanoparticles (SLN) produced by their lab. A 10-fold lower concentration was anticipated to inhibit NF-κB and AP-1. This is due to the ability of this nanoparticle technology to deliver low drug doses compared to the conventional dose. In addition, it was also not feasible to use higher concentration due to the volume that is required to reach 32μM (>100μl).

The first experiment was to determine the time to entry of the nanoparticle into myocytes by incubating myocytes with fluorescence tagged 15dPGJ2-SLN and imaged using confocal fluorescence microscopy. Myocytes were cultured on a bottom glass chamber overnight. Cells were washed with PBS and incubated with 3.2μM 15dPGJ2-loaded SLN RHB-tagged fluorophore (red; 560/583) in fluorobrite serum free DMEM for 1hr. Cells with medium alone were used as a negative control. Images were captured at intervals of 15 minutes up to 1 hour.

The representative image shows an accumulation of the nanoparticle towards the cells at 30 minutes represented by the increased fluorescence signal seen around the cells at 30 minutes (see 4^{th} row, 4^{th} lane) compared to the control (see 2^{nd} row, 4^{th} lane) (Figure 6.4).

An enlarged image of myocytes incubated with 15dPGJ2-loaded SLN shows increased intensity of the red fluorescence signal from 0 hour up to 1 hour (Figure 6.5).

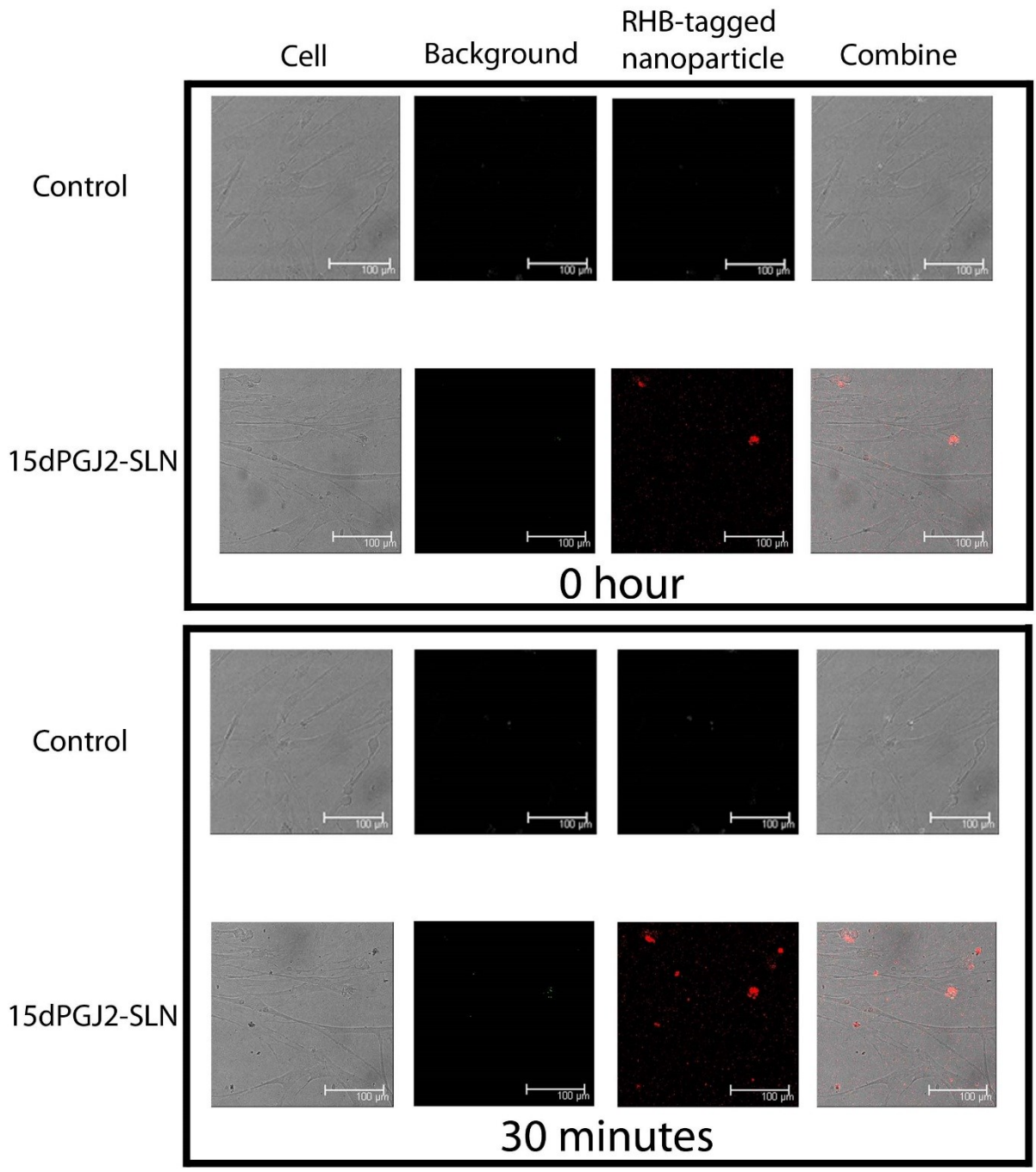


Figure 6.4 15dPGJ2-loaded solid lipid nanoparticle (SLN) accumulated the myocytes after 30 minutes. Myocytes were pre-incubated with 3.2μM of SLN, 15dPGJ2 loaded SLN and 15dPGJ2 for 60 minutes. Images were taken at 0 hour and 30 minutes under differential interference contrast (DIC) technique. The representative images of 3 independent biological replicates was shown above. The nanoparticle compound tagged with RHB-red signal (4th row, 4th lane) was seen to accumulate around the cells at 30 minutes compared to the control (2nd row, 4th lane).

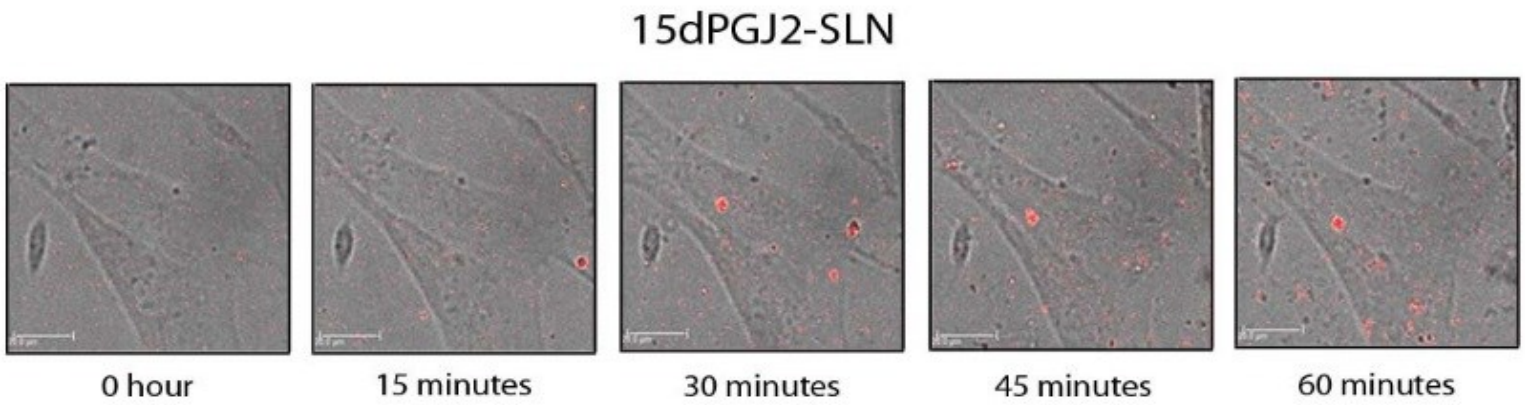


Figure 6.5 Enlarged image on the accumulation of 15dPGJ2-loaded solid lipid nanoparticle (SLN) on a myocyte over time. Myocytes were pre-incubated with 3.2μM of 15dPGJ2 loaded SLN for 60 minutes. The enlarged image from 0 hour to 60 minutes from the same experiment is presented above. The nanoparticle compound tagged with RHB-red signal was seen to accumulate around the cells from 30 minutes onwards.

6.5 Assessing the release of 15dPGJ2 from the solid lipid nanoparticle (SLN)

Based on the accumulation of the nanoparticle around myocytes at 30 minutes compared to 0 hour (Figure 6.4 and Figure 6.5), the concentration of 15dPGJ2 was quantified in the medium to examine whether 15dPGJ2 was released from the SLN core and/or being taken up by the cells. Myocytes or just the medium alone were incubated with 3.2μM of 15dPGJ2-loaded SLN, SLN or 3.2μM 15dPGJ2 alone for 2 hours. Medium was extracted at 0 hour, 30 minutes and 2 hours. 15dPGJ2 was then analysed by ELISA.

The ELISA concentrations of 15dPGJ2 in media alone was no different over time in either the 15dPGJ2 -SLN samples or the pure 15dPGJ2 samples, indicating the stability of 15dPGJ2 over time. However, there was a notably lower concentration in the 15dPGJ2- SLN compared to the pure 15dPGJ2, suggesting that not all of the 15dPGJ2 was released by the nanoparticle at 30 minutes or at 2.5 hours.

Given that the concentration of 15dPGJ2 was unchanged in the media taken from cells incubated with either 15dPGJ2-SLN and pure 15dPGJ2 over the time course, this implied that there was no detectable uptake of 15dPGJ2 into the cells.

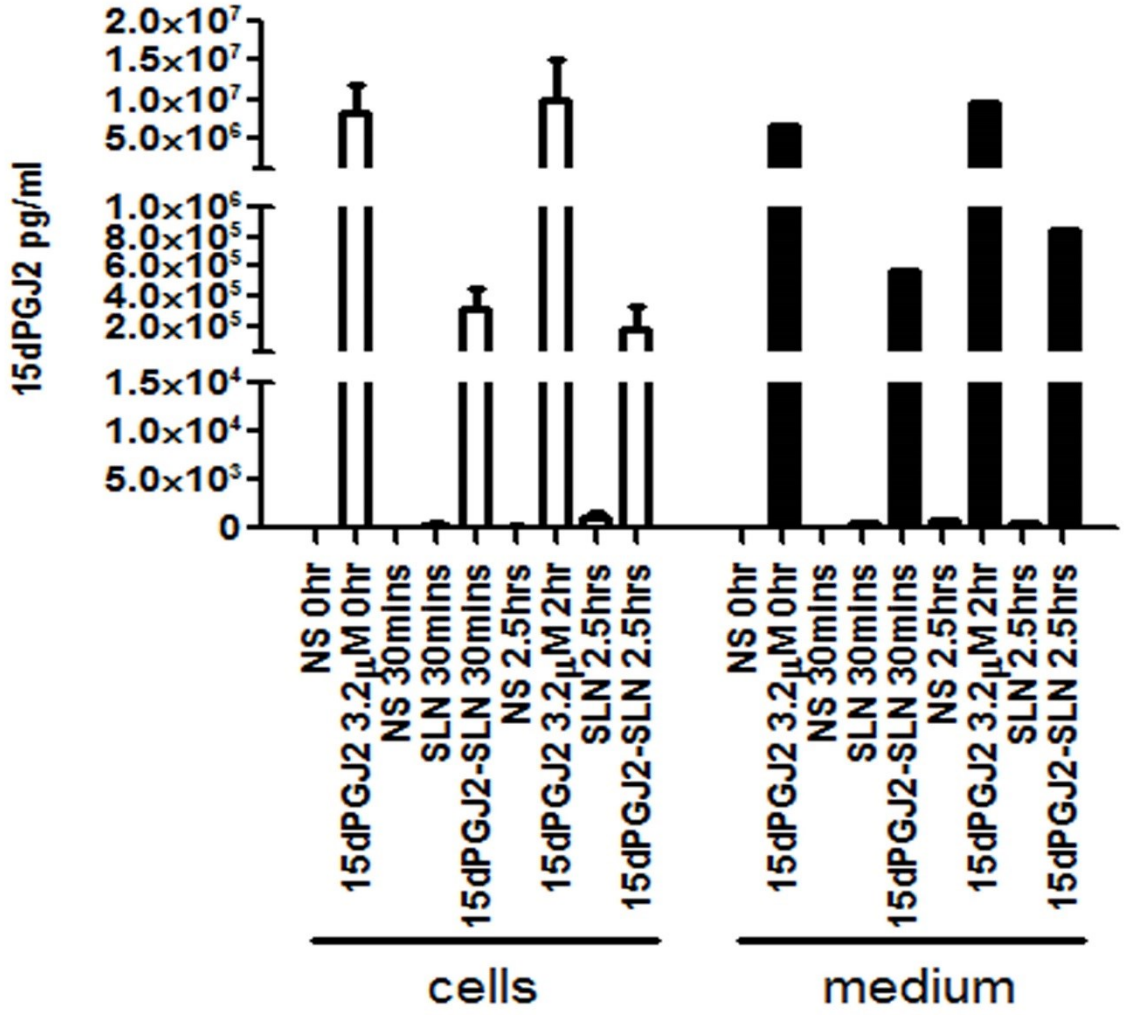


Figure 6.6 Assessing the release of 15dPGJ2 from the solid lipid nanoparticle. Myocytes or the medium alone were incubated with 15dPGJ2-loaded SLN for 30 minutes. The medium extracted from cultured myocytes cultured or medium alone was run on an ELISA. For statistical analysis, Two-way ANOVA with *Bonferroni's* multiple comparison test was used. n=3.

6.6 15dPGJ2-SLN does not attenuate IL-1β- stimulated NF-κB or AP-1 in myocytes

Having shown that the 15dPGJ2-loaded SLN accumulated over the cells (Figure 6.4) and that 15dPGJ2 could be detected at 30 minutes following addition of the 15dPGJ2-SLN (Figure 6.5), the effect of 15dPGJ2-SLN on IL-1β stimulated NF-κB and AP-1 was then assessed. Myocytes were incubated with 15dPGJ2-SLN for 2.5 hours prior to stimulation with IL-1β. This time point was chosen in order to allow 30 minutes for the nanoparticle to enter the cell and be released from the SLN and 2 hours for the 15dPGJ2 to inhibit NF-κB and AP-1 (as previously demonstrated by our group [64, 369]).

The advantage of the nanoparticle technology is the ability to use lower concentrations of a drug in the drug delivery system. Therefore, 3.2μM of 15dPGJ2-SLN was used, which is a 10-fold lower concentration to what is required by pure 15dPGJ2 to inhibit NF-κB activation.

IL-1β significantly increased the activation of NF-κB and AP-1 but no inhibition was observed with pre-incubation of 15dPGJ2 loaded SLN on NF-κB (Figure 6.7A) or AP-1 (Figure 6.7B).

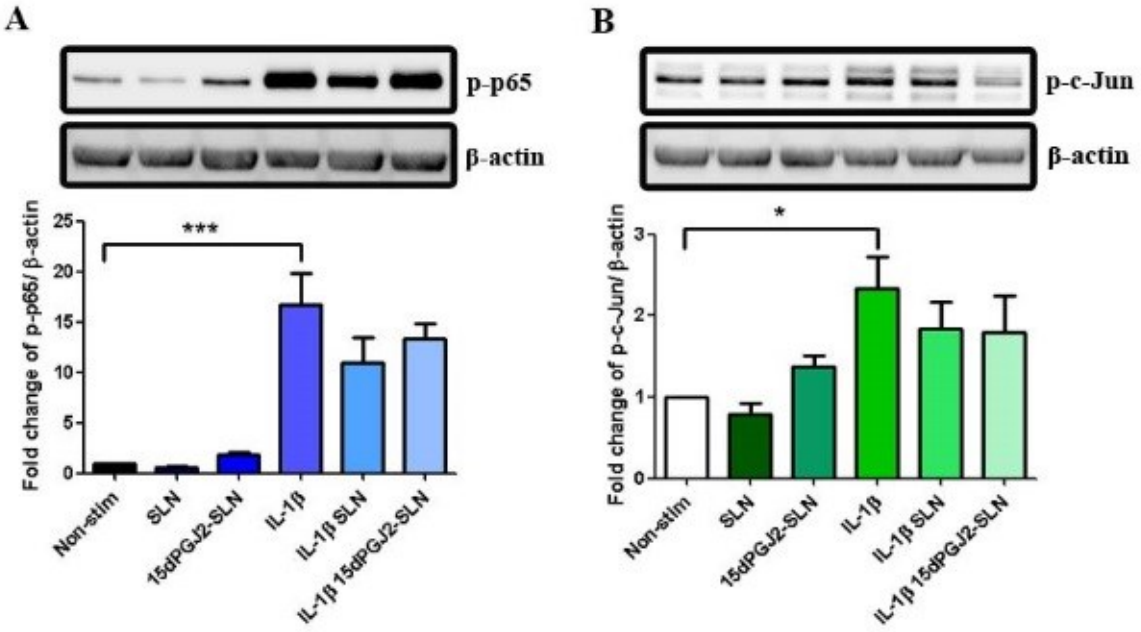


Figure 6.7 Effect of 15dPGJ2-loaded SLN on NF-κB and AP-1. Myocytes were pre-incubated with 3.2μM of 15dPGJ2 loaded SLN 30 minutes to allow the nanoparticle to enter the cells and a further 2 hours incubation to allow the 15dPGJ2 to act on the NF-κB and AP-1 pathway. The cells were then stimulated with 1ng/ml of IL-1β for 15 minutes. Whole cell lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) as a marker of NF-κB activation and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as a marker of AP-1 activation via western immunoblotting. A representative blot was shown above each graph with β-actin as a loading control. IL-1β stimulation significantly increased NF-κB (***p<0.001) and AP-1 (*p<0.05) activation. However, no inhibition was observed with 15dPGJ2 loaded SLN pre-incubation on IL-1β stimulated **(A)** NF-κB or **(B)** AP-1. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used. n=5.

The effect of 15dPGJ2 on amniocytes and vaginal epithelial cells

6.7 15dPGJ2 inhibits IL-1β-stimulated activation of NF-κB, but augments AP-1 expression in amniocytes and VEC

The effect of 15dPGJ2 on pro-inflammatory and pro-labour transcription factors was examined in amnion epithelial cells and vaginal epithelial cell lines. Since ascending infection is one of the most common causes of preterm labour, and since inflammation is key to the process of cervical ripening and membrane rupture, the effect of 15dPGJ2 on amniocytes and VEC was determined. Pre-labour amnion was used to isolate and culture amniocytes, and a vaginal epithelial cell line was used for the following experiments. 15dPGJ2 has been shown by our group to inhibit IL-1β-stimulated NF-κB in amniocytes [64, 369]. Both cell types were pre-incubated with 32μM of 15dPGJ2 for 2 hours, and stimulated with 1ng/ml of IL-1β for 15 minutes. NF-κB and AP-1 activation status were examined via western immunoblotting with antibodies directed to phospho-p65 and phospho-c-jun.

IL-1β stimulated NF-κB activation in VEC (***p<0.001) (Figure 6.8 A) and amniocytes (**p<0.01) (Figure 6.8 B) and this effect was significantly downregulated by 15dPGJ2 (***p<0.001 VEC; *p<0.05 amniocytes).

However, surprisingly, a 30-fold increase in AP-1 activation was seen in VECs pre-incubated with 15dPGJ2 (Figure 6.8 C). AP-1 activation also increased in amniocytes pre-incubated with 15dPGJ2 (3-fold), with a further increase when stimulated with IL-1β (**p<0.01) (Figure 6.8 D).

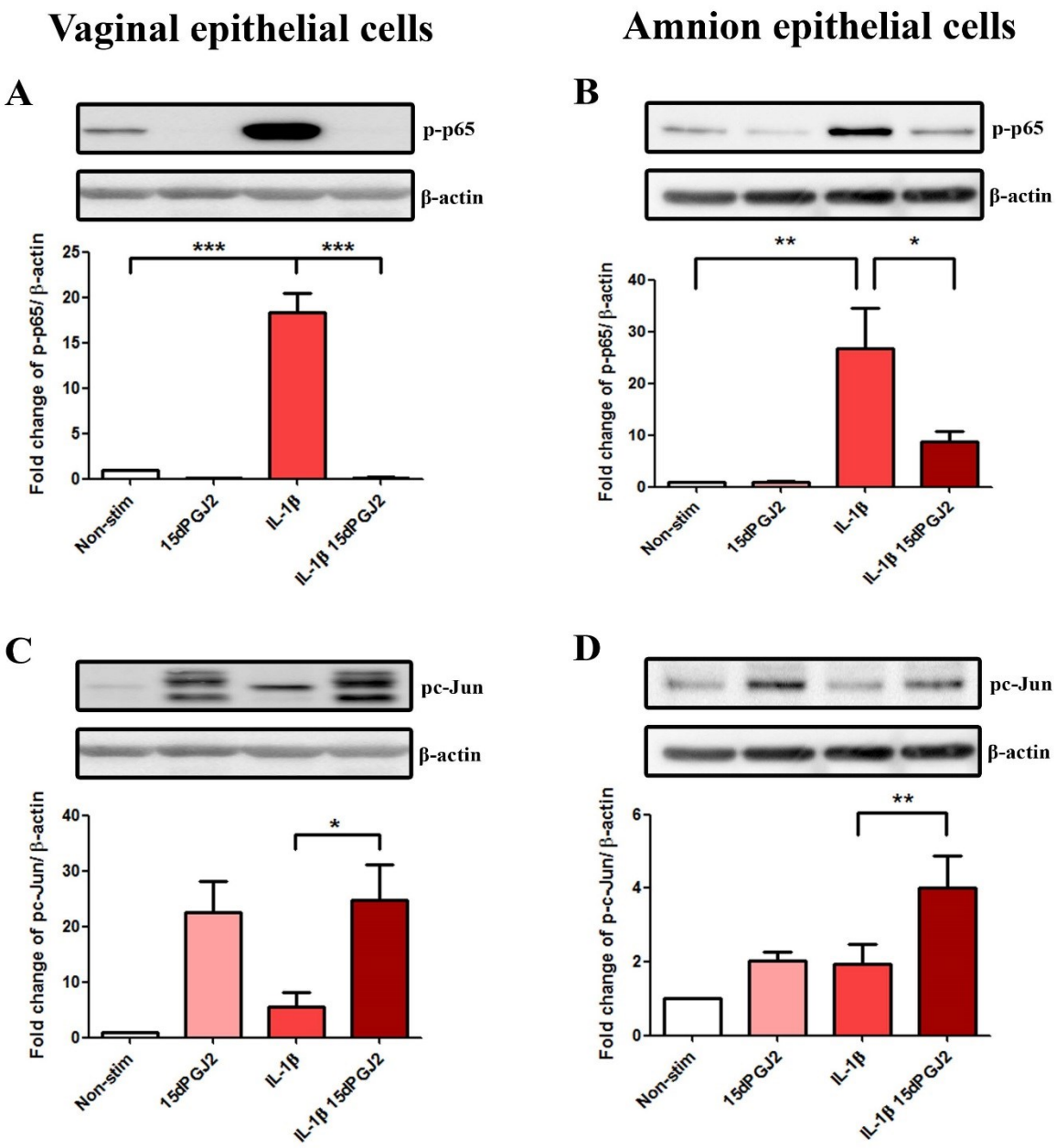


Figure 6.8 Effect of 15dPGJ2 on NF-κB and AP-1 in VEC and amniocytes. VEC and amniocytes were pre-incubated with 32μM of 15dPGJ2 for 120 minutes then stimulated with 1ng/ml of IL-1β. Whole cell lysate was used to determine serine 536-phosphorylated p65 (Ser536-p-p65) as a marker of NF-κB activation and serine 73-phosphorylated c-Jun (Ser73-p-c-Jun) as a marker of AP-1 via western immunoblotting. A representative blot was shown above each graph with β-actin as a loading control. Pre-incubation with 15dPGJ2 significantly inhibited IL-1β stimulated NF-κB in **(A)** VEC (***p<0.001) and **(B)** amniocytes (*p<0.05). AP-1 however significantly **(C)** increased after pre-incubation with 15dPGJ2 in VEC (30-fold) and **(D)** with IL-1β after pre-incubation with 15dPGJ2 in amniocytes (**p<0.01). For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; *p<0.05, **p<0.01, ***p<0.001. n=4-6.

6.8 15dPGJ2 increases AP-1 activation through p-p38 but not p-ERK in VEC and amniocytes

The mechanism by which 15dPGJ2 increased AP-1 was explored by determining its effect on the MAPK pathway in VECs and amniocytes. The same protein lysate used in the NF-κB and AP-1 quantification (section 6.7) was used to quantify phosphorylated-JNK, phosphorylated-p38 and phosphorylated-ERK.

In VECs, 15dPGJ2 significantly increased the basal level of p-JNK (15-fold, $^{**}p<0.01$) (Figure 6.9 A) and p-p38 (110-fold, $^{**}p<0.01$) (Figure 6.9 B). However, no further increase was seen with IL-1β treatment. In contrast, p-ERK was increased with IL-1β yet pre-incubation with 15dPGJ2 significantly inhibited this effect ($^{**}p<0.01$) (Figure 6.9 C).

In amniocytes, no difference in p-JNK was observed (Figure 6.9 D). 15dPGJ2 however, increased the basal level of p-p38 (30-fold) (Figure 6.8 E) and p-ERK (2-fold) (Figure 6.9 F). Stimulation with IL-1β increased p-p38 and p-ERK expression ($^{*}p<0.001$) (above and beyond 15dPGJ2 alone), yet pre-incubation with 15dPGJ2 inhibited this increase ($^{*}p<0.05$).

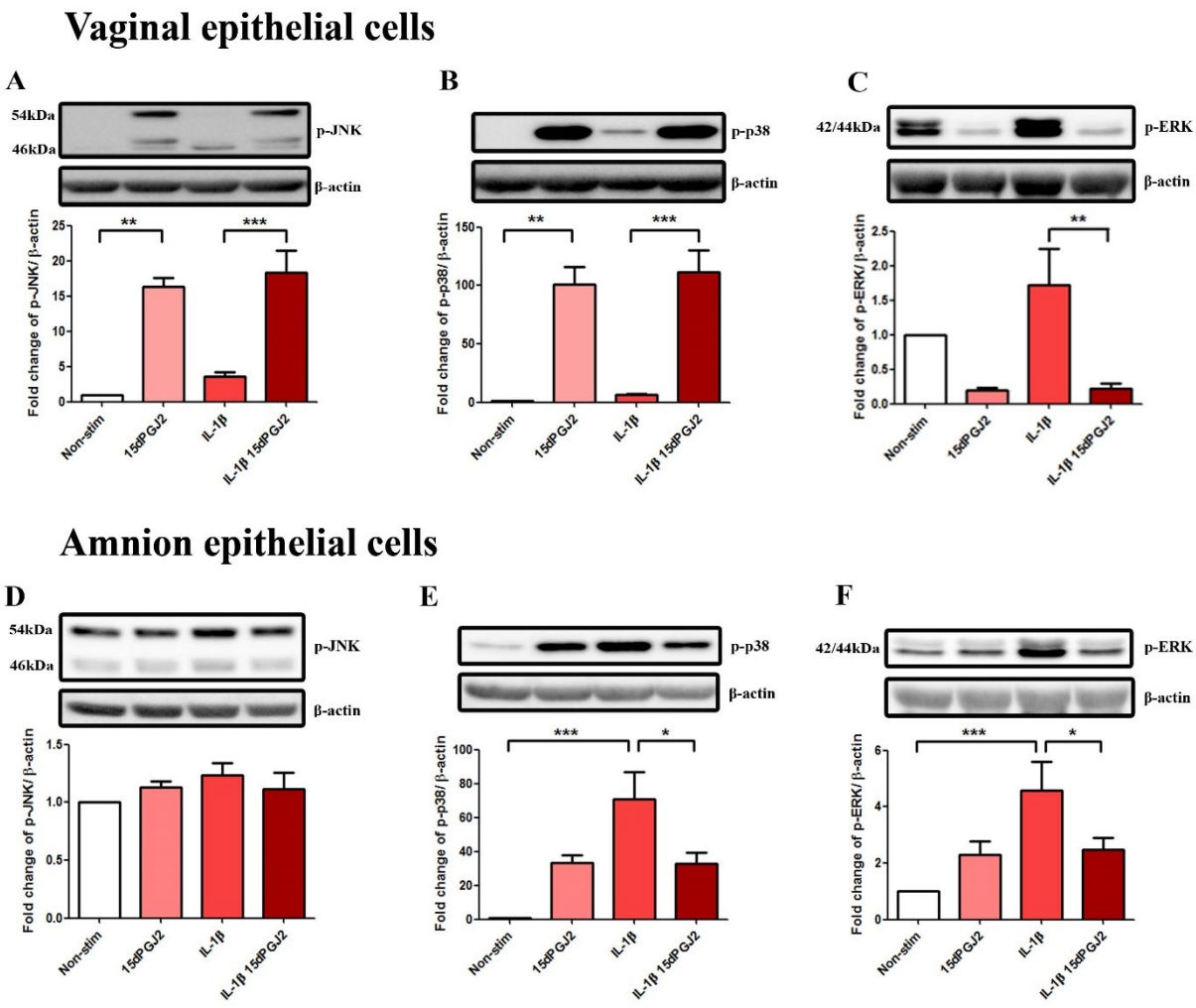


Figure 6.9 Effect of 15dPGJ2 on MAPK proteins in VEC and amniocytes. VEC and amniocytes were pre-incubated with 32μM of 15dPGJ2 for 120 minutes then stimulated with 1ng/ml of IL-1β. Whole cell lysate was used to determine phosphorylated-JNK1/JNK2 (Thr183/Tyr185), phosphorylated-p38 (Thr180/Tyr182) and phosphorylated-ERK (Thr202/Tyr204) via western immunoblotting. A representative blot was shown above each graph with β-actin as a loading control. In VEC, pre-incubation with 15dPGJ2 for 2 hours significantly increased the basal level of **(A)** p-JNK (***p*<0.01) and **(B)** p-p38 (***p*<0.01). **(C)** p-ERK on the other hand was inhibited with 15dPGJ2 after IL-1β stimulation (***p*<0.01). Pre-incubation with 15dPGJ2 in VEC **(D)** had no effect on p-JNK but increased the basal level of **(E)** p-p38 (40-fold). However, **(F)** IL-1β stimulation prior to 15dPGJ2 pre-incubation inhibited IL-1β induced p-ERK (**p*<0.05). One-way ANOVA with *Bonferroni's* multiple comparison test was used; **p*<0.05, ***p*<0.01, ****p*<0.001. n=4-6.

6.9 15dPGJ2 inhibits IL-1β stimulated COX-2 protein expression but not PGE2 production in VEC and amniocytes

Although 15dPGJ2 shows contrasting effects on NF-κB and AP-1 in VEC and amniocytes, the effect of 15dPGJ2 on pro-labour COX-2 and PGE2 in these cell types were investigated.

Both cell types were pre-incubated with 32μM of 15dPGJ2 for 2 hours before 1ng/ml of IL-1β stimulation for 4 hours to examine COX-2 protein expression. COX-2 protein increased with IL-1β stimulation and 15dPGJ2 significantly downregulated this effect in both VECs and amniocytes, (*p<0.05) (see Figure 6.10 A for effect in VEC and Figure 6.10 B for the effect in amniocytes).

In order to examine the effect of 15dPGJ2 on PGE2 production, VECs and amniocytes were pre incubated with either vehicle or 15dPGJ2 prior to 1ng/ml of IL-1β for 18 and 24 hours. Contrary to what was anticipated, PGE2 production increased with 2 hours of 15dPGJ2 pre-incubation significantly at 18 (*p<0.05) and 24 (***p<0.001) hours compared to the basal level in VEC (Figure 6.10 C). Although the same effect was detected on the amniocytes, 2 hours pre-incubation with 15dPGJ2 did not significantly increased the PGE2 production at 18 hours but significantly at 24 hours (***p<0.001) (Figure 6.10 D). Stimulation with IL-1β had no effect on PGE2 production compared to basal level in both cell types. Pre-incubation with 15dPGJ2 before IL-1β stimulation did not inhibit PGE2 production.

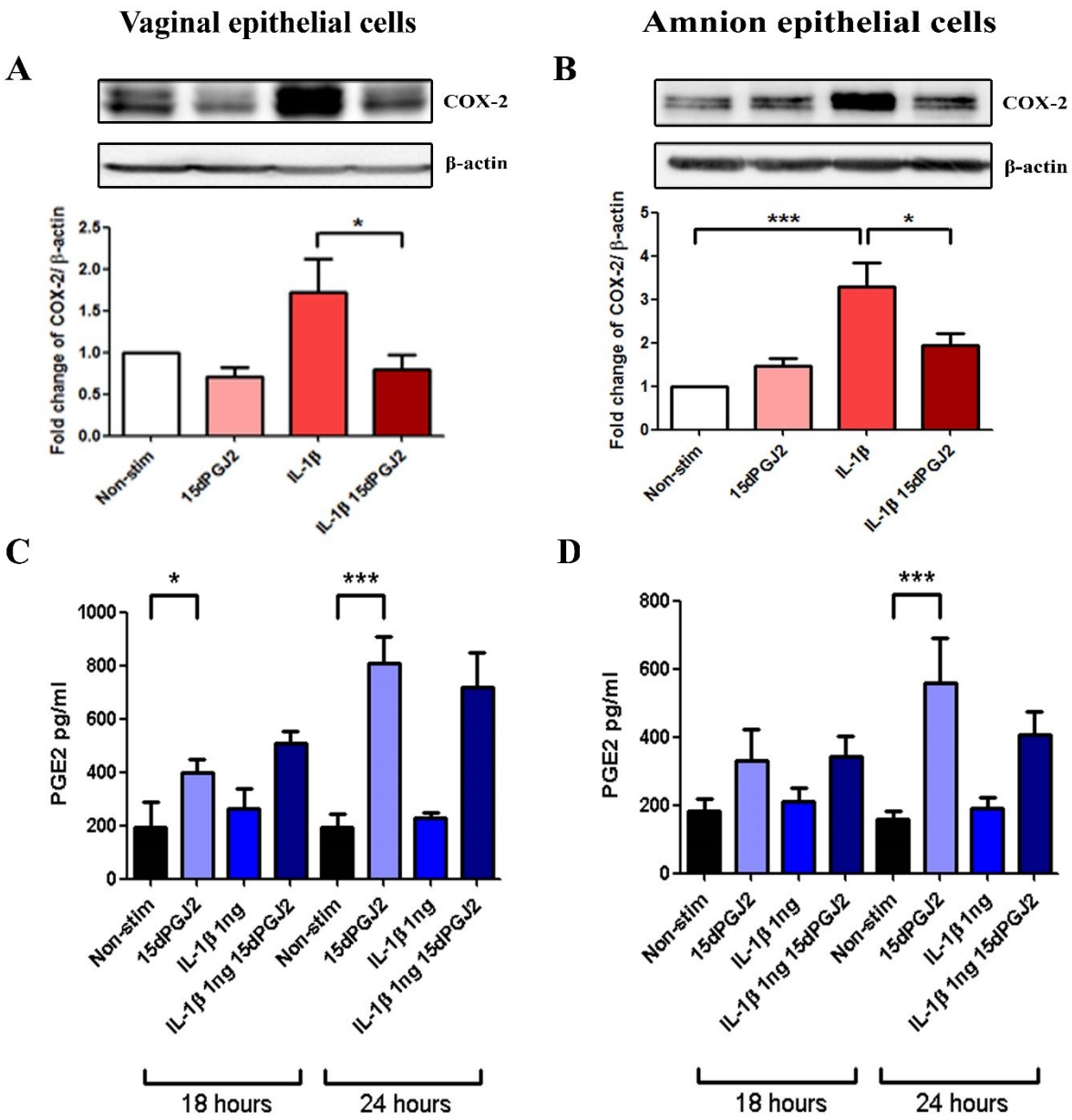


Figure 6.10 Effect of 15dPGJ2 on COX-2 and PGE2 in VEC and amniocytes. VEC and amniocytes were pre-incubated with 32μM of 15dPGJ2 for 120 minutes then stimulated with 1ng/ml of IL-1β for COX-2 protein expression after 4 hours and 18 and 24 hours for PGE2 production. Whole cell lysate was used to determine COX-2 protein expression and ELISA was used to determine PGE2 concentration. IL-1β stimulation after 15dPGJ2 pre-incubation significantly inhibited COX-2 protein expression in **(A)** VEC (**p*<0.05) and **(B)** amniocytes (**p*<0.05) after 4 hours. Pre-incubation with 15dPGJ2 for 2 hours significantly increased PGE2 production in **(C)** VEC at 18 (**p*<0.05) and 24 hours (****p*<0.001) compared to the non-stim. The same increased observation was detected in **(D)** amniocytes after 18 hours but did not reached statistical significant and significantly at 24 hours (****p*<0.001). No effect with 1ng/ml of IL-1β stimulation was seen on PGE2. For statistical analysis One-way ANOVA with *Bonferroni's* multiple comparison test was used; n=4-6.

6.10 15dPGJ2 shows variable effect on the pro-inflammatory cytokines IL-6, IL-8 and TNF-α mRNA expression in VEC and amniocytes

Pro-inflammatory cytokines are regulated by NF-κB and AP-1 transcription factors. In VEC and amniocytes, 15dPGJ2 downregulate the former and upregulate the latter transcription factor. Therefore, we sought to determine the effect of 15dPGJ2 on IL-6, IL-8 and TNF-α. Both cells were pre-incubated with 32μM of 15dPGJ2 for 2 hours (-2 hour) before addition of IL-1β (0 hour) for 24 hours. Total RNA was extracted, and quantitative PCR was performed to observe the changes in the pro-inflammatory cytokines mRNA over time.

In VECs, IL-6 expression increased at 4 and 24 (**p<0.01, ***p<0.001) hours following treatment with IL-1β. However, although there was a trend to suggest a possible inhibition with 15dPGJ2, this did not reach statistical (Figure 6.11 A). IL-1β stimulation induced IL-8 mRNA expression in VEC at 4, 8, 12 (***p<0.001, $^{\dagger\dagger\dagger}p$<0.001) and 24 (**$p$<0.01, $^{\dagger}p$<0.05) hours and this was significantly inhibited by 15dPGJ2 (Figure 6.11 B). 15dPGJ2 also significantly inhibited IL-1β-stimulated TNF-α mRNA expression in VEC at 4, 8, 12 (***p<0.001, $^{\dagger\dagger\dagger}p$<0.001) and 24 hours (*$p$<0.05, $^{\dagger}p$<0.05) (Figure 6.11 C).

In amniocytes, IL-6 mRNA expression increased after IL-1β stimulation at 8, 12 and 24 hours (***p<0.001) (Figure 6.11 D) in which 15dPGJ2 inhibited this effect ($^{\dagger\dagger\dagger}p$<0.001). TNF-α on also increased significantly with IL-1β stimulation at 4 hours (***p<0.001), however no statistically significant inhibition was achieved with 15dPGJ2 (Figure 6.10 F). Contrary to what was anticipated, 15dPGJ2 treatment alone increased IL-8 mRNA expression compared to IL-1β alone ($^{\dagger\dagger}p$<0.01) (Figure 6.11 E) at 8 hours.

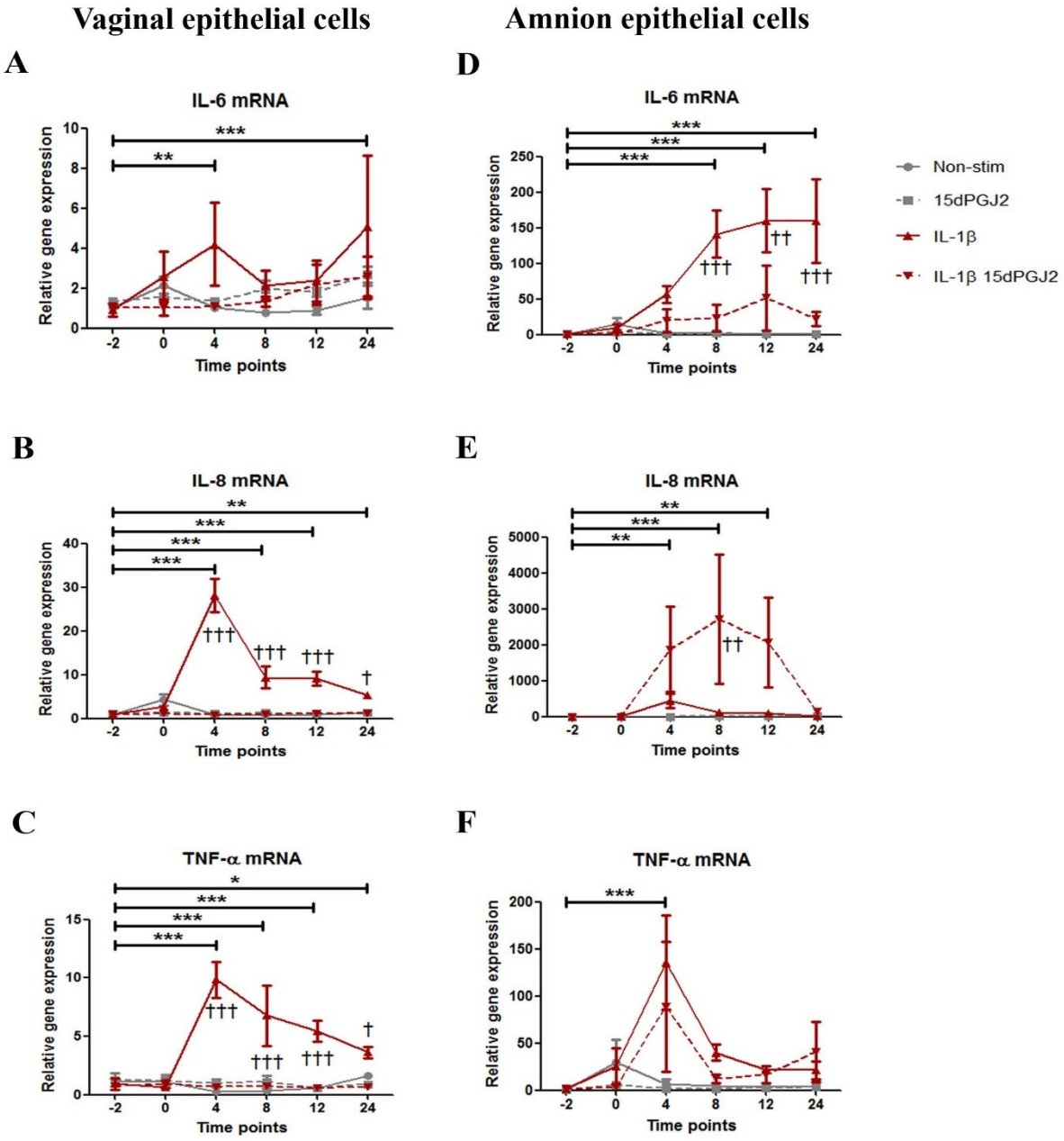


Figure 6.11 Effect of 15dPGJ2 on IL-6, IL-8 and TNF-α mRNA in VEC and amniocytes. VEC and amniocytes were pre-incubated with 32μM of 15dPGJ2 for 120 minutes then stimulated with 1ng/ml of IL-1β. Cells were harvested, and total RNA was quantified after -2, 0, 4, 8, 12 and 24 hours. Quantitative PCR was performed looking at IL-6, IL-8 and TNF-α mRNA level. IL-1β stimulation increased **(A)** IL-6 mRNA expression in VEC at 4 (**p<0.05) and 24 (***p<0.001) hours but no statistically significant inhibition was achieved with 15dPGJ2 pre-incubation. **(B)** IL-8 mRNA expression increased with IL-1β stimulation and this effect was inhibited by 15dPGJ2 at 4, 8, 12 (***p<0.001, †††p<0.001) and 24 (**p<0.01, †p<0.05) hours. **(C)** TNF-α mRNA also increased with IL-1β stimulation and this effect was inhibited by 15dPGJ2 at 4, 8, 12 (***p<0.001, †††p<0.001) and 24 (*p<0.05, †p<0.05) hours. In amniocytes, IL-1β stimulation significantly increased **(D)** IL-6 expression in which this effect was inhibited at 8 (***p<0.001, †††p<0.001), 12 (***p<0.001, ††p<0.01) and 24 (***p<0.001, †††p<0.001) hours. Pre-incubation of 15dPGJ2 before IL-1β stimulation increased **(E)** IL-8 mRNA compared to IL-1β alone (†††p<0.001) at 8 hours. **(F)** TNF-α mRNA expression increased at 4 hours with IL-1β stimulation (***p<0.001) but the inhibition by 15dPGJ2 did not reach statistical significance. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3. *p<0.05, **p<0.01, ***p<0.001 = effect of IL-1β compared to -2 non-stim time point. †p<0.05, ††p<0.01, †††p<0.001 = effect of IL-1β 15dPGJ2 compared to IL-1β.

6.11 15dPGJ2 attenuates pro-inflammatory cytokines IL-6, IL-8 and TNF-α protein expression in VEC and amniocytes

An ELISA was performed on the supernatant from cultured cells. A U-Plex human cytokine panel was used to detect IL-8, IL-6 and TNF-α production at basal, 12, 18 and 24 hours of IL-1β stimulation following 2 hours of pre-incubation with 15dPGJ2 or vehicle control. The ELISA was performed as per manufacturer's guidance for MSD as described in section 2.2.12.1 in materials and method.

IL-6 and IL-8 expression increased significantly after 12, 18 and 24 hours (***p<0.001) with IL-1β stimulation and this effect was significantly inhibited by 15dPGJ2 ($^{\dagger\dagger}p$<0.01, $^{\dagger\dagger\dagger}p$<0.001) in VECs (Figure 6.12 A and B). Despite the trend for increased TNF-α production following IL-1β stimulation, this did not reach statistical significance. Furthermore, pre-incubation with 15dPGJ2 led to a significantly lower TNF-α concentration at 12, 18 and 24 hours ($^{\dagger\dagger}p$<0.01, $^{\dagger\dagger\dagger}p$<0.001), compared to the concentration in IL-1β treated cells (Figure 6.12 C).

In amniocytes, IL-1β stimulation increased IL-6, IL-8 and TNF-α significantly after 12 (***p<0.001), 18 (***p<0.001) and 24 (***p<0.001) hours. Pre-incubation with 15dPGJ2 significantly inhibited IL-6 at 12 ($^{\dagger\dagger}p$<0.01), 18 and 24 ($^{\dagger\dagger\dagger}p$<0.001) hours (Figure 6.12 D).

The same inhibition was also observed in IL-8 (Figure 6.12 E) at 12 ($^{\dagger\dagger\dagger}p$<0.001), 18 ($^{\dagger\dagger\dagger}p$<0.001) and 24 ($^{\dagger\dagger\dagger}p$<0.001) hours.

15dPGJ2 also inhibited IL-1β-stimulated TNF-α (Figure 6.12 F) at 12 ($^{\dagger\dagger\dagger}p$<0.001), 18 ($^{\dagger\dagger\dagger}p$<0.001) and 24 ($^{\dagger\dagger\dagger}p$<0.001) hours.

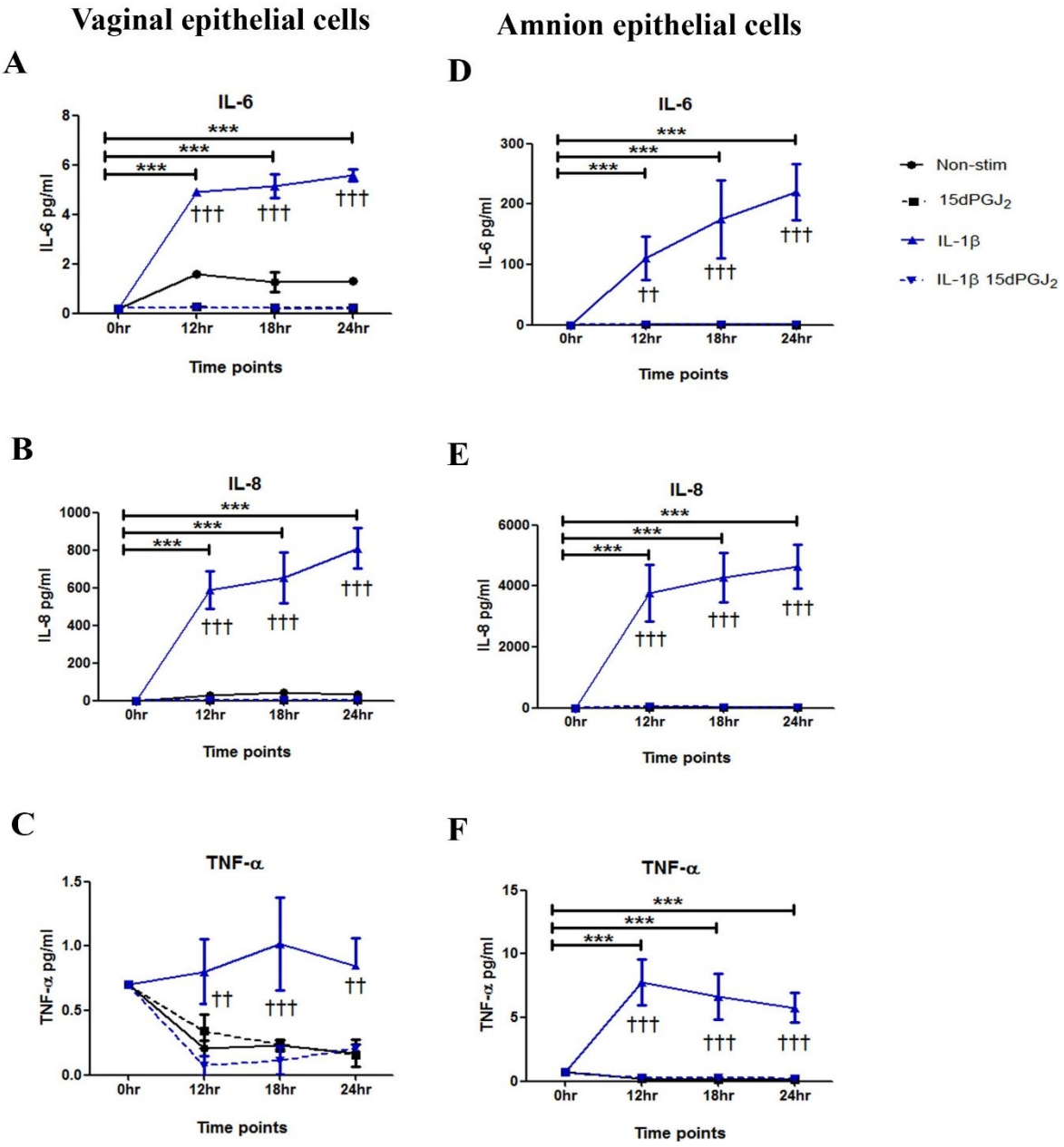


Figure 6.12 Effect of 15dPGJ2 on IL-6, IL-8 and TNF-a protein in VEC and amniocytes. VEC and amniocytes were pre-incubated with 32μM of 15dPGJ2 for 120 minutes then stimulated with 1ng/ml of IL-1β for 12, 18 and 24 hours. Supernatant was collected, and ELISA was performed to look at IL-6, IL-8 and TNF-α concentration. In VEC, IL-1β stimulation increased **(A)** IL-6 and **(B)** IL-8 expression and this effect was significantly inhibited by 15dPGJ2 at all time points (***p<0.001, †††p<0.001). 15dPGJ2 pre-incubation significantly inhibited IL-1β stimulation of **(C)** TNF-α expression at 12 (††p<0.01), 18 (†††p<0.001) and 24 (††p<0.01) hours. In amniocytes, IL-1β stimulation significantly increased **(D)** IL-6, **(E)** IL-8 and **(F)** TNF-α expression in which this effect was significantly inhibited at 12 (***p<0.001, ††p<0.01), 18 (***p<0.001, †††p<0.001) and 24 (***p<0.001, †††p<0.001) hours. For statistical analysis Two-way ANOVA with *Bonferroni's* multiple comparison test was used; n=3. ***p= effect of IL-1β compared to 0 hr non-stim time point. ††p, †††p = effect of IL-1β 15dPGJ2 compared to IL-1β.

Discussion

15dPGJ2 exhibits anti-inflammatory properties in myocytes by inhibiting IL-1β-induced inflammation. However, these properties were not replicated in VEC and amniocytes. Additionally, 15dPGJ2 in a nanoparticle form could not be applied as a prevention strategy in preterm labour.

Since inflammation is known to play a role in preterm labour and adverse effects to the neonate, developing an anti-inflammatory agent would be attractive for the use in women at risk of preterm labour. 15dPGJ2 inhibits IL-1β-stimulated NF-κB [64], a transcription factor key to regulating the expression of pro-labour and pro-inflammatory mediators. I therefore explored further the capacity of 15dPGJ2 to inhibit pro labour and pro inflammatory mediators in myocytes, vaginal epithelial cells (VECs) and amniocytes, and the potential of 15dPGJ2 encapsulated nanoparticle with a view to develop a new therapeutic agent for the prevention of preterm labour. Despite 15dPGJ2 showing promise in myocytes by inhibiting NF-κB, AP-1, cPLA2-α, COX-2, PGE2, IL-6, IL-8, TNF-α and CCL2, an unexpected increase in AP-1 and PGE2 production was seen in VECs and amniocytes. Furthermore, no effect was seen with the 15dPGJ2 nanoparticle. If this drug was to be considered for further development, targeted delivery to myocytes would need to be achieved. Additionally, the results from my study support further research into the role of antivirals and TLR2 antagonists in the prevention of multi-pathogen induced preterm labour.

Conclusion:

The effects of 15dPGJ2 are cell type specific. Although beneficial effects were seen in myocytes, the increase in AP-1 and PGE2 production in amniocytes and VEC could lead to augmentation of labour through rupture of membranes and cervical ripening if used *in vivo*. In view of this, and there being no inhibitory effect with 15dPGJ2-SLN it is unlikely that these compounds would be effective in preventing preterm labour in the human.

CHAPTER 7

General discussion and final conclusion

In support of my original hypothesis that viral infections that stimulate the TLR3 receptor during pregnancy could sensitise gestational tissues to subsequent bacterial infection, thus leading to increased susceptibility to preterm labour, I conclude that TLR3 priming leads to an enhanced pro-inflammatory response to the TLR2/6 agonist in amniocytes, VECs, myocytes and PBMCs, but not in placenta. Additionally, and contrary to my initial hypothesis, 15dPGJ2 nanoparticles can not be used as a novel therapeutic strategy for the prevention of infection/inflammation induced preterm labour due to the lack of evidence to support uptake or effect.

7.1 TLRs expression in the gestational tissues

As the first line defence, the innate immune response is required to protect the mother and fetus from infection. Toll-like receptors are important in recognising non-self and serve to allow pregnancy to progress to term. However, both spatial and temporal changes in expression have been demonstrated. For example, in trophoblasts, the functional expression of TLR2, 4 and 6 is not constant, but rather temporal and spatial. TLR6 expression is absent in first trimester [219] yet present in the third trimester [220]. Additionally, TLR2 and TLR4 exhibit a spatial expression whereby they are expressed in the term placenta [249, 409] but are absent in the first trimester placenta [219]. This spatial and temporal expression of TLRs suggests that (i) the placenta in early pregnancy may be less responsive to pathogen stimuli compared to term tissue to protect the growing fetus and (ii) the regulated responses of the TLRs are according to the gestational age. The same spatial TLR expression was also reported along the reproductive tract with a total lack of TLR4 expression in the vagina.

There is now growing interest in looking at the role of the viral TLR3 in pregnancy, however, most reported data utilises murine gestational samples [232, 333]. In humans, expression of TLR3 at the mRNA and protein level has been demonstrated in the placenta and cultured primary trophoblasts [410]. However, published information is lacking on expression patterns between pre-labour and in-labour gestational tissue or its expression in response to the synthetic agonist poly I:C.

In this study, RT-PCR and RT-QPCR revealed detectable levels of TLR2, 3, 4 and 6 mRNA in both pre-labour and in-labour samples. The difficulty in getting biopsies of vaginal tissue and culturing human primary VEC, prompted this study to use a vaginal epithelial cell line to detect the expression of TLR2, 3, 4 and 6 using standard PCR.

7.1.1 TLR2 mRNA expression

TLR2 showed significantly increased expression in in-labour compared to pre-labour amnion and myometrium tissues. However, this increased expression did not persist in cultured amniocytes and myocytes.

Studies analysing the difference in gene expression between myometrial tissue and cultured myocytes reported the same alteration in other genes. Some of the altered genes include PR and estrogen receptors that are expressed in the human myometrium [411]. The decreased fold change of TLR expression between tissue and the cultured cells might suggest that culturing the cells from tissues profoundly changes the transcriptional activity of the genes since the cellular environment is crucial as a driver of gene expression. However, a study from our group demonstrated that the cells are capable of remembering their origin. For example, using pre-labour amnion close to labour with activated NF-κB shows consistent activation at 48 hours in the cultured amniocytes [66]. In this chapter, amniocytes were cultured for 4-5 days prior to protein extraction. The cells differentiate beyond 5 days from the original cuboidal shape to form spindle-like shapes, and therefore are not used beyond this point [412, 413]. In contrast, myocytes were passaged to make multiple experiments feasible and were used within passage 3-4. Our group has demonstrated that protein expression of key pro-labour mediators are not influenced by passaging the cells (Lee *et al*, unpublished).

Studies using in-labour and pre-labour human myometrium [214], cervix [387] and placental explants [218] also recorded the same increased expression of TLR2 in the in-labour samples compared to pre-labour. The mechanism of the increased TLR2 expression during labour is still unclear, however, several reports postulate that the increase may be driven by hormones. Endogenous catecholamine, an acute stress-released hormone, is produced during labour to enhance the innate immune response [414, 415]. An increased detection of this hormone along with cortisol and endorphin also has been detected in the cord blood of newborns [416, 417]. In another study, increased TLR2 and TLR4 expression has been demonstrated in the cord blood of newborns [418]. Through the findings from these two studies, it could be postulated that the production of hormones during labour have an immunomodulatory role to

enhance TLR2 and TLR4 expression, as seen in the in-labour amnion, myometrium and placental explants in this study.

Initiation of labour is also partly signalled by the growing fetus. At the end of gestation, the matured fetus lung released surfactant A, a collectin, acts as a signal to the body to initiate labour. This surfactant is an endogenous ligand for TLR2 [419, 420] and TLR4 [421] and it is involved in the induction of the transcriptional and translational regulation of these TLRs [419]. Hence, the increased TLR2 and TLR4 mRNA expression seen in amnion, myometrium and placental explants in labour could be induced by this lung collectin, as one of the major factors for determining the *ex utero* survival of the fetus to enhance labour progress.

Infiltration of leukocytes during labour into the myometrium [57], cervix [69] and fetal membrane [388] occurs. Leukocytes express all TLRs including TLR2 and TLR4 [422]. Thus, the infiltrating leukocytes could explain the increase expression of TLR2 and TLR4 seen in the in-labour myometrium and amnion in this study.

In PBMCs, no significant change was observed between in-labour and pre-labour samples in TLR2, 3, 4 and 6 mRNA level. In contrast to this, neutrophils show a significant increase of TLR2 mRNA expression in-labour compared to pre-labour [423]. This leukocyte fraction, however, was not examined in this study. No increase in TLR expression in the PBMC at term might suggest there is less systemic inflammation in term labour compared to preterm labour.

7.1.2 TLR4 mRNA expression

TLR4 was not detected in the VECs, which is consistent with previously published reports [215, 241]. It is plausible that the lack of TLR4 expression in VECs serves as a protective mechanism to avoid excessive inflammation, which could be detrimental to the defence function of this mucosal surface during pregnancy.

However, some controversy exists in the literature since other studies have presented data to suggest mRNA is expressed [242-244]. The discrepancy between these studies may possibly be due to several factors. In one of the studies, the TLR4 amplification product from PCR was shown on an agarose gel without a water control, therefore there was no confirmation of lack of contamination during cDNA synthesis [242]. It is therefore arguable that the amplification reported in the study could be due to

contamination and is not an amplification of TLR4 gene. In the same study, TLR4 protein level was determined using western immunoblotting however, the blot showed multiple bands (which may represent nonspecific binding), and there is no indication of the correct molecular size of the protein in the text or in the figure [242]. Using vaginal tissue in another study, RT-QPCR was performed to amplify the TLR4 gene. A low TLR4 copy number was reported, which could be an indication of low or no gene expression in the sample. Furthermore, it could have been derived from infiltrating immune cells. The same tissue was then immortalised in-house using retroviral transduction to generate immortalised VECs, the same cells used in this study. RT-QPCR was then performed on the immortalised VECs and a high TLR4 copy number was reported as compared to the initial tissue. This could suggest a disturbance from the retroviral insertion during the conversion process [243]. In one of the studies, the β-actin value is very high (>27) despite the wide use of this gene as one of the housekeeping gene due to its abundant expression in the cells. A high Ct value in this study indicates low gene copy number (a low Ct value means high gene copy number). This could indicate that the primers used in the study were not properly optimised and thus, the TLR4 mRNA amplification reported in the study could be dubious [244]. The results in this study may be limited due to the use of a cell line therefore, the use of primary vaginal cells could designate a difference in expression.

In amnion and amniocytes, there was no significant change in TLR4 mRNA expression in labour compared to pre-labour. Amnion and chorion together make up the fetal membrane tissue, and in contrast to amnion, chorion expresses higher levels of TLR4. The expression is significantly decreased in the fetal membrane as pregnancy progresses in human [424], and the same is also documented in murine placenta [425]. Using a murine TLR4-knockout to study the role of TLR4, it has been shown that TLR4 is necessary for the degradation of prostaglandin but not for prostaglandin synthesis [258]. The important role of prostaglandin is for membrane rupture [426]. Thus, a lower TLR4 expression at term in the fetal membrane is important to avoid the degradation of this protein since the fetal membrane is a prostaglandin producer during labour [427].

In myometrium, TLR4 mRNA expression significantly increased in-labour compared to pre-labour. Although the same trend was observed in the myocytes, this was not statistically significant. An explanation for the increase seen in tissue rather than cells,

could reflect the presence of TLR4 positive leukocytes trafficking in the myometrium at the onset of labour [57].

7.1.3 TLR6 mRNA expression

TLR6 is not able to elicit a signal on its own and requires TLR2 to heterodimerise with. TLR2 mRNA is present in the VECs and thus, it was expected that TLR6 mRNA is also expressed by these cells, concurrent with findings from another published report [215].

In amnion, TLR6 mRNA expression showed an increasing trend in labour compared to pre-labour although no statistical significance was achieved. The same trend, however, did not persist in the cultured amniocytes, the same observation as seen in TLR2 expression. Due to the need to heterodimerise with TLR2, concurrent with the increase in TLR2 expression in amnion, increased TLR6 expression could have been anticipated.

In myometrium, a statistically significant increase in TLR6 mRNA expression was observed in labour compared to pre-labour, and this increase persisted in the cultured myocytes. This increase is also concurrent with the increase seen in TLR2 mRNA expression. The heterodimerisation of TLR2 with TLR1 or TLR6 has been demonstrated to give different downstream effects. TLR2/1 dimerization in the trophoblast induces cell-death whereas TLR2/6 shifts the cell-death response to cytokine production through NF-κB activation [223]. The same modulating response is also observed in murine gut whereby the presence of TLR6 induces an inflammatory response to *C. albicans* [428]. Aligned with the mechanism of labour as an inflammatory process, the increased expression of TLR6 may lead to increasing inflammation in the myometrium to contribute to uterine contractility and delivery as a protective mechanism.

7.1.4 TLR3 mRNA expression

Other studies have established mRNA expression of TLR3 in the placenta [218], amnion epithelial cells [216], VECs [215] and PBMC [271], with no data on myometrium or myocytes. The difference between pre-labour and in-labour TLR3 expression has not been examined in amnion and amniocytes, myometrium and myocytes or PBMC.

TLR3 mRNA is expressed in VECs suggesting that the lower reproductive tract is able to recognise invading TLR3 ligand-viruses and eventually signal an appropriate mechanism to either combat or cause further inflammation. HSV and HPV are two of the viruses that can infect the cervix, the upper region of vagina, and are established risk factors for PTB in women [174, 175, 429]. The underlying mechanism of HSV in causing preterm birth has been demonstrated in mice to be via the modulation of the cervical region mimicking cervical ripening through increased collagen remodelling and increased hyaluronic acid (HA) synthesis. To examine whether the same regulation ensued in human cells, ectocervical cells were infected with HSV which resulted in the same increased synthesis of HA [188]. This suggests that viral infection at the lower reproductive tract changes the morphology of the cells to mimic the structural changes that occur in labour.

In the in-labour amnion and placental explants, TLR3 mRNA is significantly increased as compared to pre-labour, however, this expression does not persist in the cultured amniocytes. The physiological changes that occur during labour such as plasma membrane rupture and placental separation from the endometrial walls involve apoptosis or necrosis [430]. The endogenous RNA produced from these apoptosis and necrosis processes has been shown to increase TLR3 mRNA expression, similar to the TLR3 ligand poly I:C [282, 283]. Therefore, this could explain the increased expression in the in-labour placenta and amnion tissue samples seen in this study.

No significant difference in TLR3 mRNA expression was seen in myometrium or myocytes. However, this is not consistent with the increase in TLR3 mRNA that occurs with increasing gestation and labour in murine uterine tissue. [279]. The discrepancy between both studies could be due to the different mechanisms in controlling the onset of labour between mice and humans.

7.1.5 Basal and poly I:C-treated TLR3 protein expression

The first part of this chapter showed that all gestational tissues express TLR3 mRNA at the basal level. Therefore, the protein level was assessed, and results showed that all gestational tissue express TLR3 proteins at the basal level except for amnion tissue. The peculiar observation in amnion cannot be explained by technical error since positive controls were used (placental tissue), and the experiment was performed with a total of 6 biological replicates. The same biological replicates were repeated twice

to account for technical error. Since the mRNA level of TLR3 was detected in this tissue, it is plausible that the protein concentration was not high enough in the tissue samples to detect the receptor at basal levels. Amnion tissue is also made up of mesenchymal cells, therefore, it is plausible that this cell type does not express TLR3, which would have weakened the signal in the tissue lysis buffer. It is also possible that the protein expression was lost due to high protein turnover or an induced protein modification that the antibody is unable to recognize. Furthermore, it could be an inducible protein rather than one being constitutively expressed at the basal level, in order to serve as a protective mechanism to the mother.

The in-labour TLR3 protein level in the placental explants is significantly increased compared to the pre-labour samples. The same in-labour increase compared to pre-labour was observed in the mRNA level showing that it is translated into protein. The differential TLR3 protein level in gestational tissues has never been reported in the literature and this is the first study to examine it. Labour is a process involving inflammation in each of the gestational tissues [431] and also necrosis and apoptosis as the membranes rupture and placental detachment occur at term. The heterologous mRNA, released from or associated with necrotic cells is an endogenous ligand to TLR3 [282, 283] thus, explaining the increase of TLR3 protein in the in-labour placental samples. The induction of type I IFN also ensues after TLR3 activation [307] and this mediator is important as a brake for inflammation. This is reported in a study using wild type and IFN receptor knockout ($IFNAR^{-/-}$) mice whereby the significance of IFN-β in inhibiting pro-inflammatory IL-1β, TNF-α and decreasing rate of preterm delivery was observed [432]. Using this observation, the increase of TLR3 protein in the placenta could have an important role in modulating labour-induced inflammation through the induction of type I IFN. In Chapter 5, using a placental explants model, this protein has been shown to be highly inducible in a poly I:C infection-induced model.

In PBMCs on the other hand, variation of TLR3 protein expression was noted within in-labour samples. The high TLR3 expression in the 2 patients in-labour could be due to other underlying mechanisms activating the receptor. One possible explanation could be that the pregnant mother had a viral infection such as a TLR3-activating flu virus, hence the high TLR3 protein level. The association of the influenza virus (also called the flu virus) activating TLR3 pathway has been proved using human lung

epithelial cells, inducing inflammatory responses [330, 331]. This aligns with a study using flow cytometry showing increased TLR3 expression in human PBMCs with naturally occurring influenza virus [433].

Interestingly, simulation with the TLR3 agonist, poly I:C significantly increased TLR3 protein expression. This has been successfully performed in other cell types demonstrating concurrent increase of the transcription factor NF-κB [383, 384]. In VECs, amniocytes, myocytes, PBMC and placental explants, this is the first report demonstrating increased TLR3 protein expression with poly I:C stimulation. This provides evidence of the potential ability of the gestational tissue to respond to dsRNA viruses via the TLR3 receptor during pregnancy. However, signalling relies on the fact that TLR3 possesses a functional role as either mediating the antiviral state in preventing virus replication and mitigating the disease severity or facilitating an unregulated and excessive immune response towards the infection thus harming the host. The latter however, has a negative impact during pregnancy as shown by preterm delivery in poly I:C-treated mice compared to TLR3 knockout mice [333]. In humans, the effect is still unknown, but it may have implication on human preterm labour.

One notable difference when these cells were stimulated with poly I:C was the inducibility of the TLR3 protein measured at different time points. The cells that are responsive towards ascending infection namely VECs, amniocytes and myocytes showed a delayed increase at 12 hours. In contrast, placental explants and PBMCs, representing the cells involved in the haematogenous spread of infection, showed slightly higher TLR3 protein expression (although not statistically significant) with poly I:C stimulation at around 30 minutes to 4 hours respectively. Higher concentrations of poly I:C stimulation may have led to a statistically significant increase in expression. This has been reported in PBMCs using 10µg/ml poly I:C stimulation, in which increased TLR3 protein at 30 minutes was observed [383]. This early increase is important for prompt innate immune system detection to develop to the long-formed adaptive immune system to combat the pathogens. An immunohistochemistry study found TLR3 staining in both cytosolic and membrane in the second trimester villous cytotrophoblasts [434]. The same expression could be assumed to persist in the term placenta, hence the early TLR3 protein detection with poly I:C stimulation since TLR3 also resides in the membrane of villous

cytotrophoblast. This feature is important to detect viral pathogens that have breached the placenta layer before reaching the fetus as a means of protection.

Unlike in PBMC and placental explants, TLR3 protein increases significantly at 12 hours post poly I:C stimulation in the VECs, amniocytes and myocytes. This is likely to be due to the feature of these cells exhibiting an endosomal TLR3 rather than the membrane-bound receptor. In the dose response study, the fold-change of TLR3 protein with 5μg/ml poly I:C stimulation is much higher in the amniocytes and myocytes as compared to the VECs. The difference in the response may suggest that TLR3 receptor may not be the main receptor against viral infection in the vaginal epithelial cells unlike amniocytes and myocytes. Since the vagina has the direct opening to the external environment, this organ releases its own potent molecules to fight invading pathogens such as human beta defensin (HBD), mannose-binding lecithin (MBL) and secretory leukocytes protease inhibitor (SLPI). The latter has been shown to function as a blocking agent for HIV action [435]. Therefore, a higher response of TLR3 may not be needed in viral infections in the vagina.

7.2 Poly I:C (viral TLR3 agonist) stimulation effect

VEC, amniocytes and myocytes show a pro-inflammatory response to the TLR3 agonist, with increased NF-κB/AP-1 activation and production of pro-inflammatory cytokines and chemokines. The TLR3 pathway can be activated by viruses shown in Table 1.2 (Chapter 1, section 1.7.3) and has been shown to have a dual function, either protective [436] or detrimental to the host. Undetected anti-inflammatory cytokines and anti-viral response such as IFN-α/β in the cell types examined in this study suggest that TLR3 has a potentially detrimental role in ascending infection model. The presence of these viral particles in the cervical vaginal fluid, amniotic fluid or within the cells could lead to the production of both pro-inflammatory and pro-labour mediators *in vivo*. The impact of this could lead to cervical remodelling and dilation, membrane rupture and uterine contractility, and ultimately preterm labour.

In PBMCs, TLR agonists alone increased the activation of NF-κB (Figure 5.1). However, AP-1 was not detected in these samples, despite troubleshooting. Troubleshooting involved using a new batch of antibody, different antibody dilutions and a more sensitive ECL substrate. It is possible that PBMCs may have different posttranslational modifications resulting in AP-1 being phosphorylated at Ser 63

[437], in which the antibody used detected phosphorylation at Ser 73. The early activation of NF-κB at 30 minutes suggested the peripheral blood immune cells are able to act quickly in response to systemic exposure to pathogens.

Placental explant stimulation was performed according to literature especially the 25μg/ml concentration and 4 hours time point for NF-κB effect with poly I:C stimulation [218, 333] and based on the manufacturer's datasheet for TLR2, 4 and 2/6 agonists. The expression of TLR3 receptor in the placenta has been cited in the literature [218] however, the exact role of this receptor has not been well-explored. It is likely that the TLR receptor functions to detect dsRNA viruses in the placenta, yet it is unclear if this would lead to a protective role, or to an inflammatory response that could be detrimental in causing preterm labour. An association between the presence of the adenovirus genome in the placenta and preterm labour has been reported [169].

These confirm the expression of bacterial TLRs in the gestational tissue and PBMCs, yet in-labour there is variation in the expression seen between tissue types. This demonstrates that these specific tissues are able to recognise potential bacterial pathogens which may be implicated in PTL. Furthermore, TLR3 expression is shown to have some cell-specific changes during labour, which could reflect a novel role for this receptor in the placenta and amnion during labour. The TLR3 receptor is also shown to be inducible by its own ligand but is cell-type specific. A reduced response was seen in the vaginal epithelial cells, whereas it is not inducible in the PBMCs or placental explants. This implies a differential response which is dependent on tissue type and may have implications dependent on the presence of local or systemic viral infection.

7.3 Effect of TLR3 viral priming prior to ascending bacterial agonist stimulation model

Compelling evidence for the role of viruses in preterm labour in the mouse exists since mice treated with the TLR3 agonist, poly I:C deliver preterm [333], suggesting that bacteria are not the only cause of infection induced preterm labour. With this concept in mind, the same group demonstrated the polymicrobial (or double-hit) hypothesis in which a primary viral agonist treatment increases preterm labour with an exposure to secondary bacterial infection in mice, compared to single treatments alone [334]. Using the synthetic stimulus to TLR2, 3, 4 and 6, this chapter aimed to examine

whether the polymicrobial effect is replicated in local human VEC, amniocytes and myocytes in an *in vitro* model of ascending infection. The concentration and time points used for each TLRs on each cell types is tabulated in Table 4.1 and the summary on the effect of priming is tabulated in Table 4.2, which can be find in Chapter 4.

7.3.1 TLR3/2

Priming with TLR3 prior to TLR2 agonist stimulation had no additive effect on the production of pro-inflammatory or pro-labour mediators in all cell types except in amniocytes, whereby increased AP-1 activation was seen (Figure 4.24). This was surprising since TLR3/TLR2 agonist treatment in the mouse leads to an additive increase in PTB rate. [339]. These studies are not directly comparable, *in vitro* versus *in vivo*, the route of administration in the mouse is a model of systemic spread of infection, and both agonists were given at the same time in the mouse. In contrast, in this study, the cells were primed with poly I:C before subsequent stimulation with HKLM, a synthetic stimulus for TLR2, rather than exposure to both agonists (poly I:C and PGN) at the same time. It is plausible that the potential augmentation effect of TLR3 could have worn off in the *in vitro* studies if given in advance of the TLR2 agonist. Additionally, the type of TLR2 agonist and concentration of agonist was not the same as in the murine study and therefore, could offer an explanation for the unexpected result. The dose used (10^5 cells/ml) was low when compared to the suggested working dose in the datasheet from the manufacturers (10^8 cells/ml), however this dose was selected as it was the smallest dose required to activate NF-κB.

In addition to the use of different agonists in both studies, the lack of enhanced priming response seen in the cytokines, chemokines and PGE2 in this study in these cell types could be due to the time point chosen. TLR2 agonist stimulation at 1 hour increased both NF-κB and AP-1 activation in all cell types and it is well defined that these transcription factors regulate the production of pro-inflammatory [438] and PGE2 [27] mediators. Hence, the actual time frame window of the synergistic augmented cytokine and PGE2 production might have been lost after 24 hours of stimulation. In support of this, an increase in PGE2 at 6 hours [439] and pro-inflammatory cytokines at 1 hour [440] is seen with 10^8 cells/ml of HKLM stimulation in macrophages. Therefore, earlier time points before 24 hours could be studied in the future.

Additionally, a ligand recognition by TLR2 results in a subsequent dimer arrangement either with itself, TLR1 or TLR6. The potency of the engagement with either TLR1 or TLR6 [441] could also elicit different responses depending on the various TLR2 agonists used [442]. TLR2/6 has been shown to be very potent in inducing high preterm delivery rate in mice using PGN [232] and Pam2Cys [443] agonists. While Pam3Csk, a TLR2/1 agonist, showed more enhanced pro-inflammatory cytokines production compared to HKLM [444]. This suggests that the interaction of the TLRs on the cells with different bacterial components is essential in determining the specific immune response. HKLM has been proved to be a TLR2 agonist without needing any dimerization with either TLR1 or TLR6 [445]. Using live *L. monocytogenes*, TLR2 and CD14 have been shown to be important in inducing TNF production in human monocytes [446] and cytokines in primary macrophages [447, 448]. Interestingly, the latter two studies showed that both CD14 and TLR2 are important in the host for controlling and resisting *L. monocytogenes* infection. The same involvement of both TLR2 and CD14 could be the mechanism underlying the resistance of VEC, amniocytes and myocytes to HKLM stimulation displayed by the unresponsiveness of these cells in up-regulating cytokine and PGE2 production.

In general, the results from this study suggest that there would be no additive increased risk of preterm labour in women exposed to both TLR3 inducing viral infections and TLR2 bacterial agonists that are present at the cervical-vaginal interface, the amniotic cavity or myometrium.

7.3.2 TLR3/4

TLR4 agonist stimulation alone increased NF-κB and AP-1 transcription factors in amniocytes and myocytes and in VECs. Priming with the TLR3 agonist prior to TLR4 agonists showed no effect on NF-κB or AP-1 in all cell types but PGE2 was augmented in amniocytes (Figure 4.31) and myocytes (Figure 4.50) but not in VECs. This was expected in the VECs since this study and others have failed to demonstrate the TLR4 expression in this cell type (Figure 3.2, Chapter 3) [215, 241], despite some studies suggesting otherwise [243]. If TLR4 was expressed in the vaginal epithelial cells, a continued stimulation by bacteria through pregnancy could cause sustained local inflammation leading to cervical dilation and preterm labour. Therefore, the lack of expression in the vagina suggests that it is a protective mechanism of immunological

tolerance of local microbiota at the cervical vaginal interface. Care was taken to use ultrapure LPS at a low concentration (0.1µg/ml) to avoid stimulating other TLRs since concentrations above 1µg/ml are known to reach plateau effect and are highly toxic to the cells (based on information from Invivogen technical support). Therefore, the assumption can be made that AP-1 activation seen with ultrapure LPS 055 stimulation could be an erroneous effect of VECs with the agonist or it could be that the agonist is also stimulating other TLRs at 0.1µg/ml concentration in the VECs.

No augmentation of pro-inflammatory cytokines was seen in VECs with TLR3 viral priming prior to TLR4 agonist stimulation, as anticipated due to lack of TLR4 mRNA expression. This is consistent with the unresponsiveness of VECs to vaginal inoculation with MHV-68 and LPS in mice. This study showed no augmented production of IL-1α, IL-1β, RANTES, IL-6, MIP-1α/β in the upper region of the vagina [338].

In contrast, augmented production of pro-inflammatory cytokines was observed in amniocytes and myocytes. An augmented IL-6 response was also observed in a human fetal membrane explant study [337]. The fetal membrane explant was stimulated with poly I:C as a viral stimulus for 24 hours before stimulation with LPS 0111:B4. An augmented response was seen in IL-6, MIP-1α and RANTES. In this study, the amniocytes had an augmented pro-inflammatory response, including TNF-α. TNF-α was not augmented in the fetal membranes from the Cross et al study [337]. It is possible that this difference in effect is due to the different LPS serotypes used. In this study, LPS 055:B5 was used rather than LPS 0111:B4 in the explant study. Murine studies from our group have previously shown that different LPS serotypes have different responses [253]. The latency of the labour onset effect seen in mice after LPS injection was markedly different between the serotypes used thus suggesting that the structural variances in the O-antigen, that confers the specificity of the molecule, changes the interaction of the LPS molecule with the TLR4 receptor complex. This could eventually result in the activation of different downstream inflammatory pathways [449] as seen in this study and the fetal membrane explant study.

The close contact of the fetal membranes with the myometrium could also lead to ascending infection causing an inflammatory response in myocytes. This also might expose *E. coli* to the myometrium during ascending infection. A study incubating

myometrium with LPS to mimic local effects of chorioamnionitis showed increased inflammation and apoptosis [450]. In a contractility study, LPS also increased uterine contractility using primary cultured uterine myocytes [451]. The effect of viral stimulation on myocytes is under researched. This study and Lim at al study [310] showed the evidence that myocytes are capable of eliciting pro-inflammatory cytokine and chemokines responses when stimulated with poly I:C.

Taking these data together, the presence of viral and bacterial particles in amnion and myometrium could result in premature rupture of fetal membrane and uterine contractility during pregnancy.

The augmented pro-inflammatory cytokine production seen in amniocytes and myocytes in this study occurred despite no significant increase in NF-κB or AP-1, implying that another transcription factor is involved in modulating the augmented cytokine response. TLR4 is unique in that it can elicit signalling via both MyD88 and TRIF pathways, the latter pathway is also utilised by TLR3 [452]. Activated TRIF pathway leads to phosphorylation of several transcription factors namely IRF3, thus, up-regulating an array of mediators including type 1 IFN [308] and pro-inflammatory cytokines via crosslinking with NF-κB [453]. Poly I:C also has been shown to up-regulate p-IRF3 [454] thus it may play a role in the priming effect in amniocytes and myocytes.

7.3.3 TLR3/6

Stimulation with the TLR2/6 agonist alone increased NF-κB and AP-1 activation in all cell types. Priming with TLR3/TLR2/6 increased pro-inflammatory cytokines, chemokines and PGE2 in VECs and amniocytes. In myocytes, PGE2 was not augmented with TLR3/TLR2/6 priming. This implies that the presence of pathogens that stimulate these receptors would have an additive effect *in vivo*.

In intrauterine infection, *Ureaplasma* species, consisting of *Ureaplasma parvum* and *urealyticum* [455-457], and *Mycoplasma hominis* [458] are a few of the most frequently isolated microorganisms from fetal membranes and amniotic fluid in association with MIAC and cervical insufficiency [459]. These bacteria act on the TLR2/6 ligand [455, 460] and are also associated with preterm labour [9] and fetal inflammatory response syndrome in neonates [461]. Pro-inflammatory cytokines play

a major role in labour [462], thus, the increase seen with in TLR3/TLR2/6 priming could lead to premature activation of the inflammatory cascade associated with preterm labour. Increased IL-6 concentrations in the amnion, VECs and myocytes could activate the macrophages and natural killer cells activity to invade cervical and endometrial tissue for labour progression [61]. Uterine contractility could be enhanced by IL-6 which has been shown to stimulate the oxytocin receptor to increase its responsiveness to oxytocin in myocytes [463]. Prostaglandins are crucial for myometrial contractility and cervical ripening [464] and the production is increased in response to IL-6 in amnion [465, 466], decidua [467, 468] and myometrium [469, 470]. IL-8, aids cervical ripening by inducing neutrophil migration to the cervix. Increases in IL-1, IL-6 and TNF-α production in myocytes and VECs could also activate MMP (matrix metallopeptidase), a degradation protein, to add to membrane rupture [471-473] and cervical remodelling [474, 475].

In addition to cytokines, chemokines also play an important role in the regulation of immune responses. This study showed augmented chemokine levels with TLR3/TLR2/6 priming. Macrophage inflammatory protein-1α (MIP-1α) and MIP-1β and RANTES (also known as CCL5) are from a chemokine subfamily that attracts monocytes, macrophages, basophils and other immune effector cells to amplify inflammatory responses [476-479]. MIPs concentrations have been shown to correlate with cervical dilation in women with chorioamnionitis at term [480] and preterm [481] gestation, whereas in amniotic fluid, RANTES [482] and MIP-1α [480] concentration is increased in term and preterm women with and without MIAC. Besides attracting leukocytes, RANTES also is able to induce basophils to release histamines [477, 483]. Histamines have been shown to be potent uterotonics resulting in uterine contraction in studies using isolated human pregnant and non-pregnant myometrium [483-486]. Therefore, the endogenous release of histamines could be dangerous during pregnancy by activating uterine contractility leading to preterm labour. An *in vitro* study of human primary decidual cells [487] and non gestational tissue [488] cultures also showed that histamines can act as local hormones in regulating prostaglandin production. Thus, in combination with the augmented PGE2 released in amniocytes and VECs with TLR3/TLR2/6 priming, these could lead to premature membrane rupture and cervical dilatation. In contrast, although PGE2 is not augmented in myocytes with priming, prostaglandin release regulated by histamines due to enhanced

RANTES production might imply a danger signal from TLR3/TLR2/6 priming to the myocytes.

Despite the increase of cytokines and chemokines in these cells, priming with a TLR3 agonist prior to TLR2/6 agonist stimulation had no effect on NF-κB or AP-1. This implies that there is another transcription factor that may be responsible for augmenting these mediators. NF-κB has 5 different members in its family, including RelB which has been shown to be expressed in human placenta [489] and amnion [66]. This protein could be implicated in the regulation of the cytokines and chemokines in this priming. Another transcription factor that is expressed in human gestational tissues is CEBP/β, which could also be associated with the effect seen in this priming. However, no studies have looked at the effect of poly I:C stimulation on CEBP/β in these cell types. However, CEBP/β mRNA expression of murine airway epithelial cells was induced when infected with influenza A (a TLR3 ligand) [490]. An upregulation of CEBP/β using TLR2 agonists has also been demonstrated [491].

The interferon regulatory factor (IRF) family of transcription factors has been characterised as mediators of the anti-viral response by producing type 1 interferon (IFN). Among the family members, IRF5 has been shown to be recruited to the inflammatory promoter gene via RelA [492] to mediate pro-inflammatory cytokine production [493, 494] at serine 446 via IKKβ [495]. The activation of IRF5 has been demonstrated with poly I:C stimulation [495] and FSL-1 [496]. With the recent evidence that IRF5 is increased in term labouring myometrium compared to term non-labouring myometrium [496], this transcription factor could be involved in the induction of pro-labour mediators in TLR3/TLR2/6 priming.

7.4 Effect of TLR3 viral priming prior to haematogenous bacterial agonist stimulation model

The role of viruses in haematogenous spread of infection and preterm labour is an under-researched area. However, some evidence does exist to support their role. For example, a case-control study looking at peripheral blood and extravillous placental viral load of HPV DNA reveal higher levels in women who have preterm deliveries and miscarriages compared to term deliveries [193]. Another study looking at the maternal blood collected during early pregnancy revealed that mothers who had second trimester miscarriage or very preterm birth showed higher levels of Parvovirus

B19 and human herpes virus [497]. These viruses have been shown to cross the placenta [498] and can be detrimental to the fetus [499]. Taken together, this supports the need for increased research into the role of viral infections in adverse pregnancy outcomes.

Viral infection is also known to predispose to an increased number of bacterial infections. This is supported by the increased sensitivity of lymphocytic choriomeningitis virus (LCMV)/ indiana vesiculovirus (VSV) and poly I:C-treated mice to adverse effects of LPS [500-502]. Comorbidity studies of viral infection before subsequent bacterial infection has led to the introduction of a concept of viral priming. A proposed mechanism for this is that priming leads to an immune response, which then lowers the threshold required from invading bacteria to cause an augmented response [334, 335, 339]. However, the majority of studies testing the effects of viral priming on bacterial responses have either been conducted in the mice or in human first trimester trophoblast cells. The question remains whether the human placenta is more susceptible to bacteria following viral priming either from a systemic multipathogen infection, or from the effects of local placental infection as a result of haematogenous spread. The concentration and time points used for each TLRs on PBMCs and placental explants is tabulated in Table 5.1 and the summary on the effect of priming is tabulated in Table 5.2, which can be find in Chapter 5.

7.4.1 PBMC

Stimulation of PBMCs with the TLR3 agonist alone increased the activation of NF-κB. However, the p-c-Jun and p-IRF3 were not detected. Most studies looking at the expression of AP-1 in the PBMC focused on the upstream protein expression such as p-p38, p-JNK and p-ERK. The protein concentration used to detect these proteins was as high as 50μg using western immunoblotting [503, 504] compared to 15μg of protein used in this study. Therefore, the low protein lysates used may contribute to the low protein of interest expression in the membrane to detect the presence of c-Jun protein.

Viral priming using poly I:C prior to subsequent TLR2 and TLR2/6 agonist stimulation in PBMCs had an additive effect on the production of pro-inflammatory and chemokines. This result implies that pregnant women that are exposed to both systemic viral and bacterial infections capable of activating TLR2 or TLR6 receptors may be detrimental due to the resulting further increase in systemic pro-inflammatory

cytokines. The augmented response seen with TLR3/2 and TLR3/TLR2/6 priming could be due to the increased of TLR2 mRNA at <30 minutes. Although this time point was not studied in PBMCs, the unchanged TLR2 mRNA in the placental explant (which showed lack of priming effect) and the increased TLR2 mRNA seen in VEC, amniocytes and myocytes (all of which did show a priming effect) strengthens this possibility.

In contrast to TLR2 and TLR2/6 agonists, TLR4 agonist stimulation prior to priming had no added effect on the pro-inflammatory cytokines and chemokines. The signalling of TLR can be supressed by another receptor, called as a suppressor. One of the family of suppressors that could be involved in the suppression of the TLR3/4 priming effect is the TAM receptor family that comprises of Tyro3, Axl and Mer. These receptors trigger a kinase cascade that leads to type I IFN and other inflammatory cytokines [505]. This is a feature of TLR4 signalling since it is able to elicit both type I IFN and pro-inflammatory cytokines [194]. The initial burst of type I IFN and inflammatory cytokines induces the expression of the TAM receptors and this receptor upregulates the expression of suppressor of cytokine signalling 1 (SOCS1) and SOCS3. These SOCS proteins lead to the dampening of the inflammatory response by inhibiting TLR4 and cytokine receptor cascade [505, 506]. This event could be the unique mechanism for there being no augmented response in the TLR3/4 priming experiments in PBMCs and placenta. However, this does not explain how there is an augmented pro-inflammatory cytokine response observed in amniocytes and myocytes with TLR3/4 priming (Chapter 4). A loss of TAM receptor expression after culture could explain the differential outcome of the double-hit poly I:C and LPS response seen in this study with different cell types. Another possibility could be the cell type specific action of TAM receptor. A study demonstrated that stimulation of poly I:C and LPS on the fetal membrane explants (which contain amnion mesenchymal cells and chorion) disabled the TAM receptor pathway thus, inhibiting the function of TAM as a brake for cytokines cascade.

Besides the pro-inflammatory cytokines, IL-10 production was also augmented with TLR3 priming prior to TLR2 and TLR2/6 stimulation but not TLR4. This anti-inflammatory mediator is produced by activated immune cells namely lymphocytes [507-509], macrophages [510, 511] and dendritic cells [511]. IL-10 is the key anti-inflammatory modulator to counterbalance the synthesis of a cascade of pro-

inflammatory cytokines [512]. In animal models, IL-10 has been shown to be protective, and is vital in providing resistance towards LPS-induced preterm labour [513, 514]. The induction of IL-10 with TLR3/2 and TLR3/TLR2/6 priming suggests that the gram positive bacterial components (HKLM and FSL-1 agonist) used in this study may trigger the production of this cytokine as an intrinsic negative feedback mechanism.

PGE2 is important in labour and delivery as this protein aids myometrial contractility and cervical ripening for the progression of labour. An increase in PGE2 production was observed with TLR3/2, TLR3/4 and TLR3/TLR2/6 priming. It is thus possible that the systemic circulating PGE2 could then stimulate the myometrial contractility and also lead to cervical ripening. Therefore, the increase in this protein in concert with the increased of pro-inflammatory cytokines with TLR3/2 and TLR3/TLR2/6 priming might be detrimental during pregnancy to induce preterm labour.

The collective response of PBMCs exposed to multipathogen infection is likely to be detrimental in the context of pregnancy and preterm labour. The impact of increased pro-inflammatory mediators and PGE2 in the circulation could increase the exposure of myometrium, cervix and fetal membranes. This could ultimately lead to triggering contractility, cervical dilation and fetal membrane rupture, all processes that are involved in preterm labour.

7.4.2 Placental explants

Stimulation of placenta with TLR3 and TLR4 agonists alone increased the activation of NF-κB but had no effect on AP-1 expression. Expression was not augmented with priming, consistent with there being no augmentation in the production of pro-inflammatory mediators.

In contrast to PBMC, placental explants showed a very restrictive response towards TLR3 viral priming prior to TLR2, 4 or 2/6 agonist stimulation with no enhanced pro-inflammatory cytokine production. The response seen in this study is not in agreement with a previous study showing augmented pro-inflammatory IL-6 in the primary cultures of human first trimester trophoblast cells using MHV68 as the viral stimulus and LPS as the bacterial stimulus [335]. The differences seen could be due to the timing and type of TLR agonist used, cellular responses being different to explant

responses, or the gestation of placental sampling. The difference of concentration and in LPS serotype might explain the different effects seen in the studies. Our models also differ in that a longer exposure mimicking a chronic exposure of infection was conducted in the aforementioned study, whereas a shorter exposure was performed in this study. My results imply that the third trimester placenta is less susceptible to double-hit in an acute setting rather than a chronic setting.

Despite there being no augmented response seen with the pro-inflammatory cytokines, TLR3 viral priming prior to bacterial TLR2, 4, 2/6 agonists resulted in augmented chemokine production namely RANTES and MIP-1α. Since RANTES and MIP-1α share the same receptor [515, 516], it is thought that the mode of action is the same in inducing chemotaxis of monocytes and macrophages [515, 517]. Macrophages are important in the immune system for foreign antigen processing and presenting them for T cells recognition as a process of microbial clearing. This activity can be further enhanced by the activation and differentiation of monocytes into macrophages via adhesion to the matrix components [518], which is aided by RANTES [519]. Meanwhile, MIP-1α increases when monocytes binds to the adhesion molecule [520]. Therefore, increased RANTES and MIP-1α observed in the placenta could be a mechanism of pathogens clearing to protect the fetus. To support this notion, an increase of RANTES [481, 482] and MIP-1α [521] is also seen in the amniotic fluid in term and preterm deliveries complicate by microbial invasion of the amniotic cavity which might suggest the same microbial clearing response [481, 482, 521]. RANTES also plays a role in inducing histamine release from basophils, which leads to prostaglandin production. However, prostaglandin production might be limited in the placenta by the high expression of diamine oxidase (DOA) (or histaminase) (an enzyme that inactivates histamine) in the placenta [522-524]. This may explain why PGE2 production is not augmented in the placenta with TLR3/2, TLR3/4 and TLR3/TLR2/6.

The most striking augmented response of anti-inflammatory cytokines with IL-10 following TLR3/2 and TLR3/TLR2/6 priming conditions was observed in the placental explants. Besides being produced by the immune cells, cytotrophoblast, syncytiotrophoblast [525, 526] and decidual mononuclear cells [527, 528] are additional sources of IL-10 during pregnancy. This protein during pregnancy is important, shown by LPS-stimulated preterm delivery in an IL-10 knockout mice

study. The loss of IL-10 increased the number and placental infiltration of uNK cells that leads to placental cells death hence preterm labour. The preterm delivery effect was restored with a depletion of uNK cells [529].

To further support the haematogenous route of infection on the placenta, there are several studies supporting the migration of opportunistic pathogens to placenta leading to adverse pregnancy outcome. Oral periodontitis bacteria including *Fusobacterium nucleatum, Campylobacter rectus, Peptostreptococcus micros, Prevotella nigrescens, Prevotella intermedia, Porphyromonus gingivalis, Bacteroides forsythus and Treponema denticola* have been studied in relation to pregnancy [530]. Among all, *Fusobacterium nucleatum* has been isolated from the amniotic fluid with intact membranes suggesting that the bacteria disseminates through blood, crossing the placenta to reach the amniotic sac [531]. In support of the adverse effect of the periodontal bacteria on preterm labour, intravenous injection of *F. nucleatum* into pregnant mice results in preterm birth [532]. Furthermore, *Porphyromonas gingivalis* has also been found in the placenta [533] within preterm placenta villous mesenchyme [534] suggesting that this bacteria contribute to preterm birth. Examination of specific periodontal bacteria in cord blood from preterm neonates presenting higher IgM levels of specific periodontal bacteria than the term neonates [535]. This implies that transplacental passage of infection occurs during pregnancy and may play a role in the pathogenesis of preterm labour.

In general, the increased IL-10 production seen in these priming experiments in the placenta further supports a potential protective mechanism by the placenta under conditions of multipathogen infection, by suppressing further enhancement of pro-inflammatory mediator production would lead to preterm labour.

7.5 Effect of systemic multipathogen inflammation on placental explants

Due to the systemic inflammation that can occur with the presence of systemic infections, and the controversy that exists over the existence of a placental microbiome, another model was used to examine the effects of multipathogen systemic infection on the placenta. In this model, supernatant from stimulated PBMCs described in results section 5.2, 5.3 and 5.4 was used as the culture medium for fresh placental explants. The total volume of the supernatant from the PBMC priming experiment was calculated based on the plasma volume circulating the placenta and

the mean production of cytokines with the priming experiment. This was to mimic the physiological mediators production at chorioamnionitis setting to be used to treat the fresh placenta.

Fresh placental explants cultured using media derived from TLR3-primed and TLR treated PBMC showed augmented IL-6 and IL-8 cytokine production. This suggests that if pregnant women are exposed to systemic multipathogen infection, the inflammatory mediators can potentially lead to an augmented inflammatory reaction in the placenta. This ultimately could lead to an increased risk of preterm labour.

Intrauterine infection during pregnancy that is associated with preterm labour is associated with higher levels of IL-6 and IL-8 in placenta, amniotic fluid, cervix and myometrium [61, 96, 536]. To elucidate whether IL-6 is important to cause preterm labour, neutralising IL-6 and the effect on LPS stimulation was performed in a murine study. LPS stimulation prior to IL-6 neutralisation prolonged the gestational period and significantly lower preterm delivery rates occurred compared to in control mice with un-neutralised IL-6 [537]. IL-6 null mutant mice also showed the same prolonged delivery time, reduced oxytocin receptor and prostaglandin synthesis than the wild type mice. The mutant mice were also resistant towards low dose of LPS stimulation. These effects, however, were reversed by the administration of recombinant IL-6 [538]. Increased IL-8 in the placenta is also a characteristic of labouring placenta by recruiting and activating neutrophils at the placental site [539]. Therefore, this could suggest that if placenta is being exposed to the circulating IL-8 mediators constitutively by the blood, this event could increase further risk of preterm labour.

In addition to the presence of bacteria in the periodontal diseases colonising the gum area in the oral cavity, these bacteria also have been shown to be able to produce a significant amount of pro-inflammatory mediators including IL-1β, IL-6, PGE2 and TNF-α [540]. These mediators can circulate systemically, enter the peripheral circulation, then reaching the placenta and contributing to preterm labour. With the possibility of these bacteria and the pro-inflammatory mediators produced to travel systemically, a prior encounter with viral systemic illness could enhance pro-inflammatory mediator production by the placenta.

The challenge for this study was to find the perfect model to address the haematogenous spread of multipathogen infection concept. The PBMCs and placental

explants used in this study were unmatched samples as placental explants must be left to equilibrate for 5 days prior to treatment. After 5 days, it is difficult to trace the patients to get the blood samples as the patients were all recruited in the labour ward before having their caesarean section. However, the volume used from then PBMC media to treat the fresh placenta was calculated to mimic the physiological concentration. In addition, placentas were taken from women at term pre labour (rather than pre term in the absence of infection). However, it was felt that this was more appropriate in order to gain more control on the basal immune status and to eliminate any potential pathological confounders for preterm delivery that would be present in preterm placentas.

7.6 Poly I:C effect on transcription factor p-IRF3

Poly I:C increases p-IRF3 transcription factor in VECs, amniocytes, myocytes and placental explants in this study. A meta-analysis study analysing the pathway associated with PPROM and spontaneous PTB (sPTB) revealed that in addition to NF-κB, IRF3 transcription factor is also unique in regulating genes involved in sPTB [541]. IRF3 has been shown to be phosphorylated at different serine sites namely Ser396, 398, 402, 404, and 405 and second phosphorylation at Ser385 or Ser386, which are required for IRF3 dimerization with itself or IRF7 [542]. However, phosphorylation at Thr75 and Thr253 residues hinder the dimerization process [543]. Using Western immunoblotting technique and an antibody detecting p-IRF3 at Ser396, amniocytes and VECs showed a band at 45 kDa, which is the anticipated molecular weight. In myocytes, a band was detected at 40 kDa which suggests that myocytes could be phosphorylated differently, yielding a different molecular weight. It is plausible that this transcription factor was responsible for downstream activation of cytokines and chemokines with TLR3 priming experiments, however, due to time constraints this experiment was not performed in this study.

In PBMCs, p-IRF3 protein was not detected with the stimulation of TLR3 agonist alone. The expression of p-IRF3 has been demonstrated in the literature with poly I:C stimulation in the PBMC using western immunoblotting [544, 545]. These studies showed a clear band of p-IRF3, however, the molecular weight and the detail of the antibody used was missing in the text and in the figure. While another study showed a clear band of p-IRF3 using different antibody, the phosphorylation site was not

reported [546]. These studies also failed to include a positive control that could be used as an indicator to differentiate between non-specific band and an actual band. Although NF-κB activation increased with the agonist stimulation, none of the transcription factors including AP-1 and p-IRF3 were responsible for the augmentation effect on cytokine production seen with TLR3/2 and TLR3/TLR2/6 priming in PBMC. Other transcription factors such as STAT, IRF5/7 or other subunits of NF-κB could have been responsible for the priming effects, however these were not studied due to the time constraints of the PhD.

The increased expression of p-IRF3 in the placental explants with poly I:C stimulation detected using western immunoblotting is a novel finding of this study. The responsiveness of p-IRF3 with poly I:C stimulation could suggest that this is involved in the regulation of the anti-inflammatory mediators seen in the placental explant experiments. IRF3 regulates the expression of the type I IFN mediators, namely IFN-α and IFN-β, a feature where only placental explants showed the detection level. In VECs, amniocytes and myocytes, although p-IRF3 protein increased with poly I:C stimulation, no detection level of IFN-α/β was observed. This shows the cell type dependent regulation of IFN by p-IRF3. Strong induction of IFN-β rather than IFN-α occurred in the placental explants, which strengthens the possibility that IRF3 is involved in the induction of these mediators, since IRF3 is a potent inducer of IFN-β rather than IFN-α [547, 548]. The single band observed using western immunoblotting in the placental explants however is at the same molecular weight as in myocytes (Chapter 4) but not in VECs and amniocytes. This could be due to the phosphorylation at other serine residues [542]. The induction of IFN-α/β has been reported to be vital during pregnancy. A model of IFN loss in a murine model through knockout heightens the sensitivity of mice to LPS stimulation resulting in placental inflammation and a 50% preterm birth rate [432].

7.7 Poly I:C effect on TLRs mRNA expression

Poly I:C stimulation showed an increase in TLR3 and TLR2 mRNA in all VECs, amniocytes and myocytes. A similar increase in TLR3 and TLR2 mRNA has been demonstrated in normal human bronchial epithelial cells [549], primary lung epithelial cells [550] and human airway smooth muscle cells [551] when stimulated with poly I:C. The finding from our study and other studies suggests that detection of dsRNA

leads to overall sensitization of the host to TLR2 activating ligands. One study proposes that this is due to an increase in the TLR2 adaptor proteins, MyD88 and TIRAP [549].

Bioluminescence resonance energy transfer assay has been widely used to analyse the interaction between the TLRs. Among TLRs, TLR2 has the ability to form different heterotypic formation with different molecules to activate its signalling with specific stimuli [208, 552]. This raises the question whether these formations pre-exist at the cell membrane. Previous studies confirmed that TLR2/1 and TLR2/6 heterodimers pre-exist at the cell membrane but are not stable and there is no downstream signalling if not bound to a ligand [207, 208]. However, upon recognition with a specific ligand, these heterodimers are recruited within the membrane microdomains (or lipid raft, a specialised area of the plasma membrane), their TIR domains come together and are internalised in a lipid-raft dependent pathway to be directed to the Golgi apparatus [209].

Using the same assay as above with labelled TLR2 [553, 554] and TLR6 [209], these TLRs were recruited into the lipid raft upon bacterial stimulation. However, there are still some unanswered questions about the preformed dimers especially before they bind to their respective ligands. The most anticipated characteristic is the half-life or the stability of the dimer over time and the percentage of TLR2/1 and TLR2/6 on the cell membrane. Despite the use of fixed cells (FRET) [209, 553] and live cells photobleaching technique (FRAP) [553, 554] to examine the intracellular distribution of TLR2 and TLR6, the questions remained unanswered. This is due to the restrictions of these techniques in examining the dynamics of the dimers. However, these findings reflect the abundance and higher percentage of TLR2 on the cell membrane. Therefore, on top of the readily available TLR2, poly I:C stimulation further increases TLR2 expression on the cell membrane to possibly increase the sensitivity of the host to detect gram-positive bacteria and thus activating the TLR2/6 dimer. This fits well with the pro-labour and pro-inflammatory cytokine augmentation seen with TLR2/6 agonist, FSL-1 in VECs, amniocytes and myocytes. This study also clearly demonstrates that poly I:C stimulation does not lead to an increase in TLR4 or TLR6 expression in VEC, amniocytes and myocytes. Recent study using FRET assay successfully examined intracellular interaction between poly I:C and TLR10 [555].

By utilising the same technique, it would be interesting to see intracellular poly I:C response on TLR3 and TLR2 receptors in VECs, amniocytes and myocytes.

In contrast to VECs, amniocytes and myocytes, poly I:C stimulation only showed increased expression of TLR3 mRNA in the PBMC and only TLR2 mRNA in the placental explants.

An increase of TLR3 and TLR2 mRNA was expected in the PBMC due to the augmented effect observed with the pro-labour mediators that utilise both the TLR3 and TLR2 pathways in the priming experiments. If an increase in TLR2 expression was required for the TLR3/TLR2 and TLR2/6 priming effect to occur, it is plausible that an early increase in TLR2 mRNA was missed in the time course experiment. It is also possible that PBMC expressed higher basal levels of TLR2 as compared to the VECs, amniocytes and myocytes. This could explain the reason PBMC does not need/respond to further augmentation of TLR2 to exert the augmented effect with priming. This fits with the lower Ct value in PBMCs compared to the Ct value in VECs, amniocytes and myocytes after 12 hours poly I:C stimulation.

In the placental explants, poly I:C stimulation increased TLR2 mRNA but no changes on TLR3 mRNA expression were seen. Despite there being no studies looking at the effect of poly I:C on TLR expression in human placental explants, the observation in this study is in concordance with the effect seen in human first trimester trophoblast infected with MHV68 [334]. Although there is an increase in the TLR2 mRNA expression, no augmentation on the pro-inflammatory cytokines with priming was observed in the placental explants. Since an increase in TLR3 expression is accompanied by a priming effect on cytokine production in all other cell, this suggests that an increase in TLR3 expression is crucial for mediating the response.

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7.8 Effect of 15dPGJ2

There are several lines of evidence to support the anti-inflammatory effects of 15dPGJ2. Since inflammation plays a significant role in preterm labour, I explored the potential anti-inflammatory effects of 15dPGJ2 in human gestational tissue. Although my group have previously published on the ability of 15dPGJ2 to inhibit NF-κB in amniocytes and myocytes, there are no detailed studies on the downstream effects of

this inhibition. Therefore, I explored the effect of 15dPGJ2 on IL-1β induced pro labour and pro inflammatory mediators in myocytes, amniocytes and vaginal epithelial cells in order to assess its potential as a novel therapeutic agent for the prevention of infection/inflammation induced PTL.

7.8.1 15dPGJ2 and NF-κB

15dPGJ2 has been shown to inhibit IL-1β-induced NF-κB in amniocytes and myocytes *in vitro* [64]. These findings were confirmed in myocytes (Figure 6.1 A) and amniocytes (Figure 6.8 B). The same inhibition was observed in VECs (Figure 6.8 B), in which to our knowledge is the first study looking at the effect of 15dPGJ2 in this cell type. Other studies have shown that 15dPGJ2 has the same anti-inflammatory effect on other human cell types such as macrophages [556], synovial fibroblasts [557] and in rat cells such as mesangial cells [558, 559] and synovial fibroblasts [560]. 15dPGJ2 is one of the peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands and has been shown to inhibit iNOS expression via a PPARγ-dependent mechanism [556]. However, a previous study by our group has shown that 15dPGJ2 inhibits IL-1β-stimulated NF-κB in amniocytes and myocytes in a PPARγ-independent mechanism [64]. The inhibitory effect is due to the cyclopentenone ring in the chemical structure of 15dPGJ2 [64, 368]. Molecular studies have shown that the ring contains a highly reactive electrophilic carbon that can form covalent bonds by the means of Michael adducts. This has been shown to be responsible for its receptor-independent inhibitory effects at multiple steps in the NF-κB pathway. It can block the IκBα degradation, represses IKK phosphorylation and 26S proteasome, and inhibits translocation of the p50/p65 subunit dimer into the nucleus by directly targeting the dimer by modifying the p50 subunit [352, 353]. Therefore, as expected, the same inhibition was observed in IL-1β-induced NF-κB activation in VEC.

7.8.2 15dPGJ2 and AP-1 activation via MAPK

Besides NF-κB, AP-1 has been shown to play a role as a terminal mediator in LPS induced preterm labour in the mouse [113]. This study by our group reported that AP-1 activation occurs at the onset of labour. Using a preterm labour model of infection induced PTL, various LPS serotypes were shown to selectively activate AP-1 or NF-κB. Myometrial AP-1 activation can also drive the production of pro-inflammatory and pro-contractile genes [113]. A similar finding of increase in AP-1 has also been

observed in human myometrial tissue. Term in labour samples express higher c-Jun, c-Fos and JunB compared to term not in labour tissue [108, 561, 562]. The importance of this protein is further supported by data from our group. Mutation of the IL-8 binding site within AP-1 shows significantly lower IL-8 production compared to the non mutated control, implying the role of AP-1 in IL-8 production [112]. Data in this chapter shows that 15dPGJ2 has dual effects on AP-1, which is cell type specific. 15dPGJ2 significantly inhibited IL-1β-stimulated AP-1 in myocytes, however, it led to increased AP-1 activation in VECs and AECs.

15dPGJ2 has previously been reported to have variable effects on AP-1 activation depending on the cell type of interest. It has been shown to inhibit IL-1β-treated AP-1 in rat mesangial cells independent of PPARγ [558] and human chondrocytes in both a PPARγ –independent [563] and –dependent [564] mechanism. LPS-stimulated AP-1 was also inhibited by 15dPGJ2 in rat synovial fibroblasts, independent of PPARγ [560]. In contrast to the inhibition effect seen with other studies, an increase of AP-1 activation was observed with the presence of 15dPGJ2 in human astrocytes and microglial cells [565], which is consistent with our findings seen in VEC and amniocytes. This increase could be explained by the direct post-translational modification effect of 15dPGJ2 on the proteins. The ability of 15dPGJ2 to induce c-Jun cross-linking has been reported in a study which showed c-Jun dimerization [354]. This same mechanism could occur in VEC and amniocytes.

AP-1 is activated by the three well characterised subfamilies of mitogen-activated protein kinases (MAPKs): extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 MAPK [109, 110]. Since AP-1 activation increases in VECs and amniocytes with 15dPGJ2, these MAPK proteins were examined to further elucidate the mechanism of activation. 15dPGJ2 increased the basal level of p-p38 in both VEC and amniocytes while p-JNK only increased in VEC. In contrast, p-ERK was inhibited by 15dPGJ2 in both cell types. It is therefore possible that 15dPGJ2 induced AP-1 activation is via p-p38 in VECs and AECs. Studies have shown that 15dPGJ2 is capable of inducing the activation of MAPK proteins. This has been shown in both human cells using pancreatic cancer cell line and in animal cells using cultured rat astrocytes [566, 567] while some studies showed that 15dPGJ2 has an inhibitory effect on p-ERK proteins [568]. p-ERK pathway is a regulator for cellular growth and proliferation [569] and the phosphorylation of ERK caused by oxidative stress was

inhibited when pre-treated with 15dPGJ2 [570, 571]. The same inhibition on p-ERK was observed in our IL-1β-stimulated VEC and amniocytes.

In terms of parturition, p-p38 and AP-1 have been implicated in the regulation of MMP-9 to promote cervical ripening before labour [572]. MMP-9 also has been shown to cause extracellular matrix degradation in the fetal membrane [573-578] and myometrium [579] which occur during the process of parturition. Therefore, taken together, the impact of increasing p-p38 and AP-1, and thus increasing the mediators involved in labour, this compound may not be suitable as an agent for preventing human preterm labour. The 100-fold and 40-fold difference between p-p38 and the basal level, respectively in VECs and amniocytes after 2 hours incubation of 15dPGJ2 could suggest that the use of this compound could increase the risk of cervical ripening and fetal membrane rupture, which are a pre-requisite for preterm labour.

7.8.3 15dPGJ2 effect on COX-2 and PGE2 production

Because COX-2 is under transcriptional control of NF-κB and AP-1, the effects of 15dPGJ2 on both COX-2 and prostaglandin E_2 (PGE2) were examined. In myocytes, IL-1β-induced COX-2 and PGE2 production were inhibited with 15dPGJ2 pre-treatment. The inhibited effect was observed from the upstream precursor level of cPLA2-α. These data are in support of the previously published inhibitory effect of 15dPGJ2 on COX-2 in myocytes from our group [64]. The potential impact of these findings could relate to 15dPGJ2 being used as a tocolytic.

The assessment on the PGE2 production with 15dPGJ2 in myocytes, VEC and amniocytes is a novel finding in this study. IL-1β-stimulated PGE2 was significantly inhibited with pre-incubation of 15dPGJ2 in the myocytes. PGE2 is the downstream protein of COX-2 pathway and is relevant in term and preterm labour as it is involved in softening of the cervix, fetal membrane rupture and stimulation of myometrial contractions [580]. The response seen in PGE2 shows that 15dPGJ2 is an attractive potential therapeutic preventative strategy for uterine contractility.

Despite the inhibition of PGE2 in myocytes, 15dPGJ2 did not inhibit myometrial contractility unlike the Indomethacin positive control (see Appendix C Figure S1 and S2 (Sykes, unpublished work)). The lack of inhibitory effect on contractility could be explained by the fact that PGE2 inhibition by 15dPGJ2 is not seen until 24 hours.

Since strips cease to contract in the *ex-vivo* model by 12 hours, it was not feasible to determine if inhibition of contractions could be achieved with 15dPGJ2. In a clinical setting however, if the inhibitory effect of 15dPGJ2 on PGE2 is not demonstrated until 24 hours, it would not be likely to be an effective prevention in the acute setting.

In VECs and amniocytes, although IL-1β-induced COX-2 was inhibited by 15dPGJ2, incubation of the 15dPGJ2 alone led to an increase in PGE2 production. The effects of COX-2 on amniocytes has been shown previously by our group but the PGE2 production was not investigated [64]. However, no previous studies have examined the effects of 15dPGJ2 on COX-2 expression and PGE2 production in VECs. The differential effect seen in PGE2 in these cells compared to myocytes could be explained by the possibility of 15dPGJ2 up-regulating COX-2 production at a longer time point than what was examined. In this study, COX-2 was evaluated after 4 hours and the PGE2 effects were determined at 18 and 24 hours after pre-incubation with 15dPGJ2. Therefore, it could be possible that 15dPGJ2 may have induced COX-2 protein expression after 4 hours and this could have been observed if a time course study was performed at 12, 18 and 24 hours.

In a different study using human osteoarthritic chondrocytes (HOC), an 18 hours incubation of 15dPGJ2 up-regulated the basal level of COX-2 and PGE2 production. In a separate experiment, although 18 hours of IL-1β stimulation after 15 minutes of 15dPGJ2 pre-incubation almost completely reduced the PGE2 to its basal level, COX-2 protein was not inhibited to its basal level [581]. It is possible that 15dPGJ2 has a direct effect on COX-2. For example, in one study, COX-2 protein expression was shown to increase with 15dPGJ2. An investigation on the COX-2 promoter translational start region reveals that it contains a consensus site for peroxisome proliferator response element (PPRE). This site has been shown to be responsible for the increase of COX-2 presence in the presence of 15dPGJ2 [582]. Therefore, it is possible that 15dPGJ2 binds to the PRE element to induce the expression of COX-2 at a later time point than 4 hours. This then could explain the high fold change in production of PGE2 in VEC and amniocytes post 18 and 24 hours of incubation with15dPGJ2.

It is also possible that AP-1 could play a role in the increase in PGE2 production with 15dPGJ2 pre-incubation. This is aligned with the increase of AP-1 protein in VEC and amniocytes with 15dPGJ2 pre-incubation.

7.8.4 15dPGJ2 effects on pro-inflammatory cytokines

The two transcription factors NF-κB and AP-1 regulate the production of pro-inflammatory cytokines. These transcription factors can bind to the promoter regions of IL-8, IL-6, TNF-α and CCL2 [66, 112, 253, 583-585]. It was therefore expected that these pro-inflammatory cytokines would be inhibited in myocytes, VEC and amniocytes prior to 15dPGJ2 pre-treatment.

In myocytes, IL-1β treatment increased the pro-inflammatory mediators IL-6, IL-8, TNF-α and CCL2 these were downregulated by 15dPGJ2. IL-6 is an important mediator for increasing OTR expression and gap junction Con 43 expression to stimulate uterine activation [586]. IL-8 and CCL2 on the other hand are a potent chemokines and facilitate immune cell activation to further increase inflammation [587]. In addition, TNF-α functions as a stimulator for arachidonic acid release, which is the precursor for PGE2 production [588]. The inhibition of these pro-inflammatory cytokines and chemokines would be potentially beneficial to help prevent pathophysiological changes that are important for preterm labour.

In VEC, although IL-6 at the mRNA level only showed a trend of inhibition with 15dPGJ2, a statistically significant inhibition was achieved at the protein level. The same inhibition was also observed in IL-8 and TNF-α. These proteins are important as the mediators to aid in the cervical ripening and cervical remodelling for labour progression [61, 474, 475].

The peculiar observation was seen in the amniocytes of the increase in IL-8 mRNA expression in IL-1β /15dPGJ2 treated cells. This result was so surprising that the experiment was repeated with a further 3 biological replicates in case of human error in the initial experiments, however the same results were obtained. A previous study with human THP-1 macrophages showed that 15dPGJ2 increased the expression of IL-8. The increase of IL-8 mRNA was persistent even when the NF-κB was inhibited using an inhibitor. This study thus suggested that the increase in IL-8 mRNA expression/production was via the MAPKs when NF-κB was inhibited [589]. This

could be the same mechanism seen in the VEC and amniocytes with the increase in MAPKs proteins p-p38 and p-JNK. At the protein level however, IL-8 was significantly inhibited by 15dPGJ2. The inhibition of 15dPGJ2 seen in the IL-8 protein is almost to the basal level. This result was surprising, however it was clear that the rise in mRNA was not translated into a rise in the protein. This implies that there is a form of post-transcriptional regulation that may explain the discrepancy in the 15dPGJ2 mediated effect on IL-8 mRNA and protein expression.

15dPGJ2 had initially shown promise as a potentially favourable compound to prevent preterm labour compared to other anti-inflammatory compounds such as sulfasalazine (SASP). SASP is safe to take during pregnancy [590, 591] and our group has also has shown that it inhibits IL-1β-induced NF-κB in amniocytes and myocytes [372]. However, in the same study by our group, SASP increased pro-inflammatory cytokines production and was toxic to both type of cells at the concentrations required to inhibit NF-κB [372]. Although this study demonstrated that the effects of 15dPGJ2 remained promising in myocytes some limitations were uncovered in its effects on VECs and AECs with increased MAPKs and AP-1 activation.

7.8.5 15dPGJ2-loaded solid lipid nanoparticle (SLN)

The 15dPGJ2-loaded solid lipid nanoparticle (SLN) has been shown to inhibit neutrophil chemotaxis and pro-inflammatory cytokines production using methylated bovine serum albumin (mBSA), LPS and carrageenan- induced inflammation in a murine model [360, 592].

The 15dPGJ2- loaded SLN compound used in this study was obtained from our collaborators in Sao Paulo, Brazil [360]. It was anticipated that this compound would have the potential to inhibit IL-1β-stimulated NF-κB in myocytes in a lower concentration than 32μM, the concentration that was used with pure 15dPGJ2 in previous studies from our group.

With the advantage of nanoparticle to use at a reduced concentration, 3.2μM of 15dPGJ2-loaded SLN tagged with RHB fluorophore (red signal) was incubated on the myocytes for 1 hour. The red signal intensity was higher around the cells after 30 minutes compared to the control at 0 hour. This shows that the nanoparticle accumulated around the cells. The question then remained whether the 15dPGJ2 was

being released from the SLN core and taken up by myocytes. After 2.5 hours of pre-incubation with 15dPGJ2-loaded SLN, quantification of 15dPGJ2 in the media showed a possible small reduction between the 15dPGJ2-SLN in the media alone and 15dPGJ2-SLN cultured with myocytes, however it must be emphasised that this did not reach statistical significance, and is likely not to be of scientific relevance, especially since 15dPGJ2 is also produced endogenously by cells through enzymatic degradation of PGD2 [593]. Although addition of 3.2μM 15dPGJ2 to the cells might disrupt the concentration gradient of 15dPGJ2 either side of the cell membrane, it can safely be assumed any small uptake of 15dPGJ2 by the cells was not enough to inhibit NF-κB and AP-1. Consistent with this finding, there was no inhibition of IL-1β-stimulated NF-κB and AP-1 with the 15dPGJ2-SLN.

This observation could mean that the active electrophilic ring molecular structure of 15dPGJ2 released from the SLN core might have been changed either during the incorporation into the nanoparticle, or via the release into media. A study conducted using a disrupted cyclopentenone ring, 9,10-dihydro-15d-PGJ2 shows that the analogue is not able to reproduce the same inhibitory effect on NF-κB as 15dPGJ2, although still retains PPARγ agonist activity [64]. In a murine study performed by our collaborator, 30 minutes of subcutaneous injection of 15dPGJ2-loaded SLN prior to LPS stimulation successfully inhibited IL-1β [360]. This confirms that the 15dPGJ2-loaded SLN compound is an active compound and can function *in vivo* as an anti-inflammatory agent.

The other possibility for not seeing an effect with the 15dPGJ2-SLN could be due to the agglomeration effect seen with the nanoparticle. This agglomeration is reported to happen based on studies looking at the effect on pH and the culture media conditions. Due to the high ionic strength and the neutral pH needed for the biological experiments, the nanoparticle tends to agglomerate in PBS and cell culture media [594-597]. This agglomeration can alter their physicochemical properties and colloidal behaviour, which may affect their cellular association.

Based on the release profile experiment, a small amount of 15dPGJ2 was released even at 30 minutes although 50% of the released was achieved at 100 minutes. For future experiments, the concentration of 15dPGJ2 in the 15dPGJ2-loaded SLN at 0 hour should be quantified. This would help to confirm if the 15dPGJ2 concentration

quantified at 30 minutes in my study reflected an increase due to the release of 15dPGJ2 from the SLN or reflected the background reading of 15dPGJ2 from the 15dPGJ2- SLN solution.

7.9 Final conclusion

This study shows that cells of the reproductive tract exposed to ascending infection are susceptible to a 'double-hit' hypothesis and pregnant women who are exposed to systemic viral illness are likely to be more susceptible to bacteria activating the TLR2 pathway. The multipathogen combination with the greatest effect on augmented pro-inflammatory and pro-labour mediators is seen with the combination of TLR3 and TLR2/6 agonists. The circulating pathogens and the inflammatory mediators could lead to a heightened inflammatory response in the placenta thus, leading to a higher risk of preterm labour and adverse effects to the neonate following exposure to local inflammation. This double-hit effect could lead to a pathological trigger for premature membrane rupture, cervical ripening and uterine contractility and ultimately preterm labour.

The conflicting effects of 15dPGJ2 in human cells would make it difficult to predict its overall effect on humans if used in clinical trials. Although it significantly inhibited pro-labour and pro-inflammatory mediators in myocytes, it increased PGE2 in VECs and amniocytes. Additionally, it activated p-p38 and AP-1 in VECs and amniocytes. These results were surprising, given the beneficial effects on delaying preterm labour and increasing pup survival previously demonstrated in LPS treated mice. Nevertheless, this study demonstrates the importance of human *in vitro* studies.

Appendix A

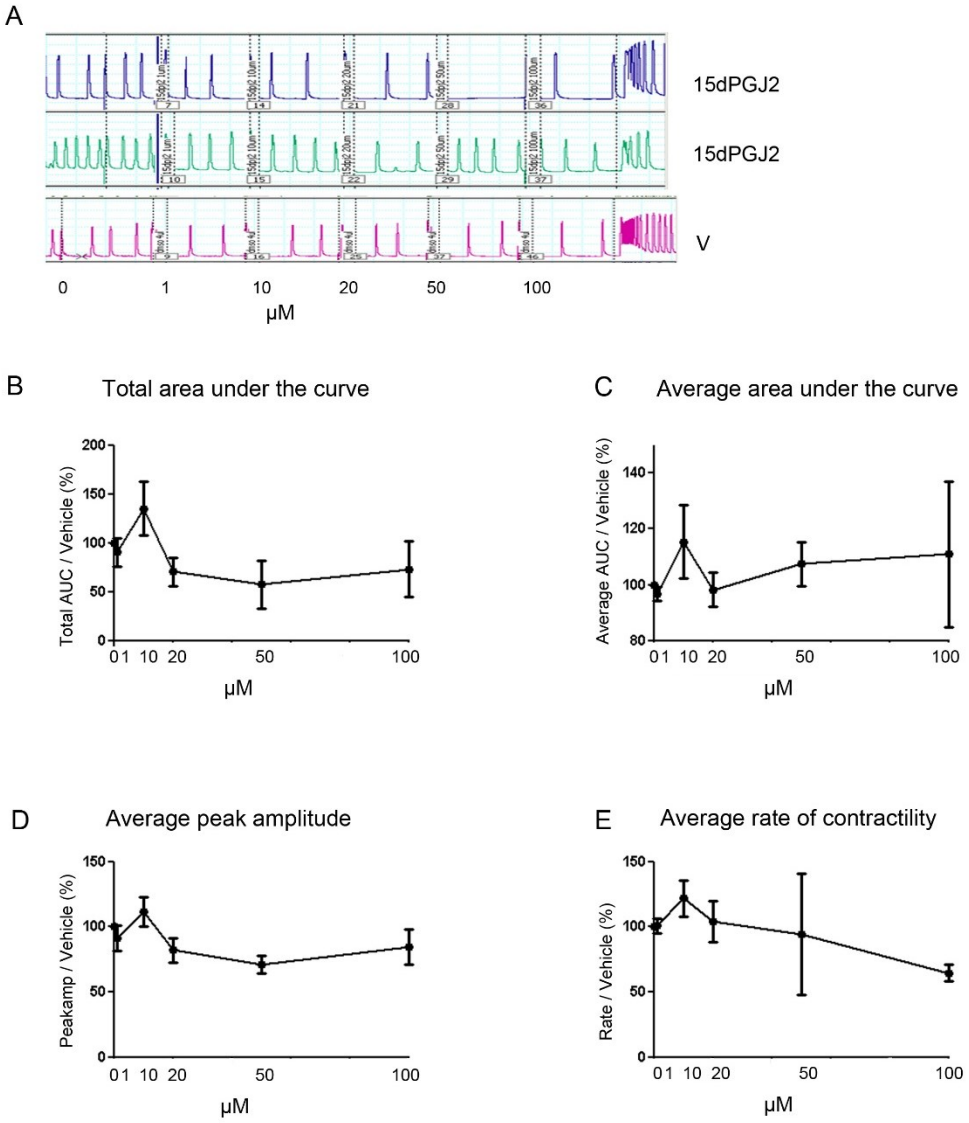


Figure S1 The effect of 15dPGJ$_2$ on myometrial contractility. Non-labouring myometrial biopsies were dissected and mounted on the myograph (n=3 subjects with 2-3 strips each). A representative trace is shown for 15dPGJ$_2$ treated strips and vehicle control (A). A cumulative dose response with 15dPGJ$_2$ 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.

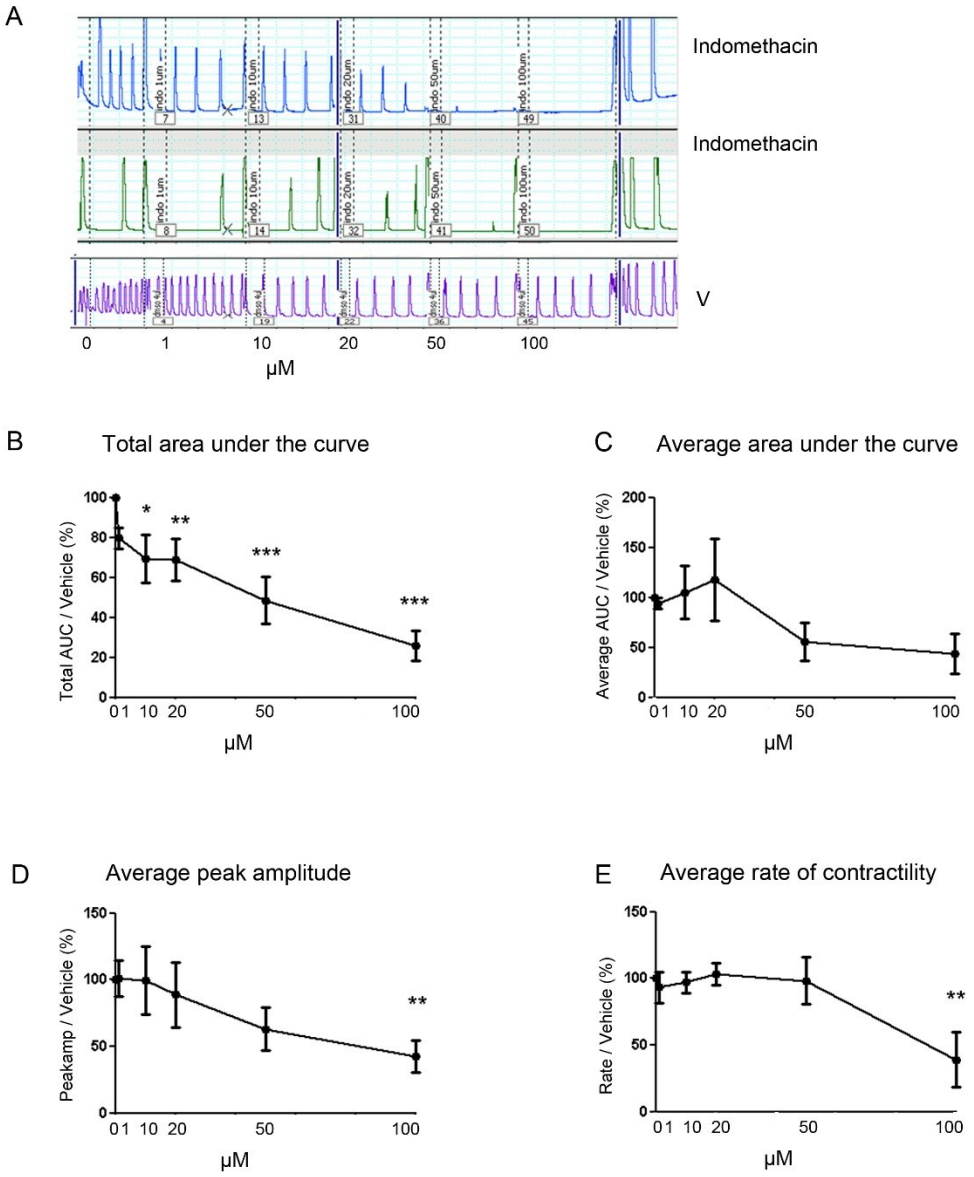


Figure S2 The effect of Indomethacin on myometrial contractility. Non-labouring myometrial biopsies were dissected and mounted on the myograph (n=3 subjects with 2-3 strips each). A representative trace is shown for indomethacin treated strips and vehicle control (A). A cumulative dose response with 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).

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