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The effect of statin treatment on preterm labour

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Abstract

Preterm labour (PTL) is defined as labour before 37 completed weeks of gestation. Despite advances in medical research, PTL remains a major clinical problem. Preterm birth (PTB) rates range from approximately 5-18% worldwide. Importantly, PTB is the leading cause of childhood morbidity and mortality. PTL is difficult to predict and the aetiology is poorly understood but infection and inflammation are believed to be major factors. It has been suggested that the presence of intrauterine infection or inflammation may initiate the pathological, preterm activation of the inflammatory cascade associated with term labour. Therefore, PTL therapeutics should aim to inhibit these inflammatory pathways. Statins, 5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are potent inhibitors of cholesterol biosynthesis, which act on the mevalonate pathway. In addition to their lipid-lowering effects, statins also have anti-inflammatory and anti-contraction properties.

The hypothesis of this thesis was that statins will prevent PTB by reducing inflammation. The aims of this thesis were firstly to investigate the effect of the statins, simvastatin and pravastatin, on inflammation and contractility in a pregnant human myometrial cell line. Secondly, to determine whether simvastatin and/or pravastatin can prevent PTB or improve neonatal outcome in a lipopolysaccharide (LPS)-induced mouse model of PTB.

Myometrial cells were either co-treated with LPS and simvastatin/pravastatin, pre-treated with simvastatin/pravastatin or treated with simvastatin/pravastatin post-LPS stimulation. The effect of statin treatment on the mRNA expression and the release of inflammatory mediators was then investigated. Simvastatin treatment reduced LPS-induced inflammation by both lowering the expression of pro-inflammatory mediators and increasing the expression of anti-inflammatory mediators. Pravastatin treatment did not alter the expression of inflammatory mediators following LPS stimulation.

The effect of simvastatin on the contraction of myometrial cells was investigated by embedding the cells in rat tail collagen to form gels. As these are smooth muscle cells, basal contraction was observed causing the gel size to reduce. When LPS was introduced, this caused the gels to contract further than the vehicle treated gels.

Simvastatin attenuated the contraction of the myometrial cells, both alone and in the presence of LPS. These effects were reversed by the addition of mevalonate pathway metabolites, mevalonate and geranylgeranyl pyrophosphate (GG-PP) but not by farnesyl pyrophosphate (F-PP). Simvastatin also lowered levels of phosphorylated myosin light chain (pMLC) in the myometrial cells, which is essential for smooth muscle contraction. Again, this effect was abolished by mevalonate and GG-PP but not F-PP. It is hypothesised that simvastatin attenuated myometrial cell contraction by inhibiting Rho isoprenylation by GG-PP, preventing Rho-associated kinase (ROCK) activation, which then prevented the phosphorylation of MLC.

A mouse model of intrauterine LPS-induced PTB was utilised to investigate the effect of statin treatment on PTB and fetal survival. Mice received an intraperitoneal injection of pravastatin (10µg) or simvastatin (20µg or 40µg) on gestational day (D)16. This was followed by ultrasound-guided intrauterine injection of LPS (1µg) on D17 and another pravastatin/simvastatin treatment two hours later. When mice were treated with LPS, 77.8% of mice delivered preterm. When mice received LPS and 20µg simvastatin, 50% delivered preterm. However, when mice were treated with LPS and 40µg simvastatin, 40% delivered preterm, more pups were born alive and uterine pro-inflammatory mRNA expression was downregulated. Conversely, pravastatin did not prevent PTB or improve the percentage of live born pups.

In summary, simvastatin treatment exerted anti-inflammatory and anti-contraction effects on human myometrial cells *in vitro*. The anti-contractile properties were likely due to the inhibition of the Rho/ROCK pathway. Furthermore, in our LPS-induced mouse model of PTB, fewer mice delivered preterm with simvastatin treatment, simvastatin attenuated LPS-induced pup mortality and reduced uterine inflammatory gene expression. These results suggest that statin therapy may be a novel treatment for PTL.

Lay summary

Preterm labour is when labour begins before 37 weeks of pregnancy. Premature babies are not fully developed, which can lead to a number of health complications. Common causes of preterm labour are infection and inflammation. Statins are drugs that are used for the prevention of heart disease, as they lower cholesterol. However, they are also believed to have other beneficial effects, such as reducing inflammation.

The hypothesis of this thesis was that statins will prevent early birth by reducing inflammation. This thesis investigated the effect of two statins, simvastatin and pravastatin, on inflammation and contraction in human myometrial cells. Furthermore, the effect of these statins was investigated in a mouse model of preterm birth.

The myometrium is the smooth muscle tissue found in the uterus. This tissue contracts during labour. Myometrial cells, donated from a woman at the end of pregnancy, were given lipopolysaccharide (LPS), which is a bacterial component that causes inflammation. These cells were treated with simvastatin and pravastatin. Simvastatin reduced the expression of genes that are linked to promoting inflammation. Simvastatin also increased the expression of genes that are associated with reducing inflammation. Conversely, pravastatin treatment did not increase or decrease genes associated with inflammation.

To study the contraction of the myometrial cells, the cells were mixed with collagen to form a solid gel. As the cells contracted, this caused the gel to shrink in size. When the cells were treated with simvastatin, the gel did not reduce in size suggesting that simvastatin stopped the contraction.

The effects of simvastatin and pravastatin were also investigated in a mouse model where preterm birth was induced by causing inflammation in the uterus with LPS. Simvastatin prevented preterm delivery in some mice, as well as increasing the number of pups that were born alive. Simvastatin treatment also reduced inflammation in the uterus of the mouse. Pravastatin treatment did not prevent the mice delivering early, nor did it increase the number of pups born alive.

In summary, simvastatin treatment may be a novel therapy for preterm labour as it reduced inflammation and prevented the contraction of human myometrial cells. In addition, simvastatin treatment prevented preterm birth in some mice, increased pup survival and reduced inflammation in the uterus.

Declaration

The composition of this thesis and the studies undertaken were the unaided work of the author, except where due acknowledgement is made by reference. No part of the work described in this thesis has been previously accepted for or is currently being submitted for another degree or qualification.

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Publications, Presentations and Posters

PUBLICATIONS

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ORAL PRESENTATIONS

Statin treatment in a novel infection-induced mouse model of preterm birth. ESRI, Oxford, September 2015.

Measuring simvastatin, simvastatin hydroxy acid and progesterone in maternal serum from a mouse model of preterm birth. SRI Scientific Symposium, Edinburgh, August 2017.

POSTERS

Simvastatin treatment significantly reduces inflammatory mediator secretion and contractility of a pregnant human myometrial smooth muscle cell line. SRI, Montreal, March 2016.

Simvastatin treatment reduces the incidence of preterm birth in an infection-induced mouse model. SRI, Orlando, March 2017.

Simvastatin attenuates myometrial smooth muscle contraction by inhibiting the RhoA/ROCK pathway. SRI, Orlando, March 2017.

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Abbreviations

11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AP-1	Activator protein 1
ATP	Adenosine triphosphate
B-cells	B-lymphocytes
BCP	1-bromo-3-chloropropane
β-ME	Beta-mercaptoethanol
BMI	Body mass index
BPD	Bronchopulmonary dysplasia
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
C/EBPε	CCAAT/enhancer binding protein ε
Ca^{2+}	Calcium
$[Ca^{2+}]i$	Intracellular Ca^{2+} concentration
CaM	Calmodulin
CCL2/MCP-1	Chemokine ligand 2/Monocyte chemoattractant protein 1
CCR2	C-C chemokine receptor 2
Cdc42	Cell division control protein 42 homolog
cDNA	Complementary DNA
CH_3OH	Methanol
CLP	Cecal ligation and puncture
CO_2	Carbon dioxide
COX	Cyclooxygenase

CPI-17	Protein phosphatase 1 regulatory subunit 14A
CRP	C-reactive protein
CSAIDs	Cytokine suppressive anti-inflammatory drugs
CT	Threshold cycle
CX43	Connexin 43
CYP	Cytochrome P450
DAG	Diacylglycerol
dH_2O	Deionized water
DMEM	Dulbecco's modified eagle medium
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
dNTPs	Deoxyribonucleotide triphosphates
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERα	Oestrogen receptor alpha
EtOH	Ethanol
FBS	Fetal bovine serum
FDA	US Food and Drug Administration
FIRS	Fetal inflammatory response syndrome
F-PP	Farnesyl pyrophosphate
FTI	Farnesyl transferase inhibitor
G418	Geneticin
GAPs	GTPase-activating proteins
G-CSF	Granulocyte-colony stimulating factor

GDIs	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide exchange factors
GG-PP	Geranylgeranyl pyrophosphate
GGTI	Geranylgeranyl transferase inhibitor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G-protein-coupled receptors
G-PP	Geranyl pyrophosphate
GTP	Guanosine trisphosphate
H_2O	Water
H_2SO_4	Sulfuric acid
HCl	Hydrogen chloride
HDL	High-density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-Coenzyme A
HO-1	Heme oxygenase 1
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule 1
IDL	Intermediate-density lipoprotein
iE-DAP	γ-D-glutamyl-meso-diaminopimelic acid
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IKK	IκB kinase
IL	Interleukin
IL-1Ra	Interleukin 1 receptor antagonist
IL-6R	Interleukin 6 receptor
IP_3	Inositol trisphosphate

I-PP	Isopentenyl pyrophosphate
IUGR	Intrauterine growth restriction
IκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
JAK	Janus kinase
K^+	Potassium
kDa	Kilodalton
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low-density lipoprotein
LFA-1	β2-integrin lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC II	Major histocompatibility complex class II
MIP	Macrophage inflammatory protein
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MTBE	Methyl tertiary butyl ether
MTT	3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide
NAC	N-acetyl cysteine
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NBDI	NEMO-binding domain inhibitor

NBF	Neutral buffered formalin
NEMO	NF-κB essential modulator
NF-κB	Factor nuclear factor kappa B
NK cells	Natural killer cells
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
NTCO	Named training and competency officer
NVS	Named veterinary surgeon
OATP1B1	Organic anion transporter protein-1B1
P/S	Penicillin/streptomycin
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline TWEEN® 20
PECAM	Platelet endothelial cell adhesion molecule
PG	Prostaglandin
PGF	Placental growth factor
PHM1-41 cells	Pregnant human myometrial 1-41 cells
PI3K	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PLC	Phospholipase C
pMLC	Phosphorylated myosin light chain
pPROM	Preterm premature rupture of membranes
PR	Progesterone receptors
PTB	Preterm birth
PTL	Preterm labour
qRT-PCR	Quantitative real-time polymerase chain reaction
Rho	Ras homology

RNA	Ribonucleic acid
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
RT	Reverse transcription
SEM	Standard error of the mean
sENG	Soluble endoglin
sFlt-1	Soluble fms-like tyrosine kinase
SP-A	Surfactant protein-A
SR	Sarcoplasmic reticulum
SRC	Steroid receptor coactivators
SSZ	Sulfasalazine
StAmP	Statins to Ameliorate early onset Preeclampsia
STAT	Signal transducer and activator of transcription
TBST	Tris buffered saline TWEEN® 20
T-cells	T-lymphocytes
TDI	Tissue Doppler Imaging
Th cells	T helper cells
TIMPs	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
Tregs	Regulatory T-cells
Tyk	Tyrosine kinase
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein

Chapter 1: Literature review

1.1 The role of inflammation in labour

Inflammation is a highly coordinated process, which is characterised by the infiltration of leukocytes and plasma proteins into affected tissues. The major cells of the immune system, such as monocytes, macrophages, neutrophils, basophils, dendritic cells, mast cells, T-lymphocytes (T-cells) and B-lymphocytes (B-cells), are involved in the complex regulation of inflammation (Turner et al. 2014). In the instance of microbial infection, inflammation is triggered by receptors, such as Toll-like receptors (TLR). This results in the production of a range of inflammatory mediators, for example chemokines and cytokines (Medzhitov 2008).

Human labour is an inflammatory process, associated with the influx of immune cells into the utero-placental compartment and the increase of mediator production locally (Bollapragada et al. 2009). Labour is initiated by the shift from a quiescent to a pro-inflammatory environment. This instigates a three-step process, characterised by myometrial contractility, cervical ripening and fetal membrane rupture (Romero et al. 1994; Rinaldi et al. 2011). Cytokines are essential for the initiation and regulation of this process (Bowen et al. 2002).

1.1.1 Inflammation of maternal tissues

1.1.1.1 Myometrium

The association between myometrial inflammation and labour was recognised in the 1980s and there is a significant body of work reporting that spontaneous term labour is associated with the large influx of inflammatory cells into the myometrium (Junqueira et al. 1980; Azziz et al. 1988). For example, macrophages and neutrophils are abundant in the human myometrium during labour, compared to women not in labour. These cells are present predominantly in the glandular epithelium of the lower uterine segment (Thomson et al. 1999; Kemp et al. 2002a). Infiltrating leukocytes are the source of interleukins (IL), such as IL-6 and IL-8, as well as tumour necrosis factor (TNF) and the leukocyte enzymes, matrix metalloproteinases (MMP)-8 and MMP-9, in the human labouring myometrium (Osmers et al. 1995; Elliott et al. 2000; Young et al. 2002).

A transcriptional profile of human myometrial samples, comparing women experiencing spontaneous labour at term and women not in labour, was determined by gene microarray analysis. A wide range of genes linked to inflammatory signalling were upregulated with labour, many of which were chemokines. For example, chemokines with the C-X-C motif, such as *CXCL3*, *CXCL5*, *CXCL8/IL-8*, and *CCL2* and *CCL20* with the C-C motif, were upregulated. *CXCL8/IL-8*, which plays an important role in neutrophil chemotaxis, was the most highly expressed gene (Bollapragada et al. 2009).

During labour, the endothelial cell adhesion molecules, E-selectin and vascular cell adhesion molecule 1 (VCAM-1), are upregulated in the blood vessels of the human lower uterine segment. These molecules mediate leukocyte adhesion to cytokine-activated vessel endothelium during inflammation. This suggests that the increase of these molecules may be an important step in stimulating the infiltration of leukocytes into the lower uterine segment during labour (Winkler et al. 1998). Intercellular adhesion molecule 1 (ICAM-1) mRNA expression is upregulated in the myometrium during labour. This expression has been localised to the vascular endothelium and in leukocytes. Platelet endothelial cell adhesion molecule (PECAM) mRNA expression is also significantly upregulated in myometrium during pregnancy, compared to non-pregnant samples, but this expression does not change at labour onset. PECAM has been localised to the vascular endothelium and has been identified on leukocytes. Therefore, these molecules may facilitate the infiltration of leukocytes into the myometrium prior to the onset of labour (Ledingham et al. 2001).

During pregnancy in the mouse, leukocytes also infiltrate the myometrium prior to the onset of labour. This is accompanied by the mRNA upregulation of inflammatory mediators, such as *Tnf*, *Il-1β*, *Il-6*, *Cxcl1* and *Cxcl2*, during labour (Shynlova et al. 2013).

1.1.1.2 Peripheral circulation

Monocytes and neutrophils are primed in the peripheral blood in both term and preterm labour (PTL) (Yuan et al. 2009). Specifically, there is an increase in nonclassical/intermediate monocytes in the peripheral blood of pregnant women

compared to non-pregnant women (Melgert et al. 2012). These cells are thought to replenish tissue-resident macrophages and produce pro-inflammatory mediators, such as IL-1β, IL-8, chemokine ligand (CCL)-2 and CCL5 (Gordon and Taylor 2005; Ancuta et al. 2009; Hamilton et al. 2013). Total leukocyte counts were found to be significantly higher in women who had a spontaneous preterm birth (PTB), compared to those who did not (Heng et al. 2014).

In a study performed on pregnant rats, the chemotactic responsiveness of peripheral leukocytes increased later in gestation, prior to the onset of labour. These leukocytes had increased *Ccl2* mRNA expression (also referred to as monocyte chemotactic protein 1; *Mcp-1*). The chemotactic activity of the uterus increased at term, with an upregulation of *Ccl2* mRNA expression. This suggests that peripheral leukocyte responsiveness increases prior to term to facilitate their infiltration into the uterine tissues in order to stimulate the labour process (Gomez-Lopez et al. 2013b).

Interestingly, when neutrophils are depleted before term in the mouse, the timing and success of labour are unaffected (Timmons and Mahendroo 2006). Specifically, female mice with a null mutation in transcription factor CCAAT/enhancer binding protein ε (C/EBPε), which is expressed in lymphoid cells and granulocytes such as neutrophils, eosinophils and basophils, cannot generate functional neutrophils but they can reproduce normally (Yamanaka et al. 1997). Furthermore, gestational length is not affected by blocking chemokine receptor 2 (CCR2) in mice, suggesting CCR-dependent leukocyte recruitment is not essential for successful labour (Menzies et al. 2012). Therefore, neutrophil infiltration does not appear to be essential for the induction of labour in mice.

Alternatively, studies have looked at the importance of immune cells in the context of PTB in mouse models. Shynlova et al. (2014) targeted the activation of mouse peripheral maternal immune cells with a broad spectrum chemokine inhibitor. A reduced incidence of lipopolysaccharide (LPS)-induced PTB was reported in addition to reducing both immune cell infiltration into maternal tissues and inflammatory mediator secretion. Rinaldi et al. (2014) found that neutrophil depletion did not delay delivery in an LPS-mediated PTB mouse model despite having an important role in the inflammatory response in the intrauterine tissues. Filipovich et al. (2015) further

corroborated this finding by reporting that the depletion of all granulocytes in an *Escherichia coli* (*E. coli*) model of PTB did not prevent early birth. Therefore, neutrophil infiltration is not essential for the induction of infection-induced PTB in the mouse. However, macrophage depletion was found to successfully prevent PTB in a mouse model induced by LPS (Gonzalez et al. 2011). This suggests that macrophages play a crucial role in PTB in the mouse. This may also relate to term labour.

1.1.1.3 Amniotic fluid

IL-1β, IL-6, IL-8, granulocyte-colony stimulating factor (G-CSF) and interferon gamma (IFN-γ) concentrations are all increased in the amniotic fluid during labour (Romero et al. 1990; Romero et al. 1991; Olah et al. 1996; Kemp et al. 2002b). The bioavailability of chemokine macrophage inflammatory protein 3α (MIP-3α)/CCL20, which is involved in the chemotaxis of immune dendritic cells, effector/memory T-cells and B-cells, is also increased in the amniotic fluid with spontaneous term and PTL (Hamill et al. 2008). The abundance of these mediators is further elevated by intrauterine infection. The presence of IL-1β in amniotic fluid, in particular, has been associated with PTB (Saito et al. 1993). Elevated IL-6 levels in the amniotic fluid can be indicative of chorioamnion microbial colonisation, even if an amniotic fluid culture is negative (Andrews et al. 1995).

1.1.1.4 Amnion/chorion

Cytokine abundance is increased in the fetal membranes during both term and PTL (Keelan et al. 1999). There is a significant increase in leukocyte recruitment to human fetal membranes during parturition, with an increase in IL-1β, IL-6, IL-8, MIP-1α and TNF release (Elliott et al. 2001b; Young et al. 2002; Gomez-Lopez et al. 2009). IL-8 has been specifically localised to leukocytes in the chorion but was not detected in the amnion of the membranes (Young et al. 2002). Furthermore, *in vitro* studies with fetal membrane explants from labouring women suggested that chemotaxis is selective for specific leukocyte subpopulations, such as monocytes, T-cells, B-cells and natural killer (NK) cells (Gomez-Lopez et al. 2009). Normal and premature rupture of membranes differ in regional chemotactic activity and related chemokine/cytokine production, which may suggest differential mechanisms of rupture. For example, the rupture of membranes during term labour is associated with an increase in T-cell

attraction, exhibiting elevated levels of IL-8. Conversely, during the premature rupture of membranes there is an increase in CXCL10 and granulocyte-macrophage colony-stimulating factor (GM-CSF), which is linked to granulocyte attraction (Gomez-Lopez et al. 2013a).

1.1.1.5 Choriodecidua

An important role for decidual activation in the initiation of labour was first suggested in the 1980s (Casey and MacDonald 1988). The choriodecidua is reported to be responsible for granulocyte, T-cell, monocyte and NK cell chemoattraction (Gomez-Lopez et al. 2011). Leukocytes infiltrate the decidua during term labour and PTL and play a role in stimulating inflammatory mediators involved in decidual activation (Osman et al. 2003; Hamilton et al. 2012). Macrophage numbers are increased in term and PTL but neutrophil abundance is only significantly increased in the decidua in PTL with infection (Hamilton et al. 2012). Elevated IL-6 and IL-8 concentrations have been identified in the chorionic-decidual tissues with labour (Keelan et al. 1999).

In a study of human choriodecidual samples there was an increase in the production of chemokines, such as IL-8, CCL2, CCL4, CCL5 and CXCL10, with term labour. The expression of chemokines was most commonly observed in the decidual stromal cells. IL-8, CCL2, CCL5, and CXCL10 were identified in the immune cells within the decidual stroma. In PTL, *CCL4*, *CCL5*, *CXCL1* and *CXCL6* mRNA concentrations were upregulated. The upregulation of these mediators suggests that chemokines may regulate decidual leukocyte recruitment during labour (Hamilton et al. 2013).

1.1.1.6 Placenta

There is limited evidence of placental inflammation. Cytokine concentrations were not altered in placental tissues collected from women following term labour (Keelan et al. 1999). However, an *in vitro* study investigating the effect of bacterial products on placental explants found an elevation of IL-1β, IL-6, IL-8 and IL-10 release (Griesinger et al. 2001).

In summary, labour is associated with an influx of leukocytes into the maternal-fetal tissues, such as the myometrium, amnion/chorion and the choriodecidua. There is also

an increase in inflammatory cytokines and chemokines in these tissues, as well as in the peripheral circulation and the amniotic fluid.

1.1.2 Inflammatory mediators

Extracellular molecular regulators, for example cytokines and chemokines, are responsible for immune cell recruitment as well as controlling the intracellular signalling that characterises inflammation (Turner et al. 2014).

1.1.2.1 Cytokines

Cytokines work through a network of interactions to modulate both acute and chronic inflammation. Cytokines can be classified by whether they act on cells of the innate or adaptive immune response, or promote or antagonise inflammation. Furthermore, they can be classified depending on their signalling receptors. Pro-inflammatory cytokines include IL-1β, IL-6, TNF and the interferon (IFN) subsets of cytokines, whereas IL-10, IL-19 and IL-20 are considered anti-inflammatory cytokines (Turner et al. 2014). Examples of cytokines associated with adaptive immunity include IL-2, IL-3, IL-4, IL-5, IL-13, IL-15 and GM-CSF. The inflammatory mediators most commonly associated with labour include IL-1, IL-6, IL-8 and TNF (Farina and Winkelman 2005).

Key pro-inflammatory cytokines, such as IL-1 and TNF, signal through type 1 cytokine receptors, which are structurally divergent from other cytokine receptors (Turner et al. 2014). IL-1β and TNF are early responders to infection, followed by IL-6 and later, IL-10 to reduce inflammation (Arango Duque and Descoteaux 2014; Friedrich et al. 2015). IL-1β is mainly produced by macrophages, B-cells and dendritic cells and targets B-cells, NK cells and T-cells. This pro-inflammatory cytokine promotes the proliferation and differentiation of cells (Turner et al. 2014). TNF is produced by and acts on macrophages to activate phagocytosis (Turner et al. 2014).

IL-6 production is stimulated by TNF and IL-1β release (Friedrich et al. 2015). IL-6 is produced by many cells, including T helper cells (Th) cells, macrophages, endothelial cells and fibroblasts. This inflammatory cytokine targets activated B-cells and plasma cells, causing immunoglobulin G (IgG) production and the differentiation of B-cells into antibody-producing plasma cells. Classical IL-6 receptor signalling takes place

via the IL-6 receptor (IL-6R) α chain (gp80, CD126) and the signal transducing component, gp130 (CD130). Gp130 is ubiquitously expressed but the IL-6R is restricted to lymphocytes and hepatocytes (Peters et al. 1998; Turner et al. 2014). The IL-6R can also be released from the cell surface in a soluble form to bind IL-6 and this complex then signals through gp130. This trans-signalling allows IL-6 to target cells that do not have the IL-6R. Gp130 activates Jak kinase (Jak)-1, Jak2 and tyrosine kinase (Tyk)-2 to activate the signal transducer and activator of transcription (STAT) and p38 mitogen-activated protein kinase (MAPK) signalling cascades (Hunter and Jones 2015). IL-6 is generally considered to be a pro-inflammatory cytokine, although it can also have anti-inflammatory effects (Tilg et al. 1997). The pro-inflammatory activities of IL-6 are mediated by the more common trans-signalling, while the anti-inflammatory effects of IL-6 are mediated by classical signalling (Scheller et al. 2011).

The anti-inflammatory cytokine IL-10 can inhibit the activation and function of T-cells, B-cells, monocytes and macrophages. IL-10 can also mediate the differentiation and function of regulatory T-cells (Tregs), which are key to controlling immune responses (Moore et al. 2001). IL-10 inhibits cytokine production and mononuclear cell function to exert anti-inflammatory effects (Turner et al. 2014). IL-10 signals through a type II cytokine receptor, which is a heterotetrameric receptor complex composed of two α and two β subunits. The binding of IL-10 to this receptor activates the JAK/STAT signalling pathway (Shouval et al. 2014).

1.1.2.2 Chemokines

Chemokines are small proteins (8-12kDa), which are sub-divided by the position of N-terminal cysteine residues. The most well-known are the C-X-C family, where the cysteine residues are separated by an amino acid, and the C-C family, with adjacent cysteine residues (Turner et al. 2014). The primary function of chemokines is to recruit leukocytes to the site of infection or injury but these mediators can also play a role in homeostatic functions (Turner et al. 2014). Signal transduction of chemokines is via members of the seven-transmembrane, G-protein-coupled receptor (GPCR) family (Moser et al. 2004). Chemokines act by inducing the expression of integrins, such as β2-integrin lymphocyte function-associated antigen 1 (LFA-1), which stimulate the diapedesis of leukocytes through the vascular endothelium (Constantin et al. 2000).

The migration and tissue specificity of leukocytes is due to the expression of particular chemokines, receptors and adhesion molecules.

CXCL8/IL-8, in particular, has been extensively studied. The primary role of IL-8 in inflammation is the recruitment of neutrophils and it is the most highly expressed chemokine in the myometrium during labour (Bollapragada et al. 2009; Turner et al. 2014). Excessive signalling of cytokines and chemokines can result in chronic conditions and autoimmune diseases, such as rheumatoid arthritis, type I diabetes, psoriasis and asthma (Pope et al. 2005; Park and Pillinger 2007; Rosa et al. 2008; Portugal-Cohen et al. 2012).

1.1.2.3 Prostaglandins

Prostaglandins, such as PGE_2 and $PGF_{2\alpha}$, are another class of inflammatory mediators that play an important role in the initiation of labour and the stimulation of uterine contractions (Crankshaw and Dyal 1994; Gibb 1998). Prostaglandins are synthesised by the release of arachidonic acid by phospholipase A2, which is then converted to PGH_2 by cyclooxygenase 1 and 2 (COX-1, -2). COX-2 expression is upregulated in the human myometrium and fetal membranes prior to the onset of labour, suggesting a role for this enzyme in the initiation of parturition (Slater et al. 1999a; Slater et al. 1999b). Specific prostaglandins are then produced from the conversion of PGH_2 (Simmons et al. 2004; Sykes et al. 2014).

Prostaglandin synthesis can be stimulated in the myometrium by inflammatory cytokines. However, prostaglandins themselves also have pro-inflammatory actions (Hertelendy et al. 1993; Molnar et al. 1993; Pollard and Mitchell 1996). $PGF_{2\alpha}$, which is produced by the decidua, can activate the decidua as well as directly stimulating myometrial contractions and upregulating decidual MMP-2 and MMP-9 expression. This action of $PGF_{2\alpha}$ may then enhance leukocyte migration into the decidua and stimulate the release of IL-1β (Schonbeck et al. 1998; Ulug et al. 2001). PGE_2 plays a role in cervical remodelling by reducing collagen concentration, as well as increasing the synthesis of proteoglycans in the cervix (Ekman et al. 1986; Norman et al. 1993). PGE_2 also stimulates IL-8 release from the cervix (Denison et al. 1999). Furthermore,

PGE_2 has been linked to the rupture of fetal membranes by stimulating an increase in MMP-9 (McLaren et al. 2000).

1.1.2.4 MMPs

MMPs are a family of zinc-dependent proteinases (Nagase and Woessner 1999). They are secreted into the extracellular matrix as proenzymes and their activity is tightly regulated by a range of mechanisms, such as proteolytic activation, the action of inducers and direct inhibition by tissue inhibitors of metalloproteinases (TIMPs) (Van Wart and Birkedal-Hansen 1990; Qin et al. 1997; Riley et al. 1999; Ulug et al. 2001). MMPs play an important role in the remodelling of the myometrial extracellular matrix to allow for uterine contraction (Roh et al. 2000). For example, MMP-9 is upregulated by IL-1β and TNF in the remodelling of the human myometrium during labour (Roh et al. 2000). MMP-2 and MMP-9 activity increase dramatically during labour and their activity is even higher in cases of PTL (Xu et al. 2002; Yonemoto et al. 2006). MMPs also facilitate both normal and pathological fetal membrane rupture (Tsatas et al. 1999).

To summarise, inflammatory mediators, such as cytokines, chemokines, prostaglandins and MMPs, are responsible for the shift from an anti-inflammatory to pro-inflammatory environment. Therefore, these mediators are implicated in the initiation of labour and stimulation of myometrial contractions.

1.1.3 Transcription factors in labour

1.1.3.1 NF-κB

A key regulator of the inflammatory pathways associated with labour is the transcription factor nuclear factor kappa-B (NF-κB). A multitude of inflammatory mediators, such as IL-1β, IL-6, IL-8 and TNF, contain NF-κB recognition elements in their promotor regions (Lindstrom and Bennett 2005). NF-κB dimers are anchored in the cytoplasm by the IκB inhibitory proteins. The NF-κB canonical pathway is triggered by microbial products as well as pro-inflammatory cytokines, such as IL-1 and TNF. This causes rapid phosphorylation of IκBα and IκBβ, followed by polyubiquitination and degradation through the 26S proteasome. The NF-κB dimer is

then free to translocate to the nucleus, where it activates gene transcription (Zandi et al. 1997).

NF-κB activity has been identified in both the upper and lower myometrium, as well as the amnion, prior to the onset of term labour (Khanjani et al. 2011; Lim et al. 2012). NF-κB activity is associated with an increase of COX-2 and MMP-9 production in term human myocytes (Choi et al. 2007). In addition, NF-κB is crucial for increased expression of the *IL-8* gene in cells of the human amnion and cervical epithelium (Elliott et al. 2001a).

1.1.3.2 AP-1

The activator protein 1 (AP-1) family of transcription factors also play a role in the regulation of inflammation. AP-1 is made up of dimeric basic leucine zipper (bZIP) proteins consisting of Fos (cFos, FosB, Fra-1, Fra2) and Jun (cJun, JunB, JunD) activating transcription factor subunits (Karin et al. 1997; Shaulian and Karin 2002).

In a study of the rat myometrium, c-fos, fosB, fra-1, fra-2 and junB were upregulated during labour. AP-1 transcription factor mRNA levels were also upregulated when labour was induced with progesterone receptor antagonist, RU486 (Mitchell and Lye 2002). AP-1 activation occurs at labour onset in the mouse and drives the production of pro-inflammatory mediators in an LPS-induced mouse model of PTB (MacIntyre et al. 2014).

The role of AP-1 in human parturition has recently emerged. TNF stimulated an increase in AP-1 activity in human myometrial cells. In addition, cFos and JunB mRNA and protein expression were increased in the human myometrium at term (Lim and Lappas 2014). Mechanical stretch of human myometrial cells and amnion epithelial cells has also been associated with AP-1 activation (Shynlova et al. 2002; Mohan et al. 2007).

1.1.4 Steroid hormones

1.1.4.1 Progesterone

Progesterone, a steroid hormone acting through nuclear progesterone receptors (PR), is crucial for myometrial quiescence during pregnancy. Administration of the PR

antagonist RU486 causes pregnancy loss in both humans and mice. In humans, progesterone is initially produced by the corpus luteum and then by the placenta. In a number of species, such as the mouse, the withdrawal of progesterone initiates the labour process. However, a systemic drop in progesterone levels is not observed in humans. Although, it has been proposed that there is a functional progesterone withdrawal, which may be mediated by a shift in the ratio of PR subtypes (Allport et al. 2001; Condon et al. 2003; Merlino et al. 2007).

Progesterone responsiveness is mediated by the relative expression of PR-A and PR-B receptor subtypes in the human myometrium at term and functional progesterone withdrawal may result from an increase in the ratio of PR-A to PR-B (Mesiano et al. 2002; Merlino et al. 2007). Another study reported a reduction in PR coactivator expression in the mouse and human uterus prior to term, which may impair PR function causing a functional progesterone withdrawal (Condon et al. 2003).

The PR itself has an anti-inflammatory role by inhibiting NF-κB activation and subsequent COX-2 gene expression, which leads to the inhibition of myometrial contractility (Hardy et al. 2006). A mutual repressive action has been identified between NF-κB and the PR (Kalkhoven et al. 1996).

Progesterone also promotes uterine quiescence by affecting the electrical activity of myometrial cells by increasing the expression of calcium (Ca^{2+}) and potassium (K^{+}) voltage-gated channels. This dampens the electrical activity of the myometrial cells, downregulating contraction-associated proteins, such as connexin 43 (CX43), and the proteins involved in actin and myosin interactions (Zhao et al. 1996; Soloff et al. 2011).

1.1.4.2 Oestrogen

Pregnancy has been described as a hyperestrogenic condition. The concentration of oestrogen increases with gestation and the placenta is the main source of this steroid hormone (Kota et al. 2013). The increased expression of oestrogen receptor alpha (ERα) in the term myometrium may induce functional oestrogen activation and may also be linked to functional progesterone withdrawal (Mesiano et al. 2002). Oestrogens induce a number of changes to the myometrium during pregnancy, such as increasing

PGE_2 and $PGF_{2\alpha}$ synthesis, increasing oxytocin activity, increasing the synthesis of CX43 and upregulating calmodulin, which is involved in the contraction of smooth muscle (Pinto et al. 1964; Ham et al. 1975; Petrocelli and Lye 1993).

1.1.5 Myometrial contractions

Uterine contractility is a complex and dynamic process that occurs in both non-pregnant and pregnant states. Contractions are a function of the myometrial layer of the uterus, which is composed of smooth muscle cells. The non-pregnant myometrium contracts to enhance the sloughing of the endometrium during menses and to assist the passage of sperm. Throughout pregnancy the uterus remains quiescent until labour, when the uterus contracts vigorously to expel the fetus. As the balance between oestrogen and progesterone shifts, morphological changes to the myometrium initiate active contractions. Contractions vary in frequency, amplitude and tone and are predominantly regulated by intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) (Pehlivanoglu et al. 2013).

1.1.5.1 The role of inflammation

As previously discussed, labour is associated with the increase of pro-inflammatory mediators, such as IL-1β, IL-6, IL-8 and TNF, and an influx of leukocytes into the myometrium (Young et al. 2002). These mediators play an important role in the stimulation of myometrial contractions.

When mouse uterine strips were stimulated with LPS, the amplitude, but not the frequency, of contraction increased (Mackler et al. 2003). Human myometrial cell contraction was also enhanced *in vitro* by LPS. This effect was mediated by the Ras homology (Rho)/Rho-associated kinase (ROCK) pathway (Hutchinson et al. 2014). In a co-culture of human monocytes and myometrial cells, there was evidence of synergistic pro-inflammatory cytokine secretion, while myometrial cell contraction was enhanced (Rajagopal et al. 2015). IL-1β stimulation of human myometrial cells increased Ca^{2+} entry into cells *in vitro*, increasing their excitability. This suggests that inflammatory mediators can affect Ca^{2+} flux in myometrial cells, which may impact on myometrial contractility (Tribe et al. 2003).

The anti-inflammatory actions of both dexamethasone and IL-10 inhibited IL-1β-induced uterine contractions in pregnant rhesus monkeys. Specifically, oestradiol concentration was reduced by dexamethasone treatment and IL-10 reduced the release of the pro-inflammatory cytokine TNF and inhibited leukocyte migration (Sadowsky et al. 2003).

Therefore, myometrial contractions can be stimulated by inflammatory mediators as well as inhibited by anti-inflammatory agents. This suggests that inflammation is key to the initiation of myometrial contractions.

1.1.5.2 Oxytocin

Oxytocin, a peptide hormone, is critical to the regulation of the myometrium during pregnancy. The expression and production of the oxytocin receptor increases as pregnancy progresses and peaks during labour (Fuchs et al. 1991; Kimura et al. 1996). Oxytocin increases myometrial contractility by stimulating an increase in $[Ca^{2+}]i$ (Sanborn et al. 1998). Stimulation of human decidua explants with IL-1β has been shown to indirectly increase the secretion of oxytocin via COX-2 and prostaglandin production (Friebe-Hoffmann et al. 2007). Furthermore, oxytocin can activate NF-κB in human myometrial cells, via the oxytocin receptor, and upregulate IL-8, CCL6, IL-6 and COX-2 gene expression (Kim et al. 2015). The production of these inflammatory mediators will then contribute to the stimulation of contractions.

1.1.5.3 CX43

The formation of gap junctions allows for intercellular communication. These junctions are composed of connexin (CX) proteins (Sohl and Willecke 2004). CX43 (43kDa) is a contraction-associated protein localised to smooth muscle cells and plays a key role in the establishment of synchronous contractions (Oyamada et al. 1994; Hutchings et al. 2009). Parturition is delayed when *Cx43* is ablated in mouse myometrial smooth muscle cells, emphasising the importance of intercellular communication for uterine contractility (Doring et al. 2006). *CX43* mRNA expression increases with gestation in both the human and animal myometrium and increases further during labour (Chow and Lye 1994; Orsino et al. 1996). CX43 abundance is also increased in the human and mouse myometrium during PTL (Balducci et al. 1993;

Chang et al. 2012). Therefore, CX43 is considered important for synchronous contractions in both term and PTL.

1.1.6 Excitation contraction coupling

The excitation-contraction coupling of smooth muscle cells can be stimulated by two main mechanisms: by the activation of Ca^{2+} signalling cascades or by the Rho/ROCK pathway, where Ca^{2+} sensitivity of the cell is altered (Berridge 2008).

1.1.6.1 Ca^{2+} signalling cascades

Myometrial cell contraction is facilitated by the cross-bridging of the myofilaments actin and myosin. This is instigated by the increase of $[Ca^{2+}]i$ and completed by the phosphorylation of myosin light chain (pMLC) (Figure 1.1). $[Ca^{2+}]i$ can increase by the influx of Ca^{2+} from the extracellular fluid via voltage-gated L-type Ca^{2+} channels and/or Ca^{2+} can be released from the sarcoplasmic reticulum (SR), which contains the intracellular stores of Ca^{2+} (Pehlivanoglu et al. 2013).

The main source of Ca^{2+} in the myometrium is by extracellular entry through voltage-gated L-type Ca^{2+} channels (Shojo and Kaneko 2001; Kupittayanant et al. 2002). Ca^{2+} can also be released from the SR by the binding of a uterine agonist to a specific GPCR anchored to the plasma membrane (Phaneuf et al. 1993). This stimulates membrane phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol trisphosphate (IP_3). This causes the release of Ca^{2+} from the SR into the cytoplasm (Taylor et al. 1991; Luckas et al. 1999). The increase in $[Ca^{2+}]i$ generates an action potential, of which two have been detailed in the myometrium. Simple action potentials are characterised by depolarisation followed by rapid repolarisation. More complicated action potentials involve depolarisation with a sustained plateau (Khan et al. 2001; Bursztyn et al. 2007). Alternatively, a feed-forward loop has also been identified in the myometrium whereby increasing $[Ca^{2+}]i$ sensitises other Ca^{2+} channels to open (Shojo and Kaneko 2001; Kupittayanant et al. 2002; Wray et al. 2003).

When there is a substantial increase in $[Ca^{2+}]i$ this activates calmodulin, a protein located in the cytosol. Calmodulin forms a complex with four Ca^{2+} ions, which leads to the activation of the enzyme myosin light chain kinase (MLCK) (Johnson et al.

1996). MLCK then phosphorylates 20kDa MLCs, leading to actin and myosin cross-bridge formation (Hai and Murphy 1989; Shojo and Kaneko 2001).

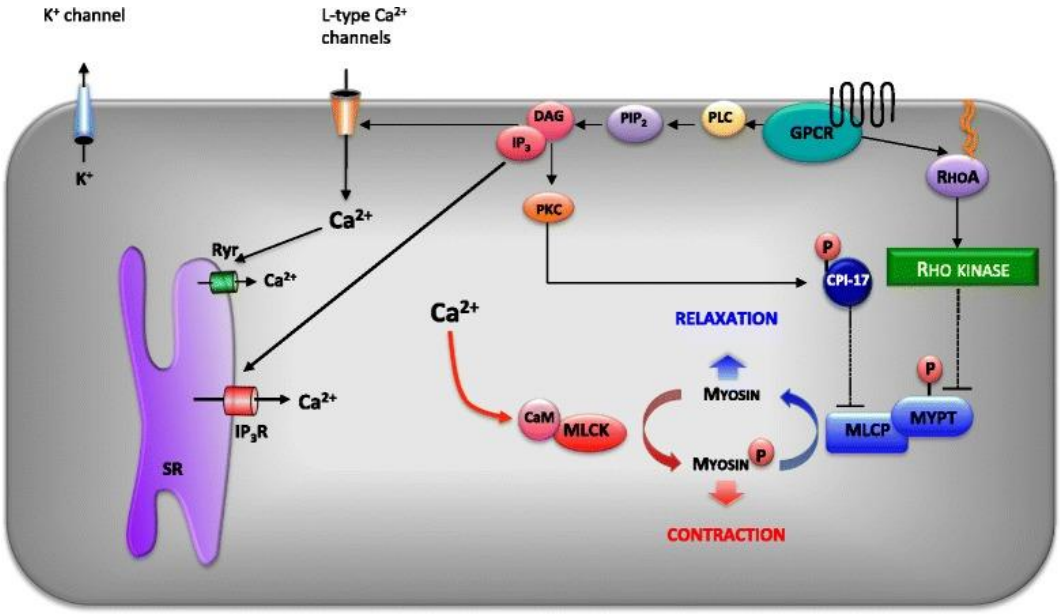

Figure 1.1. The molecular pathways in smooth muscle contraction. Actin and myosin cross-bridge formation is essential for smooth muscle contraction. This relies on the phosphorylation of myosin light chains (MLC) by myosin light-chain kinase (MLCK). G protein-coupled receptors (GPCR) activate the contractile pathways. Ca^{2+} enters the cytosol from either the extracellular space, through voltage-gated Ca^{2+} channels, or by IP_3-dependent release from the sarcoplasmic reticulum (SR). A Ca^{2+}/calmodulin (CaM) complex forms and activates MLCK. Relaxation is favoured by myosin light-chain phosphatase (MLCP) as the active subunit removes the phosphoryl group from the MLC. When MLCP is inhibited, this allows contraction. MLCP can be repressed via direct inhibition by PKC-dependent CPI-17 or inhibitory phosphorylation of the myosin phosphatase target subunit (MYPT) by Rho kinase (ROCK). PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol trisphosphate. Reprinted from Reddi et al. (2015) under a Creative Commons license.

1.1.6.2 Calcium sensitisation and the Rho/ROCK pathway

Rho proteins are members of the small GTPase family, which includes the three isoforms RhoA, RhoB and RhoC. RhoA is the most studied of the isoforms as it has an important function in smooth muscle contraction (Figure 1.1) (Wheeler and Ridley 2004). The inactive form of RhoA is bound to guanosine diphosphate (GDP) and found in the cell cytosol. Active RhoA is bound to guanosine trisphosphate (GTP) and is located in the cell membrane. GTPase-activating proteins (GAPs) facilitate the binding

of GDP to RhoA, whereas guanine nucleotide exchange factors (GEFs) regulate RhoA-GTP binding (Van Aelst and D'Souza-Schorey 1997; Moon and Zheng 2003). Guanine nucleotide dissociation inhibitors (GDIs) form a complex with RhoA facilitating its diffusion between the cytosol and the cell membrane (Michaelson et al. 2001).

ROCK is a serine/threonine-specific kinase that is found in the cell cytoplasm. It has two isoforms, ROCK I and ROCK II, which are both expressed in smooth muscle cells and ROCK II has been the most implicated in contraction. ROCK is an effector of RhoA. When RhoA becomes activated, ROCK migrates to the cell membrane to form a complex with the active RhoA-GTP, causing autophosphorylation and ROCK activation (Leung et al. 1995; Matsui et al. 1996; Nakagawa et al. 1996).

It is well established that RhoA/ROCK is a key molecule in GPCR-mediated Ca^{2+} sensitisation, leading to smooth muscle contraction (Kureishi et al. 1997; Uehata et al. 1997; Puetz et al. 2009). Ca^{2+} sensitisation takes place when an agonist, such as oxytocin or $PGF_{2\alpha}$, induces an increase in $[Ca^{2+}]i$ (McKillen et al. 1999; Woodcock et al. 2004; Woodcock et al. 2006; Shmygol et al. 2007). Following the stimulation of GPCRs by an agonist, the Rho/ROCK pathway is activated. ROCK phosphorylates myosin light chain phosphatase (MLCP), inhibiting this enzyme from having a relaxation effect on MLC (Kitazawa et al. 1989; Noda et al. 1995; Aguilar and Mitchell 2010; Aguilar et al. 2012). ROCK inhibits MLCP by phosphorylating its myosin phosphatase target subunit 1 (MYPT1) or via the inhibitory protein CPI-17. Alternatively, ROCK can phosphorylate MLC directly, by MLCK-like activity, to induce contraction (de Godoy and Rattan 2011).

1.1.7 Initiation of labour

1.1.7.1 Uterine stretch

As pregnancy progresses, the uterus must remodel and increase dramatically in size while maintaining quiescence. Intrauterine pressure increases but the uterus must adapt to counteract any wall tension. It has been hypothesised that labour is initiated when this adaptive mechanism is overcome. A study inducing mechanical stretch to rat myometrial cells caused an increase in CCL2 release. This effect was attenuated by

progesterone pre-treatment, suggesting antagonism between mechanical stretch and progesterone on cytokine release in the uterus (Shynlova et al. 2008).

An *in vitro* study investigating the effect of human myometrial cell mechanical stretch found an elevation in the release of the inflammatory mediators IL-6, IL-8 and CXCL1. There was also an increase in leukocyte adhesion to the endothelium of vessels in the uterus. This suggests that mechanical stretch can lead to leukocyte recruitment to the myometrium (Lee et al. 2015).

Uterine overdistension was modelled in non-human primates by the inflation of an intra-amniotic balloon. There was a significant increase in IL-1β, TNF, IL-8, CCL2, PGE_2 and $PGF_{2\alpha}$ in the amniotic fluid preceding labour. When this stress on the uterus is very great, it can cause PTL (Adams Waldorf et al. 2015). Therefore, uterine stretch may stimulate the initiation of the labour process by increasing immune cell infiltration into the uterus and increasing the production of pro-inflammatory mediators.

1.1.7.2 Fetal signalling

It has also been proposed that the fetus produces signals that initiate the labour process. Surfactant protein-A (SP-A) is secreted by the fetal lungs into the amniotic fluid. In mice, SP-A abundance increases in the amniotic fluid as term approaches and this is thought to signal parturition. However, in relation to humans, reports in the literature disagree on the concentration of SP-A in the amniotic fluid towards term and at the onset of labour (Chaiworapongsa et al. 2008; Reinl and England 2015).

In SP-A deficient mice, labour is only delayed for approximately 12 hours (Montalbano et al. 2013). Gao et al. (2015) investigated the effect of steroid receptor coactivators 1 and 2 (SRC-1, -2), which are transcriptional regulators of SP-A. It was found that labour was substantially delayed in dams with SRC-1/2-deficient fetuses. These mothers exhibited reduced NF-κB activation, reduced $PGF_{2\alpha}$ levels and a reduction in contraction-associated genes. Therefore, the fetal lung production of SP-A, mediated by SRC-1/2, is essential for parturition to progress normally in mice. The results from this model highlight the importance of fetal signalling for the initiation of parturition in the mouse, which may be relatable to humans (Gao et al. 2015).

1.2 Preterm labour

1.2.1 Epidemiology

The World Health Organisation definition of PTB is delivery before 37 completed weeks of gestation. This can be further subdivided into extremely preterm (<28 weeks), very preterm (28-32 weeks) and moderate to late PTB (32-36 weeks) (WHO 1977). PTB is a serious public health problem, which accounts for 11.1% of births worldwide. This rate varies from approximately 5% of births in European countries to 18% in some African countries (Blencowe et al. 2012). Furthermore, PTB is currently the leading cause of mortality in children under five years of age (Harrison and Goldenberg 2015). In England and Wales, the economic burden of neonatal intensive care followed by ongoing health and educational support is estimated to be >£2.9 billion (Mangham et al. 2009; Howson et al. 2013).

In high income countries, approximately one third of PTBs are medically indicated. This occurs when there is risk to the mother or fetus that outweighs the benefit of continuing the pregnancy. For example, in conditions such as preeclampsia and diabetes. The remainder of PTBs are spontaneous, where women can present with PTL with cervical dilation or preterm premature rupture of membranes (pPROM) (Rubens et al. 2014).

Spontaneous PTL is very difficult to predict. Maternal risk factors include previous PTB, extremes in maternal age and body mass index (BMI), multiple gestation, use of assisted reproductive technologies and low socioeconomic status (Rubens et al. 2014). Race is also an important risk factor as women classified as black, African-American or Afro-Caribbean are at greater risk of PTB than other racial groups (Goldenberg et al. 2008).

The aetiology of PTL is poorly understood. It can be initiated by multiple mechanisms, such as infection, decidual senescence, uterine overdistension, cervical disease, stress or the breakdown of maternal-fetal tolerance (Romero et al. 1994; Goldenberg et al. 2008; Romero et al. 2014).

1.2.2 Infection and inflammation as a cause of PTB

Intrauterine infection is believed to be present in 25-40% of PTBs. However, this is likely to be an underestimate as infections are often sub-clinical and cannot be detected by conventional culturing techniques (Goldenberg et al. 2008). Chorioamnionitis is the most common presentation of intrauterine inflammation and is characterised by inflammation of the chorion, amnion and placenta. This inflammatory condition is usually a result of bacterial infection (Goldenberg et al. 2008).

Exposure to intrauterine infection and/or inflammation can cause fetal inflammatory response syndrome (FIRS), which is responsible for fetal organ injury, resulting in neonatal morbidity and mortality (Gotsch et al. 2007). Bacteria or inflammatory mediators may reach the fetal circulation by placental transmission into the umbilical cord or indirectly via the amniotic fluid, causing damage to multiple fetal organ systems (Figure 1.2) (Adams Waldorf and McAdams 2013). Chorioamnionitis is associated with an increased risk of respiratory distress syndrome, bronchopulmonary dysplasia (BPD), intraventricular haemorrhage, patent ductus arteriosus and necrotising enterocolitis in exposed neonates (Been et al. 2013; Galinsky et al. 2013; Seliga-Siwecka and Kornacka 2013).

The most common route of infection is via microbial ascension from the vagina. The microbes proliferate in the amniotic fluid and invade the chorioamniotic membranes (Kim et al. 2009). However, it is also possible that there could be a haematogenous dissemination of infection or microorganisms could be introduced during an invasive procedure or originate in the fallopian tubes (Bastek et al. 2011). Various bacterial organisms, and in rare cases viral and fungal organisms, are associated with the pathogenesis of chorioamnionitis. Examples of bacteria found in the amniotic fluid are species of *Ureaplasma*, *Mycoplasma*, *Fusobacterium*, *Streptococcus*, *Bacteroides* and *Prevotella*. (DiGiulio et al. 2010; DiGiulio 2012). Genital mycoplasmas, *Ureaplasma urealyticum* and *Mycoplasma hominis*, are the most frequent microbes in cases of culture-confirmed chorioamnionitis (Waites et al. 2005). Chorioamnionitis is polymicrobial in most cases (DiGiulio 2012).

PTL may be triggered by a fetal and/or maternal response to chorioamnionitis. The invading bacteria release endotoxins and exotoxins, which are recognised by TLRs presented on the surface of cells such as leukocytes and epithelial cells (Holmlund et al. 2002). This leads to the activation of transcription factors, such as NF-κB and AP-1, which in turn stimulate the production of the cytokines and chemokines IL-1β, IL-6, IL-8 and TNF, leading to the production of prostaglandins and leukocyte chemotaxis to the uterine tissues (Goldenberg et al. 2000). Infection prevents prostaglandin dehydrogenase from inactivating prostaglandins, allowing the bioactive prostaglandins to prematurely initiate contractions (van Meir et al. 1997). These events initiate the labour process, whereby prostaglandins stimulate myometrial contractions and MMPs assist in cervical ripening and fetal membrane rupture. PTL has been associated with a shift from an anti-inflammatory to a pro-inflammatory state at the maternal-fetal interface. It has also been suggested that an imbalance between innate and adaptive immune cells occurs at this interface prior to PTL (Arenas-Hernandez et al. 2016).

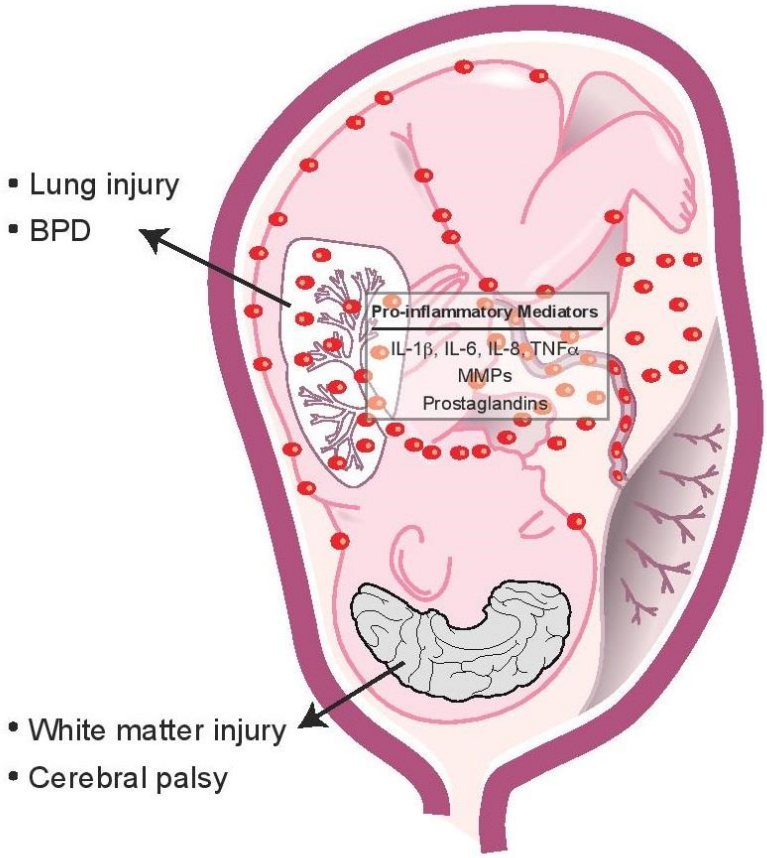

Figure 1.2. Intrauterine inflammation and fetal injury. Human parturition is an inflammatory process stimulated by mediators such as cytokines, chemokines and prostaglandins. However, the presence of inflammation earlier in gestation can cause PTL and exposure of the fetus to an adverse environment. Inflammatory mediators may reach the fetal circulation by placental transmission into the umbilical cord or indirectly via the amniotic fluid. Amniotic fluid-exposed tissues, such as the fetal skin and lung, can drive the fetal inflammatory response. Prolonged exposure to inflammatory mediators can result in fetal injury. The fetus is susceptible to lung injury and bronchopulmonary dysplasia (BPD), as well as brain white matter lesions and neurological conditions, such as cerebral palsy. Reprinted from Boyle et al. (2017) under a Creative Commons license.

1.2.3 Fetal injury

Premature neonates are at risk of a number of health complications due to the immaturity of multiple organ systems (Rubens et al. 2014). The severity of these health problems inversely correlate with gestational age at birth (WHO 1977). Premature

babies have an increased risk of cognitive and neurological impairment, for example cerebral palsy, as well as an increased risk of respiratory and gastrointestinal complications (Marlow et al. 2005; Goldenberg et al. 2008). Prematurity also predisposes to chronic diseases in adulthood such as obesity, diabetes and hypertension (Rubens et al. 2014). Interestingly, there are also sex-specific differences as male sex is an independent risk factor for adverse pregnancy outcome, including PTB (Di Renzo et al. 2007; Ferrero et al. 2016). It has been suggested that this could be due to the larger size and birthweight of males. Furthermore, factors associated with male sex may predispose to infection-mediated PTB (MacGillivray and Davey 1985; McGregor et al. 1992). Premature males are at greater risk of morbidity and mortality, with poorer neurological and respiratory outcomes than females at two years (Peacock et al. 2012).

As previously mentioned, fetal injury can result from the exposure of the fetus to inflammatory mediators. Brain white matter lesions have been associated with the presence of pro-inflammatory mediators in the amniotic fluid. Elevated levels of IL-1β and IL-6 in the amniotic fluid have been used to identify premature neonates at risk of these lesions (Yoon et al. 1997a). TNF signalling has been described as toxic to developing oligodendrocytes *in vitro* (Li et al. 2008). In a mouse model of intrauterine inflammation, fetal brain injury was reported even in the absence of PTB, with altered fetal neuronal morphology and neurotoxicity identified following this *in utero* insult (Burd et al. 2011; Elovitz et al. 2011). Intrauterine inflammation also causes long term changes to the mouse brain, with morphological alterations identified by MRI and effects on behaviour observed into adulthood (Dada et al. 2014). Inflammation may cause brain injury by direct insult to oligodendrocytes and neurons or as secondary injury via microglial cell activation. This would result in the secretion of pro-inflammatory cytokines, causing damage to the surrounding cells (Burd et al. 2012).

Fetal lung injury can also be caused by elevated levels of inflammatory mediators in the amniotic fluid. For example, high levels of TNF, IL-1β, IL-6 and IL-8 have been found in the amniotic fluid of women who had babies with the chronic lung disease, BPD (Yoon et al. 1997b; Ambalavanan et al. 2011). However, a meta-analysis found only a modest association between chorioamnionitis and BPD (Hartling et al. 2012). Infection-induced fetal lung injury has been studied in some detail in the sheep. In a

chronically catheterised sheep model of the extreme preterm period, intra-amniotic LPS exposure caused fetal skin and lung inflammation, as well as systemic inflammation (Kemp 2014; Kemp et al. 2016). The fetus rapidly generated a robust inflammatory response driven by amniotic fluid-exposed tissues, such as the lung. Fetal blood cells responded to the systemic inflammation but these cells did not contribute to the acute production of inflammatory mediators (Kemp et al. 2016). Furthermore, a primate model of transient choriodecidual infection confirmed indirect lung injury as a result of elevated inflammatory mediators. This led to the downregulation of pathways for angiogenesis, morphogenesis and cellular growth and development in the fetal lung (McAdams et al. 2012).

1.2.4 Prevention and treatment of PTL

1.2.4.1 Tocolytics

As PTL is very difficult to predict, treatments have focussed on tocolytic therapies that aim to temporarily inhibit myometrial contractions. Tocolytic administration increases the likelihood of completing a 48 hour corticosteroid treatment to accelerate fetal lung development (Alston et al. 2016). However, tocolytic treatments are largely ineffective at substantially delaying PTB and fail to improve the outcome of the premature neonates (Haas et al. 2012; Haas et al. 2014). Commonly used tocolytics include betamimetics, calcium channel blockers, oxytocin receptor antagonists and COX inhibitors. Magnesium sulphate may also be administered as it is thought to have neuroprotective properties (Haas et al. 2014).

Calcium channel blockers, such as nifedipine, inhibit contractions by blocking the influx of Ca^{2+} into myometrial cells, preventing the phosphorylation of MLC. They are superior to treatment with betamimetics due to their ability to prolong pregnancy and are associated with fewer neonatal and maternal adverse effects. These drugs may also offer some benefit over magnesium sulphate and oxytocin receptor antagonists (Flenady et al. 2014a; Flenady et al. 2014b). Overall, calcium channel blockers are considered the preferred first-line tocolytic in terms of multiple key outcomes (Haas et al. 2009; Haas et al. 2012). A recent Cochrane review found that magnesium sulphate did not postpone delivery nor did it have any beneficial neonatal or maternal outcomes. This was despite a previously pooled analysis of five trials, with 6145

infants, finding a statistically significant neuroprotective effect (Doyle et al. 2009; Crowther et al. 2014). Therefore, this treatment is not currently recommended for tocolysis as further trials are needed to establish optimal dosing and treatment regimens for any potential beneficial effects (McNamara et al. 2015). Another recent Cochrane review did not find a benefit for the use of COX inhibitors, in comparison to other tocolytics, despite this treatment having some benefit in postponing birth compared to placebo (Reinebrant et al. 2015). Importantly, tocolytic treatments do not target the underlying cause of PTL and there is no benefit of prolonging a pregnancy if the *in utero* environment is adverse for the fetus (Haas et al. 2012).

1.2.4.2 Antibiotics

As intrauterine infection is a common cause of PTB, antibiotics can be administered. Two randomised clinical trials investigated the short and long term effects of certain antibiotics.

The ORACLE I randomised trial investigated the effect of broad spectrum antibiotics on women with pPROM. Treatment with erythromycin was associated with prolonged pregnancy and neonatal health benefits, such as fewer cerebral abnormalities prior to discharge. However, co-amoxiclav was associated with neonatal necrotising enterocolitis (Kenyon et al. 2001a). The children from the ORACLE I trial were followed up at seven years of age. There was little effect of antibiotic treatment on the health of these children at this age (Kenyon et al. 2008a). Due to the prolongation of pregnancy and improvements of short-term neonatal morbidities, the routine prescription of antibiotics was recommended for the treatment of pPROM in the 2013 Cochrane review, with the exception of co-amoxiclav (Kenyon et al. 2013).

The ORACLE II randomised trial investigated the effect of erythromycin and co-amoxiclav on women with spontaneous PTL with intact membranes but with no evidence of clinical infection. Antibiotic treatment was associated with a lower occurrence of maternal infection but this treatment did not lower the rates of the primary outcome measures, which included neonatal death, chronic lung disease and major cerebral abnormality. It was concluded that antibiotics should not be prescribed for spontaneous PTL without infection (Kenyon et al. 2001b). The seven year follow-

up study for ORACLE II presented adverse outcomes in the children from mothers treated with antibiotics. Erythromycin treatment was associated with an increased risk of functional impairment in these children. Furthermore, both erythromycin and co-amoxiclav treatment increased the risk of cerebral palsy (Kenyon et al. 2008b). The results of this review highlight the need for the assessment of long term neurodevelopmental outcome. This is particularly important in cases of intrauterine infection as delaying delivery could cause additional harm by prolonging exposure of the fetus to an adverse, infectious/inflammatory environment leading to increased fetal injury.

1.2.4.3 Progesterone

Progesterone is crucial for the maintenance of pregnancy. The potential of progesterone as a treatment for threatened PTL was first suggested in 1960 (Fuchs and Stakemann 1960). A multitude of clinical trials have investigated the effects of several progesterone derivatives, routes of administration and dosage regimens, with variable outcomes.

Vaginal progesterone was reported to reduce uterine contractions in high risk women and reduce PTB incidence (da Fonseca et al. 2003). Intramuscular injection of progesterone was also shown to reduce PTB in a trial of women who had a history of PTB (Meis et al. 2003). Trials have also reported that vaginal progesterone reduces PTB in women with a short cervix (Fonseca et al. 2007; Hassan et al. 2011; Romero et al. 2012).

In contrast, the administration of vaginal progesterone to women at high risk of PTB attenuated cervical shortening but did not prolong gestation compared to the placebo group (O'Brien et al. 2007; O'Brien et al. 2009). A small randomised trial for the use of weekly progesterone treatment in women with pPROM did not prolong gestation (Briery et al. 2011). Furthermore, neither vaginal nor intramuscular injection of progesterone prevented PTB in multiple gestations (Briery et al. 2009; Norman et al. 2009; Combs et al. 2010; Combs et al. 2011; Rode et al. 2011).

Meta-analyses have concluded that prophylactic progesterone treatment may reduce the incidence of PTB in women at high risk but there is limited information on long

term neonatal outcome. Therefore, larger randomised controlled trials investigating long term outcomes are required (Dodd et al. 2005; Mackenzie et al. 2006; Rode et al. 2009). The 2013 Cochrane review further emphasised the need for longer term childhood outcomes and to refine the timing, dosage and administration of progesterone treatment (Dodd et al. 2013).

The most convincing results thus far come from a recent, large, double-blind, randomised, placebo controlled trial of prophylactic vaginal progesterone treatment. The outcome of this trial was that the risk of PTB was not reduced by progesterone nor was neonatal outcome improved in women who were at high risk. The trial did not find any long term benefit of this treatment in children at 2 years of age (Norman et al. 2016). This is the largest study to date comparing the obstetric, neonatal and childhood outcomes of vaginal progesterone treatment in women at high risk of PTB. The results of the trial have instigated an update to the NICE guidelines regarding the use of prophylactic progesterone for the prevention of PTB.

1.2.4.4 Anti-inflammatory agents

Targeting the inflammation commonly associated with PTL may be an ideal approach to delay delivery and, importantly, prevent fetal injury. Inflammatory cascades are most commonly activated as a result of bacterial invasion. Bacteria activate pattern recognition receptors, such as TLRs, leading to the activation of NF-κB and MAPK signalling pathways, which initiates an inflammatory cascade. These steps promote the pathological, preterm initiation of labour (Ng et al. 2015). Below is a summary of some inhibitors of these pathways, which may be novel treatments for the prevention of PTB.

Non-specific NF-κB inhibitors

Antioxidant, *N*-acetyl cysteine (NAC) inhibits the activity of NF-κB (Zafarullah et al. 2003). In an LPS-induced mouse model of PTB, NAC reduced PTB rates, reduced uterine inflammation and prevented white matter injury (Chang et al. 2011). NAC also attenuated NF-κB activation in the mouse uterus and reduced the expression of contraction-associated protein COX-2 (Chang et al. 2012). NAC inhibited NF-κB DNA-binding activity as well as reducing the release of inflammatory mediators, such

as IL-6, IL-8 and TNF, in LPS-stimulated human fetal membranes *in vitro* (Lappas et al. 2003). NAC has also been tested clinically in women with a previous PTB who had been treated for bacterial vaginosis. Oral NAC treatment reduced the recurrence of PTB in this small cohort (Shahin et al. 2009). Further human trials are required to validate these results.

NF-κB activation can also be blocked by directly inhibiting the activity of the IκB kinase (IKK) complex. Sulfasalazine (SSZ) is a salicylate drug that is commonly used to treat the pain and swelling associated with rheumatoid arthritis (Weber et al. 2000). SSZ is safe for use in pregnancy (Rahimi et al. 2008). SSZ treatment reduced rates of early delivery in an *E. coli*-induced mouse model of PTB, as well as increasing the litter size and weight of the pups (Nath et al. 2010). Co-treatment of human fetal membranes with LPS and SSZ *ex vivo* inhibited the nuclear translocation of NF-κB and attenuated LPS-induced cytokine accumulation in both the maternal and fetal compartments. However, chronic apoptosis and loss of function of the tissue was observed following SSZ treatment, raising concerns of prolonged treatment with this drug (Keelan et al. 2009).

Novel cytokine suppressive anti-inflammatory drugs (CSAIDs)

CSAIDs inhibit cytokine production by directly targeting the signalling molecules of the NF-κB and p38 MAPK pathways. This makes these agents more specific for reducing inflammation in the context of PTL than other anti-inflammatory drug classes, such as non-steroidal anti-inflammatory drugs (NSAIDs).

The p38 MAPK inhibitor, SKF-86002, reduced PGE_2 gene and protein expression, reduced IL-1β production and reduced *COX-2* expression in LPS-stimulated human fetal membranes *in vitro* (Sullivan et al. 2002). A more specific p38 MAPK inhibitor, SB202190, which acts by competitively inhibiting adenosine triphosphate (ATP) binding, reduced LPS-induced IL-6, TNF, PGE_2 and $PGF_{2\alpha}$ release from human placenta and fetal membranes (Lappas et al. 2007). Another inhibitor, SB239063, inhibited γ-irradiation-killed *E. coli*-induced IL-6, TNF and PGE_2 release from both the maternal and fetal compartments of human fetal membranes (Stinson et al. 2014). However, MAPKs are also involved in cellular functions, such as motility, and

inhibiting this pathway may affect trophoblast migration and differentiation in the placenta (Qiu et al. 2004; LaMarca et al. 2008; Vaillancourt et al. 2009). Furthermore, these drugs are not yet approved for human use.

For classical NF-κB activation, the regulatory subunit NF-κB essential modulator (NEMO) is required in addition to the IKKα and IKKβ subunits. IKKα and IKKβ have NEMO-binding domains, which allows regulation of the IKK complex assembly (Solt et al. 2009). A NEMO-binding domain inhibitor (NBDI) peptide can disrupt the binding of IKKβ and NEMO. In an ovine model, NBDI has been shown to prevent both LPS and *Ureaplasma parvum*-induced production of PGE_2 by the fetal membranes. However, there was no effect of NBDI on inflammatory mediator release from human fetal membranes stimulated by γ-irradiation-killed *E. coli* (Stinson et al. 2014).

Other specific IKKβ inhibitors include TPCA-1 and parthenolide, which were found to inhibit cytokine production in the human choriodecidua when stimulated with LPS with no cytotoxicity side effects (De Silva et al. 2010). Intra-amniotic administration of TPCA-1 in an ovine model of chorioamnionitis reduced PGE_2 production in the amniotic fluid but did not reduce the systemic fetal inflammation induced by LPS (Ireland et al. 2015).

TLR4 antagonists

TLRs are key recognition components of pathogen-associated molecular patterns (Poltorak et al. 1998). TLR4 antagonism is only applicable in cases of PTL that have been induced by Gram-negative bacteria. For example, TLR4 plays a role in LPS-induced PTB in mice and antagonism of this receptor delays PTB and prevents perinatal death in mice (Elovitz et al. 2003; Li et al. 2010; Chin et al. 2016). A TLR4 antagonist also attenuated LPS-induced uterine contractility, as well as reducing IL-8, TNF, PGE_2 and $PGF_{2\alpha}$ release in the amniotic fluid of rhesus monkeys (Adams Waldorf et al. 2008). TLR4 can be antagonised by molecules such as eritoran tetrasodium and TAK-242 (resatorvid) but neither of these have been studied in the context of PTL (Hawkins et al. 2004; Matsunaga et al. 2011). TLR antagonists may be a novel treatment of PTL, in association with antibiotics, to treat intrauterine infection.

TNF biologics

TNF inhibitor drugs are routinely used to treat inflammatory conditions such as rheumatoid arthritis, psoriasis and ulcerative colitis. There are conflicting reports regarding the efficacy of using antibodies against TNF to prevent LPS-induced PTB and improve neonatal outcome in mice (Fidel et al. 1997; Holmgren et al. 2008). There is little evidence of congenital abnormalities following anti-TNF treatment in pregnancy. However, clinical cases suggest that anti-TNF treatments can cross the placenta after the second trimester, reaching the fetal circulation, and are detectable in the neonatal system for weeks after birth. This may increase the risk of infection in theses infants and alter their response to vaccines (Djokanovic et al. 2011; Nielsen et al. 2013). Small levels of anti-TNF drugs have also been detected in breast milk (Gisbert and Chaparro 2013). Therefore, antagonising TNF may not be an ideal method of reducing inflammation in cases of threatened or active PTL.

IL-1 receptor antagonists

IL-1β is a key inflammatory cytokine associated with PTL. It is produced in gestational tissues and is linked to the local upregulation of proteins associated with myometrial contraction. Administration of IL-1 to pregnant mice has be shown to induce PTB (Romero and Tartakovsky 1992). Therefore, a number of studies have investigated the effect of IL-1 receptor antagonists on infection-induced PTB, with varying outcomes.

An early study demonstrated that pre-treatment with an IL-1 receptor antagonist (IL-1Ra) prevented IL-1-induced PTB in mice (Romero and Tartakovsky 1992). However, the IL-1Ra did not prevent PTB in an LPS-induced mouse model (Fidel et al. 1997). In addition, PTB was not prevented in transgenic mice overexpressing the IL-1Ra when labour was induced by IL-1β or LPS (Yoshimura and Hirsch 2005).

Although the IL-1Ra has been unable to prevent PTB in a number of mouse models, there is evidence to suggest that this antagonist has neuroprotective effects. For example, the IL-1Ra prevented LPS-induced fetal mortality, white matter damage and motor behavioural alterations in rats and reduced neurotoxicity in a mouse model of intrauterine inflammation (Girard et al. 2010; Leitner et al. 2014).

Recently, a novel, small, non-competitive IL-1R-biased ligand (rytvela/101.10) was found to delay PTB in mouse models induced by IL-1β, LPS or lipoteichoic acid, without inhibiting NF-κB. This treatment lowered the expression of pro-inflammatory and contraction-associated genes in the uterus. This specific IL-1R-biased ligand reduced the expression of c-jun, a component of the AP-1 transcription factor, and inhibited the Rho/ROCK pathway (Nadeau-Vallee et al. 2015).

These studies emphasise the complexity of cytokine signalling during pregnancy and labour. It is unlikely that the inhibition of one cytokine will be sufficient to inhibit preterm delivery and reduce adverse effects to the fetus. Shynlova et al. (2014) targeted the activation of maternal peripheral immune cells with a broad spectrum chemokine inhibitor in an LPS-induced mouse model of PTB. This treatment reduced immune cell infiltration into maternal tissues, reduced inflammatory mediator secretion and attenuated PTB. This strategy may hold more promise than targeting specific pro-inflammatory mediators.

IL-10 treatment

IL-10 may be a useful therapeutic for reducing the inflammation associated with PTL. It is well documented that IL-10 is an anti-inflammatory cytokine that is capable of suppressing the production of pro-inflammatory mediators, such as IL-1β, IL-6, IL-8, TNF and CCL2 (Couper et al. 2008). IL-10 is expressed at the maternal-fetal interface and is thought to have an immunoregulatory role during pregnancy (Lin et al. 1993; Bennett et al. 1997).

IL-10 has an important role in labour and neonatal outcome, as evidenced by a study using IL-10 null mice. Fetal loss was elicited in IL-10 null mice using a 10-fold lower dose of LPS compared to wild-type mice (Robertson et al. 2006). Fetal growth restriction was observed in more of the surviving fetuses of IL-10 null mice, in comparison to wild-type mice, and an increase in pro-inflammatory mediators was identified in the maternal serum, uterus and placenta. These effects were reversed in the IL-10 null mice by the administration of exogenous IL-10. Interestingly, when IL-10 was administered to the wild-type mice, this prevented LPS-induced fetal loss (Robertson et al. 2006).

In a rat model of LPS-induced fetal growth restriction and fetal loss, IL-10 treatment reduced fetal death, protected against growth restriction and reduced TNF release in the uterus (Rivera et al. 1998). IL-10 treatment prevented PTB and normalised pup weight in another rat model stimulated by LPS. This treatment was effective when given simultaneously with LPS and also when given 24 hours after LPS (Terrone et al. 2001). This anti-inflammatory cytokine may also have neuroprotective effects as IL-10 treatment prevented white matter lesions in pups born to rats inoculated intrauterine with *E. coli* (Rodts-Palenik et al. 2004). This may have been as a result of IL-10 suppressing the activation of microglia or by suppressing macrophage infiltration (Pang et al. 2005). In non-human primates, IL-10 attenuated IL-1β-induced uterine contractions by reducing prostaglandin production and TNF levels were also reduced in the amniotic fluid (Sadowsky et al. 2003). Therefore, exogenous IL-10 treatment may be useful for reducing inflammation in cases of threatened or active PTL. However, additional studies are required to validate these findings.

An alternative to supplementing with just one anti-inflammatory cytokine would be to administer a mediator that is specific to resolving inflammation. Rinaldi et al. (2015a) investigated the use of a key anti-inflammatory and pro-resolution mediator, 15-epi-lipoxin A_4, in an LPS-induced mouse model of PTB. Although PTB was not prevented, 15-epi-lipoxin A_4 reduced rates of fetal death. Therefore, promoting the resolution of inflammation may be a novel method to prevent fetal injury.

As well as effective treatments, improved methods of identifying at risk mothers and fetuses are also required. One strategy that shows promise is the use of new ultrasound techniques, such as Tissue Doppler Imaging (TDI), to identify signs of fetal inflammation (Di Naro et al. 2010; Stock et al. 2016). Genotype may influence the therapeutics used for PTB prevention and treatment and although the evidence to date is limited, there is increasing indication that pharmacogenomics may play a role in PTB. By integrating clinical information, environmental influences and genotype, a more comprehensive strategy to personalised medicine could be optimised (Manuck 2016).

In summary, tocolytic therapies are useful for delaying labour to allow for the administration of corticosteroids but these treatments do not treat the underlying cause

of PTL and there is no benefit to delaying birth if it does not improve neonatal outcomes. Recent clinical trial reports have questioned the efficacy of vaginal progesterone treatment, which did not delay PTB in high risk women or improve the long term health of the premature offspring when assessed at two years of age. In cases of intrauterine infection, it may be appropriate to treat women with antibiotics. However, the addition of an anti-inflammatory therapeutic or CSAIDs may help to reduce the local inflammation and prevent fetal injury. Effective therapies for the prevention and treatment of PTL are urgently required. These must target the underlying mechanisms, such as inflammation. Importantly, they must prevent neonatal morbidity and mortality.

1.2.5 Animal models of PTB

Animal models are crucial for exploring the underlying mechanisms of spontaneous PTB and for the development of therapeutics as it is difficult to perform such studies in humans. As animals rarely experience natural spontaneous PTB, models must be created where PTB is induced, usually by the administration of bacterial agents. Animal models of PTB vary in their physiology, the methods of labour induction and fetal physiology. The advantages and disadvantages of different animal models have been extensively reviewed (Elovitz and Mrinalini 2004; Mitchell and Taggart 2009; Nielsen et al. 2016). This information is summarised below.

1.2.5.1 Sheep

Sheep are a useful model due to their large size. The uterine cavity is similar to that of a human, as is the fetal weight at birth and the gestational length (144-152 days) is more comparable to human gestation than in other animal models, such as rodents. There has also been substantial development of surgical techniques that allow catheters to be implanted into to the fetus *in utero* so that both maternal and fetal serum levels can be monitored during a study. Myometrial contractility can also be assessed directly (Elovitz and Mrinalini 2004). During pregnancy in the sheep, the primary source of progesterone is the corpus luteum, before the placenta takes over, which is comparable to humans. However, sheep do experience progesterone withdrawal, which humans do not, and the placental morphology is very different between these species (Mitchell

and Taggart 2009). Furthermore, sheep are expensive, which means group numbers for studies may be limited (Elovitz and Mrinalini 2004).

In the sheep, the fetus plays a critical role in the timing of parturition. Glucocorticoid concentrations increase exponentially in the fetal plasma in the last month of gestation (Poore et al. 1998). The initial studies highlighting the importance of the fetal pituitary–adrenal axis in pregnancy were performed by Liggins in the 1960s. These studies confirmed that the administration of cortisol or glucocorticoids could induce labour in the sheep (Liggins 1968; Liggins 1969). However, glucocorticoids do not have a major role in parturition in humans and do not induce labour in women.

In recent years, interest has changed from the role of hormones to the role of inflammation in studies of PTB. Consequently, many studies have investigated the effect of intra-amniotic infection on the ovine fetal inflammatory response using inflammatory and infectious agents such as LPS, *E. coli*, *Ureaplasma parvum* and *Candida albicans* (Kallapur et al. 2001; Kramer et al. 2002; Collins et al. 2010; Kemp 2014; Stock et al. 2016). Therefore, the ovine model is very useful for the understanding of fetal response to infection and fetal maturation but this is not considered an ideal model for the study of parturition.

1.2.5.2 Rodents

Rodents are useful models as they are inexpensive and tolerate surgery well (Elovitz and Mrinalini 2004). Unlike humans, they have a very short gestation but this allows for a number of experiments to be performed in a shorter period of time (Mitchell and Taggart 2009). Mice can also be genetically manipulated, which is useful when investigating the mechanisms associated with parturition. In contrast to humans, the corpus luteum is a continuous source of progesterone in rodents and both rats and mice experience progesterone withdrawal, which precedes labour (Mitchell and Taggart 2009). Another dissimilarity with the human physiology is that rodents have a bicornuate uterus. In terms of fetal differences, myelination occurs later in rodents than in humans (Elovitz and Mrinalini 2004). Rodent offspring are less mature than humans at birth and based on some parameters, the murine neonatal period is used to model the third trimester of human fetal development (Clancy et al. 2001). Rats are also more

resistant to inflammatory insult than humans and some studies have failed to reliably induce PTB with LPS or *E. coli* in these animals (Fang et al. 2000; Hirsch et al. 2009).

A number of bacteria and bacterial components have been utilised in the study of infection-induced PTB in mice. For example, LPS, *E. coli*, lipoteichoic acid, *Chlamydia*, *Fusobacterium*, *Ureaplasma*, ligands for TLR2 and TLR3 and bacterial γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) (Nielsen et al. 2016). The anti-progesterone agent RU486 has also been used to induce PTB and investigate non-infectious mechanisms of PTB (Dudley et al. 1996). Other methods of PTB induction in mice include $PGF_{2\alpha}$, SF-A and fetal fibronectin (Nielsen et al. 2016).

1.2.5.3 Non-human primates

As the most similar to humans, non-human primates are very useful when modelling human parturition. Gestational length is similar to that of humans and there is no progesterone withdrawal in maternal serum (Mitchell and Taggart 2009). The placenta is the source of progesterone during pregnancy, as it is in humans, and fetal maturation is also comparable. Catheters can be implanted for continuous monitoring of the fetus and myometrial contractions can be assessed directly (Elovitz and Mrinalini 2004).

Primates are useful for the study of potential tocolytic treatments, as well as investigating the effect of inflammatory cytokines and infectious agents (Gravett et al. 1994; Sadowsky et al. 2003; Sadowsky et al. 2006). For example, PTL can be induced in rhesus monkeys by LPS, IL-1β or TNF. PTL can be inhibited by dexamethasone, IL-10 and TLR4 antagonists in these models (Sadowsky et al. 2006; Adams Waldorf et al. 2008). Although primates are the most relatable to humans, these experiments are costly, they come with difficulties regarding ethics and can be impractical due to the housing and professional care that these animals require.

1.2.5.4 Routes of administration of agents to induce PTB

Infectious or inflammatory agents can be administered systemically or locally in models of PTB. The main systemic route is by intraperitoneal injection to the mother. Intraperitoneal administration of LPS can induce PTB in mice (Robertson et al. 2006; Salminen et al. 2008; Shynlova et al. 2014). However, this model is more representative of conditions such as sepsis or pneumonia. Women experiencing PTL

do not normally have symptoms or evidence of systemic infection. Therefore, animal models of localised infection or inflammation more accurately mimic the human condition.

The administration routes of localised infection/inflammation include intra-amniotic, intrauterine and intravaginal (Gravett et al. 1994; Elovitz et al. 2003; Gonzalez et al. 2011). Elovitz et al. (2004) have discussed the advantages and disadvantages of these methods in some detail. Mouse models of intrauterine infection have been widely utilised in the last decade. This involves a laparotomy procedure where the uterus is removed from the dam and an intrauterine injection of a bacterial agent or an endotoxin, such as LPS, is performed between two amniotic sacs. This procedure has recently been refined by the use of ultrasound to guide the intrauterine injection, establishing a less invasive model (Rinaldi et al. 2015b). This new procedure has now been utilised for the intra-amniotic administration of inflammatory agents (Gomez-Lopez et al. 2016). To date, there have been mixed reports regarding the reliability of intravaginal LPS administration to induce PTB in mice and it is unknown how LPS could circumvent the strong physical and immunological barrier of the cervix (Gonzalez et al. 2011; Gonzalez et al. 2014; Rinaldi et al. 2015b).

In conclusion, PTB is still a significant public health problem. Despite huge advancements in medical research, PTL remains difficult to predict and treatments are ineffective. PTB, in many cases, results in neonatal mortality or a lifetime of varying degrees of morbidity for the offspring. Useful and relatable animal models will be key to development of much needed novel therapeutics that must target the inflammation associated with PTL and thus, prevent fetal injury.

1.3 Statins

Statins are 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors. These drugs are potent inhibitors of cholesterol biosynthesis and reduce the risk of cardiovascular events. Statins also have antioxidant, immunomodulatory, anti-inflammatory and anti-contraction properties. As a result of these pleiotropic effects, statins may be ideal candidates for the treatment for pregnancy disorders. In the following section the development of statins, their pharmacokinetics and their

pleiotropic effects will be discussed. In addition, their application in pregnancy disorders to date and their safely profile will be covered.

1.3.1 Cholesterol

Cholesterol is defined as an alicyclic, unsaturated alcohol, which is present in all cells and is essential for the functioning of all human organs. Cholesterol is a precursor of bile acids, as well as the precursor to many adrenal and steroid hormones. The liver is the principal site of endogenous cholesterol production but cholesterol can also be absorbed from foods that are high in fat (Cornforth and Popjak 1958).

Following its production in the liver, cholesterol is released into the circulation. Lipoproteins transport cholesterol in plasma, delivering it to cells around the body (Fredrickson et al. 1967). Lipoproteins are categorised by density: very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Olson 1998). Two types of lipoproteins are predominantly responsible for carrying cholesterol to and from cells, LDL and HDL. High levels of LDL particles or "bad cholesterol" increase the risk of developing atherosclerosis, which is the leading cause of cardiovascular disease. The LDL receptor is found in coated pits in most cells and is responsible for the endocytosis of LDL into the cell. Free cholesterol is released into the cytoplasm, which inhibits the synthesis of new LDL receptors and inhibits the synthesis of more cholesterol by preventing the production of HMG-CoA reductase (Goldstein and Brown 1977; Olson 1998). In contrast, HDL is anti-atherosclerotic and anti-inflammatory. HDL is often referred to as "good cholesterol" as this particle removes free cholesterol from cells and delivers it to the liver for excretion (Elshourbagy et al. 2014).

1.3.2 Cholesterol biosynthesis: The mevalonate pathway

The mevalonate pathway is a key metabolic pathway that produces isoprenoids. These naturally occurring, organic chemicals are crucial for a multitude of cellular functions, such as cholesterol biosynthesis and electron transport, as well as the synthesis of glycoproteins, intracellular molecules and steroid hormones (Goldstein and Brown 1990).

The main stages of cholesterol biosynthesis are detailed as follows (Figure 1.3). Acetoacetyl-Coenzyme A (CoA) condenses with acetyl-CoA to form HMG-CoA. The enzyme HMG-CoA reductase catalyses the conversion of HMG-CoA into mevalonate, as three acetate units condense to form this six-carbon intermediate. This is the rate-limiting step of the pathway and thus the common site of therapeutic intervention with drugs, such as statins. Mevalonate is then converted into activated isoprene units (Goldstein and Brown 1990). Isoprenoid precursors include isopentenyl pyrophosphate (I-PP), geranyl pyrophosphate (G-PP), farnesyl pyrophosphate (F-PP) and geranylgeranyl pyrophosphate (GG-PP) (Buhaescu and Izzedine 2007). F-PP is the last compound in the isoprenoid metabolic pathway with the potential to be incorporated into either sterol or non-sterol end products (Bradfute and Simoni 1994). F-PP is converted and dimerised to form squalene. Finally, cyclisation of squalene then forms a steroid nucleus, with a final series of changes, including oxidations, removal or migration of methyl groups, to produce cholesterol (Cornforth and Popjak 1958; Bloch 1965; Buhaescu and Izzedine 2007).

Statins also have cholesterol-independent effects due to their inhibition of F-PP and GG-PP synthesis. F-PP and GG-PP play important roles in the post-translational modification of intracellular signalling molecules, such as the small GTPases. This includes Rho, Rac and cell division control protein 42 homolog (Cdc42), which are associated with superoxide production, cell proliferation, cell growth, gene expression, protein glycosylation and remodelling of the actin cytoskeleton (Buhaescu and Izzedine 2007; Wang et al. 2008).

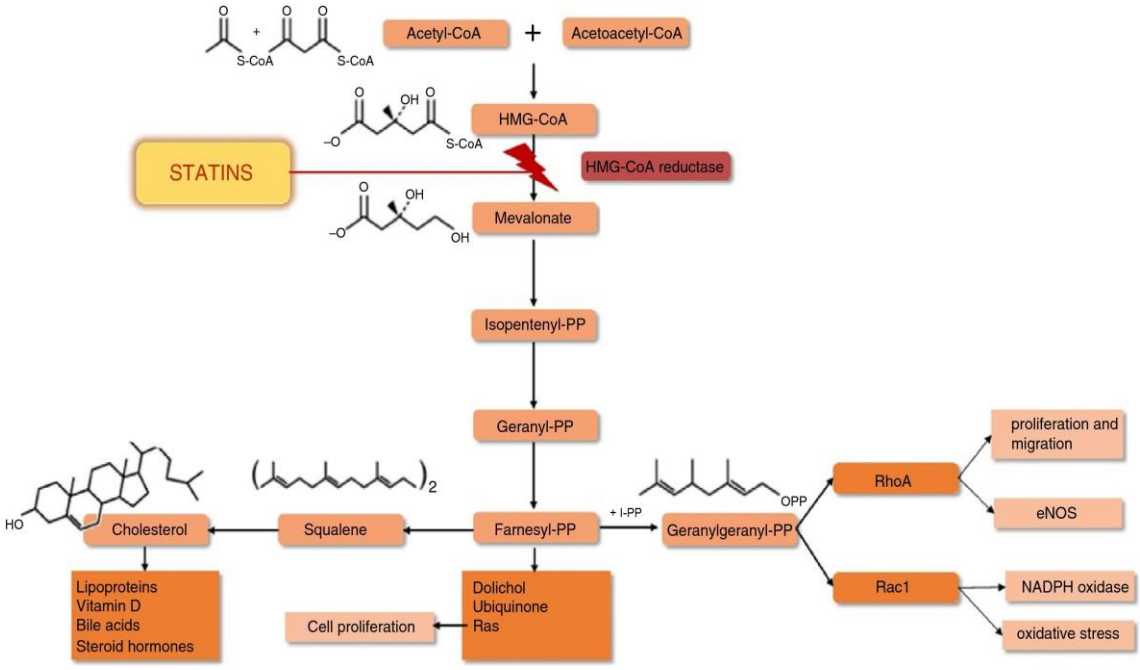

Figure 1.3. The mevalonate pathway. The pathway begins by acetoacetyl-CoA condensing with acetyl-CoA to form HMG-CoA. The conversion of HMG-CoA to form mevalonate, catalysed by HMG-CoA reductase, is the rate-limiting step of this pathway. Statins are competitive inhibitors of HMG-CoA reductase. Cholesterol is synthesised from the isoprenoid component F-PP. F-PP is also the precursor to GG-PP. F-PP and GG-PP are involved in the isoprenylation of small GTPases, such as Rho, Rac and Ras. These intracellular molecules regulate cellular processes, such as proliferation, migration and oxidative stress. eNOS: endothelial nitric oxide synthase. Adapted from Costa et al. (2016).

1.3.3 The discovery of statins

Akira Endo first identified inhibitors of cholesterol biosynthesis in 1976. He derived two compounds, ML-236A and ML-236B, from the *Penicillium citrinum* mold that infects certain Japanese oranges (Endo et al. 1976b). Endo reported that ML-236B competitively inhibited HMG-CoA reductase, the enzyme that is the rate limiting step in cholesterol biosynthesis. ML-236B was capable of inhibiting the activity of HMG-CoA reductase at nanomolar concentrations (Endo et al. 1976a). This compound was later named compactin or mevastatin (Endo 2008).

Endo et al. (1977) reported that cholesterol synthesis was reduced in rodents by the uptake of compactin by the liver. However, compactin did not lower plasma

cholesterol levels in rodents as most circulating cholesterol in these animals is not in the LDL form. In addition, compactin treatment increased hepatic HMG-CoA reductase abundance 8-10 fold, which counteracted the inhibitory effect of the drug. Although, compactin did lower the plasma levels of LDL cholesterol in hens, rabbits and dogs (Endo et al. 1979).

In 1978, Brown et al. demonstrated that compactin could inhibit the activity of HMG-CoA reductase in fibroblasts collected from patients with familial hypercholesterolemia (Brown et al. 1978). In the first human application of compactin, the drug reduced serum cholesterol levels by an average of 27% in 11 patients with primary hypercholesterolemia, over a period of 4-8 weeks (Yamamoto et al. 1980). However, the clinical development of compactin was discontinued later that year. This was due to toxicity concerns following a study in dogs where the drug was believed to have caused lymphomas, albeit at exceptionally high doses (Endo 2010).

Merck Research Laboratories also began screening HMG-CoA reductase inhibitors and isolated a compound with a similar structure to compactin from the fungus *Aspergillus terreus*. This compound was called mevinolin but would later be renamed lovastatin (Alberts et al. 1980; Endo 2010). When lovastatin was given to dogs there was an increase in liver LDL receptors, which subsequently led to a marked reduction in plasma LDL levels (Brown and Goldstein 1981). Further studies by Merck Research Laboratories demonstrated that circulating LDL cholesterol concentration was reduced by lovastatin treatment in healthy men, while HDL concentration was unaffected (Tobert et al. 1982).

Large scale clinical trials administering lovastatin to high risk patients began in 1984. The results demonstrated a dramatic reduction in LDL cholesterol levels with limited side effects. US Food and Drug Administration (FDA) approval was given to lovastatin in 1987 (Endo 2010).

Following the development of lovastatin, six additional statins have been introduced. This includes two semi-synthetic statins, simvastatin and pravastatin, and four synthetic statins, fluvastatin, atorvastatin, rosuvastatin and pitavastatin. Another

synthetic statin, cerivastatin, was discontinued due to reports of myopathy (Endo 2008).

The landmark clinical trial, the Scandinavian Simvastatin Survival Study (1994), established the association between statin-reduced cholesterol levels and the reduction in cardiovascular events. The results of this trial and others have consistently reported that statins lower plasma LDL cholesterol levels by 25-35% and reduce the risk of cardiovascular events by 25-30% (Shepherd et al. 1995).

Statins are the largest selling class of drug worldwide (Endo 2010). The initiation of statin use in the UK increased rapidly from 1995 (0.51 per 1,000 person-years at risk) peaking in 2006 (19.83 per 1,000 person-years). This declined by 2013 (10.76 per 1,000 person-years). The prevalence of prescriptions increased hugely from 1995 (2.36 per 1,000 person-years) to 2013 (128.03 per 1,000 person-years) (O'Keeffe et al. 2016).

1.3.4 Pharmacokinetics of statins

Due to uncertainties regarding the efficacy of lovastatin, semi-synthesised statins were developed. Lovastatin had an additional methyl group added to its structure to create simvastatin (Figure 1.4). This increased the hydrophobicity of the molecule, leading to more potent inhibition of HMG-CoA reductase (Mol et al. 1986). The differences in chemical structures of statins influence their absorbance, distribution, metabolism and their excretion from the body (Schachter 2005). Lovastatin and simvastatin are both inactive prodrugs due to their closed lactone ring structures. These molecules are mainly activated in the liver or, occasionally, in the gastrointestinal tract (Sirtori 2014). Then came the development of the semi-synthetic molecule, pravastatin, which was the first statin to have an open ring structure (Yoshino et al. 1986). The synthetic statins, fluvastatin, atorvastatin, rosuvastatin and pitavastatin, also have open ring structures that are all in the classical position of the mevalonic acid-analog moiety (Sirtori 2014).

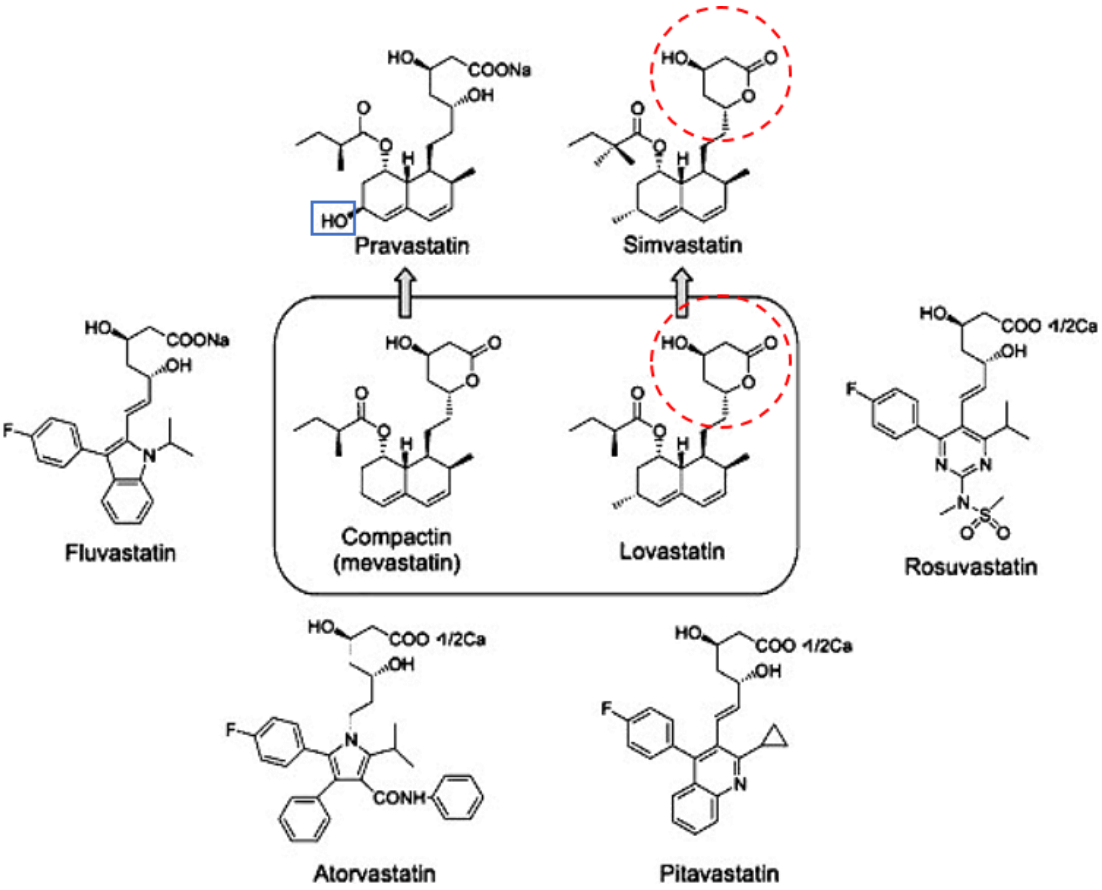

Figure 1.4. Statin structures. The original statins, compactin and lovastatin, were modified to produce the semi-synthetic statins, pravastatin and simvastatin. The synthetic statins, fluvastatin, atorvastatin, pitavastatin and rosuvastatin, were created later. Lovastatin and simvastatin have closed lactone ring structures (red circles), whereas the other statins have open ring structures. Pravastatin contains a polar hydroxyl group (blue rectangle), which makes this compound very hydrophilic. The other statins are more lipophilic. Adapted from Sirtori (2014).

Statins inhibit the activity of HMG-CoA reductase by binding to its active site, sterically preventing HMG-CoA from binding to the enzyme. The hydrophobic compounds of statins block the HMG-binding pocket, as well as a portion of the CoA-binding site. Statins bind tightly to HMG-CoA reductase due to a larger number of van der Waals interactions, with a much higher affinity than HMG-CoA (Istvan and Deisenhofer 2001). The binding properties of the individual statins may account for their potency. For example, rosuvastatin has the most binding interactions with HMG-CoA reductase. Clinical trials have suggested that rosuvastatin is the most effective statin for reducing LDL cholesterol, followed by atorvastatin, simvastatin then

pravastatin. Simvastatin and pravastatin are the most effective statins for increasing the percentage of HDL cholesterol, which they increase by an average of 12% (Schachter 2005).

Statins have both hydrophilic and hydrophobic components to their structure. Simvastatin, fluvastatin and atorvastatin are highly lipophilic and can readily enter cells by directly interacting with the plasma membrane. Rosuvastatin exhibits intermediate behaviour, due to a hydrophilic methane sulphonamide group. Pravastatin is hydrophilic, as it contains a polar hydroxyl group, and requires selective uptake into cells by a sodium-independent organic anion transporter protein-1B1 (OATP1B1). This transporter protein is exclusively expressed in the liver and so entry into other cells types is limited (Hamelin and Turgeon 1998; Corsini et al. 1999). The most important property of statins is their hepatoselectivity given that most endogenous cholesterol production takes place in the liver. The hydrophilic statins, pravastatin and rosuvastatin, are more hepatoselective than lipophilic statins (Schachter 2005).

Most of the statins are metabolised initially in the liver, lowering systemic bioavailability. These statins are metabolised by the cytochrome P450 (CYP) system, most commonly by the highly active CYP3A4 enzyme. In contrast, pravastatin and rosuvastatin undergo minimal metabolism due to their hydrophilic structure (Sirtori 2014). Pravastatin is degraded in the stomach, independently of the CYP enzymes, and is excreted by the kidneys and liver (Schachter 2005).

The bioavailability of statins differs widely. Lovastatin and simvastatin have low bioavailability of 5%. Pitavastatin's bioavailability is much greater at 60%. Pravastatin has a bioavailability of 18%. All statins, with the exception of pravastatin, have high binding affinity to plasma proteins. Therefore, systemic exposure to unbound, pharmacologically active drug is low. Active pravastatin levels in the circulation are higher than the other statins but uptake into other tissues is limited, due to its hydrophilic structure. The elimination half-life of lovastatin and the semi-synthetic statins, pravastatin and simvastatin, range from 1-3 hours. The half-lives of the synthetic statins, atorvastatin, fluvastatin, pitavastatin and rosuvastatin, range from 1 hour for fluvastatin to 19 hours for rosuvastatin (Schachter 2005).

Therefore, the differing chemical structures and hydrophobicity of statins can influence their potency, as well as their absorbance, distribution, metabolism and their excretion from the body. These factors should be considered when selecting a statin for therapeutic use.

1.3.5 Pleiotropic effects of statins

Over the last decade, clinical trial outcomes have suggested that statins have benefits beyond their lipid lowering properties. Statins have been shown to be immunomodulatory, anti-inflammatory, anti-proliferative, apoptotic and can reduce oxidative stress (Kavalipati et al. 2015). These effects have been widely reviewed (Liao and Laufs 2005; Wang et al. 2008; Zhou and Liao 2010; Kavalipati et al. 2015).

As the lipophilic statins are more likely to enter non-hepatic cells than the hydrophilic statins, it could be hypothesised that these statins may have more pleiotropic benefits than the hydrophilic statins. However, studies suggest that the hydrophilic statins, such as pravastatin, also exert similar pleiotropic effects. Therefore, it has now been proposed that these effects may be mediated by HMG-CoA reductase inhibition in the liver, which subsequently reduces circulating isoprenoid levels. This hypothesis may explain how hydrophilic statins can have beneficial effects in cells that these drugs can not directly enter (Zhou and Liao 2010).

1.3.5.1 Prevention of endothelial dysfunction and oxidative stress

The endothelium, the inner lining of blood vessels, responds to environmental factors and maintains haemostatic balance by regulating blood vessel contraction and leukocyte trafficking. Endothelial dysfunction is characterised by the increase of reactive oxygen species (ROS), which leads to a reduction in the protective molecules, endothelial nitric oxide synthase (eNOS) and nitric oxide (NO).

Statins reduce endothelial dysfunction and oxidative stress in patients with hyperlipidaemia and metabolic syndrome (Thallinger et al. 2005; Alber et al. 2007; Murrow et al. 2012). Statins have this effect independently of LDL cholesterol reduction. This was addressed in patients with chronic heart failure who were prescribed simvastatin or ezetimibe, a cholesterol absorption inhibitor. Both treatments

lowered LDL cholesterol levels to a similar degree. However, simvastatin treatment increased the number of functionally active endothelial progenitor cells and increased the activity of the enzyme that catalyses the dismutation of superoxide radicals, which was examined as a marker of oxidative stress (Landmesser et al. 2005). In a similar comparison study, simvastatin and ezetimibe again lowered LDL cholesterol levels to the same extent in patients with coronary artery disease but simvastatin also improved vasodilator function (Fichtlscherer et al. 2006).

The antioxidant effect of statins may be due to the inhibition of the small GTPase Rac. Statins can reduce oxidative stress and vascular remodelling in the context of cardiac hypertrophy by inhibiting the isoprenylation of Rac (Brown et al. 2006). Atorvastatin treatment reduced ROS production in rat aortic vascular smooth muscle cells that had been stimulated with angiotensin II, a peptide hormone that causes vasoconstriction. The mRNA expression and abundance of the angiotensin receptor was also reduced. Atorvastatin inhibited the activity and membrane translocation of Rac1 (Wassmann et al. 2001). Simvastatin treatment inhibited angiotensin II-induced rat cardiomyocyte hypertrophy, reduced Rac1 activity and lowered the production of the superoxide anion, suggesting simvastatin was having an antioxidant effect via Rac1 inhibition. This effect was reversed by supplementation with mevalonate and GG-PP but not by F-PP or cholesterol (Takemoto et al. 2001). Both pravastatin and atorvastatin inhibited Rac1 activity in myocardial explants from patients with chronic heart failure (Maack et al. 2003). These studies highlight the importance of statin-induced Rac inhibition for reducing oxidative stress.

Statins regulate eNOS through multiple pathways, for example the Rho/ROCK pathway as RhoA negatively regulates eNOS expression and activity. Statins increase the stability of eNOS mRNA, by inhibiting Rho geranylgeranylation, which leads to increased expression of eNOS (Laufs and Liao 1998). Statins can also regulate eNOS through the serine-threonine protein kinase, Akt. Statins activate this protein in endothelial cells by upregulating phosphatidylinositol-3 kinase (PI3K) signalling, which promotes the phosphorylation of eNOS and also increases angiogenesis (Kureishi et al. 2000). The inhibition of the Rho/ROCK pathway also activates the PI3/Akt pathway (Wolfrum et al. 2004). Alternatively, statins can promote eNOS

activity by reducing the expression of caveolin-1, which is a membrane protein that directly binds to eNOS in caveolae and inhibits its activity (Pelat et al. 2003).

Statins induce the production of heme oxygenase 1 (HO-1), which is a stress response protein that is essential for heme catabolism. HO-1 cleaves heme to form biliverdin and carbon monoxide. Biliverdin is then converted to the antioxidant, bilirubin. As well as having antioxidant effects, HO-1 has anti-inflammatory effects, it can prevent the development of atherosclerosis in mice and has been upregulated by statin treatment in mouse models of preeclampsia and PTB (Juan et al. 2001; Hinkelmann et al. 2010; Leung et al. 2011; Gonzalez et al. 2014; Ramma and Ahmed 2014).

1.3.5.2 Immunomodulation

Previous studies have suggested that statins may mediate their anti-inflammatory and anti-atherosclerotic effects by inhibiting CCL2 expression, which could consequently affect monocyte chemotaxis (Martinez-Gonzalez et al. 2001; Kleemann et al. 2003; Veillard et al. 2006). Atorvastatin, simvastatin and fluvastatin suppressed human monocyte chemotaxis and these statins also reduced CCL2 expression in human aortic endothelial cells by inhibiting JAK/STAT signalling cascades. These effects were both blocked by mevalonate and GG-PP but not F-PP, suggesting they were cholesterol-independent (Jougasaki et al. 2010). Conversely, in animal models of inflammatory skin conditions and wound healing, the topical application of simvastatin has been shown to reduce inflammation by promoting macrophage infiltration to the site of injury (Adami et al. 2012; Asai et al. 2012). This suggests that statins have complex effects on immune regulation.

Simvastatin treatment can inhibit major histocompatibility complex class II (MHC II) expression and subsequent T-cell activation. MHC II is upregulated in inflammatory conditions such as myocarditis, multiple sclerosis and rheumatoid arthritis. For antigens to be presented on cell surfaces, changes in the actin cytoskeleton are required to facilitate endocytosis of the antigen, internal processing and presentation of the MHC II molecules. Small GTPases control the actin cytoskeleton and statins inhibit the isoprenylation of these proteins (Kwak et al. 2000; Kwak et al. 2001). Other small

GTPases, Cdc42 and Rac, play a role in dendritic cell antigen presentation to T-cells (Nobes and Marsh 2000).

Statin treatment mediates a shift from Th1 to Th2 phenotype, producing anti-inflammatory effects. This Th2 bias is characterised by an increase in Th2 cytokines, such as IL-4, IL-5 and IL-10, and an inhibition of Th1 cytokine secretion, such as IFN-γ and TNF (Youssef et al. 2002; Aprahamian et al. 2006). In a mouse model of inflammatory arthritis, simvastatin suppressed Th1-related immune responses and IFN-γ release from mononuclear cells (Leung et al. 2003). In another study, atorvastatin stimulated the differentiation of Th2 cells, reduced immune cell infiltration into the central nervous system and inhibited the expression of MHC II in mice with autoimmune encephalomyelitis. This work highlights the potential for statin treatment for other Th1-mediated autoimmune diseases (Youssef et al. 2002). Th17 cells have also been identified as an important target of statins when modulating immune responses in autoimmune diseases such as multiple sclerosis. Simvastatin had a Th17-polarising effect on cytokine secretion from dendritic cells isolated from multiple sclerosis patients (Zhang et al. 2013).

Statins alter circulating Tregs in humans, with simvastatin and pravastatin increasing Treg circulation in hyperlipidaemia patients (Mausner-Fainberg et al. 2008). Atorvastatin increased the frequency and modulation of Tregs in a mouse model of acute allergic asthma, which was associated with reduced immune cell infiltration into the lungs and increased IL-10 production (Blanquiceth et al. 2016). Statins have also been found to have inflammation-independent immunomodulatory effects as lovastatin and atorvastatin increased the frequency of circulating CD4+ FOXP3+ Tregs in heathy males (Rodriguez-Perea et al. 2015). Overall, these immunomodulation properties aid the anti-inflammatory effects of statins.

1.3.5.3 Anti-inflammatory properties

The anti-inflammatory effects of statins were first identified in cases of atherosclerosis and cardiovascular disease. Atherosclerosis is an inflammatory process, which is characterised by leukocyte recruitment, excessive pro-inflammatory mediators and lipid accumulation. Statins have been shown to exert anti-inflammatory effects on the

vascular wall in cases of atherosclerosis and the resulting cardiovascular diseases (Libby 2002).

C-reactive protein (CRP) is a non-specific marker of inflammation and is also an independent predictor of cardiovascular disease. The PRINCE trial was an important development in the recognition of statin-mediated anti-inflammatory effects. This trial investigated the effect of pravastatin treatment on plasma levels of CRP. After 24 weeks of treatment, CRP levels had reduced both in patients with cardiovascular disease and in the control subjects who had no history of cardiovascular disease (Albert et al. 2001). The JUPITER randomised trial concluded that rosuvastatin treatment reduced CRP levels and reduced the overall incidence of major cardiovascular events in healthy individuals with elevated CRP but without hypercholesterolemia (Ridker et al. 2008). This anti-inflammatory effect was observed in a number of other studies performed in subjects with type 2 diabetes, carotid artery plaques and hypercholesterolemia (Crisby et al. 2001; Kalela et al. 2001; Solheim et al. 2001; Sommeijer et al. 2004). Furthermore, in hypercholesteraemic patients, serum IL-6, IL-8 and CCL2 were all reduced following 6 weeks of simvastatin treatment (Rezaie-Majd et al. 2002).

To investigate the anti-inflammatory effects of statins further, healthy volunteers inhaled LPS to induce acute lung inflammation. Pre-treatment with simvastatin attenuated LPS-induced TNF, MMP-7, MMP-8, MMP-9 and CRP production in the bronchoalveolar lavage fluid of the volunteers. LPS-induced CRP levels were also reduced in the plasma of simvastatin-treated subjects. LPS stimulated the nuclear translocation of NF-κB in monocyte-derived macrophages, which was inhibited by simvastatin pre-treatment (Shyamsundar et al. 2009).

Arguably, the quintessential example of an excessive innate immune response is sepsis. In a cecal ligation and puncture (CLP) mouse model exhibiting polymicrobial sepsis, both pre-treatment and post-treatment with statins substantially prolonged survival and reduced the adhesion of leukocytes to endothelial cells (Merx et al. 2004; Merx et al. 2005). Simvastatin pre-treatment also improved CLP-induced acute kidney injury, prevented renal vasculature damage and hypoxia, as well as reducing systemic inflammation (Yasuda et al. 2006). Randomised, controlled trials have not found a

reduction in pro-inflammatory markers with acute statin treatment of patients with sepsis compared to placebo (Kruger et al. 2011; Kruger et al. 2013). However, previous statin treatment is associated with lower baseline IL-6 concentration and continued statin treatment improves survival rates in patients with sepsis (Kruger et al. 2013). Previous statin therapy is also associated with a reduced risk of bacterial infections and statin users who do develop bacterial infections have a reduced hospital stay and a lower mortality rate compared to non-statin users (Nassaji et al. 2015). Statin therapy has also been associated with reduced mortality in patients with viral infections, most likely because statins reduce their inflammatory response (Vandermeer et al. 2012). However, it has been argued that these results reflect bias from "healthy user" effects as statin users are less likely to have co-morbidities and may practice more healthy behaviours (van den Hoek et al. 2011). Recent meta-analyses and systematic reviews have reported that the effects of statins on infection are still inconclusive (Tralhao et al. 2014; Shrestha et al. 2016).

A multitude of *in vitro* studies have investigated the anti-inflammatory effects of statins. Simvastatin treatment reduced the synthesis of inflammatory mediators from various cell types in culture, such as human umbilical vein endothelial cells, human primary macrophages, vascular smooth muscle cells and synoviocytes collected from patients with rheumatoid arthritis (Rezaie-Majd et al. 2002; Dichtl et al. 2003; Veillard et al. 2006; Xu et al. 2006). Simvastatin lowered the pro-inflammatory cytokine TNF and increased IL-10 production from macrophages, as well as significantly reducing IκBα degradation and NF-κB translocation *in vitro* (Leung et al. 2011). Pravastatin significantly reduced LPS-stimulated IL-6, IL-8 and GM-CSF protein production in human bronchial epithelial cells (Iwata et al. 2012). Pravastatin concentration-dependently reduced CRP, IL-6 and TNF at the gene and protein level in rat vascular smooth muscle cells. Pravastatin also downregulated the release of pro-inflammatory mediators, TNF and CCL2, from human monocytes (Grip et al. 2000).

Statins have differential effects on the inflammatory response. For example, an *in vitro* study assessed the ability of six statins to inhibit LPS-induced NF-κB binding activity in human monocytes. All statins reduced the phosphorylation and degradation of IκB with cerivastatin being the most potent, followed by atorvastatin, simvastatin,

pravastatin, lovastatin then fluvastatin. This should be taken into account when selecting a statin with the aim of anti-inflammatory benefits (Hilgendorff et al. 2003).

Statin administration has also been associated with the induction of pro-resolving lipid mediators with strong anti-inflammatory properties, such as 15-epi-lipoxin-A4. Atorvastatin increased the production of 15-epi-lipoxin-A4 levels in the rat myocardium (Birnbaum et al. 2006). In a mouse model of airway mucosal injury and inflammation, lovastatin treatment increased the production of 15-epi-lipoxin-A4 and consequently, reduced lung inflammation (Planaguma et al. 2010). Lovastatin and simvastatin also stimulated the biosynthesis of 15-epi-lipoxin-A4 during interactions between human neutrophils and airway epithelial cells *in vitro* (Planaguma et al. 2010).

Although the mechanisms by which statins have anti-inflammatory effects are not fully elucidated, they are thought to involve the inhibition of protein isoprenylation in the mevalonate pathway (Wang et al. 2008).

1.3.5.4 Anti-contraction properties

Recent studies have demonstrated that statins have anti-contractile effects. Most of these studies have focused on the cardiovascular system, where statins have been shown to reduce the contractile response of vascular smooth muscle cells *in vitro* (Kuzuya et al. 2004; Mraiche et al. 2005; Perez-Guerrero et al. 2005; Nasu et al. 2009; Gonzalez et al. 2014). Statins also have a relaxant effect on rat aortic rings (Mraiche et al. 2005; Sonmez Uydes-Dogan et al. 2005; Rossoni et al. 2011; Alp Yildirim et al. 2016; Chen et al. 2016).

This anti-contractile effect has been reported in other cell types, such as endometriotic stromal cells and lung fibroblasts (Nasu et al. 2009; Ra et al. 2011). In addition, organ bath studies have reported anti-contractile effects of simvastatin. For instance, pre-treatment with simvastatin reduced the frequency of spontaneous and C5a-induced contraction of human myometrial samples, as well as in myometrial tissue collected from simvastatin-treated mice (Gonzalez et al. 2014). Rosuvastatin induced relaxation in cardiac veins isolated from calf hearts (Nurullahoglu-Atalik et al. 2015).

These properties have been associated with mevalonate-related pathways, more specifically, the inhibition of Rho geranylgeranylation and the subsequent inhibition of ROCK activation (Mraiche et al. 2005; Sonmez Uydes-Dogan et al. 2005; Nagaoka et al. 2007; Alp Yildirim et al. 2016). The anti-contraction effect has also been demonstrated further downstream of the Rho/ROCK pathway as statin treatment reduces the phosphorylation of MLC, which is crucial to facilitate smooth muscle contraction. Zeng et al. (2005) reported that simvastatin inhibited the contraction of glomerular endothelial cells by preventing RhoA activation and subsequent MLC phosphorylation. Lovastatin treatment prevented endothelial cell motility *in vitro* due to Rho inactivation and actin depolymerisation (Xiao et al. 2012). Similar studies have been performed in multiple cell types, which have demonstrated that the mechanism by which statins exert their anti-contraction pleiotropic effects is by inhibiting Rho/ROCK, thus preventing MLC phosphorylation (Alvarez de Sotomayor et al. 2001; Mraiche et al. 2005; Kuhlmann et al. 2006; Nagaoka et al. 2007; Kidera et al. 2010; Wu et al. 2011; Xiao et al. 2012; Alp Yildirim et al. 2016).

Furthermore, statins can alter Ca^{2+} flux, which may be another mechanism by which statins affect contractility. Simvastatin altered the excitation-contraction coupling of rat skeletal muscle fibres by increasing cytosolic Ca^{2+} levels (Pierno et al. 1999). Fluvastatin and atorvastatin impaired the release of Ca^{2+} from the SR in rat skeletal muscle fibres resulting in elevated resting cytosolic Ca^{2+} concentration, which altered Ca^{2+} homeostasis (Liantonio et al. 2007). One study found that simvastatin impaired vascular contractility by modifying the influx of Ca^{2+} and disturbing RhoA activity (Kang et al. 2014). Simvastatin relaxed rat superior mesenteric artery rings *in vitro*, which was associated with the opening of voltage-dependent K^{+} channels and the blocking of extracellular Ca^{2+} influx (Chen et al. 2016). Simvastatin, atorvastatin, fluvastatin and rosuvastatin also had antagonistic effects on voltage-gated Ca^{2+} channels, inhibiting the contraction of gastrointestinal tissue in an organ bath study (Ali et al. 2016).

1.3.5.5 Anti-proliferative and pro-apoptotic properties

Statins can inhibit cell proliferation and induce cell apoptosis. These pleiotropic affects have proved beneficial in the development of cancer therapies. Recently, it was

reported that long term statin use is associated with a reduced risk of many haematological malignancies (Pradelli et al. 2015). A large scale study assessing the relationship between statin use and cancer-related mortality in the Danish population found reduced cancer-related mortality with statin use (Nielsen et al. 2012). Statin users also have a lower risk of breast cancer recurrence and there is a reduced risk of malignancy-related death in post-menopausal women (Sakellakis et al. 2016; Wang et al. 2016). Furthermore, statin treatment is associated with reduced cancer-related mortality in prostate cancer patients (Raval et al. 2016).

Statins exert pro-apoptotic, anti-proliferative, anti-inflammatory and immunomodulatory effects, which may prevent tumour growth and metastasis (Pisanti et al. 2014). Statins have pro-apoptotic effects in cancer cell lines, but some cell lines are more sensitive than others. This treatment can affect angiogenesis by downregulating vascular endothelial growth factor (VEGF) and preventing endothelial cell proliferation (Vallianou et al. 2014).

Studies have demonstrated anti-proliferative and pro-apoptosis effects in cancer of the breast, colon, prostate, lung and liver with statin treatment. These effects were attributed to statins inhibiting small GTPases, such as RhoA, and pathways such as PI3K/Akt. Anti-invasion and anti-migration effects have been reported in breast, prostate, renal and colon cancer. In addition, atorvastatin and lovastatin were associated with overcoming drug resistance to chemotherapeutic drugs in lung cancer xenograft models (Pisanti et al. 2014).

To summarise, statins have a number of properties in addition to lowering LDL cholesterol concentration. They can treat endothelial dysfunction, they have anti-oxidative stress properties as well as immunomodulatory, anti-inflammatory and anti-contraction properties. Furthermore, they have recently been investigated as a treatment for a multitude of cancers as a result of their anti-proliferative and pro-apoptotic properties.

1.3.6 Statin treatment for obstetric complications

Of late, animal and human studies have described the use of statins to prevent pregnancy complications, such as intrauterine growth restriction (IUGR),

antiphospholipid syndrome, recurrent miscarriage, preeclampsia and PTB (Redecha et al. 2008; Redecha et al. 2009; Gonzalez et al. 2014; Costantine et al. 2016; Lefkou et al. 2016).

Simvastatin and pravastatin treatment prevented fetal loss and IUGR in a mouse model of antiphospholipid-induced pregnancy loss. The expression of tissue factor, a crucial mediator in antiphospholipid-induced pregnancy loss, was downregulated on neutrophils collected from statin treated mice. Statin treatment also prevented neutrophil activation and reduced ROS production, preventing placental oxidative damage (Redecha et al. 2008).

Pravastatin treatment also prevented pregnancy loss in a model of recurrent spontaneous miscarriage generated by mating CBA/J and DBA/2 mice. Pravastatin prevented fibrin deposition in the placentas of these mice and reduced tissue factor expression, which led to reduced plasma levels of the angiogenic molecule soluble fms-like tyrosine kinase (sFlt-1) in these mice. This restored placental blood flow and prevented oxidative stress by increasing NO levels (Redecha et al. 2009). This CBA/J x DBA/2 mouse model has also been suggested as a model of preeclampsia (Ahmed 2011).

Preeclampsia is one of the major causes of maternal and neonatal morbidity. Factors contributing to this syndrome are thought to include an imbalance of angiogenic factors, such as VEGF, placental growth factor (PGF), sFlt-1 and soluble endoglin (sENG), resulting in insufficient trophoblast invasion into the uterus and remodelling of the spiral arterioles. This causes placental hypoxia, endothelial dysfunction and the release of inflammatory mediators and vasoactive factors that induce maternal effects, such as hypertension and proteinuria (Costantine et al 2010).

One study reported that simvastatin reduced VEGF-induced sFlt-1 release from normal, term placental villous explants. Simvastatin also reduced VEGF-induced sFlt-1 release and upregulated HO-1 in human umbilical vein endothelial cells *in vitro*. This suggested a potential role for statins in the treatment of preeclampsia (Cudmore et al. 2007). Brownfoot et al. (2016) investigated the effect of simvastatin, rosuvastatin and pravastatin on sFlt-1 and sENG secretion on primary human umbilical vein endothelial

cells, trophoblast cells and preterm preeclamptic placental explants. Simvastatin was the most effective inhibitor of sFlt-1 secretion in these three models. All statins increased sENG secretion from endothelial cells and upregulated HO-1. This increase in sENG is concerning as sENG contributes to the pathogenesis of preeclampsia. However, reassuringly, sENG secretion was not increased in the placental explants following statin treatment (Brownfoot et al. 2016).

In a *sFlt-1* overexpression mouse model of preeclampsia, pravastatin lowered serum sFlt-1 levels and improved vascular function when the carotid arteries of these mice were subjected to *in vitro* reactivity experiments (Costantine et al. 2010). Kumasawa et al. (2011) developed a unique model of preeclampsia by transplanting blastocysts transduced with a lentiviral vector expressing human *sFLT-1* into pseudopregnant mice, producing a placenta-specific expression system. This model exhibited hypertension, proteinuria and IUGR. Pravastatin ameliorated these preeclampsia-like symptoms and induced both placental and non-placental *Pgf* expression, which may have aided the prevention of endothelial dysfunction (Kumasawa et al. 2011).

Pravastatin treatment also has fetal protective effects in mouse models of preeclampsia. The offspring from a *sFlt-1* overexpression model displayed poor vestibular function, balance and coordination. These side effects were prevented when preeclamptic mothers received pravastatin during gestation (Carver et al. 2014). In this model, offspring also exhibited delayed growth and pravastatin normalised this (McDonnold et al. 2014).

Two clinical trials have investigated the effect of pravastatin for the prevention and treatment of preeclampsia; one trial involved women at high risk of preeclampsia and the other trial was assessing women with early onset preeclampsia.

The first study performed a randomised controlled trial to investigate the safety and pharmacokinetics of pravastatin treatment in 20 women; 10 treated with pravastatin and 10 treated with placebo. Women at high risk of preeclampsia were treated with 10mg/day pravastatin between 12 and 16 weeks gestation until delivery. Four subjects out of ten in the placebo group developed preeclampsia, whereas none of the ten subjects in the pravastatin group developed preeclampsia. The placebo group also had

five indicated preterm deliveries compared to only one in the pravastatin group. Maternal cholesterol levels were reduced by the treatment but the levels of cholesterol in the umbilical cord were unaltered and birthweights were not different between groups. No differences in drug side effects or congenital malformations were found between the pravastatin and placebo group. Overall, no safety risks were identified with this treatment (Costantine et al. 2013; Costantine et al. 2016).

The second study, a proof of concept trial, investigated the effect of 40mg/day pravastatin treatment on early onset preeclampsia when prescribed between 24 and 31+6 weeks gestation. This trial assessed the ability of pravastatin to restore angiogenic balance by measuring adverse biomarkers associated with preeclampsia in the maternal circulation, as well as collecting safety data. Recruitment of women for this trial is complete. However, the results are still to be reported (Statins to Ameliorate early onset Preeclampsia (StAmP); www.controlled-trials.com/ISRCTN23410175).

Pravastatin treatment also improves pregnancy outcomes in women with anti-phospholipid syndrome. Eleven women who were receiving conventional antithrombotic treatment for antiphospholipid syndrome were prescribed pravastatin on the diagnosis of preeclampsia or IUGR. Women treated with both pravastatin and antithrombotic treatment exhibited increased placental blood flow and preeclamptic features were improved, compared to subjects receiving antithrombotic treatment only. The addition of pravastatin treatment resulted in live births that occurred close to term in all subjects. Importantly, there were no fetal abnormalities reported (Lefkou et al. 2016).

Statins have also demonstrated anti-inflammatory effects in a mouse model of systemic maternal infection. When pregnant mice were treated with simvastatin prior to an intraperitoneal injection of LPS, *Il-1β* and *Il-6* mRNA expression were reduced in the uterus, as well as *Il-6* and *Tnf* in the cervix, compared to LPS only treated mice (Basraon et al. 2012). A wide range of LPS-induced inflammatory cytokines were also reduced in the maternal serum by the simvastatin treatment. Pravastatin treatment reduced LPS-induced *Il-1β* and *Il-6* mRNA expression in the uterus and cervix, respectively and serum levels of IL-1β and GM-CSF were also reduced (Basraon et al. 2012).

Pravastatin and simvastatin treatment prevented PTB in a mouse model of ascending infection. The authors described a reduction in MMP activity and collagen I content in the cervix, indicating that statin treatment prevented cervical remodelling. They also reported a reduction in complement-induced contraction of mouse and human myometrial strips with simvastatin and pravastatin treatment (Gonzalez et al. 2014). However, the total sample size of mice in this study was relatively small, with group numbers of 5-7 mice per treatment, and the mechanisms of action of statins were not fully elucidated. In addition, the key role of inflammation was not addressed in these studies. A similar study using the same PTB model found that statins had neuroprotective properties following fetal exposure to LPS. This treatment reportedly prevented fetal brain cortical abnormalities, evidenced by a reduction in caspase-3 staining, and LPS-induced cortical neuron disruption was ameliorated. These effects were associated with the Akt/PKB signalling pathway (Pedroni et al. 2014).

Pravastatin also displayed fetal protective effects in a mouse model of glucocorticoid overexposure by treating placental vascular defects. In mice lacking 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), there was a reduction in umbilical blood velocity and the fetuses exhibited signs of cardiac dysfunction. Pravastatin treatment increased the vascularisation, increased the placental fetal capillary volume and improved umbilical cord velocity. These effects resulted in normalised fetal weight and improved fetal cardiac function. This suggests that statin treatment may be a useful therapeutic for fetal growth restriction as it may improve the development of the placental vasculature (Wyrwoll et al. 2016).

1.3.7 The safety profile of statins

In general, statins have few serious side effects and are relatively well tolerated. The most reported side effect of statin use is myalgia, in which rates vary from 1-10% of users. The most serious side effect is rhabdomyolysis, which occurs in less than 0.1% of users (Ramkumar et al. 2016). Some statin users report side effects such as muscle weakness, muscle pain or aching, stiffness, muscle tenderness, cramps and arthralgia, which implies that statins may induce myotoxicity. However, the prevalence of statin-induced myotoxicity has been debated due to unclear definitions of the condition,

clinical reports are hugely variable and large randomised clinical trials are not always representative of clinical practice (du Souich et al. 2017).

The risk factors for statin-induced myopathy are hypothyroidism, drug-drug interactions, reduced activity of liver membrane transporters and conditions such as mitochondrial damage or increased ROS. Polypharmacy is an issue with statin treatment due to a number of therapeutics interacting with the CYP enzyme group. Inhibitors of CYP enzymes may lead to an elevation of statin levels, which increases the risk of toxicity. Common causes of interactions include gemfibrozil, a fibrate that also lowers lipids and interacts with CYP3A4 enzyme, and grapefruit juice, which potently inhibits CYP3A4. Conversely, anti-epileptic drugs are inducers of the CYP3A4 enzyme, which can reduce statin concentration and therefore, their effectiveness. As pravastatin is metabolised independently of the CYP system, it does not have many drug interactions (Ramkumar et al. 2016).

A recent meta-analysis concluded that while high doses of statins reduce the risk of cardiovascular disease, they could increase the risk of type 2 diabetes. These differential metabolic effects cannot currently be explained and further research must be performed to fully understand these additional statin effects (Lim et al. 2014). However, the benefits of cardiovascular protection and reduced mortality with statin treatment outweigh the risk of developing type 2 diabetes (Castro et al. 2016).

Overall, statin treatment is considered to be safe for most people. Statins should be prescribed with caution to those with multiple co-morbidities. In cases of polypharmacy, pravastatin may be the ideal statin as it has fewer drug interactions.

1.3.7.1 Statin use in pregnancy

Statins are defined as category X by the FDA for use in pregnancy and are generally contraindicated for pregnancy use worldwide. Currently, safety data obtained during pregnancy comes from women using statins during the early weeks of an unidentified or unplanned pregnancy.

The FDA categorisation was based on early animal studies that demonstrated teratogenic potential with the use of exceptionally high concentrations of statins

(Minsker et al. 1983). However, the fetal skeletal abnormalities in these studies have been attributed to maternal toxicity-induced nutritional deficiency rather than teratogenic effects (Lankas et al. 2004). There are also concerns that statin treatment may affect cholesterol biosynthesis in the developing fetus. In addition, the inhibition of isoprenoids and other factors involved in intracellular signalling are associated with the insulin-like growth factor system. This system is necessary for placental development and its disruption could also contribute to adverse outcomes (Forbes et al. 2008; Forbes et al. 2015).

Most teratogenic evidence is associated with the older, more commonly prescribed, lipophilic statins lovastatin and simvastatin. There is limited information on the effects of pravastatin and the newer statins, rosuvastatin and pitavastatin, in pregnancy (Godfrey et al. 2012).

A retrospective observational study by Edison and Muenke (2004) reported that there was an increase in fetal limb defects after simvastatin exposure during the first trimester. The authors associated these defects with defective cholesterol metabolism. However, these findings have since been criticised and challenged by larger studies, which found that there was no greater risk when comparing to population baseline levels of fetal malformations (Gibb and Scialli 2005; Petersen et al. 2008; Bateman et al. 2015).

The most recent evidence comes from a well-designed epidemiological study performed by Bateman et al. (2015). This study assessed pregnancy and childbirth in data collected from a large cohort of 1152 women in the USA between 2000 and 2007. They assessed the risk of major congenital malformations, as well as organ specific malformations, in the offspring of women who used statins during the first trimester of pregnancy. High density propensity scoring was used to control for confounding factors, such as maternal medical condition, particularly diabetes, the use of other medication, as well as demographic factors. The unadjusted results suggested a slight increase in the risk of congenital malformation with first trimester statin use. However, this risk disappeared once confounders were taken into account. There was no increased risk of organ specific malformations found. This analysis was performed on low income women and so cannot be generalised. However, as these women are at

greater risk of poor pregnancy outcomes, these results can also be regarded as reassuring for those from higher income surroundings (Haramburu et al. 2015). A limitation of such a study is that it relies on treatment compliance, as the criteria of "statin use" is based only on dispensed prescriptions. There was also no information on issues during pregnancy, such as medical terminations and miscarriages, or long term effects (Bateman et al. 2015).

As statins are not prescribed during pregnancy, there is no safety data on their use later in gestation when treatments for pregnancy disorders are likely to be given. Furthermore, absence of risk is more difficult to prove. Therefore, more safety information is required. Pilot trials for pravastatin use in cases of preeclampsia are beginning to address this on a small scale, with group numbers of 10-11 women in these studies (Costantine et al. 2016; Lefkou et al. 2016). However, it is still too early to assess the long term effects of this exposure. Obstetric complications, such as PTB and preeclampsia, can result in life-long morbidity as well as maternal and neonatal mortality. Therefore, it can be argued that the possible benefits of statin treatment outweigh the risks in these instances.

In conclusion, statins are well-tolerated drugs that potently inhibit the synthesis of cholesterol. These drugs also prevent the development of cardiovascular disease. Statins vary in their structure and this affects their potency, absorption and metabolism. All statins competitively inhibit the HMG-CoA reductase enzyme within the mevalonate pathway, inhibiting cholesterol production, as well as the synthesis of isoprenoids. This prevents the isoprenylation of small GTPases, which are intracellular signalling molecules responsible for cellular processes, such as proliferation, cell growth and remodelling of the actin cytoskeleton. Therefore, statins have anti-inflammatory, immunomodulatory, antioxidant and anti-contraction effects. For this reason, statin treatment has been suggested for a range of inflammatory and autoimmune diseases and more recently as a cancer therapy. Statins show promise for the treatment of pregnancy disorders, such as IUGR, miscarriage, preeclampsia and antiphospholipid syndrome. One study has also suggested the use of statins to prevent PTB. However, this requires further investigation. Statin use is currently

contraindicated in pregnancy but large epidemiological studies have not found a teratogenic effect. Further safety investigations are required to confirm these findings.

1.4 Summary

This chapter firstly addressed the role of inflammation in labour. Term labour is an inflammatory process in which immune cells are primed in the circulation and infiltrate the intrauterine tissues. There is an increase in pro-inflammatory mediators, such as cytokines, chemokines and prostaglandins, at the maternal-fetal interface. These mediators initiate labour by stimulating myometrial contractions, cervical ripening and fetal membrane rupture. Uterine stretch and fetal signalling may also play a role in the initiation of labour. However, when this inflammatory cascade begins earlier in gestation this can lead to the pathological, preterm initiation of the labour process. PTL is difficult to predict and current treatments are ineffective at preventing PTB. As PTB is the leading cause of mortality in children, treatments must aim to protect the fetus from injury and improve neonatal outcome. Although the aetiology of PTL is unclear, the majority of cases have been associated with infection and inflammation. Tocolytic drugs do not substantially delay delivery and they do not improve the outcome of the premature babies. In addition, the efficacy of vaginal progesterone application has recently been questioned. Anti-inflammatory agents may be key to the successful prevention and treatment of PTL but many of these compounds are only at the early stage of testing and are not approved for human use. Drug development is extremely expensive and time-consuming and few drugs successfully complete the process. Many of these limitations may be overcome by repurposing a drug that is already FDA-approved. Thus, statin treatment, with its many pleiotropic effects, may be an ideal therapeutic for PTL.

Statins are used to reduce cholesterol levels and prevent cardiovascular disease. By inhibiting the mevalonate pathway, statins also inhibit the isoprenylation of small GTPases, which are involved in a multitude of cellular functions. The result is that statins are capable of regulating the immune system, reducing inflammation and also inhibiting the contraction of cells. These properties suggest statins could potentially target the underlying causes of PTL as well as inhibiting myometrial contraction.

Human studies have already reported the success of statin treatment in cases of preeclampsia. Therefore, statin therapy could be a novel approach for treating PTL.

1.5 Hypothesis and aims

The work presented in this thesis was based on the hypothesis that statins will prevent PTB by reducing inflammation.

In order to address this hypothesis, the aims of the thesis were as follows:

- To investigate the effect of statins on inflammation in the reproductive tract *in vitro*
- To examine the effect of statins on the contractility of the myometrium in a cell line
- To investigate the mechanisms by which statins affect either contractility or inflammation in PTL
- To determine whether statins can delay/prevent PTB or improve neonatal outcome in an LPS-induced mouse model of PTB.

Chapter 2: Materials and Methods

2.1 Myometrial cell culture

2.1.1 Pregnant human myometrial 1-41 (PHM1-41) cell line

PHM1-41 cells are commercially produced myometrial smooth muscle cells, which were isolated from a pregnant, non-labouring woman at 39 weeks gestation. The cells were immortalised and selected by resistance to Geneticin (G418) by Monga et al. (1996). The cells were originally selected for smooth muscle cell morphology but they were not derived by limiting dilution cloning. Therefore, this PHM1-41 myometrial cell line may be a polyclonal population of cells. These cells are morphologically and phenotypically similar to cultured primary myometrial cells and have previously been used for studies investigating gene expression, ion channel activity, oxytocin signalling pathways and contraction-associated pathways (Monga et al. 1996; Hutchinson et al. 2014; Rajagopal et al. 2015; Makieva et al. 2016). The cell line was purchased at passage 12 and used up to passage 22 (PHM1-41 ATCC® CRL-3046™, LGC Standards, Teddington, UK). In the experiments outlined in this chapter, the PHM1-41 myometrial cells were cultured in 4.5g/L high glucose Dulbecco's Modified Eagle Medium (DMEM; BE12-709F, Lonza, Slough, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Paisley, UK), 1% Penicillin/Streptomycin (P/S; Sigma-Aldrich, Poole, UK), 1% L-Glutamine (Sigma-Aldrich) and 0.1mg/ml G418 sulfate (Geneticin, Gibco, Life Technologies). During experiments, G418 sulfate was omitted from the culture medium. Partial serum starvation was carried out in 4.5g/L high glucose DMEM supplemented with 5% FBS, 1% P/S and 1% L-Glutamine. All incubations were carried out at 37°C, in a humidified, 5% carbon dioxide (CO_2) atmosphere.

2.1.2 Sub-culturing myometrial cells

To sub-culture the PHM1-41 myometrial cells, medium was removed and cells were washed twice with sterile phosphate buffered saline (PBS; Life Technologies). Trypsin-Ethylenediaminetetraacetic acid (EDTA; Lonza) was added to the flask (Sarstedt, Nümbrecht, Germany) and incubated for 2-3 minutes, until cells detached. Cells were collected in 10% FBS DMEM, with 1% P/S, 1% L-Glutamine and

0.1mg/ml G418, to neutralise the trypsin-EDTA and cells were counted using a haemocytometer. The cell suspension was centrifuged at 300 x g for 5 minutes. Cells were then resuspended in complete culture medium and seeded at 1-3x10^6 cells/T75 flask or 2-4x10^6 cells/T162 flask. Medium was changed every 2 days until the cells were confluent.

2.1.3 Mycoplasma testing

Before beginning a new set of experiments, the myometrial cells were routinely monitored for mycoplasma, using the MycoAlert™ Mycoplasma Detection Kit (Lonza). This assay detects the presence of enzymes released from lysed mycoplasma, which react with the MycoAlert™ substrate, catalyzing the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). The ATP level is measured before and after MycoAlert™ substrate addition. Elevated ATP levels signify the presence of mycoplasmal enzymes. Mycoplasma testing was performed by Dr Forbes Howie (Specialised Assay Service, University of Edinburgh).

Cells were seeded at 1.5x10^5 cells/ml (3x10^5 cells in 2ml) in 6-well plates and cultured in 10% FBS DMEM, omitting P/S and G418, for 48 hours. An aliquot of medium was removed and centrifuged at 200 x g for 5 minutes, to remove cell debris and 100µl of supernatant was collected for analysis. The MycoAlert™ reagent was then added in an equal volume of 100µl and the samples were incubated for 5 minutes at room temperature. The first luminescence reading was then taken on a FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany). Then, 100µl MycoAlert™ substrate was added to the sample, followed by a 10 minute incubation at room temperature. The luminescence was then read again and the ratio between the two readings was calculated. Ratios greater than 1.2 suggested mycoplasma contamination.

2.2 Treatment preparation

Pravastatin (P4498; Sigma-Aldrich) was dissolved directly in sterile PBS and stored in a stock solution of 5mg/ml at 4°C for up to 1 month. A simvastatin (S6196-25MG; Sigma-Aldrich) stock solution was prepared by dissolving 4mg of the drug in 100µl of ethanol (EtOH) and 150µl of 0.1N sodium hydroxide (NaOH) and incubated at 50°C for 2 hours. The stock solution was adjusted to pH7 with 5N hydrogen chloride (HCl).

The volume was made up to 1ml using sterile PBS, which was filter sterilised using a 0.22µm filter (Millipore, Billerica, MA, USA) and stored at 4°C for up to 1 month. Lipopolysaccharide (LPS; *E. coli* 0111:B4, L3024, Sigma-Aldrich) was reconstituted in sterile PBS, aliquoted and stored at -20°C. (±)- Mevalonolactone (M4667; Sigma-Aldrich) was diluted 1 in 10 in EtOH to produce a stock solution, as required. Farnesyl pyrophosphate ammonium salt (F-PP; F6892, Sigma-Aldrich) and Geranylgeranyl pyrophosphate ammonium salt (GG-PP; G6025, Sigma-Aldrich) were purchased in solutions of 2500µM and 2000µM, respectively, in methanol (CH_3OH). Mevalonate, F-PP and GG-PP were all stored at -20°C. All treatment dilutions were performed with sterile PBS to achieve final treatment concentrations.

2.3 Pravastatin and simvastatin treatment of myometrial cells

Cells were seeded at $1.5x10^5$ cells/ml ($3x10^5$ cells in 2ml) in 6-well plates and then partially serum-starved in DMEM containing 5% FBS, 1% P/S and 1% L-Glutamine, for 24 hours at 37°C. Cells were stimulated with 100ng/ml LPS and treated in duplicate either simultaneously with pravastatin (10µM) or simvastatin (0.1µM, 10µM, 50µM), or pre-treated or post-treated with pravastatin/simvastatin (Figure 2.1) and incubated for 24 hours at 37°C. Control wells were included with PBS only, LPS (100ng/ml) only and pravastatin (10µM) or simvastatin (10µM) only, performed in duplicate.

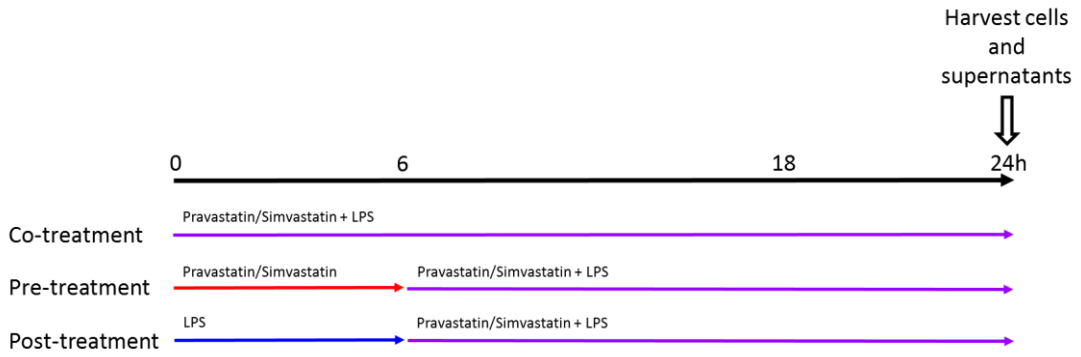

Figure 2.1. Statin and LPS treatments of myometrial cells. Cells received one of three treatment regimens, each with a total incubation time of 24 hours. LPS and statins were either co-administered or cells were first pre-treated with statins (6 hours) or received statin treatment 6 hours post-LPS stimulation.

2.4 Myometrial cell image capture

Images of the cells were captured following 24 hours of co-treatment, pre-treatment and post-treatment of statins and LPS, to assess any effects on cell morphology. Image capture was performed using a Leitz Labovert microscope with a x10 objective lens and a Zeiss AxioCam ICc1 camera. Imaging was performed using Zeiss ZEN (2011) software.

2.5 MTT metabolic activity assay

Cell metabolic activity was assessed for all treatments by measuring the ability of myometrial cells to metabolise 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich). The metabolism of yellow tetrazolium MTT by living cells results in purple formazan, which can be solublised and quantified by a spectrophotometer. The cells were seeded at 10^4 in a 96-well plate and treated with pravastatin (1, 10, 100µM), simvastatin (0.1, 1, 10, 25, 50, 100µM), LPS (25, 100ng/ml), mevalonate (200µM), GG-PP (10µM) or F-PP (10µM) for 24-48 hours, in triplicate. MTT solution was applied for the final 4 hours of the 24-48 hour treatments, at 37°C. The medium was removed and 100µl acidified isopropanol was added for 20 minutes, then light absorbance was the measured at 540nm (LabSystems Multiskan EX).

2.6 Collagen gel contraction assay

2.6.1 Collagen gel preparation

A collagen gel contraction assay was utilised to assess the effect of simvastatin on the contraction of myometrial cells and was performed as previously described by our laboratory (Hutchinson et al. 2014). The collagen utilised was a kind gift from Dr Alex Henke (Shire Pharmaceuticals, Lexington, MA, USA), which was prepared from rat tails, as previously described (Bell et al. 1979). Briefly, frozen rat tails were thawed in 70% EtOH for 20 minutes. The tendons were then removed, minced and placed in dilute acetic acid (250ml/tail) for 48 hours, with gentle agitation, at 4°C. The resulting solution was centrifuged at 14, 000 x g for 1 hour. The supernatant was removed and this crude collagen solution was refined by the addition of 0.1M NaOH in a 6:1 ratio. This neutralised the acetic acid and caused the collagen to precipitate. The mixture was

centrifuged at 300 x g for 5 minutes, the supernatant was discarded and an equal volume of fresh acetic acid was added to resolubilise the collagen. This solution was stored at 4°C until required for the collagen gel contraction assay. Simvastatin concentration response experiments used the remainder of this collagen solution. Therefore, all other experiments were performed using commercially available Type I rat tail collagen (2ml; A1048301, ThermoFisher Scientific, Hemel Hempstead, UK).

2.6.2 Collagen gel casting and contraction assay

Myometrial cells were washed twice in PBS and trypsinised, then resuspended in 10% FBS DMEM with 1% P/S and 1% L-Glutamine at $1x10^6$ cells/ml. Non-treated 24-well plates were utilised, to prevent cell adherence (3738, Costar®, Corning, NY, USA). NaOH (0.1M) was prepared in water (H_2O), sterile filtered using a 0.22µM filter and syringe, and stored at 4°C until required. To seed a full non-treated 24-well plate, 2.4ml of the cell suspension was transferred to a fresh, chilled tube on ice and 5.6ml medium was added (10^5 cells/well). To the cells, 1.2ml 0.1M NaOH was added and the cell suspension was divided evenly. The following steps were carried out quickly on ice. Rat tail collagen (either the crudely extracted or commercially available type I rat tail collagen) was added to each tube in a volume of 2ml, pipetting gently to mix, while avoiding bubbling, to prevent polymerisation. The collagen cell suspension (500µl) was added to a 24-well plate, working vertically, up and down columns for symmetric distribution. The collagen gels were allowed to polymerise overnight, incubated at 37°C. The collagen gels were then partially serum-starved for 2 hours with medium containing 5% FBS, 1% P/S and 1% L-Glutamine. The collagen gels were gently detached and treatments were added in 6 technical replicates. Treatments included simvastatin (0.1, 1, 10, 25, 50, 100µM), LPS (25, 50, 100, 200, 300, 400ng/ml), mevalonate (100, 200µM), GG-PP (10, 20, 40µM) and F-PP (10, 20, 40, 80, 100µM). The plates were incubated at 37°C and photographed at 0, 24 and 48 hours using a Leica MZ6 light microscope/camera (Mayfair, UK) on the ICD and 2x2 colour binning settings. Images were captured using Leica Firecam software (v3.4.1). ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to measure collagen gel area.

2.7 In-Cell Western assay

In-Cell Western analyses (Figure 2.2) were performed to quantify pMLC in myometrial cells, as previously described (Hutchinson et al. 2014). In brief, cells were seeded at $2x10^4$ cells/well in a volume of 200µl 10% FBS DMEM with 1% P/S and 1% L-Glutamine in black 96-well, clear base plates (6005225; Perkin Elmer, Waltham, MA, USA) and incubated overnight at 37°C. The cells were partially serum starved with 5% FBS DMEM containing 1% P/S and 1% L-Glutamine for 2 hours, then treatments (Figure 2.2) were applied in triplicate for 48h, at 37°C. Following incubation, treatments were removed by inverting the plates and cells were fixed with 4% formaldehyde (Sigma-Aldrich) for 15 minutes at room temperature. The fixative was removed and the cells were washed and permeablised 3 times with 0.1% Triton X-100 (BDH Laboratory Supplies, Poole, UK) in PBS for 5 minutes on a rocker at room temperature. Plates were blocked with Odyssey® blocking buffer (PBS) (P/N 927-40000; LI-COR Biosciences, Lincoln, NE, USA) for 1 hour at room temperature. Primary antibodies of interest (Table 2.1) were diluted in Odyssey® blocking buffer then applied in a volume of 50µl/well, followed by an overnight incubation at 4°C. The plates were then washed in 0.5% PBS TWEEN® 20 (PBST), 3 times for 15 minutes, on a rocker at room temperature. The secondary antibodies (Table 2.1) were diluted in Odyssey® blocking buffer and 50µl/well was added for 2 hours at room temperature, in the dark. The plates were washed again, as before with PBST, in the dark. The Odyssey® CLx Imaging System (LI-COR Biosciences) was used to read the plates and measure the signal in each well. The intensity of pMLC fluorescence was quantified relative to α-Tubulin within the same well.

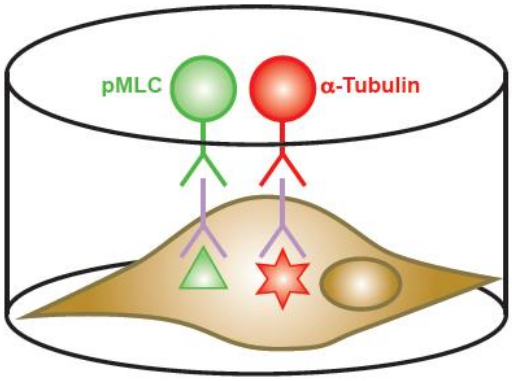

Figure 2.2. Schematic representation of the In-Cell Western assay. Cells were treated with simvastatin (10μM, 50μM) and supplemented with LPS (25ng/ml), mevalonate (200μM), GG-PP (10μM) or F-PP (10μM), then fixed and incubated with primary antibodies for pMLC and α-Tubulin, secondary LI-COR antibodies and then fluorescence was read.

Target	**Primary antibody**	**Supplier**	**Secondary antibody**	**Supplier**
α-Tubulin	Monoclonal mouse anti-α-Tubulin (1:5000)	Sigma-Aldrich (T9026)	Polyclonal donkey anti-mouse 680RD (1:10,000)	LI-COR Biosciences (926-68072)
pMLC	Polyclonal rabbit anti-pMLC 2 (Ser19) (1:200)	Cell Signaling Technology, MA, USA (3671)	Polyclonal donkey anti-rabbit 800CW (1:10,000)	LI-COR Biosciences (926-32213)

Table 2.1. Primary and secondary antibodies for In-Cell Western analyses

2.8 Mouse model of preterm birth

2.8.1 Animal studies

All animal studies were conducted under a UK Home Office licence to Professor J.E. Norman (70/8927) in line with the Animal Scientific Procedures Act (1986). To carry out procedures, all researchers were required to have a personal licence and maintain up-to-date training records on procedures. All new practical work was reviewed with the Named Training and Competency Officer (NTCO) and a Named Veterinary Surgeon (NVS) prior to commencing any training. Practical training was carried out under the supervision of an appropriate trainer until deemed competent to perform the procedure unsupervised. All new experiments were reviewed by the NVS before mice were ordered from Charles River Laboratories (Margate, UK). The principles of "the three Rs", reduction, replacement and refinement, were adhered to as far as possible. Annual Returns of Procedures were completed and sent to the Home Office providing details of procedures carried out, the severity of the procedures and the number of animals used.

Animals were acclimatised for 10 days prior to timed-mating, while housed in groups of 5 or 6 and provided with food and water. Timed-matings and plug checks were carried out by animal house technician, Mike Dodds. Gestational day one (D1) was designated when the vaginal plug was found. After procedures were performed, the pregnant mice were housed separately and monitored for signs of delivery. Temperature (19-23°C) and humidity (~55%) were tightly controlled, with constant light/dark cycles (12 hours/12 hours).

Animals were sacrificed in accordance with Schedule 1 (Animals Scientific Procedures Act, 1986). Dams were sacrificed by the inhalation of rising concentrations of CO_2 into a sealed chamber. Death was confirmed by cervical dislocation, using a blunt instrument, or exsanguination, where the blood was drained. Pups were sacrificed by cervical dislocation.

2.8.2 Ultrasound procedure

The ultrasound procedure was performed by Mr Adrian Thomson (Preclinical Ultrasound Facility, University of Edinburgh, PIL: IC1F2EA12), as described by

Rinaldi et al. (2015b). On gestational D17, mice were randomly assigned to the treatment groups, then anesthetised with inhalation of isoflurane (5% for induction, 1.5% for maintenance in oxygen) and positioned supine on the ultrasound stage. Abdominal hair was first clipped, then removed with depilatory cream. Warm ultrasound gel was then applied to the abdomen. Scans were performed with the Vevo 770 high-frequency ultrasound scanner (FUJIFILM VisualSonics, Inc., Toronto, ON, Canada) with a RMV 707B probe (center frequency, 30 MHz). Temperature and heart rate were monitored throughout all procedures (Figure 2.3).

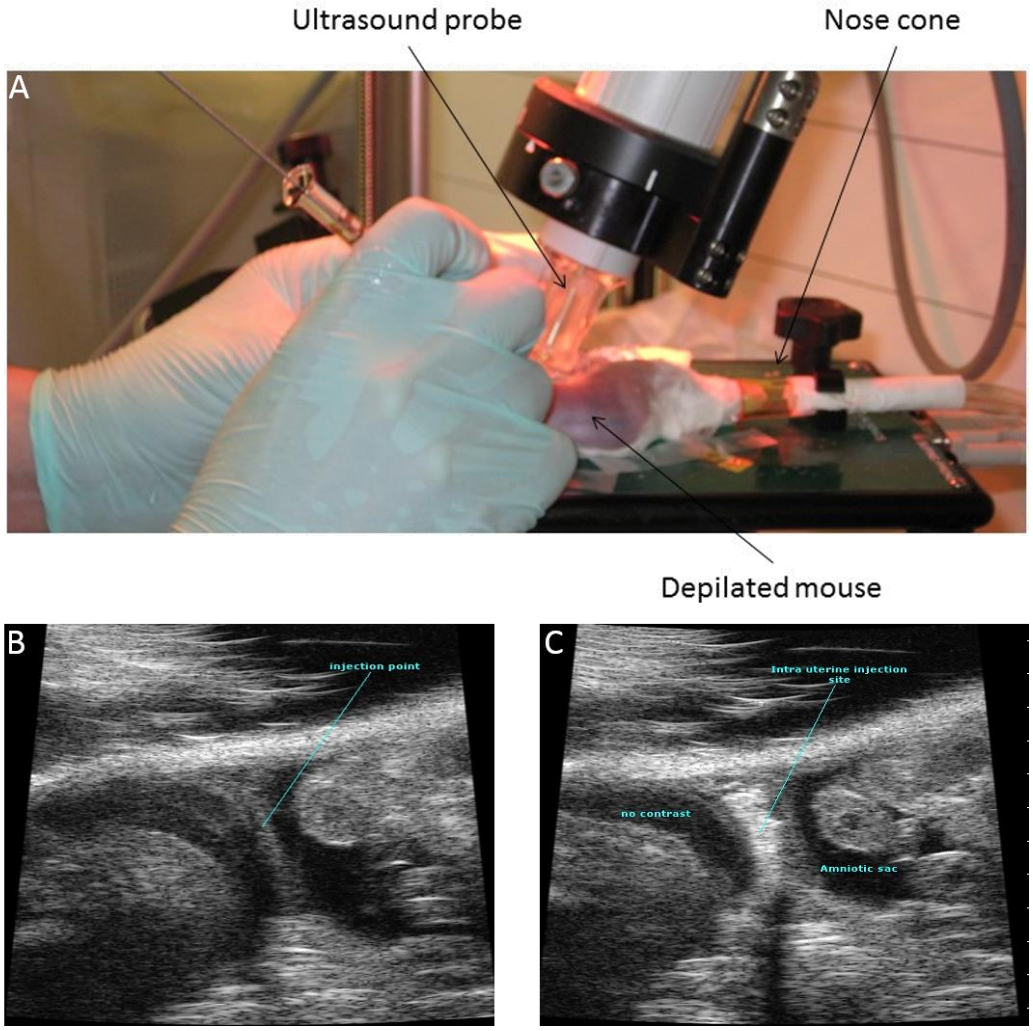

Figure 2.3. Ultrasound-guided intrauterine LPS-induced PTB mouse model. (A) An example of a CD1 mouse lying supine on the ultrasound stage, receiving isoflurane through the nose cone, as the ultrasound probe guides the intrauterine injection. (B) An ultrasound image showing two amniotic sacs and the injection point in the intrauterine space. (C) Contrast agent was injected into the intrauterine space, between two amniotic sacs, to show feasibility. Images curtesy of Adrian Thomson or adapted from Rinaldi et al. (2015b).

2.8.3 LPS dose response trial for the ultrasound-guided intrauterine LPS-induced PTB mouse model

Firstly, ultrasound was used to count the number of viable pups, then used to guide injection of LPS (0.3µg, 1µg, 3µg, 10µg, 15µg, 20µg in 25µl PBS; from *E. coli* 0111:B4) or PBS (25µl) directly into the uterine lumen, between 2 gestational sacs, using a 33-gauge Hamilton syringe (Figure 2.3). Care was taken not to enter the amniotic cavity. Mice were kept at 30°C until recovered from surgery, then transferred to individual cages to be continuously monitored using individual closed-circuit television cameras and a digital video recorder. Time to delivery was recorded as the number of hours from the time of intrauterine injection of LPS/PBS to the delivery of the first pup. The number of live/dead pups was recorded within 24 hours of their delivery. The percentage of live born pups per litter was calculated by dividing the number of live pups found within 24 hours of delivery by the number of viable pups counted via ultrasound on D17.

2.8.4 Statin treatment of the LPS-induced PTB mouse model

On gestational D16, mice were restrained by grasping the scruff of the neck between the thumb and forefinger, while maintaining a grip on the tail, and given an intraperitoneal injection of simvastatin (20µg or 40µg in 200µl PBS), pravastatin (10µg in 200µl PBS) or PBS (200µl), using a sterile 0.5ml BD Micro-Fine Ultra™ 0.3mm x 8mm (30 gauge) pen needle (Oxford, UK). This was performed by positioning the needle parallel to the line of the thigh, pushing through the centre of the posterior quadrant of the abdomen into the peritoneal cavity. By positioning the needle along the line of the leg, this avoids the bladder in the posterior abdomen and the liver anteriorly. Penetration was shallow so that only the tip of the needle entered the peritoneal cavity. These intraperitoneal injections were performed by Dr Heather MacPherson (PIL: I27C06758). Mice were then anaesthetised on D17 of pregnancy by the inhalation of isoflurane and ultrasound was used to confirm the number of viable fetuses. Following this, ultrasound was utilised to guide injection of LPS (1µg or 20µg in 25µl PBS) or PBS (25µl) into the uterus, between 2 gestational sacs. Two hours later, the mice received another intraperitoneal treatment of pravastatin/simvastatin or PBS, administered as on D16 (Figure 2.4). Animals were allowed to recover from the

anaesthesia and were monitored for signs of labour and delivery of pups. All animals were randomly assigned to each group. Cage cameras were used to monitor animals remotely with time to delivery and the number of live/dead born pups recorded, as described above.

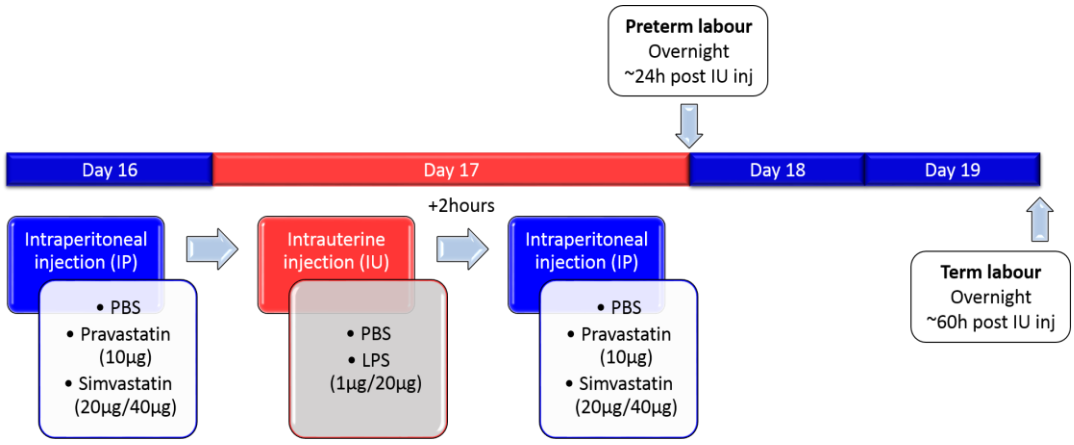

Figure 2.4. Statin and LPS treatments for the PTB mouse model. Gestational day one (D1) was designated when the vaginal plug was found. On gestational D16, mice received an intraperitoneal injection of PBS, pravastatin or simvastatin. D17, either PBS or LPS was administered, as a control or to instigate PTB, respectively. This was followed by another intraperitoneal treatment 2 hours later.

2.8.5 Tissue collection and storage

In a separate cohort of mice, tissues and fluids were harvested 6 hours after intrauterine injection of PBS/LPS (Table 2.2). Dams were sacrificed by the inhalation of CO_2, followed by exsanguination. Approximately 1ml of maternal blood was taken from the vena cava using a 25 gauge needle and 1ml syringe and allowed to clot in a 1.5ml Eppendorf for 1-2 hours at room temperature. The blood was then centrifuged at 2000 x g for 20 minutes and the serum aliquoted and stored at -80°C. Maternal serum was also collected from another cohort of mice for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. These mice received one intraperitoneal injection of either 20µg simvastatin, 40µg simvastatin or PBS on gestational D17. Serum was collected 1 hour and 2 hours after treatment.

Amniotic fluid was collected in a 1.5ml Eppendorf from each gestational sac using a pasteur pipette and pooled, resulting in one sample per dam. The amniotic fluid was stored on wet ice, then centrifuged at 8000 x g for 10 minutes at 4°C, aliquoted and stored at -80°C.

As the location of the intrauterine injection site was unknown due to the ultrasound procedure, uterus, placenta and fetal membrane samples were collected from both the left and right horn of each mouse. When analysing mRNA concentration, the average of the right and left horn tissues was taken per mouse. The cervix was cut in half to provide one sample for gene expression and one for histology. Fetal heart, lung and brain samples were also collected. Fetal plasma was collected in capillary blood collection tubes (Microvette® CB 300 µl, K2 EDTA, Sarstedt) and centrifuged at 2000 x g for 5 minutes. The plasma was stored at -80°C. Samples taken for mRNA expression analysis were collected in RNA*later* (Sigma-Aldrich). These samples were kept at 4°C overnight, then the RNA*later* was removed and the samples were stored at -80°C until required. Samples for protein analysis were immediately frozen on dry ice, then stored at -80°C until required. Samples taken for histology were collected in neutral buffered formalin (NBF, Sigma-Aldrich) and then changed to EtOH (70%) after 24 hours.

Tissue/fluid	**Gene expression (qRT-PCR)**	**Protein production (ELISA/Western blotting/LC-MS/MS)**	**Histology (Immuno-histochemistry)**
Maternal serum	-	✓	-
Fetal plasma	-	✓	-
Amniotic fluid	-	✓	-
Cervix	✓	-	✓
Uterus left/right horn	✓	✓	✓
Placenta left/right horn	✓	✓	✓
Fetal membranes left/right horn	✓	✓	✓
Maternal liver	✓	✓	✓
Maternal lung	✓	✓	✓
Whole fetus left/right horn	-	-	✓
Fetal heart	✓	✓	✓
Fetal lung	✓	✓	✓
Fetal brain	✓	✓	-

Table 2.2. Samples collected 6 hours after intrauterine injection of LPS/PBS (n=6/treatment group)

2.9 RNA extraction and reverse transcription

2.9.1 RNA extraction from cells

Cells were lysed using Buffer RLT (Qiagen Ltd., Maryland, USA) with beta-mercaptoethanol (β-ME; Sigma-Aldrich) and further homogenised by repeated passage through a blunt 20-gauge needle (0.9mm diameter; BD Microlance™) in an RNase-free syringe (BD Plastipak™). An RNAeasy mini kit (Qiagen) was used to extract total RNA with the solutions supplied. EtOH (70%) was added to the homogenised lysate (v/v) and mixed well by pipetting. The sample was added to an RNeasy spin column within a 2ml collection tube and centrifuged for 15 seconds at 8000 x g (10, 000 rpm). Columns were washed with 350µl Buffer RW1 by centrifugation for 15 seconds at 8000 x g (10, 000 rpm) to remove carbohydrates and proteins. DNase digestion, to eliminate genomic DNA contamination, was performed by adding 10µl DNase I stock, in 70µl Buffer RDD (Qiagen), directly onto the spin column membrane for each sample and these were then incubated for 15 minutes at room temperature. The columns were washed with RW1 as before. This was followed by two Buffer RPE washes, to remove salts, at 8000 x g (10, 000 rpm) for 15 seconds and 2 minutes, respectively. A further centrifugation was performed for 1 minute at full speed to eliminate any possible Buffer RPE carryover. RNA was eluted from the spin column membrane using 30µl RNase-free H_2O by centrifuging for 1 minute at 8000 x g (10, 000 rpm). Extracted RNA was stored at -80°C until required.

2.9.2 RNA extraction from tissue

Tissue was lysed in 1ml TRI reagent® (Sigma-Aldrich) with one sterile 5mm stainless steel bead (Qiagen) per Eppendorf/sample. The tissue was lysed at 25Hz using a Tissue Lyser II (Qiagen), for 3 minutes, twice, with tubes rotated half way through. The samples were then incubated for 15 minutes at room temperature, then centrifuged at 14, 000 x g for 10 minutes at 4°C. The supernatant was transferred to a 2 ml phase-lock tube (5-PRIME, Hamburg, Germany) and 200µl 1-bromo-3-chloropropane (BCP; Sigma-Aldrich) added to each sample. The samples were vigorously shaken for 15 seconds, incubated for 10 minutes at room temperature, then centrifuged at 14, 000 x g for 15 minutes at 4°C. The upper aqueous phase was transferred to a new 2ml Eppendorf and 550µl of EtOH (70%) added. The samples were then added to RNeasy

mini spin columns and RNA extraction carried out as described in section 2.9.1. The extracted RNA was again stored at -80°C until required.

2.9.3 RNA quantification

Nucleic acid concentration was quantified by measuring the absorbance of the sample at 260nm on a NanoDrop 2000c (ThermoFisher Scientific). RNA absorbs at 260nm, while contaminants, such as protein, absorb closer to 280nm. Therefore, the ratio of the absorbance at 260nm and 280nm indicated RNA purity. Ratios of 1.8-2.1 were considered acceptable.

2.9.4 Reverse Transcription (RT) – Complementary DNA (cDNA) preparation

Total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technologies) (Table 2.3). A 2X RT mastermix was prepared on ice. This included the following reagents; 10X RT buffer, 25X deoxyribonucleotide triphosphates (dNTPs), 10X random primers, RNase inhibitor, MultiScribe™ RT enzyme and RNA-free H_2O. RNA (300ng/µl) was added to the mastermix and pipetted gently to mix, then centrifuged at 10, 000 rpm for 30 seconds to remove any air bubbles. A "no reverse transcriptase" control was included, omitting the enzyme, to control for genomic DNA contamination. A "no template" control, omitting the RNA, was included to control for general contamination of reagents. A G-Storm GS1 Thermal Cycler (G-Storm, Somerset, UK) was set to perform the following cycles; 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, then held at 4°C. Samples were stored at -20°C until required for quantitative real time polymerase chain reaction (qRT-PCR) analysis.

Reagent	Volume (µl) per sample for 2X RT mastermix
10X RT Buffer	2
25X dNTPs	0.8
10X Random primers	2
RNase inhibitor	1
MultiScribe™ RT enzyme	1
RNA-free H_2O	3.2
RNA (300ng)	10

Table 2.3. RT reagent mixture

2.10 Quantitative real time polymerase chain reaction (qRT-PCR)

qRT-PCR was carried out with predesigned gene expression assays from Applied Biosystems, to quantify the mRNA expression of specific genes of interest. Details of the reagent mixture and target genes are shown in Table 2.4 and Table 2.5, respectively. qRT-RCR was performed in a 384-well plate (Applied Biosystems) with all samples and controls added in duplicate. A reagent mixture was prepared and 14µl was added to each well, together with 1µl of cDNA sample. In addition to the cDNA samples, wells containing the following controls were included; "no reverse transcriptase", "no template" and finally an H_2O control, replacing cDNA, to determine any reagent contamination. Plates were carefully sealed with optical adhesive film (MicroAmp®, Applied Biosystems) and then centrifuged at 500 x g for 1 minute. All qRT-PCR analyses were performed on an Applied Biosystems 7900HT instrument set to perform the following cycles; 50°C held for 2 minutes, 95°C held for 10 minutes, then 95°C for 15 seconds and 60°C for 1 minute, repeated for 40 cycles. Target gene expression was normalised for RNA loading using *β-actin* (ACTB VIC, Human: 4326315E, Mouse: 4352341E, Applied Biosystems). Previous studies from

our laboratory found this endogenous gene to be consistent during late pregnancy in the mouse and consistent in the human myometrial cell line (Hutchinson et al. 2014; Rinaldi et al. 2014; Rinaldi et al. 2015a; Rinaldi et al. 2015b). The expression in each sample was calculated relative to a calibrator sample (vehicle control) using the $2^{-\Delta\Delta}$ threshold cycle (CT) method of analysis.

Reagent	**Volume (µl) per sample for TaqMan reagent mixture**
TaqMan Universal Master Mix II	7.5
Primer/probe or *β-actin*	0.75
RNA-free H_2O	5.75

Table 2.4. TaqMan® reagent mixture for a 384-well plate

Gene	Species	Code
IL-6	Human	Hs00985639_m1
IL-8	Human	Hs00174103_m1
IL-10	Human	Hs00961622_m1
IL-13	Human	Hs00174379_m1
Ccl2	Mouse	Mm00441242_m1
Cox-2	Mouse	Mm00478374_m1
Cxcl1	Mouse	Mm04207460_m1
Cxcl2	Mouse	Mm00436450_m1
Cx43	Mouse	Mm01179639_s1
Il-1β	Mouse	Mm00434228_m1
Il-6	Mouse	Mm00446190_m1
Il-10	Mouse	Mm01288386_m1
Tlr4	Mouse	Mm00445273_m1
Tnf	Mouse	Mm00443258_m1

Table 2.5. Pre-designed TaqMan® gene expression assay IDs

2.11 Enzyme-linked immunosorbent assay (ELISA)

To perform a sandwich ELISA, the plates are first coated with a specific monoclonal capture antibody directed against the protein of interest (Figure 2.5). The standards, samples and controls are then added to the plate and any protein recognised by the monoclonal capture antibody will be bound. Unbound proteins are removed by washing and a biotinylated detection antibody is added, which also detects the protein of interest that is immobilised by the capture antibody. The enzyme streptavidin-horseradish peroxidase (HRP) is applied, which binds to the detection antibody. Following a further wash to remove unbound antibody-enzyme conjugates, a substrate solution of hydrogen peroxide and tetramethylbenzidine (TMB) is added to detect peroxidase activity. This induces a colour change which can be measured by spectrophotometry. The colour intensity is proportional to the concentration of protein present. A standard curve is used to extrapolate protein concentration. The specific procedures performed are explained in detail below.

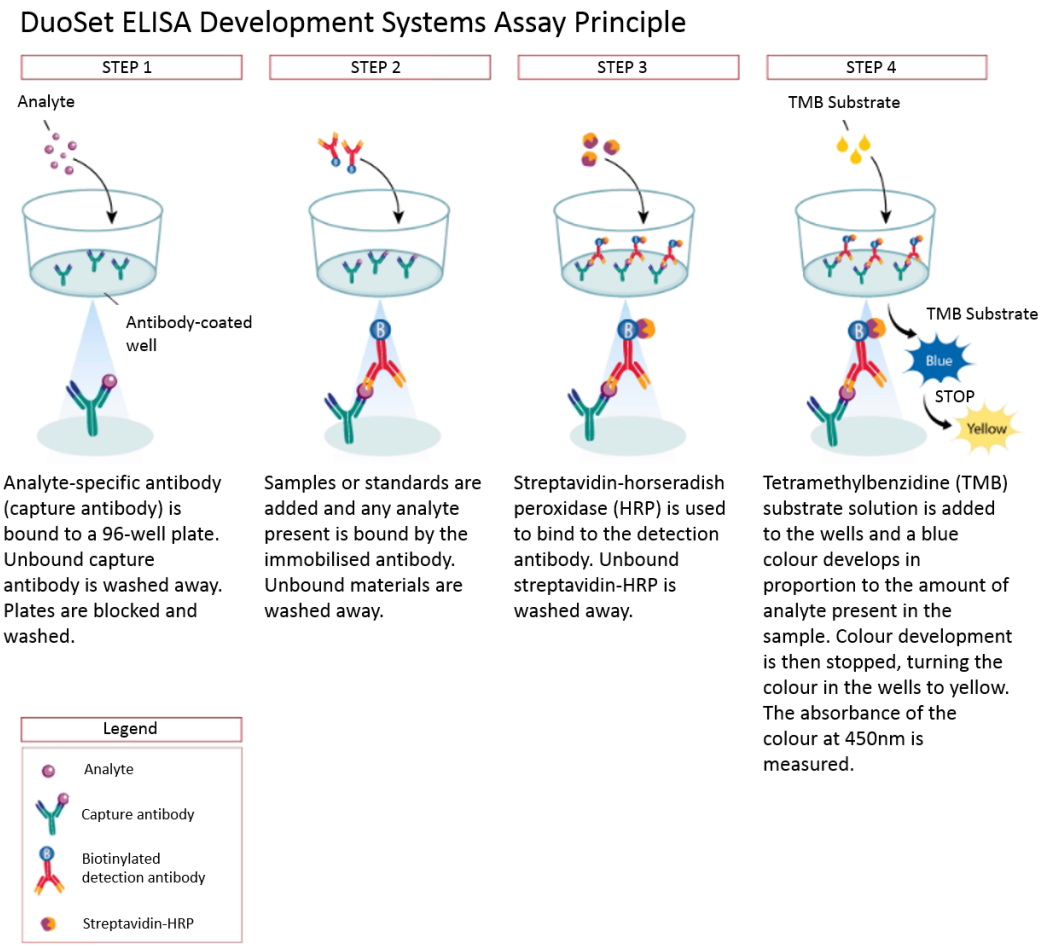

Figure 2.5. DuoSet sandwich ELISA protocol. ELISAs were performed following the principles described above. Figure edited from: (RnDSystems) https://www.rndsystems.com/resources/technical/duoset-elisa-development-systems-assay-principle.

2.11.1 DuoSet ELISA solution preparation

Preservatives, required for the dry coat solution, ELISA buffer and substrate buffer, were prepared with 2 methylisothiazolone (20%; 2g) and bromonitrodioxane (20%; 2g) dissolved in a 10ml solution of dimethylsulfoxide (DMSO; 5ml) and dimethylformamide (DMF; 5ml) and stored in the dark at room temperature. The dry coat solution, applied to plates to immobilise the primary antibody, was prepared by adding polyvinyl pyrollidone (2%; 20g), bovine serum albumin (BSA; 0.5%), EDTA (5mM; 1.68g), tris base (50mM; 6.05g) and preservatives (0.1%; 1ml). ELISA buffer (1x) was prepared by adding Tris base (100mM; 12.11g), sodium chloride (NaCl) (0.9%; 9g), EDTA (2mM; 0.744g), BSA (0.2%; 2g), phenol red solution (0.03%;

300µl), TWEEN® 20 (0.03%; 300µl) and 1ml preservatives to 800ml deionized water (dH2O) and dissolved. The pH was adjusted to 7.2 with 5N HCl and the volume made up to 1L. Wash buffer (20x) was prepared by adding NaCl (0.9%; 360g), Tris base (10mM; 48.4g), TWEEN® 20 (0.05%; 20ml) to 1.7L dH_2O, adjusted to pH 7.5 and made up to 2L. This was diluted 1 in 20 in dH_2O for use. Substrate buffer was prepared by adding sodium acetate anhydrous (100nM; 4.1g) and the above preservatives (0.1%; 0.5ml) to 405ml dH_2O, adjusting to pH6 using glacial acetic acid before making the solution up to 500ml. Solution A, for adding to the substrate buffer, was TMB (0.3%; 0.3g) dissolved in 100ml DMF and stored in the dark at room temperature. Solution B was prepared by dissolving urea hydrogen peroxide (0.5%; 0.5g) in 100ml 50mM sodium acetate buffer. Directly before use, 1ml each of solution A and B were added to 10ml stock substrate buffer and mixed well. ELISA stop solution (sulfuric acid; 2N H_2SO_4) was prepared by adding 36N concentrated acid into dH_2O.

2.11.2 DuoSet ELISA plate coating

Mouse anti-human IL-6/IL-8 capture antibodies (Table 2.6; R&D Systems, Inc. Abingdon, UK) were reconstituted in sterile PBS, aliquoted and stored at -20°C until use. When required, each antibody was diluted in PBS and 100µl was added to each well of a 96-well plate. The plate was then sealed and incubated overnight at 4°C. The following day, the contents of the plate were removed and the plate blotted. Dry coat (100µl) was added to each well and incubated for 1 hour at room temperature. The contents were again removed, the plate blotted dry and then incubated for 3-4 hours at room temperature to dry completely.

Reagent	Dilution	Diluent
Capture antibody	1:120	PBS
Detection antibody	1:60	ELISA buffer
Streptavidin HRP	1:40	ELISA buffer

Table 2.6. IL-6 and IL-8 DuoSet Human ELISA reagents

2.11.3 Method for DuoSet human IL-6 and IL-8 ELISA

Plates were first washed 4 times with wash buffer and blotted dry. A 7-point standard curve, diluted in ELISA buffer (IL-6: 600, 300, 150, 75, 37.5, 18.75, 9.375pg/ml, IL-8: 2000, 1000, 500, 250, 125, 62.5, 31.25pg/ml) was added, with ELISA buffer as a zero standard (0pg/ml). Experimental samples (myometrial cell supernatants) were diluted as necessary in ELISA buffer and a quality control was added. All standards, samples and controls were applied in duplicate. The plate was sealed and incubated overnight at 4°C. The following day, the contents were flicked out and the plate washed and blotted 4 times. The detection antibody (100µl) diluted in ELISA buffer (Table 2.6) was added per well and incubated on a plate shaker for 1 hour at room temperature. The contents were then flicked out and the plate washed and blotted 4 times, as before. DuoSet Streptavidin HRP solution (Table 2.6) was then diluted in ELISA buffer and 100µl added to each well. Plates were incubated for 20 minutes at room temperature on the plate shaker, protected from direct sunlight. The solution was removed and the plate washed 4 times. Finally, 100µl of the substrate solution, containing solutions A and B (described in section 2.11.1) was added to each well and incubated for 20 minutes at room temperature. Then 50µl 2N H_2SO_4 stop solution was added to quench colour development. The plates were read immediately at 450nm using the absorbance mode of Thermomax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) and Softmax® Pro software (v5.0, Molecular Devices). A standard curve for each plate was generated and the sample concentrations were determined from this curve using the Softmax® Pro software.

2.11.4 Quantikine mouse IL-6 ELISA

An IL-6 ELISA (M6000B; Quantikine, R&D Systems) was performed using maternal serum and amniotic fluid collected from mice. The 96-well plates were purchased pre-coated with a monoclonal antibody specific for mouse IL-6. The mouse IL-6 positive control was reconstituted in 1ml dH_2O. The wash buffer was prepared by adding 20ml wash buffer concentrate to 500ml dH_2O. The substrate solution was prepared by combining equal volumes of colour reagents A and B, no more than 15 minutes prior to use. The mouse IL-6 standard was reconstituted with Calibrator Diluent RD5T, to produce a stock solution of 500pg/ml. The stock solution was then diluted to achieve the dilution series; 500, 250, 125, 62.5, 31.3, 15.6, 7.8pg/ml. The Calibrator Diluent, RD5T, acted as the zero standard (0pg/ml).

Following the preparation of the above reagents, 50µl of assay diluent, RD1-14, was added to each well, followed by 50µl of the appropriate standard, control, maternal serum or amniotic fluid sample. The plate was incubated for 2 hours at room temperature and covered with an adhesive strip. The wells were then aspirated, washed five times and blotted. The mouse IL-6 conjugate (100µl) was added to each well, an adhesive strip applied and the plate was incubated for 2 hours at room temperature. The plate was aspirated and washed 5 times. The substrate solution was then prepared and 100µl was added per well. The plate was protected from light and incubated for 30 minutes at room temperature. The stop solution (100µl/well) was added and the plate was tapped gently to ensure thorough mixing. The plate was then read within 30 minutes at 450nm, as described in section 2.11.3.

2.12 Western blotting

2.12.1 Protein extraction from tissue

Tissue was placed in a 2ml Eppendorf tube with one sterile, stainless steel homogenising bead (Qiagen). RIPA lysis buffer was purchased in a ready-to-use solution containing 150mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS and 50mM Tris at pH8.0 (R0278; Sigma-Aldrich). On the day of the experiment, the lysis buffer was prepared with 10ml RIPA buffer and one protease inhibitor tablet (cOmplete™ Protease Inhibitor Cocktail Tablet, 04693132001, Roche, Basel, Switzerland) and 500-700µl was added per tube

depending on tissue size and texture. Samples were homogenised using a Tissue Lyser II (Qiagen) at 25Hz for 3 minutes, twice. The samples were then incubated on ice for 5 minutes and centrifuged at 10, 000 x g for 10 minutes at 4°C. The supernatant was aliquoted and stored at -80°C.

2.12.2 Protein quantification

The BIO-RAD DC™ protein assay (Bio-Rad Laboratories Ltd, Watford, UK) is a colorimetric assay which measures protein concentration. It is a similar reaction to the Lowry assay but it is more time-efficient (http://www.bio-rad.com/en-uk/product/dc-protein-assay). The protein reacts with an alkaline copper tartrate solution and Folin reagent. Protein first reacts with the copper in an alkaline solution and then reduces the Folin reagent, causing a blue colour to develop. The intensity of colour is proportional to the quantity of protein present. The absorbance can be measured at 650nm.

A 4 point standard curve (1.37, 0.685, 0.342, 0.171mg/ml) was prepared by performing a two-fold dilution using the BIO-RAD DC™ top standard diluted in the protein extraction buffer (RIPA buffer, see 2.12.1). The RIPA buffer was used as a blank (0mg/ml). Working reagent A was prepared by the addition of 20µl reagent S (surfactant solution) in 1ml of reagent A (alkaline copper tartrate solution). Standards and samples were added to a 96-well plate, in duplicate, in a volume of 5µl, followed by 25µl of working reagent A. Reagent B (200µl, dilute Folin Reagent) was then added to each well. The plate was incubated for 15 minutes at room temperature and then read using a Thermomax microplate reader at 650nm. If the sample concentration was higher than the top standard, the assay was repeated with samples diluted in RIPA buffer.

2.12.3 Fluorescent Western blot

Fluorescent Western blot was performed to quantify CX43 in the mouse uterus. Protein samples were mixed with NuPAGE® LDS Sample Buffer and NuPAGE® Sample Reducing Agent (ThermoFisher Scientific) and heated to 70°C for 10 minutes to denature the protein (G-Storm GS1 Thermal Cycler). Firstly, Precision Plus Protein™ Prestained Standard molecular weight marker (BIO-RAD) was loaded on a 4-12%

(1mm, 12 well) Bis-Tris precast NuPAGE® gel in 1X NuPAGE® MOPS running buffer (Table 2.7). Denatured protein samples were then loaded (20µg of protein/sample) and the gel was run at 180 V for 70 minutes, to separate the proteins. The proteins were transferred to Immobilon-FL PVDF membranes (Millipore) in 1X transfer buffer, using a wet-transfer system, at 100 V for 90 minutes (Table 2.7). The membranes were then blocked with 5% non-fat dry milk in 0.5% TWEEN® 20 tris buffered saline (TBST; Life Technologies) to minimise non-specific binding. The membrane was then incubated with primary antibodies against CX43 and α-Tubulin, diluted in 5% non-fat dry milk in 0.5% TBST, overnight at 4°C (Table 2.8). The following day, membranes were washed in TBST for 5 minutes, three times. Membranes were then incubated with secondary antibodies (LI-COR Biosciences) diluted in 5% non-fat dry milk in 0.5% TBST (Table 2.8) for 2 hours and washed again for 5 minutes, repeated 3 times. Membranes were scanned using the LI-COR Odyssey® Fc Imaging System. The intensity of each CX43 band (43kDa) was normalised to α-Tubulin (50kDa), as a loading control, and then quantified using the Odyssey analysis software.

2.12.4 Chemiluminescent Western blot

Chemiluminescence was performed to quantify IL-6 (23kDa) in the mouse uterus. Western blot was performed as described in section 2.12.3, with minor alterations which are outlined as follows. Subsequent to gel separation, the protein (45µg of protein/sample) was transferred to Immobilon-P PVDF membranes (Millipore) and blocked with 5% BSA (Sigma-Aldrich) in TBST. After primary and secondary antibody incubation (Table 2.8), the membranes were incubated with Amersham ECL Western blotting detection reagent (GE Healthcare Life Sciences, Marlborough, MA) for 30 seconds (α-Tubulin) and 5 minutes (IL-6), then scanned and analysed as described in 2.12.3. Recombinant Human IL-6 Protein (21kDa; R&D Systems) was included as a positive control.

Buffer	**Recipe**
1X NuPAGE® MOPS SDS Running Buffer	50ml 20X NuPAGE® MOPS SDS Running Buffer 950ml dH_2O
10X Transfer Buffer	30g Tris 144g Glycine 900ml dH_2O
1X Transfer Buffer	100ml 10X transfer buffer 800ml dH_2O 100ml CH_3OH

Table 2.7. Buffers used for Western blotting

Target	Primary antibody	Supplier	Secondary antibody	Supplier
α-Tubulin (Fluorescence)	Monoclonal mouse anti-α-Tubulin (1:5000)	Sigma-Aldrich (T9026)	Polyclonal donkey anti-mouse 680RD (1:10,000)	LI-COR Biosciences (926-68072)
α-Tubulin (Chemiluminescence)	Monoclonal mouse anti-α-Tubulin (1:5000)	Sigma-Aldrich (T9026)	Polyclonal goat anti-mouse HRP (1:1000)	Dako, Agilent, CA, USA (P0447)
CX43 (Fluorescence)	Polyclonal rabbit anti-CX43 (1:5000)	Abcam, Cambridge, UK (ab11370)	Polyclonal donkey anti-rabbit 800CW (1:10,000)	LI-COR Biosciences (926-32213)
IL-6 (Chemiluminescence)	Monoclonal rabbit anti-IL-6 (1:1000)	Cell Signaling Technology (12912)	Polyclonal swine anti-rabbit HRP (1:3000)	Dako (P0399)

Table 2.8. Primary and secondary antibodies for Western Blot analyses

2.13 Liquid chromatography tandem-mass spectrometry (LC-MS/MS)

LC-MS/MS involves first separating the sample components by liquid chromatography, which occurs as a result of the interactions of the sample with the mobile and stationary phases. The components are then introduced to the mass spectrometer, which creates and detects charged ions. This technique can provide information about the molecular weight, structure, identity and quantity of specific sample compounds. In this case, it was used to quantify progesterone in the serum collected from mice treated with simvastatin, with/without LPS.

2.13.1 Extraction of progesterone from pregnant mouse serum

The following protocol and analyses were performed by Mr George Just in the Mass Spectrometry Core laboratory of the Clinical Research Facility, Edinburgh.

D9-progesterone (1.5ng; C/D/N Isotopes Inc, Canada) was included as an internal standard and was prepared at 1 mg/ml in CH_3OH. To give a 10µg/mL stock internal standard solution, 10µl of 1mg/ml internal standard solution was added to a 3.5ml vial with 970µl of 10mM ammonium acetate (pH 4.5). Then, 19.98ml of 10mM ammonium acetate (pH 4.5) was added to 20µl of each 10µg/ml stock internal standard solution to give 20ml of 0.01µg/ml working internal standard solution. Progesterone (1mg/ml in CH_3OH) was used to prepare a set of stock standards, as detailed in Table 2.9.

For extraction, standards were prepared as outlined in Table 2.10. The liquid was evaporated using a flow of oxygen-free nitrogen in a dri-block set to 40°C (Techne, Cole Parmer, UK) then 50µl of control plasma or biological serum/amniotic fluid sample was added to individual 5ml glass centrifuge tubes. Working internal standard solution was added in a volume of 150µl to each sample and standard. The tubes were vortexed and the standards were transferred to a 96-well (200µl) Biotage SLE+ plate (Biotage, Sweden) for liquid extraction. An Isolute vacuum manifold (Biotage) was applied for 15 seconds to initiate loading. Once loaded, samples were incubated for 5 minutes to completely absorb. Samples were then eluted into a Waters 96-well (2ml) collection plate (Manchester, UK) using 1ml of Methyl tertiary butyl ether (MTBE) and then once again evaporated to dryness using a flow of oxygen-free nitrogen. Precipitate was resuspended in 100µl of water/acetonitrile (50/50, v/v) and 10µl was injected for LC-MS/MS quantification.

Volume of each stock standard added (ml)	Stock standard used	Amount of CH_3OH added (ml)	Progesterone concentration
0.01	1 mg/ml	0.97	10 µg/ml
0.5	10 µg/ml	0.5	5 µg/ml
0.2	10 µg/ml	0.8	2 µg/ml
0.1	10 µg/ml	0.9	1 µg/ml
0.1	5 µg/ml	0.9	0.5 µg/ml
0.1	2 µg/ml	0.9	0.2 µg/ml
0.1	1 µg/ml	0.9	0.1 µg/ml
0.1	0.5 µg/ml	0.9	0.05 µg/ml
0.1	0.2 µg/ml	0.9	0.02 µg/ml
0.1	0.1 µg/ml	0.9	0.01 µg/ml
0.1	0.05 µg/ml	0.9	0.005 µg/ml
0.1	0.02 µg/ml	0.9	0.002 µg/ml
0.1	0.01 µg/ml	0.9	0.001 µg/ml

Table 2.9. Preparation of stock standards

Stock standard identifier	Volume of stock standard added (ml)	Stock standard used (µg/ml)	Progesterone absolute amount (ng)
S0	N/A	N/A	0
S1	0.01	0.001	0.01
S2	0.01	0.002	0.02
S3	0.01	0.005	0.05
S4	0.01	0.01	0.1
S5	0.01	0.02	0.2
S6	0.01	0.05	0.5
S7	0.01	0.1	1
S8	0.01	0.2	2
S9	0.01	0.5	5
S10	0.01	1	10
S11	0.01	2	20
S12	0.01	5	50

Table 2.10. Preparation of standards for extraction

2.13.2 LC-MS/MS quantification

Progesterone was measured by LC-MS/MS, using a Sciex QTRAP® 5500 (Warrington, UK), with a Waters Acquity™ UPLC (Manchester, UK). Mass spectral conditions are demonstrated in Table 2.11.

Analyte separation was performed at 40°C on an Ace® Excel C18 (100x2.1mm, 1.7µm) column (Advanced Chromatography Technology Ltd, Aberdeen, UK) using a gradient solvent system (50:50 of water with 0.1% formic acid and acetonitrile with 0.1% formic acid) with a gradient run of 6.5 minutes as demonstrated in Table 2.12. Analysis was performed using MultiQuant® Software provided by Sciex (Warrington, UK).

	Molecular Weight (Da)	**Precursor ion (*m*/*z*)**	**Product ion** **Quan; Qual**	**Declustering potential (V)**	**Collision energy (V)** **Quan; Qual**	**Cell exit potential (V)** **Quan; Qual**
Progesterone	314.462	315.0	97.0; 109.0	146	29; 37	10; 18
D9-Progesterone	323.52	324.1	100.0	151	31	38

Table 2.11. Mass spectral conditions for analysis of progesterone and the internal standard, D9-Progesterone, utilising positive and negative electrospray. Da (Daltons), Quan (quantifier ion), Qual (qualifier ion), V (volts).

Time (minutes)	Mobile Phase A: Water (0.1% formic acid, v/v)	Mobile Phase B; Acetonitrile (0.1% formic acid, v/v)
0	50	50
1	50	50
4	0	100
5	0	100
5.1	50	50
6.5	50	50

Table 2.12. Chromatographic conditions (Flow Rate 0.5 ml/min)

2.14 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) and were analysed using GraphPad Prism (Version 7; GraphPad Software Inc., San Diego, CA). For cell studies, "n" denotes the number of individual experiments performed, with the replicate number per experiment indicated in the figure legends. For mouse studies, "n" represents the number of individual dams treated. Time to delivery data were analysed using Kruskal-Wallis, with Dunn's post hoc test. The percentage data for live born pups and collagen gel contraction were analysed by performing an arcsine transformation on the proportions, followed by a one-way analysis of variance (ANOVA), with either Dunnett's or Holm-Sidak post hoc test. All gene expression, ELISA, In-Cell Western and LC-MS/MS data were square root transformed, if necessary, then analysed by one-way ANOVA, followed by Dunnett's or Tukey's post hoc test. A p value <0.05 was considered statistically significant.

Chapter 3: Investigating the effect of statin treatment on inflammation in a pregnant human myometrial cell line

3.1 Introduction

Labour is an inflammatory event, initiated by the shift from a quiescent to a pro-inflammatory environment. This instigates a three step process, characterised by myometrial contractility, cervical ripening and fetal membrane rupture (Romero et al. 1994; Rinaldi et al. 2011). Leukocytes, such as monocytes and neutrophils, infiltrate the myometrium and cervix, stimulating an increase in inflammatory mediators including cytokines and chemokines. Pro-inflammatory mediators, such as *IL-1β*, *IL-6*, *IL-8* and *TNF* are all upregulated in these tissues during active labour (Bokstrom et al. 1997; Thomson et al. 1999; Osman et al. 2003). If these events occur earlier in gestation, they can result in the pathological, preterm initiation of this labour cascade. In addition, inflammatory mediators may reach the fetal circulation, resulting in fetal injury (Adams Waldorf and McAdams 2013).

Approximately 70% of PTBs are spontaneous (Rubens et al. 2014). Intrauterine infection is believed to be present in 25-40% of PTBs. However, this is likely to be an underestimate, as infections are often sub-clinical and cannot be detected by current culturing techniques (Goldenberg et al. 2008). Existing therapies are ineffective at preventing PTB and do not treat the underlying causes (Romero et al. 2014). Ideally, a treatment for PTL would target the local inflammation, having the dual benefit of delaying the labour process and reducing fetal injury.

Statins are an established treatment for the reduction of LDL cholesterol synthesis and subsequently, for the prevention of cardiovascular disease. In addition to lowering lipids, statins also exert anti-inflammatory properties (Zhou and Liao 2010). As a result, these drugs have been investigated for use in multiple inflammatory conditions such as sepsis, rheumatoid arthritis and asthma (Zeki et al. 2013; Dobesh and Olsen 2014; Das et al. 2015). The anti-inflammatory properties of statins have been widely investigated *in vitro*, mostly in vascular smooth muscle cells, where they have been shown to mediate the reduction of IL-6 and IL-8 (Ito et al. 2002a; Ito et al. 2002b).

Statins can also inhibit human neutrophil and monocyte chemotaxis, while reducing the release of pro-inflammatory mediators such as IL-6, IL-8 and CCL2 (Rezaie-Majd et al. 2002; Maher et al. 2009; Jougasaki et al. 2010).

The pharmacokinetics of statins vary. For example, pravastatin is hydrophilic and requires selective uptake by the sodium-independent transporter protein, OATP1B1. This transporter protein is exclusively expressed in the liver and so, entry into other cells types is limited (Hamelin and Turgeon 1998; Corsini et al. 1999). Simvastatin is a lipophilic drug, which readily enters cells by passive diffusion (Schachter 2005).

As statins have anti-inflammatory properties, they may be an ideal therapeutic to target the inflammation associated with PTL. Therefore, the hypothesis of this chapter was that statins will reduce LPS-induced inflammation in human myometrial cells.

3.2 Methods

3.2.1 Myometrial cell culture and treatment

Myometrial cells were cultured as described in 2.1. They were seeded at $1.5x10^5$ cells/ml in 6-well plates and treated with LPS (100ng/ml) and pravastatin (10µM) or simvastatin (0.1µM, 10µM, 50µM). For inflammatory mediator analysis, the cells were either co-treated with LPS and statins (n=6), pre-treated with statins for 6 hours before LPS (pravastatin only n=5, all other treatments n=6) or treated with statins 6 hours after LPS stimulation (n=6), as outlined in 2.3. All treatments lasted for 24 hours and were performed in duplicate. Pravastatin and simvastatin experiments were performed simultaneously. Therefore, the same vehicle and LPS treatment data were utilised for both pravastatin and simvastatin analyses. For clarity, the results are displayed separately for each statin, with the control group data represented in each graph.

3.2.2 Myometrial cell image capture

Images of myometrial cells were captured following 24 hours of co-treatment, pre-treatment and post-treatment with statins and LPS, to assess any effects on cell morphology, as described in 2.4. Treatments were performed in duplicate and images were captured from 3 individual experiments (n=3).

3.2.3 MTT metabolic activity assay

The metabolic activity of the myometrial cells was assessed as a measurement of cell viability, following statin and LPS treatment. This was performed using an MTT assay, as detailed in 2.5. The co-, pre- and post-treatments were performed in triplicate for 24 hours (n=6).

3.2.4 qRT-PCR

The mRNA expression of the inflammatory markers *IL-6, IL-8, IL-10* and *IL-13* were assessed by qRT-PCR analysis, following 24 hour statin and LPS treatment, as described in 2.10. The mRNA expression of these genes of interest was normalised to the endogenous control gene, *β-actin*, within each individual sample. Samples were then compared to the vehicle control group (PBS) using the $2^{-\Delta\Delta}$ CT method of analysis, as detailed in 2.10. The results are presented as fold change relative to the vehicle control group.

3.2.5 ELISA

ELISAs were performed with myometrial cell supernatants to quantify the levels of IL-6 and IL-8 secreted by these cells during treatment with statins and LPS. Details of the assays are explained in 2.11.3. The supernatants were collected following 24 hour incubation with statin and LPS treatments, at the same time as cells were harvested for qRT-PCR analyses.

3.2.6 Statistical analysis

Data are presented as mean ± SEM and were analysed using GraphPad Prism. In these studies, "n" denotes the number of individual experiments performed, with the replicate number per experiment indicated in the figure legends. Where data were not normally distributed, they were square root transformed prior to analysis. Gene expression (CT values) and ELISA data were analysed by one-way ANOVA, followed by Dunnett's post-hoc test. A p value <0.05 was considered statistically significant.

3.3 Results

3.3.1 The effect of statin treatment on the morphology of human myometrial cells

Myometrial cells are smooth muscle cells, which have a fibroblast-like, elongated, spindle-shaped morphology. To investigate whether statin treatment may affect the morphology of these cells, they were treated with pravastatin or simvastatin, with and without LPS stimulation. The myometrial cells were either co-treated with LPS and pravastatin/simvastatin, pre-treated with pravastatin/simvastatin or treated with pravastatin/simvastatin post-LPS stimulation. Images were captured after the cells were incubated for 24 hours with the treatments.

3.3.1.1 The effect of pravastatin treatment on myometrial cell morphology

When cells were treated with 10μM pravastatin, there was no noticeable difference in their morphology (Figure 3.1). LPS alone did not appear to have an effect on the shape of these cells. Additionally, there was no apparent effect of treating with both pravastatin and LPS. These results did not vary between the co-, pre- and post-treatment group.

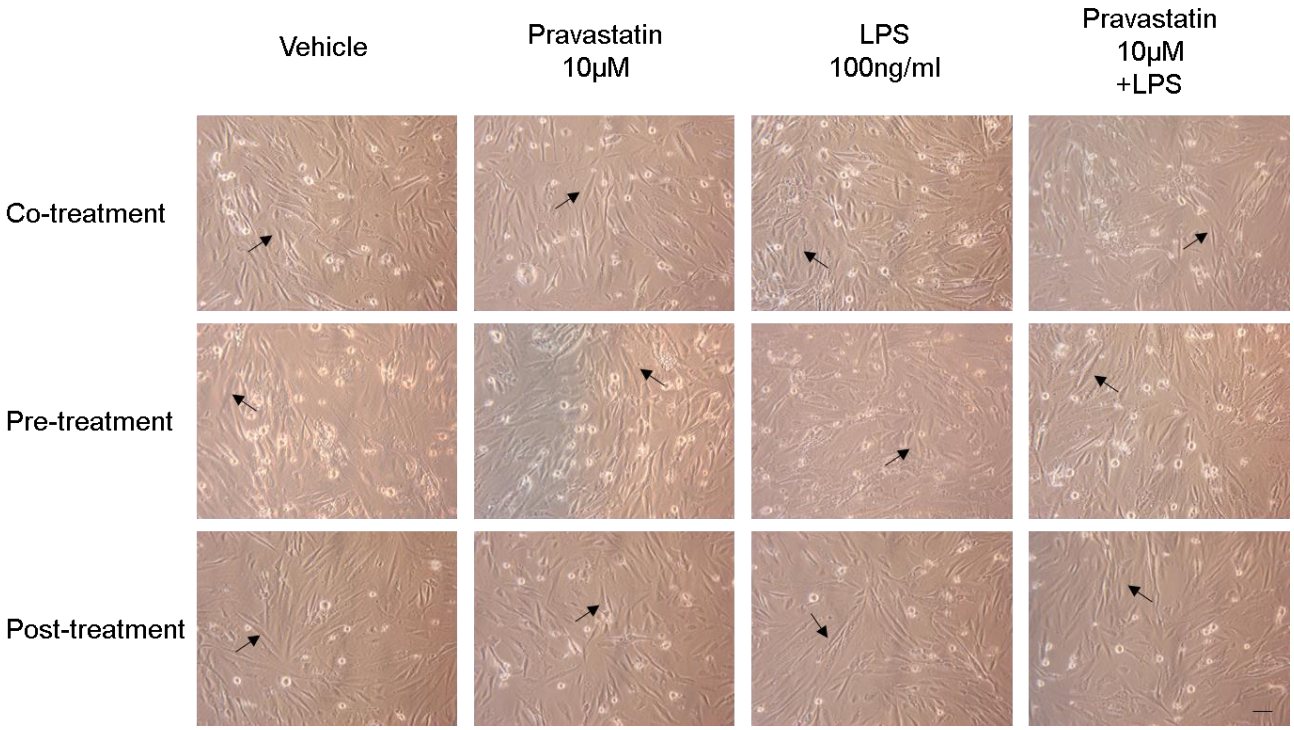

Figure 3.1. Myometrial cell morphology following pravastatin and LPS treatment. Representative images show myometrial cells which were either co-treated with pravastatin and LPS, pre-treated with pravastatin for 6 hours before LPS or given pravastatin 6 hours post-LPS stimulation. All incubations lasted for 24 hours. Spindle-shaped myometrial smooth muscle cells are shown in the vehicle control images (arrows). n=3 (in duplicate), vehicle (PBS) and LPS data shared for pravastatin/simvastatin experiments. Scale bar shows 50μm. Arrows indicate examples of normal myometrial cell morphology. All images were taken with a x10 objective lens.

3.3.1.2 The effect of simvastatin treatment on myometrial cell morphology

When the myometrial cells were treated with 10μM simvastatin alone, the cells appeared marginally more elongated (Figure 3.2). When the myometrial cells were treated with 0.1μM simvastatin and LPS, there was no effect on cell morphology, when compared to the cells treated with vehicle. However, when the cells were treated with LPS and either 10μM or 50μM simvastatin, they again looked slightly more elongated. This possible morphology change was visible in the co-, pre- and post-treatment groups. However, this was not quantified.

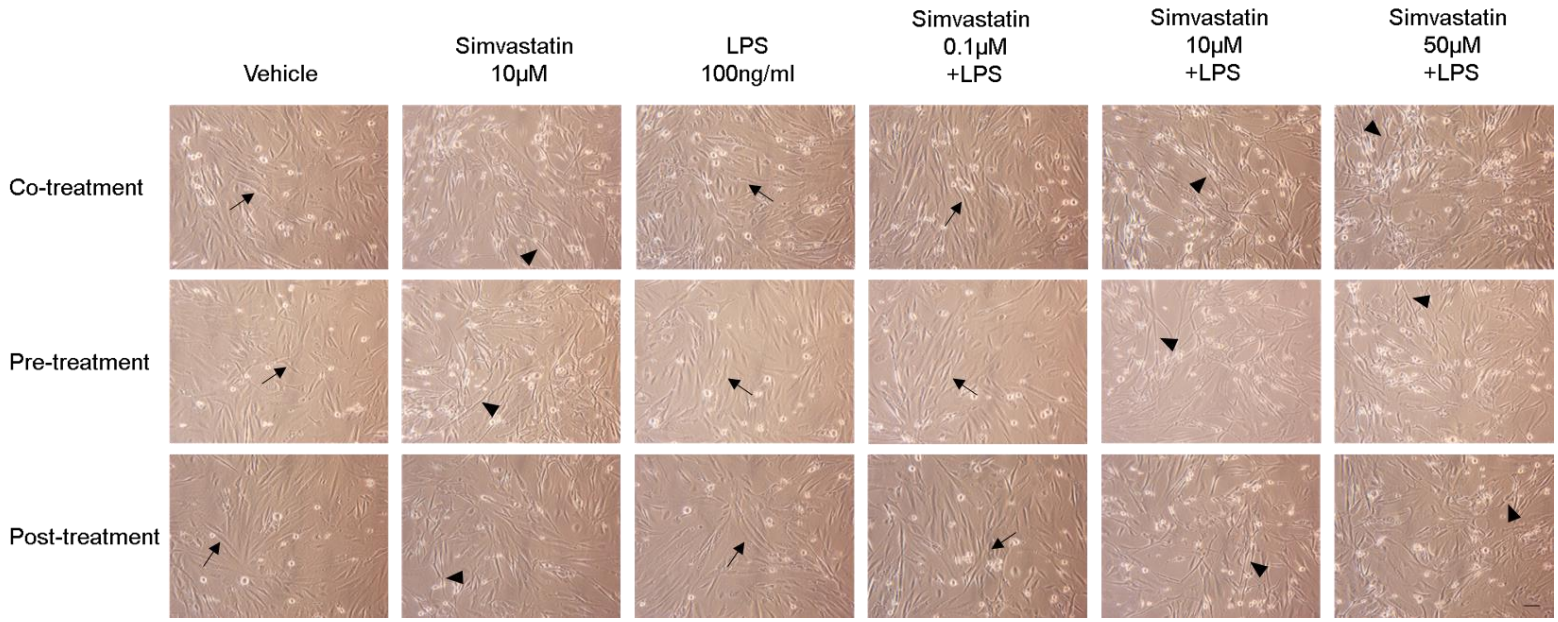

Figure 3.2. Myometrial cell morphology following simvastatin and LPS treatment. Representative images show myometrial cells which were either co-treated with simvastatin and LPS, pre-treated with simvastatin for 6 hours before LPS or given simvastatin 6 hours post-LPS stimulation. All incubations lasted for 24 hours. Myometrial smooth muscle cells are spindle-shaped, as evidenced in the vehicle control images (arrows). However, when given 10μM simvastatin treatment, in each treatment regimen, the cells became slightly thinner (arrowheads). Treatments were performed in duplicate (n=3), vehicle (PBS) and LPS images shared for pravastatin/simvastatin experiments. Scale bar shows 50μm. Arrows indicate examples of normal myometrial cell morphology. Arrowheads indicate more elongated myometrial cells. All images were taken with a x10 objective lens.

3.3.2 The effect of statin treatment on the metabolic activity of a human myometrial cell line

MTT assays were performed to investigate the effect of pravastatin, simvastatin and LPS treatment on cell metabolic activity. This was undertaken to give a preliminary indication of the functionality and viability of the myometrial cells following treatment. The myometrial cells were either co-, pre- or post-treated with pravastatin/simvastatin and/or LPS for 24 hours. The metabolic activity per treatment was made relative to the mean metabolic activity of the vehicle control (PBS).

Myometrial cell metabolic activity was unaffected by concentrations of up to 100μM of pravastatin (Figure 3.3) and simvastatin (Figure 3.4), after treatment for 24 hours. The inclusion of LPS had no additional effect on cell metabolism, irrespective of whether the cells received, co-, pre- or post-LPS statin treatment.

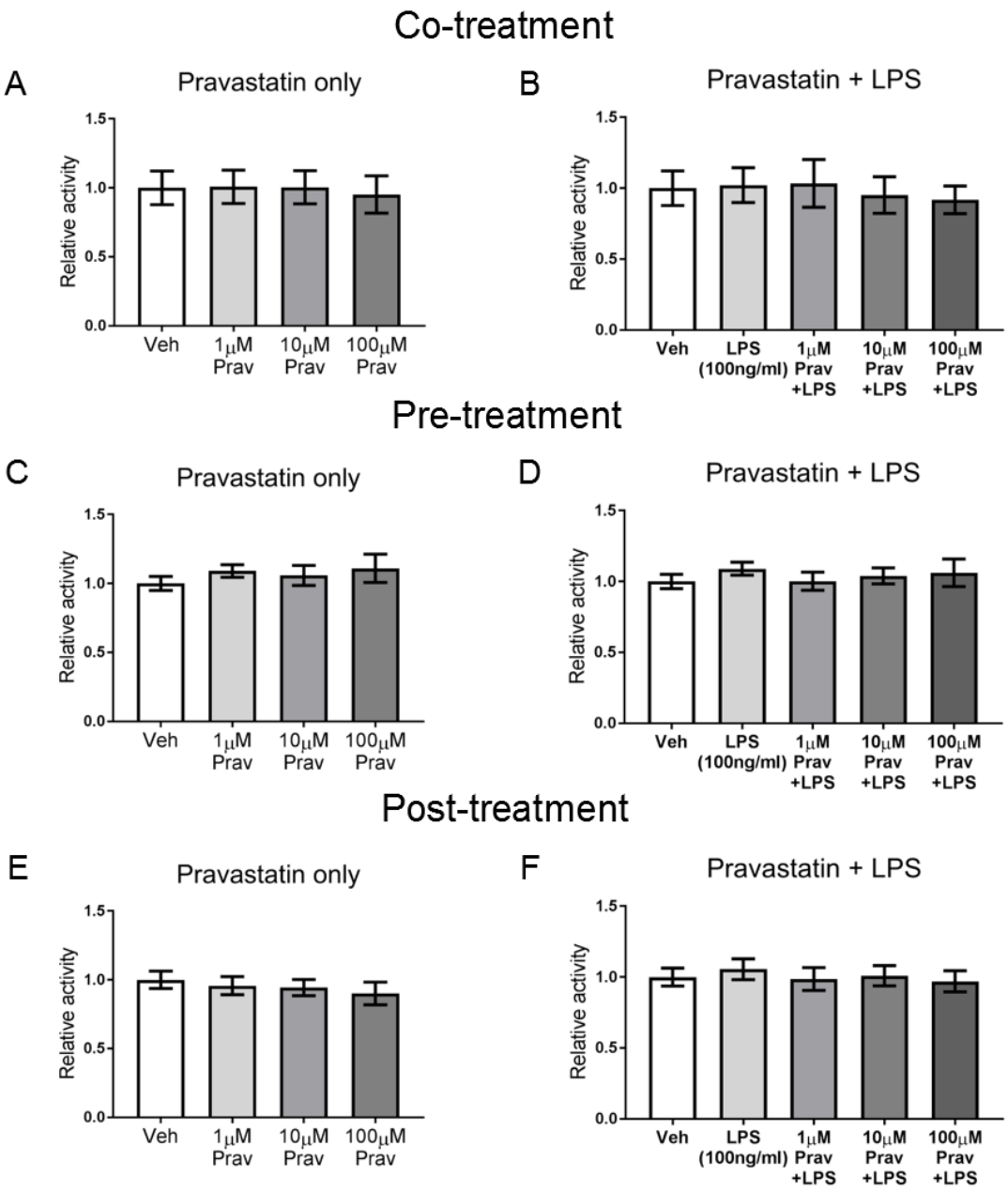

Figure 3.3. Myometrial cell metabolic activity following pravastatin and LPS treatment (24 hours). (A-F) Myometrial cell metabolic activity was unaffected by 24 hours of up to 100μM pravastatin and LPS (100mg/ml) co-, pre- and post-treatment. n=6 (in triplicate), vehicle (veh; PBS) group shared for pravastatin only (prav) and pravastatin + LPS, mean ± SEM**.**

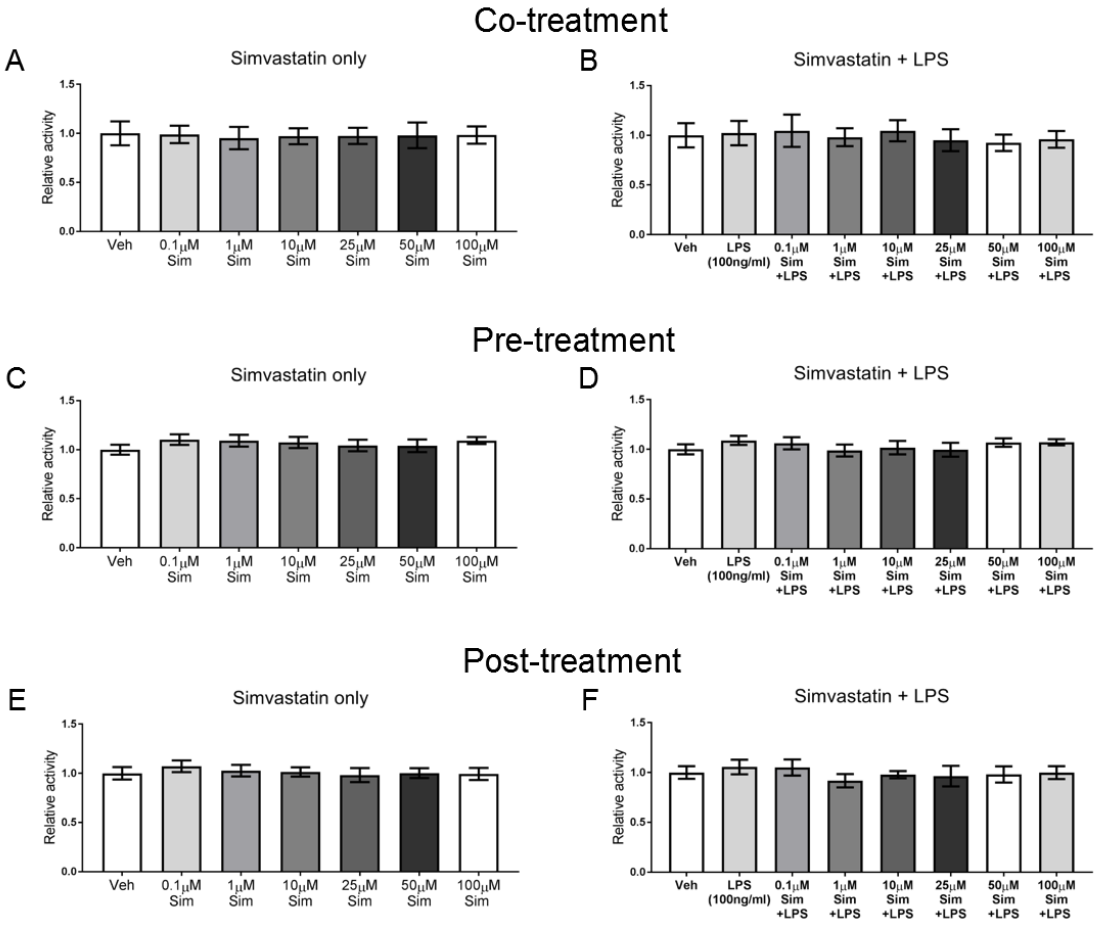

Figure 3.4. Myometrial cell metabolic activity following simvastatin and LPS treatment (24 hours). (A-F) Myometrial cell metabolic activity was unaffected by up to 100μM simvastatin and LPS (100ng/ml) co-, pre- and post-treatment for 24 hours. n=6 (in triplicate), vehicle (veh; PBS) group shared for simvastatin only (sim) and simvastatin + LPS, mean ± SEM.

3.3.3 The effect of statin treatment on inflammatory mediator mRNA expression and protein production in myometrial cells stimulated with LPS

To establish the effect of pravastatin and simvastatin treatment on the expression of inflammatory mediators in myometrial cells, mRNA expression and protein secretion of the pro-inflammatory mediators IL-6 and IL-8, were determined by qRT-PCR and ELISA, respectively. In addition, the mRNA expression of anti-inflammatory cytokines, *IL-10* and *IL-13*, was also investigated. LPS-stimulated myometrial cells were either co-, pre- or post-treated with pravastatin/simvastatin for 24 hours.

3.3.3.1 The effect of pravastatin on pro-inflammatory mediator expression and protein secretion in myometrial cells

LPS upregulated concentrations of *IL-6* mRNA 6.7 fold in the co-treatment group ($p<0.0001$ vs vehicle), which was validated at the protein level (mean 2207 ± 489pg/ml vs vehicle mean 88.3 ± 18pg/ml; $p<0.0001$; Figures 3.5A, 3.6A). LPS also increased *IL-6* mRNA and IL-6 protein in comparison to pravastatin treatment alone ($p<0.0001$). Co-treatment with pravastatin did not affect these LPS-stimulated levels of *IL-6* mRNA or IL-6 protein. *IL-8* expression was robustly upregulated 50.2 fold by LPS ($p<0.0001$ vs vehicle), as was IL-8 secretion from the myometrial cells (mean 4704 ± 893pg/ml vs vehicle mean 81.3 ± 23pg/ml; $p<0.0001$; Figures 3.5B, 3.6B). The same effect was observed in comparison to pravastatin treatment alone ($p<0.0001$). However, co-treatment with pravastatin and LPS did not alter *IL-8* expression or IL-8 protein levels compared with LPS alone.

In the pre-treatment group, LPS stimulated *IL-6* to increase 11.5 fold ($p<0.0001$ vs vehicle) and the IL-6 protein levels were increased (mean 2417 ± 715pg/ml vs vehicle mean 78.9 ± 16pg/ml; $p<0.0001$; Figures 3.5C, 3.6C). These levels were also significantly increased in comparison to pravastatin treatment alone ($p<0.0001$). Pre-treatment with pravastatin before LPS did not impact on *IL-6* mRNA or IL-6 secretion. LPS induced an 87.6 fold increase in *IL-8* expression ($p<0.0001$ vs vehicle), which was confirmed by an increase in IL-8 production by the myometrial cells (mean 6689 ± 1502pg/ml vs vehicle mean 105.4 ± 42pg/ml; $p<0.0001$; Figures 3.5D, 3.6D). Again, these levels were also significantly increased compared to pravastatin only treatment ($p<0.0001$). However, pravastatin pre-treatment did not alter the levels of *IL-8* mRNA or IL-8 protein compared with LPS only.

In the post-treatment group, *IL-6* expression increased 7.3 fold with LPS stimulation ($p<0.0001$ vs vehicle) and IL-6 protein also increased (mean 2678 ± 528pg/ml vs vehicle mean 138.3 ± 32pg/ml; $p<0.0001$; Figures 3.5E, 3.6E). These levels were significantly higher compared to pravastatin treatment alone ($p<0.0001$) but pravastatin treatment post-LPS stimulation did not affect *IL-6* RNA or IL-6 protein secretion. LPS stimulated a 51.5 fold increase in *IL-8* expression ($p<0.0001$ vs vehicle) and robustly increased IL-8 protein secretion from the myometrial cells (mean 6161 ±

902pg/ml vs vehicle mean 164.1 ± 31pg/ml; $p<0.0001$; Figures 3.5F, 3.6F). This increase in IL-8 gene and protein production was also significantly higher than pravastatin alone ($p<0.0001$). Again, post-treatment with pravastatin after LPS did not induce any alteration of *IL-8* mRNA or IL-8 protein levels compared with LPS alone.

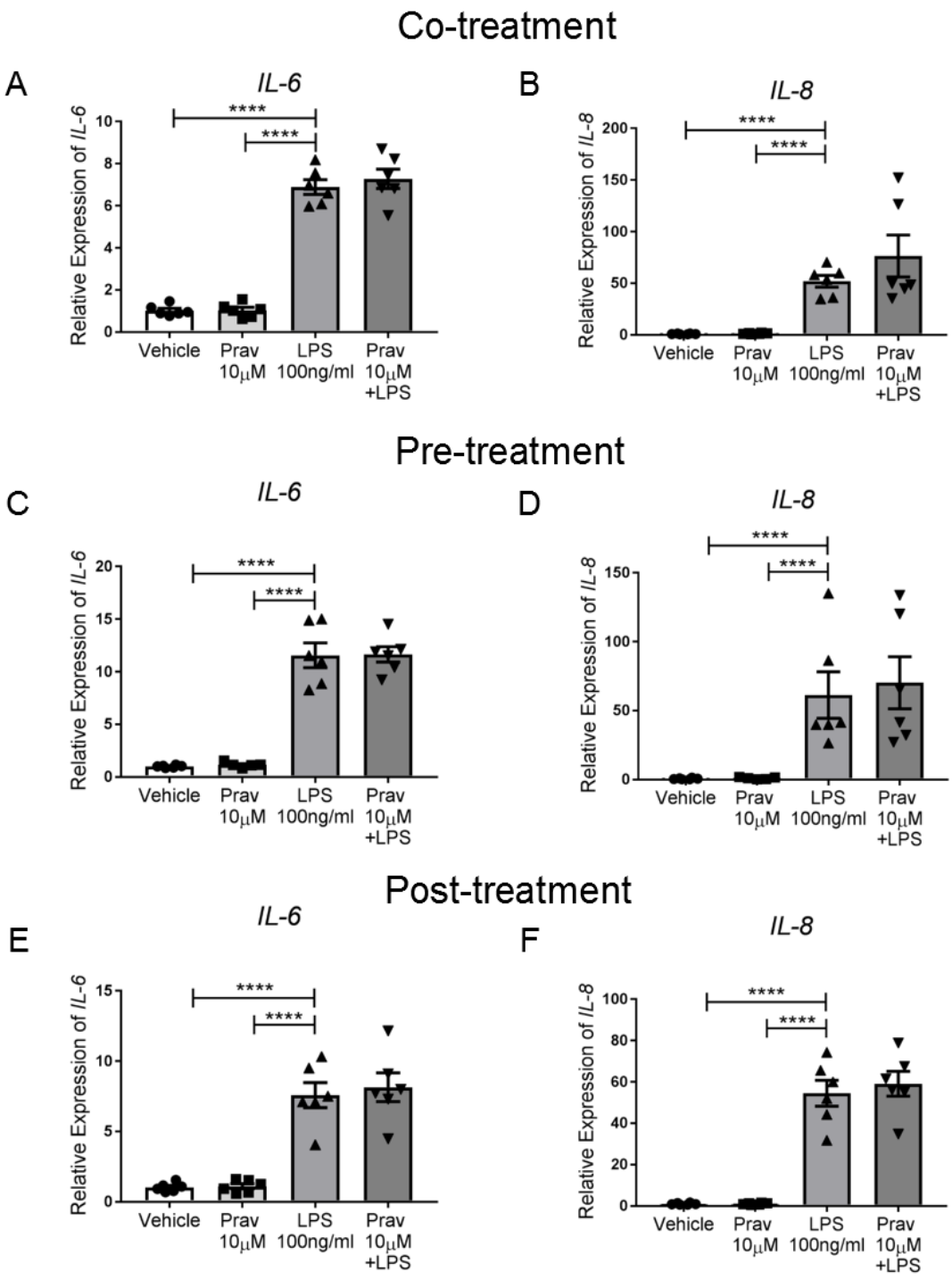

Figure 3.5. The effect of pravastatin treatment on pro-inflammatory mRNA expression in human myometrial cells following LPS stimulation (24 hours). Co-, pre- and post-treatment regimens with pravastatin and LPS in human myometrial cells. (A-F) LPS increased the expression of *IL-6* and *IL-8* from myometrial cells. This upregulation was unaltered by the co-, pre- or post-treatment with pravastatin. n=5-6 (in duplicate), vehicle (PBS) and LPS data shared for pravastatin/simvastatin experiments, ****p<0.0001, mean fold change ± SEM, one-way ANOVA with Dunnett's post hoc test.

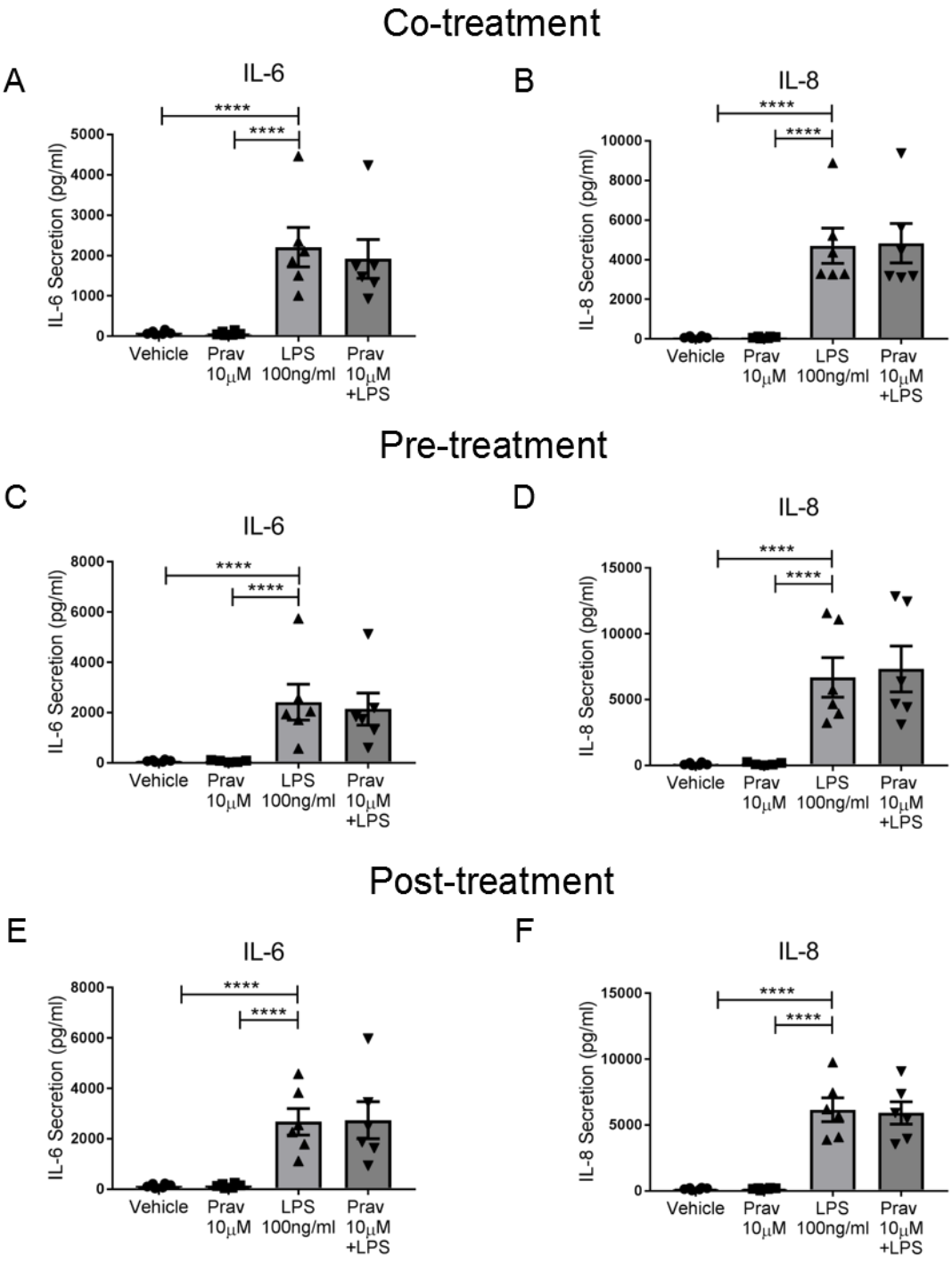

Figure 3.6. The effect of pravastatin treatment on pro-inflammatory mediator secretion from LPS-stimulated human myometrial cells (24 hours). Co-, pre- and post-treatment regimens with pravastatin and LPS in human myometrial cells. (A-F) LPS increased the secretion of IL-6 and IL-8 from myometrial cells. This increase was unaltered by the co-, pre- or post-treatment with pravastatin. n=5-6 (in duplicate), vehicle (PBS) and LPS data shared for pravastatin/simvastatin experiments, ****p<0.0001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

3.3.3.2 The effect of pravastatin on anti-inflammatory gene expression in myometrial cells

Pravastatin treatment did not alter the pro-inflammatory mediators, IL-6 and IL-8, so the effect of pravastatin treatment on the anti-inflammatory genes, *IL-10* and *IL-13* was then investigated. LPS administration did not significantly alter *IL-10* or *IL-13* expression following any treatment regimen. Similarly, pravastatin treatment did not alter the expression of these anti-inflammatory genes in the co-, pre- or post-treatment groups (Figure 3.7).

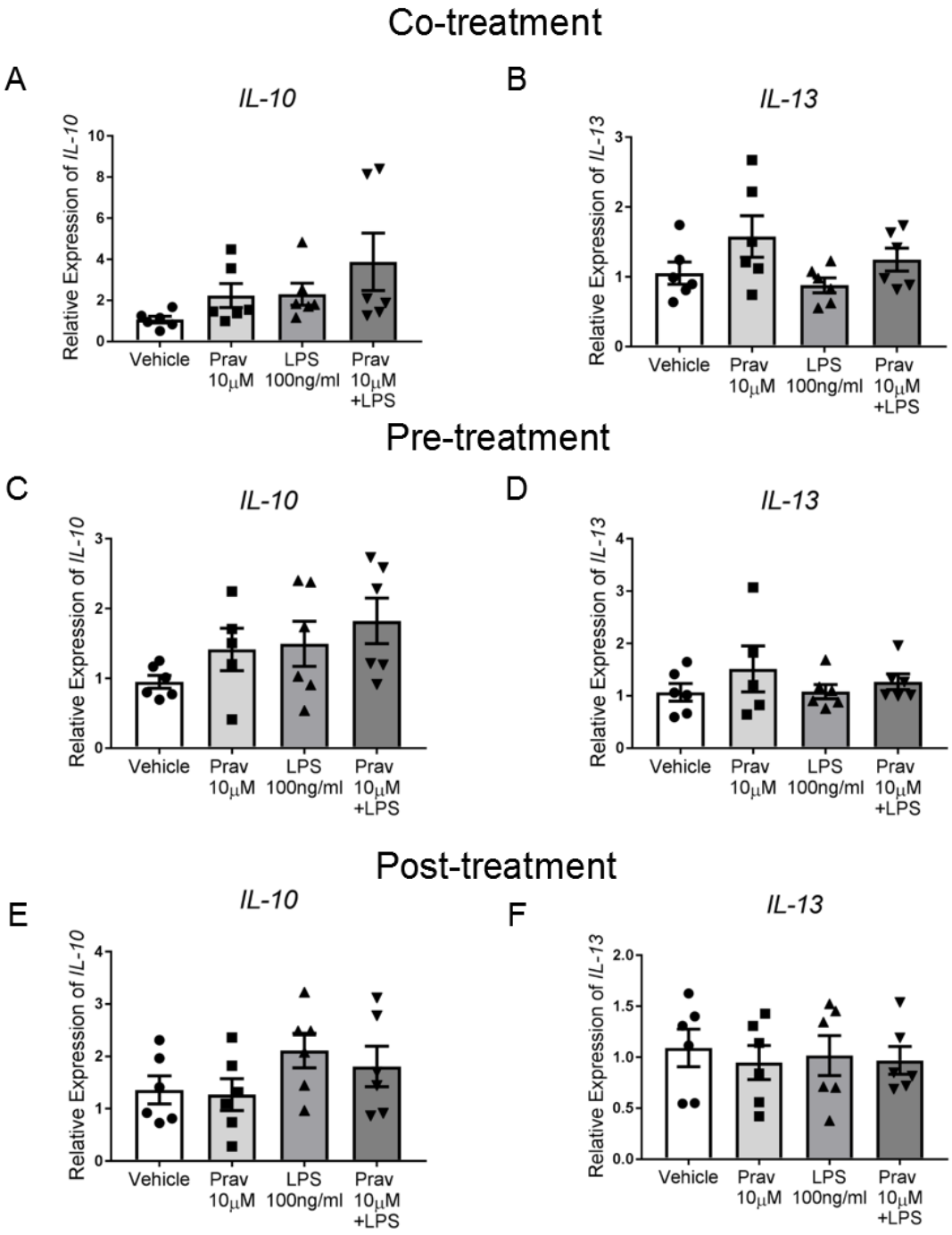

Figure 3.7. The effect of pravastatin treatment on anti-inflammatory gene expression in human myometrial cells (24 hours). Co-, pre- and post-treatment regimens with pravastatin and LPS in human myometrial cells. *IL-10* and *IL-13* expression were unaffected by pravastatin or LPS treatment. n=5-6 (in duplicate), vehicle and LPS data shared for pravastatin/simvastatin experiments, mean fold change ± SEM.

3.3.3.3 The effect of simvastatin on pro-inflammatory mediator mRNA expression and protein production in myometrial cells

Given the lack of effect of pravastatin on LPS-induced inflammatory mediator expression, an alternative statin, simvastatin, was investigated. In the co-treatment group, LPS alone stimulated upregulation of *IL-6* expression, in comparison to vehicle and simvastatin only treated cells ($p<0.0001$; Figure 3.8A). This was validated by a significant increase in the secretion of IL-6 into the cell supernatant, in comparison to the vehicle group, as described in 3.3.3.1, and the simvastatin only group ($p<0.0001$; Figure 3.9A). When cells were co-treated with 50μM simvastatin and LPS, the *IL-6* fold change was significantly lower compared to LPS alone (2.4 fold vs 6.7 fold, $p<0.0001$) and IL-6 secretion was also significantly reduced, compared to when LPS was given alone (mean 617.3 ± 163pg/ml vs LPS mean 2207 ± 489pg/ml; $p=0.0015$). LPS stimulation increased *IL-8* mRNA expression and IL-8 protein production robustly, in comparison to the vehicle and simvastatin only groups ($p<0.0001$; Figures 3.8B, 3.9B). *IL-8* mRNA was lower when the cells were co-treated with 50μM simvastatin and LPS, compared to LPS alone (30.3 fold vs 50.2 fold, $p=0.0312$). IL-8 protein expression was also lower with 50μM simvastatin and LPS co-treatment, compared to LPS alone (2295 ± 266pg/ml vs LPS mean 4704 ± 893pg/ml; $p=0.0207$). Interestingly, co-treatment with 10μM simvastatin and LPS upregulated *IL-8* mRNA expression ($p=0.0243$ vs LPS) but this was not observed at the protein level.

In the pre-treatment group, again LPS stimulated a significant increase in *IL-6* ($p<0.0001$) and IL-6 protein secretion was significantly increased by LPS, compared to vehicle and simvastatin only treated cells ($p=0.0001$, $p<0.0001$, respectively; Figures 3.8C, 3.9C). When the cells were pre-treated with 50μM simvastatin prior to LPS stimulation, *IL-6* mRNA expression was significantly lower, when compared to LPS alone (5.5 fold vs 11.5 fold, $p=0.0007$). Although simvastatin pre-treatment showed a trend to reduce IL-6 at the protein level, this was not statistically significant. LPS also increased *IL-8* mRNA expression ($p<0.0001$) and IL-8 secretion from the myometrial cells, in comparison to the vehicle and simvastatin only treated cells ($p<0.0017$; Figures 3.8D, 3.9D). Pre-treatment with simvastatin did not alter *IL-8* mRNA or IL-8 protein production, compared to the LPS group.

In the post-treatment group, LPS induced *IL-6* upregulation, compared to the vehicle and simvastatin only treated cells ($p<0.0001$; Figures 3.8E, 3.9E). This was also observed at the protein level for IL-6 ($p=0.0004$). When the myometrial cells were first stimulated with LPS, then post-treated with 50μM simvastatin, *IL-6* mRNA was lower, compared to LPS stimulation alone (2.8 fold vs 7.3 fold, $p=0.0003$). Although, IL-6 secretion was not significantly reduced. LPS stimulated a large increase in *IL-8* expression and IL-8 secretion, compared to vehicle and simvastatin only treated cells ($p<0.0001$; Figures 3.8F, 3.9F). When cells received 50μM simvastatin post-LPS treatment, *IL-8* mRNA levels were significantly reduced (30.0 fold vs 51.4 fold, $p=0.041$) and IL-8 secretion was significantly lowered, in comparison to LPS stimulation alone (mean 3640 ± 655pg/ml vs LPS mean 6161 ± 902pg/ml; $p=0.0427$).

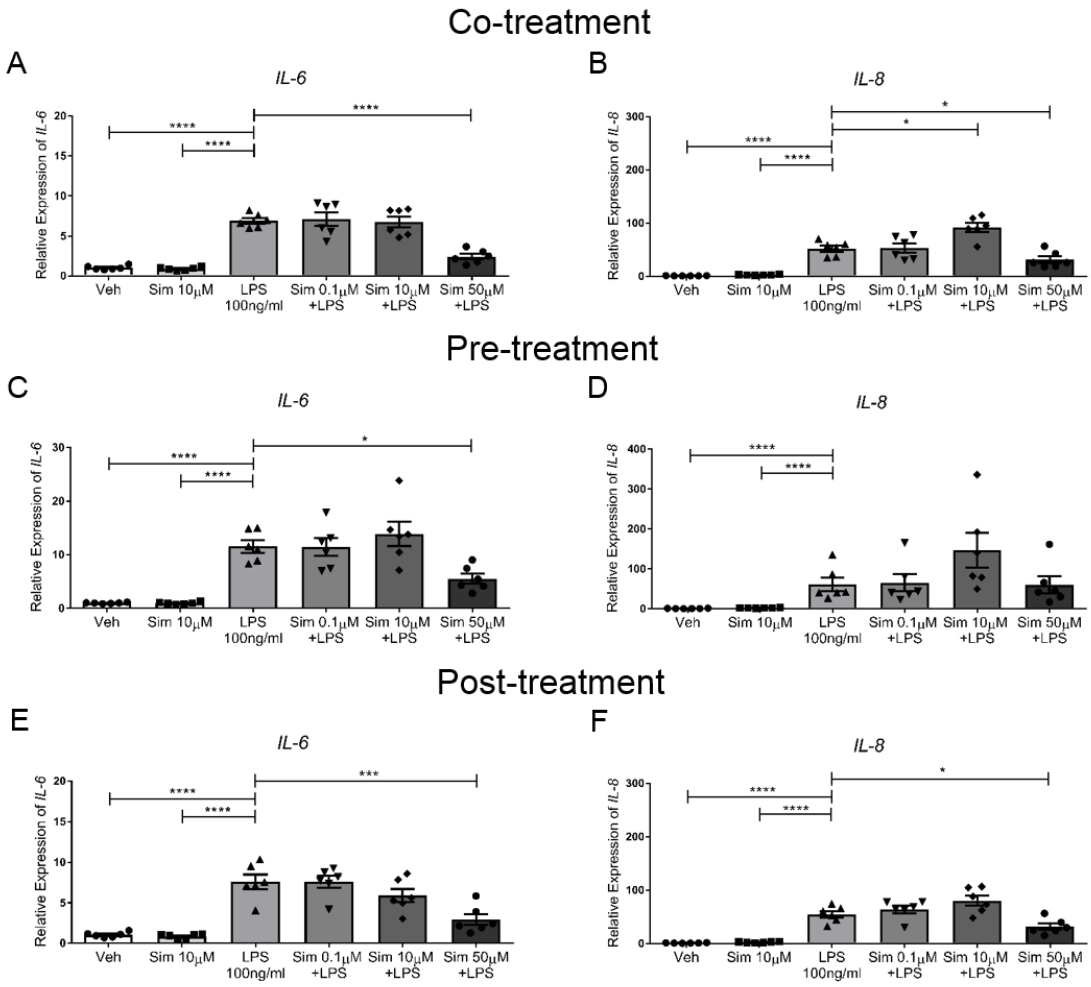

Figure 3.8. The effect of simvastatin treatment on pro-inflammatory mRNA expression in LPS-stimulated human myometrial cells (24 hours). Co-, pre- and post-treatment regimens with simvastatin and LPS in human myometrial cells. (A-F) LPS robustly upregulated *IL-6* and *IL-8* expression in human myometrial cells. (A, B) Co-treatment with LPS + 50μM simvastatin downregulated *IL-6* and *IL-8*. (C, D) Pre-treatment with 50μM simvastatin downregulated *IL-6* but not *IL-8* expression. (E, F) Post-LPS treatment with 50μM simvastatin downregulated *IL-6* and *IL-8* expression. n=6 (in duplicate), vehicle (PBS) and LPS data shared for pravastatin/simvastatin experiments, *p<0.05, ***p<0.001, ****p<0.0001, mean fold change ± SEM, one-way ANOVA with Dunnett's post hoc test.

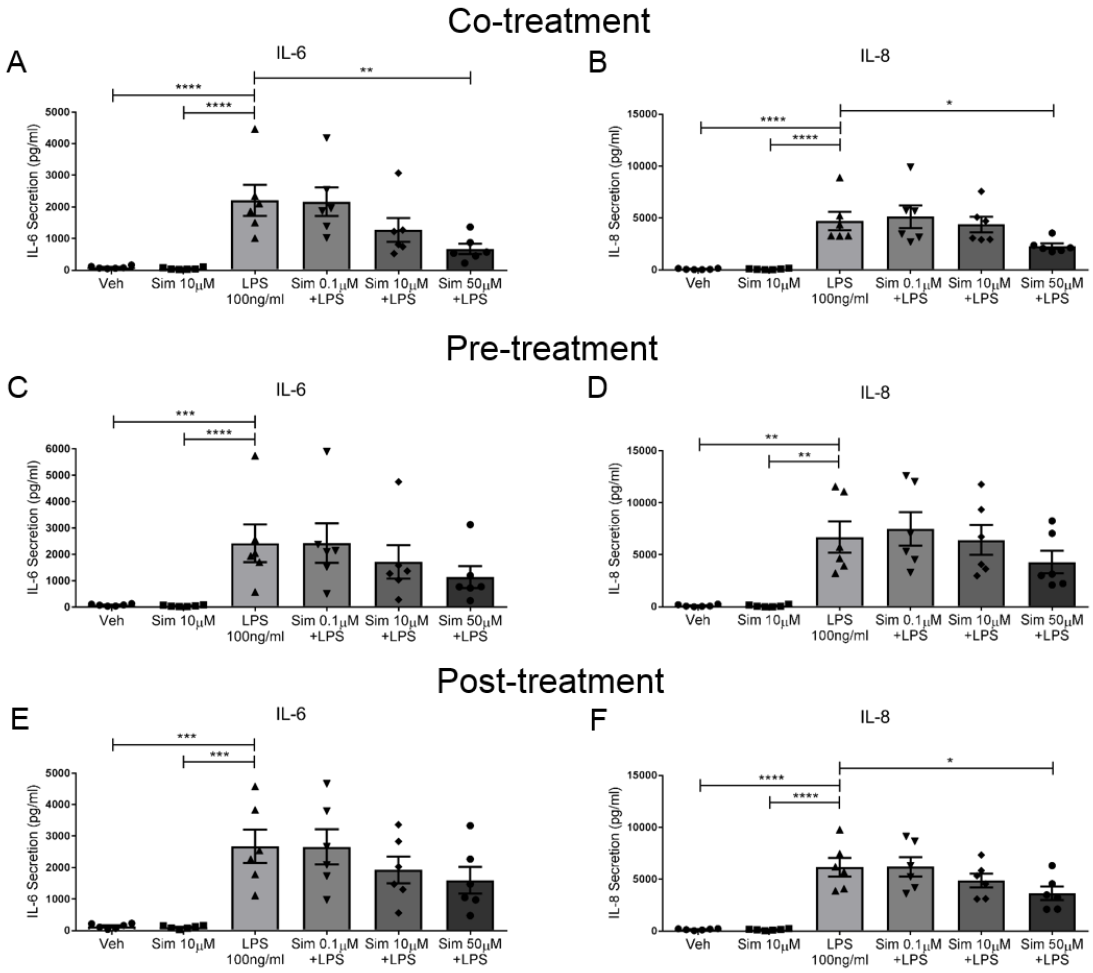

Figure 3.9. The effect of simvastatin on pro-inflammatory mediator secretion in LPS-stimulated human myometrial cells (24 hours). (A-F) LPS treatment increased IL-6 and IL-8 secretion from human myometrial cells. (A, B) Co-treatment with LPS + 50μM simvastatin reduced IL-6 and IL-8 secretion. (C, D) Pre-treatment with simvastatin did not affect LPS-induced IL-6 or IL-8 secretion at 24 hours. (E-F) Post-LPS treatment with 50μM simvastatin reduced IL-8 but not IL-6 levels. n=6 (in duplicate), vehicle (PBS) and LPS data shared for pravastatin/simvastatin experiments, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

3.3.3.4 The effect of simvastatin on anti-inflammatory cytokine mRNA expression in myometrial cells

In the co-treatment group, 10μM simvastatin alone significantly upregulated *IL-10* expression in the myometrial cells, compared to cells treated only with LPS (5.7 fold vs 2.2 fold LPS, p=0.0003; Figure 3.10A). Co-treatment with 10μM and 50μM simvastatin and LPS also upregulated *IL-10* expression (7.3 fold, p<0.0001; 5.7 fold, p=0.0003 vs LPS, respectively). This co-treatment effect was also observed with *IL-*

13 mRNA expression, where there was a 1.8 fold upregulation of *IL-13* mRNA concentration by 10µM simvastatin alone (p=0.0008 vs LPS; Figure 3.10B). Additionally, co-treatment with LPS and 10µM simvastatin upregulated *IL-13* 1.5 fold (p=0.0064) and 50µM simvastatin upregulated the gene 1.7 fold (p=0.0021), which was significantly elevated in comparison to LPS treatment alone.

When the myometrial cells were pre-treated with 10µM and 50µM simvastatin before LPS stimulation, *IL-10* was significantly upregulated (7.3 fold, p=0.0165; 7.1 fold p=0.0027 vs LPS, respectively; Figure 3.10C). There was a modest 1.7 fold upregulation of *IL-13* expression by 10µM simvastatin alone (p=0.0235 vs LPS; Figure 3.10D). *IL-13* was also increased by 10µM and 50µM simvastatin pre-treatment (1.7 fold, p=0.0172; 1.9 fold, p=0.0031 vs LPS, respectively).

Treatment with 10µM simvastatin post-LPS stimulation upregulated *IL-10* expression 4.5 fold (p=0.0018 vs LPS) but *IL-13* was unaltered by post-treatment with simvastatin (Figures 3.10E, F).

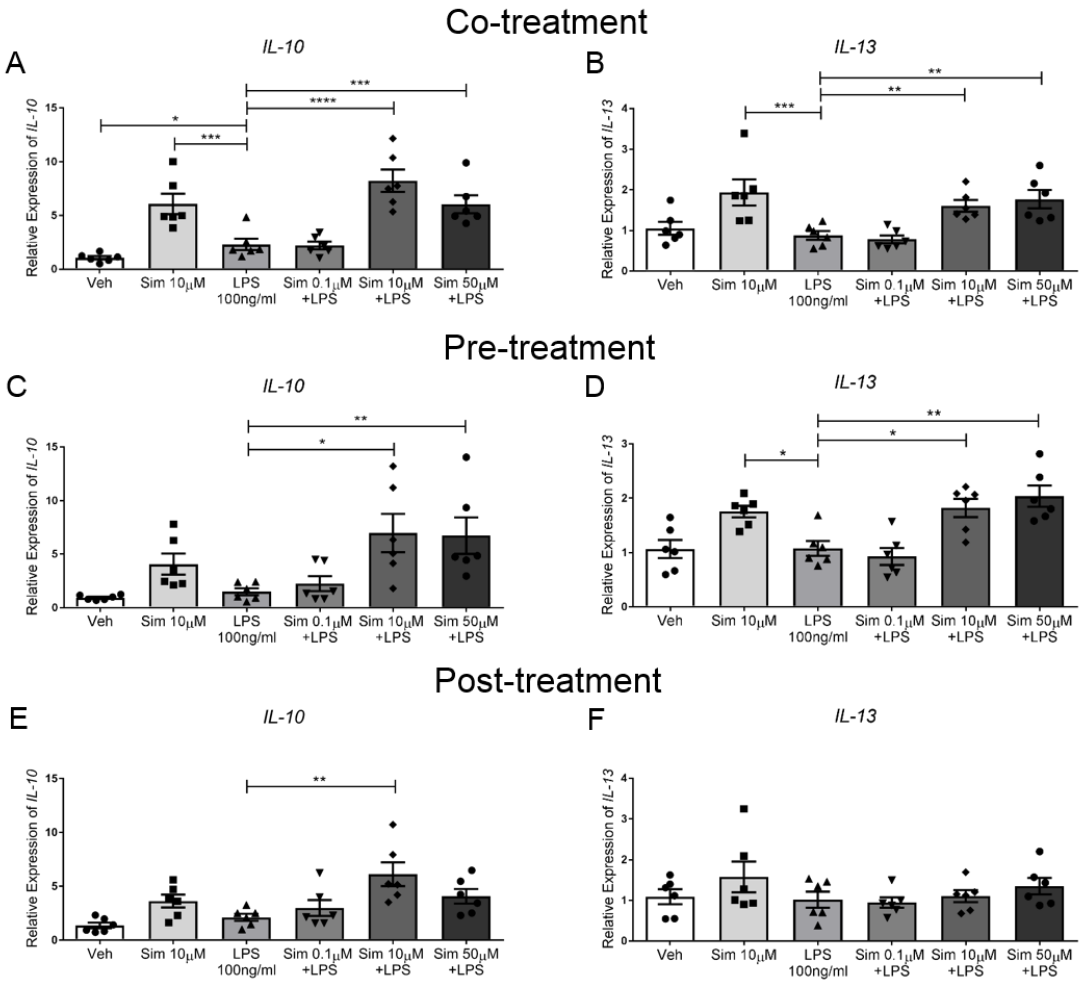

Figure 3.10. The effect of simvastatin treatment on anti-inflammatory mRNA expression alone and in combination with LPS stimulation of human myometrial cells at 24 hours. (A, B) Simvastatin treatment alone significantly upregulated *IL-10* and *IL-13* expression. Co-treatment with LPS and 10μM and 50μM simvastatin also upregulated *IL-10* and *IL-13* expression. (C) Pre-treatment with 10μM and 50μM simvastatin upregulated *IL-10* expression. (D) Simvastatin alone and pre-treatment with 10μM and 50μM simvastatin before LPS stimulation upregulated *IL-13* expression. (E) Post-LPS treatment with 10μM simvastatin upregulated *IL-10*. (F) *IL-13* levels were unaltered by post-LPS treatment with simvastatin. n=6 (in duplicate), vehicle (PBS) and LPS data shared for pravastatin/simvastatin experiments, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, mean fold change ± SEM, one-way ANOVA with Dunnett's post hoc test.

3.4 Discussion

3.4.1 The effect of statin treatment on myometrial cell morphology

3.4.1.1 Pravastatin

Pravastatin is a commonly used statin, as it has limited side effects (del Sol and Nanayakkara 2008). This drug is hydrophilic and requires selective uptake by a specific transporter protein, which is exclusively expressed in the liver. Therefore, pravastatin is highly hepatoselective (Ziegler and Hummelsiep 1993; Hatanaka 2000).

In these experiments, 10μM pravastatin did not affect myometrial cell morphology. The inclusion of LPS did not have any additional effect on the cell shape. This was true for co-, pre- and post-treatment groups. The cells, by appearance, seemed healthy, implying that the treatment was not having an adverse effect. However, the appearance alone cannot be relied upon for assessing cell function.

These results are in agreement with multiple studies which found that, even at high concentrations, pravastatin did not alter the morphology of cells such as aortic smooth muscle cells, mesenchymal stem cells and various cancer cell lines (Lee et al. 2004; Martinet et al. 2008; Menter et al. 2011). However, pravastatin was found to have a pronounced effect on hepatocyte morphology, due to its facilitated uptake into these cells (Menter et al. 2011). It has been proposed, that while most lipophilic statins do affect the morphology of certain cells, the reason pravastatin does not is because the drug does not gain entry into the cells (Menter et al. 2011). It is highly possible that these myometrial cells do not express the transporter protein required for pravastatin to enter the cell.

The myometrial cells could be treated with higher concentrations of pravastatin to further confirm that this treatment does not have an effect on cell morphology. However, this effect may be due to pravastatin not actually being taken up by the cells. As no one else has investigated statin treatment on these myometrial cells, it is unknown if they possess the transporters required, particularly as they are immortalised cells, which may not function and contain exactly the same machinery as they would *in vivo*.

3.4.1.2 Simvastatin

As a lipophilic drug, simvastatin readily diffuses across cell membranes and alters a variety of genes, namely in the mevalonate pathway, affecting cell cycle progression, DNA replication, apoptosis and cytoskeleton signalling (Gbelcova et al. 2017).

In this study, simvastatin treatment may have had a concentration-dependent effect on cell morphology. There was no effect using 0.1µM simvastatin but at concentrations of 10µM and 50µM, cell morphology appeared marginally altered, as evidenced by the cells becoming slightly more elongated. However, there did not appear to be an additive effect of LPS treatment. Although changes in cell shape are not ideal, as it suggests the drug is affecting the regulation of the cell in some way, these possible changes were only slight and were not quantified. Furthermore, morphology alone does not give an indication of functionality.

It has been well documented that simvastatin is capable of altering the morphology of cells. Simvastatin induced morphology changes in human endometriotic stromal cells at concentrations equivalent to those used in this chapter, as well as inhibiting proliferation and preventing the contraction of these cells (Nasu et al. 2009). Another study on endometrial stromal cells also reported a disruption in the cytoskeleton of cells, as a result of simvastatin treatment. F-actin fibres became disorganised and disassembled after 24 hours of 10µM simvastatin treatment, which was believed to have been caused by the inhibition of isoprenylation within the mevalonate pathway (Sokalska et al. 2010).

One study investigated the effect of simvastatin on both normal and cancer cell lines. Simvastatin caused morphology changes in prostate and colon cancer cells, where the mitochondria redistributed to the cellular processes. In a pancreatic cancer cell line, cellular processes began to extend just 1 hour after treatment with 10µM simvastatin. In hepatocytes, simvastatin disrupted caveolae, small lipid rafts associated with actin filaments, causing cytoplasmic process extension, resulting in morphology changes. Again, these changes to the cytoskeleton were attributed to the inhibition of isoprenylation (Menter et al. 2011). In contrast, 10µM simvastatin did not alter the morphology of rabbit aorta smooth muscle cells. However, when these cells were

treated with a high concentration of simvastatin (100µM), they began to detach, changing from elongated to globular, spherical cells (Martinet et al. 2008).

This experiment could be repeated and quantified at different concentrations, to determine if there is any true morphology change and to give an indication of the concentration at which an effect is first observed. Further experiments could also be performed to investigate the location or distribution of cytoskeletal proteins.

3.4.2 The effect of statin treatment on myometrial cell metabolic activity

3.4.2.1 Pravastatin

When assessing cell metabolic activity as a measure of cell viability, there was no effect of pravastatin treatment up to concentrations of 100µM, both alone and in combination was LPS treatment. This implies that pravastatin treatment is not negatively affecting the activity of the myometrial cells at 24 hours.

Many other studies looking at a variety of cell types, such as macrophages, smooth muscle cells and multiple cancer cell lines, also found that pravastatin did not affect cell viability (Guijarro et al. 1999; Sindermann et al. 2000; Martinet et al. 2008; Menter et al. 2011). However, as mentioned previously, these studies highlighted the possibility that pravastatin is perhaps too hydrophilic to enter into these cells.

3.4.2.2 Simvastatin

Simvastatin did not affect myometrial cell metabolic activity at concentrations up to 100µM. There was also no effect of adding LPS treatment, in the co-, pre- and post-treatment groups, at 24 hours. This suggests that simvastatin treatment does not affect the viability of the myometrial cells at the concentrations used in the experiments outlined in this chapter, which is reassuring.

There is varying evidence regarding the effect of simvastatin on cell viability, which appears to be cell-dependent (Morikawa et al., 2004). For example, simvastatin induced concentration-dependent cell death in ovarian, endometrial and cervical cancer cell lines but with limited or no cytotoxic effect on normal cells (Kato et al., 2010). Menter et al. (2011) found that simvastatin was more effective at inducing cell

death in poorly differentiated cancer cells, than those which were well differentiated. Interestingly, these effects were observed using lower concentrations of simvastatin than were used in the experiments within this chapter. Another study found that, while 30μM simvastatin treatment lowered the cell viability of the J774A.1 macrophage cell line, primary mouse peritoneal macrophages and rabbit aortic smooth muscle cells were virtually unaffected (Croons et al., 2010). In addition, 50μM simvastatin had no effect on human macrophage cell viability at 24 hours, which agrees with the results in this chapter (Tuomisto et al., 2008). Therefore, it appears that simvastatin can affect cell viability but mostly in cancer cells, where this effect can be considered beneficial.

Other than cell type, other explanations for this differing effect of simvastatin propose that cell culture environment could play a role. For instance, sub-confluent cells were found to be less sensitive to simvastatin treatment, compared to cells seeded at a low density (Sindermann et al., 2000). Reduction in cell viability was also more common when cells were cultured in medium containing low serum concentration, which is a common procedure to synchronise the growth phase of the cells for experimentation, forcing them to enter the quiescent G0/G1 phase (Croons et al., 2010). The experiments in this chapter were performed on sub-confluent cells, which were partially serum-starved by reducing the serum concentration from 10% (v/v) to 5% (v/v) FBS in DMEM. Other studies perform serum starvation at levels as low as 0.05% (Pirkmajer and Chibalin, 2011). However, previous experimentation in our laboratory found that these myometrial cells require a degree of serum at all times in order to remain healthy and function consistently.

The focus of these experiments was to assess the viability of the cells, to ensure they were alive and metabolically active at different statin concentrations, which was confirmed. However, further experiments could be performed to assess the function of the cells in more detail, such as looking at proliferation, caspase activity or DNA fragmentation.

3.4.3 The effect of statin treatment on inflammation in a human myometrial cell line

3.4.3.1 Pravastatin

Pravastatin treatment did not affect the mRNA or protein encoded by either pro-inflammatory mediators, IL-6 or IL-8, or the mRNA expression of anti-inflammatory mediators, *IL-10* or *IL-13*, in the co-, pre- or post-treatments of LPS-stimulated human myometrial cells.

This is surprising, as there is extensive evidence from human studies that pravastatin can reduce inflammation. A key study addressing the anti-inflammatory effect of statins was the PRINCE trial. This trial assessed the effect of pravastatin on the plasma levels of the inflammatory biomarker, CRP. Pravastatin lowered CRP levels in subjects with cardiovascular disease, as well as in the subjects with no history of cardiovascular disease, after 24 weeks. This effect was independent of LDL cholesterol levels (Albert et al. 2001). Pravastatin also reduced the plasma levels of CRP in a small cohort of patients with type 2 diabetes (Sommeijer et al. 2004). As well as lowering inflammation in patients with carotid artery plaques and men with hypercholesterolemia (Crisby et al. 2001; Kalela et al. 2001; Solheim et al. 2001).

Evidence of the anti-inflammatory effect of pravastatin is also available from a wealth of *in vitro* studies. For instance, pravastatin significantly reduced LPS-stimulated IL-6, IL-8 and GM-CSF protein production in human bronchial epithelial cells (Iwata et al. 2012). Pravastatin concentration-dependently reduced CRP, IL-6 and TNF at the gene and protein level in rat vascular smooth muscle cells. However, this affect was only observed at pravastatin concentrations above 30μM (Guo et al. 2012; Lu et al. 2015). Pravastatin downregulated the release of pro-inflammatory mediators, TNF and CCL2 but not IL-6, from human monocytes but only at very high concentrations of 100μM and 500μM (Grip et al. 2000).

The choice of pravastatin concentration (10μM) was made initially as it is one of the most commonly selected concentrations used for investigating the pleiotropic effects of statins *in vitro*, albeit higher than therapeutically relevant concentrations (Bjorkhem-Bergman et al. 2011; Iwata et al. 2012; Chang et al. 2014; Statt et al. 2015).

Nevertheless, in this instance there was no effect of pravastatin treatment on LPS-induced inflammation in myometrial cells. An important point to consider is that *in vitro* conditions are not wholly representative of the *in vivo* environment. For example, there are animal and human studies that suggest pravastatin could improve pregnancy outcomes in women with anti-phospholipid syndrome (Redecha et al. 2008; Lefkou et al. 2016). However, in a human trophoblast cell line, pravastatin treatment did not reduce inflammation or inhibit anti-phospholipid antibody-mediated changes (Odiari et al. 2012).

The experiments in this chapter could be repeated with a higher concentration of pravastatin but it is likely that the concentration would need to be increased substantially in order to see any affect. The presence of transport protein, OATP1B1, which facilitates pravastatin uptake, could be investigated in these cells. As discussed in the previous sections, the hydrophilic nature and hepatoselectivity of pravastatin prevents it from acting on many cell types, particularly *in vitro*, when only one cell type is present. Therefore, it is possible that pravastatin may still have anti-inflammatory properties on the myometrium *in vivo*, which is worth investigating.

3.4.3.2 Simvastatin

Simvastatin treatment significantly reduced the mRNA expression and protein production of IL-6 and IL-8, when cells were stimulated with LPS. Specifically, the higher concentration of simvastatin (50µM) significantly reduced these mediators at both the gene and protein level, when cells were co-treated with simvastatin and LPS. Pre-treatment with 50µM simvastatin significantly reduced IL-6 mRNA expression but this didn't reach significance at the protein level and there was no effect of pre-treatment with simvastatin on IL-8 gene or protein expression. This was interesting, as the pre-treatment group was incubated with LPS for the least amount of time (18 hours), while simvastatin was present for the whole 24 hours. However, there was also a lot of variation between individual experiments, which may have prevented statistical significance. Higher passage number, for example, could have affected the responsiveness of these cells, as all other variables remained constant. Following 6 hours of LPS stimulation, post-treatment with 50µM simvastatin significantly reduced

IL-6 gene expression, as well as *IL-8* gene expression and IL-8 secretion from the myometrial cells.

Interestingly, simvastatin alone was capable of increasing the expression of *IL-10* and *IL-13*, both of which are classed as anti-inflammatory genes. This increase in expression was seen during co-treatment experiments, when the cells were exposed to statins for the full 24 hour incubation. Simvastatin alone increased *IL-13* but not *IL-10* in the pre-treatment group. Again, the variation between samples of the simvastatin only group may explain why *IL-10* was not significantly increased in comparison to the LPS group. There was no effect of simvastatin alone on *IL-10* and *IL-13* in the post-treatment group. This may be because simvastatin was only present for 18 hours of the incubation, as the cells were being stimulated with LPS for the first 6 hours of the 24 hour incubation. *IL-10* gene expression was significantly upregulated after post-LPS treatment with 10μM simvastatin but there was no significant effect on *IL-13* expression. Ideally, it would helpful to have protein data for the anti-inflammatory cytokines, to see whether this reinforces the changes observed at the gene level. Unfortunately, as *IL-10* is expressed in very low levels, the protein was undetectable by our ELISA assay. Presumably *IL-13*, which is expressed at even lower levels, would be difficult to measure other than perhaps by an especially sensitive assay.

These anti-inflammatory properties of simvastatin have been widely investigated in human and animal studies, as well as *in vitro* (Schonbeck and Libby 2004; Jain and Ridker 2005; Loppnow et al. 2011). For example, in hypercholesteraemic patients, serum IL-6, IL-8 and CCL2 were reduced following 6 weeks of simvastatin treatment (Rezaie-Majd et al. 2002). In mice, simvastatin significantly reduced the mRNA expression of *Il-1β* and *Il-6* in the uterus of a pregnant mouse that had received LPS (Basraon et al. 2012). Treatment with simvastatin has also resulted in reduced synthesis of inflammatory mediators from various cells types in culture, such as human umbilical vein endothelial cells, primary human macrophages, vascular smooth muscle cells and even synoviocytes obtained from patients with rheumatoid arthritis (Rezaie-Majd et al. 2002; Dichtl et al. 2003; Veillard et al. 2006; Xu et al. 2006). In one study, simvastatin lowered the pro-inflammatory cytokine, TNF and increased IL-10 production from RAW264.7 macrophages, at 10μM and 30μM respectively, as well

as significantly reducing IκBα degradation and NF-κB translocation (Leung et al. 2011). As a future experiment, it would be interesting to investigate whether simvastatin treatment is affecting the transcription factor, NF-κB, in these myometrial cells.

The rationale for performing co-, pre- and post-treatments was to aid better understanding of the potential clinical application of statin treatment; whether it be used as a preventative measure for high risk patients or given at the onset of PTL. Co-treatment with simvastatin had greater anti-inflammatory effects than either the pre-treatment or post-treatment. However, this may not be a clinically relevant treatment regimen. Pre-treatment induced a greater increase in anti-inflammatory genes and post-treatment showed a greater reduction in the pro-inflammatory mediators. It is important to note that variation within the groups perhaps prevented the trends achieving statistical significance and also the slightly shorter incubation time with simvastatin for the post-treatment group, may have affected the results. In a similar study, simvastatin was shown to reduce the secretion of a range of pro-inflammatory cytokines from LPS-stimulated human fetal membranes. In this study, pre-treatment with simvastatin 6 hours before LPS stimulation appeared more effective at reducing inflammatory cytokine release and soluble cytokine receptors than co-treatment or post-treatment. Co-treatment was the least effective method, as it did not significantly reduce IL-1β, IL-6 or TNF secretion, compared to the LPS group (Basraon et al. 2015). Further experimentation is required to tease out the most effective statin treatment protocol for reducing inflammation.

The anti-inflammatory effects described in some of the above studies were achieved by lower concentrations of simvastatin than the experiments presented in this chapter. For the current experiments, concentrations of simvastatin were chosen based on previous studies which describe therapeutic concentrations (0.1μM), a common mid-range concentration used in cell culture (10μM) and a higher concentration (50μM) (Lilja et al. 1998; Dichtl et al. 2003; Leung et al. 2003; Bjorkhem-Bergman et al. 2011; Chang et al. 2014; Basraon et al. 2015). It is possible that responsiveness to simvastatin therapy may be influenced by the specific cell type. Interestingly, in these myometrial cells, simvastatin only reduced pro-inflammatory mediators at 50μM but 10μM

simvastatin was sufficient to increase the expression of anti-inflammatory genes. It would be informative to treat the myometrial cells with lower concentrations of simvastatin to gain a better understanding of the specific concentrations required to see an effect. Importantly, despite these higher concentrations, the metabolic activity of the cells was not compromised by 50μM simvastatin.

In summary, pravastatin treatment did not affect myometrial cell morphology or cell metabolic activity. Pravastatin treatment also did not alter the expression of inflammatory mediators, following LPS stimulation. However, it is likely that the myometrial cells do not possess the transporter protein that pravastatin requires for cell entry. Simvastatin may have induced some slight morphology changes in the myometrial cells but cell metabolic activity was unaffected. In contrast to pravastatin, simvastatin treatment was capable of reducing LPS-induced inflammation, by both lowering the expression of pro-inflammatory mediators and increasing the expression of anti-inflammatory mediators.

Chapter 4: Determining the effect of simvastatin treatment on human myometrial cell contraction and the mechanisms contributing to this effect

4.1 Introduction

Myometrial contractions are key to the labour process, as the uterus switches from quiescent to active (Romero et al. 1994). These contractions are mainly regulated by calcium flux (Figure 4.1). Briefly, a substantial increase in intracellular Ca^{2+} concentration activates calmodulin, a Ca^{2+}-dependent protein found in the cytoplasm. Calmodulin can bind four Ca^{2+} ions. This complex subsequently activates MLCK, leading to the cross-bridging of actin and myosin and a prominent increase in the phosphorylation of MLC. This action determines the amplitude and duration of the contraction (Haeberle et al. 1985; Csabina et al. 1986; Word et al. 1993). The small GTPase, RhoA, is a major regulator of calcium sensitisation within cells. It acts through its effector, ROCK, to inactivate the enzyme, MLCP, potentiating the contractile effects of elevated intracellular Ca^{2+} (Uehata et al. 1997; Hartshorne et al. 1998). Therapies to target PTL aim to inhibit biochemical reactions along this pathway.

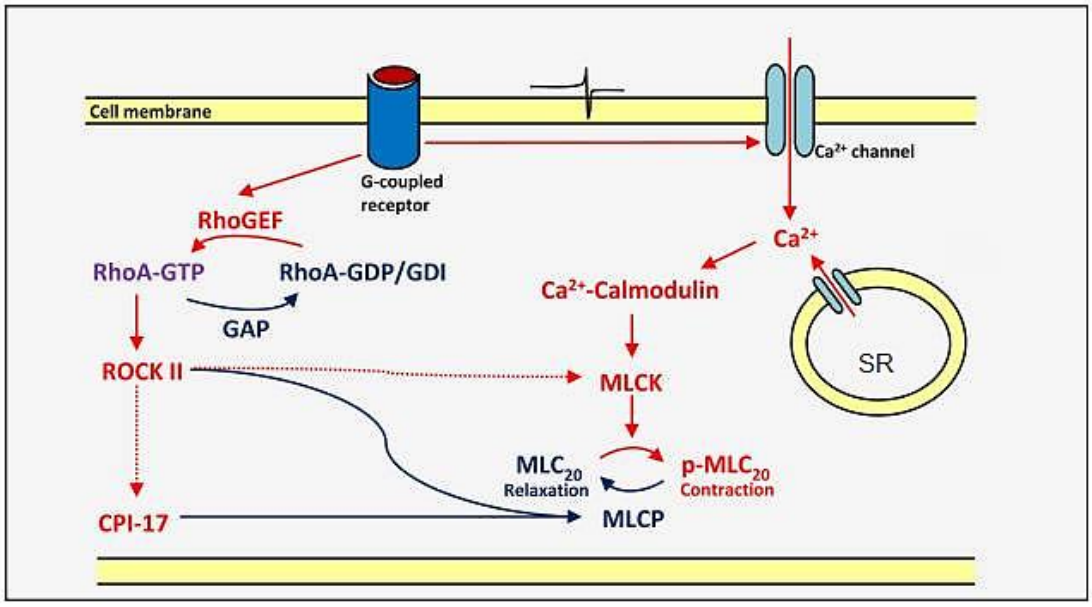

Figure 4.1. The molecular pathways in smooth muscle contraction. Intracellular Ca^{2+} concentration increases through Ca^{2+} influx into the cells or by Ca^{2+} release from intracellular stores, such as the SR. The Ca^{2+}/calmodulin complex forms and activates MLCK. MLCK phosphorylates MLC (20kDa), causing smooth muscle contraction. MLCP dephosphorylates MLC to induce relaxation. RhoA-GTP activates ROCK, which can inhibit the actions of MLCP directly or by protein phosphatase 1 regulatory subunit 14A (CPI-17) phosphorylation. ROCK may also phosphorylate MLC directly, similarly to MLCK. ROCK: Rho-kinase. MLC: Myosin light chain. MLCP: Myosin light chain phosphatase. MLCK: Myosin light chain kinase. SR: sarcoplasmic reticulum Adapted from de Godoy & Rattan (2011).

There is growing evidence to suggest that statins have anti-contractile actions. For example, they reduce the contractile response of vascular smooth muscle cells and endometriotic stromal cells (Kuzuya et al. 2004; Mraiche et al. 2005; Perez-Guerrero et al. 2005; Nasu et al. 2009; Gonzalez et al. 2014).

Statins exert their effects by targeting the mevalonate pathway, a key metabolic pathway, important in multiple cellular processes (Buhaescu and Izzedine 2007). Statins competitively inhibit HMG-CoA reductase (Figure 4.2). This enzyme catalyses the conversion of HMG-CoA to mevalonate, an early rate-limiting step in cholesterol synthesis. Mevalonate is then converted to sterol isoprenoids, as well as non-sterol isoprenoids, of which F-PP and GG-PP are components. These intermediate

metabolites play important roles in the post-translational modification of proteins involved in intracellular signalling, cell growth, gene expression, protein glycosylation and cytoskeletal assembly (Buhaescu and Izzedine 2007). F-PP is the last product of the isoprenoid metabolic pathway with the potential to be incorporated into either sterol or non-sterol end products (Bradfute and Simoni 1994). Isoprenylation of GG-PP is crucial for the activation of small GTPases, such as Rho, Rac and Cdc42. Many studies have proposed that the mechanism by which statins exert their anti-contraction effect is by inhibiting RhoA, which has an important role in the regulation of the actin cytoskeleton. Inhibiting the prenylation of RhoA prevents the downstream phosphorylation of MLC (Alvarez de Sotomayor et al. 2001; Mraiche et al. 2005; Kuhlmann et al. 2006; Nagaoka et al. 2007; Kidera et al. 2010; Wu et al. 2011; Xiao et al. 2012; Alp Yildirim et al. 2016).

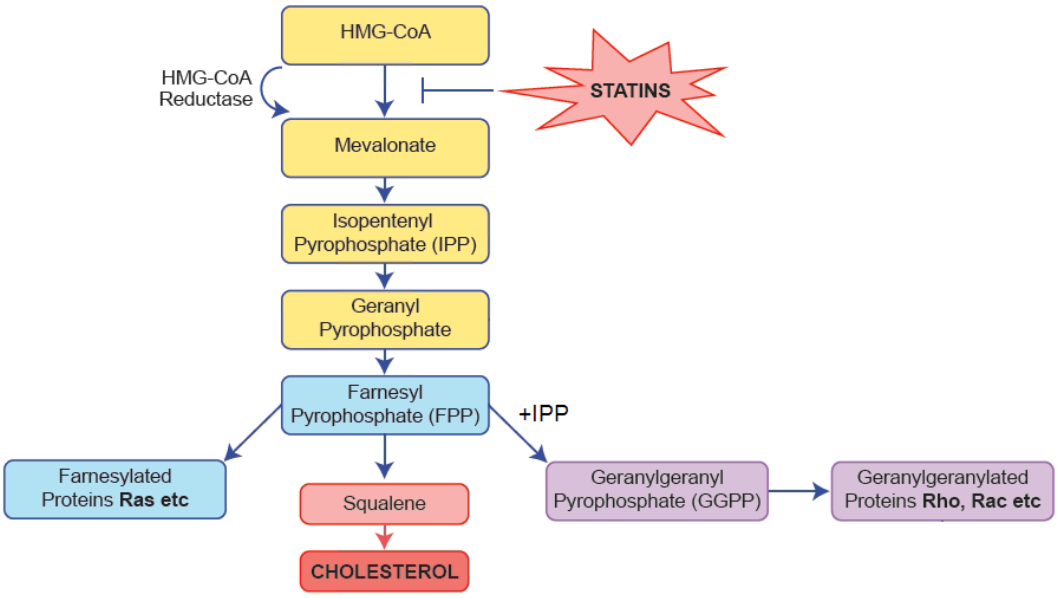

Figure 4.2. The mevalonate pathway. Statins inhibit HMG-CoA reductase from catalysing the conversion of HMG-CoA to mevalonate. This inhibits the production of cholesterol, as well as non-sterol isoprenoid components, such as F-PP and GG-PP, which are important for post-translational protein modification.

Chapter 3 demonstrated that simvastatin could reduce LPS-induced inflammation in human myometrial cells. Therefore, these results suggested that simvastatin treatment could reduce the pro-inflammatory environment associated with PTL initiation. As

myometrial contraction is a key process in term labour and PTL, and the tocolytic drugs currently available are ineffective, this chapter investigated whether simvastatin could inhibit myometrial contractile response. The hypothesis was that simvastatin will prevent myometrial cell contraction *in vitro*. The aim of this chapter was to investigate the effect of simvastatin on the contractile ability of a human myometrial cell line. Furthermore, mevalonate, GG-PP and F-PP were supplemented to simvastatin treatments to decipher a mechanism of action.

4.2 Methods

4.2.1 MTT metabolic activity assay

Analysis of myometrial cell metabolic activity was performed using an MTT assay, as detailed in 2.5. Myometrial cells were treated with simvastatin (10, 50µM) and supplemented with LPS (25ng/ml), mevalonate (200µM), GG-PP (10µM) or F-PP (10µM), in triplicate. Cells were incubated with treatments for 48 hours (n=4).

4.2.2 Collagen gel contraction assay

Simvastatin concentration response experiments were performed using collagen, which was extracted from rat tails, as described in 2.6.1. All other experiments were performed using commercially purchased rat tail collagen. Collagen gel contraction assays were performed by embedding myometrial cells in rat tail collagen and seeding non-treated 24-well plates at 10^5 cells/well, as described in 2.6.2. The gels were allowed to polymerise overnight and were then detached and treated with simvastatin (10, 50µM), in the presence or absence of LPS (25ng/ml), mevalonate (200µM), GG-PP (10µM) or F-PP (10µM). The plates were incubated at 37°C and photographed at 0, 24 and 48 hours using a Leica MZ6 light microscope/camera. Treatments were added in 6 technical replicates. For treatment concentration selection, n=1-6: simvastatin (0.1-100µM), LPS (25-400ng/ml), mevalonate (100, 200µM), GG-PP (10-40µM) or F-PP (10-100µM). For final experiments, n=6-11. Results are presented as a percentage of the vehicle mean gel size at baseline (0 hours or 24 hours, experiment dependent).

4.2.3 In-Cell Western

In-Cell Western analyses were performed to quantify pMLC in myometrial cells. Cells were seeded at $2x10^4$ cells/well, in black, clear base 96-well plates, as outlined in 2.7. Cells were treated in triplicate with simvastatin (10, 50µM) and supplemented with LPS (25ng/ml), mevalonate (200µM), GG-PP (10µM) or F-PP (10µM). The treatments were removed after 48 hours and the cells were fixed in formaldehyde**,** incubated with primary antibodies for pMLC and α-Tubulin, followed by secondary LI-COR antibodies and then fluorescence was read on the Odyssey® CLx Imaging System (n=5). Results are presented as intensity ratios of α-Tubulin:pMLC, within the same well.

4.2.4 Statistical analysis

Data are presented as mean ± SEM and were analysed using GraphPad Prism. In these studies, "n" denotes the number of individual experiments performed, with the replicate number per experiment indicated in the figure legends. The percentage data for collagen gel contraction were analysed by performing an arcsine transformation on the proportions, followed by a one-way ANOVA, with either Dunnett's or Holm-Sidak post hoc test. In-Cell Western data were analysed by one-way ANOVA, followed by Dunnett's post hoc test.

4.3 Results

4.3.1 The effect of simvastatin, LPS, mevalonate, GG-PP and F-PP treatments on myometrial cell metabolic activity

As discussed in Chapter 3, simvastatin and LPS treatment did not affect the metabolism of the myometrial cells at 24 hours. In this chapter, treatment incubations for the collagen gel assay and the In-Cell Western assay lasted 48 hours. Therefore, MTT assays were performed to investigate the effect simvastatin and LPS have on the cells after 48 hours. In addition, MTT assays were carried out for the metabolites of the mevalonate pathway, mevalonate, GG-PP and F-PP, which the cells were supplemented with to investigate a mechanism of action.

Following 48 hours of treatment, 10µM and 50µM simvastatin did not affect myometrial cell metabolic activity (Figure 4.3). This was also true for LPS treatment alone (25ng/ml) and when the cells were co-treated with LPS and 10µM and 50µM simvastatin (Figure 4.3A). Furthermore, mevalonate (200µM), GG-PP (10µM) and F-PP (10µM) did not affect cell metabolic activity, when given alone or in combination with simvastatin, for 48 hours (Figure 4.3B-D).

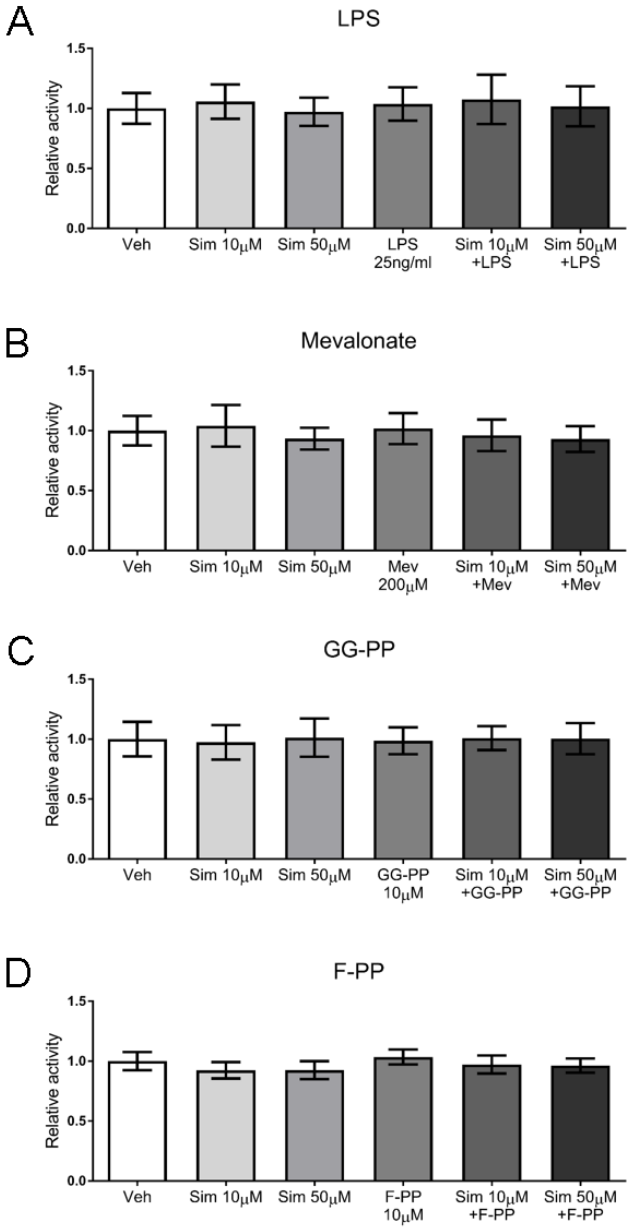

Figure 4.3. Metabolic activity of myometrial cells when treated with simvastatin, LPS, mevalonate, GG-PP or F-PP for 48 hours. (A-D) Simvastatin (10, 50µM) treatment did not alter myometrial cell metabolic activity. (A) LPS, (B) mevalonate, (C) GG-PP or (D) F-PP did not affect myometrial cell metabolic activity when given alone or with simvastatin treatment. n=4 (in triplicate), vehicle (veh): (A) PBS, (B) EtOH diluted in PBS, (C, D) CH_3OH diluted in PBS, mean ± SEM.

4.3.2 The influence of simvastatin treatment on myometrial cell contractility, alone and with LPS stimulation

4.3.2.1 The effect of simvastatin on the basal contraction of myometrial cells

Experiments were performed to establish the effect of simvastatin on myometrial cell contractility over a range of concentrations. The myometrial cells were embedded in collagen gel and the gel sizes were analysed at 24 and 48 hours.

As these are smooth muscle cells, the vehicle (PBS) treated gels established a basal contraction, causing the gel size to reduce over time (Figure 4.4). Following 24 hours of 10μM simvastatin treatment, the mean gel size was significantly larger than the vehicle mean gel size, suggesting that contraction was attenuated (mean gel size 117.5 ± 3.4% vs vehicle mean 100 ± 3.1%, p=0.0068). Simvastatin, at concentrations of 25μM and 50μM, also prevented contraction at 24 hours (115.2 ± 3.7%, p=0.023 and 118.3 ± 4.3%, p=0.004, respectively).

At 48 hours, the mean size of the vehicle gel had reduced to 85.6 ± 4.8% of the mean gel size at 24 hours, displaying further basal contraction (Figure 4.4B). When the cells were treated with 10μM simvastatin, the mean gel size was again significantly larger than the vehicle (114.6 ± 3.1%, p=0.0003). The mean gel sizes were also significantly larger than the vehicle when they were treated with 25μM simvastatin (116.8 ± 2.5%, p<0.0001), 50μM simvastatin (119.9 ± 3.5%, p<0.0001) and 100μM simvastatin (110.1 ± 5.7%, p=0.0104). There was no significant difference between the vehicle and gels treated with 0.1μM or 1μM simvastatin after 48 hours. Simvastatin concentrations of 10μM and 50μM were selected for further experimentation.

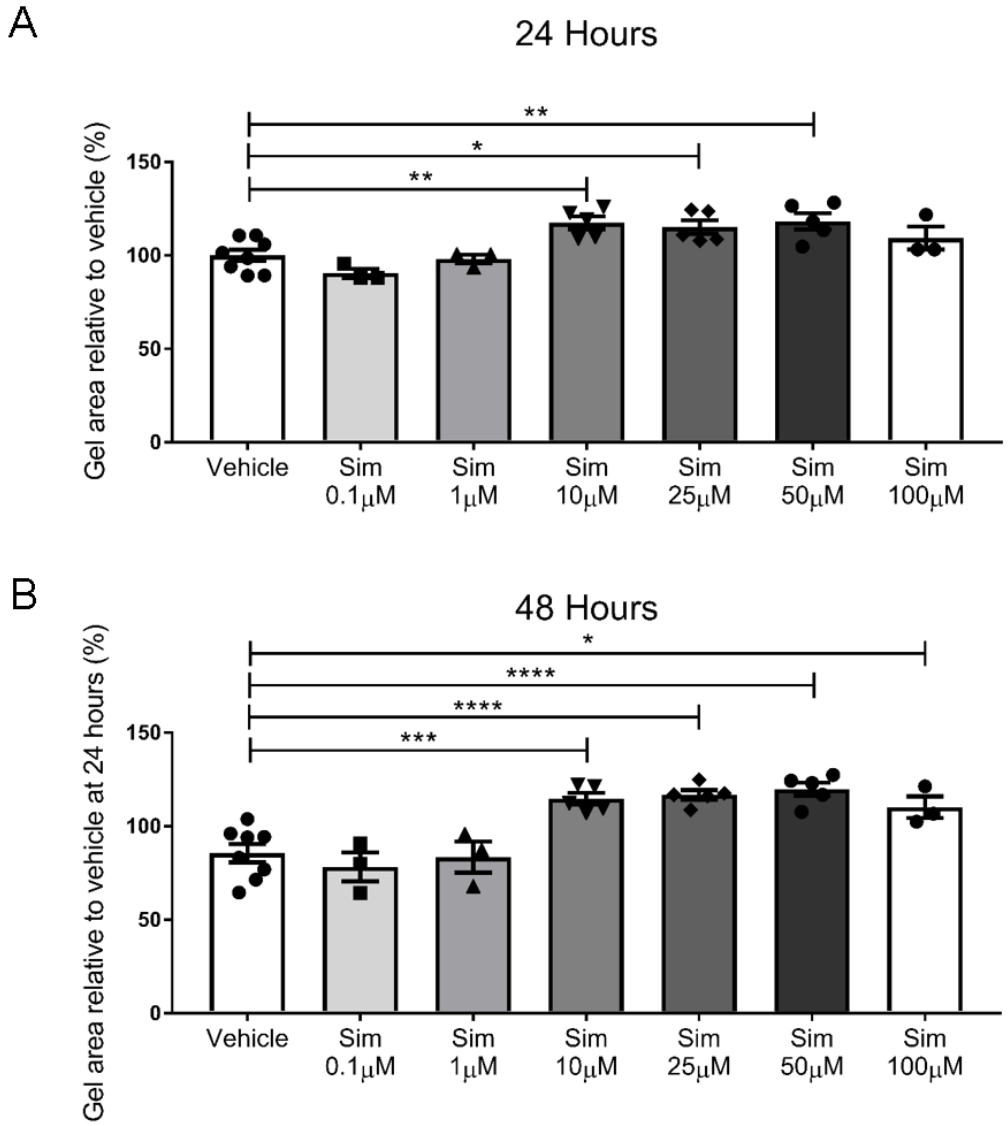

Figure 4.4. The effect of simvastatin treatment on basal myometrial cell contraction. (A) At 24 hours, when gels were treated with 10, 25 and 50μM simvastatin, the mean gel size was significantly larger than the vehicle. (B) After 48 hours, gels treated with 10, 25, 50 and 100μM simvastatin were significantly larger in size than the vehicle. n=3-8 (4-6 replicates), vehicle = PBS, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

4.3.2.2 The effect of LPS stimulation on myometrial cell contraction

Firstly, a concentration response experiment was performed to determine whether LPS could induce further contractions over the vehicle. Secondly, the anti-contractile effect of simvastatin within an LPS-induced inflammatory environment was investigated. Gels were analysed at 0, 24 and 48 hours.

4.3.2.2.1 LPS concentration response

An LPS concentration response experiment was performed to investigate the effect of LPS on the contraction of the myometrial cells. After 24 hours, contraction was evident in the vehicle (PBS) group by the reduction of the mean gel size to 61.4 ± 2.6% of its original size at 0 hours (Figure 4.5B). However, concentrations of LPS from 25-400ng/ml did not significantly increase (or decrease) the contraction of the gels, as they were similar in size to the vehicle gels.

By 48 hours, the vehicle mean gel size had reduced to 45.6 ± 2.7% of its original size (Figure 4.5C). Again, there was no significant effect of LPS on the gel size, compared to the vehicle. However, there was a slight trend appearing, as the gel sizes from treatments with 25, 50 and 100ng/ml LPS looked marginally reduced. The largest difference in gel size was with 25ng/ml LPS, where the mean gel size was 8% smaller than the vehicle ($p=0.1679$). Although there was no statistical difference, this concentration was chosen for use in the following simvastatin experiments.

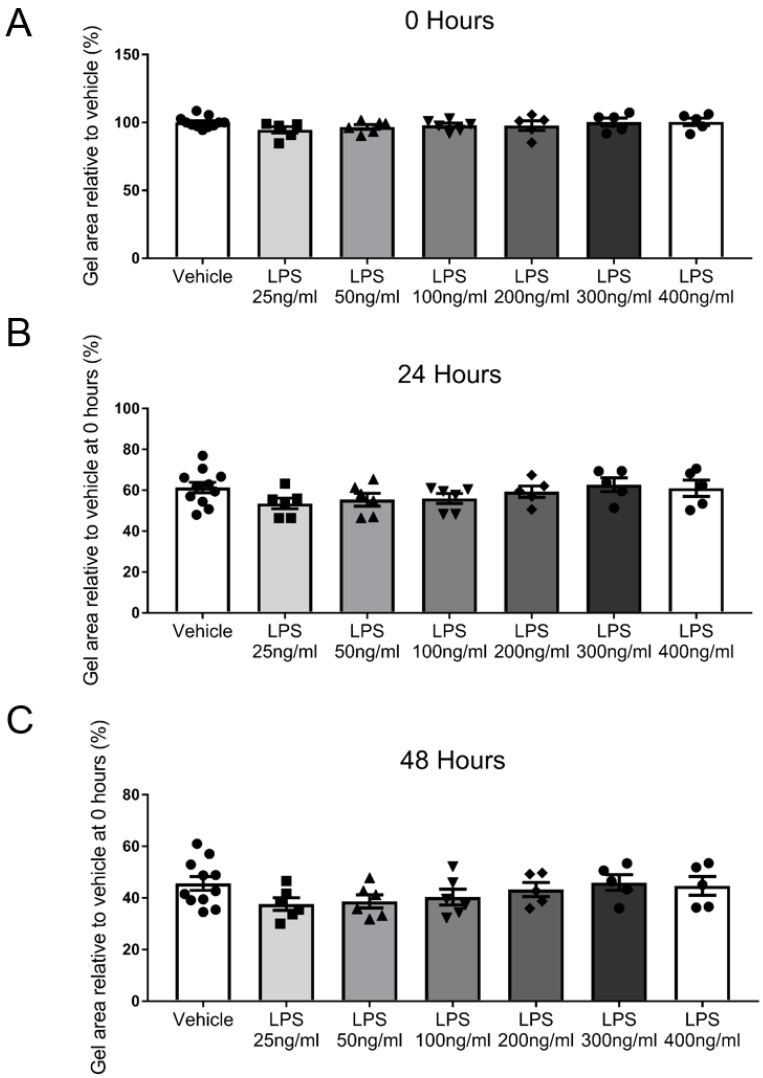

Figure 4.5. The effect of LPS on myometrial cell contraction. (A-C) LPS concentrations from 25-400ng/ml did not significantly induce further contraction of the myometrial cells, when compared to the vehicle mean gel size. (C) However, a trend to reduce mean gel size was observed for the lower concentrations, particularly 25ng/ml. n=5-11 (4-6 replicates), vehicle = PBS, mean ± SEM.

4.3.2.2.2 The effect of LPS and simvastatin on myometrial cell contraction

The basal contraction of the myometrial cells was evident within 24 hours, as the mean gel size reduced to 59.8 ± 1.9% of the baseline gel size (Figure 4.6A-C). Gels treated with 10µM simvastatin were 16.6% larger than the mean vehicle size (69.7 ± 3.1%, p=0.0059). Gels treated with 50µM simvastatin were also significantly larger than the vehicle (67.5 ± 2.0%, p=0.027). LPS treatment caused the mean gel size to reduce to

54.2 ± 1.8%, making it 10.3% smaller than the vehicle (p=0.0293). Even in the presence of LPS, 10µM simvastatin attenuated the contractile ability of the cells, as the mean gel size was 17.4% larger than the vehicle (70.2 ± 1.8%, p=0.0059). This was also observed with 50µM simvastatin, where the mean gel size was 70.3 ± 1.7%, 17.6% larger than the vehicle (p=0.0059).

After 48 hours, the mean vehicle gel size had further reduced to 43.4 ± 1.6% of its original size (Figure 4.6A, D). When gels were treated with 10µM simvastatin alone, the mean gel size was 64.4 ± 2.3%, 48.4% larger than the vehicle (p<0.0001). Following treatment with 50µM simvastatin, gel sizes were 54.8% larger than the vehicle (67.2 ± 2.0%, p<0.0001). The LPS treated gels were still significantly smaller than the vehicle gels (37.8 ± 1.6%, p=0.0247). When the gels were treated with both 10µM simvastatin and LPS, the simvastatin treatment once again inhibited the contraction of the gels, as they were 52.1% larger than the vehicle (66.0 ± 2.4%, p<0.0001). This was also observed following 50µM simvastatin and LPS treatment, as the mean gel size was 62.9% larger than the vehicle mean (70.7 ± 1.7%, p<0.0001).

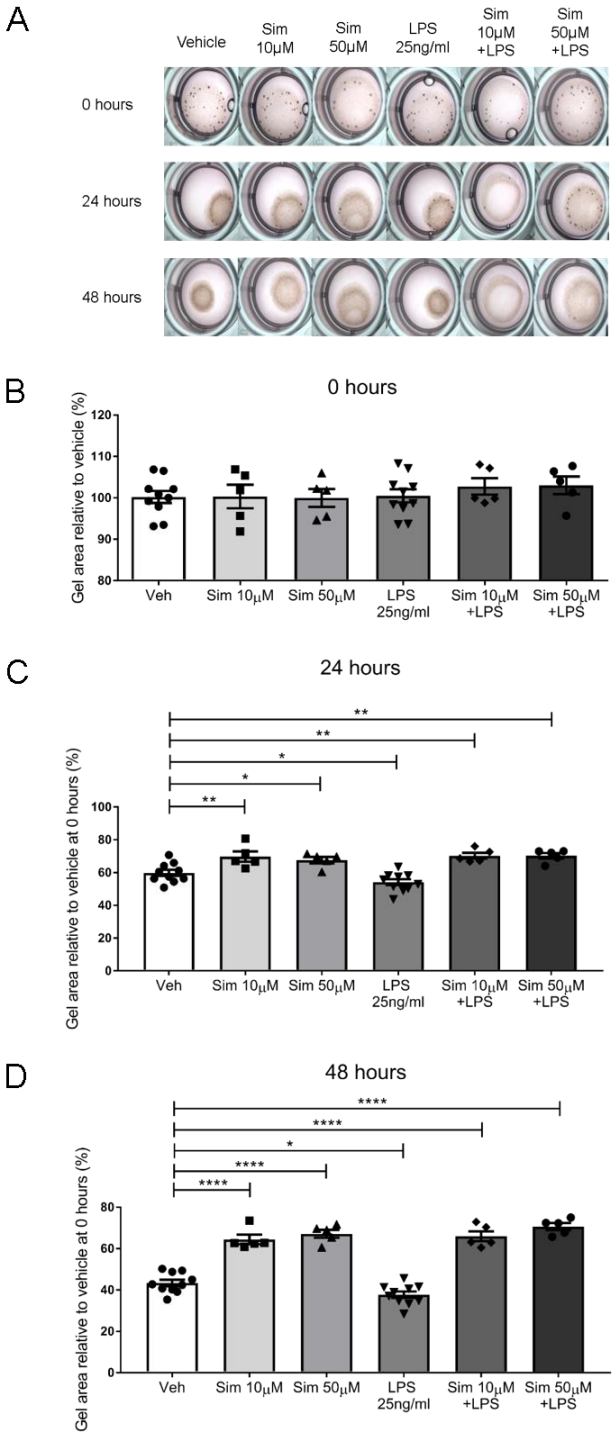

Figure 4.6. The effect of simvastatin treatment on basal and LPS-induced myometrial cell contraction. (A) Representative gel images showing gel size at 0, 24 and 48 hours. (B-D) Vehicle gel size reduced over time. This contraction was attenuated by simvastatin treatment (10, 50μM). LPS treatment (25ng/ml) caused further reduction in gel size, compared to the vehicle. Simvastatin treatment (10, 50μM) inhibited the contraction of the gels, even in the presence of LPS. n=5-10 (4-6 replicates), vehicle (veh) = PBS, *p<0.05, **p<0.01, ****p<0.0001, mean ± SEM, one-way ANOVA with Holm-Sidak post hoc test.

4.3.3 The effect of mevalonate, GG-PP and F-PP supplementation on simvastatin-induced contractile attenuation

4.3.3.1 Mevalonate

The mevalonate pathway is the main target of statins. By inhibiting HMG-CoA reductase, statins prevent the reduction of HMG-CoA to mevalonate by nicotinamide adenine dinucleotide phosphate (NADPH) (Friesen and Rodwell 2004). By supplementing simvastatin-treated gels with mevalonate, any reversal of the impact of simvastatin would imply that these anti-contractile actions were as a result of inhibiting the mevalonate pathway, rather than an alternative pathway.

4.3.3.1.1 Mevalonate concentration response

Firstly, preliminary experiments were carried out to choose a concentration of mevalonate (n=2). Both 100µM and 200µM mevalonate are widely used in the literature (Qi et al. 2013; Alarcon and Marikawa 2016). In these concentration response experiments, both concentrations appeared to abolish the effect of 10µM simvastatin (Figure 4.7). For use in the following experiments, 200µM mevalonate was chosen, as it appeared to inhibit 10µM simvastatin marginally more and it was predicted that this concentration may be more effective at inhibiting 50µM simvastatin.

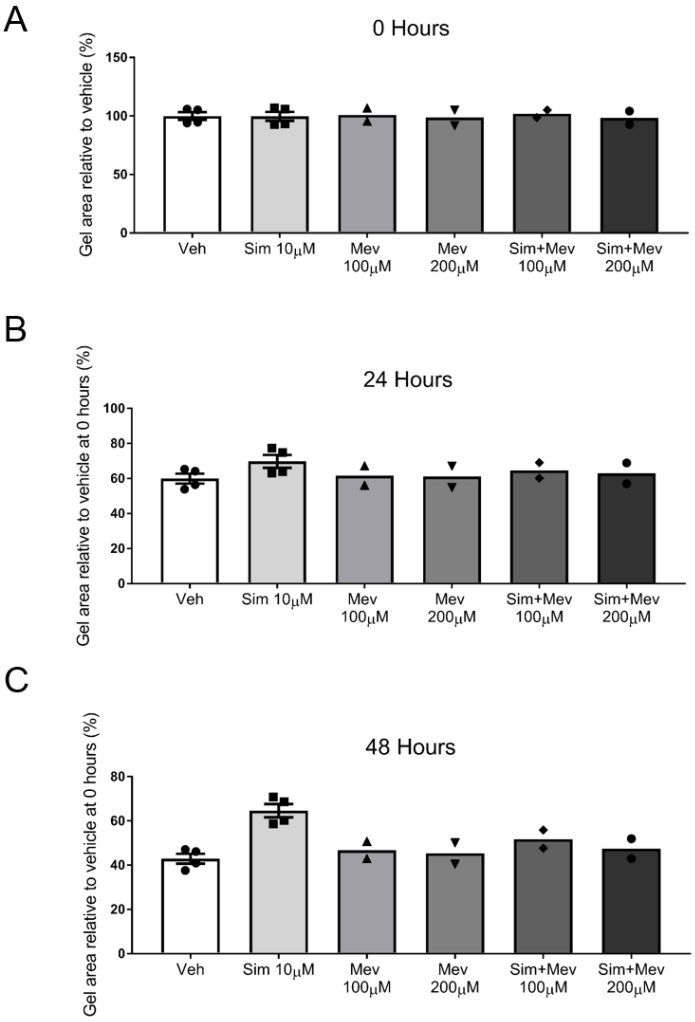

Figure 4.7. Selection of mevalonate concentration. (A-C) Mevalonate at 100μM and 200μM showed a trend to reduce the anti-contractile effect of simvastatin (10μM), allowing the gel size to decrease. (C) Mean gel size after simvastatin and 200μM mevalonate appeared marginally more similar to the vehicle gel size at 48 hours. n=2-4 (4-6 replicates), vehicle (veh) = EtOH diluted in PBS, mean ± SEM.

4.3.3.1.2 The effect of mevalonate on the anti-contractile action of simvastatin

After 24 hours, vehicle gels had reduced to a mean size of 55.7 ± 1.4% (Figure 4.8A-C). When the gels were treated with 10μM and 50μM simvastatin, the mean gel sizes were 20.8% and 17.2% larger than the vehicle (67.3 ± 2.2%, $p<0.0001$; 65.3 ± 0.7%, $p=0.0021$, respectively). Mevalonate treatment alone allowed the gels to contract and the mean gel size reduced to 55.2 ± 1.4%, similar to the vehicle. When gels were treated with either 10μM or 50μM simvastatin and mevalonate, the gels reduced in

size, abolishing the anti-contractile effect of simvastatin (mean gel sizes 58.2 ± 2.4% and 56.1 ± 0.9%, respectively).

Following 48 hours of treatment, the vehicle mean gel size had reduced further to 39.6 ± 1.2% of its original size (Figure 4.8A, D). Simvastatin (10μM, 50μM) treated gels were both 60.1% larger than the vehicle mean (63.4 ± 1.7% and 63.4 ± 1.9%, $p<0.0001$). Again, when gels were treated with 10μM and 50μM simvastatin and supplemented with mevalonate, gel sizes reduced, inhibiting the anti-contractile action of simvastatin (mean gel sizes 43.9 ± 1.8% and 41.7 ± 0.9%, respectively).

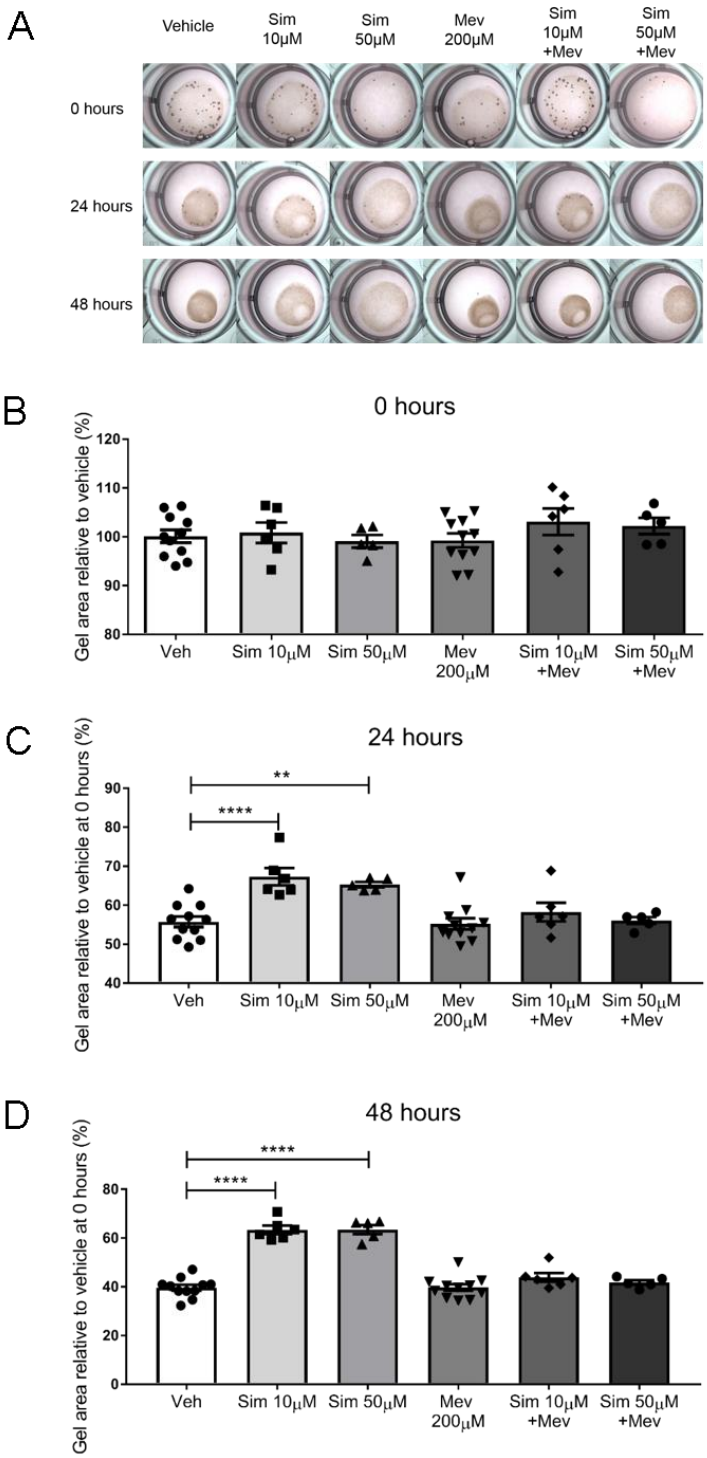

Figure 4.8. The effect of mevalonate supplementation on the anti-contractile effect of simvastatin on myometrial cells. (A) Representative gel images at 0, 24 and 48 hours. (B-D) Vehicle gels reduced in size over time. Simvastatin treatment (10, 50μM) attenuated this contraction, resulting in significantly larger gel sizes. When gels received simvastatin and mevalonate (200μM), the effect of simvastatin was inhibited and the gel size reduced. n=5-11 (4-6 replicates), vehicle (veh) = EtOH diluted in PBS, **p<0.01, ****p<0.0001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

4.3.3.2 GG-PP

GG-PP is an intermediate metabolite of the mevalonate pathway, which is key to the post-translational modifications of small GTPases, such as Rho, Rac and Cdc42 (Nurenberg and Volmer 2012).

4.3.3.2.1 GG-PP concentration response

A preliminary concentration response experiment was carried out to choose a concentration of GG-PP (n=1). The most commonly used concentration cited in the literature is 10µM (Qi et al. 2013; Alarcon and Marikawa 2016). This appeared to inhibit the action of 50µM simvastatin and so, was used in the following experiments (Figure 4.9).

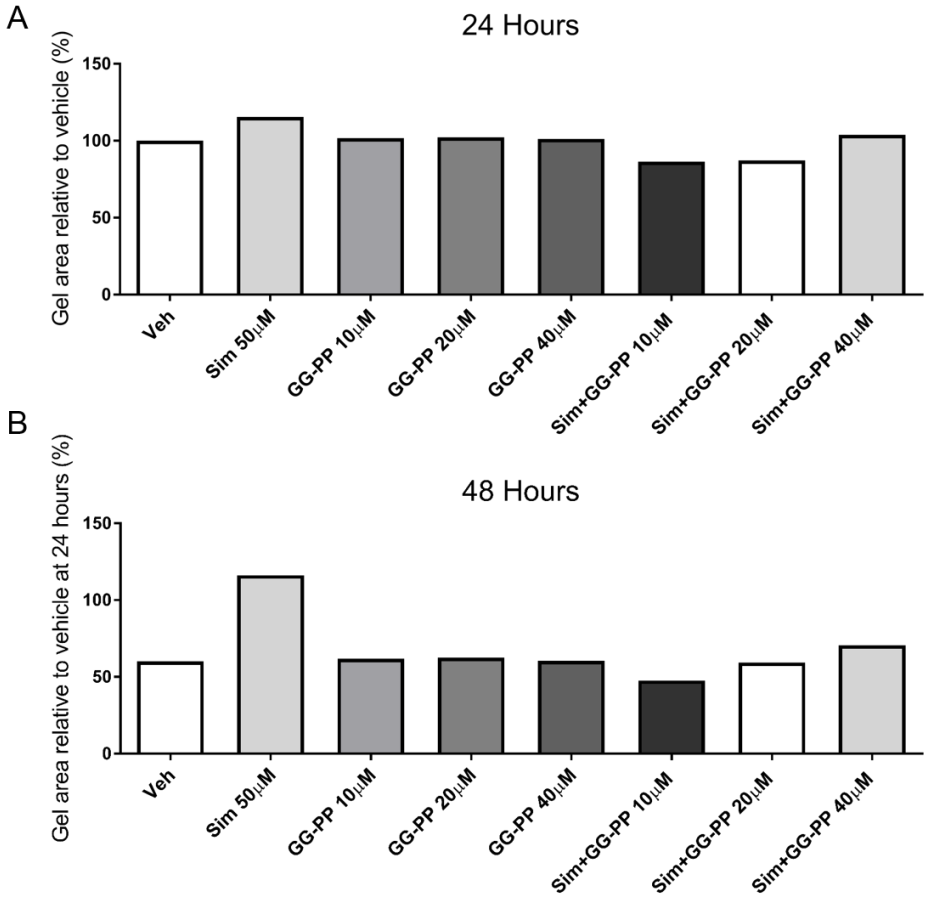

Figure 4.9. Selection of GG-PP concentration. (A, B) GG-PP appeared to inhibit simvastatin action at 10, 20 and 40µM. n=1 (4-6 replicates), vehicle (veh) = CH_3OH diluted in PBS.

4.3.3.2.2 The effect of GG-PP on the anti-contractile action of simvastatin

At 24 hours, the vehicle mean gel size had reduced to 50.9 ± 1.6%, compared to the mean gel size at 0 hours, showing basal contraction of the cells (Figure 4.10A-C). Treating the gels with 10µM simvastatin resulted in gels 16.5% larger than the vehicle mean (59.3 ± 3.6%, p=0.0483). This affect was also observed with 50µM simvastatin, where the gels were 20.6% larger than the vehicle (61.4 ± 2.9%, p=0.0107). GG-PP treatment alone did not affect the contraction of the gels, which were a similar size to the vehicle (52.5 ± 1.7%). However, when the gels received 10µM and 50µM simvastatin and GG-PP, the gels contracted, abolishing the effect of simvastatin (mean gel sizes 48.1 ± 2.0% and 49.8 ± 3.4%, respectively).

The same effects were observed at 48 hours (Figure 4.10A, D). The vehicle mean gel size reduced to 36.0 ± 1.6% and simvastatin (10, 50µM) alone resulted in larger gel sizes (10µM: 50.3 ± 3.6%, p=0.0027; 50µM: 63.1 ± 3.3%, p<0.0001). When gels received 10µM and 50µM simvastatin and GG-PP, the gel sizes reduced and were similar in size to the vehicle gels (34.1 ± 2.3% and 35.1 ± 3.4%, respectively).

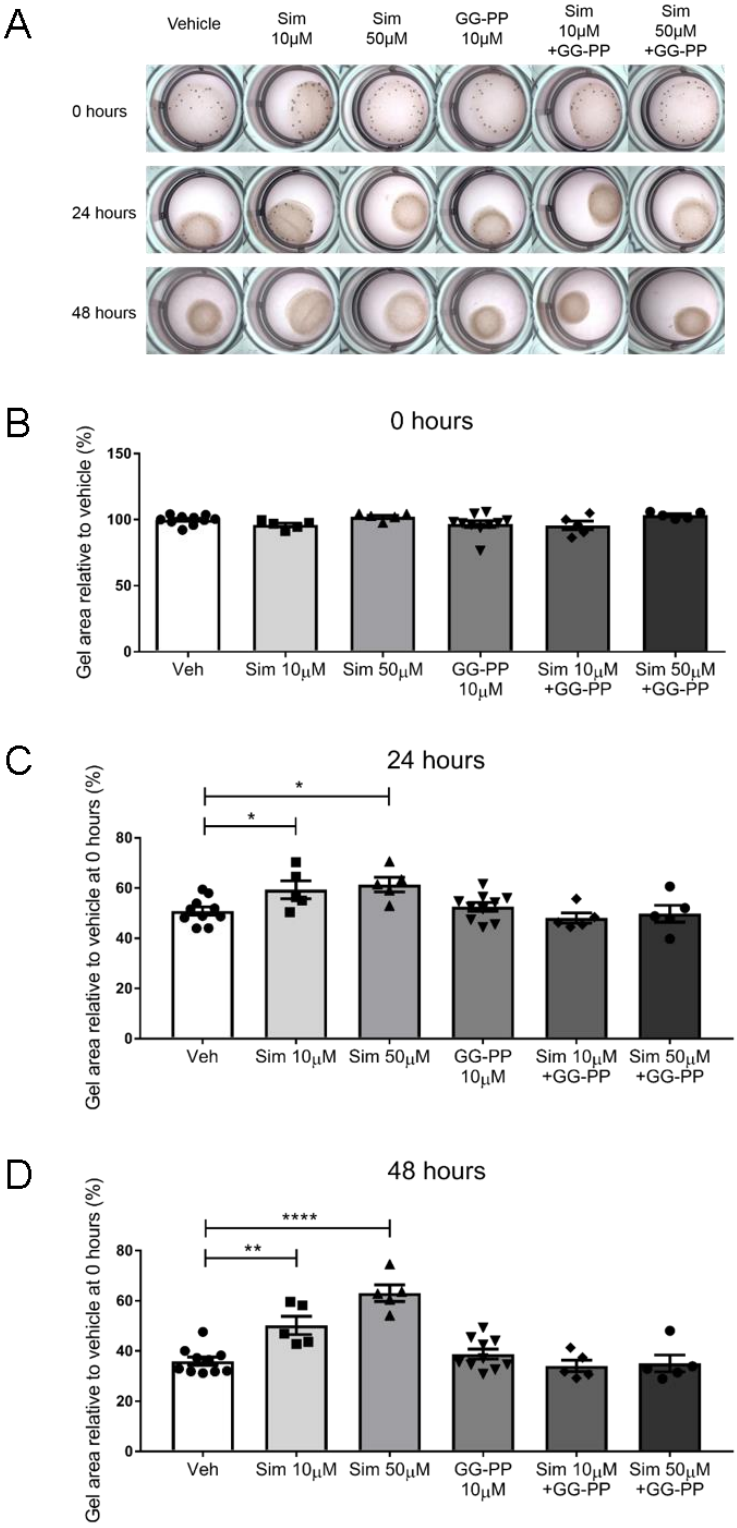

Figure 4.10. The effect of GG-PP supplementation on the anti-contractile effect of simvastatin. (A) Representative gel images at 0, 24 and 48 hours. (B-D) Vehicle gels reduced in size over time. Simvastatin treatment (10, 50μM) attenuated this reduction, resulting in significantly larger gel sizes. When gels received simvastatin and GG-PP (10μM), the gel size reduced and the effect of simvastatin was inhibited. n=5-10 (4-6 replicates), vehicle (veh) = CH_3OH diluted in PBS, *p<0.05, **p<0.01, ****p<0.0001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

4.3.3.3 F-PP

F-PP is another metabolite of the mevalonate pathway, which branches out into a sterol pathway, for products such as cholesterol, and a non-sterol pathway, for the post-translational modifications of small GTPases, such as Ras (Nurenberg and Volmer 2012).

4.3.3.3.1 F-PP concentration response

A preliminary concentration response was carried out to choose a concentration of F-PP (n=1). The most commonly used concentration in the literature is 10µM (Qi et al. 2013; Alarcon and Marikawa 2016). Concentrations up to 100µM were tested (Figure 4.11). However, none of these affected the anti-contractile effect of simvastatin and so, as the most commonly cited concentration, 10µM was used for the following experiments.

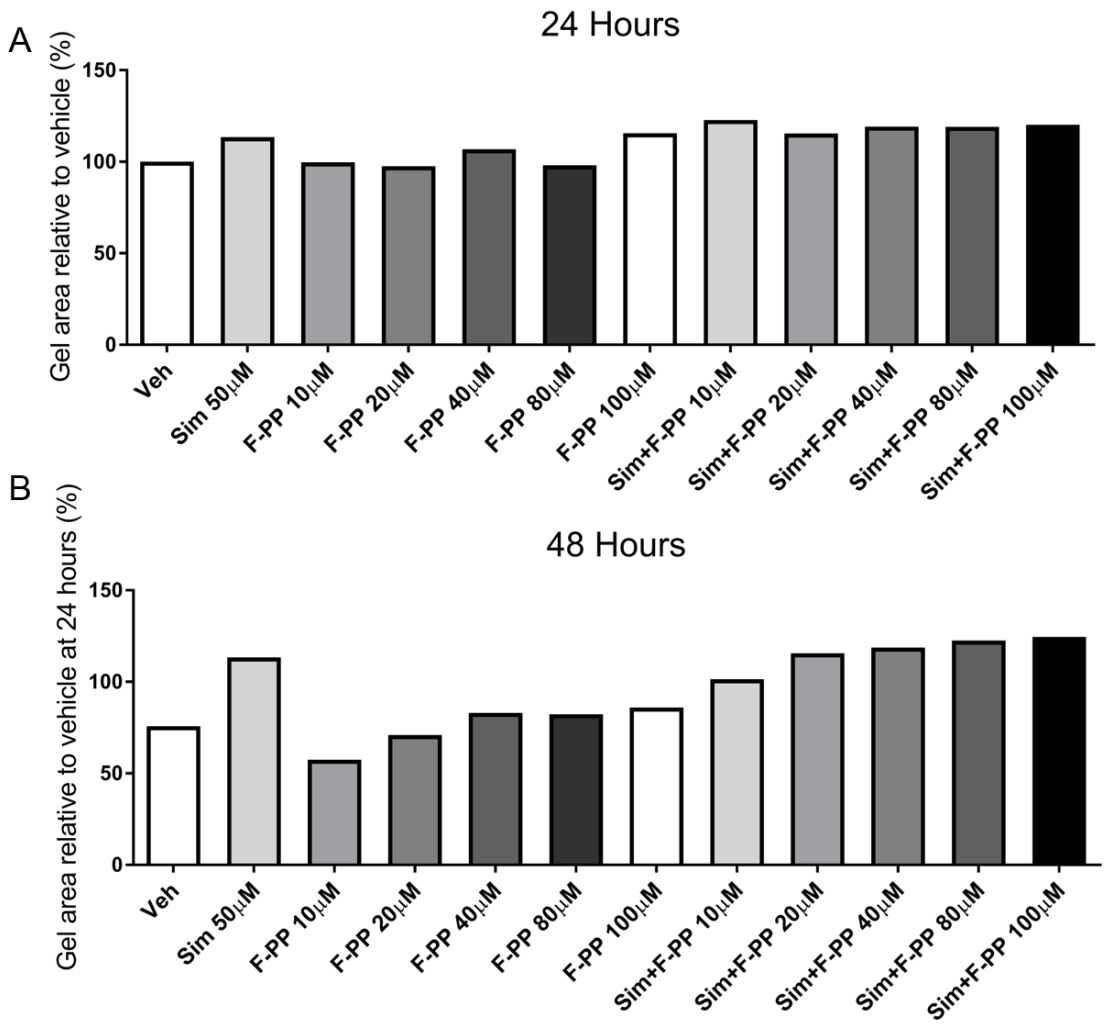

Figure 4.11. Selection of F-PP concentration. (A, B) F-PP did not appear to affect simvastatin action at 10, 20, 40, 80 or 100μM. n=1 (4-6 replicates), vehicle (veh) = CH_3OH diluted in PBS.

4.3.3.3.2 The effect of F-PP on the anti-contractile action of simvastatin

After 24 hours, the mean vehicle gel size had reduced to 50.8 ± 1.2% of the baseline size (Figure 4.12A-C). Simvastatin, at both 10μM and 50μM, attenuated the contraction of the gels with sizes of 58.1 ± 2.5% and 62.0 ± 2.1% (p=0.032 and p=0.0005 vs vehicle, respectively). F-PP alone did not affect the basal contraction of the gels, as they reduced to 50.5 ± 1.2%, similar in size to the vehicle. When gels were treated with 10μM simvastatin and F-PP, they were 19.1% larger than the vehicle gels (60.5 ± 3.3%, p=0.0024). This was also observed with 50μM simvastatin and F-PP,

where the mean gel size was 26.8% larger than the vehicle (64.1 ± 1.4%, $p<0.0001$), showing that simvastatin was still having an anti-contractile affect.

Following 48 hours of incubation, the vehicle mean gel size reduced further to 33.9 ± 0.9% of its original size (Figure 4.12A, D). Treatment with 10µM and 50µM simvastatin still attenuated gel contraction, resulting in significantly larger gels than the vehicle (50.1 ± 4.1%, $p=0.0001$ and 58.2 ± 1.9%, $p<0.0001$, respectively). When gels received 10µM simvastatin and F-PP, the gel size was 50.5 ± 4.7%, 50.0% larger than the mean vehicle gel size ($p<0.0001$). Furthermore, 50µM simvastatin and F-PP treatment resulted in a mean gel size 72.3% larger than the vehicle (58.4 ± 2.3%, $p<0.0001$).

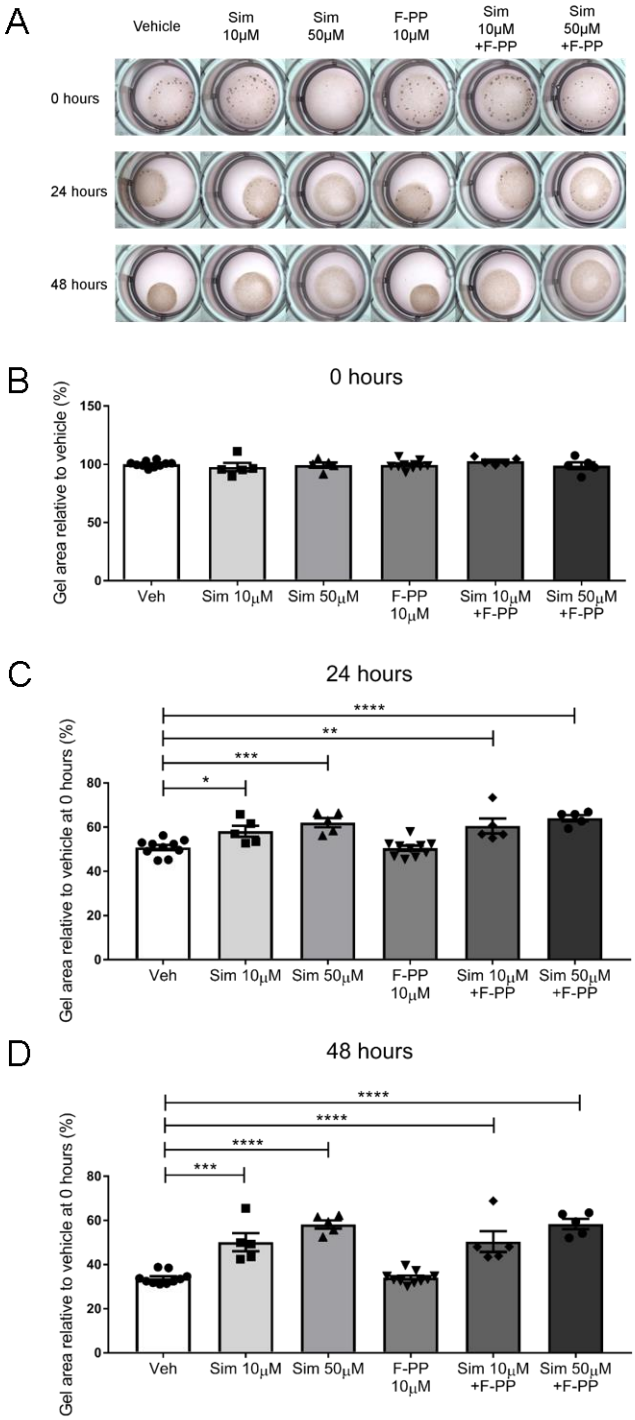

Figure 4.12. The effect of F-PP supplementation on simvastatin's anti-contractile effect. (A) Representative gel images at 0, 24 and 48 hours. (B-D) Vehicle gels reduced in size over time. Simvastatin treatment (10, 50μM) attenuated this reduction, resulting in significantly larger gel sizes. When gels received simvastatin and F-PP (10μM), gel contraction was still inhibited, as the gel sizes remained significantly larger than the vehicle. n=5-10 (4-6 replicates), vehicle (veh) = CH_3OH diluted in PBS, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test**.**

4.3.4 The influence of simvastatin, LPS and the mevalonate pathway metabolites on the phosphorylation of MLC in myometrial cells

Smooth muscle contraction is mediated by the phosphorylation of MLC via MLCK and/or ROCK (Schaafsma et al. 2008). The In-Cell Western technique was utilised to investigate the effect simvastatin has on pMLC levels.

Simvastatin treatment significantly reduced pMLC levels, both alone (10µM $p=0.0112$, 50µM $p<0.0001$ vs vehicle) and in the presence of inflammatory stimulation with LPS (10µM $p=0.0493$, 50µM $p<0.0001$ vs vehicle; Figure 4.13, Figure 4.14A). LPS alone did not affect the expression of pMLC ($p=0.7905$). Once again, when the simvastatin treated cells received either mevalonate or GG-PP supplementation, the effect of simvastatin was abolished and the pMLC levels were akin to the vehicle treated cells (Figure 14.3, Figure 14.4B, C). When the simvastatin treated cells were supplemented with F-PP, pMLC levels were significantly reduced compared to the vehicle (10µM $p=0.0452$, 50µM $p=0.0008$; Figure 14.3, Figure 14.4D). Thus, F-PP addition did not abolish the pMLC lowering effect of simvastatin. Complementing the results of the collagen gel contraction assay, mevalonate and GG-PP, but not F-PP, abolished the effects of simvastatin on pMLC levels.

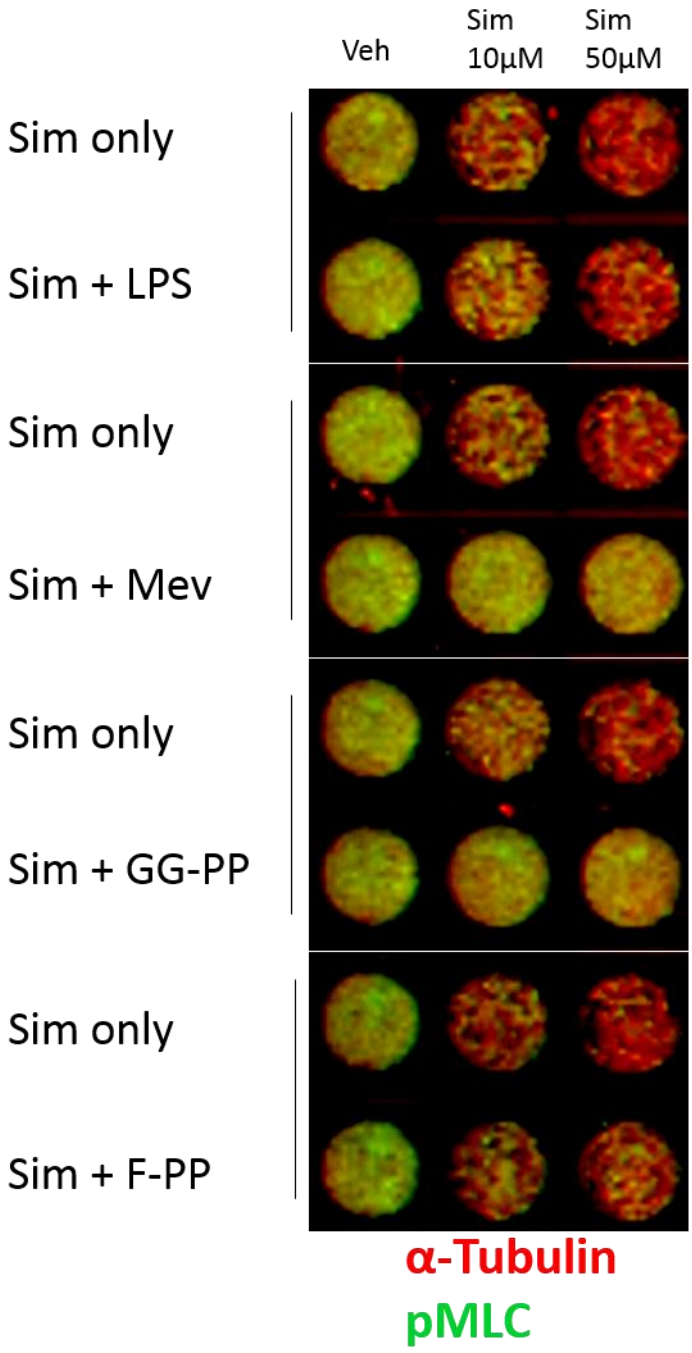

Figure 4.13. Levels of pMLC following simvastatin treatment (48 hours). This In-Cell Western representative image shows that 10µM and 50µM simvastatin concentration-dependently reduced pMLC (green) levels in myometrial cells. When 200µM mevalonate and 10µM GG-PP were added, pMLC levels were restored and the well appeared more green. However, 10µM F-PP did not affect pMLC levels, as α-Tubulin (red) is more dominant in these wells, as observed in the simvastatin-treated wells. n=5 (in triplicate).

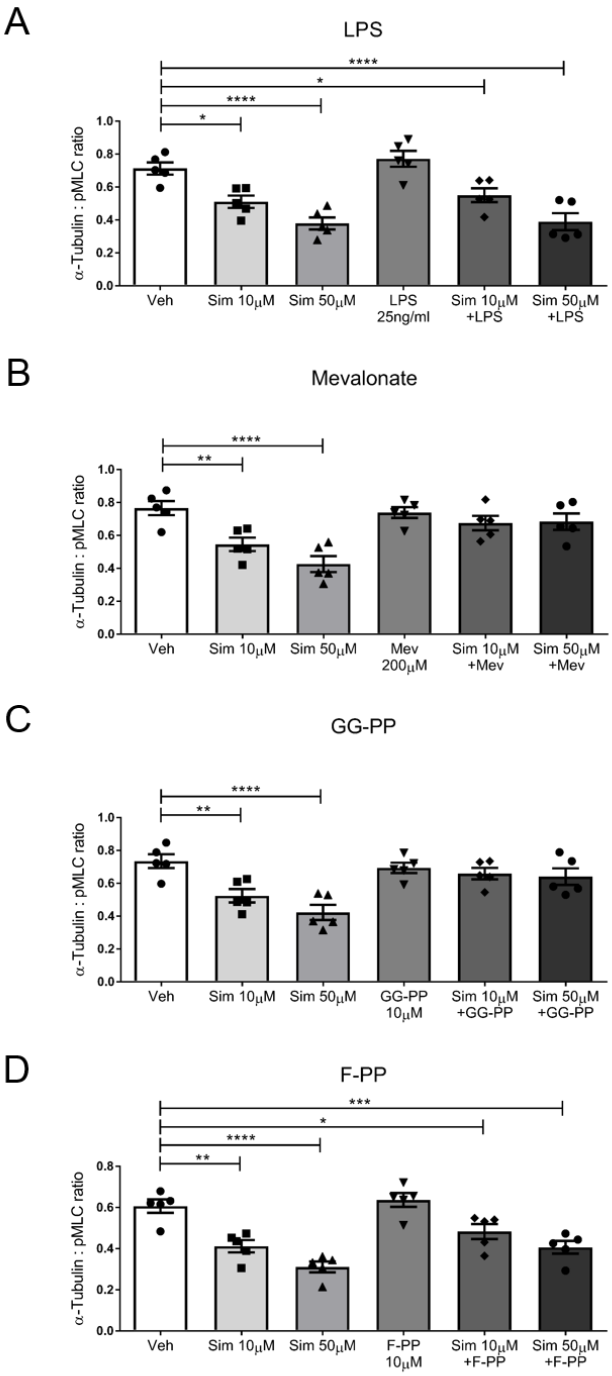

Figure 4.14. The effect of supplementation with LPS, mevalonate, GG-PP and F-PP on pMLC levels (48 hours). Signal intensity ratios (α-Tubulin:pMLC) (A) Simvastatin (10, 50μM) significantly lowered levels of pMLC, alone and in the presence of LPS. LPS did not affect pMLC levels. (B, C) Mevalonate (200μM) and GG-PP (10μM) rescued pMLC levels when given with simvastatin. (D) F-PP did not affect pMLC levels, when cells received simvastatin treatment. n=5 (in triplicate), vehicle (veh): (A) PBS, (B) EtOH diluted in PBS, (C, D) CH_3OH diluted in PBS, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

4.4 Discussion

4.4.1 Simvastatin attenuates the basal contraction of human myometrial cells

In the collagen gel assay, the gels exerted basal contraction, due to the contractile ability of the smooth muscle cells, as previously described (Hutchinson et al. 2014; Makieva et al. 2016). This led to the gel area reducing, which was evident after 24 hours in all experiments. Simvastatin treatment was able to lessen the contraction, resulting in significantly larger gel sizes, when comparing to the vehicle. This effect was shown at both 24 hours and 48 hours of treatment with simvastatin.

The anti-contractile effect of simvastatin has been previously described at similar concentrations as used in this chapter. Simvastatin reduced the contraction of human lung fibroblasts embedded in collagen gels, both alone and in the presence of TNF, IL-1β and neutrophil elastase (Ra et al. 2011). Simvastatin treatment also inhibited vascular contraction in rat aortic rings (Mraiche et al. 2005; Rossoni et al. 2011; Chen et al. 2016). In addition, organ bath studies have reported anti-contractile effects of simvastatin. For instance, simvastatin reduced the frequency of spontaneous and C5a-induced contraction in human myometrial samples pre-treated with simvastatin, as well as in myometrial tissue collected from simvastatin-treated mice (Gonzalez et al. 2014).

The simvastatin concentration response experiments were performed with collagen, which was crudely extracted from rat tails within the laboratory. However, commercially obtained collagen was used for all further experiments. This did not seem to limit the experiment, as the ability of simvastatin to attenuate contraction was unaffected by this change in material.

In the simvastatin concentration response experiments, there did not appear to be a concentration-dependent effect. Simvastatin had no effect on contraction at 0.1µM or 1µM but inhibited contraction at concentrations of 10µM and above, with little difference between 10, 25 and 50µM. The highest concentration of 100 µM simvastatin, only reduced contraction at 48 hours. For consistency, 10µM and 50µM simvastatin were chosen for further experimentation, so comparisons could be drawn

with the results outlined in Chapter 3. Interestingly, in these experiments, both 10µM and 50µM simvastatin consistently attenuated contraction, with no obvious difference in response between concentrations, whereas in Chapter 3 the anti-inflammatory properties of 50µM simvastatin were superior to those of the 10µM concentration.

Simvastatin may affect the shape of cells, as discussed in Chapter 3. This may have affected the contractile ability of the myometrial cells. In a study examining the effect of simvastatin on endometriotic stromal cell contraction, simvastatin inhibited collagen gel contraction and also altered the morphology of the cells. The collagen gels were fixed following contraction analysis and observed by laser scanning microscopy. Untreated, dendritic-shaped cells adhered to collagen fibres and contracted to form a dense, tissue-like structure. In contrast, the cells that received simvastatin had become rounded and did not readily adhere to the collagen fibres (Nasu et al. 2009).

It would have been ideal to use primary myometrial cells or tissue to perform these investigations, as an immortalised myometrial cell line may be less representative of the myometrium *in vivo*. However, these resources are limited. It would also have been informative to perform organ bath studies with human myometrial strips. However, these experiments are not only demanding of human samples, but are unable to determine long term effects, as contractions in an organ bath are unreliable after 24 hours. They are also less suitable for teasing out which particular element of the pathway is targeted by a drug. Other studies have previously shown that simvastatin inhibits the frequency of myometrial contractions, but intensity was not formally quantified (Gonzalez et al. 2014).

4.4.2 Simvastatin attenuates LPS-induced contraction of human myometrial cells

Labour is an inflammatory process, which requires the influx of leukocytes into the myometrium and an increase in the production of inflammatory mediators, to stimulate contractions. In addition, it is thought that infection is the cause of 25-40% of PTBs (Goldenberg et al. 2008). Therefore, LPS was added to the collagen gel assay to model this. LPS was shown to stimulate the gels to contract even further. This makes the collagen assay a highly relevant myometrial contraction assay system. Remarkably,

simvastatin was able to attenuate contraction even in the presence of LPS. This is an interesting and important finding as, by stopping myometrial contractions within an inflammatory environment, simvastatin could be a useful therapeutic for PTL. A previous study from our laboratory also found that LPS was capable of inducing these myometrial cells to contract more than the vehicle treated cells (Hutchinson et al. 2014). Although, these experiments utilised a different serotype of LPS.

In the LPS concentration response experiment, none of the concentrations significantly reduced the gel size in comparison to the vehicle. Although, the lower concentrations appeared to slightly reduce the mean gel size. Perhaps the variation and multiple groups within the analysis prevented statistical significance, as 25µg LPS did significantly reduce the gel size in later experiments. It was surprising that higher concentrations of LPS did not affect the contraction of the cells. It would be interesting to lower the concentration of LPS, to see if this would induce more contraction. A possible explanation for the difficulty in inducing increased contraction is that the gels already reduced greatly in size due to the basal contraction of the cells. By 48 hours, the gels were very small. It is likely that they were so densely packed, that they could not reduce much more in size.

4.4.3 Mevalonate and GG-PP but not F-PP, abolish the anti-contractile effect of simvastatin

Statins are inhibitors of the mevalonate pathway. Mevalonate is the first metabolite of which production is inhibited when statins competitively inhibit HMG-CoA reductase. In the collagen assay experiments, mevalonate supplementation reversed the anti-contractile effects of simvastatin. This implies that simvastatin is, in fact, affecting the contraction of the myometrial cells by targeting the mevalonate pathway, rather than an alternative pathway.

Isoprenoid pyrophosphates, F-PP and GG-PP, are branching points in the mevalonate pathway. F-PP further branches out into a sterol pathway, to produce cholesterol for instance, and a non-sterol pathway, for the post-translational modifications of proteins. Small GTPases are isoprenylated by covalent attachment to F-PP, for Ras, or GG-PP, for Rho, Rac and Cdc42 (Nurenberg and Volmer 2012). In the contraction assay, GG-PP abolished the anti-contractile actions of simvastatin. This suggests that the anti-

contractile effect of simvastatin was via the inhibition of geranylgeranylation of small GTPases. In contrast, F-PP supplementation did not reverse the effects of simvastatin. This implies that these pleiotropic effects were independent of both cholesterol and farnesylated protein inhibition.

Although F-PP is the precursor to GG-PP, F-PP failed to reverse simvastatin effects. To produce GG-PP, both F-PP and I-PP must be present (Nurenberg and Volmer 2012). When F-PP alone is added back into a statin treatment experiment, I-PP is not present, thus GG-PP cannot be produced in this artificial setting. This explains how the differential effects of these intermediates can be identified *in vitro*. It can be hypothesised that by supplementing simvastatin experiments with both F-PP and I-PP, G-PP may be produced and the actions of simvastatin could be reversed. This experiment could be performed in the future.

The results of these mechanism studies confirmed and extend those in the literature. Mraiche et al. (2005) reported that simvastatin induced relaxation in vascular smooth muscle cells by preventing Rho geranylgeranylation (Mraiche et al. 2005). Similar studies also attributed simvastatin-induced vasodilation of endothelial cells and the relaxation of vascular smooth muscle cells to the inhibition of the mevalonate/ROCK pathway (Alvarez de Sotomayor et al. 2001; Nagaoka et al. 2007).

This mechanism has also been observed with other statins. For example, fluvastatin reduced the contraction of rat myofibres. These effects were reversed by GG-PP but not F-PP and mimicked by a specific geranylgeranyl transferase inhibitor (GGTI-289) (Tanaka et al. 2010). A study investigating endothelial junction integrity found that atorvastatin prevented the translocation of Rho to the endothelial cell membrane. This resulted in cytoskeletal rearrangement, which was due to the inhibition of geranylgeranylation and not farnesylation (Xiao et al. 2013).

In contrast to these findings, which have utilised drugs renowned for their cholesterol-lowering properties, one study suggested that high levels of cholesterol may reduce uterine activity. The addition of cholesterol to myometrial strips dissected from pregnant rats reduced the frequency and amplitude of contractions (Smith et al. 2005). The authors suggest that this may explain the difficulties obese/dyslipidaemic women

can have when labouring and how low cholesterol levels may lead to conditions such as PTB. However, there has been little evidence since to support these claims. Cholesterol levels could be investigated in the myometrial cells following simvastatin treatment. However, it is unlikely that cholesterol is impacting the results described in this chapter, as the anti-contractile effects appear to be as a result of the GG-PP branch of the pathway, not cholesterol precursor, F-PP.

4.4.4 The effect of simvastatin treatment on pMLC levels

In order to investigate the anti-contractile mechanism of simvastatin further, the effect this statin has on pMLC levels was assessed. The Rho/ROCK pathway has long been associated with smooth muscle contraction. Rho binds to ROCK following geranylgeranylation, conversion of GDP to GTP and membrane anchoring (Matsui et al. 1996). Activated ROCK can interfere with MLCK, MLCP or phosphorylate MLC directly at the Ser-19 site (Schaafsma et al. 2008). This facilitates the actin activation of myosin ATPase, leading to actin-myosin contraction (Somlyo and Somlyo 1994; Amano et al. 1996; Samizo et al. 1999).

In this chapter, the effect of simvastatin on the abundance of pMLC was investigated by an In-Cell Western assay. Unsurprisingly, levels of pMLC were highest in the basally contracting vehicle groups. Simvastatin treatment concentration-dependently reduced the phosphorylation of MLC, validating its anti-contractile effect at a molecular level. Again, mevalonate and GG-PP, but not F-PP, abolished the effects of simvastatin. This suggests that simvastatin attenuated myometrial cell contraction by inhibiting the isoprenylation of Rho by GG-PP, preventing the activation of ROCK, which subsequently prevented the phosphorylation of MLC (Figure 4.15).

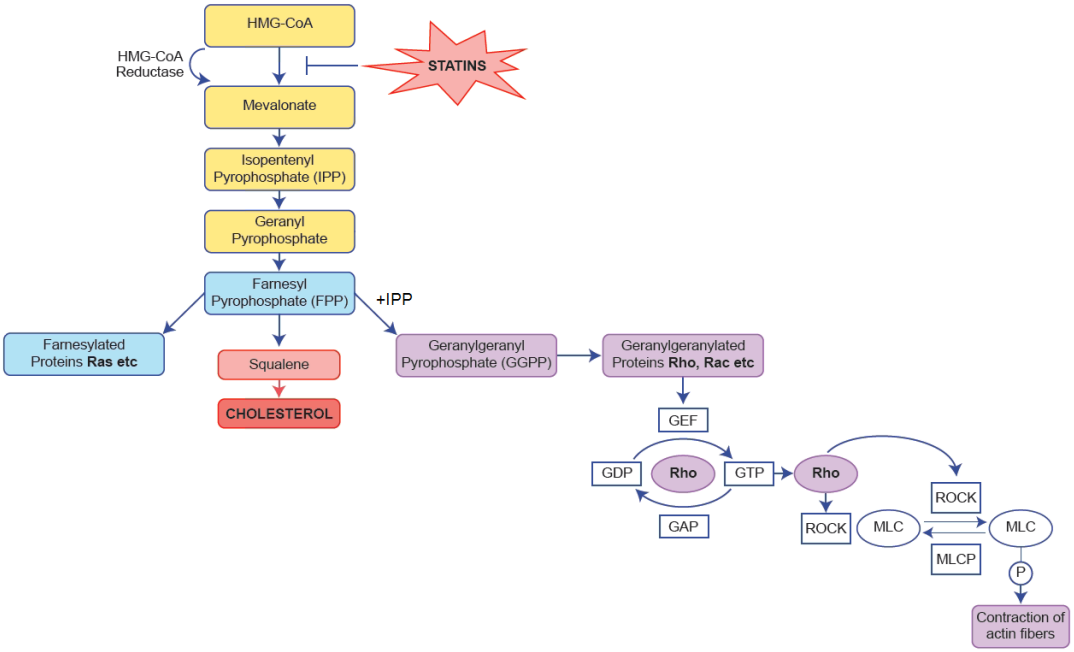

Figure 4.15. Simvastatin reduces myometrial cell contraction by inhibiting the Rho/ROCK pathway via the attenuation of GG-PP production. Mevalonate and GG-PP, but not F-PP, supplementation abolished the anti-contractile effects of simvastatin. By attenuating the production of GG-PP, simvastatin inhibited the geranylgeranylation of Rho. Thus, simvastatin indirectly prevented the phosphorylation of MLC by ROCK, which subsequently inhibited actin fibre contraction. ROCK: Rho-kinase. MLC: Myosin light chain. MLCP: Myosin light chain phosphatase.

Many studies are in agreement regarding simvastatin-induced attenuation of pMLC levels. During an investigation of proliferative vitreoretinal diseases, simvastatin inhibited the contraction of bovine hyalocytes embedded in collagen gel by reducing the phosphorylation of MLC (Kawahara et al. 2008). Simvastatin also reduced pMLC levels and RhoA activity in a bovine aortic endothelial cell line (Lampi et al, 2016). As matrix stiffness increased, RhoA activity increased. Interestingly, simvastatin reduced levels of active RhoA but total RhoA expression was unaffected. The authors also utilised Traction Force Microscopy and found that simvastatin reduced the contractile force of the endothelial cells, as well as altering endothelial cell focal adhesions and cell cytoskeletal organisation, resulting in more elongated cells (Lampi et al, 2016). Zeng et al. (2005) reported that simvastatin inhibited the contraction of glomerular endothelial cells, by preventing RhoA activation and subsequent MLC phosphorylation. Similar studies, in multiple cell types, have also proposed that the

mechanism by which statins exert their anti-contraction, pleiotropic effects is by inhibiting Rho/ROCK and subsequently, preventing MLC phosphorylation (Alvarez de Sotomayor et al. 2001; Mraiche et al. 2005; Kuhlmann et al. 2006; Nagaoka et al. 2007; Kidera et al. 2010; Wu et al. 2011; Xiao et al. 2012; Alp Yildirim et al. 2016). Other statins, such as lovastatin and atorvastatin, have also been shown to inhibit contractility and reduce pMLC levels (Wu et al, 2011; Xiao et al, 2012).

The importance of ROCK-mediated MLC phosphorylation for myometrial contractility has been previously been identified (Moran et al. 2002; Hudson et al. 2012). The Rho/ROCK pathway is believed to be an important tocolytic target, when there have been so few novel therapies is recent decades (Mitchell et al. 2013). For instance, ROCK inhibitors have been demonstrated to prolong gestation in PTB mouse models (Tahara et al. 2005; Goupil et al. 2010).

Furthermore, studies have also found that simvastatin can affect Ca^{2+} flux, which may be another anti-contractile mechanism. One study found that simvastatin impaired vascular contractility by altering the influx of Ca^{2+} and disturbing RhoA activity, leading to structural remodelling (Kang et al. 2014). Simvastatin relaxed rat superior mesenteric artery rings *in vitro*, which was associated with the opening of voltage-dependent K^+ channels and the blocking of extracellular Ca^{2+} influx (Chen et al. 2016). Simvastatin also had antagonistic effects on voltage-gated calcium channels, inhibiting the contraction of gastrointestinal tissue, in an organ bath study (Ali et al. 2016). It would be interesting to assess the effect of simvastatin treatment on Ca^{2+} flux in the myometrial cells. If Ca^{2+} flux was inhibited, this could provide an additional explanation for the downstream effect on MLC phosphorylation. This could be performed using a colorimetric assay to measure $[Ca^{2+}]i$ changes.

The next step in deciphering the pathway in full would be to add inhibitors of farnesyl transferase (FTI) and geranylgeranyl transferase (GGTI) to the collagen contraction assay. These enzymes catalyse the isoprenylation of small GTPases. GGTI should mimic the effect of simvastatin, inhibiting contraction and FTI would not affect the cell basal contraction, to confirm the findings of this chapter. As the hypothesis is that simvastatin inhibits myometrial cell contraction by inhibiting the Rho/ROCK pathway, the next experiment could be to quantify RhoA. An experiment was

attempted to immunoprecipitate active RhoA in myometrial cells. However, active RhoA is very lowly expressed. It was difficult to quantify in vehicle treated cells and, as simvastatin is hypothesised to inhibit RhoA, a large number of cells would be required to conclusively state that RhoA had been reduced. However, other studies have successfully addressed the effect of statins on RhoA. For example, performing a colorimetric RhoA assay or assessing the effect of statins on the translocation of RhoA (Xiao et al. 2013; Lampi et al. 2016). These experiments could be performed in the future. Additionally, a previous study from our laboratory showed that ROCK inhibitors can prevent the contraction of myometrial cells. Therefore, this experiment was not repeated, as it would merely mimic the effect of simvastatin (Hutchinson et al. 2014).

A possible limitation in the design of these experiments is that concentration response experiments for GG-PP and F-PP were only performed once, or twice for mevalonate. As there are a multitude of studies that have performed similar experiments in the literature and the concentrations used are consistent, these experiments were performed briefly, in the interest of time and conserving materials. The myometrial cells are slow growing and can only be used up to passage 22 (purchased at passage 12) and the contraction assays require a lot of collagen to form gels solid enough to set and detach from the wells. When F-PP did not affect myometrial contraction at the concentration cited in the literature, the concentrations were increased to confirm that F-PP did not reverse the effect of simvastatin, rather than the concentration being incorrect. It would be ideal to repeat this experiment at least three individual times but this would require many more cells, collagen and a large volume of F-PP to reach such high concentrations. Given that 10µM F-PP is most commonly used and there was no effect up to 100µM, the evidence from one concentration response experiment was considered relatively convincing in this case.

In summary, simvastatin attenuated the contraction of human myometrial cells and lowered pMLC levels in these cells, both alone and in the presence of LPS. These effects were reversed by the addition of mevalonate and GG-PP but not by F-PP. This suggests that the mechanism by which simvastatin reduced myometrial contractions was via inhibition of the Rho/ROCK pathway.

Chapter 5: Investigating the effect of statin treatment on an LPS-induced mouse model of PTB

5.1 Introduction

PTL is defined as labour before 37 completed weeks of gestation (WHO 1977). Despite considerable medical advances, PTB occurs in 5-18% of pregnancies annually and importantly, it is the leading cause of mortality in children under 5 years (Blencowe et al. 2012; Harrison and Goldenberg 2015).

Treatments targeting PTL are limited and have focused on tocolytic therapies, such as NSAIDs, COX-2 selective inhibitors, calcium channel blockers and oxytocin receptor inhibitors, which attempt to impede myometrial contractions. Delaying birth allows for corticosteroids to be administered for fetal lung development. However, these tocolytic treatments are largely ineffective at substantially delaying PTB and fail to improve the outcome of the premature neonates (Haas et al. 2012; Haas et al. 2014). Above all, these treatments do not target the underlying cause of the PTL and there is no benefit of prolonging a pregnancy if the *in utero* environment is adverse for the fetus (Haas et al. 2012).

PTB is most commonly associated with infection/inflammation (Romero et al. 2014). Inflammation initiates labour by stimulating myometrial contractions, fetal membrane rupture and cervical ripening (Rinaldi et al. 2011). For this reason, anti-inflammatory treatments are now being considered for delaying PTB and reducing fetal injury. For example, non-specific NF-κB inhibitors, TLR4 antagonists, TNF biologics and novel CSAIDs have all been investigated (Holmgren et al. 2008; Li et al. 2010; Stinson et al. 2014; Ng et al. 2015). Progesterone administration is commonly used to maintain pregnancy and reduce inflammation. However, there is conflicting evidence regarding the benefits of this treatment for reducing the rates of PTB and improving neonatal outcome (da Fonseca et al. 2003; Fonseca et al. 2007; Hassan et al. 2011; Dodd et al. 2013; Norman et al. 2016). The demand for an effective therapy prompted the investigation of statin treatment due to the increasingly documented pleiotropic properties associated with these drugs.

Statins are reported to reduce inflammation and have anti-contractile effects (Kuzuya et al. 2004; Mraiche et al. 2005; Perez-Guerrero et al. 2005; Nasu et al. 2009; Zhou and Liao 2010; Gonzalez et al. 2014). Recently, statins have been suggested for the prevention and treatment of pregnancy disorders, such as preeclampsia and anti-phospholipid syndrome (Kumasawa et al. 2011; Lefkou et al. 2016).

Chapter 3 and Chapter 4 described the anti-inflammatory and anti-contraction effects of simvastatin on human myometrial cells, respectively. These are beneficial attributes for a PTL therapeutic. Pravastatin did not have anti-inflammatory effects on the LPS-induced myometrial cells. A possible explanation for this was that this statin could not enter these cells as it necessitates specific transporter uptake. Consequently, pravastatin was not included in the myometrial cell contraction experiments performed in Chapter 4. However, this statin may still have beneficial effects *in vivo*. Therefore, it was hypothesised that statin treatment will prevent PTB in an LPS-induced mouse model. The aim of this chapter was to investigate the effect of pravastatin and simvastatin treatment on an intrauterine LPS-induced PTB mouse model. Furthermore, to assess the effect of this treatment on maternal inflammation.

5.2 Methods

5.2.1 Ultrasound-guided LPS-induced mouse model of PTB

Mice received an intrauterine injection of LPS (0.3-20µg) or PBS on gestational D17, under ultrasound guidance, as described in 2.8.2 (Rinaldi et al. 2015b). For statin experiments, mice received an intraperitoneal injection of pravastatin (10µg) or simvastatin (20µg or 40µg) on D16 and again 2 hours after intrauterine injection of LPS/PBS, as explained in 2.8.4. The mice were monitored by CCTV for signs of delivery. Time to delivery and the percentage of live born pups were recorded, as detailed in 2.8.3. The "n" numbers for individual experiments are outlined in the figure legends.

5.2.2 Tissue collection

In a separate cohort of pregnant mice, maternal serum, amniotic fluid, uterus and placenta samples were collected 6 hours after intrauterine injection of LPS/PBS, as

outlined in 2.8.5. Tissue was collected from mice receiving PBS (n=6), 20µg simvastatin (n=6), 40µg simvastatin (n=6), 1µg LPS (n=6), 20µg simvastatin and LPS (n=6) and 40µg simvastatin and LPS (n=6).

Maternal serum was also collected from a separate cohort of mice for LC-MS/MS analysis. These mice received one intraperitoneal injection of either 20µg simvastatin, 40µg simvastatin or PBS on D17. Serum was collected 1 hour (n=6) or 2 hours (n=3) after treatment to assess the acute effect of treatment on progesterone concentration, as simvastatin has a half-life of 2 hours (Schachter 2005).

5.2.3 qRT-PCR

The mRNA expression of multiple genes in the mouse uterus and placenta were assessed by qRT-PCR analysis, as described in 2.10. Uterus and placenta samples were collected from both the right and left horns, 6 hours after intrauterine injection of LPS/PBS. In the uterus, inflammatory markers *Tnf*, *Il-1β*, *Il-6*, *Il-10*, *Cxcl1*, *Cxcl2*, *Ccl2* and *Tlr4* were investigated, as well as contraction-associated genes *Cx43* and *Cox-2* (n=6). In the placenta, *Tnf*, *Il-1β* and *Il-10* were investigated (n=6).

The mRNA expression of these genes of interest was normalised to the endogenous control gene, *β-actin*, within each individual sample. Samples were then compared to the PBS control group using the $2^{-\Delta\Delta}$ CT method of analysis, as detailed in 2.10. The results are presented as fold change relative to the PBS control group.

5.2.4 ELISA

An ELISA was performed to quantify the concentration of IL-6 in the maternal serum (n=3-6) and in pooled amniotic fluid samples (n=5-6), which were collected from dams 6 hours after intrauterine injection with LPS/PBS. Details of the assay are explained in 2.11.4.

5.2.5 Western blot

Western blot analyses were performed to detect and quantify IL-6 and CX43 in the mouse uterus, collected from the PTB mouse model on D17. Samples were collected 6 hours after intrauterine injection of LPS/PBS. Protein was extracted from uterus samples collected from the right horn of the uterus of each mouse and performed as

described in 2.12.1. Western blot was carried out as described in 2.12.3 and 2.12.4. CX43 was detected using fluorescence (n=6). Due to low levels, IL-6 was amplified using an HRP enzyme and detected using chemiluminescence (n=6). The intensity of each band was normalised to loading control, α-Tubulin, then quantified using the Odyssey analysis software.

5.2.6 LC-MS/MS

LC-MS/MS was performed in the Mass Spectrometry Core laboratory to quantify progesterone in the mouse maternal serum, as outlined in 2.13. This analysis was carried out in two cohorts of mice. The first cohort were mice that received one intraperitoneal injection of 20µg or 40µg simvastatin or PBS on D17; samples were collected 1 hour and 2 hours later, to assess the acute impact of treatment. The second cohort of mice were those in the PTB model studies, treated with 20µg or 40µg simvastatin on D16 and LPS/PBS on D17, followed by a second intraperitoneal injection of 20µg or 40µg simvastatin 2 hours later. Samples were then collected 4 hours later. If the compounds were below the level of detection, they were assigned the lowest detection value of 0.05ng.

5.2.7 Statistical analysis

Data are presented as mean ± SEM and were analysed using GraphPad Prism. In these studies, "n" represents the number of individual dams treated. Time to delivery data were mainly analysed using Kruskal-Wallis, with Dunn's post hoc test, as data were not normally distributed and could not be transformed. LPS dose response time to delivery data successfully underwent logarithmic transformation, which was then followed by one way ANOVA analysis, with Dunnett's post hoc test. The percentage data for live born pups were analysed by performing an arcsine transformation on the proportions, followed by a one-way ANOVA, with Dunnett's post hoc test. All gene expression (CT values), ELISA, Western blot and LC-MS/MS data were square root transformed to achieve normal distribution (if necessary), then analysed by one-way ANOVA, followed by Dunnett's or Tukey's post hoc test. A p value <0.05 was considered statistically significant.

5.3 Results

5.3.1 The effect of statin treatment on an LPS-induced mouse model of PTB

The laparotomy procedure is widely performed to administer bacterial products, such as LPS, into the uterus to model intrauterine infection-induced PTB in mice. However, due to the invasive nature of this surgery, researchers within our laboratory developed a novel, less invasive model using ultrasound to guide the intrauterine injection (Rinaldi et al. 2015b). This model was used for the following experiments.

5.3.1.1 Time to delivery

Time to delivery was calculated as the time from intrauterine injection of 20µg LPS/PBS to the delivery of the first pup. Term was defined as ≥45 hours after intrauterine injection of 20µg LPS/PBS. When mice were treated with PBS, pravastatin or simvastatin alone, the mice all delivered at term (59.5 ± 2.0 hours; 61.3 ± 2.1 hours; 61.5 ± 3.1 hours, respectively; Figure 5.1). When mice received an intrauterine injection of 20µg LPS, they delivered much earlier (28.1 ± 10 hours), with 5 out of 6 mice delivering preterm. However, given the small group size, this was not statistically significant. As the data were not normally distributed, a Kruskal-Wallis test was performed, which is less powerful than a one-way ANOVA. When mice were given 10µg pravastatin on D16 and 2 hours after 20µg LPS, the mean delivery time was 27.1 ± 9.3 hours. Interestingly, 1 of the 5 mice delivered at term following this treatment. When mice were treated with 20µg simvastatin on D16 and 2 hours after 20µg LPS, all of the mice delivered preterm, with a mean delivery time of 18.0 ± 1.3 hours (p=0.046 vs PBS).

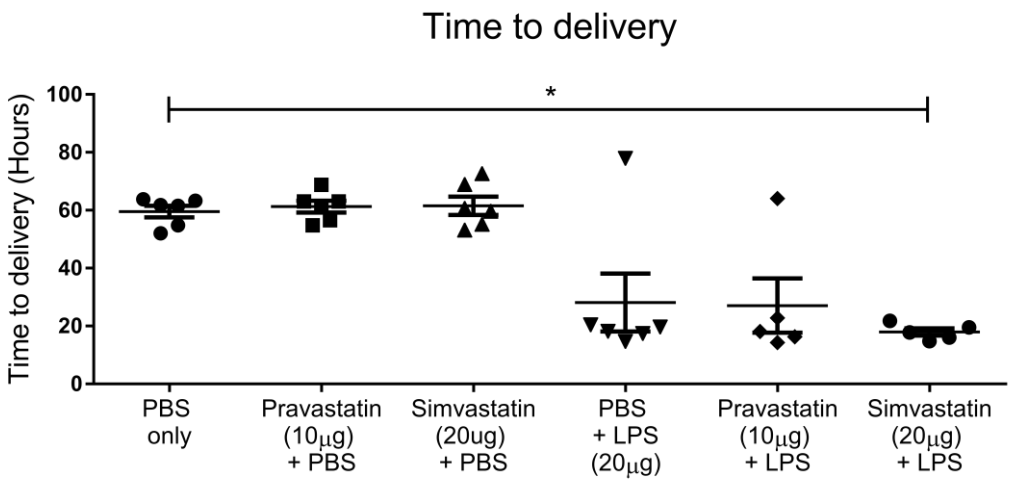

Figure 5.1. Time to delivery. Mice received PBS, pravastatin or simvastatin on D16, then intrauterine 20μg LPS/PBS on D17, followed by another PBS, pravastatin or simvastatin treatment 2 hours later. Time to delivery was calculated as the time from intrauterine injection of 20μg LPS/PBS on D17 until the delivery of the first pup. When mice were given intrauterine PBS, they delivered at term (~60 hours after PBS). When mice received intrauterine 20μg LPS, they delivered preterm (~24 hours after LPS), irrespective of statin treatment. Although, only the mean time to delivery of the simvastatin and LPS group was found to be significantly reduced compared to the PBS group. n=5-6, *$p<0.05$, mean ± SEM, Kruskal-Wallis with Dunn's post hoc test.

5.3.1.2 Percentage of live born pups

The percentage of live born pups was calculated by dividing the number of live pups found in the cage by the number of viable fetuses counted via ultrasound on D17, with an individual data point representing each dam/litter (Figure 5.2).

In the PBS control group, the mean percentage of pups born alive was 91.1 ± 5.7%. In line with expectations based on time to delivery, pup survival was also similar after administration of pravastatin or simvastatin with intrauterine PBS treatment (95.8 ± 2.6%, 82.6 ± 12.4%, respectively). When pups were delivered preterm by the mice that received an intrauterine injection of 20μg LPS, the mean percentage of live born pups was significantly lower (12.5 ± 12.5%, $p<0.0001$). This effect on the pup survival was also observed when mice were given pravastatin or simvastatin after injection of 20μg LPS (20%, $p=0.0075$; 0%, $p<0.0001$, respectively).

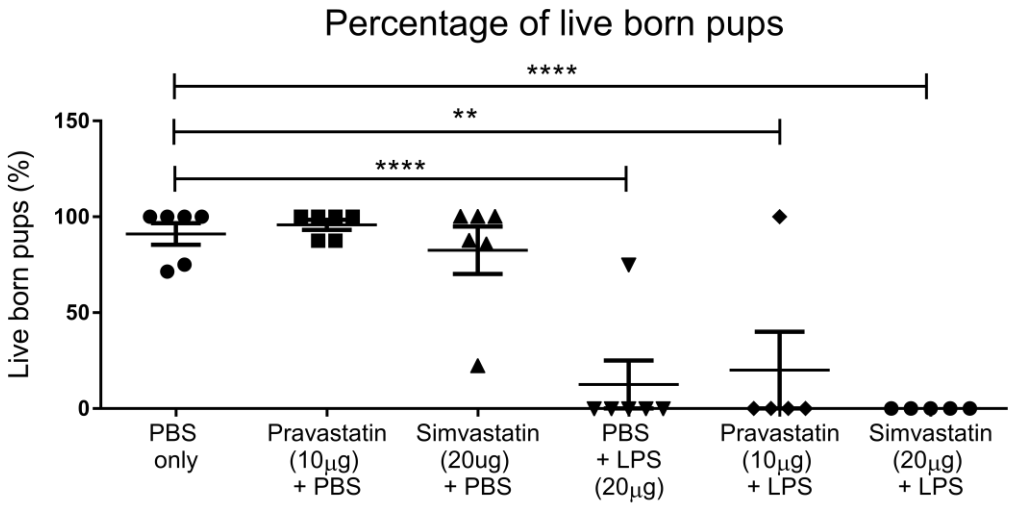

Figure 5.2. Percentage of live born pups. The percentage of live born pups per litter was calculated by dividing the number of live pups delivered by the number of viable fetuses counted using ultrasound on D17. When mice received intrauterine PBS, with either PBS, pravastatin or simvastatin treatment, there was a high percentage of live born pups. However, 20μg LPS administration significantly reduced the percentage of pups born alive, irrespective of whether the mice received pravastatin or simvastatin treatment. n=5-6, **p<0.01, ****p<0.0001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

5.3.2 Ultrasound-guided LPS dose response experiment to induce PTB in mice

In previous studies using the laparotomy model to perform intrauterine injections, 20μg LPS was required to induce PTB (Rinaldi et al. 2014). This concentration was also sufficient to induce PTB in the ultrasound model (Rinaldi et al. 2015b). However, as a dose response experiment had not previously been carried out, the impact of 0.3-20μg LPS on PTB was investigated.

5.3.2.1 Time to delivery

The mean time to delivery in the PBS control group was 61.2 ± 3.4 hours (Figure 5.3). When an intrauterine injection of 0.3μg LPS was given, only 1 out of 4 mice delivered preterm (mean delivery time 56.6 ± 8.3 hours). When the mice received 1μg LPS, all mice delivered preterm (23.7 ± 0.6 hours, p=0.014 vs PBS). Following an intrauterine injection of 3μg LPS, 3 out of 4 mice delivered preterm. However, there was no significant difference compared to the PBS group (32.5 ± 11.2 hours, p=0.0577). When the mice were given 10μg LPS, all dams delivered preterm (20.9 ± 0.4 hours, p=0.0049

vs PBS). With 15µg LPS, all mice delivered preterm (22.2 ± 0.5 hours, p=0.0168 vs PBS). Finally, when mice received 20µg LPS, 6 out of 7 mice delivered preterm (24.8 ± 8.7 hours, p=0.0004 vs PBS). It was notable that even at the higher doses of LPS, there were some mice where delivery was not preterm, a result that was replicated in other study groups in this chapter.

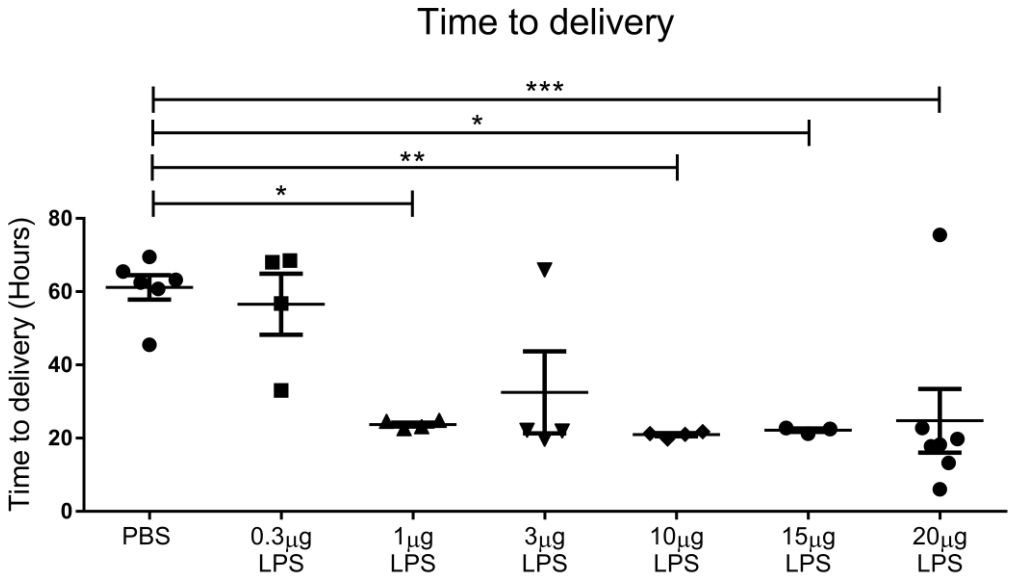

Figure 5.3. Time to delivery. Mice received intrauterine PBS or LPS (0.3-20µg) to induce PTB. 0.3µg LPS did not cause PTB. However, concentrations of 1, 10, 15 and 20µg significantly reduced the time from intrauterine injection to the delivery of the first pup, inducing PTB, compared to the PBS group. PBS and 20µg LPS data were collected previously by Dr Sara Rinaldi, n=3-7, *p<0.05, **p<0.01, ***p<0.001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

5.3.2.2 Percentage of live born pups

In the PBS group, the mean percentage of live born pups from each litter was 90.5 ± 4.8% (Figure 5.4). The mean percentage of pups born alive after 0.3µg LPS was 68.8 ± 23.1%, which was not significantly different compared to the PBS group. When the mice received 1µg LPS, none of the pups from the 4 litters survived birth (0%, p=0.0004 vs PBS). Following an intrauterine treatment of 3µg LPS, the mean percentage of live born pups was 25 ± 25%, which was significantly lower than the PBS group (p=0.0122). The percentage of live born pups was also reduced in the

groups that received 10µg LPS (7.1 ± 7.1%, p=0.004), 15µg LPS (4.8 ± 4.8%, p=0.0022) and 20µg LPS (11.4 ± 11.4%, p=0.0004), compared to the PBS group.

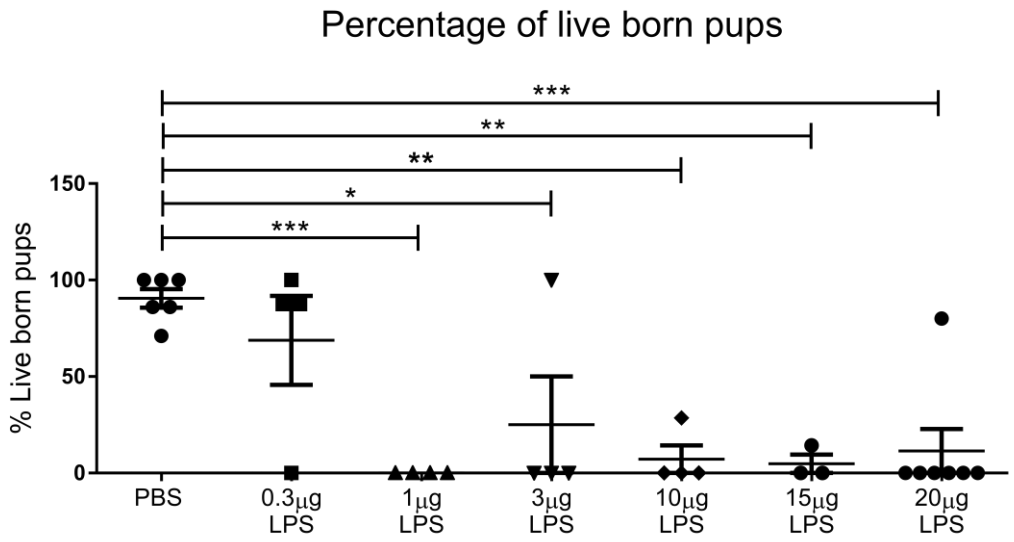

Figure 5.4. Percentage of live born pups. A dose response experiment was performed using 0.3-20µg LPS. When mice were given 0.3µg LPS, the percentage of live born pups was not significantly different from the PBS group. However, when mice received 1-20µg LPS, the percentage of pups born alive was significantly reduced compared to the PBS group. PBS and 20µg LPS data were collected previously by Dr Sara Rinaldi, n=3-7, *p<0.05, **p<0.01, ***p<0.001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

5.3.2.3 Inflammatory mRNA expression in the mouse uterus

It was established that lowering the concentration of LPS to 1µg was still sufficient to cause PTB. To further explore the impact of LPS on inflammatory mechanisms implicated in PTB, the effects of 20µg and 1µg LPS on *Cxcl1*, a homologue of human *IL-8*, and *Il-6* expression were compared.

When mice received 20µg LPS, there was a robust upregulation of *Cxcl1* mRNA in the whole uterus, including both endometrium and myometrium, which increased 366.9 fold, when comparing to the PBS group (p<0.0001; Figure 5.5A). Lowering the concentration of LPS to 1µg also stimulated a significant upregulation of *Cxcl1* compared to the PBS group (27.4 fold, p<0.0001) but the relative expression of *Cxcl1* was significantly lower than in the 20µg LPS group (p=0.0004).

This pattern was also observed when investigating *Il-6* expression (Figure 5.5B). At the higher dose of 20µg LPS, there was a large increase in *Il-6* expression in the uterus (312.9 fold, p<0.0001 vs PBS). Again, 1µg LPS increased *Il-6* expression compared to PBS (18.8 fold, p=0.0003) but this was significantly lower when compared to the 20µg treatment (p=0.0011).

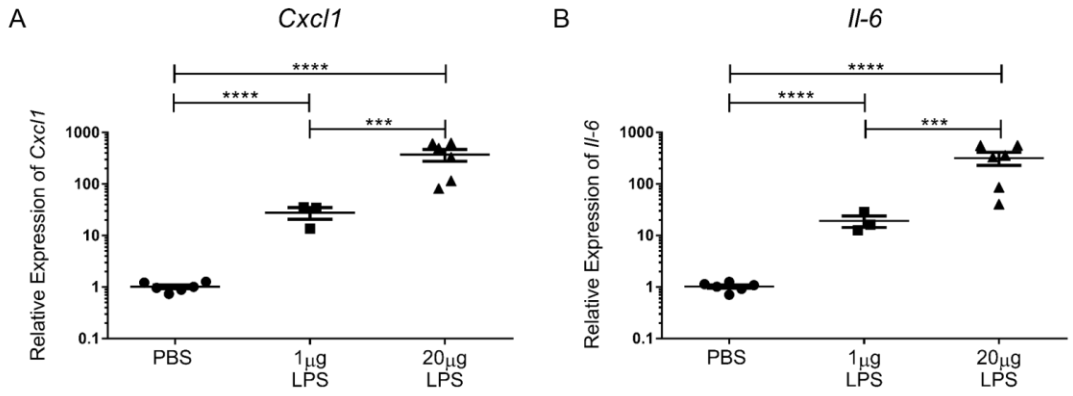

Figure 5.5. Uterine inflammatory gene expression. (A) When mice received 1µg and 20µg LPS, *Cxcl1* was significantly upregulated in the uterus, compared to the PBS group. By lowering the concentration of LPS from 20µg to 1µg, *Cxcl1* expression was significantly reduced. (B) *Il-6* expression was also significantly increased with LPS administration, compared to the PBS group. The upregulation of *Il-6* was significantly lower with 1µg LPS, compared to 20µg LPS. PBS and 20µg LPS samples were provided by Dr Sara Rinaldi. n=3-6, ***p<0.001, ****p<0.0001, mean ± SEM, one-way ANOVA with Tukey's post hoc test.

5.3.3 The effect of statin treatment on a refined LPS-induced model of PTB

Following the dose response studies, experiments were repeated to establish whether pravastatin or simvastatin treatments could delay PTB or improve the percentage of live born pups. In these studies, PTB was induced using a 1µg LPS dose in the intrauterine infection mouse model rather than the dose of 20µg LPS. For clarity, pravastatin and simvastatin results are displayed separately. As some pravastatin and simvastatin experiments were performed simultaneously, PBS and LPS controls were frequently shared. Thus, some control data will appear on both pravastatin and simvastatin graphs.

5.3.3.1 Pravastatin

Mice were treated with an intraperitoneal injection of pravastatin (10µg) or PBS on D16, followed by ultrasound-guided injection of 1µg LPS or PBS on D17 and another pravastatin or PBS treatment 2 hours later. Time to delivery and the percentage of live born pups were recorded.

5.3.3.1.1 Time to delivery

Mice given only PBS had a mean delivery time of 61.8 ± 1.8 hours (Figure 5.6). Mice that received pravastatin and intrauterine PBS had a similar mean time to delivery of 61.3 ± 2.1 hours. In the LPS group, 8 out of the 9 mice delivered preterm, with a mean time to delivery of 25.3 ± 4.4 hours after intrauterine injection (88.9% PTB, p=0.0039 vs PBS; p=0.0364 vs pravastatin + PBS). When mice received LPS and pravastatin treatment, PTB was prevented in 2 of the 8 mice (75% PTB). The mean delivery time was 33.0 ± 7.9 hours, which was not statistically different compared to the PBS or LPS group.

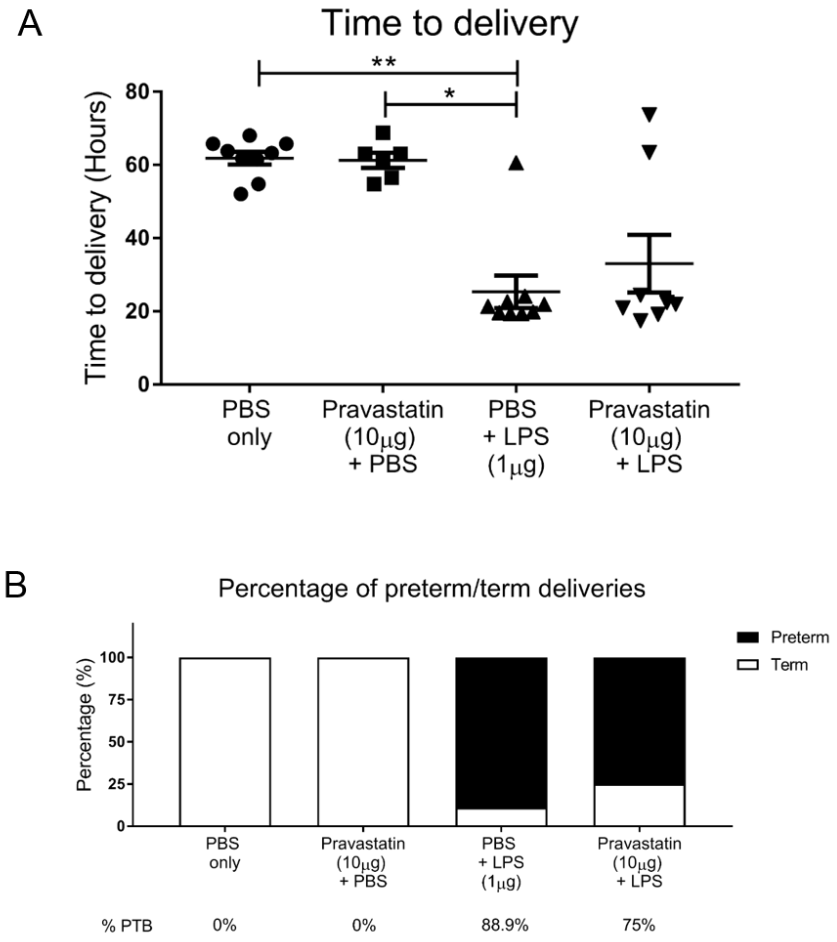

Figure 5.6. Time to delivery. Mice were treated with PBS or pravastatin on D16, followed by 1µg LPS/PBS on D17 and another PBS or pravastatin treatment 2 hours later. (A, B) When mice were given intrauterine PBS, they delivered at term (0% PTB). When mice received intrauterine LPS, time from intrauterine injection to delivery was significantly reduced, compared to the PBS only and pravastatin control groups and the PTB rate was 88.9%. When pravastatin and LPS were given, PTB was prevented in 2 of the 8 mice, with a PTB rate of 75%. There was no significant difference between this group and the PBS group or LPS group. PBS and LPS control data shared for pravastatin/simvastatin experiments, n=6-9, *p<0.05, **p<0.01, mean ± SEM, Kruskal-Wallis with Dunn's post hoc test.

5.3.3.1.2 Percentage of live born pups

In the PBS group, 88.5 ± 6.2% of pups were born alive (Figure 5.7). When mice were treated with pravastatin and intrauterine PBS, the mean percentage of live born pups was 95.8 ± 2.6%. With LPS treatment, this percentage was greatly reduced to 11.1 ± 11.1% (p<0.0001 vs PBS). When mice received pravastatin and LPS, the mean

percentage of live born pups was also significantly reduced (21.7 ± 14.2%, p=0.0002 vs PBS).

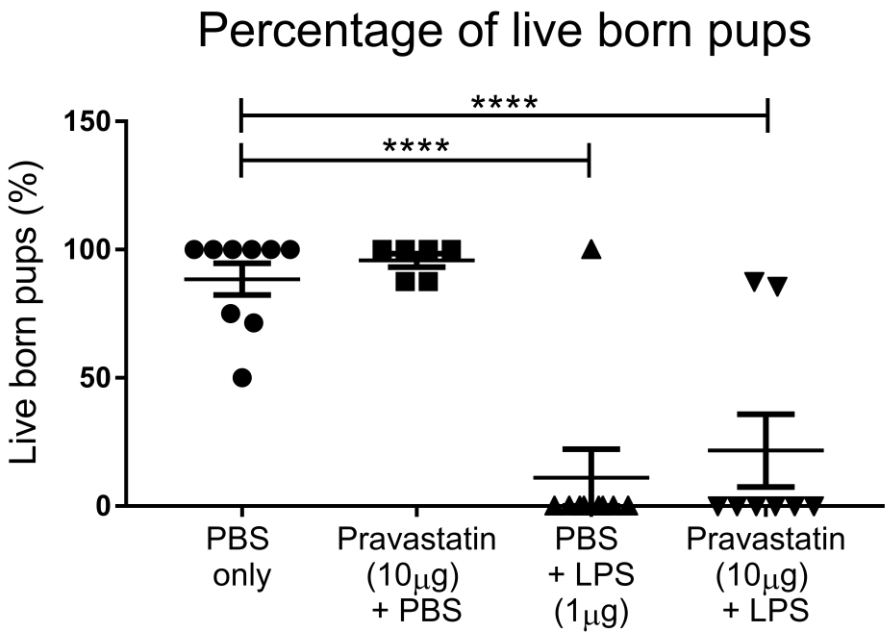

Figure 5.7. Percentage of live born pups. Treatment with 1μg LPS significantly reduced the percentage of pups born alive. This percentage was also significantly reduced when mice received pravastatin and LPS, compared to the PBS group. PBS and LPS control data shared for pravastatin/simvastatin experiments, n=6-9, ****p<0.0001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

5.3.3.2 Simvastatin

The effect of 20μg simvastatin on 1μg LPS-induced PTB and pup survival was investigated. The increased concentration of 40μg simvastatin was also included in these studies.

5.3.3.2.1 Time to delivery

In the PBS group, all mice delivered at term (61.8 ± 1.4 hours; Figure 5.8). When mice received 20μg simvastatin and intrauterine PBS, the mean delivery time was 59.1 ± 2.2 hours and with 40μg simvastatin and PBS, this was 51.7 ± 4.1 hours. When mice were given 1μg LPS, 14 out of 18 mice delivered preterm (77.8% PTB), with a mean delivery time of 30.6 ± 4.3 hours, which was significantly earlier than the PBS group (p=0.0033). When mice received 20μg simvastatin and intrauterine LPS, 8 out of 16

The effect of statin treatment on preterm labour

mice delivered at term, reducing PTB to 50% (Figure 5.8B). The mean delivery time was 46.9 ± 6.5 hours, which was not statistically significant compared to the PBS or LPS groups. Of the 10 mice given 40µg simvastatin and LPS, 6 mice delivered at term (40% PTB) and the mean delivery time for this group was 47.9 ± 6.3 hours.

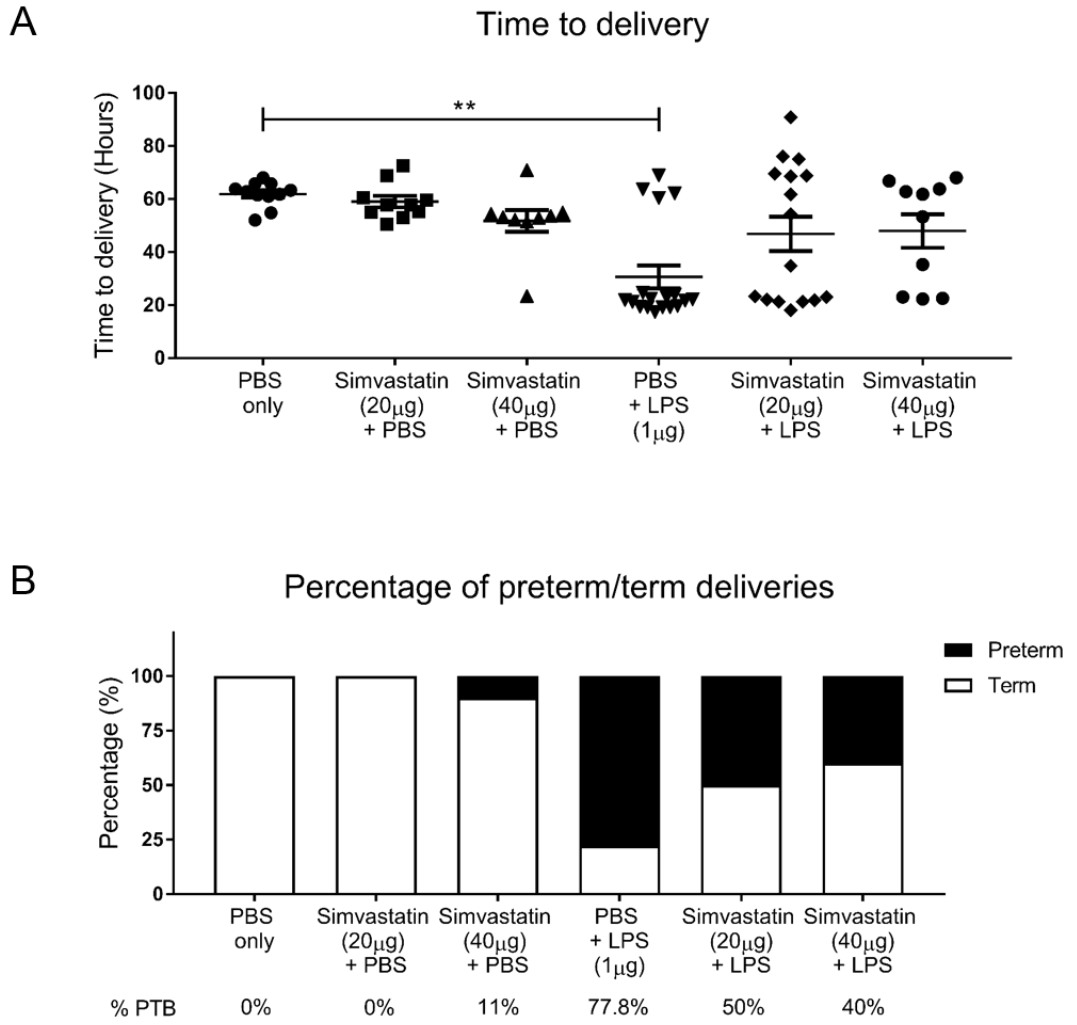

Figure 5.8. Time to delivery. Mice were treated with PBS or simvastatin (20µg or 40µg) on D16, followed by 1µg LPS on D17 and another PBS or simvastatin (20µg or 40µg) treatment 2 hours later. (A-B) When mice received intrauterine PBS, they delivered at term, with one exception when given 40µg simvastatin and PBS. In the LPS group, PTB was induced 77.8% of the time and time to delivery was significantly reduced, compared to the PBS group. When mice received 20µg simvastatin and LPS, PTB rate was reduced to 50% and the time to delivery was no longer significantly different when compared to the PBS group. In the 40µg simvastatin and LPS group, PTB was further reduced to 40% and the mean time to delivery was not significantly different to the PBS group. PBS and LPS control data was shared for pravastatin/simvastatin experiments, n=9-18, **p<0.01, mean ± SEM, Kruskal-Wallis with Dunn's post hoc test.

5.3.3.2.2 Percentage of live born pups

The percentage of pups born alive was similar when mice received intrauterine PBS with intraperitoneal treatments of PBS, 20µg and 40µg simvastatin (90.6 ± 5.2%, 88.3 ± 7.6% and 76.1 ± 10.1%, respectively; Figure 5.9). There was a significant reduction in the LPS group, where the mean percentage of pups surviving birth was only 21.4 ± 9.8% ($p<0.0001$ vs PBS). There was also a significant reduction in live born pups when mice received 20µg simvastatin and LPS (40.4 ± 10.2%, $p=0.0016$ vs PBS). However, there was no significant reduction in pup survival when mice were given 40µg simvastatin and LPS, compared to the PBS group (56.7 ± 15.6%).

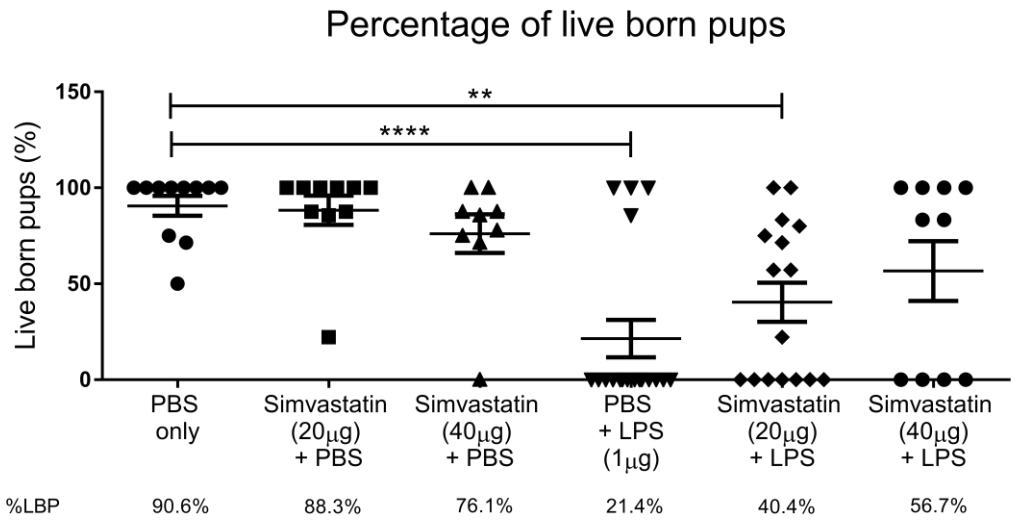

Figure 5.9. Percentage of live born pups. In the LPS group, the mean percentage of pups born alive was significantly reduced to 21.4%, compared to the PBS group. When mice received 20µg simvastatin and LPS, the percentage was also significantly lower than the PBS group. However, when mice were treated with 40µg simvastatin and LPS, the percentage of live born pups was higher, at 56.7%, which was not significantly different to the PBS group. PBS and LPS control data shared for pravastatin/simvastatin experiments, n=9-18, **p<0.01, ****p<0.0001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

5.3.3.2.3 IL-6 concentration in the maternal serum and amniotic fluid

The effect of simvastatin administration on circulating IL-6 concentrations was investigated in the maternal serum of dams (Figure 5.10A). Treatment with 1µg LPS significantly increased the concentration of IL-6 in maternal serum, when compared

to the PBS group, (4067 ± 1168pg/ml vs PBS mean 7.6 ± 2.8pg/ml, p<0.0001), 20µg simvastatin and PBS (5.9 ± 1.1pg/ml, p<0.0001) and the 40µg simvastatin and PBS group (12.4 ± 4.7pg/ml, p<0.0001). When mice were given 20µg simvastatin and LPS, mean IL-6 concentration lowered to 2857 ± 883.3pg/ml but this was not significantly lower than the LPS group. However, IL-6 concentration was significantly reduced when mice received 40µg simvastatin and LPS (1201 ± 535.8pg/ml, p=0.0213 vs LPS).

IL-6 was also measured in the amniotic fluid of these mice (Figure 5.10B). In contrast to the results in the maternal serum, none of the treatments had a significant impact on concentrations of IL-6.

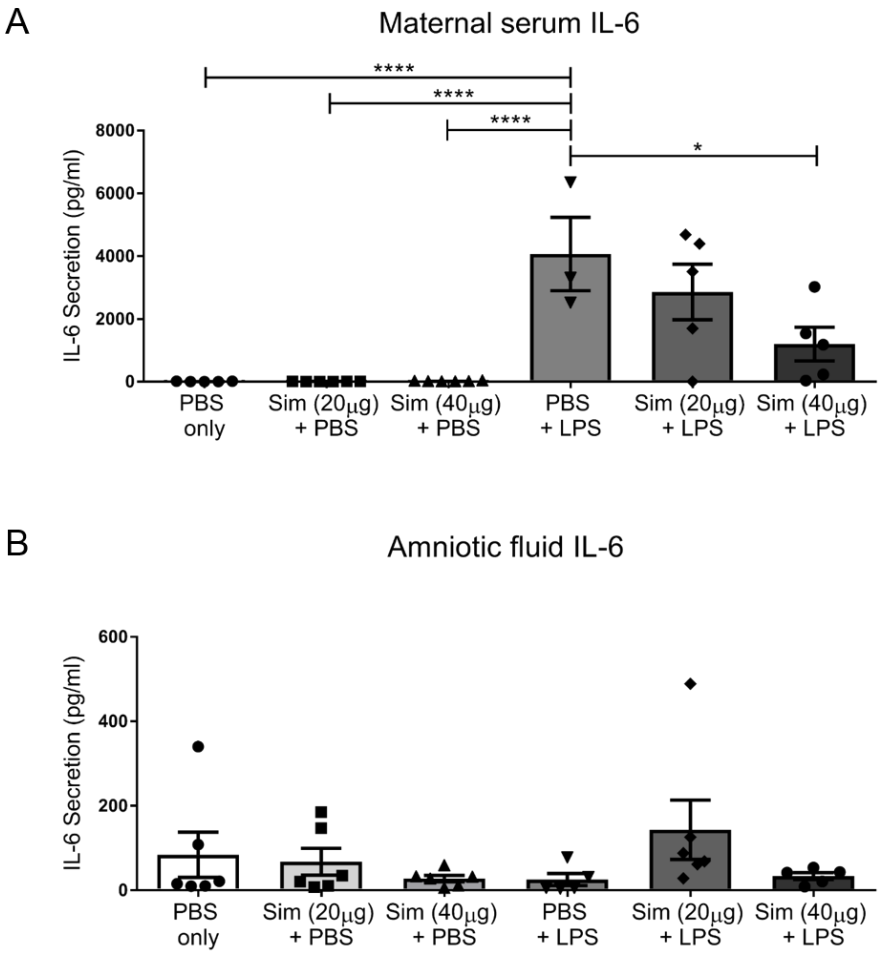

Figure 5.10. IL-6 concentration in the maternal serum and amniotic fluid. (A) 1µg LPS robustly increased IL-6 concentration in the maternal serum. This was significantly reduced when mice received 40µg simvastatin and LPS. (B) IL-6 concentration in the amniotic fluid was unchanged by LPS or simvastatin treatment. n=3-6, *p<0.05, ****p<0.0001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

5.3.3.2.4 mRNA concentrations in the uterus

As simvastatin treatment prevented PTB, as well as lowering the circulating concentration of IL-6, uterine inflammatory gene expression was then investigated.

Firstly, inflammatory cytokine mRNA expression was determined (Figure 5.11). Intrauterine LPS (1µg) administration upregulated *Tnf* expression 6.1 fold in the uterus, compared to PBS treated mice (p=0.0059; Figure 5.11A). This upregulation was also significant in comparison to PBS control mice receiving 20µg simvastatin (p=0.0017) and 40µg simvastatin (p=0.0031) treatment. However, neither 20µg nor 40µg simvastatin treatment affected these levels when given with LPS.

This pattern was also observed with *Il-1β* expression, where LPS upregulated the expression 16.3 fold, which was significantly elevated compared to the groups that received intrauterine PBS; PBS (p=0.0002), 20µg simvastatin (p<0.0001) and 40µg simvastatin (p<0.0001; Figure 5.11B). However, when mice were treated with 20µg or 40µg simvastatin and LPS, there was no effect on *Il-1β* expression compared to the LPS group.

LPS significantly elevated *Il-6* expression 24.7 fold, compared to the PBS group (p=0.0005; Figure 5.11C). This elevation was also significant when compared to the simvastatin control groups (p<0.0001). Interestingly, when mice were given LPS and 40µg simvastatin, *Il-6* expression was 8.9 fold, which was significantly lower in comparison to the LPS group (p=0.0095).

LPS upregulated *Il-10* expression in the uterus compared to the PBS group (6.7 fold, p=0.0366), 20µg simvastatin (p=0.0269) and the 40µg simvastatin control groups (p=0.0397; Figure 5.11D). When 40µg simvastatin and LPS were given, *Il-10* expression was lowered to 2.9 fold (p=0.0328 vs LPS).

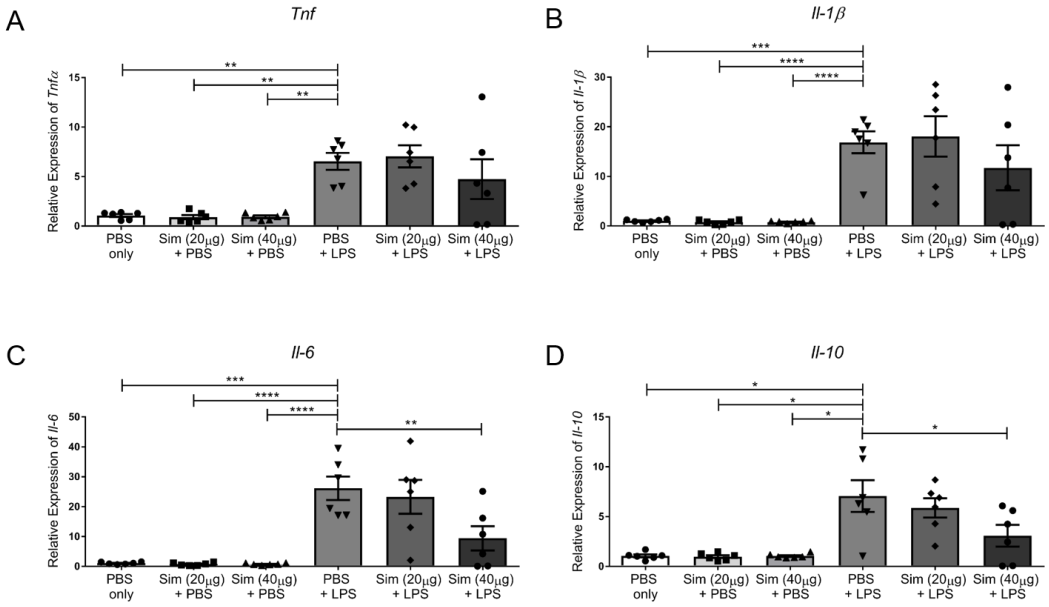

Figure 5.11. Inflammatory cytokine mRNA expression in the uterus. (A) *Tnf* was upregulated in the mice treated with 1µg LPS alone but this was not changed by simvastatin treatment. (B) *Il-β* was upregulated by LPS but these expression levels were unaffected by simvastatin treatment. (C) LPS increased the expression of *Il-6*. These levels were significantly reduced in the 40µg simvastatin and LPS group. (D) *Il-10* expression was increased by LPS and reduced by 40µg simvastatin. n=6, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, mean fold change ± SEM, one-way ANOVA with Dunnett's post hoc test.

In addition to cytokines, the effect of these treatments on a selection of chemokines was investigated in the same uterus samples (Figure 5.12). For instance, 1µg LPS upregulated *Cxcl1* expression 22.7 fold (p=0.0005 vs PBS; Figure 5.12A). This mRNA expression was also upregulated in comparison to the 20µg simvastatin (p=0.0002) and 40µg simvastatin (p=0.001) control groups. Interestingly, treatment of LPS stimulated mice with 40µg simvastatin resulted in a significantly lower concentration of *Cxcl1* compared with LPS alone (14.9 fold, p=0.0464 vs LPS).

This anti-inflammatory effect of simvastatin was not observed for *Cxcl2*, although LPS alone robustly upregulated this gene 39.0 fold, which was significant in comparison to the PBS control groups; PBS (p<0.0001), 20µg simvastatin (p<0.0001) and 40µg simvastatin (p<0.0001; Figure 5.12B).

LPS administration significantly increased the amount of *Ccl2* mRNA, a monocyte chemoattractant, 14.4 fold compared to the controls (PBS p=0.0002, 20µg simvastatin p<0.0001, 40µg simvastatin, p<0.0001; Figure 5.12C). When mice were treated with 40µg simvastatin in addition to LPS, the fold change was 5.8 fold, which was significantly lower than the LPS treatment group (p=0.0342).

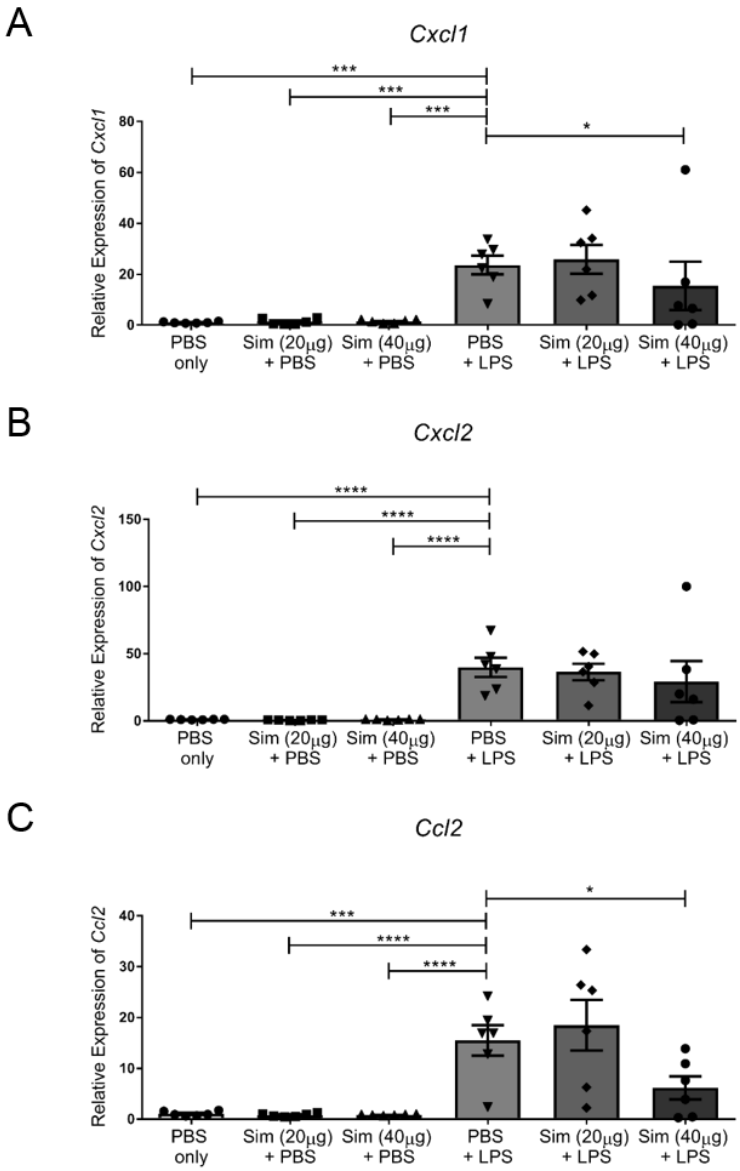

Figure 5.12. Inflammatory chemokine mRNA expression in the uterus. (A) *Cxcl1* was increased by 1µg LPS and this was significantly reduced by 40µg simvastatin. (B) *Cxcl2* was upregulated by 1µg LPS but these mRNA expression levels were unaffected by simvastatin treatment. (C) 1µg LPS increased the expression of *Ccl2*. These expression levels were significantly reduced in the 40µg simvastatin and LPS group. n=6, *p<0.05, ***p<0.001, ****p<0.0001, mean fold change ± SEM, one-way ANOVA with Dunnett's post hoc test.

As TLR4, an innate immune recognition receptor, has been found to play a role in LPS-induced PTB, the expression of the *Tlr4* gene was also investigated (Elovitz et al. 2003; Li et al. 2010). However, neither LPS nor the addition of simvastatin altered *Tlr4* mRNA levels (Figure 5.13).

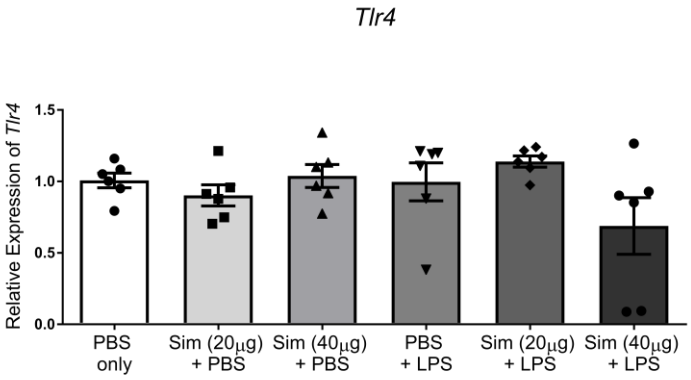

Figure 5.13. *Tlr4* mRNA expression in the uterus. *Tlr4* expression was unaltered by 1µg LPS or simvastatin treatment. n=6, mean fold change ± SEM.

Messenger RNAs encoded by genes associated with uterine contraction were also investigated (Figure 5.14). LPS administration did not significantly alter the concentration of *Cx43*, a gap junction gene, in comparison to any of the control groups, although there was a trend for increase in the LPS group. Treatment of mice administered LPS with 40µg simvastatin significantly lowered *Cx43* concentration compared to the LPS group (0.86 fold vs 1.7 fold, p=0.0143; Figure 5.14A). The expression of *Cox-2*, a gene involved in prostaglandin synthesis, was also investigated but this gene was unaltered by the different treatment groups (Figure 5.14B).

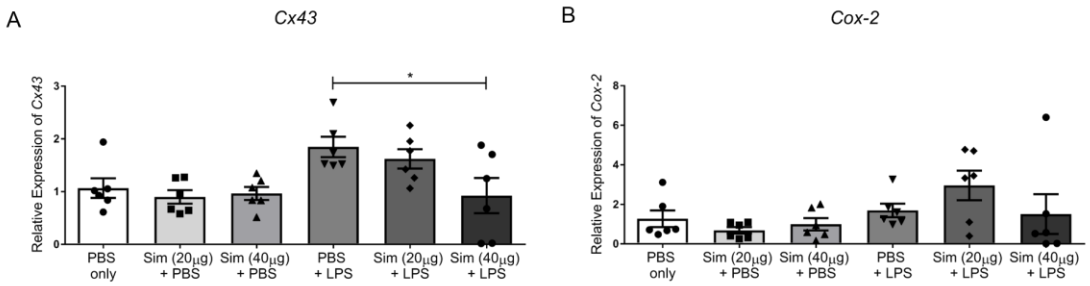

Figure 5.14. Contraction-associated mRNA expression in the uterus. (A) *Cx43* was not significantly upregulated by 1µg LPS but the mRNA expression was significantly reduced by 40µg simvastatin and LPS. (B) *Cox-2* expression was unaltered by LPS or simvastatin treatment. n=6, *p<0.05, mean fold change ± SEM, one-way ANOVA with Dunnett's post hoc test.

5.3.3.2.5 Protein production in the uterus

As 40µg simvastatin treatment attenuated the LPS-induced upregulation of a number of genes, an inflammatory gene, *Il-6*, and a contraction-associated gene, *Cx43*, were investigated at the protein level. However, the production of IL-6 and CX43 was unaltered by the different treatment groups (Figure 5.15, Figure 5.16).

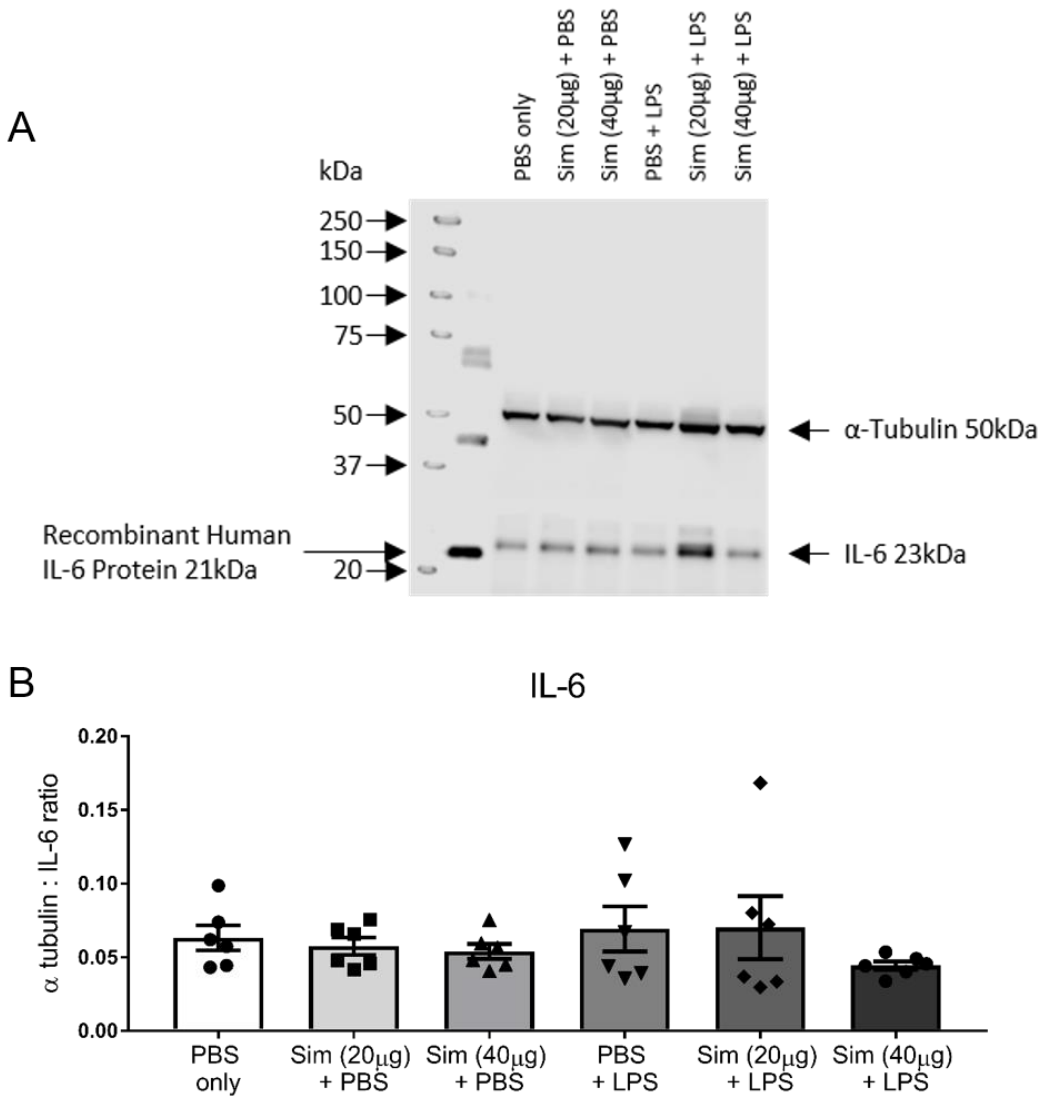

Figure 5.15. IL-6 protein production in the uterus. (A) Representative chemiluminescent image. (B) IL-6 concentration was not altered by LPS or simvastatin treatment in the uterus 6 hours after intrauterine PBS/LPS administration. Recombinant human IL-6 protein was included as a positive control. Representative image was stitched together, as α-Tubulin and IL-6 protein required different ECL exposure times (30 seconds and 5 minutes, respectively). n=6, mean ± SEM.

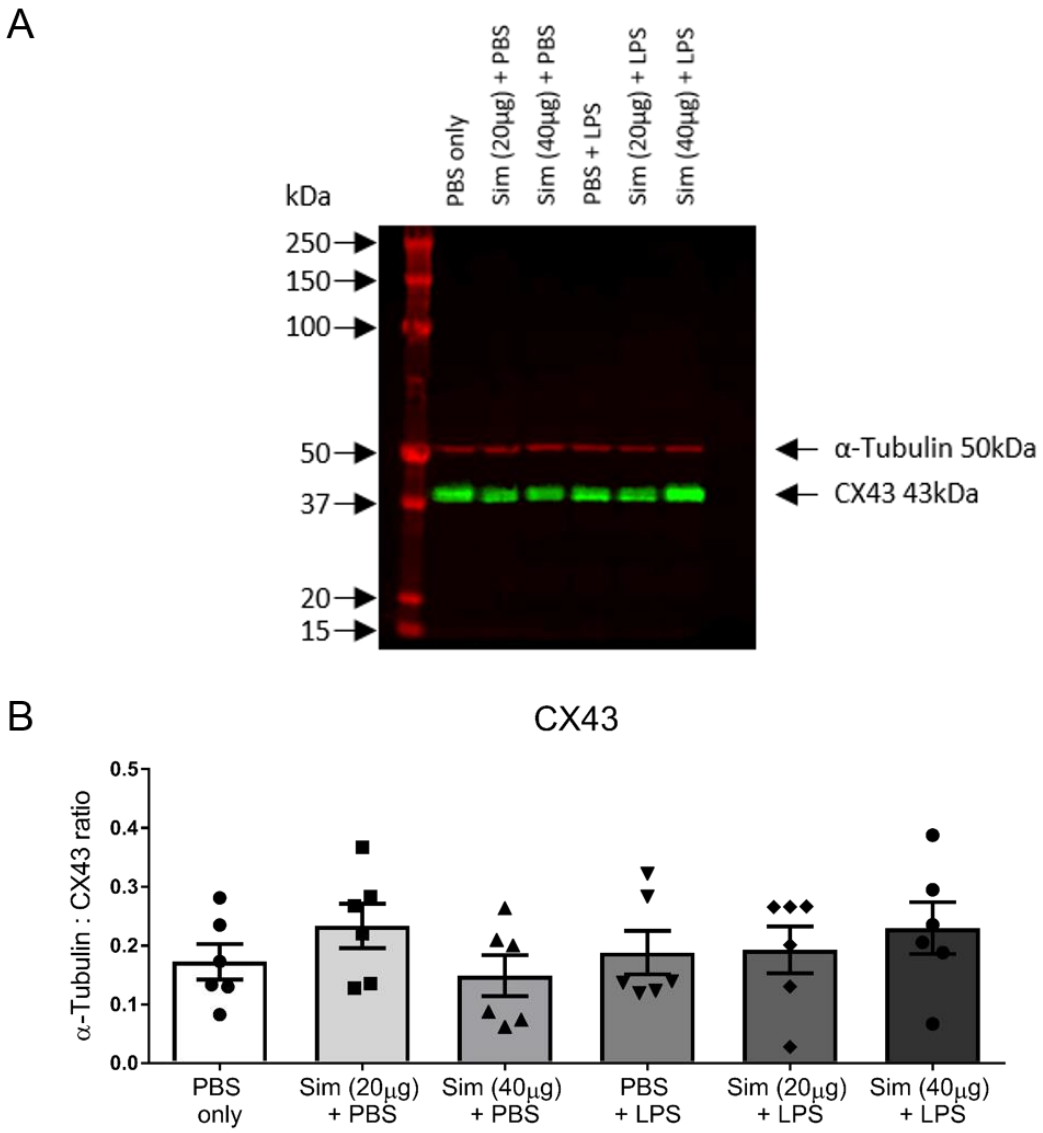

Figure 5.16. CX43 protein production in the uterus. (A) Representative fluorescent image. (B) Neither LPS nor simvastatin impacted on CX43 protein concentration 6 hours after intrauterine PBS/LPS administration. n=6, mean ± SEM.

5.3.3.2.6 mRNA concentration in the placenta

Inflammatory mRNA expression of *Tnf*, *Il-1β* and *Il-10* was also investigated in the placenta (Figure 5.17). LPS alone did not upregulate any of these genes. However, when mice were treated with 40µg simvastatin in addition to LPS, the relative concentrations of mRNA encoded by these genes were all significantly lower than in the LPS group; *Tnf* (p=0.0153), *Il-1β* (p=0.0374) and *Il-10* (p=0.028).

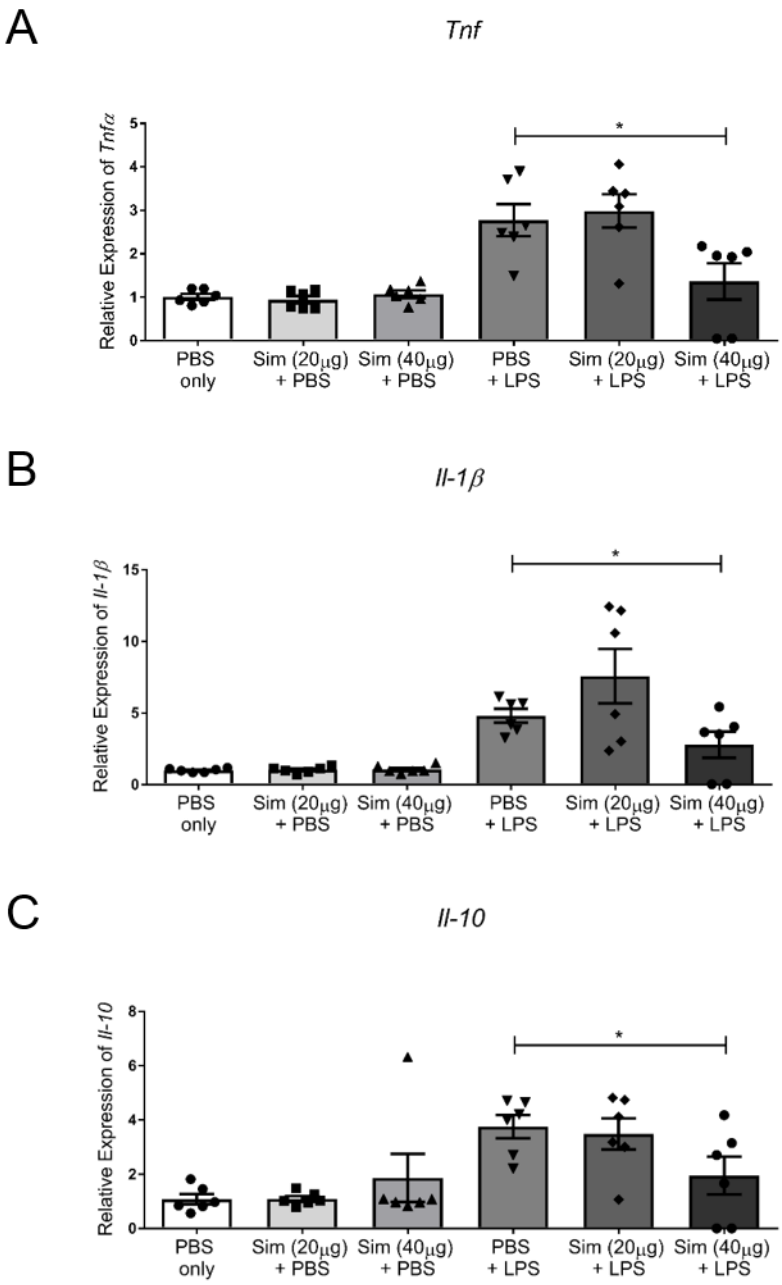

Figure 5.17. Inflammatory cytokine mRNA expression in the placenta. (A-C) *Tnf*, *Il-1β* and *Il-10* expression were all significantly reduced in the 40μg simvastatin and LPS group, compared to the LPS group. n=6, *p<0.05, mean fold change ± SEM, one-way ANOVA with Dunnett's post hoc test.

5.3.3.2.7 The acute effect of simvastatin treatment on progesterone concentration in the maternal serum of D17 pregnant mice

Progesterone plays a crucial role in the maintenance of pregnancy (Csapo and Wiest 1969). As statins inhibit the production of cholesterol, which is the precursor for all

steroid hormones, this may affect the synthesis of progesterone (Cornforth and Popjak 1958). Therefore, progesterone concentrations were investigated in D17 pregnant mice in samples that were collected 1 hour and 2 hours after the intraperitoneal administration of simvastatin, to establish if simvastatin altered the production of this hormone.

After 1 hour, mean serum progesterone concentration was 45.0 ± 7.9ng/ml in the PBS treated mice (Figure 5.18). There was no significant difference between the progesterone concentration of the PBS group and the mice that received 20µg simvastatin (38.5 ± 1.9ng/ml) or 40µg simvastatin (40.6 ± 3.6ng/ml).

After 2 hours, the PBS group had a mean serum progesterone concentration of 29.6 ± 7.8ng/ml. Again, the simvastatin treatment did not alter the progesterone concentration in comparison to the PBS group; 20µg simvastatin (43.9 ± 0.7ng/ml), 40µg simvastatin (41.6 ± 7.4ng/ml).

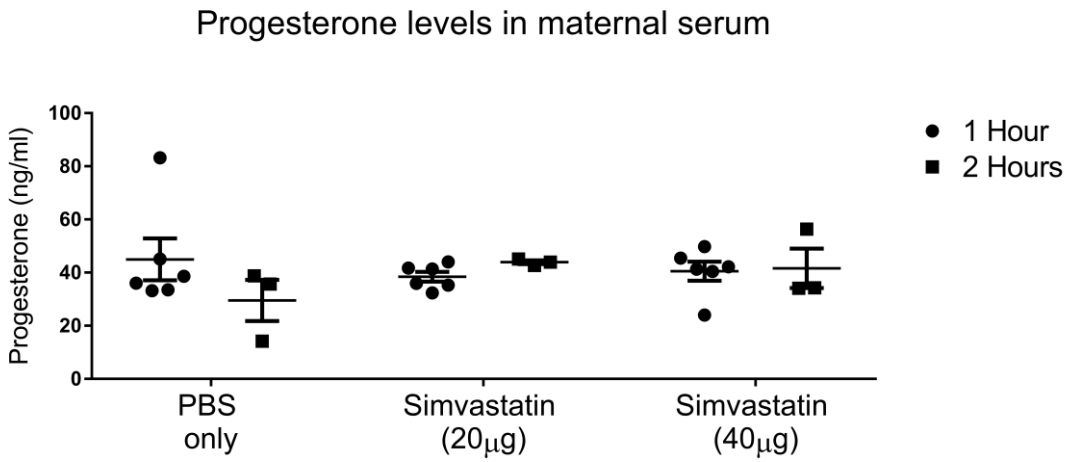

Figure 5.18. Progesterone concentration in the D17 pregnant mouse following simvastatin treatment. Progesterone concentrations were unaffected by simvastatin treatment (20µg, 40µg), 1 hour and 2 hours after administration.

5.3.3.2.8 The effect of simvastatin treatment on maternal serum progesterone concentration in an LPS-induced mouse model of PTB

Progesterone concentration was measured in the LPS-induced mouse model of PTB to determine the effect of LPS and simvastatin treatment. These samples were collected 6 hours after PBS/LPS intrauterine administration. In the PBS group, mean serum progesterone concentration was 67.9 ± 5.8ng/ml (Figure 5.19). This was similar for the 20µg simvastatin (49.5 ± 3.4ng/ml) and 40µg simvastatin control groups (59.1 ± 6.5ng/ml), showing again that simvastatin treatment alone does not affect progesterone concentration. However, when mice received LPS, the mean serum progesterone concentration dropped to 10.2 ± 1.8ng/ml ($p<0.0001$ vs PBS). Simvastatin treatment appeared to have a dose-dependent effect on LPS-reduced serum progesterone concentration. When mice were treated with 20µg simvastatin and LPS, serum concentration of progesterone was slightly higher than in the LPS group but significantly lower compared to the PBS group (16.2 ± 8.8ng/ml, $p=0.0001$). Following treatment with 40µg simvastatin and LPS, mean serum progesterone concentration was slightly higher again at 32.7 ± 10.3ng/ml, although still significantly reduced compared to the PBS group ($p=0.0039$).

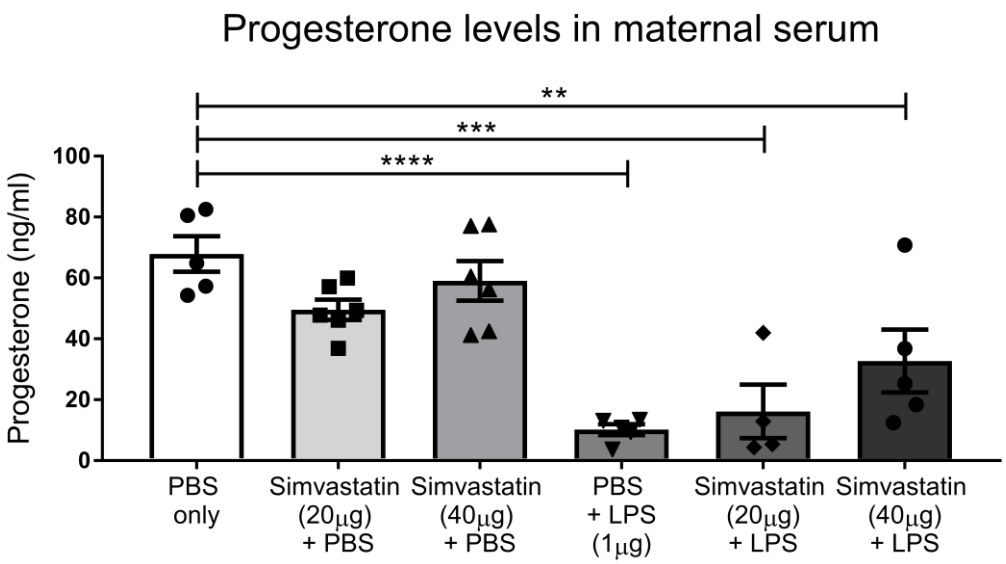

Figure 5.19. Progesterone concentration in the maternal serum of a simvastatin-treated mouse model of PTB. LPS significantly reduced progesterone concentration. These levels were also reduced in mice that received simvastatin (20µg, 40µg) and LPS treatment. n=4-6, **p<0.01, ***p<0.001, ****p<0.0001, mean ± SEM, one-way ANOVA with Dunnett's post hoc test.

5.4 Discussion

In this chapter, the impact of statins was explored in a mouse model where PTB was induced by intrauterine LPS administration under ultrasound guidance. The two statins chosen for this *in vivo* study were simvastatin and pravastatin. It was demonstrated in Chapter 3 and Chapter 4 that simvastatin reduced LPS-induced inflammation in human myometrial cells and attenuated both basal and LPS-induced myometrial cell contraction. These results suggest that simvastatin may be an effective treatment for PTL. Therefore, this hypothesis was investigated in a mouse model of PTB. Although pravastatin treatment did not reduce LPS-induced inflammation in the myometrial cells, this drug has been widely used to treat pregnancy disorders in animal models and more recently, in humans (Girardi 2009; Redecha et al. 2009; Costantine et al. 2013; Gonzalez et al. 2014; Costantine et al. 2016). Therefore, pravastatin treatment was included in this chapter, to assess its effect *in vivo*.

5.4.1 Refining the ultrasound-guided LPS-induced mouse model of PTB

While previous studies from our group and others have administered 10-20µg of the 0111:B4 LPS serotype into the uterus to induce PTB, the low rate of pup survival and concerns that the LPS might be overwhelming the immune system prompted us to perform dose response experiments (Elovitz et al. 2003; Rinaldi et al. 2014; Migale et al. 2015; Rinaldi et al. 2015a; Edey et al. 2016). The novel findings of this study included the observation that, while 0.3µg LPS did not consistently cause PTB in these mice, any dose of LPS greater than or equal to 1µg was sufficient to induce PTB in the majority of dams. However, PTB was still associated with the delivery of dead pups, even at reduced doses of LPS.

To compare the inflammatory differences between the 20µg and 1µg concentrations of LPS, pro-inflammatory genes, *Cxcl1* and *Il-6*, were investigated in the uterus. By lowering the dose of LPS to 1µg, this substantially lowered the mRNA concentration of these genes compared to the 20µg dose, while still causing some elevation of the genes compared to PBS. This is ideal, as inflammation is required to initiate the labour process but it should not be overpowering.

The results from this dose response experiment allowed our PTB mouse model to be refined. This also improved the clinical relevance of the model as, in humans, many cases of intrauterine infection are sub-clinical. Therefore, by lowering the dose of LPS as much as possible, the model became more similar to the human condition. However, clinically, the majority of babies born prematurely are born alive, so having 100% mortality at birth is a slight limitation of the model, as it does not fit the human paradigm. Nevertheless, the pravastatin and simvastatin experiments were repeated, to establish if these treatments would impact gestational length or the survival of the pups, at this lower dose of LPS.

5.4.2 Statin treatment in a refined LPS-induced mouse model of PTB

5.4.2.1 Pravastatin

Pravastatin treatment prevented PTB in 2 out of 8 mice that had received LPS. However, LPS was not 100% effective, as one mouse in the LPS group delivered at term. Therefore, too much cannot be read into this result. The pups from the preterm deliveries in the pravastatin and LPS group were not born alive. This suggests that pravastatin did not have any additional fetal protective effects when the pups were delivered preterm.

These results contradict the findings of Gonzalez et al. (2014) who reported that pravastatin prevented PTB in their intravaginal mouse model. However, the sample size in their study was smaller than the experiments performed in this chapter and the effect of pravastatin treatment on pup survival was not reported. In addition to the differential route of LPS administration, Gonzalez et al. (2014) treated their mice with a less severe serotype of LPS (O55:B5) (Migale et al. 2015).

Pravastatin is the most commonly investigated statin for use in other pregnancy disorders. For example, pravastatin treatment has been extensively researched for the prevention and treatment of preeclampsia (Kumasawa et al. 2011; Carver et al. 2014; McDonnold et al. 2014; Ramma and Ahmed 2014). Two clinical trials have investigated the effect of pravastatin; one in women at high risk of preeclampsia and another in women with early onset preeclampsia. In high risk women, maternal

cholesterol levels were reduced by pravastatin treatment but the concentration of cholesterol in the umbilical cord was unaltered and the birthweights were not different between groups. No safety risks were identified with this treatment (Costantine et al. 2013; Costantine et al. 2016). The other trial investigated the effect of 40mg/day pravastatin treatment on early onset preeclampsia (StAmP; 23410175). However, the results are still to be reported.

In addition, pravastatin treatment has been found to improve pregnancy outcomes in women with anti-phospholipid syndrome, when taken from the diagnosis of preeclampsia or IUGR. Pravastatin increased placental blood flow, subsequently easing the complications associated with preeclampsia. Importantly, there were no fetal abnormalities reported (Lefkou et al. 2016). Pravastatin was also shown to protect against IUGR in a mouse model of glucocorticoid overexposure (Wyrwoll et al. 2016).

Pravastatin, a first generation statin, is one of the least potent statins and it was not believed to cross the placenta, as it necessitates specific transporter uptake into cells (Kapur and Musunuru 2008). However, more recently, two small *ex vivo* placental perfusion studies reported the transplacental transfer of pravastatin, although limited and slow (Nanovskaya et al. 2013; Zarek et al. 2013). One study found that the transfer of pravastatin was higher in the fetal-maternal direction, suggesting that efflux transporters may be responsible for reducing the transfer of pravastatin across the placenta (Nanovskaya et al. 2013).

As pravastatin is reportedly effective in other obstetric complications, it warrants further investigation for PTB prevention. In future studies, the dose of pravastatin could be increased to establish if this could prevent PTB or improve neonatal outcome. The 10µg dose given in these experiments is equivalent to a human therapeutic dose of 20mg, which is relatively low (Kumasawa et al. 2011). Additionally, the timing of the treatment could be optimised.

5.4.2.2 Simvastatin

In the simvastatin studies, when LPS-stimulated mice were treated with 20µg or 40µg simvastatin, a higher number of pregnancies progressed to term and the mean time until delivery was no longer significantly different to the PBS group. However, there

was not a significant difference between these groups and the LPS group. Due to the non-normal distribution of the time to delivery data, the non-parametric Kruskal-Wallis statistical analysis was performed. This test has less power than a one-way ANOVA. Ideally, simvastatin treatment would significantly prolong gestation in comparison to the LPS group but power calculations revealed that too many mice would be required for there to be a statistical difference. Despite the lack of statistical significance, simvastatin is rather convincingly having an effect on the rate of PTB in this mouse model. Future experiments should focus on establishing the mechanisms involved and optimising the treatment protocol for this to be effectively translated to humans.

Interestingly, an "all or nothing" effect was observed, where the simvastatin treatment either prevented PTB or the mice delivered preterm. This suggests that there was a threshold that simvastatin had to overcome in order to prevent PTB. One possible hypothesis for this "all or nothing" effect is the sex of the fetuses. Male sex is an independent risk factor for adverse pregnancy outcome, including PTB (Di Renzo et al. 2007; Ferrero et al. 2016). It has been suggested that this could be due to the larger size and birthweight of males. Furthermore, factors associated with male sex may predispose to infection-mediated PTB (MacGillivray and Davey 1985; McGregor et al. 1992). For instance, low concentration of IL-1 receptor antagonist was reported in the amniotic fluid of premature males (Bry et al. 1995). Alternatively, this phenomenon could be linked to sex hormone differences (Cooperstock and Campbell 1996). However, the mechanism behind this is not fully understood and it is unknown if this would impact on mouse models of PTB. It could be speculated that the sex of the pups could have influenced whether simvastatin was able to rescue the pregnancy. For example, a high proportion of male fetuses in a litter could increase the threshold that simvastatin would need to overcome in order to prevent PTB. The sex of the pups was not recorded in this study but future experiments could address this hypothesis.

The effect of simvastatin on PTB has been assessed in a similar study. Gonzalez et al. (2014) reported that 20µg simvastatin prevented PTB in an intravaginal mouse model. The experiments in this chapter were performed with a larger cohort and also included information on the survival of the pups immediately after delivery, as well as

addressing the effect of simvastatin on maternal inflammation. The key difference between these studies is the model used. The most common route of intrauterine infection is believed to be ascension from the vagina and cervix. However, this is not well understood and opinions differ on the route of infection. It is also possible that there could be a haematogenous dissemination of infection, microorganisms could be introduced during an invasive procedure or originate in the fallopian tubes (Bastek et al. 2011). Previous attempts to establish an intravaginal mouse model of PTB in our laboratory have failed (Rinaldi et al. 2015b). Although the route of infection in our model may not mirror the *in vivo* situation entirely, by delivering the LPS directly into the uterus, we can be assured that intrauterine infection is induced, making this a clinically relevant model. Furthermore, myometrial transcriptomic studies reported that intrauterine LPS-induced PTB in the mouse closely resembles human labour (Migale et al. 2016).

In comparison to pravastatin, fewer studies have investigated the effect of simvastatin in other pregnancy disorders. Simvastatin treatment inhibited sFlt-1 secretion from endothelial cells, trophoblast cells and preterm preeclamptic placental explants (Brownfoot et al. 2016). Simvastatin also prevented pregnancy loss in a mouse model of anti-phospholipid syndrome (Redecha et al. 2008). These few studies reinforce the potential of simvastatin as a therapeutic for obstetric complications.

The mean percentage of live born pups was increased when mice were administered 40µg simvastatin and LPS. Consistently in these studies, if mice delivered preterm, the pups did not survive delivery. It is unknown if this was due to the LPS causing fetal death or because the pups were too premature to survive. In most cases, the dam would not remove the pup from the amniotic sac, suggesting that the fetus died *in utero*. In a study by Migale et al. (2015), the authors reported that this particular serotype of LPS (O111:B4) caused 100% fetal death, which was observed 6 hours after intrauterine LPS (20µg) administration. However, in this chapter, when the tissue collection studies were performed 6 hours after 1µg LPS was given, the pups were still alive. An observation from another study in our laboratory suggested that fetal death may begin roughly 12 hours after LPS administration. Although, this has not been quantified. In another study, a reduced dose of 10µg LPS (O111:B4) resulted in a slight reduction in

fetal demise. There was no evidence of fetal brain inflammation in these pups prior to death. The authors proposed that fetal death may be a result of reduced placental blood flow (Edey et al. 2016). If this hypothesis is correct, simvastatin may be able to restore this blood flow to the fetus as statins have vasodilatory properties and improved placental vascularisation in a mouse model of glucocorticoid overexposure (Pereira et al. 2003; Wyrwoll et al. 2016). Due to the evidenced severity of the O111:B4 LPS serotype, it is all the more remarkable that simvastatin was capable of counteracting this inflammatory insult in the majority of cases, preventing PTB and leading to the delivery of live pups.

As previous studies have suggested that simvastatin can reduce inflammation, circulating IL-6 concentration, as well as the mRNA expression of multiple cytokines and chemokines, were investigated in the uterus. LPS-induced IL-6 concentration was reduced in the maternal serum with 40µg simvastatin treatment. *Il-6*, *Cxcl1* and *Ccl2* expression were significantly reduced with 40µg simvastatin, when compared to the LPS group, suggesting that simvastatin treatment was capable of reducing LPS-induced inflammation in the uterus. Interestingly, *Il-10* was also significantly reduced by 40µg simvastatin, compared to the LPS group. This was unexpected as, in the *in vitro* studies in Chapter 3, simvastatin treatment upregulated the expression of this anti-inflammatory gene in human myometrial cells. An important point to take into consideration is that it is unknown which, if any, of these mice would have delivered preterm. It is interesting to see a spread in the data collected from 40µg simvastatin and LPS treated mice, as it may give a clue to which pregnancies may have been rescued if the mice had been left to deliver. This may also give an indication of the influence of the anti-inflammatory effect of simvastatin in the prevention of PTB in this model.

As outlined in section 2.8.5, many other tissues were collected from the PTB mouse model. These could be utilised to investigate the effect of simvastatin on the other processes of labour, such as fetal membrane rupture and cervical remodelling. In addition, the effect of simvastatin treatment on neutrophil and macrophage infiltration into the uterus could be investigated. Fetal tissues, such as brain, heart and lung, were

also collected. They could be examined for signs of fetal inflammation or injury following LPS administration and the effect of simvastatin on this.

The anti-inflammatory effects of simvastatin outlined in this chapter are in agreement with another study where pregnant mice were pre-treated with simvastatin prior to intraperitoneal LPS administration. Basraon et al. (2012) reported lowered *Il-1β* and *Il-6* mRNA expression levels in the uterus, reduced *Il-6* and *Tnf* mRNA expression in the cervix and a multitude of inflammatory mediators were reduced in the maternal serum, with simvastatin treatment. In contrast to the results in this chapter, the authors also found IL-6 concentration was increased in the amniotic fluid 6 hours after LPS administration and this was reduced by the addition of simvastatin treatment (Basraon et al. 2012).

TLRs are key recognition components of pathogen-associated molecular patterns. *Tlr4* expression was investigated in the mouse uterus, as TLR4 is the signalling receptor for LPS (Poltorak et al. 1998). A TLR4 antagonist has recently been shown to delay PTB and prevent perinatal death in a mouse model (Chin et al. 2016). There was no difference in the mRNA expression of *Tlr4* with LPS stimulation or with simvastatin treatment. However, the O111:B4 serotype of LPS is not TLR4 specific, as it may also bind to TLR2. It would be interesting to investigate if *Tlr2* expression was altered, as both *Tlr2* and *Tlr4* have been associated with the timing of labour (Montalbano et al. 2013; Wahid et al. 2015). Furthermore, simvastatin has been shown to inhibit TLR4-mediated LPS signalling, subsequently reducing cytokine release (Hodgkinson and Ye 2008). Simvastatin treatment also prevented LPS-induced upregulation of TLR2 and TLR4 on the surface of human monocytes *in vivo* (Niessner et al. 2006). By investigating the effect of simvastatin on TLR2, this may provide a mechanism by which this drug may be inhibiting LPS-induced inflammation and the subsequent initiation of labour.

In addition to inflammatory genes, contraction-associated gene *Cx43* was significantly reduced by 40µg simvastatin treatment, compared to the LPS group. CX43 gap junctions increase in size and abundance during parturition. They are crucial for the coordination of synchronous contractions in the myometrium (Doring et al. 2006). This suggests that simvastatin could be inhibiting myometrial contractions by

downregulating genes associated with this process. These results are in agreement with Chapter 4, where it was demonstrated that simvastatin treatment attenuated the LPS-induced contraction of human myometrial cells.

Progesterone concentration was investigated in the serum of D17 pregnant mice to assess whether simvastatin treatment would alter these levels. It is well established that progesterone has a crucial role in maintaining pregnancy and statins primarily inhibit the synthesis of cholesterol, the precursor to progesterone. The results were reassuring that, after 1 hour and 2 hours, simvastatin treatment did not affect progesterone concentration, when comparing to the PBS group. Progesterone concentration was also comparable to values reported in the literature at this stage of gestation in the mouse (Virgo and Bellward 1974).

Progesterone concentrations were also determined in the serum collected from the PTB mouse model, which has not previously been investigated in our ultrasound-guided intrauterine model of PTB. Progesterone withdrawal initiates parturition in the mouse (Hall 1956). Therefore, it was interesting to observe proof of reduced progesterone concentration following LPS treatment in this mouse model of PTB. Progesterone concentration was also significantly reduced in the mice that received 20µg and 40µg simvastatin in addition to LPS, compared to PBS levels. Interestingly, there appeared to be a dose effect, where treatment with 40µg simvastatin and LPS resulted in a lesser reduction in progesterone concentration than observed in the LPS group. This suggests that simvastatin treatment may have been working to counteract the effects of LPS and prevent PTB in some of these mice. However, the mechanism of this is unknown. It would be interesting to investigate this in more detail, perhaps by assessing the histology of the corpus luteum, as it has been suggested that LPS induces apoptosis of the corpus luteum, causing progesterone levels to drop (Luttgenau et al. 2016). However, this is not directly comparable to humans as, in human parturition, circulating progesterone levels do not reduce. Alternatively, there is thought to be functional progesterone withdrawal, which may be mediated by a shift in progesterone receptor ratio (Allport et al. 2001; Condon et al. 2003; Merlino et al. 2007). Although, these results do allow us to better characterise our model of PTB.

A major obstacle for the application of statins to prevent PTB is that these drugs are contraindicated during pregnancy. A retrospective observational study by Edison and Muenke (2004) reported that there was an association with fetal limb defects after simvastatin exposure during the first trimester. However, this finding has since been criticised and challenged by larger studies, which found that there was no greater risk when comparing to population baseline levels of fetal malformations (Gibb and Scialli 2005; Petersen et al. 2008; Bateman et al. 2015). Therapeutics for the prevention of PTB would likely be given in the second or third trimester, when organogenesis is mostly complete. In addition, PTB itself can result in life-long morbidity, as well as neonatal mortality. Therefore, it can be argued that the possible benefits of statin treatment outweigh the risks.

Studies *in vitro* have raised concerns over the effect of simvastatin on the placenta during the first trimester, suggesting this treatment affects trophoblast proliferation and migration, as well as causing cytotrophoblast apoptosis (Kenis et al. 2005; Tartakover-Matalon et al. 2007; Forbes et al. 2015). However, as previously discussed, PTB prevention would not take place during the first trimester. Furthermore, the results in this chapter suggest that simvastatin treatment may reduce inflammation in the placenta. Future studies could investigate the effects of simvastatin on the histology of the placenta and additional genes/protein could be assessed, as these tissues have been collected. To date, studies have not investigated the possibility that simvastatin could cross the placenta. However, it is likely given the ease at which simvastatin can enter cells. There is also a possibility that statin treatment would actually protect against fetal injury by reducing fetal inflammation. One study described protective effects of both pravastatin and simvastatin against brain injury, in fetuses from LPS-treated dams, evidenced by a reduction in the expression of cell death and neurodegeneration markers (Pedroni et al. 2014).

In the PTB studies presented in this chapter, there appeared to be a dose-dependent effect of simvastatin treatment. The higher dose of 40µg simvastatin was more effective than the 20µg treatment at preventing PTB and resulted in more live born pups. A possible explanation for this may be due to this higher dose reducing the expression of uterine inflammatory and contraction-associated genes. However,

despite doubling the dose of simvastatin, the higher dose was only slightly more effective than the 20µg treatment at preventing PTB. Therefore, the timing of the simvastatin treatment needs to be further investigated to fully understand the optimal treatment regimen. These mice received one dose of statins the day before LPS and an additional dose 2 hours after LPS. This treatment protocol was chosen due to its previously reported success in other mouse models of pregnancy disorders (Redecha et al. 2008; Gonzalez et al. 2014; Pedroni et al. 2014). It is unknown which treatment played a more important role in these experiments. For example, the dose on gestational D16 could have primed the mice for an inflammatory insult or the treatment 2 hours after LPS administration could have reduced the LPS-induced inflammation. It was shown in Chapter 3 that both pre- and post-treatment with simvastatin can have anti-inflammatory properties. In a mouse model of sepsis, simvastatin treatment was able to improve survival in both a pre-treatment cohort and in a post-treatment cohort, providing additional evidence that the anti-inflammatory benefits of this drug are not restricted to certain treatment windows (Merx et al. 2004; Merx et al. 2005). Performing such experiments will be important for determining the best clinical application of this treatment.

Finally, the mechanism by which simvastatin is preventing PTB also needs to be investigated. Other studies in animal models of inflammatory conditions reported that simvastatin reduced NF-κB activation (Jacobson et al. 2005; Fraunberger et al. 2009). Although, it has been reported that PTB induced with O111:B4 LPS is due to AP-1 activation rather than NF-κB activation (MacIntyre et al. 2014; Migale et al. 2015). It is also possible to investigate the mechanism of action by supplementing components of the mevalonate pathway, as performed in Chapter 4. For example, mevalonate reversed the anti-inflammatory effect of simvastatin in a mouse model of allergic airway inflammation (Zeki et al. 2009).

In summary, simvastatin treatment was capable of reducing the incidence of PTB in our refined intrauterine LPS-induced mouse model. Treatment with 40µg simvastatin also improved the survival of the pups. Pravastatin treatment did not prevent PTB or improve the percentage of live born pups. Simvastatin treatment alone did not affect maternal serum progesterone concentration. Progesterone withdrawal was evident in

the LPS-induced mouse model of PTB and simvastatin treatment concentration-dependently counteracted this LPS-induced reduction in serum progesterone.

The results of this chapter provide further evidence that simvastatin may be an effective therapeutic for the treatment of PTB. In addition to preventing PTB and improving pup survival, simvastatin reduced the expression of inflammatory and contraction-associated genes in the uterus, which is in agreement with the findings of Chapter 3 and Chapter 4. Furthermore, these results confirm the overall hypothesis of this thesis, that statin treatment would prevent PTB by reducing inflammation. Therefore, future experiments should refine the treatment regimen, as well as investigate the mechanisms of these effects.

Chapter 6: Discussion

6.1 Summary of findings

PTB remains a major clinical problem. It affects approximately 5-18% of births worldwide and is the leading cause of childhood morbidity and mortality (Blencowe et al. 2012; Harrison and Goldenberg 2015). Intrauterine infection and inflammation are implicated in at least 25-40% of PTB cases (Goldenberg et al. 2008). Despite advancements in medical research, PTL is still difficult to predict and current treatments are ineffective at preventing PTB and improving the outcomes of premature neonates (Haas et al. 2014). The development of new treatments should aim to target the underlying mechanisms of PTL. Statins may be an ideal candidate treatment for PTL, as these drugs exhibit anti-inflammatory effects in conditions such as hyperlipidaemia and atherosclerosis (Zhou and Liao 2010). In addition, they can inhibit the contraction of smooth muscle cells (Kuzuya et al. 2004; Perez-Guerrero et al. 2005; Nasu et al. 2009).

The hypothesis of this thesis was that statin treatment will prevent PTB by inhibiting the inflammatory processes that trigger PTL. This thesis investigated the effect of statin treatment on PTL, with specific aims to (i) assess the effects of statins on inflammation in human myometrial cells *in vitro*, (ii) examine the effect of statins on the contractility of a human myometrial cell line, (iii) investigate the mechanisms by which statins affect either contractility or inflammation in PTL and (iv) determine whether statins can prevent PTB or improve neonatal outcome in an LPS-induced mouse model of PTB. The main findings are summarised below:

- Pravastatin treatment of human myometrial cells did not increase or decrease LPS-induced inflammatory mediator mRNA concentration or protein production.
- Simvastatin treatment reduced LPS-induced inflammation in myometrial cells by downregulating the mRNA concentration and protein production of pro-inflammatory mediators, as well as increasing the mRNA expression of anti-inflammatory cytokines.

- Simvastatin attenuated the basal contraction of myometrial cells *in vitro*. Simvastatin also reduced LPS-induced contraction of myometrial cells. This effect was reversed by the addition of mevalonate and GG-PP but not F-PP. Therefore, this was a cholesterol-independent effect. Simvastatin treatment inhibited the phosphorylation of MLC by inhibiting the Rho/ROCK pathway.
- The ultrasound-guided, intrauterine LPS-induced mouse model of PTB was refined by lowering the concentration of LPS from 20µg to 1µg. Lowering the dose of LPS led to the reduction of pro-inflammatory mRNA concentration in the uterus.
- Simvastatin treatment prevented PTB and increased the percentage of live born pups in the refined 1µg LPS-induced PTB mouse model. Simvastatin also reduced LPS-induced circulating IL-6 concentration and attenuated the expression of pro-inflammatory and contraction-associated genes in the uterus. Pravastatin treatment did not prevent PTB or increase the percentage of live born pups.
- Progesterone withdrawal took place in the 1µg LPS-induced mouse model of PTB. Simvastatin treatment concentration-dependently counteracted this LPS-induced reduction in serum progesterone. Simvastatin treatment alone did not alter maternal serum progesterone concentration.

6.2 The effect of statins on inflammation

Inflammatory processes have been associated with both term and PTL. There is an influx of leukocytes into the uterus and an increase of pro-inflammatory mediators, such as cytokines and chemokines (Thomson et al. 1999; Young et al. 2002). Therefore, statins may be a novel approach to prevent PTB, as these drugs have anti-inflammatory properties. We investigated the effect of pravastatin and simvastatin treatment on inflammation in human myometrial cells and assessed the effect of simvastatin treatment on inflammation in an LPS-induced mouse model of PTB.

Pravastatin did not alter LPS-induced inflammatory mediator expression in myometrial cells. This was most likely because pravastatin requires specific uptake

into cells by the transporter protein, OATP1B1, which is exclusively expressed in the liver (Hamelin and Turgeon 1998; Corsini et al. 1999). Simvastatin treatment reduced pro-inflammatory mediator gene expression and release, as well as upregulating anti-inflammatory gene expression. These findings concur with other studies that demonstrate the anti-inflammatory effect of simvastatin in a number of cells types, such as vascular smooth muscle cells, macrophages and epithelial cells (Rezaie-Majd et al. 2002; Dichtl et al. 2003; Veillard et al. 2006; Xu et al. 2006; Iwata et al. 2012). Most studies have focused on cells associated with the cardiovascular system, as statins are normally used for the treatment of high cholesterol, which is associated with atherosclerosis and cardiovascular disease. The mechanisms by which statins have anti-inflammatory effects are not fully elucidated. However, it is likely that these effects are a result of the inhibition of protein isoprenylation within the mevalonate pathway (Wang et al. 2008).

There have also been studies that have investigated the effect of statins on inflammation in tissues more relevant to our studies. For example, in the endometrium, the inner lining of the uterus, in the context of endometriosis. Simvastatin reduced *CCL2* expression in human endometriotic cells in a nude mouse model with endometriotic implants (Cakmak et al. 2012). Simvastatin also reduced the secretion of a range of pro-inflammatory cytokines from LPS-stimulated human fetal membranes. In this study, pre-treatment with simvastatin prior to LPS stimulation was more effective at reducing inflammatory cytokine release than co-treatment or post-treatment (Basraon et al. 2015). However, the results presented in this thesis are the first, to our knowledge, to assess the effect of statins on inflammation in human myometrial cells. As these cells were collected from a pregnant women at term, they have already been exposed to an increasingly pro-inflammatory environment and hormonal changes in preparation for labour and these cells will have already experienced a degree of stretch, making this a highly relevant *in vitro* model of the pregnant myometrium at term.

Future studies should focus on determining the mechanisms by which simvastatin is having anti-inflammatory effects. Metabolites of the mevalonate pathway, such as mevalonate, GG-PP and F-PP, can be supplemented into the cultures to determine

which branch of the pathway is implicated to produce these anti-inflammatory effects, as performed in the contraction experiments in Chapter 4. Other studies in the literature have demonstrated that statins can inhibit the binding activity of the transcription factor, NF-κB, and inhibit the nuclear translocation of NF-κB, in cells stimulated by LPS (Hilgendorff et al. 2003; Shyamsundar et al. 2009). This could be investigated in the human myometrial cells by performing an electrophoretic mobility shift assay to assess binding activity. Alternatively, the degradation and phosphorylation of the inhibitor protein, IκBα, can be assessed by Western blotting.

Furthermore, as chemokines and the chemotaxis of leukocytes have a prominent role in labour, it would be of interest to investigate the effect of simvastatin on the production of other chemokines. As reported in this thesis, LPS-induced IL-8 secretion from the myometrial cells was attenuated by simvastatin treatment *in vitro*, so it can be hypothesised that simvastatin would reduce the expression and production of other chemokines. Furthermore, inflammation can also be increased by uterine overdistension, inducing PTL (Adams Waldorf et al. 2015). Therefore, the effect of simvastatin on inflammation caused by mechanical stretch of the human myometrial cell line could also be investigated.

In the PTB mouse model, 40μg simvastatin treatment reduced the concentration of LPS-induced IL-6 in the maternal serum, as well as reducing the mRNA concentration of cytokines and chemokines in the uterus. These results are broadly consistent with other studies, where simvastatin and other statins have been shown to reduce inflammation in mouse models of sepsis (Merx et al. 2004; Merx et al. 2005). Statins also have an immunomodulatory effect in some animal models, whereby leukocyte chemotaxis is altered and there is a shift in Th1 to Th2 phenotype. For example, in mouse models of inflammatory arthritis and allergic asthma (Leung et al. 2003; McKay et al. 2004). This could be addressed in the PTB mouse model by investigating the infiltration of leukocytes, such as neutrophils and monocytes/macrophages, in the myometrium by immunohistochemistry.

A study by Basraon et al. (2012) investigated the effect of simvastatin pre-treatment in a mouse model of PTB induced by intraperitoneal administration of LPS, creating a

model of systemic infection. This study reported that simvastatin treatment reduced inflammation in the maternal serum, uterus and cervix of LPS-treated mice, which agrees with our findings described in Chapter 5.

Inflammatory mediators have also been associated with cervical ripening and fetal membrane rupture, which are important processes for successful labour. Future studies could assess the anti-inflammatory effect of simvastatin on other tissues, such as the cervix and fetal membranes, which have already been collected from these mice. As well as investigating inflammatory mediators in these tissues, the effect of simvastatin on MMPs and collagen degradation can be determined, to assess whether simvastatin also prevented cervical ripening and fetal membrane rupture. Fetal tissue, such as the heart, lung and brain, was also collected and could be investigated to determine if simvastatin prevented any LPS-induced fetal inflammation or injury.

Deciphering the underlying mechanisms of simvastatin anti-inflammatory properties is a key next step, as discussed in relation to the myometrial cell *in vitro* experiments. Previous studies have demonstrated the reversal of statin effects in other mouse models by the administration of mevalonate (Christensen et al. 2006; Sharyo et al. 2008). However, it may be more informative to concentrate on specific mechanisms downstream of the mevalonate pathway. Both transcription factors, NF-κB and AP-1, have been implicated in the upregulation of inflammatory pathways in term and PTL. The effect of simvastatin on LPS-induced activity of these transcription factors could be investigated in the uterus of the PTB mouse model by Western blot analyses of the transcriptionally active, phosphorylated forms of the NF-κB subunit, p65, and the AP-1 subunit, c-Jun. However, one study reported that the serotype of LPS used in this thesis (O111:B4) selectively activates AP-1, not NF-κB in CD1 mice (MacIntyre et al. 2014; Migale et al. 2015). It is unknown if this mechanism applies to our C57Bl/6 mice.

In summary, simvastatin, but not pravastatin, reduced LPS-induced inflammation in a human myometrial cell line and simvastatin reduced inflammation in a mouse model of PTB. These are the first studies to characterise the anti-inflammatory effects of statins in human myometrial cells and in an intrauterine LPS-induced PTB mouse

model. These results suggest that simvastatin treatment could inhibit the inflammatory cascade associated with PTL. Future experiments should focus on elucidating a mechanism for these anti-inflammatory effects.

6.3 The effect of simvastatin on myometrial contraction

Uterine contraction is a key process during labour, required to safely expel the fetus. In recent years, the role of inflammation in the stimulation of contractions has emerged (Mackler et al. 2003; Hutchinson et al. 2014). During labour there is an increase in pro-inflammatory mediators, such as cytokines and prostaglandins as well as contraction-associated proteins, in the uterus (Crankshaw and Dyal 1994; Young et al. 2002). Tocolytic drugs are administered to inhibit contractions. However, they only delay labour for a short time (Haas et al. 2012; Haas et al. 2014). In Chapter 4, we demonstrated that simvastatin can attenuate basal myometrial cell contraction, as well as LPS-induced contraction and finally, we described the mechanism involved. This provides further evidence that simvastatin could be a useful tocolytic treatment for PTL.

In the collagen gel contraction assay, simvastatin prevented the contraction of the myometrial cells embedded in the gels, both alone and in the presence of LPS. This was reversed by mevalonate and GG-PP but not F-PP, which suggests that this anti-contraction effect was due to the inhibition of Rho geranylgeranylation. Simvastatin treatment also reduced the levels of pMLC in the myometrial cells, which is the final step required for actin and myosin cross-bridging. This confirmed the anti-contraction effect at a molecular level. These results again highlighted the importance of GG-PP inhibition for anti-contractile effects, suggesting that statins prevent contraction by inhibiting the Rho/ROCK pathway (Figure 4.15).

The anti-contractile effect of statins has not previously been assessed in myometrial smooth muscle cells. However, our findings are in agreement with some other studies that have mostly reported anti-contractile effects in vascular smooth muscle cells and aortic rings, which is not unexpected due to the primary use of statins being to lower

LDL cholesterol concentration and prevent cardiovascular diseases (Kuzuya et al. 2004; Mraiche et al. 2005; Perez-Guerrero et al. 2005; Alp Yildirim et al. 2016; Chen et al. 2016). Previous studies have also identified the inhibition of the Rho/ROCK pathway and the subsequent prevention of MLC phosphorylation, as a mechanism of these effects, adding strength to our findings (Zeng et al. 2005; Nagaoka et al. 2007; Kidera et al. 2010; Alp Yildirim et al. 2016). The most relevant cells to have undergone contraction studies following statin treatment are endometriotic stromal cells. However, a mechanism was not elucidated for the anti-contractile effect of these uterine cells (Nasu et al. 2009). Notably, the effect of statins on LPS-induced contraction has not previously been investigated.

As it is well established that the influx of Ca^{2+} into cells is integral for smooth muscle contraction, future studies could investigate the effect of simvastatin on $[Ca^{2+}]i$ in the myometrial cells by performing a colorimetric assay, using calcium indicator dye. Recent studies have suggested that statins can affect $[Ca^{2+}]i$ (Kang et al. 2014; Ali et al. 2016; Chen et al. 2016). This could also provide another mechanism to explain the anti-contractile effects we measured using myometrial cells. To extend these cellular assays, organ bath studies could also be performed on human myometrial strips treated with simvastatin. Some experiments have been reported in the literature but these did not investigate the impact of statins on the intensity of myometrial contractions (Gonzalez et al. 2014). Chapter 4 demonstrated how simvastatin prevented the phosphorylation of MLC by inhibiting the Rho/ROCK pathway, via GG-PP inhibition. However, understanding of the effect of simvastatin on this pathway could be strengthened by specifically investigating the effect on RhoA-GTP, which should be reduced by simvastatin treatment.

In our refined mouse model of PTB, 40µg simvastatin treatment significantly reduced the mRNA concentration of the contraction-associated gene, *Cx43,* in the uterus. The effect of statins on *Cx43* expression has not been assessed in other animal models of PTB. As treatment with simvastatin reduced the incidence of PTB, we speculate that this might be due to a reduction in myometrial contractions. When considering the mechanisms that might contribute to this observation, the reduction in LPS-induced inflammation resulting from simvastatin treatment is one of several possibilities. The

expression of other proteins, such as $PGF_{2\alpha}$ and the oxytocin receptor, both of which have been implicated in the regulation of contractions, could be investigated in the uterus of the simvastatin-treated PTB mouse model. In addition, uterine pressure could be assessed directly by telemetry studies, where transmitters are implanted into the uterine horn. However, the equipment for such studies is costly and would require additional surgery and careful validation to avoid off-target impacts of the presence of the transmitters.

To summarise, simvastatin attenuated both the basal and the LPS-induced contraction of human myometrial cells and reduced the expression of contraction-associated gene, *Cx43*, in an LPS-induced mouse model of PTB. These data provide further rationale to support the proposal that simvastatin treatment could be a novel treatment for PTL, as this drug has anti-contraction effects, as well as reducing inflammation.

6.4 Statins as a treatment for PTL

This thesis describes for the first time the effect of statins on an intrauterine model of LPS-induced PTB. Simvastatin treatment prevented PTB in many cases and more pups were born alive, as a result. These findings extend those of another study where PTB was prevented in mice given an intravaginal injection of LPS serotype 055:B5 on D15 of pregnancy, when treated with 20µg simvastatin 24 hours before and 2 hours after LPS (Gonzalez et al. 2014). Notably, in our mouse model, we used a different serotype of LPS (O111:B4), as well as a different route of administration. We reported a positive outcome of simvastatin treatment on the delivery of live born pups, as well as maternal inflammation. Furthermore, our studies were performed in a larger cohort of mice.

In our study, pravastatin treatment did not prevent PTB in our mouse model, which is in contrast to the results reported by Gonzalez et al. (2014). It is unlikely that this was just because of the inability of pravastatin to enter certain cells, as the pleiotropic effects of pravastatin are thought to be due to a reduction of circulating isoprenoid concentration (Zhou and Liao 2010). The concentration of pravastatin and the treatment regimen could be modified in future experiments. Pravastatin has been the preferred statin for preeclampsia treatment due to its safety profile. Therefore, it

warrants further investigation as a potential treatment for PTL, using additional approaches to those described in this thesis.

A limitation of both the original and our refined PTB mouse model described in this thesis is that there is 100% fetal death, which does not make it relatable to the human condition, where premature babies are mostly born alive (Rinaldi et al. 2015b). A study that analysed the effect of different LPS serotypes in an intrauterine PTB mouse model found the O111:B4 serotype used in our studies to be the most severe, as PTB was induced more rapidly in pregnant mice and there was increased fetal demise, in comparison to other serotypes (Migale et al. 2015). Although, this does highlight the efficacy of the simvastatin treatment, as it can overcome this insult by preventing PTB and preventing fetal death, in our refined mouse model. The relevancy of our model to humans could be improved by using a different serotype of LPS, which does not cause widespread fetal death, to determine whether simvastatin treatment has more subtle fetal protective effects that were being lost with the severity of the O111:B4 LPS. It is plausible that statins would have beneficial effects on fetuses, as other studies have reported that statins can prevent fetal death in mouse models of recurrent miscarriage and antiphospholipid syndrome (Redecha et al. 2008; Redecha et al. 2009). Alternatively, combination therapy may be a possibility. For example, a previous study from our laboratory found the pro-resolution mediator, 15-epi-lipoxin A_4, to have fetal protective effects but this treatment was not sufficient to prevent PTB (Rinaldi et al. 2015a). Although, these experiments were performed on the original laparotomy mouse model, utilising 20µg LPS to induce PTB. Nevertheless, treating mice with both simvastatin and 15-epi-lipoxin A_4 could improve pup survival, even if the mice deliver preterm.

Future studies should investigate the fetal outcomes from this refined mouse model in more detail. This could be performed by examining fetal tissues for signs of injury following LPS administration and whether this is ameliorated by simvastatin treatment. In addition, behavioural tests could be conducted. A recent article presented a number of neonatal motor tests that can be performed in mice from post-natal day 2-14. This includes grasping reflex, surfacing righting and hindlimb suspension. This particular study assessed motor function in a neonatal mouse model of cerebral palsy,

induced by inflammation, ischemia and hypoxia, and found hindlimb weakness and defects in fine motor skills (Feather-Schussler and Ferguson 2016). This is particularly relevant to our model where inflammation plays an important role in instigating PTB. Moreover, prematurity in humans is associated with long term behavioural and neurological disorders, such as cerebral palsy. Performing these experiments would allow us to characterise motor defects associated with our intrauterine model of PTB and whether simvastatin could prevent these adverse neonatal outcomes.

Further experiments are required to refine the concentration and timing of simvastatin treatment required to prevent PTB. Interestingly, 40µg simvastatin was not substantially more effective than 20µg simvastatin at preventing PTB and improving neonatal outcome. Although, the higher concentration was more effective at reducing inflammation. Therefore, treatment timing may have been a contributing factor. The treatment regimen for our PTB mouse experiments involved both a pre-treatment and a post-treatment with statins. This protocol was selected due to previously demonstrated success in other mouse models of obstetric complications (Redecha et al. 2008; Gonzalez et al. 2014; Pedroni et al. 2014). However, it is unknown which of these treatments was more influential in lowering inflammation and delaying the labour process until term. It can be hypothesised that the post-treatment with simvastatin may have been the more influential treatment, given the short, two hour half-life of simvastatin. Notably, Basraon et al. (2012) reported that pre-treatment with pravastatin and simvastatin reduced inflammation in an intraperitoneal LPS-induced mouse model of PTB. However, there was no information in their paper as to whether this treatment prevented PTB in these mice. As there have been no follow up studies since this paper was published in 2012, one can speculate that statin pre-treatment did not prevent PTB in that model.

An alternative route of treatment should also be investigated, as statins would normally be administered orally in women. In mice, oral treatments can be given via gavage. Although, this may be stressful for the mouse, particularly during pregnancy. Alternatively, treatments can be hidden in palatable mixtures, such as flavoured gelatine, and ingested voluntarily by the mouse.

The next step would then be to perform safety testing and assess pharmacokinetics in pregnant women during a normal pregnancy. This could assess how well statins are tolerated and investigate any gene, protein or histological changes of maternal-fetal tissues, such as the uterus, placenta and fetal membranes, with this treatment. Small trials have already treated women at high risk of preeclampsia with statins and no safety concerns have been reported to date (Costantine et al. 2016; Lefkou et al. 2016). Human placental perfusion experiments have been conducted with pravastatin but not simvastatin (Nanovskaya et al. 2013; Zarek et al. 2013). This experiment could also be performed to examine how simvastatin is transferred between the maternal and fetal compartments and this could also indicate the concentration of simvastatin that the fetus may be exposed to.

In the current study, LC-MS/MS revealed progesterone withdrawal had occurred in our PTB mouse model, something which had not been investigated before when LPS was administered by ultrasound-guided intrauterine injection. Although this is not directly relatable to human labour, it was reassuring that simvastatin treatment alone did not affect circulating progesterone levels, despite cholesterol being the precursor to this essential pregnancy hormone. Simvastatin treatment appeared to partially reduce LPS-induced progesterone withdrawal, which suggests a mechanism by which PTB may have been prevented and merits further study. It is unknown how the drug would have this effect but the histology of the corpus luteum could be assessed in the future to determine if simvastatin protects against LPS-induced damage of this tissue. The effect of simvastatin treatment on PTB induced by the progesterone receptor antagonist, RU486, could also address this. This study would determine the effect of simvastatin treatment on non-infection induction of PTB and would aid better understanding of the mechanisms responsible for these effects.

In summary, simvastatin treatment reduced the number of preterm deliveries in our refined intrauterine LPS-induced mouse model of PTB and more pups were born alive. Pravastatin treatment did not have these beneficial effects in either the 20µg LPS or the 1µg LPS, refined mouse model of PTB. Further studies are required to establish the underlying mechanisms leading to the prevention of PTB by simvastatin. Although, we hypothesise that the reduction in maternal inflammation may have

played a key role in preventing labour. The treatment regimen must also be refined to improve the efficacy and to fully understand the optimal concentration and timing of treatment, so that this can be translated for human use.

6.5 Conclusions

The studies described in this thesis investigated the effect of statin treatment on PTL by utilising pregnant human myometrial cells as an *in vitro* model and in an ultrasound-guided intrauterine LPS-induced mouse model of PTB. The experiments performed produced a number of novel and interesting findings. Simvastatin treatment reduced LPS-induced inflammation in myometrial cells, as well as attenuating both basal and LPS-induced contraction of these cells. These findings were further validated in our mouse model, where simvastatin prevented PTB and more pups were born alive as a result of this treatment. In addition, simvastatin treatment reduced LPS-induced inflammation in the uterus. Pravastatin treatment did not exhibit anti-inflammatory properties or have beneficial effects in the mouse studies. Simvastatin treatment demonstrated a number of useful properties, suggesting this drug would be an ideal candidate for the treatment of PTL, by targeting underlying inflammation, inhibiting myometrial contractions and subsequently preventing PTB.

Chapter 7: References

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Appendix

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Preterm birth: Inflammation, fetal injury and treatment strategies

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ABSTRACT

Preterm birth (PTB) is the leading cause of childhood mortality in children under 5 and accounts for approximately 11% of births worldwide. Premature babies are at risk of a number of health complications, notably cerebral palsy, but also respiratory and gastrointestinal disorders. Preterm deliveries can be medically indicated/elective procedures or they can occur spontaneously. Spontaneous PTB is commonly associated with intrauterine infection/inflammation. The presence of inflammatory mediators *in utero* has been associated with fetal injury, particularly affecting the fetal lungs and brain. This review will outline (i) the role of inflammation in term and PTB, (ii) the effect infection/inflammation has on fetal development and (iii) recent strategies to target PTB. Further research is urgently required to develop effective methods for the prevention and treatment of PTB and above all, to reduce fetal injury.

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1. Introduction

The World Health Organisation defines preterm birth (PTB) as delivery before 37 completed weeks of gestation. PTB can be subdivided into extremely preterm (<28 weeks), very preterm (28–32 weeks) and moderate to late PTB (32–36 weeks) (WHO, 1977). In 2010, 14.9 million babies were born preterm, accounting for 11.1% of all births worldwide. Rates of PTB range from approximately 5% of births in European countries, to 18% in certain African countries (Blencowe et al., 2012). PTB is currently the leading cause of childhood mortality in children under 5 years (Harrison and Goldenberg, 2015). The economic burden of PTB to the public sector is estimated to be >£2.9 billion (Mangham et al., 2009). Much of the economic burden can be attributed to neonatal intensive care, often followed by ongoing health care and educational requirements, in addition to the emotional impact experienced by families (Howson et al., 2013).

PTB can be medically indicated/iatrogenic or spontaneous. Medically indicated PTB accounts for approximately a third of PTBs in high income countries (Rubens et al., 2014). This occurs when the risk to the fetus or mother outweighs the benefit of continuing the pregnancy, for instance in conditions such as preeclampsia and diabetes. Approximately 70% of PTBs are spontaneous, with women presenting with preterm labour (PTL) with cervical dilation or preterm premature rupture of membranes (Rubens et al., 2014).

PTL can be initiated by multiple mechanisms, including infection or inflammation, uteroplacental ischaemia or haemorrhage, uterine overdistension or stress (Goldenberg et al., 2008; Romero et al., 1994). Maternal risk factors include extremes in maternal age, body mass index (BMI), multiple gestation, the use of assisted reproductive technologies, history of PTB and low socioeconomic status (Rubens et al., 2014). Race is also an important risk factor, with women classified as black, African-American or Afro-Caribbean at greater risk of PTB than other ethnic groups (Goldenberg et al., 2008).

Due to the immaturity of multiple organ systems, premature newborns are at risk of a number of health complications (Rubens et al., 2014). As you would expect, the severity of complications inversely correlates with gestational age (WHO, 1977). Children born prematurely have an increased risk of cognitive and neurological impairment such as cerebral palsy, as well as respiratory and gastrointestinal complications (Goldenberg et al., 2008; Marlow et al., 2005). There is also the increased risk of chronic diseases in adulthood, such as obesity, diabetes and hypertension (Rubens et al., 2014). Interestingly, neonatal outcomes are sex-specific. Premature males are at greater risk of morbidity and mortality than females. In one premature cohort, the males had poorer neurological and respiratory outcomes, when followed up at 2 years of age (Peacock et al., 2012).

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2. Inflammation in labour and preterm labour

Human parturition is an inflammatory process (Bollapragada et al., 2009). Labour is initiated by the shift from a quiescent to a pro-inflammatory environment, instigating a three step process, characterised by uterine contractility, cervical ripening and membrane activation/rupture (Rinaldi et al., 2011; Romero et al., 1994). Cytokines are essential in the initiation and regulation of this process (Bowen et al., 2002).

Monocytes and neutrophils are primed in the peripheral blood in association with both term and PTL (Yuan et al., 2009). These cells infiltrate the human myometrium and cervix during spontaneous term labour, which is associated with a significant increase in interleukin (*IL*)-*1β*, *IL-6* and *IL-8* gene expression (Bokstrom et al., 1997; Osman et al., 2003; Thomson et al., 1999). Stromal cells of the maternal and fetal tissues are also responsible for the release of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 and tumour necrosis factor alpha (TNFα) (Young et al., 2002). Myometrial contractions are stimulated by the increase of IL-1β and TNFα, which increase calcium entry into these myometrial smooth muscle cells (Barata et al., 2004; Tribe et al., 2003). Prostaglandins, $PGF_{2\alpha}$ and PGE_2 are also involved in the stimulation of myometrial contractions (Olson, 2003). Cervical ripening is an inflammatory event in both term and PTB (Osman et al., 2003; Tornblom et al., 2005). Pro-inflammatory cytokines stimulate matrix metalloproteinase (MMP) expression, such as MMP-9, which promotes extracellular matrix degradation and cervical remodelling (Larsen and Hwang, 2011).

There is also a significant increase in leukocyte recruitment to the fetal membranes during parturition, with an increase in IL-1β, IL-8, and TNFα (Gomez-Lopez et al., 2009). Normal and preterm rupture of membranes differ in regional chemotactic activity and related chemokine/cytokine production, which may suggest differential mechanisms of rupture (Gomez-Lopez et al., 2013). The choriodecidua is responsible for granulocyte, T-cell lymphocyte, monocyte and NK cells chemoattraction (Gomez-Lopez et al., 2011). Leukocytes infiltrate the decidua during labour at term and in PTL and play a role in stimulating inflammatory mediators involved in decidual activation (Hamilton et al., 2012; Osman et al., 2003). Macrophage numbers are increased in term and PTL but neutrophil abundance is only significantly increased in the decidua in PTL with infection (Hamilton et al., 2012).

Nuclear factor-κB (NF-κB) is a pleiotropic transcription factor which is commonly associated with inflammation, as it is activated by and regulates pro-inflammatory cytokines. NF-κB activity is central to labour-associated pathways. It is required for both prostaglandin synthesis and for the regulation of MMP expression and thus, important for the stimulation of uterine contractions and cervical ripening. The premature or pathological activation of NF-κB could subsequently contribute to the initiation of PTL (Lindstrom and Bennett, 2005).

3. Fetal injury

Intrauterine infection/inflammation is a common cause of PTL. Presence of such an adverse *in utero* environment can lead to fetal injury. Chorioamnionitis, characterised by inflammation of the fetal membranes as a result of bacterial infection, is a risk factor for conditions such as cerebral palsy, necrotising enterocolitis and patent ductus arteriosus (Been et al., 2013; Park et al., 2015; Wu and Colford, 2000). Bacteria or inflammatory mediators may reach the fetal circulation by placental transmission into the umbilical cord or indirectly via the amniotic fluid, resulting in fetal injury (Adams Waldorf and Mcadams, 2013) (Fig. 1).

Intra-amniotic inflammation is also associated with lung injury. Elevated levels of inflammatory mediators such as TNF-α, IL-1β, IL-6 and IL-8 in the amniotic fluid have been found in women who had babies with bronchopulmonary dysplasia (BPD), a chronic lung disease affecting infants (Ambalavanan et al., 2011; Yoon et al., 1997b). However, a meta-analysis found only a modest association between chorioamnionitis and BPD (Hartling et al., 2012). Intra-amniotic endotoxin administration in a sheep model led to fetal lung injury, identified by evidence of inflammation, cell death and remodelling (Kramer et al., 2002). Exposure to endotoxin *in utero* also affects the development of the fetal ovine lung. One study observed an increase in alveolar volume, while the total number of alveolar was significantly reduced. Thinning of the alveolar walls was also observed (Willet et al., 2000). In a chronically catheterised sheep model of the extreme preterm period, lipopolysaccharide (LPS) exposure caused fetal skin and lung inflammation, as well as systemic inflammation (Kemp, 2014; Kemp et al., 2016). The premature fetus rapidly generated a robust inflammatory response to intra-amniotic LPS, which was driven by amniotic fluid-exposed tissues. Fetal blood cells responded to systemic inflammation but didn't contribute to the acute production of inflammatory mediators (Kemp et al., 2016). Furthermore, a primate model of transient choriodecidual infection confirmed indirect lung injury as result of elevated inflammatory mediators. This led to the downregulation of pathways for angiogenesis, morphogenesis and cellular growth and development in the fetal lung (Mcadams et al., 2012). In addition to conditions such as neonatal BPD, the treatment premature babies receive can have an adverse effect on pulmonary development and lead to long term, altered lung function. A follow up study reported evidence of airway obstruction from mid-childhood to adulthood in a preterm-born cohort (Vollsaeter et al., 2013).

Prematurity has also been associated with an increased incidence of brain damage. This can result in neurological conditions such as cerebral palsy, which is often observed in conjunction with intraventricular haemorrhage or periventricular leukomalacia (Chang, 2015). Additionally, inflammation during fetal and neonatal development has been linked to other neuropathologies such as schizophrenia and autism. It has been suggested that schizophrenia may be as a result of post-acute latent inflammation, whereas autism may be due to persistent inflammation (Meyer et al., 2011).

The release of pro-inflammatory mediators can play a role in the generation of brain white matter lesions. Evidence of elevated levels of IL-1β and IL-6 in the amniotic fluid has been used to identify premature newborns at risk of developing these lesions (Yoon et al., 1997a). TNFα signalling, in particular, has been described as toxic to developing oligodendrocytes (Li et al., 2008). The isolation of low-virulence microorganisms from preterm placentas, such as common skin microflora, has been associated with brain lesions and cerebral palsy in these infants. Evidence of placental inflammation alone, is a predictor of brain injury (Leviton et al., 2010). Even in the absence of PTB, fetal brain injury was reported in a mouse model of intrauterine inflammation (Elovitz et al., 2011). The same group reported damage to fetal neurons, demonstrated by a significant reduction in microtubule-associated protein 2 (*Map2*) gene expression, as well as altered neuronal morphology and neurotoxicity, in this model (Burd et al., 2011). Dada et al. (2014) found that following intrauterine inflammation, brain injury is not only acute. Long term changes in MRI and behaviour are observed even in adulthood. Chronic brain inflammation was linked to eventual neuronal loss. In addition, these effects were found to be sex specific, with male mice more susceptible to long-term neurologic injury. Mallard et al. (2003) exposed fetal sheep to LPS during mid-gestation and reported microglia activation, astrocyte damage and oligodendrocyte death. Inflammation may cause brain injury as a result of direct insult to oligodendrocytes and neurons or as secondary injury via microglial cell activation. This subsequently results in the secretion

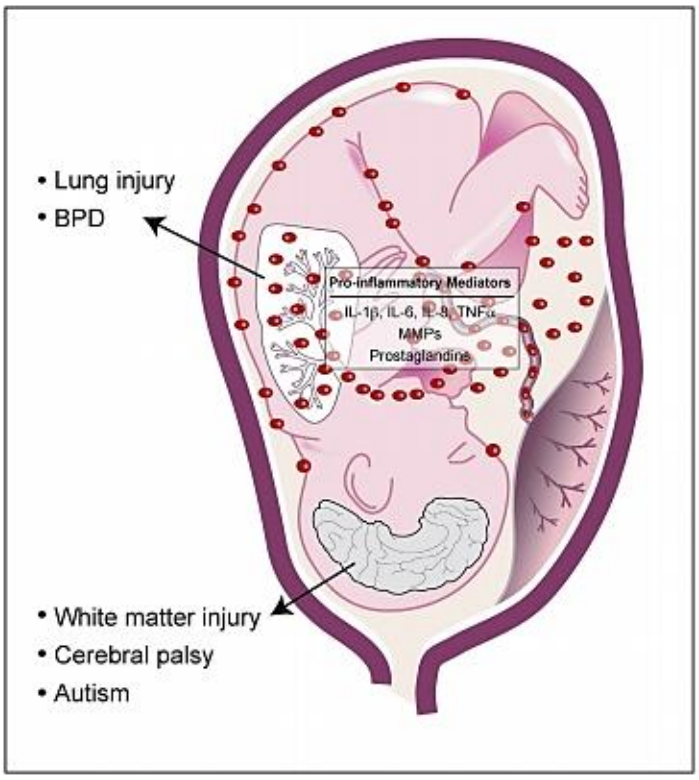

Fig. 1. Intrauterine inflammation and fetal injury.
Human parturition is an inflammatory process. However, the presence of inflammation earlier in gestation can result in the preterm, pathological initiation of labour, with adverse consequences for the fetus. Inflammatory mediators may reach the fetal circulation by placental transmission into the umbilical cord or indirectly via the amniotic fluid. Amniotic fluid exposed tissues, such as the fetal skin and lung, can drive the fetal inflammatory response. Prolonged in utero exposure to inflammatory mediators can result in fetal injury. The fetus is susceptible to lung injury and bronchopulmonary dysplasia (BPD), as well as brain white matter lesions and neurological conditions such as cerebral palsy and autism.

of pro-inflammatory cytokines, causing damage to surrounding cells (Burd et al., 2012).

4. Prevention and treatment of PTL

In general, tocolytic therapies are largely ineffective at substantially delaying delivery and reducing neonatal mortality (Haas et al., 2012). Several trials have reported the benefits of vaginal progesterone administration for reducing the rates of PTB and improving neonatal outcome. However, these studies had limited information on the long term outcome of these infants (Da Fonseca et al., 2003; Dodd et al., 2013; Fonseca et al., 2007; Hassan et al., 2011). Recently, a large, double-blind, randomised, placebo controlled trial of vaginal progesterone treatment reported that the risk of PTB was not reduced by progesterone nor was neonatal outcome improved, in women who were at risk of PTB. The trial did not find any long term benefit of this treatment in children by the time they were 2 years of age (Norman et al., 2016).

As intrauterine infection is a common cause of PTL, antibiotics have previously been considered a logical method of treatment. However, the use of antibiotics to prevent PTB has been associated with neonatal enterocolitis, as well as an increased risk of cerebral palsy (Kenyon et al., 2001; Kenyon et al., 2008). As parturition is associated with leukocyte influx into the intrauterine tissues, immunomodulation has been investigated in mouse models of PTB, using leukocyte depletion. Rinaldi et al. (2014) found that neutrophil depletion did not delay delivery in an LPS-mediated PTB mouse model, despite having an important role in the inflammatory response in the intrauterine tissues. Filipovich et al. (2015) further corroborated this finding by reporting that polymorphonuclear leukocyte depletion in an *E. coli* model of PTB did not prevent premature birth. Therefore, neutrophil infiltration is not essential for the induction of murine infection-induced PTB. However, macrophage depletion was found to successfully prevent both RU486 and LPS-induced PTB in mice (Gonzalez et al., 2011).

Targeting the inflammation commonly associated with PTB may be a suitable approach to delay the onset of parturition and prevent fetal injury. A recent review outlined the possible targets for such treatments including non-specific NF-kB inhibitors, toll-like receptor 4 (TLR4) antagonists, TNFα biologics and novel cytokine suppressive anti-inflammatory drugs (CSAIDs) (Ng et al., 2015; Stinson et al., 2014). Shynlova et al. (2014) targeted the activiation of murine peripheral maternal immune cells with a broad spectrum chemokine inhibitor. A reduced incidence of LPS-induced PTB

was reported, in additon to reducing both immune cell infiltration into maternal tissues and inflammatory mediator secretion. Other novel, naturally produced or alternative therapies such as folic acid, melatonin and statins, have recently been investigated for the treatment of PTB and fetal loss in mouse models (Boyle et al., 2015; Dominguez Rubio et al., 2014; Gonzalez et al., 2014; Zhao et al., 2013). Antigen capture therapy has been described in an ovine model of intrauterine infection/inflammation. This cationic peptide antibiotic, polymyxin B, binds to *E. coli*, reducing inflammation in the amniotic fluid and fetal lung (Saito et al., 2014). Rinaldi et al. (2015) investigated the use of a key anti-inflammatory and pro-resolution mediator, 15-*epi*-lipoxin A_4, in an LPS-induced PTB mouse model. Although PTB was not prevented, 15-*epi*-lipoxin A_4 reduced rates of fetal death.

It is unlikely that one treatment will be successful in preventing the multifactorial syndrome of PTB. However, it should be considered that combination therapies, which have both tocolytic and fetal protective effects, would be advantagous. Improved methods of identifying at risk mothers and fetuses are also required to effectively target treatments and avoid unnecessary harm. Using new ultrasound techniques, such as Tissue Doppler Imaging (TDI), to identify signs of fetal inflammation is one strategy that shows promise (Di Naro et al., 2010; Stock et al., 2016). The influence genotype may have on the therapeutics used for PTB prevention and treatment, has recently been discussed. Although the evidence to date is limited, there is increasing indication that pharmacogenomics may play a role in PTB. By integrating clinical information, environmental influences and genotype, a more comprehensive strategy to personalised medicine could be optimised (Manuck, 2016).

5. Conclusion

PTB is still a significant public health problem. This syndrome is multifactorial and its etiology is not well understood. Inflammation is necessary to stimulate term labour. However, its presence earlier in pregnancy contributes to fetal injury, particularly to the lung and brain. Evidence of inflammatory mediators in the amniotic fluid identifies infants at risk of lung damage and brain white matter lesions. Delaying PTB, as well as improving the outcome of the babies, has proved a great challenge. Current therapies, such as vaginal progesterone treatment, are ineffective. In addition, the use of antibiotics to target intrauterine infection is potentially harmful to the developing fetus, increasing the risk of cerebral palsy. Animal models are currently being utilised to investigate potential therapeutic agents which may target the intrauterine inflammation with the intention of protecting against fetal injury. The possibility of developing more personalised medicine should also be considered in the effort to reduce the rates of fetal mortality and morbidity, as a result of this clinical condition.

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