Intrauterine cooperativity: amplification between ligands, cells, and tissues to transition the uterus for parturition

by

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Abstract

Birth is a complex biological event requiring genetic, cellular and physiological changes to the uterus, resulting in a uterus *activated* for completing the physiological processes of labour. We define the change from the state of pregnancy to the state of parturition as *uterine transitioning*, which requires the actions of inflammatory mediators and localized paracrine interactions between intrauterine tissues. In the human, the birth cascade involves positive feedback interactions between a series of parallel events integrating both pro-inflammatory and pro-contractile systems, which accumulate until reaching a 'critical mass' or threshold that triggers a labouring state. An activated uterus is marked by increased expression of uterine activation proteins (UAPs) resulting in the increased sensitivity of the myometrium to coordinated contraction. In addition to the actions of steroid hormones, key inflammatory mediators, IL-1β and $PGF_{2\alpha}$, regulate the expression of critical uterine activation proteins (UAPs) *FP* and *COX2* and pro-inflammatory cytokines and chemokines in cultured primary human myometrium smooth muscle cells (HMSMC). We found that in addition to regulation of the $PGF_{2\alpha}$ receptor FP, IL-1β and $PGF_{2\alpha}$ also upregulate the IL-1 receptor system, including *IL1R1*, *IL1R2*, *IL1RAcP* and *IL1RAcPBb*. HMSMC treated with IL-1β then $PGF_{2\alpha}$ (or vice versa) produce amplified increases in IL-6 and COX-2 mRNA and protein compared to treatment with either agonist alone. These large increases were unique to myometrium and not observed with hFM.

Uterine transition not only comprises interactions between different inflammatory pathways, but also paracrine interactions between neighbouring cells and tissues. Few studies have examined the *in vitro* interactions between fetal and maternal gestational tissues within this pro-inflammatory environment. Thus we designed a co-culture model to address this gap,

incorporating primary term human myometrium smooth muscle cells (HMSMC) with human fetal membrane (hFM) explants to study interactions between the tissues. We hypothesized that crosstalk between tissues at term promotes pro-inflammatory expression and uterine transitioning for parturition. Outputs of 40 cytokines and chemokines encompassing a variety of pro-inflammatory roles were measured; all but one increased significantly with co-culture. Eighteen of the 39 cytokines increased to a higher abundance than the sum of the effect of each tissue cultured separately. In addition, *COX2* and *IL6*, but not *FP* and *OTR* mRNA abundance significantly increased in both HMSMC and hFM in response to co-culture. These data suggest that synergistic pro-inflammatory upregulation within intrauterine tissues is involved with uterine transitioning.

The series of experiments described in this dissertation together present cooperativity between ligands, cells and tissues as a hallmark of human parturition. Our work establishes $PGF_{2\alpha}$ and IL-1β as key triggers or upstream drivers of this process and IL-6 and COX-2 as key targets, promoting inflammatory amplification in the myometrium through positive feedback and synergy. We surmise that, if similar events occur *in vivo*, this large increase in mediators could reflect a critical event in the transition of the uterus from the state of pregnancy to the state of parturition.

Preface

This thesis is original work completed by Kelycia B. Leimert. This dissertation is part of a research project that received ethics approval by the University of Alberta Ethics Approval Board, Project name "The immunological regulation of term and preterm labour: the pivotal roles of peripheral leukocytes and chemotactic factors, Study ID Pro00069209, expiry date Februrary 25, 2019.

Portions of chapter four of this thesis are included in a manuscript currently under review at the journal Biology of Reproduction. KB Leimert, X Fang and DM Olson designed and planned the experiments, and the manuscript was written by KB Leimert and DM Olson. HMSMC culture was performed by KB Leimert and R Nemati and hFM culture by A Messer and KB Leimert. Quantitative RT-PCR was performed by KB Leimert, BSE Verstraeten, A Messer, R Nemati and K Blackadar. Protein assays (Western Blotting, multiplex) and statistical analysis was completed by KB Leimert. SA Robertson and S Chemtob edited and gave final approval of the manuscript, and all authors approved the final manuscript.

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Dedication

For my mom,

who taught me that strong women can accomplish anything they set their minds to.

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I have been so very blessed over the past five and a half years with the most fantastic support system one could ask for. Six years ago, I was an undergraduate student who stumbled across a paper on inhibition of small-conductance Ca^{2+}-activated K^{+} channels to delay preterm birth in mice while doing some reading for a physiology discussion course. I knew nothing about preterm birth, and was riveted when I realized how much was still unknown about it. I wrote a paper on the subject for my course, and somewhere along the way I was hooked. As a fourth-year undergraduate student with no research experience, I found David Olson's website online. I reached out and asked him to accept me as a fourth-year research project student mere days before the start of my last undergraduate semester. Little did I know, that one-semester research project would turn into an undergraduate summer student position, then an MSc project, and eventually a transfer to the PhD program.

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List of abbreviations

ACTH	Adrenocorticotropic hormone
AF-3	Transcription activation domain 3
AKR1B1	Aldo-keto reductase family 1, member B1
AL-8810	Orthosteric FP antagonist
ANOVA	Analysis of variance
AP-1	Activator protein 1
AP-2	Activator protein 2
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CCL2	C-C motif chemokine ligand 2 (alternate name for MCP-1)
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT-enhancer-binding protein
CO_2	Carbon dioxide
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2 (Alternate name: PGHS-2)
CX	Connexin
CX-43	Connexin 43
CXCL8	C-X-C motif chemokine ligand 8
CRE	cAMP-response element
CRH	Corticotropin-releasing hormone

DAG	Diacylglycerol
DAMP	Damage associated molecular pattern
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ERE	Estrogen response element
ET-1	Endothelin-1
FBS	Fetal bovine serum
FDA	United States Food and Drug Administration
FP	Prostaglandin $F_{2\alpha}$ receptor
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA	Transcription factor binds GATA
Gp130	Glycoprotein 130
GPCR	G-protein coupled protein receptor
GR	Glucocorticoid receptor
GRE	Transcription elongation factor
GTP	Guanosine triphosphate
HBSS	Hank's balanced salt solution

HCl	Hydrochloric acid
hFM	Human fetal membranes
HMGB-1	High-mobility group box-1
HMSMC	Human myometrium smooth muscle cells (primary)
HPA	Hypothalamic pituitary adrenal axis
IκBα	Nuclear factor kappa-light-chain-enhancer of activated B cells inhibitor α
IL-1β	Interleukin-1beta
IL-1R1	Interleukin-1 receptor 1
IL-1R2	Interleukin-1 receptor 2
IL-1ra	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
iNOS	Inducible nitric oxide synthase
IRAK4	Interleukin-1 receptor-associated kinase 4
JAK	Janus kinase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
MAPS	Modular accumulation of physiological systems
MCP-1	Monocyte chemoattractant protein 1 (alternate name for CCL2)
MFI	Median fluorescence intensity
MLCK	Myosin light chain kinase

MLCP	Myosin light chain phosphatase
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MYD88	Myeloid differentiation primary response 88
NFIL-6	Nuclear factor for IL-6 expression (in C/EBP family)
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nucleotide-binding oligomerization domain (NOD)-like receptor
NmUR2	Neuromedin U receptor 2
NSAID	Non-steroidal anti-inflammatory drug
nPR	Nuclear progesterone receptor
OT	Oxytocin
OTR	Oxytocin receptor
P45017C	17 α-hydroxylase cytochrome 450
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PDC113.824	Pharmaceutical Development Corporation 113.824 (FP antagonist)
PEA-3	ETS domain transcription factor
PG	Prostaglandin
PGDH	15-Hydroxyprostaglandin dehydrogenase
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin F_{2alpha}
PGHS-2	Prostaglandin endoperoxide synthase-2 (alternate name: COX-2)
PHM1-41	Immortalized human myometrial derived cells

PLC	Phospholipase C
PMSF	Phenyl methane sulfonyl fluoride
PKA	Protein kinase A (cAMP-dependent protein kinase)
PKC	Protein kinase C
PRR	Pattern recognition receptor
PTB	Preterm birth
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
RLC	Regulatory light chain
RU486	Mifepristone (progesterone receptor antagonist)
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP-1	Specificity protein 1
sTL	Spontaneous term labour
STAT	Signal transducer and activator of transcription
TCZ	Tocilizumab
THG113	TheraTechnologies 113 (FP antagonist)
TIMP-1	TIMP metallopeptidase inhibitor 1
TIR	Toll/Interleukin-1 receptor homology domain
TL	Term labouring
TLR	Toll-like receptor

TNFα	Tumor necrosis factor alpha
TNL	Term non-labouring
T_{reg}	Regulatory T cell
UAP	Uterine activation protein
ULTR	Immortalized human myometrial derived cells
uNK	Uterine natural killer cell
ZAM	Zone of altered morphology

Chapter 1
General introduction

1.1 Preterm birth: a global health problem

Preterm birth (PTB), defined as birth before the 37th completed week of gestation, affects 5-18% of births in 184 countries worldwide, resulting in 15 million babies born preterm each year (1). Of those, 1.1 million do not survive (1, 2). Those who do survive may face a spectrum of lifelong disabilities, including cerebral palsy, intellectual impairment, chronic lung disease, vision and hearing loss (3). Globally, PTB complications are the leading cause of newborn death and the second greatest cause of death in children. In the majority of countries with reliable data, PTB rates are rising (1). The PTB rate in the United States increased by more than 30% over 30 years, culminating in 12.1% of births in 2002 and an economic burden of over $26 billion in 2005 alone (3). In spite of all of the medical advancements of our era and the increasing success of neonatologists in saving babies born at earlier and earlier gestational dates, we still do not have an effective method of prevention for this global problem. A new PTB therapeutic has not been introduced to the market in over 30 years (4), so although we have seen progress in survival rates of preterm infants no intervention advancements have been made, resulting in escalating healthcare costs (5). The most recent Canadian report shows that from 2005 to 2014 the incidence of PTB (excluding Quebec) wavered between 8.0 and 8.3% (6). In the United States, one in ten births in 2017 were preterm (9.93%) (7).

Nearly half of all PTB is idiopathic or spontaneous (8). Both environmental and genetic factors contribute to PTB, influenced by stress, infection, cervical or placental complications, multiple fetuses, and more. Pre-existing medical conditions such as obesity (9), being underweight (10), diabetes (11), hypertension (12), advanced maternal age (13), exposure to air pollution (14) or smoking during pregnancy (15) can increase the risk of PTB. Regardless of the cause, we are now fairly confident that a series of common pathways are implicated in the

signalling cascade culminating in birth whether term or preterm, characterized by pro-inflammatory amplification[1] (Fig. 1.1) (16). Toll-like receptors (TLRs) are activated by pathogen-associated molecular patterns (PAMPs) due to infection, or damage-associated molecular patterns (DAMPs) from the increasingly physiologically stressed uterus, inducing assembly of the inflammasome and the production of pro-inflammatory cytokines and chemokines. Cytokine production results in leukocyte activation, and migration of maternal peripheral leukocytes into the gestational tissues. Following amplification of inflammatory mediators, the uterus is *activated*[2] for the labour process, upregulating expression of uterotonic receptors, gap junction proteins, prostaglandins and matrix metalloproteinases resulting in a myometrium with increased sensitivity to contractile agonists and capacity for contraction. Following activation, the uterus is primed for parturition and the presentation of labour symptoms occur: myometrial contractility, rupture of membranes and cervical ripening.

[1] Pro-inflammatory amplification: a cascade of inflammatory upregulation, propagated by the release of pro-inflammatory cytokines and chemokines driving positive feedback interactions to further upregulate their own pro-inflammatory effects and products.

[2] Uterine activation: an activated uterus is characterized by increased sensitivity to contractile agonists and the increased capacity for contraction, as a result of myometrial upregulation of uterotonic receptors, gap junction proteins, prostaglandins and matrix metalloproteinases.

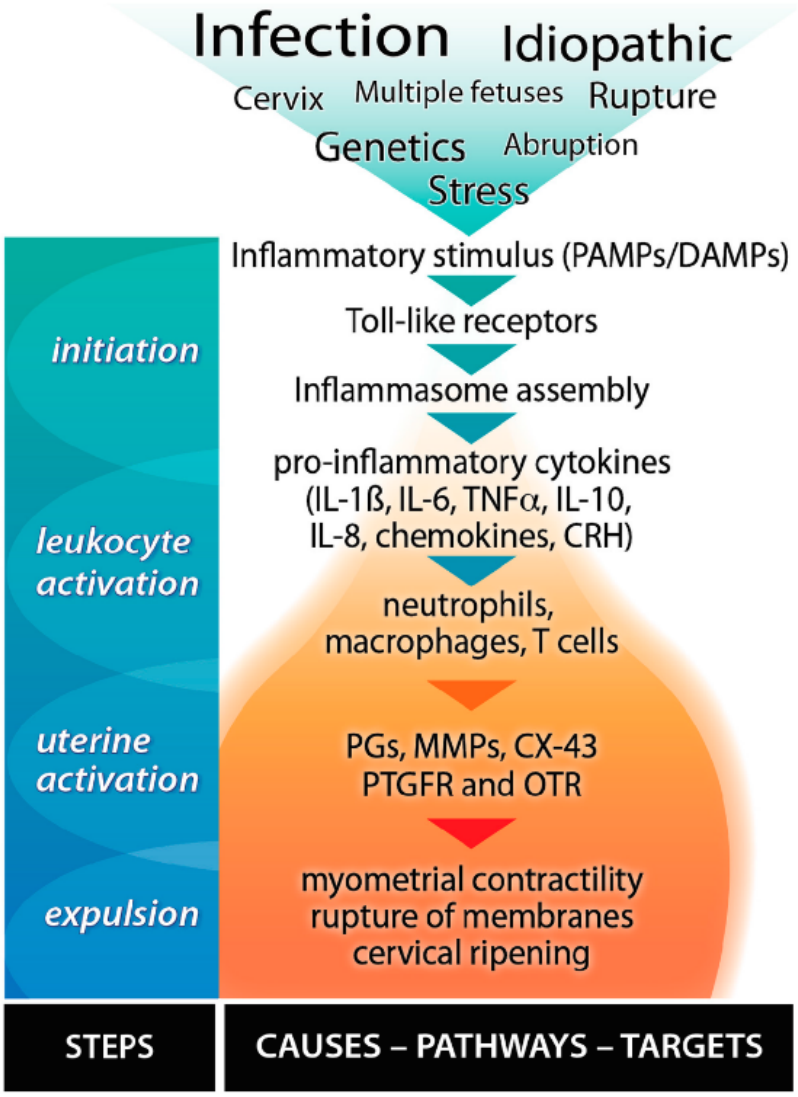

Figure 1.1. Birth cascade: inflammatory amplification resulting in parturition; presented with permission from Olson *et al.* 2015 (16).

Existing drugs for PTB management are tocolytics, which aim to suppress uterine contraction by targeting myometrial contraction. Clinicians administer tocolytics hoping to delay PTB by 24-48 hours, which is the time required for glucocorticoids to stimulate fetal lung maturation (5, 17, 18). The transition from pregnancy to parturition involves the communication and amplification between many molecules; tocolytics are unsuccessful in suppressing PTB because their targets occur too late in the signalling cascade and excessive upstream amplification and activation has already ensued at the time of inhibition (5, 18, 19). David Olson once described this dilemma as "an approach likened to trying to stop a speeding locomotive by standing on the tracks" (5). We must focus on the study and development of inhibition of potential therapeutic targets closer to the top of the birth cascade so suppression can occur before parturition progresses past an irreversible point. In order to accomplish this, it is crucial for us to develop our understanding of upstream interactions and inflammatory mechanisms at term.

1.2 Uterine transitions for labour

1.2.1 Uterine transition requires cellular and physiological changes.

The uterus is a unique and dynamic organ that must undergo considerable changes in function throughout the reproductive cycle. Every month the uterus prepares itself for possible implantation through proliferation of the uterine endometrium, upregulation of endometrial secretions, and neovascularization under the control of ovarian steroid hormones 17β-estradiol and progesterone. When implantation does not occur, the fall in levels of those hormones results in the coordinated shedding of the proliferated endometrial layer through menstruation (20). When implantation does occur resulting in pregnancy, the uterus transforms, maintaining pregnancy through processes such as decidualization that we will be discussing in the next

section. The uterus must then expand to accommodate the rapidly growing fetus, increasing more than 15x in size over the 40-week gestation period from an approximate weight of 75 g to 1300 g (21). For most of pregnancy, the uterus actively maintains a relaxed or quiescent state, sheltering, protecting, and sustaining the fetus as it grows. Nearing the end of the gestational period, the uterus must undergo extensive transition over a period of just days into an active and contractile organ capable of performing the physiology of delivery. After labour, the uterus undergoes another transitional period termed *uterine involution*, resulting in a return to the non-pregnant cycling state and original size. Although the uterus endures multiple transformative periods over the course of a reproductive cycle and pregnancy, the focus of this doctoral thesis is the pervasive transition from the state of pregnancy to the state of labour or delivery. We call the sum total of cellular, genetic and physiological changes that the uterus must undergo during this period *uterine transition*[3] (Fig. 1.2).

The core tenet of human physiology is centered on maintaining homeostatic balance, or equilibrium of the body's internal environment. The principle of negative feedback maintains homeostasis; one of the most well-known examples of this principle is the hypothalamic pituitary adrenal (HPA) axis mediating the central stress response. When sufficient levels of cortisol have been produced in response to a stressor, cortisol induces a repression of corticotropin-releasing hormone (CRH) release from the hypothalamus and adrenocorticotropic hormone (ACTH) release from the pituitary, limiting further cortisol production and restoring a state of equilibrium within the system. Negative feedback mechanisms impede most uncontrolled upregulation, so

[3] Uterine transition: The sum total of all cellular, genetic and physiological changes that the uterus (and all intrauterine tissues) must undergo to change from a state of pregnancy to a state of normal menstrual cycling.

uninhibited positive feedback or feedforward interactions are predominantly observed in deregulated disease states, such as tumour growth in cancer (22-24), and autoimmune/auto-inflammatory diseases (25). However, maintenance of pregnancy past term compromises ongoing viability of the mother and her fetus(es), forcing deviation of uterine physiology from normal homeostasis to ensure delivery. Positive feedback mechanisms are implicated to achieve adequate amplification to facilitate change at the cellular level, enabling extensive transitioning of the uterus over a short time period.

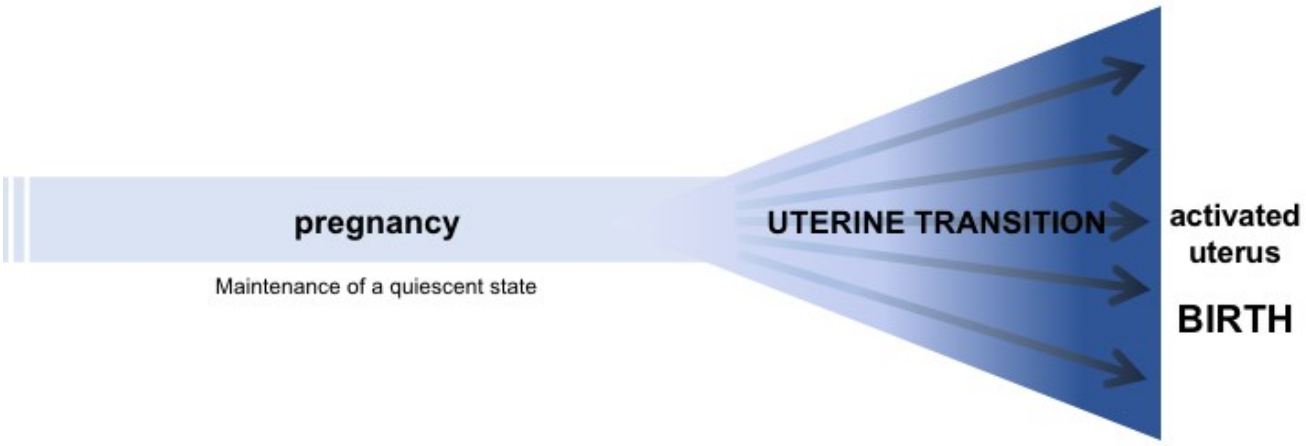

Figure 1.2. Parturition is a complex physiological event requiring biochemical and physiological transition of the uterus.

1.2.2 Uterine transition is inflammatory and encompasses multiple gestational tissues

Every birth, whether term or preterm, is an inflammatory event. Infection is associated with only 11% of all preterm births and about 2% of term births (26), hence in the majority of cases the inflammatory events of parturition occur without an infectious process (26, 27). Nearing parturition, DAMPs released by the maturing fetus, ageing placenta and increasingly

physiologically stressed uterus (26, 28) stimulate expression of pro-inflammatory cytokines, chemokines, prostaglandins and their receptors (29-31). Human microarray studies demonstrate that the transition to labour is an inflammatory event not limited to a single gestational tissue (32-36). 471 genes change in preparation for term labour in the human myometrium and 86% of the altered pathways were identified as inflammatory in nature (33). Dominant gene changes in the term labouring myometrium and cervix are involved in leukocyte movement, intercellular communication and cytokine signalling (34). 796 genes increase or decrease in choriodecidua for term labour, with the activation of inflammatory or immune pathways leading the changes to the transcriptome (35). Amnion 'activated' for labour, characterized by high NFκB activity, increased the expression of 919 genes including pro-contractile and pro-inflammatory targets compared to non-activated amnion (37). Term labouring fetal membranes upregulate genes involved in neutrophil and monocyte recruitment while no inflammatory gene expression changes occurred with labour in peripheral blood (36), affirming that uterine transition is a localized intrauterine inflammatory response.

1.3 Intrauterine environment in pregnancy and parturition

Within the pregnant uterus, intimate interactions exist between maternal and fetal gestational tissues, intersecting at the placenta (Fig. 1.3). Paracrine interactions between the uterus, decidua, and fetal membranes, consisting of amnion and chorion, are integral in localized changes for labour. Parturition requires a coordinated effort involving all gestational tissues to complete the five separate yet physiologically interrelated events of parturition: membrane rupture, cervical dilatation, myometrial contractility, placental separation and uterine involution (38). To understand the paracrine interactions in and between intrauterine tissues in this thesis,

we must first discuss the different maternal and fetal gestational tissues, their biology and composition, and how they change for labour.

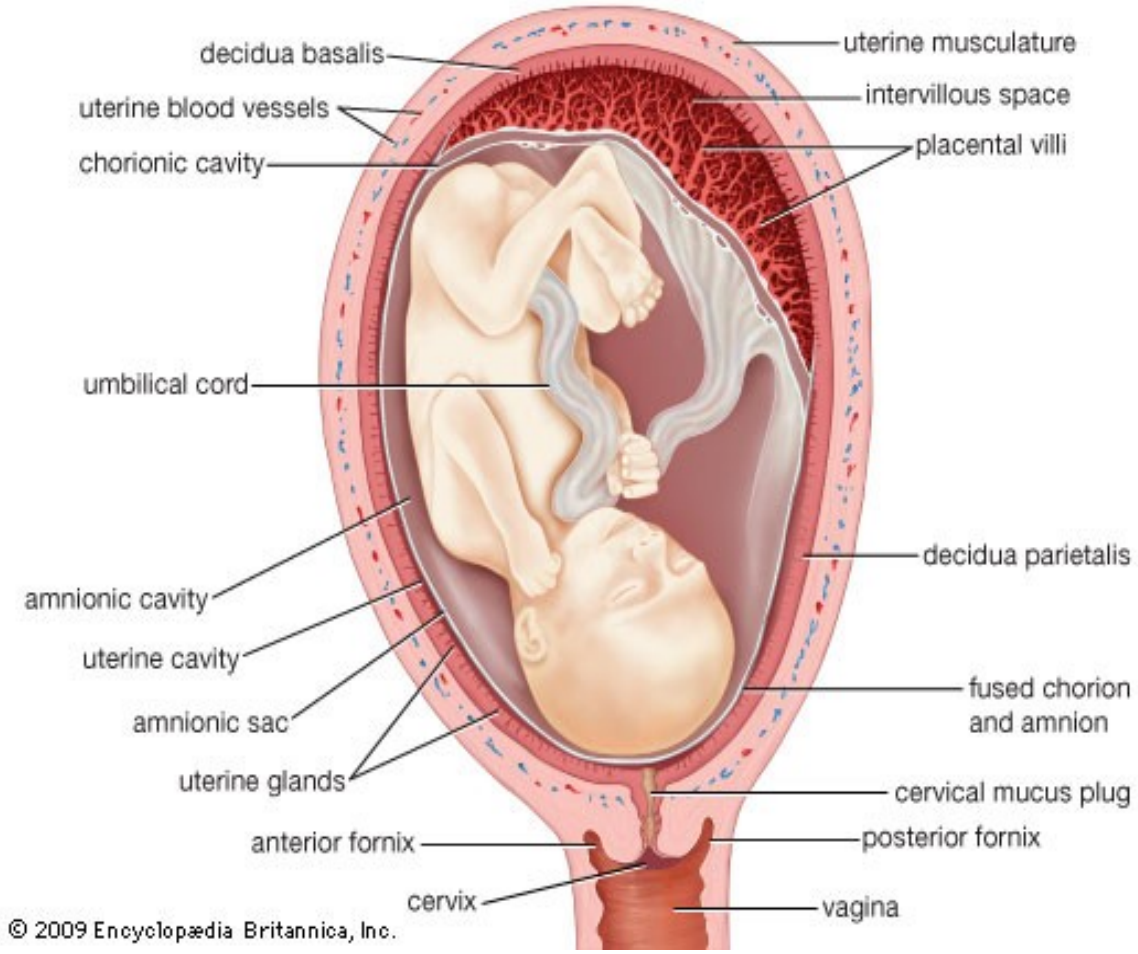

Figure 1.3. Maternal and fetal gestational tissues in the pregnant intrauterine environment, used with permission from Britannica eReader.com, a service of Encyclopaedia Britaanica, Inc.

1.3.1 Maternal tissues

1.3.1.i Myometrium

The myometrium, or uterine musculature, is comprised of fusiform-shaped uterine smooth muscle cells. The uterine myocyte contractile apparatus, like other muscle cells, consists of actin filaments and myosin 'motor' proteins. However, unlike the sarcomeres of skeletal muscle, uterine smooth muscle cells have a structural framework of dense bodies and intermediate filaments distributed throughout the cytosol of the cell (39), and contain six times more actin than myosin (40). An increase in intracellular calcium results in activation of calmodulin, forming a calcium-calmodulin complex which activates the myosin light chain kinase (MLCK) enzyme, resulting in phosphorylation of the regulatory light chain (RLC) at S19 (41). This activates ATPase on the myosin heavy chain, causing the hydrolysis of adenosine triphosphate (ATP) and conformational change of myosin (42). Actin can then bind myosin and form actin-myosin cross bridges; actin filaments slide along myosin heavy chains, initiating cell shortening/contraction of the smooth muscle unit. The RLC can be diphosphorylated at both S19 and T18 (43) by rho-associated protein kinase (ROCK), initiating an alternative myosin conformation and higher actin-induced ATPase action allowing for sustained contraction (44, 45). This diphosphorylation is unique to uterine smooth muscle and is not observed in vascular smooth muscle (45). Relaxation of the smooth muscle unit occurs through disassociation of actin and myosin, by decreasing intracellular calcium levels, inactivating MLCK, or de-phosphorylating RLC by myosin phosphatase (MLCP) (39). In addition to diphosphorylation of RLC, RhoA/ROCK signalling promotes contraction through inhibition of MLCP activity. The initial increase in intracellular calcium responsible for the induction of uterine contraction is generated by activation of $G_{\alpha q/11}$ and phospholipase C (PLC) by uterotonic agonists such as

oxytocin (OT) or prostaglandin (PG) $F_{2\alpha}$, which we will be discussing in detail later in this background chapter.

Negative regulation of contraction is mediated through activation of $G_{\alpha s}$. $G_{\alpha s}$ activates adenylate cyclase, resulting in activation of cyclic AMP (cAMP) and cAMP-dependent kinase (PKA). PKA phosphorylates Rho, blocking Rho/ROCK-mediated inhibition of MLCP and upregulating MLCP-mediated inhibition of contraction (46). $G_{\alpha i}$ antagonizes $G_{\alpha s}$ signalling through inhibition of adenylate cyclase, resulting in decreased cAMP production from ATP and suppressed PKA activation.

For the myometrium to contract as a coordinated unit, electrical coupling between individual smooth muscle cells is essential. Electrophysiological stimuli flow from cell to cell through gap junctions formed by groups of connexin proteins. A gap junction consists of two connexons, which each contain six connexin proteins. These gap junctions allow intercellular communication and propagation of action potentials between smooth muscle cells, enabling contraction of a synchronized uterine musculature through excitation-contraction coupling (47, 48). Connexin proteins, such as Connexin (CX) 43, increase in expression at labour and are stimulated by estrogens (49-51). In more than 80% of mice with a deletion of smooth muscle-specific *Cx43,* labour is delayed due to impaired intercellular communication/coupling (52). In addition to an increase in the connectivity of uterine smooth muscle with labour, uterine sensitivity to contractile agonists is also increased. This development is a result of the altered expression of a series of proteins labelled *uterine activation proteins* (UAPs) (29), including important enzymes and uterotonic receptors like the $PGF_{2\alpha}$ receptor FP and oxytocin receptor OTR, which we will be discussing in further detail later on in this introduction.

1.3.1.ii Cervix

The cervix is the lower section or 'neck' of the uterus, connecting the uterus to the vaginal canal. The cell composition of the cervix includes columnar mucous-producing glandular epithelial cells, squamous epithelial cells, cervical fibroblasts and smooth muscle cells. However, the cervix is predominantly composed of extracellular matrix, containing collagen, elastin, and glycoproteins. The uterus grows and stretches during pregnancy to accommodate the growing fetus. The cervix acts as the 'uterine gatekeeper', and has an important role during pregnancy of maintaining rigidity to retain the fetus despite increasing tension, pressure and weight. To accomplish this feat of physical integrity, the cervix must remain rigid and closed, assisted by its high collagen content.

For birth to occur, the cervix must transition from a closed and rigid state to a dilated and soft state, stretching to accommodate the passage of the fetus through the birth canal. This occurs in two stages: cervical softening and ripening. Softening occurs gradually through gestation beginning near the end of the first trimester, whereas ripening is a more accelerated process occurring over the last days to weeks of human pregnancy that results in the maximized loss of cervical integrity (53). Cervical collagen is remodeled as part of this process, mediated by increasing levels of collagenases and re-organization of matrix structure (54-56). Glycosaminoglycans (GAGs) are involved in organization and structure of collagen and increase through pregnancy, rearranging collagen for increased cervical distensibility (57). In addition, elevated concentrations of collagenases lead to increased degradation of collagen, resulting in only 30% of non-pregnant levels of collagen at term (58). Cervical ripening is influenced by steroid hormones, growth factors and invading leukocytes (59). It was first proposed to be an inflammatory process by Prof. GC Liggins in 1981; leukocytes and their products are involved in mediating changes to the cervical extracellular matrix (55, 60-63). However, conflicting reports

debate the necessity of leukocyte infiltration for cervical ripening, and suggest the role of invading leukocytes could instead be designated for post birth tissue repair and remodeling (64, 65).

1.3.1.iii Decidua

The non-pregnant uterus is lined by endometrium, consisting of endometrial stromal cells. In preparation for possible implantation following ovulation each month, rising progesterone and cyclic adenosine monophosphate (cAMP) levels induce functional change of these cells; the stromal cells enlarge due to accumulation of lipids, glycogens and released secretions (66). The transforming cells produce prolactin and insulin-like growth factor binding protein-1, as well as numerous growth factors and cytokines (67-69). The differentiation of endometrial stromal cells into decidual cells is called *decidualization,* and begins around terminal spiral arteries. In the human, the blastocyst implants into the decidua and is completely enveloped. After successful implantation, the decidua continues to grow and foster the invasion of trophoblasts and support placentation, with decidua eventually encompassing the entire endometrium. A very important aspect of decidualization is the remodeling of the vasculature at the fetal-maternal interface. In addition to the roles of estrogen and progesterone, the invasion of decidual leukocytes, specifically uterine natural killer (uNK) cells, have important roles in stimulating this vascular growth and angiogenesis (70). In addition to uNK cells, decidual macrophages, uterine dendritic cells, and regulatory T (T_{reg}) cells are present in decidua and involved in adaptive immune roles suppressing anti-embryo maternal immune responses (71, 72). The decidua is essential in the establishment of pregnancy, and continues to maintain and support pregnancy throughout gestation through the production of important hormones and growth factors.

Nearing labour, like other gestational tissues, the physiology of the decidua changes. Like the myometrium, the decidua upregulates UAPs and undergoes *activation*. In late gestation and labour, the decidua is a major producer of $PGF_{2\alpha}$, and expresses the uterotonic receptors OTR and FP (73, 74). FP decidual expression levels increase four-fold with labour, while decidual expression of all progesterone receptor (PR) isoforms decrease with labour (75, 76). In addition, term decidua increases pro-inflammatory expression. *Ex vivo* organ culture of decidua stimulated with $PGF_{2\alpha}$ upregulated expression of matrix metalloproteinase (MMP) -2 and -9, enzymes involved in degradation of adjoining fetal membranes in labour, while decreasing expression of the tissue inhibitor of metalloproteinases-1 (TIMP-1) (77).

1.3.2 Fetal tissues

The human fetal membranes (hFM), consisting of chorion and amnion layers, envelope and protect the fetus throughout pregnancy. Chorionic trophoblasts invade the maternal decidua and form a highly integrated choriodecidua maternal-fetal interface, thus close interactions exist between intrauterine fetal and maternal tissues (78). To accommodate a rapidly growing fetus and the increasing volume of amniotic fluid over 40 weeks, the amniochorion doubles in size and is able to withstand increasing fetal movements through its unique viscoelastic properties (79-81). By mid-pregnancy, the fetal membranes line the entire uterine cavity. The amnion is only one third the thickness of the chorion, but is able to withstand five times more tensile stress than the chorion (82). However, together the two tissues are stronger than either alone, as additional strength is provided by the chorion and dense connective tissue connecting the two membranes (83, 84).

During pregnancy, as the growing fetus kicks, stretches, and moves, the fetal membranes remain elastic to withstand the movements and avoid rupture, containing and protecting the fetus and surrounding amniotic fluid. This attribute must change in preparation for labour, as the membranes must rupture to allow passage of the fetus through the birth canal. Membrane rupture is facilitated by biomechanical changes to the amnion and chorion combined with the mechanical force of uterine contractions at labour. During pregnancy, tissue extensibility of amnion and chorion are evenly matched and act in parallel. With labour, tissue extensibility of the chorion increases twice as much of that of the amnion, resulting in the easier separation of amnion and chorion and a loss of fetal membrane tensile strength, demonstrated by altered load-strain curves (85). Multiple mechanisms are responsible for the loss of strength, or weakening, of amnion and chorion. Matrix metalloproteinases have a role in the remodeling of the fetal membrane extracellular matrix, specifically MMP-2 and MMP-9, gelatinase enzymes upregulated with labour that are involved in the degradation of collagen IV. Collagen IV is a prominent collagen in the basement membrane of the amnion, in chorionic cytotrophoblasts and in connective tissue (86, 87). As labour approaches, products in the amniotic fluid stimulate amniotic fibroblasts to increase production of collagenases (88) and amniotic collagen content significantly decreases (89). Chorionic trophoblasts release lytic enzymes, and leukocytes at the membrane rupture line release leukocyte elastase, contributing to local biomechanical weakening of the membranes (90).

1.3.2.i Chorion

The chorion consists of two components: chorionic trophoblast cells, which penetrate into the adjacent decidua, and the chorionic reticular layer, directly adjacent to the amnion (91). Initially after hFM formation, a ‘chorionic cavity’ exists between the amnion and chorion. First

trimester fetal growth results in obliteration of the chorionic cavity and the chorion's reticular layer adheres to the amnion. The chorionic layer surrounding the developing fetus is called the *chorion leave* and does not contain chorionic villi. The chorionic portion of the placenta is called the *chorion frondosum* and has many villi infiltrating the decidua creating the fetal side of the maternal/fetal interface. The chorion is the major site of progesterone synthesis, producing 100x more progesterone than decidua or amnion (78).

1.3.2.ii Amnion

The amnion is the innermost layer of the fetal membrane that forms the sac containing the fetus. When first established the amnion is formed in close contact to the embryo, and then as the production of amniotic fluid begins and then increases in volume, fluid fills the sac and creates an amniotic cavity where the fetus is suspended in amniotic fluid. The amnion is in direct contact with amniotic fluid and consists of a single layer of cuboidal epithelial cells and a layer of connective tissue. The fluid-filled cavity protects the fetus through enabling free movement of the fetus for proper bone and tissue development and shock absorption, among many roles (92).

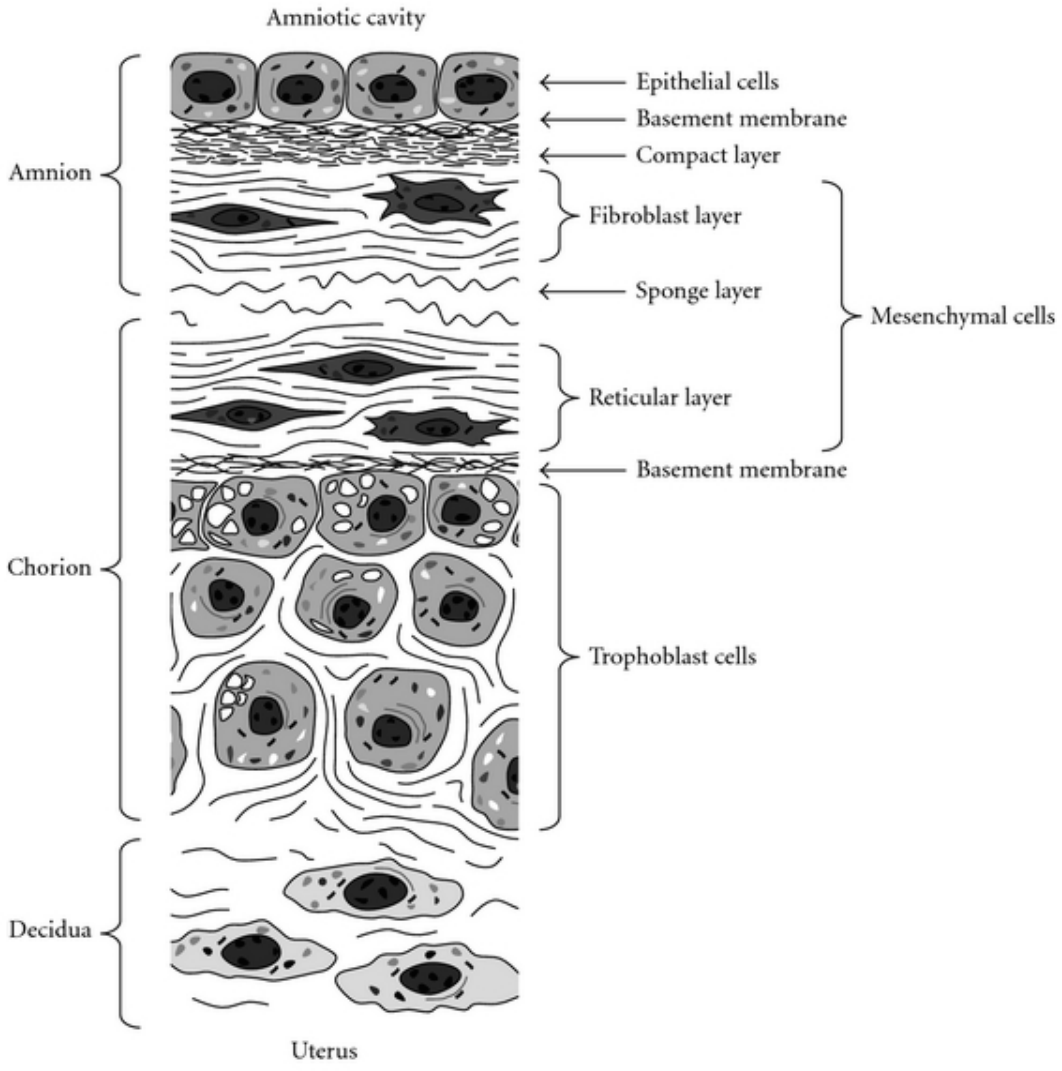

Figure 1.4. Diagrammatic representation of the human fetal membranes, as published in Uchide *et al.* 2012 (93).

1.4 An activated uterus is marked by the upregulation of UAPs

Uterine transition results in an *activated* uterus primed for activity and capable of performing the physiology of labour. The activated myometrium demonstrates increased excitability, connectivity, and sensitivity to contractile agonists due to upregulation of connexins, cyclooxygenases, receptors and agonists of contractile stimulators. These markers of activation are termed uterine activation proteins (UAPs), which change in expression at labour to increase systems of uterine contractility or suppress uterine relaxation. As described by Nadeau-Vallée, a UAP demonstrates four key characteristics: 1) change in expression for labour that returns to baseline postpartum; 2) uterocontractile effects that are gestational age-dependent; 3) expression is induced by pro-inflammatory stimuli; and 4) contribute to the process of labour (94). Many UAPs have been identified in both human and animal models, nine of these were classified in the rat by Arthur *et al*. (95). Nonetheless, more UAPs are still being characterized. During the completion of data collection for this dissertation, I collaborated with a group at the Université de Montréal on a discovery project characterizing the neurological receptor protein, neuromedin U receptor 2 (NmUR2), as a newly described UAP with a role in uterine activation and labour (manuscript included in appendix).

Table 1.1. Uterine activation proteins change their expression for labour.

Uterine activation protein	Role	Change for labour
Cyclooxygenase 2 (COX-2)	Enzyme catalyzing the rate limiting step in prostaglandin synthesis	↑
$PGF_{2\alpha}$ receptor (FP)	Uterotonic G-protein coupled receptor (GPCR)	↑
Oxytocin receptor (OTR)	Uterotonic G-protein coupled receptor (GPCR)	↑
Oxytocin (OT)	Neuropeptide, uterotonic agonist	↑
Connexin 43 (CX-43)	Gap junction protein essential for myometrial connectivity	↑
Endothelin 1 (ET-1)	Vasoconstrictor, uterotonic agonist	↑
ET-1 receptor ET_A	Uterotonic G-protein coupled receptor (GPCR)	↑
Inducible nitric oxide synthase (iNOS)	Enzyme catalyzing production of nitric oxide (NO)	↓
Prostaglandins (PGs): $PGF_{2\alpha}$ and PGE_2	Uterotonic agonists, pro-inflammatory mediators	↑
NmUR2	Neuropeptide, uterotonic agonist	↑

The shift in UAP expression profiles at labour is regulated by pro-inflammatory mediators, such as Interleukin-1β (IL-1β) or IL-6. Many markers of uterine activation have binding sites for cytokine-induced transcription factors such as nuclear factor (NF)κB in their promoter regions. For example, FP's promoter region contains binding sites for both NFκB and NFIL-6, supporting more than one route of inflammatory transcriptional regulation of FP (96). In immortalized human myometrial-derived ULTR cells, IL-1β induces FP expression via NFκB (97). In addition to FP, pro-inflammatory cytokines have been shown to regulate expression of COX-2 (98-102), OTR (103-105), CX-43 (106), ET-1 (107, 108), iNOS (109), PGs (110-112),

and NmUR2 (94). These studies emphasize the importance of pro-inflammatory amplification in converting the uterus from the physiological state of pregnancy to that of delivery.

Steroid hormones estrogen and progesterone are also important regulators of UAPs. Progesterone, named for its pro-gestational actions promoting maintenance of pregnancy, acts to antagonize NFκB via NFκB inhibitor alpha (IκBα) and suppress UAP expression (113, 114). In addition to UAP modulation through deoxyribonucleic acid (DNA) binding, progesterone represses OTR through direct binding of the receptor and the subsequent prevention of inositol phosphate production and calcium ion (Ca^{2+}) mobilization (115, 116). Estrogen has the contrary effect, and promotes UAP expression via estrogen response elements on many UAP promoter regions. The promoter region of the CX-43 gene contains estrogen response elements (117), and CX-43 expression is increased by estrogen stimulation (49, 118). Oxytocin and its receptor OTR both increase in response to estrogen (119, 120). Androstenedione infusion in the last third of pregnancy increased systemic 17 beta-estradiol and myometrial activity in the rhesus monkey, supporting a role for estrogen in regulating myometrial contractility in late gestation (121). Corticotropin-releasing hormone (CRH) also stimulates NFκB and release of C-C motif chemokine ligand 2 (CCL2), C-X-C motif chemokine ligand 8 (CXCL8), Interleukin (IL)-1β, Interleukin (IL)-6, and tumor necrosis factor alpha (TNFα) by human term uterine smooth muscle cells, which in turn regulate UAPs (122).

In addition to pro-inflammatory cytokines and steroid hormones, the stretching of the uterus to accommodate the growth and development of the fetus through gestation modulates UAP expression. Mechanical stretch of cultured human myometrial cells and fetal membranes results in induction of a series of pro-inflammatory cytokines and chemokines (123-127). Additionally, stretch of immortalized human myometrial cells was also found to enhance the

recruitment of peripheral leukocytes into uterine tissues, both by upregulating endothelial cell adhesion proteins and enhancing vascular permeability (124). It is not surprising then that uterine stretch results in increased UAP expression as well. Stretch induced COX-2 in primary human uterine myocytes through transcription factor activator protein 1 (AP-1) (128) and induced OTR through CCAAT-enhancer-binding protein (C/EBP) (129). The inflation of an intrauterine balloon (150 mL) in the uterus of non-labouring women at term resulted in increased prostaglandin synthesis and labour induction (130). In animal models, mechanical stretch increased CX-43 and OTR in the rat (131, 132), and OTR and COX-2 in the sheep (133).

Many changes are occurring and contributing to activation in preparation for labour. We track three messenger ribonucleic acid (mRNA) species and their proteins as proxies for the many markers of activation. The $PGF_{2\alpha}$ receptor FP, the oxytocin receptor OTR, and COX-2 will be discussed as outcome measures throughout the remainder of this dissertation, and therefore will be described in further detail below.

1.4.1 UAPs: $PGF_{2\alpha}$ receptor (FP)

FP is a G-protein coupled receptor (GPCR) with seven transmembrane domains encoded by the *PTGFR* gene. *PTGFR* is highly regulated; the promoter region includes both enhancer and repressor regions as well as binding sites for NFκB, NF-IL-6, AP-1, GATA, signal transducer and activator of transcription (STAT), and cAMP-response element (CRE) (96). FP is highly expressed in the uterine myometrium, decidua, cervix and fetal membranes, as well as the ovary, eye, and kidney. FP is coupled to $G_{\alpha q/11}$ and $G_{\alpha 12}$, resulting in the active contraction of uterine smooth muscle through $G_{\alpha q}$-induced inositol triphosphate (IP_3) and intracellular Ca^{2+} mobilization, and Rho/ROCK mediated cytoskeletal rearrangements. FP's most potent ligand,

$PGF_{2\alpha}$, is characterized as a uterotonic agonist involved in the stimulation of uterine contraction. More recently, a non-contractile role for $PGF_{2\alpha}$/FP in parturition has been introduced, in myometrial induction of UAPs and pro-inflammatory cytokines (134-136). $PGF_{2\alpha}$'s non-contractile roles in uterine transition for labour will remain a main theme examined and discussed throughout this dissertation.

During pregnancy FP expression declines, with 45% lower FP mRNA abundance in pregnant from non-pregnant human myometrium (137). FP receptor abundance, as well as other contractile PGE2 receptors, is suppressed with increasing gestational age but then significantly increases at labour (138-141). It is likely that this decline of uterotonic receptor expression during pregnancy is involved in the active maintenance of uterine quiescence during pregnancy. Animal models also depict a significant upregulation in FP prior to parturition (30, 142, 143). In the mouse, FP levels significantly increased prior to PTB and term birth, and remained low when labour was extended by progesterone (142).

Interference of FP signalling through the use of receptor antagonists adds further endorsement to the importance of its roles in parturition. THG113 (also known as PDC31) is a specific FP allosteric receptor antagonist that blocks FP from interacting with $G_{\alpha q}$, therefore inhibiting the subsequent increase in intracellular calcium leading to contraction (144). THG113 successfully delays lipopolysaccharide (LPS)-induced preterm labour in mice (144) and mifepristone (RU486)-induced preterm birth in sheep (145). PDC113.824 is a more selective derivative of THG113, which is able to negatively regulate signalling through the Rho/ROCK pathway while positively regulating signalling through the mitogen-activated protein kinase (MAPK) system. PDC113.824 not only delays normal parturition in mice, but blocks LPS and $PGF_{2\alpha}$-induced preterm birth as well (146). Additionally, AL-8810, a specific FP antagonist that

blocks all signalling from the receptor, reverses $PGF_{2\alpha}$-induced UAP expression changes in primary human myometrial smooth muscle cells (HMSMC) (134). Mice with a null mutation for FP (FP-/-) do not deliver because luteolysis does not occur, preventing progesterone withdrawal (147). When ovariectomies are performed on FP-/- mice on gestational day 19, progesterone levels fall and the mice proceed with normal term labour (148).

1.4.2 UAPs: Oxytocin receptor (OTR)

OTR is a GPCR with seven transmembrane domains encoded by the *OXTR* gene. Like *PTGFR, OXTR* is highly regulated. The *OXTR* promoter contains transcription factor binding sites for STAT3, NF-IL6, NFκB, AP-1, AP-2, GATA-1, and specificity protein 1 (SP-1) (149, 150), conferring immune regulation from multiple pathways. In addition, there are two half estrogen response elements (EREs) present on the 5' end and one on the 3' end, demonstrating estrogen responsiveness (151). Interestingly, the ligand for OTR, OT, is also transcriptionally regulated by estrogen via EREs on the *OXT* gene promoter (152). OTR is abundantly expressed in human myometrium and mammary tissues, as well as brain, kidney, and cardiovascular tissues. OTR is coupled to $G_{\alpha q}$ and $G_{\alpha i}$ (153, 154). $G_{\alpha i}$ inhibits adenylate cyclase and decreases cyclic AMP. $G_{\alpha q}$ activates PLC, producing IP_3 and diacylglycerol (DAG). IP_3 production induces intracellular Ca^{2+} increase activating the signalling cascade resulting in uterine contraction, whereas DAG activates protein kinase C (PKC) and the MAPK system, involved in the production of prostaglandins. There are many PKC isozymes, and PKC can be activated by DAG and Ca^{2+}, DAG alone or phosphotidyl serine in conventional, novel, and atypical activation pathways. Many myometrial studies measure PKC involvement in OT-induced signalling by measuring OT-stimulated outcome measures with or without a PKC inhibitor, such as COX-2

induction (155) and myometrial contraction (156, 157). These PKC inhibitors are specific to many isoforms of PKC. BIS1 blocks isozymes involved in conventional and novel activation pathways: PKCα, β1, β2, γ, δ, and ε (155). Additional study is required to confirm specific PKC isozymes activated as a result of OT/OTR interaction and their contributions in the resulting signalling cascade. Of all the uterotonic agonists, oxytocin acting through OTR is the most potent stimulator of uterine contraction (158).

Both OT and OTR increase in abundance at labour in humans and all animal models of parturition (73, 159-165). Unlike FP, there is no suppression of OTR during pregnancy, instead gradual increases are observed through gestation followed by a large increase in abundance at labour (73). In human myometrium, OTR increased twelve-fold from mid-pregnancy to term, and a total of thirty-fold from mid pregnancy to its peak levels at labour (166). However, pregnant mice with null mutations of *OXTR*-/- or *OXT*-/- were unable to eject milk for lactation, but experienced normal deliveries, suggesting that OT/OTR is one of multiple pathways resulting in uterine contraction and labour (167-170). OT analogues, like Atosiban, competitively bind OTR and are successful at suppressing uterine contraction, as demonstrated in human clinical trials, non-human primate models and rodents (4, 171-176). Despite effectively blocking contraction, Atosiban has been shown to have some pro-inflammatory effects in primary amniocytes (177). Atosiban is currently licensed as a tocolytic for acute PTB in Europe. In North America, Atosiban did not pass Food and Drug Administration (FDA) approval after a placebo-controlled clinical trial showed no significant increase in prolongation of pregnancy (4); interpretation of the trial outcomes was complicated by inclusion of rescue tocolytic therapies (allowing alternative tocolytic therapy after one hour), reluctance to participate, and distribution of gestational ages in the trial population.

1.4.3 UAPs: Cyclooxygenase (COX)-2

COX-2 (or prostaglandin endoperoxide H synthase, PGHS-2) is a homodimeric enzyme encoded by the *PTGS2* gene that catalyzes the rate limiting step in PG synthesis, the conversion of arachidonic acid to PGH2. There are two forms of COX with distinct genes; COX-1 is constitutively expressed in most tissues, whereas COX-2 is inducible. Unlike the COX-1 gene *PTGS1*, the *PTGS2* gene promoter has a multitude of regulatory factors; transcription factor binding sites have been identified for NFκB, CRE, Sp1, AP-2, NF-IL6, transcription elongation factor (GRE), ETS domain transcription factor (PEA-3) and C/EBP (102, 178, 179). COX has two distinct active sites that catalyze two enzymatic reactions. Arachidonic acid first undergoes oxygenation to PGG2, followed by reduction to endoperoxide intermediate PGH2. PGH2 is then converted by specific PG synthase enzymes into a distinct prostaglandin type (D_2, E_2, $F_{2\alpha}$, I_2, TXA_2). COX-2 expression increases prior to labour in the human myometrium and fetal membranes but not decidua, culminating in the shift in PG levels observed in intrauterine tissues amid the transition from pregnancy to labour (180-186).

Inhibition of COX-2 makes an attractive target for preterm birth prevention; PGs are involved in many pro-inflammatory positive feedback interactions in the birth cascade, so suppression of PG synthesis suppresses both inflammation and contraction. Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit COX, and when administered to preterm labouring sheep, NSAIDs abolished PG release and delayed PTB (187). Double blind human trials demonstrated a prolongation of pregnancy with oral NSAIDs, increasing the average gestational age and birth weight from 31.2 weeks and 2028 g to 36.4 weeks and 2833 g (188). However, blocking COX results in widespread inhibition of the synthesis of all PGs, and many PGs have important roles

in fetal organ development. *COX2* mRNA is expressed in the fetal brain, ductus arteriosus, lung, kidney, and small intestine. NSAIDs including indomethacin cross the placenta and are poorly metabolised by the fetus, promptly accumulating in fetal tissues (189). After initially promising results, studies began to associate NSAID use with the increased risk of serious fetal health complications, including necrotizing enterocolitis, patent ductus arteriosus, intracranial hemorrhage (190), renal failure (191, 192) and persistent pulmonary hypertension of the newborn (193). The Tocox randomised double-blind placebo trial confirmed that COX inhibition affected fetal renal function and ductus arteriosus, had no effect on preterm birth incidence earlier than week 30 of gestation, and actually increased PTB rates for delivery in high risk groups before 37 weeks (194). Trials of two other NSAIDs also demonstrated increased risk for impairment of renal function and ductus arteriosus constriction (195). Therefore, widespread inhibition of all PGs is not the answer to PTB prevention. Inhibition of the activity of specific PGs that are not required for essential fetal development, like $PGF_{2\alpha}$, will be discussed later in this background chapter as a potential PTB therapy.

1.5 Parturition: physiological differences in humans and animals

Medical innovation has progressed exponentially over the past few decades, driven by continuously accelerating technological advances. Most of these breakthroughs would not have been possible without the incorporation of animal models into research studies. Due to obvious ethical guidelines in human research, we are limited to *in vitro* or *ex vivo* work. Before testing a therapy in human clinical trials, it is necessary to complete thorough studies in a living subject to test for efficacy and toxicity. For almost all medical fields, the answer to this problem is the use of 'representative' animal models to study how specific proteins and systems work *in vivo*. The

mouse, for example, shares 95% gene homology with the human (196, 197). No animal will ever depict a fully accurate representation of all the mechanisms of the human body, but model organisms do provide a highly valuable contribution to pre-clinical trial testing. In parturition research the dichotomy between animals and the human body is enhanced, as many of the basic biological principles governing parturition vary markedly from species to species. A model organism precisely modeling the physiology of human parturition does not exist. Due to the lack of a better experimental option for *in vivo* modeling, we still use animal models to test agonists and potential therapies during pregnancy and parturition. We must however take these physiological differences into account when discussing and comparing outcomes of animal model studies to human parturition. In this section, we will briefly discuss the main physiological differences between animal models and humans in reference to pregnancy and parturition. Although the experimental methodology presented in this dissertation involve human *in vitro* modeling only, it remains important to include this dialogue into the background chapter as many referenced studies throughout this thesis will include animal models. For more detailed explanations of the physiological mechanisms of parturition in each animal model, an excellent review by Profs Mitchell and Taggart further characterizes these differences (198).

Characteristic	Rabbit	Sheep	Rat	Mouse	Guinea pig	Human
Gestation, days	32±3	147±4	22±1	20±1	67±3	266±14
Usual litter size, no.	8±4	1–2	10±6	10±5	3±2	1
Placental morphology	Hemodichorial, labyrinthine	Epithelial-chorial, cotyledonary	Hemotrichorial, labyrinthine	Hemotrichorial, labyrinthine	Hemomonochorial, labyrinthine, discoid	Hemomonochorial, villous, discoid
Source of progesterone	Corpus luteum	Corpus luteum, then placenta	Corpus luteum	Corpus luteum	Corpus luteum, then placenta	Corpus luteum, then placenta
Progesterone withdrawal?	Yes	Yes	Yes	Yes	Partial at *days 40–50*; not at parturition	No
Induction of preterm birth	Antiprogestin, ovariectomy	Fetal ACTH, glucocorticoid, antiprogestin	Antiprogestin, ovariectomy	Antiprogestin, ovariectomy, LPS	Antiprogestin plus oxytocin	Cervical ripening (PGE_2 or antiprogestin) plus oxytocin

Values are means ± SD.

Table 1.2. Reproductive characteristics of animal models compared with the human, as published in a review by Mitchell and Taggart, 2009 (198).

1.5.1 Progesterone: synthesis and withdrawal

In addition to the obvious basic biological differences between these different animals, such as length of gestation, number of offspring and the type of placentation, physiological differences exist between these animals in the hormonal control of parturition, labour initiation, and the regulation of these mechanisms.

During ovulation, the mature ovum is released from the ovarian follicle and begins its passage to the uterus through the Fallopian tube. The remaining ovarian follicle develops into the corpus luteum. If fertilization does not occur, the corpus luteum degrades and a new one forms during the next monthly ovulation period. If the ovum is fertilized producing a zygote, the corpus luteum continues to mature and is responsible for maintenance of the pregnancy through ample secretion of pro-gestational hormone progesterone, among others. The corpus luteum is the primary source of progesterone in the early pregnancy stages in all animals and humans (199). In the rabbit, rat, and mouse, the corpus luteum continues as the primary source of progesterone throughout the entire gestational period (Table 1.2). In the sheep, guinea pig, and human, the placenta replaces the corpus luteum as the dominant source of progesterone once it reaches a sufficient stage of development. In women, this 'luteal-placental shift' in hormone source occurs at 5-6 weeks of gestation (199, 200).

Progesterone is a potent relaxant, so its presence must decrease in order for stimulation of contraction to progress. This concept was originally described by Prof. Arpad Csapo as the 'progesterone block' theory (201). Labour occurs following the systemic withdrawal of progesterone for most animals (Fig 1.5) (198, 202, 203). Humans are fairly unique in that no systemic progesterone withdrawal occurs, yet birth still progresses. Progesterone levels continue to rise throughout pregnancy, and don't decrease until after delivery of the fetus and the placenta.

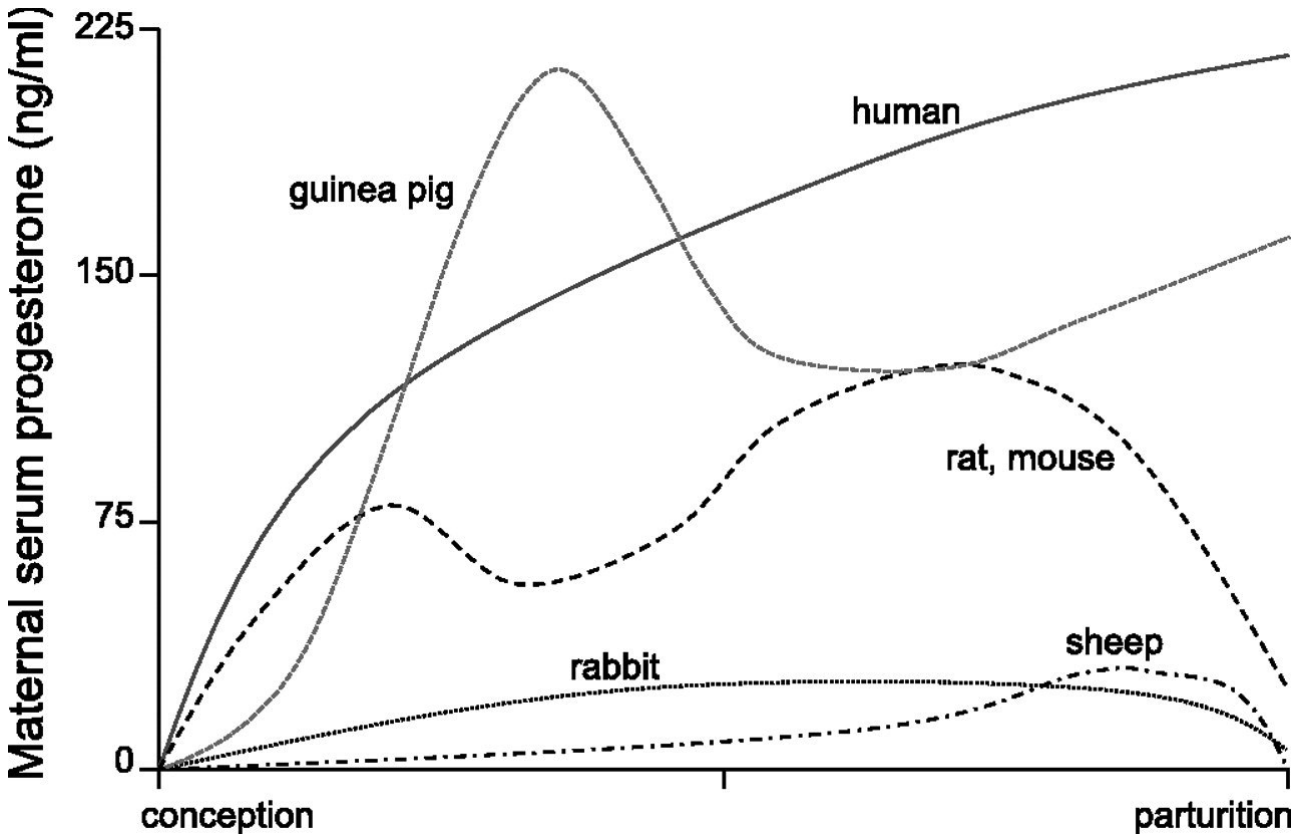

Figure 1.5. Maternal serum concentrations of progesterone through gestation, as published in Mitchell and Taggart, 2009 (198).

In animals that depend on the corpus luteum as their primary progesterone source throughout gestation, including rats, mice, and rabbits, $PGF_{2\alpha}$ is the initial stimulus that begins the cascade of labour. In these animals, $PGF_{2\alpha}$ initiates the process of luteolysis or degradation of the corpus luteum, resulting in the withdrawal of systemic progesterone and labour (204). Labour can therefore be induced in these animals at any point during pregnancy by blocking progesterone activity (201, 205, 206). RU486, a progesterone receptor antagonist mimics progesterone withdrawal *in vivo* through inhibition of progesterone (207). Knockout of the gene encoding the $PGF_{2\alpha}$ receptor FP in mice results in the inability to deliver, as luteolysis and the resulting progesterone withdrawal does not occur. When these mice lacking FP undergo ovariectomy on gestational day 19 delivery occurs normally, demonstrating that $PGF_{2\alpha}$ is required for initiation of luteolysis but birth is dependent on progesterone withdrawal (147).

In the sheep, parturition is initiated by the withdrawal of progesterone as in others, but the dominant progesterone source is the placenta instead of the corpus luteum, and progesterone withdrawal is stimulated by a fetal signal instead of $PGF_{2\alpha}$ (60, 208). As the fetal sheep matures, the HPA axis matures and begins producing hypothalamic CRH, stimulating production of ACTH at the pituitary, which induces the fetal adrenal gland to produce cortisol. Fetal cortisol stimulates synthesis of the enzyme 17 α-hydroxylase cytochrome P450 (P45017C) in the sheep placenta, which catalyzes conversion of progesterone to estrogen, resulting in progesterone withdrawal and estrogen-induced uterine activation. Concurrently, cortisol also stimulates maturation of the fetal lungs. Therefore, labour in the sheep can be initiated by infusion with CRH, ACTH, or cortisol that results in increased fetal cortisol levels, or by blocking progesterone activity to mimic progesterone withdrawal with RU486 (60, 209, 210).

Unlike the sheep, rabbit, rat, or mouse, the timing of human labour does not depend on the systemic withdrawal of progesterone, and treatment with glucocorticoids have no effect on birth timing (211). In addition to humans, nonhuman primates and guinea pigs also do not experience a fall in systemic progesterone (Fig. 1.5). The nonhuman primate is a more physiologically representative animal model for the study of preterm and term parturition, as well as inflammation-induced PTB and the testing of therapeutic agents for PTB (212-217). However, the nonhuman primate is an arduous model, as the animals are expensive to attain, care for, and house, and receiving ethics approval is a difficult process. The guinea pig is considered to be one of the best animal models for parturition, as they are more cost effective, pregnancy is maintained by placental progesterone production with a luteal-placental shift similar to humans, and they do not experience systemic progesterone withdrawal (198).

Even without systemic withdrawal, progesterone still has an essential role in the maintenance of human pregnancy. Mifepristone, or RU-486, is a competitive PR or glucocorticoid receptor (GR) antagonist, binding PR so progesterone is unable to. RU-486 is able to induce birth at any point in gestation in every species, conveying that pregnancy cannot continue in the absence of progesterone (218, 219). The current theory is that instead of a systemic progesterone withdrawal in humans there is alternatively a 'functional withdrawal', through the alteration of progesterone activity instead of a fall in maternal serum concentration (220). As described by Tan *et al.* (2012), there are four major mechanisms that could contribute to the functional withdrawal of progesterone at term: 1) Changes in expression levels of nuclear progesterone receptor (nPR) isoforms 2) A decrease in interactions between nPRs and their target gene promoters 3) Changes in expression levels of coregulators interacting with nPRs and 4) Increasing expression of an endogenous nPR antagonist (221).

To elucidate the first of the four mechanisms, the human *nPR* gene's transcription is controlled by 2 different promoters, resulting in two nPR isoforms: PR-A and PR-B. PR-A is a truncated form of PR-B, so although both isoforms have transcription activation domains 1 and 2, only PR-B has transcription activation domain 3 (AF-3), thereby resulting in differing cellular and genomic actions for the isoforms (222-224). During pregnancy, ratios of PR-A to PR-B expression are low (~0.5). With advancing labour there is a shift in PR-A:PR-B nPR dominance and PR-A:PR-B ratios are much higher (~3) (225). Previously, PR-B was thought to be responsible for the majority of the genomic actions of progesterone, whereas the role of PR-A was considered to be solely inhibition of PR-B activity. We now know that in addition to this role, PR-A mediates its own separate genomic actions as well. Cells manipulated to express a low (0.5) PR-A:PR-B ratio representative of levels during pregnancy had decreased expression of COX-2, IL-8 and IL-1β, whereas cells with a high (3) PR-A:PR-B ratio had increased expression levels of COX-2, IL-8 and IL-1β (221). Functional progesterone withdrawal adds a further level of complexity to the balance of pro-inflammatory mechanisms and myometrial contractility in preparation for parturition.

1.5.2 Labour induction: endocrine or paracrine regulation

Labour induction initiated by the withdrawal of systemic progesterone relies on the recession of a single mediator or 'trigger' under endocrine regulation. For those species that do not undergo this form of progesterone withdrawal, the regulation is more complex. Instead, localized changes are occurring in the intrauterine environment under paracrine regulation. Unlike endocrine regulation, which involves hormones travelling through the circulation system to act on their target cells, paracrine regulation involves local actions and signals between nearby

cells. In human parturition, intrauterine transition for labour is driven by paracrine interactions and localized inflammatory amplification, with minimal change occurring systemically. Human maternal serum progesterone levels remain high, and while the chorioamniotic membranes upregulate inflammatory gene expression in preparation for labour, these inflammatory changes are not detected systemically in the maternal blood (36).

In their 2009 review, Mitchell and Taggart described a concept defined as the "Modular Accumulation of Physiological Systems" (MAPS) (198). MAPS depicts the accumulation of a series of distinct parallel events upregulated concurrently to induce human labour instead of a single stimulus. The accumulation of these upregulatory events eventually reaches a 'critical mass' endpoint that results in parturition. More recently another concept has been introduced by Talati *et al.* (226), termed "inflammatory load". Through the gestational period pro-inflammatory mediators steadily increase in abundance, stimulated by DAMPs released by the maturing fetus, ageing placenta and increasingly physiologically stressed uterus (28). Human parturition occurs when pro-inflammatory mediators are upregulated and amplified until their signals exceed a threshold level whereby they stimulate functional progesterone withdrawal and complete uterine transition to its activated state for labour (226). The interweaving of these two similar concepts creates a portrait of human parturition governed by localized paracrine interactions involving cooperativity between pro-inflammatory and pro-contractile systems. At term these intricately entwined systems together drive amplification to reach a critical threshold level, at which point the accumulation of a series of parallel events together act as a trigger for parturition.

As inflammation is a key player in both of these concepts, there is value in comparing inflammatory mechanisms at term between endocrine and paracrine controlled parturition models. A 2016 study compared uterine temporal transcriptomics through pregnancy and labour

of mice in three different birth cohorts: spontaneous term labour (sTL), RU486-induced PTB modeling early progesterone withdrawal, and LPS-induced PTB modeling inflammation-induced birth (32). They found that sTL and RU486 birth groups had a similar transcriptomic profile (60%), whereas the LPS-PTB group had a distinct gene profile. Another study also showed similar expression patterns in sTL and RU486-PTB mice compared to LPS-PTB mice (227). Although sTL did result in upregulation of a gene network involved in chemotaxis and inflammation (32) and increased leukocyte extravasation into mouse myometrium (227), other mouse models have shown that the depletion of specific immune cell subsets such as neutrophils (64, 228), mast cells (229) and total polymorphonuclear leukocytes (including neutrophils, eosinophils and basophils) (230) had no effect on birth timing. Collectively, this suggests that in mice there is an involvement of inflammatory processes in labour but these processes are dispensable (unlike progesterone withdrawal). The primary role of inflammation and leukocyte infiltration in mouse parturition could possibly be related to uterine remodelling post birth and not labour induction.

The same 2016 study then compared the gene profiles of the three mouse birth cohorts to human transcriptomic data collected before and after spontaneous term labour. Interestingly, the transcriptomic profile of the human data set most closely paralleled the LPS-PTB mouse model, modeling inflammation induced PTB (32). Pro-inflammatory cytokine and chemokine abundance in human intrauterine tissues and amniotic fluid increases at labour (27, 63, 231), and leukocyte infiltration into myometrium (232), decidua (233), cervix (63, 234) and fetal membranes (235) has been characterized at term. Inflammation in human tissues has suggested a mechanism for the antagonism of progesterone-mediated gene changes involved in functional progesterone withdrawal. Inflammatory mediators like $PGF_{2\alpha}$ and IL-1β increase the PR-A/PR-B

ratio of progesterone receptor isoforms and increase the abundance and stability of PR-A (236-238), promoting pro-inflammatory expression and pro-labour transitioning (221). Cultured primary term uterine myocytes showed downregulation of genes typically upregulated by progesterone when stimulated with IL-1β (239). Computational analysis using directed graphs identified inflammatory processes as the most likely stimulus for human labour onset (240).

Therefore, unlike models of progesterone withdrawal where endocrine control of a single parturition trigger exists, human parturition encompasses accumulation of a series of interlinked parallel events resulting in amplification reaching a 'critical mass' or 'inflammatory load' threshold level that terminates in parturition. Inflammatory mechanisms appear to be integral to these events, and it seems that evolution has optimized the use of one biological system for both the process of labour and immunological uterine remodelling post birth. The failure of tocolytics to achieve successful PTB prevention to date may be due to the multitude of paracrine systems working in parallel to induce human labour. Much more complex than the inhibition of a single labour trigger, inhibition of one pathway will only result in short term or no inhibition followed by compensatory mechanisms from the other contributing parallel systems. For that reason, PTB prevention may require a multifaceted therapeutic approach.

1.6 Mediators of Parturition

1.6.1 $PGF_{2\alpha}$: role in parturition

Prostaglandins are produced by mother and fetus in the intrauterine tissues, and have a multifarious role in pregnancy and parturition. PGs have been described as the 'triggers' of labour (38) as they increase in abundance in tissues and fluids with approaching parturition (180-184, 241-244), their inhibition delays birth (187, 245), and exogenous PG treatment initiates

contraction of the myometrium (139-141). There are many types of prostaglandins, but $PGF_{2\alpha}$ is of particular interest to us in regard to its diverse roles in parturition acting through its receptor FP. Since we have already discussed both COX-2, the enzyme catalyzing the rate limiting step in $PGF_{2\alpha}$ synthesis, and FP, the $PGF_{2\alpha}$ receptor, at length in the uterine activation section above, in certain cases in the following subchapter I will refer back to these sections in the interest of avoiding repetition.

1.6.1.i Synthesis and metabolism

PGs are produced in all tissues of the body, as their precursor arachidonic acid is a component of cell membrane phospholipids. PG synthesis begins with the release of arachidonic acid from membrane phospholipids by enzymes of the phosopholipase A2 family. The released arachidonic acid undergoes oxygenation to PGG2, followed by reduction to endoperoxide intermediate PGH2 via the enzyme COX. The resulting PGH2 is then converted by specific PG synthase enzymes into a distinct prostaglandin type (D_2, E_2, $F_{2\alpha}$, I_2, TXA_2). Aldo-keto reductase family 1 member B1 (AKR1B1), a PGF synthase enzyme, is upregulated with labour in term human fetal membranes and co-localized with COX-2 at the chorion and decidua (246).

Prostaglandins are metabolized into their inactive 15-keto PG derivative forms by 15-hydroxy prostaglandin dehydrogenase (PGDH), which is also regulated throughout gestation. PGDH expression is promoted by high levels of progesterone, resulting in high levels of PG metabolism and low levels of active PGs throughout pregnancy (247, 248). In term chorion, PGDH activity decreased with labour, resulting in increased levels of active PGs. In preterm labour compared to term PGDH activity was downregulated, and activity was downregulated further with infectious preterm labour (249). Pro-inflammatory cytokines such as IL-1β decrease

the expression of PGDH, thereby increasing proportions of active PGs nearing parturition when levels of pro-inflammatory cytokines increase (250, 251).

1.6.1.ii Role in uterine contraction

$PGF_{2\alpha}$ is FP's most potent ligand, and acts as a uterotonic agonist involved in the stimulation of myometrial contraction. $PGF_{2\alpha}$ binds FP, a GPCR, resulting in activation of $G_{\alpha q}$ and IP_3, stimulation of intracellular Ca^{2+} mobilization and the active contraction of uterine smooth muscle. Additionally, $PGF_{2\alpha}$/FP can also activate $G_{\alpha 12}$ which signals through the Rho/ROCK pathway to induce cytoskeletal rearrangement and inhibit myosin light chain phosphatase, preventing dephosphorylation of the myosin light chain kinase resulting in sustained uterine contraction.

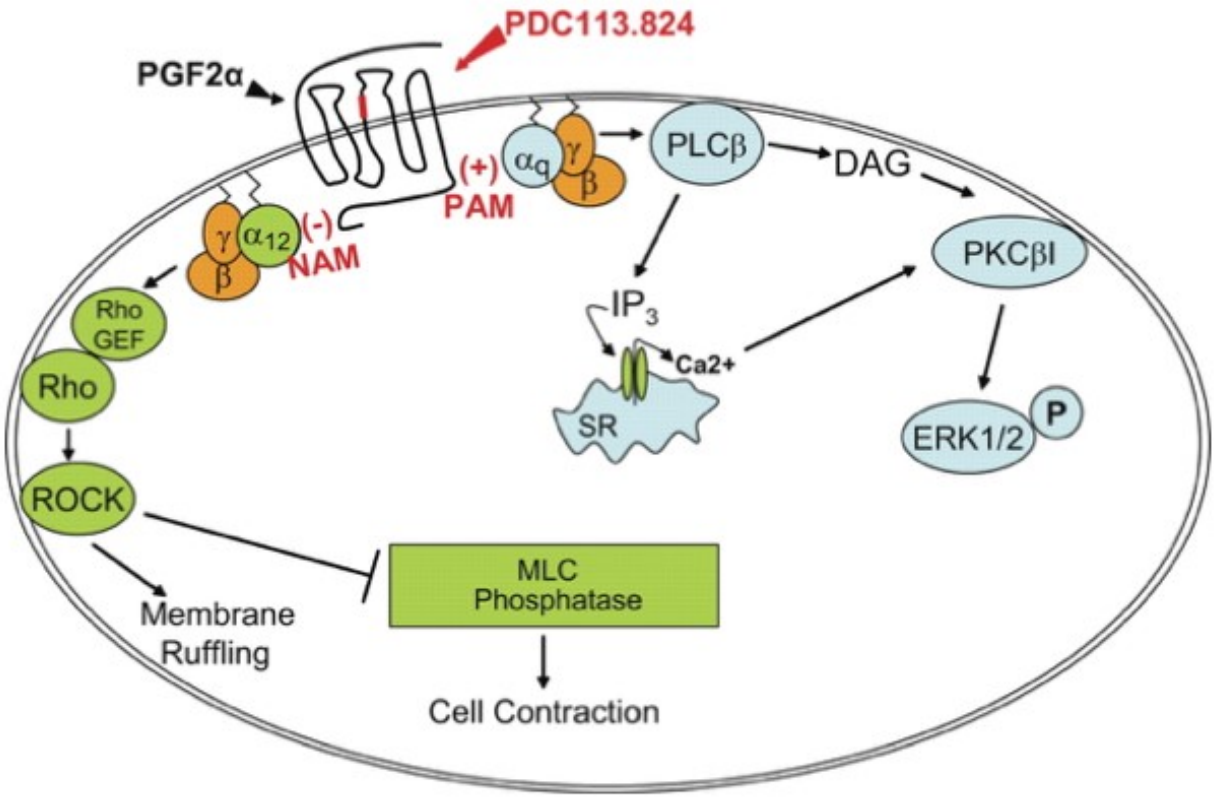

Figure 1.6. $PGF_{2\alpha}$ binds FP and activates $G_{\alpha q}$ and $G_{\alpha 12}$-dependent signalling pathways, as published in Goupil *et al.* 2010 (146). PDC113.824 is a biased allosteric FP antagonist that induces biased signalling from FP, increasing induction of the MAPK system through $G_{\alpha q}$ and decreasing Rho/ROCK signalling through $G_{\alpha 12}$.

1.6.1.iii Non-contractile roles of $PGF_{2\alpha}$ in parturition

In addition to its uterotonic actions, $PGF_{2\alpha}$ is involved in many intrauterine non-contractile signalling interactions, contributing to amplification of pro-inflammatory mediators and uterine activation for labour. In fact, $PGF_{2\alpha}$ is involved in all five physiological events of parturition: membrane rupture, cervical dilatation, myometrial contractility, placental separation and uterine involution (38).

In decidua, $PGF_{2\alpha}$ stimulates the production of MMP-2 and -9 while decreasing expression of their inhibitor TIMP-1 by 70% (77). The increased levels of MMP-2 and MMP-9 activity results in degradation of collagens 1, 4 and 5 in the gestational membranes, inducing cell apoptosis leading to the weakening and subsequent rupture of the amniotic sac (77, 86, 252, 253). Both PGE (E_1 and E_2) and $PGF_{2\alpha}$ contribute to cervical ripening, and prostaglandins are often clinically administered to induce cervical ripening in patients not successfully proceeding with labour (254-256). Placental separation in humans correlates with a peak in $PGF_{2\alpha}$ levels (241), and $PGF_{2\alpha}$ normally separated bovine placentas have higher levels of $PGF_{2\alpha}$ than non-separated placental cotyledons (257). Cows with a longer duration of post-partum uterine $PGF_{2\alpha}$ production complete uterine involution over a shorter period of time, suggesting that the presence of $PGF_{2\alpha}$ therefore promotes uterine involution (258). In addition, exogenous $PGF_{2\alpha}$ has been used to facilitate uterine involution in cows and Kundhi buffalo (259, 260).

In term primary HMSMC, $PGF_{2\alpha}$ up-regulates CX-43 expression, enhancing cell-to-cell communication and coordination for synchronized uterine contraction. Additionally, $PGF_{2\alpha}$ up-regulates COX-2, therefore stimulating its own synthesis in a positive feedback loop (134). In both sheep and rat, sensitivity of the myometrium to oxytocin decreases when PG synthesis is blocked by a COX-2 inhibitor and is restored with the administration of $PGF_{2\alpha}$, suggesting a role

for $PGF_{2\alpha}$ in oxytocin's actions in the myometrium (261-263). In primary HMSMC, $PGF_{2\alpha}$ stimulates upregulation of the oxytocin receptor OTR (134). In turn, oxytocin promotes the production of $PGF_{2\alpha}$ and other prostaglandins in the pregnant uterus and decidua (264, 265). IL-6, IL-1β and TNF-α can induce production of COX-2, therefore increasing the synthesis of prostaglandins (102, 111, 266). The upregulation of PGs by pro-inflammatory cytokines results in further cytokine production, as $PGF_{2\alpha}$ increases activity of MMP-2 and -9 which are able to process active IL-1β independently from caspase-1 (77, 267). $PGF_{2\alpha}$ also increases production of IL-6, IL-8 and CCL2 in primary HMSMCs (135).

1.6.1.iv FP antagonists delay preterm birth

After the disappointing Tocox trial outcomes, where COX-2 specific inhibitor rofecoxib negatively influenced fetal renal function and ductus arteriosus blood flow without significantly improving birth timing (194), inhibition of FP developed into a more desirable therapeutic strategy for PTB prevention. We now know that broad spectrum inhibition of COX-2 activity is not a successful therapy, as the fetal kidney, brain, cardiovascular system, lung, and gastrointestinal tract need prostaglandins like PGE_2 for proper development. However, of all the prostaglandins $PGF_{2\alpha}$/FP exhibits the strongest uterotonic effects. Inhibition of FP would block not only contractility but also pro-inflammatory upregulation and stimulation of UAPs, which makes it an appealing candidate for intervention. The first non-competitive FP receptor antagonist, THG113 (also known as PDC31), was synthesized from the second extracellular loop of FP and in the mouse inhibited generation of IP3, decreased spontaneous myometrial contraction *in vitro*, and prolonged delivery in RU486 and LPS-induced PTB (144). In the sheep, THG113 decreased both circular and longitudinal $PGF_{2\alpha}$-induced uterine muscle contraction *in vitro*, and delayed RU486-induced preterm birth (145). Human myometrial contractility assays *in*

vitro demonstrated a concentration-dependent reduction in spontaneous contraction and oxytocin-induced contraction by THG113 in pregnant and non-pregnant uterine tissue, but no reduction in $PGF_{2\alpha}$-induced contraction (268). A phase I clinical trial was completed with PDC31 (THG113) in healthy women with dysmenorrhea, and the compound was found to be safe with no dose limiting toxicity, and resulted in decreased intrauterine pressure and a reduction in pain (269).

Based on THG113, an improved peptidomimetic FP antagonist was designed, PDC113.824, which non-competitively binds to a site distinct from the $PGF_{2\alpha}$ FP binding site. PDC113.824 binding induces a small conformational change that increases the binding affinity to $G_{\alpha q}$ and decreases efficacy of binding to $G_{\alpha 12}$ (Figure 1.6). This results in the induction of biased signalling, increasing MAPK signalling through $G_{\alpha q}$ while decreasing Rho/ROCK signalling, cell contraction and cytoskeletal rearrangements. In mice, PDC113.824 prolongs delivery significantly in both LPS- and $PGF_{2\alpha}$-induced PTB, and delays normal birth past term, as well as inhibiting both spontaneous contraction and $PGF_{2\alpha}$-induced contraction assays (146).

1.6.2 IL-1β: role in parturition

So far, we have discussed the interweaving of pro-inflammatory and pro-contractile systems in human parturition, and the role of inflammation in uterine transition for labour. Of all the pro-inflammatory cytokines involved in these processes, IL-1β has the most pervasive influence in pregnancy and parturition. IL-1β is a member of the IL-1 cytokine family, consisting of 11 cytokines. There are two isoforms of IL-1, IL-1α and IL-1β (270). IL-1α and IL-1β are encoded from different genes, but bind the same receptors resulting in similar physiological effects. IL-1α is constitutively expressed and is recognized as a DAMP, as it is released during

sterile inflammation, for example, cell necrosis (271). IL-1β is not constitutive but inducible, and is transcriptionally regulated by DAMPs, PAMPs and other cytokines.

1.6.2.i Synthesis of IL-1β.

DAMPs or PAMPs interact with pattern recognition receptors (PRRs) for the generation of mature IL-1β, including TLRs and NOD-like receptors (NLRs). First, TLR activation results in IL-1β transcription. IL-1β is synthesized as pro-IL-1β, an inactive precursor that requires proteolytic modification into its mature and active form. DAMPs/PAMPs also interact with NLRs, which oligomerize and form the inflammasome complex through a series of protein-protein interactions, activating caspase-1. Caspase-1, a pro-inflammatory cysteine protease, is responsible for the cleavage of pro-IL-1β into its mature form, IL-1β. MMP-2 and MMP-9 have also been implicated in the processing of pro-IL-1β into active IL-1β independent of caspase-1 (267).

1.6.2.ii IL-1β signal transduction

IL-1β signals through a heterodimeric complex; the ubiquitously expressed functional ligand binding chain IL-1R1 binds IL-1 and complexes with its accessory protein (IL-1RAcP). IL-1R1 and IL-1RAcP have intracellular toll/interleukin-1 receptor homology (TIR) domains that interact. TIR domain interaction results in protein-protein interactions between myeloid differentiation primary response 88 (MYD88) and interleukin-1 receptor-associated kinase 4 (IRAK4), which stimulate a downstream cascade that results in the activation of transcription factors AP-1 and NFκB. The second IL-1 receptor, IL-1R2, also binds IL-1 ligand and IL-1RAcP but is lacking a TIR domain, functioning instead as a decoy receptor, thereby scavenging IL-1 without the production of signal transduction (272). Until recently, a ‘b’ isoform of the IL-1

accessory protein, labeled IL-1RAcPb, had only been identified in the central nervous system (273). Our group's recent publication demonstrated that IL-1RAcPb is expressed in the pregnant rat uterus, and its mRNA abundance increases at delivery (31).

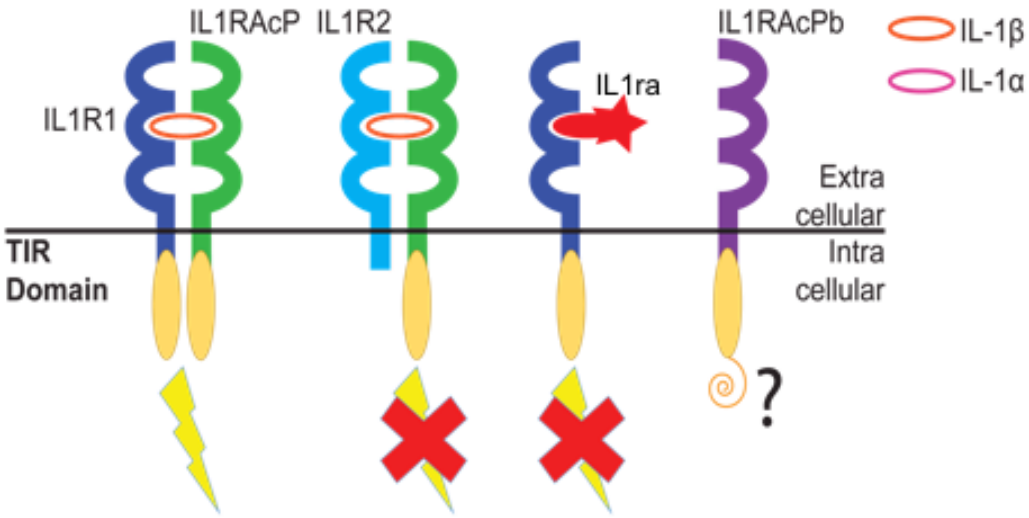

Figure 1.7. Regulation of IL-1 signal transduction: IL-1 receptors and accessory proteins, reproduced with permission from Dr. Barbara SE Verstraeten.

IL-1 is a potent cytokine, able to induce sizable biological responses at low concentrations. A maximum cellular response to IL-1 occurs at only 1% occupancy of the IL-1R1 receptors (274). Therefore, IL-1 activity must be highly regulated through negative feedback and involves the induction of endogenous anti-inflammatory mechanisms. The endogenous IL-1R antagonist (IL-1ra) competes with IL-1 ligand to bind IL-1R1, but does not result in signal transduction (275). High levels of IL-1ra in both secretory and intracellular forms are expressed endogenously; bioavailability of IL-1ra ligand is typically several thousand times higher than IL-

1 ligand (276). And, in addition to IL-1R2, soluble forms of IL-1R1 and IL-1RAcP also exist as decoy receptors to sequester IL-1 ligand without signal transduction or cellular response.

1.6.2.iii Uterine IL-1β in pregnancy and parturition

In human pregnancy at term labour, IL-1β is upregulated even without any presence of infection, in amniotic fluid (277, 278) and intrauterine tissues including myometrium, decidua, cervix and fetal membranes (27, 63, 279). Endogenous antagonist IL-1ra decreases in cervicovaginal fluid at spontaneous term labour onset (280). IL-1β has an influential role in the gene regulation of pro-inflammatory and pro-labour mediators in gestational tissues. A single hour of IL-1β stimulation upregulates 98 inflammatory genes in PHM1-41 uterine myocytes by at least 3-fold, including COX-2 (7.9-fold), NFκB (10.9-fold) and genes involved in extracellular matrix (ECM) remodelling, cell adhesion and angiogenesis (281). Microarray data of IL-1β-stimulated decidual cells demonstrated a significant upregulation of 350 transcripts, a downregulation of 78 transcripts, as well as the activation of a predicted 57 transcription factors and inhibition of 22 (282).

IL-1β is a potent stimulator of prostaglandin synthesis in all gestational tissues via COX-2 induction (98, 282-285). In addition, IL-1β induces upregulation of the $PGF_{2\alpha}$ receptor FP (97). Mice administered an intrauterine injection of IL-1β significantly increased mRNA expression of genes encoding for COX-2, FP, OTR, CX-43, MMP-1, 3, 9, IL-1β, IL-6, IL-8, CCL2 and IL-1R1 in the myometrium compared to vehicle-injected animals (286). RU486-induced rat deliveries exhibited increased uterine expression of IL-1R1, IL-1R accessory proteins AcP and AcPb, and decreased IL-1R2 (31). IL-1β treatment increased excitability of primary human myometrial cells, increasing basal calcium entry and spontaneous calcium transients

(287). It is then no surprise that IL-1 injection is sufficient to induce preterm birth in a range of animal models, such as the mouse (286, 288, 289), the rabbit (in combination with TNFα) (290), and the nonhuman primate (212).

1.6.2.iv IL-1β antagonists delay PTB

In the existing drug market, there are a series of competitive IL-1 receptor antagonists approved by the FDA for non-pregnancy related medical conditions like rheumatoid arthritis. However, these drug compounds are orthosteric antagonists that block all signal transduction from the receptor, resulting in complete inhibition of IL-1 signalling. IL-1 activation of IL-1R1 results in activation of a vast signalling network, inducing physiological interactions with a wide range of different roles in the cell. NFκB is only one of the transcription factors that IL-1 activates, and it regulates the expression of over 400 genes, many of which are important for normal biological function (291). In human pregnant myometrium, NFκB regulates at least 38 genes (292). Consequently, complete inhibition of IL-1R and all its downstream mediators may have detrimental effects. Furthermore, although IL-1-induced PTB in mice was prevented by treatment with endogenous IL-1ra (293), Kineret, one of the commercially available orthosteric IL-1R antagonists, did not successfully delay IL-1-PTB (286).

Rytvela, or 101.10, is an IL-1R non-competitive antagonist that allows for functional selectivity instead of complete inhibition of IL-1 signalling (294). 101.10 selectively inhibits signalling through AP-1 without affecting NFκB pathways (286). During the completion of this PhD dissertation, I collaborated with a research group at the Université de Montréal that measured the efficacy of 101.10 in mouse models of preterm birth induced by IL-1β, LPS and lipoteichoic acid (LTA) (manuscript included in the appendix). 101.10 significantly prolonged pregnancy in all three models and suppressed the expression of UAPs and pro-inflammatory

mediators in the mouse uterus. Moreover, a more recent publication by this group demonstrated that in addition to increasing gestational length, 101.10 prevented IL-1-induced fetal brain inflammation and improved fetal developmental outcomes (295).

1.6.3 IL-6: role in parturition

IL-6 is a complex immune mediator that is involved in a wide range of biological functions, including both local and systemic immune responses. The IL-6 cytokine family all have both pro- and anti-inflammatory actions. In the central nervous system, IL-6 is a critical cytokine in the acute phase response, with roles in fever induction, regulation of acute phase proteins, vascular permeability and leukocyte extravasation. Overproduction of IL-6 is linked to a range of pathologic diseases, including cancer and systemic autoimmune diseases like Crohn's, rheumatoid arthritis and lupus (296). IL-6 also however has an important role in implantation, pregnancy and parturition, and is produced in abundance by gestational tissues in preparation for labour (63). Abnormal IL-6 is associated with infertility, fetal loss and many other pregnancy disorders, as discussed in a review by Prins *et al.*, 2012 (297).

1.6.3.i IL-6 transcriptional regulation.

Two of the most important mediators involved in the transcriptional regulation of IL-6 are IL-1 and TNFα. IL-6 is encoded by the human *IL6* gene, and although the gene promoter has many regulatory elements the most important are AP-1, NFκB and NFIL-6. These three transcription factors are all involved in inflammation, and all three are inducible by both IL-1 and TNFα (292, 298, 299). IL-6 transcription may be cooperatively regulated, as multiple regulatory elements can synergize to induce greater levels of IL-6 induction. NFIL-6 and NFκB

individually both induced a 2-fold increase in IL-6 (as measured by luciferase activity), but together the transcription factors interacted with both promoter binding sites to induce over 40-fold increases in IL-6 (300). Additionally, all three transcription factors can interact and induce an even greater effect. AP-1, NFκB and NFIL-6 can form a complex and interact with the IL-6 promoter, resulting in nearly 300 times the level of IL-6 induction, much greater than the three regulatory elements separately, or even paired combinations (301).

1.6.3.ii IL-6 signal transduction

Like IL-1, IL-6 signalling is complex and signal transduction occurs as a result of multiple protein-protein interactions instead of a single ligand/receptor interaction. IL-6 receptor (IL-6R) has a ligand binding site but does not have a cytoplasmic domain for signal transduction, and glycoprotein 130 (gp130) has cytoplasmic domains but no ligand binding capabilities. IL-6 binds IL-6R, inducing homodimerization of gp130 and subsequent binding of IL-6R to gp130 to form the functional hexameric receptor complex. The receptor complex contains two IL-6 ligands, two IL-6Rs and two gp130 proteins (302). The homodimerization and activation of gp130 results in the mutual transactivation of tyrosine kinase JAK (Janus kinase). JAK phosphorylates tyrosine residues on gp130 cytoplasmic domains, which recruit proteins involved in two key signalling pathways (303). The phosphorylated gp130 segment recruits SHP-2, which is phosphorylated by JAK. Phospho-SHP-2 binds Grb-2 which is associated with a Ras-guanosine triphosphate (GTP), resulting in the activation of MAPK pathways terminating in activation of the transcription factor NF-IL-6. In the second pathway, the phosphorylated gp130 recruits STAT1 or STAT3 proteins, which are phosphorylated by JAK and form STAT heterodimers or homodimers, which translocate to the nucleus and bind to DNA response elements (296).

Similar to IL-1, both membrane-bound and soluble forms of the receptor complex exist biologically. IL-6 ligand can bind membrane-bound IL-6R and membrane-bound gp130 (mgp130) and induce the signal transduction pathways described above. Alternatively, IL-6 ligand can bind soluble IL-6R (sIL-6R) and sIL-6R can still bind and activate mgp130, termed trans-signalling. Membrane-bound gp130 is ubiquitously expressed, so IL-6 trans-signalling can occur anywhere. However, negative regulation of IL-6 activity in the form of decoy receptors also exists. Soluble gp130 (sgp130) will bind IL-6/sIL-6R complexes to prevent binding to mgp130. IL-6/sIL-6R/sgp130 complexes do not result in signal transduction (Fig. 1.8).

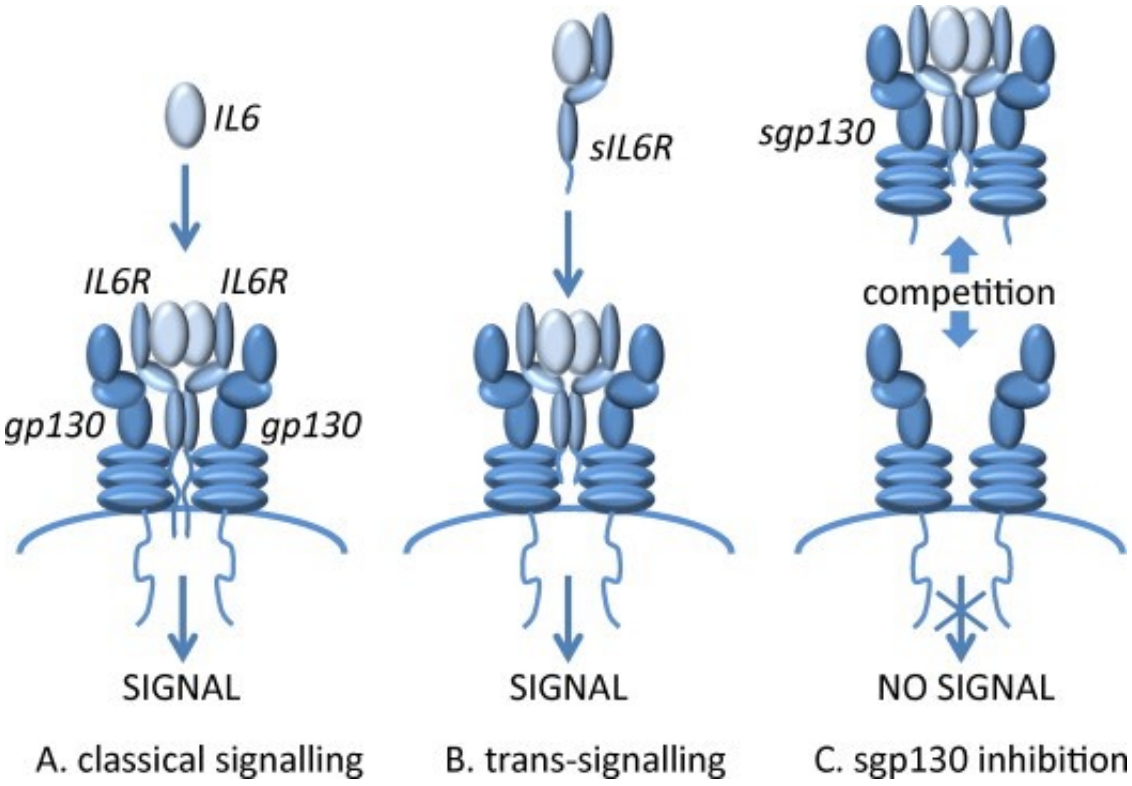

Figure 1.8. Regulation of IL-6 signal transduction: IL-6 receptor and gp130 interactions. Reprinted from the Journal of Reproductive Immunology, Vol 95, Authors Jelmer Prins, Nardhy Gomez-Lopez, and Sarah A Robertson, “Interleukin-6 in pregnancy and gestational disorders”, Pages 1-14, Copyright (2012), with permission from Elsevier (297).

1.6.3.iii Uterine IL-6 in pregnancy and parturition

There is still much to learn about the roles of IL-6 in pregnancy and parturition. We do know that the role of IL-6 is multifaceted, as atypical IL-6 levels are associated with a range of pregnancy disorders, from infertility and recurrent miscarriage to preeclampsia and preterm birth (297). Before and during labour, IL-6 output is increased in human myometrium, cervix and choriodecidua (63). IL-6 is measured in amniotic fluid in low concentrations beginning in the second trimester. At term, IL-6 in amniotic fluid increases from 399 to 4800 pg/mL with labour onset (304). Likewise, at term the soluble form of gp130 decreases in amniotic fluid, suggesting a diminishment in negative regulation of IL-6 (305). Human amnion and decidual cells stimulated by physiological concentrations of IL-6 (as measured in amniotic fluid at term) upregulate the production of prostaglandins (111). Fetal membrane explants stimulated with IL-6 upregulate the release of TNFα, IFNγ, CCL21 and IL-1β protein (Yin and Olson, unpublished), illustrating another positive feedback loop as the resulting IL-1β can induce further IL-6 production from both myometrium and fetal membranes. In the rat, IL-6 increases expression of uterotonic receptor OTR in the uterus (105). Mice with a null mutation for *Il6* (*Il6-/-)* deliver 24 hours later than mice with an IL-6 presence due to a 24-hour delay in the upregulation of UAPs, and normal birth timing is restored with exogenous IL-6 administration (306). Although *Il6-/-* mice deliver late and IL-6 levels are upregulated in LPS-induced preterm birth mouse models (286), IL-6 stimulation on its own is not sufficient to induce preterm labour (307). Nonetheless, IL-6 has been pursued as a diagnostic marker for preterm birth. Women who had spontaneous preterm births less than 35 weeks had significantly inflated IL-6 in their cervical fluid at 24 weeks of gestation (308), and elevated IL-6 in amniotic fluid in second trimester (309).

In addition to the effects of IL-6 in tissues and amniotic fluid, Robertson's group has demonstrated the importance of IL-6 in mediating T-cell population changes in late pregnancy in the mouse, contributing to the progression to parturition (310). IL-6 mediates $CD4^+$ T cell differentiation into Th17 cells, and $CD8^+$ T cell differentiation into $Foxp3^+$ T regulatory cells. Exogenous IL-6 administration restored T cell populations by 60% (310). Unlike IL-1, IL-6 does not stimulate uterine contraction (311). It is possible that the high levels of IL-6 produced by the intrauterine tissues at term are not acting on the uterine musculature itself, but on leukocytes or other cell types in the surrounding gestational tissues. Uterine myocytes released much greater IL-6 outputs when co-cultured with primary monocytes isolated from peripheral blood collected from pregnant women at term compared to the additive effect of each cell type cultured individually (312).

1.6.3.iv IL-6 antagonists delay PTB

Due to the multifaceted role of IL-6 in pregnancy and the 24-hour delay in birth in *Il6-/-* mice, inhibition of IL-6 also has potential as a PTB therapeutic. The involvement of IL-6 in numerous amplification pathways in preparation for labour suggests that suppression of this mediator may be sufficient to dampen this upregulation and prevent uterine transition. A human anti-IL-6R monoclonal antibody that inhibits both soluble and membrane bound forms of IL-6R, Tocilizumab (TCZ), is FDA-approved and currently in use as a therapy for rheumatoid arthritis (313). More anti-IL-6R compounds are currently in clinical trials, testing treatment efficacies for cancer and other systemic autoimmune diseases. In primary human amniotic epithelial cells, TCZ suppressed IL-6 induced PGE_2 release. MR16-1, another anti-IL-6 receptor antibody, effectively prolonged pregnancy in a LPS-induced PTB mouse model, increasing gestational length from 18.4 days to 19.8 days (314).

However, like IL-1, broad spectrum inhibition of all IL-6-mediated signalling could have detrimental effects in pregnancy. Allosteric modulation resulting in functional selectivity of IL-6 signal transduction represents the most promising path for future therapeutic development. An allosteric peptide targeting IL-6R, 633, is able to inhibit IL-6-induced STAT3 signalling without affecting Akt or MAPK pathways. In an LPS-induced PTB mouse model, 633 improved neonatal weight and survival, and suppressed pro-inflammatory gene expression in the uterus, fetal membranes and maternal peripheral leukocytes. IL-6-stimulated IL-1β expression was suppressed in the fetal membranes and caspase-1 and TNFα expression declined in the uterus (315).

Chapter 2
Hypotheses and Objectives

It is evident from the discussion throughout the background chapter that a better understanding of labour physiology, and especially the role of inflammatory mechanisms, is crucial. Nearly all we know about the involvement of inflammatory mediators such as IL-1β and $PGF_{2\alpha}$ in parturition has derived from animal models that may not relate to human physiology, or human *in vitro* studies examining each mediator in isolation. Yet the gene promoters of many pro-labour genes, including UAPs, contain response elements for many different inflammatory transcription factors such as NFκB, NFIL-6, AP-1 and STAT3. This infers transcriptional regulation by many different inflammatory pathways. In fact, studies have shown that NFκB, NFIL-6, and AP-1 can form a complex and interact with the *IL6* gene promoter to together induce much higher levels of IL-6 than their individual effects (301). Human amniotic fluid and intrauterine tissues at term exhibit elevated levels of a series of cytokines, chemokines, and prostaglandins. Such data, when combined, strongly suggest that neither IL-1β, $PGF_{2\alpha}$, or IL-6 act in isolation, but rather in concert to affect UAP and cytokine expression thereby amplifying the pro-inflammatory process that terminates pregnancy.

Furthermore, human uterine transition is a localized intrauterine inflammatory response involving paracrine interactions. While hundreds of genes change in the intrauterine environment in preparation for labour, no associated changes are measured in the peripheral blood (36). If these paracrine mediators are being released by intrauterine tissues to act on neighbouring cells and tissues, it is likely that the interactions between adjacent gestational tissues are consequential in labour transition as well. We have learned so much about parturition from studying one cell and one tissue at a time. Now we need to widen the lens and pursue a broader view of the cooperative interactions occurring in the intrauterine environment in preparation for parturition, which is ultimately the intended objective of this dissertation.

2.1 Hypotheses

2.1.1 $PGF_{2\alpha}$/IL-1β cooperativity in HMSMC

In HMSMC, both $PGF_{2\alpha}$ and IL-1β upregulate IL-6 and COX-2, and IL-1β also upregulates the $PGF_{2\alpha}$ receptor FP. Based on that information, we hypothesized that:

- $PGF_{2\alpha}$ upregulates IL-1β activity in HMSMC through stimulation of IL-1R1 and IL-1RAcPs.
- IL-1β upregulates its own activity in positive feedback through upregulation of its own receptor system.
- Comparable to IL-1β and $PGF_{2\alpha}$, IL-6 upregulates UAP expression in HMSMC.
- $PGF_{2\alpha}$ and IL-1β act cooperatively in the birth cascade to promote pro-inflammatory amplification of UAPs and cytokines in the uterus.
- $PGF_{2\alpha}$ and IL-1β cooperativity is unique to myometrium and is not observed in hFM.

2.1.2 Tissue cooperativity in co-culture

To the best of our knowledge, no studies have examined the in vitro interactions between fetal and maternal gestational tissues within this pro-inflammatory environment at term.

- Paracrine crosstalk between term intrauterine tissues, measured *in vitro* with co-culture of HMSMC and hFM explants, promotes pro-inflammatory expression and UAP induction in the birth cascade.
- Pro-inflammatory stimulation by $PGF_{2\alpha}$ or IL-1β in co-culture further upregulates pro-inflammatory expression and UAP induction.

- Co-culture pairs isolated before and after labour exhibit different levels of pro-inflammatory amplification: hFM from TL deliveries co-cultured with TNL HMSMC will induce more inflammatory amplification than TNL hFM paired with TNL HMSMC.

2.2 Study objectives

- To assess the roles of $PGF_{2\alpha}$ and IL-1β in upregulating expression of the IL-1 receptor system for labour.
- To examine closely the combined roles of $PGF_{2\alpha}$ and IL-1β in regulating the transition of the uterus for parturition using a validated primary human myometrium smooth muscle cell model in culture and human fetal membrane explants.
- To develop a co-culture model that enables us to study communication between maternal and fetal gestational tissues, using term HMSMC and hFM explants.
- To measure crosstalk between HMSMC and hFM in co-culture in this pro-inflammatory environment at term. Moreover, to use this model to examine the interaction between these tissues related to mediator expression/output and measure responsiveness to pro-inflammatory insult by $PGF_{2\alpha}$ or IL-1β treatment.

Chapter 3
Methodology

3.1 Culture methods

3.1.1 Primary human myometrium smooth muscle cells (HMSMC): isolation and culture

HMSMCs were isolated from lower uterine segment myometrial biopsies collected from non-labouring pregnant women undergoing elective caesarean sections at term (>37 weeks gestational age) at the Royal Alexandra Hospital in Edmonton, AB, using a validated and published protocol (45, 134, 135, 316). Ethics approval was received from the University of Alberta Research Ethics Board. Myometrial tissue was washed, dissected into small pieces, and dissociated using Hank's Balanced Salt Solution (HBSS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 2.0 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO, USA), 200 µg/mL deoxyribonuclease (DNase) I (Roche Diagnostics, Basel, Switzerland), and 1x antibiotic/antimycotic (100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B, HyClone, GE Healthcare Life Sciences, Mississauga, ON, Canada). Following 20 min of digestion at 37ºC with agitation, supernatant was discarded and replaced with 10 mL fresh dissociation medium. Incubation then continued for 2.5 h before the remaining solution was filtered through a 100 µm filter and centrifuged at 1,250 x *g* for 5 min. The resulting cell pellet was washed twice with Dulbecco's Modified Eagle Medium (DMEM, HyClone, GE Healthcare Life Sciences) before resuspension. The cell solution was then plated in a 25 cm^2 flask maintained at 37°C and 5% carbon dioxide (CO_2) in DMEM containing 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific) and 1x antibiotic/antimycotic as described above. After 15 min incubation at 37°C, the HMSMC-containing solution was moved to a new flask. Upon reaching confluence, cells were passaged using 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) according to the manufacturer-supplied protocol (Gibco, Thermo Fisher Scientific). At the 7^{th} passage, cells were plated into 6-well plates at a density of 2 x 10^5 cells/mL; once

reaching ~80-90% confluency they were starved in serum-free DMEM for 24 h before undergoing cell treatments. Additional validation information about the cell model and the number of passages used in these experiments can be found in the appendix.

3.1.2 Human fetal membrane (hFM) explants: extraction and culture

Intact placentas were obtained with consent from pregnant women (>37 weeks gestational age) undergoing elective caesarean sections at term (term non-labouring, TNL) or spontaneous vaginal deliveries (term labouring, TL). Following a protocol outlined by Yin *et al*. (317, 318), intact fetal membranes were removed from the placenta. Fetal membrane tissue explants were then excised using a 6 mm tissue punch and washed in HBSS. As demonstrated with histology in Yin *et al.* 2017 (317) and presented in Fig 3.1, the hFM explants contain intact amnion, chorion, and some *decidua vera*. This methodology is well characterized through the literature, and has been validated to maintain tissue structural and metabolic integrity for at least 72 h (78). Explants were plated with choriodecidua facing the myometrium to best represent tissue orientation *in vivo* in 12-well Netwell™ transwells (Corning Life Sciences, Tewksbury, MA, USA) in DMEM Nutrient Mixture F12 (HyClone, GE Healthcare Life Sciences) containing 15% FBS and 1x antibiotic/antimycotic. Fortunato *et al.* (1994) found that cytokine release in response to the tissue punch returned to baseline after 48 h in culture (319). Therefore, after the tissue punch hFM acclimated to culture for 48 h at 37°C and 5% CO_2 (with fresh medium every 24 h) before the start of the 6-24 h experimental treatments, well within the timeframe of tissue viability.

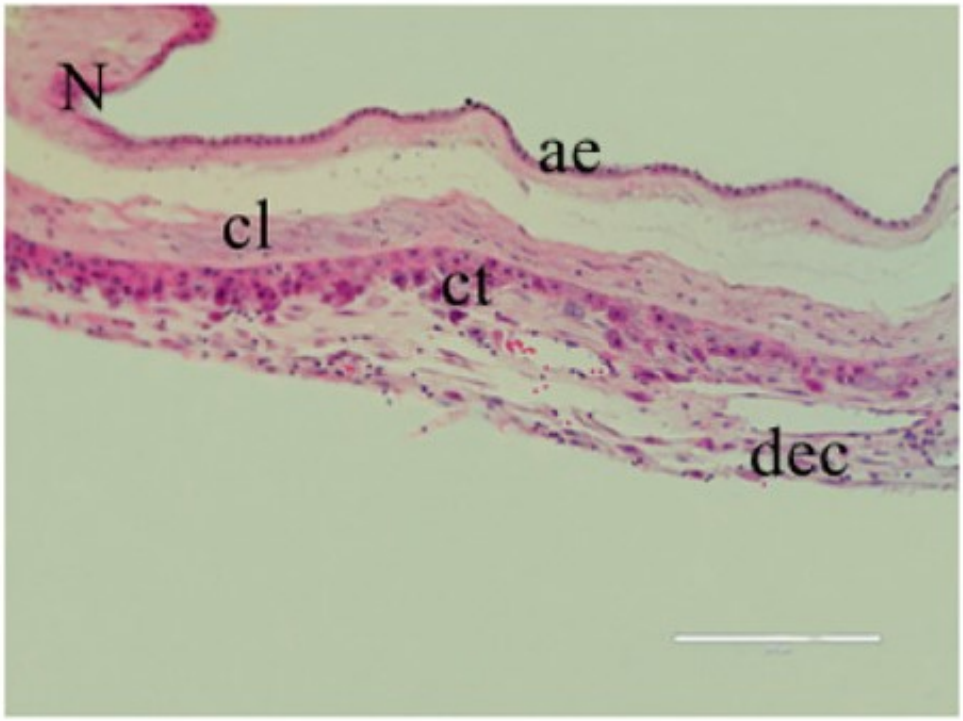

Figure 3.1. Haemotoxylin and Eosin staining of a fetal membrane explant. Human FM explants were fixed with methanol and stained with Haemotoxylin, then counterstained with Eosin. Labels: ae- amnion epithelium; cl- connective tissue layer; ct- chorionic trophoblast layer; dec- decidua. Scale bar: 200 μm. Reprinted from International Immunopharmacology, Vol 50, Authors Nanlin Yin, Hanbing Wang, Hua Zhang, Huisheng Ge, Bing Tan, Yu Yuan, Xiaofang Luo, David M Olson, Philip N. Baker, and Hongbo Qi, "IL-27 induces a pro-inflammatory response in human fetal membranes mediating preterm birth", Pages 361-369, Copyright (2017), with permission from Elsevier (317).

3.2 Treatment protocols

3.2.1 HMSMC single and double agonist treatment protocols

We first treated cells for 6 h or 12 h with single agonists, $PGF_{2\alpha}$ (0.1, 1 or 10 μM), IL-1β (1 or 5 ng/mL) or IL-6 (5 or 15 ng/mL) in order to demonstrate their effects alone and determine optimal concentrations for subsequent treatments ($PGF_{2\alpha}$: Cayman Chemical Company, Ann Arbor, MI, USA; IL-1β and IL-6: Millipore Sigma, Etobicoke, ON, Canada). All treatment solutions were diluted into serum-free DMEM containing 0.1% albumin (Sigma-Aldrich).

Then we treated HMSMC with two agonists to explore their effects in tandem (Fig. 3.2). The double agonist protocol group were treated according to one of the following schedules: 1) pre-stimulation with IL-1β for 24 h (5 ng/mL), a brief wash with HBSS, then subsequent 6 h treatment with $PGF_{2\alpha}$ (10 μM), or 2) pre-stimulation for 24 h with $PGF_{2\alpha}$ (10 μM), a brief wash with HBSS, then a second 6 h treatment of IL-1β (5 ng/mL). All treatment concentrations were selected after testing a range of concentrations and consulting the literature, and represent physiological concentrations in the cell milieu (134, 268, 320, 321).

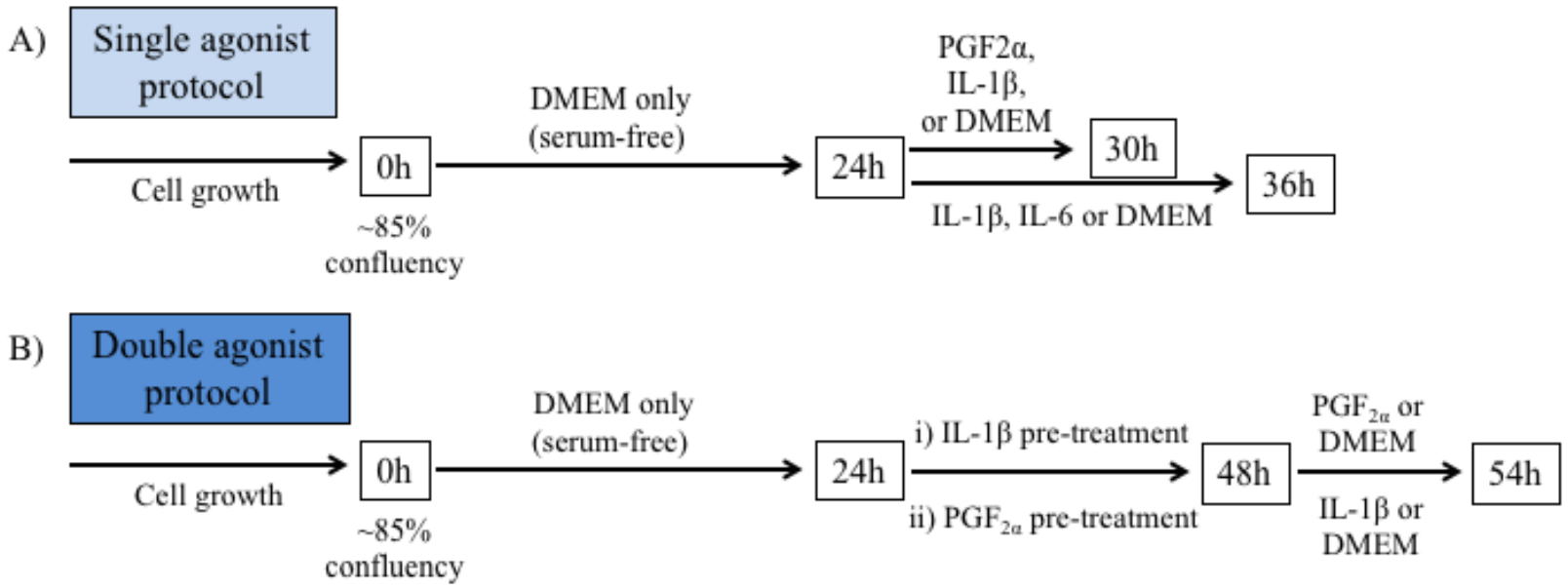

Figure 3.2. HMSMC single and double agonist treatment protocol.

3.2.2 HMSMC and hFM explant co-culture

Transwells containing hFM were placed into 12-well plates containing HMSMC with a mesh filter allowing for free passage between compartments via a shared culture medium (Fig. 3.3). These are heterologous tissue pairs, as it is not possible for primary HMSMCs to grow to confluence from matched myometrial biopsies within the viability period of the hFM tissues. TNL HMSMC were paired with TNL hFM explants, and TNL HMSMC were also paired with TL hFM explants for comparison. Each tissue was cultured in the same conditions in isolation (monoculture) as well as in co-culture. Monocultures and co-cultures were incubated i) in the absence of exogenous stimulation (serum-free DMEM F12 only), ii) with 10 μM $PGF_{2\alpha}$ (Cayman Chemical Company, Ann Arbor, MI, USA), or iii) 5 ng/mL IL-1β (Millipore Sigma, Etobicoke, Ontario, Canada) for 6 h or 24 h periods. These concentrations of agonists produce an optimal response in HMSMC and hFM (134, 320, 322).

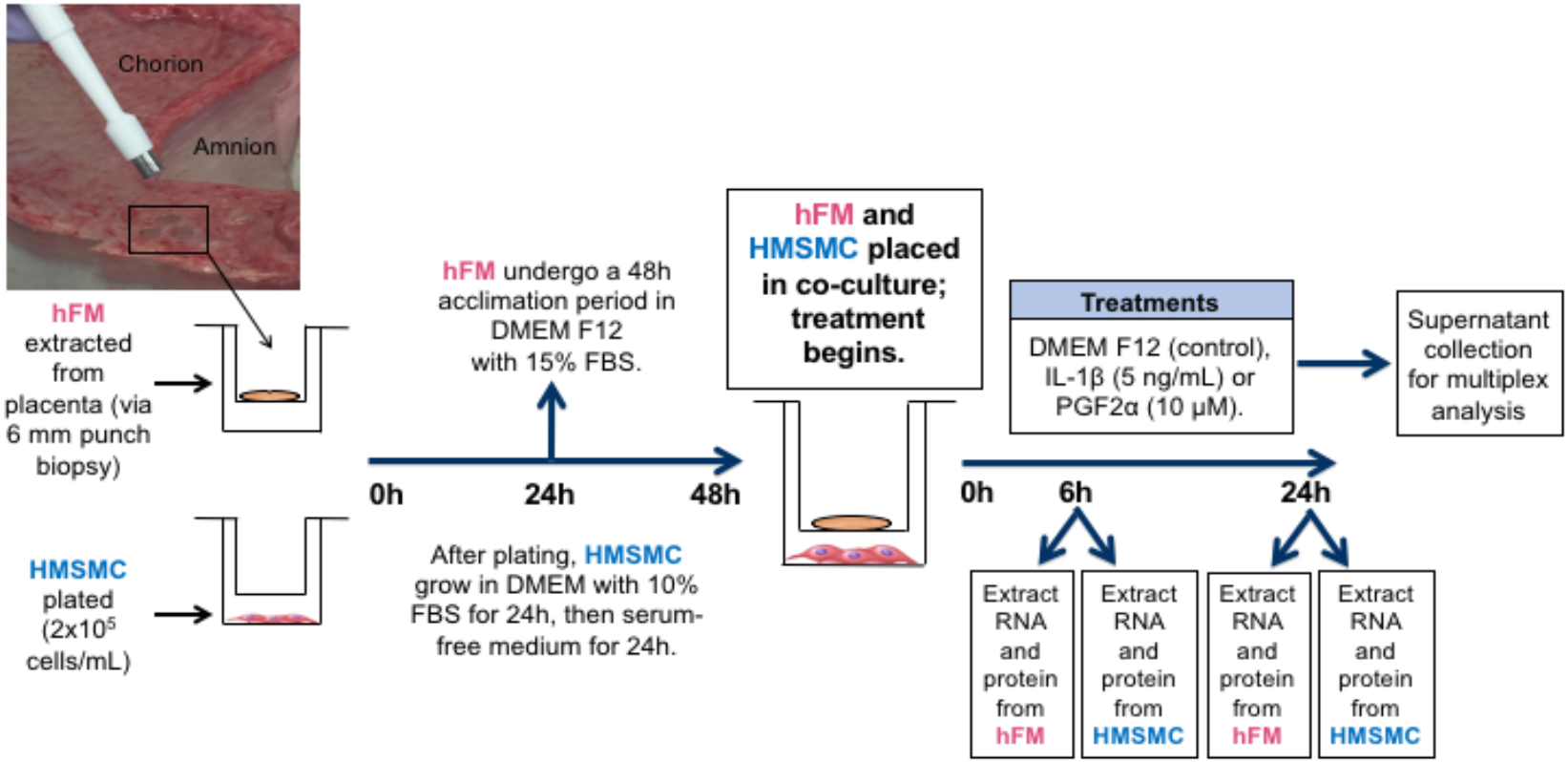

Figure 3.3. HMSMC and hFM co-culture protocol.

3.3 RNA experimental protocols

3.3.1 RNA isolation from HMSMC and hFM

Immediately following treatment, hFM tissues were removed, weighed and snap frozen, and HMSMCs were washed twice with phosphate-buffered saline (PBS, Gibco, Thermo Fisher Scientific) before RNA extraction in Trizol™ reagent (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). Trizol™ reagent (Ambion, Thermo Fisher Scientific) was used for RNA extraction following HMSMC and/or hFM treatments using the manufacturer-supplied protocol.

3.3.2 Quantitative polymerase chain reaction (PCR)

Five hundred ng of total RNA was reverse transcribed using qScript™ cDNA SuperMix (Quanta Biosciences, Beverly, MA, USA) also using the manufacturer-supplied protocol, resulting in a total reaction volume of 20 µL. The resulting complementary DNA (cDNA) was used in quantitative polymerase chain reactions (qPCR) (25 ng/µL). Human *IL6*, *COX2*, *FP*, *OTR*, *IL1R1*, *IL1R2*, *IL1RAcP*, *IL1RAcPb* and *GAPDH* primer sequences, product sizes and accession numbers are provided in Table 3.1. The annealing temperature of all primers was 60°C, except for IL-6, which was 58°C. To ensure amplification of template cDNA and not genomic DNA, all 3' and 5' primers were designed to span exon-exon boundaries, therefore impeding the primer binding to genomic DNA due to the intron presence.

Table 3.1. Primer sequences used in quantitative polymerase chain reaction (qPCR).

Target gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Size of PCR product (bp)	Accession number
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226	BC025925
IL6	CAAAGATGGCTGAAAAAGATGGA	CTGTTCTGGAGGTACTCTAGGT	118	NM_000600
COX2 (PGHS2)	GCTGGAACATGGAATTACCCA	CTTTCTGTACTGCGGGTGGAA	98	NM_000963
FP	TCCTGTATTTGTTGGAGCCCATTTCTGGTTAC	TCCATGTTGCCATTCGGAGAGCAAAAAG	115	BC112965
OTR	ATGGACAAGAACGAGTGTCGGTGAG	GAGTGGCATTCCTGGGTCATATGG	155	X64878
IL1R1	AGAGGAAAACAAACCCACAAGG	CTGGCCGGTGACATTACAGAT	106	KJ891450
IL1R2	TGGCACCTACGTCTGCACTACT	TTGCGGGTATGAGATGAACG	112	KJ892439
IL1RAcP	GGGCAGGTTCTGGAAGCA	GCTAGACCGCCTGGGACTTT	64	AH009309
IL1RAcPb	TCCAAGCACCGAGGGAAGT	AGGTGATTCTCTCCTTCACAGTAGGT	71	FJ998418

Each 20 µL reaction was run in duplicates and included 1 µL of cDNA, 10 µL of 2x PerfeCTa SYBR Green FastMix for iQ (Quanta Biosciences), 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer and 8 µL water. With the use of *i*Cycler IQ technology and software (Bio-Rad Laboratories, Hercules, CA, USA) 2-step quantitative PCR was completed under the following conditions: 10 min at 95°C, 45 cycles of 15 s at 95°C and 1 min at the annealing temperature. Following amplification, melt curve analysis was performed for each plate to ensure that amplification of non-specific products did not occur. PCR products from the primers used in this study have been confirmed previously in our lab by gel electrophoresis followed by sequencing to verify amplification of the correct products. Standard curves for target genes and *GAPDH* were generated by serial dilutions of cDNA samples and analyzed with iCycler IQ software (Bio-Rad Laboratories). The amplification efficiency for each primer set was determined manually by converting the slope of the standard curve using the algorithm $E = 10^{-1/\text{slope}}$ in a Microsoft Excel spreadsheet (Microsoft Corporation, Redmond, WA). The mean threshold cycle for each gene was calculated from duplicate reactions, then corrected for the efficiency of the reaction and expressed relative to a vehicle-treated control sample for each experiment. Target gene levels were then expressed relative to *GAPDH* levels using the following formula (323): $Ratio = \frac{E_{FP}^{\Delta Ct(Control-Sample)}}{E_{GAPDH}^{\Delta Ct(Control-Sample)}}$. GAPDH is very consistently expressed in HMSMC, remains unchanged with treatment, and has been selected as the housekeeping gene for analysis of myometrial tissue and HMSMC in previous publications (94, 134, 324).

3.4 Protein assay protocols

3.4.1 Analysis of cell lysates

3.4.1.i Extraction of cell lysates from HMSMC and hFM

Following treatment, HMSMC were washed twice with PBS and placed on ice on a shaker for 15 min with radioimmunopreciptation assay (RIPA) buffer containing 0.05% Tris, 0.01% EDTA, 0.001% Triton-X-100, 0.005% phenyl methane sulfonyl fluoride (PMSF), and 1x Halt^{TM} protease inhibitor cocktail (Thermo Fisher Scientific). Total cell lysates were then collected using a cell scraper and centrifuged at 4°C for 10 min at 12,000 x *g* to remove cell debris. HFM tissues were removed, weighed, and snap frozen in liquid nitrogen. Tissue samples were crushed using a mortar and pestle, placed in RIPA buffer, and homogenized through high speed shaking using the TissueLyser II (Qiagen, Hilden, Germany). The cell lysate is then collected into a new tube and centrifuged at 4°C for 10 min at 12,000 x *g* to remove cell debris. Total protein concentrations (for samples from both tissues) were calculated using Precision Red Advanced Protein Assay Reagent (Cytoskeleton Inc, Denver, CO, USA) using a Nanodrop 1000 spectrophotometer system (Thermo Fisher Scientific).

3.4.1.ii Western blot protocol

Total protein (50 μg) from each sample was combined with 1x loading buffer (250 mM Tris-hydrochloric acid (HCl) containing 4% sodium dodecyl sulfate (SDS), 10% glycerol, 2% β-mercaptoethanol, and 0.002% bromophenol blue) and heated for 5 min at 95°C. Protein lysates were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using polyacrylamide gels containing acrylamide and bisacrylamide, and transferred to nitrocellulose membranes by electrophoretic transfer. Membranes were incubated at room temperature for 2 h with Bløk fluorescent blocking buffer (EMD Millipore, Etobicoke, ON, Canada) before incubation with specific primary antibodies: anti-COX-2 at 1:1000 (sc-1745, Santa Cruz Biotechnology, Dallas, TX, USA), anti-FP at 1:1000 (Item #101802, Cayman Chemical Company), anti-OTR at 1:1000

(sc-33209, Santa Cruz Biotechnology), and anti-GAPDH at 1:5000 (PA1-987, Pierce Protein Biology, Thermo Fisher Scientific). Membranes were washed 3 times with filtered PBS containing 0.1% Tween 20 (Sigma-Aldrich) and incubated for 45 min with 1:2500 secondary IRDye 680LT or 800CW antibody (LI-COR Biosciences, Lincoln, NE, USA) at room temperature. COX-2, FP, OTR, and GAPDH were then detected and quantified using the Odyssey LI-COR Biosciences Infrared Imaging System and application software V3.0 (LI-COR Biosciences). Relative protein expression levels were calculated by obtaining a ratio of the target protein to GAPDH band intensities via densitometry and expressed relative to a vehicle-treated control sample for each patient group.

3.4.2 Analysis of cell culture supernatants

3.4.2.i Multiplex assay

Supernatants were collected at the end of treatment and pooled with their experimental duplicate, then immediately stored at -80 °C. The Bio-Rad custom human cytokine multiplex kits were used as per manufacturer's instructions with a Bio-Plex® 200 suspension array system and corresponding Bio-Plex® 200 software, version 6.1 (Bio-Rad Laboratories). Briefly, magnetic beads coated with antibodies targeting the cytokines of interest were added to each well of the 96-well plate and incubated with supernatant samples and cytokine standards provided by the manufacturer. The beads were incubated with biotinylated detection antibodies followed by streptavidin tagged with a phycoerythrin fluorescent reporter, which strongly binds to the biotinylated detection antibody. Beads were resuspended in assay buffer for quantification of analytes using the Luminex-based reader in the Bio-Plex system. Each analyte's concentration was calculated by measuring the median fluorescence intensity (MFI) signal of the phycoerythrin

fluorescent reporter per bead (at least 50 beads per analyte). MFI signals were compared to a standard curve generated by the manufacturer-supplied cytokine standards. The quantified concentration outputs were then normalized for each tissue to account for any sample to sample variability. A ratio of total explant weight was calculated, with the median explant weight per patient group set as 1. Fetal membrane cytokine outputs were normalized to this ratio to account for any weight discrepancies. HMSMC outputs were normalized to a ratio of cell density (2×10^5 cells/mL).

3.5 Statistical Analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad Prism, La Jolla, CA, USA). Results are expressed as mean ± SEM, and data were $\log_{10}$-transformed to conform to normality.

3.5.1 HMSMC (single tissue)

3.5.1.i Single comparison analysis: independent t-test

Data sets comparing only two treatment groups were analyzed using the independent samples t-test on $\log_{10}$-transformed data. Significance is indicated with asterisks, $*p<0.05$, $**p<0.01$, $***p<0.001$.

3.5.1.ii Multiple comparisons: one way analysis of variance (ANOVA)

Data sets comparing the means of multiple cell culture treatment groups were $\log_{10}$-transformed and analyzed by one-way analysis of variance (ANOVA). When significance was achieved ($p<0.05$), Tukey's post-hoc test followed to discriminate between treatments. Different letters denote significant differences at $p<0.05$. If there is overlap between letter groups (such as

AB versus *A*), post-hoc differences between those two groups are not significantly different. If no overlap is depicted between letters (such as *A* versus *B* and *C*), then the group is significantly different from all other groups.

3.5.2 Co-culture (multiple tissues)

3.5.1.i Multiple comparisons: two-way analysis of variance (ANOVA)

As there were two independent variables included in the co-culture model, culture condition (monoculture versus co-culture) and treatment (time in culture or exogenous inflammatory stimulus), the data sets were analyzed by two-way ANOVA. The significance of the main effects, culture and treatment conditions, are presented directly in the figures on the graph legend (referring to the y-axis) or below the x-axis, respectively, $*p<0.05$, $**p<0.01$, $***p<0.001$. In very few cases, a significant interaction was identified between the main effects. In these circumstances, Bonferroni post-hoc testing was performed to discriminate between groups, and the degree of significance was depicted by stars over the applicable bars ($*p<0.05$, $**p<0.01$, $***p<0.001$). In most figures, no significant interaction was identified between the main effects, and therefore only the statistical outcomes of the main effects are presented.

For multiplex outputs, when the culture condition (monoculture vs co-culture) main effect was found to reach significance ($p<0.05$), Bonferroni post-hoc testing was used to indicate whether both monoculture groups were significantly different from the co-culture group. If the co-culture output was significantly higher than both the HMSMC output and hFM output individually, the group's output was designated as synergistic upregulation.

Chapter 4
Combined effects of $PGF_{2\alpha}$ and IL-1β on IL-6 and COX-2 expression in HMSMC: an example of inflammatory amplification.

Portions of chapter four of this thesis are included in a manuscript currently under review at the journal Biology of Reproduction. KB Leimert, X Fang and DM Olson designed and planned the experiments, and the manuscript was written by KB Leimert and DM Olson. HMSMC culture was performed by KB Leimert and R Nemati and hFM culture by A Messer and KB Leimert. Quantitative PCR was performed by KB Leimert, BSE Verstraeten, A Messer, R Nemati and K Blackadar. Protein assays (Western Blotting, multiplex) and statistical analysis was completed by KB Leimert. SA Robertson and S Chemtob edited and gave final approval of the manuscript, and all authors approved the final manuscript.

4.1 Introduction

The core tenet of human physiology is centered on maintaining homeostatic balance or equilibrium of the body's internal environment. However, pregnancy and parturition force uterine physiology to deviate from normal homeostasis to enable extensive transitioning over a short time period. Positive feedback mechanisms are involved in the transition from uterine quiescence to labour because maintenance of pregnancy beyond term gestation (>40 weeks) compromises the health of the mother and her fetus(es).

Birth is a complex physiological event; it has recently been shown that 471 (33) and 796 (35) genes change in expression (increase or decrease) in preparation for labour in the human myometrium and choriodecidua, respectively. We refer to the physiological change from the state of pregnancy to the state of parturition as *uterine transitioning*. As a result, the uterus becomes *activated* to perform the physiology of labour. During pregnancy, a high progesterone to estrogen ratio supports a state of growth and myometrial relaxation (201). Near the end of gestation, there is functional withdrawal of progesterone signalling in humans (220) and an upregulation of uterine activation proteins (29) regulated by an increase in estrogen and pro-inflammatory mediators. There are many UAPs that contribute to uterine activation; nine of these that increase or decrease at term were studied by Arthur *et al.* (95). Of these, we routinely track the mRNA expression of three genes and their proteins that increase at term as markers of activation: COX-2, an inducible enzyme catalyzing a key intermediate step in the synthesis of prostaglandins (30), as well as the $PGF_{2\alpha}$ receptor FP (29) and the oxytocin receptor OTR (325), receptors for uterotonic contractile stimulators.

Parturition is an inflammatory event; without the presence of intrauterine infection (26, 27) pro-inflammatory cytokines, chemokines, prostaglandins and their receptors (29-31) increase

in expression near to parturition. Stimulated by DAMPs released by the maturing fetus, ageing placenta and increasingly physiologically stressed uterus (28), their consequent 'inflammatory load', as defined by Talati *et al.*, steadily increases throughout the gestational period. Parturition occurs when pro-inflammatory mediators are upregulated and amplified until their signals exceed a threshold level whereby they stimulate functional progesterone withdrawal and complete uterine transition to its activated state for labour (226).

Two very powerful mediators exert considerable control over expression of uterine activation proteins in human myometrium, interleukin (IL)-1β and $PGF_{2\alpha}$. IL-1β is a pro-inflammatory cytokine that promotes the expression of numerous pro-labour genes, regulates UAP expression and amplifies the pro-inflammatory response. *Rytvela* (101.10), an allosteric IL-1 receptor antagonist, prolongs gestation in mouse models of preterm birth induced by IL-1β, LPS, and lipoteichoic acid (LTA) (286) and improves neonatal and fetal developmental outcomes (295). IL-1β induces many pro-inflammatory cytokines and chemokines, including IL-6 (326, 327). Mice with a null mutation in *Il6* deliver 24 h later than wild-type mice due to a 24 h delay in the upregulation of UAPs (306). Among other pro-inflammatory cytokines, IL-1β upregulates COX-2 expression resulting in increased prostaglandin synthesis (99, 100).

Prostaglandins have been described as the 'triggers' of labour (245) as they increase in abundance in gestational tissues and fluids when approaching parturition (180-182, 184, 242-244), the inhibition of their synthesis delays birth (187, 245), and exogenous prostaglandin treatment initiates contraction of the myometrium (139-141). $PGF_{2\alpha}$ is a key signalling mediator in parturition as it is involved not only in stimulating uterine contraction but also in mediating uterine transition through the regulation of UAP expression and amplification of pro-

inflammatory cytokine and chemokine production (134, 135). Moreover, allosteric modulation of the FP receptor delays preterm birth in both mice and sheep (145, 146).

Nearly all we know about the involvement of IL-1β and $PGF_{2\alpha}$ in parturition has derived from studies examining each mediator in isolation. Yet the human $PGF_{2\alpha}$ receptor, FP, gene promoter encoding *PTGFR* contains four NFκB transcription factor binding sites and two NF-IL-6 binding sites, suggesting transcriptional regulation of FP by both IL-1β and IL-6 (96, 97). Such data strongly suggest that neither IL-1β nor $PGF_{2\alpha}$ act in isolation, but rather, in concert to affect UAP and possibly cytokine expression thereby amplifying the pro-inflammatory process that terminates pregnancy.

4.2 Objective

The intent of the present study was to examine closely, and for the first time, the combined roles of $PGF_{2\alpha}$ and IL-1β in regulating the transition of the uterus for parturition using a validated primary human myometrium smooth muscle cell model in culture and human fetal membrane explants. We hypothesized that $PGF_{2\alpha}$ and IL-1β act cooperatively in the birth cascade to promote pro-inflammatory amplification of UAPs and cytokines in the uterus.

4.3 Results

4.3.1. Regulation of mRNA abundance of IL6 and UAPs.

We tested a series of agonist concentrations for $PGF_{2\alpha}$ and IL-1β on UAPs in HMSMC to determine the concentration at which they achieved their maximum effect to be used in subsequent tests of both agonists together. $PGF_{2\alpha}$-stimulated a concentration-dependent increase

in *IL6* and *COX2* abundance after 6 h, resulting in an 8.8- and 5.9-fold upregulation, respectively (Fig. 4.1.). From this, we selected 10 μM as our $PGF_{2\alpha}$ concentration for subsequent tests.

IL-1β treatment also induced a dose-dependent increase in *IL6* and *COX2* mRNA by 41.9- and 28.9-fold, respectively (Fig. 4.2). We selected 5 ng/mL as the IL-1β concentration to use in the subsequent dual treatment studies, as this concentration demonstrated the maximal effect. $PGF_{2\alpha}$ and IL-1β had opposite effects on *FP* mRNA expression: 10 μM $PGF_{2\alpha}$ downregulated *FP* from 1 to 0.44 whereas 5 ng/mL IL-1β significantly increased *FP* mRNA expression 3-fold. *OTR* mRNA expression did not respond to $PGF_{2\alpha}$ at any concentration but decreased from 1 to 0.44 in response to 5 ng/mL IL-1β (Fig. 4.1, 4.2).

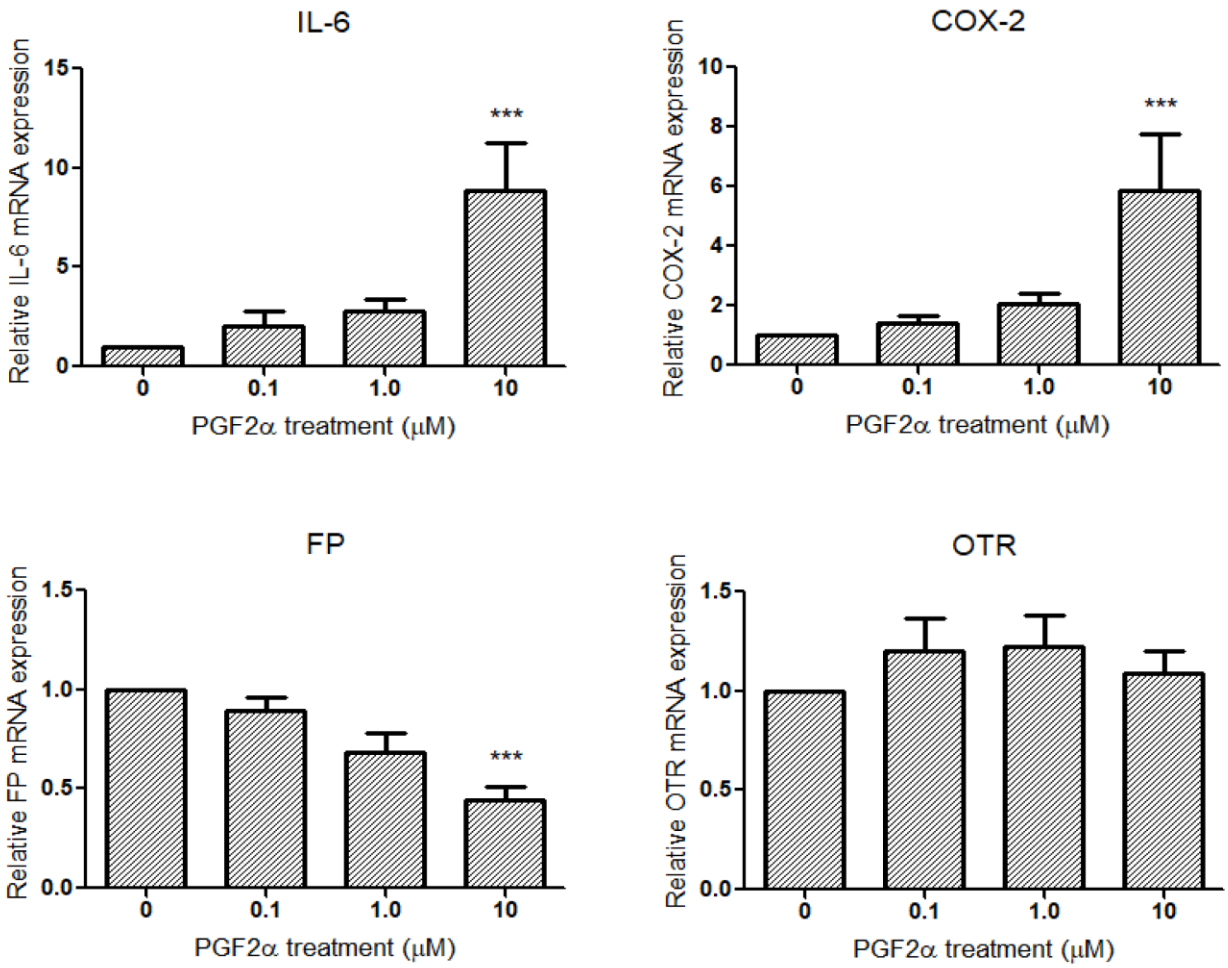

Figure 4.1. $PGF_{2\alpha}$ regulates HMSMC mRNA expression of pro-inflammatory cytokine *IL6* and UAPs *COX2* and *FP*. HMSMC were stimulated for 6 h with $PGF_{2\alpha}$ (0.1, 1 or 10 μM). Data are presented as relative change (x-fold) from control values, mean ± SEM. N=7 patients. One-way ANOVA on log-transformed data followed by Tukey post-hoc analysis, *p<0.05, **p<0.01, ***p<0.001.

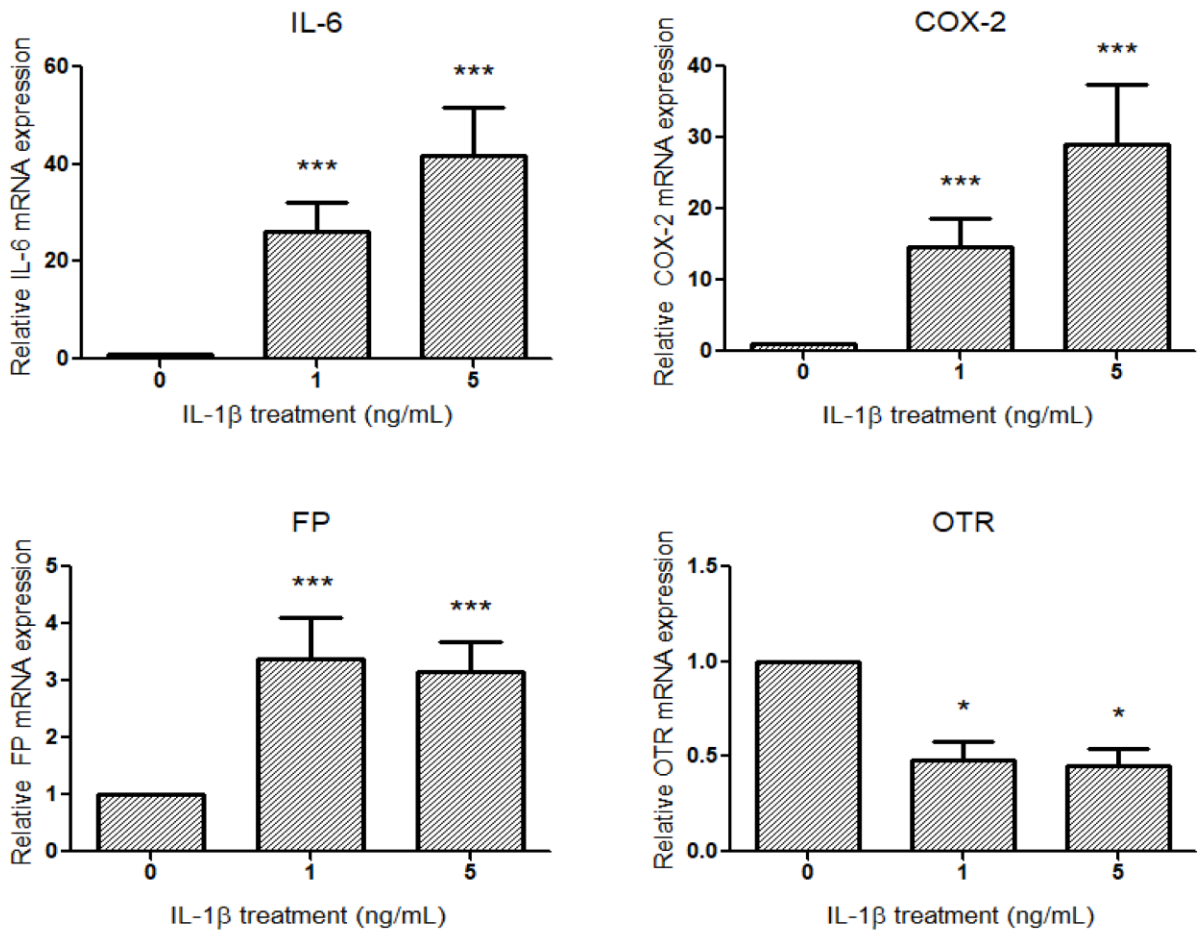

Figure 4.2. IL-1β regulates HMSMC mRNA expression of pro-inflammatory cytokine *IL6* and UAPs *COX2*, *FP* and *OTR*. HMSMC were stimulated for 6 h with IL-1β (1 or 5 ng/mL). Data are presented as relative change (x-fold) from control values, mean ± SEM. N=5 patients. One-way ANOVA on log-transformed data followed by Tukey post-hoc analysis, *p<0.05, **p<0.01, ***p<0.001.

4.3.2. Regulation of the IL-1 receptor system.

In another study, we reported that IL-1β administration to pregnant rats upregulated the IL-1 receptor and accessory protein system in the uterus near term (31). We therefore tested the effects of $PGF_{2\alpha}$ and IL-1β on the IL-1 receptor and accessory protein in HMSMC. After 6 h of $PGF_{2\alpha}$ treatment at 10 μM, mRNA abundance of *IL1R1 and IL1R2* increased 3.4-fold and 4.4-fold, respectively (Fig. 4.3). *IL1RAcP* mRNA increased 1.35-fold (not significant (NS)), while *IL1RAcPb* reached a maximal increase of 4.3-fold at 0.1 μM $PGF_{2\alpha}$. IL-1β also regulated mRNA abundance of the IL-1 receptor system. While IL-1β increased both *IL1R1* and *IL1R2* by 2.6-fold (NS), a significant 7.7- and 11-fold increase in mRNA abundance of *IL1RAcP* and *IL1RAcPb* was elicited (Fig. 4.4).

When interpreting this data, it is important to take into account the basal myometrial expression differences in *IL1R1* and *IL1R2*. A single PCR plate was assayed containing HMSMC samples isolated from nine myometrial biopsies collected from women undergoing elective caesarean sections at term. The mean threshold cycle measurement of *IL1R1* was 19.85 ± 0.24 and *IL1R2* was 34.22 ± 0.70. The standard curve included on the plate involved three-fold serial dilutions of cDNA resulting in a 1.5x change in threshold cycle per dilution. Assuming that the dilutions continue to follow a linear trajectory, we can approximate that HMSMC contain a basal abundance of *IL1R1* mRNA that is 0.6 million times higher than *IL1R2* abundance, with a difference of 15 cycles between the two genes and 1.5x change in threshold cycle per 3x dilution. Although we measured upregulation of relative mRNA abundance of both *IL1R1* and *IL1R2* with $PGF_{2\alpha}$ and IL-1β stimulation, HMSMCs exhibit a basal pro-inflammatory dominance with much higher abundance of *IL1R1*/*IL1R2*.

Unlike $PGF_{2\alpha}$ and IL-1β, stimulation of HMSMC with IL-6 (at 5 or 15 ng/mL) did not substantially alter mRNA expression of UAPs *COX2*, *FP* or *OTR*, or the IL-1 receptors and accessory proteins (Fig. 4.5A) thereby demonstrating the specificity of the effects with $PGF_{2\alpha}$ and IL-1β. IL-6 stimulation did result in a small upregulation in *IL1β* mRNA expression, but IL-1β protein outputs into cell culture supernatant showed no change (Fig 4.5B).

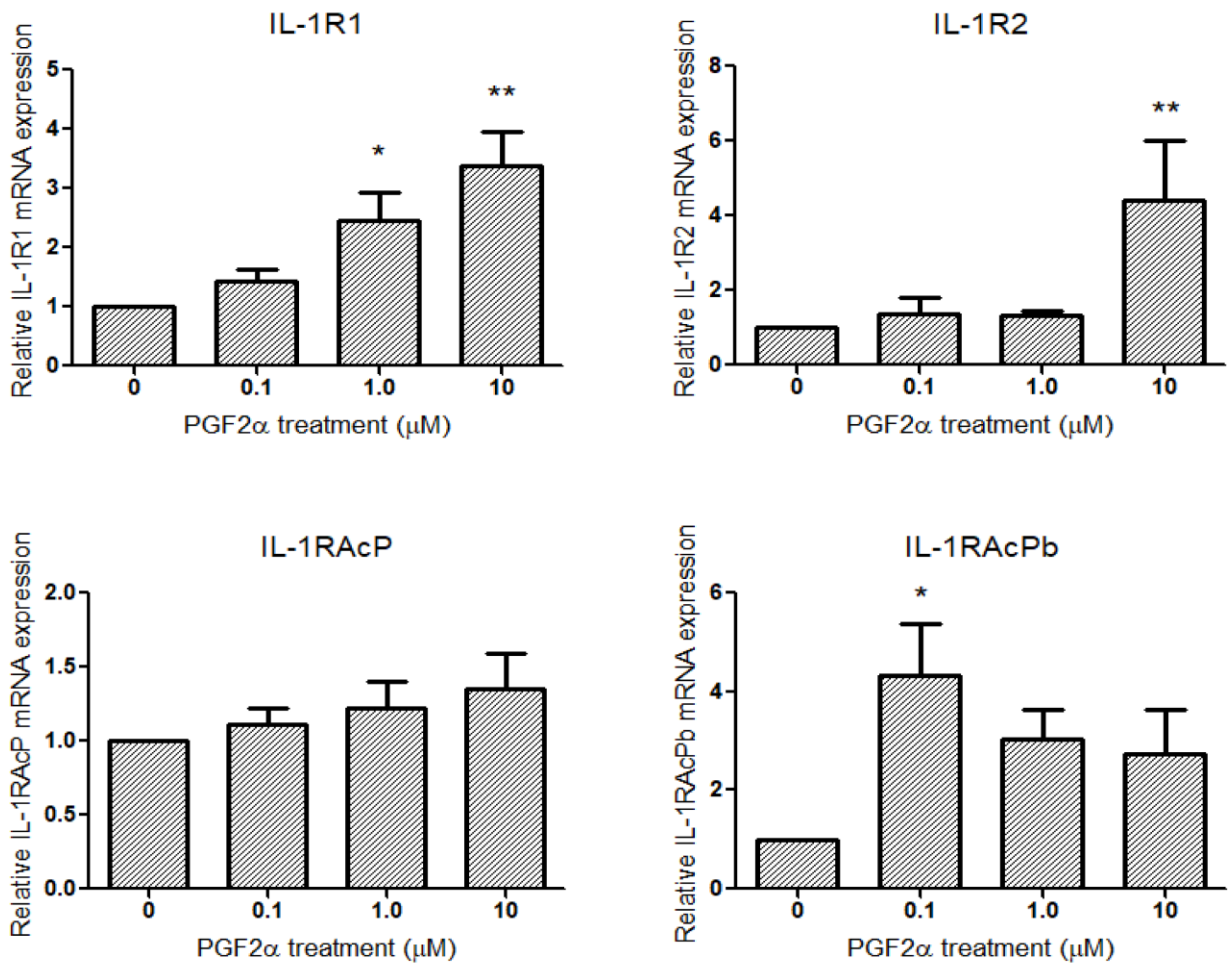

Figure 4.3. $PGF_{2\alpha}$ regulates HMSMC mRNA expression of the IL-1 receptor system. HMSMC were stimulated with $PGF_{2\alpha}$ (0.1, 1 or 10 μM) for 6 h. Data are presented as relative change (x-fold) from control values, mean ± SEM. N=7-9 patients. One-way ANOVA on log_{10}-transformed data followed by Tukey post-hoc analysis, *p<0.05, **p<0.01, ***p<0.001.

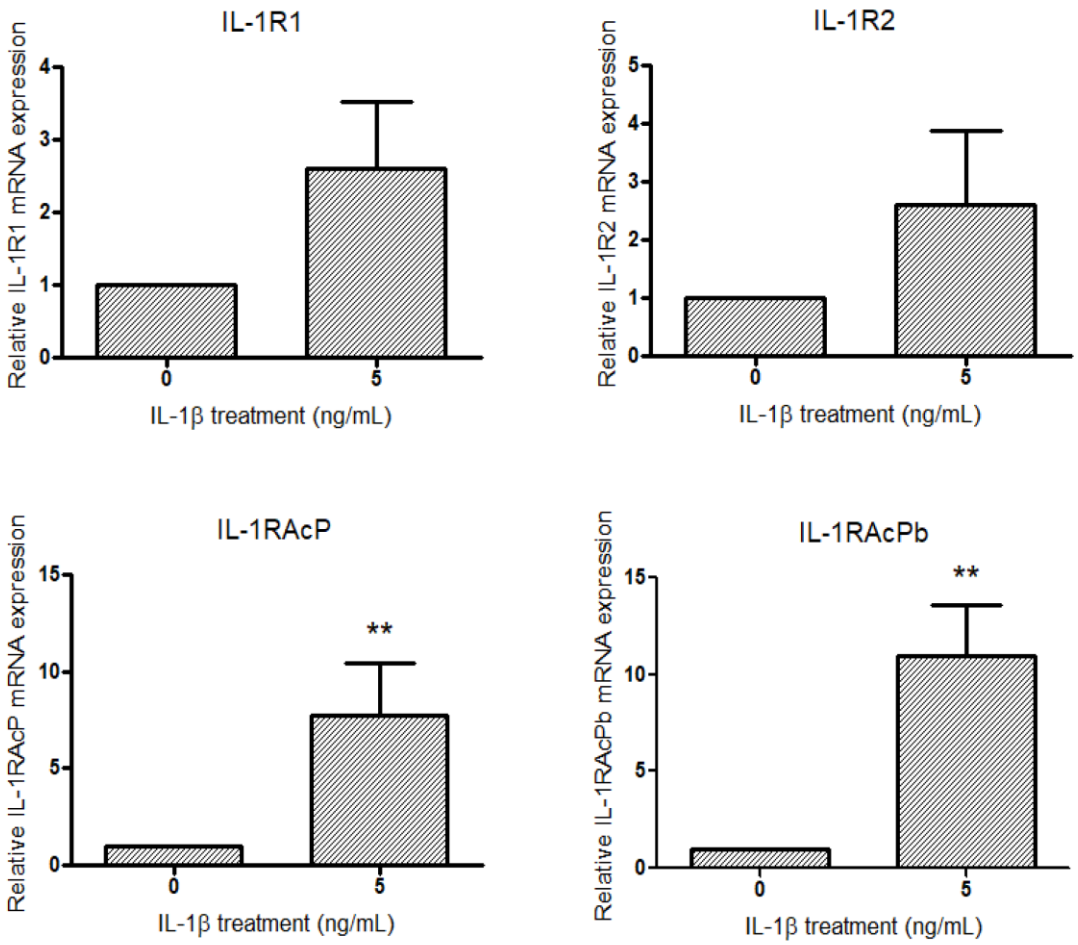

Figure 4.4. IL-1β regulates HMSMC mRNA expression of the IL-1 receptor system. HMSMC were stimulated with 5 ng/mL IL-1β for 12 h. Data are presented as relative change (x-fold) from control values, mean ± SEM. N=7-9 patients. Independent samples t-test; *p<0.05, **p<0.01, ***p<0.001.

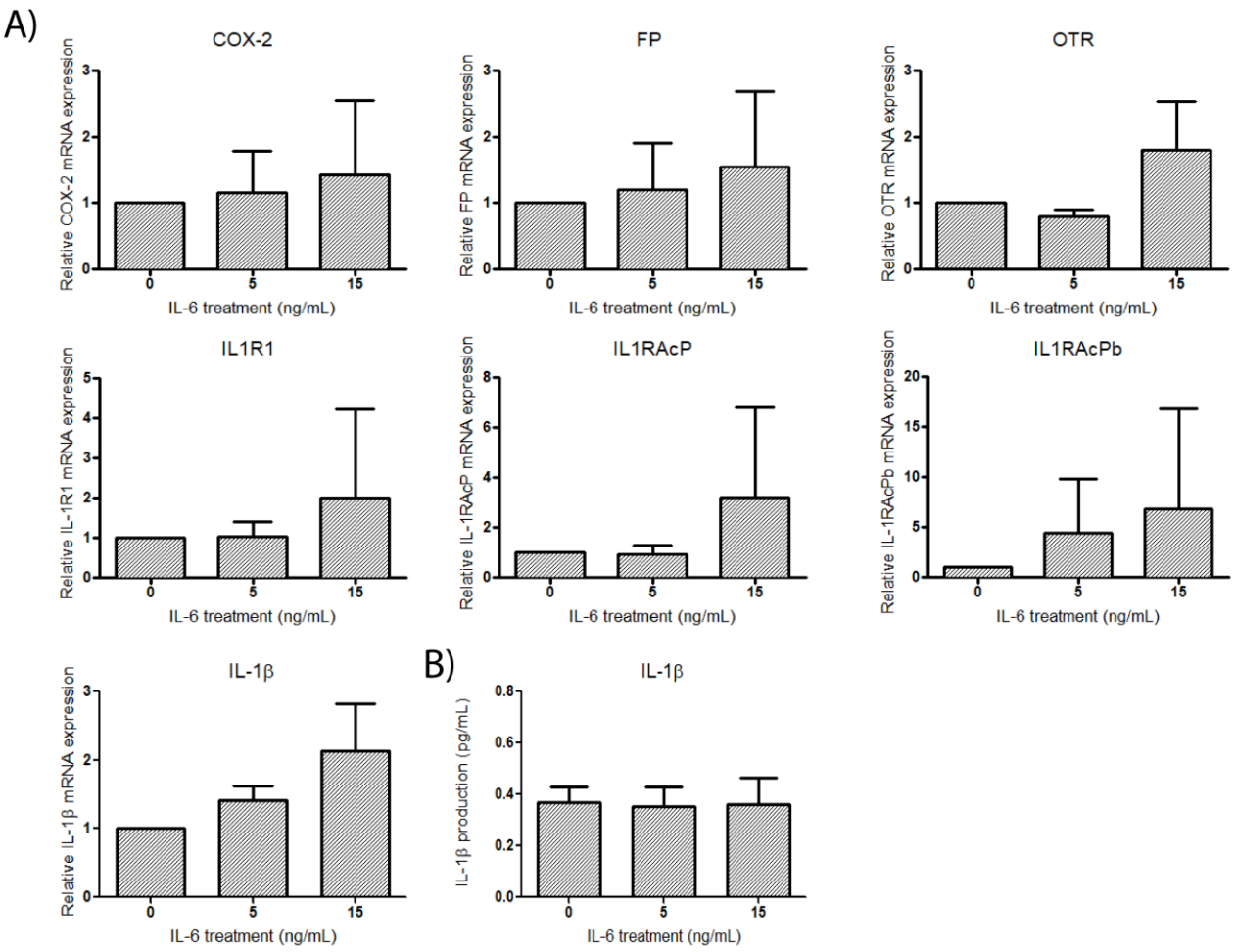

Figure 4.5. IL-6 treatment does not alter HMSMC mRNA expression of UAPs, *IL1β* or the IL-1 receptor system. HMSMC were stimulated for 12 h with IL-6 (5 or 15 ng/mL). **(A)** Target mRNA data are presented as relative change (x-fold) from control values, mean ± SEM, n=6-9. **(B)** IL-1β protein levels were measured in supernatants via multiplex, presented as mean ± SEM concentration output (pg/mL), n=4. One-way ANOVA was performed on $\log_{10}$-transformed data followed by Tukey post-hoc analysis, $p>0.05$.

4.3.3. Sequential treatments of HMSMC with IL-1β and $PGF_{2\alpha}$.

Since it is likely that *in vivo* $PGF_{2\alpha}$ and IL-1β act in concert and each is a powerful stimulant of IL-6 mRNA and protein expression, we systematically explored this possibility by careful sequential treatment of HMSMC with the two agonists and appropriate controls. HMSMC were stimulated for 24 h with 5 ng/mL IL-1β followed by DMEM or 10 μM $PGF_{2\alpha}$ for 6 h (Fig. 4.6A). IL-1β treatment alone upregulated *IL6* mRNA abundance 36-fold. $PGF_{2\alpha}$ treatment (6 h) increased *IL6* abundance by only 8-fold when administered on its own. However, IL-1β treatment for 24 h followed by $PGF_{2\alpha}$ treatment for 6 h produced a 104-fold increase in *IL6* mRNA abundance (Fig. 4.6A). Reversing the order of treatments with $PGF_{2\alpha}$ for 24 h then IL-1β for 6 h, we observed a similarly large response. $PGF_{2\alpha}$ for 24 h alone induced a 1.9-fold increase in *IL6* abundance whereas just IL-1β for 6 h produced a 42-fold increase in *IL6*. $PGF_{2\alpha}$ followed by IL-1β however stimulated an 81-fold increase in *IL6* (Fig. 4.6B).

We observed a less pronounced effect of IL-1β and $PGF_{2\alpha}$ treatments on IL-6 protein outputs from HMSMC compared to *IL6* mRNA. The 24 h IL-1β treatment increased IL-6 output by HMSMC from 2.3 ± 0.5 ng/mL to 14.7 ± 4.0 ng/mL. $PGF_{2\alpha}$ stimulation for 6 h produced an output of 8.1 ± 1.5 ng/mL, but the combined treatment sequence together produced 26 ± 8.2 ng/mL IL-6 (Fig. 4.7A). Again, reversing the treatment order, 24 h $PGF_{2\alpha}$ treatment alone led to an IL-6 output of 2.3 ± 0.5 ng/mL and 6 h IL-1β stimulated 12.5 ± 1.9 ng/mL. However, $PGF_{2\alpha}$ then IL-1β produced an IL-6 protein output of 21.8 ± 6.1 ng/mL from HMSMC (Fig. 4.7B).

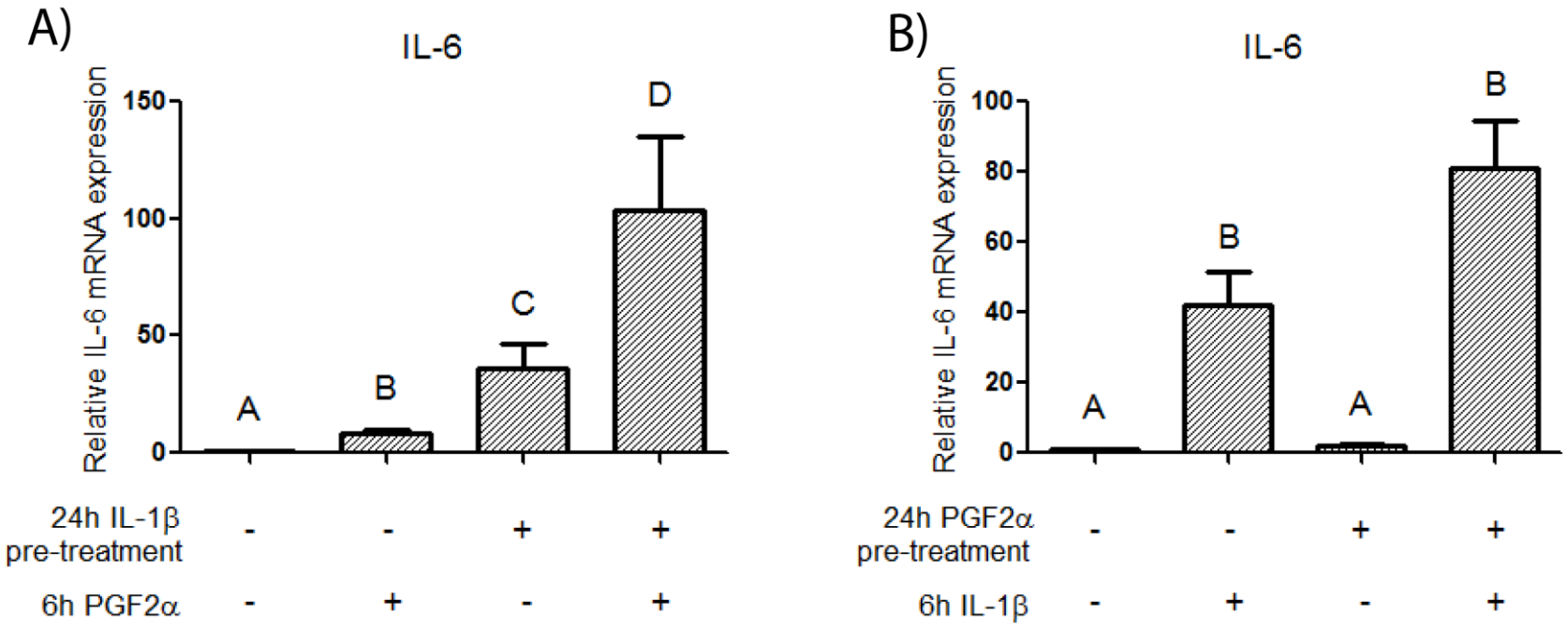

Figure 4.6. Sequential stimulation of HMSMC with $PGF_{2\alpha}$ and IL-1β induces amplified upregulation of *IL6*. HMSMC were either 1) pre-stimulated for 24 h with IL-1β (5 ng/mL), washed and subsequently stimulated with $PGF_{2\alpha}$ (10 µM) for 6 h, OR 2) pre-stimulated for 24 h with $PGF_{2\alpha}$, washed and stimulated with IL-1β. *IL6* mRNA data are presented as relative change (x-fold) from control values, mean ± SEM, n=5-7 patients. One-way ANOVA statistical testing was performed on log-transformed data followed by Tukey post-hoc analysis. Groups with statistically significant post-hoc differences are represented with different letter designations, $p<0.05$.

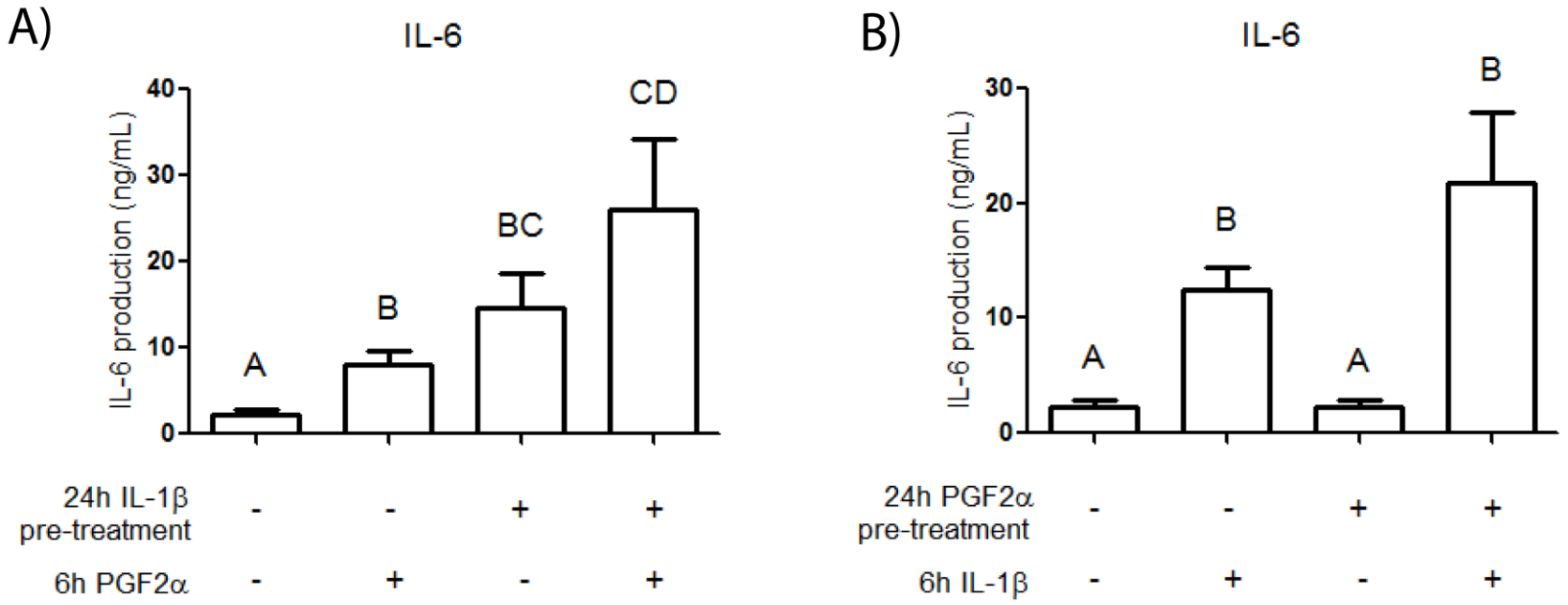

Figure 4.7. Sequential stimulation of HMSMC with $PGF_{2\alpha}$ and IL-1β induces amplified upregulation of IL-6 protein. HMSMC were either 1) pre-stimulated for 24 h with IL-1β (5 ng/mL), washed and subsequently stimulated with $PGF_{2\alpha}$ (10 μM) for 6 h, OR 2) pre-stimulated for 24 h with $PGF_{2\alpha}$, washed and stimulated with IL-1β. IL-6 protein levels in cell culture supernatant are presented as concentration output (ng/mL), mean ± SEM. N=6 patients. One-way ANOVA statistical testing was performed on log-transformed data followed by Tukey post-hoc analysis. Groups with statistically significant post-hoc differences are represented with different letter designations, $p<0.05$.

Given the substantial responses in IL-6 expression, we tested IL-1β plus $PGF_{2\alpha}$ on COX-2 expression. IL-1β alone for 24 h stimulated *COX2* mRNA abundance 19.4-fold from control levels; 6 h of $PGF_{2\alpha}$ on its own increased *COX2* abundance by 4.8-fold, whereas IL-1β then $PGF_{2\alpha}$ stimulated a 35.4-fold increase in *COX2* abundance (Fig. 4.8A). Reversing the order of administration, we observed a similar response (Fig. 4.8B). HMSMC stimulated by $PGF_{2\alpha}$ for 24 h experienced a 2.35-fold increase in *COX2* abundance, and 6 h IL-1β induced a 28.9-fold increase in *COX2* expression. Exposure to both however induced a large 55.4-fold increase in *COX2* mRNA abundance.

Additionally, we observed the same response for COX-2 protein from cell lysates collected in response to both agonists. IL-1β increased COX-2 protein 29-fold, while 6 h $PGF_{2\alpha}$ treatment alone increased relative COX-2 protein abundance 5-fold. Together, they stimulated a large 66.2-fold increase (Fig. 4.8C). When the treatment order was reversed, similar protein expression changes were measured. $PGF_{2\alpha}$ alone increased COX-2 protein mass 2.4-fold, while 6 h IL-1β on its own increased COX-2 protein expression 21.1-fold from control levels. Together, $PGF_{2\alpha}$ then IL-1β increased COX-2 protein abundance by 46-fold (Fig. 4.8D).

Combined stimulation of HMSMC with a mixture of $PGF_{2\alpha}$ and IL-1β for 30 h instead of sequential stimulation of IL-1β followed by the $PGF_{2\alpha}$ (and vice versa), does not result in synergistic upregulation in *COX2* or *IL6* mRNA expression (Fig. 4.9). Pre-stimulation of HMSMC with IL-1β (or $PGF_{2\alpha}$) seems to have a priming effect on the cells, resulting in a heightened subsequent response to the second agonist.

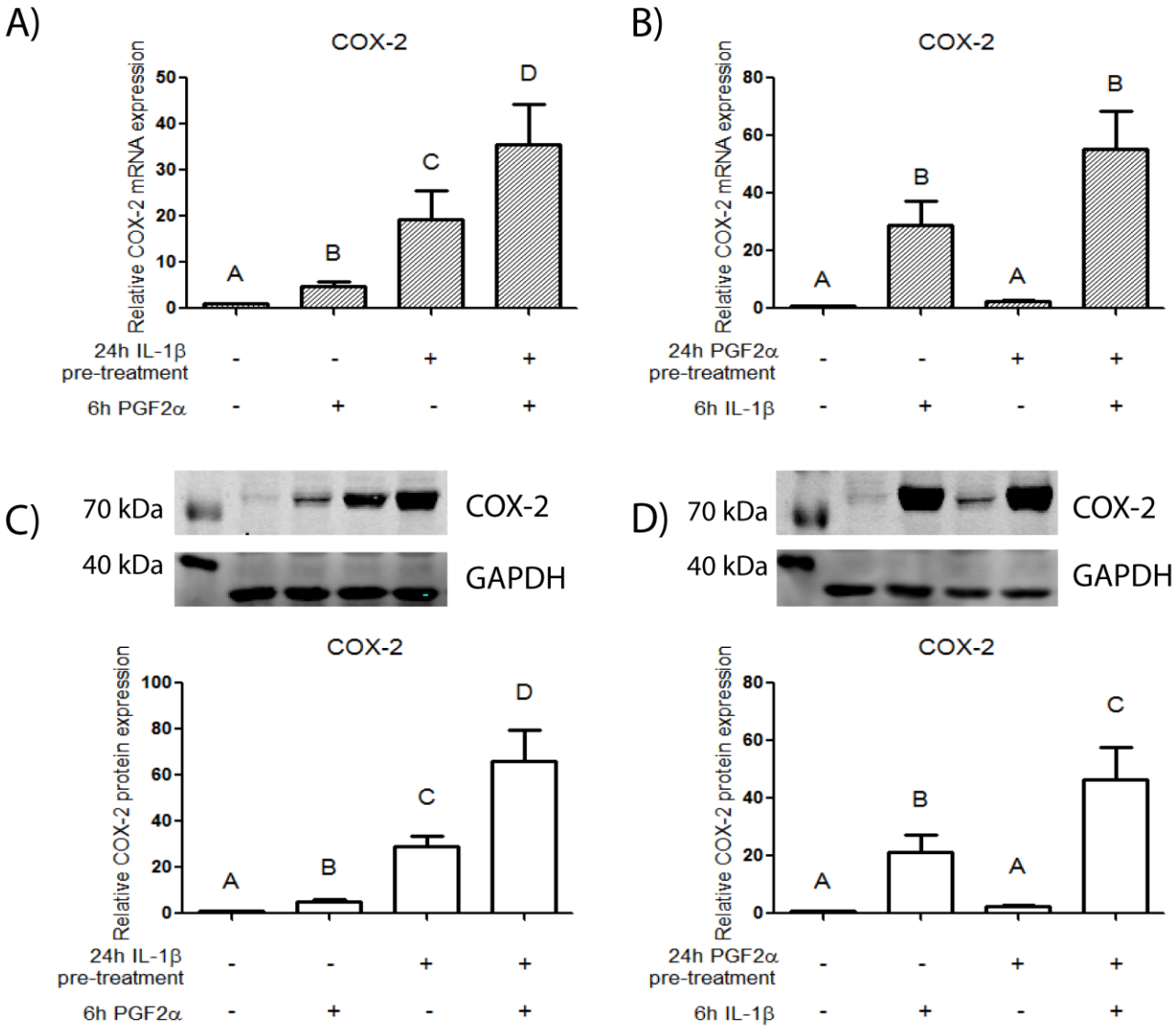

Figure 4.8. Sequential stimulation of HMSMC with $PGF_{2\alpha}$ and IL-1β induces amplified upregulation of *COX2* mRNA and protein expression. HMSMC were either 1) pre-stimulated for 24 h with IL-1β, washed, and subsequently stimulated with $PGF_{2\alpha}$ for 6 h, OR 2) pre-stimulated for 24 h with $PGF_{2\alpha}$, washed, and stimulated with IL-1β. **(A, B)** *COX2* mRNA; **(C, D)** COX-2 protein expression (representative blots included). Data are presented as relative change (x-fold) from 6h control values (first lane), mean ± SEM. N=5-7 patients. One-way ANOVA on log_{10}-data followed by Tukey post-hoc analysis. Groups with statistically significant post-hoc differences are represented with different letter designations, $p<0.05$.

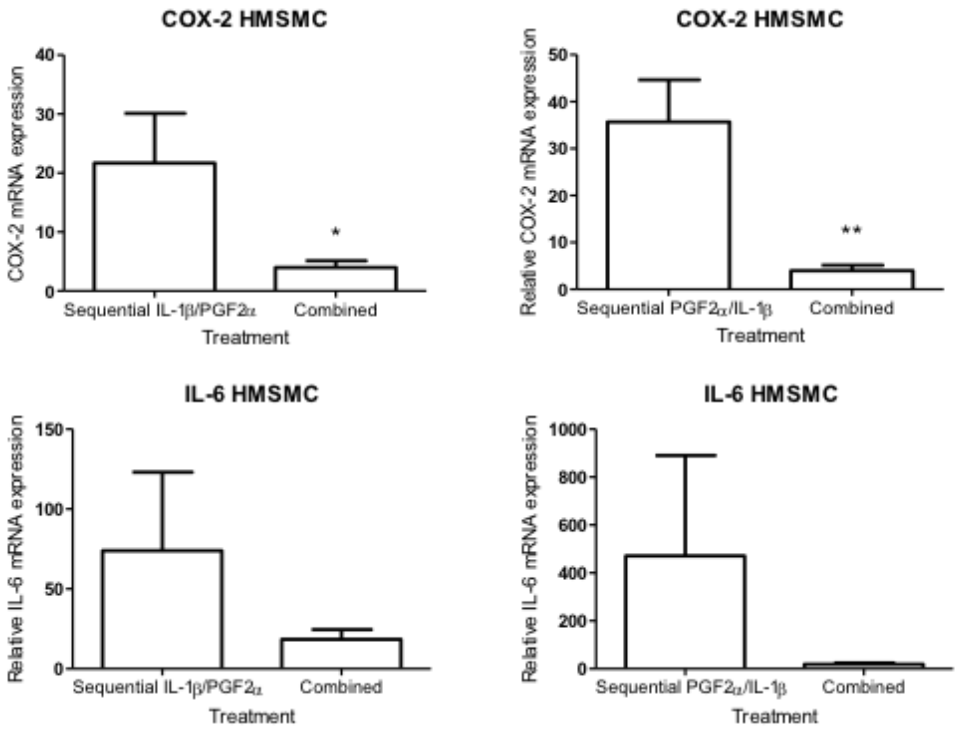

Figure 4.9. Comparison of sequential versus combined IL-1β/$PGF_{2\alpha}$ stimulation of HMSMC. HMSMC were either 1) pre-stimulated for 24 h with IL-1β, washed and subsequently stimulated with $PGF_{2\alpha}$ for 6 h, 2) pre-stimulated for 24 h with $PGF_{2\alpha}$, washed and stimulated with IL-1β, OR 3) stimulated for 30 h with a mixture of $PGF_{2\alpha}$ and IL-1β (combined). Data are presented as relative change (x-fold) from non-treated sample for each patient set, mean ± SEM. N=4 patients. Independent samples t-test; *p<0.05, **p<0.01.

Unlike their cumulative stimulatory effects on IL-6 and COX-2 expression, sequential IL-1β and $PGF_{2\alpha}$ treatment, regardless of order of treatment, did not result in elevated levels of *FP* and *OTR* induction (Fig. 4.10). IL-1β treatment alone upregulated *FP* 2-fold whereas 6 h $PGF_{2\alpha}$ treatment decreased *FP* expression from 1 to 0.36. Together, they decreased *FP* mRNA expression to 0.68 of control. When the treatment order was reversed, 24 h of $PGF_{2\alpha}$ stimulated a 1.6-fold increase in *FP* abundance whereas IL-1β treatment alone increased *FP* abundance 3.2-fold. Consecutively, they exhibited a 4-fold increase in *FP* mRNA expression. Sequential stimulation of HMSMC by IL-1β and $PGF_{2\alpha}$ led to a downregulation of *OTR* abundance regardless of order of administration (Fig. 4.10).

Since IL-6 was highly responsive to the combination of $PGF_{2\alpha}$ then IL-1β, we investigated whether other pro-inflammatory cytokines and chemokines were regulated in a similar way. IL-8 and CCL2 protein synthesis, as measured by multiplex assay, did not significantly change when treated with $PGF_{2\alpha}$, IL-1β, or either sequential combination of the two (Fig. 4.11). $PGF_{2\alpha}$ treatment alone for 24 h had no effect on TNFα output, although 6 h of IL-1β treatment on its own increased TNFα from 5.0 ± 2.2 pg/mL to 107.3 ± 46.7 pg/mL. Interestingly, $PGF_{2\alpha}$ stimulation before IL-1β suppressed the IL-1β effect on TNFα (Fig. 4.11B).

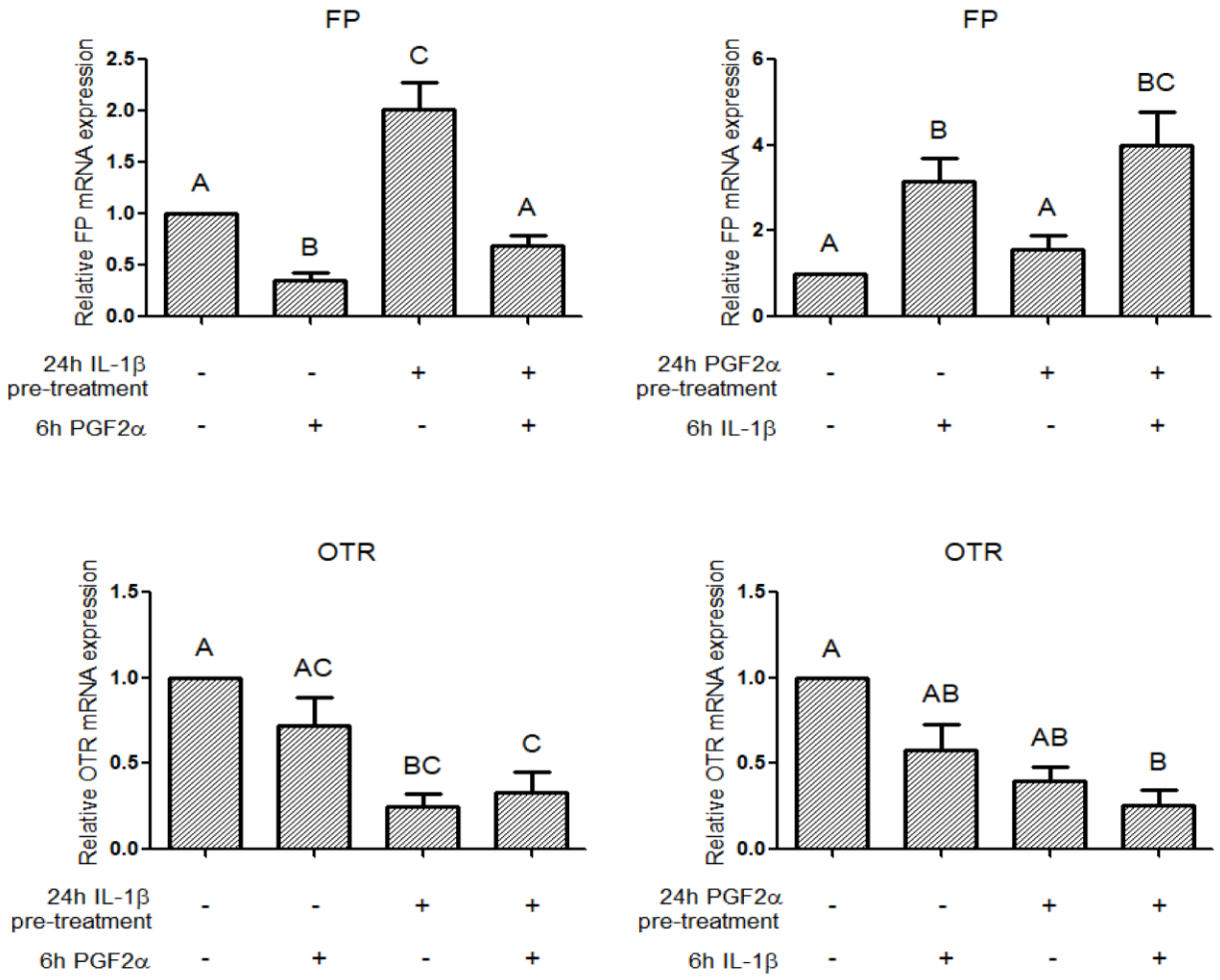

Figure 4.10. Sequential stimulation of HMSMC with $PGF_{2\alpha}$ and IL-1β does not induce amplified upregulation of *FP* and *OTR* mRNA expression. HMSMC were either 1) pre-stimulated for 24 h with IL-1β, washed and stimulated with $PGF_{2\alpha}$ for 6 h, OR 2) pre-stimulated for 24 h with $PGF_{2\alpha}$, washed and stimulated with IL-1β. Data are presented as relative change (x-fold) from control values, mean ± SEM. N=5 patients. One-way ANOVA statistical testing was performed on log-transformed data followed by Tukey post-hoc analysis. Groups with statistically significant post-hoc differences are represented with different letter designations, $p<0.05$.

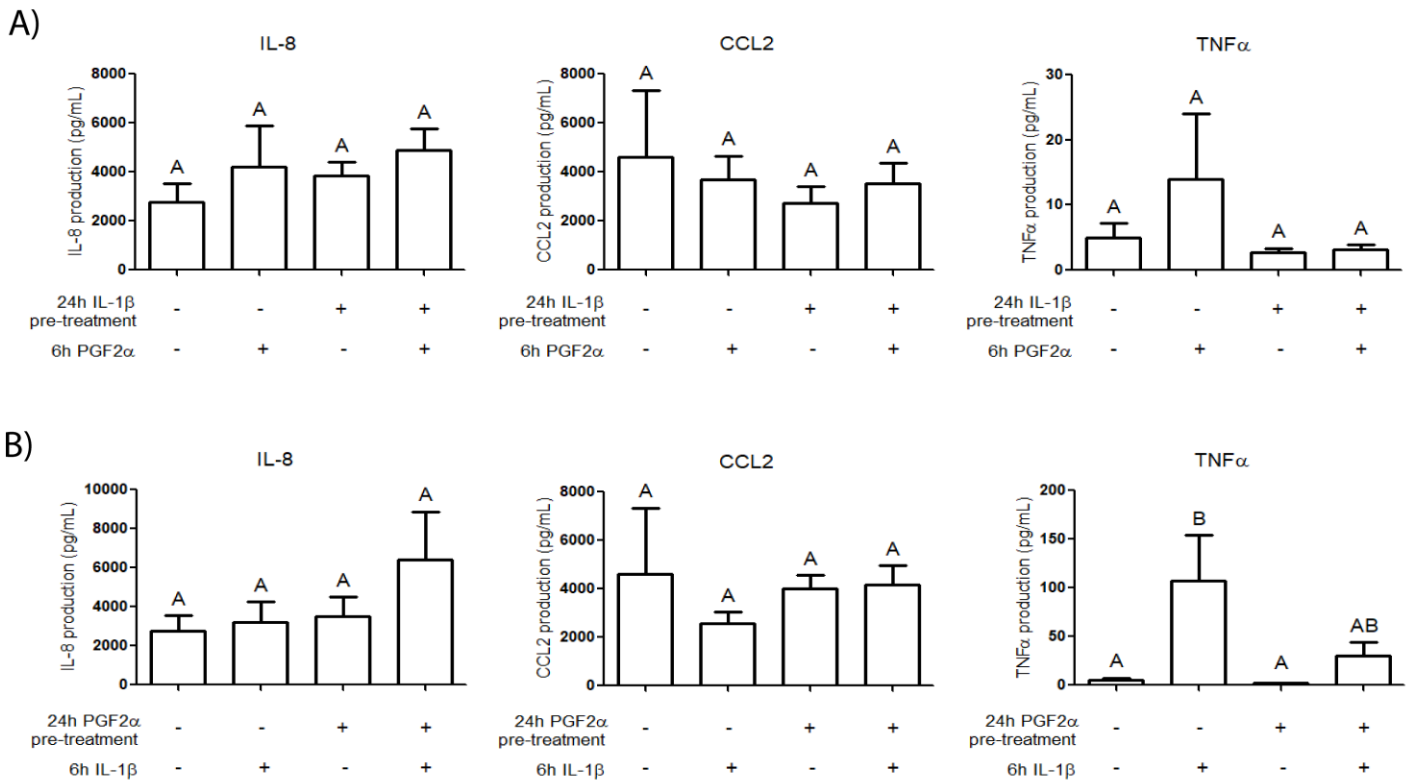

Figure 4.11. Unlike IL-6, sequential stimulation of HMSMC with $PGF_{2\alpha}$ and IL-1β does not induce synergistic upregulation of IL-8, CCL2 and TNFα. HMSMC were either A) pre-stimulated for 24 h with IL-1β (5 ng/mL), washed and stimulated with $PGF_{2\alpha}$ (10 μM) for 6 h, OR B) pre-stimulated for 24 h with $PGF_{2\alpha}$, washed and stimulated with IL-1β. Data are presented as concentration output (pg/mL) into cell culture supernatant, mean ± SEM. N=3 patients. One-way ANOVA statistical testing was performed on log-transformed data followed by Tukey post-hoc analysis. Groups with statistically significant post-hoc differences are represented with different letter designations, $p<0.05$.

4.3.4. IL-1β and $PGF_{2\alpha}$ sequential treatment of hFM explants.

A logical question that some may ask is, are these extremely large responses of COX-2 and IL-6 to combined IL-1β plus PGF2α treatments unique to the myometrium or do other intrauterine tissues respond similarly? We addressed this question using hFM explants because this excellent model contains the other intrauterine tissues, amnion, chorion, and some *decidua vera*, all together. The result indicates, however, that the very large response relationship involving IL-1β plus $PGF_{2\alpha}$ stimulation is apparently unique to the myometrium, since hFM explants treated according to the same protocol did not demonstrate significant upregulation of *COX2* or *IL6* (Fig. 4.12). *COX2* mRNA decreased from 1 to 0.8 with $PGF_{2\alpha}$ treatment. IL-1β treatment upregulated COX2 by only 2.3-fold whether alone or followed by a second treatment of $PGF_{2\alpha}$ (Fig. 4.12). $PGF_{2\alpha}$ (6 h) increased *IL6* abundance by only 1.3-fold, and IL-1β treatment alone upregulated *IL6* mRNA abundance 12-fold. IL-1β treatment followed by $PGF_{2\alpha}$ yielded a 6.7-fold increase in *IL6* mRNA abundance (Fig. 4.12).

Reversing the order of treatments with $PGF_{2\alpha}$ then IL-1β, we observed that *IL6* and *COX2* increased with 6h IL-1β stimulation but not 24h $PGF_{2\alpha}$ treatment alone (Fig. 4.13). 24h IL-1β treatment alone increased *FP* mRNA expression in hFM, but no other treatments resulted in significant changes in *FP* or *OTR* (Fig. 4.13). The effects of sequential IL-1β/$PGF_{2\alpha}$ compared to 30 h treatment with a mixture of IL-1β and $PGF_{2\alpha}$ were not significantly different (Fig. 4.14).

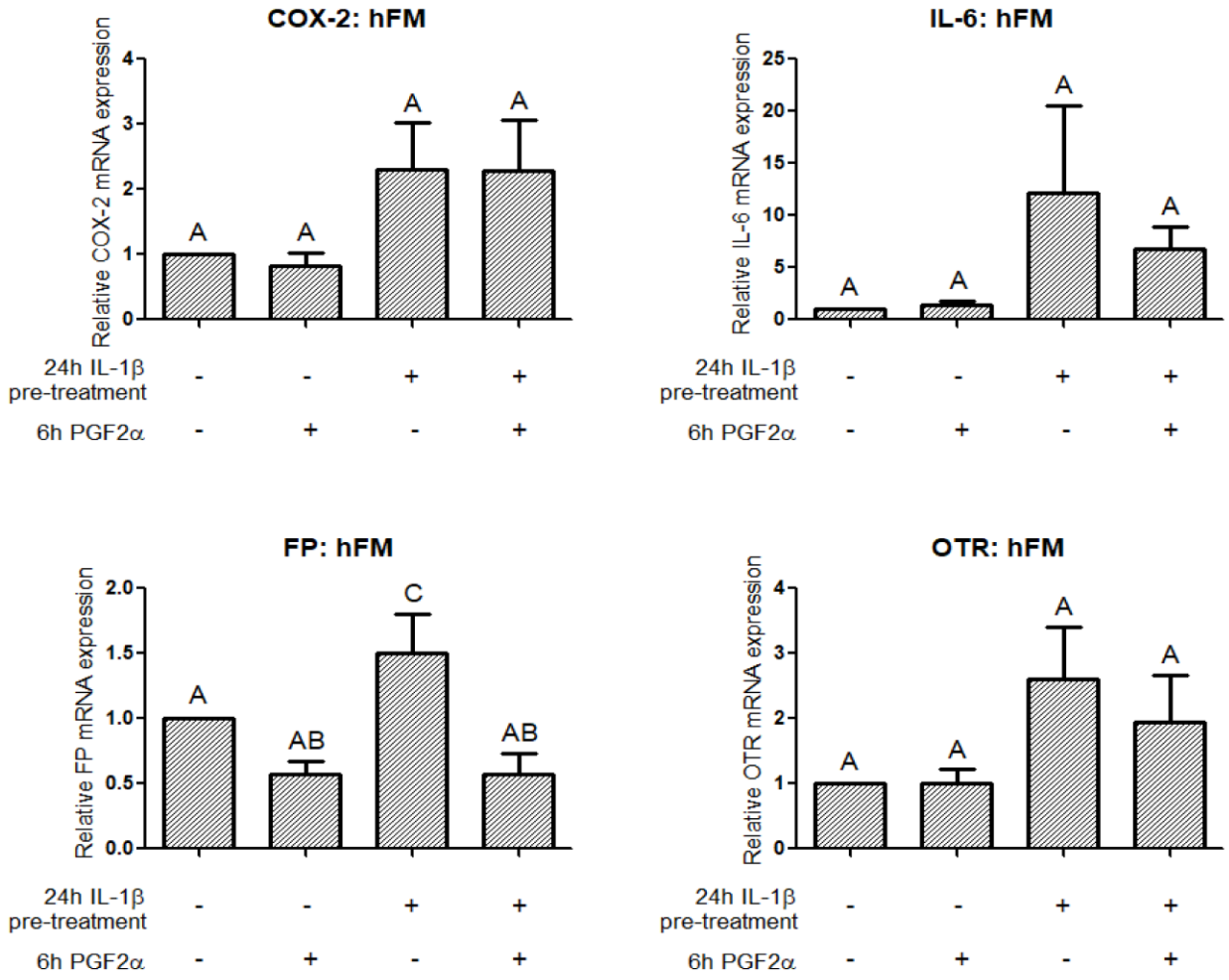

Figure 4.12. Unlike HMSMC, sequential stimulation of hFM explants with IL-1β then $PGF_{2\alpha}$ does not induce amplified upregulation of *IL6* and *COX2* mRNA expression. 6 mm hFM explants were pre-stimulated for 24 h with IL-1β (5 ng/mL), washed and stimulated with $PGF_{2\alpha}$ (10 μM) for 6 h. Data are presented as relative change (x-fold) from control values, mean ± SEM. N=6 patients. One-way ANOVA statistical testing was performed on log-transformed data followed by Tukey post-hoc analysis. Groups with statistically significant differences are represented with different letter designations, $p<0.05$.

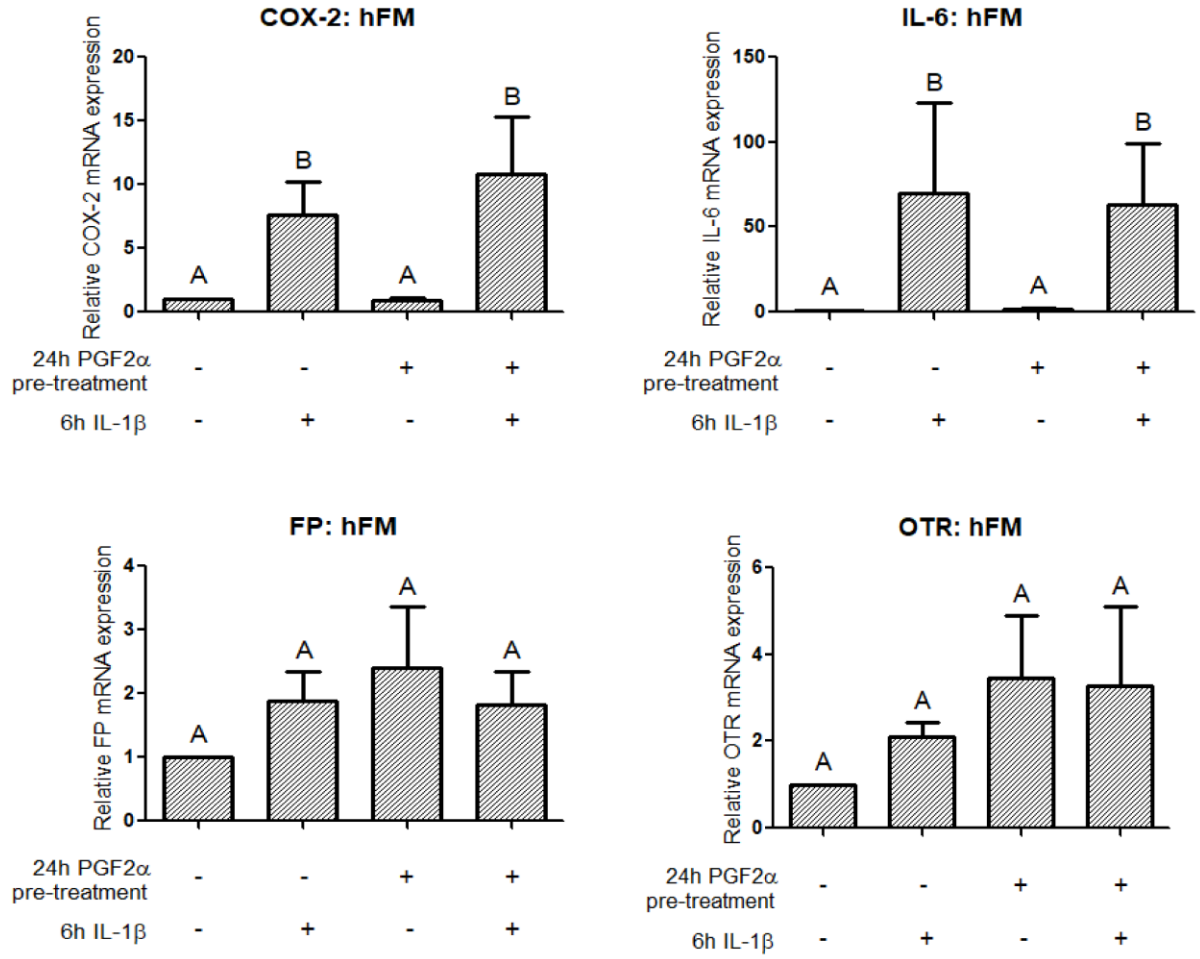

Figure 4.13. Unlike HMSMC, sequential stimulation of hFM explants with $PGF_{2\alpha}$ then IL-1β does not induce synergistic upregulation of *IL6* and *COX2* mRNA expression. 6 mm hFM explants were pre-stimulated for 24 h with $PGF_{2\alpha}$, washed and stimulated with IL-1β. Data are presented as relative change (x-fold) from control values, mean ± SEM. N=6 patients. One-way ANOVA statistical testing was performed on log-transformed data followed by Tukey post-hoc analysis. Groups with statistically significant differences are represented with different letter designations, $p<0.05$.

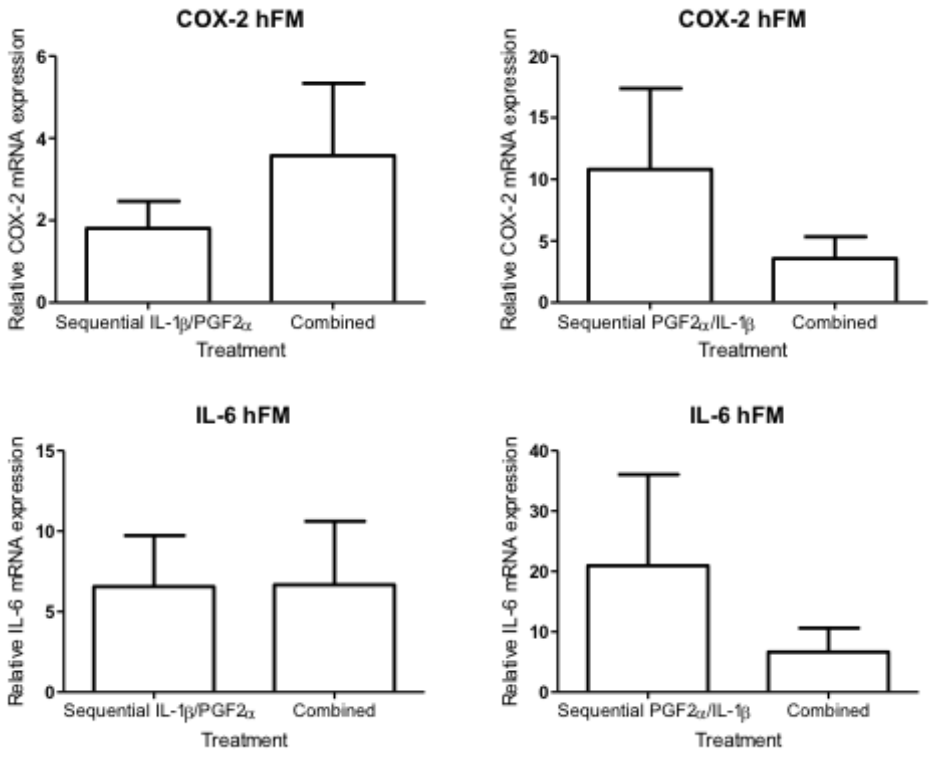

Figure 4.14. Comparison of sequential versus combined IL-1β/$PGF_{2\alpha}$ stimulation of hFM. Explants were either 1) pre-stimulated for 24 h with IL-1β, washed and subsequently stimulated with $PGF_{2\alpha}$ for 6 h, 2) pre-stimulated for 24 h with $PGF_{2\alpha}$, washed and stimulated with IL-1β, OR 3) stimulated for 30 h with a mixture of $PGF_{2\alpha}$ and IL-1β (combined). Data are presented as relative change (x-fold) from non-treated sample for each patient set, mean ± SEM. N=4 patients. Independent samples t-test; $p>0.05$.

4.4 Discussion

In this study we confirmed our earlier observation (134, 135), that $PGF_{2\alpha}$ and IL-1β are individually important pro-inflammatory mediators in the myometrium. But here we considerably expand upon that observation to prove they are acting in concert to drive positive feedback interactions that amplify the pro-inflammatory cascade required to induce labour. Sequential stimulation of HMSMC by $PGF_{2\alpha}$ plus IL-1β (in either order) results in a tremendous upregulation of IL-6 and COX-2, and this appears to be exclusive to the myometrium. In addition we made the intriguing discovery that IL-1β and $PGF_{2\alpha}$ each stimulate increases in members of the broad IL-1 family in HMSMC including the IL1R1 receptor and its accessory proteins that confirm our *in vivo* observations (31). Together, these salient observations demonstrate clearly that positive feedback or feed-forward relationships amplify the inflammatory load to the uterus and are likely critical for expressing the UAPs that convert the uterus from a physiological state of pregnancy to a state of parturition.

IL-1β has an influential role in orchestrating the gene regulation of pro-inflammatory and pro-labour mediators in gestational tissues. One hour of IL-1β stimulation upregulates 98 genes in PHM1-41 uterine myocytes by at least 3-fold (281). Microarray data of decidual cells stimulated with IL-1β demonstrated a significant upregulation of 350 transcripts, a downregulation of 78 transcripts, as well as the predicted activation of 57 transcription factors and inhibition of 22 (282). Ishiguro *et al.* demonstrated that changes in uterine sensitivity to IL-1β occur at term in the rat through an upregulation of *IL1R1* and accessory proteins *IL1RAcP* and *IL1RAcPb* and a downregulation in *IL1R2* (31). An IL-1β-induced preterm birth mouse model exhibited increased *IL1R1* and *IL1RAcP* in the myometrium, and a highly specific antagonist to IL-1R1, rytvela, blocked IL-1β- and infection-induced preterm delivery in mice (286). In the

rhesus macaque, *IL1R1*, *IL1R2,* and *IL1RAcP* were upregulated in amniochorion and decidua after intra-amniotic IL-1β injection (328). Our model corroborates these findings in the human, as HMSMC treated with IL-1β upregulated mRNA expression of *IL1R* accessory proteins, and showed increasing trends in *IL1R1* and *IL1R2*.

Similar to IL-1β, $PGF_{2\alpha}$ also stimulated an increase in mRNA abundance of *IL1R1*, *IL1R2* and *IL1RAcPb* in HMSMC. IL-1RAcPb is an isoform of the IL-1 accessory protein that when complexed with IL-1β and IL-1R1 does not activate NFκB as does IL-1RAcP, but instead acts through p38 MAPK and Src phosophorylation (329). Previously, it had only been identified in the central nervous system (273) until we described an increase in its expression in the pregnant rat uterus at delivery (31). Here we show that *IL1RAcPb* is expressed in the pregnant human uterus (HMSMC) and is regulated by both IL-1β and $PGF_{2\alpha}$.

This intriguing effect is an excellent example that $PGF_{2\alpha}$ has many more roles than its stimulation of myometrial contractility (134, 135). For instance, we confirmed that $PGF_{2\alpha}$ stimulates COX-2 expression in HMSMC as it does in amnion, myometrium and endometrial adenocarcinoma tissue (134, 330, 331). In the decidua, PGF2α increases the concentration of matrix metalloproteinases (MMP)-2 and -9 while decreasing the concentration of their tissue inhibitor, TIMP-1 (77). MMP-2, -3 and -9, in turn, catalyze the inactive pro-IL-1β into biologically active IL-1β independent of caspase-1 thereby further amplifying the pro-inflammatory response through positive feedback (267). In HMSMC, IL-6 is upregulated by both $PGF_{2\alpha}$ and IL-1β, corroborating previous findings that pro-inflammatory cytokine release is increased by $PGF_{2\alpha}$ (135). Completing the feed-forward cycle, we showed that IL-1β stimulates upregulation of the $PGF_{2\alpha}$ receptor FP (97, 134). Hence there is ample evidence that both $PGF_{2\alpha}$

and IL-1β are mutually stimulatory and thereby contribute considerably to the expression of UAPs that transition the pregnant uterus to the parturient uterus.

But without question the most significant observation of this study is that when the two agonists stimulate HMSMC sequentially, in either order, a profound increase in the output of IL-6 and COX-2 occurs. The process of synergy is defined generally as the "interaction or cooperation of two or more organizations, substances, or other agents to produce a combined effect greater than the sum of their separate effects" (332). Our data clearly demonstrate that $PGF_{2\alpha}$ and IL-1β together produce a synergistic effect using this definition. For example in Fig. 4.7 COX-2 protein levels increased 29-fold after 24 h IL-1β, and 5-fold with 6 h $PGF_{2\alpha}$ stimulation. Instead of a 34-fold additive response to IL-1β then $PGF_{2\alpha}$ stimulation, COX-2 increased 66-fold. Reversing the order of treatments led to a $PGF_{2\alpha}$- stimulated increase of COX-2 protein by 2.4-fold and an IL-1β-stimulated increase of 21-fold, but together they stimulated an increase of COX-2 protein levels of 46-fold – not the additive 23-fold. Similar synergistic outputs were observed for IL-6 but not for other cytokines, and this effect was not observed in fetal membrane explants suggesting it is unique for myometrium. However, it is possible that the outcomes are different between the tissues due to methodological differences, as one model involves intact tissue explants and the other primary cultured cells. To be able to truly compare between the tissues in the future, this experiment should be repeated using myometrial explants.

The concept of synergy is well established in the literature. In a study of gastrointestinal tumour growth, chemokines CXCL6 and CCL2 together stimulated a significantly higher mobilization of leukocyte migration than the sum of each alone (333). IL-8 synergizes with a series of chemokines including CCL2 to induce amplified neutrophil chemotaxis (334). In chondrocytes, inflammatory synergy results in cartilage destruction; high-mobility group box-1

(HMGB-1), a DAMP, synergizes with IL-1β to produce high levels of MMPs (335), and IL-1α works in concert with glucocorticoids and mineralocorticoids to synergistically increase lipocalin-2 and MMP-13 (336). The challenges and complications of experimental demonstration of *pharmacological synergy* in a system is thoroughly described in a review by Foucquier *et al.* (337). To clearly affirm pharmacological synergy in a system, large data sets are required including extended concentration response curves with many more than the 3 concentration points included in this study, as well as the completion of multiple mathematical frameworks. We are not arguing that the results presented in this paper constitute this definition of pharmacological synergy. Additionally, our agonists were not administered in combination but sequentially, instead modelling a priming effect. We contend that the amplification of IL-6 and COX-2 presented in this paper in response to sequential $PGF_{2\alpha}$/IL-1β stimulation is not modeling classical 'pharmacological synergy' of drug combination but cooperative inflammatory amplification. It is possible that there are multiple individual steps or net increments that are additive, and only more investigation will be able to tell. We demonstrate clearly, however, that in many situations described in our study, the overall output effect is higher than the additive effects of $PGF_{2\alpha}$ and IL-1β individually.

We do not know the full range of mechanisms involved in our observed synergistic responses, but one element is surely that both $PGF_{2\alpha}$ and IL-1β stimulate expression increases in the others' receptor as we demonstrate here. Other studies suggest additional mechanistic detail. Amnion-derived WISH cells displayed synergistic upregulation of COX-2 induced by co-treatment of IL-1β with epidermal growth factor (EGF). Inhibition of NFκB decreased IL-1β/EGF-induced COX-2 by only 44% suggesting that at least one signalling mechanism other than NFκB is involved in the synergistic relationship (338). IL-6 and COX-2 were both

synergistically upregulated in aortic smooth muscle cells by combined treatments of cytokines Oncostatin M and IL-1β due to both transcriptional and posttranscriptional regulation (339). IL-6 transcription may be cooperatively regulated, as multiple regulatory elements can synergize to induce greater levels of IL-6 induction. NFIL-6 and NFκB individually both induced a 2-fold increase in IL-6 (as measured by luciferase activity), but together the transcription factors interacted with both promoter binding sites to induce over 40-fold increases in IL-6 (300). Additionally, all three transcription factors can interact and induce an even greater effect. AP-1, NFκB and NFIL-6 can form a complex and interact with the IL-6 promoter, resulting in nearly 300 times the level of IL-6 induction, much greater than the three regulatory elements separately, or even paired combinations (301). IL-1β and $PGF_{2\alpha}$ stimulate several intracellular pathways in uterine tissues, and it is likely that synergistic responses could occur through intricate regulation of several of these (102, 135, 136, 340-343). Interestingly, the COX-2 gene promoter also contains NFκB and NFIL-6 regulatory elements (178).

While IL-1β was a potent stimulator of pro-inflammatory mediator output from HMSMC, IL-6 did not significantly alter mRNA expression of *COX2*, *FP*, *OTR*, *IL1R1*, *IL1RAcP* or *IL1RAcPb*. We established that the IL-6R mRNA was expressed in these cells (data not shown). In contrast, we found that IL-6 stimulation of the human fetal membrane explants used to compare against the synergistic effects of HMSMC produced several cytokines (TNFα, IFNγ, CCL21 and IL-1β protein) (Yin and Olson, unpublished), demonstrating specificity of tissue responsiveness. It is possible that the high levels of IL-6 produced by the myometrium are not acting on the uterine musculature itself, but on local leukocytes or other cell types in the surrounding gestational tissues.

Unlike their combined effects on IL-6 or COX-2, sequential IL-1β and $PGF_{2\alpha}$ treatment did not result in amplified *FP* and *OTR.* This is not surprising as $PGF_{2\alpha}$ and IL-1β have opposing effects on *FP* expression in HMSMC, and both downregulate (or have no effect) on *OTR*. Increases in receptors of contractile mediators (uterotonic receptors) are upregulated in the final step of the birth cascade; in rats, OTR does not increase until just a few hours before delivery (162). Culmination of the inflammatory load may result in the triggering of functional progesterone withdrawal (220) and uterine activation, including FP and OTR increase. In the myometrium, $PGF_{2\alpha}$ upregulates the PR-A/PR-B ratio (236) and IL-1β increases the abundance and stability of the PR-A protein (237, 238), contributing to functional progesterone withdrawal.

In conclusion, our work demonstrates that inflammatory amplification contributes to the facilitation of uterine transition from pregnancy to parturition over a short period of days or even hours. We established $PGF_{2\alpha}$ and IL-1β as key triggers or upstream drivers of this process, and IL-6 and COX-2 as key targets. Existing preterm birth therapies target mechanisms occurring at the final stages of the birth cascade, when excessive amplification has already taken place. IL-1β and IL-6 are upstream mediators in the birth cascade and now we have also demonstrated that $PGF_{2\alpha}$ is also an upstream mediator. There they interact to induce amplification of other pro-labour mechanisms. Alteration of their activity through targeting their receptors, for example by novel allosteric modulators (146, 286, 315), may suppress inflammatory amplification and uterine transition. Targeting not only uterine contraction but also inflammatory amplification and uterine transition presents a promising path for therapeutic development for preterm birth prevention or delay.

Chapter 5
Maternal and fetal intrauterine crosstalk promotes pro-inflammatory amplification and uterine transition.

Portions of chapter five of this thesis are included in a manuscript currently under review at the Journal of Immunology. KB Leimert, X Fang and DM Olson designed and planned the experiments, and the manuscript was written by KB Leimert and DM Olson. HMSMC and hFM culture was performed by KB Leimert, A Messer and T Gray. Quantitative RT-PCR was performed by KB Leimert and A Messer. Multiplex assay and statistical analysis was completed by KB Leimert. S Chemtob edited and gave final approval of the manuscript, and all authors approved the final manuscript.

5.1 Introduction

During the transition from pregnancy to parturition, intrauterine maternal and fetal tissues lie adjacent to one another communicating via paracrine processes and diffusion. These tissues, the maternal myometrium and decidua and the fetal amnion and chorion, are responsible for both the signals and the physiological responses to these signals that evolve into the process of labour. The primary mediators, prostaglandins, growth factors, steroid hormones, pro-inflammatory cytokines and chemokines (112, 165, 344-347), are produced by these tissues and participate in the sequence of events we call the birth cascade. It culminates in an *activated* uterine myometrium that produces the coordinated and forceful contractions necessary for birth of the fetus(es). This derives from the altered expression of many genes, including uterine activation proteins (UAPs), responsible for transitioning the uterus from pregnancy to parturition (32-36). We track three of these mRNA species and their proteins as proxies for the changing UAPs that comprise activation (29, 95). They are COX-2, an inducible enzyme catalyzing a key intermediate step in the synthesis of prostaglandins (30), the $PGF_{2\alpha}$ receptor FP (29), and the oxytocin receptor OTR (325); these are receptors whose responsiveness to uterotonic contractile stimulators increases late in gestation.

Despite their juxtaposition within the uterine ultrastructure, no studies have examined the interactions between fetal and maternal gestational tissues in regard to developing this pro-inflammatory cascade so essential for labour to occur. We therefore designed a co-culture model to address this gap, incorporating primary HMSMC with full thickness human fetal membrane explants in transwells to study paracrine crosstalk between the tissues at term. The transwell allows free passage of paracrine mediators between upper and lower compartments with consequent interactions between maternal and fetal tissues. A separate analysis of responses in

each tissue, or combined analysis of inflammatory outputs in shared supernatant, can be made at the end of an experiment.

5.2 Objective

The intent of this project was to establish our model for studying communication between term HMSMC and hFM explants and then use it to examine the interaction between these tissues in terms of mediator expression and output and responsiveness to IL-1β or $PGF_{2\alpha}$ treatment. We selected these agonists as our first test substances due to their central importance in the birth cascade (134, 146, 286). We hypothesized that paracrine crosstalk between tissues at term promotes pro-inflammatory expression in the birth cascade. Our data demonstrated that co-incubation of these tissues led to a very large increase of inflammatory mediators in the medium and tissues, suggesting a significant contribution to the uterine 'inflammatory load' (226) necessary for uterine transition to labour. We surmise that *in vivo* the myometrium and adjacent fetal membrane tissues interact via paracrine signalling to amplify pro-inflammatory signals to a level necessary to activate the uterus and trigger labour onset.

5.3 Results

5.3.1. Effect of co-culture on COX2 and IL6 in HMSMC and hFM.

After 48 h acclimation in culture for each tissue, HMSMC and hFM were combined in culture for 6 or 24 h to assess whether communication between compartments was sufficient to induce a change in *IL6* abundance or UAP mRNA in each tissue. As demonstrated in Fig. 5.1, HMSMC co-cultured with hFM explants increased relative abundance of *COX2* and *IL6* in HMSMC compared to HMSMC cultured alone. 6 h of co-culture upregulated *COX2* 3.9-fold,

and 24 h in co-culture increased *COX2* 18-fold from the respective monoculture controls. *IL6* was upregulated 12.5-fold in HMSMC after both 6 h and 24 h of co-culture. 6 h in co-culture increased *OTR* expression 2.7-fold, but after 24 h there was no difference in *OTR* between culture conditions (Fig. 5.1). *FP* abundance in HMSMC did not change with the addition of hFM.

HFM explants also increased relative mRNA abundance of *COX2* and *IL6* when co-cultured with HMSMC compared to hFM alone; 6 and 24 h of co-culture resulted in a 4.4-fold and 5-fold increase in *COX2* mRNA, respectively (Fig. 5.2). 6 h of co-culture upregulated *IL6* 22.4-fold, and 24 h increased *IL6* 6.4-fold from respective monoculture controls. *FP* and *OTR* mRNA abundance did not change whether hFM were cultured alone or in co-culture. No significant differences in FP and OTR protein expression in hFM were measured as a result of co-culture with HMSMC (Fig. 5.3).

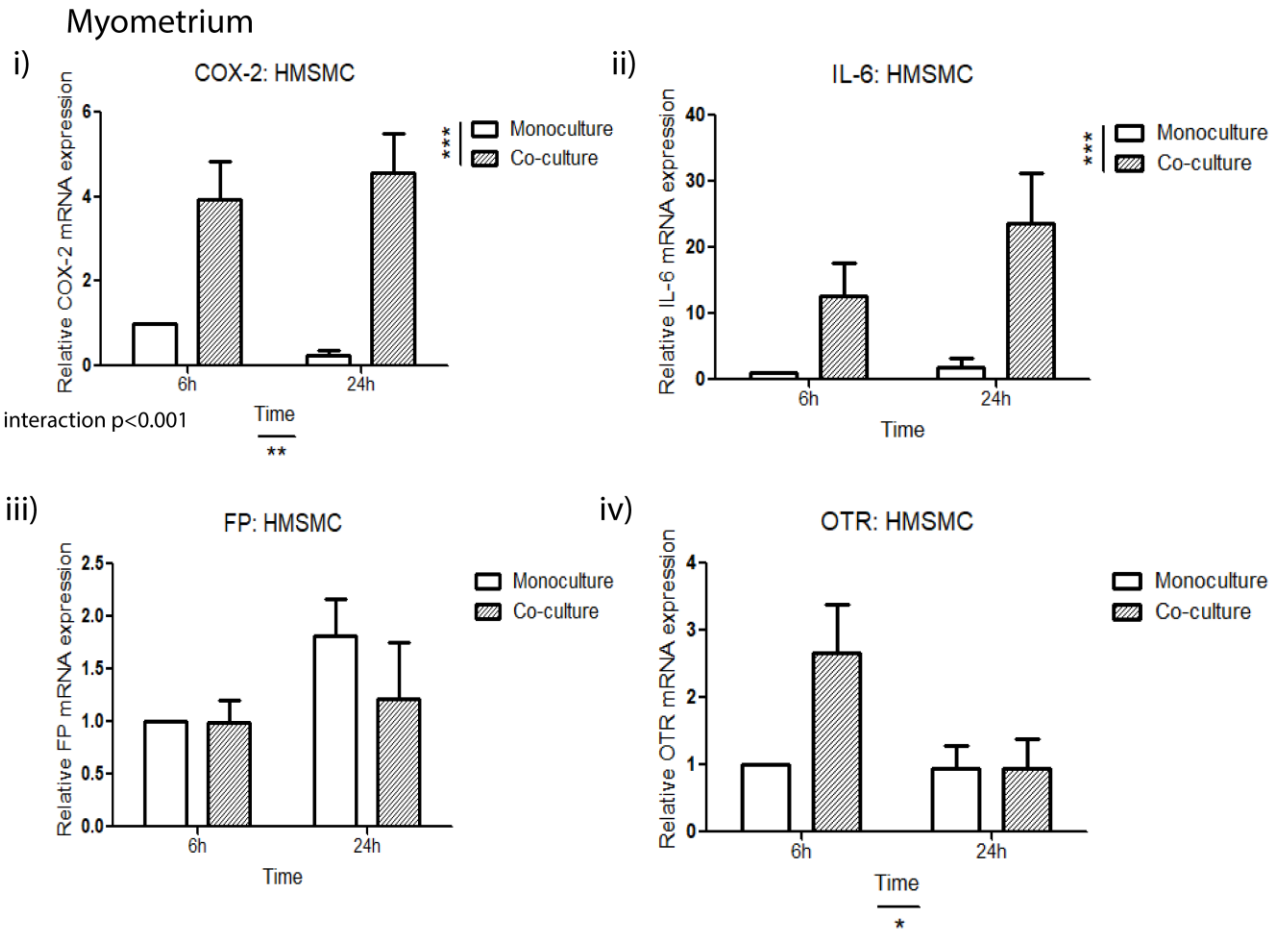

Figure 5.1. Co-culture of HMSMC and fetal membrane explants (hFM) up-regulates mRNA abundance of *COX2* (i) and *IL6* (ii) but not *FP* (iii) and *OTR* (iv) in HMSMC. HMSMC (2 x 10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture). Both culture conditions received fresh serum-free media for 6 h or 24 h before collection, n=7 different subjects. Data are presented as relative change (x-fold) from 6 h monoculture values, mean ± SEM. Two-way ANOVA was performed on $\log_{10}$-transformed data; significance of main effects is presented in the axes: *p<0.05, **p<0.01, ***p<0.001. When the interaction between culture condition and time was significant, Bonferroni *post-hoc* testing outcomes are indicated on the figure over the applicable bars.

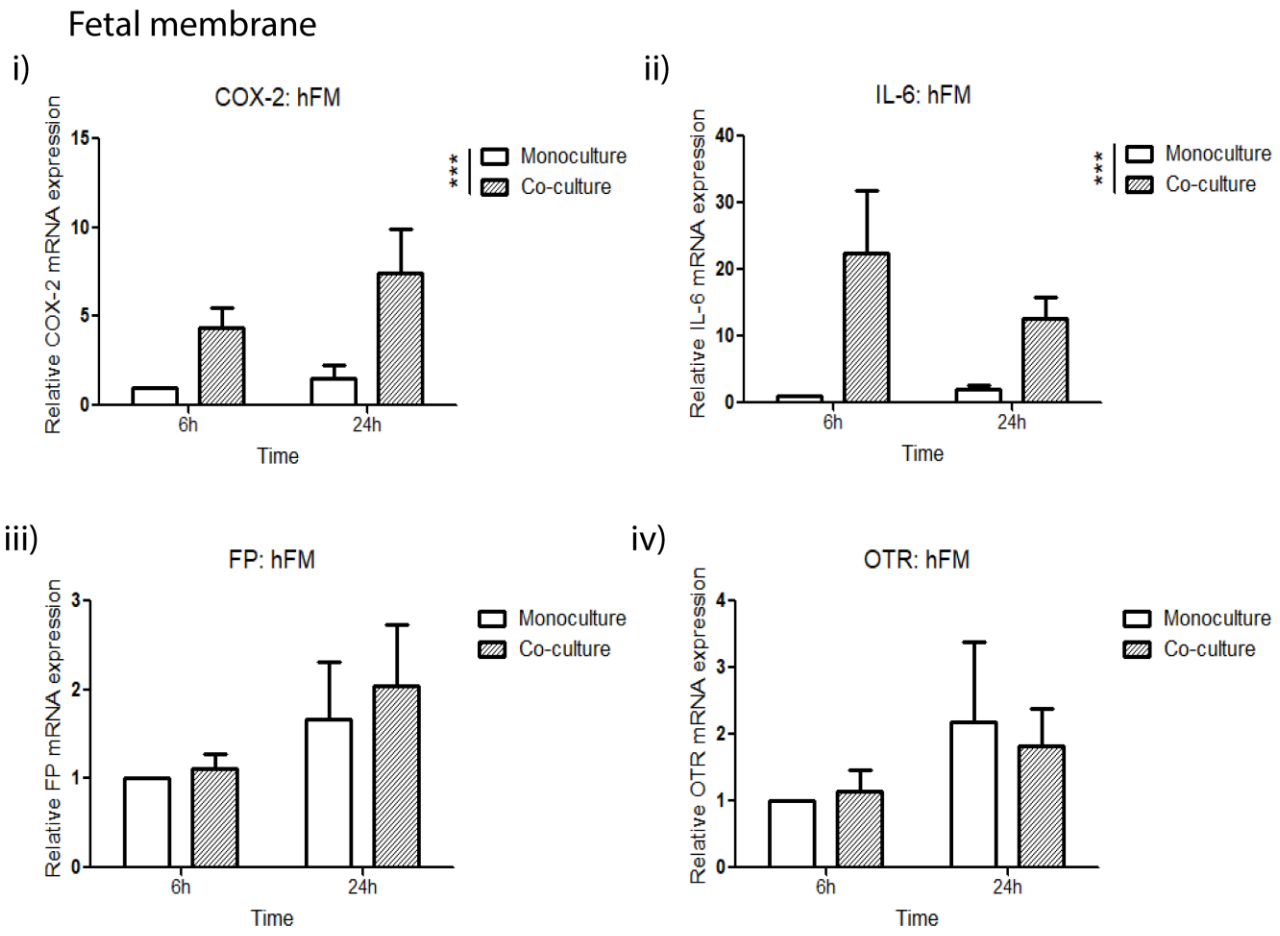

Figure 5.2. Co-culture of HMSMC and fetal membrane explants (hFM) up-regulates mRNA abundance of *COX2* (i) and *IL6* (ii) but not *FP* (iii) and *OTR* (iv) in hFM. HMSMC (2 x 10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture). Both culture conditions received fresh serum-free media for 6 h or 24 h before collection, n=7 different subjects. Data are presented as relative change (x-fold) from 6 h monoculture values, mean ± SEM. Two-way ANOVA was performed on $\log_{10}$-transformed data; significance of main effects is presented in the axes: *p<0.05, **p<0.01, ***p<0.001.

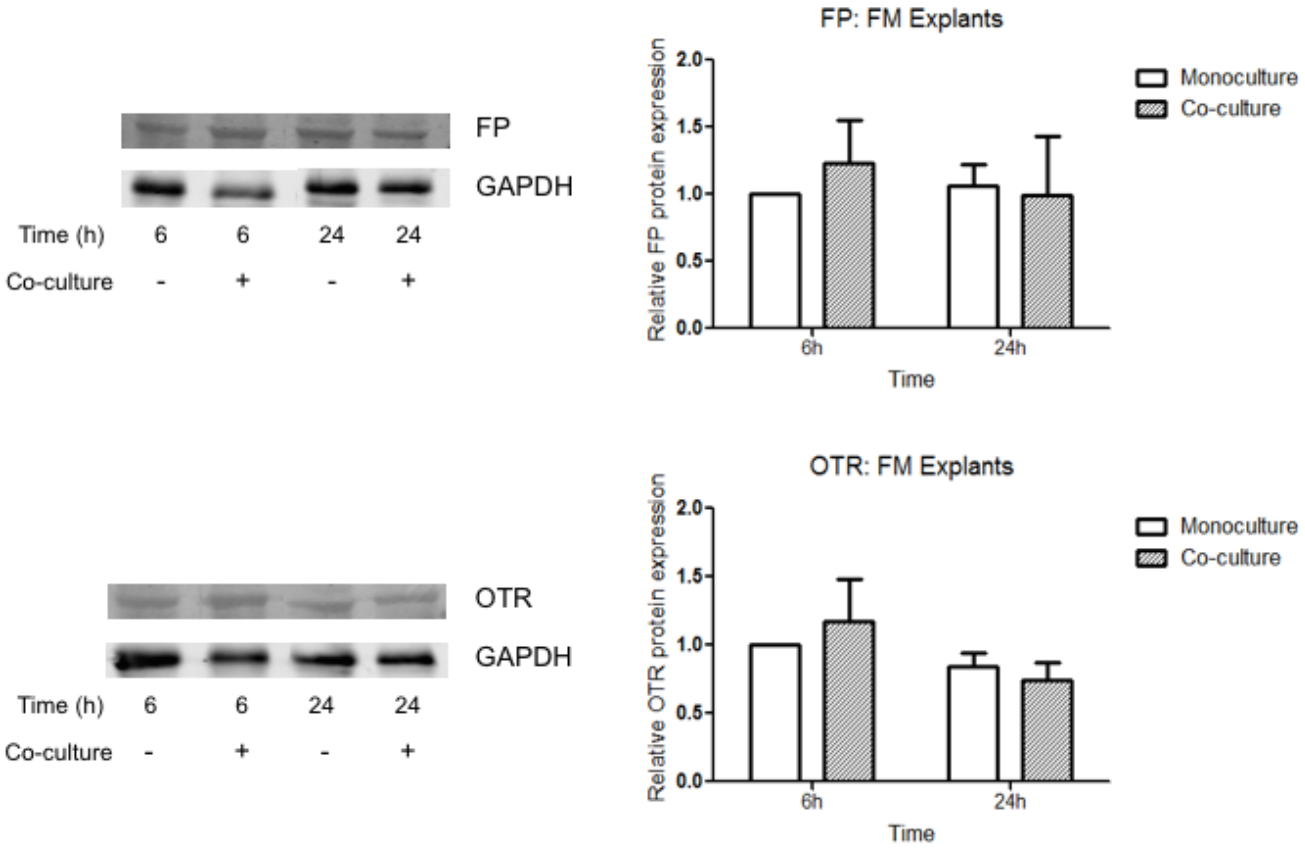

Figure 5.3. Co-culture of HMSMC and fetal membrane explants (hFM) does not change hFM FP or OTR protein expression. HMSMC (2 x 10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture). Both culture conditions received fresh serum-free media for 6 h or 24 h before collection, n=5 different subjects. Data are presented as relative change (x-fold) from 6 h monoculture values, mean ± SEM. Two-way ANOVA was performed on $\log_{10}$-transformed data, $p>0.05$.

5.3.2. Concentration outputs of cytokines and chemokines in monoculture and co-culture.

We determined the concentration of 40 cytokines and chemokines in cell culture supernatants of HMSMC monocultures, hFM monocultures and HMSMC/hFM co-cultures (Tables 5.1 and 5.2). After 6 h we measured a 0.2 ± 0.1 ng/mL IL-6 output by HMSMC alone and a 1.9 ± 1.1 ng/mL output by hFM alone. Co-cultures released a synergistic 11.6 ± 4.4 ng/mL IL-6, 5.6x higher than the sum of each tissue alone (2.1 ng/mL). After 24 h the synergy was even more pronounced: 41.4 ± 15.4 ng/mL IL-6 was released by co-cultures, 15.3x higher than the additive effect of both monocultures (2.7 ng/mL). In addition to IL-6, co-culture induced synergistic outputs of cytokines TNFα, IL-2 and a series of 15 chemokines: CCL2, CCL3, CCL13, CXCL5, CXCL6, CXCL2, CXCL13, CCL22, CCL20, CCL11, CX3CL1, CCL7, CXCL11, CCL8 and CCL15 (Table 5.1). For example, after 24 h in co-culture the CXCL6 output was 590.0 ± 131.3 pg/mL, 46.9x higher than the sum of the outputs of each monoculture (12.6 pg/mL). Out of the 40 cytokines and chemokines measured, 39 out of 40 (all except CCL24) were significantly upregulated by co-culture. We define synergism as an output that is greater in the co-cultures than the sum of the constituent cultures. However, unlike the first 18, for the other 22 cytokines Bonferroni post-hoc testing depicted that inflammatory outputs from HMSMC and hFM monocultures were not both found to be statistically different from co-culture outputs (Table 5.2). The ‘fold change co-culture effect’ measurement is calculated as the co-culture concentration output divided by the sum of HMSMC and hFM monoculture outputs for each cytokine. For the 18 cytokines and chemokines that are synergistically upregulated (Table 5.1), the fold change co-culture effect value ranges from 1.8 to 16.2 times higher after 6 hours of co-culture, and 2.5 to 66.5 times higher after 24 h.

Table 5.1. Co-culture of HMSMC and fetal membrane explants (hFM) produce synergistic outputs of 18 inflammatory mediators. HMSMC (2 x 10^5 cells/mL) and hFM (6mm) were cultured alone (monoculture) or together (co-culture) in duplicate for 6 h or 24 h. Cytokine protein outputs were measured via multiplex: data are presented as concentration output (pg/mL), mean ± SEM. N=6 for IL-6 and CCL2, n=3 for remaining 16 analytes. Fold change co-culture effect is calculated as the co-culture output divided by the sum of HMSMC and hFM monoculture outputs. Two-way ANOVA was performed on $\log_{10}$-transformed data, culture condition main effect ***$p<0.001$ for all analytes. The data presented here all have the same statistical outcome as measured by *post-hoc* Bonferroni testing: HMSMC alone and hFM alone are both significantly different than the co-culture output group, *$p<0.05$.

	Time in culture: 6h (pg/mL)				Time in culture: 24h (pg/mL)			
Cytokine	**HMSMC**	**hFM**	**Co-culture**	**Fold change co-culture effect**	**HMSMC**	**hFM**	**Co-culture**	**Fold change co-culture effect**
IL-6	221.7 ± 118.8	1861.8 ± 1059.8	11554.4 ± 4375.8	**5.6**	177.9 ± 56.5	2529.5 ± 1186.6	41359.9 ± 15422.0	**15.3**
CCL2	445.3 ± 266.7	2605.3 ± 810.4	18684.8 ± 7329.3	**6.1**	358.0 ± 127.0	4894.7 ± 1605.4	29256.6 ± 7185.6	**5.6**
TNFα	4.6 ± 3.0	16.7 ± 8.5	206.4 ± 87.4	**9.7**	3.1 ± 1.1	33.7 ± 4.7	139.0 ± 19.6	**3.8**
IL-2	0.8 ± 0.4	3.1 ± 1.0	6.5 ± 1.4	**1.7**	1.1 ± 0.1	3.1 ± 1.0	9.6 ± 1.8	**2.3**
CCL3	1.8 ± 0.6	41.4 ± 12.4	397.9 ± 68.7	**9.2**	0.9 ± 0.2	88.6 ± 45.2	1169.2 ± 282.0	**13.1**
CCL13	1.2 ± 0.5	20.6 ± 9.5	175.8 ± 76.0	**8.1**	1.0 ± 0.2	36.6 ± 18.1	296.4 ± 58.2	**7.9**
CXCL5	253.2 ± 39.8	869.8 ± 247.0	3090.1 ± 692.7	**2.8**	235.1 ± 14.5	1171.1 ± 481.3	6375.6 ± 1682.5	**4.5**
CXCL6	3.6 ± 3.4	6.3 ± 2.8	87.9 ± 26.5	**8.9**	3.0 ± 1.3	9.6 ± 4.3	590.0 ± 131.3	**46.9**
CXCL2	2.7 ± 2.7	38.9 ± 18.7	394.2 ± 188.8	**9.5**	0.4 ± 0.4	71.7 ± 37.0	1368.3 ± 801.9	**19.0**
CXCL13	< 0.1	0.3 ± 0.2	1.4 ± 0.2	**4.5**	< 0.1	0.5 ± 0.2	2.2 ± 0.6	**5.0**
CCL22	< 0.5	7.6 ± 4.0	20.0 ± 7.3	**2.6**	< 0.5	12.0 ± 6.4	79.1 ± 0.4	**6.6**
CCL20	< 0.1	6.1 ± 4.3	17.0 ± 10.2	**2.8**	< 0.1	2.8 ± 1.5	96.9 ± 84.3	**34.7**
CCL11	4.5 ± 2.0	16.7 ± 4.5	41.8 ± 8.7	**2.0**	1.7 ± 0.8	17.9 ± 6.1	337.4 ± 80.0	**17.3**
CX3CL1	5.0 ± 1.6	52.1 ± 31.5	105.0 ± 43.0	**1.8**	5.6 ± 0.2	33.8 ± 13.3	318.9 ± 192.4	**8.1**
CCL7	16.7 ± 2.6	20.5 ± 4.2	602.4 ± 465.2	**16.2**	13.0 ± 1.8	23.4 ± 6.7	2423.3 ± 1670.7	**66.5**
CXCL11	< 0.05	0.7 ± 0.2	1.7 ± 0.3	**2.4**	< 0.05	0.4 ± 0.2	3.4 ± 1.5	**9.0**
CCL8	1.3 ± 0.8	75.7 ± 47.4	170.0 ± 34.0	**2.2**	0.6 ± 0.2	71.0 ± 35.5	1829.5 ± 1451.8	**25.5**
CCL15	3.2 ± 0.5	15.1 ± 4.1	33.5 ± 4.4	**1.8**	3.3 ± 0.2	19.1 ± 8.0	56.5 ± 2.9	**2.5**

Table 5.2. Concentration outputs of 22 cytokines and chemokines from HMSMC and fetal membrane explant (hFM) monocultures and co-culture. HMSMC (2 x 10^5 cells/mL) and hFM (6mm) were cultured alone (monoculture) or together (co-culture) in duplicate for 6 h or 24 h. Cytokine/chemokine protein concentrations were measured via multiplex, data are presented as concentration output (pg/mL), mean ± SEM. N=6 for IL-8, CXCL1 and IL-10, n=3 for other 19 cytokines. Fold change co-culture effect is calculated as the co-culture output divided by the sum of HMSMC and hFM monoculture outputs. Two-way ANOVA was performed on $\log_{10}$-transformed data, culture condition main effect ***$p<0.001$ for all analytes. The data presented here all show the same statistical significance as measured by *post-hoc* Bonferroni testing: HMSMC monoculture is significantly different from co-culture, *$p<0.05$, but hFM monocultures are not, $p>0.05$.

	Time in culture: 6h (pg/mL)				Time in culture: 24h (pg/mL)			
Cytokine	**HMSMC**	**hFM**	**Co-culture**	**Fold change co-culture effect**	**HMSMC**	**hFM**	**Co-culture**	**Fold change co-culture effect**
IL-1β	0.2 ± 0.06	2.9 ± 1.0	8.0 ± 2.0	**2.6**	0.3 ± 0.05	14.1 ± 8.7	42.4 ± 12.7	**3.0**
IL-10	1.0 ± 0.8	12.7 ± 3.7	41.7 ± 12.8	**3.1**	1.4 ± 0.4	18.2 ± 5.8	45.8 ± 14.4	**2.3**
IL-8	641.8 ± 456.6	22180.0 ± 10591.8	473400.0 ± 252485.8	**20.7**	323.3 ± 111.1	18700.0 ± 5308.6	416600.0 ± 319890.0	**21.9**
CCL21	21.2 ± 4.4	291.2 ± 181.8	162.8 ± 3.0	**0.5**	21.6 ± 3.2	226.7 ± 122.3	867.4 ± 600.5	**3.5**
CCL27	0.4 ± 0.2	4.9 ± 2.1	12.7 ± 2.2	**2.4**	0.4 ± 0.2	5.9 ± 2.6	15.9 ± 1.4	**2.6**
IL-4	1.1 ± 0.8	4.5 ± 1.5	11.1 ± 2.7	**2.0**	1.6 ± 0.3	5.3 ± 2.7	18.0 ± 3.2	**2.6**
CCL1	5.3 ± 5.3	18.0 ± 4.2	44.8 ± 12.6	**1.9**	6.1 ± 3.1	18.5 ± 9.5	68.6 ± 28.3	**2.8**
IL-16	1.5 ± 1.5	32.8 ± 7.6	66.8 ± 16.8	**2.0**	< 0.8	39.3 ± 20.2	101.4 ± 11.5	**2.6**
CXCL12	22.2 ± 11.5	56.5 ± 6.4	108.6 ± 20.6	**1.4**	21.2 ± 2.8	53.4 ± 19.1	142.9 ± 13.0	**1.9**
CCL19	3.9 ± 1.8	19.2 ± 1.4	44.4 ± 8.3	**1.9**	1.6 ± 0.9	23.5 ± 8.3	78.3 ± 18.3	**3.1**
CCL23	0.9 ± 0.4	20.0 ± 13.8	45.5 ± 18.9	**2.2**	1.6 ± 0.3	31.7 ± 25.0	50.4 ± 26.4	**1.5**
CXCL1	83.1 ± 36.1	839.5 ± 189.8	4714.7 ± 724.3	**5.1**	87.1 ± 40.5	2576.9 ± 639.9	33488.3 ± 16359.5	**12.6**
CCL17	0.6 ± 0.2	4.2 ± 2.0	10.3 ± 2.3	**2.3**	< 1.1	3.7 ± 1.9	14.6 ± 3.0	**4.0**
CCL25	8.0 ± 4.6	46.4 ± 14.5	115.0 ± 26.6	**2.1**	8.9 ± 4.7	51.0 ± 26.1	159.5 ± 30.5	**2.7**
CXCL16	1.3 ± 0.3	58.5 ± 10.5	79.0 ± 16.2	**1.3**	1.1 ± 0.09	171.7 ± 87.5	208.3 ± 60.2	**1.2**
CXCL10	4.4 ± 2.5	104.2 ± 77.8	115.3 ± 32.2	**1.0**	3.8 ± 0.7	74.8 ± 56.1	292.8 ± 158.1	**3.7**
MIF	410.7 ± 53.3	1651.8 ± 845.5	1598.8 ± 414.2	**0.8**	374.9 ± 33.8	1423.4 ± 606.8	2071.8 ± 642.9	**1.2**
IFNγ	1.9 ± 1.2	8.3 ± 3.5	23.0 ± 5.6	**2.2**	1.9 ± 0.4	8.7 ± 3.6	40.9 ± 6.3	**3.8**

CXCL9	< 1.1	253.6 ± 190.6	245.6 ± 167.1	**1.0**	< 1.1	282.7 ± 263.7	316.4 ± 87.1	**1.1**
CCL24	95.0 ± 95.0	82.2 ± 4.2	98.7 ± 4.7	**0.6**	92.6 ± 2.4	83.1 ± 10.5	92.4 ± 13.8	**0.5**
GM-CSF	145.3 ± 6.7	154.3 ± 14.3	214.7 ± 33.7	**0.7**	135.6 ± 6.9	149.6 ± 15.6	388.7 ± 132.5	**1.4**
CCL26	4.0 ± 0.4	4.1 ± 0.4	6.7 ± 0.8	**0.8**	3.1 ± 0.1	3.8 ± 1.1	7.6 ± 1.1	**1.1**

5.3.3. $PGF_{2\alpha}$ stimulation of monocultures and co-culture.

Since we published that myometrial cells increase expression of cytokines, chemokines, and UAPs following $PGF_{2\alpha}$ stimulation (134, 135), we selected $PGF_{2\alpha}$ as a stimulus for the co-culture model. When both culture conditions were stimulated with $PGF_{2\alpha}$, myometrial *IL6* and *COX2* abundance were increased further, whereas *FP* and *OTR* were not. The interaction between $PGF_{2\alpha}$ treatment and culture condition was not, however, found to be statistically significant. HMSMC monocultures upregulated *COX2* mRNA 4.9-fold in response to $PGF_{2\alpha}$ (Fig. 5.4). $PGF_{2\alpha}$-stimulated co-cultures increased myometrial *COX2* 10.1-fold, 2.1x higher than $PGF_{2\alpha}$-treated monocultures and 2.9x higher than co-cultures without exogenous $PGF_{2\alpha}$. HMSMC incubated with $PGF_{2\alpha}$ increased *IL6* 9.8-fold, whereas co-cultures stimulated with $PGF_{2\alpha}$ upregulated *IL6* in HMSMC by 23.1-fold, 2.4x more. *FP* mRNA abundance was downregulated by more than half in response to $PGF_{2\alpha}$ regardless of culture condition. *OTR* did not respond to $PGF_{2\alpha}$ treatment in monoculture or co-culture.

Monocultures of hFM stimulated with $PGF_{2\alpha}$ did not change expression of *COX2*, *IL-6* or *OTR*. HFM cultured with HMSMC increased *COX2* by 5.6-fold, and co-cultures stimulated with $PGF_{2\alpha}$ increased *COX2* 8.1-fold, 1.4x more (Fig. 5.5). Co-culture with and without $PGF_{2\alpha}$ increased *IL6* 25.3 and 24.7-fold, respectively. Similar to HMSMC, *FP* was downregulated in response to $PGF_{2\alpha}$ in both hFM monoculture and co-culture, and fetal membrane *OTR* did not change.

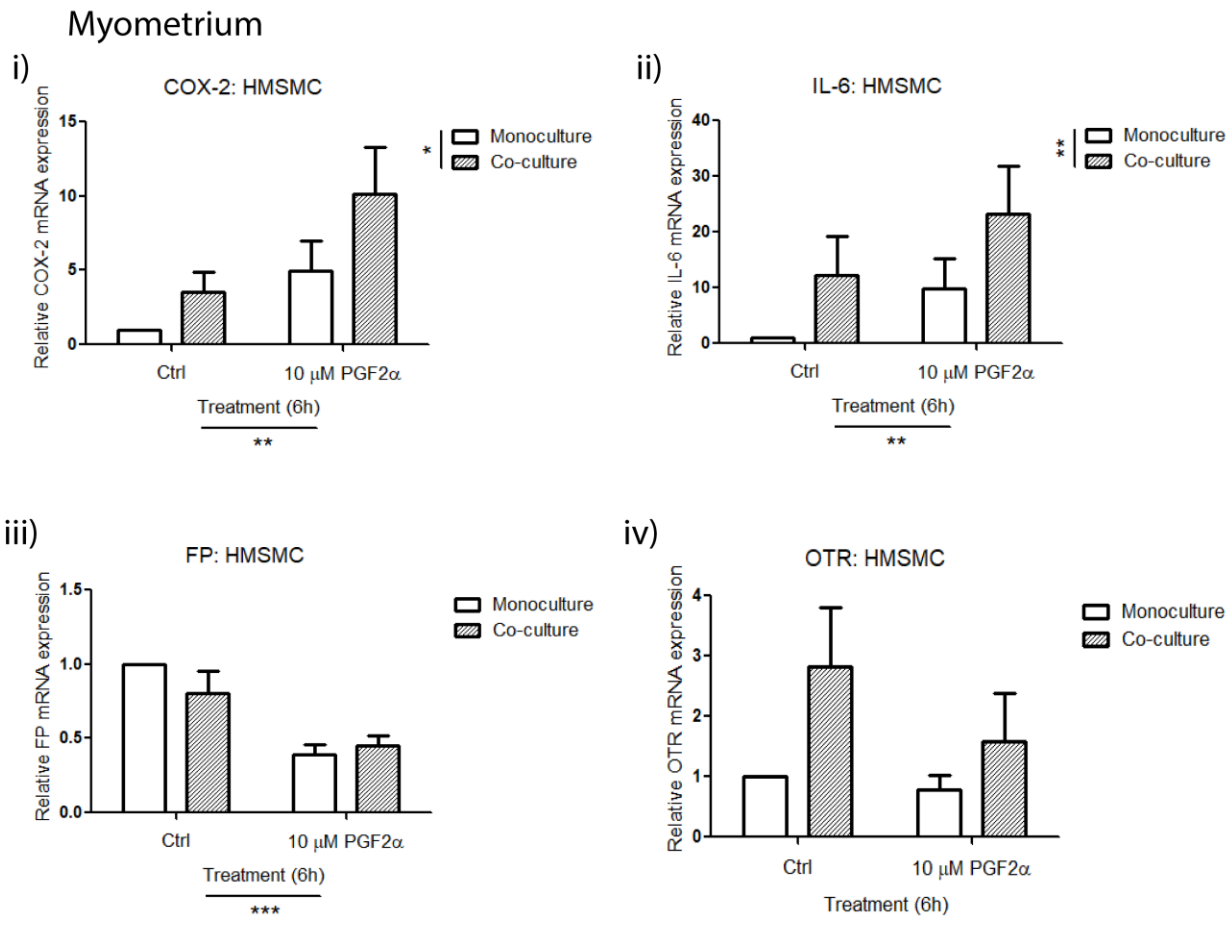

Figure 5.4. $PGF_{2\alpha}$ stimulation of co-cultures induced further upregulation of *COX2* (i) and *IL6* (ii) but not *FP* (iii) and *OTR* (iv) in HMSMC. HMSMC (2 x 10^5 cells/mL) and hFM (6mm) were cultured alone (monoculture) or together (co-culture). Both culture conditions were treated for 6 h with serum-free DMEM F12 or 10μM $PGF_{2\alpha}$, n=5. Data are presented as relative change (x-fold) from 6 h monoculture values, mean ± SEM. Two-way ANOVA was performed on log_{10}-transformed data; significance of main effects is presented in the axes: *p<0.05, **p<0.01, ***p<0.001.

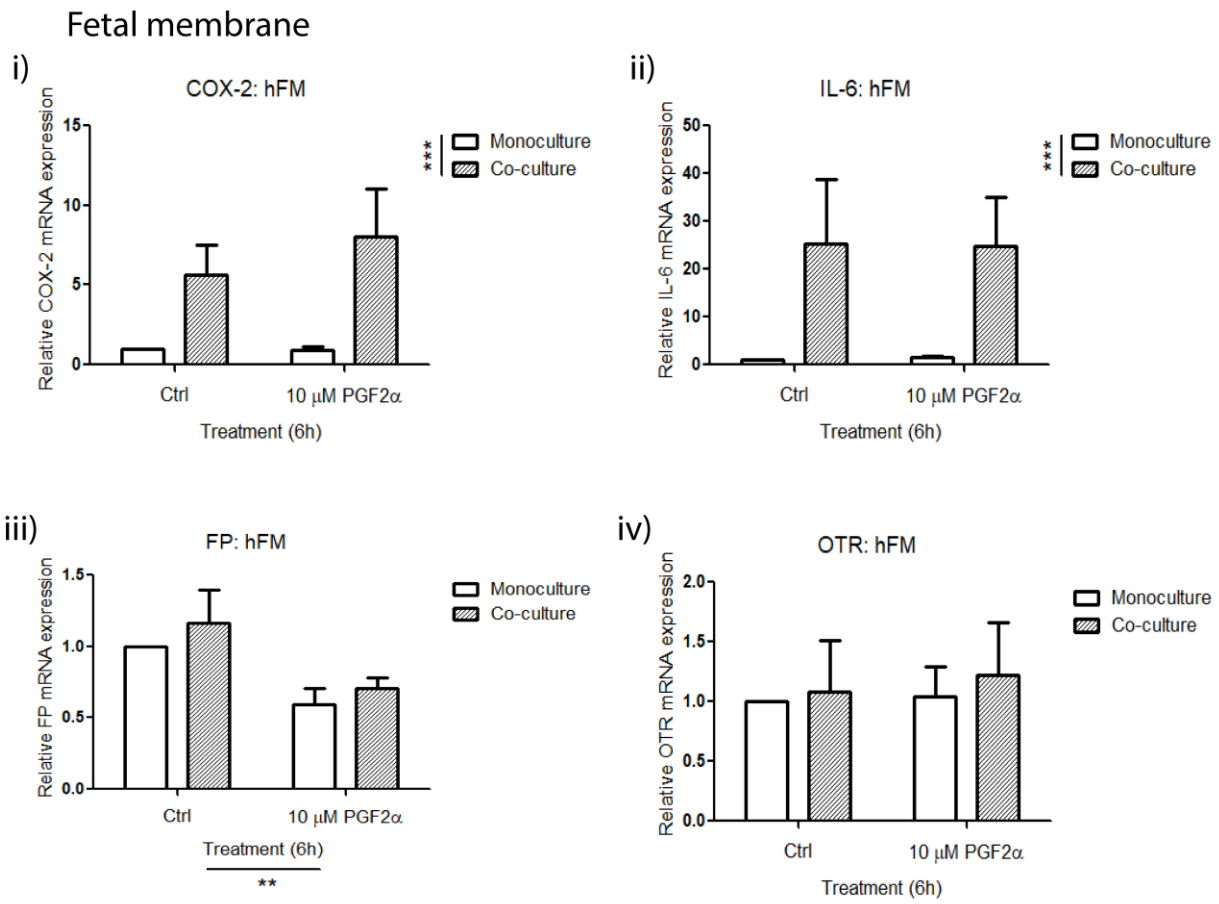

Figure 5.5. $PGF_{2\alpha}$ stimulation of co-cultures induced downregulation of *FP* (iii) but has no effect on *COX2* (i), *IL6* (ii), and *OTR* (iv) in hFM. HMSMC (2 x 10^5 cells/mL) and hFM (6mm) were cultured alone (monoculture) or together (co-culture). Both culture conditions were treated for 6 h with serum-free DMEM F12 or 10μM $PGF_{2\alpha}$, n=5. Data are presented as relative change (x-fold) from 6 h monoculture values, mean ± SEM. Two-way ANOVA was performed on log_{10}-transformed data; significance of main effects is presented in the axes: $^{**}p<0.01$, $^{***}p<0.001$.

5.3.4. IL-1β stimulation of monocultures and co-culture.

We, and others, have previously shown that IL-1β upregulates a range of pro-inflammatory mediators and UAPs in the myometrium (97, 102, 134, 281), which promoted our interest in testing IL-1β in co-culture. IL-1β significantly increased *COX2*, *IL6*, and *FP* in HMSMC, and *COX2*, *IL6*, *FP* and *OTR* in hFM. *COX2* mRNA abundance increased 15.6-fold in response to IL-1β in HMSMC monoculture (Fig. 5.6). Co-cultures stimulated with IL-1β upregulated *COX2* 43.6-fold in HMSMC, 12.5x higher than the effect of co-culture alone and 2.8x higher than the effect of IL-1β alone. *IL6* in HMSMC increased 49.6-fold with IL-1β stimulation in co-culture, 4.1x higher than the effect of co-culture alone and 2.3x higher than IL-1β alone. The interaction between culture condition and IL-1β stimulation was not statistically significant. *FP* mRNA abundance in HMSMC was not affected by culture condition, but increased 2.7-fold and 1.6-fold with IL-1β in monoculture and co-culture, respectively. *OTR* did not respond to IL-1β treatment.

IL-1β had similar effects on hFM with or without the presence of HMSMC in culture (Fig. 5.7). *COX2* mRNA increased 8.3-fold in response to IL-1β in hFM alone and 10.7-fold in response to IL-1β in co-culture (Fig. 5.7). *IL6* was upregulated 16.8-fold and 26.9-fold with IL-1β in monoculture or in co-culture, respectively, but no more than the effect of co-culture alone on *IL6*. IL-1β treatment induced a small but significant increase in fetal membrane *FP* and *OTR*, while culture condition had no effect. Neither $PGF_{2\alpha}$ or IL-1β stimulation significantly changes FP or OTR protein abundance in hFM explants (Fig. 5.8).

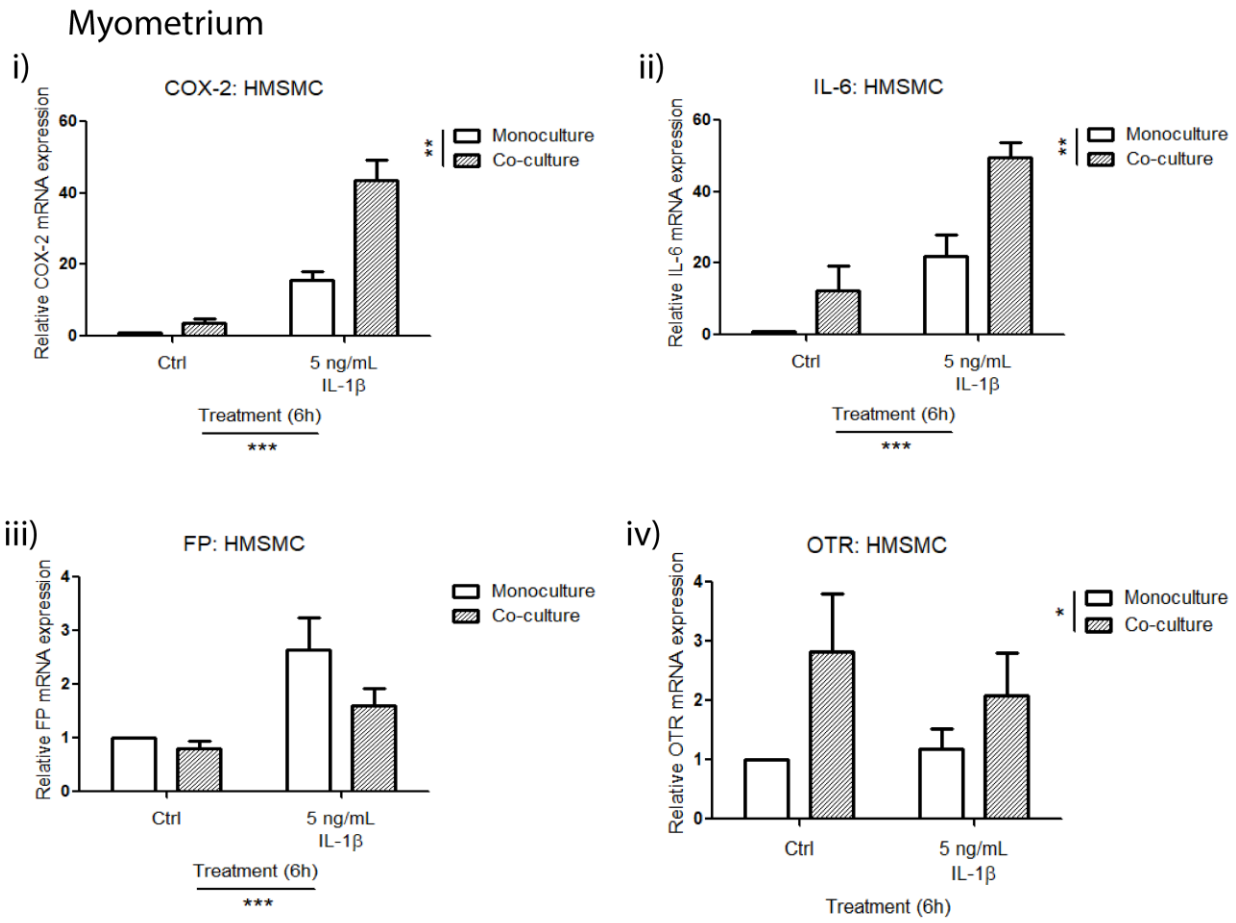

Figure 5.6. IL-1β stimulation of co-cultures induced further upregulation of *COX2* (i) and *IL6* (ii) but not *FP* (iii) and *OTR* (iv) in HMSMC. HMSMC (2 x 10^5 cells/mL) and hFM (6mm) were cultured alone (monoculture) or together (co-culture). Both culture conditions were treated for 6 h with serum-free DMEM F12 or 5 ng/mL IL-1β, n=5. Data are presented as relative change (x-fold) from 6 h monoculture values, mean ± SEM. Two-way ANOVA was performed on $\log_{10}$-transformed data; significance of main effects is presented in the axes: *p<0.05, **p<0.01, ***p<0.001.

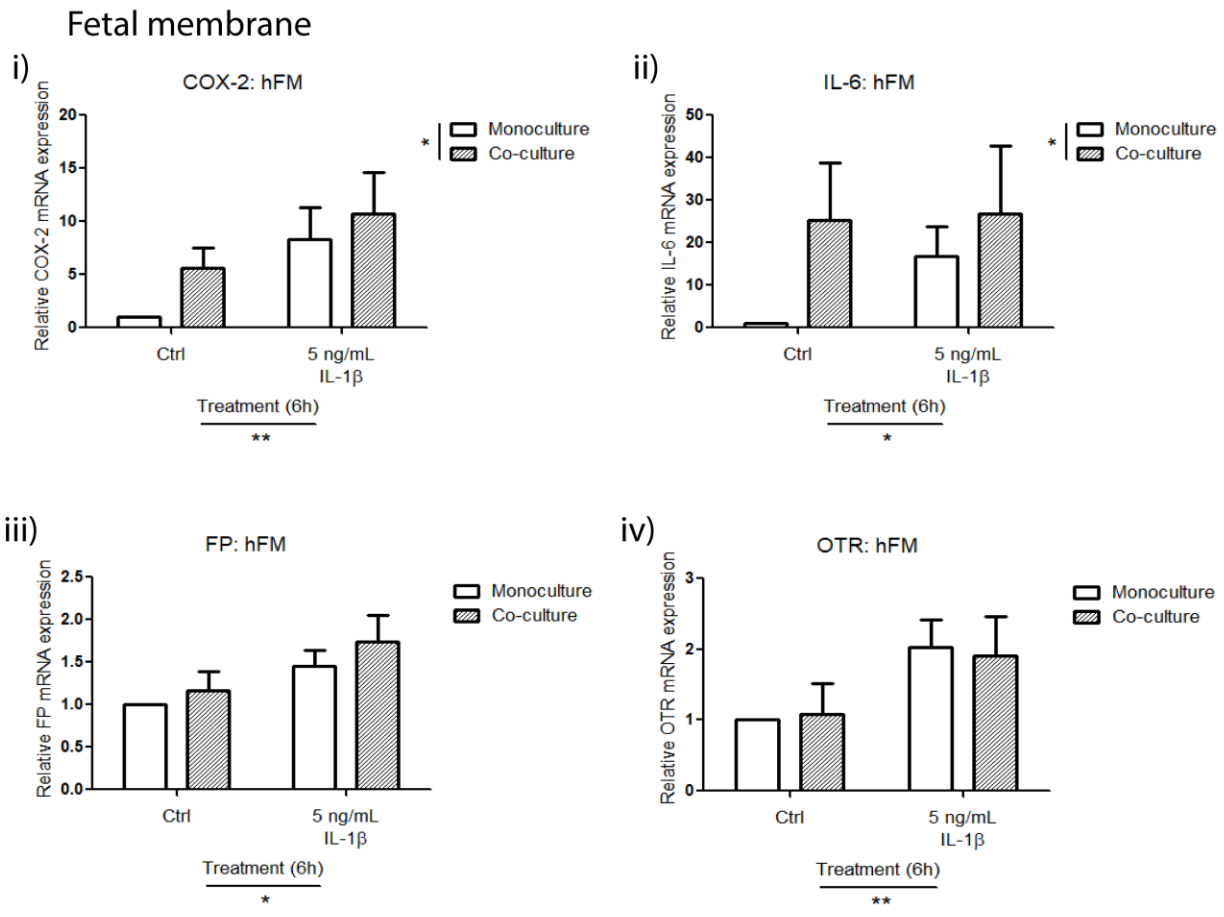

Figure 5.7. IL-1β stimulation has a similar effect on hFM expression of *COX2* (i), *IL6* (ii), *FP* (iii), and *OTR* (iv) with or without the presence of HMSMC. HMSMC (2 x 10^5 cells/mL) and hFM (6mm) were cultured alone (monoculture) or together (co-culture). Both culture conditions were treated for 6 h with serum-free DMEM F12 or 5 ng/mL IL-1β, n=5. HMSMC mRNA is displayed in **(A)** and hFM mRNA in **(B)**. Data are presented as relative change (x-fold) from 6 h monoculture values, mean ± SEM. Two-way ANOVA was performed on $\log_{10}$-transformed data; significance of main effects is presented in the axes: *p<0.05, **p<0.01.

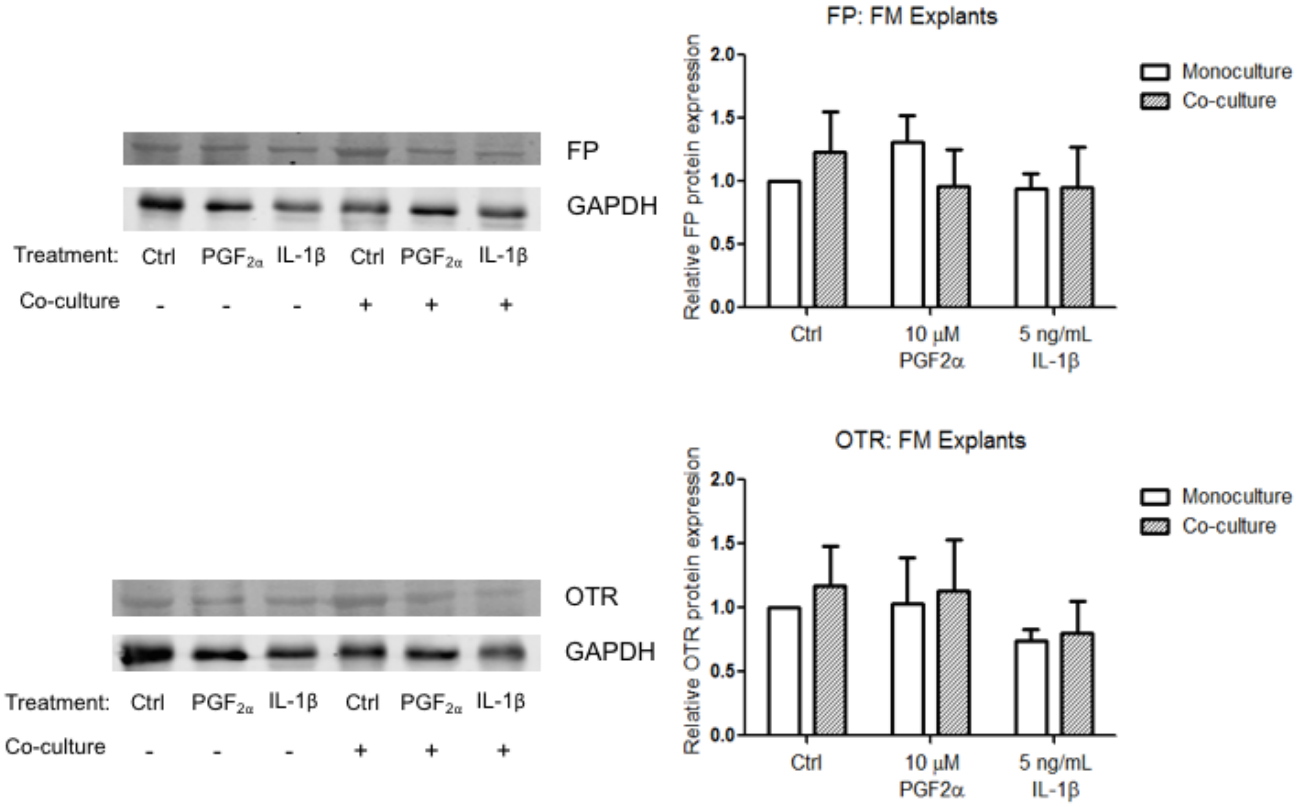

Figure 5.8. Stimulation of co-culture with $PGF_{2\alpha}$ or IL-1β does not change hFM FP or OTR protein expression. HMSMC (2 x 10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture). Both culture conditions were treated for 6 h with serum-free DMEM F12, 10µM $PGF_{2\alpha}$, or 5 ng/mL IL-1β, n=5. Data are presented as relative change (x-fold) from 6 h monoculture values, mean ± SEM. Two-way ANOVA was performed on log_{10}-transformed data, $p>0.05$.

5.3.5. IL-1β and $PGF_{2\alpha}$ effects on co-culture inflammatory protein outputs.

Cytokine/chemokine outputs were also measured in supernatants of HMSMC, hFM, and co-cultures when stimulated with $PGF_{2\alpha}$ or IL-1β for 6 h (Tables 5.3 and 5.4). Outcomes of $PGF_{2\alpha}$ stimulation were dependent on tissue. HMSMC monocultures stimulated with $PGF_{2\alpha}$ led to either no change or increased cytokine and chemokine outputs (Table 5.3). $PGF_{2\alpha}$ stimulation of hFM alone instead suppressed many cytokine outputs. Co-cultures seemingly combine the responses of the two individual tissues, resulting in cytokine outputs that were mostly unchanged in response to $PGF_{2\alpha}$. Unlike $PGF_{2\alpha}$, the majority of IL-1β's effects were stimulatory in all culture conditions. IL-1β treatment was found to significantly affect IL-6, CXCL1, IL-10, GM-CSF, CCL11, CXCL2, IFNγ, IL-2, IL-16, and CCL20 (Table 5.4). IL-1β stimulated a 6-fold increase in IL-6 output by HMSMC from 0.2 ± 0.1 ng/mL to 1.3 ± 0.6 ng/mL, and a 1.6-fold increase in IL-6 output by hFM from 1.9 ± 1.1 ng/mL to 2.90 ± 0.3 ng/mL. In co-culture, IL-1β induced a 3.2-fold increase in IL-6 output, from 11.6 ± 4.4 ng/mL to 36.9 ± 17.2 ng/mL. Similarly, CXCL1 outputs increased 5.3-fold by HMSMC with IL-1β stimulation (0.08 ± 0.04 ng/mL to 0.4 ± 0.2 ng/mL), 1.9-fold by hFM (0.8 ± 0.2 ng/mL to 1.62 ± 0.4 ng/mL), and 2.1-fold in co-culture (4.71 ± 0.7 ng/mL to 10.0 ± 2.2 ng/mL). Many of the remaining 29 cytokines and chemokines demonstrated increasing trends with IL-1β treatment (NS).

Table 5.3. Concentration outputs of 40 cytokines and chemokines in HMSMC/hFM co-culture and monocultures when stimulated with $PGF_{2\alpha}$. HMSMCs (2×10^5 cells/mL) and hFM (6mm) were cultured alone (monoculture) or together (co-culture) in duplicate for 6 h, treated for 6 h with serum-free DMEM F12 with or without 10μM $PGF_{2\alpha}$. Cytokine/chemokine protein concentrations were measured via multiplex, data are presented as concentration output (pg/mL), mean ± SEM. N=6 for IL-6, CXCL1, IL-10, IL-8, CCL2, n=3 for remaining cytokines. Two-way ANOVA was performed on $\log_{10}$-transformed data. The data presented here all show the same statistical significance: culture condition had a statistically significant effect on concentration output for every cytokine, $^{**}p<0.01$, but the effect of $PGF_{2\alpha}$ was not statistically significant $p>0.05$.

	HMSMC (pg/mL)		hFM (pg/mL)		Co-culture (pg/mL)	
Cytokine	**Control**	**$PGF_{2\alpha}$**	**Control**	**$PGF_{2\alpha}$**	**Control**	**$PGF_{2\alpha}$**
IL-6	221.7 ± 118.8	449.6 ± 246.9	1861.8 ±1059.8	604.4 ± 148.7	11554.4 ± 4375.8	10638.1 ± 3141.2
CXCL1	83.1 ± 36.1	80.5 ± 26.4	839.5 ± 189.8	755.3 ± 299.3	4714.7 ± 724.3	5059.9 ± 1029.6
IL-10	1.0 ± 0.8	1.8 ± 1.2	12.7 ± 3.7	9.8 ± 2.3	41.7 ± 12.8	36.3 ± 10.6
GM-CSF	145.3 ± 6.7	147.5 ± 7.4	154.3 ± 14.3	167.0 ± 17.3	214.7 ± 33.7	211.4 ± 18.2
CXCL5	253.2 ± 39.8	257.6 ± 37.2	869.8 ± 247.0	617.7 ± 59.3	3090.3 ± 692.7	2714.0 ± 535.0
CCL11	4.5 ± 2.0	5.0 ± 1.5	16.7 ± 4.5	11.3 ± 0.3	41.8 ± 8.7	38.1 ± 5.2
CXCL2	2.7 ± 2.7	5.7 ± 2.9	38.9 ± 18.7	17.7 ± 6.8	394.2 ± 188.8	303.8 ± 74.4
IFNγ	1.9 ± 1.2	0.8 ± 0.7	8.3 ± 3.5	6.0 ± 1.4	23.0 ± 5.6	23.7 ± 4.0
IL-2	0.8 ± 0.4	1.0 ± 0.3	3.1 ± 1.0	1.9 ± 0.3	6.5 ± 1.4	5.4 ± 0.8
IL-16	1.5 ± 1.5	2.8 ± 2.8	32.8 ± 7.6	18.4 ± 8.3	66.8 ± 16.8	69.3 ± 8.3
CCL20	< 0.1	0.3 ± 0.3	6.1 ± 4.3	1.8 ± 0.4	17.0 ± 10.2	12.6 ± 4.1
IL-1β	0.1 ± 0.06	0.2 ± 0.2	2.4 ± 1.0	1.1 ± 0.5	6.7 ± 2.0	5.1 ± 1.1
CXCL12	22.2 ± 11.5	21.4 ± 14.1	56.5 ± 6.4	53.2 ± 6.3	108.6 ± 20.6	110.0 ± 10.6
CCL21	21.2 ± 4.4	28.0 ± 6.8	291.2 ± 181.8	64.5 ± 11.7	162.8 ± 3.0	160.3 ± 26.4
CCL27	0.4 ± 0.2	0.9 ± 0.6	4.9 ± 2.1	2.5 ± 0.3	12.7 ± 2.2	11.4 ± 2.4
IL-4	1.1 ± 0.8	1.3 ± 1.3	4.5 ± 1.5	3.4 ± 0.2	11.1 ± 2.7	10.4 ± 0.7
CCL1	5.3 ± 5.3	7.6 ± 4.6	18.0 ± 4.2	15.6 ± 1.1	44.8 ± 12.6	36.8 ± 4.9
CX3CL1	5.0 ± 1.6	5.8 ± 1.9	52.1 ± 31.5	16.7 ± 4.5	105.0 ± 43.0	74.9 ± 17.8
CCL19	3.9 ± 1.8	3.6 ± 2.5	19.2 ± 1.4	19.8 ± 5.9	44.4 ± 8.3	52.9 ± 10.3
CCL23	0.9 ± 0.4	1.8 ± 0.9	20.0 ± 13.9	10.8 ± 4.1	45.5 ± 18.9	47.8 ± 19.7
CCL7	16.7 ± 2.6	14.5 ± 3.2	20.5 ± 4.2	17.6 ± 1.7	602.4 ± 465.2	305.8 ± 213.2
CCL17	0.6 ± 0.2	0.9 ± 0.9	4.2 ± 2.0	2.3 ± 0.1	10.3 ± 2.2	9.3 ± 1.5
CCL25	8.0 ± 4.6	14.7 ± 6.7	46.4 ± 14.5	37.1 ± 7.6	115.0 ± 26.6	83.3 ± 4.8
CXCL11	< 0.05	0.06 ± 0.02	0.7 ± 0.2	0.3 ± 0.05	1.7 ± 0.3	1.2 ± 0.3
CXCL16	1.3 ± 0.3	1.3 ± 0.4	58.5 ± 10.5	51.5 ± 3.4	79.0 ± 16.2	66.7 ± 16.9
CXCL10	4.4 ± 2.5	5.5 ± 1.3	104.2 ± 77.8	13.6 ± 2.4	115.3 ± 32.2	37.3 ± 9.4
MIF	410.7 ± 53.3	798.0 ± 461.6	1651.8 ± 845.5	1128.1 ± 309.5	1598.8 ± 414.2	1476.9 ± 348.3
CXCL9	< 1.1	< 1.1	253.6 ± 190.6	40.3 ± 16.8	245.6 ± 167.1	47.2 ± 16.9

CCL26	4.0 ± 0.4	3.2 ± 0.6	4.1 ± 0.4	4.3 ± 0.5	6.7 ± 0.8	6.5 ± 0.9
CCL8	1.3 ± 0.8	1.5 ± 1.0	75.7 ± 47.4	26.8 ± 2.5	169.9 ± 34.0	136.5 ± 45.2
IL-8	641.8 ± 456.6	416.0 ± 169.7	22180.0 ± 10591.8	12060.5 ± 2649.2	473467.5 ± 252485.8	1236074.0 ± 932589.9
TNFα	4.6 ± 3.0	4.9 ± 3.0	16.7 ± 8.5	7.9 ± 0.4	206.4 ± 87.4	90.0 ± 25.7
CCL15	3.2 ± 0.5	3.6 ± 0.1	15.1 ± 4.1	12.8 ± 1.0	33.5 ± 4.4	29.4 ± 4.2
CCL3	1.8 ± 0.6	1.7 ± 0.2	41.4 ± 12.4	30.5 ± 9.5	397.9 ± 68.7	264.2 ± 41.0
CXCL6	3.6 ± 3.4	6.3 ± 5.3	6.3 ± 2.8	4.7 ± 1.3	87.9 ± 26.5	92.8 ± 27.3
CCL13	1.2 ± 0.5	1.5 ± 0.8	20.6 ± 9.5	11.5 ± 1.2	175.8 ± 76.0	119.9 ± 45.7
CXCL13	< 0.1	< 0.1	0.3 ± 0.2	0.1 ± 0.08	1.4 ± 0.2	0.9 ± 0.2
CCL22	< 0.5	1.3 ± 1.3	7.6 ± 4.0	2.6 ± 1.4	20.0 ± 7.3	14.8 ± 3.8
CCL24	95.0 ± 1.8	93.8 ± 2.5	82.2 ± 4.2	100.6 ± 7.8	98.7 ± 4.7	100.1 ± 8.9
CCL2	445.3 ± 266.7	404.8 ± 267.3	2605.3 ± 810.4	1451.7 ± 171.1	18684.8 ± 7329.3	9828.0 ± 3823.8

Table 5.4. Concentration outputs of 39 cytokines and chemokines in HMSMC and hFM monocultures and HMSMC/hFM co-culture when stimulated with IL-1β. HMSMCs (2 x 10^5 cells/mL) and hFM (6mm) were cultured alone (monoculture) or together (co-culture) for 6 h in duplicate, treated for 6 h with serum-free DMEM F12 or 5 ng/mL IL-1β. Cytokine/chemokine protein concentrations were measured via multiplex, data are presented as concentration output (pg/mL), mean ± SEM. N=6 for IL-6, CXCL1, IL-10, IL-8, CCL2, n=3 for remaining cytokines. Two-way ANOVA statistical testing on $\log_{10}$-transformed data. Culture condition had a statistically significant effect on concentration output for every cytokine, $^{**}p<0.01$, but the statistical output representing the main effect of IL-1β is displayed here: $^{***}p<0.001$: IL-6, $^{**}p<0.01$: IL-10, CCL11, IL-16, $^{*}p<0.05$: CXCL1, GM-CSF, CXCL2, IFNγ, IL-2, CCL20, $p>0.05$: all other cytokines.

	HMSMC (pg/mL)		hFM (pg/mL)		Co-culture (pg/mL)	
Cytokine	**Control**	**IL-1β**	**Control**	**IL-1β**	**Control**	**IL-1β**
IL-6	221.7 ± 118.8	1337.5 ± 609.5	1861.8 ±1059.8	2904.6 ± 328.4	11554.4 ± 4375.8	36862.2 ± 17216.0
CXCL1	83.1 ± 36.1	444.5 ± 152.4	839.5 ± 189.8	1618.3 ± 409.7	4714.7 ± 724.3	10048.7 ± 2167.8
IL-10	1.0 ± 0.8	6.0 ± 2.2	12.7 ± 3.7	17.1 ± 2.3	41.7 ± 12.8	44.5 ± 10.9
GM-CSF	145.3 ± 6.7	153.2 ± 14.8	154.3 ± 14.3	198.6 ± 10.9	214.7 ± 33.7	300.7 ± 46.0
CCL11	4.5 ± 2.0	11.0 ± 3.7	16.7 ± 4.5	21.9 ± 2.3	41.8 ± 8.7	52.5 ± 5.3
CXCL2	2.7 ± 2.7	34.4 ± 31.1	38.9 ± 18.7	158.8 ± 74.3	394.2 ± 188.8	1078.1 ± 318.0
IFNγ	1.9 ± 1.2	6.9 ± 0.8	8.3 ± 3.5	15.6 ± 1.9	23.0 ± 5.6	39.4 ± 4.6
IL-2	0.8 ± 0.4	1.7 ± 0.7	3.1 ± 1.0	4.1 ± 0.3	6.5 ± 1.4	8.6 ± 0.8
IL-16	1.5 ± 1.5	11.0 ± 4.8	32.8 ± 7.6	56.5 ± 11.7	66.8 ± 16.8	106.1 ± 8.0
CCL20	< 0.1	2.9 ± 2.0	6.1 ± 4.3	8.8 ± 2.3	17.0 ± 10.2	44.8 ± 18.1
CXCL12	22.2 ± 11.5	38.9 ± 10.9	56.5 ± 6.4	79.6 ± 5.5	108.6 ± 20.6	150.8 ± 7.5
CCL21	21.2 ± 4.4	37.5 ± 7.1	291.2 ± 181.8	106.3 ± 14.5	162.8 ± 3.0	205.0 ± 16.8
CCL27	0.4 ± 0.2	1.0 ± 0.6	4.9 ± 2.1	5.8 ± 0.5	12.7 ± 2.2	14.4 ± 2.2
IL-4	1.1 ± 0.8	2.6 ± 1.2	4.5 ± 1.5	7.7 ± 0.8	11.1 ± 2.7	15.6 ± 2.1
CCL1	5.3 ± 5.3	15.3 ± 5.7	18.0 ± 4.2	26.8 ± 3.3	44.8 ± 12.6	47.0 ± 3.3
CX3CL1	5.0 ± 1.6	14.0 ± 8.0	52.1 ± 31.5	34.2 ± 6.0	105.0 ± 43.0	179.8 ± 45.1
CCL19	3.9 ± 1.8	9.8 ± 4.8	19.2 ± 1.4	29.7 ± 6.8	44.4 ± 8.3	110.3 ± 33.9
CCL23	0.9 ± 0.4	2.6 ± 2.0	20.0 ± 13.9	18.8 ± 5.2	45.5 ± 18.9	49.8 ± 26.3
CCL7	16.7 ± 2.6	25.7 ± 8.0	20.5 ± 4.2	34.3 ± 5.3	602.4 ± 465.2	600.1 ± 463.3
CCL17	0.2 ± 0.2	2.0 ± 1.5	4.2 ± 2.0	4.8 ± 0.6	10.3 ± 2.2	12.1 ± 1.8
CCL25	8.0 ± 4.6	22.1 ± 9.4	46.4 ± 14.5	69.3 ± 11.4	115.0 ± 26.6	152.2 ± 14.8
CXCL11	< 0.05	0.08 ± 0.08	0.7 ± 0.2	0.6 ± 0.03	1.7 ± 0.3	2.2 ± 0.5
CXCL16	1.3 ± 0.3	2.0 ± 0.6	58.5 ± 10.5	50.4 ± 10.2	79.0 ± 16.2	75.5 ± 16.1
CXCL10	4.4 ± 2.5	10.5 ± 2.9	104.2 ± 77.8	30.6 ± 1.8	115.3 ± 32.2	97.2 ± 19.6
MIF	410.7 ± 53.3	897.0 ± 458.9	1651.8 ± 845.5	2199.9 ± 532.6	1598.8 ± 414.2	2879.6 ± 987.2
CXCL9	< 1.1	2.6 ± 0.9	253.6 ± 190.6	67.4 ± 15.1	245.6 ± 167.1	119.3 ± 60.8
CCL26	4.0 ± 0.4	3.0 ± 0.9	4.1 ± 0.4	4.9 ± 0.2	6.7 ± 0.8	7.8 ± 0.9

CCL8	1.3 ± 0.8	1.8 ± 1.2	75.7 ± 47.4	63.3 ± 9.5	169.9 ± 34.0	194.5 ± 47.5
IL-8	641.8 ± 456.6	1855.7 ± 815.2	22180.0 ± 10591.8	34818.9 ± 11607.4	473467.5 ± 252485.8	594710.4 ± 341999.9
TNFα	4.6 ± 3.0	23.3 ± 21.0	16.7 ± 8.5	16.8 ± 1.9	206.4 ± 87.4	142.1 ± 42.4
CCL15	3.2 ± 0.5	4.2 ± 0.9	15.1 ± 4.1	21.7 ± 4.6	33.5 ± 4.4	38.9 ± 2.5
CCL3	1.8 ± 0.6	3.4 ± 1.8	41.4 ± 12.4	63.4 ± 6.3	397.9 ± 68.7	299.6 ± 24.6
CXCL6	3.6 ± 3.4	6.6 ± 4.5	6.3 ± 2.8	9.6 ± 2.4	87.9 ± 26.5	83.9 ± 11.7
CCL13	1.2 ± 0.5	4.4 ± 3.3	20.6 ± 9.5	18.1 ± 3.4	175.8 ± 76.0	202.9 ± 52.1
CXCL13	< 0.1	< 0.1	0.3 ± 0.2	0.1 ± 0.06	1.4 ± 0.2	1.9 ± 0.3
CCL22	< 0.5	2.7 ± 2.7	7.6 ± 4.0	7.2 ± 1.7	20.0 ± 7.3	21.0 ± 2.3
CCL24	95.0 ± 1.8	86.4 ± 8.8	82.2 ± 4.2	90.6 ± 3.3	98.7 ± 4.7	96.2 ± 4.0
CCL2	445.3 ± 266.7	1478.0 ± 620.5	2605.3 ± 810.4	3077.3 ± 434.5	18684.8 ± 7329.3	24381.9 ± 9417.6

5.3.6. Co-culture of HMSMC with term non-labouring (TNL) hFM versus term labouring (TL) hFM.

As both HMSMC and hFM were isolated from elective caesarean sections (TNL), we repeated the experiment using the same TNL HMSMC but instead paired the cells with hFM extracted from spontaneous term labouring births (TL) to investigate whether this change influenced the upregulation of *IL6* and *COX2*. HMSMC co-cultured with TNL hFM explants for 24h increased *COX2* 25.1-fold from HMSMC monocultures, whereas HMSMC co-cultured with TL hFM explants increased *COX2* 23.5-fold (Fig. 5.9). HMSMC with TNL hFM increased *IL6* 25.4-fold and HMSMC with TL hFM increased *IL6* 48.3-fold from their respective HMSMC monoculture controls. *FP* and *OTR* myometrial mRNA expression did not change significantly whether co-cultured with TNL or TL hFM.

There were no basal differences in mRNA abundance of *COX2*, *IL6*, *FP* or *OTR* in hFM explants cultured alone from TNL and TL placentas (Fig. 5.10). Fetal membrane *IL6* and *COX2* upregulation with co-culture was slightly less in TL hFM up-regulating *COX2* and *IL6* 12.9-fold and 4.4-fold instead of 15.5-fold and 7.3-fold, respectively (NS). *FP* and *OTR* in hFM did not change when co-cultured with HMSMC, whether hFM were extracted from TNL or TL placentas.

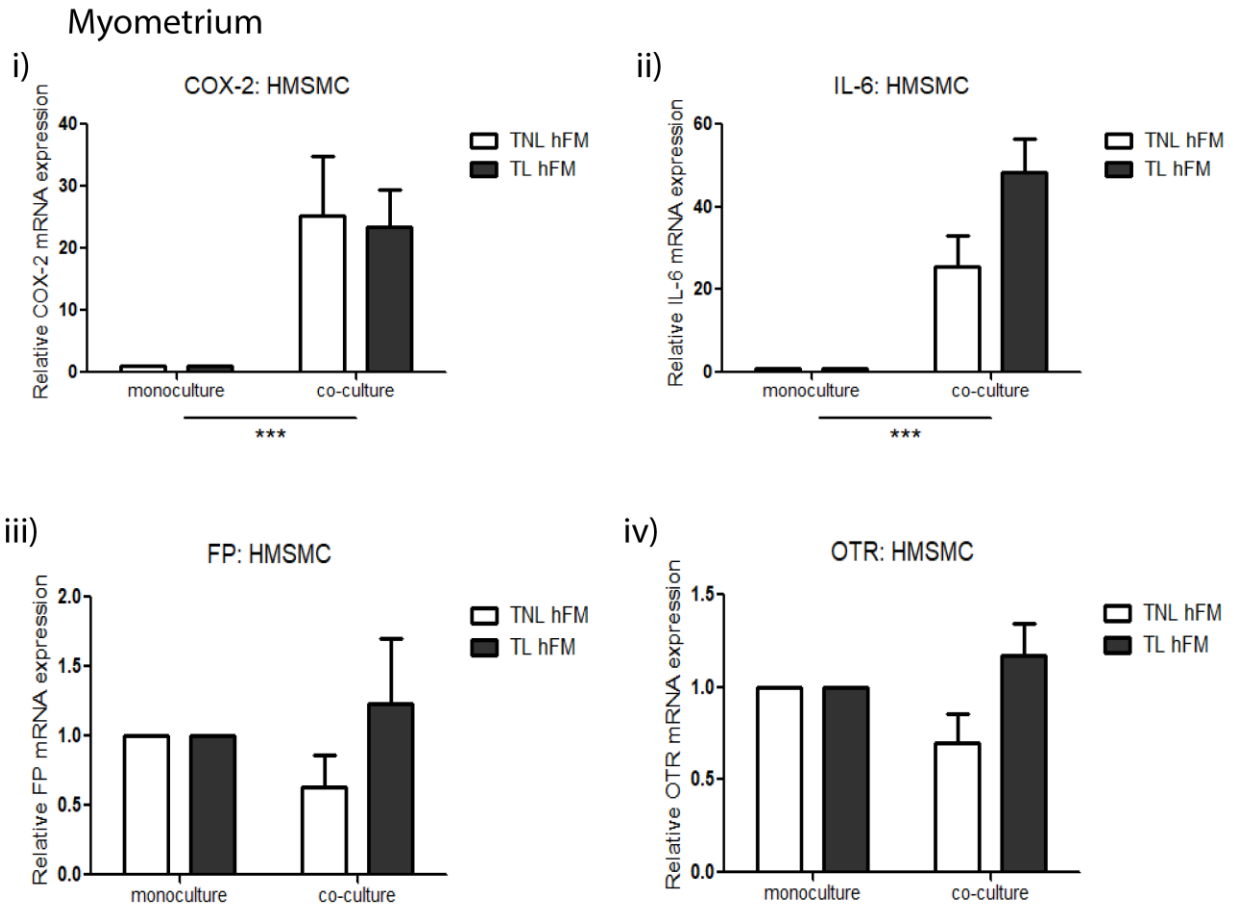

Figure 5.9. *COX2* (i), *IL6* (ii), *FP* (iii) and *OTR* (iv) mRNA in HMSMC monocultures and HMSMC/hFM co-cultures using hFM extracted from term non-labouring (TNL) placentas collected from elective caesarean sections or term labouring (TL) placentas collected from spontaneous deliveries. HMSMC (2 x 10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture) for 24 h. Messenger RNA was quantified using qRT-PCR in each tissue; target gene mRNA levels were normalized to housekeeping gene GAPDH. Data are presented as relative change (x-fold) from the monoculture control, mean ± SEM. N=5, two-way ANOVA was performed on $\log_{10}$-transformed data, ***p<0.001.

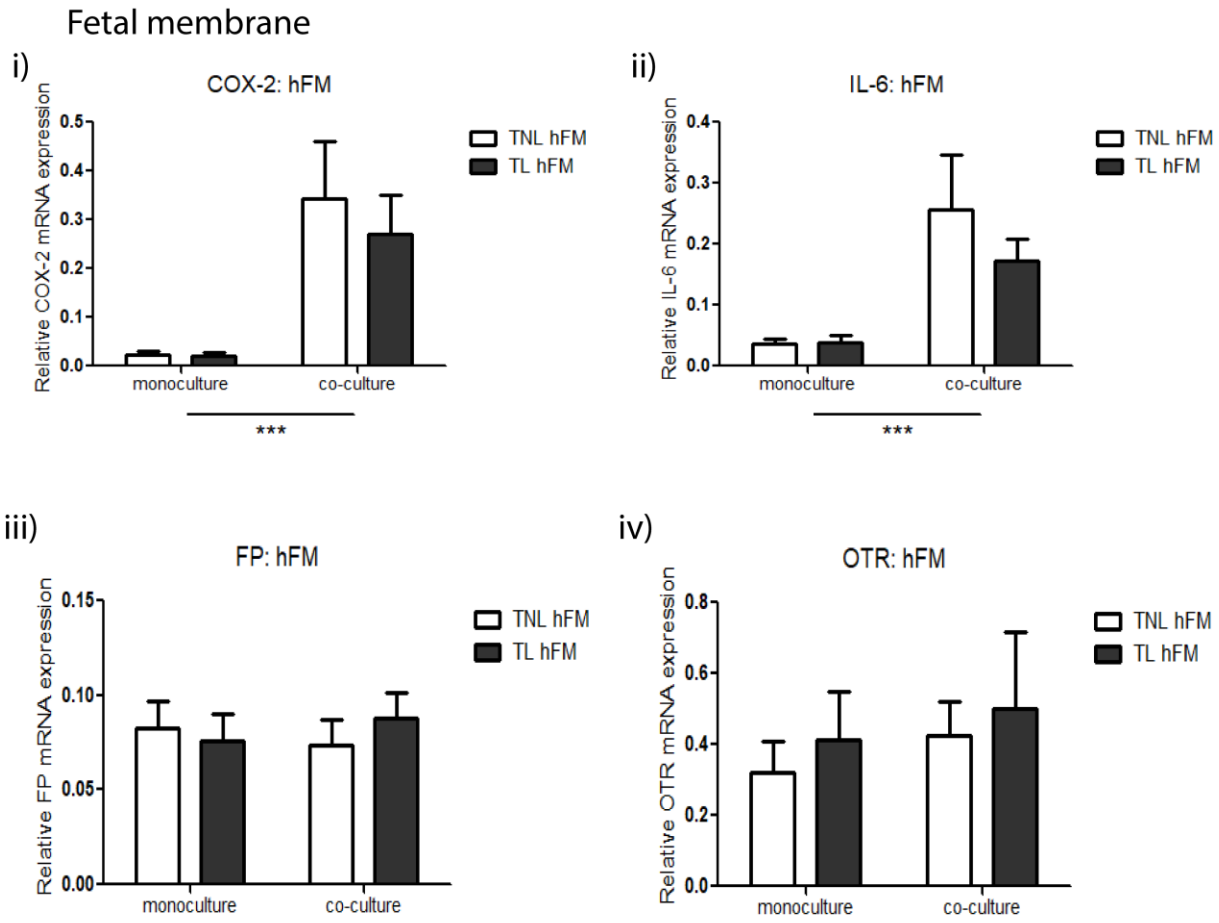

Figure 5.10. *COX2* (i), *IL6* (ii), *FP* (iii) and *OTR* (iv) mRNA in hFM monocultures and HMSMC/hFM co-cultures using hFM extracted from term non-labouring (TNL) placentas collected from elective caesarean sections or term labouring (TL) placentas collected from spontaneous deliveries. HMSMC (2 x 10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture) for 24 h. Messenger RNA was quantified using qRT-PCR in each tissue; target gene mRNA levels were normalized to housekeeping gene GAPDH. Data are presented as relative change (x-fold) from a pooled sample, mean ± SEM. N=5, two-way ANOVA was performed on $\log_{10}$-transformed data, ***p<0.001.

5.4 Discussion

To our knowledge, this is the first demonstration of co-culturing of human intrauterine tissues comprising maternal myometrium and fetal chorion and amnion. Our primary finding is that mRNA abundance and protein output of many cytokines and chemokines is considerably amplified when the tissues are co-cultured in comparison to when the tissues are separately cultured. The data strongly suggest that a tissue 'cooperativity' exists within the intrauterine tissues that significantly increase the levels of pro-inflammatory mediators. We surmise that, if similar events occur *in vivo*, this large increase in mediators could reflect a critical event in the transition of the uterus from the state of pregnancy to the state of parturition.

Every birth, whether term or preterm, is an inflammatory event. Infection is associated with only 11% of all preterm births and about 2% of term births (26), hence in the majority of cases the inflammatory events of parturition occur without an infectious process (26, 27). In most cases at term, it appears that DAMPs released by the ageing placenta and fetal membranes and the increasingly physiologically stressed uterus (28) promote expression of pro-inflammatory cytokines, chemokines, prostaglandins and their receptors via stimulation of toll-like receptors (29-31). These events lead to the physiological change, or *transition,* of the uterus from the state of pregnancy to the state of parturition or delivery characterized by increased myometrial contractile activity. Human microarray studies support this concept that the transition to labour is an inflammatory event and not limited to a single tissue (32-36). There is a substantial change in gene expression at term labour in human myometrium, with 86% of the altered pathways involving inflammation, leukocyte movement, intercellular communication and cytokine signalling (33, 34). The activation of immune pathways dominates changes to the choriodecidual transcriptome for term labour resulting in 796 altered genes (35). Although term labouring fetal

membranes upregulate genes involved in leukocyte recruitment, no inflammatory gene expression changes occur with labour in peripheral blood (36). These studies affirm that uterine transition for labour is not limited to the uterine musculature alone, but rather encompasses multiple gestational tissues and paracrine interactions in a localized intrauterine inflammatory response. As hFM contains enzymes for steroid hormone metabolism and produces growth factors and cytokines *in vivo*, the tissues are likely a determinant of myometrial transitioning and contractility. We paired term hFM explants with HMSMC, a primary cell model that our group has validated to model the myometrium *in vitro* (134, 135, 316), Leimert *et al.* 2018, submitted).

Placing hFM explant transwells into culture wells containing HMSMC had an immediate and expansive pro-inflammatory effect without any exogenous stimulation; after only 6 h, the co-cultures had released synergistic outputs of 18 different cytokines and chemokines, and those outputs increased further in 24h. The highest response levels (>15x higher than the sum of the monoculture outputs) were exhibited by CCL7, CXCL6, CCL20, CCL8, IL-8 (CXCL8), CXCL2 and IL-6. Three of these mediators, IL-8, IL-6 and CCL7, also had the highest total concentration outputs alongside CCL2, CXCL1 and CXCL5. Interestingly, microarray analysis of the myometrium also characterized IL-8 and CXCL6, which share homology, as the most upregulated genes for labour (33). Gene ontology biological process categories identified six genes that were upregulated in concert (by 5 to 6.5-fold) in term labour in fetal membranes (36); these six were all CXC chemokines involved in neutrophil recruitment, including IL-8, CXCL6, CXCL1, CXCL2, CXCL3 and CXCL5, a very similar list to what we see upregulated by our co-culture model (36). The upregulation of chemokines also dominated gene changes in myometrium and cervix (34), and CC chemokines that increase with labour (involved in migration of monocytes, eosinophils, basophils and T lymphocytes) were also upregulated by co-

culture (34-36). Therefore, the most profound genetic changes measured in the intrauterine environment at term labour are upregulated *in vitro* by the crosstalk between term hFM and HMSMC, corroborating that hFM/HMSMC co-culture is a valid model for gestational transition to labour. The upregulated cytokines and chemokines may possibly interact for further inflammatory amplification; IL-8 synergizes with CC and CXC chemokines, including CCL2, to induce increased chemotaxis (334), and CXCL6 synergizes with CCL2 in gastrointestinal tumors to increase neutrophil migration by more than 10-fold (333).

Coinciding with the increase in chemotactic chemokines in reproductive tissues nearing parturition, peripheral maternal leukocytes upregulate markers of functional activation and extravasate into intrauterine tissues (63, 232, 348-350). In rats, increased chemotactic ability of peripheral leukocytes coincides with increased expression of *Ccl2* (348), and CCL2 increases expression of integrins involved in facilitation of leukocyte migration (351). In addition to the chemotactic roles of CXC and CC chemokines, IL-6 has been identified as a key regulator of T cell populations in the pregnant decidua in preparation for parturition (310). Mice with a null mutation in *Il6* had decreased numbers of CD8+Foxp3+ regulatory T cells and an increase in IL9+CD4+ Th9 cells; both populations recovered with exogenous recombinant IL-6 treatment (310). Additionally, mice with a null mutation in *Il6* deliver 24h later than wild-type mice due to a 24h delay in the upregulation of UAPs (306), confirming a rate-limiting role for IL6 in the transition to labour. In HMSMC/hFM co-culture, *IL6* mRNA was highly upregulated in each tissue as well as IL-6 protein release into cell culture supernatant. Uterine myocytes cultured in the presence of primary monocytes isolated from peripheral blood samples of term pregnant women also synergistically release IL-6 and IL-8 (312), demonstrating another circumstance of tissue co-culture resulting in a greater inflammatory effect. CRH-induced upregulation of

myometrial UAPs CX-43, OTR, and FP was also further upregulated with the presence of THP-1 monocytes in co-culture (122). In addition, vascular smooth muscle cells produce synergistic outputs of IL-6 (and CCL2) when co-cultured with monocytes, an effect that was suppressed entirely with a combination of inhibitors for STAT3, p38 and NFκB, leading the authors to conclude that amplified production of IL-6 and CCL2 was mediated by IL-1, IL-6 and TNFα signalling (352). IL-6 outputs of vascular smooth muscle cell/monocyte co-cultures were also completely inhibited by combinations of indomethacin (a COX inhibitor) and statins (353). In HMSMC/hFM co-cultures, IL-1β treatment had some stimulatory effects on cytokine outputs, but not to the same degree as the monoculture responses, possibly due to co-culture upregulation of inflammatory outputs in part involving IL-1-dependent signalling mechanisms. It is likely that inflammatory amplification induced by HMSMC/hFM co-culture is not due to a single mediator but a cocktail of soluble factors. Further work is required to unravel the underlying molecular mechanisms using selective inhibitors and the analysis of intracellular pathways.

COX-2, the rate-limiting step in prostaglandin synthesis, is induced by inflammatory mediators (98, 281, 285, 354), and is responsible for changes in prostaglandin levels in the human fetal membranes amid the transition from pregnancy to labour (180, 181, 355). $PGF_{2\alpha}$ stimulated *COX2* in HMSMC monocultures and induced further *COX2* mRNA upregulation in HMSMC when co-cultured with hFM, corroborating previous results depicting a positive feedback loop between $PGF_{2\alpha}$ and COX-2 (134, 330). $PGF_{2\alpha}$ stimulation did not however increase *COX2* mRNA or cytokine outputs in hFM explants, when cultured alone or with HMSMC. This is likely due to the high metabolic activity of the chorion. PGDH, the initial enzymatic step in the predominant metabolic pathway of prostaglandins, is expressed more highly by chorion than decidua, myometrium or amnion (356-358). The chorion can be viewed

as a barrier that sequesters hFM-produced prostaglandins to prevent their access to the myometrium and decidua due to its high PGDH activity (249).

In the birth cascade, upstream inflammatory amplification increases the inflammatory load to reach the inflammatory threshold required to trigger functional progesterone withdrawal (220) and activation of the uterus for labour. The ratio of progesterone receptor isoforms (PR-A/PR-B) and the abundance and stability of PR-A are increased by inflammatory mediators including $PGF_{2\alpha}$ and IL-1β (236-238), contributing to functional progesterone withdrawal and pro-labour transitioning. Contractile mediators and uterotonic receptors, especially FP and OTR, are upregulated in the final step of the birth cascade (29, 138); in rats, OTR does not increase until just a few hours before delivery (162). For this reason, it is not surprising that mRNA expression of *FP* and *OTR* were unaltered by co-culture conditions while upstream *COX2*, *IL6* and many other cytokines and chemokines were amplified. Inflammatory mediators have differing effects on FP and OTR expression, including some of the only negative feedback mechanisms observed in uterine transition. Like many G-protein coupled receptors, $PGF_{2\alpha}$ negatively regulates its receptor FP (134), and has been shown to either have no effect (Leimert *et al.* 2018, submitted) or upregulate OTR (134). IL-1β upregulates the $PGF_{2\alpha}$ receptor FP (97, 134), and has been shown to downregulate OTR (103, 104). Rat uterine explants from late term pregnancies stimulated with IL-6 upregulated OTR, but explants from non-pregnant rats did not (105). We predicted that physiological differences exist between term non-labouring (TNL) and term labouring (TL) tissues as well, and compared the co-culture responses of TNL HMSMC paired with TL hFM to TNL hFM pairs to explore the differences in tissue crosstalk before and after spontaneous labour. Co-culture still significantly upregulated *IL6* and *COX2* from monoculture levels, but there was no significant difference in *IL6, COX2, FP,* or *OTR* mRNA

abundance in either tissue whether HMSMC were cultured with TNL vs TL hFM. As the birth cascade culminates in an endpoint of a pro-contractile and *activated* myometrium for labour, comparing co-culture outputs of TNL and TL HMSMC instead of just hFM would be especially valuable, notably in reference to uterotonic receptors like FP and OTR. Unfortunately, we are not currently able to collect those samples at our institution.

The model we have described has considerable potential for more detailed studies. First, it can be used to define the stimuli and mechanisms for the large increase in pro-inflammatory mediators. The mechanism may be similar to ovarian and testicular estrogen and testosterone synthesis, where cell cooperativity might be necessary for substrate and/or metabolizing enzymes to synthesize the final product (359). Cell cooperativity between maternal and fetal tissues may be required for the optimal synthesis of prostaglandins and growth factors such as EGF, as well as for optimal chemokine output. The metabolism of mediators, such as metabolizing $PGF_{2\alpha}$ to 13.14-dihydro $PGF_{2\alpha}$ via its chorionic metabolizing enzyme, PGDH, may be regulated by tissue cooperativity. This model could be used for studying the regulation of these factors. In addition, this model may have utility for studying the processes of autophagy, cell and tissue apoptosis and necrosis, and the production of DAMPs or PAMPs which stimulate the birth cascade and lead to term and preterm birth (26, 28). Finally, this combined primary human tissue method may be a valuable *in vitro* model for the testing of therapeutic candidates for pre-clinical trial characterization. Upstream inflammatory amplification is inherent to uterine transitioning and the birth cascade, so identifying therapeutic targets involved in this process with the potential to suppress both contraction and inflammatory amplification would be valuable in the pursuit of commercialization. Now that the co-culture model is established and we have characterized the

reciprocal stimulatory relationship between HMSMC and hFM, future work should focus on mechanism, the directionality of interactions, and the specific contributions of each tissue.

Chapter 6
General Discussion and Future Directions

6.1 Summary of most significant results

6.1.1. $PGF_{2\alpha}$ and IL-1β act cooperatively to amplify IL-6 and COX-2 in HMSMC.

We found that sequential stimulation of HMSMC by IL-1β plus $PGF_{2\alpha}$ (in either order) results in much higher upregulation of IL-6 and COX-2 than the additive effect of each agonist separately. This sequential effect appears to be exclusive to the myometrium, as hFM explants stimulated following the same treatment protocol do not demonstrate this amplification. In addition, we made the complementary discovery that IL-1β and $PGF_{2\alpha}$ each stimulate increases in IL-1 activity in term HMSMC through the upregulation of the IL-1R1 receptor and its accessory proteins. This supports our *in vivo* observations that rat uterine IL-1R1 and AcPs increase with labour (31). Since IL-1β also upregulates the $PGF_{2\alpha}$ receptor in HMSMC (97), these reinforcing observations expand our knowledge about the network of positive feedback pathways involving $PGF_{2\alpha}$ and IL-1β. They demonstrate the importance of $PGF_{2\alpha}$ and IL-1β in amplifying the inflammatory load of the uterus, a crucial element in converting the uterus from the physiological state of pregnancy to a state of parturition.

6.1.2. Crosstalk between HMSMC and hFM in co-culture induces pro-inflammatory amplification.

To our knowledge, our co-culture model is the first *in vitro* model comprised of maternal and fetal gestational tissues at term. This is not however the first time that co-culture models have been studied to address reproductive tissues. Co-culture was employed to demonstrate cell cooperativity in estrogen and testosterone synthesis between ovarian granulosa cells and testicular Sertoli cells (359), and myometrial smooth muscle cells were co-cultured with

peripheral monocytes to demonstrate cooperative inflammatory upregulation (122, 312). This co-culture method permits the free passage of medium through the mesh filter of the transwell to create a single culture supernatant allowing crosstalk between hFM and HMSMC. Our primary finding is that as a result of this tissue crosstalk, protein output of many cytokines and chemokines is considerably amplified in comparison to when the tissues are separately cultured. Of the eighteen cytokines and chemokines that were synergistically upregulated by co-culture, seven of them were upregulated more than 15x higher than the sum of the two monoculture outputs. Additionally, we measured an abundant upregulation of *COX2* mRNA expression in both tissues when cultured together without any exogenous stimulation. Co-cultures stimulated with $PGF_{2\alpha}$ or IL-1β induced additional increases in *IL6* and *COX2* mRNA expression in HMSMC but not hFM, reaching mRNA abundance levels 2-fold higher than the effect of co-culture alone. Pairing TNL HMSMC with hFM explants isolated from TL deliveries resulted in a minor suppression in *IL6* and *COX2* upregulation compared to TNL/TNL co-culture pairs. The data strongly suggest that a tissue 'cooperativity' exists within the intrauterine tissues that significantly increases the levels of pro-inflammatory mediators at term.

6.2 Significance of results

Evolution seems to have prepared the human for the contractile events of labour and the immune effects of giving birth within a single interlaced system. Instead of an individual trigger initiating birth, what we instead see are a series of increasing interactions involving both contractile and immune pathways. The MAPS concept introduced by Mitchell and Taggart depicts a series of increasing distinct parallel events that accumulate and amplify, eventually reaching a 'critical mass' endpoint that terminates with parturition. Similarly, the inflammatory

load (226, 360) or inflammatory burden (361) concepts illustrate a series of pro-inflammatory events that increase throughout gestation, accumulating until they reach a threshold in which the scales are tipped and functional progesterone withdrawal is induced resulting in labour. Both fetal membrane senescence and uterine stretch contribute to this escalating pro-inflammatory profile. Fetal membrane aging through the gestational period results in increasing amounts of oxidative stress and cell senescence, prompting the release of sterile inflammatory mediators of parturition, like DAMPs (362).

We propose that the pro-inflammatory amplification measured in HMSMC and hFM and presented in this dissertation is an intrinsic part of this accumulative process, contributing to the increasing inflammatory load or burden in late gestation until reaching that critical mass resulting in labour induction. This concept was introduced in 1995 by Olson *et al.*, who suggested that "an intrauterine communication system involving steroids, cytokines, and PGs may play a major role in coordinating the events of parturition" (38). Characterizing the key players within this pro-inflammatory network influencing contractility and uterine activation is of the utmost importance. Previous studies showed that $PGF_{2\alpha}$ and IL-1β were involved in positive feedback interactions and pro-inflammatory upregulation in the myometrium, but what we learned from the experimental work in this thesis is that a cooperative relationship occurs between these two ligands, resulting in synergistic upregulation of IL-6 and COX-2. IL-6 is involved in a range of pro-inflammatory events in parturition, and contributes to birth timing through the induction of an inflammatory phenotype accelerating parturition by increasing proportions of Th17 and T regulatory cell populations (310). Increased COX-2 activity results in the escalated synthesis of prostaglandins, which have widespread pro-labour roles.

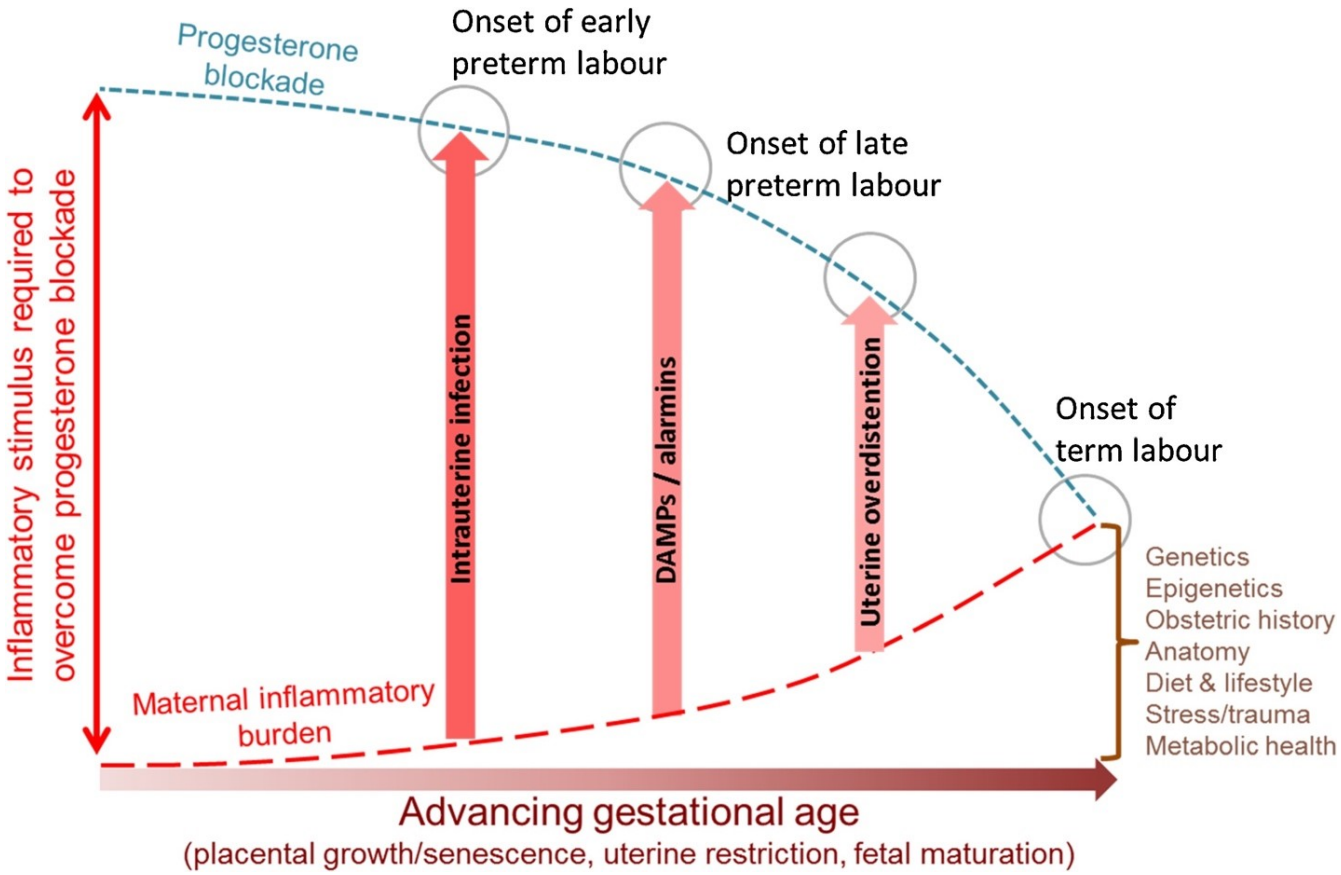

Figure 6.1. Birth timing involves inflammatory amplification overcoming the progesterone blockade and tipping the scale towards labour. Reprinted from the Journal of Reproductive Immunology, Vol 125, Author: Jeff Keelan, "Intrauterine inflammatory activation, functional progesterone withdrawal, and the timing of term and preterm birth", Pages 89-99, Copyright (2018), with permission from Elsevier (361).

Additionally, for the first time, we have shown that human myometrial cells and hFM explants communicate to drive pro-inflammatory processes. This highlights and supports the importance of paracrine interactions within the intrauterine environment at term. Of the 18 cytokines and chemokines synergistically upregulated in co-culture, CCL7, CCL20, CCL8, IL-6, IL-8, CXCL6 and CXCL2 all increased more than fifteen times higher than the sum of HMSMC and hFM monoculture outputs (Fig. 6.2). The highest total concentration outputs generated by co-cultures were CCL7, CCL2, IL-6, IL-8, CXCL1, and CXCL5. Interestingly enough, many of the mediators listed here as our top HMSMC/hFM co-culture products (either by fold-change or total concentration output) are the same or very similar to the lists of mediators that were identified as the most altered gene pathways in preparation for labour in the human microarray studies (33-36), supporting that this method is modeling pro-inflammatory amplification in uterine transition for labour. CXC chemokines are primarily involved in neutrophil recruitment, and CC chemokines are involved in migration of monocytes, eosinophils, basophils and T lymphocytes. While peripheral leukocytes are not attracted by IL-8 and CCL2 individually, in combination the chemokines induce leukocyte migration (Olson, unpublished observation), inferring that interactions between chemokines may contribute to inflammatory upregulation. This co-culture model could have great value for the future study of intrauterine tissue cooperativity relating to optimal synthesis of prostaglandins, growth factors, hormones and inflammatory markers.

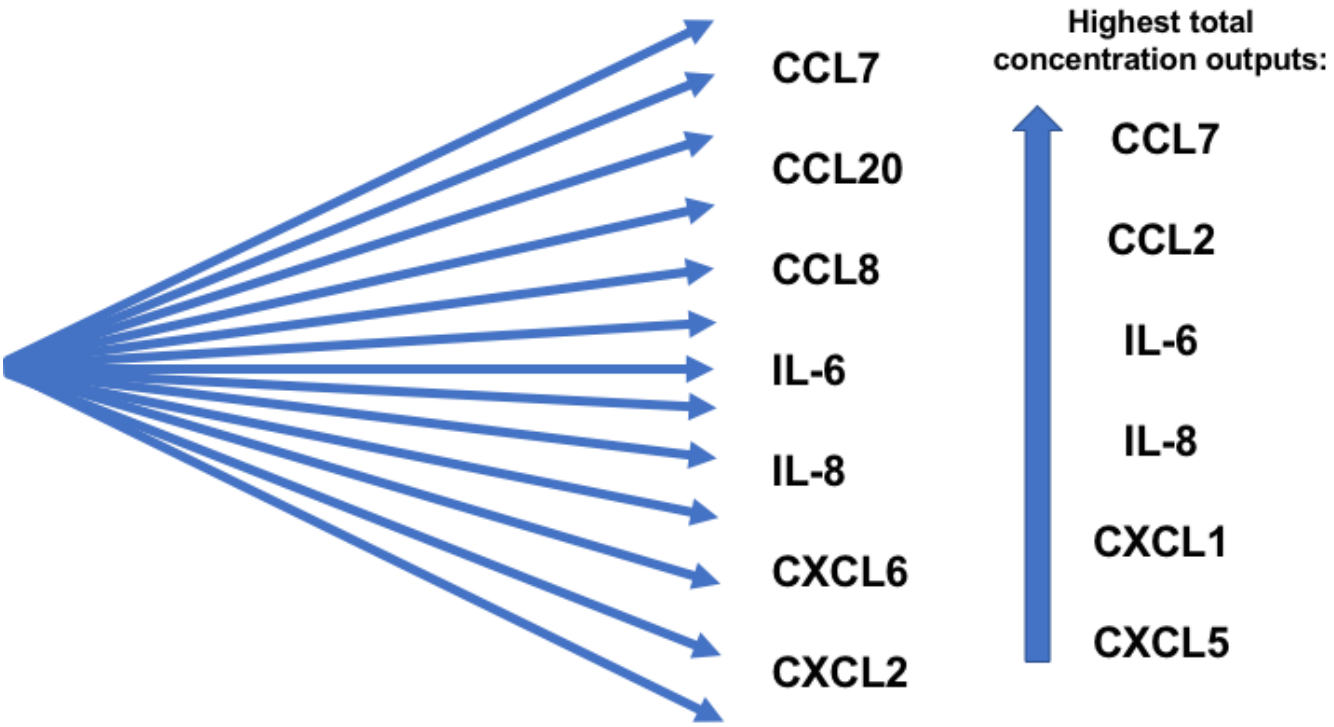

Figure 6.2. Pro-inflammatory amplification as a result of hFM/HMSMC crosstalk *in vitro.* Cytokines and chemokines listed on the left are upregulated by co-culture more than 15x higher than the sum of the individual monocultures, and mediators on the right are those with the highest total concentration outputs by co-culture.

One could depict the processes of human labour as being synonymous with a series of small gears together turning one large wheel. The small components begin to move and then pick up speed, moving quicker and quicker as upregulation continues to be amplified through intrinsic and extrinsic inflammatory interplay. As these gears turn, their motion affects neighbouring gears and initiates movement in the next grouping of wheels. This interaction represents synergy between adjacent tissues. As layer upon layer of gears turn, the speed increases and it becomes more and more difficult to stop the momentum. The cooperativity and amplification depicted in this analogy explains why we continue to fail at preventing preterm birth in humans, even when we succeed in animal models. This is not a sequence of events, but a series of parallel events occurring concurrently and gradually accumulating over time. We must slow the motion of this wheel before it reaches 'the point of no return' and its momentum becomes too strong to suppress. Current tocolytics target a wheel already rotating at top speeds and attempt to slow it down, resulting in temporary delay (or no delay) until other parallel pathways compensate for the one that is suppressed.

As the initiation of the inflammatory cascade occurs by activation of TLRs by DAMPs or PAMPs, TLRs represent an ideal target to antagonize to suppress this amplification. Wildtype mice treated with naloxone, a TLR4 antagonist, or Tlr4-null mice (*Tlr4-/-*) both deliver late due to delayed inflammatory upregulation and UAP induction (363). A broad-spectrum chemokine inhibitor, BSCI, which blocks receptors for a series of chemokines, also delays LPS-induced PTB in mice and decreases uterine inflammatory expression (364). However, the broader the inhibition, the greater the potential for possible adverse consequences for mother and child. Inhibition of TLR4 suppresses an important component of the maternal innate immune system, the body's first line of defense against foreign pathogens. Instead, this dissertation presents a

view into the extensive inflammatory amplification that involves $PGF_{2\alpha}$, IL-1β, and IL-6 (Figure 6.3). We suggest that specific allosteric modulation of these mediators results in much lower risk for adverse consequences. $PGF_{2\alpha}$, IL-1β, and IL-6 are positioned below TLRs on the birth cascade, but still upstream from current tocolytics. Due to the extent of positive feedback interactions and synergy between these pathways, suppression of the activity of one or more of these mediators may be sufficient to suppress amplification before reaching the 'critical mass' endpoint or 'inflammatory load threshold.' The optimal therapeutic targeting preterm birth would inhibit both pro-inflammatory and pro-contractile systems. $PGF_{2\alpha}$, IL-1β, and IL-6 represent points of convergence of these systems in the parturition process. One of the controversial issues regarding administration of tocolytics is their use when maternal inflammation or possibly infection is present. In this situation is it better for the fetus to be delivered or not? Some insight can be derived from mouse studies where pro-inflammatory agents (IL-1β, LPS, LTA) were used to stimulate maternal inflammation and preterm labour. 101.10, the IL-1R1 allosteric modulator, decreased the levels of maternal cytokines, blocked the preterm delivery and protected the fetuses from inflammatory harm (286, 295). Taken together, the significance of this dissertation is that therapeutic targets, including $PGF_{2\alpha}$, IL-1β, and IL-6, with the potential to suppress both contraction and inflammatory amplification and thereby blocking both PTB and fetal inflammation, will be valuable biomarkers able to assess the effectiveness of interventions that diminish maternal inflammation. These would be key efficacy indicators for the commercialization of new drugs for delaying preterm delivery and prolonging pregnancy. Our novel co-culture model, involving two term primary human intrauterine tissues, represents a valuable platform for the *in vitro* testing of potential PTB therapeutics in the future.

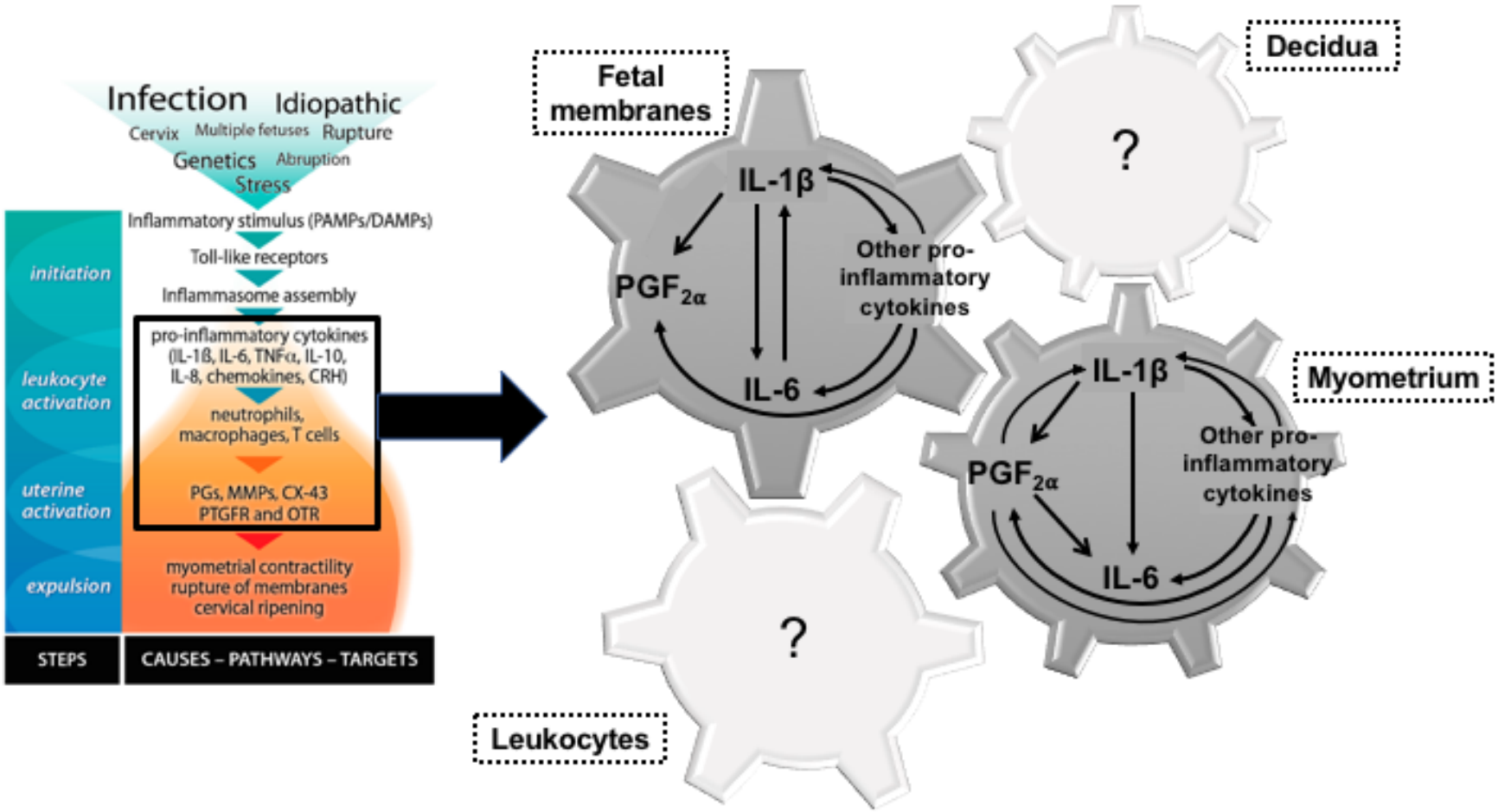
Infection
Idiopathic
Cervix
Multiple fetuses
Rupture
Genetics
Abruption
Stress
Inflammatory stimulus (PAMPs/DAMPs)
Toll-like receptors
Inflammasome assembly
pro-inflammatory cytokines (IL-1ß, IL-6, TNFα, IL-10, IL-8, chemokines, CRH)
neutrophils, macrophages, T cells
PGs, MMPs, CX-43 PTGFR and OTR
myometrial contractility rupture of membranes cervical ripening
initiation
leukocyte activation
uterine activation
expulsion
STEPS
CAUSES – PATHWAYS – TARGETS
Fetal membranes
IL-1β
PGF2α
Other pro-inflammatory cytokines
IL-6
Decidua
?
Myometrium
IL-1β
PGF2α
Other pro-inflammatory cytokines
IL-6
?
Leukocytes

Figure 6.3. Pro-inflammatory amplification is a hallmark of uterine transition involving cooperative interactions between ligands, cells, and tissues. Uterine transition can be presented as a series of small gears together turning one large wheel. The small components begin to move and then pick up speed, moving quicker and quicker as upregulation continues to be amplified through positive feedback involving $PGF_{2\alpha}$, IL-1β, and IL-6. As these gears turn, their motion affects neighbouring gears and initiates movement in the next grouping of wheels, representing synergy between adjacent tissues. This dissertation focuses on the interactions between the myometrium and fetal membranes, but the potential roles of decidua and migrating peripheral leukocytes in pro-inflammatory amplification during uterine transition should also be characterized.

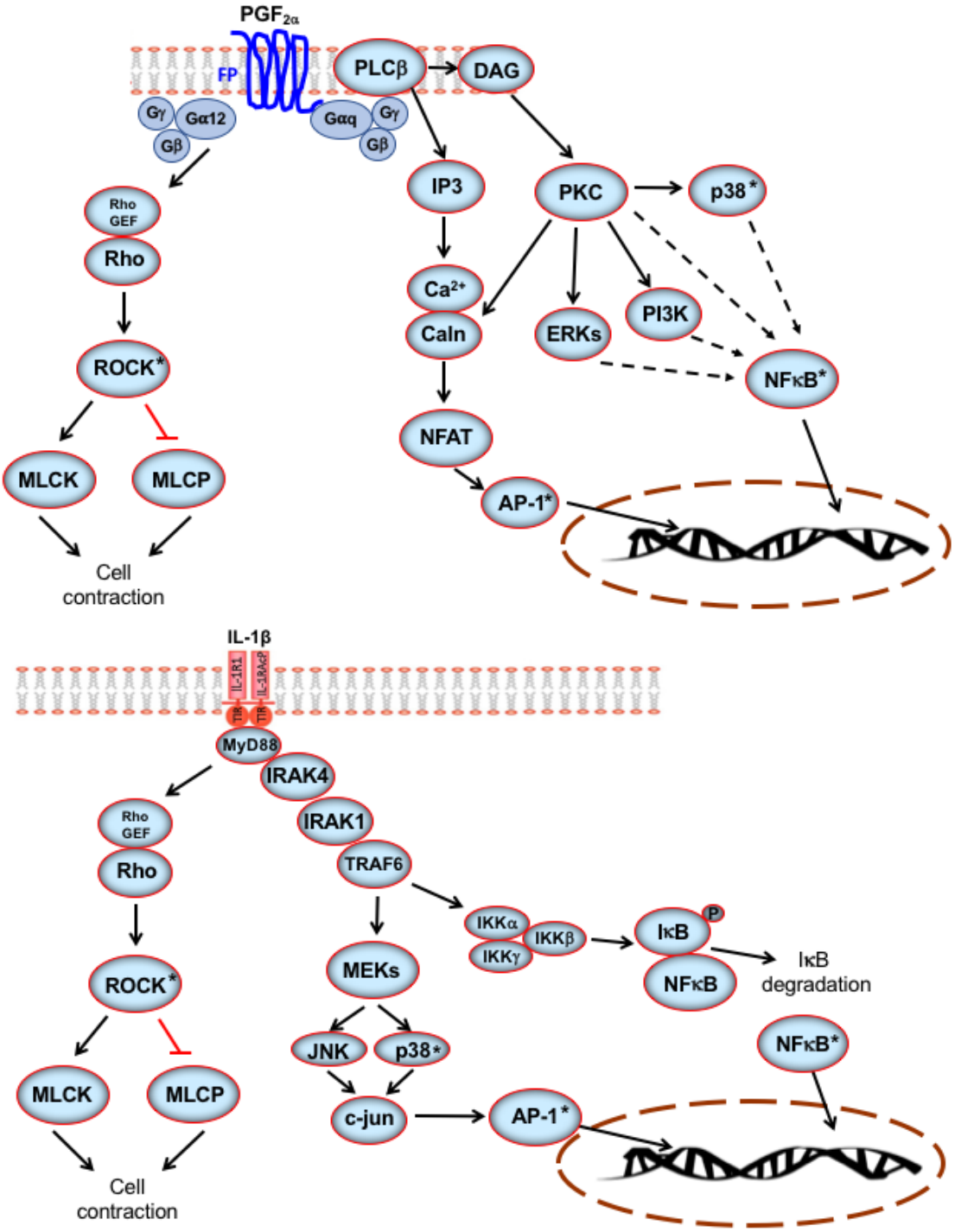

Figure 6.4. $PGF_{2\alpha}$ and IL-1β signalling in myometrium. Common signalling mediators between the two agonist-induced pathways are highlighted with an asterisk* to showcase potential interactions between the two signalling cascades.

Glucocorticoids, such as dexamethasone, are anti-inflammatory and are used to treat a multitude of inflammatory diseases. They bind GR and induce a series of anti-inflammatory genes, including IL-1ra, NFκB's inhibitor IκBα, and the IL-1 decoy receptor IL-1R2. In addition, GR has trans-repressive effects through direct binding of inflammatory transcription factors such as NFκB, AP-1 and STATs (365). Glucocorticoids are administered to women at risk of preterm birth to induce maturation of fetal organs, especially the fetal lungs. Interestingly, although glucocorticoids such as dexamethasone are anti-inflammatory, their administration has no influence on birth timing (211, 366). In a group of women delivering early as a result of preterm premature rupture of the membranes, dexamethasone increased maternal white blood cell counts but had no effect on maternal serum concentrations of infection markers (367). As dexamethasone upregulates IL-1ra and IL-1R2 and represses IL-1-induced transcription factors NFκB and AP-1, it is likely that dexamethasone would have some suppressive effects on the $PGF_{2\alpha}$ and IL-1β-induced amplification described in this thesis. However, as glucocorticoids do not influence birth timing it is likely that other regulatory systems are in place and further study is required.

6.3 Future Directions

The knowledge gained from these studies introduces several intriguing opportunities for the exploration and characterization of intrauterine paracrine interactions occurring at term. The first efforts should focus on identifying the intervening pathway steps, where convergence of pathways occurs, and the unique contributions of each tissue to the overall process.

6.3.1. Paracrine interactions in uterine transition for labour

6.3.1.i Homogeneous versus heterogeneous co-culture pairs

Human uterine transition involves a localized inflammatory response involving paracrine interactions. This is confirmed by our results that crosstalk between term HMSMC and hFM *in vitro* stimulates pro-inflammatory amplification. Uterine transition involves not only paracrine interactions between adjacent gestational tissues, but also paracrine interactions between neighbouring cells within a tissue. To further define the interactions of the paracrine system at term, and to differentiate between cellular crosstalk within a tissue and crosstalk between tissues, hFM explants should be co-cultured with hFM explants and HMSMC should be co-cultured with HMSMC. Inflammatory outputs in supernatants and UAP and cytokine receptor expression levels of each homogeneous tissue co-culture pair can then be compared to the heterogeneous pairs.

6.3.1.ii Labouring versus non-labouring tissues

We hypothesized that TNL HMSMC co-cultured with hFM explants isolated from TL deliveries would demonstrate increased pro-inflammatory amplification compared to HMSMC/TNL hFM pairs. Our hypothesis was disproved when only a small shift in expression was measured between HMSMC/TNL hFM and HMSMC/TL hFM, instead resulting in the slight suppression of *IL6* and *COX2* in the TL group. In labour physiology, the myometrium represents the endpoint and site of action in that amplification between tissues and pathways eventually accumulate, resulting in an activated uterine smooth muscle capable of expelling the fetus from the uterine cavity. Inflammatory signalling in hFM tissues at term and at term labour may not differ significantly after all. It is possible that pro-inflammatory upregulation in hFM in preparation for labour has already occurred by the time point of TNL membrane collection.

Throughout pregnancy, parturition, and uterine involution, myometrium is constantly in a state of transition. Due to the involvement of uterine smooth muscle in uterine contraction, cell culture of HMSMCs and/or myometrial explants isolated from TNL versus TL deliveries would likely exhibit physiological differences from hFM. Repetition of $PGF_{2\alpha}$/IL-1β sequential treatment experiments and hFM co-culture with paired TNL and TL myocytes would provide considerable insight into processes of uterine transition for labour. We unfortunately do not have access to myometrial biopsies from TL deliveries at our institute. The question persists whether differences exist in UAP and cytokine induction between TNL and TL myometrium once the tissue biopsies are isolated into primary cells and cultured. I addressed this question for a short 3-month period at Imperial College London in the laboratory of Prof P. Bennett where I obtained and cultured a few TL myometrial biopsies. Although the number was too few to draw hard and fast conclusions, the data indicated that indeed TL HMSMC had a heightened pro-inflammatory state compared to TNL HMSMC (Appendix). Future studies should complete this data set and delve further into the paracrine interactions involving labouring HMSMC.

6.3.1.iii The parsing of a complex whole into its simpler parts

We chose to utilize fully intact human fetal membrane explants in the HMSMC/hFM co-culture model as we desired the most representative possible impression of the *in vivo* situation. Beyond the incorporation of fully intact hFM in co-culture, we also designed the model to include a single supernatant with free passage between culture compartments to allow for constant crosstalk between tissues over the length of the treatment period. The intent was to study the overall effect of all concurrent paracrine interactions taking place during a set length of time. However, now that we have confirmed the occurrence of pro-inflammatory upregulation in response to intrauterine tissue crosstalk, it is time to facilitate separation of model components to

identify the individual contributions of each tissue layer present in hFM and characterize a directionality to these interactions.

As decidua lies directly adjacent to the myometrium, undergoes activation at term (35, 75), and has been characterized to have a central role in production of a variety of hormones, growth factors, prostaglandins and inflammatory mediators (38, 73, 76), it would be prudent to co-culture HMSMC with decidua alone to decipher the impact of crosstalk between these neighbouring maternal tissues. Additionally, the mechanical removal of decidua from hFM would result in the ability to study isolated fetal amniochorion crosstalk with maternal HMSMC, or amniochorion crosstalk with maternal decidua.

To isolate the directionality of these amplification interactions between tissues, there are a few methods in which we could direct future studies. To study tissue-tissue responses with one way directionality of treatment, conditioned media collected from cell culture of HMSMC could stimulate hFM explants, and HMSMC treated with hFM explant conditioned media. Supernatant collected from choriodecidual explants administered to HMSMC induced SP-A-dependent Rho/ROCK signalling in HMSMC, resulting in myocyte cytoskeletal rearrangements and formation of stress fibres (368). Incorporation of an Ussing chamber into the model would allow us to distinctly measure outputs from amnion and choriodecidual sides of hFM. The Ussing chamber was used by Prof. J.A. Keelan's group to separately look at amnion and chorion products as well as measure transfer function between the tissue layers (369). LPS administration to the maternal side created a cytokine chain reaction that ultimately terminated in increased cytokine production and PG outputs from the fetal side. Conditioned media collected from HMSMC culture may be administered to the maternal side of hFM, while measuring both maternal and fetal responses separately. This model would be highly valuable for the study of

potential therapeutics like 101.10, to test the outcome of maternal administration on suppressing fetal inflammation.

6.3.1.iv Mechanism for amplification

Large amounts of pro-inflammatory cytokines and chemokines are being produced by HMSMC and hFM when cultured in combination. Certainly, a series of future experiments including specific inhibitors for AP-1, NFκB, p38 MAPK, and STAT signalling would be necessary to parse the precise steps involved in this phenomenon. Co-cultures should be treated with each of these specific inhibitors separately and in combination. Transcription factor complexes have been described in the literature that are able to induce much higher levels of transcriptional induction than respective individual or pairwise effects. Assessment of activation levels of important second messengers and intracellular mediators should be measured in tissues cultured separately and tissues cultured together.

We measured concentration outputs of 40 inflammatory mediators, mRNA expression levels of *IL6*, *COX2*, *FP*, and *OTR*, and protein expression of FP and OTR in monocultures compared to co-cultures. However, these outcome measures represent the very tip of the iceberg in terms of potential involvement in this pro-inflammatory amplification at term. In the future, mRNA and protein expression abundance of sex steroid receptors such as nPRs, glucocorticoid receptor (GR), and estrogen receptors (ERs) should be quantified in both tissues, with and without the addition of a pro-inflammatory stimulus. Additionally, progesterone, estrogen, prostaglandins, growth factors, and DAMPs such as HMGB-1 and IL-1α should be measured in the shared tissue supernatant in co-culture and compared to monoculture outputs.

6.3.2. Functional Regionalization of Intrauterine Tissues

6.3.2.i Myometrium

The uterus has a unique and critical role at term, in that it must forcefully contract to successfully expel the fetus from its cavity. While the fundus or upper uterine segment is forcefully contracting, the lower uterine segment exhibits a more relaxatory phenotype in order to allow the passage of the fetus from the uterine cavity. One of the first studies introducing the functional regionalization of the myometrium at term demonstrated that upper uterine fundal myometrial strips responded to $PGF_{2\alpha}$ and PGE_2 by stimulating contraction, whereas the contraction of lower uterine strips was inhibited by PGE_2 and showed no response to $PGF_{2\alpha}$ (370). Primary HMSMC cultured from paired upper and lower segment myometrial biopsies expressed higher FP and OTR in the upper segment, but exhibited no regionalized difference in COX-2 (134). A second study using the same culture model demonstrated that lower segment HMSMC exhibited higher pro-inflammatory induction than upper segment cells, including higher HMSMC outputs of IL-6 and IL-1β (135); this study is attached in the appendix as I am included as a co-author. HMSMC studies presented in chapters 4 and 5 involve HMSMCs isolated from lower segment myometrial biopsies only. As the objectives of the study were to study pro-inflammatory amplification at term and we no longer had the access to matched upper/lower segment biopsy pairs, it was logical to utilize lower segment HMSMCs. Nonetheless it is important to keep these functional differences in mind as we consider the implications and interpretation of the work, especially as we discuss FP and OTR. It is notable that in our studies, unlike pro-inflammatory outcome measures IL-6 and COX-2, FP and OTR were not significantly upregulated by sequential IL-1β/$PGF_{2\alpha}$ treatments or co-culture with hFM. Repetition of these experiments using upper segment HMSMCs to examine whether these regionalized phenotypic differences result in different FP/OTR outcomes would be valuable to our knowledge of labour

physiology and uterine transition. It is possible that co-culture of upper segment HMSMCs with hFM explants may instead result in more upregulation of uterotonic receptors and less pro-inflammatory amplification.

6.3.2.ii hFM

Like the myometrium, the fetal membranes also exhibit functional regionalization at term. A zone of altered morphology (ZAM) or weakening is present in the fetal membrane tissues overlying the cervix, characterized by the thickening of connective tissue layers and thinning layers of chorionic trophoblasts and decidua (371, 372). The ZAM is the hFM rupture site, and is characterized by increased inflammatory gene activation and altered decidual leukocyte populations (373). In our model, hFM explants are extracted via tissue punch, and often up to 30 different explants are extracted from a single placenta. Unlike Marcellin *et al.* (373), the placentas we receive from elective caesareans are not ZAM-marked at delivery, so the location of the weakening zone remained unknown to us. Explants were excised from fully intact areas of similar thickness while avoiding proximity to the edges of the tissue. Tissue explants are placed into a container of HBSS for washing before plating in 12-well plates, which essentially randomizes the order and location of explant extraction. According to a study by Marcellin *et al.* (373), areas closer to the ZAM show increased immune activation than areas more distant from the ZAM, which could impact inflammatory outputs from hFM explants. Tissue staining of hFM explants using the same protocol, as displayed in Fig. 3.1, present a tissue with more morphological similarities to the mid-zone hFM as presented by Malek *et al*. than the ZAM hFM phenotype (371). Future experiments should include both the identification and marking of the ZAM by the operating team, and the paired culture of hFM explants isolated from areas distant from the ZAM and adjacent to the ZAM. Explants proximal to the hFM tissue overlying the

cervix would be co-cultured with lower segment HMSMC, and explants distant from the ZAM would be co-cultured with upper segment HMSMC, to better represent and explore functional regionalization at term in reference to inflammatory amplification and UAP induction.

6.3.3. Migration of peripheral leukocytes into gestational tissues

A very important part of inflammatory amplification is interaction with leukocytes that drive upregulation of a series of inflammatory mediators. Nearing labour, the release of chemoattractant by gestational tissues such as the fetal membranes (235) attracts circu lating peripheral leukocytes to the uterine tissues where they infiltrate, attach and release additional mediators. Homogenized term fetal membranes induce the migration of peripheral maternal leukocytes through a Boyden chamber. The responsiveness of migrating leukocytes to hFM is increased in peripheral blood of women in term or preterm labour compared to not in labour (350). Interestingly, chemokines involved in the chemoattraction of monocytes, neutrophils, basophils, eosinophils, T lymphocytes and more were significantly upregulated in HMSMC/hFM co-culture. Supernatant from HMSMC alone, hFM alone and HMSMC/hFM co-culture should be tested in a Boyden chamber to measure leukocyte migration. Groups of cytokines and chemokines, including IL-8, CCL2 and CXCL6 have been shown to synergistically increase leukocyte migration together compared to their individual chemotactic roles (333, 334). Tissue crosstalk between HMSMC and hFM at term strongly upregulated the aforementioned chemokines as well as many more, and may therefore induce augmented leukocyte migration as well, representing another level of intrauterine inflammatory amplification in preparation for labour.

6.4 Study Limitations

6.4.1 Primary HMSMC model

There is some controversy surrounding the phenotypic stability of passaged primary cells in culture, as under certain circumstances they can depart from their original *in vivo* phenotype the longer they are cultured or passaged. However, our group has published a characterization and validation study of this model, confirming the retention of a myometrial phenotype from passage 0 through passage 10 (316). In addition, colleagues have completed experiments testing cell passage versus outcome in HMSMC for the types of experimental questions we are asking (Appendix). Combined, these validation studies clearly demonstrated to us that expression of COX-2, FP, OTR, and CX-43 is conserved and the responsiveness to IL-1β remains consistent at the passage used for HMSMC treatment in this dissertation. As always, the most physiologically representative *in vitro* model for the myometrium is the intact tissue explant. A valuable future addition to this study would be the repetition of sequential IL-1β/$PGF_{2\alpha}$ treatments and hFM co-culture with myometrial explants instead of HMSMC. However, the incorporation of myometrial explants for that number of different treatment groups would require more myometrial tissue than is currently available from our institute. HMSMC therefore continues to be the most representative myometrial model available to us for the study of these experimental variables and outcome measures of interest.

6.4.2. In vivo study

All experimental results are based on *in vitro* passaged cells and explants. Repeating the key HMSMC experiments on freshly isolated cells or myometrial explants would present the opportunity for further validation, including the co-culture of hFM with myometrial explants. *In vivo* confirmation using animal models with specific inhibitors of $PGF_{2\alpha}$ or IL-1β action, or

combinations of inhibitors, would more precisely define the process of pro-inflammatory amplification.

6.4.3. Confirmation of mRNA data at the protein level

We assessed mRNA abundance of the IL-1 receptors and accessory proteins in response to stimulation by IL-1β or $PGF_{2\alpha}$, and found that both mediators induce the upregulation of IL-1 receptor system. However, protein level data was not completed for these outcome measures, so the interpretation is based on measurements at the mRNA level only. Our intention was to strengthen the data set with the addition of protein data via Western blotting of the IL-1 receptor system, but as IL-1RAcPb is a relatively new discovery, an antibody is not yet commercially available that can differentiate between accessory protein isoforms. Existing IL-1RAcP antibodies target a sequence common to both IL-1RAcP and IL-1RAcPb, so measurement of their individual protein expression profiles is not yet possible. Antibodies were purchased for IL-1R1 and IL-1R2, but they were such poor quality we were not able to produce any quality data. For these reasons, we decided to focus on the mRNA abundance instead.

Although we successfully quantified the synergistic upregulation of COX-2 protein in HMSMC in response to sequential $PGF_{2\alpha}$/IL-1β treatments, we were unfortunately unable to quantify COX-2 protein in the co-culture model. Cell lysate was extracted from each tissue and Western blots were performed using the identical anti-COX-2 antibody and optimized protocol. The antibody in question is a Santa Cruz antibody, which due to the presence of gelatin in solution cannot be frozen and instead must be stored long-term at 4°C. Over the years, our group (and others) have found that these antibodies work well at the start but have short lasting effects; they cease to function after one or two years, well before the expiry date. One year after

completing the COX-2 protein analysis for sequential IL-1β/$PGF_{2\alpha}$ treatments, blots of co-culture samples resulted in either entirely blank membranes or membranes with many randomly located non-specific bands, with no reproducibility from blot to blot. A new vial of the same antibody was ordered, but its production was discontinued by Santa Cruz. We tried a new anti-COX-2 antibody from a different source company (Cayman Chemicals), but after significant troubleshooting we could not produce any quality data. Next we attempted to quantify COX-2 protein through enzyme-linked immunosorbent analysis (ELISA) of the cell (HMSMC) and tissue (hFM) lysates using a prepared COX-2 ELISA kit from R&D Systems (Minneapolis, MN, USA). Unfortunately, the RIPA buffer in the cell lysates reacted with the ELISA kit and produced a matrix effect. Even after multiple dilutions, we could not reverse the matrix effect.

The co-culture samples were extracted from 12-well plates and cultivated a limited volume of HMSMC cell lysate. The completion of a series of protein assays in an endeavor to quantify COX-2 upregulation used the majority of the volume of HMSMC cell lysates. For that reason, we quantified FP and OTR protein expression in the hFM samples, but were unable to quantify myometrial FP, OTR and COX-2 or hFM COX-2. In the future, more co-culture patient sets should be completed and this protein data should be assessed. It is important to quantify protein as changes in mRNA expression are not always reflected at the protein level.

6.5 Conclusions

The series of experiments described in this dissertation together present cooperativity between ligands, cells and tissues as a hallmark of human parturition. These studies indicate, as others have proposed through the MAPS and inflammatory load hypotheses, that the

accumulation of an increasing volume of stimulatory interactions are involved in transitioning the state of pregnancy to the state of parturition.

Our work establishes $PGF_{2\alpha}$ and IL-1β as key triggers or upstream drivers of this process and IL-6 and COX-2 as key targets, promoting inflammatory amplification in the myometrium through positive feedback and synergy contributing to the facilitation of uterine transition from pregnancy to parturition. In addition, to our knowledge, this dissertation includes the first demonstration of co-culturing of human intrauterine tissues comprising maternal myometrium and fetal chorion and amnion. Our primary finding is that protein output of many cytokines and chemokines and mRNA abundance of *IL6* and *COX2* is considerably amplified when the tissues are co-cultured in comparison to when the tissues are separately cultured. The data strongly suggest that a tissue 'cooperativity' exists within the intrauterine tissues that significantly increase the levels of pro-inflammatory mediators. Mitchell and Taggart in reference to human parturition said: "Rather than seeking the trigger, experiments need to be designed to investigate synergistic interactions among a variety of physiological systems and tissues" (198). We surmise that, if similar events occur *in vivo*, this large increase in mediators could reflect a critical event in the transition of the uterus from the state of pregnancy to the state of parturition.

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Chapter 8
Appendix

Appendix 8.3.1 was originally published as "Xu C, Liu W, You X, Leimert K, Popowycz K, Fang X, Wood SL, Slater DM, Sun Q, Gu H, Olson DM, Ni X. $PGF_{2\alpha}$ modulates the output of chemokines and pro-inflammatory cytokines in myometrial cells from term pregnant women through divergent signaling pathways. *Molecular Human Reproduction*. 2015; 21(7):603-14, by permission of Oxford University Press." https://academic.oup.com/molehr/article/21/7/603/2459734.
KB Leimert contributed to cell culture and data generation.

Appendix 8.3.2 was originally published in *The Journal of Immunology*, as "Nadeau-Vallée M, Quiniou C, Palacios J, Hou X, Erfani A, Madaan A, Sanchez M, Leimert K, Boudreault A, Duhamel F, Rivera JC, Zhu T, Noueihed B, Robertson SA, Ni X, Olson DM, Lubell W, Girard S, Chemtob S. 2015. Novel noncompetitive IL-1 receptor-biased ligand prevents infection- and inflammation-induced preterm birth. *Journal of Immunology* 195(7):3402-15." Copyright © [2015] The American Association of Immunologists, Inc, http://www.jimmunol.org/content/195/7/3402.long.
KB Leimert contributed to experimental planning and data generation.

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KB Leimert contributed to data generation.

8.1 Validation of HMSMC model

One of the inherent problems with studying human myometrial cells in culture is that the amount of starting material, a small myometrial biopsy obtained following a caesarean section at term (not in labour), produces too few cells in the initial culture to perform adequately powered or controlled tests. Hence, it becomes necessary to increase the number of cells (about 2 x 10^5 cells per well are required and a minimum of 36 wells per experiment are necessary) to perform an experiment with a minimum of 2 replicate wells per treatment for RNA and protein extraction. This requires the splitting and passaging of cells to achieve a large enough number for a well-controlled experiment. However, passaging can produce its own set of problems. The dilemma that occurs with passaging of primary cells is they can change phenotypes from their original *in situ* phenotype as demonstrated in examples related to adipose-derived stem cells (374) and rheumatoid arthritis synovial fibroblasts (375). In these specific, non-myometrial cell examples, passage number altered the outcomes of the questions being asked. Therefore, being mindful of this possibility, we initially tested whether our cells changed their phenotype (in terms of the key outputs we wanted to examine) up to passage seven, which produces enough cells for a rigorous experimental protocol, before we embarked on any experimentation. Unpublished characterization studies in HMSMC were completed by Dr. Sheena Fang (Figs 8.1 and 8.2) in addition to the validation work published in BMC Pregnancy and Childbirth (Fig 8.3) (316). We determined that the phenotypic expression of enzymes, receptors and cytokines was uniform through passage 7. We then published three papers using these cells at passage 7 in excellent peer-reviewed journals (134, 135, 316).

While our cells perform uniformly, culture and passage of primary HMSMC results in the absence of progesterone receptor, which must be transfected into primary cells and all

subsequent passages as described by Georgiou *et al.* 2016 (324). In our case we are not addressing the role of progesterone or its receptor, rather, our study is specific to the actions of $PGF_{2\alpha}$ and IL-1β and their roles in inflammatory amplification and induction of UAPs. These validation studies clearly demonstrated to us that mRNA expression of the UAPs, *OTR* and *CX43*, in response to IL-1β administration is conserved from passage 2 through at least passage 7 (10 ng/mL IL-1β) (Fig. 8.1). Two different housekeeping genes were used to confirm the consistency of the qPCR results in Fig. 8.1 and 8.2, *GAPDH* (the housekeeping gene used in chapters 4 and 5) and *β-actin*. Expression of the $PGF_{2\alpha}$ receptor *FP* was also shown to be conserved in culture (Fig. 8.2), and the responsiveness of *FP* mRNA abundance to IL-1β stimulation increased with more passages. This indicates that passage 7 HMSMC would be a more sensitive model to use to study pro-inflammatory effects of $PGF_{2\alpha}$ *in vitro*. In addition to the stable expression of mRNA for the UAPs, *FP*, *OTR*, and *CX43*, our group also demonstrated that HMSMC maintain conserved pro-inflammatory responsiveness to IL-1β (1 ng/mL) from passage 1 to at least passage 10 as demonstrated by the conserved induction of *COX2* mRNA expression and pro-inflammatory cytokine protein release of IL-8 measured by ELISA assay (Fig. 8.3) (316). This is especially relevant for this dissertation, as the most abundant changes measured in HMSMC with $PGF_{2\alpha}$/IL-1β were pro-inflammatory cytokines and COX-2.

In summary, since our study is focused on the responsiveness of the myocytes to $PGF_{2\alpha}$ and IL-1β and our outcome measures include the expression of pro-inflammatory mediators and UAPs, we believe that our previously published method using passage 7 HMSMCs is quite appropriate for this type of study. The reason we passaged the cells 7 times was to obtain sufficient numbers for all the treatments in this study. Such a study would not be possible if the

cells' phenotype changes in culture restricted us to passage 2 or 3; there would not be enough cells for all the comparisons and controls required.

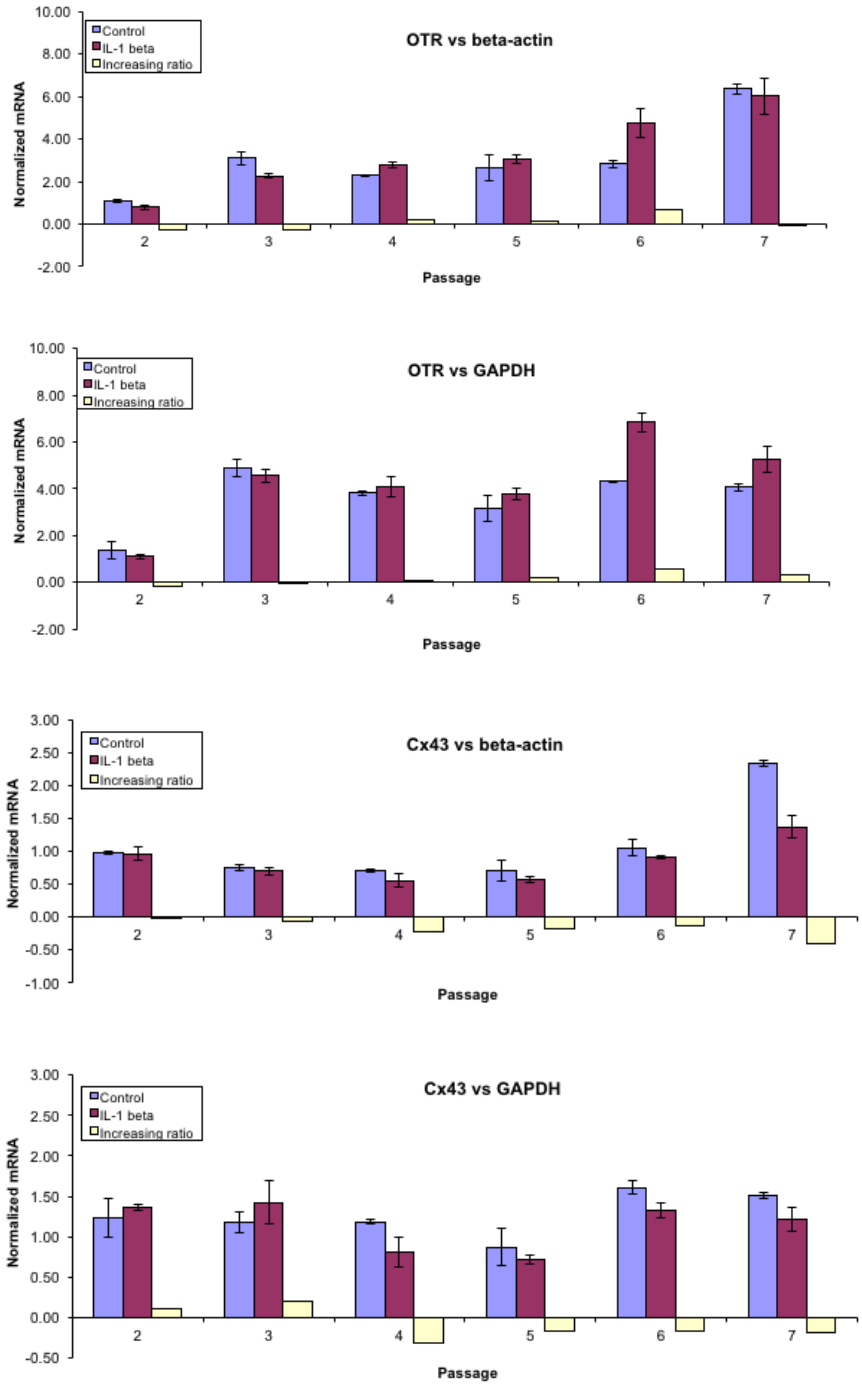

Figure 8.1. *OTR* and *CX43* mRNA expression is conserved in HMSMC culture from passages 2 through 7. HMSMC were stimulated with DMEM alone (purple bars) or DMEM with 10 ng/mL IL-1β (pink bars). Messenger RNA was normalized to two different housekeeping genes, GAPDH and β-actin, and presented relative to a pooled sample.

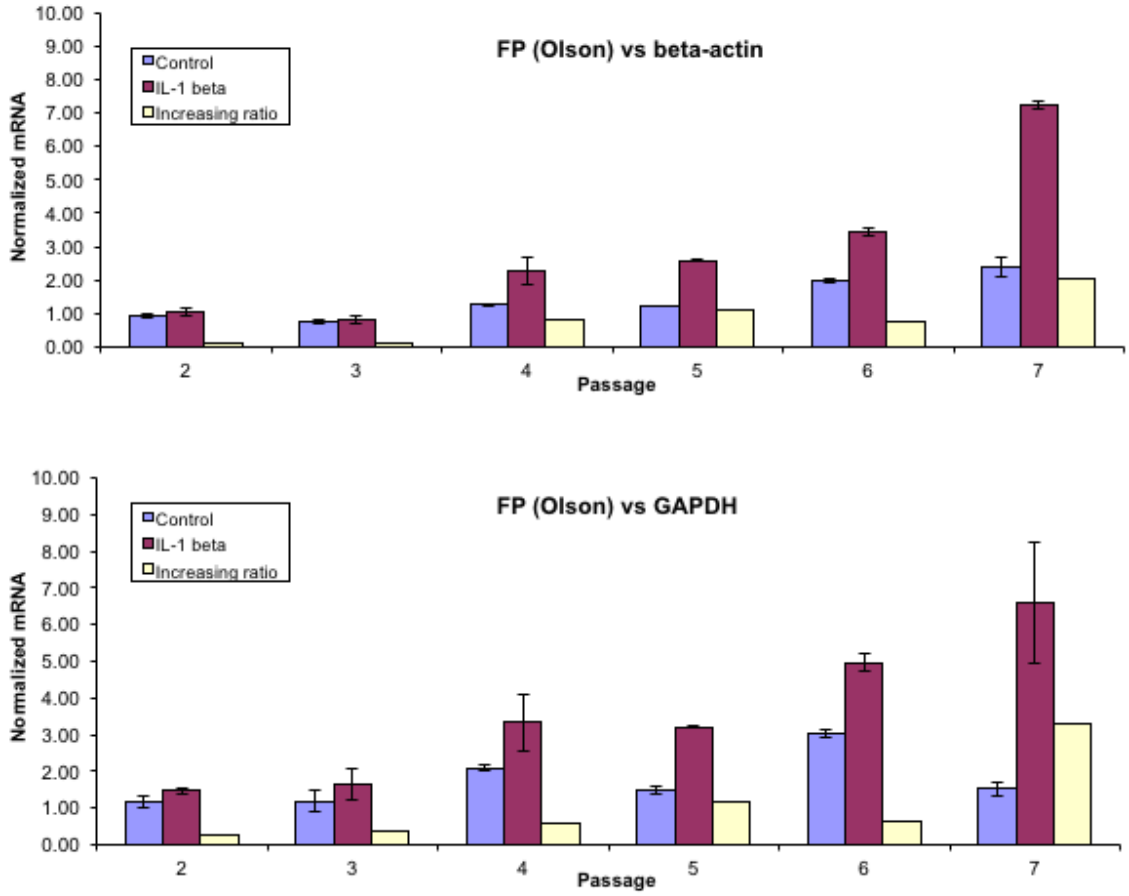

Figure 8.2. *FP* mRNA expression is conserved in HMSMC culture from passages 2 through 7. HMSMC were stimulated with DMEM alone (purple bars) or DMEM with 10 ng/mL IL-1β (pink bars). Messenger RNA was normalized to two different housekeeping genes, GAPDH and β-actin, and presented relative to a pooled sample.

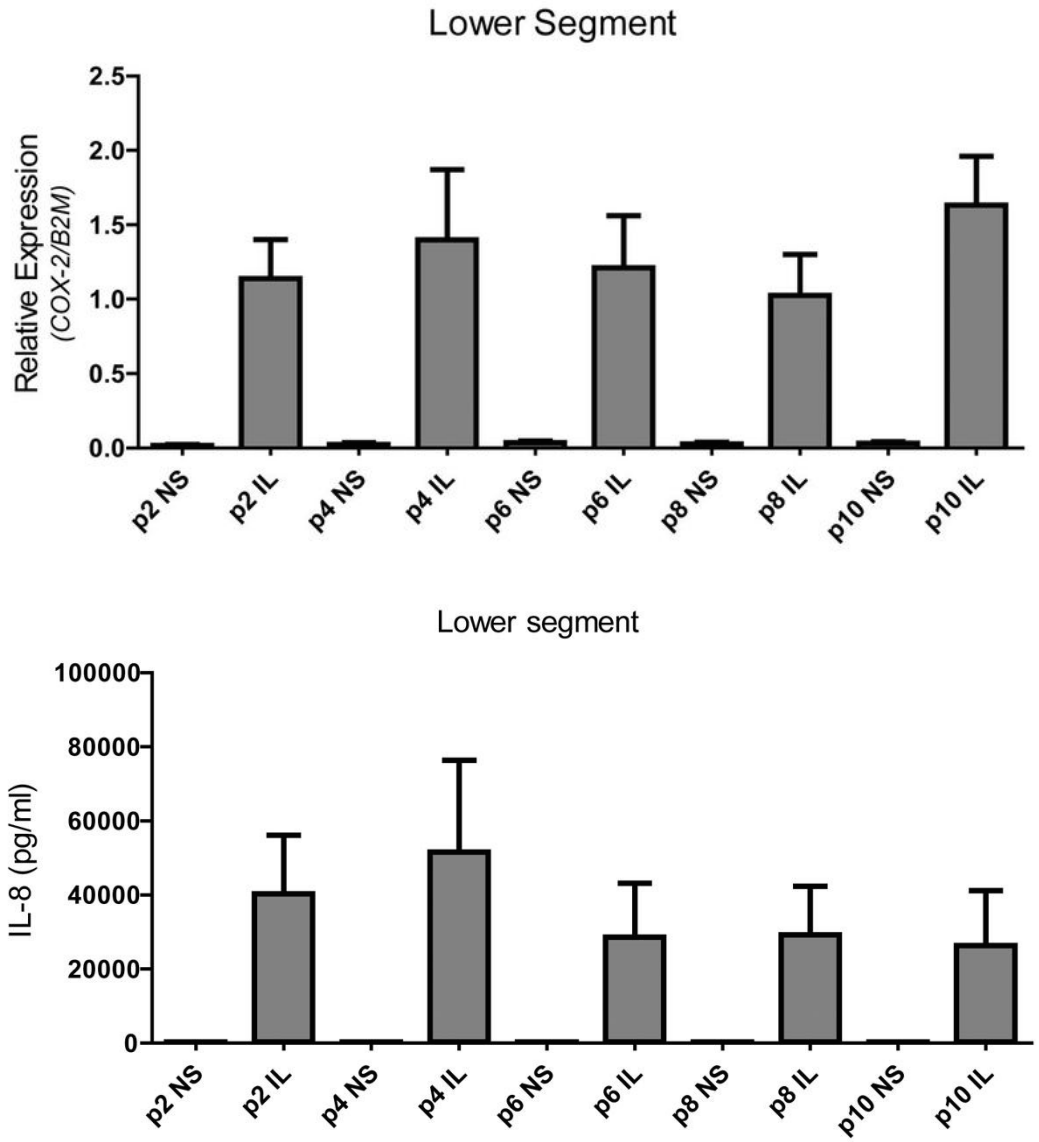

Figure 8.3. *COX2* mRNA expression and IL-8 protein output is conserved in HMSMC culture from passages 2 through 10. Responsiveness of *COX2* and IL-8 to 1 ng/mL IL-1β stimulation is conserved. Messenger RNA was normalized to β2-microglobulin, and presented relative to a pooled sample. IL-8 protein output was quantified with an ELISA kit (316).

8.2 Comparative profiles of primary HMSMC cultured from labouring and non-labouring myometrial biopsies.

8.2.1 Histological profiles of TNL and TL HMSMC

All myometrial data presented in chapters 4 and 5 involved HMSMC cultures isolated from TNL myometrial biopsies collected from women undergoing elective caesarean sections. These cells are fusiform-shaped single nucleated smooth muscle cells that grow in organized patterns neatly and evenly filling the space that is provided in culture (Fig. 8.4). HMSMC isolated from labouring deliveries (TL HMSMC) exhibit visually distinct patterns in organization and growth in culture. These observed morphological differences, as viewed and photographed with a light microscope at 10X magnification, can be characterized into three main groups (Fig. 8.5). The first difference is that the spindle-shape of TL HMSMCs has a propensity to be thinner and more elongated than TNL HMSMCs, more closely resembling the morphology of a 'stressed' TNL myocyte. The second is that TL HMSMCs exhibit less organized growth patterns, often resulting in overlapped growth with adjacent cells, which I have never before observed in culture of TNL HMSMC. The third difference is that TL HMSMCs are less inclined to fill the space provided in culture, often leaving very sparse gaps in growth while densely overcrowding adjacent areas. All three morphological phenotypes were not observed concurrently in all cells cultured from all five labouring myometrial biopsies, but from passage 0 to passage 8 in all five groups the same visual characteristics were observed on numerous occasions.

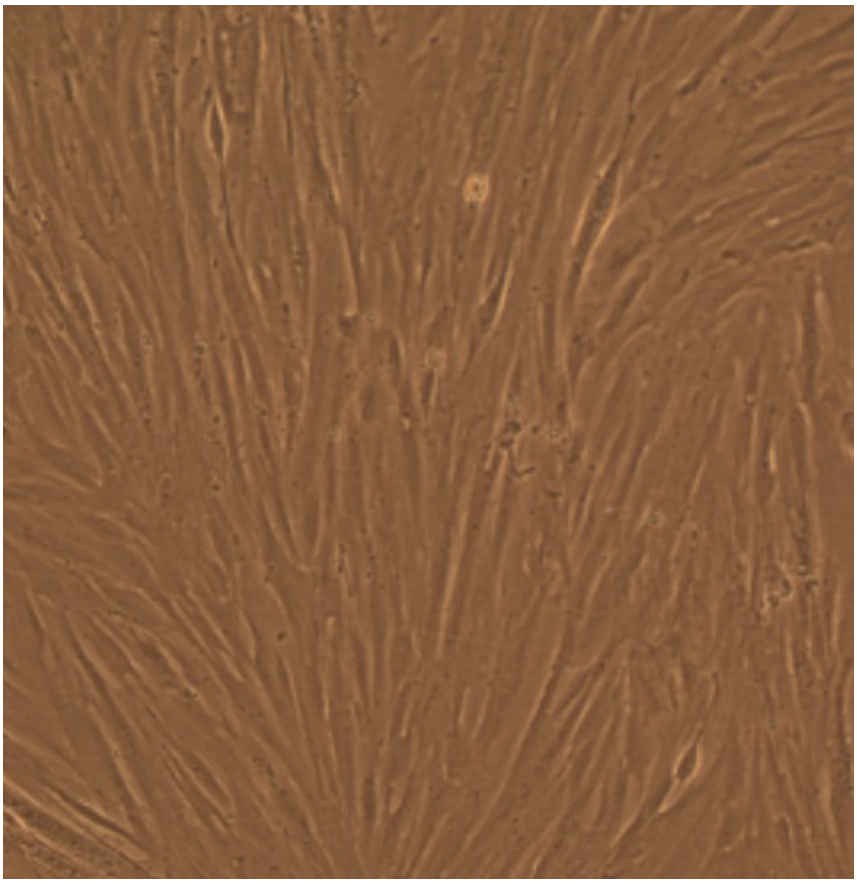

Figure 8.4. Morphology of fusiform-shaped TNL HMSMCs in culture.

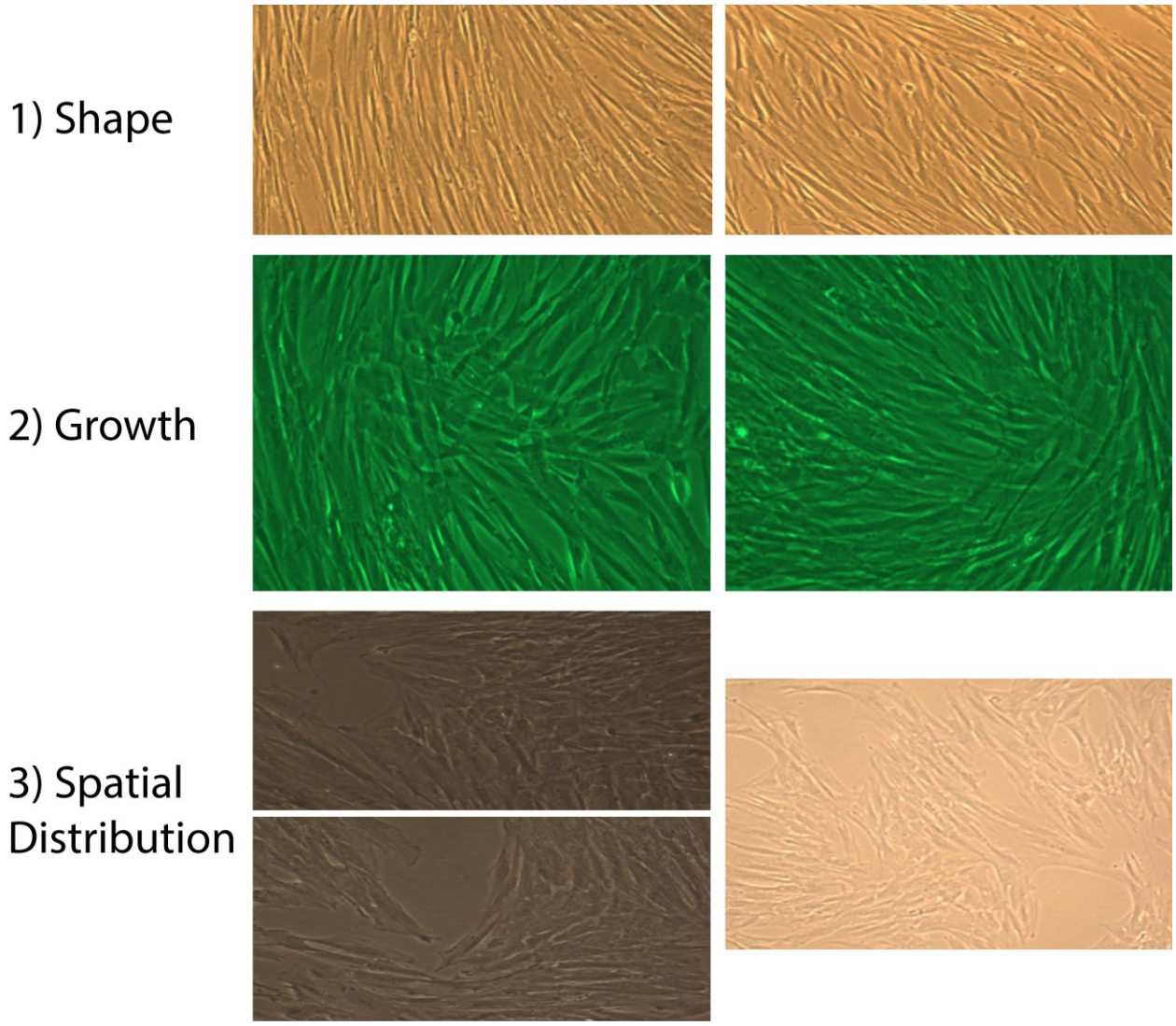

Figure 8.5. Cultured HMSMCs isolated from myometrial biopsies extracted from spontaneous labouring TL deliveries exhibit three main morphological phenotypic differences from TNL HMSMCs, as presented by light microscopy images at 10X. Image coloration is not indicative of any intended differences in imaging protocols, other than inadequate equipment training.

8.2.2 Cytokine and UAP mRNA profiles of TNL and TL HMSMC

Basal mRNA expression levels of UAPs and pro-inflammatory mediators differ between HMSMC and TL HMSMC. Increased inflammatory expression was demonstrated in TL HMSMCs, with increased mRNA expression of *IL6*, *FP*, *OTR*, *IL1RAcP* and *IL1RAcPb* (Fig. 8.6). However, results were not statistically significant due to small sample size and limited power (Table 8.1). IL-1β stimulation of TL HMSMC resulted in higher *IL6*, *COX2*, and *FP* but not *OTR* mRNA abundance from IL-1β-induced upregulation in TNL HMSMC (Fig. 8.7). Labouring HMSMC did not demonstrate upregulated IL-1β-induced *IL1R1*, *IL1RAcP* or *IL1RAcPb* (Fig. 8.8). Unlike the enhanced response to IL-1β stimulation, TL HMSMC did not demonstrate increased responsiveness to $PGF_{2\alpha}$, except for $PGF_{2\alpha}$-induced *IL6* induction which was upregulated. Interestingly, $PGF_{2\alpha}$-induced *COX2* induction was actually suppressed in TL HMSMC compared to TNL HMSMC (Fig. 8.9), a conflicting response from the upregulated IL-1β-induced *COX2* in TL HMSMC (Fig. 8.7).

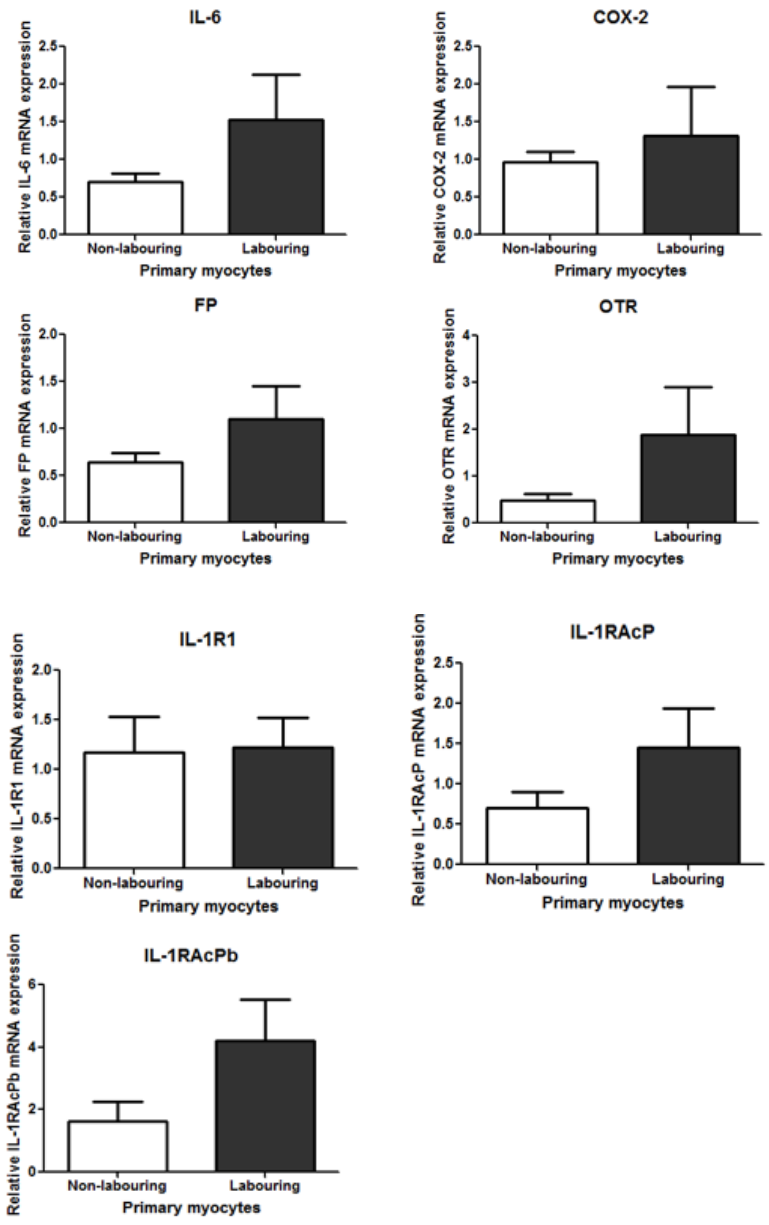

Figure 8.6. Basal levels of *IL6*, *FP*, *OTR*, *IL1RAcP* and *IL1RAcPb* mRNA expression are higher in HMSMCs isolated from labouring than non-labouring biopsies. HMSMC underwent four passages. Data are presented as relative change (x-fold) from a pooled sample, mean ± SEM. Non-labouring HMSMC, n=3, labouring HMSMC, n=5. Independent samples t-test, $p>0.05$.

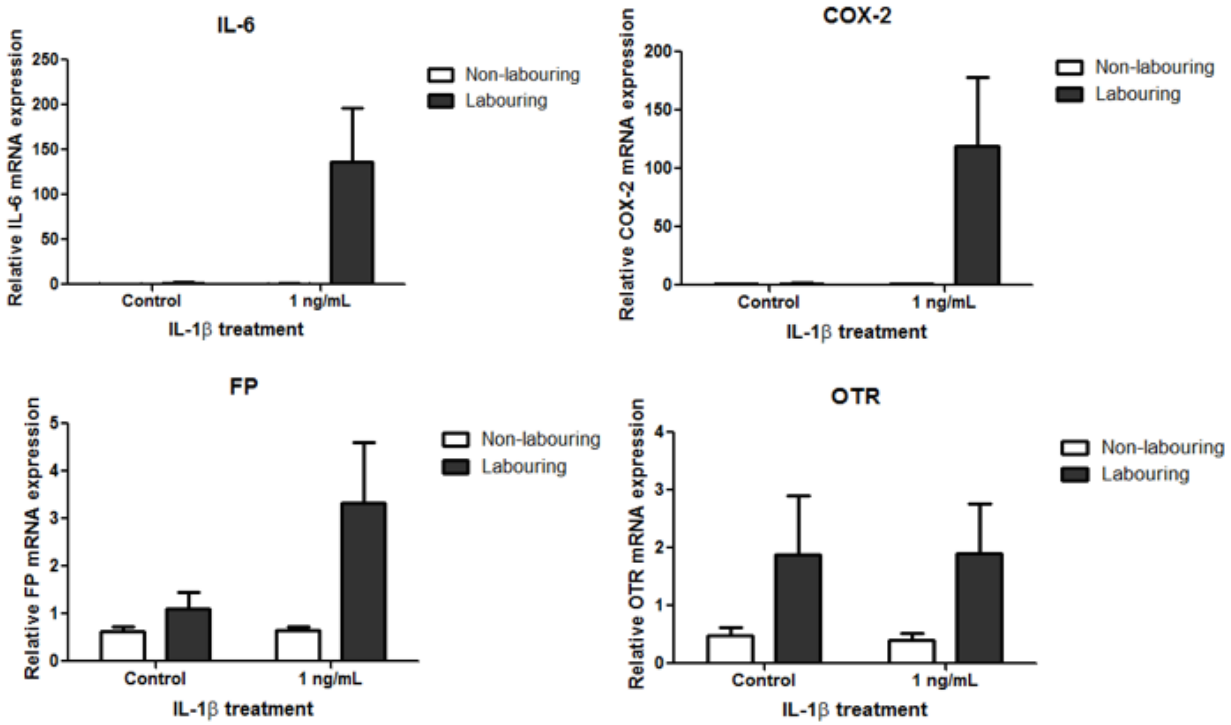

Figure 8.7. Labouring HMSMCs demonstrate increased *IL6*, *COX2* and *FP* responses to IL-1β, but not *OTR*. HMSMC underwent four passages. Data are presented as relative change (x-fold) from a pooled sample, mean ± SEM. Non-labouring HMSMC, n=3, labouring HMSMC, n=5. Two-way ANOVA was performed on $\log_{10}$-transformed data, $p>0.05$.

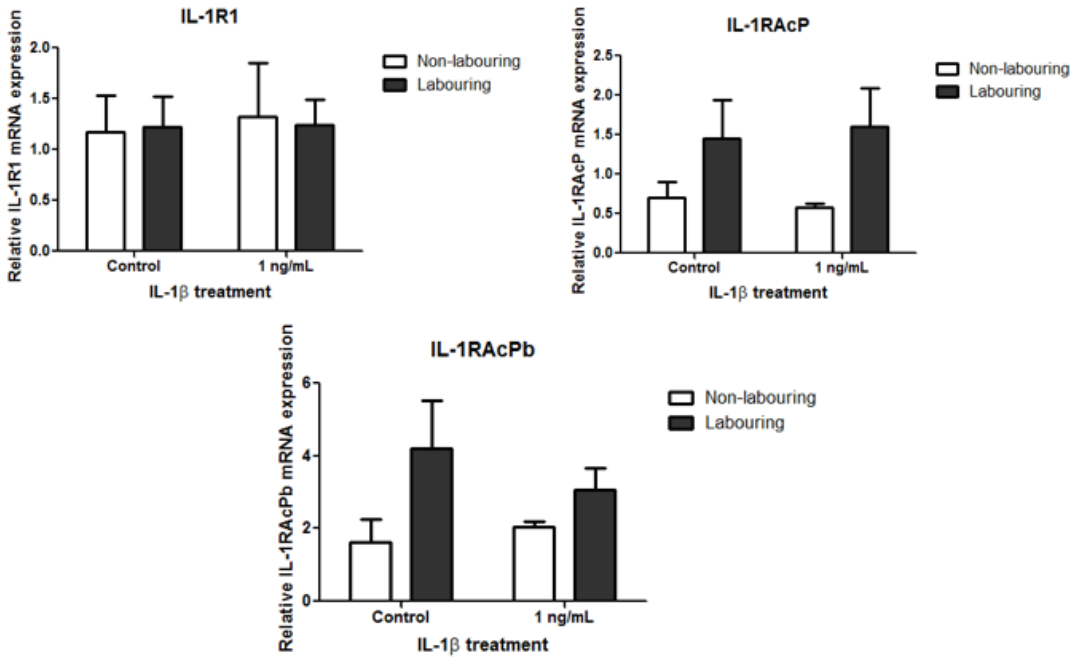

Figure 8.8. Labouring HMSMCs do not upregulate *IL1R1*, *IL1RAcP* or *IL1RAcPb* in response to IL-1β. HMSMC underwent four passages. Data are presented as relative change (x-fold) from a pooled sample, mean ± SEM. Non-labouring HMSMC, n=3, labouring HMSMC, n=5. Two-way ANOVA was performed on $\log_{10}$-transformed data, $p>0.05$.

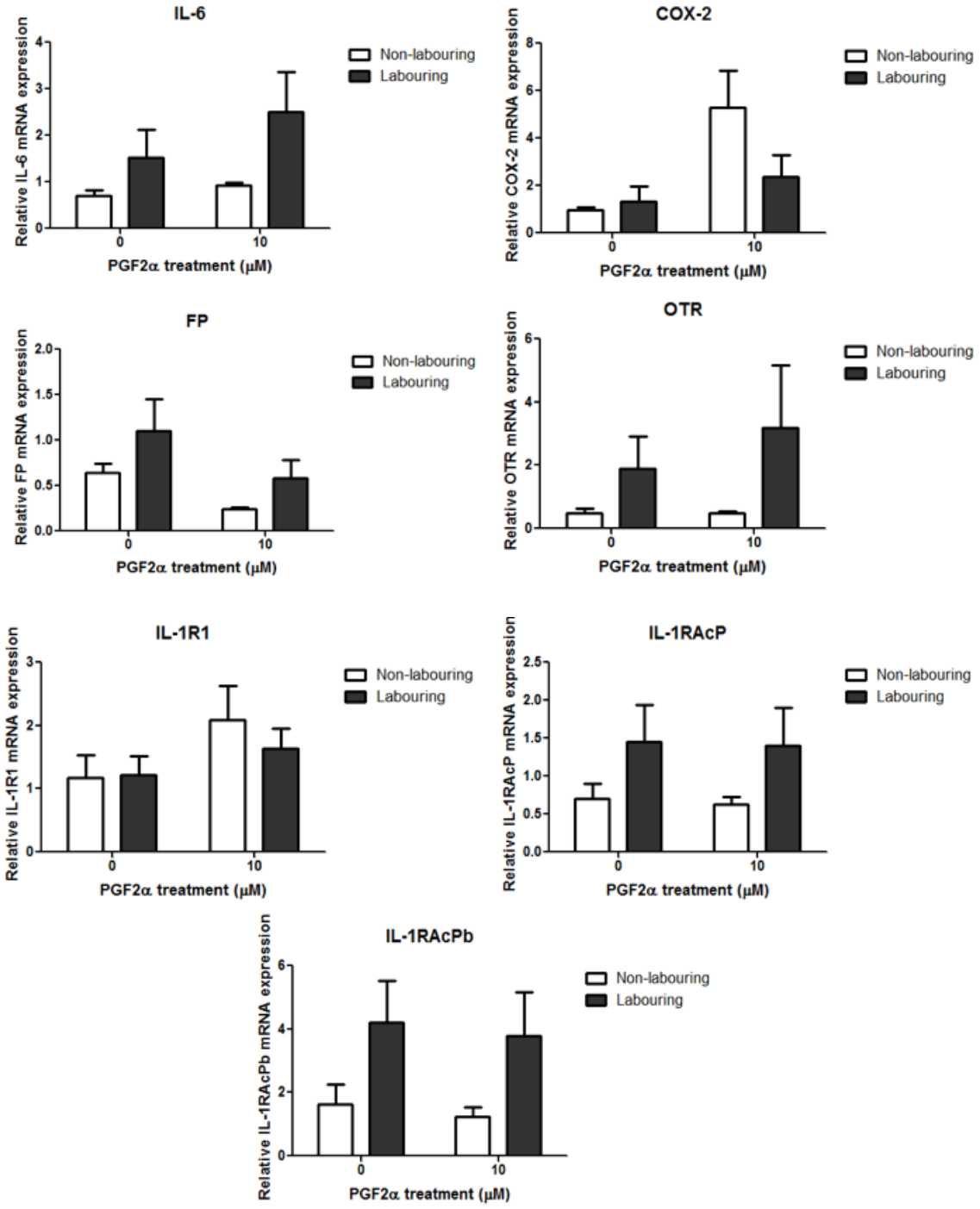

Figure 8.9. Labouring HMSMCs demonstrate increased *IL6* and decreased *COX2* responses to $PGF_{2\alpha}$ stimulation. HMSMC were passaged four times. Data are presented as relative change (x-fold) from a pooled sample, mean ± SEM. Non-labouring HMSMC, n=3, labouring HMSMC, n=5. Two-way ANOVA was performed on $\log_{10}$-transformed data, $p>0.05$.

Table 8.1. Limitations of comparative study of labouring versus non-labouring HMSMC primarily characterized by lack of sample availability during short duration project period. Sample size is too small to power statistical conclusions, with culture of HMSMC from five labouring and 3 non-labouring myometrial biopsies. The distribution of sample availability from passage to passage is unequally distributed among patient samples. Passages 4 and 5 are the only passages with n=5 for TL HMSMC.

Labouring					**Non-labouring**		
#1	#2	#3	#4	#5	#1	#2	#3
N/A	N/A	Passage 2	Passage 2	Passage 2	N/A	N/A	N/A
N/A	N/A	Passage 3	Passage 3	Passage 3	N/A	N/A	N/A
Passage 4	Passage 4	Passage 4	Passage 4	Passage 4	Passage 4	Passage 4	Passage 4
Passage 5	Passage 5	Passage 5	Passage 5	Passage 5	Passage 5	Passage 5	Passage 5
Passage 6	Passage 6	Passage 6	N/A	N/A	Passage 6	Passage 6	Passage 6
Passage 7	Passage 7	Passage 7	N/A	N/A	Passage 7	Passage 7	Passage 7
Passage 8	Passage 8	Passage 8	N/A	N/A	Passage 8	Passage 8	Passage 8

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molecular human reproduction

ORIGINAL RESEARCH

$PGF_{2\alpha}$ modulates the output of chemokines and pro-inflammatory cytokines in myometrial cells from term pregnant women through divergent signaling pathways

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ABSTRACT: Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) plays a critical role in the initiation and process of parturition. Since human labor has been described as an inflammatory event, we investigated the role of $PGF_{2\alpha}$ in the inflammatory process using cultured human uterine smooth muscle cells (HUSMCs) isolated from term pregnant women as a model. Using a multiplex assay, HUSMCs treated with $PGF_{2\alpha}$ changed their output of a number of cytokines and chemokines, with a distinct response pattern that differed between HUSMCs isolated from the upper and lower segment region of the uterus. Confirmatory enzyme-linked immunosorbent assays (ELISAs) showed that $PGF_{2\alpha}$ stimulated increased output of interleukin (IL) 1β, IL6, IL8 (CXCL8) and monocyte chemotactic protein-1 (MCP1, also known as chemokine (c-c motif) ligand 2, CCL2) by HUSMCs isolated from both upper and lower uterine segments. In contrast, $PGF_{2\alpha}$ inhibited tumor necrosis factor α (TNFα) release by HUMSCs from the lower uterine segment while the output of TNFα was undetectable in the upper segment. Small interfering (si) RNA mediated knockdown of the $PGF_{2\alpha}$ receptor prevented the changes in cytokine and chemokine output by the HUSMCs. Since the $PGF_{2\alpha}$ receptor (PTGFR) couples via the Gq protein and subsequently activates the phospholipase C (PLC) and protein kinase C (PKC) signaling pathways, we examined the role of these pathways in $PGF_{2\alpha}$ modulation of the cytokines. Inhibition of PLC and PKC reversed the effects of $PGF_{2\alpha}$. $PGF_{2\alpha}$ activated multiple signaling pathways including extracellular signal-regulated kinases (ERK) 1/2, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), P38, calcineurin/nuclear factor of activated T-cells (NFAT) and NF-κB signaling. Inhibition of ERK reversed $PGF_{2\alpha}$-induced IL1β, IL6 and CCL2 output, while inhibition of PI3K blocked the effect of $PGF_{2\alpha}$ on IL6, CXCL8 and CCL2 output and inhibition of NF-κB reversed $PGF_{2\alpha}$-induced IL1β and CCL2 output. NFAT was involved in $PGF_{2\alpha}$ modulation of CCL2 and TNFα output. In conclusion, our results support a role of $PGF_{2\alpha}$ in creating an inflammatory environment during the late stage of human pregnancy.

Key words: $PGF_{2\alpha}$ / inflammation / myometrium / pregnancy / labor

Introduction

Prostaglandins (PGs), a family of hormones produced in all tissues of the body, modulate various functions via endocrine, paracrine and autocrine mechanisms. In female reproductive systems, PGs are involved in many events including ovulation, blastocyst transport, implantation, pregnancy maintenance, luteolysis and parturition. In most mammalian species, PGs produced by gestational tissues play a central role in the initiation and

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progression of labor being involved in all aspects of parturition including ripening of the cervix, membrane rupture and induction of uterine contraction (Lundin-Schiller and Mitchell, 1990; Olson et al., 1995).

Accumulating evidence demonstrates that human labor is an inflammatory process, characterized by increased leukocyte infiltration into uterine tissues and increased expression and release of numerous cytokines and chemokines, including interleukin 6 (IL 6), IL1, interleukin 8 (also known as chemokine (c-x-c motif) ligand 8, CXCL8) and CCL2 (Goldenberg et al., 2000; Osman et al., 2003). The increased cytokines and chemokines further promote the recruitment of leukocytes into uterine tissues in a feed-forward fashion thereby creating an 'inflammatory microenvironment' (Kobayashi, 2008; White et al., 2013). Within the uterus, such inflammatory cascades result in the up-regulation of uterine activation proteins (UAPs), thereby leading to the onset of parturition (Hertelendy et al., 1993; Young et al., 2002).

It is well known that PGs can serve as pro-inflammatory mediators due to their high expression in inflamed tissues and ability to induce inflammatory symptoms. One such example, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), is produced by gestational tissues with high levels observed at parturition (Olson et al., 1995; Fortier et al., 2008; Maddipati et al., 2014), and has a recognized physiological role in stimulating myometrial contractions. However, increased $PGF_{2\alpha}$ concentrations observed in the maternal circulation occur early on in, or precede, the labor process (Kinoshita et al., 1977), suggesting that $PGF_{2\alpha}$ is involved in additional parturition events besides uterine contraction. We previously reported that $PGF_{2\alpha}$ may contribute to transformation of the relatively quiescent uterus of gestation, to the powerful contractile uterus of parturition, by up-regulating expression of the UAPs (Xu et al., 2013). Moreover, $PGF_{2\alpha}$ can also serve as an inflammatory mediator, for example, in the female reproductive system, $PGF_{2\alpha}$ induces CCL2 in the ovary (Luo et al., 2011) and CXCL8 in endometrial adenocarcinoma cells (Pollard and Mitchell, 1996). The $PGF_{2\alpha}$ signal is mediated by the $PGF_{2\alpha}$ receptor (PTGFR), which couples to the G protein Gq to activate multiple signaling pathways which include phospholipase C/protein kinase C (PLC/PKC), mitogen activated protein kinase (MAPKs), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and calcineurin/nuclear factor of activated T-cells (NFAT) signaling pathways (Sales et al., 2009; Goupil et al., 2010; Kondo et al., 2012).

Human uterine smooth muscle cells (HUSMCs) isolated from the pregnant uterus synthesize and secrete chemokines and cytokines such as IL1β, IL6, CCL2 and CXCL8 (Hua et al., 2012; Shynlova et al., 2013a). In addition, HUMSCs demonstrate increased expression of UAPs such as connexin 43(CX43) and cyclo-oxygenase-2 (COX-2, also known as PTGS-2) in response to treatment with $PGF_{2\alpha}$ (Xu et al., 2013). Thus, we hypothesize that $PGF_{2\alpha}$ regulates chemokine and cytokine output in the myometrium during pregnancy, thereby amplifying the inflammatory responses within uterus.

Finally, in the human uterus, the concept of a functional regionalization has been proposed. This suggests that the upper segment (US) displays a relaxed state during gestation to accommodate the growing fetus but at labor contracts to expel the baby, while the lower segment (LS) maintains a state conducive to passage of the fetal head during labor (Luckas and Wray, 2000). Our previous study demonstrated that $PGF_{2\alpha}$-induced changes in UAP abundance differed in HUSMCs isolated from the upper and lower uterine segments, indicating potential differentiation of roles between US and LS in pregnancy and parturition (Xu et al., 2013). However, some studies show no difference in contractility between US and LS myometrium. The expression pattern of some UAPs such as PTGFR in US and LS during labor is similar (Hay et al., 2010) whereas some other UAPs, for instance, the PGE_2 receptors, PTGER2 and PTGER3, display different expression levels in US and LS (Grigsby et al., 2006). Thus, investigating the role of $PGF_{2\alpha}$ in the regulation of cytokine and chemokine output comparing US and LS myometrial cells will expand our knowledge about functional regionalization in the human uterus.

The objectives of the present study are to (i) assess if $PGF_{2\alpha}$ participates in the regulation of cytokine and chemokine output in the uterus during pregnancy and (ii) define the signaling pathways involved in the $PGF_{2\alpha}$ mediated regulation of chemokine and cytokine output. Our study will provide insight into the mechanisms of human parturition and indicate new strategies for development of tocolytics.

Materials and Methods

Isolation and culture of HUSMCs

This study was approved by the specialty committee on ethics of biomedicine research, Second Military Medical University, Shanghai, China as well as the Conjoint Health Research Ethics Board, University of Calgary. Written informed consent was obtained from all the patients involved in this study.

Biopsies of LS human myometrium ($n = 11$) were obtained from pregnant women undergoing elective cesarean section at term (the average gestational age was 38 weeks, with a range of 37–42 weeks) in Changhai hospital, Shanghai. Among the patients who were recruited in this study, cesarean section was performed due to breech presentation, previous cesarean section, cephalopelvic disproportion or maternal request. Women who had evidence of underlying disease, such as hypertension, diabetes, pre-eclampsia, intrauterine growth restriction, were not included in this study. Biopsies were excised from the middle portion of upper edge of the incision line in the lower uterine segment. HUSMCs from LS were isolated by enzymatic dispersion as described previously (Xu et al., 2011). Briefly, myometrial pieces were incubated with phenol-red-free Dulbecco's modified Eagles medium (DMEM) containing 1 mg/ml collagenase type II (Invitrogen, Grand Island, NY), and 1 mg/ml deoxyribonuclease I (Invitrogen) at 37°C for 45 min. Following filtration by 100 μm cell strainer (Corning), the cell suspension was centrifuged at 600*g* for 10 min, and the cell pellet resuspended in DMEM containing 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were then plated into 25-cm^2 flasks and kept at 37°C in 5% CO_2-95% air humidified atmosphere until confluent (~2 weeks) and all experiments were performed with these cells at passage 2. For the treatment experiments, when the cell density is up to 90% confluence, 0.05% trypsin was used to disperse cells and placed in 6-well plates with DMEM containing 10% FCS. After the cells had grown to ~80% confluence, the media was changed to DMEM without FCS. Subsequently, cells were treated with various concentrations of $PGF_{2\alpha}$ (Sigma-Aldrich, US) in the presence or absence of kinase inhibitors, including PLC inhibitor (U73122), PKC inhibitor (chelerythrine), ERK inhibitor (PD98059), PI3K inhibitor (LY294002), Calcineurin inhibitor (CsA), the blocker of calcineurin and NFAT interaction (Inca-6), NFAT-AP1 complex inhibitor (RA), P38 inhibitor (SB202190) or NFκB inhibitor (PDTC), and incubated for 24 h. The vehicle control was treated with same volume of solvent (ethanol, ≤0.1% v/v). Concentration of the above inhibitors was determined according to the literature (Pollard and Mitchell, 1996) and our previous studies (You et al., 2012; Xu et al., 2015). All the above inhibitors were purchased from Sigma-Aldrich.

Seven paired biopsies from both US and LS uterine segments were collected from pregnant women undergoing elective cesarean sections at term. The upper segment biopsies were all taken from the side opposite

the placenta on the anterior or posterior aspect of the upper segment. Palpation and visualization of the uterus determined where the upper segment began, and the decidual layer was dissected away before a small piece of myometrium was grasped with fine forceps and dissected with Iris scissors. Biopsies of LS and US were dispersed and cultured as described above. Cells were cultured to passage 7 and placed in 6-well plates with DMEM containing 10% FCS and 1 × antimycotic (100 units/ml Penicillin g sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B) at 37°C with 5% CO_2. Following growth to ~80% confluence, cells were serum deprived overnight then treated with various concentration of $PGF_{2\alpha}$ (10^{-8}–10^{-5} M) and incubated for 24 h. After incubation, supernatant and cells were collected. The *in vitro* characteristics of the cells from upper (US-HUSMCs) and lower (LS-HUSMCs) segments were maintained to at least 10 passages as described previously (Mosher *et al.*, 2013).

RNA interference

For knockdown of PTGFR, sequence-specific small interfering RNA (siRNA) targeting human PTGFR (sense 5′-GGUGUAUUGGAGUCACAAAtt-3′; antisense 5′-UUUGUGACUCCAAUACACCgc-3′) was purchased from Santa Cruz, US (sc-44987). The following nonsense siRNA (sense 5′-GAAU CUGGGAUGUUAACCAtt-3′; antisense 5′-UGGUUAACAUCCCAGAU UCtg-3′) was also provided by Santa Cruz and used as the negative control. Cultured HUSMCs were transfected with PTGFR siRNA or control siRNA using Lipofectamine™ RNAi MAX (Invitrogen) for 6 h, followed by 18 h of incubation with DMEM only. The cells were treated with increasing concentrations of $PGF_{2\alpha}$ (10^{-8}–10^{-5} M) for 24 h.

Multiplexed fluorescent bead-based immunoassays

The multiplex immunoassays built on magnetic beads were custom-designed and obtained from Eve® Technologies (Calgary, Canada). In total, 42 cytokines, chemokines and growth factors were evaluated in the cell supernatants from primary HUSMCs in absence and presence of 10^{-6} M $PGF_{2\alpha}$. The multiplex assay was carried out by the manufacturer Eve® Technologies (Calgary, Canada).

Enzyme-linked immunosorbent assay

The concentrations of IL6, CCL2, CXCL8, IL1β and tumor necrosis factor α (TNFα) in culture media of myometrial cells were determined with specific enzyme-linked immunoassays (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Western blotting analysis

HUSMCs were harvested in the presence of M-Per lysis buffer (Pierce Biotechnology, US) and the protein extracted following the manufacturer's protocol. Seventy μg protein was separated by SDS (10% w/v)-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Subsequently, membranes were incubated with specific antibodies including PTGFR, p65, phospho-p65(ser-529), ERK1/2, phospho-ERK1/2, NFATC1, p38, phospho-p38, PI3K and phospho-PI3K(Tyr508) overnight at 4°C. After incubation with a secondary horseradish peroxidase-conjugated antibody, membranes were visualized using enhanced chemiluminescence (Santa Cruz). The intensities of light-emitting bands were detected and quantified using Sygene Bio Image system (Synoptics Ltd, UK). The levels of phospho-PI3K, phospho-p65, phospho-ERK1/2 and phospho-p38 were normalized to the unphosphorylated type of these proteins, while the level of NFATC1 was normalized to β-actin. The information of all antibodies including manufacture, catalog number and dilution is shown in Supplementary Table SI.

Statistics

The results for all protein determinations are presented as the mean ± SEM. Data were tested by SPSS software and found to be normally distributed. Data were then analyzed by two-way ANOVA followed by the Least Significant Difference (LSD) multiple comparison method. Significance was achieved at $P \leq 0.05$.

Results

$PGF_{2\alpha}$ regulates output of chemokines and cytokines in US and LS HUSMCs

We first examined the cytokine and chemokine output, in response to $PGF_{2\alpha}$ treatment, comparing paired US and LS HUSMCs, using a Multiplex assay. One micromolar $PGF_{2\alpha}$ modulated the secretion of a number of chemokines and cytokines during a 24-h incubation. $PGF_{2\alpha}$ robustly stimulated granulocyte-macrophage colony stimulating factor (GM-CSF), IL6, interferon (IFN) α, CXCL8 and CCL2 output in both US and LS cells (Supplementary Fig. S1). However, the response pattern of some cytokines differed between US and LS, for example, $PGF_{2\alpha}$ induced fibroblast growth factor (FGF) 2, IL12 and IL1β in LS-HUSMCs, but inhibited FGF2 and IL12 output, and had no effect on IL1β output by US-HUSMCs. In general, we observed that $PGF_{2\alpha}$ induced more pro-inflammatory cytokines in the LS-HUSMCs compared with the US-HUSMCs.

To confirm the effect of $PGF_{2\alpha}$ on cytokines, we used ELISAs, to measure several pro-inflammatory cytokines, whose secretion was stimulated by $PGF_{2\alpha}$ in both the LS and US cells in the above experiment. As shown in Fig. 1A, $PGF_{2\alpha}$ (10^{-8}–10^{-6} M) treatment for 24 h increased the levels of CCL2, CXCL8 and IL6 in culture media of both LS and US cells in a dose-dependent manner.

The effects of $PGF_{2\alpha}$ are mediated by PTGFR

To investigate whether the modulation of cytokine output by $PGF_{2\alpha}$ occurred as a direct effect via its own receptor, we used sequence-specific siRNA targeting PTGFR to knockdown the levels of PTGFR in the cells. The efficiency of interference with for PTGFR siRNA reached about 72% (Supplementary Fig. S2). As shown in Fig. 1B, knockdown of PTGFR reversed the $PGF_{2\alpha}$ induced up-regulation of CXCL8, CCL2 and IL6 output in both US and LS cells.

The intracellular signaling pathways involved in $PGF_{2\alpha}$ regulation of cytokine and chemokine output in the LS HUSMCs

We then investigated the signaling pathways involved in $PGF_{2\alpha}$ regulation of cytokine and chemokine output in human pregnant myometrium. Considering that the amount of US biopsies was limited, the following study was performed with the LS myometrial cells. Multiplex results showed that $PGF_{2\alpha}$ stimulated IL1β output but inhibited TNFα output in LS cells. These results were confirmed using ELISA. As shown in Fig. 2, $PGF_{2\alpha}$ inhibited TNFα output (Fig. 2C) but stimulated IL1β output (Fig. 2A) by the LS cells in a dose-dependent manner, and as expected, $PGF_{2\alpha}$ stimulated IL6, CXCL8 and CCL2 output (Fig. 2B, D and E).

$PGF_{2\alpha}$ activation of the PTGFR, a G protein coupled receptor, leads to Gq coupling and subsequent activation of downstream signaling pathways which include phospholipase C β (PLCβ), and protein kinase C

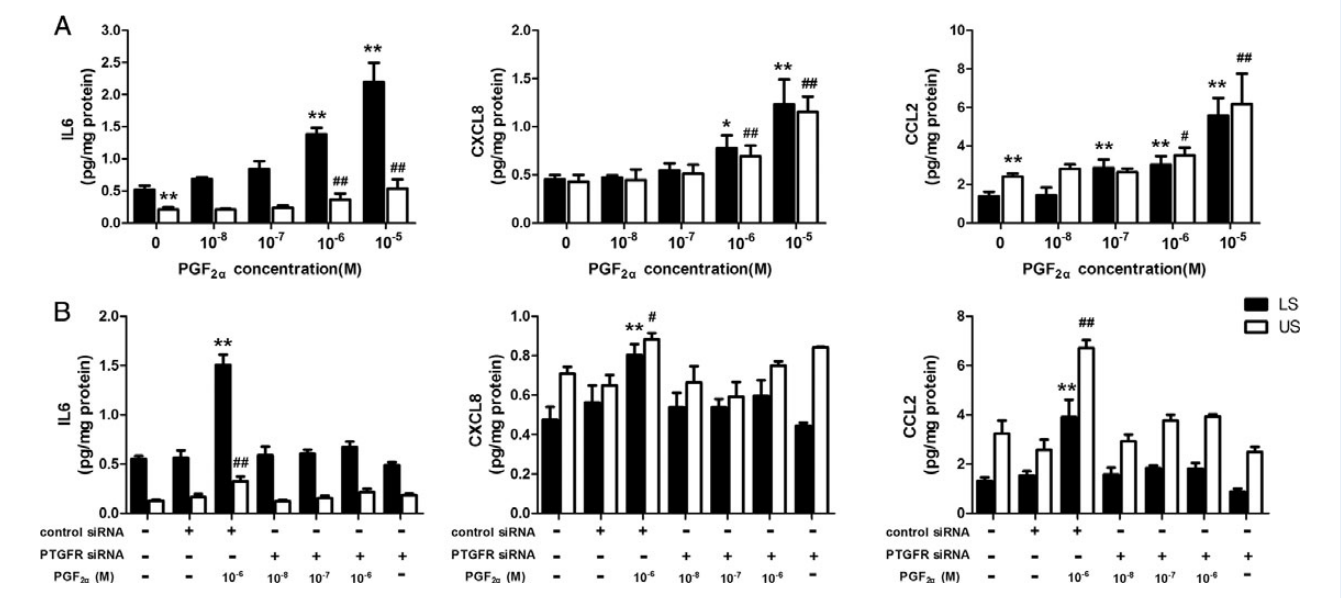

Figure 1 Role of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its receptor (PTGFR) in mediating cytokine and chemokine output in paired upper segment (US) and lower segment (LS) myometrial cells. (**A**) Human uterine smooth muscle cells (HUSMCs) isolated from paired US and LS were treated with increasing concentrations of $PGF_{2\alpha}$ (10^{-8}–10^{-5} M) for 24 h. Following incubation, the cell culture media supernatants were collected for enzyme-linked immunosorbent assay (ELISA) to determine concentration of interleukin-6 (IL-6), CXCL8 and monocyte chemoattractant protein (CCL-2). (**B**) Sequence-specific small interfering RNA (siRNA) targeting PTGFR to confirm the role of PTGFR in regulating cytokine and chemokine output. HUSMCs were transfected with specific PTGFR siRNA and treated with $PGF_{2\alpha}$ as indicated. Following a 24-h incubation, cell media supernatants were collected for ELISA to determine IL-6, CXCL8 and CCL-2. Values are presented as mean ± SEM. $n = 7$ (from seven patients). $^{**}P < 0.01$ compared with vehicle control in HUSMCs of LS. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus vehicle control in HUSMCs of US.

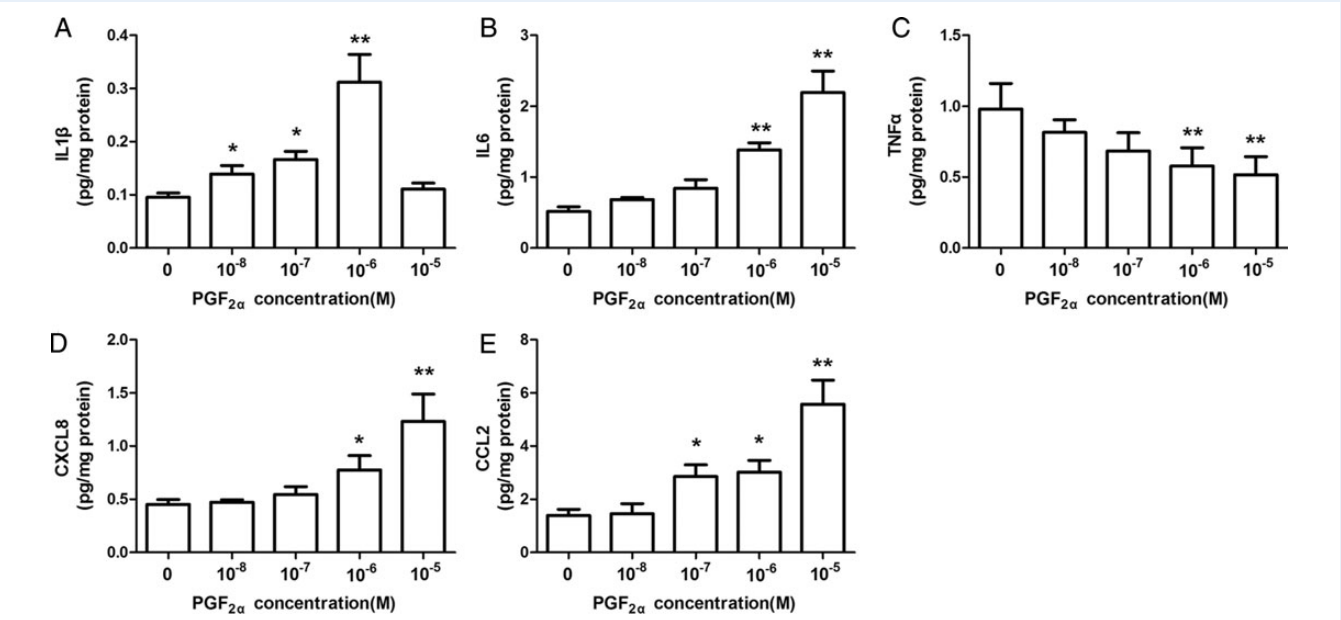

Figure 2 Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) modulates interleukins 1β, 6 and 8 (IL-1β, IL-6, CXCL8) tumor necrosis factor α (TNFα), and monocyte chemoattractant protein (CCL-2) output by lower segment (LS) myometrial cells. Isolated LS human uterine smooth muscle cells (HUSMCs) were treated with $PGF_{2\alpha}$ (10^{-8}–10^{-5} M) for 24 h. Enzyme-linked immunosorbent assays (ELISA) were used to determine concentrations of (**A**) IL-1β, (**B**) IL-6, (**C**) TNFα, (**D**) CXCL8 and (**E**) CCL-2. Values are presented as mean ± SEM. $n = 7$ (from seven patients). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle control.

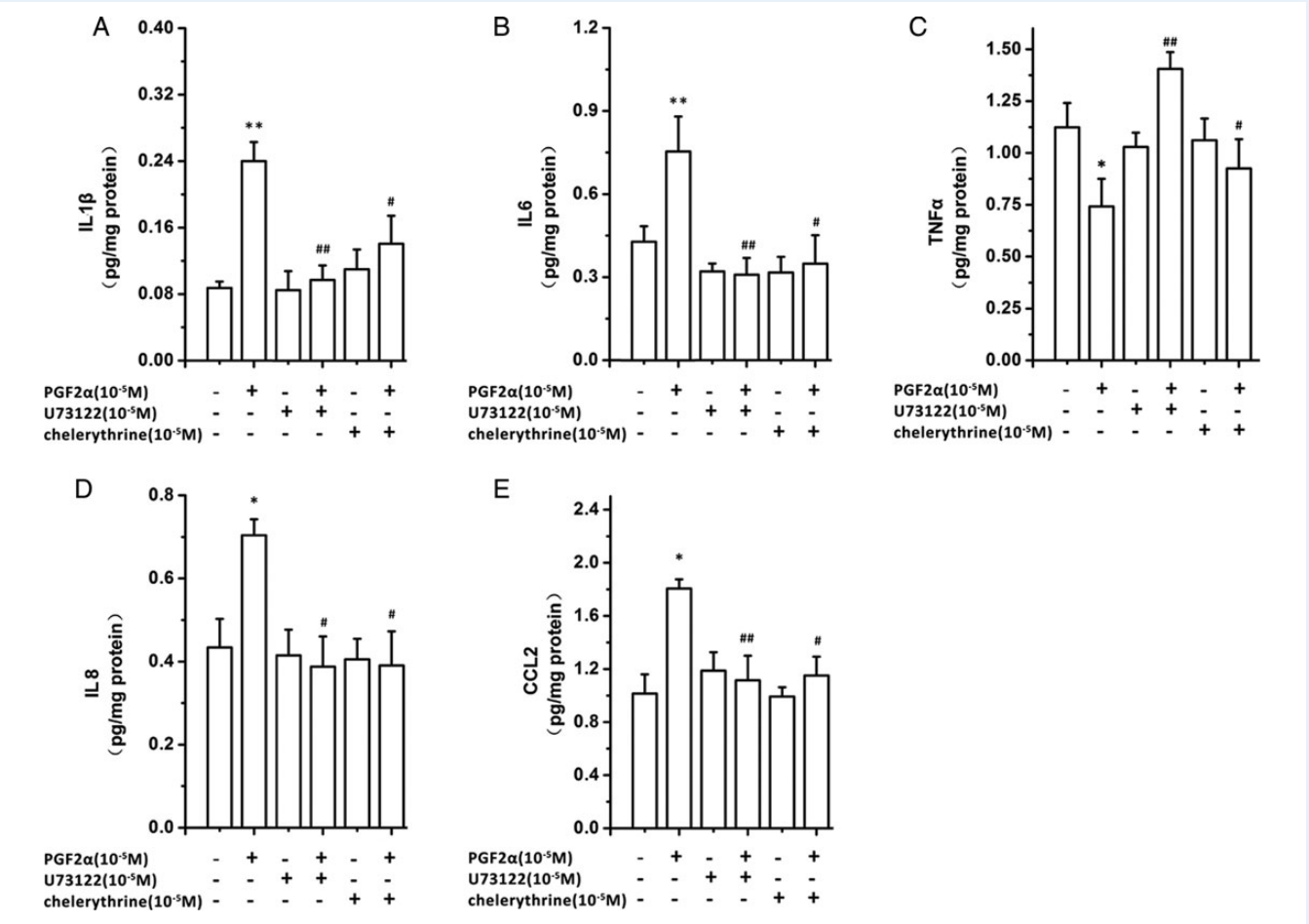

Figure 3 Inhibition of phospholipase C (PLC) and protein kinase C (PKC) modulates the effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induced cytokine and chemokine output. (**A**) Human uterine smooth muscle cells (HUSMCs) from lower segment (LS) were cultured and incubated for 24 h with the PLC inhibitor (U73122) or the PKC inhibitor (chelerythrine), in presence or absence of $PGF_{2\alpha}$ (10^{-6} M). The supernatants were collected for enzyme-linked immunosorbent assays (ELISA) to determine concentration of; (**A**) IL-1β, (**B**) IL-6, (**C**) TNFα, (**D**) CXCL8 and (**E**) CCL-2. Values are presented as mean ± SEM. $n = 4$ (from four patients). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with $PGF_{2\alpha}$ 10^{-6} M.

(PKC) (Goupil *et al.*, 2010; Kondo *et al.*, 2012). To determine the role of these downstream signaling pathways in the $PGF_{2\alpha}$-induced modulation of chemokine and cytokine output, we utilized PLC and PKC inhibitors. Inhibition of either PLC with U73122 (10^{-5} M) or PKC with chelerythrine (10^{-5} M) blocked the $PGF_{2\alpha}$-induced IL1β, IL6, CXCL8, and CCL2 output as well as $PGF_{2\alpha}$ inhibition of TNF-α output (Fig. 3).

$PGF_{2\alpha}$ has been shown to activate PI3K, ERK1/2 and P38 signaling pathways (Goupil *et al.*, 2010; Kondo *et al.*, 2012). We therefore confirmed these effects in LS HUSMCs. As shown in Fig. 4A–C, $PGF_{2\alpha}$ (10^{-6} M) increased the levels of phospho-ERK1/2, phospho-PI3K and phospho-p38 in a time-dependent manner. Inhibition of ERK with PD98059 (10^{-5} M) blocked $PGF_{2\alpha}$-induced IL1β, IL6 and CCL2, but not CXCL8 output (Fig. 5A–E). Inhibition of PI3K with LY294002 (10^{-5} M) blocked $PGF_{2\alpha}$-induced IL6, CXCL8 and CCL2, but not IL1β output. Neither PD98059 nor LY294002 affected $PGF_{2\alpha}$ inhibition of TNFα output. The inhibitor of P38, SB 202190 (10^{-5} M) reversed the increased output of IL6, CXCL8 and CCL2 by $PGF_{2\alpha}$. However, treatment of cells with LY294002 alone inhibited IL6 output, while SB202190 treatment caused an increase in IL1β output.

NF-κB, the archetypal inflammatory transcription factor, is known to drive chemokine and cytokine production. $PGF_{2\alpha}$ (10^{-6} M) increased the levels of phospho-p65 in time-dependent manner (Fig. 4D). The NF-κB inhibitor, PDTC (10^{-5} M), reversed the $PGF_{2\alpha}$-induced IL1β and CCL2 output, but not that of IL6, TNFα or CXCL8 (Fig. 5A–E). Notably, PDTC itself significantly stimulated IL6 ($P < 0.01$ versus vehicle) and CXCL8 output ($P < 0.05$ versus vehicle).

Stimulation of the Gq/PLC signaling pathway leads to Ca^{2+} release from intracellular calcium stores and subsequently activation of the calcineurin/NFAT pathway. Five members of the NFAT family of transcription factors have been isolated: NFATC2 (NF-AT1/p), NFATC1 (NF-AT2), NFATC4 (NF-AT3), NFATC3 (NF-AT4/x) and NFAT5 (TonEBP) (Rao *et al.*, 1997). Normally, Ca^{2+} induces activation of calcineurin which leads to NFAT dephosphorylation. In human myometrium, NFATC1 has been shown to be activated by Ca^{2+} signaling (Pont *et al.*, 2012). As shown in Fig. 6A, $PGF_{2\alpha}$ (10^{-6} M) time-dependently increased the level of NFATC1. A series of inhibitors were then applied to explore the role of the calcineurin/NFAT pathway in $PGF_{2\alpha}$ regulation of cytokine and chemokine outputs. With the administration of calcineurin

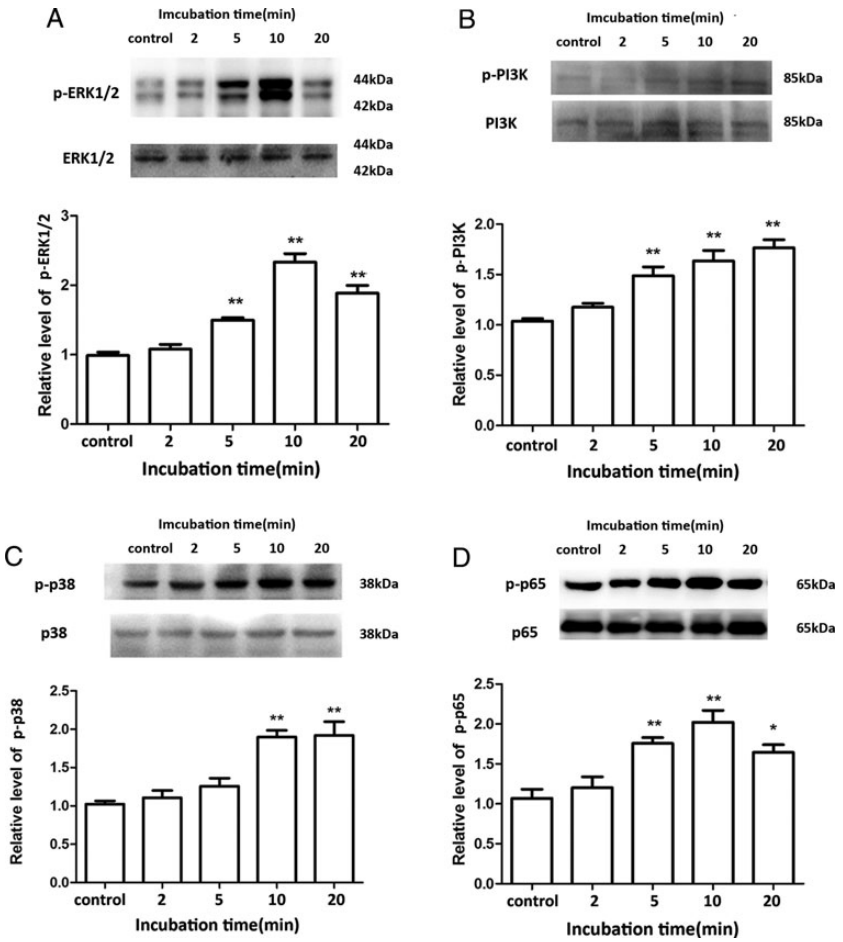

Figure 4 Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) activates phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), extracellular signal receptor kinase (ERK1/2), P38 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways. Human uterine smooth muscle cells (HUSMCs) from lower segment (LS) were treated with $PGF_{2\alpha}$ (10^{-6} M) for the indicated time. The levels of (**A**) phospho-PI3K, (**B**) phospho- ERK1/2, (**C**) phospho-P38 and (**D**) phospho-p65 were determined by western blotting. Representative blots are presented at the top of corresponding diagram. Values are presented as mean ± SEM. $n = 4$ (from four patients). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle control.

inhibitor, CsA, the robust stimulation of CCL2 output by $PGF_{2\alpha}$ was reversed (Fig. 6B). A similar trend was confirmed by the application of Inca-6, a blocker of calcineurin and NFAT interaction, and RA, an inhibitor of NFAT-AP1 complex. $PGF_{2\alpha}$-induced suppression of TNFα output was also blocked by CsA, Inca-6 and RA.

Discussion

Human parturition is an inflammatory event characterized by increased communication between uterine chemotactic signals and leukocytes in late gestation, which peaks at parturition and leads to leukocyte invasion of the uterus at every delivery (Zourbas *et al.*, 2001; Tornblom *et al.*, 2005; Golightly *et al.*, 2011; Singh *et al.*, 2011; Gomez-Lopez *et al.*, 2013). Even though there is an absence of infection, pro-inflammatory cytokines and chemokines are increased in both of preterm and term birth (Romero *et al.*, 1990; Osmers *et al.*, 1995; Young *et al.*, 2002; Esplin *et al.*, 2005). The invading leukocytes promote a positive feed-forward cycle by secreting pro-inflammatory cytokines and chemokines such as IL1β, IL6, CXCL8 and CCL2 that drive PG synthesis (Golightly *et al.*, 2011). Previous studies mostly consider that an increase in PG concentration induced by cytokines triggers uterine contractility (Young *et al.*, 2002; Keelan *et al.*, 2003). However, $PGF_{2\alpha}$ is more than just a potent stimulator of myometrial contraction. $PGF_{2\alpha}$ has an important signaling role during parturition, aiding in the transformation of the uterus of gestation to the uterus of delivery near the end of pregnancy. In this study, we extend our knowledge regarding the involvement of $PGF_{2\alpha}$ in parturition by clearly showing it as one of the mediators that promote the establishment of a pro-inflammatory intrauterine environment by stimulation of pro-inflammatory cytokine and chemokine production in myometrium, leading to the initiation of labor. In this sense, the circle becomes complete; pro-inflammatory cytokines promote synthesis of $PGF_{2\alpha}$ and its receptor, PTGFR, and $PGF_{2\alpha}$ promotes cytokine and chemokine synthesis via its receptor.

A number of studies have demonstrated that the rise of IL1β and IL6 in gestational tissues, amniotic fluid and maternal blood prior to labor indicates a role in parturition (Romero *et al.*, 1990; Osman *et al.*, 2003; Shynlova *et al.*, 2013b; Maddipati *et al.*, 2014). IL6 and IL1β can stimulate PGE_2 and $PGF_{2\alpha}$ production in gestational tissues such as myometrium,

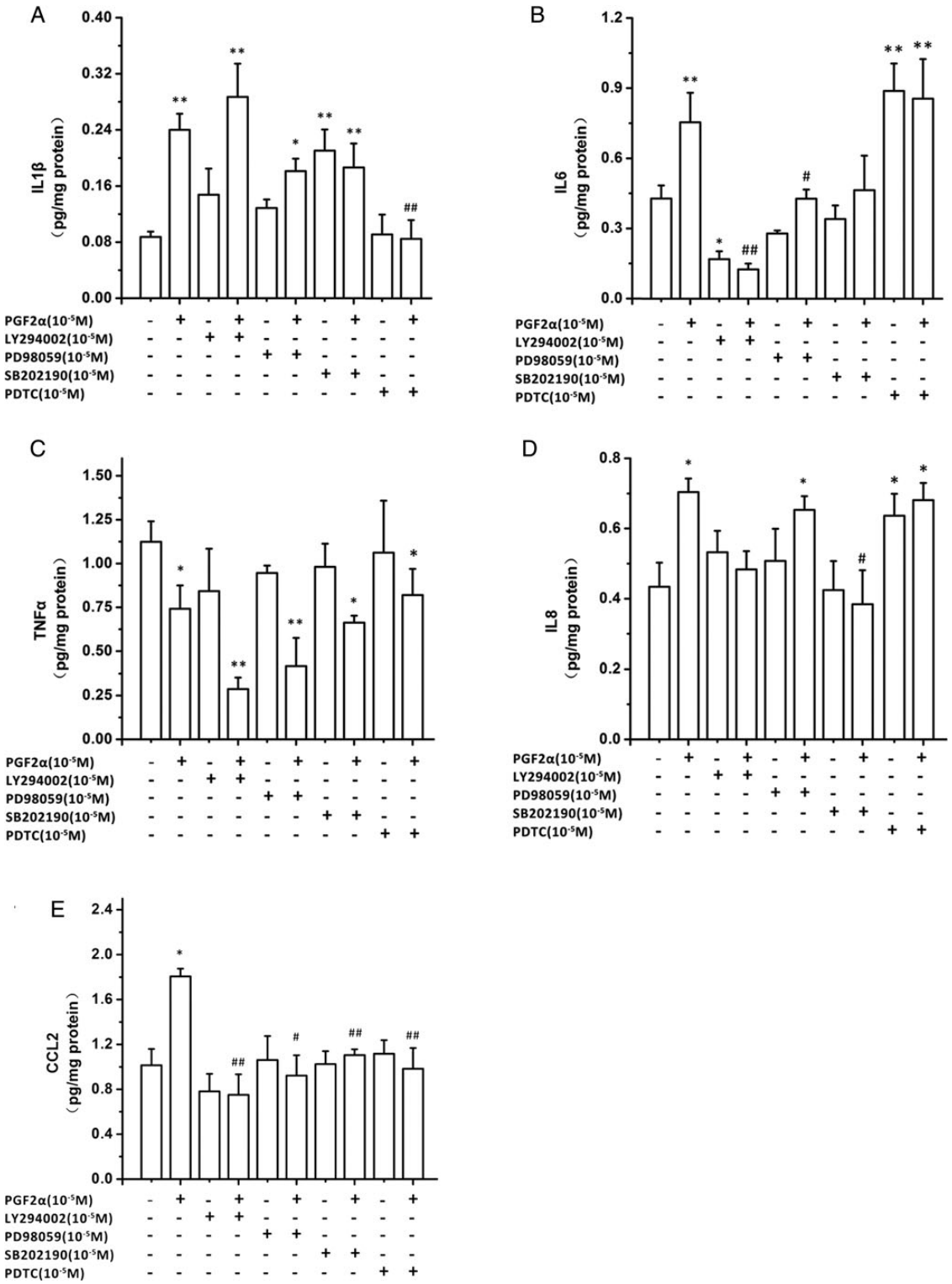

Figure 5 The role of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), extracellular signal receptor kinase (ERK1/2), P38 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways in prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) modulation of cytokine outputs. Human uterine smooth muscle cells (HUSMCs) from lower segment (LS) were treated with PI3K inhibitor (LY294002), and ERK inhibitor (PD98059), P38 inhibitor (SB202190) and NF-κB inhibitor (PDTC) in the presence or absence of $PGF_{2\alpha}$ (10^{-6} M). Supernatants were collected and analyzed by enzyme-linked immunosorbent assays (ELISA) to determine concentration of (**A**) interleukin (IL)-1β, (**B**) IL-6, (**C**) tumor necrosis factor α (TNFα), (**D**) interleukin 8 (CXCL8) and (**E**) monocyte chemoattractant protein (CCL-2). Values are presented as mean ± SEM. $n = 4$ (from four patients). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with $PGF_{2\alpha}10^{-6}$ M.

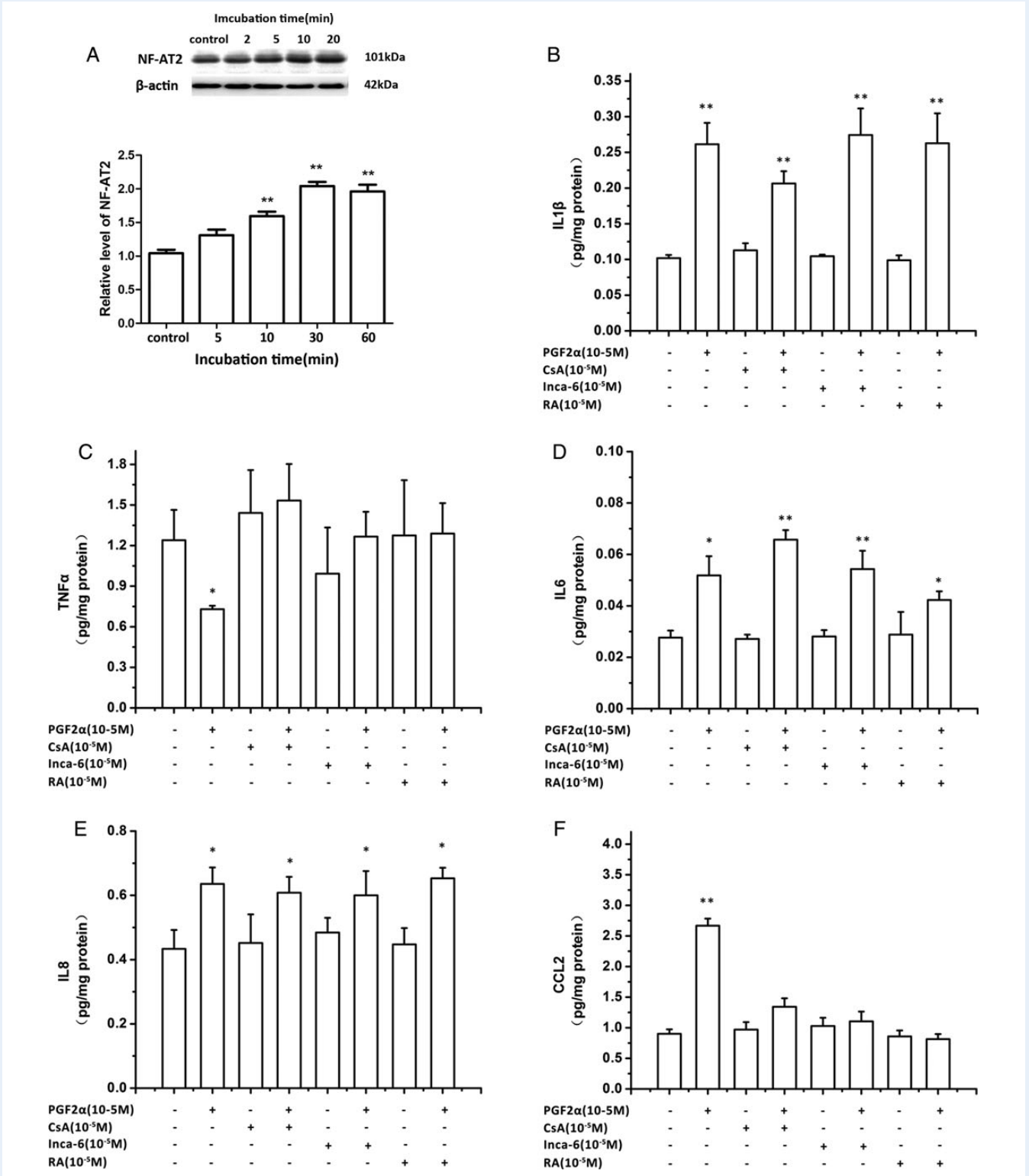

Figure 6 The role of calcineurin/nuclear factor of activated T-cells (NFATC1) pathway in prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) modulation of cytokine output. (**A**) Human uterine smooth muscle cells (HUSMCs) from lower segment (LS) were treated with $PGF_{2\alpha}$ (10^{-6} M) for the time indicated. The level of NFATC1 was determined by western blotting. (**B**–**F**) HUSMCs were treated with calcineurin inhibitor (CsA), the blocker of calcineurin and NFAT interaction (InCA-6), NFAT-AP1 complex inhibitor (RA) in presence or absence of $PGF_{2\alpha}$ (10^{-6} M). The supernatants were collected for enzyme-linked immunosorbent assays (ELISA) to determine concentration of; (B) IL-1β, (C) IL-6, (D) TNFα, (E) CXCL8 and (F) CCL-2. Values are presented as mean ± SEM. $n = 4$ (from four patients). *$P < 0.05$, **$P < 0.01$ compared with vehicle control.

amnion and decidual cells (Mitchell *et al.*, 1991; Keelan *et al.*, 2003). In the uterus, IL6 and IL1β also induce labor onset by up-regulating expression of UAPs in myometrium (Young *et al.*, 1997; Fang *et al.*, 2000; Rauk *et al.*, 2001). Our study demonstrated that $PGF_{2\alpha}$ administration triggered an up-regulation of IL6 and IL1β output in myometrial cells. Taken together, it may suggest that a positive interaction between $PGF_{2\alpha}$ and the pro-inflammatory cytokines IL1β and IL6 might occur within uterus, which amplifies inflammation and uterine activation as parturition is initiated.

Both CXCL8 and CCL2 are responsible for the recruitment of monocytes, memory T cells and dendritic cells to sites of inflammation (Xu *et al.*, 1996; Kobayashi, 2008; White *et al.*, 2013). CXCL8 is not only secreted from placental and decidual tissues (Saito *et al.*, 1994; el Maradny *et al.*, 1996; Denison *et al.*, 1998) but higher expression is detected in myometrium at labor (Osmers *et al.*, 1995). CCL2 is also expressed in human myometrium and greatly increased after onset of labor (Esplin *et al.*, 2005). Our data indicate that $PGF_{2\alpha}$ significantly enhances CXCL8 and CCL2 production in HUSMCs which may suggest that $PGF_{2\alpha}$ is involved in the process of uterine activation at end of gestation.

In the present study, we found that, unlike IL1β and IL6, TNFα output was suppressed by $PGF_{2\alpha}$ in HUSMCs. It is hard to know the significance of $PGF_{2\alpha}$ inhibition of TNFα. Previous studies have investigated the expression of TNFα in relation to labor in a variety of gestational tissues with inconsistent results (Winkler *et al.*, 2001; Tattersall *et al.*, 2008; Thomakos *et al.*, 2010; Alexander *et al.*, 2012). TNFα expression has been shown to be increased in amnion, chorion, and isolated choriodecidua with labor (Thomakos *et al.*, 2010). Some studies have reported that TNFα protein concentrations were low in decidua, fetal membranes, and myometrium, and that they did not change during the onset of labor (Winkler *et al.*, 2001; Tattersall *et al.*, 2008; You *et al.*, 2014). Given that TNFα can be induced by other pro-inflammatory cytokines such as IL1β and IL6, our findings that $PGF_{2\alpha}$ suppressed TNFα production in myometrium might partly explain why the expression of TNFα is not changed during labor even though the level of IL1β and IL6 is higher.

PTGFR, a member of the G-protein coupled receptor superfamily, principally couples to Gq protein leading to the activation of PLCβ/Ca^{2+}/PKC signaling pathways. Our data indicate that $PGF_{2\alpha}$ modulation of chemokine and cytokine production is dependent on the PLC/PKC signaling pathway. It is known that Ca^{2+} triggers calcineurin/NFAT signaling. $PGF_{2\alpha}$ up-regulates CXCL8 production via calcineurin/NFAT signaling pathway in endometrial cancer (Sales *et al.*, 2009). In the present study, we show that calcineurin/NFAT signaling pathway is involved in $PGF_{2\alpha}$ stimulation of CCL2 and suppression of TNFα, but not CXCL8

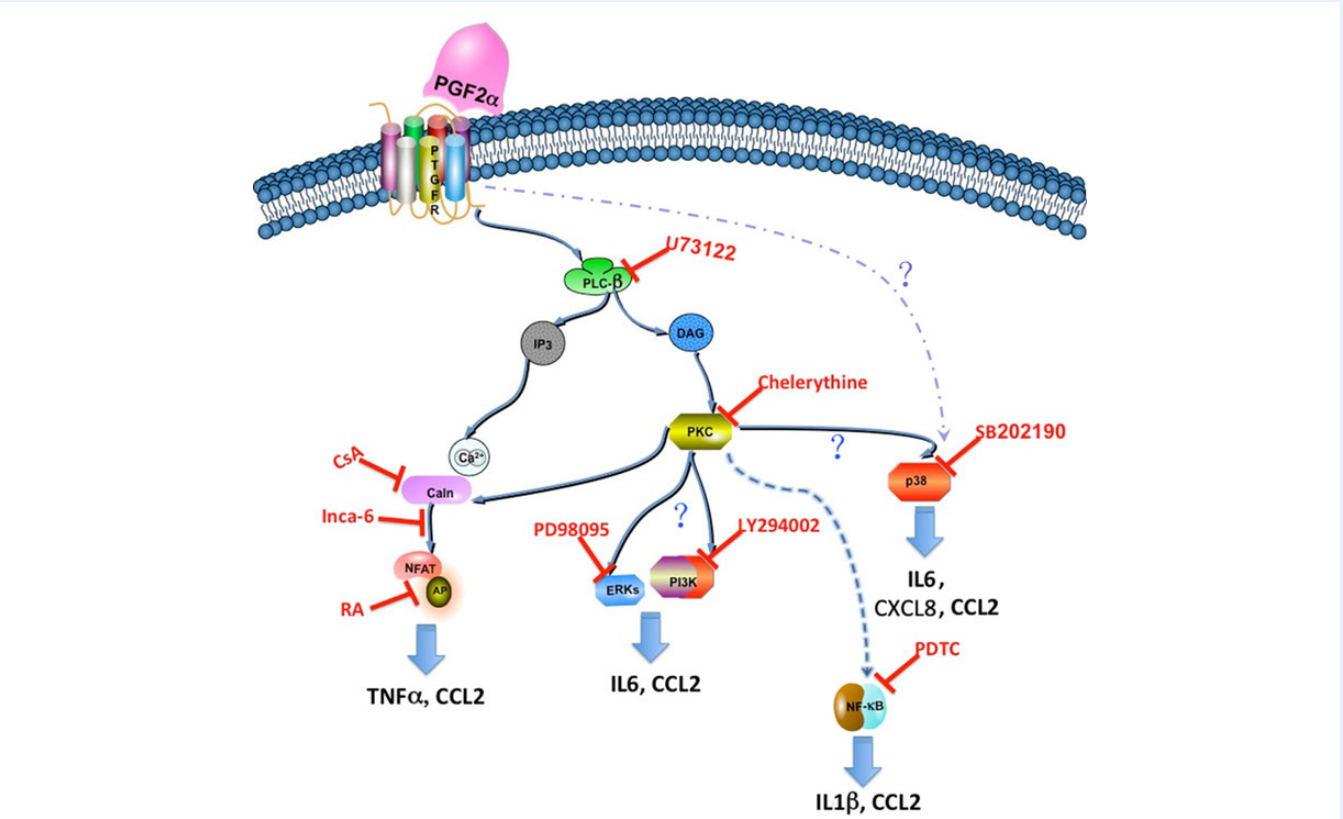

Figure 7 Scheme illustrating the signaling pathway involved in the production of chemokines and cytokines induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Prostaglandin $F_{2\alpha}$ receptor (PTGFR) primary couples to Gq protein. PTGFR can activate phospholipase β (PLCβ), which catalyzes the hydrolysis of membrane phosphoinositol lipids and leads to the release of inositol-1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG), which subsequently activate protein kinase C (PKC) and trigger the release of Ca^{2+} from endoplasmic reticulum (ER). PKC then activates phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), extracellular signal receptor kinase (ERK), P38 and nuclear factor-kappa light-chain-enhancer of activated B cells (NFκB) signaling pathways. Ca^{2+} activates calcineurin/nuclear factor of activated T-cells (NFAT) pathway and eventually activates AP-1 signaling.

production, suggesting that different signaling pathways are responsible for $PGF_{2\alpha}$ modulation of cytokines in different cells. Moreover, our data showed that $PGF_{2\alpha}$ regulation of IL1β and CCL2 is through NF-κB activation while its regulation of IL6 production is dependent on ERK, PI3K and P38 signaling pathways and CXCL8 secretion is through P38 signaling. Interestingly, $PGF_{2\alpha}$ regulation of CCL2 output occurs via multiple signaling molecules including ERK, PI3K, P38 and NF-κB. Taken together, we suggest that divergent signaling pathways mediate $PGF_{2\alpha}$ modulation of chemokines and cytokines in myometrium (Fig. 7).

Notably, the present study has shown that several kinase inhibitors themselves have corresponding effects on cytokine output, such as P38 inhibitor stimulated IL1β output while PDTC increased IL6 and CXCL8 output. Currently, it is unknown why these reagents increased secretion of the above cytokines. However, some studies have demonstrated the interaction between P38 and NF-κB signaling. Kanaji *et al.* (2012) have shown that P38 inhibitor enhances the level of phospho-p65 in endothelial cells, while PDTC has also been shown to induce P38 activation in vascular smooth muscle cells (Moon *et al.*, 2004). Interestingly, in the present study, we found that NF-κB activation leads to an increase in IL1β output while P38 signaling mediates $PGF_{2\alpha}$ stimulation of IL6 and CXCL8 output. Nevertheless, whether PDTC enhancing IL6 and CXCL8 output is through P38 signaling and P38 inhibitor increasing IL1β output is associated with NF-κB activation remains to be further elucidated.

Our previous study demonstrated that $PGF_{2\alpha}$-induced changes in UAP abundance differ between US and LS HUMSCs (Xu *et al.*, 2013). Our findings that the response pattern of some cytokines to $PGF_{2\alpha}$ was different between LS and US cells confirm our earlier observation that the responsiveness to $PGF_{2\alpha}$ can vary between US and LS cells, and indicate potential differential roles of $PGF_{2\alpha}$ in US and LS during pregnancy and parturition. Although the mechanisms underlying different responsiveness to $PGF_{2\alpha}$ in US and LS are unknown, different PTGFR receptor densities in the two regions, different intracellular pathways, or the possibility that $PGF_{2\alpha}$ stimulates other mediators with varying effects in each region might attribute to the discrepancy of $PGF_{2\alpha}$ effects in US and LS.

In conclusion, our study systematically demonstrated that $PGF_{2\alpha}$ modulates chemokine and cytokine output in human pregnant myometrium no matter upper and lower segment. Multiple signaling pathways are involved in $PGF_{2\alpha}$ regulation of chemokine and cytokine output in myometrium. Our findings corroborate the hypothesis that the initiation and amplification of non-infectious inflammation is a positive feedback reaction. This complex interrelationship of PGs, cytokines and chemokines, activates the uterus and completes its transfer to a contractile state.

Accessing research data

Data can be accessed via email by contacting nixin@smmu.edu.cn and david.olson@ualberta.ca

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

The authors were responsible for the following aspects of the study. C.X. and W.L.: study design, cell culture and treatment, acquisition, analysis and interpretation of data from ELISA, manuscript draft preparation; X.Y.: cell culture and treatment, acquisition, analysis and interpretation of data from western blotting; K.L. and K.P.: cell culture, siRNA transfection and ELISA; X.F., D.M.S., S.L.W, Q.S. and H.G. patient recruitment, tissue isolation, and clinical data collection. D.M.O. and X.N.: study design and coordination, data interpretation. X.N. and D.M.S.: critical revision of manuscript. All of the authors critically revised the manuscript and approved the final version.

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Conflict of interest

None declared.

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Novel Noncompetitive IL-1 Receptor–Biased Ligand Prevents Infection- and Inflammation-Induced Preterm Birth

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Preterm birth (PTB) is firmly linked to inflammation regardless of the presence of infection. Proinflammatory cytokines, including IL-1β, are produced in gestational tissues and can locally upregulate uterine activation proteins. Premature activation of the uterus by inflammation may lead to PTB, and IL-1 has been identified as a key inducer of this condition. However, all currently available IL-1 inhibitors are large molecules that exhibit competitive antagonism properties by inhibiting all IL-1R signaling, including transcription factor NF-κB, which conveys important physiological roles. We hereby demonstrate the efficacy of a small noncompetitive (all-d peptide) IL-1R–biased ligand, termed rytvela (labeled 101.10) in delaying IL-1β–, TLR2-, and TLR4-induced PTB in mice. The 101.10 acts without significant inhibition of NF-κB, and instead selectively inhibits IL-1R downstream stress-associated protein kinases/transcription factor c-jun and Rho GTPase/Rho-associated coiled-coil–containing protein kinase signaling pathways. The 101.10 is effective at decreasing proinflammatory and/or prolabor genes in myometrium tissue and circulating leukocytes in all PTB models independently of NF-κB, undermining NF-κB role in preterm labor. In this work, biased signaling modulation of IL-1R by 101.10 uncovers a novel strategy to prevent PTB without inhibiting NF-κB. ***The Journal of Immunology*****, 2015, 195: 3402–3415.**

Preterm birth (PTB; delivery before 37 wk of gestation, also referred to as prematurity) affects >1 of 10 infants worldwide, and is the leading cause of infant death in the United States and globally (1, 2). The onset of labor is a gradual process that begins several weeks before delivery and is characterized by changes in myometrium contractility and in cervical composition. Many causes have been suggested to explain preterm labor; in this context inflammation has been firmly linked to PTB (3–6).

Of various inflammatory cytokines implicated in PTB, IL-1 in particular has been identified as a key inducer of inflammation in PTB by binding to its ubiquitously expressed receptor IL-1RI, thus promoting activation and amplification of the inflammatory cascade. The major role of IL-1 in the onset of preterm labor is substantiated by the following evidence: 1) IL-1 alone is sufficient to induce labor in several animal models, and inhibition of its receptor prevents labor induction (7–9); 2) elevated IL-1β blood concentrations in humans is associated with PTB (10); 3) polymorphisms in human IL-1β gene (*IL1B*) and endogenous IL-1R antagonist gene (*IL1RN*) are associated with spontaneous preterm deliveries (11); and 4) IL-1β stimulates uterine activation protein (UAP) expression (12), and this effect is markedly amplified in the presence of $PGF_{2\alpha}$ in human myometrial cells (13).

Currently available tocolytics are at best only modestly effective compared with placebo; additionally, some of them present undesired maternal and/or fetal side effects (14, 15). Despite scientific evidence pointing to a major role for IL-1 in labor, preclinical studies using

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The online version of this article contains supplemental material.

Abbreviations used in this article: G, gestational day; IKK, inhibitor of NF-κB kinase; LTA, lipoteichoic acid; PTB, preterm birth; ROCK, Rho GTPase/Rho-associated coiled-coil–containing protein kinase; SAPK, stress-associated protein kinase; SMC, smooth muscle cell; UAP, uterine activation protein.

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IL-1–targeting agents reveal modest efficacy (16–18). At present there are three large molecule anti–IL-1 drugs approved for clinical use, as follows: the IL-1R antagonist Anakinra (Kineret), the soluble decoy receptor Rilonacept (Arcalyst), and the neutralizing anti–IL-1β mAb Canakinumab (Ilaris). As anticipated, these IL-1–targeting therapies inhibit all IL-1–signaling pathways, including NF-κB (19, 20). However, NF-κB, a major transcription factor for proinflammatory cytokines including IL-1, conveys important physiological roles such as cytoprotection and immune surveillance, particularly relevant in the vulnerable fetus. A recent study has reported deleterious (proapoptotic) effects of inhibiting NF-κB in pregnancy (21); accordingly, it has been suggested that complete blockade of NF-κB action would be undesirable (22).

Over the past few years, a new class of pharmacological agents termed allosteric modulators has been described. Allosteric compounds show functional selectivity by differently modulating signaling pathways induced by the binding of a natural ligand on a receptor, inhibiting some signals and/or preserving or enhancing others. Functional selectivity is a desirable approach in developing IL-1–targeting therapies in pregnancy because it does not inhibit all receptor-coupled response, contrary to that seen with orthosteric antagonists (23). Hence, functional selectivity could potentially minimize NF-κB inhibition and still inhibit other relevant IL-1 signaling. The host laboratory recently developed a small stable (all-d peptide) biased ligand modulator of IL-1R, specifically rytvela (labeled 101.10), which selectively binds to IL-1R and displays noncompetitive properties and functional selectivity toward specific pathways (24). The peptide rytvela has also been shown to be effective in numerous models of inflammation-linked diseases, including inflammatory bowel disease, contact dermatitis, hypoxic–ischemic newborn brain injuries, and ischemic retinopathies (24, 25). We hereby propose a hitherto unexplored strategy of delaying infection- and inflammation-induced PTB using 101.10, which selectively inhibits IL-1R downstream stress-associated protein kinase (SAPK)/c-jun and Rho/Rho GTPase/Rho-associated coiled-coil–containing protein kinase (ROCK) pathways without significantly affecting NF-κB activation.

Materials and Methods

Animals

Timed-pregnant CD-1 mice were obtained from Charles River at gestational day 12 and were allowed to acclimatize for 4 d prior to experiments. Animals were used according to a protocol of the Animal Care Committee of Hôpital Sainte-Justine along the principles of the *Guide for the Care and Use of Experimental Animals* of the Canadian Council on Animal Care. The animals were maintained on standard laboratory chow under a 12:12 light:dark cycle and allowed free access to chow and water.

Chemicals

Chemicals were purchased from the following manufacturers: human rIL-1β (200-01B; PeproTech), lipoteichoic acid (LTA; L3265; Sigma-Aldrich), LPS (L2630; Sigma-Aldrich), murine M-CSF (315-02; PeproTech), 101.10 (Elim Biopharmaceuticals, Hayward, CA), Kineret (Sobi, Biovitrum Stockholm, Sweden), SC-514 (10010267; Cayman Chemical), SR-11302 (2476; Tocris Bioscience), Y27632 (Y0503; Sigma-Aldrich), β-estradiol (2758; Sigma-Aldrich), and human rIL-1α (200-01A; PeproTech).

Cell culture

The myometrial smooth muscle cell (SMC) line (hTERT-C3) was provided by S. Laporte (University of McGill, Montréal, Canada). The RAW-Blue mouse macrophage reporter cell line and the HEK-blue IL-33/IL-1β cells were purchased from InvivoGen (San Diego, CA) and used at passages under 15. RAW-Blue mouse macrophages and HEK-BLUE cells were cultured in DMEM growth medium supplemented with 10% serum, 50 U/ml penicillin, 50 mg/ml streptomycin, and 200 μg/ml zeocin. Myometrial cells were cultured in DMEM/F12 growth medium supplemented with 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, and 0.1 mg/ml gentamicin. Cells were propagated in regular conditions (37°C, 5% CO_2). For in vitro experiments, cells were serum starved overnight and treated with 1 μg/ml IL-1β, LPS, or LTA for 15 min. The 101.10, Kineret (1.5 mg/ml), SC-514 (10 μM), or Y27632 (1 μM) was allowed to reach equilibrium for 30 min prior to the experiments. Cell lysis was performed in ice-cold radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Samples were stored in Laemmli buffer at −20°C or used fresh for Western blotting.

Intrauterine IL-1β–induced PTB model and i.p. LPS- and LTA-induced PTB models

Timed-pregnant CD-1 mice at 16.5 d of gestation were anesthetized with isoflurane and received an i.p. injection of either LTA (3 × 3-h interval injections of 12.5 mg/kg in 100 μL saline), LPS (a single dose of 0.5 μg in 100 μL saline), or a single intrauterine injection of IL-1β (1 μg). Doses of IL-1β, LPS, and LTA and frequencies of administration used were selected on the basis of reported documentation (8, 16, 26, 27) and on in vivo dose-response experiments we performed that would induce PTB in a reproducible manner. For the IL-1β–induced PTB model, animals were steadily anesthetized with an isoflurane mask. After body hair removal from the peritoneal area, a 1.5-cm–tall median incision was performed with chirurgical scissors in the lower abdominal wall. The lower segment of the right uterine horn was then exposed, and 1 μg IL-1β was injected between two fetal membranes with care of not entering the amniotic cavity. The abdominal muscle layer was sutured, and the skin was closed with clips. A total of 100 μL 101.10 (1 mg/Kg/12 h), Kineret (4 mg/Kg/12 h), SR-11302 (1 mg/Kg/12 h), Y27632 (0.5 mg/Kg/12 h), or vehicle was injected s.c. in the neck 30 min before stimulation with IL-1β, LPS, or LTA (to allow distribution of drugs to target tissues, in line with a first efficacy preclinical study); all doses used were based on reported efficacy (17, 24, 25, 28, 29). Mice delivery was assessed every hour until term (G19–G19.5). Immediately after delivery (<30 min postpartum), female adults were anesthetized and an intracardiac puncture was performed to collect systemic blood in heparin to prevent blood clotting. Blood plasma was isolated by centrifugation and immediately snap frozen in liquid nitrogen. The remaining blood cell pellet was treated with RBC lysis buffer (Norgen Biotek) and EDTA, according to the manufacturer protocol, and then centrifuged to isolate WBCs. Resulting WBC pellet in addition to myometrium fragments cut from the lower part of the right uterine horn was snap frozen in liquid nitrogen and kept at −80°C for subsequent RNA purification or protein extraction.

RNA extraction and real-time quantitative PCR

Myometrium fragments were thawed and rapidly preserved in RIBOzol (AMRESCO), whereas cells from in vitro experiments were treated for 6 h with IL-1β with or without 101.10 or Kineret and collected directly into RIBOzol. RNA was extracted according to manufacturer's protocol, and RNA concentration and integrity were measured with a NanoDrop 1000 spectrophotometer. A total of 500 ng RNA was used to synthetize cDNA using iScript Reverse Transcription SuperMix (Bio-Rad, Hercules, CA). Primers were designed using National Center for Biotechnology Information Primer Blast (Table I). Quantitative gene expression analysis was performed on Stratagene MXPro3000 (Stratagene) with SYBR Green Master Mix (Bio-Rad). Gene expression levels were normalized to 18S universal primer (Ambion Life Technology, Burlington ON, Canada). Dissociation curves were also acquired to test primer specificity, and amplicon length was verified by electrophoresis of product on a 2% agarose gel (data not shown). Genes analyzed include the following: *IL1B*, *IL4*, *IL6*, *IL8*, *IL10*, *TNFA*, *CCL2* (chemokine ligand 2), *CRP* (C-reactive protein), *MMP1A*, *MMP3*, *MMP9*, *PTGHS2* (PG H synthetase 2 or cyclooxygenase-2), *PTGFR* (PG F receptor), *OXTR* (oxytocin receptor), *IL1R1* (IL-1R1), *GJA1* (connexin 43), *IL1RA* (IL-1R endogenous antagonist), and *IFNB1* (IFN β1). Detailed primer sequences are shown in Table I.

Semiquantitative PCR

Cells were pretreated with 10^{-6} M 101.10 or vehicle for 30 min and then stimulated with 50 ng/ml IL-1α for 24 h. Total RNA was isolated with RNeasy mini kit (Qiagen, Germantown MD). RT-PCR was performed, as described previously (30). QuantumRNA universal 18S standard primers (Ambion) were used as internal standard references.

Western blotting

Proteins from homogenized myometrium fragments and cell samples lysed in radioimmunoprecipitation assay buffer (containing protease and phosphatase inhibitors) were quantified using Bradford's method (Bio-Rad). A total of 50 μg protein sample was loaded onto SDS-PAGE gel and electrotransferred onto polyvinylidene difluoride membranes. After blocking, membranes were incubated with either an Ab against IL-1R1 (sc-689; Santa Cruz Biotechnology, Dallas, TX), OxtR (ab101617; Abcam, Toronto, ON, Canada), α-actin

Table I. Primers used for real-time quantitative PCR

Mouse Primers	
IL1B-F: 5′-AGATGAAGGGCTGCTTCCAAA-3′	IL1B-R: 5′-GGAAGGTCCACGGGAAAGAC-3′
IL4-F: 5′-AACGAAGAACACCACAGAGAG-3′	IL4-R: 5′-GTGATGTGGACTTGGACTCA-3′
IL6-F: 5′-CAACGATGATGCACTTGCAGA-3′	IL6-R: 5′-TCTCTCTGAAGGACTCTGGCT-3′
IL8-F: 5′-TGCTTTTGGCTTTGCGTTGA-3′	IL8-R: 5′-GTCAGAACGTGGCGGTATCT-3′
IL10-F: 5′-TAACTGCACCCACTTCCCAG-3′	IL10-R: 5′-AGGCTTGGCAACCCAAGTAA-3′
TNFA-F: 5′-GCCTCTTCTCATTCCTGCTTG-3′	TNFA-R: 5′-CTGATGAGAGGGAGGCCATT-3′
CRP-F: 5′-TCTGCACAAGGGCTACACTG-3′	CRP-R: 5′-ATCTCCGATGTCTCCCACCA-3′
IFNB1-F: 5′-AGCACTGGGTGGAATGAGAC-3′	IFNB1-R: 5′-GAGTCCGCCTCTGATGCTTA-3′
MMP1A-F: 5′-CAGGACTTATATGGACCTTCCC-3′	MMP1A-R: 5′-TAAATTGAGCTCAGGTTCTGGC-3′
MMP3-F: 5′-GTGACCCCACTCACTTTCTC-3′	MMP3-R: 5′-TTGGTACCAGTGACATCCTCT-3′
MMP9-F: 5′-TCAAGGACGGTTGGTACTGG-3′	MMP9-R: 5′-CTGACGTGGGTTACCTCTGG-3′
OXTR-F: 5′-TGTGTCTCCTTTTGGGACAA-3′	OXTR-R: 5′-GGCATTTCAGAATTGGCTGT-3′
PGHS2-F: 5′-ACCTCTCCACCAATGACCTGA-3′	PGHS2-R: 5′-CTGACCCCCAAGGCTCAAAT-3′
PTGFR-F: 5′-AGCTGGACTCATCGCAAACA-3′	PTGFR-R: 5′-GTGGGCACAAGCCAGAAAAG-3′
GJA1-F: 5′-GCACTTTTCTTTCATTGGGGG-3′	GJA1-R: 5′-GGGCACCTCTCTTTCACTTA-3′
IL1R1-F: 5′-CTTGAGGAGGCAGTTTTCGT-3′	IL1R1-R: 5′-ACATACGTCAATCTCCAGCG-3′
IL1RA-F: 5′-TGGGAAGGTCTGTGCCATA-3′	IL1RA-R: 5′-CCAGATTCTGAAGGCTTGCAT-3′
CCL2-F: 5′-GCTCAGCCAGATGCAGTTA-3′	CCL2-R: 5′-TGTCTGGACCCATTCCTTCT-3′
Human Primers	
IL1B-F: 5′-AGCTGGAGAGTGTAGATCCCAA-3′	IL1B-R: 5′-ACGGGCATGTTTTCTGCTTG-3′
IL6-F: 5′-TTCAATGAGGAGACTTGCCTGG-3′	IL6-R: 5′-CTGGCATTTGTGGTTGGGTC-3′
IL8-F: 5′-CTCTGTGTGAAGGTGCAGTTTT-3′	IL8-R: 5′-TGCACCCAGTTTTCCTTGGG-3′
MMP1-F: 5′-AGAATGATGGGAGGCAAGTTGA-3′	MMP1-R: 5′-TGGCGTGTAATTTTCAATCCTGT-3′
MMP3-F: 5′-TGCTGTTTTTGAAGAATTTGGGTT-3′	MMP3-R: 5′-AGTTCCCTTGAGTGTGACTCG-3′
CCL2-F: 5′-CAGCCAGATGCAATCAATGCC-3′	CCL2-R: 5′-TTTGCTTGTCCAGGTGGTCC-3′
PGHS2-F: 5′-ATATTGGTGACCCGTGGAGC-3′	PGHS2-R: 5′-GTTCTCCGTACCTTCACCCC-3′

(ab5694; Abcam), F4/80 (ab6640; Abcam), Lamin B1 (ab16048; Abcam), NF-κB p65 (sc-372; Santa Cruz Biotechnology), IL-1R accessory protein (ab8110; Abcam), or β-actin (sc-47778; Santa Cruz Biotechnology). After washing, membranes were incubated for 1 h with their respective secondary Abs conjugated to HRP (Sigma-Aldrich). For kinases, membranes were incubated with an Ab against either phospho-JNK (9251; Cell Signaling Technology, Whitby, ON, Canada), phospho–c-jun (9261; Cell Signaling Technology), phospho-p38 (4511; Cell Signaling Technology), phospho-ROCK2 (PA5-34895; Thermo Fisher Scientific), phospho-IκBα (2859; Cell Signaling Technology), JNK (9252; Cell Signaling Technology), c-jun (9165; Cell Signaling Technology), p38 (9212; Cell Signaling Technology), or ROCK2 (PA5-21131; Thermo Fisher Scientific, Waltham, MA). ECL (GE Healthcare, Little Chalfont, U.K.) was used for detection using the ImageQuant LAS-500 (GE Healthcare), and densitometric analysis was performed using ImageJ. Resulting values were normalized first with total proteins and then with the control sample.

Rhotekin-rho binding domain bead pull-down assay

Rho activation was assessed using a Rho Activation Assay Biochem Kit (Cytoskeleton). hTERT-C3 cells were plated in 150-mm petri dishes and serum starved at ∼50% confluence for 16 h prior to the experiment. The 101.10 or Kineret was administered 30 min before the IL-1β stimulation to allow the system to equilibrate. After 15 min of IL-1β stimulation, cells were rapidly lysed with ice-cold lysis buffer, and cell debris were removed by centrifugation at 4°C. A small amount of every sample was collected on ice for protein quantitation using Bradford's method, and the remaining cell lysate was snap frozen in liquid nitrogen and conserved at −80°C for ∼1 h during protein quantitation. After thawing, 800 μg of each sample was incubated on a rocking platform with 50 μg rhotekin-rho binding domain beads (high affinity for GTP-bound RhoA) for 1 h at 4°C. As a positive control, 800 μg cell lysate was incubated for 15 min with 200 μM GTPγS (a nonhydrolyzable GTP analog) prior to the bead pull down. After washing steps, samples were centrifuged and bead lysates were loaded on SDS-PAGE gel in 2× Laemmli buffer. Samples were electrotransferred on polyvinylidene difluoride membranes, blocked, and incubated with an anti-RhoA mAb (ARH03; Cytoskeleton) overnight at 4°C. The membrane was then incubated with a HRP-conjugated anti-mouse secondary Ab (Sigma-Aldrich) and revealed using an ECL solution (GE Healthcare). Total RhoA expression and β-actin were assessed using 50 μg of the samples that were set aside on ice before pull down. Densitometric analysis was performed using ImageJ.

Circulating leukocyte RNA purification

As described before, WBCs were isolated from systemic blood of female mice (<30 min postpartum) and total leukocyte RNA was extracted using a leukocyte RNA purification kit (Norgen Biotek, Thorold, ON, Canada). Briefly, the WBC pellet was lysed and passed through a RNA-binding column. After several washing procedures, the RNA was eluted from the column and equal amount of RNA was used to synthetize cDNA using iScript Reverse Transcription SuperMix (Bio-Rad). Quantitative RT-PCR was then performed on the samples, as previously described.

NF-κB QUANTI-Blue assay

Hek-Blue cells (InvivoGen) were pretreated with different concentrations of 101.10 (10^{-9}–10^{-5} M) and Kineret (1.5 mg/ml) for 30 min, followed by treatment with constant concentration of IL-1β (1 μg/ml), and then incubated at 37°C for 4 h. Levels of secreted alkaline phosphatase in cell culture supernatant were determined by the use of QUANTI-Blue, according to manufacturer instruction (InvivoGen). Alkaline phosphatase activity was assessed by reading the OD at 620–655 nm with a micro plate reader (EnVision Multilabel reader; PerkinElmer, Waltham, MA). Data are representative of five experiments (each with $n = 6$).

Ex vivo uterine contraction experiment

Timed-pregnant CD-1 mice at G18.5 were given a single dose of either saline or 101.10 (1 mg/kg in 100 μL saline). Within 30 min, mice were injected i.p. with IL-1β (1 μg/mouse). Seventeen hours after, uterine tissues were collected under anesthesia (2.5% isoflurane). Briefly, a midline abdominal incision was made, and the uterine horns were rapidly excised and carefully cleansed of surrounding connective tissues. Longitudinal myometrial strips (2–3 mm wide and 10 mm long) were dissected free from uterus and mounted isometrically in organ tissue baths, and initial tension was set at 2 g. The tissue baths contain 20 ml Krebs buffer of the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 0.9 $MgSO_4$, 1 KH_2PO_4, 11.1 glucose, and 23 $NaHCO_3$ (pH 7.4). The buffer was equilibrated with 95% oxygen/5% carbon dioxide at 37°C. Isometric tension was measured by a force transducer and recorded by BIOPAC data acquisition system (BIOPAC MP150). Experiments began after 1-h equilibration. Mean tension of spontaneous contractions was measured using a BIOPAC digital polygraph system (AcqKnowledge); the same parameters were also determined after addition of $PGF_{2\alpha}$. At the start of each experiments, mean tension of spontaneous myometrial contractions was considered as a reference response. Increase in mean tension (%) was expressed as percentages of (X/Y) − 100, where X is changes in mean tension (g) induced by $PGF_{2\alpha}$ and Y is the initial reference response (g).

Primary myometrial SMC isolation and culture

Primary myometrial SMC were isolated using modifications of a method previously described (31). Briefly, a single s.c. injection of 50 μg 17β-estradiol was administered to mice 24 h prior to the experiment. The day

after, mice were sacrificed by cervical dislocation and sprayed with 70% ethanol. The whole uterus was excised under sterile hood and placed in buffer A (HBSS [pH 7.4], 0.098 g/L magnesium sulfate, 0.185 g/L calcium chloride, 2.25 mmol/L I-HEPES, 100 U/ml penicillin-streptomycin [Life Technologies, Grand Island, NY], and 2.5 μg/ml amphotericin B [Sigma-Aldrich]). The uterine horns were cleansed of fat and vessels and then transferred into buffer B (buffer A without magnesium sulfate or calcium chloride) for several washes by gentle flushing. Afterward, the uterine horns were cut into 1-mm–wide fragments and transferred into a volume of 10 ml/g tissue of digestion buffer (1 mg/ml collagenase type II [Sigma-Aldrich], 0.15 mg/ml DNase I [Roche Diagnostics, Mannheim, Germany], 0.1 mg/ml soybean trypsin inhibitor [Sigma-Aldrich], 10% FBS, and 1 mg/ml BSA [Sigma-Aldrich] in buffer B). Enzymatic digestion was performed at 37˚C with agitation (100 rev/min) for 30 min. The homogenate (still containing undigested myometrium fragments) was then poured through a 100-μm cell strainer. The resulting filtered solution was centrifugated at 200 × *g* for 10 min, and the pellet was resuspended in complete DMEM and plated in a T-25 dish. The remaining myometrium fragments were reused in an enzymatic digestion, and the whole digestion–centrifugation process was repeated for a total of five times. First two digestion results were discarded because they contained mostly fibroblasts. The three other SMC-containing dishes were subjected to a differential adhesion technique to selectively enrich for uterine myocytes. Briefly, 30–45 min after the cells were first plated, the medium was removed and dispensed in another T-25 culture dish to separate quickly adhering fibroblast from slowly adhering myocytes. Cells were further analyzed in immunohistochemistry to assess culture purity with the SMC marker α-actin.

Primary bone marrow–derived macrophage isolation and culture

CD-1 mice were sacrificed with cervical dislocation and then sprayed with 70% ethanol. Both femurs and tibias were prelevated under sterile hood by gently removing the muscles and then cutting the epyphyses. Bone marrow was extruded by flushing it with a 25-gauge syringe containing sterile RPMI 1640 culture medium supplemented with 10% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin. Resulting medium containing the bone marrow–derived progenitor cells was then homogenized, filtered through a 70-μm nylon web, and seeded in T-25 plates. A total of 20 ng/ml rM-CSF was added prior to incubation, and cells were allowed to differentiate for 6 d. Cells were further analyzed in immunohistochemistry to assess culture purity with the macrophage marker F4/80.

Murine IL-1β ELISA

The ELISA was performed using a mouse IL-1β Quantikine ELISA kit (R&D Systems), according to the manufacturer's protocol. Briefly, 50 μL either plasma samples, mouse rIL-1β positive control, or decreasing concentrations of a mouse rIL-1β standard were loaded into a 96-well plate precoated with an anti-mouse IL-1β mAb and incubated for 2 h at ambient temperature. Wells were washed five times and incubated with an enzyme-linked mouse polyclonal Ab specific to murine IL-1β for 2 h. After another washing step, a substrate solution was added. The enzymatic reaction was stopped after 30 min, and the plate was read at 450 nm, with wavelength correction set to 570 nm.

Immunohistochemistry

Cells were plated on coverslips precoated with poly-D-lysine and fixed in 4% paraformaldehyde. After blocking, cells were incubated overnight with 101.10-FITC or FITC alone (Sigma-Aldrich) and a primary Ab of rabbit anti–IL-1RI, rabbit anti–α-actin, or rat anti-F4/80, and then for 1 h at ambient temperature with a secondary Ab conjugated with Alexa Fluor 594 (red) or 647 (white) (Sigma-Aldrich). For tissue immunohistochemistry, mice were treated with a single s.c. 1 mg/Kg 101.10-FITC injection and animals were euthanized after 1 h of incubation. Uterine tissues were cleansed of fat and vessels. Myometrium fragments and placentas were fixed in 4% paraformaldehyde for 1 d and transferred in 30% sucrose for another day. Localization of 101.10 was determined on 14-μm uterine sagittal cryosections or longitudinal placenta cryosections. Sections blocked with 1% BSA, 1% goat serum, and 0.1% Triton X-100 (T-8787; Sigma-Aldrich) in PBS were subsequently incubated overnight with the primary Abs. Secondary Abs conjugated with Alexa Fluor (Molecular Probes) directed against rabbit or rat were incubated for 2 h at ambient temperature. Nuclei were stained with DAPI (Invitrogen; 1/5000). Images were captured using 10× (for myometrium tissues) or 30× (for cells and magnified placenta images) objective with Eclipse E800 (Nikon) fluorescence microscope. Whole placenta images were captured at 10× using a Zeiss AxioObserver.Z1 (Zeiss, San Diego, CA). Images were merged into a single file using the MosiaX option in the AxioVision software version 4.6.5 (Zeiss).

Statistical analysis

Groups were compared using one-way ANOVA. Dunnett's multiple comparison method was employed when treatments were compared with a single control. Tukey's multiple comparison test was used in Fig. 1G. A p value <0.05 was considered statistically significant. Data are presented as means ± SD.

Results

The 101.10 prevents IL-1β–induced preterm birth and associated inflammatory-triggered uterine activation

We first determined whether 101.10 was effective at delaying PTB induced specifically by intrauterine IL-1β. A total of 1 μg IL-1β was injected in the right uterine horn of pregnant mice at G16.5 to induce PTB; births between G16.5 and G18.5 were considered premature because normal term for CD-1 mice is G19.2 based on data of our group (32) (Fig. 1A). Twenty-four hours after the intrauterine injection, mice uterine horns were inspected to confirm the presence of macroscopic inflammation (edema, hemorrhage). Notably, IL-1β–treated mice exhibited frankly observable inflammation of uteri (Fig. 1B, *middle panel*) in comparison with sham animals (Fig. 1B, *left panel*); this inflammation was alleviated by 101.10 (1 mg/Kg/12-h s.c. injections; Fig. 1B, *right panel*). Accordingly, IL-1β–treated mice receiving vehicle ($n = 16$) rapidly went into premature labor, with 56% delivering within 24 h after IL-1β administration, whereas only 12% of IL-1β–treated mice receiving 101.10 (n = 17) delivered before G19 (Fig. 1C, *bottom panel*). In contrast, systemic (s.c.) administration of the competitive IL-1 inhibitor Kineret ($n = 11$) was ineffective at reducing prematurity (Fig. 1C, *top panel*) and increasing gestational duration (Supplemental Fig. 1A). A group simply treated with 101.10 (without IL-1) served for gross toxicity evaluation; there were no gross teratogenic changes detected in all major organs examined.

Analysis of myometrium samples collected within 30 min of pup delivery revealed that 101.10 diminished IL-1β–triggered induction of mRNA of numerous proinflammatory and/or prolabor genes (see Table I for primer sequences), including many UAP genes (such as *CCL2*, *OXTR*, *PTGFR*, *MMP9*, *GJA1*, and *PTGS2*; see Fig. 1D); 101.10 also decreased IL-1β–induced (protein) expression of IL-1R (Supplemental Fig. 2A) and oxytocin receptor (Supplemental Fig. 2B), but not of the IL-1R accessory protein (Supplemental Fig. 2C). Two genes of relevance to myometrial activation drew our attention, *OXTR* (Fig. 1F, *left panel*) and *PTGFR* (Fig. 1F, *right panel*), which respectively encode for oxytocin receptor and PG $F_{2\alpha}$ receptor; both were significantly suppressed by 101.10 in the myometrium of IL-1β–treated mice. Concordantly, 101.10 ($n = 4$) attenuated contractile tension in response to oxytocin (Fig. 1G, *left panel*) and $PGF_{2\alpha}$ (Fig. 1G, *right panel*) in myometrium of IL-1β–treated mice compared with controls ($n = 6$).

Consistent with its functional inefficacy, Kineret was ineffective in altering IL-1β–induced myometrial gene expression (Fig. 1D, 1F); similar results were observed on gene expression in placenta (Supplemental Fig. 3A). In contrast, gene expression profile of circulating leukocytes collected <30 min postpartum revealed comparable inhibition of intrauterine IL-1β–induced genes with 101.10 and Kineret (Fig. 1E). These observations support the concept that activated leukocytes responding to an inflammatory locus (uteroplacental unit in this case) are a significant source of IL-1, which in turn amplifies the inflammatory response (25, 33); accordingly, the systemically administered large molecule Kineret (17.5 kDa) is effective on blood leukocytes, but contrary to 101.10 seems to have limited access to intrauterine/placental IL-1R wherein inflammation is triggered (by IL-1), consistent with documentation on IL-1 (~17.5 kDa) (34, 35).

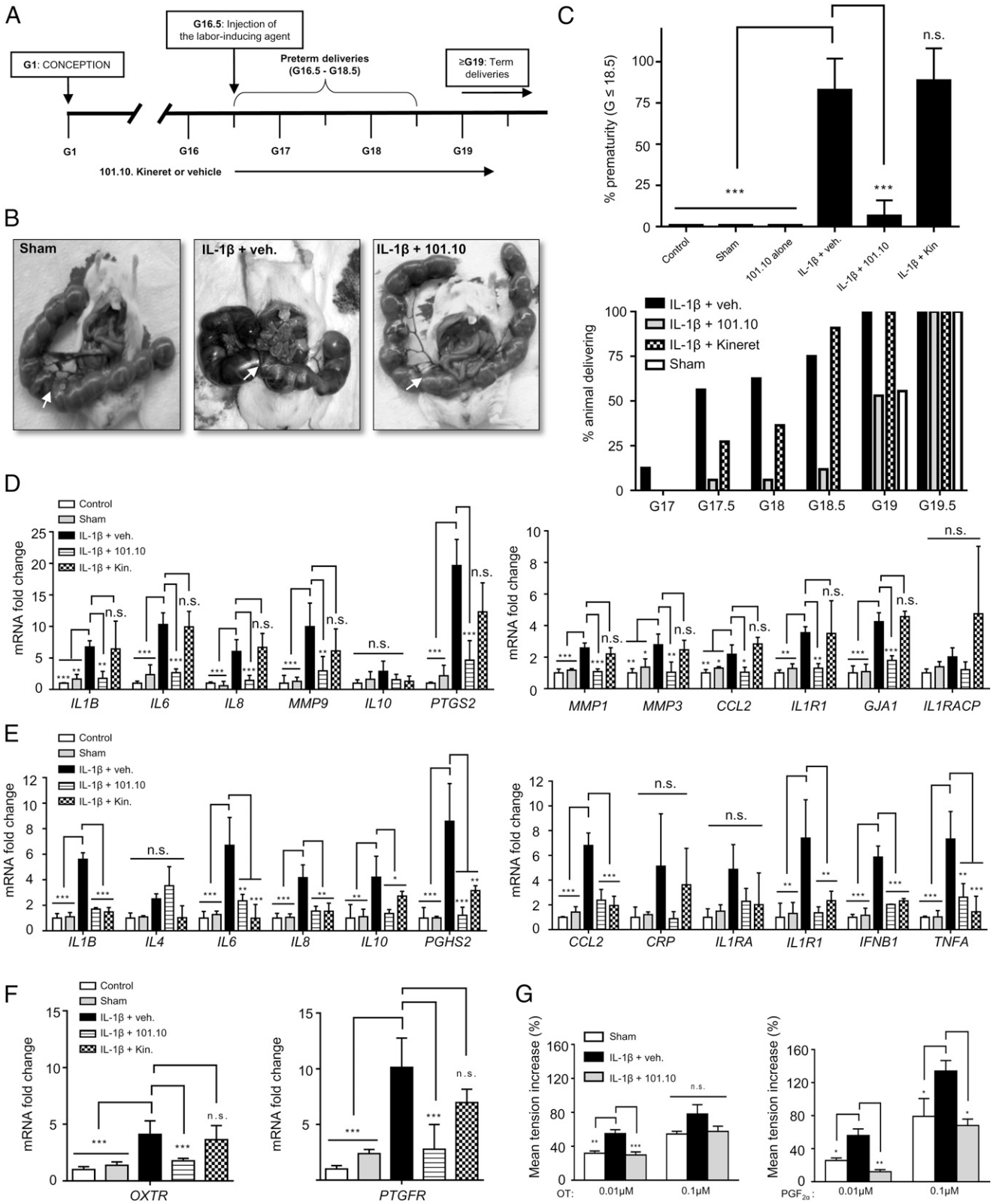

FIGURE 1. The 101.10 prevents IL-1β–induced preterm birth and curbs inflammation-induced uterine activation. (**A**) The labor-inducing agent is injected at G16.5, and spontaneous deliveries happening between G16.5 and G18.5 are considered as premature. Subcutaneous injections of 101.10 (1 mg/Kg/12 h), Kineret (4 mg/Kg/12 h), or vehicle are given twice per day until delivery. (**B**) Representative picture of uteri 24 h after the intrauterine IL-1β injection. *Left panel*, Sham; *middle panel*, IL-1β–induced uterine inflammation; *right panel*, 101.10 decreases clinical signs of IL-1β–induced uterine inflammation. (**C**) The 101.10 prevents IL-1β–induced preterm birth in mice. *Top panel*, Percentage of prematurity (≤G18.5) following 1 μg intrauterine IL-1β injection; *bottom panel*, percentage of animals having delivered plotted against gestational age. Control mice did not receive any treatment, whereas sham animals received an intrauterine dose of vehicle at G16.5. (**D**) Quantitative PCR from myometrium tissue of mice treated in (C), collected postpartum (<30 min following parturition). Results are normalized with 18S and are relative to control. (**E**) Quantitative PCR from leukocytes isolated from systemic blood of mice treated in (C) and collected postpartum (<30 min). Results are normalized with 18S and are relative to control. (**F**) The 101.10 decreases the (*Figure legend continues*)

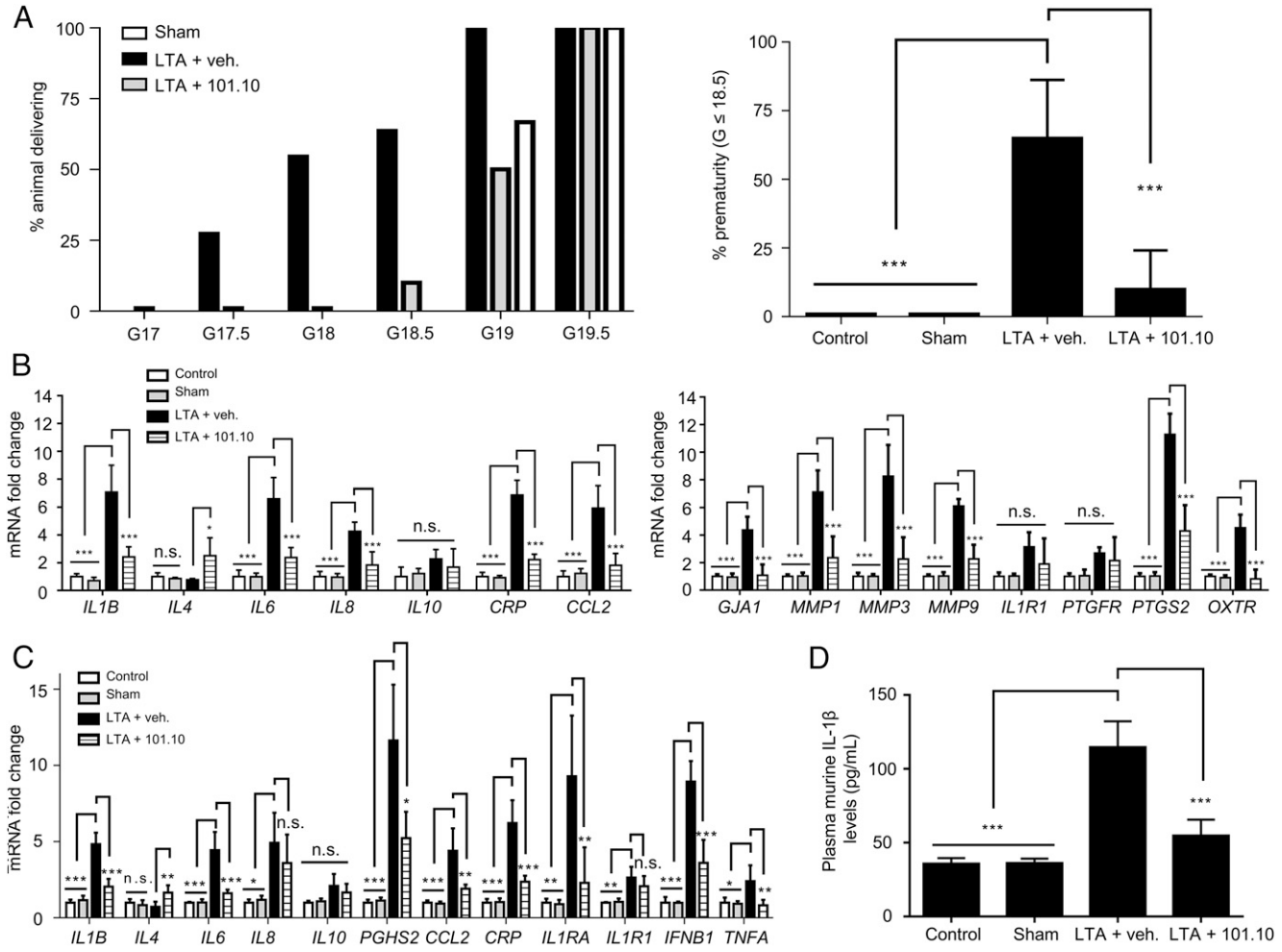

FIGURE 2. The 101.10 decreases TLR2-induced preterm birth. (**A**) The 101.10 decreases preterm birth induced by the TLR2 agonist LTA in mice. *Left panel*, Percentage of animals having delivered following three 3-h interval i.p. LTA injections (12.5 mg/Kg); *right panel*, percentage of premature deliveries. Control mice did not receive any treatment, whereas sham animals received three i.p. doses of vehicle over a period of 9 h at G16.5. The 101.10 (1 mg/Kg/12 h) or vehicle was injected s.c. twice per day until delivery. (**B**) Quantitative PCR from myometrium tissue of mice treated in (A) and collected postpartum (<30 min). Results are normalized with 18S and are relative to control. (**C**) Quantitative PCR from leukocytes isolated from systemic blood of mice treated in (A) and collected postpartum (<30 min). Results are normalized with 18S and are relative to control. (**D**) Murine IL-1β ELISA performed on plasma from mice treated in (A) and collected postpartum (<30 min). Values are presented as mean ± SD. Data are representative of 3–11 animals per group. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test compared with LTA + vehicle group.

The 101.10 distributes to myometrial SMC, macrophages, and placenta

We next determined whether 101.10 localized in blood leukocytes, myometrium, and, more importantly, placenta. The 101.10 labeled with FITC was injected s.c.; no loss of function was ensued by the labeling, as 101.10-FITC was still efficient at delaying LTA-induced PTB (data not shown). The 101.10-FITC localized on SMC (colocalization with SMC marker α-actin) (Supplemental Fig. 4A), on macrophage (marker F4/80) (Supplemental Fig. 4C), as well as in placenta (Supplemental Fig. 3D); FITC-alone fluorescence was not detected on these cells and tissues, suggesting binding specificity (Supplemental Figs. 3C, 4B, 4D); of note, fluorescence in placentas from FITC-treated mice did not differ from the autofluorescence of unlabeled placentas (Supplemental Fig. 3B).

We previously showed that actions of 101.10 required presence of the ubiquitous IL-1RI (25). Accordingly, in this study again 101.10-FITC colocalized by immunohistochemistry with IL-1RI on the myometrial cell line hTERT-C3 and the macrophage cell line RAW-Blue mouse macrophages (Supplemental Fig. 4E–H); FITC alone did not colocalize with IL-1R1.

The 101.10 delays TLR2- and TLR4-induced preterm birth (by acting downstream of TLR signaling)

The efficacy of 101.10 was also tested in PTB models that mimic relevant Gram$^+$ and Gram$^-$ infections, by stimulating corresponding TLR2 and TLR4, respectively, with LTA and LPS. The 101.10 was particularly effective in (i.p.) LTA-induced PTB (Fig. 2A), as it prolonged gestation (Supplemental Fig. 1B). The 101.10 also nearly normalized LTA-induced expression of all genes screened

expression of oxytocin receptor (*left panel*) and FP receptor (*right panel*) in the myometrium of mice treated in (C). (**G**) Ex vivo myometrium contraction in pharmacological baths performed with uterine tissues from mice treated as indicated. Uterotonic agents oxytocin (*left panel*) and $PGF_{2\alpha}$ (*right panel*) were used to induce dose-dependent contractions of the myometrium. Values are presented as mean ± SD. Data are representative of 3–17 animals per group. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test compared with IL-1β + vehicle group.

in myometrium (Fig. 2B) and blood leukocytes (Fig. 2C), with the exception of IL-1RI and the anti-inflammatory IL-4, which was increased; plasma levels of IL-1β were also decreased by 101.10 (Fig. 2D).

The 101.10 also reduced prematurity rate and prolonged gestation shortened by TLR4 stimulation with (i.p.) LPS (Fig. 3A, Supplemental Fig. 1C) and reduced LPS-induced gene induction on myometrium and blood leukocytes (Fig. 3B, 3C), as well as albeit modestly, plasma levels of IL-1β (Fig. 3D).

The 101.10 acts independently of IL-1β–induced NF-κB activation

To better understand how 101.10 regulates IL-1 activity, we determined the effects of 101.10 on IL-1R–coupled intracellular signaling in myometrial and macrophage cell lines hTERT-C3 and RAW-Blue macrophages. NF-κB is often been regarded as a key pathway for IL-1 signaling; the translocation of NF-κB to the nucleus is constitutively inhibited by IκB proteins in the cytosol, which when phosphorylated by inhibitor of NF-κB kinases (IKKs) results in its ubiquitination and subsequent degradation, hence promoting NF-κB activation (36). The 101.10 (dose dependently) did not affect IL-1β–induced IκBα phosphorylation in myometrial cells, whereas Kineret completely inhibited its activation (Fig. 4A, 4B). Likewise, in HEK-Blue cells engineered with a NF-κB–dependent promoter for secretory alkaline phosphatase, 101.10, contrary to Kineret, was again ineffective in altering IL-1β–induced secretion of alkaline phosphatase, and thus was NF-κB independent (Fig. 4C). Moreover, the critically important nuclear translocation of NF-κB upon IL-1 stimulation in myometrial cells was unaffected by 101.10, but was markedly inhibited by Kineret and the IKKβ inhibitor SC-514 (positive control) (Fig. 4D, 4E). Collectively, these data indicate that effects of 101.10 are independent of NF-κB.

The 101.10 inhibits SAPK p38 and JNK, transcription factor c-jun, and Rho/ROCK pathways in myometrial cells and in macrophages

The effect of 101.10 on other IL-1–triggered signaling pathways was investigated. Given their reported involvement in labor (37, 38), we

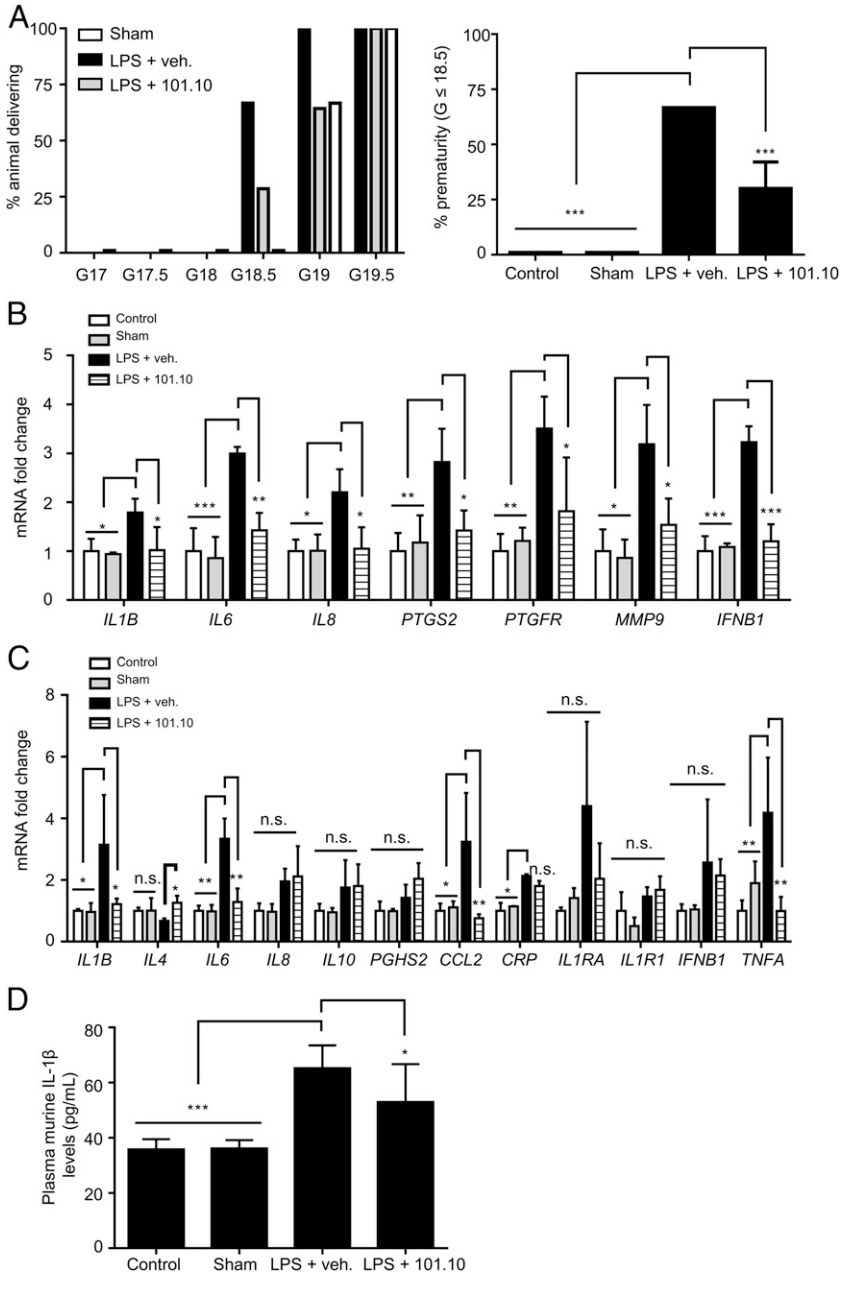

FIGURE 3. The 101.10 decreases TLR4-induced preterm birth. (**A**) The 101.10 decreases preterm birth induced by the TLR4 agonist LPS in mice. *Left panel*, Percentage of animals having delivered following a single i.p. LPS injection (0.5 μg per mice); *right panel*, percentage of premature deliveries. Control mice did not receive any treatment, whereas sham animals received a single i.p. dose of vehicle at G16.5. The 101.10 (1 mg/Kg/12 h) or vehicle was administered s.c. twice per day until delivery. (**B**) Quantitative PCR from myometrium tissue of mice treated in (A) and collected postpartum (<30 min). Results are normalized with 18S and are relative to control. (**C**) Quantitative PCR from circulating leukocytes isolated from systemic blood of mice treated in (A) and collected postpartum (<30 min). Results are normalized with 18S and are relative to control. (**D**) Murine IL-1β ELISA performed on plasma from mice treated in (A) and collected postpartum (<30 min). Values are presented as mean ± SD. Data are representative of 3–14 animals per group and of 4 in vitro experiments. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test compared with LPS + vehicle group.

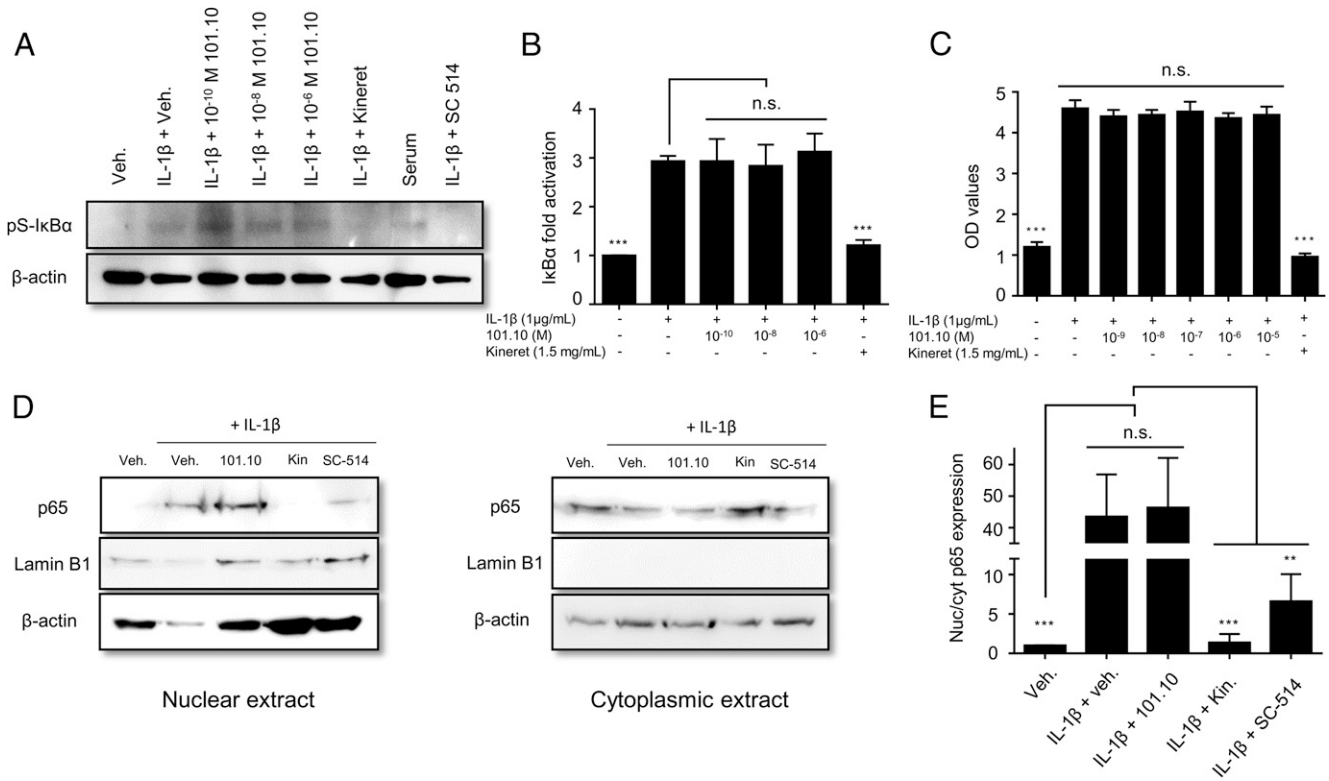

FIGURE 4. The 101.10 has no significant effect on IL-1β–induced NF-κB activation. (**A**) Myometrial SMC (hTERT-C3 cell line) were treated with IL-1β (1 μg/ml) in presence or absence of increasing doses of 101.10 for 1 h, and lysates were run on SDS-PAGE and blotted against pS-IκBα or β-actin. Kineret (1.5 mg/ml) and SC-514 (10 μM) were used as negative controls, and FBS (10%) was used as a metabolic positive control. (**B**) Densitometric analysis of protein bands showing no significant effect of 101.10 on IL-1β–induced NF-κB activity. (**C**) HEK-Blue cells were treated with IL-1β in presence or absence of increasing doses of 101.10 for 4 h, and levels of secreted alkaline phosphatase in cell culture supernatant were assessed by reading the absorbance (OD values) at 620–655 nm. Kineret was used as a negative control. (**D**) hTERT-C3 cells were treated with IL-1β (1 μg/ml) with or without 101.10 (10^{-6} M), and Western blot was performed on extracted nuclei or cytoplasmic lysate and blotted against NF-κB p65 or the nuclear maker lamin B1. Presence of NF-κB p65 in the nucleus was used as a measurement of NF-κB activation. (**E**) p65/β-actin quantification values from nuclear extracts were normalized with those for cytoplasmic extract. Kineret (1.5 mg/ml) and SC-514 (10 μM) were used as negative controls, and β-actin was used as a loading control. Values are presented as mean ± SD. Data are representative of 3–5 experiments. $^{**}p < 0.005$, $^{***}p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test compared with IL-1β + vehicle group.

examined SAPK/c-jun and small GTPase Rho/ROCK pathways, which both lead to the activation of the transcriptional factor AP-1 (Fig. 5A), respectively, using myometrial and macrophage cell lines described above. The 101.10 dose dependently decreased IL-1β–induced phosphorylation of p38, JNK, and the transcription factor c-jun in both cell types (Fig. 5B–E); Kineret was also effective. The 101.10 (like Kineret) also decreased IL-1β–triggered induction of several proinflammatory and/or prolabor genes in vitro (Fig. 5F, 5G), as previously observed in vivo (Figs. 1–3); IL-1β–triggered induction of *PGHS2* was dose dependently inhibited by 101.10 (IC_{50} = 15.1 nM; see Fig. 5H). Additionally, in myometrial cells where RhoA is important in cell function, 101.10 inhibited RhoA activation and decreased downstream ROCK2 phosphorylation (Fig. 5I, 5J).

Based on data obtained in cell lines (Fig. 5), we proceeded to study the effects of 101.10 on IL-1 signaling in primary myometrial SMC. Primary myometrial SMC were obtained by digesting CD-1 mice uterine horns and cultured; immunohistochemical staining with α-actin assessed purity at >95% of cells (Supplemental Fig. 4I, 4J). The 101.10 dose dependently inhibited the activation of p38, JNK, and c-jun (Fig. 6A, 6B) and decreased the induction of several proinflammatory and/or prolabor genes in primary myometrial SMC (Fig. 6C); effects of Kineret were comparable. Moreover, 101.10 inhibited IL-1β–induced p38, JNK, and c-jun activation in myometrial tissue freshly isolated from pregnant mice (Fig. 6D, 6E).

We performed similar experiments on primary bone marrow–derived macrophages; >95% of the cells positively stained for the macrophage marker F4/80 (Supplemental Fig. 4L, 4M). Once again, 101.10 inhibited the activation of p38, JNK, and c-jun in primary bone marrow–derived macrophages (Fig. 6F, 6G) and decreased the induction of several proinflammatory genes triggered by IL-1β (Fig. 6H). Finally, 101.10 (and Kineret) was selective to these signaling pathways induced by IL-1, but not by LTA and LPS, whereupon 101.10 (and Kineret) was ineffective (Supplemental Fig. 1E, 1F).

Inhibiting AP-1 delays inflammation-induced preterm birth

Because our in vitro and ex vivo studies suggest that 101.10 acts by inhibiting IL-1R SAPK/c-jun and Rho/ROCK pathways leading to AP-1 assembly without modulating NF-κB activity, we wanted to validate this mechanism of action in vivo. Therefore, we subjected pregnant mice to intrauterine IL-1β–induced PTB model with a group of mice receiving a selective AP-1 inhibitor, SR-11302 (n = 9) and another group receiving SR-11302 in combination with the ROCK inhibitor Y27632 (n = 7), to mimic the proposed signaling mechanism of action of 101.10. SR-11302 alone or in combination with Y27632 was comparably effective to 101.10 in reducing preterm delivery (Fig. 7A, 7B) and increasing gestational length (Supplemental Fig. 1D).

Discussion

Inflammation plays a critical role in labor (39). Various major proinflammatory cytokines, including IL-1, upregulate UAP in gestational tissues and are associated with the onset of labor in animal models and in humans. However, available IL-1–targeting agents all cause a nonselective inhibition of the entire IL-1R–coupled

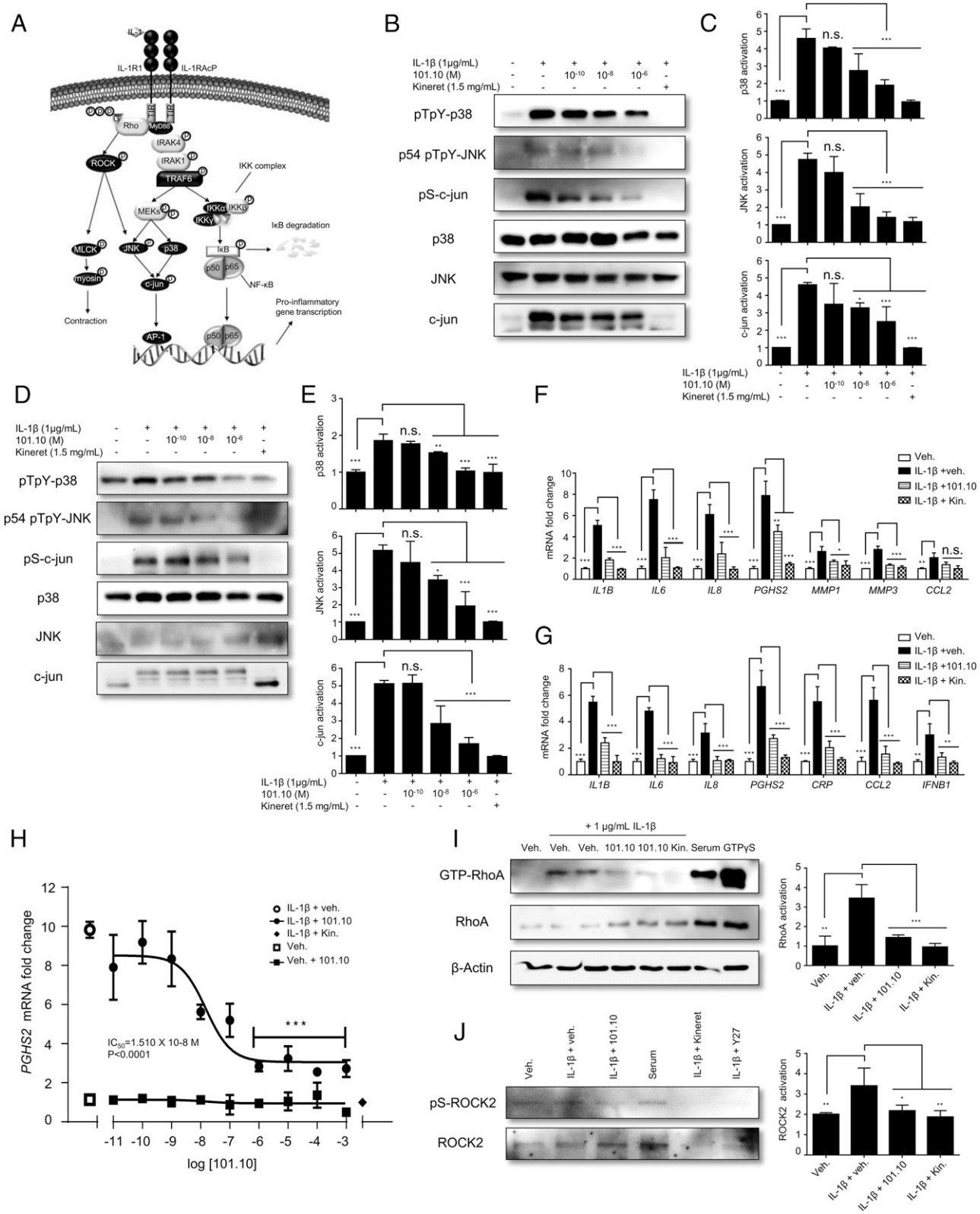

FIGURE 5. The 101.10 inhibits SAPK/c-jun and RhoA/ROCK signaling pathways in both myometrial and macrophage cell lines. (**A**) Simplified IL-1R intracellular signaling pathways. The activation of p38, JNK, or Rho/ROCK leads to the phosphorylation and translocation of the transcriptional factor c-jun to the nucleus and further assembling of the heterodimeric transcriptional factor AP-1. (**B** and **C**) Myometrial SMC (hTERT-C3 cell line) were treated with IL-1β or vehicle with or without increasing concentrations of 101.10, and Western blot was performed on lysates and blotted against indicated Abs (B). Densitometric analysis was used to quantify protein bands, and results were normalized with total proteins and plotted as fold over control (C). Kineret was used as a negative control. (**D** and **E**) RAW-Blue macrophages were treated with IL-1β (1 μg/ml) or vehicle with or without increasing concentrations of 101.10, and Western blot was performed on lysates and blotted against indicated Abs (D). Densitometric analysis was used to quantify protein bands, and results were normalized with total proteins and plotted as fold over control (E). Kineret (1.5 mg/ml) was used as a negative control. (**F**) Quantitative PCR of hTERT-C3 cells treated with IL-1β (1 μg/ml) or vehicle with or without 101.10 (10^{-6} M) or Kineret (1.5 mg/ml) for 6 h. Results are normalized with 18S and are relative (*Figure legend continues*)

signaling pathways, including NF-κB, which has an important role in cytoprotection and immune surveillance (40–42). In this work, we describe the efficacy of a noncompetitive stable (all-d peptide) modulator of IL-1R at delaying murine PTB models induced by IL-1β, LTA (TLR2 ligand), and LPS (TLR4 ligand). The 101.10 exhibited biased ligand properties by inhibiting IL-1–triggered SAPK/c-jun and Rho/ROCK pathways, without affecting NF-κB activity.

Inflammation is now considered a converging pathway toward labor (43, 44). It is believed that the initial inflammatory stimulus, such as pathogen-associated molecular patterns or danger-associated molecular patterns, activates innate immunity by binding on TLRs. This signal promotes cytokine production from cells of the innate immune response, which in turn activates adaptive immunity. Resulting inflammatory cascade leads to the induction of UAP and promotes the onset of labor. Accordingly, data from our laboratory and others show that acute inflammatory events increase UAP expression in the myometrium and other uterine tissue (12, 15, 45). This notion is supported by data obtained in the current study; IL-1β and TLR ligands induced various UAP, including *OXTR*, *PTGFR*, *PGHS2*, *CCL2*, and *GJA1* in myometrium. Products of these proinflammatory genes amplify the initial insult. Hence, targeting proinflammatory cytokines and their receptors accountable for expansion of the initial inflammatory trigger is a justifiable approach to prevent/arrest premature induction of UAP and ensued onset of PTB.

The present study focused on the role of IL-1β, a major mediator of inflammation, which can sustain the inflammatory cascade that results in preterm labor (46–49). Effects of IL-1β were antagonized by 101.10 (in a NF-κB–independent manner). Another IL-1R agonist ligand is IL-1α, which remains mostly intracellular and is released in the extracellular milieu upon cell lysis; IL-1α has been linked to sterile intra-amniotic inflammation (50). Of note, as observed for IL-1β, 101.10 is also capable of inhibiting actions of IL-1α (Supplemental Fig. 2E).

In this study, to our knowledge, we report for the first time the efficacy of a small noncompetitive inhibitor of IL-1R termed 101.10, in PTB. The peptide showed better efficacy than the competitive IL-1R antagonist Kineret in delaying IL-1β–induced PTB (Fig. 1C). Accordingly, intrauterine IL-1β triggered an inflammatory response locally, in the placenta/myometrial unit and systemically (increase in leukocyte cytokines), implicating mediators other than IL-1 partaking in amplified myometrial induction of various inflammatory factors; the dose of IL-1β used is consistent with that reported (8, 16), and, although higher than that used to stimulate human tissue, the exact concentration in human is not known, but most likely several fold higher in the immediate vicinity of cytokine-releasing cells. Contrary to 101.10, Kineret did not interfere with myometrial gene induction. Because Kineret did reduce blood leukocyte induction of inflammatory genes, the selective IL-1R antagonist Kineret is pharmacologically effective, but as a molecule as large as IL-1 per se (≈17.5 kDa), which does not cross the placental barrier (34, 35) (and with whom it competes for the ligand binding site on IL-1RI), Kineret has limited bioavailability to the placenta—the trigger locus of inflammation that in turn affects myometrium and systemic inflammation. In counterpart, the small molecule 101.10 (≈0.85 kDa) does distribute to placenta and myometrium, as seen with 101.10-FITC, and is able to diminish amplified inflammation in those tissues and in turn delay birth induced by IL-1β. The findings also infer that the local utero/placental inflammation surpasses in importance systemic inflammation in stimulating PTB.

NF-κB is a prominent downstream signal of inflammatory mediators. NF-κB has been implicated in the normal process of labor (22, 44), but its inhibition may be deleterious. Hence, reluctance to develop a NF-κB–targeted therapy to prevent PTB includes the following: 1) NF-κB plays an important role in cytoprotection, and its inhibition can increase rates of apoptosis (51); accordingly, the antibiotic sulfasalazine, which also inhibits NF-κB, has been associated with an increase in proapoptotic cells in human chorionic membranes (21) and an increased risk of adverse pregnancy outcomes (52). 2) Hypoxia-induced NF-κB activation might be implicated in preventing sequelae from myometrial contraction-induced ischemia (22). 3) NF-κB inhibition can hamper immune surveillance and potentially increase the risk of infection including during pregnancy (51, 53). In this regard, 101.10 offers a unique alternative to currently available IL-1 inhibitors by avoiding NF-κB inhibition while still interfering with other IL-1R–coupled pathways involved in the assembly of the transcription factor AP-1.

The notion that AP-1 partakes in labor is relatively new. Recent data demonstrate that labor is associated with changes in the AP-1 family members in the uterus and fetal membranes (54–56). Moreover, a causal role of JNK/AP-1 was recently described wherein AP-1 activation alone was sufficient to induce labor and inhibition of JNK was sufficient to delay LPS-induced PTB (37). Correspondingly, SAPK and their target c-jun/c-fos (AP-1) have been shown to be activated in human uterine cervix at term and after delivery, suggesting a concomitant function for AP-1 in cervical ripening (57). Our study markedly bolsters the evidence toward a crucial role of AP-1 in labor: we showed that 101.10 prevented PTB without significantly affecting NF-κB, but rather by inhibiting pathways upstream of AP-1, including c-jun. We further confirmed that inhibiting AP-1 alone was sufficient to delay IL-1β–induced PTB in mice. Notwithstanding that NF-κB controls expression of numerous genes implicated in inflammation, many proinflammatory and/or prolabor genes have both AP-1 and NF-κB binding sites, including *PGHS2* (58), *IL6* (58), *IL8* (59), and *CCL2* (60); in addition, the regulatory region of human *OXTR* displays binding sites for AP-1 (61), and AP-1 is a key regulator of MMP (62) and *CX43* (63–65). Hence, inhibition of either AP-1 or NF-κB appears to be sufficient to interfere with expression of these genes implicated in labor; this claim is supported by the comparable efficacy of 101.10 and AP-1 inhibitor SR-11302.

This study has some limitations, particularly as it relates to translation of all findings in rodents to humans. We focused on IL-1

to control. (**G**) Quantitative PCR of RAW-Blue macrophages treated with IL-1β (1 μg/ml) or vehicle with or without 101.10 (10^{-6} M) or Kineret (1.5 mg/ml) for 6 h. (**H**) Quantitative PCR of *PGHS2* induction in hTERT-C3 cells treated with IL-1β (●; 1 μg/ml) or vehicle (■) with increasing concentrations of 101.10 or with Kineret (▲; 1.5 mg/ml) for 2 h. Results are normalized with 18S and are relative to control. ***$p < 0.001$ relative to higher plateau. (**I**) hTERT-C3 cells were treated with IL-1β or vehicle with or without 101.10 (10^{-6} M), and lysates were incubated with affinity beads specific to GTP-bound RhoA. Beads and total proteins were then loaded on SDS-PAGE and blotted against RhoA or β-actin. Kineret (1.5 mg/ml) was used as a negative control, and FBS (10%) and GPTγS (200 μM) were used as positive controls. Quantification of protein bands was normalized with total RhoA and plotted as fold over control. (**J**) Western blot of hTERT-C3 cells treated with IL-1β (1 μg/ml) or vehicle with or without 101.10 (10^{-6} M) and blotted against pS-ROCK2 or ROCK2. Kineret (1.5 mg/ml) and Y27632 (10^{-6} M) were used as a negative control and FBS (10%) as a positive control. Quantification of protein bands was normalized with ROCK2 and plotted as fold over control. Values are presented as mean ± SD. Data are representative of three to four experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test compared with IL-1β + vehicle group.

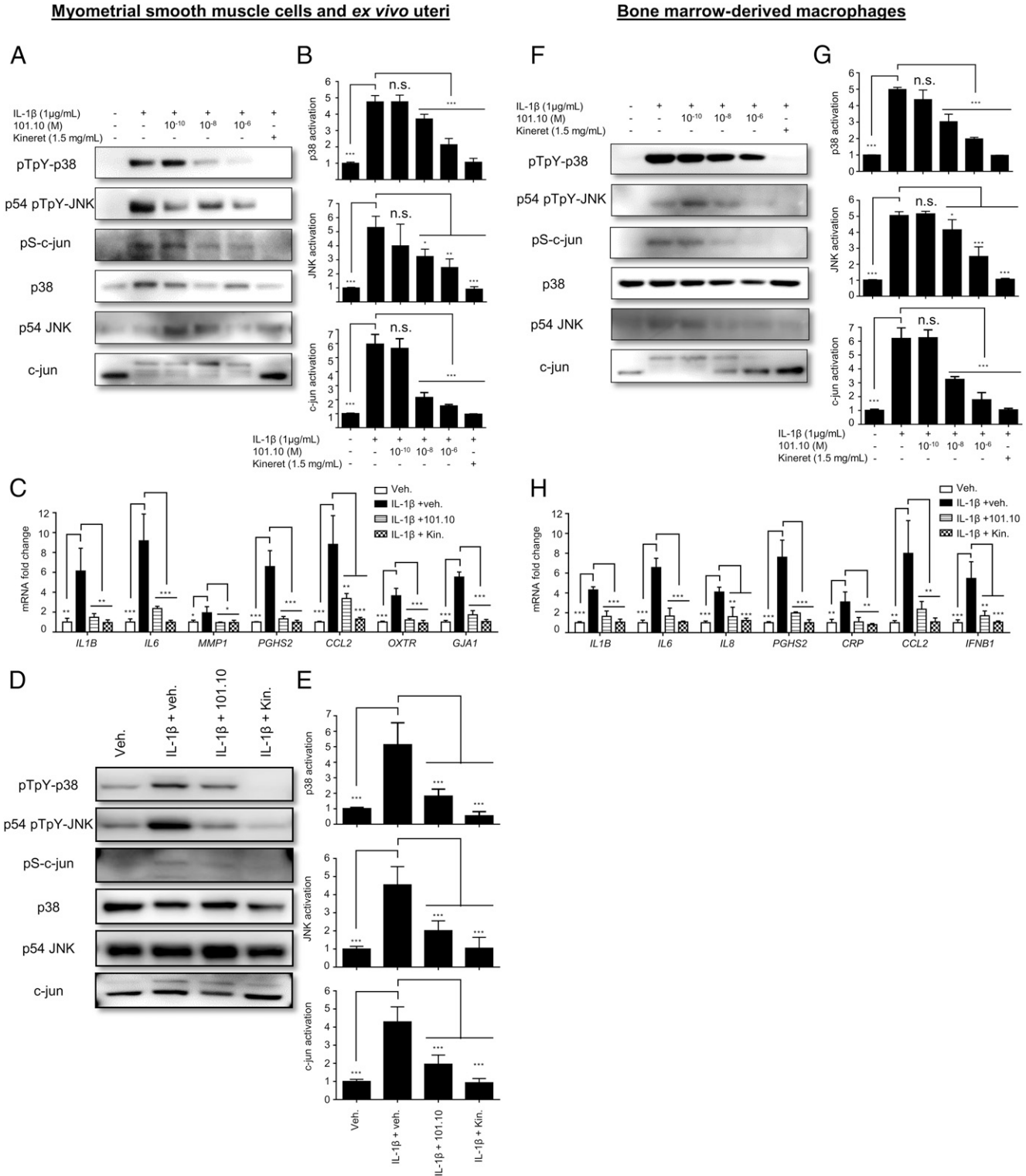

FIGURE 6. The 101.10 inhibits SAPK/c-jun signaling pathway in CD-1 mice primary myometrial SMC, in ex vivo myometrium fragments, and in bone marrow–derived macrophages. (**A** and **B**) Primary myometrial SMC were treated with IL-1β or vehicle with or without increasing concentrations of 101.10, and Western blot was performed on lysates and blotted against indicated Abs (A). Densitometric analysis was used to quantify protein bands, and results were normalized with total proteins and plotted as fold over control (B). Kineret was used as a negative control. (**C**) Quantitative PCR of primary myometrial SMC treated with IL-1β (1 μg/ml) or vehicle with or without 101.10 (10^{-6} M) or Kineret (1.5 mg/ml) for 6 h. Results are normalized with 18S and are relative to control. (**D** and **E**) Myometrium fragments were collected from CD-1 mice and incubated in serum-free medium for 1 h prior to stimulation with IL-1β (1 μg/ml) or vehicle with or without increasing concentrations of 101.10. Western blot was performed on lysates and blotted against indicated Abs (D). Densitometric analysis was used to quantify protein bands, and results were normalized with total proteins and plotted as fold over control (E). Kineret (1.5 mg/ml) was used as a negative control. (**F** and **G**) Primary bone marrow–derived macrophages were treated with IL-1β or vehicle with or without increasing concentrations of 101.10, and Western blot was performed on lysates and blotted against indicated Abs (F). Densitometric analysis was used to quantify protein bands, and results were normalized with total proteins and plotted as fold over control (G). Kineret was used as a negative control. (**H**) Quantitative PCR of primary bone marrow–derived macrophages treated with IL-1β (1 μg/ml) or vehicle with or without 101.10 (10^{-6} M) or Kineret (1.5 mg/ml) for 6 h. Results are (*Figure legend continues*)

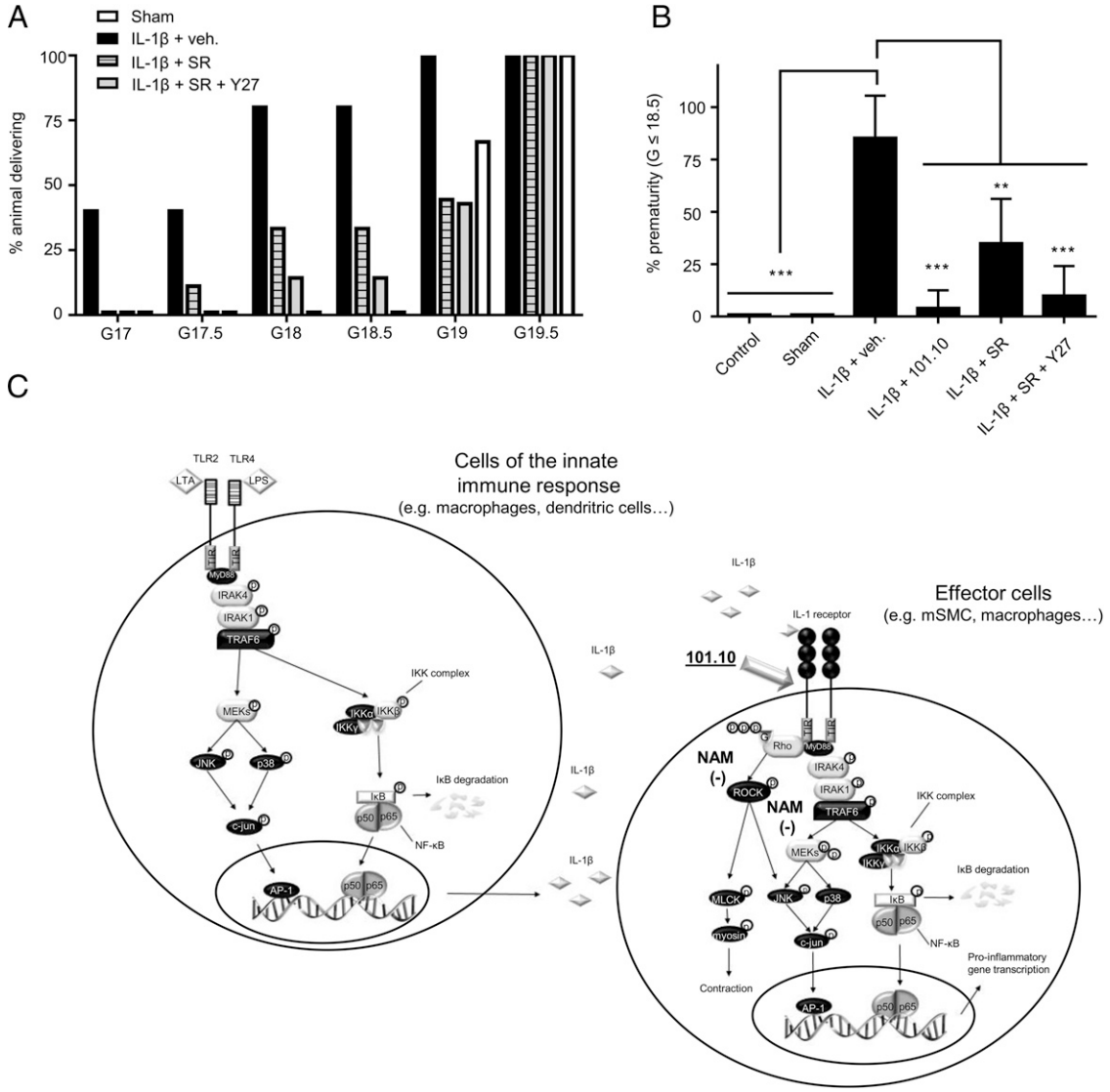

FIGURE 7. Inhibiting AP-1 protects against inflammation-induced preterm birth. (**A**) Percentage of animals having delivered following 1 μg intrauterine IL-1β injection and (**B**) percentage of prematurity. Control mice did not receive any treatment, whereas sham animals received an intrauterine dose of vehicle at G16.5. The 101.10 (1 mg/Kg/12 h), SR11302 (1 mg/Kg/12 h), Y27632 (1 mg/Kg/12 h), or vehicle was administered s.c. twice per day until delivery. (**C**) Proposed mechanism of action of 101.10. Effector cells comprise a wide range of possible cells, although the focus has been made on myometrial SMC and macrophages in this study. Values are presented as mean ± SD. Data are representative of 3–10 animals per group. $^{**}p < 0.005$, $^{***}p < 0.001$. NAM, negative allosteric modulator by one-way ANOVA with Tukey's multiple comparison test compared with IL-1β + vehicle group.

and upstream TLR4 and TLR2 (66) pathways proposed by an abundance of literature to be implicated in triggering human PTB (67–70); also, efficacy of 101.10 was shown in a relevant human cell line. Concordantly, in the current study, several mediators of inflammation were induced in our rodent models (e.g., IL-6, IL-8, and cyclooxygenase-2); yet, specific inhibition of IL-1R by 101.10 reduced PTB induced by every stimulus (IL-1, LPS, LTA) tested, highlighting its critical role. Extrapolation of these findings to humans does not exclude a role for other pathways. This inference has been proposed for IL-6 (71), IL-8 (72), FOX01 (73), and other mediators implicated in labor of humans. Although biologic effects of (heat-inactivated) Gram$^+$ bacteria are not fully reproduced by LTA (74), the latter do elicit many features of the bacteria (75); a similar argument can be made for (heat-inactivated) Gram$^-$ bacteria and LPS (76), including as it applies to placental/fetal biology (66). Ideally, anti-inflammatory drugs should be administered at an earlier time point than currently applied; accordingly, appropriate diagnostic markers are also needed for effective prevention of PTB in humans. Overall, our findings on the role of IL-1 concur with those previously reported by authors of this paper (17, 77).

Small biased ligands offer therapeutic advantages, which cannot be mimicked by currently available orthosteric inhibitors. Small peptide or peptidomimetics are likely to exhibit better bioavailability and a therapeutic index due to selective and partial modulation of specific (and not all) receptor-coupled signaling pathways. Advantages of 101.10 over available IL-1–targeting therapies in PTB comprise the following: 1) 101.10 avoids the inhibition of IL-1–induced NF-κB activation and therefore offers a novel way to prevent premature uterine activation, by acting as a negative allosteric biased ligand, in line with its reported actions on other cells (24);

normalized with 18S and are relative to control. Values are presented as mean ± SD. Data are representative of three to four experiments. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ by one-way ANOVA with Tukey's multiple comparison test compared with IL-1β + vehicle group.

2) due to enhanced pharmacological selectivity, 101.10 could be deprived of major adverse effects; 3) 101.10 is more likely to have increased bioavailability and less invasive route of administration [101.10 has been reported to exhibit enteral bioavailability (24)]; and 4) cost of goods for 101.10 is most likely less compared with recombinant proteins and Abs; the latter provides a more suitable therapeutic option for developing countries, where prematurity is a main cause of mortality (78).

In summary, to our knowledge, we hereby describe the first noncompetitive biased modulator of a cytokine receptor showing efficacy in delaying the onset of preterm birth. The 101.10 acts desirably without inhibiting IL-1β–induced NF-κB activation, albeit by dose dependently inhibiting relevant IL-1β–induced phosphorylation of SAPK p38 and JNK, transcription factor c-jun, as well as Rho/ROCK pathway. Hence, 101.10 acts independently of NF-κB in delaying IL-1β–, TLR2- and TLR4-induced PTB in mice, thus undermining the role of NF-κB activation in labor.

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Disclosures

S.C., C.Q., and W.L. hold a patent on composition of matter for the use of 101.10 (Interleukin-1 receptor antagonists, compositions, and methods of treatment, United States patent no. USPTO8618054, 2005, May 05). The other authors have no financial conflicts of interest.

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Uterotonic Neuromedin U Receptor 2 and Its Ligands Are Upregulated by Inflammation in Mice and Humans, and Elicit Preterm Birth[1]

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ABSTRACT

Uterine labor requires the conversion of a quiescent (propregnancy) uterus into an activated (prolabor) uterus, with increased sensitivity to endogenous uterotonic molecules. This activation is induced by stressors, particularly inflammation in term and preterm labor. Neuromedin U (NmU) is a neuropeptide known for its uterocontractile effects in rodents. The objective of the study was to assess the expression and function of neuromedin U receptor 2 (NmU-R2) and its ligands NmU and the more potent neuromedin S (NmS) in gestational tissues, and the possible implication of inflammatory stressors in triggering this system. Our data show that NmU and NmS are uterotonic ex vivo in murine tissue, and they dose-dependently trigger labor by acting specifically via NmU-R2. Expression of NmU-R2, NmU, and NmS is detected in murine and human gestational tissues by immunoblot, and the expression of NmS in placenta and of NmU-R2 in uterus increases considerably with gestation age and labor, which is associated with amplified NmU-induced uterocontractile response in mice. NmU- and NmS-induced contraction is associated with increased NmU-R2-coupled Ca^{++} transients, and Akt and Erk activation in murine primary myometrial smooth muscle cells (mSMCs), which are potentiated with gestational age. NmU-R2 is upregulated in vitro in mSMCs and in vivo in uterus in response to proinflammatory interleukin 1beta (IL1beta), which is associated with increased NmU-induced uterocontractile response and Ca^{++} transients in murine and human mSMCs; additionally, placental NmS is markedly upregulated in vivo in response to IL1beta. In human placenta at term, immunohistological analysis revealed NmS expression primarily in cytotrophoblasts; furthermore, stimulation with lipopolysaccharide (LPS; Gram-negative endotoxin) markedly upregulates NmS expression in primary human cytotrophoblasts isolated from term placentas. Correspondingly, decidua of women with clinical signs of infection who delivered preterm display significantly higher expression of NmS compared with those without infection. Importantly, in vivo knockdown of NmU-R2 prevents LPS-triggered preterm birth in mice and the associated neonatal mortality. Altogether, our data suggest a critical role for NmU-R2 and its ligands NmU and NmS in preterm labor triggered by infection. We hereby identify NmU-R2 as a relevant target for preterm birth.

calcium, contraction, infection, inflammation, neuromedin S, neuromedin U, NmU-R2, preterm birth, preterm labor, uterine labor

INTRODUCTION

Uterine labor is characterized by vigorous myometrial contractions required to expulse the fetus from the uterus. A number of genes and their translated proteins, referred to as uterine activation proteins (UAPs), stimulate and coordinate myometrial contractions during labor. The expression of UAPs is induced by uterotrophins (uterine activators) [1]. As pregnancy nears the end, uterotrophins (such as estrogen, CRH, and proinflammatory cytokines [e.g., interleukin 1β {IL1β}, IL6, and tumor necrosis factor α {TNFα}]) induce the expression of many UAPs in the uterus, including oxytocin receptor (OXR), prostaglandin $F_{2\alpha}$ receptor (FP), connexin-43, prostaglandin-endoperoxide synthase 2 (COX-2) [1–5], and many others [6]. The uterus then becomes increasingly sensitive to uterotonins, which are proteins responsible for inducing uterine contractions, including oxytocin (OT) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Preterm labor results from the unscheduled induction of UAPs by stressors, including inflammation with or without infection. Hence, UAPs are interesting targets to arrest preterm labor.

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Neuromedin U (NmU; U for uterus) was named for its ability to induce contractions on ex vivo uterine strips; yet, there is a growing list of functions associated with NmU that includes regulation of appetite, diminution of insulin secretion, release of different hormones, and smooth muscle contraction (of various organs: blood vessels, gut, and uterus) [7, 8]. NmU exerts its actions by binding to two G protein-coupled receptors: neuromedin U receptor 1 (NmU-R1; FM-3 or GPR66) and NmU-R2 (FM-4) [8–12]. Both NmU receptors are coupled to G_i and $G_{q/11}$-phospholipase C_β [13]. The activation of the latter leads to the intracellular release of calcium and induces contractions of smooth muscle cells. Another ligand of NmU receptors, neuromedin S (NmS), is expressed in the hypothalamus, spleen, and testis, and has been described to be more specific and exhibit higher affinity to NmU-R2 [14]. NmU has been extensively shown to exert pleiotropic effects in the brain. Notwithstanding its known procontractile effects on rodent uterus [15], which have been suggested to be mediated by NmU-R2 [16], little is known regarding its mechanism of action in uterus and potential role in labor, but it may be relevant in the context that neuromedin B, a neuropeptide of the same family, was recently shown to induce labor in mice via its receptor NmBR [17].

We therefore studied the effects and mode of action of NmU and NmS, and their cognate receptor NmU-R2, in uterus, and their implication in term and preterm labor. Our findings reveal expression of NmU and NmS in human and murine placenta, and of their receptor NmU-R2 in human and murine myometrium, with a significant increase in the expression of NmU-R2 and NmS near the end of gestation and during labor. Studies in mice suggest that NmU-R2, once activated by its ligand NmU or NmS, shares all major characteristics that are common to UAPs, by 1) exhibiting increased expression with onset of labor, 2) exerting gestational age-dependent uterocontractile effects, 3) contributing endogenously to inflammation-triggered preterm birth (PTB), and 4) being induced by uterotrophins [1, 4, 5, 18–20]; the latter was again corroborated on decidual biopsies of women with clinical evidence of infection. More importantly, NmU and NmS administration in pregnant mice was found to shorten gestation, and *Nmur2* knocked-down mice had significantly lower PTB induced by lipopolysaccharide (LPS). Hence, NmU-R2 is a potential new UAP that appears important for PTB associated with infection.

MATERIALS AND METHODS

Ethics Approval

Approval was obtained from North West Research Ethics Committee in Manchester, United Kingdom (ref.: 08/H1010/55), for decidual samples, provided by Dr. Rebecca L. Jones, and the Sainte-Justine Hospital Ethics Board (ref.: 4058 and 3988) for placental samples and placenta from uncomplicated (normal) term pregnancies for cytotrophoblast isolation (see below). Myometrium tissue biopsies were collected in part from women undergoing cesarean delivery at the Royal Alexandra Hospital in Edmonton, Alberta, with ethics approval received from the University of Alberta Research Ethics board, and in part collected from women undergoing cesarean delivery at the MacDonald Women's Hospital, University Hospitals, Cleveland, with Institutional Review Board approval (no. 11-04-06), provided by Dr. Sam Mesiano. All participants provided written informed consent.

Animals

Timed-pregnant CD-1 mice were obtained from Charles River Inc. at different gestational ages and were allowed to acclimatize for 4 days prior to experiments. Animal studies were approved by the Animal Care Committee of Hôpital Sainte-Justine along the principles of the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care. The animals were maintained on standard laboratory chow under a 12L:12D cycle and were allowed free access to chow and water.

Chemicals

Chemicals were purchased from the following manufacturers: rhIL1β (no. 200-01B; PeproTech), Neuromedin U-23 (no. NMU72-P; Alpha Diagnostic International), Neuromedin S (no. 045-88; Phoenix Pharmaceuticals), Neuromedin U-25 (no. 17617; Cayman Chemical), Prostaglandin $F_{2\alpha}$ (no. 16010; Cayman Chemical), Oxytocin (no. 66-0-52; American Peptide), W-7 (no. A3281; Sigma), U73122 (no. U6756; Sigma), and LPS from *Escherichia coli* 0111:B4 (L2630; Sigma).

Protein Extraction from Human Myometrial Biopsies

Myometrial biopsies were flash frozen in liquid nitrogen and stored at −80°C. Frozen myometrial tissues wrapped in aluminum foil were later crushed using a mortar and pestle in liquid nitrogen, and 0.1–0.2 g of myometrial tissue was placed in a round-bottomed tube with a 7-mm bead and 0.5 ml of lysis buffer containing 0.05% Tris, 0.01% ethylene diamine tetraacetic acid, 0.001% Triton X-100, 0.005% PMSF, and 0.1% protease inhibitor. Tissues were then lysed by shaking the tubes at a high speed (frequency 25/sec) using the TissueLyser II (Qiagen). Tissue lysates were centrifuged at 12 000 × *g* for 10 min at 4°C, and the supernatants were collected for Western blot analysis.

Primary Murine Myometrial Smooth Muscle Cell Isolation and Culture

Primary murine myometrial smooth muscle cells (mSMCs) were isolated as previously described [21] and used at fewer than three passages. Briefly, pregnant mice (at Gestational Day 10 [G10] or 19) were killed by cervical dislocation and sprayed with 70% ethanol. The whole uterus was excised under sterile hood and placed in buffer A (Hanks balanced salt solution [HBSS], pH 7.4; 0.098 g/L magnesium sulfate; 0.185 g/L calcium chloride; 2.25 mmol/L I-HEPES [*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid]; 100 U/ml penicillin-streptomycin [Gibco]; and 2.5 μg/ml amphotericin B [Sigma]). Placentas, fetal membranes, and products of conception were discarded and the uterine horns were cleansed of fat and vessels and then transferred into buffer B (buffer A without magnesium sulfate or calcium chloride) for several washes by gentle flushing. Afterward, the uterine horns were cut into 1-mm-wide fragments and transferred into a volume of 10 ml/g of tissue of digestion buffer (1 mg/ml collagenase type II [Sigma], 0.15 mg/ml deoxyribonuclease I [Roche Diagnostics GmbH], 0.1 mg/ml soybean trypsin inhibitor [Sigma], 10% fetal bovine serum [FBS], and 1 mg/ml bovine serum albumin [Sigma] in buffer B). Enzymatic digestion was performed at 37°C with agitation (100 revolutions per minute) for 30 min. The homogenate (still containing undigested myometrium fragments) was then poured through a 100-μm cell strainer. The resulting filtered solution was centrifuged at 200 × *g* for 10 min, and the pellet was resuspended in complete Dulbecco modified Eagle medium (DMEM) and plated in a T-75 dish. The remaining myometrium fragments were reused in an enzymatic digestion, and the whole digestion-centrifugation process was repeated for a total of five times. First, two digestion results were discarded because they mostly contained fibroblasts. The other three SMC-containing dishes were subjected to a differential adhesion technique to selectively enrich for uterine myocytes. Briefly, 30–45 min after the cells were first plated, the medium was removed and dispensed in another T-75 culture dish to separate quickly adhering fibroblasts from slowly adhering myocytes. Purity of the cells was assessed by immunohistochemistry using the smooth muscle cell marker α-actin and was always maintained above 95%.

Cell Culture

Primary murine mSMCs or human mSMCs (hTERT cell line) were cultured in DMEM growth medium supplemented with 10% serum, 50 U/ml penicillin, and 50 mg/ml streptomycin. Cells were propagated in regular conditions (37°C, 5% CO_2). For immunoblotting and PCR experiments, cells were serum starved overnight and treated with various concentrations of NmU or 5 ng/ml IL1β for 10 min. Cells were collected in ice-cold RIPA buffer containing a cocktail of protease/phosphatase inhibitors and cleared from debris by centrifugation. Samples were stored in Laemmli buffer at −20°C or used fresh for Western blotting.

Induction of Birth

Timed-pregnant mice were injected intraperitoneally with NmU, NmS, $PGF_{2\alpha}$, or oxytocin twice a day for 2 consecutive days. Injections were made at G13–G14, G15–G16, or G17–G18 twice a day for a total of four injections. Doses used for NmU and NmS correspond to those previously used to induce

TABLE 1. Sequences used for the design of shRNA against *Nmur2*.

Clone ID	Sequences
TRCN0000026236	TAAGTTGGGTGTTGTGGATGG
TRCN0000026273	TATACACCCATATGGGTTTGG
TRCN0000026279	AACAGGAAGGGATAATTGTGC
TRCN0000026293	TTTAGCCCTGATTTAGAGAGC
TRCN0000026323	ATTCACAGTCACTTTGTCTGC

labor in mice with neuromedin B [17]. Mice were checked every 2 h for any signs of labor/delivery, such as vaginal bleeding or delivery of at least one pup.

Intrauterine IL1β and LPS Injection

Timed-pregnant mice at G9 were steadily anesthetized with isoflurane. Body hair was removed and peritoneal skin was sterilized with 70% ethanol and then covered with povidone-iodine 7.5% (Atlas Laboratory). A 1.5-cm-tall median incision was made in the abdominal wall of the lower abdomen. The lower segment of the right uterine horn was then exposed and 1 μg of IL1β, LPS, or an equivalent volume of saline (for sham animals) was injected between two gestational sacs without entering the amniotic cavity. The abdominal muscle layer was sutured and the skin closed with clips. Twenty-four hours after the intrauterine injection (at G10), mice were killed with CO_2, and placenta and lower (cervical end) uterus samples were collected and preserved at −80°C for subsequent RNA purification and Western blotting. For contraction experiments ex vivo, fresh uterine fragments were used immediately after killing.

Lentivirus Production

We produced infectious lentivirus (LV) by transiently transfecting lentivector and packaging vectors into 293FT cells (Invitrogen) as previously described [22]. We used five different small-hairpin RNA (shRNA) sequences against *Nmur2* (see Table 1 for sequences) and selected the most effective sequence for further experiments (see Supplemental Fig. S1, A and B, for efficacy and variability of NmU-R2 knockdown using LV.shNmU-R2; Supplemental Data are available online at www.biolreprod.org). In vivo infections were performed on G13 or G15 mice via a single intrauterine injection under the same protocol as described above. Lentivirus was allowed to infect cells/tissues for at least 72 h. Lentiviral syringes were coded; hence, the person injecting was blinded to treatment attribution.

LPS-Induced Preterm Birth Model

Timed-pregnant mice pretreated for 72 h with an intrauterine injection of lentivirus or saline received 10 μg of intraperitoneal LPS or an equivalent volume of saline at G16. Signs of delivery were assessed every 2 h (as described above). Survival of pups was assessed at the moment of birth (±2 h after completion of delivery).

RNA Extraction and Real-Time Quantitative PCR

Myometrium fragments were thawed and rapidly preserved in Ribozol (AMRESCO), whereas cells from in vitro experiments were treated for 6 h with 5 ng/ml IL1β and collected directly into Ribozol. RNA was extracted according to the manufacturer's protocol and RNA concentration and integrity were measured with a NanoDrop 1000 spectrophotometer. A total of 500 ng of RNA was used to synthetize cDNA using iScript Reverse Transcription SuperMix (Bio-Rad). Primers were designed using NCBI Primer Blast (Table 2). Quantitative gene expression analysis was performed on Stratagene MXPro3000 (Stratagene) with SYBR Green Master Mix (Bio-Rad). Gene expression levels were normalized to 18S universal primer (Ambion Life Technology). Dissociation curves were also acquired to test primer specificity. Genes analyzed include: *Nmu*, *Nms*, *Nmur1*, *Nmur2*, *Ptgfr* (prostaglandin F receptor), and *Oxtr* (oxytocin receptor). Detailed primer sequences are shown in Table 2.

Western Blotting

Proteins from homogenized myometrium fragments and cell samples lysed in RIPA buffer were quantified using Bradford method (Bio-Rad). A total of 50 μg of protein sample was loaded onto SDS-PAGE gel and electrotransferred onto PVDF membranes. After blocking, membranes were incubated with either an antibody against NmU (no. sc-368069; Santa Cruz Biotechnology), NmS (no. PAA828Mu01; Cloud-Clone Corp.), NmU-R1 (no. sc-47241; Santa Cruz Biotechnology), NmU-R2 (no. sc-47250; Santa Cruz Biotechnology), cyclophilin B (no. ab16045; Abcam), anti-GAPDH (PA1-987; Pierce Protein Biology, Thermo Scientific), or β-actin (no. sc-47778; Santa Cruz Biotechnology); although NmU and NmS antibodies detect the corresponding propeptides [7], expression patterns were corroborated throughout the study by nearly identical mRNA profiles. Membranes were then washed with PBS containing 0.1% Tween 20 (Sigma-Aldrich) and incubated for 1 h with their respective secondary antibodies conjugated to horseradish peroxidase (HRP; Sigma). For kinases, membranes were incubated with an antibody against either phospho-JNK (no. 9251; Cell Signaling Technology), phospho-c-jun (no. 9261; Cell Signaling Technology), phospho-p38 (no. 4511; Cell Signaling Technology), phospho-Akt (no. 9271; Cell Signaling Technology), phospho-Erk (no. 9101; Cell Signaling Technology), JNK (no. 9252; Cell Signaling Technology), c-jun (no. 9165; Cell Signaling Technology), p38 (no. 9212; Cell Signaling Technology), Akt (no. 9272; Cell Signaling Technology), or Erk (no. 4695; Cell Signaling Technology). Enhanced chemiluminescence (GE Healthcare) was used for detection using the ImageQuant LAS-500 (GE Healthcare), and densitometric analysis was performed using ImageJ (National Institutes of Health). Resulting values were normalized first with the loading controls (β-actin, GAPDH, or cyclophilin B) and then as a ratio of the control samples.

Calcium Assay

A total of 40 000 mSMCs per well were cultured overnight in 96-well clear-bottomed black plates (no. 3603; Corning) prior to the calcium assay performed according to manufacturer's protocol (F36206; Life Technologies). In brief, medium was changed for a probenecid-containing assay buffer, and plates were read using a microplate reader (EnVision Multilabel reader, PerkinElmer), in response to on-time stimulations with NmU, NmS, $PGF_{2\alpha}$, or OT (using apparatus injectors). Five readings were taken before the injection (basal readings), and 2-sec interval postinjection readings were automatically stopped after 30 sec. Assay buffer was used as a negative control. Values are presented as a ratio between means of readings and means of basal readings.

Ex Vivo Uterine Contraction Experiment

Timed-pregnant mice at different gestational ages were killed with CO_2, and uterine tissue fragments were collected. Briefly, a midline abdominal incision was made, and the uterine horns were rapidly excised and carefully cleansed of surrounding connective tissues. Longitudinal myometrial strips (2–3 mm wide and 10 mm long) were dissected free from uterus, mounted isometrically in organ tissue baths, and initial tension was set at 2 g. The tissue baths contained 20 ml of Krebs buffer of the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 0.9 $MgSO_4$, 1 KH_2PO_4, 11.1 glucose, and 23 $NaHCO_3$ (pH 7.4). The buffer was equilibrated with 95% oxygen:5% carbon dioxide at 37°C. Isometric tension was measured by a force transducer and recorded by a BI-OPAC data acquisition system (BI-OPAC MP150). Experiments began after 1 h of equilibration. The mean tension of spontaneous contractions was measured using a BI-OPAC digital polygraph system (AcqKnowledge); the same parameters were also determined after the addition

TABLE 2. Mouse primers used for real-time quantitative PCR.

Gene	Forward sequence	Reverse sequence
Nmu	CACGCTGAGGGACAGCTAAA	TATTGGCACACCTTTGCAAGC
Nms	CCAACCTAAGGAAAACCAGGATG	GATGGACCGGAGCAAACTCA
Nmur1	TGACTACCGCACTGCTCTTC	ACAACCAGTGCAAACAGCATC
Nmur2	TACATCCTCCCGATGACCCT	CACGAGGACCAAGACAAACAG
Ptgfr	AGCTGGACTCATCGCAAACA	GTGGGCACAAGCCAGAAAAG
Oxtr	TGTGTCTCCTTTTGGGACAA	GGCATTTCAGAATTGGCTGT

of NmU, NmS, $PGF_{2\alpha}$, or oxytocin. At the start of each experiment, mean tension of spontaneous myometrial contractions was used as the reference response. Increases in mean tension (%) were expressed as percentages of (X/Y) − 100, where X is change in mean tension (g) induced by NmU, NmS, oxytocin, or $PGF_{2\alpha}$, and Y is the initial reference response (g).

Histological Analysis of Placental Villous Tissue

Villous tissue biopsies were fixed in 10% neutral buffered formalin and paraffin embedded. For immunohistochemistry, 5-μm-thick sections were obtained using a microtome and processed as previously described [23]. The following antibodies were used: NmU (1:50; Santa Cruz Biotechnology) and NmS (1:10; Cloud Corp.), with matched HRP-conjugated secondary antibodies (goat anti-rabbit-HRP; Bio-Rad). Staining was revealed using 3,3-diaminobenzidine (Amresco) and slides were counterstained with hematoxylin and mounted. Primary antibodies were omitted for negative controls. Images were obtained with a slide scanner (Axioscan; Zeiss) using Zen2 program.

Primary Cytotrophoblast Isolation and Culture

Primary cytotrophoblasts were isolated from term placentas from uncomplicated pregnancies obtained after cesarean delivery using a well-established method developed by Kliman et al. [24]. Briefly, villous tissue was dissected, minced, and rinsed in PBS prior to three enzymatic digestions in HBSS with trypsin and DNase. Cytotrophoblasts were obtained from these digestions after separation by centrifugation on a discontinuous Percoll gradient. Cytotrophoblasts were plated at 2×10^6 cells per milliliter in DMEM-F12 supplemented with 10% FBS and penicillin/streptomycin and washed with PBS to removed nonadherent cells after 12 h. Cells were treated with LPS (1 μg/ml; Sigma-Aldrich) for 24 or 48 h in Opti-MEM (Life Technologies). Cells were collected in lysis buffer (PBS with 1% Triton X-100 and protease inhibitor cocktail), they were centrifuged at 13 000 rpm for 10 min at 4°C, and supernatant was collected and kept at −20°C until it was used for analysis.

Immunocytochemistry

Cells were plated on coverslips precoated with poly-D-lysine and fixed in 4% paraformaldehyde. After blocking, cells were incubated overnight with a primary antibody for rabbit anti-α-actin and for 1 h at ambient temperature with a secondary antibody conjugated with Alexa Fluor 488 (Sigma). Nuclei were stained with 4′,6-diamidino-2-phenylindole (1:5000; Invitrogen). Images were captured using a 30× objective with an Eclipse E800 (Nikon) fluorescence microscope.

Statistical Analysis

Groups were compared by two-tailed Student *t*-test or one-way analysis of variance (ANOVA). Dunnett multiple-comparison method was employed when treatments were compared to a single control. Tukey multiple-comparison test was used for ex vivo contraction assays. A value of $P < 0.05$ was considered statistically significant. Data are presented as means ± SEM.

RESULTS

NmU-R2 Is Expressed in Murine Uterus and Induces Uterine Contractions and Labor upon Stimulation with NmU and NmS

We confirmed the expression of NmU-R2 in murine pregnant uterus at term gestation (G19; Fig. 1A) and the uterocontractile effects of NmU (EC_{50} = 15 nM; Fig. 1B). Effects of NmU were NmU-R2 dependent, because uterine contraction was markedly diminished after *Nmur2* knockdown (Fig. 1C; Supplemental Fig. S1, A–C) performed with an intrauterine injection of lentiviral-encoded shRNA-*Nmur2*. In contrast to *Nmur2* knockdown, that of *Nmur1* did not alter critical contraction-associated uterine smooth muscle NmU-triggered Ca^{++} mobilization (Supplemental Fig. S1, D and E), which is consistent with the documented NmU-R1-independent uterotonic effects of NmU [16]. Additionally, NmU-R2-specific ligand NmS [14] also increased uterine contractility, comparably to NmU (at ~EC_{50} value; Fig. 1D). Hence, NmU exerts its effects on the uterus specifically via NmU-R2.

NmU injected in vivo intraperitoneally twice daily on G17 and G18 to pregnant mice dose-dependently accelerated delivery, equivalent to that observed with established uterine contractile agonist $PGF_{2\alpha}$, but less effectively than oxytocin (Fig. 1E); shortened gestation induced by NmU was again NmU-R2 dependent, because it was abrogated in mice with uterine knockdown of *Nmur2* (Fig. 1F); shRNA-*Nmur2* did not significantly affect length of intact gestation. The NmU-R2-specific ligand NmS [14] exerted a modest shortening of gestation of 12 h, comparable to that by NmU (Fig. 1G).

Expression of NmS (but Not NmU) in Human and Murine Placenta and of NmU-R2 in Uterus Markedly Increases Near Term and During Labor, and Is Associated with Increased Uterocontractile Response

Given the labor-inducing action of NmU and NmS, we studied the endogenous expression of these NmU-R2 ligands in placenta, which is considered a key organ in the regulation of on-time labor [25]. We found placental protein and mRNA expression of NmU (Fig. 2A and Supplemental Fig. S2A) and NmS (Fig. 2B and Supplemental Fig. S2B); NmU expression was consistent throughout pregnancy, whereas NmS was exclusively and consistently expressed during spontaneous labor in mice. Study of human placental biopsies collected from women at term not in labor (TNL) or in established labor (TL) paralleled murine data, revealing expression of both NmU and NmS (Fig. 2, C and D), with a marked increase of NmS during labor.

We also studied the expression of procontractile NmU-R2 in murine and human uterus. In murine uterus, NmU-R2 protein and mRNA expression rose near term, peaked during labor, and rapidly declined 24 h postpartum (Fig. 2E and Supplemental Fig. S2D). Study of human myometrial biopsies was again rigorously consistent with murine data, revealing a marked (>7-fold) and gradual increase of NmU-R2 protein expression in pregnant women at term and preterm (TL, n = 5; TNL, n = 9; preterm without any clinical sign of labor [PTNL], n = 4) compared with nonpregnant women (n = 4; Fig. 2F and Supplemental Fig. S2G). In contrast, NmU-R1 protein and gene expression in murine uterus did not increase during gestation or labor (Supplemental Figs. S1F and S2, C–F). Concordant with the gestational age-dependent rise in NmU-R2 expression, ex vivo uterine contractile response to NmU also significantly rose (dose dependently) with advancing gestation (Fig. 2G); NmU potency was comparable to that of $PGF_{2\alpha}$ at any gestational age. Correspondingly, NmU, as seen with $PGF_{2\alpha}$, accelerated delivery when administered (intraperitoneally) in late but not early gestation (Table 3), confirming the gestational age-dependent effect of NmU.

NmU-R2-Coupled Contraction-Associated Intracellular Signaling in Response to NmU Is Gestational Age Dependent

Primary murine mSMCs isolated at G10 (G10 mSMCs) and G19 (G19 mSMCs) were used to study NmU- and NmS-induced signaling responses. One first notes the purity of our cell isolate cultures (>95% of cells immunoreactive to α-actin), and significantly greater mRNA and protein expression of NmU-R2, but not NmU-R1, in cells isolated at G19 compared with G10 (Supplemental Fig. S3), as seen directly on uterine samples (Fig. 2E). NmU induced greater calcium transients on G19 mSMCs than G10 mSMCs (Fig. 3A); a similar profile was

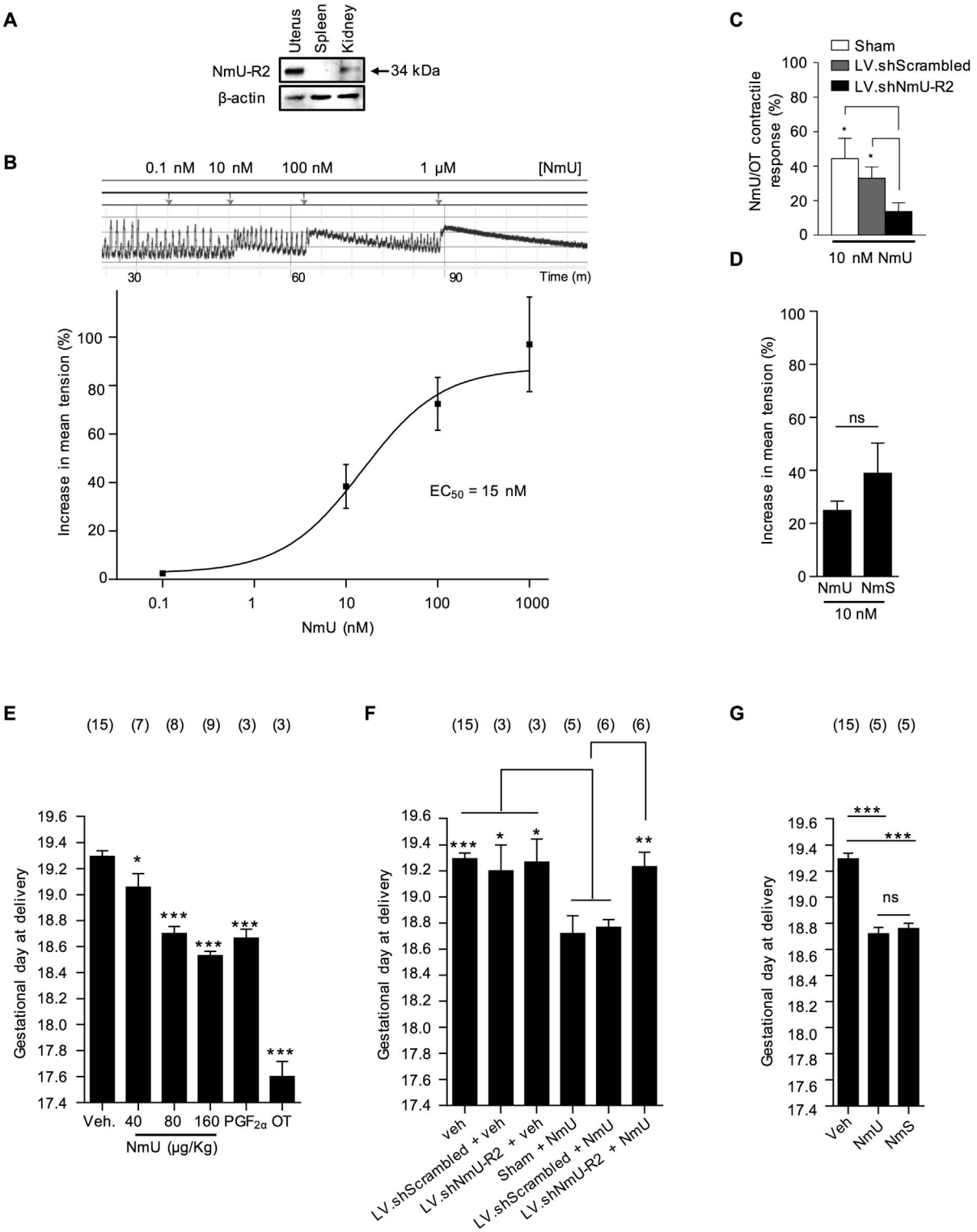

FIG. 1. NmU and NmS induce uterine contractions and labor via NmU-R2. **A**) NmU-R2 immunoblot from term uterus. Spleen and kidney samples were used as negative and positive controls, respectively. **B**) Representative ex vivo contraction assay performed on a myometrium fragment from a pregnant mouse at term in response to increasing NmU concentrations (top) and dose-response curve (bottom). **C**) Pregnant mice were pretreated with LV.shNmU-R2 or LV.shScrambled at G15, and their uteri were collected at term for a contraction assay in response to NmU and OT. For each uterine strip, the contractile response to NmU was normalized to its response to OT (to control for interindividual variability). $*P < 0.05$. **D**) Ex vivo contraction assay on G19 uteri in response to 10^{-8} M NmU or NmS. **E**) Pregnant mice were injected intraperitoneally with increasing NmU doses twice a day from G17 to G18. Control animals were given an equivalent volume of saline. $PGF_{2\alpha}$ and OT were used as positive controls at a dose of 160 μg/kg. $*P < 0.05$, $***P < 0.001$ compared with vehicle only. **F**) Pregnant mice were pretreated with LV.shNmU-R2 or LV.shScrambled at G15 and then treated with 160 μg/kg NmU twice a day from G17 to G18. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with NmU or NmU + LV.shScrambled. **G**) Pregnant mice were injected intraperitoneally with 160 μg/kg NmS or NmU twice a day from G17 to G18. $***P < 0.001$ compared with vehicle only. Data for **A**–**D** are representative of four to five mice per group. The number of mice used in **E**–**G** is displayed above each group, and mice treated with vehicle (control mice) were pooled together and repeatedly shown in each graph. Values are presented as mean ± SEM. Data were statistically analyzed using one-way ANOVA with comparison to control groups using Dunnett multiple-comparison test; ns, not significant.

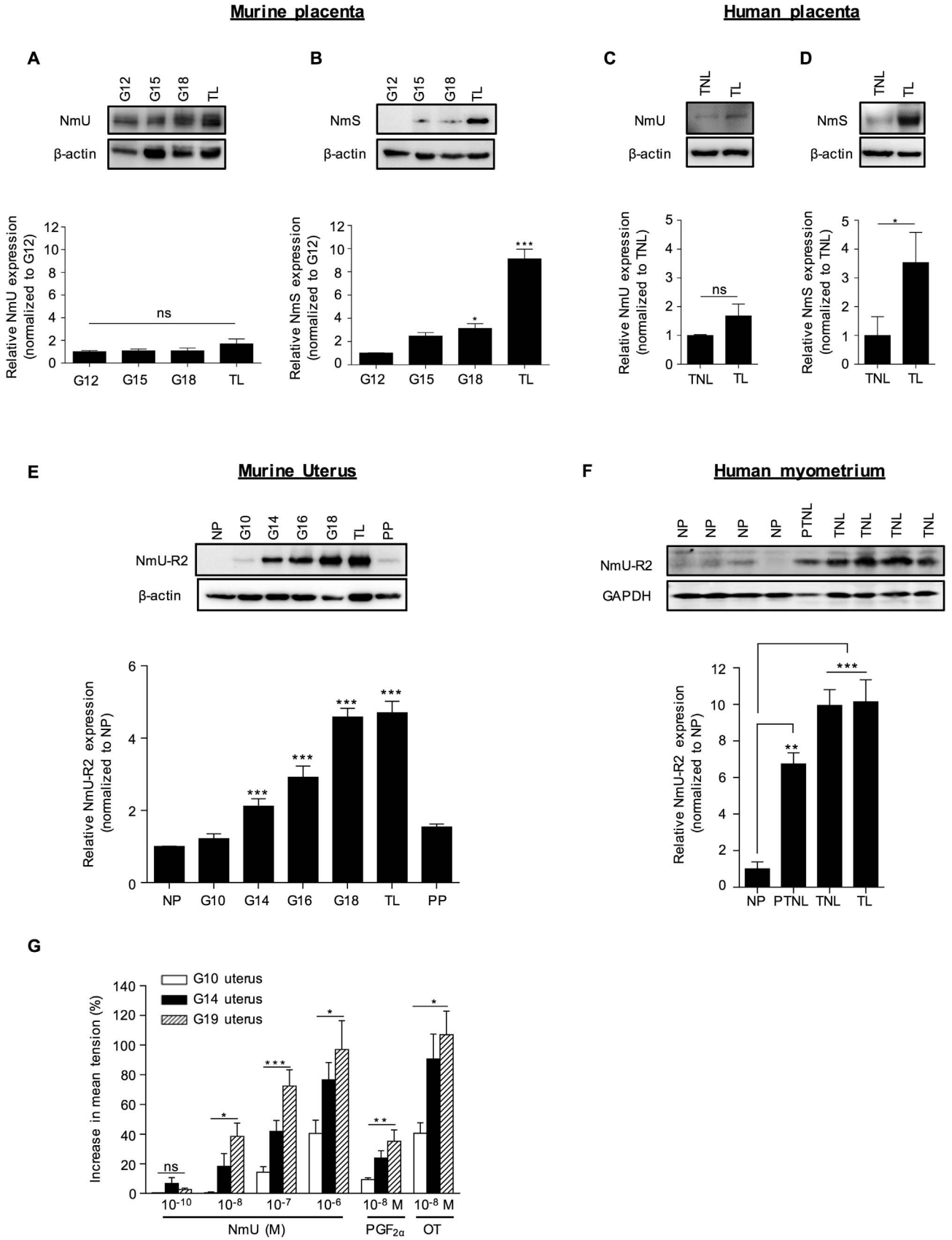

FIG. 2. The expression of NmS in placenta and of NmU-R2 in uterus increases near term and during labor in mice and humans, which is associated with increased NmU-induced myometrial contractility at term. **A** and **B**) NmU (**A**) and NmS (**B**) representative immunoblots of murine placentas collected at different gestational ages (G) and during spontaneous term labor (TL). Lower panels show densitometric analysis of protein bands normalized with β-actin and plotted as fold change versus the control group (G12). **C** and **D**) NmU (**C**) and NmS (**D**) immunoblots of human placenta biopsies from women at term not in labor (TNL) or in established labor (TL). Lower panels show densitometric analysis of protein bands normalized with β-actin and plotted as fold

TABLE 3. NmU and $PGF_{2\alpha}$ efficacy to induce labor is dependent on gestational age.

Substance injected (doses per injection)	Gestational days of injections (no. of mice)	Gestational age at delivery ± SD (term delivery = 19.2)
NmU (160 μg/kg)	G13-14 (n = 4)	19.3 ± 0.34
	G15-16 (n = 4)	19.4 ± 0.19
	G17-18 (n = 9)	18.5 ± 0.10*
$PGF_{2\alpha}$ (160 μg/kg)	G13-14 (n = 3)	19.4 ± 0.20
	G15-16 (n = 3)	19.3 ± 0.12
	G17-18 (n = 3)	18.7 ± 0.12*

* $P < 0.05$.

observed upon stimulation with known uterotonins $PGF_{2\alpha}$ and oxytocin. As expected, NmU-induced calcium transients were NmU-R2 dependent (Fig. 3B and Supplemental Fig. S1E); the extent and duration of calcium transients are consistent with those observed for other G protein-coupled receptors [26, 27]. Exploration of NmU-R2 downstream mechanisms in G19 mSMCs revealed that NmU does not activate (by phosphorylation) p38, JNK, and c-jun (Supplemental Fig. S4A), but it does activate Akt and Erk (Fig. 3, C and D, and Supplemental Fig. S4, B and C); no effect was seen in G10 mSMCs. NmU-triggered Akt activation, but not that of Erk, was inhibited by calmodulin inhibition (W-7) and by phospholipase C_β inhibition (U73122; Supplemental Fig. S4, D and E). *Nmur2* knockdown abrogated NmU-induced Akt and Erk activation in G19 mSMCs (Fig. 3, E and F). A schematic diagram of NmU-R2 signaling pathway is presented in Supplemental Figure S4F. NmS, which acts specifically on NmU-R2 [14], also elicited calcium transients, as well as Akt and Erk activation, comparable to those seen with NmU (Fig. 3, G–I).

IL1β Induces Uterine Expression of NmU-R2 and Potentiates Its Uterotonic Effects; Proinflammatory Infectious Stimuli Trigger NmS Expression in Human Decidua and Placenta

Chorioamnionitis is a major factor in triggering PTB [4–6]. We determined whether NmU-R2 is regulated by inflammatory factors. In line with the important role for IL1β in eliciting uterine inflammation and PTB [28–31], we stimulated G10 mSMCs with IL1β and measured the mRNA expression of *Nmur1* and *Nmur2*, as well as *Ptgfr* and *Oxtr*; expression of the last three genes (but not *Nmur1*) was upregulated within 6 h by IL1β (Fig. 4A). Of relevance herein, progesterone (when anti-inflammatory PR-B is dominant [32]) had no effect on expression of these genes (data not shown). We then proceeded to explore whether inflammation (IL1β-induced) could reproduce these effects in vivo. Consistent with changes observed in mSMCs, IL1β (1 μg intrauterine, at G9 [Fig. 4B]), shown to amplify uteroplacental inflammation [30], induced protein and mRNA uterine expression of NmU-R2 (Fig. 4C and Supplemental Fig. S2I), but not NmU-R1, in mice (Supplemental Figs. S1G and S2H). Correspondingly, uteri isolated from pregnant mice (G10) treated with IL1β displayed increased uterocontractile response to NmU ex vivo (Fig. 4D). Concordantly, calcium transients triggered by NmU were significantly increased in murine mSMCs isolated at G10 and pretreated with IL1β (Fig. 4E); this effect was also observed in human mSMCs (Fig. 4F).

Given the strong and consistent effect of IL1β in triggering NmU-R2 expression and actions in uterus, we determined whether IL1β triggered expression of NmU-R2 ligands in placenta. IL1β was found to increase placental expression of the NmU-R2-specific agonist NmS, but not NmU, in mice (Fig. 4, G and H). A similar induction of placental NmS was observed when mice were stimulated intrauterinely with known inflammasome-activating TLR4 ligand LPS (arising from Gram-negative bacteria) to mimic an infectious stimulus (Fig. 4I). Consistent with murine data, NmS expression was found to be markedly augmented (>8-fold) in decidual biopsies from women who delivered preterm with clinical evidence of infection (PTLi, n = 3) compared with those without (PTL, n = 3; Manchester, U.K., tissue bank; Fig. 4J). In human placenta, NmS was primarily expressed by cytotrophoblasts and Hofbauer (placental macrophage) cells (within placental villi), whereas NmU was specifically expressed by syncytiotrophoblasts (external layer of placental villi; Fig. 4K). Therefore, we stimulated primary human cytotrophoblasts isolated from term placenta with LPS for 24 and 48 h; LPS induced NmS expression from cytotrophoblasts (Fig. 4L), validating human and murine data (presented in Fig. 4, H–J).

NmU-R2 Plays a Key Role in Infection-Induced PTB

Previous results strongly suggest that NmS in placenta and NmU-R2 in uterus are regulated by inflammatory stimuli with or without infection. We proceeded to study the role of NmU-R2 in PTB associated with infection. For this purpose G16 mice were injected with LPS (to mimic infection), in animals previously treated or not treated intrauterinely with lentivirus encoded with shRNA against procontractile *Nmur2* (or scrambled shRNA). Gestation was significantly prolonged in LPS-treated *Nmur2* knocked down animals, by an average of ~28 h (Fig. 5A); this effect was associated with improved neonatal survival rate (Fig. 5B).

DISCUSSION

Inflammation, with or without infection, is considered to be implicated in more than 50% of PTBs, and its onset is often subclinical. Administration of bacteria or bacterial endotoxins in pregnancy triggers uterine activation pathways that can induce labor in rodents and nonhuman primates [30, 33–36]. Our findings indicate that NmU-R2 and its specific ligand NmS are regulated by inflammation in humans and animals, and play a critical role in infection-associated PTB. First, we found in

change versus the control group (TNL) of all patients screened (TNL, n = 6; TL, n = 6). **E**) NmU-R2 representative immunoblot of murine uteri collected at different gestational ages (G), during spontaneous term labor (TL) and 24 h postpartum (PP). The lower panel shows densitometric analysis of protein bands normalized with β-actin and plotted as fold change versus the control group (NP). **F**) Representative immunoblot of NmU-R2 from human myometrial tissue biopsies from four nonpregnant (NP) women (hysterectomy), one pregnant women at preterm without any clinical sign of labor (PTNL), and four pregnant women at term without any clinical sign of labor (TNL). The lower panel shows densitometric analysis of protein bands normalized with β-actin and plotted as fold change versus the control group (NP) of all patients screened (NP, n = 4; PTNL, n = 4; TNL, n = 9; TL, n = 5), as presented in **F** and Supplemental Figure S2G. **G**) Ex vivo contraction assay in response to increasing doses of NmU performed on myometrium fragments from pregnant mice at G10, G14, or G19. Uteri collected at G19 were only considered if the mice were still undelivered. $PGF_{2\alpha}$ and OT were used as positive controls at a dose of 10^{-8} M. Data are representative of four to five mice per group and at least three independent experiments. Values are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by one-way ANOVA with comparison to control groups using Dunnett multiple-comparison test; ns, not significant.

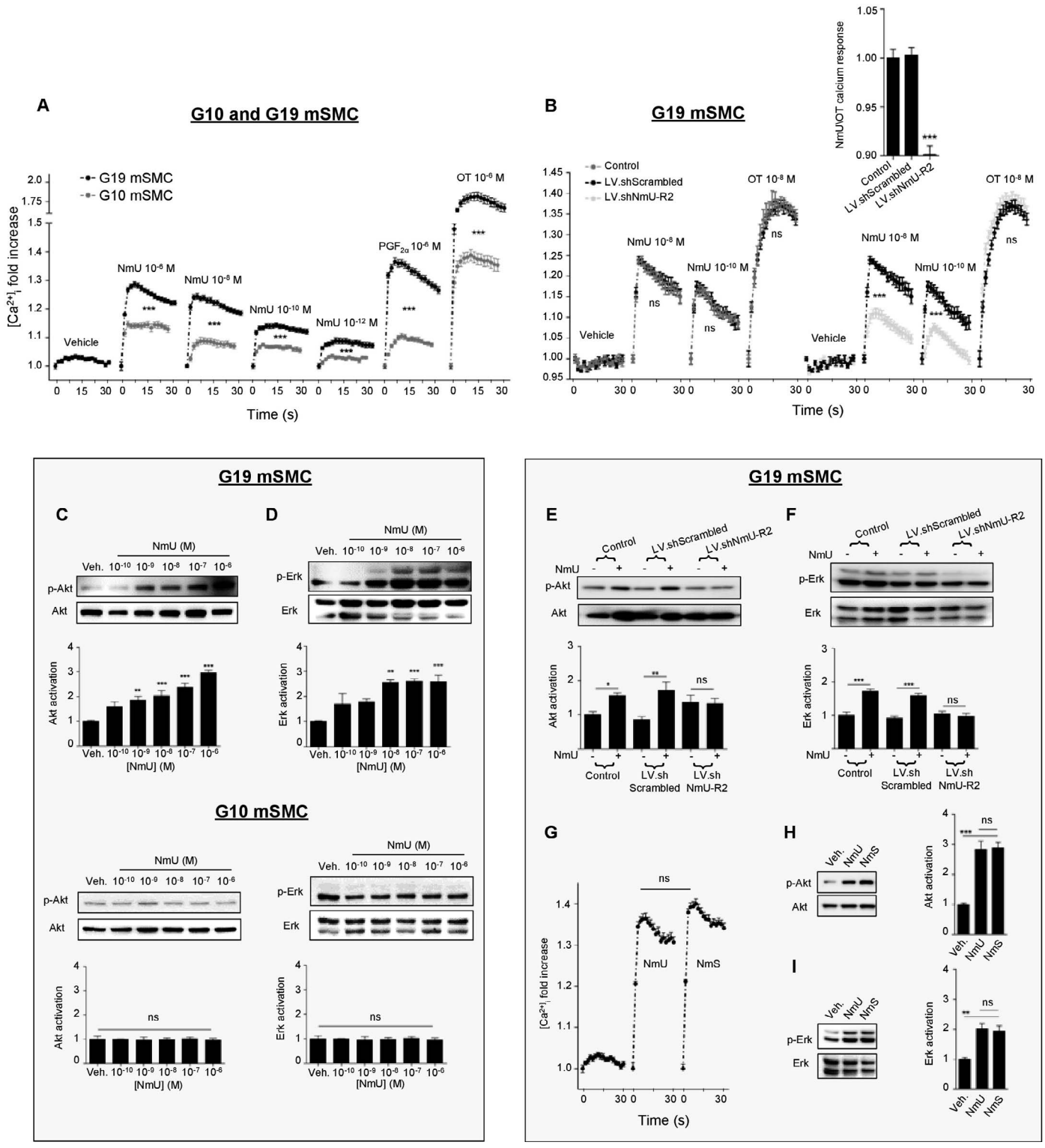

FIG. 3. NmU-R2-associated signaling in mSMCs is potentiated by gestational age. **A**) Calcium assay performed on primary mSMCs from pregnant mice at either G10 or G19. $PGF_{2\alpha}$ and OT were used as positive controls at a concentration of 10^{-6} M; n = 12–45 in each group. **B**) G19 myometrial cells were pretreated with LV.shNmU-R2 or LV.shScrambled for 72 h and then used in a calcium assay in response to 10^{-8} M NmU or OT. The calcium mobilization response to NmU is also presented when normalized with the response to OT (inset); n = 6–12 in each group. **C** and **D**) Akt (**C**) and Erk (**D**) phosphorylation immunoreactivity on primary mSMCs from pregnant mice at G10 or G19 stimulated with increasing concentrations of NmU for 10 min. Data are representative of four to five independent experiments. **E** and **F**) Akt (**E**) and Erk (**F**) immunoreactivity on G19 myometrial cells pretreated with LV.shNmU-R2 or LV.shSrambled for 72 h and then stimulated with 10^{-6} M NmU for 10 min. Data are representative of four to five independent experiments. **G**) Calcium assay of G19 myometrial cells treated with 10^{-6} M NmU or NmS; n = 6–45 in each group. **H** and **I**) Akt (**H**) and Erk (**I**) phosphorylation immunoblot of G19 myometrial cells treated with 10^{-6} M NmU or NmS for 10 min. Data are representative of four to five independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA with comparison to control groups using Dunnett multiple-comparison test; ns, not significant.

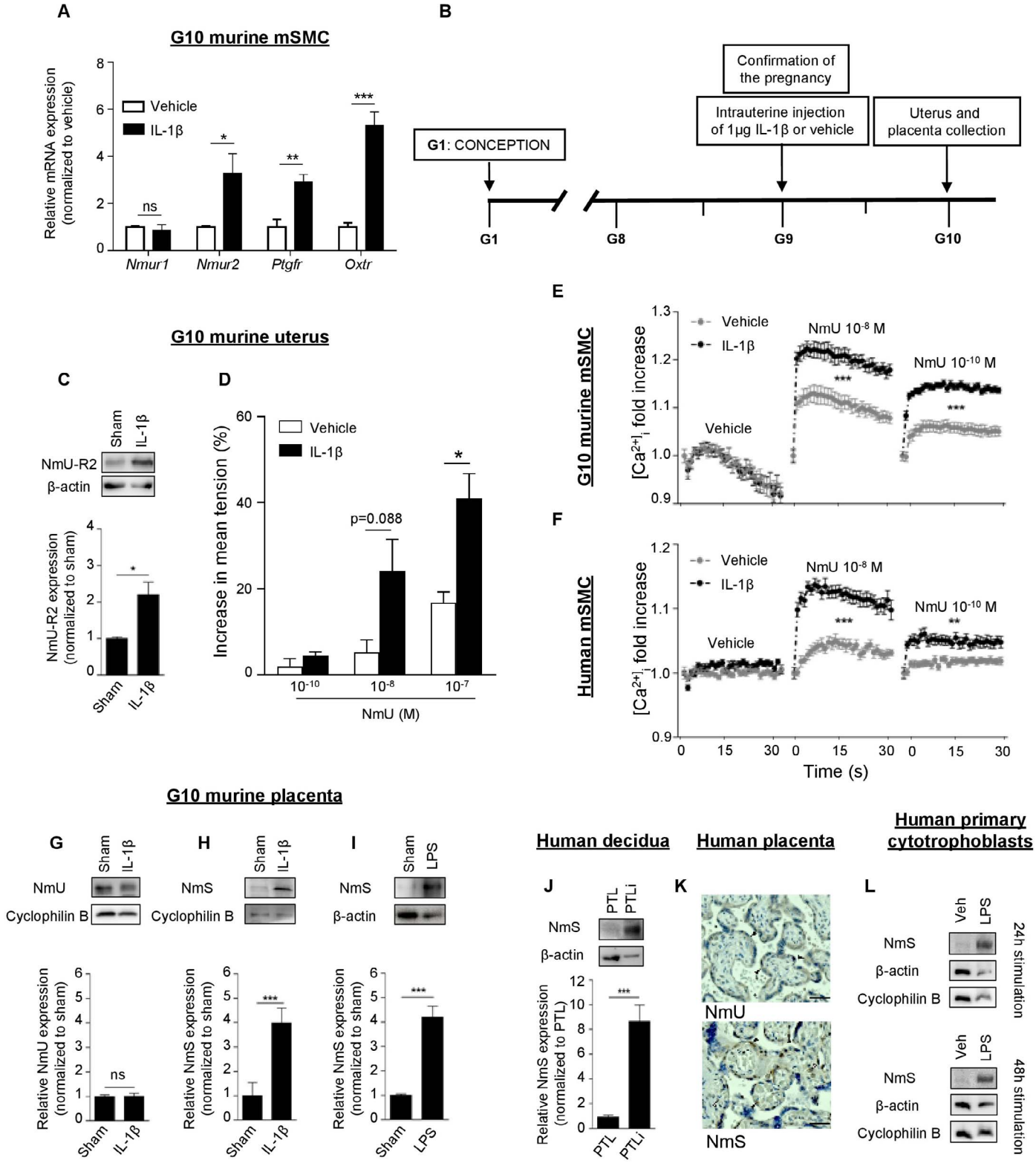

FIG. 4. NmU-R2 in uterus and NmS in placenta are under the control of inflammation in mice and humans. **A**) Quantitative PCR performed on primary mSMCs collected from pregnant mice at G10 and stimulated with 5 ng/ml IL1β for 6 h. Results are relative to 18S and plotted as fold over the control group (vehicle). **B**) Schematic representation of the in vivo model used. **C**) Representative NmU-R2 immunoblot (top) and densitometric analysis (bottom) of uteri of pregnant mice collected 24 h after an intrauterine injection of saline (sham) or IL1β. **D**) Ex vivo contraction assay performed on uteri from pregnant mice exposed for 24 h to saline (sham) or IL1β. Data are representative of three to four mice per group. **E** and **F**) Pregnant mouse (G10) mSMCs or human mSMCs (hTERT-C3 cell line) were treated for 24 h with IL1β or vehicle, and were used in a calcium assay; n = 6–12 in each group. **G** and **H**) Immunoblots (top) and relative densitometric analysis of protein bands (bottom) showing NmU (**G**) and NmS (**H**) expression in placenta of pregnant mice 24 h after an intrauterine injection of either saline (sham) or IL1β. **I**) Immunoblot (top) and relative densitometric analysis of protein bands (bottom) showing NmS expression in placenta of pregnant mice 24 h after an intrauterine injection of either saline (sham) or LPS. **J**) NmS immunoblot from human decidual biopsies from women in preterm labor with (PTLi, n = 3) and without (PTL, n = 3) clinical evidence of infection. Bottom panel shows

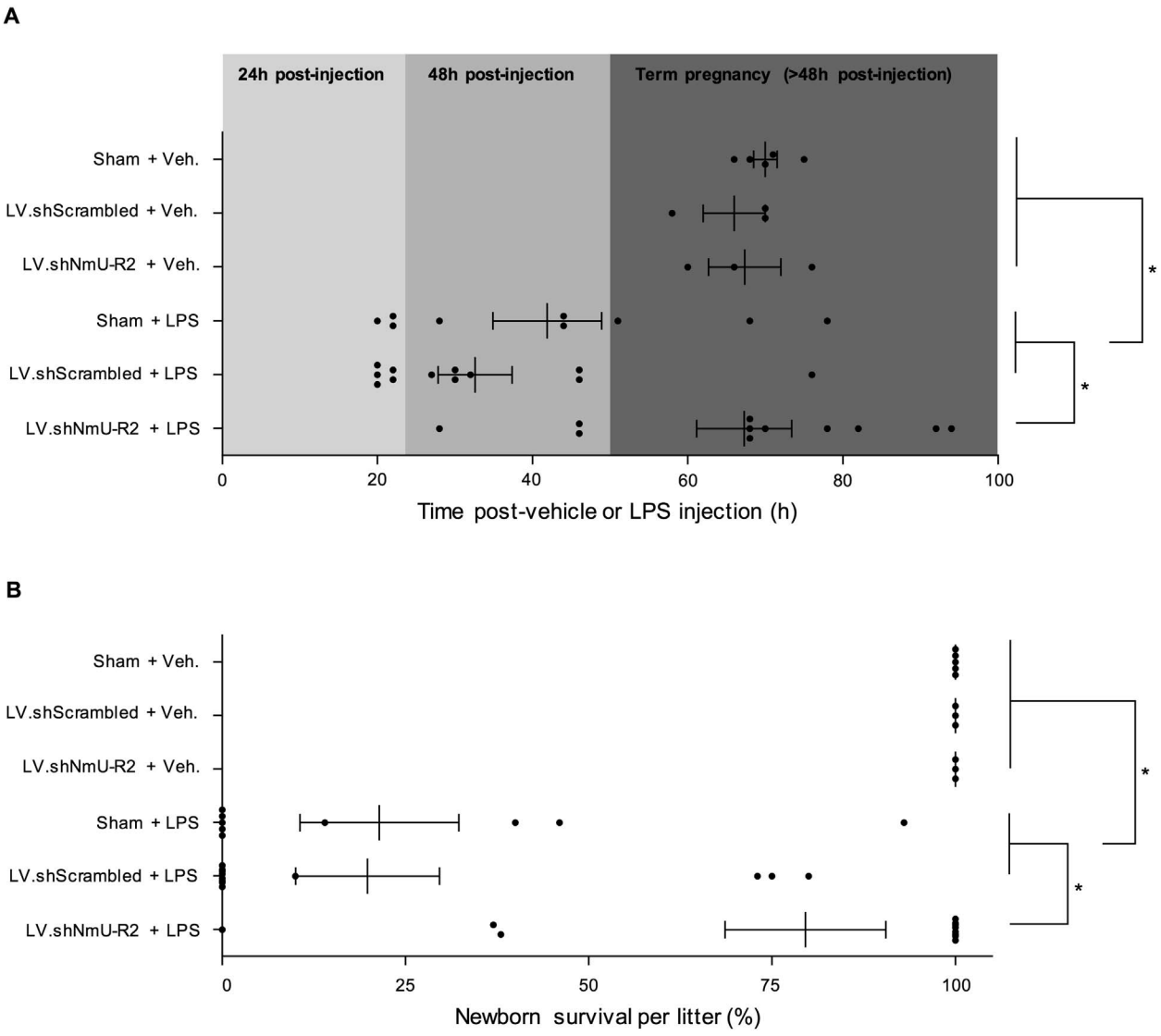

FIG. 5. NmU-R2 is important for infection-induced adverse gestation outcomes. **A** and **B**) Pregnant mice were pretreated with intrauterine injections of vehicle (sham), LV.shScrambled, or LV.shNmU-R2 at G13 (one injection in each uterine horn), and then treated with a single intraperitoneal injection of 10 μg of LPS or an equivalent volume of saline at G16. The timing of birth (**A**) and the survival of pups at birth per litter (**B**) were rigorously assessed. Values are presented as mean ± SEM. $^*P < 0.05$ by one-way ANOVA with comparison to control groups using Dunnett multiple-comparison test.

murine gestational tissues that the expression of NmU-R2 and NmS was markedly increased upon intrauterine treatment with the major proinflammatory labor-inducing cytokine IL1β. Correspondingly, treatment with IL1β significantly increased the uterotonic effect and associated calcium transients coupled to NmU-R2 in murine and human mSMCs. Second, the inflammation-driven NmU-R2 upregulation observed in animals was also observed in human gestational tissues, because NmS was upregulated in women with clinical signs of infection; correspondingly, NmS was induced by LPS stimulation of primary human cytotrophoblasts isolated from term placenta. This suggests that the bacterial trigger needs to penetrate placental villi to induce NmS production from placenta and in turn promote labor. Interestingly, NmU-R2 is another labor-associated protein present in the central nervous system, as is the case for OXTR and IL-1RAcPb [37]. Third, knockdown of NmU-R2 in uterus significantly delayed preterm labor induced by Gram-negative bacterial product LPS. For these reasons, we suggest that infectious and noninfectious proinflammatory stimuli in pregnant gestational tissues, as well as advanced gestational age (in case of physiological term labor), trigger: 1) NmU-R2 upregulation in myometrium, thereby increasing the uterocontractile sensitivity to NmS; and 2) NmS production in placenta (specifically in cytotropho-

densitometric analysis of protein bands normalized with β-actin and plotted as fold over the control group (PTL). **K**) Immunohistochemistry analysis performed on term human placentas blotted against NmU and NmS. For the NmU panel, black arrows represent syncytiotrophoblasts; for the NmS panel, black arrows represent cytotrophoblasts and white arrows Hofbauer cells. Bar = 40 μm. **L**) Primary human cytotrophoblasts were stimulated with LPS for 24 h (top) or 48 h (bottom), and cell lysates were blotted against NmS. β-Actin and cyclophilin B were used as loading controls. Values are presented as mean ± SEM. Data are representative of at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA with comparison to control groups using Dunnett multiple-comparison test; ns, not significant.

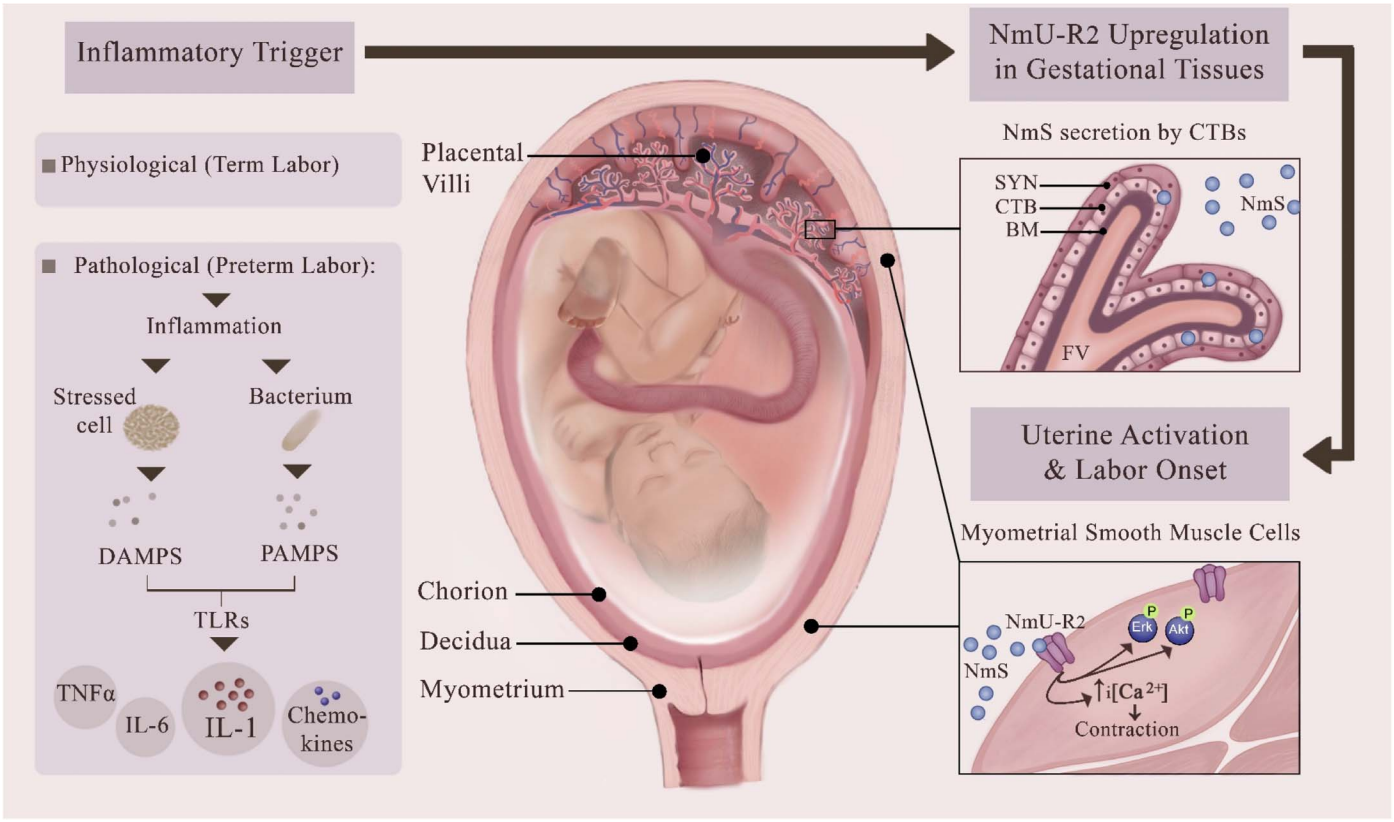

FIG. 6. Proposed role of NmU-R2 and its ligand NmS in physiological term labor and in pathological PTB. The schema provides a current view of inflammation-associated preterm birth whereby pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) activate pattern recognition receptors in uterus, predominantly Toll-like receptors (TLRs), to trigger an inflammatory cascade characterized by the local maturation and release of proinflammatory, prolabor cytokines, leading to uterine activation and PTB. Our study reveals that uterotonic NmS and its cognate receptor NmU-R2 are endogenously produced in human placenta and myometrium, respectively, and are upregulated by the PAMP LPS and by its downstream mediator IL1. We propose that this neuromedin system is implicated in sustaining or initiating uterine contractions in both physiological labor and pathological preterm labor associated with inflammation. This figure was made exclusively for this manuscript by the authors.

blasts). This uterotonic system may contribute to the establishment of functional labor at term and preterm in the case of a pathological activation of an intrauterine inflammatory cascade (Fig. 6). Hence, NmU-R2 constitutes an interesting target for the prevention of inflammation-associated PTB.

Preterm birth remains a challenge for clinicians because there are no available pharmacological agents sufficiently effective at prolonging preterm gestation by more than 48 h and improving newborn outcomes. The development of preventive therapies is limited because diagnostic tools with successful positive predictive values are also lacking, which hinders the identification of women at risk of preterm labor. Current therapies administered to women in labor (tocolytics) are largely ineffective and in many cases (e.g., indomethacin, nifedipine) are used off-label [38]. Only one tocolytic drug specifically designed to target uterine contraction has been approved in the last 30 yr, the oxytocin receptor antagonist atosiban. Although atosiban was demonstrated to be as efficacious as β-mimetics (which remains limited) and much better tolerated by women [39], its usage is limited to Europe, because it failed to gain U.S. Food and Drug Administration approval. Hydroxyprogesterone is effective for women with either short cervical length or prior history of preterm labor [40]. Yet there is still an unmet medical need for an effective tocolytic. Uterine activation proteins are interesting targets for the prevention of preterm birth because their induction directly precedes labor, and their functions are critical for successful labor.

Herein, we characterized a potential UAP, NmU-R2 (and its ligands NmU and NmS), in labor. NmU-R2 is upregulated in human and animal gestational tissue at term and during labor; NmS is concomitantly upregulated during parturition. The properties of NmU-R2 correspond to those of known UAPs: 1) its expression increases near labor and decreases in the immediate postpartum period; 2) it exhibits gestation age-dependent uterocontractile effects; 3) it is induced by proinflammatory uterotrophins, and its expression correspondingly increases during preterm labor with clinical evidence of infection; and 4) it contributes to the process of labor. In this study, NmU-R2 was found to be important for the onset of preterm labor associated with infection, but not necessary for labor at term, as seen with our NmU-R2 knocked-down model displaying normal parturition at term. This may be due to redundant mechanisms that are present in term uterus to ensure successful delivery, as is seen with the unaltered gestation length in germ line gene knockout mice for other critically important proteins, including IL1R1, TNFα, OT, COX-1, and others [19].

NmU-induced ex vivo uterine contractions have already been reported to be unaltered by NmU-R1 deficiency (using gene knockout mice), and thus are independent of NmU-R1 [16]. The present study is concordant and clarifies actions of NmU by showing that its contractile-associated effects (calcium transients) in uterus are specifically mediated by

NmU-R2 (but not NmU-R1). In addition, uterine NmU-R2 knockdown considerably attenuated receptor-coupled signaling (calcium, Erk, and Akt), uterocontractile and prolabor effects induced by NmU, as well as preterm labor induced by inflammatory/infectious stimuli. In our murine experiments, NmU and NmS had similar efficacy to induce calcium, Erk, and Akt signaling in mSMCs, as well as uterine contraction and labor. Interestingly, only the NmU-R2-specific ligand NmS was endogenously induced by inflammation and upregulated during physiological labor, suggesting that NmU is not as important as NmS in initiating or sustaining labor contractions.

In summary, we hereby describe a procontractile, prolabor human and murine system wherein neuromedins U and (more importantly) S induce calcium (and other downstream) signals in mSMCs to promote potent uterine contractions and labor via NmU-R2 at term and particularly before term. NmU-R2, NmU, and NmS are expressed in human and murine gestational tissues, and NmU-R2 and NmS are upregulated (and potentiated) by gestation age, infection, and inflammation. Correspondingly, this system is important for the onset of inflammation-induced preterm labor in mice, which may plausibly apply to human labor because NmS is markedly upregulated in gestation with clinical evidence of infection. Overall, the present study expands our understanding of the physiological mechanisms underlying labor, and it uncovers new targets for potential therapeutic intervention to delay preterm delivery, and more opportunities to identify biomarkers to predict women at risk of preterm birth. Specifically, NmU-R2 antagonists may provide benefits to prolong gestation in threatened pregnancies.

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