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# Regulation of Interleukin-1 Governs Acute Intrauterine Inflammation to Improve Gestational and Neonatal Outcome

Par Mathieu Nadeau-Vallée

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*Présentée par*

**Mathieu Nadeau-Vallée**

*A été évaluée par un jury composé des personnes suivantes*

**Martin G. Sirois**
Président-rapporteur

**Sylvain Chemtob**
Directeur de recherche

**Sylvie Girard**
Codirectrice

**Édouard Kouassi**
Membre du jury

**Daniel Dufort**
Examinateur externe

# Abstract

Preterm birth (PTB; birth before 37 weeks' gestation) is the leading cause of neonatal mortality and morbidity worldwide, and surviving infants are at risk of long-lasting functional impairments. The pathophysiology of spontaneous preterm labor has been largely attributed to intrauterine inflammatory processes independent of aetiology, gestation age at delivery, and presence of infection. Importantly, intrauterine inflammation can propagate to the fetus whereupon the initial trigger is amplified to cause fetal organ injury, thereby compromising neonatal outcome. Of all pro-inflammatory mediators, interleukin-1 (IL-1) stands out as a major player in PTB and fetal inflammation. Therefore, modulating its action antenatally may be key to achieve better gestational and neonatal outcomes. Herein, we show that antenatal regulation of IL-1 by lactate (or 3,5-dihydroxybenzoic acid [3,5-DHBA]) *via* activation of anti-inflammatory GPR81 in myometrium, or by an heptapeptide noncompetitive antagonist of IL-1 receptor developed in our laboratory and termed 101.10, prevents PTB and improves neonatal survival in intrauterine-inflammatory and systemic-infectious murine models of PTB. Specifically, we show that antenatal maternal administration of 101.10 prevents premature triggering of uterine, choriodecidual, placental, amniotic, and fetal inflammation, thereby decreasing organ injury and functional impairment in progeny. In this setting, 101.10 has shown superior efficacy as compared to anakinra (Kineret), a competitive IL-1 receptor antagonist, especially to prevent PTB and neonatal mortality. Further, we demonstrate that 101.10 exhibits functional selectivity by inhibiting IL-1-induced signals transducers p38, c-Jun N-terminal kinase (JNK), c-jun, and Rho GTPase/ Rho-associated coiled-coil-containing protein kinase (ROCK), while desirably preserving IL-1-induced activation of IκBα and nuclear factor-kappa

B (NF-κB). In a second set of experiments, we uncover a novel uterine negative feedback mechanism whereby the anaerobic metabolism solicitated during active labor produces large levels of lactate, which in turn activates GPR81 in myometrium to decrease IL-1-induced acute inflammatory cascade. Correspondingly, GPR81$^{-/-}$ mice display increased uterine inflammation during labor and increased rates of labor dystocia, whereas inversely administration of the GPR81 agonist 3,5-DHBA decreases the uterine inflammatory response to IL-1 and prevents lipopolysaccharide (LPS, gram(-) bacteria endotoxin)-induced PTB. Altogether, this data points to a major role of antenatal IL-1 in eliciting PTB and long-lasting fetal organ injury, and describes a novel therapeutic approach to inhibit IL-1 receptor antenatally while preserving important physiological inflammatory signaling pathways.

**Keywords**: Preterm birth, preterm labor, interleukin-1, anti-IL-1, inflammation, neonatal morbidity, functional selectivity, lactate, GPR81.

# Résumé

La naissance prématurée (NP; naissance avant 37 semaines de gestation) est la cause principale de mortalité et de morbidité néonatale à travers le monde, et les nourrissons survivants sont à risque de déficits fonctionnels à long-terme. La physiopathologie du travail préterme spontané est largement attribuable aux processus inflammatoires intra-utérins indépendamment de l'étiologie, de l'âge gestationnel à l'accouchement, et de la présence d'infection. De façon importante, l'inflammation intra-utérine se propage au fœtus, après quoi le déclencheur initial est amplifié pour causer des dommages aux organes fœtaux, compromettant ainsi l'issue néonatal. De tous les médiateurs pro-inflammatoires, l'interleukine-1 (IL-1) se démarque comme étant un joueur majeur dans la NP et l'inflammation fœtale. Ainsi, de moduler son action durant la gestation pourrait être essentiel afin d'atteindre une meilleure issue néonatale. Dans la présente, nous démontrons que la régulation anténatale de l'IL-1 par le lactate (ou l'acide 3,5-dihydroxybenzoïque [3,5-DHBA]) *via* le récepteur myométrial anti-inflammatoire GPR81, ainsi qu'un heptapeptide antagoniste non-compétitif du récepteur de l'IL-1 développé dans notre laboratoire et nommé 101.10, préviennent la NP et améliorent la survie néonatale dans des modèles murins de NP induite par l'inflammation intra-utérine ou l'infection systémique. Spécifiquement, nous montrons que l'administration maternelle anténatale de 101.10 prévient le déclenchement prématuré de l'inflammation utérine, choriodéciduale, placentaire, amniotique et fœtale, diminuant ainsi les dommages aux organes fœtaux et les déficits fonctionnels chez la progéniture. Dans ce contexte, 101.10 a démontré une efficacité supérieure à anakinra (Kineret), un antagoniste compétitif du récepteur de l'IL-1, surtout pour prévenir la NP et la mortalité néonatale. De plus, nous démontrons que le 101.10 agit par sélectivité

fonctionnelle en inhibant les signaux de transduction activés par l'IL-1, spécifiquement p38, *c-Jun N-terminal kinase* (JNK), c-jun, et Rho GTPase/ *Rho-associated coiled-coil-containing protein kinase* (ROCK), tout en préservant désirablement l'activation de IκBα et de *nuclear factor-kappa B* (NF-κB) induite par l'IL-1. Dans un second ensemble d'expériences, nous découvrons un mécanisme inédit de rétroaction négative intra-utérine par lequel le métabolisme anaérobique sollicité durant la phase de travail utérin actif produit des niveaux élevés de lactate, ce qui en retour active GPR81 dans le myomètre pour diminuer la cascade inflammatoire aigüe induite par IL-1. De façon correspondante, les souris GPR81$^{-/-}$ présentent plus d'inflammation utérine durant le travail et des taux plus élevés de dystocie utérine, alors qu'à l'inverse l'administration de l'agoniste de GPR81 3,5-DHBA diminue la réponse inflammatoire utérine à l'IL-1 et prévient la NP induite par le lipopolysaccharide (LPS, une endotoxine provenant des bactéries gram(-)). En somme, nos données démontrent un rôle majeur de l'IL-1 anténatale à éliciter la NP et les dommages à long-terme aux organes fœtaux, en plus de décrire une approche thérapeutique inédite pour inhiber le récepteur de l'IL-1 avant la naissance tout en préservant les voies de signalisation inflammatoires physiologiquement importantes.

**Mots-clés:** Naissance prématurée, travail préterme, interleukine-1, anti-IL-1, inflammation, morbidité néonatale, sélectivité fonctionnelle, lactate, GPR81.

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# List of abbreviations and acronyms

ADAM, A Disintegrin and Metalloprotease; AF, amniotic fluids; AHCA, acute histological chorioamnionitis; AP-1, activator protein-1; $Ca^{++}$, calcium; cAMP, cyclic adenosine monophosphate; CCA, clinical chorioamnionitis; CCL2, chemokine ligand 2; CCR, C-C chemokine receptor; CD, cluster of differentiation; cffDNA, cell-free fetal deoxyribonucleic acid; CMV, cytomegalovirus; COX, cyclo-oxygenase; CRH, corticotrophin-releasing hormone; CRH-R1, corticotrophin-releasing hormone receptor-1; CSAIDs, cytokine suppressive anti-inflammatory drugs; CX-43, connexin-43; CYP450, cytochromes P450; DAMPs, damage-associated molecular patterns; DCs, dendritic cells; DNA, deoxyribonucleic acid; EBV, Epstein-Barr virus; ECM, extracellular matrix; e.g., exemplī grātiā; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; FADD, Fas-Associated protein with Death Domain; FDA, Food and Drug Administration; FIRS, fetal inflammatory response syndrome; FP, prostaglandin $F_{2\alpha}$ receptor; GLP-1, glucagon-like peptide-1; GPR, G protein-coupled receptor; gp130, glycoprotein 130; GTPase, guanosine-5'-triphosphatase; HIV, human immunodeficiency virus; HMGB1, high–mobility group box 1; HPA, hypothalamic–pituitary–adrenal; HSPs, heat-shock proteins; HSV, herpes simplex virus; icIL-1Ra, intracellular interleukin-1 receptor antagonist; i.e., id est; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; IKK, IκB kinase; IL, interleukin; IL-1R, interleukin-1 receptor; IL-1Ra, interleukin-1 receptor antagonist; IL-1RAcP, interleukin-1 receptor accessory protein; IL-6R, interleukin-6 receptor; iNOS, inducible nitric oxide synthase; IRAK, interleukin-1 receptor–activated protein kinase; i.v., intravenous; JNK, c-Jun N-terminal kinase; kDa, kilodalton; LPS, lipopolysaccharide; LTA, lipoteichoic acid; M-As, meta-analyses;

MAPK, mitogen-activated protein kinases; MLCK, myosin light-chain kinase; MMPs, matrix metalloproteinases; MSU, monosodium urate; MYD88, myeloid differentiation primary response gene 88; NAC, N-acetyl cysteine; NOD, nucleotide oligomerization domain; NK, natural killer; NF-κB, nuclear factor-kappa B; NLRs, NOD-like receptors; NLRP3, NOD-like receptor family, pyrin domain containing 3; Nm, neuromedin; NmU-R2, neuromedin U receptor 2; OXR, oxytocin receptor; PAMPs, pathogen-associated molecular patterns; PAR, protease-activated receptor; PG, prostaglandin; PGHS-2, prostaglandin H synthetase-2; poly(I:C), polyinosinic:polycytidylic acid; pPROM, preterm premature rupture of membranes; PR, progesterone receptor; PRRs, pattern recognition receptors; PTB, preterm birth; RAGE, receptor for advanced glycation endproducts; RCTs, randomized clinical trials; RNA, ribonucleic acid; RSV, respiratory syncytial virus; SAPK, stress-associated protein kinases; SCTMs, syncytiotrophoblast microvesicles; sIL-1Ra, soluble interleukin-1 receptor antagonist; sIL-1RacP, soluble interleukin-1 receptor accessory protein; sIL-6R, soluble interleukin-6 receptor; STAT, signal transducers and activators of transcription; TIMPs, tissue inhibitors of metalloproteinases; TIR, Toll- and IL-1R–like; TLRs, Toll-like receptors; TNF, tumor necrosing factor; TNFR, tumor necrosing factor receptor; TRAF, tumor necrosis factor–associated factor; TRP, transient receptor potential channel; UAPs, uterine activation proteins; USA, United States of America; USD, United States dollar; VEGF, vascular-endothelial growth factor; WHO, World Health Organization.

*If I have seen further, it is by standing on the shoulders of giants*

-Sir Isaac Newton

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# Part A: Introduction[1]

# 1. Preterm birth :

## 1.1. Epidemiology and definitions

Preterm birth (PTB; delivery before 37 weeks of gestation) affects 1 out of 10 newborn, which corresponds to approximately 15 million babies worldwide each year, and in many countries this rate increases unabatedly each year (1). PTB is a leading cause of infant mortality and morbidity worldwide and surviving infants are at risk of lifelong complications, which constitutes a major social and economic burden (2). Although major advances have been made in the past decades, to this date no pharmacological compound has been successful in arresting uterine labor after its onset, or to prolong gestation by more than a week in symptomatic (i.e. in labor) women. Accordingly, the rate of PTB in the United States has increased since 1990 (from 10.62 % in 1990 to 11.72% in 2011) suggesting that PTB remains an important clinical challenge despite advances made (3). Importantly, annual cost of PTB was estimated to $26.2

[1] The introduction contains figures, tables, and text sections originally published in:
a) Cytokine & Growth Factor Reviews. Nadeau-Vallée M, Obari D, Quiniou C, Lubell WD, Olson DM, Girard S, Chemtob S. A critical role of interleukin-1 in preterm labor. Cytokine Growth Factor Rev. 2016 Apr;28:37-51. Copyright © [2015] Elsevier Ltd.
b) Reproduction. Nadeau-Vallée M, Obari D, Palacios J, Brien MÈ, Duval C, Chemtob S, Girard S. Sterile inflammation and pregnancy complications: a review. Reproduction. 2016 Dec;152(6):R277-R292. Copyright © [2016] Society for Reproduction and Fertility.
c) Current Pharmaceutical Design. Nadeau-Vallee M, Obari D, Beaudry-Richard A, Sierra EM, Beaulac A, Maurice N, Olson DM, Chemtob S. Preterm Birth and Neonatal Injuries: Importance of Interleukin-1 and Potential of Interleukin-1 Receptor Antagonists. Curr Pharm Des. 2017;23(40):6132-6141 Copyright © [2017] Bentham Science Publishers.
d) University of Ottawa Journal of Medicine. Mai-Vo TA, Nadeau-Vallée M, Beaudry-Richard A. Preterm Birth: An Inflammatory Syndrome, Not Just a Myometrial Disorder. 2017 Dec 18; 7(2):61-69. Copyright © [2017] Thuy-An Mai-Vo, Mathieu Nadeau-Vallée, and Alexandra Beaudry-Richard.

billion (USD) in 2005, and this estimation does not include further health problems that premature infants might suffer (2). Thus, the global burden of PTB on maternal and child health calls for an urgent need to develop effective treatments to reduce the incidence of PTB.

Not all PTBs are equivalent in terms of medical risks. The risk of neonatal morbidity and long-term complications is inversely proportional to gestational age[1] at birth, with severe preterm infants (<28 weeks, accounting for <5%) being the most at risk, followed by very preterm infants (28-$31^{6/7}$ weeks, 20%) and late preterm infants (32-$36^{6/7}$ weeks, 70-80%) (4). About one-quarter of PTBs are iatrogenic deliveries usually due to life-threatening fetal or maternal pregnancy-associated health condition, whereas the remainder are due to spontaneous preterm labor. Risk factors for PTB include low parity, cigarette smoking, use of alcohol and street drugs (especially cocaine), environmental stress, multiple gestation, and poor nutrition. Additionally, women who were born preterm have increased risk to deliver preterm (5). Therefore, undergoing spontaneous preterm labor or developing a pregnancy-associated condition severe enough to force iatrogenic delivery seems to be multifactorial, with aetiologies spanning genetic, social/environmental, epigenetic, and fetal reprogramming factors. Interestingly, the highest reported rates of PTB independent of gestation age at birth are found in the USA, sub-Saharan Africa, and Southeast Asia (4), whereas the highest rate of severe PTB is found in sub-Saharan Africa and Southeast Asia (1).

[1] Gestational age refers to the length of pregnancy from the first day of last menstrual period, whereas conceptional age refers to the length of pregnancy from the date of conception.

## 1.2. Spontaneous preterm birth

As mentioned, spontaneous PTB (as opposed to iatrogenic PTB) represents approximately 75% of PTBs and is the result of spontaneous preterm labor. The onset of labor is a gradual process that begins several days before delivery with changes in gestational tissues, culminating in powerful contractions to expulse the conceptus. Term and preterm labor (i.e. labor before 37 weeks' gestation) share a common (patho)physiological process, including activation of the membranes/decidua (detachment of the chorioamniotic membranes from the decidua and rupture of the membrane), uterine contractility (shift from irregular contractions to functional contractions) and cervical ripening (dilatation and effacement of the cervix due to changes in cervical composition and increasing myometrial contractility) (6). It has been suggested that while term labor is a result of a physiological activation of this pathway, preterm labor is on the other hand the result of a pathological activation of the same process (7-9).

### 1.2.1. Etiology

Many causes of spontaneous PTB have been identified and include infection, fetal growth disorders, ischemia, uterine over-distension, cervical incompetence, fetal and maternal stress, hemorrhage, placental abruptio, and several others (10). For this reason, PTB is not seen as a single disease entity, but is referred to as a syndrome (7, 11). A definitive cause is rarely identified, but based on the predominant health condition, the most prevalent causes are (independent of gestational age): multiple gestation (10.4%), extrauterine infection (7.7%), chorioamnionitis (intrauterine infection; 7.6%), mid/late-pregnancy bleeding (6.2%), and suspected fetal growth restriction (5.8%), with almost the third of PTBs having no predominant

condition identified (12). The most prevalent cause in extreme and very preterm infants is chorioamnionitis, which is found (in placental pathology) in 35.4% at 29-32 weeks, and in as much as 94% at 21-24 weeks (13).

### 1.2.2. Pathophysiology

It is hypothesized that all the different aetiologies share a common final pathway leading to preterm labor, which involves inflammatory processes and uterine activation. Different mechanisms *upstream* of this common pathway have been proposed for each etiology. For instance: a) maternal and fetal stress is thought to trigger preterm labor *via* the excessive release of cortisol which induces the release of placental corticotrophin-releasing hormone (CRH) (in contrast to its inhibitory feedback effect on hypothalamic CRH) in turn acting as an uterotrophin (uterine activator) (14, 15); b) placental abruptio and decidual hemorrhage is thought to induce preterm labor in part *via* intrauterine thrombin activation, which is a potent uterotonin (uterine contractant) *via* protease-activated receptors (PAR) independent of its effect on hemostasis (16-19); and more importantly c) the local release of alarmins in response to sterile stressors and cell injury, and subsequent invasion of the uterus by leukocytes, is probably implicated in most, if not all PTB aetiologies as as been suggested for ischemia and hemorrhage (20-25). However, of all aetiologies identified, only intraamniotic infection has been causally linked to spontaneous PTB, whereas others are mostly based on associations reported by epidemiologic, placental pathologic, or experimental studies (26). During infection, microbial endotoxins activate pattern-recognition receptors (PRRs), a class of phylogenetically conserved receptors ubiquitously expressed by mammalian immune and non-immune cells, leading to nuclear

translocation of nuclear factor-kappa B (NF-κB), amplification and maintenance of uterine inflammation, activation of the common parturition pathway, and onset of spontaneous preterm labor. Further elaboration on the common parturition pathway and on the role of inflammation is presented in Chapter 2.

## 1.2.3. Treatment

Most of the clinically-available therapeutic drugs used for PTB target the myometrium to arrest or delay labor in symptomatic women and are referred to as tocolytics (from the Greek tokos, childbirth; and lytic, dissolving). Tocolytics are used to gain sufficient time for administration of corticosteroids (to accelerate lung maturation and surfactant production) or transport to a tertiary care unit. Because tocolytics have not been shown to improve neonatal outcome, and because they convey limited efficacy to prolong gestation and exhibit numerous side effects, routine use of tocolytics is controversial (27, 28). Only progesterone (and cervical cerclage, a minor surgical procedure wherein the internal cervical os is sewn closed) has been used in asymptomatic (non-laboring) women at risk of PTB to prevent or delay PTB.

### 1.2.3.1. Therapeutic approach for asymptomatic women

To date, only two treatment regimens are recommended for the prevention of PTB: 1) progesterone administered daily *via* vaginal suppository (e.g., Endometrin®; Ferring Pharmaceuticals) from the 24th to the 34th week of gestation in women with a short cervix (trans-vaginal sonographic cervical length of <25 mm) and no history of PTB, and 2) 17α-

hydroxyprogesterone caproate (Makena®; AMAG Pharmaceuticals, Inc) administered weekly *via* intramuscular injection from the 16th to the 36th week of gestation in women with a history of PTB. These recommendations are based on the results of numerous clinical studies, which have shown a reduction in the risk of both PTB and neonatal mortality and morbidity (29-31). In contrast, the more recent and elaborated OPPTIMUM study suggests that the use of progesterone in women at risk of PTB is not associated with a reduction in PTB, or improvement in a composite neonatal outcome (including death, brain injury, or bronchopulmonary dysplasia), or any long-term positive effects on cognitive score at 2 years of age. However, single components of the composite neonatal score, namely neonatal death and neonatal brain injuries, were both significantly (statistically) reduced (32), which would be expected to translate into long-term beneficial effects contrary to what has been observed in the study (33). Correspondingly, the PROLONG study, a multicenter, multinational, placebo-controlled randomized clinical trial of >1700 pregnant women, failed to show efficacy of antenatal progestins to prevent PTB and improve neonatal outcome (34). Further studies are warranted to design effective diagnostics to expand the identifiable population at risk of PTB (current clinical and sonographic criteria only identify approximately 12% of the women at risk (35)). Effective diagnostics could help clarify the role of antenatal progestins or other antenatal therapeutics that are in development. The physiological role of progesterone will be discussed in Chapter 2.

In women with cervical insufficiency, cervical cerclage has been consistently shown to decrease the rate of PTB (36). However this subset of women represents less than 1% of the obstetrical population (37).

### 1.2.3.2. Therapeutic approach for symptomatic women (tocolytics)

Numerous tocolytic agents are used to prolong the gestation of women in spontaneous preterm labor. The most vastly used are ritodrine, nifedipine, atosiban, magnesium sulphate, and indomethacin.

**Ritodrine:** Numerous β-mimetics have been used as tocolytics (e.g. terbutaline, ritodrine, salbutamol), but the most used is ritodrine (28). β-mimetics bind and activate β-adrenergic receptors on myometrial cells. β-adrenergic receptors are $G_s$ protein-coupled receptors and therefore activate adenylyl cyclase-induced production of cAMP, in turn reducing intracellular $Ca^{++}$ levels and promoting the inactivation of myosin light-chain kinase (MLCK; a group of enzymes important for contraction) in myometrial smooth muscle cells (28).

Several randomized control trials and meta-analyses concur to the efficacy of ritodrine to prolong gestation by at least 48h (38, 39). However, there is no evidence for improvement of neonatal outcomes (38). The current rationale for using ritodrine (and other tocolytics) is to gain enough time for corticosteroid action and transfer to a tertiary care facility.

However, because β-adrenergic receptors are widely expressed throughout the human body, ritodrine (and other β-mimetics) have numerous maternal and fetal adverse effects (see Table I). Since other similarly effective tocolytics have been shown to cause less significant side effects, ritodrine is no longer marketed in the USA (28).

**Nifedipine:** Calcium is an essential signal transducer of pro-contractile intracellular targets by binding to and activating calmodulin. The resulting complex activates MLCK, in turn promoting actomyosin interaction and contraction. Nifedipine blocks calcium channels, thereby reducing intracellular calcium levels and reducing actomyosin activity in smooth muscle cells. A meta-analysis published in 2002 showed that if calcium channel blockers are administered

before 34 weeks of gestation, they can prolong gestation by at least 7 days (40). This is a much longer period as compared to β-mimetics. Calcium channel blockers, specifically nifedipine, have also been shown to have fewer side effects and a lower neonatal morbidity rate (41). However, nifedipine is associated with higher rates of adverse effects in women with cardiovascular disease, congenital cardiac malformations or pulmonary hypertension (41).

**Atosiban:** Atosiban was the first drug developed specifically for preterm labor (as opposed to already existing drugs used off-label) and is largely used in Europe. It is the first member of a new class of tocolytics, the oxytocin receptor antagonists. When oxytocin binds to its receptor in the myometrium, it activates the phospholipase C/ inositol 1,4,5-trisphosphate pathway, leading to the release of intracellular calcium which causes contractions. Atosiban inhibits this pathway, thereby preventing myometrial contractions (42).

In a large multi-centre randomized clinical trial, atosiban was found to be as effective as β-mimetics in prolonging gestation, with fewer side effects than β-mimetics (43). However, in a large placebo-controlled randomized clinical trial conducted in the USA, numerous hurdles were encountered. Most significantly, there was biased distribution of pregnant women in the two treatment groups, leading to a markedly higher number of women at low gestational age (< 26 weeks) in the atosiban group. In this subgroup, the mortality was significantly higher than in those treated with β-mimetics. However, in the subgroup that delivered >28 weeks, atosiban was more effective than placebo to prolong gestation (44). Because the data of women that delivered <26 weeks were inconclusive and other reasons, the FDA has not yet approved the use of atosiban. Atosiban is currently the most used tocolytics drug in Europe (28). Numerous new oxytocin receptor antagonists are being considered for acute tocolysis (e.g. barusiban).

**Magnesium sulfate:** Magnesium is a divalent cation that competes with $Ca^{++}$ for: 1) entry into the cell *via* calcium channels, and 2) binding to calmodulin (which precedes MLCK activation). Based on this rationale, magnesium sulphate is used as a tocolytic agent, but it lost in popularity after numerous randomized clinical trials and meta-analyses revealed its inefficacy to prolong gestation, in addition to an increased risk of fetal and neonatal mortality (45). Because of the withdrawal of β-mimetics from the American market and the failure of atosiban to obtain FDA approval, magnesium sulfate has been used extensively in the USA as a first-line tocolytic (28). Magnesium sulfate is still used antenatally for its fetal neuroprotective effect (46); however, this topic is controversial and possibly population-dependent (47).

**Indomethacin:** Indomethacin is a non-steroidal, anti-inflammatory drug that reversibly inhibits cyclo-oxygenase (COX)-2, thereby inhibiting the production of uterotonic prostaglandins. Indomethacin is widely used in Canada for acute tocolysis. Although it has been shown to prolong gestation (48), its prolonged use (>48h) has been associated with severe neonatal complications, including premature closure of ductus arteriosus, renal toxicity, necrotizing enterocolitis, intraventricular hemorrhage, and periventricular leukomalacia (49) . It therefore must be used with utmost caution. There is currently no evidence that indomethacin has any advantage as a first-line tocolytic over calcium channel blockers or oxytocin antagonists, each of which have better side effect profile (50). Treatments for symptomatic and asymptomatic women are detailed in Table I.

Table I. Most used therapeutic molecules for the treatment and prevention of PTB

| **Therapeutic agents** | **Mechanism of action** | **Efficacy** | **Adverse effect profile** | **RCTs[a] and M-As[b]** |
|---|---|---|---|---|
| Ritodrine | $\beta_2$-adrenergic agonist: | - Prolong gestation for 48h vs placebo | High frequency of potentially life- | (38) |

| | | | | |
|---|---|---|---|---|
| | ↑cAMP ($G_s$); ↓ intracellular $Ca^{++}$ ↓ MLCK activation in myocytes | - Elicits no improvement of perinatal outcome | threatening maternal and fetal side effects including: palpitations, tremor, nausea, headaches, and chest pain (51). | |
| Nifedipine | Calcium channel blocker | - More effective than Ritodrine to prolong gestation<br>- Decreases rates of severe neonatal morbidity | Fewer maternal side effects than ritodrine. Includes: flushing, headache, dizziness, nausea, and transient hypotension. Possible neonatal side effects include: tachycardia, hypoglycemia, and hypocalcemia. | (40), (41) |
| Atosiban | Oxytocin receptor antagonist | As effective as Ritodrine to prolong gestation | Lower side-effect profile than ritodrine and most tocolytics. | (45) |
| Magnesium sulphate | -Competes with Ca ++ for entry into the cell; -Blocks calmodulin-induced activation of MLCK | Not more effective than placebo | Lower side-effect profile than most other tocolytic agents. | (43) |
| Indomethacin | COX-2 inhibitor: ↓ $PGF_{2\alpha}$ ↓$PGE_2$ | Insufficient level of evidence for firm conclusions | Major side effects on fetal kidney development and cardiovascular system | (50) |
| Vaginal progesterone prophylaxis | Promotes uterine quiescence | Insufficient level of evidence for firm conclusions | Lower side-effect profile than most tocolytic agents. | (52), (53) |
| 17α-hydroxyprogesterone caproate prophylaxis (synthetic progestin) | Promotes uterine quiescence | Insufficient level of evidence for firm conclusions | Lower side-effect profile than most tocolytic agents. Its use has been associated with increased incidence of gestational diabetes | (54) |

[a]RCTs: randomized control trials
[b]M-As: meta-analyzes

## 1.3. Iatrogenic preterm birth

Iatrogenic PTB refers to medically indicated PTB. In this context, the risks of continuing a pregnancy must be carefully weighed against the risks of prematurity. Indications for iatrogenic delivery include maternal conditions such as preeclampsia, diabetes and heart disease, and fetoplacental conditions such as prolonged (post-term) pregnancy, intrauterine growth restriction, rhesus type incompatibility, preterm premature rupture of membranes (pPROM; rupture of membranes before term) and chorioamnionitis (55). Delivery can be provoked medically with a combination of intravaginal prostaglandins (for cervical ripening) and i.v. oxytocin (for uterine contractility), or surgically by caesarean section. The global incidence of iatrogenic PTB is increasing (56).

Elective delivery refers to the induction of delivery prompted by a request from the patient. Elective delivery accounts for approximately 10-15% of births under 39 weeks of gestation (57), and has been performed as early as 35-37 weeks of gestation (58), despite longstanding recommendations by the American College of Obstetricians and Gynecologists against this practice (59). Therefore, elective delivery accounts for a small part of late PTBs.

## 1.4. Prognosis

Major advances in perinatal care over the last few decades have drastically improved neonatal mortality at the cost of a rise in neonatal morbidity and long-term disabilities related to prematurity, largely attributable to the increased survival of extremely preterm infants. Premature infants are born with immature homeostatic and defense mechanisms and are additionally exposed to relative supraphysiological concentrations of stressors and to numerous

invasive procedures. This includes oxidative stress from oxygen supplementation (or even room air oxygen which is hyperoxic relative to the intrauterine environment); barostress from mechanical ventilation; hypoxia-ischemia from neonatal asphyxia and vascular fragility/immaturity; inflammatory stress; and commonly, infectious stress including sepsis. This storm of stressors likely induces permanent cellular adaptation and reprogramming, as suggested by increased risks of chronic disorders in adulthood following PTB, including of hypertension, diabetes, and obesity (60); chronic lung malfunction (61); and interestingly, increased risk of delivering prematurely (5).

The fetal (and neonatal) brain (especially the white matter) is particularly vulnerable to injury between 20 and 32 weeks after conception. The incidence of cerebral palsy and other adverse neurodevelopmental outcomes increases with decreasing gestational age at birth (62). Correspondingly, of all infants born before 26 weeks of gestation, half of them will suffer from neurological disabilities after 30 months, and a quarter will have severe neurological impairments (63, 64). Hypoxia-ischemia (65) and antenatal inflammation (66-68) both contribute to neonatal brain injury.

The advent of antenatal corticosteroid therapy and exogenous surfactant therapy, and the improvement of neonatal intensive cares, has improved management of neonatal respiratory distress syndrome, which had been the leading cause of infant mortality in preterm infants (69). However, the rate of chronic lung disease, defined as the need for ventilation or oxygen supplementation after 36 weeks of post-conceptional age, has continued to increase. Correspondingly, approximately 1 in 3 infants born prematurely are later hospitalized for a respiratory condition before their first 2 years of life (70), and a similar amount will have evidence of respiratory symptoms, lung function impairments, or radiological abnormalities that

persist until early adulthood (61, 71, 72). Other neonatal conditions associated with prematurity include necrotizing enterocolitis, sepsis, hyperbilirubinemia, and hypoglycemia.

Overall, this corollary suggests that treating premature infants postnatally has its limitations, in part because of the extreme immaturity (and vulnerability) of their organs, and in part because of the abrasive nature of the treatment required to maintain them alive. Neonatal injuries most frequently begin antenatally and therefore the most desirable therapeutic approach is antenatal treatment (73-75). Currently, antenatal treatment of PTB is limited to tocolysis and progesterone. The former targets the myometrium to minimally prolong gestation in a suboptimal intrauterine environment which results in no demonstrable favorable outcome, whereas the latter shows promise to improve neonatal outcome in a specific subset of identifiable women at risk of PTB (which only represents a small percentage of women at risk), although long-term benefits have not yet been clearly demonstrated. None of these treatments address uterine inflammation despite its clear contribution to fetal/neonatal injury.

The next Chapters will elaborate on the role of inflammation in PTB, and on how an antenatal anti-inflammatory therapeutic approach could safely improve the fetal environment, in turn preventing PTB, fetal organ injury, and improving long-term functional outcome.

# 2. The role of inflammation in preterm birth:

## 2.1. Physiopathological and clinical manifestations of inflammation during pregnancy

As mentioned previously, clinical data accumulated to date concur in the inefficacy of tocolytic agents to prolong gestation by > 7 days or to improve neonatal outcome (28). This corollary first suggests that tackling uterine contractions without addressing the underlying physiological cascade resulting in increased myometrial contractility is insufficient to definitively shut down labor; and second, that simply delaying preterm labor does not confer significant protection to the fetus. The latter inferences are supported by: 1) compelling clinical evidence pointing to inflammation as a strong risk factor of neonatal injuries independent of gestational age (76, 77); 2) murine studies showing that neonatal injuries observed with inflammation-triggered PTB are not observed when PTB is induced by non-inflammatory progesterone inhibition (78), and 3) animal studies revealing that inflammatory stimulation initiated at term results in poor neonatal outcomes akin to when inflammation is triggered earlier in gestation eliciting PTB (79, 80). This suggests that it is less the inherent immaturity of organs than rather the burden of stressors to which the fetus/neonate is exposed that really explains neonatal outcome. Additionally and importantly, converging lines of evidences suggest that inflammation plays a significant role in all labors, regardless of the presence of infection, other etiology, or timing of delivery (25, 77). Consequently, an emergent area of research focuses on the development of effective anti-inflammatory therapeutics to prevent and treat PTB and its consequences (81).

Inflammation is an essential physiological process by which tissues respond to insults in order to return to a state of homeostasis. The clinical signs of inflammation are calor (heat), dolor (pain), rubor (redness), tumor (swelling), and as later introduced, function laesa (impaired function) (82), which reflect the effects of cytokines, chemokines, and other mediators on adjacent blood vessels and tissues.

Every birth, whether term or preterm, is associated with inflammatory mediators that promote a feed-forward amplification cascade leading to delivery. In a healthy term birth, inflammatory factors are part of the normal physiological process and are not harmful to mothers or babies. In most spontaneous PTBs, microorganisms or sterile insults including ischemia, tissue injury and necrotic cell death, prematurely trigger a common parturition pathway, in addition to eliciting fetal inflammation and injury.

Some PTBs are associated with the presence of neutrophils in the human fetal membranes (amnion and chorion), a condition termed “acute histological chorioamnionitis.” Acute histological chorioamnionitis (AHCA) is generally not accompanied by an infectious process. Hence, AHCA can only be diagnosed after delivery and is therefore not treated. AHCA is associated with 94% of preterm births at 21-24 weeks of gestation, 10.7% of births at 33-36 weeks, and only 4% of births at term (13).

In contrast to AHCA, clinical chorioamnionitis (CCA), which includes the conditions of preterm premature rupture of membranes and funisitis (13, 83-85) is caused by the invasion of the gravid uterus and amniotic fluids (AF) by bacteria, viruses or parasites that cause an inflammatory response with stimuli termed Pathogen Associated Molecular Patterns (PAMPs) (86). CCA is characterized clinically by maternal fever (temperature >37.8°C) plus two of the

following symptoms: maternal leukocytosis >18,000 cells/cm3, maternal or fetal tachycardia, uterine sensitivity, purulent discharge, or serum C-reactive protein (CRP) >0.8 U/mL. Clinical management of CCA includes administration of antibiotics and delivery of the fetus, because the infectious process can lead to fetal inflammatory response syndrome (FIRS) which causes outright sepsis and risk of fetal and newborn brain white matter damage, chronic lung disease, necrotizing enterocolitis and in the long-term, neurodevelopmental (including cerebral palsy), bronchopulmonary, cardiovascular and growth disorders (83). No fetal organs escape risk from CCA and FIRS. Careful studies have shown however that CCA occurs relatively rarely, in only 2% of term delivery and 5-10% of preterm delivery; reports suggest that CCA is over diagnosed.

## 2.2. The inflammatory cascade leading to preterm labor

Birth reflects transition from a pro-pregnancy state and immunological tolerance towards the fetus allograft, to a pro-labor/pro-inflammatory state. Notwithstanding the role of hormones, pro-inflammatory cytokines are thought to orchestrate the on-time synchronization of myometrial contractility, cervical ripening and membrane weakening through the induction of uterine activation proteins (UAPs, described in the next section) and matrix metalloproteinases (MMPs) (87-89). This converging inflammatory pathway precedes the onset of both term and preterm labor (90, 91).

All aetiologies of PTB converge to a common pathway which inevitably begins with the local or systemic release of Damage (or Danger)-associated molecular patterns (DAMPs, also known as alarmins; e.g. uric acid) or PAMPs (e.g. endotoxins). Whereas DAMPs are produced endogenously and mediate sterile inflammation, PAMPs are produced by pathogens. Both

DAMPs and PAMPs are biomolecules that bind to PRRs, notably Toll-like receptors (TLRs). TLRs are expressed abundantly in the decidua, placenta, and membranes throughout pregnancy, in immune and non-immune cells (92). Their activation leads to a local release of proinflammatory cytokines, chemokines, and products of arachidonic acid, resulting in increased vascular permeability, leukocyte activation and transmigration from peripheral blood to gestational tissues, and amplification of the initial inflammatory response (93). Leukocyte extravasation has been observed in the decidua, the cervix, the placenta, the fetal membranes, and the AF in human and in animal models, and is principally mediated by cytokines and chemokines, including IL-1, IL-6, IL-8 and TNFα (94-101). As more and more leukocytes invade the uterus, the ensuing uterine exudate is enriched in inflammatory proteins (interleukins [IL], complement effectors, kinins) whose concentration increases markedly during the acute inflammatory phase, particularly in the immediate vicinity of myometrial smooth muscle cells, trophoblasts and decidual cells (97-99) (Fig. 1). Of importance, factors such as IL-1, IL-6 and TNFα induce phenotypic changes in myometrial smooth muscle cells to promote contractility and intercellular connectivity. Specifically, the plasma membrane of myometrial smooth muscle cells becomes saturated in uterotonin (pro-contractile) receptors (e.g. oxytocin receptor [OXR], prostaglandin $F_{2\alpha}$ receptor [FP]) and gap junctions (89), concomitant with cytoskeleton changes in cytoplasm (102, 103), which altogether results in a pro-contractile phenotype. Simultaneously, the decidual cells, uterine fibroblasts, resident macrophages and chorion laeve trophoblasts increase their production of MMPs and other extracellular matrix (ECM) proteases (104-106) and decrease their production of tissue inhibitor of metalloproteinases (TIMPs) (107), which favors structural weakening of fetal membranes, while trophoblasts secrete high concentrations of uterotonins to promote contractions and cervical ripening (108). These

inflammation-driven modifications underlie the three hallmarks of uterine preparedness to labor: 1) increased myometrial contractility; 2) cervical ripening; and 3) weakening of fetal membranes (Fig. 2). Correspondingly, several studies have correlated the increase in pro-inflammatory cytokines with the risk of PTB (109-111) and inversely, antenatal inhibition of key mediators of inflammation prevents uterine activation and preterm labor (112-114).

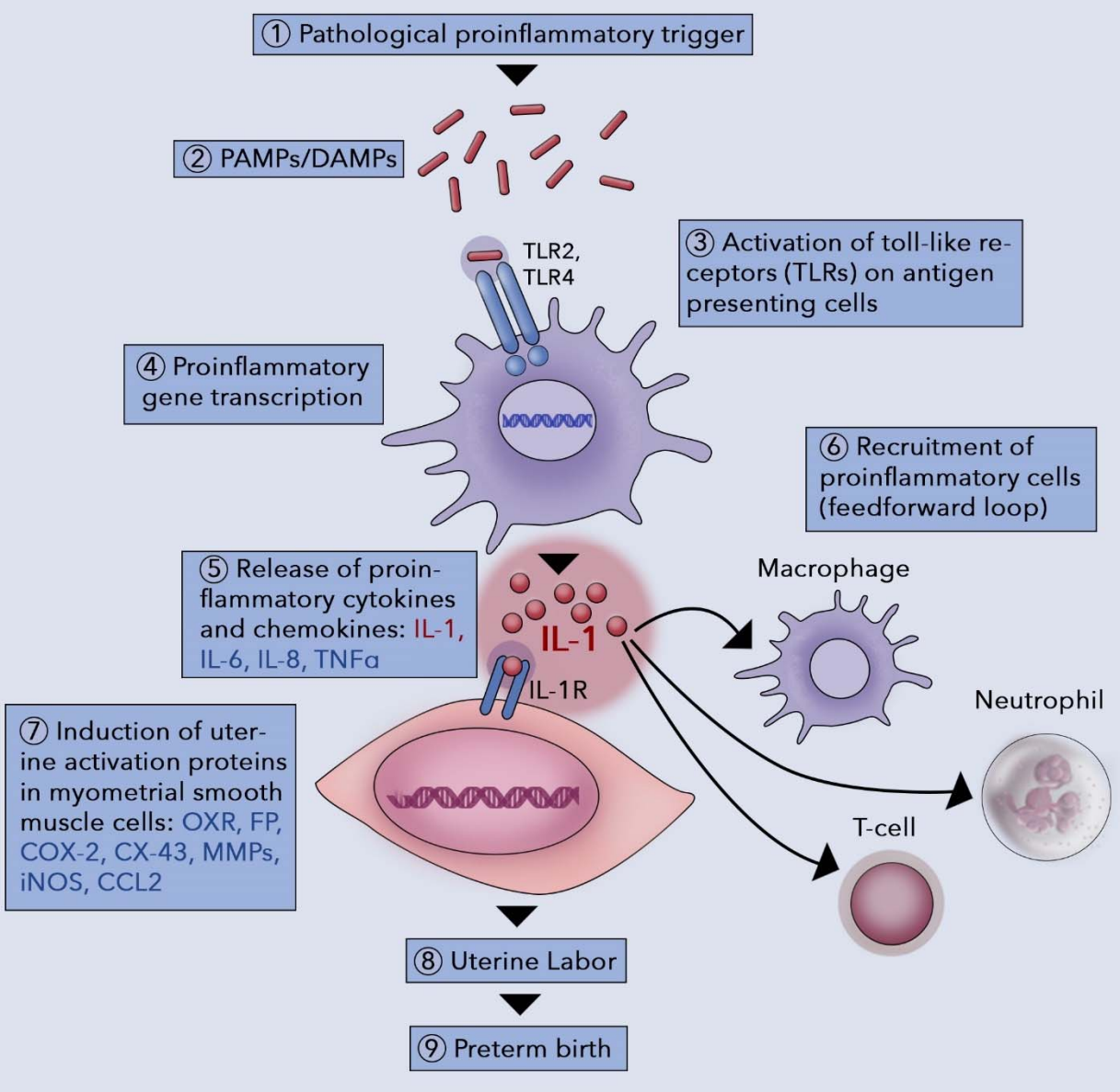

Figure 1. **Proposed common upstream mechanism leading to uterine preparedness and labor**. TLR, toll-like receptor; OXR, oxytocin receptor; FP, prostaglandin $F_{2\alpha}$ receptor; COX-2, cyclooxygenase-2; CX-43, connexin-43; MMP, matrix metalloproteinase; iNOS, inducible nitric oxide synthase; CCL2, chemokine ligand 2.

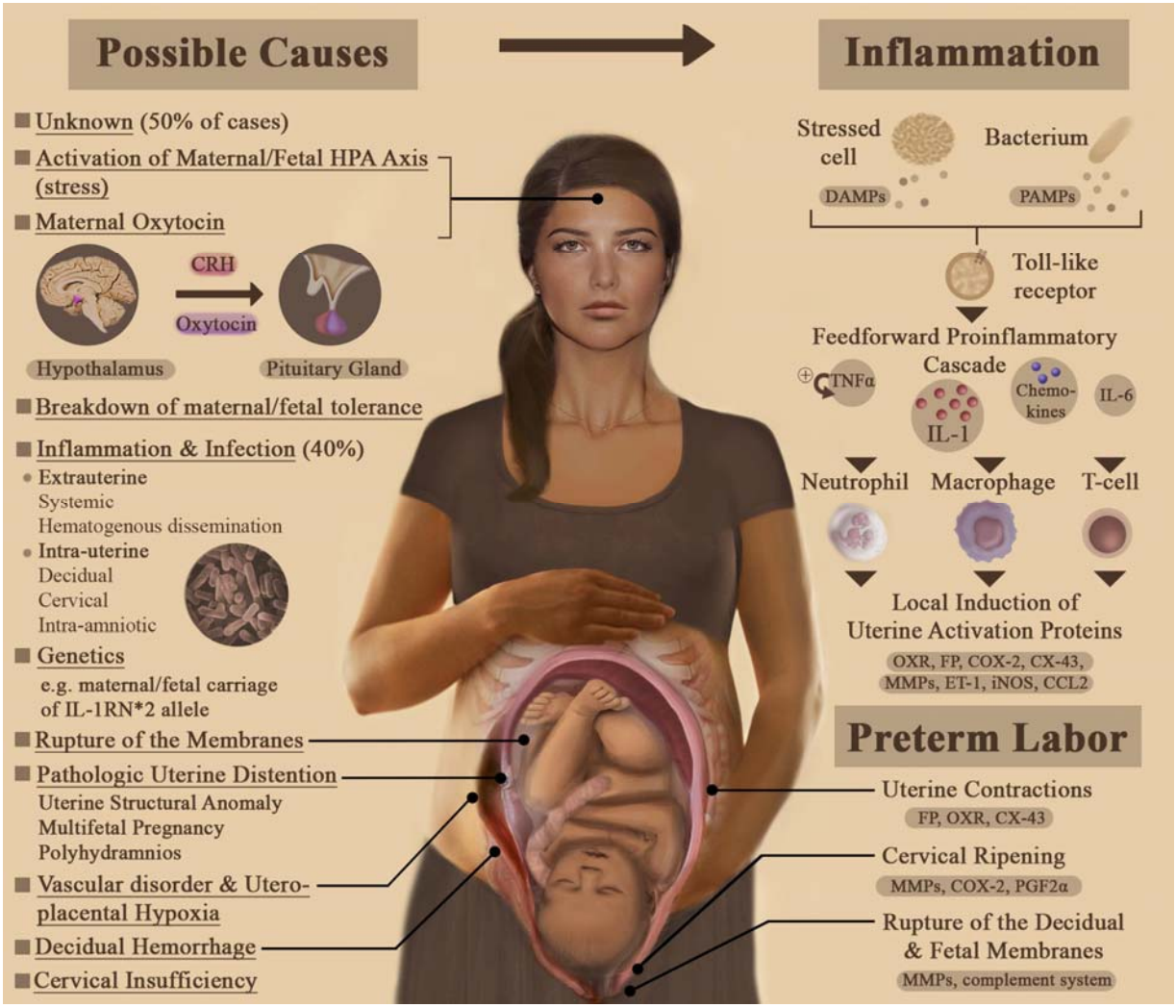


Figure 2. **Inflammatory pathway to preterm labor**. All possible causes of preterm labor (such as infections, genetics, cervical insufficiency…) are thought to invariably lead to an inflammatory cascade wherein cytokines (primarily IL-1) trigger uterine activation and preterm labor. The initial insult may arise from the environment (infection) or can be maternally-produced by stressed cells (alarmins), the former being a source of PAMPs and the latter a source of DAMPs. PAMPs and DAMPs can stimulate the innate immune response by activating Toll-like receptors, which subsequently leads to the production of pro-inflammatory cytokines and chemokines from immune cells (neutrophils, macrophages, T-cells). If this cascade is triggered in (or reaches) the uterus, cytokines (primarily IL-1) can

act *via* their receptors to activate gestational tissue in preparation for labor. This activation is driven by a class of proteins referred to as uterine activation proteins (UAPS; such as OXR, COX-2, MMPs, CX-43…). The induction of UAPs by inflammatory processes increases myometrial contractility and drives cervical ripening and the rupture of membranes, which promotes unscheduled labor onset. CRH, corticotrophin-releasing hormone; ET-1, endothelin-1.

## 2.3. Uterotrophins, uterine activation proteins, and uterotonins

In humans, mice and other viviparous mammals, parturition can be separated into four different phases: 1) uterine quiescence, mediated mainly *via* progesterone and relaxin; 2) uterine activation, mediated by uterine activation proteins (which are upregulated by uterotrophins); 3) active labor, triggered and maintained through uterotonins action; and 4) involution of the uterus (Fig.3).

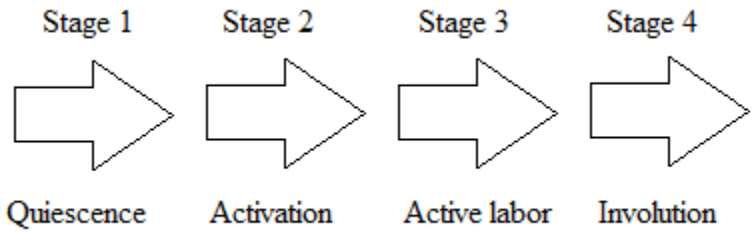


Figure 3. **Depiction of the four stages of the uterus during pregnancy**.

Uterotrophins induce UAPs in gestational tissues. Estrogen, pro-inflammatory cytokines (e.g. IL-1β, IL-6 and TNFα), and (hypothalamic but mostly placental) CRH and are the most important uterotrophins. The action of estrogen is inhibited by progesterone, and therefore progesterone withdrawal amplifies its action. Further, as mentioned before placental CRH is produced in response to increased levels of maternal or fetal cortisol, whereas cytokines are produced in response to DAMPs/PAMPs. Of all uterotrophins, IL-1 exerts a predominant role,

as it governs the induction of the other uterotrophins by promoting progesterone withdrawal and placental CRH release (115-119).

UAPs include enzymes (e.g. COX-2, MMPs), cytokines (e.g. chemokine ligand 2 [CCL2]), uterotonin receptors (e.g. OXR), gap junction proteins (e.g. connexin 43 [CX-43]) and other contraction-associated proteins. As a general principle, the induction of UAPs increases the biological activity of uterotonins, and therefore prepares the uterus for labor. The cardinal features of UAPs are: a) exhibiting increased expression at the onset of (normal and pathological [i.e. preterm]) labor; b) eliciting gestational age-dependent pro-labor effects (i.e. increase in efficacy and potency with gestation age); c) contributing to or being necessary for inflammation-induced PTB; and d) being inducible by uterotrophins (87, 89, 120-124). Although numerous molecules may correspond to this definition, the best described UAPs are CCL2, COX-2, CX-43, FP, and oxytocin receptor. Other candidates include (PAR)-1 (18, 125), neuromedin B receptor (126), neuromedin U receptor 2 (NmU-R2) (127), melatonin receptor type II (128, 129), and iNOS (130).

Uterotonins are a wide family of molecules that initiate and sustain myometrial contractions during labor. Uterotonins include the eicosanoids $PGF_{2\alpha}$ and $PGE_2$, the (neuro)peptides oxytocin, neuromedin S and endothelin-1 (ET-1), the enzyme thrombin, and many other emerging molecules (89, 121). The redundancy in endogenous molecular pathways to initiate labor may explain the inefficacy of current tocolytics, especially atosiban. Inflammation (131, 132) and intrauterine bleeding (133, 134) may lead to the premature release of uterotonins and to spontaneous preterm labor especially in a pre-activated uterus.

To summarize, uterotrophins are molecules that activate the uterus by upregulating UAPs. Once the uterus is activated, uterotonins are much more effective to initiate labor. As one could expect from a physiological mechanism governed by numerous redundant pathways, not all uterotrophins, UAPs and uterotonins are essential for effective (and on-time) delivery. Accordingly, gene knockout murine studies reveal that: a) normal delivery is seen in CRH- (135), IL-1R1- (136), tumor necrosing factor (TNF)-α- (137), COX-1-(138), and oxytocin- (139) devoid mice; b) parturition disturbances (dystocia or delayed [i.e. post-term] delivery) are seen in steroid 5α-reductase- (140), phospholipase A2- (141), IL-6 (142), TLR4- (143), and $PGF_{2\alpha}$ receptor- (144) devoid mice; and c) infertility is observed in estrogen receptor- (145), progesterone receptor (PR)- (146), CYP450 aromatase (CYP19A1)- (147) and COX-2- (148) devoid mice. However, this only relates to physiological term parturition and may not apply to pathological spontaneous preterm labor, inasmuch as blocking one redundant pathway may be sufficient to avoid reaching a key threshold level of pathological preterm uterine stimulation. This is suggested for instance by the efficacy of blocking (non-essential) TNF-α to prevent lipopolysaccharide (LPS; a TLR4 ligand)-induced PTB in mice (165).

## 2.4. Progesterone and inflammation (briefly)

Progesterone maintains human pregnancy (*progest*ation) and its withdrawal induces labor (149). Unlike numerous other species, there is no significant decrease in plasma progesterone preceding the onset of human labor . Rather, human pregnancy culminates with a functional progesterone withdrawal wherein the ratio between PR-A and PR-B increases in the nucleus of myometrial cells, resulting in a progesterone-insensitive uterus (150, 151). New

evidence strongly suggests that myometrial intranuclear stabilisation of PR-A is governed by inflammatory pathways (118). Therefore, human functional progesterone withdrawal represents a relatively downstream (late) event in the common inflammatory cascade leading to PTB. As mentioned previously, antenatal exogenous progestin administration has not been shown to improve neonatal or long-term health outcome (32, 34), likely because progesterone only exerts modest anti-inflammatory effects in the uterus (152).

## 2.5. Important mediators of inflammation implicated in preterm birth

### 2.5.1. TNF-α

TNF-α is an endotoxin-inducible acute-phase cytokine that causes necrosis of tumors *in vitro* (153). TNF-α is pyrogenic, pro-apoptotic, pro-inflammatory, anti-tumorigenic, and pro-cachexia. It is expressed mostly by monocytes/macrophages, T cells and neutrophils, but also by NK cells and mast cells. It has been implicated in the pathogenesis of a wide spectrum of diseases, including sepsis, diabetes, cancer, rheumatoid arthritis, and inflammatory bowel diseases.

TNF-α is a homotrimer that is biologically active under two forms: a transmembrane protein form (154) and a soluble homotrimeric form available through ADAM17-mediated cleavage of the transmembrane protein (155). TNF-α elicits its action *via* two receptors: TNFR-1 (CD120a) and TNFR-2 (CD120b). TNFR1 is ubiquitously expressed whereas TNFR2 is mostly found in cells of the immune system. Activation of TNFRs trigger nuclear translocation

of NF-κB, phosphorylation of JNK, p38, ERK, and recruitment of death signaling molecules such as FADD and caspase-8 (156).

The role of TNF-α in preterm labor is equivocal, in part because of a lack of fundamental animal studies and preclinical studies of anti-TNF agents, and in part because of conflicting evidence. In summary, studies show that: 1) an association between TNF-α (-308G/A) polymorphism and PTB has been found in some studies (157, 158), whereas others could not detect such association (159-161); 2) elevated maternal serum levels of TNF-α have been observed in women undergoing spontaneous preterm labor in some studies (162), whereas baseline levels have been reported in others (163); 3) detectable AF levels of TNF-α are found in >90% women with a positive AF culture, and in <25% of women undergoing preterm labor with a negative AF culture or undergoing labor at term (164); 4) Etanercept, a decoy receptor binding to and ensnaring TNF-α, shows modest efficacy (30% decrease) to prevent shiga toxin type 2-induced PTB (165) and no efficacy to prevent LPS-induced PTB (166), in contrast to the moderate efficacy (50% decrease) of an experimental mouse-specific monoclonal antibody against TNF-α in a murine model of LPS-induced PTB (167); 5) *Il1r1*/*Tnfrsf1a* double-knockout mice are protected against heat-killed *E.coli*-induced PTB (168); 6) TNF-α induces MMPs and suppresses TIMPs in human chorionic cells, in addition to promoting the production of uterotonic $PGE_2$ (169) and amplifying uterine inflammation (167); and 7) intra-amniotic infusion of TNF-α induces preterm labor and delivery in nonhuman primates (170). In the light of this information, one may conclude that TNF-α is sufficient to trigger PTB, but not (by itself) necessary. Accordingly, although the difference in efficacy of the different antagonists may be due to pharmacological properties inherent to each compound, none achieves high efficacy. Additionally, TNF-α is not a consistent marker to predict spontaneous preterm labor, suggesting

that it may only be implicated in a subset of PTBs (e.g. infection). Hence, its role in PTB remains ill-defined. Interestingly, post-natal administration of Etanercept improves outcome in experimental necrotizing enterocolitis (a neonatal condition associated with prematurity) by decreasing intestinal inflammation and oxidative stress (171), suggesting a potential post-natal therapeutic role.

### 2.5.2. Interleukin-6

IL-6 is an endotoxin-inducible acute-phase cytokine, but in contrast to TNF-α and IL-1, IL-6 can display context-dependent pro- and anti-inflammatory properties. IL-6 is produced by almost all immune and non-immune cells, including adipocytes and myocytes (172), and plays numerous role in immune-surveillance, lipid metabolism, insulin resistance, mitochondrial activity, hematopoiesis, and bone homeostasis (172). IL-6 deficiency leads to impaired innate and adaptive immunity to viral, parasitic, and bacterial infection (173-175).

IL-6 cis-signaling refers to the binding of IL-6 to the membrane-bound cytokine α-receptor subunit IL-6R (CD126), which triggers the recruitment of the universally expressed signal-transducing β-receptor subunit gp130 (CD130). This IL-6-IL-6R-gp130 complex signals *via* janus kinases (Jak)/STAT, Akt and Erk (176). In contrasts, IL-6 trans-signaling refers to the binding of IL-6 to a soluble IL-6R (sIL-6R), followed by activation of a membrane-bound gp130 subunit. Because gp130 is ubiquitously expressed, IL-6 trans-signaling widens the range of action of IL-6 particularly as it applies to prolonging T cell-mediated response, supporting recruitment (and apoptosis) of leukocytes, and activating stromal tissues (177).

IL6 is associated with PTB and fetal inflammation: a) increased concentrations of IL-6 in vaginal secretions and AF have reproducibly been reported in women that deliver before term (178, 179); b) *Il6* null mutation delays delivery and expression of UAPs by 24h and is restored to normal upon IL-6 administration (142); c) anti-IL-6R neutralizing antibodies block LPS-induced PTB in mice (114), curb IL-6-stimulated amnion cell $PGE_2$ output (180), and neutralize LPS-induced permeability of fetal blood-brain barrier (181); d) the single nucleotide polymorphism rs1800795 located within the *IL6* promoter results in reduced production of IL-6 and decreased rate of PTB (182); and e) preliminary data from our laboratory show that a single systemic infusion of IL-6 consistently induces labor in mice (Marin Sierra E and Chemtob S, unpublished data). Because its levels drastically increase prior to and during preterm labor, IL-6 has received a marked interest as a possible biomarker to predict PTB (183, 184).

Different therapeutic modalities exist to counteract IL-6 action (177). However, most of them (i.e. the two drugs that are FDA-approved [for rheumatoid arthritis] and most of those in development) consist of monoclonal antibodies which albeit promising in term of efficacy (114), convey unfavorable pharmacokinetic and pharmacodynamic properties for the treatment of acute/subacute PTB, including long-action immunosuppressive undesirable side effects. These issues will be addressed in Chapter 3 and 4.

### 2.5.3. Chemokine ligand 2

Chemokine (C-C motif) ligand-2 (CCL2, also known as monocyte chemoattractant protein 1) is a small (13 kDa) monomeric polypeptide secreted by endothelial cells, fibroblasts, smooth muscle cells, astrocytes, monocytes, macrophages, and dendritic cells (DCs) upon

activation by oxidative stress, growth factors or cytokines such as IL-1 and TNF-α (185-187). It activates C-C chemokine receptor (CCR) 2 and 4 on monocytes primarily, but also on memory T cells and NK cells, to recruit them to the site of inflammation (188, 189). CCR-2-null mice develop normally but are severely immuno-compromised (190, 191). CCL2 plays a role in innumerable diseases including cancer, atherosclerosis, multiple sclerosis, infection including by human immunodeficiency virus (HIV) and *mycobacterium tuberculosis*, recurrent miscarriage, and many others (192).

Macrophages are major producers of cytokines in preparation to labor (99, 101). Correspondingly, premature infiltration of gestational tissues by macrophages heralds the onset of preterm labor (101, 193). Therefore, CCL2 is a major player in parturition. CCL2 is upregulated in human AF and choriodecidua during preterm labor independent of infection (194, 195), and in rat myometrium during labor (196), in mouse decidua in term labor, as well as in RU486- (mifepristone; a progesterone receptor antagonist) and LPS-induced preterm labor (197). Upregulation of CCL2 is rapidly followed by infiltration of gestational tissues by myeloid cells (197). The release of CCL2 seems to be controlled by both progesterone withdrawal and uterine stretch, independently of each other (196). Blocking CCL2 with specific inhibitors has been shown to improve outcome in murine models of steatohepatitis (198) and lupus nephritis (199), suggesting an important anti-inflammatory therapeutic potential. To our knowledge, specific CCL2 antagonists have not been tested for the prevention of PTB. However, a recent murine study showed that administration of a broad spectrum of chemokine inhibitors significantly decreased neutrophil infiltration in the myometrium, and in turn reduced PTB induced by LPS (200). Further studies are needed to establish the potential therapeutic benefit of anti-CCL2 therapies.

### 2.5.4. Interleukin-1

Interleukin-1 (also known as leukocytic pyrogen and lymphocyte activating factor) exists under two distinct forms, IL-1α and IL-1β, which bind the same receptors. Once formed, the IL-1-IL-1R complex recruits membrane-bound IL-1 Receptor accessory protein (IL-1RacP) which in turn leads to the nuclear translocation of NF-κB and AP-1, two major pro-inflammatory transcription factors (201-205). These pathways will be further elaborated in Chapter 3.

Because IL-1R is ubiquitously expressed, IL-1 has a broad range of action. Once reaching inflammatory concentrations, IL-1 acts on immune cells to induce activation, proliferation, differentiation, chemotaxis, phagocytosis, and cytokine production (including itself, and TNF-α and IL-6); it promotes hematopoiesis particularly neutrophilia and thrombocytosis; production of acute-phase proteins and cortisol; cartilage degradation and bone resorption; fever; fatigue (and decreased REM sleep); and anorexia (206, 207). It is therefore a major player in mounting and orchestrating an acute inflammatory response in response to both infectious and sterile stressors. Correspondingly, mutations eliciting IL-1 hypersecretion or IL-1 receptor antagonist (IL-1Ra) deficiency lead to autoinflammatory diseases (208).

Of all cytokine candidates, IL-1 stands out as a major player in PTB and fetal inflammation. This applies to both sterile (mostly *via* IL-1α) and microbial inflammation independently of the aetiology and timing of birth (209-211). Due to the ubiquitous expression of its cognate receptor IL-1R1, IL-1 exerts a wide range of effects during gestation, ranging from premature uterine activation to marked fetal brain cytotoxicity. Although some beneficial effects have been described, studies concur to a detrimental role of IL-1 in both PTB and

neonatal morbidity (209, 210, 212-214), which is also supported by the higher levels of IL-1 in human cervicovaginal fluids (215) and AF (211) of patients delivering preterm.

Since the discovery that IL-1 expression rises in term deliveries without infection (216) as well as in preterm deliveries (209), it is thought that IL-1 overproduction heralds term and preterm labor. Not only does IL-1 induce labor in various animal species (170, 217), but also fetal and maternal carriers of polymorphisms in genes of the IL-1 system are associated with PTB in humans (218-220). Furthermore, elevated IL-1β blood concentrations in human neonates are associated with PTB and adverse neonatal outcome (221, 222) and post-natal anti-IL-1 therapy protects against antenatal LPS-induced neonatal brain injury (68) and experimental bronchopulmonary dysplasia (214). Taken together, this body of evidence suggests that uterine and fetal/neonatal inflammation are both governed by IL-1. For these reasons and others (addressed in Chapter 3), IL-1 is now considered a key inducer of inflammation in PTB.

## 2.6. Infections associated with preterm birth

### 2.6.1. Bacterial infections

Bacterial infection within the uterus is termed chorioamnionitis when it occurs between the decidua and the fetal membranes or within the fetal membranes; villitis when it occurs within the placental villi (rare, mostly found in cases of malaria); amnionitis when infection reaches AF, and funisitis when bacteria are found within the umbilical cord. Bacteria can also be found within the fetus and cause neonatal brain injury independent of the timing of delivery (223).

Bacteria gain access to the uterine cavity through: a) migration from the abdominal cavity into the salpinges; b) needle contamination during chorionic-villus sampling or amniocentesis; c) hematogenous spread into the placenta *via* spiral arteries; or d) vaginal ascending pathway (224). Because organisms isolated from the amniotic cavity are similar from those found in the lower genital tract, the latter is considered the most frequent route of infection (11). In this case, bacteria can either ascend through the choriodecidual space or directly cross intact membranes (224, 225).

The bacteria that are most commonly isolated in women in spontaneous preterm labor with intact membranes are *Ureaplasma urealyticum* and *Mycoplasma hominis* (both from the family mycoplasmataceae [small-sized, cell wall-free]), *Gardnerella vaginalis* (Gram-variable, facultative anaerobic coccobacillus), peptostreptococcus species (Gram-positive anaerobic bacteria), and bacteroides species (Gram-negative anaerobic bacteria). On the other hand, group B streptococci and *Escherichia coli* are the most commonly found bacteria in women with pPROM (226-228). On rare occasions, *Neisseria gonorrhoeae, Chlamydia trachomatis*, *Treponema pallidum* (syphilis), and bacteria from the oral microflora are isolated. The presence of bacterial vaginosis, defined as a decrease in lactobacillus species concomitant to an increase in other organisms, doubles the risk of PTB (229, 230). Interestingly, recent studies suggest that the human placenta is not sterile, but rather harbors a unique microbiota with similar flora to the oral community, and that chorioamnionitis and funisitis alters the placental microbiota and predispose to PTB independently of antibiotic treatment (231, 232).

Antibiotics have long been considered as potential treatment for PTB. For women with intact membranes and with symptoms of preterm labor, antibiotics do not delay preterm delivery, do not reduce the risk of preterm delivery, and do not elicit any improvement of

neonatal outcome (233). As alluded earlier, the failure of antibiotics to prolong gestation is likely due to their administration too late in the infectious process, when bacteria have already reached the uterine cavity wherein their demise only contributes to the massive release of pro-labor PAMPs upon which antibiotics have no effects. Of all PAMPs released during colonization, endotoxins (constituents of bacterial walls) are the major stimuli in infection-associated PTB (234, 235). Correspondingly, most models of intrauterine infection employ exogenous endotoxins (e.g. LPS, lipoteichoic acid [LTA; a TLR2 agonist]) or heat-killed bacteria, because they are as effective as live bacteria to induce PTB (236). There are two exceptional clinical settings in which antibiotics are effective to prevent PTB. First, in a subset of women with a history of PTB and with bacterial vaginosis diagnosed in the second trimester, treatment for at least 7 days with metronidazole, and perhaps with erythromycin, results in a marked reduction of PTB (237), again possibly because this intervention precedes and prevents significant choriodecidual colonization. Nevertheless, there is little to no benefit to screening pregnant women for bacterial vaginosis especially in low-risk populations, and therefore only symptomatic women (i.e. women with persistent and bothersome vaginal discharge and odor) may benefit from this treatment, whereas others (>75%) are unlikely to be identified (238). There is currently no consensus regarding screening and treatment of bacterial vaginosis during pregnancy. Second, antibiotics treatment for syphilis results in positive outcomes for both the mother and her baby (i.e. prevention of vertical transmission, stillbirth, and PTB); therefore, screening for syphilis is offered to all pregnant woman and confirmed cases are treated (239). Syphilis infection during pregnancy is rare (less than 1 case per 100 000 live births per year).

Animal studies suggest that cytokines are essential mediators of intrauterine bacterial endotoxins (114, 167, 240) and may therefore represent more effective targets than bacteria *per*

*se*. TLR4 antagonists have also been used effectively to prevent LPS-induced PTB in mice (112), but given the phylogenetically-demonstrated importance of TLRs in innate immune defense in addition to established susceptibility of $TLR4^{-/-}$ mice to gram(-) sepsis, anti-TLR agents may not be safe to administer during pregnancy.

### 2.6.2. Viral infections

Maternal viral infection during pregnancy may have two outstanding consequences to fetal health: 1) mother-to-child transmission; and 2) adverse perinatal outcomes. HIV is of particular importance especially in developing countries where access to anti-retroviral drugs is limited. HIV-positive women are approximately 4 times more likely to deliver prematurely and to give birth to low birth weight newborns (241, 242), and this risk grows with elevation of plasma markers of inflammation (243, 244). Data from longitudinal studies show that the prevalence of PTB and low birth weight in HIV-exposed infants drastically decreased in the era of maternal anti-retroviral drugs (245). Rubella infection during pregnancy, especially during the 1st trimester, causes spontaneous abortion or PTB, and serious congenital anomalies in almost all surviving infants (246). There is no effective treatment. Although mass immunizations campaigns have largely decreased the susceptible population in developed countries, Rubella infection remains endemic in the developing world (247). Further, Ebola virus, a major cause of maternal and neonatal mortality, was until recently (2017) almost universally lethal to infected newborns (248, 249). Chronic hepatitis B virus (HBV) infection also increases the risk of PTB (250, 251).

Other viruses have been implicated more sparsely in PTB. Although viral DNA from adenovirus, cytomegalovirus (CMV), Epstein-Barr virus (EBV), enterovirus and respiratory syncytial virus (RSV) is commonly found in AF samples of anatomically normal fetuses from asymptomatic women, particularly during summer and late winter (252), there is a body of evidence pointing to an association between AHCA/PTB and placental infection by adenovirus (253), CMV (254, 255), herpes simplex virus (HSV) (256), and EBV (257-259). Severe pandemic influenza A (H1N1) has also been associated with PTB and low birth weight (260). Recently, an association between PTB and higher vaginal viral diversity (i.e. richer DNA virome) independent of the strain was discovered, with the highest rate of PTB in women with high levels of vaginal diversity in both viruses and bacteria (261). Hence, viruses are increasingly recognized as contributors to PTB worldwide.

The pathogenesis of virus-induced inflammation and PTB is intricate. Two theories co-exist to date. First, viruses can activate PRRs (including TLR-2,3,9) in placenta and mount an inflammatory response resulting in PTB (262). Concordantly, intrauterine (263) and intraperitoneal (264) administration of polyinosinic:cytidylic acid [poly(I:C)], a synthetic analog of double-stranded RNA (which is a replication intermediate in the life cycle of most viruses), induces intrauterine cytokine production and PTB in mice *via* TLR3. Second, viral infection during pregnancy may predispose to other infections by altering immune-tolerance (265). This is suggested by increased rate of LPS-induced PTB and neonatal adverse outcomes in mice exposed to gammaherpesvirus 68, independent of transplacental virus passage (266, 267). Noteworthy, both pathways involve cytokine production.

The placenta exerts a crucial role in protecting the fetus from viral infection (268) and accordingly, transplacental viral passage is a rare event (269). The reason why viruses reach the

AF in some pregnancies and do not in others is still unclear, but this may be associated to viral load, timing of infection and virulence of the strain implicated, as some viruses have been shown to induce trophoblast death (*via* apoptosis) suggesting placental dysfunction and transplacental passage susceptibility (270). The link between exposure to virulent viruses (e.g. CMV, EBV, HSV) during pregnancy and adverse neonatal outcome, especially brain injury, is clearly established (271-273). Interestingly, exposure to viruses during pregnancy is highly investigated as a possible initiator of fetal reprogramming sequences underlying the onset of numerous children- and adult-onset diseases including schizophrenia (274), depression (275), childhood leukemia (276), and Parkinson's disease (277). Despite the increasingly recognized burden of viral infections in pregnancy, there is no consensus regarding antenatal routine screening and treatment of most of these viruses (e.g. CMV, HSV). Recommendations exist regarding the routine screening of rubella (to identify women at risk and instigate preventive measures including post-natal maternal vaccination), HBV (to prevent vertical transmission by administering post-natal vaccination and immunoglobulins to the newborn) and HIV (to prevent vertical transmission by administering antenatal antiretroviral therapy). Antenatal treatment of HIV reduces PTB (245), which encourages the development of other antiviral agents to treat this subset of PTBs.

### 2.6.3. Parasitic infections

Because of the relative state of immunosuppression conveyed by pregnancy, parasitic infections are more common in pregnant women (278). Living in or travelling to an endemic area prior to or during pregnancy increases the risk of contracting a parasitic infection during pregnancy.

Infection with malaria parasites (279), amoeba (279), flagellated protozoan parasites including *Trichomonas vaginalis* (280) and *leishmania* (281), toxoplasma gondii (282), and fish-borne intestinal nematodes (283) all increase the risk of PTB and adverse neonatal outcome. Malaria is of particular importance because the malaria parasites exhibit affinity for red blood cells and placental tissue, and therefore are a major cause of anemia, intrauterine growth restriction, low birth weight, and long-term adverse pediatric outcomes in endemic areas (284, 285), with a prevalence in pregnant women ranging from 10 to as much as 65% (286). Erythrocytes infected with *Plasmodium falciparum* – the species responsible for millions of deaths annually – can sequester in the microvasculature of organs such as the placenta, potentially leading to vascular obstruction, tissue hypoxia, and placental dysfunction (287, 288). Interestingly, maternal helminth infection has been associated with lower cognitive scores during infancy (289), suggesting that being the host of intestinal parasites during pregnancy may also cause long-term complications for children.

Parasitic infections typically induce a type 2 immune response characterized by the production of IL-4, 5, 9, 10 and 13 by T helper 2 lymphocytes (290), but other pathways may also be involved, such as TLRs 2, 3 and 9, especially as it applies to malaria parasites (288). Accordingly, increased production of IL-1β, TNF-α and a panel of other cytokines associated with a type 1 immune response have been observed in children and adults infected with malaria parasites (291). Nevertheless, how parasites induce PTB remains unresolved.

## 2.7. Sterile inflammation: alarmins implicated in preterm birth

Many of the components of the inflammatory cascade leading to premature uterine activation, if not all, can be triggered in absence of microbial stimulation. Along these lines, although infection constitutes a defined aetiology of PTB, most women in preterm labor do not meet the clinical/pathological criteria for the diagnosis of AHCA or CCA. Accordingly, sterile intra-amniotic inflammation is observed significantly more often than microbial-associated intra-amniotic inflammation in patients with preterm labor and intact membranes (25), and as mentioned above antibiotics are vastly ineffective to prevent preterm labor (28), suggesting a predominant pro-labor effect of inflammation (self) over infection (non-self). Additionally, DAMPs are elevated in placentas (292, 293) and blood (209, 294-297) of women with high risk pregnancy, and sterile administration of such DAMPs has been shown to induce intrauterine inflammation, PTB, and fetal demise (23, 217, 298, 299); of relevance, the effects of DAMPs are mediated to a significant extent *via* pro-inflammatory cytokines. For these reasons and others, sterile inflammation has been considered as a potential initiating or early event in the cascade to PTB and neonatal injuries.

Alarmins are released as a result of sterile tissue stress (including tissue injury, hypoxia/ischemia, and cellular senescence) and trigger an inflammatory cascade *via* PRRs and other receptors including RAGE and IL-1R. PRRs include TLRs 1-11, scavenger receptors, C-type lectins, and NOD-like receptors (NLRs), and are expressed abundantly in decidua, placenta, membranes and myometrium throughout pregnancy, in immune and non-immune cells (92, 300, 301). Therefore, the uteroplacental compartment is a sensor of ″danger″ and ″stranger″ inflammatory stressors.

Because DAMPs are endogenous intracellular molecules primarily released as a result of non-programmed cell death to convey danger cues in the first few hours of an injury, they are also referred to as alarmins (302). Of all candidate alarmins studied in the context of spontaneous PTB, high mobility group box 1 (HMGB1), uric acid, IL-1α and cell-free fetal DNA (cffDNA) are predominant (Fig. 4). Their mechanism of action and respective role in PTB will be presented sequentially.

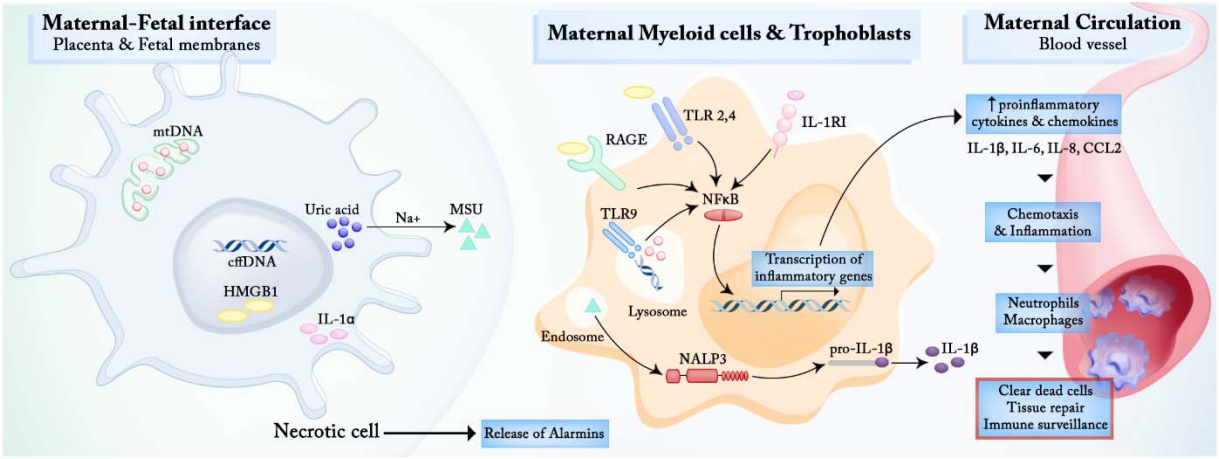


Figure 4. **Mechanism of action of HMGB1, uric acid, IL-1α and cell-free DNA at the maternal-fetal interface.** The general mechanism of action of alarmins at the maternal–fetal interface is shown, with alarmins (uric acid/MSU, HMGB1, cffDNA and IL-1α) being released from cells of the maternal–fetal interface (i.e., placenta and fetal membranes) following a stimulus or necrosis. They act on placental cells (primarily trophoblasts) and maternal myeloid cells to induce an inflammatory response. The inflammatory cascade leads to the secretion/release of cytokines/chemokines, which stimulate the recruitment of immune cells from the maternal circulation.

## 2.7.1. HMGB1

HMGB1 is a highly conserved non-histone protein (25 kDa) with cytokine-like activity in the extracellular space. HMGB1 is abundantly and ubiquitously expressed in nucleus where

it plays a role in DNA replication, transcription and repair, and nucleosome stabilization (303-305). HMGB1 is structured into two DNA-binding domains, HMG box A and B and an aspartic and glutamic acids-rich C-terminal tail. Although originally discovered in nucleus, HMGB1 is also found in cytosol, mitochondria and on membrane surface, and can be released to the extracellular milieu through active (secretion) and passive pathways (306): (i) active pathways are triggered by pathogenic products (e.g. bacteria, viruses) or other stressors (e.g. oxidative stress, cytokines), which has been shown in immune cells and non-immune cells (307-309); whereas (ii) passive release is observed following tissue injury and cell death, especially necrosis (310) and in specific cases of apoptosis (311) – including when triggered by sterile injury events (e.g. hypoxia, senescence, autoimmune disease). The latter happens immediately (310), whereas the former is a slower mechanism mediated by cellular signal transduction (312). Once HMGB1 accumulates in the extracellular milieu, it conveys danger signals by triggering inflammatory pathways, including NF-κB, ERK and p38, in neighboring cells *via* numerous cell-surface receptors such as TLRs 2, 4 and 9, RAGE, CD24 and others (313). This leads to the activation of innate and adaptive immunity, cytokine, chemokine, and metalloproteases release and ensued pro-migration, pro-inflammatory outcomes (310, 314-316). Of interest, HMGB1 has also been shown to form complexes with many pro-inflammatory mediators and enhance their respective actions in a synergistic manner (317). Furthermore, HMGB1 levels are elevated in multiple animal models of sterile injurious events (312), and in humans with acute organ injury, autoimmune diseases or cancer (318, 319). *In vitro* and *in vivo*, HMGB1 administration induces inflammation (320) and inversely, HMGB1 antagonism protects against sepsis (321). This evidence highlights a critical alarmin role of HMGB1 as an endogenous sterile driver of inflammation.

### 2.7.2. cell-free DNA

Circulating cell-free DNA refers to double-stranded, cell-unbound DNA fragments in the blood of humans. Cell-free DNA originates from genomic or mitochondrial DNA released subsequently to cell death. Cell-free DNA is present in small amount in the blood of healthy individuals, but its concentration is increased in patients suffering from chronic diseases. In this context, studies suggest that it acts as a contributor to chronic diseases by inducing inflammation *via* TLR9, a PRR classically activated by unmethylated CpG motifs-containing bacterial and viral DNA fragments (322-324). Mitochondrial DNA also triggers TLR9 to induce inflammation (325).

Circulating blood of pregnant women contains an additional type of cell-free DNA, referred to as cell-free fetal DNA (cffDNA) which originates from the placenta. Evidence for the placental origin of cffDNA include: 1) it is detected in anembryonic gestation (326); 2) it is still detected after therapeutic abortion in which placenta is incompletely removed, albeit undetectable after normal delivery (327, 328); 3) it is detected in cases of invasive placenta, a postpartum pregnancy complication wherein trophoblasts invade myometrium (329); and 4) it carries the placental genotype in patients with confined placental mosaicism (330). In contrast with maternal cell-free DNA, of which 32% of the fragments are >356 bp, cffDNA are short hypomethylated fragments (<313 bp) and potent inducer of sterile inflammation (23, 331, 332). The release of cffDNA is a physiological process present in all mammals, but its possible roles and implications in normal pregnancy (and more importantly parturition) remain poorly understood. Placental growth involves proliferation, differentiation, and syncytial fusion of

cytotrophoblasts which is associated with significant release (grams per day) of microvesicles-encapsulated, cffDNA-containing apoptotic trophoblasts content into maternal circulation (333-337). These microparticles, also referred to as syncytiotrophoblast microvesicles (SCTMs), were first described more than 100 years ago in lung capillaries of women who died from preeclampsia (338), and were later described as a feature of normal pregnancy, though increased in preeclampsia (339). SCTMs as well as cffDNA alone are pro-inflammatory (340-342). Interestingly, once pregnancy is past 20 weeks, the levels of cffDNA in maternal circulation consistently increase of 1% per additional week of gestation to abruptly rise (up to 13 folds) when gestation nears the end (343-345). This evidence, along with the established pro-inflammatory effects of cffDNA, underpins the theory that cffDNA may represent a common trigger to parturition in mammals (346). Furthermore, elevated cffDNA in the maternal circulation has been observed in pathological pregnancies (292, 347, 348) in association with placental dysfunction and inflammation. For these reasons and others, cffDNA is increasingly used for diagnostic purposes to decrease the use of invasive amniocentesis.

Mechanistically, cffDNA can bind to TLR9 to induce a conformational change in the homodimers of the receptor resulting in the close apposition of the Toll/Interleukin-1R (TIR) signaling domains and downstream activation of NF-κB and transcription of inflammatory cytokines genes (349). Importantly, this TLR9-, NF-κB-dependent pro-inflammatory effect of cffDNA was shown in pregnant mice and is characterized by IL-6 production in human peripheral blood mononuclear cells (23). Classically, TLR9 is localized intracellular in endoplasmic reticulum (ER), endosomes, and lysosomes (350). Therefore, cffDNA must be transported by endocytosis inside immune cells in order to convey inflammatory effects *via* TLR9; this is likely occurring through phagocytosis of cffDNA-containing SCTMs by placental

or circulating granulocytes. Given the half-life of cffDNA (16.3 minutes in humans) (327), this inflammatory stimulation is short-lived, but likely sustained by unabated trophoblast turnover.

### 2.7.3. Uric acid

Uric acid (160 Da) is a product of the metabolic breakdown of purine nucleotides by xanthine oxidase, with normal blood concentration range between 40-60 μg/ml. Upon achieving concentrations >70 μg/ml, uric acid forms needle-like, immunostimulatory monosodium urate (MSU) crystals, which cause the acute inflammation of gout. In the last few years, uric acid has been vastly regarded as an alarmin of sterile inflammation because of the high cytosolic concentration (≈ 4 mg/ml) released upon cell death, which reacts with extracellular sodium to form MSU in the immediate vicinity of cellular injury (351). Transport of MSU inside antigen-presenting cells through phagocytosis promotes its interaction with NLRP3 inflammasome and induces IL-1β maturation and release thereby triggering an inflammatory response (352, 353). This is an important step in sterile inflammation that enables immune cells to sense injuries. Concordantly, administration of MSU causes acute inflammation (354) and in mice, blocking uric acid is sufficient to inhibit the immunological and inflammatory response associated with cellular death or injury in numerous cell types and tissues (351, 355).

### 2.7.4. Interleukin-1 alpha

The interleukin-1 family comprises 11 cytokines which regulate inflammatory response to injuries and stressors. Two major members of the family are IL-1α and IL-1β. They bind to

ubiquitous IL-1R1 to activate the translocation of transcription factors NF-κB and AP-1, thereby triggering the expression of numerous cytokines including itself and initiating or sustaining an inflammatory response (356, 357). Although IL-1α and IL-1β bind to the same receptor and convey similar biological effect, the two cytokines are encoded by different genes and have distinct mode of action. Unlike IL-1β, IL-1α is not actively secreted but instead translocates to the nucleus to participate in the regulation of gene transcription (358). Furthermore, while IL-1β precursor requires exogenous (or endogenous in rare cases) signals to trigger its transcription and to initiate its inflammasome-dependent cleavage into a functional cytokine, IL-1α precursor is on the other hand ubiquitously expressed in cytoplasm of healthy cells, in the form of a biologically active precursor (but can also be induced by inflammatory stimuli). Consequently, only IL-1α is released in a functional form upon necrosis (207, 359); therefore, IL-1α is regarded as an alarmin whereas IL-1β is not (360, 361). Accordingly, sterile cell death-induced neutrophil inflammatory response in mice requires both IL-1α and IL-1R, but not IL-1β (362), suggesting that IL-1β is not essential for the mounting of a functional sterile inflammatory response to cell death. However, evidence shows that both IL-1α and IL-1β are implicated in sterile inflammation, but have distinct timing of effect and roles (363), suggesting that IL-1β contributes to sterile inflammation, not as an initiator but as a redundant mechanism to amplify the initial trigger. Accordingly, it is documented that IL-1β can be produced to contribute to sterile inflammation in response to non-cytotoxic sterile stressors as those released upon necrosis (361). This was also recently reported for IL-1α (364). Interestingly, the release of IL-1α is tightly regulated during programmed cell death *via* chromatin sequestration, which significantly reduces its pro-inflammatory effect during apoptosis; this is not observed during necrosis (365). These data suggest a critical role of IL-1α in sterile inflammation, and a

contributive, albeit non-essential role of IL-1β. The major role of IL-1α in sterile inflammation has been reviewed elsewhere (361).

Notably, IL-1α is also detected in activated monocytes as a membrane-bound form. This form does not require cell lysis to become available for receptor binding. Binding of either the membrane form (through cell-cell contact) or the released form to the IL-1 receptor, will result in the initiation of an inflammatory cascade (366-368).

### 2.7.5. Other potential alarmins

Different levels of evidence have been accumulated suggesting that many other intracellular factors can induce acute inflammation once released in their environment and therefore may represent potential alarmins. These include S100 proteins (369, 370), nucleosomes (371), purines (372), sIL-6R (172), heat-shock proteins (373), saturated fatty acids (374) and antimicrobial peptides (375-377). Interestingly, possible alarmin activity has been reported for molecules of mitochondrial provenance such as mitochondrial DNA (325), *N*-formylated mitochondrial peptides (378) and others (379), which could arise from their probable prokaryote origin. Noteworthy, the role and mode of action of the aforementioned candidates *in vivo* are mostly unknown. Along these lines, the possible alarmin activity of heat-shock proteins is still debated. Early studies have shown that purified HSPs activate DCs *ex vivo* (373) and *in vivo* (380) to trigger an inflammatory response. This pro-inflammatory effect has latter been attributed to bacterial contaminant (381, 382) and the enthusiasm of a possible alarmin role of HSPs was consequently severely dampened.

By definition, any pro-inflammatory endogenous molecule physiologically expressed in low concentrations in the extracellular milieu, that is upregulated and released during pathological events could be considered as an alarmin candidate, and therefore many other mediators could potentially be included in this category, such as glucose (383).

### 2.7.6. Role of alarmins in the onset of spontaneous preterm labor

Although most of data linking a rise in alarmin levels and the onset of preterm labor are correlational, causal, and mechanistic data have also been documented, especially for cffDNA and HMGB1. First, administration of cffDNA was found to induce placental inflammation and fetal resorption *via* TLR9 when injected i.p. in pregnant mice, contrastingly with the lack of effects of adult DNA (23). This suggests that high levels of circulating cffDNA, as achieved in women with preterm or term labor (referenced above), can trigger pathological inflammation in gestational tissue *via* TLR9. Accordingly, hypomethylated CpG fragments, the TLR9-responsive element in cffDNA, have been found to induce prompt (24-48h) leukocyte migration to uterus, TNFα production, and preterm labor/birth in IL-10-deficient mice (384). Second, recent evidence shows that stimulation with HMGB1 induces the expression of uterine activation genes including *Tnfa*, *Il6* and *Pghs2* in human myocytes (24), and labor in mice when administered intra-amnion (298); and correspondingly, an association between high HMGB1 amniotic levels and earlier deliveries in patients with intra-amniotic sterile inflammation has been reported (25, 385). Furthermore, HMGB1 administration *ex vivo* in human fetal membranes induces p38-mediated IL-6 and IL-8 production (386). In this setting, a potential role in labor for the HMGB1 pathway was reported using transcriptomics and bioinformatics

analysis (24, 387). Additionally, HMGB1 and its receptors RAGE, TLR2 and TLR4 are found in cervix, and extranuclear fraction of HMGB1 increases with labor onset at term and preterm (21), suggesting that HMGB1 may play a role in cervical ripening. Interestingly, stimulation with endotoxins triggers HMGB1 expression and release *in vitro* in human fetal membranes (386) and *in vivo* in murine fetuses when endotoxins are administered in dams (i.p.) (20); concordant, women with intra-amniotic infection/inflammation and women with chorioamnionitis have higher AF levels of HMGB1 (22, 388). The latter suggests that HMGB1 may also have an implication in the infectious etiology of preterm birth.

IL-1α was the first alarmin to be associated with preterm labor and labor at term (209). However, because IL-1β is released in response to infection by immune and non-immune cells in the uteroplacental compartment, its role has been primarily investigated rather than IL-1α. As previously mentioned, IL-1β and IL-1α bind to the same receptor and have similar effects. Evidence linking IL-1α to preterm labor are: 1) stimulation of IL-1R induces the transcription of numerous pro-labor genes *via* MAPK p38, JNK, c-jun, small GTPase Rho in myometrial smooth muscle cells which results in increased myometrial contractility (102, 389); and preterm labor in mice and non-human primates (170, 217); 2) antagonism of IL-1R prevents all of these events (390); 3) IL-1α AF levels are elevated in women that deliver preterm (391); 4) preterm labor is associated with increased IL-1α activity in AF (209); 5) maternal polymorphisms and haplotypes in the IL-1α gene (392), as well as fetal polymorphism in the endogenous IL-1R antagonist (219), have been associated with increased risk of preterm birth.

Interestingly, sterile inflammation has been suggested to induce a common inflammatory pathway leading to labor at term in normal pregnancies (346, 393). Accordingly, transcriptomic analysis of choriodecidual tissue collected at term predicted HMGB1 as a potential upstream

regulator of parturition (387). As previously mentioned, cffDNA is another alarmin candidate initiator of labor at term (346).

### 2.7.7. Role of alarmins in the onset of fetal inflammatory response syndrome

Recently Romero *et al.* indicated that FIRS can occur by the non-infectious DAMPs pathway by which no evidence of a microbial invasion of the amniotic cavity is observed (394). DAMPS are released in response to cellular stress or death as seen in vascular disorders, autoimmune disorders, inflammation, and exposure to environmental stressors (395), and can stimulate an insidious, silent, non-infectious inflammatory response in the maternal uterine tissues and in the fetal membranes and placenta. A plausible mechanism could be that cytokines generated from DAMP stimulation create a cytokine chain reaction that causes progressive self-amplification leading to their transfer into fetal fluid compartments (396). Once cytokine levels in the fetus are aberrantly elevated, fetal cells become susceptible to cytokine signals and suffer inappropriate terminal differentiation that ultimately retards normal organogenesis. Babies are thus in danger when the maternal immune response becomes great enough to increase inflammatory cytokines within the fetus.

### 2.7.8. Alarmins as potential targets for diagnosis and treatment of preterm birth

Efforts are underway to identify the most early or upstream event in this cascade to develop effective preventive treatments. The release of alarmins represents an initiating step in

sterile inflammation following an injury, and therefore alarmins may represent interesting candidate factors initiating the onset of preterm labor in women without infection (i.e. presumably the majority of women undergoing preterm labor). Accordingly, recent advances have found increased expressions of alarmins in maternal serum or gestational tissue of women at risk of preterm labor or having delivered preterm, specifically of cffDNA (294-296), HMGB1 (386), interleukin-1 (209, 211), uric acid (297, 397) and S100B (398). This consistent increase in alarmins could represent the missing link between numerous aetiologies of preterm labor wherein tissue injury and cell death are implicated, and the initiation of labor *per se*. This is plausible for numerous aetiologies such as: placental and uterine senescence, breakdown of maternal/fetal tolerance, uterine and cervical structural insufficiency, hemorrhage, multiple pregnancy, vascular disorders, and hypoxia/ischemia (Fig.5). The establishment of such a link could convey major implications for the development of effective therapeutics and diagnostic tests. Along these lines, cell-free DNA fraction $\geq 95^{th}$ percentile as screened between 14-20 weeks' gestation has been suggested as an effective biomarker to assess risk of preterm birth (399).

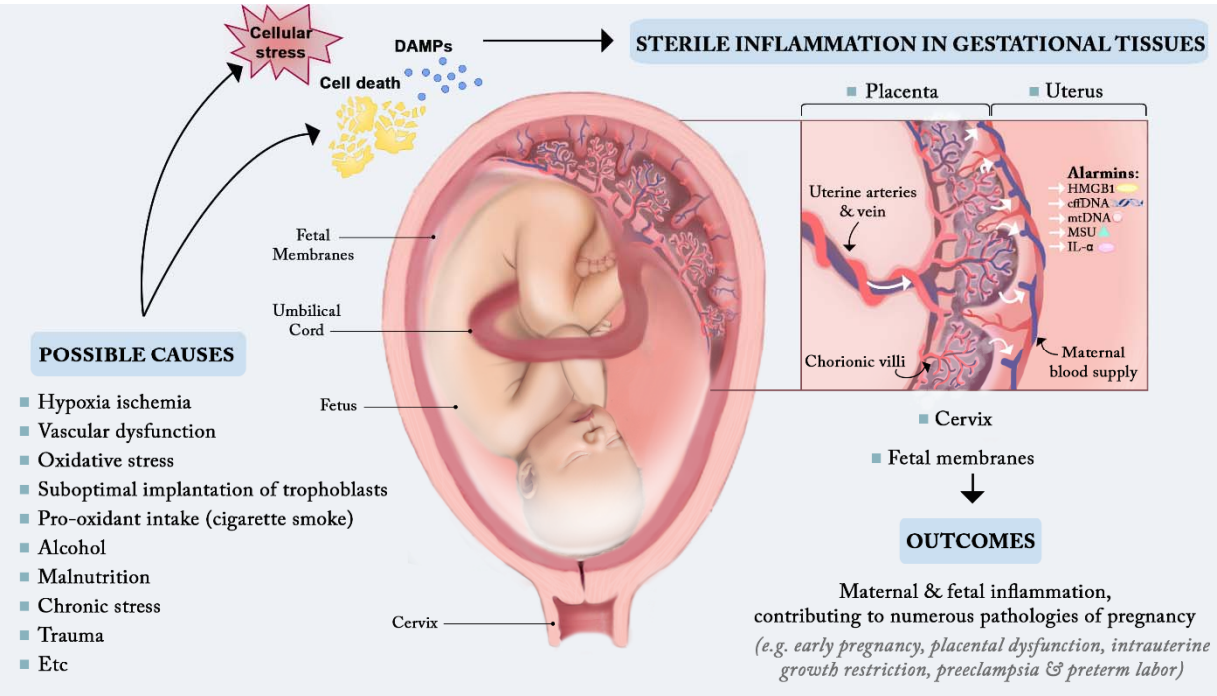


Figure 5. **Principal sites of release and actions of alarmins in pathological pregnancy.** Multiple causes of cellular stress will affect cell viability and lead to the release of DAMPs (alarmins) by the fetal membranes and the placenta. These DAMPs will then act not only on the placenta itself but also on the uterus, cervix and fetal membranes inducing inflammation and contributing to many complications of pregnancy.

Data accumulated to date converge toward a deleterious contribution of alarmins to the pathophysiology of PTB. Hence, effectively blocking alarmins could potentially result in favorable outcomes. This strategy has yielded positive outcomes in other inflammatory diseases, as it applies to HMGB1 (400), uric acid (401), IL-1 (402) and cffDNA (23). Therapies effective to block HMGB1, uric acid, IL-1α and cffDNA are summarized in Table II. Further efforts are also needed to develop specific and potent antagonists of uric acid and cffDNA to gain better knowledge in their role during physiological and pathological labor.

Table II. Therapeutic molecules targeting the alarmin activity of HMGB1, uric acid, IL-1α and cell-free DNA

| Target | Therapeutic molecule | Description | Mode of action | References |
|---|---|---|---|---|
| **HMGB1 /RAGE** | Recombinant box A | Truncated N-terminal domain of HMGB1 (~10 kDa) | Competitive antagonist of the receptor RAGE | (403, 404) |
| | S100P-derived RAGE antagonistic peptides | Small peptides inhibitors derived from S100P, a RAGE ligand (~1 kDa) | Binds with RAGE and inhibits HMGB1-mediated NF-κB activation | (405) |
| | Ethyl pyruvate | Derivative of pyruvate (~116 Da) | Downregulates the HMGB1-RAGE axis *in vitro* and *in vivo* | (406, 407) |
| | Methotrexate | Antimetabolite and antifolate drug used in treatment of cancer and autoimmune diseases (~454 Da) | Binds to HMGB1 and prevents its interaction with RAGE | (408) |
| | Neutralizing HMGB1 antibody | Polyclonal antibody against the B box domain of HMGB1 | Binds to HMGB1 and prevents its interaction with RAGE | (404) |
| | Anti-HMGB1 mAB | Monoclonal antibody against HMGB1 (IgG2b 2G7) | Binds to HMGB1 and prevents its interaction with RAGE | (409) |
| | Glycyrrhizin | Natural anti-inflammatory and antiviral triterpene in clinical use (~822 Da) | Binds to HMGB1 and reduces its activity. | (410) |
| | Quercetin | Plant-derived flavonoid (302 Da) | Inhibits the cytokine activity of HMGB1 | (411) |
| | Lycopene | Natural carotenoid (~536 Da) | Inhibits the cytokine activity of HMGB1 | (412) |
| | Vasoactive intestinal peptide (VIP) and urocortin | Endogenous neuropeptides (~2,8 kDa and 4,7 kDa, respectively) | Inhibits HMGB1 secretion | (413) |
| | Pituitary adenylate cyclase-activating polypeptide (PACAP) | Endogenous neuropeptide (~4,5 kDa) | Inhibits HMGB1-induced cytokine release *in vitro* and *in vivo* | (414) |
| | *chim 2A* | Kinked oligonucleotide duplexes (18bp) | Interacts with HMGB1; potently blocks a number of HMGB1 extracellular effects | (415) |
| | Others | | | (316, 416) |
| **Uric acid** | Allopurinol | Uric acid analogue (~136 Da) | Reduces uric acid production by xanthine oxidase | (351, 355) |

| | | | | |
|---|---|---|---|---|
| | Uricase, also known as urate oxidase | Homotetrameric enzyme specific to uric acid (~33 kDa) | Breaks down uric acid to allantoin | (351, 355) |
| | Sodium bicarbonate | Salt composed of sodium ions and bicarbonate ions (~84 Da) | Increases urine pH, thus increasing the dissolution and excretion of uric acid and decreasing its plasma concentrations | (401) |
| | Benzbromarone | Small organic molecule (~424 Da) | Uricosuric agent and non-competitive inhibitor of xanthine oxidase | (417) |
| **IL-1α /IL-1R** | Anakinra | Recombinant version of the interleukin 1 receptor antagonist (IL-1Ra) (~17 kDa) | IL-1R competitive antagonist | (359) |
| | Rilonacept also known as IL-1 Trap | Dimeric fusion protein (~251 kDa) | Soluble decoy IL-1R; competitive antagonist | (359) |
| | 101.10, also known as rytvela | Small peptide (all-d) (~850 Da) | Noncompetitive IL-1R antagonist, negative allosteric modulator of IL-1R | (418, 419) |
| **Cell-free DNA including cffDNA and mtDNA / TLR9** | ODN TTAGGG (A151) | Synthetic oligonucleotide | Interacts with TLR9; neutralizes the stimulatory effect of CpG-containing oligonucleotides | (420) |
| | chloroquine | Small organic molecule (~320 Da), diprotic weak base | Reduces NF-κB and AP-1 activation induced by CpG oligonucleotides; also exerts other anti-inflammatory effects | (421) |
| | AT791 and E6446 | Small organic molecules | Inhibit DNA-TLR9 interaction and TLR9 signaling *in vitro*; *in vivo* efficacy also reported | (422) |

## 2.8. Fetal injury associated with exposure to inflammation *in utero*

Chorioamnionitis has been firmly associated with the development of FIRS (defined as fetal plasma concentrations of IL-6 >11pg/mL)(423). The most plausible pathophysiological

mechanism of neonatal injuries is the paracrine propagation of the initial labor-inducing inflammatory response from the uterus to the placenta, and in more severe cases to the AF and fetus, mostly because direct transplacental passage of cytokines is highly unlikely (424, 425). A body of evidence suggests that the fetus can mount its own inflammatory response, characterized by neutrophil and monocyte activation (426); rises in plasma IL-6 and CRP (427); production of immunoglobulin M in response to congenital viral infections (428); and activation of a subset of type 1 helper T cells in response to perinatal intrauterine infection (429) and pPROM (430). This response amplifies the initial stimulus, in turn leading to systemic fetal dissemination of inflammation, diffuse fetal tissue injury, and multiorgan failure (425, 431, 432). The organs most severely affected are the hematopoietic system, the adrenals, heart, brain, lungs, and skin (433).

FIRS is particularly damageable to the fetal brain. Inflammation alters the permeability of the blood-brain barrier facilitating access of microorganisms and cytokines inside the brain (434). Accordingly, fever in pregnant woman confers a 7-to-9-fold increased risk of cerebral palsy (435, 436), as does a diagnosis of CCA or AHCA (433, 437). A recent study found that differences in the levels of inflammatory markers are associated with alterations in functional connectivity between numerous neonatal brain networks, and later to working memory scores at age 2 (73). FIRS has also been associated with neonatal sepsis, pneumonia, bronchopulmonary dysplasia, intraventricular hemorrhage, periventricular leukomalacia, necrotizing enterocolitis, neonatal respiratory distress syndrome, multiorgan failure, and death (438, 439), independent of infection (11, 25, 433). Neither antenatal tocolytics nor progesterone are likely to play a significant role once FIRS is already established. On the other hand, the co-administration of antibiotics and dexamethasone + indomethacin has shown promise to decrease

intraamniotic inflammation in a nonhuman primate model of intraamniotic infection-induced preterm labor (440). Noteworthy, single use of ampicillin was not effective to decrease uterine activity, premature delivery, and amniotic fluid cytokines, prostaglandins, and MMP-9 (440). However, corticosteroids have numerous undesirable effects that preclude their prolonged use during pregnancy. In another study, intraamniotic infusion of rhIL-1Ra protected against intra-amniotic LPS-induced amnion inflammation (441). Overall, this suggests a key role for anti-inflammatory molecules for the antenatal protection of the fetus against overt inflammation. If administered early enough, anti-inflammatory molecules may prevent the propagation of inflammation from the uterus to the fetal environment.

## 2.9. The resolution of inflammation - an unavoidable postpartum salvaging process

There are two major possible outcomes to acute inflammation: 1) the transition to chronic inflammation, wherein the site of inflammation is slowly invaded by long-lived leukocytes (e.g. T cells, macrophages) and undergo simultaneous destruction and repair; and 2) the resolution of inflammation. The resolution of inflammation is not a passive process, but rather involves anti-inflammatory (more accurately described as pro-resolution) cells and molecules, including regulatory T cells (442), resolvins/protectins (443), lipoxins (444), 15d-$PGJ_2$ (445), IL-10 (446), gases (e.g. nitric oxide, hydrogen sulfide and carbon monoxide) (447), adenosine (448), PAR-2 (449) and other candidates such as lactate. Pro-resolution mediators have numerous effects: 1) they exert an opposing effect to cytokines and chemokines, limiting leukocyte extravasation and activation (450, 451); 2) they reduce pain (452); 3) they switch off

important signaling pathways implicated in leukocytes survival (453); 4) they skew macrophage polarization toward an anti-inflammatory phenotype (454); 5) they promote the clearance of tissue debris by macrophages (455); and 6) they promote healing without fibrosis (453). Unresolved inflammation may result in significant tissue damage, in turn transitioning to self-perpetuating chronic inflammation and organ failure (456, 457); this has been demonstrated for numerous chronic diseases and processes including atherosclerosis (458), myocardial infarction and stroke (459), chronic pulmonary inflammation (460), cancer (461), and Alzheimer's disease (462). Accordingly, pro-resolution molecules analogs have shown great promise as therapeutic agents in numerous chronic inflammatory diseases (463).

As alluded earlier, pro-resolution is different than anti-inflammation. Anti-inflammation essentially involves inhibitory effects (e.g. blocking the nuclear translocation of NF-κB thereby decreasing the production of inflammatory mediators), whereas resolution involves the activation of specific processes (e.g. apoptosis, efferocytosis, etc.) regulated at multiple levels (464). The resolution process begins during the first few hours of an acute injury (465) with a lipid mediator class switching (*via* enzyme regulation) from PGs/leukotrienes to lipoxins, resolvins and protectins (466). Lipoxins are metabolites of arachidonic acid, whereas resolvins and protectins are metabolites of omega-3 fatty acids. Consequently, the resolution process involves a polarized metabolism of fatty acid precursors present in exudates in a temporally orchestrated way. Once produced, pro-resolution lipids act *via* specific G protein-coupled receptors, leading to alternative activation of NF-κB and other pathways (467, 468). In line, whereas the proinflammatory p65/p50 heterodimer is the predominant form of activated NF-κB during acute inflammation, a switch toward activated anti-inflammatory p50/cRel, p65/cRel,

and p50/p50 occurs at the onset of resolution (467-470). Interestingly, the p50/p50 homodimer has been shown to compete with p65/50 heterodimer for DNA binding.

Very few is known on the resolution of inflammation during and after labor (i.e. postpartum). Postpartum refers to the final stage of parturition wherein the uterus rapidly involutes to return to its pre-pregnant state. Because the laboring uterus undergoes mechanical stress and physical injuries, and bathes in an overabundance of labor-inducing inflammatory mediators, one could expect that termination of inflammation during the postpartum period is critical to salvage uterine tissue in preparation for subsequent pregnancies. However, the literature on postpartum resolution of inflammation is essentially inexistent. One study found an upregulation of lipoxin receptor FRP2/ALX in neutrophils and myometrial cells, and of maternal serum lipoxin A4 in pregnant women near term as compared to non-pregnant women (471). Other studies suggested an overall decrease in pro-resolution pathways at the onset of labor (472, 473). None of these studies have studied the postpartum period.

One candidate mediator of resolution during labor and during the postpartum period is lactate. More than just an inert end-product of the anaerobic metabolism, lactate exerts anti-inflammatory actions *via* GPR81 (474), but only at significantly elevated concentrations (the $EC_{50}$ of GPR81 is 4,8 mM (475)). These high concentrations are likely to only be reached during extensive anaerobic activity, such as observed in moderate-to-severe exertion (476), ischemia (477), cancer (478), and uterine labor (479). Because anaerobic metabolism is the predominant source of energy of the myometrium, serum and AF lactate levels increase markedly and proportionally to myometrial effort during labor (479, 480). A study conducted in foals and mares suggests that these increased levels of lactate may persist for up to 12h postpartum especially following dystocic parturition (481). Therefore, one could expect that the lactate

produced during labor acts on anti-inflammatory GPR81 to dampen inflammation and restore tissue homeostasis. Interestingly, M1-polarized macrophages, which are highly active during acute inflammation, obtain energy *via* glycolysis, and therefore produce high amounts of lactate early in inflammation when the resolution program begins, potentially contributing to its onset. On the other hand, M2-polarized macrophages, which are active relatively late, obtain energy *via* oxidative phosphorylation and produce minimal amount of lactate (482).

# 3. The role of interleukin-1 in uterine inflammation, preterm birth, and neonatal outcome:

## 3.1. Overview and mechanism of action of the interleukin-1 system

The IL-1 family of cytokines comprises 11 proteins (IL-1F1 to IL-1F11) encoded by 11 distinct genes which are important mediators of the innate immune response. IL-1α (IL-1F1) and IL-1β (1L-1F2) constitute the major inducer of this pathway and are thus tightly regulated by other members of the family, of which IL-1Ra (IL-1F3) is the prototype endogenous antagonist. IL-1α and IL-1β have similar biological effects and bind to the same receptors but are encoded by different genes (483).

### 3.1.1. Interleukin-1α

IL-1α has been introduced in Chapter 2.

### 3.1.2. Interleukin-1β

IL-1β expression is inducible and not constitutive, unlike IL-1α. IL-1β is mostly produced by hematopoietic cells such as DCs, blood monocytes and tissue macrophages. The induction of its transcription is triggered by PAMPs, DAMPs or pro-inflammatory cytokines, including itself. The IL-1β precursor protein (31 kDa) is cleaved by caspase-1, a cytosolic cysteine protease part of the NLRP3 inflammasome, into its functional mature form (17.5 KDa). Inflammatory stimuli such as PAMPs and DAMPs generate two signals on cells of the innate

immune response to promote IL-1β expression and maturation that are mediated by: 1) TLRs to promote transcriptional induction of pro-IL-1β and; 2) NOD-like receptors which upon activation oligomerize and complex with caspase-1 to form the inflammasome which promotes IL-1β maturation. The active form of IL-1β is then released in the extracellular space, where it can bind to its receptor and subsequently initiate or sustain an inflammatory response (359, 367, 368). The maturation process of IL-1β *via* the inflammasome is depicted in Fig. 6.

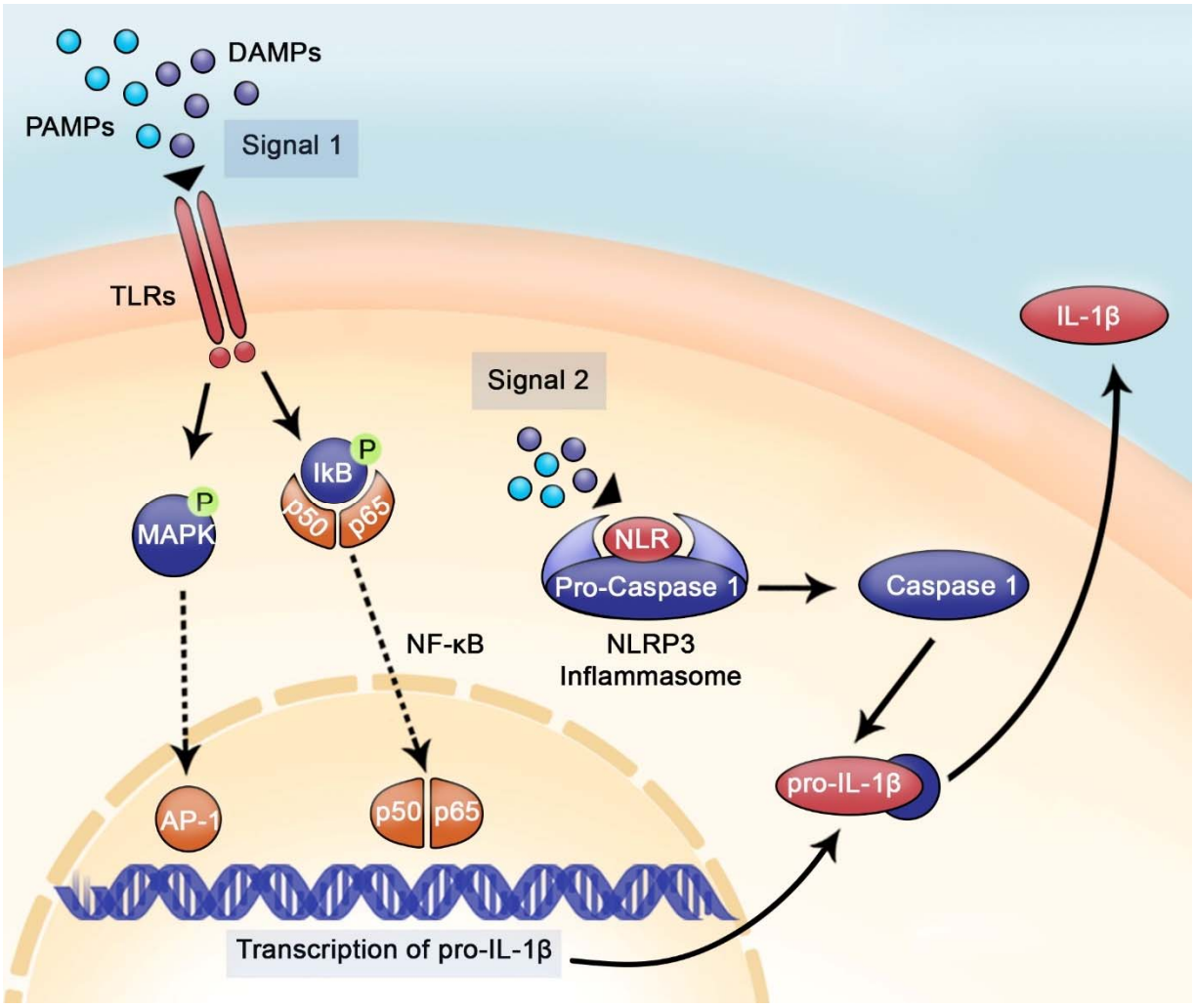


Figure 6. **IL-1β synthesis and maturation *via* the inflammasome in response to DAMPs and PAMPs**. The inflammasome is an active component of the innate immune response to infection and allows the maturation of IL-1β into its functional form. Signal 1, TLR agonists

(e.g. PAMPs and DAMPs) promote pro-IL-1β transcriptional induction *via* transcription factors AP-1 and NF-κB; signal 2, NLR agonists (also include PAMPs and DAMPs) induce inflammasome activation and IL-1β maturation. NLRP3; NOD-like receptor family, pyrin domain containing 3.

### 3.1.3. Interleukin-1 receptor antagonist

IL-1 is a potent cytokine active at low concentrations; 1% of IL-1R1 occupancy is sufficient to induce maximum biological response (484). For this reason, IL-1 signaling is tightly regulated through an endogenous inhibitor feedback system. IL-1Ra is an endogenous inhibitor of IL-1α and IL-1β which acts by competitively binding to IL-1RI without activating it (485). Two structural variants have been identified: a secreted glycosylated form (sIL-1Ra) and an intracellular form (icIL-1Ra) (483, 486). IL-1Ra is secreted by most cell types including myeloid and lymphoid cells (487-491). A 100 fold or greater excess in IL-1Ra over IL-1 is required to prevent IL-1R1 activation (492). Interestingly, data from displacement binding assays reveal that IL-1β has similar affinity than IL-1Ra, and both have slightly better affinity than IL-1α. The potency of the IL-1α precursor and of its mature form have been shown to be equivalent in inducing IL-6 *in vitro* (493). Altogether, IL-1Ra, the soluble form of IL-1RAcP (sIL-1RAcP), the membrane-bound and secreted IL-1 receptor II (sIL-1RII), and the soluble naturally occurring "shed" domains of each of the extracellular IL-1R chains, help to dampen IL-1 effects and therefore represent endogenous inducible anti-inflammatory mechanisms (494, 495).

### 3.1.4. Interleukin-1 receptors

The IL-1 receptor family comprises 11 receptors composed of extracellular immunoglobulin-like domains and intracellular TIR domains, and two have been identified to

bind IL-1: IL-1 receptor type 1 (IL-1RI) and IL-1 receptor type II (IL-1RII). IL-1RI is ubiquitously expressed and binds to IL-1 to produce an inflammatory response, whereas IL-1RII is found primarily in B lymphocytes, neutrophils and monocytes and is unable to transduce the signal due to its lack of a signaling-competent cytoplasmic tail (496-499). For this reason, IL-1RII has been identified as a decoy receptor for IL-1, limiting its action (500). Binding of IL-1 to IL-1RI induces a conformational change in the first extracellular loop of the receptor and further facilitates the interaction of IL-1RI with IL-1RAcP, a transmembrane protein required for signal transduction (501, 502). IL-1RAcP and IL-1RII also exist in soluble form, and act mostly as decoys. Another isoform of IL-1RAcP was recently discovered and referred to as IL-1RAcPb (503). Although IL-1RAcPb expression has been described to be restricted to neurons, our group was able to amplify its mRNA from rat gestational tissues (mostly uterus, but also cervix, ovaries and placenta) (504) and human uterus (Olson DM, unpublished).

### 3.1.5. Interleukin-1 receptor canonical pathway

IL-1R1/IL-1RAcP complex possesses conserved intracellular regions, the TIR domains (505), which mediate the protein-protein interaction of two signaling proteins, myeloid differentiation primary response gene 88 (MYD88) and interleukin-1 receptor–activated protein kinase (IRAK) 4 (506, 507). Knock-out mice for either MYD88 or IRAK4 have dysfunctional IL-1 signaling (508). IRAK4 can phosphorylate itself, which allows it to phosphorylate IRAK1 and IRAK2. Subsequently, tumor necrosis factor receptor–associated factor (TRAF) 6 is recruited (509, 510). Downstream cascades activated by TRAF6 eventually lead to the nuclear translocation of the transcription factor nuclear factor-kappa B (NF-κB) (201, 511) and to the

phosphorylation of mitogen-activated protein kinase (MAPK) p38 and stress-activated protein kinase (SAPK) JNK, which in turn leads to the activation of proteins that form transcription factor activator protein-1 (AP-1) (512). NF-κB and AP-1 are jointly involved in the expression of numerous pro-inflammatory genes including: *PGHS2* (513), *IL6 (204)*, *IL8* (205) and *CCL2* (514). NF-kB (515, 516) and more recently AP-1 (517, 518) have been associated with PTB, and inhibition of either NF-kB (Nadeau-Vallée M and Chemtob S, unpublished) or AP-1 (419, 519) with selective inhibitors is sufficient to reduce IL-1- and LPS-induced PTB in mice. Hence, the role of IL-1 in the onset of parturition is mainly mediated by NF-kB and AP-1 through specific transcription of target genes implicated in inflammation.

## 3.2. Role of interleukin-1 in normal term labor

As previously mentioned, term and preterm labor share similarities, and most of the major players in preterm labor are also implicated in term labor. The onset of labor is a complex process independently of the timing of delivery and is less likely to be caused by a single trigger; rather labor is the result of an interaction of various contributors. In this context, IL-1 has been suggested to exert an important contribution towards the onset of labor at term mainly because of its role in the induction of prostaglandin production by intrauterine tissues (209). Term delivery without infection is associated with a rise of IL-1β mRNA expression in decidua and placenta (483) in addition to an increase in the levels of IL-1β in the amniotic (216) and cervicovaginal (215) fluids. IL-1α (and IL-1β) concentrations also increase in cervicovaginal fluid within 2 weeks prior to term labor, and is associated with a decrease in IL-1Ra, which are significantly correlated with labor onset (520).

Interestingly, studies in mice uncovered a signal of parturition arising from fetal lungs, which in turn induces the production of IL-1 at term. Surfactant protein A concentration in AF rises near term and is associated with increased IL-1β and NF-κB expression in AF-resident macrophages, increased macrophage migration to the maternal uterus, and increased levels of IL-1 in uterine tissues, which is thought to herald labor (521). SP-A-deficient and steroid receptor coactivator-1 and -2 (acting upstream by regulating SP-A transcription)-deficient mice fail to produce IL-1 at term, and their parturition is delayed by 12 hours and 38 hours, respectively. However, as mentioned previously, germline knockout mice with a disrupted IL-1 system (IL-1β null mice (522), IL-1β converting enzyme null mice (523), and IL-1 receptor type 1 null mice (524)) are fully fertile and deliver at term (136), suggesting complex interplays between redundant mechanisms conceivably preserved throughout mammalian evolution to guarantee birth at term, thereby circumventing placental senescence and associated adverse outcomes.

## 3.3. Role of interleukin-1 in preterm labor

IL-1 was the first cytokine to be implicated in the mechanism of PTB associated with infection or acute inflammation as well as spontaneous delivery at term in humans (209, 216). Evidence that IL-1 plays a role in physiological and pathological labor of humans comprise the following: 1) human parturition has been associated with increased levels of IL-1β in the cervix, the myometrium and in fetal membranes (99), regardless of the presence of infection (210); 2) IL-1β concentration and bioactivity increases in AF of women with preterm labor and infection (209), and seems to be associated with PTB (211); 3) elevated maternal plasma levels of IL-1β

are associated with spontaneous preterm labor (115); 4) IL-1β is produced in response to bacterial endotoxins in *ex vivo* gestational tissues (525, 526); and 5) IL-1β stimulation of human uterine-derived cells induces UAP genes including COX-2 (and upstream uterotonic $PGE_2$ and $PGF_{2\alpha}$) (527-531), thereby promoting cervical ripening (532, 533) and myometrial contractions (534, 535). Evidence linking IL-1α and PTB have been presented in Chapter 2.

Animal studies support human data and further provide more insight into the mechanism of action of IL-1 in gestational tissues during labor. The main findings obtained with animals include: 1) systemic, intrauterine, and intraamniotic IL-1β dose-dependently induces PTB in multiple animal models including in mouse (217, 390, 536), rabbit (537) and nonhuman primate (170, 538), and this effect is reversible with IL-1Ra co-treatment (390); 2) preterm delivery after LPS administration is preceded by the appearance of dramatic increases in maternal serum and AF concentrations of IL-1 (235); 3) intraamniotic infusion of IL-1β in nonhuman primate prematurely activates the uterus in mice and increases myometrial contractility more effectively than TNF-α, IL-6 and IL-8 (170). IL-1 also plays key roles in eliciting fetal injury (539-542). Collectively, the evidence presented underscores the importance of IL-1 in (human and animal) labor.

As mentioned earlier, IL-1β is activated following stimulation of cells of the innate immune response by DAMPs or PAMPs. Although IL-1 crosses negligibly the placenta (424, 543), it can trigger an inflammatory chain reaction on the maternal side of the placenta, which progresses through the placental villi by activating Hofbauer cells to secrete more IL-1 and amplify the initial response (544), thus eventually reaching the fetus. This mode of action is supported by the sequential onset of placental inflammation and fetal inflammatory response resulting from intrauterine administration of IL-1β (396). However, inflammation may also

spread because of changes in placental factors that affect perfusion and cellular transporters, thereby limiting the flow of nutrients to the fetus and causing stress; alternatively, during infection inflammation can spread through microbial infiltration of the amniotic cavity. Regardless of the mode of dissemination, chorioamnionitis is firmly linked to the onset of FIRS (433), which is dependent of IL-1 (545). Hence, the effects of IL-1 will be presented sequentially as inflammation spreads, i.e. from maternal to fetal tissues, although other modes of dissemination have been described as well (546).

### 3.3.1. Effects of interleukin-1 on pre-placental tissues

Effects of IL-1 on pre-placental tissues (i.e. excluding placenta and fetus) include myometrial activation, cervical ripening, weakening of fetal membranes, leukocyte extravasation inside the uterine walls, and eventually production of prostaglandins and labor. As a general principle, these effects predominantly affect maternal tissues and normally exert minimal impact on the fetus *per se* other than to hasten birth and to constitute a locus for the spread of inflammation to the fetus.

Low concentrations of IL-1 activates the myometrium in preparation of labor and therefore IL-1 is considered a potent uterotrophin. Studies show that stimulation of human myometrial smooth muscle cells (102) or decidual cells (547) with 1 ng/ml of IL-1β for short periods of time leads to substantial alterations in the transcription of genes implicated in labor, such as *CXCL2*, *IL6*, *PTGS2*, *NFKB1*, *TNF*, *PLAU*, *TNC* and many others. Essentially, these genes encode for enzymes catalyzing the conversion of arachidonic acids to prostaglandins, membrane-bound receptors coupled to calcium signaling, proteins implicated in cytoskeleton

remodeling and actomyosin-dependent contraction, signal transducers, chemokines, and many other relevant proteins (102, 547).

Myometrial contractility is amplified by induction of $G_{\alpha q}$ protein-coupled receptors in the membrane of myometrial smooth muscle cells; these receptors include FP (548), OXR (42), and PAR-1 (activated by proteases such as thrombin and MMP-1) (549); whereas the coordination of laboring myocytes is attributable to the presence of gap junctions, which are specialized intercellular connexions that allow the transmission of $Ca^{++}$ between cells and intercellular coordination (550). In myometrium, IL-1 upregulates CX-43 (protein component of gap junctions) (419), COX-2 (enzyme catalyzing the formation of prostaglandins from arachidonic acid) (551), and FP (552), thereby inducing the transition from uterine quiescence to uterine activation. Additionally, IL-1 has been shown to upregulate the expression of the putative calcium entry channel TRP-3 (553), and to increase reticular calcium storage (389) in myometrial smooth muscle cells. However, the effect of IL-1 on OXR expression is controversial; while OXR reaches its maximal expression in term pregnancies when IL-1 levels are highest, *in vitro* evidence shows that stimulation of myocytes with IL-1 decreases mRNA levels of *Oxtr* (554). Regardless, the demonstrated consequence of IL-1 on myometrial contractility is increased potency and efficacy of uterotonic molecules (419, 555).

Myometrial contractions of labor, which are potentiated by IL-1 (170), convey mechanical stress that promotes cervical ripening (556). In addition to this effect, IL-1 directly affects cervical ripening and integrity of fetal membranes by inducing the production of ECM proteases from endometrial fibroblasts (106, 107). The activity of these enzymes results in the reorganization of collagen fibril structure with a gradual loss of tensile strength in cervix, and in the degradation of the ECM connecting the amniochorion layers together. Further, IL-1

indirectly contributes to cervical ripening by upregulating COX-2 and promoting prostaglandin production (108, 527, 531). Correspondingly, preterm labor is associated with higher concentrations of *Il1b* mRNA in human cervix (557); and contrary to the requirement of administering both an agent to ripe the cervix (i.e. prostaglandins) and an agent to promote uterine contractility (i.e. oxytocin) in women who require induction, IL-1 is sufficient on its own to induce labor in numerous animal models including non-human primate (170, 217). Interestingly, the rat uterus at term expresses increased levels of IL-1R1 relative to the reduced expression of the decoy receptor IL-1R2 (another endogenous inhibitor of IL-1 activity), suggesting that the uterine sensitivity to IL-1 rises near the end of gestation (558). Interestingly, the uterine quiescence hormone progesterone can suppress IL-1R1 upregulation and prolong gestation; conversely, administration of the progesterone receptor inhibitor RU-486 in late pregnant rats facilitates a precocious rise in uterine IL-1R1 predisposing to preterm labor (558).

Another important UAP induced by IL-1 is CCL2, a potent monocyte chemoattractant. Macrophages infiltrate the decidua in human and rodent prior to term and preterm labor (101), and perform key roles by releasing cytokines, prostaglandins and proteases in cervix and uterus when gestation nears the end (559). Our group has shown in mice that the administration of IL-1β induces a >10-fold increase in *Ccl2* expression in placenta and fetal membranes, and >5-fold increase in circulating leukocytes (396, 419); this upregulation in *Ccl2* is also observed in human myometrial cultures (560).

### 3.3.2. Effects of interleukin-1 on placenta

The placenta is a unique organ of fetal origin that infiltrates the decidua early in gestation to form a feto-maternal interface, thereby providing the fetus with nutrients and $O_2$ while maintaining a physical and immunological barrier for the fetus. Hemochorial placentation is present in multiple species (e.g. human, non-human primates, rodents). Numerous studies suggest that the placenta can coordinate the onset of labor at term or preterm in response to stressors (15, 87, 561). Although the placenta usually generates very low levels of IL-1 in normal conditions, the release of IL-1 (α and β) is markedly induced in inflammatory conditions generated by endotoxins or sterile cellular death (293, 562); this has been shown to amplify the initial inflammatory response (563). As discussed earlier, the placental inflammatory response, if unresolved, may spread to the fetus through paracrine effects of cytokines; this pathway remains to be explored. Further, endotoxins have been shown to drastically reduce placental perfusion in rats in an IL-1-dependent manner (240); such detrimental hemodynamic effect bears important implications for fetal growth (564).

### 3.3.3. Interleukin-1 in spontaneous preterm labor associated with infection

Infection is implicated in approximatively 40% of PTB and is often subclinical. Studies in mice found that TLRs are essential mediators of bacterial stimuli leading to PTB (263, 565, 566). Specifically, endotoxins from Gram-negative bacteria bind to TLR4, whereas exotoxins from Gram-positive bacteria bind to TLR2 (567). In humans, TLR2 and TLR4 are found in the cervix, endometrium and fallopian tubes (568), in the placenta (569) and in other cells at the

fetal-maternal interface (92), and are up-regulated in cases of chorioamnionitis and during parturition (570).

The pathophysiology of preterm labor in presence of infection has been attributed to the release of pro-inflammatory cytokines, principally IL-1 (571). The activation of TLRs leads to induction of downstream inflammatory cascade (including NF-κB activation), which promotes cytokine production. Accordingly, LPS has been shown to induce IL-1 production from human gestational tissues *ex vivo* (525, 526). In human pregnancies, decidual IL-1 levels increase in presence of microorganisms and bacterial products (235, 572). Also, in pregnant women with infection, increased concentration and bioactivity of IL-1 is observed in AF (209, 573). Further, a disproportionate increase in the IL-1β/IL-1Ra ratio in response to Gram-negative infection has been found to correlate with PTB (574).

Germinal cell knockout mice deficient in IL-1 receptor or IL-1β are not protected against endotoxin-induced PTB, likely because of compensatory mechanisms; however, these animals do exhibit lower inflammatory responses to endotoxins (575, 576). Moreover, mice lacking both IL-1 receptor and TNF receptor are protected against endotoxin-induced PTB (168), suggesting a complementary role of those cytokines that would be altogether essential. On the other hand, one study reported that treatment with both IL-1Ra and a TNF receptor antagonist did not prevent endotoxin-induced PTB (577); this discrepancy between genetic and pharmacological approaches may be due to the nature of antagonists used, route of administration, and doses utilized. In an extensive study conducted by Girard *et al.*, a central role of IL-1 in placental defects induced by bacterial endotoxins was demonstrated (240). More recently, rhIL-1Ra was found to protect against LPS-induced uterine inflammation in nonhuman primates (441). Together, these data suggest a key role for IL-1 in infection-induced PTB.

### 3.3.4. Polymorphisms in interleukin-1-related genes associated to preterm birth

The genes coding for IL-1β (*IL1B*) and IL-1Ra (*IL1RN*) are both located adjacent to each other on chromosome 2. Different alleles in intron 2 of *IL1RN* are associated with varying levels of IL-1β and IL-1Ra (578, 579). Expression by the fetus of the *IL1RN**2 allele was associated with enhanced mid-trimester intra-amniotic IL-1β production and high IL-1β/IL-1Ra ratio, and was associated with increased risk for PTB ($p<0.0001$) (219). Also, fetal carriage of *IL1B+3953*1* and *IL1RN*2* alleles was associated with risk for PTB in African and Hispanic populations (218). Previous data suggest that some polymorphisms in the fetal IL-1 system are likely to predispose to PTB in case of an intra-amniotic pro-inflammatory immune response (580).

Maternal carriage of at least one copy of *IL1RN**2 is also associated with increased risk to PTB (581, 582). Whereas *IL1B*+3953C>T, a common *IL1B* polymorphism which has been shown to elevate the capacity to produce IL1B *in vitro* (583), has yielded controversial observations on the risk reduction for PTB (584). While the rare allele for the *IL1B* promoter region (IL1B-31T>C) was found to be associated with PTB (220).

### 3.3.5. Prediction of preterm birth with levels of interleukin-1 and interleukin-1 receptor antagonist in gestational tissues and fluids

It has been abundantly suggested that IL-1 can be a promising potential predictor of PTB for two main reasons: 1) its levels in AF are elevated in mid-trimester and positively associated with preterm birth (211) and microbial penetration of the amnion (573); 2) mRNA levels of IL-1β are elevated in the human cervix during PTL (557). Several studies have correlated PTB with levels of IL-1β in human cervicovaginal fluids (215), in AF (211) and in premature neonate blood (221). Moreover, measurements of IL-1Ra levels in maternal blood (585) and in cervicovaginal fluids (111) of women in mid-term gestation was able to accurately predict PTB and was associated with increased rate of spontaneous preterm labor (586). Since IL-1Ra counterbalances the pro-inflammatory action of IL-1β, the IL-1β/IL-1Ra ratio is important in the initiation of the inflammatory cascade that leads to the onset of labor. Interestingly, in cervicovaginal fluid, IL-1Ra levels decrease as labor approaches, while IL-1β rises, indicating a pro-inflammatory shift in the IL-1β/IL-1Ra balance (520). Accordingly, the IL-1β/IL-1Ra ratio was significantly higher in decidual samples of women with spontaneous labor compared to women without labor, as the increase of IL-1β in the decidua turned out to be the major cause of the ratio change (483). Also, IL-1α and IL-1β concentration are also significantly higher in placenta from pregnancies at high risk of PTB in association with placental dysfunction (292), which may be of interest for the identification of biomarkers of placental dysfunction and PTB.

### 3.3.6. Implication of interleukin-1 in hormonal regulation related to parturition

As mentioned above, progesterone is implicated in human pregnancy maintenance by promoting uterine quiescence and in human, a functional progesterone withdrawal occurs when pregnancy nears its term. IL-1β has been shown to facilitate the conversion of progesterone to the inactive metabolite 20α-hydroxyprogesterone in human cervical fibroblasts (116). Interestingly, IL-1β was also shown to inhibit progesterone production by primate luteal cells *in vitro* (117). More recently, IL-1β has been shown to stabilize PR-A in myometrial smooth muscle cells *via* post-translational mechanisms, thereby decreasing the response to progesterone (118). Altogether, these studies suggest that IL-1 decreases progesterone bioactivity at the end of gestation, and therefore acts upstream of the functional progesterone withdrawal described in human.

CRH is a peptide hormone part of the HPA axis which leads to cortisol production from the adrenal cortex in response to stress. CRH plays an important role in coordinating and regulating parturition (15, 587, 588), and appears to be an important player in PTB associated to stress (14, 589). Since IL-1 is central to many pro-labor pathways, its possible role in modulating CRH has been explored. IL-1β was found to induce CRH expression in human placenta (590) and its receptor CRH-R1 in human myometrium (591). Along these lines, it has been hypothesized that IL-1β could act as a trigger to placental CRH release in humans thereby eliciting labor (115). Moreover, IL-1β has been shown to inhibit placental 11 beta-hydroxysteroid dehydrogenase type 2, an enzyme responsible for the inactivation of cortisol, a recognized trigger to placental CRH release, in human placental villi explants (592). These

studies suggest that IL-1β triggers the release of placental CRH, decreases the catabolism of CRH-inducing cortisol, and upregulates CRH receptor in intrauterine tissues.

A role for IL-1 in the regulation of other hormones implicated in labor have also been suggested as it applies to oxytocin (593), ET-1 (102, 594) and estrogen (which antagonizes the effects of progesterone) (595).

### 3.3.7. Modulation of other factors implicated with labor

In a genome-wide expression profiling study using human myometrial cells in response to IL-1β, Chevillard *et al.* identified enhanced expression of many genes implicated in labor including cell adhesion factors (such as *VCAM1* and *ICAM1*), angiogenesis modulators and several ECM remodeling enzymes (including *TNC* and *PLAU*) (102). Another study from a different group found that IL-1β up-regulated ECM remodelling enzymes, precisely MMPs in human uterine cells (106), while it down-regulated the expression of TIMP-2 in the human cervix (107), which further promotes MMPs accumulation. MMPs contribute to cervical ripening during labor (596). In a recent study monitoring the global inflammatory transcriptional profile in human term decidual cells, treatment with IL-1β elicited a regulation of 428 transcripts of mRNA (including cytokines, chemokines and other inflammatory mediators genes) and micro RNA (547), highlighting the vast scope of its effect in gestational tissue.

Vascular-endothelial growth factor (VEGF) is important for growth and maintenance of the decidua. The expression of VEGF mRNA is significantly increased in the chorio-decidua from women undergoing spontaneous PTL compared to those going into spontaneous term labor

(597). Interestingly, the expression of VEGF is increased *in vitro* in human decidual stromal cells stimulated with IL-1β (598).

## 3.4. Effects of interleukin-1 on the fetus and its environment - post-placental effects

Inflammatory concentrations of IL-1 exert a wide range of deleterious effects on fetal and neonatal tissue. This has been demonstrated in animals using postnatal administration of IL-1 (599), and using antenatal administration of IL-1 through the fetal compartments (600). Consistently, severe perinatal complications are associated with higher levels of IL-1β in cord blood of human neonates, and not of other major cytokines such as TNFα and IL-6 (222).

The premature fetus/newborn is exquisitely sensitive to inflammatory stimuli. If inflammation is not rapidly resolved, fetal/neonatal cells can undergo persistent phenotypical changes which often has life-long implications; this applies particularly to the brain (601, 602). Correspondingly, IL-1β has been shown to elicit neuro-microvascular decay (603), inhibit hippocampal neuron differentiation (604), and in turn lead to seizures (605), in addition to inducing hypomyelination (606) and learning deficits (212). Conversely, blockade of IL-1 using pharmacological or genetic approaches is neuroprotective (68, 212, 607), as it improves long-term learning ability (212). In addition, animal studies on neonatal injuries to other organs such as the lungs, eyes and intestines, also concur to a preponderant role of IL-1 in neonatal tissue injury (214, 608-613). Concordantly, studies in human newborns affected by injury to these

organs point to a robust association with the levels of IL-1β (539-542, 614), which correlates with severity of the disease (615). Other than evidence through correlation, these neonatal injuries can be reproduced in rodent and ovine models by overexpressing or administering IL-1 (599, 608, 609). A recent study by Kallapur *et al*. revealed that intraamniotic infusion of IL-1β in late-pregnant rhesus monkeys caused histological chorioamnionitis and lung inflammation characterized by neutrophil infiltration of fetal airways and proinflammatory gene induction (600). Similarly, a recent study has shown that intraamniotic infusion of rhIL-1Ra prevents intraamniotic LPS-induced amnion inflammation in nonhuman primate, thereby preventing choriodecidual infiltration by neutrophils (441). Overall, this body of pre-clinical and clinical data points to detrimental and long-lasting outcomes of antenatal exposure to IL-1.

Although evidence indicates detrimental effects of high inflammatory levels of IL-1, this cytokine may also exert some beneficial effects in the context of PTB. Accordingly, a recent study suggests that IL-1 accelerates fetal lung maturation by inducing surfactant protein synthesis prior to PTB (616). Interestingly, as mentioned previously, surfactant protein A has been shown to initiate parturition in mice (521). Along these lines, post-placental effects of IL-1 may be linked to the onset of PTB (inside out pathway). Accordingly, levels of IL-1 in AF are associated with preterm labor (211), which also manifests when the fetus expresses the *IL1RN**2 allele associated with increased intra-amniotic production of IL-1β (219). Antenatal exposure to IL-1 may also convey other physiological effects, such as on immune response (600) and skeletal growth (617). In light of the overall detrimental effects of IL-1 in the second half of pregnancy, IL-1-targeting therapeutic interventions are warranted to protect the fetus.

## 3.5. Therapies available to oppose the effects of interleukin-1

Available pharmacological strategies to antagonize the action of IL-1 include IL-1 receptor competitive antagonists, non-specific NF-κB inhibitors, and cytokine suppressive anti-inflammatory drugs (CSAIDs) (see Fig.7).

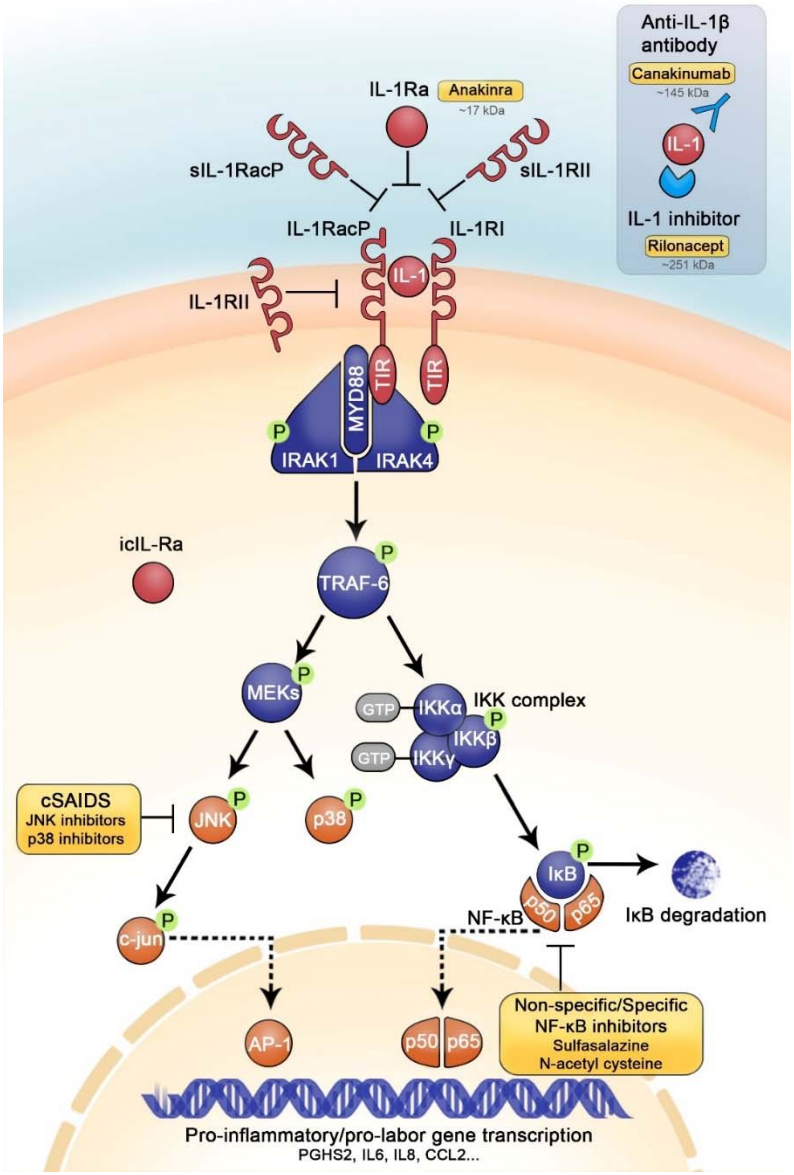

Figure 7. **Signaling pathways of the IL-1 system and available antagonists of the IL-1 receptor.** The IL-1 system is composed of IL-1RI and IL-1RAcP, which form a functional complex with IL-1 (α or β). This ligand-receptor complex signals *via* two canonical pathways: the kinase pathway leading to the activation of transcription factor AP-1, and the NF-κB pathway. The nuclear translocation of these transcription factors promotes the transcription of key pro-inflammatory, pro-labor genes including PGHS2, IL6, IL8 and CCL2. This system is tightly regulated by the endogenously-produced IL-1 inhibitors IL-1Ra, sIL-1RAcP, IL-1RII (soluble and membrane-bound), and others (not shown). Three IL-1 receptor antagonists are currently available to counter IL-1 action: Anakinra, Canakinumab and Rilonacept. Other available strategies to counter IL-1 action include CSAIDs and NF-κB inhibitors. MEKs, mitogen-activated protein kinase kinases

### 3.5.1. Interleukin-1 competitive antagonists

Three IL-1 targeting agents are approved for clinical use to this day: the IL-1 antagonist anakinra (Kineret), the soluble decoy receptor rilonacept (Arcalyst), and the neutralizing monoclonal anti-IL-1β antibody canakinumab (Ilaris). These pharmacological agents are FDA-approved for Cryopyrin-Associated Periodic Syndromes (CAPS) and rheumatoid arthritis (only Kineret) but are also being considered for other inflammatory diseases such as gout and type-2 diabetes (359, 618). None of these IL-1 blocking therapies are approved to treat disorders of pregnancy. All three agents antagonize IL-1 action in a competitive manner by preventing the binding of IL-1 to its receptor and therefore blocking all downstream signal transduction, including NF-κB activation. Anakinra is efficacious in numerous animal models of acute inflammation (367) and its potential has been explored in pre-clinical studies of PTB (607), intraamniotic infection/inflammation (441), neurobehavioral impairments following oxidative or inflammatory stressor (68, 240), and neonatal injuries of the lung (214, 545), brain (68, 240, 607), and eye (603, 619). These studies revealed that antenatal delivery of Anakinra to dams treated with LPS elicits limited improvement of PTB outcomes and inflammatory status in pre-placental gestational tissues (607), while it protects against placental injuries in response to

endotoxins (240), pulmonary insults (545), and long-term neurological and motor behavioral impairments (240, 607), and choriodecidual activation in response to intraamniotic endotoxins (inside-out signals) (441). Postnatal administration of Anakinra improves antenatal inflammatory- and/or post-natal hypoxia/ischemia-driven neurological and behavioral impairments (68), and retinal injury to hyperoxic exposure (603). Since Anakinra seems to protect the fetus without significant effect on prematurity, the aforementioned data supports a clear contribution of inflammation to neonatal injuries independent of gestation age at birth. The inefficacy of Anakinra in protecting pre-placental tissue and preventing PTB may in part be dose-related. To our knowledge, rilonacept and canakinumab have not been used in preclinical studies of PTB.

Reluctance to using competitive IL-1-targeting therapies in PTB include: 1) large size and immunogenicity; 2) high costs; 3) limited efficacy to prevent infection-induced PTB in pre-clinical studies (536, 577, 607); 4) undesirable (long) half-life of Rilonacept and Canakinumab (>3 weeks) for acute/sub-acute treatment of women in labor; 5) inevitable inhibition of NF-κB-mediated cytoprotection, antioxidant response, and contribution to the resolution of inflammation; 6) minimal (at best) desirable passage through the amniotic epithelial barrier and placental barrier (441); and 7) hindrance in immune-surveillance. The latter can subject the fetus and the mother at risk of infections (620, 621), and could compound on the relative state of immunosuppression established during pregnancy (622-624). In addition, since IL-1 has a role in the physiological process of labor, complete inhibition of IL-1 signals *per se* could influence the normal parturition process. For these reasons, optimization of the pharmacological and biochemical properties of available IL-1 receptor antagonists is desirable for the treatment of PTB.

### 3.5.2. Non-specific NF-κB inhibitors

Because IL-1 signals in part *via* NF-kB, NF-kB inhibitors can partly counter IL-1 action. FDA-unapproved selective NF-κB inhibitors (e.g. TPCA-1, parthenolide, and SC-514 [selective IKK complex inhibitors]) may seem promising to prevent PTB but interfere with desirable properties of NF-κB activation; FDA-approved NF-κB inhibitors (e.g. Sunitinib, Lestaurtinib) lack selectivity to the pathway (625) and thus undesirably increase off-target effects. Despite the clear contribution of NF-kB to the pathophysiology of PTB (515), NF-kB is a crucial signaling effector of pro-resolution mediators and its inhibition impedes resolution, in turn leading to detrimental protraction and amplification of the initial response (626). Some studies show efficacy of NF-kB inhibitors to prevent inflammation, but also describe major adverse effects. Along these lines, a recent study using sulfasalazine to inhibit NF-κB pointed out pro-apoptotic effects on human fetal membranes, despite marked efficacy in decreasing inflammation (627). This study prompted reactions from the scientific community, and overall a complete blockade of NF-κB activity is seen as undesirable in pregnancy (516). In a placebo-controlled randomized clinical trial of pregnant women treated for bacterial vaginosis, NAC was shown to significantly decrease PTB rates and neonatal mortality/morbidity (628). However, NAC is a non-specific NF-kB inhibitor; its other known effects include inhibition of JNK, p38 and AP-1; NAC acts mostly as an anti-oxidant (629). Nonetheless, NAC may appear to exhibit potential promise for the prevention of PTB.

### 3.5.3. Cytokine suppressive anti-inflammatory drugs

CSAIDs constitute a new class of drugs which inhibits cytokine-mediated events, and are elegantly reviewed in (113). These drugs include MAPK/SAPK and NF-κB inhibitors (addressed in the section above) and are FDA-approved for cancer. MAPK inhibitors (e.g. Trametinib, Dabrafenib) and JNK inhibitors (e.g. Sorafenib) partly inhibit IL-1 signaling without affecting NF-kB. However, they also inhibit IL-1-independent MAPK and JNK pathway, thereby largely increasing off-targets and potential adverse effects. Because the targets of CSAIDs are involved in placental growth and differentiation (630, 631), and in fetal growth, these agents are teratogenic and could not be safely used to treat PTB (632).

## 3.6. Summary

Of all inflammatory mediators, IL-1 is central to the pathophysiology of PTB and neonatal injuries. Due to the ubiquitous expression of its receptor, inflammatory concentrations of IL-1 act on virtually all types of cells and tissues: in myometrium, it promotes contractility; in cervix and fetal membranes, it promotes loosening of ECM; in placenta, it promotes an acute inflammatory response and vasoconstriction; and in fetal organs, it promotes injury and long-term adverse consequences. The development of therapeutic modalities for the treatment and prevention of PTB and its complications is needed as there is at present no effective agent available. Although IL-1 is a major player partaking in the pathological induction of PTB, currently approved IL-1-targeting therapies have failed to show expected efficacy in pre-clinical studies. In addition, their competitive mechanism of action inhibits all IL-1 receptor-associated signals which promotes immunosuppression and other undesired effects. Alternative IL-1- and

IL-1R-targeting therapies include CSAIDs and NF-κB inhibitors, but these agents are not safe to use during gestation. Allosteric modulators of IL-1 receptor, which will be discussed in Chapter 4, may represent a safe and effective alternative to modulate IL-1.

# 4. Biased antagonism to treat preterm birth:

## 4.1. Inhibition of specific transduction pathways using functional selectivity

The use of peptides and peptidomimetics as drugs is increasingly attracting attention (reviewed by (633)). This is not only true for natural ligands (e.g. octreotide, glucagon, GLP-1, Egrifta, etc) but also for modified peptides that exert inhibitory actions (e.g. atosiban, PDC31, etc). Over 60 peptide drugs have reached the market, and approximately 140 are currently evaluated in clinical trials (634).

Small peptidomimetics (<12 amino acids) can be derived from the sequence of specific regions of a receptor (or enzyme) thereby interfering with its activity, as amply documented (635-637). Because these molecules interact with regions remote from the natural orthosteric binding site, they exhibit allosteric properties, such as a noncompetitive mode of action, and therefore are likely to exert functional selectivity (638). Functional selectivity is the selective modulation of specific signaling pathways triggered by a ligand-receptor interaction, resulting in inhibition of some signals and/or enhancement of others. Functional selectivity provides concrete solutions to the different issues that competitive IL-1-targeting therapies bear and therefore represents a promising option for the development of IL-1 targeting therapies for the prevention of PTB. For example, it provides a solution to avoid NF-κB inhibition while inhibiting other IL-1-related pathways implicated in the labor-associated inflammatory response, such as MAPK p38 and SAPK JNK. Circumventing NF-κB inhibition may be safer considering the concerns mentioned above, including the crucial role of NF-κB for efficient

resolution of inflammation (626). An allosteric modulator, PDC31, blocks myometrial contractions by acting as a biased agonist (i.e. an agonist displaying functional selectivity) of FP, thereby selectivity modulating $PGF_{2\alpha}$-mediated signaling pathways. Administration of PDC31 to pregnant mice prolongs normal gestation and prevents PTB induced by $PGF_{2\alpha}$ and LPS (639). PDC31 has now successfully completed Phase 1b human clinical trial, which confirmed its efficacy and safety to decrease myometrial contractions in women with primary dysmenorrhea (640). Hence, PDC31 represents an example of how biased agonism could be used safely and effectively.

### 4.1.1. Noncompetitive interleukin-1 receptor antagonists

Based on the implication of IL-1 in a broad range of diseases, and urgent need to develop an effective and safe IL-1-targeting molecule for the treatment of PTB and fetal inflammation, our team designed a series of small peptide derived from the extracellular regions of IL-1RacP known to interact with IL-1R1 and to share interspecies homology with human, rat, and mouse. Based on their differential efficacy and potency to decrease IL-1-induced $PGE_2$ formation and p38 phosphorylation *in vitro*, the all-d peptide 101.10 (sequence: rytvela) was selected (418). All d-peptides are protease-resistant and thus more stable (641).

101.10 binds specifically to IL-1R1 (as suggested by failure of 101.10 to bind on cells collected from IL-$1R1^{-/-}$ mice), without blocking the binding (i.e. orthosteric) site of IL-1, thereby altering intracellular coupling toward specific signaling pathways (418). These properties offer numerous advantages over available competitive anti-cytokine receptor drugs and signal-specific CSAIDs for the treatment of inflammatory conditions (including increased selectivity as illustrated in Fig. 8). Accordingly, 101.10 has been shown to be at least as effective

to IL-Ra in a murine model of retinopathy of prematurity (603), and superior to anti-inflammatory corticosteroids and IL-1Ra in animal models of hyperthermia, inflammatory bowel disease as well as topically in contact dermatitis (418), as well as effective in a rodent model of osteoarthritis (642). Small noncompetitive peptides offer numerous advantages over large competitive drugs especially for the treatment of PTB (see section below).

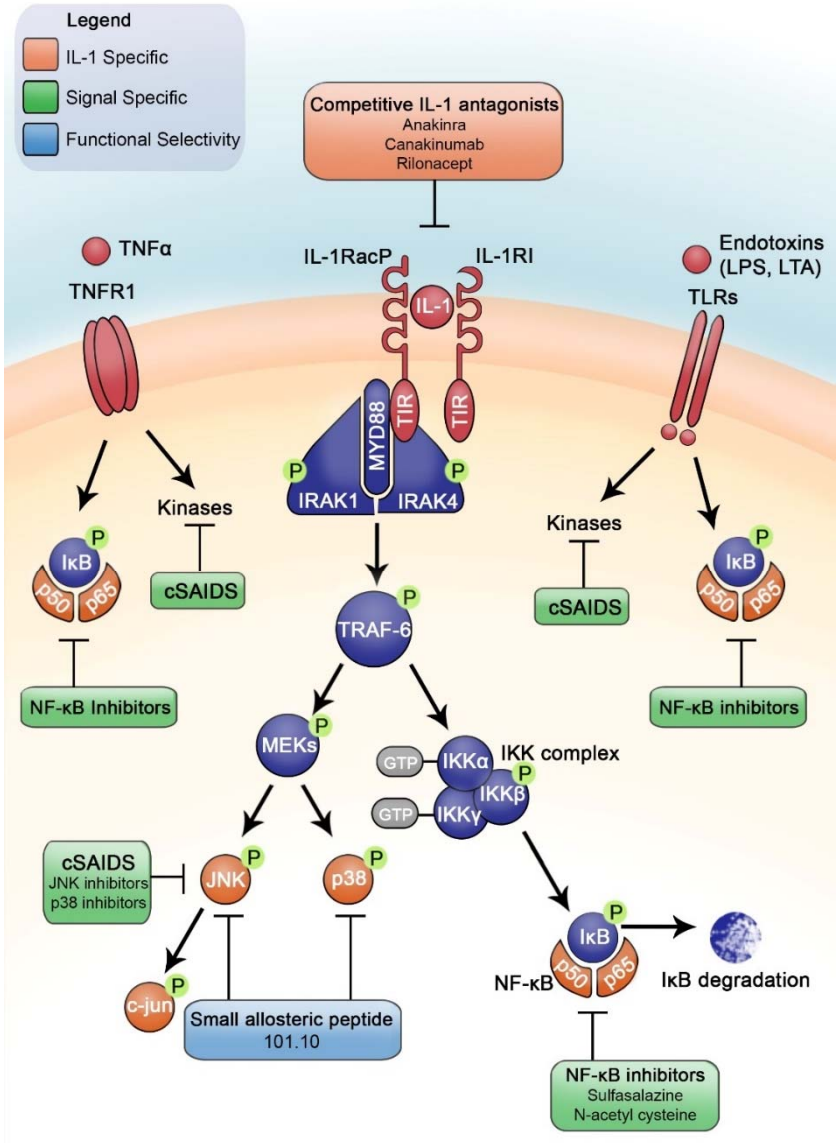

Figure 8. **Three distinct pharmacological strategies to inhibit IL-1 signals and their possible effects on other important systems.** Simplified representation of the interaction of different IL-1 modulators with three major components of the human immune response: the IL-1, TNFα and TLR systems. A therapy that would provide minimal hindrance of these systems is more likely to benefit from less adverse effects, notably in immuno-surveillance. IL-1-specific inhibitors (depicted in red) affect all signaling pathways that are downstream of IL-1 receptor activation (e.g. IL-1-induced AP-1 and NF-κB). Signal-specific inhibitors (depicted in green) affect a single signal without specificity for the receptor triggering it (depicted are tumor necrosis factor receptor 1 [TNFR1] activated by TNFα and TLRs activated by endotoxins). Whereas agents that exhibit functional selectivity (depicted in blue) affect specific IL-1 signals (e.g. IL-1-induced AP-1 or NF-κB), providing an unparalleled advantage in specificity.

## 4.2. Advantages of small noncompetitive peptides over large competitive molecules

Functional selectivity results in numerous benefits for small allosteric modulators over large competitive molecules, which include: a) negligible immunogenicity; b) high bioavailability and potential to be converted into orally bioavailable peptide mimics or small molecules; c) anticipated high therapeutic index (due to pathway specificity); d) not metabolized by (polymorphic) cytochrome P450; e) high potency; and f) economical costs (particularly relative to biologics) and ease of synthesis especially compared to costly antibodies and other biologics (such as anakinra, rilonacept and canakinumab). The latter is of particular interest considering the epidemiologic disparity between countries regarding PTB. In high income countries, almost 95% of babies born between 28 to 32 weeks of gestation survive, with more than 90% surviving without impairment. In contrast, in many low-middle income countries, only 30% of those born between 28 to 32 weeks of gestation survive, with almost all those born at less than 28 weeks dying in the first few days of life (1). Hence, based on the tremendous specificity to target recognition of peptidomimetic allosteric modulators, and the versatility of

their mechanisms by which they can interfere with protein functions, the development of peptides such as 101.10 into drugs offers desirable benefits and promise.

## 4.3. Hypotheses

Thus, based on the body of evidence presented above, we surmise that the noncompetitive IL-1R biased agonist 101.10 is effective in decreasing acute inflammation generated by IL-1, which constitutes a key event in both sterile and infectious PTB, thereby preventing the dissemination of inflammation to the fetus, and in turn preventing long-lasting fetal organ injury and functional impairment. We also hypothesize that lactate/GPR81 alleviates the response to IL-1 *in utero* during labor in a novel endogenous negative feedback mechanism to prevent detrimental excessive uterine inflammation.

# Part B: Articles

# Article 1: Novel Non-competitive Interleukin-1 Receptor Biased Ligand Prevents Infection- and Inflammation-induced Preterm Birth[1]

Mathieu Nadeau-Vallée[1,2], Christiane Quiniou[1], Julia Palacios[1], Xin Hou[1], Atefeh Erfani[1], Ankush Madaan[1,3], Mélanie Sanchez[1,3], Kelycia Leimert[4], Amarilys Boudreault[1], François Duhamel[1,2], José Carlos Rivera[1,5], Tang Zhu[1], Baraa Noueihed[1], Sarah A. Robertson[6], Xin Ni[7], David M. Olson[4], William Lubell[8], Sylvie Girard[9] and Sylvain Chemtob[1,5]

[1]Departments of Pediatrics, Ophthalmology and Pharmacology, CHU Sainte-Justine Research Center, Montréal, Canada, H3T 1C5; [2]Department of Pharmacology, Université de Montréal, Montréal, Canada H3C 3J7; [3]Department of Pharmacology and Therapeutics, McGill University, Montréal, Canada, H3G 1Y6; [4]Departments of Obstetrics and Gynecology, Pediatrics and Physiology, University of Alberta, Edmonton, AB, Canada; [5]Maisonneuve-Rosemont Hospital, Research Center, Montreal, Canada, H1T 2M4; [6]Department of Obstetrics and Gynecology, University of Adelaide, Adelaide, Australia; [7]Department of Obstetrics and Gynecology, Second Military Medical University, Shanghai, China; [8]Department of Chemistry, Université de Montréal, Montréal, Québec, Canada; [9]Departments of Obstetrics and Gynecology, Physiology & Pediatrics, CHU Sainte-Justine Research Centre, Montréal, Canada, H3T 1C5.

Running head: Non-competitive IL-1 receptor inhibitor delays preterm birth

Abbreviations: G, gestational day; IKK, inhibitor of NF-kB kinase; LTA, lipoteichoic acid; PTB, preterm birth; ROCK, Rho GTPase/Rho-associated coiled-coil–containing protein kinase;

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[1] Originally published in The Journal of Immunology. 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. Novel Noncompetitive IL-1 Receptor-Biased Ligand Prevents Infection- and Inflammation-Induced Preterm Birth. J Immunol. 2015 Oct 1;195(7):3402-15. 

SAPK, stress-associated protein kinase; SMC, smooth muscle cell; UAP, uterine activation protein.

Corresponding authors: Sylvain Chemtob, M.D., Ph.D. or Sylvie Girard, PhD. CHU Sainte-Justine, Research Center, Departments of Pediatrics, Ophthalmology and Pharmacology. 3175 Chemin Côte Ste-Catherine, Montréal, Québec, Canada, H3T 1C5. Fax: 1-514-345-4801, Tel: 1-514-345-4931 ext. 2978. E-mail: sylvain.chemtob@umontreal.ca or sylvie.girard@recherche-ste-justine.qc.ca

**Abstract**

Preterm birth (PTB) is firmly linked to inflammation independent of infection. Pro-inflammatory cytokines including interleukin (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 which exhibit competitive antagonism properties by inhibiting all IL-1 receptor signaling, including transcription factor NF-κB which conveys important physiological roles. We hereby demonstrate the efficacy of a small non-competitive (all-d peptide) IL-1 receptor biased ligand, termed rytvela (labelled 101.10) in delaying IL-1β-, Toll-Like Receptor (TLR) 2- and TLR4- induced PTB in mice. 101.10 acts without significant inhibition of NF-κB, and instead selectively inhibits IL-1 receptor downstream stress-associated protein kinases (SAPK) / transcription factor c-jun and Rho GTPase/ Rho-associated coiled-coil-containing protein kinase (ROCK) signaling pathways. 101.10 is effective at decreasing pro-inflammatory and/or pro-labor genes in myometrium tissue and circulating leukocytes in all PTB models independently of NF-κB, undermining NF-κB role in preterm labor. Herein, biased signaling modulation of IL-1 receptor by 101.10 uncovers a novel strategy to prevent PTB without inhibiting NF-κB.

Keywords: Preterm birth, Interleukin-1, Inflammation, LPS

## Introduction

Preterm birth (PTB; delivery before 37 weeks of gestation, also referred to as prematurity) affects more than 1 out 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-1 receptor antagonist (IL-1Ra) gene (*IL1RN*) are associated with spontaneous preterm deliveries (11); and 4) IL-1β stimulates uterine activation proteins (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 to 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, pre-clinical studies using IL-1 targeting agents reveal modest efficacy (16-18). At present there are three large molecule anti-IL-1 drugs approved for clinical use: the IL-1 receptor antagonist Anakinra (Kineret), the soluble

decoy receptor Rilonacept (Arcalyst) and the neutralizing monoclonal anti-IL-1β antibody 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 pro-inflammatory 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 (pro-apoptotic) 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 have 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 since 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-1 receptor, specifically rytvela (labelled 101.10), which selectively binds to IL-1 receptor and displays non-competitive 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-1 receptor

downstream SAPK/c-jun and Rho/ROCK pathways without significantly affecting NF-κB activation.

## Materials and methods

*Animals*

Timed-pregnant CD-1 mice were obtained from Charles River Inc at gestational day (G) 12 and were allowed to acclimatize for 4 days 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: rhIL-1β (#200-01B; PeproTech), lipoteichoic acid (LTA)(#L3265; Sigma), LPS (#L2630; Sigma), murine M-CSF (#315-02; PeproTech), 101.10 (Elim Biopharmaceuticals, Hayward, California), Kineret (Sobi, Biovitrum Stockholm, Sweden), SC-514 (#10010267; Cayman Chemical), SR-11302 (#2476; Tocris Bioscience), Y27632 (#Y0503; Sigma), β-estradiol (#2758; Sigma), rhIL-1α (#200-01A; PeproTech).

*Cell Culture*

The myometrial smooth muscle cell (SMC) line (hTERT-C3) was kindly provided by Dr. Stéphane 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 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 200µg/ml zeocin. Myometrial cells were cultured in DMEM/F12 growth medium supplemented with 10% fetal bovine serum (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 mins. 101.10, Kineret (1.5 mg/mL), SC-514 (10µM) or Y27632 (1µM) were allowed to reach equilibrium for 30 mins prior to the experiments. Cells lysis was performed in ice-cold RIPA 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 intraperitoneal LPS- and LTA-induced PTB models*

Timed-pregnant CD-1 mice at 16.5 days of gestation were anesthetized with isoflurane and received an intraperitoneal injection of either LTA (3x 3-hours interval injections of 12.5 mg/kg in 100µL saline), LPS (a single dose of 0.5µg in 100µL saline), or a single intrauterine injection of IL-1β (1µg). 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 of 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 closed with clips. One hundred μL of 101.10 (1mg/Kg/12h), Kineret (4mg/Kg/12h), SR-11302 (1mg/Kg/12h), Y27632 (0.5mg/Kg/12h) or vehicle was injected subcutaneously in the neck 30 mins before stimulation with IL-1β, LPS or LTA (to allow distribution of drugs to target tissues, in line with a first efficacy pre-clinical study); all doses utilized were based on reported efficacy (17, 24, 25, 28, 29). Mice delivery were assessed every hour until term (G19-G19.5). Immediately after delivery (<30 mins postpartum), female adults were anesthetized and an intra-cardiac 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 red blood cell lysis buffer (Norgen Biotek Corporation) and EDTA according to the manufacturer protocol and then centrifuged to isolate white blood cells. Resulting white blood cell pellet in addition of myometrium fragments cut from the lower part of the right uterine horn were snap-frozen in liquid nitrogen and kept at -80°C for subsequent RNA purification or protein extraction.

*RNA extraction and Real-Time quantitative Polymerase Chain Reaction (RT-qPCR)*

Myometrium fragments were thawed and rapidly preserved in RIBOzol (AMRESCO, Solon OH, United States), whereas cells from *in vitro* experiments were treated for 6 hours 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 was measured with a NanoDrop 1000 spectrophotometer. Five hundred ng of RNA was used to synthetize cDNA using iScript Reverse Transcription SuperMix (Bio-Rad, Hercules CA, United

States). Primers were designed using NCBI Primer Blast (Table I). Quantitative gene expression analysis was performed on Stratagene MXPro3000 (Stratagene) with SYBR Green Master Mix (BioRad). 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: *IL1B*, *IL4*, *IL6*, *IL8*, *IL10*, *TNFA*, *CCL2* (chemokine ligand 2), *CRP* (C-reactive protein), *MMP1A*, *MMP3*, *MMP9*, *PTGHS2* (Prostaglandin H synthetase 2 or COX-2), *PTGFR* (prostaglandin F receptor), *OXTR* (oxytocin receptor), *IL1R1* (IL-1 receptor 1), *GJA1* (connexin 43), *IL1RA* (IL-1 receptor endogenous antagonist) and *IFNB1* (interferon β1). Detailed primer sequences are shown in Table I.

*Semiquantitative PCR*

Cells were pre-treated with $10^{-6}$ M of 101.10 or vehicle for 30min, then stimulated with 50 ng/mL IL-1α for 24h. Total RNA was isolated with RNase TM mini kit (Qiagen, Germantown MD, United States). 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 RIPA buffer (containing protease and phosphatase inhibitors) were quantified using Bradford's method (Bio-Rad). Fifty μg of protein sample were loaded onto SDS-PAGE gel and

electrotransfered onto PVDF membranes. After blocking, membranes were incubated with either an antibody against IL-1R1 (#sc-689; Santa Cruz Biotechnology, Dallas TX, United States), OxtR (#ab101617; abcam, Toronto ON, Canada), α-actin (#ab5694; abcam), F4/80 (#ab6640; abcam), Lamin B1 (#ab16048; abcam), NF-κB p65 (#sc-372, Santa Cruz Biotechnology), IL-1RacP (#ab8110; abcam) or β-actin (#sc-47778; Santa Cruz Biotechnology). After washing, membranes were incubated for 1 hour with their respective secondary antibodies conjugated to HRP (Sigma). For kinases, membranes were incubated with an antibody 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, United States). Enhanced chemiluminescence (GE Healthcare) was used for detection using the ImageQuant LAS-500 (GE Healthcare, Little Chalfont, United Kingdom) 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 (RBD) 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 approximatively 50% confluence for 16 hours prior to the experiment. 101.10 or Kineret were administered 30 mins

before the IL-1β stimulation to allow the system to equilibrate. After 15 mins 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 approximatively 1 hour during protein quantitation. After thawing, 800 µg of each samples were incubated on a rocking platform with 50µg of rhotekin-RBD beads (high affinity for GTP-bound RhoA) for one hour at 4°C. As a positive control, 800 µg of cell lysate was incubated for 15 mins with 200µM GTPγS (a non-hydrolysable GTP analog) prior to the bead pull-down. After washing steps, samples were centrifuged and bead lysates were loaded on SDS-PAGE gel in 2X Laemmli buffer. Samples were electrotransfered on PVDF membranes, blocked and incubated with an anti-RhoA monoclonal antibody (#ARH03; Cytoskeleton) overnight at 4°C. The membrane was then incubated with an HRP-conjugated anti-mouse secondary antibody (Sigma) and revealed using an enhanced ECL chemiluminescence solution (GE Healthcare). Total RhoA expression and β-actin were assessed using 50 µg of the samples that was set aside on ice before pull-down. Densitometric analysis was performed using ImageJ.

*Circulating leukocyte RNA purification*

As described before, white blood cells were isolated from systemic blood of female mice (<30 mins postpartum) and total leukocyte RNA was extracted using a Leukocyte RNA purification kit (Norgen Biotek Corporation, Thorold ON, Canada). Briefly, the white blood cell pellet was lysed and passed through an 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). RT-qPCR was then performed on the samples as previously described.

*NF-κB Quanti-Blue assay*

Hek-Blue cells (InvivoGen) were pre-treated with different concentrations of 101.10 ($10^{-9}$ M to $10^{-5}$ M) and Kineret (1.5 mg/ml) for 30 mins followed by treatment with constant concentration of IL-1β (1 μg/ml), then incubated at 37°C for 4 hours. Levels of secreted alkaline phosphatase (AP) in cell culture supernatant were determined by the use of QUANTI-Blue according to manufacturer instruction (InvivoGen, San Diego CA, United States). AP activity was assessed by reading the OD at 620-655 nm with a micro plate reader (EnVision Multilabel reader, PerkinElmer, Waltham MA, United States). Data are representative of 5 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 (1mg/kg in 100μL saline). Within 30 mins, mice were injected intraperitoneally with IL-1β (1μg/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 to 3mm wide and 10mm long) were dissected free from uterus, mounted isometrically in organ tissue baths and initial tension was set at 2 g. The tissue baths contain 20 ml of Krebs

buffer of the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 0.9 MgSO4, 1 KH2PO4, 11.1 glucose, and 23 NaHCO3 (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 hour equilibration. Mean tension of spontaneous contractions were 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 were 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 subcutaneous injection of 50µg 17β-estradiol was administered to mice 24h 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 (Hank's balanced salt solution, 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, Grand Island, NY], and 2.5 µg/mL amphotericin B [Sigma]). 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 1mm 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, Mannheim, Germany], 0.1 mg/mL soybean trypsin inhibitor [sigma], 10% FBS, and 1 mg/mL bovine serum albumin [BSA, Sigma] in buffer B). Enzymatic digestion was performed at 37°C with agitation (100 rev/min) for 30 mins. The homogenate (still containing undigested myometrium fragments) was then poured through a 100 µm cell strainer. The resulting filtered solution was centrifugated at 200g for 10 mins, the pellet was resuspended in complete DMEM medium and plated in a T-25 dish. The remaining myometrium fragments were re-used in an enzymatic digestion and the whole digestion-centrifugation process was repeated for a total of 5 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 mins 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 analysed in immunohistochemistry to assess culture purity with the SMC marker α-actin.

*Primary Bone Marrow-derived Macrophages (BMM) isolation and culture*

CD-1 mice were sacrificed with cervical dislocation 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 RPMI1640 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. 20 ng/ml of rM-CSF was added prior to incubation, and cells were allowed to differentiate for 6 days. Cells were further analysed in immunohistochemistry to assess culture purity with the macrophage marker F4/80.

*Murine IL-1β ELISA assay*

The ELISA assay was performed using a mouse IL-1β Quantikine ELISA kit (R&D systems) according to the manufacturer's protocol. Briefly, 50 μL of either plasma samples, recombinant mouse IL-1β positive control or decreasing concentrations of a recombinant mouse IL-1β standard were loaded into a 96-well plate pre-coated with a monoclonal anti-mouse IL-1β antibody and incubated for 2 hours at ambient temperature. Wells were washed 5 times and incubated with an enzyme-linked mouse polyclonal antibody specific to murine IL-1β for 2 hours. After another washing step, a substrate solution was added. The enzymatic reaction was stopped after 30 mins and the plate was red at 450 nm, with wavelength correction set to 570 nm.

*Immunohistochemistry*

Cells were plated on cover slips pre-coated with poly-D-lysine and fixed in 4% paraformaldehyde. After blocking, cells were incubated overnight with 101.10-FITC or FITC alone (Sigma) and a primary antibody of rabbit anti-IL-1RI, rabbit anti-α-actin or rat anti-F4/80 and then for 1 hour at ambient temperature with a secondary antibody conjugated with Alexa Fluor 594 (red) or 647 (white)(Sigma). For tissue immunohistochemistry, mice were treated

with a single subcutaneous 1mg/Kg 101.10-FITC injection and animals were euthanized after 1 hour of incubation. Uterine tissues were cleansed of fat and vessels. Myometrium fragments and placentas were fixed in 4% paraformaldehyde for 1 day 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% bovine serum albumin, 1% goat serum and 0.1% TritonX-100 (T-8787; Sigma) in PBS were subsequently incubated overnight with the primary antibodies. Secondary antibodies conjugated with Alexa Fluor (Molecular Probes) directed against rabbit or rat were incubated for 2 hours at ambient temperature. Nuclei were stained with Dapi (Invitrogen; 1/5000). Images were captured using 10X (for myometrium tissues) or 30x (for cells and magnified placenta images) objective with Eclipse E800 (Nikon) fluorescence microscope. Whole placenta images were captured at 10X 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 analysis of variance (ANOVA). Dunnett's multiple comparison method was employed when treatments were compared to a single control. Tukey's multiple comparison test was used in figure 1G. A value of $p<0.05$ was considered statistically significant. Data are presented as means +/- S.D.

## Results

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

We first determined if 101.10 was effective at delaying PTB induced specifically by intra-uterine IL-1β. One μg of 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 since 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 to sham animals (Fig.1B left panel); this inflammation was alleviated by 101.10 ([1mg/Kg/12h subcutaneous injections], Fig.1B right panel). Accordingly, IL-1β-treated mice receiving vehicle (n=16) rapidly went into premature labor, with 56% delivering within 24h after IL-1β administration. Whereas in IL-1β-treated mice that received 101.10 (n=17), only 12% delivered before G19 (Fig.1C bottom panel). In contrast, systemic (subcutaneous) administration of the competitive IL-1 inhibitor Kineret (n=11) was ineffective at reducing prematurity (Fig.1C top panel) and increasing gestational duration (Suppl. Fig.1A). A group simply treated with 101.10 (without IL-1) served for gross toxicity evaluation; there was no gross teratogenic changes detected in all major organs examined.

Analysis of myometrium samples collected within 30 mins of pup delivery revealed that 101.10 diminished IL-1β-triggered induction of mRNA of numerous pro-inflammatory and/or pro-labor genes, including many uterine activation protein (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-1 receptor (Suppl. Fig. 2A) and oxytocin receptor (Suppl. Fig. 2B), but not of the IL-1 receptor accessory protein (IL-1RacP) (Suppl. 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 prostaglandin $F_{2\alpha}$ 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 to controls (n=6).

Consistent with its functional inefficacy, Kineret was ineffective in altering IL-1β-induced myometrial gene expression (Fig. 1D,F); similar results were observed on gene expression in placenta (Suppl. Fig.3A). On the other hand, gene expression profile of circulating leukocytes collected <30 mins 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 (utero-placental 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).

*101.10 distributes to myometrial SMC, macrophages, and placenta*

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

We previously showed that actions of 101.10 required presence of the ubiquitous IL-1RI (25). Accordingly, here again 101.10-FITC co-localized by immunohistochemistry with IL-1RI on the myometrial cell line hTERT-C3 and the macrophage cell line RAW-Blue mouse macrophages (Suppl. Fig.4E-H); FITC alone did not co-localize with IL-1R1.

*101.10 delays Toll-Like Receptor (TLR) 2- 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. 101.10 was particularly effective in (intraperitoneal) LTA-induced PTB (Fig.2A), as it prolonged gestation (Suppl. Fig. 1B). 101.10 also nearly normalized LTA-induced expression of all genes screened 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).

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

*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 IKK kinases results in its ubiquitination and subsequent degradation hence promoting NF-κB activation (36). 101.10 (dose-dependently) did not affect IL-1β-induced IκBα phosphorylation in myometrial cells, whereas Kineret completely inhibited its activation (Fig.4A and 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 and 4E). Collectively, these data indicate that effects of 101.10 are independent of NF-κB.

*101.10 inhibits stress-associated protein kinase (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 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. 101.10 dose-dependently decreased IL-1β-induced phosphorylation of p38, JNK and of the transcription factor c-jun in both cell types (Fig.5B-E); Kineret was also effective. 101.10 (like Kineret) also decreased IL-1β-triggered induction of several pro-inflammatory and/or pro-labor genes *in vitro* (Fig.5F,G), as previously observed *in vivo* (Fig. 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,J).

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 more than 95% of cells (Suppl. Fig.4I,J). 101.10 dose-dependently inhibited the activation of p38, JNK and c-jun (Fig.6A,B), and decreased the induction of several pro-inflammatory and/or pro-labor 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,E).

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

*Inhibiting AP-1 delays inflammation-induced preterm birth*

Since our *in vitro* and ex-vivo studies suggest that 101.10 acts by inhibiting IL-1 receptor 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, were comparably effective to 101.10 in reducing preterm delivery (Fig.7A,B) and increasing gestational length (Suppl. Fig.1D).

**Discussion**

Inflammation plays a critical role in labor (39). Various major pro-inflammatory cytokines including IL-1 upregulate uterine activation proteins (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 non-selective inhibition of the entire IL-1R-coupled signaling pathways, including NF-κB which has an important role in cytoprotection and immune-surveillance (40-42). Herein, we describe the efficacy of a non-competitive stable (all-d peptide) modulator of IL-1 receptor at delaying murine PTB models induced by IL-1β, LTA (TLR2 ligand) and LPS (TLR4 ligand). 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 towards labor (43, 44). It is believed that the initial inflammatory stimulus, such as PAMP or DAMPs activates innate immunity by binding on Toll-like receptors. 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 promote the onset of labor. Accordingly, data from our lab 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 present study; IL-1β and TLR ligands induced various UAP including *OXTR*, *PTGFR*, *PGHS2*, *CCL2* and *GJA1* in myometrium. Products of these pro-inflammatory genes amplify the initial insult. Hence, targeting pro-inflammatory 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 an NF-κB-independent manner). Another IL-1 receptor 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 (Suppl. Fig. 2E).

In this study we report for the first time the efficacy of a small non-competitive inhibitor of IL-1 receptor termed 101.10, in PTB. The peptide showed better efficacy than the competitive IL-1 receptor 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 likely several fold higher in the immediate vicinity of cytokine-releasing cells. Contrary to 101.10, Kineret did not interfere with myometrial gene induction. Since Kineret did reduce blood leukocyte induction of inflammatory genes, the selective IL-1 receptor 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 which 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 include: 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 pro-apoptotic 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 sequelæ from myometrial contraction-induced ischemia (22); and 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-1 receptor-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 that 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 pro-inflammatory and/or pro labor 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-kB 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 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 present study, several mediators of inflammation were induced in our rodent models (e.g. IL-6, IL-8 and COX-2); yet specific inhibition of IL-1 receptor by 101.10 reduced PTB induced by every stimulus (IL-1, LPS, LTA) tested, highlighting its critical role. Extrapolation of these findings to human 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-positive 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-negative 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: 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); 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 likely less compared to recombinant proteins and antibodies; the latter provides a more suitable therapeutic option for developing countries, where prematurity is a main cause of mortality (78).

In summary, we hereby describe the first non-competitive biased modulator of a cytokine receptor showing efficacy in delaying the onset of preterm birth. 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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**Contribution**

Participated in research design: Nadeau-Vallée, Quiniou, Hou, Olson, Chemtob; conducted experiments: Nadeau-Vallée, Quiniou, Hou, Palacios, Madaan, Boudreault, Erfani, Leimert, Rivera, Zhu, Noueihed; performed data analysis: Nadeau-Vallée, Quiniou, Hou, Erfani, Leimert, Duhamel, Chemtob; wrote or contributed to the writing of the manuscript: Nadeau-Vallée, Rivera, Sanchez, Quiniou, Robertson, Ni, Lubell, Olson, Girard, Chemtob. All authors participated in the critical review of the manuscript and all approved the final manuscript.

**Disclosures**

None.

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**Figure legends**

Figure 1. **101.10 prevents IL-1β-induced preterm birth and curbs inflammation-induced uterine activation.** A, The labor-inducing agent is injected at gestational day (G) 16.5 and spontaneous deliveries happening between G16.5 and G18.5 are considered as premature. Subcutaneous injections of 101.10 (1mg/Kg/12h), Kineret (4mg/Kg/12h) or vehicle are given twice a day until delivery. B, Representative picture of uteri 24 hours after the intrauterine IL-1β injection. Left panel: sham; central panel: IL-1β-induced uterine inflammation; right panel: 101.10 decreases clinical signs of IL-1β-induced uterine inflammation C, 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 mins 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 mins). Results are normalized with 18S and are relative to control. F, 101.10 decreases the expression of oxytocin receptor (left panel) and FP (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 ± S.D. 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 to IL-1β + vehicle group.

Figure 2. **101.10 decreases Toll-Like Receptor (TLR) 2-induced preterm birth.** A, 101.10 decreases preterm birth induced by the TLR2 agonist LTA in mice. Left panel: percentage of animals having delivered following three 3-hours interval intraperitoneal LTA injection (12.5 mg/Kg); right panel: percentage of premature deliveries. Control mice did not receive any treatment, whereas sham animals received three intraperitoneal doses of vehicle over a period of 9 hours at G16.5. 101.10 (1mg/Kg/12h) or vehicle were injected subcutaneously twice a day until delivery. B, quantitative PCR from myometrium tissue of mice treated in A and collected postpartum (<30 mins). 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 mins). 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 mins). Values are presented as mean ± S.D. 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 to LTA + vehicle group.

Figure 3. **101.10 decreases Toll-Like Receptor (TLR) 4-induced preterm birth.** A, 101.10 decreases preterm birth induced by the TLR4 agonist LPS in mice. Left panel: percentage of animals having delivered following a single intraperitoneal 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 intraperitoneal dose of vehicle at G16.5. 101.10 (1mg/Kg/12h) or vehicle were administered subcutaneously twice a day until delivery. B, quantitative PCR from myometrium tissue of mice treated in A and collected postpartum (<30 mins). 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 mins). 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 mins). Values are presented as mean ± S.D. Data are representative of 3-14 animals per group and of 4 *in vitro* experiments (F). *, p<0,05; **, p<0,005; ***, p<0,001 by one-way ANOVA with Tukey's multiple comparison test compared to LPS + vehicle group.

Figure 4. **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 hour 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 fetal bovine serum (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 hour and levels of secreted alkaline phosphatase in cell culture supernatant was 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 ± S.D. Data are representative of 3-5 experiments. ***, p<0,001 by one-way ANOVA with Tukey's multiple comparison test compared to IL-1β + vehicle group.

Figure 5. **101.10 inhibits SAPK/c-jun and RhoA/ROCK signaling pathways in both myometrial and macrophage cell lines.** A, Simplified IL-1 receptor 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-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 antibodies (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-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 antibodies (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 hours. Results are normalized with 18S and are relative 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 hours. 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 hours. 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 fetal bovine serum (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 fetal bovine serum (10%) as a positive control. Quantification of protein bands was normalized with ROCK2 and plotted as fold over control. Values are presented as mean ± S.D. Data are representative of 3-4 experiments. *, p<0,05; **, p<0,005; ***, p<0,001 by one-way ANOVA with Tukey's multiple comparison test compared to IL-1β + vehicle group.

Figure 6. **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-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 antibodies (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 hours. Results are normalized with 18S and are relative to control. D-E, Myometrium fragments were collected from CD-1 mice and incubated in serum-free medium for 1 hour 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 antibodies (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-G, primary bone marrow-derived macrophages (BMM) 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 antibodies (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 BMM 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 hours. Results are normalized with 18S and are relative to control. Values are presented as mean ± S.D. Data are

representative of 3-4 experiments. *, p<0,05; **, p<0,005; ***, p<0,001 by one-way ANOVA with Tukey's multiple comparison test compared to IL-1β + vehicle group.

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. 101.10 (1mg/Kg/12h), SR11302 (1mg/Kg/12h), Y27632 (1mg/Kg/12h) or vehicle were administered subcutaneously twice a day until delivery. C, proposed mechanism of action of 101.10. Effector cells comprise a wide range of possible cells, though the focus has been made on myometrial SMC and macrophages in this study. Values are presented as mean ± S.D. Data are representative of 3-10 animals per group. *, p<0,05; **, p<0,005; ***, p<0,001. NAM, negative allosteric modulator by one-way ANOVA with Tukey's multiple comparison test compared to IL-1β + vehicle group.

**Supplementary figure legends**

Figure S1. **Gestational length of mice from 3 different PTB models treated as indicated.** A and D, intrauterine IL-1β-induced PTB; B, intraperitoneal LTA-induced PTB; C, intraperitoneal LPS-induced PTB. E-F, immunoblot (E) and relative quantification (F) of protein bands of RAW-Blue macrophages treated with LTA or LPS for 5 mins in presence or in absence of 101.10 or Kineret and blotted against pTpY-p38 or p38 showing that 101.10 does not affect direct toll-like receptor signaling. G, simplified inflammatory cascade leading to premature labor. Values are presented as mean ± S.D. Data are representative of 3-4 experiments. One-way-ANOVA analysis was used: *, p<0,05; **, p<0,005; ***, p<0,001 by one-way ANOVA with Tukey's multiple comparison test. Values are presented as mean ± S.D. Data are representative of 3-14 animals per group. ROM, rupture of membrane.

Figure S2. **101.10-FITC administered subcutaneously reaches placental tissue.** Immunoblot of myometrium tissue from mice treated as indicated displaying the expression of IL-1R1 (A), OxtR (B) and IL-1RAcP (C) and relative quantification of protein bands (bottom panels). β-actin was used as a loading control. D, Murine IL-1β ELISA performed on plasma from mice treated as indicated and collected postpartum (<30 mins). IL-1β was used as a negative control since the test is highly specific to murine IL-1β and 150 pg/mL of murine IL-1β was used to assess precision. E, Representative semi-quantitative PCR of cells treated with IL-1α for 24 hours with and without 101.10. Values are presented as mean ± S.D. Data are representative of 3-12 experiments. One-way-ANOVA analysis was used: ***, p<0,001 by one-way ANOVA with Tukey's multiple comparison test compared to IL-1β + vehicle group.

Figure S3. **101.10 reaches the placenta and curbs IL-1-induced placental inflammation.** A, quantitative PCR performed on placentas collected 24h after a single IL-1β intrauterine injection from mice treated with 3 subcutaneous injections of either 101.10 (1 mg/Kg/12h), Kineret (4mg/Kg/12h) or vehicle. Results are normalized with 18S and are relative to sham. Values are presented as mean ± S.D and data are representative of 6 placentas from 6 different animals per group. The placenta selected for each animal was perfusing the first fetus in the proximal right uterine horn. B-D, representative images of longitudinal cryosections of placentas from non-treated mice (B), mice treated with FITC alone (C) or 101.10-FITC (D) 1h prior to sacrifice. 101.10-FITC was found in all parts of the placenta, but its localization was more pronounced in

the junctional zone (F and H), in the fetal membranes (E) and near fetal vessels (G). One-way-ANOVA analysis was used: *, p<0,05; **, p<0,005; ***, p<0,001 by ANOVA. Scale for A and B, 1000µM; scale for C-F, 100 µM. jcz, junctional zone; lb, labyrinth; bv, blood vessel; dc, decidua; fm, fetal membranes.

Figure S4. **101.10 binds to myometrial smooth muscle cells (SMC) and macrophages.** A, representative confocal image of myometrium sagittal cryosections immunohistochemically stained with 101.10-FITC (green) and the smooth muscle cell marker α-actin (white) and B, negative control showing no binding of FITC (green) alone. DAPI was used to stain nuclei. Scale bar, 100µM. C, representative confocal image of myometrium sagittal cryosections immunohistochemically stained with 101.10-FITC (green) and the macrophage marker F4/80 (red) and D, negative control showing no binding of FITC (green) alone. DAPI was used to stain nuclei. Scale bar, 100µM. E, 101.10-FITC (green) co-localizes with IL-1R1 (white) in myometrial cells culture (hTERT-C3 cell line) and F, negative control showing no co-localization with FITC (green) alone. DAPI was used to stain nuclei. Scale bar, 100µM. G, 101.10-FITC (green) co-localizes with IL-1R1 (white) in macrophages culture (RAW-Blue cell line) and H, negative control showing no co-localization with FITC (green) alone. I, approximately 95% of freshly isolated primary myometrial smooth muscle cells (SMC) culture was immunoreactive to α-actin (green). J, negative control showing no binding of the Alexa Fluor secondary antibody alone. DAPI was used to stain nuclei. Scale bar, 100µM. K, α-actin (M.W.:42 kDa) primary antibody used for immunohistochemistry binds to a 42 kDa protein in a primary myometrial SMC immunoblot. L, approximately 95% of freshly isolated primary bone marrow-derived macrophages (BMM) culture was immunoreactive to F4/80 (red). M, negative control showing no binding of the Alexa Fluor secondary antibody alone. DAPI was used to stain nuclei. Scale bar, 100µM. N, F4/80 (predicted M.W.:102 kDa) primary antibody used for immunohistochemistry show 3 different bands in a primary BMM immunoblot, most likely because F4/80 is heavily glycosylated. DAPI was used to stain nuclei. Scale bar, 100µM. Data are representative of at least 3 experiments.

## Figures

Figure 1

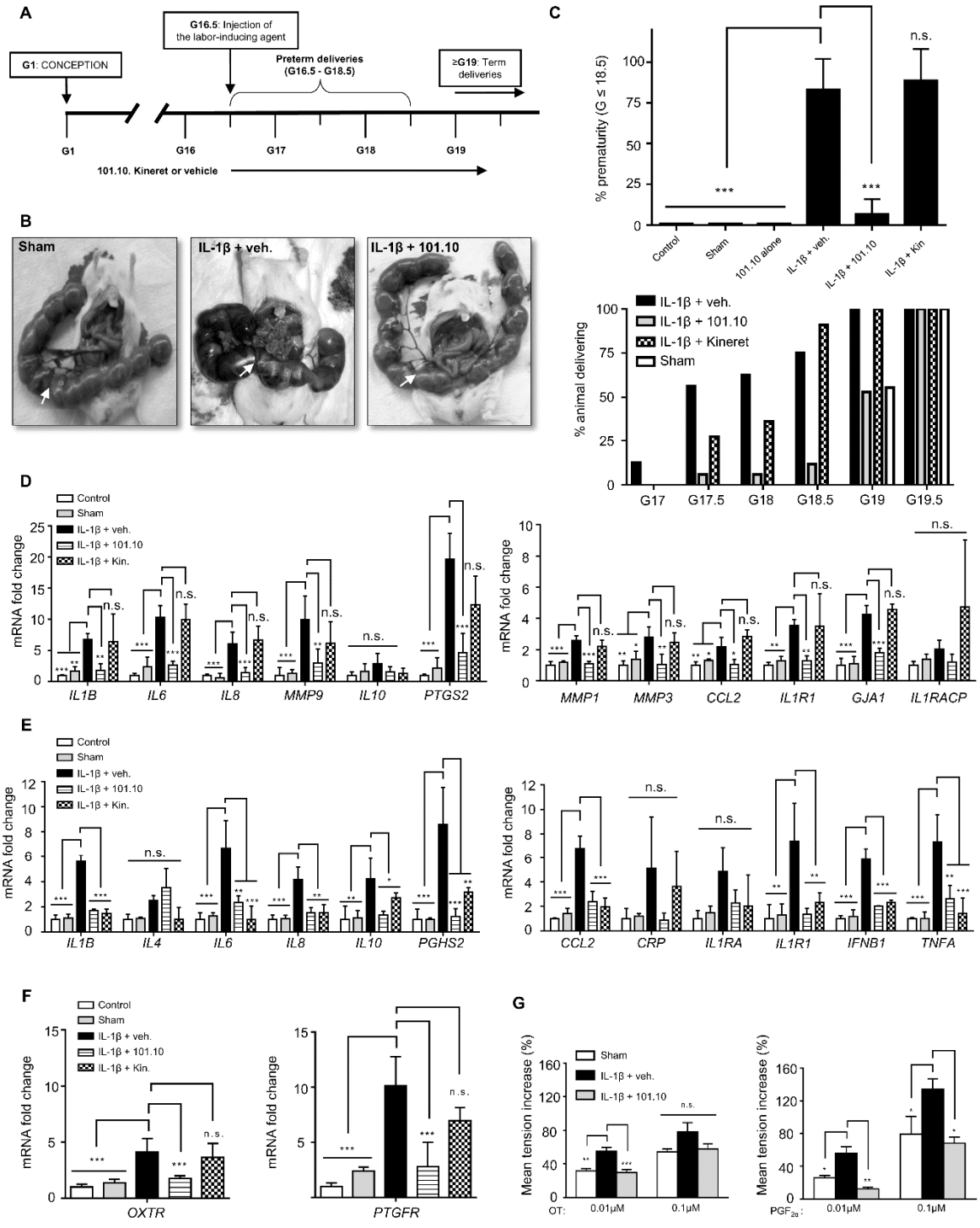
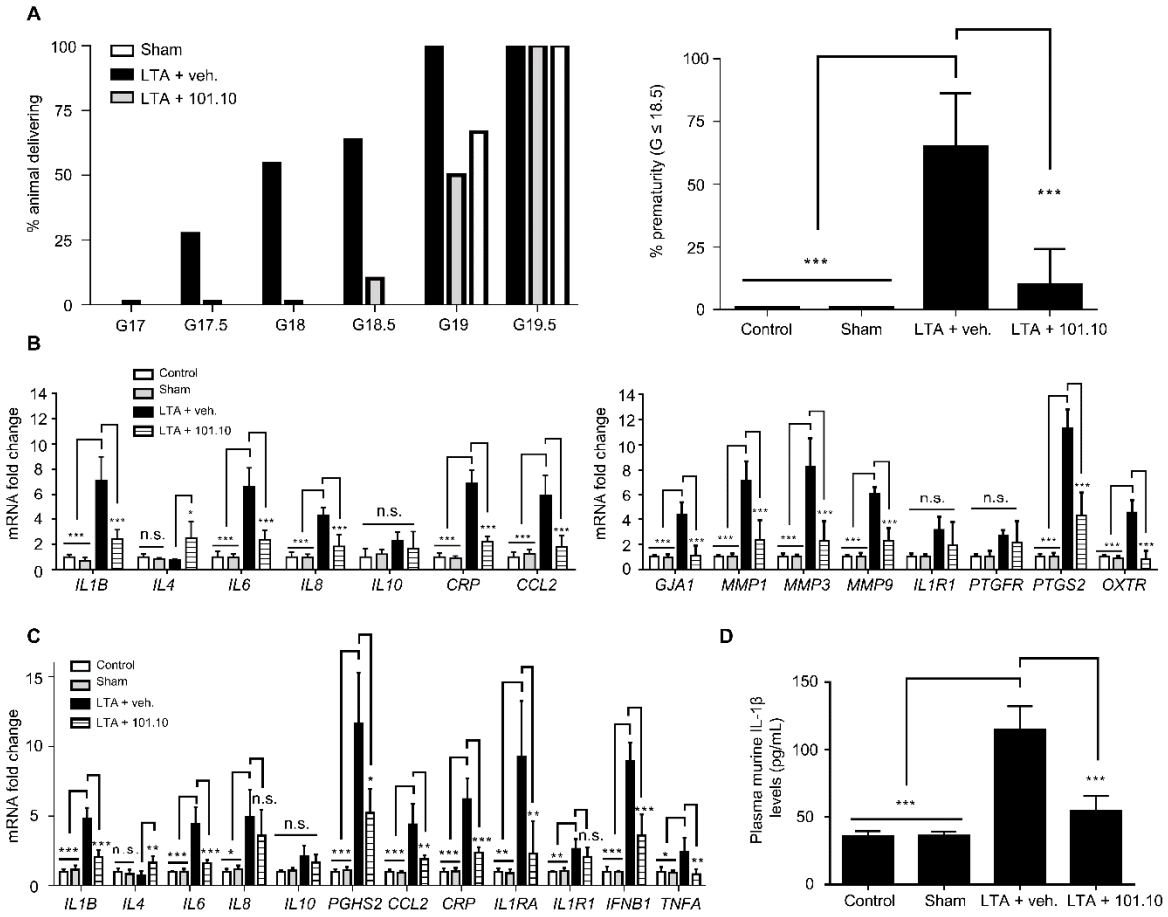


Figure 2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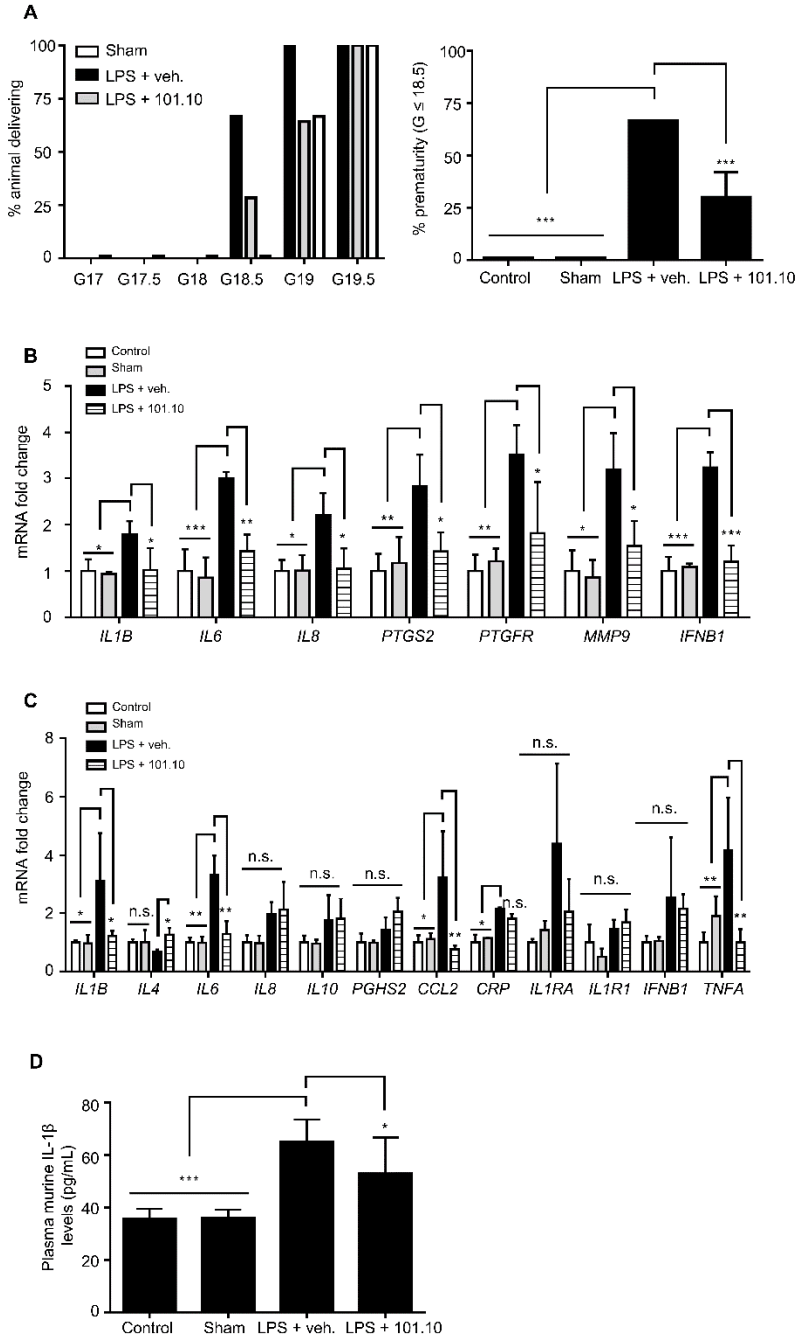
Sham
LTA + veh.
LTA + 101.10
% animal delivering
G17
G17.5
G18
G18.5
G19
G19.5
% prematurity (G ≤ 18.5)
Control
Sham
LTA + veh.
LTA + 101.10
B
mRNA fold change
IL1B
IL4
IL6
IL8
IL10
CRP
CCL2
GJA1
MMP1
MMP3
MMP9
IL1R1
PTGFR
PTGS2
OXTR
n.s.
C
PGHS2
CCL2
IL1RA
IFNB1
TNFA
D
Plasma murine IL-1β levels (pg/mL)

Figure 3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LPS + veh.
LPS + 101.10
% animal delivering
G17
G17.5
G18
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G19
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% prematurity (G ≤ 18.5)
Control
Sham
LPS + veh.
LPS + 101.10
B
mRNA fold change
IL1B
IL6
IL8
PTGS2
PTGFR
MMP9
IFNB1
C
mRNA fold change
n.s.
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IL4
IL6
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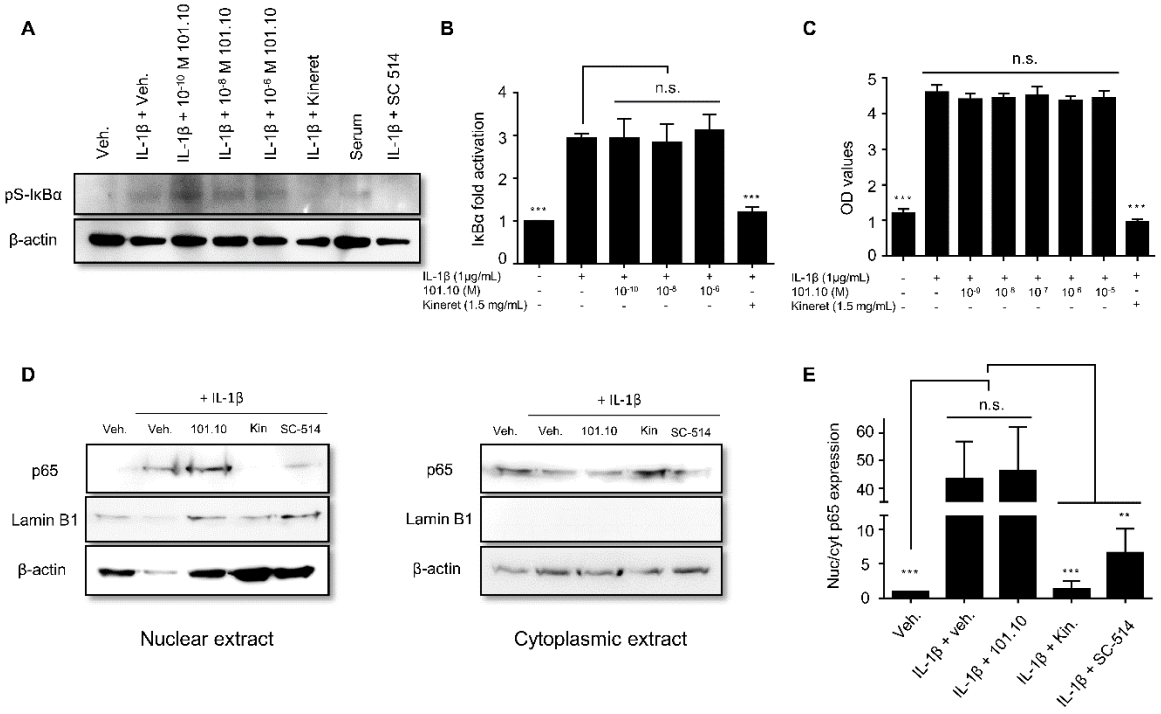
TNFA
D
Plasma murine IL-1β levels (pg/mL)

Figure 4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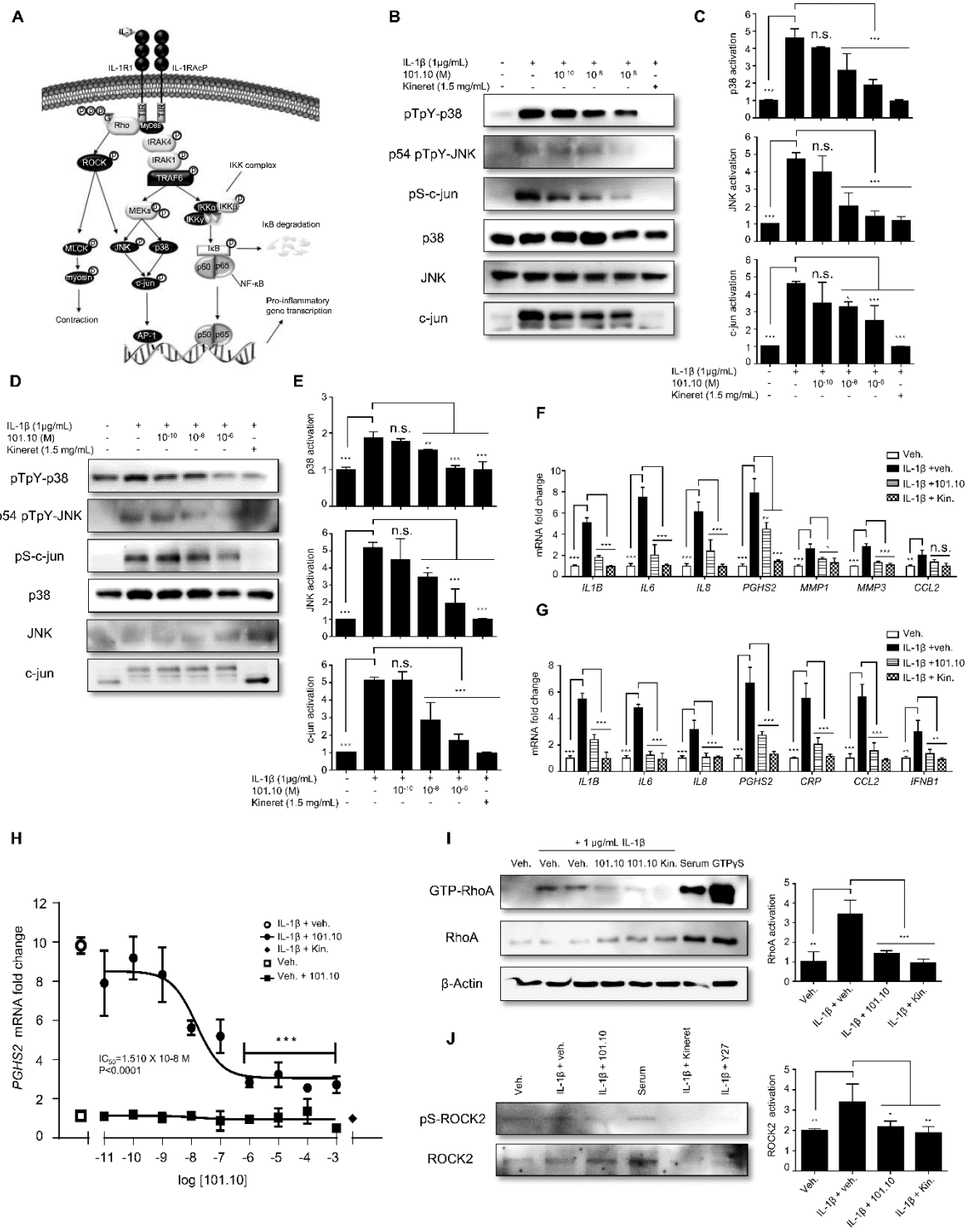
Veh.
IL-1β + Veh.
IL-1β + 10-10 M 101.10
IL-1β + 10-8 M 101.10
IL-1β + 10-6 M 101.10
IL-1β + Kineret
Serum
IL-1β + SC 514
pS-IκBα
β-actin
B
IκBα fold activation
n.s.
***
IL-1β (1μg/mL)
101.10 (M)
Kineret (1.5 mg/mL)
C
OD values
n.s.
***
IL-1β (1μg/mL)
101.10 (M)
Kineret (1.5 mg/mL)
D
+ IL-1β
Veh.
Veh.
101.10
Kin
SC-514
p65
Lamin B1
β-actin
Nuclear extract
Cytoplasmic extract
E
Nuc/cyt p65 expression
n.s.
***
**
Veh.
IL-1β + veh.
IL-1β + 101.10
IL-1β + Kin.
IL-1β + SC-514

Figure 5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IRAK1
TRAF6
ROCK
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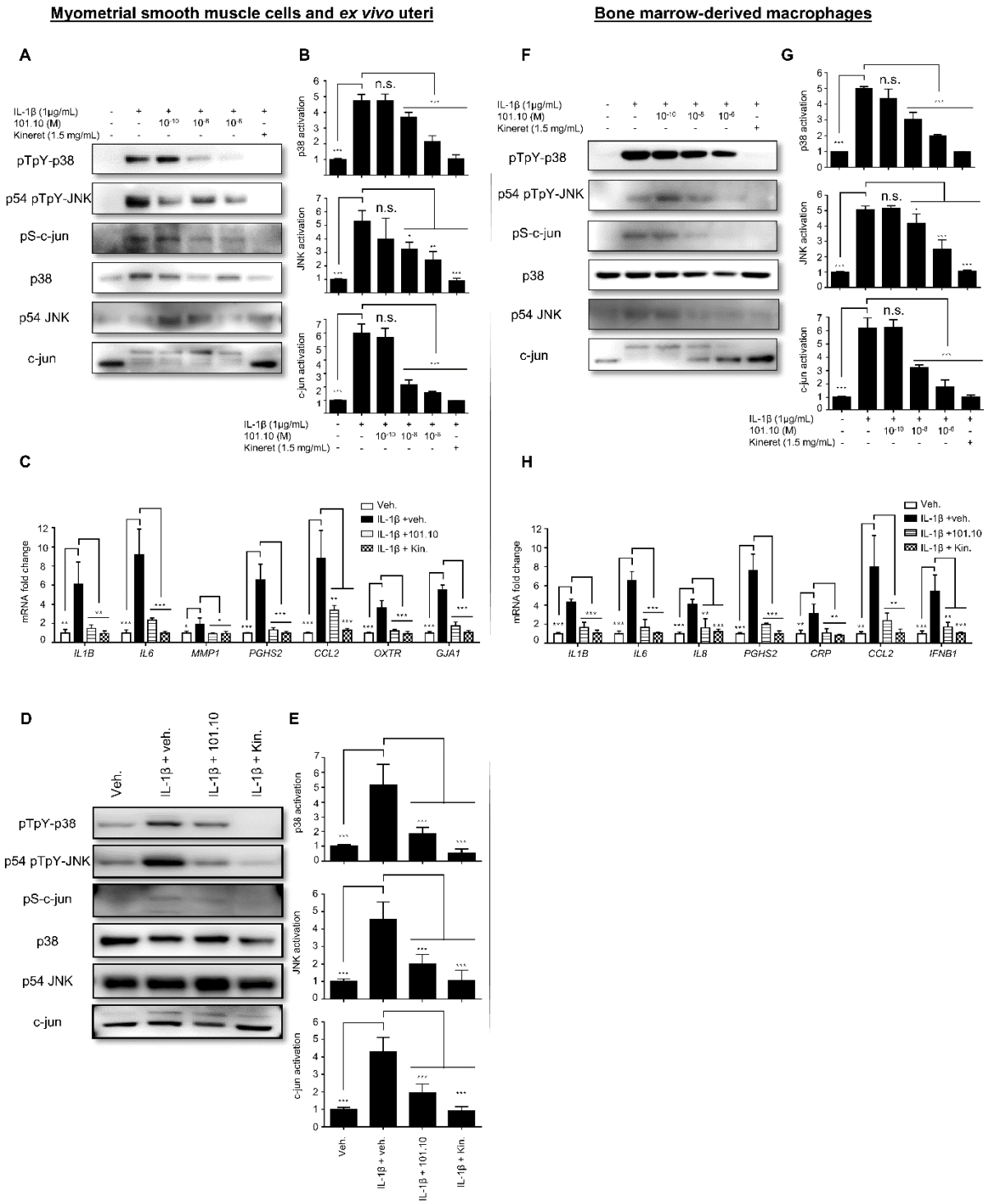
MEKs
IKKα
IKKβ
IKKγ
IκB degradation
MLCK
JNK
p38
IκB
p50
p65
myosin
c-jun
NF-κB
Pro-inflammatory gene transcription
Contraction
AP-1
B
IL-1β (1μg/mL)
101.10 (M)
Kineret (1.5 mg/mL)
pTpY-p38
p54 pTpY-JNK
pS-c-jun
p38
JNK
c-jun
C
p38 activation
JNK activation
c-jun activation
n.s.
D
E
F
mRNA fold change
Veh.
IL-1β +veh.
IL-1β +101.10
IL-1β + Kin.
IL1B
IL6
IL8
PGHS2
MMP1
MMP3
CCL2
G
CRP
IFNB1
H
PGHS2 mRNA fold change
IL-1β + veh.
IL-1β + 101.10
IL-1β + Kin.
Veh.
Veh. + 101.10
IC50=1.510 X 10-8 M
P<0.0001
log [101.10]
I
+ 1 μg/mL IL-1β
Veh. Veh. Veh. 101.10 101.10 Kin. Serum GTPγS
GTP-RhoA
RhoA
β-Actin
RhoA activation
J
Veh.
IL-1β + veh.
IL-1β + 101.10
Serum
IL-1β + Kineret
IL-1β + Y27
pS-ROCK2
ROCK2
ROCK2 activation

Figure 6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Bone marrow-derived macrophages
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B
C
D
E
F
G
H
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p54 pTpY-JNK
pS-c-jun
p38
p54 JNK
c-jun
n.s.
p38 activation
JNK activation
c-jun activation
mRNA fold change
Veh.
IL-1β +veh.
IL-1β +101.10
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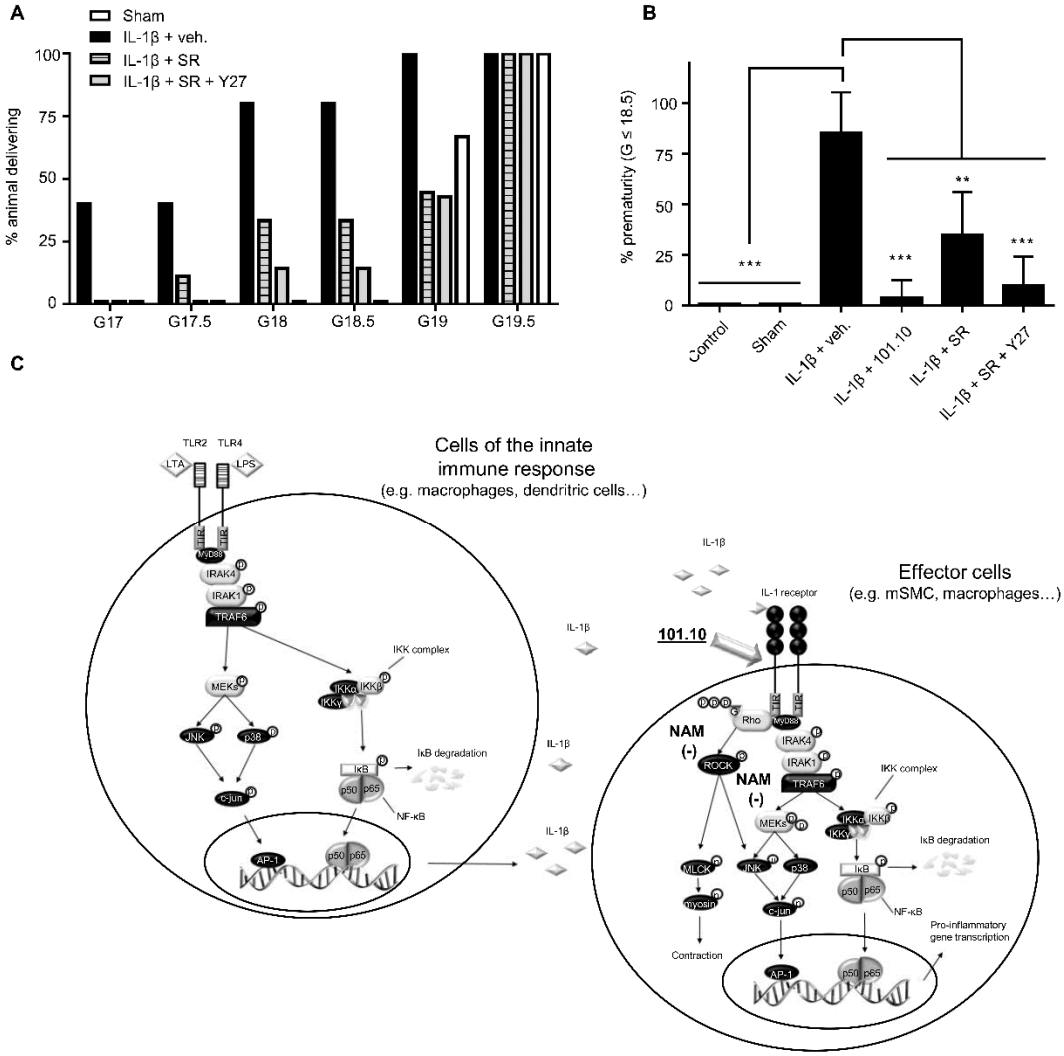
IL8
CRP
IFNB1

Figure 7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IL-1β + veh.
IL-1β + SR
IL-1β + SR + Y27
% animal delivering
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75
50
25
0
G17
G17.5
G18
G18.5
G19
G19.5
B
% prematurity (G ≤ 18.5)
100
75
50
25
0
***
***
**
***
Control
Sham
IL-1β + veh.
IL-1β + 101.10
IL-1β + SR
IL-1β + SR + Y27
C
TLR2
TLR4
LTA
LPS
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IRAK4
IRAK1
TRAF6
IKK complex
MEKs
IKKα
IKKβ
IKKγ
JNK
p38
IκB degradation
IκB
p50
p65
c-Jun
NF-κB
AP-1
Cells of the innate
immune response
(e.g. macrophages, dendritic cells…)
IL-1β
IL-1 receptor
101.10
Effector cells
(e.g. mSMC, macrophages…)
NAM
(-)
ROCK
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MLCK
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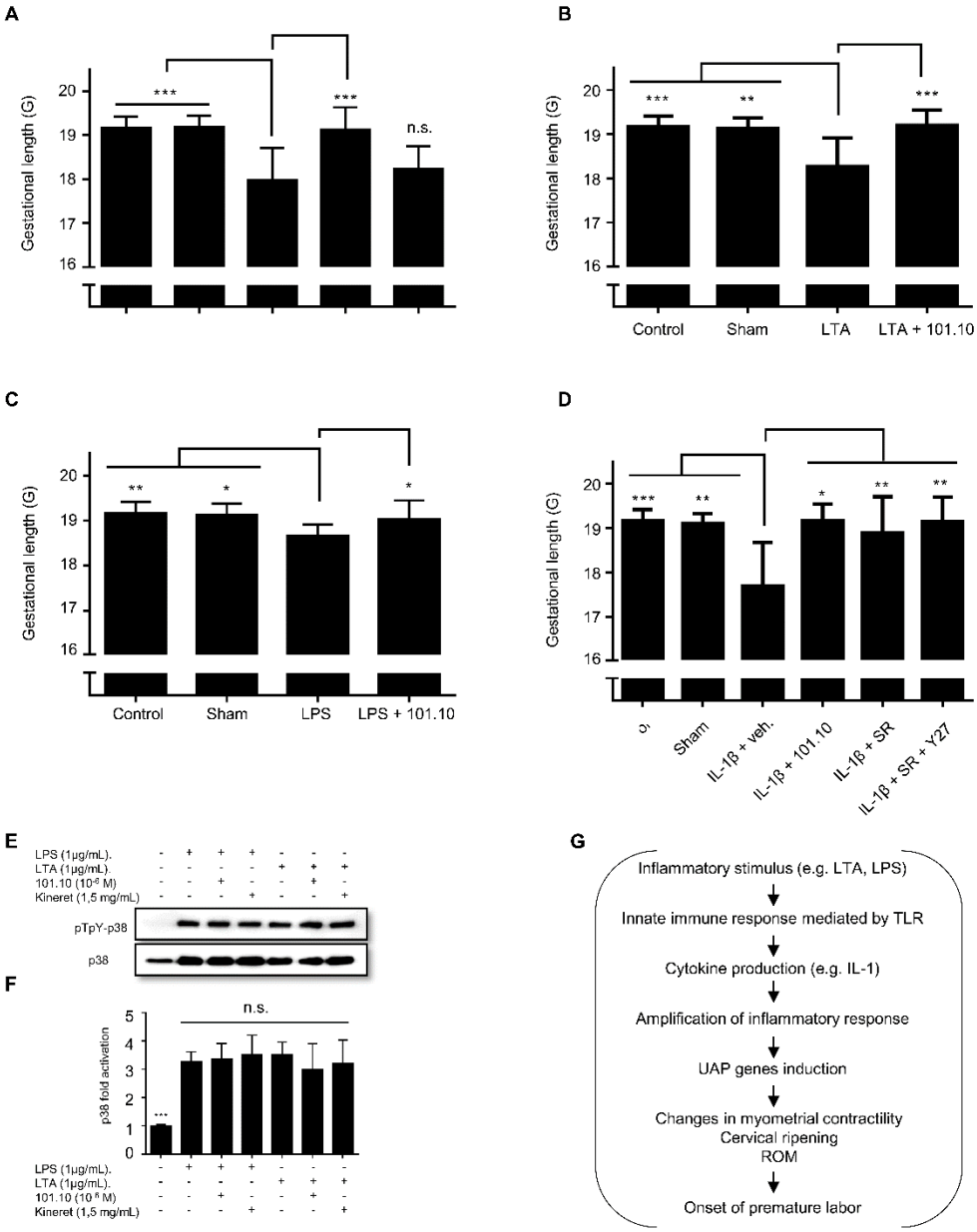
Contraction
Pro-inflammatory
gene transcription

## Supplementary Figures

Figure S1



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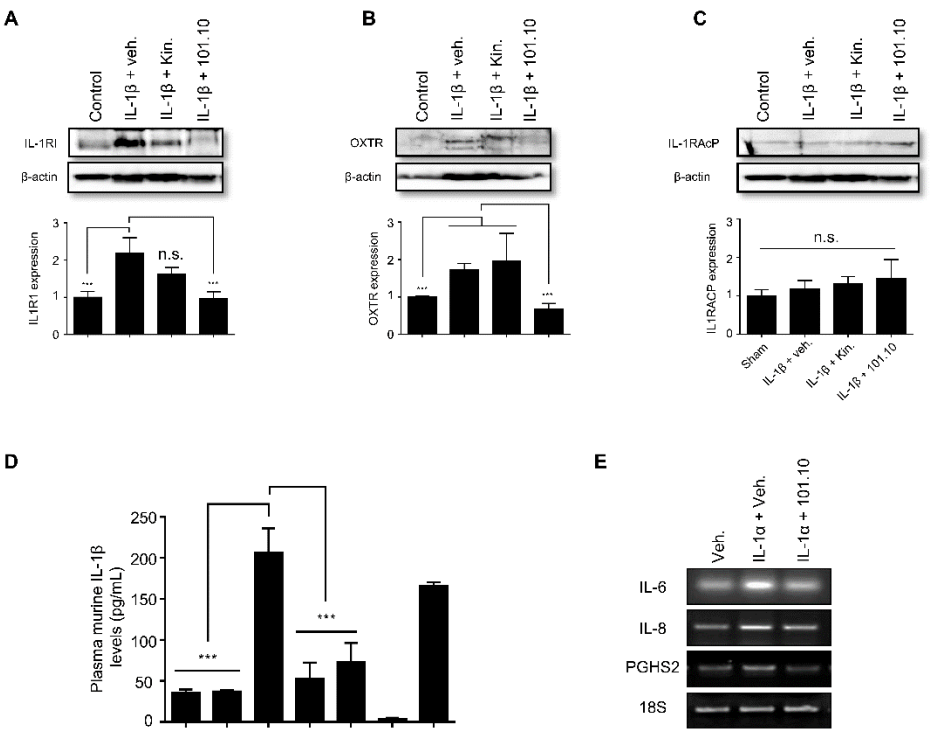
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β-actin
IL1R1 expression
n.s.
B
OXTR
β-actin
OXTR expression
C
IL-1RAcP
β-actin
IL1RACP expression
n.s.
Sham
D
Plasma murine IL-1β levels (pg/mL)
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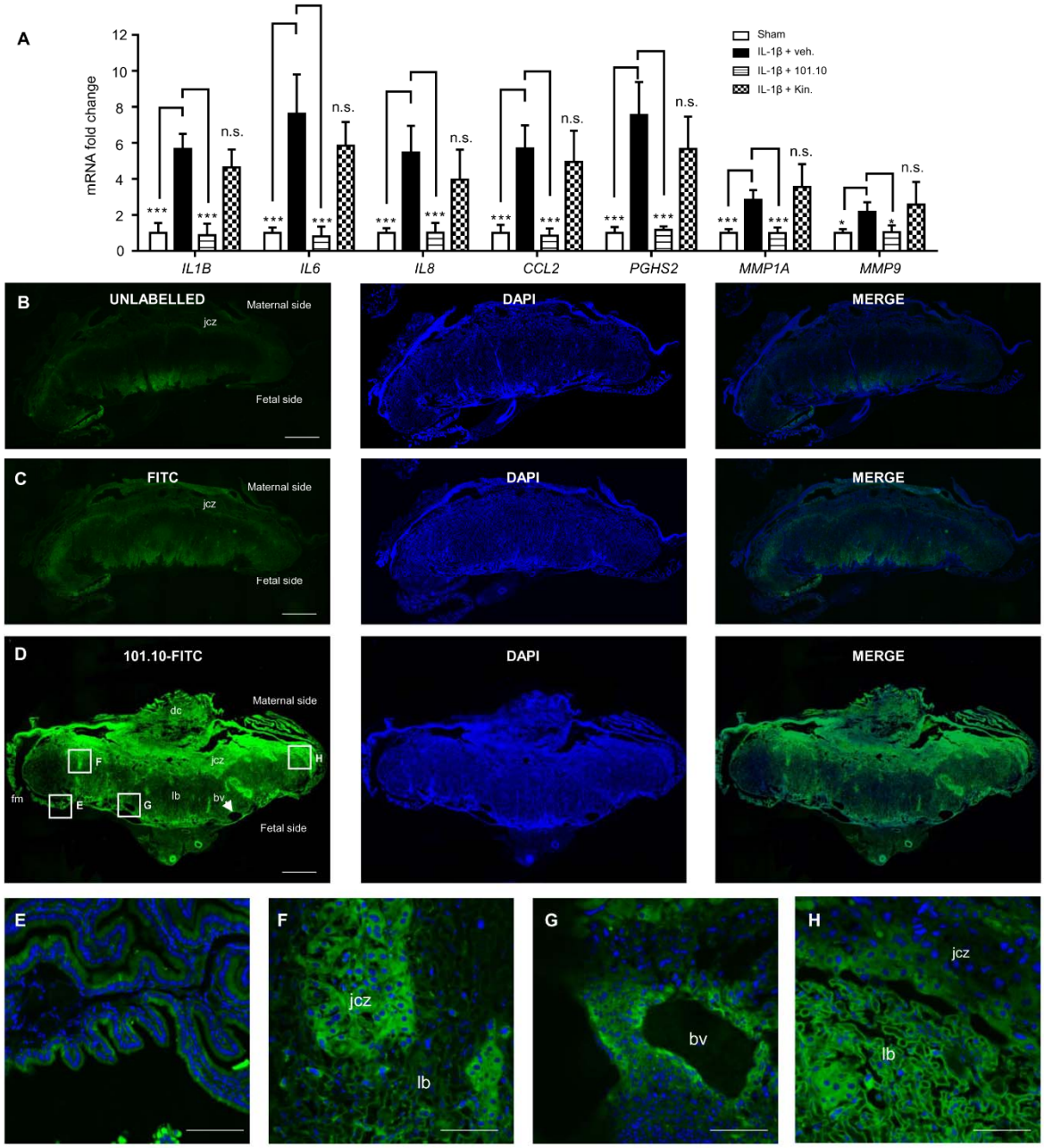
IL-1α + Veh.
IL-1α + 101.10
IL-6
IL-8
PGHS2
18S

Figure S3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IL-1β + 101.10
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IL1B
IL6
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Maternal side
jcz
Fetal side
DAPI
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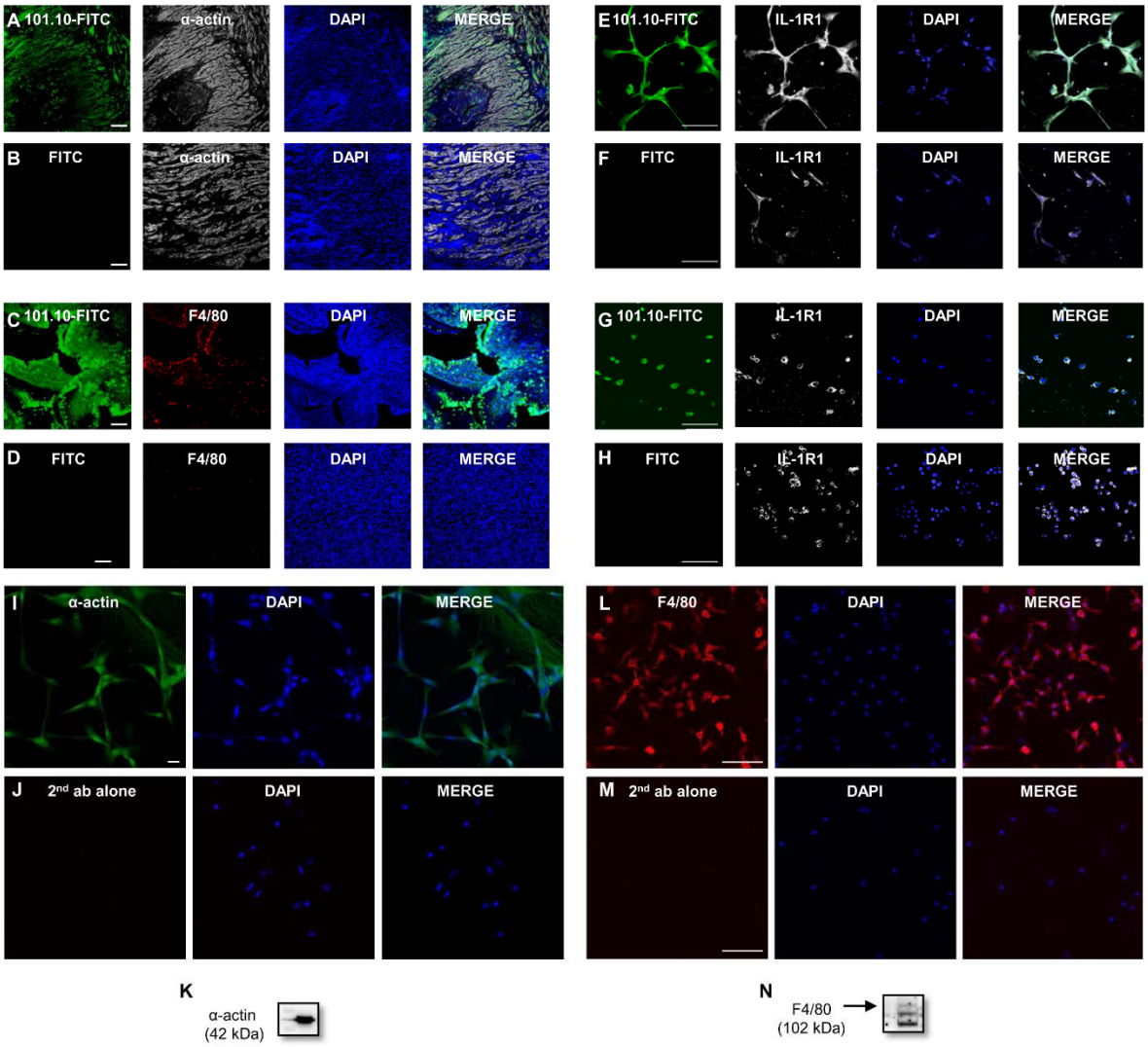
E
G
lb
bv
E
F
G
H

Figure S4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α-actin
DAPI
MERGE
B FITC
α-actin
DAPI
MERGE
C 101.10-FITC
F4/80
DAPI
MERGE
D FITC
F4/80
DAPI
MERGE
E 101.10-FITC
IL-1R1
DAPI
MERGE
F FITC
IL-1R1
DAPI
MERGE
G 101.10-FITC
IL-1R1
DAPI
MERGE
H FITC
IL-1R1
DAPI
MERGE
I α-actin
DAPI
MERGE
J 2nd ab alone
DAPI
MERGE
L F4/80
DAPI
MERGE
M 2nd ab alone
DAPI
MERGE
K
α-actin
(42 kDa)
N
F4/80
(102 kDa)

## Tables

Table I: Primers used for Real-Time qPCR

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

# Article 2: Antenatal suppression of interleukin-1 protects against inflammation-induced fetal injury and improves neonatal and developmental outcomes in mice[1]

Mathieu Nadeau-Vallée[1,2], Peck-Yin Chin[3], Lydia Belarbi[1], Marie-Ève Brien[4], Sheetal Pundir[1,5], Martin H. Berryer[6], Alexandra Beaudry-Richard[1], Ankush Madaan[1,5], David J. Sharkey[3], Alexis Lupien-Meilleur[6], Xin Hou[1], Christiane Quiniou[1], Alexandre Beaulac[1], Ines Boufaied[4], Amarilys Boudreault[1], Adriana Carbonaro[4], Jean-Sebastien Joyal[1,2], William D. Lubell[7], David M. Olson[8], Sarah A. Robertson[*,3], Sylvie Girard[*,4] and Sylvain Chemtob[*,1,2,5].

[1]Departments of Pediatrics, Ophthalmology and Pharmacology, CHU Sainte-Justine Research Center, Montreal, Canada, H3T 1C5; [2]Department of Pharmacology, Université de Montréal, Montreal, Canada, H3T 1J4; [3]Department of Obstetrics and Gynecology, Adelaide Medical School and Robinson Research Institute, University of Adelaide, Adelaide, Australia, SA 5005; [4]Department of Obstetrics and Gynecology; Microbiology, Infectiology and Immunology, Université de Montréal, CHU Sainte-Justine Research Center, Montreal, Canada, H3T 1J4; [5]Department of Pharmacology and Therapeutics, McGill University, Montréal, Canada, H3G 1Y6; [6]Department of Neurosciences, CHU Sainte-Justine Research Center, Université de Montréal, Montreal, Canada, H3T 1J4; [7]Department of Chemistry, Université de Montréal, Montréal, Québec, Canada, H3T 1J4; [8]Departments of Obstetrics and Gynecology, Pediatrics and Physiology, University of Alberta, Edmonton, Canada, T6G 2R3.

Running title: IL-1 blockade improves neonatal outcome

Abbreviations: BPD, bronchopulmonary dysplasia; FIRS, fetal inflammatory response syndrome; GD, gestational day; IL, interleukin; NEC, necrotizing enterocolitis; PT, post-term day; PTB, preterm birth; VEP, visual evoked potential.

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Corresponding authors: Sylvain Chemtob, M.D., Ph.D. CHU Sainte-Justine, Research Center, Departments of Pediatrics, Ophthalmology and Pharmacology, 3175 Chemin Côte Ste-Catherine, Montréal, Québec, Canada, H3T 1C5. Fax: 1-514-345-4801, Tel: 1-514-345-4931 ext. 2978. E-mail: sylvain.chemtob@umontreal.ca; and Sylvie Girard, Ph.D. CHU Sainte-Justine, Research Center, Departments of Obstetrics and Gynecology, 3175 Chemin Côte Ste-Catherine, Montréal, Québec, Canada, H3T 1C5. Tel: 1-514-345-4931 ext. 4133. E-mail: sylvie.girard@recherche-ste-justine.qc.ca; and Sarah A. Robertson, Ph.D. Robinson Research Institute and Adelaide Medical School, The University of Adelaide, Adelaide, SA 5005 Australia. Tel: 61-8-83134094. E-mail: sarah.robertson@adelaide.edu.au

**Abstract**

Preterm birth (PTB) is commonly accompanied by *in utero* fetal inflammation, and existing tocolytic drugs do not target fetal inflammatory injury. Of the candidate proinflammatory mediators, interleukin-1 (IL-1) appears central and is sufficient to trigger fetal loss. Therefore, we elucidated the effects of antenatal IL-1 exposure on post-natal development, and investigated two IL-1 receptor antagonists, the competitive inhibitor Kineret (anakinra) and a potent noncompetitive inhibitor 101.10, for efficacy in blocking IL-1 actions. Antenatal exposure to IL-1β induced *Tnfa*, *Il6*, *Ccl2*, *Pghs2* and *Mpges1* expression in placenta and fetal membranes, and elevated AF IL-1β, IL-6, IL-8 and $PGF_{2\alpha}$, resulting in PTB and marked neonatal mortality. Surviving neonates had increased *Il1b*, *Il6*, *Il8*, *Il10*, *Pghs2*, *Tnfa* and *Crp* expression in white blood cells with elevated plasma IL-1β, IL-6 and IL-8, increased IL-1β, IL-6 and IL-8 in fetal lung, intestine and brain, and morphological abnormalities including disrupted lung alveolarization, atrophy of intestinal villus and colon-resident lymphoid follicle, and brain microvascular degeneration and atrophy with visual evoked potential anomalies. Late gestation treatment with 101.10 abolished these adverse outcomes, whereas Kineret exerted only modest effects and no benefit for gestation length, neonatal mortality or placental inflammation. In a LPS-induced model of infection-associated PTB, 101.10 prevented PTB, neonatal mortality and fetal brain inflammation. There was no substantive deviation in postnatal growth trajectory or adult body morphometry after antenatal 101.10 treatment. The results implicate IL-1 as an important driver of neonatal morbidity in PTB and identify 101.10 as a safe and effective candidate therapeutic.

Keywords: Interleukin-1, neonatal morbidity, inflammation, preterm birth, antenatal

## Introduction

Preterm birth (PTB; birth <37 weeks of gestation) is a leading cause of infant mortality and morbidity worldwide and often results in lifelong complications for surviving children (1). Inflammation is implicated in a significant proportion of PTB regardless of the presence of infection (2) and is associated with the onset of fetal inflammatory response syndrome (FIRS) (3), and further represents an independent risk factor for neonatal morbidities (4-6). Increases in proinflammatory cytokines are readily detected in AF and umbilical cord blood in such cases, and herald the onset of neonatal morbidities (4, 7). Physiologically, cytokines in the fetal circulation rapidly spread and affect organs that are particularly vulnerable to inflammatory stressors at an early stage of development, especially in the premature newborn, by triggering intracellular signaling cascades resulting in organ injuries and neonatal morbidity (8, 9) with vulnerability primarily observed in lung, intestine and brain (10-13). Correspondingly, key features of tissue (lung, intestine, and brain) injury of common neonatal diseases can be reproduced in animals following administration of inflammatory stressors (14-16); conversely, tissue integrity can be preserved by anti-inflammatory agents (17-19). Despite this compelling evidence and unequivocal need to tackle inflammation for preterm birth and neonatal injury (20), tocolytics are the only treatments available for preterm labor, but these tackle myometrial contractions and have no impact on the inflammatory mediators implicated in fetal inflammatory injury.

To date, there is no therapeutic molecule available to prevent/alleviate pathological inflammatory processes in pregnant women at risk of PTB. Of all mediators implicated in gestational inflammation and the onset of neonatal morbidities, interleukin (IL)-1 exerts a major detrimental role, as suggested by a broad body of evidence including: 1) increased levels of IL-

1β and IL-1Ra are early markers of neonatal injuries of the lung, intestine and brain (21-24) and such injuries can be recreated in rodent and ovine models *via* overexpression or administration of IL-1 (25-27); 2) antagonism of IL-1 receptor, IL-1β, or inhibition of the cleavage and release of IL-1β by targeting caspase-1 activity provides improvement in outcomes of perinatal injuries to the aforementioned organs including when triggered by upstream proinflammatory stressors (17, 19, 28-32); and 3) inflammatory concentrations of IL-1β elicit neuro-microvascular decay (33), curtail hippocampal neuron differentiation (34) and consequently leads to seizures wherein IL-1β further contributes to brain injury (35). Therefore, IL-1 represents a target of high interest and potential to improve health outcomes in premature infants. However, data accumulated to date mainly describe a harmful role of IL-1 in the postnatal period whereas its antenatal contribution to neonatal diseases is not well described, which hinders the development of a therapeutic administered preferably during pregnancy at the onset of chorioamnionitis. This is particularly relevant considering that IL-1β levels are elevated in women with chorioamniotis (36) which constitutes an early event in the onset of perinatal complications in human and animal (37-39) and that administration of IL-1β in pregnant rodent and non-human primate induces PTB (40-43).

Therefore, we sought to investigate the effects exerted by antenatal exposure to IL-1β on the development of offspring. We focused on changes induced by intrauterine exposure to IL-1β, particularly in the placenta, fetal membranes and AF and its association with the onset of a fetal inflammatory response and gestation outcome. Furthermore, we studied litters postnatally to assess growth and development of surviving offspring, with specific consideration of the morphology of lung parenchyma and intestine villi, and microvascular development in brain and normal cortex function, which are cardinal features of common neonatal morbidities. To

evaluate the utility of suppressing IL-1 signaling to protect the fetus from inflammatory injury, we used a commercially-available IL-1R competitive antagonist Kineret (anakinra), in addition to a small peptide noncompetitive IL-1R antagonist (termed 101.10) (44) that is proven effective at decreasing IL-1-induced uterine inflammation in pregnant mice *via* inhibition of IL-1-induced MAPK p38 and JNK, c-jun and Rho GTPase (42) upstream of transcription factor AP-1 implicated in cytokine induction (45-47) and labor (48). Our data in IL-1β- and LPS-induced models of PTB uncover a major detrimental role of antenatal IL-1 on the development of adverse perinatal, neonatal and developmental outcomes in progeny, and suggest that 101.10 represents an effective therapeutic candidate for administration preferably during pregnancy to decrease neonatal morbidities including in cases of infection.

## Methods

*Animals*

IL-1β model: Timed-pregnant CD-1 mice were obtained from Charles River Inc at different gestation 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 12:12 light:dark cycle and allowed free access to chow and water.

LPS model: C57Bl/6 (B6) mice were bred and housed in the specific pathogen-free University of Adelaide Laboratory Animal Services facility under a 12:12 h light-dark cycle. Food and water were provided ad libitum. Animals were utilised in accordance with the NHMRC Australian Code of Practice for the care and use of animals for scientific purposes, and all experiments were approved by the University of Adelaide Animal Ethics Committee. One to three virgin female mice of 8-12 weeks of age were housed with a proven fertile B6 male and checked daily between 0800-1000 h for vaginal plugs, as evidence of mating. The morning of vaginal plug detection was designated gestational day (GD) 0.5. Females were then removed from the male and housed individually. Data presented herein using B6 mice are concordant with those previously reported with CD-1 mice (42).

*Chemicals*

Chemicals were purchased from the following manufacturers: recombinant human IL-1β (#200-01B; PeproTech), LPS from Salmonella typhimurium (Sigma-Aldrich, St. Louis, MO,

USA), 101.10 (Elim Biopharmaceuticals, Hayward, California; and synthetized as previously reported (49, 50)) and Kineret (Anakinra: Sobi, Biovitrum Stockholm, Sweden).

*IL-1β-induced PTB model*

Timed-pregnant CD-1 mice were steadily anesthetized with an isoflurane mask for the complete procedure. After body hair removal from the peritoneal area, a 1.5 cm medial incision was performed with surgical scissors in the lower abdominal wall. The lower segment of the right uterine horn was exposed and 1 μg of IL-1β was injected between two fetal membranes with care to not enter the amniotic cavity. The abdominal muscle layer was sutured and the skin closed with clips. One hundred μL of 101.10 (1 mg/Kg/12h), Kineret (4 mg/Kg/12h) or vehicle was injected subcutaneously in the neck 30 mins before stimulation with IL-1β. Time of parturition and newborn outcome was assessed every 2 h until term (GD19 - GD19.5). A subset of pregnant mice was sacrificed 24 h after the IL-1β injection to collect fluid and tissue samples of AF, fetal membranes and placenta for biochemical analysis, and fetuses for gross fetal growth assessment. Another subset was killed immediately after delivery (± 2 h postpartum) and samples of brain, lung, intestine and white blood cells (as described below) were collected and stored at -80 ˚C for biochemical analysis. Pups (up to 8 per litter) were kept with dams and weighed every 2-3 days, then killed on post-term day (PT)15 (representing adolescent pups in term of brain development) and PT30 (at the stage of young adulthood in term of brain development) for further histological and electrophysiological analysis, respectively.

*Circulating leukocyte RNA isolation*

Newborn blood was collected by decapitation, pooled together for each litter and immediately transferred into heparin-containing tubes to prevent clotting. White blood cells were isolated by centrifugation after a treatment with red blood cell lysis buffer (Norgen Biotek Corporation, Thorold ON, Canada) and EDTA according to the manufacturer's protocol, and the resulting pellets were stored at -80˚C. For RNA isolation, pellets were thawed, lysed and passed through a RNA-binding column using a leukocyte RNA isolation kit according to the manufacturer's protocol (Norgen Biotek Corporation). Briefly, after washing, RNA was eluted from the columns and quantified with using a NanoDrop 1000 spectrophotometer. Equal amounts of RNA were used to synthetize cDNA using iScript Reverse Transcription SuperMix (Bio-Rad). RT-qPCR was then performed on the samples as described below.

*RNA extraction and Real-Time quantitative Polymerase Chain Reaction (RT-qPCR)*

Tissues were thawed and rapidly preserved in Ribozol (AMRESCO, Solon OH, United States). RNA was extracted according to manufacturer's protocol and sample were DNAse-treated using Ambion DNA-free™ Kit according to the manufacturer's instructions. RNA concentration and integrity were measured with a NanoDrop 1000 spectrophotometer, and samples with a A260:A280 ratio of 1.6-1.8 were used in PCR analysis after RNA integrity was verified by denaturing agarose electrophoresis. Five hundred ng of RNA was used to synthetize cDNA using iScript Reverse Transcription SuperMix (Bio-Rad, Hercules CA, United States). Primers were designed using NCBI Primer Blast (see Table I). Quantitative gene expression analysis was performed on Stratagene MXPro3000 (Stratagene) with SYBR Green Master Mix (BioRad). PCR products were subjected to High Resolution Melt (HRM) analysis to assess

primer specificity. Gene expression levels were normalized to 18S universal primer (Ambion Life Technology, Burlington ON, Canada) or β-actin.

*Murine ELISA assays*

The ELISA assays were performed using the following ELISA kits according to the manufacturers' protocol: mouse IL-1β/IL-1F2 Quantikine (#MLB00C; R&D systems), mouse IL-6 Quantikine (#M6000B; R&D systems), mouse IL-8 (#MBS261967; Mybiosource; recognizes the IL-8 homologue CXCL2) and mouse $PGF_{2\alpha}$ (#MBS264160; Mybiosource). Briefly, tissues were lysed in RIPA buffer (containing proteases inhibitors) and equal amounts of proteins (assessed using Bradford method) or 50 µL of fetal plasma or amniotic fluids were loaded into a 96-wells plate pre-coated with specific primary antibodies and incubated for 2 hours at ambient temperature. Wells were then washed 5 times and incubated with enzyme-linked polyclonal secondary antibodies for 2 hours. After another washing step, a substrate solution was added. The enzymatic reaction was stopped after 30 mins and plates were read at 450 nm, with wavelength correction set to 570 nm.

*Western blotting*

Proteins from homogenized placenta lysed in RIPA buffer (containing proteases inhibitors) were quantified using Bradford's method (Bio-Rad). Fifty µg of protein sample were loaded onto SDS-PAGE gel and electrotransfered onto PVDF membranes. After blocking, membranes were incubated with either an antibody against phospho-JNK (#9251; Cell Signaling Technology, Whitby ON, Canada), IL-6 (#sc-1264; Santa Cruz Biotechnology) or β-actin (#sc-

47778; Santa Cruz Biotechnology). Membranes were then washed with PBS containing 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO) and incubated for 1 hour with their respective secondary antibodies conjugated to HRP (Sigma). Enhanced chemiluminescence (GE Healthcare) was used for detection using the ImageQuant LAS-500 (GE Healthcare, Little Chalfont, United Kingdom) and densitometric analysis was performed using ImageJ (ImageJ, NIH, http://rsb.info.nih.gov/ij/). Resulting values were normalized first with β-actin, and then as a ratio of the control samples.

*Tissue collection and fixation*

Pups were sacrificed at PT15, intubated *via* the trachea and perfused with 10% formalin (Fisher Scientific) at a pressure of 20 cm. After 10 mins, lung, intestine and brain were collected. Briefly, the cranium was opened with surgical scissors (following the sagittal suture from sigma to bregma) and the brain was carefully extracted. Then, the lower intestine (1 cm above the caecum to the rectum) and lungs were excised and all tissues were fixed in 10% formalin for at least 24 h and subsequently transferred to PBS at 4˚C.

*Lung, intestine and brain histology*

Five μm-thick sections were performed on paraffin-embedded lungs (at three levels from the apex to base), intestines (ileum to colon) and brains and stained with H&E (lungs and brains) or hematoxylin-phloxin-safran (intestine). Images were acquired using 20X objective with a high-resolution slide scanner (Axioscan, Zeiss, ON, Canada).

*Histological analysis*

Analysis were performed with Zen 2 software or ImageJ by evaluators blinded to group identification. Tissues were obtained from 2 pups/dam from 6-8 dams per group (total of 28 dams and 56 pups). A post-analysis was performed to determine if the morphological differences observed were dependent on the sex of pups; no significant differences were noted (data not shown).

Lung. Alveolar count was obtained from the mean of two 1 mm2 sections in each tissue section analyzed. Alveolar size and parenchymal thickness were obtained from the mean of 10 alveoli per tissue section from 2 different areas. The number of intercepts between a 1 mm straight line (generated with Zen 2 software) and lung structure was used as an index of alveolar counts (51). The results are presented as an absolute number per mm of the mean from 4 distinct 1 mm2 sections that were free of blood vessels.

Intestine. Villus height was measured from the basal layer of the submucosa to the ending of the villus in the jejunum-ileum; atrophied villi were arbitrarily defined as villi measuring <400 μM, which corresponds to a 2-fold decrease in mean villus height of controls at same age. In colon, lymphoid follicle count was divided by the length of the tissue analyzed and plotted as count/mm. The surface of lymphoid follicles was measured on all follicles encountered.

Brain. Immunohistochemistry was performed as previously described (52). Immunostaining for lectin (vasculature, shown in brown) was separated from the purple hematoxylin using the color deconvolution function in Image J, and staining density was determined using ImageJ analysis software as previously described (53). Staining threshold was then set to detect only specific

lectin staining and then applied to all samples, allowing semi-quantitative comparisons of the vascular density.

*Immunocytochemistry*

Pregnant mice (GD17) injected subcutaneously with 1 mg/Kg 101.10-FITC, FITC alone (Sigma) or vehicle were euthanized after 1 h to analyze tissue distribution of the fluorescent-tagged compounds. Placentas and fetuses were collected and fixed in 4% paraformaldehyde for 1 day and transferred in 30% sucrose for another day. Localization of 101.10 was determined on 14 µm longitudinal placenta and fetus cryosections. Nuclei were stained with DAPI (Invitrogen; 1:5000). Images of the complete sections were captured using a 10X objective with Zeiss AxioObserver.Z1 (Zeiss, San Diego, CA) and merged into a single file using the MosiaX option in the AxioVision software version 4.6.5 (Zeiss).

*Flow cytometry*

Samples were lysed and filtered obtain a single cell suspension, then analysed on a BD FACSAria flow cytometer (BD Bioscience, USA) equipped with 488 nm, 405 nm and 633 nm lasers and routinely calibrated with CS&T beads (BD Biosciences). Data were processed using BD FACS Diva (BD Biosciences) and detection of FITC emission was collected through a 530/30 band pass filter. A minimum of 10 000 total events were acquired for each sample. Data analysis was performed with FlowJo solfware (tree star Inc, Ashland, OR) and results were reported as a percentage of positive cells in the tested sample.

*Visual Evoked Potential*

Visual evoked potential (VEP) is a reliable and sensitive parameter to evaluate neurologic functional alterations. VEPs were recorded at P30. Mice were anesthetized using a mixture of 80 mg/kg ketamine and 20 mg/kg xylazine. A subcutaneous needle electrode (Diagnosys, LLC, US) was inserted under the scalp at the lambda suture and served as the active electrode, whereas reference and ground electrodes (Diagnosys, LLC, US) were placed in cheek and tail, respectively. Impedance were maintained below 5 kΩ. Visual stimuli were generated by a mini-Ganzfield stimulator (3 cd.s/m²). Flash VEP were elicited by a brief flash (≤5 ms) in the visual field presented in a dark room (red light) without pre-stimulus. Analogue high pass and low pass filters were set at ≤ 1 Hz and at ≥ 100 Hz, respectively. Photic stimulation was delivered 100 times at a frequency of 1 Hz. The robust components of flash VEP are N2 and P2 peaks. Measurement of P2 amplitude was made from positive P2 peak and preceding N2 negative peak. Each response represents an average of 100 sweeps (performed with Espion E3 systems).

*LPS-induced PTB model, progeny growth trajectory and body composition*

C57Bl/6 (B6) pregnant mice at GD16.5 were injected with 101.10 (1 mg/kg) or vehicle intraperitoneally (ip) 30 mins prior to injection of 0.5 μg LPS in 200 μl PBS. Mice were then administered additional doses of 101.10 or vehicle on GD 17.0, 17.5 and 18.0. A subset of pregnant females was killed by cervical location 4 h post-treatment and the uterus, decidua, placenta and fetal brain were dissected from two fetuses (one from each horn), snap-frozen in liquid nitrogen and stored at -80ºC for mRNA isolation. Another cohort of female mice was

monitored until the time of parturition. Gestation length and the number of pups were recorded. Viable pups were weighted within the first 24 h of life. Pups were then sexed at weaning (3 weeks of age) when they were weighted again. All offspring were weighed at 4 weeks of age and then every 2 weeks until 20 weeks of age. At 20 weeks, progeny was anaesthetised (Avertin; Sigma-Aldrich) and approximately 1 ml of blood was collected by cardiac puncture before mice were weighed and killed by cervical dislocation for full body composition analysis. The following tissues were excised and weighed individually; brain, heart, lungs (left and right), kidneys (left and right), liver, adrenal glands (left and right), thymus, spleen, testes (males, left and right), seminal vesicle (males), epididymis (males), ovaries (females, left and right), uterus (females), quadriceps (left and right), triceps (left and right), biceps (left and right), gastrocnemius muscle (left and right), retroperitoneal fat, peri-renal fat, epididymal fat (males, left and right) and parametrial fat (females). Weights of tissues and organs present on both the left and right sides were summed. Total muscle weight was calculated by adding the combined weights of the quadriceps, triceps, and biceps and gastrocnemius muscles. Total fat weight was calculated by adding the combined weights of the retroperitoneal fat, peri-renal fat and epididymal fat (for males) or parametrial fat (for females). Total muscle and total fat weights were used to calculate the muscle:fat ratio. Total fat weight was subtracted from total body weight to calculate the total lean weight.

*Serum Preparation*

Immediately following collection, serum was prepared by first allowing blood to clot at room temperature for 30 mins, then serum was separated by centrifugation at 4000 rpm for 5

mins, removed and divided into small individual aliquots to avoid multiple freeze-thaw cycles. Serum aliquots were immediately stored at -80°C until assay.

*Mouse Luminex Assays*

Adiponectin and Leptin were quantified by Luminex multiplex microbead assay (Millipore, Australia), according to the manufacturer's instructions. For adiponectin, serum samples were diluted 1 in 5000 in assay buffer, as recommended by the manufacturer, whereas for leptin, samples were tested neat. The minimum detectable threshold was 3.0 pg/ml and 4.2 pg/ml for adiponectin and leptin, respectively.

*Statistical analysis*

All data was analysed using SPSS Version 20.0 software (SPSS Inc, Chicago, IL) or Graphpad Prism version 6.0 software (Graphpad Software, San Diego, CA). Groups were tested for normality using a Shapiro–Wilk test. One-way analysis of variance (ANOVA) or two-tailed Student's t-test was employed when data were normally distributed. Dunnett's multiple comparison method was utilized when data were compared to a single control. A Kruskal-Wallis test followed by a Mann-Whitney U-test was used when the data were not normally distributed. Body composition data is expressed as estimated marginal mean ± S.E.M. and analysed as a Mixed Model Linear Repeated Measures ANOVA and post-hoc Sidak test, with litter size as a covariant. A value of $p<0.05$ was considered statistically significant. Data are presented as means ± S.E.M for large sample size and individual values + median for small sample sizes.

## Results

*Administration of IL-1β in utero induces adverse perinatal outcomes*

To study the implications of antenatal exposure to IL-1β, we administered IL-1β intrauterine in late gestation (GD16; normal gestation length =19.5 days) (Fig.1A) to induce uterine inflammation and preterm delivery (42, 54). IL-1R competitive antagonist Kineret and noncompetitive IL-1R antagonist (small all-d peptide) 101.10 (44) were administered subcutaneously to additional groups of IL-1β-treated dams twice daily from GD16 to GD18. IL-1β shortened gestation length (Fig.1B) and induced substantial neonatal mortality (Fig.1C,D), whereas co-administration of 101.10, but not Kineret, significantly improved these outcomes. The majority of pups alive after birth survived the first week of life in all treatment groups (Fig.1E).

Given the high neonatal mortality rate, we examined the fetal response to IL-1β by conducting gross and histological examination of fetuses after 24 h of exposure to IL-1β *in utero*. We found that a majority of fetuses from IL-1β-treated dams displayed an underdeveloped anatomy in addition to noticeable autolysis (Fig. 1F, arrows). In contrast, none of these features were observed in fetuses from IL-1β-treated dams receiving 101.10, in line with improved neonatal survival as previously described (see Fig. 1C). In fetuses that developed normally, no significant difference in morphological parameters between groups was found after 24h exposure to IL-1β *in utero*, as measured by histological analysis (Fig.1G; Table 2). Correspondingly, the weight of viable newborns was not significantly different between groups (Fig.1H), suggesting that short-term exposure to (non-lethal) inflammation is insufficient to affect late prenatal growth, although a slight tendency is observed for weight.

*Uterine IL-1β induces an inflammatory response in placenta, fetal membranes and amniotic fluids which propagates to newborn*

To characterize the maternal and fetal inflammatory response triggered by IL-1β in pregnant uterus and to further explore the link between maternal-onset inflammation and the adverse perinatal outcomes observed herein, we performed biochemical analysis on feto-maternal tissues (placenta, fetal membranes and amniotic fluids collected 24 h after IL-1β injection) of normally developed fetuses (see Fig.1F-H and Table 2). Placental expression of genes encoding key proinflammatory factors, including TNFα, IL-6, CCL2 and COX-2, was upregulated in IL-1β-treated dams (Fig. 2A). This upregulation was blocked by 101.10, which is readily able to access the placenta (Fig. S1A-D), and to a lesser extent Kineret as previously reported for other inflammatory genes including Il1b and Il8 (42); anti-inflammatory IL-10 and IL-4 were unaffected by IL-1β in absence or presence of 101.10 or Kineret (Fig. 2A). The strong induction of placental *Il6* by IL-1β (and its downregulation by 101.10) was reflected in protein abundance (Fig.2B) and was associated with activation (by phosphorylation) of the IL-1R-induced stress kinase JNK (Fig. S2A). A similar proinflammatory profile was observed in fetal membranes (Fig. S2B). Furthermore, proinflammatory mediators associated with parturition (IL-1β, IL-6, IL-8 and $PGF_{2\alpha}$) in amniotic fluids were concurrently elevated in IL-1β-treated dams (Fig.2C-F), suggesting propagation of the initial inflammatory response into the fetal compartment as expected (3, 55). Again, 101.10 blocked this effect with more efficacy than Kineret (at recommended doses, effective on maternal inflammation (42)). The inefficacy of Kineret on fetal-placental inflammation was dose-related as higher doses reduced inflammation and preterm birth (Fig. S2C-E).

To confirm dissemination of maternal inflammation to the fetus, we quantified proinflammatory mRNAs and proteins in white blood cells and plasma of neonates (collected within an hour of birth). A significant increase in *Il1b*, *Il6*, *Il8*, *Il10*, *Pghs2*, *Tnfa* and *Crp* was observed in circulating white blood cells (Fig.2G), associated with elevated levels of IL-1β, IL-6 and IL-8 in plasma (Fig.2H-J). Again, 101.10 abrogated this increase with higher efficacy than Kineret. Notably, FITC-coupled 101.10 was not detectable in fetal tissues when administered s.c. to dams (Fig. S1E-H), suggesting that its protective effects on the fetus may be mediated *via* suppression of gene expression in placenta and gestational tissue, as opposed to direct suppression of IL-1β signaling within the fetus (see Fig. 2A,B, Fig. S2A,B and Fig. S1A-D).

Given the strong elevation in plasma cytokines IL-1β, IL-6 and IL-8 in newborns exposed to IL-1β *in utero*, we assessed if the lung, intestine and brain, which are well-perfused organs particularly vulnerable to inflammatory insults (10-13), were affected by the systemic inflammatory response. We found significant elevation in IL-1β, IL-6 and IL-8 in lung (Fig.3A-C), of IL-1β and IL-8, but not IL-6 in intestine (Fig.3D-F) and of IL-1β, IL-6 and IL-8 in brain (Fig.3G-I). Newborns from IL-1β-treated dams administered 101.10 were protected, whereas Kineret had only a modest effect on some factors.

*In utero exposure to IL-1β induces marked morphological alterations and malformations in lung, intestine and brain of developing offspring*

To determine if the systemic inflammatory response triggered in newborns from IL-1β-treated dams was associated with abnormalities in organ development, as is observed in human (3, 56), we studied litters from birth to PT15. Concordant with the unaffected neonatal weight

observed between groups in viable pups (see Fig. 1H), growth from PT1 to PT13 was unaffected by treatments (Fig. S2F). However, histological analysis of the lung, intestine and brain revealed marked morphological alterations. Lungs of pups exposed to IL-1β displayed a grossly atypical lung parenchyma histology featuring disrupted alveolarization (Fig.4A). Semi-quantitative analysis of lung morphology revealed a 2-fold decrease in alveolar count induced by antenatal exposure to IL-1β associated with a 2-fold increase in alveolar size, a significant decrease in septation count (Fig.4D) and a 2-fold increase in parenchymal thickness (Fig.2E). This phenotype was not observed in pups born from dams administered 101.10, whereas Kineret conveyed only partial improvement.

The intestine of pups from IL-1β-treated dams exhibited an abnormal shortening in villi (Fig.5A,B) associated with an increased incidence of villous atrophy (Fig.5C) in the jejunum-ileum. In the upstream intestine, this abnormality was associated with a marked loss in the quantity of colon-resident lymphoid follicles (Fig.5D,E). The remaining follicles exhibited a significantly smaller size (Fig.5F), suggesting compromised colon immunity. Treatment with 101.10 and Kineret both protected against jejunum-ileum and colon injury; however, Kineret was ineffective in normalizing the quantity of lymphoid follicles (see Fig.5E).

In the brain, systemic perinatal inflammation impairs angiogenesis (57) to elicit major lifelong pathophysiological implications (58, 59). Perinatal brain injury is generally widespread throughout the brain inferring an abnormality in vascularization (60). In pups exposed to IL-1β *in utero*, we found a significant microvascular degeneration in the cortex, cingulum, hypothalamus (CA3 and dentate gyrus), but not in the striatum (Fig. 6A-F). This was associated with decreased brain weight that persisted to adulthood (Fig. 6G-H). 101.10 and Kineret both

prevented these injuries, with the exception that Kineret did not improve microvascular degeneration in the CA3 region of the hypothalamus.

Given the vascular impairment in the developing cortex of pups exposed *in utero* to IL-1β, and the loss in total brain mass which is indicative of decay in cortical structure and function in human (60), we conducted electrophysiological measurements of visual evoked potential (VEP) to objectively assess cortical function in young adults. We found that young adults (PT30) exposed to IL-1β in the antenatal phase presented severe abnormalities in VEP performance, with a decreased amplitude and delayed latency of key N2 and P2 components (Fig. 7A-C). These and other VEP anomalies were noted in 100% (6/6) of young adults exposed to IL-1β analyzed. Among the anomalies, absent VEP (characterized by unrecognizable P or N component) was observed in 50% of animals exposed to IL-1β (Fig.7D; Fig. S3A), suggesting a compromised cortical function that is concordant with observations in human infants with neurological disorders (61). Antagonism of IL-1R by either 101.10 or Kineret prevented these outcomes.

*101.10 prevents adverse obstetrical and perinatal outcomes triggered by LPS*

Our previous data suggest that IL-1 is sufficient to trigger major adverse obstetrical, perinatal, and developmental outcomes and identify 101.10 as an effective tool to prevent its action, whereas Kineret exhibits sporadic and attenuated efficacy (42, 62). We proceeded in investigating if 101.10 exhibits effective therapeutic potential in a clinically-relevant model triggered by bacterial products, an important upstream cause of uterine inflammation associated with poor neonatal outcomes (63); 0.5 μg of LPS, a dose shown previously to trigger robust IL-

1 release (64), was administered (ip) at GD16.5 (Fig.8A) and induced PTB (Fig.8B), as amply documented by our group and others (42, 65). Accordingly, 101.10 decreased PTB induced by LPS with comparable efficacy as previously reported (42).

We found that 101.10 significantly improved LPS-induced survival at birth and at 1 week of age (Fig.8C-E). Viable pups did not display significant differences in weight at birth (Fig.8F). Biochemical analysis of feto-maternal tissue collected 4 h after LPS exposure revealed marked and consistent activation of major proinflammatory mRNA transcripts in the uterus (Fig.9A), decidua (Fig.9B), placenta (Fig.9C) and fetal brain (Fig.9D). The 101.10 significantly decreased activation of these genes, with the exception of uterine and placental *Il12b* ($p=0.10$).

In a comprehensive analysis of offspring phenotype, growth trajectory from week 3 to week 20 (Fig. S3B-C), as well as body morphometry of >20 tissues (Table S1A-B) and serum adipocytokines assessed at week 20 of life to assess metabolic function (Fig. S3D-E), were unaltered by treatment with 101.10 regardless of sex. Although 101.10 treatment caused a small reduction in body weight in male and female pups at 4 weeks of age after treatment, this was not seen when 101.10 was administered in combination with LPS. The only difference in adult offspring exposed to 101.10 plus LPS *in utero* was a 12% reduction in epididymis weight in males.

## Discussion

Inflammation is an essential physiological mechanism employed by complex organisms to respond to infection and non–infectious insults including oxidative stress, hypoxia-ischemia and senescence. Inflammatory processes can become pathological depending on their location, timing, intensity, and chronicity. Overt inflammation, triggered by several stressors encountered by preterm infants, is a common upstream pathway observed in major perinatal diseases in the presence and absence of infection (10, 12, 13, 66, 67), including when excessive inflammation is triggered before birth (68). Accordingly, a causal relationship between inflammation and various neonatal diseases is firmly established (3, 14, 17-19).

Systemic inflammation of the fetus and newborn is referred to as fetal inflammatory response syndrome (FIRS) and is clinically defined by elevated levels of IL-6 and other pro-inflammatory cytokines in fetal blood. FIRS is an independent risk factor of neonatal morbidity that affects multiple organs particularly the lung, intestine and brain (3, 56, 66). In our study, we have shown that the inflammatory response to IL-1β *in utero* spreads from the uterine cavity to placenta and fetal membranes to induce a systemic fetal response characterized by >4-fold increase in fetal plasma levels of IL-1β, IL-6 and IL-8 paralleled by leukocyte-mediated transcriptional induction of Il1b, Il6, IL8 and other proinflammatory genes. This fetal inflammatory response is known to result in morphological anomalies and injuries to lung, intestine and brain (10-13).

*Major organ injuries of the newborn associated with inflammation*

Several tissues in the neonate are particularly prone to inflammatory damage which may begin before birth and be exacerbated by treatments and conditions to which the neonate is exposed. The immature lung of the premature infant is vulnerable to proinflammatory insults such as infection, hyperoxia and mechanical stress and can therefore easily be injured by oxygen therapy, ventilation or other insults in the first hours after birth. Pathological inflammation induces severe lung injuries in newborns, particularly those born preterm, characterized by loss in alveolar septation, reduced maturation of epithelial cells, parenchymal thickening, and diminished capillary density (27, 69, 70). During gestation, amniotic fluid is inhaled by the fetus and acts as an additional carrier of cytokines and other proinflammatory mediators to the fetal alveoli. Thus, antenatal exposure to intra-amniotic inflammation represents a strong and independent risk factor for the development of bronchopulmonary dysplasia (BPD) (4), an alveolar and vascular damage which results in pronounced disruption in alveolarization.

Inflammation is well-recognized as a final common pathway to BPD wherein IL-1 is a critical contributor (17). Consistent with this, elevated cytokines (IL-1β, IL-6 and TNFα) in the airway of premature newborns is associated with the onset of BPD (24, 71) and postnatal administration of IL-1 induces a BPD-like phenotype in mice (27). Pathological inflammation can begin before birth in the form of chorioamnionitis or related conditions and stemming antenatal inflammation may improve of the prospect for BPD (17). Herein, we observed that lungs of pups born from IL-1β-treated dams displayed an increase in concentrations of various cytokines leading to severe morphological changes, consistent with features of BPD (70).

The gastrointestinal tract harbours the largest lymphoid tissue in the body consisting of resident lymphocytes grouped in follicles, but also of cells of the innate immune response such

as macrophages and DCs localized in the intestinal mucosa; these phagocytes present antigens to follicle-resident lymphocytes, coordinating immunologic defense mechanisms (72).

Inflammation is a primary cause of necrotizing enterocolitis (NEC) (73). NEC is characterized by increased circulating and intestinal cytokines levels, including IL-1β, in neonates (21, 74) and a decreased expression of endogenous IL-1Ra 2-3 is seen weeks prior to the onset of NEC (75). Correspondingly, targeting the inflammatory response in mice protects against experimental NEC (18).

We found that exposure to IL-1β during gestation induced an increase in intestinal levels of IL-1β and IL-8 in newborns leading to intestinal anomalies later in life. Specifically, villus integrity in the jejunum-ileum was severely compromised in young adolescent progeny exposed to IL-1, as reported in other models of neonatal intestinal injury (76, 77). Furthermore, a significant decrease in the size and number of lymphoid follicles was observed, possibly predisposing to inadequate immune-surveillance. Transcription factor NF-κB exerts a crucial role in maintaining intestinal immune-surveillance (78) and interestingly, NF-κB activity is preserved by 101.10, but abolished by Kineret (42), which may explain the inconsistent protective efficacy of Kineret in the colon.

Chorioamnionitis and antenatal exposure to inflammation causes major detriment to cerebral development (79). Numerous meta-analyses have linked chorioamnionitis to impairments of the newborn brain, including periventricular leukomalacia and cerebral palsy (80). Correspondingly, antenatal exposure of the fetus to inflammation is a strong and independent risk factor of cerebral palsy (5). Of all the cytokines implicated IL-1 stands out, as pre-clinical and clinical evidence suggests it directly induces neurotoxicity (81), whereas its

blockade using pharmacological or genetic approaches exerts neuroprotective effects in animal models (19, 62). A systematic review of 47 studies with a positive correlation between cytokine and neonatal infection or neurological insults concurs that levels of IL-1β in cord or neonatal blood are augmented in 100% of patients with neurological insults (82).

Fetal and neonatal inflammation is widespread throughout the neural vascular network (33, 83) resulting in generalized microvascular degeneration which in turn causes diffuse injury to the brain resulting in globally reduced brain mass, volume, and function, as observed in similar rodent studies (33, 84) as well as in extreme premature infants (60).

VEP provides an objective assessment of brain function and is regularly utilized in infants to identify brain pathology (85). In this context, a study by Kato *et al.* reported VEP anomalies (including absent VEP) in all infants suffering from periventricular leukomalacia (61). In line with this, pups exposed to IL-1β presented VEP anomalies and absent VEP was observed in 50% of cases. *In utero* antagonism of IL-1R abrogated IL-1-induced cerebral inflammation at birth and its consequences for the vascular network, brain weight and VEP, demonstrating an efficacy comparable to that of neuroprotective therapies delivered to the neonate (62, 86). A similar efficacy of 101.10 was observed in relevant LPS-treated animals (see Fig.9).

*Pathophysiological contribution to neonatal diseases: inflammation versus prematurity*

It is not clear whether the consequences of intrauterine inflammation in infants eventuate due to shorter gestation and immaturity at birth, or directly due to detrimental effects of inflammation on tissues (3, 86). Compelling clinical evidence points to antenatal inflammation

in tissue injury independent of gestation length (3-6, 56, 66, 87). This body of evidence is complemented with pre-clinical data clearly demonstrating that: 1) induction of preterm birth in mice using intrauterine infusion of proinflammatory LPS at GD15 induces an elevation in cerebral cytokine levels and neurological injury to the fetus, whereas none of these features are observed when preterm birth is induced using a non-inflammatory model of progesterone inhibition (88); 2) administration of LPS to pregnant mice at GD15 and GD18 induces comparable acute injury to fetal brain, despite that pregnant mice treated at GD15 deliver prematurely whereas those treated at GD18 deliver at term (89); 3) intrauterine administration of LPS in pregnant mice at term induces inflammation in the fetal brain and causes neurotoxicity (90) consistent with clinical evidence that chorioamnionitis at term can impair neurobehavioral outcome in infants (91-94); and 4) administration of Kineret to pregnant mice treated with LPS improves neurological outcomes without preventing preterm birth (62), as reaffirmed in the present study. Overall, this suggests that both preterm labor and inflammation need to be tackled by effective and targeted therapeutics to improve gestation outcome. This study uncovers a pivotal contribution of IL-1 in this process, and shows that targeting IL-1 is effective in preventing fetal inflammatory injury in an infection model.

*Contrast in efficacy between 101.10 and Kineret*

Although both 101.10 and Kineret elicit improved outcomes for tissue integrity of progeny after IL-1 exposure, 101.10 is more consistently effective than Kineret in other aspects particularly in inhibiting *in utero* inflammation, preventing PTB and improving neonatal mortality. The inefficacy of Kineret to block *in utero* inflammation and PTB is related to dose,

suggesting that the uterine inflammation responsible for preterm labor is more pronounced and difficult to tackle than the placental response leading to neonatal inflammatory injury. This is supported by the fact that Kineret (standard dose of 4 mg/Kg/12h) elicits a modest (albeit not statistically significant) inhibition of essentially all inflammatory mediators which leads to lower levels of cytokines in the fetal amniotic fluid as well as in fetal tissues per se. Correspondingly, these observations on Kineret in fetal-placental inflammation also apply to LPS-induced PTB (62). Accordingly, lower (standard) doses of Kineret are able to reduce placental inflammation sufficiently to convey protection to the fetus (62, 86); on the other hand, prevention of preterm birth needs much higher doses of Kineret to inhibit inflammatory factors to a greater degree in the utero-placental compartment. This disparity in potency may be due to pharmacological considerations, including: a) competitive antagonists rely on high concentrations at the site of action to establish a favorable antagonist/agonist ratio, whereas non-competitive antagonists bind to a site remote from the natural ligand binding site and their effects are for the most part independent of agonist concentrations (95); and 2) small molecules such as 101.10 (0.85 kDa) have increased access and distribution to tissue. Yet, neither 101.10 (as shown herein) nor Kineret (96) crosses the placenta in significant amounts even under inflammatory conditions, suggesting that their therapeutic effects on pups are mediated indirectly through actions in the maternal gestational tissues which spread inflammation into the fetal placental and tissue compartments.

## Conclusion

In summary, we herewith report harmful effects of antenatal exposure to IL-1 on the development of progeny from intrauterine life to adulthood, and demonstrate that suppression of IL-1 signaling using a novel peptide inhibitor of the IL-1 receptor is efficacious in rescuing pups from injury in an LPS-induced model of fetal inflammatory insult. This work has implications for the development of therapeutic molecules for pregnancy disorders wherein IL-1 plays a pathophysiological role such as chorioamnionitis, which affects a significant proportion of PTB and is associated with adverse neonatal outcomes independently of the duration of gestation. Our work substantiates the increasing evidence suggesting that it is insufficient simply to tackle uterine contractions in preterm labor to ultimately improve neonatal outcome. Suppressing the harmful effects of an excessive antenatal exposure to IL-1 using 101.10 during pregnancy appears to be a safe, potent, and effective therapeutic modality to protect the fetus exposed to intrauterine inflammation. As a small molecule able to access the placental tissue, 101.10 may have therapeutic advantages over the currently available drug Kineret.

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**Competing interests**

SC, CQ, and WDL 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.

**Author contributions**

MNV, SP, SAR, SG and SC designed the research studies; MNV, LC, LB, MEB, SP, MHB, AM, ABR, DS, ALM, XH, AB, AC, IB and SG conducted experiments; MNV, LC, LB, MEB, SP, MHB, AM, ABR, XH and SG analyzed data; WDL provided reagents; MNV, ABR, CQ, JSJ, AB, DMO, SAR, SG and SC wrote or contributed to the writing of the manuscript. All authors participated in the critical review of the manuscript and all approved the final manuscript.

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**Figure legends**

Figure 1. **Adverse gestational and perinatal outcomes are induced by antenatal exposure to IL-1β, and corrected by 101.10.** A, 101.10 (1mg/Kg/12h), Kineret (4 mg/Kg/12h) or vehicle are administered subcutaneously for 2 consecutive days and IL-1β (1µg) is administered intrauterine at GD16.5. B-D, gestation length (B), viable pup count (C) and pup survival rate as determined by counting breathing and non-breathing pups at birth (D). E, pup survival rate at one week (denominator is viable pups at birth). F, representative images of late gestation fetuses recovered from dams administered IL-1β with or without 101.10, analyzed after autopsy at GD17.5 (24 h after intrauterine IL-1β injection). Pups displaying gross morphological or developmental anomalies are indicated with arrows. G, representative micrograph of the morphology measurements (shown in Table 2) in a normally developed pup at GD17.5. H, pup weight at 24 h post-birth. n=18-50 dams/grp for gestational and neonatal outcome data, and 8-15 dams/grp for post-birth data. Values are presented as mean ± S.E.M. ***p<0.001 by one-way ANOVA with Dunnett's post-analysis.

Figure 2**.** **Inflammatory cytokines induced in the placenta, amniotic fluid and neonatal blood are induced by antenatal exposure to IL-1β, and corrected by 101.10.** A-B, placentas were collected 24 h after uterine exposure to IL-1β to perform quantitative PCR (A) and immunoblots against IL-6 (B). PCR results are relative to 18S and plotted as fold change vs. the control groups. Immunoblot quantification was normalized with β-actin and plotted as fold change vs. sham. n=3-4 dams/grp. C-F, cytokine (IL-1, IL-6 and IL-8) and $PGF_{2\alpha}$ levels in amniotic fluids collected 24h after IL-1β intrauterine exposure. n=4 sacs/grp. G, quantitative PCR performed on isolated white blood cells from newborn pups. The blood of 4-8 newborns per litter was pooled together to achieve sufficient mRNA levels, n= 5-7 dams/grp. H-J, levels of IL-1, IL-6 and IL-8 in plasma samples from newborn pups. The blood of 4-8 newborns per litter was pooled together to achieve sufficient mRNA levels, n= 7-9 dams/grp. Individual values are presented with median. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Dunnett's post-analysis.

Figure 3. **Inflammatory cytokine gene expression in the newborn lung, intestine and brain is induced by antenatal exposure to IL-1β, and corrected by 101.10.** A-P, cytokines levels in lung (A-C), intestine (D-F) and brain (G-I) of newborns. n=4 newborns/grp. Individual values

are presented with median. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Dunnett's post-analysis.

Figure 4. **Lung injury in adolescent offspring is induced by antenatal exposure to IL-1β, and corrected by 101.10.** A, representative image of the lung parenchyma stained with H&E. Lungs were collected following *in vivo* formalin perfusion. Scale, 250µM. B-E, measurement of alveolar count (A), alveolar size (B), alveolar septation count (C) and parenchymal thickness (D) performed on full tissue slides using Zen2 software. Data were collected on adolescent pups (PT15) from 6-8 dams/grp. Values are presented as mean ± S.E.M. ***p<0.001 by one-way ANOVA with Dunnett's post-analysis.

Figure 5. **Morphological anomalies in intestine of adolescent offspring is induced by antenatal exposure to IL-1β, and corrected by 101.10.** A, representative images of intestinal villi integrity as assessed in HPS-stained jejunum-ileum. Scale, 1000µM. B-C, villi height was quantified using Zen2 software (B); atrophied villi were defined as villi measuring < 400µM and plotted as a percentage (C). D, representative images of colon-resident lymphoid follicles. Scale, 250µM. E-F, quantification of the number of lymphoid follicles (E) and their surface (F) was performed using Zen2 software. Data were collected on adolescent pups (PT15) from 6-8 dams/grp. Values are presented as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Dunnett's post-analysis.

Figure 6. **Microvascular degeneration in brain of adolescent offspring associated with cortical malfunction in adulthood is induced by antenatal exposure to IL-1β, and corrected by 101.10.** A-E, representative images and quantification of vessel density in cortex (A), cingulum (B), hypothalamus (C,D) and striatum (E) of adolescent pups. F, negative control showing nonspecific staining. G, the areas quantified are represented on the full cerebral right hemisphere micrograph. H-I, brain weight of adolescent pups (H) and young adults (I). Data were collected on adolescent pups (PT15) from 6-8 dams/grp and from young adults (PT30) from 3-4 dams/grp (total of 6-8 young adults/grp). Values are presented as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Dunnett's post-analysis.

Figure 7. **Cerebral functional impairment in adulthood is induced by antenatal exposure to IL-1β, and corrected by 101.10.** A, representative VEP measured on young adults (PT30)

that were exposed during gestation to IL-1β with or without treatment with 101.10 or Kineret. B-C, amplitude (B) and latency (C) or the N2 and P2 components of the VEP. D, % of VEP anomalies and absent VEP in each group. VEP anomalies include significantly delayed latency or decreased amplitude of the P2 component, or absent VEP (See Figure S2D). Data were collected from 6 mice/grp. Values are presented as mean ± S.E.M. **p<0.01, ***p<0.001 by one-way ANOVA with Dunnett's post-analysis.

Figure 8. **Therapeutic effect of 101.10 on gestational and perinatal outcomes following LPS treatment.** A, pregnant females were given either LPS or vehicle i.p. on GD16.5, then 101.10 or vehicle at 12 h intervals on G 16.5, 17.0, 17.5 and 18.0. B-D, gestation length (B), viable pup count (C) and pup survival rate as determined by counting breathing and non-breathing pups at birth (D). E, pup weight at 12-24 h post-birth. F, pup survival rate at one week (denominator represents viable pups at birth). n=10 dams/grp. Values are presented as mean ± S.E.M. *p<0.05, ***p<0.001 by one-way ANOVA with Dunnett's post-analysis.

Figure 9. **101.10 protects against LPS-mediated induction of proinflammatory cytokines in gestational and fetal tissues.** A-D, Uterus (A), decidua (B), placenta (C) and fetal brain (D) were recovered from dams treated with LPS. Relative expression of *Il1a*, *Il1b*, *Il6*, *Tnf*, *Il10* and *Il12b* mRNA transcripts were determined in each tissue by quantitative PCR normalized to *Actb*. Two implantation sites were collected per dam, n = 12 (6 dams/group). Values are presented as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Dunnett's post-analysis.

**Supplementary figure legends**

Figure S1. **Biodistribution of 101.10 to placenta and fetus.** A-D, placentas were collected 1 h after subcutaneous injection of 101.10-FITC, FITC alone or vehicle and FITC expression was assessed using fluorescence microscopy (A-C) or flow cytometry (D). Scale, 1000μM. E-H, fetuses were collected 1 h after subcutaneous injection of 101.10-FITC, FITC alone or vehicle and FITC expression was assessed using fluorescence microscopy (A-C) or flow cytometry (D). Scale, 2500μM. Values are presented as mean ± S.E.M. **p<0.01, ***p<0.001 by one-way ANOVA with Dunnett's post-analysis.

Figure S2. **Proinflammatory effects of IL-1β on placenta and fetal membranes, and growth trajectory of developing pups**. A, immunoblot was performed on placentas collected 24 h after IL-1β intrauterine exposure. Membranes were incubated with antibodies against phospho-JNK or β-actin as a loading control. Densitometric analysis of protein band density was normalized with β-actin and plotted as fold change vs. sham. n=3 dams/grp. B, fetal membranes were collected 24 h after IL-1β intrauterine injection to perform quantitative PCR. Results are relative to 18S and plotted as fold change vs. the control groups. Individual data are presented with median. C-D, uteri were collected 24 h after IL-1β intrauterine injection to perform quantitative PCR. Results are relative to 18S and plotted as fold change vs. the control groups. Individual data are presented with median. E, gestation length after an i.u. injection of IL-1β and s.c. treatment with 101.10, Kineret or vehicle at the indicated doses. F, Growth trajectory of pups until adolescent age. n=6-10 pups/grp. Values are presented as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Dunnett's post-analysis compared to IL-1β+ Veh.

Figure S3. **Cerebral function impairment in adult mice exposed to IL-1 *in utero* and growth trajectory and metabolism in male and female offspring from LPS-treated dams.** A, cerebral functional impairment in adulthood is induced by antenatal exposure to IL-1β, and corrected by 101.10. Representative VEP measured on young adults (PT30) showing absent VEP in young adults exposed to IL-1β *in utero*. B-C, growth trajectories of male (A) and female (B) progeny of LPS-treated dams receiving 101.10 or vehicle. Values are presented as mean ± S.E.M. of n=10-22 male and n=11-22 female offspring in each group. Data was analysed by a Mixed Model Linear Repeated Measures ANOVA and post-hoc Sidak test, *p<0.05. D-E, leptin

and adiponectin levels in serum samples of male (C) and female (D) progeny at 20 weeks of age. Data were analysed by one-way ANOVA with Dunnett's post-analysis. Values are presented as mean ± S.E.M. of n = 10 male and 10 female progeny per treatment group. A p value <0.05 was considered significant.

## Figures

Figure 1

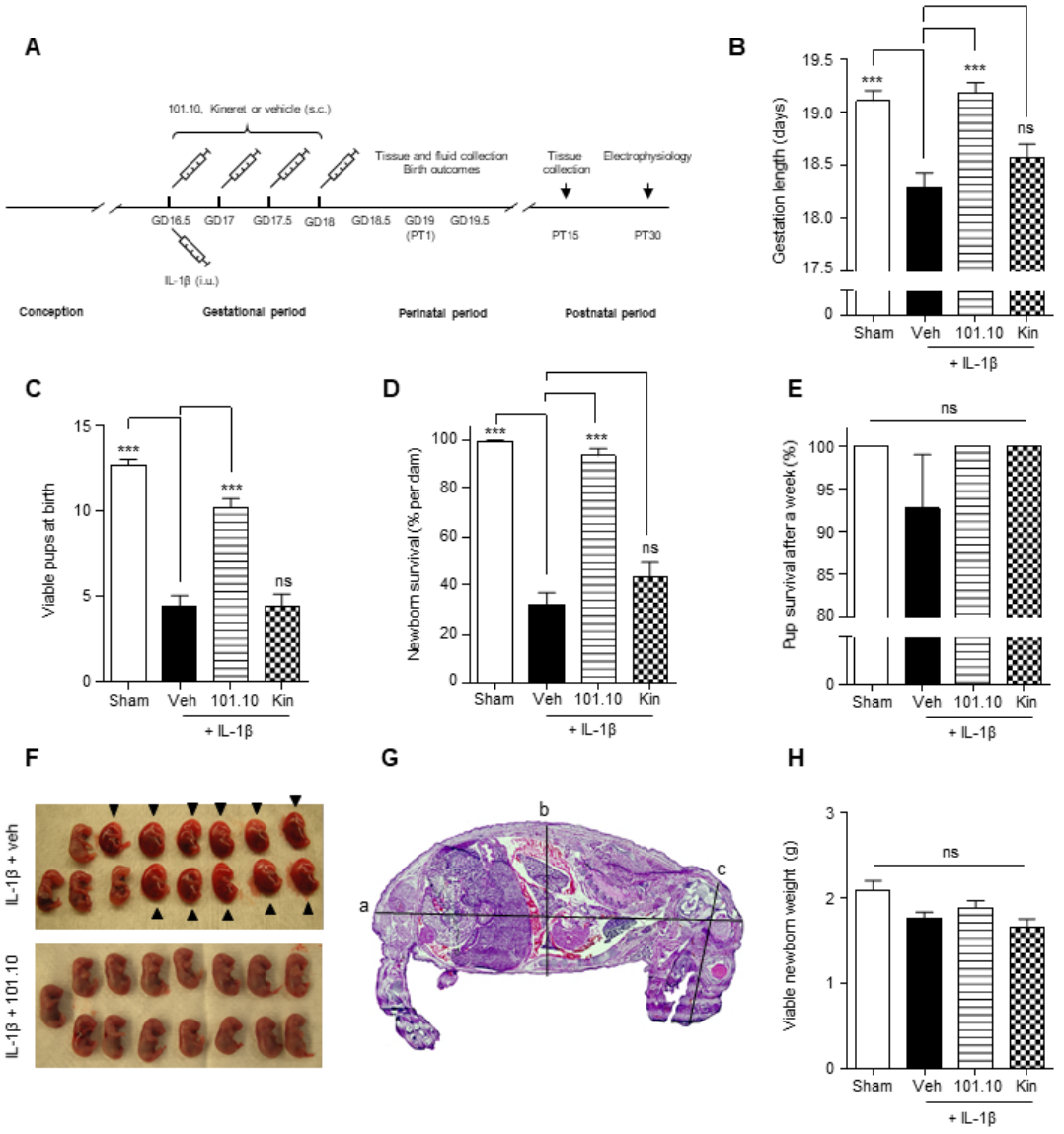


Figure 2

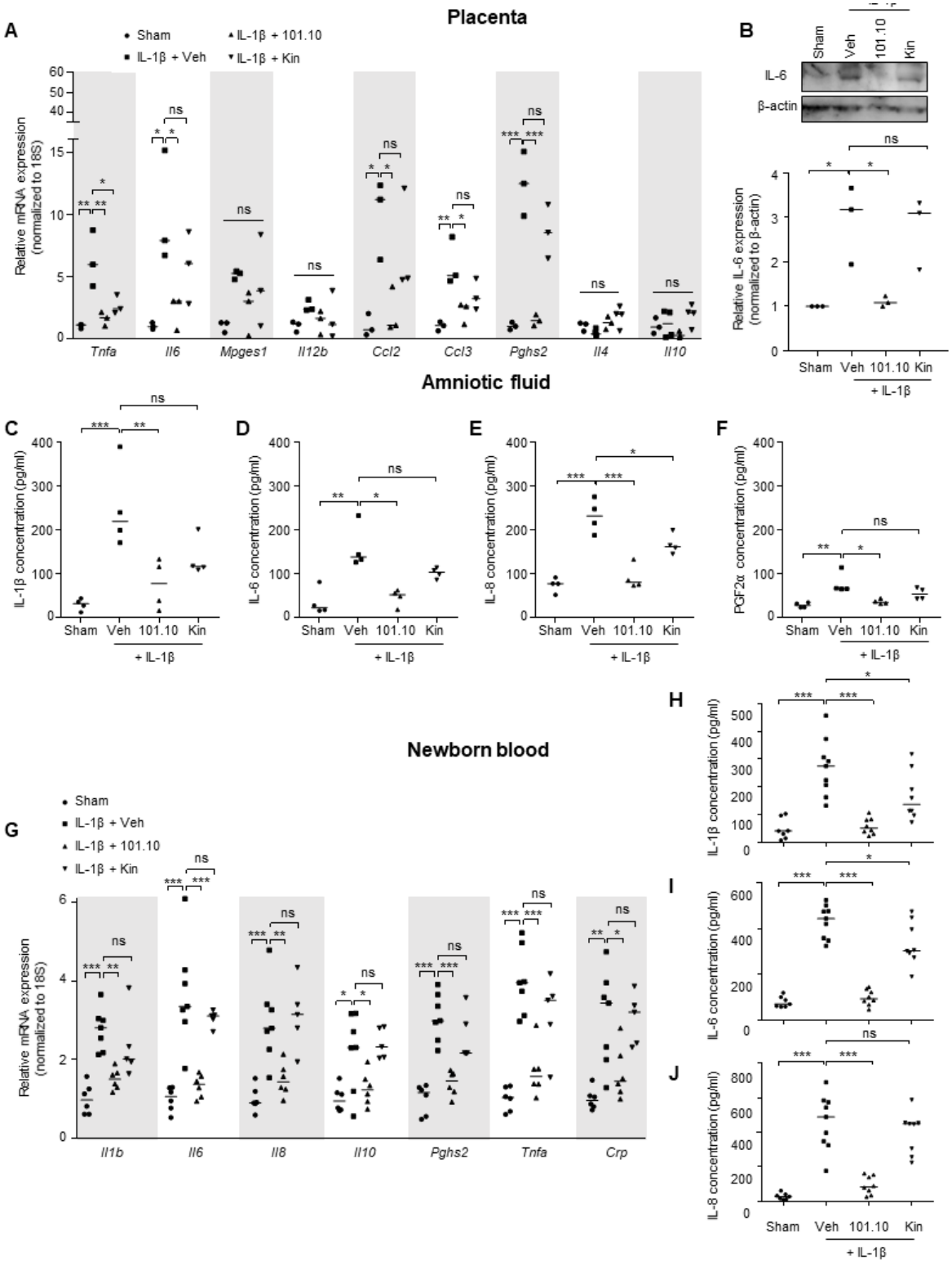

Placenta
A
Sham
IL-1β + Veh
IL-1β + 101.10
IL-1β + Kin
Relative mRNA expression (normalized to 18S)
Tnfa
Il6
Mpges1
Il12b
Ccl2
Ccl3
Pghs2
Il4
Il10
B
Sham
Veh
101.10
Kin
IL-6
β-actin
Relative IL-6 expression (normalized to β-actin)
+ IL-1β
Amniotic fluid
C
IL-1β concentration (pg/ml)
D
IL-6 concentration (pg/ml)
E
IL-8 concentration (pg/ml)
F
PGF2α concentration (pg/ml)
Newborn blood
G
Il1b
Il6
Il8
Il10
Pghs2
Tnfa
Crp
H
I
J
ns

Figure 3

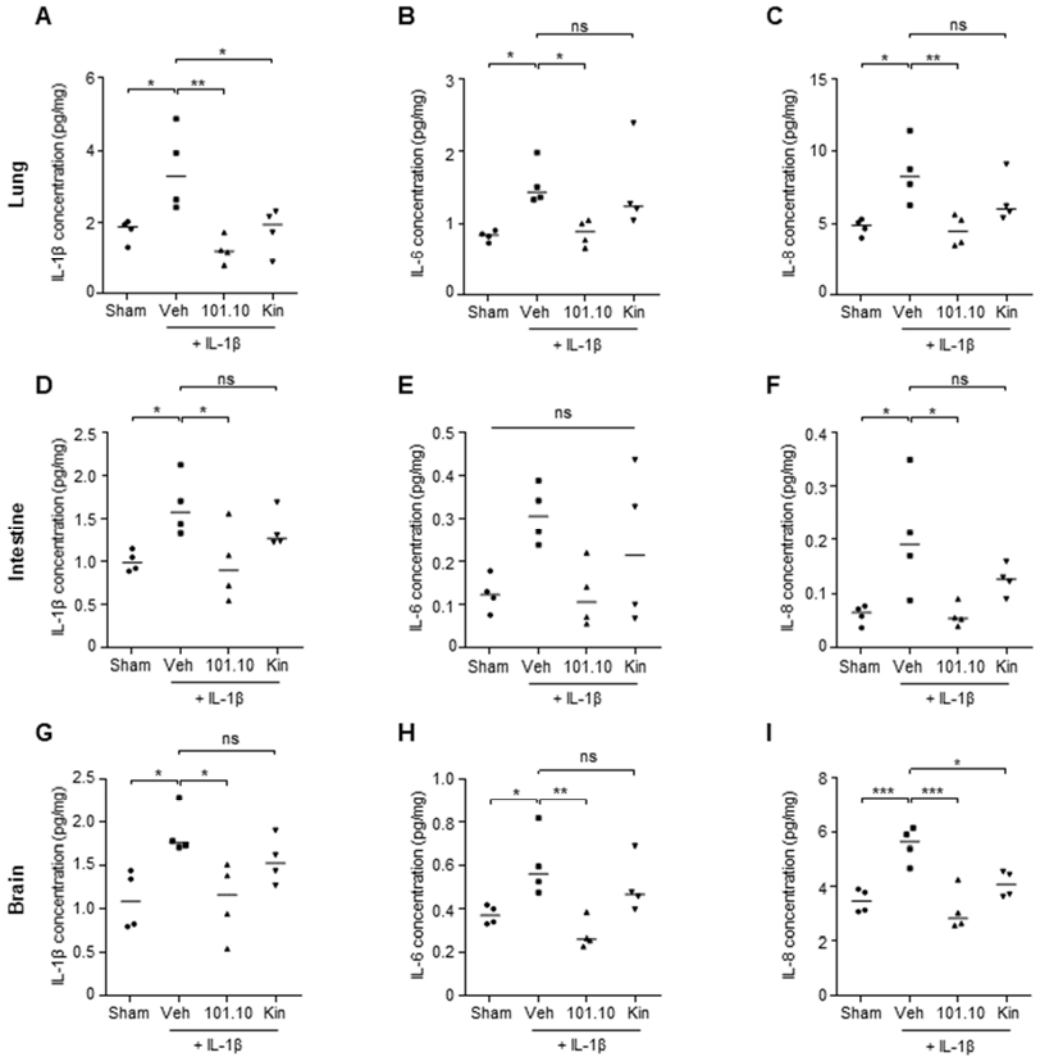

A
B
C
D
E
F
G
H
I
Lung
Intestine
Brain
IL-1β concentration (pg/mg)
IL-6 concentration (pg/mg)
IL-8 concentration (pg/mg)
Sham
Veh
101.10
Kin
+ IL-1β
ns

Figure 4

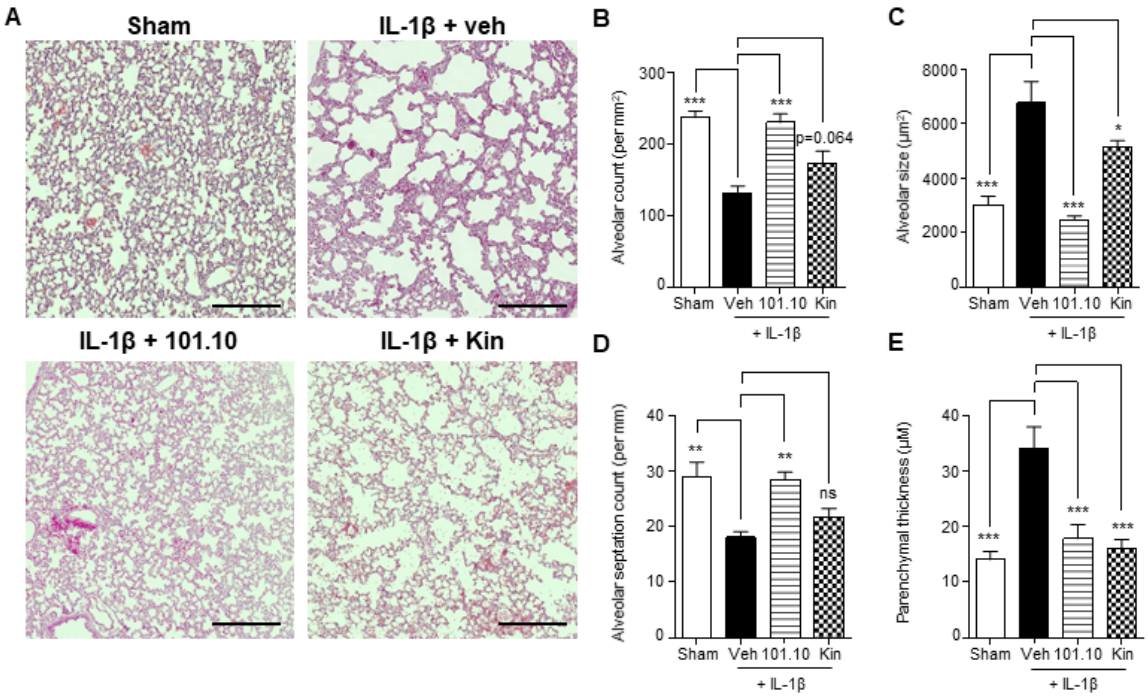

A
Sham
IL-1β + veh
IL-1β + 101.10
IL-1β + Kin
B
Alveolar count (per mm²)
300
200
100
0
***
***
p=0.064
Sham
Veh
101.10
Kin
+ IL-1β
C
Alveolar size (µm²)
8000
6000
4000
2000
0
***
***
*
Sham
Veh
101.10
Kin
+ IL-1β
D
Alveolar septation count (per mm)
40
30
20
10
0
**
**
ns
Sham
Veh
101.10
Kin
+ IL-1β
E
Parenchymal thickness (µM)
40
30
20
10
0
***
***
***
Sham
Veh
101.10
Kin
+ IL-1β

Figure 5

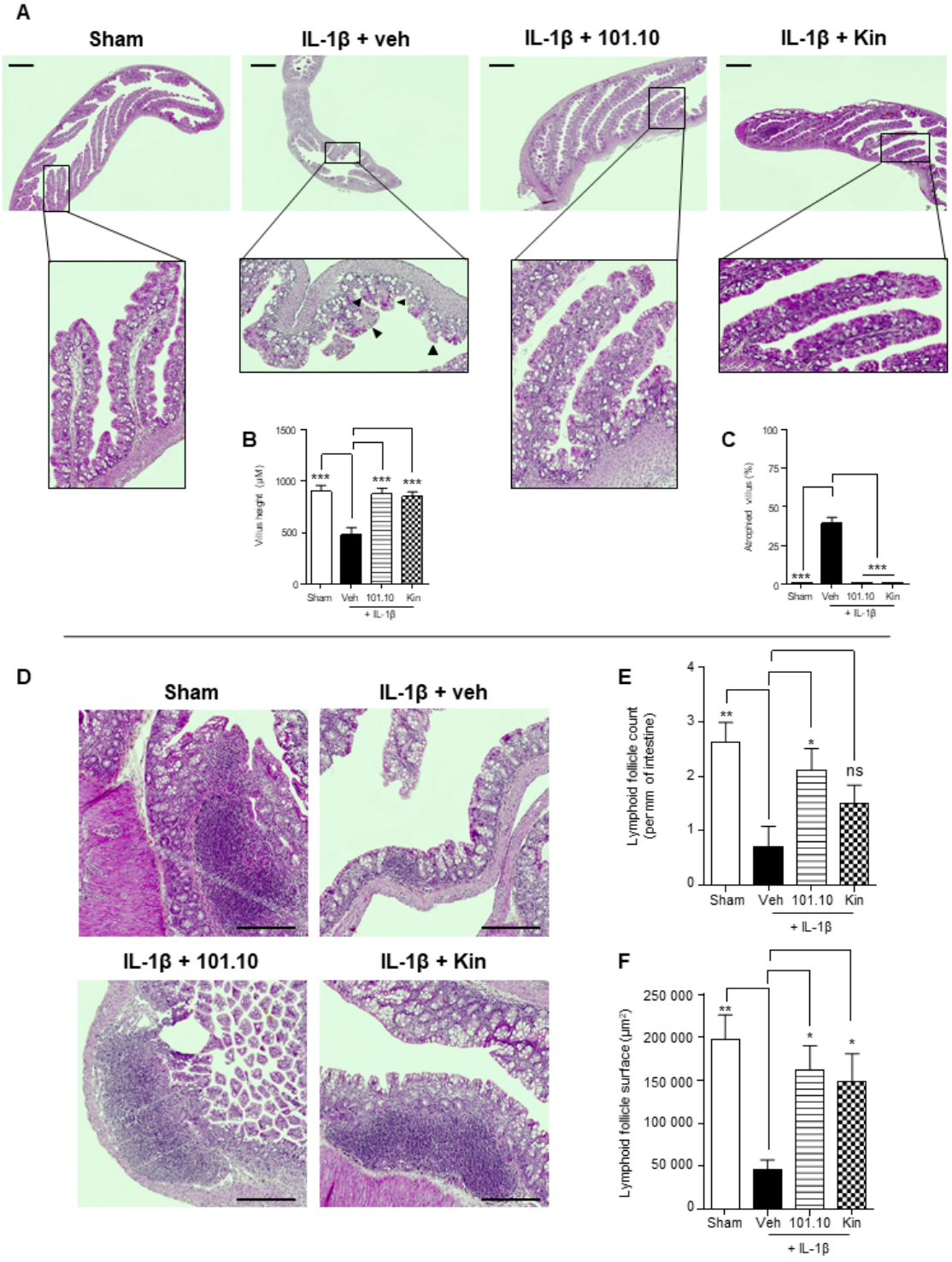

A
Sham
IL-1β + veh
IL-1β + 101.10
IL-1β + Kin
B
Villus height (µM)
1500
1000
500
0
***
***
***
Sham
Veh
101.10
Kin
+ IL-1β
C
Atrophied villus (%)
100
75
50
25
0
***
***
Sham
Veh
101.10
Kin
+ IL-1β
D
Sham
IL-1β + veh
IL-1β + 101.10
IL-1β + Kin
E
Lymphoid follicle count (per mm of intestine)
4
3
2
1
0
**
*
ns
Sham
Veh
101.10
Kin
+ IL-1β
F
Lymphoid follicle surface (µm²)
250 000
200 000
150 000
100 000
50 000
0
**
*
*
Sham
Veh
101.10
Kin
+ IL-1β

Figure 6

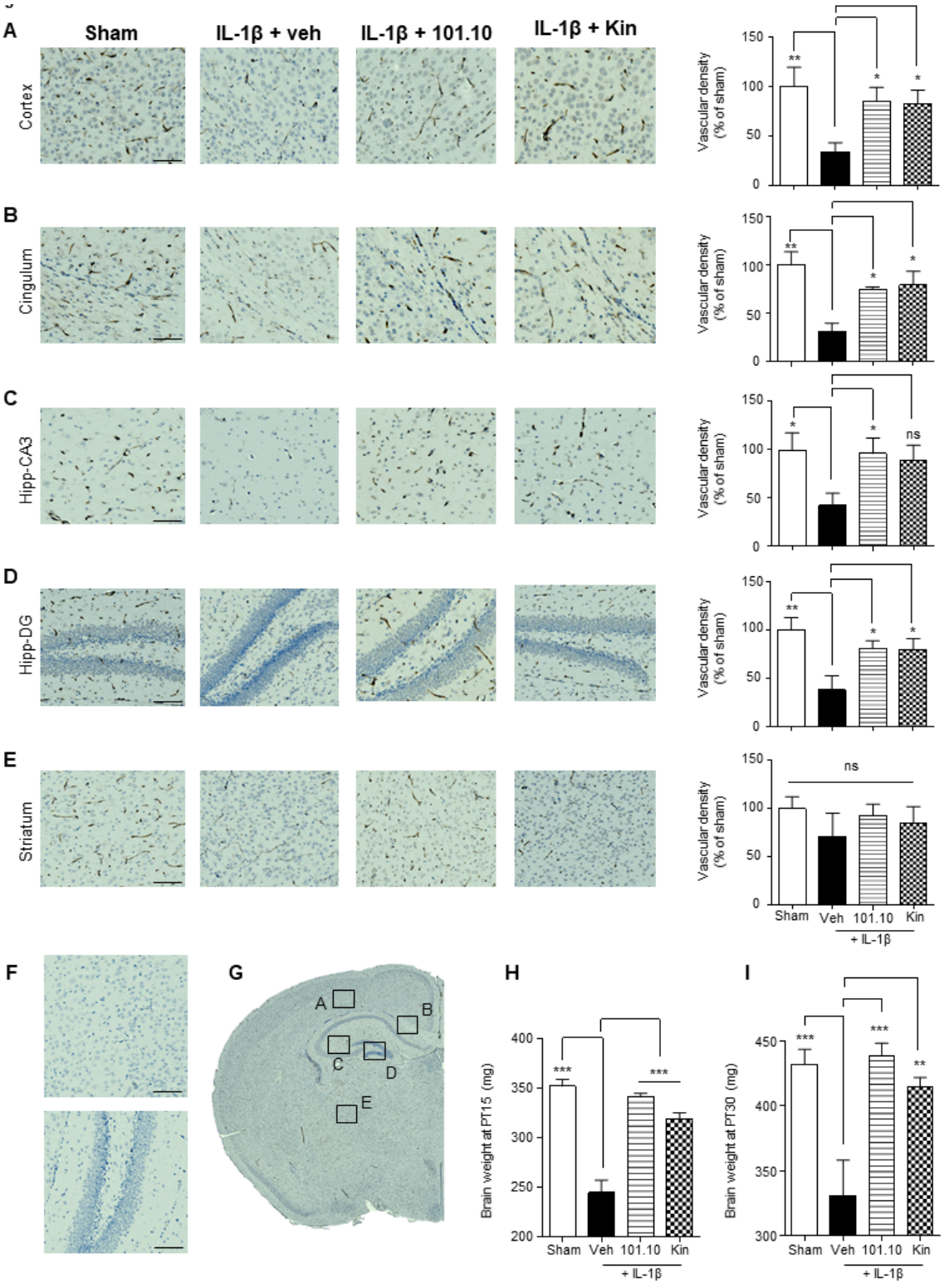

A
Sham
IL-1β + veh
IL-1β + 101.10
IL-1β + Kin
Cortex
B
Cingulum
C
Hipp-CA3
D
Hipp-DG
E
Striatum
Vascular density (% of sham)
150
100
50
0
**
*
ns
Sham
Veh
101.10
Kin
+ IL-1β
F
G
A
B
C
D
E
H
Brain weight at PT15 (mg)
400
350
300
250
200
***
I
Brain weight at PT30 (mg)
450
400
350
300
***
**

Figure 7

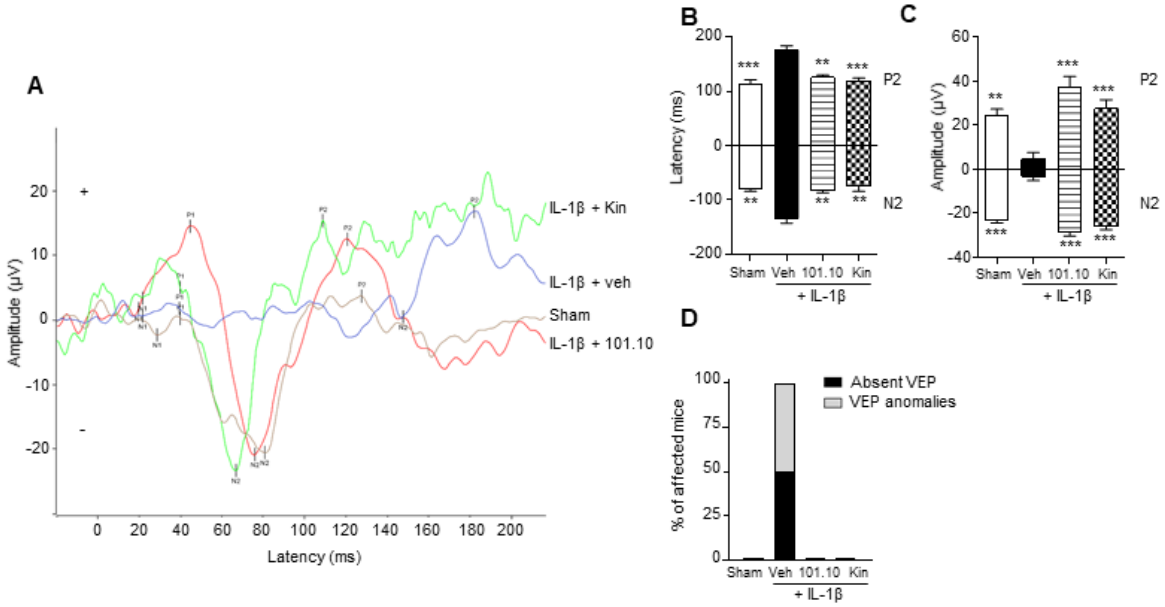

A
Amplitude (μV)
Latency (ms)
IL-1β + Kin
IL-1β + veh
Sham
IL-1β + 101.10
B
Latency (ms)
P2
N2
Sham
Veh
101.10
Kin
+ IL-1β
C
Amplitude (μV)
P2
N2
Sham
Veh
101.10
Kin
+ IL-1β
D
% of affected mice
Absent VEP
VEP anomalies
Sham
Veh
101.10
Kin
+ IL-1β

Figure 8

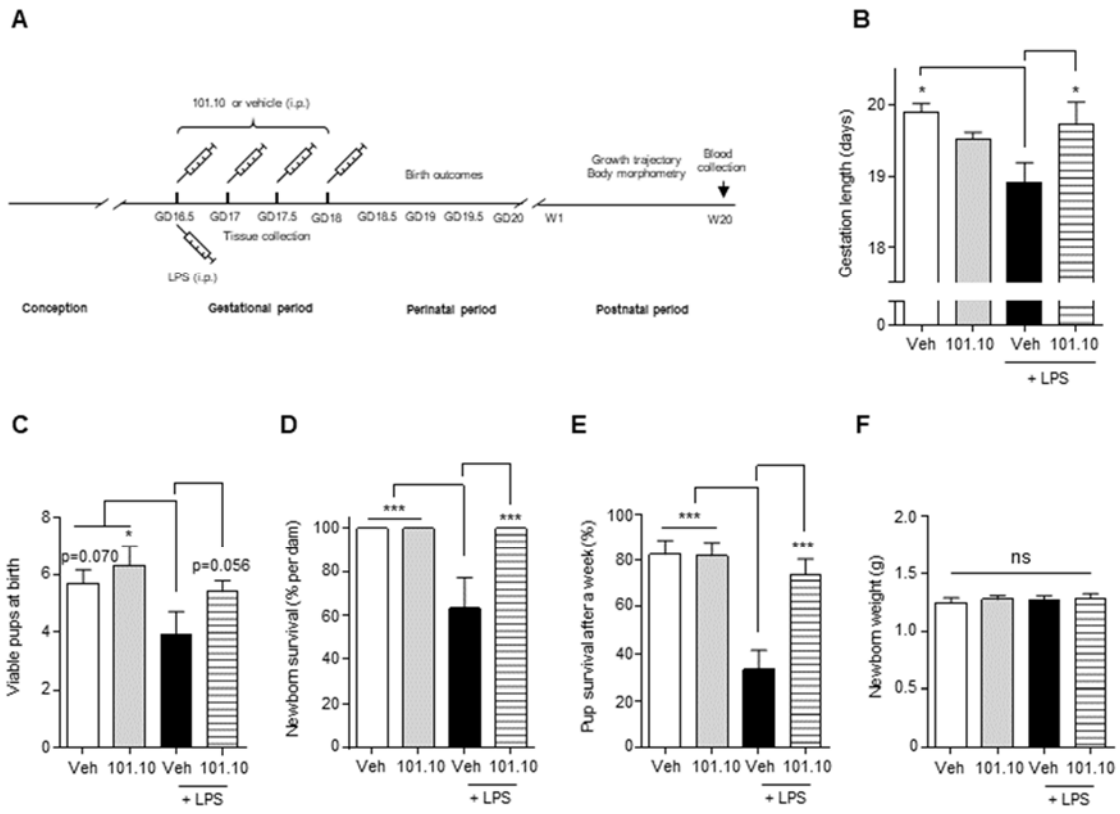
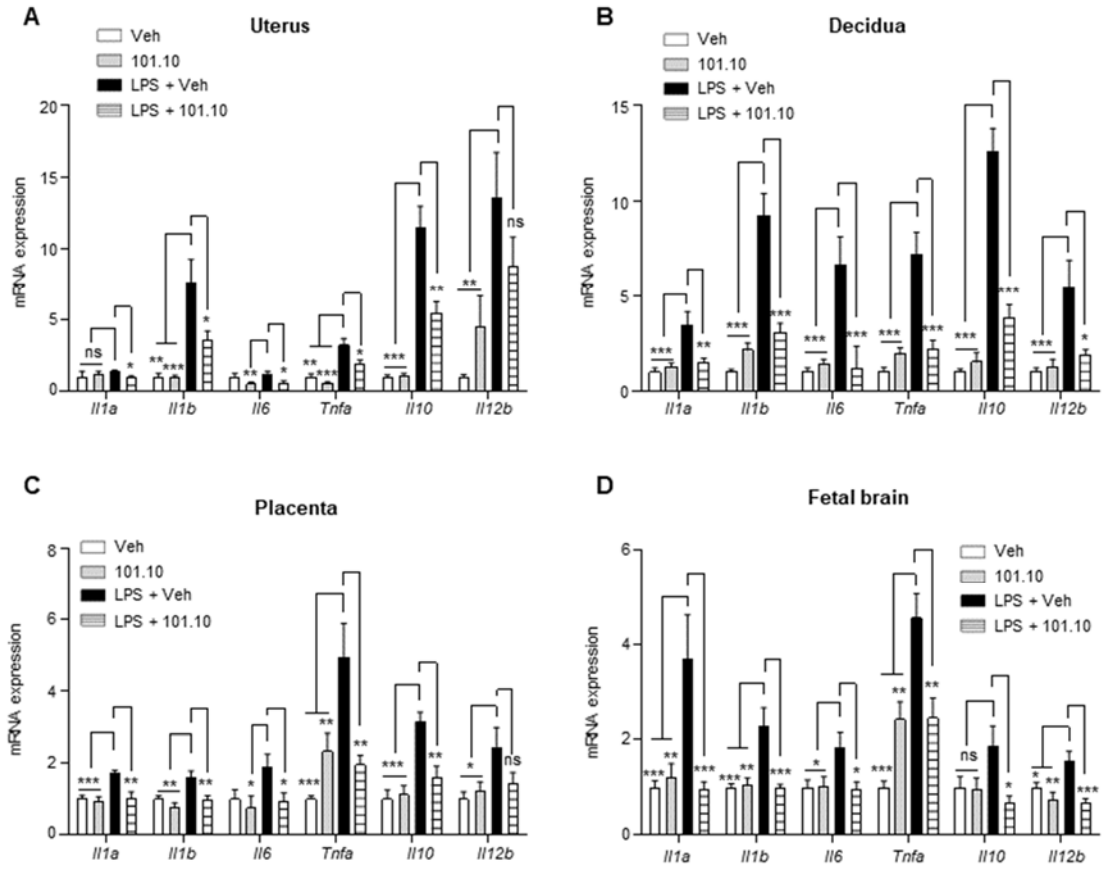

A
101.10 or vehicle (i.p.)
Birth outcomes
Growth trajectory
Body morphometry
Blood collection
GD16.5
GD17
GD17.5
GD18
GD18.5
GD19
GD19.5
GD20
W1
W20
Tissue collection
LPS (i.p.)
Conception
Gestational period
Perinatal period
Postnatal period
B
Gestation length (days)
Veh
101.10
Veh
101.10
+ LPS
C
Viable pups at birth
p=0.070
p=0.056
D
Newborn survival (% per dam)
E
Pup survival after a week (%)
F
Newborn weight (g)
ns

Figure 9


A
Uterus
B
Decidua
C
Placenta
D
Fetal brain
Veh
101.10
LPS + Veh
LPS + 101.10
mRNA expression
Il1a
Il1b
Il6
Tnfa
Il10
Il12b
ns

## Supplementary Figures

Figure S1

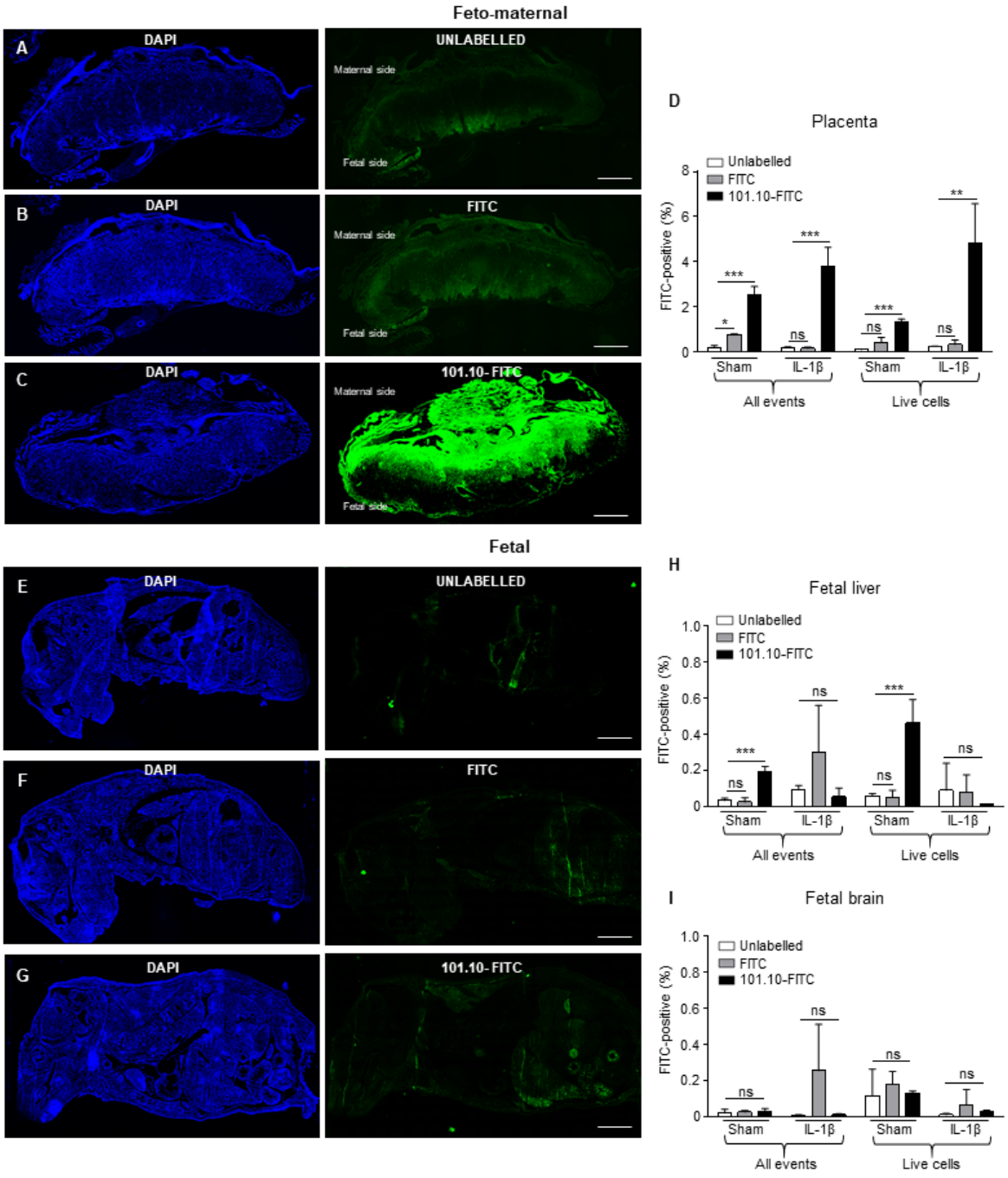


Figure S2

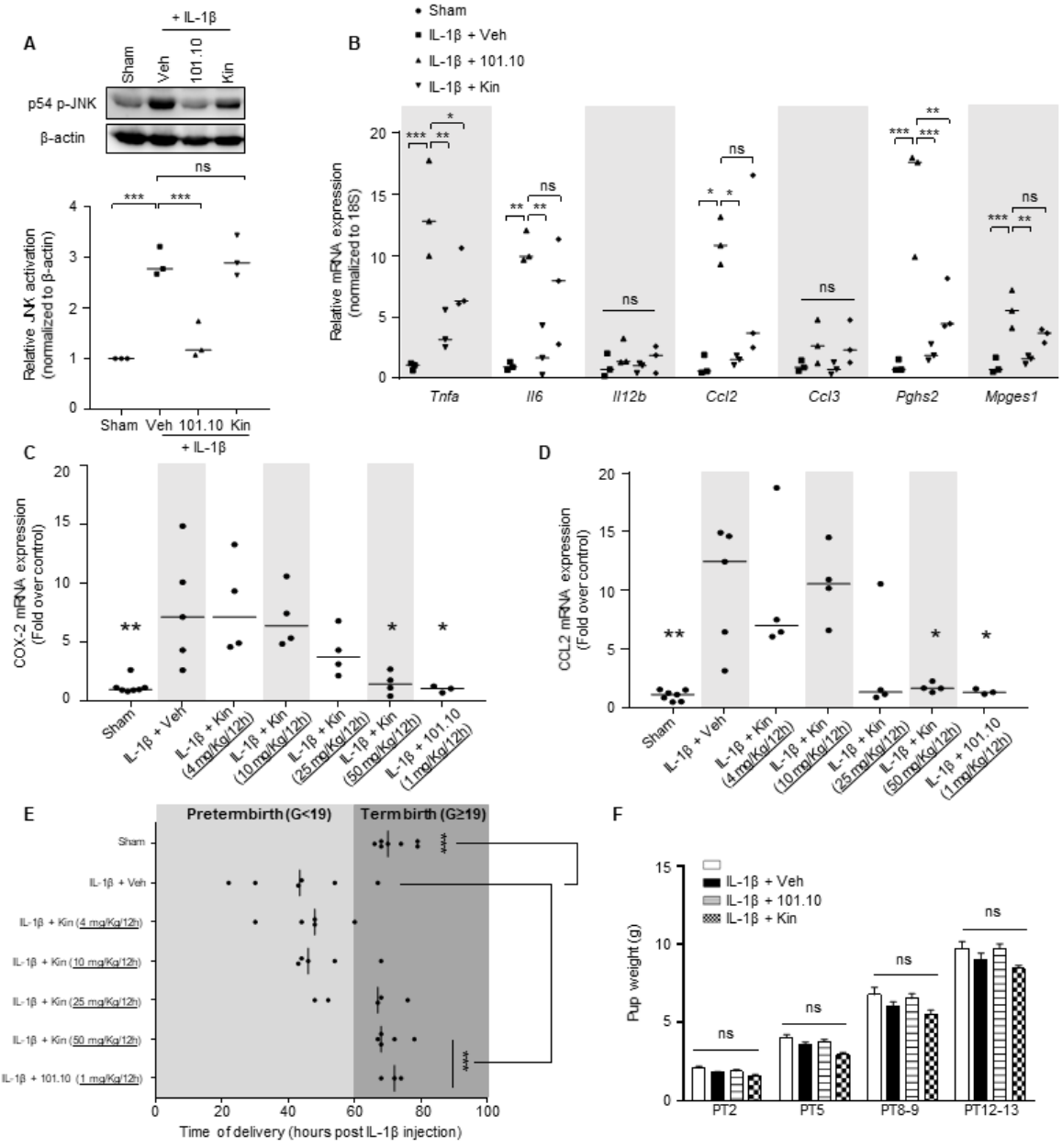
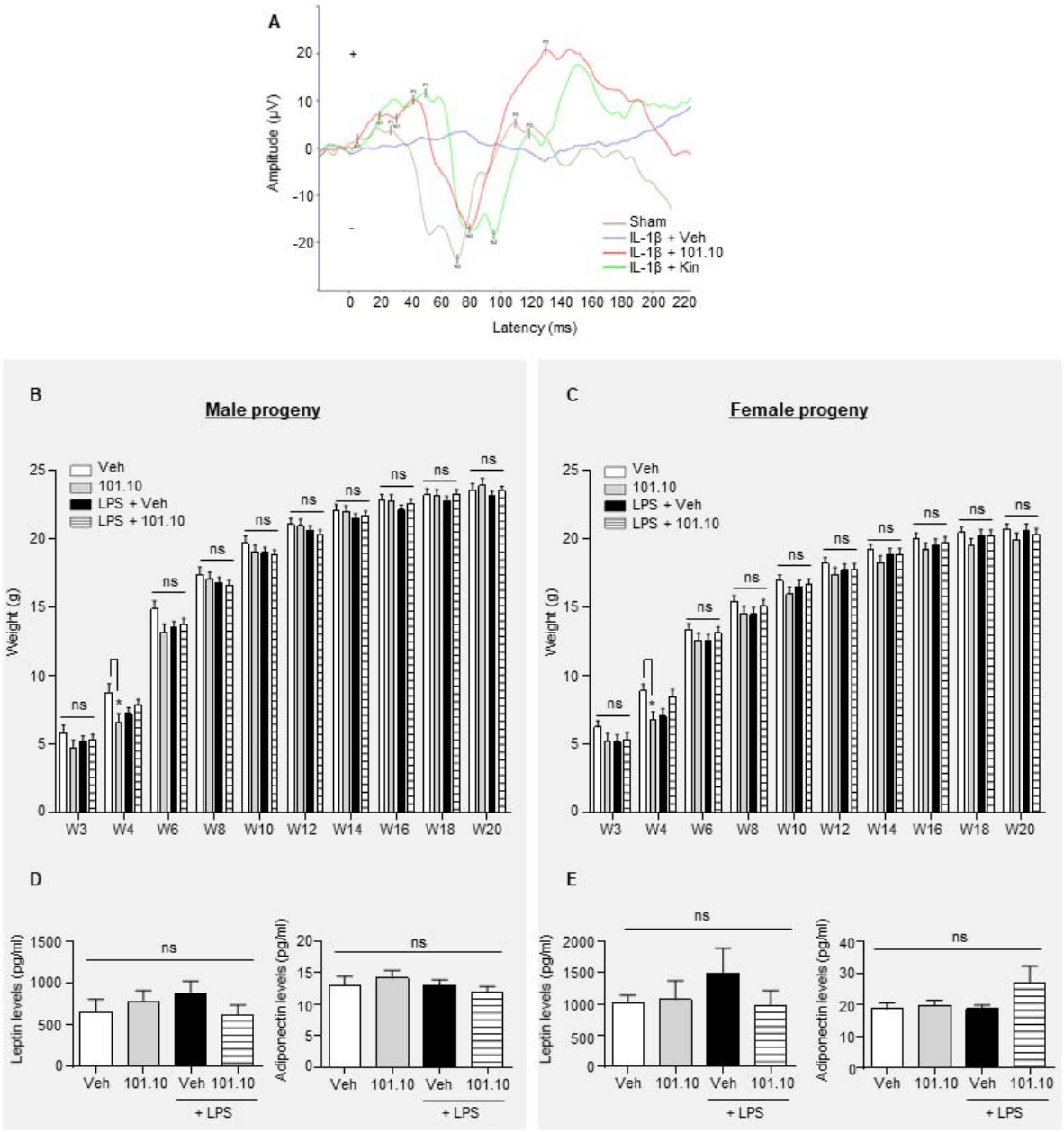

A
+ IL-1β
Sham
Veh
101.10
Kin
p54 p-JNK
β-actin
Relative JNK activation (normalized to β-actin)
B
Sham
IL-1β + Veh
IL-1β + 101.10
IL-1β + Kin
Relative mRNA expression (normalized to 18S)
Tnfa
Il6
Il12b
Ccl2
Ccl3
Pghs2
Mpges1
C
COX-2 mRNA expression (Fold over control)
IL-1β + Kin (4 mg/Kg/12h)
IL-1β + Kin (10 mg/Kg/12h)
IL-1β + Kin (25 mg/Kg/12h)
IL-1β + Kin (50 mg/Kg/12h)
IL-1β + 101.10 (1 mg/Kg/12h)
D
CCL2 mRNA expression (Fold over control)
E
Pretermbirth (G<19)
Termbirth (G≥19)
Time of delivery (hours post IL-1β injection)
F
Pup weight (g)
PT2
PT5
PT8-9
PT12-13

Figure S3


A
Amplitude (µV)
Latency (ms)
Sham
IL-1β + Veh
IL-1β + 101.10
IL-1β + Kin
B
Male progeny
C
Female progeny
Veh
101.10
LPS + Veh
LPS + 101.10
Weight (g)
W3 W4 W6 W8 W10 W12 W14 W16 W18 W20
ns
D
E
Leptin levels (pg/ml)
Adiponectin levels (pg/ml)
Veh 101.10 Veh 101.10
+ LPS

## Tables

Table I. Primers for mRNA expression analysis

| Gene | Forward and Reverse Prime Sequence | GeneBank accession # |
|---|---|---|
| *Il1a* | F-*5' CCGACCTCATTTTCTTCTGG 3',*<br>R-*5' GTGCACCCGACTTTGTTCTT 3'* | NM_010554.4 |
| *Il1b* | F-*5'* CCAAAGCAATACCCAAAGAAA *3'*<br>R-*5'* GCTTGTGCTCTGCTTGTGAG *3'* | NM 008361.3 |
| *Il1b (second pair)* | F-*5'* AGATGAAGGGCTGCTTCCAAA *3'*<br>R-*5'* GGAAGGTCCACGGGAAAGAC *3'* | NM 008361.3 |
| *Il4* | F-*5'* CCATATCCACGGATGCGACA *3'*<br>R-*5'* CTGTGGTGTTCTTCGTTGCTG *3'* | NM 021283 |
| *Il6* | F-*5' ACAACCACGGCCTTCCCTAC 3',*<br>R-*5' TCCACGATTTCCCAGAGAACA 3'* | NM 031168.1 |
| *Il6 (second pair)* | F-*5' CAACGATGATGCACTTGCAGA 3',*<br>R-*5' TCTCTCTGAAGGACTCTGGCT 3'* | NM 031168.1 |
| *Il10* | F-*5' AGGCGCTGTCATCGATTTCT 3'*<br>R-*5' TGGCCTTGTAGACACCTTGGT 3'* | NM 010548.2 |
| *Il10 (second pair)* | F-*5' TAACTGCACCCACTTCCCAG 3'*<br>R-*5' AGGCTTGGCAACCCAAGTAA 3'* | NM 010548.2 |
| *Il12b* | F-*5' TGACACGCCTGAAGAAGA 3'*<br>R-*5' AGAGACGCCATTCCACAT 3'* | NM 008352.2 |
| *Il12b (second pair)* | F-*5' TGGGAGTACCCTGACTCCTG 3'*<br>R-*5' AGGAACGCACCTTTCTGGTT 3'* | NM 008352.2 |
| *Tnf* | F-*5' GTAGCCCACGTCGTA 3'*<br>R-*5' TCCACGATTTCCCAG 3'* | NM 013693.3 |
| *Tnf (second pair)* | F-*5' GCCTCTTCTCATTCCTGCTTG 3'*<br>R-*5' CTGATGAGAGGGAGGCCATT 3'* | NM 013693.3 |
| *Actb* | F-*5' CGTGGGCCGCCCTAGGCACCA 3'*<br>R-*5' ACACGCAGCTCATTGTA 3'* | NM 007393.3 |
| *Crp* | F-*5' TCTGCACAAGGGCTACACTG 3'*<br>R-*5' ATCTCCGATGTCTCCCACCA 3'* | NM 007768 |
| *Pghs2* | F-*5' ACCTCTCCACCAATGACCTGA 3'*<br>R-*5' CTGACCCCCAAGGCTCAAAT 3'* | NM_011198.4 |
| *Ccl2* | F-*5' GCTCAGCCAGATGCAGTTA 3'*<br>R-*5' TGTCTGGACCCATTCCTTCT 3'* | NM_011333 |
| *Ccl3* | F-*5' CCCAGCCAGGTGTCATTTTC 3'*<br>R-*5' GTGGCTACTTGGCAGCAAAC 3'* | NM_011337.2 |
| *Mpges1* | F-*5' GCTGCGGAAGAAGGCTTTTG 3'*<br>R-*5' GGTTGGGTCCCAGGAATGAG 3'* | NM_022415 |

Table II. Body morphometry in fetuses 24 h after exposure to IL-1β and vehicle, 101.10 or Kineret

| | Sham<br>N = 4 | IL-1β + veh<br>N = 4 | IL-1β + 101.10<br>N = 4 | IL-1β + Kin<br>N = 4 |
|---|---|---|---|---|
| Head length (mm) | 7078 ± 148 | 7405 ± 293 | 7498 ± 115 | 7176 ± 293 |
| Body length (mm) | 20535 ± 194 | 20587 ± 764 | 20658 ± 438 | 20217 ± 694 |
| Thorax length (m | 5623 ± 334 | 5889 ± 436 | 5824 ± 238 | 5677 ± 238 |

All data are presented as estimated marginal means ± SEM and analysed using One-way ANOVA with Dunnett's multiple comparison test. Differences between treatment and control groups are considered significant when of $p < 0.05$.

**Supplementary Tables**

Table S1A. Body morphometry in 20 week old adult male progeny after exposure to LPS and/or 101.10

| Absolute weight | PBS N = 20 | LPS N = 10 | PBS + 101.10 N = 16 | LPS + 101.10 N = 22 |
|---|---|---|---|---|
| Lean body weight (g) | 22.67 ± 0.46 | 22.73 ± 0.37 | 23.59 ± 0.51 | 23.04 ± 0.42 |
| Muscle:fat ratio | 1.30 ± 0.16 | 1.53 ± 0.14 | 1.28 ± 0.18 | 1.39 ± 0.16 |
| Total Central Fat (mg) | 662 ± 42 | 633 ± 33 | 669 ± 46 | 625 ± 37 |
| Epididymal Fat (mg) | 243 ± 19 | 240 ± 15 | 244 ± 20 | 224 ± 16 |
| Retroperitoneal Fat (mg) | 347 ± 27 | 318 ± 23 | 353 ± 30 | 334 ± 26 |
| Peri-renal Fat (mg) | 70 ± 7 | 68 ± 5 | 72 ± 7 | 65 ± 6 |
| Combined Muscle (mg) | 833 ± 21 | 828 ± 17 | 840 ± 23 | 848 ± 18 |
| Gastrocnemius (mg) | 249 ± 8 | 249 ± 6 | 241 ± 9 | 260 ± 7 |
| Quadriceps (mg) | 309 ± 9 | 302 ± 7 | 318 ± 10 | 313 ± 8 |
| Biceps (mg) | 58 ± 3 | 63 ± 3 | 67 ± 4 | 58 ± 3 |
| Triceps (mg) | 217 ± 7 | 214 ± 6 | 215 ± 8 | 217 ± 6 |
| Brain (mg) | 414 ± 6 | 409 ± 5 | 404 ± 7 | 413 ± 5 |
| Heart (mg) | 128 ± 3 | 122 ± 3 | 131 ± 4 | 128 ± 3 |
| Lungs (mg) | 170 ± 5 | 167 ± 4 | 181 ± 5 | 173 ± 4 |
| Thymus (mg) | 60 ± 3 $^{ab}$ | 51 ± 3 $^{a}$ | 53 ± 4 $^{ab}$ | 63 ± 3 $^{b}$ |
| Kidneys R (mg) | 172 ± 5 | 158 ± 4 | 162 ± 6 | 159 ± 5 |
| Kidneys L (mg) | 157 ± 5 | 147 ± 4 | 156 ± 6 | 149 ± 5 |
| Adrenals R (mg) | 4 ± 2 | 4 ± 1 | 6 ± 2 | 3 ± 1 |
| Adrenals L (mg) | 4 ± 0 | 4 ± 0 | 5 ± 0 | 4 ± 0 |
| Liver (mg) | 1098 ± 41 | 1051 ± 35 | 1141 ± 47 | 1097 ± 40 |
| Spleen (mg) | 67 ± 5 $^{a}$ | 72 ± 4 $^{ab}$ | 87 ± 5 $^{b}$ | 82 ± 4 $^{ab}$ |
| Seminal Vesicle (mg) | 255 ± 11 | 217 ± 9 | 233 ± 12 | 218 ± 9 |
| Testes R (mg) | 87 ± 3 | 85 ± 3 | 87 ± 3 | 85 ± 3 |
| Testes L (mg) | 86 ± 3 | 84 ± 2 | 85 ± 3 | 84 ± 3 |
| Epididymis (L + R) (mg) | 116 ± 3 $^{a}$ | 108 ± 2 $^{ab}$ | 112 ± 4 $^{ab}$ | 102 ± 3 $^{b}$ |

All data are presented as estimated marginal means ± SEM and analysed as a Mixed Model Linear Repeated Measures ANOVA and post-hoc Sidak test, with litter size as a covariate. Differences between treatment and control groups are considered significant when of $p < 0.05$.

Table S1B. Body morphometry in 20 week old adult female progeny after exposure to LPS and/or 101.10

| Absolute weight | PBS N = 22 | LPS N = 11 | PBS + 101.10 N = 14 | LPS + 101.10 N = 11 |
|---|---|---|---|---|
| Lean body weight (g) | 19.73 ± 0.44 | 19.91 ± 0.47 | 19.85 ± 0.54 | 19.44 ± 0.53 |
| Muscle:fat ratio | 1.19 ± 0.05 | 1.12 ± 0.06 | 1.18 ± 0.06 | 1.23 ± 0.06 |
| Total Central Fat (mg) | 623 ± 33 | 629 ± 36 | 589 ± 40 | 562 ± 41 |
| Parametrial Fat (mg) | 202 ± 16 | 210 ± 18 | 192 ± 20 | 180 ± 20 |
| Retroperitoneal Fat (mg) | 345 ± 15 | 342 ± 16 | 326 ± 18 | 317 ± 18 |
| Peri-renal Fat (mg) | 78 ± 6 | 76 ± 7 | 71 ± 7 | 66 ± 7 |
| Combined Muscle (mg) | 731 ± 19 | 692 ± 22 | 690 ± 24 | 673 ± 25 |
| Gastrocnemius (mg) | 226 ± 6 | 219 ± 7 | 215 ± 8 | 207 ± 8 |
| Quadriceps (mg) | 274 ± 9 | 255 ± 11 | 254 ± 12 | 254 ± 13 |
| Biceps (mg) | 52 ± 3 | 54 ± 3 | 51 ± 3 | 48 ±3 |
| Triceps (mg) | 180 ± 5 | 164 ± 6 | 170 ± 7 | 165 ± 7 |
| Brain (mg) | 415 ± 7 | 417 ± 8 | 406 ± 9 | 408 ±9 |
| Heart (mg) | 109 ± 3 | 111 ± 3 | 109 ± 3 | 105 ± 4 |
| Lungs (mg) | 173 ± 9 | 173 ± 9 | 174 ± 11 | 165 ± 10 |
| Thymus (mg) | 64 ± 4 | 60 ± 4 | 64 ± 5 | 70 ± 5 |
| Kidneys R (mg) | 132 ± 5 | 129 ± 5 | 122 ± 6 | 127 ± 6 |
| Kidneys L (mg) | 124 ± 4 | 119 ± 5 | 112 ± 5 | 120 ± 5 |
| Adrenals R (mg) | 4 ± 0 | 4 ± 0 | 4 ± 0 | 4 ± 0 |
| Adrenals L (mg) | 5 ± 0 | 4 ± 0 | 4 ± 0 | 4 ± 0 |
| Liver (mg) | 975 ± 30 | 975 ± 33 | 945 ± 37 | 955 ± 38 |
| Spleen (mg) | 90 ± 6 | 92 ± 6 | 93 ± 7 | 86 ± 7 |
| Uterus (mg) | 64 ± 6 | 72 ± 7 | 63 ± 7 | 66 ± 8 |
| Ovary R (mg) | 16 ± 1 | 14 ± 1 | 16 ± 1 | 13 ± 1 |
| Ovary L (mg) | 17 ± 2 | 16 ± 2 | 16 ± 2 | 13 ± 2 |

All data are presented as estimated marginal means ± SEM and analysed as a Mixed Model Linear Repeated Measures ANOVA and post-hoc Sidak test, with litter size as a covariate. Differences between treatment and control groups are considered significant when of $p < 0.05$.

# Article 3: Lactate Produced during Labor Modulates Uterine Inflammation *via* GPR81[1]

Ankush Madaan[1,2,*], Mathieu Nadeau-Vallée[1,3,*]; Jose Carlos Rivera[4]; Dima Obari[3]; Xin Hou[1]; Estefania Marin Sierra[1,2]; Sylvie Girard[5]; David M. Olson[6]; Sylvain Chemtob[1,2,3,4]

[1]Departments of Pediatrics, Ophthalmology and Pharmacology, CHU Sainte-Justine Research Center, Montréal, Canada, H3T 1C5, and Université de Montréal, Montréal, Canada H3C 3J7; [2]Department of Pharmacology and Therapeutics, McGill University, Montréal, Canada, H3G 1Y6; [3]Department of Pharmacology, Université de Montréal, Montréal, Canada H3C 3J7; [4]Maisonneuve-Rosemont Hospital, Research Center, Montreal, Canada, H1T 2M4; [5]Departments of Obstetrics and Gynecology, CHU Sainte-Justine research center, Montréal, Canada, H3T 1C5; 6Departments of Obstetrics and Gynecology, Pediatrics and Physiology, University of Alberta, Edmonton, AB, Canada.

*Ankush Madaan and Mathieu Nadeau-Vallée contributed equivalently to this work.

Running title. Lactate/GPR81 curbs uterine inflammation during labor

Correspondence. Sylvain Chemtob, M.D., Ph.D. CHU Sainte-Justine, Research Center, Departments of Pediatrics, Ophthalmology and Pharmacology. 3175 Chemin Côte Ste-Catherine, Montréal, Québec, Canada, H3T 1C5. Fax: 1-514-345-4801, Tel: 1-514-345-4931 ext. 2978. E-mail: sylvain.chemtob@umontreal.ca

[1] Originally published in American Journal of Obstetrics & Gynecology. Madaan A, Nadeau-Vallée M, Rivera JC, Obari D, Hou X, Sierra EM, Girard S, Olson DM, Chemtob S. Lactate produced during labor modulates uterine inflammation *via* GPR81 (HCA(1)). Am J Obstet Gynecol. 2017 Jan;216(1):60.e1-60.e17.

**Abstract**

**Background**: Uterine inflammatory processes trigger pro-labor pathways and orchestrate on-time labor onset. Although essential for successful labor, inflammation needs to be regulated to avoid uncontrolled amplification and resolve postpartum. During labor, myometrial smooth muscle cells generate ATP mainly *via* anaerobic glycolysis, resulting in accumulation of lactate. Aside from its metabolic function, lactate has been shown to activate a G protein-coupled receptor, GPR81, reported to regulate inflammation. We therefore hypothesize that lactate produced during labor may act *via* GPR81 in uterus to exert in a feedback manner anti-inflammatory effects, to resolve or mitigate inflammation. **Objective**: To investigate the role of lactate produced during labor and its receptor, GPR81 in regulating inflammation in uterus. Study design: We investigated the expression of GPR81 in uterus and the pharmacological role of lactate acting *via* GPR81 during labor, using shRNA-GPR81 and GPR81$^{-/-}$ mice. **Results**: 1) Uterine lactate levels increased substantially from 2 mM to 9 mM during labor. 2) Immuno-histological analysis revealed expression of GPR81 in uterus with high expression in myometrium. 3) GPR81 expression increased during gestation, and peaks near labor. 4) In primary mSMC and *ex vivo* uteri from wild type mice, lactate decreased interleukin (IL)-1β-induced transcription of key pro-inflammatory *Il1b*, *Il6*, *Ccl2* and *Pghs2*; suppressive effects of lactate were not observed in cells and tissues from GPR81$^{-/-}$ mice. 5) Conversely, pro-inflammatory gene expression was augmented in uterus at term in GPR81$^{-/-}$ mice and wild type mice treated intrauterine with lentiviral-encoded shRNA-GPR81; GPR81 silencing also induced pro-inflammatory gene transcription in uterus when labor was induced by endotoxin (lipopolysaccharide). 6) Importantly, administration to pregnant mice of a metabolically stable specific GPR81 agonist, 3,5-dihydroxybenzoic acid, decreased endotoxin-induced uterine

inflammation, preterm birth and associated neonatal mortality. **Conclusions**: Collectively, our data uncover a novel link between the anaerobic glycolysis and the control of uterine inflammation wherein the high levels of lactate produced during labor act on uterine GPR81 to down-regulate key pro-inflammatory genes. This discovery may represent a novel feedback mechanism to regulate inflammation during labor, and conveys a potential rationale for the use of GPR81 agonists to attenuate inflammation and ensued preterm birth.

Keywords. GPR81; Inflammation; Labor; Spontaneous Labor; Myometrium; Parturition; Preterm labor; Lactate; Lactic Acid; Pyruvate; Mouse; Lipopolysaccharide; Chorioamnionitis; Endotoxin; Chemokine; Cytokine; Interleukin; IL-1; IL-6; CCL2; PGHS2

## Introduction

The onset of uterine labor is the culmination of a gradual uterine activation wherein physiological inflammation induces a common pro-labor pathway characterized by increased myometrial contractility, weakening of fetal membrane integrity and cervical ripening. Pathological pro-inflammatory stimuli, as observed in numerous aetiologies of preterm birth (PTB), can induce one or more of the components of this common pathway resulting in preterm labor and is sufficiently pronounced to induce fetal/neonatal damage as has often been reported (1,2); PTB is a leading cause of neonatal mortality and morbidity worldwide (3-5).

During labor metabolic demand increases beyond tissue oxygenation capacity (6). To sustain the vigorous contractions of the myometrium, glycogen and glucose are utilized by myometrial smooth muscle cells (mSMC) to produce ATP under relative anaerobic conditions, leading to the accumulation of intermediates of carbohydrate metabolites, including lactate (7). This anaerobic glycolytic metabolic pathway is extremely active (high lactate/pyruvate ratio) in myometrium during labor (8). Accordingly, blood lactate levels of laboring women increase considerably as a function of the duration of labor (9).

Lately, lactate has been demonstrated to activate a G protein-coupled receptor, GPR81 (also labelled $HCA_1$) (10). This suggests that lactate has unexpected signaling functions beyond its traditional metabolic role. Along these lines, a role for lactate in the regulation of inflammation has been reported wherein lactate-induced stimulation of GPR81 in leukocytes specifically inhibited the inflammasome (11), an important pro-inflammatory system active during labor (12); correspondingly, a tocolytic function for lactate has been postulated (13). We thus proceeded to study the role of lactate and GPR81 in uterus during labor. Herein, we describe

a novel role for lactate in regulating inflammation during labor *via* activation of GPR81 in uterus.

## Materials and methods

*Experimental design*

This study was designed to address the pharmacological role of lactate *via* its cognate receptor GPR81 in uterus during labor, with a particular focus on its potential contribution to the intrauterine inflammatory environment of labor. Loss-of-function experiments were designed to acquire direct evidence of the effect of GPR81 in uterus using GPR81$^{-/-}$ and GPR81 knocked-down mice. Specifically, we investigated the expression of GPR81 in uterus during pregnancy using antibody-based methods, namely immunohistochemistry and immunoblotting. The transcriptional induction of genes of key inflammatory mediators (e.g. *Il6*, *Ccl2*, *Pghs2*) in response to GPR81 stimulation (with lactate or the specific GPR81 agonist 3,5-DHBA), and with the addition of the major pro-inflammatory stimulant IL-1β, was measured *ex vivo* in uterus explants, *in vitro* in isolated mSMC and *in vivo* in pregnant mice in labor. This set of experiments was designed to assess the potential anti-inflammatory effect of uterine GPR81 when stimulated with exogenous lactate and 3,5-DHBA, or endogenous lactate during labor. A widely used lipopolysaccharide (LPS)-induced PTB model was utilized to investigate the therapeutic potential of GPR81 stimulation as a mean to decrease uterine inflammation and consequently prevent preterm labor and neonatal mortality.

*Animals*

Timed-pregnant CD-1 mice were obtained from Charles River Inc. (Montreal, PQ) at G11 and were allowed to acclimatize for 2 days prior to experiments. Animals were used according to a protocol of the Animal Care Committee of Hospital 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. GPR81$^{-/-}$ mice were obtained from Lexicon Pharmaceuticals (Texas, USA). The gestational time of GPR81$^{-/-}$ and WT mice was monitored every 2 h.

*Chemicals*

Chemicals were purchased from the following manufacturers: rhIL-1β (#200-01B; PeproTech), lactate (#L1750; Sigma), β-estradiol (#2758; Sigma), 3,5-dihydroxybenzoic acid (3,5-DHBA) (#54965; Sigma), lipopolysaccharide E. coli strain 0111:B4 (#L2360; Sigma)

*Lentivirus production and intrauterine injection*

We produced infectious lentivirus (LV) by transiently transfecting lentivector and packaging vectors into 293FT cells (Invitrogen) as previously described (14). We used five different small hairpin RNA sequences against *Gpr81* (RMM4534-EG243270; Dharmacon) (see Table II for sequences) and selected the most effective (see Suppl. Fig. 4A). *In vivo* infections were performed in pregnant mice at G13 with a single intrauterine injection. Briefly, pregnant mice were steadily anesthetized with an isoflurane mask. After body hair removal from the peritoneal area, a 1.5 cm-long median incision was performed with surgical scissors in the lower abdominal wall. Fifty µL of vehicle, LV.shGFP or LV.shGPR81 was injected in the lower segment of both uterine horns (100 µL total) between two fetal membranes with care of not

entering the amniotic cavity. The abdominal muscle layer was then sutured and the skin closed with clips. Lentivirus were allowed to infect the uterus for 72 h.

*LPS-induced preterm birth model*

Timed-pregnant CD-1 mice were carefully randomized using simple randomization by generating computer aided randomized numbers for total animals which were subsequently assigned to the indicated treatment groups as explained before (15). Mice were pre-treated at G13 with an intrauterine injection of vehicle (n=16), LV.shGFP (n=12) or LV.shGPR81 (n=15) (described in the section above). At G16, these animals were anesthetized with isoflurane and received an intraperitoneal injection of Escherichia-Coli-derived lipopolysaccharide (LPS, single dose of 10 μg in 100 μL saline); space limitations precluded performing these experiments in GPR81-knock out mice, as these studies require large number of colonies. In other experiments, mice received only the LPS injection without a prior lentivirus intrauterine injection. One hundred μL of pH-balanced 3,5-dihydrobenzoic acid (3,5-DHBA; 25 mg/Kg/8h) (n=20) or vehicle (n=36) was injected to these animals subcutaneously (in the neck skin) 30 min before LPS or vehicle stimulation (to allow distribution of the drug to target tissues in a pre-clinical efficacy study). The LPS-induced PTB model used was selected on the basis of reported documentation (16). For both experiments, deliveries were monitored hourly until term (>G19). During labor (as confirmed with vaginal bleeding and newborns in the nest), female adults were anesthetized and uterine fragments from their lower uterus (cervical side) were collected, snap-frozen in liquid nitrogen and kept at -80°C for subsequent RNA purification. In some cases (e.g.

Fig. 5), randomized mice (selected on the same basis described above) were allowed to fully deliver to assess neonatal mortality.

*Intrauterine IL-1β-induced PTB model*

Timed-pregnant CD-1 mice at G16 were steadily anesthetized with an isoflurane mask. After body hair removal from the peritoneal area, a 1.5 cm-long median incision was performed with surgical scissors in the lower abdominal wall. The lower segment of the right uterine horn was then exposed and 1 μg of 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 closed with clips.

*Primary myometrial smooth muscle cell and uterine explant isolation and culture*

Primary myometrial smooth muscle cells (mSMC) and uterine explants were isolated from WT and GPR81$^{-/-}$ animals using modifications of a method previously described (17). Briefly, a single subcutaneous injection of 50 μg 17β-estradiol was administered to mice 24h prior to the experiment. The day after, mice were sacrificed by cervical dislocation and sprayed with 70% ethanol. The whole uterus was excised under a sterile hood and placed in Hank's balanced salt solution (HBSS), 100 U/mL penicillin-streptomycin (Gibco, Grand Island, NY), and 2.5 μg/mL amphotericin B (Sigma). The uterine horns were cleansed of fat and vessels and washed by gentle flushing. For explant culture, the uterine horns (including endometrium) were cut into 5mm long fragments and immediately incubated in DMEM medium supplemented with 10% serum for 1 h at 37°C and 5% $CO_2$ (with sufficient volume to completely cover the tissue).

Explants were then serum-starved for an additional h and stimulated with 5 ng/ml IL-1β and/or 10 mM lactate (pH-balanced) or 100 μM 3,5-DHBA for 8 h and frozen at -80ºC for subsequent mRNA isolation. Lactate and 3,5-DHBA were added 30 min prior to IL-1β stimulation. For primary myometrial smooth muscle cell culture, the uterine horns were cut into 1mm 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, Mannheim, Germany], 0.1 mg/mL soybean trypsin inhibitor [sigma], 10% FBS, and 1 mg/mL bovine serum albumin [Sigma] in HBSS). 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 centrifuged at 200g for 10 min, the pellet was resuspended in complete DMEM medium and plated in a T-25 dish. The remaining myometrium fragments were re-used in an enzymatic digestion and the whole digestion-centrifugation process was repeated for a total of 5 times. The 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 analysed in immunohistochemistry to assess culture purity with the smooth muscle cell marker α-actin (see Suppl. Fig. 2).

*Cell Culture*

Primary murine mSMC or human myometrial smooth muscle cells (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 *in vitro* experiments, cells (serum-starved overnight) or freshly-isolated uterine fragments from WT or GPR81$^{-/-}$ mice were treated with 5 ng/ml IL-1β and/or 10 mM lactate (pH-balanced) or 100 μM 3,5-DHBA for 8 h. Lactate and 3,5-DHBA were added 30 min prior to IL-1β stimulation. Cells and tissues were then collected in Ribozol (AMRESCO, Solon OH, United States) and stored at -80ºC for mRNA extraction.

*RNA extraction and Real-Time quantitative Polymerase Chain Reaction (RT-qPCR)*

RNA from tissues (from *ex vivo* stimulation [described above] or collected during pregnancy) or cells was extracted according to manufacturer's protocol. The RNA concentration and integrity were measured with a NanoDrop 1000 spectrophotometer. Five hundred ng of RNA was used to synthesize cDNA using iScript Reverse Transcription SuperMix (Bio-Rad, Hercules CA, United States). Primers were designed using NCBI Primer Blast (see Table I). Quantitative gene expression analysis was performed on Stratagene MXPro3000 (Stratagene) with SYBR Green Master Mix (BioRad). Gene expression levels were normalized to 18S universal primer (Ambion Life Technology, Burlington ON, Canada). Dissociation curves were also acquired to test primer specificity. Gene analyzed include: *Il1b*, *Il4*, *Il6*, *Il8*, *Ccl2* (chemokine ligand 2), *Ptghs2* (Prostaglandin H synthetase 2 or COX-2), *Oxtr* (oxytocin receptor), *Mmp9* (metalloproteinase 9), *Crp* (C-reactive protein), *Gja1* (connexin 43) and *Gpr81*.

*Semiquantitative PCR*

hTERT myometrial cells were pre-treated with LV.shGFP or three different forms of shRNA encoded in lentivirus (LV.shGPR81A,B,C) for 72h to verify efficacy of the latter in knocking down GPR81. Total RNA was isolated with RNase TM mini kit (Qiagen, Germantown MD, United States). RT-PCR (only used to verify efficacy of shRNA) was performed as described previously (18). QuantumRNA universal 18S standard primers (Ambion) were used as internal standard references. LV.shGPR81B was used *in vivo* to effectively knock down GPR81. Note that all other measurements of mRNA were performed by qPCR.

*Western blotting*

Proteins from homogenized myometrium fragments collected during pregnancy lysed in RIPA buffer were quantified using Bradford's method (Bio-Rad). Fifty µg of protein sample were loaded onto SDS-PAGE gel and electrotransferred onto PVDF membranes. After blocking, membranes were incubated with an antibody against GPR81 (#sc-689; Santa Cruz Biotechnology, Dallas TX, United States). After washing, membranes were incubated for 1 h with their respective secondary antibodies conjugated to HRP (Sigma). Enhanced chemiluminescence (GE Healthcare) was used for detection using the ImageQuant LAS-500 (GE Healthcare, Little Chalfont, United Kingdom).

*Lactate quantification assay*

Age-matched laboring pregnant mice at term (TL) or non-laboring pregnant mice at term (TNL) were sacrificed and their uteri were snap-frozen in liquid nitrogen and stored at -80ºC for less than a month. A fragment of 400 mg of each uterus was homogenized and used to quantify tissue lactate concentration using a colorimetric assay following manufacturer's protocol (K627; BioVision). Readings were made on a microplate reader (EnVision Multilabel reader, PerkinElmer, Waltham MA, United States) adjusted for 450 nm. A standard curve of nmol/well vs. OD450nm was plotted and sample readings were applied to it and calculated using C = La/Sv (nmol/µl or mM); La = lactic acid amount (nmol) of sample from standard curve, Sv = sample volume (µl) added into the well. Results were then converted into a concentration unit (mM) using the above standard curve. Inter-assay variation (for standards) is 0.5 mM (based on laboratory and manufacturer).

*Immunohistochemistry*

Myometrial smooth muscle cells were plated on coverslips pre-coated with poly-D-lysine and fixed in 4% paraformaldehyde. After blocking, cells were incubated overnight with rabbit anti-α-actin (#ab5694; abcam) and then for 1 h at ambient temperature with a secondary antibody conjugated with Alexa Fluor 488 (green) (Sigma). For tissue immunohistochemistry, uteri from pregnant mice in labor and non-pregnant mice were cleansed of fat and vessels and fixed in 4% paraformaldehyde for 1 day and transferred in 30% sucrose for another day. Localization of GPR81 was determined on 14 µm uterine sagittal cryosections. Sections blocked with 1% bovine serum albumin, 1% goat serum and 0.1% TritonX-100 (T-8787; Sigma) in PBS were subsequently incubated overnight with the primary antibodies. Secondary

antibodies conjugated with Alexa Fluor (Molecular Probes) directed against rabbit were incubated for 2h at ambient temperature. Nuclei were stained with Dapi (Invitrogen; 1/5000). Images were captured using 10X (for whole uterus imaging) or 30x (for cells and magnified uterus images) objective with Eclipse E800 (Nikon) fluorescence microscope. Whole uterus images were captured 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 by one-way analysis of variance (ANOVA). Dunnett's multiple comparison method was employed when treatments were compared to a single control. A value of $p<0.05$ was considered statistically significant. Data are presented as means +/- S.E.M.

**Results**

*The lactate receptor GPR81 is expressed in uterus and its expression increases during pregnancy; lactate levels are highest during labor*

GPR81 was found in uterus of pregnant mice in labor (G19) and non-pregnant mice within the myometrium layer (Fig. 1A,B); no immunoreactivity to GPR81 was found in uteri from GPR81$^{-/-}$ mice (Suppl. Fig. 1), demonstrating specificity; GPR81 was also localized intracellularly, as reported for many other GPCRs (19,20).

mRNA expression of Gpr81 in uterus increased at G14 (approx. 75% of normal murine gestation length) and remained high (compared to non-pregnant mice) until labor (albeit borderline significantly increased in term labor), after which it decreased at postpartum (Fig. 2A). Concordantly, translated protein expression of GPR81 in uterus was highest towards the end of gestation at G16 and remained so till parturition (Fig. 2B). Consistently, uterine lactate concentration markedly increased during labor from 2 mM to 9 mM (Fig. 2C).

*Lactate exerts anti-inflammatory effects on uterine tissue and myometrial cells via GPR81*

We investigated if lactate/GPR81 modulated inflammation in uterus. Uterine explants (from non-pregnant mice) were stimulated with interleukin-1β (IL-1β), a major pro-inflammatory cytokine implicated in labor (21,22), with and without (pH-buffered) lactate (10 mM; comparable to concentrations achieved during labor [see Fig. 2C] and consistent with $EC_{50}$ of lactate [5 mM] 10). IL-1β caused comparable induction of pro-inflammatory and pro-labor genes in non-pregnant mouse uterus from wildtype (WT) and GPR81-null mice (Fig. 3A,B). As expected, lactate significantly attenuated expression of the numerous IL-1β-induced pro-

inflammatory and pro-labor genes in uterus from WT animals, but was ineffective on uterus from GPR81-null mice (Fig. 3A,B). Effects of lactate on pro-inflammatory genes were corroborated on primary mSMC (see Suppl. Fig. 2 for purity of cells) from WT and GPR81-null mice (Suppl. Fig. 3A). Hence, lactate acting *via* GPR81 conveys anti-inflammatory effects on myometrial cells.

*GPR81 suppresses inflammation in the uterus in labor*

We further explored the role of GPR81 on uterine inflammation of animals in labor (at start of pup expulsion). Increased expression of pro-inflammatory *Il1b*, *Il6*, *Pghs2*, *Ccl2*, *Mmp9* and *Crp*, but not of anti-inflammatory *Il4* was detected in GPR81-null compared to WT mice (Fig. 4A). To rule out possible attenuating compensatory mechanisms that can occur in germ-line gene knockout mice, we performed similar studies after knocking down GPR81 in the uterus using a lentiviral-encoded shRNA targeting *Gpr81* (found to be effective in hTERT cells [used to select efficacious shRNA, Suppl Fig. 4A], and confirmed *in vivo* [Suppl Fig. 4B]), which was injected intra-uterine to WT mice in intact and in LPS-treated animals (Fig. 4B,C). An increase in pro-inflammatory gene transcripts in uterus during labor analogous to that observed in GPR81-null mice was detected in GPR81-knocked down mice (Fig. 4B); lentiviral-encoded GFP exerted no effect. Since the increased uterine inflammation in GPR81-silenced mice occurs during labor process, gestation length was not affected (Suppl. Fig. 4C,E).

In addition to term labor, we studied the role of GPR81 in preterm labor triggered by pro-inflammatory Escherichia Coli-derived LPS (to mimic infectious/inflammatory stimulus of preterm labor) administered intraperitoneally (23,24). LPS-induced inflammatory profile in

uterus (collected in labor) was further augmented in *Gpr81* knocked-down mice (Fig. 4C; LV.shGPR81 was effective in reducing GPR81 expression [Suppl. Fig. 4D]); again *Gpr81* silencing did not affect gestation length (Suppl. Fig. 4E). Concordant with increased uterine GPR81 expression at term gestation (Fig. 2A,B), GPR81 expression was also increased during preterm labor induced by LPS (Suppl. Fig. 4D and Fig. 5A) and IL-1β (Suppl. Fig. 3B). Altogether, data suggest that lactate/GPR81 suppresses inflammation during labor (term and preterm).

*Selective stimulation of GPR81 attenuates inflammation and prevents LPS-induced preterm birth*

Based on anti-inflammatory properties of GPR81 in uterus we proceeded to establish whether stimulation of GPR81 prevents PTB. For this purpose, we utilized a metabolically stable and selective agonist of GPR81, 3,5-DHBA, which is also more potent ($EC_{50} \approx 150$ μM) than metabolizable lactate ($EC_{50} \approx 5$ mM) (25). We first confirmed the anti-inflammatory properties of (pH-buffered) 3,5-DHBA on IL-1β-induced transcripts of *Il6*, *Pghs2* and *Ccl2* on *ex vivo* uteri (Suppl. Fig. 4F); 3,5-DHBA had no effects on GPR81-null uteri. Likewise, uterine inflammation in mice treated with LPS (at G16) was abrogated by (sc) 3,5-DHBA (Fig. 5A), which was also effective in prolonging gestation shortened by LPS (Fig. 5B) as well as normalizing neonatal survival (Fig. 5C). Of interest, anti-inflammatory properties of 3,5-DHBA were also observed in human mSMC (Suppl. Fig. 4F inset).

**Comment**

*Principal findings of the study*

Herein, we describe the glycolytic product lactate as a novel endogenous anti-inflammatory signaling molecule in the uterus acting *via* GPR81, thus providing an unprecedented mechanistic link between metabolism and inflammation in reproductive tissue; a schematic diagram of this concept is presented in Fig. 6. In line with high concentrations of lactate generated during labor (~10 mM), corresponding concentrations are shown to exert anti-inflammatory effects *via* GPR81 in myometrium, and conversely *in vivo* silencing of GPR81 amplifies uterine inflammation during labor. Concordantly, selective stimulation of GPR81 attenuates inflammation induced by pro-inflammatory agents and in turn normalizes gestational length and neonatal survival.

*Meaning of the findings*

Uterine contractions that occur during labor are intense and associated with recurrent periods of hypoxia, leading to the accumulation of lactate from pyruvate during glycolysis (26). Glucose is the main energy substrate of the human myometrium during pregnancy (8,27), and expression of lactate dehydrogenases responsible for the conversion of lactate from pyruvate is found to increase near term in myometrium, inducing a shift toward an anaerobic profile (28-30). Of interest, anaerobic metabolism of glucose is significantly more active in myometrium than in striated muscles (8) known to generate lactate during exercise. Accordingly, lactate production is expected to rise abruptly during labor especially with sustained uterine contractions (9,31), causing maternal (blood) and fetal (blood, amniotic fluids) lactate levels to

increase in parturient women and animals and their progeny (9,32,33), consistent with the murine data presented herein.

Once lactate is produced, its transport (into or out of the cellular compartment) is facilitated by monocarboxylate transporters (MCTs). During anaerobic conditions outward flow of lactate dominates over intracellular transport in mSMC (34). Hence during labor lactate flows out of the mSMC to bind to membrane-bound GPR81, at concentrations consistent with the affinity of GPR81 for lactate (~5 mM) (10); the inherently low affinity of GPR81 for its ligand is consistent with other GPCRs of the same family, notably GPR109A and GPR109B (35). High uterine lactate concentrations as observed during parturition are not likely to be achieved under other physiological circumstances, which may restrict the role of GPR81 to labor only. This claim is consistent with the amplified uterine inflammation observed in GPR81$^{-/-}$ mice during labor, as well as in *Gpr81* knocked-down mice in labor at term or at preterm (induced by inflammatory stimuli); whereas uterus from non-pregnant animals responds similarly to an inflammatory stimulus in WT and GPR81-null mice. Our data further suggest that GPR81 has a limited role during pregnancy before the onset of labor, as evidenced by unaffected gestational length in GPR81$^{-/-}$ mice and in GPR81 knocked-down mice treated with LPS. Again, this is most likely due to insufficient local lactate concentrations during gestation in the quiescent uterus as well as during the inflammatory preparatory phase of the yet inactive uterus. Whereas exogenous stimulation of GPR81 with a pharmacologic agonist can reduce inflammation and prevent PTB as observed with 3,5-DHBA. The deletion of a gene coding for an important endogenously-produced anti-inflammatory mediator favoring uterine quiescence during pregnancy would be expected to predispose to PTB when induced by inflammation, as observed with *Il10*-null mice (36); yet this does not apply to *Gpr81*, which is consistent with our

hypothesis that GPR81 is specifically active during labor when lactate levels are increased. Altogether, our data suggest that lactate accumulates in uterus during labor to act *via* GPR81 thereby regulating inflammation and restore tissue function (postpartum). The anti-inflammatory action of lactate *via* GPR81 described herein is concordant with the recent finding that lactate/GPR81 inhibits TLR4-induced activation of NF-κB and the inflammasome, and the downstream transcription of pro-IL-1β and pro-IL-18 (and their maturation) in macrophages, monocytes, other types of cells, and with a similar effect *in vivo* (11); GPR81 may thus represent a novel target for prevention of PTB and ensuing neonatal complications.

*Clinical Implications*

This work may convey clinical implication as it suggests that lactate is pharmacologically active in uterus and that high concentrations of lactate regulate inflammation during labor to restore normal tissue function thereafter. This is consistent with an early study by Quenby *et al.* suggesting that women with dysfunctional labor have lower hemoglobin saturation with $O_2$ and higher lactate levels in capillaries from the lower segment of the uterus (37). Accordingly, more recent studies have demonstrated that high (≥ 5.0 mmol/L) amniotic fluid lactate levels accurately predict labor disorders (38,39). Although sustained reduction in oxygenation of the uterus during labor directly leads to impaired fetal oxygenation and lactate accumulation in amniotic fluids, whether amniotic fluid and myometrial levels of lactate correlate is not yet known. Nonetheless it is interesting to point out that these high lactate concentrations correspond to >$EC_{50}$ of GPR81 implying plausible activation of GPR81. In this context, lactate was suggested to exert tocolytics effects (13), in addition to its desirable anti-

inflammatory effect reported herein. Pharmacological stimulation of GPR81 may provide benefits by directly attenuating excessive detrimental inflammation for the fetus/newborn.

*Research implications*

The onset of labor is an intricate phenomenon orchestrated by an acute/sub-acute uterine inflammatory response (40,41), particularly relevant in a high proportion of PTB, especially at 21-24 weeks where the rates are higher than 94% (42). In preparation for labor, the uterus is invaded by myeloid cells (e.g. neutrophils, macrophages) which actively produce pro-inflammatory mediators (such as cytokines, chemokines, prostaglandins) in the vicinity of decidual, chorionic and myometrial cells (43-45). Combined with specific hormonal changes (e.g. progesterone withdrawal), these factors directly lead to induction of uterotonic proteins in gestational tissue, which is a prerequisite to the on-time onset of labor (3). Although inflammation conveys critical functions before and during labor, inflammation needs to be controlled and tissue function must be restored postpartum.

Lactate/GPR81 plausibly partakes in the resolution of inflammation that occurs postpartum. Inflammation needs to be terminated to avoid negative maternal outcomes, such as postpartum endometritis (46). The resolution of inflammation is an active process that involves the action of anti-inflammatory and pro-resolution modulators such as lipoxins, resolvins, protectins, IL-10 and 15d-PDJ2 (47). The involvement and importance of this process in gestational tissues is still ill-defined. Recently, Maldonado-Perez *et al.* described lipoxin A4 as a potential pro-resolution factor active after labor in both physiological and pathological human labor (48). This study revealed the anti-inflammatory effects of lipoxin A4 on *ex vivo* uterine

biopsies, in addition to an increased expression of the lipoxin A4 receptor FRP2 in laboring myometrium. Interestingly, our observations presented herein on lactate/GPR81 in myometrium complement those related to other mediators.

*Strengths and Limitations*

Strengths. This study provides converging *in vitro*, *ex vivo* and *in vivo* evidence that lactate exhibits GPR81-mediated anti-inflammatory effects in uterus during labor. To our knowledge, this is the first study addressing the pharmacological role of an intermediate of carbohydrate metabolites in pregnant uterus and therefore opens new areas of investigation which possibly expand beyond labor. Intermediates of carbohydrate metabolites accumulate under hypoxic conditions in the body (49); such condition is observed in uterus during physiological labor, as well as in pathological conditions (e.g. preterm labor, cancer, primary dysmenorrhea, preeclampsia) (50,51). Other than lactate, succinate and α-ketoglutarate are two citric acid cycle intermediates that accumulate during hypoxia and which exhibit pharmacological activity *via* GPR91 and GPR99, respectively (52); notably angiogenic properties of GPR91 have been documented (53). Therefore, intermediates of carbohydrate metabolites may represent a new mechanistic link between hypoxia and the pathophysiology of certain diseases of the female reproductive tract.

Limitations. Although mice and humans share similarities in some physiological and mechanistic aspects of their gestation and parturition, differences exist particularly as it applies to endocrinology and immunology (54;55). Notwithstanding dissimilarities, GPR81 mRNA is

found in human uterus (56) and our data suggest that GPR81 stimulation of human mSMC exerts similar anti-inflammatory effects than those observed in mice.

## Conclusion

In summary, we report an anti-inflammatory role of lactate *via* its receptor GPR81 in the uterus in labor. GPR81 expression increases as gestation nears the end, whereas lactate concentration considerably increases during labor. Lactate acts as an anti-inflammatory signaling molecule on myometrium. These findings suggest a novel link between the anaerobic glycolysis - the main source of ATP during labor - and the control of uterine inflammation, whereby lactate augmented by vigorous uterine contractions muscle activates GPR81 to down-regulate transcription of key pro-inflammatory genes in uterus, in what seems to be a feedback regulatory mechanism.

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**Disclosure**.

All authors have participated in the critical review of the article and have approved the final version of the manuscript submitted. The authors report no conflict interest.

**Authors contributions**

MNV, AM and SC participated in research design. MNV, AM, XH and EMS conducted experiments. MNV and AM performed data analysis. MNV, AM, SG, DMO and SC wrote or participated in the writing of the manuscript. JCR and DO produced the illustration. All authors participated in the critical review of the manuscript and all approved the final manuscript.

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**Figure legends**

Figure 1. **GPR81 is expressed in the uterus on myometrial smooth muscle cells.** A-B, representative images of cryosections of uterus from non-pregnant mice (A) and pregnant mice in labor (B) revealing immunoreactivity to GPR81 and α-actin. The secondary antibodies alone were used as a negative control (Suppl. Fig. 1A). Images are representative of 3 experiments. DAPI (blue) was used to stain the nuclei. Scale, 20 μm.

Figure 2. **GPR81 mRNA and protein expression increases near labor; lactate levels in the uterus increase during labor.** A-B, mRNA (measured by quantitative PCR) (A) and immunoblot (B) of uterine samples collected on non-pregnant (NP) female mice, throughout gestation (G10-G16), at term without labor (TNL), at term during labor (TL), and 24 h postpartum (PP). qPCR (mRNA) results are normalized with 18S and are relative to control. Lower panel in B shows densitometric analysis of protein bands normalized with β-actin and plotted as fold change vs. the control group (NP). C, lactate levels were measured in uterus from pregnant mice at term before labor (TNL) and during labor (TL). Values are presented as mean ± S.E.M. n=3-4. *, $p<0.05$; ***, $p<0.001$ by one-way ANOVA with Tukey's multiple comparison test compared to NP or TNL.

Figure 3**. Lactate acts *via* GPR81 to inhibit IL-1β-induced transcription of numerous pro-inflammatory genes.** A-B, quantitative PCR of pro-inflammatory (A) and pro-labor (B) mRNA transcripts performed on *ex vivo* uteri from non-pregnant mice treated with IL-1β (5 ng/ml) or vehicle with or without lactate (10 mM) for 8 h. Results are normalized with 18S and are relative to control. Values are presented as mean ± S.E.M. n=3. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ by one-way ANOVA with Tukey's multiple comparison test compared to IL-1β.

Figure 4. **GPR81 is an important regulator of inflammation during labor.** A, mRNA (qPCR) of pro- and anti-inflammatory genes in uterus collected during term labor from WT and GPR81$^{-/-}$ mice. Results are normalized with 18S and are relative to control. Values are presented as mean ± S.E.M. of n=4 per group. B-C, pregnant mice were pre-treated with intrauterine injections of vehicle (sham), LV.shGFP or LV.shGPR81 at G13 (1 injection in each uterine horn) and then treated with a single intraperitoneal injection of 10 μg LPS or an equivalent volume of saline at G16 to induce preterm labor. At time of labor, uteri were collected to

measure mRNA (by qPCR). The LV.shGFP was used as a (negative) control. Results are normalized with 18S and are relative to control (sham [not LPS]-treated). Values are presented as mean ± S.E.M. Data are representative of 3-5 animals per group. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ with Tukey's multiple comparison test compared to LV.shGFP, sham or WT, as indicated.

Figure 5. **GPR81 specific agonist 3,5-DHBA decreases inflammation and prevents PTB induced by LPS.** A, mRNA expression (qPCR) of *Il6*, *Pghs2*, *Ccl2* and *Gpr81* from uterine samples of mice in labor induced by LPS (from experiments described in B); results are normalized with 18S and are relative to control. Values are presented as mean ± S.E.M. Data are representative of 3-7 animals per group. B, pregnant mice were pre-treated with a single intraperitoneal injection of 10 µg LPS or an equivalent volume of saline at G16. Mice were randomly selected to receive 3,5-DHBA (25 mg/Kg/8h) or vehicle 30 min before LPS, injected subcutaneously three times a day until delivery; timing of birth was closely monitored. Values are presented as mean ± S.E.M of n=4-22 mice per group. C, neonatal survival at birth assessed by counting breathing pups per litter. Values are presented as mean ± S.E.M of n=4-16 litters per group. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ by one-way ANOVA with Tukey's multiple comparison test compared to IL-1β + Veh or LPS + Veh.

Figure 6. **Schematic representation of anti-inflammatory action of lactate acting *via* GPR81 in uterus during labor.** A-B, schematic representation (A) and representative image (B) of a murine pregnant uterus. C, schematic representation of the proposed mechanism of action of uterine lactate/GPR81 wherein anaerobic glycolysis, the main generator of ATP in myometrium during labor, produces lactate that diffuses out of the mSMC to activate its cognate receptor GPR81, thereby decreasing the transcription of pro-inflammatory genes. D, expression of GPR81, levels of lactate and inflammatory profile in the murine pregnant uterus during gestation and labor.

**Supplementary figure legends**

Figure S1. **GPR81 is expressed in the uterus on myometrial smooth muscle cells.** A, representative images of circular cryosections of uterus from pregnant mice revealing immunoreactivity to GPR81; the secondary antibodies alone were used as a negative control. DAPI was used to stain the nuclei. Scale for main images, 1000 μm; scale for insets, 40 μm. B, absence of selective immunoreactivity to GPR81 antibody on uterus from GPR81$^{-/-}$ pregnant mice. DAPI was used to stain nuclei. Images are representative of 4 experiments. Scale for 4X images, 300 μm; scale for 10X images, 150 μm.

Figure S2. **Primary mSMC culture purity assessment.** A, >95% of freshly isolated primary mSMC culture was immunoreactive to α-actin (green). B, absence of binding of the Alexa Fluor secondary antibody alone. DAPI was used to stain nuclei. Scale bar, 100 μm. Images are representative of 4 experiments.

Figure S3. **Lactate acts *via* GPR81 to inhibit IL-1β-induced transcription of numerous pro-inflammatory genes in primary murine mSMC.** A, mRNA (measured by qPCR) of indicated genes in primary mSMC treated with IL-1β (5 ng/ml) or vehicle with or without lactate (10 mM) for 8 h. Results are normalized with 18S and are relative to control. Values are presented as mean ± S.E.M. n=4. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ by one-way ANOVA with Tukey's multiple comparison test compared to IL-1β. B, uterine expression of *Gpr81* mRNA increases during preterm labor induced by intrauterine injection of IL-1β at G16. mRNA (qPCR) results are normalized with 18S and are relative to control. Values are presented as mean ± S.E.M. Data are representative of 4-7 animals per group. *, $p<0.05$ by one-way ANOVA with Tukey's multiple comparison test compared to vehicle (Veh).

Figure S4. **Duration of gestation of GPR81$^{-/-}$ mice and GPR81 knocked-down (shRNA-GPR81) mice.** A, shRNA-GPR81 encoded lentivirus efficacy in knocking down GPR81 mRNA (RT-PCR) and protein (immunoblot) expression in hTERT cells. β-actin was used as a loading control. B, shRNA-GPR81(B) encoded lentivirus injected *in vivo* was effectively used to knock down *Gpr81* mRNA expression (qPCR) in murine uterine tissue. C, timing of birth monitored in WT and GPR81$^{-/-}$ mice. D, uterine expression of Gpr81 mRNA increases during preterm labor induced by intraperitoneal injected LPS at G16; LV.shGPR81 (at G13) suppresses this increase.

mRNA (qPCR) results are normalized with 18S and are relative to control. Values are presented as mean ± S.E.M. Data are representative of 3-5 animals per group. *, $p<0.05$ by one-way ANOVA with Tukey's multiple comparison test compared to vehicle (Veh). E, pregnant mice were pre-treated with intrauterine injections of vehicle (sham), LV.shGFP or LV.shGPR81 at G13 (1 injection in each uterine horn) and then treated with a single intraperitoneal injection of 10μg LPS or an equivalent volume of saline at G16; timing of birth was closely monitored; the LV.shGFP was used as a control. Values are presented as mean ± S.E.M. n=4-11 animals per group. F, mRNA expression (by qPCR) of pro-inflammatory genes performed on *ex vivo* WT of KO murine uteri or human mSMC (hTERT cell line) (inset) treated with IL-1β (5 ng/ml) or vehicle with or without 3,5-DHBA (100 μM) for 8 h. Results are normalized with 18S and are relative to control. Values are presented as mean ± S.E.M. n=3-6. ***, $p<0.001$ with Tukey's multiple comparison test compared to WT, sham, LV.shGFP or LV.shGPR81 as indicated.

## Figures

Figure 1

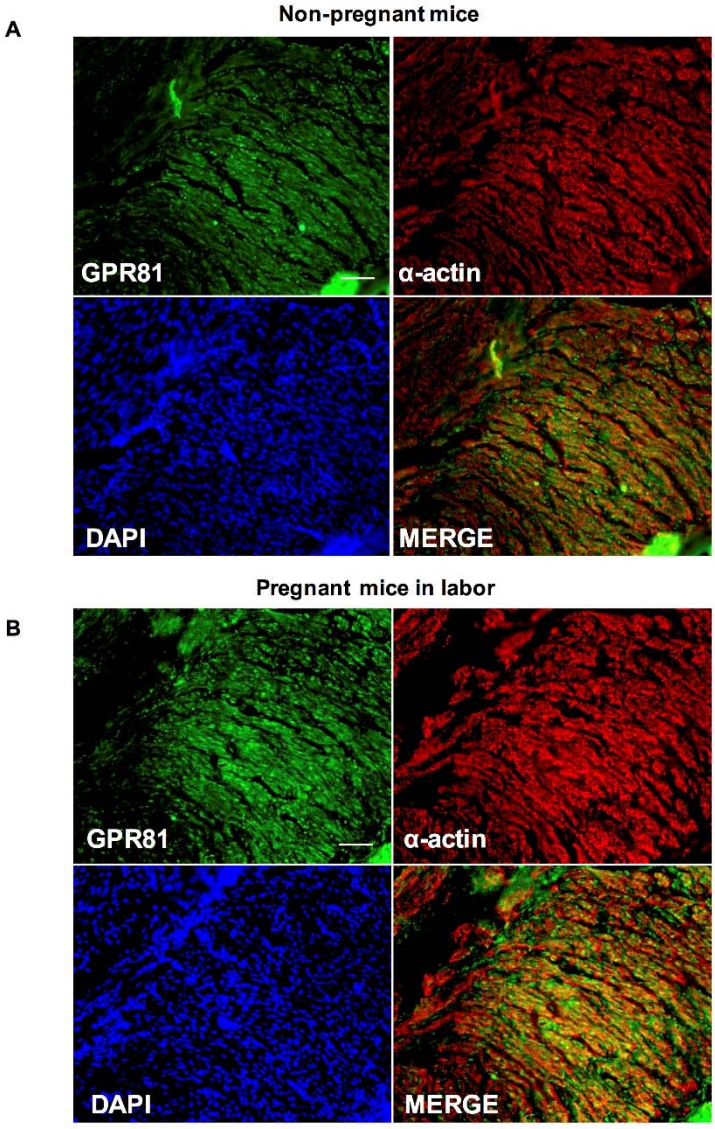


Figure 2

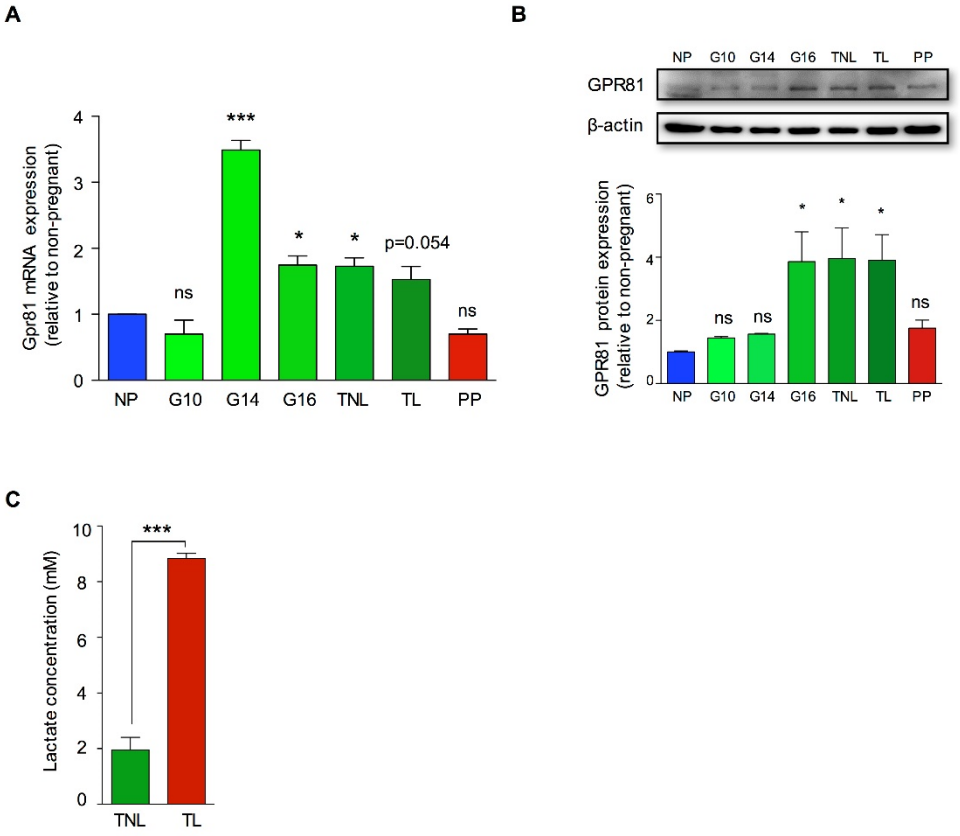

A
Gpr81 mRNA expression (relative to non-pregnant)
***
*
*
p=0.054
ns
ns
NP G10 G14 G16 TNL TL PP
B
NP G10 G14 G16 TNL TL PP
GPR81
β-actin
GPR81 protein expression (relative to non-pregnant)
ns ns
* * *
ns
C
Lactate concentration (mM)
***
TNL TL

Figure 3

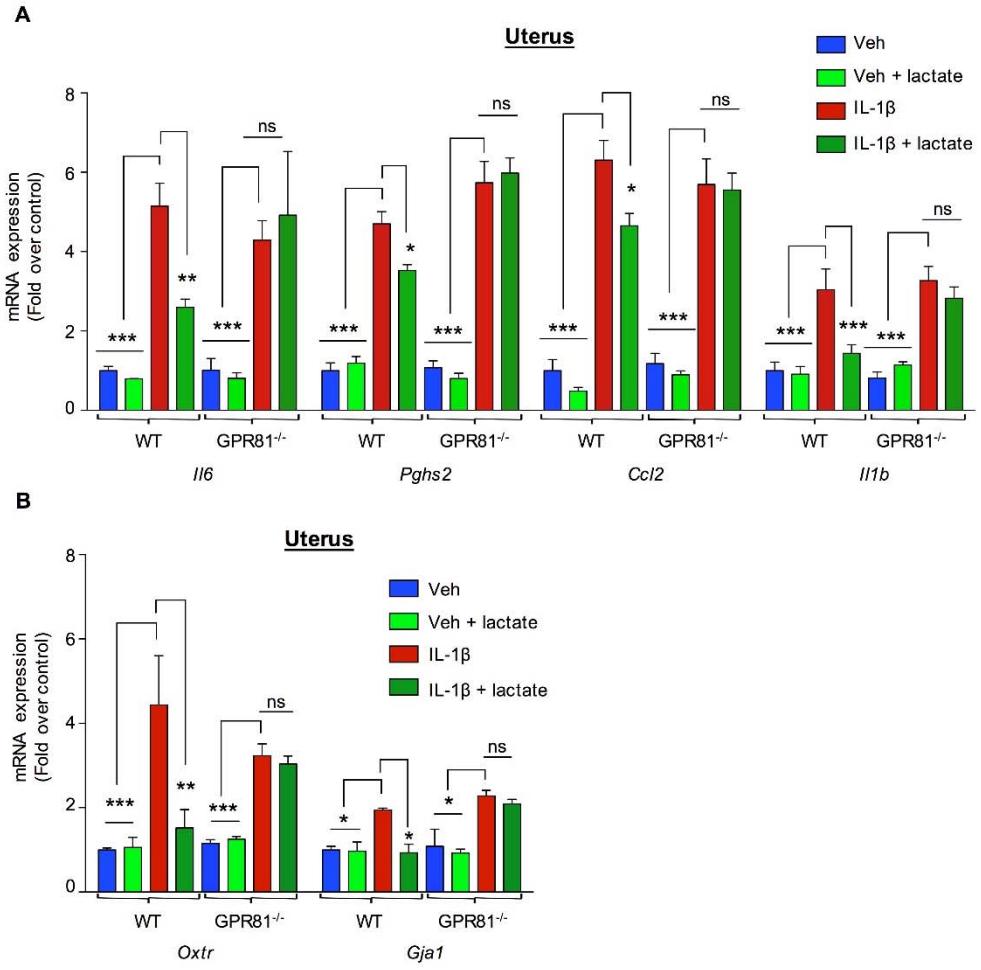

A
Uterus
Veh
Veh + lactate
IL-1β
IL-1β + lactate
mRNA expression
(Fold over control)
ns
***
**
*
WT
GPR81-/-
Il6
Pghs2
Ccl2
Il1b
B
Uterus
Oxtr
Gja1

Figure 4

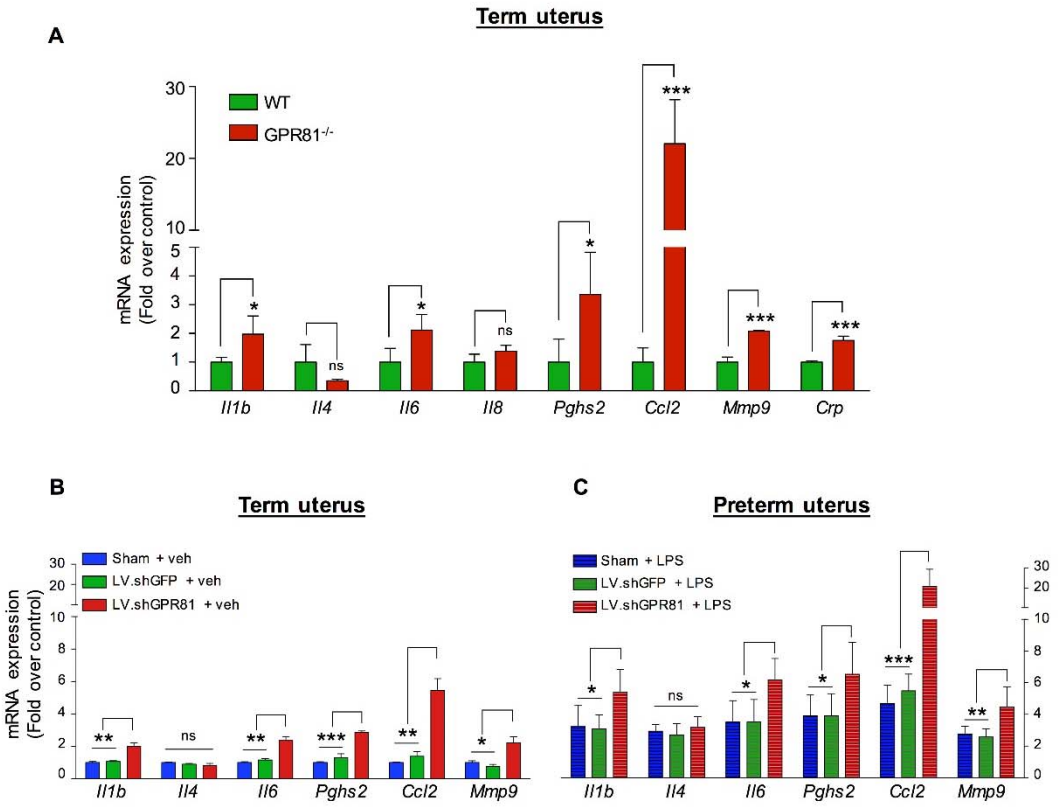

A
Term uterus
WT
GPR81-/-
mRNA expression
(Fold over control)
Il1b
Il4
Il6
Il8
Pghs2
Ccl2
Mmp9
Crp
B
Term uterus
Sham + veh
LV.shGFP + veh
LV.shGPR81 + veh
C
Preterm uterus
Sham + LPS
LV.shGFP + LPS
LV.shGPR81 + LPS

Figure 5

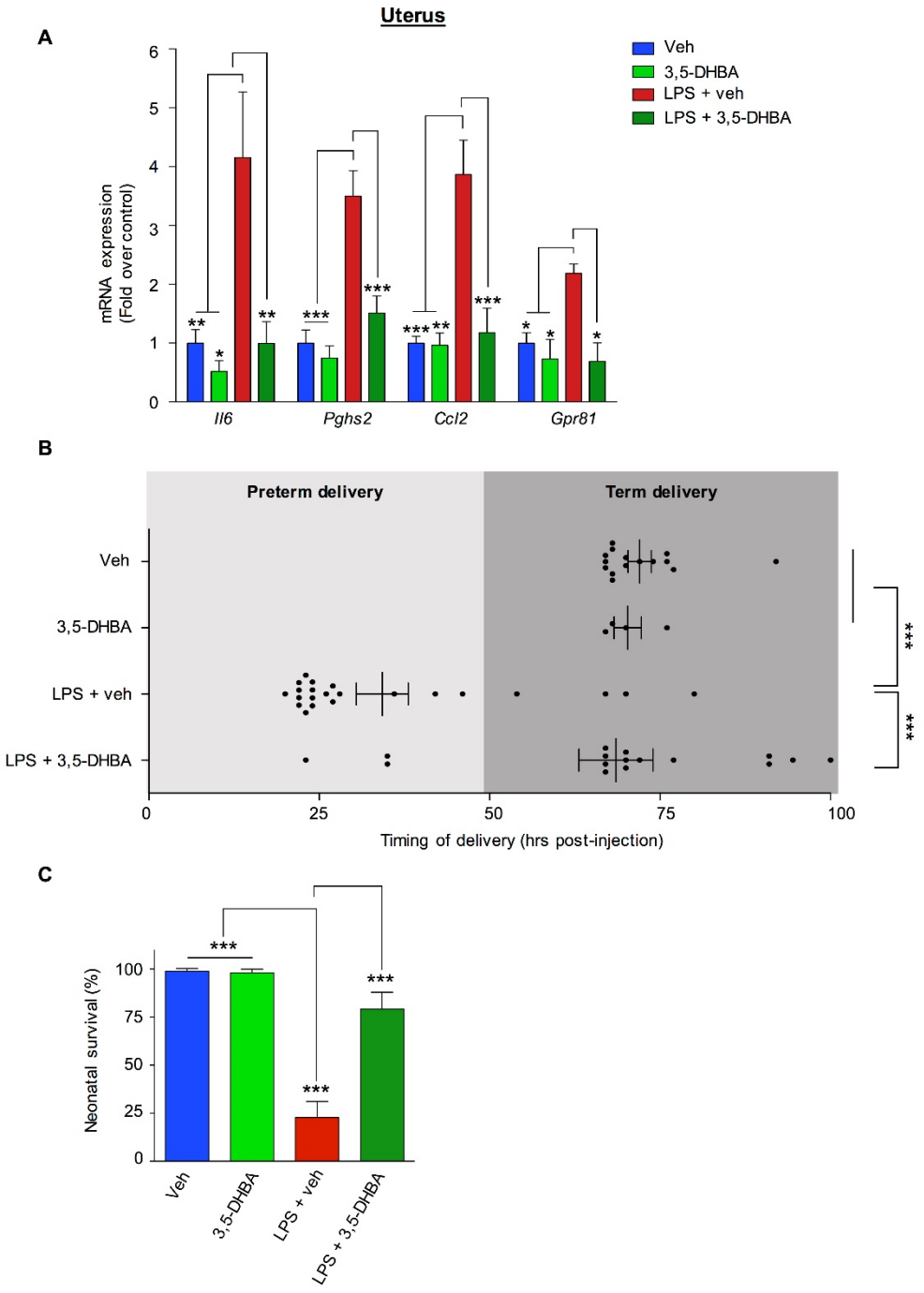
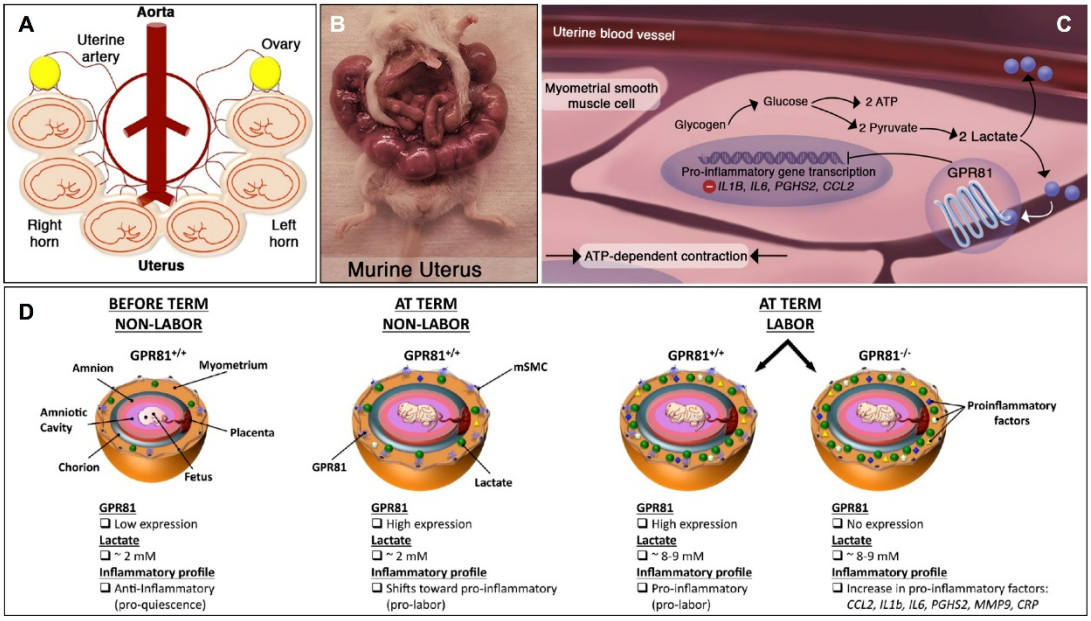

A
Uterus
Veh
3,5-DHBA
LPS + veh
LPS + 3,5-DHBA
mRNA expression
(Fold over control)
Il6
Pghs2
Ccl2
Gpr81
B
Preterm delivery
Term delivery
Veh
3,5-DHBA
LPS + veh
LPS + 3,5-DHBA
Timing of delivery (hrs post-injection)
C
Neonatal survival (%)
Veh
3,5-DHBA
LPS + veh
LPS + 3,5-DHBA

Figure 6


A
Aorta
Uterine artery
Ovary
Right horn
Left horn
Uterus
B
Murine Uterus
C
Uterine blood vessel
Myometrial smooth muscle cell
Glycogen
Glucose
2 ATP
2 Pyruvate
2 Lactate
Pro-inflammatory gene transcription
IL1B, IL6, PGHS2, CCL2
GPR81
ATP-dependent contraction
D
BEFORE TERM NON-LABOR
AT TERM NON-LABOR
AT TERM LABOR
GPR81+/+
GPR81-/-
Amnion
Myometrium
Amniotic Cavity
Placenta
Chorion
Fetus
mSMC
Lactate
Proinflammatory factors
GPR81
❑ Low expression
Lactate
❑ ~ 2 mM
Inflammatory profile
❑ Anti-Inflammatory (pro-quiescence)
GPR81
❑ High expression
Lactate
❑ ~ 2 mM
Inflammatory profile
❑ Shifts toward pro-inflammatory (pro-labor)
GPR81
❑ High expression
Lactate
❑ ~ 8-9 mM
Inflammatory profile
❑ Pro-inflammatory (pro-labor)
GPR81
❑ No expression
Lactate
❑ ~ 8-9 mM
Inflammatory profile
❑ Increase in pro-inflammatory factors: CCL2, IL1b, IL6, PGHS2, MMP9, CRP

**Supplementary Figures**

Figure S1

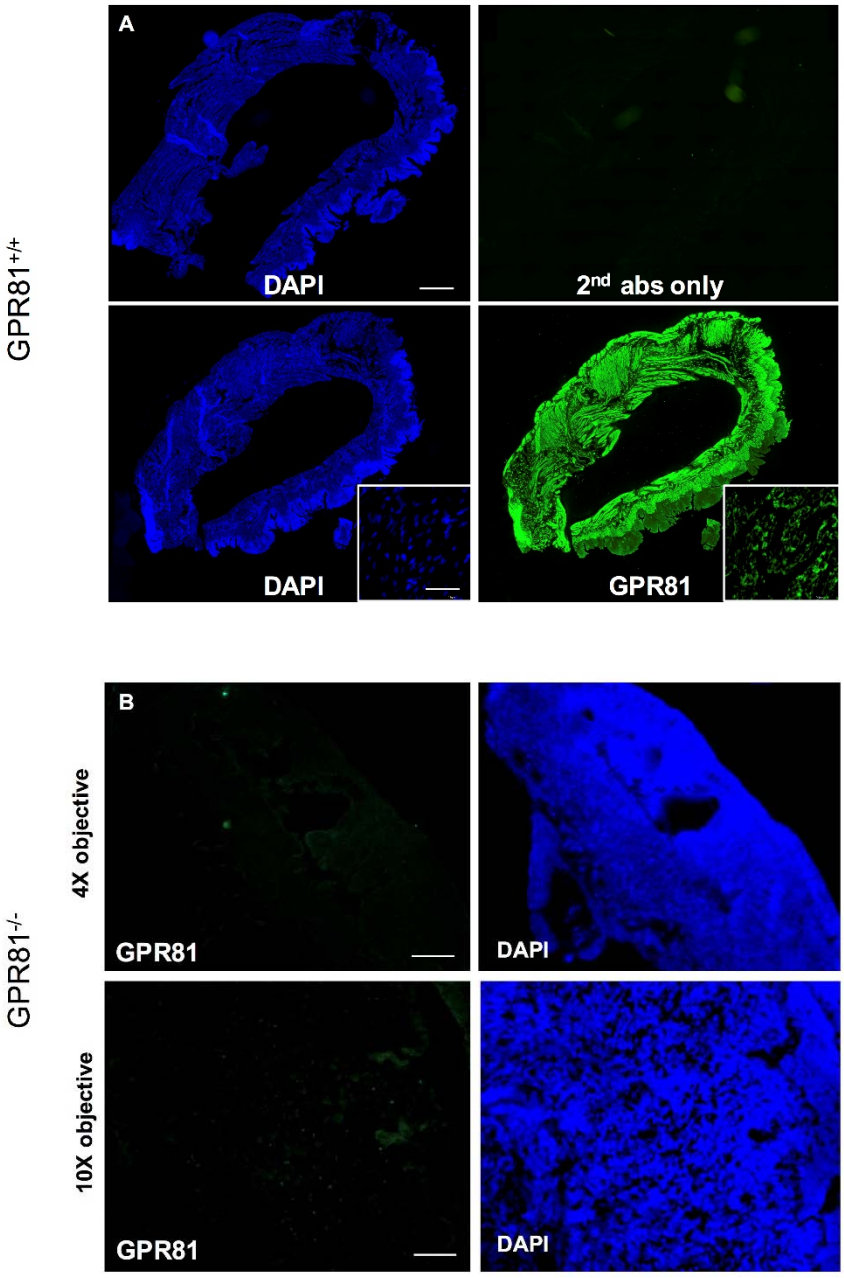


Figure S2

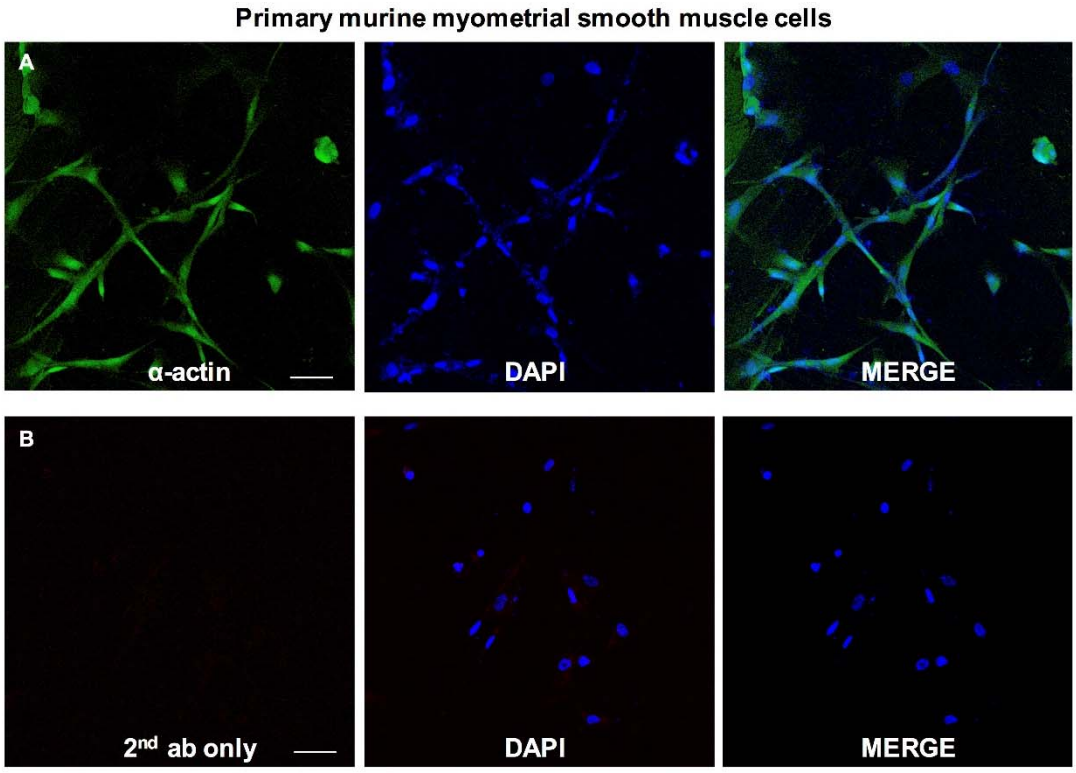

Primary murine myometrial smooth muscle cells
A
α-actin
DAPI
MERGE
B
2nd ab only
DAPI
MERGE

Figure S3

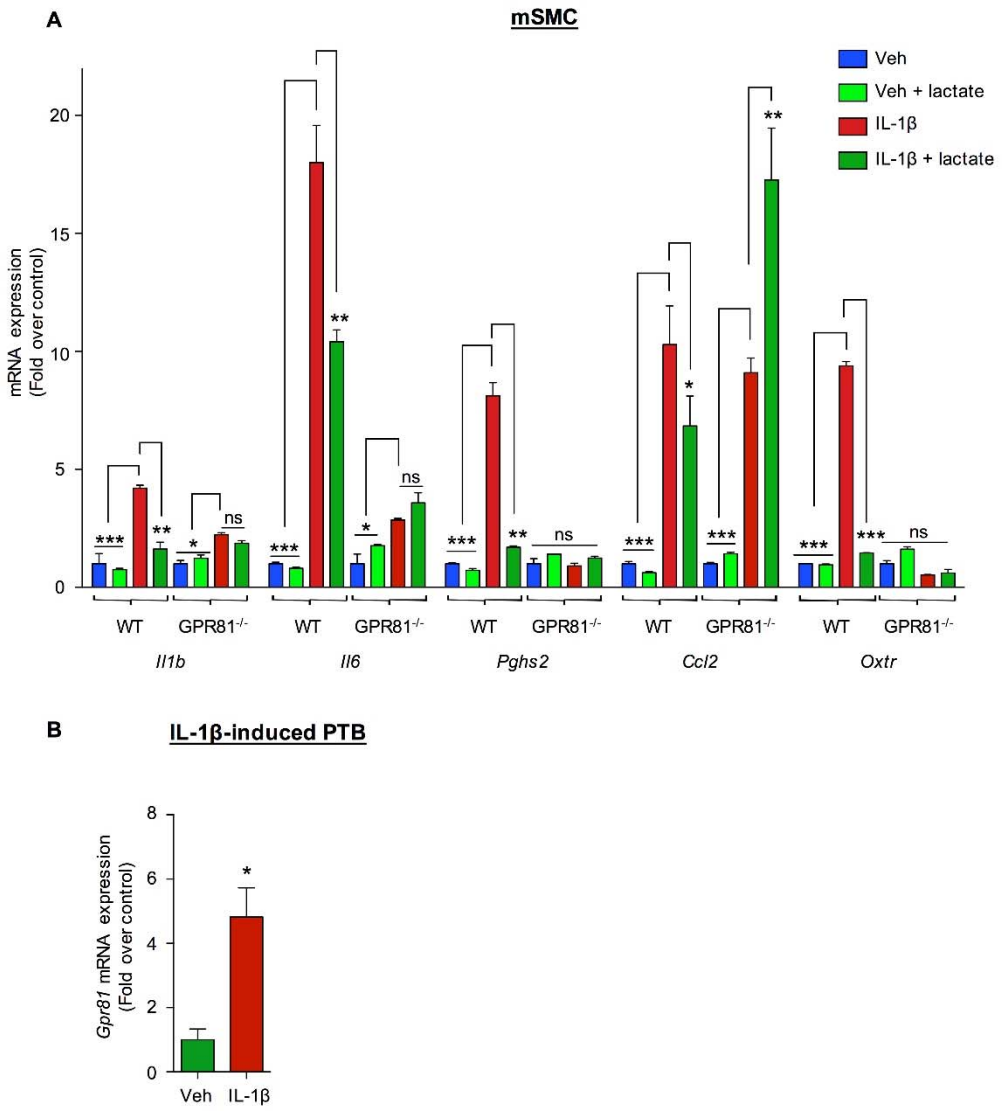
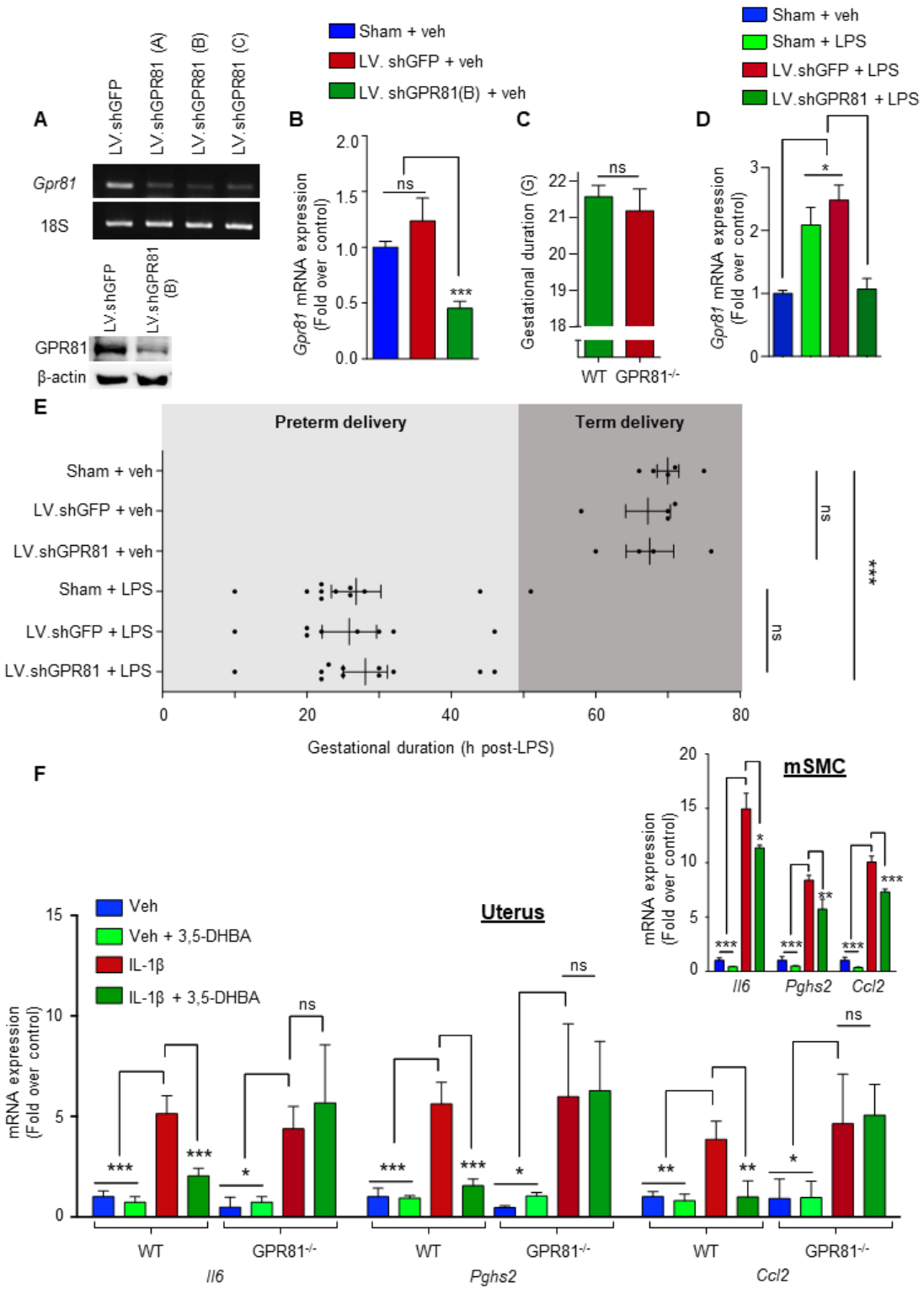

A
mSMC
Veh
Veh + lactate
IL-1β
IL-1β + lactate
mRNA expression
(Fold over control)
0
5
10
15
20
***
**
*
ns
WT
GPR81-/-
Il1b
Il6
Pghs2
Ccl2
Oxtr
B
IL-1β-induced PTB
Gpr81 mRNA expression
(Fold over control)
0
2
4
6
8
Veh
IL-1β

Figure S4


A
LV.shGFP
LV.shGPR81 (A)
LV.shGPR81 (B)
LV.shGPR81 (C)
Gpr81
18S
LV.shGFP
LV.shGPR81 (B)
GPR81
β-actin
B
Sham + veh
LV. shGFP + veh
LV. shGPR81(B) + veh
Gpr81 mRNA expression (Fold over control)
ns
***
C
Gestational duration (G)
ns
WT
GPR81-/-
D
Sham + veh
Sham + LPS
LV.shGFP + LPS
LV.shGPR81 + LPS
Gpr81 mRNA expression (Fold over control)
*
E
Preterm delivery
Term delivery
Sham + veh
LV.shGFP + veh
LV.shGPR81 + veh
Sham + LPS
LV.shGFP + LPS
LV.shGPR81 + LPS
ns
ns
***
Gestational duration (h post-LPS)
F
Veh
Veh + 3,5-DHBA
IL-1β
IL-1β + 3,5-DHBA
Uterus
mRNA expression (Fold over control)
ns
WT
GPR81-/-
Il6
Pghs2
Ccl2
mSMC
mRNA expression (Fold over control)
Il6
Pghs2
Ccl2

## Tables

Table I: Primers used for Real-Time qPCR

| **Mouse primers** | |
|---|---|
| *IL1B*-F: AGATGAAGGGCTGCTTCCAAA | *IL1B*-R: GGAAGGTCCACGGGAAAGAC |
| *IL4*-F: AACGAAGAACACCACAGAGAG | *IL4*-R: GTGATGTGGACTTGGACTCA |
| *IL6*-F: CAACGATGATGCACTTGCAGA | *IL6*-R: TCTCTCTGAAGGACTCTGGCT |
| *IL8*-F: TGCTTTTGGCTTTGCGTTGA | *IL8*-R: GTCAGAACGTGGCGGTATCT |
| *IL10*-F: TAACTGCACCCACTTCCCAG | *IL10*-R: AGGCTTGGCAACCCAAGTAA |
| *TNFA*-F: GCCTCTTCTCATTCCTGCTTG | *TNFA*-R: CTGATGAGAGGGAGGCCATT |
| *MMP1A*-F: CAGGACTTATATGGACCTTCCC | *MMP1A*-R: TAAATTGAGCTCAGGTTCTGGC |
| *MMP3*-F: GTGACCCCACTCACTTTCTC | *MMP3*-R: TTGGTACCAGTGACATCCTCT |
| *MMP9*-F: TCAAGGACGGTTGGTACTGG | *MMP9*-R: CTGACGTGGGTTACCTCTGG |
| *OXTR*-F: TGTGTCTCCTTTTGGGACAA | *OXTR*-R: GGCATTTCAGAATTGGCTGT |
| *PGHS2*-F: ACCTCTCCACCAATGACCTGA | *PGHS2*-R: CTGACCCCCAAGGCTCAAAT |
| *PTGFR*-F: AGCTGGACTCATCGCAAACA | *PTGFR*-R: GTGGGCACAAGCCAGAAAAG |
| *GJA1*-F: GCACTTTTCTTTCATTGGGGG | *GJA1*-R: GGGCACCTCTCTTTCACTTA |
| *CCL2*-F: GCTCAGCCAGATGCAGTTA | *CCL2*-R: TGTCTGGACCCATTCCTTCT |
| *GPR81*-F: CCGGTTCATCATGGTGGTGGCT | *GPR81*-R: CTCTTCTGACCTCCGCGTCTTC |

Table II: shRNA sequences used for *Gpr81* knockdown

| | |
|---|---|
| **A forward** : TTGACCGAGCAGAACAAGATG | **A reverse** : CATCTTGTTCTGCTCGGTCAA |
| **B forward** : AAGATGACCAAAGTCCAGAGG | **B reverse** : CCTCTGGACTTTGGTCATCTT |
| **C forward** : AAATAGTGCTTGACTTCCAGG | **C reverse** : CCTGGAAGTCAAGCACTATTT |

# Part C: Discussion

Spontaneous preterm labor (11), fetal inflammation/injury (433), and long-term impairments associated with prematurity (643), are all governed by inflammatory processes independent of infection (388, 644) and gestation age (80, 607, 645). Correspondingly, in our murine model of PTB, a single intrauterine (maternal) injection of IL-1β during gestation was sufficient to trigger a full-blown inflammatory cascade in gestational tissue and fetus, thereby inducing preterm labor and delivery; activating monocytes circulating in the blood of neonates; and causing long-lasting organ injury in pups and in turn functional impairment. This data, as well as converging evidence in human (209, 216, 218-221, 646) and rodents and larger animals (102, 170, 209, 216, 240, 441), points to a critical role of IL-1 in the inflammatory process leading to PTB.

Here, we have described the therapeutic potential of antagonizing IL-1 (*via* agonism of anti-inflammatory GPR81 [with lactate and 3,5-DHBA] and antagonism of IL-1R [with Kineret and 101.10]) to achieve better obstetrical, neonatal, and developmental outcomes, thereby confirming its essential role in PTB and fetal injury. Specifically, antenatal maternal delivery of 101.10 resulted in: 1) prevention of preterm labor induced by IL-1β (i.u.), lipoteichoic acid (TLR2 ligand, i.p.) and LPS (TLR4 ligand, i.p.); 2) inhibition of numerous proinflammatory and uterotrophic mRNA transcripts from circulating leukocytes, fetal membranes, decidua, placenta, and myometrium, *in vivo* as well as *in vitro* in response to IL-1β and IL-1α; 3) reduction of oxytocin-induced and $PGF_{2\alpha}$-induced myometrial contractile response in IL-1β-treated dams; and 4) reduced levels of endogenous IL-1β in maternal blood. Furthermore, 101.10 reached the

placenta and protected the fetus *via*: 5) inhibition pro-inflammatory mRNA transcripts in placenta and fetal brain; 6) down-regulation of pro-inflammatory mRNA transcripts in circulating leukocytes of newborns, resulting in decreased plasma levels of IL-1β, IL-6 and IL-8; and 7) inhibition of the inflammatory response in neonatal lung, intestine and brain, in turn leading to the recovery of normal tissue architecture integrity, and recovery of normal brain mass at adulthood accompanied by improved cortical function assessed by visual evoked potentials. These results were confirmed and further extended in a recent study that revealed the efficacy of antenally-administered 101.10 to protect the retina and sub-retina of progeny exposed to IL-1β (647). As for GPR81 agonism, it resulted in a significant reduction in: 1) IL-1-induced myometrial production of major proinflammatory mRNA transcripts; 2) PTB rates; and 3) neonatal mortality. However, because of the inherent elevated $EC_{50}$ of GPR81 agonists, which translates into the necessity to administer very high doses to attain therapeutic goals, GPR81 agonists are not likely to represent interesting drug candidates. Lactate (90 Da, 3 carbons) and 3,5-DHBA (154 Da, 7 carbons) are small non-protein molecules and are therefore non-specific. Altogether, this compelling evidence points to 101.10 as an effective therapeutic candidate and corroborates the physiological anti-inflammatory role of lactate during labor.

Our studies have revealed the mechanism of action of 101.10, which has been confirmed using different *in vitro* and *ex vivo* models. 101.10 inhibits IL-1-induced phosphorylation of MAPK p38, SAPK p54 (JNK), transcription factor c-jun, and inhibits the small GTPase Rho (by reducing the GTP-bound form). This has been demonstrated in human cell lines and primary murine cultures of myometrial smooth muscle cells and macrophages, and further confirmed using murine uterine explants. Interestingly, the signal transducers inhibited by 101.10 all act upstream of transcription factor AP-1, whose major role in labor has recently been uncovered

(517, 518, 648), and whose signal-specific inhibition leads to the prevention of IL-1-induced PTB, as demonstrated herein. Additionally, Rho plays a critical role in actomyosin cytoskeleton contractility (649). Remarkably, 101.10 does not exert significant effects on IL-1-induced NF-kB nuclear translocation. Although inhibition of NF-kB in gestational tissue is effective to reduce inflammation, this pharmacological strategy has revealed deleterious (pro-apoptosis) effects (627), consistent with the role of NF-kB in transcription of genes important for cytoprotection. Further, NF-kB is crucial for immune-surveillance (516, 650) and resolution of inflammation (626). Therefore, 101.10 may convey advantages over competitive antagonists of IL-1 such as Anakinra by sparing the IL-1-induced NF-kB pathway. Although postnatal (68) and antenatal intraamniotic (441) use of Kineret has resulted in favorable outcome, this is only partly true when Kineret is administered systemically during gestation (607), the most desirable route and timing of administration for treatment of PTB and its complications. On the other hand, 101.10 displays increased efficacy as compared to Kineret, especially to prevent PTB and uterine inflammation. It is also noteworthy that 101.10 is comparably effective to Kineret in a murine model of retinopathy of prematurity (603), but is superior to anti-inflammatory corticosteroids and Kineret in animal models of hyperthermia, inflammatory bowel disease, as well as topically in contact dermatitis (418). Further, reported side effects of Kineret in patients with rheumatoid arthritis include neutropenia and increased risks of infection (651). Hence, a possible side effect resulting from acute/subacute inhibition of IL-1 in the setting of pregnancy is transient compromise in immunocompetence. A combination of various anti-inflammatory strategies, although potentially more effective altogether, may further predispose to immunosuppression. Small peptide antagonists such as 101.10 provide increased specificity of action and may reduce the risk of such adverse effect, a claim that remains to be explored,

although normal growth trajectory and no major adverse effects have been observed in 20-week-old mice exposed antenatally to 101.10. Yet, further research in larger animals is required to evaluate the safety of 101.10. Clearly, the pharmacological properties of 101.10 makes it a unique drug candidate for the treatment of PTB.

Our data also describe for the first time a link between anaerobic metabolism and uterine inflammation. Intrauterine lactate reaches very high levels during labor to activate anti-inflammatory GPR81, resulting in decreased production of numerous cytokines including IL-1β. It may plausibly represent a feedback mechanism specific to labor wherein inflammation leads to increased contractility which in turn leads to anti-inflammation. This may serve two purposes: 1) it may be a first step in the resolution of inflammation necessary to heal uterine tissue in the postpartum period; and 2) it may serve to prevent excessive inflammation which could lead to uterine dystocia and ensued fetal demise (especially as it could have occurred before the advent of modern medicine and safe caesarian section procedures, which hints evolutionary pressure), and to dissemination of the excessive inflammation to the fetus independent of dystocia. Concordant, although inflammation is a key pathway to delivery, excessive inflammation prolongs the active phase of labor and increases the rate of caesarean sections (652), suggesting overall decreased efficacy of uterine contractions. Comparably to its effect on myometrial smooth muscle cells, excessive inflammation paralyses intestinal smooth muscle cells and lead to paralytic ileus (653). Along these lines, labor dystocia (i.e. prolonged or difficult labor) was found in 22 of 235 litters (approximately 10%) of $GPR81^{-/-}$ mice as compared to less than 1% in control mice (Madaan, Nadeau-Vallée, Chemtob, unpublished data). Further research is needed to corroborate these claims.

*Other pressing issues related to PTB*

Developing an effective anti-inflammatory agent is necessary to decrease the incidence of PTB and improve neonatal outcome, but insufficient by itself, in part because: a) there is an imperative need for the development of a diagnostic test to largely identify the population at risk; b) there is a lack of investment into the commercial development of therapeutics and diagnostics for PTB; and less importantly c) there is also a need for an effective and safe tocolytic agent which could provide sufficient time (perhaps > 7day) for the anti-inflammatory agent to elicit its pro-quiescence effect in the already activated and inflammatory uterus of symptomatic women who could not be identified early.

A. Diagnostic test: PTB is difficult to predict because no clinical criteria exist for its early diagnosis, which is inconvenient for the design of a randomized clinical trial (since there is no discriminator for enrolling women) or for the determination of the timing of the treatment. Current medical evaluation of preterm labor means overt display of preterm birth symptoms of women in labor. This is too late for effective intervention. Hence, combining an effective diagnostic that predicts risk before symptoms appear would achieve several benefits. First, it will identify those at risk who may not have been characterized using other assessment tools thereby targeting more women earlier in their pregnancies. Second, it will reduce the number of women treated unnecessarily. This will lead to starting treatments earlier, before symptoms and labor begins, with better effectiveness and possibly with more courses of treatment. From a global perspective, more women in more populations may be diagnosed and treated; and from a health standpoint, better pregnancy outcomes will ensue.

As of now, only the cervicovaginal fetal fibronectin test (fFN) and the clinical (sonographic) assessment of cervical length (CL) are used for diagnostic purposes. The negative predictive value of fFN ranges from 90% to 99,5%, and its positive predictive value ranges from 17% to 29%, depending on the clinical setting and study population (654). The negative predictive value and positive predictive value of CL is highly dependent of the study population and on the cut-off value used, but rarely exceeds 98% and 66%, respectively (655, 656). Therefore, the fFN is only used to determine which symptomatic women will not deliver and can be sent at home, whereas CL is inaccurate and could lead to the unnecessary treatment of at best 33% of women tested positive. Additionally, ultrasound is not always affordable in low-income countries. For these reasons, numerous diagnostic tests are being developed with the goal of improving positive predictive value (657, 658). Hundreds of biomarkers have been investigated as potential predictors of PTB, but none were found reliable (659, 660). However, in a recent study by Ngo *et al.* published in the journal *Science*, non-invasive measurement of a combination of nine placental cell-free RNA transcripts in maternal blood predicted PTB at lower cost than ultrasounds, with a positive predictive value of 75-80%, in a study population in which the fFN and CL yielded a positive predictive value of 21% and 17%, respectively (657). Hence, there are encouraging advances in the development of a valid diagnostic test for asymptomatic PTB.

B. Investment into clinical trials: A major problem in pregnancy health is that even though new, safe, and effective diagnostics and therapeutics are being developed to assess earlier in pregnancy those women at risk of a preterm delivery, there is practically no industry to support their commercial development. This derives, in part, from a lack of awareness of the size of the international market for diagnostics and therapeutics, fear of an adverse outcome for

treating two patients with potential consequent litigation or negative effect upon sales or reputation of other company products, the perceived high cost of clinical trials and knowing who to treat (because some trials will be in asymptomatic women), and the lack of investors willing to support Phase 1 and 2 testing. New diagnostic tests with high positive predictive values could 'de-risk' (and incentivise) the investment into clinical trials (35) by identifying the women who should be enrolled and possibly monitoring the effectiveness of the therapeutic. Using a diagnostic test and a new effective therapeutic (such as 101.10) in tandem could hasten their arrival for clinical use, vastly identifying the target population, improving treatment efficacy (originally geared to target population), decreasing global perinatal health care costs, increasing global market size, and thus providing an incentive for needed pharmaceutical/biotech partners for this unmet medical need.

C. Effective tocolysis: As detailed in the Introduction, tocolytics are only modestly effective at prolonging gestation and, aside from Atosiban which is not approved in the USA, they have numerous undesirable effects. Hence, the development of more effective and safer tocolytics could improve the management of preterm labor by allowing more time to administer an anti-inflammatory agent to protect the fetus and restore uterine quiescence.

Neuromedin U and S are two neuropeptides implicated in appetite, CRH release, adrenocortical axis regulation, puberty onset, energy metabolism, and intestinal and uterine smooth muscle cells contractility (661). We have identified a novel UAP, neuromedin U receptor 2 (NmU-R2), which could represent an interesting target for tocolysis. This study, presented in Appendix A, reveals that the placenta and decidua produce neuromedin S (NmS) at term or in response to labor-inducing doses of proinflammatory stimuli including IL-1 and LPS, which activates its (G protein-coupled) receptor NmU-R2 on myometrial cells resulting in

immediate intracellular calcium release, uterine contraction, and labor. In humans and in mice, the myometrial expression of NmU-R2 increases at term in preparation of incoming labor, and in response to IL-1 *in vitro*. Importantly, the neuromedin system is also activated in women in preterm labor with chorioamnionitis. In mice, NmU-R2 activation results in preterm labor and inversely, intrauterine shRNA-containing lentivirus-mediated gene knockdown of NmU-R2 prolongs gestation and reduces prematurity and neonatal mortality in mice treated with LPS. Overall, our study, presented in Appendix A, suggests a major implication of the intrauterine neuromedin system in the onset of term and preterm labor in both rodents and humans. Along these lines, in a set of peptide scrambled-controlled preliminary experiments from our lab, we have shown that a new peptide drug, temporarily called Peptide 5, dose-dependently decreases $Ca^{++}$ influx in myometrial smooth muscle cells ($IC_{50}$=23nm), and blocks uterine contactions in uterine explants, with more efficacy than a neutralizing anti-NmU-R2 antibody (662). Therefore, NmU-R2 represents an interesting novel target for tocolysis.

# Conclusion

In summary, PTB is a common syndrome that afflicts millions of families over the world. It is the leading cause of neonatal mortality and morbidity worldwide, and one of the most important unmet medical needs as identified by the WHO. In line with numerous evidence accumulated for over 30 years, our results point to a critical role for IL-1 in triggering maternal and fetal inflammation, leading to PTB and long-lasting fetal organ injury regardless of gestation age and infection. Correspondingly, modulating key IL-1 signaling pathways with 101.10 uncovers a novel therapeutic approach wherein PTB is prevented without unnecessary inhibition of important NF-κB, with the added benefit of preserving its desirable effects on immunosurveillance and resolution of inflammation. With the concomitant development of a valid and precise diagnostic test, this novel therapeutic approach could directly translate into better cares to pregnant women and better health outcome for babies worldwide.

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# Appendix A: Supplementary Articles

## Article S1: Uterotonic neuromedin U receptor 2 and its ligands are upregulated by inflammation in mice and humans, and elicit preterm birth[6]

Mathieu Nadeau-Vallée[1,2], Amarilys Boudreault[1], Kelycia Leimert[3], Xin Hou[1], Dima Obari[2], Ankush Madaan[1,4], Raphael Rouget[1], Tang Zhu[1], Lydia Belarbi[1], Marie-Ève Brien[5], Alexandra Beaudry-Richard[1], David M. Olson[3], Sylvie Girard[5] and Sylvain Chemtob[1,2,4]

[1]Departments of Pediatrics, Ophthalmology and Pharmacology, CHU Sainte-Justine Research Centre, Montréal, Canada, H3T 1C5; [2]Department of Pharmacology, Université de Montréal, Montréal, Canada, H3G 1Y6; [3]Departments of Obstetrics and Gynecology, Pediatrics and Physiology, University of Alberta, Edmonton, AB, Canada; [4]Department of Pharmacology & Therapeutics, McGill University, Montreal, QC, Canada; [5]Departments of Obstetrics and Gynecology, CHU Sainte-Justine research center, Montréal, Canada, H3T 1C5.

Running title: Inflammation controls uterotonic NmU-R2

List of abbreviations: G, gestational day; IL, interleukin; LV, lentivirus; mSMC, myometrial smooth muscle cells; NmS, neuromedin S; NmU, neuromedin U; NmU-R, neuromedin U receptor; NP, non-pregnant; OT, oxytocin; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PTB, preterm birth; PTL, preterm labor; PTLi, preterm labor with clinical evidence of infection; PTNL, preterm non-labor; TNL, term non-labor; TL, term labor; UAPs, uterine activation proteins;

Corresponding author: Sylvain Chemtob, M.D., Ph.D. CHU Sainte-Justine, Research Center, Departments of Pediatrics, Ophthalmology and Pharmacology. 3175 Chemin Côte Ste-Catherine, Montréal, Québec, Canada, H3T 1C5. Fax: 1-514-345-4801, Tel: 1-514-345-4931 ext. 2978. E-mail: sylvain.chemtob@umontreal.ca

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## Study significance

In this article, we uncover a novel pro-uterocontractile, pro-labor human and murine system tightly regulated by gestation age, infection, and inflammation. Placental neuromedin (Nm) S, and its cognate receptor Neuromedin U Receptor 2 (NmU-R2) in myometrium, are upregulated by IL-1 and lipopolysaccharide (LPS) in mice, by chorioamnionitis in human pregnancy, and physiologically at term in both mice and humans to initiate and maintain uterine contractions at term and preterm. Preterm birth remains a challenge for clinicians as there are no available therapeutic agent sufficiently effective to prolong preterm gestation by more than 48 h and improve newborn outcomes. The NmU system that we hereby uncover in human and murine uterus appears causal, necessary, and sufficient for preterm labor associated with inflammation/infection, and therefore represents an interesting new target for tocolysis.

**Abstract**

Uterine labor requires the conversion of a quiescent (pro-pregnancy) uterus into an activated (pro-labor) 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 utero-contractile 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 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 utero-contractile 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 (mSMC), which are potentiated with gestational age. NmU-R2 is upregulated *in vitro* in mSMC and *in vivo* in uterus in response to pro-inflammatory IL-1β, which is associated with increased NmU-induced utero-contractile response and $Ca^{++}$ transients in murine and human mSMC; additionally, placental NmS is markedly upregulated *in vivo* in response to IL-1β. In human placenta at term, immunohistological analysis revealed NmS expression primarily in cytotrophoblasts; furthermore, stimulation with LPS (gram– endotoxin) markedly upregulates NmS expression in primary human cytotrophoblasts isolated from term placentas. Correspondingly, decidua of women with clinical signs of infection that delivered preterm display significantly higher

expression of NmS compared to those without infection. Importantly, *in vivo* knock-down of NmU-R2 prevents LPS-triggered PTB 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.

Keywords: Preterm birth; Inflammation; Neuromedin U; Neuromedin S; NmU-R2; Preterm labor; Uterine labor; Infection; Contraction; Calcium.

## Introduction

Uterine labor is characterized by vigorous uterine 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 uterine 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 pro-inflammatory cytokines [e.g. IL-1β, IL-6 and 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 in 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.

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 which 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: NmU-R1 (FM-3 or GPR66) and NmU-R2 (FM-4) [8-12]. Both NmU receptors are coupled to Gi and Gq/11 – phospholipase Cβ [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 pro-contractile effects on rodent uterus [15] that has 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 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 a) exhibiting increased expression with onset of labor, b) exerting gestational age-dependent utero-contractile effects, c) contributing endogenously to inflammation-triggered PTB, and d) 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 LPS. Hence, NmU-R2 is a potential new UAP that appears important for PTB associated with infection.

## Material and Methods

### *Ethical approval*

Approval was obtained from North West Research Ethics Committee in Manchester, UK (Ref: 08/H1010/55) for decidual samples, provided by Dr. Rebecca L. Jones, and the Sainte-Justine Hospital Ethic 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 caesarean section 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 caesarean section at the MacDonald Women's Hospital, University Hospitals, Cleveland with IRB approval (#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 12:12 light:dark cycle and allowed free access to chow and water.

### *Chemicals*

Chemicals were purchased from the following manufacturers: rhIL-1β (#200-01B; PeproTech), Neuromedin U-23 (#NMU72-P; Alpha Diagnostic International), Neuromedin S (#045-88; Phoenix Pharmaceuticals), Neuromedin U-25 (#17617; Cayman Chemical), Prostaglandin $F_{2\alpha}$ (#16010; Cayman Chemical), Oxytocin (#66-0-52; American peptide), W-7 (#A3281; Sigma), U73122 (#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 aluminium 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 7mm bead and 0.5 mL of lysis buffer containing 0.05% Tris, 0.01% EDTA, 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, Germantown, MD). Tissue lysates were centrifuged at 12000g for 10 minutes at 4°C, and the supernatants were collected for Western blot.

*Primary murine myometrial smooth muscle cell isolation and culture*

Primary murine myometrial smooth muscle cells (mSMC) were isolated as previously described [21] and used at less than 3 passages. Briefly, pregnant mice (at gestational day [G] 10 or 19) were sacrificed 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, 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, Grand Island, NY], 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 transfered into buffer B (buffer A without magnesium sulfate or calcium chloride) for several washes by gentle flushing. Afterwards, the uterine horns were cut into 1mm wide fragments and transfered 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, Mannheim, Germany], 0.1 mg/mL soybean trypsin inhibitor [sigma], 10% FBS, and 1 mg/mL bovine serum albumin [BSA, Sigma] 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 200g for 10 min, the pellet was resuspended in complete DMEM medium and plated in a T-75 dish. The remaining myometrium fragments were re-used in an enzymatic digestion and the whole digestion-centrifugation process was repeated for a total of 5 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 mSMC or human mSMC (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 of IL-1β 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 (i.p.) with NmU, NmS, $PGF_{2\alpha}$ or oxytocin twice a day for 2 consecutive days. Injections were made at G13-G14, G15-16 or G17-G18 twice a day for a total of 4 injections. Doses used for NmU and NmS correspond to those previously used to induce labor in mice with neuromedin B [17]. Mice were checked every 2h for any signs of labor/delivery such as vaginal bleeding or delivery of at least one pup.

*Intrauterine IL-1β and LPS injection*

Timed-pregnant mice at 9 days of gestation (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 IL-1β, 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 h after the intrauterine injection (at G10), mice were sacrificed 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 sacrifice.

*Lentivirus production*

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

*Lipopolysaccharide (LPS)-induced preterm birth model*

Timed-pregnant mice pre-treated for 72h with an intrauterine injection of lentivirus or saline received 10 µg of intraperitoneal LPS or an equivalent volume of saline at 16 days of gestation (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 Polymerase Chain Reaction (RT-qPCR)*

Myometrium fragments were thawed and rapidly preserved in Ribozol (AMRESCO, Solon OH, United States), whereas cells from *in vitro* experiments were treated for 6 h with 5ng/mL of IL-1β and collected directly into Ribozol. RNA was extracted according to manufacturer's protocol and RNA concentration and integrity was measured with a NanoDrop 1000 spectrophotometer. Five hundred ng of RNA was used to synthetize cDNA using iScript Reverse Transcription SuperMix (Bio-Rad, Hercules CA, United States). Primers were designed using NCBI Primer Blast (see Suppl. Table I). Quantitative gene expression analysis was performed on Stratagene MXPro3000 (Stratagene) with SYBR Green Master Mix (BioRad). Gene expression levels were normalized to 18S universal primer (Ambion Life Technology, Burlington ON, Canada). Dissociation curves were also acquired to test primer specificity. Genes analyzed include: *Nmu*, *Nms*, *Nmur1*, *Nmur2*, *Ptgfr* (prostaglandin F receptor), *Oxtr* (oxytocin receptor). Detailed primer sequences are shown in Table II.

*Western blotting*

Proteins from homogenized myometrium fragments and cell samples lysed in RIPA buffer were quantified using Bradford's method (Bio-Rad). Fifty µg of protein sample were loaded onto SDS-PAGE gel and electrotransfered onto PVDF membranes. After blocking, membranes were incubated with either an antibody against NmU (#sc-368069; Santa Cruz Biotechnology, Dallas TX, United States), NmS (#PAA828Mu01; Cloud-Clone Corp., Houston TX, United States), NmU-R1 (#sc-47241; Santa Cruz Biotechnology), NmU-R2 (#sc-47250;

Santa Cruz Biotechnology), cyclophilin B (#ab16045; abcam), anti-GAPDH (PA1-987, Pierce Protein Biology, Thermo Scientific, Rockford, IL) or β-actin (#sc-47778; Santa Cruz Biotechnology); (although NmU and NmS antibodies detect the corresponding pro-peptides [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, St. Louis, MO) and incubated for 1 hour with their respective secondary antibodies conjugated to HRP (Sigma). For kinases, membranes were incubated with an antibody 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-Akt (#9271; Cell Signaling Technology), phospho-Erk (#9101; Cell Signaling Technology), JNK (#9252; Cell Signaling Technology), c-jun (#9165; Cell Signaling Technology), p38 (#9212; Cell Signaling Technology), Akt (#9272; Cell Signaling Technology), or Erk (#4695; Cell Signaling Technology). Enhanced chemiluminescence (GE Healthcare) was used for detection using the ImageQuant LAS-500 (GE Healthcare, Little Chalfont, United Kingdom) and densitometric analysis was performed using ImageJ. 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*

40 000 mSMC/well were cultured overnight in 96-wells clear-bottomed black plates (#3603; Corning, New York NY, United States) 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, Waltham MA, United States), 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 2s-interval post-injection readings were automatically stopped after 30 s. Assay buffer was used as a negative control. Value 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 age were sacrificed 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 to 3mm wide and 10mm long) were dissected free from uterus, mounted isometrically in organ tissue baths and initial tension was set at 2 g. The tissue baths contain 20 ml of Krebs buffer of the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 0.9 MgSO4, 1 KH2PO4, 11.1 glucose, and 23 NaHCO3 (pH 7.4). The buffer was equilibrated with 95% oxygen/5% carbon dioxide at 37°C. Isometric tension is measured by a force transducer and recorded by BIOPAC data acquisition system (BIOPAC MP150). Experiments began after 1 hour of equilibration. The mean tension of spontaneous contractions was measured using a BIOPAC digital polygraph system (AcqKnowledge); the same parameters were also determined after addition 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, five micrometers thick sections were obtained using a microtome and processed as previously described [23]. The following antibodies were used: NmU (1:50; Santa Cruz Biotechnology, Dallas, TX, USA), NmS (1:10; Cloud Corporation, Cedarlane, ON, Canada) with matched HRP-conjugated secondary antibodies (goat anti-rabbit-HRP, BioRad, ON, Canada). Staining was revealed using 3, 3-diaminobenzidine (DAB; Amresco, VWR, ON, Canada) and slides were counterstained with hematoxylin and mounted. Primary antibodies were omitted for negative controls. Images were obtained with a slide scanner (Axioscan, Zeiss, ON, Canada) using Zen2 program.

*Primary cytotrophoblast isolation and culture*

Primary cytotrophoblasts were isolated from term placentas from uncomplicated pregnancies obtained after caesarean section using a well-established method developed by Kliman et al. [24]. Briefly, villous tissue was dissected, minced and rinsed in phosphate-buffered saline (PBS) prior to 3 enzymatic digestions in Hanks' balanced salt solution (HBSS) with trypsin and DNase. Cytotrophoblasts were obtained from these digestions after separation by centrifugation on a discontinuous Percoll gradient. Cytotrophoblasts were plated at 2x106 cells/ml in DMEM-F12 supplemented with 10% FBS and penicillin/streptomycin and washed

with PBS to removed non-adherent cells after 12h. Cells were treated with lipopolysaccharide (LPS; 1µg/ml, Sigma-Aldrich, ON, Canada) for 24h or 48h in Opti-MEM (Life Technologies, ON, Canada). Cells were collected in lysis buffer (PBS with 1% Triton X-100 and protease inhibitors cocktail), centrifuged at 13000rpm for 10min at 4ºC and supernatant collected and kept at -20ºC until used for analysis.

*Immunocytochemistry*

Cells were plated on cover slips pre-coated 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 1h at ambient temperature with a secondary antibody conjugated with Alexa Fluor 488 (Sigma). Nuclei were stained with Dapi (Invitrogen; 1/5000). Images were captured using 30x objective with Eclipse E800 (Nikon) fluorescence microscope.

*Statistical analysis*

Groups were compared by two-tailed Student's t -test or one-way analysis of variance (ANOVA). Dunnett's multiple comparison method was employed when treatments were compared to a single control. Tukey's 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 +/- S.E.M.

## 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 utero-contractile effects of NmU ($EC_{50}$=15 nM) (Fig. 1B). Effects of NmU were NmU-R2-dependent, as uterine contraction was markedly diminished after *Nmur2* knockdown (Fig. 1C; Suppl Fig. 1A-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 (Suppl. Fig. 1D,E), consistently 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 (ip) 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, as 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 utero-contractile 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, Suppl. Fig. 2A) and NmS (Fig. 2B, Suppl. Fig. 2B); 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. 2C-D), with a marked increase of NmS during labor.

We also studied the expression of pro-contractile 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 (PP) (Fig. 2E, Suppl. Fig. 2D). 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; PTNL, n=4) as compared to non-pregnant women (NP; n=4) (Fig. 2F; Suppl. Fig. 2G). In contrast, NmU-R1 protein and gene expression in murine uterus did not increase during gestation or labor (Suppl. Fig. 1F,2C-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 (ip) in late but not early gestation (Table III), 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 myometrial smooth muscle cells isolated at G10 (G10 mSMC) and G19 (G19 mSMC) 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 to G10 (Suppl. Fig. 3), as seen directly on uterine samples (see Fig. 2E). NmU induced greater calcium transients on G19 mSMC than G10 mSMC (Fig. 3A); a similar profile was observed upon stimulation with known uterotonins $PGF_{2\alpha}$ and oxytocin. As expected, NmU-induced calcium transients were NmU-R2-dependent (Fig. 3B, Suppl. Fig. 1E); the extent and duration of calcium transients is consistent with that observed for other G protein-coupled receptors [26, 27]. Exploration of NmU-R2 downstream mechanisms in G19 mSMC revealed that NmU does not activate (by phosphorylation) p38, JNK and c-jun (Suppl Fig. 4A), but does activate Akt and Erk (Fig. 3C,D; Suppl Fig. 4B,C); no effect was seen in G10 mSMC. NmU-triggered Akt activation, but not that of Erk, was inhibited by calmodulin inhibition (W-7) and by phospholipase Cβ inhibition (U73122) (Suppl Fig. 4D,E). *Nmur2* knockdown abrogated NmU-induced Akt and Erk activation in G19 mSMC (Fig. 3E,F). A schematic diagram of NmU-R2 signaling pathway is presented in Suppl. Fig. 4F. 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. 3G-I).

*IL-1β induces uterine expression of NmU-R2 and potentiates its uterotonic effects; pro-inflammatory infectious stimuli trigger NmS expression in human decidua and placenta*

Chorioamnionitis is a major factor in triggering PTB [4-6]. We determined if NmU-R2 is regulated by inflammatory factors. In line with the important role for IL-1β in eliciting uterine inflammation and PTB [28-31], we stimulated G10 mSMC with IL-1β and measured the mRNA expression of *Nmur1*, *Nmur2*, as well as *Ptgfr* and *Oxtr*; expression of the latter 3 genes (but not *Nmur1*) was upregulated within 6 h by IL-1β (Fig. 4A); of relevance herein, progesterone (when anti-inflammatory PR-B is dominant [32]) had no effect on expression of these genes (not shown). We then proceeded to explore if inflammation (IL-1β-induced) could reproduce these effects *in vivo*. Consistent with changes observed in mSMC, IL-1β (1 μg intrauterine, at G9 [Fig.4B]), shown to amplify utero-placental inflammation [30], induced protein and mRNA uterine expression of NmU-R2 (Fig. 4C; Suppl. Fig.2I), but not NmU-R1 in mice (Suppl. Fig.1G; Suppl. Fig. 2H). Correspondingly, uteri isolated from pregnant mice (G10) treated with IL-1β displayed increased utero-contractile response to NmU *ex vivo* (Fig. 4D). Concordantly, calcium transients triggered by NmU were significantly increased in murine mSMC isolated at G10 and pre-treated with IL-1β (Fig. 4E); this effect was also observed in human mSMC (Fig. 4F).

Given the strong and consistent effect of IL-1β in triggering NmU-R2 expression and actions in uterus, we determined if IL-1β triggered expression of NmU-R2 ligands in placenta.

IL-1β was found to increase placental expression of the NmU-R2-specific agonist NmS, but not NmU, in mice (Fig. 4G-H). A similar induction of placental NmS was observed when mice were stimulated intrauterine with known inflammasome-activating TLR4 ligand LPS (arising from G(-) 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 that delivered preterm with clinical evidence of infection (PTLi, n=3) compared to those without (PTL, n=3) (Manchester UK, tissue bank) (Fig. 4J). In human placenta, NmS was primarily expressed by cytotrophoblasts and Hofbauer (placental macrophage) cells (within placental villi), while 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 h and 48 h; LPS induced NmS expression from cytotrophoblasts (Fig. 4L), validating human and murine data (presented in Fig. 4H-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 intrauterine with lentivirus encoded with shRNA against pro-contractile *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 PTB, and its onset is often subclinical. Administration of bacteria or bacterial endotoxins in pregnancy triggers uterine activation pathways which can induce labor in rodents and non-human primates [30, 33-36]. Our findings indicate that NmU-R2 and its specific ligand NmS are regulated by inflammation in human and animal, and play a critical role in infection-associated PTB. First, we found in murine gestational tissues that the expression of NmU-R2 and NmS was markedly increased upon intrauterine treatment with the major pro-inflammatory labor-inducing cytokine IL-1β. Correspondingly, treatment with IL-1β significantly increased the uterotonic effect and associated calcium transients coupled to NmU-R2 in murine and human mSMC. Second, the inflammation-driven NmU-R2 upregulation observed in animals was also observed in human gestational tissues, as 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, knock-down of NmU-R2 in uterus significantly delayed preterm labor induced by Gram(-) bacterial product LPS. For these reasons, we suggest that infectious and non-infectious pro-inflammatory stimuli in pregnant gestational tissues, as well as advanced gestation age (in case of physiological term labor), trigger: 1) NmU-R2 upregulation in myometrium thereby increasing the utero-contractile sensitivity to NmS; and 2) NmS production in placenta (specifically in cytotrophoblasts). This uterotonic system may contribute to the establishment of functional labor at term and preterm

in 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 as there are no available pharmacological agent sufficiently effective to prolong preterm gestation by more than 48 h and improve 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 years, 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, as it failed FDA-approval. Hydroxy-progesterone 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. UAPs are interesting targets for the prevention of preterm birth as 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 utero-contractile effects; 3) it is induced by pro-inflammatory 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 seen with the unaltered gestation length in germ-line gene knockout mice for other critically important proteins including IL-1R1, 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), thus 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 knock-down considerably attenuated receptor-coupled signaling (calcium, Erk and Akt), utero-contractile and pro-labor 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 mSMC, 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.

## Conclusions

In summary, we hereby describe a pro-contractile, pro-labor human and murine system wherein neuromedins U and (more importantly) S induce calcium (and other downstream) signals in mSMC 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 as 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 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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**Conflict of interest**

The authors have declared that no conflict of interest exists.

**Authors contribution**

MNV, SC and SG participated in research design. MNV, AB, KL, XH, RR, TZ, MEB, AM, ABR and SG conducted experiments. MNV, AB, XH, KL, RR, LB and SG performed data analysis. MNV, SC, DMO, AM, DO, SG and AB wrote or participated in the writing of the manuscript. DO produced the illustration. All authors participated in the critical review of the manuscript and all approved the final manuscript.

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**Figure legends**

Figure 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 panel) and dose-response curve (bottom panel). C, pregnant mice were pre-treated 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 inter-individual 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 to vehicle only. F, pregnant mice were pre-treated with LV.shNmU-R2 or LV.shScrambled at G15 and then treated with 160 µg/Kg of NmU twice a day from G17 to G18. *p<0.05, **p<0.01, ***p<0.001 compared to NmU or NmU + LV.shScrambled. G, pregnant mice were injected intraperitoneally with 160 µg/Kg of NmS or NmU twice a day from G17 to G18. ***p<0.001 compared to vehicle only. Data for A-D are representative of 4-5 mice per group. The number of mice used in E-G is displayed above each groups and mice treated with vehicle (control mice) were pooled together and repeatedly shown in each graph. Values are presented as mean ± S.E.M. Data were statistically analysed using one-way ANOVA with comparison to control groups using Dunnett's multiple comparison test.

Figure 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-B, NmU (A) and NmS (B) representative immunoblots of murine placentas collected at different gestation age (G) and during spontaneous term labor (TL). Lower panels show densitometric analysis of protein bands normalized with β-actin and plotted as fold change vs. the control group (G12). C-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 change vs. 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 gestation age (G), during spontaneous term labor (TL) and 24h postpartum (PP). The lower panel shows densitometric analysis of protein bands normalized with β-actin and plotted as fold change vs. the control group (NP). F, representative immunoblot of NmU-R2 from human myometrial tissue biopsies from 4 non-pregnant (NP) women (hysterectomy), 1 pregnant women at preterm without any clinical sign of labor (PTNL), and 4 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 vs. the control group (NP) of all patients screened (NP, n=4; PTNL, n=4; TNL, n=9; TL, n=5) as presented in Fig. 2F and Suppl. Fig. 2G. 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 4-5 mice per group and at least 3 independent experiments. Values are presented as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with comparison to control groups using Dunnett's multiple comparison test.

Figure 3. **NmU-R2-associated signaling in mSMC is potentiated by gestation age.** A, calcium assay performed on primary mSMC from either pregnant mice at 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 pre-treated 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-D, Akt (C) and Erk (D) phosphorylation immunoreactivity on primary mSMC from pregnant mice at G10 or G19 stimulated with increasing concentrations of NmU for 10 min. Data are representative of 4-5 independent experiments. E-F, Akt (E) and Erk (F) immunoreactivity on G19 myometrial cells pre-treated with LV.shNmU-R2 or LV.shSrambled for 72 h and then stimulated with $10^{-6}$ M of NmU for 10 min. Data are representative of 4-5 independent experiments. G, calcium assay of G19 myometrial cells treated with $10^{-6}$ M NmU or NmS. n=6-45 in each group. H-I, Akt (H) and Erk (I) phosphorylation immunoblot of G19 myometrial cells treated with $10^{-6}$ M of NmU or NmS for 10 min. Data are representative of 4-

5 independent experiments. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with comparison to control groups using Dunnett's multiple comparison test.

Figure 4. **NmU-R2 in uterus and NmS in placenta are under control of inflammation in mice and humans.** A, Quantitative PCR performed on primary myometrial smooth muscle cells collected from pregnant mice at G10 and stimulated with 5 ng/mL of interleukin-1β for 6h. 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 panel) and densitometric analysis (lower panel) of uteri of pregnant mice collected 24h after an intrauterine injection of saline (sham) or IL-1β. D, *ex vivo* contraction assay performed on uteri from pregnant mice exposed for 24h to saline (sham) or IL-1β. Data are representative of 3-4 mice per group. E-F, mSMC from pregnant mice (G10) or human mSMC (hTERT-C3 cell line) were treated for 24h with IL-1β or vehicle and were used in a calcium assay. n=6-12 in each group. G-H, immunoblots (top panels) and relative densitometric analysis of protein bands (lower panels) showing NmU (G) and NmS (H) expression in placenta of pregnant mice 24h after an intrauterine injection of either saline (sham) or IL-1β. I, immunoblot (top panel) and relative densitometric analysis of protein bands (lower panel) showing NmS expression in placenta of pregnant mice 24h 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. The lower panel shows 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. Scale bar, 40μM. L, primary human cytotrophoblasts were stimulated with LPS for 24h (top panel) or 48h (lower panel) and cell lysates were blotted against NmS. β-actin and cyclophilin B were used as loading controls. Values are presented as mean ± S.E.M. Data are representative of at least 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with comparison to control groups using Dunnett's multiple comparison test.

Figure 5. **NmU-R2 is important for infection-induced adverse gestation outcomes.** A-B, pregnant mice were pre-treated with intrauterine injections of vehicle (sham), LV.shScrambled

or LV.shNmU-R2 at G13 (1 injection in each uterine horn) and then treated with a single intraperitoneal injection of 10 μg LPS or an equivalent volume of saline at G16. The timing of birth (A) and the survival of pups at birth per litter (B) was rigorously assessed. Values are presented as mean ± S.E.M. *$p<0.05$ by one-way ANOVA with comparison to control groups using Dunnett's multiple comparison test.

Figure 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 pro-inflammatory, pro-labor 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 IL-1. 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.

**Supplementary figure legends**

Figure S1. **NmU-R1 is not implicated in the utero-contractile effects of NmU.** A, 5 different shRNA designed to target *Nmur2* were tested in primary mSMC to test their efficacy to knockdown NmU-R2. The arrow points at the shRNA that was selected for all experiments. B, pregnant mice were treated with a single intrauterine injection of either LV.shScrambled or LV.shNmU-R2 for 72h, and their uteri were collected for analysis of protein expression of NmU-R2. C, representative image of a contraction assay in uteri from mice treated with lentivirus or with vehicle (sham). D, siRNA-mediated NmU-R1 knock-down was confirmed using qPCR and immunoblot. E, G19 myometrial cells were pre-treated with siNmU-R1 or siControl for 72h and then used for a calcium assay in response to $10^{-8}$ M of 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. F, representative NmU-R1 immunoblot of murine uteri collected at different gestation age. The lower panel shows densitometric analysis of protein bands normalized with β-actin and plotted as fold change vs. the control group (NP). G, representative NmU-R1 immunoblot (top panel) and densitometric analysis (lower panel) of uteri of pregnant mice collected 24h after an intrauterine injection of saline (sham) or IL-1β. Values are presented as mean ± S.E.M. Data are representative of 3 independent experiments. *$p<0.05$, **$p<0.01$ by one-way ANOVA with comparison to control groups using Dunnett's multiple comparison test.

Figure S2. **Quantitative PCR analysis of gestational tissue samples**. A-B, quantitative PCR of Nmu (A) and Nms (B) performed on placentas collected at different gestation age (G) and during spontaneous term labor (TL). C-F, quantitative PCR of *Nmur1* (C) and *Nmur2* (D) performed on uteri collected at different gestation age (G), during spontaneous term labor (TL) and 24h postpartum (PP). *Ptgfr* (E) and *Oxtr* (F) were used as positive controls of UAP genes known to be upregulated near term. G, representative immunoblot of NmU-R2 from human myometrial tissue biopsies from 5 pregnant women at term without any clinical sign of labor (TNL; samples from women different than those presented in immunoblot of Fig. 2F) and 5 pregnant women at term in labor (TL). Densitometric analysis of all patients screened is presented in Fig.2F. H-K, quantitative PCR of *Nmur1* (H) and *Nmur2* (I) performed on uteri of mice treated for 24 h with an intrauterine injection of saline (sham) or IL-1β. *Ptgfr* (J) and *Oxtr*

(K) were used as positive controls. Results are relative to 18S and plotted as fold change vs. the control groups. Murine data are representative of 4-5 mice in each group. Values are presented as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with comparison to control groups using Dunnett's multiple comparison test.

Figure S3. **Purity assessment of primary isolated myometrial smooth muscle cells.** A, approximately 95% of freshly isolated and cultured primary myometrial smooth muscle cells were immunoreactive to α-actin (green). B, negative control showing no binding of the Alexa Fluor secondary antibody alone. DAPI was used to stain nuclei. Scale bar, 100μM. C-D, quantitative PCR of *NMUR1* (C) and *NMUR2* (D) performed on myometrial smooth muscle cells collected from either pregnant mice at G10 or at G19. Results are relative to 18S and plotted as fold over the G10 cells. E-F, NmU-R1 (E) or NmU-R2 (F) immunoblots performed on the same cells. Data are representative of at least 3 independent experiments. Values are presented as mean ± S.E.M. ***p<0.001 by one-way ANOVA with comparison to control group (G10 mSMC) using Dunnett's multiple comparison test.

Figure S4. **Mechanism of action of NmU in primary myometrial smooth muscle cells**. A-D, MAPK p38, SAPK JNK and downstream transcription factor c-jun (A, quantified in right panel), Akt (B) and Erk (C) immunoblots performed on cells treated or not with $10^{-6}$ M of NmU for different periods of time. IL-1β (5ng/mL) was used as a positive control for stress associated protein kinases. D-E, Akt (D) or Erk (E) immunoblots on cells pre-treated for 30 min with either the phospholipase Cβ inhibitor U73122, the calmodulin inhibitor w-7 or vehicle and then treated for 10 min with or without $10^{-6}$ M of NmU. Right panel shows densitometric analysis of protein bands normalized with β-actin and plotted as fold over the control group. The non-treated and the treated groups were first normalized with β-actin, and then divided by the non-treated group value. F, proposed signaling pathway of NmU in myometrial smooth muscle cells adapted from FP mechanism of action [26]; * indicates signaling steps uncovered herein. PLC, phospholipase; IP3, inositol triphosphate; MLCK, myosin light chain kinase. Data are representative of at least 3 independent experiments. Values are presented as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with comparison to control groups using Dunnett's multiple comparison test.

## Figures

Figure 1

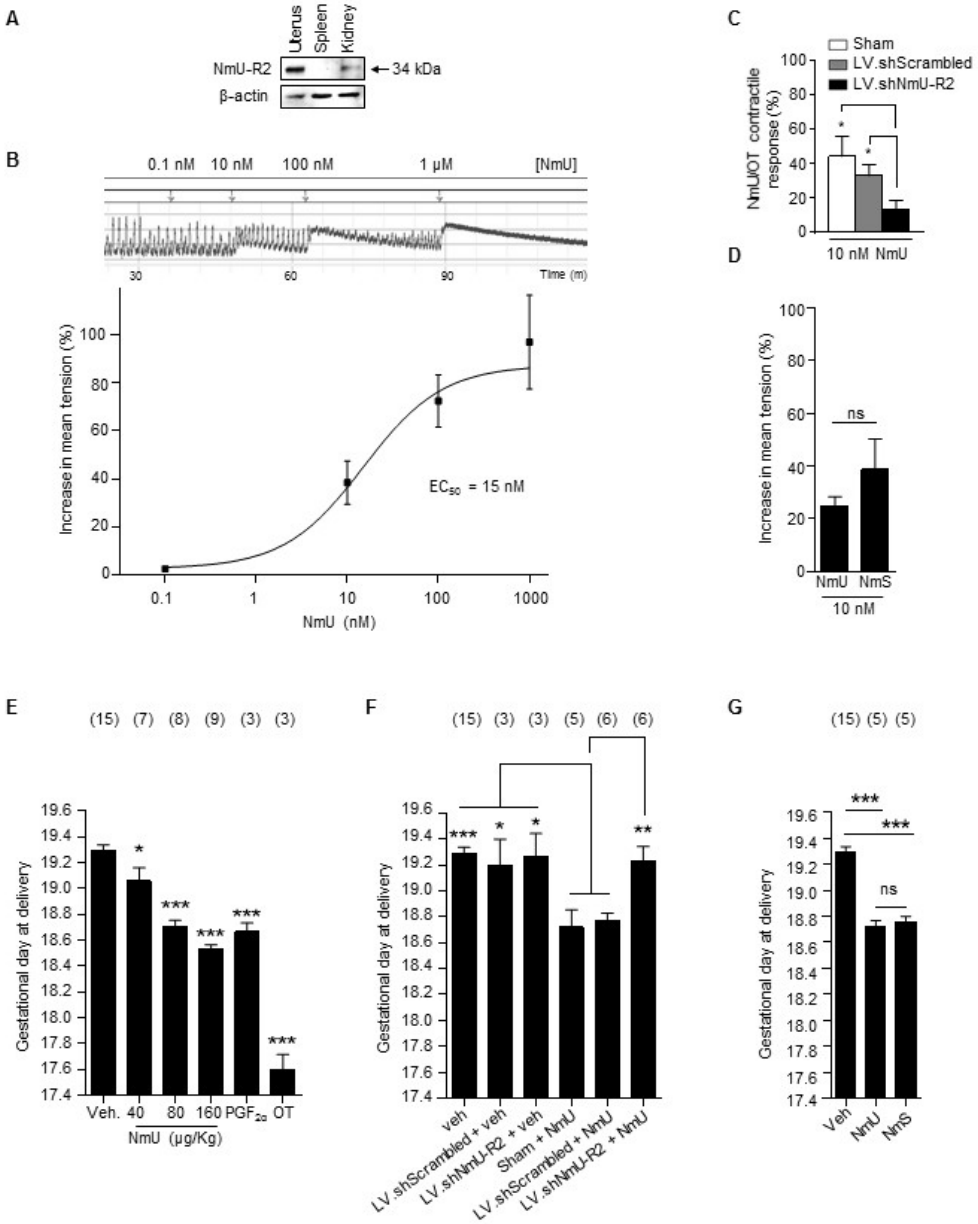


Figure 2

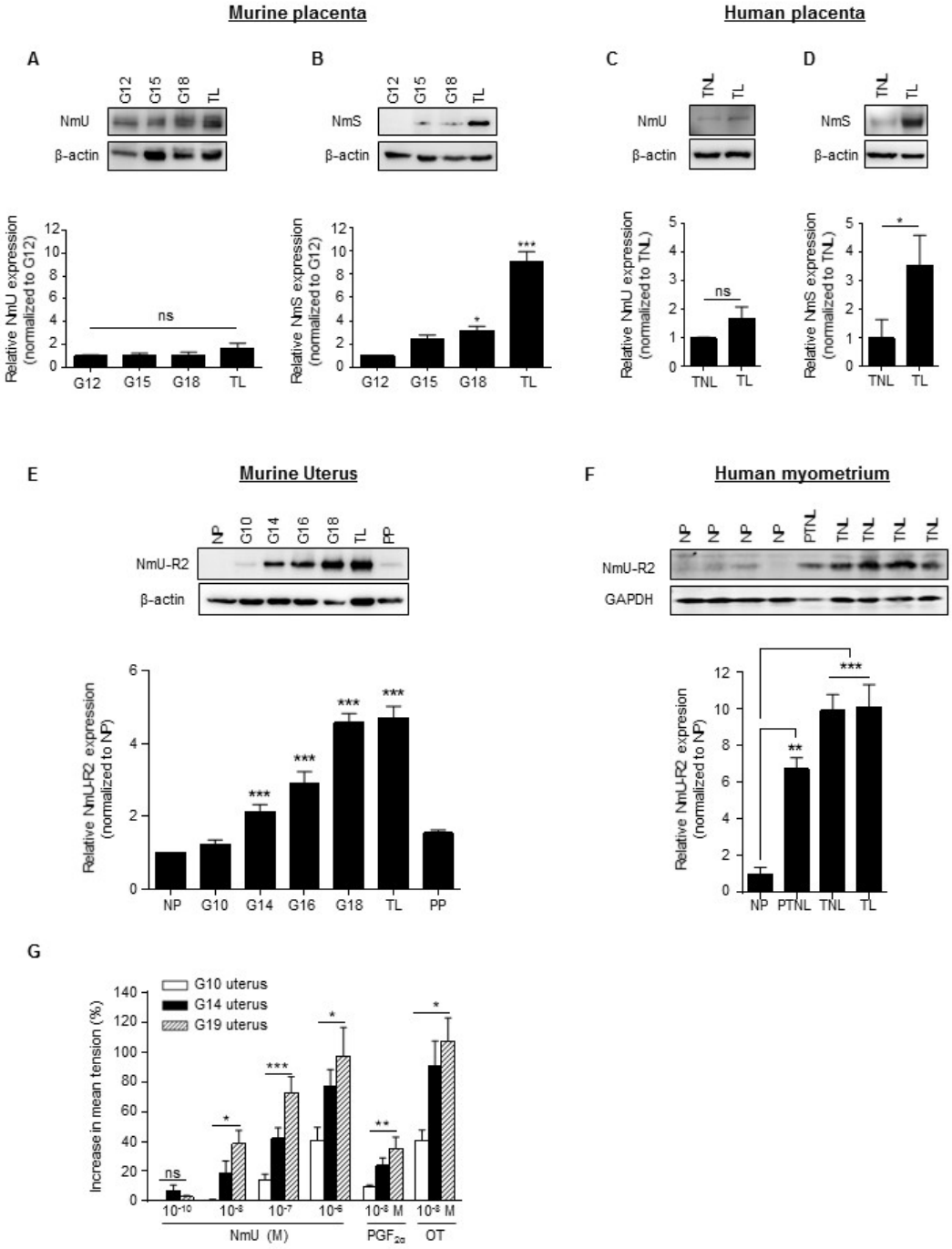

Murine placenta
Human placenta
A
B
C
D
G12
G15
G18
TL
TNL
NmU
NmS
β-actin
Relative NmU expression (normalized to G12)
Relative NmS expression (normalized to G12)
Relative NmU expression (normalized to TNL)
Relative NmS expression (normalized to TNL)
ns
*
***
E
F
Murine Uterus
Human myometrium
NP
G10
G14
G16
PP
PTNL
NmU-R2
GAPDH
Relative NmU-R2 expression (normalized to NP)
**
G
G10 uterus
G14 uterus
G19 uterus
Increase in mean tension (%)
NmU (M)
PGF2α
OT

Figure 3

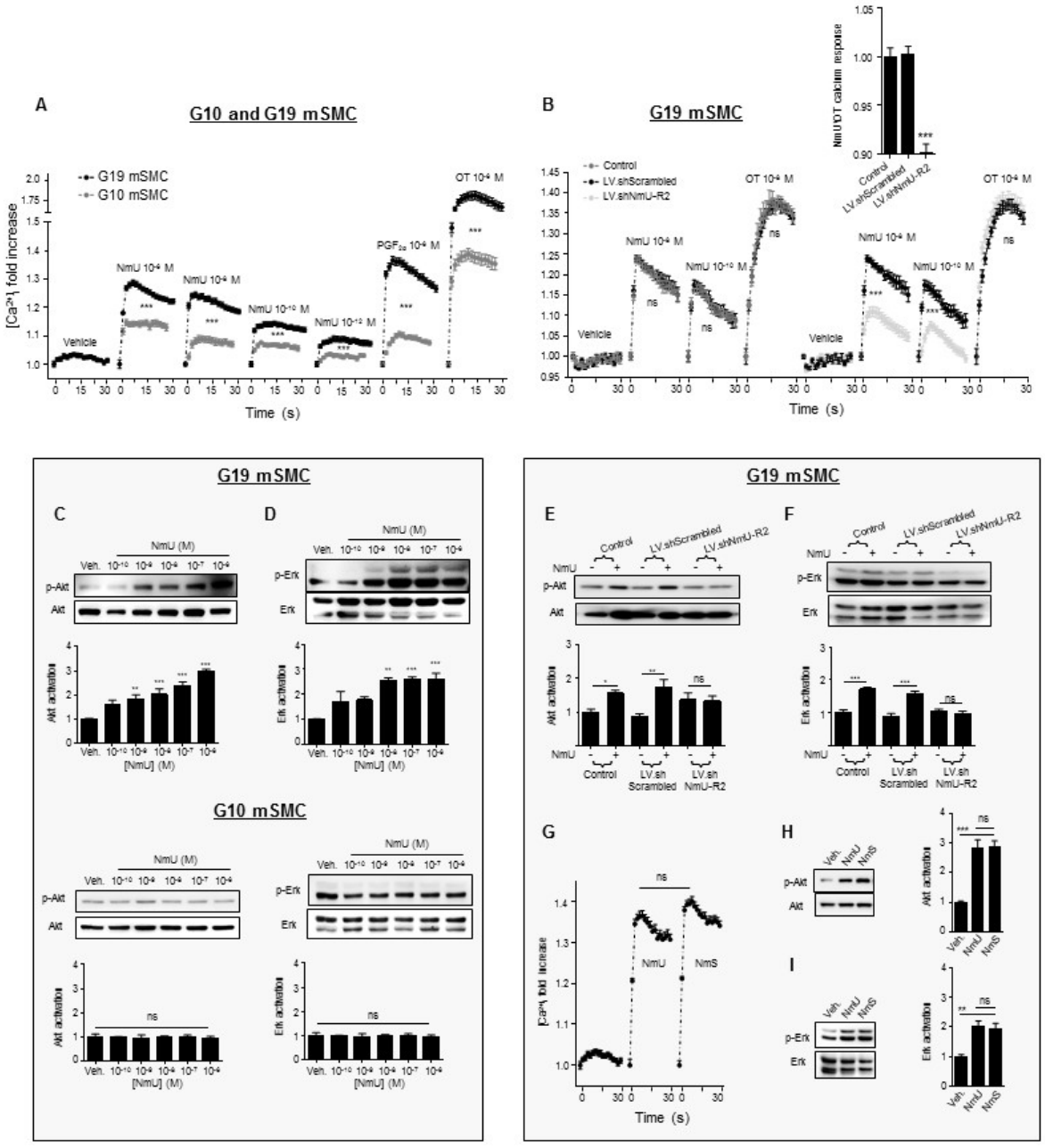

A
G10 and G19 mSMC
G19 mSMC
G10 mSMC
[Ca2+]i fold increase
Vehicle
NmU 10-8 M
NmU 10-9 M
NmU 10-10 M
NmU 10-11 M
PGF2α 10-4 M
OT 10-4 M
Time (s)
B
G19 mSMC
Control
LV.shScrambled
LV.shNmU-R2
NmU OT calcium response
Vehicle
NmU 10-8 M
NmU 10-10 M
OT 10-4 M
ns
Time (s)
C
G19 mSMC
NmU (M)
Veh. 10-10 10-9 10-8 10-7 10-6
p-Akt
Akt
Akt activation
[NmU] (M)
D
p-Erk
Erk
Erk activation
G10 mSMC
E
Control
LV.shScrambled
LV.shNmU-R2
NmU
Akt activation
Control
LV.sh Scrambled
LV.sh NmU-R2
F
Erk activation
G
[Ca2+]i fold increase
NmU
NmS
Time (s)
H
Veh.
NmU
NmS
I

Figure 4

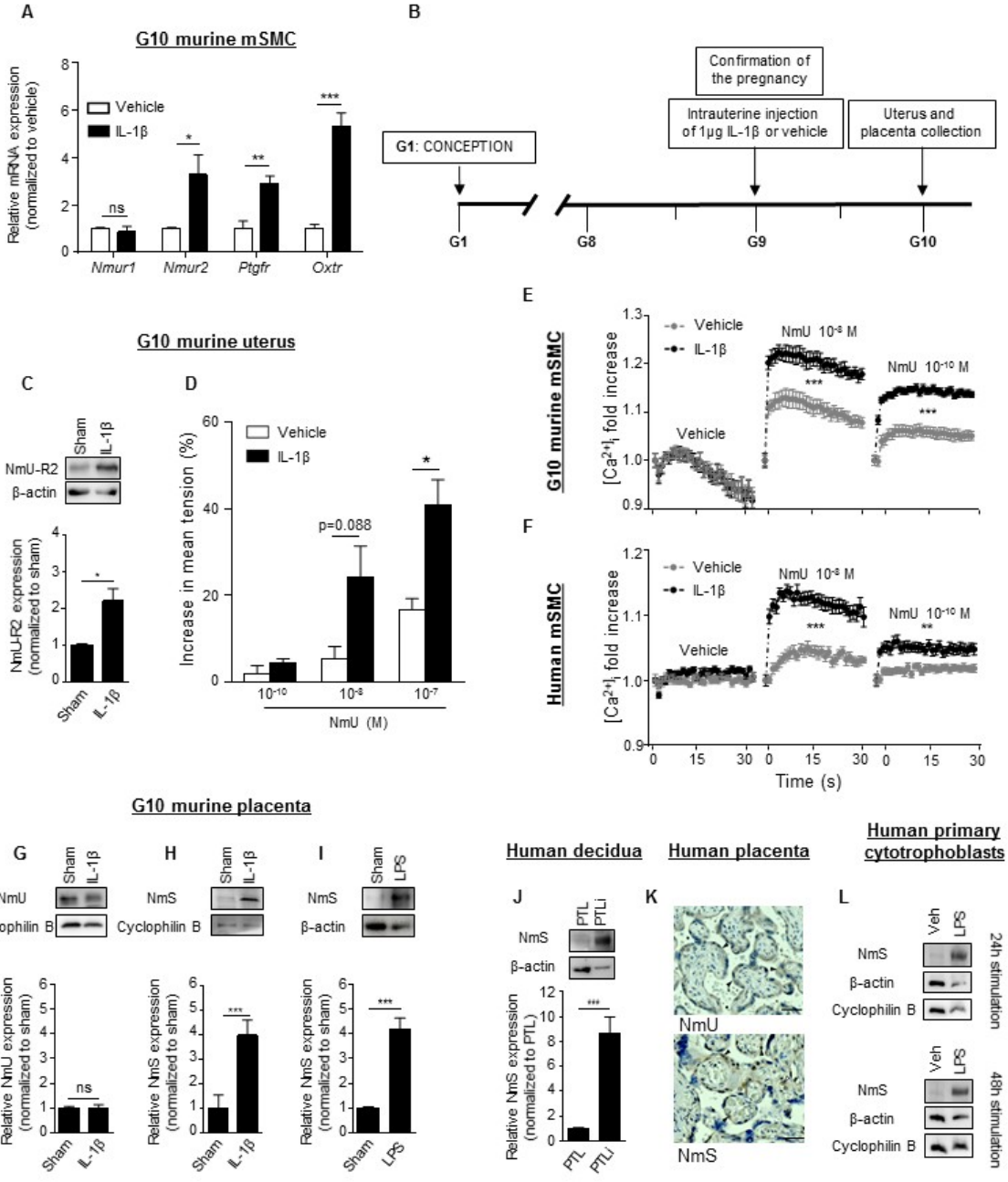

A
G10 murine mSMC
Relative mRNA expression (normalized to vehicle)
Vehicle
IL-1β
ns
*
**
***
Nmur1
Nmur2
Ptgfr
Oxtr
B
Confirmation of the pregnancy
Intrauterine injection of 1µg IL-1β or vehicle
Uterus and placenta collection
G1: CONCEPTION
G1
G8
G9
G10
G10 murine uterus
C
Sham
IL-1β
NmU-R2
β-actin
NmU-R2 expression (normalized to sham)
D
Increase in mean tension (%)
p=0.088
10-10
10-8
10-7
NmU (M)
E
G10 murine mSMC
[Ca2+]i fold increase
NmU 10-8 M
NmU 10-10 M
F
Human mSMC
Time (s)
G10 murine placenta
G
NmU
Cyclophilin B
Relative NmU expression (normalized to sham)
H
NmS
Relative NmS expression (normalized to sham)
I
LPS
β-actin
Human decidua
Human placenta
Human primary cytotrophoblasts
J
PTL
PTLi
Relative NmS expression (normalized to PTL)
K
NmU
NmS
L
Veh
LPS
24h stimulation
48h stimulation

Figure 5

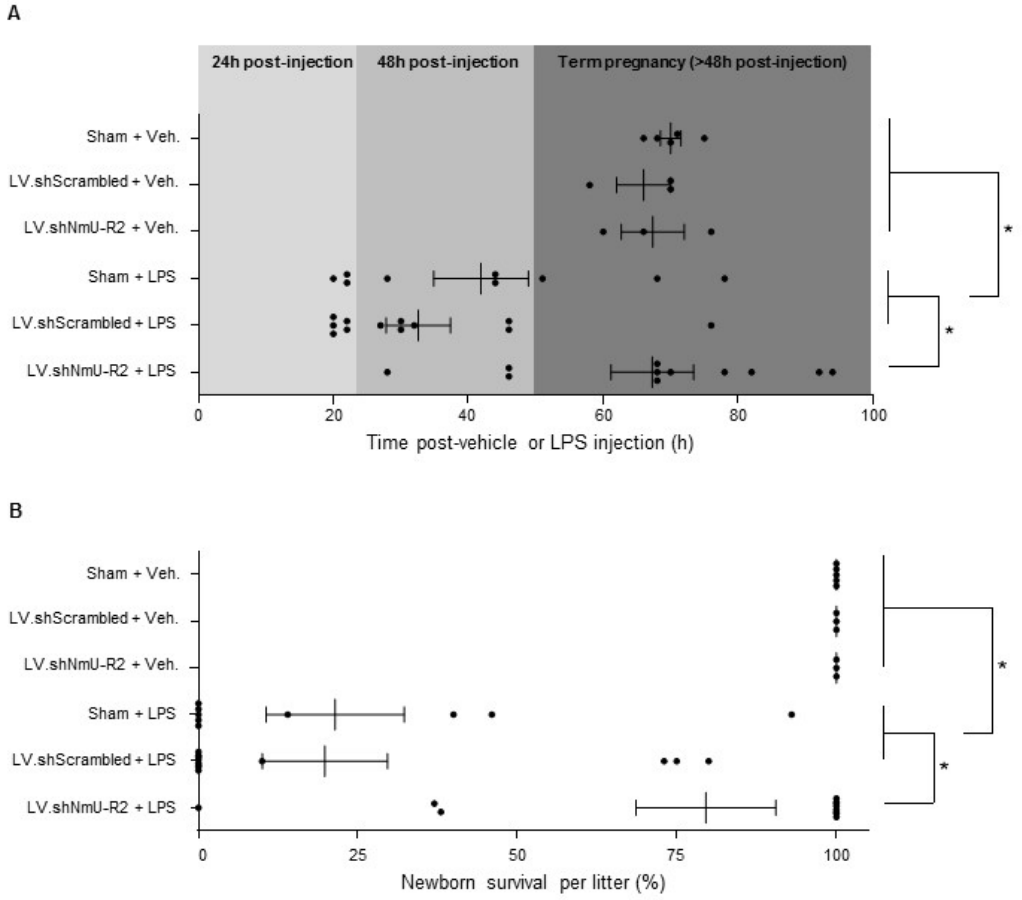
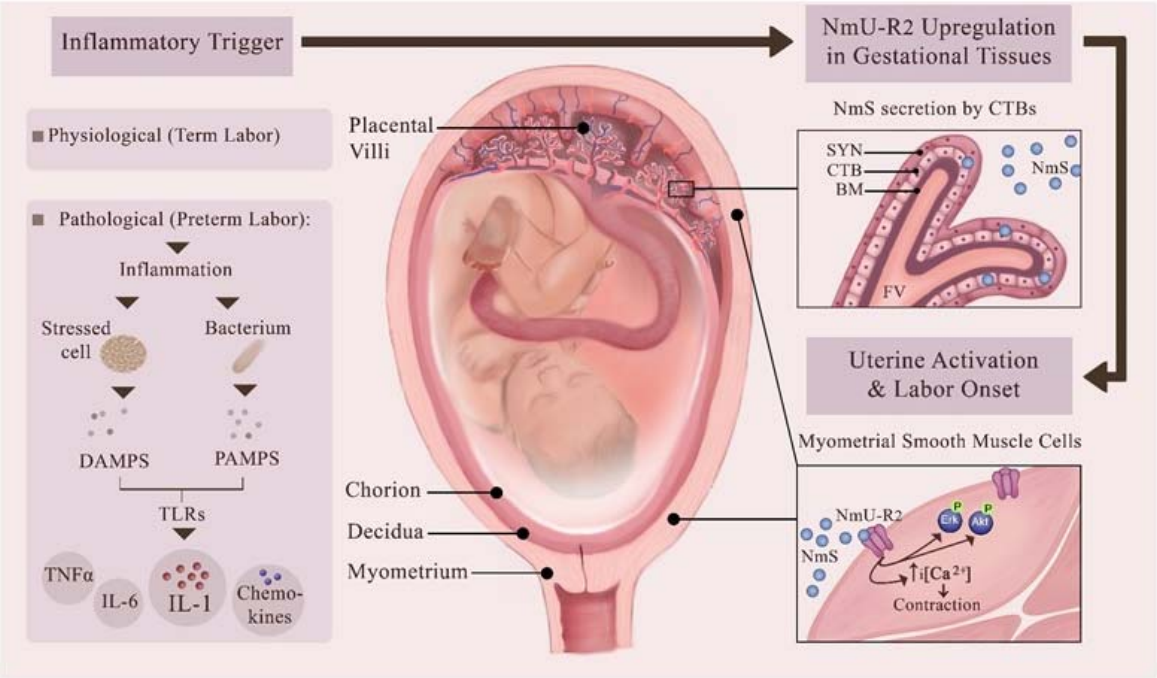

A
24h post-injection
48h post-injection
Term pregnancy (>48h post-injection)
Sham + Veh.
LV.shScrambled + Veh.
LV.shNmU-R2 + Veh.
Sham + LPS
LV.shScrambled + LPS
LV.shNmU-R2 + LPS
0
20
40
60
80
100
Time post-vehicle or LPS injection (h)
*
*
B
Sham + Veh.
LV.shScrambled + Veh.
LV.shNmU-R2 + Veh.
Sham + LPS
LV.shScrambled + LPS
LV.shNmU-R2 + LPS
0
25
50
75
100
Newborn survival per litter (%)
*
*

Figure 6


Inflammatory Trigger
NmU-R2 Upregulation in Gestational Tissues
Physiological (Term Labor)
Pathological (Preterm Labor):
Inflammation
Stressed cell
Bacterium
DAMPS
PAMPS
TLRs
TNFα
IL-6
IL-1
Chemo-kines
Placental Villi
Chorion
Decidua
Myometrium
NmS secretion by CTBs
SYN
CTB
BM
NmS
FV
Uterine Activation & Labor Onset
Myometrial Smooth Muscle Cells
NmU-R2
NmS
Erk
Akt
P
P
↑i[Ca2+]
Contraction

## Supplementary Figures

Figure S1

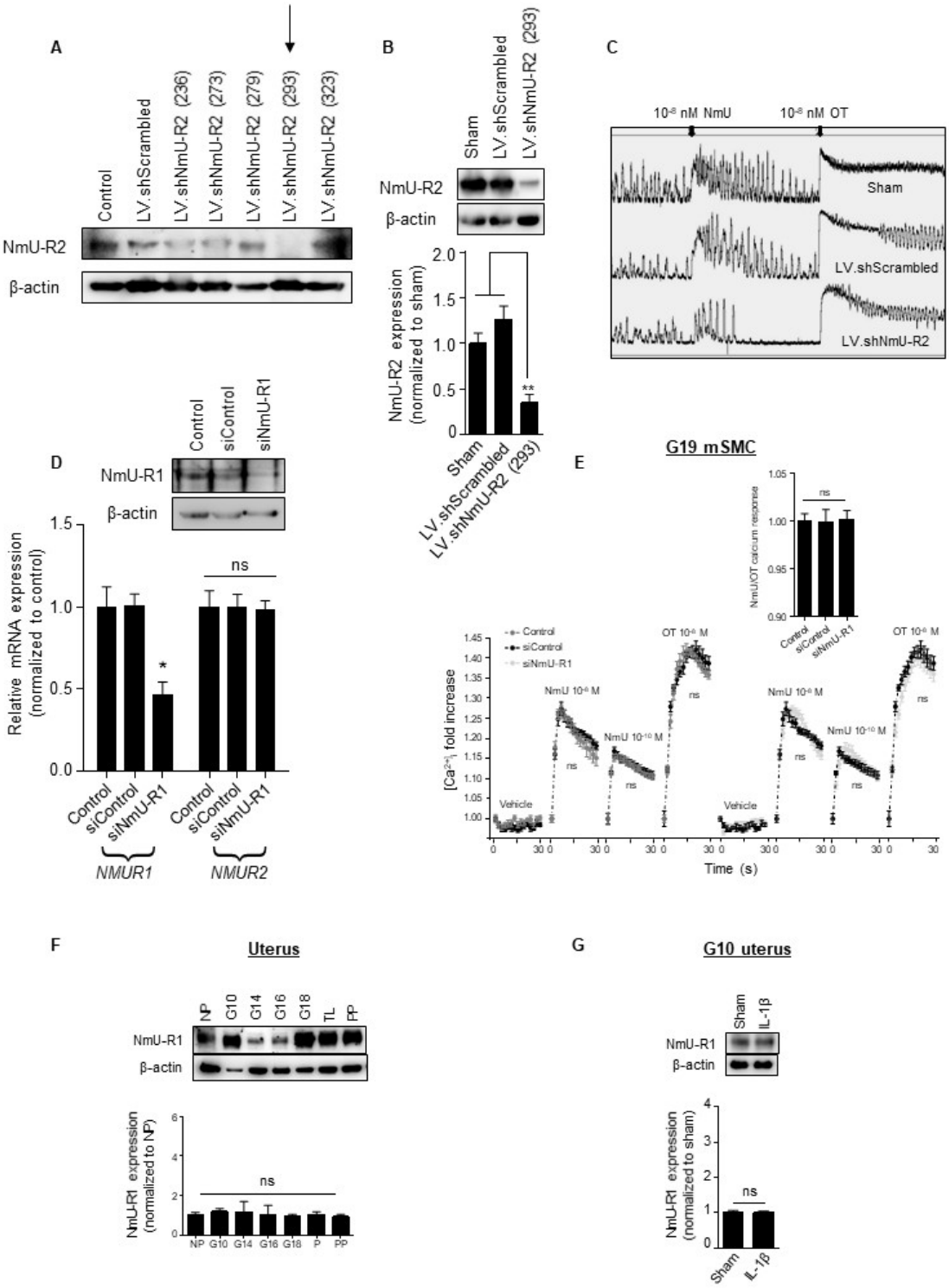


Figure S2

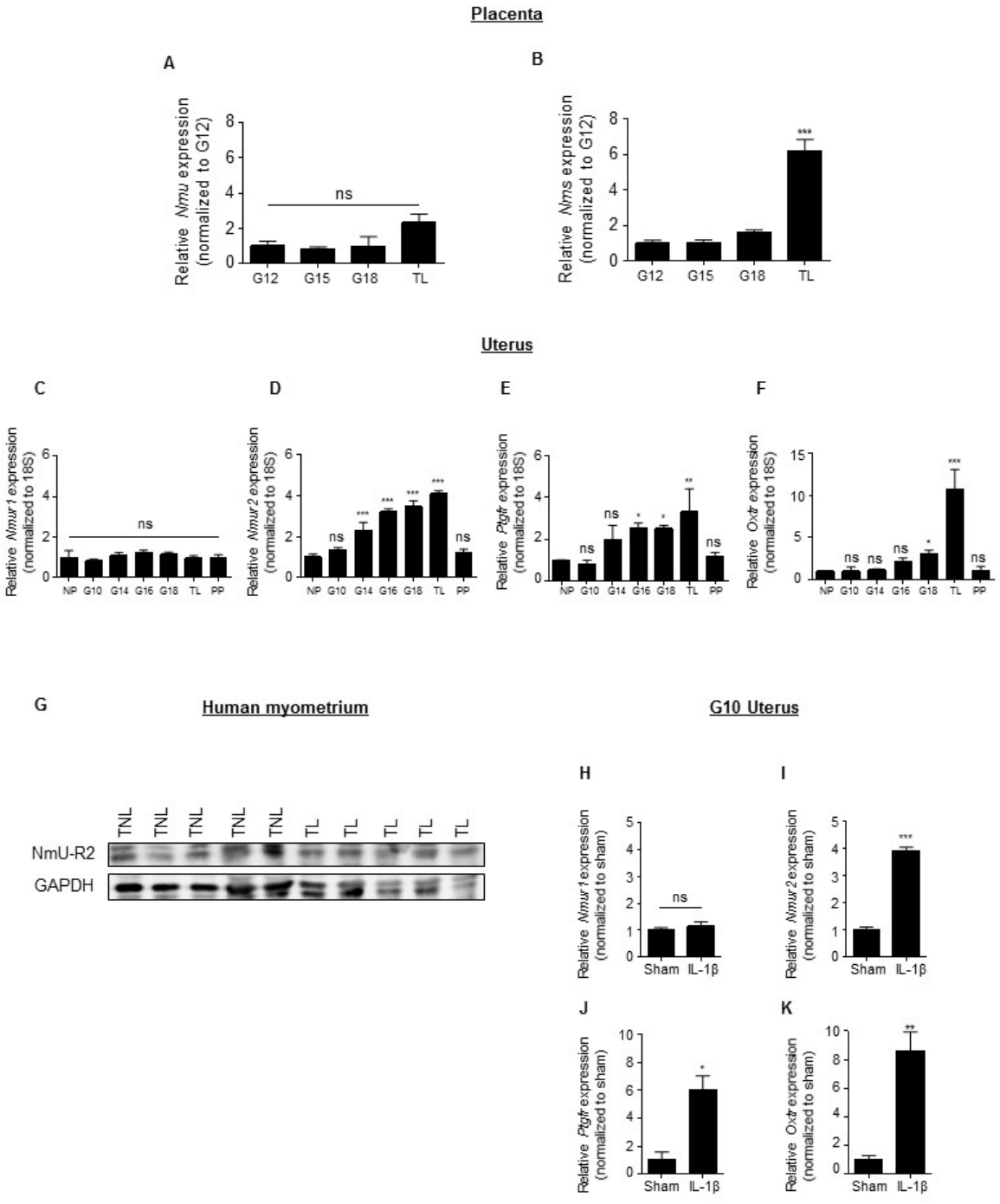

Placenta
A
Relative Nmu expression (normalized to G12)
ns
G12 G15 G18 TL
B
Relative Nms expression (normalized to G12)
***
G12 G15 G18 TL
Uterus
C
Relative Nmur1 expression (normalized to 18S)
ns
NP G10 G14 G16 G18 TL PP
D
Relative Nmur2 expression (normalized to 18S)
E
Relative Ptgfr expression (normalized to 18S)
F
Relative Oxtr expression (normalized to 18S)
G
Human myometrium
TNL TNL TNL TNL TNL TL TL TL TL TL
NmU-R2
GAPDH
G10 Uterus
H
Relative Nmur1 expression (normalized to sham)
Sham IL-1β
I
Relative Nmur2 expression (normalized to sham)
J
Relative Ptgfr expression (normalized to sham)
K
Relative Oxtr expression (normalized to sham)

Figure S3

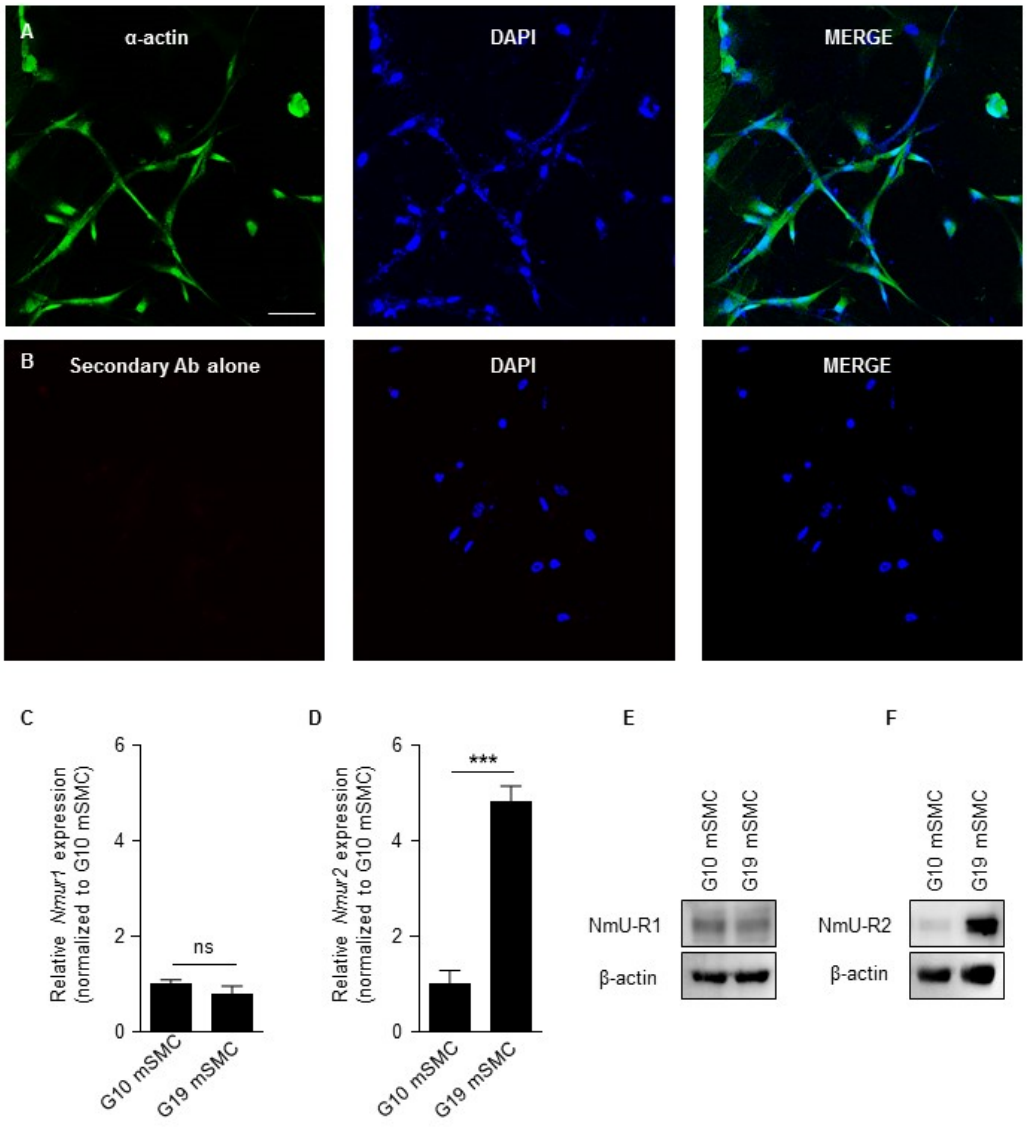
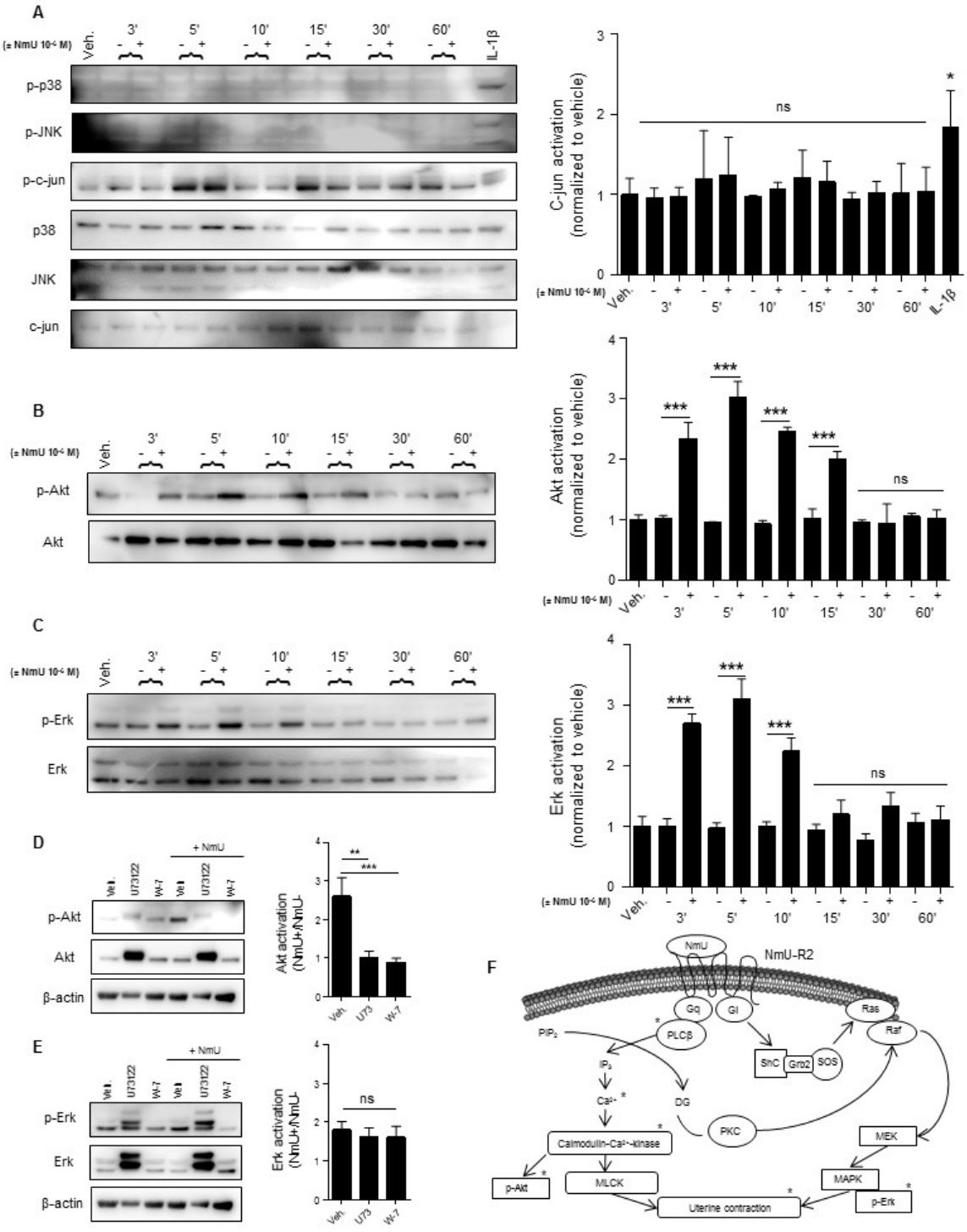

A
α-actin
DAPI
MERGE
B
Secondary Ab alone
DAPI
MERGE
C
Relative Nmur1 expression (normalized to G10 mSMC)
6
4
2
0
ns
G10 mSMC
G19 mSMC
D
Relative Nmur2 expression (normalized to G10 mSMC)
6
4
2
0
***
G10 mSMC
G19 mSMC
E
G10 mSMC
G19 mSMC
NmU-R1
β-actin
F
G10 mSMC
G19 mSMC
NmU-R2
β-actin

Figure S4


A
(± NmU 10⁻⁶ M)
Veh.
3'
5'
10'
15'
30'
60'
IL-1β
p-p38
p-JNK
p-c-jun
p38
JNK
c-jun
C-jun activation (normalized to vehicle)
ns
*
B
p-Akt
Akt
Akt activation (normalized to vehicle)
***
ns
C
p-Erk
Erk
Erk activation (normalized to vehicle)
D
+ NmU
U73122
W-7
β-actin
Akt activation (NmU+/NmU-)
**
E
Erk activation (NmU+/NmU-)
F
NmU
NmU-R2
Gq
Gi
Ras
Raf
PLCβ
PIP2
IP3
DG
Ca2+
SHC
Grb2
SOS
PKC
MEK
Calmodulin-Ca2+-kinase
MAPK
p-Akt
MLCK
p-Erk
Uterine contraction

## Tables

Table I: Sequences used for the design of shRNA against *Nmur2*

| **shRNA against *Nmur2* sequences** | |
|---|---|
| **Clone ID** | **Sequences** |
| TRCN0000026236 | TAAGTTGGGTGTTGTGGATGG |
| TRCN0000026273 | TATACACCCATATGGGTTTGG |
| TRCN0000026279 | AACAGGAAGGGATAATTGTGC |
| TRCN0000026293 | TTTAGCCCTGATTTAGAGAGC |
| TRCN0000026323 | ATTCACAGTCACTTTGTCTGC |

Table II: Primers used for Real-Time qPCR

| **Mouse primers** | |
|---|---|
| *Nmu*-F: CACGCTGAGGGACAGCTAAA | *Nmu*-R: TATTGGCACACCTTTGCAAGC |
| *Nms*: CCAACCTAAGGAAAACCAGGATG | *Nms*-R: GATGGACCGGAGCAAACTCA |
| *Nmur1*-F: TGACTACCGCACTGCTCTTC | *Nmur1*-R: ACAACCAGTGCAAACAGCATC |
| *Nmur2*-F: TACATCCTCCCGATGACCCT | *Nmur2*-R: CACGAGGACCAAGACAAACAG |
| *Ptgfr*-F: AGCTGGACTCATCGCAAACA | *Ptgfr*-R: GTGGGCACAAGCCAGAAAAG |
| *Oxtr*-F: TGTGTCTCCTTTTGGGACAA | *Oxtr*-R: GGCATTTCAGAATTGGCTGT |

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

| Substance injected (doses per injection) | Gestational days of injections (number of mice) | Gestational age at delivery ± S.D. (Term delivery = 19.2) |
|---|---|---|
| NmU (160µg/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 * |
| $PGF_{2\alpha}$ (160µg/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 * |