Author/s:
Liong, S;Lim, R;Nguyen-Ngo, C;Barker, G;Parkington, HC;Lappas, M

Title:
The immunoproteasome inhibitor ONX-0914 regulates inflammation and expression of contraction associated proteins in myometrium

Date:
2018-08-01

Citation:
Liong, S., Lim, R., Nguyen-Ngo, C., Barker, G., Parkington, H. C. & Lappas, M. (2018). The immunoproteasome inhibitor ONX-0914 regulates inflammation and expression of contraction associated proteins in myometrium. European Journal of Immunology, 48 (8), pp.1350-1363. https://doi.org/10.1002/eji.201747458.

The immunoproteasome inhibitor ONX-0914 regulates inflammation and expression of contraction associated proteins in myometrium

Stella Liong[1,2], Ratana Lim[1,2], Caitlyn Nguyen-Ngo[1,2], Gillian Barker[1,2], Helena C Parkington[3], and Martha Lappas[1,2]

[1]*Obstetrics, Nutrition and Endocrinology Group, Department of Obstetrics and Gynaecology, University of Melbourne, Victoria, Australia*

[2]*Mercy Perinatal Research Centre, Mercy Hospital for Women, Heidelberg, Victoria, Australia*

[3]*Department of Physiology and Biomedicine Discovery Institute, Monash University, Victoria, Australia*

Correspondence:

Associate Professor Martha Lappas

Department of Obstetrics and Gynaecology, University of Melbourne

Mercy Hospital for Women, Level 4/163 Studley Road

Heidelberg, 3084, Victoria, Australia

Ph: 61-3-8458 4370; Fax: 61-3-8458 4380

E-mail: mlappas@unimelb.edu.au

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/eji.201747458.

Keywords: immunoproteasome; LMP7; human labour; myometrium; inflammation

ABSTRACT

There is currently no effective treatments to prevent spontaneous preterm labour. The precise upstream biochemical pathways that regulate the transition between uterine quiescence during pregnancy and contractility during labour remain unclear. It is well known however that intrauterine inflammation, including infection, is commonly associated with preterm labour. In this study, we identified the immunoproteasome subunit low-molecular-mass protein (LMP)7 mRNA expression to be significantly upregulated in labouring human myometrium. Silencing LMP7 using siRNA targeted knockdown of LMP7 and its inhibitor ONX-0914 in human myometrial cells and tissues decreased pro-inflammatory cytokines (IL-6), cell chemotaxis (CXCL8, CCL2 expression and THP-1 migration), cell to cell adhesion (ICAM1 expression and myometrial adhesion), contraction-associated proteins (PTGS2, FP, PGE2 and PGF2α), as well as suppressing contractions in myometrial cells and in myometrial tissues obtained from labouring women. In addition, LMP7 silencing reduced NF-κB RelA activity. ONX-0914 alleviated inflammation (CCL3, CXCL1, PTGS2, and IL-6) in myometrium, placenta, fetal brain, amniotic fluid and maternal serum induced by LPS in pregnant mice. Collectively, our data suggest a novel role for ONX-014 to suppress uterine activation and contractility associated with preterm labour.

Treatment with ONX-0914 or LMP7 siRNA knockdown in myometrium *in vitro* reduces inflammation, cell adhesion and contractions. ONX-0914 treatment in pregnant mice reduces LPS-induced myometrial and fetal brain inflammation *in utero*.

INTRODUCTION

Preterm birth (< 37 weeks gestation) affects approximately 15 million babies globally each year [1], and is associated with severe neonatal morbidity and mortality rates [2]. Birthing outcome data collected between 1990-2010 in the United States have shown that, despite considerable medical advancements in materno-fetal care, the incidence of preterm birth has not diminished and remains high [3]. Although survival rates of preterm birth has improved in recent times, premature infants are at increased risk of infection [4] and long-term developmental disabilities [5] compared with their term delivered counterparts. Of clinical significance, 40% of preterm births are a result of infection-induced preterm labour [6]. There is strong evidence that bacterial and viral infections, which are responsible for the majority of spontaneous preterm birth, play a central role in regulating inflammation and the production of pro-labour mediators [7].

Although the precise mechanisms of labour onset remain largely unknown, pro-inflammatory cytokines and chemokines, cyclooxygenase-2 (PTGS2), prostaglandins, and NF-κB play an important role [8-12]. During infection, the host immune response is initiated via recognition of pathogen-associated molecular patterns (PAMPs) by toll-like receptors (TLRs) [13]. Activation of TLRs upregulates the expression of pro-labour effectors via the NF-κB signalling pathway [14]. The bacterial products lipopolysaccharide (LPS), a TLR4 ligand, and flagellin, a TLR5 ligand, enhance the production of various pro-inflammatory cytokines and pro-labour mediators via the NF-κB signalling pathway in human myometrium [14]. Similarly, activation of TLR3 by the viral dsRNA analogue polyinosinic polycytidilic acid (poly (I:C)) induces pro-labour mediators in human myometrium [15]. Excessive intrauterine inflammation has also been shown to be a strong indicator of poor perinatal neurological

outcomes among preterm children, such as cerebral palsy, cognitive disabilities and developmental delays [5]. Studies on mice models of inflammation-induced preterm birth has been shown to induce altered neuronal morphology and increased expression of inflammation and markers of neuronal damage in the fetal brain [16].

Proteasomes are large multi-subunit protein complexes responsible for the majority of non-lysosomal proteolysis occurring in cells [17]. Structurally, proteasomes are comprised of 14 distinct subunits which are subdivided into α subunits (α1–α7) and β subunits (β1–β7). Distinct inducible proteasome subunits can, however, replace components of the constitutively expressed proteasome to form what has been termed the 'immunoproteasome' [17]. There is strong evidence that the inducible immunoproteasome subunit, low-molecular-mass protein LMP7, can increase cytokine production by macrophages, T cells, and other immune cell types [18] and can be induced following exposure to certain inflammatory cytokines [19]. Recent studies have shown that in vivo administration of the LMP7 inhibitor ONX-0914, attenuates the progression of experimental colitis [20], arthritis [18] and autoimmune encephalomyelitis [21].

LMP7 has been detected in human placenta [22], and expression of LMP7 mRNA can be induced by IFN-β, IFN-γ and TNF in human placental choriocarcinoma JEG-3 cells [22]. However, the role of LMP7 in regulating inflammation associated with preterm birth has yet to be described. Thus, we first sought to characterise the labour-associated changes in LMP7 expression in myometrium. Second, we determined the effect of ONX-0914 treatment and siLMP7 knockdown on pro-inflammatory cytokines, leukocyte chemotaxis and cell adhesion in human myometrium. Third, contraction associated proteins (CAPs) expression and myometrial contraction studies were

performed to determine ONX-0914 as a potential tocolytic. Finally, the effect of LMP7 on uterine and fetal brain inflammation in a mouse model of LPS-induced inflammation was assessed.

RESULTS

LMP7 expression is upregulated in labouring myometrium

To determine LMP7 expression in myometrium during labour, qRT-PCR was performed (Figure 1). LMP7 mRNA expression was significantly elevated in myometrial tissue obtained from term labouring women compared to term non-labouring women at time of Caesarean section delivery.

ONX-0914 treatment and siLMP7 knockdown reduces pro-inflammatory cytokines in human myometrium

To determine if LMP7 was involved in the genesis of pro-inflammatory and pro-labour mediators in human myometrium, loss-of-function studies were carried out using the LMP7 inhibitor ONX-0914 and LMP7 silencing using siRNA. For the explant studies, LPS (TLR4 ligand) and flagellin (TLR5 ligand) was used to stimulate the production of pro-inflammatory cytokines, chemokines in the context of infection mediated preterm labour. As shown in Figure 2, LPS significantly increased IL-6 mRNA expression and release. Pre-incubation with ONX-0914 significantly suppressed LPS-induced IL-6 mRNA expression and release (Figures 2A,B). ONX-0914 also significantly reduced IL-6 mRNA expression and secretion in myometrium stimulated with flagellin (Supplementary Table 1).

The effect of LMP7 inhibition on pro-cytokines and chemokines was confirmed using siRNA silencing of LMP7 in primary myometrial cells. The efficacy of siLMP7 is demonstrated in Supplementary Figure 2; there was a 60% decrease in LMP7 mRNA expression and a 70% decrease in LMP7 protein expression. For subsequent experiments, after siRNA transfection, cells were treated with IL-1β and

TNF, flagellin, and poly(I:C); the data for IL-1β is presented in Figure 2. As expected, in siCONT transfected myometrial cells, IL-1β induced a significant increase in IL-6 mRNA expression (Figure 2C) and release (Figure 2D). In siLMP7 transfected cells, this increase in IL-6 mRNA expression and release was significantly reduced (Figures 2C,D). Similar results were obtained in myometrial cells stimulated with TNF, flagellin or poly(I:C) (Supplementary Table 2).

ONX-0914 treatment and siLMP7 knockdown reduces cell chemotaxis in myometrium

We next sought to assess whether ONX-0914 and siLMP7 knockdown alters myometrial chemokine production and cell migration. In myometrium, ONX-0914 significantly decreased LPS-induced protein expression of CXCL8 but not CCL2 (Figures 3A,B). ONX-0914 had no effect on LPS-induced CXCL8 or CCL2 secretion (Figures 3E,F). ONX-0914 significantly reduced CXCL8 mRNA expression and secretion in myometrium stimulated with flagellin (Supplementary Table 1).

In siCONT transfected myometrial cells, IL-1β significantly induced CXCL8 and CCL2 mRNA expression (Figures 3C,D) and release (Figures 3G,H). In siLMP7 transfected cells, this increase in CXCL8 mRNA expression and release was significantly reduced (Figures 3C,G). LMP7 siRNA knockdown significantly reduced IL-1β-induced CCL2 release (Figure 3D), however there was no change in mRNA expression (Figure 3H). Similar results were obtained for CXCL8 in myometrial cells stimulated with TNF, flagellin or poly(I:C) (Supplementary Table 2).

Decreased secretion of chemokines suggests that LMP7 inhibits secretion of factors that induce leukocyte recruitment to myometrial cells. Transwell migration assays were performed to assess whether LMP7 blockade by ONX-0914 could inhibit leukocyte recruitment to myometrial cells. Conditioned medium collected from myometrial cells stimulated with IL-1β (Figure 3I) or TNF (Figure 3J) significantly induced THP-1 monocyte migration (424% and 183% compared with basal,

respectively). In comparison, conditioned medium from myometrial cells co-treated with ONX-0914 significantly reduced IL-1β- and TNF-induced THP-1 migration by 29% and 45%, respectively (Figures 3I,J). This suggest ONX-0914 inhibits secretion of factors that induce leukocyte recruitment to myometrial cells.

ONX-0914 treatment suppresses myometrial cell adhesion

Secretion of pro-inflammatory cytokines and chemokines by myometrial cells can induce the attraction and chemotaxis of migratory leukocytes resulting in the localised augmentation of myometrium inflammation [23]. Expression of cell adhesion molecules such as ICAM1 have been shown to be upregulated in labouring human myometrium [24]. Thus, we next sought to assess the effect of ONX-0914 on myometrial cell adhesion. As expected, LPS significantly elevated ICAM1 mRNA expression in myometrium (Figures 4A). Treatment with ONX-0914 significantly reduced ICAM1 mRNA expression induced by LPS (Figure 4A).

Cell adhesion assays were next performed to assess the effect of ONX-0914 on myometrial cell adhesion. IL-1β and TNF increased myometrial cell adhesion compared to basal by 34% and 27%, respectively (Figures 4B,C). ONX-0914 significantly reduced myometrial cell adhesion induced by IL-1β or TNF by 26% and 30%, respectively (Figures 4B,C).

ONX-0914 treatment and siLMP7 knockdown suppresses contractions in human myometrium

To determine the potential usefulness of ONX-0914 as a tocolytic for preterm labour, we assessed the effect of ONX-0914 on uterine contractility. Increased expression of contraction associated proteins such as PTGS2, leads to increased prostaglandin production which induces uterine contractions via the $PGF_{2\alpha}$ receptor (FP). In myometrial tissues explants, ONX-0914 significantly inhibited LPS-induced PTGS2 and FP mRNA expression (Figures 5A,B) and $PGF_{2\alpha}$ and PGE_2 secretion

(Figures 5C,D). Similar results were obtained in myometrium stimulated with flagellin (Supplementary Table 1). In addition, LMP7 siRNA knockdown in myometrial cells showed a significantly reduced PTGS2 and FP mRNA expression (Figures 5E,F) and $PGF_{2\alpha}$ and PGE_2 secretion secretion (Figures 5G,H) induced by IL-1β. Similar results by siLMP7 were obtained in TNF-, flagellin- and poly(I:C)-treated myometrial cells (Supplementary Table 2).

Collagen gel contraction assays were performed to assess whether ONX-0914 could inhibit myometrial cell contraction (Figures 5I,J). As expected, stimulation of primary human myometrial cells with IL-1β or TNF induced contraction of the collagen gels (as indicated by the reduced gel area) after 24 h (17% and 32% decrease, respectively). This IL-1β- and TNF-induced contraction was significantly reversed in cells treated with ONX-0914 (24% and 20% increase compared to IL-1β and TNF respectively).

Following on from our in vitro findings, we next sought to determine whether ONX-0914 could suppress contractions in myometrium from laboring women ex vivo. As shown in Figure 5K, exposure to oxytocin induced a concentration-dependent increase in contractility in uterine smooth muscle strips obtained from women in labour. Pre-treatment with ONX-0914 significantly suppressed the maximum oxytocin-induced contractility in paired strips from the same women, from $79 \pm 9\%$ to $48 \pm 9\%$. There was no change in the sensitivity to oxytocin (pD_2 8.88 versus 8.83).

siLMP7 knockdown regulates NF-κB RelA transcriptional activity

There is extensive evidence to show that the classical NF-κB pathway is an important regulator of the terminal effector pathways of labour and delivery [10-12]. However, there have been conflicting reports on the role of immunoproteasome subunits to affect NF-κB activity differently from the constitutive proteasome subunits [25-28]. Thus, to determine whether LMP7 regulates pro-labour

mediators in primary myometrial cells through NF-κB, we utilised a luciferase activity assay. As shown in Figure 6, siLMP7 significantly decreased NF-κB luciferase activity in myometrial cells stimulated with IL-1β, TNF, flagellin or poly(I:C).

ONX-0914 treatment decreases inflammation *in vivo* in a mouse model of LPS-induced preterm birth

Given the potent anti-inflammatory properties of ONX-0914 in vitro, we next sought to determine the efficacy of ONX-0914 in suppressing inflammation in vivo. For these studies, pregnant mice at GD 15.5 were intraperitoneally injected with either saline (vehicle control), LPS or LPS with ONX-0914 at 10 mg/kg body weight. After 6 h, maternal serum and amniotic fluid were collected and circulating levels of pro-inflammatory cytokines were measured using a mouse multiplex assay. Myometrium and fetal brains were also harvested after 6 h and assessed for mRNA expression of pro-inflammatory markers. As shown in Figure 7, LPS significantly increased IL-1α, IL-1β, CCL3, CXCL1 and PTGS2 mRNA expression in mouse myometrium. ONX-0914 had no effect on LPS-induced IL-1α and IL-1β mRNA expression (Figures 7A,B). In contrast, ONX-0914 significantly decreased LPS-induced CCL3, CXCL1 and PTGS2 mRNA expression induced in mouse myometrium (Figures 7C-E). In mouse placenta, LPS induced IL-1α and IL-1β mRNA expression was subsequently reversed with ONX-0914 treatment (Supplementary Figure 3). In fetal brain, LPS-induced CXCL1 and CCL3 mRNA expression was also reversed following ONX-0914 treatment (Figures 7F,G). In maternal serum and amniotic fluid, ONX-0914 significantly decreased LPS-induced IL-6 levels (Figures 7H,I). TNF, IL-2 and IL-4 levels in maternal and amniotic fluid were below the detection limit of the assay.

DISCUSSION

To date, there has been no molecular target that is effective in preventing intrauterine inflammation and myometrial contractions associated with preterm labour and delivery. This is further confounded by our lack of understanding in the mechanisms involved in transitioning the myometrium from a quiescent state during pregnancy to a contractile state during labour. In this study, we report that myometrial LMP7 expression is upregulated with human labour. We found that silencing LMP7 as well as treatment with ONX-0914 significantly decreased pro-inflammatory cytokines, chemotaxis, cell adhesion and contraction in myometrium exposed to bacterial and viral products and pro-inflammatory cytokines We have also found that LPS-induced pro-inflammatory and pro-labour mediators in maternal and fetal tissues was significantly alleviated in GD 15.5 pregnant mice administered with ONX-0914. Thus, our data demonstrates an important role of the immunoproteasome as an upstream regulator of pro-inflammatory mediators and contraction-associated proteins in human myometrium and resulting in the myometrial activation and preterm labour.

Over the last few decades, in vitro and in vivo studies have shown how inflammation triggered by infection or by sterile pro-inflammatory insults, originating either locally or systemically, can induce preterm birth [9, 14, 15, 23, 29-31]. This leads to activation of the maternal immune system which produces pro-inflammatory cytokines and chemokines [24, 32]. Chemokines activate maternal peripheral leukocytes and induce their infiltration into uterine tissue. This is facilitated by the action of cellular adhesion molecules such as ICAM1. The leukocytes that have infiltrated are a rich source of cytokines which in turn leads to further production of cytokines and chemokines plus the production of CAPs such as PTGS2 and the resultant generation of uteronics such as the prostaglandin PGF2α, involved in the onset of labour. Thus, to assess the role of ONX-0914 on

inflammation in myometrium, we performed LMP7 loss-of-function studies using siRNA or ONX-0914, a chemical inhibitor of LMP7. In our study, LMP7 inhibition with ONX-0914 significantly reduced LPS-induced IL-6 mRNA expression and secretion in myometrial tissue. In contrast, although LMP7 siRNA knockdown induced a small reduction in LPS-induced IL-6 mRNA expression in myometrial cells, IL-6 secretion was markedly reduced in siLMP7 transfected cells. ONX-0914 treatment and siLMP7 knockdown also reduced CXCL8 and CCL2 in myometrium. Of note, although IL-1β-induced CCL2 secretion was reduced following LMP7 siRNA knockdown, there was no significant reduction on CCL2 mRNA expression. This suggests that the mechanism which LMP7 regulates CCL2 secretion may not be through regulating mRNA transcription. Evidence for transcriptional control of IL-1β-induced CCL2 production has also been demonstrated by other studies. For example, CCL2 production in human myometrial cells by prostaglandin PGE2 significantly suppressed IL-1β-induced CCL2 secretion with no change in CCL2 mRNA expression. Although beyond the scope of this study, further investigations is warranted on the mechanism of which IL-1β induces CCL2 production in myometrial cells. It is thought that IL-6 and CXCL8 may potentiate myometrial contractility. For example, IL-6 increases myometrial responsiveness to oxytocin by up-regulating expression of oxytocin receptors [33], whereas CXCL8 induces neutrophil chemotaxis and activation [34]. In addition, we have shown ONX-0914 to significantly reduce ICAM1 expression, as well as reducing IL-1β- and TNF-induced adhesion properties in myometrial cells. Co-treatment of ONX-0914 with IL-1β or TNF in myometrial cells significantly decreased THP-1 monocyte chemotaxis. Taken together, these findings suggest that ONX-0914 can impair leukocyte recruitment and infiltration resulting in reduced inflammation to the myometrium.

PTGS2 is the enzyme responsible for labour-associated increase in prostaglandin (PGE_2 and $PGF_{2\alpha}$) synthesis. FP is the receptor that mediates the biological actions of $PGF_{2\alpha}$ to induce uterine contractions that can result in preterm birth in humans [9]. In our study, siLMP7 knockown

significantly reduced PTGS2 and FP mRNA expression and prostaglandin (PGE_2 and $PGF_{2\alpha}$) release in myometrium. ONX-0914 treatment significantly decreased the magnitude of oxytocin-induced contractions. The suppression of contraction was not associated with a change receptor sensitivity (pD_2 remained unchanged). This could reflect an effect on oxytocin receptor signalling, rather than a change in oxytocin receptor density. ONX-0914 treatment also reversed the effect of IL-1β- and TNF-induced collagen gel contraction in myometrial cells in vitro. Taken together, treatment with ONX-0914 and siLMP7 knockdown reduces the expression of these contraction-associated proteins and suppresses myometrial cell contractility. Thus, we propose that LMP7 in human myometrium could be a tocolytic target to suppress preterm labour.

Multiple studies in human gestational tissues have shown that expression of pro-labour mediators such as pro-inflammatory cytokines and prostaglandins are regulated by NF-κB [10-12]. Moreover, labour is associated with increased NF-κB nuclear translocation in the cervix, fetal membranes and myometrium [35-37]. The role of immunoproteasome subunits to modulate NF-κB in non-gestational tissue has been controversial. For example, human lymphocytes lacking LMP7 also demonstrated reduced NF-κB activation and were highly susceptible to apoptosis when stimulated with TNF [38]. Experimental colitis was also attenuated in LMP7 knockout mice as a result of reduced NF-κB signalling [39]. In contrast, other studies have found no influence of the immunoproteasome on the canonical pathway of NF-κB activation [27, 28, 40]. Thus, it was of interest to determine if LMP7 may be regulating pro-inflammatory and pro-labour mediators through NF-κB in human myometrium. To do this, we performed a luciferase assay to measure NF-κB activity. We found siRNA knockdown of LMP7 significantly diminished NF-κB RelA transcriptional activity when stimulated with IL-1β and TNF or the TLR ligands flagellin or poly(I:C). Thus, we propose that the pro-

inflammatory actions of LMP7 in human myometrium may be mediated via the canonical NF-κB pathway.

Recent *in vivo* studies have reported that treatment with ONX-0914 attenuated the progression of various autoimmune diseases including rheumatoid arthritis, multiple sclerosis, Hashimoto's thyroiditis, inflammatory bowel disease and lupus in experimental mice models [18, 21, 41, 42]. Thus, we used an in vivo approach to assess the effect of ONX-0914 on LPS-induced inflammation in pregnant mice. In agreement with our in vitro findings, ONX-0914 significantly reduced LPS-induced CCL3, CXCL1 and PTGS2 mRNA expression in myometrium, as well as decreasing LPS-induced IL-6 levels in maternal plasma and amniotic fluid in pregnant mice after 6 h post i.p. administration of LPS. There is also strong in vivo evidence using mouse models of intrauterine inflammation which showed intrauterine infusion of LPS increased IL-10, IL-1β, TNF and IL-6 mRNA expression in whole fetal brain and also disrupted fetal neuronal morphology and growth, whereas in non-inflammatory models of preterm birth using RU486, there was no adverse effect on fetal brain development or neuroinflammation in mice offspring [16]. In our study, LPS-induced CXCL1 and CCL3 mRNA expression in fetal brain was significantly decreased in pregnant mice administered ONX-0914. These findings are of particular clinical significance given that adverse neurological outcomes are associated with preterm birth in infants, resulting in long-term behavioural, social and learning disabilities in childhood and adolescence [43]. Thus, ONX-0914 may be useful in alleviating and preventing inflammation-induced fetal brain injury associated with preterm birth.

Although we have shown that ONX-9014 significantly reduces myometrial inflammation and contractions, a significant limitation of this study was that we did not confirm ONX-0914 at the concentration used (0.5 μM) to selectively inhibit LMP7 incorporation into the immunoproteasome complex in myometrial tissues. Therefore, we are unable to rule out the effects observed by ONX-

0914 to be strictly caused by LMP7 blockade and are not contributed by off-target effects by also inhibiting the constitutive beta 5 subunit. Previous studies have shown ONX-0914 at 0.5 µM concentration will also inhibit the constitutive beta 5 subunit in cells. Although penetration of chemical inhibitors into cells versus tissues are not directly comparable, our findings with ONX-0914 in myometrial tissue should be interpreted with caution. Further studies using beta 5 and LMP7 selective fluorogenic substrates (Ac-WLA-AMC and Ac-ANW-AMC, respectively) or using a specific assay to confirm that beta 5 is not inhibited will be required to determine the specificity of ONX-0914 at 0.5 µM in myometrium tissue. Performing these assays will clarify the mechanism of ONX-014 action to alleviate inflammation. Notwithstanding this limitation, our LMP7 siRNA data in myometrial cells does support similar modulation of the inflammatory response with ONX-0914 in myometrial tissue. Another limitation of this study was that although we found LMP7 mRNA expression to be significantly upregulated in term labouring human myometrium, we were unable to accurately measure LMP7 protein expression by Western blotting (data not shown). Our attempts to measure LMP7 protein expression by Western blotting resulted in the detection of multiple bands which could be reflective of the LMP7 subunit at different stages of processing and maturation (i.e. precursor, intermediate and mature forms). Studies have shown that the mature but not the precursor form of LMP7 is incorporated into the 20S proteasome complex [44]. Therefore, more studies will be required to determine whether our observation of increased LMP7 mRNA expression in labouring myometrium is reflective of increased LMP7-subunit-containing proteasome complexes.

Sterile inflammation- and/or infection-mediated inflammation is a major causative factor of spontaneous preterm labour [8, 45]. Therefore, understanding the upstream mechanisms involved in modulating inflammation in gestational tissues is crucial in the development of interventions to delay spontaneous preterm delivery and to improve fetal outcomes. The studies presented here demonstrate a use for ONX-0914 to prevent preterm labour. Notably, pre-clinical trials are in

development to evaluate the potential clinical applications of ONX-0914 in treatment of autoimmune disorders, such as rheumatoid arthritis, lupus and inflammatory bowel disease [46]. Other next-generation of LMP7 inhibitors have recently been developed (e.g. KZR-616, PR-924 and ONX-0912) and these have the added benefit of being more selective and potent in their inhibition of LMP7 than ONX-0914 [47-49]. Thus, future studies are required to assess these new inhibitors as potential tocolytic drugs for women who would otherwise deliver preterm.

MATERIALS AND METHODS

Tissue collection for expression studies

The Research Ethics Committee of Mercy Hospital for Women, Mercy Health, approved this study. Written, informed consent was obtained from all participating women. All tissues were obtained from women who delivered healthy, singleton infants. Tissues were brought to the research laboratory and processed within 15 mins of delivery. Women with any underlying medical conditions such as diabetes, asthma, polycystic ovarian syndrome, preeclampsia and macrovascular complications were excluded. Additionally, women with evidence of an active infection, multiple pregnancies, obese women, fetuses with chromosomal abnormalities were excluded.

For expression studies by qRT-PCR, myometrium was obtained from women at the time of term caesarean delivery (≥37 weeks gestation). Myometrial biopsies were collected from two groups of women: (i) pregnant women undergoing elective Caesarean section in the absence of labour (n=8 patients; mean gestational age 39.4±0.3 weeks), and (ii) pregnant women who were delivered during active labour; labour was defined as the presence of regular uterine contractions (every 3–4

min) resulting in cervical effacement and dilation (n=8 patients; mean gestational age 39.8±0.2 weeks). A myometrial biopsy was obtained from the upper margin of the lower uterine segment incision during Caesarean section. There was no difference in maternal age and body mass index, parity, or gestational age of the patients recruited. In the labouring group, none of the patients received any medications to augment or induce labour, and the average length of labour was 10 h ± 6 h 40 min. Tissue samples were snap frozen in liquid nitrogen and immediately stored at -80^{o}C for analysis by qRT-PCR as detailed below.

Tissue explant culture

Tissue explants were performed to determine the effect of the LMP7 inhibitor ONX-0914 on pro-labour mediators in myometrium treated with the bacterial products LPS derived from E. coli strain 02:6B6 (Sigma-Aldrich; St. Louis, MO) or flagellin (Life Research; Scoresby, Vic, Australia). Tissue explants were performed as previously described [30] on myometrium obtained from non-labouring women at the time of term Caesarean section. Exclusions criteria for these studies are as detailed for the expression studies above. Briefly, fresh myometrium (collected as detailed above) were placed in DMEM at 37^{o}C in a humidified atmosphere of 21% O_2 and 5% CO_2 for 1 h. Tissues were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (50 mg wet weight per well). The explants were incubated in 1 ml DMEM containing 100 U/ml penicillin G and 100 µg/ml streptomycin. Tissues were incubated in the absence or presence of 0.5 µM ONX-0914 for 60 min before the addition of 10 µg/ml LPS or 1 µg/ml flagellin for 20 h. After final incubation, tissue and media were collected separately and stored at -80^{o}C for further analysis as detailed below. The concentrations of ONX-0914 were based on previous studies [18] and an initial dose response (Supplementary Figure 1). Experiments were performed on myometrium obtained from six patients at term gestation.

Knockdown of LMP7 with siRNA in primary myometrial cells

Primary myometrial cells were used to investigate the effect of siRNA-mediated silencing of LMP7 on pro-inflammatory and pro-labour mediators. Term myometrium was obtained in the absence of labour and cells were isolated and cultured as previously described [50]. Cells between passages 3 and 8 were used for experiments. At approximately 50% confluence, cells were transfected using Lipofectamine 3000 according to manufacturer's guidelines (Life Technologies; Mulgrave, Victoria, Australia) and as we have previously described [51]. LMP7 siRNA (siLMP7) was obtained from Integrated DNA Technologies (IDT; Coralville, IA, USA), and the negative control siRNA (siCONT) was obtained from Sigma (Sigma-Aldrich). Cells were transfected with 200 nM siLMP7 or siCONT in DMEM/F-12 for 48 h. The medium was then replaced with DMEM/F-12 (containing 0.5% (w/v) BSA) with or without 1 ng/ml IL-1β, 10 ng/ml TNF, 1 μg/ml flagellin or 5 μg/ml poly(I:C) and the cells were incubated at 37°C for an additional 20 h. After final incubation, cells and media were collected separately and stored at -80^{o}C for further analysis as detailed below. Cell viability was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) proliferation assay. Experiments were performed from myometrium obtained from five patients.

NF-κB RelA luciferase assay

A luciferase assay was utilised to determine possible interactions between LMP7 and NF-κB, as previously described [51]. Primary myometrial cells, prepared as described above, at approximately 50% confluence, were transfected with 0.1 μg NF-κB RelA reporter construct (Qiagen; Chadstone Centre, Vic, Australia) with FuGENE HD transfection reagent (Promega; Alexandria NSW, Australia). After 6 h, cells were transfected with 200 nM siLMP7 or siCONT (as detailed above) for 48 h. The medium was then replaced with DMEM/F-12 with 0.5% (w/v) BSA, with or without 1 ng/ml IL-1β, 10 ng/ml TNF, 1 μg/ml flagellin or 5 μg/ml poly(I:C), and the cells incubated at 37°C for an additional 20 h. The cells were harvested in lysis buffer, and luminescence activity was measured using a

Luciferase Reporter Assay Kit (Life Research) and Renilla Luciferase Flash Assay kit (Thermo Fisher Scientific; Scoresby, Vic, Australia) as instructed. For each treatment, experiments were performed using myometrium obtained from five patients.

Myometrial cell gel contraction assay

Cell contraction assays were performed as previously described [52] with minor modifications. Primary myometrial cells were re-suspended in 0.25 ml DMEM/F12 (containing 10% FBS) and mixed with 40 µl collagen (3 mg/ml collagen I from rat protein solution; Gibco™) and 1 µl 1 M NaOH by gently pipetting. The mixture was transferred to 48-well tissue culture plates, incubated in 37°C to allow polymerization (approx. 15 min) and then treated with 1 ng/ml IL-1β or 10 ng/ml TNF in the absence or presence of 0.25 µM ONX-0914. The gel matrix was gently detached from the well, incubated for 36 h at 37°C, and the area of the gel was determined using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA). Experiments were performed from myometrium obtained from 5 patients.

Cell adhesion assay

The cell adhesion assay was adapted from [52] and optimised for our studies. Primary myometrial cells were seeded into a 48-well tissue culture plate. At 95-100% cell confluence, media was replaced with DMEM/F-12 containing 0.5% (w/v) BSA, with 1 ng/ml IL-1β in the absence or presence of 0.25 µM ONX-0914 and cells were incubated for 20 h at 37°C. The conditioned medium was retained for the THP-1 cell migration assay. Cells were resuspended in serum-free DMEM/F-12 and then re-seeded into 48-well tissue culture plates pre-blocked with 2% BSA in PBS and incubated for 1h at 37°C. Non-attached cells were washed away with PBS. The remaining attached cells were fixed with 4% paraformaldehyde, stained with crystal violet and counted by absorbance at 560 nm in a

microplate reader (Bio-Rad Laboratories). Experiments were performed using myometrium obtained from five patients.

THP-1 cell migration assays

THP-1 monocyte cells (1× 10^5/well) were suspended in serum-free DMEM/F-12 medium and seeded into 24-well Corning® Transwell® culture plate inserts (Sigma-Aldrich). Conditioned medium retained from the cell adhesion assays were added to the bottom chamber and cells were then incubated at 37°C for 24 h. Cells that had migrated to the bottom chamber were collected and transferred into a 96-well plate. Experiments were performed using conditioned medium obtained from three patients.

Myometrial contractile studies

For these studies, myometrial tissues were obtained from the Royal Women's Hospital, Parkville, Victoria, Australia, with approval of the Royal Women's Hospital Research Ethics Committee. Participants gave informed written consent for collection of myometrial samples before surgery and tissue collection. Women with singleton pregnancies undergoing term (37–40 weeks gestation) elective caesarean delivery following spontaneous labour (≥4cm cervical dilation) (n=4). Exclusion criteria were the same as those described above (Tissue collection). Myometrial smooth muscle contractile studies were conducted as previously described [53]. Briefly, myometrium samples (approximately 2x1.5x1.5cm in tissue size) were obtained from the upper lip of the lower uterine segment during caesarean delivery, immediately placed into HEPES buffered saline and transported to Monash University, and contraction studies were commenced within 1 h. Strips of muscle, ~10×2×2 mm, were cut from the tissue, and placed into wells of a tissue culture plate containing DMEM/F-12, 100 U/ml penicillin G and 100 μg/ml streptomycin, and incubated for 30 min in 5% CO_2 at 37°C. Incubation medium was then removed and replenished with fresh medium and incubated

for another further 30 min. Tissues were then pre-incubated with fresh solution containing 0.5 μM ONX-0914 for 60 min and co-incubated with 10 μg/ml LPS for an additional 14 h. Control tissues included strips exposed to vehicle alone. Following treatment incubation, spontaneous contraction and responses to oxytocin were recorded in the myometrial strips. Each strip was mounted in a 20 ml organ bath filled with physiological saline solution (PSS) containing (in mM): 119 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1 KH_2PO_4, 2.5 $CaCl_2$, 11 glucose, gassed with 95% O_2:5%CO_2 at 35°C. One end of the strip was attached to an isometric force transducer (FT03C, Grass Instruments; MA, USA) and hence to a LabChart 7.3.7 amplifier (ADInstruments; Australia). After 20 min equilibration the strips were exposed to high-potassium PSS (isotonic replacement of NaCl with 100mM KCl) for 5 min in order to elicit a standard contraction [41]. Following a further 30 min equilibration, oxytocin (2 μl aliquots of aqueous stock solutions, 20 min per concentration) was added to the bath in increasing concentrations, 10^{-11} to 10^{-8}M. The area under the contraction curve was analysed (ADInstruments software) and was expressed as a % of the high-potassium contraction.

Mouse model of LPS-induced inflammation

Animal studies were conducted with approval of the Austin Health's Animal Ethics Committee (A2015/05268). Time mated C57BL/6 mice were obtained from the Austin BioSciences Resource Facility on day 12.5 of gestation and allowed to acclimatise for 3 days prior to experiments. They received food and water ad libitum and were on a 12-hour light/dark cycle. Female mice were mated overnight with males and the day of vaginal plug detection was designated gestational day (GD) 0.5 of pregnancy.

The LPS used for this study was isolated from Escherichia coli, serotype 026:B6 (Sigma-Aldrich, St Louis, MO). Based on our studies and by others [29, 54-56], 10–75 μg of LPS per mouse was administered intraperitoneally, with the 50 μg/mouse dose being the final concentration used. The

concentration of ONX-0914 used was 10 mg/kg, based on other studies [18, 21]. On GD 15.5, mice received an intraperitoneal (i.p.) injection (150 µl total volume) of saline (n = 6), LPS (n = 6), or LPS with 10 mg/kg ONX-0914 (n = 6). As ONX-0914 was solubilised in DMSO, equal amounts of DMSO (20% v/v) were included in the injections for control and LPS groups. Six hours after injection, mice were euthanised by cervical dislocation. Myometrium and fetal brain from pups were harvested and flash frozen in liquid nitrogen then stored at −80°C until further analysis by qRT-PCR as detailed below. Maternal serum and amniotic fluid were also collected and stored at −80°C until further analysis by mouse multiplex assay as detailed below.

RNA extraction and quantitative RT-PCR (qRT-PCR)

RNA extractions and qRT-PCR was performed as previously described [31]. RNA quality and integrity were measured using a NanoDrop ND1000 and determined via the A_{260}/A_{280} ratio. RNA (0.5 µg for tissues and 0.2 µg for cells) was converted to cDNA using the Tetro cDNA synthesis kit (Bioline; Alexandria, NSW, Australia) according to the manufacturer's instructions. The RT-PCR was performed using the CFX384 Real-Time PCR detection system (Bio-Rad Laboratories) using 100 nM of pre-designed and validated QuantiTect primers (Qiagen; Chadstone Centre, Vic, Australia). Average gene C_T values were normalised against two housekeeping genes: 14-3-3 protein zeta/delta (YWHAZ) and succinate dehydrogenase complex subunit A (SDHA) (cells); 18S and SDHA (tissue explants); or actin and GAPDH (mice studies). Of note, there was no effect of experimental treatment on the housekeeping genes used. Fold differences were determined using the comparative Ct method.

Cytokine and prostaglandin assays

Assessment of IL-6 and CXCL8 cytokine release was performed using CytoSet™ sandwich ELISA (Life Technologies). Assessment of CCL2 and ICAM1 release was performed using DuoSet™ sandwich ELISA (R&D Systems Inc., Minneapolis, MN). Assessment of PGE_2 and $PGF_{2\alpha}$ secretion was performed

using a competitive enzyme immunoassay kit (Kookaburra Kits from Sapphire Bioscience, NSW, Australia). Assessment of IL-6, TNF, IL-4 and IL-2 cytokine levels in mouse maternal serum and amniotic fluid was performed using the Mouse Cytokine 4-Plex Array according to the manufacturer's instructions (Quansys Biosciences, Logan, UT, USA).

Statistical analysis

All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Student's (unpaired) t-test was used for two sample comparisons. For multiple comparisons, the homogeneity of data was assessed by Bartlett's test, and when significant, the data were logarithmically transformed before further analysis using a repeated measures one-way ANOVA (with LSD post-hoc testing to discriminate among the means). For the myometrial contraction studies (Figure 5G), a sigmoid curve was filled to the concentration-contraction curves using the lease squares method. The concentration of oxytocin that evoked half of a maximal response (EC_{50}), pD_2 ($-\log EC_{50}$) and the maximal response were compared using 2-way ANOVA, followed by Tukey's post hoc testing. Data are expressed as mean $\pm$ SEM, with N as the number of women contributing. Statistical significance was accepted as $P<0.05$.

ACKNOWLEDGEMENTS

The following are gratefully acknowledged: clinical research midwives Genevieve Christophers, Gabrielle Fleming, and Rachel Murdoch for sample collection; and the obstetrics and midwifery staff of the Mercy Hospital for Women for their co-operation. ML is supported by a Career Development Fellowship from the National Health and Medical Research Council (NHMRC; grant no. 1047025) and Research Fellowship from the University of Melbourne. SL was supported by a Glyn White Research Fellowship by the Royal Australian and New Zealand College of Obstetricians and Gynaecologists (RANZCOG) Research Foundation. CNN was supported by an Australian Government Research

Training Program (RTP) Scholarship. This study was supported by the Norman Beischer Medical Research Foundation, The University of Melbourne and the Mercy Research Foundation. Part of this work was supported by the National Health & Medical Research Council (NHMRC) project grants (1058786 to ML 1042478 to HCP).

CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

REFERENCES

1 **March of Dimes, P., Save the Children, WHO,** Born Too Soon: The Global Action Report on Preterm Birth. In **Howson, C. P., Kinney, M. V. and Lawn, J. E.** (Eds.). World Health Organization, Geneva 2012.

2 **Liu, L., Johnson, H. L., Cousens, S., Perin, J., Scott, S., Lawn, J. E., Rudan, I. et al,** Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *Lancet* 2012. **379**: 2151-2161.

3 **Kuehn, B. M.,** Center takes broader look at preterm birth. *JAMA* 2012. **308**: 2448.

4 **Lawn, J. E., Kerber, K., Enweronu-Laryea, C. and Cousens, S.,** 3.6 million neonatal deaths--what is progressing and what is not? *Semin Perinatol* 2010. **34**: 371-386.

5 **Mwaniki, M. K., Atieno, M., Lawn, J. E. and Newton, C. R.,** Long-term neurodevelopmental outcomes after intrauterine and neonatal insults: a systematic review. *Lancet* 2012. **379**: 445-452.

6 **Lawn, J. E., Cousens, S. and Zupan, J.,** 4 million neonatal deaths: When? Where? Why? *Lancet* 2005. **365**: 891-900.

7 **Goldenberg, R. L., Culhane, J. F., Iams, J. D. and Romero, R.,** Epidemiology and causes of preterm birth. *Lancet* 2008. **371**: 75-84.

8 **Christiaens, I., Zaragoza, D. B., Guilbert, L., Robertson, S. A., Mitchell, B. F. and Olson, D. M.,** Inflammatory processes in preterm and term parturition. *J Reprod Immunol* 2008. **79**: 50-57.

9 **Olson, D. M.,** The role of prostaglandins in the initiation of parturition. *Best Pract Res Clin Obstet Gynaecol* 2003. **17**: 717-730.

10 **Lappas, M., Permezel, M., Georgiou, H. M. and Rice, G. E.,** Nuclear factor kappa B regulation of proinflammatory cytokines in human gestational tissues in vitro. *Biol Reprod* 2002. **67**: 668-673.

11 **Lappas, M. and Rice, G. E.,** The role and regulation of the nuclear factor kappa B signalling pathway in human labour. *Placenta* 2007. **28**: 543-556.

12 **Lindstrom, T. M. and Bennett, P. R.,** The role of nuclear factor kappa B in human labour. *Reproduction* 2005. **130**: 569-581.

13 **Vasselon, T. and Detmers, P. A.,** Toll receptors: a central element in innate immune responses. *Infect Immun* 2002. **70**: 1033-1041.
14 **Lim, R., Barker, G. and Lappas, M.,** The TLR2 ligand FSL-1 and the TLR5 ligand Flagellin mediate pro-inflammatory and pro-labour response via MyD88/TRAF6/NF-kappaB-dependent signalling. *Am J Reprod Immunol* 2014. **71**: 401-417.
15 **Liong, S. and Lappas, M.,** The Stress-responsive Heme Oxygenase (HO)-1 Isoenzyme is Increased in Labouring Myometrium where it Regulates Contraction-associated Proteins. *Am J Reprod Immunol* 2015. **74**: 62-76.
16 **Burd, I., Bentz, A. I., Chai, J., Gonzalez, J., Monnerie, H., Le Roux, P. D., Cohen, A. S. et al,** Inflammation-induced preterm birth alters neuronal morphology in the mouse fetal brain. *J Neurosci Res* 2010. **88**: 1872-1881.
17 **Navon, A. and Ciechanover, A.,** The 26 S proteasome: from basic mechanisms to drug targeting. *J Biol Chem* 2009. **284**: 33713-33718.
18 **Muchamuel, T., Basler, M., Aujay, M. A., Suzuki, E., Kalim, K. W., Lauer, C., Sylvain, C. et al,** A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat Med* 2009. **15**: 781-787.
19 **Mishto, M., Ligorio, C., Bellavista, E., Martucci, M., Santoro, A., Giulioni, M., Marucci, G. et al,** Immunoproteasome expression is induced in mesial temporal lobe epilepsy. *Biochem Biophys Res Commun* 2011. **408**: 65-70.
20 **Basler, M., Dajee, M., Moll, C., Groettrup, M. and Kirk, C. J.,** Prevention of experimental colitis by a selective inhibitor of the immunoproteasome. *J Immunol* 2010. **185**: 634-641.
21 **Basler, M., Mundt, S., Muchamuel, T., Moll, C., Jiang, J., Groettrup, M. and Kirk, C. J.,** Inhibition of the immunoproteasome ameliorates experimental autoimmune encephalomyelitis. *EMBO Mol Med* 2014. **6**: 226-238.
22 **Roby, K. F., Yang, Y., Gershon, D. and Hunt, J. S.,** Cellular-distribution of proteasome subunit Lmp7 messenger-RNA and protein in human placentas. *Immunology* 1995. **86**: 469-474.
23 **Sivarajasingam, S. P., Imami, N. and Johnson, M. R.,** Myometrial cytokines and their role in the onset of labour. *J Endocrinol* 2016. **231**: R101-R119.
24 **Ledingham, M. A., Thomson, A. J., Jordan, F., Young, A., Crawford, M. and Norman, J. E.,** Cell adhesion molecule expression in the cervix and myometrium during pregnancy and parturition. *Obstet Gynecol* 2001. **97**: 235-242.
25 **Hayashi, T. and Faustman, D.,** NOD mice are defective in proteasome production and activation of NF-kappaB. *Mol Cell Biol* 1999. **19**: 8646-8659.
26 **Hayashi, T. and Faustman, D. L.,** Development of spontaneous uterine tumors in low molecular mass polypeptide-2 knockout mice. *Cancer Res* 2002. **62**: 24-27.
27 **Kessler, B. M., Lennon-Dumenil, A. M., Shinohara, M. L., Lipes, M. A. and Ploegh, H. L.,** LMP2 expression and proteasome activity in NOD mice. *Nat Med* 2000. **6**: 1064; author reply 1065-1066.
28 **Runnels, H. A., Watkins, W. A. and Monaco, J. J.,** LMP2 expression and proteasome activity in NOD mice. *Nat Med* 2000. **6**: 1064-1065; author reply 1065-1066.
29 **Lim, R., Morwood, C. J., Barker, G. and Lappas, M.,** Effect of silibinin in reducing inflammatory pathways in in vitro and in vivo models of infection-induced preterm birth. *PLoS One* 2014. **9**: e92505.
30 **Lim, R., Barker, G., Wall, C. A. and Lappas, M.,** Dietary phytophenols curcumin, naringenin and apigenin reduce infection-induced inflammatory and contractile pathways in human placenta, foetal membranes and myometrium. *Mol Hum Reprod* 2013. **19**: 451-462.
31 **Lappas, M.,** KLF5 regulates infection- and inflammation-induced pro-labour mediators in human myometrium. *Reproduction* 2015. **149**: 413-424.

32 **Tattersall, M., Engineer, N., Khanjani, S., Sooranna, S. R., Roberts, V. H., Grigsby, P. L., Liang, Z. et al,** Pro-labour myometrial gene expression: are preterm labour and term labour the same? *Reproduction* 2008. **135**: 569-579.

33 **Rauk, P. N., Friebe-Hoffmann, U., Winebrenner, L. D. and Chiao, J. P.,** Interleukin-6 up-regulates the oxytocin receptor in cultured uterine smooth muscle cells. *Am J Reprod Immunol* 2001. **45**: 148-153.

34 **Romero, R., Ceska, M., Avila, C., Mazor, M., Behnke, E. and Lindley, I.,** Neutrophil attractant/activating peptide-1/interleukin-8 in term and preterm parturition. *Am J Obstet Gynecol* 1991. **165**: 813-820.

35 **Choi, S. J., Oh, S., Kim, J. H. and Roh, C. R.,** Changes of nuclear factor kappa B (NF-kappaB), cyclooxygenase-2 (COX-2) and matrix metalloproteinase-9 (MMP-9) in human myometrium before and during term labor. *Eur J Obstet Gynecol Reprod Biol* 2007. **132**: 182-188.

36 **Stjernholm-Vladic, Y., Stygar, D., Mansson, C., Masironi, B., Akerberg, S., Wang, H., Ekman-Ordeberg, G. et al,** Factors involved in the inflammatory events of cervical ripening in humans. *Reprod Biol Endocrinol* 2004. **2**: 74.

37 **Vora, S., Abbas, A., Kim, C. J., Summerfield, T. L., Kusanovic, J. P., Iams, J. D., Romero, R. et al,** Nuclear factor-kappa B localization and function within intrauterine tissues from term and preterm labor and cultured fetal membranes. *Reprod Biol Endocrinol* 2010. **8**: 8.

38 **Hayashi, T. and Faustman, D.,** Essential role of human leukocyte antigen-encoded proteasome subunits in NF-kappaB activation and prevention of tumor necrosis factor-alpha-induced apoptosis. *J Biol Chem* 2000. **275**: 5238-5247.

39 **Schmidt, N., Gonzalez, E., Visekruna, A., Kuhl, A. A., Loddenkemper, C., Mollenkopf, H., Kaufmann, S. H. et al,** Targeting the proteasome: partial inhibition of the proteasome by bortezomib or deletion of the immunosubunit LMP7 attenuates experimental colitis. *Gut* 2010. **59**: 896-906.

40 **Bitzer, A., Basler, M., Krappmann, D. and Groettrup, M.,** Immunoproteasome subunit deficiency has no influence on the canonical pathway of NF-kappaB activation. *Mol Immunol* 2017. **83**: 147-153.

41 **Ichikawa, H. T., Conley, T., Muchamuel, T., Jiang, J., Lee, S., Owen, T., Barnard, J. et al,** Beneficial effect of novel proteasome inhibitors in murine lupus via dual inhibition of type I interferon and autoantibody-secreting cells. *Arthritis Rheum* 2012. **64**: 493-503.

42 **Nagayama, Y., Nakahara, M., Shimamura, M., Horie, I., Arima, K. and Abiru, N.,** Prophylactic and therapeutic efficacies of a selective inhibitor of the immunoproteasome for Hashimoto's thyroiditis, but not for Graves' hyperthyroidism, in mice. *Clin Exp Immunol* 2012. **168**: 268-273.

43 **Wood, N. S., Costeloe, K., Gibson, A. T., Hennessy, E. M., Marlow, N., Wilkinson, A. R. and Group, E. P. S.,** The EPICure study: associations and antecedents of neurological and developmental disability at 30 months of age following extremely preterm birth. *Arch Dis Child Fetal Neonatal Ed* 2005. **90**: F134-140.

44 **Frentzel, S., Kuhn-Hartmann, I., Gernold, M., Gott, P., Seelig, A. and Kloetzel, P. M.,** The major-histocompatibility-complex-encoded beta-type proteasome subunits LMP2 and LMP7. Evidence that LMP2 and LMP7 are synthesized as proproteins and that cellular levels of both mRNA and LMP-containing 20S proteasomes are differentially regulated. *Eur J Biochem* 1993. **216**: 119-126.

45 **Romero, R., Espinoza, J., Goncalves, L. F., Kusanovic, J. P., Friel, L. and Hassan, S.,** The role of inflammation and infection in preterm birth. *Semin Reprod Med* 2007. **25**: 21-39.

46 **Basler, M., Mundt, S., Bitzer, A., Schmid, M. and Groettrup, M.,** The immunoproteasome: a novel drug target for autoimmune diseases. *Clin Exp Rheumatol* 2015. **33 (Suppl 92)**: S74-S79.

47 **J, L., J, A., CJ, K., J, W. and D, B.,** KZR-616, a Selective Inhibitor of the Immunoproteasome, Shows a Promising Safety and Target Inhibition Profile in a Phase I, Double-Blind, Single (SAD) and Multiple Ascending Dose (MAD) Study in Healthy Volunteers. *Arthritis Rheumatol* 2017. **69 (suppl 10)**.

48 **AV, S., M, B., MA, A., CJ, K., DE, H., N, R., D, C. et al,** PR-924, a selective inhibitor of the immunoproteasome subunit LMP-7, blocks multiple myeloma cell growth both in vitro and in vivo. *Br J Haematol* 2011. **152**.

49 **Zhou, H. J., Aujay, M. A., Bennett, M. K., Dajee, M., Demo, S. D., Fang, Y., Ho, M. N. et al,** Design and synthesis of an orally bioavailable and selective peptide epoxyketone proteasome inhibitor (PR-047). *J Med Chem* 2009. **52**: 3028-3038.

50 **Lim, R., Barker, G., Riley, C. and Lappas, M.,** Apelin is decreased with human preterm and term labor and regulates prolabor mediators in human primary amnion cells. *Reprod Sci* 2013. **20**: 957-967.

51 **Lim, R., Barker, G. and Lappas, M.,** A Novel Role for FOXO3 in Human Labor: Increased Expression in Laboring Myometrium, and Regulation of Pro-Inflammatory and Pro-Labor Mediators in Pregnant Human Myometrial Cells. *Biol Reprod* 2013. **88**: 156.

52 **Li, H., Yu, Y., Shi, Y., Fazli, L., Slater, D., Lye, S. and Dong, X.,** HoxA13 Stimulates Myometrial Cells to Secrete IL-1beta and Enhance the Expression of Contraction-Associated Proteins. *Endocrinology* 2016. **157**: 2129-2139.

53 **Parkington, H. C., Stevenson, J., Tonta, M. A., Paul, J., Butler, T., Maiti, K., Chan, E.-C. et al,** Diminished hERG K^+ channel activity facilitates strong human labour contractions but is dysregulated in obese women. *Nature Communications* 2014. **5**: 4108.

54 **Lee, P. R., Kim, S. R., Jung, B. K., Kim, K. R., Chung, J. Y., Won, H. S. and Kim, A.,** Therapeutic effect of cyclo-oxygenase inhibitors with different isoform selectivity in lipopolysaccharide-induced preterm birth in mice. *Am J Obstet Gynecol* 2003. **189**: 261-266.

55 **Kaga, N., Katsuki, Y., Obata, M. and Shibutani, Y.,** Repeated administration of low-dose lipopolysaccharide induces preterm delivery in mice: a model for human preterm parturition and for assessment of the therapeutic ability of drugs against preterm delivery. *Am J Obstet Gynecol* 1996. **174**: 754-759.

56 **Gross, G., Imamura, T., Vogt, S. K., Wozniak, D. F., Nelson, D. M., Sadovsky, Y. and Muglia, L. J.,** Inhibition of cyclooxygenase-2 prevents inflammation-mediated preterm labor in the mouse. *Am J Physiol Regul Integr Comp Physiol* 2000. **278**: R1415-1423.

FIGURE LEGENDS

Figure 1. Expression of LMP7 in human myometrium

Human myometrium was obtained from term non-labouring and labouring women at time of Caesarean section. LMP7 mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to non-labouring women group. Data are shown as mean + SEM (n=8 patients per group). Samples of individual patients were collected and processed over a period of time and qRT-

PCR data are from a single experiment with 8 patient samples per group. *P<0.05 vs. Term No Labour (Student's t-test).

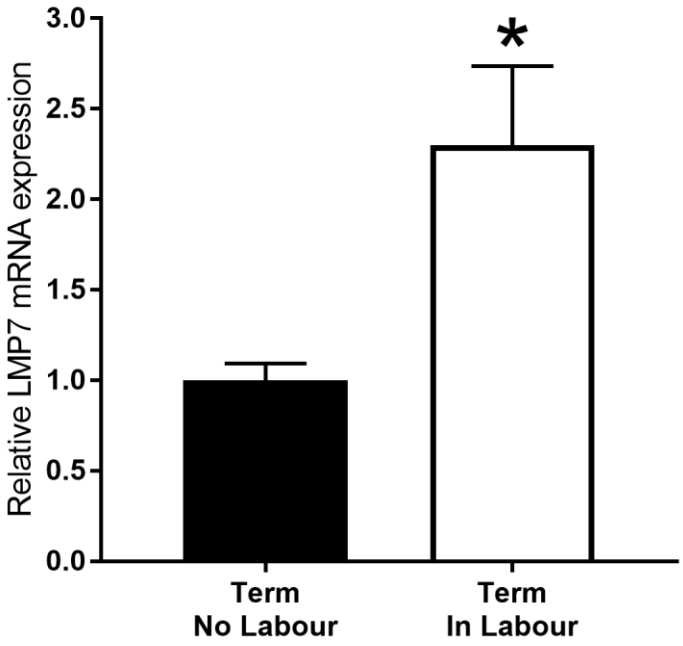

Figure 2. Effect of LMP7 silencing and ONX-0914 treatment on pro-inflammatory cytokines in human myometrium

(A,B) Human myometrium was incubated in the absence or presence of ONX-0914 (ONX) and then treated with LPS (n=6 patients). IL-6 mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to LPS. The incubation medium was assayed for the concentration of IL-6 by ELISA. Data are shown as mean + SEM. Samples were from 6 in vitro individual experiments with 1 patient sample per experiment. ELISA and qRT-PCR data are from a single experiment with 6 patient samples per group. *P<0.05 vs. LPS (one-way ANOVA). **(C,D)** Human primary myometrial cells were transfected with or without siLMP7 or siCONT 48 h and then treated with IL-1β (n=5 patients). IL-6 mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to LPS. The incubation medium was assayed for the concentration of IL-6 by ELISA. Data are shown as mean + SEM. Samples were collected from 5 in vitro individual experiments with 1 patient sample per

experiment. ELISA and qRT-PCR data are from a single experiment with 5 patient samples per group.

**P<0.05 vs. siCONT + IL-1β (one-way ANOVA).

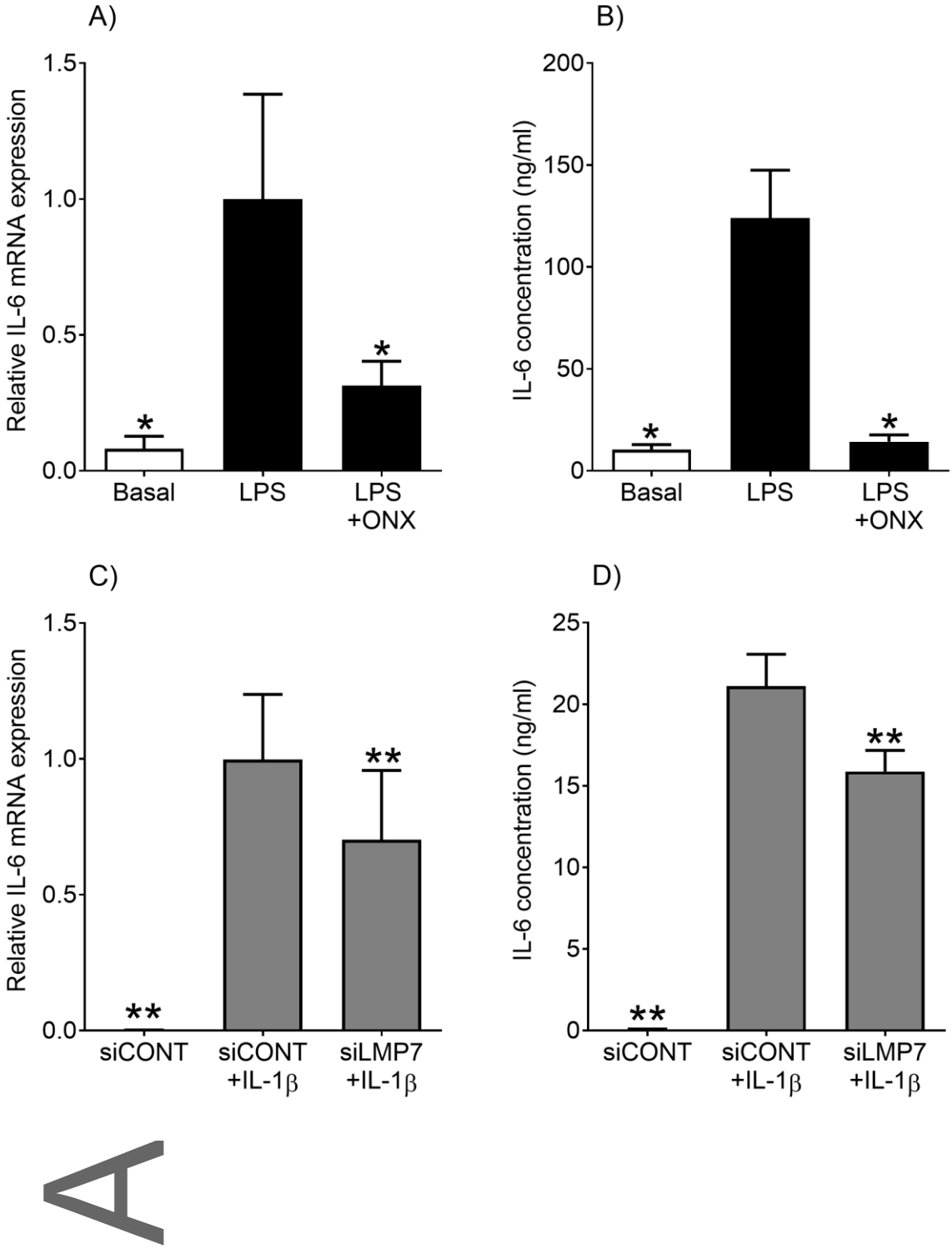

Figure 3. Effect of LMP7 silencing and ONX-0914 treatment on pro-inflammatory chemokines and chemotaxis in human myometrium

(A,B,E,F) Human myometrium was incubated in the absence or presence of ONX-0914 (ONX) and then treated with LPS (n=6 patients). CXCL8 and CCL2 mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to LPS. The incubation medium was assayed for the concentration of CXCL8 and CCL2 by ELISA. Data are shown as mean + SEM. Samples were from 6 in vitro individual experiments with 1 patient sample per experiment. ELISA and qRT-PCR data are from a single experiment with 6 patient samples per group. *P<0.05 vs. LPS (one-way ANOVA). **(C,D,G,H)** Human primary myometrial cells were transfected with or without siLMP7 or siCONT and then treated with IL-1β (n=5 patients). CXCL8 and CCL2 mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to LPS. The incubation medium was assayed for the concentration of CXCL8 and CCL2 by ELISA. Data are shown as mean + SEM. Samples were from 5 in vitro individual experiments with 1 patient sample per experiment. ELISA and qRT-PCR data are from a single experiment with 5 patient samples per group. **P<0.05 vs. siCONT + IL-1β (one-way ANOVA). **(I,J)** THP-1 cell migration were performed using conditioned media from human primary myometrial cells treated in the absence or presence of ONX-0914 (ONX) with IL-1β or TNF. Fold change was calculated relative to IL-1β or TNF. Data are shown as mean + SEM. Samples were from 3 in vitro individual experiments with 1 patient sample per experiment. Migration data are from a single experiment with 3 patient samples per group. #P<0.05 vs. IL-1β; ***P<0.05 vs. TNF (one-way ANOVA).

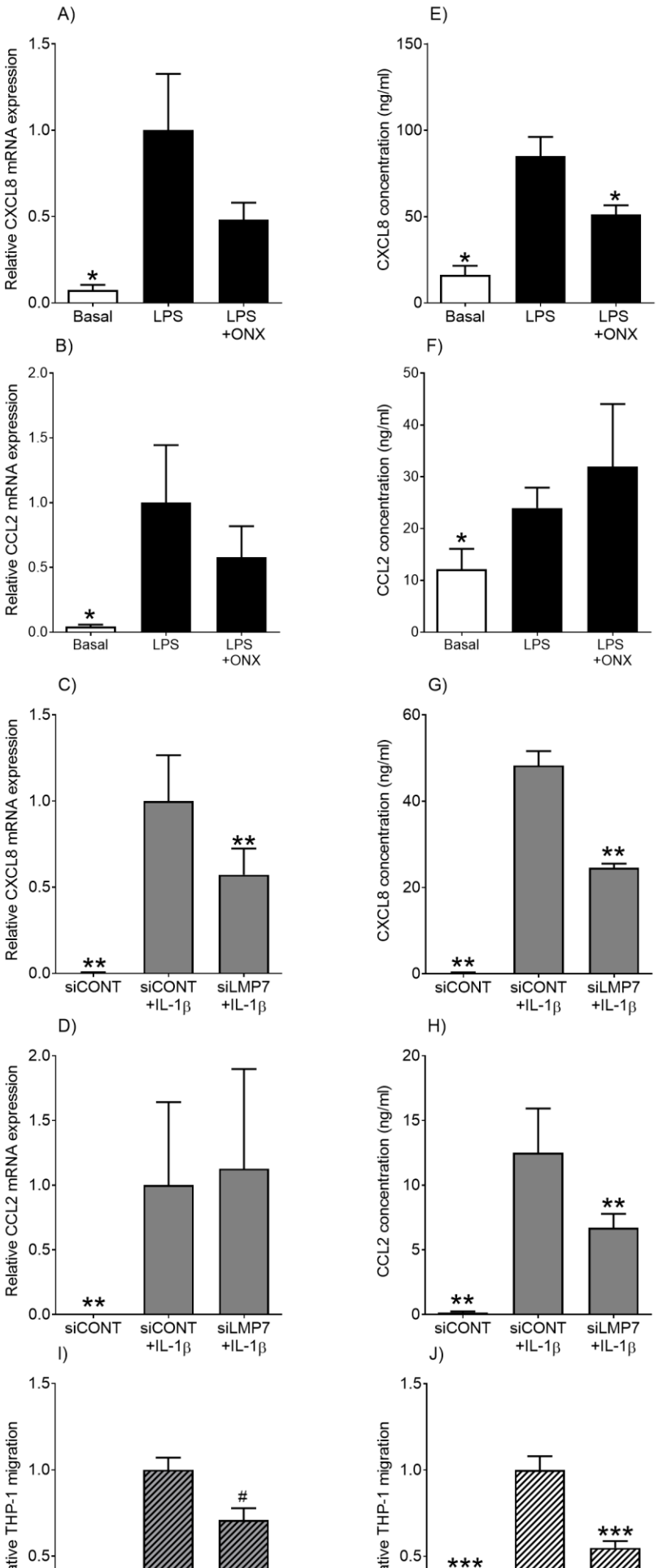
A)
Relative CXCL8 mRNA expression
1.5
1.0
0.5
0.0
*
Basal
LPS
LPS +ONX
E)
CXCL8 concentration (ng/ml)
150
100
50
0
*
*
Basal
LPS
LPS +ONX
B)
Relative CCL2 mRNA expression
2.0
1.5
1.0
0.5
0.0
*
Basal
LPS
LPS +ONX
F)
CCL2 concentration (ng/ml)
50
40
30
20
10
0
*
Basal
LPS
LPS +ONX
C)
Relative CXCL8 mRNA expression
1.5
1.0
0.5
0.0
**
**
siCONT
siCONT +IL-1β
siLMP7 +IL-1β
G)
CXCL8 concentration (ng/ml)
60
40
20
0
**
**
siCONT
siCONT +IL-1β
siLMP7 +IL-1β
D)
Relative CCL2 mRNA expression
2.0
1.5
1.0
0.5
0.0
**
siCONT
siCONT +IL-1β
siLMP7 +IL-1β
H)
CCL2 concentration (ng/ml)
20
15
10
5
0
**
**
siCONT
siCONT +IL-1β
siLMP7 +IL-1β
I)
ative THP-1 migration
1.5
1.0
0.5
#
J)
ative THP-1 migration
1.5
1.0
0.5
***
***

Figure 4. Effect of ONX-0914 treatment on cell adhesion in human myometrium

(A) Human myometrium was incubated in the absence or presence of ONX-0914 (ONX) and then treated with LPS for an additional (n=6 patients). ICAM1 mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to LPS. The incubation medium was assayed for the concentration of ICAM1 by ELISA. Data are shown as mean + SEM. Samples were from 6 in vitro individual experiments with 1 patient sample per experiment. ELISA and qRT-PCR data are from a single experiment with 6 patient samples per group. *P<0.05 vs. LPS (one-way ANOVA). **(B,C)** Cell adhesion assays were performed using human primary myometrial cells pre-treated in the absence or presence of ONX-0914 (ONX) with IL-1β or TNF (n=5 patients). Fold change was calculated relative to IL-1β or TNF. Data are shown as mean + SEM. Samples were from 5 in vitro individual experiments with 1 patient sample per experiment. Adhesion data are from a single experiment with 5 patient samples per group. #P<0.05 vs. IL-1β; ***P<0.05 vs. TNF (one-way ANOVA).

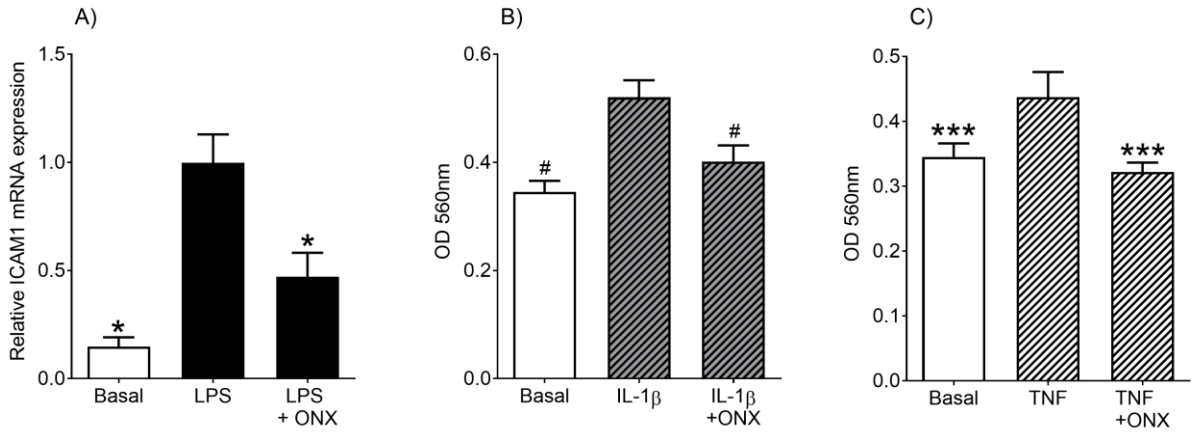

Figure 5. Effect of LMP7 silencing and ONX-0914 treatment on myometrial contraction

(A-D) Human myometrium was incubated in the absence or presence of ONX-0914 (ONX) and then treated with LPS for an additional (n=6 patients). PTGS2 and FP mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to LPS alone. The incubation medium was assayed for the concentration of PGE_2 and $PGF_{2\alpha}$ by ELISA. Data are shown as mean + SEM. Samples were from 6 in vitro individual experiments with 1 patient sample per experiment. ELISA and qRT-PCR data are from a single experiment with 6 patient samples per group. *P<0.05 vs. LPS (one-way ANOVA). **(E-H)** Human primary myometrial cells were transfected with or without siLMP7 or siCONT and then treated with IL-1β (n=5 patients). PTGS2 and FP mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to IL-1β-stimulated siCONT transfected cells. The incubation medium was assayed for concentrations of PGE_2 and $PGF_{2\alpha}$ by ELISA. Fold change was calculated relative to IL-1β-stimulated siCONT transfected cells. Data are shown as mean + SEM. Samples were from 5 in vitro individual experiments with 1 patient sample per experiment. ELISA and qRT-PCR data are from a single experiment with 5 patient samples per group. **P<0.05 vs. IL-1β-stimulated siCONT transfected cells (one-way ANOVA). **(I-J)** Collagen gels containing human primary myometrial cells (n=5) were treated in the absence or presence of ONX-0914 (ONX) with IL-1β or TNF. Fold change was calculated relative to IL-1β or TNF. A representative image of the collagen gels is shown. Data are shown as mean + SEM from 2 in vitro individual experiments with 2-3 patient sample per experiment. #P<0.05 vs. IL-1β; ***P<0.05 vs. TNF (one-way ANOVA). **(K)** Human myometrium was obtained from in-labour women (n=4) and incubated in ONX-0914 (ONX) or vehicle

control (Veh control) and then treated with LPS. Oxytocin-induced myometrial contractions were then recorded. The area under the contraction curve was expressed as % of the contraction to high-potassium solution (HiK, standardization for muscle mass in the tissue). Data are shown as mean + SEM from 4 in vitro individual experiments with 1 patient sample per experiment. Responses following exposure to ONX-0914 versus vehicle control were compared using 2-way ANOVA.

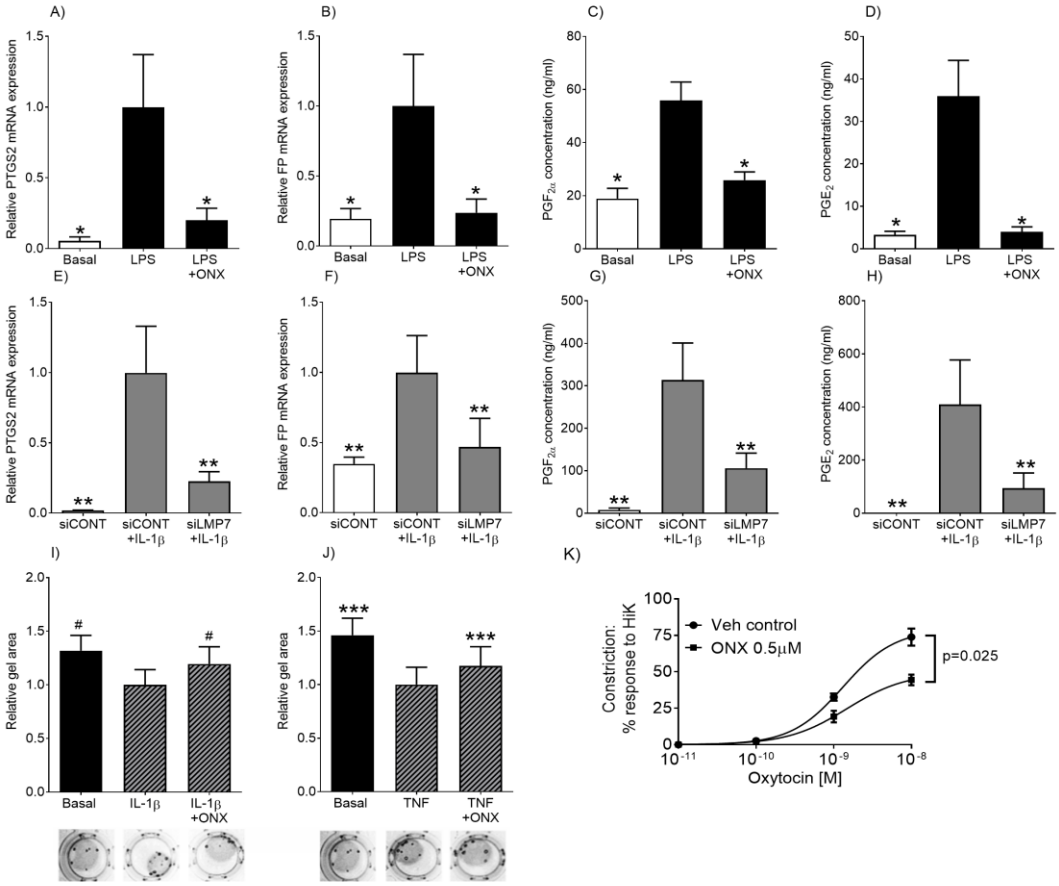

Figure 6. Effect of LMP7 silencing on NF-κB RelA activity in human primary myometrial cells

Human myometrial cells were co-transfected with NF-κB RelA reporter construct, along with siLMP7 or siCONT. Cells were then treated with **(A)** IL-1β, **(B)** TNF, **(C)** poly(I:C), or **(D)** flagellin (n=5 patients). Promoter activity (normalised with Renilla expression) is expressed as a ratio of luciferase activity of NF-κB RelA reporter plus siCONT plus treatment group. Data are shown as mean + SEM. Samples

were from 5 in vitro individual experiments with 1 patient sample per experiment. NF-κB RelA data are from a single experiment with 5 patient samples per group. *P<0.05 vs. siCONT + IL-1β (one-way ANOVA); †P<0.05 vs. cells transfected with siCONT + TNF (one-way ANOVA); §P< 0.05 vs. cells transfected with siCONT + poly(I:C); **P<0.05 vs. cells transfected with siCONT + flagellin (one-way ANOVA).

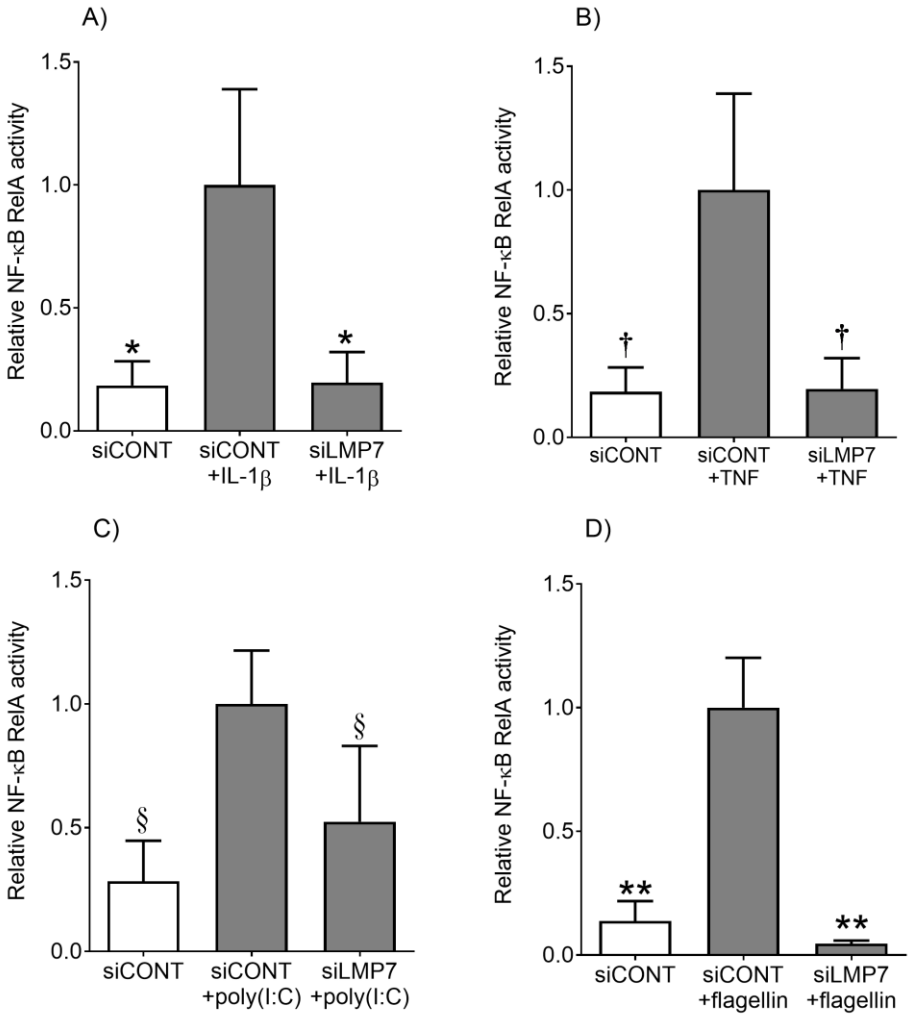

Figure 7. Effect of ONX-0914 on maternal and fetal inflammation in a mouse model of inflammation

Time-mated pregnant C57BL/6 mice were intraperitoneally injected at day 15.5 with either saline (control), LPS, or LPS with ONX-0914 (n=6 mice per treatment group). Myometrium, fetal brain,

maternal serum and amniotic fluid were collected after 6 h post i.p. **(A-E)** IL-1α, IL-1β, CCL3, CXCL1 and PTGS2 mRNA expression was analysed by qRT-PCR in myometrium. **(F,G)** CCL3 and CXCL1 mRNA expression were analysed by qRT-PCR in fetal brain. **(H,I)** IL-6 concentrations in maternal serum and amniotic fluid were measured by a mouse multiplex assay. The fold change was calculated relative to LPS expression. Data are shown as mean + SEM. Samples were from 6 in vivo individual experiments with 1 mouse per experiment. ELISA and qRT-PCR data are from a single experiment with 6 mice per group *P<0.05 vs. LPS (one-way ANOVA).