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NOD-like receptor pyrin domain containing 3 (NLRP3) regulates inflammation-induced pro-labour mediators in human myometrial cells

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ABSTRACT

Problem: Inflammation plays a major role in preterm birth. Nucleotide-binding oligomerization domain-like receptor pyrin domain-containing-3 (NLRP3) plays a role in inflammatory diseases. The aims of this study were to determine the effect of term labor on the expression of NLRP3 in human myometrium, and the effect of NLRP3 silencing on pro-labor mediators in myometrial cells.

Method of Study: NLRP3 expression was assessed in myometrium from non-laboring and laboring women by qRT-PCR and Western blotting. Human primary myometrial cells were transfected with NLRP3 siRNA (siNLRP3), treated with pro-inflammatory cytokines and toll-like receptor (TLR) ligands, and assayed for pro-inflammatory mediators expression.

Results: NLRP3 expression was higher in myometrium after term spontaneous labor and byTNF, IL1B, fsl-1 and flagellin. In siNLRP3 transfected cells, there was a significant decrease in the expression of pro-inflammatory cytokines (IL1A, IL6), chemokines (CXCL8, CCL2), and adhesion molecules (ICAM1 and VCAM1) stimulated with IL1B, TNF or TLR ligands; decrease in IL1B-stimulated PTGS2 and PTGFR mRNA expression and $PGF_{2\alpha}$ release; and increase in TNF-stimulated myometrial gel shrinkage as assessed by an in vitro cell contraction assay.

Conclusions: ~~NLRP3 regulates pro-inflammatory and pro-labour mediators in human myometrium.~~ NLRP3 is increased with labor in myometrial and knockdown of NLRP3 is associated with an attenuation of inflammation-induced expression of pro-inflammatory and pro-labor mediators in human myometrium.

Key words: NLRP3; myometrium; pro-labor mediators; inflammation; preterm birth

INTRODUCTION

Preterm birth continues to be a major contributor to neonatal deaths and morbidity [1]. Compared to those born at term, children born prematurely have increased risk of cerebral palsy, neurodevelopmental delays, and respiratory illnesses, health issues that can extend into adult life [2]. The many costs of preterm birth places an enormous burden on both families and the healthcare system [3].

Spontaneous preterm birth accounts for up to 80% of all preterm births; prelabor rupture of the membranes (PPROM) occurs in up to 30% of cases, and up to 45% are due to unexplained preterm labor [4]. Based on microbiological studies, infection is associated with up to 40% of all preterm

births [5]; due to limitation of methods of detection, this number is likely to be higher. Infection and/or inflammation activates the maternal immune system, eventually resulting in myometrial contractions, cervical ripening and rupture of fetal membranes, thus spontaneous preterm birth. Pro-inflammatory cytokines, chemokines, cell adhesion molecules (CAMs), contraction associated proteins such as cyclooxygenase-2 (PTGS2), prostaglandins and their receptors, and extracellular matrix (ECM) remodelling enzymes such as matrix metalloproteinase 9 (MMP9) have been shown to play a role in labor associated processes [6, 7].

Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs) recognise pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). NLR pyrin domain-containing-3 (NLRP3; also known as NALP3 or cryopyrin), a key component of the NLRP3 inflammasome, is activated by ligation of TLRs and/or cytokine receptors [8, 9]. A second activation step, mediated by DAMPs, is required for inflammasome assembly and resultant caspase-1 mediated secretion of mature IL1B and/or IL18. Notably, however, a number of studies have shown that NLRP3 can induce inflammation independent of inflammasome assembly [10-13]. Furthermore, *in vivo*, ablation of NLRP3 prevents inflammation in various animal models of disease [14-19]. In line with this, aberrant activation of NLRP3 has been associated to the pathogenesis of numerous inflammatory disorders including inflammatory bowel disease [20], gouty arthritis [17], atherosclerosis [16] and obesity [15].

A role for NLRP3 has recently been implicated in spontaneous term labor in chorioamniotic membranes. Specifically, chorioamniotic membranes from women who underwent labor had higher concentrations of NLRP3 protein compared to those who delivered at term without labor [22]. Notably, the expression was even higher in chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis compared to those without acute histologic chorioamnionitis [23]. Whether NLRP3 is also increased in labouring myometrium is not known. Thus, an aim of this study was to characterise the expression of NLRP3 in myometrium from non-laboring and laboring women.

Incubation of the chorioamniotic membranes with the alarmin high-mobility group box-1 (HMGB1) increased the mRNA abundance and protein expression of NLRP3 [24]. In the uterus of pregnant mice, the expression of NLRP3 is increased by peptidoglycan (PGN; a TLR2 agonist) and polyinosinic-polycytidylic acid (poly(I:C); a TLR3 agonist) [25]. Thus, another aim was to determine the effect of pro-inflammatory mediators on NLRP3 expression in cells isolated from human myometrium.

NLRP3 has been shown to contribute to inflammation in a number of *in vitro* and *in vivo* models of disease [14-19]. Whether NLRP3 is involved in the genesis of pro-inflammatory and pro-labor mediators in human myometrium is not known. Therefore, we sought to determine if NLRP3 contributes to cytokine- or TLR-induced expression of pro-labor mediators in primary cells isolated from human myometrium. The pro-inflammatory cytokines IL1B and TNF, the TLR2/6 ligand fibroblast-stimulating lipopeptide (fsl-1) and the TLR5 ligand flagellin were used to mimic inflammation associated with preterm labor [26, 27].

MATERIALS AND METHODS

Tissue collection

Human myometrium was obtained (with institutional Research and Ethics Committee approval) from the upper margin of the lower uterine segment incision during Caesarean section. Myometrium was brought to the research laboratory and processed within 15 mins of delivery. Myometrial tissues were washed in PBS to remove excess blood, cleared of serosa, fibrous or damaged tissue and visible blood vessels, and then dissected into smaller pieces. Tissues were immediately snap frozen in liquid nitrogen and stored at -80^{o}C for expression studies, or used immediately for cell culture experiments.

For all studies, women with any underlying medical conditions such as diabetes, asthma, polycystic ovary syndrome, preeclampsia and macrovascular complications were excluded. Additionally, women with multiple pregnancies, obese women, and fetuses with chromosomal abnormalities were excluded.

For the expression studies, myometrial biopsies were collected from women at term Caesarean section from in the absence of labor (n=8 patients; mean gestational age 39.4±0.3 weeks) or during active spontaneous term labor (n=8 patients; mean gestational age 39.8±0.2 weeks). Indications for Caesarean section in the absence of labor were breech presentation and/or previous Caesarean section. Indications for Caesarean section in the laboring samples were for placenta praevia, fetal distress and delayed or failure to progress. Labor was defined as the presence of regular uterine contractions (every 3–4 min) resulting in cervical effacement and dilation. In the laboring group, none of the patients received any medications to augment or induce labor, and the average length of labor was 10 h ± 6 h 40 min.

For the cell culture studies, fresh myometrium was obtained from women who delivered healthy, singleton infants at term (37-40 weeks gestation) undergoing elective Caesarean section in the absence of labor. Myometrial cells were isolated and cultured as previously described [28].

Primary myometrial cell culture

To determine the effect of pro-inflammatory mediators on NLRP3 expression, myometrial cells at approximately 80% confluence were incubated in the absence or presence of 1 ng/ml IL1B (PeproTech; Rocky Hill, NJ, USA), 10 ng/ml TNF (PeproTech; Rocky Hill, NJ, USA), 250 ng/ml fsl-1 (InVivoGen; San Diego, California, USA) or 1 μg/ml flagellin (purified flagellin from *Salmonella typhimurium;* InVivoGen; San Diego, California, USA) for 20 h. Cells were collected and stored at −80°C until assayed for NLRP3 mRNA expression by qRT-PCR as detailed below. Experiments were performed from myometrium obtained from 5 patients.

To determine the effect of NLRP3 siRNA on pro-labor mediators, transfection of primary myometrial cells with siRNA was performed as previously described [28]. Briefly, myometrial cells at approximately 50% confluence were transfected using Lipofectamine 3000 according to manufacturer's guidelines (Life Technologies; Mulgrave, Victoria, Australia). NLRP3 siRNA (siNLRP3) and negative control siRNA (siCONT) were obtained from Ambion (Thermo Fisher Scientific; Scoresby, Vic, Australia). Cells were transfected with 50 nM siNLRP3 or 50 nM siCONT in DMEM/F-12 for 48 h. The medium was then replaced with DMEM/F-12 (containing 0.5% BSA) with or without 1 ng/ml IL1B, 10 ng/ml TNF, 250 ng/ml fsl-1 or 1 μg/ml flagellin and the cells were incubated at 37°C for an additional 20 h. After final incubation, cells and conditioned media were collected separately. The cells were used immediately for cell contraction assays or stored at -80°C for RNA extraction. The conditioned media was stored at -80°C until analysis of endpoints by ELISA. Data is presented as fold change in expression relative to the expression level in IL1B, TNF-, fsl-1- or flagellin-stimulated cells. Experiments were performed from myometrium obtained from 7 patients.

Cell contraction assay

Cell contraction assays were performed as previously described [29]. Cells (collected from experiments 2 and 3 above) 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 at 37°C to allow polymerization (approx. 15 min) and then treated with 10 ng/ml TNF. The gel matrix was gently detached from the well, incubated for 36 h at 37°C, and the area of the gel was

140 determined using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA). Experiments were performed from myometrium obtained from 5 patients.

142

RNA extraction and qRT-PCR

144 RNA extractions and qRT-PCR was performed as previously described [30]. RNA concentration and purity were measured using a NanoDrop ND1000 (Thermo Fisher Scientific; Scoresby, Vic,
146 Australia). RNA was converted to cDNA using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific; Scoresby, Vic, Australia) according to the manufacturer's instructions.
148 The RT-PCR was performed using the CFX384 Real-Time PCR detection system (Bio-Rad Laboratories; Gladesville, NSW, Australia) using 100 nM of pre-designed and validated QuantiTect
150 primers (primer sequences not available) (Qiagen; Chadstone Centre, Vic, Australia). Average gene Ct values were normalised to the average YWHAZ and succinate dehydrogenase (SDHA) Ct values
152 of the same cDNA sample. Fold differences were determined using the comparative Ct method.

154

156 **Western blotting**

Western blotting was performed as previously described [28]. Blots were incubated in a 1/1000
158 dilution of rabbit monoclonal anti-NLRP3 (cat no. ab109314; Abcam, Cambridge, UK) prepared in blocking buffer (5% skim milk in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) with 0.05%
160 Tween-20) for 16 h at 4°C. Semi-quantitative analysis of the relative density of the bands in Western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad
162 Laboratories, Hercules, CA, USA). Protein normalisation was performed as previously described [31]; the level of NLRP3 was normalised to Ponceau S stain; a section of the Ponceau S stained
164 membrane was chosen which did not show variation with labor status.

166 **Enzyme Immunoassays**

Assessment of cytokine and chemokine release of IL6 and CXCL8 was performed using the
168 CytoSet™ sandwich ELISA according to the manufacturer's instructions (Life Technologies; Mulgrave, Vic, Australia). The release of CCL2, sICAM1 and sVCAM1 was performed by
170 sandwich ELISA from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions. The release of $PGF_{2\alpha}$ into the incubation medium was assayed using a commercially
172 available competitive enzyme immunoassay kit according to the manufacturer's specifications (Cayman Chemical Company; Ann Arbor, MI, USA). The interassay and intraassay coefficients of
174 variation for all assays were less than 10%.

176 **Statistical analysis**

All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA,
178 USA). For two sample comparisons, either a paired or unpaired Student's t-test was used to assess statistical significance between normally distributed data; otherwise, the nonparametric Mann-
180 Whitney U (unpaired) or the Wilcoxon (matched pairs) tests were used. For all other comparisons, the homogeneity of data was assessed by Bartlett's test, and when significant, data were
182 logarithmically transformed before analysis by a repeated measures one-way ANOVA (with LSD post-hoc testing to discriminate among the means). Statistical significance was ascribed to a *P* value
184 <0.05. Data is expressed as mean ± SEM.

RESULTS

186

Expression of NLRP3 in human myometrium

188 The first aim of this study was to characterise the expression of NLRP3 in myometrium form non-laboring and laboring women. The mRNA abundance and protein expression of NLRP3 was
190 assessed by qTR-PCR and Western blotting, respectively, and the data is presented in Figure 1. NLRP3 mRNA and protein expression was significantly higher in myometrium after term
192 spontaneous labor when compared to gestational-age matched tissues from non-laboring women (Figure 1A,B).

194

We also wanted to determine whether inflammatory insults induce a similar increase in NLRP3
196 expression. We thus assessed the effect of TNF or IL1B, two pro-inflammatory cytokines increased during labor, on NLRP3 expression in primary human myometrial cells.. We also assessed the
198 effect of TLR activation on NLRP3 expression. For these studies, we used the TLR ligands and bacterial products fsl-1 (TLR2/6 ligand) and flagellin (TLR5 ligand). All treatments significantly
200 increased NLRP3 mRNA expression (Figure 1C) with TNF inducing the largest increase in NLRP3 mRNA expression (16-fold). IL1B and fsl-1 induced a 5-fold increase in NLRP3 mRNA
202 expression, while flagellin increased NLRP3 mRNA expression 2-fold.

204 **Effect of siNLRP3 on pro-inflammatory cytokines in primary myometrial cells**

The pro-inflammatory cytokines IL1B and TNF, and the TLR ligands fsl-1 and flagellin are
206 commonly used to induce inflammation associated with preterm labor [26, 27]. Thus, we manipulated the expression of NLRP3 to determine if it is involved in IL1B-, TNF-, fsl-1- or flagellin-induced
208 pro-inflammatory and pro-labor mediators. To do this, we utilised primary cells isolated from human myometrium and knocked down NLRP3 by siRNA. The efficacy of siNLRP3 transfection

was assessed by qRT-PCR. When compared to siCONT transfected cells, there was an 85% decrease in NLRP3 mRNA expression in siNLRP3 transfected cells. There was no effect of siNLRP3 on cell viability as determined by MTT cell viability assay (data not shown).

The result of siNLRP3 on IL1A, IL1B and IL6 mRNA expression and IL6 secretion is represented in Figure 2. As expected, treatment with IL1B significantly increased IL1A and IL6 mRNA expression and secretion of IL6. In cells transfected with siNLRP3, there was a significant decrease in IL1B-induced IL1A and IL6 mRNA expression and release of IL6. Treatment with TNF significantly increased IL1A, IL1B and IL6 mRNA expression and release of IL6. The effect of siNLRP3 was a significant decrease in TNF-induced IL1B and IL6 mRNA expression and release of IL6. There was no effect of siNLRP3 on TNF-induced IL1A mRNA expression. Treatment with fsl-1 significantly increased IL1A, IL1B and IL6 mRNA expression and release of IL6. The effect of siNLRP3 was a significant decrease in fsl-1-induced IL1A, IL1B and IL6 mRNA expression and release of IL6. Treatment with flagellin significantly increased IL1A, IL1B and IL6 mRNA expression and release of IL6. There was a significant decrease in flagellin-induced IL1B mRNA expression and release of IL6. The decrease in flagellin-induced IL6 mRNA expression was not statistically significant (P=0.07). There was no effect of siNLRP3 on flagellin-induced IL1A mRNA expression. IL1A and IL1B levels are not detectable in the incubation media from human primary myometrial cells and thus not assessed.

Effect of siNLRP3 on chemokines in primary myometrial cells

Chemokines are involved in leukocyte activation and recruitment into the uterus during labor [32]. Thus, it was of interest to determine the effect of siNLRP3 on the expression and secretion of the chemokines CXCL8 and CCL2. As shown in Figure 3, treatment with IL1B, TNF, fsl-1 and flagellin all significantly increased CXCL8 and CCL2 mRNA expression and secretion. The effect of siNLRP3 was a significant decrease in IL1B-, fsl-1- and flagellin-induced CXCL8 and CCL2 mRNA expression and release. There was a significant decrease in TNF-induced CXCL8 mRNA expression and release, but no effect on TNF-induced CCL2 mRNA expression and release in siNLRP3 cells.

Effect of siNLRP3 on adhesion molecules in primary myometrial cells

Adhesion molecules, which play an important role in leukocyte recruitment into tissues, are increased in labor in the myometrium [33]. We have previously shown that the expression of the adhesion molecules ICAM1 and VCAM1 can be induced by pro-inflammatory cytokines and TLR ligands [27]. Thus, we investigated the role of NLRP3 in the expression and secretion of ICAM1 and

VCAM1 using siRNA. As shown in Figure 4, treatment with IL1B, TNF, fsl-1 and flagellin significantly increased ICAM1 and VCAM1 mRNA expression and release. In siNLRP3 cells, there was a significant decrease in only TNF-induced ICAM1 mRNA expression; there was no effect of siNLRP3 on IL1B-, fsl-1- and flagellin-induced ICAM1 mRNA expression. However, there was a significant reduction in the release of sICAM1 by all treatments. The effect of siNLRP3 was a significant decrease in IL1B-, fsl-1 and flagellin-induced VCAM1 mRNA expression and secretion. There was a significant decrease in TNF-induced VCAM1 mRNA expression, but no change in sVCAM1 secretion.

Effect of siNLRP3 on contraction-associated proteins and myometrial cell contractility

In preparation for labor, the myometrium transitions from a quiescent to a contractile state. This transition is associated with an increase in the expression of contraction-associated proteins such as PTGS as well as an increase in prostaglandin signalling. Thus, to assess if NLRP3 regulates myometrial contractility, we firstly assessed the effect of siNLRP3 on PTGS2 expression and PGF2α signalling (i.e. $PGF_{2\alpha}$ secretion and expression of its receptor PTGFR). As shown in Figure 5, IL1B treatment significantly increased PTGS2 and PTGFR mRNA expression and release of $PGF_{2\alpha}$. The effect of siNLRP3 was a significant attenuation of IL1B-induced PTGS2 and PTGFR mRNA expression and release of $PGF_{2\alpha}$.

Following on from these studies, we wanted to determine if NLRP3 could also regulate myometrial cell contractility *in situ*. To carry out these studies, we used an *in vitro* gel contraction assay in primary myometrial cells deficient in NLRP3 were embedded into 3D collagen gel matrices so that the cells could contract within the matrix in response to TNF. As shown in Figure 5D, TNF induced the contractile activity of myometrial cells, as evidenced by increased myometrial cell gel shrinkage (approximately 35% shrinkage compared to cells without TNF). TNF-induced shrinkage was significantly suppressed in siNLRP3 transfected cells (Figure 5D).

DISCUSSION

In extension to the studies in fetal membranes [22], herein we report that spontaneous term labor is also associated with increased NLRP3 mRNA and protein expression in myometrium. All the myometrial samples from the laboring group were obtained from women in established labor and thus we do not know if the increase in NLRP3 during labor is a cause or consequence of labor. Whether NLRP3 expression is augmented earlier in pregnancy was unable to be determined, given the great difficulty in obtaining myometrial biopsies from early to mid-gestation. In this respect, animal studies would prove useful to clarify the expression of NLRP3 in myometrium throughout

pregnancy. Nevertheless, the increase in NLRP3 is most likely a consequence of inflammation, as the pro-inflammatory cytokines IL1B and TNF, which are higher during labor in myometrium [34, 35], significantly increased NLRP3 mRNA expression in myometrial cells.

TNF and IL1B, acute phase pro-inflammatory cytokines produced by the leukocytes that invade the uterus during labor [32, 35], have key roles in the processes of human labor and delivery. IL1B and TNF, through a feed-forward mechanism, can amplify the inflammatory environment by inducing chemokines [36] and cellular adhesion molecules [33] to promote an influx of leukocytes into myometrium. IL1B and TNF can also increase pro-MMP9 expression [37], which is higher in myometrium during human labor [37] and postulated to participate in uterine remodelling during parturition. Finally, IL1B and TNF are important for the induction of uterine contractions. They do this by up-regulating the expression of contraction-associated proteins such as PTGS2 and PTGFR [38-40], stimulating the production of uterotonic prostaglandins [41], and potentiating myometrial contractions by inducing the entry of calcium into myometrial smooth muscle cells [42].

Studies in non-gestational tissues have shown that NLRP3 can induce pro-inflammatory mediators independent of inflammasome assembly [10-13]. Thus, we performed functional studies, using siRNA, to determine if NLRP3 is involved in the genesis of pro-labor mediators induced by IL1B or TNF. We found that in siNLRP3 transfected cells, the response to IL1B and TNF was diminished. That is, compared to siCONT transfected cells, cells transfected with siNLRP3 displayed a decrease in the expression of the pro-inflammatory cytokine IL6, chemokines (CXCL8, CCL2), adhesion molecule VCAM1, and contraction associated proteins (*PTGS2, PTGFR,* $PGF_{2\alpha}$) when treated with IL1B. Similarly, when treated with TNF, in siNLRP3 transfected cells there was a decrease in cytokines and chemokines (IL6, IL1B and CXCL8), adhesion molecule ICAM1, and myometrial contractions as seen in the *in vitro* cell contraction assay. Collectively, suppression of NLRP3 using siRNA, significantly reduced inflammation and pro-labor mediators when treated with IL1B and TNF, and contraction capability in human myometrial cells treated with TNF. Overall, our data suggests that NLRP3 positively regulates the activation of myometrium.

One of the main causes of preterm birth, especially those before 32 weeks gestation, is infection [43]. Bacterial and/or viral pathogens bind to discrete TLRs to induce an inflammatory response that results in preterm birth [44]. In fetal membranes, the expression of NLRP3 is higher from women who underwent spontaneous labor at term with acute histologic chorioamnionitis compared to those without acute histologic chorioamnionitis [23]. We could not assess the effect of infection on NLRP3 expression in myometrium due to the difficulty in obtaining these samples. Notwithstanding this

limitation, incubation of myometrial cells with the bacterial products and TLR ligands fsl-1 (TLR2/6 ligand) and flagellin (TLR5 ligand) significantly increased NLRP3 mRNA expression by 5- and 2-fold, respectively. Likewise, NLRP3 expression is increased in the uterus of pregnant mice by TLR2 or TLR3 ligands [25].

We have previously shown that in primary human myometrial cells, fsl-1 and flagellin can induce pro-inflammatory and pro-labor mediators [26, 27]. Thus, it was of interest to determine if NLRP3 is involved in TLR signalling in human myometrial cells. We found that fsl-1- or flagellin-induced expression and secretion of pro-inflammatory cytokines IL1A, IL1B and IL6, the chemokines CXCL8 and CCL2, the adhesion molecules ICAM1 and VCAM1, were significantly blunted in siNLRP3 transfected cells when compared to siCONT transfected cells. These findings are in agreement with our studies using IL1B and TNF, suggesting that NLRP3 is involved in the genesis of pro-inflammatory and pro-labor mediators induced by inflammation.

Tocolytic therapies, such as prostaglandin inhibitors and calcium channel blockers, have some clinical use in delaying preterm labor for high risk women [45], long enough for administration of antenatal corticosteroids or transport of the mother to a tertiary care facility. However, use of these therapies have not been proven to benefit the preterm baby for both short and long term outcomes. Additionally, many preterm births occur in women that do not present the established risk factors for preterm labor. It is thus essential to develop new therapies for preterm birth, which would stem from a better understanding of the mechanisms that drive human labor and delivery. The findings of these studies point to a novel role of NLRP3 in the genesis of inflammation-induced pro-inflammatory and pro-labor mediators in human myometrium (Figure 6). Infection and/or inflammation activate TLRs in myometrium to upregulate the expression of NLRP3 leading to increased production of pro-inflammatory cytokines, chemokines, adhesion molecules and prostaglandins leading to increased uterine contractility and preterm labor, which then culminates in spontaneous preterm birth. This cascade of events is further augmented by pro-inflammatory cytokines. While numerous animal models of disease have demonstrated an essential role for NLRP3 in inflammation [14-19], future studies, using mouse models of preterm birth, are required to investigate if inhibition NLRP3 can delay preterm birth and suppress intrauterine inflammation.

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CONFLICT OF INTEREST

The authors have nothing to declare.

REFERENCES

1 Liu L, Johnson HL, Cousens S, Perin J, Scott S, Lawn JE, Rudan I, Campbell H, Cibulskis R, Li M, Mathers C, Black RE, Child Health Epidemiology Reference Group of WHO, Unicef: Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *Lancet* 2012;**379**:2151-2161.

2 Saigal S, Doyle LW: An overview of mortality and sequelae of preterm birth from infancy to adulthood. *Lancet* 2008;**371**:261-269.

3 Petrou S, Eddama O, Mangham L: A structured review of the recent literature on the economic consequences of preterm birth. *Archives of Disease in Childhood-Fetal and Neonatal Edition* 2011;**96**:F225-F232.

4 Goldenberg RL, Culhane JF, Iams JD, Romero R: Preterm birth 1 - Epidemiology and causes of preterm birth. *Lancet* 2008;**371**:75-84.

5 Lettieri L, Vintzileos AM, Rodis JF, Albini SM, Salafia CM: Does "idiopathic" preterm labor resulting in preterm birth exist? *Am J Obstet Gynecol* 1993;**168**:1480-1485.

6 Sivarajasingam SP, Imami N, Johnson MR: Myometrial cytokines and their role in the onset of labour. *The Journal of endocrinology* 2016;**231**:R101-r119.

7 Christiaens I, Zaragoza DB, Guilbert L, Robertson SA, Mitchell BF, Olson DM: Inflammatory processes in preterm and term parturition. *Journal of reproductive immunology* 2008;**79**:50-57.

8 Chen GY, Nunez G: Sterile inflammation: sensing and reacting to damage. *Nature reviews Immunology* 2010;**10**:826-837.

9 Strowig T, Henao-Mejia J, Elinav E, Flavell R: Inflammasomes in health and disease. *Nature* 2012;**481**:278-286.

10 Kinoshita T, Imamura R, Kushiyama H, Suda T: NLRP3 Mediates NF-κB Activation and Cytokine Induction in Microbially Induced and Sterile Inflammation. *PLOS ONE* 2015;**10**:e0119179.

11 Zuurbier CJ, Jong WMC, Eerbeek O, Koeman A, Pulskens WP, Butter LM, Leemans JC, Hollmann MW: Deletion of the Innate Immune NLRP3 Receptor Abolishes Cardiac Ischemic Preconditioning and Is Associated with Decreased Il-6/STAT3 Signaling. *PLOS ONE* 2012;**7**:e40643.

12 Shigeoka AA, Mueller JL, Kambo A, Mathison JC, King AJ, Hall WF, Correia JdS, Ulevitch RJ, Hoffman HM, McKay DB: An Inflammasome-Independent Role for Epithelial-Expressed Nlrp3 in Renal Ischemia-Reperfusion Injury. *The Journal of Immunology* 2010;**185**:6277-6285.

13 Wang W, Wang X, Chun J, Vilaysane A, Clark S, French G, Bracey NA, Trpkov K, Bonni S, Duff HJ, Beck PL, Muruve DA: Inflammasome-Independent NLRP3 Augments TGF-β Signaling in Kidney Epithelium. *The Journal of Immunology* 2013;**190**:1239-1249.

14 Youm YH, Grant RW, McCabe LR, Albarado DC, Nguyen KY, Ravussin A, Pistell P, Newman S, Carter R, Laque A, Munzberg H, Rosen CJ, Ingram DK, Salbaum JM, Dixit VD: Canonical Nlrp3 inflammasome links systemic low-grade inflammation to functional decline in aging. *Cell metabolism* 2013;**18**:519-532.

15 Vandanmagsar B, Youm Y-H, Ravussin A, Galgani JE, Stadler K, Mynatt RL, Ravussin E, Stephens JM, Dixit VD: The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nat Med* 2011;**17**:179-188.

16 Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, Abela GS, Franchi L, Nunez G, Schnurr M, Espevik T, Lien E, Fitzgerald KA, Rock KL, Moore KJ, Wright SD, Hornung V, Latz E: NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 2010;**464**:1357-1361.

17 Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J: Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 2006;**440**:237-241.

18 Komada T, Usui F, Kawashima A, Kimura H, Karasawa T, Inoue Y, Kobayashi M, Mizushina Y, Kasahara T, Taniguchi Si, Muto S, Nagata D, Takahashi M: Role of NLRP3 Inflammasomes for Rhabdomyolysis-induced Acute Kidney Injury2015;**5**:10901.

19 Kobayashi M, Usui-Kawanishi F, Karasawa T, Kimura H, Watanabe S, Mise N, Kayama F, Kasahara T, Hasebe N, Takahashi M: The cardiac glycoside ouabain activates NLRP3 inflammasomes and promotes cardiac inflammation and dysfunction. *PLOS ONE* 2017;**12**:e0176676.

20 Neudecker V, Haneklaus M, Jensen O, Khailova L, Masterson JC, Tye H, Biette K, Jedlicka P, Brodsky KS, Gerich ME, Mack M, Robertson AAB, Cooper MA, Furuta GT, Dinarello CA, O'Neill LA, Eltzschig HK, Masters SL, McNamee EN: Myeloid-derived miR-223 regulates intestinal inflammation via repression of the NLRP3 inflammasome. *J Exp Med* 2017;**214**:1737-1752.

21 Kim EH, Park M-J, Park S, Lee E-S: Increased expression of the NLRP3 inflammasome components in patients with Behçet's disease. *Journal of Inflammation* 2015;**12**:41.

22 Romero R, Xu Y, Plazyo O, Chaemsaithong P, Chaiworapongsa T, Unkel R, Than NG, Chiang PJ, Dong Z, Xu Z, Tarca AL, Abrahams VM, Hassan SS, Yeo L, Gomez-Lopez N: A Role for the Inflammasome in Spontaneous Labor at Term. *American journal of reproductive immunology (New York, NY : 1989)* 2016.

23 Gomez-Lopez N, Romero R, Xu Y, Plazyo O, Unkel R, Than NG, Chaemsaithong P, Chaiworapongsa T, Dong Z, Tarca AL, Abrahams VM, Yeo L, Hassan SS: A Role for the Inflammasome in Spontaneous Labor at Term with Acute Histologic Chorioamnionitis. *Reproductive sciences (Thousand Oaks, Calif)* 2017;**24**:934-953.

24 Plazyo O, Romero R, Unkel R, Balancio A, Mial TN, Xu Y, Dong Z, Hassan SS, Gomez-Lopez N: HMGB1 Induces an Inflammatory Response in the Chorioamniotic Membranes That Is Partially Mediated by the Inflammasome. *Biology of reproduction* 2016;**95**:130.

25 Jaiswal MK, Agrawal V, Mallers T, Gilman-Sachs A, Hirsch E, Beaman KD: Regulation of apoptosis and innate immune stimuli in inflammation-induced preterm labor. *Journal of immunology (Baltimore, Md : 1950)* 2013;**191**:5702-5713.

26 Lim R, Barker G, 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. *American journal of reproductive immunology (New York, NY : 1989)* 2014;**71**:401-417.

27 Lim R, Barker G, Lappas M: TLR2, TLR3 and TLR5 regulation of pro-inflammatory and pro-labour mediators in human primary myometrial cells. *Journal of reproductive immunology* 2017;**122**:28-36.

28 Lim R, Barker G, Lappas M: A novel role for FOXO3 in human labor: increased expression in laboring myometrium, and regulation of proinflammatory and prolabor mediators in pregnant human myometrial cells. *Biology of reproduction* 2013;**88**:156.

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

30 Lappas M: KLF5 regulates infection- and inflammation-induced pro-labour mediators in human myometrium. *Reproduction* 2015;**149**:413-424.

31 Lim R, Tran HT, Liong S, Barker G, Lappas M: The Transcription Factor Interferon Regulatory Factor-1 (IRF1) Plays a Key Role in the Terminal Effector Pathways of Human Preterm Labor. *Biology of reproduction* 2015.

32 Osman I, Young A, Ledingham MA, Thomson AJ, Jordan F, Greer IA, Norman JE: Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. *Molecular human reproduction* 2003;**9**:41-45.

33 Ledingham MA, Thomson AJ, Jordan F, Young A, Crawford M, Norman JE: Cell adhesion molecule expression in the cervix and myometrium during pregnancy and parturition. *Obstetrics and gynecology* 2001;**97**:235-242.

34 Tattersall M, Engineer N, Khanjani S, Sooranna SR, Roberts VH, Grigsby PL, Liang Z, Myatt L, Johnson MR: Pro-labour myometrial gene expression: are preterm labour and term labour the same? *Reproduction* 2008;**135**:569-579.

35 Young A, Thomson AJ, Ledingham M, Jordan F, Greer IA, Norman JE: Immunolocalization of proinflammatory cytokines in myometrium, cervix, and fetal membranes during human parturition at term. *Biology of reproduction* 2002;**66**:445-449.

36 Hua R, Pease JE, Sooranna SR, Viney JM, Nelson SM, Myatt L, Bennett PR, Johnson MR: Stretch and inflammatory cytokines drive myometrial chemokine expression via NF-kappaB activation. *Endocrinology* 2012;**153**:481-491.

37 Roh CR, Oh WJ, Yoon BK, Lee JH: Up-regulation of matrix metalloproteinase-9 in human myometrium during labour: a cytokine-mediated process in uterine smooth muscle cells. *Molecular human reproduction* 2000;**6**:96-102.

38 Rauk PN, Chiao JP: Interleukin-1 stimulates human uterine prostaglandin production through induction of cyclooxygenase-2 expression. *American journal of reproductive immunology (New York, NY : 1989)* 2000;**43**:152-159.

39 Erkinheimo TL, Saukkonen K, Narko K, Jalkanen J, Ylikorkala O, Ristimaki A: Expression of cyclooxygenase-2 and prostanoid receptors by human myometrium. *The Journal of clinical endocrinology and metabolism* 2000;**85**:3468-3475.

40 Liang Z, Sooranna SR, Engineer N, Tattersall M, Khanjani S, Bennett PR, Myatt L, Johnson MR: Prostaglandin F2-alpha receptor regulation in human uterine myocytes. *Molecular human reproduction* 2008;**14**:215-223.

41 Pollard JK, Mitchell MD: Intrauterine infection and the effects of inflammatory mediators on prostaglandin production by myometrial cells from pregnant women. *Am J Obstet Gynecol* 1996;**174**:682-686.

42 Tribe RM, Moriarty P, Dalrymple A, Hassoni AA, Poston L: Interleukin-1β Induces Calcium Transients and Enhances Basal and Store Operated Calcium Entry in Human Myometrial Smooth Muscle1. *Biology of reproduction* 2003;**68**:1842-1849.

43 Romero R, Espinoza J, Kusanovic JP, Gotsch F, Hassan S, Erez O, Chaiworapongsa T, Mazor M: The preterm parturition syndrome. *BJOG : an international journal of obstetrics and gynaecology* 2006;**113 Suppl 3**:17-42.

44 Ilievski V, Lu SJ, Hirsch E: Activation of toll-like receptors 2 or 3 and preterm delivery in the mouse. *Reproductive sciences (Thousand Oaks, Calif)* 2007;**14**:315-320.

45 Haas DM, Caldwell DM, Kirkpatrick P, McIntosh JJ, Welton NJ: Tocolytic therapy for preterm delivery: systematic review and network meta-analysis. *BMJ* 2012;**345**.

FIGURE LEGENDS

Figure 1. Expression of NLRP3 in human myometrium

(A,B) Human myometrium was obtained at term Caesarean section from women at term in the absence of labor (term no labour, n=8 patients) or during labor at term (term in labor, n=8 patients). **(A)** NLRP3 mRNA abundance was analysed by qRT-PCR. Data are displayed as mean ± SEM. **P*<0.05 vs. no labor (Student's t-test). **(B)** NLRP3 protein expression was analysed by Western blotting. Representative Western blot from 5 patients per group is shown. Data are displayed as mean ± SEM. **P*<0.05 vs. no labor (Student's t-test). **(C)** Human myometrial cells were incubated in the absence or presence of 1 ng/ml IL1B, 10 ng/ml TNF, 250 ng/ml fsl-1, 5 µg/ml poly(I:C) or 1 µg/ml flagellin for 20 h (n=6 patients per treatment). NLRP3 mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to basal. Data are displayed as mean ± SEM. **P*<0.05 vs. basal (paired sample comparison).

Figure 2. Effect of siNLRP3 on pro-inflammatory cytokines in primary myometrial cells

Human primary myometrial cells were transfected with 50 nM siCONT or 50 nM siNLRP3 for 48 h and then treated with **(A-C)** 1 ng/ml IL1B, **(D-G)** 10 ng/ml TNF, **(H-K)** 250 ng/ml fsl-1, or **(L-O)** 1 µg/ml flagellin (flag) for an additional 20 h (n=7 patients). **(A,B,D-F,H-J,L-N)** IL1A, IL1B and IL6 mRNA expression was analysed by qRT-PCR. **(C,G,K,O)** The concentration of IL6 in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to IL1B, TNF, fsl-1 or flag treated cells, and displayed as mean ± SEM. **P*<0.05 vs. siCONT + IL1B (one-way ANOVA); ***P*<0.05 vs. siCONT + TNF (one-way ANOVA); #*P*<0.05 vs. siCONT + fsl-1 (one-way ANOVA); §*P*<0.05 vs. siCONT + flag (one-way ANOVA).

Figure 3. Effect of siNLRP3 on chemokines in primary myometrial cells

Human primary myometrial cells were transfected with 50 nM siCONT or 50 nM siNLRP3 for 48 h and then treated with **(A,E)** 1 ng/ml IL1B, **(B,F)** 10 ng/ml TNF, **(C,G)** 250 ng/ml fsl-1, or **(D,H)** 1 µg/ml flagellin (flag) for an additional 20 h (n=7 patients). CXCL8 and CCL3 mRNA expression was analysed by qRT-PCR. The concentration of CXCL8 and CCL2 in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to IL1B, TNF, fsl-1 or flag treated cells, and displayed as mean ± SEM. **P*<0.05 vs. siCONT + IL1B (one-way ANOVA);

**P<0.05 vs. siCONT + TNF (one-way ANOVA); #P<0.05 vs. siCONT + fsl-1 (one-way ANOVA); §P<0.05 vs. siCONT + flag (one-way ANOVA).

Figure 4. Effect of siNLRP3 on cell adhesion molecules in primary myometrial cells

Human primary myometrial cells were transfected with 50 nM siCONT or 50 nM siNLRP3 for 48 h and then treated with **(A,E)** 1 ng/ml IL1B, **(B,F)** 10 ng/ml TNF, **(C,G)** 250 ng/ml fsl-1, or **(D,H)** 1 µg/ml flagellin (flag) for an additional 20 h (n=7 patients). ICAM1 and VCAM1 mRNA expression was analysed by qRT-PCR. The concentration of sICAM1 and sVCAM1 in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to IL1B, TNF, fsl-1 or flag treated cells, and displayed as mean ± SEM. *P<0.05 vs. siCONT + IL1B (one-way ANOVA); **P<0.05 vs. siCONT + TNF (one-way ANOVA); #P<0.05 vs. siCONT + fsl-1 (one-way ANOVA); §P<0.05 vs. siCONT + flag (one-way ANOVA).

Figure 5. Effect of siNLRP3 on myometrial cell contractions

(A-C) Human primary myometrial cells were transfected with 50 nM siCONT or 50 nM siNLRP3 for 48 h and then treated with 1 ng/ml IL1B for an additional 20 h (n=7 patients). **(A,B)** *PTGS2* and *PTGFR* mRNA expression was analysed by qRT-PCR. **(C)** The concentration of $PGF_{2\alpha}$ in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to IL1B treated cells, and displayed as mean ± SEM. *P<0.05 vs. siCONT + IL1B (one-way ANOVA). **(D)** Cell contraction assays were performed using collagen gels made from human primary myometrial cells transfected with 50 nM siCONT or 50 nM siNLRP3 for 48 h (n=4 patients). The collagen gels were then treated with or without 10 ng/ml TNF for 36 h, and the area of gel was determined. Representative gel contraction image from 1 patient is also shown. For all data, the fold change was calculated relative to IL1B or TNF treated cells, and displayed as mean ± SEM. *P<0.05 vs. siCONT + IL1B (one-way ANOVA); **P<0.05 vs. siCONT + TNF (one-way ANOVA).

Figure 6. A model explaining the potential role of NLRP3 in preterm labor

Bacterial infection acts via TLRs to upregulate the expression of NLRP3 in myometrium. This leads to the increased production of pro-inflammatory cytokines, chemokines, adhesion molecules and contraction associated proteins to activate the myometrium leading to preterm labor and ultimately preterm birth. Additionally, NLRP3-induced induction of pro-inflammatory cytokines IL1B and TNF can further upregulate NLRP3 expression and augment the inflammatory cascade leading to preterm labor.

Figure 1

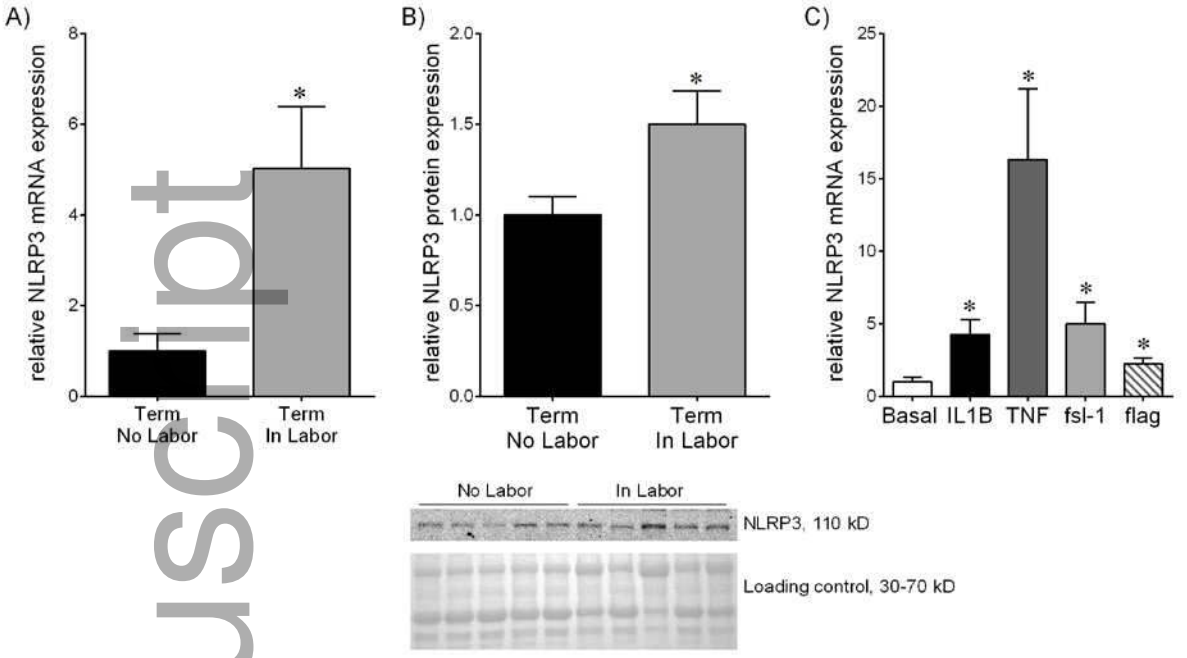


Figure 2

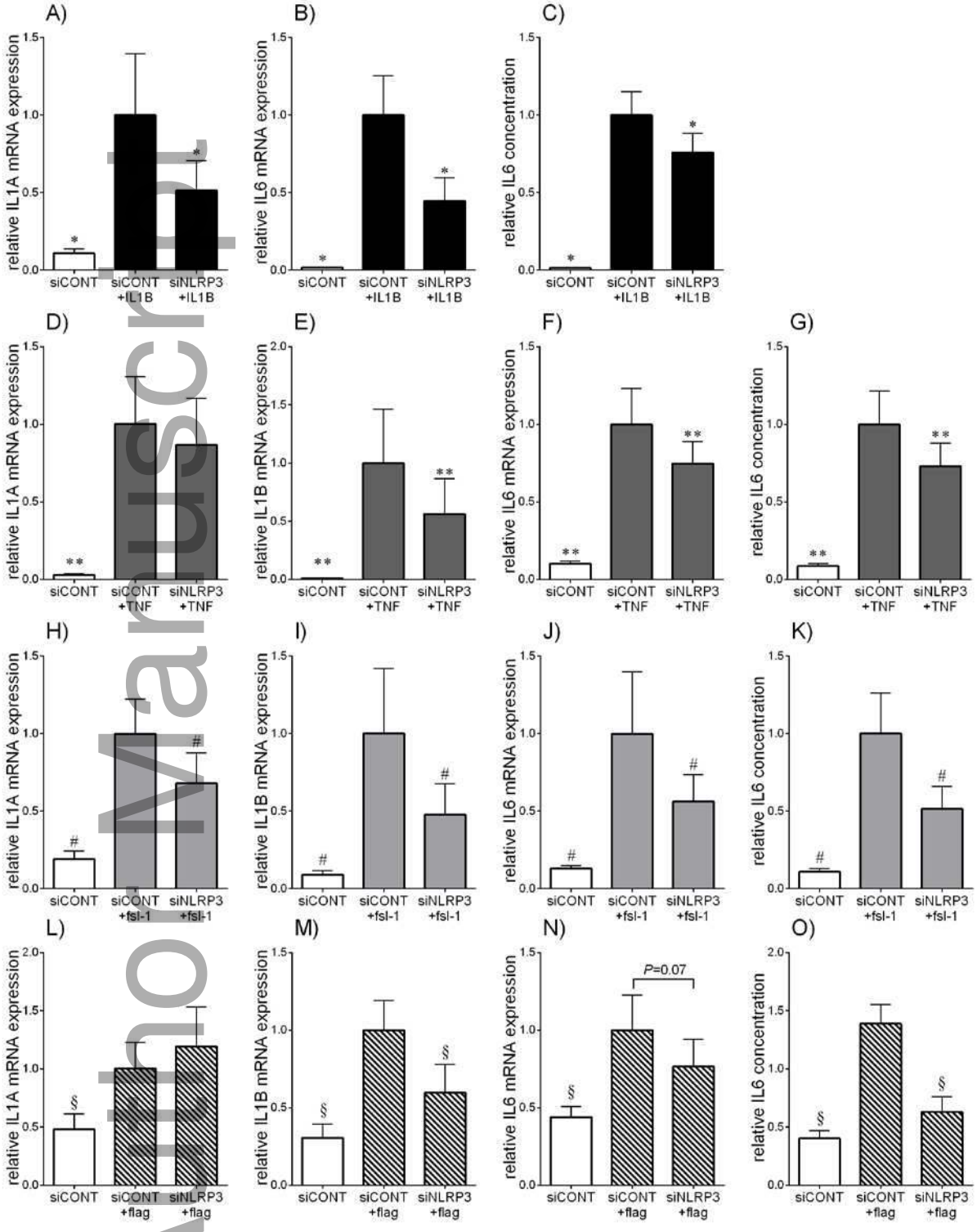


Figure 3

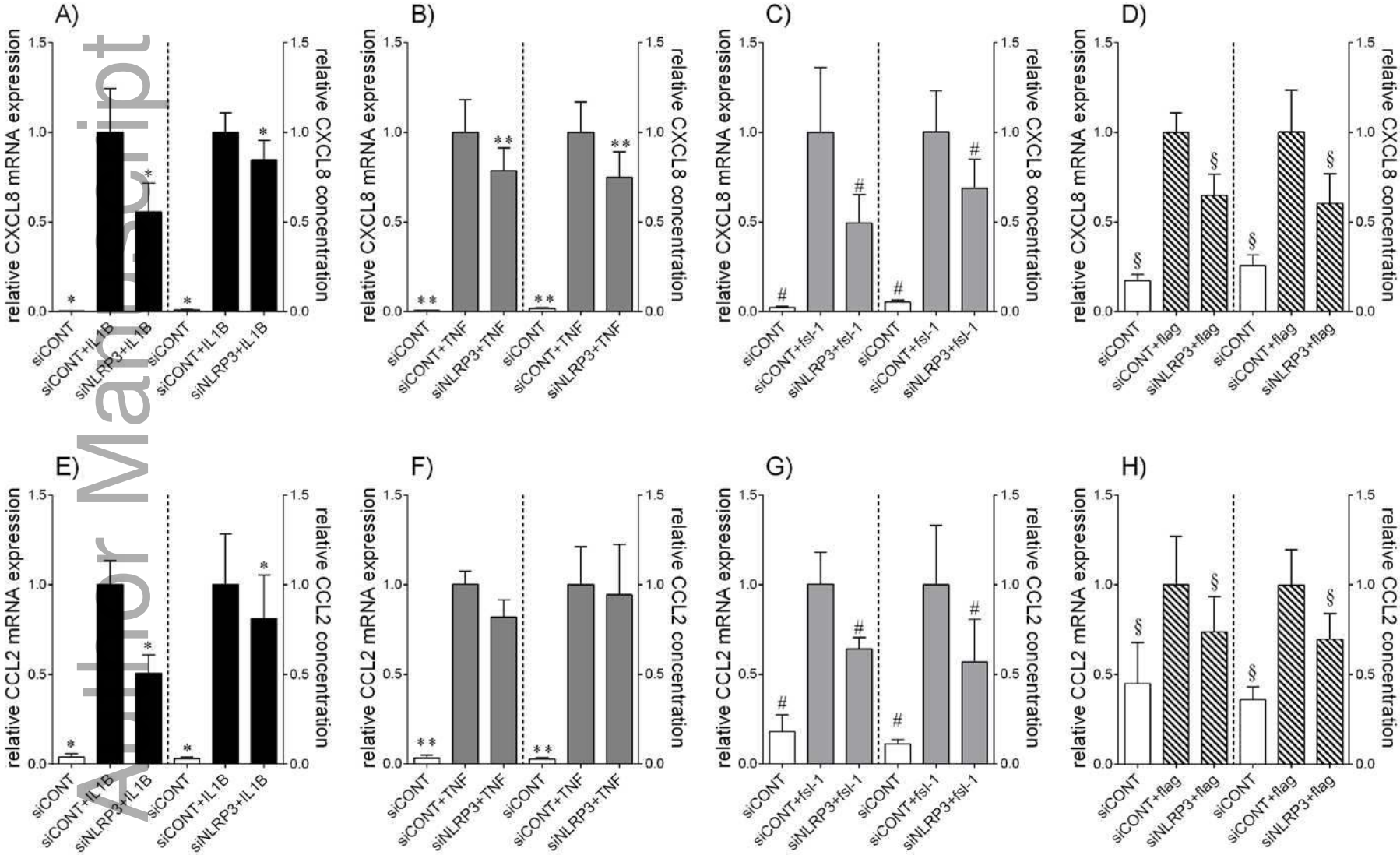


Figure 4

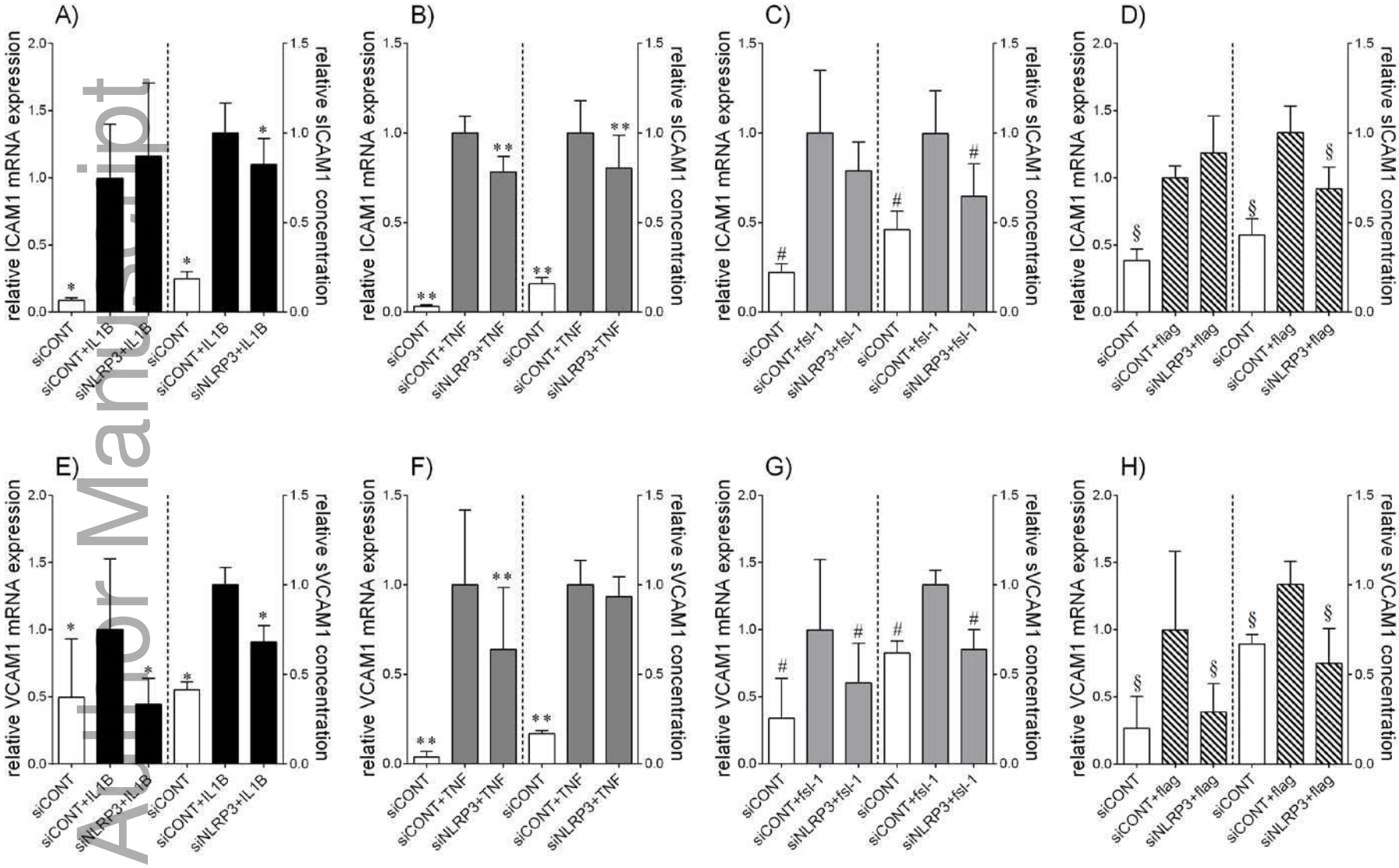


Figure 5

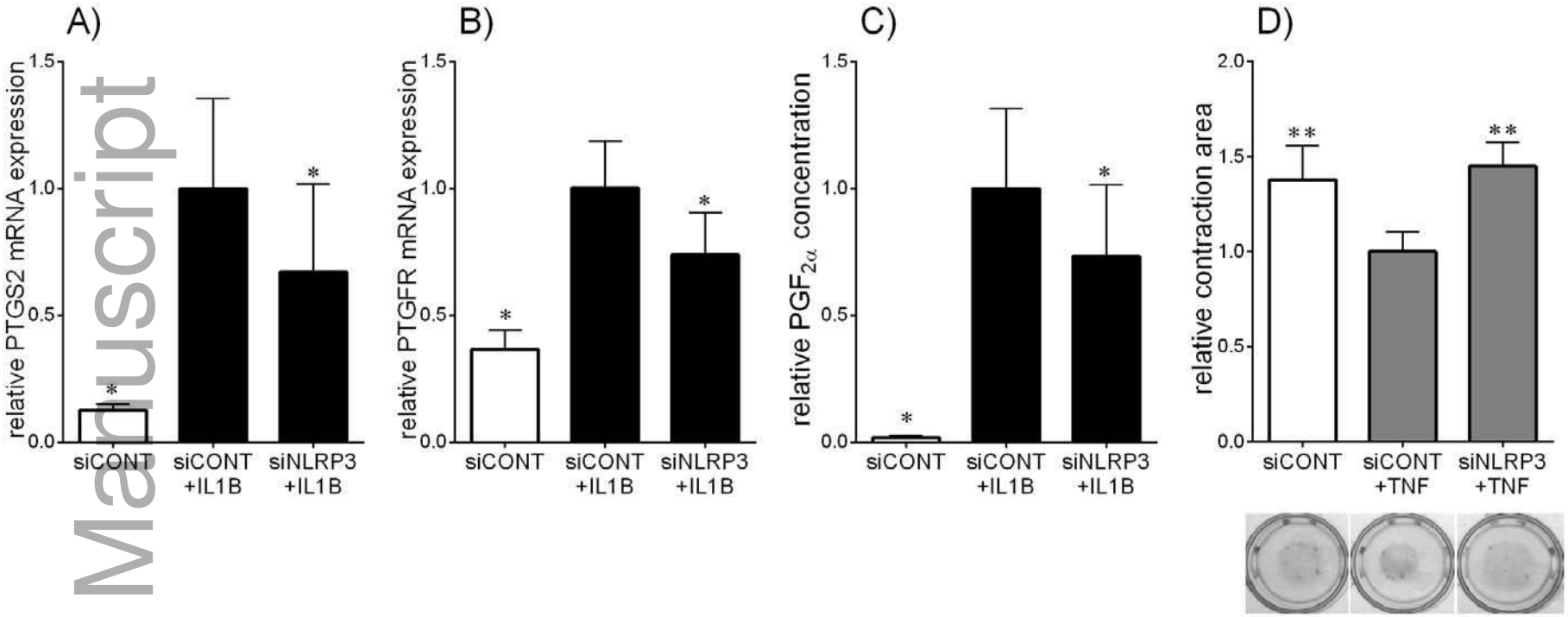
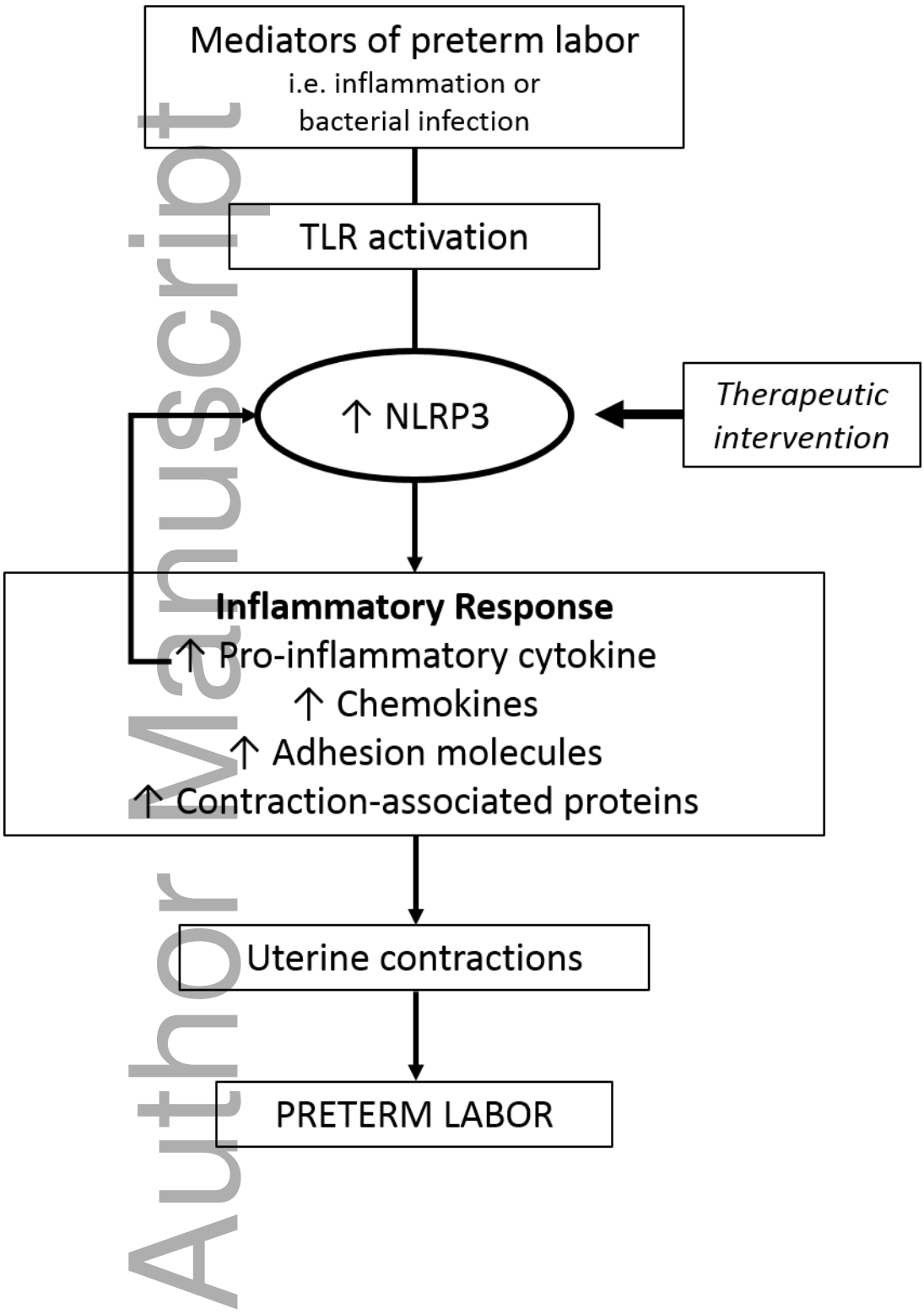


Figure 6



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