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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

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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.

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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.

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Results: NLRP3 expression was higher in myometrium after term spontaneous labor and by TNF, 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_{2α} release; and increase in TNF-stimulated myometrial gel shrinkage as assessed by an in vitro cell contraction assay.

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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.

22
Key words: NLRP3; myometrium; pro-labor mediators; inflammation; preterm birth

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INTRODUCTION

26

Preterm birth continues to be a major contributor to neonatal deaths and morbidity¹. 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 life². The many costs of preterm birth places an enormous burden on both families and the healthcare system³.

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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 labor⁴. Based on microbiological studies, infection is associated with up to 40% of all preterm

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36 births⁵; 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
38 contractions, cervical ripening and rupture of fetal membranes, thus spontaneous preterm birth. Pro-
inflammatory cytokines, chemokines, cell adhesion molecules (CAMs), contraction associated
40 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
42 to play a role in labor associated processes^{6,7}.

44 Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs)
recognise pathogen associated molecular patterns (PAMPs) and damage associated molecular
46 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
48 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,
50 however, a number of studies have shown that NLRP3 can induce inflammation independent of
inflammasome assembly¹⁰⁻¹³. Furthermore, *in vivo*, ablation of NLRP3 prevents inflammation in
52 various animal models of disease¹⁴⁻¹⁹. In line with this, aberrant activation of NLRP3 has been
associated to the pathogenesis of numerous inflammatory disorders including inflammatory bowel
54 disease²⁰, gouty arthritis¹⁷, atherosclerosis¹⁶ and obesity¹⁵.

56 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
58 concentrations of NLRP3 protein compared to those who delivered at term without labor²².
Notably, the expression was even higher in chorioamniotic membranes from women who
60 underwent spontaneous labor at term with acute histologic chorioamnionitis compared to those
without acute histologic chorioamnionitis²³. Whether NLRP3 is also increased in labouring
62 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.

64 Incubation of the chorioamniotic membranes with the alarmin high-mobility group box-1 (HMGB1)
increased the mRNA abundance and protein expression of NLRP3²⁴. In the uterus of pregnant
66 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
68 the effect of pro-inflammatory mediators on NLRP3 expression in cells isolated from human
70 myometrium.

72 NLRP3 has been shown to contribute to inflammation in a number of *in vitro* and *in vivo* models of
disease¹⁴⁻¹⁹. Whether NLRP3 is involved in the genesis of pro-inflammatory and pro-labor
74 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
76 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
78 inflammation associated with preterm labor^{26,27}.

MATERIALS AND METHODS

80

Tissue collection

82 Human myometrium was obtained (with institutional Research and Ethics Committee approval)
from the upper margin of the lower uterine segment incision during Caesarean section.

84 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
86 damaged tissue and visible blood vessels, and then dissected into smaller pieces. Tissues were
immediately snap frozen in liquid nitrogen and stored at -80°C for expression studies, or used
88 immediately for cell culture experiments.

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

94

For the expression studies, myometrial biopsies were collected from women at term Caesarean
96 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
98 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
100 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,
102 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.

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106 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 described²⁸.

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Primary myometrial cell culture

110 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
112 (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
114 *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.
116 Experiments were performed from myometrium obtained from 5 patients.

118 To determine the effect of NLRP3 siRNA on pro-labor mediators, transfection of primary
myometrial cells with siRNA was performed as previously described²⁸. Briefly, myometrial cells at
120 approximately 50% confluence were transfected using Lipofectamine 3000 according to
manufacturer's guidelines (Life Technologies; Mulgrave, Victoria, Australia). NLRP3 siRNA
122 (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
124 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
126 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
128 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
130 in IL1B, TNF-, fsl-1- or flagellin-stimulated cells. Experiments were performed from myometrium
obtained from 7 patients.

132

Cell contraction assay

134 Cell contraction assays were performed as previously described²⁹. Cells (collected from
experiments 2 and 3 above) were re-suspended in 0.25 ml DMEM/F12 (containing 10% FBS) and
136 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
138 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 described³⁰. 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

Western blotting

156 Western blotting was performed as previously described²⁸. 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 described³¹;
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.

Enzyme Immunoassays

166 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_{2α} 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**

178 All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA,
USA). For two sample comparisons, either a paired or unpaired Student's t-test was used to assess
180 statistical significance between normally distributed data; otherwise, the nonparametric Mann-
Whitney U (unpaired) or the Wilcoxon (matched pairs) tests were used. For all other comparisons,
182 the homogeneity of data was assessed by Bartlett's test, and when significant, data were
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 from 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

210 was assessed by qRT-PCR. When compared to siCONT transfected cells, there was an 85%
212 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).

214 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
216 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
218 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
220 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
222 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
224 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
226 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
228 primary myometrial cells and thus not assessed.

230 **Effect of siNLRP3 on chemokines in primary myometrial cells**

Chemokines are involved in leukocyte activation and recruitment into the uterus during labor³².
232 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
234 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
236 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
238 siNLRP3 cells.

240 **Effect of siNLRP3 on adhesion molecules in primary myometrial cells**

Adhesion molecules, which play an important role in leukocyte recruitment into tissues, are
242 increased in labor in the myometrium³³. We have previously shown that the expression of the
adhesion molecules ICAM1 and VCAM1 can be induced by pro-inflammatory cytokines and TLR
244 ligands²⁷. 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
246 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
248 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
250 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
252 sVCAM1 secretion.

254 **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
256 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
258 myometrial contractility, we firstly assessed the effect of siNLRP3 on PTGS2 expression and
PGF_{2α} signalling (i.e. PGF_{2α} secretion and expression of its receptor PTGFR). As shown in Figure
260 5, IL1B treatment significantly increased PTGS2 and PTGFR mRNA expression and release of
PGF_{2α}. The effect of siNLRP3 was a significant attenuation of IL1B-induced PTGS2 and PTGFR
262 mRNA expression and release of PGF_{2α}.

264 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
266 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
268 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
270 significantly suppressed in siNLRP3 transfected cells (Figure 5D).

272 **DISCUSSION**

In extension to the studies in fetal membranes ²², herein we report that spontaneous term labor is
274 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
276 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
278 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

280 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},
282 significantly increased NLRP3 mRNA expression in myometrial cells.

284 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
286 TNF, through a feed-forward mechanism, can amplify the inflammatory environment by inducing
chemokines³⁶ and cellular adhesion molecules³³ to promote an influx of leukocytes into
288 myometrium. IL1B and TNF can also increase pro-MMP9 expression³⁷, which is higher in
myometrium during human labor³⁷ and postulated to participate in uterine remodelling during
290 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
292³⁸⁻⁴⁰, stimulating the production of uterotonic prostaglandins⁴¹, and potentiating myometrial
contractions by inducing the entry of calcium into myometrial smooth muscle cells⁴².

294
Studies in non-gestational tissues have shown that NLRP3 can induce pro-inflammatory mediators
296 independent of inflammasome assembly¹⁰⁻¹³. 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.
298 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
300 expression of the pro-inflammatory cytokine IL6, chemokines (CXCL8, CCL2), adhesion molecule
VCAM1, and contraction associated proteins (*PTGS2*, *PTGFR*, *PGF_{2α}*) when treated with IL1B.
302 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
304 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
306 contraction capability in human myometrial cells treated with TNF. Overall, our data suggests that
NLRP3 positively regulates the activation of myometrium.

308
One of the main causes of preterm birth, especially those before 32 weeks gestation, is infection⁴³.
310 Bacterial and/or viral pathogens bind to discrete TLRs to induce an inflammatory response that
results in preterm birth⁴⁴. In fetal membranes, the expression of NLRP3 is higher from women who
312 underwent spontaneous labor at term with acute histologic chorioamnionitis compared to those
without acute histologic chorioamnionitis²³. We could not assess the effect of infection on NLRP3
314 expression in myometrium due to the difficulty in obtaining these samples. Notwithstanding this

316 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
318 by TLR2 or TLR3 ligands ²⁵.

320 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
322 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
324 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
326 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.

328 Tocolytic therapies, such as prostaglandin inhibitors and calcium channel blockers, have some
330 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
332 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
334 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
336 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
338 inflammation activate TLRs in myometrium to upregulate the expression of NLRP3 leading to
increased production of pro-inflammatory cytokines, chemokines, adhesion molecules and
340 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
342 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
344 investigate if inhibition NLRP3 can delay preterm birth and suppress intrauterine inflammation.

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352

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358

CONFLICT OF INTEREST

360 The authors have nothing to declare.

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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 \pm 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 \pm 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 μ g/ml poly(I:C) or 1 μ g/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 \pm 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 μ g/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 \pm 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 μ g/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 \pm 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 $\text{PGF}_{2\alpha}$ 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

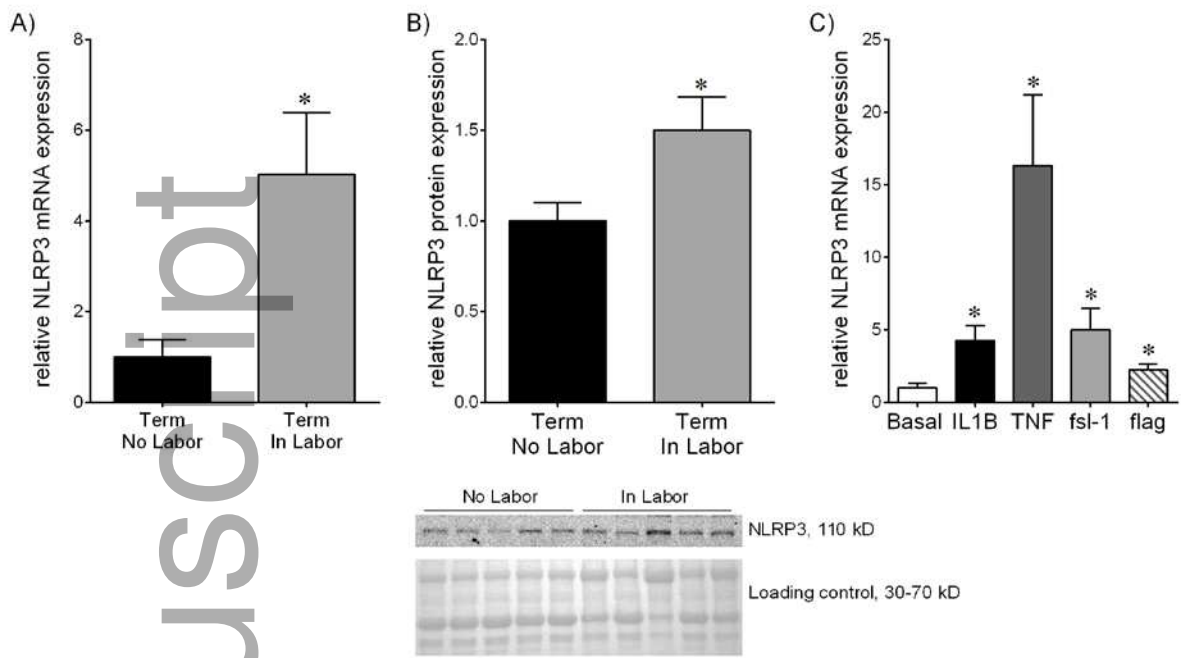


Figure 2

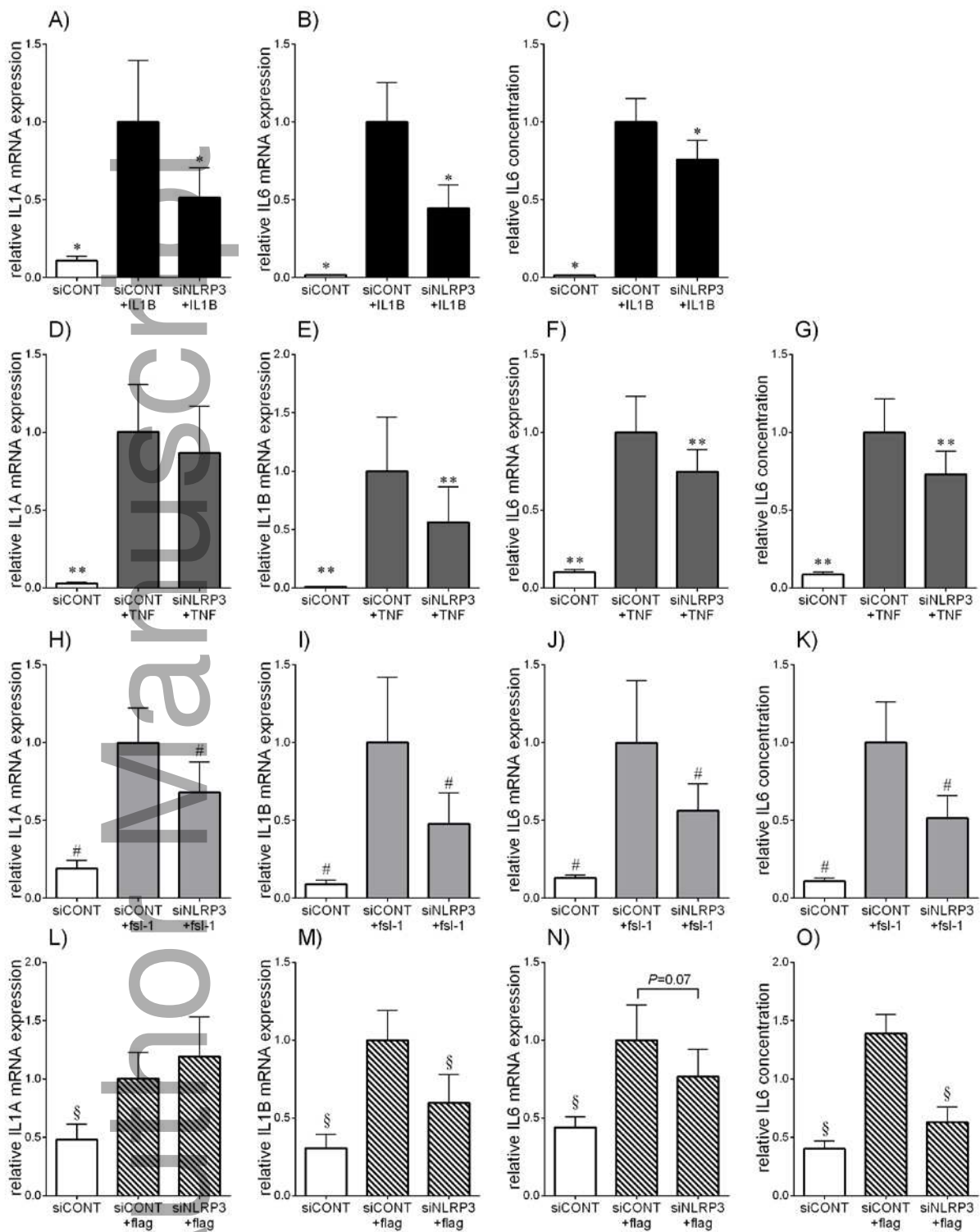


Figure 3

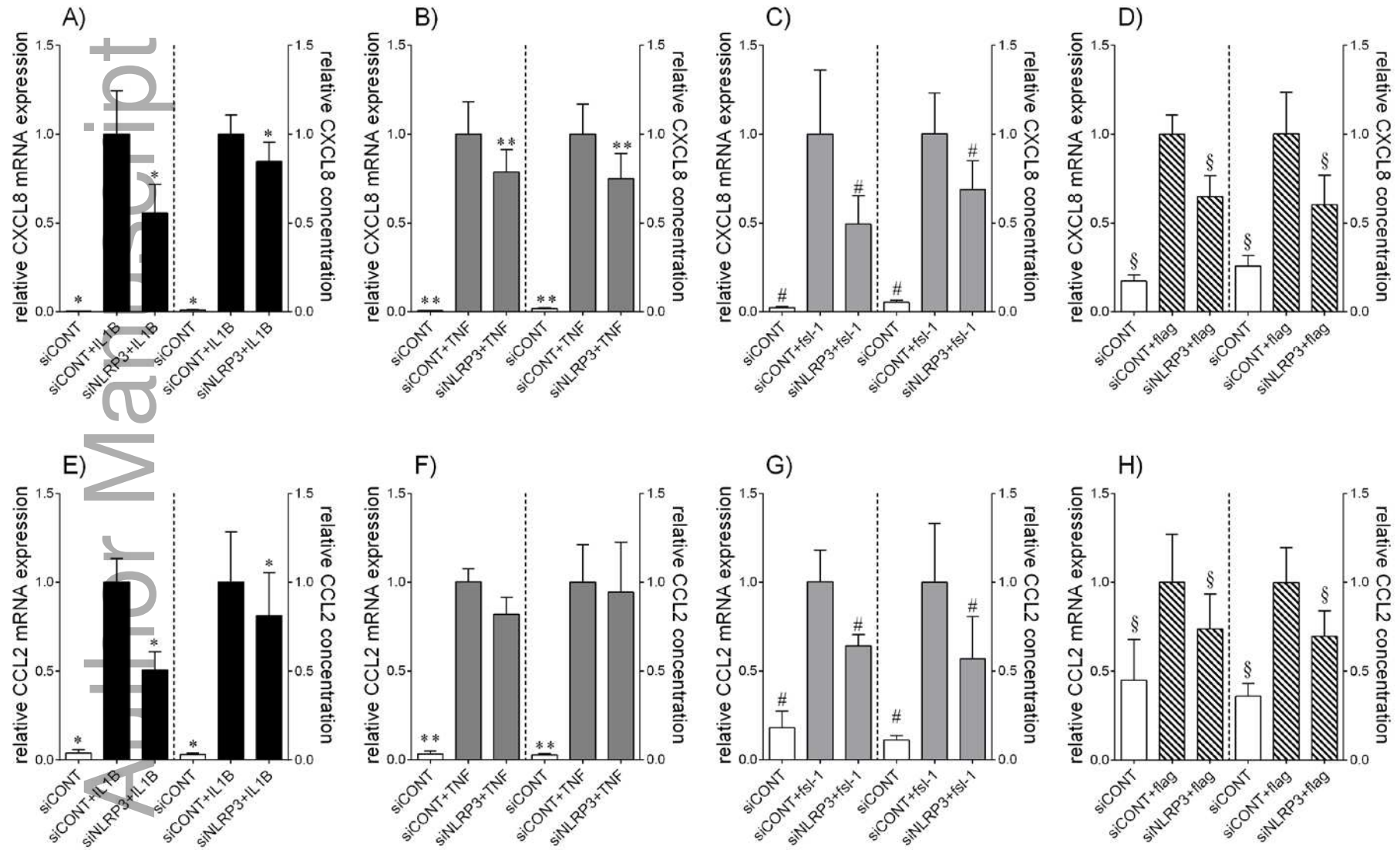


Figure 4

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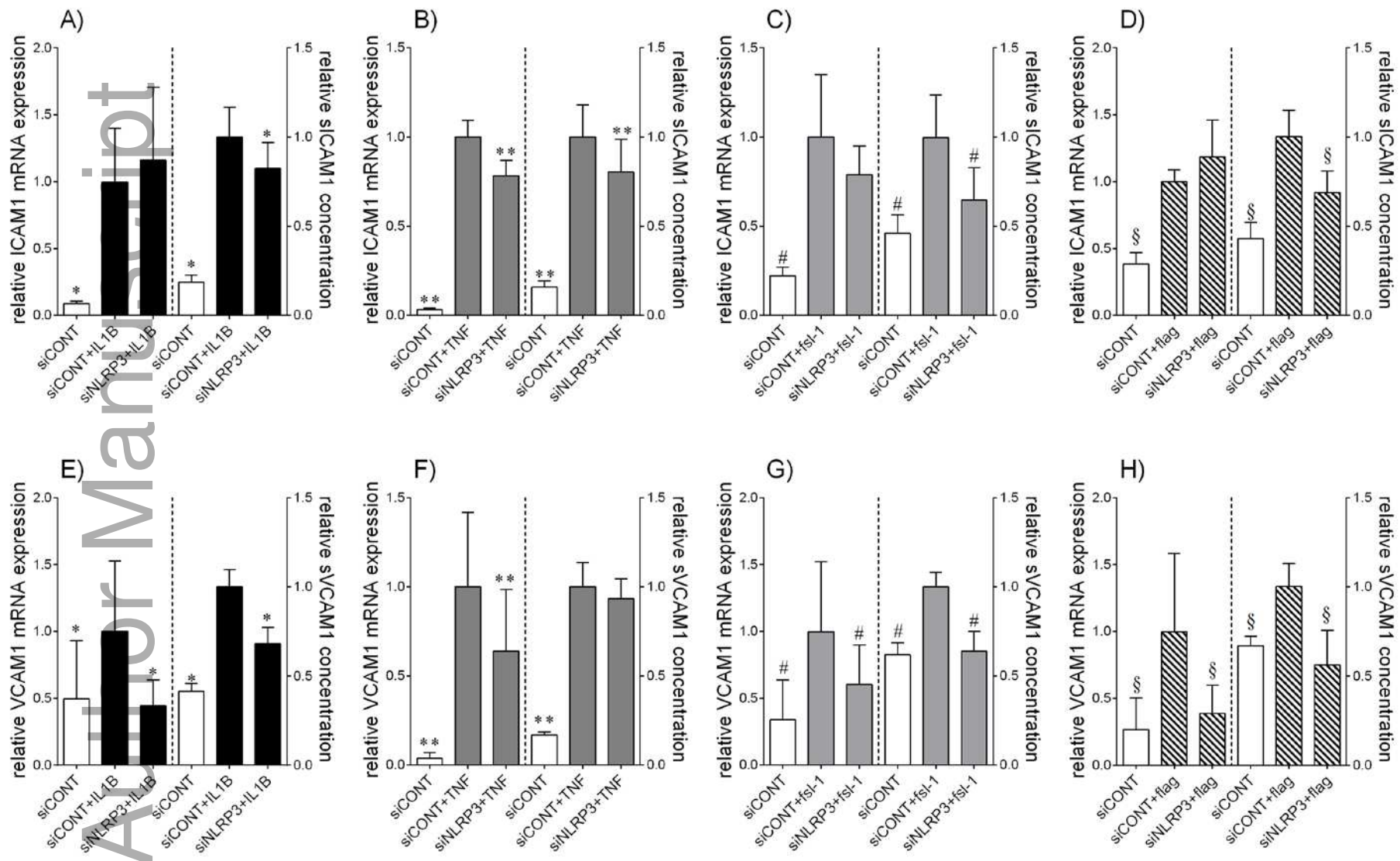


Figure 5

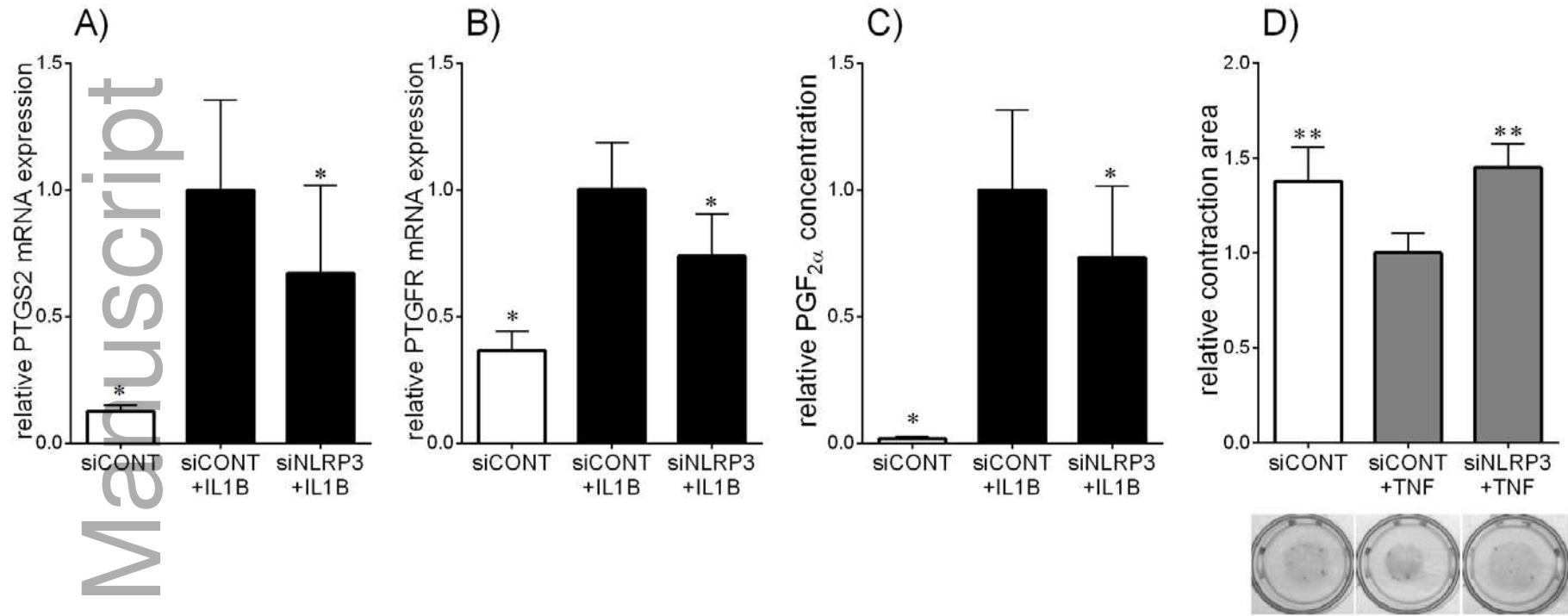


Figure 6

