Author/s:
Lim, R;Lappas, M

Title:
Expression and function of macrophage-inducible C-type lectin (Mincle) in inflammation driven parturition in fetal membranes and myometrium

Date:
2019-07-01

Citation:
Lim, R. & Lappas, M. (2019). Expression and function of macrophage-inducible C-type lectin (Mincle) in inflammation driven parturition in fetal membranes and myometrium. Clinical and Experimental Immunology, 197 (1), pp.95-110. https://doi.org/10.1111/cei.13281.

Persistent Link:
https://hdl.handle.net/11343/285575

DR. MARTHA LAPPAS (Orcid ID : 0000-0002-4744-6929)

Article type : Original Article

Expression and Function of Macrophage-Inducible C-type Lectin (Mincle) in Inflammation Driven Parturition in Fetal Membranes and Myometrium

Short title: Mincle and labor

Ratana Lim and Martha Lappas

Mercy Perinatal Research Centre, Mercy Hospital for Women, Heidelberg, Victoria, Australia
Obstetrics, Nutrition and Endocrinology Group, Department of Obstetrics and Gynaecology, University of Melbourne, Victoria, Australia

Correspondence and Reprint Requests to:
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

ABSTRACT

The pivotal role of inflammatory processes in human parturition is well known, but not completely understood. We have performed a study to examine the role of Macrophage-inducible C-type lectin (Mincle) in inflammation-associated parturition. Using human samples, we show that spontaneous labor is associated with upregulated Mincle expression in the myometrium and fetal membranes. Mincle expression was also increased in fetal membranes and myometrium in the presence of pro-labor mediators, the pro-inflammatory cytokines IL1B and TNF, and toll-like receptor (TLR) ligands fsl-1, poly(I:C), LPS and flagellin. These clinical studies are supported by mouse studies, where an inflammatory challenge in a mouse model of preterm birth increased Mincle expression in the uterus. Importantly, elimination of Mincle decreased the effectiveness of pro-inflammatory cytokines and TLR ligands to induce the expression of pro-labor mediators, namely pro-inflammatory cytokines and chemokines, contraction-associated proteins and prostaglandins, and extracellular matrix remodelling enzymes, matrix metalloproteinases. The data presented in this study suggests that Mincle is required when inflammatory activation precipitates parturition.

Keywords: Mincle; human labor; myometrium; fetal membranes; inflammation

INTRODUCTION

Pattern recognition receptors (PRRs) are located on cell surfaces and recognise pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) such as proteins, nucleic acids, carbohydrates and lipids. PAMPs and DAMPs bind to PRRs to activate signal transduction pathways resulting in activation of multiple signalling pathways including NF-κB. Activation of these pathways leads to transcription of pro-inflammatory cytokines and chemokines, which in turn attract leukocytes such as neutrophils to the site of pathogen invasion or tissue damage. There are four main classes of PPRs; toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs). TLRs and NLRs have been shown to play a role in regulating the inflammatory processes associated with human labor (1-8). There are, however, no studies on the expression and function of CLRs in parturition.

CLRs are a large family of proteins that are divided into 17 groups based on functional and structural characteristics. They contain an extracellular recognition domain that recognises a wide range of microbial and fungal carbohydrates and lipids (9-11). CLRs also contain a signal transmembrane domain and a cytoplasm domain. Macrophage-inducible C-type lectin (Mincle; gene name CLEC4E) is a CLR that was first identified in 1999 as a downstream target of the

transcription factor NF-IL6 in macrophages (12). Mincle expression is strongly induced in response to several inflammatory stimuli and stresses such as LPS, TNF, IL6 and saturated fatty acids (12-15) and in various diseases characterised by increased inflammation including obesity, rheumatoid arthritis, allergic contact dermatitis, ischemic stroke, traumatic brain injury, and hepatitis (13, 16-20).

Mincle binding to its ligands initiates a cascade of events that leads to the recruitment of spleen tyrosine kinase (Syk) which activates a cascade of signalling events that leads to the induction of inflammatory genes via NF-κB, mitogen activated protein kinase (MAPK), activator protein 1 (AP-1) or nuclear factor of activated T-cells (NFAT) (21-27). Numerous studies have implicated Mincle in regulating inflammation. Mincle deletion, blockade or pharmacologic inhibition attenuates the inflammatory response in vivo (13-15, 18, 20, 28) and in vitro (14, 17, 18, 25).

Therefore, we hypothesised that Mincle expression would be upregulated during labor and with infection in fetal membranes and myometrium. Furthermore, we hypothesised that pro-inflammatory cytokines and TLR ligands would induce Mincle expression leading to upregulated expression and secretion of mediators involved in the processes of labor. To examine this, we (i) characterised the expression of Mincle in human myometrium and fetal membranes from laboring and non-laboring women at term and preterm; (ii) investigated the effect of pro-inflammatory cytokines, and bacterial and viral products on Mincle expression in fetal membranes and myometrium; and (iii) determined the effect of Mincle siRNA knockdown on inflammation induced pro-labor mediators in human primary myometrial and amnion cells.

METHODS

Tissue collection

Placenta (with attached fetal membranes) and myometrium was collected at the time of delivery from women delivering a singleton baby. Written informed consent was obtained from all women. Myometrium was obtained from the upper margin of the lower uterine segment incision during Caesarean section. Tissue samples were obtained from women who delivered healthy, singleton infants. Criteria for recruited patients were as we have previously described (29) and excluded women with any underlying medical conditions (e.g. diabetes, asthma, preeclampsia).

Tissues were processed within 15 mins of delivery as previously described (30, 31). For the Mincle expression studies, placenta and myometrium was obtained from laboring and non laboring women at term or preterm as detailed below. Tissue samples were immediately snap frozen in liquid

nitrogen and stored at -80^{o}C until processed by RT-qPCR as detailed below. For the tissue explant and cell culture experiments, placenta and myometrium was obtained from non laboring women at the time of term Caesarean section and used immediately as detailed below.

For the Mincle expression studies, myometrium was obtained from women at term Caesarean section (i) in the absence of labor or (ii) during active spontaneous labor (n=8 patients per group) as we have previously described (29). Labor was defined as the presence of regular uterine contractions (every 3–4 min) resulting in cervical effacement and dilation. Fetal membranes (amnion and choriodecidua) were obtained from women at preterm or term (i) undergoing elective Caesarean section in the absence of labor or (ii) after spontaneous labor and vaginal delivery (n=9 patients per group) as we have previously described (29). Fetal membranes from the non-laboring group were obtained from the area overlying the cervix (i.e. supracervical site, SCS) and from the site of membrane rupture in the after labor group as previously described (32). For the term samples, indications for Caesarean section in the absence of labor were breech presentation and/or previous Caesarean section. Indications for Caesarean section in the term laboring samples were for fetal malpresentation, fetal distress and delayed or failure to progress. Indications for preterm delivery (in the absence of labor) were for placenta praevia, vasa praevia, placental abruption or antepartum haemorrhage. None of the patients received any medications to augment or induce labor, had pre-labor rupture of membranes, or any clinical signs of infection. The relevant clinical characteristics of the patients used for these studies are detailed in Supplementary Tables 1 and 2.

To characterise infection associated changes in Mincle expression, amnion was collected from women undergoing Caesarean section in the absence of labor (i) with histologically confirmed acute chorioamnionitis or (ii) without histologically confirmed acute chorioamnionitis (n=8 patients per group) as previously described (33). As previously described (33), choriodecidual tissue could not be collected from the samples with histologically confirmed acute chorioamnionitis as it was degraded. The relevant clinical characteristics of the patients used for these studies are detailed in Supplementary Table 3.

Tissue Explants

To elucidate the effect of pro-inflammatory insults on Mincle expression, tissue explants were performed as previously described (34). Briefly, fresh fetal membranes and myometrium (n=6 patients) were dissected, equilibrated in a humidified atmosphere of 8% O_2 (fetal membranes) or 21% O_2 (myometrium) for 1 h, and then incubated in DMEM containing 100 U/ml penicillin G and 100 μg/ml streptomycin (50 mg tissue/ml) with or without 10 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), 20 μg/ml poly(I:C) (Sigma-Aldrich; St. Louis, MO, USA), 10 μg/ml LPS (derived from E. coli strain 026:B6; Sigma-Aldrich; St. Louis, MO) or 1 μg/ml flagellin (purified flagellin from *Salmonella typhimurium;* InVivoGen; San Diego, California, USA). After 20 h, tissues were collected and stored at −80°C until assayed for Mincle mRNA expression by RT-qPCR as detailed below.

Primary myometrial and amnion epithelial and mesenchymal cell culture

In order to elucidate if Mincle regulates pro-inflammatory mediators, we performed siRNA knockdown experiments in primary myometrial and amnion cells (n=6-7 patients per experiment). Myometrial cells were isolated and cultured as previously described (30). Isolation of amnion epithelial cells and mesenchymal cells was performed as described (35) with minor modifications. Briefly, approximately 3 g of amnion fragments were incubated in 10 ml of DMEM/F-12 with 1% penicillin-streptomycin and 0.25% trypsin for 35 min at 37°C with gentle agitation. After straining through a 100 μm cell strainer, the eluate was neutralised with 1% FBS and remaining fragments were further digested in another 10 ml of DMEM/-F12 with 0.25% trypsin for 35 min at 37°C. Eluate was combined with previous digest and epithelial cells were pelleted by centrifugation at 500 x *g* for 10 min, resuspended in complete media (DMEM/F-12 containing 10% FBS and 1% penicillin-streptomycin) and plated in tissue culture flasks. Flasks were incubated at 37°C, 5% CO_2 and 8% O_2 with media changes 4 h after initial plating, then 24-48 h thereafter until confluent. The de-epithelialized amnion fragments were washed extensively in PBS and then incubated in 10 ml DMEM/F-12 containing 1% penicillin-streptomycin and 0.125% collagenase A at 37°C for 1 h with gentle agitation, until fragments had dissolved. After straining, eluate was centrifuged at 500 x *g* for 10 min. Pelleted mesenchymal cells were plated in complete media (DMEM/F-12 containing 10% FBS and 1% penicillin-streptomycin) in tissue culture flasks. Upon confluency (3-5 days), epithelial and mesenchymal cells were trypsinized and plated in 48-well plates in complete media for siRNA transfection experiments.

Mincle siRNA (siCLEC4E) and negative control siRNA (siCONT) (Ambion; Thermo Fisher Scientific; Scoresby, Vic, Australia) were transfected in amnion and myometrial cells using RNAiMax (Life Technologies; Mulgrave, Victoria, Australia) according to manufacturer's guidelines. Cells were transfected with 50 nM siCLEC4E or 50 nM siCONT for 48 h, followed by treatment with or without 1 ng/ml IL1B, 10 ng/ml TNF, 250 ng/ml fsl-1 or 5 μg/ml poly(I:C) for an additional 20 h. After final incubation, cells and media were collected and stored at −80°C until

analysed as detailed below. MTT assay was used to assess cell viability as we have previously described (36).

Cell contraction assay

To determine the effect of siCLEC4E on myometrial cell contractility, gel contraction assays were performed as previously described (37). The area of the gel was determined using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA). Experiments were performed in myometrium obtained from 6 patients.

Mice studies

To determine the effect of preterm labor on Mincle mRNA expression, a mouse model was utilised, injecting LPS on gestational day (gd) 15.5 mice and collecting myometrium at delivery of first pup. Animal studies (conducted with approval from the Austin Health's Animal Ethics Committee) were performed as we have previously described (38). Briefly, gd 15.5 mice were injected i.p. with LPS (serotype O26:B6; 15 μg in 50 μl of PBS; Sigma) or sterile PBS (vehicle control). In the LPS group, mice delivered the first pup between 18-22 h post-LPS treatment. None of the vehicle-injected mice went into labor. The mice were killed on the birth of one pup, and time-matched controls were killed directly afterward. Myometrial tissue was washed in PBS, flash frozen and stored at -80°C until further analysis by RT-qPCR as detailed below.

RNA extraction and RT-qPCR

RNA extractions, cDNA synthesis and RT-qPCR was performed as previously described (39) using 100 nM of pre-designed and validated QuantiTect primers (primer sequences not available) (Qiagen; Chadstone Centre, Vic, Australia). For normalisation of data, the average Ct of two housekeeping gene, YWHAZ and succinate dehydrogenase (SDHA), was used and the fold differences determined using the comparative Ct method.

Enzyme immunoassays

Commercial assays were used to measure the levels of IL6, CCL2, CXCL1, CXCL8, sICAM1 (R&D Systems; Minneapolis, MN, USA) and $PGF_{2\alpha}$ (Cayman Chemical Company; Ann Arbor, MI, USA) in the incubation media. The interassay and intraassay coefficients of variation for all assays were less than 10%.

Gelatin zymography

Incubation media was also collected and assessment of MMP9 was performed by gelatin zymography as previously described (40). Gels were scanned using a ChemiDoc XRS system (Bio-Rad Laboratories; Gladesville, NSW, Australia), inverted, and densitometry performed using Quantity One image analysis software (Bio-Rad Laboratories; Gladesville, NSW, Australia).

Statistical analysis

All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Normality of the data was assessed using the Shapiro-Wilk test. For Figure 1, an unpaired Student's t-test was used to assess statistical significance between normally distributed data, otherwise the Mann-Whitney U test was used. For Figure 2, the Wilcoxon test was used to assess statistical significance. For all other comparisons, data were analysed by a repeated measures one-way ANOVA (with LSD post-hoc testing to discriminate among the means); non-normally distributed data were logarithmically transformed before analysis. Statistical significance was ascribed to a P value ≤ 0.05. Data is expressed as mean ± SEM.

RESULTS

Mincle expression is upregulated in laboring fetal membranes and myometrium

The first aim was to determine whether the expression of Mincle mRNA changed with respect to labor. In human fetal membranes, Mincle mRNA expression was significantly increased with term labor (Figure 1A) and preterm labor (Figure 1B), compared to non-laboring tissues. There was also a significant increase in Mincle mRNA expression in amnion collected from preterm women with histological chorioamnionitis (Figure 1C), compared to preterm controls without histological chorioamnionitis. In myometrium, there was a significant increase in Mincle mRNA with human term labor (Figure 1D) compared to non-laboring tissue. To determine the effect of preterm labor on Mincle mRNA expression, a mouse model was utilised, injecting LPS on gestational day 15.5 mice and collecting myometrium at delivery of first pup. Figure 1E demonstrates that Mincle mRNA expression is significantly increased in mouse myometrium with preterm (LPS) labor.

Pro-inflammatory mediators increase Mincle expression in fetal membranes and myometrium

To further elucidate the effect of labor on Mincle expression, tissue explants were incubated in the presence of known labor mediators, namely pro-inflammatory cytokines IL1B and TNF, and bacterial and viral products associated with TLR signalling; fsl-1 (TLR2/6), poly(I:C) (TLR3), LPS (TLR4) and flagellin (TLR5). All six treatments significantly increased Mincle mRNA expression in fetal membranes (Figure 2A) and myometrium (Figure 2B).

Knockdown of Mincle suppresses pro-inflammatory cytokines and chemokines in human primary myometrial and amnion mesenchymal cells *in vitro*

Subsequent experiments investigated the effect of Mincle suppression, using siRNA, in the presence of inflammatory, pro-labor stimuli. To determine if Mincle regulates cytokine-induced expression of pro-labor mediators, primary cells isolated from human myometrium and amnion (epithelial and mesenchymal cells), were transfected with Mincle siRNA (siCLEC4E). The efficacy of transfection was assessed by RT-qPCR and there was >75% decrease in Mincle mRNA expression in siCLEC4E transfected cells. There was no effect of siCLEC4E on cell viability as determined by MTT cell viability assay (data not shown).

Firstly, in amnion mesenchymal cells, treatment with IL1B significantly increased the expression of pro-inflammatory cytokines IL1A and IL6 mRNA expression and the secretion of IL6 (Figure 3A-C) and chemokines CCL2 and CXCL8 mRNA expression and secretion (Figure 3D-G), compared to siCONT transfected cells. In siCLEC4E transfected cells, there was a significant decrease in IL1B induced IL1A, IL6, CCL2 and CXCL8 mRNA expression (Figure 3A-B,D,F) and release of IL6 and CXCL8 (Figure C,G), but not CCL2 (Figure 3E). Similarly in amnion mesenchymal cells treated with TNF, siCLEC4E transfected cells displayed a significant decrease in TNF-induced IL1A, IL1B, IL6, CCL2 and CXCL8 mRNA expression (Figure 3H-J,L,N) and release of IL6 and CXCL8 (Figure 3K,O), but not CCL2 release (Figure 3M).

Figures 4-6 demonstrates the effect of siCLEC4E transfection in myometrial cells in the presence of pro-labor mediators; pro-inflammatory cytokine IL1B (Figure 4), TLR2/6 ligand fsl-1 (Figure 5) and TLR3 ligand poly(I:C) (Figure 6). Treatment with IL1B significantly increased TNF, IL1A, IL6, CCL2, CXCL1 and CXCL8 mRNA expression (Figure 4A-C,E,G,I) and release of IL6, CCL2, CXCL1 and CXCL8 (Figure 4D,F,H,J). While there was no effect of siCLEC4E transfection on IL1B-induced TNF mRNA expression (Figure 4A), there was a significant decrease in IL1A mRNA expression (Figure 4B) and IL6, CCL2, CXCL1 and CXCL8 mRNA expression and release (Figure 4C-J). Treatment of myometrial cells with fsl-1 (Figure 5) and poly(I:C) (Figure 6) displayed very similar results; the expression of TNF, IL1A, IL1B, IL6, CCL2, CXCL1 and CXCL8 mRNA and release of IL6, CCL2, CXCL1 and CXCL8 was significantly decreased in siCLEC4E transfected cells, compared to siCONT cells treated with fsl-1 or poly(I:C).

Knockdown of Mincle suppresses myometrial cell contractility *in vitro*

Pro-inflammatory cytokines can participate in myometrial activation by inducing contraction associate proteins such as PTGS2 (41) and promote the production of the uterotonic prostaglandin

$PGF_{2\alpha}$ (42) which acts via the prostaglandin F receptor (PTGFR) (43). Thus, it was interest to explore the effect of siCLEC4E on expression of contraction associated proteins (Cx43, OXTR, PTGS and PTGFR), $PGF_{2\alpha}$ and myometrial cell contractility in situ. Treatment of siCONT cells with IL1B and TNF significantly decreased Cx43 mRNA expression (Figure 7A,D), and significantly increased PTGFR mRNA expression and secretion of $PGF_{2\alpha}$, (Figure 7B-C,E-F), compared to basal cells. While there was no effect of siCLEC4E on IL1B-induced Cx43 mRNA expression (Figure 7A), there was a significant decrease in TNF-induced Cx43 mRNA expression (Figure 7D). In siCLEC4E transfected cells, there was a significant decrease in IL1B- and TNF-induced PTGFR mRNA expression and secretion of $PGF_{2\alpha}$ (Figure 7B-C,E-F). There was no change with PTGS2 or OXTR mRNA expression in siCLEC4E transfected cells (data not shown). To accompany these experiments, we determined whether Mincle can regulate myometrial contractility *in situ* by utilising an *in vitro* gel contraction assay; primary myometrial cells deficient in Mincle were embedded into 3D collagen gel matrices where the cells could contract in response to TNF. As shown in Figure 7G, TNF induced cell contractility, evidenced by increased gel shrinkage, compared to siCONT cells without TNF. In siCLEC4E transfected cells, TNF-induced gel shrinkage was significantly suppressed.

Knockdown of Mincle suppresses expression of ECM remodelling enzymes

The pro-inflammatory cytokines IL1B and TNF play important roles in the initiation of labor, particularly the rupture of membranes. The two constituent cell populations of the amnion, epithelial and mesenchymal cells, were both investigated, as they independently respond to different stimuli to produce different matrix metalloproteinases (MMPs); for example, amnion epithelial cells, but not mesenchymal cells, participate in IL1B-induced MMP9 release (35), and in mesenchymal cells, but not epithelial cells, thrombin can induce both MMP1 and MMP9 expression (44).

In amnion epithelial cells, we found that IL1B significantly increased MMP9 mRNA expression (Figure 8A) and release of pro MMP9 (Figure 8B). In siCLEC4E transfected cells, there was a significant decrease in IL1B-induced MMP9 mRNA expression (Figure 8A) and pro MMP9 secretion (Figure 8B). Amnion mesenchymal cells were transfected with or without siCLEC4E and treated with TNF. Treatment with TNF significantly increased MMP1, MMP8 and MMP9 mRNA expression (Figure 8C-E). In siCLEC4E transfected cells, there was a significant attenuation in TNF-induced MMP1 and MMP8 mRNA expression (Figure 8C,D), but there was no change with MMP9 mRNA expression (Figure 8E).

DISCUSSION

This study adds to the growing literature of the role of PRRs in human labor. This study, however, is the first to describe a role for the CLR in human myometrium and fetal membranes in the context of parturition. Here, we show that the expression of the receptor Mincle (CLEC4E) is upregulated in myometrium and/or fetal membranes after spontaneous labor at preterm and term and in response to intrauterine infection. Furthermore, Mincle expression is highly induced by pro-inflammatory and pro-labor insults such as pro-inflammatory cytokines (IL1B, TNF), bacterial products (LPS, fsl-1, flagellin) and viral products (poly(I:C)). Knockdown of Mincle in primary amnion and myometrial cells causes the suppression of mediators involved in regulating the processes of labor and delivery including pro-inflammatory cytokines (TNF, IL1A, IL1B), chemokines (CCL2, CXCL1, CXCL8) and ECM degrading enzymes (MMP1, MMP8, MMP9). In addition, our study reveals a role for Mincle in regulating myometrial activation as assessed by suppression of contraction associated proteins (Cx43, PTGFR) and secretion the uterotonic $PGF_{2\alpha}$ in cells deficient in Mincle. Finally, using an *in situ* model of myometrial contractility, the absence of Mincle leads to a significant augmentation of the magnitude of inflammation-induced collagen gel contractility. Taken together, our results demonstrate a previously unconsidered important role of Mincle in regulating inflammation induced pro-labor mediators.

Human labor at term in myometrium, and at preterm and term in fetal membranes was associated with upregulated Mincle expression when compared to non-laboring tissues. Likewise, Mincle expression was also upregulated in myometrium during labor in a mouse model of inflammation induced preterm birth and in amnion from women with preterm chorioamnionitis compared to amnion from women without preterm chorioamnionitis. Inflammation is a hallmark of labor, with an influx of leukocytes and increased expression of cytokines, chemokines and other inflammatory markers observed in uterus, cervix, placenta and fetal membranes during labor (45, 46). Chorioamnionitis is an acute inflammation that occurs within the fetal membranes in response to the presence of pathogenic organisms (47). To test the possibility that increased Mincle expression is a result of these inflammatory responses, we evaluated the effect of the pro-inflammatory cytokines on Mincle expression. We found that IL1B and TNF, two acute phase pro-inflammatory cytokines that are secreted by leukocytes, significantly increased Mincle expression in fetal membranes and myometrium. Collectively, these studies demonstrate that Mincle expression is regulated by inflammatory insults in myometrium and fetal membranes, and may thus may play a role in human labor.

IL1B and TNF are two acute phase cytokines that have vital roles during parturition. For example, in various animal species, injection of IL1B or TNF can induce preterm labor (48-51) and increase levels of pro-inflammatory cytokines, chemokines, prostaglandins, MMP9 and leukocytes in amniotic fluid (49). These animal studies are supported by *in vitro* studies whereby IL1B or TNF can further increase cytokine production, upregulate the expression of chemokines, activate ECM matrix remodelling enzymes, induce expression of contraction associated proteins and stimulate the ongoing release of prostaglandins (52-54). As Mincle was induced by IL1B and TNF, we investigated if Mincle is involved in the genesis of pro-inflammatory and pro-labor mediators induced by IL1B or TNF. Indeed, using siRNA knockdown experiments, we found that Mincle regulates IL1B or TNF induced pro-inflammatory cytokines (TNF, IL1A, IL1B), chemokines (CCL2, CXCL1, CXCL8) and ECM degrading enzymes (MMP1, MMP8, MMP9) in human primary myometrial and amnion cells. Further, Mincle regulates IL1B or TNF induced expression of contraction associated proteins (Cx43, PTGFR), secretion the uterotonic $PGF_{2\alpha}$, and myometrial contractility *in situ*. Taken together, these results suggest that Mincle is involved in the genesis of IL1B or TNF induced mediators involved myometrial contractions and rupture of fetal membranes.

In addition to pro-inflammatory cytokines, TLRs can also induce Mincle expression in numerous species (12, 14, 15, 55). Likewise, in this study, the TLR2/6 ligand fsl-1, TLR3 ligand poly(I:C), TLR4 ligand LPS and the TLR5 ligand flagellin significantly increased Mincle expression in fetal membranes and myometrium. This has important implications given that infection is the most common cause of early preterm birth (56) and that pathogens and bacterial and viral products engage TLRs to induce preterm labor in mice (6-8, 57).

Induction of Mincle by TLRs activates a cascade of events leading to the transcriptional regulation of pro-inflammatory genes (12, 14, 15, 55). Our previous studies have shown that ligation of TLR2/6 ligand fsl-1 and the TLR3 ligand poly(I:C) can induce pro-labor mediators in human myometrial and amnion cells (1, 3). Thus, it was of interest to determine if Mincle is also involved in TLR signalling. Using primary myometrial cells as a model system, and akin to the studies using IL1B and TNF, we found that siRNA inhibition of Mincle decreased the effectiveness of the TLR2/6 ligand fsl-1 and the TLR3 ligand poly(I:C) to induce the expression and secretion of pro-inflammatory cytokines and chemokines. Collectively, the results presented in this paper suggest that the expression and secretion of pro-labor mediators can be induced by TLRs through the Mincle pathway.

The downstream pathways involved in Mincle induced regulation of pro-labor mediators is not known. We found no effect of Mincle siRNA knockout on NF-κB transcriptional activity in myometrial cells, suggesting that Mincle regulates pro-inflammatory and pro-labor mediators in gestational tissues by other downstream transcription factors. NFAT, MAPK and AP-1 have also been shown to be involved in Mincle induced gene transcription (21-23). Notably, a few recent studies indicate a potential role for NFAT in mediating stretch or oxytocin induced gene expression in myometrium (58, 59). A role for MAPK and AP-1 proteins in regulating mediators involved in myometrial contractility and degradation of fetal membranes has also been reported (60-63). Further studies are therefore required to determine the functional role of NFAT, MAPK and AP-1 in Mincle signalling.

A limitation of this study is that we could not assess Mincle protein expression due to non-specificity of commercial antibodies (data not shown). Studies looking at Mincle expression and localisation in myometrium throughout gestation would further clarify other potential functional contributions Mincle makes in regulating myometrial contractility in pregnancy. Likewise, further studies are required to determine the regional localisation of Mincle in fetal membranes, i.e. site over the cervix prone to rupture versus distal areas, throughout gestational ages would shed light on the role of Mincle in fetal membrane rupture. In this study, all experiments were performed in the presence of an inflammatory insult. It would also be worth determining if Mincle plays a role in parturition in the absence of inflammation.

In conclusion, the expression of Mincle is upregulated after labor and infection in fetal membranes and myometrium. Furthermore, pro-inflammatory cytokines, and bacterial and viral products increased Mincle expression. Knockdown of Mincle reduced the expression and secretion of mediators involved in active labor and delivery. Importantly, in the absence of Mincle, the effectiveness of pro-inflammatory cytokines and TLR ligands to induce the expression and or secretion of pro-labor mediators was reduced. These data suggest that Mincle is part of the process by which inflammation induces parturition.

ACKNOWLEDGEMENTS

The following are gratefully acknowledged: Clinical Research Midwives Gabrielle Pell, Genevieve Christophers and Rachel Murdoch for sample collection; and the Obstetrics and Midwifery staff of the Mercy Hospital for Women for their co-operation.

FUNDING

Associate Professor Martha Lappas is supported by a Research Fellowship from the Department of Obstetrics and Gynaecology (University of Melbourne) and a Faculty Fellowship from the University of Melbourne. Funding for this study as provided by the NHMRC (grant no. 1058786), Norman Beischer Medical Research Foundation, the University of Melbourne and the Mercy Research Foundation.

DISCLOSURE SUMMARY

The authors have nothing to declare.

REFERENCES

1. Lim R, Barker G, Lappas M. TLR2, TLR3 and TLR5 regulation of pro-inflammatory and pro-labour mediators in human primary myometrial cells. J Reprod Immunol. 2017;122:28-36.
2. Lim R, Lappas M. NOD-like receptor pyrin domain-containing-3 (NLRP3) regulates inflammation-induced pro-labor mediators in human myometrial cells. Am J Reprod Immunol. 2018;79(4):e12825.
3. 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. Am J Reprod Immunol. 2014;71(5):401-17.
4. Lappas M. NOD1 and NOD2 regulate proinflammatory and prolabor mediators in human fetal membranes and myometrium via nuclear factor-kappa B. Biol Reprod. 2013;89(1):14.
5. Hoang M, Potter JA, Gysler SM, Han CS, Guller S, Norwitz ER, et al. Human fetal membranes generate distinct cytokine profiles in response to bacterial Toll-like receptor and nod-like receptor agonists. Biol Reprod. 2014;90(2):39.
6. Elovitz MA, Wang Z, Chien EK, Rychlik DF, Phillippe M. A New Model for Inflammation-Induced Preterm Birth. Am J Pathol. 2003;163(5):2103-11.
7. Ilievski V, Lu SJ, Hirsch E. Activation of toll-like receptors 2 or 3 and preterm delivery in the mouse. Reprod Sci. 2007;14(4):315-20.
8. Koga K, Cardenas I, Aldo P, Abrahams VM, Peng B, Fill S, et al. Activation of TLR3 in the trophoblast is associated with preterm delivery. Am J Reprod Immunol. 2009;61(3):196-212.
9. Braganza CD, Teunissen T, Timmer MSM, Stocker BL. Identification and biological activity of synthetic macrophage inducible C-type lectin ligands. Front Immunol. 2018;8(1940).
10. Goyal S, Castrillón-Betancur JC, Klaile E, Slevogt H. The interaction of human pathogenic fungi With C-type lectin receptors. Front Immunol. 2018;9(1261).
11. Williams SJ. Sensing lipids with mincle: structure and function. Front Immunol. 2017;8(1662).
12. Matsumoto M, Tanaka T, Kaisho T, Sanjo H, Copeland NG, Gilbert DJ, et al. A novel LPS-inducible C-type lectin is a transcriptional target of NF-IL6 in macrophages. J Immunol. 1999;163(9):5039-48.
13. Ichioka M, Suganami T, Tsuda N, Shirakawa I, Hirata Y, Satoh-Asahara N, et al. Increased expression of macrophage-inducible C-type lectin in adipose tissue of obese mice and humans. Diabetes. 2011;60(3):819-26.

14. Lv LL, Tang PM-K, Li CJ, You YK, Li J, Huang X-R, et al. The pattern recognition receptor, Mincle, is essential for maintaining the M1 macrophage phenotype in acute renal inflammation. Kidney Int. 2017;91(3):587-602.
15. Miyake Y, Oh-hora M, Yamasaki S. C-type lectin receptor MCL facilitates mincle expression and signaling through complex formation. J Immunol. 2015;194(11):5366.
16. Suzuki Y, Nakano Y, Mishiro K, Takagi T, Tsuruma K, Nakamura M, et al. Involvement of Mincle and Syk in the changes to innate immunity after ischemic stroke. Sci Rep. 2013;3:3177.
17. de Rivero Vaccari JC, Brand FJ, 3rd, Berti AF, Alonso OF, Bullock MR, de Rivero Vaccari JP. Mincle signaling in the innate immune response after traumatic brain injury. J Neurotrauma. 2015;32(4):228-36.
18. Greco SH, Torres-Hernandez A, Kalabin A, Whiteman C, Rokosh R, Ravirala S, et al. Mincle signaling promotes Con A hepatitis. J Immunol. 2016;197(7):2816-27.
19. Nakamura N, Shimaoka Y, Tougan T, Onda H, Okuzaki D, Zhao H, et al. Isolation and expression profiling of genes upregulated in bone marrow-derived mononuclear cells of rheumatoid arthritis patients. DNA Res. 2006;13(4):169-83.
20. Kostarnoy AV, Gancheva PG, Lepenies B, Tukhvatulin AI, Dzharullaeva AS, Polyakov NB, et al. Receptor Mincle promotes skin allergies and is capable of recognizing cholesterol sulfate. Proc Natl Acad Sci U S A. 2017;114(13):E2758-e65.
21. Honjoh C, Chihara K, Yoshiki H, Yamauchi S, Takeuchi K, Kato Y, et al. Association of C-type lectin mincle with FcεRIβγ subunits leads to functional activation of RBL-2H3 cells through Syk. Sci Rep. 2017;7:46064.
22. Lee W-B, Kang J-S, Choi WY, Zhang Q, Kim CH, Choi UY, et al. Mincle-mediated translational regulation is required for strong nitric oxide production and inflammation resolution. Nat Commun. 2016;7:11322.
23. Lee WB, Kang JS, Yan JJ, Lee MS, Jeon BY, Cho SN, et al. Neutrophils promote mycobacterial trehalose dimycolate-induced lung inflammation via the mincle pathway. PLoS Pathog. 2012;8(4):e1002614.
24. Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T. Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nat Immunol. 2008;9:1179.
25. Ishikawa E, Ishikawa T, Morita YS, Toyonaga K, Yamada H, Takeuchi O, et al. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. J Exp Med. 2009;206(13):2879-88.
26. Werninghaus K, Babiak A, Groß O, Hölscher C, Dietrich H, Agger EM, et al. Adjuvanticity of a synthetic cord factor analogue for subunit *Mycobacterium tuberculosis* vaccination requires FcRγ–Syk–Card9–dependent innate immune activation. J Exp Med. 2009;206(1):89-97.

27. Schoenen H, Bodendorfer B, Hitchens K, Manzanero S, Werninghaus K, Nimmerjahn F, et al. Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. J Immunol. 2010;184(6):2756-60.
28. Yamasaki S, Matsumoto M, Takeuchi O, Matsuzawa T, Ishikawa E, Sakuma M, et al. C-type lectin Mincle is an activating receptor for pathogenic fungus, Malassezia. Proc Natl Acad Sci U S A. 2009;106(6):1897-902.
29. Lim R, Barker G, Lappas M. PARK7 regulates inflammation-induced pro-labour mediators in myometrial and amnion cells. Reprod. 2018;155(2):207-18.
30. 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. Biol Reprod. 2013;88(6):156.
31. Lappas M, Permezel M, Georgiou HM, Rice GE. Type II phospholipase A_2 in preterm human gestational tissues. Placenta. 2001;22(1):64-9.
32. Lappas M, Mitton A, Lim R, Barker G, Riley C, Permezel M. SIRT1 is a novel regulator of key pathways of human labor. Biol Reprod. 2011;84(1):167-78.
33. Lim R, Barker G, Lappas M. Inhibition of PIM1 kinase attenuates inflammation-induced pro-labour mediators in human fetal membranes in vitro Mol Hum Reprod. 2017.
34. Liong S, Lim R, Barker G, Lappas M. Hepatitis A virus cellular receptor 2 (HAVCR2) is decreased with viral infection and regulates pro-labour mediators Am J Reprod Immunol. 2017;78(1).
35. Moore RM, Novak JB, Kumar D, Mansour JM, Mercer BM, Moore JJ. Alpha-lipoic acid inhibits tumor necrosis factor-induced remodeling and weakening of human fetal membranes. Biol Reprod. 2009;80(4):781-7.
36. Lim R, Barker G, Lappas M. TREM-1 expression is increased in human placentas from severe early-onset preeclamptic pregnancies where it may be involved in syncytialization. Reprod Sci. 2014;21(5):562-72.
37. Lappas M. Runt-related transcription factor 1 (RUNX1) deficiency attenuates inflammation-induced pro-inflammatory and pro-labour mediators in myometrium. Mol Cell Endocrinol. 2018.
38. Lappas M. Runt-related transcription factor 1 (RUNX1) deficiency attenuates inflammation-induced pro-inflammatory and pro-labour mediators in myometrium. Mol Cell Endocrinol. 2018;473:61-71.
39. Lim R, Barker G, Lappas M. SIRT6 is decreased with preterm labor and regulates key terminal effector pathways of human labor in fetal membranes. Biol Reprod. 2013;88(1):17, 1-0-, 1-0.

40. Lim R, Barker G, Wall CA, 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(7):451-62.
41. Belt AR, Baldassare JJ, Molnar M, Romero R, Hertelendy F. The nuclear transcription factor NF-kappaB mediates interleukin-1beta-induced expression of cyclooxygenase-2 in human myometrial cells. Am J Obstet Gynecol. 1999;181(2):359-66.
42. O'Brien WF. The role of prostaglandins in labor and delivery. Clin Perinatol. 1995;22(4):973-84.
43. Fortier MA, Krishnaswamy K, Danyod G, Boucher-Kovalik S, Chapdalaine P. A postgenomic integrated view of prostaglandins in reproduction: implications for other body systems. J Physiol Pharmacol. 2008;59 Suppl 1:65-89.
44. Mogami H, Keller PW, Shi H, Word RA. Effect of thrombin on human amnion mesenchymal cells, mouse fetal membranes, and preterm birth. J Biol Chem. 2014;289(19):13295-307.
45. Osman I, Young A, Ledingham MA, Thomson AJ, Jordan F, Greer IA, et al. Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. Mol Hum Reprod. 2003;9(1):41-5.
46. Bollapragada S, Youssef R, Jordan F, Greer I, Norman J, Nelson S. Term labor is associated with a core inflammatory response in human fetal membranes, myometrium, and cervix. Am J Obstet Gynecol. 2009;200(1):104.e1-11.
47. Redline RW. Inflammatory response in acute chorioamnionitis. Semin Fetal Neonatal Med. 2012;17(1):20-5.
48. Romero R, Mazor M, Tartakovsky B. Systemic administration of interleukin-1 induces preterm parturition in mice. Am J Obstet Gynecol. 1991;165(4 Pt 1):969-71.
49. Sadowsky DW, Adams KM, Gravett MG, Witkin SS, Novy MJ. Preterm labor is induced by intraamniotic infusions of interleukin-1beta and tumor necrosis factor-alpha but not by interleukin-6 or interleukin-8 in a nonhuman primate model. Am J Obstet Gynecol. 2006;195(6):1578-89.
50. Romero R, Tartakovsky B. The natural interleukin-1 receptor antagonist prevents interleukin-1-induced preterm delivery in mice. Am J Obstet Gynecol. 1992;167(4 Pt 1):1041-5.
51. Nadeau-Vallee M, Chin PY, Belarbi L, Brien ME, Pundir S, Berryer MH, et al. Antenatal suppression of IL-1 protects against inflammation-induced fetal injury and improves neonatal and developmental outcomes in mice. J Immunol. 2017;198(5):2047-62.
52. Lim R, Barker G, Lappas M. TRADD, TRAF2, RIP1 and TAK1 are required for TNF-alpha-induced pro-labour mediators in human primary myometrial cells. Am J Reprod Immunol. 2017;78(1).

53. Kumar D, Fung W, Moore RM, Pandey V, Fox J, Stetzer B, et al. Proinflammatory cytokines found in amniotic fluid induce collagen remodeling, apoptosis, and biophysical weakening of cultured human fetal membranes. Biol Reprod. 2006;74(1):29-34.
54. Lappas M. The IL-1β signalling pathway and its role in regulating pro-inflammatory and pro-labour mediators in human primary myometrial cells. Reprod Biol. 2017;17(4):333-40.
55. Zoccola E, Kellie S, Barnes AC. Immune transcriptome reveals the mincle C-type lectin receptor acts as a partial replacement for TLR4 in lipopolysaccharide-mediated inflammatory response in barramundi (Lates calcarifer). Mol Immunol. 2017;83:33-45.
56. Romero R, Espinoza J, Kusanovic JP, Gotsch F, Hassan S, Erez O, et al. The preterm parturition syndrome. BJOG. 2006;113 Suppl 3:17-42.
57. Wang H, Hirsch E. Bacterially-induced preterm labor and regulation of prostaglandin-metabolizing enzyme expression in mice: the role of toll-like receptor 4. Biol Reprod. 2003;69(6):1957-63.
58. Chin-Smith EC, Willey FR, Slater DM, Taggart MJ, Tribe RM. Nuclear factor of activated T-cell isoform expression and regulation in human myometrium. Reprod Biol Endocrinol. 2015;13:83-.
59. Pont JN, McArdle CA, Lopez Bernal A. Oxytocin-stimulated NFAT transcriptional activation in human myometrial cells. Mol Endocrinol. 2012;26(10):1743-56.
60. Lappas M, Riley C, Lim R, Barker G, Rice GE, Menon R, et al. MAPK and AP-1 proteins are increased in term pre-labour fetal membranes overlying the cervix: regulation of enzymes involved in the degradation of fetal membranes. Placenta. 2011;32(12):1016-25.
61. Khanjani S, Terzidou V, Johnson MR, Bennett PR. NFκB and AP-1 drive human myometrial IL8 expression. Mediators Inflamm. 2012;2012:504952-.
62. Lappas M, Permezel M, Rice GE. Mitogen-activated protein kinase proteins regulate LPS-stimulated release of pro-inflammatory cytokines and prostaglandins from human gestational tissues. Placenta. 2007;28(8-9):936-45.
63. MacIntyre DA, Lee YS, Migale R, Herbert BR, Waddington SN, Peebles D, et al. Activator protein 1 is a key terminal mediator of inflammation-induced preterm labor in mice. Faseb j. 2014;28(5):2358-68.

FIGURE LEGENDS

Figure 1. Expression of Mincle in myometrium and fetal membranes

(A) Fetal membranes were obtained from women at term Caesarean section in the absence of labor (term no labor, n=9 patients) or from women after term spontaneous labor onset and delivery (term after labor, n=9 patients). **(B)** Fetal membranes were obtained from women at preterm Caesarean section in the absence of labor (preterm no labor, n=9 patients) or from women after preterm spontaneous labor onset and delivery (preterm after labor, n=9 patients). **(C)** Amnion was obtained from women at preterm Caesarean section without histological chorioamnionitis (preterm no CAM, n=8 patients) or from women at preterm Caesarean section with histological chorioamnionitis (preterm CAM, n=8 patients). **(D)** Human myometrium was obtained from women at term Caesarean section in the absence of labor (term no labor, n=8 patients) or from women at term Caesarean section during labor (term in labor, n=8 patients). **(E)** Myometrium was obtained from mice during LPS-induced labor at gestational day 15.5 and time-matched vehicle-injected mice (n=4 mice/group). For all data, Mincle mRNA expression was analysed by RT-qPCR. Individual data points represent different patients and the horizontal line represents the mean ± SEM of each group. *$P \leq 0.05$, Mann-Whitney U test.

Figure 2. Effect of pro-inflammatory mediators on Mincle expression in fetal membranes and myometrium

Human **(A)** fetal membranes and **(B)** myometrium were incubated in the absence or presence of 10 ng/ml IL1B, 10 ng/ml TNF, 250 ng/ml fsl-1, 20 μg/ml poly(I:C), 10 μg/ml LPS or 1 μg/ml flagellin for 20 h (n=6 patients/treatment). Mincle mRNA expression was analysed by RT-qPCR. Individual data points represent 6 independent experiments and displayed as mean ± SEM. *$P \leq 0.05$, Wilcoxon test.

Figure 3. Effect of Mincle siRNA knockdown on pro-inflammatory cytokines and chemokines in human primary amnion mesenchymal cells

Human primary amnion mesenchymal cells were transfected with 50 nM siCONT or 50 nM siCLEC4E and then treated with 1 ng/ml IL1B or 10 ng/ml TNF (n=6 patients). **(A,B,D,F,H-J,L,N)** IL1A, IL1B, IL6, CCL2 and CXCL8 mRNA expression was analysed by RT-qPCR. **(C,E,G,K,M,O)** The concentration of IL6, CCL2 and CXCL8 in the incubation medium was assayed by ELISA. Individual data points represent 6 independent experiments and displayed as mean ± SEM. *$P \leq 0.05$ (repeated measures one-way ANOVA).

Figure 4. Effect of Mincle siRNA knockdown on IL1B-induced pro-inflammatory cytokines and chemokines in human primary myometrial cells

Human primary myometrial cells were transfected with 50 nM siCONT or 50 nM siCLEC4E and then treated with 1 ng/ml IL1B (n=7 patients). **(A-C,E,G,I)** TNF, IL1A, IL6, CCL2, CXCL1 and CXCL8 mRNA expression was analysed by RT-qPCR. **(D,F,H,J)** The concentration of IL6, CCL2, CXCL1 and CXCL8 in the incubation medium was assayed by ELISA. Individual data points represent 6 independent experiments and displayed as mean ± SEM. *$P \leq 0.05$ (repeated measures one-way ANOVA).

Figure 5. Effect of Mincle siRNA knockdown on fsl-1-induced pro-inflammatory cytokines and chemokines in human primary myometrial cells

Human primary myometrial cells were transfected with 50 nM siCONT or 50 nM siCLEC4E and then treated with 250 ng/ml fsl-1 (n=6 patients). **(A-D,F,H,J)** TNF, IL1A, IL1B, IL6, CCL2, CXCL1 and CXCL8 mRNA expression was analysed by RT-qPCR. **(E,G,I,K)** The concentration of IL6, CCL2, CXCL1 and CXCL8 in the incubation medium was assayed by ELISA. Individual data points represent 6 independent experiments and displayed as mean ± SEM. *$P \leq 0.05$ (repeated measures one-way ANOVA).

Figure 6. Effect of Mincle siRNA knockdown on poly(I:C)-induced pro-inflammatory cytokines and chemokines in human primary myometrial cells

Human primary myometrial cells were transfected with 50 nM siCONT or 50 nM siCLEC4E and then treated with 5 µg/ml poly(I:C) (n=6 patients). **(A-D,F,H,J)** TNF, IL1A, IL1B, IL6, CCL2, CXCL1 and CXCL8 mRNA expression was analysed by RT-qPCR. **(E,G,I,K)** The concentration of IL6, CCL2, CXCL1 and CXCL8 in the incubation medium was assayed by ELISA. Individual data points represent 6 independent experiments and displayed as mean ± SEM. *$P \leq 0.05$ (repeated measures one-way ANOVA).

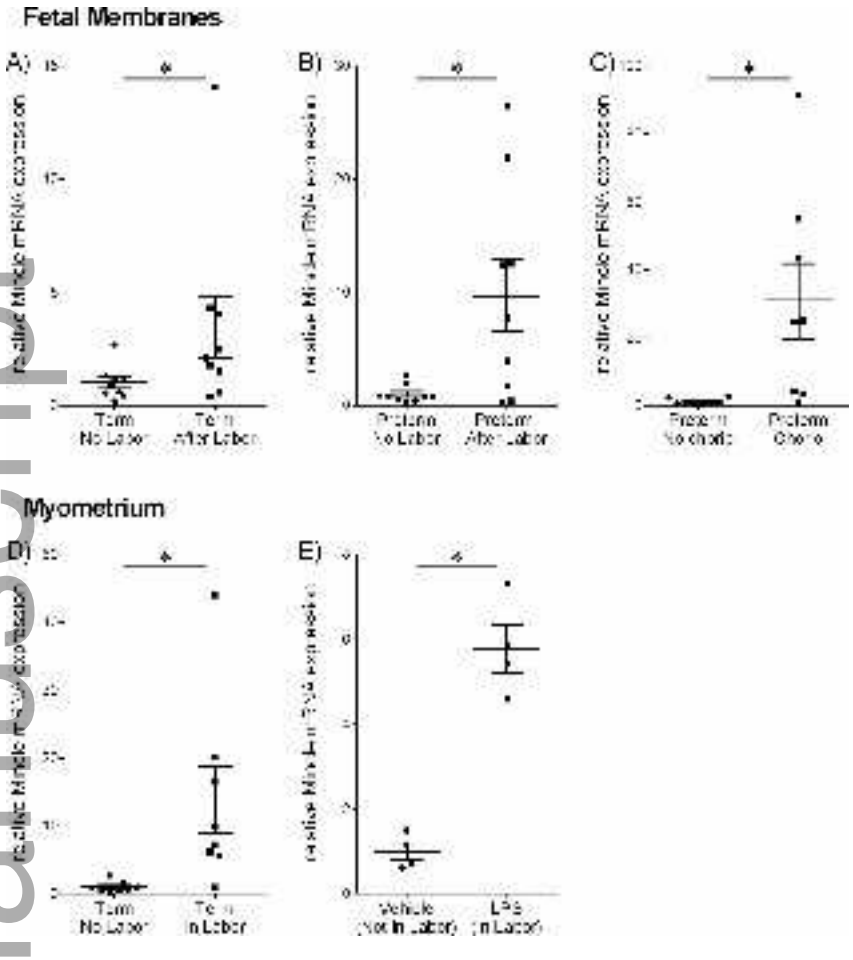
Figure 7. Effect of Mincle siRNA knockdown on myometrial contractility

(A-F) Human primary myometrial cells were transfected with 50 nM siCONT or 50 nM siCLEC4E and then treated with (A-C) 1 ng/ml IL1B or (D-F) 10 ng/ml TNF (n=8 patients). (A,B,D,E) PTGS2 and PTGFR mRNA expression was analysed by RT-qPCR. (C,F) The concentration of $PGF_{2\alpha}$ in the incubation medium was assayed by ELISA. Individual data points represent 6 independent experiments and displayed as mean ± SEM. *$P \leq 0.05$ (repeated measures one-way ANOVA). **(G)** Cell contraction assays were performed using collagen gels made from human primary myometrial cells transfected with 50 nM siCONT or 50 nM siCLEC4E for 48 h (n=6 patients). The collagen gels were then treated with or without 10 ng/ml TNF for 36 h, and the area of gel was determined.

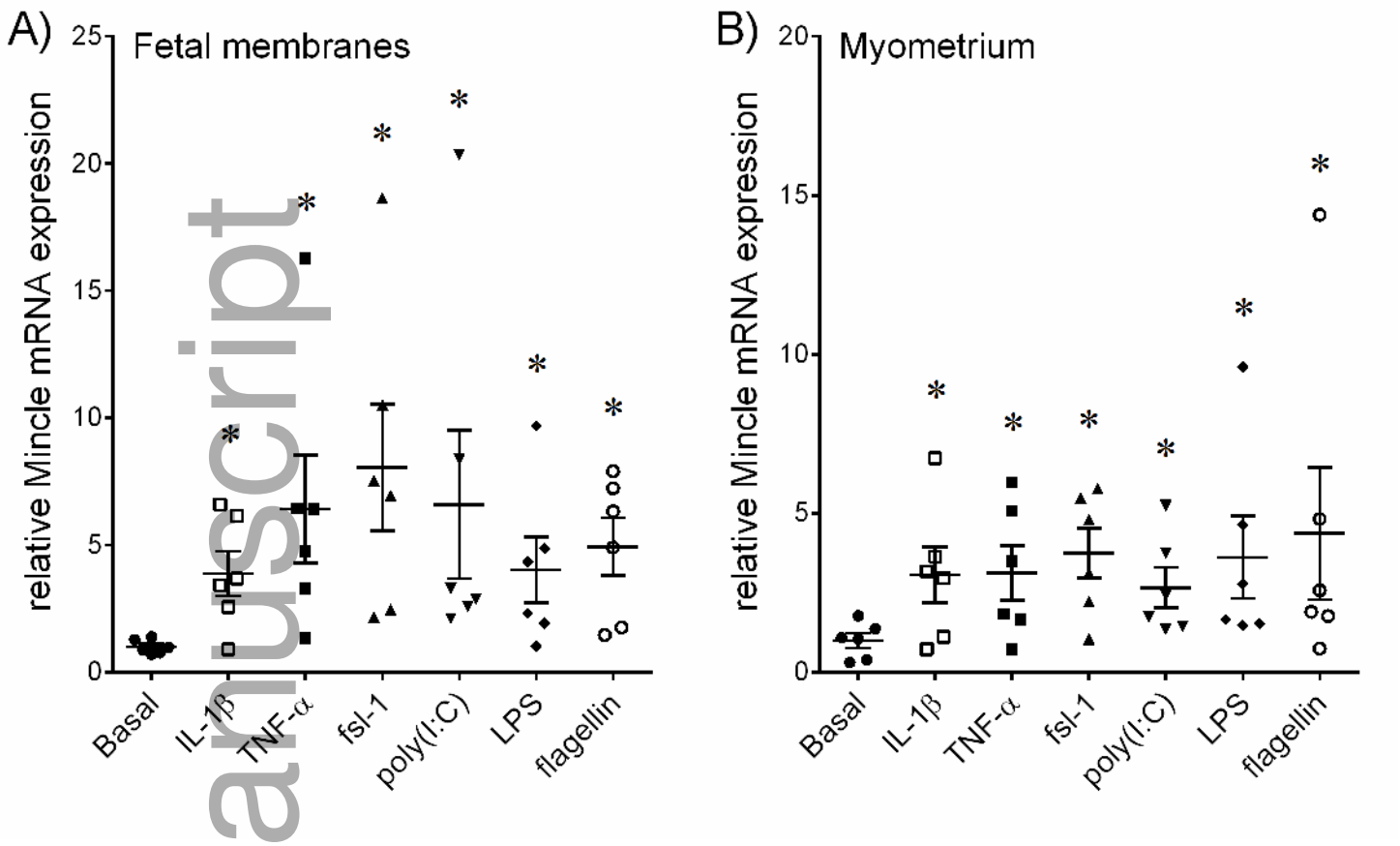
Representative gel contraction image from 1 patient is also shown. *$P \leq 0.05$ (repeated measures one-way ANOVA).

Figure 8. Effect of Mincle siRNA knockdown on the expression of ECM degrading enzymes in primary amnion cells

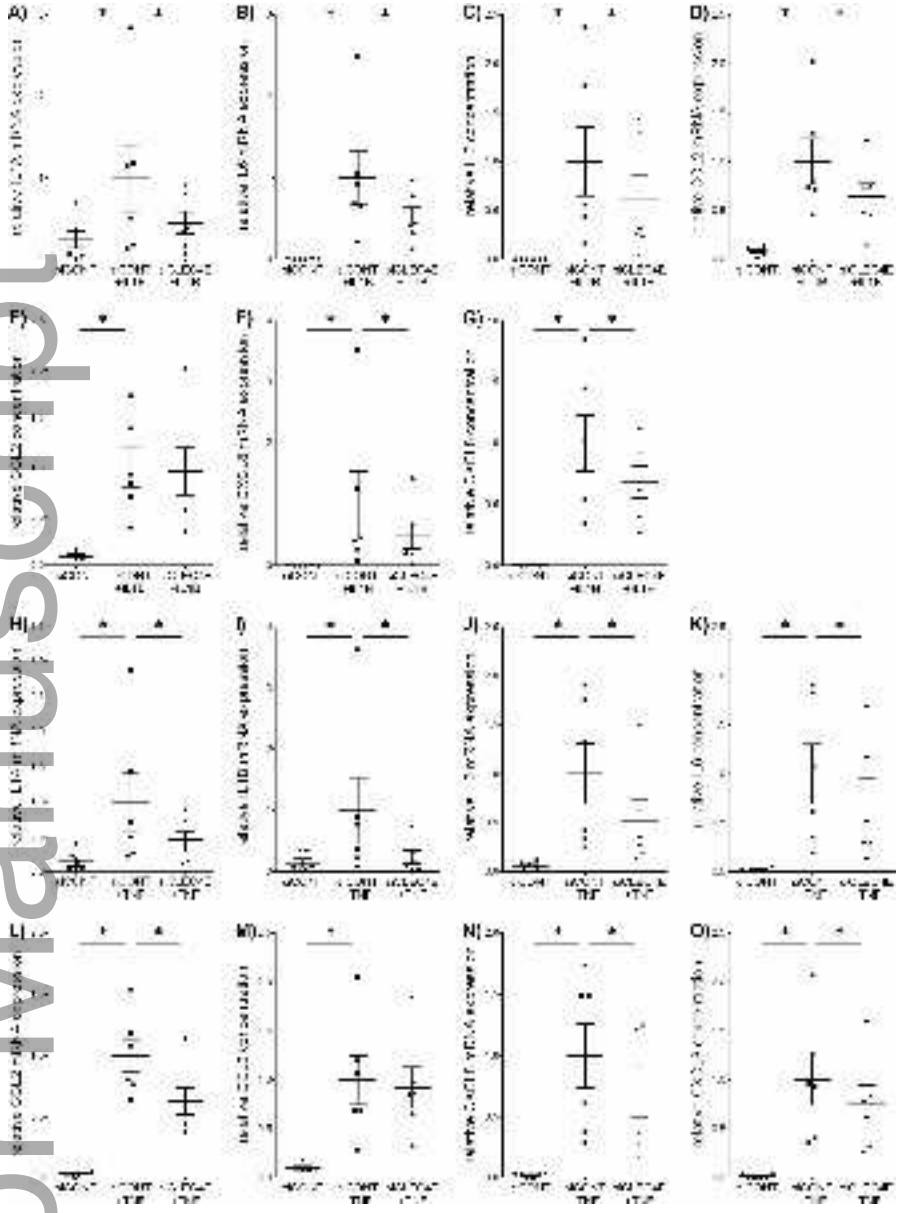
(A,B) Human primary amnion epithelial cells were transfected with 50 nM siCONT or 50 nM siCLEC4E and then treated with 1 ng/ml IL1B (n=6 patients). **(A)** MMP9 mRNA expression was analysed by RT-qPCR. **(B)** The concentration of pro MMP9 in the incubation medium was assayed by zymography. Representative zymography image from one patient is also shown. Individual data points represent 6 independent experiments and displayed as mean ± SEM. *$P \leq 0.05$ (repeated measures one-way ANOVA). **(C-E)** Human primary amnion mesenchymal cells were transfected with 50 nM siCONT or 50 nM siCLEC4E and then treated with 10 ng/ml TNF (n=6 patients). **(A)** MMP1, **(B)** MMP8, and **(C)** MMP9 mRNA expression was analysed by RT-qPCR. Individual data points represent 6 independent experiments and displayed as mean ± SEM. *$P \leq 0.05$ (repeated measures one-way ANOVA).



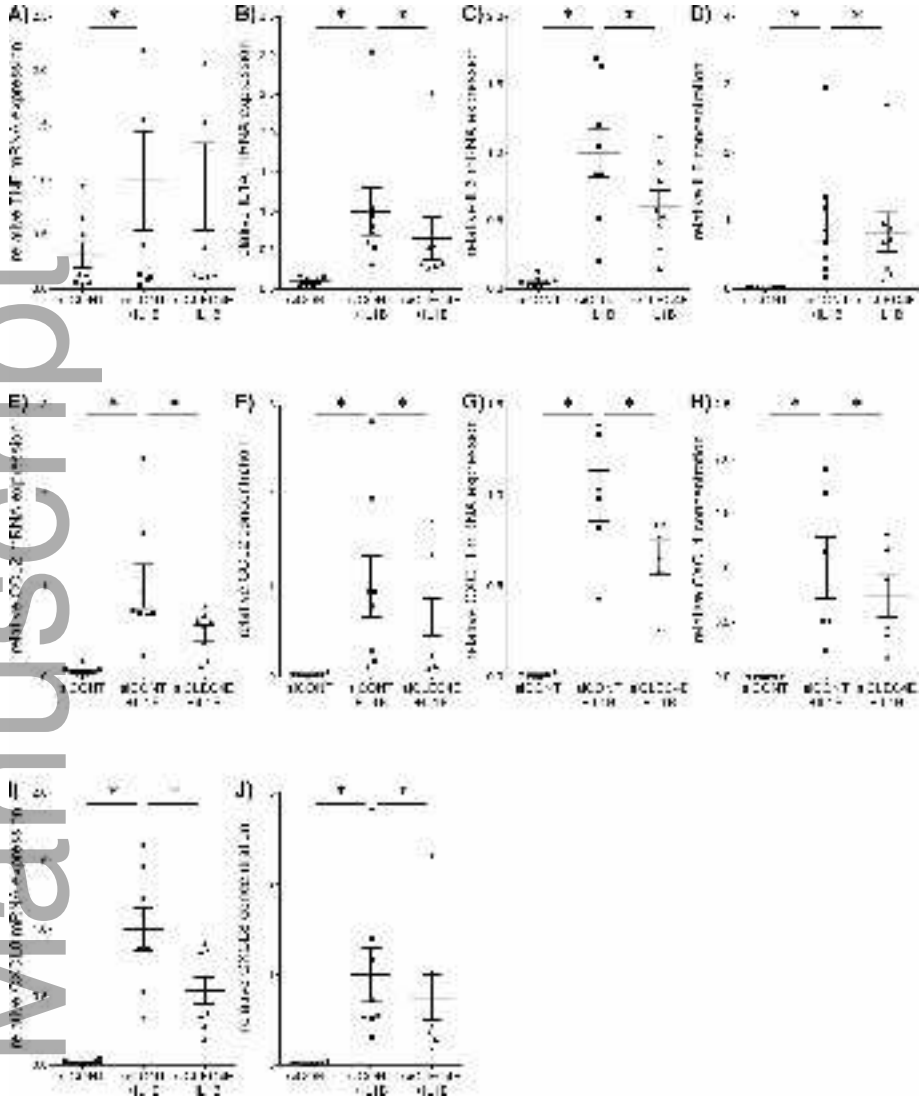
cei_13281_f1.tif



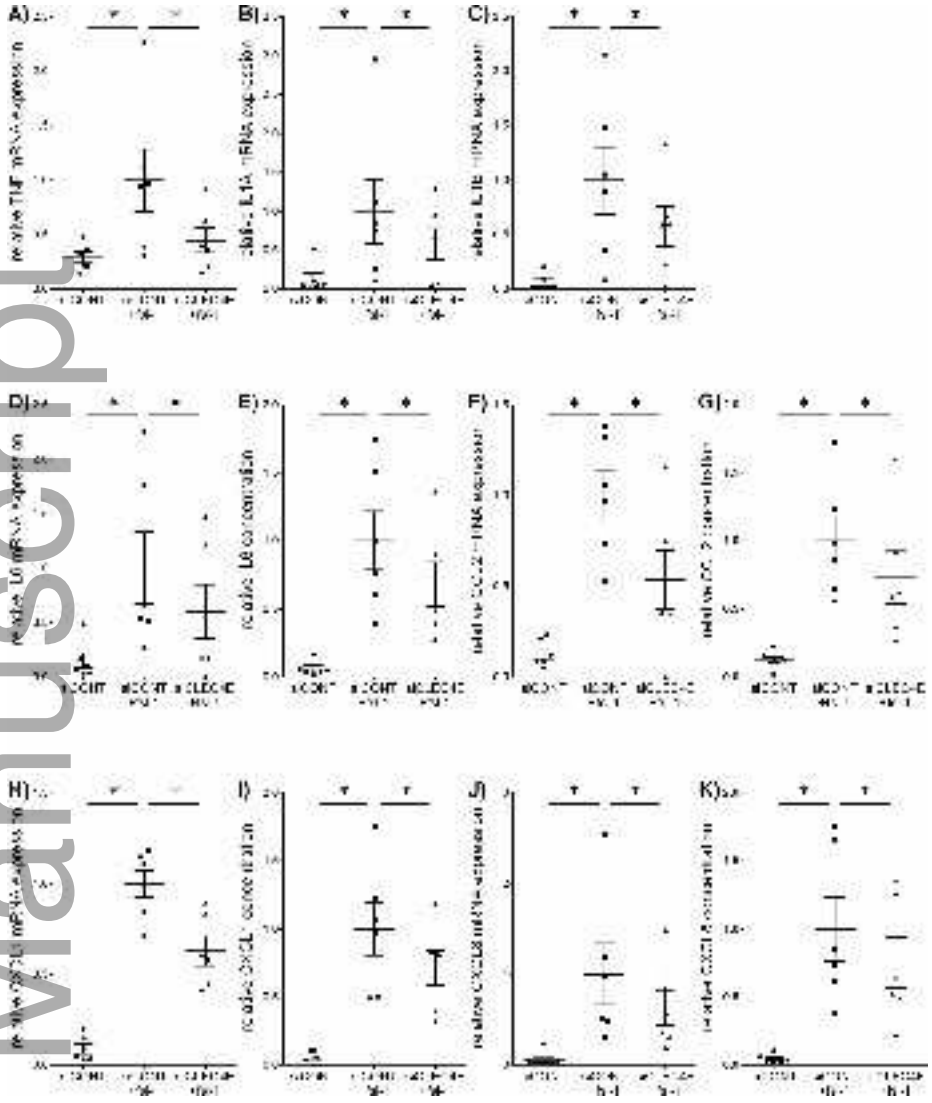
cei_13281_f2.tif



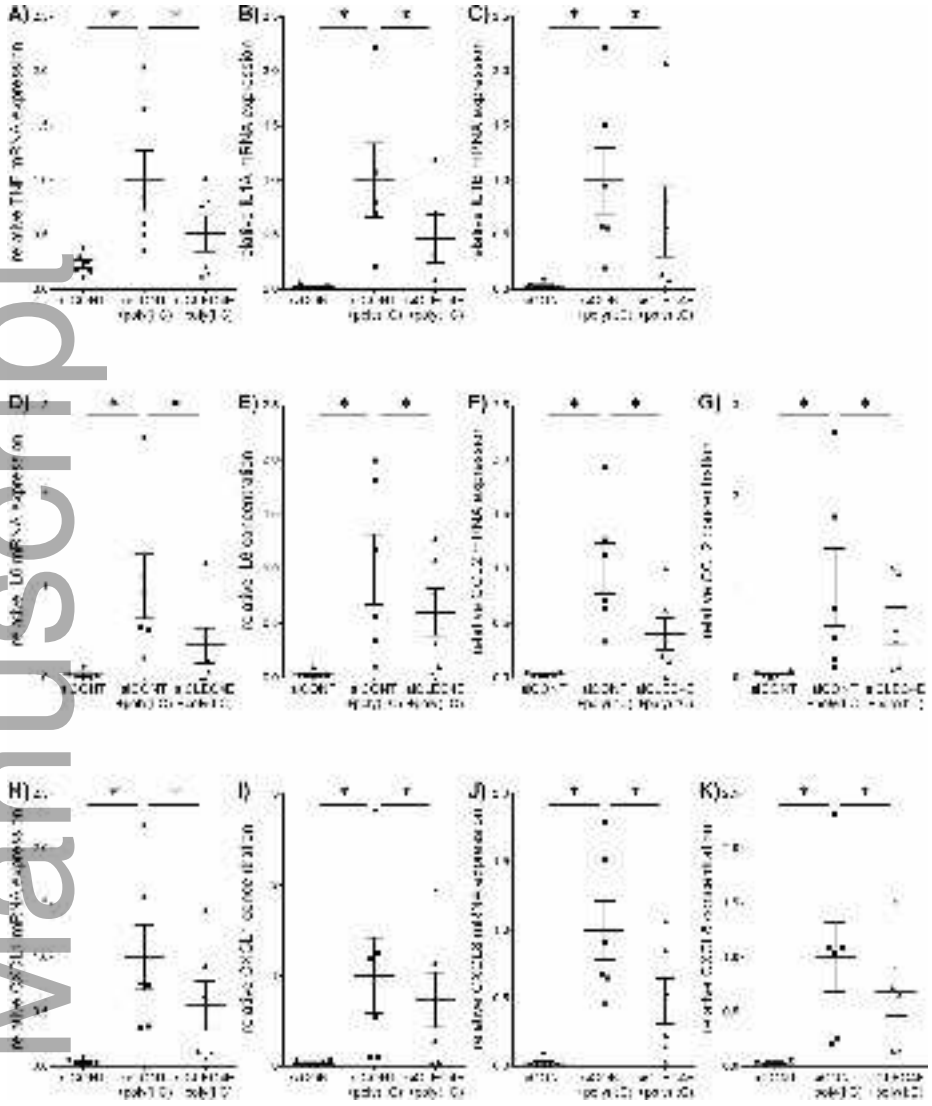
cei_13281_f3.tif



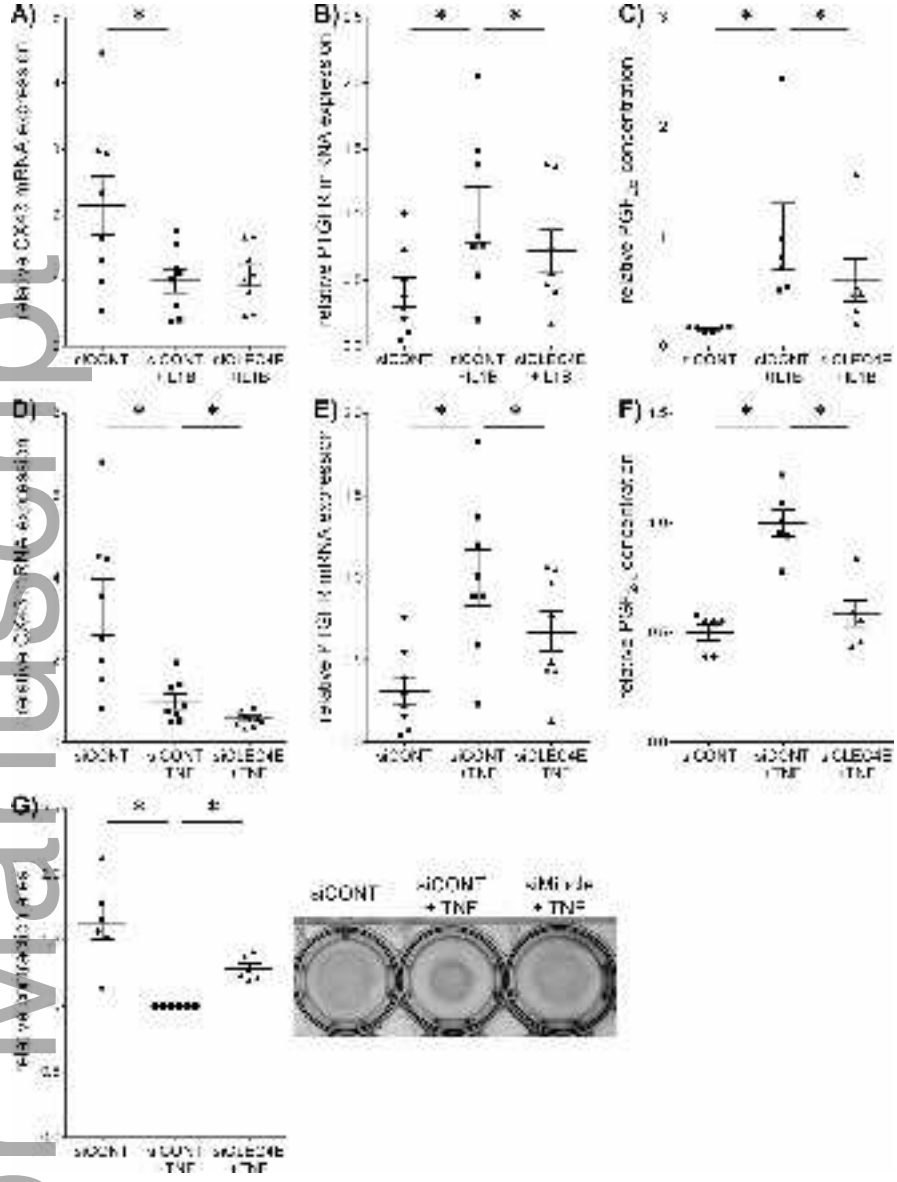
cei_13281_f4.tif



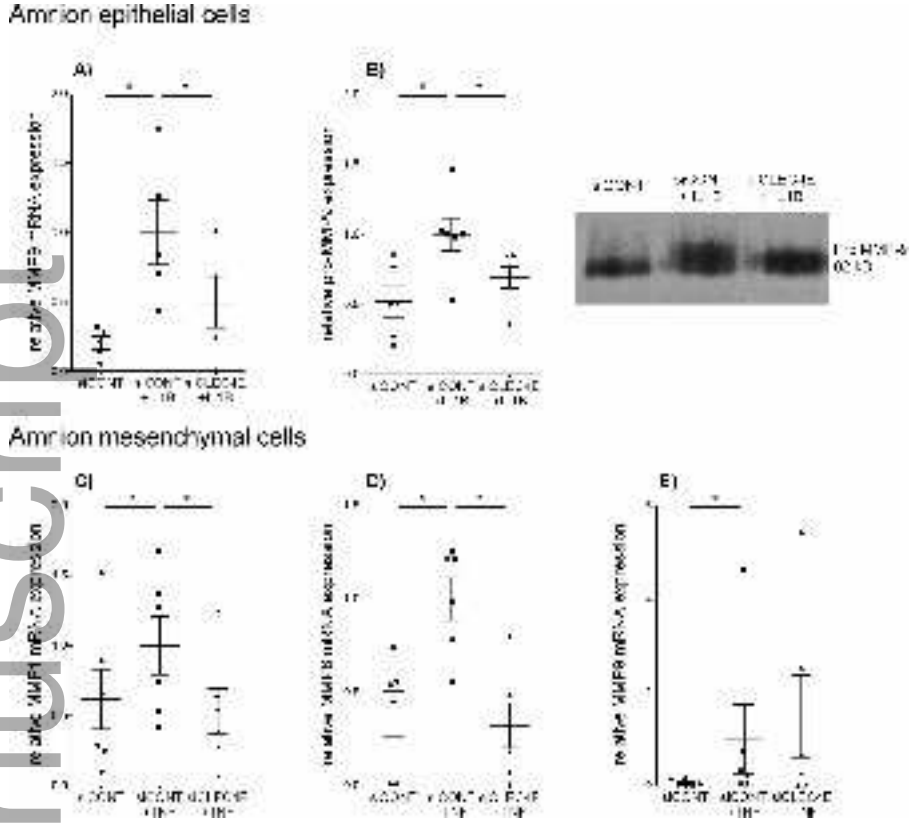
cei_13281_f5.tif



cei_13281_f6.tif



cei_13281_f7.tif



cei_13281_f8.tif