IRF5 is increased in labouring myometrium and regulates pro-labour mediators

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Abstract

Preterm birth continues to be the leading cause of neonatal mortality and morbidities that can extend into adult life. Few treatment options stem from our incomplete understanding of the mechanisms of human labour and delivery. Activation of the inflammatory response in gestational tissues by inflammation and/or infection leads to the production of pro-inflammatory and pro-labour mediators, thus preterm birth. Interferon regulatory factor 5 (IRF5) has recently emerged as an important pro-inflammatory transcription factor involved in acute and chronic inflammation. The aims of this study were to determine the expression of IRF5 in human myometrium from labouring and non-labouring women, and whether IRF5 is involved in the genesis of pro-inflammatory and pro-labour mediators induced by pro-inflammatory cytokines or toll-like receptor (TLR) ligands. IRF5 mRNA and protein expression was significantly higher in human myometrium after spontaneous term labour, compared to non-labouring tissues. *IRF5* mRNA expression was also significantly higher in primary myometrial cells treated with the pro-inflammatory cytokines IL1B or TNF. In primary myometrial cells, IRF5 knockdown by siRNA (siIRF5) was associated with significantly decreased expression and or secretion of pro-inflammatory cytokines (IL1A, IL6), chemokines (CXCL8, CCL2), adhesion molecules (ICAM1, VCAM1) and contraction-associated proteins *PTGS2*, *PGF_{2α}* and *PTGFR* when in the presence of IL1B, TNF, fsl-1 (TLR2/6 ligand) or flagellin (TLR5 ligand). siIRF5-transfected cells also displayed decreased NF-κB RELA transcriptional activity in the presence of these preterm birth mediators. Our study suggests a novel role for IRF5 in the regulation of the inflammatory response in human myometrium.

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Introduction

Preterm birth continues to be a major obstetric healthcare situation (Liu *et al.* 2012), despite decades of research (Rubens *et al.* 2014). Of the 15 million babies born premature each year worldwide, over 1 million will die (Beck *et al.* 2010) and a significant proportion will experience health issues that extend into adult life (Saigal & Doyle 2008, Mwaniki *et al.* 2012). Specifically, of 22,161 neonates born preterm, one-third had an impairment, such as learning difficulties or developmental delay (59%), cerebral palsy (21%) and hearing impairment (20%) (Mwaniki *et al.* 2012). In Sweden, the prevalence of cerebral palsy in neonates born less than 28 weeks was 85.5 per 1000, but only 1.3 per 1000 for those born at least 37 weeks. The human and financial burden of preterm birth on the family and healthcare system is enormous (Petrou *et al.* 2011), costing the USA \$26.2 billion in 2005 alone. While up to one third of preterm births are indicated due to maternal or foetal reasons (Goldenberg *et al.* 2008), the remaining are due to spontaneous preterm births. Of all spontaneous preterm births, approximately 45%

are due to preterm labour (Goldenberg *et al.* 2008), i.e. myometrial contractions with intact membranes. There are no treatments that can stop these contractions; tocolytics today alone do not extend the pregnancy beyond 48h, have not reduced the incidence of preterm birth, nor improved long-term outcomes for the preterm baby (Mitchell *et al.* 2013, Illanes *et al.* 2014, van Vliet *et al.* 2014). Tocolytics can only be improved by understanding the physiology of labour and the pathological processes involved in preterm labour; the myometrium, responsible for these uterine contractions, is thus an important area of study.

It is known that inflammation is central to preterm labour and can be induced by sterile inflammatory insults or from infection (Romero *et al.* 2006, Christiaens *et al.* 2008). Subsequent activation of the maternal immune system (Yuan *et al.* 2009) can ultimately lead to cervical ripening, foetal membrane rupture and myometrial contractions, thus spontaneous preterm birth. It has been shown that pro-inflammatory cytokines, chemokines and the cell adhesion molecule ICAM1 play important roles in these labour processes (Christiaens *et al.* 2008, Nadeau-Vallee *et al.* 2016, Sivarajasingam *et al.* 2016).

Many pro-inflammatory cytokines and chemokines have been implicated in the inflammatory process of labour, including IL6, CXCL8 and CCL2. IL6 upregulates the expression of oxytocin receptors on myometrial cells, increasing their responsiveness to oxytocin stimulation and thus initiating contractions (Rauk *et al.* 2001). CXCL8 is a potent chemokine for neutrophils while CCL2 exhibits a chemotactic activity for monocytes and basophils. In myometrium, these cytokines and chemokines are consistently and markedly upregulated in preterm and term labour (Mittal *et al.* 2010, Shynlova *et al.* 2013, Sivarajasingam *et al.* 2016) and by stretch (Shynlova *et al.* 2013). Additionally, prostaglandin synthase 2 (PTGS2)-dependent prostaglandin PGF_{2α} is an important regulator of myometrial contractility (Olson 2003). Importantly, many of these pro-inflammatory and labour-associated genes are regulated by nuclear factor-kappa B (NF-κB) (Barton & Medzhitov 2003, Lindstrom & Bennett 2005, Lappas & Rice 2007). In human gestational tissue, NF-κB is activated by a broad range of stimuli including the pro-inflammatory cytokines TNF (Lim *et al.* 2017b) and IL1B (Lappas 2017b), and toll-like receptor (TLR) ligands such as the bacterial products fibroblast-stimulating lipopeptide (fsl-1) and flagellin (Lim *et al.* 2014, 2017a).

The interferon (IFN) regulatory factor (IRF) family are a group of nine transcription factors (IRF1–9). They are critical for regulating immune and inflammatory responses, and as such, defects in IRF expression and/or function are linked to numerous metabolic, cardiovascular and neurological diseases (Honda & Taniguchi 2006, Zhao *et al.* 2015). We have previously shown that IRF1 is increased in term labour in foetal membranes and myometrium where it is involved in the genesis of IL1B- and TLR-induced pro-inflammatory cytokines, chemokines and contraction-associated proteins (Lim *et al.* 2016b). Numerous lines of evidence have now identified an important role for IRF5 in driving the inflammatory response. *In vitro* gene knockout or overexpression studies have demonstrated an essential role of IRF5 in the production of pro-inflammatory cytokines in response to numerous pathogens and TLR ligands (Takaoka *et al.* 2005, Krausgruber *et al.* 2010, Hedl & Abraham 2012, 2011, Ren *et al.* 2014). In keeping with this, IRF5-knockout mice are resistant to lethal shock induced by TLR ligands (Takaoka *et al.* 2005), accumulate fewer neutrophils at the site of inflammation due to the reduced levels of chemokines (Xu *et al.* 2012, Weiss *et al.* 2015) and show reductions in serum and/or tissue levels of inflammatory cytokines (Paun *et al.* 2008, Feng *et al.* 2012, Duffau *et al.* 2015, Oriss *et al.* 2017). Moreover, myeloid deficiency of IRF5 protects mice from liver fibrosis and alters hepatic macrophage transcriptome response to experimental fibrosis (Alzaid *et al.* 2016) while cells derived from IRF5-knockout mice produce lower levels of pro-inflammatory cytokines and/or chemokines in response

to TLR ligands (Tada *et al.* 2011, Duffau *et al.* 2015, Weiss *et al.* 2015). Notably, ChIP seq studies have shown that IRF5 regulates many inflammatory genes in cooperation with the RELA subunit of NF-κB (Saliba *et al.* 2014) while IRF5 knockdown suppresses RELA activation in human monocyte-derived cells (Hedl & Abraham 2012). Finally, IRF5 gene polymorphisms are associated with a number of inflammatory diseases including rheumatoid arthritis (Lee *et al.* 2013), systemic lupus erythematosus (Salloum & Niewold 2011), inflammatory bowel disease (Gathungu *et al.* 2012), pulmonary fibrosis (Zhao *et al.* 2017) and asthma (Wang *et al.* 2012).

The role of IRF5 in regulating inflammation associated with labour is not known. Thus, the aims of this present study were to characterise the expression of IRF5 in myometrium from non-labouring and labouring women and to determine if IRF5 is involved in the genesis of pro-inflammatory and pro-labour mediators induced by pro-inflammatory cytokines or TLR ligands. The pro-inflammatory cytokines IL1B and TNF, the TLR2/6 ligand fsl-1 and the TLR5 ligand flagellin were used to mimic inflammation associated with preterm labour (Lim *et al.* 2014, 2017a,b, Lappas 2017b).

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 min 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°C for expression studies or used immediately for cell culture experiments.

For all studies, tissue samples were obtained from women who delivered healthy, singleton infants. 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 labour or during active spontaneous term labour (*n*=9 patients per group). Indications for Caesarean section in the absence of labour were breech presentation and/or previous Caesarean section. Indications for Caesarean section in the labouring samples were for foetal malpresentation, foetal distress and delayed or failure to progress. Labour was defined as the presence of regular uterine contractions (every 3–4 min) resulting in cervical effacement and dilation. None of the patients received any medications to augment or induce labour. Relevant clinical details for the patients are detailed in Table 1.

Table 1 Clinical characteristics of patients.

	No labour (n=9 patients)	In labour (n=9 patients)
Maternal age (years)	31 (1.6)	31 (1.5)
Pre-pregnancy maternal BMI	22.4 (1.1)	22.6 (0.9)
Gravida	2.1 (0.3)	2.0 (0.4)
Parity	1.0 (0.3)	0.6 (0.3)
Gestational age (weeks)	38.9 (0.3)	39.4 (0.5)
Foetal gender	2 female; 7 male	4 female; 5 male
Birthweight (g)	3562 (161)	3542 (84)
Labour		
No labour (%)	100	0
Spontaneous (%)	0	100
Duration of labour (min)	N/A	642 (89)
Membrane rupture		
SROM (%)	0	22
PROM (%)	0	22
ARM (%)	100	56

Values represent mean ($\pm$ S.E.M.) unless otherwise specified.

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 labour. Myometrial cells were isolated and cultured as previously described (Lim *et al.* 2013a).

IRF5 siRNA transfection in primary myometrial cells

To determine the effect of pro-inflammatory mediators on IRF5 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), 250 ng/mL fsl-1 (InVivoGen; San Diego, CA, USA) or 1 μ g/mL flagellin (purified flagellin from *Salmonella typhimurium*; InVivoGen) for 20 h. Cells were collected and stored at -80°C until assayed for IRF5 mRNA expression by qRT-PCR as detailed below. Experiments were performed from myometrium obtained from five patients.

To determine if IRF5 regulates pro-labour mediators, transfection of primary myometrial cells with siRNA was performed as previously described (Lim *et al.* 2015). Briefly, myometrial cells at approximately 50% confluence were transfected using Lipofectamine 3000 according to manufacturer's guidelines (Life Technologies). IRF5 siRNA (siIRF5) and negative control siRNA (siCONT) were obtained from Ambion (Thermo Fisher Scientific). Cells were transfected with 50 nM siIRF5 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 μ g/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 adhesion and cell contraction assays or stored at -80°C for RNA extraction. The conditioned media was stored at -80°C for analysis of endpoints by ELISA. Data are presented as fold change relative to IL1B, TNF-, fsl-1- or flagellin-stimulated cells. Experiments were performed from myometrium obtained from seven patients.

RELA luciferase assay

To determine the effect of siIRF5 on RELA transcriptional activity, a luciferase assay was performed as previously described (Lim *et al.* 2016b). Briefly, primary myometrial cells were transfected with 300 ng/mL RELA reporter construct (Qiagen) using FuGENE HD transfection reagent (Promega). After 6 h, cells were transfected with 50 nM of siIRF5 or siCONT (as detailed above) 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 μ g/mL flagellin and the cells incubated at 37°C for an additional 20 h. After final incubation, cells were harvested in lysis buffer, and luminescence activity was measured using a luciferase reporter assay kit (Life Research; Scoresby, Vic, Australia) and Renilla luciferase flash assay kit (Thermo Fisher Scientific) as instructed. The ratio of the firefly luciferase level to the Renilla luciferase level was determined, and the results are expressed as a ratio of normalised luciferase activity. The experiments were performed from myometrium obtained from six patients.

Mice studies

Animal studies were conducted with approval from the Austin Health's Animal Ethics Committee. Eight-week-old timed pregnant C57BL/6 female mice were purchased from WEHI (Melbourne, Australia). Timed pregnancies were achieved by housing female mice with male mice overnight and mice found to have vaginal plugs the next morning were considered to be 0.5 day post-coitum (dpc). Studies were performed as previously described (Lappas 2018). For LPS-induced preterm labour, 15.5 dpc timed pregnant C57BL/6 female mice were intraperitoneally injected with LPS (serotype O26:B6; 15 μ g in 50 μ L of PBS; Sigma) or sterile PBS (vehicle control). This model of preterm labour results in a high rate of preterm delivery (18–22 h post LPS treatment) and does not cause maternal mortality. None of the vehicle-injected mice went into labour. 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 qRT-PCR as detailed below.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin sections as described previously (Lim *et al.* 2015) using the IHC Select HRP Detection Set (Merck Millipore). Briefly, sections were deparaffinised followed by an antigen retrieval step (boiled in 10 mM Tris, 1 mM EDTA, pH 9.0 for 10 min followed by 20-min incubation) and then endogenous peroxidases were inactivated by adding 3% hydrogen peroxide for 10 min. After blocking (blocking reagent: normal goat serum in PBS) for 5 min, sections were incubated with 2 μ g/mL rabbit polyclonal anti-IRF5 (catalogue number GTX113477, GeneTex; Irvine, CA, USA) in 1% (wt/vol) bovine serum albumin in PBS and incubated in a humidity chamber at 4°C overnight. Binding sites were labelled with biotin-conjugated rabbit anti-goat IgG antibody followed by the streptavidin-HRP. Haematoxylin was used for nuclear staining. Negative control slides, where

primary antibody was replaced with rabbit IgG, were also performed. Positive controls, which were composite slides with tonsil, breast tumour and ovarian tumour, were included in each run.

RNA extraction and qRT-PCR

RNA extractions and qRT-PCR was performed as previously described (Lim *et al.* 2015). RNA concentration and purity were measured using a NanoDrop ND1000 (Thermo Fisher Scientific). RNA was converted to cDNA using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The RT-PCR was performed using the CFX384 Real-Time PCR detection system (Bio-Rad Laboratories) using 100 nM of pre-designed and validated QuantiTect primers (primer sequences not available) (Qiagen). For the human samples, gene Ct values were normalised to the average YWHAZ and succinate dehydrogenase (SDHA) Ct values of the same cDNA sample. For the mouse samples, gene Ct values were normalised to the average actin and GAPDH Ct values of the same cDNA sample. Fold differences were determined using the comparative Ct method.

Western blotting

Western blotting was performed as previously described (Lim *et al.* 2015). Blots were incubated in a 1 µg/mL rabbit polyclonal anti-IRF5 (catalogue number GTX113477, GeneTex; Irvine, CA, USA) prepared in blocking buffer (5% skim milk in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) with 0.05% 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 Laboratories). Protein normalisation was performed as previously described (Lim *et al.* 2016b). For the expression studies, the level of IRF5 was normalised to Ponceau S stain; a section of the Ponceau S-stained membrane was chosen, which did not show variation with labor status.

Enzyme immunoassays

Assessment of cytokine and chemokine release of IL6 and CXCL8 was performed using the CytoSet sandwich ELISA according to the manufacturer's instructions (Life Technologies). The release of CCL2, sICAM1 and sVCAM1 was performed by 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 available competitive enzyme immunoassay kit according to the manufacturer's specifications (Cayman Chemical Company). The interassay and intraassay coefficients of variation for all assays were less than 10%.

Statistical analysis

All statistical analyses were undertaken using GraphPad Prism (GraphPad Software). For Figure 1 and Tables 1, 2, a paired or unpaired Student's *t*-test was used to assess the statistical

significance between normally distributed data; otherwise, the nonparametric Mann-Whitney *U* (unpaired) or the Wilcoxon (matched pairs) tests were used. For Figs 2, 3, 4, 5 and 6, 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

Figure 1 Expression of IRF5 in human myometrium. (A, B and C) Human myometrium was obtained at term Caesarean section from women at term in the absence of labour (term no labour, $n=8$ patients) or during labour at term (term in labour, $n=8$ patients). (A) Immunohistochemical expression of IRF5. These sections are representative of one patient sample per group. Negative control is also displayed. Original magnification $\times 100$. Scale bar 100 µm. (B) *IRF5* mRNA abundance was analysed by qRT-PCR. Data are displayed as box-and-whisker plots (median, interquartile range). $*P \leq 0.05$, Mann-Whitney *U* test. (C) IRF5 protein expression was analysed by Western blotting. Representative Western blot from four patients per group is shown. Data are displayed as box-and-whisker plots (median, interquartile range). $*P \leq 0.05$, Mann-Whitney *U* test. (D) Mice were injected with a single i.p. injection of 15 µg of LPS at 15.5 dpc. Mice were considered in labour on birth of one pup. LPS-treated mice were killed on the birth of one pup, and time-matched vehicle-injected mice were killed directly afterward ($n=4$ mice/group). IRF5 mRNA expression in myometrium was analysed by qRT-PCR and the fold change was calculated relative to vehicle group. All data are displayed as mean $\pm$ S.E.M. $*P < 0.05$, unpaired Student's *t*-test. (E) Human myometrial cells were incubated in the absence or presence of 1 ng/mL IL1B or 10 ng/mL TNF for 20 h ($n=6$ patients per treatment). *IRF5* mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to basal. Data are displayed as mean $\pm$ S.E.M. $*P \leq 0.05$, paired Student's *t*-test.

post hoc testing to discriminate among the means). Statistical significance was ascribed to a *P* value ≤ 0.05 . Data are expressed as mean $\pm$ S.E.M.

Results

IRF5 expression in human myometrium

The localisation of IRF5 in human term myometrium was determined by immunohistochemistry. Within sections of myometrium, there was strong cytoplasmic and some nuclear IRF5 protein localised to longitudinal and transverse smooth muscle fibres. IRF5 staining was stronger in tissues obtained from labouring women compared to non-labouring women (Fig. 1A). Of note, there was no staining present in the negative controls. The mRNA and protein expression was also investigated in myometrium from non-labouring and labouring women at term. As depicted in Fig. 1B and C, IRF5 mRNA and protein expression was significantly higher in myometrium after spontaneous term labour when compared to non-labouring tissues.

To determine whether a comparable upregulation in IRF5 mRNA expression was associated with preterm labour, we utilised a mouse model of preterm labour. In this model, LPS was injected into 15.5 dpc mice and mice were killed on the birth of one pup (between 18 and 22 h post LPS injection). A time-matched vehicle-injected mouse was killed directly afterward; none of the vehicle-injected mice delivered preterm. LPS significantly increased IRF5 mRNA expression in the mouse myometrium when compared with vehicle-injected controls (Fig. 1D).

We next sought to determine if the pro-inflammatory cytokines IL1B and TNF, which are increased in labouring myometrium (Young *et al.* 2002), may be causing the increase in IRF5. To do this, we treated primary cells isolated from human myometrium, in the absence or presence of IL1B or TNF for 20 h. As shown in Fig. 1E, IL1B or TNF treatment induced a significant increase (2-fold increase) in IRF5 mRNA expression.

IRF5 regulates cytokine- and TLR-induced pro-inflammatory cytokines in primary myometrial cells

To determine if IRF5 regulates cytokine and TLR-induced pro-labour mediators, primary cells were isolated from human myometrium and IRF5 was knocked down by siRNA. The efficacy of siIRF5 transfection was assessed

by qRT-PCR; when compared to siCONT-transfected cells, there was a 77% decrease in IRF5 mRNA expression in siIRF5-transfected cells (1.00 ± 0.22 vs 0.23 ± 0.05). There was no effect of siIRF5 on cell viability as determined by MTT cell viability assay (data not shown).

Figure 2 depicts the effect of siIRF5 on IL1A and IL6 mRNA expression and IL6 secretion. As expected, in cells transfected with siCONT, treatment with IL1B, TNF, fsl-1 and flagellin significantly increased IL1A and IL6 mRNA expression and release of IL6. In siIRF5-transfected cells, there was a significant decrease in IL1A and IL6 mRNA expression and IL6 release when stimulated with IL1B (Fig. 2A, B and C) and fsl-1 (Fig. 2G, H and I). While there was no effect of siIRF5 on TNF and flagellin-stimulated IL1A mRNA expression (Fig. 2D and J), there was a significant decrease in IL6 mRNA expression and release when stimulated with TNF (Fig. 2E and F) and flagellin (Fig. 2K and L). As shown in Table 2, there was no effect of siIRF5 on IL1A and IL6 mRNA expression and IL6 secretion under basal conditions. As previously reported (Lim *et al.* 2016b), IL1A and IL1B levels are not detectable in the incubation media from human primary myometrial cells and thus not assessed.

IRF5 regulates cytokine- and TLR-induced chemokines in primary myometrial cells

The effect of siIRF5 on chemokine expression of CXCL8 and CCL2 is depicted in Fig. 3. Expectedly, in cells transfected with siCONT, treatment with IL1B, TNF, fsl-1 and flagellin significantly increased CXCL8 and CCL2 mRNA expression and release. In siIRF5-transfected cells, there was a significant decrease in IL1B-induced CXCL8 and CCL2 mRNA expression, but there was no effect on IL1B-induced CXCL8 and CCL2 release (Fig. 3A, B, C and D). There was a significant decrease in TNF-induced CCL2 mRNA expression, but no effect on TNF-induced CXCL8 mRNA expression and release, and release of CCL2 (Fig. 3E, F, G and H). In siIRF5-transfected cells, there was a significant decrease in fsl-1- and flagellin-induced CXCL8 and CCL2 mRNA expression and release (Fig. 3I, J, K, L, M, N, O and P). As shown in Table 2, there was no effect of siIRF5 on CXCL8 and CCL2 mRNA expression and secretion under basal conditions.

Table 2 Effect of siIRF5 on basal mRNA expression and secretion of pro-labour mediators in human primary myometrial cells.

	IL1A mRNA expression	IL6 mRNA expression	IL6 release (ng/mL)	CXCL8 mRNA expression	CXCL8 release (ng/mL)	CCL2 mRNA expression	CCL2 release (ng/mL)	ICAM1 mRNA expression	sICAM1 release (ng/mL)	VCAM1 mRNA expression	sVCAM1 release (ng/mL)
siCONT	0.04 $\pm$ 0.02	0.06 $\pm$ 0.02	1.26 $\pm$ 0.61	2.12 $\pm$ 0.99	3.46 $\pm$ 0.92	1.95 $\pm$ 1.10	1.75 $\pm$ 0.70	0.15 $\pm$ 0.05	0.34 $\pm$ 0.11	0.20 $\pm$ 0.06	0.31 $\pm$ 0.17
siIRF5	0.04 $\pm$ 0.02	0.06 $\pm$ 0.03	0.92 $\pm$ 0.43	1.93 $\pm$ 0.98	3.20 $\pm$ 1.06	1.50 $\pm$ 0.86	1.59 $\pm$ 0.67	0.16 $\pm$ 0.06	0.31 $\pm$ 0.10	0.18 $\pm$ 0.06	0.26 $\pm$ 0.15

Data displayed as mean $\pm$ S.E.M.

Figure 2 Effect of siIRF5 on pro-inflammatory cytokines in primary myometrial cells. Human primary myometrial cells were transfected with 50 nM siCONT or 50 nM siIRF5 for 48 h and then treated with (A, B and C) 1 ng/mL IL1B, (D, E and F) 10 ng/mL TNF, (H and I) 250 ng/mL fsl-1 or (J, K and L) 1 µg/mL flagellin (flag) for an additional 20 h ($n = 5-7$ patients). (A, B, D, E, G, H, J and K) *IL1A* and *IL6* mRNA expression was analysed by qRT-PCR. (C, F, I and L) 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. Individual data points represent 5–7 independent experiments and displayed as mean $\pm$ S.E.M. * $P \leq 0.05$ (repeated-measures one-way ANOVA).

IRF5 regulates cytokine- and TLR-induced adhesion molecules in primary myometrial cells

The effect of siIRF5 on the expression of adhesion molecules ICAM1 and VCAM1 is demonstrated in Fig. 4. Treatment of siCONT-transfected cells with IL1B, TNF, fsl-1 and flagellin expectedly increased *ICAM1* and *VCAM1* mRNA expression and release of sICAM1 and sVCAM1. In siIRF5-transfected cells, there was no effect on *ICAM1* mRNA expression when treated with IL1B, TNF, fsl-1 or flagellin (Fig. 4A, E, I and M). In contrast, there was a significant decrease in the release of sICAM1 in siIRF5-transfected cells with stimulated with

all treatments (Fig. 4B, F, J and N). In siIRF5-transfected cells, there was a significant decrease in IL1B, TNF, fsl-1 and flagellin-induced VCAM1 mRNA expression and release (Fig. 4C, D, G, H, K, L, O and P). As shown in Table 2, there was no effect of siIRF5 on *ICAM1* and *VCAM1* mRNA expression and sICAM1 and sVCAM1 secretion under basal conditions.

IRF5 regulates IL1B-induced contraction-associated proteins in primary myometrial cells

Primary myometrial cells treated with IL1B were used to determine the effect of siIRF5 on the expression of mediators in the PTGS2-prostaglandin pathway. IL1B significantly increased *PTGS2* and *PTGFR* mRNA expression and release of $\text{PGF}_{2\alpha}$ in myometrial cells transfected with siCONT (Fig. 5). The effect of siIRF5 was a significant decrease in IL1B-induced *PTGS2* and *PTGFR* mRNA expression and $\text{PGF}_{2\alpha}$ secretion.

IRF5 regulates NF- κ B RELA transcriptional activity

A luciferase assay was used to determine the effect of IRF5 silencing on NF- κ B RELA transcriptional activity. In siCONT-transfected myometrial cells, treatment with IL1B, TNF, fsl-1 or flagellin significantly increased NF- κ B RELA transcriptional activity (Fig. 6). In cells transfected with siIRF5, there was a significant attenuation of NF- κ B RELA transcriptional activity when stimulated with all treatments.

Discussion

The findings of this study demonstrate that IRF5 is increased in myometrium obtained from labouring women when compared to myometrium obtained from non-labouring women at term. Additionally, using a mouse model of preterm labour induced by LPS, we found that LPS significantly upregulated IRF5 mRNA expression in the maternal myometrium as compared with vehicle-injected controls. Functional studies in primary human myometrial cells demonstrated a pro-inflammatory and pro-labour role for IRF5. Specifically, silencing of IRF5 using siRNA showed a significant attenuation of cytokine- or TLR-induced pro-inflammatory cytokines (IL1A, IL6), chemokines (CCL2, CXCL1, CXCL8), adhesion molecules (ICAM1, VCAM1) and contraction-associated proteins (*PTGS2*, *PTGFR*, $\text{PGF}_{2\alpha}$). Additionally, NF- κ B RELA transcriptional activity was significantly reduced in siIRF5-transfected myometrial cells, suggesting that IRF5 may regulate pro-inflammatory and pro-labour mediators via NF- κ B signalling.

Increased expression of IRF5 in labouring myometrium, which is characterised by increased inflammation (Bollapragada et al. 2009, Weiner et al. 2010), is in agreement with studies in non-gestational

Figure 3 Effect of siIRF5 on chemokines in primary myometrial cells. Human primary myometrial cells were transfected with 50 nM siCONT or 50 nM siIRF5 for 48 h and then treated with (A, B, C and D) 1 ng/mL IL1B, (E, F, G and H) 10 ng/mL TNF, (I, J, K and L) 250 ng/mL fsl-1 or (M, N, O and P) 1 µg/mL flagellin (flag) for an additional 20 h ($n=5-7$ patients). (A, C, E, G, I, K, M, O) CXCL8 and CCL2 mRNA expression was analysed by qRT-PCR. (B, D, F, H, J, L, N, P) 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. Individual data points represent 5–7 independent experiments and displayed as mean $\pm$ S.E.M. * $P \leq 0.05$ (repeated-measures one-way ANOVA).

tissues; higher IRF5 is observed in a number of diseases associated with inflammation including asthma (Oriss *et al.* 2017), arthritis (Weiss *et al.* 2013) and liver fibrosis (Alzaid *et al.* 2016). It is worth noting, that for the human samples, the majority of labouring cases were obtained from women who were delivered by Caesarean section for failure to progress in labour. Interestingly, a recent study showed that there is an exaggerated inflammatory response in myometrium obtained from labouring women who had a Caesarean delivery because they failed to progress in labour when compared to those who had a Caesarean delivery due to foetal distress or foetal malpresentation (Chaemsathong *et al.* 2013). Thus, it is possible that the increase in IRF5 expression during labour may be due to dysfunctional myometrial signalling in the failure to progress group. However, our studies in mice, whereby IRF5 mRNA expression was increased in maternal myometrium during preterm labour, reassuringly suggests that IRF5 expression is increased by mechanisms associated with labour and

not because of dysfunctional myometrial signalling due to failure to progress group.

Whether increased IRF5 observed in labour in this study is a cause or consequence of labour is not known. It would therefore be of interest to determine if temporal changes occur in IRF5 expression in myometrium; however, obtaining myometrial biopsies from early/mid pregnancy is extremely difficult. Regardless, the mechanisms leading to increased IRF5 expression in labouring myometrium are not known but most likely a consequence of the inflammatory response associated with labour. In non-gestational tissues, IRF5 expression is higher in leukocytes of inflammatory states (Swindell *et al.* 2010, Krausgruber *et al.* 2011, Wang *et al.* 2012, Weiss *et al.* 2013). Although recent studies suggest that leukocyte infiltration and/or activation are not a prerequisite for labour initiation (Singh *et al.* 2017), higher expression of leukocytes are observed in labouring myometrium (Osman *et al.* 2003). Thus, it is possible that the differences seen in labouring myometrium may

Figure 4 Effect of siIRF5 on cell adhesion molecules in primary myometrial cells. Human primary myometrial cells were transfected with 50 nM siCONT or 50 nM siIRF5 for 48 h and then treated with (A, B, C and D) 1 ng/mL IL1B, (E, F, G and H) 10 ng/mL TNF, (I, J, K and L) 250 ng/mL fsl-1 or (M, N, O and P) 1 µg/mL flagellin (flag) for an additional 20 h ($n=5-7$ patients). (A, C, E, G, I, K, M, O) ICAM1 mRNA expression was analysed by qRT-PCR. (B, D, F, H, J, L, N and P) 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. Individual data points represent 5–7 independent experiments and displayed as mean $\pm$ S.E.M. * $P \leq 0.05$ (repeated measures one-way ANOVA).

be attributed to the infiltration of leukocytes observed in labouring tissues. Leukocytes are a rich source of IL1B and TNF, two acute phase pro-inflammatory cytokines that are also upregulated in myometrium during labour (Young *et al.* 2002, Tattersall *et al.* 2008). In our studies, treatment of primary human myometrial cells with IL1B or TNF significantly increased *IRF5* mRNA expression. Collectively, this suggests that the increase in *IRF5* expression seen in labouring myometrium may be a consequence of the inflammatory response associated with labour.

Inflammation induces a cascade of events that activates the myometrium culminating in preterm labour. Inflammation can originate either locally or systemically and is triggered by infection or by sterile pro-inflammatory insults. Pro-inflammatory cytokines such as TNF and IL1B and bacterial products such as fsl-1 and flagellin can induce an inflammatory response in myometrium (Rauk & Chiao 2000, Liang *et al.* 2008, Lim *et al.* 2014, 2017a,b, Lappas 2017b). They can induce chemokines

and cellular adhesion molecules, which can stimulate an influx of leukocytes into myometrium. They can also induce uterine contractions by increasing the expression of contraction-associated proteins such as PTGS2 and PTGFR as well as inducing the production of uterotonic prostaglandins such as PGF_{2α}. IRF5 has emerged as an important pro-inflammatory transcription factor involved in acute and chronic inflammation (Takaoka *et al.* 2005, Paun *et al.* 2008, Krausgruber *et al.* 2010, 2011, Tada *et al.* 2011, Feng *et al.* 2012, Hedl & Abraham 2012, Xu *et al.* 2012, Ren *et al.* 2014, Duffau *et al.* 2015, Weiss *et al.* 2015, Alzaid *et al.* 2016, Oriss *et al.* 2017). Thus, functional studies were then performed to investigate if IRF5 may play a role in regulating pro-inflammatory and pro-labour mediators induced by the pro-inflammatory cytokines IL1B and TNF or bacterial products and TLR ligands fsl-1 and flagellin.

We found that, for the most part, IRF5 silencing was associated with a blunting of the inflammatory response in myometrial cells suggesting that that IRF5

Figure 5 Effect of siIRF5 on contraction-associated proteins in primary myometrial cells. Human primary myometrial cells were transfected with 50 nM siCONT or 50 nM siIRF5 for 48 h and then treated with 1 ng/mL IL1B for an additional 20 h ($n=6$ patients). (A and 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. Individual data points represent 6 independent experiments and displayed as mean $\pm$ S.E.M. * $P \leq 0.05$ (repeated measures one-way ANOVA).

plays an important role in cytokine and TLR signalling pathways associated with preterm labour. Specifically, the expression and/or secretion of pro-inflammatory cytokines (IL1A, IL6), chemokines (CCL2, CXCL1, CXCL8), adhesion molecules (ICAM1, VCAM1) and contraction-associated proteins (*PTGS2*, *PTGFR*, $\text{PGF}_{2\alpha}$) was significantly suppressed in siIRF5-transfected cells when compared to siCONT-transfected cells. The reduction in mRNA expression or secretion of pro-labour mediators by siIRF5 ranged from 20% to 50%. The biological relevance of this decrease cannot be inferred from these studies as all data were from *in vitro* siRNA experiments using cells isolated from myometrium. Regardless of this limitation, our *in vitro* studies suggest that in a setting of increased IRF5 expression, myometrial inflammation is increased. This infers that myometrial IRF5 transcriptionally induces pro-inflammatory and pro-labour mediators in myometrium.

In non-gestational tissues, IRF5 has been shown to regulate inflammation by interacting with the RELA subunit of NF- κ B. Specifically, IRF5 forms a protein

complex with RELA to drive a sustained induction of the human TNF gene (Krausgruber *et al.* 2010) while IRF5 genome recruitment to inflammatory genes is aided by RELA (Saliba *et al.* 2014). Furthermore, IRF5 has also been shown to directly induce the activation of RELA; IRF5 knockdown profoundly decreases RELA pathway activation (Hedl & Abraham 2012). Numerous studies over the last 20 years have shown that NF- κ B plays a central role in regulating pro-inflammatory and pro-labour mediators in human gestational tissues (Lindstrom & Bennett 2005, Lappas & Rice 2007). Thus, we hypothesised that IRF5 may also regulate pro-labour mediators in primary myometrial cells through NF- κ B. We addressed this by determining the effect of siIRF5 on NF- κ B RELA transcriptional activity using a luciferase assay. The effect of siIRF5 was a significant suppression of NF- κ B RELA activation induced by the pro-inflammatory cytokines IL1B and TNF and the bacterial products fsl-1 and flagellin. This suggests that in the absence of IRF5, primary myometrial cells display hypoactive RELA, which would result in decreased transcription of pro-inflammatory and pro-labour genes. Collectively, these findings suggest that in human myometrium, IRF5 is a mediator of the NF- κ B signalling pathway, corroborating the potential of IRF5 as a therapeutic target for the prevention of preterm labour.

Much research over the last decade has been focussed on the identification of inflammatory molecules that can be targeted to mitigate the inflammatory response associated with preterm labour. In addition to NF- κ B, we have identified a number of regulators of inflammation including A20 (Lappas 2017a), Kruppel-like factor 5 (KLF5) (Lappas 2015), sirtuins (SIRT5) (Lappas *et al.* 2011, Lim *et al.* 2013b, 2016a, Poljak *et al.* 2014) and forkhead box O (FOXO) proteins (Lim *et al.* 2013a). Whether IRF5 may interact with these signalling proteins to regulate pro-labour mediators is not known and an avenue for further research.

In conclusion, we have identified a novel role of IRF5 in regulating the inflammatory response in human myometrium. Specifically, IRF5 expression is increased

Figure 6 Effect of siIRF5 on NF- κ B RELA transcriptional activity. Human myometrial cells were transfected with 0.75 ng NF- κ B RELA reporter construct. After 6 h, cells were transfected with 50 nM siCONT or 50 nM siIRF5 for 48 h, then treated with (A) 1 ng/mL IL1B, (B) 10 ng/mL TNF, (C) 250 ng/mL fsl-1 or (D) 1 μ g/mL flagellin (flag) for an additional 20 h ($n=6$ patients). RELA promoter activity (normalised to Renilla) is expressed as a ratio of luciferase activity of IL1B-, TNF-, fsl-1- or flag-stimulated siCONT transfected cells. Individual data points represent 6 independent experiments and displayed as mean $\pm$ S.E.M. * $P \leq 0.05$ (repeated-measures one-way ANOVA).

in myometrium with labour and the coincident pro-inflammatory cytokines IL1B and TNF. Increased IRF5 expression is associated with an exaggerated inflammatory response that is characterised by increased expression of pro-inflammatory cytokines, chemokines, adhesion molecules, contraction-associated proteins and prostaglandins leading to labour. This cascade of events is further amplified by pro-inflammatory cytokines. Further studies are required in order to elucidate the role of IRF5 in the processes of labour. In particular, it would be of interest to determine the role of IRF5 in foetal membranes as they also play an important role in releasing pro-inflammatory mediators that can contribute to labour (Menon 2016). Furthermore, the relationship between IRF5 and progesterone receptors should be examined given a recent report suggesting a direct correlation between loss of IRF5 expression and progesterone receptor expression (Pimenta *et al.* 2015). This is of importance as progesterone receptors mediate the actions of progesterone and thus play an important role in controlling myometrial contractility and the parturition process (Mesiano *et al.* 2010).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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