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Interleukin 1 signalling is regulated by Leukemia Inhibitory Factor (LIF) and is aberrant in *Lif* $^{-/-}$ mouse uterus[1]

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Summary statement:

Components of the Interleukin 1 system are misregulated during the peri-implantation period in *Lif* $^{-/-}$ mice; in vitro LIF stimulates apical secretion of IL1A by LE in co-culture with stromal cells but not alone

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Key words: Leukemia Inhibitory Factor, uterus, implantation, Interleukin 1, prostaglandin

Abstract

This study addresses the regulation of the Interleukin 1 (IL1) system in the murine uterine luminal epithelium (LE) and stroma by leukemia inhibitory factor (LIF). Using RT-PCR we compared expression of *Il1a, Il1b, Il1rn, Il1r1* and *Il1r2* during the pre- and peri-implantation periods of pregnancy in wild type (wt) and LIF null LE and stroma. In wt LE, *Il1a* transcripts were down-regulated on day (D) 4am with renewed expression by D4pm. In *Lif* $^{-/-}$ LE there was a gradual decrease in expression from D2 which became undetectable by D6. *Il1b* and *Il1r1* expression were similar in wt and null mice, but *Il1rn* expression was almost completely lost during the peri-implantation period in *Lif* $^{-/-}$ LE. In the stroma *Il1a* was sharply down-regulated on D4 am reappearing on D4 pm, but in the null mice was only expressed on D3 and D5. Stromal *Il1r1* and *Il1r2* were also misregulated. *Il1rn* showed constitutive expression in null stroma in contrast to the loss of expression on D4am in the wt mouse. In *Lif* deficient mice, immunostaining indicated a reduction of endometrial IL1A at the time of implantation and of Il1B in stroma. LE-stromal co-culture revealed that LIF stimulated apical secretion of both IL1A and PTGES2 by LE cells without affecting basal secretion of IL1A and with only a small effect on basal PTGES2 secretion. We conclude that *Il1a* and *Il1rn* in LE and *Il1a, Il1rn* and *Il1r1* in stroma are regulated by LIF which stimulates apical secretion of IL1A by LE.

Introduction

Embryo implantation involves a complex and dynamic interaction between the trophoblast, the uterine epithelium and the stroma which must occur within a specific temporal 'window' during which the uterine endometrium is receptive to the embryo. Although it is well established that this 'window of implantation' is primarily controlled by the steroid hormones estrogen and

progesterone (P_4) [1;2], recent evidence has shown that a plethora of other molecules including growth factors and cytokines mediate and modulate the actions of these steroid hormones [3;4]. Uterine LIF is expressed in two transient peaks during early pregnancy. Firstly, on day 1 (D1) of pregnancy (vaginal plug = D1 of pregnancy) LIF expression is stimulated by ovulatory estrogen in both luminal and glandular epithelium. Secondly, on D4, nidatory estrogen stimulates expression of both *Lif* mRNA and protein in the glandular epithelium (GE) [5-7]. This second peak of LIF expression is essential for successful embryo implantation into the uterus on the evening of D4 of pregnancy [8]. The cellular target of LIF in the uterus during pregnancy appears to be the luminal epithelium (LE) and *Lif* receptor (*Lifr*) transcripts and protein have been found to be present predominantly in the LE during D3-D5 of pregnancy [9;10]. It has been known for some time that uteri of *Lif* deficient mice are unable to support embryo implantation [6]. However, *Lif* $^{-/-}$ blastocysts can undergo implantation when transferred into pseudopregnant recipients and develop to term demonstrating that the implantation defect is maternal. Rescue of implantation can be achieved by exogenous delivery of LIF on D4 of pregnancy in the homozygous mutants [6;8;11]. The importance of LIF for successful embryo implantation in the mouse may be of general significance to all mammals and other species. Indeed, increased levels of LIF during pregnancy have been shown to be conserved in several species including humans and rhesus monkeys [12-15], while low levels of *Lif* have been correlated with infertility in women [16-19].

Furthermore, the uteri of *Lif* deficient mice do not undergo decidualisation, a process involving the differentiation of the uterine stroma essential to support the implanting embryo [6-8]. Decidualisation is triggered by a number of molecules and is first discerned by an increase in vascular permeability at the site of implantation [1;20]. Amongst the best candidates for roles in the initiation of decidualisation are prostaglandins (PGs), which increase at the time of implantation. PTGES2 is a central PG involved in the initiation of uterine vascular permeability

[21-23]. PGs are produced by both uterine epithelial and stromal cells and their synthesis is induced by Interleukin 1 (Il1), also produced by the uterine epithelium, as well as by other cell types including macrophages [24]. The IL1 system is composed of two agonists IL1A and IL1B, one antagonist IL1RN and two membrane bound receptors, IL1 receptor type one (IL1R1) and type two (IL1R2) [25;26]. Endogenous control of secreted IL1 activity is achieved by regulation of IL1 synthesis and processing and release from intracellular and membrane bound stores [26]. This control of IL1 bioavailability is further regulated by a unique receptor antagonist (IL1RN), which binds with high affinity to IL1 receptors thus preventing access by IL1 ligands and inhibiting signalling [27]. In mouse, IL1R1 protein is reported to be induced in uterine LE cells during the preimplantation period and subsequent blockade of IL1 signalling by injection of IL1RN during early pregnancy prevents attachment of the blastocyst to the LE [28;29].

Epithelial derived IL1A has been previously reported to upregulate the synthesis of PTGES2 and $PGF_{2\alpha}$ in mouse and rat uterine stromal cells [30;31] and other studies *in vitro* have shown that IL1A increases levels of mRNA for *Ptgs2* (a rate limiting enzyme for PG synthesis) in rat uterine stromal cells [32]. Evidence from *in vivo* studies has demonstrated that mRNA and protein levels of PTGS2 are reduced in the uterine stroma of *Lif* deficient mice at the implantation site [7;33]. We have shown, however, that LIF does not directly promote the synthesis of PTGES2 by uterine stromal cells *in vitro* suggesting that PTGES2 is not a direct target of LIF here [34]. In human endometrial epithelial cells, IL1B upregulates *LIFR* and this effect is abrogated by inhibition of IL1R1 [35]. This suggests that in human and murine endometrium it is likely that feedback loops exist between LIF and IL1 in uterine epithelial cells. Together with the reduction of PTGS2 expression at the implantation site in *Lif* $^{-/-}$ females these findings support a signalling

cascade involving LIF induction of IL1 in the LE that triggers the onset of the decidual response via PGs. Therefore using a co-culture system we have investigated the effects of LIF on IL1A production and gene expression by cultured mouse uterine LE and stromal cells in a physiologically relevant model. We have also shown that IL1 and its associated molecules are precisely regulated in LE and stroma during early pregnancy *in vivo.* Moreover the temporal sequence of changes in *Il1* related gene expression (specifically *Il1a* and *Il1rn*) during uterine LE development for implantation is seriously altered in *Lif* $^{-/-}$ mice indicating that a close relationship exists between LIF and IL1A in the regulation of endometrial cells as demonstrated *in vitro*.

Materials and methods

Animals

All mice were maintained under conditions in accordance with the UK Home Office as in Fouladi-Nashta et al., [7] and procedures were in accordance with our UK Home Office licence. MF1 (wild type outbred) female mice (Harlan Olac Ltd, Bicester, UK) between 7-9 weeks of age were placed with MF1 males overnight for mating and pregnancy was confirmed by the presence of a vaginal plug (D1 of pregnancy). MF1 female mice used for *in vitro* culture were induced to ovulate by an intraperitoneal injection of a single dose of 5 IU eCG (Intervet, Milton Keynes, UK), followed by a single injection of 5IU hCG (Intervet) 48h later. Mating was confirmed by the observation of a vaginal plug the following morning. Mice were killed by cervical dislocation on D2 of pregnancy (48h following hCG) and uterine tissues processed as below. The *Lif* $^{-/-}$ MF1 founder mice were provided by Dr Andrew Sharkey (University of Cambridge) from an original colony generated at the Institute for Stem Cell Research, University of Edinburgh [36]. Since *Lif* $^{-/-}$ females are infertile, propagation of *Lif* $^{-/-}$ mice was achieved by

breeding from null males and heterozygote females as previously described [7;37]. Genotyping for identification of *Lif* $^{-/-}$ mice was carried out by PCR on DNA samples from progeny following weaning as previously reported by us [7;37]. Animals were killed by cervical dislocation on the required day of pregnancy and uterine tissue processed as detailed below. Uteri were harvested in the morning between 0900h-1000h and on D4 also in the evening between 2100h-2200h.

Reagents

All reagents were purchased from Sigma (Dorset, UK) unless otherwise indicated. Primary antibodies were used as follows: Goat anti-mouse IL1A (2µg/ml; R&D systems, Oxfordshire, UK), rabbit anti-mouse IL1B (1µg/ml; Santa Cruz Biotechnology, Heidelberg, Germany), monoclonal 11-5F against desmoplakin (1:10; courtesy of Prof. D Garrod, University of Manchester), rabbit anti-mouse TJP1 (1µg/ml; Zymed, Cambridge UK), rat anti-mouse f4/80 (Serotec, Oxford, UK), fluorescein isothiocyanate (FITC) conjugated donkey anti goat, rat or rabbit IgG secondary antibodies were used at 4µg/ml (Jackson Immunoresearch Laboratories, PA, USA) or alternatively an Alexa 488 conjugated donkey anti goat IgG (10µg/ml; Molecular Probes, Invitrogen, Paisley, UK) or a biotinylated goat anti rabbit IgG (7.5µg/ml; Vector Laboratories, Peterborough, UK) was used. Texas red-X phalloidin was used at 1:50 (Molecular Probes). Normal goat serum (NGS) was used at a 1:20 dilution to minimise non-specific binding. Mouse IL1A used as the standard in ELISA was purchased from Chemicon, (Hampshire, UK). For use in culture LIF was obtained courtesy of Dr A Vernallis (Aston University) and its activity calibrated by the proliferation response of BAF cells (gift from Dr A Vernallis). The LIF inhibitor (hLIF-05), a LIFR antagonist was used at 10 times the concentration of supplemented LIF [34;38;39].

Uterine epithelial cell layer dissociation for RNA extraction

Uterine horns were dissected from wt or *Lif* null females on D2-6 of pregnancy and the LE cell 'tube' dissociated from the stroma and gently squeezed out according to [40]. The uterine horns were then slit longitudinally and stromal cells scraped from LE depleted horns using a cell scraper (BD Biosciences, Oxfordshire, UK). The samples were centrifuged at 3000xg for 3 mins. Total RNA was isolated from the cells using the RNeasy Kit, (Qiagen, West Sussex, UK) according to manufacturer's instructions. Briefly, the tissue was lysed by drawing 10 times through a 21 gauge needle (BD Biosciences) in either 350µl (epithelial extracts) or 600µl (stromal extracts) of guanidine isothiocyanate (GITC) and 0.1% (v/v) β mercaptoethanol. To ensure complete homogenisation of the tissue, the samples were added to a Qiashredder column (Qiagen) following manufacturer's instructions. RNA preparations were quantified by absorbance at 260nm (A_{260}) using a Nanodrop spectrophotometer (Labtech Intl., E. Sussex, UK) or Genequant (Amersham Bioscience, Amersham, UK) spectrophotometer. Purity was calculated from the A_{260}/A_{280} ratio.

Isolation of total RNA from cultured uterine epithelial and stromal cells

The stromal cells were detached from the wells using a cell scraper (Corning) and the cell suspensions were centrifuged at 1000g for 5 min. The supernatant culture medium was removed and the pellet was stored in liquid nitrogen. The LE cells attached to the membranes were transferred directly to the lysis buffer. RNA was isolated from all samples using RNeasy mini kit (Qiagen, West Sussex, UK) as above.

Reverse Transcription-Polymerase Chain Reaction

Relative changes in *Il1a, Il1b, Il1rn, Il1r1* and *Il1r2* mRNA were examined in uterine LE and stromal isolates on D2-6 of pregnancy in wt and *Lif* null females using reverse transcription

polymerase chain reaction (RT-PCR). Samples from a minimum of 3 independent animals were used in each case Changes in PCR products obtained for *Il1* were normalised by comparison with an endogenous house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), expression of which has been shown to be consist in the uterus [40]. Briefly, 2µg total RNA from each sample was reverse transcribed using Superscript II first strand cDNA synthesis (Invitrogen, Paisley, UK) following manufacturer's instructions with omission of reverse transcriptase run in parallel in all reactions. PCRs were assembled to a final volume of 25µl containing 0.5µl of cDNA template, 10pmol (final concentration) primers and Red Taq PCR Buffer reaction mix (Sigma). No template and a reverse transcriptase negative control were assembled in parallel. Optimal annealing temperatures and cycle number are shown in table 1. Cycle conditions were as follows: initial denaturation at 94°C for 1 min, then cycles of the following, 30s at 94°C, annealed for 30s at a temperature determined as optimum and extended at 72°C for 30s. PCR products were resolved on a 2% (w/v) agarose gel and the results visualised under UV trans-illumination (GRI, Essex, UK). PCRs were also taken to saturation (40 cycles) to determine if transcripts were weakly expressed or absent. The PCR products were verified by automated capillary gel electrophoresis by Manchester Sequencing Services using an ABI Prism 377 sequencer (Applied Biosystems, Cheshire, UK) and products confirmed by a BLAST search.

Immunolocalisation of IL1A/IL1B

Uterine horns were fixed in either 4% paraformaldehyde (PFA) for 4h at room temeperature or in Carnoy's fixative for 30 mins at room temperature and dehydrated through an ethanol series before being embedded in paraffin wax and sectioned. Deparaffinised sections were either processed for antigen retrieval by microwave treatment (750W) with TEG buffer (1.2 g/l Tris,

0.190 g/l EGTA in distilled water, pH: 9) (IL1A) as previously described [7] or, following exposure to 0.3% (v/v) hydrogen peroxide in methanol for 12 mins, subjected to antigen retrieval with 0.01M citrate buffer (pH 6.0) for 6 minutes (IL1B). After cooling, non-specific binding was blocked in 10% (v/v) NGS and 0.1% (w/v) BSA in PBS (blocking solution).

For immuno-peroxidase staining (IL1B), endogenous biotin was blocked using an avidin/biotin blocking kit as per manufacturers' instructions (Vector Laboratories). The primary rabbit anti-IL1B or irrelevant control antibodies were diluted 1:50 in blocking solution and incubated overnight at 4ºC. Following washing, the sections were incubated in the appropriate biotinylated secondary antibody for 45 mins at room temperature. ABC reagent (Vector Laboratories) was applied to the sections for 30 mins and positive immunoreactivity was detected using a diaminobenzidine peroxidase (DAB) substrate kit (Vector Laboratories). Nuclei were counterstained with Harris' haematoxylin and sections mounted in a permanent mountant (CellPath, Newtown, Powys). To determine macrophage and IL1A immunoreactivity, uterine tissue from D4 of pregnancy was placed into aluminium foil containers of cryo-embedding compound OCT (Raymond A Lamb Laboratories, Sussex, UK). The samples were then flash-frozen in liquid nitrogen and stored at –80 °C. Serial sections (7μm) were taken using a cryostat (Leica UK Ltd, Milton Keynes, UK) and fixed for 10 minutes in ice-cold acetone at -20° C. The sections were rehydrated in 0.1%w/v BSA, 0.1%v/v Tween20 in PBS. Normal goat serum (NGS) at a 1:20 dilution was used to block non specific binding. The diluted primary antibody (1:50 for both IL1A and f4/80) was added to each section and left overnight at 4°C. Following washing, the sections were incubated with the appropriate fluorescein FITC conjugated secondary antibody for 45 mins at RT. The sections were mounted in Vectashield with 1.5μg/ml DAPI (Vector Laboratories, Peterborough, UK) and stored in the dark at 4°C. For all

198 experiments relevant isotypes were used as negative controls and carried out in parallel. A
199 secondary antibody only control was also used to check for non specific secondary antibody
200 binding.

201 ***Isolation and culture of uterine luminal epithelial and stromal cells***

202 Briefly fat-trimmed uteri were cut longitudinally to expose the lumen. They were placed in
203 trypsin dissociation solution (0.5% Type II bovine trypsin and 0.165% pancreatin in Hanks
204 Balanced Salt Solution (HBSS: Invitrogen) for 1h at 4°C followed by 1h at room temperature.
205 The medium was removed from the uteri, discarded and replaced with ice cold DNase medium
206 (1μg/ml DNAse [Type II from bovine pancreas], 10mM $MgCl_2$ and 0.1% fetal calf serum
207 [HIFCS: Invitrogen] in HBSS) before vortexing for 10 s at medium speed. The supernatant cell
208 suspension was transferred to a 50 ml Falcon tube on ice. The whole process was repeated and
209 the supernatants pooled for isolation of LE cells. The remaining uteri were washed with HBSS
210 and used for isolation and culture of stromal cells as described below.

211 ***Isolation and culture of uterine LE cells***

212 Preparation and culture of epithelial cells was as developed by Blissett and Kimber [41] modified
213 from [42]. The epithelial cell suspension was centrifuged at 200g for 5 min at 4ºC. The
214 supernatant was removed and the cell pellet was re-suspended in 10 ml ice cold DNAse medium
215 for 1 min before re-centrifugation. This procedure was repeated 3 times. DNAse medium was
216 replaced with HBSS and the Falcon tube placed at a 45° angle (15 min on ice) to allow LE cell
217 plaques to separate under gravity. The supernatant was removed and the epithelial cells re-
218 suspended in 10ml ice cold HBSS. The process was repeated for a total of 4 gravitational
219 separations before adjusting cell density to 8.0×10^5 cells / ml in LE culture medium [1 :1 Ham's
220 F12:Dulbecco's modified essential medium (DMEM) (Gibco BRL Life Technologies Ltd,

Paisley UK) containing 0.1% bovine serum albumin (BSA ; Fraction V Albumin, ICN), 100mg/ml pen/strep, 2.5% NuSerum (Collaborative Research Inc, Bedford, UK), 2.5% HIFCS, 15mM Hepes buffer and 200mM L-glutamine]. LE cells were cultured on Cellagen membranes (ICN-Flow Thame UK) as previously described [43;44]. Cellagen discs were pre-incubated with culture medium. After pre-incubation, media in the apical compartment was replaced with 250µl cell suspension and the basal compartment with 450µl LE culture medium (Fig 1a). Cells grown on these membranes are cuboidal and show a semi-polarised phenotype, intermediate between the highly polarised LE morphology seen *in vivo* at D1-3 of pregnancy and the flattened morphology seen for cells grown on plastic. The transepithelial resistance (TER) of the cultures was measured using a Millicell -ERS transepithelial resistance meter (Millipore Watford UK). All cultures used in these experiments had a TER above 400cm^2.

Isolation and culture of uterine stromal cells

Uterine stromal cells were isolated and cultured as previously described [34]. Upon removal of LE from the uterine tissue (see above), ten glass beads were added to the remaining LE denuded endometrium extract, together with stromal trypsin dissociation solution (0.05% trypsin and 0.02% EDTA (BDH) in HBSS). The tubes containing cell extracts were incubated for 20 min at 37ºC and vortexed at medium speed for 10s every 10 mins. This was process was repeated by incubation at room temperature. The content of the tube was passed through a 70µm gauze filter (Falcon) and the enzymatic digestion stopped (2% Soybean trypsin inhibitor in HBSS) after filtration. The cell suspension was then centrifuged at 400g for 10min at 4ºC. The pellet was washed in stromal cell culture medium: 1:1 mixture of DMEM and Ham's F12 medium (Invitrogen) supplemented with 1.2g/l of sodium bicarbonate, 100IU/ml penicillin streptomycin (Invitrogen), 2% Heat Inactivated Fetal Calf Serum (HIFCS, Invitrogen) and centrifuged for 10

244 min at 4ºC. The pellet was re-suspended in culture medium and live cells were assessed by
245 trypan blue exclusion using a Neubauer haemocytometer. We have already shown that cells
246 stained with epithelium-specific antibody marker (H001) were less than 2% of cells [34] and
247 leukocytes were < 1% by 48 h under these conditions.
248 Isolated stromal cells were cultured in 24 well dishes (Nunc) at 1.5 x 10^5 cells/ml in 5% CO_2 in
249 air at 37ºC. Evaluation was undertaken on a minimum of 3 cultures in each case. For co-culture,
250 uterine stromal cells were cultured in the basal compartment and LE cells introduced on to the
251 inserts at the time of stromal seeding. Media in both compartments were changed at 48 h and 96
252 h. Culture media from both compartments were collected and stored at -80°C for IL1A and
253 PTGES analysis in triplicateAll experiments were repeated on a minimum of 3 separate
254 occasions.
255 ***ELISA for IL1A***
256 IL1A secretion into the culture media by uterine stromal and LE cells was measured using a
257 mouse IL1A ELISA module set (BMS611MST; Medsystems Diagnostic GmbH, Vienna,
258 Austria) according to manufacturer's instruction. Briefly, Microwell plates (Maxisorb) were
259 coated with rabbit anti-mouse IL1A (3 μg/ml) overnight at 4°C. Non-specific binding was
260 blocked with 250μl of assay buffer (5mg/ml % BSA, 0.05% Tween 20 in PBS) for 2 h at room
261 temperature. Serial dilutions of mIL1 standard protein in PBS were added in duplicate to the
262 standard wells (for construction of a standard curve). Wells were then incubated with Biotin-
263 Conjugate (1 in 10000) for 2 h at room temperature. They were washed 3 times in wash buffer
264 (0.05% Tween 20 in PBS), Streptavidin-HRP added and incubated for 1 h at room temperature.
265 After washing TMP substrate solution (1:2 mixture of H_2O_2 and Tetramethylbenzidine) was
266 added and shaken for 20 min in the dark. The enzyme reaction was stopped by 100μl 4N

Sulphuric Acid and the colour intensity read on a microplate reader at 450nm to calculate IL1A concentrations.

Prostaglandin E radioimmunoassay (RIA)

The concentration of PTGES2 was measured in the culture media as in [34] using Sigma RIA and standards (0-100pg/ml) prepared in RIA buffer (0.01M PBS, pH 7.4 containing 0.1% BSA and 0.1% sodium azide). One hundred µl of sample or standards and 500µl of antibody working solution were added to 1.5 ml Eppendorf tubes, vortexed, incubated for 3 min at 4˚C and then ^{3}H prostaglandin E (Amersham), diluted in RIA buffer to give 6000 cpm in 700µl, was added. The tubes were vortexed and incubated for 1h at 4˚C and 200µl cold dextran-coated charcoal suspension (0.1% dextran, 1% activated charcoal (100-400 mesh) in RIA buffer) added. After shaking, the tubes were centrifuged at 800g for 15 min at 4˚C and the supernatants transferred into scintillation vials with 4 ml of scintillation cocktail (Optiphase Hisafe 2, Wallac). Radioactivity was measured with a ß counter (Wallac-M1214) and the sample concentration extrapolated from the standard curve. The values were considered reliable only in the logit interval of ±2.2 when the unlabelled molecules displace between 10 and 90% of maximum radioactivity bound [45].

Immunofluorescence staining of junctional proteins in cultured LE cells

Cellagen discs were removed from culture wells and the membranes (carrying LE cells) were detached from the supports and cut in two pieces. One half of each membrane was used for isolation of total RNA and the other half was fixed and deposited on a coverslip for immunofluorescence staining of junctional proteins including Z0-1, desmoplakin as in [7]. Primary antibodies and controls were as above. The coverslips were incubated for 2h at room temperature with an appropriate affinity-purified FITC-conjugated secondary antibody (green)

containing 10μg/ml phalloidin (red), washed, and incubated for 5mins in 5μg/ml bizbenzimide (Hoescht (33342, blue staining) before mounting in hydrophilic mounting media containing anti-fading reagent, Gelvatol.

RT-PCR for the Il1a in cultured cells

A one-step RT-PCR kit (Qiagen) was used according to the manufacturer's instructions for RT and amplification of a 220bp product. One μg of RNA was used for reverse transcription and PCR over 30 cycles with an annealing temperature of 60˚C and 5 min extension. For experiments where *Il1a* mRNA transcripts were compared between different groups, the tubes were removed from the cycler (Eppendorf) every 2 cycles after the 18^{th} cycle (amplification cycles in the linear range). Extension was then continued in another machine. The cycle number at which *Actb* was first detected was used to normalise for cDNA quantities.

Statistical analysis

Data are presented as mean ± S.E.M. Statistical analysis was performed with the SPSS 13.0 program to carry out a two-way analysis of variance using General Linear model (GLM) procedure. Effects in the linear model consisted of batch effects and the effects of time and LIF treatments. A *post hoc* test was then used to analyse the difference between control and treatments. Tukey's test was also used to reveal the differences between each treatment.

Results

Il1 family members are regulated at the transcript level in peri-implantation uterus

Characterisation of *Il1a, Il1b, Il1rn, Il1r1* and *Il1r2* mRNA expression on D2-D6 of pregnancy in wt and *Lif* null females was performed by RT-PCR (Fig 2) on RNA extracted separately from uterine stromal and LE isolates. Transcript patterns shown are representative of 3 separate animals at each stage and genotype.

Both ligands, *Il1a* and *Il1b* showed temporal regulation in the uteri of wt mice during early pregnancy (Fig 2 A,B). Specifically, transcripts bands were observed on D2 of pregnancy in both LE and stromal isolates and intensity of bands appeared to then decrease such that on the morning of D4 of pregnancy (0900h) no transcripts could be detected for *Il1a* (even when PCRs were taken to saturation), although a very faint band was seen for *Il1b* in LE and stroma. However, by the evening of D4 (2200h), which follows elevated levels of estrogen and LIF, mRNAs for *Il1a* and *Il1b* in both LE and stromal isolates were again detected as seen on D2. Although, *Il1a* mRNA was continually expressed up until D6 in both the stroma and LE, *Il1b* mRNA was undetectable on D6 in both the LE and stroma, suggesting only transient re-expression on D4 evening and D5 of pregnancy. Moreover the pattern of disappearance of *Il1a* on the morning of D4 in wt uteri was not paralleled in the uteri of *Lif* deficient mice on D2-D6 of pregnancy. *Il1a* mRNA levels appeared to decline progressively from D2 onwards in the LE, whereas stromal expression of *Il1a* transcripts were only detected on D3 and D5 of pregnancy in *Lif* null mice. Interestingly, in null females, the pattern of *Il1b* expression in the LE was parallel to that seen in wt mice, but stromal expression of *Il1b* was markedly different. Obvious stromal *Il1b* mRNA signal was detected on D2, D3, D4 morning and D6 of pregnancy but was undetectable on D4 evening and D5 morning when it was readily detectable in wt stroma.

Transcriptional expression of *Il1r1* was similar to that seen for *Il1b* in wt mice where a reduction in detectable transcripts was identified on the morning of D4 in the stroma and LE (Fig 2D). On D5, stromal transcript levels declined and on D6 of pregnancy no transcripts could be detected in the LE and little in the stroma. *Il1r2* transcripts were consistently detected in the LE from D2 onwards (Fig 2E). Strong signal for *Il1r2* mRNA was seen on the evening of D4 and morning of

D5 of pregnancy with lowest levels being on D4 morning. By D5 no stromal expression of *Il1r2* mRNA could be detected. Similar patterns of gene expression were seen in *Lif* null uteri for *Il1r1* and *Il1r2* in the LE to that in wt uteri. However, stromal expression of *Il1r1* mRNA appeared to be delayed relative to wt, with strong signal on D3, D4 morning and D6, but barely detectable signals on D2, D4 evening and D5 morning. In the null uterus *Il1r2* transcripts were only detected in the stroma on D3 and D5.

Il1rn transcripts were consistently expressed throughout D2-D6 of pregnancy in both LE and stromal isolates from wt mice, with only a transient but marked reduction on D4 morning in the stromal isolate (Fig 2C). In LE of *Lif* nulls, *Il1rn* mRNA could only be reliably detected on day 2 and D6 of pregnancy. In the stroma however, *Il1rn* transcripts were consistently expressed through D2-D6 of pregnancy with no loss of expression on day 4 as in the wt stroma.

IL1A protein expression is reduced in the Lif null uterus at implantation

Transcript analysis revealed that *Il1a* was regulated differently during early pregnancy in the uteri of wt and *Lif* null animals. To investigate whether similar changes occurred in protein expression, immunohistochemistry was performed on uterine sections from both wt and *Lif* null mice (three females for each genotype) on D3-D6 of pregnancy using an antibody to IL1A (Fig 3). Immunoreactive IL1A was not restricted to the site of embryo attachment/invasion in either wt or lif null uterus, so sections were stained at and adjacent to the implantation site in wt mice and presumptive implantation sites in *Lif* null uteri. In wt mice, the protein profile was similar to that seen for mRNA. On D3 of pregnancy, IL1A protein was identified in LE cells and staining of a higher intensity was observed in the stroma. The IL1A positive cells in the stroma (particularly on D3 of pregnancy) were interspersed with non-stained cells and appeared to be larger in size than adjacent stromal cells and may be macrophages. Attempts at double staining

for IL1A and macrophage markers were hampered by the different antigen-antibody requirements. However, staining on sequential frozen uterine sections suggested both macrophages and IL1A protein are in the same areas with distinct expression for IL1A to that of macrophage distribution (Fig3). By the morning of D4 of pregnancy only very weak staining was observed in the stroma, but, by the evening of D4, IL1A was detected in the LE and decidualising stromal cells. Intense punctate staining could also be seen in the uterus on D5 of pregnancy, particularly in the decidualised stroma and the embryo itself. On D6 of pregnancy, IL1A was still detectable in the primary decidual zone around the embryo and in the outer decidual cells at the mesometrial pole of the uterus. In contrast, overall levels of immunoreactive IL1A appeared greatly reduced in the uteri of *Lif* null mice compared to wt mice from D4 morning onwards. Thus on the morning of both D3 and D4 of pregnancy, IL1A protein was present in the LE, stroma and glands, but by the evening of D4 IL1A staining was barely detectable, with only small sporadic patches of IL1A positive stromal cells visible on D5. By D6 no IL1A was apparent in either the LE or stroma.

IL1B protein is only transiently expressed on the evening of D4 in Lif null uteri

The cellular expression of IL1B was also investigated by immunohistochemistry in wt and *Lif* null mice on D4 and D5 of pregnancy (Fig 4). These days were chosen based upon the RT-PCR analysis, showing that changes in expression of *Il1b* transcripts were greatest around the time of implantation. On the morning of D4 of pregnancy, faint IL1B immunoreactivity was observed in the cells of the LE and GE in wt mice. By the evening of D4, intense staining of IL1B was observed in the LE, GE and stromal cells and a similar pattern of expression was detected on D5, but the staining was of a lower intensity. In contrast, in *Lif* null mice, immunoreactive IL1B was predominantly observed in the cells of the luminal and glandular epithelia on the evening of D4

of pregnancy, although some faint staining was also evident in the sub-luminal stroma. Immunoreactive IL1B was not detected on D4 morning or D5 in *Lif* null uteri.

Establishment of co-culture system

Since our data suggested that the changing expression of IL1 and associated molecules is disrupted in the *Lif* null uterus, we investigated the effect of LIF on stromal and LE cells *in vitro*. For this purpose we used our co-culture system in which LE cells are grown on suspended membranes. LE cells proliferated and formed a pavement-like epithelium on Cellagen membranes. They became confluent after 4 days of culture at which time the TER plateaued at or above 400Ω cm^2 indicative of a tight junctional network. The LE cells were immunostained for the tight junctional protein TJP1, and desmosomal protein, desmoplakin, together with cytoplasmicstaining for actin and examined by confocal microscopy demonstrated intact junctional complexes with neighbouring cells (Fig 5).

Influence of LIF on production of PTGES2 and IL1A by LE and stromal cells in vitro

For co-culture experiments, uterine LE cells from D2 of pregnancy were cultured on Cellagen membranes with stromal cells in the culture well as described in methods (Fig 1). Preliminary experiments using increasing concentrations of LIF showed that 50ng/ml LIF had a stimulatory effect on release of IL1A by LE cells into the apical compartment, an effect that was prevented when the LIF inhibitor (LIF05) was added to the medium (Fig 6). Subsequent experiments were carried out using this concentration of LIF. LIF and/or the inhibitor were added to the culture media in both compartments and the medium was collected at 24 h and then every 48h up until 120h and used for measurements of IL1A and PTGES2. LIF significantly ($p<0.01$) increased secretion of both IL1A and PTGES2 in the apical medium from the LE compartment of the co-culture system at both 72 and 120 h while IL1A was also increased at 24 h (Figs 7A and 8A).

By 120h in culture IL1A concentration increased more than twice in LIF cultures compared to inhibitor or LIF plus inhibitor cultures, while the PTGES2 concentration in cultures with LIF had tripled compared to all other groups. When the effect of LIF on the concentration of IL1A and PTGES2 was analysed in the lower chamber (adjacent to stromal cells), no significant difference was found during the entire culture (Figs 7B and 8B). In addition, LIF had no significant effect on IL1A production by LE cells cultured on membranes without co-cultured stromal cells (Figs 9A-B). However, LIF increased PTGES2 concentration only in the apical compartment of LE cells (Fig 9C)

Effects of LIF on expression of mRNA for Il1a

In order to assess effects of LIF on mRNA for *Il1a* LE cells and stromal cells were cultured in the co-culture system above and RNA extracted. Preliminary observations confirmed presence of *Il1a* mRNA in all cell types (data not presented). Semi-quantitative RT-PCR showed no differences between treatments after any of 2, 4 or 6 (data not shown) days of culture (Figure 10A, B).

Discussion

LIF expression on D4 of pregnancy is critical for successful implantation [5;8] and in its absence the expression of a variety of molecules is affected in both the LE and stroma [7;8;11;33;46] reviewed in [10]. Uteri of *Lif* deficient mice are unable to support embryo implantation or to mount a decidual response [6]. However, the precise mechanisms by which LIF exerts its effects on implantation are still largely undefined. The identification of LIF regulated molecules in the endometrium around the time of implantation will provide insights into the mechanisms that underlie uterine receptivity and decidualisation.

LIF is known to influence cells of the immune system [47; 48] in addition to cells in the reproductive tract. The cytokine IL1 and its associated molecules also play well established roles in inflammatory processes in the body [49]. Indeed the changes associated with implantation have been compared to an inflammatory reaction [50; 51]. Moreover several studies have suggested that IL1 expression is regulated by the local effects of estrogen and P_4 and estrogen also regulates *Lif* [8;48].

We found that components of the *Il1* system are spatially and temporally regulated during the pre- and peri-implantation period. Abundant transcripts for *Il1a, Il1b, Il1rn* and *Il1r2* were detected in the LE from both wt and *Lif* null uteri on D2 of pregnancy suggesting that at least at the transcript level, the ability of the *Lif* deficient mouse to induce a pro-inflammatory response in the early stages of pregnancy is not comprised. However we have shown previously that the proportions and distribution of leukocytes, particularly macrophages and NK cells are already disrupted in *Lif* null uteri by day 3 of pregnancy [37].

Increased transcript signals for *Il1a, Il1b, Il1rn* and *Il1r1* and *Il1r2* were observed in wt mice at the time of implantation on the evening of D4 of pregnancy in both LE and stroma. These findings agree with previous studies where *Il1a* and *Il1b* mRNAs and their bioavailability were shown to peak between D4 and D5 of pregnancy [52]. Wood and colleagues [53] also reported that mRNA levels of uterine *Il1a* and *Il1b* decreased, from peak levels on D1 and D2, to very low levels on D3 of pregnancy but expression increased in the peri-implantation period. We have further shown that *Il1rn* mRNA is consistently expressed up until the evening of D4 of pregnancy in the LE but barely detectable on D5 while stromal expression was uninterrupted, except for a transient loss of transcript on the morning of D4 of pregnancy. A similar pattern was observed for stromal *Il1r2* transcripts. That expression of *Il1rn* in the LE is continuous during

448 the pre- implantation phase suggests that IL1RN in the LE may play a role in inhibiting IL1
449 receptor activation up until just after implantation has been initiated. In line with this,
450 experiments have suggested that IL1 signalling is crucial to embryo implantation *in vivo*, as
451 functional blockade of IL1R1 by repeated IL1RN administration i.p. from D3-D9 of pregnancy
452 resulted in reduced implantation rates [28]. This inhibition of embryo implantation was
453 attributed to a down regulation of integrins α4 and β3 on the LE by IL1RN [54]. However,
454 Abbondanzo and co-workers [55] found administration of IL1RN to C57BL/6 X 129Sv hybrids
455 had no effect on implantation. This difference may have a methodological source but it casts
456 uncertainty on the absolute requirement for IL1 signalling for implantation. Our data suggest
457 that IL1 signalling is likely to be functional in the later stages of implantation
458 Since activation of the IL1R2 by any IL1 ligand does not elicit a biological response, it is
459 proposed to function by limiting the bioavailability of IL1A and IL1B by acting as a decoy
460 receptor; reducing the amount of free IL1 [56; 57]. Examination of *Il1r2* mRNA levels in the wt
461 peri-implantation uterus revealed that stromal transcript expression of *Il1r2* was low with
462 detection only on d3, d4pm and d5 and only d3 and d5 in the null stroma. In contrast LE extracts
463 on D2-D6 of pregnancy consistently gave bands both in wt and null animals. Therefore,
464 transcriptional regulation of stromal *Il1r2* during the peri-implantation period may play a role in
465 regulating the bioavailability of IL1A and IL1B but it is little affected by LIF.
466 The spatio-temporal expression of several components of the IL1 system in the *Lif* null uterus
467 was altered from that of wt uteri from D3 of pregnancy onwards. Transcripts for *Il1a* in the LE
468 from *Lif* null uteri were low except for a strong band on D2, in contrast to wt LE, where signal
469 for *Il1a* was high on the evening of D4 of pregnancy following the nidatory burst of LIF
470 expression. This suggests that LIF does either directly or indirectly regulate *Il1a* over the peri-

implantation period. Stromal expression of *Il1a* mRNA in the nulls was restricted to D3 and D5 suggesting major misregulation in transcriptional timing.

Interestingly PCR signal for *Il1rn* in the *Lif* null LE similarly declined from D2 onwards, with barely detectable signal by D4-D5. The low levels of *Il1rn* in the uteri of *Lif* null compared to wt mice could contribute to the implantation defect. The premature reduction in antagonism of IL1A and IL1B may result in over-stimulation of the IL1 signalling cascade potentially altering the inflammatory response [20]. Huang and colleagues [58] postulated that an appropriate ratio of IL1 to IL1RN is crucial during embryo implantation. Furthermore, work from our laboratory has shown that both macrophage number and distribution (a primary source of IL1) were altered in the uteri of *Lif* deficient mice from D3 of pregnancy compared to wt [37]. It has also been confirmed in the present study that IL1 is synthesised in luminal and probably glandular epithelium as well as stromal cells, and that leukocytes are by no means the only source of IL1 ligands in the pre-implantation uterus. Surprisingly, there were no significant differences between the expression of *Il1b* transcripts in the uteri of wt and *Lif* null mice, indicating that the strong *Il1b* bands seen on the evening of D4 are not a direct result of LIF production on D4.

No significant alterations were observed in the gene expression of *Il1r1* or *Il1r2* between uterine LE of wt and *Lif* null mice during early pregnancy in the LE suggesting these receptors are not regulated by LIF. However null stromal *Il1r1* was expressed in a pattern at variance with that in wt, with loss of transcripts 12-24 h after the known peak of *Lif* expression in wt animals. Lack of proper regulation of epithelial derived IL1A in the *Lif* null uterus may lead secondarily to lack of IL1B in the glands observed by immunocytochemistry on day 5. This may reflect a failure of normal LE to stromal signalling. Indeed, it is likely that the stromal misexpression of several Il1 components in the uteri of *Lif* null mice compared to wt may be partly attributed to the lack of

494 secondary signalling between unstimulated LE and the stroma in the null uteri, particularly on
495 D4 or D5.
496 On D3 of pregnancy in both wt and *Lif* null uteri, immunoreactive IL1A was detected in the LE,
497 GE and some stromal cells. IL1A positive cells within the stroma appeared larger than other
498 stromal cells and may represent IL1A producing immune cells such as macrophages but it was
499 not possible to confirm this definitively because of the differnences in fixation requirement of the
500 various antibodies. Previous studies by our laboratory have shown that there are increasing
501 numbers of macrophages at this time which are known to be one of the major producers of IL1
502 [37]. Intense staining of IL1A was observed in the decidua on D5 and D6 of pregnancy but
503 markedly reduced in *Lif* null uteri from the morning of D4 onwards. Since these animals lack
504 decidualisation and IL1 is known to induce decidualising molecules [30; 31] the lack of IL1A
505 here is consistent with it being a mediator of LIF induced decidualisation. *In vitro* studies in
506 murine endometrial stromal cells have also shown that the ECM glycoprotein tenascin C (TNC)
507 expression is upregulated by *Il1a* [7; 59]. Similarly, TNC has also been shown to be absent from
508 the site of implantation in the uteri of *Lif* null mice during the implantation period [7]. However,
509 whilst evidence suggests a fundamental role for IL1 signalling in embryo implantation, gene
510 deletion experiments in mice have revealed that there are no overt reproductive phenotypes in
511 mice lacking either *Il1r1* or *Il1b* [60-62]. Various members of the IL1 system have been
512 identified in the uterus including novel ligands namely $IL1F_5$ and $IL1F_7$ [63] but to date they
513 have no identifiable function. Perhaps the lack of overt phenotypes in the gene deleted mice can
514 be explained by compensatory effects of these and other novel ligands or receptors.
515 In order to obtain functional evidence for the interrelationship of IL1 and LIF we co-cultured LE
516 with stromal cells to examine the influence of LIF in a system which more closely mimics the

physiological relationship of these two cell layers than separate culture. A surprising result here was that the predominant secretion of IL1 induced by LIF in our *in vitro* culture system is directed towards the lumen, a location removed from potential tissue targets. However it is possible that this molecule is involved in leucocyte recruitment to the point of possible pathogen entry from luminal fluids. This would seem logical since the LE barrier is breached during implantation with potential risk of infection.

LIF stimulated IL1A secretion by LE cells in a dose dependent manner, an effect that was abrogated by an established LIF inhibitor. It did not stimulate IL1A secretion by the basal stromal cells. Expression of *Il1a* mRNA in cultured LE (by semi-quantitative PCR) did not appear to be affected by adding LIF to medium, or by LIF inhibition. Therefore, stimulation of secretion of this cytokine into the culture medium by LIF is likely to occur post transcriptionally at the level of protein synthesis or of the secretory apparatus. These would be consistent with an effect of the inhibitor by 24 h. Stimulation of IL1 secretion by LIF is in keeping with the increased protein seen by immunohistochemistry. Thus, this is an additional level of LIF regulation of IL1A to the regulation of LE *Il1a* transcription *in vivo,* inferred from the rapid loss of PCR signal after D2 of pregnancy in *Lif* null LE. The lack of direct transcript regulation in culture suggests that, *in vivo,* regulation may be indirect and/or on mRNA stability. Alternatively, direct transcript stimulation may be prevented under our co-culture conditions.

LIF also induced release of PTGES2 by LE cells in co-culture, an effect which was also observed in culture of LE alone. In the co-culture system, LE and stromal cells are able to establish a dialogue, resulting in modulation of cytokine production [64], thus making this model more physiologically relevant. Jacob and Carson [30] reported that IL1 induces PTGES2

539 secretion by uterine stromal cells *in vitro*. This effect is mediated through upregulation of *Ptgs2*
540 mRNA in stromal cells [32; 65]. Moreover uterine secretion of IL1A by epithelial cells increases
541 PTGS2 enzyme activity [66-68]. We have previously reported defects in PTGS2 protein
542 expression and decidualisation of uterine stroma cells in *Lif* null mice [7]. We have also shown
543 that LIF does not stimulate PTGES2 by stromal cells cultured without LE [34]. However, since
544 LIF does not directly promote the secretion of PTGES2 by uterine stromal cells *in vitro*,
545 PTGES2 is not likely to be a direct target of LIF in the stroma [34]. LIF may exert its effect
546 mainly though upregulation of intermediate messengers in the LE that in turn regulate
547 decidualisation. It is unlikely that IL1A from LE acts directly on stromal cells *per se* since our
548 results suggest it is secreted mainly apically. However IL1A from GE and/or other cells in the
549 stroma may regulate stromal PTGS2 *in vivo* and thus contribute to decidualisation and
550 angiogenesis. Furthermore, autocrine activation of IL1R1 signalling pathways in the LE is
551 likely. This may contribute to LE receptivity and susceptibility to embryonic signals that in turn
552 directly or indirectly initiate the decidual response. It should not be forgotten that the embryo
553 itself expresses IL1A [69-70 and this study], which could also interact locally with LE. Moreover
554 IL1 is known to affect the phenotype of invasive trophoblast in human [71-73] and mouse [74]
555 so one target of apically secreted IL1 could be the trophoblast as well as a possible
556 immunomodulatory one, mentioned above.
557 In LE cells LIF may induce PTGES2 production through an autocrine influence of IL1 via the
558 IL1 receptor. This may also activate basally released modulators of stromal cells to contribute to
559 the decidual response following signalling from the embryo. In addition, LIF modulates IL1
560 signalling by regulating IL1RN in the LE to dampen both the autocrine effect and any paracrine

influence on the blastocyst. Our results confirm the extreme complexity of the interacting network of secreted molecules which regulate implantation related events.

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Figure Legends

Figure 1. Schematic presentation of co-culture system. Mouse uterine luminal epithelial (LE) cells were cultured on the suspended Cellagen membrane, stroma cells were cultured on the base of the wells in a 24-well culture dish.

Figure 2. Gene expression analysis of the *Il1* associated proteins in the peri-implantation uterus of wt and *Lif* null mice. mRNAs were prepared separately from LE and stroma of wt and *Lif* null uteri on the required days of pregnancy. RT-PCRs were performed for (A) *Il1a*, (B) *Il1b*, (C) *Il1rn*, (D) *Il1r1* and (E) *I1r2*. (F) *Gapdh*. Negative controls consisting of no template (water only) and no reverse transcriptase (-RT) were assembled in parallel in each experiment (final two columns).

Figure 3. Immunolocalisation of IL1A during early pregnancy**.** Immunofluorescence detection of IL1A (green) in paraffin embedded uterine sections on D3-D6 of pregnancy in wt and *Lif* null females. Note that the overall expression of IL1A is reduced in the *Lif* null uterus compared to wt. Strong signals were also apparent in the embryo on D5 of pregnancy in wt mice. Scale bars represent 100μm. LE = luminal epithelium, S= stroma, E = embryo, G= glands.

Figure 4. Immunolocalisation of IL1B in the peri-implantation uterus from wt and *Lif* null mice. Detection of IL1B was performed on paraffin embedded uterine sections from wt and *Lif* null mice on D4 and D5 of pregnancy by immunohistochemistry. Cytoplasmic staining of IL1B (brown) can be seen in the LE, stroma and uterine glands of the wt mouse. In contrast, IL1B

immunoreactivity was only evident in the uteri of *Lif* null mice on D4 evening (2000-2200h). All nuclei were counterstained with haematoxylin (blue). Scale bars represent 100μm. LE=luminal epithelium, S=stroma, G = glands, E = embryo and L = lumen.

Figure 5. Confocal microscopic analysis of junctional proteins in the cultured luminal epithelium (LE) cells. LE cells cultured on Cellagen membranes were stained for tight junction proteins TJP1 and desmoplakin protein (11.5F) (green) and also the nuclear stain Hoescht (blue) and Texas red-conjugated phalloidin (red). A single optical section is shown. Note high number of binuclear LE cells. Scale bar = 20 μm.

Figure 6. Effects of LIF on production of IL1A by murine endometrial luminal epithelial (LE) cells co-cultured with stromal cells. In preliminary experiments, LE cells were cultured on Cellagen membranes in the absence (control) or presence of increasing concentrations of LIF with stromal cells on the floor of the wells. LIF inhibitor was also supplemented in a ten fold excess to the concentration of LIF. Concentration of IL1A in culture media increased in a dose dependent manner. The data represent average ± S.E.M. in three separate replicates. Treatments marked with different number of asterisks are significantly different from each other. * vs **$p<0.05$. LIF + Inh = 50ng/ml LIF + 500ng/ml inhibitor.

Figure 7. Effects of LIF on production of IL1A by co-cultured murine endometrial cells. Luminal epithelial (LE) cells were cultured on Cellagen membranes placed in the wells of 24 well plates and the stromal cells were cultured on the bottom of the culture wells. A) Concentration of IL1A in the LIF-treated LE culture media (apical compartment) increased significantly with time in culture. B) No significant differences were observed in the concentration of IL1A in stromal culture media (basal) between treatments. The data represent average ± S.E.M. in three separate replicates. At each time point treatments marked with different number of asterisks are

significantly different from each other. * vs ** $p<0.05$, * vs. *** $p<0.01$, ** vs. ***$p<0.05$, **** vs. *** $p<0.05$, **** vs. ** $p<0.01$, **** vs. * $p<0.01$. LIF + Inh = 50ng/ml LIF + 500ng/ml inhibitor.

Figure 8. Effects of LIF on production of PTGES2 by co-cultured murine endometrial cells. Luminal epithelial (LE) cells were cultured on Cellagen membrane and the stromal cells were cultured on the bottom of the culture wells. A) LIF increased concentration of PTGES2 in the LE culture media (apical) with time in culture. B) No differences were observed in the concentration of PTGES2 in stroma culture media (basal) between treatments after 24 and 72 h of culture but there was a significant increase compared to other groups at 120h. The data represent average ± S.E.M. in three separate experiments. At each time point treatments marked with different number of asterisks are significantly different from each other. * vs.*** $p<0.05$, **vs. *** $p<0.05$, * vs. **** $p<0.01$, ** (both control and LIF + Inh treatments at 120h) vs. **** $p<0.05$. LIF + Inh = 50ng/ml LIF + 500ng/ml inhibitor.

Figure 9. Effects of LIF on production of IL1A and PTGES2 by murine endometrial luminal epithelial (LE) cells cultured alone. LE cells were cultured on the insert Cellagen membrane in the absence (control) or presence of 50ng/ml LIF. No differences were observed in the concentrations of IL1A between apical (A) and basal (B) culture media or in the presence or absence of LIF. Similarly, no differences were observed in the concentration of PTGES2 in the basal media (D). However, LIF increased PTGES2 concentrations in the apical compartment at 72 and 120 hrs (C) ($p<0.05$). Data represents average ± S.E.M. in three separate experiments in each case.

Figure 10. Effects of LIF on expression of mRNA for *Il1a* in murine endometrial cells. Total RNA was extracted from either of freshly collected and cultured stromal and LE cells. A) Semi-

quantitative analysis of mRNA for *Il1a* in LE and stroma cells after 6 days of culture in the absence (control) or presence of LIF or LIF inhibitor. No differences were observed in the intensity of bands for *Il1a* mRNA among treatments. B) Semi-quantitative analysis of changes in the expression of mRNA for *Il1a* in LE cells in relation to time of culture. LIF + Inh = 50ng/ml LIF + 500ng/ml inhibitor.

TABLE 1.

Primer set	Sequence (5′-3′)	Cycle number	Product size (bp)	Optimized annealing temp (°C)	Accession no./ reference
Actb (f)	AAACTGGAACGGTGAAGGC	30	192	60	X03672
Actb (r)	CCTGGGCCATTCAGAAATTA				
Gapdh (f)	TCTGAGGGCCCACTGAAG	28	220	60	NM008084
Gapdh (r)	AGGGTTTCTTACTCCTTGGAGG				
Il1r1 (f)	AAATAATGAGTTACCCGAGGTCCAGTGG	30	709	60	NM008362
Il1r1 (r)	AGGCATCGTATGTCTTTCCATCTGAAGC				
Il1a (f)	CAAACTGATGAAGCTCGTCA	32	220	60	NM010554
Il1a (r)	TCTCCTTGAGCGCTCACGAA				
Il1b (f)	ATGGCAACTGTTCCTGAACTCAACT	32	563	60	NM008361
Il1b (r)	CAGGACAGGTATAGATTCTTTCCTTT				
Il1r2 (f)	GCATCATTGGGGTCAAGACT	32	156	60	BC032962
Il1r2 (r)	TGAGTACTGGGGGTGTAGCC				
Il1rn (f)	TAGCAAATGAGCCACAGACG	32	190	60	NM031167
Il1rn (r)	ACATGGCAAACAACACAGGA				

Figure 1

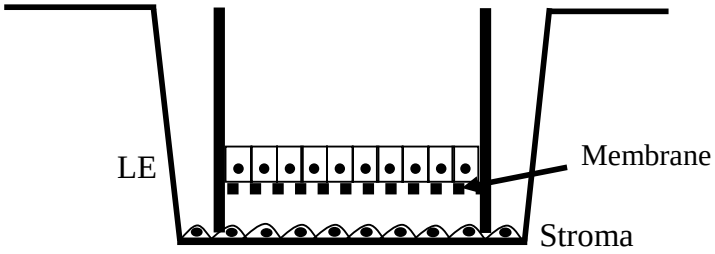


Figure 2

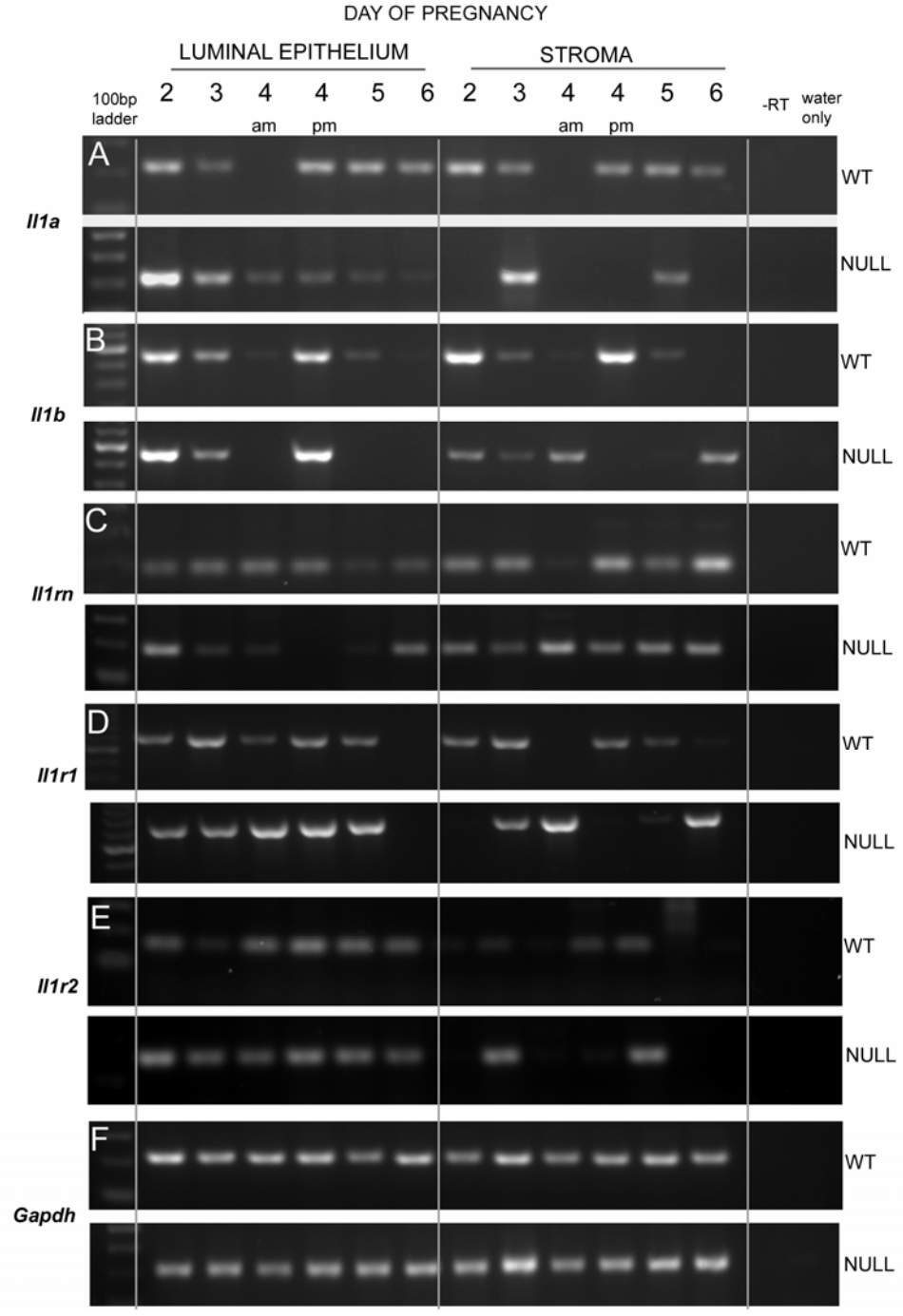
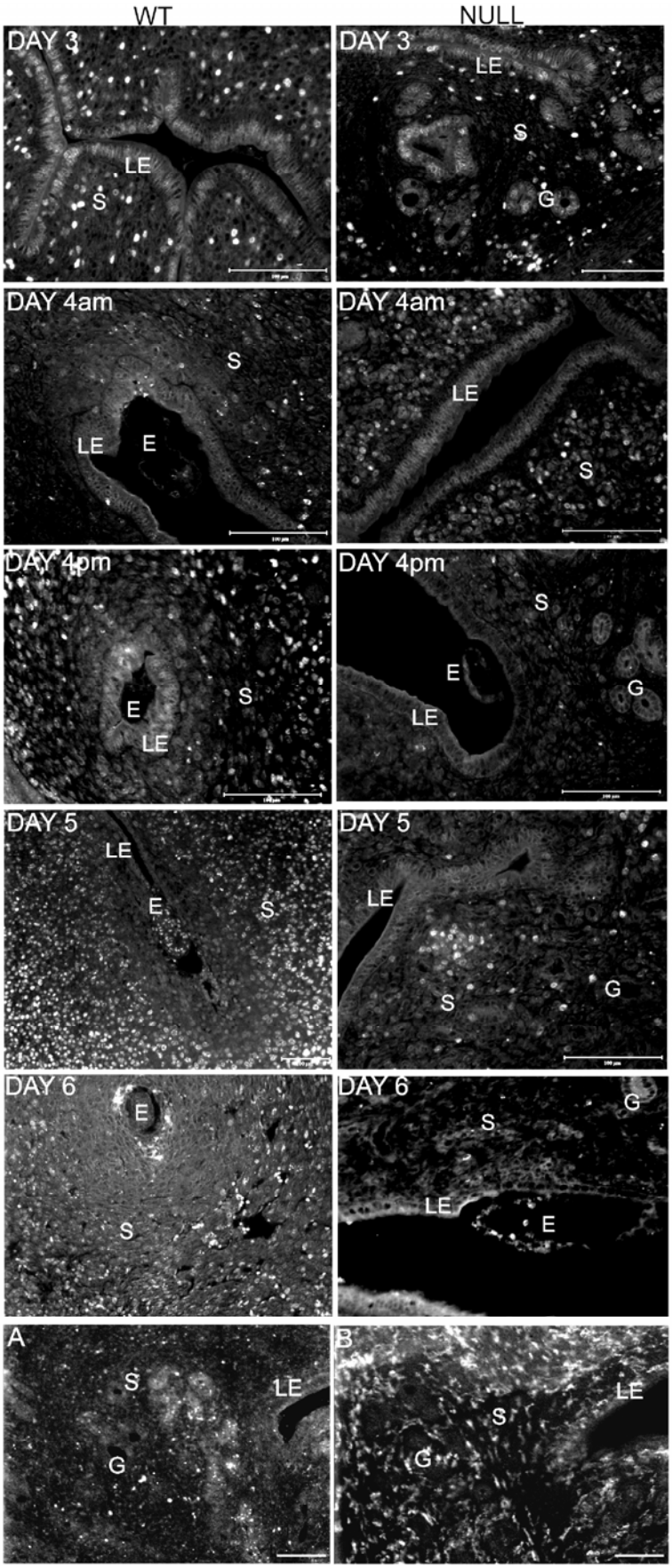


Figure 3


WT
NULL
DAY 3
LE
S
DAY 3
LE
S
G
DAY 4am
S
LE
E
DAY 4am
LE
S
DAY 4pm
E
S
LE
DAY 4pm
S
E
G
LE
DAY 5
LE
E
S
DAY 5
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A
S
LE
G
B
LE
S
G

Figure 4

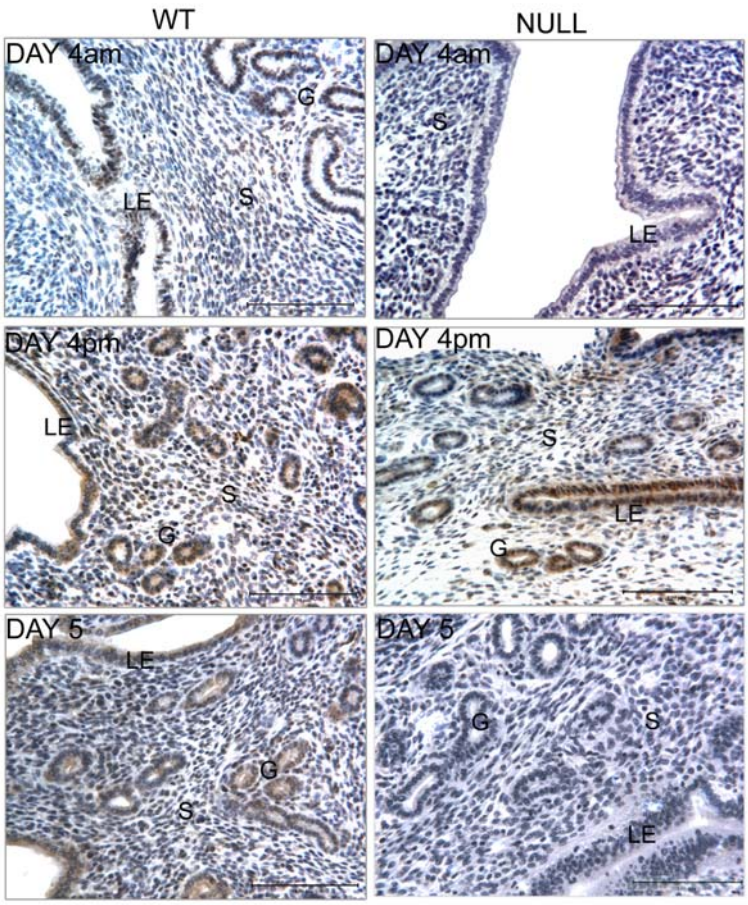
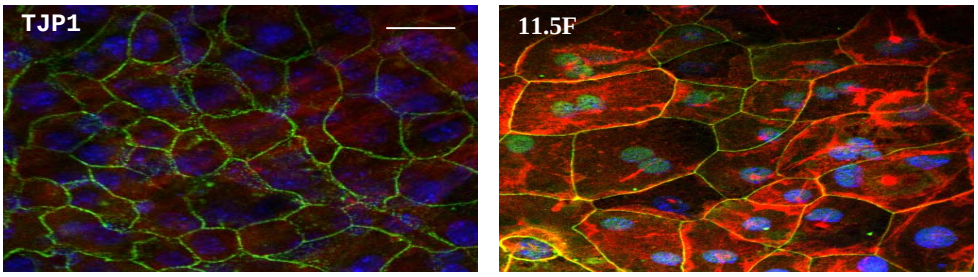


Figure 5



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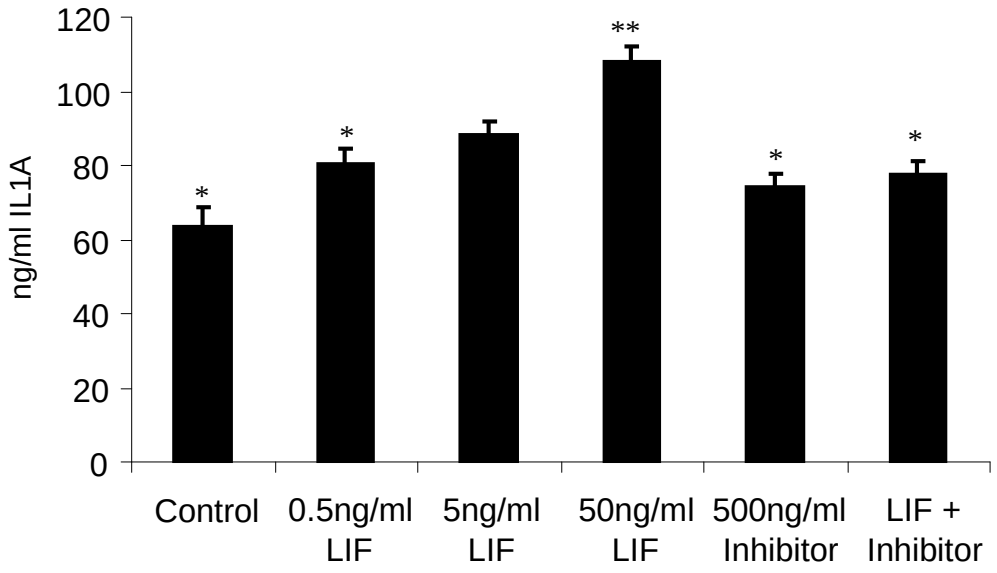
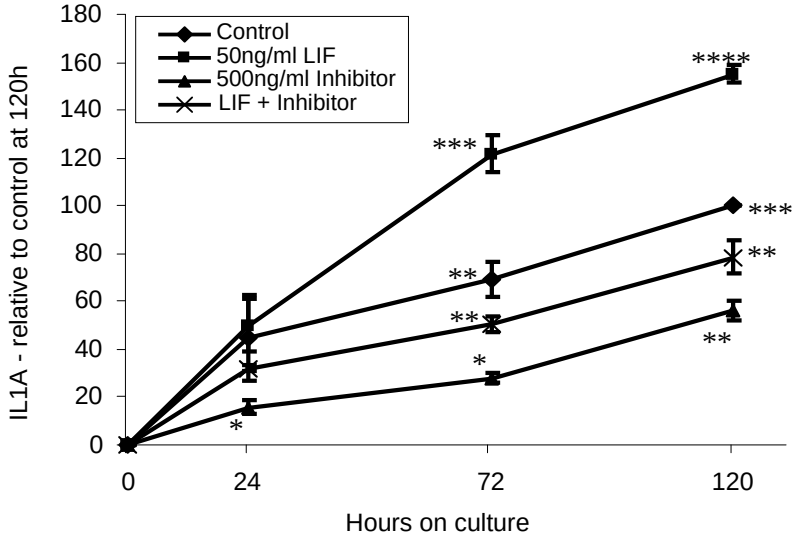


Figure 7

A)



B)

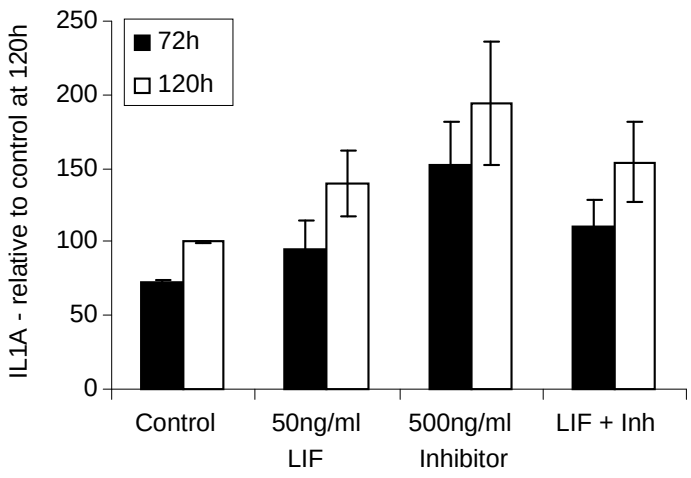
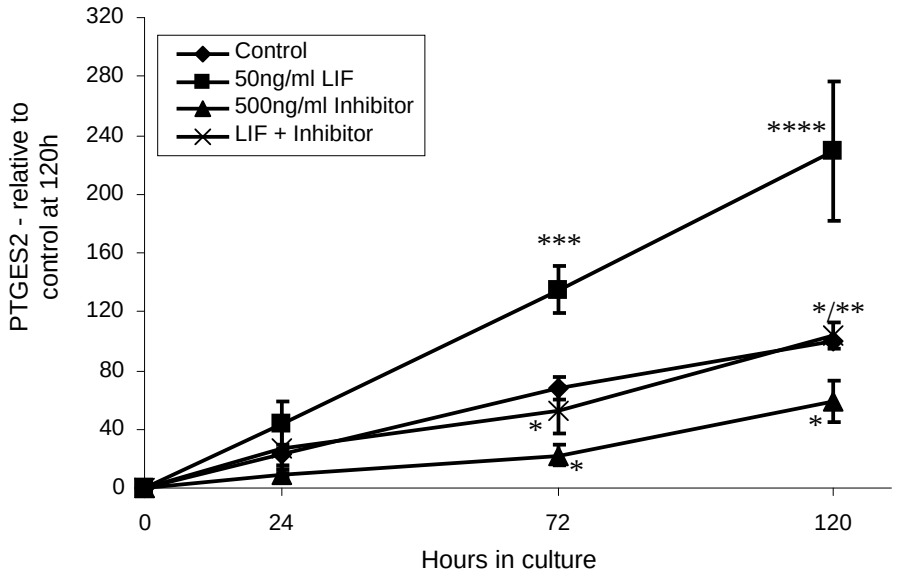


Figure 8

A)


320
280
240
200
160
120
80
40
0
PTGES2 - relative to control at 120h
Control
50ng/ml LIF
500ng/ml Inhibitor
LIF + Inhibitor
****
***
*/**
*
*
*
0
24
72
120
Hours in culture

B)

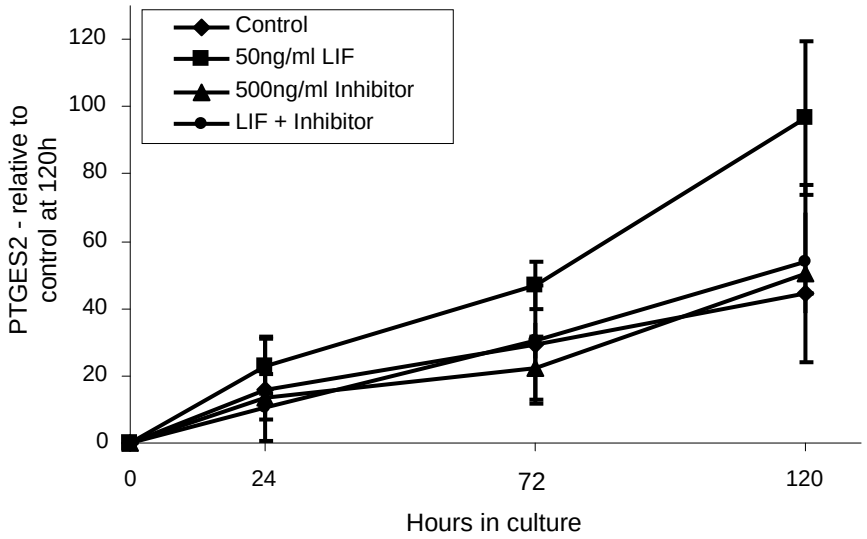
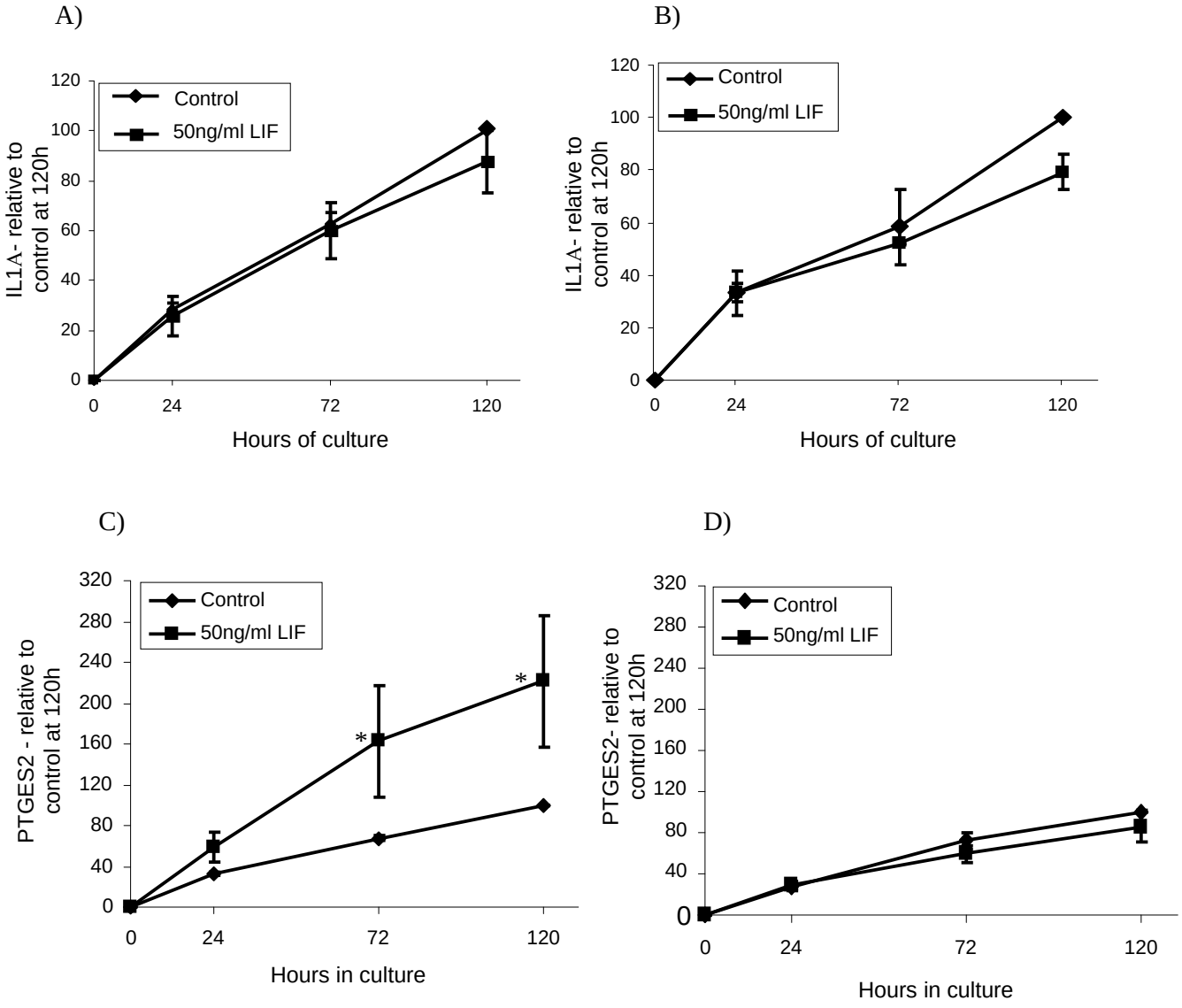
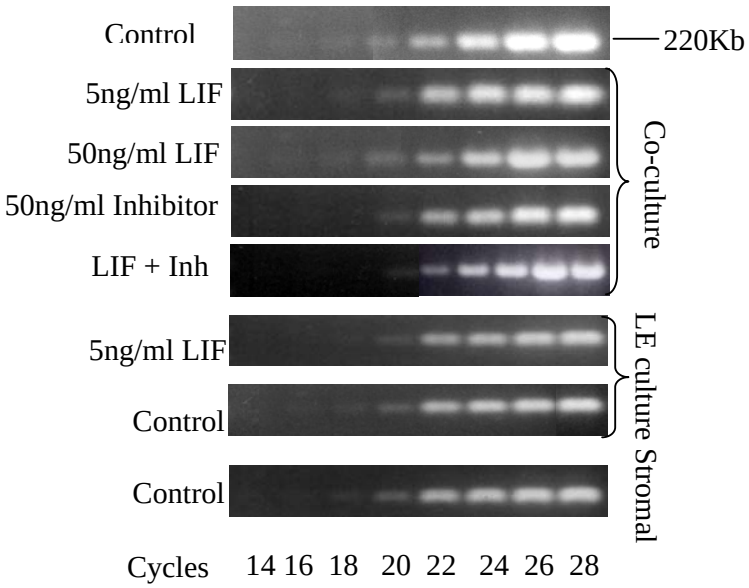

120
100
80
60
40
20
0
PTGES2 - relative to control at 120h
Control
50ng/ml LIF
500ng/ml Inhibitor
LIF + Inhibitor
0
24
72
120
Hours in culture

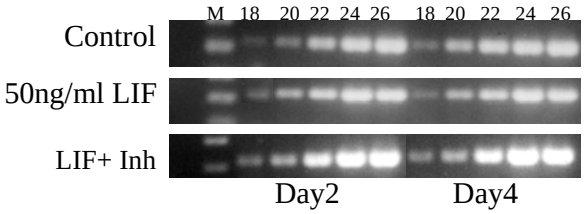
Figure 9


A)
B)
C)
D)
Control
50ng/ml LIF
IL1A- relative to control at 120h
PTGES2 - relative to control at 120h
PTGES2- relative to control at 120h
Hours of culture
Hours in culture
0
24
72
120
*

A)


Control
220Kb
5ng/ml LIF
50ng/ml LIF
50ng/ml Inhibitor
LIF + Inh
Co-culture
5ng/ml LIF
Control
LE culture Stromal
Control
Cycles 14 16 18 20 22 24 26 28

B)


M 18 20 22 24 26 18 20 22 24 26
Control
50ng/ml LIF
LIF+ Inh
Day2
Day4