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1 **Interleukin 1 signalling is regulated by Leukemia Inhibitory Factor (LIF) and is aberrant**
2 **in *Lif*^{-/-} mouse uterus¹**

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10 **Summary statement:**

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

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

15 **Abstract**

16 This study addresses the regulation of the Interleukin 1 (IL1) system in the murine uterine
17 luminal epithelium (LE) and stroma by leukemia inhibitory factor (LIF). Using RT-PCR we
18 compared expression of *Il1a*, *Il1b*, *Il1rn*, *Il1r1* and *Il1r2* during the pre- and peri-implantation
19 periods of pregnancy in wild type (wt) and LIF null LE and stroma. In wt LE, *Il1a* transcripts
20 were down-regulated on day (D) 4am with renewed expression by D4pm. In *Lif*^{-/-} LE there was
21 a gradual decrease in expression from D2 which became undetectable by D6. *Il1b* and *Il1r1*
22 expression were similar in wt and null mice, but *Il1rn* expression was almost completely lost
23 during the peri-implantation period in *Lif*^{-/-} LE. In the stroma *Il1a* was sharply down-regulated
24 on D4 am reappearing on D4 pm, but in the null mice was only expressed on D3 and D5.
25 Stromal *Il1r1* and *Il1r2* were also misregulated. *Il1rn* showed constitutive expression in null
26 stroma in contrast to the loss of expression on D4am in the wt mouse. In *Lif* deficient mice,
27 immunostaining indicated a reduction of endometrial IL1A at the time of implantation and of
28 IL1B in stroma. LE-stromal co-culture revealed that LIF stimulated apical secretion of both IL1A
29 and PTGES2 by LE cells without affecting basal secretion of IL1A and with only a small effect
30 on basal PTGES2 secretion. We conclude that *Il1a* and *Il1rn* in LE and *Il1a*, *Il1rn* and *Il1r1* in
31 stroma are regulated by LIF which stimulates apical secretion of IL1A by LE.

32 **Introduction**

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

37 progesterone (P₄) [1;2], recent evidence has shown that a plethora of other molecules including
38 growth factors and cytokines mediate and modulate the actions of these steroid hormones [3;4].
39 Uterine LIF is expressed in two transient peaks during early pregnancy. Firstly, on day 1 (D1) of
40 pregnancy (vaginal plug = D1 of pregnancy) LIF expression is stimulated by ovulatory estrogen
41 in both luminal and glandular epithelium. Secondly, on D4, nidatory estrogen stimulates
42 expression of both *Lif* mRNA and protein in the glandular epithelium (GE) [5-7]. This second
43 peak of LIF expression is essential for successful embryo implantation into the uterus on the
44 evening of D4 of pregnancy [8]. The cellular target of LIF in the uterus during pregnancy appears to
45 be the luminal epithelium (LE) and *Lif* receptor (*Lifr*) transcripts and protein have been found to be
46 present predominantly in the LE during D3-D5 of pregnancy [9;10]. It has been known for some
47 time that uteri of *Lif* deficient mice are unable to support embryo implantation [6]. However, *Lif*
48 ^{-/-} blastocysts can undergo implantation when transferred into pseudopregnant recipients and
49 develop to term demonstrating that the implantation defect is maternal. Rescue of implantation
50 can be achieved by exogenous delivery of LIF on D4 of pregnancy in the homozygous mutants
51 [6;8;11]. The importance of LIF for successful embryo implantation in the mouse may be of general
52 significance to all mammals and other species. Indeed, increased levels of LIF during pregnancy
53 have been shown to be conserved in several species including humans and rhesus monkeys [12-
54 15], while low levels of *Lif* have been correlated with infertility in women [16-19].
55 Furthermore, the uteri of *Lif* deficient mice do not undergo decidualisation, a process involving
56 the differentiation of the uterine stroma essential to support the implanting embryo [6-8].
57 Decidualisation is triggered by a number of molecules and is first discerned by an increase in
58 vascular permeability at the site of implantation [1;20]. Amongst the best candidates for roles in
59 the initiation of decidualisation are prostaglandins (PGs), which increase at the time of
60 implantation. PTGES2 is a central PG involved in the initiation of uterine vascular permeability

61 [21-23]. PGs are produced by both uterine epithelial and stromal cells and their synthesis is
62 induced by Interleukin 1 (IL1), also produced by the uterine epithelium, as well as by other cell
63 types including macrophages [24]. The IL1 system is composed of two agonists IL1A and IL1B,
64 one antagonist IL1RN and two membrane bound receptors, IL1 receptor type one (IL1R1) and
65 type two (IL1R2) [25;26]. Endogenous control of secreted IL1 activity is achieved by regulation
66 of IL1 synthesis and processing and release from intracellular and membrane bound stores [26].
67 This control of IL1 bioavailability is further regulated by a unique receptor antagonist (IL1RN),
68 which binds with high affinity to IL1 receptors thus preventing access by IL1 ligands and
69 inhibiting signalling [27]. In mouse, IL1R1 protein is reported to be induced in uterine LE cells
70 during the preimplantation period and subsequent blockade of IL1 signalling by injection of
71 IL1RN during early pregnancy prevents attachment of the blastocyst to the LE [28;29].
72
73 Epithelial derived IL1A has been previously reported to upregulate the synthesis of PTGES2 and
74 PGF_{2α} in mouse and rat uterine stromal cells [30;31] and other studies *in vitro* have shown that
75 IL1A increases levels of mRNA for *Ptgs2* (a rate limiting enzyme for PG synthesis) in rat uterine
76 stromal cells [32]. Evidence from *in vivo* studies has demonstrated that mRNA and protein
77 levels of PTGS2 are reduced in the uterine stroma of *Lif* deficient mice at the implantation site
78 [7;33]. We have shown, however, that LIF does not directly promote the synthesis of PTGES2
79 by uterine stromal cells *in vitro* suggesting that PTGES2 is not a direct target of LIF here [34].
80 In human endometrial epithelial cells, IL1B upregulates *LIFR* and this effect is abrogated by
81 inhibition of IL1R1 [35]. This suggests that in human and murine endometrium it is likely that
82 feedback loops exist between LIF and IL1 in uterine epithelial cells. Together with the reduction
83 of PTGS2 expression at the implantation site in *Lif*^{-/-} females these findings support a signalling

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

93 **Materials and methods**

94 ***Animals***

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

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

113 ***Reagents***

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

Uterine epithelial cell layer dissociation for RNA extraction

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

Isolation of total RNA from cultured uterine epithelial and stromal cells

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

Reverse Transcription-Polymerase Chain Reaction

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

152 polymerase chain reaction (RT-PCR). Samples from a minimum of 3 independent animals were
153 used in each case. Changes in PCR products obtained for *III* were normalised by comparison
154 with an endogenous house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*),
155 expression of which has been shown to be consistent in the uterus [40]. Briefly, 2µg total RNA from
156 each sample was reverse transcribed using Superscript II first strand cDNA synthesis
157 (Invitrogen, Paisley, UK) following manufacturer's instructions with omission of reverse
158 transcriptase run in parallel in all reactions. PCRs were assembled to a final volume of 25µl
159 containing 0.5µl of cDNA template, 10pmol (final concentration) primers and Red Taq PCR
160 Buffer reaction mix (Sigma). No template and a reverse transcriptase negative control were
161 assembled in parallel. Optimal annealing temperatures and cycle number are shown in table 1.
162 Cycle conditions were as follows: initial denaturation at 94°C for 1 min, then cycles of the
163 following, 30s at 94°C, annealed for 30s at a temperature determined as optimum and extended
164 at 72°C for 30s. PCR products were resolved on a 2% (w/v) agarose gel and the results
165 visualised under UV trans-illumination (GRI, Essex, UK). PCRs were also taken to saturation
166 (40 cycles) to determine if transcripts were weakly expressed or absent. The PCR products were
167 verified by automated capillary gel electrophoresis by Manchester Sequencing Services using an
168 ABI Prism 377 sequencer (Applied Biosystems, Cheshire, UK) and products confirmed by a
169 BLAST search.

170 ***Immunolocalisation of IL1A/IL1B***

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

175 0.190 g/l EGTA in distilled water, pH: 9) (IL1A) as previously described [7] or, following
176 exposure to 0.3% (v/v) hydrogen peroxide in methanol for 12 mins, subjected to antigen retrieval
177 with 0.01M citrate buffer (pH 6.0) for 6 minutes (IL1B). After cooling, non-specific binding
178 was blocked in 10% (v/v) NGS and 0.1% (w/v) BSA in PBS (blocking solution).
179 For immuno-peroxidase staining (IL1B), endogenous biotin was blocked using an avidin/biotin
180 blocking kit as per manufacturers' instructions (Vector Laboratories). The primary rabbit anti-
181 IL1B or irrelevant control antibodies were diluted 1:50 in blocking solution and incubated
182 overnight at 4°C. Following washing, the sections were incubated in the appropriate biotinylated
183 secondary antibody for 45 mins at room temperature. ABC reagent (Vector Laboratories) was
184 applied to the sections for 30 mins and positive immunoreactivity was detected using a
185 diaminobenzidine peroxidase (DAB) substrate kit (Vector Laboratories). Nuclei were
186 counterstained with Harris' haematoxylin and sections mounted in a permanent mountant
187 (CellPath, Newtown, Powys). To determine macrophage and IL1A immunoreactivity, uterine
188 tissue from D4 of pregnancy was placed into aluminium foil containers of cryo-embedding
189 compound OCT (Raymond A Lamb Laboratories, Sussex, UK). The samples were then flash-
190 frozen in liquid nitrogen and stored at -80 °C. Serial sections (7µm) were taken using a cryostat
191 (Leica UK Ltd, Milton Keynes, UK) and fixed for 10 minutes in ice-cold acetone at -20° C. The
192 sections were rehydrated in 0.1%w/v BSA, 0.1%v/v Tween20 in PBS. Normal goat serum
193 (NGS) at a 1:20 dilution was used to block non specific binding. The diluted primary antibody
194 (1:50 for both IL1A and f4/80) was added to each section and left overnight at 4°C. Following
195 washing, the sections were incubated with the appropriate fluorescein FITC conjugated
196 secondary antibody for 45 mins at RT. The sections were mounted in Vectashield with 1.5µg/ml
197 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 MgCl₂ 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 4°C. 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,

221 Paisley UK) containing 0.1% bovine serum albumin (BSA ; Fraction V Albumin, ICN),
222 100mg/ml pen/strep, 2.5% NuSerum (Collaborative Research Inc, Bedford, UK), 2.5% HIFCS,
223 15mM Hepes buffer and 200mM L-glutamine]. LE cells were cultured on Cellagen membranes
224 (ICN-Flow Thame UK) as previously described [43;44]. Cellagen discs were pre-incubated with
225 culture medium. After pre-incubation, media in the apical compartment was replaced with 250µl
226 cell suspension and the basal compartment with 450µl LE culture medium (Fig 1a). Cells grown
227 on these membranes are cuboidal and show a semi-polarised phenotype, intermediate between
228 the highly polarised LE morphology seen *in vivo* at D1-3 of pregnancy and the flattened
229 morphology seen for cells grown on plastic. The transepithelial resistance (TER) of the cultures
230 was measured using a Millicell -ERS transepithelial resistance meter (Millipore Watford UK).
231 All cultures used in these experiments had a TER above 400cm².

232 ***Isolation and culture of uterine stromal cells***

233 Uterine stromal cells were isolated and cultured as previously described [34]. Upon removal of
234 LE from the uterine tissue (see above), ten glass beads were added to the remaining LE denuded
235 endometrium extract, together with stromal trypsin dissociation solution (0.05% trypsin and
236 0.02% EDTA (BDH) in HBSS). The tubes containing cell extracts were incubated for 20 min at
237 37°C and vortexed at medium speed for 10s every 10 mins. This was process was repeated by
238 incubation at room temperature. The content of the tube was passed through a 70µm gauze filter
239 (Falcon) and the enzymatic digestion stopped (2% Soybean trypsin inhibitor in HBSS) after
240 filtration. The cell suspension was then centrifuged at 400g for 10min at 4°C. The pellet was
241 washed in stromal cell culture medium: 1:1 mixture of DMEM and Ham's F12 medium
242 (Invitrogen) supplemented with 1.2g/l of sodium bicarbonate, 100IU/ml penicillin streptomycin
243 (Invitrogen), 2% Heat Inactivated Fetal Calf Serum (HIFCS, Invitrogen) and centrifuged for 10

244 min at 4°C. 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 1.5×10^5 cells/ml in 5% CO₂ in
249 air at 37°C. 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 triplicate. All 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 H₂O₂ and Tetramethylbenzidine) was
266 added and shaken for 20 min in the dark. The enzyme reaction was stopped by 100µl 4N

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

269 ***Prostaglandin E radioimmunoassay (RIA)***

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

283 ***Immunofluorescence staining of junctional proteins in cultured LE cells***

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

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

293 ***RT-PCR for the Illa in cultured cells***

294 A one-step RT-PCR kit (Qiagen) was used according to the manufacturer's instructions for RT
295 and amplification of a 220bp product. One µg of RNA was used for reverse transcription and
296 PCR over 30 cycles with an annealing temperature of 60°C and 5 min extension. For experiments
297 where *Ill1a* mRNA transcripts were compared between different groups, the tubes were removed
298 from the cycler (Eppendorf) every 2 cycles after the 18th cycle (amplification cycles in the linear
299 range). Extension was then continued in another machine. The cycle number at which *Actb* was
300 first detected was used to normalise for cDNA quantities.

301 ***Statistical analysis***

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

307 **Results**

308 ***Ill family members are regulated at the transcript level in peri-implantation uterus***

309 Characterisation of *Ill1a*, *Ill1b*, *Ill1rn*, *Ill1r1* and *Ill1r2* mRNA expression on D2-D6 of pregnancy
310 in wt and *Lif* null females was performed by RT-PCR (Fig 2) on RNA extracted separately from
311 uterine stromal and LE isolates. Transcript patterns shown are representative of 3 separate
312 animals at each stage and genotype.

313 Both ligands, *Ill1a* and *Ill1b* showed temporal regulation in the uteri of wt mice during early
314 pregnancy (Fig 2 A,B). Specifically, transcripts bands were observed on D2 of pregnancy in
315 both LE and stromal isolates and intensity of bands appeared to then decrease such that on the
316 morning of D4 of pregnancy (0900h) no transcripts could be detected for *Ill1a* (even when PCRs
317 were taken to saturation), although a very faint band was seen for *Ill1b* in LE and stroma.
318 However, by the evening of D4 (2200h), which follows elevated levels of estrogen and LIF,
319 mRNAs for *Ill1a* and *Ill1b* in both LE and stromal isolates were again detected as seen on D2.
320 Although, *Ill1a* mRNA was continually expressed up until D6 in both the stroma and LE, *Ill1b*
321 mRNA was undetectable on D6 in both the LE and stroma, suggesting only transient re-
322 expression on D4 evening and D5 of pregnancy. Moreover the pattern of disappearance of *Ill1a*
323 on the morning of D4 in wt uteri was not paralleled in the uteri of *Lif* deficient mice on D2-D6 of
324 pregnancy. *Ill1a* mRNA levels appeared to decline progressively from D2 onwards in the LE,
325 whereas stromal expression of *Ill1a* transcripts were only detected on D3 and D5 of pregnancy in
326 *Lif* null mice. Interestingly, in null females, the pattern of *Ill1b* expression in the LE was parallel
327 to that seen in wt mice, but stromal expression of *Ill1b* was markedly different. Obvious stromal
328 *Ill1b* mRNA signal was detected on D2, D3, D4 morning and D6 of pregnancy but was
329 undetectable on D4 evening and D5 morning when it was readily detectable in wt stroma.

330 Transcriptional expression of *Ill1r1* was similar to that seen for *Ill1b* in wt mice where a reduction
331 in detectable transcripts was identified on the morning of D4 in the stroma and LE (Fig 2D). On
332 D5, stromal transcript levels declined and on D6 of pregnancy no transcripts could be detected in
333 the LE and little in the stroma. *Ill1r2* transcripts were consistently detected in the LE from D2
334 onwards (Fig 2E). Strong signal for *Ill1r2* mRNA was seen on the evening of D4 and morning of

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

341 *Il1rn* transcripts were consistently expressed throughout D2-D6 of pregnancy in both LE and
342 stromal isolates from wt mice, with only a transient but marked reduction on D4 morning in the
343 stromal isolate (Fig 2C). In LE of *Lif* nulls, *Il1rn* mRNA could only be reliably detected on day
344 2 and D6 of pregnancy. In the stroma however, *Il1rn* transcripts were consistently expressed
345 through D2-D6 of pregnancy with no loss of expression on day 4 as in the wt stroma.

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

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

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

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

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

381 of pregnancy, although some faint staining was also evident in the sub-luminal stroma.

382 Immunoreactive IL1B was not detected on D4 morning or D5 in *Lif* null uteri.

383 ***Establishment of co-culture system***

384 Since our data suggested that the changing expression of IL1 and associated molecules is
385 disrupted in the *Lif* null uterus, we investigated the effect of LIF on stromal and LE cells *in vitro*.

386 For this purpose we used our co-culture system in which LE cells are grown on suspended
387 membranes. LE cells proliferated and formed a pavement-like epithelium on Cellagen
388 membranes. They became confluent after 4 days of culture at which time the TER plateaued at or
389 above 400Ω cm² indicative of a tight junctional network. The LE cells were immunostained for
390 the tight junctional protein TJP1, and desmosomal protein, desmoplakin, together with
391 cytoplasmicstaining for actin and examined by confocal microscopy demonstrated intact
392 junctional complexes with neighbouring cells (Fig 5).

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

393 For co-culture experiments, uterine LE cells from D2 of pregnancy were cultured on Cellagen
394 membranes with stromal cells in the culture well as described in methods (Fig 1). Preliminary
395 experiments using increasing concentrations of LIF showed that 50ng/ml LIF had a stimulatory
396 effect on release of IL1A by LE cells into the apical compartment, an effect that was prevented
397 when the LIF inhibitor (LIF05) was added to the medium (Fig 6). Subsequent experiments were
398 carried out using this concentration of LIF. LIF and/or the inhibitor were added to the culture
399 media in both compartments and the medium was collected at 24 h and then every 48h up until
400 120h and used for measurements of IL1A and PTGES2. LIF significantly (p<0.01) increased
401 secretion of both IL1A and PTGES2 in the apical medium from the LE compartment of the co-
402 culture system at both 72 and 120 h while IL1A was also increased at 24 h (Figs 7A and 8A).

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

411 ***Effects of LIF on expression of mRNA for *Il1a****

412 In order to assess effects of LIF on mRNA for *Il1a* LE cells and stromal cells were cultured in
413 the co-culture system above and RNA extracted. Preliminary observations confirmed presence of
414 *Il1a* mRNA in all cell types (data not presented). Semi-quantitative RT-PCR showed no
415 differences between treatments after any of 2, 4 or 6 (data not shown) days of culture (Figure
416 10A, B).

417 **Discussion**

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

425 LIF is known to influence cells of the immune system [47; 48] in addition to cells in the
426 reproductive tract. The cytokine IL1 and its associated molecules also play well established roles
427 in inflammatory processes in the body [49]. Indeed the changes associated with implantation
428 have been compared to an inflammatory reaction [50; 51]. Moreover several studies have
429 suggested that IL1 expression is regulated by the local effects of estrogen and P₄ and estrogen
430 also regulates *Lif* [8;48].

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

438 Increased transcript signals for *Il1a*, *Il1b*, *Il1rn* and *Il1r1* and *Il1r2* were observed in wt mice at
439 the time of implantation on the evening of D4 of pregnancy in both LE and stroma. These
440 findings agree with previous studies where *Il1a* and *Il1b* mRNAs and their bioavailability were
441 shown to peak between D4 and D5 of pregnancy [52]. Wood and colleagues [53] also reported
442 that mRNA levels of uterine *Il1a* and *Il1b* decreased, from peak levels on D1 and D2, to very
443 low levels on D3 of pregnancy but expression increased in the peri-implantation period. We
444 have further shown that *Il1rn* mRNA is consistently expressed up until the evening of D4 of
445 pregnancy in the LE but barely detectable on D5 while stromal expression was uninterrupted,
446 except for a transient loss of transcript on the morning of D4 of pregnancy. A similar pattern was
447 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 $\alpha 4$ and $\beta 3$ 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-

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

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

486 No significant alterations were observed in the gene expression of *Il1r1* or *Il1r2* between uterine
487 LE of wt and *Lif* null mice during early pregnancy in the LE suggesting these receptors are not
488 regulated by LIF. However null stromal *Il1r1* was expressed in a pattern at variance with that in
489 wt, with loss of transcripts 12-24 h after the known peak of *Lif* expression in wt animals. Lack of
490 proper regulation of epithelial derived IL1A in the *Lif* null uterus may lead secondarily to lack of
491 IL1B in the glands observed by immunocytochemistry on day 5. This may reflect a failure of
492 normal LE to stromal signalling. Indeed, it is likely that the stromal misexpression of several *Il1*
493 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 differences 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 IL1F₅ and IL1F₇ [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

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

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

534 Alternatively, direct transcript stimulation may be prevented under our co-culture conditions.

535 LIF also induced release of PTGES2 by LE cells in co-culture, an effect which was also
536 observed in culture of LE alone. In the co-culture system, LE and stromal cells are able to
537 establish a dialogue, resulting in modulation of cytokine production [64], thus making this model
538 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 through 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

561 influence on the blastocyst. Our results confirm the extreme complexity of the interacting
562 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) *Il1r2*. (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 \pm 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 \pm 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 |
|------------------|------------------------------|--------------|-------------------|-------------------------------|-------------------------|
| <i>Actb</i> (f) | AAACTGGAACGGTGAAGGC | 30 | 192 | 60 | X03672 |
| <i>Actb</i> (r) | CCTGGGCCATTCAGAAATTA | | | | |
| <i>Gapdh</i> (f) | TCTGAGGGCCCACTGAAG | 28 | 220 | 60 | NM008084 |
| <i>Gapdh</i> (r) | AGGGTTTCTTACTCCTTGGAGG | | | | |
| <i>Il1r1</i> (f) | AAATAATGAGTTACCCGAGGTCCAGTGG | 30 | 709 | 60 | NM008362 |
| <i>Il1r1</i> (r) | AGGCATCGTATGTCTTTCCATCTGAAGC | | | | |
| <i>Il1a</i> (f) | CAAACCTGATGAAGCTCGTCA | 32 | 220 | 60 | NM010554 |
| <i>Il1a</i> (r) | TCTCCTTGAGCGCTCACGAA | | | | |
| <i>Il1b</i> (f) | ATGGCAACTGTTCCCTGAACCTCAACT | 32 | 563 | 60 | NM008361 |
| <i>Il1b</i> (r) | CAGGACAGGTATAGATTCTTTCCTTT | | | | |
| <i>Il1r2</i> (f) | GCATCATTGGGGTCAAGACT | 32 | 156 | 60 | BC032962 |
| <i>Il1r2</i> (r) | TGAGTACTGGGGTGTAGCC | | | | |
| <i>Il1rn</i> (f) | TAGCAAATGAGCCACAGACG | 32 | 190 | 60 | NM031167 |
| <i>Il1rn</i> (r) | ACATGGCAAACAACACAGGA | | | | |

Figure 1

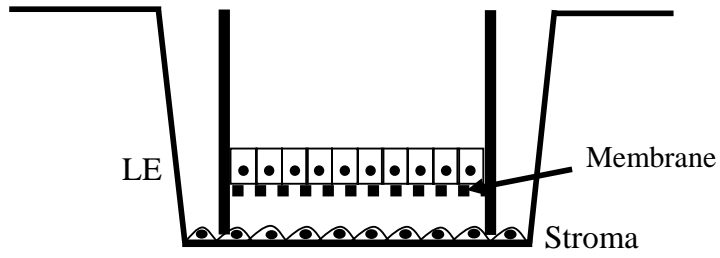


Figure 2

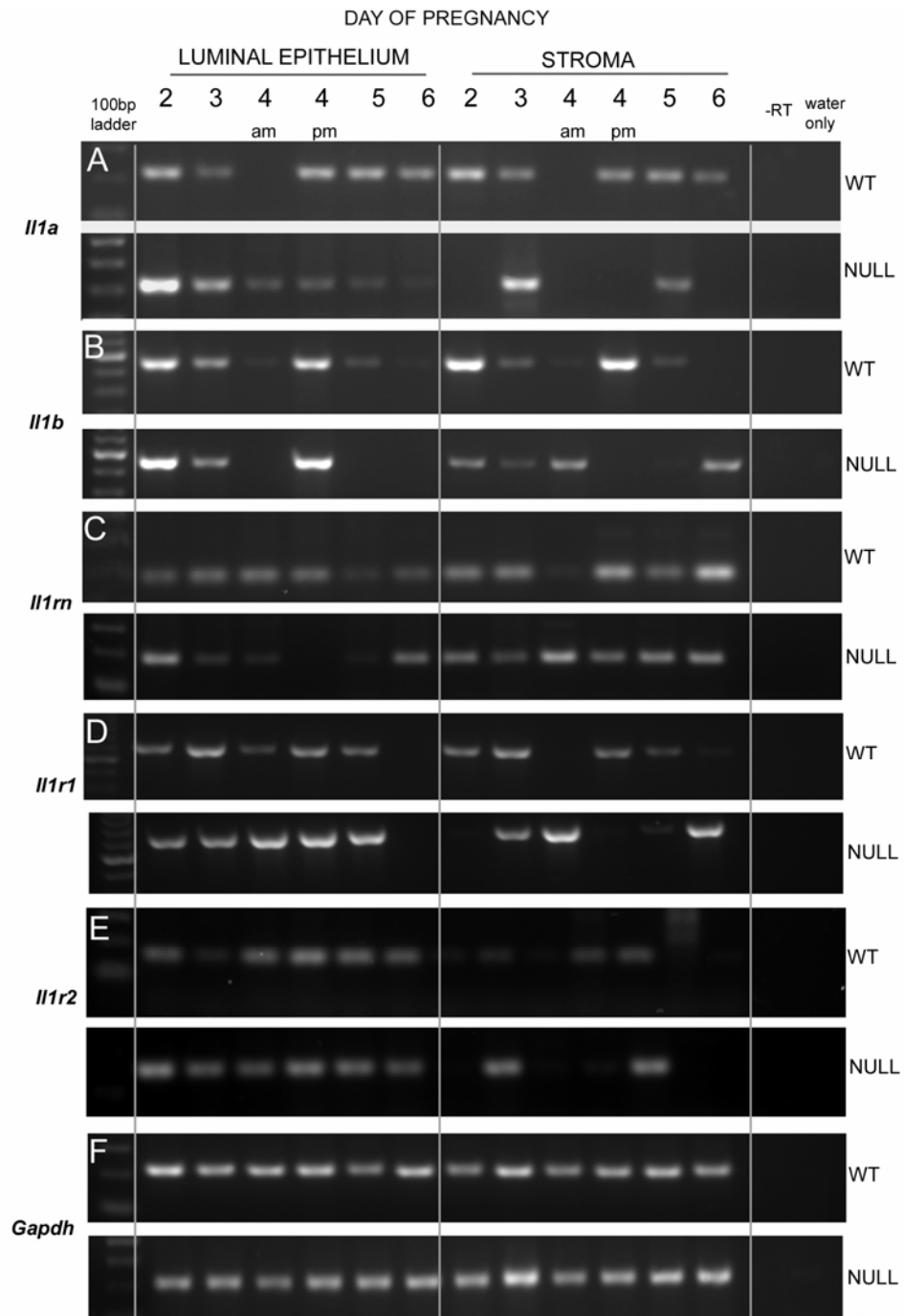


Figure 3

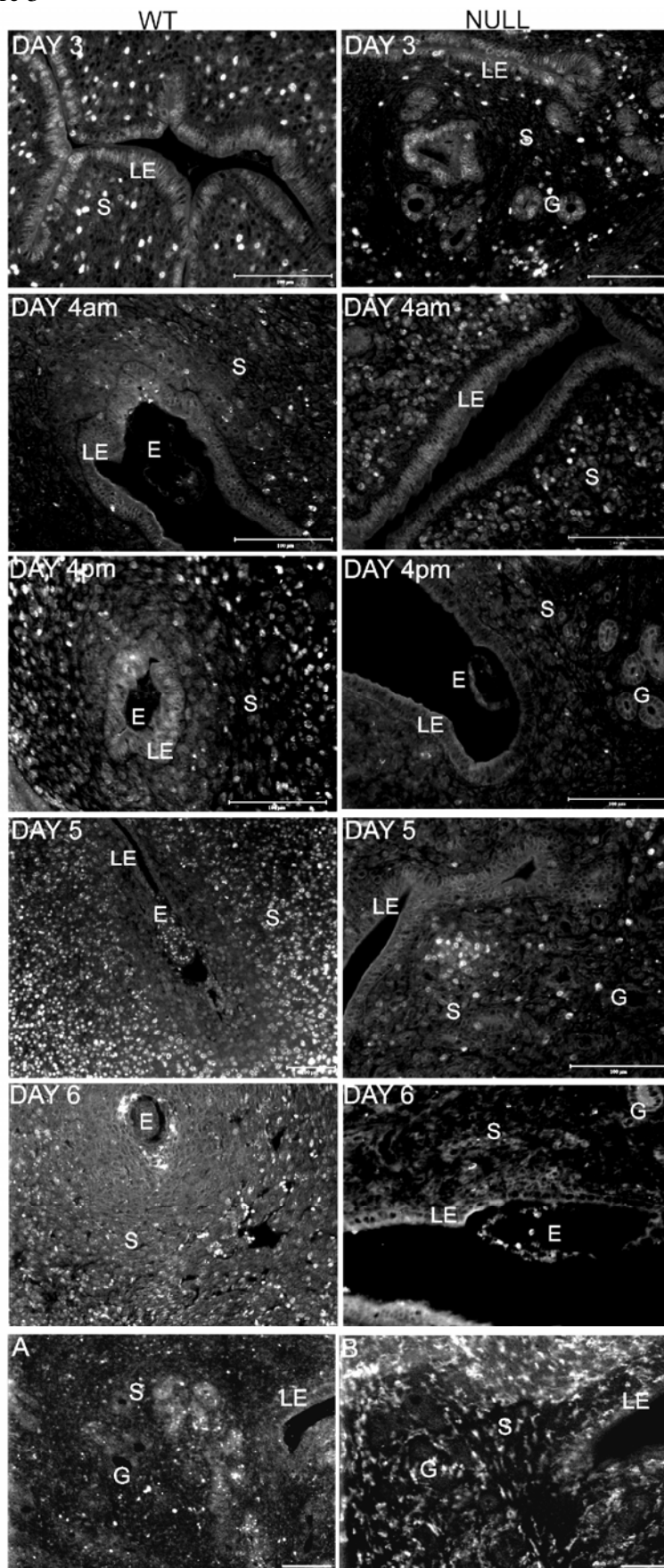


Figure 4

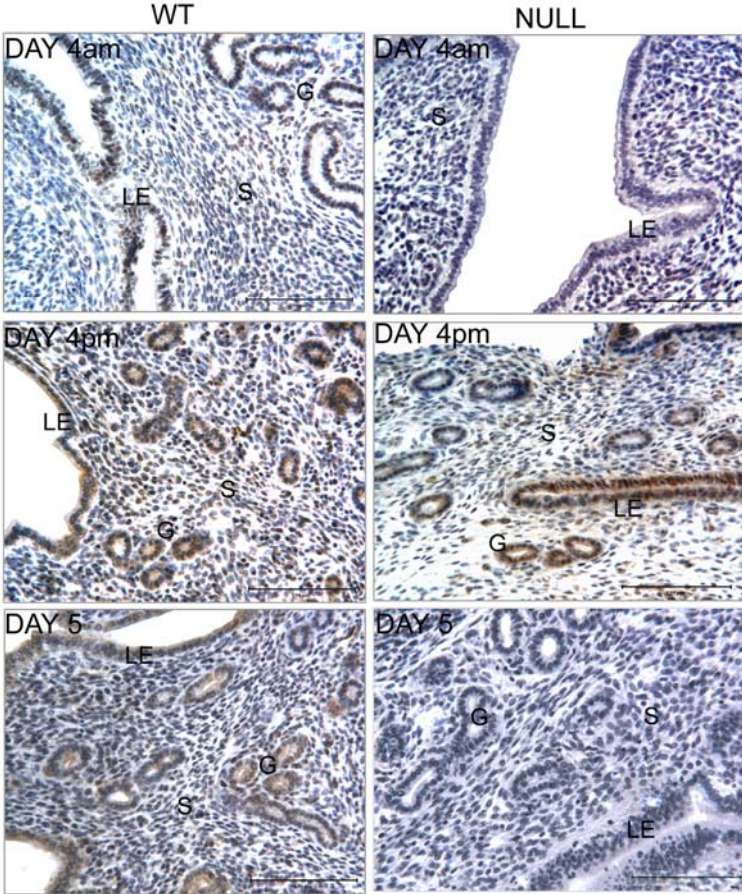


Figure 5

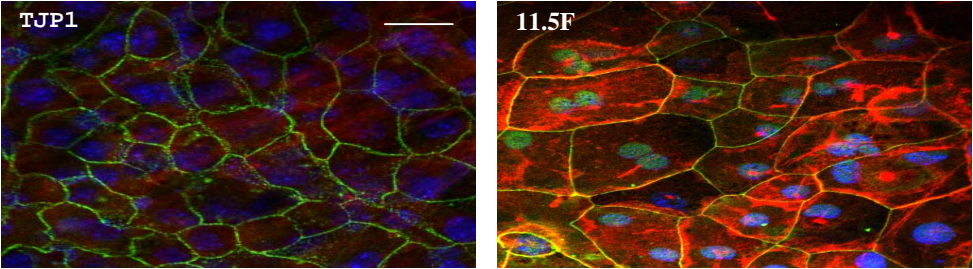


Figure 6

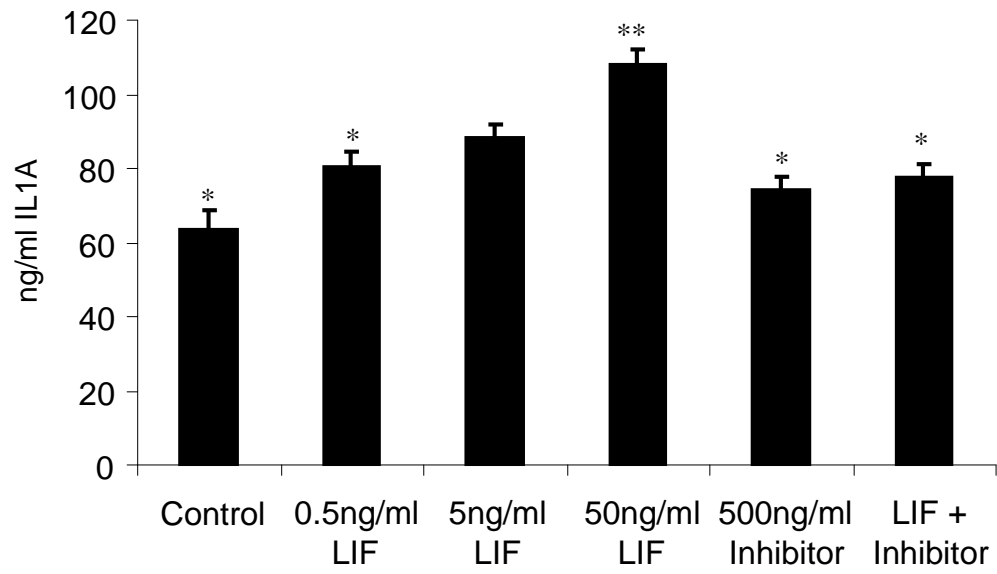


Figure 7

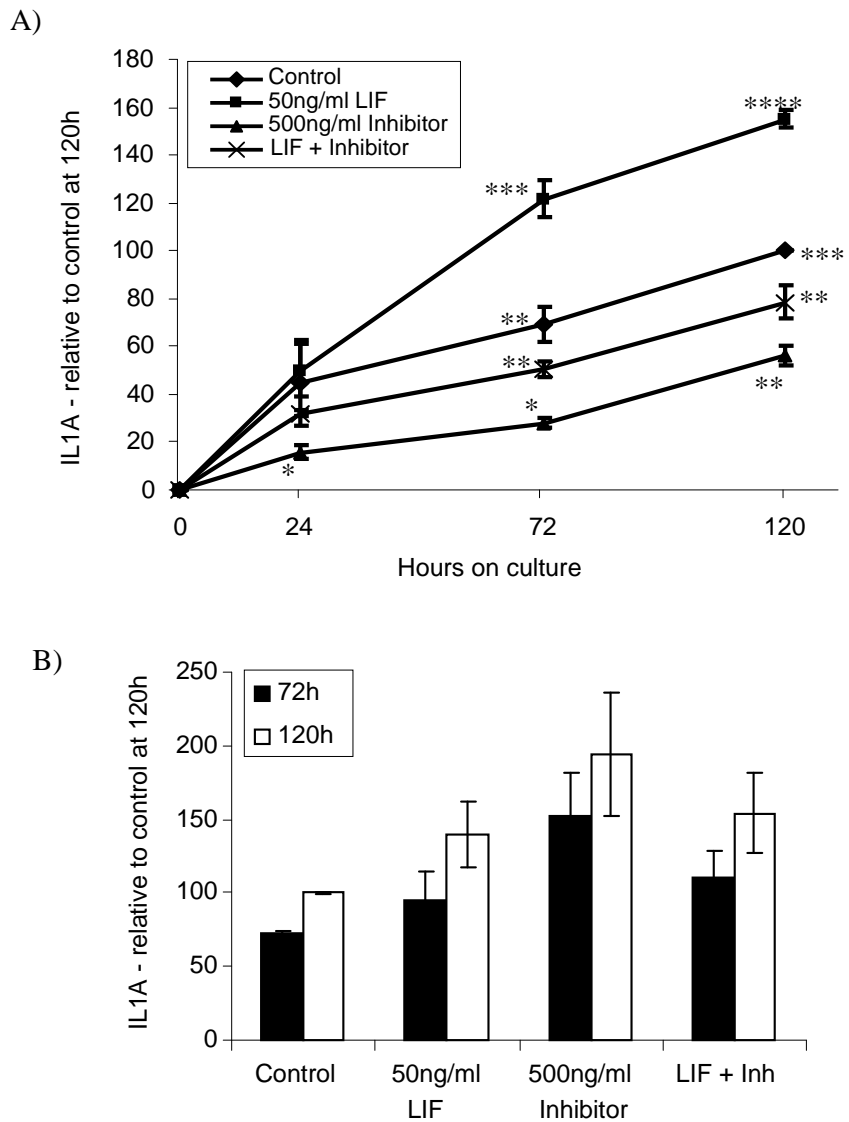


Figure 8

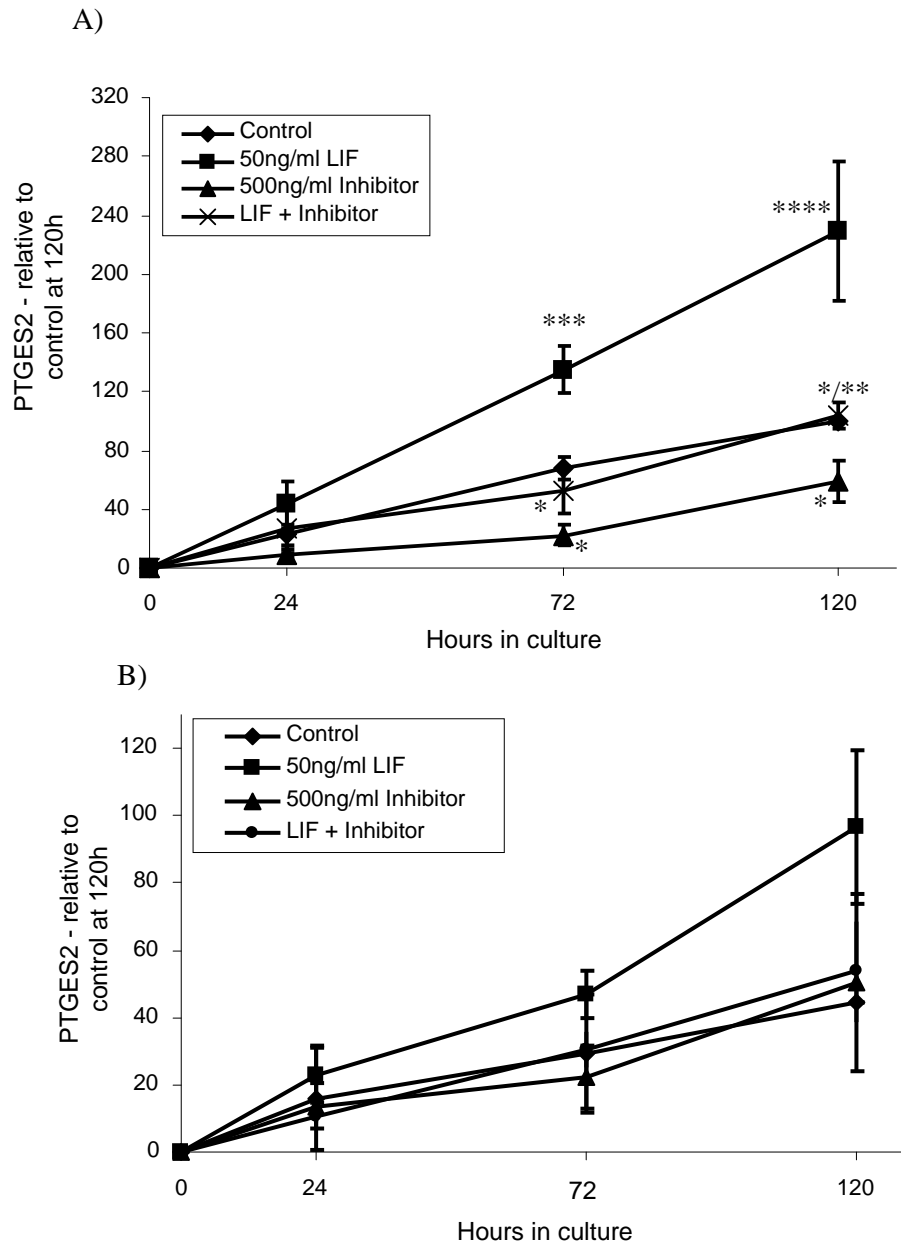
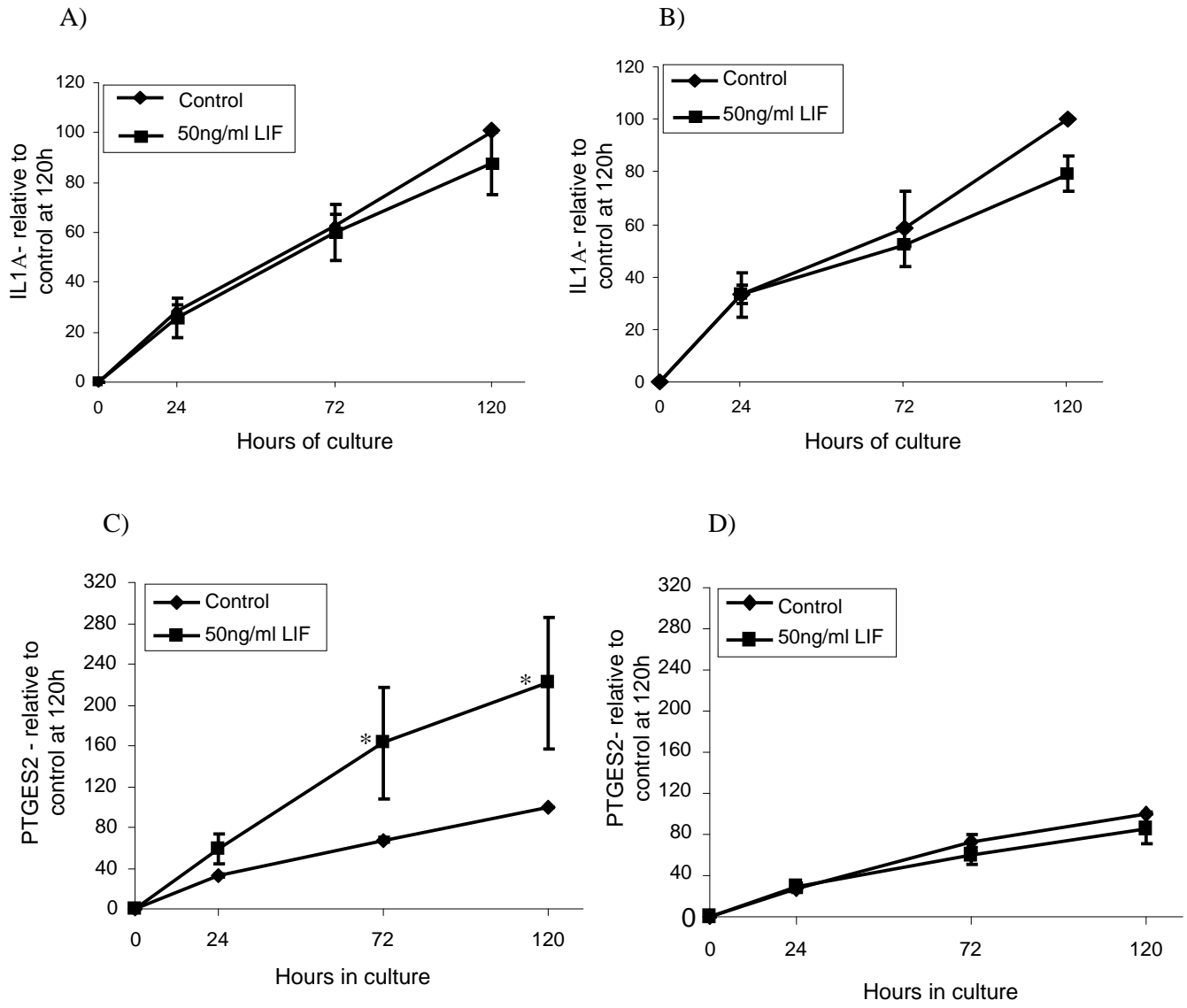
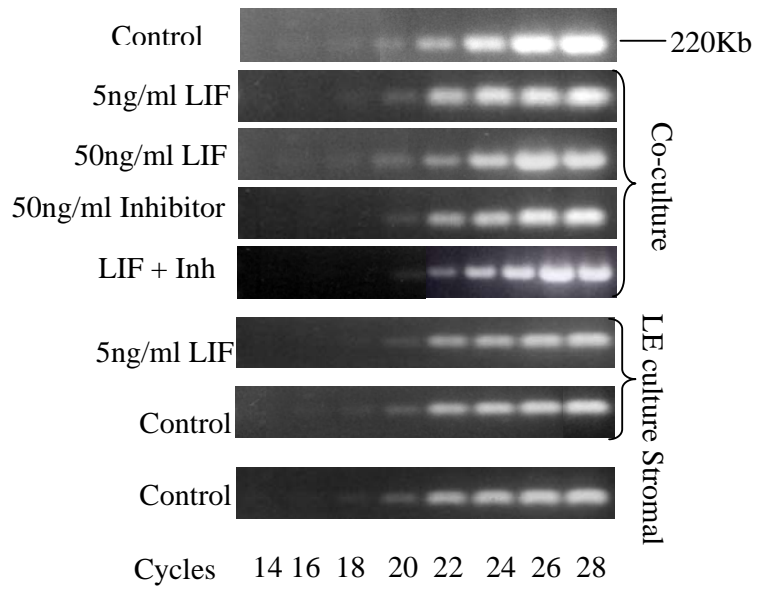


Figure 9



A)



B)

