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### Paper:

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1 **Title: Interleukin-4 and interleukin-13 down-regulate the lipopolysaccharide-**  
2 **mediated inflammatory response by human gestation-associated tissues**

3

4 **Running Title:** Anti-inflammatory activity of cytokines in the human placenta

5 **Summary Sentence:** The anti-inflammatory cytokines interleukin (IL)-4 and IL-13 can  
6 down-regulate the lipopolysaccharide (LPS) induced inflammatory response by human  
7 gestation-associated tissues in an IL-4 receptor alpha dependent manner.

8

9 **Keywords:** Inflammation; pregnancy; placenta; IL-4; IL-13

10

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**26 Abstract**

27 Inflammation is a key feature of preterm and term labour. Pro-inflammatory mediators are  
28 produced by gestation-associated tissues in response to pathogen-associated molecular  
29 patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Interleukin (IL)-4,  
30 IL-10 and IL-13 are anti-inflammatory cytokines with potential as anti-inflammatory  
31 therapies to prevent preterm birth. The objective of this study was to determine if IL-4 and  
32 IL-13 exert anti-inflammatory effects on lipopolysaccharide (LPS)-stimulated production of  
33 pro-inflammatory cytokines produced by human term gestation-associated tissues (placenta,  
34 choriodecidua and amnion). Both IL-4 and IL-13 reduced LPS-stimulated IL-1B and  
35 macrophage inflammatory protein (MIP)-1A; this effect diminished with delay to exposure to  
36 either cytokine. There was no effect on LPS-stimulated prostaglandin production. IL-4  
37 receptor alpha (IL-4RA) was expressed throughout the placenta, choriodecidua and amnion,  
38 and the inhibitory effects of IL-4 and IL-13 were IL-4RA-dependent. Combined IL-4 and IL-  
39 13 did not enhance the anti-inflammatory potential of either cytokine; however, a  
40 combination of IL-4 and IL-10 had a greater anti-inflammatory effect than either cytokine  
41 alone. These findings demonstrate that human term gestation-associated tissues are  
42 responsive to the anti-inflammatory cytokines IL-4 and IL-13, which could down-regulate  
43 LPS induced cytokine production in these tissues. Anti-inflammatory cytokines might offer  
44 an adjunct to existing therapeutics to prevent adverse obstetric outcome.

45

46

47

## 48 **Introduction**

49 Preterm birth (PTB; < 37 weeks gestation) accounts for three-quarters of perinatal mortality  
50 and half of perinatal morbidity. Premature babies are prone to higher rates of complications  
51 of the gastrointestinal, renal and respiratory systems, and neurodevelopmental impairment  
52 and these problems persist into later life [1]. Both term and preterm labour, regardless of  
53 aetiology are considered inflammatory processes. Various inflammatory mediators ranging  
54 from cytokines and chemokines such as interleukin (IL)-1 $\beta$  IL-6, tumour necrosis factor  
55 alpha (TNFA), monocyte chemoattractant protein (MCP)-1, and IL-8 to prostaglandins,  
56 complement, and matrix metalloproteases (MMPs), contribute to cervical ripening,  
57 membrane rupture and/or myometrium contractility [2]. Targeting common inflammatory  
58 pathways might curtail the inflammatory cascade before myometrial contractions and cervical  
59 changes have occurred [3].

60

61 The resolution of inflammation is essential for homeostasis. One mechanism by which this is  
62 achieved is the production of various anti-inflammatory molecules in response to an  
63 increased pro-inflammatory environment. A number of cytokines including IL-4, IL-10 and  
64 IL-13 are well recognised for their anti-inflammatory activity. Investigations into the use of  
65 anti-inflammatory cytokines to prevent PTB have focused primarily on IL-10. Animal studies  
66 have shown that IL-10 decreases lipopolysaccharide (LPS) induced placental nitric oxide,  
67 TNFA and apoptosis [4], and can prevent LPS mediated PTB in rats [5]. Ex vivo studies in  
68 humans have shown IL-10 can down-regulate LPS- and lipoteichoic acid (LTA)-induced  
69 cytokine and chemokine responses by the healthy term placenta [6], and inhibit IL-1B and  
70 prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production and cyclooxygenase-2 (COX-2) expression in intact term  
71 fetal membranes [7]. However, the impact of other anti-inflammatory cytokines in this  
72 clinical setting is limited.

73 IL-4 and IL-13 are able to down-regulate the production of a variety of cytokines and  
74 chemokines, including IL-1 $\beta$ , TNFA, IL-8 and macrophage inflammatory protein (MIP)-1A  
75 [8-11]. They share functional characteristics through a common receptor component IL-4  
76 receptor alpha (IL-4RA) and share many signalling pathways including the Janus kinases  
77 (JAK) signalling pathway to activate signal transducer and activator of transcription 6  
78 (STAT6); the JAK/STAT pathway [12]. There are two types of IL-4 receptor – type I  
79 consisting of the common gamma chain ( $\gamma_c$ ; IL-2RG) and IL-4R $\alpha$ ; and type II consisting of  
80 IL-4RA and IL-13RA1. IL-4 first binds to IL-4RA and can signal via either receptor whereas  
81 IL-13 first binds to IL-13RA1 and only signals via the type II receptor [13]. Another receptor  
82 component, IL-13RA2, has been suggested to be a decoy receptor but this remains  
83 controversial [14]. Receptor activation by IL-4 and IL-13 ultimately leads to the activation of  
84 the transcription factors STAT6 and GATA3 (GATA binding protein 3) and the induction of  
85 IL-4/13 inducible genes to suppress Th1 and enhance Th2 immunity [12,15]. This anti-  
86 inflammatory potential of both IL-4 and IL-13 has been explored therapeutically in a number  
87 of pathologies including renal cell carcinoma [16], Alzheimer's disease [17], arthritis [18] and  
88 psoriasis [19] with variable results.

89

90 As our knowledge of the biology of both IL-4 and IL-13 in gestation associated tissues is  
91 limited, we set out to examine both the activity of these cytokines and their classically  
92 described signalling pathways. We hypothesised that IL-4 and IL-13 down-regulate the  
93 inflammatory response in gestation-associated tissues as reported previously for IL-10. To  
94 examine this, an explant model of term gestation-associated tissues (placenta, choriodecidua  
95 and amnion) was utilised to determine the biological effects of IL-4 and IL-13 on the LPS  
96 induced inflammatory response by these tissues.

97

## 98 **Materials and Methods**

### 99 *Gestation associated tissues collection, preparation and stimulation*

#### 100 *Samples*

101 Healthy pregnant women (>37 weeks' gestation; n=25) scheduled for elective caesarean  
102 section (ESC) were approached in the antenatal day assessment unit at Singleton Hospital,  
103 Swansea, during their pre-anaesthetic assessment. Informed written consent was obtained.  
104 Ethical approval for this study was given by the South West Wales Research Ethics  
105 Committee Wales and was conducted in accordance with the Declaration of Helsinki  
106 (2013) of the World Medical Association. Women undergoing ECS for fetal or maternal  
107 anomalies were not recruited therefore samples were typically from women scheduled for  
108 ECS because of breach presentation, cephalo-pelvic disproportion, or emergency section at  
109 previous delivery. Samples were transported to the laboratory and processed within 90  
110 minutes of delivery. Care was taken to minimise contamination by LPS/endotoxin when  
111 handling the placenta and the attached membranes. All procedures were performed in a class  
112 II tissue culture cabinet using disposable sterile consumables. Reagents used were determined  
113 by manufacturers to be endotoxin free.

114

#### 115 *Explant Cultures*

116 Placenta. Placental explant cultures were prepared as described previously [20,21]. The  
117 decidua basalis overlaying the maternal side of the placenta was removed and 1 cm<sup>3</sup> pieces of  
118 placental tissue cut from various sites across the placenta and placed into sterile calcium and  
119 magnesium free phosphate buffered saline (PBS; Thermo Fisher Scientific, UK). Tissue was  
120 washed repeatedly with PBS to remove contaminating blood and then minced into smaller  
121 pieces and washed further. Pieces of tissue (1 mm<sup>3</sup> pieces to a total of 0.2 g) were transferred  
122 into the appropriate number of wells of a standard 12-well tissue culture plate (Greiner Bio-

123 one, Germany) containing 1ml UltraCULTURE™ medium (Lonza, Switzerland),  
124 supplemented with 2 mM GlutaMAX™ (Thermo Fisher Scientific, UK) and 1% PSF  
125 (penicillin/streptomycin sulphate/fungizone®; Thermo Fisher Scientific, UK).

126

127 Membranes. Membranes were detached from the placenta. Choriodecidua and amnion were  
128 separated from each other by blunt dissection and placed individually into PBS. Tissue was  
129 washed repeatedly with PBS to remove any contaminating blood. Explants were cut with an  
130 8mm biopsy punch (Steifel, Medisave, UK). For amnion, three pieces were transferred into  
131 each individual well of a 24-well tissue culture plate containing 0.5 ml of Advanced DMEM  
132 (Thermo Fisher Scientific, UK) supplemented with 2 mM GlutaMAX™, 2% FBS  
133 (HyClone™; Thermo Fisher Scientific, UK) and 1% PSF. For choriodecidua, two pieces  
134 were transferred into each individual well of a 24-well tissue culture plate containing 0.5 ml  
135 Advanced RPMI supplemented with 2 mM GlutaMAX™, 2% FBS, 5mM 2-mercaptoethanol  
136 (2-ME; Thermo Fisher Scientific) and 1% PSF.

137

138 Once prepared, explant cultures were stimulated with ultrapure LPS (10 ng/ml [20,21];  
139 Invivogen, USA), and/or combinations of recombinant human IL-4, IL-10, and IL-13 (all 10  
140 ng/ml [22,23]; Miltenyi Biotec, UK); an unstimulated control was always included.  
141 Cytokines were added either pre- or post-LPS stimulation as detailed in the appropriate figure  
142 legends. All cultures were incubated for 24 hours. For inhibition/neutralisation experiments  
143 cultures were incubated with the following antibodies: anti-hIL-4RA IgG2a, Mouse IgG2a  
144 isotype (both 1 µg/ml; R&D Systems) for 30 minutes prior to the addition of cytokines/LPS.

145

146 ***RNA Extraction from Gestation-Associated Tissue Samples***

147 Biopsies of tissue were preserved in TRI reagent (Sigma-Aldrich, UK) at -20°C. DNA-free  
148 RNA was prepared from homogenised tissue (FastPrep FP120A Homogeniser; Qbiogene,  
149 The Netherlands) following the TRIzol method of extraction and DNA-free DNase kit  
150 (Ambion®, Thermo Fisher Scientific, UK) as per manufacturer's instructions. Purity and  
151 concentration of RNA were measured (NanoDrop 3300 fluorescence spectrometer; NanoDrop  
152 Technologies, USA).

153

#### 154 ***Polymerase Chain Reaction (PCR)***

155 Reverse transcription was performed using the RETROscript kit (Ambion®, Thermo Fisher  
156 Scientific, UK) as per manufacturer's instructions. Polymerase chain reaction was performed  
157 using the Platinum® Taq DNA Polymerase kit and dNTP Mix (both Thermo Fisher  
158 Scientific, UK). All primers were synthesised by Thermo Fisher Scientific, using sequences  
159 obtained using primer BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). UBE2D2  
160 housekeeping primers were based on previously published sequences [24]. Primer sequences  
161 and specific conditions to each PCR are listed in Table 1.

162

#### 163 ***Cytokine and prostaglandin production***

164 IL-1 $\beta$ , MIP-1 $\alpha$  and PGE<sub>2</sub> in the tissue free supernatants of explant cultures collected after 24  
165 h were measured using commercially available immunoassay kits (IL-1 $\beta$  and MIP-1 $\alpha$ ,  
166 DuoSet; PGE<sub>2</sub>, Parameter; all R&D Systems) as per manufacturer's instructions.

167

#### 168 ***Immunohistochemistry***

169 Immunohistochemical staining for IL-4RA was performed on formalin fixed, paraffin  
170 embedded sections (4  $\mu$ m) of placenta and fetal membranes using the Ventana ULTRA  
171 automated staining instrument with the OptiView DAB detection kit. Antigen retrieval used



172 cell conditioning 1 (CC1) for 16 mins at 98°C. Mouse monoclonal anti-IL-4RA (3 µg/ml;  
173 R&D Systems, USA) primary antibody was incubated at room temperature for 24 minutes.  
174 Control slides where primary antibody was replaced with mouse IgG2a isotype control  
175 (eBioscience) at the same concentration were also included. Tonsil was used as a positive  
176 control tissue.

177

### 178 *Statistical analysis*

179 All experiments were performed a minimum of three times. Statistical analysis was  
180 performed using GraphPad Prism (Version 6, GraphPad Software Inc, USA). Agonist  
181 mediated cytokine production by non-laboured tissues was evaluated by one-way ANOVA  
182 with Tukey post hoc test for multiple comparisons. Difference between cytokine (IL-1B  
183 versus MIP-1A)/tissue (placenta versus choriodecidua versus amnion) response was  
184 evaluated by two-way ANOVA with Tukey post hoc test for multiple comparisons. A *p*-value  
185 of  $\leq 0.05$  was considered significant.

186

187

**188 Results****189 *Pre-treatment with IL-4 or IL-13 inhibits LPS induced cytokine output of placenta,***  
**190 *choriodecidua and amnion***

191 LPS treated gestational tissues produce a robust inflammatory response measurable as  
192 increased levels of various cytokines and chemokines [20,21], therefore LPS exposed tissues  
193 were used as a relevant inflammatory model. Tissue explants were pre-incubated with IL-4 or  
194 IL-13 at a standard concentration of 10 ng/ml [22] for 90 minutes prior to the addition of LPS  
195 for 24 h. For all three tissues, a significant decrease in both LPS-stimulated IL-1B and MIP-  
196 1A was observed in the presence of IL-4 or IL-13 (Figure 1). A similar anti-inflammatory  
197 effect of IL-4 or IL-13 pre-treatment when LPS was substituted for Pam3CSK4 (TLR2/1),  
198 FSL-1 (TLR2/6) and Tri-DAP (NOD1) (Supplementary Figure 1). While not significantly  
199 different IL-4 generally has a greater anti-inflammatory effect than IL-13. Despite using the  
200 same concentration for both cytokines, a molar to molar comparison of IL-4 (6.62 nM) versus  
201 IL-13 (7.94 nM) would suggest that IL-4 is more effective. A trend for a greater percentage  
202 reduction was seen for IL-1B than MIP-1A in all tissues, however with was only significant  
203 in the amnion but for both IL-4 ( $p < 0.05$ ) and IL-13 ( $p < 0.05$ ). Additionally, the greatest  
204 percentage reduction in cytokine outputs by IL-4 or IL-13 pre-treatment was in the placenta  
205 with the least in the amnion; this is only significant for the reduction of MIP-1A following  
206 IL-13 pre-treatment (placenta versus amnion;  $p < 0.001$ ).

207

**208 *The effect of IL-4 or IL-13 on gestation associated tissues is IL-4RA dependent manner***

209 Since both IL-4 and IL-13 pre-treatment has a biological effect on LPS induced cytokine  
210 production, we investigated IL-4/IL-13 signalling components in gestation-associated tissues.  
211 Expression of receptor and signalling components of the IL-4/IL-13 signalling pathways was  
212 investigated at the RNA level using conventional PCR. Transcripts for IL-4 receptor alpha

213 (*IL-4RA*), the common gamma chain ( $\gamma_c$ , *IL-2RG*), IL-13 receptor alpha 1 (*IL-13RA1*), and  
214 *STAT6* were expressed in all 5 examples of placenta, choriodecidua and amnion. *IL-13RA2*  
215 was expressed by all 5 examples of placenta and choriodecidua but showed varied expression  
216 in the amnion including 2 samples where it was not expressed (Figure 2A).

217

218 Immunohistochemistry was used to determine the localisation of IL-4RA, a common receptor  
219 subunit utilised by both IL-4 and IL-13 [13]. In the placenta, expression was localised to the  
220 trophoblast and cells within the stroma, while in the fetal membranes expression was  
221 localised to the amnion epithelial cells, chorionic trophoblast and various cells within the  
222 decidua (Figure 2B). Expression of IL-4RA was consistent in all seven examples of placenta  
223 and membranes stained. There was negligible background in the negative controls for either  
224 the placenta or fetal membranes (Figure 2B). Tonsil was used as a positive control.

225

226 To confirm the functional activity of IL-4RA within gestational tissues, IL-4RA was  
227 inhibited with a neutralising antibody. In the presence of this antibody the effect of both IL-4  
228 and IL-13 on LPS-induced IL-1B and MIP-1A was abrogated (Figure 3A-F), however, this  
229 was not significant ( $p=0.1$ ) for IL-13 in the amnion in relation to MIP-1A. This confirms the  
230 role of IL-4RA in IL-4 and IL-13 signalling in the placenta, choriodecidua and amnion.

231

232 ***IL-4 and IL-13 abrogate LPS induced cytokine production by gestation associated tissues in***  
233 ***a therapeutic ex vivo model***

234 While pre-treatment with either IL-4 or IL-13 is able to reduce LPS induced cytokine levels,  
235 this does not mimic the typical clinical setting wherein the inflammatory insult would likely  
236 precede therapeutic intervention. Therefore, the ability of both IL-4 and IL-13 to modulate  
237 cytokine production post-LPS exposure was examined. To investigate this, tissue explants

238 were treated with LPS and IL-4 or IL-13 added either 90 minutes prior to LPS (as in Figure  
239 3), concurrently, or at arbitrary time points of 90 or 240 minutes post-LPS treatment allowing  
240 for the development of an inflammatory environment to mimic a clinical setting. IL-4 and IL-  
241 13 retain inhibitory effects when added after LPS; however, the statistical significance of this  
242 response is diminished with delay to adding IL-4 or IL-13 (Figure 4). This is most apparent at  
243 240-minute post treatment where a significant reduction in cytokine response no longer  
244 occurs, with the exception of IL-4 treated placenta. Furthermore, in IL-13 treated amnion  
245 there was no significant effect with either concurrent or post-LPS treatment with the  
246 cytokine, highlighting the reduced potential of IL-13 in this setting.

247

248 *A combination of IL-4 and IL-13 does not enhance the anti-inflammatory properties of*  
249 *either cytokine*

250 Since the effect of both IL-4 and IL-13 was diminished following exposure post-LPS  
251 treatment, the effect of co-treatment with both cytokines was examined. Tissues were treated  
252 with either IL-4 alone, IL-13 alone or IL-4 and IL-13 in combination at 90 and 240 minutes  
253 post-LPS exposure. The response with IL-4 and IL-13 co-treatment did not differ  
254 significantly from the response to individual treatment with IL-4 or IL-13 (Figure 5).

255

256 *Impact of IL-4/IL-13 on prostaglandin production by gestation-associated tissues*

257 In addition to many pro-inflammatory cytokines, prostaglandins have a key role in facilitating  
258 the physiological processes of labour. Therefore, the effect of IL-4 and IL-13 alone or in  
259 combination on PGE<sub>2</sub> levels was considered. IL-4 and IL-13 had no effect on PGE<sub>2</sub> levels  
260 from LPS-treated placental and amnion explant cultures when added 90 or 240 minutes after  
261 LPS exposure (Figure 6). In the choriodecidua, both IL-4 and IL-13 reduced the levels of  
262 LPS-induced PGE<sub>2</sub>, with a combination of both cytokines resulting in a significant reduction

263 when added 90 minutes after LPS treatment. This effect was lost by 240 minutes post-LPS  
264 exposure.

265

266 ***IL-10 enhances the anti-inflammatory properties of IL-4***

267 Since IL-10 has been shown previously to inhibit cytokine production in the term placenta  
268 and fetal membranes [6], the possibility that co-treatment with IL-10 could enhance the  
269 inhibitory effect of IL-4 administered post-LPS stimulation was considered. In all three  
270 tissues, co-treatment with IL-4 and IL-10 at 90 minutes' post LPS exposure resulted in an  
271 enhanced decrease in cytokine production compared to either cytokine alone, however this  
272 was only significant in the choriondecidua for MIP-1A (IL-4+IL-10 versus IL-4 alone; Figure  
273 7). An enhanced decrease in cytokine production is still observed at 240 minutes post-LPS  
274 exposure with combined IL-4/IL-10. This was most notable in the choriondecidua and amnion  
275 where significant reduction with co-treatment was observed in relation to IL-4/IL-10 versus  
276 IL-4 (MIP-1A) and IL-4/IL-10 versus IL-10 (IL-1B) (Figure 7).

277

## 278 **Discussion**

279 Cytokine production at the maternal-fetal interface is a part of normal pregnancy. Changes in  
280 cytokine production occur with term and preterm labour [2]; whether such changes precede  
281 labour and might serve as targets for therapeutic intervention in adverse obstetric outcomes  
282 remains to be determined. Maternal and fetal indications, e.g. preeclampsia, account for up to  
283 35% of PTB but the pathophysiological mechanisms underlying the bulk of PTB are largely  
284 unknown. Evidence supports inflammatory processes: heterogeneous pro-inflammatory  
285 profiles reflecting different underlying causes of PTB, including infection. Targeting  
286 common inflammatory pathways might curtail the inflammatory cascade before myometrial  
287 contractions and cervical changes occur to prevent preterm delivery. Anti-inflammatory  
288 cytokines such as IL-4 and IL-13 but also IL-10 might offer this therapeutic potential [25]. As  
289 noted above the effects of IL-10 upon gestation-associated tissues are relatively well studied  
290 [4-6], therefore the ability of IL-4 and IL-13 to dampen the inflammatory response by  
291 gestation-associated tissues (placenta, choriodecidua and amnion) was examined.

292

293 Using LPS as a known inducer of cytokine production by gestational tissues [20,21] and a  
294 standard initiator of infection induced preterm labour in animal models [5], we showed that  
295 both IL-4 and IL-13 can down-modulate stimulated cytokine (IL-1B) and chemokine (MIP-  
296 1A) responses by all three tissue types studied. These anti-inflammatory effects of IL-4 and  
297 IL-13 were IL-4RA dependent and expression of IL-4R $\alpha$  was observed on multiple cell types  
298 throughout the placenta, choriodecidua and amnion. IL-4 and IL-13 also utilise several  
299 signalling molecule and transcription factors, including STAT6, GATA3, PI3K, MAP kinases  
300 and AMPK [26,27], likely contributing to the anti-inflammatory environment generated by  
301 these cytokines. For example, activation of AMPK in fetal membranes treated with TLR  
302 ligands is associated with a reduced pro-inflammatory output compared to TLR activation

303 without an AMPK activator [28] which might relate to the down-regulation of TLR  
304 expression or function [29]. IL-4 and IL-13 may also target common signalling components  
305 of pattern recognition receptors. Similarly to the effect on LPS, pre-treatment with IL-4 and  
306 IL-13 was shown to attenuate cytokine production by several other bacterial agonists, namely  
307 Pam3CSK4 (TLR2/1), FSL-1 (TLR2/6) and Tri-DAP (NOD1). While this effect has only  
308 been examined in the relation to bacterial agonists, both viral infection and sterile  
309 inflammation mediated by DAMPs may cause PTB [2]. However regardless of the  
310 inflammatory trigger innate signalling pathways triggered by bacteria, virus or DAMPs all  
311 utilise common signalling components such as MAP kinases and the transcription factor AP-  
312 1 which can be negatively impacted by IL-4 and IL-13 [2,30,31].

313

314

315 IL-4 and IL-13 tended to cause a greater percentage reduction in LPS-induced IL-1B than  
316 MIP-1A although this was only significant in the amnion. Since IL-4 and IL-13 down-  
317 regulate both IL-1B and MIP-1A gene expression in other settings [10,11], this difference  
318 might relate to greater non-transcriptional regulation of the inflammasome by IL-4 [32].  
319 Impaired inflammasome assembly and subsequent activation would limit the maturation of  
320 IL-1B from its pro-form to the mature secreted form. The inhibition of IL-1B by IL-13 and  
321 other anti-inflammatory cytokines likely occurs by a similar mechanism.

322

323 Although not significant, IL-4 in general had a greater inhibitory effect compared to IL-13 on  
324 LPS-induced IL-1B and MIP-1A production, with the exception of IL-1B in the  
325 choriodecidua and MIP-1 $\alpha$  in the amnion. Any differential effects of these cytokines might  
326 relate to relative expression of signalling components other than IL-4RA. While PCR  
327 analysis of the placenta, choriodecidua and amnion revealed transcripts of all components of

328 the IL-4/IL-13 signalling pathway (*IL-4RA*, *IL-2RG*, *IL-13RA1*, *IL-13RA2* and *STAT6*) a  
329 quantitative method was not used so other than the obvious variation in *IL-13RA2* in the  
330 amnion it is not possible to provide any detail on relative levels of these signalling  
331 components in each tissue. The varied expression of *IL-13RA2* in amnion could impact the  
332 effectiveness of IL-4 and IL-13 in this tissue. While classically described as a decoy receptor  
333 due to its high affinity binding, fast association rate and slow dissociation rate for IL-13, IL-  
334 13RA2 might also have some signalling capabilities including the activation of the  
335 transcription factor AP-1 in macrophages [33]. IL-13RA2 can also affect IL-4  
336 responsiveness, due to interaction of its cytoplasmic tail with IL-4RA preventing the  
337 association of JAK1 and the subsequent downstream signalling [34].

338

339 While both IL-4 and IL-13 could down regulate LPS-induced pro-inflammatory cytokine  
340 production when tissues were pre-treated with these cytokines, in a clinical setting any  
341 intervention would likely occur after the inflammatory insult. To explore if IL-4 and IL-13  
342 would be effective post insult as would occur clinically, the effect of IL-4 and IL-13 on  
343 gestation-associated tissues post-LPS exposure at arbitrary time points was determined. The  
344 anti-inflammatory effect of these cytokines was diminished with delayed treatment post-LPS  
345 exposure. When added following 240-minute exposure of the tissues to LPS the anti-  
346 inflammatory effect of both IL-4 and IL-13 was negligible. This might relate to the induction  
347 of SOCS1, a negative regulator of JAK/STAT signalling pathways by LPS in a TLR4  
348 dependent manner [12,35]. While a standard concentration of IL-4 and IL-13 was utilised here,  
349 it is unknown if any dose dependent effects would have been observed with higher  
350 concentrations potentially yielding a greater anti-inflammatory effect post-exposure to LPS.  
351 Examining any potential dose-dependent effects might aid in our understanding of how these  
352 cytokines could be used therapeutically. However, a previous study in peripheral blood



353 mononuclear cells noted a maximal suppressive effect of IL-4 on LPS-induced IL-1B at a  
354 concentration of 10 ng/ml with no enhanced effect at 100 ng/ml [36]. It should also be noted  
355 that a reverse dose response for IL-10 has been observed in placental explants, where 10  
356 ng/ml had a greater effect on macrophage migration inhibitory factor (MIF) than 25 ng/ml in  
357 human placental explants, with this effect greater in first trimester explants versus third [37].

358

359 Co-treatment with both IL-4 and IL-13 did not enhance the anti-inflammatory effects of  
360 either cytokine; this is likely due to the utilisation of similar signalling components.  
361 Furthermore, combined IL-4, IL-13 or IL-4 and IL-13 had little impact on the production of  
362 PGE<sub>2</sub> by gestation associated tissues other than for the choriodecidua 90 minutes post-LPS  
363 exposure. This suggests that while these anti-inflammatory cytokines can modulate the  
364 production of pro-inflammatory cytokines by gestational tissues, downstream molecules are  
365 not affected. To date treatment strategies for PTB have focused on inhibiting myometrial  
366 contractions utilising an array of compounds including cyclooxygenase inhibitors, oxytocin  
367 antagonists, magnesium sulphate and B-mimetics [38]. Each of these treatment strategies  
368 targets late stage events in the labour process and not the upstream causes of an inflammatory  
369 cascade. Given IL-4 and IL-13 had negligible effects on PGE<sub>2</sub>, combination therapy with an  
370 anti-inflammatory approach as described here with anti-contraction medications would likely  
371 be needed [39].

372

373 As IL-10 has been described as an effective anti-inflammatory in a similar experimental  
374 setting we investigated the effects of combination IL-4 and IL-10. A combination of IL-4 and  
375 IL-10 has been suggested as a treatment for preeclampsia with promise shown in an animal  
376 model [40]. The combination of IL-4 and IL-10 had a greater effect in reducing the levels of  
377 LPS-induced cytokine production at both 90 minutes and 240 minutes post-LPS stimulation

378 than either cytokine alone, with the greatest effect in the choriodecidua, but this was not  
379 always significant. This enhanced effect likely results from the activation of both shared and  
380 specific downstream targets of IL-4 and IL-10. For example, both IL-4 and IL-10 enhance the  
381 expression of IL-1 receptor antagonist (IL-1RA) [41], a potent anti-inflammatory cytokine  
382 involved in the inhibition of IL-1A and IL-1B [42]. In contrast, the micro RNA miR-146b,  
383 which is upregulated by IL-10 but not IL-4 or IL-13 in human monocytes, can inhibit TLR4  
384 dependent signalling [43]. Therefore, combined IL-4 and IL-10 offer a promising therapeutic  
385 approach to curtailing the inflammatory cascade at the maternal-fetal interface.

386

387 IL-4, IL-10 and IL-13 have all been reported to be produced by trophoblast, decidua and  
388 amnion and to have homeostatic roles in pregnancy [44]. While advocating a role for these  
389 cytokines as anti-inflammatory therapeutics to prevent PTB it must be noted that both IL-4  
390 [45] and IL-10 [46] are increased in amniotic fluid from women who delivered preterm both  
391 with and without evidence of intra-amniotic infection. This likely represents a compensatory  
392 mechanism that has failed and our proposed approach might augment the body's own  
393 protective mechanisms. Furthermore, understanding how these cytokines might function in  
394 preterm tissues versus term tissues, in relation to both the relative expression of signalling  
395 components in addition to their functional activity is required. The therapeutic potential of  
396 IL-4 and IL-10 in humans in other disease settings has yielded variable and often  
397 disappointing results [16,25] highlighting that the therapeutic use of highly pleiotropic native  
398 cytokines is hampered by side effects related to delivery, degradation, toxicology and off-  
399 target effects. The identification of peptides that deliver only the desired biological effect  
400 might overcome this. A 16 amino acid peptide (Ph8) based on alpha-helix C of IL-4 has been  
401 reported and had anti-inflammatory effects but no effect on the Th1/Th2 cytokine profile  
402 [47]. Clearly, more work is required to better understand the action of anti-inflammatory

403 cytokines at the maternal-fetal interface in both physiological and pathological settings and to  
 404 devise strategies to harness their therapeutic potential.

405

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410

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550

551

552

### 553 **Table Legends**

#### 554 **Table 1. Specifications for PCR.**

555 Primer sequences for IL-4/IL-14 signalling components and optimum conditions for each pair  
556 of primers.

557

### 558 **Figure Legends**

559

#### 560 **Figure 1. Effect of IL-4 and IL-13 on LPS-induced cytokine production by term non-** 561 **laboured gestation associated tissues.**

562 Cytokine levels (IL-1B & MIP-1A) from explants of (A-B) placenta (C-D) choriodecidua and  
563 (E-F) amnion in response to treatment with IL-4 or IL-13 (both 10 ng/ml) 90 minutes prior to  
564 treatment with LPS (10 ng/ml). Data shown as percentage of response to LPS alone (n=6);  
565 error bars represent SEM. Statistically significant differences compared to control treatment  
566 are shown: \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$  \*\*\*\*  $p \leq 0.0001$ .

567

#### 568 **Figure 2. Expression of the IL-4/13 signalling pathway components by term non-laboured** 569 **gestation-associated tissues.**

570 (A) PCR for each of the receptor chains involved in IL-4 and IL-13 signalling as well as the  
571 main transcription factor *STAT6*. Three representative samples of 5 are shown. Human spleen

572 (S) was used as a positive control and *UBE2D2* was used as a housekeeping gene. L = 100  
573 base pair ladder and the negative control (-ve) was with water replacing cDNA in the reaction  
574 mix. (B) Sections of formalin-fixed paraffin-embedded placenta and fetal membranes were  
575 stained with an anti-human IL-4RA antibody; samples from 7 individuals were examined; a  
576 representative is shown. Human tonsil was used as the positive control. Original  
577 magnification x 10. T – Trophoblast, S – Stroma, BV – Blood Vessel, Am – Amnion, Cd –  
578 Chorion, Dec – Decidua, GC – Germinal Centre, TC – Tonsilar Crypt, E – Epithelium.

579

580

581 **Figure 3. Anti-inflammatory effects of IL-4 and IL-13 in the placenta, choriodecidua**  
582 **and amnion are IL-4RA-dependent.**

583 Cytokine levels (IL-1B & MIP-1A) from explants of (A-B) placenta, (C-D) choriodecidua  
584 and (E-F) amnion in response to LPS (10 ng/ml) pre-treated for 90 minutes with IL-4 or IL-  
585 13 (both 10 ng/ml) in the presence or absence of mouse IgG2a anti-human IL-4RA antibody  
586 or mouse IgG2a isotype. Data are shown as % of LPS alone (n = 3); error bars represent  
587 SEM. Statistically significant differences compared to LPS + IL-4/13 treatments are shown: \*  
588  $p \leq 0.05$ , \*\*  $p \leq 0.01$ .

589

590 **Figure 4. Effect of IL-4 and IL-13 treatment over time on LPS-induced cytokine**  
591 **production by term non-laboured gestation-associated tissues.**

592 Cytokine levels (IL-1B & MIP-1A) from explants of (A) placenta (B) choriodecidua and (C)  
593 amnion in response to LPS (10 ng/ml) in the presence of IL-4 or IL-13 (10 ng/ml) added 90  
594 minutes prior to LPS (T-90), at the same time as LPS (T0), or 90 minutes (T+90) or 4 hours  
595 (T+240) after LPS (n=3). Data are shown as percentage of response to LPS alone; error bars

596 represent SEM. Statistically significant differences compared to LPS treatment are shown: \*  
597  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

598

599 **Figure 5. Combined effect of IL-4 and IL-13 treatment on LPS-induced cytokine**  
600 **production by term non-laboured gestation-associated tissues.**

601 Cytokine levels (IL-1B & MIP-1A) from explants of (A) placenta (B) choriodecidua and (C)  
602 amnion in response to LPS (10 ng/ml) in the presence of IL-4, IL-13 or IL-4 + IL-13 (all 10  
603 ng/ml) added 90 minutes (T+90) or 4 hours (T+240) after LPS (n=3). Data are shown as  
604 percentage of response to LPS alone; error bars represent SEM. Statistically significant  
605 differences compared to LPS treatment are shown: \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ .

606

607 **Figure 6. Combined effect of IL-4 and IL-13 treatment on LPS-induced PGE<sub>2</sub>**  
608 **production by term non-laboured gestation-associated tissues.**

609 PGE<sub>2</sub> from explants of (A) placenta (B) choriodecidua and (C) amnion in response to LPS  
610 (10 ng/ml) in the presence of IL-4, IL-13 or IL-4 + IL-13 (all 10 ng/ml) added 90 minutes  
611 (T+90) or 4 hours (T+240) after LPS (n=3). Data are shown as percentage of response to LPS  
612 alone; error bars represent SEM. Statistically significant differences compared to LPS  
613 treatment are shown: \*  $p \leq 0.05$ .

614

615 **Figure 7. Combined effect of IL-4 and IL-10 treatment on LPS-induced cytokine**  
616 **production by term non-laboured gestation-associated tissues.**

617 Cytokine levels (IL-1B & MIP-1A) from explants of (A) placenta (B) choriodecidua and (C)  
618 amnion in response to LPS (10 ng/ml) in the presence of IL-4, IL-10 or IL-4 + IL-10 (all 10  
619 ng/ml) added 90 minutes (T+90) or 4 hours (T+240) after LPS (n=3). Data are shown as



620 percentage of response to LPS alone; error bars represent SEM. Statistically significant  
621 differences compared to LPS treatment are shown: \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

622

623

624 Table 1

Gene	Primer	Mg <sup>2+</sup> Conc (nM)	Anneling Temp (°C)
<i>IL-4RA</i>	<i>F</i> 5' GACCTGGAGCAACCCGTATC	2.5	70
	<i>R</i> 5' CATAGCACAAACAGGCAGACG		
<i>IL-2RG</i>	<i>F</i> 5' ACGGGAACCCAGGAGACAGG	2	70
	<i>R</i> 5' AGCGGCTCCGAACACGAAAC		
<i>IL-13RA1</i>	<i>F</i> 5' GAGCTGACCAAAGTGAAGGA	3	69
	<i>R</i> 5' ATTGCACCTGCGACGATGACTG		
<i>IL-13RA2</i>	<i>F</i> 5' GGCATAGGTGATCTTCTTGA	2	60
	<i>R</i> 5' GCCAGAAACGATGCAAAGTTT		
<i>STAT6</i>	<i>F</i> 5' AGAGGGGTTGCCGAGGTGA	4	70
	<i>R</i> 5' TGTCCACCAGGCTTTCACAC		
<i>UBE2D2</i>	<i>F</i> 5' GATCACAGTGGTCTCCAGCA	3	65
	<i>R</i> 5' TCCATTCCCGAGCTATTCTG		

Figure 1

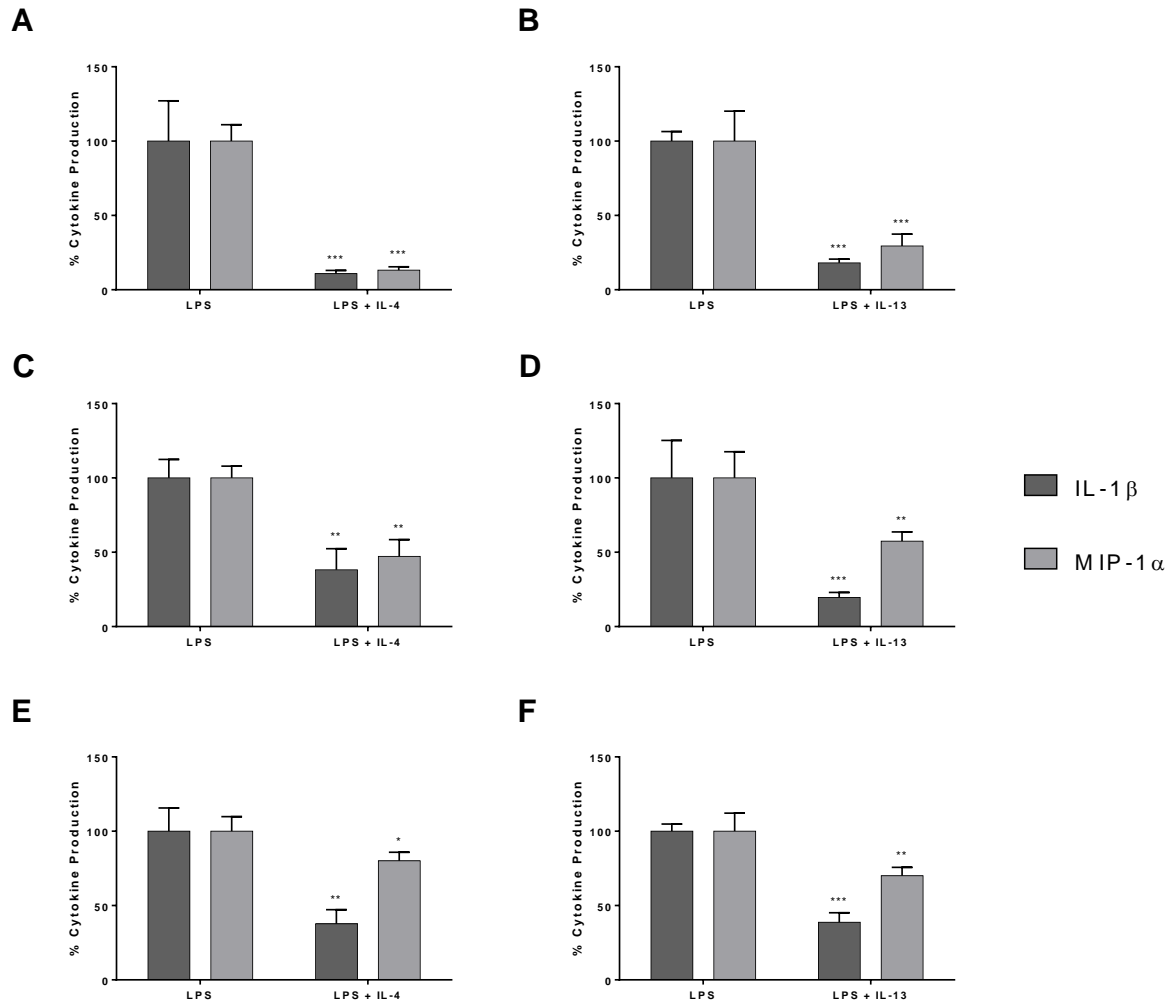


Figure 2A

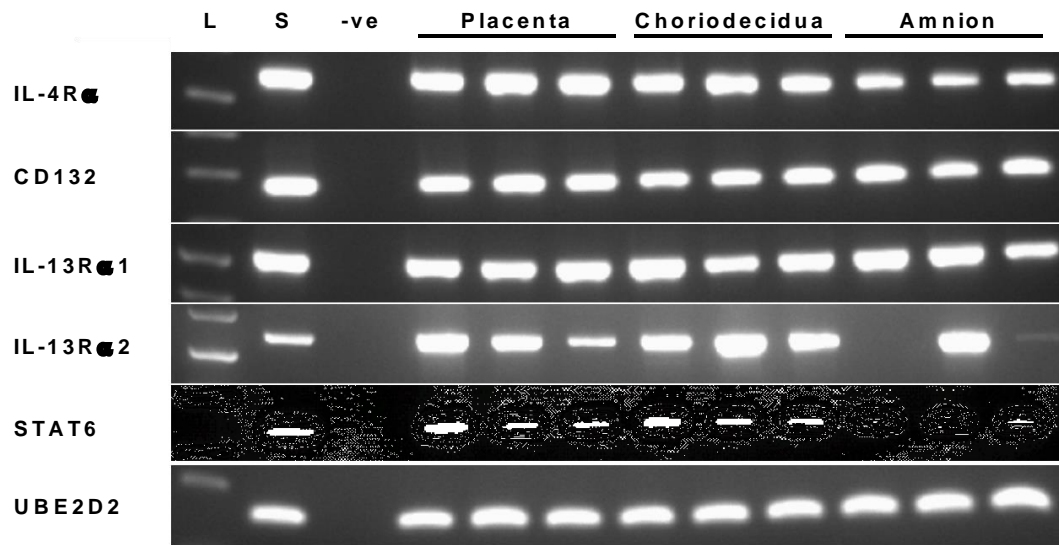


Figure 2B

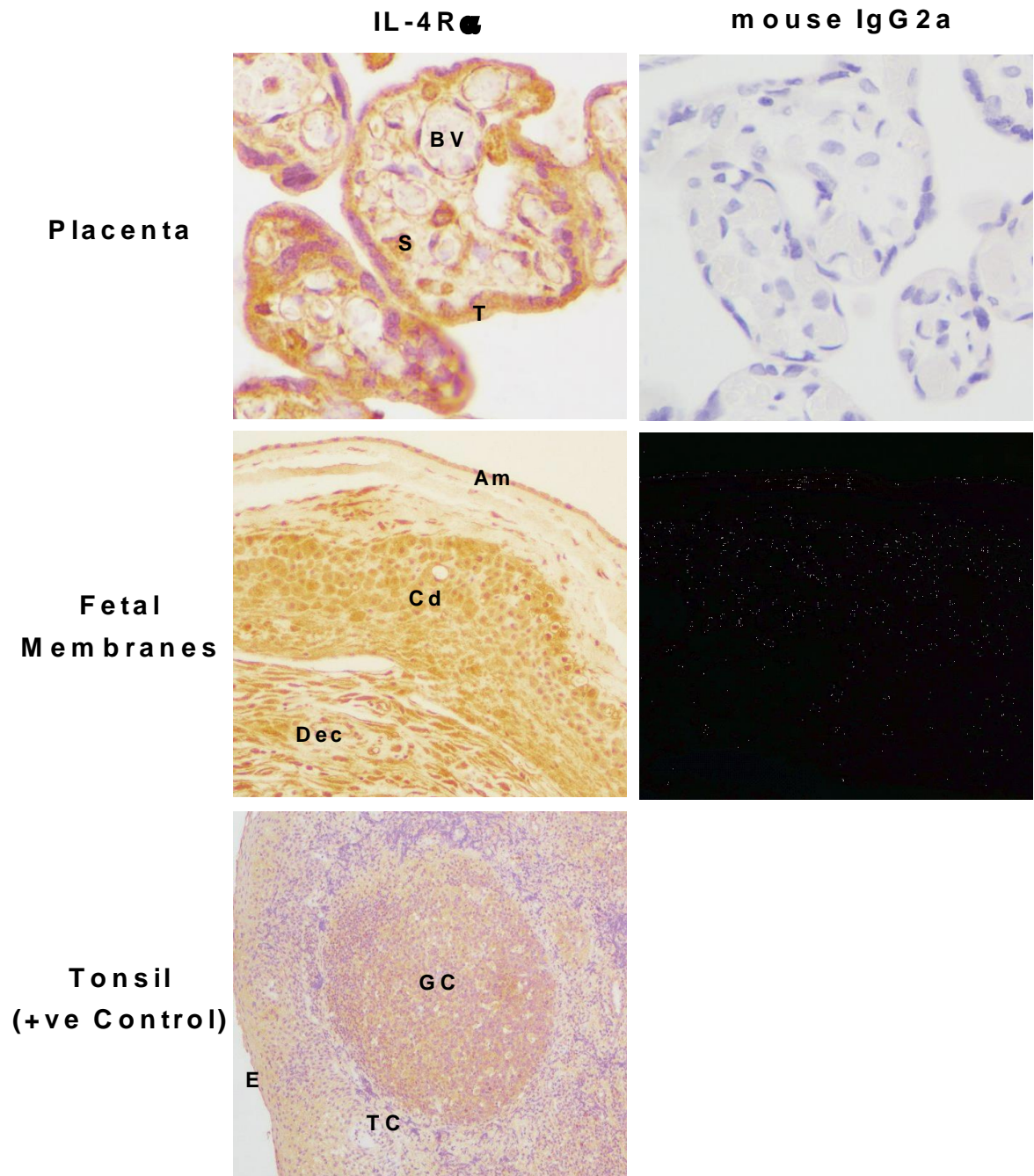


Figure 3

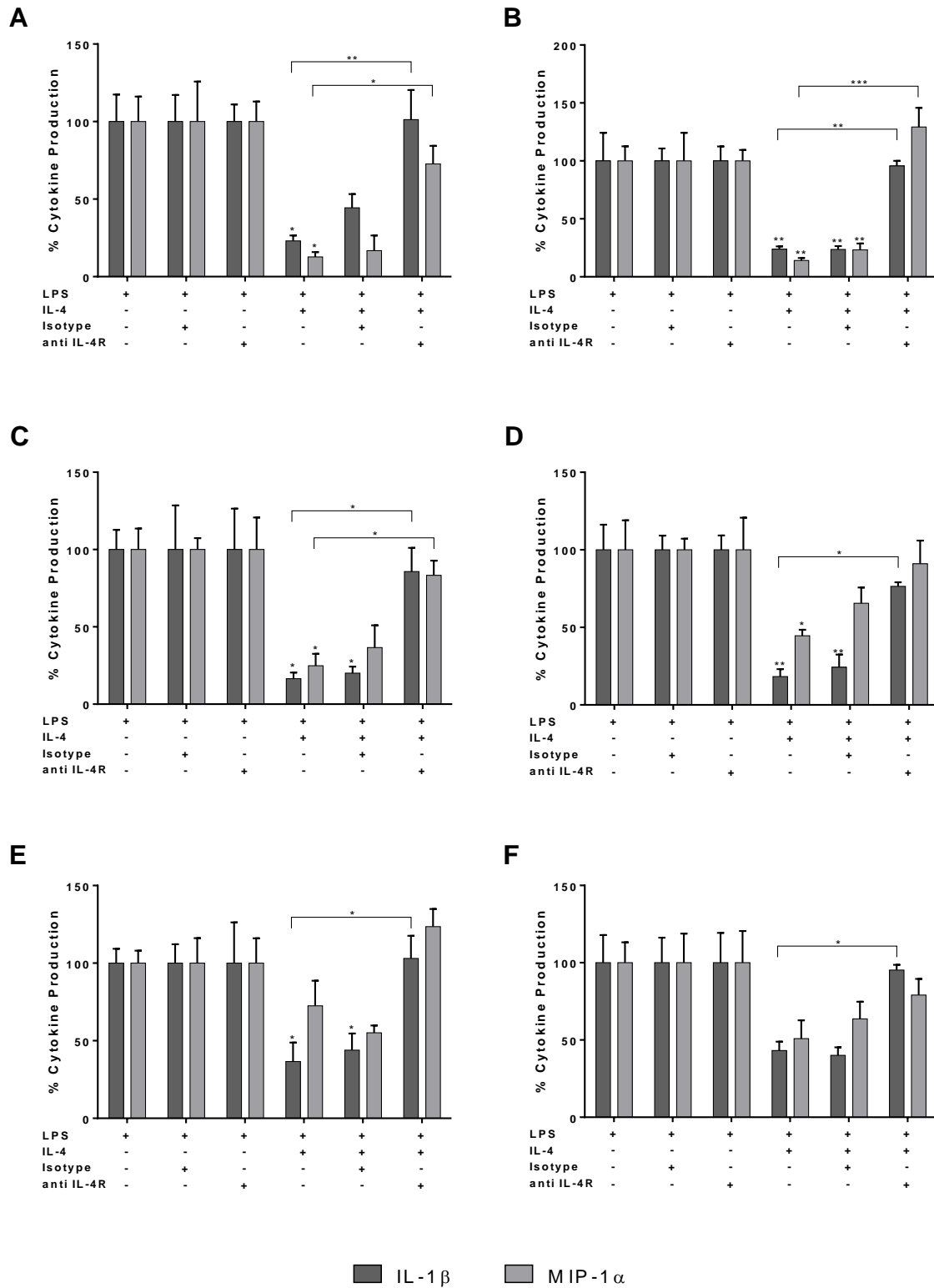


Figure 4

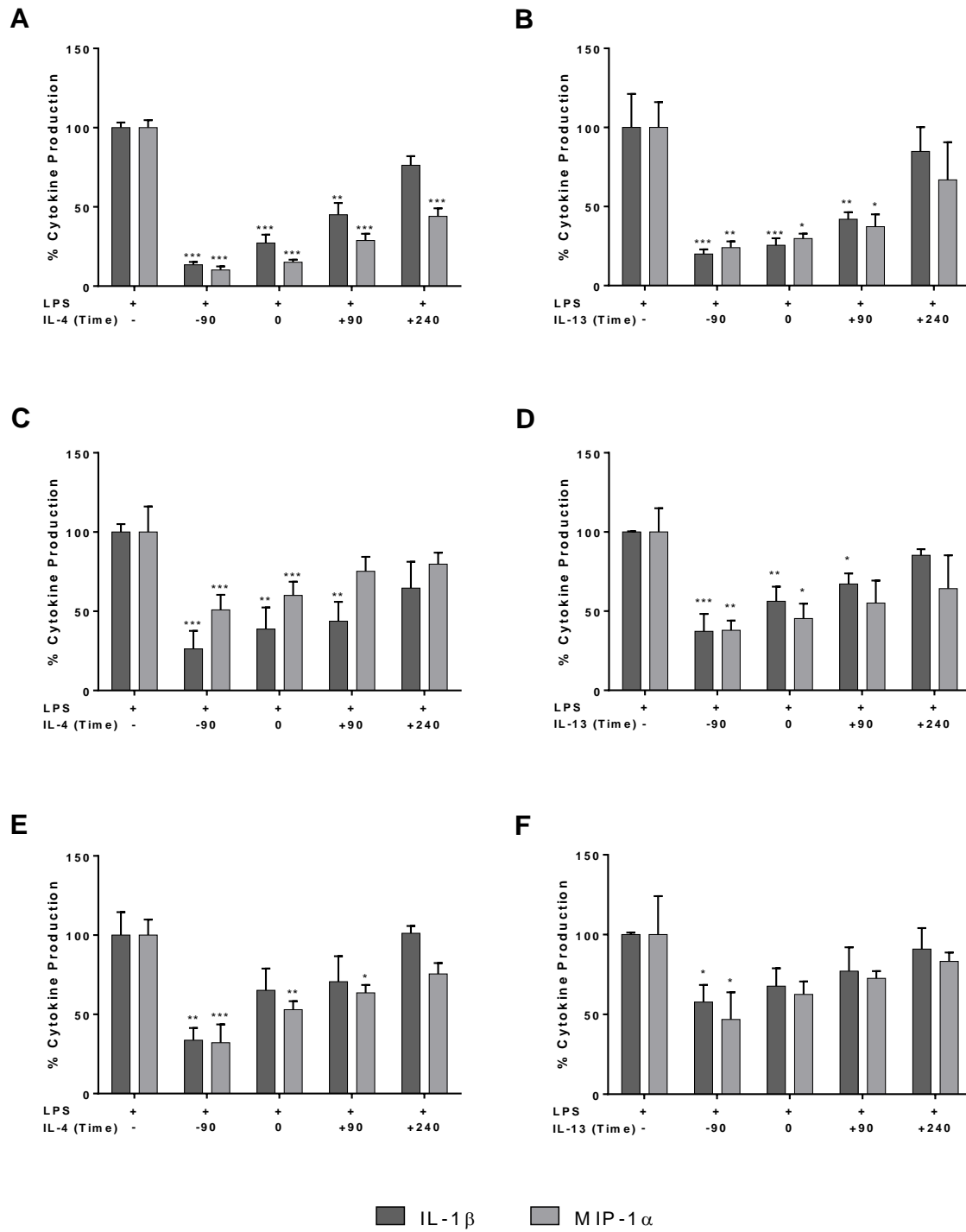


Figure 5

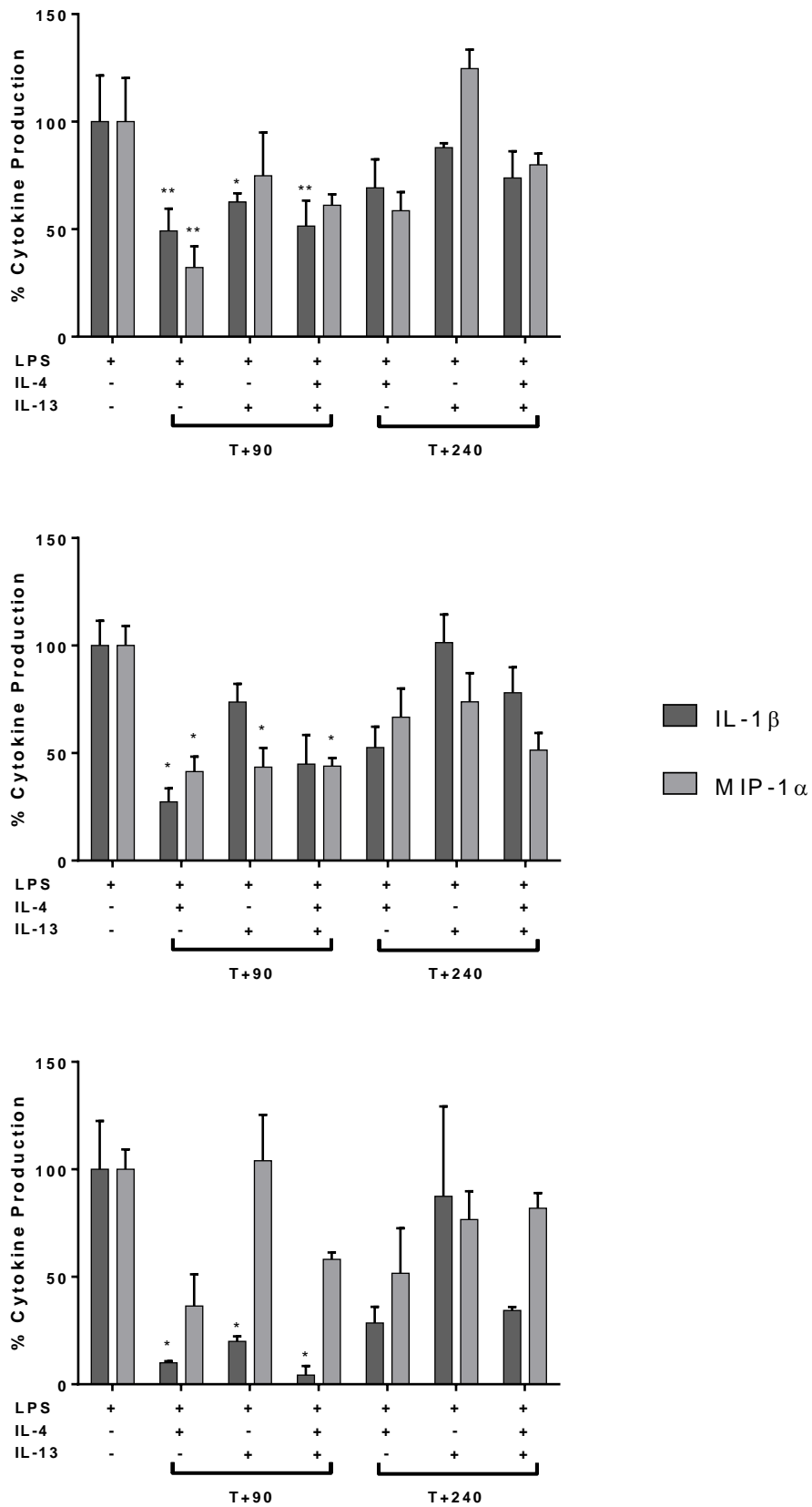




Figure 6

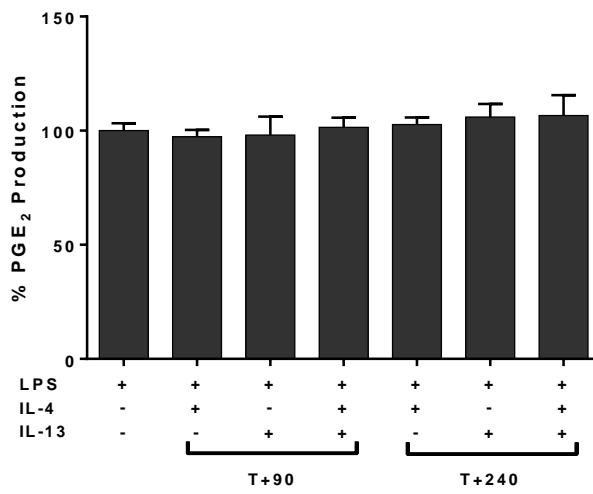
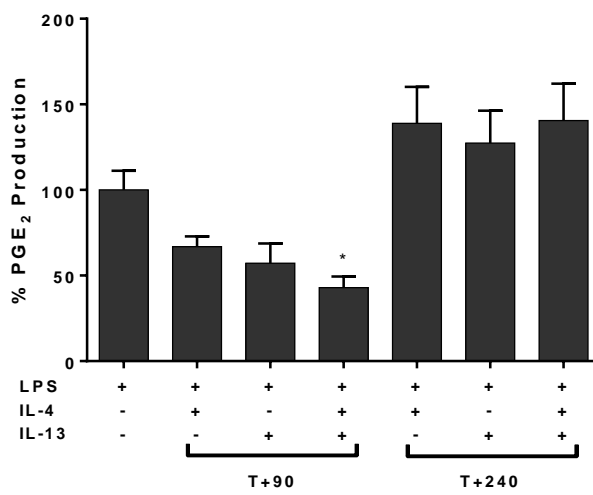
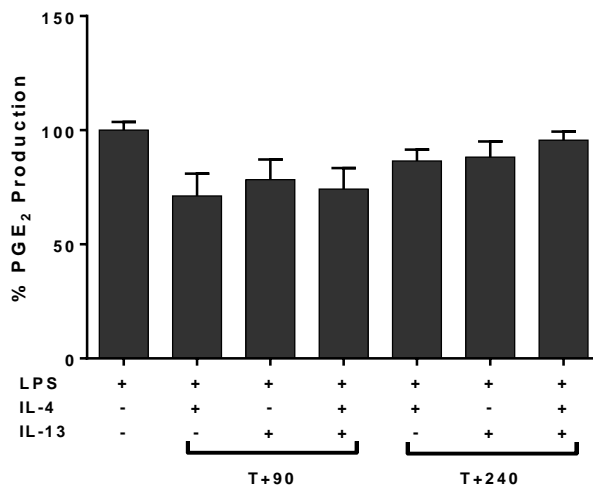
**A****B****C**

Figure 7

