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

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

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

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

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Grant Support: Funding for this research was provided by Health and Care Research Wales.

Abstract

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

Introduction

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

The resolution of inflammation is essential for homeostasis. One mechanism by which this is achieved is the production of various anti-inflammatory molecules in response to an increased pro-inflammatory environment. A number of cytokines including IL-4, IL-10 and IL-13 are well recognised for their anti-inflammatory activity. Investigations into the use of anti-inflammatory cytokines to prevent PTB have focused primarily on IL-10. Animal studies have shown that IL-10 decreases lipopolysaccharide (LPS) induced placental nitric oxide, TNFA and apoptosis [4], and can prevent LPS mediated PTB in rats [5]. Ex vivo studies in humans have shown IL-10 can down-regulate LPS- and lipoteichoic acid (LTA)-induced cytokine and chemokine responses by the healthy term placenta [6], and inhibit IL-1B and prostaglandin E_2 (PGE_2) production and cyclooxygenase-2 (COX-2) expression in intact term fetal membranes [7]. However, the impact of other anti-inflammatory cytokines in this clinical setting is limited.

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

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

Materials and Methods

Gestation associated tissues collection, preparation and stimulation

Samples

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

Explant Cultures

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

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

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

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

RNA Extraction from Gestation-Associated Tissue Samples

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

Polymerase Chain Reaction (PCR)

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

Cytokine and prostaglandin production

IL-1β, MIP-1α and PGE_2 in the tissue free supernatants of explant cultures collected after 24 h were measured using commercially available immunoassay kits (IL-1β and MIP-1α, DuoSet; PGE_2, Parameter; all R&D Systems) as per manufacturer's instructions.

Immunohistochemistry

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

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

Statistical analysis

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

Results

Pre-treatment with IL-4 or IL-13 inhibits LPS induced cytokine output of placenta, choriodecidua and amnion

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

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

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

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

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

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

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

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

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

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

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

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

In addition to many pro-inflammatory cytokines, prostaglandins have a key role in facilitating the physiological processes of labour. Therefore, the effect of IL-4 and IL-13 alone or in combination on PGE_2 levels was considered. IL-4 and IL-13 had no effect on PGE_2 levels from LPS-treated placental and amnion explant cultures when added 90 or 240 minutes after LPS exposure (Figure 6). In the choriodecidua, both IL-4 and IL-13 reduced the levels of LPS-induced PGE_2, with a combination of both cytokines resulting in a significant reduction

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

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

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

Discussion

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

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

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

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

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

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

While both IL-4 and IL-13 could down regulate LPS-induced pro-inflammatory cytokine production when tissues were pre-treated with these cytokines, in a clinical setting any intervention would likely occur after the inflammatory insult. To explore if IL-4 and IL-13 would be effective post insult as would occur clinically, the effect of IL-4 and IL-13 on gestation-associated tissues post-LPS exposure at arbitrary time points was determined. The anti-inflammatory effect of these cytokines was diminished with delayed treatment post-LPS exposure. When added following 240-minute exposure of the tissues to LPS the anti-inflammatory effect of both IL-4 and IL-13 was negligible. This might relate to the induction of SOCS1, a negative regulator of JAK/STAT signalling pathways by LPS in a TLR4 dependent manner [12,35]. While a standard concertation of IL-4 and IL-13 was utilised here, it is unknown if any dose dependent effects would have been observed with higher concentrations potentially yielding a greater anti-inflammatory effect post-exposure to LPS. Examining any potential dose-dependent effects might aid in our understanding of how these 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_2 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 $PGE_{2,}$ 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

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

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

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

Acknowledgments

We are grateful to all the staff in the antenatal day assessment unit and on the delivery suite at Singleton Hospital Swansea for their tireless help in the collection of samples. Thanks also to all the women who agreed to the use of their placentas for this study.

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Table Legends

Table 1. Specifications for PCR.

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

Figure Legends

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

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

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

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

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

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

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

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

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

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

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

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

Figure 6. Combined effect of IL-4 and IL-13 treatment on LPS-induced PGE_2 production by term non-laboured gestation-associated tissues.

PGE_2 from explants of (A) placenta (B) choriodecidua and (C) amnion in response to LPS (10 ng/ml) in the presence of IL-4, IL-13 or IL-4 + IL-13 (all 10 ng/ml) added 90 minutes (T+90) or 4 hours (T+240) after LPS (n=3). Data are shown as percentage of response to LPS alone; error bars represent SEM. Statistically significant differences compared to LPS treatment are shown: * $p \leq 0.05$.

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

Cytokine levels (IL-1B & MIP-1A) from explants of (A) placenta (B) choriodecidua and (C) amnion in response to LPS (10 ng/ml) in the presence of IL-4, IL-10 or IL-4 + IL-10 (all 10 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^{2+} Conc (nM)	Anneling Temp (°C)
IL-4RA	***F*** 5' GACCTGGAGCAACCCGTATC ***R*** 5' CATAGCACAACAGGCAGACG	2.5	70
IL-2RG	***F*** 5' ACGGGAACCCAGGAGACAGG ***R*** 5' AGCGGCTCCGAACACGAAAC	2	70
IL-13RA1	***F*** 5' GAGCTGACCAAAGTGAAGGA ***R*** 5' ATTGCACCTGCGACGATGACTG	3	69
IL-13RA2	***F*** 5' GGCATAGGTGATCTTCTTGA ***R*** 5' GCCAGAAACGATGCAAAGTTT	2	60
STAT6	***F*** 5' AGAGGGGTTGCCGAGGTGA ***R*** 5' TGTCCACCAGGCTTTCACAC	4	70
UBE2D2	***F*** 5' GATCACAGTGGTCTCCAGCA ***R*** 5' TCCATTCCCGAGCTATTCTG	3	65

Figure 1

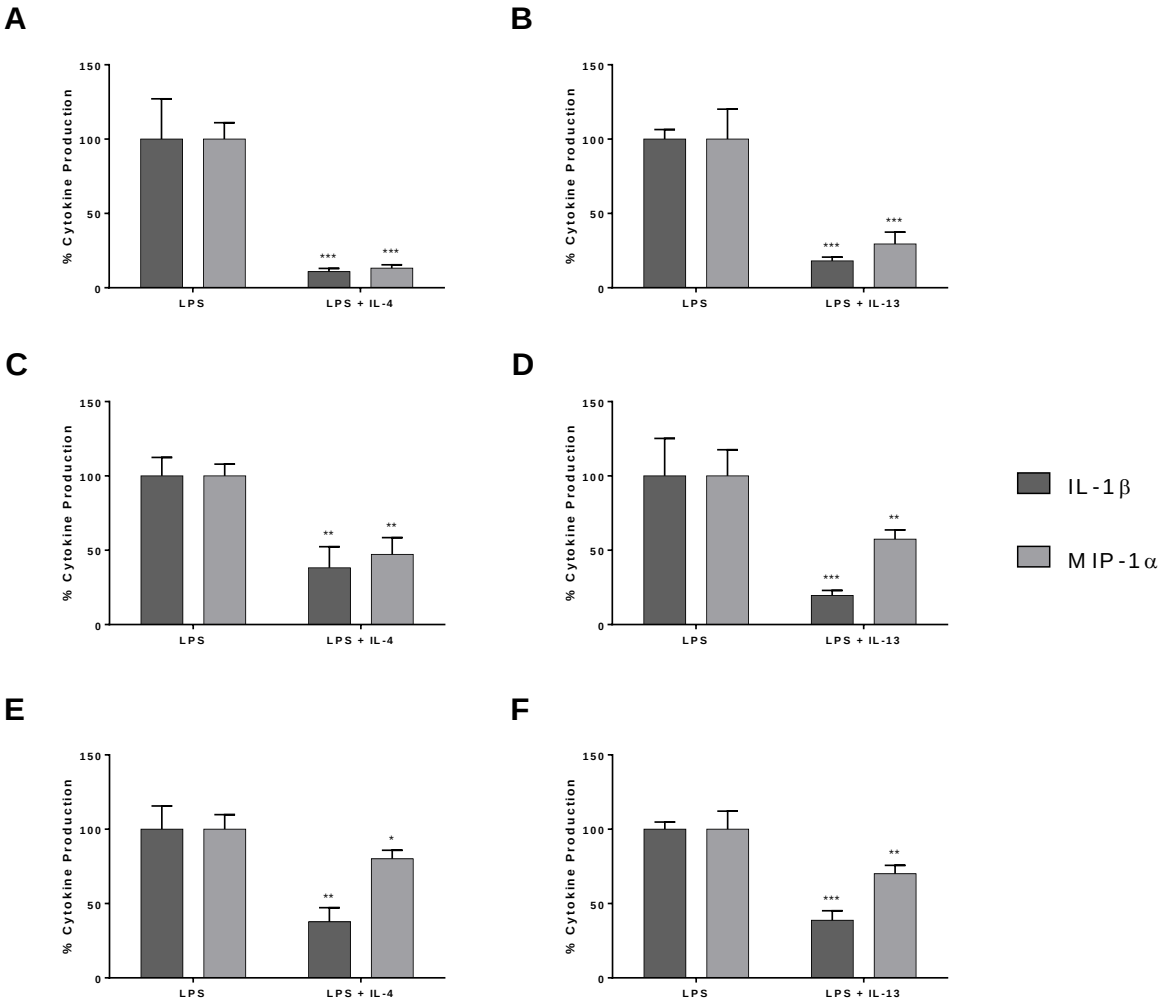

A
B
C
D
E
F
% Cytokine Production
150
100
50
0
LPS
LPS + IL-4
LPS + IL-13
***
**
*
IL-1β
MIP-1α

Figure 2A

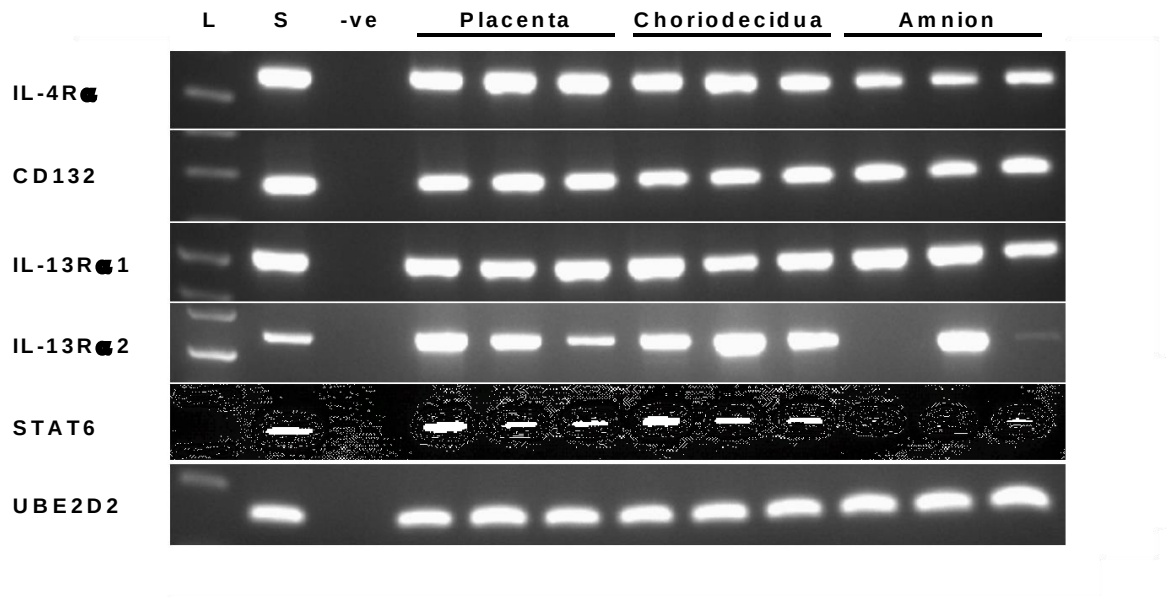

L
S
-ve
Placenta
Choriodecidua
Amnion
IL-4Rα
CD132
IL-13Rα1
IL-13Rα2
STAT6
UBE2D2

Figure 2B

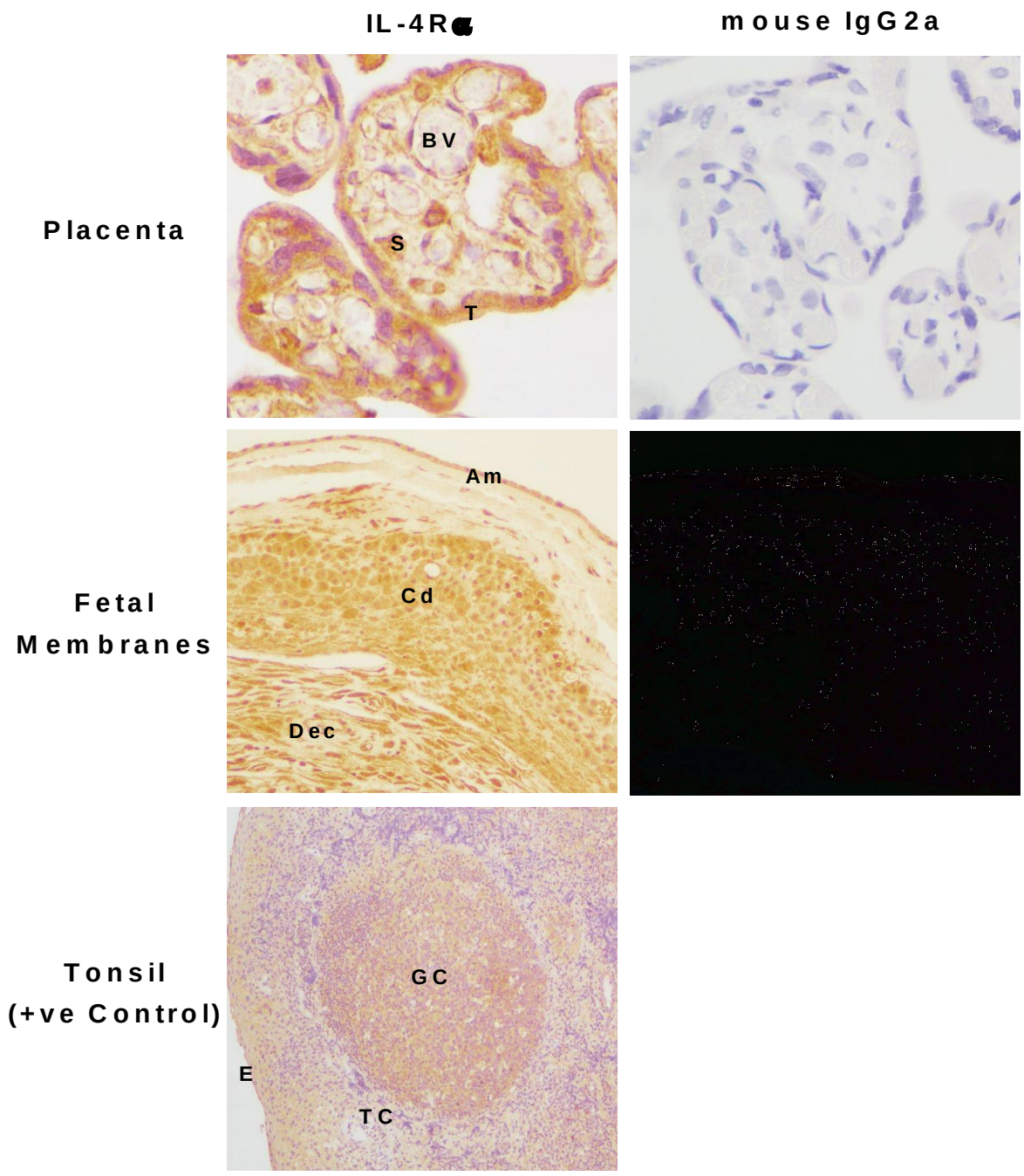

IL-4Rα
mouse IgG2a
Placenta
BV
S
T
Fetal Membranes
Am
Cd
Dec
Tonsil (+ve Control)
GC
E
TC

Figure 3

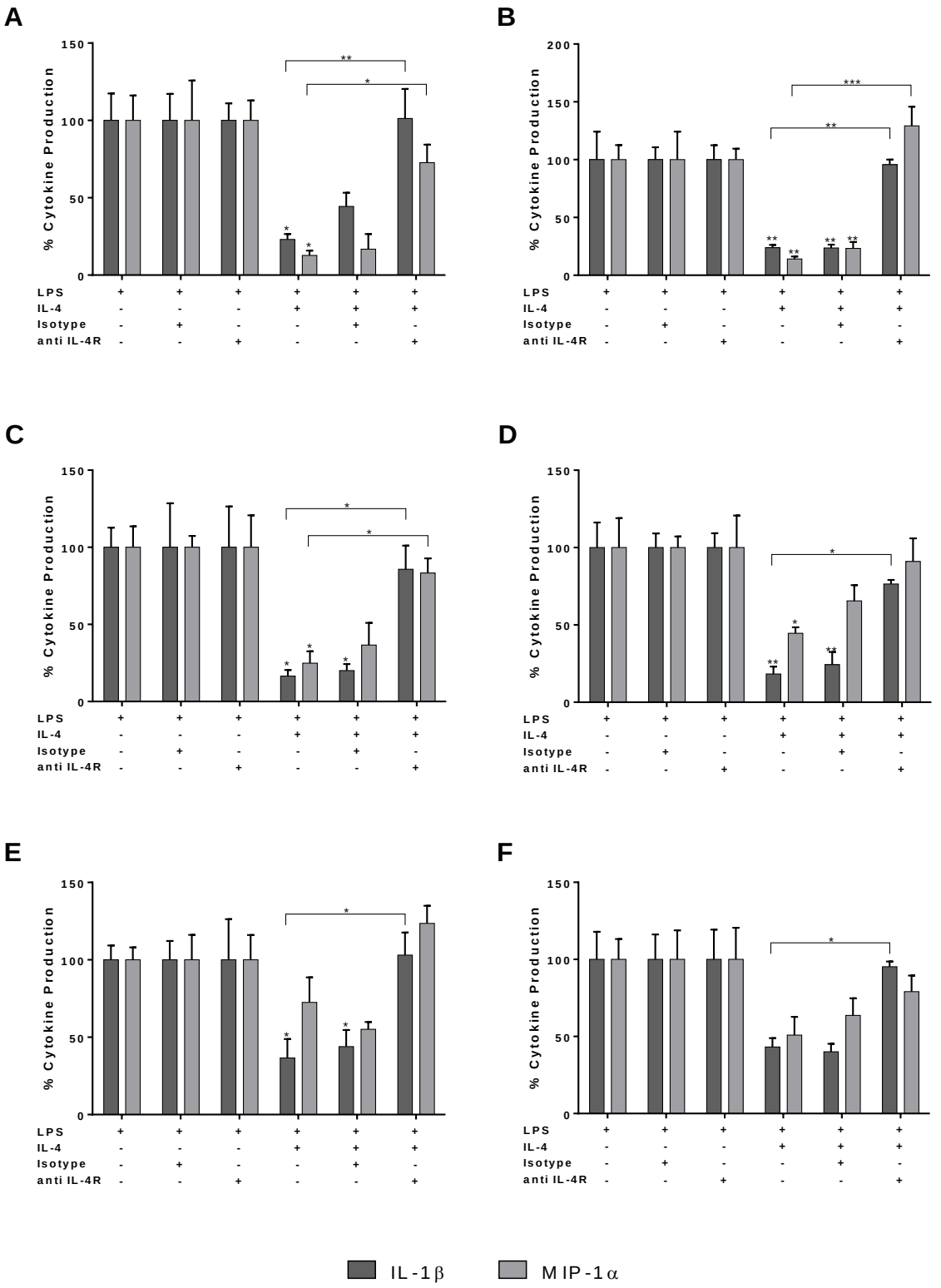

A
B
C
D
E
F
% Cytokine Production
LPS
IL-4
Isotype
anti IL-4R
IL-1β
MIP-1α

Figure 4

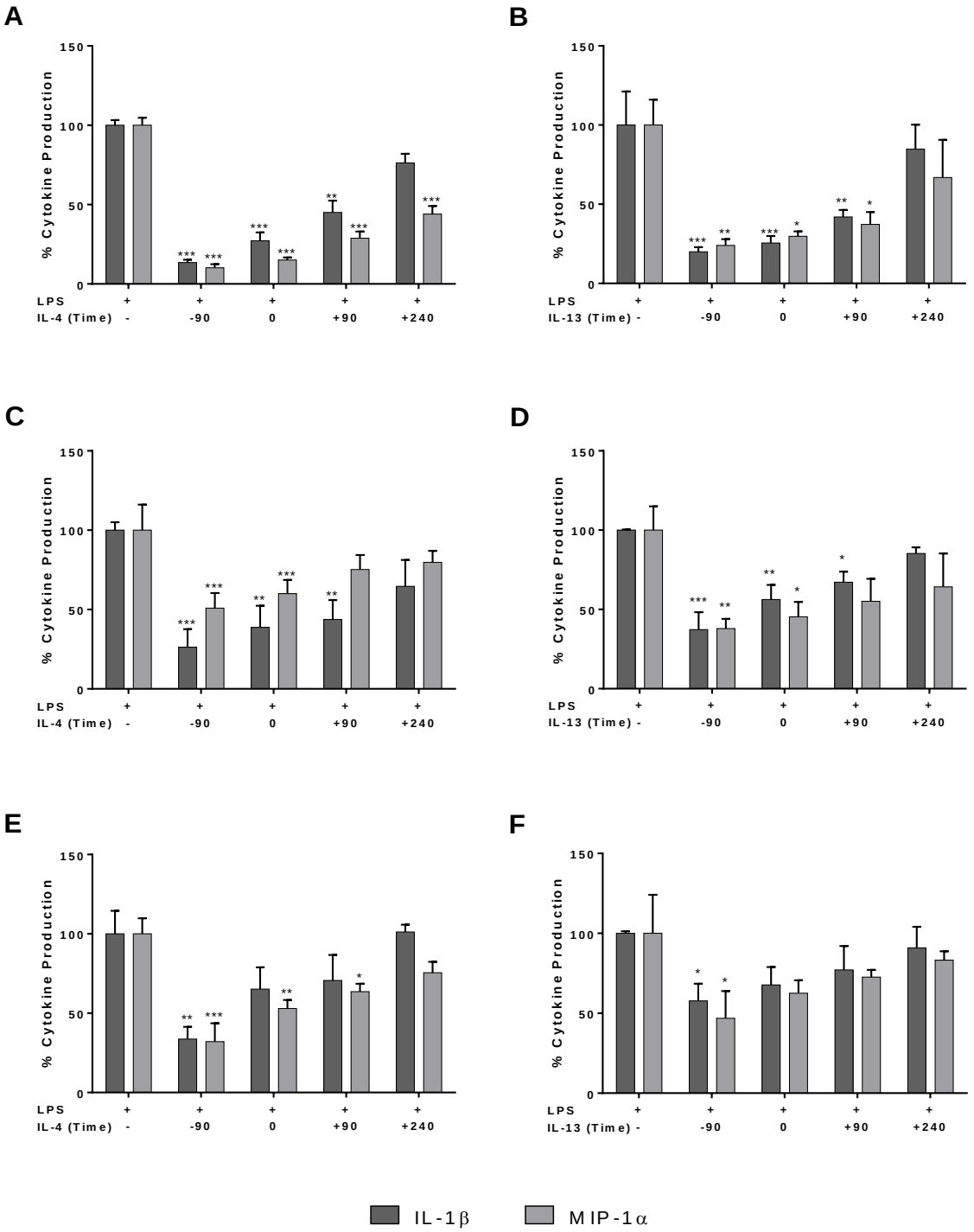

A
B
C
D
E
F
% Cytokine Production
150
100
50
0
LPS + + + + +
IL-4 (Time) - -90 0 +90 +240
IL-13 (Time) - -90 0 +90 +240
IL-1β
MIP-1α

Figure 5

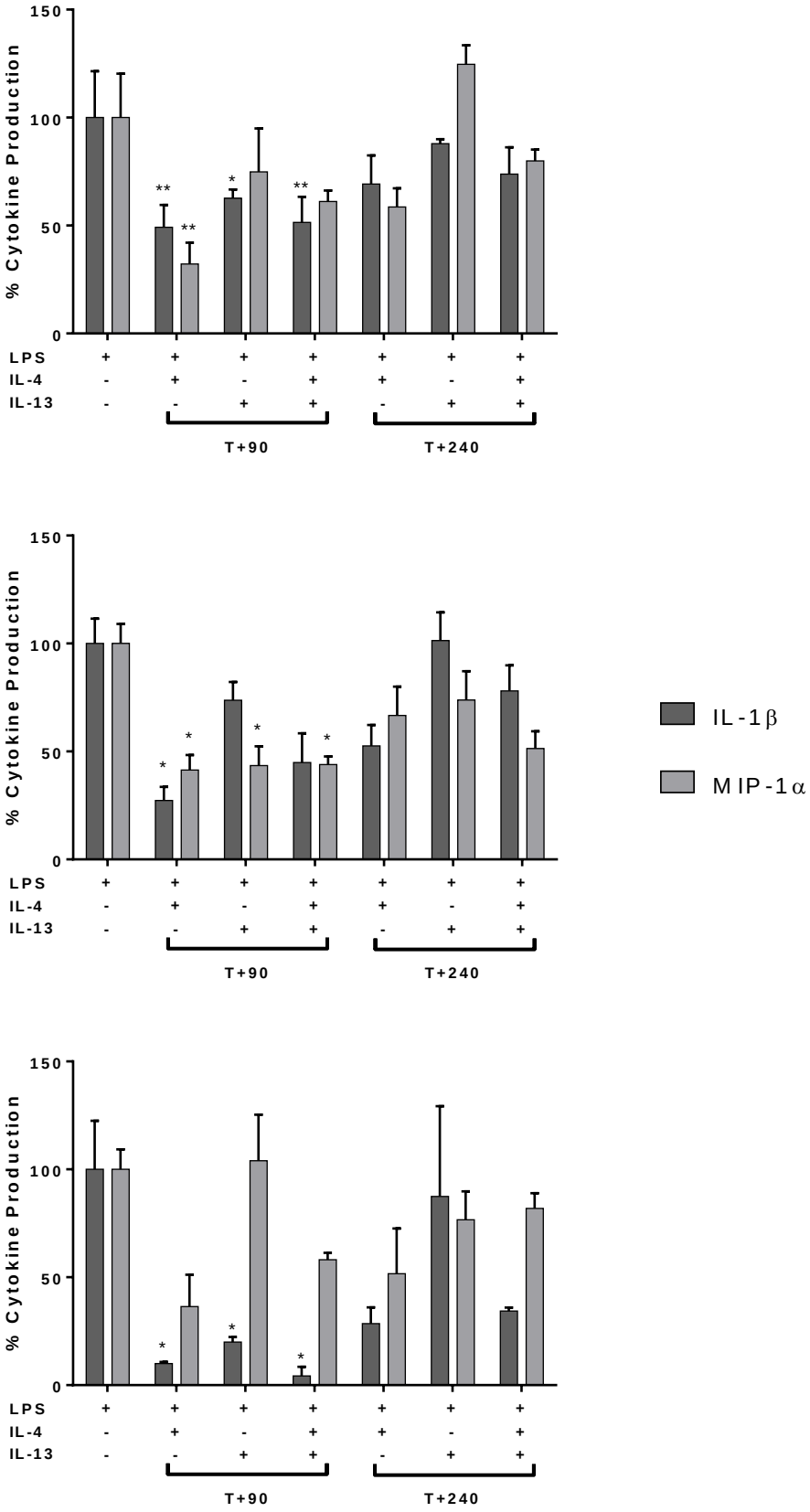

% Cytokine Production
150
100
50
0
LPS + + + + + + +
IL-4 - + - + + - +
IL-13 - - + + - + +
T+90
T+240
IL-1β
MIP-1α

Figure 6

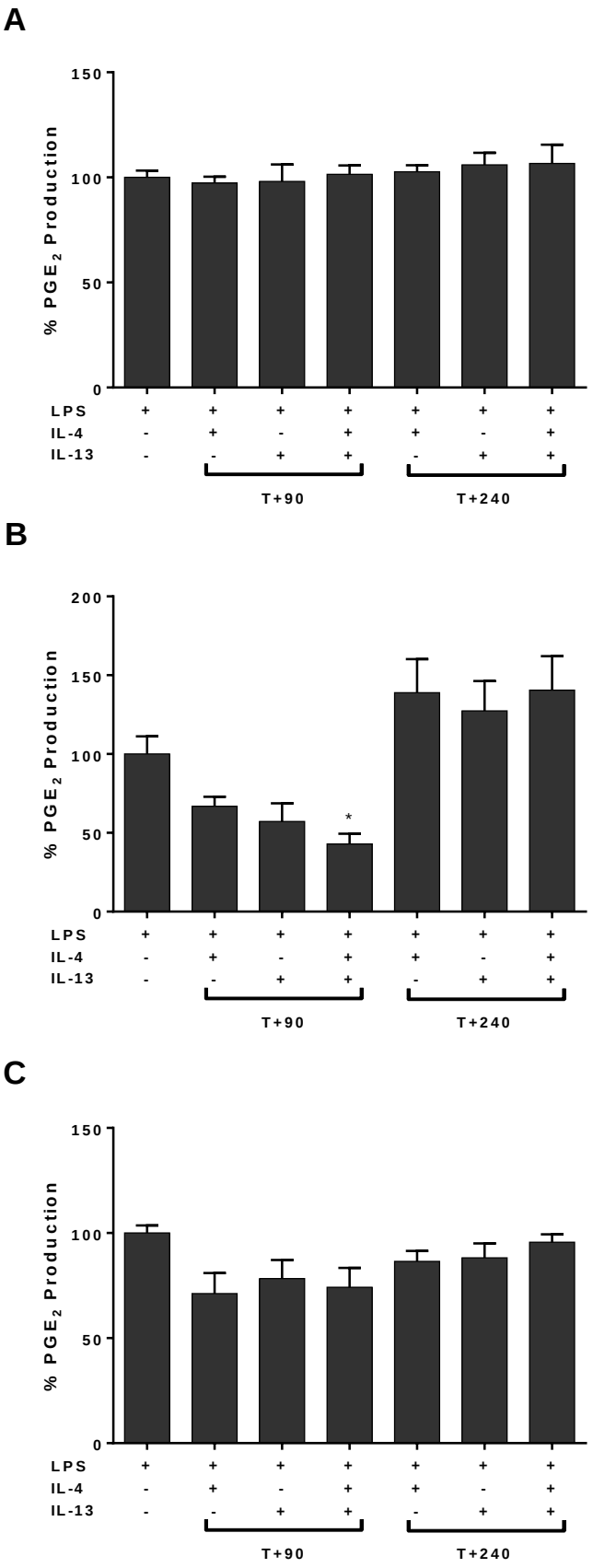
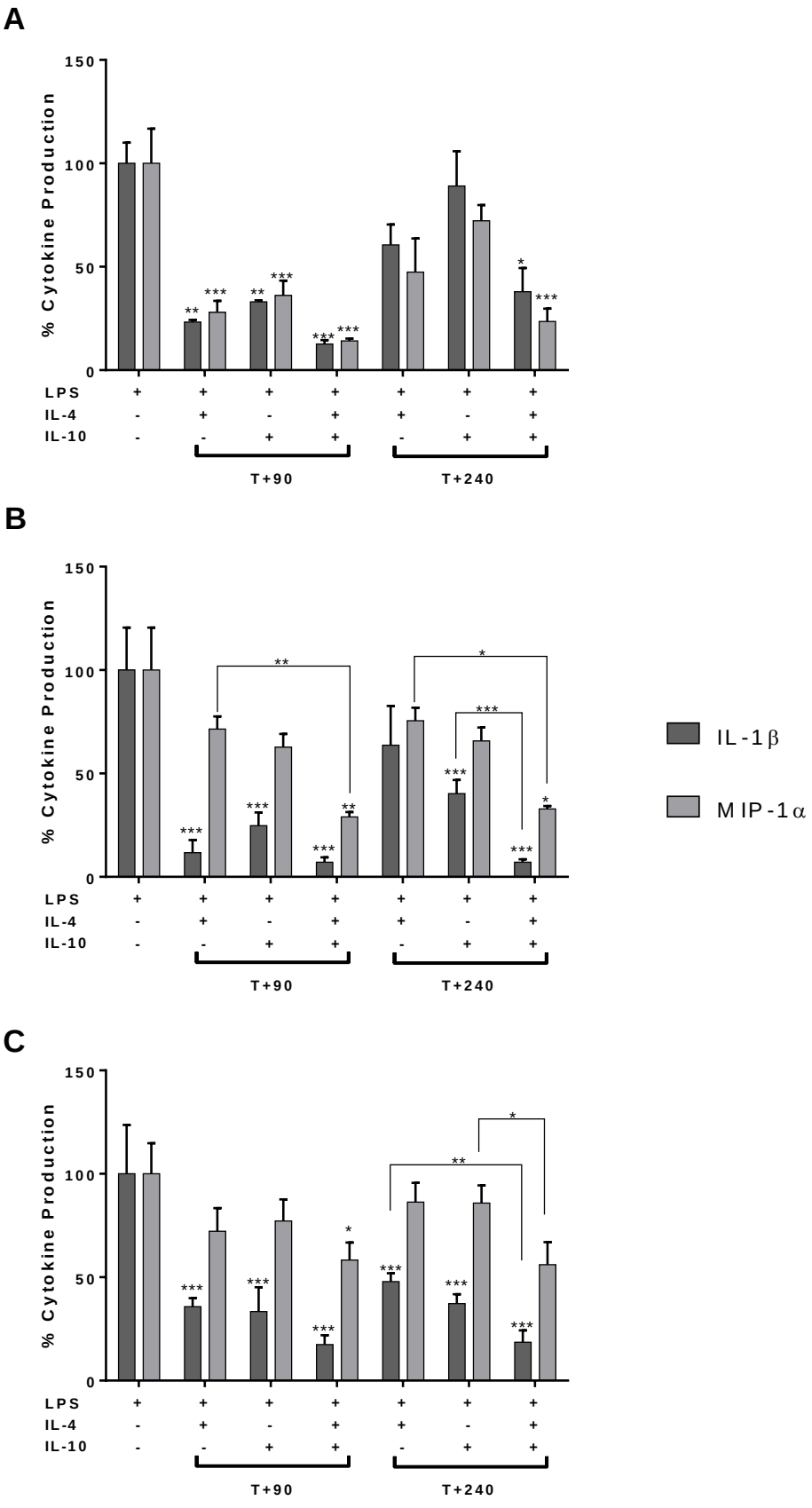

A
150
100
50
0
% PGE2 Production
LPS + + + + + + +
IL-4 - + - + + - +
IL-13 - - + + - + +
T+90
T+240
B
200
150
100
50
0
% PGE2 Production
*
LPS + + + + + + +
IL-4 - + - + + - +
IL-13 - - + + - + +
T+90
T+240
C
150
100
50
0
% PGE2 Production
LPS + + + + + + +
IL-4 - + - + + - +
IL-13 - - + + - + +
T+90
T+240

Figure 7


A
B
C
% Cytokine Production
150
100
50
0
LPS
IL-4
IL-10
T+90
T+240
IL-1β
MIP-1α