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# **The anti-inflammatory effects of prostaglandin E<sub>2</sub> on human lung macrophages are mediated by the EP<sub>4</sub> receptor**

Sharonjit K Gill, Yiwen Yao, Linda J Kay, Martin A Bewley, Helen M Marriott and Peter T Peachell

Academic Unit of Respiratory Medicine, University of Sheffield, The Medical School (Floor L), Beech Hill Road, Sheffield, S10 2RX, UK

**Author for correspondence:** Peter Peachell

**Email:** [p.t.peachell@shef.ac.uk](mailto:p.t.peachell@shef.ac.uk)

**Telephone:** +44-114-271-2063

**Fax:** +44-114-271-0275

**Running Title:** EP<sub>4</sub> receptor inhibition of macrophages

## Abstract

**Background and purpose:** Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) has been shown to inhibit cytokine generation from human lung macrophages. However, the EP receptor that mediates this beneficial anti-inflammatory effect of PGE<sub>2</sub> has not been elucidated definitively. The aim of this study was to identify the EP receptor by which PGE<sub>2</sub> inhibits cytokine generation from human lung macrophages. This was determined by using recently-developed EP receptor ligands.

**Experimental approach:** The effects of PGE<sub>2</sub> and EP-selective agonists on lipopolysaccharide (LPS) induced tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) and interleukin-6 (IL-6) generation from macrophages were evaluated. The effects of EP<sub>2</sub>-selective (PF-04852946, PF-04418948) and EP<sub>4</sub>-selective (L-161,982, CJ-042794) antagonists on PGE<sub>2</sub> responses were studied. The expression of EP receptor subtypes by human lung macrophages was determined by RT-PCR.

**Key results:** PGE<sub>2</sub> inhibited LPS-induced and Streptococcus pneumoniae-induced cytokine generation from human lung macrophages. Analysis of mRNA levels indicated that macrophages expressed EP<sub>2</sub> and EP<sub>4</sub> receptors. L-902,688 (EP<sub>4</sub>-selective agonist) was considerably more potent than butaprost (EP<sub>2</sub>-selective agonist) as an inhibitor of TNF $\alpha$  generation from macrophages. EP<sub>2</sub>-selective antagonists had marginal effects on the PGE<sub>2</sub> inhibition of TNF $\alpha$  generation whereas EP<sub>4</sub>-selective antagonists caused rightward shifts in the PGE<sub>2</sub> concentration-response curves.

**Conclusions and implications:** These studies demonstrate that the EP<sub>4</sub> receptor is the principal receptor that mediates the anti-inflammatory effects of PGE<sub>2</sub> on human lung macrophages. This suggests that EP<sub>4</sub> agonists could be effective anti-inflammatory agents in human lung disease.

## Abbreviations

COPD, chronic obstructive pulmonary disease

**FCS, foetal calf serum**

IL-6, interleukin-6

LPS, lipopolysaccharide

PBS, phosphate buffered saline

**PDE, phosphodiesterase**

PGE<sub>2</sub>, prostaglandin E<sub>2</sub>;

RT-PCR, reverse transcription-polymerase chain reaction

TNF $\alpha$ , tumour necrosis factor- $\alpha$

## Tables of Links

TARGETS	LIGANDS
GPCRs	PGE <sub>2</sub>
EP <sub>2</sub>	misoprostol
EP <sub>4</sub>	butaprost
	L-902,688
	ONO-AE1-259
	PF-04418948
	PF-04852946
	CJ 042794
	L-161,982

These tables list key protein targets and ligands in this article that are hyperlinked to corresponding entries in [www.guidetopharmacology.org](http://www.guidetopharmacology.org) the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

## Introduction

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is known to have wide-ranging effects on a variety of tissues. These effects of PGE<sub>2</sub> are mediated through specific EP receptors of which four have been identified (Coleman et al., 1994; Breyer et al., 2001; Woodward et al., 2011). In the lung, PGE<sub>2</sub> can act on airway smooth muscle to mediate bronchodilation. This had led to suggestions that targeting EP receptors may be of benefit in the treatment of respiratory diseases (Kawakami et al., 1973; Melillo et al., 1994; Gauvreau et al., 1999). An undesirable effect of PGE<sub>2</sub>, however, is that it also induces cough (Maher et al., 2011). Nonetheless, cough and bronchodilation appear to be mediated by different receptors suggesting that selective targeting of the beneficial receptor might be possible. The EP<sub>3</sub> receptor has been linked to cough (Maher et al., 2011) whereas **bronchodilation appears to be mediated by EP<sub>4</sub> receptors** (Buckley et al., 2011; Benyahia et al., 2012). Identification of the relevant EP receptor that mediates the beneficial effects of PGE<sub>2</sub> is likely to be valuable information from a clinical perspective.

The human lung macrophage plays an important role in host defence in the lung. However, aberrant activation of lung macrophages has been linked to respiratory diseases, chronic obstructive pulmonary disease (COPD) in particular (Barnes, 2008). PGE<sub>2</sub> has been shown to inhibit pro-inflammatory cytokine release from lung macrophages (Rowe et al., 1997; Ratcliffe et al., 2007; Buenestado et al., 2012). This effect of PGE<sub>2</sub> on human lung macrophages has been reported to be mediated by EP<sub>2</sub> and EP<sub>4</sub> receptors (Ratcliffe et al., 2007). However, this conclusion was drawn at a time when the availability of selective pharmacological ligands at EP<sub>2</sub> and EP<sub>4</sub> receptors was limited. The situation has now changed with the recent emergence of novel ligands such as PF-04418948, the first potent and selective EP<sub>2</sub> receptor antagonist reported (af Forselles et al., 2011). Use of these novel experimental tools has provided an opportunity to reappraise the mechanism by which PGE<sub>2</sub> stabilizes macrophage responses. In this regard, use of these tools has shown that the EP<sub>4</sub> receptor is the main receptor regulating functional responses in THP-1 cells, a human monocytic cell line (Birrell et al., 2015).

The aim of the present study was to identify the EP receptor responsible for mediating the inhibitory effects of PGE<sub>2</sub> on pro-inflammatory cytokine release from human lung macrophages. This was determined by using a variety of pharmacological ligands, principally, a range of EP<sub>2</sub>-selective and EP<sub>4</sub>-selective antagonists. These studies demonstrate that the EP<sub>4</sub> receptor is the principal receptor that mediates the anti-inflammatory effects of PGE<sub>2</sub> on human lung macrophages suggesting that EP<sub>4</sub> agonists could be effective anti-inflammatory agents in human lung disease.

## Methods

### Buffers

Phosphate buffered saline (PBS) contained (mM): NaCl 137; Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O 8; KCl 2.7; KH<sub>2</sub>PO<sub>4</sub> 1.5. PIPES buffer contained (mM): PIPES (22), NaCl (110), KCl (5) and the pH was titrated to 7.4 with NaOH.

### Preparation of compounds

Stock solutions (10 mM) of PGE<sub>2</sub>, butaprost (free acid), L-902,688, misoprostol (free acid) and indomethacin were prepared in ethanol and stored at –20 °C. ONO-AE1-259 was made up in distilled water (10 mM stock) and stored at –20 °C. All antagonists, PF-04852946, PF-04418948, CJ-042794 and L-161,982, formerly known as EP<sub>4</sub>A (Machwate et al., 2001), were prepared as stock solutions (10 mM) in dimethyl sulphoxide and stored at –20 °C. **Salbutamol was prepared as a stock solution (10 mM) dissolved in distilled water and stored at 4 °C.** Roflumilast was prepared as a stock solution (10 mM) in dimethyl sulphoxide and stored at –20 °C. LPS from *E. coli* serotype R515 (Re) was provided as a 1 mg mL<sup>-1</sup> stock solution and stored at 4 °C.

### Preparation of *Streptococcus pneumoniae*

Type 2 *S. pneumoniae* (Spn) strain D39 was grown and stored as previously described (Dockrell et al., 2001). Bacteria were opsonized by resuspending pellets in RPMI-1640 with 10% anti-pneumococcal immune serum and incubating at 37 °C for 30 min on a rotating stand. Pellets were then washed three times in PBS and resuspended in RPMI-1640 supplemented with 10% FCS (foetal calf serum) without antibiotics.

### Lung tissue

Non-lesional lung tissue was obtained from surgical resections. Most patients were undergoing surgery for carcinoma. **Sixty-two preparations were used in this study and these were derived from 31 males and 31 females.** Ages of participants ranged from 49 to 88 years with a median age of 71. The use of lung tissue in this study was approved by the National Research Ethics' Service (REC reference: 15/NW/0657). Informed written consent was obtained.

### Macrophage isolation

Lung tissue was chopped with scissors in RPMI-1640 and the tissue filtered over 100

µm nylon mesh (Incamesh, Warrington, UK) over a collection vessel. This cycle of chopping and washing was repeated. The filtrate (100-200 mL) was centrifuged (300 g, 10 min) at room temperature, the supernatant aspirated and the pellets resuspended in 40-50 mL of RPMI-1640 supplemented with 10% FCS, penicillin (25 U mL<sup>-1</sup>), streptomycin (25 µg mL<sup>-1</sup>), gentamicin (50 µg mL<sup>-1</sup>) and amphotericin B (1 µg mL<sup>-1</sup>). The cell suspensions were inverted several times and left to sediment at 4 °C for 1 h according to a protocol modified from Liu et al (1984). After sedimentation, the supernatant was aspirated and the sedimented material was resuspended in supplemented RPMI-1640. This sedimentation step at 4 °C was repeated. The sedimented material was resuspended in 30 mL PIPES buffer and centrifuged (300 g, 10 min, room temperature). The resulting pellet was resuspended in PIPES buffer and the suspension was filtered through nylon mesh before being layered on to a discontinuous Percoll gradient.

One 20 mL Percoll gradient was used for cells harvested from every 5 g of lung tissue. Isotonic Percoll (9 parts Percoll to 1 part 10x PIPES buffer) was diluted with PIPES buffer to produce an 80% Percoll gradient. The cell suspension (20 mL) was layered onto the gradient and centrifuged (400 g, 20 min, room temperature) resulting in a flocculent layer containing macrophages. The interface was harvested and two washes were performed with PIPES buffer (50 mL). Following centrifugation, (488 g, 10 min at room temperature) the resulting cell pellet was resuspended in 10 mL of supplemented RPMI-1640 (or for infection experiments, supplemented RPMI-1640 without antibiotics). The cells were counted using a haemocytometer. Macrophages were seeded at  $2 \times 10^5$  per well in a 24-well cell culture plate with 1 mL of supplemented RPMI-1640 (or for infection experiments, supplemented RPMI-1640 without antibiotics) and incubated overnight (37 °C, 5% CO<sub>2</sub>).

The purity of cell suspensions was determined by morphology using cytopins (Thermo Shandon Cytospin 3). Cytopins were stained with Quick-Diff and processed according to the manufacturer's instructions. Cell viability was assessed by erythrosin-B exclusion. In this study, macrophage purity was 85±2% and cell viability was 92±1%.

Macrophage activation protocol

After incubation overnight, medium from the wells was removed and replaced with fresh supplemented RPMI-1640 (1 mL) 2 h before the start of the experiment. Where pharmacological agents were used the cells were pre-treated with these (30 to 60 min at 37 °C, 5% CO<sub>2</sub>) before addition of stimulus. When agonists were used, macrophages were first incubated with or without indomethacin for 30 min and then with or without agonist for a further 30 min before addition of LPS. When antagonists were used, cells were incubated first with indomethacin (30 min), then with antagonist (1 h) followed by agonist (30 min) before activation. The cells were incubated (37 °C, 5% CO<sub>2</sub>) for 22 h with the stimulus. The cell culture supernatants were then harvested and centrifuged (488 g, 4 min, room temperature). The resulting supernatants were stored at -80 °C until analysis for cytokine content. TNF $\alpha$  and IL-6 were analysed using commercially-available ELISA kits (RSG kits, eBioscience, Hatfield, UK). PGE<sub>2</sub> was also analysed using a commercially-available kit (Cayman Chemical Company, Ann Arbor, MI, USA).

#### Macrophage infection protocol

After incubation overnight, medium from the wells was removed and replaced with fresh supplemented RPMI-1640 without antibiotics (1 mL) 2 h before the start of the experiment. Opsonized Type 2 Spn strain D39 were added to the cells at a multiplicity of infection (MOI) of 1 or mock infected. The cells were incubated at 4 °C for 1 h to maximize bacterial adherence followed by incubation at 37 °C for 3 h for internalization. The wells were then washed with PBS and the cell culture medium replaced with the re-addition of pharmacological agents as appropriate. The cells were incubated at 37 °C until 22 h post-infection. The cell culture supernatants were then harvested and stored at -80 °C until analysis for cytokine content.

#### Assessment of total cell cyclic-AMP

Macrophages ( $2 \times 10^5$  cells) were incubated (30 min) with or without indomethacin (1  $\mu$ M) and then with PGE<sub>2</sub> (0.5 to 5 h) in supplemented RPMI-1640 (1 mL). After incubation, the supernatants were removed and the cells solubilised by addition of ice-cold acidified ethanol and snap frozen in liquid nitrogen. After thawing, the ethanol was recovered and centrifuged (13,000 g, 2 min) to pellet any cellular debris. The supernatant was then evaporated off under vacuum using a rotary evaporator. The dried residue was reconstituted in assay buffer (250  $\mu$ L) and stored at -80 °C. Total

cell cyclic-AMP content was determined using a commercially-available kit (Cayman Chemical Company, Ann Arbor, MI, USA).

#### RT-PCR

RNA was extracted from purified macrophages (1 to  $5 \times 10^6$  cells) using Tri-Reagent (1 mL). In order to generate cDNA, samples were processed essentially as described elsewhere (Kay et al., 2013). Amplification of cDNA was performed by PCR using conditions and primer pairs for human EP receptor subtypes (Schlötzer-Schrehardt et al., 2002; Thorat et al., 2008). The house-keeping gene,  $\beta$ -actin, was also amplified. Primers were synthesised by Sigma (Poole, UK). PCR products were sequenced in-house to ensure that correct amplification had taken place as described in more detail elsewhere (Kay et al., 2013).

#### Materials

The following were purchased: indomethacin, PGE<sub>2</sub>, Percoll, salbutamol, Tri-Reagent (all Sigma, Poole, UK); gentamicin, penicillin/streptomycin, fungizone, RPMI 1640, (Invitrogen, Paisley, UK); butaprost, misoprostol, L-902,688 (Cayman Chemical Company, Ann Arbor, MI, USA); L-161,982 (Tocris Bioscience, Bristol, UK); roflumilast (Santa Cruz Biotechnology, Heidelberg, Germany); Quick-Diff (Reagen, Toivala, Finland); FCS (Promocell, Heidelberg, Germany); LPS (Enzo Life Sciences, Exeter, UK).

PF-04418948, PF-04852946 and CJ-042794 were obtained from Pfizer Global Research & Development (Sandwich, UK). PF-04418948 will be available commercially from Sigma-Aldrich, Tocris and Toronto Research Chemicals Inc (North York, ON, Canada). ONO-AE1-259 was a kind gift from Ono Pharmaceutical Company Ltd (Osaka, Japan).

#### Data analysis

Antagonist affinity ( $pK_B$ ) was determined by using the Gaddum equation:  $pK_B = \log(\text{dose ratio} - 1) - \log(\text{antagonist concentration})$  (Kenakin, 1984). Maximal responses ( $E_{\text{max}}$ ) and potencies ( $EC_{50}$ ) were determined by non-linear regression analysis (GraphPad Prism, version 5.0d, La Jolla, CA, USA). Statistical significance was performed **utilizing Student's paired t-tests or repeated measures ANOVA as**

appropriate. When analyzing data by ANOVA, post hoc tests were either Dunnett's test or Tukey's test. Comparisons were considered significant when  $P < 0.05$ . The data and statistical analyses described in this paper conform to guidelines provided by the journal (Curtis et al., 2015)

## Results

### PGE<sub>2</sub> inhibits cytokine generation from macrophages

In keeping with previous studies, PGE<sub>2</sub> was found to inhibit LPS-induced TNF $\alpha$  generation from human lung macrophages in a concentration-dependent manner. This experiment was carried out in the absence (Figure 1A) and presence (Figure 1B) of the cyclo-oxygenase (COX) inhibitor indomethacin (1  $\mu$ M). PGE<sub>2</sub> was a more potent (EC<sub>50</sub>; 3.2  $\pm$  0.6 cf 10.8  $\pm$  2.0 nM) and efficacious (E<sub>max</sub>; 77  $\pm$  1.8 cf 53.5  $\pm$  2.0 % inhibition) inhibitor of LPS-induced TNF $\alpha$  generation in the presence of indomethacin (Figure 1C). Moreover, in the presence of indomethacin (1  $\mu$ M), TNF $\alpha$  generation by LPS was significantly ( $P < 0.05$ ) higher than in its absence (2657  $\pm$  496 cf 1648  $\pm$  213 pg mL<sup>-1</sup>; n=13).

These experiments suggested that macrophages produce PGE<sub>2</sub> in response to LPS which acts in a paracrine fashion to limit TNF $\alpha$  generation. Further experiments confirmed that macrophages generate a small amount of PGE<sub>2</sub> spontaneously and larger quantities following challenge with LPS (data not shown). In order to eliminate the potentially confounding influence of endogenous PGE<sub>2</sub> generation in the context of receptor characterizations, in all subsequent functional studies, indomethacin was also included.

In further studies, the effects of PGE<sub>2</sub> on LPS-induced IL-6 as well as TNF $\alpha$  generation were determined (Figure 1D). PGE<sub>2</sub> inhibited TNF $\alpha$  and IL-6 generation with similar potency (EC<sub>50</sub>; ~1.6 nM) but PGE<sub>2</sub> was less efficacious as an inhibitor of IL-6 generation than TNF $\alpha$ .

### Macrophages express EP<sub>2</sub> and EP<sub>4</sub> receptors

Expression of EP receptors by human lung macrophages was determined by RT-PCR. The data indicate that human lung macrophages express message for EP<sub>2</sub> and EP<sub>4</sub> receptors but do not express message for EP<sub>1</sub> or EP<sub>3</sub> receptors (Figure 2).

### PGE<sub>2</sub> increases macrophage cyclic-AMP levels

Since EP<sub>2</sub> and EP<sub>4</sub> receptors are G-protein receptors coupled to adenylyl cyclase, we investigated whether exposure (30 min) of macrophages to PGE<sub>2</sub> (1  $\mu$ M) induced increases in total cell cyclic-AMP. Our data demonstrated that PGE<sub>2</sub> induced statistically significant ( $P < 0.05$ ) increases in total cell cyclic-AMP levels over basal

(Figure 3). Further studies demonstrated that PGE<sub>2</sub> maintained these increased cyclic-AMP levels in macrophages for up to 5 hours (data not shown).

EP<sub>4</sub> agonists are far more potent inhibitors than EP<sub>2</sub> agonists

The effects of alternative EP agonists on macrophage function were explored. The effects of misoprostol (non-selective), butaprost (EP<sub>2</sub>-selective) and L-902,688 (EP<sub>4</sub>-selective) on LPS-induced TNF $\alpha$  generation from macrophages were investigated. The data show that misoprostol (Figure 4A) was about 26-fold less potent than PGE<sub>2</sub> as an inhibitor of TNF $\alpha$  generation (Table 1). The EP<sub>4</sub> agonist, L-902,688 (Figure 4B), was 7-fold more potent than PGE<sub>2</sub> as an inhibitor of TNF $\alpha$  generation whereas, by contrast, the EP<sub>2</sub>-selective agonist, butaprost (Figure 4C), was over 400-fold less potent than PGE<sub>2</sub> in this system (Table 2). In further studies, the effects of an alternative EP<sub>2</sub>-selective agonist, ONO-AE1-259, were determined and ONO-AE1-259 was about 40-fold less potent than PGE<sub>2</sub> (Table 1).

EP<sub>4</sub> antagonists reverse the effects of PGE<sub>2</sub>

The effects of the antagonists PF-04418948 (EP<sub>2</sub>-selective) and CJ-042794 (EP<sub>4</sub>-selective) were investigated (Murase et al., 2008; af Forselles et al., 2011).

Macrophages were incubated with either PF-04418948 (300 nM) or CJ-042794 (300 nM) before incubation with PGE<sub>2</sub> and then challenged with LPS. CJ-042794 effectively antagonised the PGE<sub>2</sub> inhibition of TNF $\alpha$  generation (Figure 5A). No antagonism of the PGE<sub>2</sub> inhibition was seen with PF-04418948 (Figure 5B).

An alternative EP<sub>4</sub>-selective antagonist, L-161,982 (Machwate et al., 2001), was also evaluated and, in agreement with data obtained with CJ-042794, L-161,982 (300 nM) was found to be effective as an antagonist (Figure 5C). An alternative EP<sub>2</sub>-selective antagonist, PF-04852946, was also studied. PF-04852946 is structurally distinct from PF-04418948 and about ten-fold more potent than PF-04418948 at EP<sub>2</sub> receptors (Kay et al., 2013). PF-04852946 (30 nM) was found to be an ineffective antagonist of the PGE<sub>2</sub> inhibition of TNF $\alpha$  generation (data not shown).

pK<sub>B</sub> estimates for the antagonism of PGE<sub>2</sub> by CJ-042794 and L-161,982 were  $8.77 \pm 0.13$  (K<sub>B</sub>, 1.7 nM) and  $8.46 \pm 0.12$  (K<sub>B</sub>, 3.5 nM), respectively. These affinities are consistent with effects of these compounds at EP<sub>4</sub> receptors (Jones et al., 2009).

In further studies to determine whether a contribution of the PGE<sub>2</sub> effect on macrophages might be mediated by the EP<sub>2</sub> receptor, the effect of a combination of

EP<sub>2</sub>- and EP<sub>4</sub>-selective antagonists on the PGE<sub>2</sub> inhibition was investigated. The data demonstrate that combined use of PF-04418948 (300 nM) and CJ-042794 (300 nM) caused marginally greater antagonism of the PGE<sub>2</sub> response than CJ-042794 alone (Figure 5D). These data indicate that if the EP<sub>2</sub> receptor does contribute to the PGE<sub>2</sub> response in macrophages then the contribution is, at best, minimal. These data further emphasize that EP<sub>4</sub> is the principal receptor mediating the anti-inflammatory effects of PGE<sub>2</sub> on macrophages.

#### *PGE<sub>2</sub> inhibits TNF $\alpha$ generation induced by Streptococcus pneumoniae*

While LPS is an effective tool to activate macrophages, we also investigated whether the response of macrophages to a respiratory pathogen, *Streptococcus pneumoniae* (Spn), could be attenuated by PGE<sub>2</sub> (Figure 6). Preliminary studies indicated that Spn induced TNF $\alpha$  generation from macrophages in a concentration-dependent fashion with maximal levels of release at an MOI of 1 (data not shown). Further studies demonstrated that PGE<sub>2</sub> concentration-dependently inhibited TNF $\alpha$  generation induced by Spn (MOI of 1). The effects of alternative agonists, L-902,688 and butaprost on Spn-induced TNF $\alpha$  generation from macrophages were also investigated. The EP<sub>4</sub> agonist, L-902,688 (EC<sub>50</sub>; ~2 nM) was slightly more potent than PGE<sub>2</sub> (EC<sub>50</sub>; ~3 nM) as an inhibitor of TNF $\alpha$  generation whereas, by contrast, the EP<sub>2</sub>-selective agonist, butaprost, was less potent than PGE<sub>2</sub>.

#### *PGE<sub>2</sub> is more effective than either salbutamol or roflumilast*

In further studies we compared the effects of PGE<sub>2</sub> with established drugs used in the treatment of respiratory diseases. PGE<sub>2</sub> was found to be both more potent and efficacious than the  $\beta_2$ -adrenoceptor agonist salbutamol (Figure 7A) as an inhibitor of TNF $\alpha$  generation from macrophages driven by LPS. Similar studies with roflumilast, an inhibitor of the cyclic-AMP specific phosphodiesterase (PDE) PDE4, demonstrated that roflumilast was a considerably weaker inhibitor than PGE<sub>2</sub> (Figure 7B). Further studies were performed to determine whether roflumilast (30 nM) might enhance the effects of PGE<sub>2</sub>. The data show that, in the context of inhibiting LPS-induced TNF $\alpha$  generation, the effect of roflumilast on PGE<sub>2</sub> was at best additive (Figure 7C).

## Discussion

In this study, we demonstrate that PGE<sub>2</sub> is an effective inhibitor of cytokine generation from activated macrophages. Furthermore, we show that PGE<sub>2</sub> acts principally through the EP<sub>4</sub> receptor to stabilise the pro-inflammatory responses of human lung macrophages. This suggests that in lung diseases in which activated macrophages participate, EP<sub>4</sub> agonists could be effective anti-inflammatory agents.

In order to identify which EP receptors are expressed by macrophages a number of approaches were adopted. Evaluation of mRNA expression by RT-PCR demonstrated that lung macrophages express both EP<sub>2</sub> and EP<sub>4</sub> receptors but not EP<sub>1</sub> or EP<sub>3</sub> receptors. These data suggest that EP<sub>2</sub> and/or EP<sub>4</sub> receptors are involved in mediating the effects of PGE<sub>2</sub> in human lung macrophages. This was further reinforced by the finding that PGE<sub>2</sub> induced increases in total cell cyclic-AMP in macrophages. Because both EP<sub>2</sub> and EP<sub>4</sub> receptors are known to be coupled to adenylyl cyclase, increases in cyclic-AMP are consistent with the expression of EP<sub>2</sub> and/or EP<sub>4</sub> receptors in macrophages (Wilson et al., 2004).

In attempts to characterize EP receptors further, a range of EP agonists were studied for effects on cytokine generation. The non-selective agonist, misoprostol, was about 26-fold less sensitive than PGE<sub>2</sub> as an inhibitor of LPS-induced TNF $\alpha$  generation. This potency ratio is consistent with an effect of misoprostol at EP<sub>4</sub> receptors since misoprostol is about 29-fold less potent than PGE<sub>2</sub> at EP<sub>4</sub> receptors whereas at EP<sub>2</sub> receptors misoprostol is about 7-fold less potent than PGE<sub>2</sub> (Abramovitz et al., 2000). Alternative agonists were also studied and it was of interest that the EP<sub>4</sub> agonist L-902,688, was about 7-fold more potent than PGE<sub>2</sub>. This finding provides preliminary evidence that the EP<sub>4</sub> receptor is involved in mediating the effects of PGE<sub>2</sub>. Although EP<sub>2</sub>-selective agonists were active in this system the concentrations of both butaprost and ONO-AE1-259 required for inhibition were higher than those usually associated with effects at EP<sub>2</sub> receptors. In this system, butaprost was over 400-fold less potent than PGE<sub>2</sub> whereas at EP<sub>2</sub> receptors butaprost has been reported to be about 18-fold less potent than PGE<sub>2</sub> (Abramovitz et al., 2000). Also, it is noteworthy that butaprost is known to activate EP<sub>4</sub> receptors when used at high enough concentrations (Tang et al., 2000; Clarke et al., 2004; Wilson et al., 2004; Benyahia et al., 2012). Overall, these data provide

strong evidence that the EP<sub>4</sub> receptor is responsible for mediating the effects of PGE<sub>2</sub> but evidence for involvement of the EP<sub>2</sub> receptor cannot be excluded.

In order to obtain a definitive characterization of EP receptors, the effects of EP<sub>2</sub>- and EP<sub>4</sub>-selective antagonists on the PGE<sub>2</sub> response in macrophages were evaluated. It is noteworthy that the EP<sub>2</sub> antagonists, PF-04418948 and PF-04852946, that were used in this study are highly selective ligands (af Forselles et al., 2011; Kay et al., 2013) and considerably superior to AH6809 which until now was the only EP<sub>2</sub> antagonist available. Indeed, AH6909 has been used in recent studies to invoke a role for EP<sub>2</sub> receptors (O'Brien et al., 2014). However, AH6809 shows poor selectivity and potency such that data generated with this antagonist are unlikely to be reliable (Abramovitz et al., 2000; Jones et al., 2009). Neither of the two EP<sub>2</sub> antagonists used in this study had any effect on the PGE<sub>2</sub> inhibition of TNF $\alpha$  generation. By contrast, two EP<sub>4</sub> antagonists, CJ-042794 (K<sub>B</sub>; 1.7 nM) and L-161,982 (K<sub>B</sub>; 3.5 nM) effectively reversed the PGE<sub>2</sub> inhibition of TNF $\alpha$  generation with affinities consistent with antagonism at EP<sub>4</sub> receptors (Jones et al., 2009). Combining an EP<sub>2</sub> antagonist with an EP<sub>4</sub> antagonist did lead to a marginal rightward shift in the PGE<sub>2</sub> concentration response curve over that seen with an EP<sub>4</sub> antagonist alone. This could mean that a very small component of the PGE<sub>2</sub> inhibition is driven by EP<sub>2</sub> receptors. Overall, these data provide strong evidence that the principal receptor that mediates the anti-inflammatory effects of PGE<sub>2</sub> in human lung macrophages is the EP<sub>4</sub> receptor.

The suggestion has been made that the EP<sub>4</sub> receptor could be a target for respiratory diseases. This contention has been based largely on recent studies showing that PGE<sub>2</sub> mediates bronchodilation via the EP<sub>4</sub> receptor (Buckley et al., 2011; Benyahia et al., 2012). The present study has demonstrated that targeting the EP<sub>4</sub> receptor may provide desirable anti-inflammatory effects by preventing cytokine generation from macrophages. In this regard, it is of interest that PGE<sub>2</sub> attenuated the generation of both TNF $\alpha$  and IL-6 in human lung macrophages which differs to findings reported for mouse alveolar macrophages in which PGE<sub>2</sub> inhibited TNF $\alpha$  but by contrast potentiated IL-6 generation (Konya et al., 2015).

The potential therapeutic value of targeting EP receptors is reinforced by the finding that PGE<sub>2</sub> was effective at attenuating cytokine generation from macrophages activated by not only LPS but the respiratory pathogen, *S.pneumoniae*. Moreover, it is noteworthy that PGE<sub>2</sub> was considerably more efficacious and potent than either salbutamol or roflumilast as an inhibitor of LPS-induced TNF $\alpha$  generation from

macrophages. Bronchodilators such as salbutamol are  $\beta_2$ -adrenoceptor agonists that may possess some anti-inflammatory activity (Donnelly et al., 2010). The mechanism of action of the PDE4 inhibitor roflumilast is not entirely known although anti-inflammatory effects have been suggested (Giembycz and Field, 2010). However, our data suggest that EP<sub>4</sub> agonists are likely to show far greater anti-inflammatory potential than either  $\beta_2$ -adrenoceptor agonists or PDE inhibitors.

In an allied context, it was notable that the PGE<sub>2</sub> response was relatively consistent among macrophage preparations (see Supplemental Information, Figure 1). This could be important from a therapeutic perspective since the possibility exists that factors such as disease state, smoking status and age could influence macrophage functionality (Berenson et al., 2006; Hodge et al., 2007; Suzuki et al., 2008). While we were unable to stratify effectively our population according to disease state, we were able to stratify according to smoking status and age (see Supplemental Information, Figure 1). There was clearly no difference in the inhibitory response to PGE<sub>2</sub> among macrophages isolated from smokers, ex-smokers or never smokers. Moreover, there was no influence of age on the inhibitory response to PGE<sub>2</sub>. This consistency in response could be an advantage when considering the potential of targeting the EP<sub>4</sub> receptor therapeutically.

To conclude, our studies demonstrate that the EP<sub>4</sub> receptor is the principal receptor that mediates the anti-inflammatory effects of PGE<sub>2</sub> in human lung macrophages. This suggests that EP<sub>4</sub> agonists could be effective anti-inflammatory agents in lung diseases that are associated with aberrant macrophage activation.

## **Authorship Contribution Statement**

SK Gill, Y Yao, LJ Kay and MA Bewley performed the experimental work; HM Marriott and PT Peachell designed the study; PT Peachell and SK Gill wrote the manuscript.

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## **Conflicts of Interest**

None

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**Table 1** EC<sub>50</sub> and E<sub>max</sub> values for the inhibition of TNF $\alpha$  generation by EP agonists

agonist	EC <sub>50</sub> (nM)	E <sub>max</sub> (%)
PGE <sub>2</sub>	2.1 $\pm$ 0.6	77 $\pm$ 3
misoprostol	54 $\pm$ 9.1	80 $\pm$ 4
L-902,688	0.3 $\pm$ 0.1	63 $\pm$ 7
butaprost	878 $\pm$ 340	67 $\pm$ 5
ONO-AE1-259	82 $\pm$ 24	43 $\pm$ 4

Experimental details relevant to this table can be found in the legend to Figure 4.

Values are means  $\pm$  SEM from 5 (misoprostol, L-902,688, ONO-AE1-259), 6 (butaprost) and 8 (PGE<sub>2</sub>) experiments.

## Figure legends

**Figure 1** Effects of PGE<sub>2</sub> on cytokine generation from macrophages. Macrophages were pre-incubated without (A) or with (B) indomethacin (1 μM) for 30 min and then with or without PGE<sub>2</sub> for 30 min before challenge with LPS (1 ng mL<sup>-1</sup>) for 22 h after which supernatants were harvested and assayed for TNFα generation. The data in (A) and (B) were reworked as % inhibition of the control unblocked release of TNFα and this is shown in (C). In further experiments, macrophages were pre-incubated (30 min) with indomethacin (1 μM) and then with or without PGE<sub>2</sub> for 30 min before challenge with LPS (1 ng mL<sup>-1</sup>) for 22 h and both IL-6 and TNFα measured in the supernatants (D). Values are expressed as the % inhibition of control cytokine releases which were 2422 ± 510 pg mL<sup>-1</sup> of TNFα and 4992 ± 1980 pg mL<sup>-1</sup> of IL-6. Values are means ± SEM, for 9 (A, B, C) or 6 (D) experiments. Statistically significant (P<0.05) levels of inhibition compared to unblocked control levels are indicated by an asterisk.

**Figure 2** EP receptor expression in macrophages. Isolated RNA was converted to cDNA by reverse transcriptase (+) and, as a control, this reaction step was also carried out in the absence of reverse transcriptase (-). Amplification of cDNA was performed using primers specific for each of the EP receptor subtypes and β-actin. Expression profiles for three macrophage preparations (MAC1, MAC2 and MAC3) are shown. No mRNA for EP<sub>1</sub> was detected in macrophages but in separate experiments the presence of EP<sub>1</sub> could be readily demonstrated in several breast cancer cell lines, MDA-MB-468, MDA-MB-231 and ZR-75-1 (Kay et al., 2013). No mRNA for EP<sub>3</sub> was detected but, in separate experiments, EP<sub>3</sub> could be detected in the human mast cell line, LAD-2 (Kay et al., 2013). These findings are representative of a total of 5 different macrophage preparations in excess of 95% purity. Lanes at either end of each gel represent a 100 bp ladder.

**Figure 3** Effect of PGE<sub>2</sub> on cyclic-AMP. Macrophages were pre-incubated (30 min) with or without indomethacin (indo; 1 μM) and then with or without PGE<sub>2</sub> (1 μM) for a further 30 min. After this treatment, the cells were solubilised and total cell cyclic-AMP levels measured. Values are means ± SEM for 5 experiments. Statistically

significant ( $P < 0.05$ ) increases in cyclic-AMP over unstimulated control levels are indicated by an asterisk.

**Figure 4** Effects of EP agonists on macrophages. Macrophages were pre-incubated (30 min) with indomethacin ( $1 \mu\text{M}$ ) and then with or without either (A) misoprostol, (B) L-902,688, (C) butaprost or  $\text{PGE}_2$  for 30 min before challenge with LPS ( $1 \text{ ng mL}^{-1}$ ) for 22 h after which  $\text{TNF}\alpha$  was measured in the supernatants. Values are expressed as the % inhibition of control cytokine release which was  $1379 \pm 431 \text{ pg mL}^{-1}$  of  $\text{TNF}\alpha$ . Values are means  $\pm$  SEM for 5 (A, B) or 6 (C) experiments.

**Figure 5** Effects of EP receptor antagonists on  $\text{PGE}_2$ . Macrophages were pre-incubated with indomethacin ( $1 \mu\text{M}$ ) for 30 min and then without or with EP-selective antagonists (300 nM) for 1 h and then without or with  $\text{PGE}_2$  for 30 min before challenge with LPS ( $1 \text{ ng mL}^{-1}$ ) for 22 h after which  $\text{TNF}\alpha$  was measured in the supernatants. The effects on  $\text{PGE}_2$  of (A) the  $\text{EP}_4$ -selective antagonist CJ-042794, (B) the  $\text{EP}_2$ -selective antagonist PF-04418948, (C) the  $\text{EP}_4$ -selective antagonist L-161,982 and (D) CJ-042794 with and without PF-04418948 were evaluated. Values are expressed as the % inhibition of control  $\text{TNF}\alpha$  releases which were, in the absence and presence of antagonist respectively, (A)  $2646 \pm 562$  and  $2582 \pm 496 \text{ pg mL}^{-1}$ , (B)  $2912 \pm 532$  and  $2881 \pm 507 \text{ pg mL}^{-1}$ , (C)  $2756 \pm 882$  and  $2873 \pm 862 \text{ pg mL}^{-1}$  and (D)  $2672 \pm 972$  to  $2212 \pm 799 \text{ pg mL}^{-1}$ . Values are means  $\pm$  SEM for 5 (A, B, D) and 6 (C) experiments, respectively.

**Figure 6** Effects of  $\text{PGE}_2$  and alternative agonists on Spn-induced  $\text{TNF}\alpha$  generation. Macrophages were pre-incubated (30 min) with indomethacin ( $1 \mu\text{M}$ ) and then with or without either  $\text{PGE}_2$ , L-902,688 or butaprost for 30 min before challenge with Spn (MOI of 1) for 22 h after which  $\text{TNF}\alpha$  was measured in the supernatants. Values are expressed as the % inhibition of the control cytokine release which was  $1346 \pm 669 \text{ pg mL}^{-1}$  of  $\text{TNF}\alpha$ . Values are means  $\pm$  SEM for 4 experiments.

**Figure 7** Effects of salbutamol and roflumilast on macrophages. Macrophages were pre-incubated (30 min) with indomethacin ( $1 \mu\text{M}$ ) and then with or without either (A) salbutamol, (B) roflumilast or (C)  $\text{PGE}_2$  in the absence (control) or presence of a single concentration of roflumilast (30 nM) for 30 min before challenge with LPS (1

ng mL<sup>-1</sup>) for 22 h after which TNF $\alpha$  was measured in the supernatants. The horizontal grid line in (C) shows the inhibition seen with roflumilast alone ( $22 \pm 5\%$  inhibition). Values are expressed as the % inhibition of the unblocked control TNF $\alpha$  releases which ranged from  $2363 \pm 835$  to  $2208 \pm 969$  pg mL<sup>-1</sup>. Values are means  $\pm$  SEM for 5 (A, B, C) experiments.

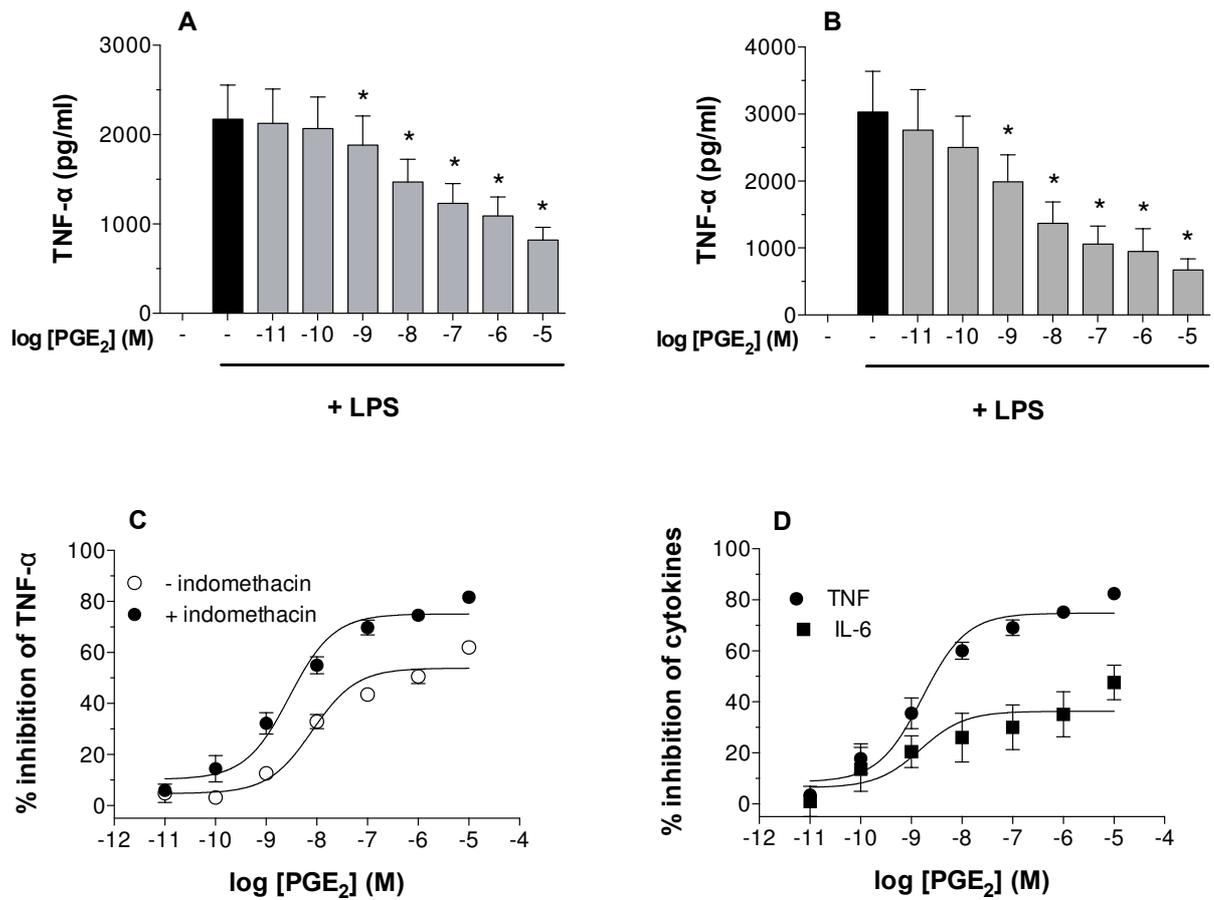


Figure 1

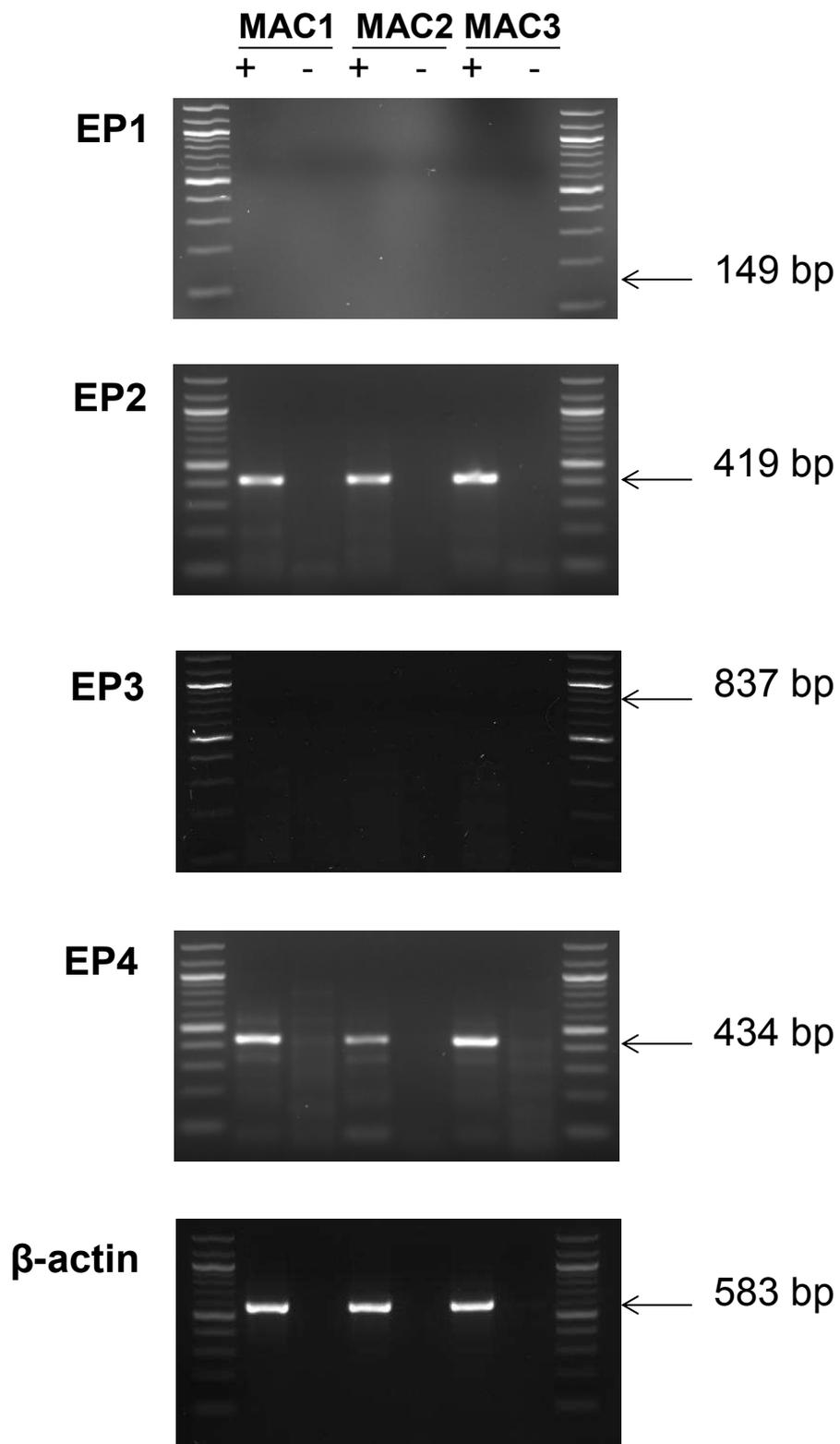


Figure 2

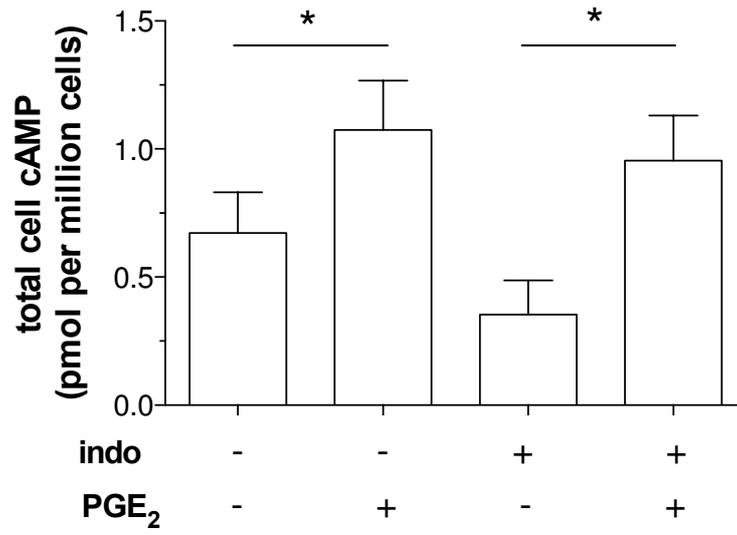


Figure 3

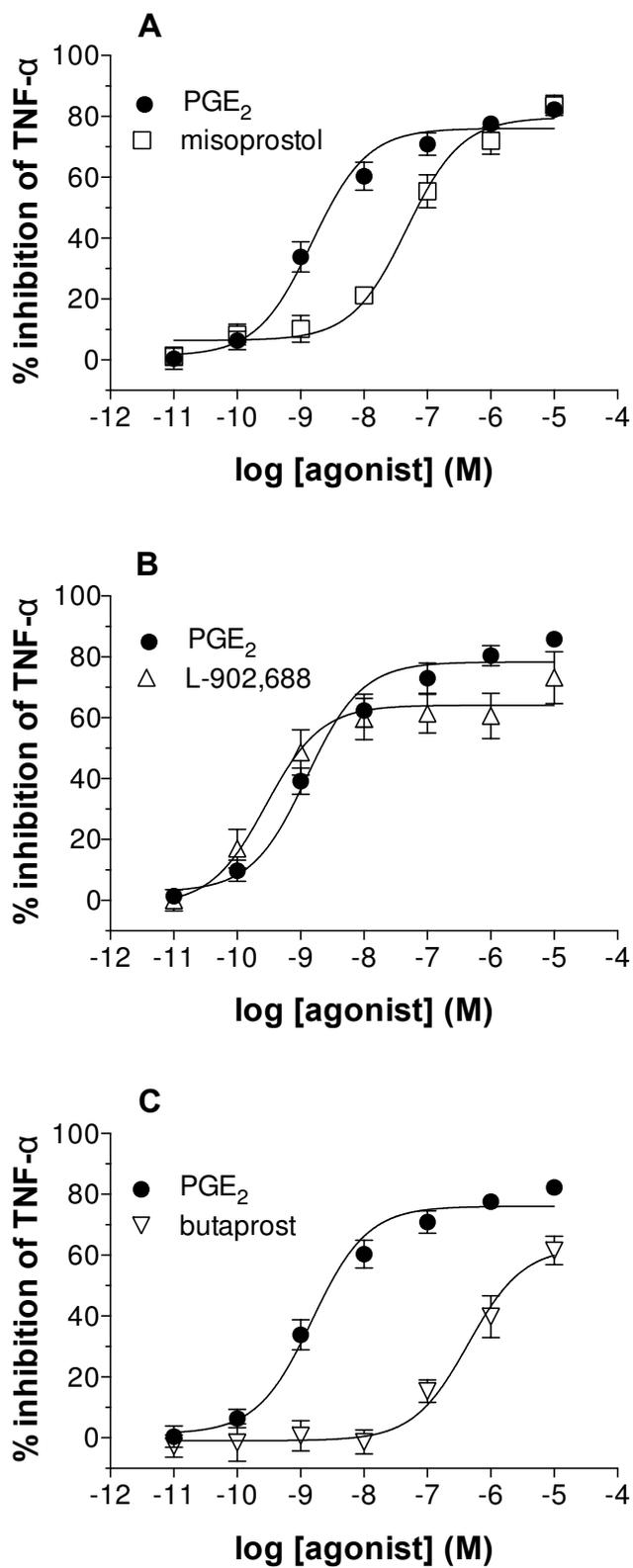


Figure 4

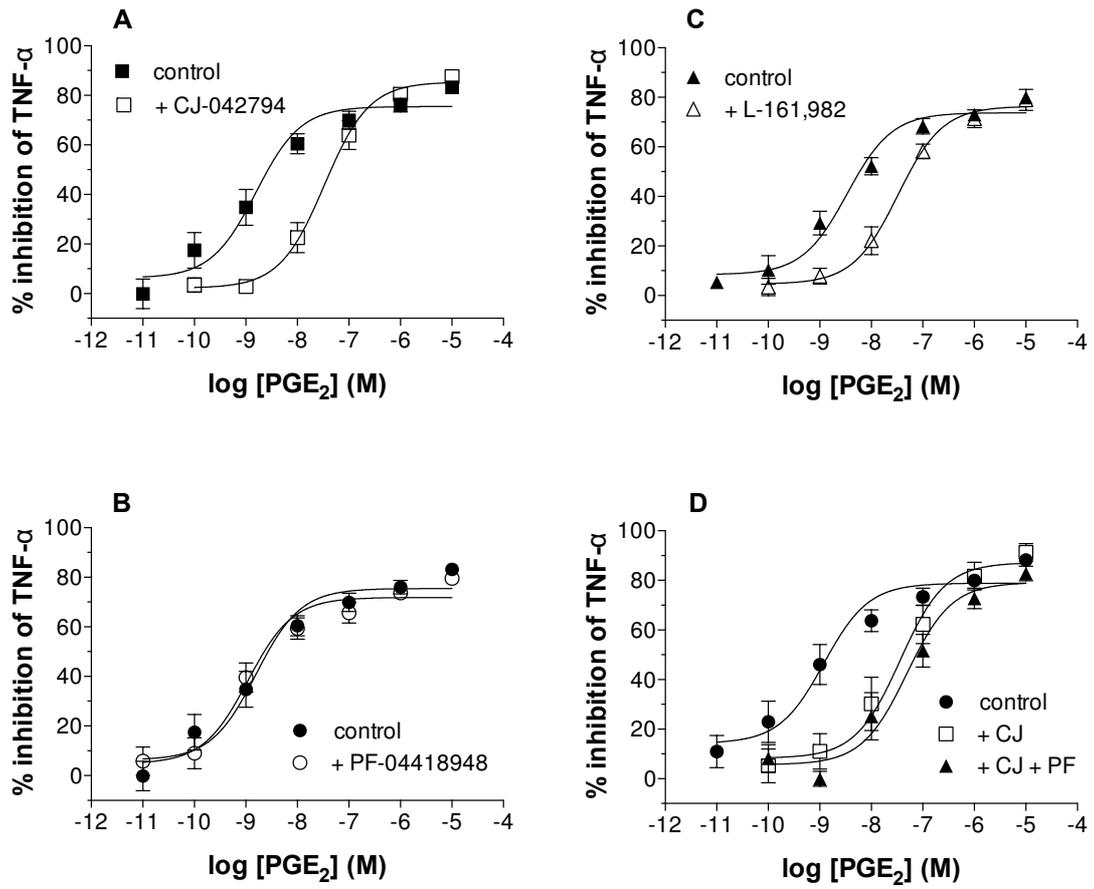


Figure 5

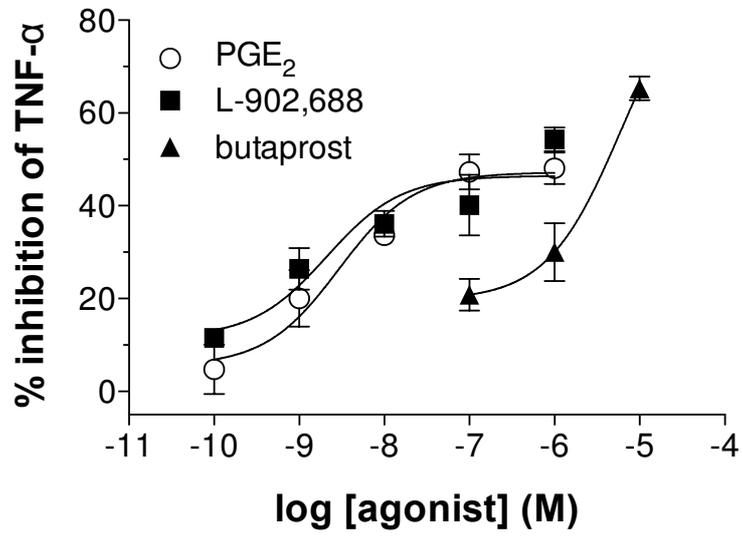


Figure 6

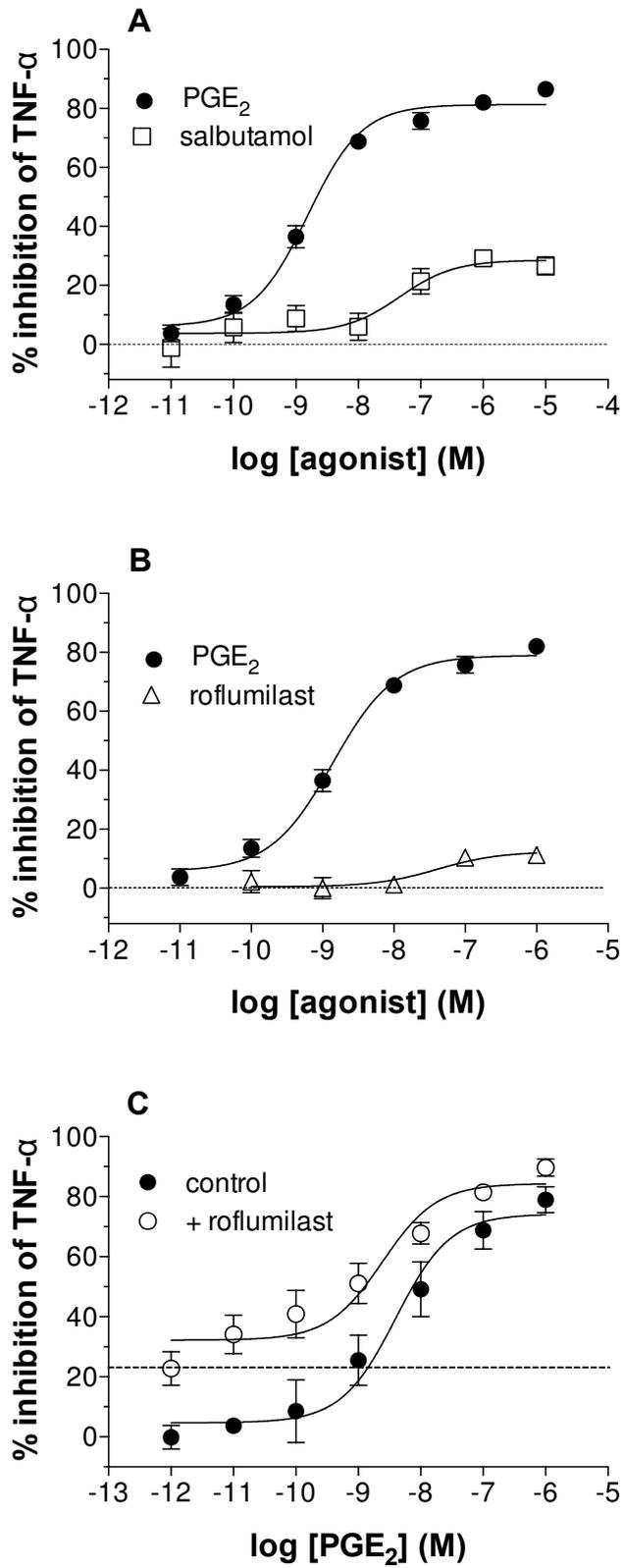


Figure 7