



# Activation of E-prostanoid<sub>4</sub> and E-prostanoid<sub>2</sub> receptors inhibits TNF- $\alpha$ release from human alveolar macrophages

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**ABSTRACT:** Prostaglandin (PG)E<sub>2</sub> has been shown to inhibit mediator release from human alveolar macrophages (AMs), but the prostanoid receptor(s) mediating this response have not yet been documented. To investigate this, the present authors conducted a range of pharmacological and expression-based studies in monocyte-derived macrophages (MDMs) and AMs.

MDMs were obtained by *in vitro* differentiation of monocytes from the peripheral blood of healthy human volunteers. Human AMs were obtained by perfusion of lung tissue from carcinoma resection patients.

In MDMs, PGE<sub>2</sub> potently inhibited lipopolysaccharide-induced tumour necrosis factor (TNF)- $\alpha$  release (p[A]<sub>50</sub> 8.51  $\pm$  0.11, maximum inhibition 95.9  $\pm$  4.8%). In human AMs, PGE<sub>2</sub> also inhibited TNF- $\alpha$  release but the observed concentration–effect curve was very flat and inhibition was incomplete. The shape of the PGE<sub>2</sub> curve in AMs suggested that its effects were mediated by activation of a heterogeneous receptor population. Expression studies combined with the use of various E-prostanoid (EP) receptor agonists and a selective EP<sub>4</sub>-receptor antagonist (Ono-AE2-227) confirmed that the inhibitory effects of PGE<sub>2</sub> in both AMs and MDMs were mediated by activation of EP<sub>4</sub> and EP<sub>2</sub> receptors.

These data indicate that both E-prostanoid<sub>4</sub> and E-prostanoid<sub>2</sub> selective agonists may have anti-inflammatory properties in lung diseases where macrophages play a role.

**KEYWORDS:** Alveolar, cytokine, human, macrophage, prostaglandin E<sub>2</sub>

**A**lveolar macrophages (AMs) are a key component of innate immune defence in the lungs. AMs remove inhaled material, including particulates and microorganisms, *via* phagocytosis and by releasing a wide range of mediators which recruit other cells, notably neutrophils, into the lungs. This pro-inflammatory response is important in clearing foreign agents but it has been hypothesised that in certain conditions it is excessive, leading to the destruction of host tissues and the development of chronic inflammatory disease [1]. This has promoted considerable interest in the discovery of pharmacological agents that dampen down the release of pro-inflammatory mediators from macrophages.

One such agent is prostaglandin (PG)E<sub>2</sub> which elicits a range of biological effects *via* its interaction with G-protein coupled receptors, designated E-prostanoid (EP)<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub> and EP<sub>4</sub> [2]. EP receptors all have a high affinity for PGE<sub>2</sub> but differ in their affinities for various synthetic agonists and antagonists [3, 4]. PGE<sub>2</sub> has been shown to affect many macrophage functions,

including tumouricidal activity [5, 6], phagocytosis [7, 8] and mediator release [9–13]. The effects of PGE<sub>2</sub> on mediator release are generally considered to be anti-inflammatory, as PGE<sub>2</sub> has been demonstrated to inhibit the release of a number of cytokines and chemokines, whilst inducing expression of the anti-inflammatory cytokine interleukin (IL)-10 [14, 15]. These observations suggest that it may be possible to develop EP receptor agonists as therapeutic drugs to treat inflammatory diseases. However, few studies have assessed the effects of such agents on human AMs. PGE<sub>2</sub>-mediated inhibitions of IL-1 [16], IL-8 [17], tumour necrosis factor (TNF)- $\alpha$  [18] and fibronectin [10] have been reported in these cells but the receptor type(s) mediating these responses were not investigated. The aim of the present study was, therefore, to address this issue and by doing so, provide information on the potential efficacy of EP agonists as anti-inflammatory drugs for pulmonary diseases, such as chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis or acute respiratory distress syndrome.

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## STATEMENT OF INTEREST

None declared.

## MATERIALS AND METHODS

### Reagents

Lipopolysaccharide (LPS; *Escherichia coli* 026:B6), 3-isobutyl-1-methylxanthine (IBMX), indomethacin and foetal calf serum (FCS) were supplied by Sigma-Aldrich (Poole, Dorset, UK). PGE<sub>2</sub>, PGE<sub>1</sub>-OH, sulprostone and butaprost (methyl ester) were supplied by Cayman Chemical (Ann Arbor, MI, USA). The selective EP<sub>4</sub> receptor antagonist, Ono-AE2-227 [19], was synthesised by the Medicinal Chemistry Department (Astra-Zeneca Research and Development Charnwood, Loughborough, UK). RPMI, Dulbecco's modified Eagle Medium (DMEM), Iscoves modified Dulbecco's medium containing GlutaMAX™ (IMDM), geneticin, L-glutamine and penicillin/streptomycin were supplied by Invitrogen Ltd (Paisley, UK).

### Generation of human embryonic kidney cells stably transfected with the human EP<sub>2</sub> or EP<sub>4</sub> receptor

The human EP<sub>2</sub> and EP<sub>4</sub> cDNA sequences were cloned into the mammalian expression vector pIRESneo2 (Clontech Laboratories Inc., Mountain View, CA, USA). Plasmid DNA was transfected into human embryonic kidney (HEK) cells using FuGENE 6 transfection reagent (Roche Molecular Systems, Inc., Alameda, CA, USA) and expressing cells were selected using 1 mg·mL<sup>-1</sup> geneticin solution. Specific cell surface expression of EP<sub>2</sub> or EP<sub>4</sub> was confirmed by antibody staining (data not shown).

### Preparation of macrophages

Human monocyte-derived macrophages (MDMs) were prepared from peripheral blood monocytes isolated from healthy human volunteers. Venous blood was layered onto lymphoprep (Axis-Shield UK, Kimbolton, UK) and centrifuged at 800 × g for 20 min with no brake. The mononuclear cell layer was removed and platelet contamination was removed by several low-speed (80 × g) centrifugation/wash steps using 5% volume (v)/v autologous serum in 0.9% weight (w)/v NaCl. Monocytes were prepared by negative selection using human monocyte isolation kit II (Miltenyi Biotec, Surrey, UK), according to the manufacturer's instructions. Cells were cultured in IMDM/10% v/v FCS/penicillin (100 U·mL<sup>-1</sup>)/streptomycin (100 mg·mL<sup>-1</sup>) with 50 ng·mL<sup>-1</sup> human macrophage-colony stimulating factor (R&D Systems, Abingdon, Oxon, UK). Medium was changed every 5 days. Cells were used 10–14 days after isolation.

Human AMs were obtained from resection tissue from lung cancer patients. Macrophages were flushed from the tissue with PBS. Macrophages were purified by 1 h adhesion to tissue culture plastic in serum-free RPMI. Contaminating cells were removed by stringent washing in RPMI. Cells were incubated for 1–3 days in RPMI/10% FCS before use in cytokine release experiments.

### Real-time quantitative RT-PCR expression studies

RNA was isolated from cell homogenates using Trizol (Invitrogen Ltd) and prepared using RNeasy miniprep column (Qiagen, Crawley, UK) with DNase. RNA was reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen Ltd) according to the manufacturer's recommended protocol. Control (-RT) reactions were performed in the absence of reverse transcriptase.

The primers used comprised of the following nucleotides based on published sequences. EP<sub>1</sub>-Forward: ATGGTGGG-CCAGCTTGTG; EP<sub>1</sub>-Reverse: GCCACCAACACCAGCATTG; EP<sub>2</sub>-Forward: GCCTGCAACTTCAGTGTTCATTG; EP<sub>2</sub>-Reverse: GTCCGCAGCGGCTTCT; EP<sub>3</sub>-Forward: GACGGCCATTGACCTTATGG; EP<sub>3</sub>-Reverse: CTGATTGAAGATCATTTCACA-TCA; EP<sub>4</sub>-Forward: ACATGTACGCGGGCTTCAG; EP<sub>4</sub>-Reverse: GCCGCACACAAGCACGTT. All primers were supplied by Eurogentec Ltd (Southampton, UK). The 18S probe had the following modification: 5'-Yakima Yellow, 3'-Black Hole Quencher 1. RT-PCR was conducted on an Mx3000P RT-PCR machine (Stratagene Europe, Amsterdam, The Netherlands). Samples were run in triplicate for each gene. PCR conditions were: 95°C for 5 min; 50 cycles of 95°C for 20 s followed by 60°C for 90 s; 95°C for 1 min, followed by dissociation curve from 55–95°C. Data were collected at the end of each cycle and throughout each dissociation curve. For each gene eight-point, two-fold standard curves were run using a human reference cDNA (Stratagene Europe) as a template. Expression values were calculated from a standard curve and normalised to 18S levels.

### Flow cytometry

MDMs and AMs were harvested by scraping, washed in PBS and EP receptor proteins labelled using a BD Pharmingen Cytofix/cytoperm™ kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Rabbit immunoglobulin (Ig)G control was supplied by Sigma-Aldrich, EP<sub>2</sub> and EP<sub>4</sub> antibodies were supplied by Cayman Chemical and secondary goat anti-rabbit IgG-Alexafluor488 antibody was supplied by Invitrogen Ltd. Primary antibodies were used at 2 µg·mL<sup>-1</sup> of the final concentration and secondary antibodies at 4 µg·mL<sup>-1</sup>. At each stage 10 µL·mL<sup>-1</sup> FcR Block (Miltenyi Biotec) was added. Fluorescence was measured using a Beckman Coulter FC500 flow cytometer (Beckman Coulter UK Ltd, High Wycombe, UK).

### Cyclic adenosine monophosphate assay

EP<sub>4</sub> or EP<sub>2</sub> receptor transfected HEK cells were harvested using Accutase™ (Innovative Cell Technologies Inc., San Diego, CA, USA) and plated at 50,000 cells·well<sup>-1</sup> in DMEM/10% FCS in poly-D-lysine coated 96-well plates (BD Labware, Bedford, MA, USA). The following day, cells were washed twice and incubated in serum-free DMEM containing IBMX (1 mM). Ono-AE2-227 was added 20 min later for 15 min. PGE<sub>2</sub> was then added for a further 20 min. Cyclic adenosine monophosphate (cAMP) in cell lysates was measured using RPN225 cAMP EIA Biotrak system (GE Healthcare Amersham Biosciences, Chalfont St Giles, UK) according to protocol 3 of the manufacturer's instructions.

### Cytokine release assay

MDMs and AMs were harvested, washed and plated at 50,000 cells·well<sup>-1</sup> in a 96-well plate in IMDM containing 0.5% FCS for 1–2 h. EP agonists were then added as indicated, followed 15 min later by LPS (100 ng·mL<sup>-1</sup> final concentration). Cells were incubated for 24 h. In experiments using Ono-AE2-227, this agent was added 20 min before PGE<sub>2</sub>. Where used, indomethacin (10 µM) was added 2 h before the addition of PGE<sub>2</sub>. Supernatants were harvested and cytokines measured using optEIA ELISA kits (BD Biosciences, Erembodegen, Belgium) according to the manufacturer's instructions.

## DATA ANALYSIS

### Logistic curve fitting

Where agonist E/[A] curve data were clearly monophasic they were fitted to the following form of the Hill equation:

$$E = \frac{\alpha[A]^{n_H}}{[A]^{n_H} + [A]_{50}^{n_H}} \quad (1)$$

in which  $\alpha$ ,  $[A]_{50}$  and  $n_H$  are the asymptote (maximum effect), location (potency) and slope parameters, respectively.  $[A]_{50}$  values were assumed to be log-normally distributed and quoted as  $p[A]_{50}$  ( $-\log[A]_{50}$ ) values.

In most instances data from individual experiments were fitted to equation 1. However, the AM data were more variable than the MDM data and in this case the mean data were fitted to equation 1.

All cytokine data were expressed as percentage inhibitions of the LPS-induced response. cAMP data were expressed as per cent maximum response to PGE<sub>2</sub>.

### Antagonist affinity estimation

$[A]_{50}$  data obtained in antagonist experiments were fitted to the following linear form of the Schild equation [20]:

$$\log_{10}[A]_{50} = \log_{10}[A]_{50}^C + \log_{10}(1 + [B]^n/K_B) \quad (2)$$

Where  $[A]_{50}^C$  is the estimated control  $[A]_{50}$  value,  $[B]$  is the concentration of the antagonist,  $K_B$  is the antagonist equilibrium constant and  $n$  is equivalent to the Schild plot slope parameter (unity for simple competition). If  $n$  was not significantly different from unity, it was constrained to unity for  $pK_B$  ( $-\log K_B$ ) estimation.

### Computer simulation of two-receptor systems

In order to interpret the data from the experiments using Ono-AE2-227 it was necessary to consider the action of a selective antagonist on a one-agonist, two-receptor system. The model of FURCHGOTT [21] was used to simulate the expectations of such an interaction. This model is an extension of the traditional model for a one-receptor system [22, 23], in which pharmacological effect (E) is assumed to be a function of stimulus (S). In the two-receptor case the combined stimulus is imparted by the interaction of the agonist at each receptor, thus:

$$S = S_1 + S_2 = e_1 p_1 + e_2 p_2 \quad (3)$$

where  $e_1$  and  $e_2$  are the efficacies of the agonist at each receptor and  $p_1$  and  $p_2$  are the respective fractional occupancies.

FURCHGOTT [21] assumed that the relationship between S and E can be described by:

$$\frac{E}{E_m} = \frac{S^n}{1 + S^n} = \frac{(e_1 p_1 + e_2 p_2)^n}{1 + (e_1 p_1 + e_2 p_2)^n} \quad (4)$$

When a competitive antagonist able to interact with each receptor is present the occupancies  $p_1$  and  $p_2$  are defined by:

$$p_1 = \frac{[A]}{K_{A1}(1 + [B]/K_{B1}) + [A]} \quad (5)$$

$$p_2 = \frac{[A]}{K_{A2}(1 + [B]/K_{B2}) + [A]} \quad (6)$$

where  $K_{A1}$ ,  $K_{A2}$ ,  $K_{B1}$  and  $K_{B2}$  are the dissociation constants of the agonist and antagonist respectively for the two receptors.

Equations 4, 5 and 6 were used to generate theoretical agonist concentration-effect curves in the presence and absence of the competitive antagonist in the MDM and AM systems. These simulations were then superimposed on to the experimental data to see how well the latter fitted the model.

All of the data-fitting procedures and simulations were carried out using Microsoft Excel. Results are expressed and plotted as mean  $\pm$  SE. Statistical differences were assessed by the use of a paired t-test or ANOVA as appropriate and considered significant at the level of  $p < 0.05$ .

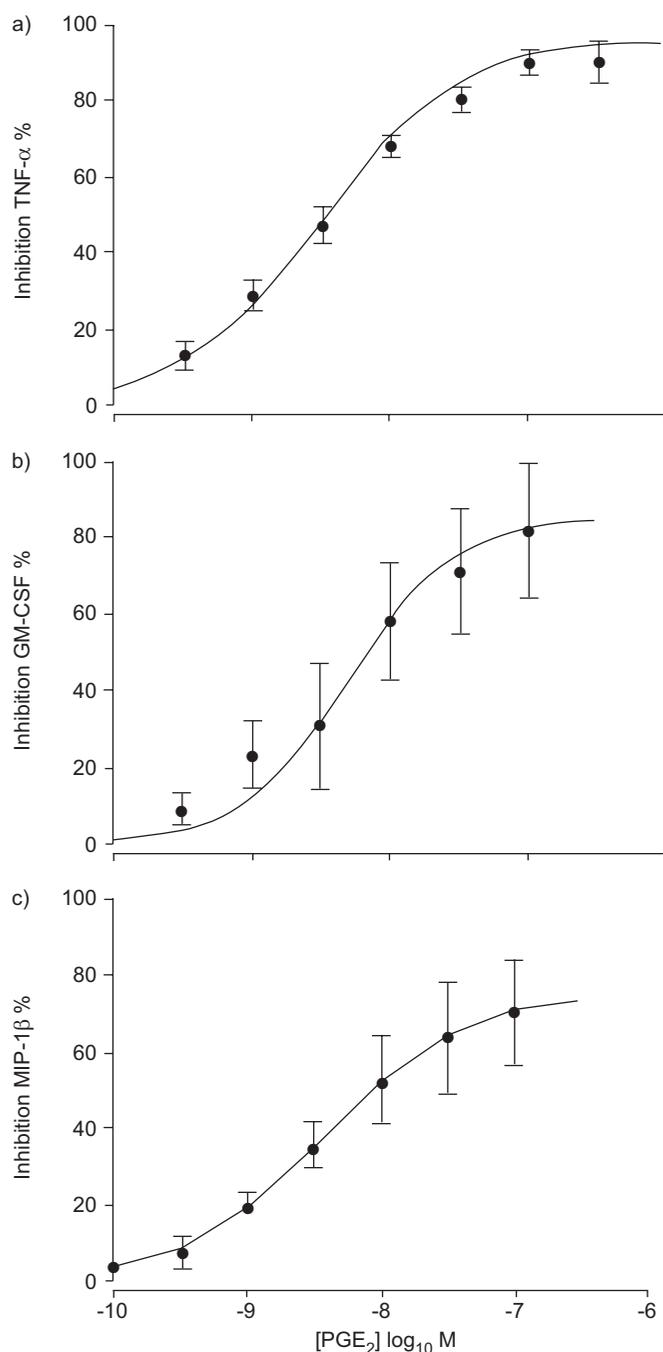
## RESULTS

### Inhibition of TNF- $\alpha$ release from human macrophages by EP-receptor agonists

Preliminary experiments revealed that PGE<sub>2</sub> potently inhibited LPS-induced release of a variety of mediators, including TNF- $\alpha$ , granulocyte macrophage-colony stimulating factor and macrophage inflammatory protein-1 $\beta$  from MDMs (fig. 1). Of these, the most robust signal was TNF- $\alpha$ , so this readout was used in all subsequent studies in both MDMs and AMs. PGE<sub>2</sub> caused a concentration-dependent inhibition of LPS-induced TNF- $\alpha$  release from both MDMs (fig. 2a) and AMs (fig. 2b). In MDMs, the E/[A] curve appeared monophasic and logistic curve fitting (equation 1) yielded a  $p[A]_{50}$  of  $8.51 \pm 0.11$  ( $n=7$ ) and a maximum inhibition ( $\alpha$ ) of  $95.9 \pm 4.8\%$ . In AMs, the PGE<sub>2</sub> E/[A] curve appeared biphasic, precluding logistic curve fitting and suggesting that more than one receptor type may be involved in the response. To further investigate this, the current authors tested the effects of a range of EP receptor agonists. PGE<sub>1</sub>-OH (EP<sub>4</sub> and EP<sub>2</sub>) [24, 25] and butaprost (an EP<sub>2</sub> receptor selective agonist) [4, 25] were active in both systems. In MDMs they produced similar maximum inhibitions of TNF- $\alpha$  as PGE<sub>2</sub> ( $95.7 \pm 1.2\%$  ( $n=7$ ) and  $94.4 \pm 4.78\%$  ( $n=4$ ), respectively) but were 10-fold and 245-fold less potent than PGE<sub>2</sub> ( $p[A]_{50}$   $7.51 \pm 0.07$  ( $n=7$ ) and  $p[A]_{50}$   $6.12 \pm 0.13$  ( $n=4$ ), respectively). By measuring cAMP elevation in HEK cells transfected with human EP<sub>4</sub> it was confirmed that PGE<sub>1</sub>-OH is a relatively potent EP<sub>4</sub> agonist ( $p[A]_{50}$   $8.95 \pm 0.15$ ;  $n=4$ ) and that butaprost is inactive at EP<sub>4</sub> up to concentrations of 10  $\mu$ M (data not shown). In AMs the PGE<sub>1</sub>-OH and butaprost E/[A] curves were not fully defined at the highest concentrations tested, again precluding logistic curve fitting, but these two agonists were clearly less potent than PGE<sub>2</sub>. Sulprostone (an EP<sub>1</sub>/EP<sub>3</sub> selective agonist) [4] did not cause significant inhibition in either system at concentrations as high as 10  $\mu$ M. The activity of butaprost suggested the presence of EP<sub>2</sub> receptors in both systems but the relatively high potency of PGE<sub>2</sub> and PGE<sub>1</sub>-OH suggested an additional receptor was involved. Given the inactivity of sulprostone, the most likely candidate was the EP<sub>4</sub> receptor.

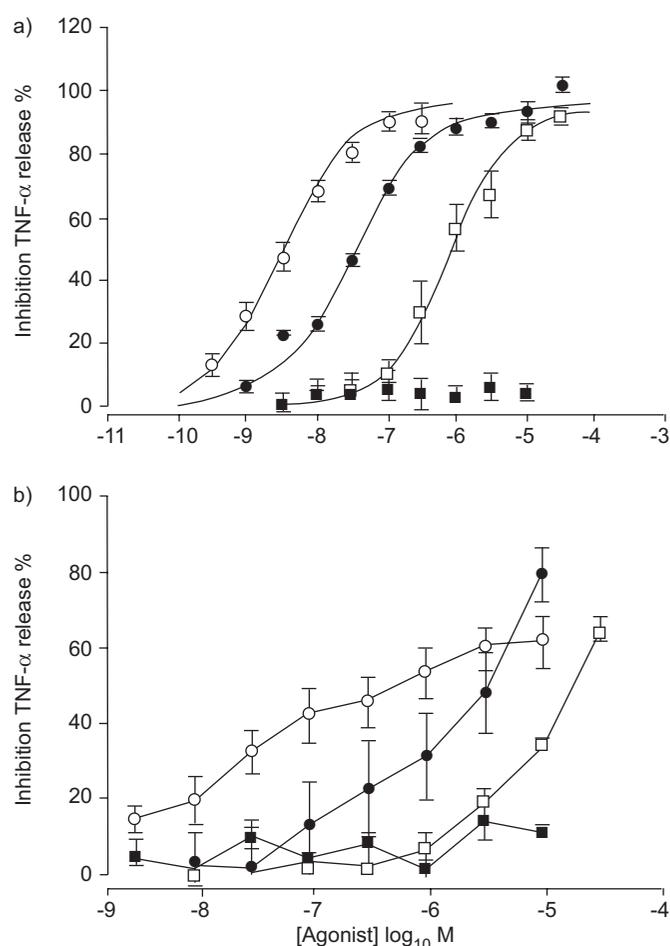
### Expression of EP receptors in human macrophages

Real-time quantitative RT-PCR was used to determine the pattern of mRNA expression for the four known EP receptors



**FIGURE 1.** E/[A] curves for prostaglandin (PG)<sub>E2</sub>-induced inhibitions of lipopolysaccharide stimulated a) tumour necrosis factor (TNF)- $\alpha$ , b) granulocyte-macrophage colony-stimulating factor (GM-CSF), and c) macrophage inhibitory protein (MIP)-1 $\beta$  release from monocyte-derived macrophages (MDMs). Data represent the mean of three to seven experiments. Vertical lines indicate the SE. Lines drawn through the MDM data are the result of curve-fitting using equation 1.

in MDMs and AMs. This analysis demonstrated significant expression of EP<sub>2</sub> and EP<sub>4</sub> receptors in both systems but little or no expression of EP<sub>1</sub> and EP<sub>3</sub> receptors (fig. 3a). EP<sub>2</sub> and EP<sub>4</sub> protein expression in MDMs and AMs was also assessed using flow cytometry (figs 3b–3e). These studies confirmed that both macrophage systems expressed significant levels of

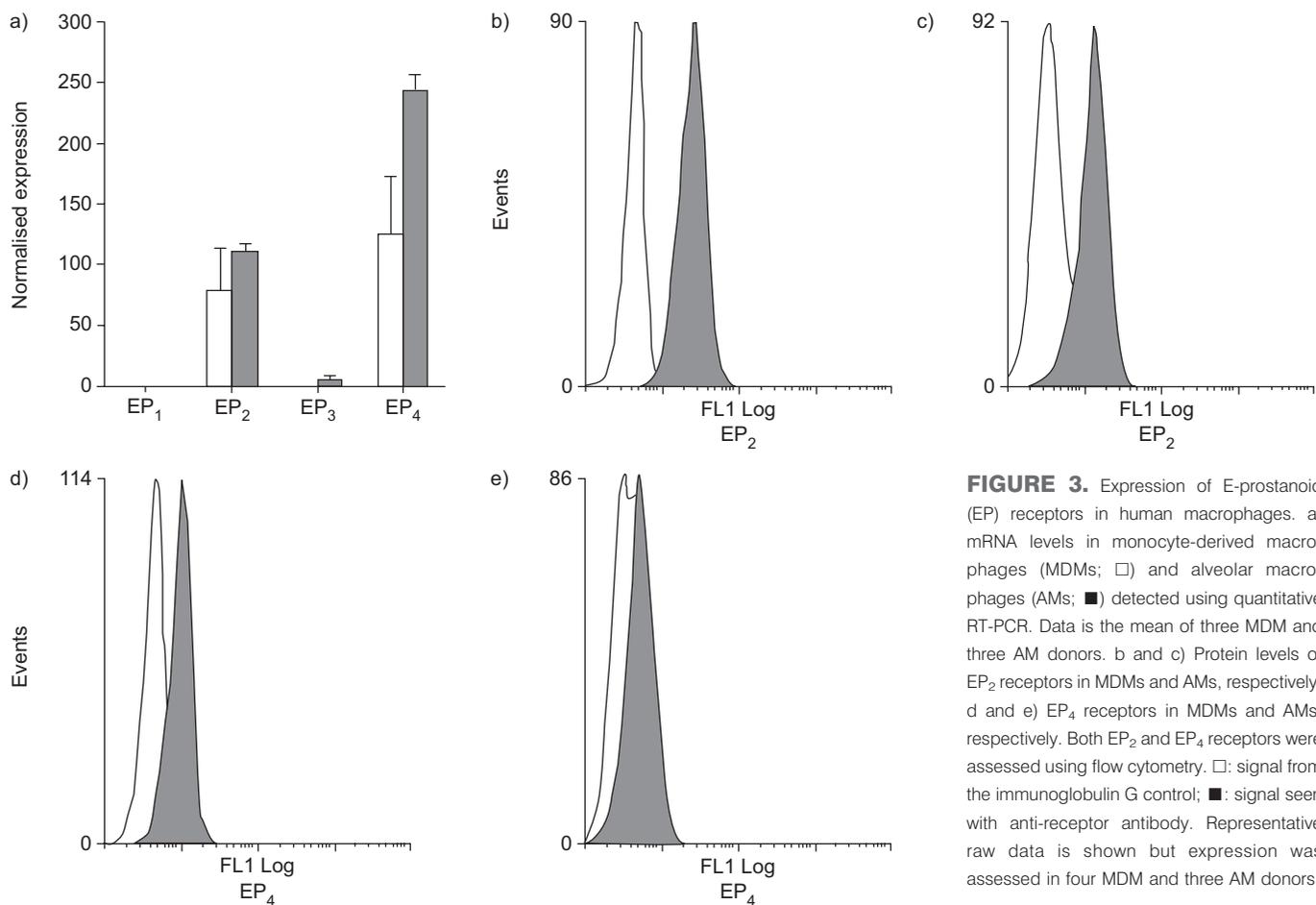


**FIGURE 2.** The effects of E-prostanoid receptor agonists in human monocyte-derived (MDMs) and alveolar macrophages (AMs). E/[A] curves for agonist-induced inhibitions of lipopolysaccharide-stimulated tumour necrosis factor (TNF)- $\alpha$  release from a) MDMs and b) AMs. Agonists used were prostaglandin (PG)<sub>E2</sub> (○), PGE<sub>1</sub>-OH (●), butaprost (□) and sulprostone (■). Data represent the mean of four to seven experiments. Vertical lines indicate the SE. Lines drawn through the MDM data are the result of curve fitting using equation 1. The AM data could not be fitted.

EP<sub>2</sub> (cells with positive expression: MDMs  $96.3 \pm 2.2\%$  (n=4); AMs  $87.7 \pm 5.8\%$  (n=3)) and EP<sub>4</sub> receptors (cells with positive expression: MDMs  $78.5 \pm 7.4\%$  (n=4); AMs  $33.0 \pm 12.4\%$  (n=3)).

#### Characterisation of Ono-AE2-227 in HEK cells stably transfected with human EP<sub>4</sub> or EP<sub>2</sub> receptors

The expression pattern and agonist potency order obtained indicated EP<sub>4</sub> and EP<sub>2</sub> receptor involvement in inhibition of TNF- $\alpha$  release from MDMs and AMs. To provide further evidence for this, a reported selective EP<sub>4</sub>-receptor antagonist, Ono-AE2-227, was synthesised and characterised in HEK cells stably expressing human EP<sub>4</sub> or EP<sub>2</sub> receptors. In the EP<sub>4</sub> system, this compound inhibited PGE<sub>2</sub>-mediated elevations of cAMP and the resulting curve displacements did not deviate significantly from parallelism (ANOVA; fig. 4a). This apparent competitive behaviour was confirmed by analysis of the computed  $[A]_{50}$  values using equation 2, which yielded a slope value of  $1.11 \pm 0.07$  (n=5). This value was not



**FIGURE 3.** Expression of E-prostanoid (EP) receptors in human macrophages. a) mRNA levels in monocyte-derived macrophages (MDMs; □) and alveolar macrophages (AMs; ■) detected using quantitative RT-PCR. Data is the mean of three MDM and three AM donors. b and c) Protein levels of EP<sub>2</sub> receptors in MDMs and AMs, respectively. d and e) EP<sub>4</sub> receptors in MDMs and AMs, respectively. Both EP<sub>2</sub> and EP<sub>4</sub> receptors were assessed using flow cytometry. □: signal from the immunoglobulin G control; ■: signal seen with anti-receptor antibody. Representative raw data is shown but expression was assessed in four MDM and three AM donors.

significantly different from unity and the resulting  $pK_B$  estimate was  $9.17 \pm 0.11$  ( $n=5$ ). Figure 4b shows an example of a Clark plot [26] generated from one of the five experiments.

In contrast, in the EP<sub>2</sub> system this compound did not block the effects of PGE<sub>2</sub> at concentrations  $\leq 1 \mu\text{M}$  (fig. 4c). These data confirmed that Ono-AE2-227 is a potent EP<sub>4</sub>-receptor antagonist with high selectivity over EP<sub>2</sub> receptors.

#### **Effect of Ono-AE2-227 on PGE<sub>2</sub>-induced inhibitions of TNF- $\alpha$ release from MDMs and AMs**

In MDMs, Ono-AE2-227 produced parallel right-ward shifts of the PGE<sub>2</sub> E/[A] curve, indicating involvement of EP<sub>4</sub> receptors in this response, but the interaction was inconsistent with simple competitive behaviour (fig. 5a; compare with fig. 4a). This was confirmed by fitting the  $[A]_{50}$  data to equation 2, which yielded a slope value ( $0.53 \pm 0.03$  ( $n=4$ )), significantly less than unity (data not shown). A theoretical model was used that describes the behaviour of an agonist in a system in which its response is mediated through two distinct receptor types (see Materials and methods section). Figure 5b shows the result of this analysis in which the model-generated curves were super-imposed on to the experimental data. The good accordance of the lines with the data points support the hypothesis: Ono-AE2-227 initially shifts the PGE<sub>2</sub> control curve by blocking EP<sub>4</sub> receptors, but as its concentration is increased the PGE<sub>2</sub> curves hit a "resistant phase", which is most likely the result of EP<sub>2</sub> receptor activation.

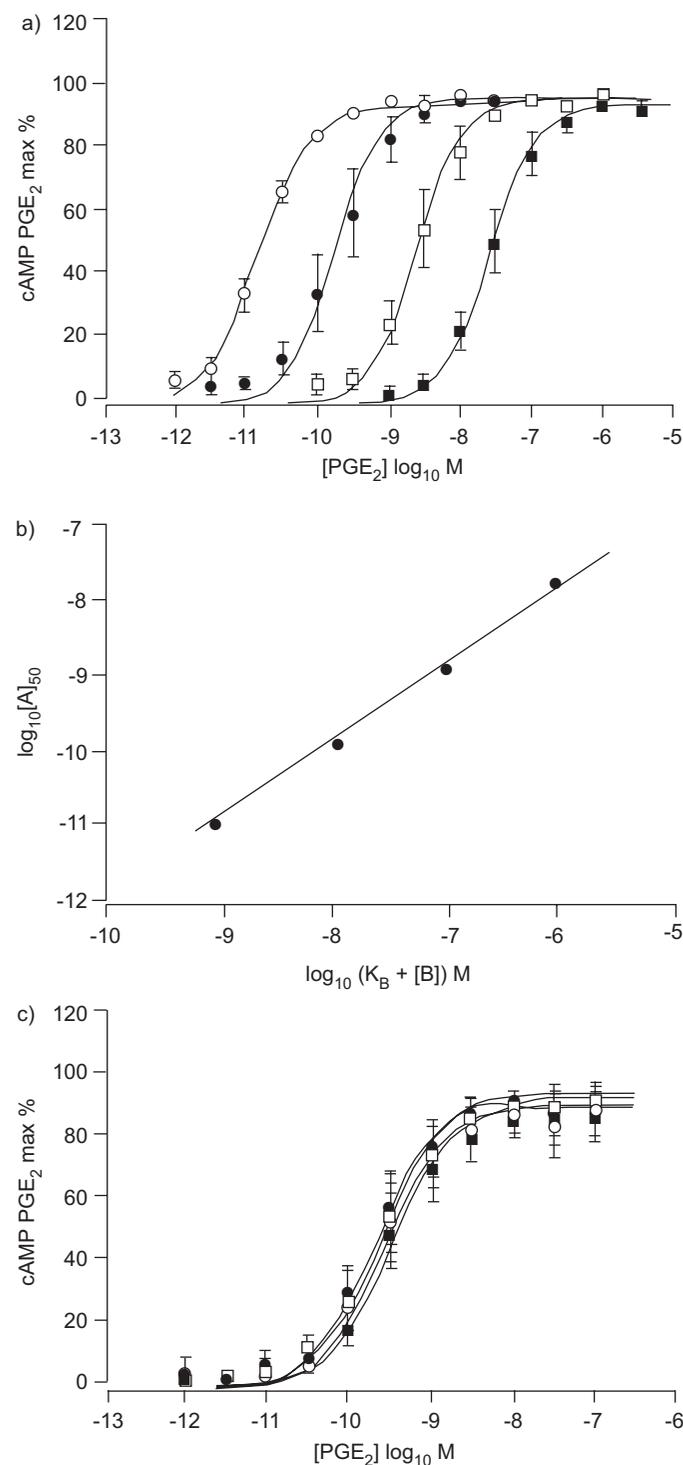
The model parameters used in figure 5b are given in table 1.

In AMs, Ono-AE2-227 amplified the response to LPS in a concentration-dependent manner. Thus, when expressed as a percentage of the response to LPS alone, Ono-AE2-227 elicited "negative inhibitions" of the LPS-induced TNF- $\alpha$  release in the presence of low concentrations of PGE<sub>2</sub> (fig. 6a). Clearly these basal effects of Ono-AE2-227 were complicating the analysis of the data. The most likely explanation for this potentiation was that endogenous PGE<sub>2</sub> released from the AMs had an inhibitory effect on cytokine release and that Ono-AE2-227 blocked this response. Based on this assumption, the experiment was repeated in AMs that had been treated with the cyclo-oxygenase inhibitor, indomethacin. This protocol eliminated the basal effects of Ono-AE2-227 and the resulting data is shown in figure 6b. Under these conditions, inhibition was seen with the EP<sub>4</sub> antagonist but the interaction was again clearly not consistent with simple competition; Clark analysis yielded a slope value of 0.66. Again the data was modelled using the one-agonist, two-receptor model and found good accordance with the experimental data (fig. 6c).

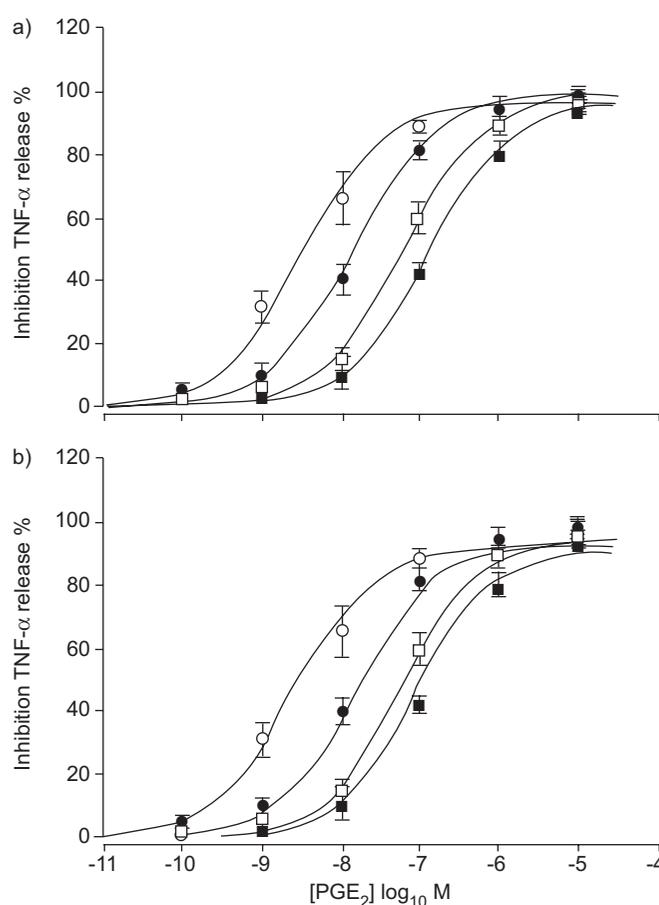
The model parameters used in figure 6b are given in table 2.

#### **DISCUSSION**

To the current authors' knowledge this is the first study to fully characterise the receptors involved in PGE<sub>2</sub>-mediated inhibition of cytokine release in both human MDMs and human



**FIGURE 4.** Effect of Ono-AE2-227 on prostaglandin (PGE<sub>2</sub>)-induced increase in cyclic adenosine monophosphate (cAMP) levels in human embryonic kidney cells stably expressing human E-prostanoid (EP<sub>4</sub> or EP<sub>2</sub>) receptors. E/[A] curves to PGE<sub>2</sub> in the presence of 10–1,000 nM Ono-AE2-227 in a) EP<sub>4</sub>- and c) EP<sub>2</sub>-expressing cells. b) Corresponding [A]<sub>50</sub> data for one of the five experiments shown in a), in Clark plot form. The adherence of the data with the unit slope drawn through them indicates consistency with simple competition. Data series are PGE<sub>2</sub> alone (○) or in the presence of 10 nM (●), 100 nM (□) or 1 μM (■) Ono AE2-227. a and c) Data represented as the mean of five experiments. Vertical lines indicate the SE. Lines drawn through the data are the result of curve fitting using equation 1. The pK<sub>B</sub> value estimated from b) was 9.15.



**FIGURE 5.** Effect of Ono-AE2-227 on prostaglandin (PGE<sub>2</sub>)-induced inhibition of lipopolysaccharide stimulated tumour necrosis factor (TNF)- $\alpha$  release from monocyte-derived macrophages. E/[A] curves to PGE<sub>2</sub> in the presence of 10–1,000 nM Ono-AE2-227. Data series are PGE<sub>2</sub> alone (○) or in the presence of 10 nM (●), 100 nM (□) or 1 μM (■) Ono-AE2-227. Data represent the mean of four experiments. Vertical lines indicate the SE. a) Lines drawn through the data are the results of curve fitting using equation 1. b) Data was fitted to the theoretical model described in the Materials and methods section.

AMs. The agonist potency order obtained in both these systems was similar (fig. 2), suggesting that MDMs are a good surrogate system for AMs. The relatively high potency of PGE<sub>2</sub> and PGE<sub>1</sub>-OH and the activity of butaprost (EP<sub>2</sub> selective) suggested that both EP<sub>4</sub> and EP<sub>2</sub> receptors were present in these cells. This assertion was supported by the biphasic nature of the PGE<sub>2</sub> E/[A] curve in AMs. Such data is consistent with the activation of a mixed receptor population, in which the agent in question exhibits partial agonism, *i.e.* PGE<sub>2</sub> submaximally activates EP<sub>4</sub> and EP<sub>2</sub> receptors in AMs leading to an apparent additive effect. The higher potency phase results from EP<sub>4</sub> receptor activation and the lower potency phase from EP<sub>2</sub> receptor activation. In contrast, the PGE<sub>2</sub> E/[A] curve appears monophasic in MDMs because it behaves as a full agonist in this system, *i.e.* maximum activation of EP<sub>4</sub> receptors at low concentrations means that subsequent activation of EP<sub>2</sub> receptors at higher concentrations cannot produce an additive effect (see tables 1 and 2 for the efficacy values used to simulate the data in MDMs and AMs, respectively).

**TABLE 1** Model parameters used to stimulate effect of Ono-AE2-227 on prostaglandin E<sub>2</sub>-induced inhibition of lipopolysaccharide-stimulated tumour necrosis factor- $\alpha$  release from monocyte-derived macrophages

	Control	10 nM	100 nM	1000 nM
E <sub>m</sub> %	100	100	100	100
n	0.9	0.9	0.9	0.9
$\epsilon_1$	1.0	1.0	1.0	1.0
$\epsilon_2$	1.0	1.0	1.0	1.0
R <sub>1</sub>	10.0	10.0	10.0	10.0
R <sub>2</sub>	10.0	10.0	10.0	10.0
K <sub>A1</sub>	30 nM	210 nM	1.8 $\mu$ M	21 $\mu$ M
K <sub>A2</sub> $\mu$ M	1	1	1	1

E-prostanoid (EP)<sub>4</sub> is receptor 1, EP<sub>2</sub> is receptor 2 in this model. E<sub>m</sub>: maximum possible effect in the system.  $\epsilon_1 = \epsilon_1 R_1$  and  $\epsilon_2 = \epsilon_2 R_2$ , where  $\epsilon$  is intrinsic efficacy and R is receptor concentration. K<sub>A1</sub> and K<sub>A2</sub> are the dissociation constants of the agonist for the two receptors. To simulate the addition of Ono-AE2-227, K<sub>A1</sub> is multiplied by the factor (1+[B]/K<sub>B1</sub>), where [B] is 10, 100 or 1,000 nM and K<sub>B1</sub> is 0.67 nM (as estimated from the experiments in EP<sub>4</sub> transfected human embryonic kidney cells). This factor was also corrected for the protein binding of Ono-AE2-227, which was measured as ~99.8% in human plasma. The effective concentrations of [B] in the presence of 0.5% volume (v)/v FCS were, therefore, 4, 40 and 480 nM. K<sub>A2</sub> is constant, as Ono-AE2-227 does not bind to EP<sub>2</sub> receptors, *i.e.* the factor (1+[B]/K<sub>B2</sub>)=1 at all concentrations used.

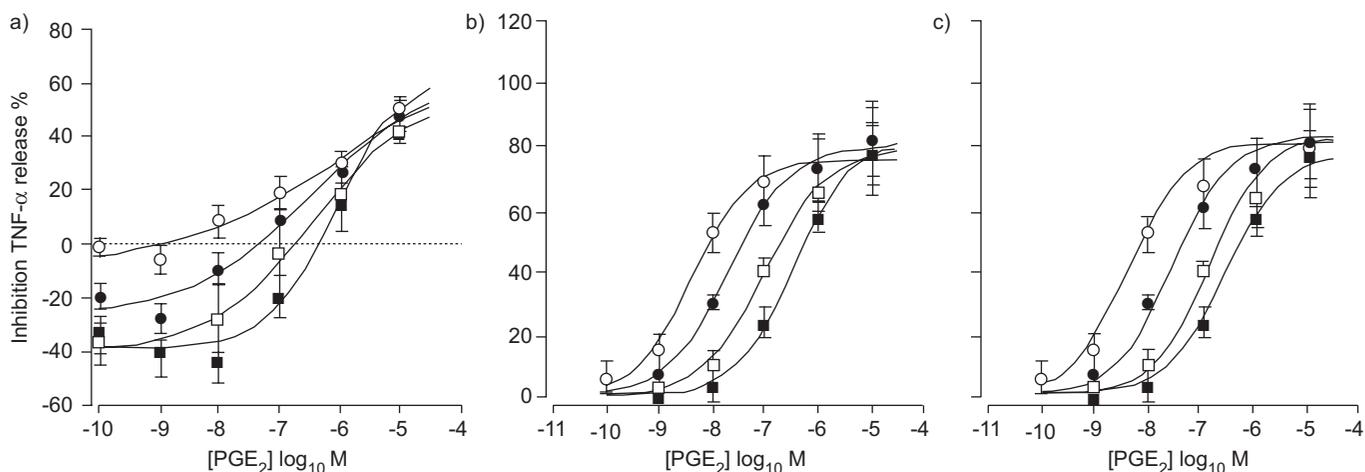
Sulprostone was inactive in both MDMs and AMs, indicating the absence of EP<sub>1</sub> and EP<sub>3</sub> receptors.

The present authors' initial pharmacological findings were supported by expression studies in these cells that demonstrated significant expression of EP<sub>4</sub> and EP<sub>2</sub> receptor mRNA and protein, but barely detectable levels of EP<sub>1</sub> and EP<sub>3</sub> receptor mRNA (fig. 3). These data are similar to those reported in other

human macrophage systems [27], although TAKAYAMA *et al.* [15] found mRNA for only EP<sub>4</sub> in human MDMs. This may be due to the increased sensitivity of the quantitative real-time RT-PCR used in this study over the static end-point RT-PCR method used in the study by TAKAYAMA *et al.* [15].

The EP<sub>4</sub> receptor selective antagonist, Ono-AE2-227, produced right-ward shifts of the PGE<sub>2</sub> E/[A] curve in MDMs that were parallel but clearly did not accord with simple competition at a homogeneous population of receptors (fig. 5a). A theoretical model describing the action of an agonist with two distinct receptor populations was able to accommodate the present data set well (fig. 5b). The finding that low concentrations of Ono-AE2-227 shifted the PGE<sub>2</sub> E/[A] curve indicate that EP<sub>4</sub> receptors are the dominant receptor type mediating the agonist responses. The PGE<sub>2</sub> E/[A] curve obtained in the presence of 1  $\mu$ M Ono-AE2-227 is likely to represent EP<sub>2</sub> activity in MDMs, since >99.9% of the EP<sub>4</sub> receptors are occupied by this concentration of antagonist (fig. 4a).

In the AM system, Ono-AE2-227 clearly potentiated the LPS response in a concentration-dependent manner (fig. 6a). This suggested that these cells were releasing endogenous PGE<sub>2</sub>, a phenomenon that has been reported by other investigators [16, 28]. The current authors did not observe such an effect in the MDM system suggesting that the activation state of these cells may differ. Addition of indomethacin eliminated this basal effect of Ono-AE2-227 in AMs, confirming that it was the result of endogenous prostanoid release. In the presence of indomethacin, Ono-AE2-227 blocked the responses to PGE<sub>2</sub>, but the curve shifts did not conform to the behaviour expected of a one-receptor system. The data could again be accommodated by the theoretical one-agonist, two-receptor model, requiring only a small alteration of the model parameters corresponding to a two- and four-fold lower protein expression level of EP<sub>4</sub> and EP<sub>2</sub> receptors, respectively (compare tables 1 and 2). The lower maximum inhibitions observed in the AMs are consistent with the lower expression of EP<sub>4</sub> and EP<sub>2</sub> receptors



**FIGURE 6.** Effect of Ono-AE2-227 on prostaglandin (PG)E<sub>2</sub>-induced inhibition of lipopolysaccharide stimulated tumour necrosis factor (TNF)- $\alpha$  release from alveolar macrophages. E/[A] curves to PGE<sub>2</sub> in the presence of 10–1,000 nM Ono-AE2-227. Data series are PGE<sub>2</sub> alone ( $\circ$ ) or in the presence of 10 nM ( $\bullet$ ), 100 nM ( $\square$ ) or 1  $\mu$ M ( $\blacksquare$ ) Ono-AE2-227. Data represent the mean of four experiments. Vertical lines indicate the se. b) Experiment was carried out in the presence of 10  $\mu$ M indomethacin. a and b) Lines drawn through the data are the results of curve fitting the mean data using equation 1. c) Data used in (b) was fitted to the theoretical model described in the Materials and methods section.

**TABLE 2** Model parameters used to stimulate effect of Ono-AE2-227 on prostaglandin E<sub>2</sub>-induced inhibition of lipopolysaccharide stimulated tumour necrosis factor- $\alpha$  release from alveolar macrophages

	Control	10 nM	100 nM	1000 nM
E <sub>m</sub> %	100	100	100	100
n	0.8	0.8	0.8	0.8
$\epsilon_1$	1.0	1.0	1.0	1.0
$\epsilon_2$	1.0	1.0	1.0	1.0
R <sub>1</sub>	5.0	5.0	5.0	5.0
R <sub>2</sub>	2.5	2.5	2.5	2.5
K <sub>A1</sub>	30 nM	210 nM	1.8 $\mu$ M	21 $\mu$ M
K <sub>A2</sub> $\mu$ M	1	1	1	1

E-prostanoid (EP)<sub>4</sub> is receptor 1, EP<sub>2</sub> is receptor 2 in this model. E<sub>m</sub>: maximum possible effect in the system.  $e_1 = \epsilon_1 R_1$  and  $e_2 = \epsilon_2 R_2$ , where  $\epsilon$  is intrinsic efficacy and R is receptor concentration. K<sub>A1</sub> and K<sub>A2</sub> are the dissociation constants of the agonist for the two receptors. To simulate the addition of Ono-AE2-227, K<sub>A1</sub> is multiplied by the factor  $(1 + [B]/K_{B1})$ , where [B] is 10, 100 or 1,000 nM and K<sub>B1</sub> is 0.67 nM (as estimated from the experiments in EP<sub>4</sub> transfected human embryonic kidney cells). This factor was also corrected for the protein binding of Ono-AE2-227, which was measured as ~99.8% in human plasma. The effective concentrations of [B] in the presence of 0.5% volume (v/v) FCS were, therefore, 4, 40 and 480 nM. K<sub>A2</sub> is constant, as Ono-AE2-227 does not bind to EP<sub>2</sub> receptors, i.e. the factor  $(1 + [B]/K_{B2}) = 1$  at all concentrations used.

suggested by the modelling and indicated by the present protein expression studies (figs 3b–3e). This may be a consequence of partial receptor downregulation [29] induced by the endogenous release of PGE<sub>2</sub> from these cells.

The current findings are consistent with previously reported data from human monocytes and rodent macrophages, where E-prostanoid<sub>2</sub> and E-prostanoid<sub>4</sub> receptors have been demonstrated to mediate the anti-inflammatory effects of prostaglandin E<sub>2</sub> [11, 12, 30]. By extending these studies to human alveolar macrophages the current authors have provided evidence to suggest that E-prostanoid<sub>4</sub>, E-prostanoid<sub>2</sub> or mixed E-prostanoid<sub>4</sub>/E-prostanoid<sub>2</sub> receptor agonists could be useful anti-inflammatory drugs in diseases such as chronic obstructive pulmonary disease and acute respiratory distress syndrome.

## REFERENCES

- Shapiro SD. The macrophage in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999; 160: 29S–32S.
- Coleman RA, Smith WL, Narumiya S. International union of pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* 1994; 46: 205–229.
- Abramovitz M, Adam M, Boie Y, et al. The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. *Biochim Biophys Acta* 2000; 1483: 285–293.
- Kiriyama M, Ushikubi F, Kobayashi T, Hirata M, Sugimoto Y, Narumiya S. Ligand binding specificities of the eight types and subtypes of the mouse prostanoid

receptors expressed in Chinese hamster ovary cells. *Br J Pharmacol* 1997; 122: 217–224.

- Schultz RM, Pavlidis NA, Stylos WA, Chirigos MA. Regulation of macrophage tumoricidal function: a role for prostaglandins of the E series. *Science* 1978; 202: 320–321.
- Schultz RM, Pavlidis NA, Stoychkov JN, Chirigos MA. Prevention of macrophage tumoricidal activity by agents known to increase cellular cyclic AMP. *Cell Immunol* 1979; 42: 71–78.
- Rossi AG, McCutcheon JC, Roy N, Chilvers ER, Haslett C, Dransfield I. Regulation of macrophage phagocytosis of apoptotic cells by cAMP. *J Immunol* 1998; 160: 3562–3568.
- Aronoff DM, Canetti C, Peters-Golden M. Prostaglandin E<sub>2</sub> inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *J Immunol* 2004; 173: 559–565.
- Christman JW, Christman BW, Shepherd VL, Rinaldo JE. Regulation of alveolar macrophage production of chemoattractants by leukotriene B<sub>4</sub> and prostaglandin E<sub>2</sub>. *Am J Respir Cell Mol Biol* 1991; 5: 297–304.
- Ozaki T, Moriguchi H, Nakamura Y, Kamei T, Yasuoka S, Ogura T. Regulatory effect of prostaglandin E<sub>2</sub> on fibronectin release from human alveolar macrophages. *Am Rev Respir Dis* 1990; 141: 965–969.
- Akaogi J, Yamada H, Kuroda Y, Nacionales DC, Reeves WH, Satoh M. Prostaglandin E<sub>2</sub> receptors EP<sub>2</sub> and EP<sub>4</sub> are up-regulated in peritoneal macrophages and joints of pristane-treated mice and modulate TNF- $\alpha$  and IL-6 production. *J Leukoc Biol* 2004; 76: 227–236.
- Treffkorn L, Scheibe R, Maruyama T, Dieter P. PGE<sub>2</sub> exerts its effect on the LPS-induced release of TNF- $\alpha$ , ET-1, IL-1 $\alpha$ , IL-6 and IL-10 via the EP<sub>2</sub> and EP<sub>4</sub> receptor in rat liver macrophages. *Prostaglandins Other Lipid Mediat* 2004; 74: 113–123.
- Renz H, Gong JH, Schmidt A, Nain M, Gerns D. Release of tumor necrosis factor- $\alpha$  from macrophages. Enhancement and suppression are dose-dependently regulated by prostaglandin E<sub>2</sub> and cyclic nucleotides. *J Immunol* 1988; 141: 2388–2393.
- Strassmann G, Patil-Koota V, Finkelman F, Fong M, Kambayashi T. Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E<sub>2</sub>. *J Exp Med* 1994; 180: 2365–2370.
- Takayama K, Garcia-Cardena G, Sukhova GK, Comander J, Gimbrone MA Jr, Libby P. Prostaglandin E<sub>2</sub> suppresses chemokine production in human macrophages through the EP<sub>4</sub> receptor. *J Biol Chem* 2002; 277: 44147–44154.
- Monick M, Glazier J, Hunninghake GW. Human alveolar macrophages suppress interleukin-1 (IL-1) activity via the secretion of prostaglandin E<sub>2</sub>. *Am Rev Respir Dis* 1987; 135: 72–77.
- Standiford TJ, Kunkel S, Rolfe MW, Evanoff HL, Allen RM, Strieter RM. Regulation of human alveolar macrophage and blood monocyte-derived interleukin-8 by prostaglandin E<sub>2</sub> and dexamethasone. *Am J Respir Cell Mol Biol* 1992; 6: 75–81.
- Rowe J, Finlay-Jones JJ, Nicholas TE, Bowden J, Morton S, Hart PH. Inability of histamine to regulate TNF- $\alpha$

- production by human alveolar macrophages. *Am J Respir Cell Molecular Biol* 1997; 17: 218–226.
- 19 Kitamura T, Itoh M, Noda T, *et al.* Combined effects of prostaglandin E receptor subtype EP1 and subtype EP4 antagonists on intestinal tumorigenesis in adenomatous polyposis coli gene knockout mice. *Cancer Sci* 2003; 94: 618–621.
  - 20 Trist DG, Leff P. Quantification of H<sub>2</sub>-agonism by clonidine and dimaprit in an adenylate cyclase assay. *Agents Actions* 1985; 16: 222–226.
  - 21 Furchgott RF. Adrenergic and dopaminergic peripheral receptors. Proceedings of the 4th Meeting on Adrenergic Mechanisms. Porto, Portugal, 1981; pp. 10–29.
  - 22 Furchgott RF. The use of B-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agpmost complexes. *Adv Drug Res* 1966; 3: 21–55.
  - 23 Stephenson RP. A modification of receptor theory. *Br J Pharmacol Chemother* 1956; 11: 379–393.
  - 24 Coleman RA, Grix SP, Head SA, Louttit JB, Mallett A, Sheldrick RLG. A novel inhibitory prostanoid receptor in piglet saphenous vein. *Prostaglandins* 1994; 47: 151–168.
  - 25 Regan JW, Bailey TJ, Pepperl DJ, *et al.* Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP2 subtype. *Mol Pharmacol* 1994; 46: 213–220.
  - 26 Stone M, Angus JA. Developments of computer-based estimation of pA<sub>2</sub> values and associated analysis. *J Pharmacol Exp Ther* 1978; 207: 705–718.
  - 27 Blaschke V, Jungermann K, Puschel GP. Exclusive expression of the Gs-linked prostaglandin E2 receptor subtype 4 mRNA in human mononuclear Jurkat and KM-3 cells and coexpression of subtype 4 and 2 mRNA in U-937 cells. *FEBS Lett* 1996; 394: 39–43.
  - 28 Beusenberg FD, Hoogsteden HC, Bonta IL, van Amsterdam JGC. Cyclic AMP enhancing drugs modulate eicosanoid release from human alveolar macrophages. *Life Sci* 1994; 54: 1269–1274.
  - 29 Nishigaki N, Negishi M, Ichikawa A. Two Gs-coupled prostaglandin E receptor subtypes, EP2 and EP4, differ in desensitization and sensitivity to the metabolic inactivation of the agonist. *Mol Pharmacol* 1996; 50: 1031–1037.
  - 30 Meja KK, Barnes PJ, Giembycz MA. Characterization of the prostanoid receptor(s) on human blood monocytes at which prostaglandin E2 inhibits lipopolysaccharide-induced tumour necrosis factor- $\alpha$  generation. *Br J Pharmacol* 1997; 122: 149–157.