

**The prostaglandin EP4 receptor is a master regulator of the  
expression of PGE<sub>2</sub> receptors following  
inflammatory activation in human monocytic cells**

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## **Abstract**

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is responsible for inflammatory symptoms. However, PGE<sub>2</sub> also suppresses pro-inflammatory cytokine production. There are at least 4 subtypes of PGE<sub>2</sub> receptors, EP1 – EP4, but it is unclear which of these specifically control cytokine production. The aim of this study was to determine which of the different receptors, EP1R - EP4R modulate production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in human monocytic cells.

Human blood, or the human monocytic cell line THP-1 were stimulated with LPS. The actions of PGE<sub>2</sub>, alongside both selective agonists of EP1 – EP4 receptors, were assessed on LPS-induced TNF- $\alpha$  release. The expression profiles of EP2R and EP4R in monocytes and THP-1 cells were characterised by RT-qPCR. In addition, the production of TNF- $\alpha$  protein was evaluated following knockdown of the receptors using siRNA and over-expression of the receptors by transfection with constructs.

PGE<sub>2</sub> and also EP2 and EP4 agonists (but not EP1 or EP3 agonists) suppressed TNF- $\alpha$  production in blood and THP-1 cells. LPS also up regulate expression of EP2R and EP4R but not EP1 or EP3. siRNA for either EP2R or EP4R reversed the suppressive actions of PGE<sub>2</sub> on cytokine production and overexpression of EP2R and EP4R enhanced the suppressive actions of PGE<sub>2</sub>.

This indicates that PGE<sub>2</sub> suppression of TNF- $\alpha$  by human monocytic cells occurs via EP2R and EP4R expression. However EP4Rs also control their own expression and that of EP2 whereas the EP2R does not affect EP4R expression. This implies that EP4 receptors have an important master role in controlling inflammatory responses.

## INTRODUCTION

PGE<sub>2</sub> is a major mediator present at sites of inflammation [1]. It is well established that PGE<sub>2</sub> contributes to the localised and systemic symptoms of inflammation. A key study by Portanova *et al.* [2] demonstrated that anti-PGE<sub>2</sub> antibodies were able to reverse the swelling and pain in localized inflammation. This directly connects PGE<sub>2</sub> to the development of inflammatory symptoms. The major pro-inflammatory cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 (IL-1), which are produced in response to inflammatory stimuli such as lipopolysaccharide, sequentially induce PGE<sub>2</sub> biosynthesis from macrophages in a concentration-dependent manner [3]. However, other early studies also showed that exogenous PGE<sub>2</sub> potently suppressed the production of TNF- $\alpha$  from monocytic cells (at the transcriptional level) and the suppression of endogenous PGE<sub>2</sub> production (using cyclooxygenase inhibitors) enhanced TNF- $\alpha$  synthesis clearly indicated that the downstream release of PGE<sub>2</sub> is a negative-feedback loop [4, 5]. It is now recognised that prostaglandins in general, and PGE<sub>2</sub> in particular, play a cytoprotective and controlling role in limiting immune and inflammatory activation and consequent pathology [6]. Thus, the suppressive action of PGE<sub>2</sub> on TNF- $\alpha$  production is an important axis in limiting the extent of an inflammatory response. The actions of PGE<sub>2</sub> are mediated via at least 4 membrane receptors termed EP1 – EP4, each with its particular signalling system some of which overlap [6]. Suppression of TNF- $\alpha$  production in monocytic cells can occur via EP2 receptors on the basis of a variety of agonists and antagonists of the respective receptors [1]. In the study of Brown *et al.* [1] it was also shown that the inhibition of TNF- $\alpha$  production by PGE<sub>2</sub> could be reversed to an extent by an EP4 receptor antagonist. However, it is very clear that these compounds have greatly overlapping activities with relatively poor selectivity between EP receptor subtypes, particularly to distinguish clearly between EP2 and EP4 receptors. Thus, an alternative approach is required to attempt to delineate which precise EP receptors are involved in controlling monocyte-derived cytokine production, particularly TNF- $\alpha$ .

Little information is available regarding the expression of the different EP receptor subtypes on

human monocytic cells, particularly those that are involved in downregulating cytokine production. The receptors are controlled by the respective genes for each receptor i.e. PTGER1 – PTGER4 for EP1 – EP4 receptors respectively. This study attempts to correlate the functional characteristics of suppressing LPS-induced TNF- $\alpha$  production in monocytes by various PGE<sub>2</sub> analogues (agonists) to the expression of PTGER genes particularly PTGER2 and PTGER4.

This study reports that PGE<sub>2</sub> can control the expression of EP2 and EP4 receptors and that EP4-selective agonists mimic this response whereas EP2 agonists are unable to do so. Coupled with the observation that overexpression of EP4 receptors enhances PGE<sub>2</sub> suppression of TNF- $\alpha$  production and siRNA silencing of EP4 receptor expression elevates TNF- $\alpha$  and IL-1 $\beta$  production but not the production of the anti-inflammatory cytokine IL-10, this implies that the regulation of EP receptor expression in monocytes occurs primarily via EP4 receptors and that they may be important controllers of inflammatory responses. In addition, it also indicates that gene expression of the receptors has direct functional consequences indicating a complex dynamic control during inflammatory responses.

## **MATERIALS AND METHODS**

### ***Cell culture***

#### ***Preparation of THP-1 cells***

THP-1 cells were incubated in flasks containing RPMI 1640 with 10% (v/v) fetal calf serum (FCS) at 37°C, 100% humidity, 5% (v/v) CO<sub>2</sub>. Cells were passaged when they reached 80-90% confluence then collected for experiments by transferring them into 30ml sterile tubes followed by centrifugation at 400 x g for 5 minutes. The supernatant was discarded and RPMI 1640 was added to resuspend the cells. THP-1 cells were used at a concentration of  $1 \times 10^6$  cells/ml. Cells were maintained at 37°C, 5% CO<sub>2</sub>.

#### ***Human blood incubation protocol***

Whole human blood (700µl) was added to sterile 1 ml microcentrifuge tubes. To each microcentrifuge tube, different compounds were added in a volume of 100 µl to yield the various concentrations indicated in the results section. The incubations were all carried out in a final total volume of 1 ml. The samples were then gently vortex mixed and placed in an incubator for 22 hours at 37°C, 100% humidity, 5% CO<sub>2</sub> with the tubes remaining opened to allow CO<sub>2</sub> access. Following incubation, the sample tubes were centrifuged at 12,000 x g for 40 seconds after which the plasma was transferred into fresh microcentrifuge tubes and stored at -20°C until required for the assay.

#### ***THP-1 cells incubation protocol***

THP-1 cells ( $1 \times 10^6$  cells/ml) were added into 24-well or 6-well culture plates. Different treatments such as LPS, PGE<sub>2</sub> etc., were always added in a volume of 50 µl (in order to yield the appropriate final concentrations indicated in the results section) in a final volume of 0.5 ml in each well of 24-well plates. In the 6-well plates, additions were adjusted to 200 µl in a final volume of 2 ml. The plates were then incubated at 37°C, 100 % humidity, 5% CO<sub>2</sub> for 22 hours.

Following incubation, all samples were transferred into microcentrifuge tubes and stored at -20°C until required for the assay.

### ***Measurement of cytokines***

The production of TNF- $\alpha$ , IL-1 $\beta$  and IL-10 was measured by sandwich ELISA assay as described previously [1].

### ***Statistical analysis of ELISA data***

All data was analysed statistically using Statview software (SAS Institute, North Carolina, USA). Levels of analytes were calculated from standard curves using 3<sup>rd</sup> order polynomial regression analysis. IC<sub>50</sub> values for the inhibition of cytokine production were obtained using GraphPad Prism Version 5.0 (GraphPad Software Inc., California, USA). ANOVA tests (Fishers PLSD) were used to ascertain statistically significant changes in responses, with P < 0.05 regarded as a significant change.

### ***Transient cell transfection***

THP-1 cells were transfected with either siRNA duplexes from a PTGER4 TriFECTa RNAi kit (Integrated DNA technologies, Leuven, Belgium) for the knock-down study or Flag-PTGER4 plasmid for the overexpression study. The nucleotide sequences for PTGER4 and negative control siRNA duplexes are shown in Table 1. THP-1 cells (1x10<sup>6</sup> cells/ml) were placed into 6-well plates and incubated with different treatment in a final volume of 2 ml with either 5  $\mu$ l of 100nM siRNA duplexes for knock-down assays or 5  $\mu$ l of FLAG-PTGER4 plasmid (1904 ng/ $\mu$ l) for overexpression assays in the presence of 3  $\mu$ l of transfection reagent (HiPerFect; Qiagen, UK)). Untransfected cells were used as controls. Also, in knock-down experiments, two negative control duplex siRNA (NC1, NC5) and a positive control were used. In overexpression experiments, an empty expression vector that did not contain a PTGER4 insert was prepared and used as a control. Plates were incubated at 37°C, 100 % humidity, 5% CO<sub>2</sub> for different time points (3h-48h). Primary optimisation experiments were performed using different concentrations of all three siRNA duplexes (1nM, 10nM and 100nM), FLAG-PTGER4 (1904

ng/ $\mu$ l, 952 ng/ $\mu$ l and 476 ng/ $\mu$ l) and empty vector (2461 ng/ $\mu$ l, 1230 ng/ $\mu$ l and 615 ng/ $\mu$ l).

Duplex No.	Sequence
PTGER 4Duplex 1	Forward 5'- rGrCrArGrUrUrGrUrArCrCrArArGrUrGrArArArUrUrArUTT-3'
	Reverse 5'- rArArArUrArArUrUrUrCrArCrUrUrGrGrUrArCrArArCrUrGrCrUrU-3'
PTGER 4Duplex 2	Forward 5'- rArGrUrGrCrUrCrArGrUrArArArGrCrArArUrArGrArGrAAG-3'
	Reverse 5'- rCrUrUrCrUrCrUrArUrUrGrCrUrUrUrArCrUrGrArGrCrArCrUrGrU-3'
PTGER 4Duplex 3	Forward 5'- rArGrArUrArUrUrArGrArArArGrGrCrUrCrUrArUrUrCrCAA-3'
	Reverse 5'- rUrUrGrGrArArUrArGrArGrCrCrUrUrUrCrUrArArUrArUrCrUrGrG-3'
NC1 Control	Forward 5'- rCrUrUrCrCrUrCrUrCrUrUrUrCrUrCrUrCrCrCrUrUrGrUGA-3'
	Reverse 5'- rUrCrArCrArArGrGrGrArGrArGrArArArGrArGrArGrGrArArGrGrA-3'
NC5 Control	Forward 5'- rCrArUrArUrUrGrCrGrCrGrUrArUrArGrUrCrGrCrGrUrUAG -3'
	Reverse 5'- rCrUrArArCrGrCrGrArCrUrArUrArCrGrCrGrCrArArUrArUrGrGrU-3'

Tale 1. Nucleotide sequences of siRNA duplexes used for transfection of THP-1 cells



### ***Flag-PTGER4 Plasmid***

One Shot chemically competent TOP10 *E.coli* cells (Invitrogen, UK)) were transformed with Flag-PTGER4 (Integrated DNA technologies, Leuven, Belgium) plasmid DNA (OmicsLink™ expression clone EX-Q0086-M11; GeneCopoeia, Rockville, USA) was obtained and propagated with Carbenicillin-selective (100 mg/ml) agar plates and Luria-bertani broth (LB). Plasmid DNA was prepared from the cultures using an EndoFree Plasmid Maxi kit (Qiagen, UK). The sequence of the Flag-PTGER4 plasmid confirmed by automated DNA sequencing (GATC Biotech, Cologne, Germany).

### **Creation of empty vector control DNA for transfection**

The Flag-PTGER4 was provided in a pReceiver-M11 expression vector. An empty expression vector transfection control DNA was needed for this study. This was created from the Flag-PTGER4 plasmid DNA using site-directed mutagenesis and restriction digestion to obtain a pReceiver-M11 plasmid DNA. The plasmid DNA was purified using an EndoFree Plasmid Maxi kit (Qiagen, Manchester, UK). The sequence of the pReceiver-M11 plasmid was confirmed by automated DNA sequencing.

### ***Gene expression analysis***

To ensure good reproducibility and reliable data, the qRT-PCR assays were conducted using practices laid out in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [7].

### ***Cell harvesting and total RNA isolation***

Confluent monocytic cells or THP-1 cells ( $2 \times 10^6$ ) were placed in 6-well plates and incubated with the different treatments to give a final volume of 2 ml. After the incubation period, total RNA was isolated from cells using the Sigma Aldrich's GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Dorset, UK) following the manufacturer's instructions. On-column DNase digestion with the RNase-Free DNase Set (Sigma-Aldrich, Dorset, UK) was used to reduce potential carryover genomic DNA contamination. RNA concentration was then

measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Paisley, UK). All of the extracted RNA samples used for PCR had  $A_{260/230}$  and  $A_{260/280}$  purity ratios of around 2.0.

### ***cDNA synthesis (Reverse Transcription (RT))***

To quantify the mRNA transcripts of target genes by PCR, total RNA was reverse-transcribed to complementary DNA (cDNA) using a Tetro cDNA Synthesis Kit (Bioline, London, UK) following the manufacturer's manual. Total RNAs were normalised to 1  $\mu$ g per synthesis and oligo-dT used to prime the synthesis reaction. The reactions were incubated for 30 min at 42°C, and the reaction inactivated by heating to 85°C for 5 min. All these samples were labelled as (RT+). The resulting cDNA was then used as the DNA template for quantitative real-time PCR for gene expression analysis. To check for the presence of any remaining contaminating genomic DNA, an additional reaction tube, labelled (RT-) was prepared in parallel for each RNA samples to be used as negative controls in the PCR assays. These samples (RT-) contained all of the cDNA synthesis components and total RNA except for the Tetro reverse transcriptase.

### ***Reverse-Transcribed Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)***

RT-qPCR primers were designed against their human mRNA targets and synthesised by Integrated DNA Technologies (Leuven, Belgium) or Eurofins MWG Operon (B2M, ACTB, HPRT1; Ebersberg, Germany). Their primers sequenced and product sizes are shown in Table 2. The RT-qPCR assay was performed by placing the samples in fast reaction PCR MicroAmp tubes (Applied Biosystems, Paisley, UK). The PCR reactions were carried out in 20  $\mu$ l volumes containing 19  $\mu$ l of PCR master mix and 1  $\mu$ l of each template cDNA sample in MicroAmp<sup>TM</sup> Fast Reaction PCR tubes (Applied Biosystems, Paisley, UK). The PCR master mix (1x) contained 1  $\mu$ l of Primer Forward (10 pmol/ $\mu$ l), 1  $\mu$ l of Primer Reverse (10 pmol/ $\mu$ l), 10  $\mu$ l of Applied Biosystems SYBR<sup>®</sup> Select Master Mix (Life Technologies, Paisley) and 7  $\mu$ l RNase-free water. Three technical and biological replicates were conducted for each assay. Thermal cycling using the SYBR Select Standard Cycling Mode with a combined Anneal and Extend temperature of 60°C over 40 cycles and detection was performed on an Applied Biosystems StepOne Plus

real-time PCR system. The gene expression levels, represented by the cycle threshold (Ct) values, were obtained for each of the reactions. Since this reaction was a SYBR Green-based chemistry, a melt–curve analysis was required to follow the amplification. This was to ensure that the PCR reaction only produced a single amplicon and not off-target, multiple products such as primer dimers or misprimers which may compromise the gene expression quantification.

### ***ΔΔCt Relative Quantification***

The quantification method selected to validate PCR results was the relative quantification (ΔΔCt) method [8]. This method normalises Ct values of the target gene to Ct values of the endogenous reference gene in order to demonstrate the fold changes ( $2^{-\Delta\Delta C_t}$ ) in gene expression among control and treated samples. A number of candidate reference genes (PPIB, TBP, GAPDH, RPL37A, B2M, ACTB and HPRT1) were screened for their stability and suitability as normalisers using RefFinder [9].

Primer Name	Sequence	PCR Product Size (bp)
PTGER1	Forward: 5'- CATGGTGGTGTGTCGTGCATC -3' Reverse: 5'- TGTACACCCAAGGGTCCAG -3'	149
PTGER2	Forward: 5'- CCTCATTCTCCTGGCTATCATG-3' Reverse: 5'- CTTTCGGGAAGAGGTTTCATTC-3'	94
PTGER3	Forward: 5'- AACTATGCATCCAGCTCCAC -3' Reverse: 5- CAGTTGCCCTCTGTATCTGAG -3'	144
PTGER4	Forward: 5'- ATCTTACTCATTGCCACCTCC-3' Reverse: 5'- TGACTTCTCGCTCCAAACTTG-3'	106

PPIB	Forward: 5'- ACCTACGAATTGGAGATGAAGATG -3' Reverse: 5'- GTCCTTGATTACACGATGGAATTTG -3'	152
TBP	Forward: 5'- CTGGTTTGCCAAGAAGAAAGTG -3' Reverse: 5'- GGTCAGTCCAGTGCCATAAG -3'	145
GAPDH	Forward: 5'- ACATCGCTCAGACACCATG-3' Reverse: 5'- TGTAGTTGAGGTCAATGAAGGG-3'	143
RPL37A	Forward: 5'- TGCATGAAGACAGTGGCTG -3' Reverse: 5'- CCAGTGATGTCTCAAAGAGTAGAG -3'	132
Flag- PTGER4	Forward: 5'- AAAGCAGGCTTGAAGGAGTTTCG - 3' Reverse: 5'- CCACGATGGCCACCAGGTTG -3'	148
B2M (Eurofins MWG Operon, Ebersberg/ Germany)	Forward: 5'- AGATGAGTATGCCTGCCGTGTGAAC-3' Reverse: 5'- CAAATGCGGCATCTTCAAACCTC-3'	163
	Forward: 5'- ATTGCCGACAGGATGCAGAA-3'	150

ACTB (Eurofins MWG Operon, Ebersberg/ Germany)	Reverse: 5'- GCTGATCCACATCTGCTGGAA-3'	
HPRT1 (Eurofins MWG Operon, Ebersberg/ Germany)	Forward: 5'- CCCTGGCGTCGTGATTAGTGATG-3' Reverse: 5'- CGAGCAAGACGTTTCAGTCCTGTCC- 3'	119

Table 2. Nucleotide sequences of the primers used for the analysis of gene expression by RT-qPCR).

***Statistical analysis of PCR data***

All data was analysed statistically using GraphPad Prism Version 5.0 (GraphPad Software Inc., California, USA). In all experiments values were mean  $\pm$  s.d. for three separate observations. ANOVA tests were used to ascertain a change with  $P < 0.05$  regarded as a significant change.

## RESULTS

### *Effect of LPS on TNF- $\alpha$ production in whole blood, peripheral blood monocytes and THP-1 cells.*

LPS induced a concentration-dependent increase in TNF- $\alpha$  production in whole blood incubations, monocytes and THP-1 cells. The maximal increases in TNF- $\alpha$  production occurred with LPS concentrations between 10  $\mu\text{g/ml}$  – 100  $\mu\text{g/ml}$  in all 3 preparations. The EC<sub>50</sub> concentrations of LPS for whole blood incubations, monocytes and THP-1 cells were 2.9  $\mu\text{g/ml}$ , 1.9  $\mu\text{g/ml}$  and 1.2  $\mu\text{g/ml}$  respectively. In all subsequent experiments LPS was used at a close-to-EC<sub>50</sub> LPS concentration of 1  $\mu\text{g/ml}$  to induce TNF- $\alpha$  production.

### *Effect of PGE<sub>2</sub> and its analogues on TNF- $\alpha$ , IL-10 and IL-1 $\beta$ production in response to LPS.*

The ability of PGE<sub>2</sub> and the various EP receptor-selective agonists of PGE<sub>2</sub> to modulate TNF- $\alpha$  production in the different cell systems in response to stimulation with LPS were compared.

Cells or blood were incubated with PGE<sub>2</sub> or analogues in the presence or absence of LPS (1  $\mu\text{g/ml}$ ) and TNF- $\alpha$  levels in cell supernatants or plasma were measured by ELISA.

The LPS-induced elevation in TNF- $\alpha$  levels in blood, normal monocytes and THP-1 cells were suppressed in a concentration-dependent manner in the presence of either, PGE<sub>2</sub>, butaprost (EP2-selective) or L-902,688 (EP4-selective). Table 3 shows the IC<sub>50</sub> values calculated from the concentration-response curve data. All 3 agonists produced a similar suppressive response on TNF- $\alpha$  production. In general PGE<sub>2</sub> showed a lower IC<sub>50</sub> than butaprost in all 3 preparations. In THP-1 cells the IC<sub>50</sub> was in the order of 2-fold lower for L-902,688 than PGE<sub>2</sub> but approximately 13-fold higher in blood (table 3).

The actions of agonists of all 4 EP receptors on the LPS-induced elevation in TNF- $\alpha$  levels were directly compared. The following selective EP-receptor agonists were used (all at 1  $\mu\text{M}$ ); sulprostone (EP1/EP3), L-902,688 (EP4) and butaprost (EP2) and compared directly to PGE<sub>2</sub>. Incubation with either L-902,688 or butaprost resulted in a decrease in the LPS-stimulated

elevation of TNF- $\alpha$  levels. However, there was no effect of sulprostone on LPS-stimulated TNF- $\alpha$  (Fig. 1).

The effects of L-902,688 and butaprost were also studied on the LPS-stimulated production of both IL-10 and IL-1 $\beta$ . Butaprost was able to suppress LPS-stimulated IL-1 $\beta$  and IL-10 production in a concentration-dependent manner (Fig. 2). Similarly, L-902,688 also inhibited LPS-stimulated IL-1 $\beta$  production in a concentration-dependent manner, however, there was little effect on LPS-stimulated IL-10 production (Fig. 2) with a small suppression at the highest concentration used (10  $\mu$ M).

#### ***Expression of mRNA for PTGER2 and PTGER4 in monocytic cells.***

The expression of the EP4 receptor (*PTGER4*) gene is shown in Fig. 3. THP-1 cells were incubated and processed as described previously. Incubation of THP-1 cells with LPS resulted in an up-regulation of *PTGER4* mRNA. PGE<sub>2</sub> induced a significant reduction in this high level of receptor expression (fold stimulation reduced from  $2.8 \pm 0.6$  to  $1.3 \pm 0.1$ ,  $p < 0.0001$ ).

In a separate experiment, to determine potential regulation of *PTGER4* by the EP4 receptor, cells were incubated with the EP4 agonist L-902,688. L-902,688 also decreased the expression of *PTGER4* in response to LPS (the fold stimulation decreased from  $7.2 \pm 0.6$  to  $0.7 \pm 0.1$ ,  $p < 0.0001$ ). However, butaprost did not affect the expression of *PTGER2* in LPS-stimulated THP-1 cells. The expression level in response to LPS alone and the expression level with butaprost (fold stimulation in response to LPS was  $20.0 \pm 3.5$ -fold and  $20.0 \pm 2.1$  in the presence of butaprost).

### ***Cross regulation of PTGER2 and PTGER4 by EP2R and EP4R agonists***

*PTGER2* expression was also evaluated in response to the EP4 agonist L-902, 688 (Fig. 3). LPS stimulation resulted in an increase in *PTGER2* expression compared to control. A downregulation ( $p < 0.0001$ ) of *PTGER2* by  $\text{PGE}_2$  was observed. This reduction in *PTGER2* mRNA (in response to LPS) was comparable to the suppression ( $P < 0.0001$ ) by L-902, 688 (Fig. 3).

Having established that L-902, 688 reduced both *PTGER2* and *PTGER4* expression, conversely it was decided to ascertain the effect of butaprost (EP2 agonist) on the expression of *PTGER4*. Although  $\text{PGE}_2$  was able to down-regulate *PTGER4* expression ( $P < 0.0001$ ) after LPS stimulation butaprost did not significantly reduce the expression of *PTGER4* (Fig. 3).

### ***The effect of PTGER4 siRNA on TNF- $\alpha$ production in THP-1 cells***

Having established that the expression of *PTGER4* could be downregulated only by EP4R agonists ( $\text{PGE}_2$  and L-902, 688) it was decided to ascertain whether this was merely at the level of gene regulation or whether this also has actual functional consequences. This was achieved by using siRNA directed toward the *PTGER4* gene and measuring TNF- $\alpha$ , IL-1 $\beta$  and IL-10 production in THP-1 cells.

The production of TNF- $\alpha$  was determined during different time points (0h, 6h, 24h and 48h) each compared to empty vector at the appropriate time points, following transfection with siRNA (Fig. 4). 0h represented TNF- $\alpha$  production immediately before transfection of THP-1 cells with siRNA. At this time point (0h), in response to LPS, the TNF- $\alpha$  level increased compared to control (empty vector). However, LPS in the presence of  $\text{PGE}_2$  decreased TNF- $\alpha$  production. After 6h siRNA post-transfection, there was no clear difference in TNF- $\alpha$  production compared to 0h (before siRNA transfection). At 24h of stimulation, in response to LPS, there was an increase in the TNF- $\alpha$  level compared to non-stimulated cells (0h), while TNF- $\alpha$  production did not change in response to LPS/  $\text{PGE}_2$ . After 48h of siRNA transfection, the TNF- $\alpha$  level was higher than earlier time points in response to both LPS and LPS/  $\text{PGE}_2$ .



The levels may have increased further but later time points were not studied. TNF- $\alpha$  production increased significantly compared to cells before transfection (Fig. 4). Using a negative control (random siRNA), there was also a small increase in TNF- $\alpha$  levels with a different time course but it was not as large an increase as occurred with the positive control. The effect of siRNA (directed toward the *PTGER4* gene) was also evaluated on both IL-1 $\beta$  and IL-10 production following transfection for 48h. All incubations included either control siRNA duplexes or siRNA toward the *PTGER4* gene. As previously observed for TNF- $\alpha$  production, LPS increased the production of both IL-10 and IL-1 $\beta$  and in both cases in the presence of PGE<sub>2</sub>, LPS-stimulated IL-10 and IL-1 $\beta$  levels were lower. *PTGER4* siRNA was able to reverse the inhibitory actions of PGE<sub>2</sub> on LPS-stimulated IL-1 $\beta$  production, however, there was no effect of on LPS-stimulated IL-10 levels (Fig. 5). This implies that *PTGER4* gene is important in the functional suppression of IL-1 $\beta$  and TNF- $\alpha$  production by PGE<sub>2</sub> but not the suppression of IL-10.

#### ***The effect of siRNA on PTGER4 expression in THP-1 cells***

The actions of siRNA on *PTGER4* mRNA were also determined to confirm effects at the level of expression. A series of experiments were carried out to determine the time course of the actions of siRNA on *PTGER4* expression. *PTGER4* expression was studied between 3 hours and 48 hours following transfection with 100 nM siRNA. Figure 6 shows transfection of THP-1 cells with siRNA for 3 hours in the presence of LPS (1  $\mu$ g/ml). At this time point, there was no knockdown observed in *PTGER4* expression using siRNA compared to non-transfected cells. Similarly after 6 hours and 12 hours post-transfection, siRNA did not induced any significant change in *PTGER4* expression compared to cells in the presence of LPS alone (untransfected cells). At 24 hours post-transfection a substantial knockdown of *PTGER4* expression was observed in LPS-stimulated cells (P <0.001). A similar effect was observed at 48h post-transfection, with a larger reduction in *PTGER4* expression (Fig. 6).

#### ***Effect of PTGER4 overexpression on LPS-induced TNF production by monocytic cells***

In order to further determine the influence of *PTGER4* expression on functional responses a converse series of experiments to those using siRNA were carried out. The overexpression of

*PTGER4* was evaluated on the production of TNF- $\alpha$  by transfecting THP-1 cells with exogenous *PTGER4* plasmids (Flag-EP4 and empty-vector - 1904ng/ $\mu$ l) for 24h in LPS-stimulated cells. LPS stimulation resulted in TNF- $\alpha$  levels which were significantly lower in cells transfected with the *PTGER4* exogenous plasmid compared to untransfected cells ( $P < 0.01$ ). The empty-vector, (used as a control without a *PTGER4* construct) did not have any significant effect on TNF- $\alpha$  production in response to LPS.

A series of experiments were performed to ascertain the time course of the actions of exogenous *PTGER4* overexpression (plasmid) on TNF- $\alpha$  production in THP-1 cells. The production of TNF- $\alpha$  was studied between 6 hours and 24 hours (Fig. 7) following transfection with *PTGER4* plasmid in comparison to untransfected cells (0h). Untransfected cells (0h) produced a high level of TNF- $\alpha$  in response to LPS. PGE<sub>2</sub> suppressed this elevated level. After 6h transfection with plasmid, there was a significant decrease in TNF- $\alpha$  production in response to LPS compared to untransfected cells ( $P < 0.01$ ). In addition, PGE<sub>2</sub> was able to induce a greater decrease in TNF- $\alpha$  level in LPS-stimulated cells (Fig. 7) compared to the corresponding untransfected cells ( $P < 0.01$ ). At 24h post-transfection with *PTGER4* plasmid, the inhibition by PGE<sub>2</sub> of LPS-stimulated TNF- $\alpha$  levels was reduced to that very close to cells incubated without LPS.

### ***The effect of PGE<sub>2</sub> and various agents on cell viability***

In order to exclude the possibility that the suppression of LPS-induced TNF- $\alpha$  production may be indirectly related to cell viability, the effect of the various compound/ reagents on THP-1 cell viability was assessed using 2 different methods, one estimating non-viable cells (Trypan blue exclusion) and the other measuring total viable cells (MTT assay). The total number of viable cells in control incubations was adjusted to 100 %  $\pm$  2.5% and the viable cells in the presence of PGE<sub>2</sub>, Butaprost, Sulprostone and L-902, 688 (all at 1  $\mu$ M) which were compared to control levels indicated 103  $\pm$  1.5%, 105  $\pm$  0.5%\*, 101  $\pm$  2.2% and 100  $\pm$  2.4% respectively (n = 3  $\pm$  s.d. both  $P < 0.05$  by ANOVA). The viability of cells (1 x 10<sup>5</sup> cells) using the MTT method was less subjective and was expressed directly as absorbance of the amount of converted dye. The control cells yielded a mean absorbance of 1.213  $\pm$  0.024 and in the presence of PGE<sub>2</sub>, Butaprost, Sulprostone and L-902, 688 the mean absorbances were 1.291  $\pm$  0.125\*, 1.411  $\pm$  0.029\*, 1.228  $\pm$  0.035 and 1.214  $\pm$  0.005 respectively (n = 3  $\pm$  s.d. both  $P < 0.05$  by ANOVA).

Both methods indicated that the prostanoid/ agonists appeared to have a mild cytoprotective action on THP-cell viability.

The effect of the various reagents used in transfection studies were also assessed for their effects on cell viability using the MTT assay and the data are shown in Table 4. Dead cells were prepared by briefly boiling viable cells in a water bath for 30s.

Treatment	Viable cells (%)
Control (untreated cells)	100 ± 1.5%
siRNA	108 ± 0.05%
Positive control	103 ± 0.02%
Negative control 1	100 ± 2.5%
Negative control 5	110 ± 0.03%
Hiperfect	100 ± 1.2%
Dead cells	0 ± 0.001%

**Table 4 Effect of transfection reagents on cell viability (MTT assay, n = 4)**

## DISCUSSION

In this study we report that PGE<sub>2</sub> is a potent suppressor of LPS-stimulated TNF- $\alpha$  production in human monocytic cells and that expression of the genes (PTGER1 - 4) for the various PGE<sub>2</sub> receptors (EP1 – EP4) is important in the suppressive response to PGE<sub>2</sub> particularly the EP4 receptor.

### *PGE<sub>2</sub> and its analogues on the suppression of cytokine production*

In the present study we confirmed the suppressive action of PGE<sub>2</sub> and various EP receptor ligands. Both PGE<sub>2</sub> and the EP2R agonist butaprost demonstrated potent suppression of LPS-stimulated TNF- $\alpha$  production in all 3 preparations used, whole human blood, normal monocytes (isolated from peripheral blood) THP-1 cells. The IC<sub>50</sub> values for both PGE<sub>2</sub> and butaprost (table 3) indicate that all 3 preparations respond in a qualitatively identical manner. THP-1 cells, a human monocyte cell line, responded to PGE<sub>2</sub>, butaprost and the EP4R agonist L-902,688 by suppressing TNF- $\alpha$  production. Both PGE<sub>2</sub> and L-902,688 showed a much lower IC<sub>50</sub> for the suppression of TNF- $\alpha$  production than butaprost (table 3). This was similar to the comparative effects of PGE<sub>2</sub> and butaprost in normal monocytes. Thus, in subsequent studies THP-1 cells were used as a plausible surrogate for normal monocyte functions and processes. This was necessary as the yield of monocytes from blood is generally very low and highly variable as the blood derives from different donors. THP-1 cells have been shown in many studies to be suitable for revealing the molecular processes of monocytes especially those focused on the control of inflammatory cytokine production and responses to lipid-derived modulators [1, 10, 11].

It is unclear what receptors (EP1 – EP4) are important in suppressing TNF- $\alpha$  production. It is well established that both EP2Rs and EP4Rs can result in suppressive action on the basis of selective ligands for each receptor. This was confirmed in the present study where butaprost and L-902,688 were comparable to PGE<sub>2</sub> in their inhibitory activity in both blood and THP-1 cells indicating EP2R and EP4R involvement (Fig. 1). In contrast, sulprostone did not have any effect on the suppression of LPS-stimulated TNF- $\alpha$  production. Sulprostone acts non-selectively via EP1/EP3 receptors [12-14]. This clearly demonstrates that neither EP1 nor EP3 receptors play any role in controlling pro-inflammatory cytokine production.

Butaprost is an EP2-selective agonist and has been shown to suppress cytokine levels in a wide variety of immune cells such as monocytes and NK cells [1, 15]. This is consistent with antagonist selectivity, the EP2 antagonist AH6809 can reverse the actions of butaprost and PGE<sub>2</sub> [1] specifically TNF- $\alpha$  production. There are many previous studies that agree with this [13, 16-18]. This shows the potent suppressive actions on TNF- $\alpha$  production mediated via EP2 receptors as butaprost has a high selectivity for the EP2R [19] and has been used extensively in functional studies. Similarly, L-902,688 is a highly selective EP4 agonist and has been shown to be >4000-fold more selective for the EP4R than the other EP receptors [20]. The involvement of the EP4 receptor in cytokine inhibition has been evaluated previously [1, 21] and the data obtained here indicate an important role. Thus, butaprost and L-902,688 were subsequently used to ascertain the receptor-selective modulation of EP receptor gene expression, specifically expression of *PTGER2* and *PTGER4* genes.

### ***Expression of EP receptors in THP-1 cells***

Each EP receptor has a unique expression pattern and is linked to different signalling pathways [6]. EP4 receptor mRNA is abundant in LPS-stimulated human monocytes [22] which implies that the EP4 receptor has a role in cytokine modulation. For example, in chronic inflammation, the expression of mRNA for the EP4 receptor is up-regulated, whereas there is a low expression of *PTGER2* and no detectable expression of *PTGER1* or *PTGER3* [22]. An earlier study also agreed with this and showed that there is an increased expression of EP4 and EP2 receptor mRNA, while the mRNA levels for EP1 and EP3 receptors are barely detectable [23]. This is in agreement with the present study which shows that LPS induced an increase in the expression of both EP4 (*PTGER4*) and EP2 receptors (*PTGER2*) receptors in THP-1 cells, whereas EP1 and EP3 receptors (*PTGER1*, *PTGER3*) were below the level of detection. In this study the expression of *PTGER4* and *PTGER2* could be downregulated by L-902,688 but butaprost only affected *PTGER2* expression implying that expression is controlled by PGE<sub>2</sub> primarily via the EP4 receptor. Additional evidence for this has been reported where the EP4 agonist (ONO-AE1-329) inhibited TNF- $\alpha$  production in EP2-receptor-deficient cells. However, the effect of EP2 agonist (ONO-AE1-259) on TNF- $\alpha$  in EP4-deficient cells was not potent as the EP4 agonist [24]. Therefore, this may indicate that the EP4 receptor is the major anti-inflammatory regulator. An important observation in the present study was the effect of *PTGER4* siRNA on the LPS-stimulated production of TNF- $\alpha$  and IL-1 $\beta$ , where the PGE<sub>2</sub>-

induced suppression of both was reversed, however, this was not the case for the anti-inflammatory cytokine IL-10 (Figs. 4 and 5). This implies that the EP4 receptor may downregulate pro-inflammatory cytokines whilst simultaneously sparing anti-inflammatory mechanisms. A genetic deletion or significant mutation of the *PTGER4* gene especially in monocytes may result in a substantial loss of suppressive activity on pro-inflammatory cytokine levels. In the case of EP4 receptor deficiency PGE<sub>2</sub> fails to have any effect on LPS-stimulated TNF- $\alpha$  induction [25]. This observation is strongly in agreement with the current data (Fig. 4) which also demonstrated that silencing *PTGER4* expression using siRNA resulted in increased TNF- $\alpha$  production indicating the functional failure of suppression in response to exogenous PGE<sub>2</sub>. This was confirmed by overexpressing *PTGER4* using an exogenous plasmid, which amplified the inhibitory actions of PGE<sub>2</sub> on TNF- $\alpha$  production (Fig. 7).

In conclusion, EP2R and EP4R ligands can both result in the inhibition of pro-inflammatory cytokine production. However, with respect to gene expression for the receptors, only the EP4 ligand was able to modulate the expression of both receptors. The functional significance of the modulation of expression of *PTGER4* was confirmed by both knockdown of the gene using siRNA and overexpression using a *PTGER4* plasmid. This clearly showed that the changes in expression are not merely at the level of mRNA but actually have functional consequences. This also indicates that modulation of pro-inflammatory production by PGE<sub>2</sub> is determined by dynamic changes in receptor-expression-coupling and is not merely determined by a static level of receptors present at any particular time. The relative lack of effect on IL-10 production by EP4R processes is an important advantage in the development of anti-inflammatory targets. This may have consequences not only for the development of therapeutic strategies but potentially also for understanding the underlying mechanisms of pathology of some inflammatory diseases which may involve a failure of EP receptor-mediated control.

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### ***Conflict of Interest***

The authors wish to confirm that there is no conflict of interest in the work described here.

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## Figures and Table Legends

### Table 3.

**IC<sub>50</sub> values for the suppressive actions of PGE<sub>2</sub>, butaprost and L-902,688 on LPS-stimulated TNF- $\alpha$  production.** Blood or cells were incubated with varying concentrations of the agonists (0.01 – 10  $\mu$ M) in the absence or presence of LPS (1  $\mu$ g/ml) and the IC<sub>50</sub> values (all nM) from the corresponding data was statistically calculated.

### Fig. 1. Direct comparison of EP receptor ligands on LPS-stimulated TNF- $\alpha$ production.

Blood (A) or THP-1 cells (B) were incubated in the absence or presence of LPS (10  $\mu$ g/ ml) with the various EP receptor agonists (all at 1  $\mu$ M); PGE<sub>2</sub>, sulprostone (sulp), L-902,688 (L-9) or butaprost (but) for 22h at 37 °C, 5 % CO<sub>2</sub> and 100 % humidity, after which the plasma or cell supernatants were collected by centrifugation. The level of TNF- $\alpha$  in samples was measured by ELISA. Values are the means of n = 3  $\pm$  s.d. \*P < 0.01 versus LPS alone.

### Fig. 2. The effect of butaprost and L-902,688 on LPS-stimulated IL-10 and IL-1 $\beta$ production.

THP-1 cells were incubated in the absence (0  $\mu$ M) or presence of various concentrations of either butaprost (circles) or L-902,688 (squares) either alone (open symbols) or together with LPS (10  $\mu$ g/ml – filled symbols). The open triangles indicated THP-1 cells incubated alone (control). Cells were incubated for 22h at 37 °C, 5 % CO<sub>2</sub> and 100 % humidity, after which the cytokine levels in cell supernatants were measured by ELISA. Values are the means of n = 3  $\pm$  s.d. \*P < 0.01 versus LPS alone and #P < 0.01 versus control incubations for each cytokine.

### Fig. 3. Expression of *PTGER2* and *PTGER4* in response to various agonists.

THP-1 cells were placed into 6-well plates after which LPS (10  $\mu$ g/ ml), PGE<sub>2</sub> (1 $\mu$ M), L-902, 688 (1 $\mu$ M) and culture medium (control) were added and cells incubated for 22h. Total RNA was prepared from the cells. After reverse transcription, quantitative PCR analysis was performed on the cDNA using selected primers for either A. *PTGER4* or B. *PTGER2* as describe previously. Relative expression levels of mRNA transcripts were normalized to the reference gene PPIB using the delta-delta Ct method as described previously (methods section). Values are the means of n = 3  $\pm$  s.d. \*\*\*P < 0.0001 versus LPS alone.

**Fig. 4. Time course of the effect of *PTGER4* siRNA on TNF- $\alpha$  production in THP-1 cells.** Cells were placed into 6-well plates after which LPS (10  $\mu$ g/ml), PGE<sub>2</sub> (1 $\mu$ M) and culture medium (control) were added and cells were transfected with siRNA duplexes using Hiperfect. The plates were incubated for various times at 37 °C, 5 % CO<sub>2</sub> and 100 % humidity, after which the level of TNF- $\alpha$  in culture medium was measured by ELISA. Values are the means of n = 3  $\pm$  s.d. \*P < 0.01 versus LPS for untransfected (0h) cells and #P < 0.01 versus LPS alone.

**Fig. 5. The effect of *PTGER4* siRNA on LPS-stimulated IL-10 and IL-1 $\beta$  production in THP-1 cells.** Cells were incubated with either LPS (10  $\mu$ g/ml), PGE<sub>2</sub> (1 $\mu$ M), culture medium (control) or a combination thereof. All cells were transfected with either control siRNA duplexes or siRNA toward *PTGER4* (indicated by siRG4 on Figs). The plates were incubated for 48h at 37 °C, 5 % CO<sub>2</sub> and 100 % humidity, after which the levels of IL-10 (A) and IL-1  $\beta$  (B) in the cell supernatants was measured by ELISA. Values are the means of n = 3  $\pm$  s.d. \*P < 0.01 versus LPS + PGE<sub>2</sub> with control siRNA, #P < 0.01 versus cells alone (both containing control siRNA) and §P < 0.01 versus LPS alone.

**Fig. 6. The effect of siRNA toward *PTGER4* on gene expression.** THP-1 cells were placed into 6-well plates after which LPS (10  $\mu$ g/ml), PGE<sub>2</sub> (1 $\mu$ M) and culture medium (control) were added and cells were transfected with siRNAs using Hiperfect. The plates were incubated for the indicated periods of time after which total RNA was prepared from the cells. After reverse transcription, quantitative PCR analysis was performed on the cDNA using primers to detect *PTGER4* (see methods section) Relative expression levels of *PTGER4* mRNA transcripts were normalized to the reference gene PPIB using the delta-delta Ct method as described previously. Values are the means of n = 3  $\pm$  s.d. \*\*P < 0.001 versus untransfected cells.

**Fig. 7. The effect of *PTGER4* overexpression on TNF- $\alpha$  production in THP-1 cells.** Cells were incubated in 6-well plates with *PTGER4* plasmid and empty-vector plasmid in the absence or presence of LPS (10 $\mu$ g/ml) or PGE<sub>2</sub> (1 $\mu$ M). The plates were incubated for various times at 37 °C, 5 % CO<sub>2</sub> and 100 % humidity, following transfection after which the level of TNF- $\alpha$  in culture medium was measured by ELISA. Values are the means of n = 3  $\pm$  s.d. \*P < 0.01 versus LPS alone and #P < 0.05 versus untransfected cells (0h).

**Table 3**

Agonist/ cells	blood	monocytes	THP-1 cells
PGE <sub>2</sub>	3.8	13.3	35.4
butaprost	55.4	330.3	200.4
L-902,688	51.6	n.d.	16.0

**Fig. 1.**

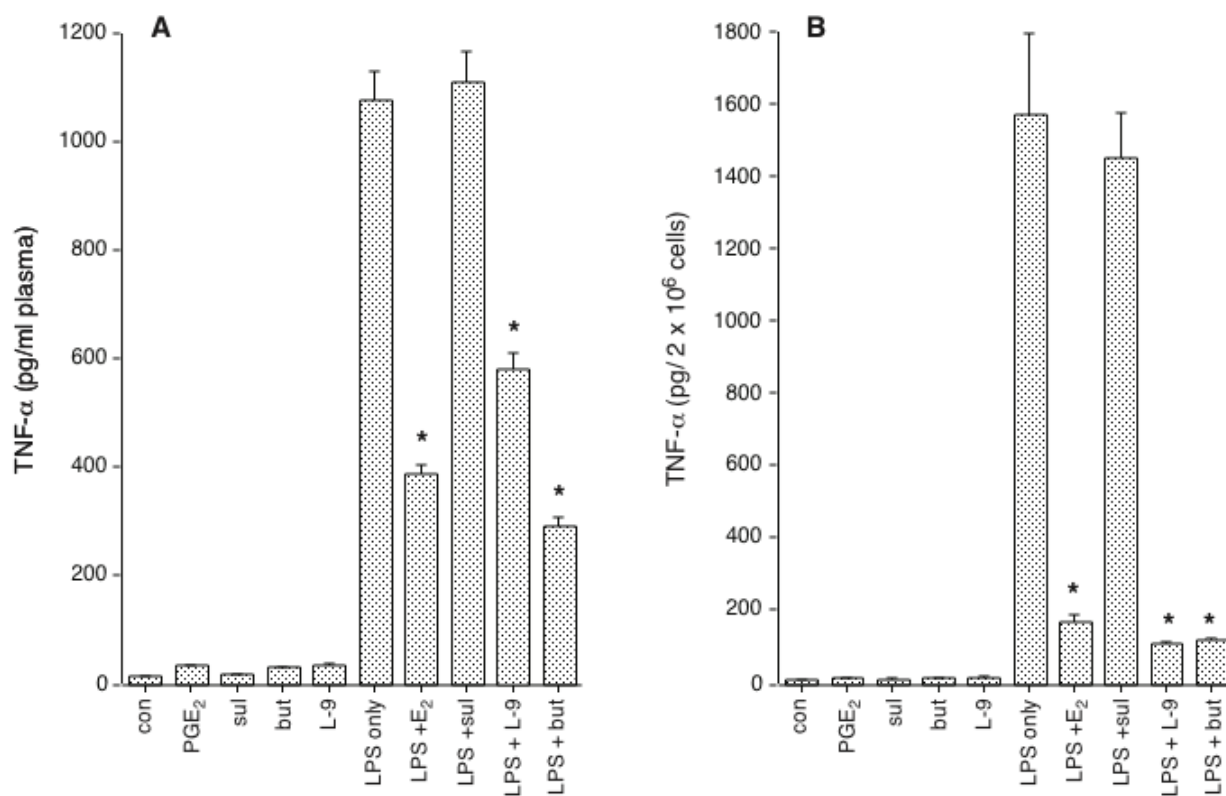
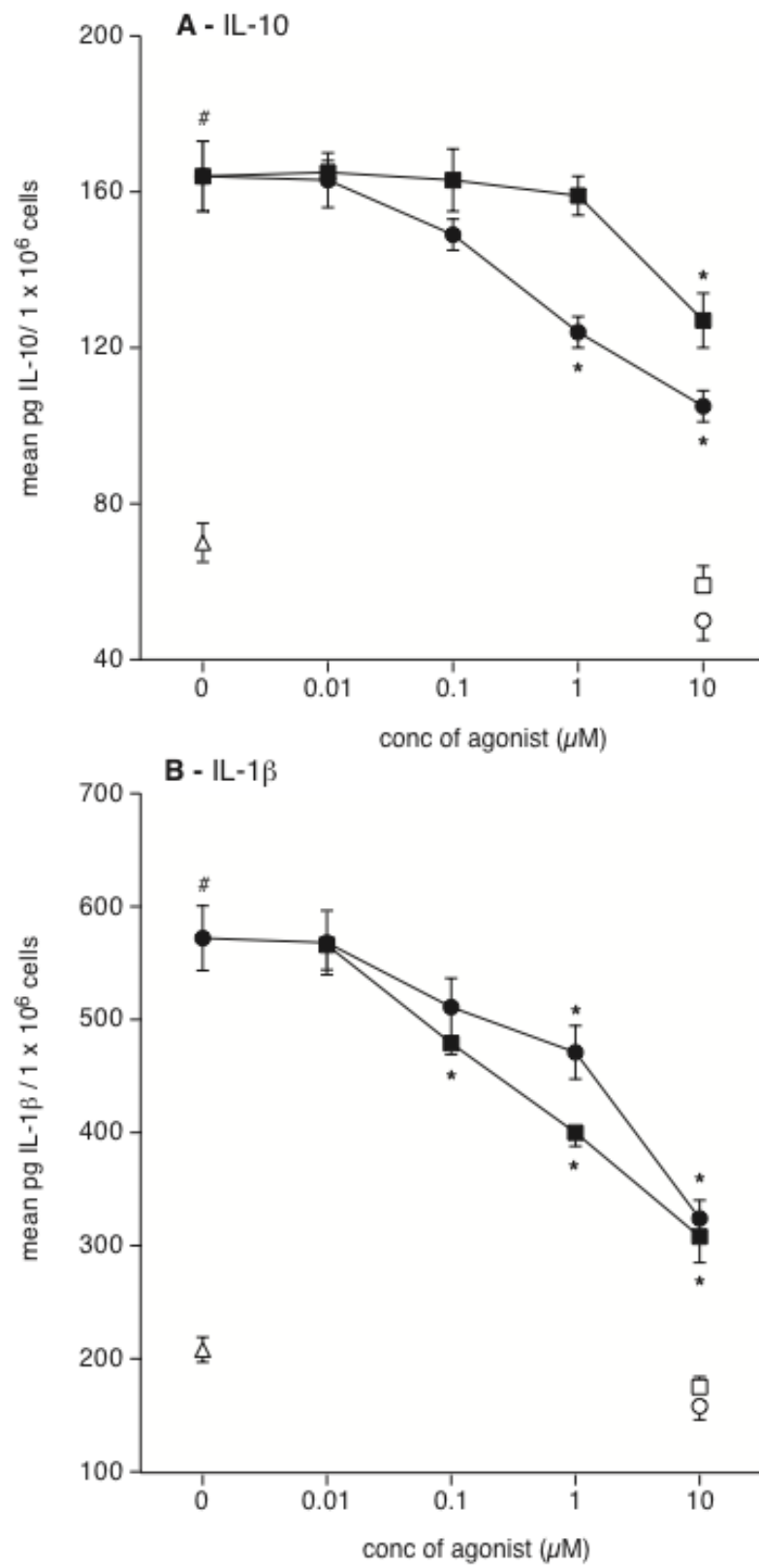
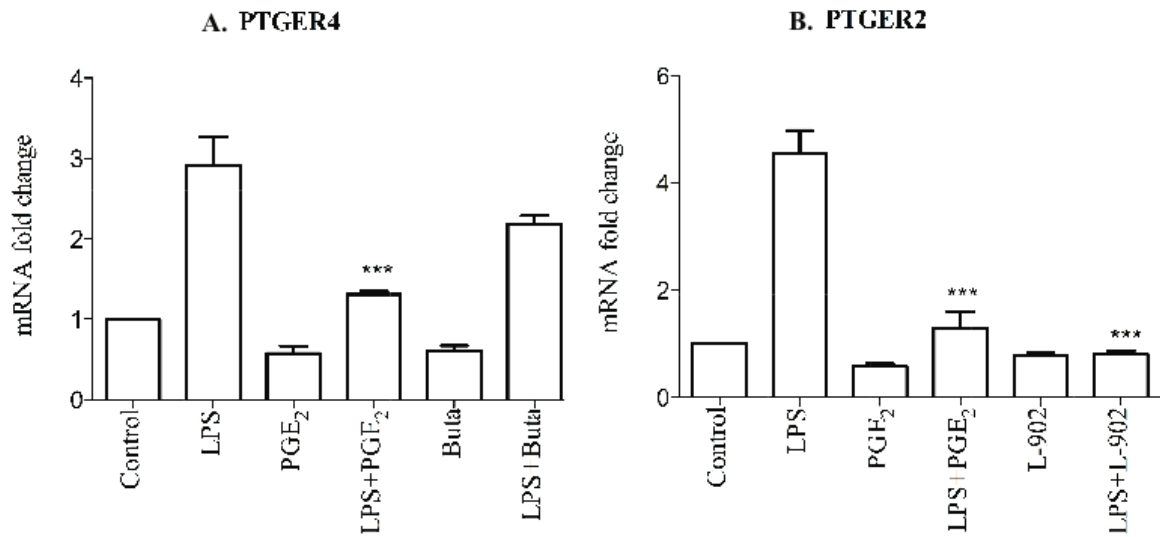


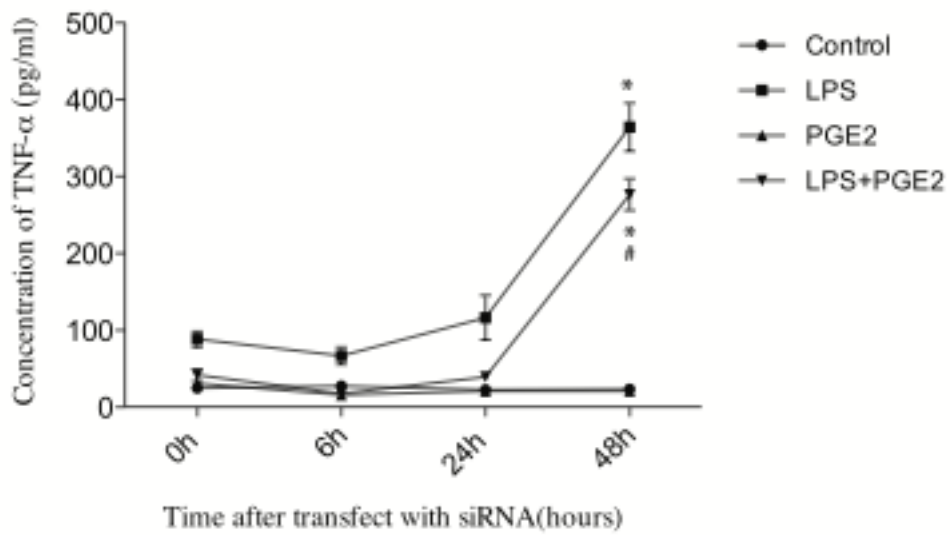
Fig. 2.



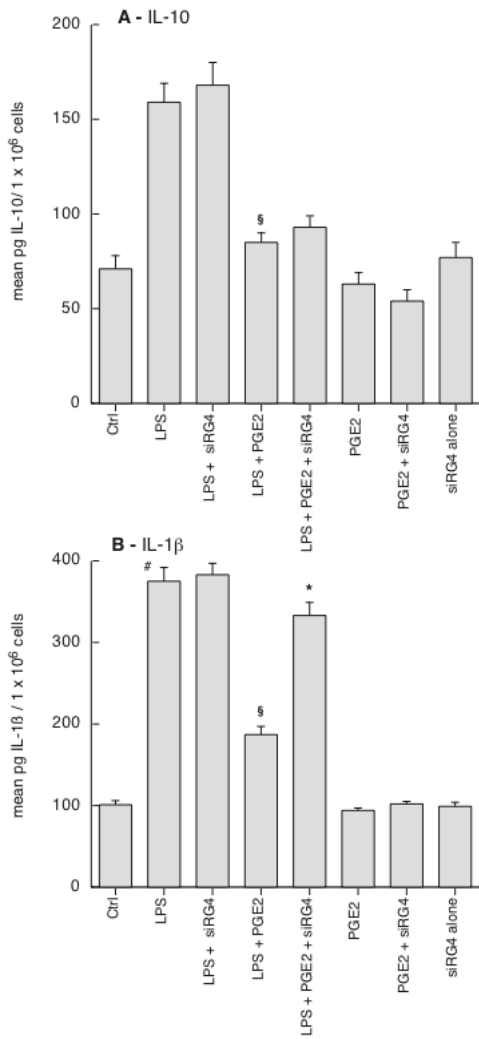
**Fig. 3.**



**Fig. 4.**



**Fig. 5.**



**Fig. 6.**

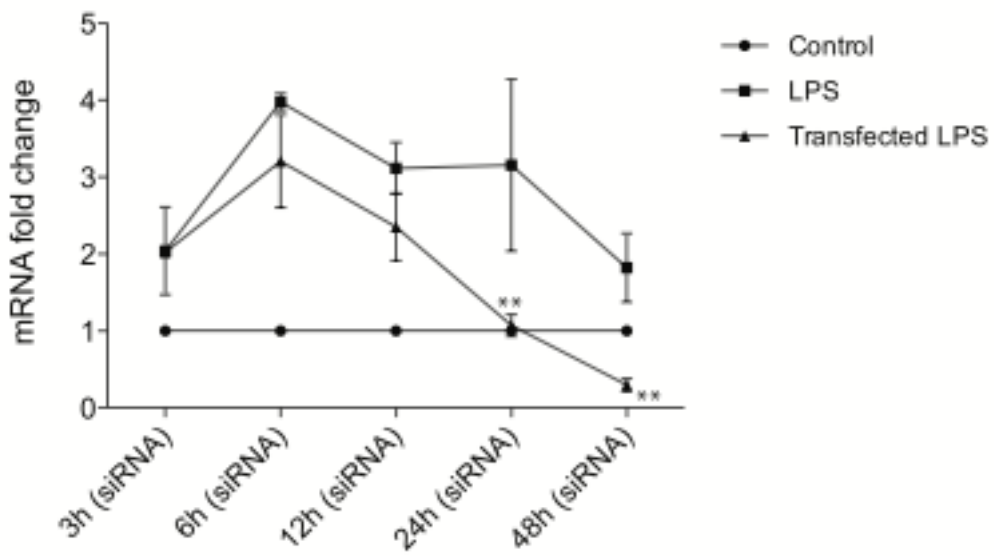


Fig. 7.

