Regulation of Prostaglandin Receptor Expression in Human Monocytic Cells Following Inflammatory Activation

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Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: Alaa A Kashmiry

Date: 20 April 2016

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Dedications

I dedicate this thesis to:

My parents (Abbas & Buthaynah), I am proud to be your daughter.

My sisters (Hanady, Hala and Dr. Heba), who means to me a beautiful example of sibling relationships.

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Abbreviations

15d- PGJ_2	15-deoxy-$\Delta^{12\text{-}14}$ prostaglandin J_2
AA	Arachidonic Acid
ACTB	Beta-Actin
AH23848	EP4/ TP receptor antagonist
AP-1	Activation Protein-1
ATP	Adenosine Triphosphate
B cells	B-lymphocytes
B2M	Beta-2-Microglobulin
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
Buta	Butaprost (EP2 receptor agonist)
BW245C	DP1 receptor agonist
BWA868C	DP1 receptor antagonist
Ca^{2+}	Calcium
cAMP	Cyclic Adenosine Monophosphate
CD14	Cluster of Differentiation 14
CD16	Cluster of Differentiation 16
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
cDNA	Complementary Deoxyribonucleic Acid
CHX	Cycloheximide
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
$cPLA_2s$	Cytosolic Phospholipases A_2
Cq	Quantitation Cycle
CREB	cAMP Response Element-Binding Protein
$CRTH_2$	Chemoattractant Receptor Homologous Molecule Expressed on T helper Type 2 Cells
CT	Cycle Threshold

CV	Coefficient of Variance
DD	Death Domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DP1	Prostaglandin D_2 Receptor -1
DP2	Prostaglandin D_2 Receptor -2
dsRNA	Double-Stranded Ribonucleic Acid
ECACC	European Collection of Cell Cultures
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	endothelial Nitric Oxide Synthase
EP1	Prostaglandin E_2 Receptor -1
EP2	Prostaglandin E_2 Receptor -2
EP3	Prostaglandin E_2 Receptor -3
EP4	Prostaglandin E_2 Receptor -4
ERK	Extracellular Signal-Related Kinase
ESB	Electrophoresis Sample Buffer
FADD	FAS Mediated Death Domain
FCS	Fetal Calf Serum
Flu	Fluprostenol (FP receptor agonist)
FP	Prostaglandin $F_{2\alpha}$ Receptor
GAPDH	Glycereraldehyde-3-Phosphate Dehydrogenase
gDNA	Genomic Deoxyribonucleic Acid
GPCRs	G-Protein-Coupled Receptors
GSK-3/β	Glycogen Synthase Kinase-3/ Beta
GW627368X	EP4 receptor antagonist
h-PGDS	Hematopoietic PGD Synthase
HBSS	Hanks' Balanced Salt Solution
HCl	Hydrochloric Acid
HETEs	Hydroxyeicosatetraenoic Acid

HLA	Human Leukocyte-associated Antigens
HPRT1	Hypoxanthine Phosphoribosyltransferase-1
I-BOP	TP receptor agonist
ID	Intermediate Domain
IDT	Integrated DNA Technologies
IFN-γ	Interferon Gamma
IKKs	IκB Kinases
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-15	Interleukin-15
IL-1β	Interleukin-1 Beta
IL-2	Interleukin-2
IL1-R	Interleukin-1 Receptor
IP	Prostaglandin I_2 Receptor
iPLA$_2$s	Independent Phospholipases A_2
IRAK	IL-1 Receptor-Associated Kinase
IRAK-1	IL-1 Receptor-Associated Kinase- 1
IRAK-2	IL-1 Receptor-Associated Kinase- 2
IRAK-4	IL-1 Receptor-Associated Kinase- 4
IRF3	Interferon Regulatory Factor 3
IRF7	Interferon Regulatory Factor 7
JNK	Jun- N Terminal Kinase
KCl	Potassium Chloride
KDO	Keto Deoxyoctonoic Acid
KH_2PO_4	Monopotassium Phosphate
L-902,688	EP4 receptor agonist
l-PGDS	Lipocalin-Type PGDS
LB	Luria-Bertani Broth
LBP	Lipid Binding Protein
LPPLA$_2$	Lipoprotein Phospholipases A_2
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat

LT	Lymphotoxin
MAL	MyD88 Adaptor Like
MAPKs	Mitogen-Activated Protein Kinases
MD2	Myloid Differentiation Protein-2
MEK	Mitogen-Activated Protein Kinase Kinase
MHC	Major Histocompatibility Complex
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MKK3	MAP Kinase Kinases-3
MKK6	MAP Kinase Kinases-6
MMTV	Mouse Mammary Tumour Virus
mPGES-1	Microsomal Prostaglandin E Synthase-1
mRNA	Messenger Ribonucleic Acid
MRP4	Multidrug Resistance Protein-4
MSK	Mitogen and Stress Activated Protein Kinase
MyD88	Myeloid Differentiation Primary-Response Gene 88
Na_2HPO_4	Disodium Hydrogen Phosphate
NaCl	Sodium Chloride
NAG	N-acetylglucosamine
NC	Negative Control
NCBI	National Centre for Biotechnology Information
NF-κB	Nuclear Factor Kappa B cells
NK cells	Natural Killer Cells
NO	Nitric Oxide
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
ONO- AE3-208	EP4 Antagonist
ONO-AE1-259	EP2 Agonist
ONO-AE1-329	EP4 Agonist
PAF	Platelet Activating Factor
PAMPs	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDTC	Pyrrolidine Dithiocarbamate
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PGs	Prostaglandins
PI3K	Phosphatidylinositol 3-Kinase
PKA	Protein Kinase A
PKC	Protein kinase C
PKR	Protein Kinase R
PLA_2	Phospholipases A_2
PLC	Phospholipase C
Poly IC	Polyinosinic-Polycytidylic Acid
PPIB	Peptidylprolyl Isomerase B
PRP	Platelet-Rich Plasma
PRRs	Pattern Recognition Receptors
PTGDR1	Prostaglandin D_2 receptor -1 gene
PTGDR2	Prostaglandin D_2 receptor -2 gene
PTGER1	Prostaglandin E_2 receptor -1 gene
PTGER2	Prostaglandin E_2 receptor -2 gene
PTGER3	Prostaglandin E_2 receptor -3 gene
PTGER4	Prostaglandin E_2 receptor -4 gene
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RIG-I	Retinoic Acid-Inducible Gene-1
RIPA	Radio Immunoprecipitation Assay
RISC	Ribonucleic Acid Induced Silencing Complex
RL37A	Ribosomal Protein L37a
RNA	Ribonucleic Acid

RNAi	Ribonucleic Acid Interference
ROI	Reactive Oxygen Intermediates
RPMI	Roswell Park Memorial Institute
RSV	Respiratory Syncytial Virus
RT	Reverse Transcription
SARM	Sterile α-and Armadillo-Motif-Containing Protein
SD	Standard Deviation
SDM	Site-Cirected Mutagenesis
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SH-SY5Y	Human Neuroblastoma Cell Line
siRNA	Small or Short Interfering Ribonucleic Acid
$sPLA_2s$	Secreted Phospholipases A_2
sTNF	Soluble Tumor Necrosis Factor
Sulp	Sulprostone (EP1/EP3 Agonist)
T cells	T lymphocytes
TAB1	TAK1-Binding Proteins
TAB2	TAK2-Binding Proteins
TAB3	TAK3-Binding Proteins
TABs	TAK Binding Proteins
TACE	TNF Alpha Converting Enzyme
TBE	Tris-Borate-EDTA
TBP	TATA-Binding Protein
TE buffer	Tris EDTA Buffer
TEMED	Tetramethylenediaamine
Th1 lymphocytes	T Helper Lymphocytes Type-1
Th2 lymphocytes	T Helper Lymphocytes Type-2
THP-1	Human Leukemia Monocytic Cell Line
TICAM-1	Toll-Like Receptor Adaptor Molecule 1
TIR	Toll-IL-1 Receptor
TIRAP	TIR-Domain-Containing Adaptor Protein
TLRs	Toll Like Receptors

Tm	Melting Temperature
TMB	Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor-α
TNFR1	TNF Receptor Type 1
TNFR2	TNF Receptor Type 2
TP	Thromboxane A_2 receptors
TRADD	TNF Receptor Associated Death Domain
TRAF3	Tumor Necrosis Factor Receptor- Associated Factor 3
TRAF6	Tumor Necrosis Factor Receptor- Associated Factor 6
TRAFs	Tumor Necrosis Factor Receptor- Associated Factors
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR-Domain Containing Adaptor Protein Inducing IFNβ
TXA_2	Thromboxane A_2
β-ME	Beta-Mercaptoethanol

Summary

Inflammatory cells such as monocytes can be activated by bacteria leading to the release of pro-inflammatory cytokines, particularly tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1β) which sequentially induce prostaglandin (PG) production. It has been shown that PGs, especially PGE_2 and PGD_2, can potently suppress the production of cytokines when added exogenously or produced endogenously by negative-feedback regulation. There are multiple receptor subtypes for these PGs and it is uncertain which subtypes are specifically involved in the suppression of cytokine production. Therefore, the aim of the present study was to ascertain the expression profile of these receptors following activation of monocytic cells in the presence of lipopolysaccharide (LPS) and the PGs, particularly PGE_2.

Monocytes were isolated from whole human blood and compared to the human monocytic cell line THP-1 which was cultured continuously with RPMI 1640 containing 10% fetal calf serum (FCS). Levels of TNF-α and IL-1β (in plasma or cell supernatants) were measured by ELISA. The expression of prostaglandin receptor genes for PGE_2 and PGD_2 (PTGER2, PTGER4, PTGDR1 and PTGDR2) were determined by quantitative real-time PCR. THP-1 cells were transfected with either siRNA duplexes for knock-down of the EP4 receptors or exogenous EP4 constructs in overexpression experiments.

LPS increased TNF-α and IL-1β levels in blood, monocytes and THP-1 cells. The LPS-stimulated increase in levels of cytokines were suppressed by PGE_2 and PGD_2. Both EP2 and EP4 receptor agonists (butaprost and L-902, 688) inhibited the

production of TNF-α but EP1/3 receptor agonist (sulprostone) did not alter TNF-α levels. Expression of the PTGER2, PTGER4, PTGDR1 and PTGDR2 genes were enhanced by LPS and inhibited by PGE_2 and PGD_2 in both monocytes and THP-1 cells. The EP4 receptor agonist (L-902, 688) inhibited both PTGER2 and PTGER4 genes expression but there was no obvious effect on PTGER2 expression using an EP2 agonist (butaprost). In contrast, the EP1/3 agonist (sulprostone) did not alter PTGER2 expression levels. Knock-down of PTGER4 resulted in a significant increase in TNF-α production and following exogenous transfection with an EP4 receptor construct, the TNF-α level was decreased.

This implies that prostaglandins such as PGE_2 and PGD_2 can limit the level of cytokines released in response to LPS. It appears that there is cross regulation between EP2 and EP4 receptors because PTGER2 and PTGER4 were suppressed by both PGE_2 and L-902, 688. This suggests that EP4 receptors play a crucial central role in the inhibition of TNF-α production.

Chapter 1

Introduction

1.1 Immune system

The immune system is responsible for protecting the host against foreign organisms. The main role of this defensive system is to eliminate those pathogens, which are usually harmful such as bacteria, by specific mechanisms in order to maintain normal body function. Moreover, the immune system can also distinguish cells which are altered by diseases e.g. virally-infected cells. Immune cells not only abolish dangerous pathogens directly but can also produce mediators to initiate other components of the immune system (Beck & Habicht, 1996).

The immune organs are distributed throughout the body. These organs are called lymphoid organs which mainly consist of lymph nodes, thymus, spleen and bone marrow. Lymph nodes are located in the neck, armpits, groin and abdomen. Thymus is responsible for T lymphocytes maturation to be circulated through blood vessels. Spleen and lymph nodes contain specific compartments where immune cells can produce defence antigens. Bone marrow is considered the fundamental source of blood cells including monocytes (Charles A Janeway *et al.*, 2001; Gonzalez *et al.*, 2011; Hampton & Chtanova, 2016).

A healthy immune system has the ability to distinguish between the body's own cells (normal self cells) and foreign antigens (non-self antigens). An antigen that triggers immune responses can be a microbe or even a part of this microbe. Disorder of the immune system can lead to either autoimmune diseases or allergic diseases. In abnormal conditions such as autoimmune diseases, the immune system can recognise self as non-self antigen whereas the most common allergic diseases are associated

with specific antibody known as IgE. Another disorder of the immune system could be immunodeficiency, when the system is missing one of its crucial components. An example of an immunodeficiency disorder is AIDS that can destroy $CD4^+$ T cells (Janeway, 2001; Chaplin, 2010; Gonzalez *et al.*, 2011).

The immune system can interact with the body's other systems and maintain their functions. For instance, the immune system can support the circulatory system, integumentary system and nervous system as described in the following table.

Table 1.1: Interaction of the immune system with other body's system.

System	Interaction with the immune system	Key reference
Circulatory system	The immune system interacts closely with the circulatory system for transportation purposes by circulating immune cells throughout the infected body.	(Charles A Janeway *et al.*, 2001)
Integumentary system	The immune system cooperates with the integumentary system by fighting against infections and protecting the body from damage. The main organ in the integumentary is skin.	(Salmon *et al.*, 1994)
Nervous system	The immune system works along with the nervous system to stimulate the brain for sending/receiving nerve impulses through the body.	(Wang & Ma, 2016)

1.2 Innate and adaptive immunity

The immune system can be categorised into two main components: the innate immune response and the adaptive immune response. Innate immunity acts rapidly against pathogens e.g. phagocytosis and secretes mediators, such as cytokines and lipid molecules including prostaglandins (PGs) as mediators to signal and regulate other cells (Borghesi & Milcarek, 2007; Chaplin, 2010; Akira, 2011).

Adaptive immunity is usually initiated after the innate response and can take much longer to develop, in the order of weeks. The adaptive response depends on the processing of antigens (usually by innate immune cells) and specific receptors for the antigen presented on T lymphocytes. Antigens can also be directly targeted by B-lymphocytes which consequently produce antibodies (Janeway, 2001). T cells have the ability to recognise foreign antigens from self-antigens by the major histocompatibility complex molecules (MHC), also known as human leukocyte-associated antigens-HLA, which preserves self-tolerance. Adaptive responses produce “memory” cells to provide life-long immunity with a much faster onset of action on subsequent challenge by antigens to which the T or B-cells have previously responded (Chaplin, 2010).

Although the mechanisms of innate and adaptive immune systems are different, both are essential for a completely effective immune response. Moreover, adaptive immunity requires innate signals for optimal activation (Chaplin, 2010).

1.3 Cells of immune system

1.3.1 Monocytes

Monocytes are circulating mononuclear phagocytes in blood and can migrate to tissues as macrophages in organs such as lymph nodes, liver, and spleen (van Furth, 1968). Monocytic cells are immune effector cells that originate from bone marrow (myeloid). During infection, monocytes migrate from blood to target tissues followed by cytokine production (Geissmann *et al.*, 2010). Monocytes are considered heterogeneous cells because there are distinct morphological and phenotypically diverse populations (Geissmann *et al.*, 2003; Ziegler-Heitbrock, 2007). Developmentally, myeloid cells lead to monoblasts that act as a precursor for pro-monocytes to develop into functional monocytes (Gordon & Taylor, 2005). Mature monocytes can directly eliminate pathogens by phagocytosis, nitric oxide (NO) and reactive oxygen intermediate (ROI) production and can also signal to the rest of the body by releasing pro- and anti-inflammatory cytokines (Serbina *et al.*, 2008). Monocytes can also participate in chronic inflammatory diseases such as rheumatoid arthritis and atherosclerosis (Nahrendorf *et al.*, 2007; Ingersoll *et al.*, 2011). Human monocytes are categorized into two main classes according to cell-surface markers: classical and Non-classical monocytes. Classical monocytes are 90-95% of the total monocytes in a healthy human and can express $CD14^{hi}$ and $CD16^{-}$ (Passlick *et al.*, 1989; Strauss-Ayali *et al.*, 2007). In contrast, non-classical monocytes, which are also identified as pro-inflammatory monocytes, represent 5-10% of the total monocytes in healthy subjects and express both $CD14^{+}$ and $CD16^{+}$. The pro-inflammatory monocytes are associated with higher levels of TNF-α production in response to TLR4 agonists (Strauss-Ayali *et al.*, 2007).

1.3.2 Cells used experimentally as monocyte models (THP-1 cells)

Monocytes and macrophages are relatively much less abundant than other leucocytes in blood. Typically they comprise in the order of 5% of the total leucocyte population. Thus, cell lines could provide a useful role by providing an abundant level of homogenous monocytic cells. Normal monocytes/ macrophages have key roles such as; detection of foreign pathogens like bacteria through binding with various Pattern Recognition Receptors (PRRs), phagocytosis and the subsequent production of cytokines (Chanput *et al.*, 2014). Early studies showed that a human leukemia monocytic cell line (THP-1 cells) acted like primary monocytes and macrophages in terms of its morphology and immunological functions (Tsuchiya *et al.*, 1982; Sakamoto *et al.*, 2001; Kramer & Wray, 2002; Ueki *et al.*, 2002; Hjort *et al.*, 2003). For this reason, THP-1 cells have been widely used to study the mechanisms and signalling pathways of monocytes (Chanput *et al.*, 2014). The following table illustrates a brief comparison between monocytes, macrophages and THP-1 cells.

Table 1.2: Comparison between monocytes, macrophages and THP-1 cells.

	Monocytes (Yang *et al.*, 2014)	**Macrophages** (Yang *et al.*, 2014)	**THP-1 cells** (Qin, 2012)
Definition	Monocytes are phagocytic white blood cells (leukocytes).	Macrophages are differentiated form of monocytes. They are large phagocytic white blood cells.	THP-1 cells are cell lines derived from the blood of a patient with acute monocytic leukemia.
Main function	➢ Initiate inflammation by producing cytokines. ➢ Develop into macrophages for phago-cytosis.	➢ Produce cytokines. ➢ Engulf foreign substances such as microbes. ➢ Stimulate other immune cells by presenting them with small parts of the invaders.	➢ Mimic the function of monocytes and macrophages (Initiate inflamm-ation and produce cytokines).
PG receptors	All these cells (Monocytes, Macrophages and THP-1) are able to couple to PG receptors especially EP receptors (Brown *et al.*, 2013b; Rogers *et al.*, 2014).		

1.3.2.1 Comparison between THP-1 cells and monocytes

Stimulation of both monocytes and THP-1 cells with LPS leads to expression of MD2, CD14 and MyD88 genes (Chanput *et al.*, 2014). The ethical and financial restrictions of using human monocytic cells isolated from blood is a major disadvantage. In addition, large variations can occur when working with individual donors and this could affect the interpretation of entire studies. In contrast, the identical genetic background of THP-1 reduces this variability (Qin, 2012). Another advantage of using THP-1 cells is that they are relatively easy to grow and maintain with a doubling time of approximately 35 to 50 hours with simple media requirements (RPMI 1640 containing 10% FBS). The quality control should be checked by using the same batch of FBS and checking LPS levels. It has been confirmed that THP-1 cells are safe to use and do not contain any infectious products (Chanput *et al.*, 2014). Daigneault's group demonstrated that THP-1 detection limits are 1.6 pg/ml and 4.8 pg/ml for IL-1β and TNF-α respectively (Daigneault *et al.*, 2010). Cultured THP-1 cells can be used up to 25 passages with no alterations in their activity. THP-1 cells can also be resuscitated easily after storage in liquid nitrogen for a number of years without affecting cell viability, contrasting with PBMC-derived monocytes which cannot be stored. A possible issue that can happen during monocyte isolation is contamination with other blood components like platelets which may disturb monocyte stimulation (Chanput *et al.*, 2014)(see Table 1.3).

Table 1.3: Advantages and disadvantages of using monocytes and THP-1 cells.

	Monocytes	**THP-1 cells**
Time/cost	Required long procedure to be isolated from blood with expensive materials.	Saving time and cost (ready to use).
Variation between samples	Large variations obtained with individual donors.	Low variability because of identical genetic background.
Safety	Can contain infectious substances.	Safe.
Storage	Used immediately and cannot be stored.	Stable for years at -80 °C.
Purity	During isolation, it is likely to be contaminated with other blood components.	Pure cells.

1.3.2.2 THP-1 cells-functional immune responses

Early studies of inflammatory diseases used THP-1 cells to mimic the functional mechanisms of human monocytes (Sakamoto *et al.*, 2001; Kramer & Wray, 2002; Ueki *et al.*, 2002; Hjort *et al.*, 2003). At sites of infection, pathogen-derived molecules, such as LPS, result in monocytes being activated resulting in cytokine production. THP-1 cells can also be activated in a similar way, by stimulation with LPS. The exposure to LPS results in the activation of NF-κB transcription factor and enhances the expression of inflammation-related genes; for instance IL-1β and TNF-α. Consequently, cytokines are released from THP-1 cells in a similar manner to normal monocytes (Sharif *et al.*, 2007; Chanput *et al.*, 2014).

1.4 Inflammation

Inflammation is the first reaction of the innate immune system to trauma or infection (Serhan & Savill, 2005). There are several clinical signs characterizing localised inflammation and are termed the Celsus signs which refer to Aulus Celsus, who was a Roman writer who lived between 30 BC and 45 AD. These are; rubor (redness), tumor (swelling), calor (heat) and dolor (pain)(Rocha e Silva, 1978; Bistrian, 2007). Inflammation is not restricted to these clinical signs, it could be manifest systemically also. Systemic inflammation can be either mild or severe. A mild form is usually represented by fever which is the most frequently used objective clinical indicator of inflammation and infection. Severe systemic inflammation occurs when the inflammatory response is overwhelming. This may lead to extremely low blood pressure (shock) and consequently organ failure and septic shock in the case of bacterial infection and may ultimately leading to death (Groeneveld *et al.*, 2001).

1.4.1 Initiation of inflammatory responses

Inflammatory reactions can be activated by different stimuli, the most common being those that are molecules derived from bacteria and viruses. Despite the fact that each stimulus may promote reactions with different characteristics, almost all inflammatory reactions have similar features (Kumar *et al.*, 2011). These are discussed below.

1.4.1.1 Lipopolysaccharide (LPS)

LPS is a component of Gram-negative bacteria membranes. Initially, LPS was recognised as an endotoxin because it plays a crucial role in the toxic manifestations and inflammation induced by Gram-negative bacteria (Rietschel *et al.*, 1994). The toxic effect of LPS was discovered more than a century ago by Richard Pfeiffer, who distinguished between exotoxin and endotoxin activity. Exotoxins are produced and released by bacteria into the surrounding environment, whereas endotoxins are maintained within the bacterial cell to be released after destruction of the cell wall (Pfeiffer, 1892). The outer membrane of gram-negative bacteria acts as a permeability barrier against external toxic agents. LPS is the central molecule which plays this function because it occupies most of the space in the outer membrane whereas glycerophospholipids are located in the inner membrane of gram-negative bacteria (Vaara & Nurminen, 1999).

Lipopolysaccharides are molecules which have a molecular weight of circa 10k. Generally, LPS consists of three regions (Fig. 1.1) the first one is a lipid component known as Lipid A. This part of LPS is the hydrophobic membrane-anchoring region

which contains a phosphorylated N-acetylglucosamine (NAG) attached to 7 saturated fatty acids. These fatty acids can bind either directly to the NAG dimer or be esterified to the 3-hydroxy fatty acids. Lipid A has a short chain of 3-hydroxy fatty acids (C10) which are attached to a glucosamine disaccharide directly by an ester or amide bond. Lipid A is responsible for the biological activity of LPS (the endotoxic/ inflammatory effect), while the immunological activity is related to the polysaccharide molecules (Raetz *et al.*, 2007).

The second region of LPS is called the Core (R) antigen. It is also known as R polysaccharide that contains a short chain of sugars. This region consists of 2 unusual sugars, heptose and 2-keto-3-deoxyoctonoic acid (KDO). KDO always exists in LPS, therefore for this reason, it can be utilized as a detector for the presence of LPS endotoxin in experimental assays (Caroff & Karibian, 2003).

The third region of LPS is known as Somatic (O) antigen. It is also termed O polysaccharide that contains 3-5 sugars of repeated oligosaccharides. Each chain of sugar has a different length. It can be up to 40 oligosaccharide units long. This part of LPS (O polysaccharide) is longer than the second region (R polysaccharide) to preserve the hydrophilic feature of the LPS molecule (Whitfield *et al.*, 1992; Kondo *et al.*, 1996; Caroff & Karibian, 2003).

In human hosts, LPS interacts with a lipid binding protein (LBP) which correlates with Toll-like receptor-4 (TLR4) activation to initiate a sequence of signalling steps which result in the production of cytokines including pro-inflammatory cytokines

such as TNF-α and IL-1β. Once bacteria are lysed via cell death, the LPS are induced to stimulate monocytes through the innate immune system and elevate the cytokines level (Leone *et al.*, 2007). The activation of monocytes by LPS is gradually decreased after 24 hours incubation, this may be linked to the production of anti-inflammatory mediators (e.g. IL-10) induced by LPS and the downstream initiation of prostaglandin biosynthesis to inhibit cytokine production (Swantek *et al.*, 1997; Shibata *et al.*, 2002).

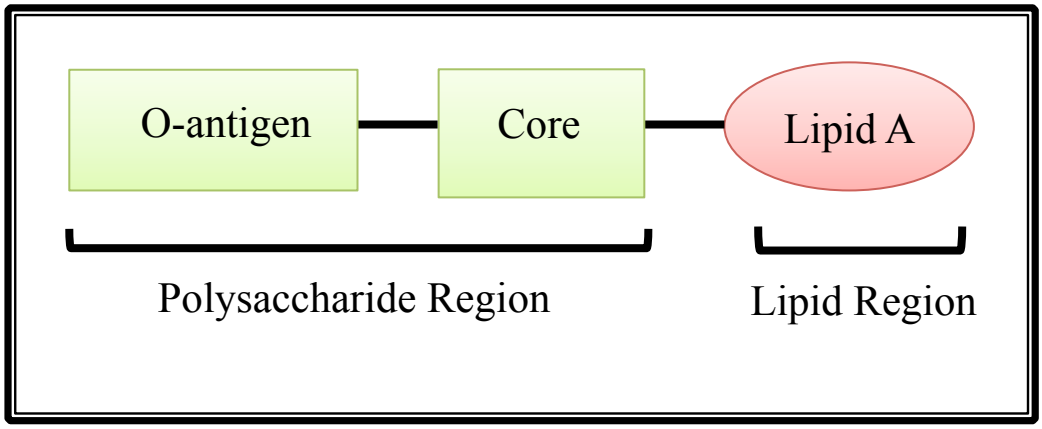


Fig. 1.1: Lipopolysaccharide (LPS) structure.

LPS is a component of the membranes of Gram-negative bacteria and contains the different components shown in the figure above.

1.4.1.2 Toll like receptors (TLRs)

Immune response is initiated once a non-self (microbe) is detected. Recognition of this foreign body is an essential step to protect the host (Beutler, 2009). In different types of innate immune cells, a distinct group of germ line encoded receptors determine the way in which microbes are recognised. These receptors were termed pattern recognition receptors (PRR) (Medzhitov & Janeway, 1997; Janeway, 2001). The family of Toll-like receptors (TLRs) is one of these PRRs classes. TLRs are fundamental for the innate immune system. They act as the first line of defense against invading microorganisms and are "conserved" receptors (Doyle & O'Neill, 2006; Uematsu & Akira, 2006). Detection of pathogen-associated molecular patterns (PAMPs) activates a wide range of signalling pathways that give rise to inflammatory gene expression and lead to cytokine production in order to abolish the infection (Kopp & Medzhitov, 2003; Takeda *et al.*, 2003; Kawai & Akira, 2005). Production of pro-inflammatory cytokines can involve stimulation of the adaptive immune system receptors (Takeda *et al.*, 2003; Kawai & Akira, 2005). Cytokines elicit other activities such as cell migration, differentiation and tissue damage repair. In cancer progression, cytokines can control cell replication and apoptosis. Cytokines can also be markers of cytotoxicity. For instance, a cytotoxic dose of drugs like paracetamol has been revealed to be associated with an increase of TNF-α which may lead to centrilobular necrosis in the liver (Foster, 2001).

TLRs are type-1 transmembrane glycoproteins containing leucine rich repeat (LRR) sequences. This distinctive structure allows receptors to recognise different types of PAMPs. The TLRs are similar to the Toll-IL-1 receptor (TIR) (Takeda & Akira,

2003a). There are 13 TLRs that have been identified in mammals (Uematsu & Akira, 2006). TLRs 1-9 can be found in both humans and mice. TLR10 is expressed by human cells but TLR11-13 appear to exist only in mice. TLRs1, 2, 4, 5 and 6 are able to identify molecules derived from bacteria, fungi, and protozoa. These 5 types of TLRs are present on the cell surface of plasma membrane. However, TLRs 3, 7, 8 and 9 are located inside the cell, they may have an important role in recognising nucleic acids derived from viruses or intracellular bacteria (Kumar *et al.*, 2009; Kawai & Akira, 2010).

1.4.1.2.1 TLRs and their ligands

TLRs are able to recognise a number of pathogens through different selective ligands (Fig. 1.2). TLR4 recognises LPS via it's lipid A which is the main component of LPS which activates cells (Shimazu *et al.*, 1999; Miller *et al.*, 2005). TLR4 has also been shown to be activated by the fusion protein of respiratory syncytial virus (RSV) and by an envelope protein in mouse mammary tumour virus (MMTV) (Beutler, 2009). TLR2 recognises a variety of components from different pathogens. These include: lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, glycosylphophatidylinositol anchors from the protozoan parasite *Trypanosoma Cruzi*, a phenol-soluble modulin similar to glycosylphophatidylinositol from Gram-positive bacteria (*Staphylococcus epidermis*), Zymosan from fungi and glycolipids from Gram-negative bacteria (*Treponema maltophilum*) (Akira *et al.*, 2001; Medzhitov, 2001; Takeda & Akira, 2003b; Beutler, 2009).

The triacyl lipopeptide in bacteria and mycobacteria is recognised by the TLR1 whereas diacyl lipopeptide in mycoplasma can be bound by TLR6 (Takeuchi *et al.*, 2001, 2002; Kumar *et al.*, 2009). Double stranded RNA (dsRNA) and Poly IC are recognised by TLR3 (Alexopoulou *et al.*, 2001; Beutler, 2009) and TLR5 can distinguish flagellin from bacteria (Kumar *et al.*, 2009; Kawai & Akira, 2010). Single-stranded RNA from viruses binds to both TLR7 and TLR8 while TLR9 recognises bacterial unmethylated CpG DNA (Takeda & Akira, 2003b; Beutler, 2009).

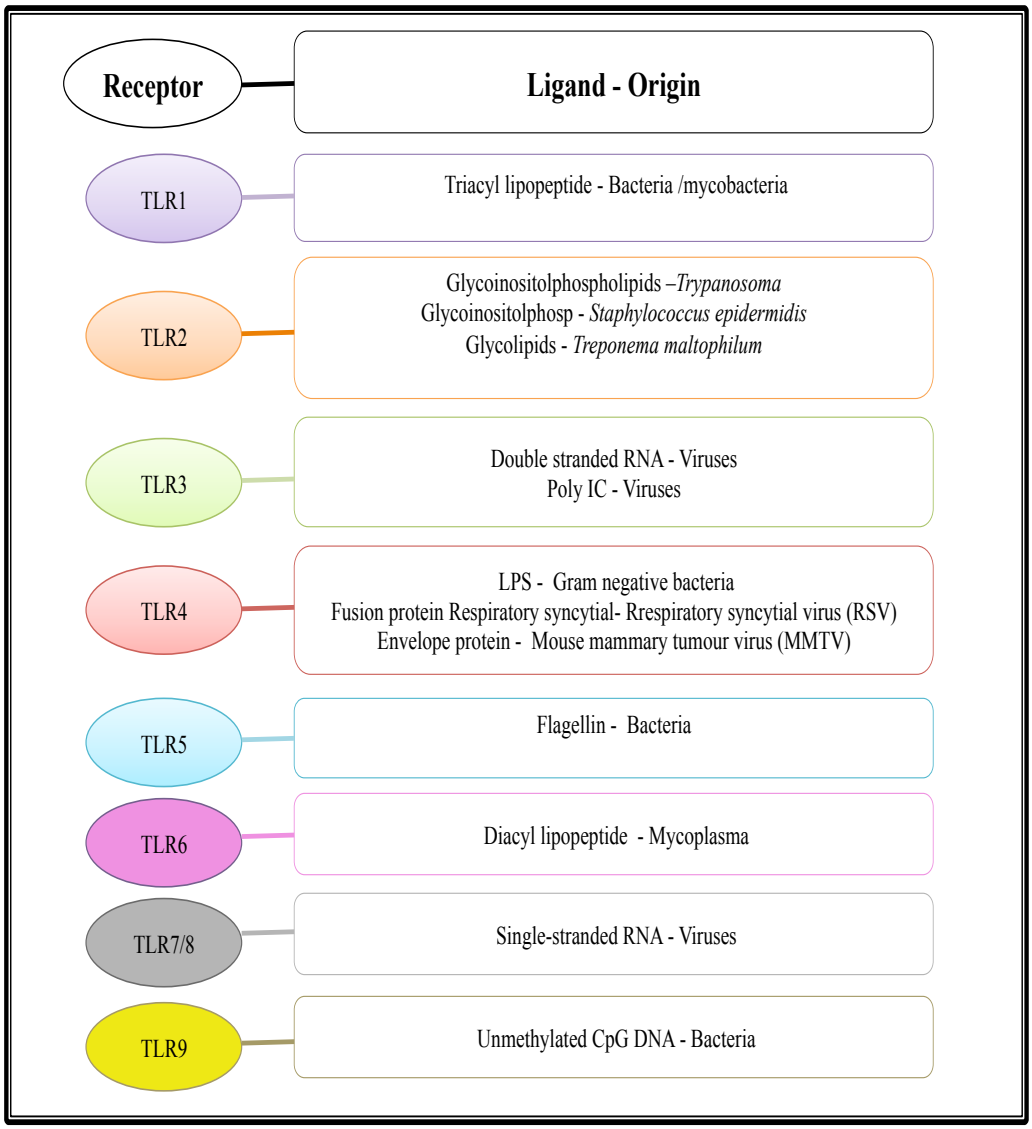


Fig. 1.2:Toll like receptors and their ligands.

In humans, Toll like receptors confer host defence against different pathogens including Gram-positive bacteria, Gram-negative bacteria, Viruses and fungi. The figure above shows the various TLRs and their selective ligands and their respective microbial origins.

1.4.1.2.2 Signalling pathways of TLRs

Recognition of any particular ligand, such as LPS, by TLR4 leads to the activation of different signalling pathways. TLR signalling depends on cytoplasmic Toll-IL-1 receptor (TIR) domains. All TLRs, except TLR3, have proline residues which are the main component of TIR domains. Negative effects on TLRs signalling can occur if proline amino acids are replaced by histidine (Hoshino *et al.*, 1999). This is because proline is vital for TLR4 signalling and responsible for dephosphorylation of IKKs through the MyD88-dependent pathway to inhibit NF-κB (Kaisho & Akira, 2002; Zhang *et al.*, 2013). Several studies proved that a mutation which replaces proline with histidine resulted in a negative impact on TLR-mediated signalling (Underhill *et al.*, 1999; Takeda & Akira, 2004; Zhang *et al.*, 2013). It has been reported that stimulation of TLR induces histidine decarboxylase in response to various cytokines to form histamine, which may contribute to innate immune responses (Funayama *et al.*, 2010).

The TIR domain has cytoplasmic adaptor molecules for various TLRs which mediate signalling events. The main adaptor protein is myeloid differentiation primary-response gene 88 (MyD88) (Newton & Dixit, 2012; Arthur & Ley, 2013). There is also TIR-domain containing adaptor protein inducing IFNβ (TRIF) also called (TICAM-1), TIR-domain-containing adaptor protein (TIRAP) also known as MyD88 adaptor-like (MAL), TRIF-related adaptor molecule (TRAM) and sterile α-and armadillo-motif-containing protein (SARM) (Uematsu & Akira, 2006; O'Neill & Bowie, 2007; Kumar *et al.*, 2009; Kawai & Akira, 2010; Takeuchi & Akira, 2010).

MyD88 associates with all members of the TLR family except TLR 3. Once MyD88 binds to a TLR with a bound ligand, this leads to activation of signalling complexes including IL-1 receptor-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor 6 (TRAF6). This leads to activation of protein kinases e.g. mitogen-activated protein kinases (MAPKs), protein kinase C, and consequently the activation-translocation of the transcription factor NF-κB and interferon regulatory factor 3 or 7 (IRF3)/(IRF7) in order to regulate the production of pro-inflammatory cytokines, chemokines and Type-1 interferons (Kumar *et al.*, 2009; Kawai & Akira, 2010; Takeuchi & Akira, 2010). This leads to the expression and production of TNF-α/ IL-1β in response to TLR4 ligands. MyD88-deficient cells do not have any signalling responses and thus lack activation of NF-κB and MAPKs. This indicates that MyD88 is a vital adapter for signalling in response to TLR activation (Kaisho & Akira, 2002).

The MyD88-dependent pathway is crucial for pro-inflammatory cytokine production i.e. the expression of TNF-α and IL-1β (Akira *et al.*, 2006). The MyD88-dependent signalling pathway is initiated by both TIRAP and MyD88 by TLRs 1, 2, 4 and 6. However, TLRs 5, 7, 9 and 11 uses MyD88 alone (Kawai & Akira, 2010; Takeuchi & Akira, 2010).

There is an additional intracellular signalling pathway known as the TRIF-dependent pathway. This signalling pathway is only activated by TLR3 and TLR4 via the TRIF adaptor molecule (Takeda & Akira, 2003a). In this pathway, type-1 interferon is produced through the stimulation of pro-inflammatory cytokines, IFN regulatory

factor 3 (IRF3) and 7(IRF7) (Yamamoto *et al.*, 2002; Hoebe *et al.*, 2003). TLR4 can use TRAM as an adaptor to stimulate the TRIF-dependent pathway, but TLR3 does not use this adaptor molecule (Oshiumi *et al.*, 2003; Yamamoto *et al.*, 2003b).

1.4.1.2.2.1 The MyD88-dependent pathway

The MyD88-dependent pathway produces a complex between the adaptor molecules and the TIR-domain to activate different signalling cascades downstream and trigger the transcription of pro-inflammatory genes (Lu *et al.*, 2008). The MyD88 molecule consists of a C-terminal region (about 150 aa residues) and an N-terminal region (about 90 aa residues). The C-terminal of MyD88 which binds with the TIR domain of TLRs is found in the intra-cytoplasmic tail of receptors. The N-terminal is known as an amino-terminal death domain (DD) which is involved in cell death. In addition to these two regions, there is an intermediate domain (ID) of MyD88 which is vital in TLR signalling via interaction with IRAK-4 (Bonnert *et al.*, 1997; Loiarro *et al.*, 2009). It has been revealed that residues in both the DD and ID of MyD88 are essentially involved in IRAK-4 signalling. Strong evidence for this is that a lack of an ID domain results in a fail interaction of MyD88 with IRAK-4 which can lead to switch off lipopolysaccharide-induced NF-κB activation (Burns *et al.*, 2003). Residues within the DD of MyD88 are important in the recruitment of molecules containing IRAK-1 and IRAK-4 which are required for the TLR/IL1-R signalling cascade (Loiarro *et al.*, 2009). Therefore, MyD88 plays an important role in innate immunity signal transduction because it is essential for NF-κB activation and MAPK signalling (Warner & Núñez, 2013).

TLR4 signalling represents a good example of the activation of the MyD88-dependent pathway. Once LPS binds to LPS binding protein (LBP), a number of interactions occur between proteins involving myloid differentiation protein-2 (MD2) and CD14 through TLR4 followed by MyD88 signalling (Fig. 1.3)(Lu *et al.*, 2008). LBP attached to LPS enhances interaction between LPS and CD14, consequently CD14 augments the binding of LPS to the TLR4/MD2 complex (Fitzgerald *et al.*, 2004; Lu *et al.*, 2008). Despite that MD2 is strongly related to TLR4, it is also able to create a complex with LPS in the absence of TLR4 (Nagai *et al.*, 2002). Another adaptor molecule known as TIRAP/MAL has a role in the activation of MyD88-TIR domain in the presence of LPS (Horng *et al.*, 2001). Both TLR2 and TLR4 use TIRAP/MAL to activate IL-1 receptor associated kinase (IRAK) (Horng *et al.*, 2002). IRAK4 is the first molecule to be initiated and then IRAK-1, while IRAK-2 is phosphorylated and separates from the receptor to associate with TRAF6. Consequently, the IRAK-TRAF6 complex dissociates followed by a number of signals to stimulate transforming growth factor-β-activated kinase-1 (TAK-1). TAK-1 is recruited and binds with TAK1-binding proteins (TABs) such as TAB1, TAB2 and TAB3. The complex produced (TAK1/TABs) can interact with IKKs which are then phosphorylated (Takeda & Akira, 2004). Then, NF-κB activation is triggered by the phosphorylated IKKs via a serial interaction involving IκBα phosphorylation, ubiquitination and degradation in the cellular proteasome. Subsequently, activated NF-κB is able to translocate to the nucleus in order to bind with the appropriate response elements on DNA which lead to the expression of pro-inflammatory cytokines (Fig. 1.4)(Arancibia *et al.*, 2007). TAK1 also can activate two members of the mitogen-activated protein kinase family

(MAPKs) which are MKK3 and MKK6. This leads to an activation of c-Jun-N terminal kinase (JNK), p38 and induction of activation protein-1 (AP-1)(Landström, 2010; Zhang *et al.*, 2013).

Other TLRs such as TLR7 and TLR9 can use MyD88. The transcription factor IRF7 is activated via different signalling proteins such as IRAK1, IRAK4, TRAF3, TRAF6 and IKKα to produce type-1 IFNs (Kumar *et al.*, 2009; Kawai & Akira, 2010).

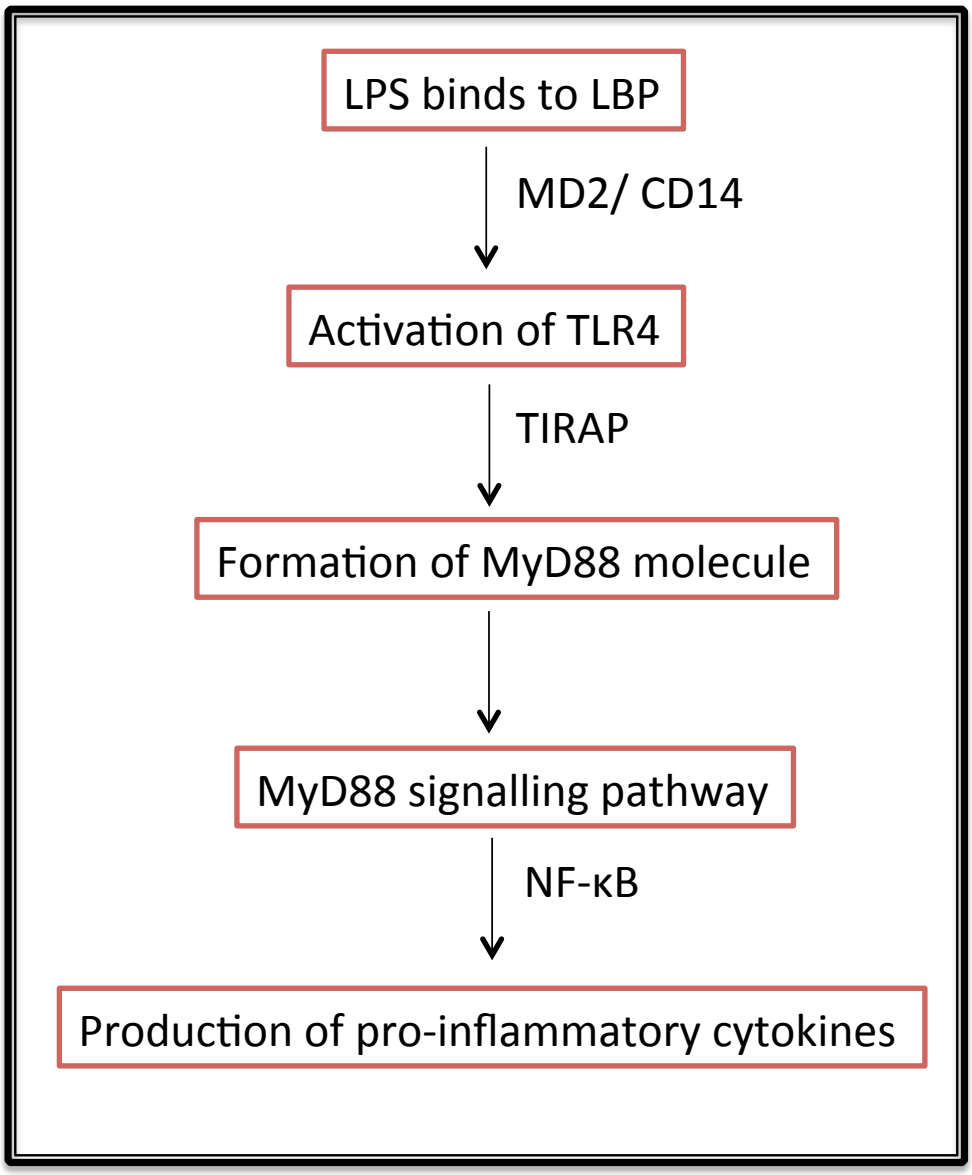


Fig. 1.3: Overview of activation sequence of LPS stimulation via TLR4.

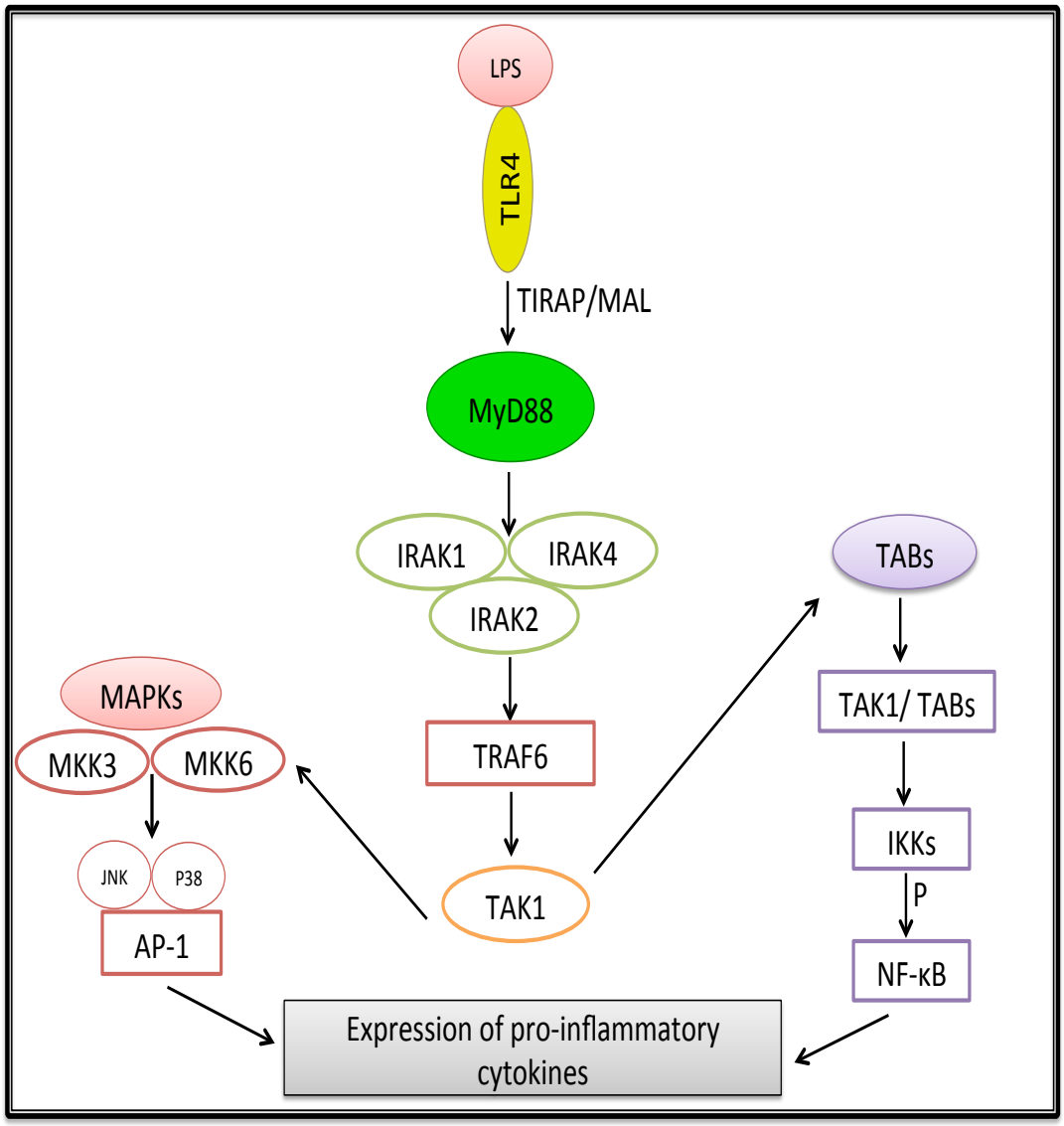


Fig. 1.4: TLR4 signalling pathway via the MyD88-dependent pathway.

TLR4 activates MyD88 signalling leading to activation of IRAK family members. TAK-1 is linked via three different pathways: 1) Production of TAK-1 by TRAF6. 2) TAK-1 binds with TABs and interacts with IKKs, Phosphorylated IKKs then trigger NF-κB to be translocated to the nucleus for the expression of pro-inflammatory cytokines. 3) TAK-1 activates MAPKs (MKK3, MKK6) which in turn stimulate JNK, p38 and AP-1.

1.5 Pro-inflammatory cytokines

Following TLR activation, immune cells such as monocytes produce and release pro-inflammatory cytokines. The main inflammatory cytokines are IL-1β and TNF-α (Dinarello, 2000; Zhang & An, 2007). TNF-α is involved in a wide range of inflammatory and infectious conditions. It is a key modulator of the host response to infection and autoimmunity (Bradley, 2008). Similarly, IL-1β plays a vital role in inflammatory cell migration and cytokine release (Cekici *et al.*, 2014). IL-1β has a crucial role in controlling immune reactions. IL-1β can also stimulate the downstream production of mediators such as prostaglandins via the activation of PLA_2 and also through the induction of COX. LPS is able to stimulate the sequential production of both IL-1β and prostaglandins. It has been demonstrated that IL-1β can control its own induction via a self-induced inhibitor (endogenous PGE_2). Both IL-1β and TNF-α can act through their respective receptors to trigger the production of secondary mediators such as prostanoids that in turn produce the symptoms of inflammation in a cascade (Fig. 1.5)(Dinarello, 2000; Molina-Holgado *et al.*, 2000; Watanabe *et al.*, 2009).

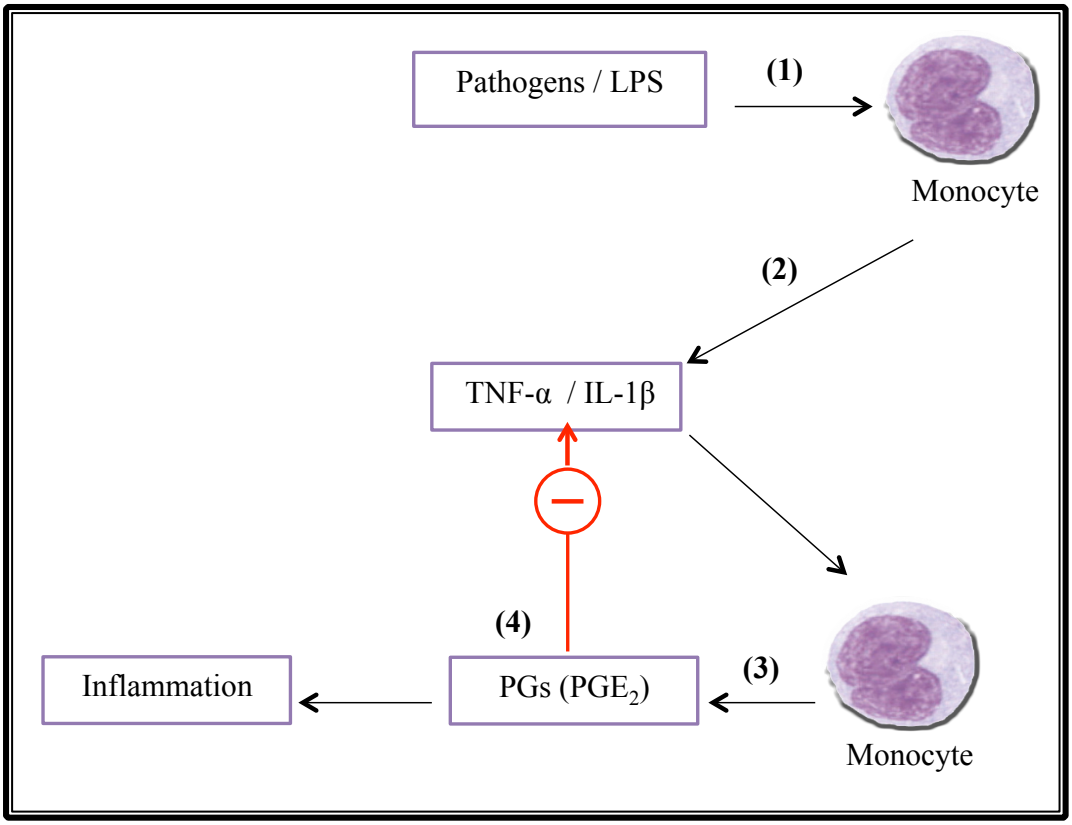


Fig. 1.5: The role of inflammatory mediators in the inflammation process.

(1) LPS can activate inflammatory cells such as Monocytes. (2) Pro-inflammatory mediators (TNF-α or IL-1β) are produced. (3) Monocytes produce PGs which induce the end symptoms of inflammation. (4) PGs (particularly PGE_2) switch off the production of cytokines in a negative feedback loop and influence vasoactive and immune processes associated with inflammation.

1.5.1 Cytokine receptors

Cytokine receptors activate immune cells such as monocytes to produce the symptoms of inflammation. The two main classes of cytokine receptors that are involved in inflammation were identified as the IL-1 family receptors and the TNF-α family receptors (Foxwell *et al.*, 1992). The IL-1 receptor family members are structurally homologous proteins which have Toll Interleukin-1Receptor domain (TIR). In addition to IL-1 receptors, TLRs have also TIR domain. Both of them induce similar signalling through the MyD88 pathway (Subramaniam *et al.*, 2004; Boraschi & Tagliabue, 2006). In contrast, TNF-α family receptors have adaptor proteins such as TNF receptor associated factors (TRAFs) and TNF receptor associated death domain (TRADD). These adaptors are crucial for determining apoptosis because they are linked to enzymes that are responsible for cell death (MacEwan, 2002; Meylan *et al.*, 2008).

1.5.2 Interleukin-1

LPS is the most effective activator of IL-1 production. However, the precise mechanism by which IL-1 production is regulated is still unclear. IL-1 production leads to a positive feedback pathway to induce additional IL-1 (Dinarello *et al.*, 1986; Ghezzi & Dinarello, 1988) and has been shown to induce its own gene expression (Dinarello *et al.*, 1987). It has been shown that human monocytic cells stimulated by bacteria can produce PGE_2 which able to inhibit further IL-1 production. Accordingly, it would appear that PGE_2 can control the production of IL-1 in a negative feedback loop (Kunkel *et al.*, 1986; Davidson *et al.*, 1998). Strong evidence in support of this is provided by the observation that, in the presence of PG

inhibitors, there is an increase in IL-1 production after stimulation with exogenous IL-1 or inducers of IL-1 such as LPS (Knudsen *et al.*, 1987; Rotondo *et al.*, 1992; Dooper *et al.*, 2002). This suggests that IL-1 production is controlled by endogenously produced PGs.

1.5.3 The TNF family

The TNF family consists of three common members: TNF-α, lymphotoxin-α (LT-α or TNF-β) and lymphotoxin-β (LT-β) which are associated with MHC proteins and then act via TNF receptors (Wiley *et al.*, 1995; MacEwan, 2002). TNF-α is an important cytokine that is produced as a result of inflammatory processes. This pro-inflammatory mediator can be overproduced in serious inflammatory diseases (Foster *et al.*, 1993; Moreira-Tabaka *et al.*, 2012). The metalloprotease, TNF alpha converting enzyme (TACE), produces soluble homotrimeric cytokine (sTNF) which is classified as type 2 transmembrane protein (Müller *et al.*, 2009). TNF-α can in turn induce the production of numerous other cytokines involved in inflammation including IL-1. Thus, both TNF-α and IL-1 are involved in similar inflammatory mechanisms (Dinarello *et al.*, 1986; Standiford *et al.*, 1990; Zwerina *et al.*, 2007).

1.5.4 TNF-α receptor signalling

Almost all cytoplasmic tails (death domains) of TNF-α receptors consist of globular domains of 70 amino acids (Zettlitz *et al.*, 2010). There are two different cell surface TNF receptors: TNF receptor type 1 (TNFR1) and TNF receptor type 2 (TNFR2). TNFR1 is activated by many TNF forms including soluble trimeric TNF-α. This receptor is expressed in most tissues and TNFR2 is expressed in lymphoid cells.

Both have a FAS mediated death domain (FADD) and TNF-α receptor associated death domain (TRADD) which are assembled once the ligand binds with the receptors. This leads to the activation of a number of cascades to stimulate NF-κB and MAP-Kinase signalling and induce cell death. In virus infections, cellular protein synthesis is suppressed due to blocked transcriptional responses. This kind of signalling pathway plays a vital role in antiviral immunity that is induced by TNF-α only when transcriptional responses are blocked (MacEwan, 2002; Meylan *et al.*, 2008).

1.5.5 TNF pharmacology

Autoimmune diseases can lead to the activation of T-cells which release other cytokines that support the persistent production of pro-inflammatory cytokines (TNF-α/ IL-1β) which result in chronic inflammation (Blanco *et al.*, 2008). Aeberli *et al*., (2002) demonstrated that the symptoms of inflammation can be abolished by neutralising the activity of TNF-α through binding by monoclonal antibodies. It has been established that infliximab is a good example of a monoclonal antibody which is able to inhibit the biological action of TNF-α. Thus, infliximab is effective in treating inflammatory diseases and results in abolishing the symptoms of inflammation (Aeberli *et al.*, 2002). Another treatment which can decrease TNF-α levels is a drug known as pirfenidone. During bacterial stimulation, pirfenidone can inhibit the synthesis of TNF-α (Hale *et al.*, 2002). However, it is possible that these treatments may have many drawbacks by causing serious bacterial and viral infections in patients treated with these drugs (Gan *et al.*, 2011). In addition, glucocorticoid hormones and their analogues such as dexamethasone also potently

inhibit cytokine production (Bleeker *et al.*, 1997). PGs appear to play the most crucial role in switching-off the upstream production of inflammatory cytokines in a negative feedback loop (Brown *et al.*, 2013b) and perturbations in this axis could be responsible for inflammatory pathogenesis.

1.6 Eicosanoids

Eicosanoids are a family of signalling molecules synthesised by the oxygenation of 20-carbon polyunsaturated fatty acids. They have the ability to regulate the function of almost every cell in the body. They are especially important mediators in controlling immunological actions especially in inflammation. The production of eicosanoid molecules can originate from omega-3 or omega-6 polyunsaturated fatty acids released from the cell membrane. Eicosanoids have four major subfamilies: prostaglandins, thromboxanes, leukotrienes and 5-hydroxyeicosatetraenoic acid (HETEs). In addition to these classes of bioactive lipids, there is another important class of arachidonate products that are bioactive, these are primarily ethanolamide derivatives termed endocannabinoids as they are thought to be the endogenous substrates for cannabinoid receptors. Endocannabinoids are derived *in situ* from dietary fatty acids. Examples of these are arachidonoylethanolamide (arachidonic acid + ethanolamine) and 2-arachidonoyl-glycerol (arachidonic acid + glycerol). These endocannabinoids can also be substrates for prostaglandin synthesis via cyclooxygenase and result in the respective prostaglandin-ethanolamide (Nomura *et al.*, 2011; Brown *et al.*, 2013a)(Fig. 1.6). This project will focus on prostaglandins which are primarily produced from arachidonic acid. Arachidonic acid plays a unique, crucial role in the immune system (Rotondo *et al.*, 1994). Although

arachidonic acid acts mainly as a precursor to produce prostaglandins, it can also be involved in signalling mechanisms through eicosanoid-independent pathways. For instance, Fc-γ receptor-mediated superoxide generation by neutrophils is regulated by arachidonic acid (Badwey *et al.*, 1981; Calder *et al.*, 1992; Rotondo *et al.*, 1994). However, many of arachidonic acid's actions are mediated through prostanoid synthesis (Coceani *et al.*, 1986; Rotondo *et al.*, 1994).

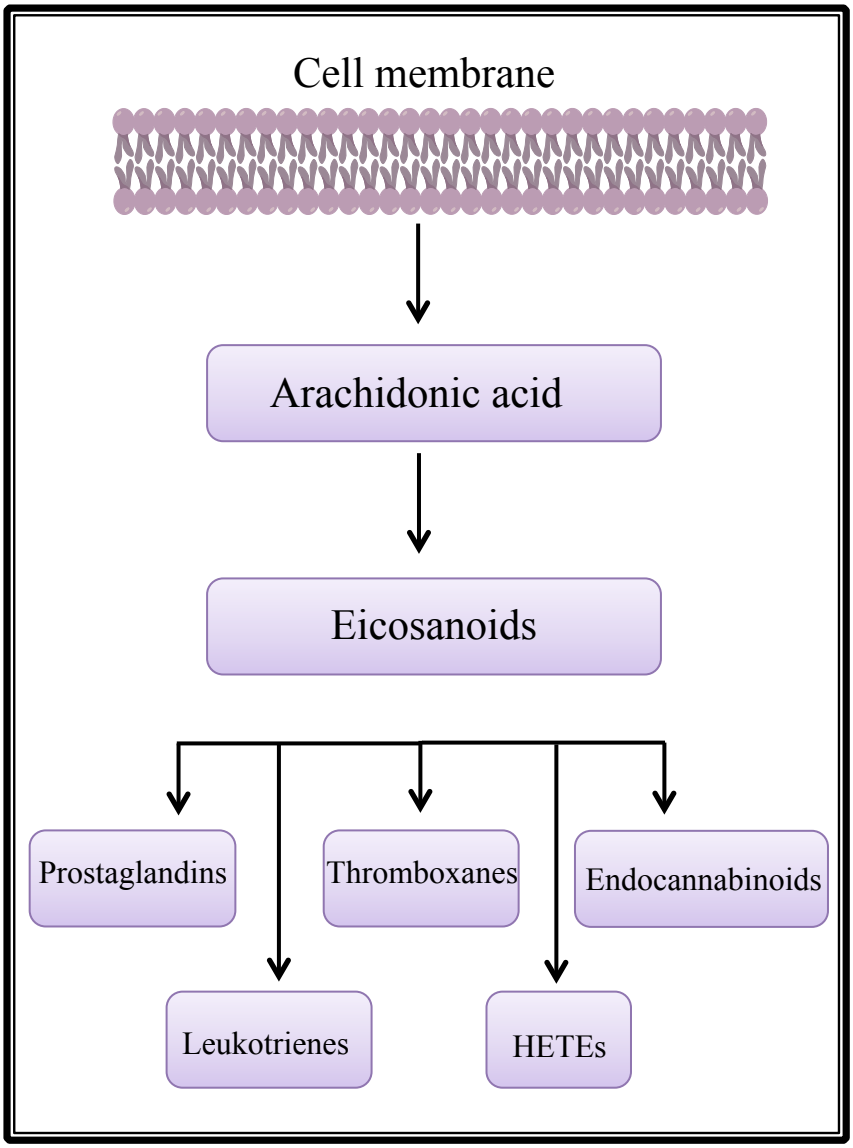


Fig. 1.6: Eicosanoids family.

Overview of eicosanoid derivation. The above figure shows the major classes of eicosanoids: (Prostaglandins, Leukotrienes, Thromboxanes, HETEs and Endocannabinoids).

1.6.1 Biosynthesis and structure of prostaglandins

In inflammation, cells can be activated by cytokines and allow arachidonic acid to be released from membrane phospholipids. Arachidonic acid is a 20-carbon tetraenoic fatty acid [(5Z,8Z,11Z,14Z)-5,8,11,14-Eicosatetraenoic acid] which is released through the activation of phospholipases. Arachidonic acid is a key fatty acid that is responsible for prostaglandin synthesis; it is converted to PGG_2 by cyclooxygenase. PGG_2 is subsequently peroxidised to PGH_2 and then produces different prostaglandins by specific synthase enzymes (Bomalaski *et al.*, 1992; Simmons *et al.*, 2004). Fig. 1.7 illustrates the synthesis of different prostaglandins from arachidonic acid.

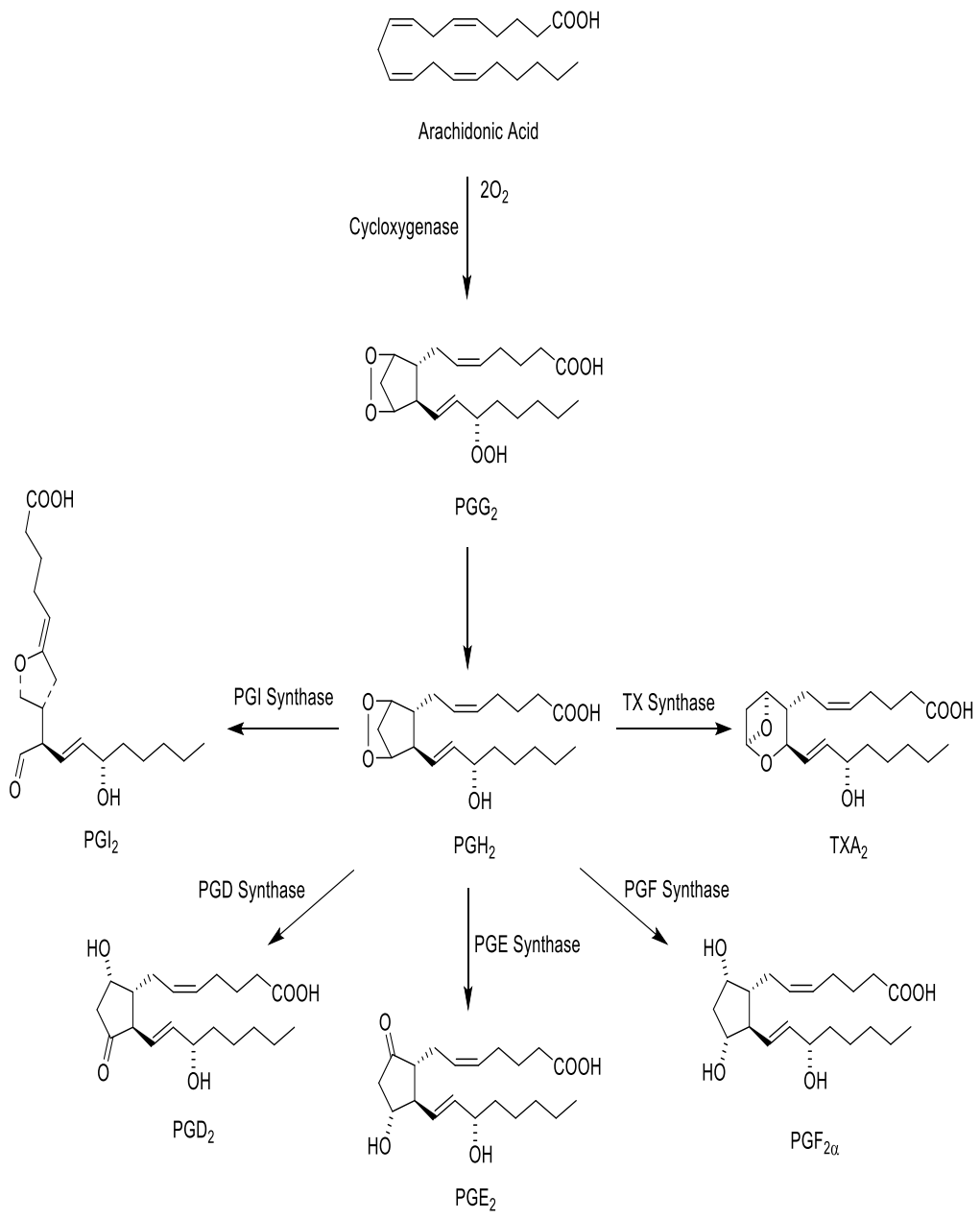


Fig. 1.7: Prostaglandin synthesis from arachidonic acid.

This figure is adapted from (Narumiya *et al.*, 1999).

1.6.2 Phospholipase A_2 (PLA_2)

Phospholipases A_2 (PLA_2) are a group of lipolytic enzymes which regulate fatty acid metabolism. They are responsible for releasing arachidonic acid by hydrolysis of the sn-2 fatty acyl bond of phospholipids from which they derive their name (Balsinde *et al.*, 2002).

PLA_2 can be classified into four main categories: secreted PLA_2 ($sPLA_2$s), cytosolic PLA_2 ($cPLA_2$s), calcium-independent $iPLA_2$s and platelet activating factor (PAF) acetyl hydrolase/oxidized lipid lipoprotein associated (Lp) PLA_2s (Fig. 1.8). Each of these classes is involved in different aspects of lipid metabolisms (Burke & Dennis, 2009).

The secreted PLA_2 ($sPLA_2$s) are characterised by a low-molecular weight (13-15 kD), this category is subdivided into three classes: I PLA_2, II PLA_2 and III PLA_2. Group II PLA_2 can be found in a soluble form in inflammatory sites such as rheumatoid arthritis. II PLA_2 is thought to be responsible for maintaining inflammatory processes. A good illustration of this is that during an inflammation, the synthesis of group II PLA_2 is triggered by cytokines. This leads to arachidonic acid release quickly followed by an increase in PGE_2 synthesis. Therefore, cytokines (IL-1β and TNF-α) tend to stimulate PGE_2 production by elevating the mRNA level for group II PLA_2 (Pfeilschifter *et al.*, 1993; Burke & Dennis, 2009). In an inflammatory reaction, intracellular PLA_2 can mobilise arachidonic acid from membrane phospholipids in order to synthesise the eicosanoids in response to fast-acting agonists (Longo *et al.*, 1999; Balsinde *et al.*, 2002).

The second PLA_2 category is high-molecular weight (60-110kD) termed cytosolic PLA_2 ($cPLA_2$s). $cPLA_2$s enzyme can display a preference for substrates containing arachidonic acid esterified at the sn-2 position of glycerophospholipids. It has been shown that cytokines such as TNF-α and IL-1β enhance $cPLA_2$ activity (Group IV). Therefore, Group IV cytosolic ($cPLA_2$) is involved in the production and mobilisation of arachidonic acid (AA) and thus generating PGE_2 (Pfeilschifter *et al.*, 1993). This was confirmed by Burke & Dennis (2009) who indicated that an absence of this enzyme (Group IV $cPLA_2$) decreases inflammatory activity significantly (Burke & Dennis, 2009). In St-Onge's study (2007), it was also shown that the inhibition of group IV $cPLA_2$ by pyrrophenone led to a 90% reduction in PGE_2 production, compared to the inhibition of other PLA_2s which had no significant effect (St-Onge *et al.*, 2007). Therefore, group IV $cPLA_2$ is considered to be a key enzyme that mediates the production of eicosanoids during many inflammatory processes (Burke & Dennis, 2009).

Calcium-independent $iPLA_2$s is the third class of PLA_2 enzymes (VI PLA_2). VI PLA_2 is similar to IV PLA_2 but not specific for arachidonic acid production. However, VII PLA_2 is a member of PAF acetyl hydrolase/ oxidized lipid $LPPLA_2$ family. This enzyme has a protective role by impeding the pro-inflammatory activity of platelet activating factor (PAF) and mediates the anti-inflammatory activity (Burke & Dennis, 2009).

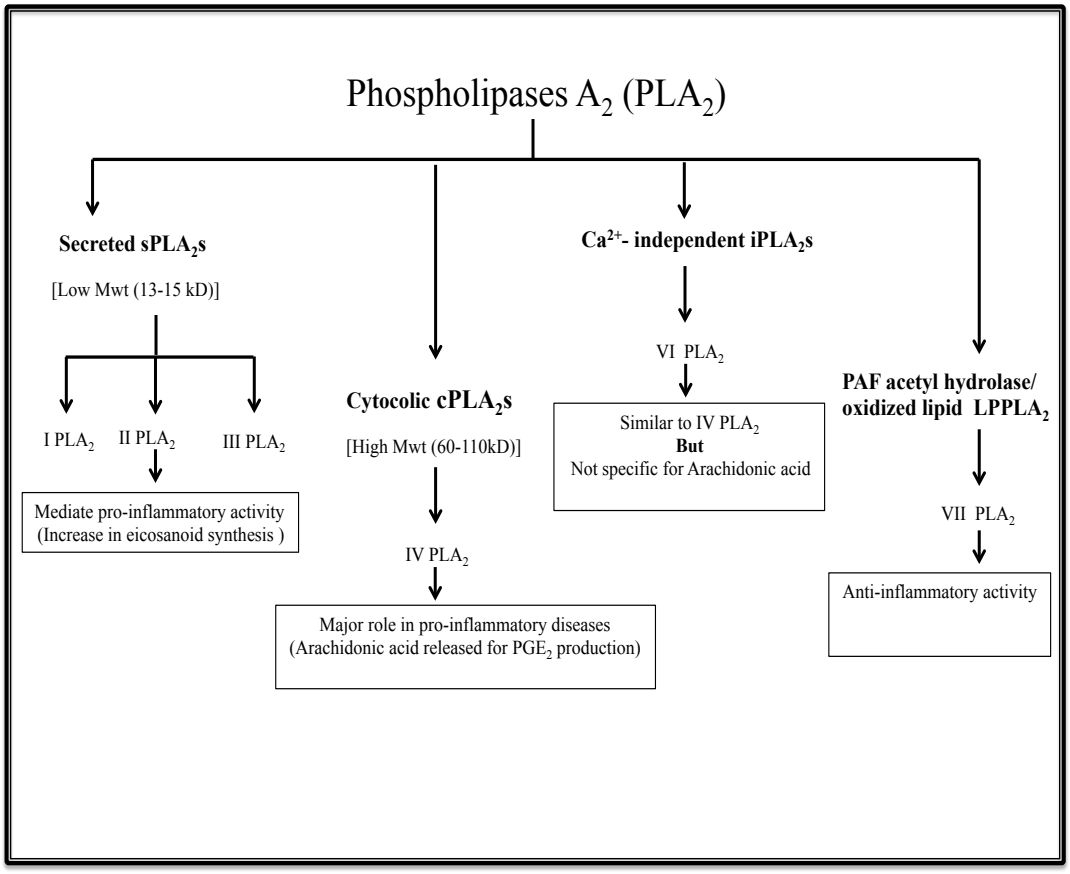


Fig. 1.8: Phospholipases A_2 (PLA_2) classification.

PLA_2 is classified into four categories: 1) Secreted $sPLA_2$s (I PLA_2, II PLA_2 and III PLA_2). 2) Cytocolic c PLA_2s (IV PLA_2). 3) Ca^{2+}-independent $iPLA_2$s (VI PLA_2). 4) Platelet activating factor (PAF) acetyl hydrolase/oxidized lipid lipoprotein associated $LPPLA_2$s (VII PLA_2).

1.6.3 Cyclooxygenase enzyme (COX)

Cyclooxygenase enzyme (COX) which is also known as prostaglandin H_2 synthase, plays a critical role in prostaglandin production. To produce prostaglandins, there are two main steps: Firstly, the oxidation of arachidonic acid by COX produces hydroperoxy endoperoxide PGG_2. Then, the hydroxy endoperoxide PGH_2 is produced by the reduction of PGG_2 through the same enzyme (COX). Finally, prostaglandins (PGE_2, $PGF_{2\alpha}$, PGD_2 and PGI_2) are synthesised by specific synthases via the conversion of PGH_2 (Vane et al., 1998). Initially in 1971, before the COX enzyme had been discovered, it was shown that NSAIDs such as ketoprofen and aspirin have the ability to suppress the enzyme activity and then inhibit PG synthesis (Fig. 1.9)(VANE, 1971; Vane, 1997; Vane & Botting, 2000; Botting, 2010).

The cyclooxygenase enzyme has two isoforms: Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 (COX-2). The COX enzyme was identified as COX-1 which is expressed in many tissues (Hemler & Lands, 1976; Miyamoto *et al.*, 1976). Subsequently, another isoform of COX was recognised as COX-2 which is expressed in several immune cells such as macrophages and monocytes (Fig. 1.9)(Simmons *et al.*, 1989). There are many similarities between these two isoforms. A good illustration of this is that both have similar structure and catalytic activity but they differ in inhibitor selectivity and in their intracellular locations. COX-1 is usually present in almost all cell types such as the kidney, stomach and platelets whereas COX-2 is located in macrophages, leukocytes and fibroblasts (Vane *et al.*, 1998; Haddad *et al.*, 2012). The COX-1 gene is on chromosome 9, while the COX-2 gene is located on chromosome 1 (Williams & DuBois, 1996). Although there are

similarities between COX-1 and COX-2, some differences have been demonstrated. In simple terms, COX-1 activity exists in almost all cell types as COX-1 is constitutively expressed with constant levels. COX-2 is absent from un-stimulated cells, but there is an increase in the protein levels once COX-2 is induced (St-Onge *et al.*, 2007). Another noticeable difference is that COX-1 is a stable protein because it is expressed in many types of cells, while COX-2 has a short half-life and it is expressed transiently because it is ubiquitinated and degraded rapidly. The degradation of COX-2 can be occur through two pathways: an activity-independent pathway which is initiated by post-translational N-glycosylation and substrate-dependent pathway of suicide inactivation (Mbonye *et al.*, 2008; Haddad *et al.*, 2012). Both COX isoforms can contribute to inflammatory responses. COX-2 acts as a major source of prostaglandin formation in inflammation and COX-1 can be expressed in resident inflammatory cells. It has been demonstrated that COX-1 is induced during LPS-mediated inflammatory response as an initial phase of an acute inflammation (Ricciotti & FitzGerald, 2011). However, COX-2 is often induced in the presence of acute and chronic inflammation (St-Onge *et al.*, 2007). This is because the expression of COX-2 enzyme is rapidly induced in response to LPS and cytokines including TNF-α and IL-1β (Williams & DuBois, 1996). Ricciotti and FitzGerald (2011) illustrated that both COX-1 and COX-2 knockout mice show impaired inflammatory responses, although the effect of gene deletions have a different time course. It has been suggested that the contribution of either COX-1 or COX-2 in inflammatory responses may depend on the kind of inflammatory stimulus (Ricciotti & FitzGerald, 2011).

COX-2 mainly acts as an early response gene, because it can be rapidly regulated through protein transcription and translation. The variety in the control of COX-2 expression suggests the involvement of some important regulatory pathways of regulation of function (Cerella *et al.*, 2010). Increased COX-2 expression seems to be attributed to both increased transcription and enhanced mRNA stability. Cytokines stimulate COX-2 transcription in the presence of many transcription regulatory elements including NF-κB. It has been revealed that increased COX-2 expression leads to an increase in PGE_2 production level (Chun & Surh, 2004). It has been demonstrated that COX-2-induced PGE_2 is produced from arachidonic acid. Caughey's research group (2001) provided evidence that COX-2 is associated with prostanoid synthesis and showed that PGE_2 concentration increased substantially (50-80 fold) in response to different doses of arachidonic acid (Caughey *et al.*, 2001).

There are two main pathways involved in COX-2 expression: NF-κB and MAP kinase pathways. NF-κB is able to control the expression of COX-2 in LPS-stimulated cells such as monocytes and macrophages (D'Acquisto *et al.*, 1997; Kojima *et al.*, 2000). LPS-induced COX-2 expression can be inhibited by the NF-κB inhibitors such as pyrrolidine dithiocarbamate (PDTC) in immune cells (D'Acquisto *et al.*, 1997; Liu *et al.*, 1999). However, it has been verified that at higher concentrations, NSAIDs can suppress the stimulation of NF-κB via a number of anti-inflammatory reactions. NSAIDs that can induce NF-κB inhibition are aspirin and sodium salicylate (Kopp & Ghosh, 1994). Therefore, the suppression of NF-κB leads to an inhibition of pro-inflammatory cytokines (TNF-α and IL-1β) (Sakurada *et al.*,

1996; Lee & Burckart, 1998). This suppression of NF-κB, which induces cytokines, block both COX-2 mRNA and protein levels of COX-2 (Xu *et al.*, 1999).

AP-1 can also impact on COX-2 expression. AP-1 is activated via MEK/ERK pathway in response to LPS. LPS also induces Tpl-2/ERK/MSK from CREB phosphorylation, this has a controlling effect on COX-2 regulation (Eliopoulos *et al.*, 2002). PGE_2 production and mRNA for COX-2 in macrophages can be inhibited by either MEK1/2 inhibitors or p38 MAP kinase inhibitors (Lo, 2003). Many previous studies have revealed that MAP kinases are involved in COX-2 expression (Paul *et al.*, 1999). This probably occurs at the COX-2 promoter that regulates the activation of transcription once the transcription co-activator p300 binds to the transcription complex-CREB, AP-1, C/EBP and NF-κB (Deng *et al.*, 2004; Tsatsanis *et al.*, 2006).

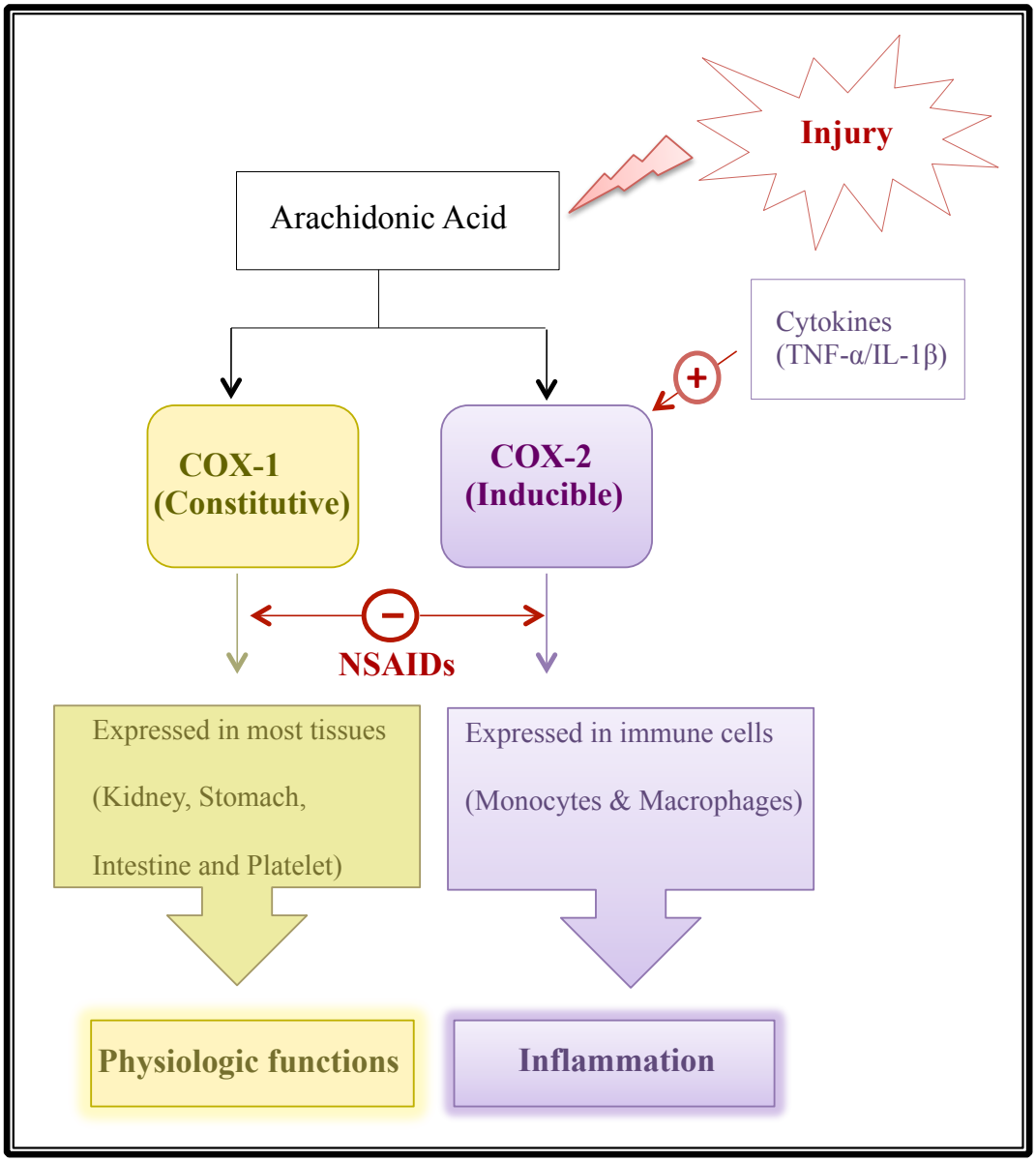


Fig. 1.9: Two isoforms of the COX enzyme.

Arachidonic acid produces COX enzymes in response to an injury. NSAIDs suppress the enzyme activity. COX-1 is expressed in most tissues, while COX-2 is expressed in immune cells and activated by cytokines in most of inflammatory cases.

1.6.4 Prostaglandin E_2 (PGE_2) and the activity of innate immune cells

PGE_2 can control the tissue inflow of cells such as neutrophils, macrophages and mast cells during inflammatory reactions and also alters cell functions. An example is NK cells, it is generally agreed that PGE_2 can suppress their cytolytic functions by inhibiting IL-2, IL-12 and IL-15 production. In addition, PGE_2 can also inhibit the production of IFN-γ by NK cells and thereby abolish the helper function of NK cells during the stimulation of T helper 1 (Th1) responses (Walker & Rotondo, 2004; Kalinski, 2012).

Activation of NK cells results in interferon-γ synthesis, which is considered another of the major pro-inflammatory cytokines. This can be associated with promoting Th1 activity and the consequent amplified activation of macrophages. This may also be an important axis in chronic inflammatory disease. PGE_2 can inhibit LPS-induced IL-12 production from cells such as monocytes. It can also reduce IL-2-driven T-cell proliferation and may subsequently inhibit the production of IFN-γ by CD4+ or CD8+ T cells (Walker & Rotondo, 2004).

PGE_2 can use EP2 receptors to modulate IFN-γ production on NK cells. EP2 receptors play an important role in the regulation of both T-cell and B-cell activity. Pro-inflammatory cytokines such as IL-12 and IL-18, which induce IFN-γ by NK cells, can be regulated by PGE_2. Interestingly, by using synthetic PGE_2 analogues and cAMP measurements, it has been revealed that PGE_2 has the ability to suppress cytokines through EP2 receptors via an elevation in intracellular cAMP levels. The

regulatory impact of PGE_2 on NK cells could prevent many pathological effects of IFN-γ overproduction via early immune activation. These findings demonstrate promising results about suppression of pro-inflammatory activity by PGE_2 and most likely has similar actions on other cells like macrophages and monocytic cells (Walker & Rotondo, 2004; Kalinski, 2012).

1.6.5 Regulation of PGE_2 production

During PGE_2 synthesis, some vital enzymes are involved. For instance, phospholipase A_2 tends to mobilise AA from cellular membranes, whereas cyclooxygenases converts AA into PGH_2 and PGE synthases that are utilised to form PGE_2. In addition, it seems clear that there are many further factors that have an impact on PGE_2 synthesis such as the availability of AA and the activity of COX-2 (Kalinski, 2012).

As previously demonstrated, PGE_2 has the ability to regulate various features of inflammation and the functions of many immune cells. Despite the fact that PGE_2 is responsible for the symptoms of active inflammation in the initial phases of the response, it is able to control the generation of suppressive IL-10 and to inhibit the production of different pro-inflammatory cytokines. This can permit it to limit inflammation thereby controlling the immune suppression (Kalinski, 2012).

PGE_2 synthesis can be inhibited at various levels by pharmacological agents: steroids suppress the release of arachidonic acid (AA), while non-steroidal anti-inflammatory drugs (NSAIDs) such as ketoprofen are able to block the enzymic activity of

COX1/2 or COX-2 specifically (Kalinski, 2012). Therefore, the presence of NSAIDs leads to a decline in inflammatory symptoms by reducing the production of endogenous PGs, primarily PGE_2, however, this allows cytokine levels to be increased (Davidson *et al.*, 1998). This indicates that endogenous PGE_2 production plays a vital role in regulating inflammation by switching-off the upstream production of cytokines in a negative feedback loop (Knudsen *et al.*, 1986; Brown *et al.*, 2013b).

1.6.6 PGE_2 and inflammation

PGE_2 plays a crucial role in inflammation and potently regulates many immune responses. It is one of the most abundant prostaglandins synthesised in the body. PGE_2 is involved in almost all immunological activities leading to clinical signs characterising localised inflammation (Funk, 2001). As mentioned earlier in Section 1.6.3, a specific synthase (prostaglandin E_2 synthase is responsible for the production of PGE_2) (Samuelsson *et al.*, 2007). Since this enzyme has a dominant function in PGE_2 generation, Ricciotti & FitzGerald (2011) demonstrated that a lack of prostaglandin E_2 synthase leads to a decrease in inflammatory responses. This suggests that prostaglandin E_2 synthase-derived PGE_2 contributes to inflammatory diseases. After PGE_2 is produced, it is either transferred via the cell membrane by the ATP-dependent multidrug resistance protein-4 (MRP4) or distributed through the plasma-membrane in order to act as a mediator at the site of inflammation (Park *et al.*, 2006).

PGE_2's role is not only restricted to pro-inflammatory processes, it is also involved in anti-inflammatory responses at a later stage in the inflammatory process (Ricciotti & FitzGerald, 2011). Frolov's study (2013) provided a good indication of how PGE_2 contributes in promoting an anti-inflammatory action via microsomal prostaglandin E synthase-1 (mPGES-1). It was found that mPGES-1 is highly up-regulated by inflammatory stimuli (LPS) and it has been noted that mPGES-1 expression level is strongly related to the production of PGE_2. This finding suggests possible evidence that PGE_2 may have an anti-inflammatory function such as suppression of the production of pro-inflammatory cytokines by inhibition of the activity of NF-κB. Therefore, PGE_2 may have a fundamental role at the intersection of innate and acquired immunity (Ogawa *et al.*, 2009; Frolov *et al.*, 2013).

1.6.7 Prostaglandin D_2 (PGD_2)

There are also other PGs that have been shown to modulate inflammatory responses in addition to PGE_2 such as PGD_2. PGD_2 is synthesised by the conversion of PGH_2 via two enzymes: hematopoietic PGD synthase (h-PGDS) and lipocalin- type PGDS (l-PGDS). Hematopoietic PGDS can be found constitutively in tissues, whereas l-PGDS is induced during bacterial infections (Maicas *et al.*, 2012).

PGD_2 can be produced by many cells including mucosal mast cells or by intestinal epithelial cells via inflammation. Basically, the impact of PGD_2 is mediated by binding to either the G protein-coupled receptors DP1, DP2 or by conversion to its metabolite 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) (Xue *et al.*, 2005). 15d-PGJ_2 can mediate anti-inflammatory responses by blocking pro-inflammatory NF-κB

signalling cascades independently of peroxisome proliferator–activated receptor, PPAR γ , via direct interactions with signalling molecules (Kim *et al*., 2007).

1.6.8 Prostaglandin receptors

Prostaglandin receptors have very wide biological functions, including immunomodulation and specifically suppression of inflammatory mediator release. By acting through PG receptor subtypes, PGs induce diverse pharmacological reactions in different tissue types (Coleman *et al.*, 1994). Long-chain omega-3 fatty acids have anti-inflammatory effects in mammalian systems and they are precursors for the 3-series of prostaglandins (Nomura *et al.*, 2011; Brown *et al.*, 2013a). Omega-3 PGs could also have a major effect on these receptors and subsequently, cytokine production (TNF-α /IL-1β) might be affected (Brown *et al.*, 2013a; Fraga *et al.*, 2016). However, the present thesis focuses mainly on the omega-6 Arachidonic acid derivatives.

The classification of prostaglandin receptors is based on prostanoid ligands (Clarke *et al.*, 2005). Nine categories of prostaglandin receptors have been established (Table 1.4). There are specific receptors for the different PGs. For example, PGD_2 acts on D receptors (termed DP), while $PGF_{2\alpha}$, PGE_2, PGI_2 and TXA_2 bind to FP, EP, IP and TP receptors respectively (Hata & Breyer, 2004; Surh *et al.*, 2012).

PGE_2 acts through its four cell surface receptors (EP1, EP2, EP3 and EP4), but it is not certain which of these receptors is important in controlling cytokine production particularly TNF-α (Davidson *et al.*, 2012). Other prostanoid receptors involved in

inflammation and immune system modulation are DP1, FP, IP, TP, DP1 and DP2 receptors (also known as the chemoattractant receptor homologous molecule expressed on T helper type 2 cells-$CRTH_2$)(Fig. 1.10). The functions of prostaglandin receptors are determined by several factors like ligand-receptor affinity, receptor expression cellular type and their intracellular signalling system. Prostaglandins can activate more than one receptor subtype on any one cell to modulate cytokine (TNF-α/IL-1β) production (Hata & Breyer, 2004). For example, although a DP1 receptor agonist (BW245C) has a high selectivity towards this receptor, it retains activity against EP2 and EP4 receptors as well. However, it has also been demonstrated that the DP1 antagonist (BWA868C) is not able to change the action of this DP1 agonist (BW245C). This may indicate that the action of BW245C was most likely mediated through EP receptors (Rangachari *et al.*, 1995; Abramovitz *et al.*, 2000).

Prostaglandin receptors are cell surface transmembrane proteins that are associated with heterotrimeric G-proteins inducing different intracellular signalling pathways (Sugimoto *et al.*, 2003). Each prostanoid receptor activates G-protein differently according to the c-terminal region of the receptors. For instance, EP2, EP4, IP and DP1 receptors can activate Gs-protein and activate adenylate cyclase thereby increasing the formation of cAMP. This is followed by stimulation of protein kinase A (Yamamoto & Suzuki, 1987; Adie *et al.*, 1992; Alexander *et al.*, 2011). Other receptors such as EP1, TP and FP stimulate Gq-protein and elevate intracellular Ca^{2+} levels (Funk, 2001; Alexander *et al.*, 2011). In addition to activated Gs and Gq, EP3 and DP2 receptors can trigger the pertussis toxin-sensitive Gi protein which inhibits

adenylate cyclase and thus decreases cAMP production (Bos *et al.*, 2004; Alexander *et al.*, 2011) (Table 1.4).

Cytokines such as TNF-α and IL-1β can induce an increase of intracellular cAMP suggesting that the cAMP pathway is a critical regulatory component in immune cells. A stimulation of PGE_2 can lead to an increase in cAMP because TNF-α can cause a phosphorylation of protein kinase A (PKA) substrates in a COX-2-dependent manner. However, it has been demonstrated that each PGE_2 receptors can modulate TNF-α differently. For instance, up-regulation of either EP2 or EP4 receptors but not EP3 by TNF-α indicates the presence of an increase of cAMP. In contrast, EP3 receptor up-regulation causes a decrease in cAMP levels which suggests that EP3 receptors are likely to be coupled to Gi proteins (Kunisch *et al.*, 2009).

The EP4 receptor can also link to the phosphatidylinositol 3-kinase (PI3K) pathway, because this signalling cascade is usually activated in many inflammatory responses by stimuli such as LPS followed by induction of cytokines (IL-1β/TNF-α). The EP3 and EP1 receptor can inhibit adenylate cyclase and activate phospholipase C (PLC) which is required for immune mediators including cytokines. This can lead to the activation of protein kinase C (PKC) via an increase in free intracellular levels of calcium (Ojaniemi *et al.*, 2003; Biswas *et al.*, 2004; Fantuzzi *et al.*, 2008; Andrade da Costa *et al.*, 2009; Haddad *et al.*, 2012).

Activated cells that produce PGD_2 lead to different pro-inflammatory effects depending on which DP receptors are expressed/ activated though both DP1 and DP2 receptors which bind PGD_2 with high affinity. DP2 receptors are expressed on

human Th2 lymphocytes which can modulate chemotaxis and cytokine production (Bos *et al.*, 2004). DP1 and DP2 receptors have different actions on cAMP production. This was demonstrated by using a selective agonist for DP1 (BW245C) and DP2 (DK-PGD_2). It has been found that activation of DP1 leads to an increase in cAMP but DP2 reduces cAMP production (Liang *et al.*, 2005).

In conclusion, different functions of prostaglandins are controlled by their receptors that induce various intracellular signals. Prostaglandin receptors are expressed in a broad range of immune cells. Each receptor is itself affected by either pro-inflammatory or anti-inflammatory pathways. The production of prostaglandins can be suppressed by NSAIDs, however, the desirable anti-inflammatory effects further upstream in the production of cytokines, could be disrupted. Thus, it would be useful to use receptor agonists which mimic the actions of specific prostaglandin receptors distinct from those involved in normal essential physiological processes (Sugimoto & Narumiya, 2007).

Table 1.4: Signal transduction pathways of different 2-series prostaglandin receptors.

Prostaglandin	Receptor type	Signalling pathway	Key reference
PGE_2 PGI_2 PGD_2	EP2/ EP4 IP DP1	Activate Gs activate adenylate cyclase increase cAMP	(Yamamoto & Suzuki, 1987; Adie *et al.*, 1992; Alexander *et al.*, 2011)
PGE_2 TXA_2 $PGF_{2\alpha}$	EP1 TP FP	Activate Gq increase intracellular Ca^{2+}	(Funk, 2001; Alexander *et al.*, 2011)
PGE_2 PGD_2	EP3 DP2	Activate Gi decrease adenylate cyclase decrease cAMP	(Bos *et al.*, 2004; Alexander *et al.*, 2011)

Different prostaglandin receptors characterised and grouped by their respective signal systems.

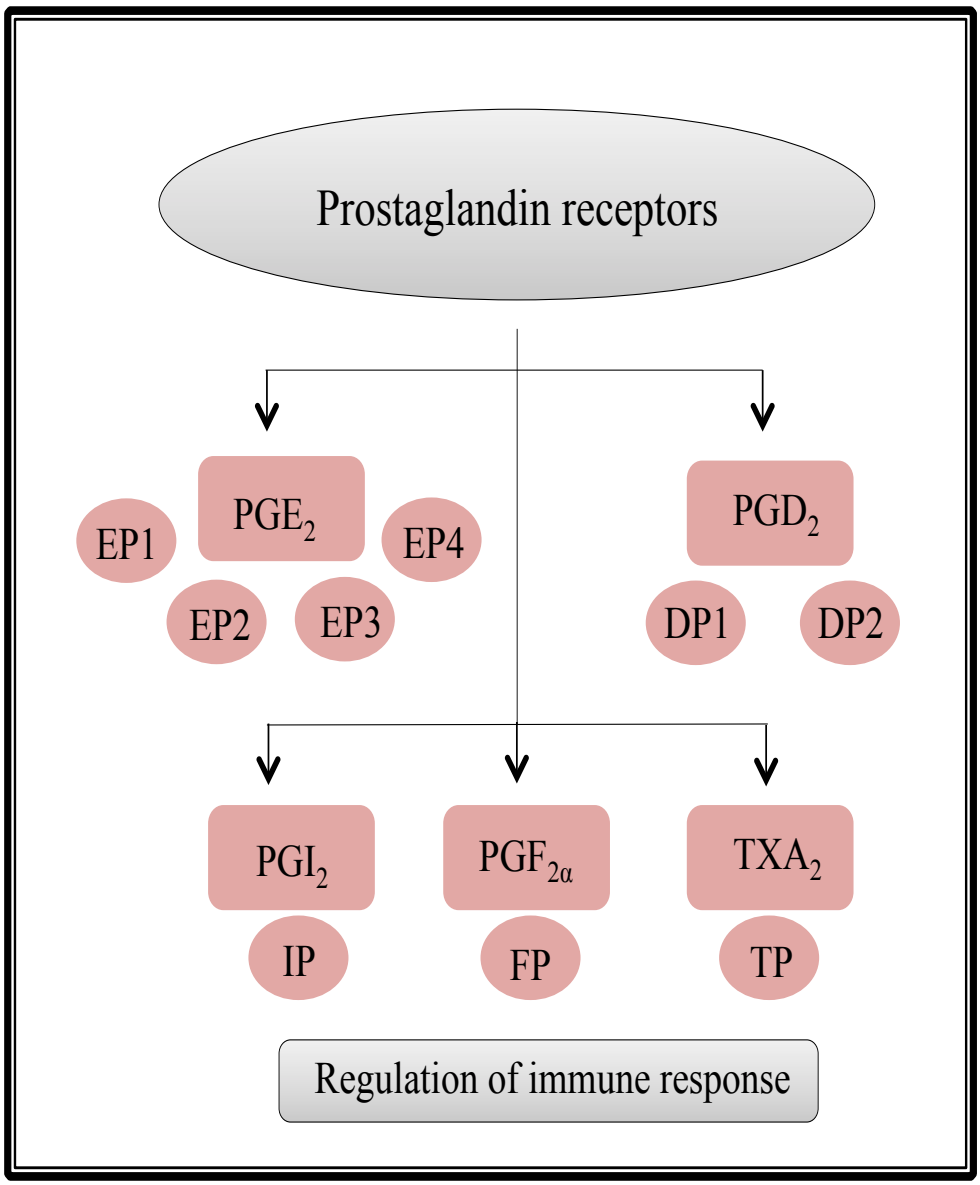


Fig. 1.10: Prostaglandins receptors.

PGE_2 receptors are EP1, EP2, EP3 and EP4. PGD_2 receptors are DP1 and DP2. PGI_2 receptor is IP. $PGF_{2\alpha}$ receptor is FP. TXA_2 receptor is TP.

1.6.8.1 Prostaglandin E_2 receptor subtypes

As previously indicated, PGE_2 can act through at least four different receptor subtypes (EP1, EP2, EP3 and EP4). The individual genes responsible for their expression are termed PTGER1, PTGER2, PTGER3 and PTGER4 respectively. The EP2 receptor (PTGER2) can suppress the oxidative damage which is caused by LPS activation, suggesting that EP2 may be a useful potential therapeutic target for this suppression related to the activation of the innate immune response. The EP4 receptor (PTGER4) can also mediate the suppression of immune cell functions by PGE_2 (Hata & Breyer, 2004).

Analyses of the expression of the four EP receptors (PTGER1, PTGER2, PTGER3 and PTGER4) have demonstrated their presence on many cell types including monocytes, reinforcing the major critical function that PGE_2 has in the immune system (Tilley *et al.*, 2001; Hata & Breyer, 2004). However, it remains unclear if any of the EP receptors is involved in actually regulating expression i.e. at the mRNA level. This study investigated the way in which EP receptors may have critical functions by modulating the expression of EP receptor genes.

EP receptors can be divided into two classes according to their binding affinities for PGE_2, high-affinity receptors [EP3 and EP4 (K_d < 1 nM)] and low-affinity receptors [EP1 and EP2 (K_d > 10 nM)] (Konger *et al.*, 2005). The low affinity receptors require considerably higher concentrations of PGE_2 for effective signalling and vice versa (Kalinski, 2012). This variation in PGE_2-binding affinities and signalling pathways

illustrates the variety of PGE_2-mediated signalling. This may also be related to the location of PGE_2 actions (Konger *et al.*, 2005).

1.6.8.2 PG receptor functions

Interestingly, each EP receptor subtype has a specific pharmacological function within any given tissue. The EP receptors were primarily categorised by their functional role. For example in smooth muscle, generally, EP1 and EP3 stimulate smooth muscle contraction while EP2 and EP4 produce a reverse effect (smooth muscle relaxation) (Ball *et al*., 2013). A possible explanation of this is that the EP2 and EP4 receptors (relaxant) can bind to Gs protein to stimulate cAMP and result in relaxation of the tissue. EP1 receptors (constrictor) can increase intracellular calcium which is required for muscle contraction. The EP3 receptor has an inhibitory effect by coupling to Gi and decreasing cAMP but EP1 is coupled to Gq (Hata & Breyer, 2004).

The function of other prostanoid receptors like DP and IP receptors is very close to the EP2 receptor in terms of being involved in smooth muscle relaxation. In addition to Ca^{2+} coupled EP1 receptor, there are other Ca^{2+} coupled prostanoid receptors such as TP and FP which are also similar to the EP1 receptor in smooth muscle constriction (Toh *et al.*, 1995).

EP4 receptors can induce an extracellular signal-related kinase 1/2 (ERK 1/2) to be phosphorylated by PI3-kinase. This leads to activation of the GSK-3/β-catenin

signalling pathway. Phosphorylation of β-catenin by GSK-3 leads to a cytosolic sequestration allowing β-catenin to be located in the nucleus. However, the EP2 receptor can activate β-catenin via a PKA-dependent, P13-kinase-independent pathway. It is possible that an increase in cAMP may reduce EP4 receptor signalling compared with signalling via the EP2 receptor (Hata & Breyer, 2004).

Although, the function of EP2 and EP4 receptors are similar, the signalling by EP2 and EP4 is prompted by different concentrations of PGE_2. To be specific, EP4 signalling is desensitised after its interaction with PGE_2. However, EP2 is resistant to ligand-induced insensitivity, involving its capability to mediate PGE_2 functions at later stages of inflammation where there are higher concentrations of PGE_2. The variations in PG receptor sensitivity and ability to stimulate many signalling pathways between various PGE_2 receptors on the same cell may permit adaptable designs of responses at different stages of immune responses depending on the concentration of PGE_2 (Kalinski, 2012).

The different prostanoid receptors have distinctive structural variations. The EP4 receptor has a longer C-terminal tail than the EP2 receptor which is required for fast agonist-induced desensitisation of the EP4 receptor. The EP4 receptor consists of 488 amino acids with serines located between 370 and 382 of these amino acids. This specific site of serines leads to a rapid desensitisation of the EP4 receptor (Bastepe & Ashby, 1999). On the other hand, the EP2 receptor contains 358 amino acids with a short C-terminal domains. The lack of exposed C terminal serine

residues may therefore be a reason that the EP2 receptor does not undergo rapid agonist-induced desensitisation (Nishigaki *et al.*, 1996).

The EP3 receptor has an important characteristic unlike other EP receptors. This feature is splicing of the C-terminal tail which produces multiple splice variants. For instance, in humans, eight EP3 receptor splice variants have been recognised. These splice variants have diverse roles in terms of signal pathway activation and agonist-induced desensitisation (Hata & Breyer, 2004).

1.6.8.3 Prostaglandin receptor ligands

Functional classification of prostaglandin receptors can be achieved using either selective agonists or antagonists. Under specific physiological conditions, some endogenous prostanoids are extremely unstable and are rapidly cleared from the bloodstream, such as PGI_2 and TXA_2. They have relatively short half-lives compared to PGE_2. The half-life of PGI_2 is approximately between 7-10 minutes (Ritter *et al.*, 1982) and TXA_2 has a half-life of 30-37 seconds (Dubin *et al.*, 1982; Ricciotti & FitzGerald, 2011), whereas PGE_2 has a much longer half-life (8+/-3 hours) (Ishihara *et al.*, 1991). Because the half-lives of some prostanoids are relatively short, a number of stable synthetic prostaglandin analogues have been developed with extended half-lives to be used for functional receptor studies. The structures of these agonists are close to their corresponding prostaglandins (Jones *et al*., 2009). Moreover, EP1 receptors have a similar structure as FP and TP receptors, while EP2 receptors show amino acid sequence similarity with IP and DP1 receptors (Toh *et al.*,

1995; Boie *et al.*, 1997). Fig. 1.11 shows chemical structures of prostaglandin receptor ligands.

Selective prostaglandin receptor analogues have been well demonstrated by earlier studies (Fig. 1.12). An example of a PGE_2 receptor agonist is butaprost, an EP2 agonist which can be used as a preliminary step in identifying the action of PGE_2 through EP2 receptors (Honda *et al.*, 1993). Sulprostone has been used as an EP1/ EP3 receptor agonist (Poeschmann *et al.*, 1991; Kennedy *et al.*, 1999; Alexander *et al.*, 2011). It has been shown that L-902, 688 acts as a selective agonist for EP4 receptors (Benyahia *et al.*, 2012; Konya *et al.*, 2013a). In addition to these PGE_2 receptor agonists, fluprostenol is one of the $PGF_{2\alpha}$ receptor analogues which only appears to bind to FP receptors (Jones *et al.*, 2009). Table 1.5 shows the EC50s of some prostanoid receptors.

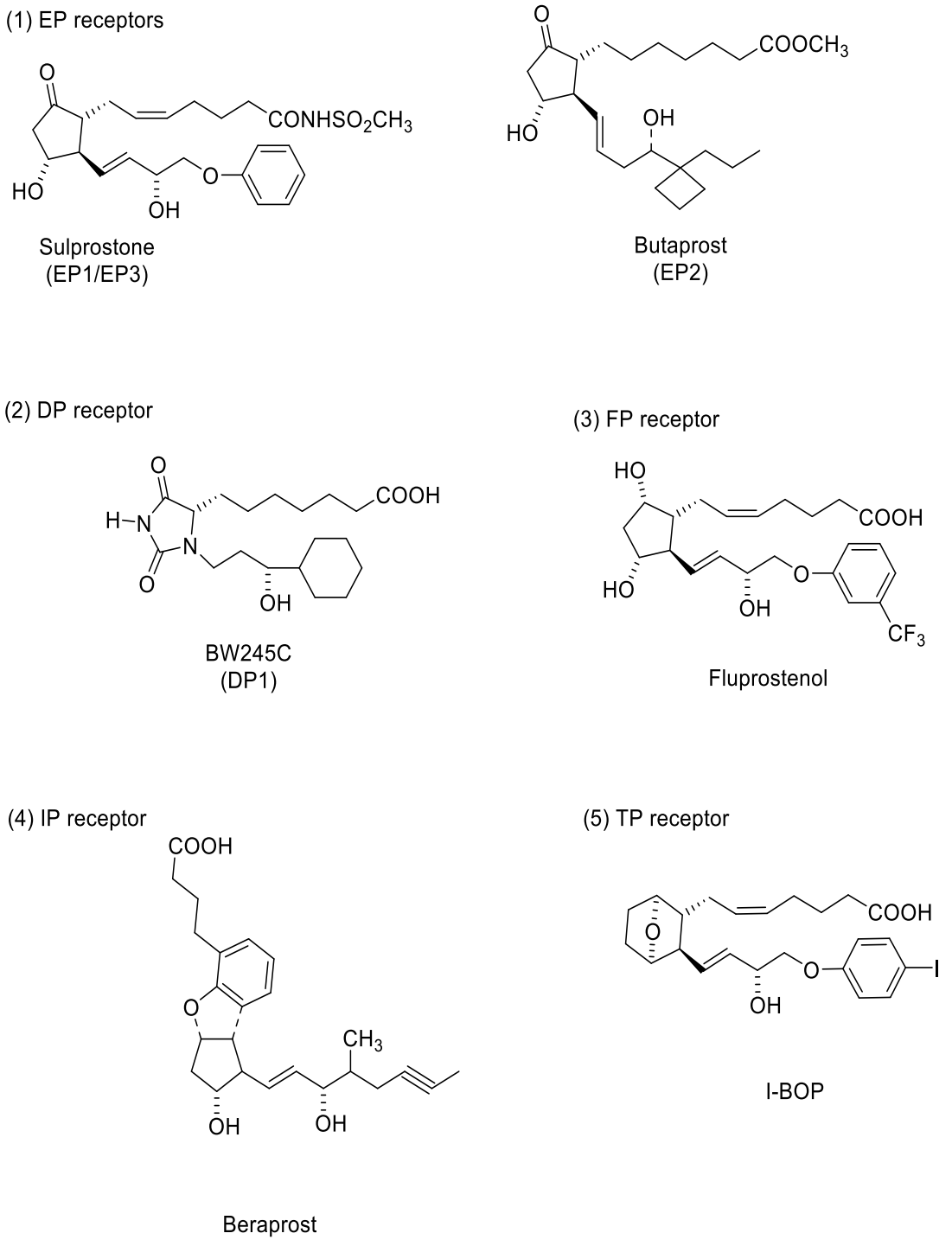


Fig. 1.11: Structures of prostaglandin receptor ligands.

This figure is adapted from (Narumiya *et al.*, 1999).

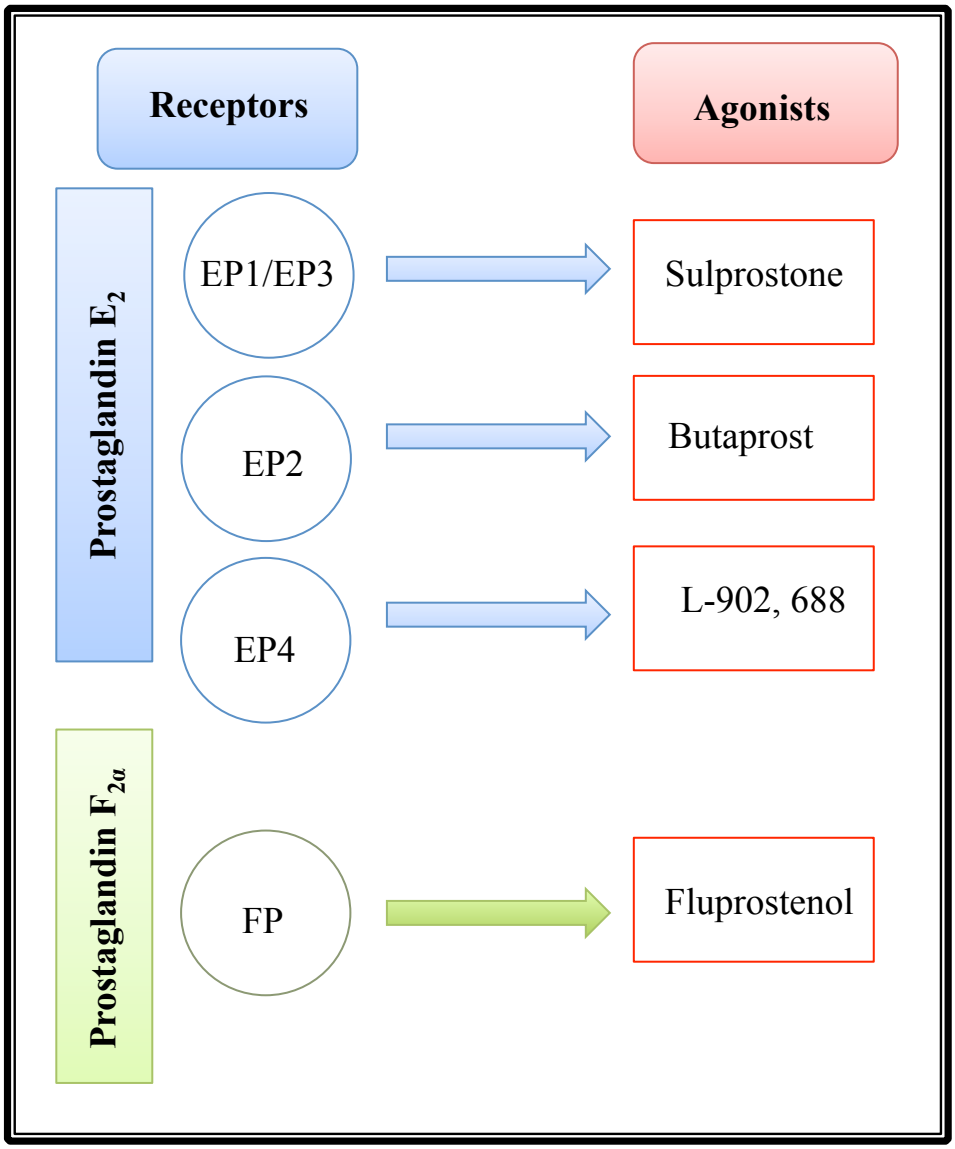


Fig. 1.12: Selective prostaglandin receptor agonists.

Table 1.5: A potency of prostanoid analogues (EC50 values).

Prostanoid receptor	EC50	Key reference
EP1/EP3 agonist (Sulprostone)	4-10 nM	(Matthews & Jones, 1993)
EP2 agonist (Butaprost)	3-10 μM	(Baba *et al.*, 2001)
EP4 agonist (L902,688)	7.76 nM	(Pantazaka *et al.*, 2013)
FP agonist (Fluprostenol)	6.1 nM	(Sharif *et al.*, 2002)

Selective prostaglandin ligands for the various EP receptors. Table shows ligands for EP receptors indicating their EC50 values.

1.7 Aims and objectives

The production of pro-inflammatory cytokines such as TNF-α and IL-1β and the subsequent production of prostaglandins, especially from monocytic cells, in response to bacterial lipopolysaccharide (LPS) is an important inflammatory function (Zhang & An, 2007). Previous studies have clearly shown that prostaglandins play a critical role in inflammation by acting in a pro-inflammatory manner from a symptomatic perspective but can also be anti-inflammatory via their ability to switch-off cytokine production upstream (Brown *et al.*, 2013b). The capacity of prostaglandins to suppress pro-inflammatory cytokines is an important negative-feedback regulatory mechanism. PGE_2 can act through at least four receptor subtypes EP1–EP4 (Davidson *et al.*, 2012). However, it is not certain which of these receptors is important in controlling cytokine production. The present study set out to clarify how these receptors are involved in an immune response, specifically the control of pro-inflammatory cytokine production from human monocytic cells. This was achieved by adopting the following approaches:

a) Using a pharmacological approach with receptor-selective agonists/ antagonist.

b) Measuring either up- or down-regulation of genes for these receptors.

c) Estimating the effects of alterations in the expression of EP receptor genes on functional end-points i.e. cytokine production.

Thus, a major aim of this study was to ascertain the expression profile of PGE_2 receptors, especially following LPS-stimulation, and also correlating this to the release of TNF-α and IL-1β. This was achieved by studying the expression of mRNA for PG receptors by qRT-PCR in human monocytes isolated from human

peripheral blood or the human monocytic cell line THP-1. Cytokine production (TNF-α and IL-1β) from human monocytic cells was measured by ELISA assay. PG-receptor-selective ligands were also used to ascertain which of these activities were regulated by specific EP receptors.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General Reagents and Materials

All materials and reagents used were of the highest commercial grade available and were obtained from the following suppliers.

Sigma-Aldrich Co. Ltd (Poole, Dorset, UK)

Acetic acid, Agarose powder, Ammonium persulfate, Bovine serum albumin (BSA), Carbenicillin, Disodium hydrogen phosphate (Na_2HPO_4), Dimethyl sulfoxide (DMSO), Dulbecco's modified eagle's medium (DMEM), Ethanol, Ethidium bromide, Ethylenediaminetetraacetic acid (EDTA), Fetal Calf Serum (FCS), Ficoll Histopaque, Formaldehyde, Glycerol, Hanks' Balanced Salt Solution (HBSS), HCl, Isopropanol, Ketoprofen, L-glutamine, Lipopolysaccharides (LPS from Salmonella), Methanol, Monopotassium phosphate (KH_2PO_4), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), N,N,N',N'tetramethylenedia-amine (TEMED), non-essential amino acid, nutrient mixture F-12 Ham's media, Penicillin/streptomycin, Phosphate buffered saline (PBS), Polyinosinic–polycytidylic acid sodium salt (Poly IC), Ponceau S red powder, Potassium chloride (KCl), Roswell Park Memorial Institute medium (RPMI-1640) with L-glutamine, Sodium chloride (NaCl), Sodium Dodecyl Sulphate (SDS), Sulphuric acid, Tris-Borate-EDTA (TBE), Triton x-100, Trypan blue, TWEEN-20 and Typsin.

Abcam Biotech Company (Cambridge, UK)

Fluoroshield Mounting Medium With DAPI.

Severn Biotech. Ltd (Worcestershire, UK)

30 % acrylamide/bisacrylamide solution.

Nunc (Roskilde, Denmark)

1ml Cryo tube vials and Tissue culture flasks (75 cm^2).

TPP Techno Plastic Products (Switzerland, Europe)

6, 24, and 96-well tissue culture plates.

Trefflab, Scotlab Bioscience Ltd (Scotland, UK)

Eppendorf microcentrifuge tubes.

Elkay Laboratory Products Ltd (Hampshire, UK)

30ml sterile tubes.

Corning Science Products (USA)

Sterile 100 mm Petri-style culture dishes.

StarLab Ltd (Milton Keynes, UK)

Graduated Tips, 0.6 ml RNase/ DNase and pyrogen safe tubes.

VWR International Ltd (Leicestershire, UK)

0.5 ml Micro tubes with cap.

Amersham International Plc (Aylsbury, Buckinhamshire, UK)

0.45µM 3MM Nitrocellulose blotting membranes and 80x 90 mm blotting paper.

Gilson (Middleton, USA)

Pipettes.

2.1.2 Prostaglandins and related compounds

Cayman Chemical Co. (USA)

Butaprost (EP2 agonist), Fluprostenol ($PGF_{2\alpha}$ agonist), L-902, 688 (EP4 agonist),

Prostaglandin D_2 (PGD_2), Prostaglandin E_2 (PGE_2), Prostaglandin E_2-biotin (PGE_2-biotin) and Sulprostone (EP3 agonist).

2.1.3 Enzyme-linked immunosorbent assay (ELISA)

Invitrogen, Life Technologies Ltd (paisley, UK)

HRP chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB), Human IL-1β ELISA Kit, Human TNF-α ELISA Kit and Streptavidin-HRP Conjugate.

2.1.4 Kits and products for molecular biology

Sigma-Aldrich Co. Ltd (Poole, Dorset, UK)

Free-RNAase water, GenElute™ Mammalian Total RNA Miniprep Kit and On-column DNase I digestion.

Life Technologies Ltd (paisley, UK)

Gene Expression Cells-to-CT Kit, PCR fast reaction MicroAmp tubes and Applied Biosystems SYBR Select.

Bioline Reagents Ltd (London, UK)

HyperLadderI/ HyperLadderII (DNA molecular weight marker) and Tetro cDNA Synthesis Kit.

QIAGEN Sample and Assay Technologies (West Sussex, UK)

HiPerFect Transfection Reagent and TE buffer.

Integrated DNA Technologies (UK)

TriFECTa RNAi kit: [Three target-specific dicer substrate siRNA duplexes (2 nmoles each), Positive Control duplex (1 nmole) and Negative control duplex (NC1) (1 nmole)].

2.1.5 Western blot

Thermo Fisher Scientific (Loughborough, UK)

FLAG™ Epitope Tag (DYKDDDDK) Monoclonal Antibody [(FG4R) (1mg/ml)] and GAPDH Loading Control Monoclonal Antibody [(GA1R)(1mg/ml)].

Bioss Antibodies (USA)

Anti-Prostaglandin E Receptor EP4 Polyclonal Antibody (1μg/μl).

Santa Cruz Biotechnology Inc. (CA, USA)

2x Electrophoresis Sample Buffer (ESB) and RIPA lysis buffer system.

Millipore Corporation (Hertfordshire, UK)

Immobilon western chemiluinescent HRP substrate.

Bioline Reagents Ltd (London, UK)

HyperPAGE prestained protein marker.

2.1.6 Cell lines and blood

Human Monocyte LS columns kit for Monocyte isolation (Miltenyi Biotec Ltd, Surrey, UK), Human monocytic leukaemic cell line THP-1 cells [European Collection of Cell Cultures (ECACC), Public Health England (Porton Down, UK)], Human neuroblastoma cell line SH-SY5Y [generously provided by Dr. Elizabeth Ellis (University of Strathclyde, Glasgow, UK)] and Whole human blood (The Glasgow and West of Scotland blood Transfusion Service, UK).

2.1.7 Transformation of plasmid DNA

Life Technologies Ltd (Paisley, UK)

Competent cells (TOP10 E.coli), FastDigest XhoI and SOC medium.

GeneCopoeia (Rockville, USA)

FLAG-PTGER4 plasmid DNA (OmicsLinkTM ORF expression clone, EX-Q0086-M11).

Bioline Reagents Ltd (London, UK)

ISOLATE II Plasmid Mini Kit and Quick-Stick Ligase kit.

BioLabs (Ipswich, UK)

DpnI and Q5® Hot start High-Fidelity DNA polymerase 2X Master Mix.

GE Healthcare (Little Chalfont, UK)

Illustra PCR DNA and gel band purification kit.

QIAGEN Sample and Assay Technologies, (Crawley-West Sussex, UK)

QIAfilter Plasmid Maxi Kit.

Sigma-Aldrich Co. Ltd (Poole, Dorset, UK)

Agar powder for microbiology and LB powder.

2.1.8 Equipment and instruments

Nikon (UK)

Epi-fluorescence microscope (consists of: Nikon eclipse E600, photometrics cool snap fx, cool LED PE-2 collimator and cool LED PE excitation system) and TMS Microscope.

Molecular Devises (USA)

Spectra Max 190 Absorbance Micro-Plate Reader.

Thermo Scientific, Labtech International Ltd (East Sussex, UK)

Nano drop Spectrophotometer (ND, 2000C).

Applied Biosystems, Life Technologies (Paisley, UK)

StepOne Plus real-time PCR system.

ATTO Corporation (Japan)

ATTO gel apparatus for western blot electrophoresis.

Bio-Rad Laboratories (Hertfordshire, UK)

Mini trans blot for transferring blot.

VWR International Ltd (Leicestershire, UK)

UV transilluminator.

PerkinElmer (UK)

DNA Thermal cycler.

Sanyo (Loughborough, UK)

CO_2 Incubator.

Heraeus (Germany)

Multifuge Centrifuge.

Eppendorf Ltd (UK)

Mini Centrifuge 5415D.

Weber Scientific (Hamilton, USA)

Haemocytometer.

2.2 Methods

2.2.1 Cell culture

All cell culture work was conducted in a Class II biological safety cabinet, following strict aseptic conditions.

2.2.1.1 Preparation of THP-1 cells

THP-1 cells were incubated in flasks containing RPMI 1640 with 10% fetal calf serum (FCS) at 37°C, 100% humidity, 5% (v/v) CO_2. Cells were passaged when they reached 80-90% confluence then collected for experiments by transferring them into 30ml sterile tubes followed by centrifugation at 400xg 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 1×10^6 cells/ml. Cells were maintained at 37°C, 5% (v/v) CO_2 and the media replaced every 4 days.

2.2.1.2 Long-term storage and recovery of THP-1 stocks

Once THP-1 cells reached 80%-90% confluence, cells were centrifuged as described in previous Section (2.2.1.1). Pelleted cells were mixed with 1 ml of freezing mixture and stored in a Cryo tube vial at -80°C until further recovery use. The freezing mixture contained 50% fetal calf serum (FCS), 40% RPMI 1640 and 10% DMSO and stored at -20°C. THP-1 cells were recovered by quick thawing at 37 °C and by re-incubating the thawed cells into fresh RPMI 1640 with 10% (v/v) FCS and incubated at 37°C, 100% humidity, 5% (v/v) CO_2 as described previously.

2.2.1.3 Preparation of SH-SY5Y cells

SH-SY5Y cells were incubated in flasks containing an equal volume (1:1) of DMEM and Ham's F12 media containing 10% FCS, 5 ml non-essential amino acids, 5 ml L-glutamine and 5 ml penicillin-streptomycin antibiotic (10,000 units) at 37°C, 100% humidity, 5% (v/v) CO_2. Cells were passaged when they reached 80-90% confluence. Cells were maintained and the media replaced every 4 days. The old media was discarded and cells were washed with 2 ml of PBS. PBS solution was removed and 1 ml of 1% trypsin was added. Cultured flask containing cells with trypsin was incubated at 37°C for 2-3 minutes. The flask was firmly shaken and the media/cells were transferred into a new culture flask containing fresh media. Trypsin was then inactivated once the media was added. The flask was incubated at 37°C.

2.2.1.4 Isolation of human peripheral blood monocytic cells (PBMCs)

Adherent human peripheral blood monocytic cells (PBMCs) were isolated by density centrifugation from whole blood. Blood (10 ml) was layered onto 10 ml Ficoll Histopaque and centrifuged at 400xg for 90 minutes at room temperature. The interface cell layer (between plasma and separation medium) was collected into sterile tubes, diluted with HBSS medium to 20 ml and centrifuged at 400xg for 5 min at room temperature. Cell pellets were resuspended in RPMI 1640 medium. Aliquots of the suspension were transferred to sterile 100 mm Petri-style culture dishes and incubated at 37°C in 100% humidified air with 5% (v/v) CO_2 for 4 hours. Non-adherent cells were discarded and culture dishes were washed 3 times and scraped into HBSS medium. The cells were centrifuged for 20 minutes at 400xg at room

temperature. The cell pellet was resuspended in RPMI 1640 medium. These peripheral blood monocytes were used at a concentration of 2×10^6 cells/ml.

2.2.1.5 Isolation of monocytes using LS columns and magnetic separation

Peripheral blood monocytic cells were isolated following the same protocol as Section 2.2.1.4 but cells were not placed into Petri dishes. The resuspended cells were used at a concentration of 2×10^8 cells/ml. Magnetic labelling requires up to 1×10^7 total cells using the Human Monocyte LS column kit (Miltenyi Biotec Ltd, Surrey, UK), therefore, 50 µl were labelled. Two ml of 2×10^8 cells were centrifuged at 400xg for 10 minutes. Cell pellets were resuspended in 2 ml of cold (4°C) PBS/BSA/EDTA buffer. This buffer (pH 7.2) contained 50 ml of 10x PBS, 2.5 g of BSA (0.5%), 372 mg of EDTA (2 mM) in 500 ml distilled water. Cell suspension (500 µl) was centrifuged at 400xg for 10 minutes. Cell pellets were then resuspended in 300 µl of the cold PBS/BSA/EDTA buffer. Cold FCR blocking-reagent and cold Biotin cocktail (both 100 µl) were then added (supplied with the Human Monocyte LS column kit). The mixture was mixed and incubated at 4°C for 10 minutes. Cold buffer (300 µl) and 200 µl of cold Anti-biotin were added. The mixture was mixed and incubated at 4°C for 15 minutes. Cells were washed with 2 ml of cold buffer and centrifuged at 400xg for 10 minutes. The cell pellets were resuspended in 500 µl of buffer. Column separation was carried out in a cold room (4°C). An LS column was placed on a magnetic stand and washed with 3 ml of the cold PBS/BSA/EDTA buffer. The eluting cells (500 µl) were collected from the column, washed with 9 ml of the cold PBS/BSA/EDTA buffer and then centrifuged at 400xg for 10 minutes and

used as monocytes. The pelleted monocytes were re-suspended with RPMI 1640 and used at a concentration of 3×10^5 cells/ml.

2.2.1.6 Preparation of mononuclear cells from human blood

Whole human blood was isolated by density centrifugation. Blood (10 ml) was layered onto 10 ml Ficoll Histopaque (Sigma-Aldrich Co. LtD (Poole, Dorset, UK)) and centrifuged at 400xg for 90 minutes at room temperature. The interface cell layer (between the plasma and separation medium) was used as the mixed mononuclear cell fraction and collected into sterile tubes, diluted with HBSS medium up to 20 ml and centrifuged at 400xg for 40 min at room temperature. Cell pellets were resuspended in RPMI 1640 medium and used at a concentration of 1×10^6 cells/ml.

2.2.1.7 Human blood incubation protocol

Human blood (700 µl) was added to sterile 1.5 ml microcentrifuge tubes. To each microcentrifuge tube, different compounds were added in a volume of 100 µl of RPMI 1640 to yield the various concentrations indicated in the Results Section. The incubations were all carried out in a final total volume of 1 ml. Control (no treatments) for this protocol was 700 µl of human blood + 300 µl of RPMI 1640. The samples were then vortex mixed and placed in an incubator for 22 hours at 37°C, 100% humidity with the tubes remaining opened to allow CO_2 access. After 22 hours incubation, the blood samples were centrifuged at 12, 000xg for 40 seconds after which the plasma was transferred into fresh microcentrifuge tubes and stored at -20°C (up to 6 months) until required for the assay.

2.2.1.8 THP-1 cells, monocytic cells and mononuclear cells incubation protocol

Either THP-1 cells, monocytic cells or mononuclear cells ($1x10^6$ cells/ml) were added into 24-well or 6-well culture plates. Different treatments such as Poly IC, LPS, PGE_2 etc., were always added in a volume of 50 µl dissolved in RPMI 1640 (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. Additions were adjusted to 200 µl of each treatments dissolved in RPMI 1640 in a final volume of 2 ml in 6-well plates. Control for this protocol was cells without any treatments dissolved in RPMI 1640. The plates were then incubated at 37°C, 5% (v/v) CO_2 for 22 hours. After the incubation period, all samples were transferred into microcentrifuge tubes and stored at -20°C (up to 6 months) until required for the assay.

2.2.1.9 SH-SY5Y cells incubation and harvesting protocol

SH-SY5Ycells were prepared as described in Section 2.2.1.3. The cell culture flask which had the control cells (without treatment) was incubated for 24h at 37°C, 100% humidity, 5% (v/v) CO_2. After the incubation period, the flask was placed on ice and washed with cold PBS. PBS was gently removed without affecting the cells and additional PBS was added. Cells were harvested by scraping in a sweeping motion and pipetted into a sterile microcentrifuge and centrifuged for 2 minutes at 19, 000xg. The supernatant was removed and pellet was stored at -20°C (up to 6 months).

2.2.2 Measurement of cytokines

2.2.2.1 ELISA solutions and buffers

Solutions and buffers were prepared as follows:

Coating buffer 10X: 40 g of NaCl, 5.6 g of Na_2HPO_4, 1 g of KH_2PO_4 and 1 g of KCl were dissolved in 500 ml dH_2O. This coating buffer was diluted 1 in 10 (working-strength buffer) when required.

Standard diluent/assay buffer: 1 g of bovine serum albumin and 200 µl of Tween-20 were dissolved in 200 ml of the coating buffer.

Blocking solution: 1 g of bovine serum albumin was dissolved 200 ml of coating buffer (0.5 %).

Wash buffer: 0.5 ml of Tween was added to 500 ml of the working-strength coating buffer.

2.2.2.2 Measurement of TNF-α

In order to measure the production of TNF-α, a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) was used. Immunosorbent modules (see Materials Section 2.1.3) were coated with 100 µl of coating/capture antibody which was diluted 1:1000 in coating buffer to each well (2 µg/ml). The plates were incubated for 18 hours at 4°C. Following this, the antibody was removed and the plate was washed 4 times with washing buffer. Next, 300 µl of blocking solution (0.5% BSA) was added into the wells and the plate was incubated at room temperature for a minimum 2 h. The blocking solution was discarded and the plate was washed 3 times with wash buffer.

The TNF-α standards were prepared as described in next section (Section 2.2.2.3) and samples were prepared as explained in Sections 2.2.1.7 and 2.2.1.8. Either 100 µl of TNF-α standards (dissolved in assay buffer) or 100 µl plasma/cultured media samples (RPMI 1640) were added to the appropriate wells. Following this 50 µl of detection antibody was diluted 1: 625 with assay buffer (0.8 µg/ml) and added to each well. Then, the plate was incubated at room temperature for 2 hours. The plate was washed 3 times with wash buffer and 100 µl of Streptavidin-HRP Conjugate, (diluted 1:5000 with assay buffer) was added. Then, the plate was incubated at room temperature for 30 minutes. The plate was washed 4 times with wash buffer then TMB chromogen substrate (100 µl) and the plate was further incubated at room temperature for 30 minutes. At 30 minutes, when a blue colour appeared, 100 µl of sulphuric acid (1 M) was added to stop the reaction. The absorbance was measured at 450 nm.

A standard curve for TNF-α was created for each assay (Fig. 2.1) using Statview software to calculate unknown concentration of TNF-α from the absorbance value, the following typical example equation was generated:

$$\mathbf{Y = -19.177 + 557.746x - 839.338x^2 + 407.229x^3}$$

X= Absorbance at 450

Y= concentration of TNF-α (pg/ml)

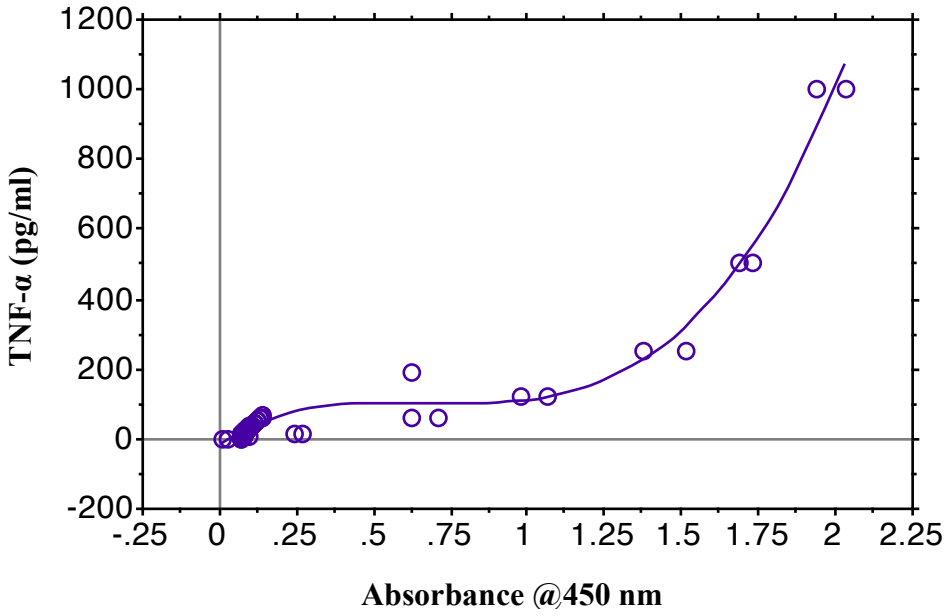


Fig. 2.1: Typical TNF-α standard curve for ELISA assay.

2.2.2.3 Preparation of TNF-α standards

TNF-α standards were prepared by taking 50 µl of TNF-α stock solution (10,000 pg/ml) into 450 µl of assay buffer to give a final concentration of 1000 pg/ml. Then, a serial dilution was carried out in order to prepare different concentrations of TNF-α (1000 pg/ml-16 pg/ml).

2.2.2.4 Measurement of IL-1β

Measurement of IL-1β followed the same steps as TNF-α Measurement (see Section 2.2.2.2).

A standard curve for IL-1β was created for each assay (Fig. 2.2) using Statview software to calculate unknown concentration of IL-1β from the absorbance value, the following typical example equation was generated:

$$Y = 139.768 - 22.624x - 232.122x^2 + 199.514x^3$$

X= Absorbance at 450

Y= concentration of IL-1β (pg/ml)

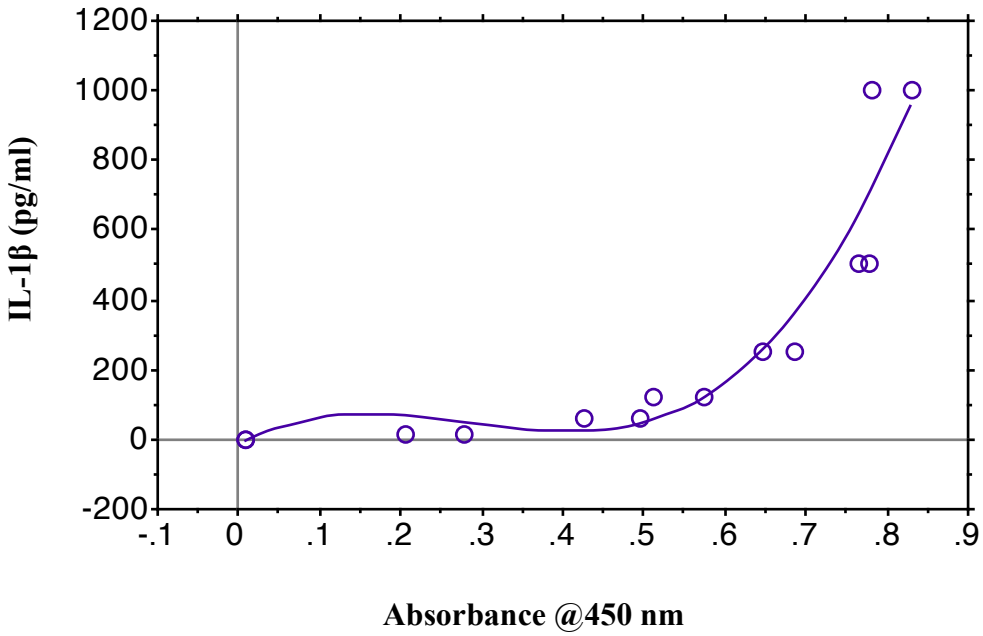


Fig. 2.2: Typical IL-1β standard curve for ELISA assay.

2.2.2.5 Preparation of IL-1β standards

IL-1β standards were prepared by the same steps as TNF-α standards (see Section 2.2.2.3).

2.2.2.6 Statistical analysis of ELISA data

All data was analysed statistically using Statview software. Levels of analytes were calculated from standard curves using 3rd order polynomial regression analysis using Statview Software. Where appropriate either ANOVA or T-tests were used to ascertain statistically significant changes in responses, with $P<0.05$ regarded as a significant change.

2.2.3 Transient cell transfection

THP-1 cells were transfected with either siRNA duplexes for the knock-down study or FLAG-PTGER4 plasmid for the overexpression study.

THP-1 cells ($1x10^6$ cells/ml) were placed into 6-well plates and incubated with different treatments in a final volume of 2 ml and 5 µl of siRNA duplexes (100 nM) (for knock-down assays) or 5 µl of FLAG-PTGER4 plasmid (1904 ng/µl) (for overexpression assays) in the presence 3 µl of transfection reagent (HiPerFect). Untransfected cells were used as controls. Also, in knock-down experiments, two negative control siRNA duplexes (NC1, NC5) and a positive control were used. However, in overexpression experiments, an empty vector that did not contain PTGER4 was prepared and used as a control (Section 2.2.16). Plates were incubated at 37°C, 5% (v/v) CO_2 for different time points (3h, 6h, 12h, 24h, and 48h).

Primary optimisation experiments were performed using different concentrations of siRNA duplexes (1 nM, 10 nM and 100 nM), FLAG-PTGER4 (1904 ng/µl, 952 ng/µl and 476 ng/µl) and empty vector (2461 ng/µl, 1230 ng/µl and 615 ng/µl).

Nucleotide sequences for siRNA duplexes are shown in the following table. However, sequence for positive siRNA duplex control was not provided (Table 2.1).

Table 2.1: Nucleotide sequences of siRNA duplexes used for transfection of THP-1 cells.

Duplex No.	Sequence
Duplex 1	Forward 5'-rGrCrArGrUrUrGrUrArCrCrArArGrUrGrArArArUrUrArUTT-3' Reverse5'-rArArArUrArArUrUrUrCrArCrUrUrGrGrUrArCrArArCrUrGrCrUrU-3'
Duplex 2	Forward 5'-rArGrUrGrCrUrCrArGrUrArArArGrCrArArUrArGrArGrAAG-3' Reverse 5'-rCrUrUrCrUrCrUrArUrUrGrCrUrUrUrArCrUrGrArGrCrArCrUrGrU-3'
Duplex 3	Forward 5'-rArGrArUrArUrUrArGrArArArGrGrCrUrCrUrArUrUrCrCAA-3' Reverse5'-rUrUrGrGrArArUrArGrArGrCrCrUrUrUrCrUrArArUrArUrCrUrGrG-3'
NC1	Forward5'- rCrUrUrCrCrUrCrUrCrUrUrUrCrUrCrUrCrCrCrUrUrGrUGA-3' Reverse5'-rUrCrArCrArArGrGrGrArGrArGrArArArGrArGrArGrGrArArGrGrA-3'
NC5	Forward 5'- rCrArUrArUrUrGrCrGrCrGrUrArUrArGrUrCrGrCrGrUrUAG -3' Reverse5'- rCrUrArArCrGrCrGrArCrUrArUrArCrGrCrGrCrArArUrArUrGrGrU-3'

The melting temperature (Tm) of duplexes was between 47–56 °C.

2.2.4 Cell viability measurement

Before THP-1 cells or monocytic cells were incubated for 22 h as described in Section 2.2.1.8. Cell viability was measured by trypan blue exclusion (estimation of non-viable cells). Trypan blue was added to cells immediately before counting (using a haemocytometer) and the percentage of blue cells was subtracted from the total cell count. In addition, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye assay (measurement of viable cells) was used to determine whether siRNA duplexes or HiPerFect induced cell toxicity. THP-1 cells ($1x10^6$ cells/ml) were placed in 96-well plates and incubated with different treatments all added in a volume of 20 µl (to yield the appropriate final concentrations indicated in the Results Section) in a final total volume of 200 µl (RPMI 1640, LPS (10 µg/ml), PGE_2 (1µM), butaprost (1µM), sulprostone (1µM), L-902, 688 (1µM), siRNAs (100 nM), HiPerFect (3 µl), NC1 (100 nM), NC5 (100 nM), and positive control (100 nM). Untransfected cells and dead cells (boiled cells) were used as controls. Plates were incubated at 37°C, 5% CO_2 for 24h. Treatment media was removed from all wells and replaced with 10 µl of 5 mg/ml MTT solution (diluted in sterile PBS and covered with foil) and left for 4-6 h in incubator at 37°C, 5% CO_2. Four hours post MTT media was removed and cells lysed with 100 µl/well DMSO. Absorbance was then measured at 570 nm in plate using a spectrophotometer.

2.2.5 Bacterial culture

Luria-bertani broth (LB) was prepared by dissolving 10 g of LB powder in 500 ml of dH_2O then autoclaved. LB agar plates were prepared by adding 1.5 g of microbiology agar to LB broth to make 1.5% (w/v) then autoclaved. The agar was

then microwaved and allowed to cool until it could be held by hand. Carbenicillin (100 mg/ml) was added (1:1000 ratio) before pouring into petri dishes and allowed to set.

2.2.6 Transformation of plasmid DNA (FLAG-PTGER4) into competent bacteria

The OmicsLink^{TM} expression clone, FLAG-PTGER4 plasmid DNA was resuspended and diluted to 10 ng/µl with TE buffer upon arrival. To propagate this plasmid, 1 µl of this diluted plasmid was added to a vial of one shot competent cells (TOP10 *E.coli*) which were thawed earlier from -80°C on ice (50 µl/vial) into 2 ml microcentrifuge tube. This mixture was incubated on ice for 30 min. The bacteria were heat-shocked for 30 seconds at 42°C in a water bath then quick chilled on ice for 5 min. 250 µl of pre-heated SOC medium at 37°C (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the tube. The tube was placed horizontally in shaking incubator at 37°C for 1 hour at 200 rpm. This was important to allow the expression of the antibiotic resistance gene coded for in the vector. 20-200 µl of the transformation plasmid was pipetted and spread on LB agar plates. The agar plates were incubated overnight at 37°C. Single colonies were picked for further analysis.

2.2.7 Maxi-prep plasmid DNA (FLAG-PTGER4) preparation

A single colony carrying FLAG-PTGER4 plasmid DNA was picked from LB agar plate (up to 3 weeks old if stored at +4°C) using a sterile loop under aseptic

conditions. This colony was added to a 30 ml sterile universal tube with 10 ml of LB Broth containing 10 µl of carbenicillin (100 mg/ml). The tube was placed horizontally in a shaking incubator at 37°C for 6 hours at 200 rpm. After 6h incubation, the broth was transferred into a 400 ml flask containing 100 ml of LB broth with 100 µl of carbenicillin (100 mg/ml). The flask was placed vertically in shaking incubator at 37°C for overnight. Next day, 0.8 ml of this overnight bacterial culture was added to 0.2 ml of sterile glycerol into a sterile microcentrifuge tube. The tube was vortexed and stored immediately at -80°C for long-term storage. 50 ml of 100 ml overnight bacterial cultures were transferred to sterile 50 ml tube and centrifuged at 3000xg for 10 min at 4°C. Supernatant was discarded, the pellet was kept and the rest of bacterial cultures (50 ml) added and the tube centrifuged again at 3000xg for further 10 min at 4°C. Supernatant was discarded and the pellet was ready to carry out plasmid DNA purification (see the following Section).

2.2.8 Purification of maxi-prep plasmid DNA

The plasmid DNA prepared in the previous Section was purified using a Qiagen Endotoxin-free Maxi plasmid purification kit following the manufacturer's instructions. These are summarised briefly in the following steps:

1. Alkaline Lysis of pelleted bacteria (obtained previously in Section 2.2.7).
2. Clear lysates by filtration using QIAfilter.
3. Treatment of clear lysate with endotoxin-removal agent.
4. DNA binding to gravity-flow anion-exchange columns.
5. Eliminate all contaminants during plasmid DNA preparations using washing buffer.

6. Elute DNA.
7. DNA precipitation by isopropanol and washing of pellet with endotoxin-free 70% Ethanol.
8. Dissolving DNA pellet with endotoxin-free TE Buffer.
9. Obtained product was ultra-pure, endotoxin-free plasmid DNA. Aliquots of this plasmid were stored at -20°C for further use.

2.2.9 Long-term storage and recovery of bacterial stocks

Aliquots (0.8 ml) of the overnight bacterial cultures used in plasmid preps were added to 0.2 ml of sterile glycerol into sterile microcentrifuge tubes. The tubes were vortexed and stored immediately at -80°C until required. When plasmid preps were running out, this glycerol stock was removed from -80°C cells scraped with a sterile inoculation loop, and then streak onto the surface of an LB agar plate containing carbenicillin (100 μg/ml). The frozen stock was returned immediately to the -80°C freezer. The plate was inverted and incubated in a 37°C incubator overnight to allow the growth of the bacterial colonies. Single isolated colonies were selected for inoculation of cultures for plasmid preparation. Agar plates were wrapped in Parafilm and stored inverted at 4°C (up to 3 weeks).

2.2.10 Site-directed mutagenesis of EX-Q0086-M11 plasmid DNA

To produce an empty vector (EX-Q0086-M11 without FLAG-PTGER4) to act as a transfection control, the sequence of the FLAG-PTGER4 EX-Q0086-M11 plasmid was examined for restrictions site that would allow the excision of the FLAG-PTGER4. Sequence information for EX-Q0086-M11 is shown in Fig. 2.3.

Unfortunately, there are no unique restriction enzymes which will excise the whole FLAG-PTGER4 insert. A double digest with *BstB*1 and *Xho*I would leave the FLAG-tag and attB1 site remaining. If the FLAG-tag remains then there could be the possibility of the cell lysates from control transfections showing a false positive signal with an anti-FLAG antibody. This might make it difficult to discriminate in blots between control transfections with the empty vector and transfections with the full FLAG-PTGER4 plasmid. Site-directed mutagenesis (SDM) could introduce an additional *Xho*I site at position 5748. This would allow a digest with *Xho*I to completely remove the FLAG-PTGER4 insert from the plasmid. With the resultant compatible ends, a simple ligation would recircularise the purified linear vector sequence to give a whole empty vector for use in transfection studies. Site-directed mutagenesis (SDM) primers introducing the additional *Xho*I restriction site are show in Table 2.2.

Table 2.2: *Xho*I EX-Q0086-M11 SDM Primer sequences.

Primer Name	Sequence
Ex-Q0086-M11 Forward	5′- CAGCCTCCGGACTCGAGCCTAGGCCGCGGAC -3′
Ex-Q0086-M11 Reverse	5′- GTCCGCGGCCTAGGCTCGAGTCCGGAGGCTG -3′

Following the Q5® Hot Start High-Fidelity DNA Polymerase 2x Master Mix protocol (New England BioLabs, Hitchin, UK), 1.25 µl of both the Forward and Reverse primers (10 pmol/ml) was added to 10 µl of purified plasmid DNA of FLAG-PTGER4 (1 ng/µl) (prepared as described in Section 2.2.8) with 12.5 µl of Q5®DNA Polymerase 2x Master Mix. The volume was made up to 25 µl with ultra-

pure water. Reagents were mixed completely and transferred to thermocycler for cycling under following conditions: Initial denaturation at 90°C for 30 sec; 18 cycles: at 90°C for 10 sec, at 60°C for 30 sec and at 72°C for 4 min. A final extension at 72°C for 4 min was followed by a cycler hold at 4°C. Once completed, the parental plasmid template was digested by adding 1 µl of *Dpn*I restriction enzyme (10 U/µl; NEB, Hitchin, UK) and incubated at 37°C for 1 hour. Transformation of the EX-Q0086-M11-SDM was performed using the same protocol as FLAG-PTGER4 transformation (see Section 2.2.6).

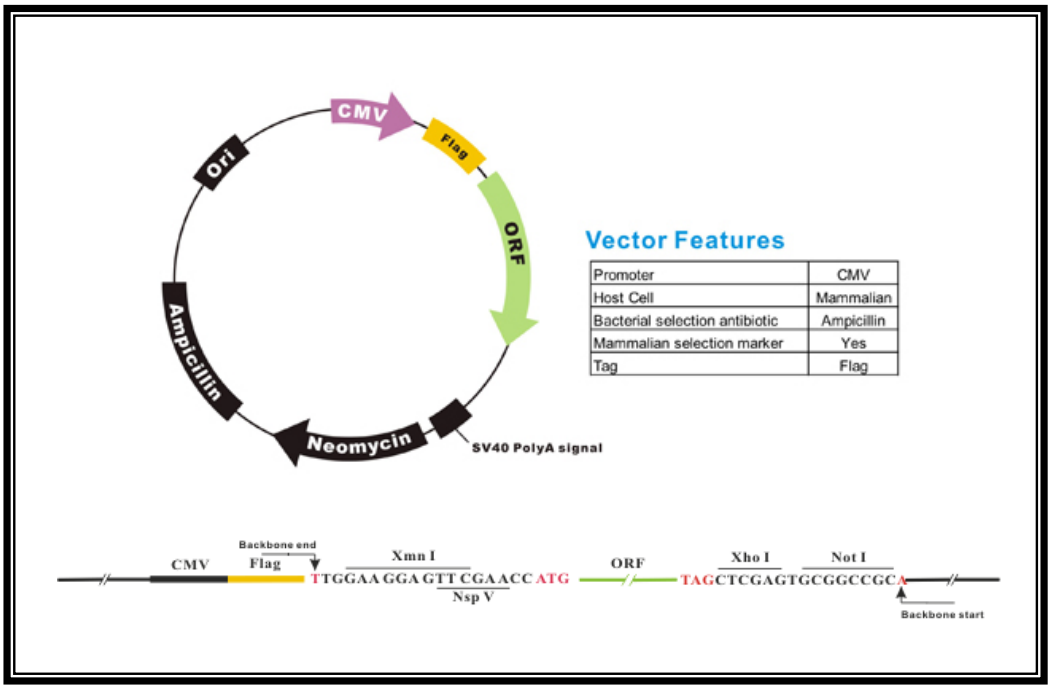


Fig. 2.3: EX-Q0086-M11 vector selective for human PTGER4.

Whole Plasmid Size is 7322 bp. FLAG-PTGER4 ORF Length is 1467 bp (This figure is adapted from GeneCopoeia OmicsLink™ Expression Clone Datasheet of EX-Q0086-M11 (Appendices 5.2).

2.2.11 Mini-prep plasmid DNA (EX-Q0086-M11-SDM) preparation

A single colony carrying EX-Q0086-M11-SDM was picked from LB agar plate using a sterile loop under aseptic conditions. This colony was added to a 30 ml sterile universal tube with 5 ml of LB Broth containing 5 µl of carbenicillin (100 µg/ml). The tube was placed horizontally in shaking incubator at 37°C for 16 hours at 200 rpm. After the incubation period, 1.5 ml of this overnight culture was centrifuged for 30 seconds at 13,000xg to pellet the cells. The supernatant was removed. This step was repeated with a further 1.5 ml of culture. The pellet was ready to carry out a plasmid DNA purification (see the following Section). A glycerol stock of EX-Q0086-M11-SDM was prepared as described in Section 2.2.9. Then plasmid DNA (EX-Q0086-M11-SDM) was purified using an ISOLATE II Plasmid Mini Kit (Bioline, London, UK) following the manufacturer's instructions. EX-Q0086-M11-SDM concentration was 100 ng/ µl measured by NanoDrop.

2.2.12 DNA sequencing

The purified EX-Q0086-M11-SDM plasmid (100 ng/µl) was sent to GATC biotech (Cologne, Germany) for automated DNA sequencing. FinchTV software (Geospiza, Seattle, USA) was used for viewing DNA sequence traces to confirm the introduction of the additional *Xho*I site.

2.2.13 EX-Q0086-M11-SDM plasmid DNA digestion

EX-Q0086-M11-SDM plasmid construct was digested using FastDigest *Xho*I (Life Technologies, UK) to remove the FLAG-PTGER4 insert and leave an empty vector.

A combination of the following reaction components was prepared: 1 µl of FastDigest *Xho*I enzyme, 5 µl of EX-Q0086-M11-SDM plasmid DNA (100 ng/µl) and 2 µl of 10x FastDigest green buffer. Nuclease-free water was added to bring the final volume up to 20 µl. The tube containing the digest was added to a 37°C water bath and incubated for 1 hour. Two aliquots (10 µl) of this reaction mixture were directly loaded on a 0.7% (w/v) TBE agarose gel containing the DNA stain, ethidium bromide (0.4 µg/ml). HyperLadderI (Bioline, London, UK) was used as DNA molecular weight marker. The gel was run at 50 volts for 1 hour. DNA fragments were separated according to their molecular weight. The empty vector fragment was 5.8 kb, whereas the FLAG-PTGER4 fragment was 1.4 kb.

2.2.14 Gel purification

The gel was visualised under UV light using a transilluminator in a dark room. Agarose bands containing the empty vectors were cut using a sterile scalpel and placed in a microcentrifuge tube. Gel-DNA fragments were weighed and the DNA extracted using an Illustra PCR DNA and gel band purification kit (GE Healthcare, Little Chalfont, UK) following the manufacturer's instructions protocol for DNA purification from TBE agarose gels. The recovered DNA was measured by a NanoDrop spectrophotometer and stored at -20°C until required for the ligation step.

2.2.15 DNA ligation

The empty, XhoI-linearised vector was re-cirularised using Quick-Stick Ligase kit (Bioline, London, UK). 5 µl of the purified vector DNA (62.5 ng) obtained from previous procedure was added to 5 µl of ligation mixture (following the Bioline

protocol). The reaction was incubated at room temperature for 15 min (allowing the empty linear vector to become circular and ready for transformation). The vector was transformed following the same protocol as Section 2.2.6. Mini-prep plasmid DNA was performed using the protocol described in Section 2.2.11. The plasmid DNA was sent off to GATC for confirmatory DNA sequencing as in Section 2.2.12. To obtain a high yield of this plasmid, a maxi-scale plasmid prep was carried out using the same kit as Sections 2.2.7 and 2.2.8.

2.2.16 Formation of the empty vector (EX-Q0086-M11 without FLAG-PTGER4)

To produce an empty vector (Fig. 2.4), site-directed mutagenesis was conducted on the FLAG-PTGER4 EX-Q0086-M11 plasmid (Section 2.2.10). The GeneCopoeia OmicsLink™ Expression Clone Datasheet of EX-Q0086-M11 (see Appendices Section 5.2) shows that introduction of an additional XhoI restriction site by site-directed mutagenesis (SDM) was used to allow completely removal of the FLAG-PTGER4 from the whole vector via a *Xho*I digestion. Following SDM of the FLAG-PTGER4 EX-Q0086-M11 plasmid, the mutated plasmid underwent DNA sequencing (Section 2.2.12)(GATC biotech, Germany) to verify the introduction of the additional *Xho*I site. Following confirmation via sequencing, the mutant plasmid (*Xho*I-FLAG-PTGER4 EX-Q0086-M11) was subjected to *Xho*I digestion (Section 2.2.13) to completely remove the FLAG-PTGER4 insert and DNA fragments were separated on an agarose gel. The empty vector fragment should have a higher molecular weight than the FLAG-PTGER4 fragment. Interestingly, as expected, it was found that the empty vector was 5.8 kb, but the FLAG-PTGER4 was 1.4 kb.

Gel-DNA fragments were recovered after purification (Section 2.2.14) in order to re-circularise the vector by DNA ligation (Section 2.2.15).

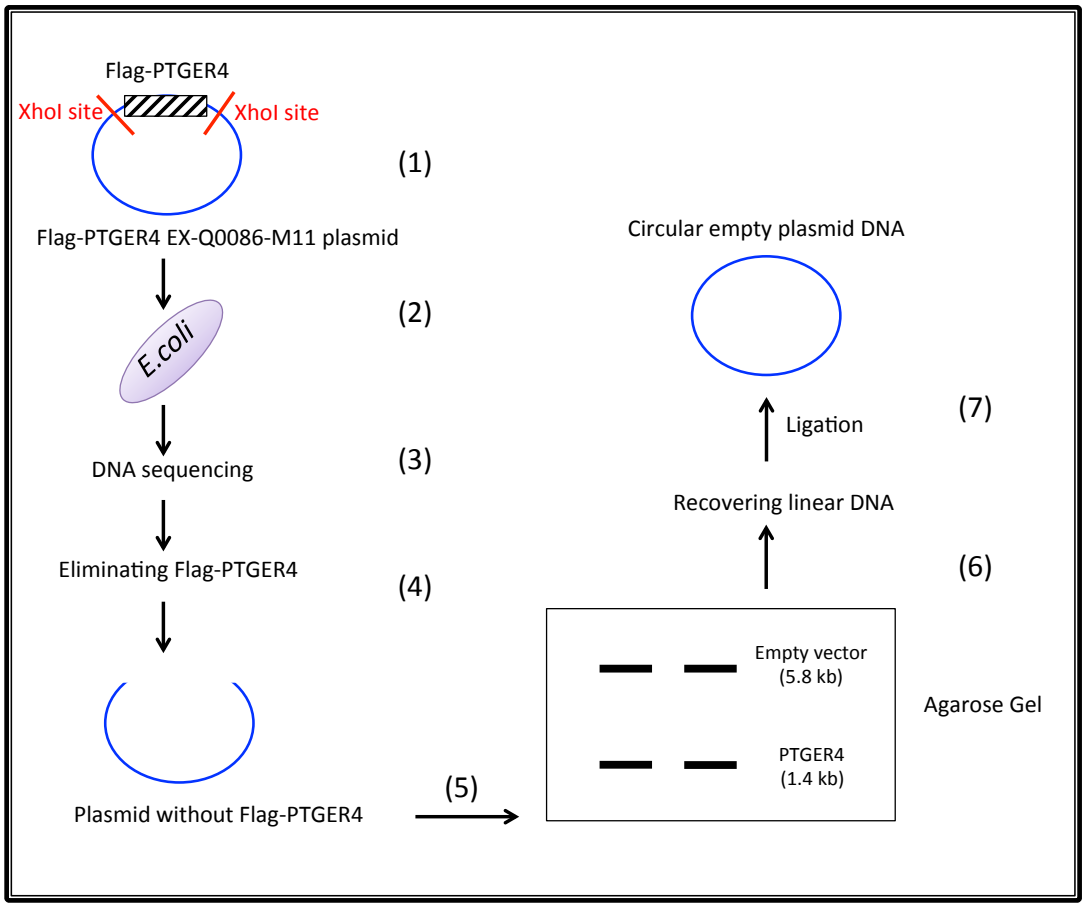


Fig. 2.4: Producing the empty vector.

(1) Introducing XhoI digestion sites into FLAG-PTGER4 EX-Q0086-M11 plasmid. (2) Transformation the plasmid into *E.coli*. (3) DNA sequencing after plasmid purification to confirm that XhoI digestion sites were introduced. (4) Removing FLAG-PTGER4 construct by Fast Digest XhoI. (5) Loading the plasmid into agarose gel to separate the DNA fragments depending on their molecular weights. (6) Recovering linear DNA after gel purification. (7) Ligation of linear DNA to produce a circular empty plasmid DNA.

2.2.17 Polymerase chain reaction (PCR)

To ensure good reproducibility and reliable data, the qRT-PCR assays were conducted using the practices laid out in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009) The MIQE checklist of experimental parameters in this study can be found in Appendics (Section 5.1).

2.2.17.1 Cell harvesting and total RNA isolation

Confluent monocytic cells or THP-1 cells (2×10^6) were placed in 6-well plates and incubated with the different treatments to give a final volume of 2 ml (incubations were described previously in Section 2.2.1.8). After the incubation period, total RNA was isolated from cells using the Sigma Aldrich's GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Adrich, Dorset, UK) following the manufacturer's instructions. Cells were harvested as follows: Cell-culture medium was aspirated from the wells using a 5 ml pipette and transferred into 1.5 ml microcentrifuge tubes followed by centrifugation at 400xg for 5 minutes. Then supernatants were discarded and pellets were lysed using the lysis buffer provided with β-mercaptoethanol (β-ME). Wells containing adherent cells were also lysed and collected together with lysed pellets to maximise RNA recovery. On-column DNase digestion with the RNase-Free DNase Set was used to eliminate potential genomic DNA contamination. RNA concentration was then measured using the NanoDrop spectrophotometer. Biological and technical replicates had *n* number of 3 each for qRT-PCR assays.

2.2.17.2 RNA concentration

Total RNA concentration was measured using a NanoDrop spectrophotometer. RNase-free water was used as a blank. Then 2 µl of each RNA sample was added after cleaning the sampling platform. The software showed the sample absorption curve and calculated RNA concentration and ratios (A_{260}nm/A_{230}nm; A_{260}nm/A_{280}nm). A ratio at A_{260}nm/A_{230}nm of around 2.0 suggested low salt contamination and the A_{260}nm/A_{280}nm ratio should also be approximately 2.0 for pure RNA. All extracted RNA samples used for PCR had acceptable purity ratios of around 2.0 for $A_{260/230}$ and $A_{260/280}$.

2.2.17.3 cDNA synthesis (Reverse Transcription (RT))

To quantify the mRNA transcripts of target genes, total RNA was reverse transcribed to complementary DNA (cDNA) using a Tetro cDNA Synthesis Kit (Bioline, London, UK) following the manufacturer's manual. The kit can transcribe a maximum of 5 µg of RNA per reaction. RNA concentrations were normalised between tubes to 1 µg. RNase-free water was added followed by 1 µl of oligo-dT primer mixture (500 ng/µl) as the first-strand synthesis primer and 1 µl of dNTP (10 mM) mixture. The template-primer mixture was denatured by heating for 5 min at 70°C, and then quick chilled on ice for 1 min. 10 µl of a reverse transcriptase master mix was added. The reverse transcriptase master mix (1x) consisted of 4 µl RT buffer (5x), 1 µl RiboSafe RNase Inhibitor, 0.25 µl tetro reverse transcriptase (200 u/µl) and diethypyrocarbonate (DEPC)-treated water (RNase-free) up to 10 µl. The reactions were incubated for 30 min at 42°C, and the reverse transcriptase was inactivated by heating to 85°C for 5 min. All these samples were labelled as (RT+).

To check for the presence of any remaining contaminating genomic DNA, an additional reaction tube, labelled (RT-) was prepared in parallel for each RNA sample. These samples (RT-) contained all of the cDNA synthesis components and total RNA except tetro reverse transcriptase. The resulting cDNA was then used as the DNA template for quantitative real-time PCR for gene expression analysis. An aliquot of (RT-) should not generate an amplicon, therefore these samples were used as negative controls in all PCR assays.

2.2.17.4 Gene expression cells-to-cycle threshold (CT) protocol

Where low cell numbers or separate column-based RNA isolation might result in very low RNA yields, an Applied Biosystems Gene Expression Cells-to-Ct Kit (Life Technologies, Paisley, UK) was used. There were three main steps in the protocol:

1. Cell lysis for RNA collection:

THP-1 cells (10^5 cells/ml) were placed in 24-well plates and incubated with different treatments as described previously in Section 2.2.1.8. After the incubation period, culture medium was aspirated from the wells and transferred into microcentrifuge tubes followed by centrifugation at 400xg for 5 minutes and wells were washed with 50 µl of cold 1x phosphate buffered saline (PBS). Both adherent cells and pellets were chilled and resuspended in 50 µl of PBS for the lysis reaction following DNase I digestion. Then, lysate samples were incubated at room temperature for 5 min. Five µl of Stop Solution (provided within the kit) was added to each reaction and incubated at room temperature for 2 min (lysates were stored at -20°C).

2. Reverse transcription (RT) reactions for PCR:

By following the manufacturer's instruction of Gene Expression Cells-to-Ct Kit protocol, a thermal cycler was programmed for 3 different incubation stages: reverse transcription stage (37°C for 60 minutes), RT inactivation stage (95°C for 5 minutes) and the last stage was hold the reaction for 5 minutes at 4°C.

3. Real-time PCR:

The resulting cDNA product from Step 2 was then used as the template for quantitative real-time PCR for gene expression analysis.

2.2.17.5 Polymerase chain reaction (PCR) amplification

cDNA was used in PCR with specific selected primers. The PCR amplifications were performed in a volume of 25 µl. The PCR reaction mix (1x) contained 1 µl of template cDNA (as described in Section 2.2.17.3), 1.25 µl of Primer Forward (10 pmol/µl), 1.25µl of Primer Reverse (10 pmol/µl), 12.5 of Q5® Hot start High-Fidelity DNA polymerase (BioLabs, UK) and 9 µl of RNase-free water. PCR was set up under the following conditions: an initial denaturation and polymerase activation at 98°C for 2 min; followed by 35 cycles with each cycle having a denaturation at 98°C for 1 min; annealing at 55°C for 30s and an elongation at 72°C for 30s. There was a final elongation at 72°C for 1 min and the reaction stopped by a 4°C incubation. The resulting PCR products were evaluated by agarose gel electrophoresis as quality control step.

2.2.17.6 Agarose gel electrophoresis

Gel electrophoresis was carried out on the PCR products performed in Section 2.2.17.5 using 2% (w/v) agarose in 50 ml Tris-Borate-EDTA (TBE) gel containing 2 µl of Ethidium Bromide (10 mg/ml). 80 ml TBE with 6 µl of Ethidium Bromide was used as runing buffer. 15 µl of PCR products with 2 µl of gel loading buffer (bromophenol blue) were pipetted into wells. 6 µl of HyperLadderII (Bioline, London, UK) was used as a DNA molecular weight marker. Samples were electrophoresed at a constant voltage of 50 V until the tracking dye reached about two-thirds down the length of the gel.

2.2.17.7 Quantitative real time polymerase chain reaction amplification (qRT-PCR)

The qRT-PCR assay was performed by placing the samples in sterile PCR fast reaction MicroAmp tubes (Applied Biosystems, Paisley, UK). The PCR reaction was carried out in a volume of 20 µl containing 19 µl of PCR master mix and 1µl of each template cDNA sample (as described in Section 2.2.17.3). The PCR master mix (1x) contained 1 µl of Primer Forward (10 pmol/µl), 1 µl of Primer Reverse (10 pmol/µl), 10 µl of Applied Biosystems SYBR Select (Life Technologies, Paisley) and 7 µl RNase-free water. Three technical and biological replicates were conducted for each assay. The thermal cycling and detection was performed on an Applied Biosystems StepOne Plus real-time PCR system (Table 2.3). 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 misprimes

which may compromise the gene expression quantification. All genes validated in this thesis resulted in a single amplicon by qRT-PCR according to melt-curve analysis. Reaction negative controls such as water blank reactions and RT-reactions gave "undetermined Ct" values as expected, i.e. no Ct value was returned for these samples over 40 cycles of amplification.

Table 2.3: The Applied Biosystems StepOne Plus thermal cycling program for use with SYBR Select MasterMix.

Hold stage	Cycling stage (40 cycles)	Final extension stage
Uracil-DNA glycosylase (UNG) activation 2 min at 50°C	**Melt step** 15 sec at 95°C	5 min at 72°C
DNA polymerase activation 10 min at 95°C	**Anneal/ Extend step** 1 min at 60°C	Cooling down at 4°C to stop PCR reaction.

2.2.17.8 The relative quantification [ΔΔCt] method for real-time PCR

The quantification method used with the PCR results was the relative quantification (ΔΔCt) method (Livak & Schmittgen, 2001). This method normalised Ct values of the target gene to Ct values of the endogenous reference gene in order to obtain the fold changes in gene expression between the control and treated samples. The PCR

efficiencies of both of the target gene and the reference gene primers under the cycling and reaction conditions was determined before using this quantification method and confirmed that they had the acceptable range of 90%–110% (see next Section for PCR efficiency). The following equations were used to compare expression levels between control and treated samples:

1. Calculation of the difference between the Cts for the target gene and the reference gene for the treated and control samples (ΔCt):

$$\Delta Ct = Ct_{\text{target}} - Ct_{\text{reference gene}}$$

2. Calculation of the difference between the ΔCts of the between the treated and control samples (ΔΔCt):

$$\Delta\Delta Ct = (Ct_{\text{target}} - Ct_{\text{reference}})\ \text{treated} - (Ct_{\text{target}} - Ct_{\text{reference}})\ \text{control}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{treated}} - \Delta Ct_{\text{control}}$$

3. Fold change (FC) in the treated sample was equal to $2^{-\Delta\Delta Ct}$

2.2.17.9 PCR efficiency

A real-time PCR standard curve of untreated samples (control) were conducted to compare the PCR efficiency of the target and reference gene reactions (Pfaffl, 2001). One in 10 serial dilutions of the control sample over 5 logs were performed. The slope of the standard curve was obtained to determine the PCR amplification efficiency (*E*) using the following next equation:

$$\text{Amplification Efficiency} = 10^{(-1\ /\ \text{slope})} - 1$$

A PCR efficiency of between 90%–110% is acceptable (i.e., a slope of between 3.1 and 3.58). The PCR efficiency in specific selected primers in the next table (Table 2.4) was between 93.9% to 110% and slope between 3.12 to 3.5.

2.2.17.10 PCR primers for SYBR® green-based real-time assays

The objective of this section was to select primers that only bound to the target gene. This was important in order to avoid primer dimers and non-specific products in all SYBR® assays which would compromise gene expression determination. All primers used in this study for the analysis of gene expression by quantitative real-time polymerase chain reaction amplification (qRT-PCR), including housekeeping or reference genes were designed as describe in the following steps:

- Gene sequences were obtained from GenBank.
- The selected sequence was imported into the PrimerQuest webtool (http://eu.idtdna.com/Primerquest/Home/Index) in the Integrated DNA Technologies (IDT) website (http://eu.idtdna.com/site) to identify potential primer pairs.
- The assay setting was chosen as ”qPCR – 2 Primers and Intercalating dye” for use with STBR-Green-based polymerase chemistries.
- Amplicons size between 94-163 bp for the amplicon was chosen to help maximise PCR efficiency.
- Primer sequences with good specificity were identified. The primer specificity was validated using the Primer-BLAST (Basic Local Alignment Search Tool) functions of The National Centre for Biotechnology Information (NCBI) Genome Browser (Ye *et al.*, 2012)) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi*)*. This step is important to ensure that the selected primers only bind to the target gene and to minimise mispriming on other targets which could give rise to false positives.

- The melting temperature (Tm) of primers kept between 58–60°C; the ΔTm between forward and reverse primers was ≤1°C.
- Primer length: 19–29 bases.
- Primer GC content: 45%–60%.
- Primers were designed to span or flank intron-exon boundaries on genomic DNA (gDNA) sequences to prevent the false positive amplification of any contaminating genomic DNA present in the cDNA samples.
- Most of the primer nucleotide sequences designed were synthesised and ordered through IDT‹ unless otherwise stated (see Table 2.4).

Table 2.4: Nucleotide sequences of the primers used for the analysis of gene expression by Quantitative Real Time Polymerase Chain Reaction amplification (qRT-PCR).

Primer Name	Sequence	PCR Product Size (bp)
PTGER1	Forward: 5'- CATGGTGGTGTCGTGCATC -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
PTGDR1	Forward: 5'- TTCACTATGTGTTCTCTGCCC-3' Reverse: 5'- GGGTCCACAATTGAAATCACA-3'	140
PTGDR2	Forward: 5'- GCTGCCTCTTGTCTAGCTG-3' Reverse: 5'- CAGAGTGGCTTCAGTGTGG-3'	115
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
TLR4	Forward: 5'- CTTGGCCCTAAACCACACAGAAGA -3' Reverse: 5'- GTAATATTAGGAACCACCTCCGTGATAAA-3'	136
TNF	Forward: 5'- CCTGCTGCACTTTGGAGTGATCG -3' Reverse: 5'- CAGCTTGAGGGTTTGCTACAACATGG -3'	149
FLAG-PTGER4	Forward: 5'- AAAGCAGGCTTGGAAGGAGTTCG -3' Reverse: 5'- CCACGATGGCCACCAGGTTG -3'	148
B2M (Eurofins MWG Operon, Ebersberg/ Germany)	Forward: 5'- AGATGAGTATGCCTGCCGTGTGAAC-3' Reverse: 5'- CAAATGCGGCATCTTCAAACCTC-3'	163
ACTB (Eurofins MWG Operon, Ebersberg/ Germany)	Forward: 5'- ATTGCCGACAGGATGCAGAA-3' Reverse: 5'- GCTGATCCACATCTGCTGGAA-3'	150
HPRT1 (Eurofins MWG Operon, Ebersberg/ Germany)	Forward: 5'- CCCTGGCGTCGTGATTAGTGATG-3' Reverse: 5'- CGAGCAAGACGTTCAGTCCTGTCC-3'	119

2.2.17.11 Selecting the qRT-PCR reference gene

The reference gene (housekeeping gene) is important for normalising differences in the cDNA concentration added in each PCR reaction. Therefore, it is important that the mRNA expression of the reference gene needs to be stably expressed between PCR samples. Reference genes such as ribosomal protein L37a (RPL37A), beta-actin (ACTB), glycereraldehyde-3-phosphate dehydrogenase (GAPDH), beta-2-microglobulin (B2M), peptidylprolyl isomerase B (PPIB), hypoxanthinephosphor-ribosyltransferase 1 (HPRT1) and TATA-binding protein (TBP) are commonly used with THP-1 qRT-PCR studies (Vandesompele *et al.*, 2002; Maess *et al.*, 2010). Concerns regarding their suitability were raised when Ct values were obtained from PCR analysis (also known as Cq, quantification cycle under MIQE nomenclature (Bustin *et al.*, 2009). Amplification of gDNA was detected using these reference genes in negative control samples even with an integral DNase-digestion during RNA extraction. Genomic DNA-derived PCR products were predicted using NCBI Primer-BLAST with primers of these reference genes. To validate selection of only the most stably expressed reference gene for this study, candidate reference primers were used in qRT-PCRs with control and treated samples and the obtained Crossing Threshold (Ct) or Quantitation Cycle (Cq) values (Bustin *et al.*, 2009) were entered into the RefFinder webtool (http://fulxie.0fees.us/?type=reference)(Xie *et al.*, 2012). RefFinder provides an estimation of reference gene stability using the GeNorm, BestKeeper and NormFinder gene stability programs (Vandesompele *et al.*, 2002; Andersen *et al.*, 2004; Pfaffl *et al.*, 2004). Only, PPIB gene showed no amplification with negative controls and mRNA expression of this reference gene was found to be

the most stably expressed reference gene within all samples in the qRT-PCR assays. Therefore, the PPIB gene was selected to be the reference gene for the present study.

2.2.17.12 qRT-PCR negative controls

For additional validation of the qRT-PCR assay, two samples were used as negative controls. These controls should not show any amplification:

1. Water control: this was a no-template control (NTC) containing PCR primers, PCR master mix, and water instead of a cDNA template (n=3). If any amplicons were detected in these reactions this would give an indication that DNA contamination of PCR reaction had taken place. However, all water controls conducted in this project did not show any amplicons.
2. RT- control: in this control, there was no reverse transcriptase added to the RNA samples through preparing cDNA (n=3). If any amplicons were identified in these reactions, this would suggest genomic DNA carryover during RNA isolation step had occurred. However, all RT- controls used in this study did not generate a product.

2.2.17.13 Statistical analysis of PCR data

All data was analysed statistically using GraphPad Prism Version 5.0 (GraphPad Software, California). For the entire experiments in this project, values were mean ± s.d. for three separate observations. Where appropriate either One-way ANOVA or Two-way ANOVA were used to ascertain a change with $P< 0.05$ regarded as a significant change.

2.2.18 Western blot

2.2.18.1 Protein sample preparation

THP-1 cells were placed into 6-well plates (1 x 10^6 cells/ml) and transfected with the FLAG-PTGER4 plasmid as described in Section 2.2.3. The plates were then placed on ice to stop the reaction. Culture media was removed from each well and transferred into microcentrifuge tubes for centrifugation at 400xg for 5 minutes. Wells were immediately washed twice with 500 µl of ice-cold 1xPBS and scraped using a rubber policeman. Both detached and pelleted cells were lysed with 100 µl of a freshly prepared RIPA lysis buffer in ice (prepared as described in Table 2.5) and 100 µl of 2x Electrophoresis Sample Buffer (ESB). The cells were then scraped from the wells and the chromosomal DNA was sheared by repeatedly pipetting up and down by syringe. Pellets from SH-SY5Y cells that had been prepared previously in Section 2.2.1.9 were also lysed by RIPA Lysis Buffer and 2x Electrophoresis Sample Buffer (ESB) exactly as for THP-1 cells as described above. The samples were then transferred to labelled microcentrifuge tubes and heated (60°C-90°C) for 5 min for protein denaturation, before storing at -20°C until required.

Table 2.5: RIPA lysis buffer preparation.

Chemical / Solution Name	Volume
PMSF solution	10 µl
Sodium orthovanadate solution	10 µl
Protease inhibitor cocktail solution	20 µl
1x RIPA lysis buffer	up to 1 ml

2.2.18.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

For running SDS-PAGE, the following buffers were prepared:

4x Resolving buffer

90.8 g of Tris base; 2 g of SDS; were dissolved in 450 ml distilled water and the pH was adjusted to 8.8 with concentrated HCl. The solution was then made up to 500 ml with distilled water and filtered through Whatman 3MM blotting paper.

4x Stacking buffer

15.14 g Tris base; 1 g SDS; were dissolved in 200 ml distilled water and the pH was adjusted to 6.8 with concentrated HCl. The solution was made up to 250 ml with distilled water and filtered through the Whatman 3MM blotting paper.

1x and 10x Running buffer

10x buffer: 30.28 g Tris base; 144 g Glycine; 10 g SDS; were dissolved in 1 L of distilled water. 1x buffer was prepared by 1:10 dilution of 10x running buffer when required.

The following gels were prepared:

Resolving Gel (10% Gel)

6.6 ml of 30% acrylamide/bisacrylamide solution, 5 ml of 4x Resolving buffer, 8.2 ml of distilled water, 100 µl of Ammonium persulfate (100 mg/ml) and 10 µl of TEMED.

Stacking Gel (5% Gel)

1.64 ml of 30% acrylamide/bisacrylamide solution, 2.5 ml of 4x stacking buffer, 5.86 ml of distilled water, 60 µl of ammonium persulfate (100 mg/ml) and 10 µl of TEMED.

Gel glass plates were cleaned with 70% ethanol before assembly, then distilled water added to check the glass plates were flush and not leaking. The plates were assembled using rubber spacers in between plates. Resolving gel (10%) was poured into the assembled plates leaving about 1cm space at the top. The gel was left to polymerise. Following polymerisation, the top of the gels were quickly washed with distilled water and dried using clean paper towels. Stacking gel (5%) was poured on top of the resolving gel and combs were inserted to create the wells. A thin layer of isopropanol was added on the top surface of gel to remove existing air bubbles and obtain a smooth surface. The gel was left to polymerise. The comb was removed and the plates were placed in an ATTO gel apparatus for electrophoresis (ATTO corporation, Japan). The tank was filled with electrophoresis buffer (1x running buffer). Aliquots of the denatured protein samples which had been prepared as detailed in Section 2.2.18.1 were added to the wells with a Hamilton syringe. HyperPAGE prestained protein markers (Bioline Reagents Ltd, UK)(to produce a ladder of known molecular weights) was run in each gel. Samples were run at a constant voltage of 125 V and 200mA for 2 hours.

2.2.18.3 Electrophoretic transfer of proteins to nitrocellulose membrane

For transferring gel, the following buffers were prepared:

1x Transfer Buffer

14.4 g glycine, 3.0 g Tris base, 200 ml Methanol and distilled water up to 1L.

10x TBS

100 ml of 1 M Tris.Cl (pH 7.5), 375 ml of 4 M NaCl and distilled water up to 1L.

1x TBSTween

100 ml of 10x TBS, 2 ml of Tween 20 (0.2% v/v) and distilled water up to 1L.

1%BSA blocking buffer

0.5 mg of BSA was dissolved in 50 ml of 1x TBSTween**.**

The proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane. The gel was pressed firmly against a nitrocellulose sheet and assembled in a transfer cassette sandwiched between two pieces of Whatman 3MM paper and two sponge pads. The cassette was immersed in a Bio-Rad transfer tank containing 1x transfer buffer and a constant current of 300 mA was applied for 2 h, whilst the tank was cooled by inclusion of an ice reservoir. The presence of SDS in the resolving gel confers a negative charge on the proteins so the cassette was oriented with the nitrocellulose towards the anode.

2.2.18.4 Immunological detection of proteins

Following transfer of the proteins from the gel to the nitrocellulose membrane, the membrane was removed into a clean plastic container containing 10 ml of blocking buffer for 1 h at room temperature with gentle agitation on a platform shaker. Then the membrane was incubated overnight at 4°C with the 1st (primary) antibody specific to the target protein (anti-FLAG, anti-GAPDH or anti-EP4) appropriately diluted 1:1000 in blocking buffer. On the following day the membrane was washed 3 times with 1 x TBS Tween for 15 min (5 min each) with gentle agitation. The membranes were then incubated for 2-4 h at room temperature with the 2nd antibody (Streptavidin-HRP Conjugate) diluted to 1:5000 in blocking buffer with gentle shaking. After incubation, the membranes were washed twice with 1x TBS Tween

and once with TBS for 15 min (5 min each). Immunoreactive protein bands were detected by enhanced chemiluminescence (ECL) reagent. The membrane was then photographed using a Las-3000 dark-box camera (Fujifilm).

2.2.18.5 Quantification proteins in membranes

Following incubation of nitrocellulose membranes with 1st and 2nd antibodies, the membrane was stained with Ponceau S red solution (0.1% Ponceau S (w/v) in 5% acetic acid) and incubated for 5 min on an agitator. Once bands were visualised, they were cut out and placed into separate wells of 96-well plates. TMB chromogen substrate (100 µl) was added to each well and the plate was further incubated at room temperature for 30 minutes. When a blue colour appeared, 100 µl of sulphuric acid (1M) was added to stop the reaction. The absorbance was measured at 450 nm using a Spectra Max 190 Absorbance Micro-plate Reader (Molecular devices, USA).

2.2.18.6 Statistical analysis of immunoblotting data

All data obtained through immunoblotting were analysed using ImageJ software (http://imagej.nih.gov/ij/) and compared relative to internal control values.

2.2.19 Dot blot

2 µl of THP-1 cells (2x10^6 cell/ml) was spotted onto 3 different nitrocellulose membrane strips. The first strip was for cells alone, the second was for PGE_2-biotin and the last nitrocellulose strip was for PGE_2. Membranes were left for 10 min to dry at room temperature and then placed in 3 different petri dishes containing 10 ml of

1 x buffer pH 7.4 (10x buffer: 40 g of NaCl, 5.6 g of Na_2HPO_4, 1 g of KH_2PO_4 and 1 g of KCl in 500 ml dH_2O, then diluted 1 in 10).

- **Petri dish 1:** containing cells dotted onto nitrocellulose membrane in 10 ml of buffer.
- **Petri dish 2:** containing cells dotted onto nitrocellulose membrane labelled with PGE_2-biotin (200 nM) in 10 ml of buffer.
- **Petri dish 3:** containing cells dotted onto nitrocellulose membrane incubated with PGE_2 (200 μM) in 10 ml of buffer.

These petri dishes were incubated for 20 min at room temperature with gentle agitation on a platform shaker. Membranes were washed three times (5 min each) with washing buffer (1x buffer containing 0.5 ml of Tween). 2 μl of streptavidin-HRP conjugate was dissolved in 10 ml of buffer and added to both of petri dish 1 and 2. Petri dish 1 and 2 were incubated for 30 min at room temperature with gentle agitation. However, petri dish 3 which had been incubated previously with PGE_2, was labelled with PGE_2-biotin (200 nM) in 10 ml of buffer before being conjugated with streptavidin-HRP and incubated for 20 min at room temperature with gentle agitation. The strips in petri dish 3 were washed 3 times with washing buffer and then 2 μl of streptavidin-HRP was dissolved in 10 ml of buffer and incubated for 30 min at room temperature with gentle agitation. After 30 min incubation of all petri dishes with streptavidin-HRP conjugate, membranes were washed twice with washing buffer (containing tween) and once with 1x buffer (did not contain tween). Dots were detected by incubation in ECL reagent for 2 min with agitation. The membrane was then photographed using a Las-3000 dark-box camera (Fujifilm).

2.2.20 Preparation of microscope slides

2.2.20.1 PGE_2-biotin labelled cells

THP-1 cells were prepared as described in Section 2.2.1.1. 100 µl of cells were spread onto a microscope slide and left to dry for 10 min. 1ml of PGE_2-biotin (200 nM) which was diluted in buffer pH 7.4 (40 g of NaCl, 5.6 g of Na_2HPO_4, 1 g of KH_2PO_4 and 1 g of KCl in 500 ml dH_2O) and this was added to the cells on the top of the slide. The slide was incubated for 30 min at room temperature and then washed 3 times with cold PBS. Streptavidin-HRP conjugate (1 µl) was dissolved in 5 ml of buffer (pH 7.4), 1ml of this solution was added onto the slide. The slide was further incubated for 30 min at room temperature. A final 3 washes with PBS were performed and 1ml of ECL reagent added. The slide was then viewed under an Epi-fluorescence microscope.

2.2.20.2 siRNA transfected cells

THP-1 cells ($1x10^6$ cells/ml) were transfected in 6-well plates with 2 different concentrations of siRNA duplex (1000 nM and 50 nM) and incubated at 37°C, 5% CO_2 for 24 hours. Culture media was transferred into microcentrifuge tubes. Wells were washed 3 times with cold PBS and added to the same tubes to be centrifuged at 400 g for 5 min. Supernatants were discarded and the pellets were resuspended with 100 µl of media. 90 µl of resuspended pellets were spread onto slides and left 10 min to dry before viewed under an Epi-fluorescence microscope.

2.2.20.3 Localisation of EP4 receptors using fluorescent anti-EP4 antibody in THP-1 cells

THP-1 cells were prepared as described in Section 2.2.1.1. 100 µl of cells were spread onto a microscope slide and left to dry for 1h. Cells on each slide were fixed with 1ml of 4% (v/v) formaldehyde in PBS (pH 7.4) for 30 min. The slide was washed then with PBS. Cells were permeabilised by incubating with 0.25% (v/v) triton X-100 in distilled H_2O for 5 min. The slide was incubated for 30 min with a blocking solution (0.5% BSA) to prevent non-specific protein binding. Cells were incubated for 15 min with fluorescent EP4 antibody-FITC conjugate (Bioss Antibodies ,USA) diluted in PBS (1:100). The slide had a final wash with PBS and was left to dry, after which a drop of slide-mount containing DAPI was added on the top of slide and covered with a cover-slip. Slides were viewed under an Epi-fluorescence microscope.

Chapter 3

Results

3.1 Cytokine production in whole human blood and by monocytic cells

3.1.1 The effect of varying concentrations of LPS on TNF-α production in human blood, THP-1 cells and monocytic cells

Previous studies have shown that LPS is one of the most effective stimuli for pro-inflammatory cytokine production particularly TNF-α (Rietschel *et al.*, 1994; Kreutz *et al.*, 1997).

Preliminary experiments were carried out to examine the effect of different concentrations of LPS on the production of TNF-α in human blood, THP-1 cells and normal monocytic cells isolated from blood. Blood and cells were incubated with varying concentrations of LPS (Methods Sections 2.2.1.7 and 2.2.1.8), and the effect on TNF-α production was investigated.

Fig. 3.1 shows the production of TNF-α in human blood. An increase in LPS concentrations resulted in an increase in TNF-α in a concentration-dependent manner. The TNF-α level was increased significantly up to 200 pg/ml with an LPS concentration between 0.01 μg/ml and 1 μg/ml. At 10 μg/ml of LPS, there was also a significant increase in TNF-α production. The maximum production of TNF-α was observed in the presence of 100 μg/ml LPS, which resulted in the order of a 550-fold rise in the production of TNF-α compared to control. Fig. 3.2 shows a similar production of TNF-α in response to LPS in THP-1 cells. The TNF-α concentration was increased with concentrations of LPS between 0.01-100 μg/ml. The effect of

LPS on cytokine production was also demonstrated in monocytic cells (Fig. 3.3). LPS (0.1-100 μg/ml) produced a concentration-dependent increase in TNF-α production.

Monocytic cells produced the highest amounts of TNF-α compared to TNF-α induced by human blood and THP-1 cells. The TNF-α concentrations were 3000 pg/ml, 600 pg/ml and 550 pg/ml produced by monocytes, THP-1 cells and blood respectively in response to 100 μg/ml of LPS.

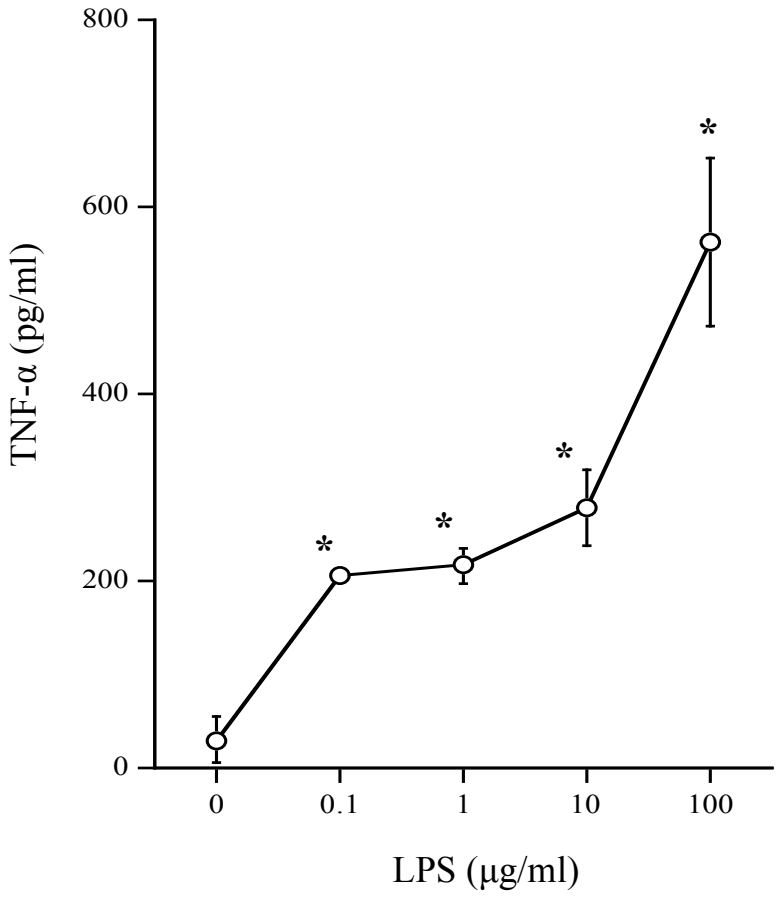


Fig. 3.1: The effect of LPS on TNF-α production in human blood.

Blood was incubated with varying concentrations of LPS. Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in plasma was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus incubations without LPS.

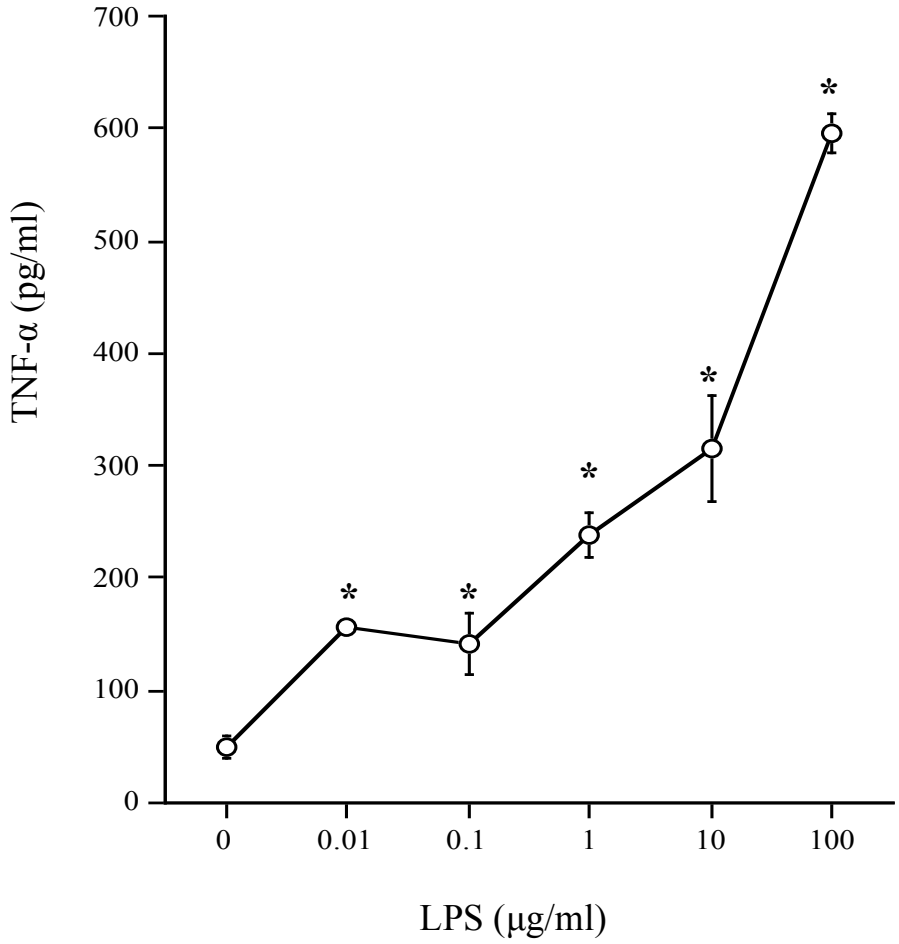


Fig. 3.2: The effect of LPS on TNF-α production in THP-1 cells.

Cells ($1x10^6$ cells/ml) were incubated with varying concentrations of LPS. Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus incubations without LPS.

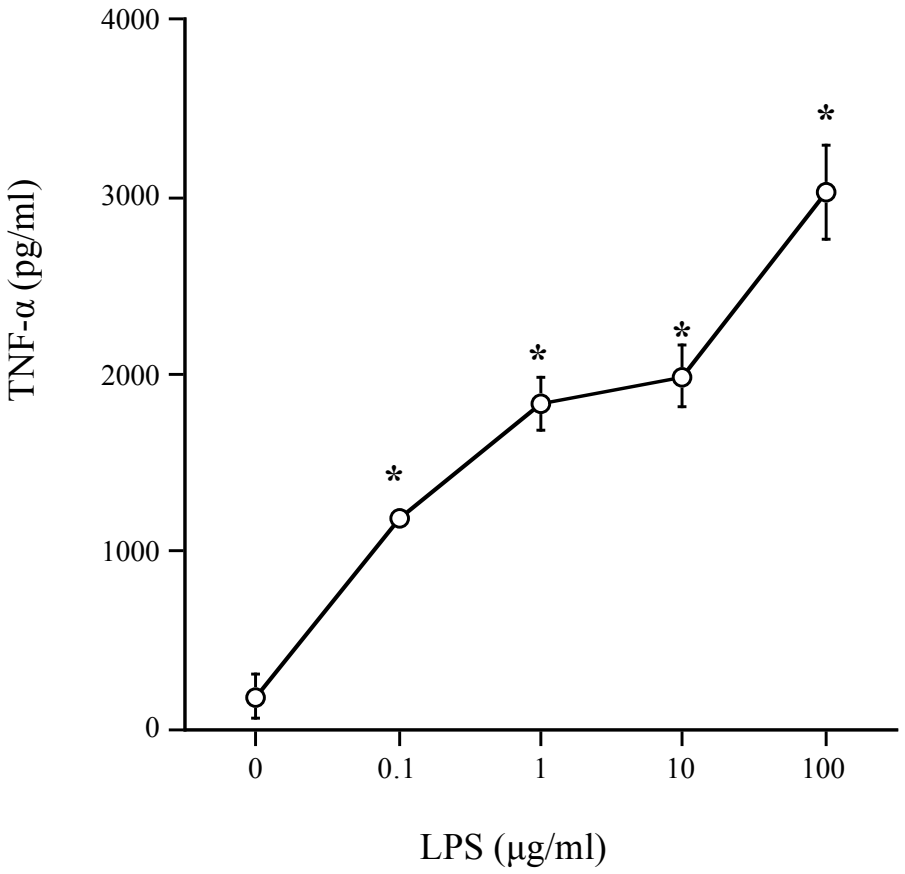


Fig. 3.3: The effect of LPS on TNF-α production in monocytic cells.

Monocytes ($1x10^6$ cells/ml) were incubated with varying concentrations of LPS. Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus incubations without LPS.

3.2 The effect of PGE_2 and its agonists on THP-1 cell viability

In order to exclude the possibility that the suppression of LPS-induced TNF-α production may be indirectly related to cell viability, the effect of different treatments on THP-1 cell viability was assessed using trypan blue exclusion and MTT assay as described in Methods Section 2.2.4. Viability was greater than 98% as determined by trypan blue (Table 3.1). MTT assay measured total viable cells incubated with PGE_2, butaprost, sulprostone and L-902, 688 (all at 1 μM) which were adjusted to 103 ± 1.5%, 105 ± 0.5%, 101 ± 2.2% and 100 ± 2.4% respectively compared to untreated cells (control) incubations (100 ± 2.5%). Dead cells (boiled cells) were used as a control (there were 0 ± 0.001% viable cells)(Table 3.2).

The control cells (untreated cells) yielded a mean absorbance of 1.213 ± 0.024. The means absorbance of cells treated with PGE_2, butaprost, sulprostone and L-902, 688 1.291 ± 0.125, 1.411 ± 0.029, 1.228 ± 0.035 and 1.214 ± 0.005 respectively. Dead cells had a mean absorbance of 0.119 ± 0.008 (All values are the means of n = 3 ± s.d, $P < 0.05$ by ANOVA).

Table 3.1: The percentage of the total viable THP-1 cells with different treatments using Trypan blue exclusion.

Treatment	Viable cells (%)
Control (untreated cells)	100 ± 1.3%
PGE_2	99 ± 0.09%
Butaprost	98 ± 1.5%
Sulprostone	99 ± 0.02%
L-902, 688	100 ± 0.04%
Dead cells	0 ± 0.03%

THP-1 cell viability ($1x10^6$ cells/ml) was measured by trypan blue exclusion (estimation of non-viable cells). Trypan blue was added to cells immediately before counting (using a haemocytometer) and the percentage of blue cells was subtracted from the total cell count (Methods Section 2.2.4). All values are the means of n = 3 ± s.d (three separate observations), $P < 0.05$ by ANOVA.

Table 3.2: The percentage of the total viable THP-1 cells with different treatments using MTT assay.

Treatment	Viable cells (%)
Control (untreated cells)	100 ± 2.5%
PGE_2	103 ± 1.5%
Butaprost	105 ± 0.5%
Sulprostone	101 ± 2.2%
L-902, 688	100 ± 2.4%
Dead cells	0 ± 0.001%

THP-1 cells ($1x10^6$ cells/ml) were placed into 96-well plates after which PGE_2, butaprost, sulprostone, L-902, 688 (all at 1 μM), control cells (untreated cells) and dead cells (boiled cells) were added. Plates were incubated at 37°C, 5% CO_2 for 24h. Treatments were removed from wells and replaced with MTT solution and left for 4 h in incubator at 37°C, 5% CO_2 and lysed with DMSO (Methods Section 2.2.4). All values are the means of n = 3 ± s.d (three separate observations), $P < 0.05$ by ANOVA.

3.3 The effect of prostaglandins on cytokine production

3.3.1 The effect of PGE_2 on TNF-α production in monocytic cells and THP-1 cells in response to LPS

Different concentrations of PGE_2 were used to determine its ability to suppress TNF-α production. Cells were incubated with PGE_2 as described previously (Methods Section 2.2.1.8). Fig. 3.4 shows a 3-fold increase in TNF-α production in LPS-stimulated monocytes in comparison to control. This elevated level of produced TNF-α was inhibited in a concentration-dependent manner by PGE_2 (0.01-10 μM). The maximal suppressive effect of PGE_2 was between 1 μM and 10 μM. PGD_2 (1 μM) was also able to decrease the TNF-α level.

Similar experiments were performed to ascertain whether PGE_2 has also a similar suppressive effect on TNF-α production in THP-1 cells. LPS (10 μg/ml) increased TNF-α production by 20-fold and PGE_2 (1 μM) induced a significant decrease in the LPS-stimulated TNF-α level. PGD_2 (1 μM) also inhibited this TNF -α level (Fig. 3.5). This figure also shows that PGE_2 is more potent than PGD_2 in TNF-α inhibition because it induced a more significant decrease compared to the effect of PGD_2.

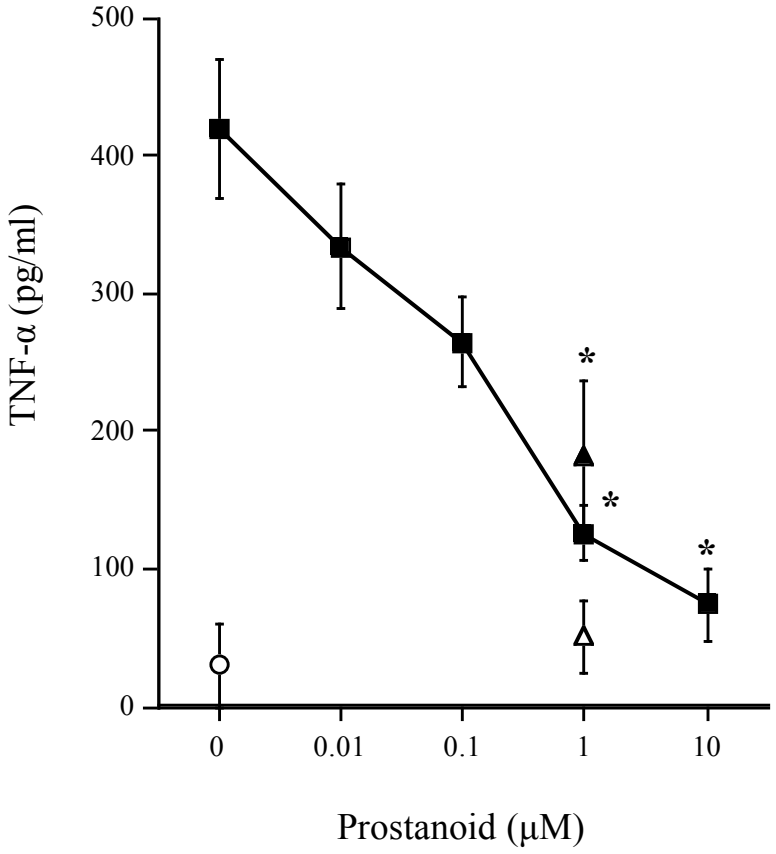


Fig. 3.4: Effect of varying concentrations of PGE_2 on TNF-α production in monocytes in response to LPS.

Monocytes ($1x10^6$ cells/ml) were incubated with varying concentrations of PGE_2 (square filled symbols), PGD_2 (1 μM) (triangle filled symbols) in the presence of LPS (10 μg/ml). Incubation without LPS is shown as open circle, with PGD_2 alone (open triangle). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

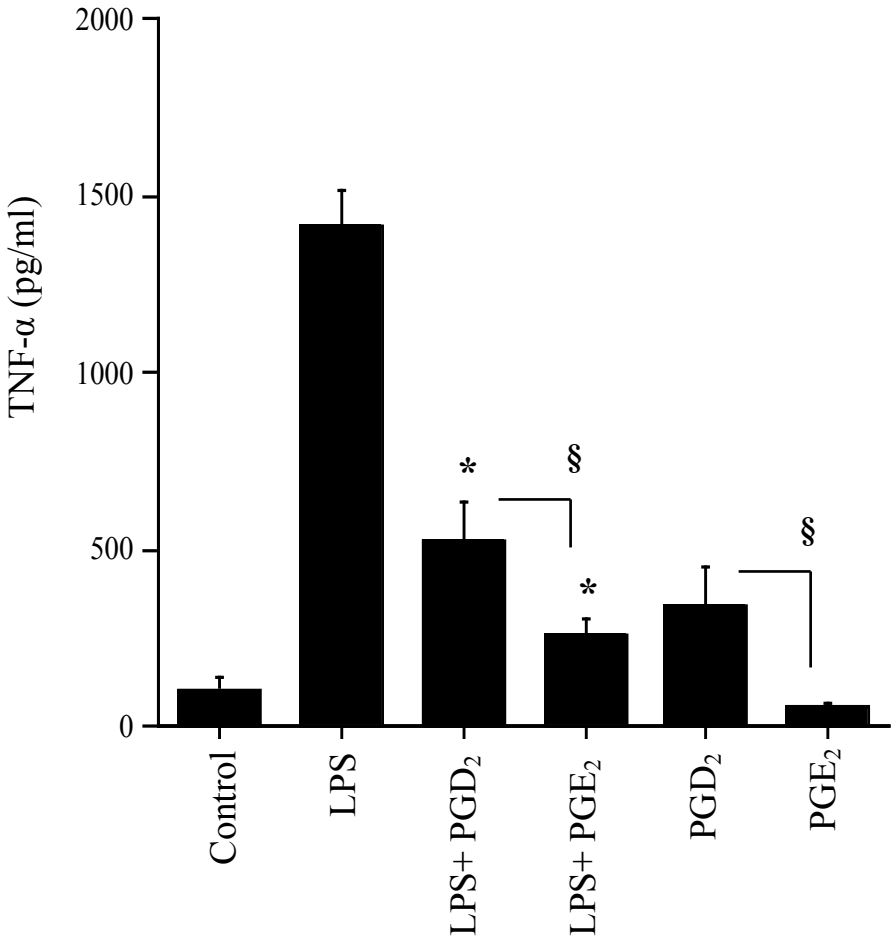


Fig. 3.5: The effect of PGE_2 and PGD_2 on TNF-α production in response to LPS in THP-1 cells.

Cells ($1x10^6$ cells/ml) were incubated with LPS (10 µg/ ml), PGE_2 (1 µM) and PGD_2 (1 µM). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone. § P < 0.01 versus PGD_2.

3.3.2 The effect of PGE_2 on IL-1β and TNF-α production in human monocytes and THP-1 cells in response to LPS

After ascertaining that the most potent concentrations of PGE_2 were 1 μM and 10 μM (Fig. 3.4), further experiments were conducted on monocytes to study whether PGE_2 had the same effect on the production of IL-1β compared to TNF-α. Cells were stimulated with LPS and the effect of PGE_2 was studied as previously indicated (Methods Section 2.2.1.8). LPS induced a relatively large increase in the production of IL-1β. There was a 6000-fold increase in IL-1β induced by LPS in monocytes. Interestingly, this LPS-stimulated level was decreased significantly by 58% by PGE_2 (1 μM) (Fig. 3.6). The effect of PGE_2 (1 μM) on the LPS-stimulated production of IL-1β produced a similar inhibition as for TNF-α levels under equivalent experimental conditions. The reduction of TNF-α in response to LPS was 57% by PGE_2 (Fig. 3.7).

In contrast to its suppressive effects in blood and monocytes, PGE_2 (between 0.1 μM to 10 μM) had no effect on the level of LPS-stimulated IL-1β production from THP-1 cells. However, at low concentration of PGE_2 (0.01 μM), there was a significant increase in IL-1β level. It was uncertain how low concentrations of PGE_2 induced this significant increase in IL-1β production in THP-1 cells. This observation requires further work in future to investigate the reason of an increase obtained by lower concentrations of PGE_2. PGD_2 had no suppressive effect on the IL-1β level from THP-1 cells (Fig. 3.8).

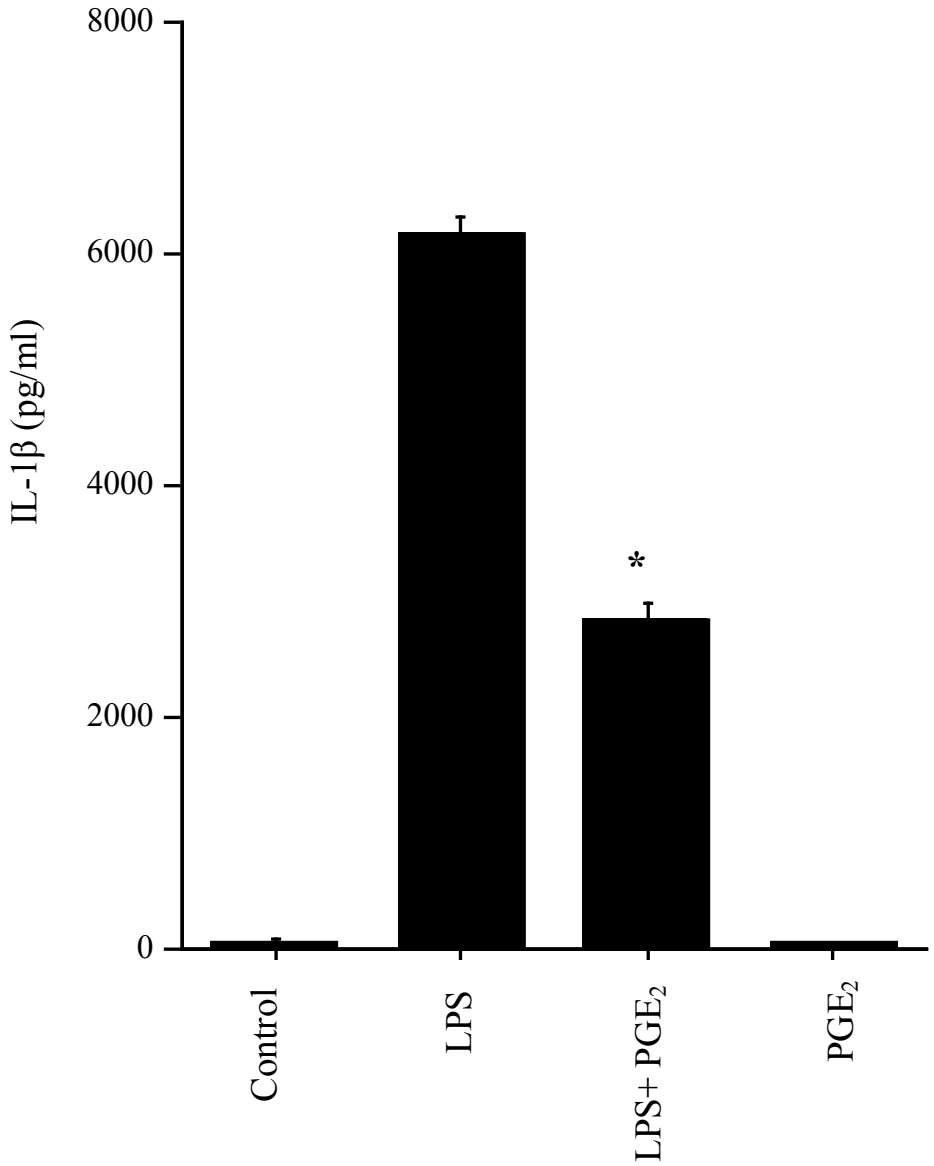


Fig. 3.6: The effect of PGE_2 on IL-1β production in human monocytes in response to LPS.

Monocytes ($1x10^6$ cells/ml) were incubated with LPS (10 μg/ml) and PGE_2 (1 μM). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of IL-1β in the culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

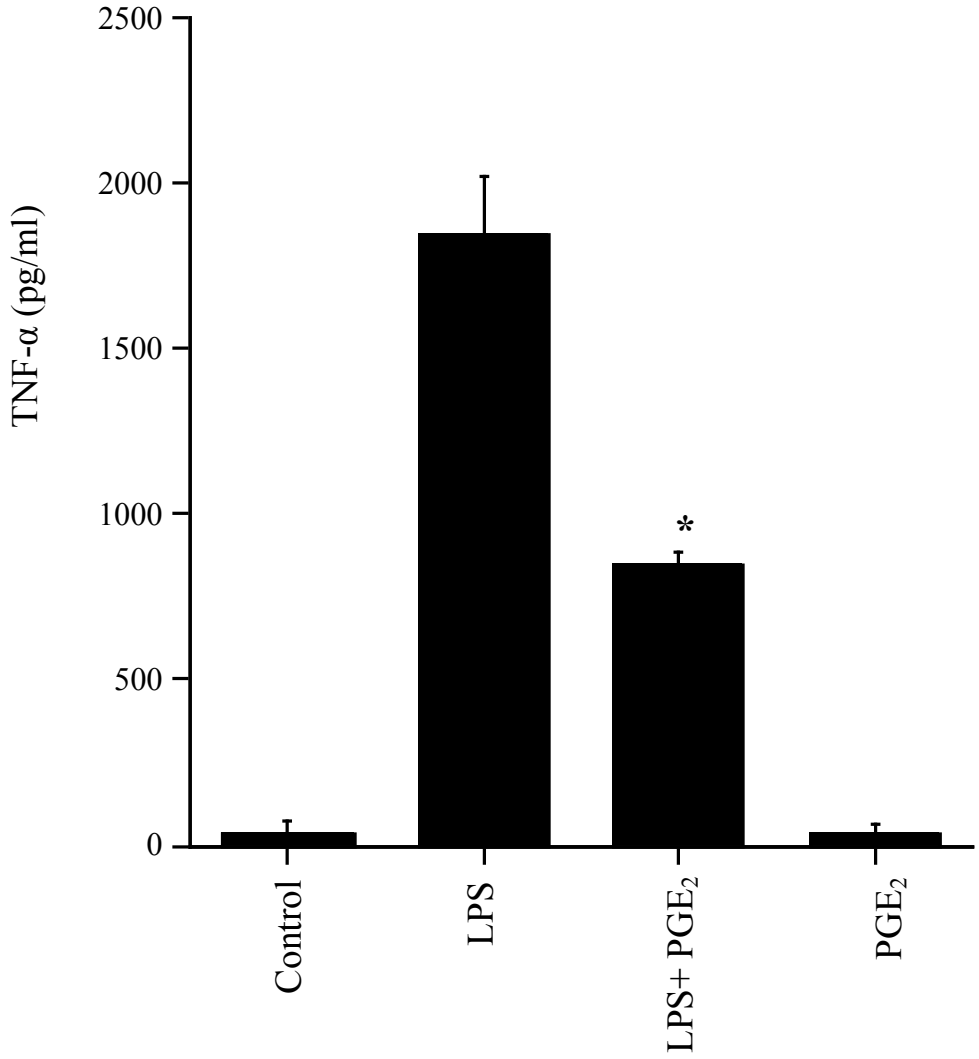


Fig. 3.7: The effect of PGE_2 on TNF-α production in human monocytes in response to LPS.

Monocytes ($1x10^6$ cells/ml) were incubated with LPS (10 μg/ml) and PGE_2 (1 μM). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in the culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

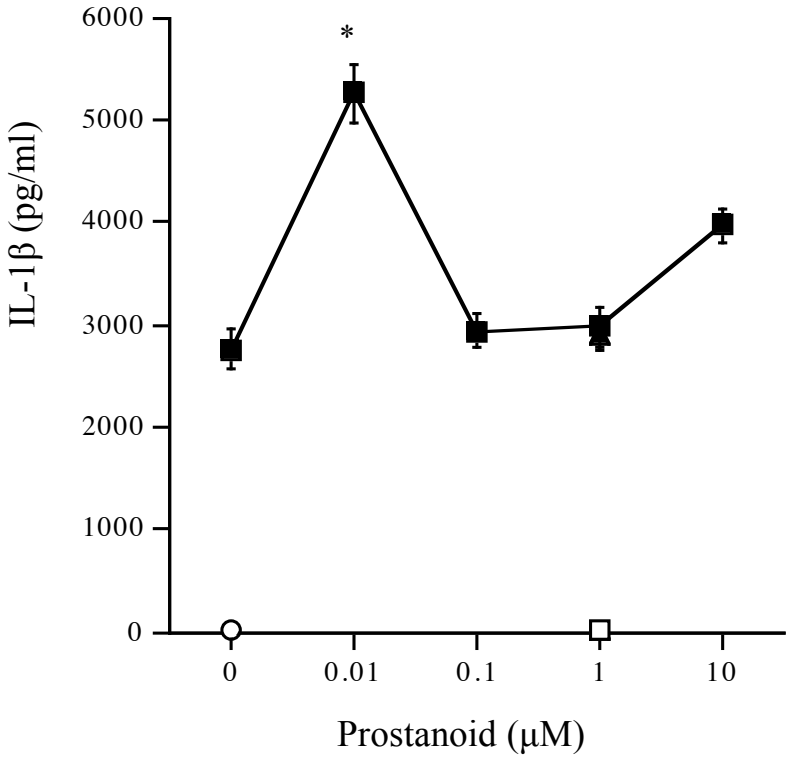


Fig. 3.8: Effect of varying concentrations of PGE_2 on IL-1β production in response to LPS in THP-1 cells.

Cells ($1x10^6$ cells/ml) were incubated with varying concentrations of PGE_2 (square filled symbols), PGD_2 (1 μM) (triangle filled symbols) in the presence of LPS (10 μg/ml). Incubation without LPS is shown as open circle, with PGD_2 alone (open triangle) and PGE_2 alone (open square). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of IL-1β in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

3.3.3 The effect of PGE_2 and PGD_2 on the LPS dose-responses for the production of both TNF-α and IL-1β in human blood

In previous experiments, PGE_2 inhibited LPS-stimulated TNF-α production in both monocytic cells and THP-1 cells (Fig. 3.5 and Fig. 3.7). The effect of PGE_2 on TNF-α production was also studied on whole human blood in response to LPS. Blood was treated with various concentrations of LPS in the presence of PGE_2 and PGD_2 (Methods Section 2.2.1.7). Incubation of blood with different concentrations of LPS (0.1 μg/ml-100 μg/ml) resulted in a concentration-dependent increase of TNF-α levels. However, in the presence of PGE_2 (1 μM), the production of TNF-α induced by LPS was significantly suppressed (Fig. 3.9).

PGD_2 (1 μM) was also studied to ascertain whether it also had a similar suppressive effect as PGE_2. The concentration-dependent increase in TNF-α levels was considerably reduced in the presence of PGD_2 (1 μM) (Fig. 3.9).

A direct comparison of the effects both PGE_2 and PGD_2 (1 μM) showed that they inhibited TNF-α production in response to 10 μg/ml of LPS (Fig. 3.10). LPS induced 3000-fold increase in TNF-α level. This LPS stimulated level was significantly decreased 75% by PGD_2 and 83% by PGE_2. The inhibitory effect of both PGE_2 and PGD_2 (1 μM) was not restricted to TNF-α. Fig. 3.11 shows that at 1 μM, PGE_2 and PGD_2 also reduced IL-1β production. There was a 40% significant reduction of IL-1β level by PGD_2 and 43% decrease by PGE_2 in response to 10 μg/ml of LPS.

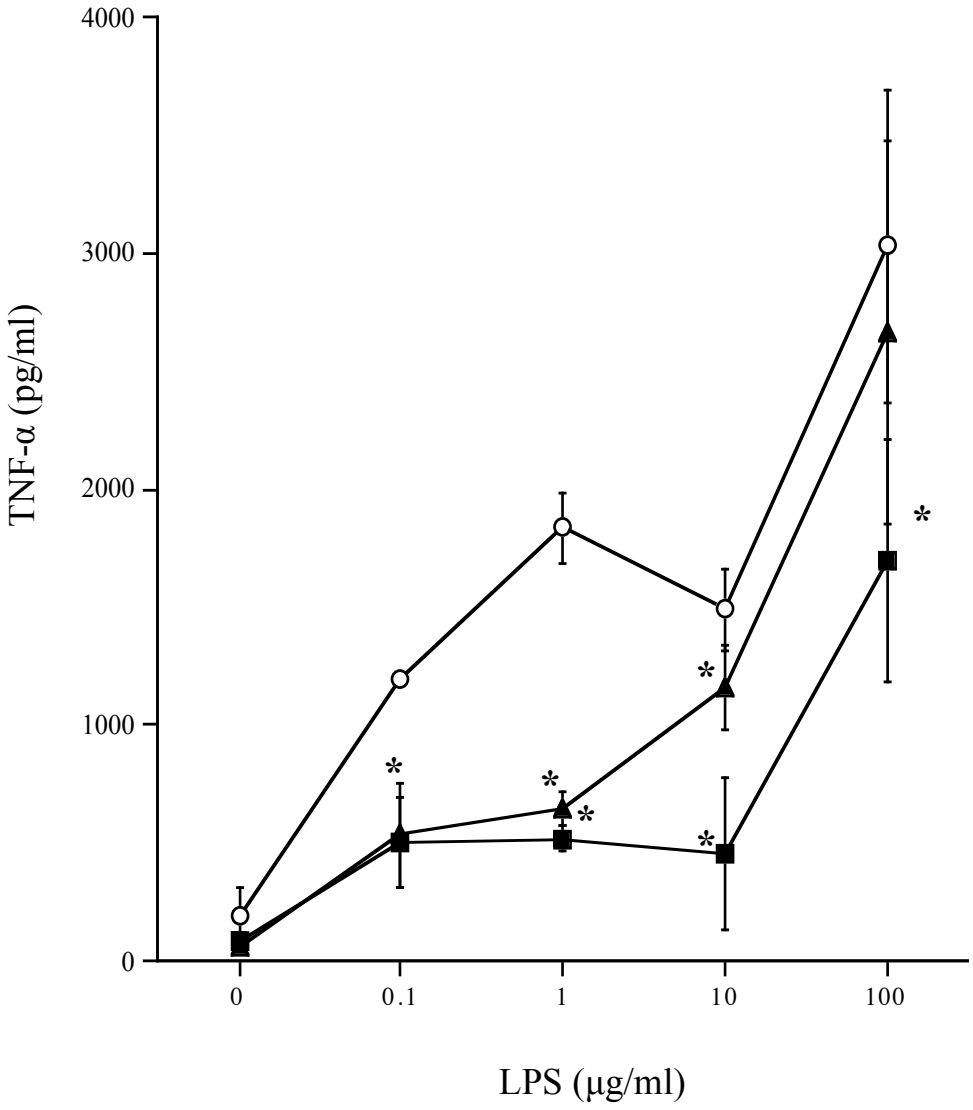


Fig. 3.9: The effect of different concentrations of LPS in the presence and absence of PGE_2 and PGD_2 on the production of TNF-α in human blood.

Blood was incubated with varying concentrations of LPS (circle open symbols), PGE_2 (1 μM) (square filled symbols) and PGD_2 (1 μM) (triangle filled symbols). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in plasma was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

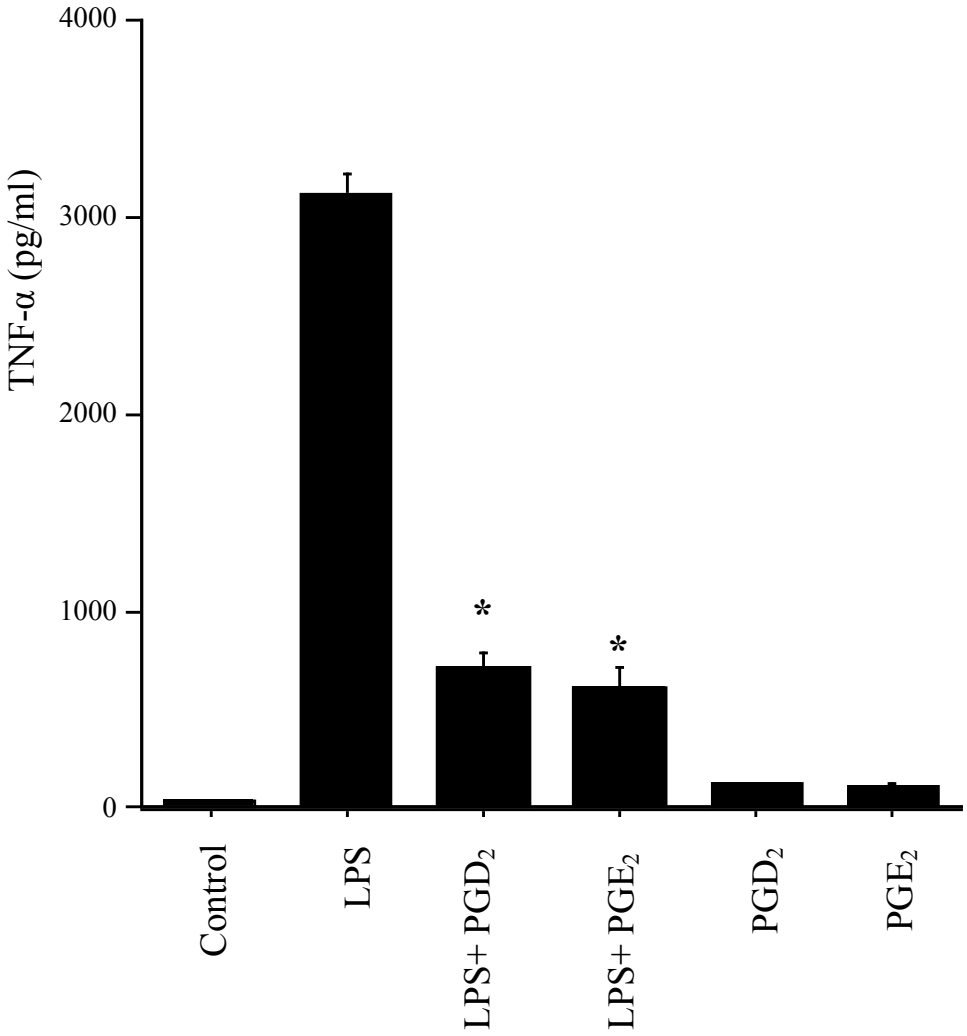


Fig. 3.10: The effect of LPS, PGE_2 and PGD_2 on the production of TNF-α in human blood.

Blood was incubated with LPS (10 μg/ml), PGE_2 (1 μM) and PGD_2 (1 μM). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in plasma was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

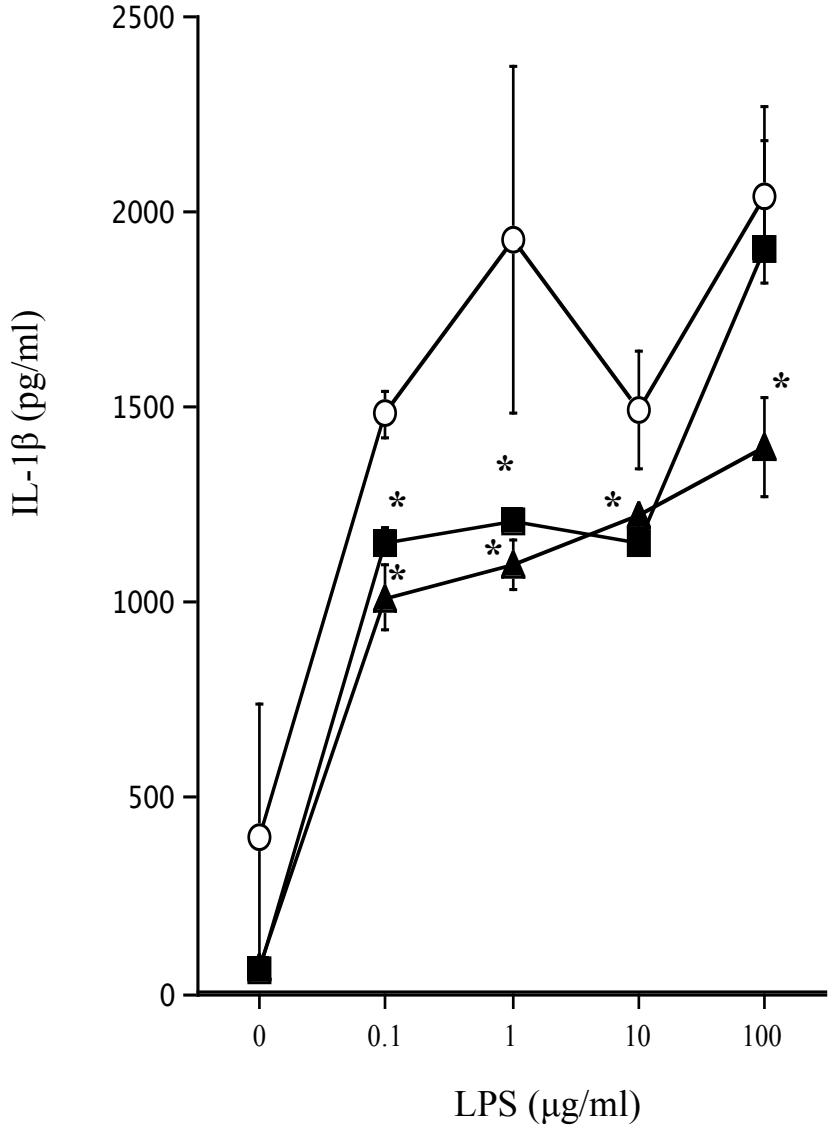


Fig. 3.11: The effect of different concentrations of LPS in the presence and absence of PGE_2 and PGD_2 on the production of IL-1β in human blood.

Blood was incubated with varying concentrations of LPS (circle open symbols), PGE_2 (1 μM) (square filled symbols) and PGD_2 (1 μM) (triangle filled symbols). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of IL-1β in plasma was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

3.3.4 The effect of PGD_2 on IL-1β production in human monocytes in response to LPS

Monocytes were incubated with LPS, and either PGE_2 or different concentrations of PGD_2 (see Methods Section 2.2.1.8). Fig. 3.12 shows that LPS induced 450-fold increase in IL-1β production in monocytes. However, different concentrations of PGD_2 were unable to inhibit IL-1β production in response to LPS. Unlike the earlier results which were obtained using human blood, there was a 40% decrease in IL-1β production by 1 μM of PGD_2 (Fig. 3.11). This suggests that monocytes might require other blood cells to inhibit the production of IL-1β.

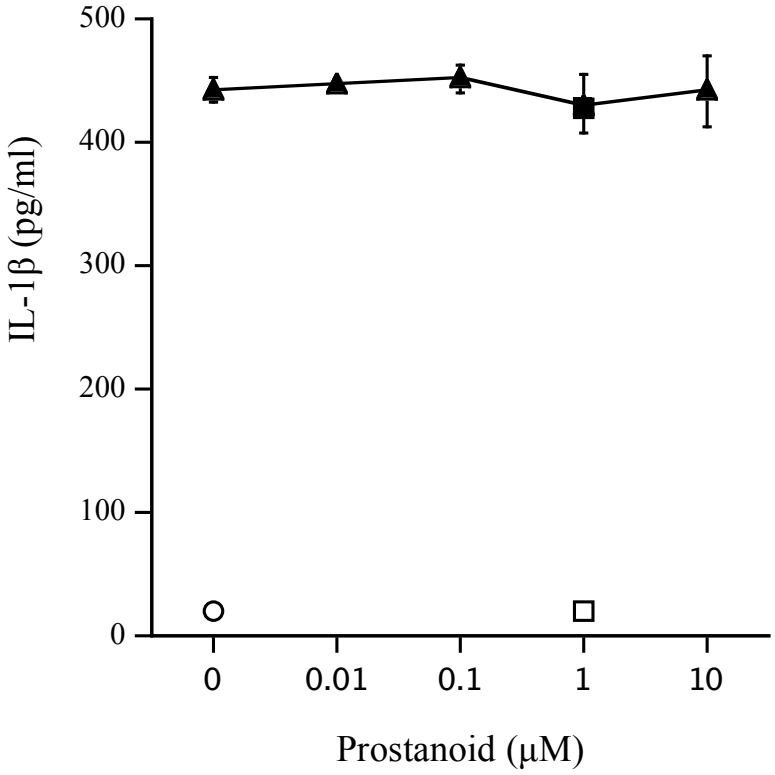


Fig. 3.12: Effect of varying concentrations of PGD_2 on IL-1β production in response to LPS in human monocytes.

Monocytes ($1x10^6$ cells/ml) were incubated with varying concentrations of PGD_2 (triangle filled symbols), PGE_2 (1 μM) (square filled symbols) in the presence of LPS (10 μg/ml). Incubations without LPS are shown as open circle, with PGD_2 alone (open triangle, behind the open square symbol) and PGE_2 alone (open square). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of IL-1β in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations).

3.4 Cytokine production by mononuclear cells

3.4.1 The effect of PGD_2 and PGE_2 on the production of both TNF- α and IL-1β from mononuclear cells

Earlier results indicated that TNF-α production was inhibited by both PGE_2 and PGD_2 in monocytes, THP-1 cells and whole human blood (Fig. 3.4, Fig. 3.5 and Fig. 3.10). However, PGE_2 and PGD_2 did not show any significant inhibition of IL-1β levels in monocytes or THP-1 cells (Fig. 3.8 and Fig. 3.12) but in whole human blood, PGE_2 and PGD_2 suppressed the production of IL-1β (Fig. 3.11). This disparity between monocytes, THP-1 cells and whole human blood in terms of the suppression of IL-1β production was further studied by designing additional experiments using a whole mononuclear cell fraction. Mononuclear cells were incubated with LPS, PGE_2 and PGD_2 as described in the method (Methods Section 2.2.1.8). In these experiments it was found that PGE_2 decreased TNF-α production compared to LPS alone, whereas PGD_2 did not decrease the level (Fig. 3.13). In contrast, both PGD_2 and PGE_2 inhibited the IL-1β level (Fig. 3.14).

In conclusion, by taking all previous cytokine production results that were obtained in previous sections into consideration (Section 3.1 to 3.4), it was found that PGE_2 was able to induce a significant decrease in TNF-α production in all cell models (monocytes, THP-1 cells, human blood and mixed mononuclear cells). PGE_2 also inhibited the IL-1β levels in all cell types except THP-1 cells, which may indicate that other cells might be involved in IL-1β inhibition (not only monocytes). Moreover, PGD_2 followed a similar inhibitory pattern as PGE_2 in these cells.

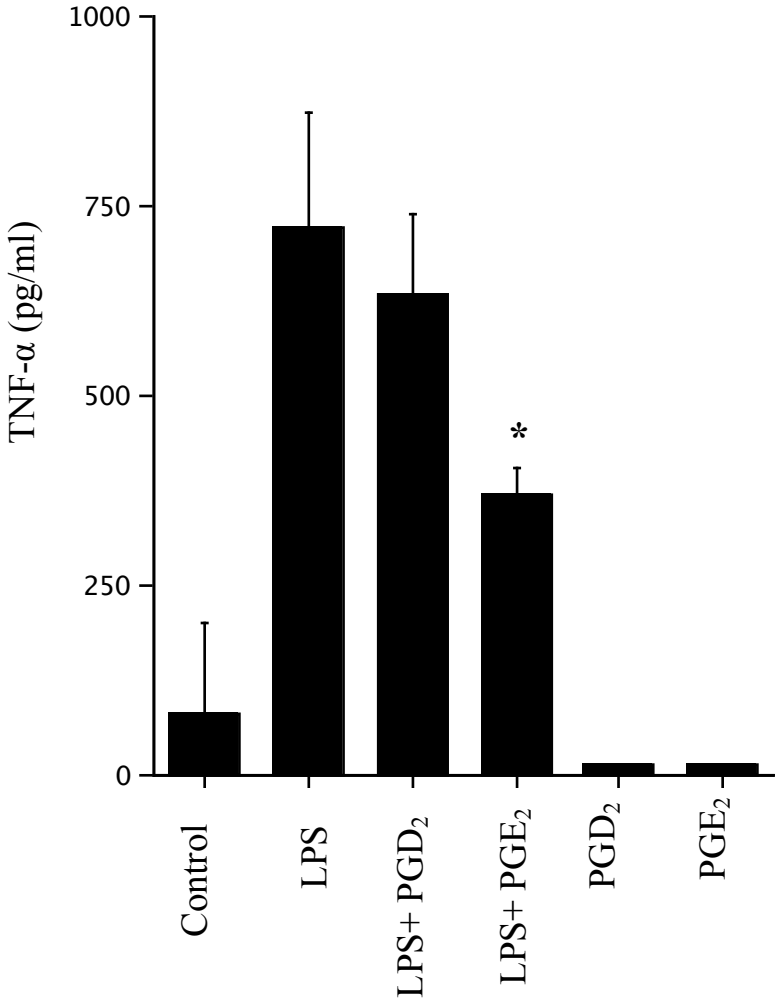


Fig. 3.13: The effect of PGD_2 and PGE_2 on the production of TNF-α in mononuclear cells.

Cells ($1x10^6$ cells/ml) were incubated with LPS (10 μg/ml), PGE_2 (1 μM) and PGD_2 (1 μM). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

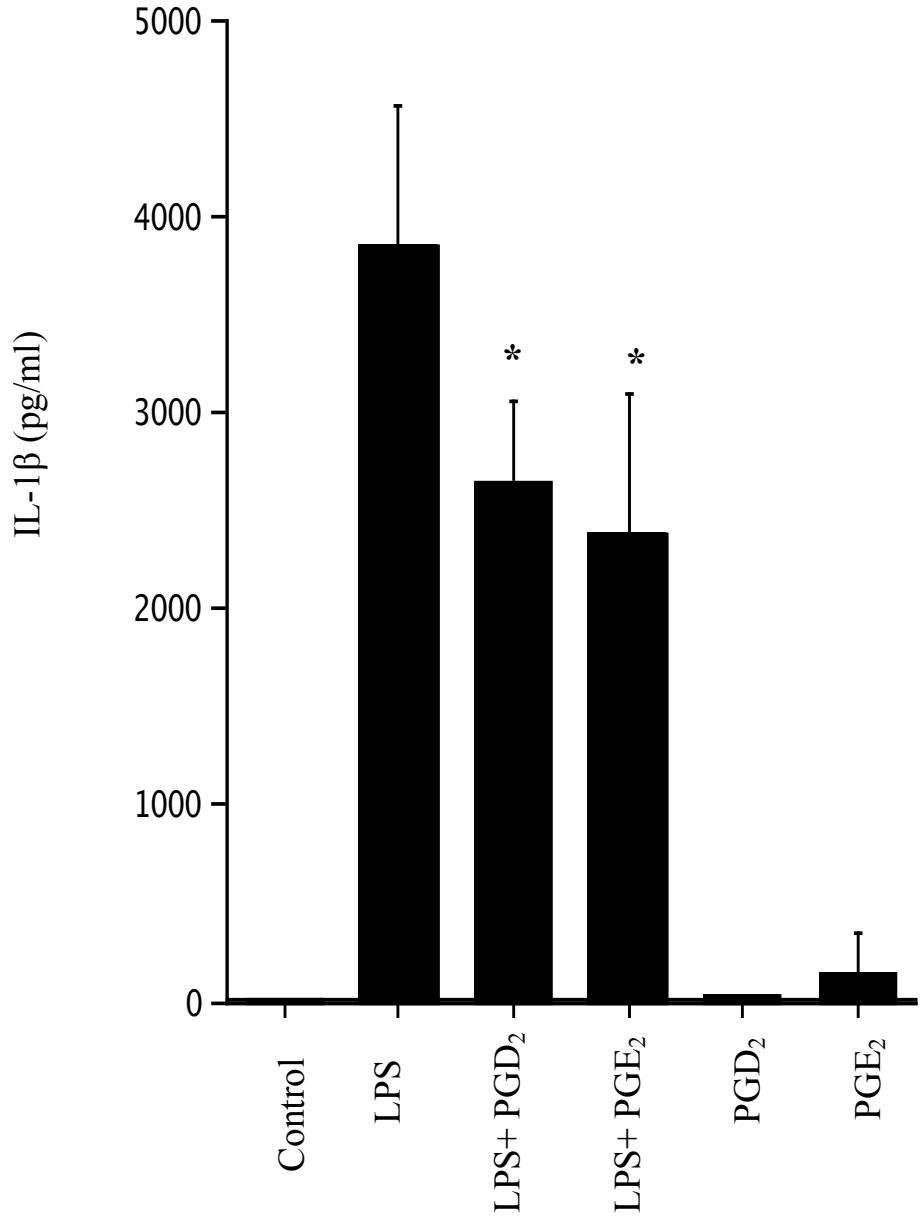


Fig. 3.14: The effect of PGD_2 and PGE_2 on the production of IL-1β in mononuclear cells.

Cells ($1x10^6$ cells/ml) were incubated with LPS (10 μg/ml), PGE_2 (1 μM) and PGD_2 (1 μM). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of IL-1β in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

3.5 The effect of prostaglandin receptor agonists on cytokine production

3.5.1 $PGF_{2\alpha}$ receptor agonist (fluprostenol)

3.5.1.1 The effect of varying concentrations of fluprostenol on TNF-α and IL-1β production in human blood in response to LPS

The effect of different concentrations of the $PGF_{2\alpha}$ analogue (fluprostenol) on LPS-stimulated TNF-α production in whole human blood was assessed. Blood was stimulated with various concentrations of fluprostenol and PGE_2 in presence of LPS as discussed in Methods Section 2.2.1.7. Fluprostenol (0.01 μM-10 μM) decreased TNF-α production in a concentration-dependent manner. A significant inhibition of TNF-α level occurred at 1 μM and 10 μM of fluprostenol (Fig. 3.15). PGE_2 (1 μM) was used as an inhibitory "landmark" in these experiments.

The effect of fluprostenol on IL-1β production in human blood was also evaluated. Blood was incubated with fluprostenol (0.01 μM-10 μM). However, there was no significant inhibition of IL-1β production, while PGE_2 suppressed the IL-1β significantly in response to LPS (Fig. 3.16).

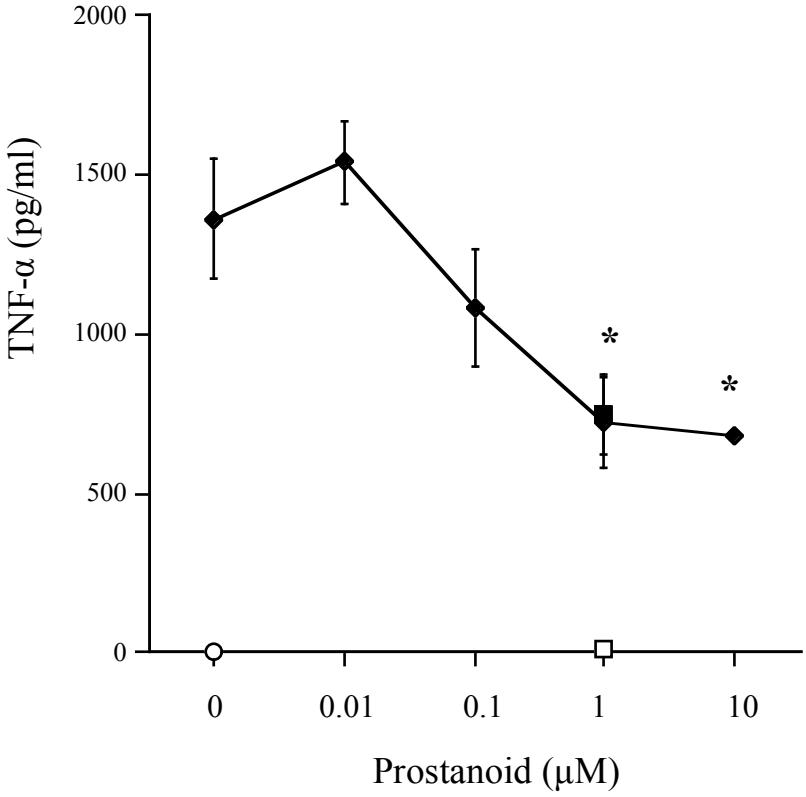


Fig. 3.15: Effect of varying concentrations of $PGF_{2\alpha}$ agonists (fluprostenol) on TNF-α production in response to LPS from human whole blood.

Blood was incubated with varying concentrations of fluprostenol (diamond filled symbols), PGE_2 (1 μM) (square filled symbols) in the presence of LPS (10 μg/ml). Incubations without LPS are shown as open circle, with PGE_2 alone (open square). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in plasma was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

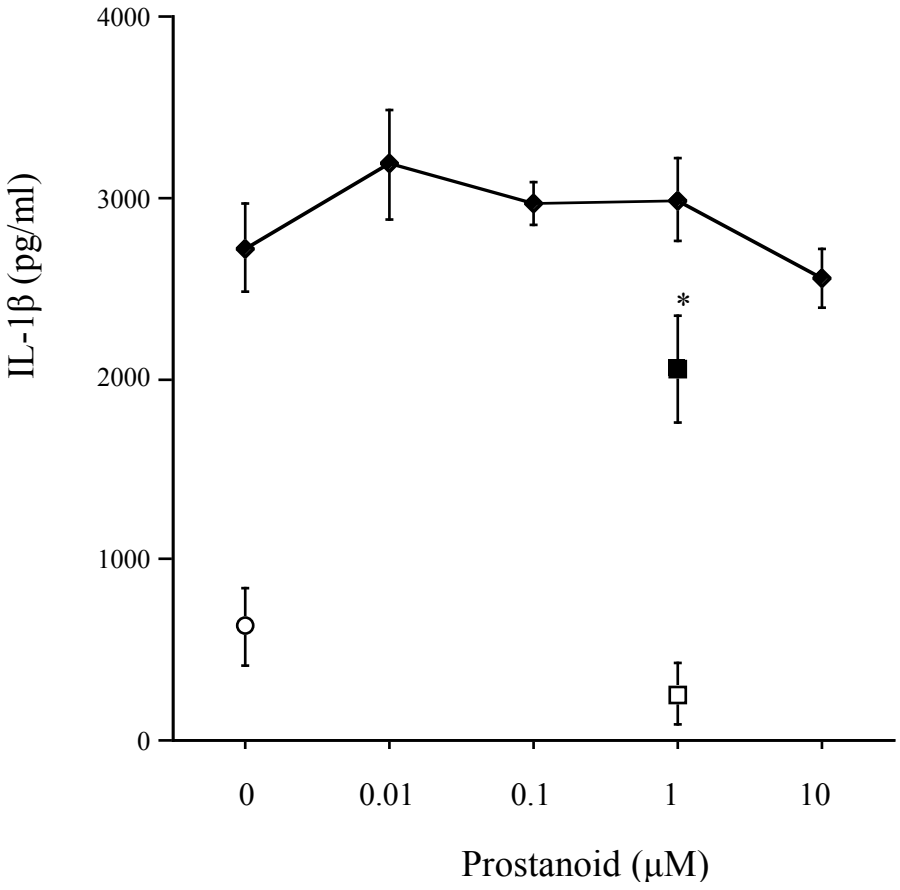


Fig. 3.16: Effect of varying concentrations of $PGF_{2\alpha}$ agonists (fluprostenol) on IL-1β production in response to LPS from human whole blood.

Blood was incubated with varying concentrations of fluprostenol (diamond filled symbols), PGE_2 (1 μM) (square filled symbols) in the presence of LPS (10 μg/ml). Incubations without LPS are shown as open circle, with PGE_2 alone (open square). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of IL-1β in plasma was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations), *P < 0.01 versus LPS alone.

3.5.1.2 The effect of fluprostenol on TNF-α and IL-1β production in THP-1 cells in response to LPS

In previous experiments it was shown that fluprostenol inhibited TNF-α production in whole human blood (Fig. 3.15). Further experiments were conducted to investigate whether fluprostenol can suppress the TNF-α release in THP-1 cells in a similar manner as in whole human blood. However, fluprostenol (0.01 μM-1 μM) had no effect on the TNF-α levels in THP-1 cells compared to LPS alone but a higher concentration of fluprostenol (10 μM) induced a significant decrease in TNF-α production in THP-1 cells. THP-1 cells were incubated with different concentrations of fluprostenol, PGE_2 and LPS (as explained in Section 2.2.1.8). PGE_2 (1 μM) was used as a marker (Fig. 3.17).

The effect of fluprostenol on IL-1β production in THP-1 cells was also evaluated. THP-1 cells were incubated with different concentration of fluprostenol (0.01 μM-10 μM). However, fluprostenol had no significant effect on IL-1β production in THP-1 cells (Fig. 3.18). This observation was comparable to the effect of fluprostenol on IL-1β production in human blood (Fig. 3.16).

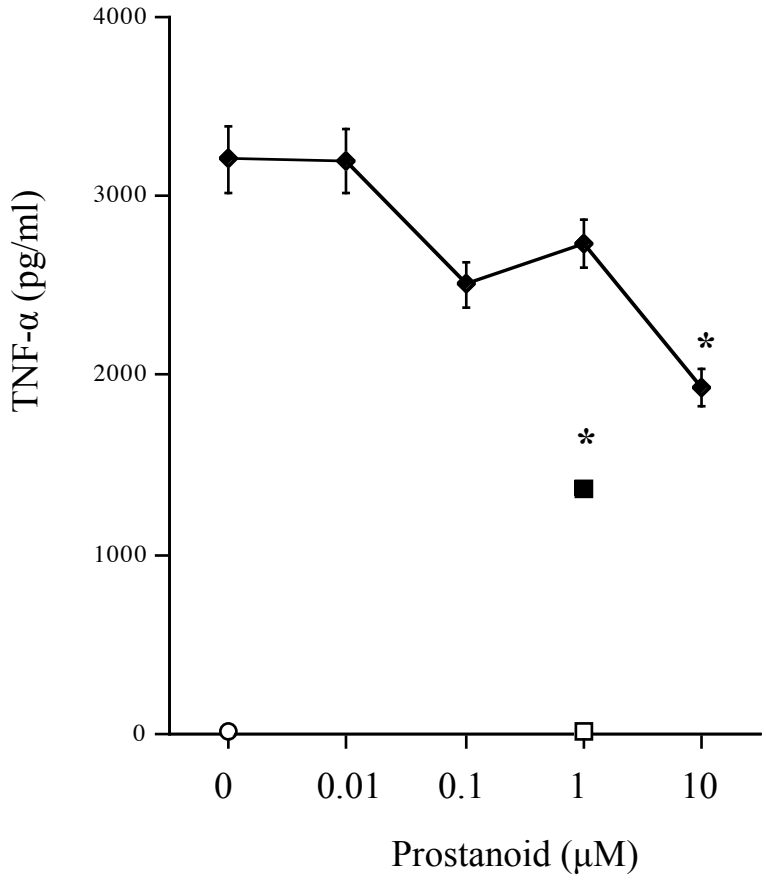


Fig. 3.17: Effect of varying concentrations of $PGF_{2\alpha}$ agonists (fluprostenol) on TNF-α production in response to LPS in THP-1 cells.

Cells ($1x10^6$ cells/ml) were incubated with varying concentrations of fluprostenol (diamond filled symbols), PGE_2 (1 μM) (square filled symbols) in the presence of LPS (10 μg/ml). Incubations without LPS are shown as open circle, with PGE_2 alone (open square). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

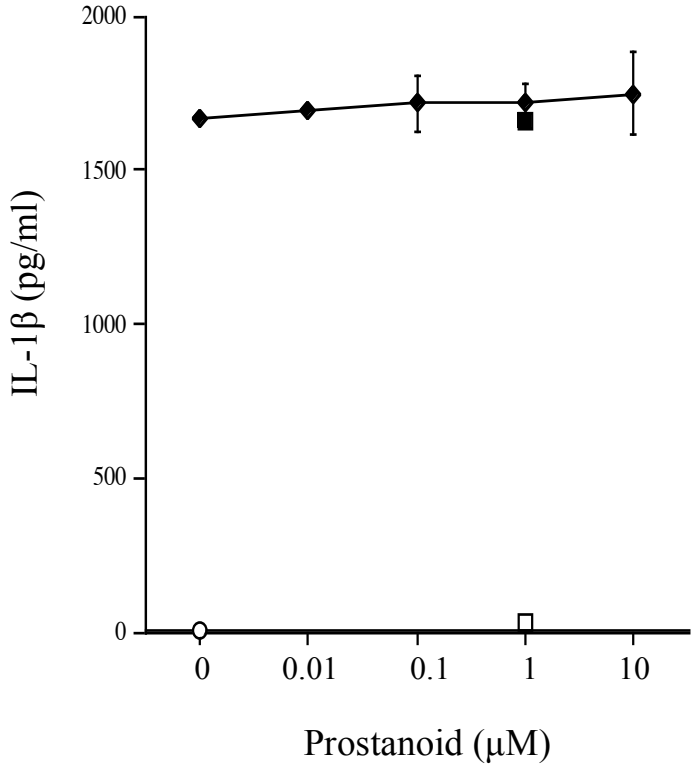


Fig. 3.18: Effect of varying concentrations of $PGF_{2\alpha}$ agonists (fluprostenol) on IL-1β production in response to LPS in THP-1 cells.

Cells ($1x10^6$ cells/ml) were incubated with varying concentrations of fluprostenol (diamond filled symbols), PGE_2 (1 μM) (square filled symbols) in the presence of LPS (10 μg/ml). Incubations without LPS are shown as open circle, with PGE_2 alone (open square). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of IL-1β in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations).

3.5.1.3 The effect of LPS, PGE_2 and fluprostenol on the production of both TNF-α and IL-1β in human blood

As previous experiments showed that fluprostenol at 1 μM inhibited TNF-α production in human blood (Fig. 3.15), the effect of this concentration (1 μM) was used with a range of LPS concentrations using the incubation protocol detailed in Methods Section 2.2.1.7. Fluprostenol (1 μM) induced a significant decrease in TNF-α production with 10 μg/ml and 100 μg/ml of LPS. The TNF-α level was suppressed with lower concentrations of PGE_2. This indicates that PGE_2 is more effective than fluprostenol in terms of the suppression of TNF-α production (Fig. 3.19).

The effect of fluprostenol (1 μM) was also studied on LPS-stimulated IL-1β production using various concentrations of LPS (0.1 μg/ml-100 μg/ml) in blood. Both fluprostenol and PGE_2 (1 μM) had a similar significant inhibitory effect on IL-1β levels throughout all LPS concentrations used (Fig. 3.20).

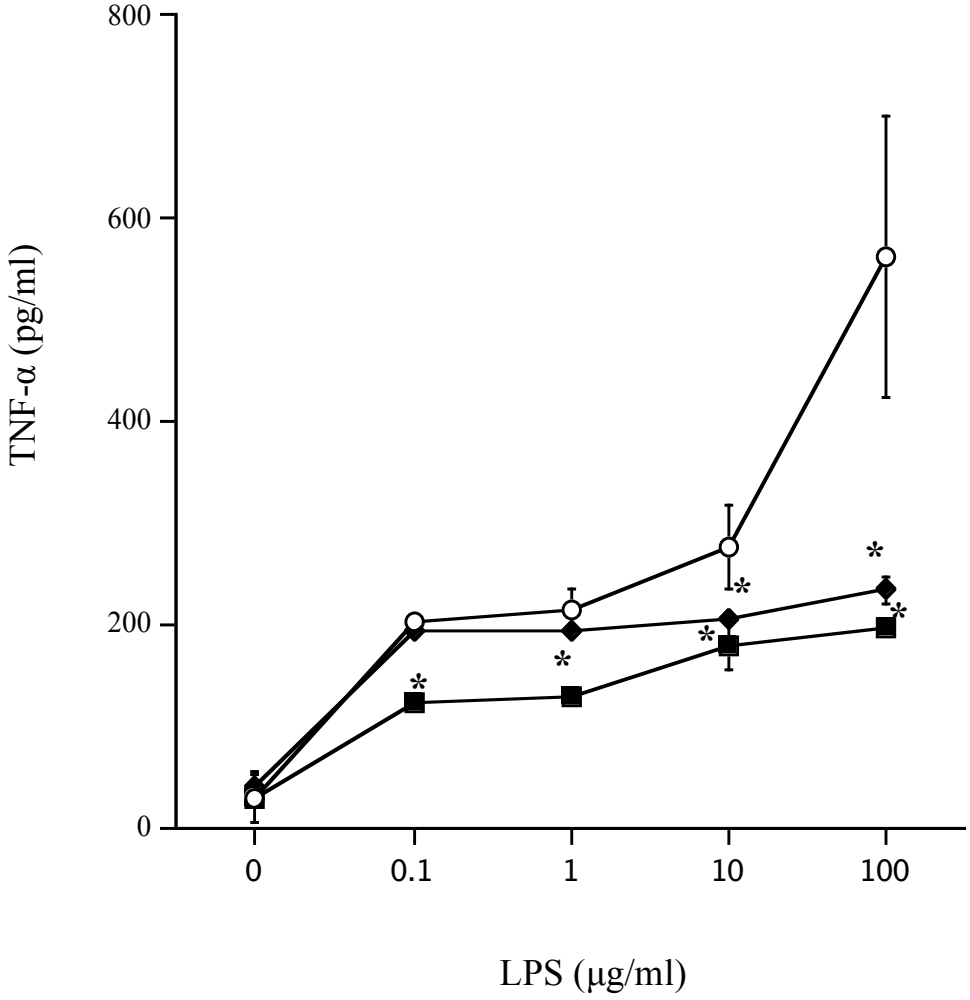


Fig. 3.19: The effect of PGE_2 and fluprostenol on TNF-α production in response to different concentrations of LPS in human whole blood.

Blood was incubated with varying concentrations of LPS (circle open symbols), fluprostenol (1 μM) (diamond filled symbols) and PGE_2 (1 μM) (square filled symbols). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in plasma was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations).*P < 0.01 versus LPS alone.

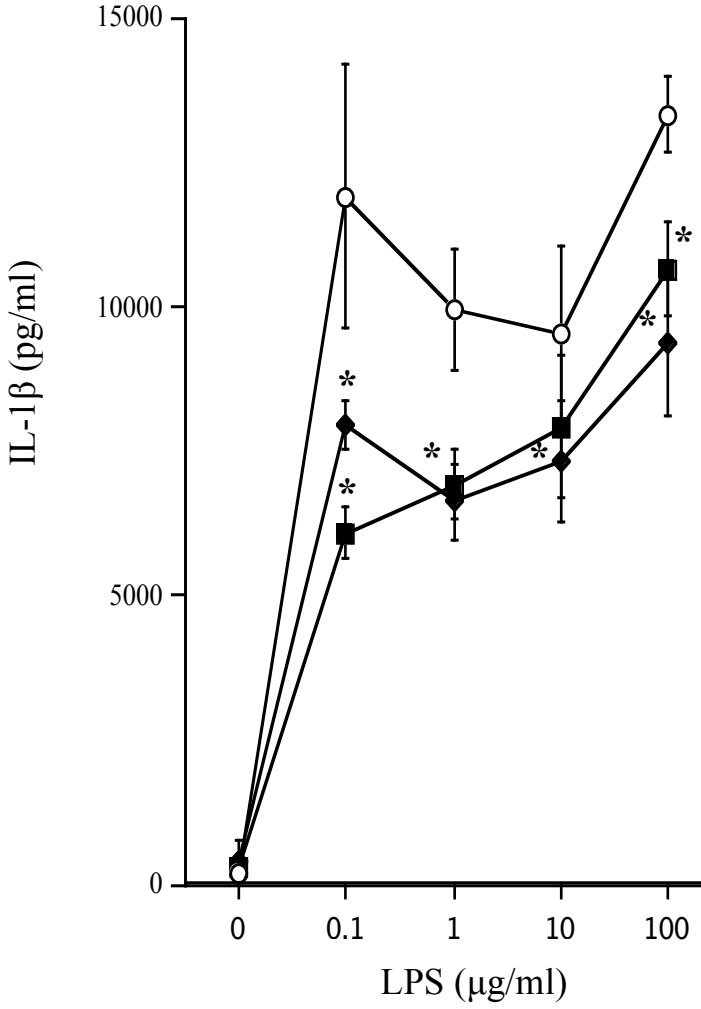


Fig. 3.20: The effect of PGE_2 and fluprostenol on IL-1β production in response to different concentrations of LPS in human whole blood.

Blood was incubated with varying concentrations of LPS (circle open symbols), fluprostenol (1 μM) (diamond filled symbols) and PGE_2 (1 μM) (square filled symbols). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of IL-1β in plasma was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations).*P < 0.01 versus LPS alone.

3.5.2 EP2 receptor agonist (butaprost)

3.5.2.1 The effect of butaprost and PGE_2 on LPS-stimulated TNF-α production in whole human blood, monocytes and THP-1 cells

A series of experiments were carried out to study the effect of the EP2 receptor agonist (butaprost) compared to PGE_2 on LPS-stimulated TNF-α production in blood, monocytes and THP-1 cells following the incubation protocol mentioned in Methods Sections 2.2.1.7 and 2.2.1.8.

Fig. 3.21 represents the effect of different concentrations of butaprost and PGE_2 on TNF-α production in whole human blood. Butaprost concentrations between 0.1 μM to 10 μM induced a significant decrease in the production of TNF-α in plasma. PGE_2 also reduced the TNF-α level significantly even at the lowest concentration used (0.01 μM).

In a parallel experiment, Fig. 3.22 shows the effect of butaprost and PGE_2 with different concentrations (0.1 μM-10 μM) on TNF-α production in monocytes. Levels of TNF-α were suppressed in a concentration-dependent manner by both butaprost and PGE_2. At 1 μM and 10 μM of butaprost, the TNF-α level decreased significantly. PGE_2 also induced a similar inhibition in TNF-α production between 0.1 μM- 10 μM of PGE_2 in monocytic cells.

The effect of butaprost and PGE_2 was also evaluated in THP-1 cells. Fig. 3.23 shows the production of TNF-α incubated with different concentrations of butaprost and PGE_2 in THP-1 cells. Both butaprost and PGE_2 produced a concentration-dependent suppression of LPS-stimulated TNF-α production.

In conclusion, it was observed that PGE_2 was more potent at lower concentrations than butaprost in reducing TNF-α production in the three models used (blood, monocytes and THP-1 cells)(Fig. 3.21, Fig. 3.22 and Fig. 3.23). In all these models, 1 μM of butaprost induced a significant decrease in TNF-α level. Other previous studies agreed with the current work by showing that 1 μM of butaprost suppressed the production of TNF-α (Brown *et al.*, 2013b; Johansson *et al.*, 2013). Thus, 1 μM of butaprost was used in all experiments conducted in this project.

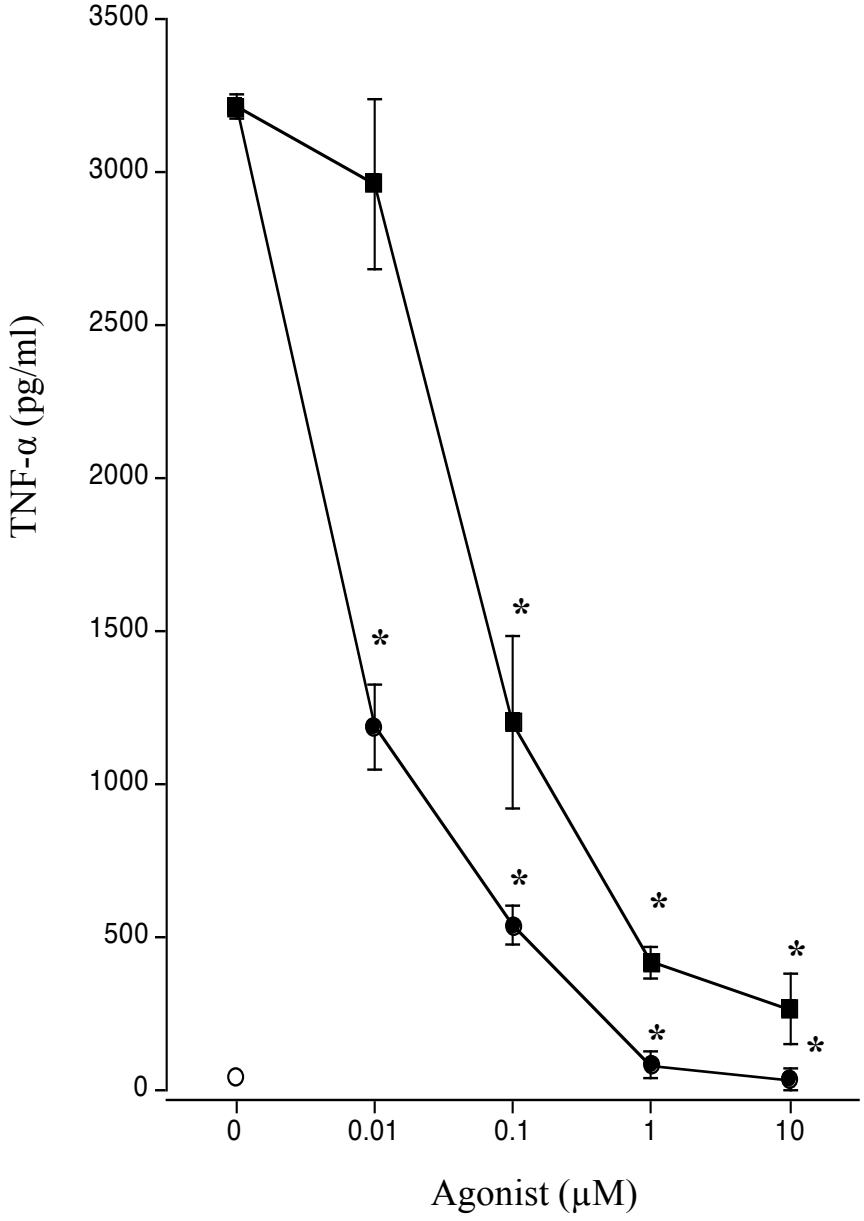


Fig. 3.21: The effect of EP2 agonist (butaprost) and PGE_2 on LPS-stimulated TNF-α production from human whole blood.

Blood was incubated with varying concentrations of either butaprost (square filled symbols) or PGE_2 (round filled symbols) in the presence of LPS (10 μg/ml). Incubations without agonist or LPS are shown as open circles. Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in plasma was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

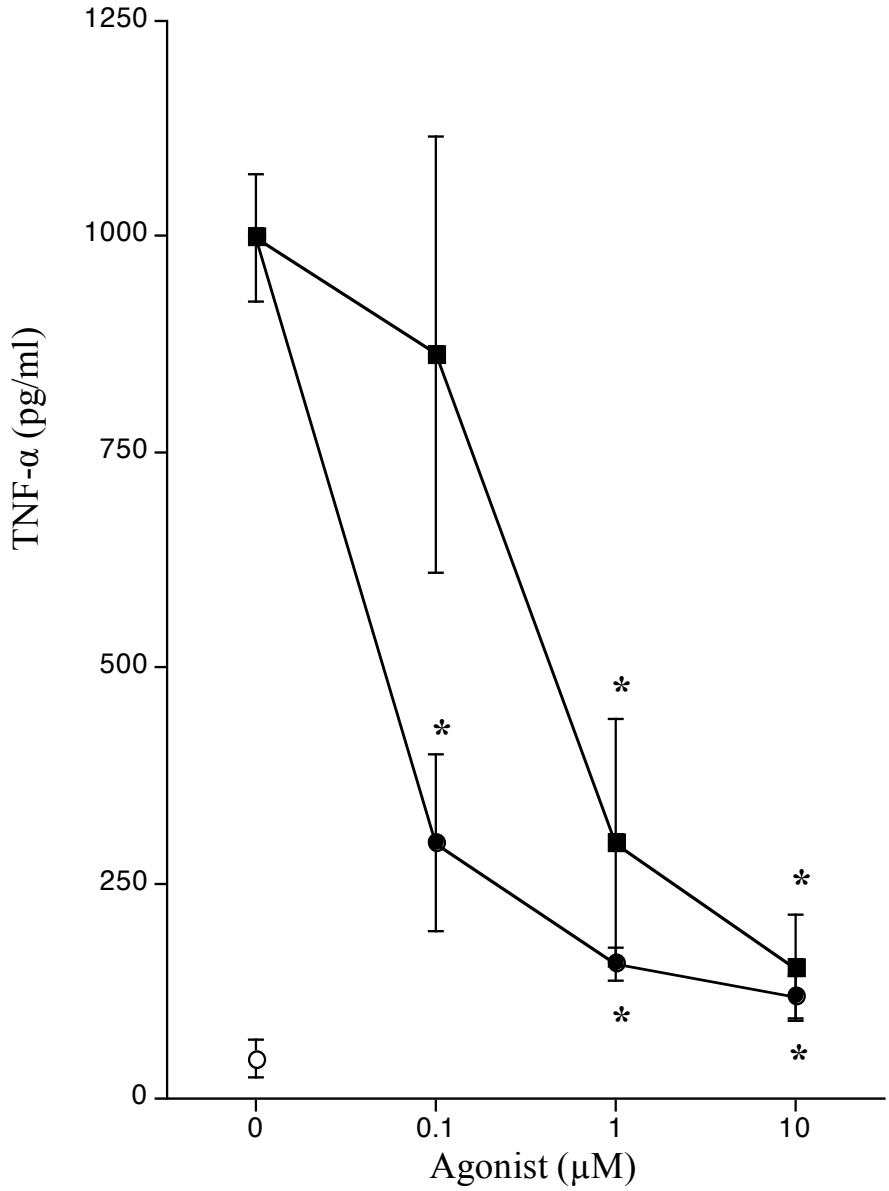


Fig. 3.22: The effect of EP2 agonist (butaprost) and PGE_2 on LPS-stimulated TNF-α production in human monocytes.

Monocytes ($1x10^6$ cells/ml) were incubated with varying concentrations of either butaprost (square filled symbols) or PGE_2 (round filled symbols) in the presence of LPS (10 μg/ml). Incubations without agonist or LPS are shown as open circles. Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

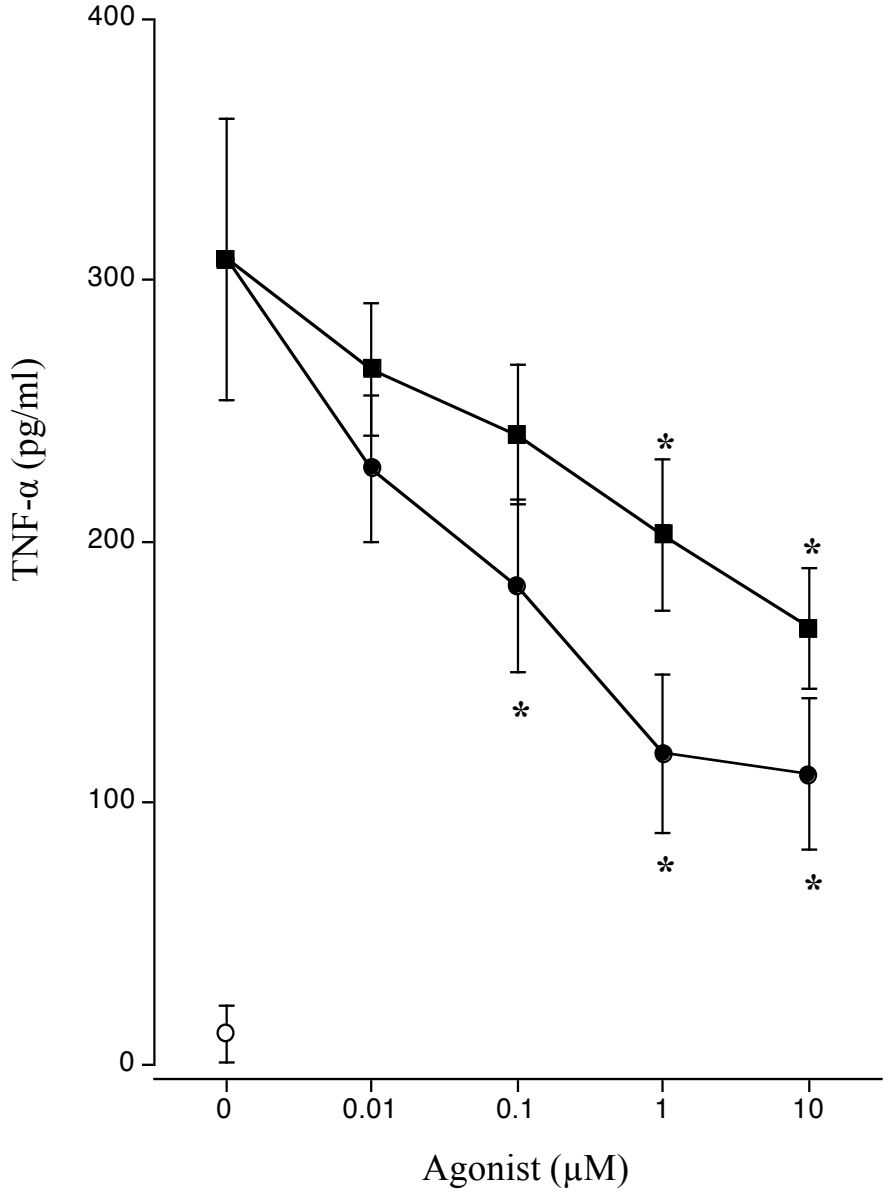


Fig. 3.23: The effect of EP2 agonist (butaprost) and PGE_2 on LPS-stimulated TNF-α production in THP-1 cells.

Cells ($1x10^6$cells/ml) were incubated with varying concentrations of either butaprost (square filled symbols) or PGE_2 (round filled symbols) in the presence of LPS (10 μg/ml). Incubations without agonist or LPS are shown as open circles. Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

3.5.3 EP4 receptor agonist (L-902, 688)

3.5.3.1 The effect of L-902, 688 on LPS-stimulated TNF-α and IL-1β production from THP-1 cells

To determine the effect of the EP4 agonist (L-902, 688) on the production of TNF-α in THP-1 cells, various L-902, 688 concentrations were incubated with cells as described in Methods Section 2.2.1.8. An increase in L-902, 688 concentrations resulted in a decrease in TNF-α in a concentration-dependent manner. There was a significant decrease in TNF-α levels between 0.1 μM-100 μM (Fig. 3.24). However, in Fig. 3.25 it shows that L-902, 688 (1 μM) had no effect on the production of IL-1β.

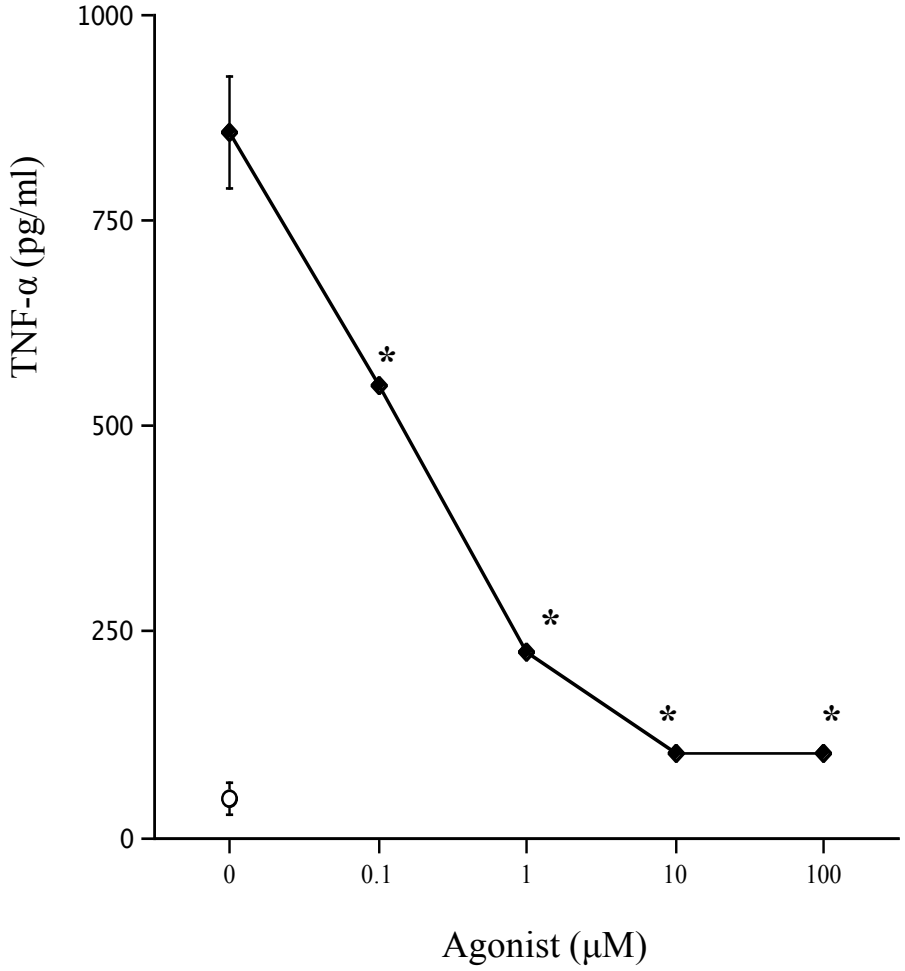


Fig. 3.24: The effect of EP4 agonist (L-902, 688) on LPS-stimulated TNF-α production in THP-1 cells.

Cells ($1x10^6$ cells/ml) were incubated with varying concentrations of L-902, 688 (diamond filled symbols) in the presence of LPS (10 μg/ml). Incubations without LPS are shown as open circle. Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

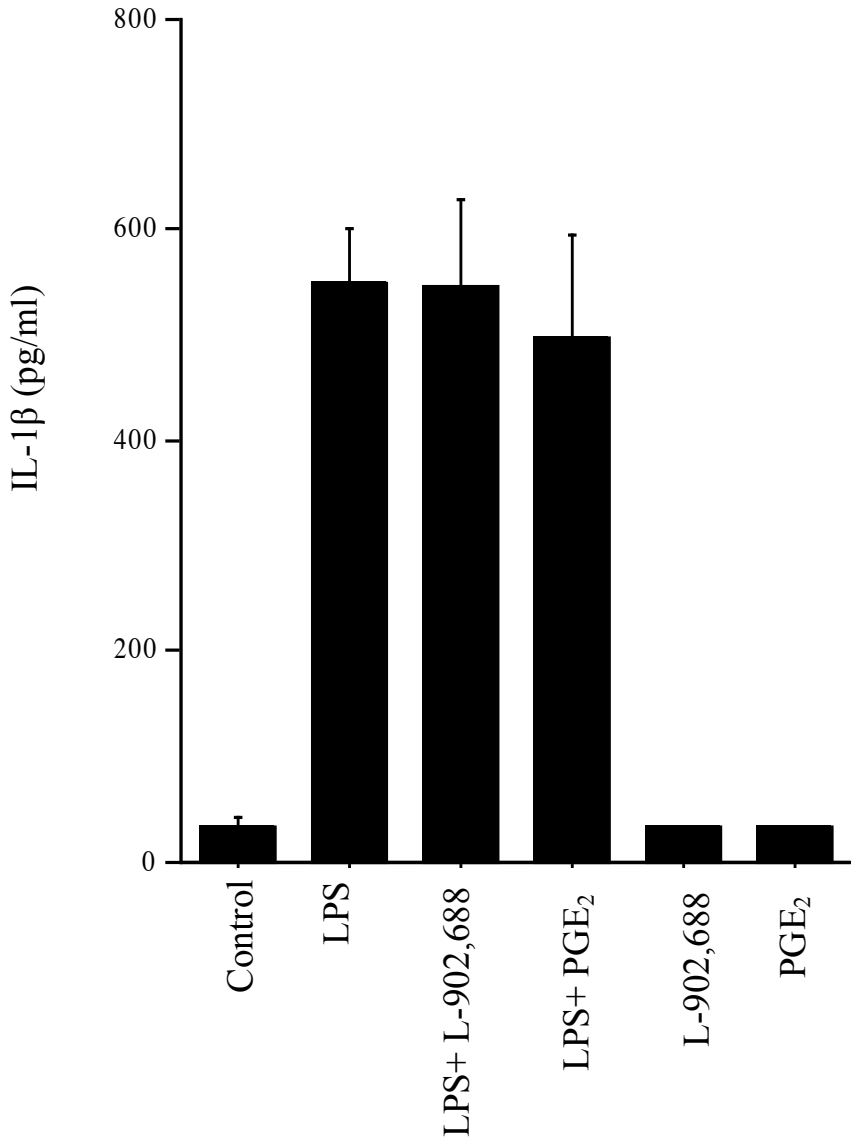


Fig. 3.25: The effect of EP4 agonist (L-902, 688) on LPS-stimulated IL-1β production in THP-1 cells.

Cells ($1x10^6$ cells/ml) were incubated with L-902, 688 (1 μM) and PGE_2 (1 μM) in the presence of LPS (10 μg/ml). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of IL-1β in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations).

3.5.4 The effect of PGE_2, EP1/ EP3 receptor agonist (sulprostone), EP4 agonist (L-902, 688) and the EP2 agonist (butaprost) on LPS-stimulated TNF-α production in whole blood and THP-1 cells

Experiments were performed to determine the effects of selective receptor agonists on TNF-α production. Sulprostone (EP1/EP3), L-902, 688 (EP4) and butaprost (EP2) were used in addition to PGE_2 (the incubation protocols were detailed in Methods Sections 2.2.1.7 and 2.2.1.8). Incubation with either L-902, 688 or butaprost in the presence of LPS resulted in a decrease in TNF-α levels compared to incubation with LPS alone. However, there was no effect of sulprostone on LPS-stimulated TNF-α production. Fig. 3.26 shows the effect of the EP-receptor agonists on LPS-stimulated TNF-α production in whole human blood and Fig. 3.27 shows the effect of the same EP-receptor agonists in THP-1 cells.

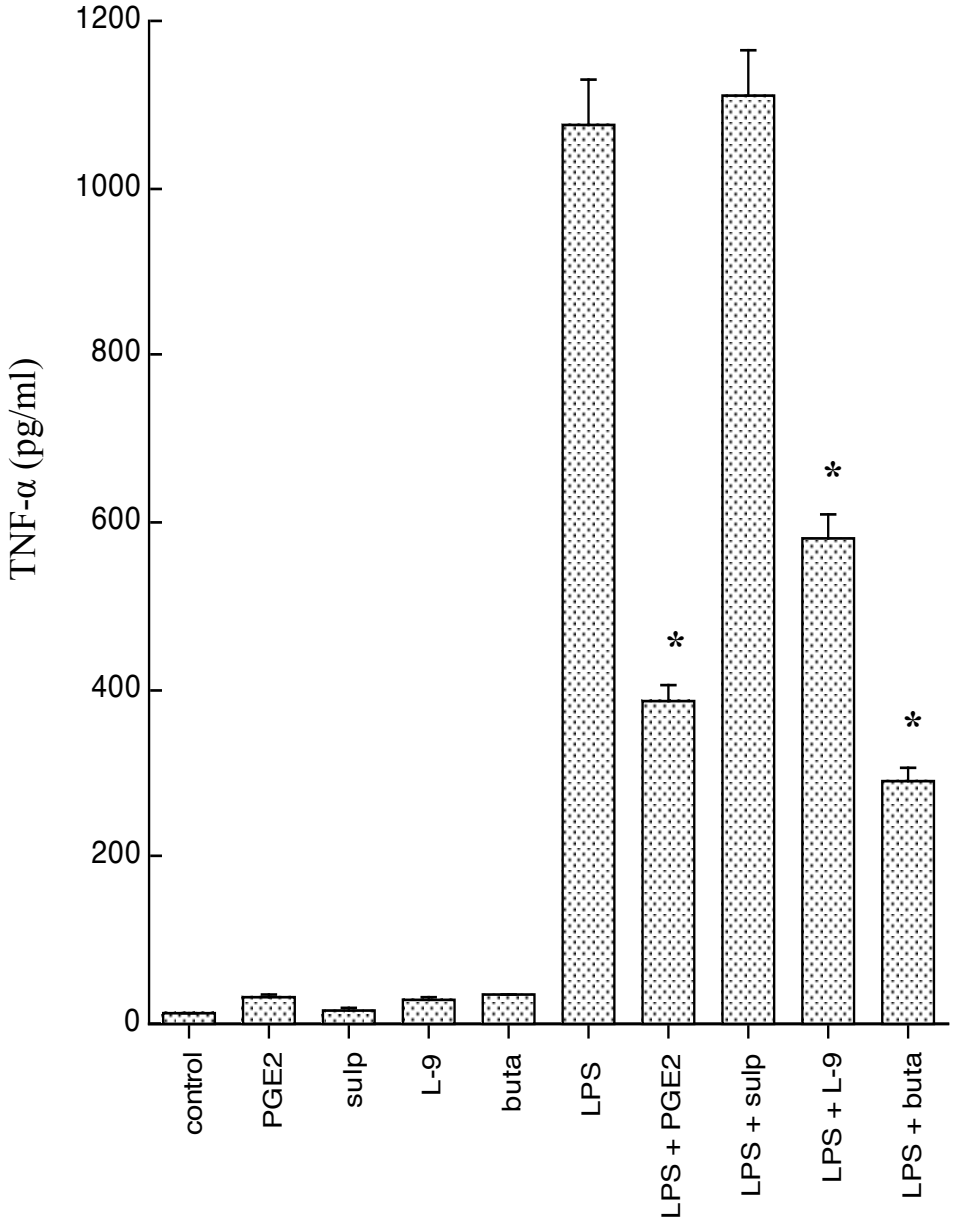


Fig. 3.26: The effect of EP-receptor agonists on LPS-stimulated TNF-α production in whole human blood.

Blood was incubated in the absence or presence of LPS (10 μg/ml) with the various EP receptor agonists (all at 1 μM); PGE_2, sulprostone (sulp), L-902,688 (L-9) or butaprost (buta) for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the plasma was collected by centrifugation. The level of TNF-α in plasma samples was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

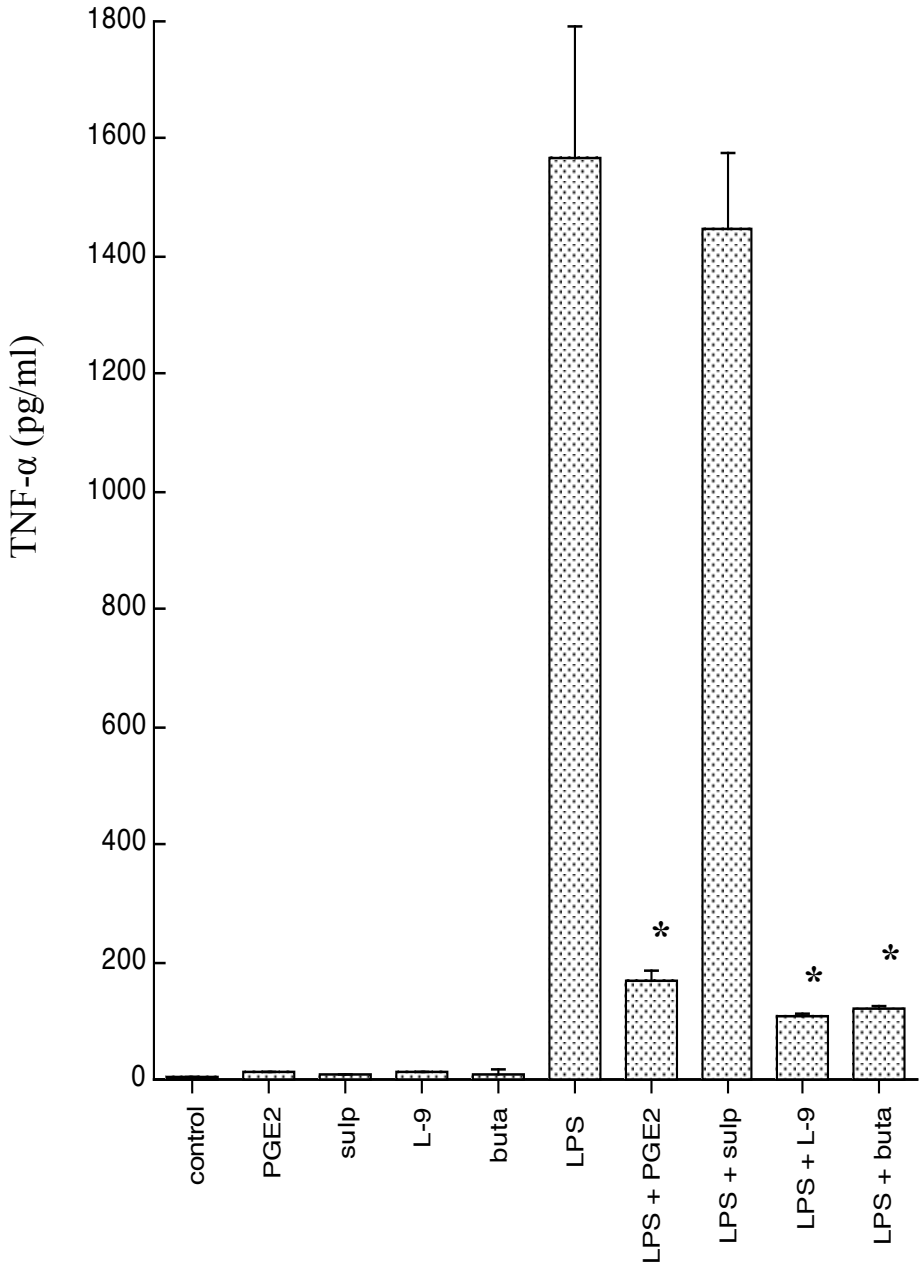


Fig. 3.27: The effect of EP-receptor agonists on LPS-stimulated TNF-α production from THP-1 cells.

Cells ($1x10^6$ cells/ml) were incubated in the absence or presence of LPS (10 µg/ml) with the various EP receptor agonists (all at 1 µM); PGE_2, sulprostone (sulp), L-902,688 (L-9) or butaprost (buta) for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the cell supernatants were collected. The level of TNF-α in supernatants was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

3.6 Reference gene stability in THP-1 cells

The stability of reference genes used in this study (PPIB, TBP, GAPDH, RPL37A, B2M, ACTB and HPRT1) was validated using a comprehensive ranking of the stability of candidate reference genes via the web-based program RefFinder (http://fulxie.0fees.us/?type=reference)(Xie *et al.*, 2012). This collates the results from the GeNorm, BestKeeper and NormFinder gene stability programs (Vandesompele *et al.*, 2002; Andersen *et al.*, 2004; Pfaffl *et al.*, 2004). It was found that PPIB was the most stable control gene ranked by all three programs (Table 3.3). Genes which have a lower mean (*M* value) with low standard deviation (SD) are considered to be the most stable reference gene calculated by geNorm and BestKeeper programs (Cao *et al.*, 2012), PPIB was deemed to be the most invariant or stably expressed out of the candidate genes tested because its *M* value was below 1.5 with SD of 0.09. The candidate reference genes tested had higher values as shown in Table 3.4.

Table 3.3: Stability of reference genes in THP-1 cells by NormFinder, geNorm and BestKeeper.

	Rank	NormFinder	geNorm	BestKeeper
Most stable genes ↑	1	PPIB	PPIB	PPIB
	2	TBP	TBP	ACTB
	3	GAPDH	HPRT1	TBP
	4	ACTB	ACTB	RPL37A
Least stable genes ↓	5	RPL37A	GAPDH	HPRT1
	6	HPRT1	B2M	GAPDH
	7	B2M	RPL37A	B2M

The stability of expression is disproportionate to the rank position. A low rank number suggest most stable, most invariantly expressed genes while a high rank number shows least stable ones. Therefore, PPIB is the most stable reference gene in the list of genes tested throughout all of the validation programs: NormFinder, geNorm and BestKeeper followed by all other genes which appear to be less stable.

Table 3.4: Stability analysis of candidate reference genes by BestKeeper (SD value) and geNorm (*M* value).

Reference gene	SD value	*M* value
PPIB	0.09	1.00
GAPDH	1.11	1.1
TBP	0.82	1.2
RPL37A	0.65	1.2
HPRT1	0.79	1.3
ACTB	1.24	1.5
B2M	1.58	1.8

The stably expressed invariant reference gene should have a standard deviation lower than 1 (SD< 1) and its *M* value is preferable to be less than 1.5 (*M* value < 1.5) (Cao *et al.*, 2012). In this list, PPIB appears to be the most stable reliable control gene followed by TBP, GAPDH, ACTB, RPL37A, HPRT1 and B2M respectively according to their SD and *M* values.

3.7 Expression of prostaglandin receptor genes

3.7.1 Expression of the PTGER4 gene (EP4 receptor) and the PTGER2 gene (EP2 receptor) in the presence of PGE_2 and the EP4 agonist (L-902, 688) in THP-1 cells

The expression of EP4 receptor (PTGER4) is shown in Fig. 3.28. THP-1 cells were incubated and processed as described in Methods Section 2.2.17.1. Incubation of THP-1 cells with LPS resulted in a 7.15 fold up-regulation of PTGER4 when compared to untreated control THP-1 cells. PGE_2 treatment of THP-1 cells induced a 2.44 fold down-regulation in PTGER4 receptor expression. Treatment of THP-1 cells with PGE_2 significantly reduced their PTGER4 expression response to LPS stimulation (PTGER4 expression fold changed from a 7.15 ± 0.58 up-regulation with LPS-only to a 1.34 ± 0.09 fold up-regulation with LPS+PGE_2, $P<0.0001$). In order to determine potential regulation of PTGER4 by the EP4 receptor, cells were incubated with the EP4 agonist L-902, 688. L-902, 688 treatment of THP-1 cells caused a 3.32 fold drop in PTGER4 expression. L-902, 688 also decreased the expression of PTGER4 significantly in response to LPS with PTGER4 expression changed from a 7.15 ± 0.58 fold up-regulation with LPS alone to a 1.52 ± 0.11 fold down-regulation with LPS+ L-902, 688 ($P<0.0001$).

Expression of the EP2 receptor (PTGER2) was also assessed in THP-1 cells as shown in Fig. 3.29. LPS stimulation resulted in an increase in PTGER2 expression from control (a fold change of 4.54 ± 0.75). A significant down-regulation of PTGER2 expression by PGE_2 was observed (a 1.3 fold ± 0.52 reduction in

expression, P<0.0001). This reduction in the EP2 receptor expression in response to LPS by PGE_2 was comparable to the suppression by the EP4 agonist (L-902, 688). L-902, 688 also significantly altered THP-1 cells LPS stimulated-PTGER2 expression from a 4.54 ± 0.758 fold up-regulation with LPS alone to a 1.23 ± 0.11 fold down-regulation (P<0.0001).

These two expression profiles (PTGER2 and PTGER4) suggest that both PGE_2 and EP4 agonist (L-902, 688) suppressed EP2 and EP4 genes effectively in LPS-stimulated THP-1 cells.

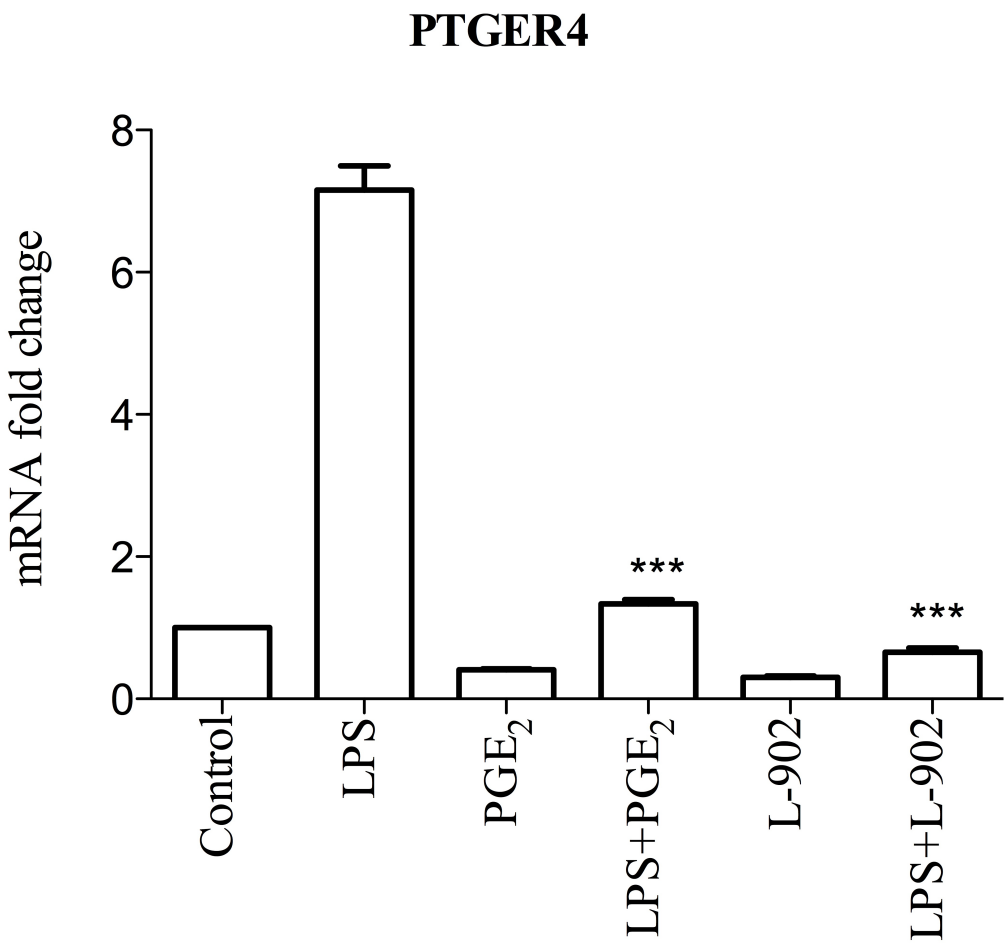


Fig. 3.28: The effect of EP4 agonist (L-902, 688) on PTGER4 expression in THP-1 cells.

Cells ($1.5x10^6$ cells/ml) were placed into 6-well plates after which LPS (10 μg/ml), PGE_2 (1 μM), L-902, 688 (1 μM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGER4 as described in the Method Section 2.2.17.10. Relative expression levels of PTGER4 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). ***P <0.0001 versus LPS alone.

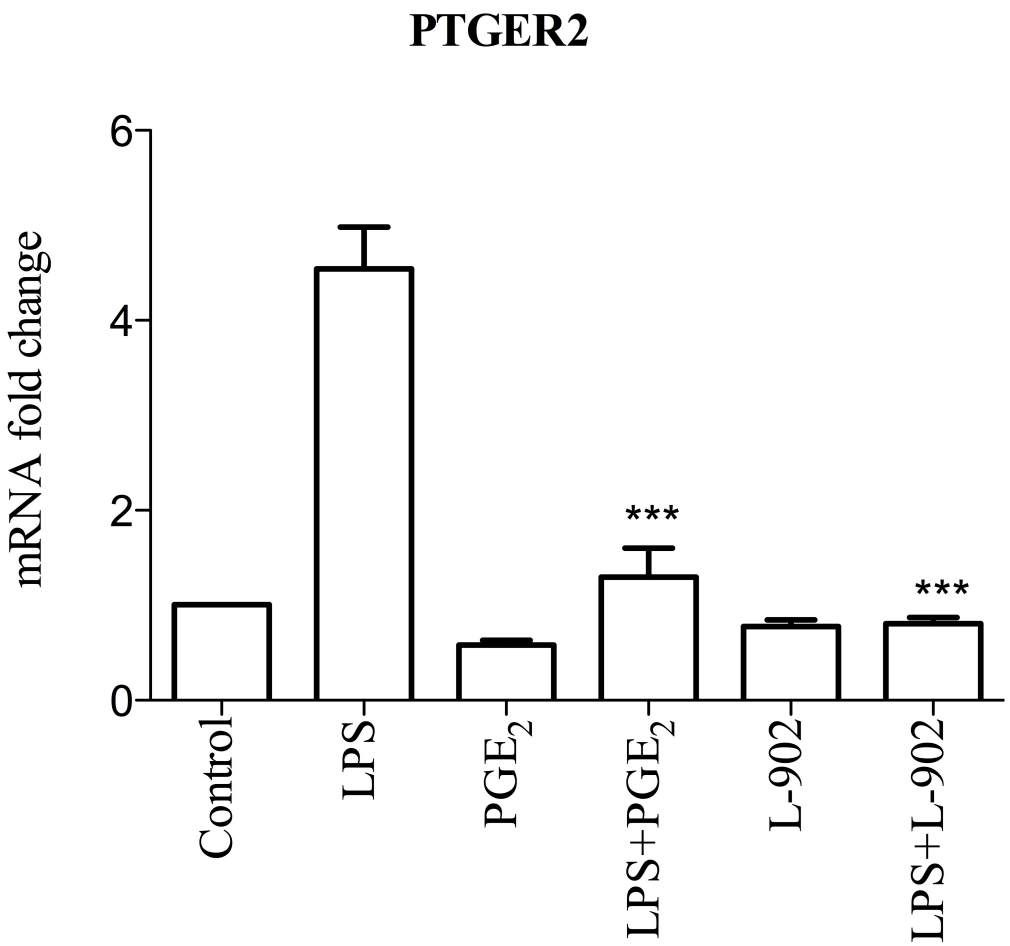


Fig. 3.29: The effect of EP4 agonist (L-902, 688) on PTGER2 expression in THP-1 cells.

Cells ($1.5x10^6$ cells/ml) were placed into 6-well plates after which LPS (10 μg/ml), PGE_2 (1 μM), L-902, 688 (1 μM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGER2 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER2 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). ***P <0.0001 versus LPS alone.

3.7.2 Expression of the PTGER4 gene (EP4 receptor) and the PTGER2 gene (EP2 receptor) in the presence of PGE_2 and the EP2 agonist (butaprost) in THP-1 cells

Having established the ability of the EP4 agonist (L-902, 688) to reduce both PTGER2 and PTGER4 expression, it was important to ascertain the effect of the EP2 agonist (butaprost). THP-1 cells were incubated and processed as described previously (Methods Section 2.2.17.1). Fig. 3.30 shows the effect of butaprost on the expression of PTGER4 in THP-1 cells. Although PGE_2 significantly reduced PTGER4 expression after LPS stimulation (PTGER4 expression fold changed from 2.92 fold ± 0.6 up-regulation with LPS-only to a 1.309 fold ± 0.072 up-regulation with LPS+PGE_2, $P<0.0001$), incubation of THP-1 cells with butaprost reduced the EP4 receptor up-regulation in the THP-1 cells response to LPS but the reduction was not significant (PTGER4 expression fold changed from 2.92 fold ± 0.6 up-regulation with LPS-only to a 2.18 fold ± 0.179 up-regulation with LPS+ butaprost, $P= 0.56$).

On the other hand, butaprost did not affect the expression of PTGER2 in LPS-stimulated THP-1 cells. Fig. 3.31 confirms that there was no obvious alteration between PTGER2 expression level in response to LPS alone and the expression level with butaprost (fold change in response to LPS was 20.01 fold ± 3.506 and 20.03 fold ± 2.094 in the presence of butaprost). In addition, PGE_2 did not significantly alter in PTGER2 expression in LPS-stimulated THP-1 cells (PTGER2 expression was up-regulated 20.01 fold ± 3.506 in response to LPS, whereas PTGER2 up-regulation in the presence of PGE_2 was 15.8 fold ± 2.33, $P= 0.06$).

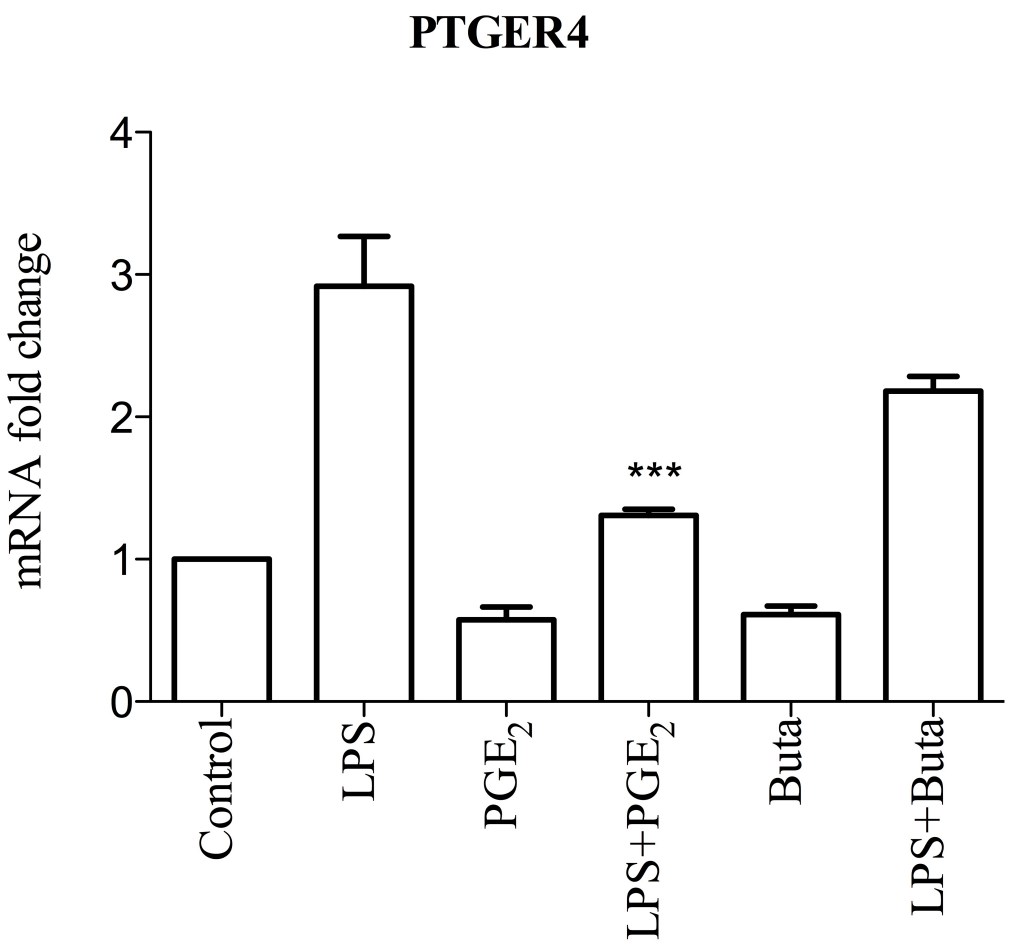


Fig. 3.30: The effect of EP2 agonist (butaprost) on PTGER4 expression in THP-1 cells.

Cells ($1.5x10^6$ cells/ml) were placed into 6-well plates after which LPS (10 μg/ml), PGE_2 (1 μM), butaprost (1 μM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGER4 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER4 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). ***P <0.0001 versus LPS alone.

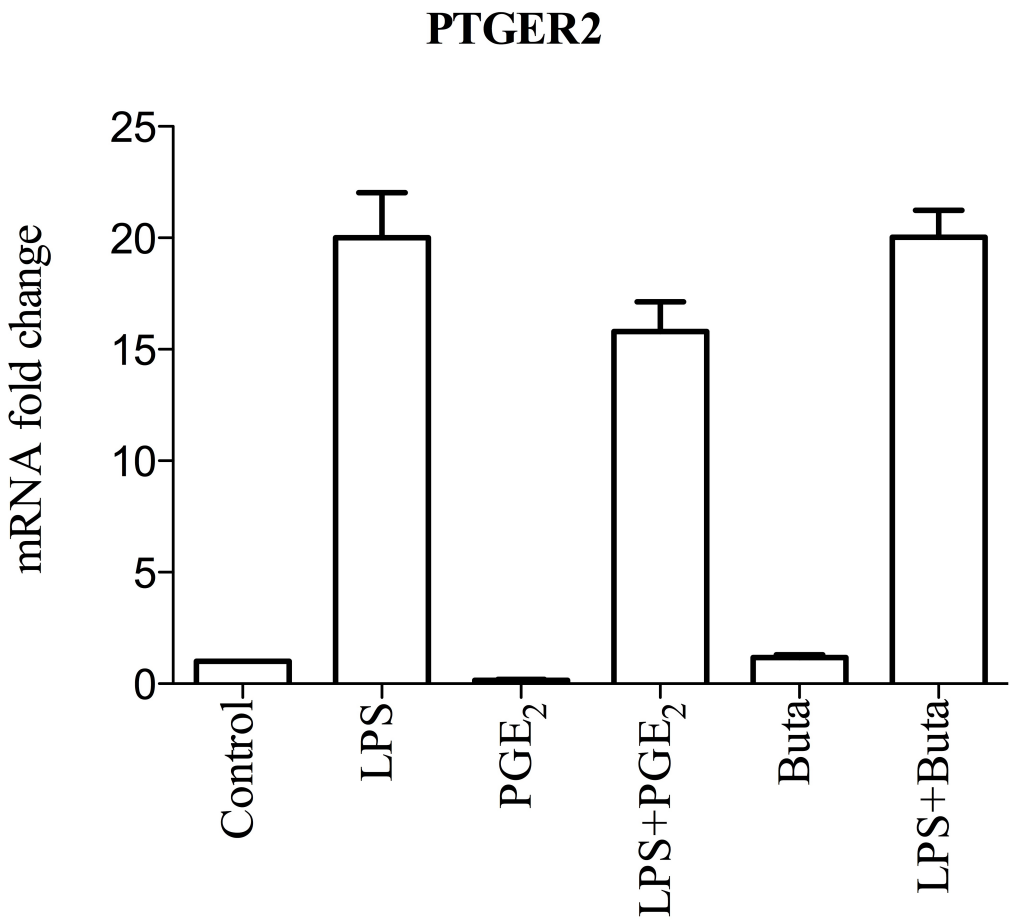


Fig. 3.31: The effect of EP2 agonist (butaprost) on PTGER2 expression in THP-1 cells.

Cells ($1.5x10^6$ cells/ml) were placed into 6-well plates after which LPS (10 μg/ml), PGE_2 (1 μM), butaprost (1 μM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGER2 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER2 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations).

3.7.3 Expression of PTGER1, PTGER2 and PTGER3 genes in the presence of the EP1/EP3 agonist (sulprostone) in THP-1 cells

The expression of both PTGER1 and PTGER3 was examined following incubation with sulprostone. THP-1 cells were incubated and processed as described in Methods Section 2.2.17.1. Neither PTGER1 nor PTGER3 was expressed in THP-1 control cells and also there was no expression observed in the presence of sulprostone agonist (Data not shown because the expression was below the level of detection of the assay).

In order to demonstrate whether sulprostone affects other PGE_2 receptors, PTGER2 expression was assessed in the presence of sulprostone. As shown in Fig. 3.32, stimulation of THP-1 cells with LPS increased PTGER2 expression up to 19.44 fold ± 7.63. Sulprostone alone caused a significant up-regulation of PTGER2 expression (6.86 fold ± 0.74). The EP1/EP3 agonist (sulprostone) did not significantly affect EP2 receptor expression level following LPS stimulation (fold stimulation was 17.79 ± 3.77, P= 0.248).

In conclusion, studying the expression of EP receptor genes in THP-1 cells in Sections (3.7.1 to 3.7.3) illustrated that mRNA of PTGER4 was significantly inhibited by both PGE_2 and L-902, 688 in LPS-stimulated cells. The suppression was not restricted to PTGER4 since PTGER2 was also decreased by PGE_2 and L-902, 688. This suggests that there is a cross-regulation between these two receptors (EP2 and EP4). However, there was no expression detected for EP1 and EP3 receptor genes (PTGER1/PTGER3) in THP-1 cells.

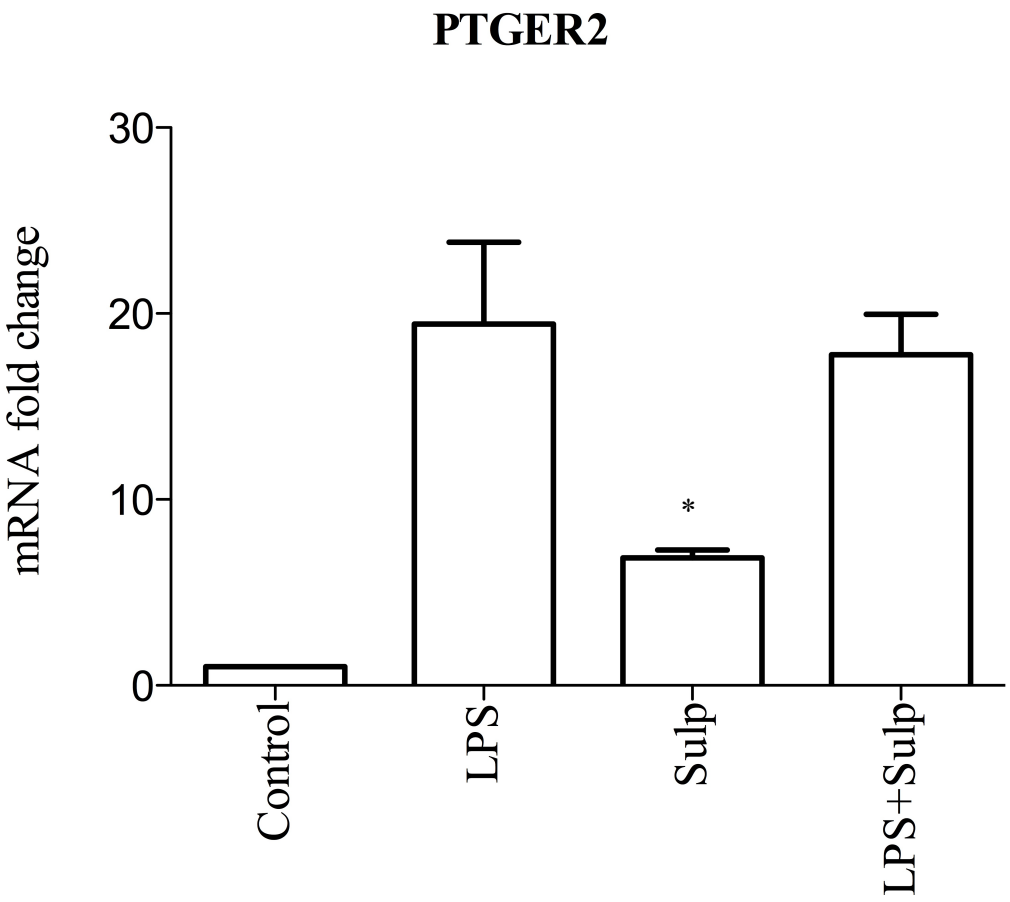


Fig. 3.32: The effect of EP1/EP3 agonist (sulprostone) on PTGER2 expression in THP-1 cells.

Cells ($41.5x10^6$ cells/ml) were placed into 24-well culture plates after which LPS (10 μg/ml), sulprostone (1 μM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGER2 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER2 mRNA transcripts were normalised to the reference gene GAPDH using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus control.

3.7.4 Expression of the PTGER2 gene (EP2 receptor) and the PTGER4 gene (EP4 receptor) in the presence of PGE_2 or the EP4 agonist (L-902, 688) in monocytes

PTGER2 expression in monocytes cells followed the same PTGER2 mRNA expression profile as in THP-1 cells. Monocytes were incubated and processed as detailed in Methods Section 2.2.17.1. There was a 2.94 fold ± 0.94 up-regulation of monocytes PTGER2 expression in response to LPS. Both PGE_2 and L-902, 688 reduced the extent of LPS up-regulation but the reduction was not significant (PTGER2 expression fold changed from 2.94 fold ± 0.94 up-regulation with LPS alone to a 1.83 fold ± 0.38 up-regulation with LPS+PGE_2 and to a 1.29 fold ± 0.72 up-regulation with LPS+ L-902, 688, P= 0.162)(Fig. 3.33).

On the other hand, there was no clear increase in PTGER4 expression in response to LPS. This suggests that endogenous PGE_2 might be produced in monocytes and this resulted in the reduced PTGER4 expression in response to LPS. However, there was an up-regulation of PTGER4 after stimulation of cells with LPS with both PGE_2 and L-902, 688 showing a 3.07 fold ± 1.48 and 1.98 fold ± 1.12 change respectively (Fig. 3.34).

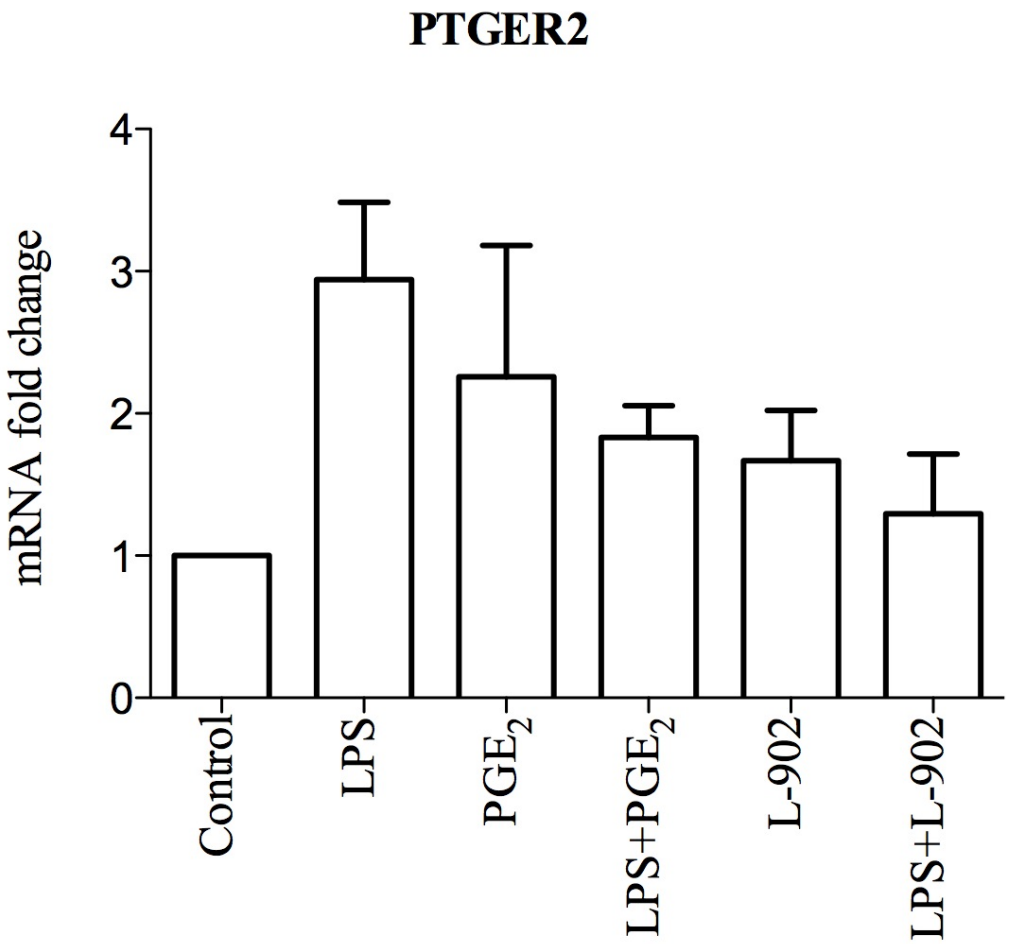


Fig. 3.33: The effect of EP4 agonist (L-902, 688) on PTGER2 expression in monocytic cells.

Cells (10 x10^6 cells/ml) were placed into 24-well plates after which LPS (10 μg/ml), PGE_2 (1 μM), L-902, 688 (1 μM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGER2 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER2 mRNA transcripts were normalised to the reference gene GAPDH using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations).

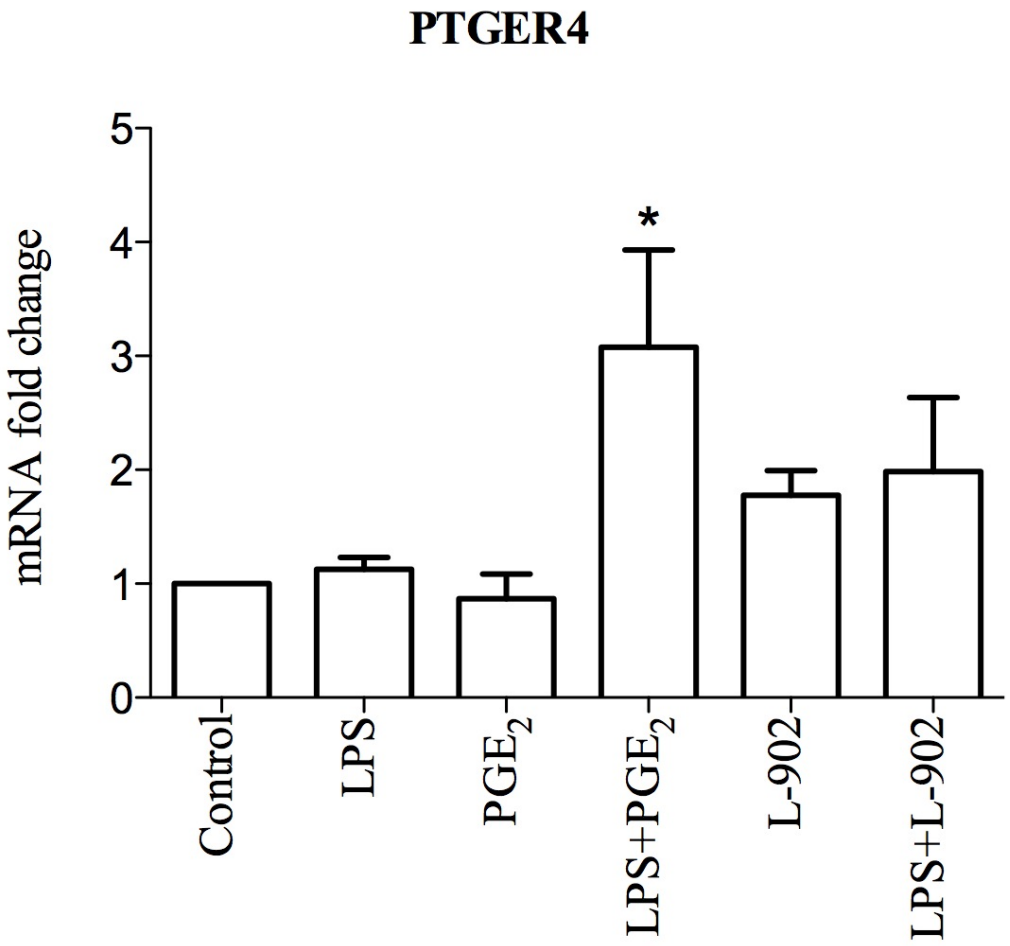


Fig. 3.34: The effect of EP4 agonist (L-902, 688) on PTGER4 expression in monocytic cells.

Cells (4.8 x10^6 cells/ml) were placed into 24-well plates after which LPS (10 μg/ml), PGE_2 (1 μM), L-902, 688 (1 μM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGER4 as described in Method Section 2.2.17.10. Relative expression levels of PTGER4 mRNA transcripts were normalised to the reference gene GAPDH using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations).*P < 0.01 versus LPS alone.

3.7.5 Expression of PTGER4 gene (EP4 receptor) in the presence of PGE_2 and PGD_2 in monocytes

As identified previously (Fig. 3.34), LPS stimulated cells did not increase the expression of PTGER4 in monocytes, whereas PGE_2 with LPS did. A further experiment was performed to see whether PGD_2 had a similar effect on PTGER4 expression as PGE_2 in LPS-stimulated monocytes. Monocytes were incubated and processed as described in Methods Section 2.2.17.1. Fig. 3.35 shows a preliminary result of incubation of monocytes with PGE_2 and PGD_2 in the presence and absence of LPS. This initial observation gives an indication that LPS with PGD_2 may increase the mRNA PTGER4 expression as LPS with PGE_2, but the obtained finding requires further validation as the data were from an individual experiment.

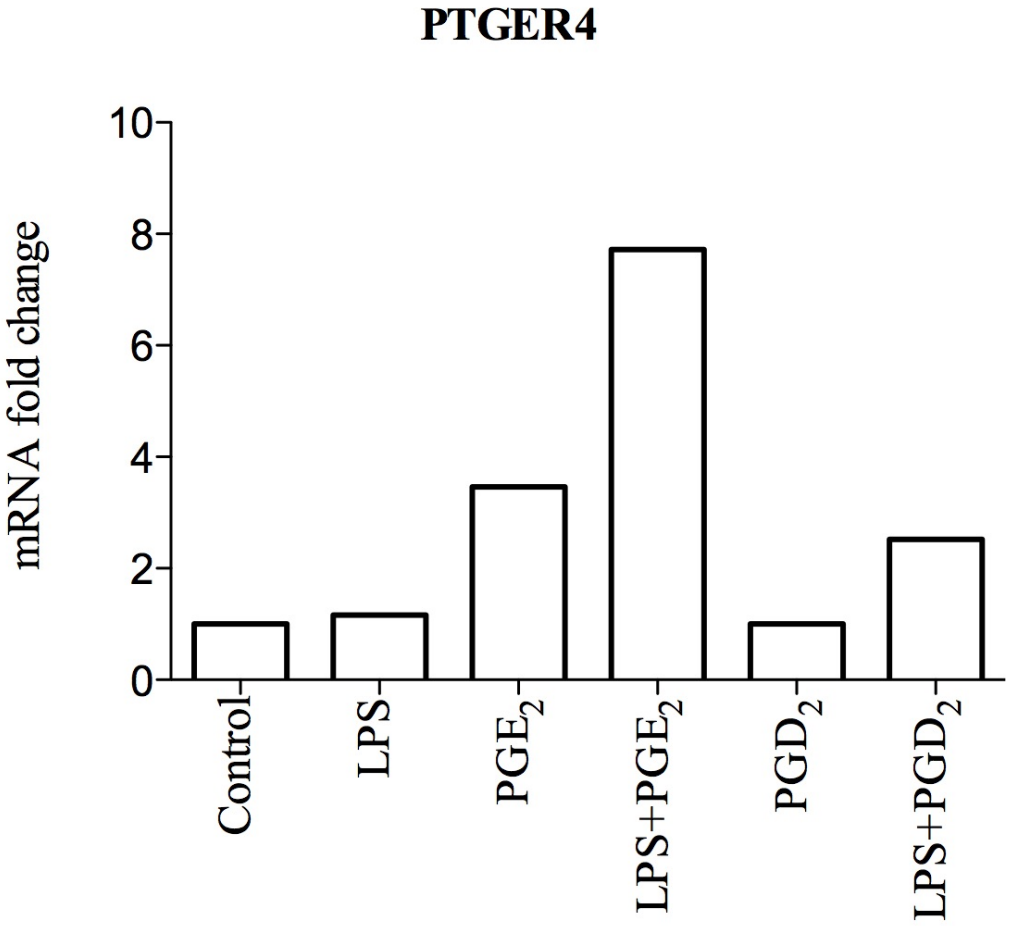


Fig. 3.35: The effect of PGE_2 and PGD_2 on PTGER4 expression in monocytic cells.

Cells ($2.5x10^6$ cells/ml) were placed into 24-well plates after which LPS (10 μg/ml), PGE_2 (1 μM), PGD_2 (1 μM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGER4 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER4 mRNA transcripts were normalised to the reference gene GAPDH using the delta-delta Ct method (Livak and Schmittgen, 2001). The figure represents the data from one individual experiment.

3.7.6 Expression of PTGER2, PTGER4, PRGDR1 and PTGDR2 in mononuclear cells

Mononuclear cells were incubated and processed as described in the Methods Section 2.2.17.1. The results using mixed mononuclear cells were obtained from one individual experiment and can only give an indication of the level of expression of theses receptors because of too few observations. This is because the major parts of this thesis focus intensively on THP-1 cells, but it would be interesting to study the EP receptor expression in mononuclear cells in future.

These initial findings suggest that LPS can increase PTGER2 and PTGER4 mRNA expression. Treatment of mixed mononuclear cells with PGE_2 may decrease their PTGER2 expression in response to LPS stimulation (Fig. 3.36). In contrast, the indicative data for PTGER4 expression shows that the receptor expression may not be affected by PGE_2 in response to LPS (Fig. 3.37). All these observations need further experimentation to clarify this interesting observation.

On the other hand, stimulation of mononuclear cells with LPS resulted in a higher expression in both PTGDR1 and PTGDR2 than in EP receptors but the significance of this elevated level cannot be measured as the data were obtained from one experiment as EP receptor's data in mononuclear cells. Another clue can be found in the expression of PTGDR1 and PTGDR2 is that PGD_2 can decrease the extent of LPS up-regulation. Interestingly, the mRNA's of both PTGDR1 and PTGDR2 were expressed in mixed mononuclear cells, unlike monocytes that did not show DP

receptor expression (Fig. 3.38 and Fig. 3.39). This suggests that monocytes may do not express DP receptors whereas other cells can involve in this expression.

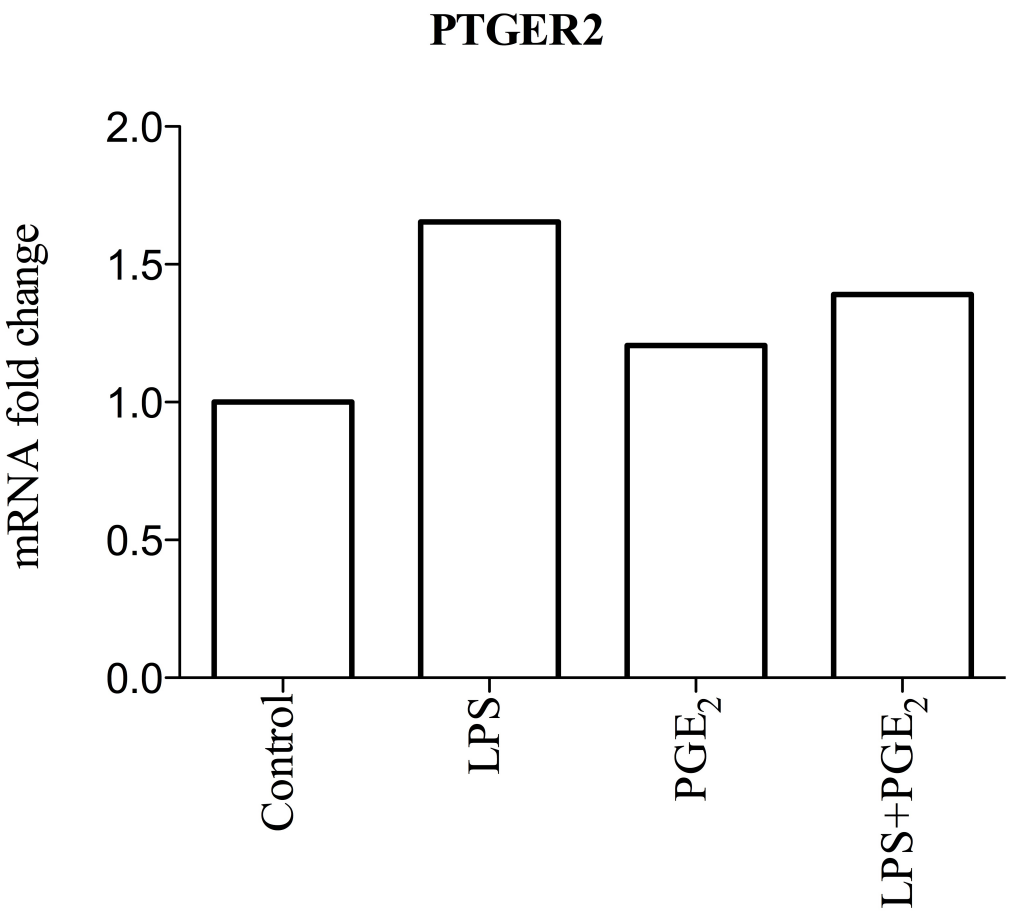


Fig. 3.36: The effect of PGE_2 on PTGER2 expression in mixed mononuclear cells.

Cells ($2.77x10^8$ cells/ml) were placed into 24-well plates after which LPS (10 μg/ml), PGE_2 (1 μM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGER2 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER2 mRNA transcripts were normalised to the reference gene GAPDH using the delta-delta Ct method (Livak and Schmittgen, 2001). The figure represents the data from one individual experiment.

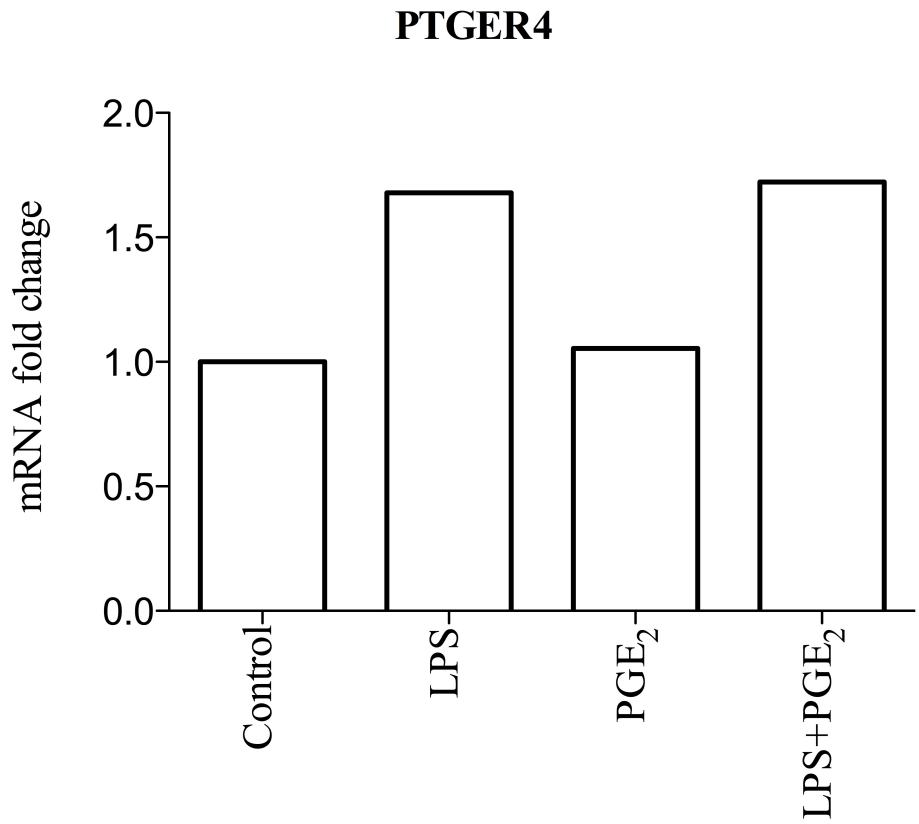


Fig. 3.37: The effect of PGE_2 on PTGER4 expression in mixed mononuclear cells.

Cells ($2.77x10^8$ cells/ml) were placed into 24-well plates after which LPS (10 μg/ml), PGE_2 (1 μM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGER4 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER4 mRNA transcripts were normalised to the reference gene GAPDH using the delta-delta Ct method (Livak and Schmittgen, 2001). The figure represents the data from one individual experiment.

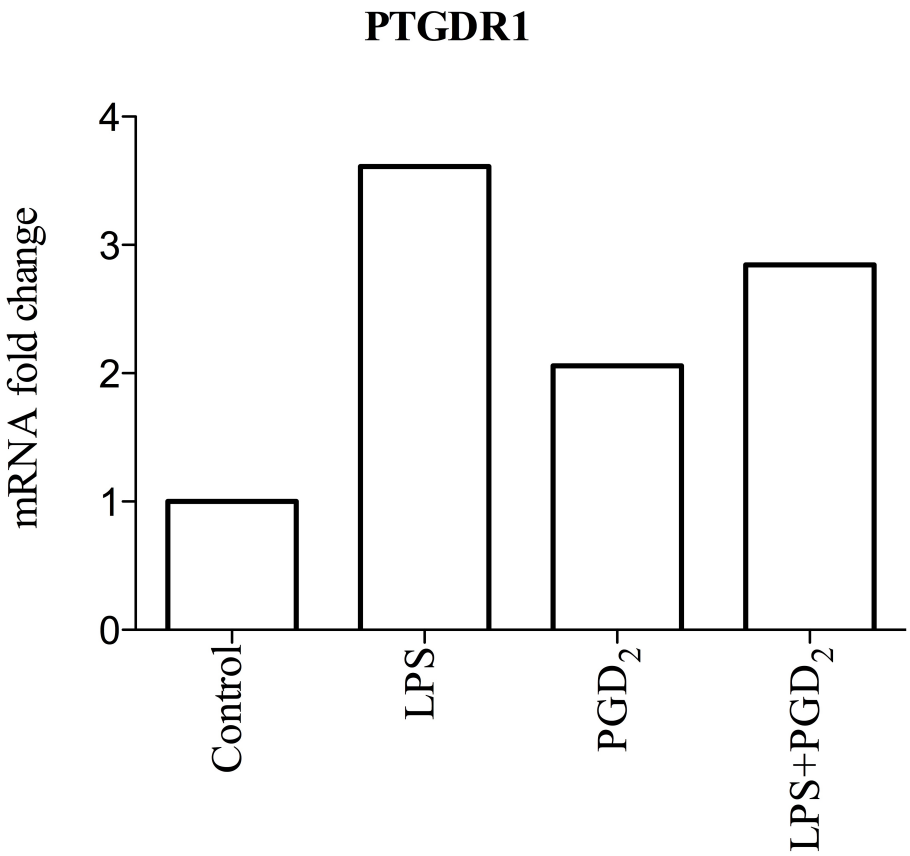


Fig. 3.38: The effect of PGD_2 on PTGDR1 expression in mixed mononuclear cells.

Cells ($2.77x10^8$ cells/ml) were placed into 24-well plates after which LPS (10 μg/ml), PGD_2 (1 μM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGDR1 as described in Methods Section 2.2.17.10. Relative expression levels of PTGDR1 mRNA transcripts were normalised to the reference gene GAPDH using the delta-delta Ct method (Livak and Schmittgen, 2001). The figure represents the data from one individual experiment.

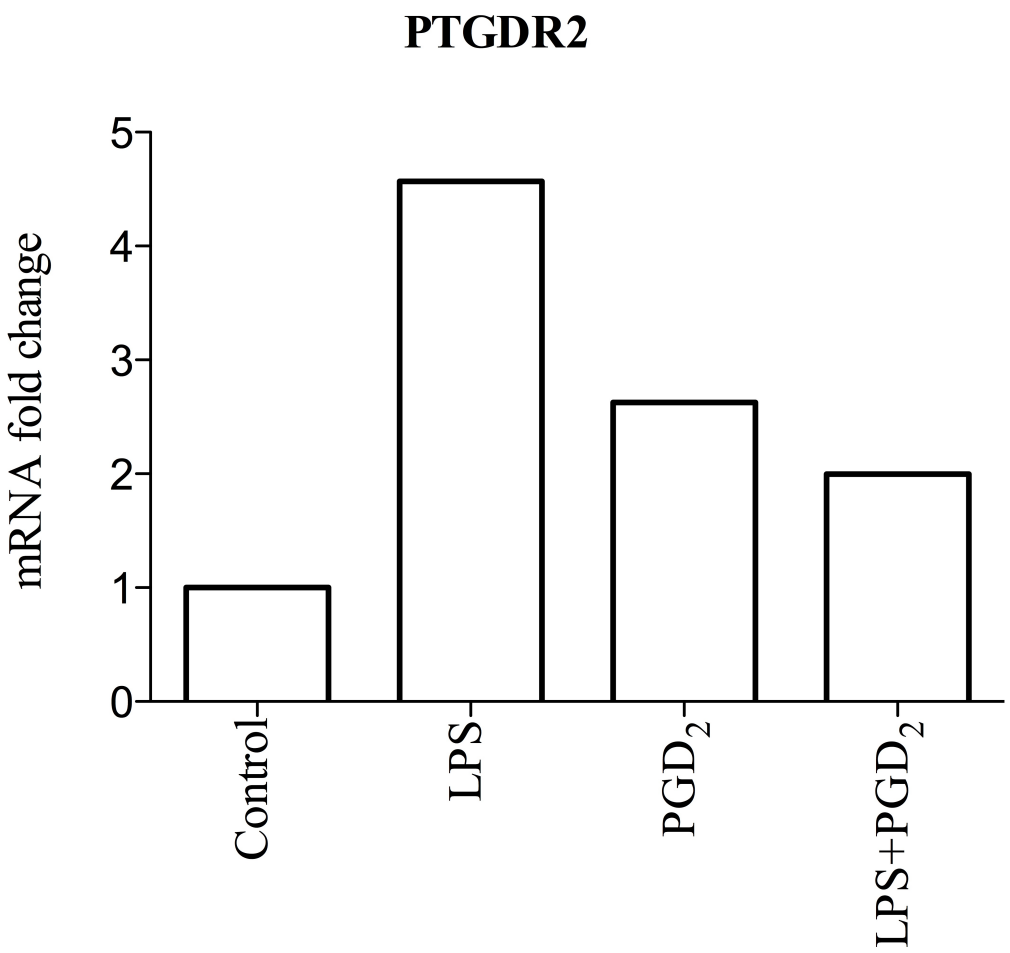


Fig. 3.39: The effect of PGD_2 on PTGDR2 expression in mixed mononuclear cells.

Cells ($2.77x10^8$ cells/ml) were placed into 24-well plates after which LPS (10 μg/ml), PGD_2 (1 μM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGDR2 as described in Methods Section 2.2.17.10. Relative expression levels of PTGDR2 mRNA transcripts were normalised to the reference gene GAPDH using the delta-delta Ct method (Livak and Schmittgen, 2001). The figure represents the data from one individual experiment.

3.8 Silencing EP4 receptors in THP-1 cells by knock-down of PTGER4 expression

3.8.1 The effect of siRNA on THP-1 cell viability

In order to determine the effect of siRNA duplexes on THP-1 cell viability of LPS-stimulated THP-1 cells, 2 different methods were assessed as described previously in Methods Section 2.2.4. The first method estimates non-viable cells (Trypan blue exclusion). The number and viability of THP-1 cells incubated with different treatments (PTGER4 siRNA, siRNA positive control, siRNA negative control 1, siRNA negative control 5 duplexes and HiPerFect only) ranged between 95% and 98% as measured by trypan blue exclusion. Addition of LPS did not reduce the viability below 95% (Table 3.5).

The second method measures total viable cells (MTT assay). The total number of viable cells in control incubations (untreated cells) was adjusted to 100 ± 1.5%. The viable cells in the presence of siRNA, positive control, negative control 1 or negative control 5 (all at100 nM) were 108 ± 0.05%, 103 ± 0.02%, 100 ± 2.5% and 110 ± 0.03% respectively. Transfection of cells using HiPerFect did not decrease the cell viability (there were 100 ± 1.2% viable cells). Dead cells (boiled cells) were used as a control (there were 0 ± 0.001% viable cells). The control cells (untreated cells) yielded a mean absorbance of 1.259 ± 0.034. The mean absorbance of cells treated with siRNA, positive control, negative control 1 or negative control 5 (all at100 nM) were 1.318 ± 0.016, 1.213± 0.029, 1.26 ± 0.01 and 1.412 ± 0.044 respectively. Mean absorbance of cells transfected with HiPerFect alone was 1.225 ± 0.019 and mean absorbance of dead cells was 0.135 ± 0.001 (Table 3.6). In some samples (such as

incubation with siRNA, positive control and negative control 5), MTT assay results show cell viability of more than 100%. This could be due to an increase in the mitochondrial enzymatic activity since MTT assay depends on a mitochondrial reductase in order to convert the tetrazole to formazan. Another possibility is a too long incubation of cells with MTT solution, but this was less likely in the current project as the incubation time for all samples with MTT solution was between 4-6 hours. For this reason, it was preferable to perform MTT experiment along with simple cell counts (Trypan blue exclusion assay) to complement the observations (Twentyman & Luscombe, 1987; Sylvester, 2011; van Meerloo *et al.*, 2011).

Table 3.5: The percentage of the total viable THP-1 cells in knock-down experiments using Trypan blue exclusion.

Treatment	Viable cells (%)
Control (untreated cells)	100 ± 0.08%
siRNA	98 ± 1.9%
Positive control	96 ± 0.03%
Negative control 1	97 ± 0.001%
Negative control 5	95 ± 1.4%
HiPerFect	97 ± 0.05%
LPS	95 ± 0.09%
Dead cells	0 ± 0.001%

THP-1 cell viability ($1x10^6$ cells/ml) was measured by trypan blue exclusion (estimation of non-viable cells). Trypan blue was added to cells immediately before counting (using a haemocytometer) and the percentage of blue cells was subtracted from the total cell count (Methods Section 2.2.4). All values are the means of n = 3 ± s.d (three separate observations), $P < 0.05$ by ANOVA.

Table 3.6: The percentage of total viable THP-1 cells in knock-down experiments using MTT assay.

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%

THP-1 cells ($1x10^6$ cells/ml) were placed into 96-well plates after which siRNA, positive control, negative control 1 or negative control 5 (all at100 nM), control cells (untreated cells), dead cells (boiled cells) and HiPerFect (3 µl) were added. Plates were incubated at 37°C, 5% CO_2 for 24h. Treatments were removed from wells and replaced with MTT solution and left for 4 h in incubator at 37°C, 5% CO_2 and lysed with DMSO (Methods Section 2.2.4). All values are the means of n = 3 ± s.d (three separate observations), $P < 0.05$ by ANOVA.

3.8.2 Time course of the effect of siRNA (duplex 3) on PTGER4 expression in THP-1 cells

A series of experiments were carried out to determine the time course of the actions of siRNA on the expression of EP4 receptors. PTGER4 expression mRNA was studied between 3 hours and 48 hours after transfection with 100 nM siRNA (Fig. 3.40).

Fig. 3.40 (A) shows transfection of THP-1 cells with siRNA duplex 3 for 3 hours in the presence of LPS (10 μg/ml). At this time point, there was no knockdown observed in PTGER4 expression using siRNA duplex 3 compared to non-transfected cells, suggesting that 3 hours siRNA transfection was insufficient for PTGER4 down-regulation. Fold change of PTGER4 in response to LPS was 2.04 fold ± 0.56 before siRNA duplex 3 transfection and 2.02 fold ± 0.05 after 3h of siRNA transfection. A down-regulation of 4.4 fold ± 0.03 of PTGER4 in LPS-stimulated cells in the presence of PGE_2 was observed before transfection and 2.35 fold ± 0.03 after 3h transfection.

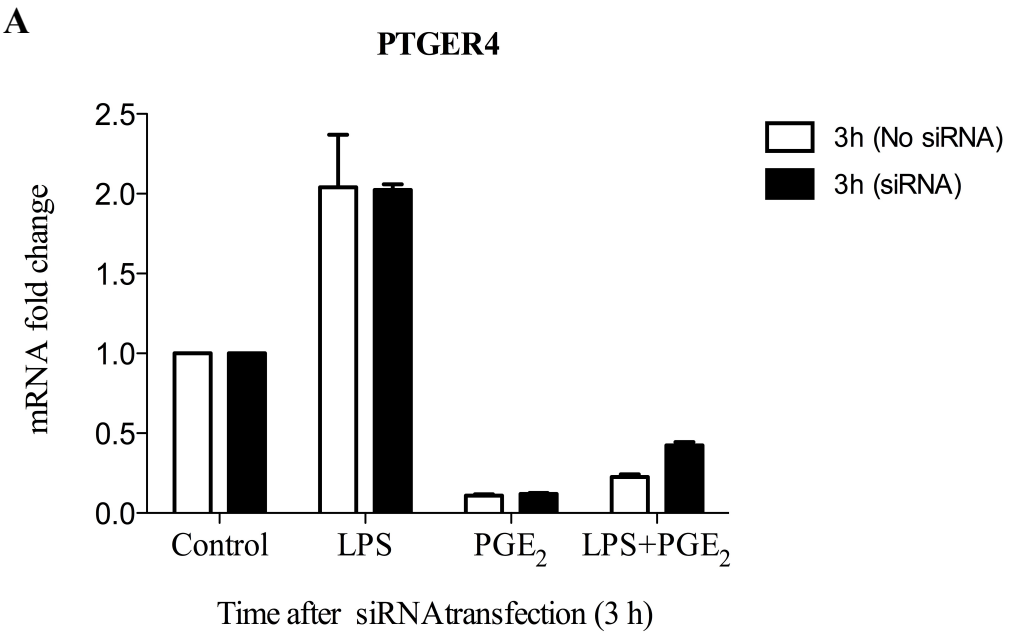
After 6 hours transfection, siRNA induced a significant reduction in the PTGER4 expression level in response to LPS from 3.97 fold ± 0.11 (non-transfected cells) to 3.20 fold ± 0.6 (transfected cells) $P < 0.001$. However, there was no difference in the expression level in the presence of LPS/PGE_2 after siRNA transfection (Fig. 3.40 (B)).

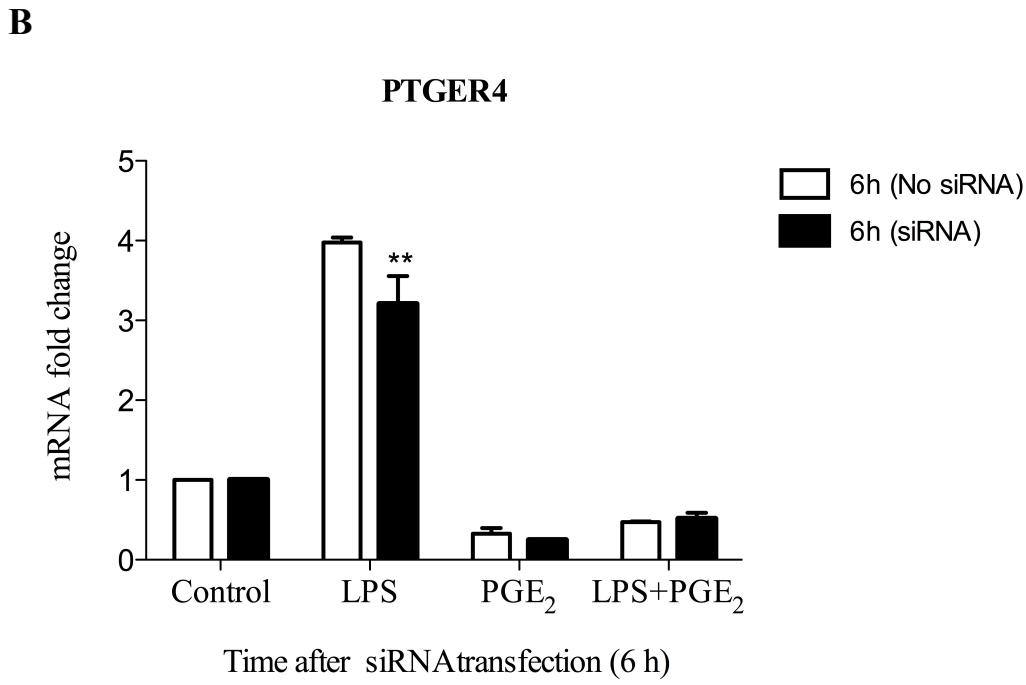
A similar knockdown pattern was observed at 12 hours post-transfection, mRNA of PTGER4 response was reduced significantly from 3.11 fold ± 0.33 up-regulation to 2.35 fold ± 0.43 up-regulation in the presence of LPS ($P < 0.001$). At the same time point (12 h), there was a 2.61 fold ± 0.13 decline in PTGER4 expression in response to LPS/PGE_2 compared to non-transfected cells ($P < 0.05$) (Fig. 3.40 (C)).

At the 24 hours of siRNA transfection, there was a substantial fold change in PTGER4 expression in response to LPS from 3.15 fold ± 1.11 up-regulation with non-transfected cells to a 1.07 fold ± 0.14 up-regulation with transfected cells ($P<0.0001$). While PTGER4 expression response to LPS/PGE_2 changed significantly from 1.02 fold ± 0.21 down-regulation to a 50 fold ± 0.002 down-regulation compared to non-transfected THP-1 cells ($P < 0.05$) (Fig. 3.40 (D)).

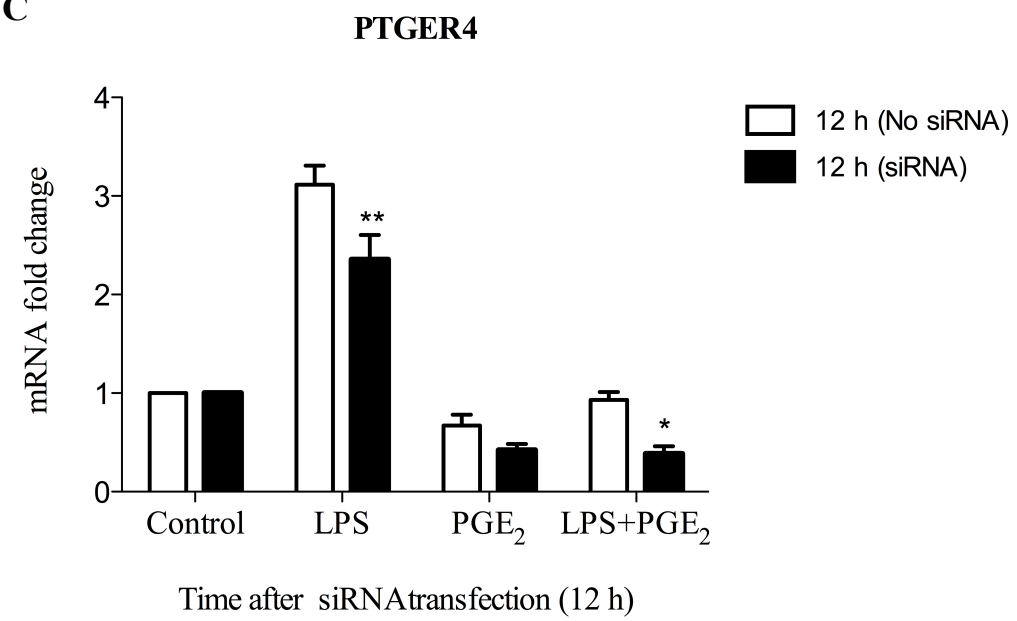
There was also a much larger PTGER4 reduction at 48 h post-transfection. A significant 3.37 fold ± 0.08 decrease occurred in mRNA of EP4 receptor with LPS-stimulated transfected cells ($P < 0.0001$). In addition, the PTGER4 expression level was knocked-down by 2.16 fold ± 0.09 ($P < 0.05$) in the presence of LPS/PGE_2 (Fig. 3.40 (E)).

Among these different time courses of siRNA transfection, the greatest PTGER4 knockdown level was observed at 24 hours and 48 hours post-transfection with the greatest effect at 48 hours.

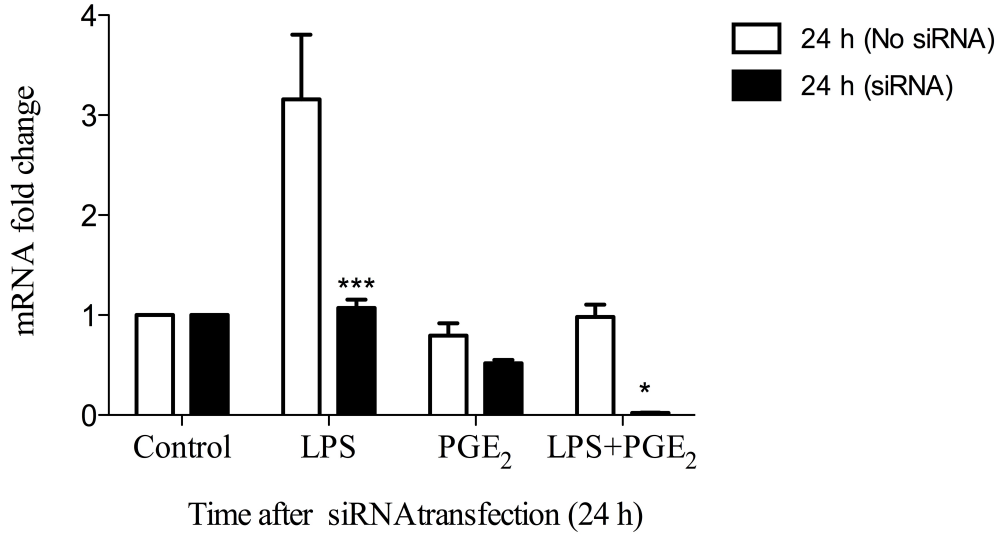

A
PTGER4
mRNA fold change
2.5
2.0
1.5
1.0
0.5
0.0
Control
LPS
PGE2
LPS+PGE2
3h (No siRNA)
3h (siRNA)
Time after siRNAtransfection (3 h)


B
PTGER4
mRNA fold change
5
4
3
2
1
0
**
Control
LPS
PGE2
LPS+PGE2
6h (No siRNA)
6h (siRNA)
Time after siRNAtransfection (6 h)

C


PTGER4
mRNA fold change
4
3
2
1
0
**
*
12 h (No siRNA)
12 h (siRNA)
Control
LPS
PGE2
LPS+PGE2
Time after siRNAtransfection (12 h)

D


PTGER4
mRNA fold change
4
3
2
1
0
***
*
24 h (No siRNA)
24 h (siRNA)
Control
LPS
PGE2
LPS+PGE2
Time after siRNAtransfection (24 h)

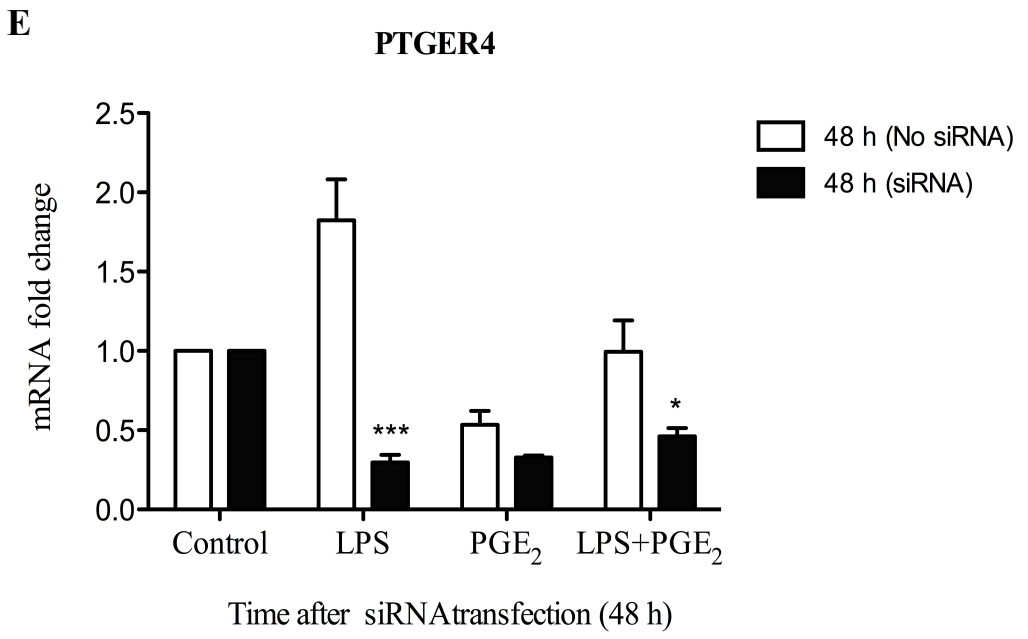


Fig. 3.40: Time course of the effect of siRNA on PTGER4 expression in THP-1 cells.

Cells ($3x10^5$ cells/ml) were placed into 6-well plates after which LPS (10 μg/ml), PGE_2 (1 μM) and culture medium (control) were added and cells were transfected with siRNA duplex 3 (100 nM) using HiPerFect. The plates were incubated for the indicated periods of time. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using primers to detect PTGER4 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER4 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak & Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). *P < 0.05, **P < 0.001 and ***P < 0.0001versus non-transfected cells. A) The effect of 3h siRNA transfection on PTGER4 expression. B) The effect of 6h siRNA transfection on PTGER4 expression. C) The effect of 12h siRNA transfection on PTGER4 expression. D) The effect of 24h siRNA transfection on PTGER4 expression. E) The effect of 48h siRNA transfection on PTGER4 expression.

3.8.3 Optimising PTGER4 gene silencing using three different siRNA duplexes

Synthetic siRNA can mimic the natural products of dicer in mammalian cells (Elbashir *et al.*, 2001a). SiRNA optimisation would require trying more than one duplex targeted at the gene of interest (PTGER4)(Mocellin & Provenzano, 2004; Huppi *et al.*, 2005). Since the TriFECTa RNA kit (specific for PTGER4 knockdown) provided 3 different siRNA duplexes, these duplexes were required to be validated by determination of the expression levels of the selected gene (PTGER4) by qRT-PCR. THP-1 cells were treated with LPS (10 μg/ml) and transfected with 3 siRNA duplexes (100 nM) as described in Methods Section 2.2.3. Quantitative RT-PCR was performed after harvesting cells (Methods Section 2.2.17.1) to ascertain any changes in gene expression. The results were normalised using the reference gene PPIB and gene expression changes determined using the ΔΔCT method (Methods Section 2.2.17.8). Values are expressed as relative fold change (FC) of stimulated over control (untreated samples).

Fig. 3.41 shows the expression of PTGER4 in the presence and absence of siRNA complexes. In the absence of siRNA, there was a 2.77 fold ± 0.39 up-regulation of PTGER4 after LPS activation in three separate expreiments. However, treatment of THP-1 cells with siRNA duplex 1 significantly reduced the PTGER4 expression response to LPS stimulation (PTGER4 expression fold changed from 2.77 fold ± 0.39 up-regulation with LPS alone to a 1.30 fold ± 0.35 down-regulation with siRNA duplex 1, $P< 0.0001$). THP-1 PTGER4 expression response to LPS significantly changed from 2.77 fold ± 0.39 up-regulation to a 14.92 fold ± 0.04 down-regulation

following siRNA duplex 3 treatment, $P< 0.0001$. This indicated that both of siRNA duplex1 and duplex 3 were able to down-regulate the expression of the EP4 receptor. In addition to these two siRNA complexes, another siRNA (duplex 2) was also transfected to cells, but it gave an undetermined CT value for PTGER4 expression (Data not shown because the expression was below the level of detection of the assay). Fig. 3.41 clarifies that PTGER4 expression in response to HiPerFect was close to control (fold change of 1.36 ± 0.57), this indicated that this transfection reagent did not significantly affect the expression level.

The findings demonstrated that siRNA duplexes for PTGER4 provided different levels of down-regulation. SiRNA duplex 2 abolished the EP4 receptor whereas siRNA duplex 1 and duplex 3 induced a significant knock-down in the expression of PTGER4 rather than receptor abolishment. In particular, siRNA duplex 3 was able to accurately quantify the knock-down response. Therefore, siRNA (duplex 3) was chosen to be used in all knock-down experiments as an efficient complex that induced a successful PTGER4 knock-down.

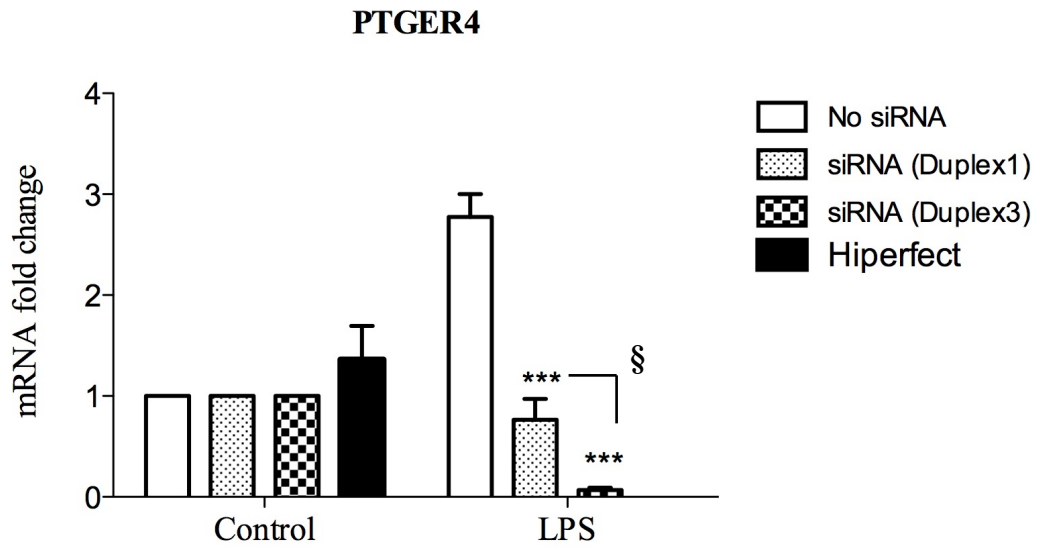


Fig. 3.41: siRNA duplexes knockdown the expression of PTGER4 in THP-1 cells.

Cells ($1x10^6$ cells/ml) were placed into 6-well plates after which either LPS (10 μg/ml) or culture medium (control) was added and cells were transfected with siRNA (100 nM) using HiPerFect. The plates were incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers to detect PTGER4 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER4 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). ***P < 0.0001 versus LPS alone in non-transfected cells. § P < 0.01 versus LPS in cells transfected with siRNA (Duplex 1).

3.8.4 Optimising PTGER4 knock-down efficiency using different concentrations of HiPerFect

After confirming that siRNA (duplex 3) was able to induce a significant knock-down in PTGER4 gene in the previous result (Fig. 3.41), various volumes of HiPerFect were used (3µl, 6µl and 9µl) during transfection of cells with siRNA (duplex 3) following Qiagen's HiPerFect optimisation guidelines. It was investigated whether siRNA with 3µl, 6µl and 9µl of HiPerFect induced the same significant level of PTGER4 knock-down after LPS stimulation (Fig. 3.42). PTGER4 expression fold reduced significantly from 4.34 fold ± 0.14 up-regulation with LPS alone to a 1.76 fold ± 0.39 up-regulation with 3µl of HiPerFect ($P < 0.0001$). There was also a significant reduction in PTGER4 expression in response to LPS from 4.34 fold ± 0.14 up-regulation to a 1.18 fold ± 0.01 up-regulation using 6µl of HiPerFect ($P < 0.0001$). Using 9µl of HiPerFect, PTGER4 expression response to LPS changed significantly from 4.34 fold ± 0.14 up-regulation to 1.8 fold ± 0.3 down-regulation ($P < 0.0001$). The effect of different concentrations of HiPerFect (3µl, 6µl and 9µl) on PTGER4 expression was also demonstrated after treatment of LPS stimulated cells with PGE_2. There was a 1.18 fold ± 0.01 up-regulation of PTGER4 in response to LPS+ PGE_2 in the absence of siRNA. However, transfection THP-1 cells with siRNA duplex 3 using HiPerFect (3µl, 6µl and 9µl) reduced the PTGER4 expression response to LPS+ PGE_2 stimulation to a 1.25 fold ± 0.18, 3.69 fold ± 0.02, and to a 1.22 fold ± 0.6 down-regulation respectively). Since 3µl of HiPerFect with siRNA effectively knocked-down the EP4 receptor with a significant decrease, it was suggested to use this volume of HiPerFect (3µl) in all subsequent knock-down assays.

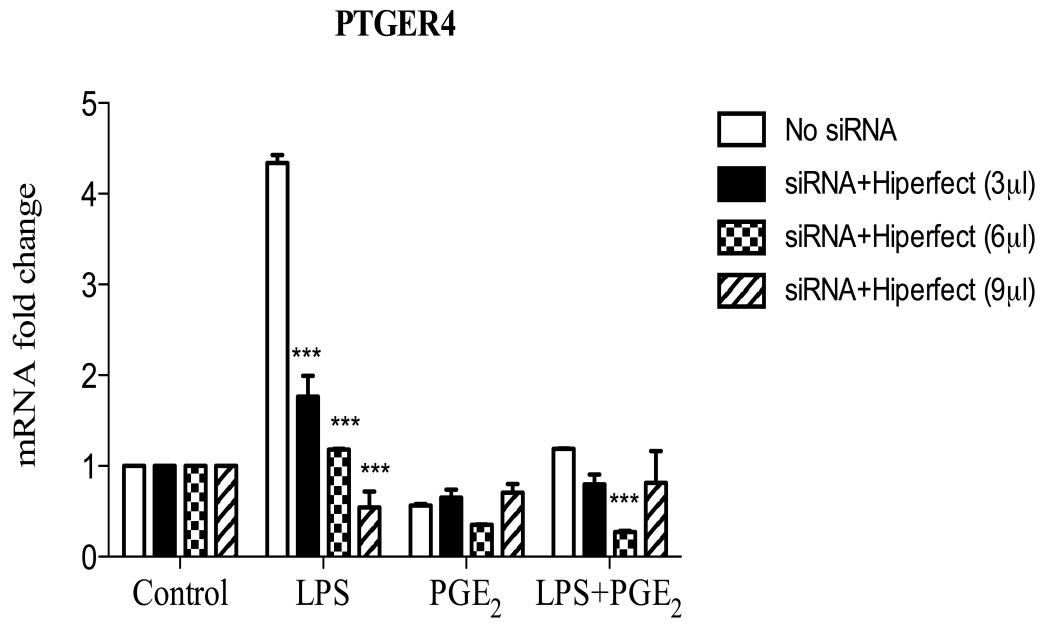


Fig. 3.42: The effect of different concentrations of HiPerFect on knockdown the expression of PTGER4 in THP-1 cells.

Cells ($1x10^6$ cells/ml) were placed into 6-well plates after which LPS (10 μg/ml), PGE_2 (1 μM), culture medium (control) were added and cells were transfected with siRNA duplex 3 (100 nM) using different volumes of HiPerFect (3μl, 6μl and 9μl). The plates were incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers to detect PTGER4 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER4 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). ***P < 0.0001 versus non-transfected cells.

3.8.5 Optimising PTGER4 knock-down efficiency using three different concentrations of siRNA (duplex 3)

Previous results suggested that siRNA duplex 3 was able to silence PTGER4 mRNA efficiently (Fig. 3.41). In order to determine the most potent concentration of this siRNA, THP-1 cells were transfected with 3 different concentrations (1 nM, 10 nM, and 100 nM).

The following graph (Fig. 3.43) shows that at 1 nM, siRNA was unable to reduce the level of PTGER4 expression compared to non-transfected cells. In response to LPS, the fold change in non-transfected cells was 2.69 fold ± 0.13 up-regulation and transfected cells with 1 nM of siRNA resulted in a 3.20 fold ± 0.44 up-regulation. However, both higher siRNA concentrations (10 nM and 100 nM) significantly reduced the extent of LPS up-regulation of PTGER4 expression from 2.69 fold ± 0.13 up-regulation in the absence of siRNA to a 1.76 fold ± 0.39 up-regulation with 10 nM siRNA ($P < 0.01$) and to a 1.57 fold ± 0.17 up-regulation with 100 nM siRNA ($P< 0.001$). The effect of different concentrations of siRNA (1 nM, 10 nM and 100 nM) had a similar effect on PTGER4 expression in LPS-stimulated cells in the presence of PGE_2. There was 2.78 fold ± 0.35 up-regulation of PTGER4 expression with LPS+ PGE_2 in the absence of siRNA. However, at 100 nM of siRNA, the expression fold changed significantly to a 1.63 fold ± 0.1 up-regulation in response to LPS+ PGE_2 stimulation ($P < 0.001$). At 10 nM, there was a significant down-regulation of PTGER4 expression by 1.25 fold ± 0.18 with LPS+ PGE_2 ($P < 0.0001$). At 1 nM, there was 2.63 fold ± 0.54 up-regulation in response to LPS+ PGE_2

stimulation. Hence, 100 nM of siRNA was used for optimum silencing of PTGER4 expression with duplex 3.

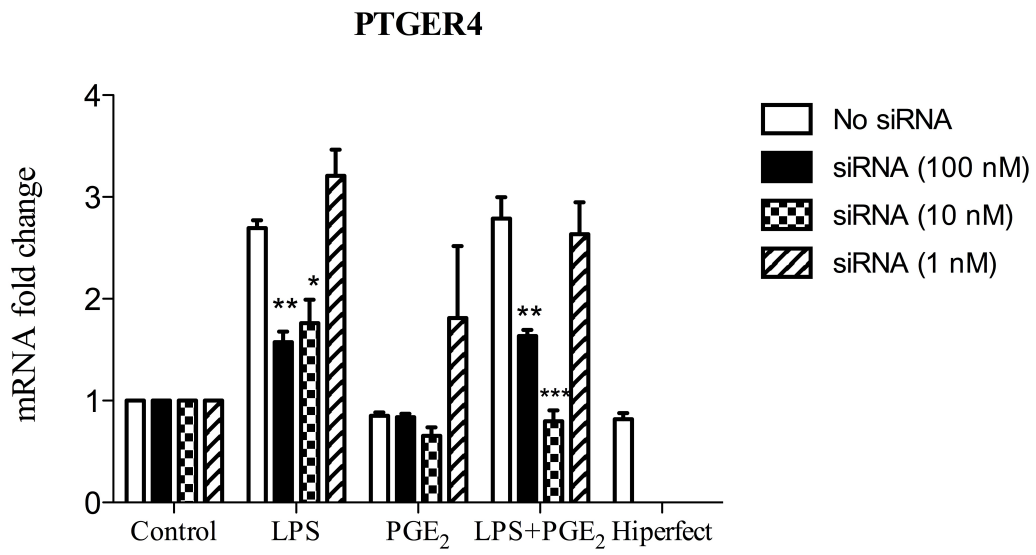


Fig. 3.43: The effect of different concentrations of siRNA duplex 3 on knockdown the expression of PTGER4 in THP-1.

Cells ($3x10^5$ cells/ml) were placed into 6-well plates after which LPS (10 μg/ml), PGE_2 (1μM), culture medium (control) and HiPerFect alone were added and cells were transfected with different concentrations of siRNA duplex 3 (1nM, 10 nM and 100 nM) using HiPerFect. The plates were incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers to detect PTGER4 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER4 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations).*P < 0.01, **P < 0.001 and ***P < 0.0001 versus non-transfected cells.

3.8.6 The effect of both positive and negative controls of siRNA duplexes on PTGER4 expression in THP-1 cells

Fig 3.44 shows that in the absence of siRNA, PTGER4 is expressed by 14.353 fold ± 2.882 up-regulation in response to LPS compared to control. This level of expression response was significantly decreased to only a 1.714 fold ± 0.821 up-regulation by siRNA duplex 3 (100 nM)($P<0.0001$), which was obsereved earlier in a similar knock-down experiment (Fig. 3.41). In this study, three control siRNAs were supplied with TriFECTa PTGER4 kit: two of them were siRNA negative controls (NC1 and NC5) (100 nM) and the third one was siRNA positive control (100 nM). Both negative controls induced a significant decrease in PTGER4 expression fold change in response to LPS from 14.353 fold ± 2.882 up-regulation (non-transfected cells) to 7.841 fold ± 0.638 and 6.831 fold ± 0.407 up-regulation (with NC1 and NC5 respectively)($P< 0.0001$). Whereas, the positive control siRNA had the same effect as siRNA duplex 3 in terms of down-regulation of PTGER4. The positive control reduced the expression of PTGER4 from 14.353 fold ± 2.882 up-regulation (non-transfected cells) to a 2.343 fold ± 0.922 up-regulation (with positive C) in response to LPS ($P<0.0001$) (Fig. 3.44).

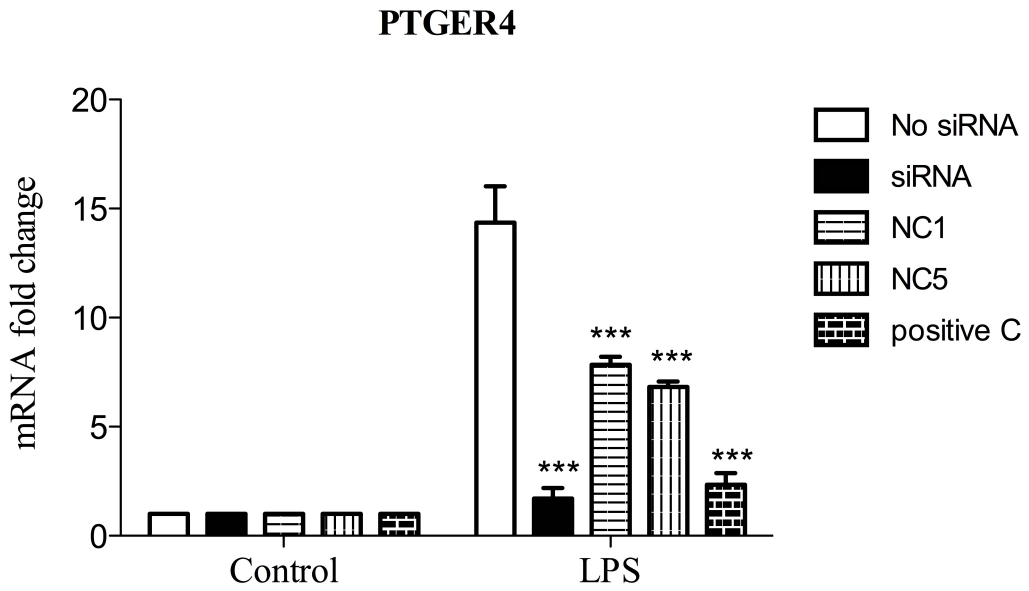


Fig. 3.44: The effect of positive and negative controls of siRNA duplexes on PTGER4 expression in THP-1 cells.

Cells ($1x10^6$ cells/ml) were placed into 6-well plates after which either LPS (10 μg/ml) or culture medium (control) was added and cells were transfected with (siRNA duplex 3, positive control, negative control 1 (NC1) and negative control 5 (NC5) (all at 100 nM)) using HiPerFect. The plates were incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using primers to detect PTGER4 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER4 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak & Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). ***P < 0.0001versus LPS alone in non-transfected cells.

3.8.7 TNF-α production using three different siRNA duplexes in THP-1 cells

As siRNA duplexes were demonstrated previously during the PTGER4 expression study, it was also important to demonstrate the effect of these siRNA complexes with respect to their ability to modulate TNF-α production in order to assess their functional capabilities. THP-1 cells transfected with different siRNA duplexes (duplex 1, 2 and 3) showed a significant increase in the level of TNF-α following LPS stimulation compared with non-transfected cells, $P < 0.01$. The production of TNF-α also increased significantly after cells were transfected with the positive control siRNA, $P < 0.01$. Transfection with the negative control 1 siRNA (NC1) induced a small increase in TNF-α production. There were no noticeable differences between the three PTGER4 siRNA duplexes during the study of TNF-α production. It was decided to choose PTGER4 siRNA duplex 3 in subsequent experiments (Fig. 3.45).

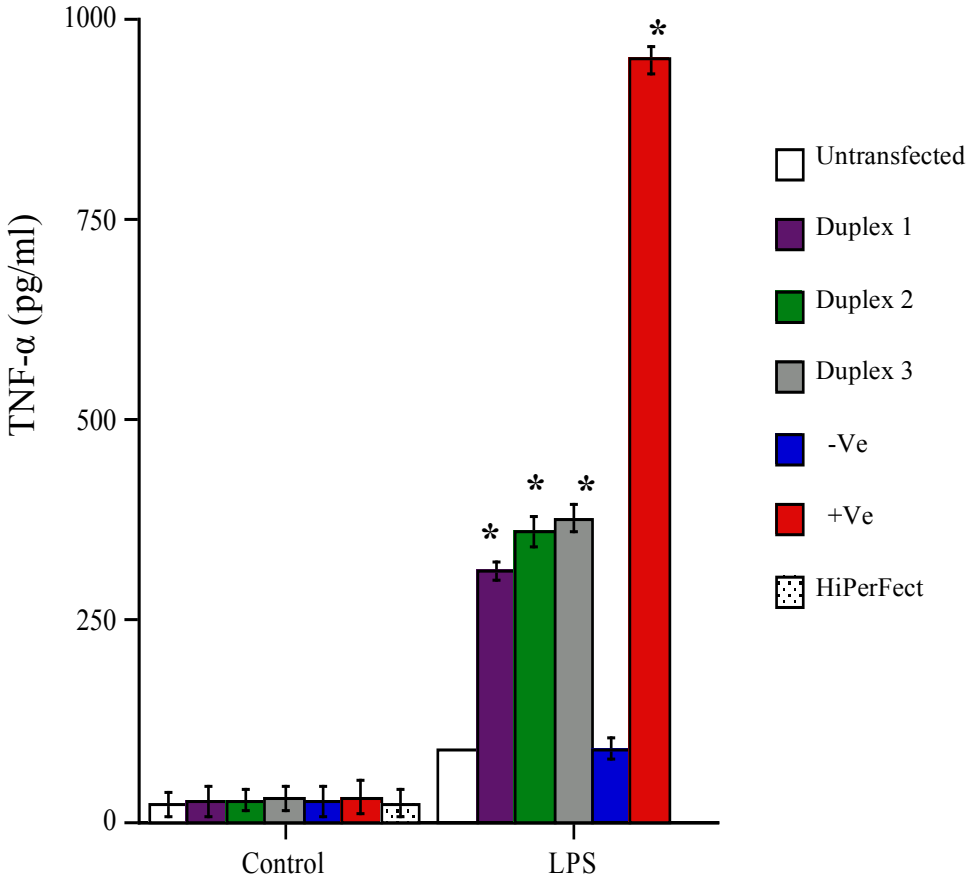


Fig. 3.45: The effect of different siRNA duplexes on TNF-α production in THP-1 cells.

Cells ($1x10^6$ cells/ml) were placed into 6-well plates after which LPS (10 μg/ml), culture medium (control) and HiPerFect alone were added and cells were transfected with (siRNA duplexes, positive control and negative control 1 (NC1)(all at 100 nM)) using HiPerFect. The plates were incubated for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS for untransfected cells.

3.8.8 Time course of the effect of PTGER4 siRNA on TNF-α production in THP-1 cells

Having established that the mRNA level for PTGER4 was successfully knocked-down by siRNA duplex 3 (100 nM) (Fig. 3.41), further experiments were performed in order to demonstrate the effect of silencing the EP4 receptor on TNF-α production. THP-1 cells were stimulated and transfected as describe in Methods Section 2.2.3. As shown in Fig. 3.46, the production of TNF-α was determined during different time points (0h, 6h, 24h and 48h) following transfection. 0h represented TNF-α production immediately in THP-1 cells that had not been transfected with siRNA. At this time point (0h), in response to LPS, the TNF-α level increased compared to control. However, PGE_2 in the presence of LPS decreased TNF-α production. After 6h siRNA post-transfection, there was no clear difference in TNF-α production compared to 0h (before siRNA transfection) since LPS was added at the same time as siRNA. At 24h of stimulation, in response to LPS, there was an increase in the TNF-α level compared to non-stimulated cells (0h), while TNF-α production did not change in response to LPS/ PGE_2. After 48h of siRNA transfection, the TNF-α level reached the maximal high concentration in response to both LPS and LPS/ PGE_2, TNF-α production increased significantly compared to cells before transfection (0h), $P < 0.01$.

Fig. 3.47 shows the effect of positive and negative controls on TNF-α production with time. Cells stimulated with positive control followed a similar TNF-α production profile as cells transfected with siRNA (Fig. 3.46). Using a negative control (NC1), there was an increase in TNF-α concentration with a different time

course but it was not as large an increase as occurred with the positive control (Fig. 3.47).

In conclusion, previous sections (3.8.1 to 3.8.8) showed results for silencing the EP4 receptor gene and its effect on the production of TNF-α. It was demonstrated that siRNA Duplex 3 (100 nM) provided the best measurable knock-down level in LPS stimulated cells. The most effective time points post-transfection were 24 hours and 48 hours to induce a significant PTGER4 knock-down. TNF-α production was evaluated after silencing PTGER4 and there was a significant increase in TNF-α in the absence of EP4 receptor thereby highlighting its pivotal role in TNF-α regulation.

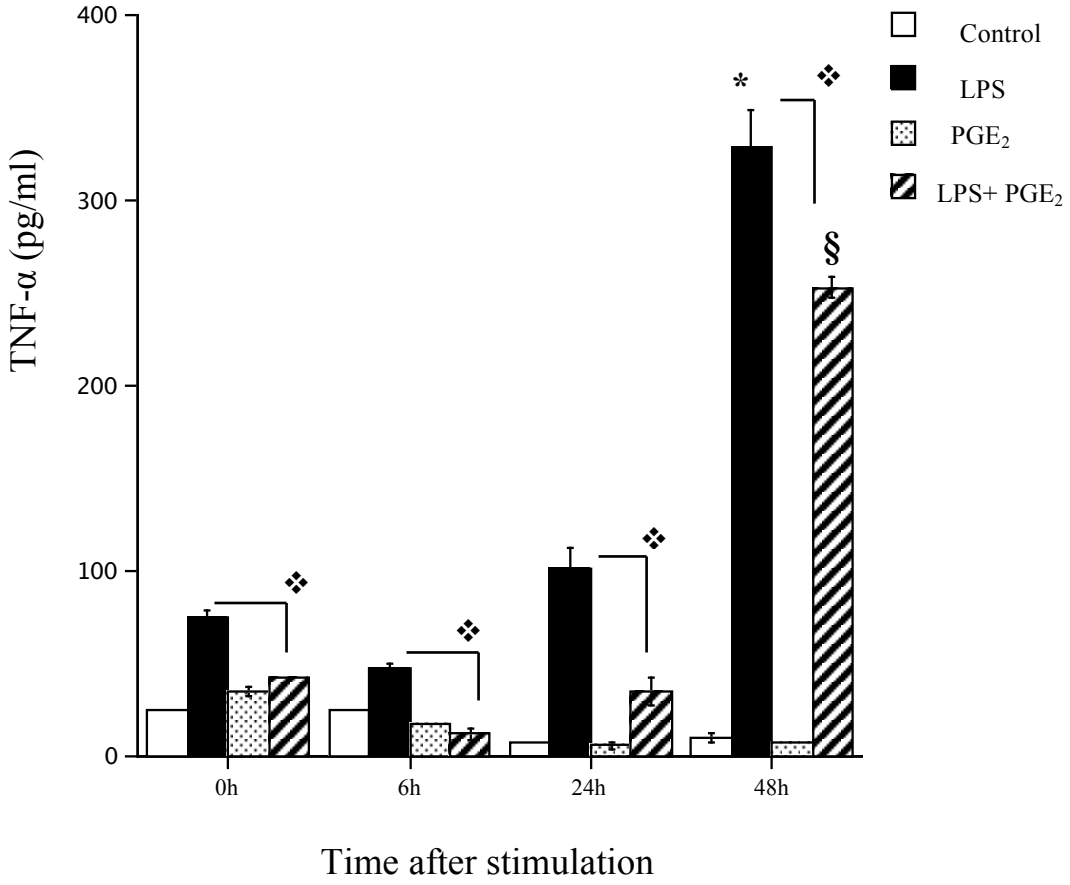


Fig. 3.46: Time course of the effect of siRNA on TNF-α production in THP-1 cells.

Cells ($1x10^6$ cells/ml) were placed into 6-well plates after which LPS (10 μg/ml), PGE_2 (1 μM) and culture medium (control) were added and cells were transfected with siRNA duplexe 3 (100 nM) using HiPerFect. The plates were incubated for 22 h before transfection then transfected with siRNA for various time (6h, 24h and 48h) at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS for untransfected cells, §P< 0.01 versus LPS with PGE_2 for untransfected cells and ❖P< 0.01 versus LPS for transfected/ untransfected cells.

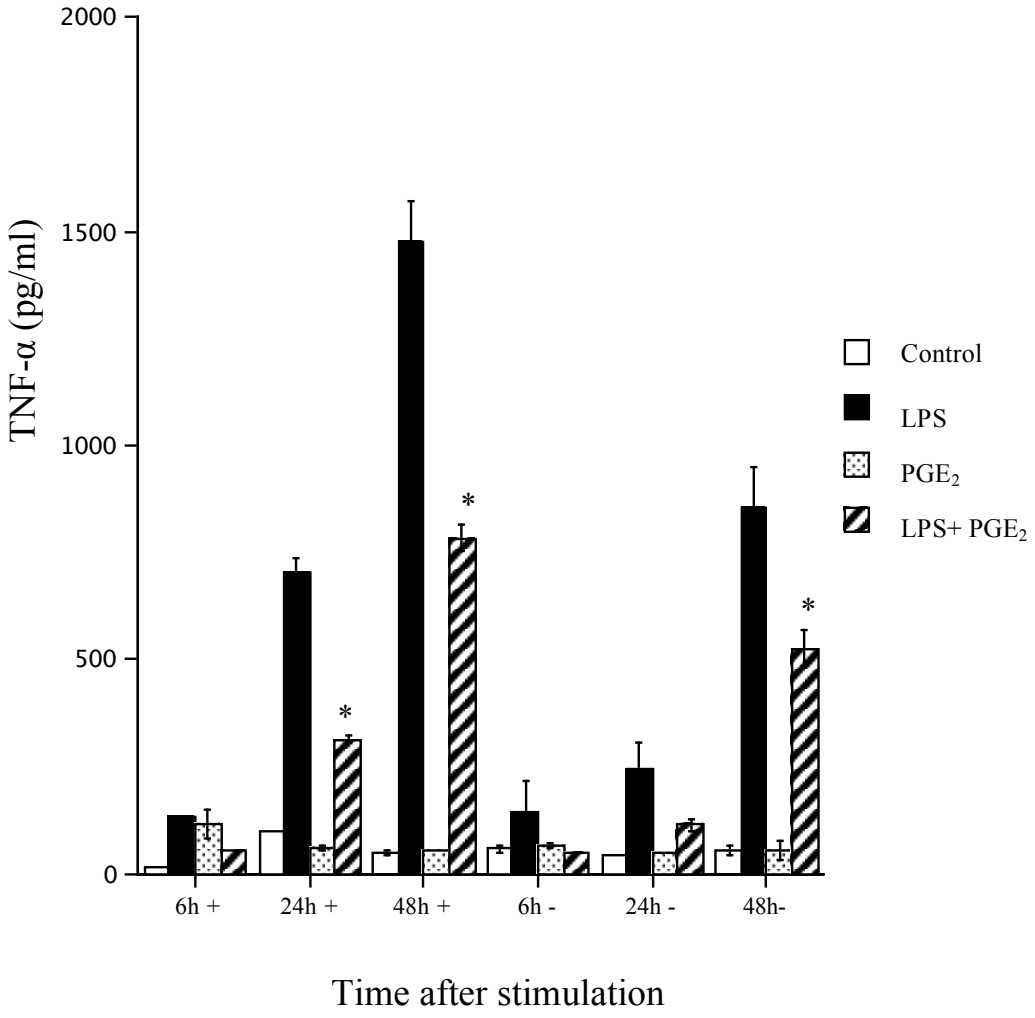


Fig. 3.47: Time course of the effect of positive and negative control of siRNA on TNF-α production in THP-1 cells.

Cells (1x10 ^{6}cells/ml) were placed into 6-well plates after which LPS (10 μg/ml), PGE_2 (1 μM) and culture medium (control) were added and cells were transfected with (siRNA positive and negative control (NC1) duplexes (all at100 nM) using HiPerFect. The plates were incubated for various time (6h, 24h and 48h) at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations), *P < 0.01 versus LPS.

3.8.9 THP-1 cell images after transfection with siRNA

THP-1 cells were transfected and spread onto microscope slides as described in Methods Section 2.2.20.2. Different images of THP-1 cells were taken after incubation with siRNA (50 nM) and (1000 nM) using various magnifications (x100, x200, x600 and x1000) (Fig. 3.48). The purpose of performing this experiment was to demonstrate that THP-1 cells were successfully labelled using siRNA transfection. Interestingly, Fig. 3.48 (B) shows mixed of negative and positive labelled cells. High colour intensity indicates that THP-1 cells were positively labelled with siRNA duplex, whereas pale cells represent negatively siRNA transfection, which suggests that these cells had not been transfected with siRNA. As seen in Fig. 3.48, the majority of THP-1 cells were transfected effectively and were positively labelled.

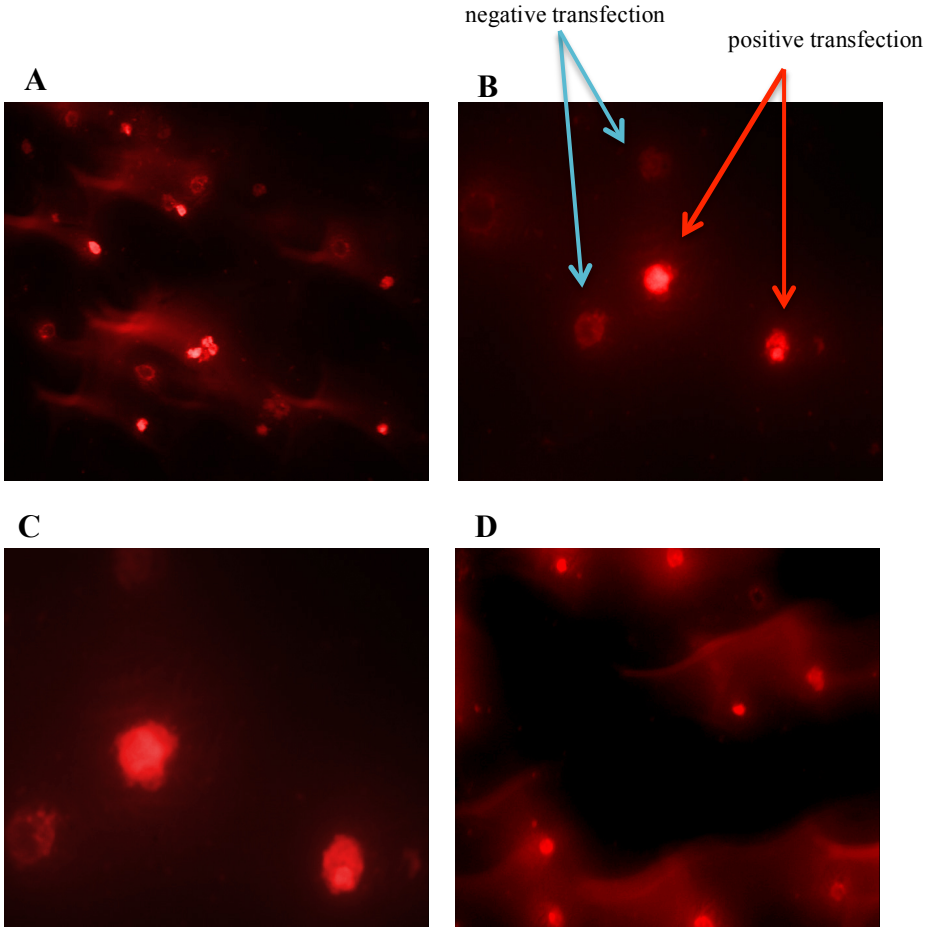


Fig. 3.48: Transfected THP-1 cells images.

Images of negative and positive siRNA transfection of THP-1 cells. THP-1 cells ($1x10^6$ cells/ml) were transfected in 6-well plates with 2 different concentrations of siRNA duplex (1000 nM and 50 nM) and incubated at 37°C, 5% CO_2 for 24 hours. A) Cells transfected with siRNA (1000 nM) x100 magnification. B) Cells transfected with siRNA (1000 nM) using x600 scale. C) Cells transfected with siRNA (1000 nM) using x1000 scale. D) Cells transfected with siRNA (50 nM) using x200 scale.

3.9 Overexpression of EP4 receptors in THP-1 cells

In the following experiments, an exogenous EP4 plasmid was transfected into THP-1 cells in order to induce overexpression of the PTGER4 gene.

3.9.1 Optimising TNF-α production using different concentrations of FLAG-EP4 plasmid

In all overexpression experiments conducted in this study, two DNA plasmids were required. The first plasmid was (FLAG-PTGER4) which had an EP4 construct and tagged with a FLAG epitope tag. The second one was an empty vector (EX-Q0086-M11 without FLAG-PTGER4) and used as a control.

In this study the FLAG-PTGER4 plasmid DNA was transformed into TOP10 chemically competent *E.coli* (Life Technologies Ltd, UK) in order to amplify the plasmid to be induced into THP-1 cells (as described in Methods Section 2.2.6). A plasmid isolation kit with an integral bacterial endotoxin removal step and endotoxin-free reagents were used to purify the FLAG-PTGER4 plasmid DNA (Methods Section 2.2.8). This step is important in order to reduce the likelihood of bacterial endotoxin release that can be carried over during the purification with the plasmid DNA affecting the mammalian cells. After obtaining ultra pure FLAG-PTGER4, it was vital to optimise the effect of different concentrations of this plasmid DNA on TNF-α production. Cells were transfected with different concentrations of FLAG-EP4 [1904 ng/µl, 952 ng/µl and 476 ng/µl (4.7 µg, 2.3 µg and 1 µg / $1x10^6$ THP-1 cells respectively)], Empty vector [2461 ng/µl, 1230 ng/µl and 615 ng/µl (6 µg, 3 µg and 1.5 µg/ $1x10^6$ THP-1 cells respectively)], and incubated for 24h as described in Methods Section 2.2.3. These specific

concentrations were chosen because the highest concentration obtained for FLAG-EP4 was 1904 ng/µl (4.7 µg/ $1x10^6$ THP-1 cells) and 2461 ng/µl (6 µg/ $1x10^6$ THP-1 cells) for the empty vector. Then serial dilutions (1 in 2) were carried out. Fig. 3.49 shows the production of TNF-α with various concentrations of FLAG-EP4 plasmid in response to LPS. LPS induced the maximum TNF-α production in the absence of the exogenous EP4 plasmid while FLAG-EP4 (1904 ng/µl) produced a significant decrease in this TNF-α level, $P < 0.01$, suggesting that introducing EP4 plasmid DNA resulted in a reduction in TNF-α levels (Fig. 3.49). A Polymerase Chain Reaction (PCR) amplification was conducted after transfection of THP-1 cells with FLAG-PTGER4 plasmid DNA in order to examine the expression of the FLAG-PTGER4 mRNA transcript produced by the EP4 plasmid. Unfortunately, the data are not shown because the FLAG-PTGER4 mRNA expression determination appeared inconsistent. Despite the instability of the FLAG-PTGER4 mRNA expression after transfection of THP-1 cells with FLAG-PTGER4 plasmid DNA, the next Section shows that TNF-α production was reduced significantly by transfection with the FLAG-PTGER4 plasmid DNA. This suggests that the EP4 plasmid had been introduced successfully into THP-1 cells.

There was no significant inhibition in TNF-α level with the lowest concentration (952 ng/µl and 476 ng/µl) in response to LPS, unlike the higher concentrations (1904 ng/µl) which suppressed the TNF-α production significantly. These three concentrations of FLAG-EP4 were also evaluated without LPS stimulation. The lowest concentration (476 ng/µl) increased the TNF-α level but did not read

significant, unlike the higher concentrations (1904 ng/µl and 952 ng/µl) which did not affect the TNF-α production (Fig. 3.49).

The effect of FLAG-PTGER4 plasmid DNA on TNF-α production had to be compared with the empty vector (EX-Q0086-M11) lacking the PTGER4 insert. This empty vector was used as control. Different concentrations of the empty vector were used to establish which of these concentrations can be used as a control in terms of its effect on TNF-α production. As the empty vector should not have affected the production of TNF-α, it seems that the best concentration was 2461 ng/µl (6 µg/1x10^6 THP-1 cells) because it did not alter the production of TNF-α in LPS-stimulated THP-1 cells (Fig. 3.50). The effect of the empty vector was in parallel with the effect of untransfected THP-1 cells in response to LPS. In the absence of LPS, both concentrations of empty-vector (2461 ng/µl and 1230 ng/µl) did not influence the production of TNF-α, whereas 615 ng/µl of empty-vector induced a significant increase in TNF-α level.

Based on these findings, both exogenous plasmids (FLAG-EP4 and empty-vector) were used at high concentrations in the overexpression assays.

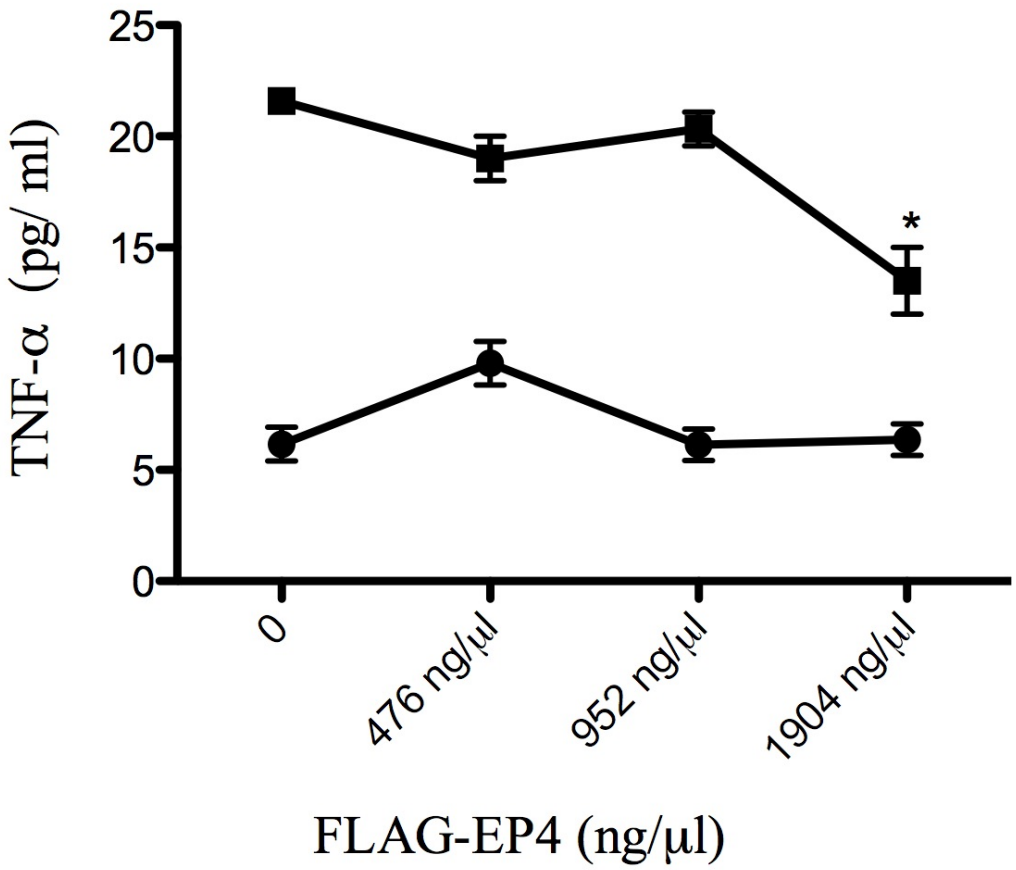


Fig. 3.49: The effect of different concentrations of FLAG-EP4 plasmid on TNF-α production in THP-1 cells.

Cells ($1x10^6$ cells/ml) were incubated in 6-well plates with varying concentrations of FLAG-EP4 plasmid [1904 ng/µl, 952 ng/µl and 476 ng/µl (4.7 µg, 2.3 µg and 1 µg / $1x10^6$ THP-1 cells respectively)] in the presence of LPS (10 µg/ml)(square filled symbols). Incubations without LPS (FLAG-EP4 plasmid only) are shown as circle filled symbols. The plates were incubated for 24 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

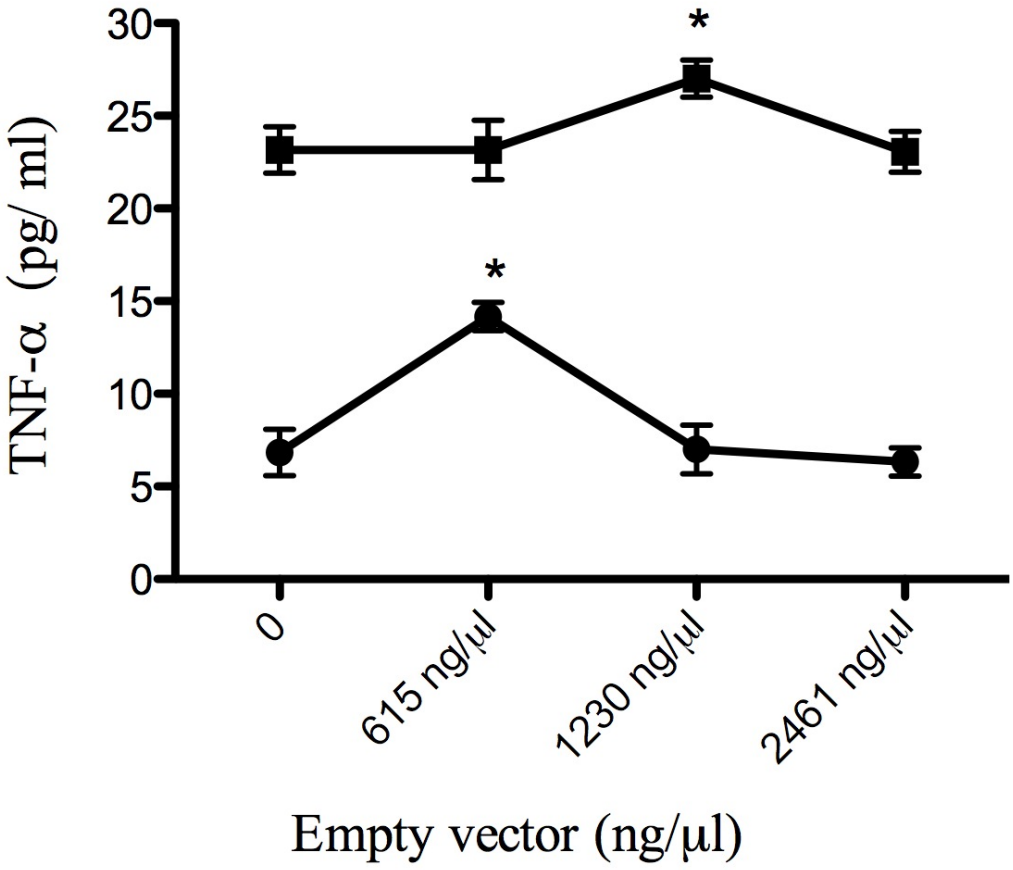


Fig. 3.50: The effect of different concentrations of empty-vector plasmid on TNF-α production in THP-1 cells.

Cells ($1x10^6$ cells/ml) were incubated in 6-well plates with varying concentrations of empty-vector plasmid [2461 ng/µl, 1230 ng/µl and 615 ng/µl (6 µg, 3 µg and 1.5 µg/ $1x10^6$ THP-1 cells respectively)], in the presence of LPS (10 µg/ml)(square filled symbols). Incubations without LPS (empty-vector plasmid only) are shown as circle filled symbols. The plates were incubated for 24 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus control.

3.9.2 The effect of PTGER4 overexpression on TNF-α production in THP-1 cells

The influence of EP4 receptor overexpression on the production of TNF-α was studied by transfecting THP-1 cells with exogenous plasmids (FLAG-EP4 and empty-vector)(1904 ng/µl) for 24h and then stimulated with LPS as described in Methods Section 2.2.3. Fig. 3.51 shows that LPS-stimulated cells produced a TNF-α level which was significantly lower in cells transfected with the EP4 exogenous plasmid, $P < 0.01$. The empty-vector, (used as a control and having no EP4 construct) did not have an effect on TNF-α production in response to LPS. The TNF-α concentration was 116 pg/ml after transfected LPS-stimulated cells with the empty-vector. This concentration was very close to the TNF-α level in response to LPS alone (115 pg/ml). The graph also shows that LPS induced a significant increase TNF-α production in the presence of both FLAG-EP4 plasmid and empty vector.

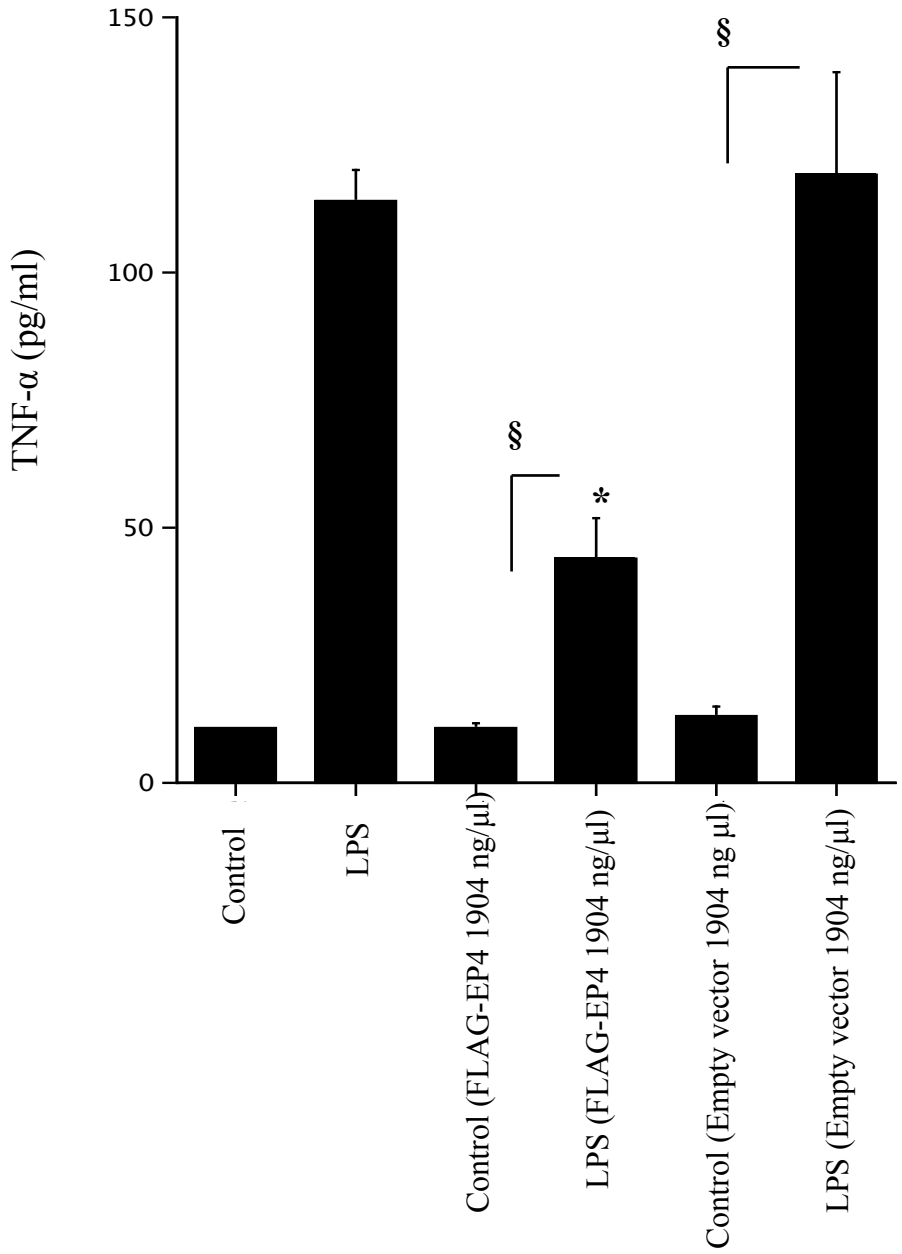


Fig. 3.51: The effect of PTGER4 overexpression on TNF-α production in THP-1 cells.

Cells ($1x10^6$ cells/ml) were incubated in 6-well plates with FLAG-EP4 plasmid and empty-vector plasmid (both at 1904 ng/µl) in the absence or presence of LPS (10 µg/ml). The plates were incubated for 24 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone, § P < 0.01 versus plasmid/vector alone.

3.9.3 Time course of the effect of PTGER4 overexpression on TNF-α production in THP-1 cells

After demonstrating that transfection of THP-1 cells with 1904 ng/µl of FLAG-PTGER4 plasmid (4.7 µg/1x10^6 THP-1 cells) resulted in a decrease in the production of TNF-α while the negative control [empty vector 2461 ng/µl (6 µg/1x10^6 THP-1 cells)] did not alter the TNF-α production, it was essential to determine the most optimal transfection time for exogenous EP4 plasmid overexpression. This was assessed by ascertaining the time course of the effects of EP4 plasmid overexpression on TNF-α production in THP-1 cells. Cells were transfected with FLAG-EP4 (1904 ng/µl) plasmid and incubated with LPS and PGE_2 as described in Methods Section 2.2.3. Fig. 3.52 illustrates the production of TNF-α throughout different time points (0h, 6h and 24h).

Untransfected cells which are represented as 0h (LPS pre-incubation period), induced a high concentration of TNF-α (570 pg/ml) in response to LPS. PGE_2 inhibited this level to 160 pg/ml. After 6h transfection of FLAG-EP4 plasmid, there was a significant decrease in TNF-α production (460 pg/ml) in response to LPS compared to untransfected cells (570 pg/ml) ($P < 0.01$). PGE_2 was able to induce a notable decrease in TNF-α level with LPS stimulated cells (95 pg/ml) compared to PGE_2 with untransfected cells (150 pg/ml) ($P < 0.01$) (see Fig. 3.52).

At 24h post-transfection with EP4 plasmid, the concentration of TNF-α reached a lowest inhibitory level. FLAG-EP4 plasmid suppressed the production of TNF-α from 570 pg/ml (untranfected cells) to 270 pg/ml ± 10.02 (24h transfected with the

plasmid) ($P < 0.01$). PGE_2 also decreased TNF-α production significantly in response to LPS from 150 pg/ml (untranfected cells) to 45 pg/ml ± 3.5 (24h transfected with the plasmid) ($P < 0.01$) (see Fig. 3.52).

Transfection of LPS-stimulated THP-1 cells with exogenous FLAG-PTGER4 plasmid DNA resulted in a significant decrease in the production of TNF-α. As EP4 receptor activation results in the suppression of TNF-α production, this suggests that overexpression of the EP4 receptor can result in further suppression, confirming that it can play an important role in controlling the production of TNF-α. Therefore, it would be a promising approach to the treatment of inflammation by enhancing the function of EP4 receptors either via highly selective ligands or interfering with expression.

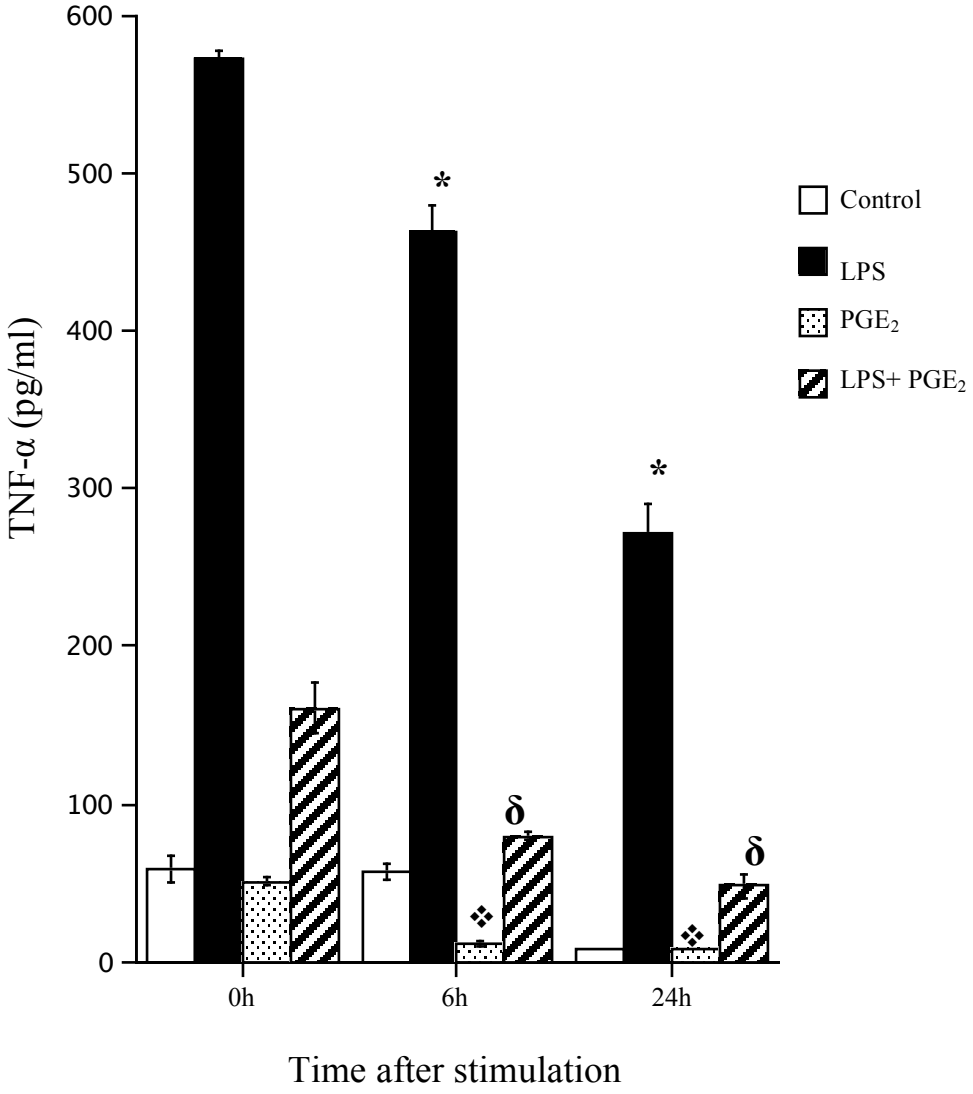


Fig. 3.52: Time course of the effect of PTGER4 overexpression on TNF-α production in THP-1 cells.

Cells ($1x10^6$ cells/ml) were stimulated in 6-well plates with LPS (10 μg/ml), PGE_2 (1 μM) and transfected with FLAG-EP4 plasmid (1904 ng/μl) for 6h and 24h. untransfected cells are represented as 0h.The plates were incubated at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS for untransfected cells (0h), §P< 0.01 versus LPS with PGE_2 for untransfected cells (0h) and ❖ P< 0.01 versus PGE_2 alone for untransfected cells (0h).

3.10 Protein expression

3.10.1 Protein expression of FLAG-tagged EP4 receptor in THP-1 cells

A study of the protein expression of FLAG-tagged EP4 receptors was attempted using two different antibodies: anti-FLAG antibody and anti- EP4 antibody. Protein was extracted from THP-1 cells and subjected to PAGE separation and immunoblotting as described in Methods Section 2.2.18.1.

With anti-FLAG antibody, it was found that staining was present but in a very low molecular weight (20 kDa) band (using HyperPage prestained molecular weight markers). Proteins were stained with anti GAPDH as a loading control (35.8 KDa). The blot shows that LPS treated cells had less expression compared to control (Fig. 3.53 (A)). Blots were quantified for both FLAG-stained and GAPDH-stained changes by normalising the blots of transfected THP-1 cells to untransfected cells as shown in Fig. 3.53 (B). There was an increase in the fold change of FLAG-staining in cells transfected with FLAG-EP4 plasmid compared to untransfected cells. The fold change of FLAG-staining in cells transfected with the empty vector plasmid was closer to the fold change of untransfected cells (Fig 3.53 B). This finding can only be considered as indicative because it was obtained from only one individual expreiment, but it suggests the possibility that cells can be successfully transfected with FLAG-EP4 plasmid because the fold stimulation with the plasmid was higher than transfected cells. This would require further investigation.

Using anti-EP4 antibody, the FLAG-EP4 protein was expressed at a higher molecular weight than anti-FLAG antibody (>20 kDa). The actual molecular weight of EP4 receptors is 53 kDa (Castleberry *et al.*, 2001). Interestingly, the FLAG-EP4 protein using anti-EP4 antibody presented the expected molecular weight of EP4 receptor (53 kDa). Proteins were also stained with anti GAPDH as a loading control (35.8 KDa) Fig. 3.54 (A). Blots were quantified for both EP4-stained and GAPDH-stained changes by normalising the blots of transfected THP-1 cells to untransfected cells (as done previously with FLAG-stained) Fig. 3.54 (B). Fold change of EP4 staining in cells transfected with EP4 plasmid showed an increase compared to untransfected cells. The blot also shows that LPS treated cells had less expression compared to controls. Cells with empty vector showed a smaller fold change compared to the fold stimulated by FLAG-EP4 (Fig. 3.54 B). This finding can only be a promising indicative result as with the anti-FLAG antibody because the values represent one individual experiment, but it may suggest that transfecting cells with FLAG-EP4 plasmid might result in a higher fold stimulation than in untransfected cells.

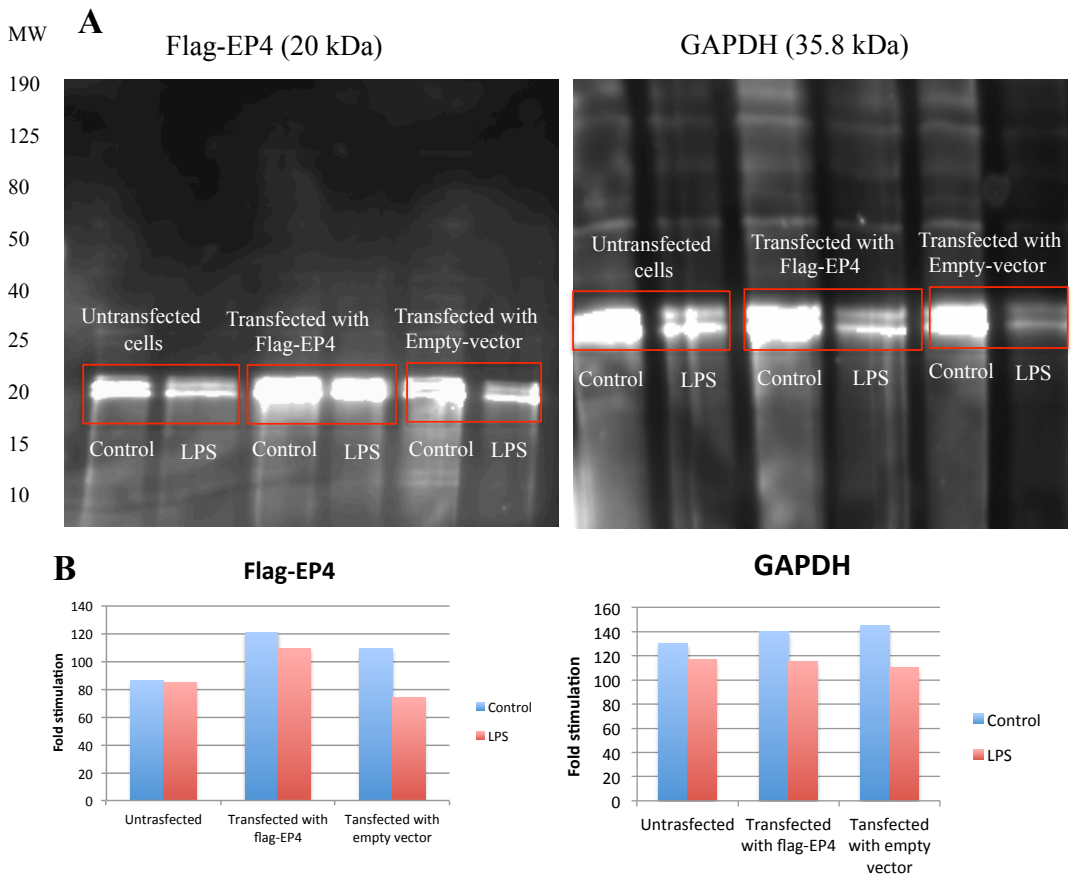


Fig. 3.53: Protein expression of FLAG tagged EP4 receptors using anti- FLAG antibody in THP-1 cells.

Cells were transfected with either FLAG-EP4 plasmid or empty vector (both at 1904 ng/µl) in the absence and presence of LPS (10 µg/ml) for 24h. Control cells (control/LPS alone) were left untransfected. Whole cell lysates were prepared for FLAG-EP4 by Western blotting as described in Methods Section 2.2.18.1. Expression of GAPDH was measured as loading control. A) Expression of FLAG-EP4 and GAPDH. B) Blots were quantified for FLAG-EP4 and GAPDH fold stimulation by normalising to untransfected cells. Values represent an individual experiment.

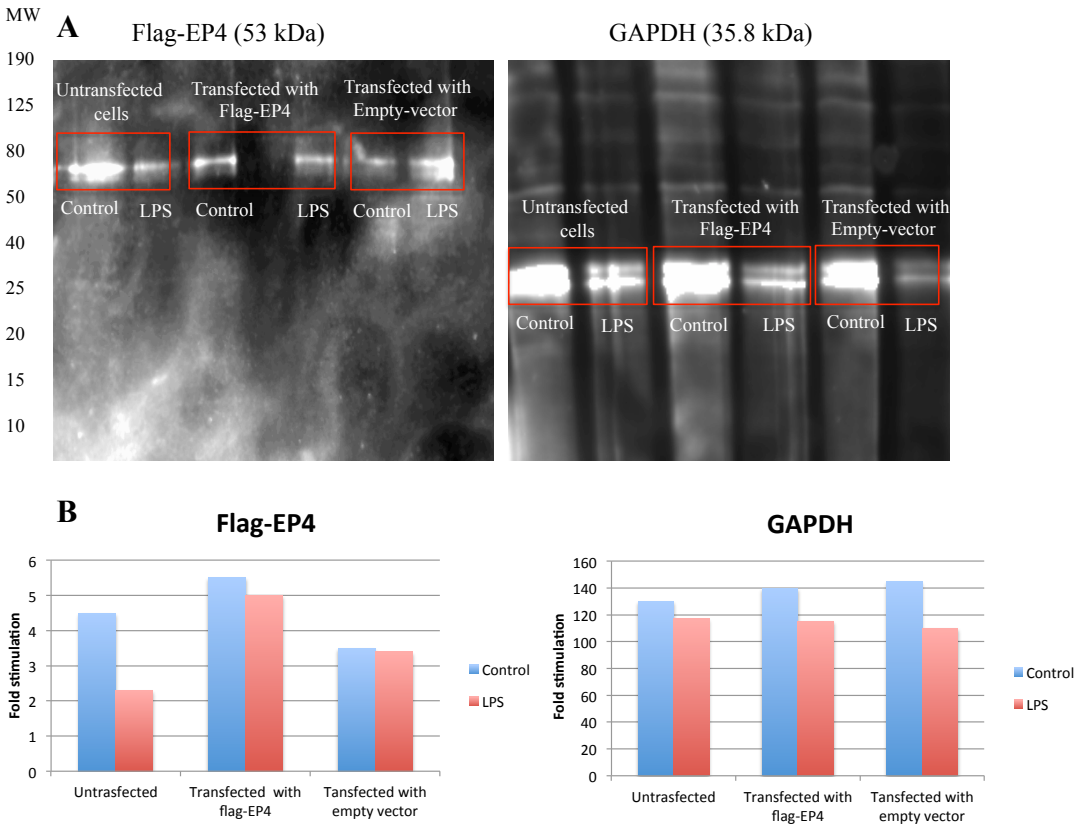


Fig. 3.54: Protein expression of FLAG tagged EP4 receptors using anti-EP4 antibody in THP-1 cells.

Cells were transfected with either FLAG-EP4 plasmid or empty vector (both at 1904 ng/µl) in the absence and presence of LPS (10 µg/ml) for 24h. Control cells (control/LPS alone) were left untransfected. Whole cell lysates were prepared for FLAG-EP4 by Western blotting as described in Methods Section 2.2.18.1. Expression of GAPDH was measured as loading control. A) Expression of FLAG-EP4 and GAPDH. B) Blots were quantified for FLAG-EP4 and GAPDH fold stimulation by normalising to untransfected cells. Values represent an individual experiments.

3.10.2 Protein staining with anti-FLAG -antibody in SH-SY5Y cells

FLAG-sequences appeared to be present at an unexpected molecular weight (20 kDa) using anti-FLAG antibody indicating either cross-reactive binding or binding to sequences similar to FLAG but unrelated to the construct. It was decided to try the anti-FLAG antibody with another cell line (SH-SY5Y) to see whether this antibody detected protein at a similar range of molecular weight. Protein was extracted from SH-SY5Y cells as described in Methods Section 2.2.18.1. Indeed, anti-FLAG staining was observed at 20 kDa as in THP-1 cells. GAPDH was used as control and expressed at 35.8 kDa as well (Fig. 3.55). As these cells had not been transfected with any construct it would appear that the anti-FLAG antibody did not bind to denovo FLAG-tagged protein.

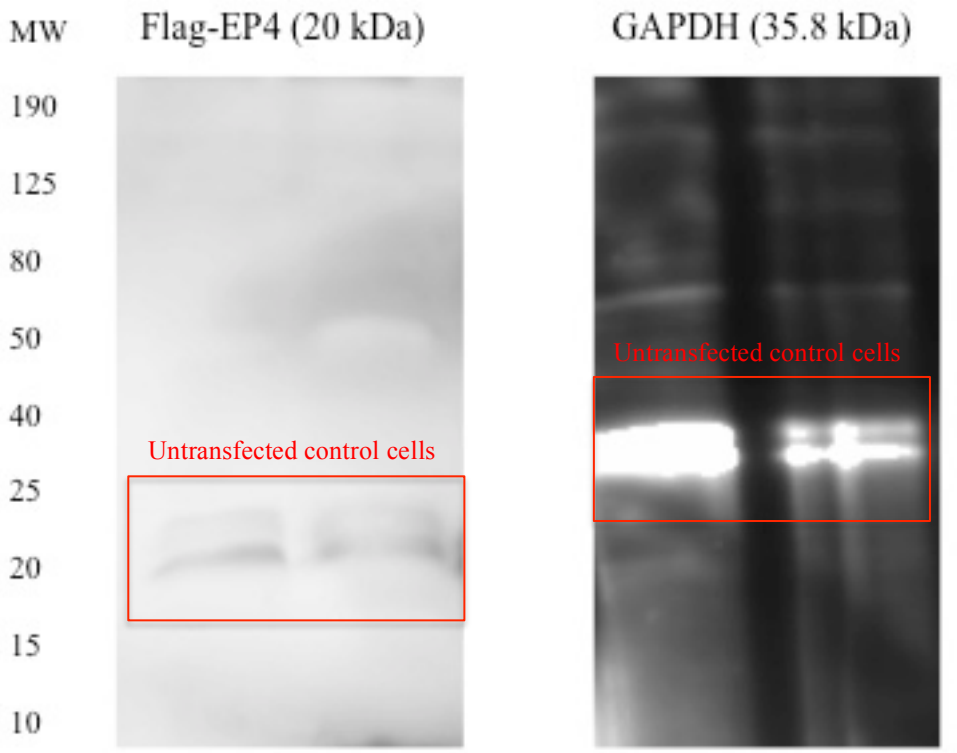


Fig. 3.55: Protein expression of FLAG tagged EP4 receptors using anti- FLAG antibody in SH-SY5Y cells.

Control cells (without any treatment) were prepared for FLAG-EP4 by Western blotting as described in Methods Section 2.2.18.1. Expression of GAPDH was detected as loading control. Values represent an individual experiment.

3.10.3 Quantification of FLAG-tagged protein in THP-1 cells

The purpose of this experiment was to attempt to quantify FLAG-tagged EP4 protein as an indicator of de novo synthesised EP4 receptors in response to transfection with FLAG-EP4 construct. The expression of FLAG-EP4, that was observed with anti-EP4 and anti-FLAG antibodies, was quantified by subjecting visualised protein bands (using Ponceau S red) to a semi ELISA procedure after staining the nitrocellulose membranes as describe in methods (Methods Section 2.2.18.5). Fig. 3.56 (A) shows a comparison between FLAG-EP4 expression using either anti-FLAG or anti-EP4 antibodies. The expression of FLAG-EP4 with anti-EP4 antibody was 53 kDa, whereas with anti-FLAG antibody it was 20 kDa using GAPDH (35.8 kDa) as a loading control. Fig. 3.56 (B) represents the quantitation analysis of FLAG-EP4 with both antibodies.

Using anti-EP4 antibody as a semi ELISA, the absorbance of bands (protein intensity) of almost all samples was around 2 (control/ LPS for untransfected cells, control/ LPS for cells transfected with FLAG, control/ LPS cells transfected with empty vector). In contrast to anti-EP4 antibody, the same samples were also quantified using anti-FLAG antibody and their protein bands show a similar protein absorbance as protein quantified with anti-EP4 antibody.

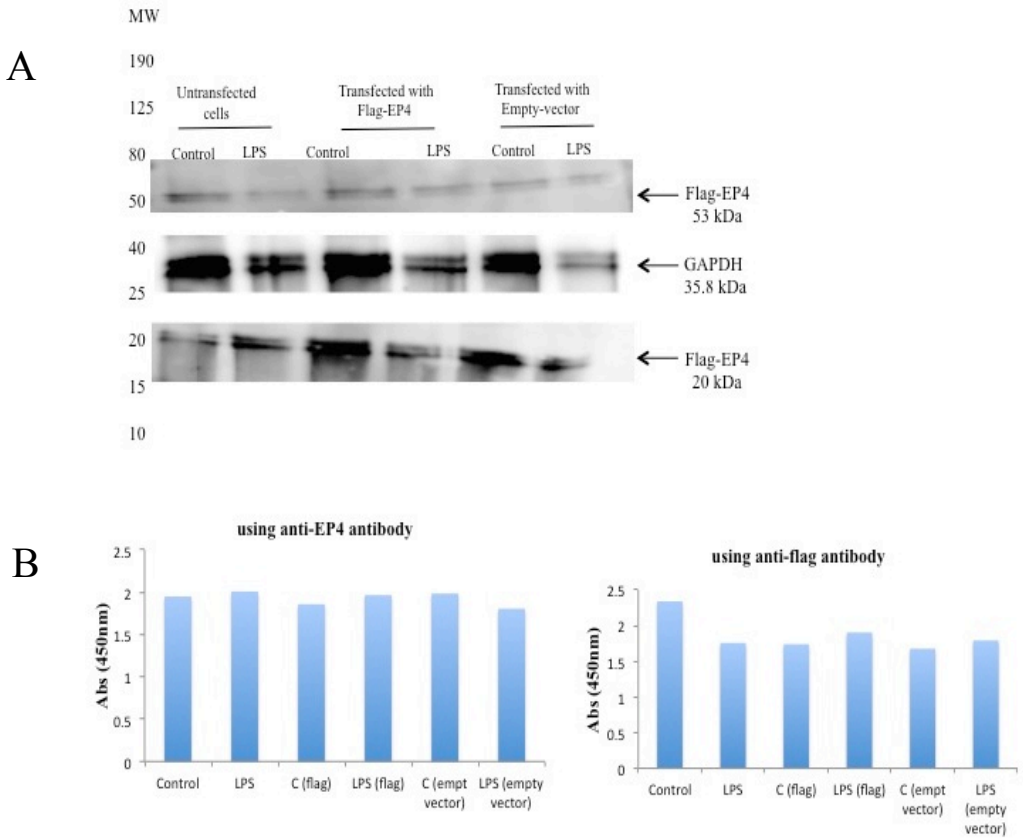


Fig. 3.56: Quantification of FLAG-PTGER4 in THP-1 cells.

Cells were transfected with either FLAG-EP4 plasmid or empty vector (both at 1904 ng/µl) in the absence and presence of LPS (10 µg/ml) for 24h. Control cells (control/LPS alone) were left untransfected. Whole cell lysates were prepared for FLAG-EP4 by Western blotting as described in Methods Section 2.2.18.1. Expression of GAPDH was measured as a loading control. A) Expression of FLAG-EP4 using anti-EP4 antibody (53 kDa), expression of FLAG-EP4 using anti-FLAG antibody (20 kDa) and expression of GAPDH (35.8 kDa) as a control. B) Absorbance of FLAG-EP4 using anti EP4 and anti-FLAG antibodies. Protein was quantified in the membrane as described in Methods Section 2.2.18.5.Values represent an individual experiment.

3.11 Biotin-labeled PGE_2 binding to THP-1 cells

THP-1 cells were spotted onto 3 different nitrocellulose membrane strips. The first membrane had spots of THP-1 cells that had not been incubated with biotin-labelled PGE_2. The second membrane had spots of THP-1 cells that were incubated with biotin-labelled PGE_2. The last membrane had spots of THP-1 cells that were pre-treated with PGE_2 followed by incubation with biotin-labelled PGE_2. All of the THP-1 cell spots were then incubated with streptavidin-HRP to be detected by enhanced chemiluminescene (ECL) reagent (as described in Methods Section 2.2.19).

THP-1 cells binding to PGE_2 labelled with biotin was demonstrated in this part of research. Fig. 3.57 (A) shows THP-1 cells, which had not been treated or labelled with biotin, had no binding signals. Fig. 3.57 (B) shows there were clear binding signals of THP-1 cells labelled with biotin-PGE_2. However, those cells were treated with PGE_2 before labelling with biotin did not show any binding signals (Fig. 3.57 (C)).

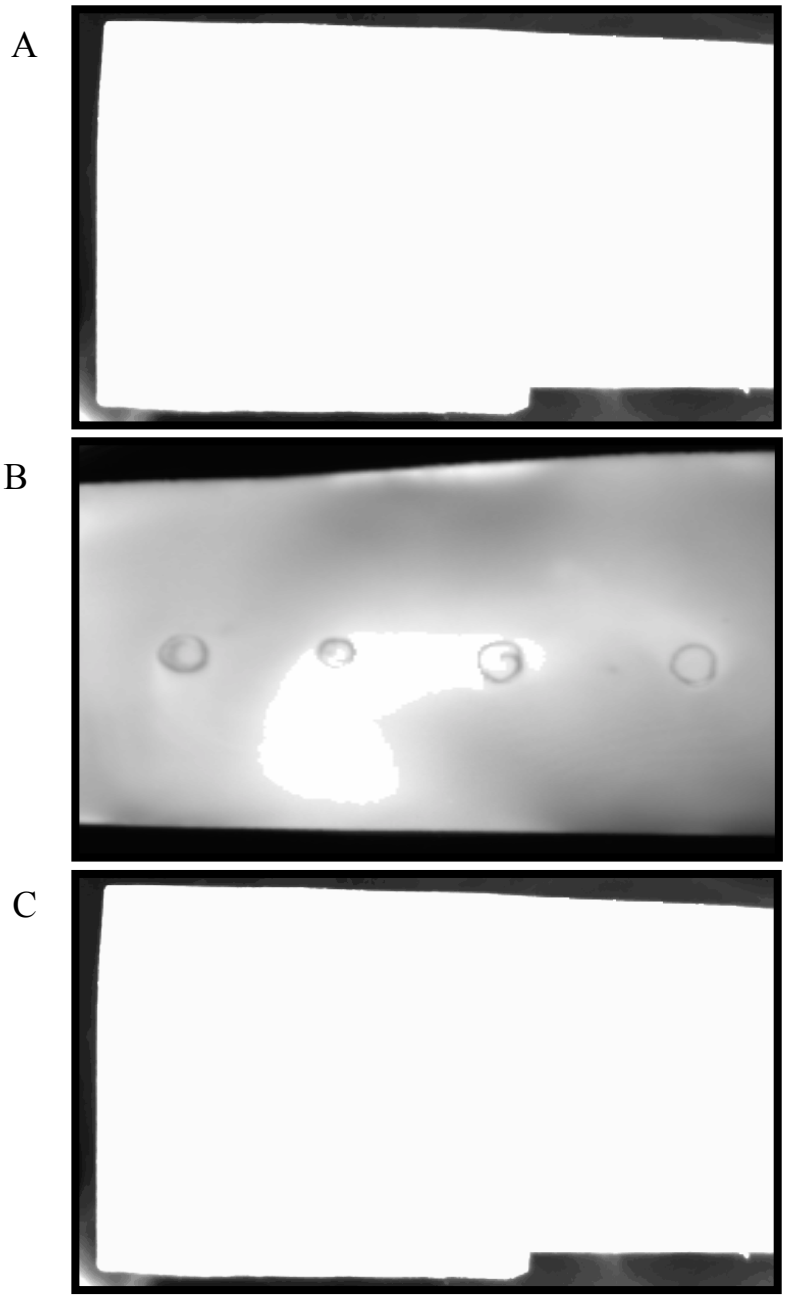


Fig. 3.57: Binding THP-1 cells to PGE_2 labeled with biotin.

A) Cells dotted onto nitrocellulose membrane. B) Cells dotted onto nitrocellulose membrane labelled with PGE_2-biotin (200 nM). C) Cells dotted onto nitrocellulose membrane incubated with PGE_2 (200 nM) then labelled with PGE_2-biotin (200 nM).

3.12 EP4 receptor localisation in THP-1 cells

Initially, it was attempted to visualise THP-1 cells by Epi-fluorescence using PGE_2-biotin labelled cells (Methods Section 2.2.20.1). However, there were no luminescence signals (images not shown). Therefore, it was decided to use an EP4 Ab-FITC conjugate to attempt to visualise EP4 receptors as described in Methods Section 2.2.20.3. THP-1 cell images were captured using different excitation were lengths for the 2 fluorophores (DAPI and FITC) at various magnifications (x200, x600 and x1000). Fluorescence (for FITC) was visualised indicating that EP4 receptors could be detected on THP-1 cells (Fig. 3.58). Interestingly, although the fluorescence was on the membrane it was not evenly distributed but appeared to be concentrated at a specific point on the membrane in each cell. There appeared to be polarisation of the fluorescence with respect to the plasma membrane in each cell.

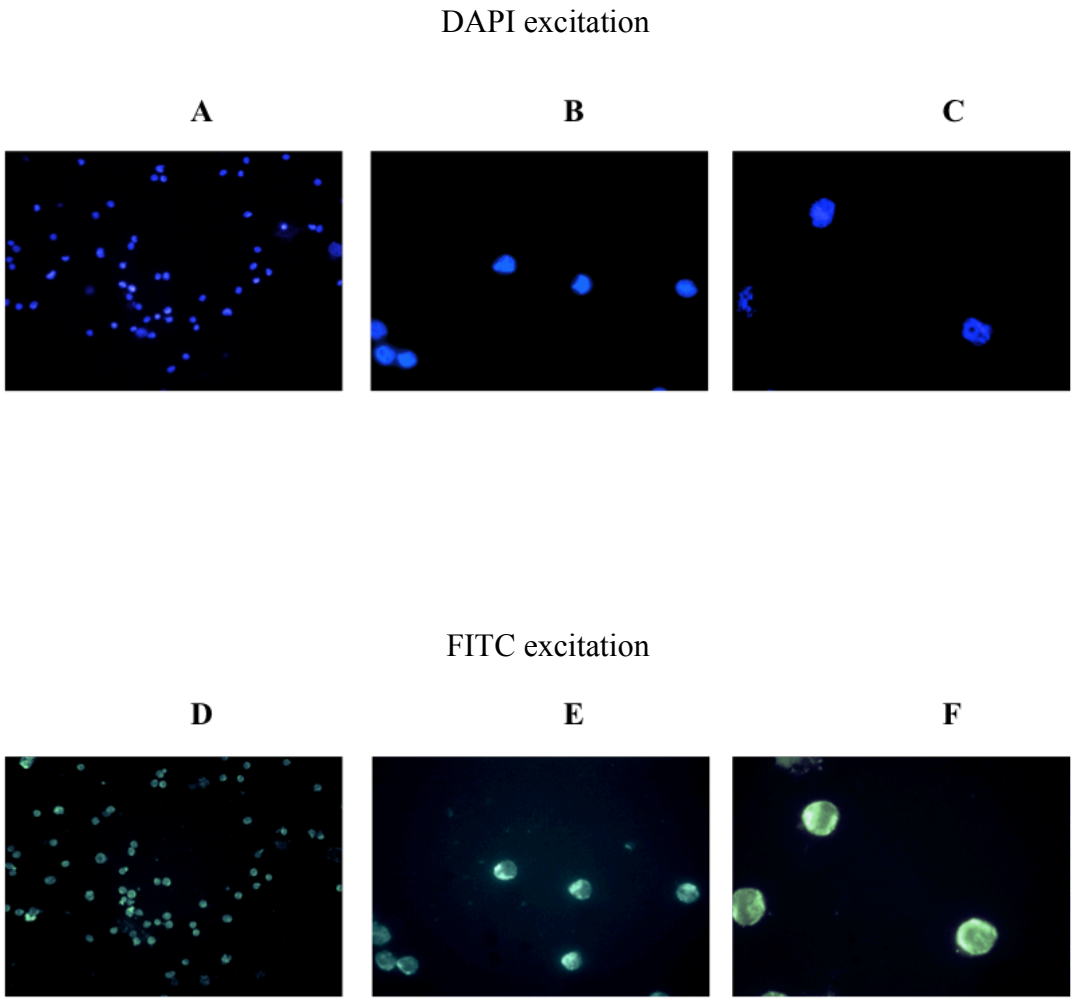


Fig. 3.58: Images for THP-1 cells tagged with fluorescent E4 Ab-FITC.

THP-1 cells were visualised using two excitations (DAPI and FITC). A) Cells with DAPI excitation x200 magnification. B) Cells with DAPI excitation x600 magnification. C) Cells with DAPI excitation x1000 magnification. D) Cells with FITC excitation x200 magnification. E) Cells with FITC excitation x600 magnification. F) Cells with FITC excitation x1000 magnification.

3.13 Expression of TLR4 in THP-1 cells

3.13.1 The effect of PGE_2 and receptor agonists (sulprostone, butaprost and L-902, 688) on TLR4 expression

The data in Section 3.7 showed that PGE_2 and its receptor agonists induce a significant reduction in the expression of PTGER2 and PTGER4 in response to LPS. Additional experiments were carried out to investigate the effect of PGE_2 and receptor agonists on TLR4 expression in THP-1 cells. This was performed to demonstrate whether TLR4 expression in response to LPS could be inhibited by PGE_2, as TLR4 is the receptor for LPS. THP-1 cells were harvested as stated in Methods Section 2.2.17.1. Fig. 3.59 illustrates TLR4 expression in THP-1 cells after being treated with PGE_2 and different agonists (sulprostone, butaprost and L-902, 688) in the presence and in the absence of LPS. Incubation of cells with LPS did not induce a noticeable change in TLR4 expression compared to control (the fold change of TLR4 in response to LPS was 1.145 fold ± 0.097 up-regulation). Treatment of THP-1 cells with PGE_2, butaprost and sulprostone significantly reduced the TLR4 expression response to LPS stimulation from 1.145 fold ± 0.097 up-regulation with LPS alone to a 1.351 fold ± 0.029 down-regulation with LPS+ PGE_2, 2.247 fold ± 0.036 down-regulation with LPS+butaprost and to a 2.57 fold ± 0.004 down-regulation with LPS+ sulprostone ($P< 0.0001$). However, L-902, 688 produced a clear increase in TLR4 expression response to LPS stimulation (TLR4 expression changed from 1.145 fold ± 0.097 up-regulation with LPS alone to 2 fold ± 0.127 up-regulation with LPS+ L-902, 688, $P < 0.0001$).

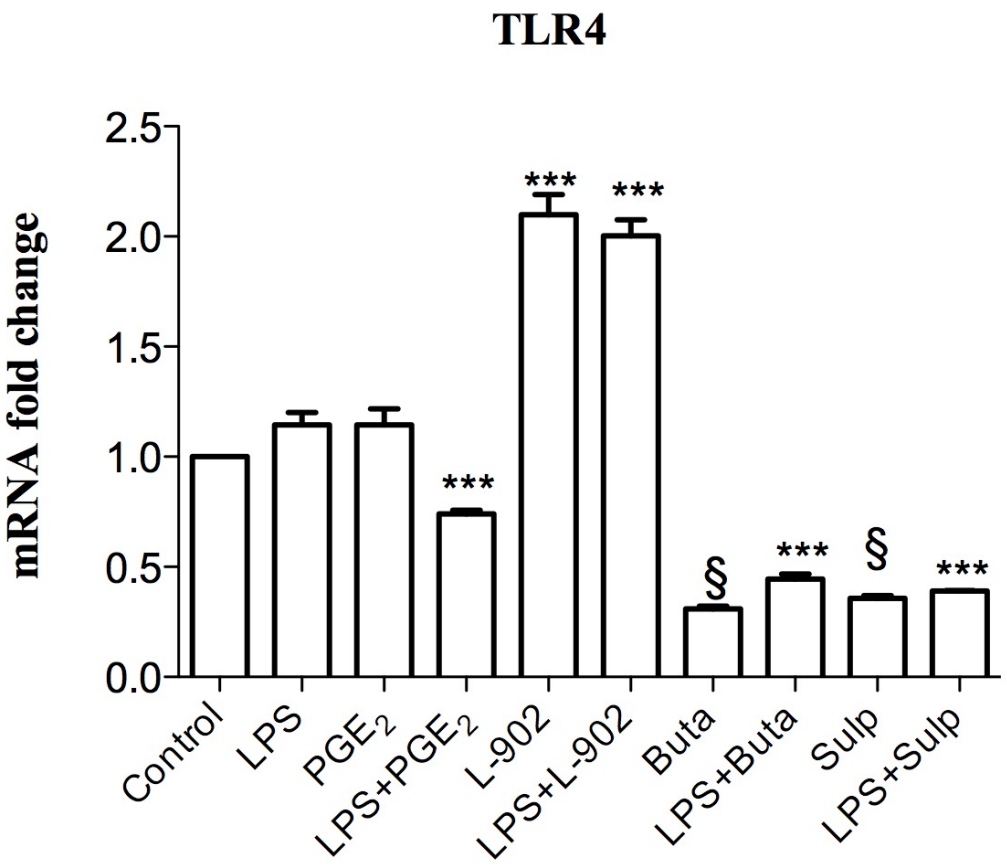


Fig. 3.59:The effect of PGE_2 and receptor agonists (sulprostone, butaprost and L-902, 688) on TLR4 expression in THP-1 cells.

Cells ($1.5x10^6$ cells/ml) were placed into 6-well plates after which LPS (10 µg/ml) and various EP receptor agonists (all at 1 µM); PGE_2), L-902,688 (L-902), butaprost (Buta) and sulprostone (Sulp) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for TLR4 as described in Methods Section 2.2.17.10. Relative expression levels of TLR4 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). ***P < 0.0001 versus LPS alone and § P < 0.0001 versus control.

3.13.2 The effect of PTGER4 knockdown on TLR4 expression in THP-1 cells

The previous Section 3.13.1 established that the expression of TLR4 was significantly inhibited by PGE_2 in LPS stimulated THP-1 cells. This suggested carrying out a further expreiment to investigate whether the lack of PGE_2 had a different effect on TLR4 expression. To study this aspect, THP-1 cells were knocked-down by transfection with PTGER4 siRNA duplex as described in Methods Section 2.2.3. Indeed, after 24h transfection, TLR4 had a different expression compared to non-transfected cells. Fig. 3.60 shows TLR4 expression before and after PTGER4 down-regulation. A deficiency of PTGER4 induced a significant up-regulation of TLR4 expression in response to LPS stimulation (TLR4 expression fold changed from 1 fold ± 0.102 up-regulation with LPS in non-transfected cells to a 1.328 fold ± 0.118 up-regulation with LPS in cells transfected with PTGER4 siRNA duplex, $P < 0.0001$). Similarly, PTGER4 down-regulation significantly increased the TLR4 expression from 2.5 fold ± 0.119 down-regulation with non-transfected cells to a 1.126 fold ± 0.07 up-regulation with cells transfected with PTGER4 siRNA duplex in response to LPS+ PGE_2 ($P < 0.0001$). However, the lack of PTGER4 did not alter TLR4 expression in the presence of either PGE_2 alone or THP-1 cells alone (control).

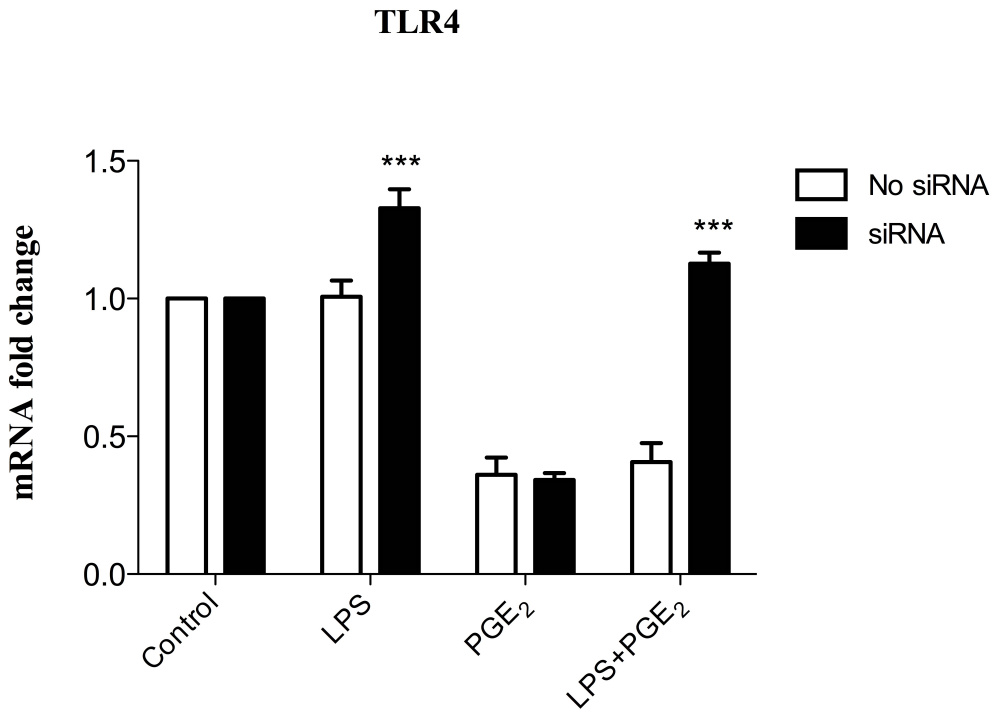


Fig. 3.60: The effect of PTGER4 knockdown on TLR4 expression in THP-1 cells.

Cells ($1x10^6$ cells/ml) were placed into 6-well plates after which LPS (10 μg/ml), culture medium (control) and PGE_2 (1 μM) were added and cells were transfected with siRNA duplex (100 nM) using HiPerFect. The plates were incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using primers to detect TLR4 as described in Methods Section 2.2.17.10. Relative expression levels of TLR4 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak & Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). ***P < 0.0001versus non-transfected cells.

3.14 Expression of TNF in THP-1 cells

3.14.1 The effect of PGE_2 and EP agonists on TNF expression

The expression of TNF was also studied in order to ascertain whether this related to the changes in actual TNF protein production in THP-1 cells as described previously. THP-1 cells were harvested as described in Methods Section 2.2.17.1. Fig. 3.61 shows that TNF expression was up-regulated 5.87 fold ± 0.13 in response to LPS (10 μg/ml). This level of TNF expression was not affected by the EP2 agonist (butaprost) but it was significantly up-regulated by the EP1/EP3 agonist (sulprostone)(TNF expression fold changed from 5.87 fold ± 0.13 up-regulation with LPS alone to 9.3 fold± 0.47 up-regulation with LPS+ sulprostone, $P < 0.0001$). However, the expression of TNF in response to the EP4 agonist (L-902, 688) was below the level of detection of the assay. For this reason, another experiment was conducted using a lower LPS concentration (1 μg/ml) rather than (10 μg/ml) (Fig. 3.62). After reducing the LPS concentration to a 1 μg/ml, TNF expression was up-regulated 36.2 fold ± 2.26 in response to LPS. Both PGE_2 and the EP4 agonist (L-902, 688) were able to inhibit this LPS stimulated expression level significantly from 36.2 fold ± 2.26 up-regulation with LPS alone to 3.85 fold ± 0.95 up-regulation with LPS+PGE_2 and to 2.77 fold ± 0.27 up-regulation with LPS+ L-902, 688 ($P < 0.0001$).

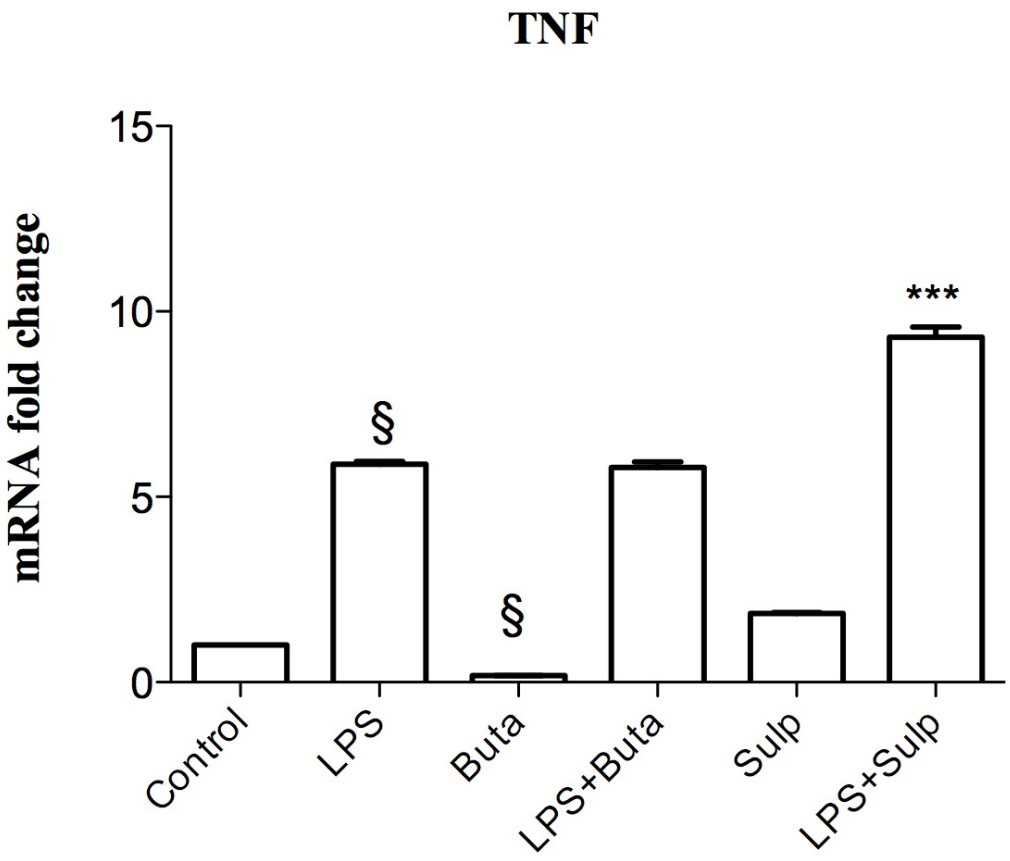


Fig. 3.61: The effect of EP agonists (butaprost and sulprostone) on TNF expression in THP-1 cells.

Cells ($1x10^6$ cells/ml) were placed into 6-well plates after which LPS (10 µg/ ml), EP receptor agonists (all at 1 µM); [butaprost (Buta) and sulprostone (Sulp)] and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for TNF as described in Methods Section 2.2.17.10. Relative expression levels of TNF mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). ***P < 0.0001 versus LPS alone and § P < 0.0001 versus control.

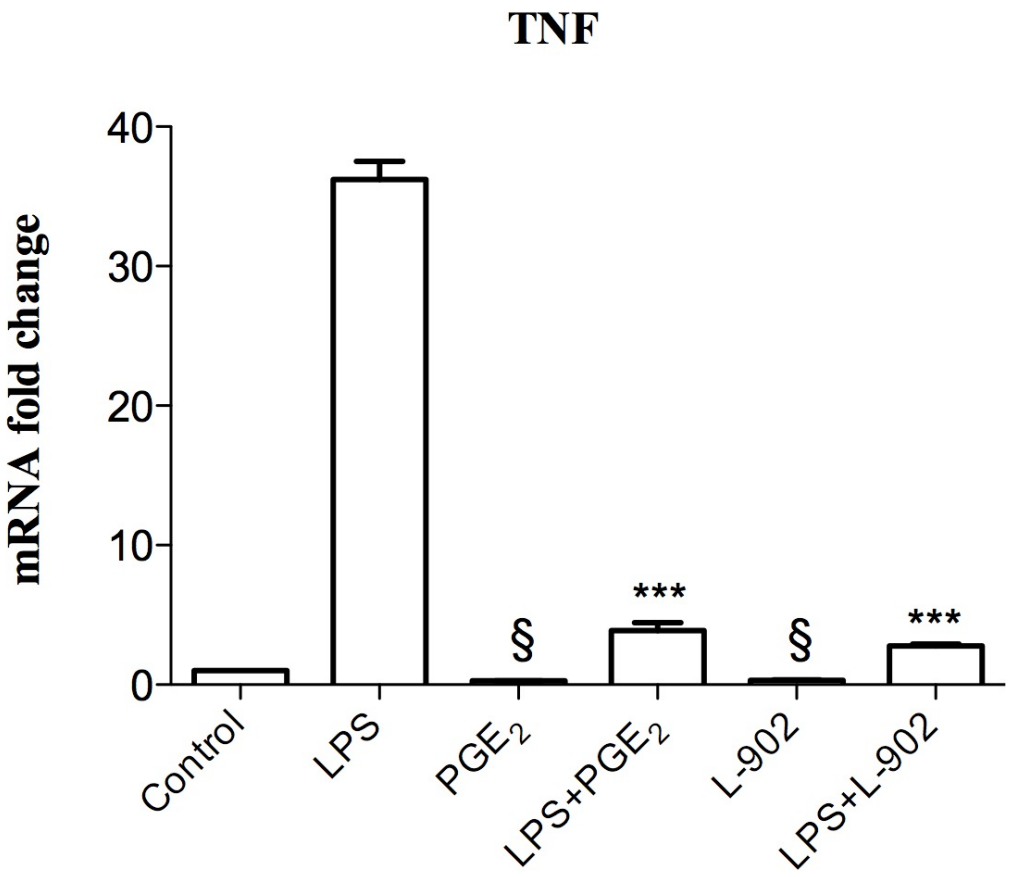


Fig. 3.62: The effect of PGE_2 and EP4 agonist (L-902, 688) on TNF expression in THP-1 cells.

Cells ($1x10^6$ cells/ml) were placed into 6-well plates after which LPS (1 µg/ ml), PGE_2 (1 µM), L-902, 688 (1 µM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for TNF as described in Methods Section 2.2.17.10. Relative expression levels of TNF mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). ***P < 0.0001 versus LPS alone and § P < 0.0001 versus control.

3.15 The effect of Poly IC on THP-1 cells and it's modulation by agonists

3.15.1 The effect of Poly IC on TNF-α production in THP-1 cells

In all previous experiments, LPS was used to stimulate TNF-α production from THP-1 cells. It was decided to compare another stimulus (Poly IC) in order to ascertain whether Poly IC had a similar effect on TNF-α production as LPS. THP-1 cells were incubated with different concentrations of Poly IC as described in Methods Section 2.2.1.8. Fig. 3.63 shows the production of TNF-α in response to Poly IC. Low concentrations of Poly IC (0.01 μg/ml, 0.1 μg/ml and 1 μg/ml) had no significant impact on the production of TNF-α. However, higher concentrations of Poly IC (10 μg/ml and 100 μg/ml) induced an increase in TNF-α production ($P < 0.01$). Therefore, 10 μg/ml of Poly IC was used in all subsequent Poly IC experiments.

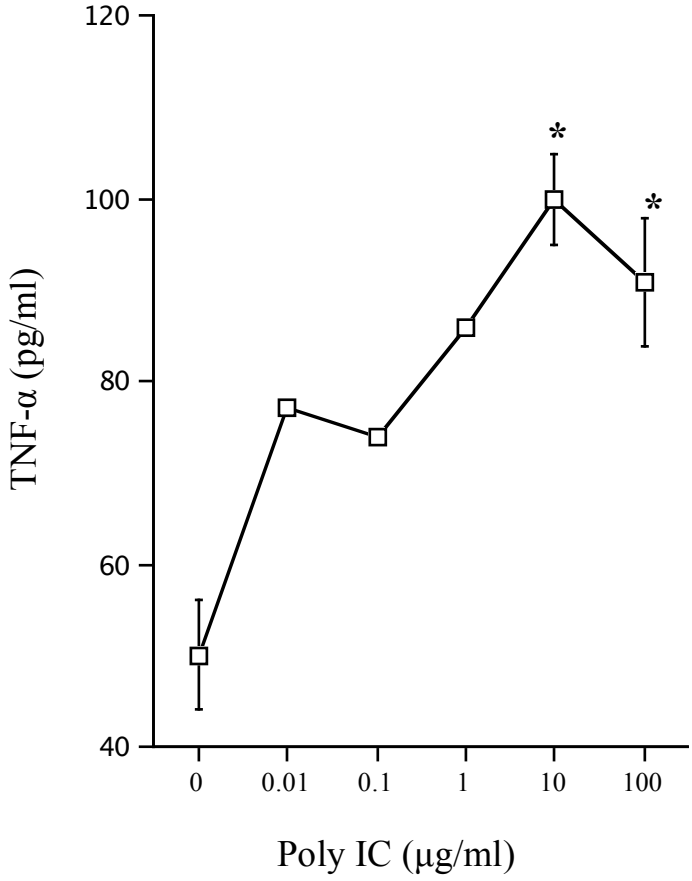


Fig. 3.63: The effect of Poly IC on TNF-α production in THP-1 cells.

Cells ($2x10^6$ cells/ ml) were placed into 6-well plates and incubated with varying concentrations of Poly IC (square open symbols). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus control.

3.15.2 The effect of Poly IC on LPS-stimulated TNF-α Production

Previous experiments showed the production of TNF-α with different concentrations of Poly IC. The effect of different Poly IC concentrations were also studied in the absence or presence of LPS (10 μg/ ml) and TNF-α production was measured from THP-1 cells as described in Methods Section 2.2.1.8. Fig. 3.64 showed that Poly IC at high concentrations can result in a decrease in TNF-α in LPS stimulated cells. 125 pg/ml of TNF-α was produced in response to LPS, this stimulated level was suppressed significantly to 85 pg/ml by 1000 μg/ml of Poly IC, $P < 0.01$. However, lower concentration of Poly IC (10 μg/ml and 100 μg/ml) did not have any effect on TNF-α levels.

By comparing the amount of TNF-α produced in the previous graph (Fig. 3.63) with the following figure, it can be clearly seen that incubation of THP-1 cells with Poly IC was not effective in terms of TNF-α production. It was found that Poly IC did not affect the TNF-α in $4x10^6$ cells/ ml (Fig. 3.64), but using lower cell concentration ($2x10^6$ cells/ ml) there was an increase in TNF-α (Fig. 3.63). This suggests that Poly IC effects need further experiments to demonstrate the actual role of Poly IC and LPS in THP-1 cells stimulation. This thesis, however, was focussed largely on LPS stimulation effects on in TNF-α in the cell types mentioned.

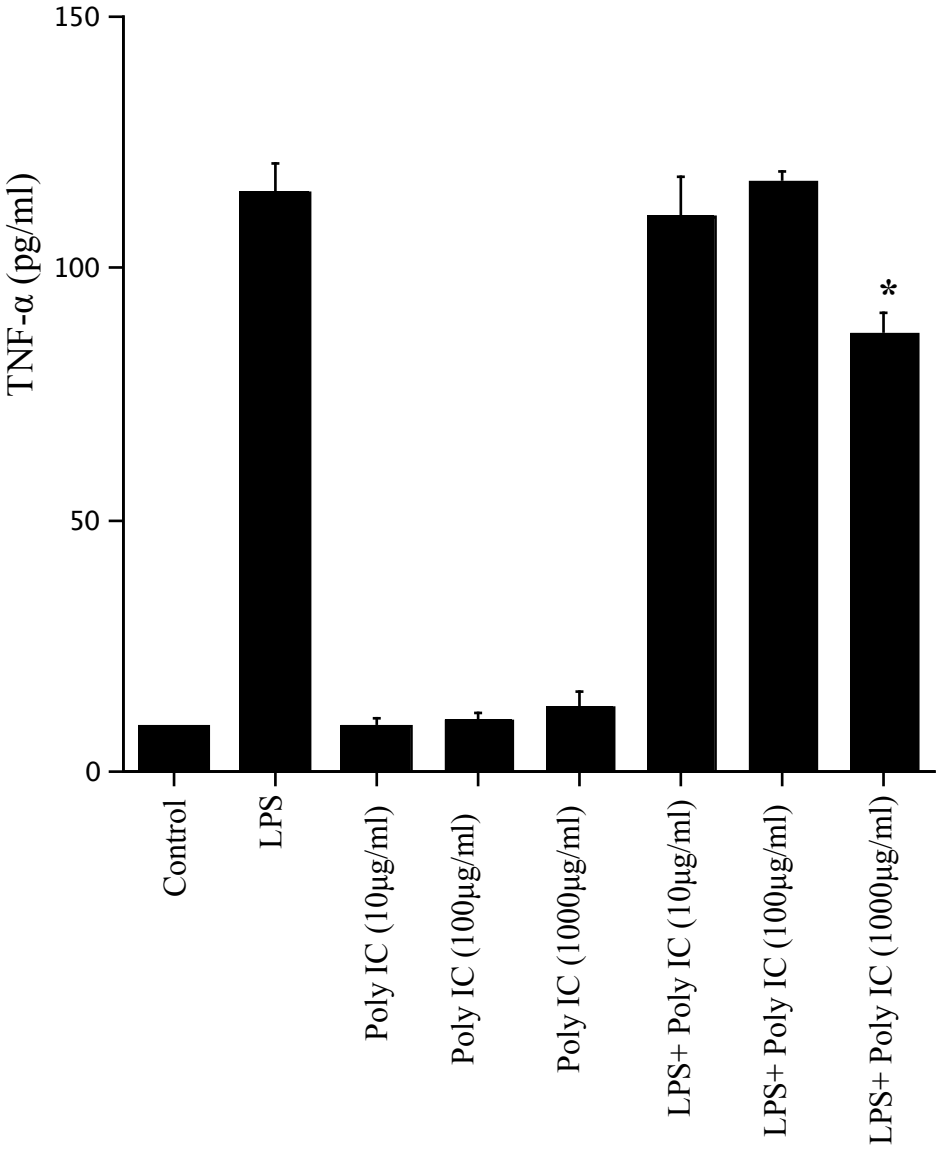


Fig. 3.64: The effect of Poly IC on LPS-stimulated TNF-α Production in THP-1 cells.

Cells ($4x10^6$ cells/ ml) were placed into 24-well plates and incubated with varying concentrations of Poly IC and LPS (10 µg/ml). Incubations were carried out for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

3.15.3 The effect of PGE_2 on Poly IC- stimulated TNF-α Production

PGE_2 has previously been shown to inhibit TNF-α production from LPS stimulated cells. It was also decided to ascertain if PGE_2 had an influence on TNF-α Production using another stimulus (Poly IC) acting via a different TLR. THP-1 cells were stimulated with either LPS or Poly IC and treated with PGE_2 as described in Methods Section 2.2.1.8. As previously shown, PGE_2 inhibited the TNF-α level significantly in response to LPS (Fig. 3.65). On the other hand, the same figure revealed that PGE_2 had no effect on TNF-α production in response to Poly IC in THP-1 cells.

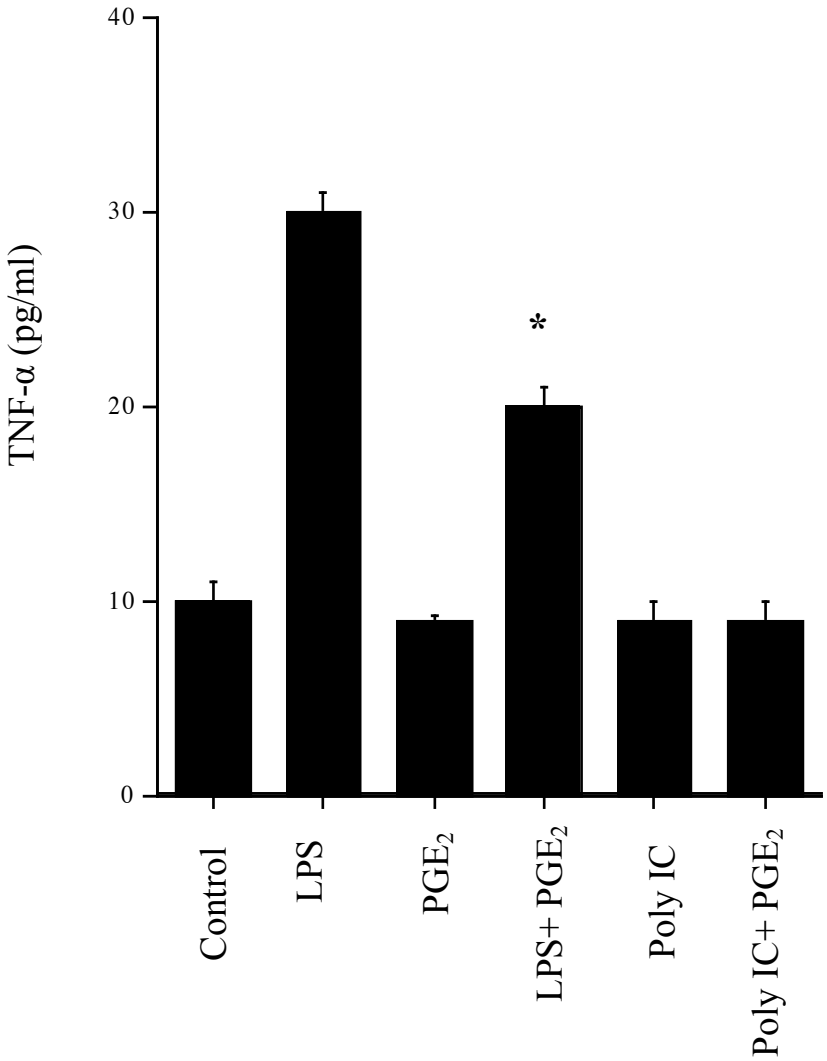


Fig. 3.65: The effect of PGE_2 on Poly IC- stimulated TNF-α Production in THP-1 cells.

Cells ($1x10^6$ cells/ ml) were placed into 6-well plates after which LPS (10 μg/ml), PGE_2 (1 μM), Poly IC (10 μg/ml) and culture medium (control) were added and cells incubated for 22 h at 37°C, 5% CO_2 and 100% humidity, after which the level of TNF-α in culture medium was measured by ELISA. Values are the means of n = 3 ± s.d (three separate observations). *P < 0.01 versus LPS alone.

3.15.4 Expression of PTGER2 and PTGER4 in response to poly IC in THP-1 cells

The production of TNF-α in Poly IC stimulated cells was validated in the last three Sections (3.15.1, 3.15.2 and 3.15.3). Since PGE_2 did not affect the TNF-α level in response to Poly IC (Section 3.15.3), it was decided to study the expression profile of EP receptors. THP-1 cells were incubated and processed as described in Methods Section 2.2.17.1. Fig. 3.66 showed EP2 receptor expression (PTGER2); stimulation cells with either LPS or LPS+PGE_2 resulted in an increase in the mRNA PTGER2. However, Poly IC had no effect on this expression level. PTGER4 expression was also studied. Poly IC did not affect the expression of EP4 receptor in THP-1 cells. However, PGE_2 significantly decreased the LPS stimulated receptor expression in these cells, $P < 0.001$ (Fig. 3.67). Therefore, Poly IC has no effect on either PTGER2 or PTGER4 expression. This was expected because Poly IC did not affect the TNF-α production in these cells as shown in Fig. 3.64; thus PGE_2 will be less likely to alter this expression in Poly IC stimulated cells.

In conclusion, previous sections (3.15.1 to 3.15.4) show that there might be a link between LPS and Poly IC in TNF-α production, because a high concentration of Poly IC (1000 μg/ml) induced a significant TNF-α inhibition in LPS-stimulated THP-1 cells. However, PGE_2 had no effect on TNF-α production in Poly IC-stimulated cells. Therefore, this finding suggests that PGE_2 will be less likely to affect EP receptor expression in the presence of Poly IC. Indeed, stimulation of THP-1 cells with Poly IC did not alter either PTGER2 or PTGER4. The reason for this suppression of the PGE_2 effect is not clear at present and warrants further investigation.

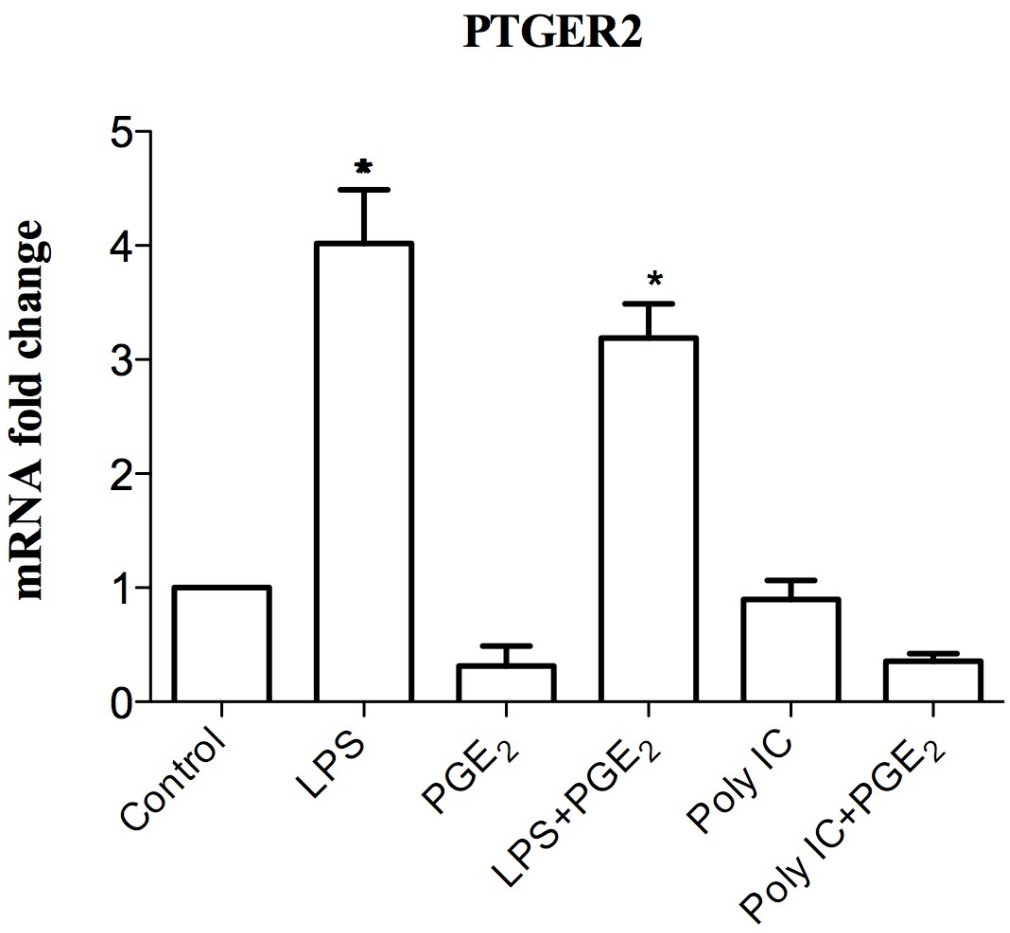


Fig. 3.66: Expression of EP2 receptor (PTGER2) in response to poly IC in THP-1 cells.

Cells ($1x10^6$ cells/ml) were placed into 6-well plates after which either LPS (10µg/ml) or Poly IC (10 µg/ ml), PGE_2 (1 µM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGER2 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER2 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations), *P < 0.01 versus control.

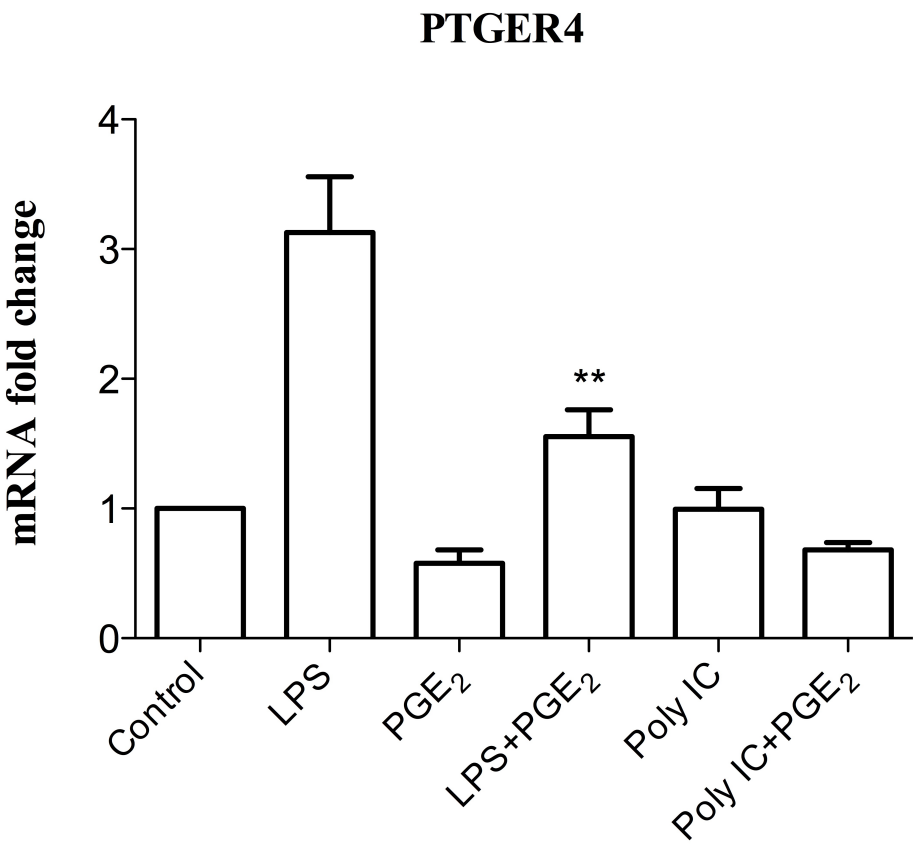


Fig. 3.67: Expression of EP4 receptor (PTGER4) in response to poly IC in THP-1 cells.

Cells ($1x10^6$ cells/ml) were placed into 6-well plates after which either LPS (10 µg/ ml) or Poly IC (10 µg/ ml), PGE_2 (1 µM) and culture medium (control) were added and cells incubated for 24 h. Total RNA was prepared from the cells. After reverse transcription, quantitative real-time PCR analysis was performed on the cDNA using selected primers for PTGER4 as described in Methods Section 2.2.17.10. Relative expression levels of PTGER4 mRNA transcripts were normalised to the reference gene PPIB using the delta-delta Ct method (Livak and Schmittgen, 2001). Values are the means of n = 3 ± s.d (three separate observations). **P < 0.001 versus LPS alone.

Chapter 4

Discussion

The aim of this study was to evaluate the role of individual PG receptors in the suppression/control of inflammatory cytokine production in human peripheral blood and in human monocytic cells including the human monocytic cell line THP-1. The prostaglandin receptors were studied both pharmacologically, using selective ligands to investigate how these receptors were involved in regulating cytokine production and this was followed by studying the expression profile of mRNA for PG receptors. This discussion will consist, in the first instance, of an evaluation of the methodologies used throughout the research work and will be followed by a discussion of the experimental data obtained in this study and compared to those from previous studies.

4.1 Evaluation of methodologies

4.1.1 The use of the human THP-1 monocytic cell line

The THP-1 monocytic cell line was originally derived from the blood of a patient with acute monocytic leukemia (Tsuchiya *et al.*, 1980). These cells are commonly used for modeling the function of human monocytes because they can display a mature monocytic phenotype (Qin, 2012). THP-1 cells follow a similar action pattern as human primary monocytes after stimulation with LPS. For instance, LPS-stimulated human primary monocytes increase production of cytokines, especially TNF-α, in a similar manner as LPS-stimulated THP-1 cells under identical experimental conditions (Schildberger *et al.*, 2013). It has been well established that at sites of inflammation, pathogen-derived molecules such as LPS result in monocytes being activated to produce pro-inflammatory cytokines (Sharif *et al.*, 2007; Chanput *et al.*, 2014). Therefore, to assess the effect of inflammatory

mediators on LPS-stimulated cells, THP-1 cells were chosen as a model for human monocytes. This was because using THP-1 cells has many advantages as described previously in the Introduction Section (Table 1.3). Isolation of monocytes from blood provided very low monocyte levels. It was also a very time consuming and labour-intensive process to isolate the monocytes from an extremely heterogenous cell population. A higher level of "pure" cells was obtained using THP-1 cells without the long laborious isolation process.

THP-1 cell lines might have some drawbacks in vitro. For instance, during differentiation of THP-1 macrophages, up-regulation of specific genes could mask the effects of particular stimuli. Another disadvantage of using THP-1 cell lines is that the sensitivity to LPS might increase through the differentiation process. It is possible that cell lines may respond differently compared to normal cells because cell lines are cultured outside their natural environment (Chanput *et al.*, 2014). However, all these drawbacks are less likely to occur in this study because the present work did not differentiate THP-1 cells. In the current study, LPS-stimulated THP-1 cells increased TNF-α (Fig. 3.2) and IL-1β production (Fig. 3.18) in a similar manner to normal monocytes and were thus deemed to be a valid model for the monocyte functions of interest in the present study.

4.1.2 Isolation of monocytic cells from whole human blood

It should be taken into consideration that some changes in gene expression might occur in cultured monocytes which may affect the LPS response. For this reason, the

present study investigated this by isolating monocytes with two common procedures: A) Using Ficoll density gradient centrifugation. B) Magnetic microbeads separation.

Several characteristics should be taken into consideration to perform an evaluation of monocyte isolation. For instance, the yield and purity of monocytes are essential throughout the isolation process. This may have an effect on the performance of isolated cells. In the current project, a concentration of 2×10^6 cells/ml was obtained by Ficoll density centrifugation. In contrast, a concentration of 3×10^5 cells/ml was obtained by magnetic separation to yield more cells with high purity (Zhou *et al.*, 2012). In general, the two procedures provided similar results in terms of cytokine production in LPS-stimulated cells. However, the low monocyte density which was obtained by magnetic separation (3×10^5 cells/ml) affected the stability of gene expression because using this low cell density to extract RNA provided a very low RNA concentration, subsequently, cDNA formation was correspondingly low also. Thus, mRNA expression data using monocytes isolated by magnetic separation were below the level of detection of the assay. On the other hand, the higher monocyte concentrations which were obtained by Ficoll density centrifugation (2×10^6 cells/ml) did provide a constant expression level. For this reason, it was decided to isolate monocytes using Ficoll density centrifugation and subsequent adherence rather than magnetic separation in the current project since it provided a high yield of cells that improved the reliability of the detection of gene expression. All monocytes used in this study were isolated by the Ficoll density method. The data for monocytes which were isolated by Magnetic microbeads separation were not shown because the mRNA expression was below the level of detection of the assay.

4.2 Lipopolysaccharide (LPS)-induced cytokine production

LPS is a major component of the outer membrane of gram-negative bacteria, which has been shown to modulate host immune responses (Raetz *et al.*, 1991; Gessani *et al.*, 1993; Rietschel *et al.*, 1994; Förster-Waldl *et al.*, 2005; Rossol *et al.*, 2011; Ngkelo *et al.*, 2012). LPS is recognised by immune cells through activation of their TLR4 which is a member of the pathogen associated molecular pattern family (PAMP) and has been characterised as one of the important sensors of the innate immune system (Medzhitov, 2001; Beutler, 2002). Binding of LPS to TLR4 triggers activation of intracellular signalling cascades including the NF-κB pathway via the adaptor molecule MyD88 (Förster-Waldl *et al.*, 2005). Following recognition, some metabolic and cellular alterations are elicited (Raetz *et al.*, 1991), in particular, the production of pro-inflammatory cytokines such as TNF-α and IL-1β which result in systemic and local inflammatory responses to infection (Underhill & Ozinsky, 2002; Heine & Lien, 2003). As monocytes play a central role in the response to bacterial infections, monocytes and THP-1 cells (a monocytic cell line) were chosen in this project to be target cells for the study of LPS-induced cytokine production.

LPS concentrations between 5-50 μg/ml have been found to induce a maximal production of TNF-α in monocytes (Kreutz *et al.*, 1997). Foster's research group (1993) also agreed that incubation of human blood with LPS in the 1-100 μg/ml range resulted in a concentration-dependant increase in TNF-α production (Foster *et al.*, 1993). Another previous study (Davidson *et al.*, 1998) indicated that incubation of monocytes with LPS caused a concentration-dependent increase in IL-1β production. LPS can also stimulate cytokines to be produced at very low

concentrations. LPS (1-10 pg/ml) may stimulate the production of these cytokines in monocytes and macrophages (Gessani *et al.*, 1993). A high level of LPS is more likely to induce sepsis as shown in Fig. 4.1. In the present study the effect of different LPS concentrations was evaluated on human blood, monocytes and THP-1 cells. The highest production of TNF-α was observed at 100 μg/ml (Fig. 3.1, Fig. 3.2 and Fig. 3.3). The present results also showed a release of IL-1β in response to LPS (10 μg/ml) (Fig. 3.6). This was in agreement with Brown *et al.* (2013) which showed that the highest increase in TNF-α production was observed with LPS concentrations between 10 μg/ml and 100 μg/ml (Brown *et al.*, 2013b). It was important to understand the dynamics of how the cells responded to LPS by producing cytokines (TNF-α/ IL-1β) for subsequent experiments studying the control of cytokine production by lipid mediators such as PGE_2. This will be discussed in detail in the following Sections.

LPS responses can be enhanced by priming monocytes with either granulocyte-macrophage colony-stimulating factor or gamma interferon. This leads to a production of IFN-α in response to LPS by monocytes which is required for their priming. After LPS stimulation, TNF expression is also increased in primed cells. TNF is readily expressed in unprimed cells as well, unlike IFN-α which can only be expressed in primed cells. Therefore, priming can affect LPS-induced gene expression at various levels in monocytes. However, in this study, isolated monocytes were used without priming since LPS does not induce IFN-α from these freshly isolated cells but LPS can produce cytokines such as TNF-α and IL-1β from unprimed monocytes as reported in previous studies (Hayes & Zoon, 1993).

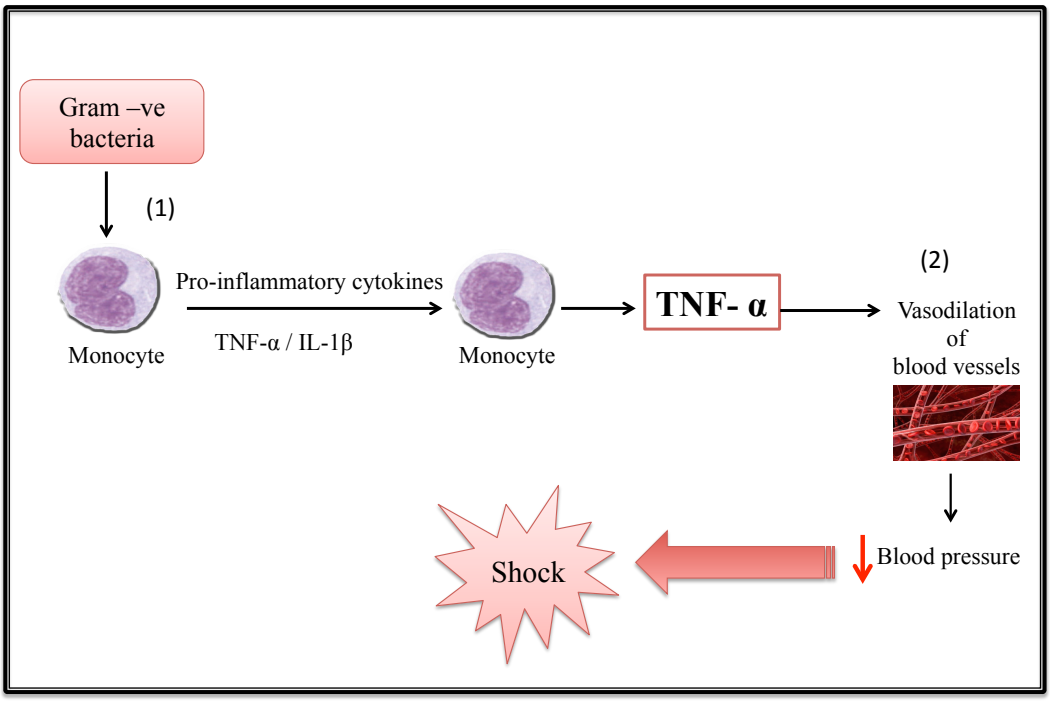


Fig. 4.1: Overview of bacterial induced septic shock.

(1) A high level of LPS can trigger the production of pro-inflammatory cytokines such as TNF-α and IL-1β. (2) The production of these cytokines causes vasodilation and ultimately leads to low blood pressure which is irrecoverable with consequent organ damage and death.

4.3 Modulation of cytokine production by prostaglandins

4.3.1 Inhibition of LPS-stimulated TNF-α and IL-1β production by prostaglandins

Inflammation is caused by a variety of stimuli including bacterial infection and immune reactions. During inflammation, immune cells such as monocytes migrate to the site of tissue injury or infection. TNF-α is one of the major mediators released from these cells and the over-production of TNF-α may lead to an inflammation-induced pathology. In the presence of infection involving Gram –ve bacteria, TNF-α is produced in response to LPS (Moreira-Tabaka *et al.*, 2012). This then leads to the synthesis of prostaglandins such as PGE_2, which are responsible for the end symptoms of inflammation. However, prostaglandins like PGE_2 tend to also inhibit LPS-stimulated TNF-α/ IL-1β production (Ignatowski *et al.*, 2000). This indicates a negative feedback regulatory loop for the control of pro-inflammatory cytokines. Many previous studies have reported that PGE_2 suppresses TNF-α production in a dose-dependent manner. It was observed that the inhibitory effect of PGE_2 was between 10 nM and 100 nM. Vassiliou's group (2003) examined the effectiveness of PGE_2 through different time courses and reported that PGE_2 can inhibit the induction of TNF-α when added 30 minutes before or at the same time as LPS. However, addition of PGE_2 30 minutes after LPS stimulation had no impact on TNF-α production (Vassiliou *et al.*, 2003). Therefore, it was decided in the current research to treat cells with PGE_2 at the same time as the LPS stimulation, as suggested in the Vassiliou *et al*., study (2003). As shown in Fig. 3.4, PGE_2 was able to induce a noticeable suppressive effect on the production of TNF-α at 1 μM and 10 μM in monocytes. Additionally, PGD_2 also inhibited this production at 1 μM. It was

decided to use 1 μM for both PGE_2 and PGD_2 in all experiments that were conducted throughout the study. The inhibition of TNF-α production in response to LPS was also determined in THP-1 cells and found that 1 μM of PGE_2 and PGD_2 reduced the cytokine's production significantly (Fig. 3.5). These data were comparable with a recent publication which showed that PGE_2 had the same potent inhibitory effect on TNF-α production in normal human monocytes and THP-1 cells (Brown *et al.*, 2013b).

Johansson's group investigated the function of PGE_2 in vivo via its EP2 receptor and it has been shown that in vivo, LPS infection resulted in cytokine production in plasma. This high level of cytokines was significantly reduced in the absence of EP2 receptors using EP2 deficient mice, suggesting that PGE_2 is a major effector of innate immune responses (Johansson *et al.*, 2013).

IL-1β is another critical inflammatory mediator produced in response to LPS (Baldie *et al.*, 1993). Davidson *et al*. (1998) confirmed that in the presence of PGE_2 (10 μM), IL-1β production was down-regulated in LPS-stimulated monocytes. It is likely that the inhibitory effect of PGE_2 could vary using different cells. A possible reason for differential PGE_2 sensitivity of monocytes and THP-1 cells is that PGE_2 binding sites may exist in some cells more than others. This suggests that the sensitivity difference to the suppressive effect of PGE_2 might depend on the number EP receptors (Kuroda *et al.*, 2000). This was investigated in this thesis by studying the effect of PGE_2 on LPS-stimulated monocytes and THP-1 cells. Indeed, the results in the present work were in agreement with these previous studies showing that LPS increased IL-1β

production and PGE_2 suppressed the induction of IL-1β in response to LPS in monocytes (Fig. 3.6). Although PGE_2 (1 μM) inhibited the IL-1β level in monocytes, different concentrations of PGE_2 were unable to induce a suppression of IL-1β production in THP-1 cells (Fig. 3.8). In addition, a wide range of PGD_2 concentrations also failed to inhibit the production of IL-1β in monocytes (Fig. 3.12).

By comparing the TNF-α data with IL-1β, it can be clearly seen that PGE_2 was more potent in suppressing the production of TNF-α in response to LPS compared to PGD_2 in mononuclear cells (Fig. 3.13). However, both PGE_2 and PGD_2 were able to supress the production of IL-1β in whole human blood (Fig. 3.11). The decrease in TNF-α/ IL-1β production by PGE_2 in both whole human blood and mononuclear cells provides an indication that the monocytic cells are an important source of these cytokines in blood and can be the target of PG-induced suppression. It appears that there was a difference in sensitivity of mononuclear cells to PGE_2 and PGD_2. A potential reason for this variance is that DP receptors are less potent in these cells than EP receptors. Another possible explanation could be that the inhibitory DP receptors are more likely to be a major PG receptors in specific cells such as mast cells (Chan *et al.*, 2000).

4.3.2 Evaluation of the use of prostaglandin analogues and their effects on cytokine production

Since PGE_2 suppressed LPS-produced TNF-α production in THP-1 cells and whole human blood, the current study evaluated which EP receptors contribute to this

inhibition by using selective EP agonists for each receptor, sulprostone for EP1/EP3, butaprost for EP2 and L-902, 688 for EP4. As shown in Fig. 3.26 and Fig. 3.27, TNF-α production was inhibited by both butaprost and L-902, 688, while sulprostone did not alter TNF-α production in whole human blood and THP-1 cells. This suggests that PGE_2 suppressed LPS-stimulated TNF-α production mainly through EP2 and EP4 receptors. This Section discusses EP receptor sub-types in greater detail.

After characterising the inhibitory effect of PGE_2 on TNF-α production, this was compared to that of PGE_2 agonists. In this study, three different EP receptor agonists (butaprost, L-902,688 and sulprostone) were used to assess which of the EP receptors played a key role in controlling cytokine production. Butaprost is a PGE_2 agonist which can act through the EP2 receptor and has been shown to supress cytokine levels (Walker & Rotondo, 2004). Baba *et al*. (2001) showed that butaprost is an adequately selective ligand for EP2 receptor with an IC_{50} between 3-10 μM as listed in Table 1.5 in the Introduction Section. Brown *et al*. (2013) imply that the EP2 receptor is mainly involved in the inhibition of TNF-α production by showing that butaprost significantly decreases the TNF-α level in response to LPS in both human blood and THP-1 cells (Brown *et al.*, 2013b). This observation was in agreement with the present work since obtained data illustrated that butaprost inhibited the TNF-α production (Fig. 3.21, Fig. 3.22 and Fig. 3.23) in all cell types studied. There are many previous studies that agree with Brown *et al.* (2013) in addition to the current research, and link the inhibition of TNF-α to the EP2 receptor. All of these authors examined the effects of butaprost on the LPS-induced production of TNF-α.

These studies are Meja *et al.*, 1997; Ikegami *et al.*, 2001; Xu *et al.*, 2008 and Johansson *et al.*, 2013. Meja *et al* demonstrated that butaprost inhibited TNF-α levels in LPS-stimulated human monocytes, whereas Ikegami *et al* found that butaprost suppressed TNF-α production in C3H/HeN macrophages. Xu *et al* showed that EP2 receptor suppressed IFN-β in response to LPS in J774A macrophages. The last study (Johansson's group) illustrated that EP2 receptors are the central mediators of innate immune responses in mice macrophages. Taken together, all these findings indicate that the EP2 receptor has a potent suppressive impact on LPS-induced TNF-α production. For this reason, it was decided to try butaprost as an EP2 agonist in the present study. The results shown here support the interpretation that the production of TNF-α is inhibited significantly by PGE_2 through the EP2 receptor in LPS-stimulated whole human blood and THP-1 cells (Fig. 3.26 and Fig. 3.27).

Some studies provide an indication that PGE_2 may also act through the EP4 receptor to inhibit cytokine production in monocytes, THP-1 cells and macrophages (Ikegami *et al.*, 2001; Brown *et al.*, 2013b; Pantazaka *et al.*, 2013). The involvement of the EP4 receptor in suppressing cytokine production was evaluated by Brown and colleagues (2013). They provided useful evidence by using an EP4 receptor-selective antagonist (AH 23848B) and found that the suppressive actions of PGE_2 were reversed by this antagonist in THP-1 cells (Brown *et al.*, 2013b). Another finding pointing to a role for EP4 receptors in the inhibition of TNF-α production was obtained using ONO-604 as an EP4 agonist which significantly decreased the TNF-α level in C3H/HeN macrophages (Ikegami *et al.*, 2001). These observations clearly implicate the EP4 receptor in the inhibitory action of PGE_2 on cytokine production.

Pantazaka *et al*. (2013) showed that L902, 688 is a selective EP4 agonist with a concentration of 10 μM and high affinity. This study revealed that the sensitivity of the functional response in inhibition of histamine-evoked Ca^{2+} signals in human smooth muscle cells (pIC50) for L902, 688 is 9.5 (10 nM) and its binding affinity (pKD) is 9.4 (Pantazaka *et al.*, 2013). This suggests that L902, 688 is an extremely selective agonist for EP4 receptors. Thus, the present study used L-902, 688 agonist to ascertain whether the EP4 receptor contributed to the suppression of TNF-α production by PGE_2. Certainly, as observed in Fig. 3.26 and Fig. 3.27, L-902, 688 produced a decrease in TNF-α levels in response to LPS in both whole human blood and THP-1 cells. Pantazaka *et al*. (2013) used L-902, 688 at 10 μM in their experiments (Pantazaka *et al*., 2013). In the current work L-902, 688 reduced the production of TNF-α in a concentration-dependent manner (Fig. 3.24). L-902, 688 at 10μM and100 μM induced a maximum inhibition effect on TNF-α level in response to LPS.

The potential action of EP1/EP3 receptors in the inhibition of TNF-α production was also evaluated using sulprostone as a PGE_2 agonist. A series of studies have shown that sulprostone acts via EP1/EP3 receptors (Matthews & Jones, 1993; Meja *et al.*, 1997; Vassiliou *et al.*, 2003; Brown *et al.*, 2013b). However, it appears that sulprostone does not suppress LPS-induced TNF-α (Vassiliou *et al.*, 2003). This finding is in parallel with other research which showed that sulprostone has no effect on the LPS-stimulated production of TNF-α in human blood (Brown *et al.*, 2013b). The current project assessed the effect of sulprostone on the production of TNF-α in whole blood and THP-1 cells in response to LPS. Sulprostone was not able to reduce

the TNF-α level in LPS-stimulated blood or THP-1 cells (Fig. 3.26 and Fig. 3.27) suggesting that monocytes do not express EP1/EP3 (Meja *et al.*, 1997). Several research groups used sulprostone with different concentrations. For instance, Matthews & Jones (1993) showed that the potency of sulprostone is about 4-10 nM in human platelet-rich plasma (PRP) (Matthews & Jones, 1993). A higher concentration of sulprostone (1 μM) was used to determine its effect on cytokine inhibition in monocytic cells (Brown *et al.*, 2013b). The present study used sulprostone at the same concentration as Brown *et al* (1 μM).

In comparing the EP receptor agonists used in the existing study (butaprost as EP2 agonist, L-902, 688 as EP4 agonist and sulprostone as EP1/EP3 agonist), it suggests that the inhibitory effect of PGE_2 on the production of TNF-α is most likely mediated through EP4 and EP2 but not EP1/EP3 receptors. Many previous publications demonstrated that EP ligands are associated with pathologies and therefore have potential therapeutic applications. These are illustrated in the following table.

Table 4.1: Potential therapeutic applications of EP analogues.

EP ligands	Experimental model	Therapeutic applications	Reference
EP1 antagonists (such as ONO-8711)	Acetic acid-induced inflammation in rat bladder	Inhibition of inflammation-related neuronal activity	(IKEDA *et al.*, 2006)
EP2 agonists (such as butaprost)	Isolated human uterine specimens from pregnant and non-pregnant donors	Prevention of preterm labor	(Duckworth , 2002)
EP3 agonists (such as TEI-3356) EP3 antagonists (such as L-826266)	Occlusion/reperfusion in rat Formalin induced hyperalgesia (formalin-injected mice)	Cardiac infarction Pain	(Zacharowski *et al.*, 1999) (Oliva *et al.*, 2006)
EP4 antagonists (such as ONO-AE3-208) EP4 agonists (such as ONO-4819)	Injection of M26 cells Ovariectomized rat	Reduction in lung and colon cancer metastasis Inhibition of osteoporosis	(Yang *et al.*, 2006) (Yoshida *et al.*, 2002)

In addition to EP receptor agonists, an FP receptor analogue (fluprostenol) was used in this project to examine its effect on cytokine production. A previous study using $PGF_{2\alpha}$ (up to 10 mM) was inactive in the inhibition of TNF-α production in human blood monocyte (Meja *et al.*, 1997). This was investigated in the current research to determine the involvement of FP receptors in the inhibition of cytokine production. Since the FP receptor binds fluprostenol with high affinity (K_i value is 3.7 nM) (Kiriyama *et al.*, 1997), Fluprostenol was chosen to be used as an FP agonist. Fig. 3.15 illustrates that fluprostenol decreased levels of TNF-α in whole blood in a concentration-dependent manner but it did not affect IL-1β production (Fig. 3.16). However, in THP-1 cells, a higher level of fluprostenol was required (10 μM) to inhibit TNF-α production significantly (Fig. 3.17). In contrast, the production of IL-1β in THP-1 cells could not be inhibited by concentrations of fluprostenol up to 10 μM (Fig. 3.18). In conclusion, fluprostenol did not affect the production of IL-1β in either whole human blood or THP-1 cells. However, it can induce a decrease in TNF-α level in blood and in THP-1 cells.

4.4 Evaluating the effect of endogenous PGE_2 on TNF-α production

Non-steroidal anti-inflammatory drugs (NSAIDs) such as Ketoprofen can block the production of endogenous prostaglandins especially PGE_2 (Vassiliou *et al.*, 2003; Brown *et al.*, 2013b). Since Ketoprofen stops the formation of endogenous PGE_2, Brown *et al*. (2013) validated the effect of Ketoprofen (50 μM) on TNF-α production in THP-1 cells and found that there was no effect indicating that there was no modulation by endogenous PGs (Brown *et al.*, 2013b). This may indicate that THP-1 cells do not produce endogenous PGE_2. Therefore, the inhibition of TNF-α

production after stimulation of THP-1 cells with LPS occurred due to the addition of exogenous PGE_2 only because any endogenous PGE_2 synthesis would already have been inhibited by ketoprofen. This finding is similar to the present results in this study, in which initial experiments were conducted to characterise the role of PGE_2 on TNF-α production in presence of ketoprofen. Monocytes and THP-1 cells were treated with LPS and exogenous PGE_2 both in the presence and absence of NSAIDs (ketoprofen 50 μM). The exogenous PGE_2 was added at the same time as LPS. It was expected to obtain a higher level of TNF-α in cells which were treated with ketoprofen more than untreated cells, because ketoprofen, by inhibiting endogenous PGE_2 production should, subsequently allow TNF-α production to be increased (Davidson *et al.*, 2008). However, it was found that the TNF-α production was at the same level both in the presence or absence of ketoprofen. Thus, it was decided that all cells used in this work would be incubated without ketoprofen.

4.5 Gene expression

4.5.1 Expression using cells-to-cycle threshold (Ct)

Gene expression quantification has become an important tool in molecular biological and biomedical research. However, consistent and reliable protocols and procedures should be selected. All expression data in this study were obtained using typical methods which are based on column RNA isolation (cell lysis, RNA extraction, RT and then qPCR). Such investigations are reliant of RNA isolation methods which will provide RNA of high-quality and of good yields. Column-based RNA extraction methods typically meet these needs. However, it was found that this assay gave low RNA yields from THP-1 cells. The inability to accurately determine low

concentration of RNAs may compromise the calculation of expression profiles. Therefore, an alternative expression assay method (Cells-to-Ct protocol, Life Technologies Ltd, UK) was attempted in order to achieve higher RNA recoveries from the cell numbers used and to see if this offered similar quality templates as qPCR. Several publications have reported that the Cells-to-Ct expression method delivers equivalent results to the traditional multi-step qRT-PCR procedure of separate RNA purification, cDNA synthesis, and qRT-PCR stages (Abruzzese *et al.*, 2010; Van Peer *et al.*, 2012). After trying both methods in the current work (Cells-to-Ct method and the traditional multi-step method), it was found that Cells-to-Ct assay was the most efficient in time and cost. The Cells-to-Ct ability to process directly through crude cell lysates eliminates many time-consuming and potentially inefficient separate steps of cell harvesting, RNA purification and cDNA synthesis. However, expression data obtained using Cells-to-Ct protocol with the THP-1 cells in this study suggested there was low RNA yield with poor quality (Data not shown because the expression was inconstant in results obtained). One possible reason for this might be the compatibility of the THP-1 cell line with the Cells-to-Ct technology. The manufacturer provides a list of 23 cell lines which are known to work with this protocol and THP-1 cells are not on the list. Therefore, all PCR expression data presented in this thesis were obtained by using the common gene expression workflow which includes an RNA isolation and not Cells-to-Ct.

4.5.2 Validation of reference genes

Reference genes, which are also commonly known as “housekeeping” genes, are likely to be stably or invariantly expressed in all cells under normal and experimental

treatment conditions. The reference genes are broadly used as internal controls and instrumental tools for calibration and normalisation of gene expression data in molecular biological studies. A number of candidate reference genes were identified by studying their expression (Eisenberg & Levanon, 2013). Quantitative real-time reverse transcription PCR (qRT-PCR) can quantify and validate the level of RNA expression of reference genes (Dheda *et al.*, 2004; Maess *et al.*, 2010; Cao *et al.*, 2012).

Glyceraldehyde-phosphate-dehydrogenase (GAPDH) is a common reference gene used in peripheral blood mononuclear cell culture and whole blood studies (Dheda *et al.*, 2004). Many studies normalise the qRT-PCR results to either GAPDH or beta actin (ACTB) in LPS-stimulated cells. However, these two reference genes (GAPDH and ACTB) have been shown to vary across samples and they are not stably expressed in many experimental settings (Maess *et al.*, 2010; Eisenberg & Levanon, 2013). An earlier study evaluated some candidate reference genes in LPS-stimulated THP-1cells including: Ribosomal Protein L37A (RPL37A), ACTB, GAPDH, Beta-2-Microglobulin (B2M), Peptidylprolyl Isomerase B (PPIB), TATA-binding protein (TBP), and Hypoxanthine Phosphoribosyltransferase1 (HPRT1) using qRT-PCR (Cao *et al.*, 2012). It was indicated that PPIB was the most stable reference gene in that research (Cao *et al.*, 2012). Another study involving LPS-stimulated monocytes emphasised the importance of validation of control genes where it was identified that PPIB and B2M are the most stably expressed genes in monocytes but ACTB is considered to be an unstable gene (Piehler *et al.*, 2010). In contrast, Maess's study (2010) which was conducted using THP-1 cells, found that ACTB is stably

expressed whereas PPIB and B2M are less stable genes (Maess *et al.*, 2010). In summary, two out of three separate studies strongly agree that PPIB is a stable reference gene (Piehler *et al.*, 2010; Cao *et al.*, 2012). However, Maess's study reveals the opposite finding which suggests that PPIB seems to have less stability (Maess *et al.*, 2010). The variation between these three publications highlights the need for precise validation of reference genes used in the current study. Validation of reference genes is critically important because the RNA expression levels of these reference genes may vary within cell types and experimental conditions i.e. stimulation (Cao *et al.*, 2012). Therefore, candidate internal reference genes in the present project were carefully evaluated before their use in gene expression studies with qRT-PCR. In order to correctly evaluate the results, a number of reference genes were chosen to be assessed. These were: PPIB, TBP, GAPDH, RPL37A, B2M, ACTB, and HPRT1 as listed in Table 2.4 (Methods Section).

Normalising the values obtained from different experiments was a challenge because improper analysis can lead to misinterpretation of entire results. However, all issues were fully addressed following an examination of their expression stability under experimental test conditions relevant to this particular study. RNA expression of these genes should not be significantly changed with the inflammatory stimulus, LPS, which was used here. The gene stability can be assessed using validation programs such as the geNorm, BestKeeper, and NormFinder which are recommended by many researchers and based on Microsoft Excel (Maess *et al.*, 2010; Cao *et al.*, 2012). The mRNA expression levels of all seven candidate reference genes used in this thesis (PPIB, TBP, GAPDH, RPL37A, B2M, ACTB and

HPRT1) were evaluated by qRT-PCR in THP-1 cells based on the above suggested conditions. It was found that PPIB is considered the most reliable reference gene compared to the rest of the genes because mRNA expression of PPIB is the most constant among different samples even in the presence of LPS. The stability of potential control genes can be determined by Normfinder, geNorm and BestKeeper using RefFinder web tool (http://fulxie.0fees.us/?type=reference)(Xie *et al.*, 2012). All reference genes used in the present study were also assessed through these validation programs (Vandesompele *et al.*, 2002; Andersen *et al.*, 2004; Pfaffl *et al.*, 2004). Normfinder is a qRT-PCR data normalisation tool which calculates the stability of each gene and ranks its expression according to the corresponding stability value. A higher value shows lower stability and vice versa (Cao *et al.*, 2012). The geNorm program outlines the expression stability of control genes by measuring the *M* value of each gene. The *M* value is a mean of the variation between a particular gene and all other reference genes used. A lower *M* value shows the most stable expression. It is generally agreed that genes with $M < 1.5$ should be considered as stable, reliable reference genes (Cao *et al.*, 2012). The other validation program is BestKeeper which defines the expression variability between a group of reference genes based on quantification cycle (Cq) values, coefficient of variance (CV) and the standard deviation (SD). It is suggested that genes with a $SD > 1$ are considered unstable control genes and should be rejected from any list (Cao *et al.*, 2012). Taking Normfinder, geNorm and BestKeeper-data analysis into account, PPIB was ranked as the most stable control gene tested in this present project because its *M* value was below 1.5 (*M* value of PPIB = 1) and had a low SD (SD of PPIB = 0.09) and all other

genes were excluded from consideration (see Table 3.3 and Table 3.4 - Results Section).

It should be noted that reference genes for THP-1 cells are not restricted to these genes only (PPIB, TBP, GAPDH, RPL37A, B2M, ACTB and HPRT1), other stable, expressed control genes could have been examined and assessed as to their suitability if time had permitted.

4.5.3 Expression of PG-receptor genes

4.5.3.1 Expression of PGE_2 receptors in THP-1 cells and monocytic cells

As discussed previously (Brown *et al.*, 2013b), PGE_2 exerts a switch-off in the upstream production of inflammatory cytokines in a negative feedback loop. PGE_2 can act via at least four receptor subtypes EP1–EP4 (Davidson *et al.*, 2012), but which of these receptors is important in controlling cytokine production still remains very unclear. This study focused on how these receptors are involved in controlling an inflammatory response to LPS by studying the expression of mRNA for the different EP receptors. EP receptor gene isoforms can be expressed by diverse inflammatory cells in response to LPS as a part of the immune system (Nataraj *et al.*, 2001; Akaogi *et al.*, 2004; Nakatani *et al.*, 2004). This project focused on EP2 and EP4 receptor genes because the current data show that their respective ligands (butaprost and L-902, 688) had a significant functional response in TNF-α suppression. The present results indicate that LPS induced an increase in the

expression of both receptor genes in THP-1 cells: EP4 receptor gene (PTGER4) and EP2 receptor gene (PTGER2)(Fig. 3.28 and Fig. 3.31), whereas EP1 and EP3 receptor genes (PTGER1, PTGER3) were below the level of detection of the assay (Data not shown). There are many previous publications that agree with the present data and reported that immune cells, which are activated by stimuli such as LPS, can result in a receptor expression up-regulation. For example, Nakatani's research (2004) demonstrated that the expression of EP4 mRNA was increased as a response to LPS stimulation in macrophages (Nakatani *et al.*, 2004). Moreover, two further studies also provided a good example of this by showing that LPS can induce an increase in the expression of both EP2 and EP4 mRNA and that PGE_2 is able to suppress the TNF-α level which is induced by LPS (Ikegami *et al.*, 2001; Treffkorn *et al.*, 2004).

Since both EP2 and EP4 gene expression are affected by LPS stimulation, the mechanisms of their regulation should be established. This could be addressed by studying the expression of their respective genes using EP receptor-selective agonists because the impact of PGE_2 on inflammation may vary depending on which EP receptor is involved. It has been suggested that EP4 and EP2 agonists might be useful for controlling the production of TNF-α as an inflammatory response (Akaogi *et al.*, 2004). Ligand studies which were conducted on THP-1 cells in this work showed that the EP4 receptor agonist (L-902, 688) resulted in a significant decrease in PTGER4 expression (Fig. 3.28). In contrast, with the EP2 receptor agonist (butaprost), there was no obvious decrease in the expression of mRNA of EP2 receptor gene (PTGER2) (Fig 3.31). The present results also imply that there is a

possibility of cross-regulation between EP2 and EP4 receptors because the EP4 receptor agonist (L-902, 688) decreased the expression of EP2 receptor gene (PTGER2) significantly in response to LPS (Fig. 3.29) and can regulate its own expression at the same time by decreasing the expression of PTGER4 (Fig. 3.28). However, the EP2 receptor agonist (butaprost) did not decrease the expression of the EP4 receptor gene (PTGER4) in response to LPS (Fig. 3.30) and also could not induce a clear decrease in its own receptor gene expression (PTGER2) (Fig 3.31). The effect of the EP1/EP3 receptor agonist (sulprostone) on EP2 receptor gene (PTGER2) was also studied in order to ascertain whether there is cross-regulation between EP1/EP3 receptor and EP2 receptor gene expression. Sulprostone did not have any effect on EP2 receptor gene expression in response to LPS (Fig. 3.32). This may indicate that there was no cross-regulation between EP1/EP3 and EP2 receptors, unlike the possible cross-regulation that appears to exist between EP2 and EP4 receptors as described above.

The expression of EP receptors in normal human monocytes was also evaluated in this study by analysing mRNA of EP receptors. LPS induced a three-fold increase in EP2 receptor gene (PTGER2) expression but neither PGE_2 nor L-902, 688 decreased this LPS-stimulated level (Fig 3.33). On the other hand, LPS stimulation did not increase the EP4 receptor gene (PTGER4) compared to the untreated sample in monocytes (Fig. 3.34). A possibility may be that endogenous PGE_2 could be released during the incubation and may have reduced the LPS-stimulated expression level. To address this issue, ketoprofen, a potent non-steroidal anti-inflammatory agent was used. Ketoprofen was added to the culture medium in order to prevent the production

of endogenous PGE_2 but this did not alter the expression level. There was no change observed in LPS-induced expression either in the absence or presence of ketoprofen. This may indicate that LPS up-regulation of PTGER4 expression does not occur in monocytes. The effect of endogenous PGE_2 on TNF-α production was discussed previously in Section 4.4.

Taken together with the findings for TNF-α production, the EP4 receptor seems to be the major inhibitory regulator for TNF-α production compared to other EP receptors as discussed previously in Section 4.3.2. Ikegami *et al*., (2001) provide strong evidence that the 2 receptors regulate TNF-α production at different times following stimulation. EP4 agonists can inhibit the early production of TNF-α (3 hours after LPS stimulation), whereas EP2 agonists have no early effect because it may require a longer time to suppress cytokine production in mouse macrophages (Ikegami *et al.*, 2001). The current project examined the effect of both EP2 and EP4 receptor agonists during a longer time of incubation (24 hours after LPS treatment). It was found that the EP4 receptor agonist (L-902, 688) is a potent inhibitor of TNF-α production during these 24 hours (Fig. 3.28), while the EP2 receptor agonist butaprost is not as effective as L-902, 688 (Fig. 3.31). It is, therefore, possible that the EP4 receptor is the major anti-inflammatory regulator because of how it suppresses the TNF-α production. Any genetic alteration such as deletion or mutation in the EP4 receptor in monocytes may result in a compromised suppressive action on TNF-α levels leading to disease (Yokoyama *et al.*, 2013). This is also supported by Nataraj's study (2001) who also indicated that PGE_2 acts mainly through the EP4 receptor to suppress cytokine production in mouse macrophages

(Nataraj *et al.*, 2001). However, in mutated EP4 cells, PGE_2 has no effect on LPS-stimulated TNF-α induction which can be referred to as a deficiency of EP4 receptor. This observation is in agreement with the current study which also evaluated the effect of silencing the EP4 receptor using siRNA on TNF-α production. It was found that there was a change in TNF-α level after EP4 knock-down. The production of TNF-α was significantly increased after down-regulation of the EP4 receptor in THP-1 cells (Fig. 3.45). The noticeable increase in TNF-α production indicates that the EP4 receptor plays a crucial role in controlling the cytokine production because in the presence of EP4 receptor, the TNF-α level was much less compared to EP4 knock-down cells.

The regulatory actions of PGE_2 in monocytic cells such as THP-1 cells may involve more than one receptor. Using a combination of pharmacological and genetic approaches in the present study, there was evidence of cross-regulation between EP2 and EP4 receptors regarding cytokine suppression. This indicates that both EP2 and EP4 receptors are likely to cooperate with each other to induce an inhibitory response toward cytokine production. However, one of the prominent suggestions in this study is that PGE_2 mainly acts through the EP4 receptor to supress TNF-α production. The EP4 receptor could be the focus of a novel therapeutic strategy in inflammation and autoimmune diseases, especially by using an L-902, 688-like agonist which was well studied in this project. EP ligands seem promising for future approaches because they tend to have therapeutic implications. For instance, in the case of chronic inflammation, the expression of mRNA of EP4 is up-regulated, whereas there is a low expression of EP2 and no noticeable gene expression of

EP1/EP3 (Yokoyama *et al.*, 2013). An early study also agreed with this and supported the finding that there is a significant gene expression of EP4 and EP2 receptor mRNA, while the mRNA levels of EP1 and EP3 are hardly detectable in human alveolar macrophages (Ratcliffe *et al.*, 2007). This suggests that EP4 mRNA is abundant in stimulated cells. Evidence for using selective EP agonists has also been reported by the Yamane group (2000) who examined the effect of both EP2 and EP4 receptor agonists on TNF-α production and determined that the EP4 agonist (ONO-AE1-329) has an effective inhibitory effect on TNF-α production in EP2-deficient cells in mouse neutrophils (Yamane *et al.*, 2000). However, the effect of EP2 agonist (ONO-AE1-259) on TNF-α in EP4-deficient cells is not as potent as the EP4 agonist. All these observations indicate that the EP4 receptor may be a dominant suppressive regulator for LPS-stimulated TNF-α production.

As demonstrated in this thesis, the EP4 receptor agonist (L-902, 688) inhibited LPS-stimulated TNF-α production. Based on a high selectivity for the EP4 receptor and a lack of side-effects (indicated by other studies), these ligands could potentially be used therapeutically for various inflammatory diseases. The EP4 receptor seems to be an effective regulator of inflammatory processes. One of the main concerns about the use of PGE_2 analogues systemically is their vasodilatory activity. EP4 receptors seem to mediate vasodilation of the human middle cerebral artery. Davis's study (2004) demonstrated the involvement of prostaglandin receptors in the relaxation and contraction of human arteries. It was shown that EP4 receptors are most likely to mediate PGE_2-induced vasodilation of human middle cerebral artery. This was reinforced in the same study by using an EP4 receptor antagonists (AH23848), it was

shown that AH23848 reduced the relaxation effects and resulted in a significant shift in the PGE_2-mediated vasodilation. In contrast, the effect of EP2 receptors on vasodilation of human artery was also verified by using EP2 agonist (butaprost) but found that butaprost failed to induce a relaxation in cerebral arteries (Davis *et al.*, 2004). It seems that vasodilation, which is caused by EP4 receptor, is not restricted to cerebral arteries. It has also been demonstrated that EP4 receptors are involved in the vasodilation of aortic rings. This was shown using both the EP4 receptor antagonist (ONO- AE3-208) and the EP4 agonist (ONO-AE1-329). It was found that there were significant relaxations of aortic rings after using the EP4 agonist (AE1–329) but this was dependent on endothelial nitric oxide synthase (eNOS), indicating that the relaxation originates from the endothelium and also requires cyclic GMP. The relaxations were abolished by the EP4 antagonist (AE3–208). In contrast, other EP receptors (EP1, EP2 and EP3) seem less likely to be involved in aorta vasodilation, because no expression of these receptors was detected (Hristovska *et al.*, 2007). This would be a disadvantage in the use of EP4 agonists and possibly therapeutic development would be focused on attempting to prevent their vasodilator actions while maintaining their cytokine-suppressing actions. However, in certain circumstances this could be advantageous in the dual treatment of inflammation and high blood pressure.

EP4 receptor agonists could also be used as therapeutic agents in other diseases. For instance, EP4 ligands could be used in treatment of airway resistance in asthma. It has been shown that PGE_2 induces relaxation of human bronchi and these relaxations are blocked by the EP4 antagonist (GW627368X). Similarly, the EP4 agonist (L-902,

688) was shown to induce relaxation of human bronchi. In comparison, using the EP2 agonist (ONO-AE1-259), there was lower relaxation observed, suggesting that the EP2 receptors are less potent in relaxing human bronchi. Therefore, EP4 receptor agonists might be used as a potential treatment for either asthma or increased airway resistance (Benyahia *et al.*, 2012). The bronchodilator actions of EP4 ligands would also be a beneficial action in the use of these compounds to treat inflammatory disease.

EP4 receptors have a wide range of other potential therapeutic uses. For this reason, EP4 drug development programs to examine EP4 receptor agonists and antagonists have been evaluated in clinical research (Konya *et al.*, 2013b). There are possible outcomes of using both EP4 receptor agonists and antagonists as novel drugs (Table 4.2). For instance, the EP4 receptor mediates vasodilation of human artery, which might be associated with headache (Zimecki, 2012). EP4 ligands could also be useful in the inhibition of platelet aggregation and thrombus formation (Zimecki, 2012). Moreover, Arthritis can be modulated via inhibition of LPS-induced TNF-α production in macrophages (Zimecki, 2012). Central nervous activity appears to be regulated by EP4 receptors; this would be promising for Alzheimer's patients (Konya *et al.*, 2013b). Konya's research (2013) provided evidence that using EP4 antagonists such as ONO-AE3-208 in a murine model of Alzheimer's disease resulted in an improvement by inducing a decrease in amyloid-β levels (Konya *et al.*, 2013b). Another consequence of using EP4 receptor ligands is the inhibition of tumor progression, EP4 receptors can act as a tumor suppressor by providing a negative feedback signal to reduce cell proliferation (Konya *et al.*, 2013b). A final

potential outcome could be for patients who have kidney diseases. A delay in the progression of chronic renal failure was induced by reducing serum creatinine levels using EP4 agonist treatment (Konya *et al.*, 2013b).

Table 4.2: Regulation of various pathologies using EP4 receptor agonists or antagonists as novel drugs.

Target	**Outcome associated with effects of using EP4 receptor ligands**
Blood vessels	Vasodilation of human artery (headache)
Platelets	Inhibition of platelet aggregation and thrombus formation
Arthritis	Inhibition of LPS-induced TNF-α production in macrophages
Alzheimer's disease	Regulation of central nervous activity (decrease amyloid-β levels)
Cancer	Inhibition of tumor progression
Kidney diseases	Inducing a delay in the progression of chronic renal failure

4.5.3.2 Expression of PGD_2 receptors in THP-1 cells and mononuclear cells

PGD_2 is another arachidonic acid metabolite released from immune cells during an inflammatory response. PGD_2 can contribute to the inflammation by enhanced cytokine production (Hata & Breyer, 2004; Xue *et al.*, 2005). PGD_2 is also involved in other roles, it can mediate many reactions such as inhibition of platelet aggregation, mucus secretion and muscle relaxation/contraction (Hata & Breyer, 2004). The pro-inflammatory effects of PGD_2 seem to be mediated by binding two different G-protein-coupled receptors (GPCRs): DP1 receptor and DP2 receptor which is also called chemoattractant receptor expressed on Th2 cells (CRTH2 receptor). PGD_2 binds to both receptors with high affinity (Hata & Breyer, 2004; Xue *et al.*, 2005; Arima & Fukuda, 2011; Ricciotti & FitzGerald, 2011). Although the CRTH2 receptor and the DP1 receptor have similar affinity for PGD_2, the CRTH2 receptor can bind to more diverse ligands compared to the DP1 receptor. For instance, the CRTH2 receptor has the ability to bind the PGD_2 metabolite (15-deoxy-$\Delta^{12, 14}$-PGJ_2) but not the DP1 receptor (Hata & Breyer, 2004).

At sites of allergic inflammation, PGD_2 is produced by mast cells to activate consequent inflammatory responses by interaction with the DP1 receptor and CRTH2 receptor (Xue *et al.*, 2005). It has been reported that PGD_2 can also be produced by other immune cells such as antigen-presenting dendritic cells and type 2 T-helper lymphocytes (Th2 cells), indicating that PGD_2 may have an antigen-specific immune system role (Hata & Breyer, 2004). The present study demonstrates the expression of DP1 receptor gene (PTGDR1) and DP2 receptor gene (PTGDR2) in

THP-1 cells and human monocytes after LPS activation to compare their expression with EP receptors and see which of these prostaglandin receptors may be involved in controlling pro-inflammatory cytokine production. However, there was no gene expression detected for either the DP1 or DP2 receptors in THP-1 cells and monocytes. Therefore, it was decided to perform a further experiment to study the expression of DP receptors in mononuclear cell preparations. These peripheral blood mononuclear cells contain a mix of immune cells such as monocytes and lymphocytes including NK cells, T cells, and B cells. Interestingly, mixed mononuclear cells expressed both receptor genes (PTGDR1 and PTGDR2) (Fig. 3.38 and Fig. 3.39), suggesting that cells other than monocytes are involved in this expression. It would therefore be possible that activation of mixed mononuclear cells can stimulate different signalling pathways depending on which DP receptors are locally expressed (Ricciotti & FitzGerald, 2011). It has been revealed that the expression of DP1 receptor gene is lower than CRTH2 expression in immune cells such as TH2 cells and monocytes (Arima & Fukuda, 2011). The present data gave an indication that the expression of DP1 receptor gene (PTGDR1) can be lower than DP2 receptor expression (PTGDR2) in response to LPS (Fig. 3.38 and Fig. 3.39); these results were considered to be from preliminary experiments because they were conducted once and need to be developed in future. Another indication obtained was that PGD_2 can induce a decrease in LPS-stimulated PTGDR2 (Fig. 3.39) but there was only a small decrease in LPS-stimulated PTGDR1 by PGD_2 (Fig. 3.38). However, all these observations can only be indicative, qualitative data for future work. This thesis mainly focuses on the expression of EP receptors.

4.5.4 Effect of PGE_2 on TLR4 and TNF-α expression in THP-1 cells

Immune cells play an important role by initiating a rapid inflammatory response towards pathogen-derived products such as the gram-negative bacterial cell wall component LPS involving the release of cytokines, especially TNF-α. Pathogens can be recognised by a family of pattern recognition receptors (PRRs) termed Toll-like receptors (TLRs). TLR4 is the receptor for LPS and any modulation of its activity or expression would have an impact on cytokine production. Lipid mediators such as PGE_2 have the ability to inhibit pro-inflammatory cytokine production as shown extensively in the present study and others (Degraaf *et al.*, 2014). Zarember and Godowski (2002) highlight the importance of TLR4 and cytokine release during the activation of innate immune systems in THP-1 cells (Zarember & Godowski, 2002). In order to determine the intracellular targets of PGE_2 action, part of the present project focused on the mRNA expression for both TLR4 and TNF-α in LPS-stimulated THP-1 cells.

A previous study reported that PGE_2 inhibited the TLR4 signalling pathway rapidly (Kim *et al.*, 2011). This was in parallel with recent research which has shown a decrease in TLR4 expression in response to LPS and this was mediated by PGE_2 (Degraaf *et al.*, 2014). Indeed, the data obtained in the current project illustrated that there was a decrease in the mRNA level of TLR4 in LPS-stimulated THP-1 cells following treatment with PGE_2 (Fig. 3.59). This graph also shows a reduction of mRNA of TLR4 by butaprost (EP2 agonist) in response to LPS. However, the EP4 agonist (L-902, 688) increased the expression of TLR4. In addition to these two EP agonists, the EP1/3 agonist (sulprostone) also resulted in a decrease in TLR4 as with

EP2. This may suggest that the inhibition of TLR4 by PGE_2 is controlled by EP2, EP1/3 and not EP4 receptors. Since, mRNA expression for TLR4 was inhibited by the EP2 agonist (butaprost) and EP1/3 (sulprostone) and not by the EP4 agonist (L-902, 688) after LPS stimulation (Fig. 3.59), it was decided to knock-down the expression of the EP4 receptor (PTGER4) by transfecting siRNA into THP-1 cells. This was to investigate whether the reduction of EP4 receptor expression had different effects on TLR4 expression. Interestingly, it was found that after silencing PTGER4, the expression profile of TLR4 was changed (Fig. 3.60). A deficiency of EP4 receptor expression resulted in an increase in the mRNA of TLR4 expression. It would therefore be possible that the EP4 receptor plays a dual function because L-902, 688 (EP4 agonist) increased TLR4 (Fig. 3.59) and knock-down EP4 also resulted in an increase of TLR4 as well (Fig. 3.60). It is likely that this particular receptor could initiate other signalling pathways that require switching-on TLR4.

This current data is consistent with other published studies. For instance, it has been demonstrated that the suppression of TLR4 was mediated via EP2 receptor not EP4 by using EP2 and EP4 antagonists in alveolar macrophages (Degraaf *et al.*, 2014). Another study has shown this also, where suppression of TLR4 expression occurs via either EP1 or EP2 receptors in microglia cells (Keene *et al.*, 2009).

It has been investigated that the decrease in TLR4 expression caused by PGE_2 was adequate to reduce TNF-α in response to LPS in macrophages (Degraaf *et al.*, 2014). On the basis of this strong correlation between TLR4 and TNF-α, it was decided that it would be interesting to evaluate the expression of mRNA for TNF-α in the present

research (Fig. 3.61 and Fig. 3.62). It would thus be useful to treat LPS-stimulated THP-1 cells with PGE_2 and the various receptor agonists. It was found that only PGE_2 and the EP4 agonist (L-902, 688) were able to reduce the expression level of TNF-α in response to LPS (Fig. 3.62). However, other EP receptor agonists, EP2 agonist (butaprost) and EP1/3 agonist (sulprostone) failed to induce a suppressive effect on TNF-α expression (Fig. 3.61). These findings indicate that PGE_2 can inhibit TNF-α produced in response to LPS through the EP4 receptor which seems to be the key component involved in this suppression.

These expression data for TNF-α were in parallel with results obtained from the measurement of TNF-α levels. The effect of PGE_2 on TNF-α concentration was also demonstrated earlier in this thesis (Fig. 3.27). Despite the observation that showed a decrease in TNF-α expression by PGE_2 and the EP4 agonist and not by other EP receptor agonists, TNF-α concentration was reduced by PGE_2 and both agonists of EP4 (L-902, 688) and EP2 (butaprost) in THP-1 cells stimulated with LPS as shown in Fig. 3.27.

As TLR4 can initiate TNF-α production in LPS-activated THP-1 cells, it was decided to evaluate the level of mRNA for TNF-α expression. Interestingly, PGE_2 can inhibit both TLR4 and TNF-α expression in response to LPS. Recent research supports these observations obtained in the present study by demonstrating that PGE_2 was produced in response to stimulation of TLR4 by LPS in alveolar macrophages (Degraaf *et al.*, 2014). The suppression of TNF-α could be via reduction in TLR4. However, this

research did not determine whether reduced TLR4 mRNA can lead to a reduction in the receptor protein.

4.6 Gene silencing

4.6.1 Gene knockdown by transfection of exogenous small interfering RNA (siRNA)

siRNA delivery systems can cause both intended effects and unintended results. The intended effect represents the silencing of the target gene, whereas unintended actions include immune stimulation, toxicity and off-target gene silencing (Kanasty *et al.*, 2012).

In 1998, Fire and his colleagues discovered that double-stranded RNA molecules (dsRNAs) had an interesting action in producing post-transcriptional gene silencing by interfering with RNA (Fire *et al.*, 1998). RNA interference (RNAi) is a useful approach in targeting the expression of specific genes in order to modulate their function. An example of an RNAi process is gene silencing which is initiated by small molecules called small or short interfering RNAs (siRNAs). The RNase III-type enzyme (Dicer) cleaves double-stranded RNA molecules (dsRNAs) into smaller 21- 23 nucleotides (siRNA) (Bernstein *et al.,* 2001; McManus & Sharp, 2002). These small dsRNAs assemble to create an RNA-induced silencing complex (RISC). RISC binds to the target RNA to form the guide-strand and silence gene expression by cleaving the target RNA. The target RNA cleavage occurs between the 10th and 11th nucleotides which are located at the 5' end of the guide-strand. Once appropriate complementarity exists between the guide and target strand in the cleavage location,

the target RNA is degraded (Fig. 4.2) (Hammond *et al.*, 2000; Bernstein *et al.*, 2001; Elbashir *et al.*, 2001b; Rana, 2007; Shan, 2010).

Elbashir *et al.,* (2001) demonstrated that 21-nucleotide siRNA duplexes play an important role in suppression of the expression of endogenous and heterologous genes in many cell lines. This can greatly enhance the study of gene functions (Elbashir *et al.*, 2001b). Studies have revealed that this potent technique of gene silencing is an efficient method to produce gene knockdown in mammalian cells (Elbashir *et al.*, 2001a; McManus & Sharp, 2002; Mocellin & Provenzano, 2004). In particular, this gene silencing method has also been shown to be effective in cultured monocytic cells such as THP-1 (Elbashir *et al.*, 2001a; Cioca *et al.*, 2003; Maratheftis *et al.*, 2007; Landry *et al.*, 2012). In this current project, this gene silencing was chosen to transfect THP-1 cells with siRNA duplexes.

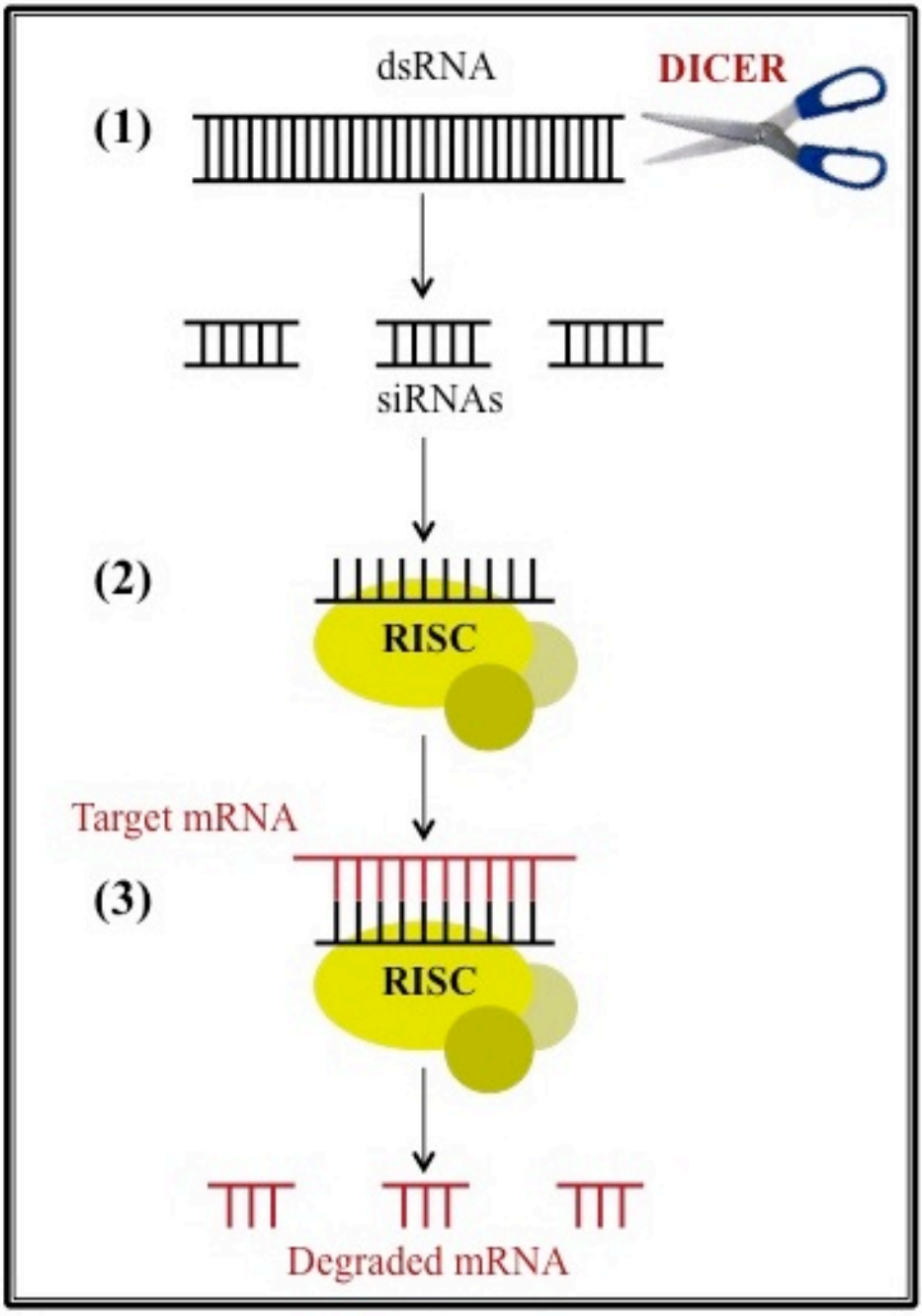


Fig. 4.2: Small interfering RNAs (siRNAs) mechanism.

(1) Dicer enzyme cleaves double stranded RNA (dsRNA) to produce siRNAs. (2) The anti-sense of siRNA binds to RISC. (3) Target mRNA was identified by RISC to be degraded.

4.6.2 Optimising siRNA duplexes and transfection solution

Elbashir and colleagues demonstrated that synthetic siRNAs (21 bp) could mimic the natural siRNAs product which result from cleavage of dsRNA with Dicer (Elbashir *et al.*, 2001a). SiRNA duplexes can be synthesised to order by a number of commercial suppliers. In this project, a TriFECTa RNAi kit (Integrated DNA Technologies, UK) was selected which contained three different siRNA duplexes (Duplex 1, 2, and 3) targeted on PTGER4 knockdown, in addition to negative and positive controls. The present data in the Results Section (Section 3.8.3/Fig. 3.41) shows that both siRNA Duplex1 and Duplex 3 were able to down-regulate EP4 receptor expression. Based on this result, Duplex 3 induced greater PTGER4 knockdown than Duplex1. However, the use of Duplex 2 resulted in the recording of an"undetermined Ct value" for PTGER4 expression as measured by qRT-PCR under the standard assay cycling conditions (results not shown). This may indicate that siRNA Duplex 2 completely abolished EP4 receptor expression. The undetermined Ct value prevents the accurate calculation of the fold change of PTGER4 expression following duplex to treatment. siRNA Duplex 3 exhibited a lesser but measurable significant knock-down on PTGER4 expression than Duplex 2 and was used in all the subsequent experiments. Additionally, a negative control (NC5) was used as the starting point for optimising the PTGER4-targeting siRNAs and comparing the effectiveness of siRNA duplexes in silencing EP4 receptors. As a negative control, it was expected that this would not affect the PTGER4 expression. Indeed, when control THP-1 cells were treated with NC1 and NC5, both had no effect on PTGER4 expression. However, when THP-1 cells were treated with LPS, the PTGER4 expression changed from 14.3 fold up-regulation (non-transfected cells) to 7.8 fold

and 6.8 fold with NC1 and NC5 respectively. As NC1 had a lesser effect on PTGER4 expression, NC1 was used as a negative siRNA transfection control in all knock-down experiments. Moreover, control THP-1 cells were also treated with positive control siRNA which had no effect on PTGER4 expression but when cells were treated with LPS, the positive control decreased the PTGER4 expression to 2.3 fold. Therefore, this positive control siRNA was used in all subsequent silencing assays (Results Section 3.8.6/ Fig. 3.44).

siRNA complexes need to be transfected into cells through the help of a transfection agent (Bertrand *et al.*, 2002; Khvorova *et al.*, 2003). Qiagen's HiPerFect transfection reagent has been used successfully for this role (Fischer *et al.*, 2010). It has been shown that HiPerFect can successfully transfect sufficient levels of siRNA into cells to produce gene silencing (Pallet *et al.*, 2008). Thus, in this study, Qiagen's HiPerFect was chosen as the transfection reagent. Following Qiagen guidelines, HiPerFect efficiency was optimised by using 3µl, 6µl, and 9µl of HiPerFect with siRNA duplexes. Since all of these three HiPerFect volumes helped siRNAs to induce a potent PTGER4 knock-down, it was suggested to use the lowest one (3µl) in all subsequent knock-down assays to minimise the potential toxicity which might occur by using a higher concentration of HiPerFect (Results Section 3.8.4/ Fig. 3.42).

4.6.3 Optimising gene silencing method

To obtain an effective transfection and gene silencing, there were essential parameters that were taken into consideration. Khvorova and colleagues (2003) established that siRNAs are considered efficient once the level of gene knock-down

reaches 70% (Khvorova *et al.*, 2003). In this study, if an undetermined Ct value from the duplex 2 treatment of THP-1 cells causes a complete abolition of PTGER4 expression, then duplex 2 has met Khvorova's criteria of siRNA capable of an efficient knock-down. The knock-down percentage was assessed for duplex 1 and duplex 3. It was found that the maximal knock-down (97.5%) was achieved by duplex 3, whereas 72.3% knock-down was obtained by duplex 1. Therefore, duplex 3 was used in all knock-down assays (Results Section 3.8.3/ Fig. 3.41).

Another important factor was the impact of HiPerFect reagent alone on gene silencing. HiPerFect should not affect the expression profile. This was assessed in (Results Section 3.8.3/ Fig. 3.41). The control expression levels of PTGER4 in either the presence/ absence of HiPerFect were similar. One of the most important criteria that had to be considered was cell toxicity. siRNAs and HiPerFect reagent should not induce toxicity in THP-1 cells. This was evaluated by measuring the amount of viable THP-1 cells using MTT assay without HiPerFect and in the presence of HiPerFect alone. Indeed, transfection of THP-1 cells with HiPerFect and siRNA duplexes did not decrease cell viability compared to the control incubations (untreated cells)(Results Section 3.8.1/ Table 3.6). Therefore, it is unlikely that either HiPerFect or siRNA duplexes induced cell toxicity.

Transfection of cells with siRNAs can result in off-target effects (Jackson & Linsley, 2010). Off-target effects of siRNAs were first recognised by Jackson's group (Jackson *et al.*, 2003). The length of dsRNA, could also contribute to off-target effects as long dsRNA may induce mammalian interferon response (Robbins &

Rossi, 2005; Reynolds *et al.*, 2006). The length of siRNAs used in this study is a typical size for commercially available siRNA duplexes. It has been recommended that it is possible to minimize off-target effects by using different concentrations of siRNA (Bridge *et al.*, 2003; Moss & Taylor, 2003; Sledz *et al.*, 2003; Cullen, 2006). For this reason, three concentrations of siRNA duplex (1nM, 10nM and 100nM) were chosen for optimisation. Fig. 3.43 (Results Section 3.8.5) shows that siRNA (100 nM and 10 nM) induced high levels of specific gene silencing.

A further vital feature should be considered during gene silencing i.e. the time point at which knock-down might occur. Qiagen's optimisation of transfection THP-1 cells with the siRNA protocol was followed. In a previous work, it has been determined that gene knock-down can be anywhere between 24-96 hours post-transfection for mammalian cells (Chang *et al.*, 2012). In the present study (Fig. 3.40 in Results Section 3.8.2), five different time points (3, 6, 12, 24, and 48 hours) were chosen to ascertain the best PTGER4 knockdown time. Among these time points, the highest gene silencing was seen at 24 hours and 48 hours which was compatible with the range (between 24-96 hours) as previous studies (Chang *et al.*, 2012).

4.6.4 siRNA and immunity

In addition to mediating siRNA through RNA interference pathways, siRNA molecules can be recognised by the innate immune system as foreign molecules. Inducing siRNAs into endosomes allow endosomal TLRs to produce immune responses, whereas delivering siRNAs into the cytoplasm leads to RNAi pathway. Toll-like receptors (TLR3, TLR7, and TLR8) and other cytoplasmic receptors

(protein kinase R (PKR) and RIG-I) are involved in this immune recognition. TLR3 recognises double stranded RNA, whereas TLR7/8 are activated by single stranded RNA (Mogensen, 2009). Other receptors such as PKR can block protein translation (Moss & Taylor, 2003). In the presence of different forms of siRNA, RIG-I induces a strong interferon response (Kanasty *et al.*, 2012). However, the activity of the immune system towards siRNA can be avoided by introducing some modifications. siRNA sequence, structure, and chemistry are important features that influence immune stimulation (Mocellin & Provenzano, 2004; Kanasty *et al.*, 2012). For instance, siRNA sequences which are rich in guanosine and uridine bases tend to induce immunostimulatory activity, but this stimulation is decreased once the presence of uridine is lowered. The innate immune response can also be reduced without affecting RNAi potency by the ribose ring located on the RNA backbone, whereas TLR7 and TLR8 are inhibited by 2'-O-methyl group (Kanasty *et al.*, 2012). siRNA circulation levels and elimination depend on its formulation. Unmodified siRNA is rapidly degraded by RNAses to be cleared from bloodstream, but modified siRNA is protected from nucleases (Kanasty *et al.*, 2012).

4.7 Research approaches using overexpression of EP4 receptors

Overexpression of target genes has become a powerful tool to identify the importance of pathway components by analysing changes in end-functional parameters. A number of molecular mechanisms ensure that genes are usually expressed at the appropriate level under specific conditions. An increase in wild-type gene expression results in modification of the target gene's function. An important factor in overexpression studies is gene dosage which can affect overexpression

mechanisms (Prelich, 2012). Therefore, in the current project, a high copy number of the EP4 gene was evaluated in order to ascertain whether this would change the receptor's function in terms of cytokine suppression.

Target proteins can be regulated via the overexpression of signalling pathways that control the targets. This could be useful in identifying potential drug targets (Prelich, 2012). The limitations of any drug activity could be overcome through overexpression of its target control protein. Another overexpression application is outlining the loss of functional phenotype of genes of interest. Three possible outcomes should be taken into consideration in terms of the loss of function. Firstly, overexpression may cause opposite phenotypes by inducing a functional deletion. Secondly, overexpression can mimic the wild-type gene at same level of function and produce an identical phenotype. The third possibility is that there is no obvious change in an overexpression phenotype possibly because of pre-existing proteins with a long half-life (Prelich, 2012).

Overexpression studies have important implications for human diseases. There are various examples that show the way in which diseases are a direct result of either an increase in gene expression (Shastry, 1995; Santarius *et al.*, 2010) or changes in the level of its expression patterns (Carroll, 2008). Therefore, determining the correct levels of expression is essential for gene therapy approaches. In this study, the EP4 receptor gene (PTGER4) was overexpressed in THP-1 cells to ascertain its functional role in cytokine production.

4.8 Protein expression of FLAG-tagged EP4 receptor (FLAG-PTGER4)

As discussed previously, the mRNA expression of exogenous EP4 plasmid DNA seemed to not be an appropriate approach to demonstrate whether the EP4 construct was overexpressed in THP-1 cells, because of its instability of expression. Therefore, it was decided to find another way to resolve this in order to ascertain that the exogenous EP4 plasmid DNA had been introduced and overexpressed effectively in cells. In the present study, Western Blot was chosen to determine the protein expression of both FLAG-PTGER4 and empty vector. By using this approach, it was intended to establish the overexpression by comparing the FLAG-PTGER4 transfected THP-1 cells with untransfected cells. It was expected that THP-1 cells transfected with FLAG-PTGER4 plasmid DNA would have higher fold stimulation than untransfected cells.

Any EP4 produced as a result of transfection with FLAG-tagged construct could be detected using two different antibody approaches. The first one targets the FLAG (anti-FLAG antibody) and is indicative of newly-expressed EP4 receptor protein and the second binds directly to EP4 receptor (anti-EP4 antibody) indicating the presence of total EP4 receptor protein (new and existing). In addition to these antibodies, anti-GAPDH antibody was used as a loading control. The predicted molecular weight obtained was 53 KDa, since the molecular weight of EP4 receptor tagged with FLAG is 53 KDa. However, protein staining with anti-FLAG antibody in THP-1 cells indicated a lower molecular weight (20 KDa)(Fig. 3.53A) though quantification of the FLAG-EP4 blot gave a promising indication that cells were transfected with

FLAG-EP4 because of the increase in its fold stimulation compared to untransfected THP-1cells; however, these values represent only an individual experiment and require further validation (Fig. 3.53 B). It appears that anti-FLAG antibody is not specific for targeting the FLAG tag that is attached to EP4 receptors because it was not of the expected molecular weight (20 KDa rather than 53 KDa). One possible reason for this mismatching may be the existence of either cross-reactive binding or binding to sequences similar to FLAG but unrelated to the EP4 construct. In order to validate this hypothesis, anti-FLAG antibody was assessed with another cell line (SH-SY5Y) which had not been transfected with any construct. Interestingly, it was found that anti-FLAG staining was also observed at 20 kDa as in THP-1 cells (Fig. 3.55). This may indicate that the anti-FLAG antibody did not bind to denovo FLAG-tagged PTGER4.

In addition to anti-FLAG antibody, anti-EP4 antibody was also used in the current research for staining protein in THP-1 cells. Indeed, by using this antibody, the FLAG-EP4 protein was detected at the expected range of molecular weight (53 KDa) (Fig. 3.54A). Blot quantification using anti-EP4 antibody shows an increase in FLAG-PTGER4 stimulation compared to untransfected THP-1cells. This could be a promising result (Fig. 3.54B), suggesting that EP4 was overexpressed in THP-1 cells. However, the data was obtained from only an individual experiment and consequently can only be regarded as indicative.

Regarding the empty vector which was synthesised to use as a negative transfection control, bands should not be observed as this plasmid had no EP4 receptor

sequences. However, surprisingly, with both antibodies (anti-FLAG and anti-EP4 antibodies) in addition to the loading control (anti-GAPDH antibody) the empty vector showed increased signal (Fig. 3.53A and Fig. 3.54A). This unexpected expression could be due to the presence of a remaining FLAG-PTGER4 insert that was not digested by the XhoI enzyme, leading to a false positive signal with an anti-FLAG antibody compared to control (untransfected cells).

After demonstrating the presence of EP4 protein using a Western Blot approach and how its quantification compared to untransfected cells, a further experiment was performed to quantify the FLAG-tagged protein in THP-1 cells using a semi-ELISA method by staining protein bands on nitrocellulose membranes with Ponceau S red dye. The stained bands were cut from the blot for protein quantitation by semi-ELISA using a biotin labelled antibody (EP4 conjugate) followed by incubation with streptavidin and TMB. This was then quantitated by measuring the absorbance as would be used for an ELISA assay (Methods Section 2.2.18.5). It was found that this method was not sufficiently precise because all protein band absorbance values were at a similar level. All absorbance was between 1.5-2 with anti-FLAG and anti-EP4 antibodies in THP-1 cells (Fig. 3.56B). However, quantification by normalising transfected THP-1 cells to untransfected cells displayed clear variations between controls and transfected samples (Fig. 3.53B and Fig. 3.54B).

It would therefore appear that protein staining using anti-EP4 antibody was more likely to indicate expression of the construct (EP4 receptor) in THP-1 cells transfected with FLAG-PTGER4, because the protein bands were expressed at their

correct predicted molecular weight (53 KDa). In contrast, anti-FLAG antibody was not specific and unable to show any expressed FLAG-PTGER4 at the required expression level in transfected THP-1 cells. It appeared to act non-specifically in other cells also which had not been transfected with any construct (SH-SY5Y cells). The protein bands were expressed at 20 KDa instead of 53 KDa. Therefore, the overexpression of EP4 plasmid determined by anti-EP4 antibody (Fig. 3.54B) would tend to indicate that there was a higher level of EP4 receptor indicating an overexpression following treatment with the EP4 plasmid.

4.9 Evaluation of binding of PGE_2 to THP-1 cells

As the general aim of the work presented here was to study the effect of PGE_2 through its receptors on the release of TNF-α in THP-1 cells, it was fundamental to examine that PGE_2 was actually binding to THP-1 cells. This was achieved by treating THP-1 cells with biotin-labeled PGE_2. Selection of biotin-labeled PGE_2 was an appropriate choice because biotin (a common vitamin that exists in cells) has the ability to bind to streptavidin-HRP conjugate protein with high affinity (Hirsch *et al.*, 2002; Holmberg *et al.*, 2005; Chivers *et al.*, 2011). THP-1 cells were not incubated with any treatment and used as control to validate this binding study. These control cells showed no binding signals (Fig. 3.57A). Once streptavidin-HRP conjugate protein attached to biotin, protein signals were visualised using enhanced chemiluminescence (ECL) (Fig. 3.57B), as this reagent is the most popular detection systems used in immunoblotting assays (Mruk & Cheng, 2011). On the other hand, THP-1 cells which were treated previously with PGE_2 alone then labelled with biotin-PGE_2 showed no binding signals when streptavidin-HRP conjugate protein

was added into the cells. This indicates that binding of PGE_2 to THP-1 cells at an early stage (before labelling) displaced biotin-PGE_2 preventing it from becoming attached (Fig. 3.57C). Therefore, this study clearly shows that PGE_2 does indeed bind to THP-1 cells.

4.10 EP receptor localisation in THP-1 cells

Initially it was attempted to visualize EP receptors on THP-1 cells by Epi-fluorescence microscopy after labelling cells with PGE_2-biotin followed by streptavidin-HRP conjugate and luminescent substrate (Methods Section 2.2.20.1) but no luminescence was detected. A possible reason for this could be that much of the PGE_2-biotin may have been washed off during washing steps, thus streptavidin-HRP conjugate would not have been bound to cells. Therefore, it was decided to use an anti-EP4 receptor antibody conjugated to FITC (Methods Section 2.2.20.3). As shown in Fig. 3.58, there were clear fluorescence signals which indicated EP4 antibody binding to EP4 receptors on THP-1 cells membrane. It appears that in Fig. 3.58, there is an aggregation of fluorescence in the cell membrane at specific points in the membrane and that it was not evenly distributed. It is not certain why this occurred. A possible explanation for this is that EP4 receptors are aggregating at localised points in the membrane. It is not clear what process would cause this. In other studies, proteins may aggregate at sites in the membrane prior to cell division. Protein aggregation might occur as a normal consequence of a cellular response to an imbalanced protein homeostasis leading to the deposition of misfolded proteins at specific sites. This protective step is essential to maintain cell function and allow protein distribution to daughter cells through cell division (Tyedmers *et al.*, 2010;

Zhou *et al.*, 2014). Since active translation is essential for protein aggregation, Zhou's research examined the existence of these aggregates by treating cells with a translation inhibitor, cycloheximide (CHX), before induction of protein aggregation and established that incubating cells with CHX led to suppression of aggregate formation. This increases the possibility that new synthesized polypeptides may involve aggregate formation (Zhou *et al.*, 2014). It is possible that in THP-1 cells the EP4 receptor may be involved in a similar manner possibly the initial stages of cell division.

4.11 Modulation of Poly IC in THP-1 cells

The current study mainly used LPS to stimulate THP-1 cells to release TNF-α. In order to ascertain whether this was restricted specifically to LPS another stimulus was also used. Poly IC was chosen as it acts via TLR3 (an intracellular TLR).

Poly IC appears to be similar to LPS in stimulating the production of TNF (North *et al.*, 1991). The effect of Poly IC on TNF-α production was also studied by Reimer *et al*. (2008) and found that incubation of human macrophages with Poly IC resulted in the production of cytokines including TNF-α , which is similar to the LPS response (Reimer *et al.*, 2008). However, Reimer's group observed that TNF-α in response to Poly IC was also released in the absence of NF-κB activation (Reimer *et al.*, 2008). This may indicate that TNF-α can be released in an indirect manner as a response to Poly IC. In comparison to previous studies the present data indicated that Poly IC stimulated THP-1 cells to increase the production of TNF-α in a concentration-dependent manner (Fig. 3.63).

Poly IC can also induce production of IFN-β following binding of Poly IC by TLR3 to activate IRF3 via TRIF cascades (a MyD88-independent signalling pathway). IFN-β can also be produced through LPS-stimulated TLR4 via the MyD88-independent pathway, because LPS is able to promote IRF3 and NF-κB to release IFN-β and TNF-α respectively (Fig. 4.3)(Yamamoto *et al.*, 2003a; Bagchi *et al.*, 2007; Reimer *et al.*, 2008). Since Poly IC and LPS have a common pathway (activation of IRF3 via TRIF), this indicates that they are related. For that purpose, an additional experiment in this work was performed to investigate the link between LPS and Poly IC in TNF-α production by stimulating THP-1 cells with LPS in the presence of different concentrations of Poly IC (Fig. 3.64). Interestingly, it was found that a high concentration of Poly IC (1000 μg/ml) suppressed LPS-induced TNF-α levels, suggesting that activation of TLR3 may antagonise the production of TNF-α via activation of TLR4 pathways. Jiang *et al.* (2005) supported this finding by establishing that Poly IC induced TLR3 activation can inhibit LPS via down-regulation of TLR4 expression (Jiang *et al.*, 2005).

It has been extensively demonstrated in this thesis that PGE_2 inhibited LPS-induced TNF-α in monocytic cells. This was compared to the effect of PGE_2 in Poly IC-stimulated THP-1 cells. However, as shown in Fig. 3.65, PGE_2 had no impact on TNF-α production in response to Poly IC indicating that there was no suppression observed in TNF-α levels. It is possible that the effect of PGE_2 on TNF-α may be mediated by the MyD88-dependent pathway and not by the TRIF pathway, because TNF-α production was inhibited by PGE_2 in response to LPS and not Poly IC, even though Poly IC can lead to TNF-α production via a different pathway [MyD88-

independent pathway (TRIF pathway)] in mouse macrophages (Fig. 4.3)(Bagchi *et al.*, 2007; Cui *et al.*, 2013) but PGE_2 did not suppress this TNF-α level in response to Poly IC as shown in Fig. 3.65. The expression of PTGER2 and PTGER4 in the presence of Poly IC was measured (Fig. 3.66 and Fig. 3.67) and there was no significant change in the expression of either of these genes in THP-1 cells.

It appears that although Poly IC and LPS are recognised by different TLRs, they can trigger the production of the same cytokine, indicating that there was an overlapping response. This is because Poly IC as well as LPS activates NF-κB to induce TNF-α release and both of them can induce IFN-β through IRF3 activation.

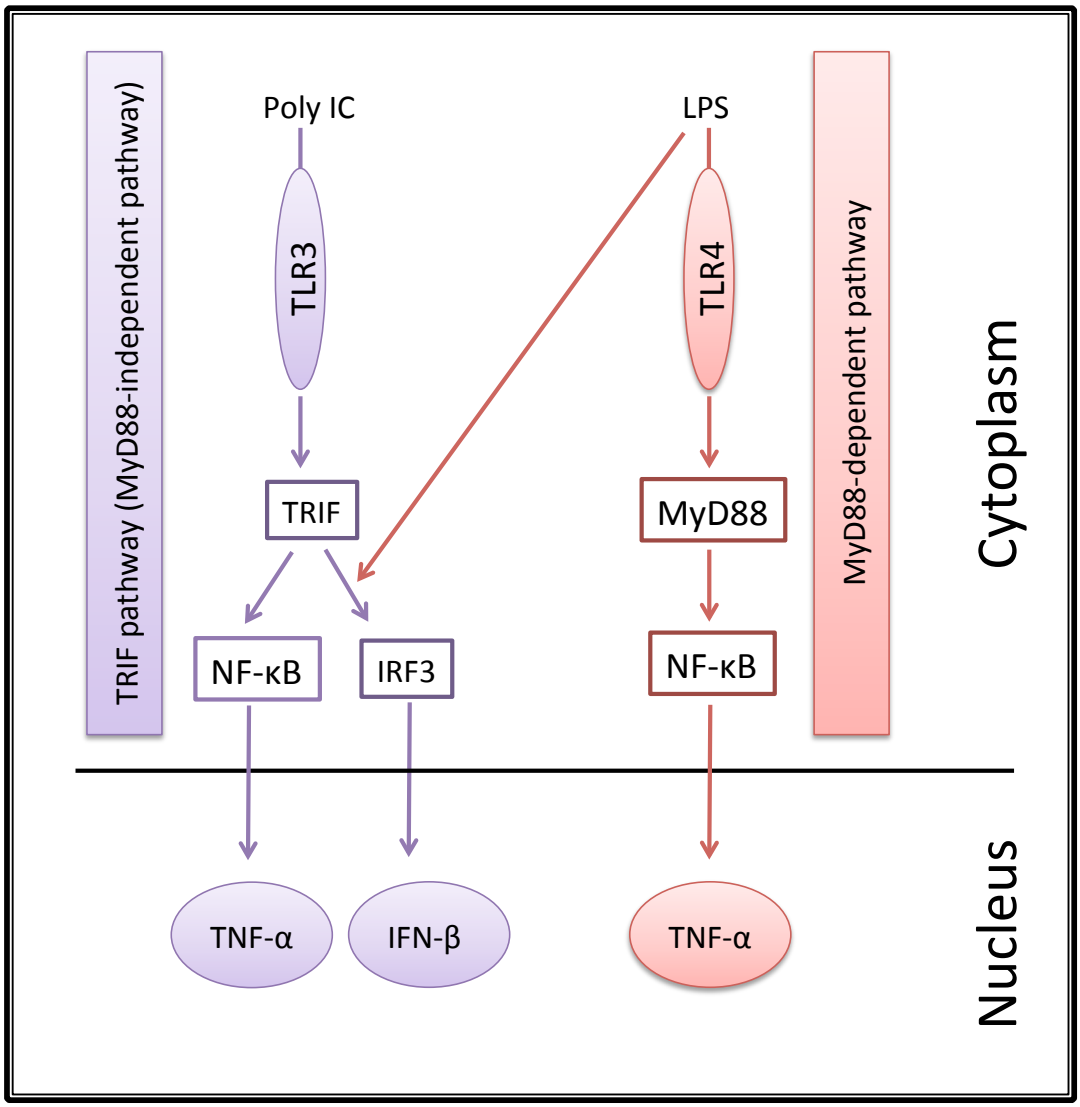


Fig. 4.3: Overview of Poly IC and LPS signalling pathways.

LPS mainly activates TLR4 through MyD88 molecule to promote NF-κB for TNF-α production. However, LPS can trigger the TRIF pathway (MyD88-independent pathway) to induce IFN-β. Poly IC is recognised by TLR3 to activate TRIF via IRF3 for IFN-β production. Poly IC can also stimulate NF-κB to induce TNF-α.

Conclusion

The work reported here shows that LPS can activate whole blood, blood monocytes and THP-1 cells to release pro-inflammatory cytokines (TNF-α/ IL-1β). Cytokine production was suppressed in the presence of PGs especially PGE_2. The production of IL-1β was inhibited by PGE_2 in whole human blood, peripheral blood monocytic cells (PBMCs) and mixed mononuclear cells. However, there was no suppression of IL-1β level observed using THP-1 cells. Prostaglandins (PGE_2/ PGD_2) were more potent in suppression of the production of TNF-α in LPS-stimulated cells compared to IL-1β. For this reason, this thesis concentrated mainly on the inhibition of TNF-α production using PGE_2 and its receptor agonists.

PGE_2 can act through any one of four receptor subtypes EP1–EP4. Functional experiments were conducted to ascertain which subtypes are specifically involved in the suppression of cytokine production using a pharmacological approach by using selective ligands for each receptor. The data in the present study showed that both EP2 and EP4 receptor agonists (butaprost and L-902, 688) had a suppressive effect on the production of TNF-α. However, sulprostone, an EP1/3 receptor agonist did not alter TNF-α levels. It was also shown that the activation of monocytic cells in the presence of LPS, indicated that PGE_2 and the various receptor-selective ligands are strongly involved in the inhibition of mRNA for EP receptors especially EP2 and EP4 receptor genes (PTGER2 and PTGER4). This is because PTGER2 and PTGER4 were inhibited by both PGE_2 and L-902, 688, which suggests that there is cross regulation between EP2 and EP4 receptors. PTGER1 and PTGER3 were below the level of detection of the assays used, indicating that these two receptors are not

expressed in monocytes or THP-1 cells suggesting that they are highly unlikely to be involved in cytokine inhibition in these cells.

Further research was conducted to ascertain which receptors were most important by characterising the effects of siRNA (for the EP4 receptor) on TNF-α production. Following knock-down of the receptor, using siRNA, the TNF-α levels significantly increased. In addition, cells transfected with an EP4 receptor construct resulted in a decrease in the production of TNF-α indicating that overexpression of the EP4 receptor is more efficient in switching-off TNF-α production. This suggests a novel role for the EP4 receptor in the suppression of cytokine production.

This study has provided new insights into the understanding of the way in which pro-inflammatory cytokines are suppressed via EP receptors especially the EP4 receptor. The following diagram illustrates the main contribution of the current thesis.

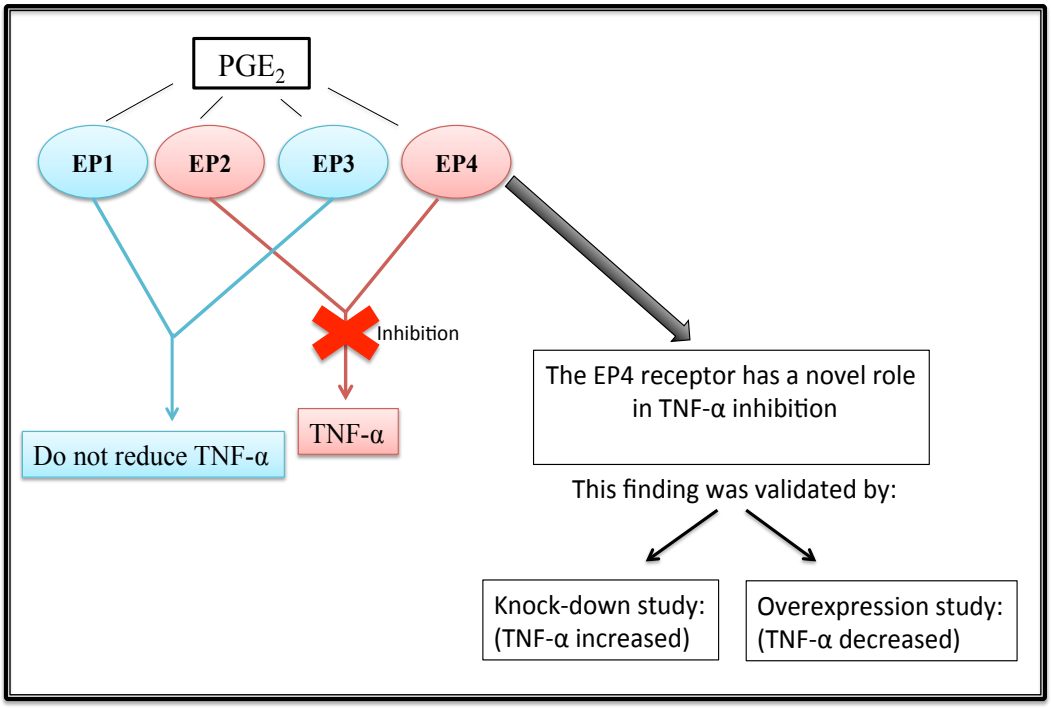


Fig. 4.4: Overview scheme of the effect of PGE_2 and its receptors on cytokine production (TNF-α).

Chapter 5

Appendices

5.1 MIQE check list (The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines)

ITEM TO CHECK	IMPORTANCE	CHECK LIST
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	**E**	✓
Number within each group	**E**	✓
Assay carried out by core lab or investigator's lab?	D	✓
Acknowledgement of authors' contributions	D	-
SAMPLE		
Description	**E**	✓
Volume/mass of sample processed	D	✓
Microdissection or macrodissection	**E**	-
Processing procedure	**E**	✓
If frozen - how and how quickly?	**E**	samples were used
If fixed - with what, how quickly?	**E**	directly
Sample storage conditions and duration (especially for FFPE samples)	**E**	-

NUCLEIC ACID EXTRACTION		
Procedure and/or instrumentation	**E**	✓
Name of kit and details of any modifications	**E**	✓
Source of additional reagents used	D	✓
Details of DNase or RNAse treatment	**E**	✓
Contamination assessment (DNA or RNA)	**E**	✓
Nucleic acid quantification	**E**	✓
Instrument and method	**E**	✓
Purity (A260/A280)	D	✓
Yield	D	✓
RNA integrity method/instrument	**E**	✓
RIN/RQI or Cq of 3' and 5' transcripts	**E**	✓
Electrophoresis traces	D	RQI
Inhibition testing (Cq dilutions, spike or other)	**E**	-
REVERSE TRANSCRIPTION		
Complete reaction conditions	**E**	✓
Amount of RNA and reaction volume	**E**	✓

Priming oligonucleotide (if using GSP) and concentration	**E**	✓
Reverse transcriptase and concentration	**E**	✓
Temperature and time	**E**	✓
Manufacturer of reagents and catalogue numbers	D	✓
Cqs with and without RT	D	-
Storage conditions of cDNA	D	✓
qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay.	**E**	Efficiency
Sequence accession number	**E**	✓
Location of amplicon	D	✓
Amplicon length	**E**	✓
In silico specificity screen (BLAST, etc)	**E**	✓
Pseudogenes, retropseudogenes or other homologs?	D	✓
Sequence alignment	D	-
Secondary structure analysis of amplicon	D	-
Location of each primer by exon or intron (if applicable)	**E**	-
What splice variants are targeted?	**E**	-

qPCR OLIGONUCLEOTIDES		
Primer sequences	**E**	✓
RTPrimerDB Identification Number	D	-
Probe sequences	D	-
Location and identity of any modifications	**E**	-
Manufacturer of oligonucleotides	D	✓
Purification method	D	✓
qPCR PROTOCOL		
Complete reaction conditions	**E**	✓
Reaction volume and amount of cDNA/DNA	**E**	✓
Primer, (probe), Mg++ and dNTP concentrations	**E**	✓
Polymerase identity and concentration	**E**	-
Buffer/kit identity and manufacturer	**E**	✓
Exact chemical constitution of the buffer	D	-
Additives (SYBR Green I, DMSO, etc.)	**E**	✓
Manufacturer of plates/tubes and catalog number	D	✓
Complete thermocycling parameters	**E**	✓
Reaction setup (manual/robotic)	D	✓
Manufacturer of qPCR instrument	**E**	✓

qPCR VALIDATION		
Evidence of optimisation (from gradients)	D	✓
Specificity (gel, sequence, melt, or digest)	**E**	✓
For SYBR Green I, Cq of the NTC	**E**	✓
Standard curves with slope and y-intercept	**E**	-
PCR efficiency calculated from slope	**E**	✓
Confidence interval for PCR efficiency or standard error	D	✓
r2 of standard curve	**E**	✓
Linear dynamic range	**E**	-
Cq variation at lower limit	**E**	-
Confidence intervals throughout range	D	-
Evidence for limit of detection	**E**	-
If multiplex, efficiency and LOD of each assay.	**E**	✓
DATA ANALYSIS		
qPCR analysis program (source, version)	**E**	✓
Cq method determination	**E**	✓
Outlier identification and disposition	**E**	✓
Results of NTCs	**E**	✓
Justification of number and choice of reference genes	**E**	✓

Description of normalisation method	**E**	-
Number and concordance of biological replicates	D	✓
Number and stage (RT or qPCR) of technical replicates	**E**	✓
Repeatability (intra-assay variation)	E	✓
Reproducibility (inter-assay variation, %CV)	D	✓
Power analysis	D	-
Statistical methods for result significance	**E**	✓
Software (source, version)	E	✓
Cq or raw data submission using RDML	**D**	✓

Table 5.1: MIQE checklist for authors, reviewers and editors.

All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available.

5.2 OmicsLink™ Expression Clone Datasheet of EX-Q0086-M11

Clone Information

Catalog No.: EX-Q0086-M11

Accession No.: NM_000958

ORF Length: 1467 bp

Whole Plasmid Size: 7322 bp

Description: Homo sapiens prostaglandin E receptor 4 (subtypeEP4) (PTGER4), mRNA.

Vector: pEZ-M11 **Antibiotic:** Ampicillin

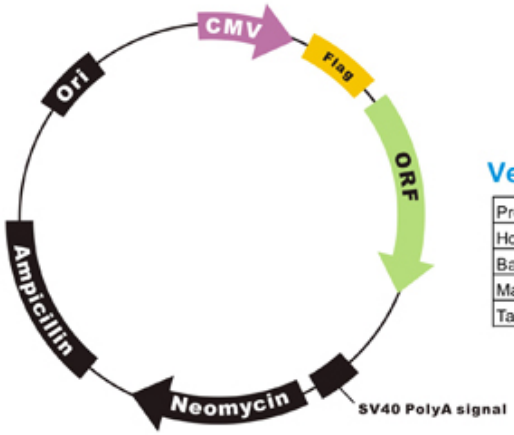
Stable Selection Marker: Neomycin

Suggested Sequencing Primers:

Forward: 5'-CAGCCTCCGGACTCTAGC-3'

Reverse: 5'-TAATACGACTCACTATAGGG-3'

Vector Information for EX-Q0086-M11



Vector Features

Promoter	CMV
Host Cell	Mammalian
Bacterial selection antibiotic	Ampicillin
Mammalian selection marker	Yes
Tag	Flag

>EX-Q0086-M11 [with 502F_B11]

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5.3 Work presented at scientific meetings

Alaa Kashmiry, Rothwelle Tate and Dino Rotondo (2014). Regulation of Prostaglandin receptor expression in human monocytic cells following inflammatory activation. SIPBS research day. University of Strathclyde, Glasgow, UK.

Alaa Kashmiry, Rothwelle Tate and Dino Rotondo (2014). Regulation of Prostaglandin receptor expression in human monocytic cells following inflammatory activation. Saudi Students Conference. Edinburgh, UK.

Alaa Kashmiry, Rothwelle Tate and Dino Rotondo (2014). Regulation of Prostaglandin receptor expression in human monocytic cells following inflammatory activation. 5th PhD Experience Conference. University of Hull, Hull, UK.

Alaa Kashmiry, Rothwelle Tate and Dino Rotondo (2014). Regulation of Prostaglandin receptor expression in human monocytic cells following inflammatory activation. International conference on the Bioscience of Lipids. Robert Gordin University, Aberdeen, UK.

Alaa Kashmiry, Rothwelle Tate and Dino Rotondo (2014). Regulation of Prostaglandin receptor expression in human monocytic cells following inflammatory activation. Metabolism and Immunity: A Rediscovered Frontier conference. Trinity College, Dublin, Ireland.

Chapter 6

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