Characterisation of the Modulation of Human Inflammatory Cytokine Production by Prostaglandin D_2 Pathways

by

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Declaration of Author's Rights

I declare that the research work reported in this myself carried out thesis at the Strathclyde Institute of Pharmacy and Biomedical sciences (SIPBS), University of Strathclyde and that any help that I received for any part of the work is duly acknowledged. I believe that the contents of this thesis are wholly original, expect where due references are made. Furthermore, the work described in this thesis has not been presented for any other degree.

Prasad Raval B

January-2009

Dedicated to Lord Krishna

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Abbreviations

AA	Arachidonic acid
APC	Antigen presenting cells
BSA	Bovine serum albumin
cAMP	Cyclic- adenosine monophosphate
C5a	Complement type 5-a
cDNA	complementary DNA
CNS	Central nervous system
CSFs	Colony stimulating factor
COX	Cyclooxygenase
$CRTH_2$	Chemoattractant homologous molecule expressed on Th2 cells
DAG	Diacylglycerol
DC	Dendritic cells
DGLA	Dihomo-gamma linolenic acid
$DKPGD_2$	13,14-dihydro-15-keto PGD_2
DMSO	Dimethyl sulphoxide
EFA	Essential fatty acids
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain protein
FCS	Foetal Calf Serum
Fc	Constant fragment (antibody)
FcR	Fc receptor
GLA	Gamma linolenic acid
GM-CSF	Granulocyte monocyte-colony stimulating factor
GSH	Glutathione
HIV	Human Immunodeficiency virus
GST	Glutathione S-transferase

HBBS	Hank's balanced salt solution
HLA	Human leukocyte antigen
MYD88	Myeloid differention primary response gene (88)
NF-κB	Nuclear factor-kappa B
NK	Natural Killer cells
NO	Nitric oxide
NOD	Nucleotide oligomerization domain
NSAIDs	Non-steroidal anti-inflammatory drugs
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PG	prostaglandin
PGE_2	Prostaglandin E_2
PGD_2	Prostaglandin D_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
15d-PGJ_2	15-deoxy Prostaglandin J_2
PKA	cAMP dependent protein kinase
PKC	Protein Kinase C
PLA	Phospholipase A
PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome proliferator-activated receptor
PUFAs	Polyunsaturated fatty acids
RBC	Red blood cells
rIL	Recombinant interleukin
RPM	Revolution per minute
rTNF	Recombinant Tumour necrosis factor
s.d.	Standard deviation
TB	Tuberculosis
TGF-β	Transforming growth factor beta

Th	T helper
TMB	Tetramethyl benzidine
TNF-α	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor Receptor
TLR	Toll-like receptor
TRADD	Tumor necrosis factor receptor 1-associated death domain protein
TRAF	Tumor necrosis factor receptor-associated factor-6
TX	Thromboxane
VCAM	Vascular cell adhesion molecule
WBC	White blood cells

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Abstract

TNF-α is the major pro-inflammatory cytokine released in response to many pathogenic stimuli. Prostaglandins are well-established secondary mediators of inflammation derived from arachidonic acid. Recently PGE_2 has been shown to be a potent suppressor of the production of the primary inflammatory mediators, specifically TNF-α possibly as a negative feedback mechanism, (Davidson *et al.,* 1998). There are also other series of prostaglandins such as $PGF_{2\alpha}$, PGD_2, and 15d-PGJ_2. In general there is a lack of information regarding the role of these other prostaglandins especially in terms of immune and inflammatory responses. In the current study, attempts were made to evaluate the effects of PGD_2 on suppression of TNF-α. Attempts were also made to elucidate the receptor pathway involved by which PGD_2 mediates its suppressive effect. The effect of the spontaneous PGD_2 metabolite, 15d-PGJ_2, was also studied on the inhibition TNF-α production.

Whole human blood and monocytes were utilised to investigate the effects of PGD_2, PGE_2, $PGF_{2\alpha}$ and 15d-PGJ_2, upon TNF-α production, which was measured by ELISA. TNF-α production was also analysed in the presence and absence of various agonists and antagonists including BWA868C (DP_1 receptor antagonist), CAY10471 (DP_2/$CRTH_2$ receptor antagonist) and BW245C (DP_1 receptor agonist). PGE_2, $PGF_{2\alpha}$, 15d-PGJ_2, and PGD_2 induced a strong suppressive effect on LPS-stimulated TNF-α production in human blood and monocytes. The suppressive effect of these prostaglandins was not altered in the presence of BWA868C (DP_1 receptor antagonist) suggesting the absence of a DP_1 receptor pathway. However, BW245C (DP_1receptor agonist) showed a dose-dependent decrease in LPS-stimulated TNF-α production in blood and monocytes. This suppressive effect was not altered by CAY10471 (DP_2/$CRTH_2$ antagonist) or BWA868C (DP_1 receptor antagonist) suggesting that BW245C may be acting via other non- PGD_2 receptors. The suppressive actions of PGD_2 and 15d-PGJ_2 on TNF-α production was inhibited in the presence CAY10471 but not by BWA868C suggesting the potential involvement of DP_2 pathways. Overall the study indicates a similarity between the actions of PGD_2 and 15d-PGJ_2 on the modulation of TNF-α production via a DP_2 receptor pathway.

1. Introduction

1.1 The Immune system

The immune system in vertebrates incorporates a wide range of defensive mechanisms which includes intracellular sensors and signalling molecules that provide protection by reorganization and killing of diverse pathogenic organisms, thereby eliminating them. (De Franco *et al*., 2007). The immune mechanism in invertebrates includes antimicrobial peptides called defensins, elements of phagocytes and the complement system (Klein & Heresy, 1997). Evolution of vertebrates such as humans has led to the evolution of a much more sophisticated immune system comprised of proteins, cells organs and tissues that interact in a dynamic network (Gregory *et al*., 1996). Optimal immunity is required to prevent hypo- or hyper- functioning of the immune system.

1.2 Optimal immunity

Immunodeficiency disorders occur when the immune system is not functioning normally and may be due to genetic diseases, or acquired immunodeficiency e.g. caused by HIV (retro virus). This could lead to, for example, insufficient production of inflammatory cytokines, (Bouma & Strober, 2003) resulting in hypo-functioning of the immune system. Conversely, The overproduction of inflammatory mediators can occur in septic shock or in autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and inflammatory bowel disease (Bouma & Strober, 2003). The adaptive and innate immune systems play an important role in the maintenance of optimal immunity.

1.3 Innate and adaptive immunity

The innate immune system acts directly through the recognition of microorganisms (De Franco *et al.,* 2007). The response is non-specific and can lead to an immediate maximal response. The innate immune response involves:

- Phagocytic cells (neutrophils, monocytes, and macrophages);
- Cells that release inflammatory mediators (basophils, mast cells, and monocytes)
- Natural killer cells (NK cells)
- Molecules such as complement proteins, acute phase proteins and cytokines.

In the adaptive immune response, the immune system adapts during infection to improve its recognition of a pathogen. This improved response is retained after the elimination of the pathogen as immunological memory, thus allowing a faster and stronger attack on subsequent encounters with the pathogen. This adaptive immune response is primarily mediated by T- Lymphocytes and B-Lymphocytes (Defranco *et al.,* 2007).

1.4 Cells of the immune system

Haematopoietic cells from bone marrow give rise to RBC and WBC via committed progenitors. Erythroid lineage cells develop into erythrocytes and megakaryocytic cells (which form platelets that initiate blood clotting). Myeloid lineage cells give rise to cells which include phagocytes that engulf pathogens and merge with lysosomes (macrophages, neutrophils, dendrites cells, mast cells, eosinophils and basophils) whereas lymphoid lineage cells give rise to T cells and B cells of adaptive immunity as well as NK cells that are specialized cytotoxic cells (Defranco *et al.,* 2007).

1.5 Inflammation

Inflammation is regarded as the first response of the immune system to infection (Serhan *et al.*, 2005). Inflammation is a complex biological response of vascular tissue to harmful stimuli such as pathogens, damaged cells or irritants (Sell, 1987). It is a protective attempt by an organism to remove an injured stimuli as well as an innate healing process for the tissue (Gallin *et al.*, 1988). The inflammatory response is the body's natural response that occurs immediately following tissue damage. Its main functions are to defend the body against harmful substances, dispose of dead or dying tissue and to promote the renewal of new tissue. The following series of events takes place during inflammatory conditions:

- Vasodilation resulting in increased blood flow
- Increased vascular permeability
- Exudation of fluid into tissue leading to swelling
- Phagocytosis (destruction of microbes)
- Tissue repair

1.5.1 Cardinal signs of Inflammation

The cardinal signs of inflammation are outlined below and are more commonly termed the "Celsus signs" of inflammation

Rubor- redness	associated with increased blood flow
Calor- heat	increased blood flow/ vasodilation
Tumor- swelling	vasodilation followed by an increase in permeability/ exudation leading to increased tissue mass
Dolor- pain	increased sensitivity of sensory nerves manifest as pain

1.5.2 Causes of inflammation

The inflammatory process can be either classified as acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli resulting in increased movement of plasma and leucocytes from blood to the injured tissue. This process leads to the progression of an inflammatory cascade. Chronic inflammation leads to a progressive shifting of cell types at the site of inflammation and a cycle of destruction and healing of the tissue from the inflammatory process. Chronic inflammation is a consequence of many human diseases. If inflammation remains unchecked it can result in a host of diseases such as rheumatoid arthritis, which has a strong risk of rheumatoid vasculitis or rheumatoid lung disease, thereby elevating the risk of cardiovascular disease. Severe systemic inflammation can also lead to a shock response e.g. septic shock. This can result in a profound drop in blood pressure due to the presence of bacteria in the blood resulting in multi-organ failure and death. It is for this reason that inflammation must be tightly regulated by the body (Serhan *et al.*, 2005). There are various diverse triggers of inflammation such as: environmental factors including air pollutants, foreign bodies including splinters and dirt, pathological factors such as infection by bacteria, burns, and immune reactions due to hypersensitivity.

1.5.3 Initiation of inflammatory responses

1.5.3.1 Lipopolysaccharide

The main component of the membrane of gram-negative bacteria is a lipopolysaccharide. Being discovered as endogenous toxins of pathogenic bacteria, they were initially named endotoxins. LPS, is a potent bacterial endotoxin and is responsible for the toxic manifestation of gram-negative infections and generalized inflammatory reactions (Rietschel *et al.,* 1993). In humans, LPS triggers an innate immune response characterized by cytokine production and immune system activation. Inflammation is a common result of cytokine production, which produces host toxicity. LPS is one of the most potent stimuli for the induction and release of pro-inflammatory cytokines especially IL-1 from monocytes (Rietschel *et al*., 1994).

LPS acts as a permeability barrier in bacteria allowing access only to low molecular weight, hydrophilic molecules. It is impermeable to larger molecules and hydrophobic compounds. Thus, LPS in hosts may impede the destruction of bacterial cells by serum components and phagocytic cells. LPS may also play a role as an adhesin, used in colonization of the host. Variations in LPS structure provide for different antigenic strains of a pathogen that may be able to bypass a previous immunological response to a related strain (Corsaro *et al.,* 2001).

Lipopolysaccharides are complex amphiphilic molecules with a molecular weight of about 10kDa that vary in chemical composition among bacterial species. In Region I: Lipid A is the lipid component of LPS (Fig. 1.1). It contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids attached to it. All fatty acids in Lipid A are saturated (Fig. 1.2). Some fatty acids are attached directly to the NAG dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. The biological activity of LPS resides in the Lipid A portion and is mostly responsible for the endotoxic activities of LPS whereas immunogenicity is associated with the polysaccharide components (Raetz *et al.,* 2007). The lipid A is characterized by relatively short chain length (C10) of the 3-hydroxy fatty acid components directly bound to a glucosamine disaccharide backbone by either amide or ester linkages.

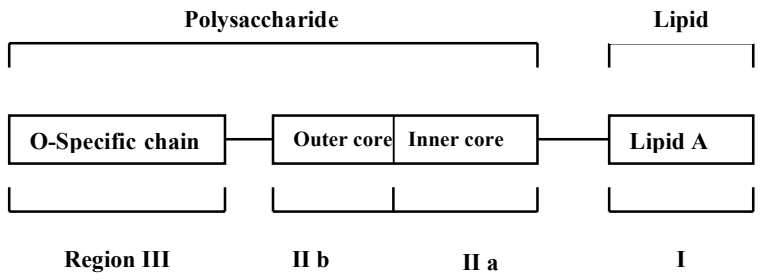


Fig: 1.1 General architecture of Lipopolysaccharide. General Structure of LPS molecules (Kenneth, 2008)

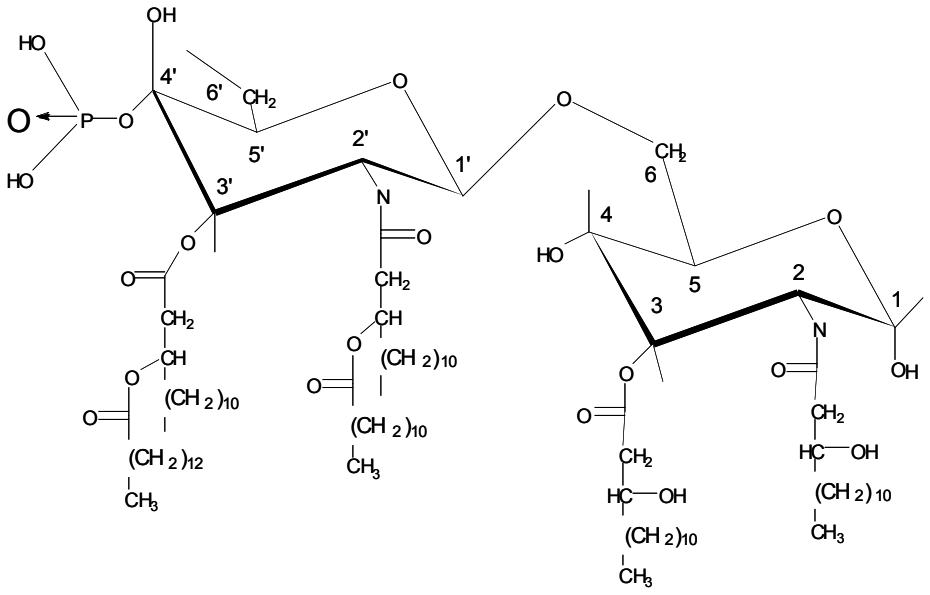


Fig: 1.2 Structure of the glucosamine disaccharide and fatty acids in the Lipid A moiety of LPS (region I from figure 1.1).

These unusual fatty acids in Lipid A are embedded into the outer membrane of the bacterium while the rest of the LPS project from the surface (Fig. 1.3).

Region II: Core (R) antigen or R polysaccharide consists of a short chain of sugars. Two unusual sugars, heptose and 2-keto-3-deoxyoctonoic acid (KDO) are usually present, in the core polysaccharide. KDO is unique and invariably present in LPS and so it has been used as an indicator in assays for LPS/ endotoxin (Corsaro *et al.,* 2001).

Region III: Somatic (O) antigen or O polysaccharide is attached to the core polysaccharide. The cell wall antigens (O antigens) of Gram-negative bacteria are components of LPS. It consists of repeating oligosaccharide subunits made up of 3 - 5 sugars. The individual chains vary in length ranging up to 40 repeat units (Fig. 1.1). The O polysaccharide is much longer than the core polysaccharide, and it maintains the hydrophilic domain of the LPS molecule. A major antigenic determinant (antibody-combining site) of the Gram-negative cell wall resides in this O polysaccharide. The presence or absence of O chains determines whether the LPS is rough or smooth respectively. The involvement of the polysaccharide chain in virulence is shown by the fact that small changes in the sugar sequences in the side chains of LPS result in major changes in virulence (Jansson, 1999).

In humans, LPS binds to a lipid binding protein (LBP) in the serum which transfers it to CD14 on the cell membrane, which in turn transfers it to another non-anchored protein, MD2, which associates with Toll-like receptor-4 (TLR4). This triggers the signaling cascade for macrophage/endothelial cells to secrete inflammatory cytokines and nitric oxide that can ultimately lead to endotoxic shock. CD14 and TLR4 are present on several immunological cells, including macrophages and dendritic cells. These can activate the production of cytokines, including IL-1, IL-6, IL-8, and TNF-α. These cytokines, in turn, stimulate the production of prostaglandins and leukotrienes. These powerful mediators of inflammation can in extreme circumstances cause life-threatening symptoms such as the loss of blood pressure control which is a hallmark of septic shock.

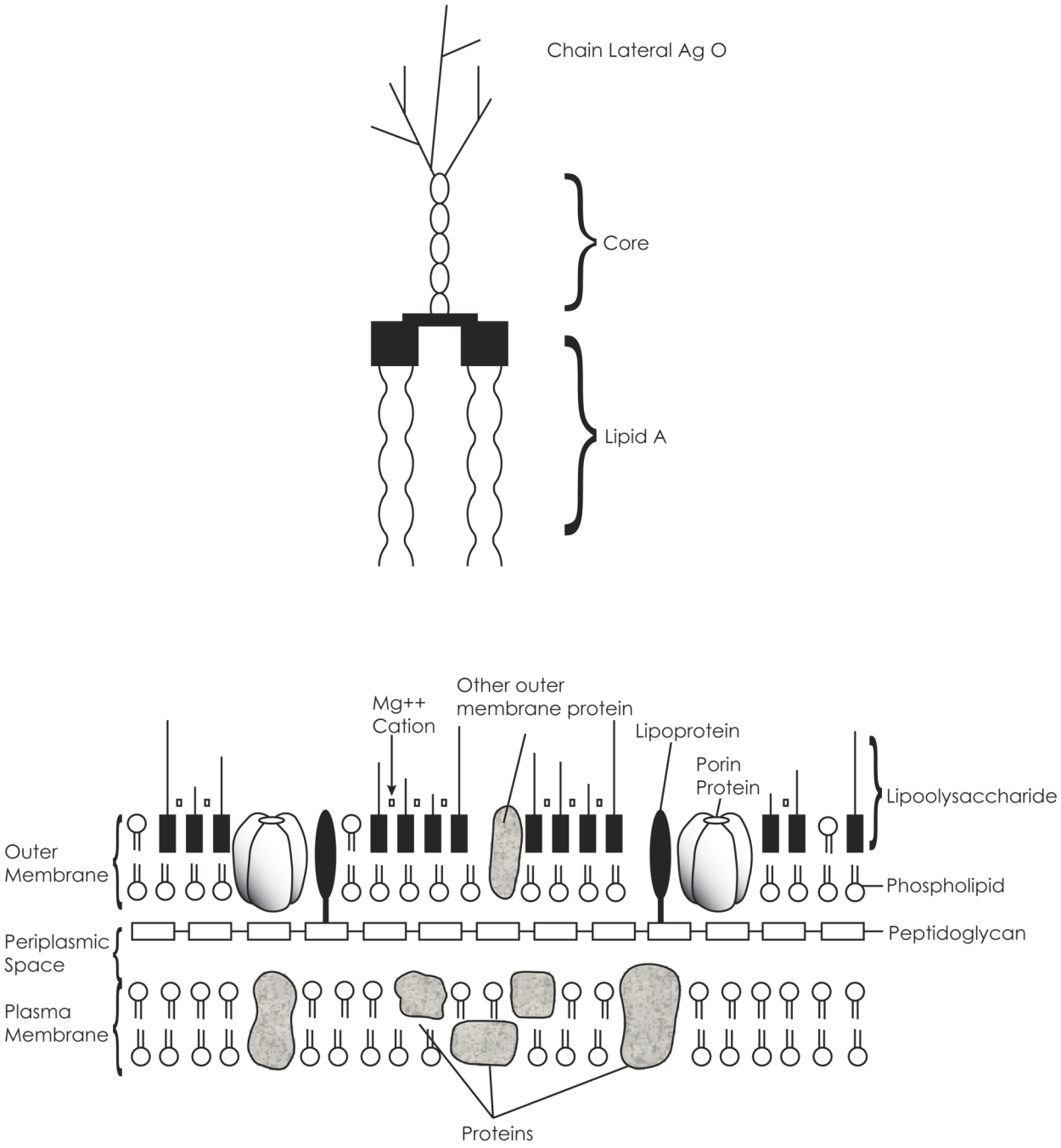


Fig. 1.3 structure of LPS *in situ* (bacterial cell wall).

When bacteria are lysed, either through cell death or division, the bacterial cell wall endotoxin (LPS) can be released and can activate monocytes to initiate acute immune responses. Tissue macrophages are the primary source of increased serum TNF-α level during endotoxaemia (Leone, *et al.,* 2007). Release of LPS either into localized regions or the circulation results in inflammatory responses (manifest as fever systemically) (Davidson *et al.,* 1998). Both this effect of the bacteria locally and systemically mediates the production of endogenous cytokines, such as interleukins especially IL-1 and TNF-α. These pro-inflammatory cytokines stimulate the production of prostanoids, primarily PGE_2, which are ultimately responsible for the symptomatic manifestations of the inflammatory response (see Fig. 1.4).

Activation of host immune cells by Gram-negative bacteria can be reproduced *in vitro* by incubating whole blood, or cells isolated from blood, with LPS. Macrophages participate actively in the onset of inflammation and immune system activation by releasing pro-inflammatory and anti-inflammatory cytokines that further enhance inflammatory stimulation, release of bioactive lipids (PGs, leukotrienes), reactive oxygen species, nitrogen species that exert a cytotoxic effect against pathogens and tumour cells (Takahiro *et al.,* 2002). Stimulation of monocytes by LPS is restricted to the first few hours, with little or no effect on longer incubations up to 24 hours; this may be due to the production of downstream mediators by LPS which counteract the actions of LPS by initiating a down-regulating response which can be induced by PGs (see Fig. 1.4). LPS induces the production of TNF -α by activation of the MAP kinase pathway via toll-like receptor 4 (Swantek *et al.*, 1997).

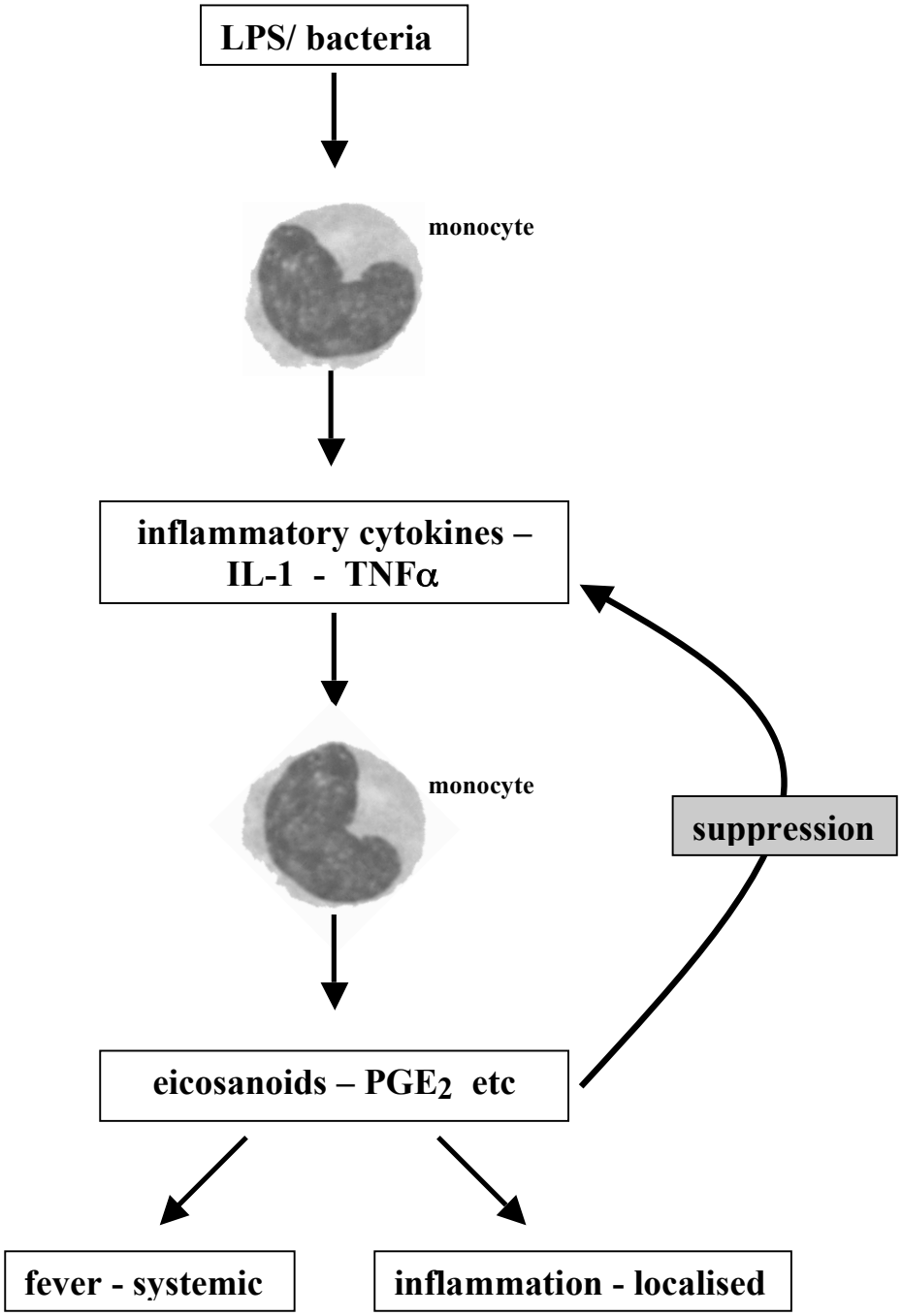


Fig. 1.4. LPS stimulation of inflammatory cascade and its regulation.

1.5.3.2 Toll like receptors

The mammalian toll-like receptors (TLRs) are expressed on cells of the immune system, which are primarily involved in innate immunity such as macrophages and dendritic cells. When microorganisms enter a tissue site, Toll-like receptors sense their presence. Pathogen recognition by TLRs results in the activation of innate immunity and induces the production of pro-inflammatory cytokines and chemokines like TNF-α, IL-1 *etc*. (Kaisho *et al*., 2002). Cytokine inducers such as LPS, polyinosinic: polycytidylic acid and IL-1 itself, activate immune cell responses by utilizing TLR receptor pathways. Lipopolysaccharide (LPS) is the major ligand for TLR-4.

1.5.3.3 Signalling pathways of TLRs

Almost all TLRs associate with the adapter protein, MyD88, which is essential for cytokine production induced by TLRs. MyD88 is a cytoplasmic adapter protein, which associates with all members of IL-1R domains and TLR families (Kaisho *et al*., 2002). Ligands for several TLRs, such as TLR2, TLR3, TLR4, TLR5, TLR6, and TLR-9 have been identified. These TLRs play an important role in inflammatory cascades and primarily signal via MyD88-dependent pathways (Mainly TLR2 and TLR9). TLR4 is essential for recognizing LPS from Gram-negative bacteria. Upon binding of TLR ligands, TLR induces cytokine production and up-regulation of co-stimulatory molecule expression in APCs, thereby conferring on them the ability to activate adaptive and innate immune responses. Ligand binding recruits MYD88 adaptor to TLR. MyD88 recruits related protein kinases such as IRAK-4 and IRAK-1 which in turn recruits the adaptor-TRAF6. The IRAK 1- TRAF6 complex dissociates allowing amplification of signal and activation of TAK-1 and MAP kinase leading to IκB phosphorylation, with the sequential disengagement/ activation of NFκB which activates inflammatory cytokine gene transcription (Kaisho *et al*., 2002).

1.5.4 Inflammatory Cytokines

Inflammation is regulated by cytokines primarily TNF-α and IL-1. These act on their respective receptors to activate the production of secondary mediators which are ultimately responsible for the symptoms of inflammation (see Fig. 1.4).

1.5.4.1 Cytokine receptors

Cytokine receptors stimulate immune responses by either promoting or inhibiting inflammation, activating macrophages, activating B and T cells, activating eosinophils and mast cells. Six major classes of cytokine receptors have been described and the major sets involved in inflammation are the IL-1 family receptors and the TNF-α superfamily receptors. IL-1 family-receptors have homologous domains to the TLRs (TLR domains) in their cytoplasmic tails and they mainly signal by interacting with the adaptor protein MyD88 and downstream signalling is also identical to TLR signalling. Receptors of the TNF receptor superfamily signal apoptosis via death domains called TRADD and FADD that link to enzymes of programmmed cell death e.g. caspases, by TNF receptor associated factors (TRAFs).

1.5.4.2 Interleukin-1

It is well established that LPS stimulates the production of IL-1. However, it is less clear how IL-1 production is controlled. IL-1 appears to produce a positive feedback mechanism whereby released IL-1 further increases IL-1 release (Dinarello *et al.,* 1986, Ghezzi *et al.,* 1988). IL-1 can induce its own synthesis by inducing gene expression (Dinarello *et al.,* 1987). In contrast, Stephen *et al.* (1987) showed that endothelial cells, stimulated with IL-1, results in the production of PGE_2 that can suppress further IL-1 production. Thus, reduced IL-1 production may be controlled by a negative feedback mechanism mediated by PGE_2 (Kunkel *et al.*, 1986). In the presence of indomethacin (a PG-synthesis inhibitor), an increased IL-1 release was detected when stimulated by exogenous IL-1 (Knudsen *et al.*, 1987; Rotondo *et al.,* 1992; Maakie *et al.,* 2002). This indicated a regulation of IL-1 production by endogenously produced PGs. Rotondo *et*

al. (1992) showed that IL-1 and LPS increase the activity of phospholipases associated with the release of arachidonic prior to the production of PGs.

1.5.4.3 Tumour necrosis factor-α

TNF-α is a cytokine which appears to be centrally involved in the development of inflammatory processes. Dysregulation and overproduction of TNF-α mediates a series of inflammatory diseases (Gallin *et al.*, 1988).

TNF molecules are homotrimers synthesized as type 2 transmembrane proteins. STNF (soluble TNF) is released via proteolytic cleavage by metalloproteases. They act distantly or locally either on neighbouring cells in a paracrine fashion (Defranco *et al.*, 2007). TNF-α then acts as a trigger that activates a cascade of cytokine production. TNF-α is released rapidly in response to inflammatory conditions that induce the production of other inflammatory cytokines such as IL-1 (Standiford *et al.,* 1990).

1.5.4.4 TNF-α receptor family

The three members of the TNF family are commonly known as TNF-α, lymphotoxin-α, (LT-α also termed TNF-β), and lymphotoxin-β (LT-β). They are encoded in three closely linked genes within the MHC (Klein and Horejsi, 1997) and act through the TNF receptor superfamily.

1.5.4.5 Signalling by the TNF-α receptor superfamily

The TNF-α receptor superfamily have a conserved 70-amino acid globular domain in their cytoplasmic tail termed the "death domain" (Defranco *et al.*, 2007). Two distinct cell-surface receptors have been described. These are TNF receptor type 1 (TNF-R1) and TNF receptor type 2 (TNF-R2). TNF-R1 is expressed in most tissues and can be fully activated by both membrane bound and soluble trimeric forms of TNF-α. TNF-R2 signals directly in lymphoid cells and has an auxiliary role in modulating TNFR1

signalling. On binding of ligand by receptor, a death domain recruits the formation of FAS mediated death domain (FADD) and TNF-α receptor associated death domain (TRADD). This results in the activation of caspases ultimately leading to cell death. During cell infection by viruses the transcriptional response is blocked, this leads to inhibition of protein synthesis. In such cases TNFR1 induces a dual mode of signalling by inhibiting apoptotic pathways but also inhibiting viral replication without inducing cytolysis. These pathways induced by TNF-α are only operational when transcription is blocked, thereby reflecting a role in antiviral immune defences (Defranco *et al.,* 2007).

1.5.4.6 Physiology of TNF

Dinarello *et al.* (1986) showed that TNF-α is an endogenous pyrogen that induces the production of IL-1, moreover rTNFα and rIFN-γ have synergistic effects on IL-1 production. However, almost all actions of TNF-α are shared by IL-1. Some of these are listed in table 1 below (Gallin *et al.,* 1988).

Table 1 Comparison of the actions of IL-1 and TNF-α

target cells	**Biological activity shared by IL-1 and TNF-α**
Endothelium	Cytokine production Leukocyte adherence Procoagulant NO synthase
B-cells	Stimulate B cells for antibody production
T-cells	Synthesis of IL2, IFN-γ and other cytokines.
Brain	Fever
Fibroblast	Interferon β synthesis
Myocyte	Causes proteolysis
Osteoclast	Bone resorption
Macrophage	production of IL-1, TNF, and IL-6

1.5.4.7 Pharmacology of TNF-α

Rheumatoid arthritis is an autoimmune disease involving activated T-cells which give rise to overproduction of IL-1 and TNF-α production resulting in chronic inflammation (Abramson & Weismann, 1989). Monoclonal antibodies such as Infliximab (Remicade) or Adalimumab (Humira) and the TNF-receptor fusion protein, Etanercept (Enbrel) can abolish the symptoms of inflammation by binding to TNF-α and neutralising its activity (Aeberli *et al.*, 2002). Alternative treatment strategies have been developed for reducing circulating levels of TNF-α other than by neutralisation with TNF-α binding proteins. An example being Pirfenidone that has been shown to inhibit *de-novo* TNF-α synthesis particularly following bacterial activation (Hale *et al.*, 2002). However, a disadvantage of these treatments is that cases of tuberculosis (TB) and other serious infections have been observed in patients treated with these medicines. Various oxysterols, particularly 25-hydroxycholesterol have been shown to decrease LPS-induced TNF-α secretion (Englund *et al.*, 2001) probably via a glucocorticoid-independent pathway. Glucorticoid analogues such as dexamethasone etc., are well established inhibitors of cytokine production, particularly TNF-α (Bleeker *et al.*, 1997). However, they have numerous undesirable clinical effects and with respect to inflammatory resposnes, also inhibit the formation of eicosanoids. This is important as PGs in particular, appear to have a negative feedback mechanism for the release of TNF-α and other pro-inflammatory cytokines (Kunkel *et al.*, 1986).

1.5.5 Eicosanoids

Eicosanoids are a group of molecules produced by the oxygenation of 20-carbon unsaturated fatty acids which can exert many complex control actions over numerous organ and cell systems but especially in inflammation and immunity. They are derived either from ω-3 or ω-6 polyunsaturated fatty acids. There are at least four broad families of eicosanoids that include prostaglandins, thromboxanes, leukotrienes and HETEs. This study will focus on prostaglandins which are predominantly derived from arachidonic acid released from membrane phospholipids. Arachidonic acid appears to have a unique and selective role in cell signalling in the immune system (Rotondo *et al.*, 1994). Although arachidonic acid is the immediate precursor for the formation of 2-series prostaglandins (Coceani *et al.*, 1986) it can also be involved in signalling via eicosanoid-independent mechanisms. An example is mediation by arachidonic acid of Fc-γ-receptor-mediated superoxide generation (Rotondo *et al.*, 1994). Arachidonic acid also appears to be an obligatory mediator for TNF-α induced cytotoxicity (Calder *et al.*, 1991) and can play an important role in G-protein activation. However, many of the actions of arachidonic acid are mediated via their conversion to prostanoids.

1.5.5.1 Structure and Biosynthesis of Prostanoids

Cells activated by mechanical trauma, cytokines, and other growth factors, release fatty acids, primarily arachidonic acid, from membrane phospholipids. The release of arachidonic acid occurs via the activation of phospholipases. The release of arachidonic acid is the rate-limiting step in PG synthesis. Arachidonic acid is further converted to PGG_2, which is peroxidised to PGH_2. This reaction is catalyzed by the enzyme cyclooxygenase (Bomalaski *et al.*, 1992). Various downstream enzymes produce the different specific prostaglandins (see Fig. 1.5).

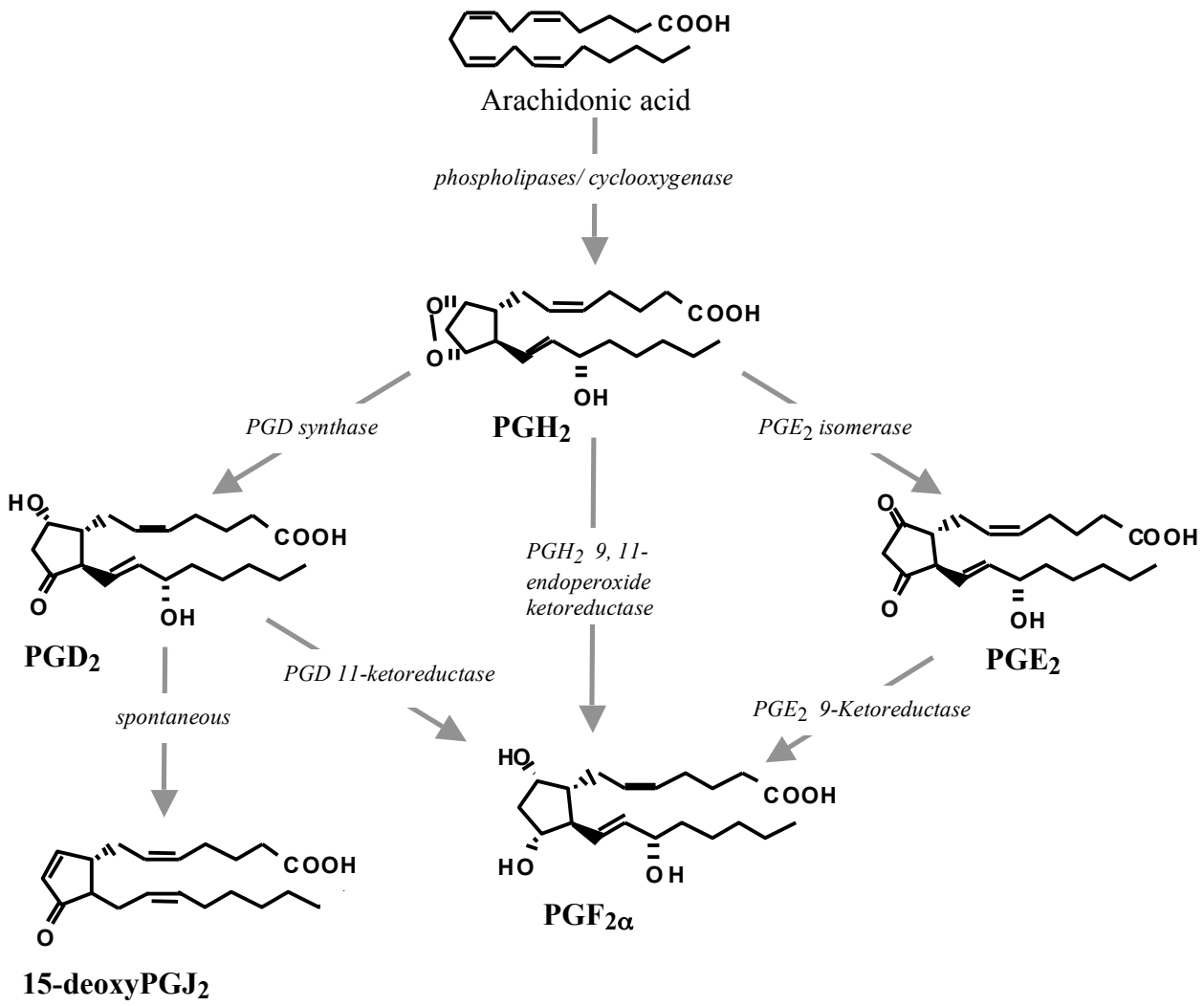


Figure 1.5 Structures of the main prostaglandins and the various enzymes which lead to the production of each specific prostanoid (adapted from Watanabe, 2002).

The prostaglandins are the most prevalent autacoids found in almost every tissue as well as body fluids. They mediate responses to numerous diverse stimuli, in minute quantities and produce a remarkably broad spectrum of effects that encompasses practically every biological function. Prostaglandins were first isolated from the semen of sheep and that of human prostate gland from where their name is derived (Goldblatt and Von Euler 1935). Chemically they are 20-carbon unsaturated fatty acids containing a cyclopentane ring. There are a number of different prostaglandins and are named as A, B, C, D, E, F etc., depending on the constituents of the cyclopentane ring. If a prostaglandin has one double bond in the hydrocarbon chain they are classified as 1-series e.g. PGE_1. If there are two double bonds in the chain they are classified as 2-series PGs e.g. PGE_2. Similarly, there may also be 3-series PGs such as PGE_3 (Sell, 1987).

1.5.5.2 Cyclooxygenase

There are 2 major isoforms of cyclooxygenase COX-1, localized in the endoplasmic reticulum and COX-2 which acts at the nuclear envelope (Morita *et al.,* 1995). Prostaglandins are produced by the sequential oxidation of arachidonic acid by cyclooxygenase, either COX-1 or COX-2 (Smith, 1992). COX-1 is expressed in most cells, thought to be responsible for baseline levels of prostaglandin production. COX-1 plays a major role in platelet aggregation and renal function. COX-2 is not constitutively present but induced by cells stimulated with pro-inflammatory agents such as cytokines e.g. IL-1 or TNFα. Inflammatory responses can be associated with COX-1 and COX-2 isoforms. Both COX-1 and COX-2 convert arachidonic acid to the various specific prostaglandins via the various prostaglandin synthases (Fig. 1.5).

1.5.5.3 Prostaglandin synthases

Prostaglandin F Synthesis. $PGF_{2\alpha}$ can be synthesized via three pathways from PGE_2, PGD_2, or PGH_2. The PGE_2 pathway for $PGF_{2\alpha}$ synthesis involves the action of 9-ketoreductase, the PGD_2 pathway is via 11-ketoreductase, and the PGH_2 pathway is via 9-11-endoperoxide reductase in the presence of NADH or NADPH (Watanabe, 2002).

Prostaglandin E Synthesis PGE_2 is mainly produced by conversion of the unstable intermediate PGH_2 via isomerisation by prostaglandin E synthase (PGES) which is also termed PGE isomerase. There exist two PGES biosynthetic routes, COX -1-cytosolic cPGES and the COX-2-membrane associated mPGES (see Fig. 1.6). Membrane associated PGES participates in various pathophysiological states in which COX-2 is involved. Murakami *et al*. (2002) showed that NSAIDs can preferentially inhibit mPGES activity.

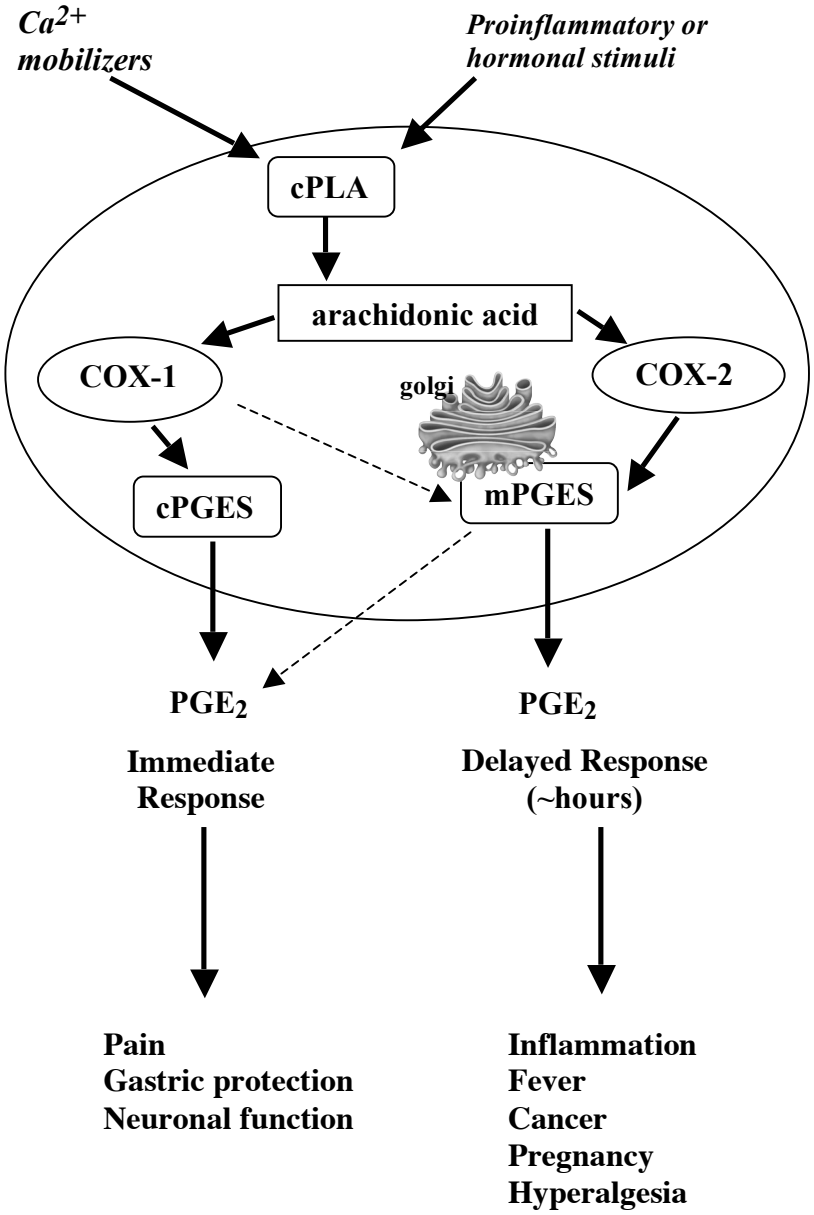


Figure 1.6 Model showing the formation of PGE_2 by two separate pathways. Ca^{2+} mobilisers elicit an immediate response whereas a delayed response can be induced by pro-inflammatory stimuli. Arachidonic acid is slowly and continuously released by $cPLA_2$, which is further metabolized to PGE_2, via COX-2 and mPGES. mPGES is capable of producing PGE_2 via COX-1 but only when explosive activation of $cPLA_2$ occurs (Murakami *et al.*, 2002).

1.5.5.4 Prostaglandin receptors

Prostanoid receptors are classified primarily on the basis of the naturally occurring prostanoid ligand and sub-classified on the basis of synthetic prostanoid agonists and antagonists (Koremu *et al.,* 1997). Nine types of prostaglandin receptors have been described (Table 2). These are a family of cell surface seven-transmembrane spanning receptors which are linked to heterotrimeric G-proteins (Sugimoto *et al.,* 2003). These receptors are termed depending on which prostanoid is the preferred ligand followed by the suffix P (e.g. EP-receptors for PGE_2) rather than any genetic relationship. The PGE receptors constitute a complete family consisting of EP_1, EP_2, EP_3, and EP_4 receptors each encoded by distinct genes (Coleman *et al.,* 1990). EP_1 receptors are structurally closely related to TP and FP receptors and EP_2 receptors are closely related to IP and DP receptors (Toh *et al.,* 1995). The type of G protein activated by different prostanoid receptors correlates well with the c-terminal region of the receptors. One category encompasses receptors that activate Gs and stimulate cyclic AMP formation by adenylate cyclase e.g. IP, EP_2, EP_4 and DP receptors (Adie *et al.,* 1992). A second category includes receptors that activate Gq thus enhancing intracellular Ca^{2+} levels by increasing phosphotidylinositol turnover e.g. TP, EP_1 FP receptors (Funk *et al.,* 1993). There is also a third category of prostanoid receptors that act via Gi which inhibits adenylate cyclase and suppresses cyclic AMP formation e.g. EP_3 receptors (Bos *et al.,* 2004).

Table: 2 Major classes of prostanoid receptors and proposed intracellular 2nd messenger systems (data from Alexander *et. al.*, 2009).

PG Receptor	Agonist	2nd messenger
DP_1	PGD_2	cAMP increase (Gs)
DP_2	PGD_2	cAMP decrease (Gi)
EP_1	PGE	IP_3/ DAG/ Ca^{2+} (Gq)
EP_2	PGE	cAMP increase (Gs)
EP_3	PGE	cAMP decrease (Gi)
EP_4	PGE	cAMP increase (Gs)
FP	$PGF_{2\alpha}$	IP_3/ DAG/ Ca^{2+} (Gq)
IP	PGI_2	cAMP increase (Gs)
TP	TXA_2	IP_3/ DAG/ Ca^{2+} (Gq)

1.5.5.5 PGE_2 and EP receptors

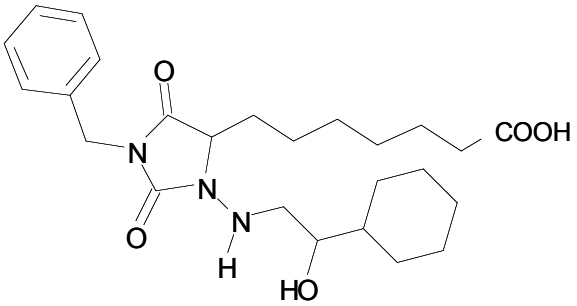
PGE_2 binds to EP receptors to mediate its many actions. EP_2 and EP_4 receptors can stimulate Gs and induce upregulation of cyclic AMP production with the subsequent activation of protein kinase A (Yamamoto *et al.,* 1987). PGE_2 via EP_2 receptors can regulate cytokine production especially TNF-α. EP_3 receptors inhibit adenylate cyclase via activation of the pertussis toxin-sensitive G protein Gi. Functional characterisation of the receptors can be achieved using various agonists and antagonists which have an overlapping selectivity for each receptor. An example is the use of Butaprost (a selective EP_2 agonist) to indicate the action of PGE_2 via EP_2 receptors (Honda *et al.*, 1993). This would be compared to the actions of the EP_1/EP_3 selective agonist Sulprostone to eliminate activity via EP_3 receptors and the EP_1 receptor antagonist SC51322 which would eliminate actions mediated by EP_1 receptors (Alexander *at al.*, 2009)

1.5.5.6 $PGF_{2\alpha}$ and FP receptors:

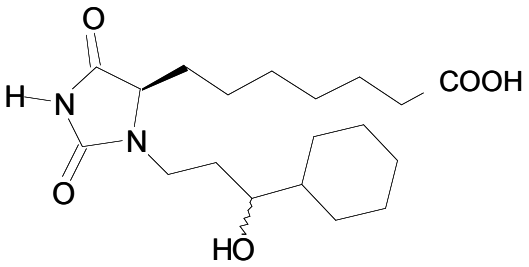
$PGF_{2\alpha}$ induces its actions via FP receptors (Breyer *et al.,* 2001). Upon binding of $PGF_{2\alpha}$ to its receptor, it results in the activation of Gq which is linked to an increase in phosphatidyl inositol turnover with a subsequent increase in intracellular calcium levels (Chen *et al.,* 1998). This pathway plays an important role in uterine contraction as well as modulating mitogenesis. There appear to be 2 selective FP receptor agonists, Fluprostenol and Latanoprost (both PG F-series analogues) and, more recently, a non-prostanoid analogue (AS604872) has been shown to be a highly selective antagonist of FP receptors (Alexander *et al.*, 2009).

1.5.5.7 PGD_2 and DP Receptors

PGD_2 can bind and activate two distinct receptors DP_1 receptors and $CRTH_2$ receptors (Chemoattractant receptor homologues molecule expressed on T-helper type 2 cells) now identified as a DP_2 receptor. DP_1 receptors are linked to adenylyl cyclase via the G protein subunit Gαs resulting in an increase in cyclic AMP levels (Hirata *et al.,* 1994, Boie *et al.,* 1995). BWA868C (see Fig. 1.7) which also binds to DP_1 receptors can completely antagonize the PGD_2-induced elevation in cyclic AMP levels indicating that BWA868C is an antagonist of the DP_1 receptor (Arimura *et al.,* 2001). Town *et al.* (1983) had previously shown that an analogue, BW245C (see Fig. 1.7), had a high affinity and selectivity towards DP_1 receptors and acted as an agonist. Abramowitz *et al.,* (2000) have since demonstrated that BW245C has an approximately 5-fold higher affinity for DP_1 receptors than PGD_2. However, although BW245C exhibits a high selectivity towards DP_1 receptors it retains activity against EP_2 and EP_4 receptors. This was demonstrated in the study of Rangachari *et al.* (1995) which showed that BW245C did not inhibit canine colonic epithelium in the same manner as PGD_2, but produced a stimulant effect instead. In addition, the DP_1 receptor antagonist, BWA868C, failed to alter the actions of BW245C which indicated that the actions of BW245C were most likely mediated via EP receptors. BW245C's affinity towards EP_2 and EP_4 receptors has also been shown by others (Abramowitz *et al*., 2000). BW245C has, nevertheless, been useful in elucidating the existence of the DP_2 receptor. It was shown by Hirai *et al.* (2001) that the PGD_2 dependent migration of blood eosinophils and basophils was not mimicked by BW245C indicating that the actions of PGD_2 on eosinophils and basophils was not mediated via the DP_1 receptor but by another PGD_2-binding receptor. This has subsequently been termed the DP_2 receptor



BWA868C (DP_1 Receptor antagonist)



BW245C (DP_1 Receptor agonist)

Figure: 1.7 Structures of Prostaglandin DP_1 receptor ligands

1.5.5.8 15-Deoxy$^{12\,14}$-prostaglandin J_2 (15d-PGJ_2)

PGD_2 readily undergoes spontaneous dehydration to yield 15-deoxy-PGJ_2, which has been suggested to exert anti-inflammatory effect *in vivo* (Takahiro *et al.,* 2002). PGD_2 *in vitro*, is a natural precursor for PGJ_2 which spontaneously converts into the cyclopentenone PGs of the J series (see Fig. 1.8), such as PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,\ 14}$-PGJ_2 (15d-PGJ_2) (Hirata *et al.,* 1988). 15d-PGJ_2 can be produced both extracellularly, via nonenzymatic conversion of PGD_2 (where it may function as an autocrine and paracrine factor during inflammatory processes) as well as intracellularly where it has thought to regulate peroxisome proliferator-activated receptors (PPARs). Both inflammatory cytokine stimulation (TNF-α and IL-1) and also cytoprotective steroids can enhance the production of 15d-PGJ_2 from human monocytes. Davidson *et al*. (2008) have shown that 7β-hydroxy-epiandrosterone, a cytoprotective steroid, which protects against experimentally-induced damage in various organs, appears to do so by upregulating 15d-PGJ_2 production. This upregulation of 15d-PGJ_2 production appears to be further enhanced in the presence of TNF-α (Davidson *et al.*, 2008).

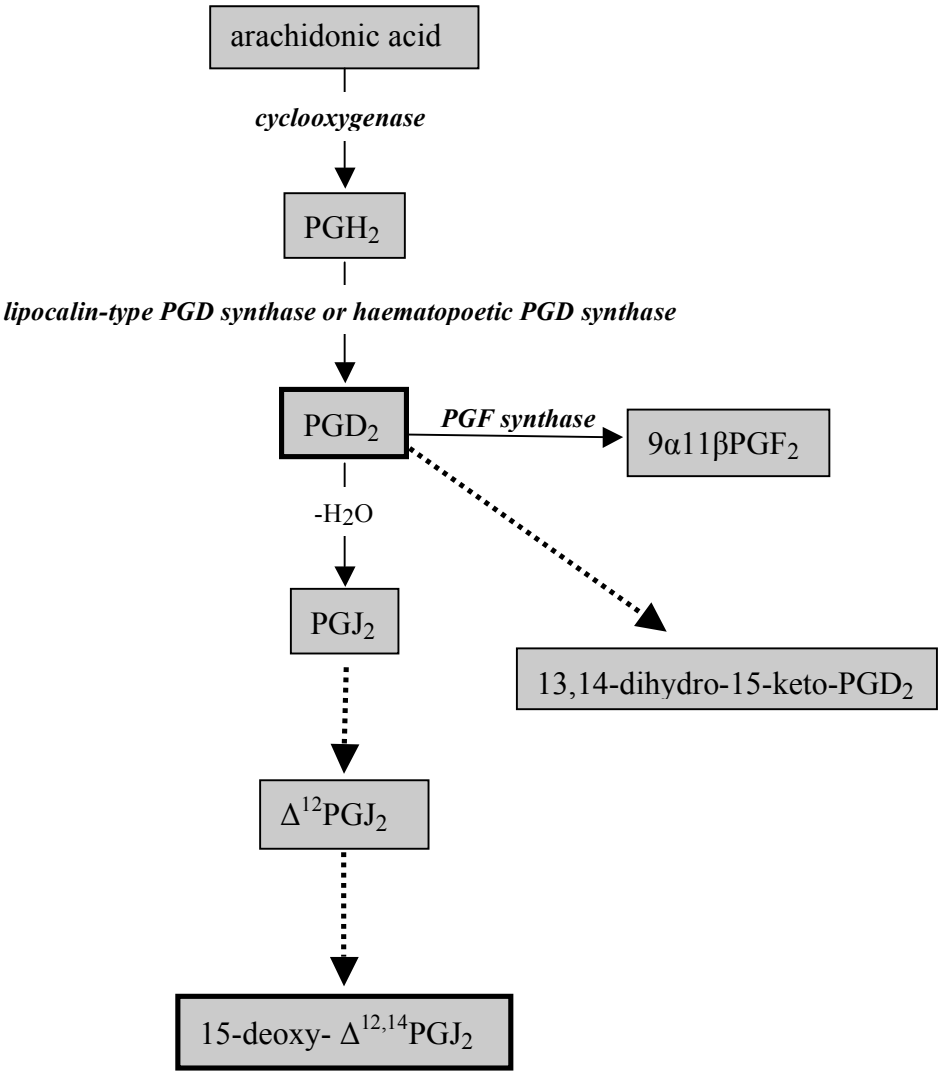


Figure 1.8 PGD_2 metabolic pathways. This shows the production of PGD_2 by two enzymic pathways (depending on cell type) and the subsequent conversion of PGD_2 to known metabolites (dashed arrows show non-enzymatic conversion). The compounds outlined in bold are those which have been used in the current study (adapted from Hirata *et al.*, 1988).

1.5.5.10 Immunopharmacology of Prostaglandins

The various PGs have many opposing and overlapping actions. An example of this is the effect of $PGF_{2\alpha}$ which produces bronchoconstriction, while PGE_2 produces bronchodilation yet they both contract muscles of the GI tract (Mathe & Hedqvist, 1975). PGE_2 is a well established mediator of inflammation as it appears to be responsible for the symptoms of inflammation (Gallin *et al.,* 1988), however, the role which other PGs play in the control or modulation of inflammatory responses, particularly TNF production, is not well characterised. This is important with respect to both the different PGs and their respective receptor subtypes. PG analogues have been useful in this regard, both agonists and antagonists have helped elucidate the PG and receptor pathways for various responses especially for PGE_2. As indicated earlier, the use of analogues which target PGs other than the one of interest are useful in eliminating "cross-reactivity" and confirming whether a receptor system is solely involved in controlling a particular response. Thus, although PGD_2 receptor pathways are the focus of the present study, analogues for PGE_2will also be briefly discussed.

1.5.5.10.1 Prostaglandin E_2 analogues

Numerous studies have shown that PGE_2 can inhibit many immune cell responses such as phagocytosis or the production of inflammatory cytokines (Davidson *et al.*, 1998). Conversely, COX inhibitors enhance these parameters indicating that endogenous prostaglandins are actively involved in the downregulation (Watabe *et al.*, 1993; Davidson *et al.*, 1998). The nature of the receptors which mediate this suppression has been studied using various synthetic PGE_2 analogues. Butaprost (an EP_2 receptor agonist) has been shown to suppress TNF production from LPS-stimulated dendritic cells (Vassiliou *et al.*, 2003) which would indicate that EP2 receptors are involved in the suppression. The lack of effect of the EP3/ EP1 agonist, sulprostone, gave a clear indication that downregulation of TNF occurs via EP2 receptors. In the same study, the authors showed that all 4 EP receptor subtypes are upregulated in the presence of LPS (Vassiliou *et al.*, 2003) clearly indicating the invaluable contribution of PG receptor

analogues in elucidating the receptors responsible for controlling specific functions. On the basis of gene expression data it would not have been possible to reveal which receptor subtype was the most important with respect to function.

1.5.5.10.2 Prostaglandin DP analogues

As indicated previously, BWA868C is an antagonist which binds to DP_1 receptors and has been shown to prevent PGD_2-induced increases in cyclic AMP levels (Arimura *et al.,* 2001). Similarly BW245C, a DP_1 receptor agonist increases intracellular levels of cyclic AMP (Town *et al.*, 1983), but, as discussed previously, it retains activity against EP_2 and EP_4 receptors. (Abramowitz *et al.,* 2000).

Recently several compounds have been developed which may be useful tools in evaluating the role of DP_2 receptors. The most promising compound is CAY10471 which is an analogue of BAY-µ3405 (ramatroban) a TP receptor antagonist which also showed antagonist activity toward DP_2 receptors (Ulven *et al.,* 2005). CAY10471 was developed by modifying the ramatroban structure (Sugimoto *et al.,* 2003). Minor structural changes such as shortening of the acidic side chain by one methylene group resulted in a compound with highly selective and potent DP_2 antagonist activity (Sugimoto *et al.*, 2003). Mathiesen *et al.* (2006) showed that three ramatroban analogues TM30642, TM30643, TM30089 (CAY10471) which were modifications of the ramatroban structure by either i) a single additional methyl group (TM30642), or ii) an acetic acid instead of propionic acid side chain (TM30634), or iii) by both modifications (TM30089- CAY10471). All three compounds inhibited PGD_2 binding to DP_2 receptors with nanomolar affinity (Mathiesen *et al.,* 2006). However, it was also shown that the 3 compounds were functionally antagonists and compared to ramatroban, this was accompanied by a decrease in maximal effect indicating that the antagonism, by CAY10471 especially, was insurmountable. It was shown, in the same study, that this was not the result of an allosteric interaction but by direct binding to DP_2 receptors. The data indicated that the reason for the insurmountable antagonism is a result of the slow rate of dissociation of from the DP_2 receptor and compared to ramatroban (which shows

surmountable antagonism) the half-life was 1.55 x 10^6 -fold longer (Mathiesen *et al.*, 2006). This clearly indicates that antagonism of the DP_2 receptor by CAY10471 is essentially irreversible and would make it an ideal research tool and, potentially, a useful therapeutic compound.

1.5.5.10 Prostaglandins in inflammation

The 2- series of prostaglandins, especially PGE_2, are generally regarded as being pro-inflammatory (Funk *et al.*, 2001). This is based on the observations that PGE_2 can induce the various symptoms of inflammation i.e. vasodilation, swelling and pain. Direct evidence for the role of PG in inflammatory responses was provided by the study of Portanova *et al.* (1996). It was shown that specific anti- PGE_2 antibodies attenuated both the oedema (swelling) and pain associated with inflammation. They showed that rat paw oedema and hyperalgesia induced by carageenan was suppressed by an anti-PGE_2 monoclonal antibody compared to treatment with a control non-specific antibody (Portanova *et al.*, 1996). These results indicate that PGE_2 plays a major role in tissue oedema and hyperalgesia. This effect was comparable to the actions of the cyclooxygenase inhibitor indomethacin in the same study (Portanova *et al.*, 1996). Thus, this is further direct evidence that PGE_2 is involved in producing the symptoms of inflammation. However, many studies have also shown that PGE_2 can potently suppress the formation of the upstream mediators of inflammation i.e. cytokines especially IL-1 and TNF-α. PGE_2 can completely suppress TNF-α production from many cells including monocytes/ macrophages and whole blood (Spengler *et al.*, 1989; Davidson *et al.*, 1998; Habeeb *et al.*, 2002). This clearly demonstrates that exogenous PGE_2 can suppress cytokine production, but cyclooxygenase inhibitors have been shown to enhance cytokine release indicating that endogenous PGs have a regulatory role in the control of cytokine production (Gilroy *et al.*, 1999). As the cylooxygenase inhibitors suppress all PG synthesis it is most likely that PGE_2 is not the only PG which can modulate cytokine production and that other major PGs produced during inflammatory responses may also play an important role such as the PGD_2 pathways evaluated in the present study.

1.6 Aims and objectives of the study

Following the stimulation of immune cells such as monocytes or lymphocytes by a wide variety of pathogenic stimuli, Interleukin-1 and TNF-α are the major pro-inflammatory cytokines released. It has been shown that prostaglandins are produced from arachidonic acid and act as secondary mediators of inflammation (Davidson *et al.*, 1998). More recently it has been recognized that PGE_2 can suppress the production of primary inflammatory mediators, specifically TNF-α. (Davidson *et al.*, 1998). There are many different series of prostaglandins in addition to PGE_2 such as PGD_2 and $PGF_{2\alpha}$. This project aimed to study the effect of prostaglandin D_2 (PGD_2) and its metabolite 15d-PGJ_2 on LPS-stimulated TNF-α production in human blood and monocytes.

It was also intended to ascertain which receptor systems were involved in mediating the effects of PGD_2 and 15d-PGJ_2. This was achieved pharmacologically, using different agonist and antagonist drugs which interfere with the 2 main receptor systems, $DP_1$and DP_2, described for PGD_2. The study will assess the actions of 15d-PGJ_2 on TNF-α production.

The specific objectives of the study were:

- To ascertain the effect of PGD_2 on LPS-stimulated TNF-α production in human blood and monocytes.
- To compare the effect of PGE_2 and $PGF_{2\alpha}$ on LPS-stimulated TNF-α production in human blood as a landmark (established) action.
- To characterise the receptor pathways for PGD_2 on LPS stimulated TNF-α production in human blood and monocytes using:
  a selective agonist for DP_1 receptors - BW245C
  selective antagonists for the 2 receptors
  BWA868C (DP_1 antagonist) and
  CAY10471 (DP_2/$CRTH_2$ antagonist)
- To compare the effect of PGD_2 to that of 15d-PGJ_2 on LPS-stimulated TNF-α production in the presence of the DP_1 and DP_2 inhibitors BWA868C and CAY10471.

2. Materials and Methods

2.1 Materials

'Analar' reagents were used throughout and any additional materials were obtained from the following sources

Cells

Human Blood- & Buffy Coat	Scottish National Blood Transfusion Service (SNBTS) Gartnavel Hospital, Glasgow UK

Drugs/ modulators

BWA868C (DP_1 antagonist)	Cayman Chemical Co., USA
BW245C (DP_1 agonist)	"
CAY10471 (DP_2 /$CRTH_2$ antagonist)	"
Prostaglandin D_2	"
Prostaglandin E_2	"
Prostaglandin $F_{2\alpha}$	"
Prostaglandin 15d-PGJ_2	"
Lipopolysaccharide (*Salmonella abortus equi*)	"

Chemicals/ Assay Materials

Bovine serum albumin	Sigma –Aldrich, Dorset, UK
Dimethyl sulphoxide	"
Di-Sodium Hydrogen Phosphate	"
Ethanol	"
Ficoll Histopaque (1.077g/l)	"
Polyethylene glycol (Av. Mol. Wt. 8000)	"
Potassium Chloride	"
Potassium dihydrogen Phosphate	"
Sodium Chloride	"
Sulphuric acid	"
Tween-20	"
3, 3', 5, 5'-tetramethylbenzidine (TMB)	"

Human TNF-α ELISA antibody pairs	Biosource Europe S.A. Belgium

Consumables/ plastics

Cell culture dish and flask	Coaring- Coaster
Eppendorf 1.5 ml tubes	Greiner Bio-one (Stonehouse, UK)
Sterile universal tubes (30ml)	”
24-well cell culture plates	”

Additional Materials

Chloroform	Fisher Scientific UK
Distilled water	ELGASTAT water purifier
Foetal calf serum	Invitrogen/ Gibco, Paisley, UK
Hank’s balanced salt solution	”
RPMI-1640	”

Equipment

Balance- Mettler AJ100

Benchtop Centrifuge- Jouan A-14

Centrifuge- Sorvall RT 6000D

ELISA plate reader – Titertek Multiskan MCC/340

pH meter –Jenway 3020

2.2 Methods

2.2.1 Preparation of test substances, chemicals and drugs

Stock solutions of PGE_2, PGD_2 and $PGF_{2\alpha}$ and BWA868C were prepared in ethanol. BW245C and CAY10471 stock solutions were prepared in Dimethyl sulphoxide (DMSO). The maximum solvent concentration did not exceed (0.1%) in final incubations and solvents were diluted in RPMI immediately before use.

2.2.2 Preparation of human blood and human monocytes for experimental use

Whole human blood was obtained from the Scottish National Blood Transfusion Service (SNBTS) Glasgow, and utilised directly from the donor bag. Human mononuclear cells and monocytes were isolated from leukocyte-rich Buffy coat fraction obtained from SNBTS.

2.2.2.1 Blood incubation protocol

Each assay was performed under aseptic conditions. Aliquots of blood (700µl) were dispensed into plastic 1.5 ml Eppendorf tubes. Sterile solutions of drugs including LPS, PGE_2, PGD_2, and $PGF_{2\alpha}$ were added in 100 µl aliquots and the volume was adjusted to yield a final volume of 1ml by the addition of RPMI-1640 or solvent. Samples were incubated in plastic 1.5ml Eppendorf tubes. Incubations were carried out for 20 h, at 37 °C, 5% CO_2 and 100% humidity. After incubation, the samples were centrifuged at 10,000 g for 30 seconds and plasma was collected. The plasma was transferred to fresh tubes and stored at -20^{o} C until assayed.

2.2.2.2 Isolation of human peripheral blood monocytes

The Leukocyte-rich Buffy coat fraction was collected from the donor bag under aseptic conditions and the mononuclear cell layer was obtained by Ficoll Histopaque (1.077 g/ l) density centrifugation. The Buffy coat was gently layered onto 10 ml of Ficoll before being centrifuged in swing-out rotor for 1 h at 400 g at 20 °C. The mononuclear cell layer was removed, washed twice with HBBS and resuspended in 16ml RPMI-1640.

Aliqouts (4 ml) of the mononuclear cell suspension (isolated as described previously) were placed into sterile 60-mm diameter plastic Petri dishes (Nunclon, Denmark) and incubated for 20 h, at 37^{o}C, 5% CO2 and 100% humidity. Adherent cells were collected by scraping the plates with a sterile rubber 'policeman' into 1 ml of HBBS followed by centrifugation at 400 g for 5mins at 20 ^{o}C and washed twice in HBBS. Finally, the cells were resuspended in RPMI 1640 at 2 x 10^{6} cells/ ml and referred to as monocytes. Monocytes were adjusted to produce a final concentration of 1x10^{6} cells/ml and placed in Eppendorf tubes. Cells were incubated in a volume of 700 µl and the various agents were added in 100 µl aliquots to yield a final volume of 1 ml. Cells were incubated at 37^{o}C, 5% CO_2 and 100% humidity. Following incubations, the samples were centrifuged at 10,000 g for 30 seconds and the resultant supernatant was transferred to fresh tubes and stored at -20 °C until assayed.

2.2.2.3 Cell counting and assessment of viability

Cell viability was estimated with cell counting using the Trypan blue exclusion test. An aliquot of collected cells was diluted 1:10 (v/ v) with RPMI-1640 before being diluted 1:1 with Trypan blue solution. The stained cells were then immediately transferred to a Neubauer improved Haemocytometer and counted under a phase contrast light microscope. Trypan blue stain excluded from viable cells, while non-viable cells are permeable appearing blue when stained. Viability was estimated by calculating the percentage of total cells within the sample that were observed to exclude the dye. Cell viability was routinely found to be greater than 95% in human peripheral mononuclear cells and human monocytes.

2.2.3 Measurement of TNF-α Production

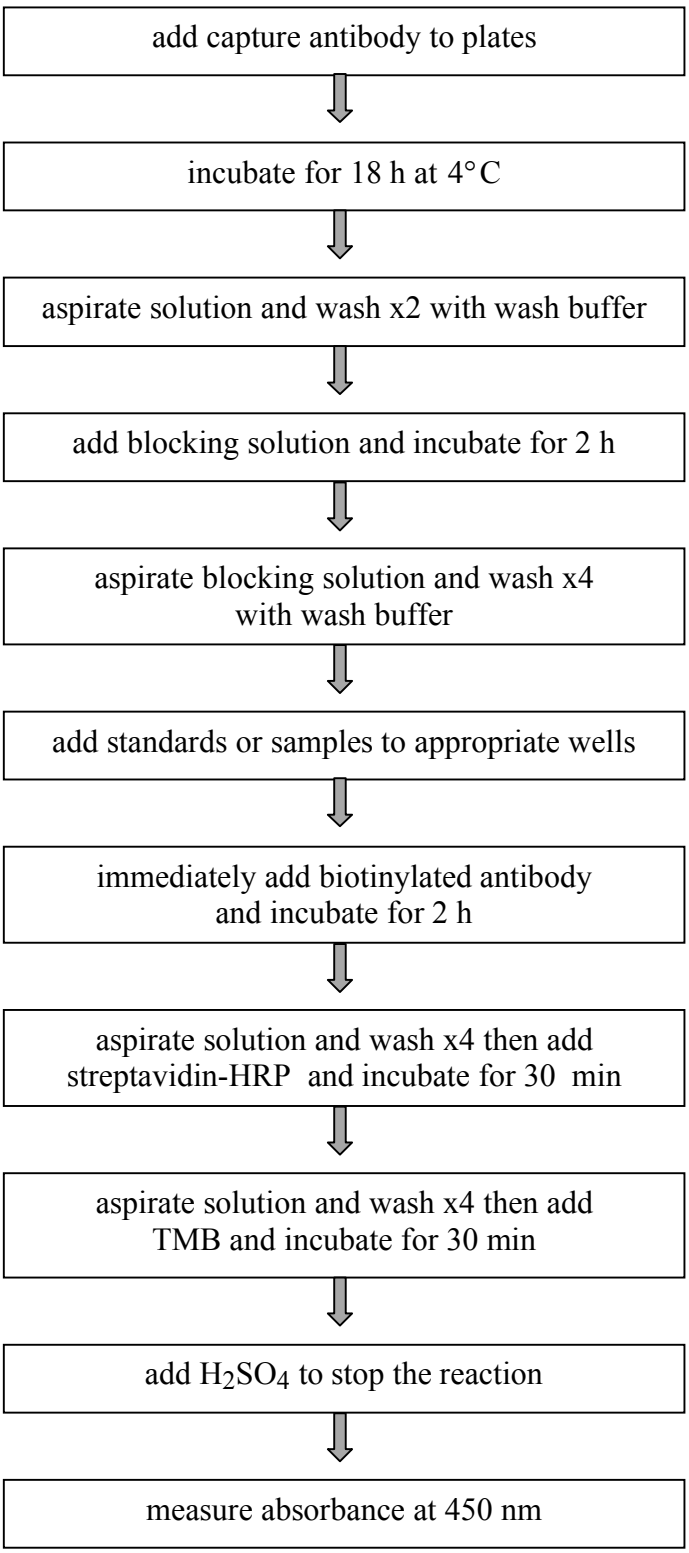
2.2.3.1 ELISA Assay Protocol

The sandwich enzyme-linked immunosorbent assay (ELISA) technique was used for the determination of TNF-α. Microtitre plates (Nunc-Imuno modules 100 µl/ well) were used coated with capture monoclonal antibody diluted to 5µgm/ml for TNF-α antibody in a coating buffer (PBS: Nacl 8g/ L, $Na_2HPO_42H_2O$ 1.13g/ l, KH_2PO_4 0. 2g/ L KCl 0. 2g/ L, 99% Distil H_2O) at pH 7.4 and incubated for 18 h at 4^0 C. Wells were then washed four times with PBS containing 0.5% (v/ v) Tween-20. Samples and TNF-α standards were diluted appropriately in standard diluent (standard diluent; PBS containing 0.5% (w/ v) BSA and 0.1% (v/ v) Tween-20). The biotinylated detection antibody was also diluted in the standard diluent to achieve a concentration of 0.8µg/ml. Aliquots of diluted samples/standards (100µl) were added to the appropriate wells before 50µl of diluted detection antibody was added to each well and incubated for 2 hrs at room temperature. After a further five washes with PBS containing 0.5% (v/ v) Tween-20, a streptavidin horseradish peroxidase (HRP) conjugate was diluted 1:625 in standard diluent and 100µl added to each well for 30 minutes at room temperature.

The substrate 3,3', 5,5'-tetramethyl-benzidine (TMB) was utilised to detect the bound HRP. The TMB substrate solution was diluted 1:3 in standard diluent and 100µl added to each well. The wells were then incubated at room temerature in the dark or 30 minutes. The reaction was stopped by the addition of 100µl of 1M H_2SO_4 to each well and the absorbance measured within 30 minutes on a Titertek Multiskan MCC/340 plate reader at an absorbance of 450 nm.

In order to determine the concentrations of TNF-α in samples, standard curves were always prepared alongside samples.

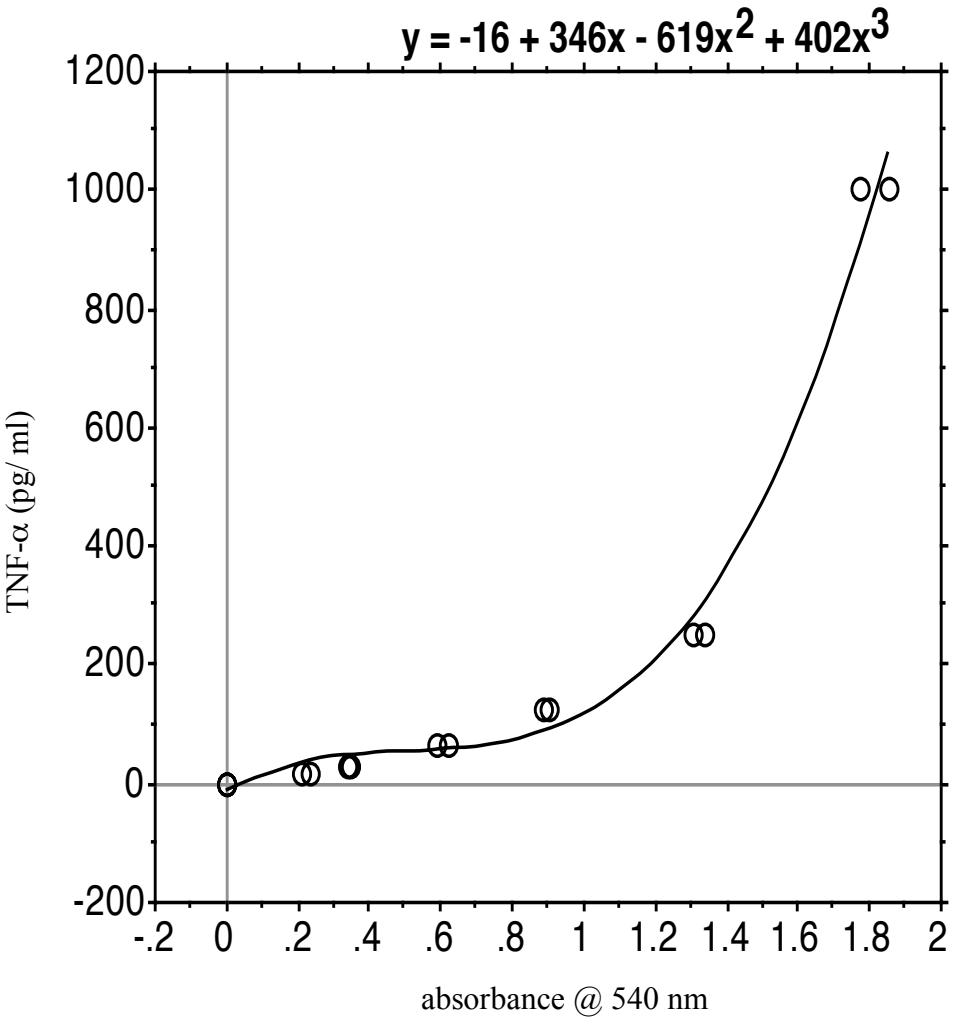
2.2.3.2 Flow Diagram for ELISA Assay of TNF-α



2.2.3.3 Data analysis

Data was analysed using Statview Student Software (Abacus Concepts, Inc.) with the TNF-α concentration present within each sample determined by 3rd order polynomial regression analysis, enabling comparison with the appropriate standard curve (Figure 2.1). In each case the values obtained were adjusted for the appropriate dilution factors. All the data are expressed as the mean of n replicates ± the standard deviation of the mean and were assessed for statistical significance using a Students unpaired t-test. The difference between control and treated groups was considered to be significant when P was less than 0.05.

Figure 2.1 Typical TNF-α standard curve for ELISA assay



A standard curve for TNF-α was generated for each assay using human recombinant TNF-α standards that were run concurrently with the samples during analysis by ELISA as described in section 2.2.3.1. The equation above the figure shows the 3rd order polynomial algorithm generated by this particular data set and used by the software to calculate unknown concentrations of TNF-α from the absorbance values.

3. Results

3 RESULTS

3.1 The effect of Varying LPS Concentrations on TNF-α Production in Human Blood

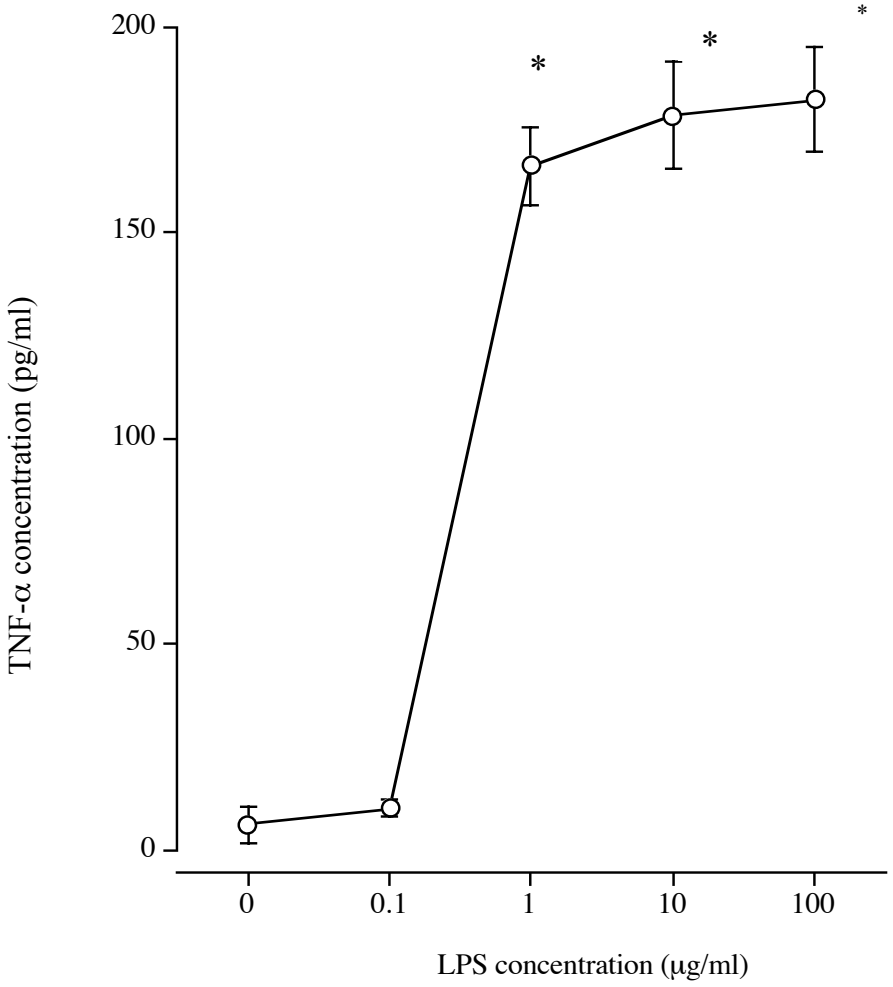
The various effects of lipopolysaccharide (LPS) are widely documented within the scientific literature for its ability to stimulate cytokine production (Mathiak *et al.*, 2002).

Initial experiments were performed to determine the effect of LPS on the production of TNF-α in human blood. The level of TNF-α was measured by ELISA assay as described in section 2.2.3 As shown in Fig. 3.1, increasing concentrations of LPS resulted in a dose-dependent increase in TNF-α production. At 0.1μg/ml LPS had no effect. The threshold level in the production of TNF-α occurred at 1μg/ml. 10μg/ml concentration of LPS was shown to induce sub-maximal production of TNF-α. The maximum production of TNF-α was observed in the presence of 10-100 μg/ml LPS, which shows a 17-18 fold increase in TNF-α levels above control.

3.2 Effect of Varying Concentrations of PGE_2 on TNF-α production in Blood in response to LPS.

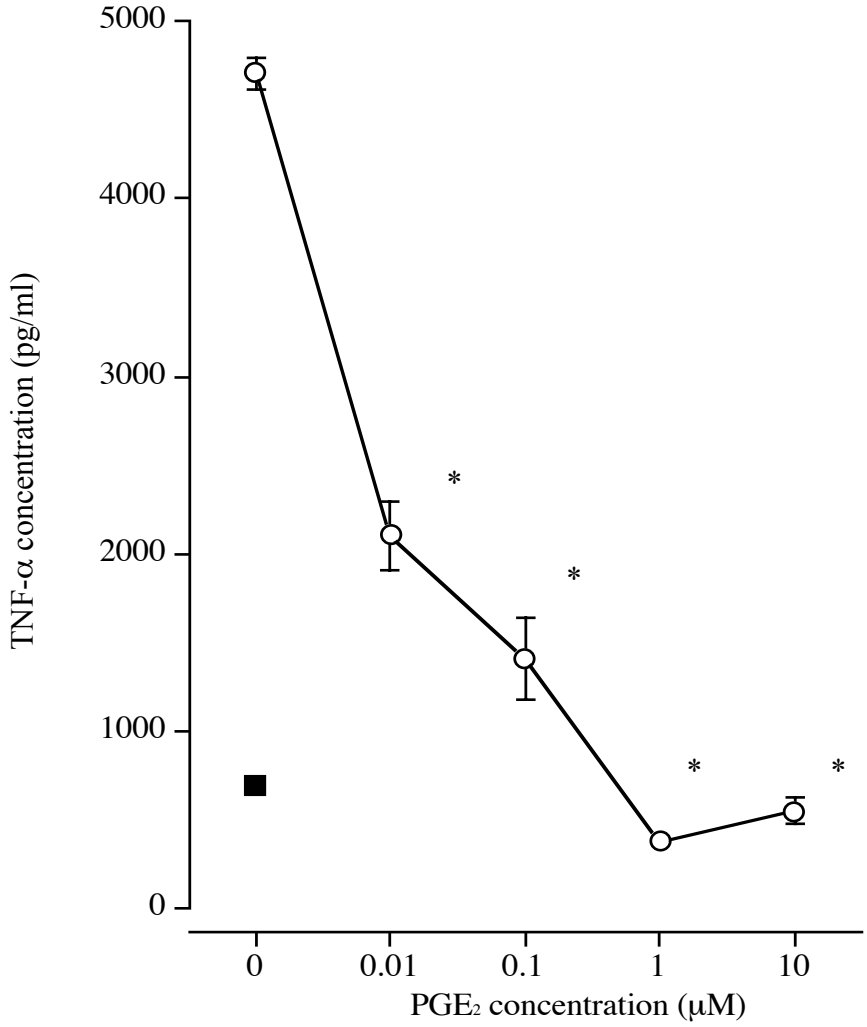
PGE_2 was used to ascertain its ability to reduce cytokine production, specifically TNF-α. Human blood was incubated with increasing concentrations of PGE_2 in the absence (control) or presence of LPS (10μg/ml) for 20 hours. The production of TNF-α within each sample was determined by ELISA assay (section 2.2.3). As shown in Fig. 3.2, blood incubated with LPS produced a 6-fold increase in TNF-α production in comparison to incubations without LPS or PGE_2. Increasing the PGE_2 concentration resulted in a dose-dependent decrease in TNF-α production having a maximum suppressive effect between 1-10μM (Fig. 3.2). The threshold level at which PGE_2 exerted a significant inhibition on TNF-α production in whole blood was 0.01 μM. The IC_{50} for PGE_2 to inhibit the production of TNF-α in blood was calculated to be 40 nM.

Figure 3.1 **The effect of Varying LPS Concentrations on TNF-α Production in Human Blood**



Blood was incubated with increasing concentrations of LPS for 20 hours at 37 °C, 100% humidity, 5% CO_2. Incubations were then centrifuged and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. *Denotes $P < 0.05$ when compared to control.

Figure 3.2 Effect of Varying Concentrations of PGE_2 on TNF-α production in Blood in response to LPS



Blood was incubated either with culture medium alone (filled square, showing basal TNF-α production) or with various concentrations of PGE_2 in the presence of LPS (10μg/ml) for 20 hours. Incubations were then centrifuged and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. *Denotes $P < 0.05$ when compared to LPS alone i.e. no PGE_2.

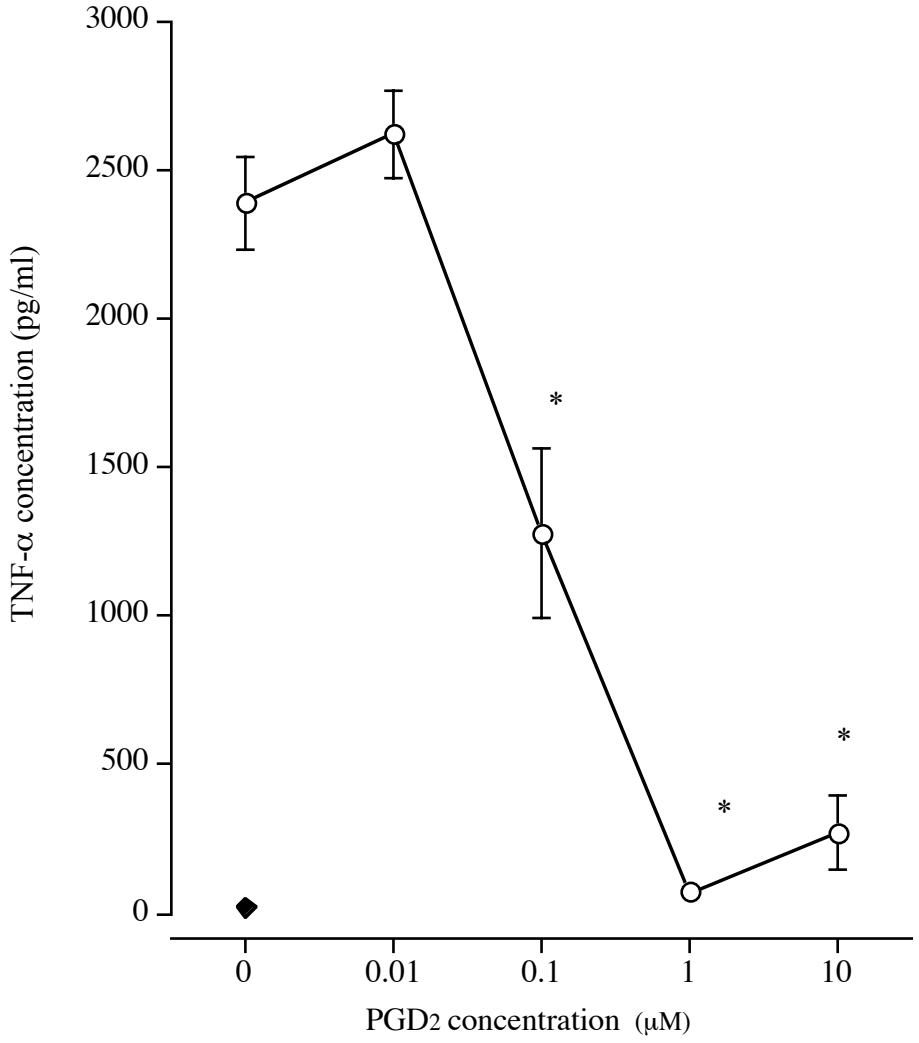
3.3 Effect of Varying Concentrations of PGD_2 on LPS-stimulated TNF-α production in blood.

In the previous experiment PGE_2 suppressed LPS-stimulated TNF-α production in blood. Thus, the effect of other prostaglandins such as PGD_2, and $PGF_{2\alpha}$ was also evaluated on LPS-stimulated TNF-α production. LPS stimulation resulted in a > 20-fold increase in of TNF-α production compared to the control incubations (Fig. 3.3). PGD_2 produced a dose-dependant decrease in TNF-α production. Maximal suppression of TNF-α was observed at 1 - 10μM PGD_2. The threshold level at which PGD_2 exerted a significant inhibition on TNF-α production in blood was 0.1 μM. The IC_{50} for PGD_2 to inhibit the production of TNF-α in whole blood was calculated to be 20 nM.

3.4 Effect of PGD_2 on LPS-stimulated TNF-α Production in Human Monocytes

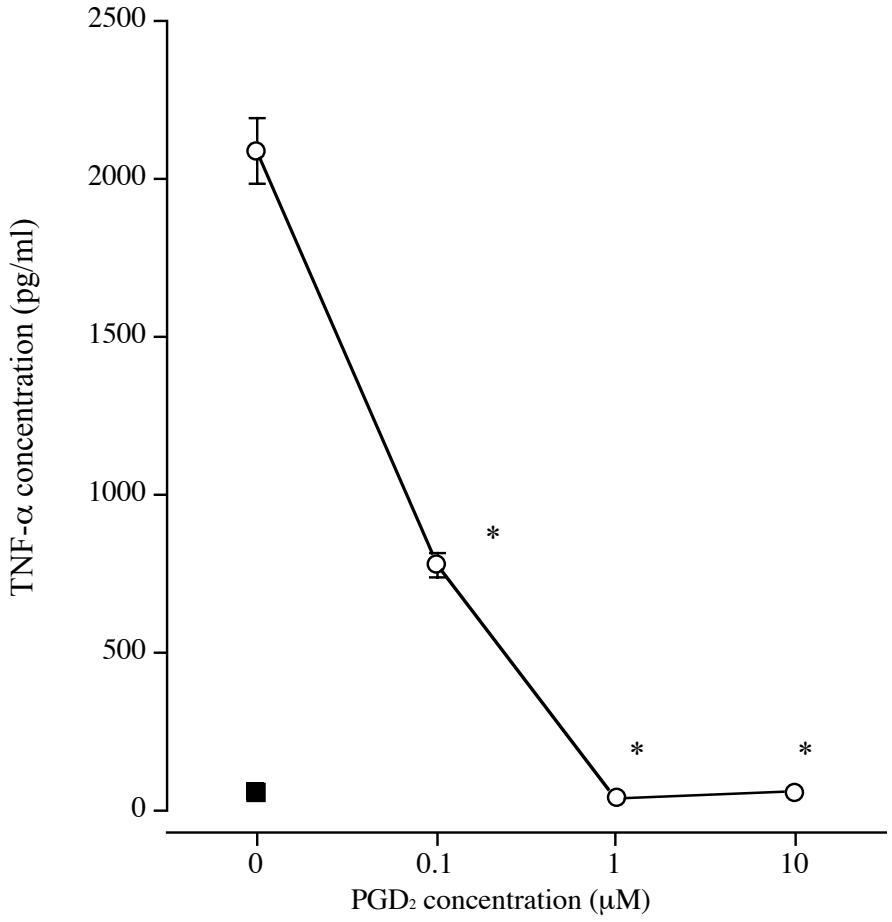
As PGD_2 was shown to have a suppressive action toward LPS-stimulated TNF-α production, experiments were performed to see whether this action of PGD_2 was directly targeted toward monocytes. This was studied on monocytes isolated from human blood. Incubation of monocytes with LPS (10 μg/ ml) resulted in a 22-fold increase in TNF-α production in comparison to control incubations (Fig. 3.4). The increase in TNF-α production was reduced with increasing PGD_2 concentrations (Fig. 3.4). The threshold level at which PGD_2 exerted a significant inhibition of LPS-stimulated TNF-α production in monocytes was 0.1 μM. The IC_{50} for this PGD_2 to inhibition in monocytes was calculated to be 30 nM. Maximum suppression of TNF-α production occurred at 1 – 10 μM (Figure 3.4).

Figure 3.3 Effect of PGD_2 on LPS-stimulated TNF-α Production in Human blood



Blood was incubated either with culture medium alone (♦ - basal TNF-α production) or with various concentrations of PGD_2 in the presence of LPS (10μg/ml) for 20 hours. Incubations were then centrifuged and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. *Denotes $P < 0.05$ when compared to incubations with LPS alone.

Figure 3.4 Effect of PGD_2 on LPS-stimulated TNF-α Production in Human Monocytes



Monocytes (1x10^6) isolated from human peripheral blood were incubated either with culture medium alone (■) or with various concentrations of PGD_2 in the presence of LPS (10μg/ml) for 20 hours. Incubations were then centrifuged and the TNF-α levels in the supernatant were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the mean of n = 3 ± s.d. *Denotes $P < 0.05$ when compared to incubations with LPS alone.

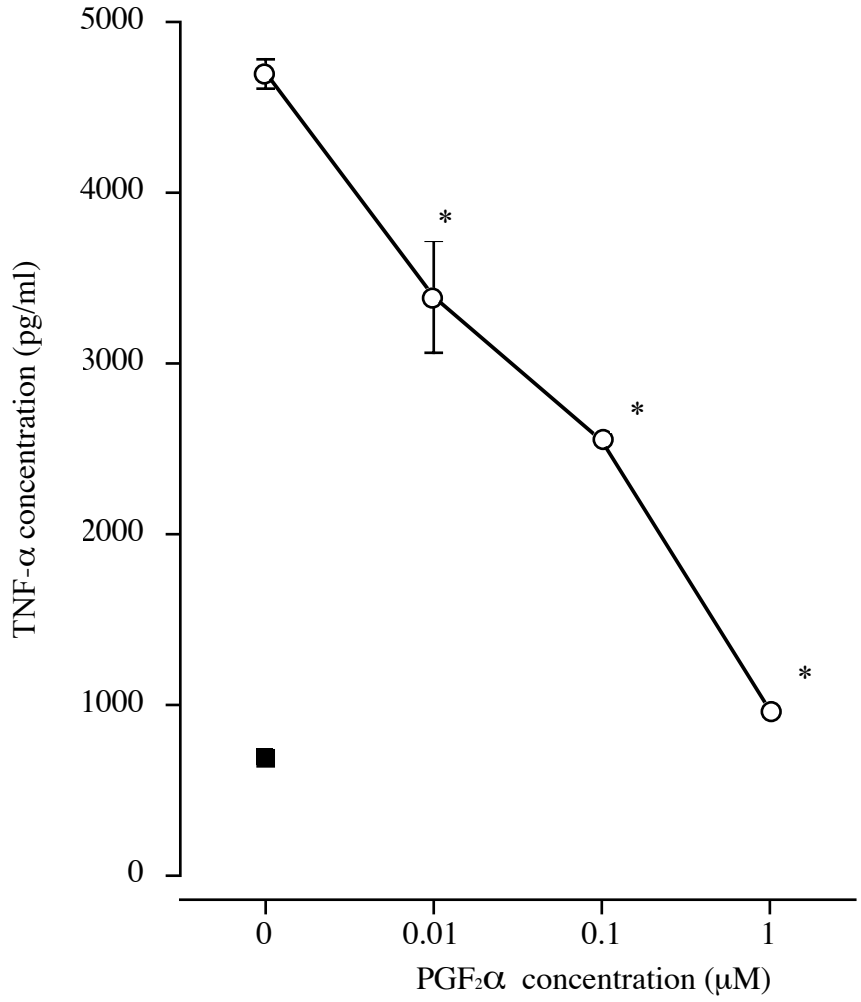
3.5 Effect of $PGF_{2\alpha}$ on LPS-stimulated TNF-α Production in Human Blood

The effect of $PGF_{2\alpha}$ on LPS-stimulated TNF-α production in whole blood was also evaluated. Blood was incubated with LPS in the presence of increasing concentrations of $PGF_{2\alpha}$ for 20 hours. TNF-α production was determined by ELISA assay (section 2.2.3). As shown in previous experiments, LPS increased TNF-α production well above the basal level. $PGF_{2\alpha}$ decreased TNF-α production in a dose dependent manner (Fig. 3.5). The threshold level at which $PGF_{2\alpha}$ exerted a significant inhibition of TNF-α production was 0.01 μM. The corresponding IC_{50} value for the $PGF_{2\alpha}$ inhibition of TNF-α production in whole blood was calculated to be 50 nM. This indicated that $PGF_{2\alpha}$was less effective than either PGE_2 or PGD_2. The remainder of the study focussed on PGD and PGD receptor pathways.

3.6 Effect of PGD_2 and BWA868C (DP_1 receptor antagonist) on LPS -stimulated TNF-α production in Human Blood.

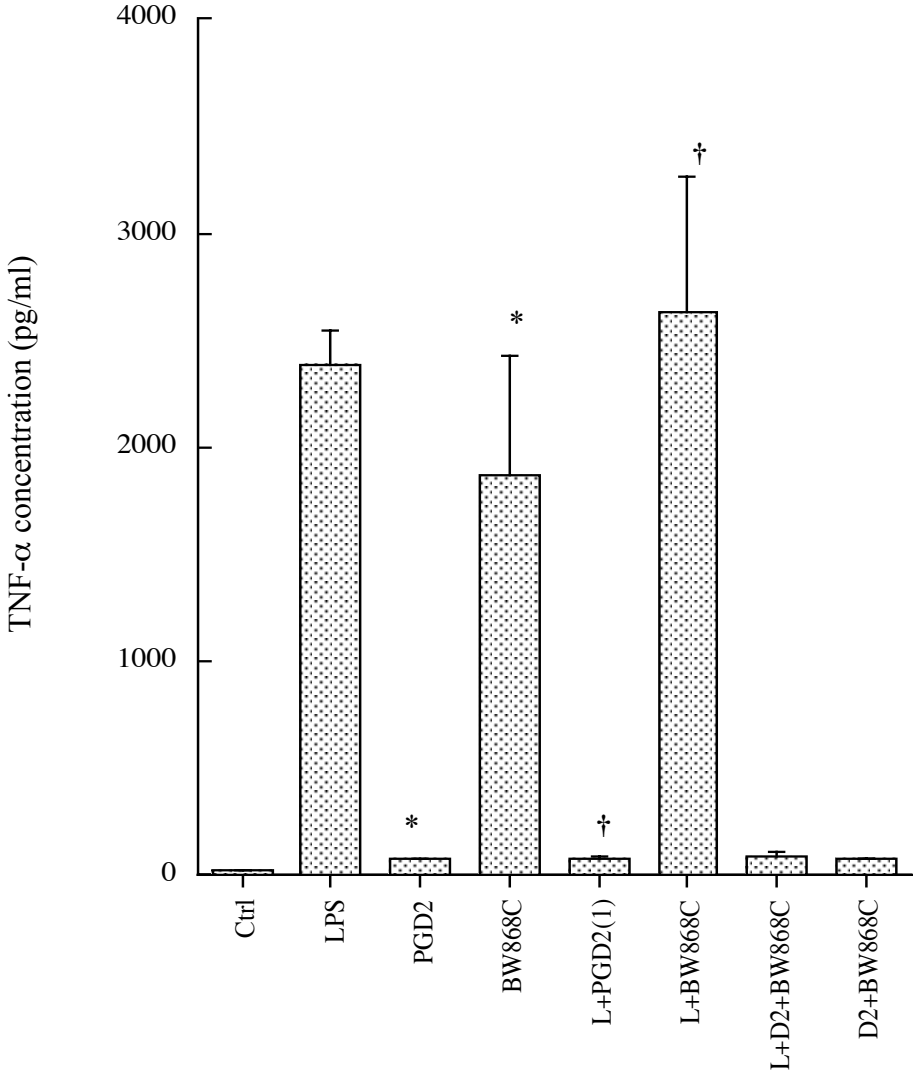
A series of experiments were carried out to ascertain the receptor pathways by which the various prostaglandins PGE_2, PGD_2, and $PGF_{2\alpha}$ induced their suppressive effects. PGD_2 was shown to suppress TNF-α production and this could be mediated by two potential receptor pathways DP_1 or $CRTH_2$/ DP_2. The effects of a DP_1 receptor antagonist (BWA868C) were evaluated on the PGD_2-induced suppression of TNF-α production. In order to ascertain the role of DP_1 receptors. Fig. 3.6 shows the effect of PGD_2 on TNF-α production in the presence of BWA868C. TNF-α production was very low in control incubations (in the absence of any agents). LPS (10μg/ml) stimulated a large increase in TNF-α production, > 40-fold. BWA868C alone also resulted in an increase in TNF-α. PGD_2 almost completely abolished the LPS-stimulated increase in TNF-α levels (Fig. 3.6). Incubation with BWA868C and LPS resulted in an increase in TNF-α compared to incubations with LPS alone. The PGD_2 –induced suppression of LPS-stimulated TNF-α production was not altered by BWA868C.

Figure 3.5 Effect of $PGF_{2\alpha}$ on LPS-stimulated TNF-α Production in Human Blood



Blood was incubated either with culture medium alone (■) or with various concentrations of $PGF_{2\alpha}$ in presence of LPS (10μg/ml) for 20 hours. Incubations were then centrifuged and the levels of TNF-α in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the mean of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to incubations with LPS alone.

Figure 3.6 **Effect of PGD_2 and BWA868C (DP_1 receptor antagonist) on LPS-stimulated TNF-α production in Human Blood.**



Blood was incubated for 20 hours either with culture medium alone (ctrl) or with LPS (10μg/ml) in absence and presence of PGD_2 (1 μM), BWA868C (50 nM) or a combination of LPS, PGD_2 and BWA868C (L+BWA868C+D2). The TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS induced TNF-α stimulation.

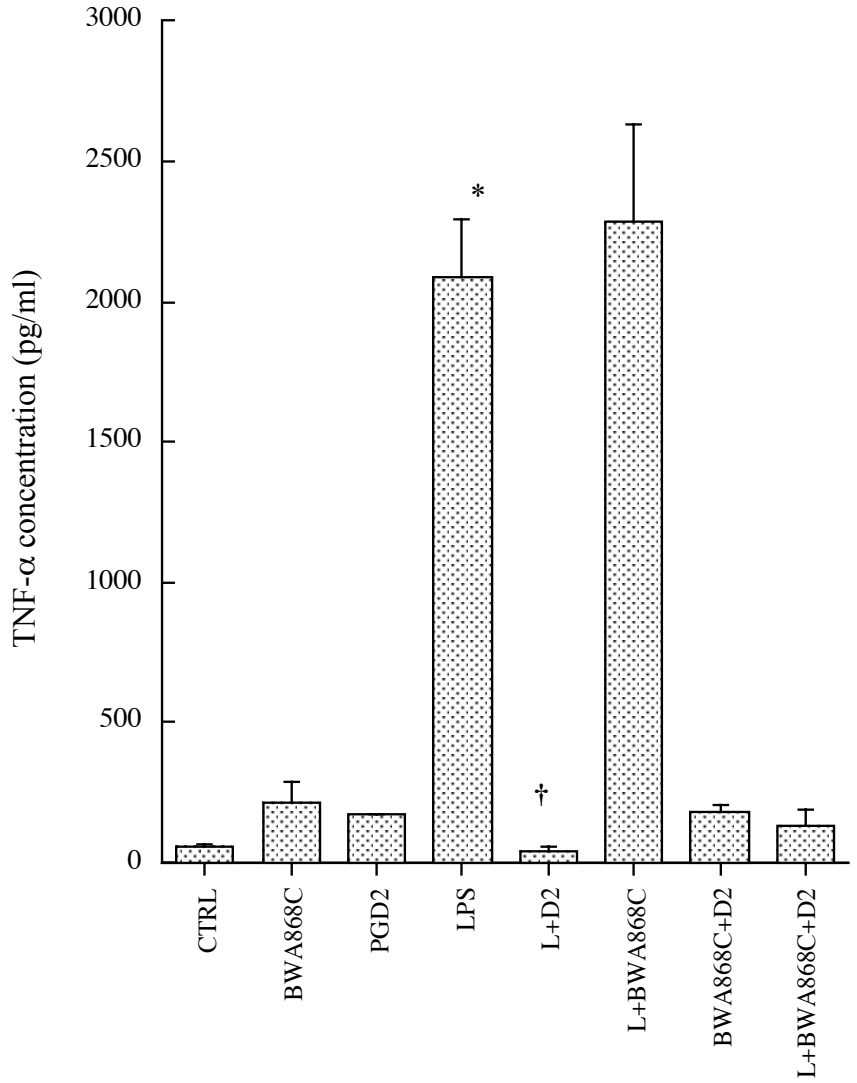
3.7 Effect of PGD_2 and BWA868C (DP_1 receptor antagonist) on LPS –stimulated TNF-α production in Human monocytes.

In whole blood PGD_2 demonstrated a profound suppression of LPS-stimulated TNF-α levels that was unaffected in the presence of BWA868C. Further experiments were performed to ascertain whether similar effects occurred in monocytes. In the presence of LPS, TNF-α production was greatly elevated and this was considerably suppressed in incubations including PGD_2 (Fig. 3.7). BWA868C did not significantly alter either the PGD_2–induced suppression of LPS-stimulated TNF-α production or the elevated level of TNF-α with LPS alone. Thus, the suppressive effect of PGD_2 was not altered by the DP_1 receptor antagonist, BWA868C.

3.8 Effect of PGE_2 on LPS-stimulated TNF-α production in Human Blood in the presence of BWA868C (DP_1 receptor antagonist)

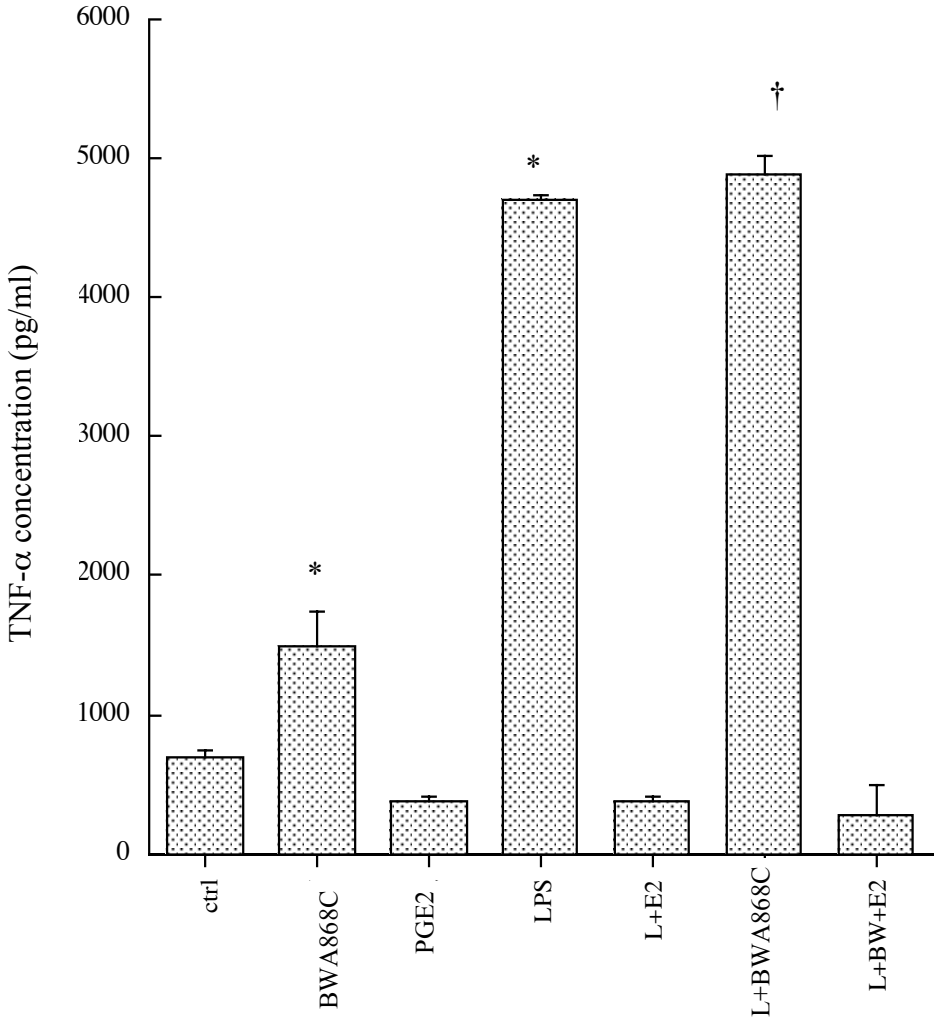
PGE_2 or $PGF_{2\alpha}$ can be interconverted by enzymatic actions in blood and possibly converted to PGD_2. The metabolites may be responsible for producing a suppressive effect on TNF-α production. To test this hypothesis, additional experiments were carried out to ascertain the effects of exogenous PGE_2 and $PGF_{2\alpha}$ via DP1 receptors on TNF-α production in the presence of a DP_1 receptor antagonist (BWA868C). Fig 3.8 shows the effect of PGE_2 on TNF-α production in the presence of BWA868C. Incubation of blood with LPS greatly increases TNF-α production in comparison to control incubations. Exogenously added PGE_2 either alone or in incubation with LPS significantly reduced TNF-α production. BWA868C either alone or in the presence of LPS produced a significant increase in TNF-α production. However, BWA868C did not alter the suppressive actions of PGE_2 (Fig. 3.8).

Figure 3.7 **Effect of PGD_2 and BWA868C (DP_1 receptor antagonist) on LPS-stimulated TNF-α production in Human monocytes**



Monocytes ($1x10^6$) isolated from Human peripheral blood were incubated for 20 hours either with culture medium alone (ctrl) or with LPS (10μg/ml) in the absence and presence of PGD_2 (1μM), BWA868C (50 nM) or a combination of both PGD_2 and BWA868C in the presence of LPS (L+BWA868C+D2). TNF-α levels in the supernatant were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the mean of n = 3 ± s.d. * Denotes $P<0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS alone.

Figure 3.8 Effect of PGE_2 on LPS-stimulated TNF-α production in Human Blood in the presence BWA868C (DP_1 receptor antagonist)



Blood was incubated for 20 hours either with culture medium alone (ctrl) or with LPS (10μg/ml) in absence and presence of PGE_2 (1μM), BWA868C (50 nM) or combination of PGE_2 and BWA868C (L+BWA868C+E2) and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the mean of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to LPS alone. † Denotes $P < 0.05$ when compared to LPS alone.

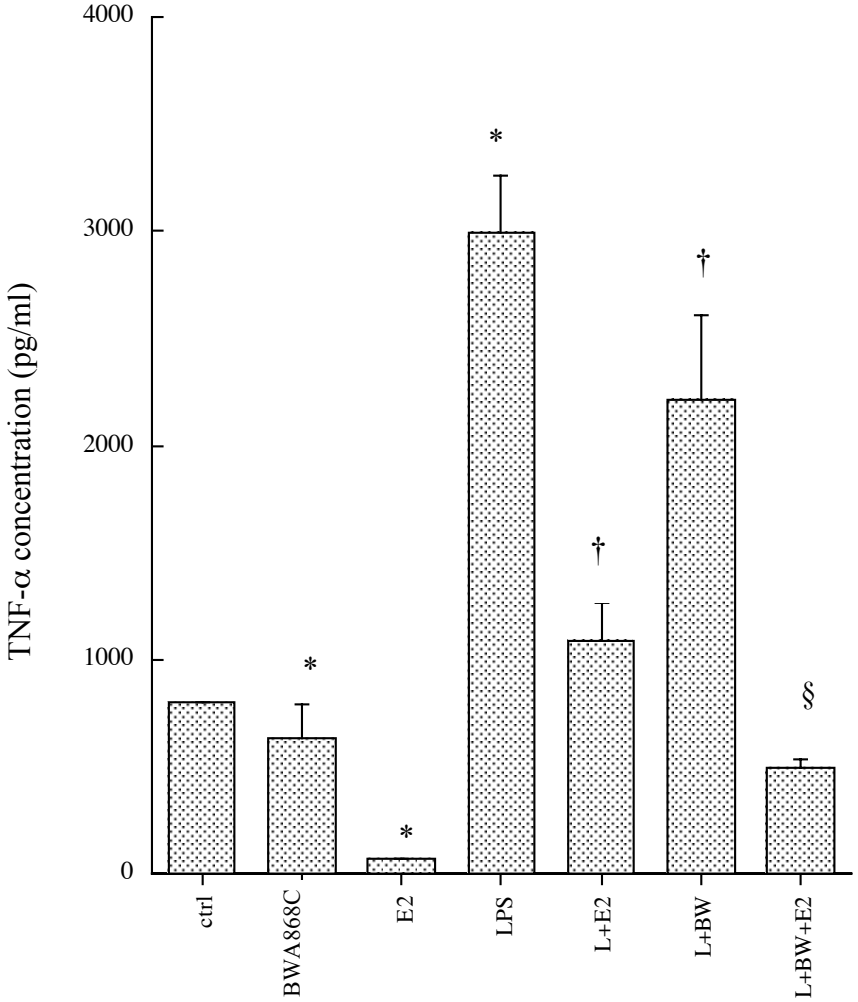
3.9 Effect of PGE_2 on LPS -stimulated TNF-α production in Human monocytes in the presence of BWA868C (DP_1 receptor antagonist)

In whole blood PGE_2 demonstrated a suppression of TNF-α production that was unaltered by BWA868C. Further experiments were designed to see whether similar effects of PGE_2 were found in monocytes. The effect of PGE_2 on TNF-α production in human monocytes is shown in Fig. 3.9. The LPS-stimulated elevation of TNF-α levels were greatly reduced by PGE_2 (1 μM). As with the blood incubations, BWA868C did not alter the effects of PGE_2.

3.10 Effect of the BWA868C (DP_1 receptor antagonist) on the $PGF_{2\alpha}$ induced suppression of the LPS-stimulated TNF-α production in Human Blood

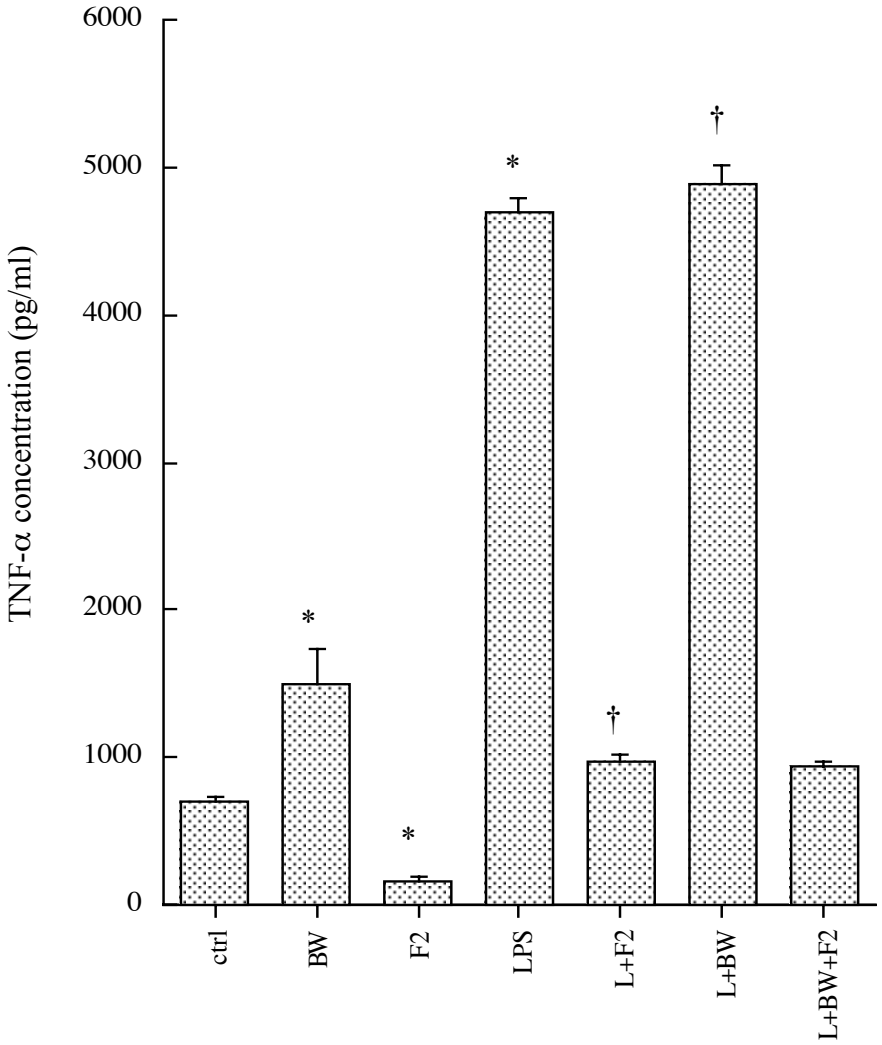
Previous experiments had shown that in human blood and monocytes, incubation with PGE_2 and PGD_2 produces a considerable suppression of TNF-α production. The effect of $PGF_{2\alpha}$ either alone or in combination with BWA868C was also evaluated. $PGF_{2\alpha}$ either alone or in the presence of LPS significantly lowers TNF-α production (Fig. 3.10). Incubation with BWA868C indicated that although it significantly increased TNF-α levels when incubated alone, it did not significantly alter either the LPS-stimulated increase or the $PGF_{2\alpha}$–induced suppression (Fig. 3.10).

Figure 3.9 Effect of PGE_2 on LPS- stimulated TNF-α production in Human monocytes in the presence of BWA868C (DP_1 receptor antagonist)



Monocytes ($1x10^6$) isolated from Human peripheral blood were incubated for 20 hours either with culture medium alone (ctrl) or with LPS (10μg/ml) in the absence and presence of PGE_2 (1μM), BWA868C (50 nM) or a combination of both PGE_2 and BWA868C (L+BWA868C+E2) and the TNF-α levels in the supernatant were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the mean of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. †$P < 0.05$ compared to LPS alone, § $P < 0.05$ when compared to LPS + PGE_2.

Figure 3.10 **Effect of BWA868C (DP_1 receptor antagonist) on $PGF_{2\alpha}$-induced suppression of LPS-stimulated TNF-α production in Human Blood**



Blood was incubated for 20 hours either with culture media alone (ctrl) or with LPS (10μg/ml) in the absence or presence of $PGF_{2\alpha}$ (1μM), BWA868C (50 nM) or a combination of both $PGF_{2\alpha}$ and BWA868C (L+BW+F2). Incubations were then centrifuged and the TNF-α levels in the plasma were determined by ELISA. Results are from of single blood donor and are representative of at least three independent experiments. Values are expressed as the mean of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ compared to LPS alone.

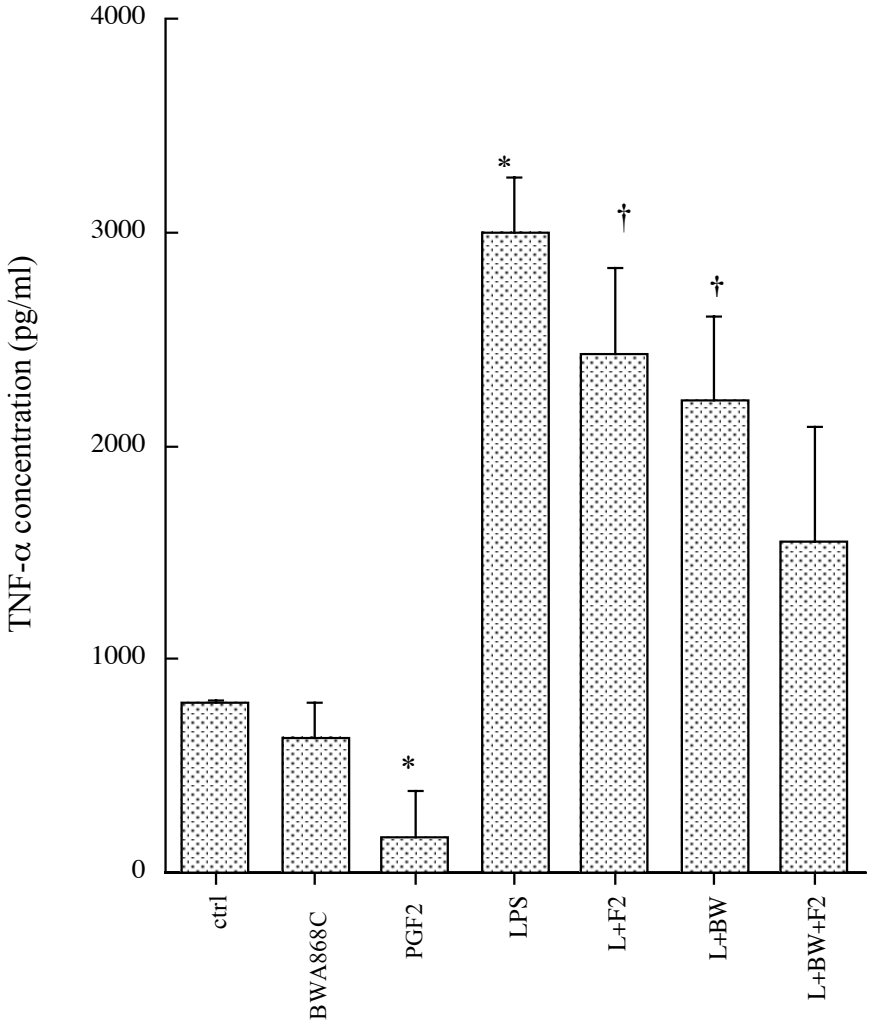
3.11 Effect of BWA868C (DP_1 receptor antagonist) on the $PGF_{2\alpha}$ induced suppression of LPS-stimulated TNF-α production in Human Monocytes

Previous experiments had shown that in human blood $PGF_{2\alpha}$ produced a considerable suppression of TNF-α production which was unaltered by BWA868C. Experiments were also carried out on monocytes to ascertain if the effects were similar. Fig. 3.11 shows the effect of the BWA868C (DP_1 receptor antagonist) on TNF-α production in the presence of $PGF_{2\alpha}$. LPS increased TNF-α levels which were reduced by $PGF_{2\alpha}$. The $PGF_{2\alpha}$–induced decreases in TNF-α production were not altered by BWA868C.

3.12 Effect of BW245C (DP_1 receptor agonist) on LPS-stimulated TNF-α production in Human Blood

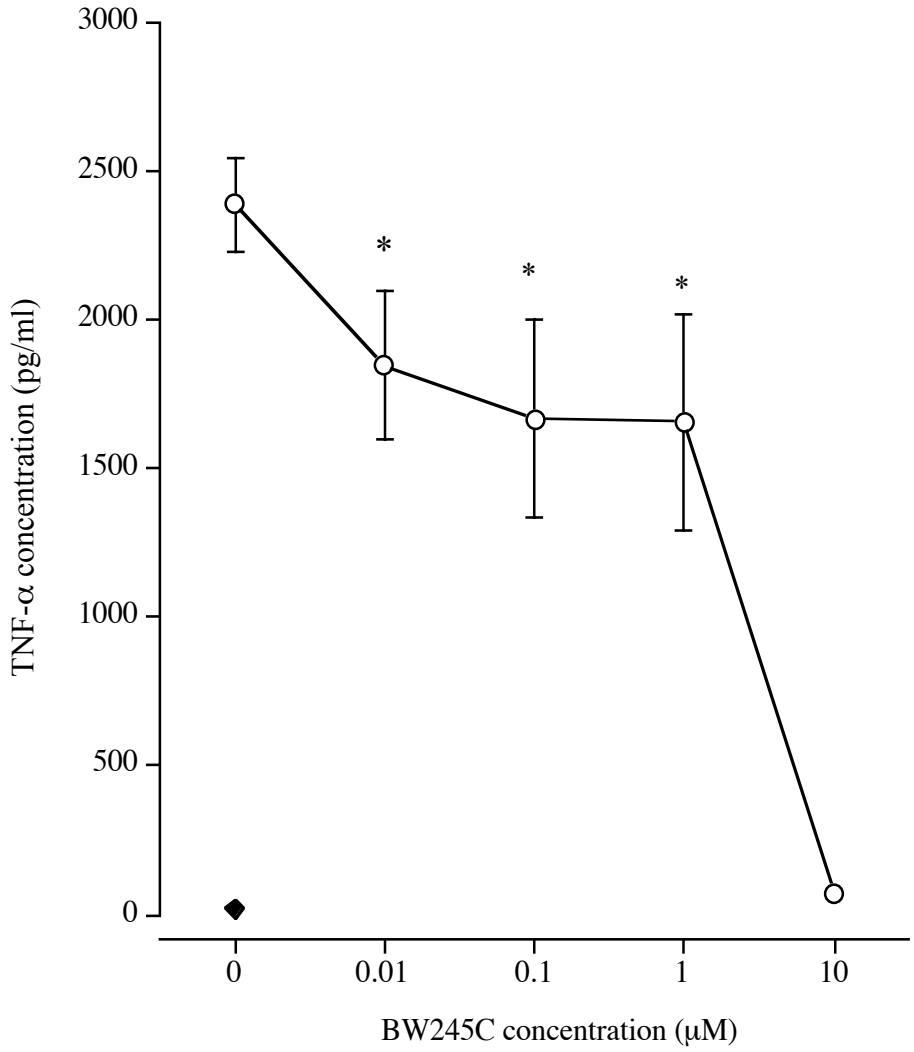
As the suppression of TNF-α production by $PGD_2$was unaltered in the presence of BWA868C (DP_1 antagonist). It was decided to further investigate the potential role of DP_1 receptor pathways by using a DP_1 agonist. The effects of the synthetic prostanoid analogue, BW245C (known to act at DP_1 receptors in other tissues) was evaluated on LPS-stimulated TNF-α production. In the presence of BW245C, LPS-stimulated TNF-α production decreased in a dose-dependent manner. The maximum suppression of TNF-α production was between 1 and 10μM (Fig. 3.12). The concentration of BW245C which yielded a threshold inhibition was 0.01μM. The IC_{50} for BW245C inhibition of TNF-α production was 0.01μM (10 nM).

Figure 3.11 Effect of BWA868C (DP_1 receptor antagonist) on the $PGF_{2\alpha}$ induced suppression of LPS-stimulated TNF-alpha production in Monocytes



Monocytes ($1x10^6$) isolated from Human peripheral blood were incubated for 20 hours either with control media alone (ctrl) or with LPS (10μg/ml) in the absence or presence of $PGF_{2\alpha}$ (1μM), BWA868C (50 nM) or a combination of both $PGF_2\alpha$ and BWA868C (L+BW+F2) and the TNF-α levels in the supernatant were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the mean of n = 3 ± s.d. * Denotes $P < 0.05$ when compared controls. † Denotes $P < 0.05$ compared to LPS alone.

Figure 3.12 **Effect of BW245C (DP_1 receptor agonist) on LPS- stimulated TNF-α production in Human Blood**



Blood was incubated for 20 hours either with control media alone (◆) or with LPS (10μg/ml) in the absence and presence of various concentrations of BW245C. Incubations were then centrifuged and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to control.

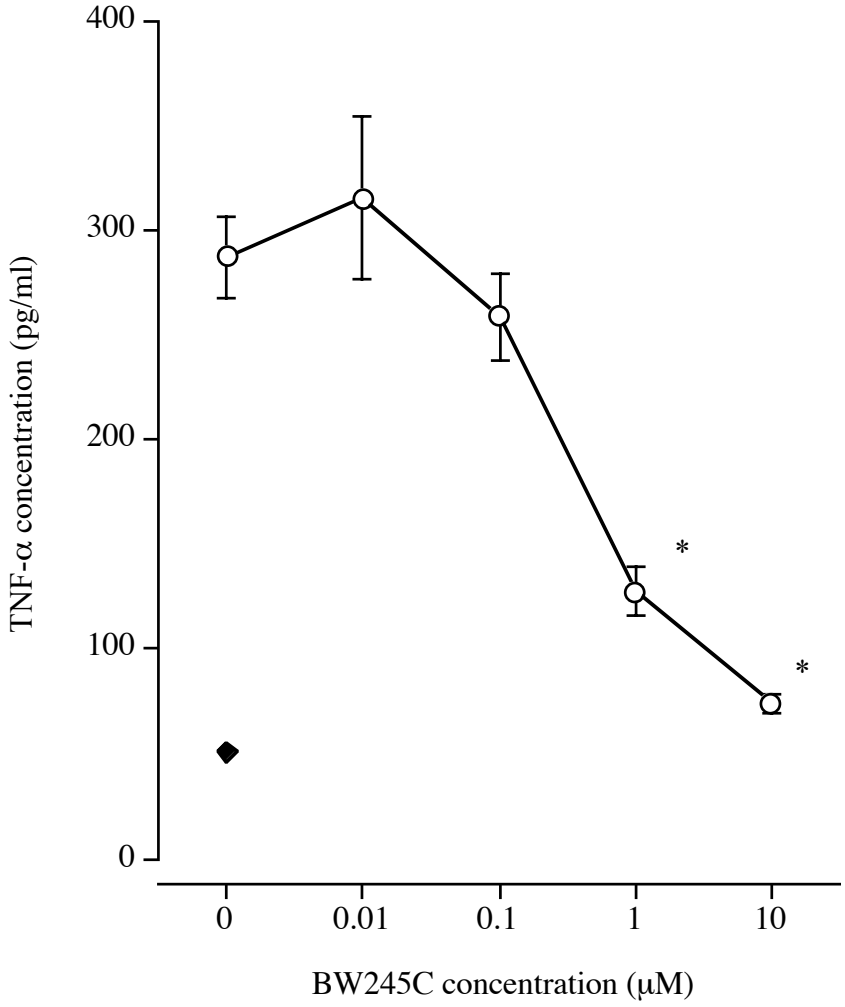
3.13 Effect of the BW245C (DP_1 receptor agonist) on LPS-stimulated TNF-α production in Human Monocytes

In whole blood BW245C showed a profound dose-dependent suppression of TNF-α production (Fig. 3.12). Thus, the effect of BW245C was also evaluated on TNF-α production from monocytes. LPS incubation produced a six-fold increase in TNF-α production in comparison to the control. As with whole blood, BW245C also suppressed TNF-α levels in a dose-dependent manner in monocytes. The maximum suppressive effect occurred at 10μM. (Fig. 3.13). The threshold effect occurred at 0.1μM BW245C. The data showed that the IC_{50} for BW245C –induced suppression of TNF-α production in monocytes was higher than that in blood - 0.09μM (90nM).

3.14 Effect of PGD_2 on LPS-stimulated TNF-α Production in Human Blood in the presence of BW245C (DP_1 receptor agonist) and BWA868C (DP_1 receptor antagonist)

In previous experiments BW245C showed a significant inhibition of TNF-α production. In order to ascertain whether this occurred via DP_1 receptors, the actions of BW245C alone or in combination with PGD_2 were investigated in the presence of BWA868C. Figs. 3.14 (a) and 3.14 (b) show the effect of PGD_2 in combination with BW245C and both agonists with BWA868C respectively. LPS produced a significant increase in TNF-α production which was suppressed by PGD_2 and BW245C individually and additionally when combined (Fig. 3.14a). The inclusion of BWA868C to the incubations did not alter the ability of either PGD_2 or BW245C to suppress TNF-α production (Fig. 3.14 b).

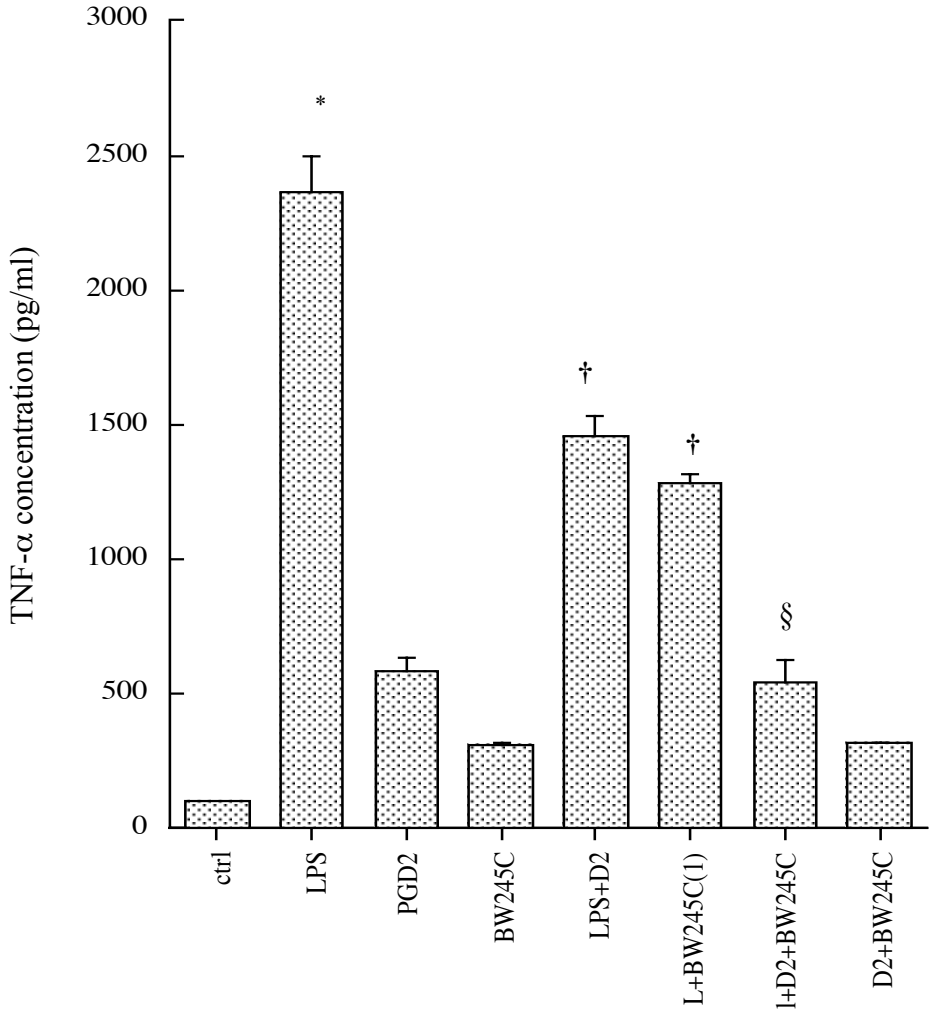
Figure 3.13 Effect of the BW245C (DP_1 receptor agonist) on LPS-stimulated TNF-α production in Human Monocytes



Monocytes ($1x10^6$) were incubated for 20 hours either with control media alone (◆) or with LPS (10μg/ml) in the absence or presence of various concentrations of BW245C. Incubations were then centrifuged and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d.

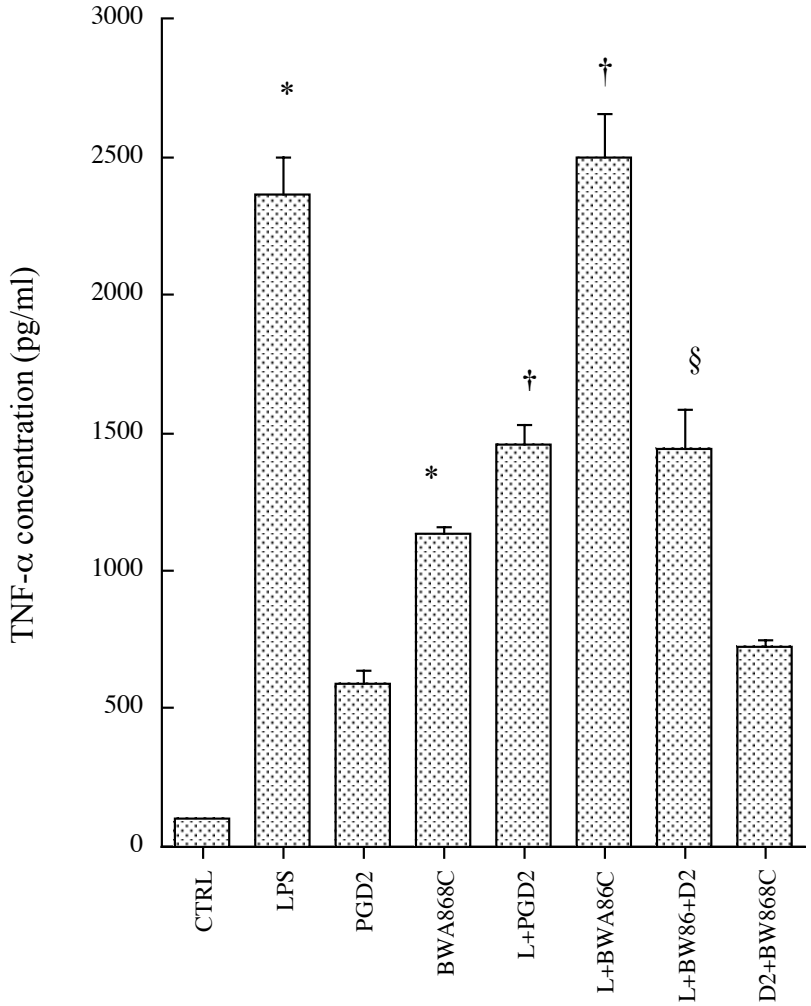
* Denotes $P < 0.05$ when compared to controls.

Figure 3.14a **Effect of PGD_2 on LPS-stimulated TNF-α Production in Human Blood in the presence of BW245C (DP_1 receptor agonist)**



Blood was incubated for 20 hours either alone (ctrl) or with LPS (10μg/ml) in the absence or presence of PGD_2 (1μM), BW245C (1μM), or in combination of PGD_2 and BW245C (L+D2+BW245) and the TNF-α levels in the plasma were determined by ELISA. Results are from the single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. *Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS-stimulated TNF-α production. § Denotes $P < 0.05$ when compared to PGD_2 induced suppression of LPS stimulated TNF-α production. (note: some error bars are extremely small and are not visible).

Figure 3.14b **Effect of PGD_2 on LPS-stimulated TNF-α Production in Human Blood in the presence of BWA868C (DP_1 receptor antagonist)**



Blood was incubated for 20 hours either alone (ctrl) or with LPS (10μg/ml) in the absence or presence of PGD_2 (1μM), BWA868C (0.05μM) or a combination of PGD_2 and BWA868C (L+ BWA868C +D2) and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS stimulated TNF-α production.

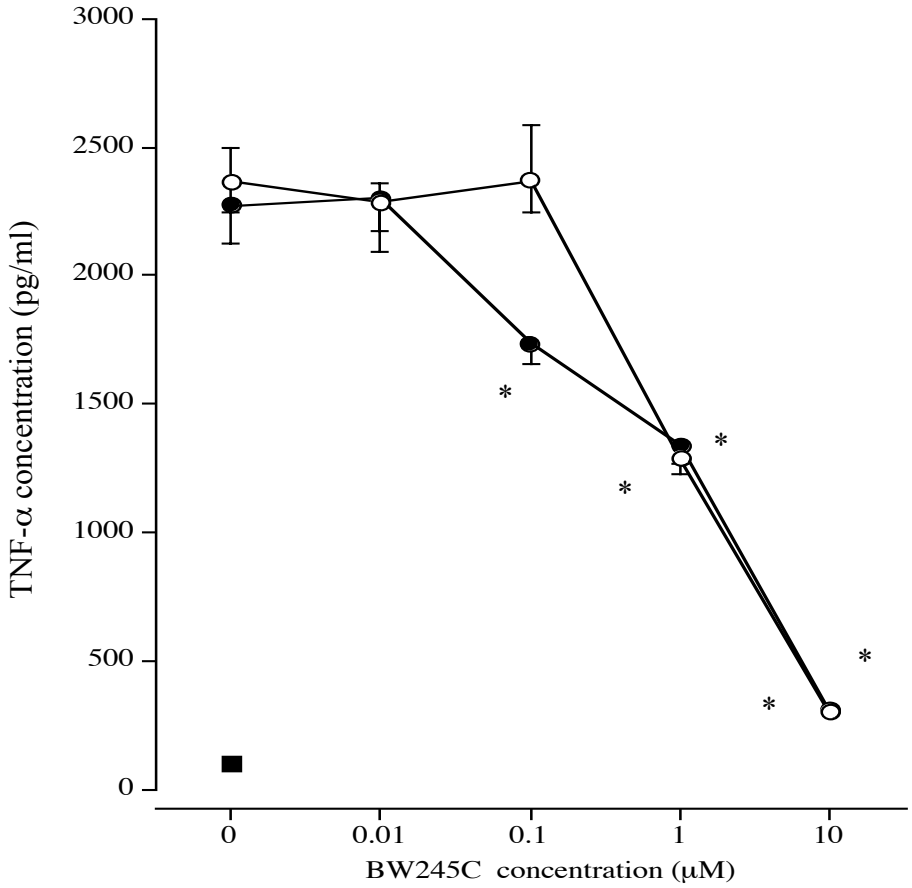
3.15 Effect of BW245C (DP_1 receptor agonist) and CAY10471 (DP_2 antagonist) on LPS-stimulated TNF-α production in Human blood

BW245C had demonstrated its ability to downregulate TNF-α production which was unaffected by the DP_1 antagonist BWA868C. It was decided to evaluate the effect of a DP_2 antagonist (CAY10471) on the BW245C-induced suppression of TNF-α production. The LPS-stimulated increases in TNF-α production was significantly reduced in a dose-dependent manner by BW245C. This inhibition was largely unaltered in the presence of CAY10471 (0.05 μM). However, only at a concentration of 0.1 μM BW245C there was a significant suppression of TNF production in the presence of CAY10471 which did not alter any other actions (Fig. 3.15). Thus, The IC_{50} for BW245C was calculated to be 0.12μM in absence of CAY10471 and slightly enhanced to 0.07μM in the presence of CAY10471.

3.16 Effect of CAY10471 (DP_2 antagonist) on BW245C (DP_1 receptor agonist) induced suppression of LPS-stimulated TNF-α production in human blood

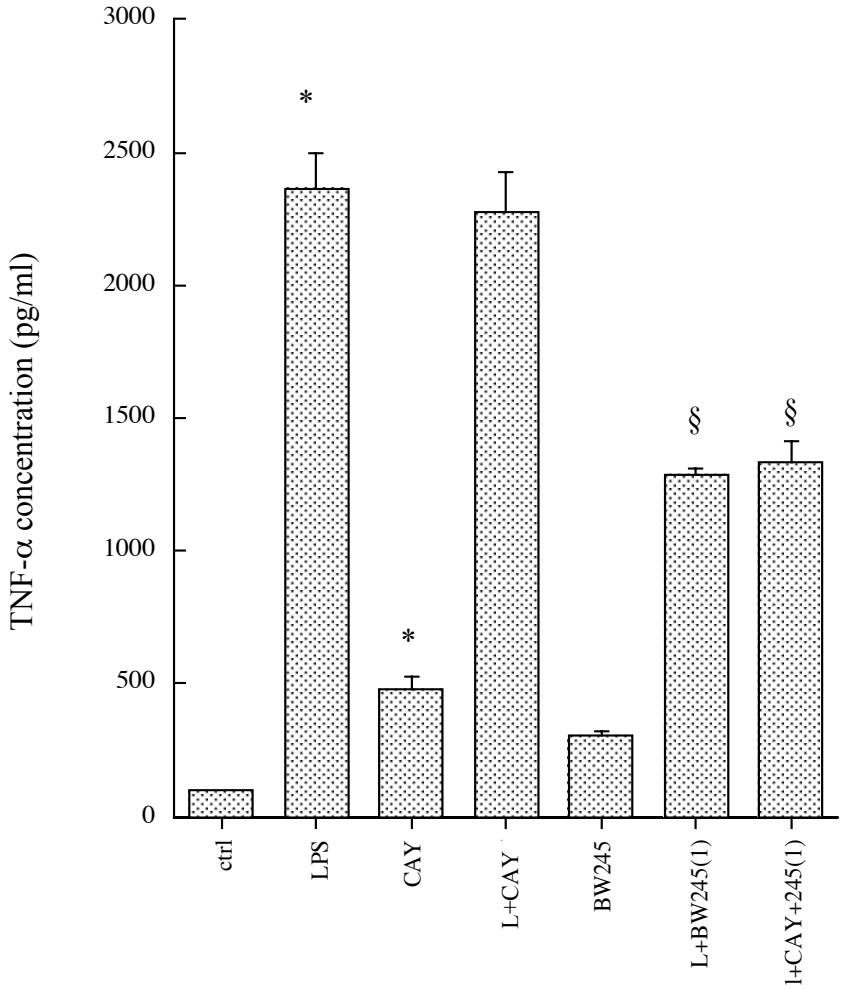
As shown previously, a single concentration of BW245C (1μM) induced a suppression of LPS-stimulated (10 μg/ ml) TNF-α production which was unaltered by CAY10471 (0.05 μM). However, it was noticed that the basal level of TNF-α production (in the absence of LPS) increased in the presence of CAY10471 (Fig. 3.16).

Figure 3.15 Effect of BW245C (DP_1 receptor agonist) and CAY10471 (DP_2 antagonist) on LPS-stimulated TNF-α production in Human blood



Blood was incubated for 20 hours either with culture media alone (–obscured by 0.05 μM CAY10471 alone- ■) or with LPS (10μg/ml) in the absence or presence of increasing concentrations of BW245C alone (open circles) or BW245C + CAY10471 (closed circles). Incubations were then centrifuged and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls.

Figure 3.16 Effect of CAY10471 (DP_2 antagonist) on BW245C (DP_1 receptor agonist) induced suppression of LPS-stimulated TNF-α production in human blood



Blood was incubated for 20 hours either with culture media alone (ctrl) or with LPS (10μg/ml) in the absence or presence of BW245C (1μM), CAY10471 (0.05μM), or a combination of BW245C and CAY10471 [L+BW245 (1) +CAY] and TNF-α levels in the plasma were determined by ELISA. Results are from the single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. *Denotes $P < 0.05$ when compared to controls. § Denotes $P < 0.05$ when compared to LPS alone. Denotes $p< 0.05$ when compared to CAY-induced stimulation of TNF-α production in LPS incubated blood.

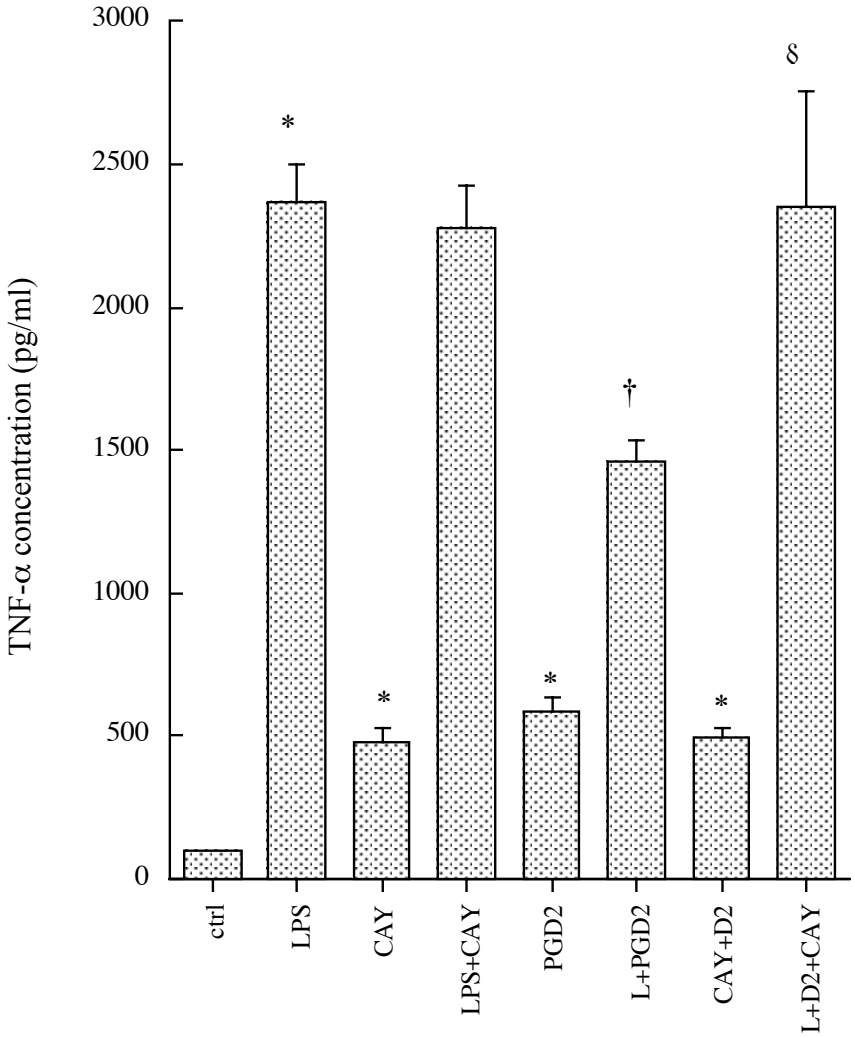
3.17 Effect of PGD_2 on LPS-stimulated TNF-α Production in Human Blood in the presence of CAY10471 (DP_2 antagonist).

PGD_2 was previously shown to effectively suppress TNF-α production and that this was not affected by the DP_1 receptor antagonist BWA868C. Thus, it was decided to evaluate the actions of the DP_2 receptor antagonist CAY10471 on the PGD_2-induced suppression of LPS-stimulated TNF-α production. In control incubations TNF-α production increased in the presence of CAY10471 (0.05μM). PGD_2 (1 μM) also resulted in a small increase in TNF-α production in comparison to control. However, PGD_2 greatly decreased levels of TNF-α stimulated by LPS (10 μg/ml). This inhibition was reversed in the presence of CAY10471 (Fig. 3.17) indicating the possible involvement of a DP_2 receptor pathway.

3.18 Direct Comparison of the effect of and BWA868C (DP_1 antagonist) and CAY10471 (DP_2 antagonist) on the BW245C (DP_1 agonist)-induced suppression of LPS-stimulated TNF-α production in human blood

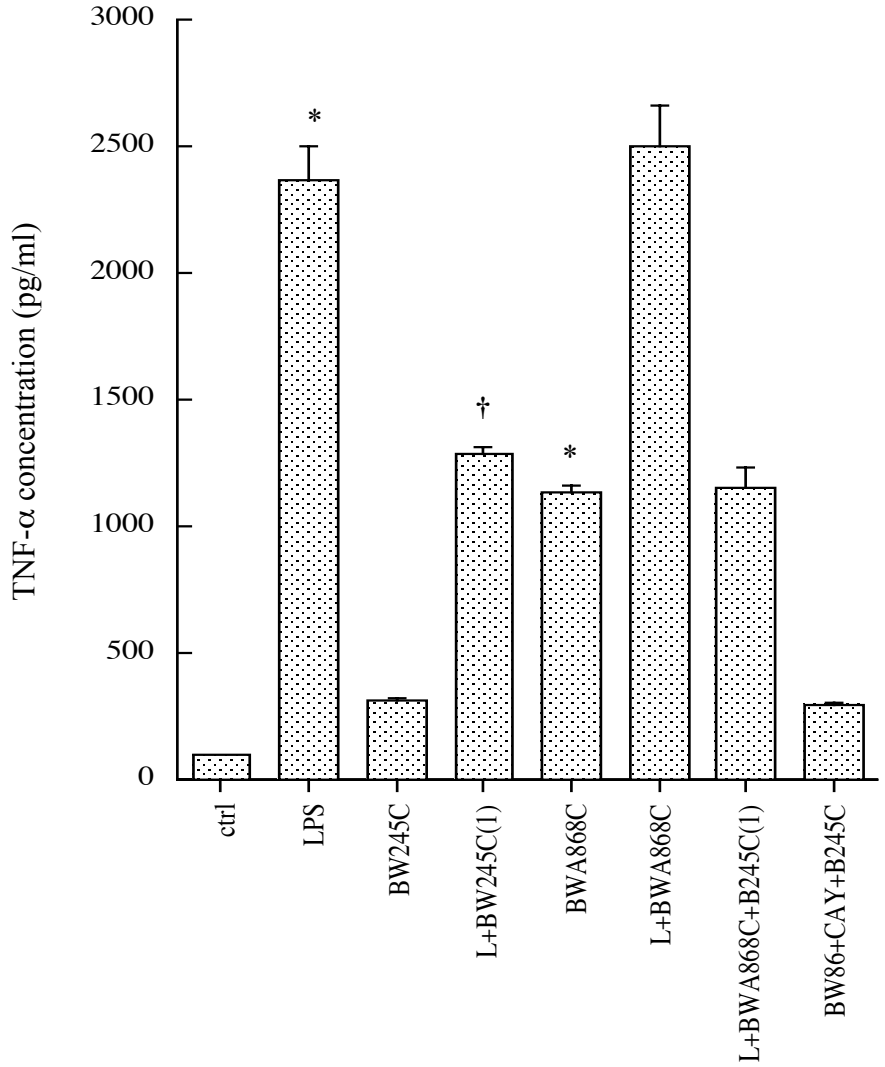
BW245C previously demonstrated a profound suppression of LPS-stimulated TNF-α levels which were unaffected by BWA868C. Thus, it was decided to directly compare the actions of BWA868C and CAY10471 on the suppressive effects of BW245C (Figs. 318 a/b). LPS (10 μg/ ml) greatly increased levels of TNF-α which were inhibited by 1 μM BW245C (Figs. 3.18a and 3.18b). This BW245C-induced inhibition was not altered by BWA868C (0.05 μM) or CAY10471 (0.05 μM).

Figure 3.17 Effect of PGD_2 on LPS-stimulated TNF-α Production in Human Blood in the presence of CAY10471 (DP_2 antagonist)



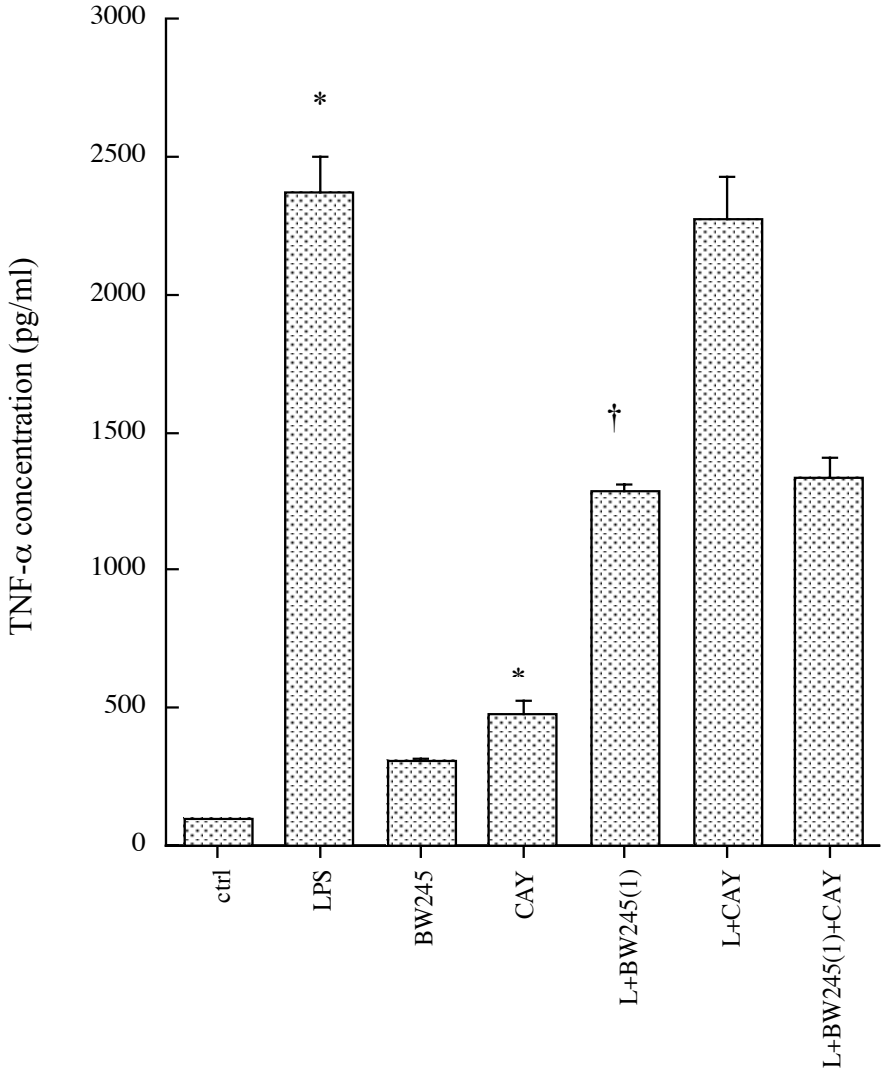
Blood was incubated for 20 hours either with culture media alone (ctrl) or with LPS (10μg/ml) in the absence or presence of PGD_2 (1μM), CAY10471 (0.05μM) or a combination of both PGD_2 and CAY10471 (L+D2+CAY). TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the mean of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS alone. § Denotes $P < 0.05$ when compared to LPS + PGD_2.

Figure 3.18a Comparison of effect of the BW245C (DP_1 receptor agonist) and BWA868C (DP_1 receptor antagonist), on LPS-stimulated TNF-α production in human blood



Blood was incubated for 20 hours either with culture media alone (ctrl) or with LPS (10μg/ml) in the absence or presence of BW245C (1μM), BWA868C (0.05μM), or a combination of BW245C and BWA868C (L+ BWA868C+B245 (1)) and TNF-α levels in the plasma was determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P<0.05$ when compared to LPS alone.

Figure: 3.18b Comparison of effect of BW245C (DP_1 agonist) and CAY10471 (DP_2 antagonist) on LPS stimulated TNF-α production in human blood



Blood was incubated for 20 hours either with culture media alone (ctrl) or with LPS (10μg/ml) in absence and presence of BW245C (BW245-1μM), CAY10471 (CAY-0.05μM), or combination of both BW245C, and CAY10471 (L+BW245 (1) +CAY) and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the mean of n=3 ± s.d. * Denotes $P<0.05$ when compared to controls. † Denotes $P<0.05$ when compared to LPS stimulated TNF-α production.

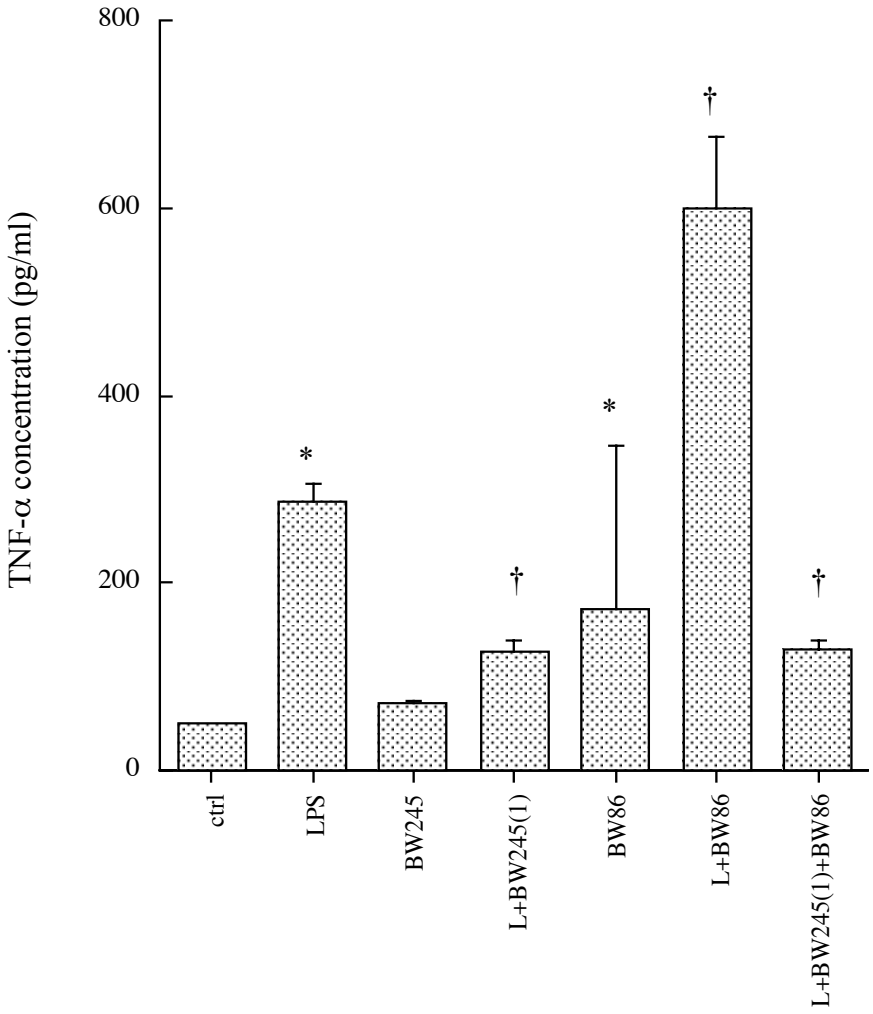
3.19 Direct Comparison of the effect of BWA868C (DP_1 antagonist) and CAY10471 (DP_2 antagonist) on BW245C (DP_1 agonist) induced suppression of LPS -stimulated TNF-α production in human monocytes

In previous experiments BWA868C and CAY10471 did not have any significant effect on the suppressive actions of BW245C toward LPS-induced TNF-α production in blood. It was decided to examine the effects of these treatments on monocytes. In monocytes, the LPS-induced elevation in TNF-α levels was reduced in the presence of BW245C (1 μM). This reduction was not altered by either BWA868C (0.05 μM) or CAY10471 (0.05 μM). However, there was an increase in both the basal and LPS-stimulated level of TNF-α (Fig. 3.19a) in the presence of BW868C (without BW245C). CAY10471 did not affect either the basal or BW245C-suppression of TNF-α production (Fig. 3.19b).

3. 20 Effect of varying concentrations of BWA868C (DP_1 antagonist) on the BW245C (DP_1 agonist)-induced suppression of LPS-stimulated production of TNF-α on induced in human blood.

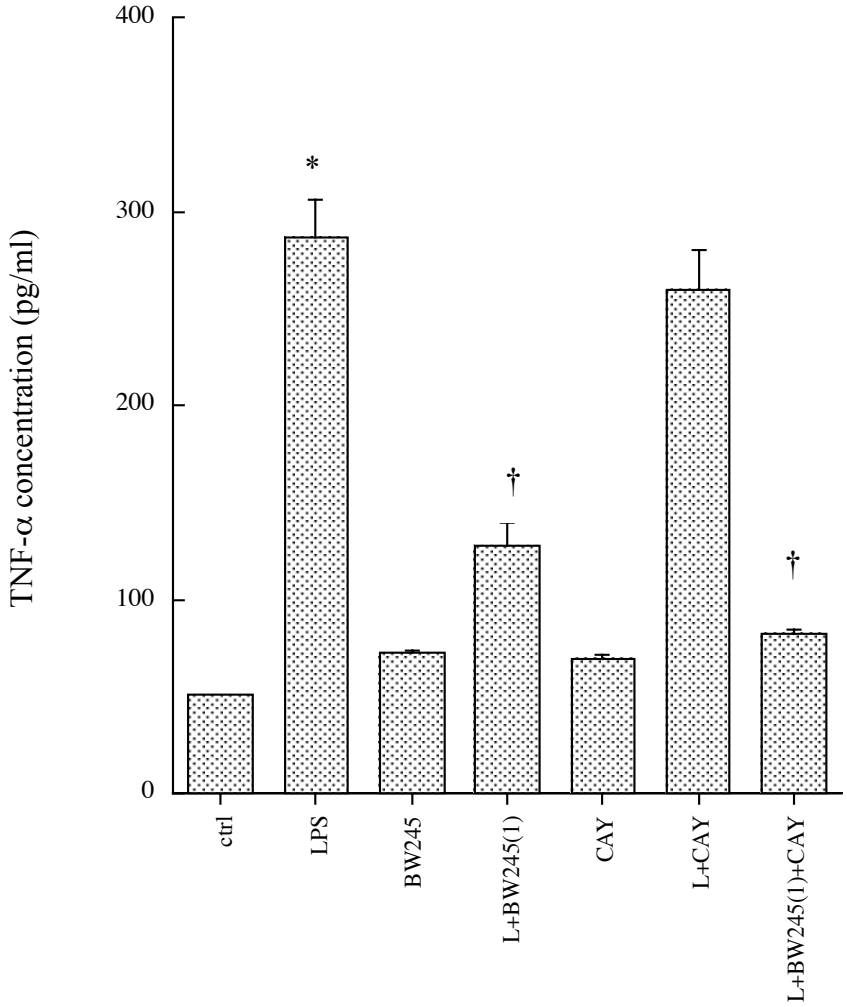
In previous experiments, where BW245C demonstrated a concentration-dependent suppression of LPS-induced TNF-α production in blood, the DP_1 antagonist BWA868C at a single concentration (0.05 μM) did not show any effect. Thus, it was decided to examine the effect of a higher range of BWA868C concentrations (0.1 – 10 μM) on the BW245C-induced suppression of TNF-α levels. The LPS-stimulated elevation in TNF-α levels was suppressed by BW245C (1 μM) as shown previously, however, this inhibition was unaltered by any of the BWA868C concentrations between 0.1 and 10 μM (Fig. 3.20).

Figure 3.19a **The effect of BW245C (DP_1 receptor agonist) on LPS-stimulated TNF-α production in the presence of BWA868C (DP_1 antagonist) in human monocytes**



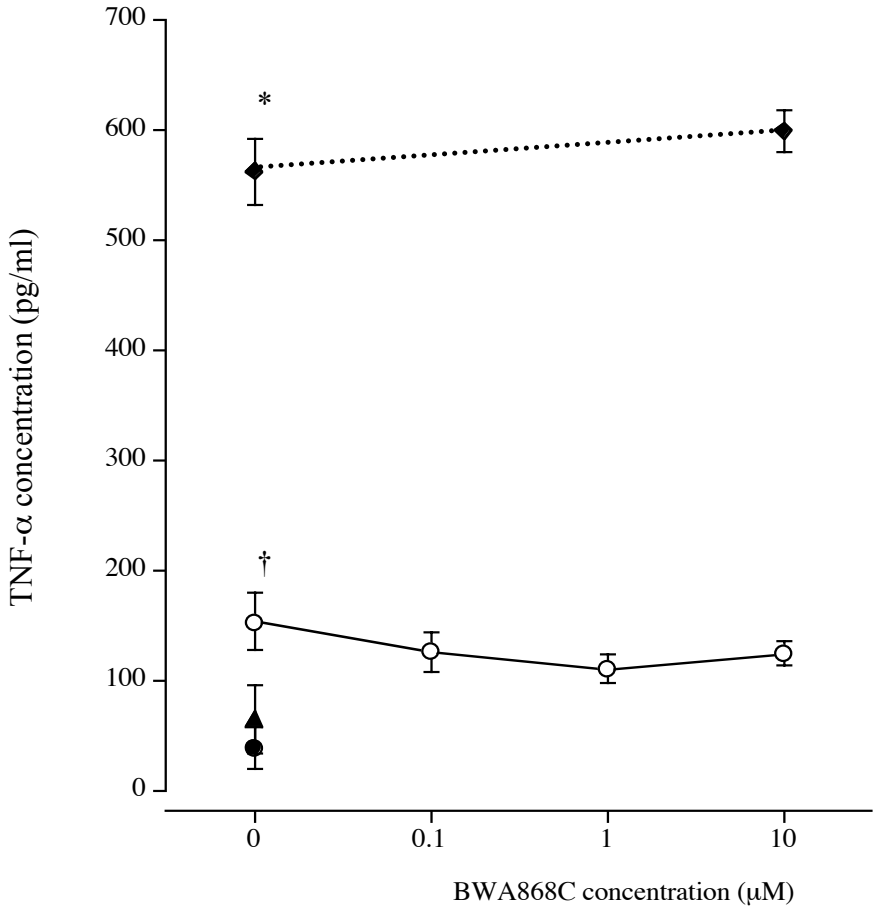
Monocytes ($1x10^6$) isolated from Human peripheral blood were incubated for 20 hours either with culture media alone (ctrl) or with LPS (10μg/ml) in the absence or presence of BW245C (1μM), BWA868C (BW86 - 0.05μM), or a combination of both BW245C, BWA868C (L+BW245 (1) + BW86) and TNF-α levels in the supernatant were measured by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS alone.

Figure: 3.19b The effect of BW245C (DP_1 agonist) on LPS-stimulated TNF-α production in the presence of CAY10471 (DP_2 antagonist) in human monocytes



Monocytes ($1x10^6$) isolated from Human peripheral blood 20 hours either with culture media alone (ctrl) or with LPS (10μg/ml) in the absence or presence of BW245C (BW245-1μM), CAY10471 (CAY-0.05μM), or a combination of BW245C, and CAY10471 (L+BW245 (1) +CAY) and TNF-α levels in the supernatant was determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS alone.

Figure 3.20 Effect of varying concentrations of BWA868C (DP_1 antagonist) on the BW245C (DP_1 agonist)-induced suppression of LPS-stimulated production of TNF-α on induced in human blood.



Blood was incubated for 20 hours either with either culture media alone (●), BW245C alone (▲) or a combination of LPS (10μM) and BWA868C (◆) or LPS + BW245C (1μM) + increasing concentrations of BW868C (○). Incubations were then centrifuged and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS alone.

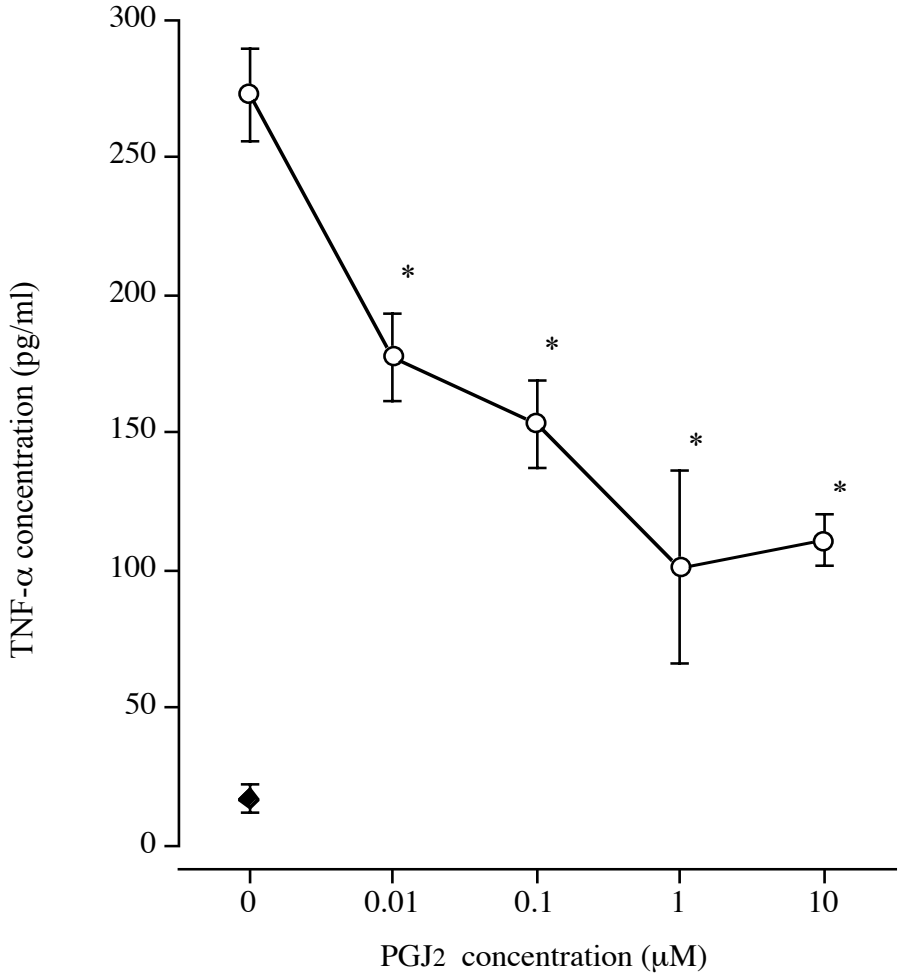
3. 21 Effect of 15d-PGJ_2 on LPS-stimulated TNF-α production in Human blood

The effect of the PGD_2 metabolite, 15d-PGJ_2, was studied in order to ascertain its effects on basal and LPS-stimulated TNF-α production in blood. As shown in Fig 3.21 LPS produced a 15-fold increase in TNF-α production in comparison to control incubations. In the presence of 15d-PGJ_2, TNF-α production decreased in a dose-dependent manner (Fig. 3.21). Maximum suppression of TNF-α was observed between 1 and 10μM 15d-PGJ_2. The threshold concentration for TNF-α suppression was 0.01 μM. The data showed that the IC_{50} value for 15d-PGJ_2 inhibition of TNF-α production was 0.01 μM (10 nM).

3.22 Comparison of the effects of PGD_2 and 15d-PGJ_2 in the presence of BWA868C (DP_1 receptor antagonist) on LPS-stimulated TNF-α production in Human blood

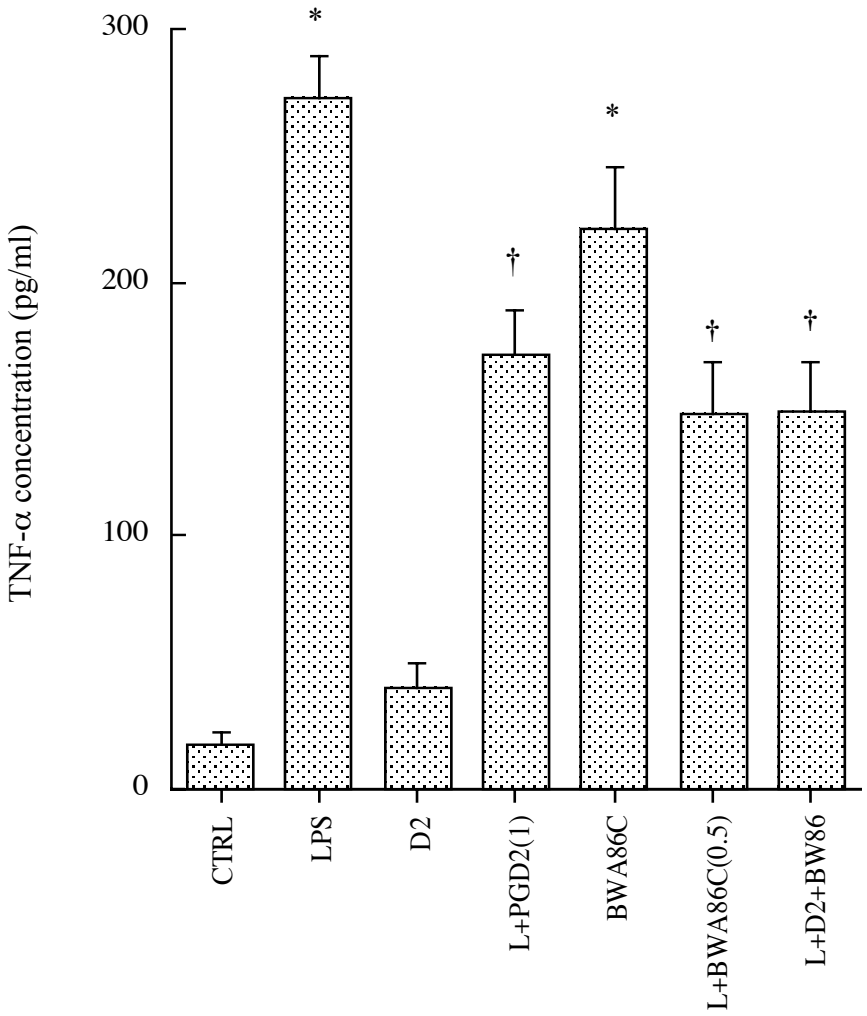
PGD_2 was shown to inhibit TNF-α production which was not affected by the DP_1 receptor antagonist BWA868C but did appear to be reversed by the DP_2 receptor antagonist CAY10471. However, it was not certain if the suppressive actions of PGD_2 occurred directly or were mediated via its conversion to the spontaneous metabolite 15d-PGJ_2. Figs. 3.22a/b shows a direct comparison of the effect of PGD_2 and 15d-PGJ_2 respectively, on TNF-α production in the presence of the DP_1 receptor antagonist BWA868C. Both PGD_2 and 15d-PGJ_2 decreased the elevated levels of TNF-α following exposure to LPS (Figs. 3.22a/b) and these decreased-levels were unaffected by BWA868C (0.5 μM). The low basal level of TNF-α was slightly increased by 15d-PGJ_2 (Fig. 3.22b). In addition, the higher level of BWA868C (0.5 μM) used in this experiment was shown to slightly reduce the LPS-stimulated level of TNF-α when incubated in the absence of either PGD_2 or 15d-PGJ_2 (Figs. 3.22 a/b).

Figure 3.21 Effect of 15d-PGJ_2 on LPS -stimulated TNF-α production in Human blood



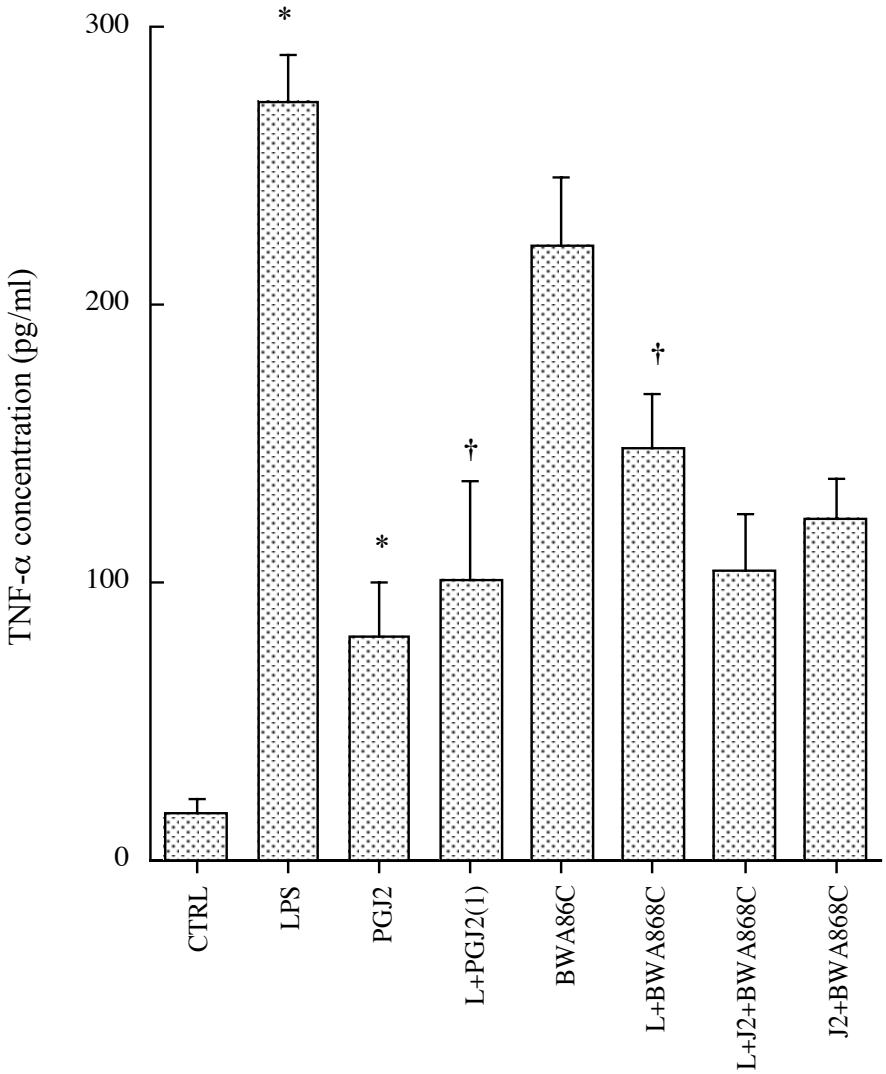
Blood samples were incubated either with culture medium alone (◆) or with increasing concentrations of 15d-PGJ_2 in the presence of LPS (10μg/ml- ○) for 20 hours. Samples were then centrifuged and TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ±s.d. *$P < 0.05$ when compared to controls.

Figure 3.22a The effects of BWA868C (DP_1 antagonist) on PGD_2 induced suppression of LPS-stimulated TNF-α production in Human blood.



Blood was incubated for 20 hours either with media alone (ctrl) or with LPS (10μg/ml) in the absence or presence of PGD_2 (D2 -1μM), and BWA868C (BWA86- 0.5 μM) or a combination of PGD_2 and BWA868C (L+ D2+BW86) and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS alone.

Figure 3.22b The effects of BWA868C (DP_1 antagonist) on 15d-PGJ_2 –induced suppression of LPS-stimulated TNF-α production in Human blood.



Blood was incubated for 20 hours either with media alone (ctrl) or with LPS (10μg/ml) in the absence or presence of 15d-PGJ_2, (PGJ2- 1μM), and BWA868C (BWA86C-0.5 μM) or a combination of 15d-PGJ_2 and BWA868C (L+ J2+BWA86C). TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared LPS alone.

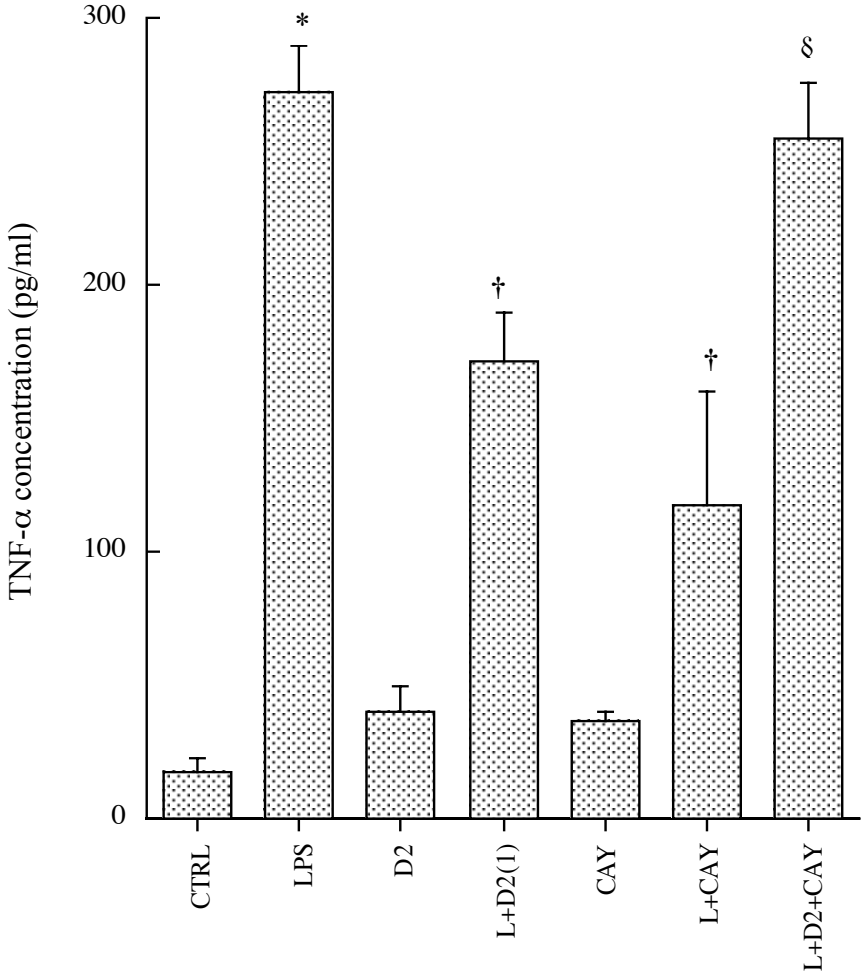
3.23 Comparison of the effects of PGD_2 and 15d-PGJ_2 in the presence of CAY10471 (DP_2/$CRTH_2$ antagonist) on LPS-stimulated TNF-α production in Human blood

As shown in previous experiments the PGD_2 and 15d-PGJ_2–induced inhibition of LPS-stimulated TNF-α production was unaffected by the DP_1 antagonist BWA868C. It was therefore decided to examine the effects of the DP_2 antagonist CAY10471 on the PGD_2 and 15d-PGJ_2–induced suppression. Figs. 3.23a and 3.23b show a direct comparison of the effect of CAY10471 on TNF-α production in the presence of PGD_2 and 15d-PGJ_2. As shown previously, incubation with LPS (10 μg/ ml) produced a large increase in TNF-α levels which were suppressed by both PGD_2 and 15d-PGJ_2 (Figs. 3.23 a/b). The suppressive action of PGD_2 (Fig. 3.23a) was significantly reversed by CAY10471 (0.05 μM). However, this concentration of CAY10471 did not significantly alter the suppressive effect of 1 μM 15d-PGJ_2 (Figure 3.23b).

3.24 Direct comparison of the effect of BWA868C (DP_1 antagonist) on PGD_2 and 15d-PGD_2-induced suppression of LPS-stimulated TNF-α production from Human monocytes.

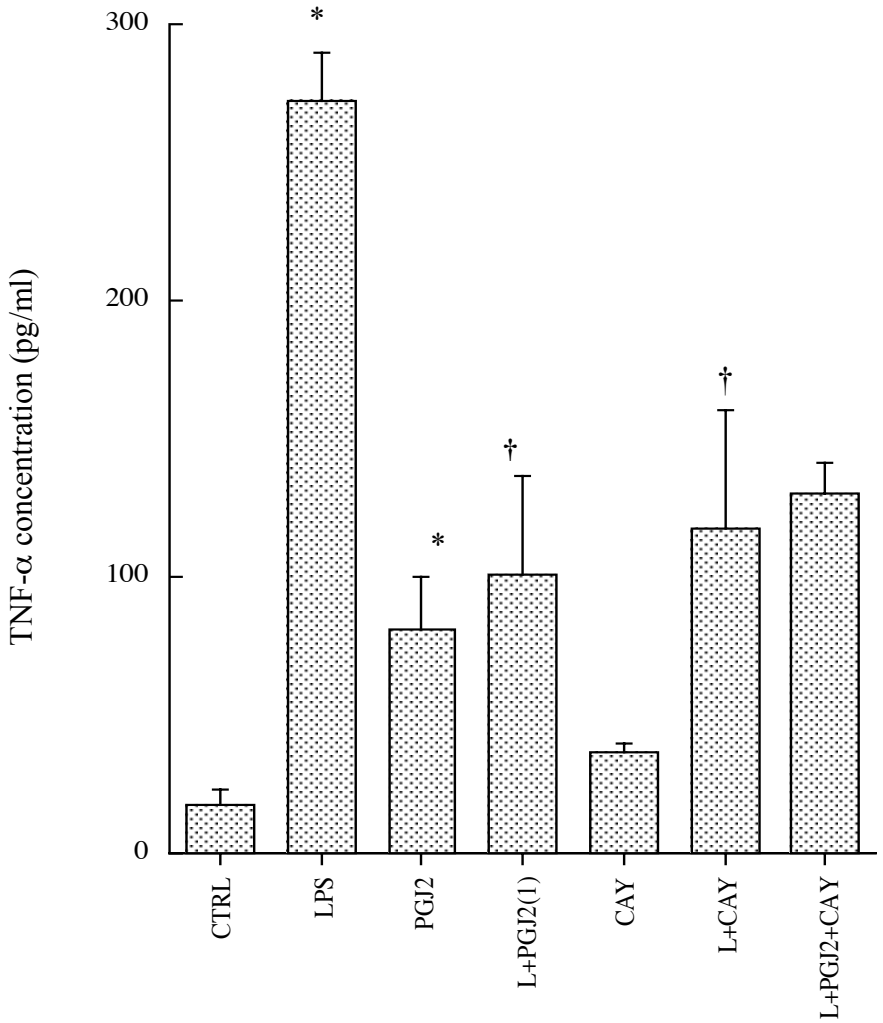
The suppressive effects of PGD_2 and 15d-PGJ_2 on LPS-stimulated TNF-α production was shown to be unaltered by BWA868C in blood. Thus, a similar series of experiments was carried out using monocytes to ascertain the actions of BWA868C on the inhibitory actions of PGD_2 and 15d-PGJ_2 (Figs. 3.24 a/b). In general, similar observations were made using monocytes compared to blood i.e. that the higher concentration of BWA868C (0.5 μM) did not alter either the PGD_2 or 15d-PGJ_2-induced suppression of LPS-stimulated TNF-α production (Figs. 3.24 a/b).

Figure 3.23a The effects of CAY10471 (DP_2 antagonist) on PGD_2- induced suppression of LPS-stimulated TNF-α production in Human blood



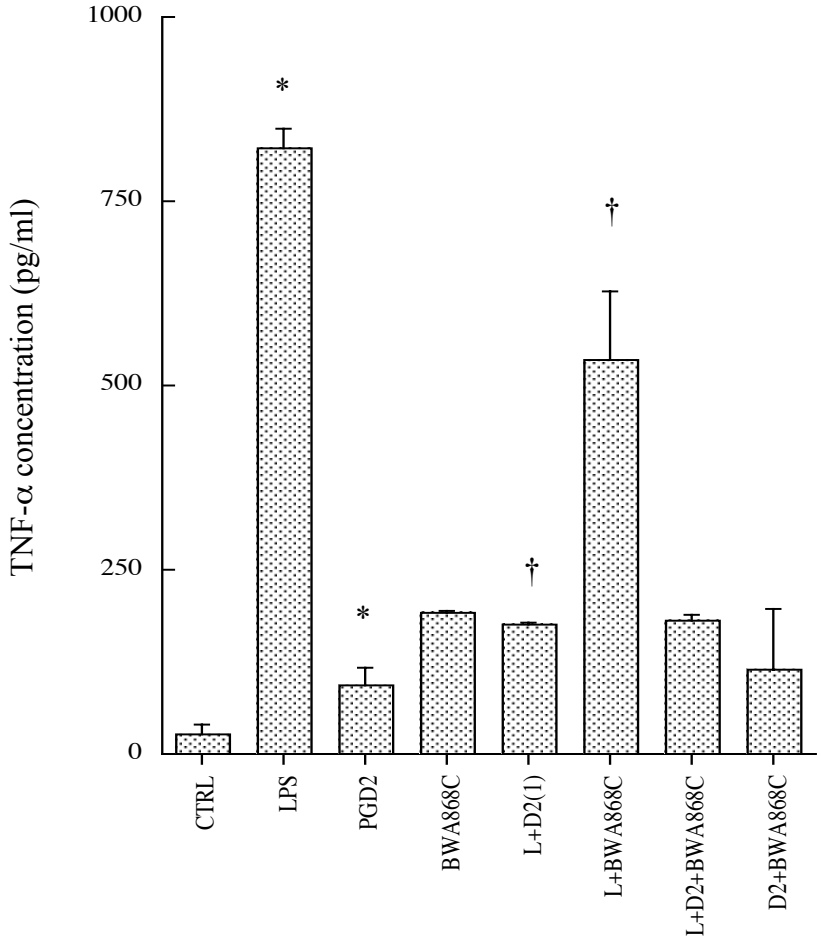
Blood was incubated for 20 hours either with media alone(ctrl) or with LPS (10μg/ml) in the absence or presence of PGD_2 (D2 -1μM), and CAY10471 (CAY -0.05μM) or a combination of both PGD_2 and CAY10471 (L+ D2+CAY). TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls**.** † Denotes $P < 0.05$ when compared to LPS alone. § Denotes $P < 0.05$ when compared to PGD_2 induced suppression of LPS stimulated TNF-α production.

Figure 3.23b The effects of CAY10471 (DP_2 antagonist) on 15d-PGJ_2-induced suppression of LPS-stimulated TNF-α production in Human blood.



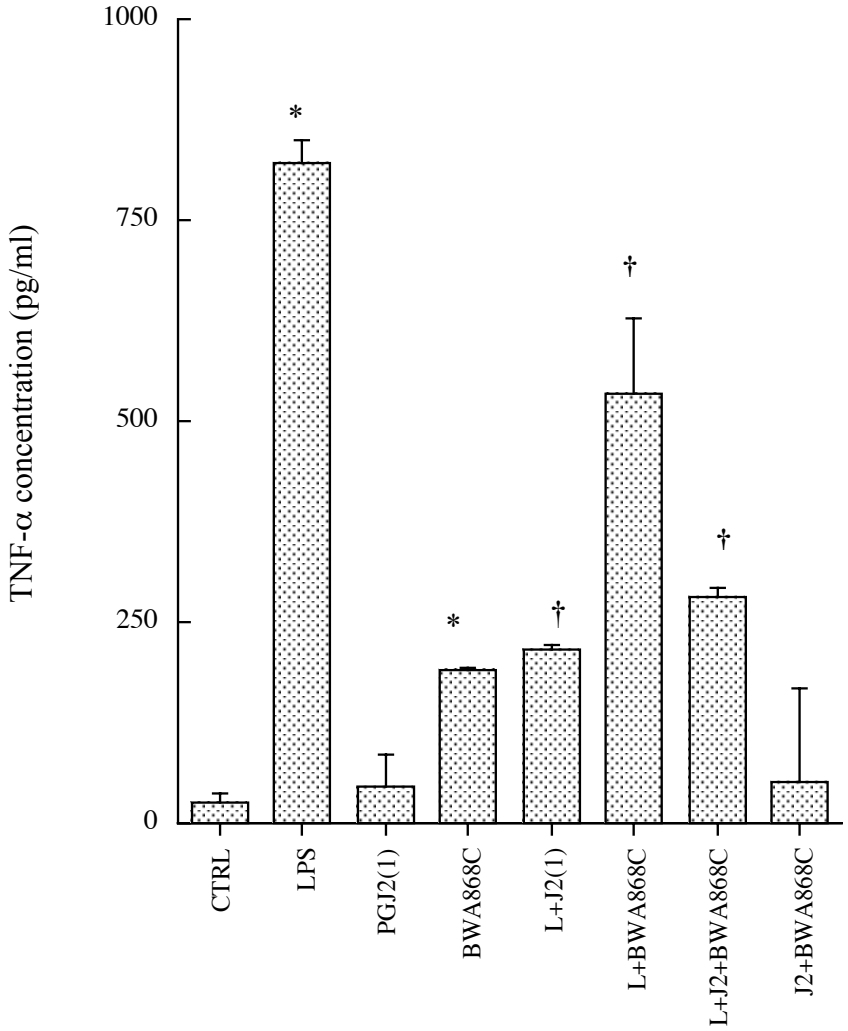
Blood was incubated for 20 hours either with media alone(ctrl) or with LPS (10μg/ml) in the absence or presence of 15d-PGJ_2, (J2- 1μM), and CAY10471 (CAY- 0.5μM) or a combination of both 15d-PGJ_2 and CAY10471 (L+ PGJ2+CAY). TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS alone.

Figure 3.24a **Effect of BWA868C (DP_1 antagonist) on PGD_2 –induced suppression of LPS-stimulated TNF-α production from Human monocytes.**



Monocytes ($1x10^6$) isolated from Human peripheral blood were incubated for 20 hours either with media alone (ctrl) or with LPS (10μg/ml) in the absence or presence of PGD_2 (1μM) and BWA868C (0.5 μM) or a combination of PGD_2 and BWA868C (L+ D2+ BWA868C) and the TNF-α levels in the supernatants were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS stimulated TNF-α production.

Figure 3.24b Effect of BWA868C (DP_1 antagonist) on 15d-PGJ_2-induced suppression of LPS-stimulated TNF-α from Human monocytes



Monocytes ($1x10^6$) isolated from Human peripheral blood were incubated for 20 hours either with media alone (ctrl) or with LPS (10μg/ml) in the absence or presence of 15d-PGJ_2 (PGJ_2-1μM), BWA868C (0.5 μM) or a combination of 15d-PGJ_2 and BWA868C (L+ J2+ BWA868C) and the TNF-α levels in the supernatants were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS alone.

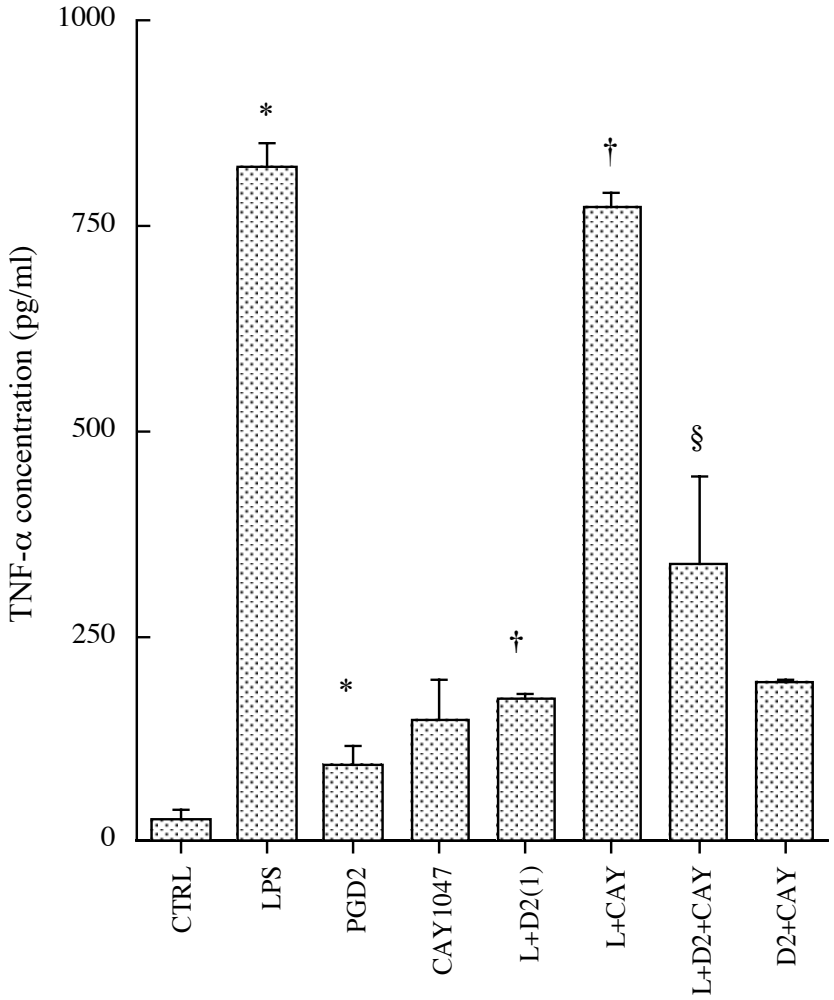
3.25 Effects of CAY10471 (DP_2 antagonist) on the PGD_2-induced suppression of LPS-stimulated TNF-α production from human monocytes.

It was previously shown in blood, that the suppressive effect of PGD_2 on TNF-α production was reversed in the presence of CAY10471. Similar experiments were carried out using monocytes. Fig. 3.25 shows the effect of PGD_2 on TNF-α production in the presence of CAY10471. As shown previously, TNF-α production was greatly increased by LPS (10 μg/ml) and this elevation was dramatically lowered when PGD_2 (1 μM) was included (Fig. 3.25). In the presence of CAY10471 (0.05 μM) the PGD_2-induced inhibition was reversed. A slightly lower level of TNF-α was observed in LPS incubations with CAY10471 compared to those with LPS alone (Fig. 3.25).

3.26 Effect of CAY10471 (DP_2 antagonist) on 15d-PGJ_2-induced suppression of LPS-stimulated TNF-α production from Human monocytes.

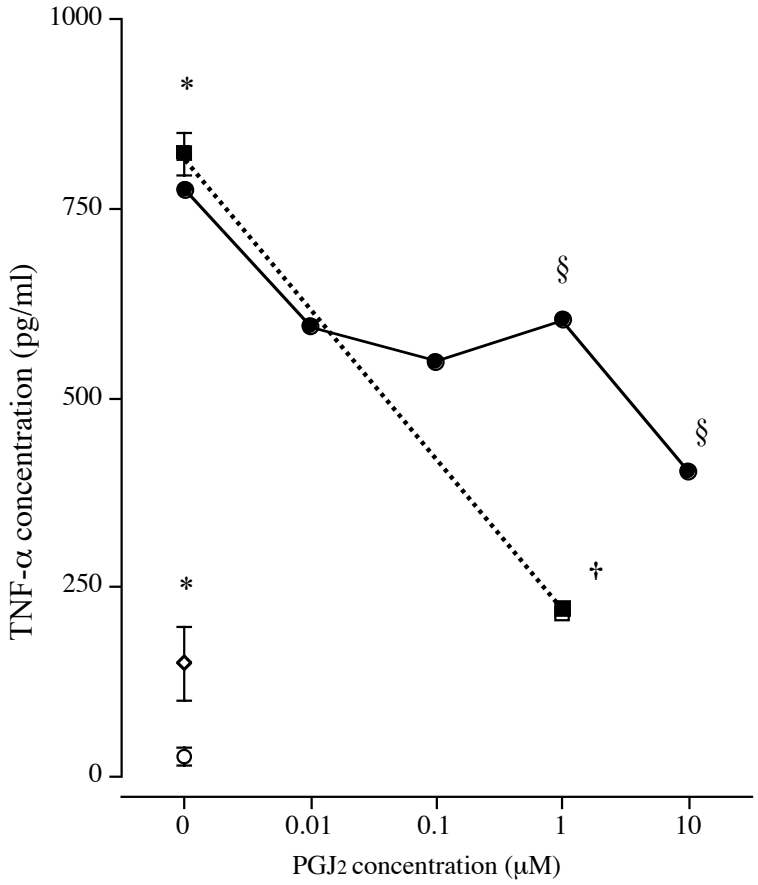
As the PGD_2-induced inhibition of TNF-α production from monocytes was reversed by CAY10471, the effects of CAY10471 were also evaluated on the 15d-PGJ_2-induced inhibition of TNF-α production from monocytes. Human monocytes were incubated with LPS, in the presence of increasing concentrations of 15d-PGJ_2 along with a fixed concentration of CAY10471 (0.05 μM). A concentration-dependent decrease in LPS-stimulated TNF-α levels was observed in response to 15d-PGJ_2 (Fig. 3.26). CAY10471 on its own produced a small increase in TNF-α production but had no significant effect on TNF-α levels stimulated by LPS. The inhibitory effect of 15d-PGJ_2 was reversed in the presence of CAY10471 (Figure 3.26). The IC_{50} value for 15d-PGJ_2 in presence of CAY10471 was calculated to be 0.08 μM (80 nM).

Figure 3.25 **Effect of CAY10471 (DP_2 antagonist) on PGD_2-induced suppression of LPS-stimulated TNF-α production from Human monocytes.**



Monocytes ($1x10^6$) isolated from Human peripheral blood were incubated for 20 hours either with media alone (ctrl) or with LPS (10μg/ml) in the absence or presence of PGD_2 (D_2- 1μM), CAY10471 (CAY- 0.05 μM) or a combination of PGD_2 and CAY10471 (L+ D2+ CAY) and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the means of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS alone. § Denotes $P<0.05$ when compared to PGD_2 induced suppression of LPS stimulated TNF-α production.

Figure 3.26 Effect of CAY10471 (DP_2 antagonist) on the suppression of LPS-stimulated TNF-α production from Human monocytes by varying concentrations of 15d-PGJ_2.



Monocytes ($1x10^6$) isolated from Human peripheral blood were incubated for 20 hours either with media alone (○), CAY10471 alone (0.05 μM) (◊), LPS + 15d-PGJ_2 (■) or a combination of LPS + 15d-PGJ_2 + CAY10471 (●). Samples were then centrifuged and the TNF-α levels in the plasma were determined by ELISA. Results are from a single blood donor and are representative of at least three independent experiments. Values are expressed as the mean of n = 3 ± s.d. * Denotes $P < 0.05$ when compared to controls. † Denotes $P < 0.05$ when compared to LPS alone. § Denotes $P < 0.05$ when compared to 15d-PGJ_2 induced suppression of TNF-α production.

3.27 Summary of the Inhibitory Actions of PGE_2, $PGF_{2\alpha}$, PGD_2, 15d-PGJ_2, BW245C and BWA868C upon LPS-stimulated TNF-α production in blood and from monocytes.

The data presented in previous figures were subjected to statistical analysis via 3rd order polynomial regression analysis to produce IC_{50} values for the various agents. These values indicated that the most potent inhibitor of LPS-stimulated TNF-α production in blood was 15d-PGJ_2, which was at least 2-fold more potent than PGD_2 and 4-5- fold more potent than either PGE_2 or $PGF_{2\alpha}$ in blood.

Table 3 **Direct comparison of IC_{50} values for different modulators/ suppressors on LPS-stimulated TNF-α production from human blood and monocytes.**

Cell system	TNF-α modulators	IC_{50}
human blood	15d-PGJ_2	0.01 μM
	PGD_2	0.02 μM
	PGE_2	0.04 μM
	$PGF_{2\alpha}$	0.05 μM
	BW245C + CAY10471	0.07 μM
	BW245C	0.12 μM
	BW245C + BWA868C	0.45 μM
human monocytes	PGD_2	0.03 μM
	15d-PGJ_2 + CAY10471	0.08 μM
	BW245C	0.09 μM
	15d-PGJ_2	0.12 μM

The IC_{50} values for the different modulators of LPS-stimulated TNF production from human blood or monocytes were derived using Statistical software using 3rd order polynomial regression analysis to calculate the concentration of each agent which produced a 50 % maximal response and are listed in order of decreasing potency for each cell system.

4. Discussion

4. Discussion

Interleukin-1 and Tumour necrosis factor α (TNF-α) are the major pro-inflammatory cytokines released in response to the stimulation of monocytes by variety of pathogenic organisms (Defranco *et al.,* 2007). This release of TNF-α from human blood or monocytes is altered by variety of lipids including arachidonic acid and prostaglandins. The present study characterised the effects of several prostaglandins such as PGE_2, $PGF_{2\alpha}$ and especially PGD_2 along with its metabolite 15d-PGJ_2 on TNF-α production with a focus on the 2 main PGD_2 receptor pathways, DP_1 and DP_2.

4.1 Bacterial endotoxin-induced TNF-α production

LPS (endotoxin) is a component of the outer membrane of Gram-negative bacteria. Many studies have shown that LPS is one of the most potent stimuli for the production of pro-inflammatory mediators especially TNF-α and IL-1 from monocytes (Rietschel *et al*., 1994). In the study of Kreutz *et al.* (1997) the effects of bacterial LPS, a synthetic lipoprotein analogue lipopeptide (Pam 3-Cys-Ala-Gly) and heat killed *Staphylococcus aureus* were compared with respect to TNF-α production in human monocytes. The data showed that LPS stimulated the production of TNF-α with higher potency compared to the other stimuli. LPS was always greatly more potent in the lower dose range compared to Pam3-Cys-Ala-Gly, or *S. aureus*. LPS at concentrations between 5- 50 μg/ ml produced a maximal production of TNF-α (Kreutz *et al.,* 1997). This is similar to the observations in the current study where the same LPS was used i.e. derived from *Salmonella Abortus equi*. In other studies (using L929 TNF-α cell-toxicity bioassay) it was shown that incubation of human blood with LPS (1-100 μg/ ml) also caused a concentration-dependent increase in TNF-α release with a maximum between 10 μg/ ml- 100 μg/ml (Foster *et al.,* 1993). This strongly correlates with the present study in that a maximal production of TNF-α occurred with concentrations between 10μg/ ml-100 μg/ml (Figure 3.1). TNF-α production was measured by ELISA assay in this study. The similarity to the data obtained by Foster *et al.* (1993) that used bioassay indicated

that the ELISA assay used in this study was able to reflect changing levels of bioactive TNF-α.

4.2 Control of TNF-α production by prostaglandins

Prostaglandins can inhibit LPS-induced TNF-α production in blood or monocytes by acting via their respective receptor pathways. Rotondo *et al.* (1993) showed that TNF-α / IL-1 are the major pro-inflammatory cytokines released from monocytes in response to LPS. Rotondo *et al.* (1988) showed that there is a dramatic increase (> 10-fold) in circulating PGE_2 levels following the administration of LPS in rabbits, indicating that the increase in PGE_2 levels which occur *in vivo* is an important component of the response to LPS stimulation. The released PGE_2 results in the systemic inflammatory response primarily manifest as fever. However, it may also provide a negative feedback mechanism suppressing the further release of cytokines (Davidson *et al.,* 1998). It had previously been shown that exogenously added PGE_2 suppressed TNF-α production from monocytes and that cyclooxygenase inhibitors enhanced production, indicating a role for endogenous prostaglandins in limiting TNF-α production (Kunkel *et al.*, 1986; Spengler *et al.*, 1989). Many other studies have also demonstrated the suppressive effects of PGE_2 (Davidson *et al.*, 1998) and $PGF_{2\alpha}$ (Kiyoshi *et al.,* 2000). However, the role of other prostaglandins has not been well characterised. In the current study, PGE_2, $PGF_{2\alpha}$ and PGD_2 were all able to decrease TNF-α production (Figs. 3.2, 3.3 and 3.5).

Although many studies have shown that there are other pathways which can suppress TNF-α production, such as Transforming Growth Factor β_2 (TGF-β_2) and that this occurs independently of PGE_2 (Dunham *et al.*, 1990), it would appear that the "master" regulators are prostaglandins (Habeeb *et al.*, 2002). It is clear that endogenous prostaglandins are involved in the suppression of inflammatory cytokine production as COX inhibitors enhance their release (Habeeb *et al.*, 2002). However, the inhibition of COX, which would result in the global inhibition of prostaglandin synthesis, does not give any indication as to which specific prostaglandins are involved. In the current

study, the suppressive actions of PGE_2 were confirmed and compared alongside the actions of the PGD_2 whose role is less well studied. Initial experiments demonstrated that PGD_2 suppressed TNF-α production in both blood and monocytes (Figs. 3.3 and 3.4). Thus, the PGD_2 receptor pathway were further studied using different agonists and antagonists that interfere with the main receptor systems for PGD_2.

4.3 The role of PGD_2 receptor pathways in the suppression of TNF-α production

PGD_2 can bind to two different receptors, the DP_1 receptor and the chemo-attractant receptor homologous molecule expressed on Th_2 cells ($CRTH_2$), now termed the DP_2 receptor. These 2 receptor pathways are discussed below.

4.3.1 The role of DP_1 receptors in the suppression of TNF-α production

DP_1 is positively linked to adenylyl cyclase via the G-protein subunit Gαs causing an increase in cyclic AMP levels (Hirata *et al.,* 1994, Boie *et al.,* 1995). Hirata *et al.* (1994) have identified a gene and cloned DNA for the mouse PGD_2 receptor. They also demonstrated that the DP_1 receptor activates adenylate cyclase. Similarly, Boie *et al.* (1995) showed that a cloned DP_1 receptor (expressed in CHO cells) resulted in the elevation of cyclic AMP, but did not lead to the generation of inositol 1,4,5-triphosphate. Both PGD_2 and BW245C (a DP_1 receptor agonist) activated adenylate cyclase with a resulting elevation in cyclic AMP levels (Boie *et al*., 1995) clearly indicating that the DP_1 receptor signals through the cyclic AMP pathway.

BWA868C binds to DP_1 receptors with high affinity and has been shown to inhibit agonist-induced cyclic AMP production in response to either PGD_2 or BW245C with a Kd value in the order of 10 nM (Sharif *et al*., 2000). In the present study BWA868C was used to evaluate the effect of PGD_2 on TNF-α production. In both blood and monocytes

PGD_2-downregulated production of TNF-α was not altered by BWA868C indicating the possibility that TNF-α levels are not regulated by DP_1 receptor pathways.

BW245C is a potent DP_1 receptor agonist and like BWA868C it binds to PGD_2 receptors with high affinity. It has been shown to inhibit human platelet aggregation with a K_i of 24- 26 nM and, compared to the potency of PGD_2 with a K_i of 80 nM this indicates that BW245C is in the order of 3-fold more potent than PGD_2 (Sharif *et al*., 2000). In the present study BW245C was used as a DP_1 agonist in order to confirm the absence of a DP_1 receptor pathway involvement in the suppression of TNF-α production. BW245C inhibited LPS-stimulated TNF-α production from both blood and monocytes (Fig. 3.12 and 3.13). However, this action of BW245C was not affected by BWA868C. These observations were interesting and contradictory, this may be possibly due to the involvement of other receptors i.e. the suppressive action of BW245C is potentially acting through receptors other than DP_1 as it has been shown that in other cells the actions of BW245C are reversed by BWA868C (Sharif *et al*., 2000). BW245C also has affinity toward EP receptors which can also downregulate TNF-α production. The actions of BW245C in other tissues has clearly shown that it can have divergent effects to PGD_2. Rangachari *et al.* (1995) showed that BW245C did not mimic the inhibitory effects of PGD_2, but produced a stimulant effect on canine colonic epithelium. BWA868C even at the highest concentration (30 μM) failed to produce any significant inhibition of BW245C-induced stimulation indicating that its activity appears to involve receptors other than the classical DP_1 receptor. In their study Rangachari *et al.* (1995) showed that BW245C produced its stimulatory effect via EP receptors as only PGE_2 was able to demonstrate cross desensitisation of the BW254C effects. This was similar to that observed in the present study where the down-regulating effect of BW245C on TNF-α production was not altered even in the presence of high concentrations of BWA868C (10 μM) (Figure 3.20). The BW245C suppression could be possible via EP receptors as BWA868C has been shown to not affect IP, EP_1, EP_2, FP and TP receptors (Giles *et al.,* 1989). Stimulation of EP receptors, in the present study, was shown to suppress TNF-α production (Fig. 3.2). This PGE_2-induced inhibition of TNF-α production was not altered by BWA868C (Figs. 3.8 and 3.9).

Clearly, human monocytes also have EP receptors which downregulate TNF-α production, but it in the present study, the nature of the EP receptors was not studied further. Synthetic PGE_2 analogues such as butaprost (EP_2/EP_4 receptor agonist) suppress pro-inflammatory cytokine synthesis. However, sulprostone, an EP_3 receptor agonist, has no effect on cytokine production. Rotondo *et al.* (2004) showed that PGE_2 strongly suppresses IL-12-induced synthesis of IFN-γ by NK cells. Butaprost suppressed IFN-γ synthesis and competes exclusively with PGE_2 for receptor binding on NK cells. Sulprostone, (EP_1/EP_3 agonist) was devoid of any effect indicating that cytokine production is regulated by EP_2 or EP_4 receptors. In the current series of experiments PGE_2 strongly suppressed TNF-α production both in blood and monocytes. BWA868C had no effect on the suppressive actions of PGE_2 (Figs. 3.8 and 3.9). Indicating that PGE_2 acts in a manner distinct from the DP_1 receptor pathway.

$PGF_{2\alpha}$ also lowered TNF-α production which was also unaffected by BWA868C (Figs. 3.10 and 3.11) confirming the view that PGE_2 & $PGF_{2\alpha}$ produce their actions without the involvement of the DP_1 receptor. It was important to evaluate the effects of $PGF_{2\alpha}$ as it can be synthesised from PGD_2. Watanabe *et al.* (2002) showed that $PGF_{2\alpha}$ is synthesized via three pathways from PGE_2, PGD_2, or PGH_2 by PGE_2 9-ketoreductase, PGD 11-ketoreductase, or PGH 9-, 11-endoperoxide reductase enzymes, respectively. The enzymatic action could be responsible for the inter-conversion of various prostaglandins (Rotondo *et al.,* 2004). PGE_2 or $PGF_{2\alpha}$ are unlikely to be converted to PGD_2, thus, suppressive effect on TNF-α production would not occur via DP receptors (Rotondo, *et al.,* 2004).

4.3.2 The role of DP_2 receptors on the suppression of TNF-α production.

The $CRTH_2$ receptor, now identified as a DP_2 receptor, has been shown to be a major PGD_2-related receptor, which may have pivotal roles in controlling the production of pro-inflammatory cytokines such as TNF-α. Unlike DP_1 receptors, Hirai *et al.* (2001) showed that DP_2 receptors inhibit adenylyl cyclase through Gαi proteins, leading to the inhibition of cyclic AMP production and an increase in intracellular calcium. Hirai *et al.* (2001) also indicated that DP_2 but not DP_1, mediates PGD_2 dependent cell migration of eosinophils and basophils. This was confirmed using DP_2 receptor knockout mice where PGD_2 had no effect on cell migration. The DP_2 receptor may also bind the PGD_2 metabolite, 15d-PGJ_2, with equal affinity to that of PGD_2 (Sawyer *et al.,* 2002). Equilibrium competition binding assays revealed that PGD_2 and its metabolite 15d-PGJ_2 bound with the high affinity to the DP_2. This raises the possibility that PGD_2 metabolites may differentially exert effects through the DP_2 receptor.

In the present study, the effect of BW245C and PGD_2 (DP_1 receptor agonists) were investigated in the presence of CAY10471 (DP_2 antagonist). CAY10471 is an analogue of BAY-μ3405 (ramatroban). BAY-μ3405 itself is a potent antagonist of DP_2 receptors (Sugimoto *et al.,* 2003; Ulven *et al.,* 2005) and minor structural changes in ramatroban results in a compound (CAY10471) with highly selective, potent and essentially non-reversible DP_2 antagonist activity. In the current study, the suppressive effect of PGD_2 on TNF-α production was reversed in the presence of CAY10471 (Fig. 3.17). Indicating that PGD_2 acts via DP_2 receptors in suppressing TNF-α production in blood and monocytes. A similar action has been reported for lymphocytes. Xue *et al.* (2005) showed that PGD_2 stimulates Th2 cytokine production from CD23/CD28 cells solely via a DP_2-dependent mechanism. The effect of PGD_2 is mimicked by the selective DP_2 agonist 13, 14-dihydro-15-keto-PGD_2 (DK-PGD_2) but the selective DP_1 agonist BW245C was without any effect, confirming that stimulation was mediated by DP_2 and not DP_1 receptors.

In the current series of experiments CAY10471, rather than reversing the action of BW245C, reduces TNF-α production further in comparison to BW245C alone (Fig. 3.15). This raises the possibility that the receptors which BW245C acts through (possibly EP_2 or EP_4 receptors as discussed previously) may be actively antagonised by DP_2 receptor pathways.

4.4 Comparison of the effects of PGD_2 and its metabolite 15d-PGJ_2 on TNF-α production

PGD_2 can be converted to 15d-PGJ_2, thus the actions of PGD_2 may not occur directly but occur via conversion to 15d-PGJ_2 (Takahiro *et al.,* 2002). It appears that the natural precursor for 15d-PGJ_2 is PGD_2. Administration of PGD_2 *in vivo* leads to a large increase in 15d-PGJ_2 (Takahiro *et al.,* 2002). In the present study exogenously added 15d-PGJ_2 suppressed LPS-stimulated TNF-α production in blood and monocytes (Figs. 3.21 and 3.26). Experiments were carried out to compare the effects of PGD_2 & 15d-PGJ_2 on TNF-α production in the presence of BWA868C (DP_1 receptor antagonist) and CAY10471 (DP_2 antagonist). In both blood and monocytes BWA868C had no effect on the ability of PGD_2 or 15d-PGJ_2 to suppress TNF-α production (Figs. 3.22a-3.22b), whereas, CAY10471 reversed the suppressive action of both PGD_2 and 15d-PGJ_2 (Figs. 3.23a-3.23b) indicating the potential involvement of DP_2 receptor pathways in the suppressive response of both prostanoids.

PGD_2 like PGE_2 has been regarded as a pro-inflammatory prostanoid as it has the ability to produce the end-functions involved in the symptoms of inflammation such as erythema etc. When it is administered *in vivo* it produces eosinophilia in the lungs of several experimental animals such as rats and dogs (Sandig *et al.,* 2007). However, PGD_2 has also been shown to have anti-inflammatory actions. An example is carageenan-induced pleurisy which is exacerbated in the presence of COX inhibitors but resolves following administration of either PGD_2 or 15d-PGJ_2 (Sandig *et al.,* 2007). It has been suggested that the opposing effects may be due to either conversion of PGD_2 to 15d-PGJ_2 or actions via either of the DP receptors. This would appear unlikely with

respect to the control of inflammatory cytokine production as DP_1 pathways appear to have no role and DP_2 pathways suppress TNF-α production in the present study. In addition, both PGD_2 and 15d-PGJ_2 are inhibitory. It would also not appear to be the case that DP_2 receptors are generally anti-inflammatory in nature. Almishri *et al*. (2004) showed that PGD_2 induced eosinophil infiltration *in vitro* through DP_2 receptors. A selective DP_2 receptor agonist, DK-PGD_2 induced similar responses. However, the current study clearly suggests the potential involvement of DP_2 receptor pathways in the suppressive control of TNF-α production in human cells.

4.5 SUMMARY AND CONCLUSIONS

- LPS stimulated TNF-α production in human blood and from human monocytes.
- PGE_2, $PGF_{2\alpha}$, PGD_2 and 15d-PGJ_2 induced a strong suppressive effect on LPS-stimulated TNF-α production in both blood and monocytes.
- The suppressive effect of either PGE_2, $PGF_{2\alpha}$, PGD_2 or 15d-PGJ_2 on LPS stimulated TNF-α production in whole blood and monocytes was not altered by BWA868C (DP_1 receptor antagonist) suggesting the absence of a DP_1 receptor pathway in this response.
- BW245C (DP_1 receptor agonist) also suppressed LPS -stimulated TNF-α production in blood and monocytes, This effect was not altered by CAY10471 (DP_2 antagonist) or BWA868C (DP_1 receptor antagonist) suggesting that BW245C may be acting via other non-PGD_2 receptors.
- The suppressive actions of PGD_2 and 15d-PGJ_2 on TNF-α production was reversed by CAY10471 suggesting the involvement of DP_2 receptor pathways.

The present study has made an attempt at elucidating the mechanism by which PGD_2 and 15d-PGJ_2 suppress TNF-α production. The most probable mechanism is via the DP_2 receptor without involvement of the DP_1 pathway. This study proposes that within blood and monocytes PGD_2 and 15d-PGJ_2 demonstrates suppression of TNF-α

production by utilization of DP_2 pathway. Further research is still required to establish the important role that PGD_2 and 15d-PGJ_2 may play in the control of inflammatory conditions. In the present study BW245C (DP_1 receptor agonist) is thought to mediate its suppressive effect via the involvement of other receptors possibly EP_2 and/ or EP_4 receptors. Further studies on the DP_2 receptor pathway are required to clarify the significance of the DP_2-mediated activities in the control of inflammatory responses.

5. References

5. References:

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