Characterization of human lung macrophage phenotype and function

Sharonjit Kaur Gill

Academic Unit of Respiratory Medicine

Department of Infection and Immunity

University of Sheffield

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Summary

Macrophages have important roles in the lung, in homeostasis and in the immune response. Inappropriate activation of macrophages can cause aberrant inflammation, although the mechanisms leading to this are not fully understood. Studying these cells could be useful for understanding their possible involvement in the pathogenesis of inflammatory lung diseases. Most studies of macrophages have involved using monocyte-derived macrophages, monocytic cell lines or murine models. In this study, macrophages were isolated from resected human lung tissue, as they may be of more pathophysiological relevance. The overarching aim of this study was to characterize the phenotype of lung macrophages, principally by investigating functional responses to disease-relevant stimuli.

Initial studies were performed to attempt to isolate macrophages from lung tissue to a high level of purity and viability, which was achieved successfully. Flow cytometry studies showed that these were mature macrophages, with expression of CD206 and low expression of CD14. The cells were found to secrete TNF-α, IL-6 and IL-8 but much lower levels of IL-10 in response to lipopolysaccharide (LPS) stimulation as measured by ELISA. Further studies using Proteome Profiler Array indicated the cells had a pro-inflammatory phenotype in response to LPS, poly(I:C) and gardiquimod. Preliminary qPCR studies assisted in suggesting that the cells were predominantly of an M1, pro-inflammatory phenotype. LPS stimulation also resulted in the release of prostaglandin E_2 (PGE_2). Endogenously-derived PGE_2 was found to act in a paracrine fashion to inhibit cytokine release, as a mechanism to limit macrophage activation. Further studies indicated that LPS-induced PGE_2 was driven by the inducible isoform of cyclooxygenase (COX), COX-2. Moreover, it was found that PGE_2 acts at the EP_4 receptor to stabilize macrophages. Agonists at the EP_4 receptor were found to be considerably more effective than both short-acting and long-acting β_2-agonists, at producing anti-inflammatory effects, suggesting that targeting the EP_4 receptor could be an effective approach to stabilize macrophages.

This study demonstrates that the isolated lung macrophage is a tractable model amenable to a range of experimental applications. Overall, the results indicate that lung macrophages express a predominantly pro-inflammatory M1 phenotype and this could be important in the context of respiratory diseases. Further work will be necessary to confirm this conclusion.

Acknowledgements

Declaration

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Abbreviations

AERD	Aspirin exacerbated respiratory disease
APS	Ammonium persulphate
ANOVA	Analysis of variance
APC	Allophycocyanin
BAL	Bronchoalveolar lavage
bp	Base pairs
cAMP	Cyclic-AMP
cDNA	Complementary DNA
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
Ct	Cycle threshold
dH_2O	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
DPX	Di-n-butyl phthalate in xylene
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FcR	Fc receptor
FCS	Foetal calf serum
FSC	Forward scatter
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
HEPES	N'-2-Hydroxyethylpiperazine-N'-2 ethanesulphonic acid
HLM	Human lung macrophage
HRP	Horseradish peroxidase
ICS	Inhaled corticosteroid

IFN-	Interferon- (eg. IFN-γ)
Ig	Immunoglobulin
iNOS	Inducible nitric oxide synthase
I-TAC	Interferon-inducible T cell alpha chemoattractant/CXCL11
kDa	Kilo Dalton
IL-	Interleukin- (eg. IL-6)
LABA	Long-acting β_2-agonist
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein 1 (CCL2)
M-CSF	Macrophage-colony stimulating factor
MFI	Mean fluorescence intensity
MIF	Macrophage migration inhibitory factor
MIP-(1α)	Macrophage inflammatory protein-(1α)
MDM	Monocyte-derived macrophage
MM	Master mix
MOI	Multiplicity of infection
MRC-1	Mannose receptor, C type 1
mRNA	Messenger RNA
MyD88	Myeloid differentiation protein 88
NIP	5-iodo-4-hydroxy- 3-nitrophenacetyl
NIP_{20}-HSA	NIP_{20}-human serum albumin
NF-κB	Nuclear factor-κ-light-chain-enhancer of activated B cells
NOS2	Nitric oxide synthase 2
NSAID	Non-steroidal anti-inflammatory drug
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
Poly(I:C)	Polyinosinic-polycytidylic acid
qPCR	Quantitative real-time PCR

RANTES	Regulated on activation normal T cell expressed and secreted (CCL5)
RNA	Ribonucleic acid
RNase	Ribonuclease
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SABA	Short-acting β_2-agonist
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SDF-1	Stromal cell-derived factor-1
SSC	Side scatter
SEM	Standard error of the mean
sTREM-1	Soluble triggering receptor expressed on myeloid cells-1
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBST	TBS-Tween
TEMED	Tetramethyl-ethylenediamine
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
UNG	Uracil-N-glycosylase

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Chapter 1: Introduction

Macrophages, also referred to as mononuclear phagocytes, are present in all tissues of the body (Chow et al., 2011). Metchnikoff, in 1882, recognized the importance of these cells that could phagocytose ("eat") and destroy bacteria, in innate immunity (Gordon, 2007, Gordon, 2008). The term 'macrophages' comes from Greek, meaning 'big eaters'. In addition to phagocytosis, macrophages have other important roles, not only in immunity but also in development, homeostasis and repair. Specialized features allow macrophages to carry out their various functions. Macrophages also exhibit phenotypic heterogeneity and plasticity, which is currently of great research interest. Different macrophage phenotypes subserve the various roles of the cells. Macrophages are implicated in various conditions, such as rheumatoid arthritis and chronic lung diseases. Furthermore, different macrophage subsets can be implicated in different conditions. Therefore, the ability to modulate phenotypes makes the cells an attractive potential target for treatments.

1.1. Macrophage origins

Haematopoietic stem cells (HSC) in the embryonic yolk sac are believed to develop into primitive macrophages that are not differentiated from monocytes (Shepard and Zon, 2000). Later, haematopoiesis in the fetal liver begins, leading to the development of monocytes, which differentiate into macrophages (Shepard and Zon, 2000, Gordon and Taylor, 2005, Metcalf, 2007). HSC from the yolk sac may also move to the fetal liver but it is not clear whether the resulting macrophages persist into adulthood (Lichanska and Hume, 2000, Hume et al., 2002).

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Macrophages in adults are derived from monocytes, which in turn originate from multipotent HSC in the bone marrow. These precursor stem cells undergo differentiation, first along the myeloid pathway (as opposed to the lymphoid pathway) producing Granulocyte/Macrophage Colony-Forming Unit (GM-CFU), then along the monocyte/macrophage pathway (Metcalf, 2007, Mosser and Edwards, 2008). Differentiation of GM-CFU in the presence of macrophage colony stimulating factor (M-CSF) produces monocytes, which initially move into the bloodstream. Migration of the monocytes into tissues is believed to result in their differentiation into tissue-specific macrophages (figure 1.1) (Murray and Wynn, 2011b, Gordon and Taylor, 2005). This system is termed the 'mononuclear phagocyte system' (van Furth et al., 1972).

Although this described system, of derivation of macrophages from circulating monocytes is generally accepted, there are some conflicting views concerning macrophages in the absence of infection (the 'steady state'). Some early studies indicated that in the steady state, certain macrophages including alveolar macrophages were mainly derived from local proliferation of colony-forming cells present in the tissue rather than from monocytes recruited from the blood (Tarling et al., 1987). A more recent report claimed that in the steady state, tissue-resident macrophages, including alveolar macrophages, are not derived from blood monocytes. Instead, the macrophages proliferate *in situ* in response to M-CSF and granulocyte macrophage (GM)-CSF (Hashimoto et al., 2013).
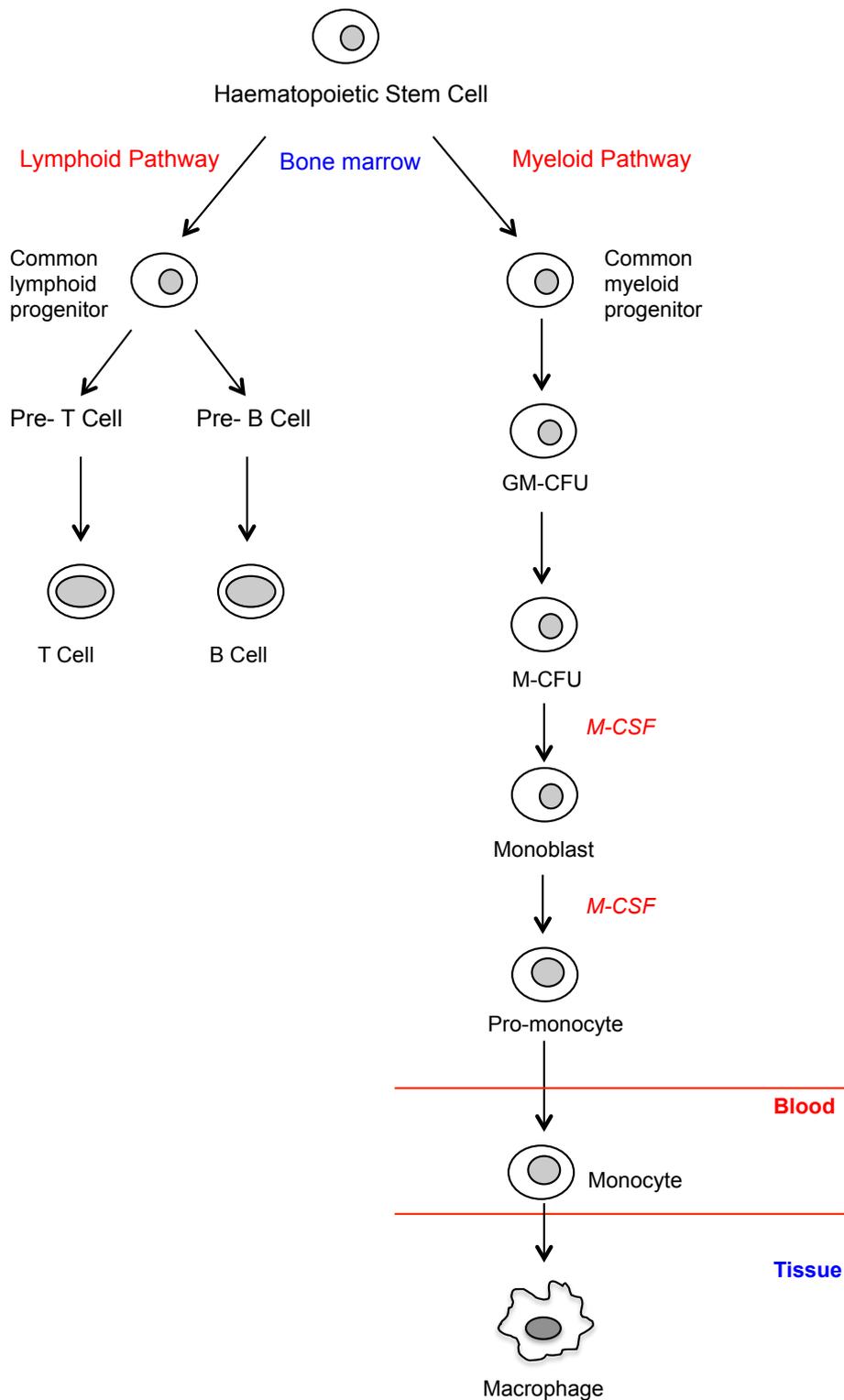
Figure 1.1. Macrophage development from haematopoietic stem cells.
A simplified figure of the development of macrophages along the myeloid pathway, as opposed to the lymphoid pathway, from bone-marrow precursor cells is shown. Monocytes are believed to move from the peripheral blood into tissues to develop into tissue-specific macrophages in adults. [GM-CFU, granulocyte macrophage-colony forming unit; M-CFU, macrophage-colony forming unit; M-CSF, macrophage-colony stimulating factor](Gordon and Taylor, 2005, Metcalf, 2007).

By contrast, the views of macrophages in infection conditions have been less conflicting. It has been understood that it is often necessary for monocytes to be recruited to infected tissues, and subsequently differentiate into macrophages, as the numbers of macrophages present in the tissues are not always sufficient to manage the infection alone (Murray and Wynn, 2011b). However, as well as differentiation from monocytes, there is evidence from a recent study in mice, of macrophages proliferating in the tissue, in certain types of infections. The study demonstrated that in response to a nematode infection, in which IL-4 is present, alternatively activated macrophages (discussed in 1.3.2) can proliferate *in situ*, although the exact mechanisms are not clear (Murray and Wynn, 2011b, Jenkins et al., 2011).

1.2. Principal roles of the macrophage

1.2.1. Phagocytosis

Phagocytosis is a method by which large particles and pathogens that require removal from the body are taken up by cells to be destroyed. Macrophages (along with monocytes and neutrophils) are termed 'professional phagocytes' because unlike many other cells that can also phagocytose, they carry out the process with a high level of efficiency (Aderem and Underhill, 1999, Grimsley and Ravichandran, 2003). The main way in which this is facilitated is through the presence of many different receptors that have affinity for a number of different particles. The density of receptors present also enables phagocytosis to occur at a high rate.

For the recognition and phagocytosis of pathogens, the receptors on macrophages are mainly Fc receptors for antibodies bound to pathogen surfaces (FcγRs), complement receptors for C3b that bind bacteria that have been opsonised by complement protein and mannose receptors which recognise mannose on pathogen surfaces (figure 1.2)

(Aderem and Underhill, 1999). Complement is a system of 20 proteins that, in response to pathogens, initiate a proteolytic cascade that leads to destruction of the pathogens (Roozendaal and Carroll, 2006). The different receptors are associated with different signalling pathways leading to phagocytosis (Aderem and Underhill, 1999). In Fc receptor mediated phagocytosis, Fc receptor cross-linking leads to phosphorylation of receptor domains by src kinase. Phagocytosis then results via a pathway involving activation of the tyrosine kinase Syk, phosphoinositide 3-kinase (PI3-kinase) and phospholipase C. Complement receptor mediated phagocytosis occurs by protein kinase C activation, requiring the presence of stimuli to do this.

The process of phagocytosis involves actin polymerisation (Aderem and Underhill, 1999). Receptor-ligand interactions on the cell surface stimulate the polymerisation of actin, resulting in the plasma membrane surrounding the pathogen, forming a phagosome. The actin is then lost by depolymerisation, leaving the vacuolar membrane free to fuse with endosomes (that have been acidified) and lysosomes (containing acid hydrolases and lysozyme), forming a mature phagolysosome in which pathogens can be destroyed (Aderem and Underhill, 1999).
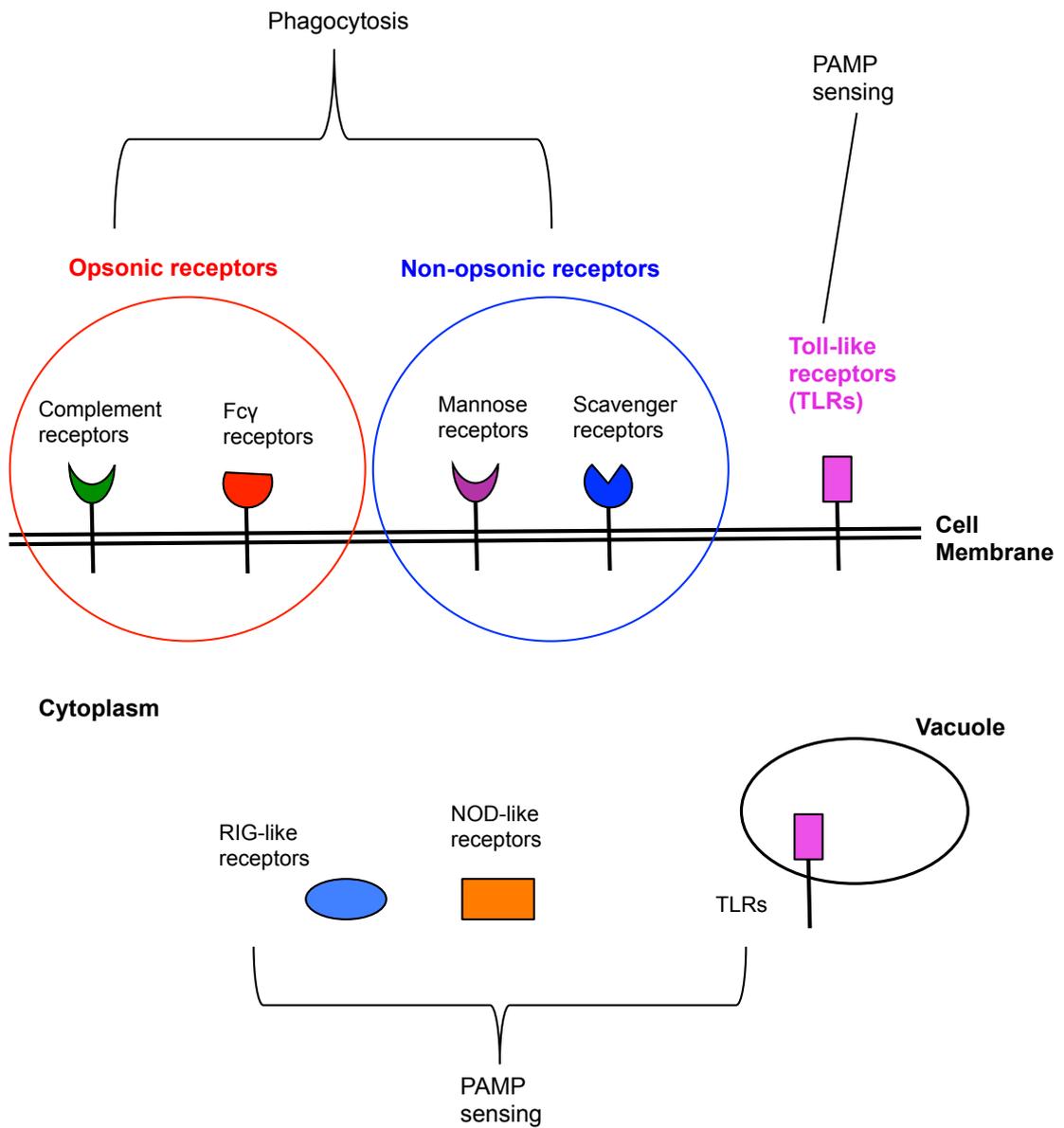
Figure 1.2. Macrophage receptors.
The macrophages receptors involved in phagocytosis are Fc receptors, complement receptors, mannose receptors and scavenger receptors. The macrophage receptors involved in detecting pathogen associated molecular patterns are Toll-like receptors, NOD-like receptors and RIG-like helicase receptors (Aderem and Underhill, 1999, Gordon, 2007)

1.2.2. Efferocytosis

A major role of macrophages in the healthy host is the removal of apoptotic cells (a process termed efferocytosis) for maintenance of tissue homeostasis and in development (Mosser and Edwards, 2008, Erwig and Henson, 2007). Removal of large numbers of erythrocytes from the blood to be replenished is one essential requirement for correct functioning of the host. In development, tissue remodelling leaves apoptotic cells that need to be removed. Neutrophils are constantly circulating short-lived cells that begin to undergo constitutive apoptosis within a matter of hours, requiring clearance to enable their replacement to occur. Apoptotic cells in the healthy host that have undergone constitutive apoptosis (as opposed to apoptosis as the result of injury or infection) need to be removed in large quantities, at a high rate, before substances that could be damaging to the surrounding tissue are leaked from the cells (Grimsley and Ravichandran, 2003). This constantly occurring process needs to take place without eliciting a pro-inflammatory response (Aderem and Underhill, 1999, Meagher et al., 1992). Macrophages are suggested to be able to recognise the difference between cells that have undergone constitutive apoptosis and those that have become apoptotic as a result of infection, then generate an appropriate pro-inflammatory response to the infected cells (Perskvist et al., 2002).

Macrophages (and other phagocytes) possess receptors on their surface that can recognise certain markers expressed by apoptotic cells that are not present on cells that are alive. Phosphatidylserine (PtdSer) is normally present in the inner leaflet of the cell membrane lipid bilayer. In apoptotic cells, it is translocated to the outer leaflet (Grimsley and Ravichandran, 2003, Savill and Fadok, 2000). In addition to PtdSer, other markers indicating apoptotic cells are changes in glycosylation of surface proteins, changes in lipids, change in the cell surface charge and modifications of molecules such

as intercellular adhesion molecule-3 (ICAM-3) and cluster of differentiation 31 (CD31 or platelet endothelial cell adhesion molecule (PECAM)-1) (Savill, 1997, Platt et al., 1998, Brown et al., 2002). Some of the receptors on macrophages that can recognise these markers are the class A scavenger receptor, the class B scavenger receptor CD36 and CD14 (figure 1.2) (Fadok et al., 2001, Febbraio et al., 2001). It has been proposed that phagocytosis of apoptotic cells does not elicit a pro-inflammatory response as occurs when pathogens are taken up because the presence of PtdSer engages phosphatidyl serine receptor in a dominant manner over the other receptors present on the macrophage (Fadok et al., 2001).

Ingesting apoptotic neutrophils results in macrophages down-regulating pro-inflammatory responses (in contrast to when ingesting pathogens) by not secreting IL-8 (which is a neutrophil chemoattractant), TNF-α (tumour necrosis factor-alpha) and MCP-1 (monocyte chemotactic protein-1) (Fadok et al., 1998). Furthermore, anti-inflammatory responses are actively produced, involving up-regulation of secretion of mediators such as prostaglandin E_2, TGF-β1, IL-10 and platelet-activating factor (Fadok et al., 2001, Fadok et al., 1998), demonstrating the importance of macrophage engulfment of apoptotic cells in tissue homeostasis.

1.2.3. Pattern recognition receptors and inflammatory responses

An important role for macrophages in disease states is the production of soluble mediators of inflammation in response to pathogens, such as cytokines and chemokines, which have important effects on other cells involved in immune and inflammatory responses (Puneet et al., 2005). Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that recognise specific pathogen-associated molecular patterns (PAMPS) (figure 1.2) (Aderem and Underhill, 1999). However, unlike with some other

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macrophage receptors, phagocytosis is not induced when pathogen binding occurs
(Gordon, 2007). Instead, intracellular signalling leads to cytokine gene activation
resulting in cytokine production and secretion, producing an inflammatory response.

TLRs are transmembrane receptors with an intracellular domain referred to as TIR
(Toll/IL-1 receptor homologous) domain and there are ten TLRs known to be present in
humans (Kaisho and Akira, 2006). Different TLRs respond to different ligands but most
of them signal via the NF-κB pathway (figure 1.3). The signalling pathway utilized
depends on the combination of TIR domain-containing adapter molecules, such as
MyD88 (myeloid differentiation primary response protein 88) and TRIF (TIR domain-
containing adapter protein inducing IFN-β), recruited from the cytoplasm (Martinez et
al., 2009, Kaisho and Akira, 2006). Some of the TLRs are expressed and detect PAMPs
at the cell surface, for example, TLR2 which is mainly activated by bacterial
lipoteichoic acid or bacterial lipoproteins and TLR4 which is mainly activated by
lipopolysaccharide (LPS) which is a component of gram-negative bacterial cell walls
(Sabroe et al., 2003, Martinez et al., 2009).

Some pathogens are phagocytosed before there is a chance for them to be detected at the
cell surface. Their PAMPs can be detected by TLRs intracellularly, as TLR2 and TLR6
form a heterodimer which can connect with phagolysosomes and detect the pathogens
within them. TLRs can also detect viral single-stranded RNA and CpG DNA present
within endosomes, for example TLRs 7 and 9, leading to the production of Interferon-α
(IFN-α) and Interferon-β (IFN-β) in addition to inflammatory cytokines (Gordon, 2007,
Kaisho and Akira, 2006, Diebold et al., 2004, Hochrein et al., 2004).
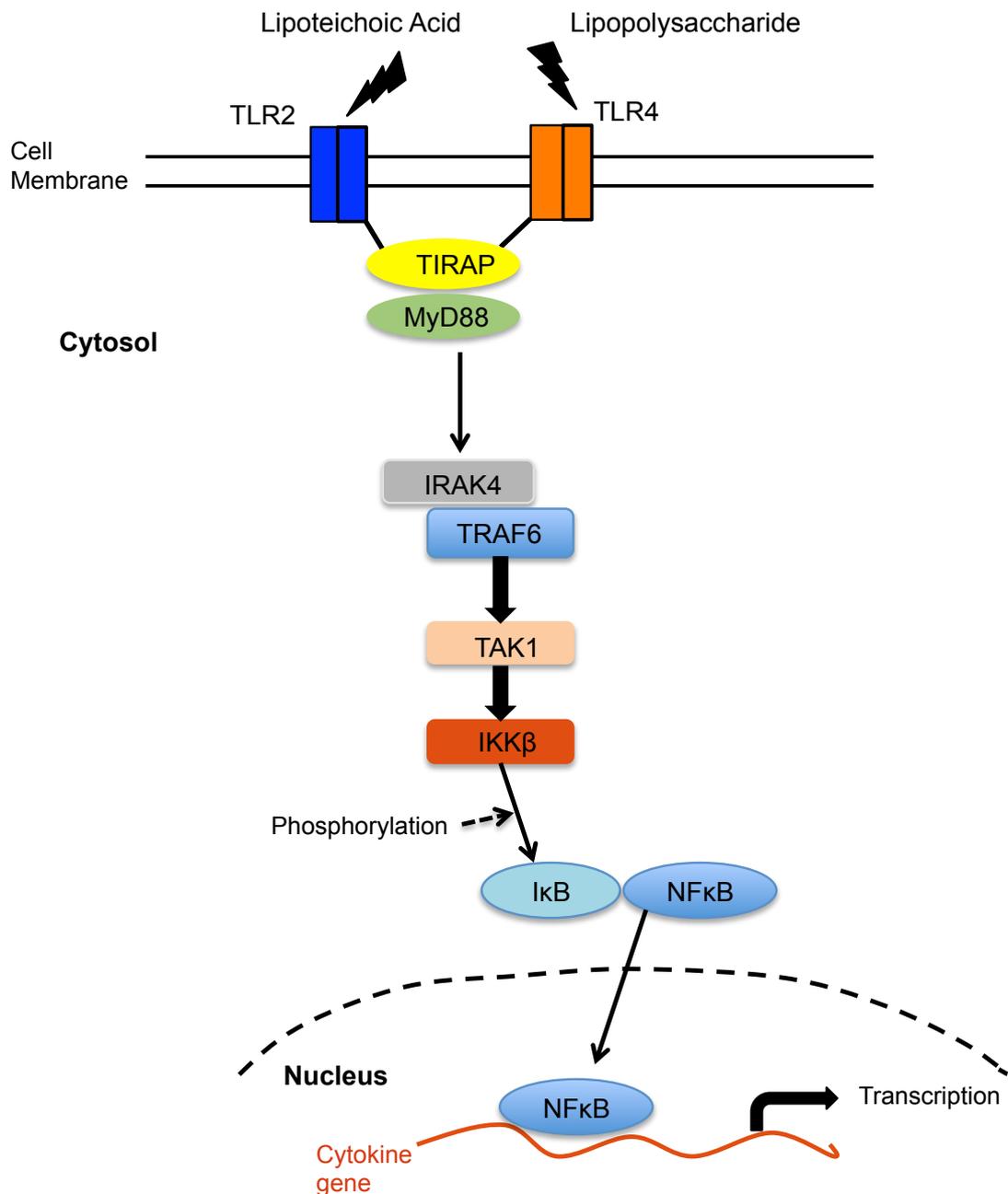
Figure 1.3. Pathway of TLR2 and TLR4 signalling via MyD88.
Stimulation by pathogens causes TLR2 and TLR4 to associate with MyD88/TIRAP.
IRAK-4 is recruited. TRAF6 recruitment leads to activation of TAK1 and IKKβ.
Phosphorylation of IκB results in its degradation, allowing NF-κB to become activated,
move to the nucleus and cause transcription of cytokine genes. [MyD88: Myeloid
differentiation primary response protein 88; TIRAP: TIR domain-containing adapter
protein; IRAK: IL-1 receptor-associated kinase; TRAF: TNF receptor-associated factor;
TAK: TGF-β-activated kinase; IKKβ: IκB kinase] (Kaisho and Akira, 2006).

In addition to TLRs, the other pathogen recognition receptors associated with macrophages are the NOD (nucleotide-binding oligomerization domain)-like receptors and RIG (retinoic acid-inducible gene 1)-like helicase receptors (Gordon, 2007). These receptors can detect viral and bacterial PAMPs in the cytosol (figure 1.2). The NOD-like receptors include NOD1, NOD2, NALP1 and NALP3 (Kawai and Akira, 2011). Inflammasomes are formed by the NALP proteins, resulting in activation of caspase-1, which is required for the release of certain pro-inflammatory cytokines, such as IL-1β. More investigation needs to be done on these receptors to bring the level of information about them up to that known about TLRs.

1.2.4. Antigen presentation to T cells

Macrophages have a further important role, acting as antigen presenters to T lymphocytes (T cells). Macrophages are considered to be, like dendritic cells, professional antigen presenting cells (APCs) (Nickoloff and Turka, 1994). Antigen presentation to T cells activates T cells, resulting in a T helper 1 (Th1) or T helper 2 (Th2) response (Martinez et al., 2009).

Pathogens binding to macrophages stimulate the macrophages to release chemokines which induce the recruitment of other immune cells that secrete either Interferon-γ (IFN-γ) (for Th1 responses) or interleukin (IL)-4 and IL-13 (for Th2 responses) depending on the macrophage receptor that is activated by the pathogen (Martinez et al., 2009). IFN-γ together with the pathogens binding stimulates the macrophage to release IL-12, whereas IL-4 or IL-13 with the pathogens stimulates IL-10 secretion from the macrophage.

Antigenic peptides from pathogens that are internalised but not able to be immediately destroyed in phagolysosomes bind to MHC (major histocompatibility complex) Class II molecules. Naive T cells that express CD4 (CD4-positive T helper cells) have affinity for MHC Class II molecules. The Peptide-MHC II complex moves to the cell membrane, displaying the antigenic peptide on the surface of the macrophage. Macrophages migrate from the site of infection to the lymph nodes (Hawrylowicz et al., 2001), where naive CD4-positive T helper cells can interact with the complex via the T cell receptor (TCR), with CD4 acting as a co-receptor. Co-stimulation for T cell activation is provided by co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) on the macrophage interacting with CD28 on the T cell (Lenschow et al., 1996, Grewal and Flavell, 1997). The cytokines (IL-12 or IL-10 released by the macrophage after pathogen binding) that are present during this process of T cell activation determine whether the naive T helper cells differentiate into Th1 or Th2 effector T cells respectively (Martinez et al., 2009).

Th1 and Th2 effector cells release different cytokines resulting in different roles (Martinez et al., 2009). Th1 release IFN-γ, which can then activate the macrophages with co-stimulation from CD40 ligand (CD40L) (which is expressed on the activated, effector T cells) binding to CD40 on the antigen-presenting macrophage (Grewal and Flavell, 1997). CD40-CD40L also up-regulates expression of the B7 co-stimulatory molecules (Peng et al., 1996, Grewal and Flavell, 1997). This Th1 response results in macrophages that are able to enhance the killing of pathogens in their phagolysosomes (Martinez et al., 2009). Th2 release IL-10 (which supresses Th1) and IL-4. Their main role is to help B cells to produce antibodies.

1.3. Macrophage heterogeneity and polarization

Macrophages demonstrate heterogeneity of function according to the requirements of their specific environment (Gordon and Taylor, 2005). Macrophages in the lung express certain receptors (discussed in 1.2.1) allowing them to recognise and remove or kill the pathogens that the lung is constantly exposed to. The cells also produce pro-inflammatory cytokines, which assists with this. These macrophages are different to those found in the lamina propria of the gut, for example, which also have the ability to phagocytose and kill harmful bacteria. However, they do not normally have a good ability to produce pro-inflammatory cytokines (Gordon and Taylor, 2005), which is important, as their other role is to ensure there is tolerance to the food antigens and resident flora constantly present in the gut (Murray and Wynn, 2011b).

Heterogeneity is also demonstrated by macrophages that reside in different locations of the same tissue. In the lung, alveolar macrophages are present in the alveoli, whereas interstitial macrophages reside in in the interstitium, which is a sterile environment, except when infection has taken place (Triggiani et al., 2004). The location of alveolar macrophages results in them easily coming into contact with inhaled foreign particles (Rankin, 1989). In line with their location-based requirements, alveolar macrophages have been reported to be better at carrying out phagocytosis than interstitial macrophages (Fathi et al., 2001) whereas a study using rhesus macaques found that interstitial macrophages acted as better antigen presenters than alveolar macrophages and were better at cytokine release (Cai et al., 2014). Macrophages in the lung have been referred to by some researchers as 'low density' macrophages or 'high density' macrophages (Triggiani et al., 2004). These have the properties described in other studies, of alveolar and interstitial macrophages respectively. Most of the studies into this area have been carried out using murine, not human, macrophages. It can also be

difficult to distinguish between the two types of macrophages, and the criteria used to discriminate between the types can vary between studies.

Tissue macrophages are generally believed to arise from circulating blood monocytes that are recruited to the tissue (Murray and Wynn, 2011b), although alternative proposals to this have been discussed in section 1.1. Monocytes are believed to exhibit heterogeneity themselves and have been reported to have either an 'inflammatory' or 'resident' phenotype, with different chemokine receptors expressed by each of the two subsets (Weber et al., 2000). Therefore, the differentiation of different monocyte subsets into macrophages could result in heterogeneous macrophage populations. The 'inflammatory' monocytes ($CD14^{hi}CD16^{-}CCR2^{+}$) are believed to be recruited (in response to pro-inflammatory CCL2 which is the ligand for CCR2) to inflamed tissues where they differentiate into macrophages that can contribute to resolution of inflammation (Gordon and Taylor, 2005, Shi and Pamer, 2011). The 'resident' monocytes ($CD14^{+}CD16^{+}CCR2^{-}$) are believed to be recruited to normal tissue, where they differentiate into macrophages to replenish the tissue macrophages. It should be noted that these reported results were partially extrapolated to humans from the results of murine studies, as there appeared to be phenotypic similarities between murine and human monocyte subsets (Geissmann et al., 2003, Ingersoll et al., 2010).

Macrophage heterogeneity can arise from differential activation. The definition of macrophage activation varies in the literature, depending on the context in which the term 'activation' is being used. The term can be used to refer to macrophages that have been stimulated in some manner resulting in them responding in a way that changes their state, or used in an immune context, where macrophages have been stimulated by a T helper cell cytokine response (Martinez et al., 2009) resulting in them acquiring a

particular phenotype. For the purposes of this discussion on macrophage activation in heterogeneity, it is used in the latter context. Macrophages can also be activated *in vitro* by various stimuli in the absence of T cells. An alternative term to 'activation' often used by many researchers is 'polarization' (Murray et al., 2014). There is considered to be a 'spectrum' of macrophage activation, with overlap in the characteristics of the different phenotypes (Mosser and Edwards, 2008). However, the major classifications of macrophages are into two groups. These are 'classically activated', by the Th1 response and 'alternatively activated', by the Th2 response, also known as M1 and M2 phenotypes respectively (Martinez et al., 2009).

1.3.1. Classically activated macrophages

Classical activation is believed to result in a macrophage phenotype that has increased intracellular pathogen killing capacity and produces increased levels of pro-inflammatory cytokines (figure 1.4) (Mosser and Edwards, 2008). The stimuli resulting in classical activation are IFN-γ and LPS. Originally it was believed that IFN-γ produced by Th1 cells during the immune response and TNF-α produced by TLR activation were both required to classically activate macrophages. However it has since been reported that LPS is one of the TLR ligands that can alone activate a pathway that results in IFN-β production, which can, instead of IFN-γ, contribute to macrophage activation along with TNF-α (Mosser and Edwards, 2008). The cytokines released by these M1 macrophages are pro-inflammatory and the broad description of M1 cells is that they produce high pro-inflammatory IL-12 and low anti-inflammatory IL-10. Much of the research into this area has been carried out using murine macrophages. The characteristics of murine macrophage phenotypes are therefore much better defined than those of human macrophages and there are some known differences between macrophages from the two species. However, attempts to better characterize human

macrophages have been made in recent studies. Recently it has been recommended that instead of describing stimuli as simply being M1 (or M2), the specific stimuli used should be reported, as for example, cells stimulated by LPS alone or by LPS+IFN-γ result in slightly different cytokine profiles (Murray et al., 2014). Human macrophages generate TNF-α, IL-6, IL-1β and the chemokines CXCL10 and IL-8 in response to LPS. In response to LPS+IFN-γ, macrophages generate IL-12, IL-23 and the chemokines CXCL10, CXCL9, CXCL11 and CCL5 (Murray et al., 2014).

M1 macrophages are believed to express certain cell surface markers, although these markers can also be expressed on cells other than macrophages. Expression of mannose receptor C type 1, MRC-1 (CD206) and FcγR II (Fc receptor for IgG) is down-regulated (Mosser, 2003). The cells are good antigen presenters to T cells, as expression of MHC II and co-stimulatory molecules (CD80 and CD86) is increased (Martinez et al., 2008). Whole pathogens can be considered to be M1 stimuli (Martinez and Gordon, 2014). It has been demonstrated that activation by bacteria can occur by the components of the bacteria, such as LPS, lipoteichoic acid, muramyl dipeptide and heat shock proteins activating TLRs. This has been shown to cause induction in gene expression of various pro-inflammatory cytokines and chemokines (Nau et al., 2002). The increased bacterial killing capacity of M1 macrophages is a result of increased production of reactive oxygen species (ROS) and nitric oxide (NO).
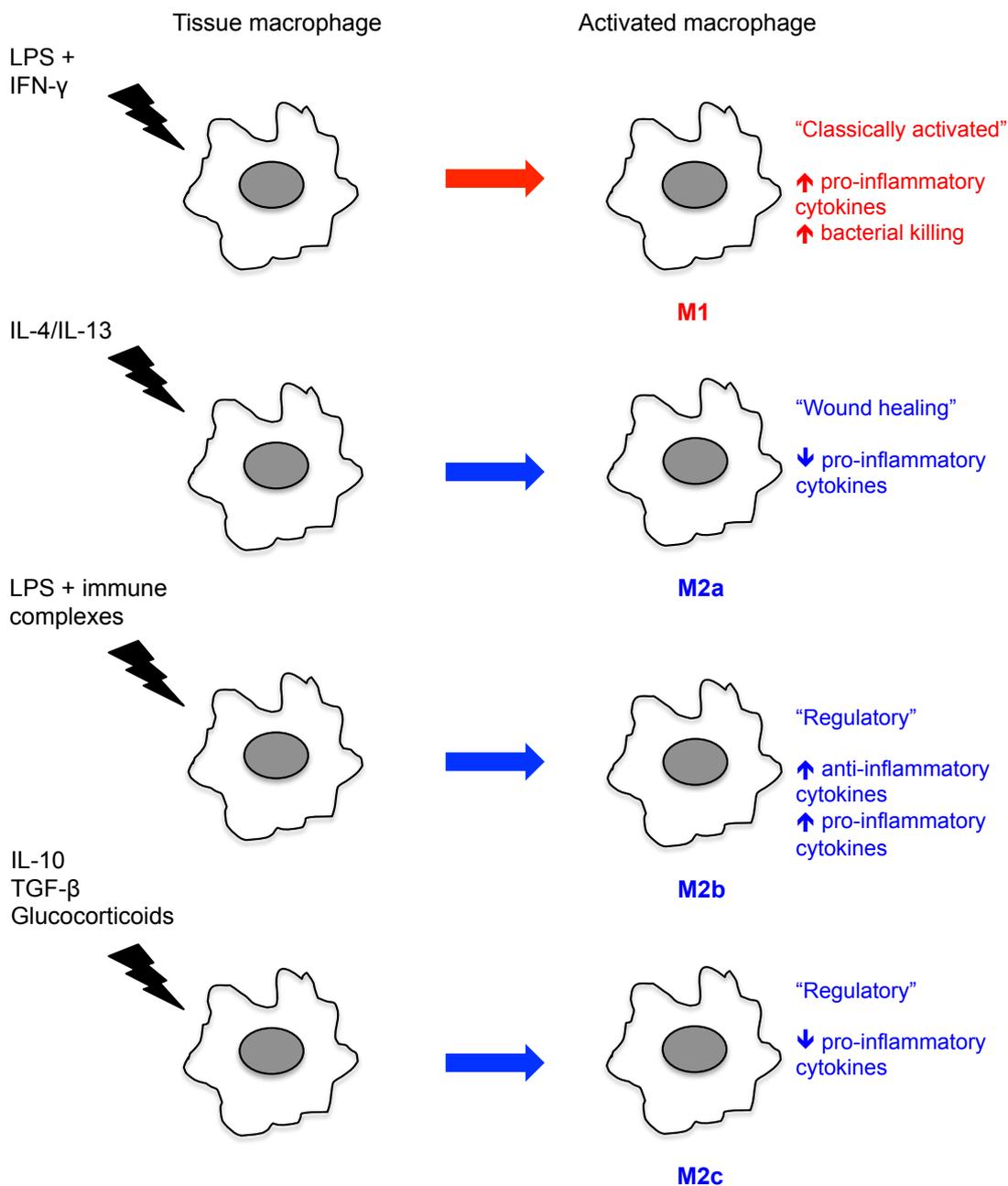
Figure 1.4. Macrophage activation producing phenotypic heterogeneity.
Activation of macrophages with different stimuli results in macrophage heterogeneity.
M1 macrophages have a pro-inflammatory 'classically activated' phenotype with the
increased ability to kill bacteria. M2a macrophages have decreased production of pro-
inflammatory cytokines and have a 'wound healing' phenotype. M2b and M2c
macrophages are both considered to have a 'regulatory' phenotype. M2b macrophages
have increased production of anti-inflammatory cytokines but can also produce
considerable levels of pro-inflammatory cytokines. M2c macrophages have decreased
production of pro-inflammatory cytokines and their phenotype is more anti-
inflammatory than that of M2a macrophages (Mosser and Edwards, 2008).

1.3.2. Alternatively activated macrophages

In addition to responding to infection, macrophages also have homeostatic roles. These are primarily the roles of the M2, 'alternatively activated' macrophages. M2 macrophages are also important in the resolution of inflammation. Classifying macrophages into just two categories has been found to be rather simplistic and not representative of all the phenotypes that can be produced. It has been proposed that the M2 macrophages category should be divided into further categories that better describe the phenotypes within (Mosser and Edwards, 2008). These categories are M2a, M2b and M2c (figure 1.4) (Martinez et al., 2008). M2a macrophages are the result of IL-4 or IL-13 stimulation and have also been referred to as 'wound-healing macrophages'. M2b and M2c macrophages have been referred to as 'regulatory macrophages' (Mosser and Edwards, 2008). M2b macrophages (also referred to as type II macrophages) are the result of stimulation by LPS or IL-1β plus immune complexes (Edwards et al., 2006). The stimuli leading to generation of M2c macrophages are IL-10, TGF-β or glucocorticoids. These can act in combination but the individual stimuli each have slightly different effects on macrophages (Martinez et al., 2008, Arango Duque and Descoteaux, 2014).

M2a macrophages are the result of the effects of IL-4 or IL-13 produced in Th2 responses (Gordon, 2003). IL-4 and IL-13 have very similar effects on macrophages. The wound-healing phenotype arises through activation of arginase, resulting in production of ornithine from L-arginine, rather than nitric oxide as is the case for M1 cells (Kreider et al., 2007, Chang et al., 1998). Ornithine is metabolized to proline which is a constituent of collagen and to polyamines which are important in cell proliferation (Varin and Gordon, 2009). However, arginase presence has been demonstrated in mice, not humans (Martinez et al., 2008). M2a macrophages express

MHC II and therefore can present antigen to T cells but not as well as M1 macrophages. Production of pro-inflammatory cytokines and efficiency at destroying intracellular pathogens is also down-regulated compared to M1 (Edwards et al., 2006, Gordon, 2003). In response to IL-4/IL-13, several pro-inflammatory cytokines are down-regulated, rather than anti-inflammatory cytokines being up-regulated (Martinez et al., 2008). Cell surface markers and genes that are up-regulated include scavenger receptor SR-A and mannose receptor MRC-1 (Gordon, 2003, Martinez et al., 2008, Murray et al., 2014). All of this indicates that wound healing appears to be the primary role of this macrophage phenotype, hence the proposed classification term.

M2b macrophages have a cytokine profile that is opposite to M1 cells, with high IL-10 and low IL-12 production (Edwards et al., 2006). However, the cells are not considered to be anti-inflammatory, as they also produce TNF-α, IL-1β and IL-6 (Martinez et al., 2008). The cells have up-regulated MHC II and therefore antigen presentation can place, resulting in a Th2 response, hence the cells being known as 'type II macrophages' (Martinez et al., 2009, Mosser, 2003). The cells have been reported to produce I-309 (CCL1), which could be important for the recruitment of regulatory T cells (Martinez et al., 2009, Arango Duque and Descoteaux, 2014).

M2c macrophages can arise when glucocorticoids stimulate macrophages, inhibiting the transcription of pro-inflammatory cytokine genes including IFN-γ-induced genes and cause up-regulation of the scavenger receptor CD163 (Martinez et al., 2009). IL-10 as a stimulus results in inhibition of production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12. IL-10 also down-regulates MHC II and therefore, antigen presentation by the M2c macrophage phenotype to T cells is inhibited (Martinez et al., 2008). These effects of glucocorticoids and IL-10 are more inhibitory than those of IL-

4/IL-13 and therefore, M2c macrophages can be considered to be further from an M1 phenotype than M2a macrophages are (Gordon, 2003, Edwards et al., 2006). IL-10 also reduces collagen synthesis, which is another example of this phenotype being different to the 'wound healing' M2a phenotype and also, the production of ROS is down-regulated. TGF-β as a stimulus regulates expression of CD163 and inhibits LPS-induced pro-inflammatory cytokines (Pioli et al., 2004, Martinez et al., 2008). In addition to being activation stimuli for M2c macrophages, IL-10 and TGF-β (transforming growth factor-β) are also produced by the cells. IL-10 and TGF-β can induce regulatory T cells and Th2 cells (Arango Duque and Descoteaux, 2014, Franchimont, 2004).

1.3.3. Macrophage plasticity

Activated macrophages have been shown to have the ability to change phenotypes depending on the environmental conditions that they are in. It is possible to produce hybrid macrophage phenotypes, for example. M2a macrophages (IL-4-stimulated) can be stimulated with TLRs and immune complexes to produce a phenotype which exhibits high IL-10 and low IL-12 secretion as would be expected from a regulatory macrophage but also expresses resistin-like molecule alpha (RELMα/ FIZZ1) in mice which is a marker of M2a macrophages (Raes et al., 2002).

Macrophages can be differentiated *in vitro* into an M1 phenotype from monocytes cultured in the presence of GM-CSF and into an M2 phenotype from monocytes cultured in the presence of M-CSF (Fleetwood et al., 2009, Ambarus et al., 2012). It has been demonstrated that these macrophages can reversibly re-differentiate into M2 and M1 phenotypes respectively when the growth factors are switched so that the M1 macrophages are cultured in M-CSF and the M2 macrophages are cultured in GM-CSF (Xu et al., 2013). Further supporting this, GM-CSF (M1)-differentiated macrophages

that were subsequently incubated with IL-4 (M2 stimulus) showed up-regulation of M2 markers. M-CSF (M2)-differentiated macrophages that were subsequently incubated with IFN-γ (M1 stimulus) showed induction of M1 markers (Ambarus et al., 2012). The plasticity of macrophages is important in disease conditions, as cytokines and other potential stimuli that are present in the tissue could alter the phenotype of the macrophages. The transient nature of macrophage phenotypes could therefore have implications for exacerbation or resolution of the clinical condition.

1.4. Macrophage model systems in research

1.4.1. Human cell systems

Monocyte-derived macrophages (MDMs) are widely used as a model for human tissue macrophages. MDMs are produced by the *in vitro* differentiation of monocytes obtained from whole blood. Peripheral blood mononuclear cells (PBMCs) are isolated from the blood by density gradient centrifugation and seeded onto cell culture plates. Monocytes from the PBMCs can be enriched before seeding, using selection columns. Alternatively most lymphocytes, which are non-adherent, can be removed after plastic adherence of the monocytes from the PBMCs to the cell culture wells. The differentiation protocols used can vary between research groups, with variations in the lengths of time deemed necessary to produce mature macrophages. A major disadvantage can be the variable yield of cells obtained from blood and the subsequent large numbers of cell loss during the differentiation process. An advantage is that venipuncture is less invasive than surgery and therefore it is possible to obtain ethical approval to recruit healthy volunteers for studies, which ensures sufficient numbers of control samples when comparing to disease conditions. As stated in 1.3.3, culturing monocytes in GM-CSF or M-CSF can result in differentiation into M1 or M2 macrophage phenotypes. Some researchers activate these differentiated macrophages further with LPS/IFN-γ or IL-

4/IL-13 for an M1 or M2 phenotype respectively (Murray et al., 2014). Alternatively, monocytes can be cultured with the cytokines without prior culture in GM-CSF or M-CSF (Ambarus et al., 2012). However, it has been shown by looking at gene expression, that differentiation of monocytes to macrophages in GM-CSF/M-CSF does not result in the exact same phenotypic markers as the markers resulting from differentiation of monocytes in the presence of the cytokines (Ambarus et al., 2012, Mia et al., 2014). This suggests that perhaps the two methods of differentiation should not be considered to be interchangeable.

Monocytic cell lines are often used as a substitute for MDMs (and ultimately, tissue macrophages), eliminating the issue of low cell yield. THP-1 cells were established from the blood of a one year-old male with acute monocytic leukaemia (Tsuchiya et al., 1980). Another monocytic cell line is U937, for which the cells were isolated from the histiocytic lymphoma of a 37 year-old male (Medeiros et al., 2012). However, it has been demonstrated that different protocols to produce differentiated, mature macrophages can result in variation in the level of similarity to MDMs. The protocols generally used involve treatment with phorbol-12-myristate-13-acetate (PMA) or 1,25-dihydroxyvitamin D3 (VD_3). A recent study was carried out comparing macrophages resulting from different THP-1 differentiation protocols to monocytes and MDMs (Daigneault et al., 2010). It was found that differentiating THP-1 cells by VD_3 resulted in cells more like monocytes in comparison to MDMs. The results of differentiating by PMA followed by resting in culture indicated that the cells more closely resembled MDMs in terms of morphology and responses to TLR ligands.

1.4.2. Murine cells

Bone marrow-derived macrophages (BMDMs) isolated from mice are commonly used as a macrophage model. BMDMs can be cultured *in vitro* like human MDMs, in GM-CSF or M-CSF and cytokines to produce M1 or M2 phenotypes (Murray et al., 2014). Other murine macrophages that are often used are peritoneal macrophages, which are obtained by peritoneal lavage. Thioglycollate injection prior to lavage can elicit a greater yield of peritoneal macrophages (Zhang et al., 2008, Murray et al., 2014). Tissue macrophages can also be obtained from the organs of mice and also alveolar macrophages, although the yield is generally low (Murray et al., 2014, Zhang et al., 2008). A major issue with the use of murine macrophages as a model for human macrophages for certain types of studies is that there are known differences in phenotypic markers between the two systems. There are phenotypic markers exhibited by murine cells, which are either not expressed by human macrophages or which have no human analogues (Murray and Wynn, 2011a, Geissmann et al., 2010, Murray and Wynn, 2011b). There are also many differences in expression of other markers such as various cytokines/chemokines and transcription factors in response to different stimuli (Murray et al., 2014).

RAW 264.7 cells have been used in many studies as models for macrophages. The cells were established from an Abselon leukaemia virus-induced tumour in a male Balb/c mouse (Medeiros et al., 2012). There are advantages to using RAW 264.7 cells. The cells can be transfected, unlike the primary cells that have been discussed, which may be useful for certain experiments. However, there is a possibility that sources of cell lines could be problematic. For example, the RAW 264.7 cells available from American Type Culture Collection (ATCC) have in the past been tested and reported to produce

ecotropic murine leukaemia virus (Hartley et al., 2008). This could affect the results obtained from certain types of experiments.

Recently, Max Planck Institute cells (MPI cells) were proposed as a model for alveolar macrophages (Fejer et al., 2013). These cells are produced from murine fetal liver cells cultured in GM-CSF and are self-renewing in the presence of GM-CSF. MPI cells are reportedly more similar to alveolar macrophages than M-CSF-cultured bone marrow-derived macrophages. This is in terms of similarities in their pro-inflammatory response to TLR ligands and pathogens such as *Mycobacterium tuberculosis* which can infect the lungs, expression of certain cell surface markers, in addition to similarities in morphology.

1.4.3. Human lung macrophages

The cell systems discussed earlier in this section can be useful models for human macrophages. There are more obstacles to overcome in order to obtain primary cells of interest from human tissue and the process can be more technically challenging. However, the cells are expected to be more relevant for studies into conditions affecting the specific tissue. In the present study, macrophages were isolated from surgically resected human lung tissue and used as a model for investigations into respiratory diseases. Alternatively, macrophages can be obtained from the human lung by bronchoalveolar lavage (BAL).

Both macrophages obtained by BAL and macrophages obtained from lung tissue are considered to be alveolar macrophages. To obtain interstitial macrophages, it is generally believed that enzymatic digestion of the tissue is required. However, even after enzymatic digestion, it has been reported that the majority of macrophages present

in the digested fraction appear to be alveolar macrophages and only a small number are interstitial (Cai et al., 2014). It has also been demonstrated in other studies that the majority of macrophages obtained from the lung are alveolar macrophages and only a small proportion are interstitial macrophages. For example, a study in mice reported that in the steady state, 93% of the total macrophages obtained from the lung were alveolar macrophages and 7% were a mixture of alveolar and interstitial (van oud Alblas and van Furth, 1979). It is difficult to distinguish between the two types of macrophages morphologically although alveolar macrophages are reported to be larger (Cai et al., 2014, Fathi et al., 2001). One study, using rhesus macaque macrophages reported that both alveolar and interstitial macrophages are HLA-DR (human leukocyte antigen-DR), CD11b and CD163 positive but in addition, alveolar macrophages are also CD206 positive whereas interstitial macrophages are CD206 negative (Cai et al., 2014). However, this selection of cell surface markers is not definitive for distinguishing between the two types of macrophages and studies by other researchers are ongoing (Dewhurst et al., 2014). This is one of the reasons why separating the two types of macrophages is difficult, for example by using a process such as fluorescence-activated cell sorting (FACS).

The macrophages used for the studies in this thesis were isolated by manual chopping and washing of lung tissue. Therefore, based on the information from other studies discussed in the previous paragraphs, it can be assumed that they are predominantly alveolar macrophages. However, as the possibility of a very small number being interstitial macrophages cannot be excluded, the cells are referred to as 'human lung macrophages'.

1.5. Macrophages in inflammatory lung diseases

Macrophages may be involved in the progression of inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD) and asthma. Different phenotypes of macrophages have been reported to be present in different diseases (Murray and Wynn, 2011b). It has been suggested that the plastic nature of macrophage phenotypes makes them susceptible to modulation by environmental factors. Risk factors for COPD and asthma include smoking and airborne particles, which macrophages in the lungs are exposed to. These factors could have adverse effects on macrophages, although the mechanisms leading to defective or exaggerated macrophage responses are not fully understood.

1.5.1. COPD

COPD is a progressive condition in which inflammation of the lungs causes airflow to be severely restricted (Sutherland and Martin, 2003). It is a condition of which the incidence throughout the world is increasing to the extent that it is predicted that by 2020 it will be the fifth biggest public health problem (Sutherland and Martin, 2003, Barnes, 2008a) and according to World Health Organization predictions, it will be the third biggest cause of death in the world in 2030 (www.who.int/en/).

The clinical manifestation of COPD reflects two major pathological conditions. The first is chronic bronchitis, which is caused by chronic inflammation of the small and medium airways and the symptoms, in addition to irreversible reduced airflow, are chronic cough and production of sputum. Fibrosis can also occur (Sarir et al., 2008). The second condition is emphysema, in which the release of proteases from inflammatory cells causes destruction of the elastic tissue in the lung and lung parenchyma, such as alveolar walls, resulting in low oxygen concentration in the blood

(Sutherland and Martin, 2003, Barnes, 2008a). Emphysema can also involve mucus blocking the airways (Sarir et al., 2008).

The most prominent inflammatory cells in COPD, in addition to neutrophils, are macrophages (Sutherland and Martin, 2003). The numbers of alveolar macrophages in COPD patients are significantly higher than normally found (Finkelstein et al., 1995, Sarir et al., 2008). This could be due to increased recruitment of monocytes from the blood into the tissue or due to decreased macrophage apoptosis (Tomita et al., 2002, Barnes, 2008b). One of the main ways in which macrophages are believed to be involved in COPD is through their ability to secrete cytokines and factors (Barnes, 2008a). In response to cigarette smoke, for example, which is a major risk factor, inappropriate or aberrant cytokine/chemokine release from macrophages in COPD may occur (Barnes, 2008a). The recruitment of monocytes and other immune cells in response to the chemokines may lead to persistent inflammation, causing damage to the airways (Barnes, 2008a). Also, in response to cigarette smoke, macrophages and recruited neutrophils can release proteases such as MMP-9 (matrix metalloproteinase-9), which can degrade elastin, causing emphysema (Lim et al., 2000, Barnes, 2008b).

It has been reported that the phagocytic ability of macrophages in COPD could be defective. This could be another way in which macrophages are implicated in the pathogenesis of COPD and contribute to exacerbations of the condition. The defects could be both in the phagocytosis of pathogens and in efferocytosis of apoptotic cells (Henson et al., 2006, Krysko et al., 2010). Dysregulated phagocytosis of pathogens could result in persistence of bacterial load and inflammation, contributing to pathogenesis and exacerbations of COPD. A previous study reported that MDMs derived from COPD patients showed a decrease in phagocytosis of *Streptococcus*

pneumoniae compared to MDMs from smokers and non-smokers without COPD

(Taylor et al., 2010). However, a study using alveolar macrophages from COPD

patients showed that there was no defective phagocytosis of *Streptococcus pneumoniae*

by these cells compared to non-COPD macrophages. However, there was defective

phagocytosis of non-typeable *Haemophilus influenzae* by alveolar macrophages from

both ex and current smokers with COPD compared to healthy non-smokers (Berenson

et al., 2013). An earlier study by the same group showed that phagocytosis of this

pathogen was defective in ex-smokers with COPD compared to ex-smokers without

COPD, indicating that smoking may not be impairing the ability of macrophages to

phagocytose *Haemophilus influenzae* in COPD (Berenson et al., 2006). It has been

shown in other studies that clearance of bronchial epithelial cells by alveolar

macrophages from BAL is impaired in patients with COPD (Hodge et al., 2003).

Furthermore, the efferocytic ability of macrophages from smokers with COPD has been

reported to be decreased compared to those from ex-smoker COPD patients and one

mechanism suggested for this is that cigarette smoke may alter the expression of

macrophage surface markers that are required for apoptotic cell detection (Hodge et al.,

2007). Further research is required for clarification but it may be possible that the effect

of smoking on macrophages may result in impairment of efferocytosis but not

impairment of pathogen phagocytosis in COPD.

It has been suggested that the dysregulation of the normal macrophage processes may

be due to the phenotype of the macrophages being altered in COPD and/or in response

to smoking. It has been observed in a previous study that in smokers with and without

COPD, down-regulation of genes for M1 macrophage-related chemokines occurs

overall, rather than marked up-regulation of M2 macrophage-related genes (Shaykhiev

et al., 2009). This study indicated that smoking causes the phenotype of alveolar

macrophages to switch to a mixed phenotype that is skewed towards an M2 phenotype. This effect is further emphasised in smokers with COPD (Shaykhiev et al., 2009). Mixed phenotypes have also been reported in another study in alveolar macrophages from healthy smokers, COPD patients and ex-smoker COPD patients, although the trends were less clear (Hodge et al., 2011). It appears that the phenotype can be altered in macrophages from smokers and COPD patients. However one particular phenotype does not seem to be associated with the pathogenesis of COPD. Rather, markers associated with both M1 and M2 phenotypes can be either up-regulated or down-regulated, which could result in the dysregulation of certain macrophage functions.

1.5.2. Asthma

Asthma has clinical manifestations that are similar to COPD but there are many differences in the underlying inflammation, distinguishing the two diseases (Barnes, 2008a). The inflammation in severe asthma, however, is closer in phenotype to that in COPD compared to that in mild asthma, for example, in terms of the numbers of certain inflammatory cells being present. The incidence of asthma is increasing globally, as is the case for COPD and according to the World Health Organization, it is estimated that 300 million people currently have the condition (www.who.int/en/). The major similarity to COPD is that asthma is also an inflammatory lung condition in which airflow is restricted but unlike in COPD, it is reversible, in response to bronchodilators (Sutherland and Martin, 2003). The differences compared to COPD are that asthmatic airways exhibit hyperresponsiveness which consists of reacting in an exaggerated manner to stimulants that are inhaled, such as pollutants (Peachell, 2005); mucus hypersecretion results in blocking of the airways (Rankin, 1989); the lung parenchyma is not destroyed as in COPD; basement membrane thickening occurs and the large airways are involved more than the small airways in asthma (Barnes, 2008a).

Asthma can be categorized as atopic (allergic) and non-atopic. Most asthma cases are atopic. The most important cells in asthma are eosinophils and mast cells (Sutherland and Martin, 2003). However, macrophages are also involved (Rankin, 1989, Gosset et al., 1999, Yang et al., 2012). The number of macrophages in the lungs are increased in asthma (Barnes, 2008b). The position of alveolar macrophages in the lung enables them to easily come into contact with allergens (Rankin, 1989). Alveolar macrophages in asthma show increased antigen (allergen) presentation compared to non-asthmatics, eliciting a Th2 cytokine response, resulting in allergic inflammation (Martinez et al., 2009, Kay, 2002). However, not only Th2 cytokines are seen in asthma. A decrease in the release of anti-inflammatory cytokine IL-10 has been seen from macrophages from asthmatics and an increase in pro-inflammatory MIP-1α (macrophage inflammatory protein-1α) and IFN-γ (John et al., 1998).

A greater percentage of macrophages from allergic asthmatics express low affinity receptors for IgE (FcεRII) than control subjects, suggesting that macrophages can be activated by allergens via IgE-dependent mechanisms (Rankin, 1989, Williams et al., 1992, Gosset et al., 1999). The release of pro- and anti-inflammatory cytokines has been reported to be increased in IgE-dependent activation in both control and asthmatic alveolar macrophages (Gosset et al., 1999). In asthmatics, release of pro-inflammatory cytokines is increased over release of anti-inflammatory cytokines. Therefore, activation by IgE receptors could result in increased inflammation in allergic asthmatics (Gosset et al., 1999). Alveolar macrophages from severe asthmatics also show reduced phagocytosis of apoptotic cells (Huynh et al., 2005). Phagocytosis of apoptotic cells is important for inducing the release of anti-inflammatory or immunosuppressive

mediators such as IL-10, TGF-β and PGE$_2$ from macrophages (Fadok et al., 1998, Huynh et al., 2005).

A higher number of M2 phenotype macrophages are present in the lungs in asthma compared to controls (Moreira and Hogaboam, 2011). M2 macrophages are important in the lungs for tissue repair and the resolution of inflammation but excessive numbers could contribute to the pathogenesis of asthma, by resulting in increased recruitment of cells, mucus secretion and airway remodelling (Moreira and Hogaboam, 2011, Liu et al., 2014). However, it has been suggested that the increased numbers of M2 macrophages could be a result of the Th2 cytokine environment after allergen challenge, rather than the M2 macrophages having an important role in actively contributing to the pathogenesis of asthma (Boorsma et al., 2013). Supporting this notion, a study using mice with macrophages lacking the receptor to respond to IL-4/IL-13 activation developed allergic airway disease that was comparable to controls (Nieuwenhuizen et al., 2012).

M1 macrophages have also been reported to be present in asthma. M1 markers have been found in macrophages from BAL fluid in severe asthma but high levels of LPS were also present (Goleva et al., 2008). Studies in humans and mice indicate that it is possible that macrophages switch to an M1 phenotype in severe asthma, releasing pro-inflammatory mediators which can contribute to damage to the airways and may contribute to exacerbations (Moreira and Hogaboam, 2011, Goleva et al., 2008, Kim et al., 2007). M1 macrophages could also contribute to the pathogenesis of asthma by recruiting neutrophils, through the release of chemokines such as IL-8. Neutrophilia in the lung is associated with severe asthma (Kamath et al., 2005).

1.6. Current therapies for COPD and asthma

The treatments available for COPD and asthma act to alleviate symptoms and to dampen the inflammation associated with the conditions. The difficulties in producing novel effective treatments arise partly because these are complex diseases involving several different cell types. Targeting a particular pathway of mediator release may be effective in one cell type but may not be effective in another. This could decrease the overall efficacy of the treatment or even produce adverse effects. Novel, more specific treatments are required, particularly for COPD, for which current treatments are often not effective.

1.6.1. Bronchodilators

One of the symptoms of both COPD and asthma is bronchoconstriction. Beta-adrenoceptor agonists (β_2-agonists) and muscarinic antagonists are available as bronchodilators. β_2-agonists act on β_2-adrenoceptors, which are G-protein (G_s)-coupled receptors (GPCRs) linked to adenylyl cyclase. Receptor activation leads to an increase in cyclic-AMP (cAMP) and activation of protein kinase A, which results in smooth muscle relaxation (Tashkin and Fabbri, 2010). The action of muscarinic antagonists is less direct. The neurotransmitter acetylcholine binds to M_3 muscarinic receptors on smooth muscle. As M_3 receptors are G-protein (G_q)-coupled, this results in an increase in intracellular calcium, causing contraction of the muscle. Muscarinic antagonists block acetylcholine binding, enabling smooth muscle relaxation (Tashkin and Fabbri, 2010).

Both types of bronchodilators are used in inhaler form in current therapies. Bronchodilator therapy has improved over the years, progressing from short-acting compounds that had effects lasting 4-6 hours to long-acting β_2-agonists (LABA) and

long-acting muscarinic antagonists (LAMA) with effects lasting 12 hours (Domingo, 2013). Some of these compounds have effects lasting up to 24 hours. Tiotropium is one such muscarinic antagonist and indacaterol is one such β_2-agonist. Vilanterol, also has effects of 24 hours but is termed an 'ultra-LABA' and has been authorized for use in treatments very recently (Domingo, 2013, Theron et al., 2013). Combinations of LABA and LAMA may also be used in treatments. The bronchodilators are generally effective for reversing airflow obstruction in asthma. In COPD, the reduction of airflow upon using the bronchodilators is not fully reversible but the treatments can provide some symptomatic relief.

1.6.2. Corticosteroids

The pathogenesis of both COPD and asthma involves inflammation of the airways. Corticosteroids are the most potent anti-inflammatory treatments available. These were initially used as effective anti-inflammatories in asthma therapy but are now also used in COPD treatment. Corticosteroids work by binding to their intracellular cognate receptor in the cytosol. The complex can then translocate to the nucleus to bind to response elements in the promoter regions of genes. This is known as 'transactivation' and can result in an up-regulation or down-regulation of gene transcription (Johnson, 2004, Taylor and Hancox, 2000). This could result in an up-regulation of anti-inflammatory cytokines and a down-regulation of inflammatory cytokines. Another mechanism is termed 'transrepression', in which the steroid-receptor complex interacts directly with transcription factors, inhibiting gene transcription, which could inhibit the production of pro-inflammatory cytokines (Johnson, 2004, Taylor and Hancox, 2000).

Corticosteroids are generally effective in asthma but are usually much less effective in COPD (Barnes, 2010). It has been suggested that corticosteroid insensitivity is a result

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of the oxidative stress that is present in COPD causing reduction of expression of histone deacetylase-2, which is a co-factor of the glucocorticoid receptor (Kirkham and Barnes, 2013, Rossios et al., 2012). This results in reduced corticosteroid function, allowing gene transcription to occur (Rossios et al., 2012, Ito et al., 2001). Inhaled corticosteroids (ICS) are used in treatments, which bypass the side-effects of oral corticosteroids to a large extent. Currently, commonly used treatments for anything but the least severe cases of asthma are combination inhalers, consisting of ICS with a LABA. It has been suggested that in future treatments, LAMA may be combined with LABA and ICS, as a triple therapy (Tashkin and Ferguson, 2013, Domingo, 2013).

1.6.3. Other therapies

Whilst β_2-agonists and ICS are the main and most effective therapies used for COPD and asthma, other therapies are available. One of the first compounds available as a bronchodilator was theophylline, which has limited use today due to its associated side effects (Barnes, 2006a). Theophylline is a non-selective phosphodiesterase (PDE) inhibitor. PDE acts to regulate the cyclic nucleotide signalling pathway by carrying out hydrolysis of cyclic nucleotides such as cAMP. PDE exists as at least 11 isoforms, although PDE4 is the one most widely expressed by inflammatory cells and specifically breaks down cAMP (Souness et al., 2000). PDE4 inhibitors are available, which can prolong the effects of cAMP (Barnes, 2003). This is beneficial in treatments as cAMP can act on smooth muscle cells to cause bronchodilation, as discussed earlier. Increases in cAMP can also lead to inhibition of cytokine release from inflammatory cells (Serezani et al., 2008, Aronoff et al., 2004). Roflumilast is one PDE4-selective inhibitor that has been approved recently as a treatment for COPD. Roflumilast has been shown in a study to reduce chemokine and cytokine release from human lung macrophages (Buenestado et al., 2012). It is this reported anti-inflammatory effect on macrophages

that makes roflumilast more effective in COPD than asthma, as macrophages are implicated to a greater extent in COPD (Peachell, 2005).

Bronchoconstriction and inflammation can be caused by eicosanoid release from inflammatory cells in COPD and asthma. In this context, cysteinyl-leukotrienes produced from the metabolism of arachidonic acid by the 5'-lipoxygenase (5'-LO) pathway play an important role. Cysteinyl-leukotrienes are mainly released from mast cells upon activation by stimuli such as allergens. Cysteinyl-leukotriene receptor antagonists are available for asthma, such as montelukast, which has been shown to be effective in patients with severe asthma, when used in addition to corticosteroids (Dahlen et al., 2002, Barnes, 2006b). A 5'-LO inhibitor, zileuton, is also available to block production of cysteinyl-leukotrienes (Holgate, 2013).

Cromones such as nedocromil and cromoglycate were used in early treatments for asthma and were introduced as mast cell stabilizers (Holgate, 2013, Peachell, 2005). However, although cromones are effective stabilizers of rodent mast cells, the evidence of such an effect on human mast cells is weak (Peachell, 2005). Therefore, their mode of action has not been determined. It has recently been suggested that one mechanism of action that results in an inhibitory effect on mast cells involves the stimulation of the release of anti-inflammatory protein annexin-A1, which can act in an autocrine or paracrine fashion to inhibit mast cell activation (Yazid et al., 2013). This is interesting, as anti-inflammatory glucocorticoids can also stimulate the synthesis and release of annexin-A1 (D'Acquisto et al., 2008). The use of cromones has now been usurped by the use of current inhaled therapies but there is interest in determining the mechanisms by which these drugs work, as it could contribute to development of other treatments (Holgate, 2013, Barnes, 2006a, Peachell, 2005).

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Antibody therapies have been developed for use in severe asthma. Omalizumab is a monoclonal antibody to IgE which binds to IgE, preventing it binding to high affinity Fcε receptors on cells such as mast cells and basophils, reducing allergic responses (Holgate, 2014). Omalizumab has been found to be effective in severe asthma that has an allergic basis, although not in all patients (Holgate, 2013). Antibody therapies have also been developed to block cytokines and chemokines. IL-5 recruits eosinophils resulting in eosinophilic inflammation seen in asthma (Holgate, 2013). Anti-IL-5 has been developed and tested in asthma but showed variable efficacy (Holgate, 2013, Haldar et al., 2009, Castro et al., 2011, Flood-Page et al., 2007).

Over the years, attempts have been made to develop novel therapies. Despite this, the frontline treatments for respiratory diseases have remained similar since the 1970s although there have been significant improvements in the selectivity and duration of action of the compounds used (Peachell, 2005, Domingo, 2013, Barnes, 2006b). β_2-agonists and corticosteroids are the mainstay therapies but are not always effective, particularly in COPD. It is also possible that some of the compounds currently used may be reaching their limit for improvement, for example, β_2-agonists have been developed to the stage of being ultra long-acting (Domingo, 2013). Novel therapies are much needed.

1.7. Macrophages as targets for therapy

Although a range of treatments is available for COPD and asthma, none specifically targets macrophages. This is despite the prominence of macrophages in these diseases, particularly in COPD. Corticosteroids have been shown to inhibit cytokine release from lung macrophages, indicating that this anti-inflammatory function could be important in

treatments for respiratory diseases. Although β_2-agonists are used as bronchodilators, it has been reported that they also have some anti-inflammatory activity. Currently, ICS can often be used at high doses in treatments but over the long-term, this can produce side-effects in patients. The ability to use β_2-agonists as anti-inflammatories and potentially enable lower doses of ICS to be used would be desirable. There are varying reports on the abilities of different β_2-agonists to inhibit cytokine release from macrophages and this may be due to the different model systems used for investigations (Donnelly et al., 2010, Theron et al., 2013, Buenestado et al., 2012, Zetterlund et al., 1998). The potential anti-inflammatory effects of a range of β_2-agonists on human lung macrophages have therefore been evaluated in the present study.

As discussed earlier, β-adrenoceptors are GPCRs coupled to adenylyl cyclase and activation of the receptors by agonists leads to cAMP increases. Elevations in cAMP are believed to underpin the inhibitory effects of β_2-agonists. Other classes of agonist can also induce cAMP increases. Prostanoids act on GPCRs and the action of certain prostanoids on GPCRs that are coupled to adenylyl cyclase can lead to cAMP increases (Sugimoto and Narumiya, 2007).

Prostanoids are lipid mediators produced as a result of the metabolism of arachidonic acid, which is a 20 carbon unsaturated fatty acid (Ricciotti and FitzGerald, 2011). Arachidonic acid is released from phospholipids in the plasma membrane of the cell by the action of phospholipase A2, upon cell activation. Arachidonic acid can be metabolized via two different pathways – the 5-lipoxygenase pathway to produce leukotrienes or via the cyclooxygenase (COX) pathway to produce prostanoids. The latter pathway involves arachidonic acid being metabolized to an intermediate, Prostaglandin H_2, by COX enzymes. Prostaglandin H_2 can then be converted by specific

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synthases to five different prostanoids: prostaglandin D_2, prostaglandin E_2, prostaglandin $F_{2\alpha}$, prostaglandin I_2 (prostacyclin) and thromboxane A_2. Different cells express each synthase at different levels, resulting in certain prostanoids being predominantly produced in certain cell types over others. Thromboxane A_2 (TXA_2) is mainly produced by activated platelets and acts on TP receptors. Prostaglandin D_2 (PGD_2) is predominantly produced by mast cells. PGD_2 acts on DP or CRTH2 receptors but can also act on TP receptors. Prostaglandin $F_{2\alpha}$ is produced mainly in the uterus and acts on FP receptors. Prostaglandin I_2 (prostacyclin) is produced by vascular endothelial cells and acts on IP receptors. PGE_2 is the prostanoid that is predominantly produced by macrophages and acts on EP_1, EP_2, EP_3 and EP_4 receptors. The prostanoid receptors that can be coupled to adenylyl cyclase are DP, TP, IP, EP_2 and EP_4 (Ricciotti and FitzGerald, 2011). Prostanoid production can be blocked by inhibition of the COX enzymes (figure 1.5). COX inhibitors include non-steroidal anti-inflammatory drugs (NSAIDs). Both non-selective inhibitors and inhibitors selective to either of the two COX isoforms, COX-1 and COX-2 are available (discussed further in Chapter 5).
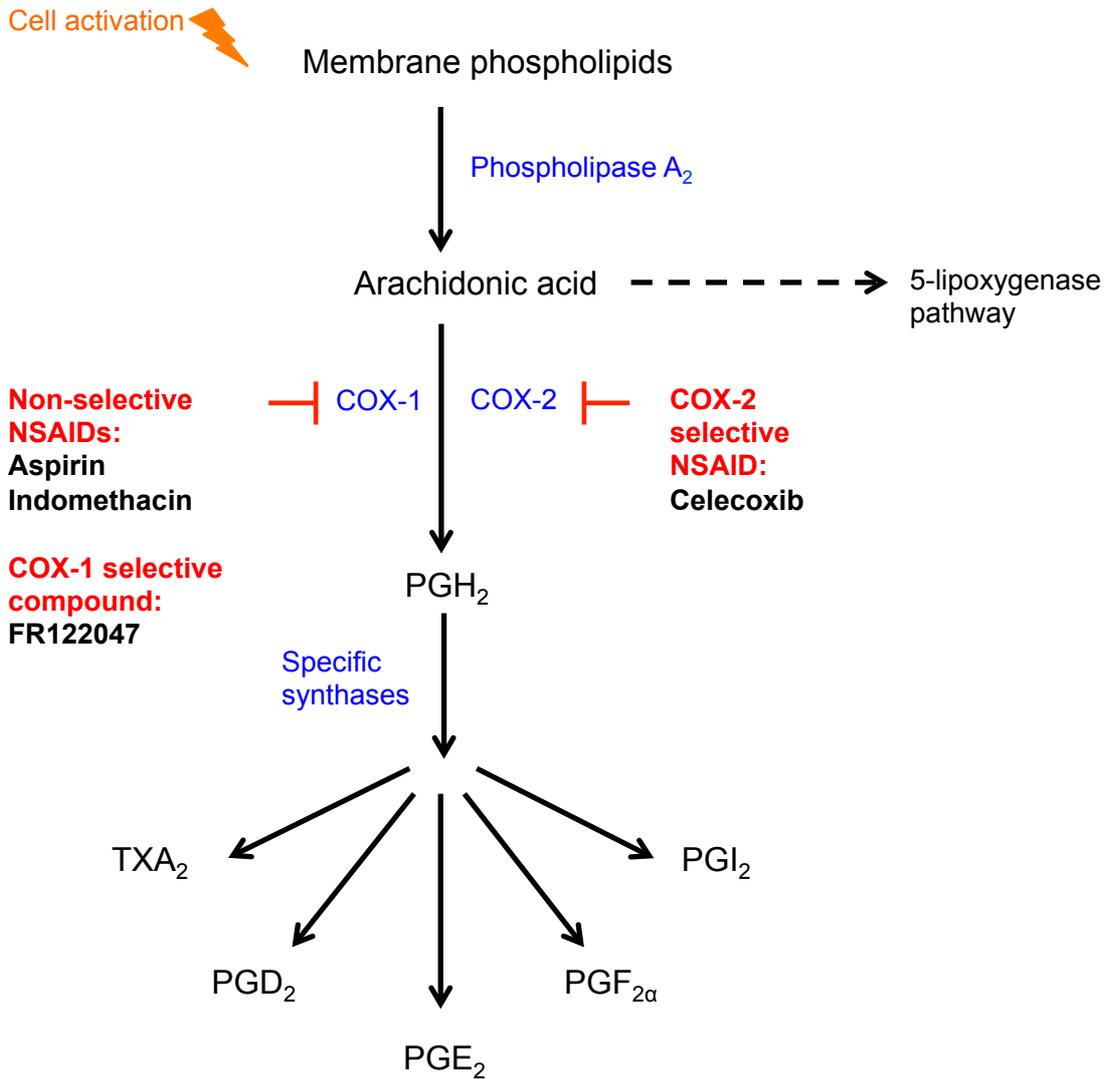
Figure 1.5. Prostanoid generation and inhibition by COX inhibitors.
A simplified figure of the generation of prostanoids from the metabolism of arachidonic acid. Prostanoid generation can be blocked by the inhibition of cyclooxygenases COX-1 and COX-2. (PGH_2: prostaglandin H_2; NSAIDs: non-steroidal anti-inflammatory drugs; TXA_2: thromboxane A_2; PGD_2: prostaglandin D_2; PGE_2: prostaglandin E_2; $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$; PGI_2: prostaglandin I_2) (Peters-Golden et al., 2006, Medeiros et al., 2012).

PGE$_2$ is the most widely found prostanoid in the body and has been shown to be important in the lung. PGE$_2$ has been shown to have bronchodilatory effects but can also induce cough, depending on which of the four possible EP receptors it acts on (Maher et al., 2011). It has been reported that the bronchodilatory effects are mediated either by the EP$_2$ receptor or the EP$_4$ receptor and it has been suggested that targeting the EP$_4$ receptor could be an effective bronchodilator treatment (Buckley et al., 2011, Norel et al., 1999, Benyahia et al., 2012). PGE$_2$ is anti-inflammatory in macrophages, although it is not clear which EP receptor mediates this effect. Investigating the EP receptor(s) that PGE$_2$ acts on in lung macrophages may enable determination of whether targeting EP receptors would be a valid strategy for potential novel anti-inflammatory therapies for respiratory diseases.

Development of more targeted treatments for respiratory diseases may be beneficial. In order to identify potential novel targets, more research is needed into the characteristics and roles of lung macrophages, which are prominent in respiratory diseases. This requirement underpins the rationale for the aims of this thesis. Much of the research into lung macrophages has involved investigating principal roles such as phagocytosis, in relation to inflammation. The role of PGE$_2$ is lesser investigated but as a mediator involved in regulation of inflammation, there is currently interest from the pharmaceutical industry in investigating its effects. There is also interest in the possible use of existing drugs for more than one purpose. As beta-agonists are one of the principal bronchodilatory treatments for respiratory diseases, ascertaining whether they have an additional anti-inflammatory role would be of great benefit in treatments.

1.8. Aims and objectives

1) Isolate macrophages from lung tissue and assess their suitability for use as a model system

2) Investigate the phenotype and functional characteristics of the isolated macrophages

3) Investigate the involvement of PGE_2 in regulation of macrophage function

4) Evaluate the anti-inflammatory effects of beta-agonists in macrophages

Chapter 2: Materials and Methods

2.1. Materials

Acrylamide	Bio-Rad, Hemel Hempstead, UK
Agarose	Melford Laboratories Ltd., Ipswich, UK
Amersham Hybond-ECL Membrane	GE Healthcare, Little Chalfont, UK
Anti-IgE	Stratech, Newmarket, UK
Butaprost (free acid)	Cayman Chemical Company, Ann Arbor, MI, USA
Celecoxib	BioVision Inc., CA, USA
CJ-042794	Pfizer Global R&D, Sandwich, UK
ColorPlus Prestained Protein Ladder, Broad Range (10-230 kDa)	New England Biolabs, Hitchin, Hertfordshire, UK
Complete, EDTA-free Protease Inhibitor Cocktail Tablets	Roche Applied Science, Mannheim, Germany
Cyclic-AMP ACE EIA Kit	Cayman Chemical Company, Ann Arbor, MI, USA
Dexamethasone	Sigma, Poole, UK
Dimethyl Sulphoxide (DMSO)	Sigma, Poole, UK
DNA-free DNase Treatment & Removal	Ambion (Life Technologies), Paisley, UK
DPX Mounting Medium	Fisher Scientific, Loughborough, UK
ECL Western Blotting Detection Reagent	GE Healthcare, Little Chalfont, UK
Ethidium Bromide	Sigma, Poole, UK
Ficoll-Paque Plus	GE Healthcare, Little Chalfont, UK
FcR Blocking Reagent	Miltenyi Biotec, Surrey, UK
Formoterol	Gift from Novartis, Surrey, UK
FR122047 (hydrate)	Cayman Chemical Company, Ann Arbor, MI, USA
Fetal Calf Serum (FCS)	Promocell, Heidelberg, Germany
Gardiquimod	InvivoGen, CA, USA
GoTaq Flexi	Promega, Southampton, UK
Human Ready-Set-Go Cytokine ELISA	eBioscience, Hatfield, UK
HEPES Buffer Solution (1M)	Lonza, Slough, UK

HI NBCS (heat inactivated newborn calf serum)	Gibco (Life Technologies), Paisley, UK
High Capacity cDNA RT Kit	Applied Biosystems, Paisley, UK
Iloprost	Cayman Chemical Company, Ann Arbor, MI, USA
Indacaterol	Gift from Novartis, Surrey, UK
Indomethacin	Sigma, Poole, UK
(-) -Isoprenaline Bitartrate	Sigma, Poole, UK
JW8-IgE	BioServ UK Ltd, Sheffield, UK
Lipopolysaccharide (LPS) from *E. coli* serotype R515 (Re)	Enzo Life Sciences, Exeter, UK
Live/Dead Fixable Dead Cell Stain Kit Near IR	Life Technologies, Paisley, UK
L-161,982	Tocris Bioscience, Bristol, UK
L-902,688	Cayman Chemical Company, Ann Arbor, MI, USA
MinElute Gel Extraction Kit	Qiagen, Crawley, UK
Misoprostol	Cayman Chemical Company, Ann Arbor, MI, USA
Mouse anti-human CD14-PE	Miltenyi Biotec, Surrey, UK
Mouse anti-human CD206-APC	Miltenyi Biotec, Surrey, UK
Mouse IgG2a R-PE Isotype Control	Life Technologies, Paisley, UK
Mouse mIgG1-APC Isotype control	Life Technologies, Paisley, UK
NIP$_{20}$-HSA	Gift from Dr Birgit Helm, University of Sheffield, UK
Quick load 100 bp DNA ladder	New England Biolabs, Hitchin, Hertfordshire, UK
ONO-AE1-259	Ono Pharmaceutical Company Ltd, Osaka, Japan
Penicillin-Streptomycin-Amphotericin B (100X)	Lonza, Slough, UK
Percoll	Sigma, Poole, UK
PF-04418948	Pfizer Global R&D, Sandwich, UK
PF-04852946	Pfizer Global R&D, Sandwich, UK
Prostaglandin E$_2$ EIA Kit - Monoclonal	Cayman Chemical Company, Ann Arbor,

	MI, USA
Prostaglandin E$_2$ (PGE$_2$)	Sigma, Poole, UK
Prostaglandin D$_2$	Biomol, Plymouth Meeting, PA, USA
Prostaglandin F$_{2\alpha}$	Biomol, Plymouth Meeting, PA, USA
Proteome Profiler Human Cytokine	
Array Panel A Kit	R&D Systems, Abingdon, UK
Poly (I:C)-LMW	InvivoGen, CA, USA
Quick-Diff	Reagena, Toivala, Finland
RNase Inhibitor	Applied Biosystems, Paisley, UK
RPMI-1640 medium with L-Glutamine	Lonza, Slough, UK
Salbutamol	Sigma, Poole, UK
Salmeterol	Gift from Glaxo R&D, Stevenage, UK
Sulprostone	Sigma, Poole, UK
Sterile H$_2$O for injections	B.Braun, Sheffield, UK
TaqMan Gene Expression Assay	Applied Biosystems, Paisley, UK
Terbutaline	Sigma, Poole, UK
Trichloroacetic acid	Sigma, Poole, UK
TRI Reagent	Sigma, Poole, UK
Tween-20	Fisher Scientific, Loughborough, UK
U-46619	Biomol, Plymouth Meeting, PA, USA
Whatman™ chromatography paper	GE Healthcare, Little Chalfont, UK

2.2. Buffers and solutions

All chemicals were from Sigma, Poole, UK unless otherwise specified.

10x PIPES:

PIPES (free acid) (7.6 g), NaCl (6.43 g), KCl (0.37 g), made up to 100 ml with ddH$_2$O and to pH 7.4 with NaOH.

1x PBS:

NaCl (8 g), Na$_2$HPO$_4$.7H$_2$O (2.16 g), KCl (200 mg), KH$_2$PO$_4$ (200 mg) (BDH, Poole, UK), made up to 1 L with ddH$_2$O.

Erythrosin-B:

Made to a stock concentration of 0.15% in 1x PIPES, with 5% FCS. Aliquots were frozen at -20 ^{0}C until required.

FACS Buffer:

1x PBS (469 ml), FCS (5 ml), 0.1 M EDTA (25 ml), 10 % Sodium Azide (1 ml), for 500 ml.

50x TAE buffer:

Tris(hydroxymethyl)aminomethane (Tris-base) (242 g) (Fisher Scientific, Loughborough, UK), Glacial acetic acid (57.1 ml) (Fisher Scientific, Loughborough, UK), 0.5 M EDTA (100 ml), made up to 1 L with H_2O.

Protein lysis and Western blotting buffers:

Tris-EDTA buffer:

H_2O (202.5 ml), 1 M Tris-HCL pH 7.4 (5 ml), 1 M NaCl (37.5 ml), 0.5 M EDTA (2.5 ml), 0.5 M EGTA (2.5 ml), for 250 ml.

Tris-EDTA-SDS lysis buffer:

H_2O (72 ml), 1 M Tris-HCl pH 7.4 (2 ml), 1 M NaCl (15 ml), 0.5 M EDTA (1 ml), 0.5 M EGTA (1 ml), 20% SDS (5 ml) (Fisher Scientific, Loughborough, UK), for 96 ml.

2x Sample loading buffer:

20% Sodium Dodecyl Sulphate (SDS) solution (200 μl), 0.5 M Tris-HCl pH 6.8 (125 μl), Glycerol (200 μl) (Fisher Scientific, Loughborough, UK), 0.2% Bromophenol blue

(20 μl), 1 M DTT (100 μl), 25x Protease Inhibitor Cocktail (1 tablet added to 2 ml H_2O) (40 μl), made up to 1 ml with H_2O. Aliquots stored at -20 °C.

10x Running buffer:

Tris(hydroxymethyl)aminomethane (30.3 g), Glycine (190 g), 20% SDS solution (50 ml), made up to 1 L with H_2O.

10x Transfer buffer:

Tris(hydroxymethyl)aminomethane (29 g), Glycine (14.5 g), 20% SDS solution (9.25 ml), made up to 400 ml with H_2O.

1x Transfer buffer:

10x Transfer buffer (100 ml), Methanol (200 ml), H_2O (700 ml).

10x Tris-buffered saline (TBS):

1 M Tris-HCl pH 8.0 (100 ml), Sodium chloride (97.3 g), made up to 1 L with H_2O.

TBS-(0.05%) Tween (TBS-Tween):

As for TBS but with Tween-20 (5 ml) (Fisher Scientific, Loughborough, UK).

5% TBS-milk solution:

Non-fat dry milk powder (5 g) (Bio-Rad, Hemel Hempstead, UK), TBS (100 ml).

2.3 Preparation of compounds

The following compounds were prepared as stock solutions of 10 mM unless otherwise stated. Indomethacin, prostaglandin E_2, misoprostol, L-902,688, prostaglandin $F_{2\alpha}$, U-

46619, prostaglandin D_2, iloprost, FR122047, celecoxib, butaprost (free acid) and sulprostone were prepared in ethanol and stored at -20 °C. L-161,982, PF-04418948, PF-04852946, CJ-042794, formoterol, indacaterol and salmeterol were prepared in dimethyl sulphoxide and stored at 4 °C. Dexamethasone was prepared as a stock solution of 100 mM in dimethyl sulphoxide and stored at 4 °C. ONO-AE1-259 was prepared in distilled H_2O and stored in aliquots at -20 °C. Salbutamol and terbutaline were prepared in distilled H_2O and stored at 4 °C. (-) -isoprenaline bitartrate was prepared in 0.05% sodium metabisulphite (dissolved in 0.9% NaCl) and stored at 4 °C. LPS from *E. coli* serotype R515 (Re) was a ready-to-use stock solution (1 mg/ml) in double distilled pyrogen-free H_2O and was stored in aliquots at 4 °C.

2.4. Human lung tissue

Macroscopically normal resected lung tissue was obtained from adult patients undergoing thoracic surgery mainly for carcinoma, at the Northern General Hospital, Sheffield, UK. The present project, 'Studies on lung cells involved in respiratory diseases', falls within the remit of the Sheffield Lung Tissue Research Bank, for which ethical approval was granted by the National Research Ethics Service (REC ref. 10/H1010/50). All participants gave informed written consent in the form of a completed questionnaire (Appendix II). Participants' smoking history and relevant clinical information (such as allergies) was noted where available. The ratio of male:female participants was approximately 50:50 and the median age was 70 years, with a range of 19-84 years. The percentage of participants that were non-smokers was 22%, the percentage of current smokers was 30% and 48% were ex-smokers.

2.5. Human lung macrophage isolation and culture

2.5.1. Tissue processing

Tissue was manually chopped in RPMI-1640 medium containing 5% fetal calf serum (FCS) and placed on to a 100 μm nylon mesh (Incamesh, Warrington, UK) over a collection vessel. The medium was used to "wash through" the cells from the tissue into the vessel. This was repeated twice, resulting in a final volume of 'wash through' of 100-200 ml in the vessel. This was decanted into 50 ml centrifuge tubes and centrifuged at 300 g x 10 min at room temperature (RT) (MSE Mistral 2000). The supernatant was carefully aspirated and the pellets resuspended in 40-50 ml of RPMI-1640 supplemented with 10% FCS, 1x Penicillin-Streptomycin-Amphotericin B antibiotic solution and HEPES buffer (25 mM). (This medium will be referred to as 'supplemented RPMI-1640' throughout the thesis). After inverting gently several times, the cell suspensions were left to sediment at 4 °C for 1 h. The supernatant was aspirated to remove debris and the sedimented material was resuspended in supplemented RPMI-1640. The sedimentation step at 4 °C was repeated. Alternatively, one of the sedimentations was substituted with an overnight sedimentation at 4 °C. The sedimented material was resuspended in 30 ml 1x PIPES and centrifuged (300 g x 10 min, RT). The resulting pellet was resuspended in 20 ml of 1x PIPES and the suspension was filtered through a 100 μm nylon mesh, before being layered onto a discontinuous Percoll gradient. This protocol was a modification of one described elsewhere (Liu et al., 1984).

2.5.2. Percoll density gradient centrifugation

One 20 ml Percoll gradient was used for cells harvested from every 5 g of tissue (pre-chopped weight). Isotonic Percoll was produced by mixing Percoll:10x PIPES at a 9:1 ratio. This isotonic Percoll was then known as '100% Percoll' and was diluted with 1x PIPES to produce an 80% Percoll gradient. The 20 ml cell suspension was layered onto

the gradient and centrifuged (400 g x 20 min, RT) resulting in a flocculent layer containing macrophages. The layer was transferred to a new 50 ml tube and 2 centrifugations were carried out (488 g x 10 min and 488 g x 7 min, both RT) after resuspensions to 50 ml with 1x PIPES, to wash the cells from the residual Percoll. The resulting cell pellet was resuspended in 10 ml of supplemented RPMI-1640. A haemocytometer cell count was performed using a light microscope (Motic SFC-100FLA). The resulting cells were seeded at 2×10^5 per well in 24-well cell culture plates with 1 ml of supplemented RPMI-1640 or at 1×10^6 per well in 6-well cell culture plates with 5 ml of supplemented RPMI-1640 and incubated overnight (37 °C, 5% CO_2).

2.5.3. Cell purity assessment

The macrophage purity of the cell suspension obtained post-Percoll density gradient centrifugation was determined by cytospin preparations. An aliquot of cell suspension at 1×10^6/ml (90 μl) was pipetted into a cytospin chamber and cytocentrifuged at 400 rpm x 3 min, RT (Thermo Shandon Cytospin 3). The cytospin slides were air-dried before fixation with 100% methanol. The cytospins were stained with Quick-Diff, according to the manufacturer's instructions and air-dried before coverslips were mounted using DPX (di-n-butyl phthalate in xylene) mounting medium. Cytospins were produced in duplicate. Macrophages were counted as a proportion of all immune cells present by morphological assessment using a light microscope (Nikon Eclipse TE300).

2.5.4. Cell viability assessment

Cell viability was assessed pre and post-experiment by erythrosin-B exclusion. To assess viability of cells in the wells, cell culture medium was aspirated and the wells washed once with 1x PBS. Erythrosin-B (stock diluted 1:1 with 1x PBS) was pipetted into each well at a volume of 200 μl and incubated for 2 min at room temperature. The

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erythrosin-B was aspirated and the wells washed once with 1X PBS before visualization using an inverted microscope (Zeiss ID 02). Viability was determined by counting the number of macrophages that had taken up the pink dye (non-viable) as a proportion of the total macrophages present. The average of duplicate wells was calculated.

2.6 Peripheral blood mononuclear cell (PBMC) isolation and culture

2.6.1. Ficoll-Paque isolation from whole blood

PBMC isolation was carried out by Jonathan Kilby (Department of Infection and Immunity). Peripheral blood was obtained from healthy volunteers who gave informed written consent as part of the study entitled 'Investigation of how macrophage responses to micro-organisms programme the innate immune response to human disease', for which ethical approval was granted by the National Research Ethics Service (REC ref. 07/Q2305/7) (Appendix II). Blood (25 ml) was layered onto Ficoll-Paque (12.5 ml) in a 50 ml tube and centrifuged (500 g x 23 min, 18 °C) (MSE Falcon 3/300). The plasma above the resulting layers of cells was discarded. The PBMC layer was transferred to a new 50 ml tube and centrifuged (225 g x 13 min, 4 °C) after resuspension to 50 ml with 1x PBS. The supernatant was discarded. The cells were washed by combining all pellets in one 50 ml tube and resuspending in 1x PBS (to 50 ml) before centrifugation (225 g x 13 min, 4 °C). The resulting cell pellet was resuspended in RPMI-1640 with newborn calf serum (HI NBCS) (10%). A haemocytometer cell count was performed using a light microscope. A cell suspension at 1×10^6/ml was pipetted into 24-well cell culture plates (1 ml/well) and incubated (37 °C, 5% CO_2) for 24 h.

2.6.2. Differentiation into monocyte-derived macrophages (MDM)

After 24 h, the media was aspirated from the 24-well plates to remove any non-adherent cells, which were likely to be cells other than monocytes. The adherent cells were

cultured in supplemented RPMI for 14 days. The media was changed at 7 days or earlier

if it was found to be changing colour due to an increase in cell confluency. At day 14,

approximately 2×10^5 MDMs were present in each well, estimated by scraping and

counting the cells. The cells were used for experiments on day 14.

2.7. Functional studies

Macrophages (2×10^5) in the wells of the 24-well cell culture plates were challenged

with various stimuli. The cell culture medium was changed 2 h before the start of the

experiment, to remove any non-adherent cells and spontaneously released cytokines.

Where pharmacological agents were used, pre-treatment of the cells for 30 min (or 1h

for antagonists) at 37 °C, 5% CO_2 was carried out before addition of stimulants to the

wells. The cells were then incubated for 22 h with the stimulants (37 °C, 5% CO_2). The

cell culture supernatants were then harvested by pipetting the cell culture medium (1

ml) from each well into a 1.5 ml microfuge tube and centrifuging (488 g x 4 min, RT) to

pellet any cells present. The resulting cell-free supernatant was transferred into a new

1.5 ml tube and stored at -80 °C until required for analysis. Investigation of IgE-

dependent responses by passive sensitization was carried out using a different

experimental design, described in schematic figure 2.1.
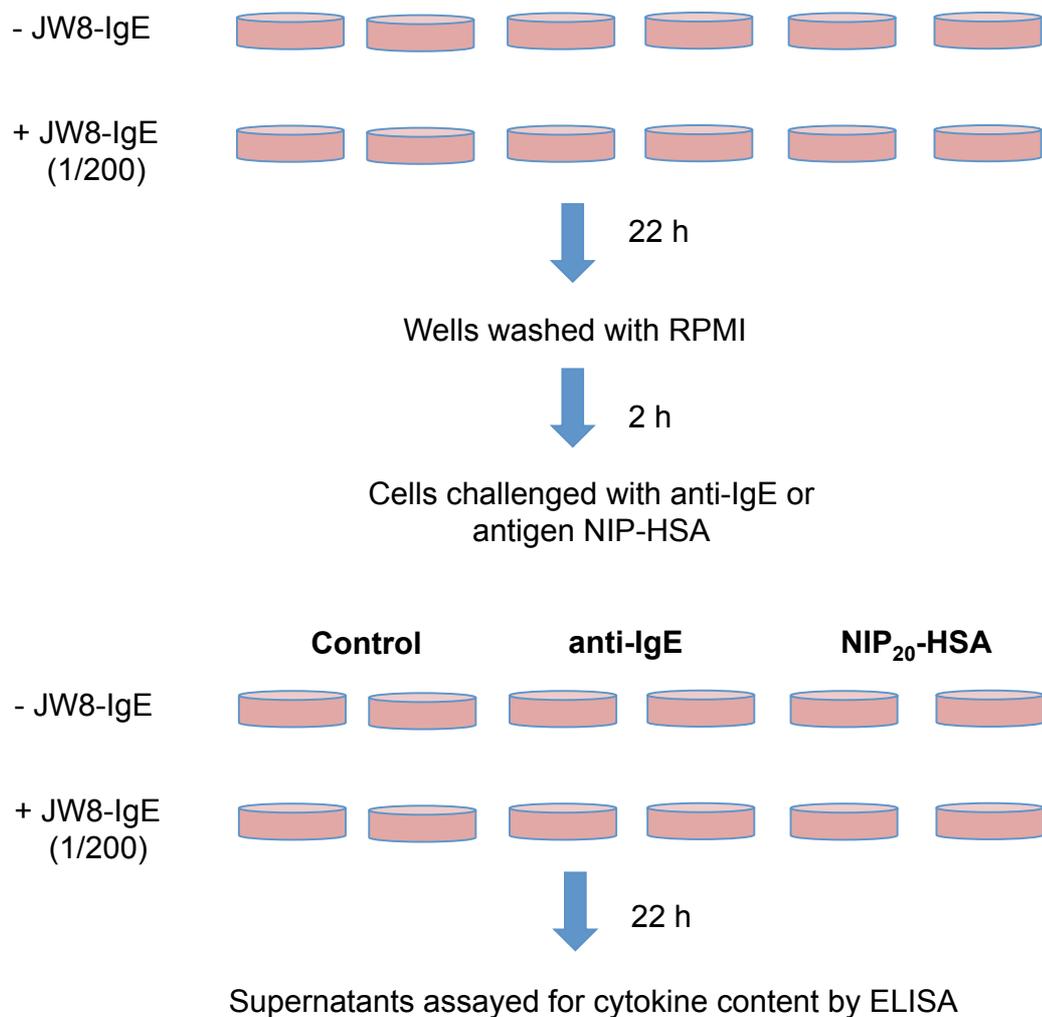
Figure 2.1. Schematic diagram of passive sensitization of macrophages with IgE.
Macrophages were incubated for 22 h without or with JW8-IgE. Wells were washed
twice with supplemented RPMI and then incubated with supplemented RPMI for 2 h.
Cells were then incubated without or with anti-IgE (2 µg/ml) or NIP_{20}-HSA (5-iodo-4-
hydroxy-3-nitrophenacetyl-Human Serum Albumin) (100 ng/ml) for 22 h after which
cell culture supernatants were assayed for TNF-α, IL-4 and IL-17a.

2.8. Cytokine/chemokine release detection

2.8.1. Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels in cell culture supernatants were detected by ELISA. Wells of 96-well high binding plates (Corning Costar 3590) were coated with capture antibody (100 μl/well) and incubated overnight at 4 °C. Wells were then blocked with 200 μl of 1x Assay Diluent for 1 h at RT. Standards and samples were diluted in 1x Assay Diluent before being pipetted onto the plate (100 μl/well). The plate was incubated for 2 h at RT or overnight at 4 °C. The wells were then incubated with detection antibody (100 μl/well) for 1 h at RT, followed by incubation with Avidin-HRP for 30 min at RT. The ELISA was developed with addition of tetramethylbenzidine (TMB) substrate (100 μl/well) for 15 min at RT. The development was stopped with 1 M H_2SO_4 (50 μl/well). The plate was read at 450 nm using a microplate reader (Thermo Multiskan EX).

During incubation steps, the plate was sealed with sealing film (Sigma, Poole, UK). After each incubation step, wells were aspirated and washed 4 times with 1x PBS containing 0.05% Tween-20. All antibodies were used at a 1/250 dilution. The limit of detection of the IL-8, TNF-α and IL-17a ELISAs was 4 pg/ml. For the IL-6, IL-10 and IL-4 ELISAs the limit was 2 pg/ml. Standard curves were produced and the concentrations of the unknowns were determined by interpolation from the curves using GraphPad Prism (figure 2.2). Before each experimental assay was carried out, selected cell culture supernatants of those to be assayed were tested at different dilutions using the above protocol. This enabled a suitable dilution to be determined for assaying the rest of the supernatants, to ensure the cytokine levels of the stimulated samples would be within the range of detection.
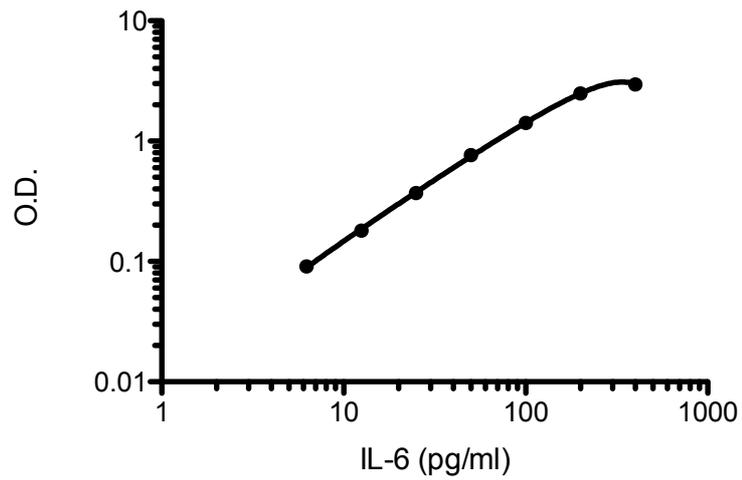
Figure 2.2. Representative cytokine ELISA standard curve.
The above standard curve for an IL-6 ELISA was produced using non-linear regression
and a third order polynomial (cubic) fit. The comfortable range of detection based on
the linear section of the curve was determined as 6.25 - 200 pg/ml. Values outside of
this were excluded as being above or below the range of detection.

2.8.2. Proteome Profiler Array

Identification of a range of cytokines present in cell culture supernatants was carried out using Proteome Profiler Human Cytokine Array Panel A Kit, which consists of nitrocellulose membranes spotted with 36 different capture antibodies and array buffers (of which the composition is unknown). The proteome profiler array was performed according to the manufacturer's instructions. Briefly, each membrane was placed into a separate well of a 4-well rectangular multi-dish and blocked in array buffer for 1 h on a rocking platform at RT. Simultaneously, cell culture supernatants (from $2x10^5$ cells) to be tested ($\leq$ 1 ml) were added to array buffers in separate tubes to obtain a final volume of 1.5 ml. Detection antibody cocktail (15 μl) was added to each diluted sample and the mixtures were incubated at RT for 1 h. Array buffer was aspirated from the wells and the sample-antibody mixtures were pipetted into the relevant wells. The dish was incubated overnight at 2-8 °C on a rocking platform. Each membrane was placed into separate containers and 3 x10 min washes were carried out with 1x wash buffer (20 ml) on a rocking platform at RT. The wells of the 4-well multi-dish were washed with dH_2O and streptavidin-HRP (2 ml) was then pipetted into each well. The membranes were incubated in the streptavidin-HRP for 30 min at RT before being washed as above. The membranes were exposed to enhanced chemiluminescence (ECL) reagents according to the manufacturer's instructions and covered in clingfilm. The membranes were developed using Bio-Rad ChemiDoc XRS+ System, resulting in spots of different densities where cytokines/chemokines were detected. Semi-quantitative analysis was carried out using Image Lab software (version 5.1, Bio-Rad). Mean background-adjusted pixel intensity (or 'density') was calculated.

2.9. Prostaglandin E$_2$ assay

Prostaglandin E$_2$ (PGE$_2$) levels in cell culture supernatants were quantified using a commercially available competitive enzyme immunoassay (Cayman Chemical EIA Kit - Monoclonal). The assay was performed according to the manufacturer's instructions. The absorbance was measured at 405 nm using a microplate reader (Thermo Multiskan EX). The data were analysed as directed by the manufacturer's instructions to provide PGE$_2$ values in pg/ml. The lower detection limit of the assay was 15 pg/ml. Before each experimental assay was carried out, a series of dilutions of selected cell culture supernatants were tested using the above protocol. This enabled a suitable dilution to be determined for assaying the rest of the supernatants, ensuring the PGE$_2$ levels would be within the range of detection.

2.10. Cyclic-AMP assay

Cyclic-AMP (cAMP) levels in cells were determined using a commercially available competitive enzyme immunoassay (Cayman Chemical ACE EIA Kit). Cell culture medium was removed from the wells post-experiment and 1 ml of acidified ethanol (1 ml hydrochloric acid: 99 ml ethanol) per well was added for 5 min. The acidified ethanol was pipetted up and down before being transferred to 1.5 ml microfuge tubes. The samples were stored at -80 °C unless proceeding immediately to the next step. The samples were centrifuged (13,000 rpm, 10 min, 4 °C) and 900 ml of the supernatant was transferred to a new 1.5 ml tube. The tubes were centrifuged with the lids removed, in a rotary evaporator (Eppendorf Concentrator 5301) until all of the ethanol had evaporated. The resulting cAMP-containing pellet/residue was resuspended in 1x EIA buffer (220 μl) and vortexed before being stored at -80 °C until analysis. The assay was then performed according to the manufacturer's instructions. The standards and samples were acetylated before being added to the cAMP-specific IgG coated 96-well plate. The

absorbance was measured at 405 nm using a microplate reader (Thermo Multiskan EX). The data was analysed as directed by the manufacturer's instructions to provide acetylated cAMP values in pmol/ml. This was used to calculate cAMP per million cells. The lower detection limit of the assay was 0.1 pmol/ml. Before each experimental assay was carried out, a series of dilutions of selected cell culture supernatants were tested using the above protocol. This enabled a suitable dilution to be determined for assaying the rest of the supernatants, ensuring the cAMP levels would be within the range of detection.

2.11. Gene expression

EP receptor and COX gene expression was determined by Polymerase Chain Reaction (PCR). RNA was converted to cDNA by Reverse Transcriptase PCR and the cDNA was amplified using EP receptor and COX subtype-specific primers. Expression of a range of macrophage M1 and M2 marker genes was measured by quantitative real-time PCR (qPCR).

2.11.1. RNA extraction and quantification

RNA was extracted from cells ($2\text{-}3 \times 10^6$) by lysis with TRI Reagent® (1 ml). For phase separation of the protein, DNA and RNA, chloroform (0.2 ml) was added. After vigorously shaking, the samples were left to stand for 10 min at RT. The RNA-containing aqueous layer was transferred to a new microfuge tube. Isopropanol (0.5 ml) was added and mixed before leaving the samples to stand for 10 min at RT. The samples were centrifuged (12,000 x g, 10 min, 4 °C). The resulting pellet was washed in 75% ethanol (1 ml) by inverting the tube, after the supernatant was removed using a syringe. The tube was centrifuged at 7500 x g for 5 min at 4 °C. The supernatant was removed using a 21G needle (BD Microlance™ 3) and 5 ml syringe (BD Plastipak™). The pellet was left to air dry by leaving the tube lid open at RT. The pellet was

resuspended in sterile H$_2$O (20 µl) and this RNA solution was stored at -80 °C unless proceeding directly to the next step.

Removal of any DNA present in the extracted RNA was done by DNase I digestion using a commercially available kit (DNA-free DNase Treatment & Removal, Ambion). DNase I buffer (10x) was added to the RNA solution at 0.1x volume along with rDNase I (1 µl) and mixed by pipetting gently. The microfuge tube was incubated at 37 °C for 25 min in a heat block (Techne Dri-Block DB-2A). DNase inactivation reagent was then added (after vortexing) at 0.1x volume to the RNA and the solution was mixed by pipetting. The tube was incubated for 1 min at RT, mixed by pipetting and incubated for a further 1 min before centrifuging (10, 000 x g, 1.5 min, RT). The top, clean layer containing the RNA was transferred to a 0.5 ml microfuge tube. The RNA concentration in ng/µl was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) and the 260/280 nm ratio enabled determination of sample purity. The RNA was stored at -80 °C unless proceeding directly to the next step.

2.11.2. Reverse transcriptase PCR (RT-PCR)

RNA (1 µg) was reverse-transcribed to cDNA. The NanoDrop concentration reading was used to calculate the volume of RNA that was equal to 1 µg and sterile H$_2$0 was added to produce a total volume of 20 µl in a PCR tube. A master mix (MM) of the components required for the PCR reaction was made up as shown in table 2.1 for the number of RNA samples plus a negative control. The MM was added to the RNA (20 µl/RNA sample). A 'no enzyme' negative control was required consisting of RNA (20 µl) and the MM (20 µl) with H$_2$O replacing the RT (reverse transcriptase) enzyme, to ensure any amplification that occurred was not of any genomic DNA in the sample. The reverse transcriptase PCR conditions were 1 cycle at 25 °C (10 min), 37 °C (120 min)

and 85 °C (5 min) followed by holding at 4 °C (∞) (DNA Engine PTC-200 Peltier Thermal Cycler). The resulting cDNA was stored at -20 °C.

Table 2.1. Master mix for reverse transcriptase PCR.

	Volume (μl) per PCR reaction
10X RT buffer	4
25X dNTPs mix (100 nM)	1.6
Multiscribe RT enzyme	2
RNase Inhibitor	2
10X RT random primers	4
H_2O	6.4

Table 2.1 shows the composition of the master mix to be added to RNA for conversion to cDNA by reverse transcriptase PCR. The cycling conditions for the reverse transcriptase PCR reactions are given in section 2.11.2.

2.11.3. Polymerase chain reaction (PCR)

The cDNA was amplified by PCR to determine the gene expression of EP1, EP2, EP3, EP4, COX-1 and COX-2. Expression of β-actin (housekeeping gene) was determined to compare to expression of the genes of interest. The MM used for all PCR reactions is shown in table 2.2. MM (24 µl) and cDNA (1 µl) were added to a PCR tube and overlayed with mineral oil (20 µl) to prevent evaporation (Techne TC-312 Thermal Cycler). Primers were designed by Dr Linda J. Kay and then synthesized by Sigma (Poole, UK). The primer sequences are shown in table 2.3A and PCR conditions are shown in table 2.3B.

Table 2.2. Master mix for standard PCR.

	Volume (µl) per PCR reaction
5X GoTAQ® Flexi Green Buffer	5
dNTPs (10 mM)	0.5
Forward primer (2 µM)	2.5
Reverse primer (2 µM)	2.5
$MgCl_2$ (25 mM)	2
Sterile H_2O	11.25
GoTAQ® Flexi DNA polymerase	0.25

Table 2.2 shows the composition of the master mix for the PCR reactions to amplify cDNA. The sequences of the primers are shown in table 2.3A and the cycling conditions for the PCR reactions are shown in table 2.3B.

Table 2.3A. Primer sequences for standard PCR

Primer pair no.	Gene	Primer sequences in 5'-3' direction	Product size (bp)
1	β-actin	ATATCGCCGCGCTCGTCGTC (Sense) TAGCCGCGCTCGGTGAGGAT (Antisense)	583
2	EP1	ATCATGGTGGTGTCGTGCAT (Sense) TACACCCAAGGGTCCAGGAT (Antisense)	149
3	EP2	CAACCTCATCCGCATGCAC (Sense) CTCAAAGGTCAGCCTG (Antisense)	419
4	EP3	CGCCTCAACCACTCCTACACA (Sense) GCAGACCGACAGCACGCACAT (Antisense)	837
5	EP4	TGGTATGTGGGCTGGCTG (Sense) GAGGACGGTGGCGAGAAT (Antisense)	434
6	COX-1	TGCCCAGCTCCTGGCCCGCCGCTT (Sense) GTGCATCAACACAGGCGCCTCTTC (Antisense)	304
7	COX-2	TTCAAATGAGATTGTGGAAAAATTGCT (Sense) AGATCATCTCTGCCTGAGTATCTT (Antisense)	305

Table 2.3A shows the sequences of the primers used for amplification of cDNA. The cycling conditions for the PCR reaction for each primer pair is given in table 2.3B.

Table 2.3B. Conditions for standard PCR

Primer pair no.	Magnesium concentration	Denaturation	Annealing	Polymerisation	Final elongation
1	1 mM	94 °C 30 sec	60 °C 45 sec	72 °C 35 sec	72 °C 7 min
2, 3, 5	1 mM	95 °C 15 sec	55 °C 30 sec	72 °C 90 sec	72 °C 10 min
4	1 mM	95 °C 15 sec	61 °C 30 sec	72 °C 90 sec	72 °C 10 min
6, 7	1.5 mM	95 °C 45 sec	60 °C 45 sec	72 °C 60 sec	72 °C 10 min

Table 2.3B shows the magnesium ($MgCl_2$) concentration required in the master mix and the thermal cycling conditions for the primers listed in table 2.3A. The number of denaturation, annealing and polymerization cycles was 35 for all of the primers.

2.11.4. Agarose gel electrophoresis

PCR products were visualized on a 2% agarose gel. Agarose (3 g) was added to 1x TAE buffer (150 ml) in a conical flask and heated in a microwave. Ethidium bromide (15 μl) was added when the melted gel was slightly cooled. The gel was cast in a cassette with a well comb inserted. After ~ 1 h the comb was removed from the set gel which was then placed into a gel tank and covered with 1x TAE buffer. Samples (5 μl/well) and a 100 base pair DNA ladder (5 μl) were loaded onto the gel. The gel was run at 100V for 50 min (Bio-Rad PowerPac 300) and the DNA bands were visualized using the Bio-Rad ChemiDoc XRS+ System. Densitometry was performed using Image Lab software (version 5.1, Bio-Rad).

In order to ensure that the correct amplification had taken place, PCR products were cut from the agarose gel and purified using a MinElute Gel Extraction Kit (Qiagen,

Crawley, UK), according to the manufacturer's instructions. Genotypic analysis was performed in-house by automated sequencing (ABI 3730 DNA Analyser; Applied Biosystems, Carlsbad, CA, USA).

2.11.5. Real-time quantitative PCR (qPCR)

Determination of the expression of a range of genes in macrophages incubated without or with a range of stimuli for 22 h was determined using qPCR. RNA was reverse-transcribed to cDNA as described in 2.11.2. A master mix (MM) of the components required for each PCR reaction was made up, consisting of 2x TaqMan Universal Master Mix including AmpErase® UNG (uracil-N-glycosylase) (5 µl), 20x Taqman Gene Expression Assay primer/probe mix (0.5 µl) and RNase-free H_2O (3 µl). The probe consisted of a 6-FAM (6-carboxy fluorescein) fluorescent reporter dye at the 5' end and a non-fluorescent quencher at the 3' end. Where TaqMan Gene Expression Assays were not available, primers and probes were designed by and obtained from Primerdesign (Southampton, UK) or from Sigma-Aldrich (Suffolk, UK). For the primers and probes from Sigma-Aldrich, the MM for each reaction consisted of 2x Universal Master Mix (5 µl), sense primer (0.1 µl), antisense primer (0.1 µl), probe (0.02 µl) and RNase-free H_2O (3.28 µl). Primer and probe sequences are shown in table 2.4.

Table 2.4. Primer/probe sequences for qPCR

Gene	Primer sequences in 5'-3' direction or primer code	Company
β-actin (ACTB)	Hs01060665_g1	Applied Biosystems
NOS2	NOS2_8296 (Primer mix/PerfectProbe)	Primerdesign
ARG	ARG1_14369 (Primer mix/PerfectProbe)	Primerdesign
IL-10	GCTGTCATCGATTCCCTG (sense) TCTATGTAGTTGATGAAGATGTCAAA (Antisense) CACCTGCTCCACGGCCTTGCTCTTCAGGTG (Probe)	Sigma-Aldrich
CD36	CCAAATGAAGAAGAACATAGGACATAC (sense) TGGATTGACCAATAGGTTGAC (Antisense) TGGATTCACTTTACAATTTGCAAAACGGCTGCAGAATCCA (Probe)	Sigma-Aldrich
IL-1β	CCATGTCCTTTGTACAAGGAGAA (Sense) GCAGGACAGGTACAGATTCTTT (Antisense) CCAAGTAATGACAAAATACCTGTGGCCTTGGGATTACTTGG (Probe)	Sigma-Aldrich
MRC-1	AGATGGGTGGGTTATTTACAAAGA (Sense) ATATTTCCATAGAAACTTCTTTTCACTT (Antisense) CACTCGCGCATTGTCCATGGTTTCCTTCTCGAGTG (Probe)	Sigma-Aldrich

Table 2.4 shows the sequences or identification codes for the commercially available and custom made primers and probes used in qPCR, along with the company they were obtained from.

The MM (8.5 μl) was added in triplicate to the wells of a 384-well plate. Each well was loaded with 37.5 ng of cDNA (1.5 μl) resulting in a total volume of 10 μl per well. The plate was sealed with a plate cover and run in a CFX384 Real-Time PCR Detection System (Bio-Rad) according to the thermal cycling conditions in table 2.5. The data was analysed using the comparative Ct method. The expression of each gene of interest was normalized to the expression of the β-actin housekeeping gene in each sample. DeltaDelta Ct values were obtained by subtracting the Delta Ct value of the non-treated control sample from the Delta Ct value of the treated samples. The fold change between the untreated sample and the treated sample was then calculated as $2^{(-DeltaDeltaCt)}$.

Table 2.5. Thermal cycling conditions for qPCR

			PCR (40 cycles)	
	UNG incubation	*Polymerase activation*	*Denaturation*	*Annealing/Extending*
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min

Table 2.5 shows the cycling conditions for the qPCR reactions for the primers and probes given in table 2.4.

2.12. Protein expression

2.12.1 Protein lysis

Protein lysis was carried out using the trichloroacetic acid precipitation method (Wang et al., BioTechniques 1996, **20** p662-668). The following steps were carried out on ice. Macrophages in 6-well plates (1×10^6/well) were washed once with 1x PBS (1 ml/well). The PBS was removed and the wells were washed once in Tris-EDTA buffer (1 ml/well). All of the Tris-EDTA was removed from the wells. Tris-EDTA-SDS lysis buffer (600 µl) with Protease Inhibitor Cocktail added at 1:25 dilution was pipetted into each well and swirled around to lyse the cells. A cell scraper was used to gently scrape the lysate from the edge of the wells. Trichloroacetic acid (6.1 N) was added (100 µl/well) and the plate swirled immediately, to precipitate the protein. The supernatant was pipetted into microfuge tubes, leaving the DNA behind and centrifuged (13,000 rpm, 5 min, 4 °C). The supernatant was discarded and the resulting pellet was washed once in 2.5% trichloroacetic acid in H_2O (500 µl) and centrifuged (13,000 rpm, 5 min, 4 °C). The supernatant was discarded. A short centrifugation was carried out to enable any residual supernatant to be removed. Tris(hydroxymethyl)aminomethane (Tris-base) (3 M) containing Protease Inhibitor Cocktail at 1:25 dilution was pipetted onto each pellet (≤ 40 µl depending on the size of the pellet). The microfuge tubes were incubated for 1 h at RT or overnight at 4 °C to solubilize the protein. A volume of sterile H_2O equal to the volume of Tris-base added before incubation was added to the microfuge tubes and pipetted up and down to mix. The resulting 1.5 M Tris-base solution was pipetted into new microfuge tubes, leaving behind any non-soluble material. The protein lysate was stored at -80 °C until required for analysis.

2.12.2. SDS-PAGE and Western blotting

Protein lysates were reduced by adding equal volume of 2x sample loading buffer and placing in a heat block at 95 °C for 10 min. The molecular weight markers (ColorPlus Prestained Protein Ladder, New England Biolabs) were also heated. The lysates were then centrifuged (13,000 xg, 1 min). Protein separation was performed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Bio-Rad Mini-PROTEAN system. Glass plates were cleaned with 70% industrial methylated spirits before being assembled for gel casting. Resolving and stacking gels were made up as follows:

10% Resolving gel (for 2 gels):

H$_2$O	6.1 ml
1.5 M Tris-HCl pH 8.8	3.75 ml
30% Acrylamide	4.95 ml
10% Sodium Dodecyl Sulphate (SDS)	150 μl
10% Ammonium persulphate (APS)	75 μl
Tetramethyl-ethylenediamine (TEMED)	18 μl

4% Stacking gel (for 2 gels):

H$_2$O	6.1 ml
0.5 M Tris-HCl pH 6.8	2.5 ml
30% Acrylamide	1.3 ml
10% SDS	100 μl
10% APS	100 μl
TEMED	20 μl

APS and TEMED were added immediately before pouring the gel. The resolving gel was poured first and overlaid with isopropanol until set. The isopropanol was decanted before the stacking gel was poured and a well comb inserted. When the stacking gel was set, the comb was removed and the gels were placed into a tank with 1x running buffer. The protein lysate samples and protein ladder were loaded into the wells. The gels were

run at 70 V 15 min until the samples migrated into the resolving gel and run at 150 V for ~1 h until the samples reached the bottom of the gel (Bio-Rad PowerPac Basic).

Wet transfer of the proteins from the gel to a nitrocellulose membrane was carried out, by sandwiching in a transfer cassette. Two sponges and four pieces of filter paper (Whatman™ chromatography paper) were soaked in a tray containing 1x transfer buffer. A transfer cassette was placed into the transfer buffer. A sponge was placed onto the black side, followed by two pieces of filter paper and then the gel (face up). The membrane was placed on top of the gel, followed by two pieces of filter paper and then a sponge. The cassette was closed, ensuring no air bubbles. The cassette was placed into a cassette holder in a transfer tank, with the black side of the cassette facing the black side of the cassette holder. An ice pack was placed into the tank, and the tank was filled with 1x transfer buffer. Transfer was carried out at 100 V for 1 h on a magnetic stirrer.

The membrane was blocked in 5% TBS-milk solution in on a shaker for 1 h at RT before being washed in TBST (2x 10 min). The primary antibody was diluted in 5% TBS-milk (5 ml). The membrane was incubated with the primary antibody in a 50 ml tube overnight at 4 °C on a rolling platform. The membrane was then washed in TBST (2x 10 min) at RT on a rolling platform. The secondary antibody was diluted in 5% TBS-milk (5 ml). The membrane was incubated with the secondary antibody for 1 h at RT on a rolling platform and then washed in TBST (2x 10 min). Antibody concentrations are shown in table 2.6.

Table 2.6. Western blotting antibody concentrations

Primary antibody	Concentration used	Secondary antibody	Concentration used
Anti-actin (Sigma A2066)	1.2 µg/ml	Donkey anti-rabbit polyclonal immunoglobulins-HRP (Dako P0448)	0.8 µg/ml
Anti-COX-1 goat polyclonal IgG (Santa Cruz Biotechnology sc-1752)	0.8 µg/ml	Donkey anti-goat IgG-HRP (Santa Cruz Biotechnology sc-2020)	0.4 µg/ml
Anti-COX-2 goat polyclonal IgG (Santa Cruz Biotechnology sc-1745)	0.8 µg/ml	Donkey anti-goat IgG-HRP (Santa Cruz Biotechnology sc-2020)	0.4 µg/ml

Table 2.6 shows the primary and the corresponding secondary antibodies used for Western blotting. The concentrations that the antibodies were used at and the companies that the antibodies were obtained from are also given.

Protein detection was carried out using ECL reagent. Reagent A was mixed with reagent B at 1:1. The membrane was placed onto clingfilm and covered with the ECL reagent for 1 min. Excess ECL reagent was removed from the membrane before placing between two pieces of plastic, ensuring no air bubbles were present. The membrane was developed using Bio-Rad ChemiDoc XRS+ System and densitometry was performed using Image Lab software (version 5.1, Bio-Rad).

2.12.3. Membrane stripping and re-probing:

To prepare for re-probing, the membrane was stripped. The membrane was incubated as follows: in H_2O (10 min), in 0.2 M NaOH (15 min), in H_2O (10 min), in TBS (15 min). The membrane was then blocked in 5% TBS-milk (1 h), followed by incubation with primary and secondary antibodies as previously described in 2.12.2.

2.13. Flow cytometry

Flow cytometry was performed on an LSRII flow cytometer (BD Biosciences). Cell populations were identified using forward and side-scatter characteristics in addition to positive staining with antibodies to CD14 (present on monocytes and macrophages) and CD206 (present on macrophages). The antibodies used were CD14-PE (clone TÜK4, Miltenyi Biotec) and CD206-APC (clone DCN228, Miltenyi Biotec). The corresponding isotype controls used were mIgG2a R-PE (Life Technologies) and mIgG1-APC respectively (Life Technologies). A total of 10,000 events were captured.

Positive staining with Live/Dead Fixable Dead Cell Stain Kit Near IR (Life Technologies) was used to eliminate dead cells from the analysis. Doublet discrimination was carried out to ensure only single cells were included in the analysis. Mean fluorescence intensity values were calculated by subtracting the geometric mean

fluorescence values for isotype control staining from the geometric mean fluorescence values for antibody staining. All analysis was performed using FlowJo® software (version 9.3.3, Tree Star, Inc.).

2.13.1. Cell-specific staining

The cell suspension obtained after Percoll gradient centrifugation was plated into 6 well cell culture plates at $1x10^6$ cells/well. The cells were incubated overnight (37 °C, 5% CO_2) and the cell culture medium was changed the next day. Cells were scraped from the wells using a rubber cell scraper (Sarstedt) in the presence of cell culture medium. The cell culture medium containing the cells was pipetted into a 50 ml centrifuge tube and centrifuged (488 g x 5 min, RT). The resulting pellet was resuspended in 1 ml of FACS buffer and a haemocytometer count was carried out. The cell suspension was adjusted as necessary with FACS buffer and pipetted equally into microfuge tubes (ideally $1x10^6$ cells/tube). The tubes were centrifuged (488 g x 5 min, RT). The resulting pellets (except in the tube to be left unstained) were resuspended in FcR Blocking Reagent (at 1:5 dilution in 100 μl final volume of FACS Buffer) for 15 min at 4 °C. Washes (2 x) were carried out in FACS buffer by resuspending in 750 μl and centrifuging (488 g x 5 min, RT). The pellets were resuspended in their respective antibody mixtures (antibodies at a 1:10 dilution in 100 μl final volume of FACS buffer) and the live/dead stain was added (1 μl for no more than $1x10^6$ cells) to each tube. The pellet to be left unstained was resuspended in 100 μl of FACS buffer alone. The tubes were incubated for 30 min on ice, in the dark. Washes (2 x) were carried out before resuspension of the pellets in 500 μl of FACS buffer and pipetting into glass sample tubes for the flow cytometer.

Compensation controls were produced using BD™ CompBeads Anti-Mouse Ig, κ /Negative Control (FBS) Compensation particles set according to the manufacturer's instructions. FACS buffer (100 μl) was added to each sample tube (one tube for each fluorochrome used and one negative control tube). The BD™ CompBeads were vortexed before use. One drop of BD™ CompBeads negative control (has no binding capacity) was added to each tube and one drop of BD™ CompBeads Anti-Mouse Ig, κ beads (binds any mouse κ light chain-bearing Ig) was added to each tube except the negative control tube. Antibodies conjugated to each fluorochrome used in the experiment were diluted 10x in FACS buffer and of the diluted antibodies (20 μl) were added to their respective tubes. Following vortexing, the tubes were incubated in the dark for 30 min. FACS buffer (2 ml) was then added to each tube, followed by centrifugation (488 g x 5 min) to wash. The supernatant was decanted and the beads were resuspended in FACS buffer (350 μl) before running on the flow cytometer to set compensation values.

The positive control for live/dead discrimination was produced by heating an aliquot of cells to 65 °C for 1 min immediately followed by incubating on ice for 1 min, before combining 1:1 with non-treated cells. This mixture of live and dead cells was then stained with the live/dead stain.

2.14. Data analysis and statistics

Data were displayed as mean ± standard error of the mean (SEM). Antagonist affinity (pK_B) was calculated using the Gaddum equation: $pK_B = \log(\text{dose ratio} - 1) - \log(\text{antagonist concentration})$. Statistical analyses involved one-way ANOVA or paired t test followed by Tukey's or Dunnett's Multiple Comparison test to determine the

statistical significance. Significant values were defined as $p<0.05$. Statistical tests were

performed using GraphPad Prism Version 6.0c (GraphPad Software, Inc.).

Chapter 3: Preliminary characterization of human lung macrophage phenotype

3.1. Introduction

Macrophages are believed to be implicated in inflammatory lung diseases, which makes them the focus of many investigations into this area (Barnes, 2008b, Pons et al., 2005, Moreira and Hogaboam, 2011). However, owing to the lack of availability or accessibility of tissue, much of the research into lung diseases is carried out using substitutes for human lung macrophages, such as monocytic cell lines or monocyte-derived macrophages (MDMs) (Daigneault et al., 2010). Whilst there are a number of advantages to using these alternatives, it is possible that human lung macrophages may provide a more accurate representation of what occurs *in vivo*. This formed the premise for this study, which comprised a new initiative in our laboratory to isolate human lung macrophages for use as a model system. This chapter outlines the initial attempts at isolating macrophages, followed by preliminary functional and phenotypical characterization to enable validation of their use for investigations into potential roles in inflammatory lung diseases.

Macrophages exhibit phenotypic heterogeneity and can be broadly classified as having an M1 (classically activated) or M2 (alternatively activated) phenotype (Mosser and Edwards, 2008). A good indicator of the phenotype is the cytokines that are released in response to activation by different stimuli. M1 macrophages are associated with release of pro-inflammatory cytokines (and low levels of anti-inflammatory cytokines) whereas M2 macrophages are associated with release of anti-inflammatory cytokines (Mosser and Edwards, 2008, Mantovani et al., 2007). In this study, the profile of cytokine

release from macrophages was determined by ELISA and Proteome Profiler Array following activation by various stimuli.

A range of TLR (toll-like receptor) agonists was employed in this study. The gram-negative bacterial cell wall component LPS (TLR4 agonist) and the synthetic triacylated lipoprotein Pam3CSK4 (TLR2 agonist) are both mimics for bacterial infection. Mimics for viral infection that were used were the double-stranded RNA analog poly(I:C) (TLR3 agonist) and the imidazoquinoline compound, gardiquimod (TLR7(/8) agonist). The macrophage response to infections *in vivo* is believed to contribute to exacerbations of chronic lung diseases (Barnes, 2008a, Berenson et al., 2014). Therefore, in addition to investigating phenotypes, TLR agonists were also useful for observing whether the cytokines released in response to bacterial mimics were different to those released in response to viral mimics.

In addition to assessment of cytokine release, expression of cell surface markers can indicate the phenotype of macrophages. Therefore experiments were carried out investigating the expression of M1 and M2 macrophage markers by flow cytometry. Another approach to investigate markers associated with a particular phenotype is to explore gene expression. A preliminary qPCR experiment was carried out to determine whether this technique could assist in phenotyping.

In addition to the preliminary characterization of the function and phenotype of the isolated macrophages, some comparative studies were also performed with the commonly used alternative cells, MDMs. This enabled investigation into whether there were any major differences in functional responses and phenotype between the two model systems.

3.2. Results

3.2.1. Isolation of macrophages from human lung tissue

Macrophages were isolated from lung tissue according to the methods described in section 2.5.1 and 2.5.2. These methods were a result of modification of the initial protocol used (Liu et al., 1984). It was discovered during early attempts to use the protocol, that there were various issues that needed to be addressed in order to adequately isolate macrophages from the 'wash through' from the chopped lung tissue.

Initially, the 'wash through' was centrifuged and the pelleted material was resuspended in supplemented RPMI-1640. This mixed cell suspension was left to sediment overnight at 4 °C in 50 ml centrifuge tubes. For comparison, some was pipetted into 6-well cell culture plates to be kept in the incubator at 37 °C overnight. In order to make a valid comparison, the cells from the two conditions were then counted using a haemocytometer and seeded into 6-well plates at the same density of 1×10^6 cells per well. (To count the cells that had been kept in plates in the incubator, the cells had to be scraped from the wells). The cells were incubated for 1 h at 37 °C before the supplemented RPMI-1640 was changed, to wash the wells to remove non-adherent cells. The cells were incubated for a further 1 h at 37 °C. This was repeated for a total of three washes and then both sets of plates were examined using an inverted microscope. The cells from the 'wash through' that had initially undergone sedimentation at 4 °C appeared to look healthier than those from the 'wash through' that had been immediately incubated at 37 °C. This test was carried out twice before it was decided that for subsequent cell isolations, sedimentations of the 'wash through' would be performed at 4 °C. An issue that was found to be common between both sets of plates was that there were a large number of erythrocytes present, in addition to many other cells that were unlikely to be macrophages based on their size, as visualized with an

inverted microscope. In order to minimize the presence of these cells, utilizing a Percoll gradient was attempted as a possible solution.

Incorporating a Percoll gradient step into the isolation procedure greatly minimized the presence of the contaminating cells. Resulting cells were seeded at a range of densities and it was determined after two tests that the most suitable density for 6-well plates was 1×10^6 cells per well and for 24-well plates, the most suitable density was 2×10^5 cells per well, based on the confluency in the wells. This coincided with the density used by monocyte-derived macrophage researchers within the department, providing further support for this decision. The 'wash through' contained debris, which interfered with cell separation on the Percoll gradient. In particular, tissue from patients who were smokers often contained large amounts of black particles. After two isolations performed using a Percoll gradient, it was determined that it was necessary to incorporate a filtering step before layering the cells onto the gradient, to minimize the effects of this issue.

As the cells were isolated using a Percoll gradient alone, it was possible that there would still be some contaminating cells present. To determine the macrophage purity, cytospins of the cell suspension that was plated into cell culture wells were produced after every cell isolation, as described in section 2.5.3. As the cell culture medium was changed before starting each experiment, it was also necessary to check the purity in the wells after this 'wash', as it was believed that some non-adherent contaminating cells would be removed. As macrophages adhere to the wells, the wash would potentially increase the macrophage purity. Although not done routinely, this was achieved by placing coverslips into two of the wells before seeding. The coverslips were removed at the time of starting an experiment then fixed and stained as in section 2.4.3 before being

mounted onto microscope slides cell side up with DPX to seal the edges. Comparison of cytospins and coverslips from the same cell preparation revealed that the cell counts were the same or very similar with both showing a high level of macrophage purity (figure 3.1). The macrophage purity was routinely 80-97 % and contaminating cells were monocytes, neutrophils and lymphocytes.

To check that the differential cell counts that were routinely performed were not being influenced by subjectivity, a selection of cytospins were blind counted by senior histopathologist Dr SK Suvarna from the Royal Hallamshire Hospital, Sheffield. The resulting assessments of macrophage purity were comparable to our assessments, providing further confidence in the method used for determination of macrophage purity. Cell preparations with a low level of macrophage purity were either discarded or used only for pilot experiments. Cell preparations were mostly of good macrophage purity and very few preparations were of low purity, represented by the images of cytospins of cell suspensions from the final protocol shown in figure 3.2.

It was necessary to determine the viability of the cells, before starting the experiments and after incubation with agents. Viability was determined using the method described in section 2.5.4. As macrophages are adherent to cell culture wells, this method was a modification of the method generally used to test the viability of cells that are in suspension. Initially, attempts were made to scrape cells or use EDTA to remove cells from the wells, to enable viability to be tested of cells in a suspension. However, the cell recovery from the wells was low, making this an unreliable method, as the cells in suspension may not have been representative of all the cells in the wells. Adapting the method to enable erythrosin-B to be added directly to the wells enabled routine, reliable checking of viability. Viability pre- and post-experiment was routinely 85-95 %.

A
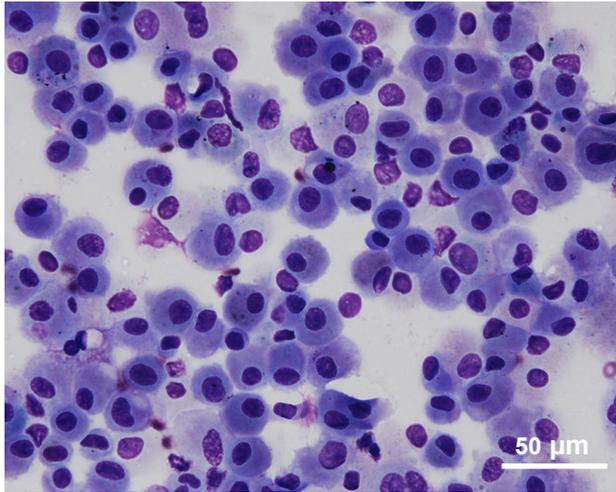
Cytospin

94% macrophages

B
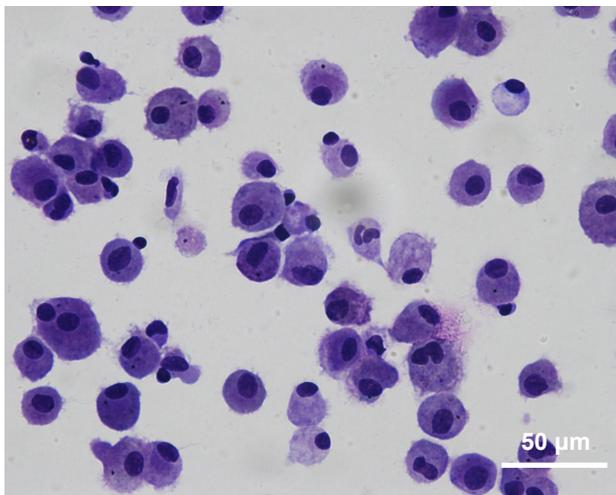
Coverslip

94% macrophages

Figure 3.1. Determination of macrophage purity.
Representative (A) cytospin (1×10^6 cells/ml) and (B) coverslip (2×10^5 cells/well) of macrophages from the same lung preparation, stained with Quick-Diff. Macrophage purity was determined by differential cell counts. The mean cell counts of duplicate cytospins and coverslips were calculated.
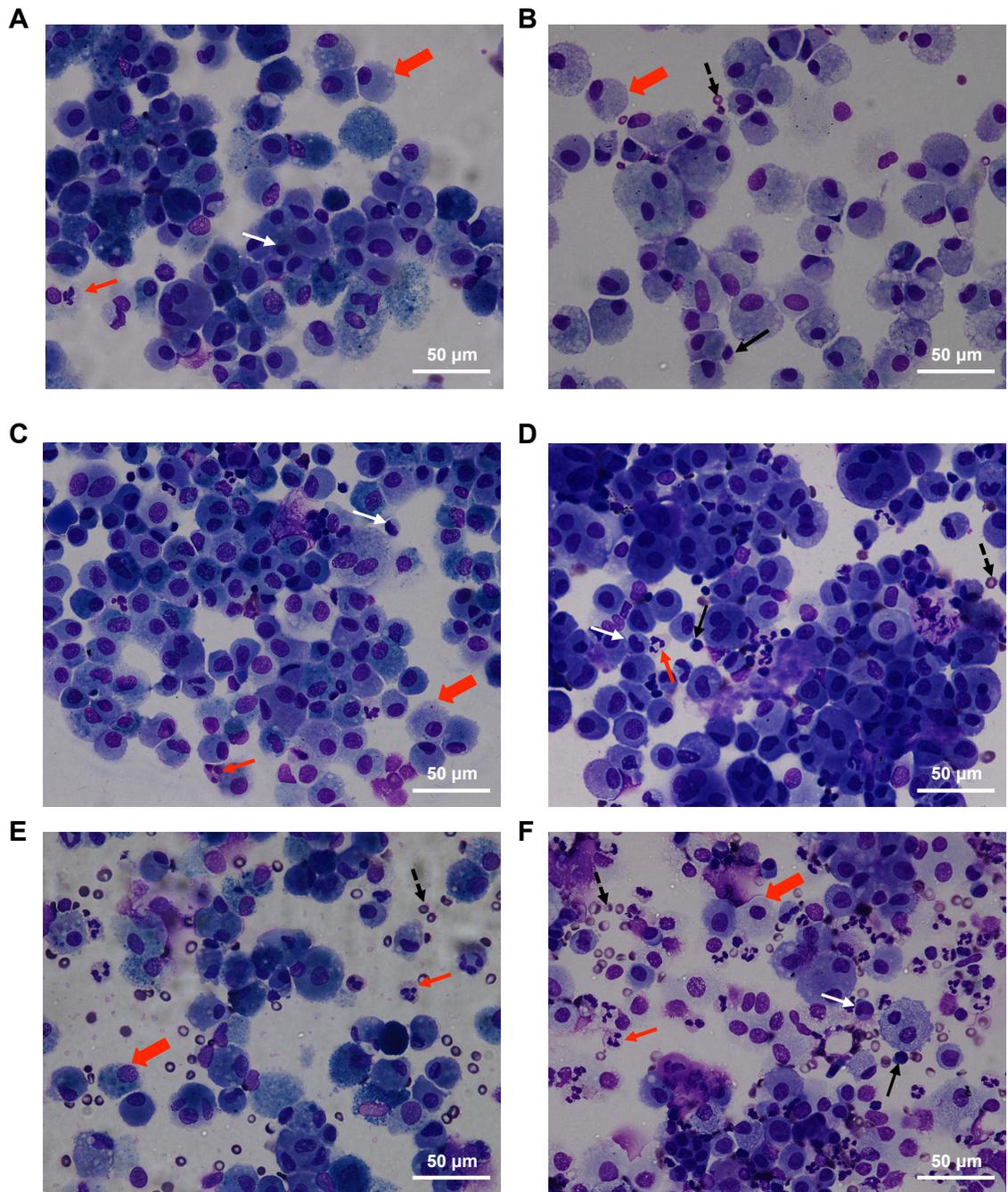
Figure 3.2. Representative cytospins of macrophage preparations.
Representative cytospins (1×10^6 cells/ml) of cells from six different lung preparations showing macrophage purities of (A) 99%, (B) 99%, (C) 92%, (D) 86%, (E) 81% and (F) 64%. Cytospins were stained with Quick-Diff. Macrophage purity was determined by differential cell counts. The mean cell counts of duplicate cytospins were calculated. Macrophage (red block arrow), neutrophil (red arrow), monocyte (white arrow), lymphocyte (black arrow), erythrocyte (dashed black arrow).

An average of two million macrophages were obtained from one gram of lung tissue. It was important to isolate the cells as soon as possible after processing the tissue. Some cell preparations contained large numbers of erythrocytes and it was found that these ideally required longer sedimentation steps before being isolated using a Percoll gradient. It was therefore possible that some cell loss occurred in those situations where the isolation procedure was started before the second sedimentation was optimally complete. This could have resulted in a less than maximum yield on occasions. Information on the lung preparations carried out in this project is provided in Appendix III.

3.2.2. Cytokine release from macrophages in response to LPS challenge

Macrophages were challenged with LPS (10 ng/ml) for 22 h, after which the release of the pro-inflammatory cytokines TNF-α, IL-6, IL-8 (figure 3.3A, B, D) and the anti-inflammatory cytokine IL-10 (figure 3.3C) into the cell culture media was measured. LPS challenge caused a significant ($p < 0.05$) increase in the release of all four cytokines compared to spontaneous cytokine release. However, quantitative differences in the extent of cytokine generation were observed. The extent of IL-8 generation was greatest (40,937±3877 pg/ml) and substantial levels of both IL-6 (7344±1534 pg/ml) and TNF-α (4558±1075 pg/ml) were generated whereas IL-10 generation was considerably lower (108±34 pg/ml). In the absence of LPS, spontaneous release of TNF-α, IL-6 and IL-10 was negligible whereas considerable levels of spontaneous IL-8 release were detected (5158±1377 pg/ml) (figure 3.3D).

Although a high release of IL-8 from lung macrophages has been reported in other studies, there was a concern that the cells were being activated spontaneously by the culture conditions. Low levels of endotoxin can be present in some types of FCS. To

investigate the possibility of this having an effect, macrophages were cultured in media containing different types of FCS for 22 h, without and with LPS challenge. As cells were routinely cultured in media containing 10% FCS from Promocell, the IL-8 release from these cells was compared to that from cells cultured under other conditions. Ultra low endotoxin FCS (10%) from Autogen Bioclear or a lower concentration of Promocell FCS (5%) resulted in no significant ($p>0.05$) difference in IL-8 release compared to 10% Promocell FCS. Only the absence of FCS resulted in a significant ($p<0.05$) 46% decrease in spontaneous IL-8 (figure 3.4A) and 70% decrease in LPS-induced IL-8 (figure 3.4B). Moreover in the absence of any FCS, cells exhibited lower viability (64% viable) than those cultured in media containing FCS (84-90% viable), as assessed by erythrosin-B exclusion.
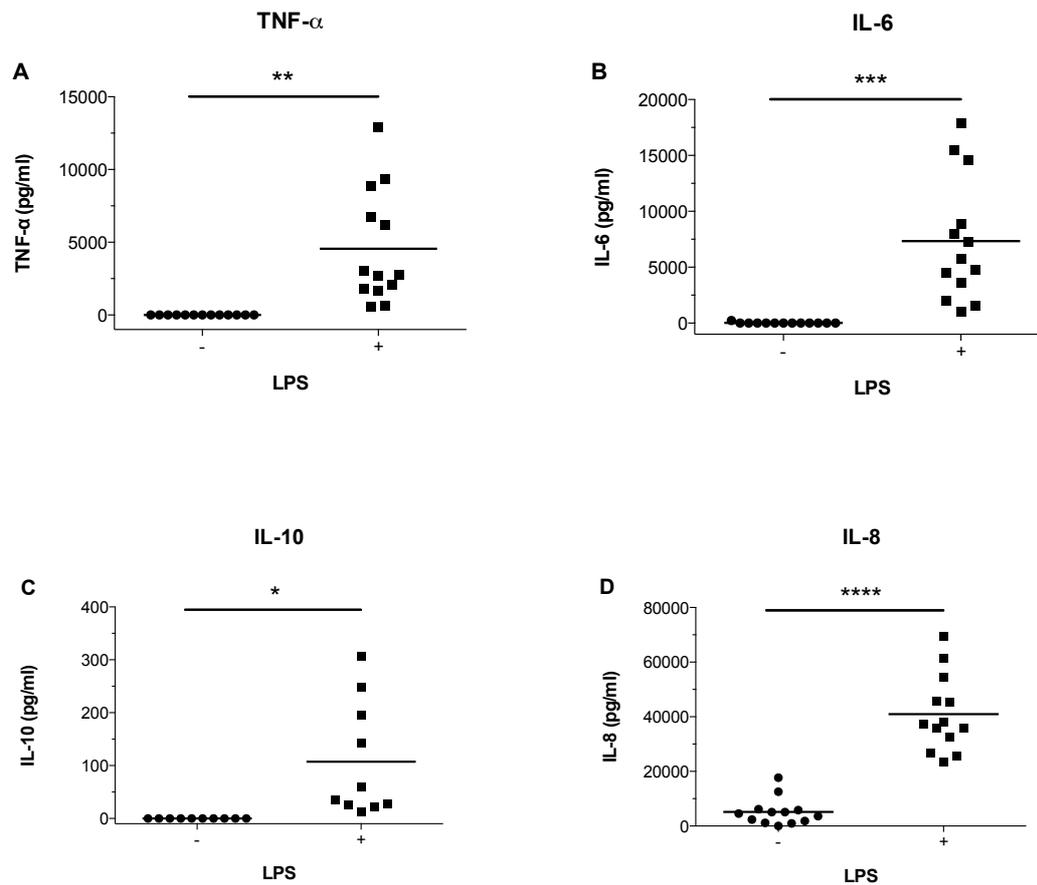
Figure 3.3. Cytokine release from LPS-stimulated macrophages. Macrophages were incubated without or with LPS (10 ng/ml). After 22 h, cell culture supernatants were harvested and assayed by ELISA for (A) TNF-α, (B) IL-6, (C) IL-10 and (D) IL-8. Solid horizontal lines represent the mean for 13 (TNF-α, IL-6, IL-8) and 10 (IL-10) individual experiments. (Paired t test between spontaneous and LPS-challenged, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

A Spontaneous

B LPS Challenge
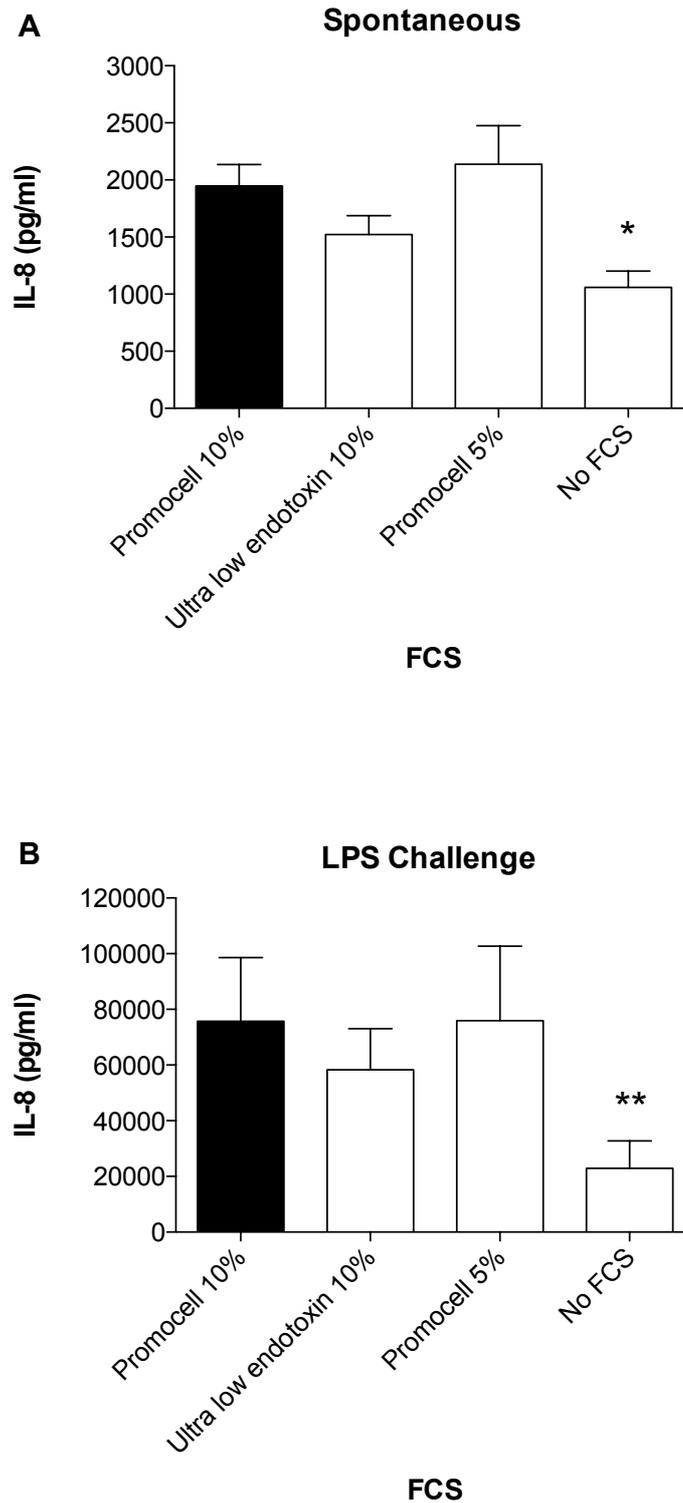
Figure 3.4. Comparison of the effect of different FCS conditions on IL-8 release from macrophages. Macrophages were cultured in RPMI containing different types and concentrations of FCS without (A) or with (B) LPS (10 ng/ml). After 22 h, cell culture supernatants were assayed for IL-8. Values are mean ± SEM for 4 individual experiments. (One-way ANOVA with Dunnett's post test, *p<0.05, **p<0.01, compared with 10% Promocell FCS).

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3.2.3. Concentration and time dependency of LPS-induced cytokine release

Macrophages were challenged with a range of LPS concentrations for 22 h after which the levels of TNF-α were measured. The lowest concentration of LPS (0.1 ng/ml) did not result in a significant ($p > 0.05$) increase in TNF-α release relative to spontaneous release. Higher concentrations of LPS (1-100 ng/ml) induced significant ($p < 0.05$) increases in TNF-α release. Although 100 ng/ml of LPS resulted in a significant release of TNF-α, the level was lower than that generated by 10 ng/ml (figure 3.5).

To investigate the release of pro-inflammatory cytokines in response to LPS over time, macrophages were challenged with LPS (10 ng/ml) for 2, 5, 16 and 22 h. The levels of TNF-α, IL-6 and IL-8 were measured. Although some variation in the levels of cytokines released at each time point was seen between individual experiments, it was possible to identify an overall pattern of release by observing the mean data. The release of TNF-α (figure 3.6A) increased up to 16 h and then decreased slightly at 22 h. A similar pattern of release overall was seen for IL-6 (figure 3.6B). By contrast, IL-8 release (figure 3.6C) continued to increase over 22 h. Spontaneous time-dependent IL-8 release was also detected. This was in contrast to the other cytokines, for which the spontaneous release was below the level of detection.
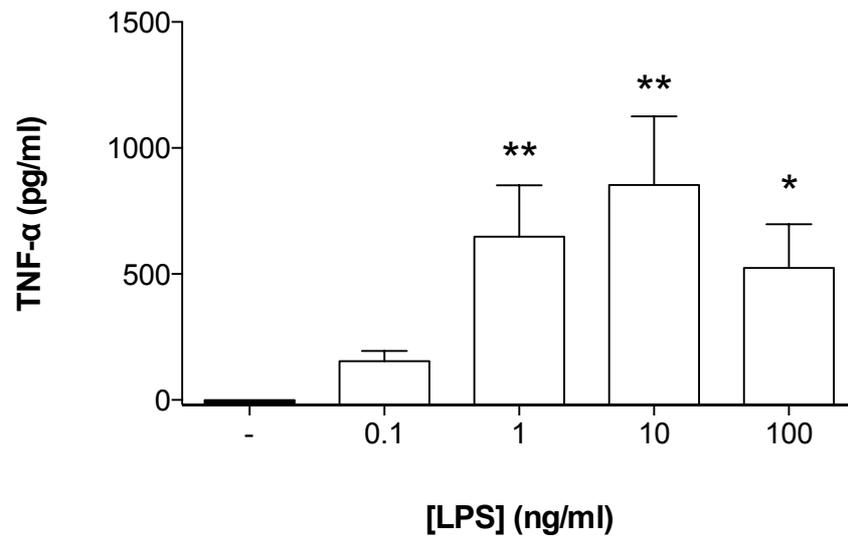
Figure 3.5. Effect of a range of concentrations of LPS on TNF-α release.
Macrophages were incubated without or with LPS (0.1 - 100 ng/ml). After 22 h, cell
culture supernatants were harvested and assayed by ELISA for TNF-α. Values are mean
± SEM for 4 individual experiments. (One-way ANOVA with Dunnett's post test,
*p<0.05, **p<0.01, compared with spontaneous release).
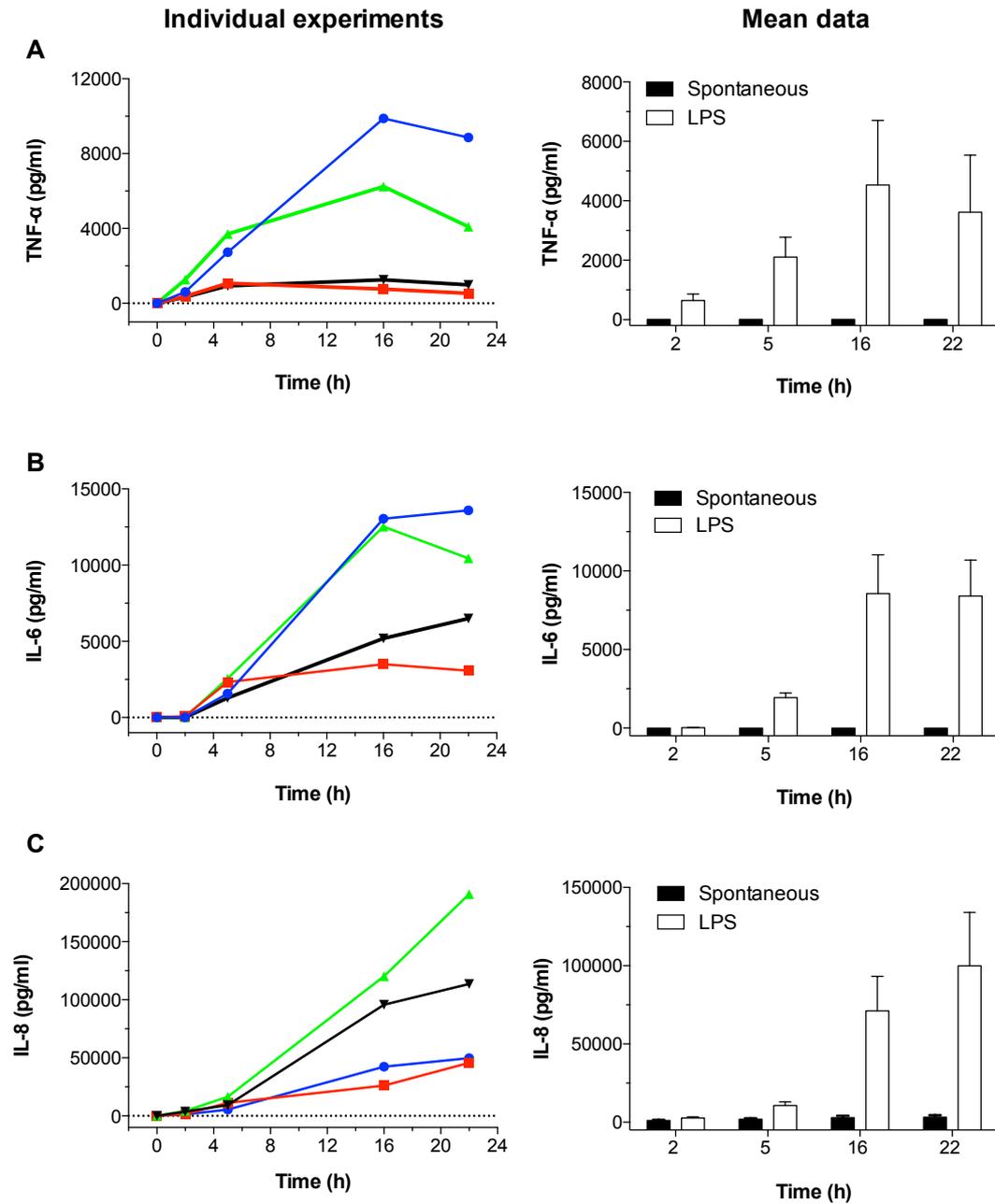
Figure 3.6. Time-dependent LPS-induced cytokine release.
Macrophages were incubated without or with LPS (10 ng/ml). At 2, 5, 16 and 22 h, cell culture supernatants were harvested and assayed for (A) TNF-α, (B) IL-6 and (C) IL-8. Data shown are of 4 individual experiments (left panels) alongside the mean ± SEM data (right panels). Spontaneous cytokine release was below the level of detection except for IL-8, for which the individual experiment values shown are net values (gross IL-8 − spontaneous IL-8).

3.2.4. Cytokine release induced by TLR agonists

To determine if TLR agonists other than LPS induced TNF-α release, the TLR3 agonist poly(I:C) (0.1-10 μg/ml) and the TLR7 agonist gardiquimod (0.1-10 μg/ml) were used to challenge macrophages in addition to LPS (0.1-10 ng/ml) for 22 h. Only the highest concentration of LPS (10 ng/ml) or poly(I:C) (10 μg/ml) induced a significant ($p<0.05$) increase in TNF-α release relative to spontaneous release (figure 3.7). The only concentration of gardiquimod that induced TNF-α release was 10 μg/ml but the increase in TNF-α relative to spontaneous release was not significant ($p>0.05$).
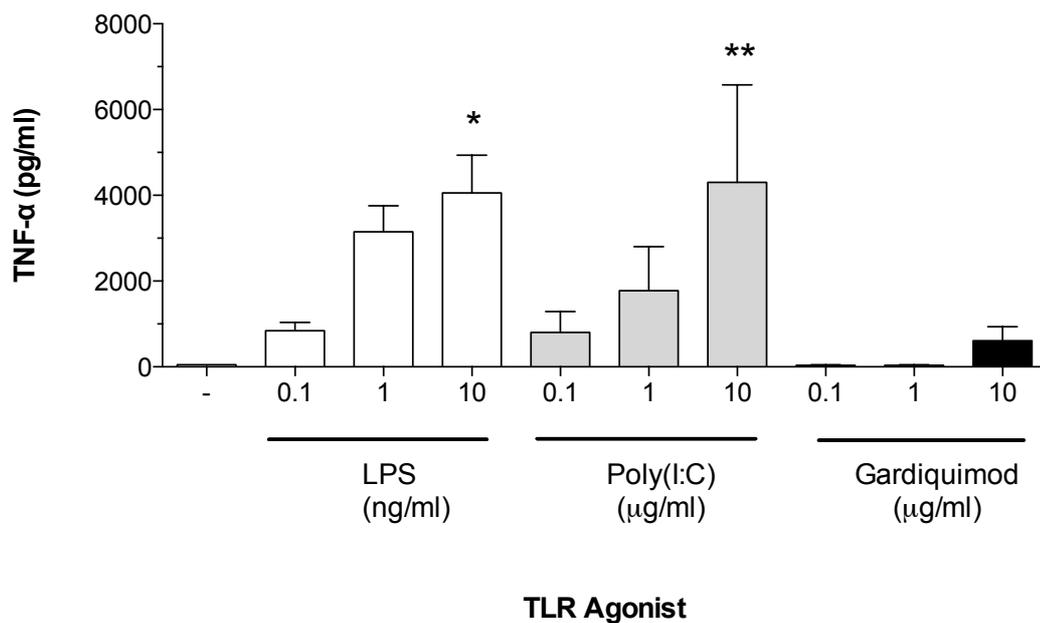
Figure 3.7. Effect of LPS, poly(I:C) and gardiquimod on TNF-α release.
Macrophages were incubated without or with LPS, poly(I:C) or gardiquimod. After 22 h, cell culture supernatants were harvested and assayed for TNF-α. Values are mean ± SEM for 4 individual experiments. (One-way ANOVA with Dunnett's post test, *$p<0.05$, **$p<0.01$, compared with spontaneous release).

Macrophages were challenged with the TLR1/2 agonist Pam3CSK4 (100 ng/ml) or LPS (10 ng/ml) for 22 h for comparison of the release of TNF-α. The Pam3CSK4-induced TNF-α was below the level of detection (figure 3.8A). To test whether a higher concentration of Pam3CSK4 was required to elicit a response, 200 ng/ml was used to challenge cells, alone or combined with LPS. Pam3CSK4 alone did not elicit release of TNF-α (figure 3.8B) and did not enhance LPS-induced TNF-α release.

Macrophages were challenged with LPS (10 ng/ml), poly(I:C) (10 µg/ml) or gardiquimod (10 µg/ml) for 22 h. TNF-α, IL-6, IL-8 and IL-10 release from five corresponding cell preparations was measured. Poly(I:C) and gardiquimod challenge, in addition to LPS challenge, resulted in the release of all four cytokines (figure 3.9A-D). Overall, the levels of cytokine release induced by both poly(I:C) and gardiquimod were lower by comparison than those induced by LPS. However, there was some variation in the cytokine release by different cell preparations, in response to the different TLR agonists. In particular, a higher level of IL-8 release was detected from three of the cell preparations, in response to gardiquimod compared to LPS (figure 3.9C). The levels of IL-10 induced by all of three TLR agonists were considerably lower than the levels of the other cytokines measured.
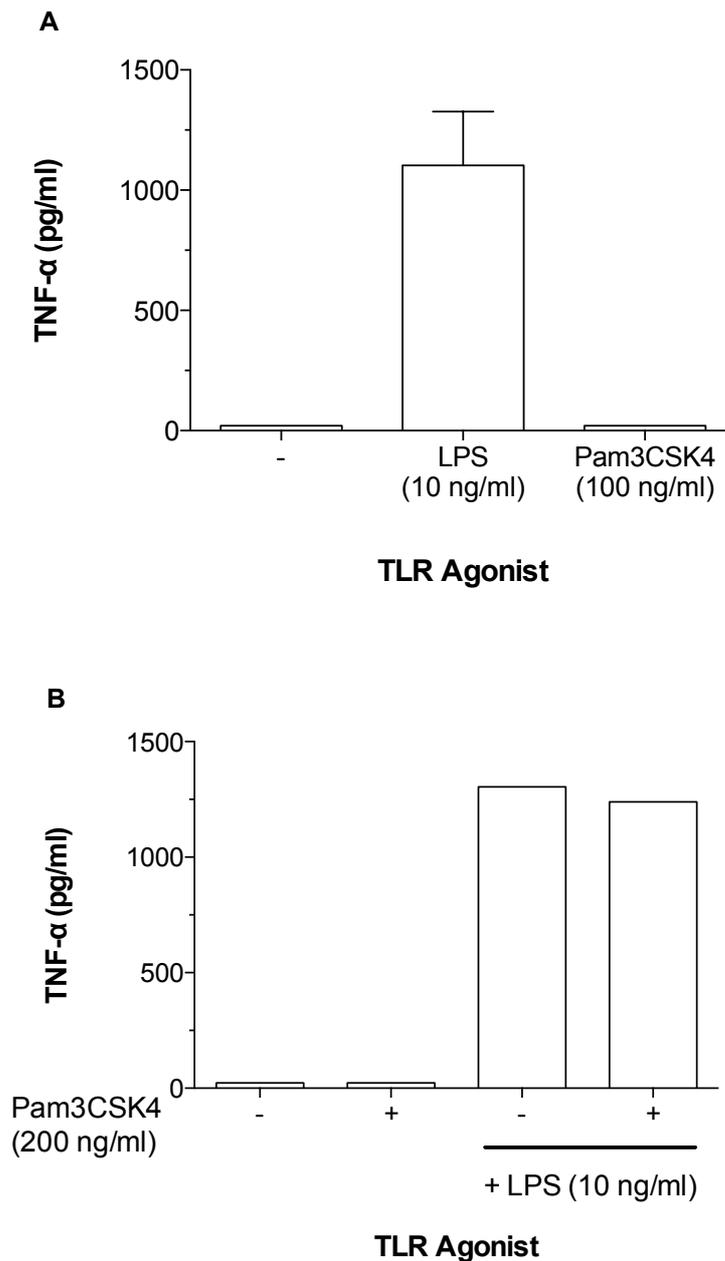
Figure 3.8. Effect of the TLR 1/2 agonist Pam3CSK4 on TNF-α release.
(A) Macrophages were incubated without or with LPS (10 ng/ml) or Pam3CSK4 (100 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for TNF-α. Values are mean ± SEM for 2 individual experiments. (B) Macrophages were incubated without or with Pam3CSK4 (200 ng/ml) combined with LPS (10 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for TNF-α. Values are for 1 experiment.
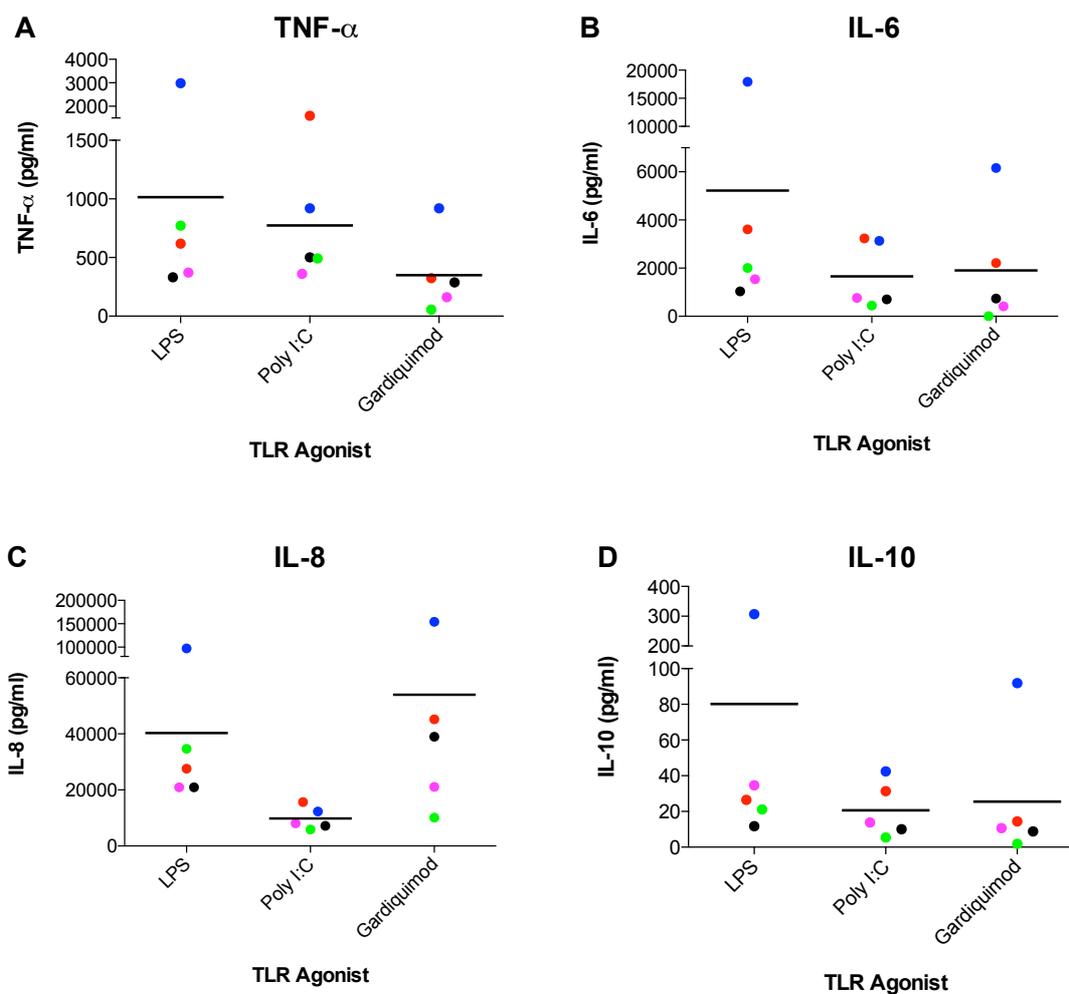
Figure 3.9. Cytokine release upon challenge with TLR agonists.
Macrophages were incubated without or with LPS (10 ng/ml), poly(I:C) (10 µg/ml) or gardiquimod (10 µg/ml). After 22 h, cell culture supernatants were harvested and assayed for (A) TNF-α, (B) IL-6, (C) IL-8 and (D) IL-10. Spontaneous cytokine release was below the level of detection except for IL-8, for which the values shown are net values. Coloured dots represent matched cell preparations. Solid horizontal lines represent the mean for 5 individual experiments.

3.2.5. Proteome profiler array of lung macrophages in response to TLR agonists

To determine if lung macrophages released a wider range of cytokines in addition to those already examined by ELISA, cell culture supernatants from macrophages (2×10^5 cells) that were challenged with LPS (10 ng/ml), poly(I:C) (10 μg/ml) or gardiquimod (10 μg/ml) for 22 h were diluted 1:3 with assay buffer (provided in the kit) and run on separate proteome profiler arrays, as were the corresponding control (spontaneous release) supernatants. The expression detected on the arrays is qualitative. Therefore, the supernatants tested on each of the arrays were also tested by ELISA for the cytokines routinely assayed in this project - TNF-α, IL-6 or IL-8. This provided an indication of the level of expression of at least one 'known' cytokine and provided a reference for the expression of the previously untested cytokines to be compared with.

The array analysis of **Human Lung Macrophages challenged with LPS** (figure 3.10C, D) compared to its corresponding control array, showed that there was an increase from spontaneous expression in GROα (CXCL1) and IL-1ra (IL-1 receptor antagonist). There was not much change in expression of MIF (macrophage migration inhibitory factor) or serpin E1 (plasminogen activator inhibitor-1). Some cytokines that were not detected in the spontaneous sample were detected upon LPS challenge. These were complement protein C5/C5a, at a low level. IP-10 (IFN-γ induced protein 10/CXCL10), MCP-1 (monocyte chemotactic protein 1/CCL2), MIP (macrophage inflammatory protein)-1α (CCL3) and MIP-1β (CCL4) were detected at higher levels. RANTES (regulated on activation normal T cell expressed and secreted/CCL5) and TNF-α were also detected upon LPS challenge. IL-8 expression after LPS challenge was detected on the array as being slightly lower than the spontaneous expression. This was a discrepancy between the array and previous ELISA data.

A

	Cytokine/Chemokine
1	C5/C5a
2	CD40L (CD154)
3	G-CSF
4	GM-CSF
5	GROα (CXCL1)
6	I-309 (CCL1)
7	sICAM-1 (CD54)
8	IFN-γ
9	IL-1α
10	IL-1β
11	IL-1ra
12	IL-2
13	IL-4
14	IL-5
15	IL-6
16	IL-8 (CXCL8)
17	IL-10
18	IL-12p70
19	IL-13
20	IL-16
21	IL-17
22	IL-17E (IL-25)
23	IL-23
24	IL-27
25	IL-32α
26	IP-10 (CXCL10)
27	I-TAC (CXCL11)
28	MCP-1
29	MIF
30	MIP-1α
31	MIP-1β
32	Serpin E1 (PAI-1)
33	RANTES (CCL5)
34	SDF-1 (CXCL12)
35	TNF-α
36	sTREM-1

Human Lung Macrophages

Figure 3.10. Proteome profiler array analysis of LPS-challenged macrophages. Potential expression of (A) 36 cytokines/chemokines was assessed in cell culture supernatants of human lung macrophages incubated without (B) or with (C) LPS (10 ng/ml) for 22 h. (D) Quantification of the pixel intensity of the detected spots. (PC, positive control; NC, negative control).

The array analysis of **Human Lung Macrophages challenged with poly(I:C)** (figure 3.11C, D) showed an increase from the spontaneous level in expression of IL-1ra. The high expression of IL-8 was not altered after poly(I:C) challenge and there was also no change in expression of MIF and serpin E1, which both remained faintly expressed. The cytokines that were not detected in the corresponding spontaneous release sample but were detected upon poly(I:C) challenge were IP-10 and MCP-1. Also only expressed upon challenge were C5/C5a, GROα and IL-6, MIP-1α and RANTES, which were all faintly expressed.

The array analysis of **Human Lung Macrophages challenged with gardiquimod** (figure 3.12C, D) showed an increase from the spontaneous level in expression of GROα, IL-1ra and MCP-1. There was no change in expression of MIF and serpin E1, which remained faintly expressed. The cytokines that were not detected in the corresponding spontaneous release sample but were detected upon gardiquimod challenge were C5/C5a, IL-6, MIP-1α, MIP-1β and TNF-α. IL-8 expression after gardiquimod challenge was detected on the array as being lower than the spontaneous expression but this was a discrepancy between the array and previous ELISA data.

The key differences between LPS, poly(I:C) and gardiquimod challenge of human lung macrophages can be summarized. IP-10 and RANTES were detected after challenge with LPS and poly(I:C) but not gardiquimod. MIP-1β and TNF-α were detected after challenge with LPS and gardiquimod but not poly(I:C).

A

	Cytokine/Chemokine
1	C5/C5a
2	CD40L (CD154)
3	G-CSF
4	GM-CSF
5	GROα (CXCL1)
6	I-309 (CCL1)
7	sICAM-1 (CD54)
8	IFN-γ
9	IL-1α
10	IL-1β
11	IL-1ra
12	IL-2
13	IL-4
14	IL-5
15	IL-6
16	IL-8 (CXCL8)
17	IL-10
18	IL-12p70
19	IL-13
20	IL-16
21	IL-17
22	IL-17E (IL-25)
23	IL-23
24	IL-27
25	IL-32α
26	IP-10 (CXCL10)
27	I-TAC (CXCL11)
28	MCP-1
29	MIF
30	MIP-1α
31	MIP-1β
32	Serpin E1 (PAI-1)
33	RANTES (CCL5)
34	SDF-1 (CXCL12)
35	TNF-α
36	sTREM-1

Human Lung Macrophages

B Spontaneous

C Poly(I:C)

D

Legend: □ Spontaneous ■ Poly(I:C)

Mean Pixel Intensity (y-axis, 0–20000)

Cytokine/Chemokine (x-axis): Positive control, Negative control, C5/C5a, GROα (CXCL1), I-309, sICAM-1, IL-1ra, IL-6, IL-8, IL-23, IL-27, IP-10 (CXCL10), I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1 (PAI-1), RANTES, TNF-α

Figure 3.11. Proteome profiler array analysis of poly(I:C)-challenged macrophages.
Potential expression of (A) 36 cytokines/chemokines was assessed in cell culture supernatants of human lung macrophages incubated without (B) or with (C) poly(I:C) (10 µg/ml) for 22 h. (D) Quantification of the pixel intensity of the detected spots. (PC, positive control; NC, negative control).

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A

	Cytokine/Chemokine
1	C5/C5a
2	CD40L (CD154)
3	G-CSF
4	GM-CSF
5	GROα (CXCL1)
6	I-309 (CCL1)
7	sICAM-1 (CD54)
8	IFN-γ
9	IL-1α
10	IL-1β
11	IL-1ra
12	IL-2
13	IL-4
14	IL-5
15	IL-6
16	IL-8 (CXCL8)
17	IL-10
18	IL-12p70
19	IL-13
20	IL-16
21	IL-17
22	IL-17E (IL-25)
23	IL-23
24	IL-27
25	IL-32α
26	IP-10 (CXCL10)
27	I-TAC (CXCL11)
28	MCP-1
29	MIF
30	MIP-1α
31	MIP-1β
32	Serpin E1 (PAI-1)
33	RANTES (CCL5)
34	SDF-1 (CXCL12)
35	TNF-α
36	sTREM-1

Human Lung Macrophages

B Spontaneous

C Gardiquimod

D
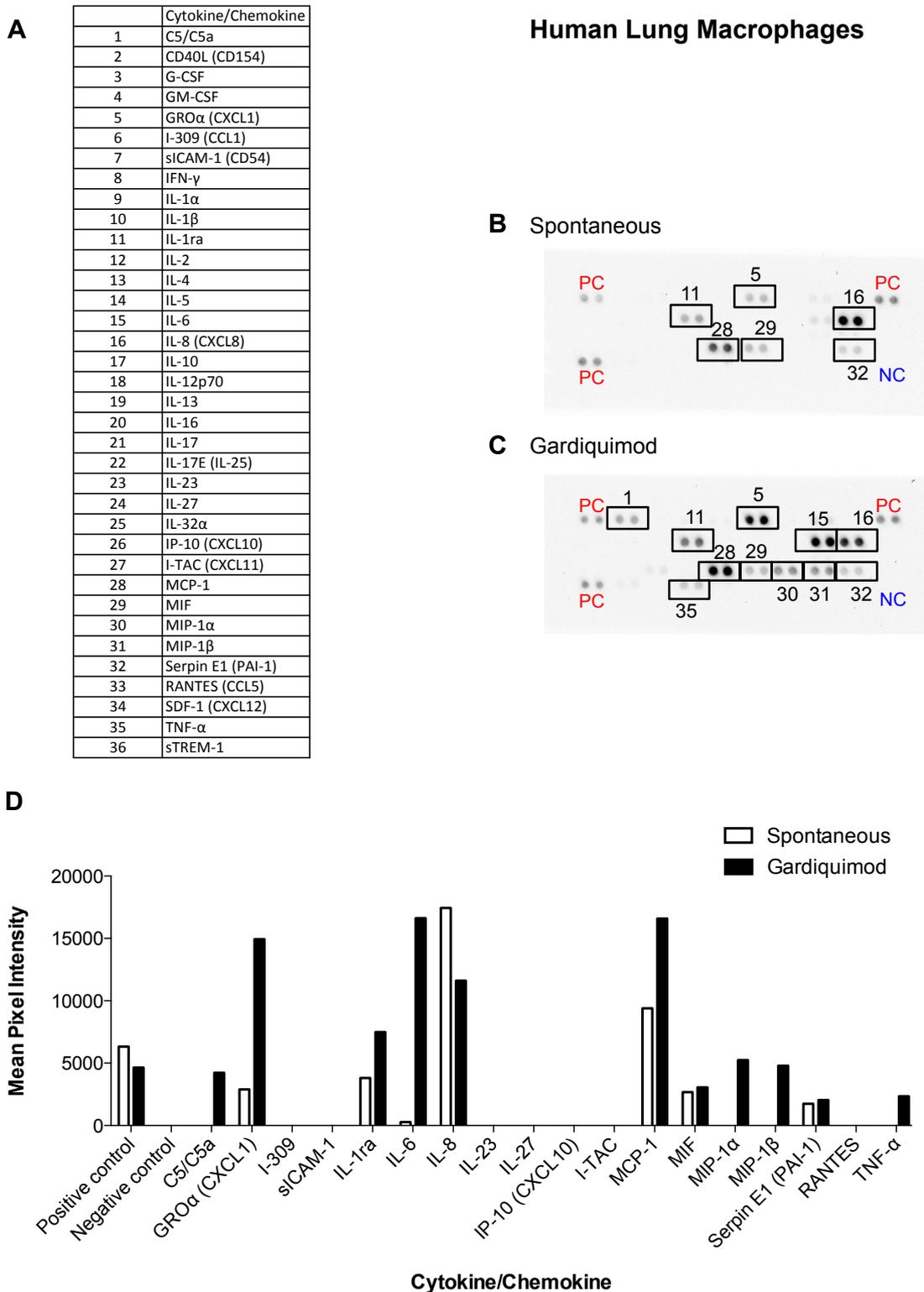
Figure 3.12. Proteome profiler array analysis of gardiquimod-challenged macrophages.
Potential expression of (A) 36 cytokines/chemokines was assessed in cell culture supernatants of human lung macrophages incubated without (B) or with (C) gardiquimod (10 μg/ml) for 22 h. (D) Quantification of the pixel intensity of the detected spots. (PC, positive control; NC, negative control).

The **spontaneous release** of cytokines/chemokines from human lung macrophages was assessed in 3 separate experiments (figures 3.10, 3.11, 3.12 B and D). In all three cell preparations, high release of IL-8 and some release of IL-1ra was detected. Low/faint expression of spontaneous MIF and serpin E1 was also detected in all three cell preparations. Some expression of GROα was detected in two of the three cell preparations (figures 3.10, 3.12 B and D). Expression of MCP-1 was detected in one cell preparation (figure 3.12 B and D) and faint expression of IL-6 was detected in one cell preparation (figure 3.10B and D).

Several cytokines and chemokines were not detected in any of the supernatants. These were CD40L, G-CSF (granulocyte-colony stimulating factor), GM-CSF (granulocyte macrophage-colony stimulating factor), IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-17E, IL-32α, SDF-1 (stromal cell-derived factor-1) and sTREM-1 (soluble triggering receptor expressed on myeloid cells-1). Although most of these cytokines/chemokines are not associated with macrophages, some are associated and it is possible that they were not present at a high enough concentration to be detected on the array. These are IL-1α, IL-1β, IL-10, IL-12p70 and IL-17. For some there is uncertainty as to whether they would be released by macrophages, such as IL-16.

3.2.6. Proteome profiler array of monocyte-derived macrophages in response to LPS

The **spontaneous release** of cytokines/chemokines from **monocyte-derived macrophages (MDMs)** was assessed in one experiment (figure 3.13B and D) for comparison to that of HLMs. As with HLMs, high release of IL-8 was detected, as was some release of GROα and IL-1ra. The cytokines that were detected with MDMs but were not seen with HLMs were C5/C5a and IP-10. High release of MCP-1 was also

detected with MDMs but only seen with one HLM preparation out of three. Serpin E1 was not seen with MDMs but was seen with HLMs.

The array analysis of **MDMs challenged with LPS** (figure 3.13C, D) showed an increase from the spontaneous level in expression of IP-10. There was not much change in expression of C5/C5a, GROα, IL-1ra, IL-8 and MCP-1 (although the mean pixel intensity values appeared to indicate that expression of IL-1ra and MCP-1 decreased slightly after LPS challenge, this is not obvious on the actual array films). The cytokines that were not detected in the corresponding spontaneous release sample but were detected upon LPS challenge were IL-6, I-TAC (Interferon-inducible T cell alpha chemoattractant/CXCL11), MIP-1α, MIP-1β, RANTES and TNF-α. Faint expression of I-309 (CCL1) and MIF was also detected after LPS challenge.

The cytokines that were expressed by MDMs after LPS challenge were the same ones as those expressed by human lung macrophages after LPS challenge, although there may have been some variation in the level of expression. The differences between LPS-challenged MDMs and human lung macrophages were that faint expression of I-309 and I-TAC was seen with MDMs but not with human lung macrophages.

A

	Cytokine/Chemokine
1	C5/C5a
2	CD40L (CD154)
3	G-CSF
4	GM-CSF
5	GROα (CXCL1)
6	I-309 (CCL1)
7	sICAM-1 (CD54)
8	IFN-γ
9	IL-1α
10	IL-1β
11	IL-1ra
12	IL-2
13	IL-4
14	IL-5
15	IL-6
16	IL-8 (CXCL8)
17	IL-10
18	IL-12p70
19	IL-13
20	IL-16
21	IL-17
22	IL-17E (IL-25)
23	IL-23
24	IL-27
25	IL-32α
26	IP-10 (CXCL10)
27	I-TAC (CXCL11)
28	MCP-1
29	MIF
30	MIP-1α
31	MIP-1β
32	Serpin E1 (PAI-1)
33	RANTES (CCL5)
34	SDF-1 (CXCL12)
35	TNF-α
36	sTREM-1

Monocyte-Derived Macrophages

B Spontaneous

C LPS

D

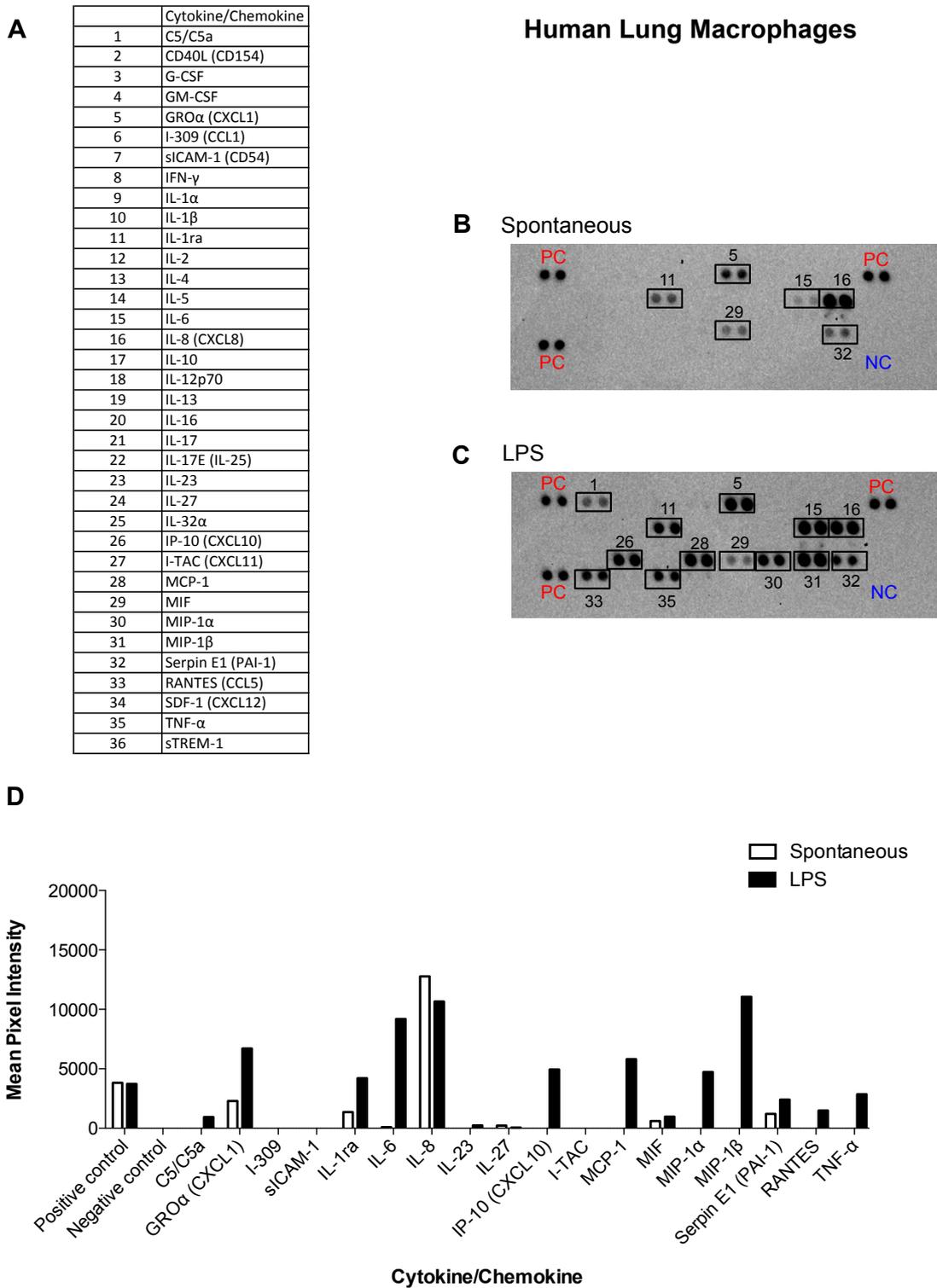**Figure 3.13. Proteome profiler array analysis of LPS-challenged MDMs.**
Potential expression of (A) 36 cytokines/chemokines was assessed in cell culture
supernatants of monocyte-derived macrophages (MDMs) incubated without (B) or with
(C) LPS (10 ng/ml) for 22 h. (D) Quantification of the pixel intensity of the detected
spots. (PC, positive control; NC, negative control).

3.2.7. The effect of long-term culture on response to LPS

Experiments investigating cytokine release were routinely initiated the day after cell isolation (day one), when the cells had been in culture for ~24 h. To determine the effect of a longer time in culture, macrophages were challenged with LPS (10 ng/ml) on day one, two and five. TNF-α release was measured 22 h after each LPS challenge. The differences in the release after stimulation on day two or day five compared to the release after stimulation on day one were not significant ($p>0.05$) (figure 3.14). The cell viability post-experiment remained high (88-93%) for all of the conditions tested.

3.2.8. Assessment of cytokine release in response to allergic stimuli

Macrophages were challenged with anti-IgE (2 μg/ml) to simulate conditions of allergy or with LPS (10 ng/ml) for 22 h after which levels of cytokines were measured. In response to LPS, all five experiments resulted in release of TNF-α, IL-6 and IL-10 (figure 3.15A). Only one of the five experiments carried out resulted in release of TNF-α (11922 pg/ml), IL-6 (15264 pg/ml) and IL-10 (309 pg/ml) after challenge with anti-IgE (figure 3.15B). These levels of release in response to anti-IgE were higher than the levels of each of the cytokines observed in response to LPS. All of the other experiments showed cytokine release to be below the level of detection after anti-IgE challenge.
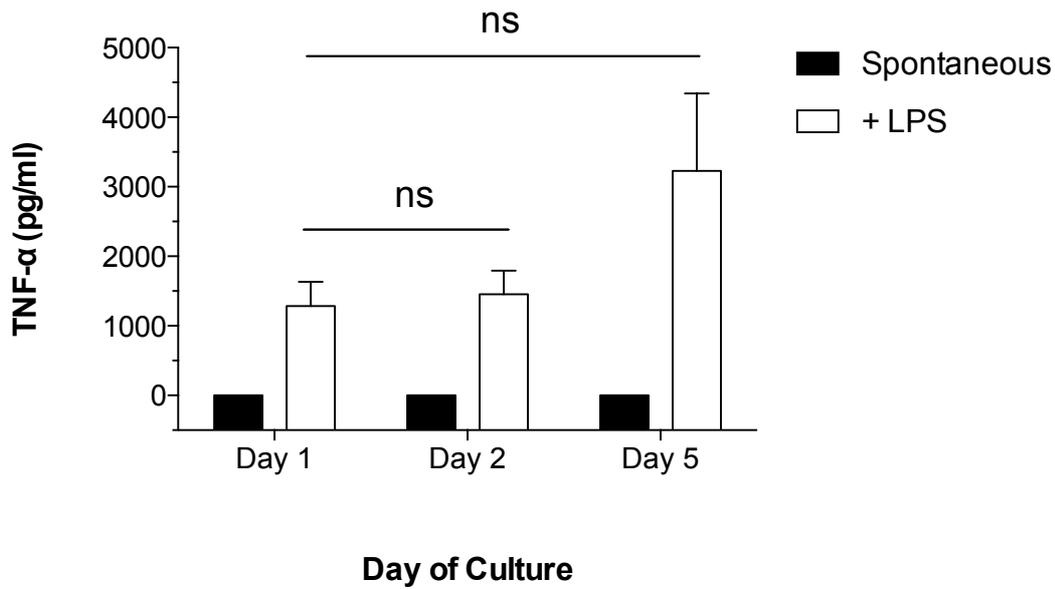
Figure 3.14. Effect of long-term culture of macrophages on LPS response.
Macrophages were cultured for 1, 2 or 5 days before incubation with or without LPS
(10 ng/ml). Cell culture supernatants were harvested at 22 h and assayed for TNF-α.
Values are mean ± SEM for 3 individual experiments. (One-way ANOVA with
Dunnett's post test, all p>0.05 compared with day 1).

A

LPS

B
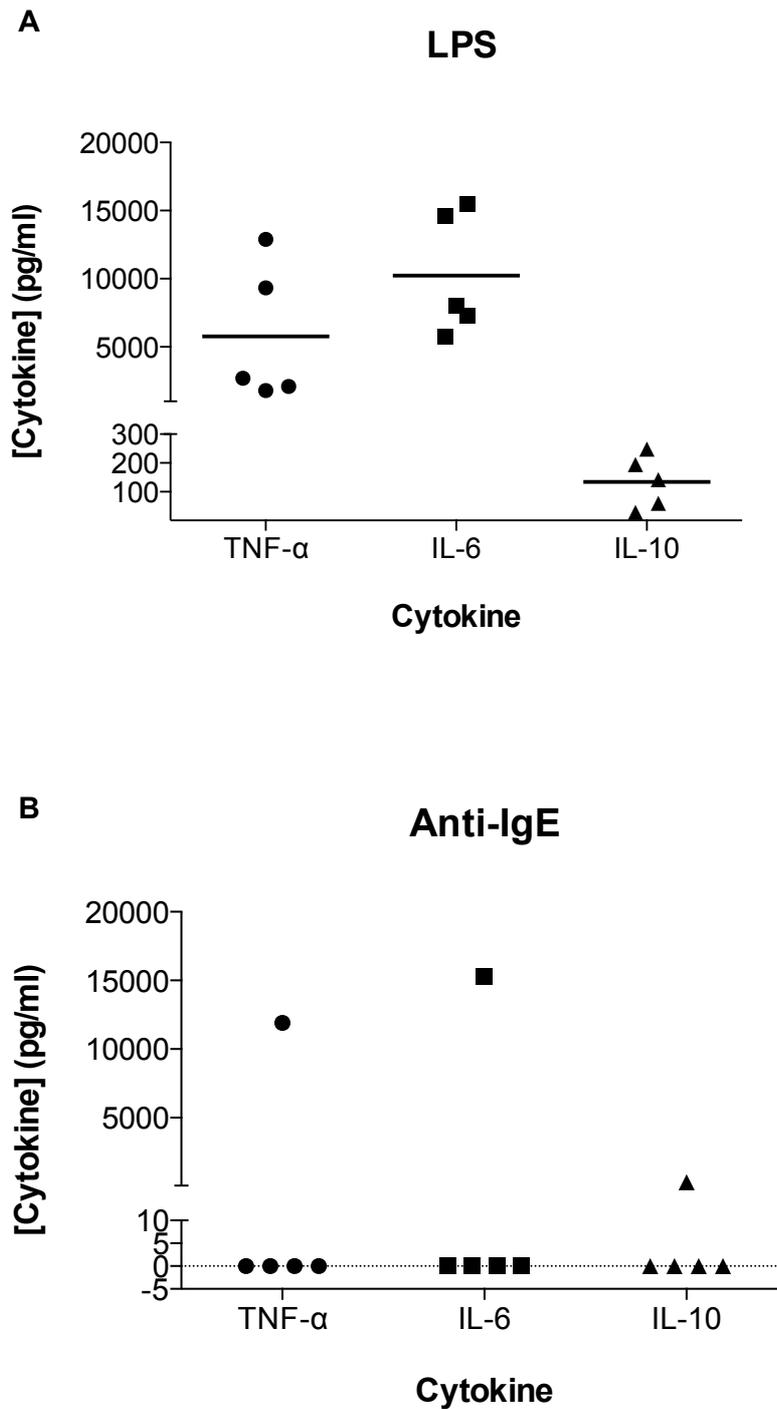
Anti-IgE

Figure 3.15. Effect of anti-IgE on cytokine release.
Macrophages were incubated without or with (A) LPS (10 ng/ml) or (B) anti-IgE (2 μg/ml). After 22 h, cell culture supernatants were assayed for TNF-α, IL-6 and IL-8. Data shown are of 5 individual experiments. Spontaneous cytokine release was below the level of detection.

Release of IL-8 was also measured from cells challenged with anti-IgE using a further five different macrophage preparations. LPS challenge of these cell preparations resulted in IL-8 release in all five experiments (38,823±1729 pg/ml). However, there was no discernible increase in IL-8 after anti-IgE challenge (8425 ± 3136 pg/ml) compared to the level of the spontaneous release (8312 ± 3088 pg/ml). In addition, the cell preparation that resulted in release of TNF-α, IL-6 and IL-10 in response to anti-IgE was also tested for IL-8 release. This cell preparation was the only one that resulted in an increase in IL-8 after anti-IgE challenge compared to the spontaneous release. As for TNF-α, IL-6 and IL-10 release, the release of IL-8 release was higher after anti-IgE challenge than LPS challenge (data not shown).

Two of the experiments in which macrophages were challenged with LPS or anti-IgE were also tested by ELISA for release of the Th2 cytokines IL-4 and IL-17a. One of these experiments was the one that resulted in release of TNF-α, IL-6 and IL-10 in response to anti-IgE. The release of IL-4 and IL-17a in both experiments was below the level of detection in response to both LPS and anti-IgE (data not shown).

To further investigate IgE dependent responses, an experiment was carried out in which macrophages were passively sensitized with NIP-specific IgE (JW8-IgE) prior to challenge with anti-IgE (2 μg/ml) or antigen NIP_{20}-HSA (100 ng/ml), as described in figure 2.1. The cell culture supernatants were assayed for TNF-α, IL-4 and IL-17a. The release of all three cytokines was below the level of detection for all of the samples (data not shown).

3.2.9. The effect of IL-4 pre-incubation on LPS-induced cytokine release

To determine whether LPS-induced TNF-α release from macrophages could be modulated by pre-incubation with a Th2 cytokine, the cells were incubated with IL-4 before challenging with LPS. The release of TNF-α in response to IL-4 alone was below the level of detection. Incubating the cells with IL-4 (10 ng/ml) for 24 h before challenging with LPS (10 ng/ml) for 24 h, resulted in a significant ($p<0.05$) 33% inhibition of the TNF-α release induced in cells challenged with LPS alone (figure 3.16A). The release of IL-10 was also measured, which followed the same pattern as the release of TNF-α. Pre-incubation with IL-4 resulted in a significant ($p<0.05$) 26% inhibition of the IL-10 release induced in cells challenged with LPS alone (figure 3.16B).

A

B
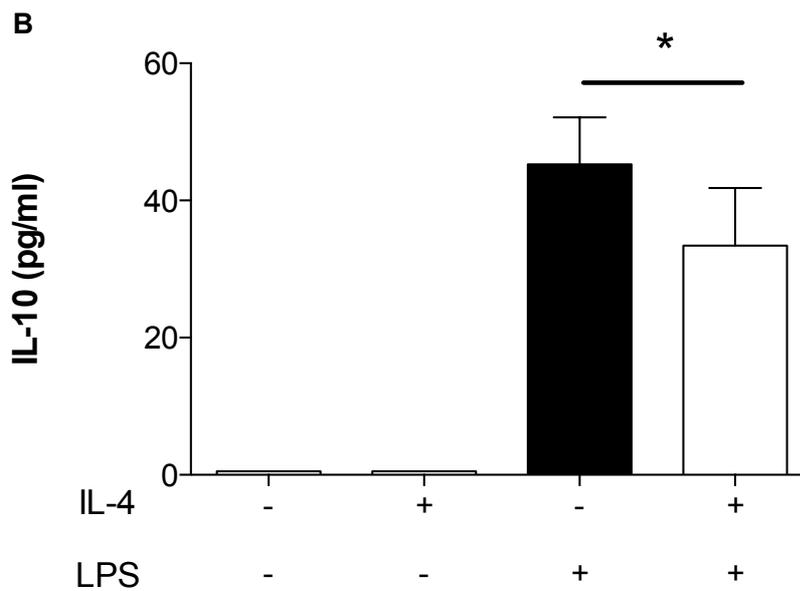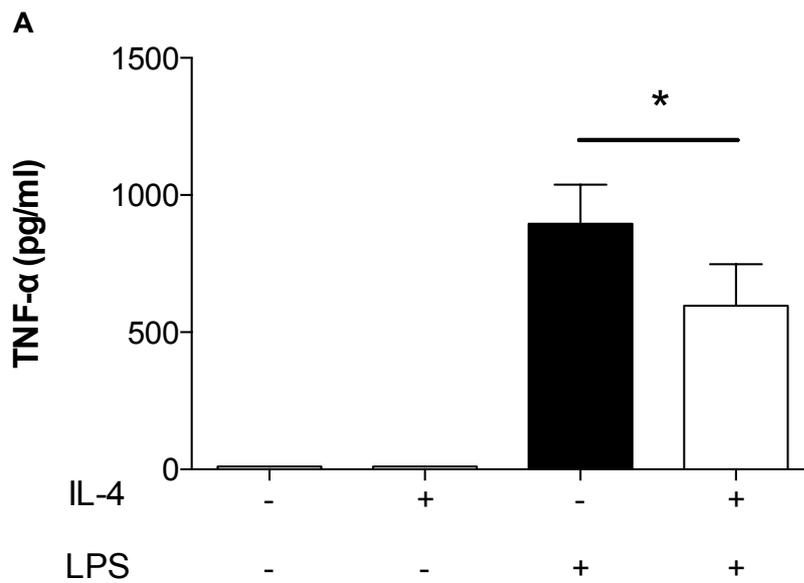
Figure 3.16. Effect of IL-4 pre-incubation on LPS-induced cytokine release.
Macrophages were incubated in the absence or presence of IL-4 (10 ng/ml) for 24 h and then without or with LPS (10 ng/ml) for a further 24 h. After a total of 48 h, cell culture supernatants were harvested and assayed for (A) TNF-α and (B) IL-10. Values are mean ± SEM for 3 individual experiments. (Paired t test, *p< 0.05, for LPS challenge after IL-4 pre-incubation compared with LPS challenge in the absence of IL-4 pre-incubation).

3.2.10. Comparison of lung macrophage and MDM cell surface marker expression

Flow cytometry was carried out to determine the CD14/CD206 cell surface marker profile of lung macrophages compared to that of MDMs. CD14 is considered to be a monocyte marker but can be expressed on macrophages. CD206 is a mannose receptor specific to macrophages but is regarded as being a marker of the M2 phenotype. It was found that most of the cells that were gated on the FSC/SSC plot (both MDMs and lung macrophages) were CD206$^+$ (figure 3.17A, B). Analysis of lung macrophage staining showed that a very low percentage of cells were CD14$^+$ (figure 3.17A). This was in contrast to the staining profile of MDMs, which showed considerable expression of CD14 (figure 3.17B). Most of the cells were CD14$^+$CD206$^+$. A corresponding cytospin of the human lung macrophage preparation that was analysed by flow cytometry showed 86% macrophage purity (figure 3.17C). As the flow cytometric analysis demonstrated that most of the cells in the preparation were CD206$^+$, it could be assumed that all of the macrophages expressed CD206. These profiles were representative of three preparations for each cell type. The expression of CD14 on CD206$^+$ lung macrophages and CD206$^+$ MDMs was then determined for the three preparations of each cell type studied (figure 3.17D). The expression of CD14 was significantly higher on MDMs than lung macrophages.

A major issue of flow cytometry studies using human lung macrophages is that the cells quite often exhibit autofluorescence, mainly due to particles taken up in the lungs of smokers. This autofluorescence can be severe, preventing detection of fluorochromes. This was the case for many of the cell preparations in the present study. It was necessary to exclude the results of experiments using these cell preparations from inclusion in this thesis, as they could not be fully analysed. Therefore, it is necessary to

use methods in addition to flow cytometry to investigate phenotypic marker expression

on human lung macrophages.
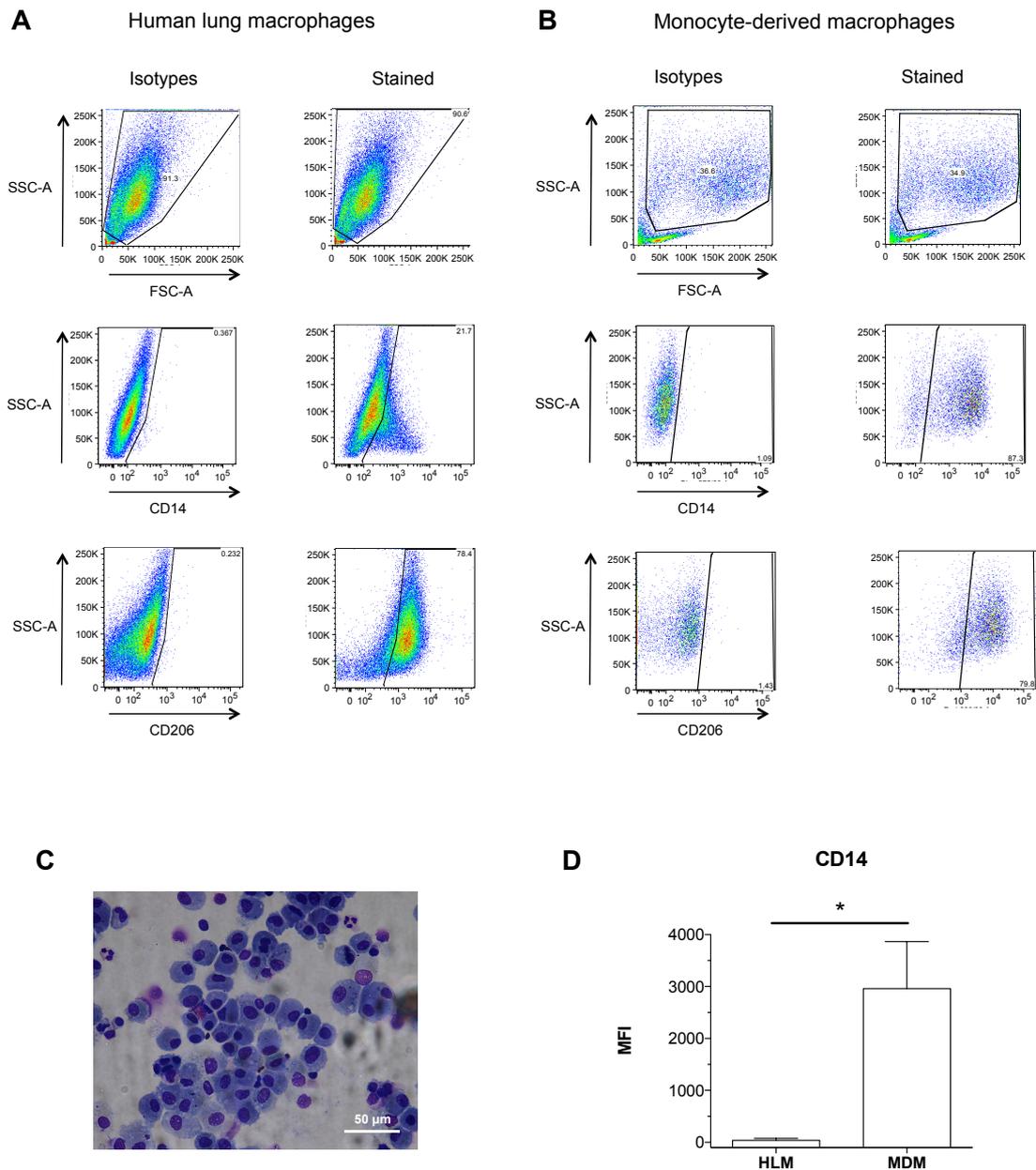
Figure 3.17. Flow cytometric analysis of CD14 expression on macrophages.
Unstimulated human lung macrophages (A) and monocyte-derived macrophages (B)
were analysed for CD14 and CD206 expression by flow cytometry. Data are
representative of a total of 3 individual experiments for each cell type. (C) Cytospin
showing 86% purity of the human lung macrophage preparation analysed. (D) Graph of
the geometric mean fluorescence intensity (MFI) of CD14 expressed on CD206^{+} human
lung macrophages compared to CD206^{+} monocyte-derived macrophages (Unpaired t
test, *p< 0.05).

3.2.11. Gene expression of M1 and M2 markers

In addition to investigating cytokine release and cell surface markers to explore phenotypes, a preliminary experiment using qPCR was performed. Macrophages were incubated for 22 h without or with LPS, IFN-γ, TNF-α, IL-13, dexamethasone or PGE$_2$ either alone or in combination. These stimuli were considered to be either pro or anti-inflammatory and in addition, some were considered to be M1 or M2-inducing. LPS, IFN-γ and TNF-α were the pro-inflammatory stimuli used. LPS and IFN-γ in combination are, in addition to being pro-inflammatory, considered to be M1-inducing. The anti-inflammatory stimuli used were IL-13 and dexamethasone, which are also considered to be M2-inducing. PGE$_2$ was also used, which has been shown to be anti-inflammatory to macrophages. Genes that could be expressed by either M1 or M2 phenotypes were examined. The M2 genes examined were for IL-10, the scavenger receptor CD36, the arginine-converting enzyme arginase I and the mannose receptor C type 1 (MRC-1). The M1 genes examined were for IL-1β and inducible nitric oxide synthase (iNOS or NOS2 gene).

One very clear association was found between pro-inflammatory M1 stimuli and M1 marker expression. LPS caused a 54-fold and LPS+IFN-γ caused a 25-fold up-regulation of IL-1β expression compared to the non-stimulated (media) control (figure 3.18C). The M2 marker genes that were investigated were up-regulated to an extent by both M1 and M2 stimuli. CD36 expression was up-regulated two-fold in response to the M2 stimulus dexamethasone but also 1.7-fold in response to the M1 stimulus IFN-γ (figure 3.18B). MRC-1 expression was up-regulated in response to the M2 stimuli IL-13 (66-fold) and dexamethasone (40-fold) (figure 3.18F). It was also up-regulated in response to the anti-inflammatory prostanoid PGE$_2$ (seven-fold). However, up-regulation of MRC-1 was also seen in response to M1 stimuli LPS (12-fold), IFN-γ (20-

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fold), LPS+IFN-γ (two-fold), and TNF-α (five-fold). Up-regulation of IL-10 (figure 3.18A), arginase (figure 3.18D) or NOS2 (figure 3.18E) expression was not seen in response to any of the stimuli, compared to the non-stimulated control.
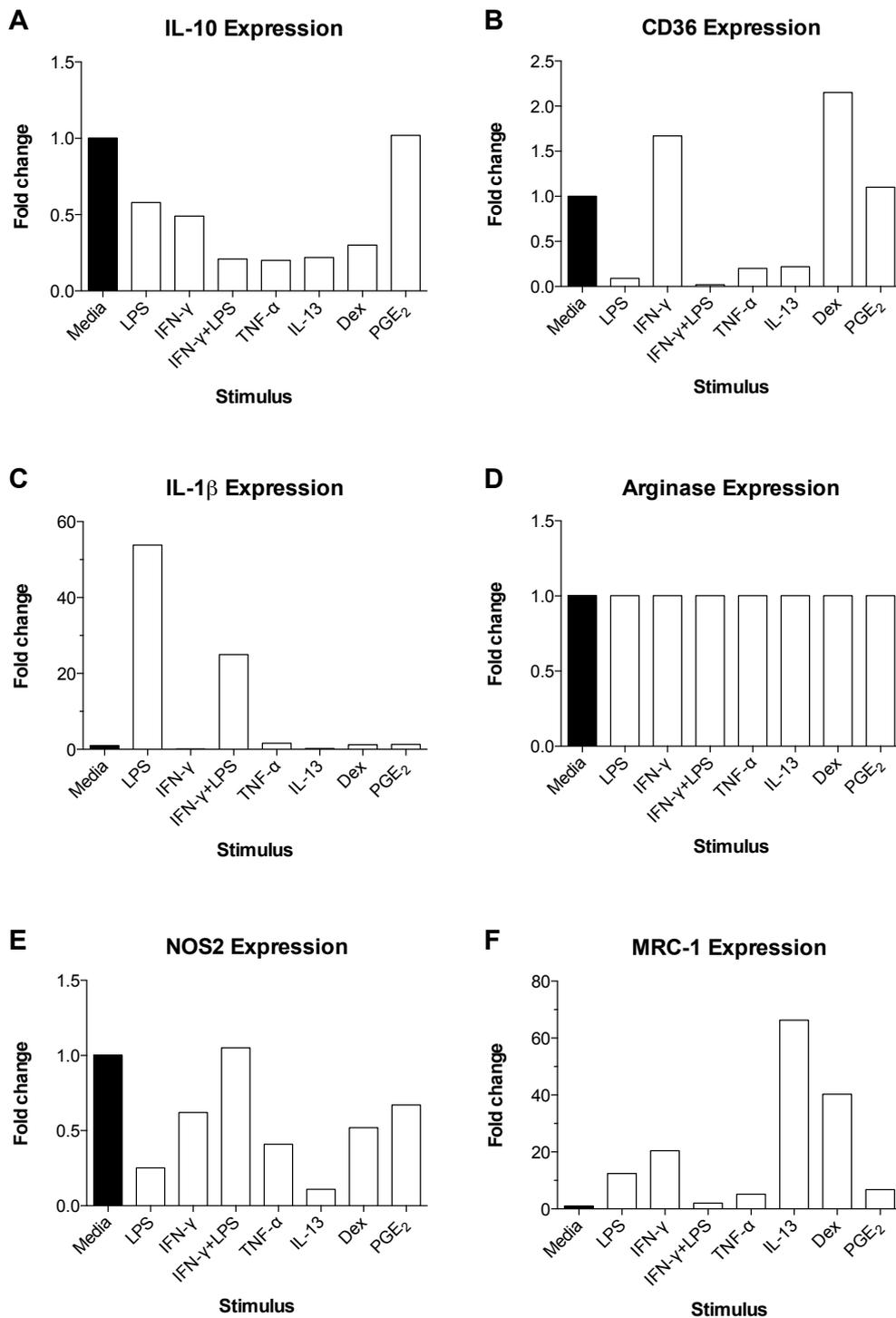
Figure 3.18. Gene expression of M1 and M2 markers in response to various stimuli. Macrophages (1 x 10^6) were incubated without or with LPS, IFN-γ, IFNγ+LPS, TNF-α, IL-13 (all 10 ng/ml), dexamethasone (Dex) (10^{-7} M) or PGE$_2$ (10^{-6} M) for 22 h. Isolated RNA was converted to cDNA in the presence (+) or absence (−) of reverse transcriptase. Amplification of cDNA was carried out using IL-10, CD36, IL-1β, Arginase, NOS2 and MRC-1 specific primers. Expression levels for each gene were normalised to the housekeeping gene β-actin. Data shown are of 1 macrophage preparation, in excess of 95% purity.

3.3. Discussion

Methods were developed to isolate macrophages from human lung tissue. The level of macrophage purity of the resulting cell preparations was high and the cells exhibited good viability. Studies were then carried out to investigate the functional responses to relevant stimuli and the phenotype of the isolated macrophages. Analysis of cytokine production and marker expression, along with selective comparisons to MDMs enabled this.

The release of key cytokines associated with macrophages was investigated in response to activation by the TLR4 agonist LPS. The release of the pro-inflammatory cytokines TNF-α, IL-6 and IL-8 was much higher than the release of anti-inflammatory IL-10. This is consistent with macrophages responding to pathogen-associated molecular patterns (PAMPs) with an inflammatory response (Taylor et al., 2005, Zhang and Mosser, 2008, Kaisho and Akira, 2006). The release of IL-8 was particularly high, both spontaneously and upon LPS challenge. Initial concerns that this was a result of endotoxin contamination in FCS were quashed by demonstrating that using ultra low endotoxin FCS or a lower percentage of FCS in the cell culture medium did not result in a significant decrease in IL-8 release, compared to release from cells cultured in the presence of the FCS that was routinely used. Other studies have also reported high release of IL-8 from lung macrophages (Thorley et al., 2007). As IL-8 is a powerful neutrophil chemoattractant, the data imply that the macrophages could play an active role *in vivo* in the recruitment of neutrophils in the lung. Neutrophilic inflammation is characteristic of COPD and higher numbers of neutrophils are found in the sputum of COPD patients as the severity of the disease increases (Keatings et al., 1996, Barnes, 2008b).

The release of TNF-α in response to LPS was concentration-dependent. However, at the highest concentration of LPS tested, a decrease in TNF-α was seen, indicating that the maximal release of TNF-α was with a concentration of LPS of ~10 ng/ml. The decrease in TNF-α seen at the higher concentration of LPS could be due to a number of reasons. Cytokine signalling can be both autocrine and paracrine (Kaisho and Akira, 2006). Release of one cytokine can lead to stimulation or inhibition of another. The higher strength of stimulus could have caused a more rapid concomitant release of other cytokines than stimulation with the lower LPS concentrations. These cytokines could have then resulted in the inhibition of TNF-α. It is possible for loss of cells, for example due to cell death during experiments, to result in a decrease in the cytokine levels detected. Variations in cell number between the wells containing the different concentrations of LPS were not measured at the end of the experiments. However, the viability was found to be unaltered from pre-experiment levels for all of the experimental conditions.

Observation of the release of pro-inflammatory cytokines over a time course revealed that overall there was a marked increase in release from five hours onwards, indicating that stimulation with LPS had resulted in initiation of protein synthesis taking place. The maximal release of cytokines was reached between 16 and 22 h, except for IL-8, which continued to increase at 22 h. This could have implications for the role of macrophages in relation to lack of resolution of inflammation in lung diseases.

In addition to bacterial proteins, macrophages *in vivo* can mount an inflammatory response to viral pathogens by activation of TLRs (Malmgaard et al., 2004). Therefore it was important to investigate the response to viral mimics. Macrophages responded to the TLR3 agonist poly(I:C) and the TLR7 agonist gardiquimod. Both agonists induced

the release of the pro-inflammatory cytokines IL-8, IL-6 and TNF-α but only low levels of IL-10, similar to the situation with LPS.

In further studies, the wider release of cytokines was investigated using proteome profiler array. In response to LPS, poly(I:C) or gardiquimod challenge, the cytokines and chemokines released from lung macrophages were all consistent with immune activation. Altogether, in addition to IL-8, IL-6 and TNF-α, these were complement protein C5/C5a, IP-10 (CXCL10), MIF, MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), GROα (CXCL1), serpin E1 and IL-1ra.

There were some differences between the cytokines released in response to each of the three stimuli and the reasons for these can be speculated. IP-10 and RANTES were detected upon challenge with LPS and poly(I:C) but not with gardiquimod. TLR signalling requires adaptor proteins, one of which is myeloid differentiation protein 88 (MyD88). Gardiquimod exerts its effects through signalling through a MyD88-dependent pathway, in contrast to LPS and poly(I:C) which can signal through MyD88-independent pathways (Bjorkbacka et al., 2004, Lundberg et al., 2007, Kaisho and Akira, 2006). The MyD88 pathway leads to transcription of type I interferons (Buitendijk et al., 2013) and does not induce transcription of IFN-γ, which is a type II interferon. This could explain the absence of IP-10 (Fitzgerald et al., 2003). RANTES was perhaps not seen in response to gardiquimod as production of RANTES is MyD88 independent (Fitzgerald et al., 2003). In response to poly(I:C) challenge, TNF-α was not detected on the proteome profiler array, despite being routinely detected in ELISAs throughout the study. The reason for this is not clear but it could be a result of cell preparation variation, where the cell preparation tested was one that did not respond strongly to the challenge, releasing TNF-α at a level too low to be detected by the array.

This could also be an explanation for the lack of detection of MIP-1β after poly(I:C) challenge.

Comparison of lung macrophages and MDMs without and with LPS challenge using proteome profiler array revealed that overall the cytokines released by the two cell types were the same. One difference was that serpin E1 (plasminogen activator inhibitor-1) was expressed by lung macrophages but not MDMs. As plasminogen activator is involved in blood clot fibrinolysis, serpin E1 could be involved in fibrosis (Liu, 2008, Wynn, 2011). Although roles in fibrosis are outside the scope of this study, this is of interest as it indicates that macrophages from the lung have some features that are not seen in the *in vitro* differentiated MDMs. There were subtle differences between the arrays. Faint expression of I-309 (CCL1), which is secreted by activated T lymphocytes was seen with MDMs but not lung macrophages. Faint expression of I-TAC (CXCL11), which is induced by interferons and is chemotactic for activated T lymphocytes (Barnes, 2008a), was also detected with MDMs but not lung macrophages. This indicates that there may have been contaminating lymphocytes in the MDM culture that were producing IFN-γ, rather than a difference between MDMs and lung macrophages themselves. The presence of lymphocytes is feasible as column purification of the PBMCs isolated from blood was not carried out, only purification by plastic adherence to the wells. This provides further reassurance of the reliability of the isolation procedure used to obtain lung macrophages, as any possible lymphocyte contamination of the lung macrophage preparations was not sufficient to result in detection of lymphocyte-associated factors.

It is interesting to note that for both macrophages and MDMs, some spontaneous release of cytokines and chemokines was detected, which then either remained at similar levels

or increased upon challenge with the TLR agonists. This indicates that prior to challenge, the lung macrophages did not exhibit any obvious marked indication of having been activated in the lung before isolation, as their cytokine generation appeared to be comparable to that of MDMs at rest. Whether the cytokine response after stimulation is more marked for lung macrophages or MDMs is difficult to determine from the proteome profiler array.

Some caution needs to be exercised in interpretation of the data from the proteome profiler arrays. The profiler arrays can only be used for semi-quantitative analysis, as the limits of cytokine detection are unknown. It is possible that some cytokines were not detected due to donor variation, for example TNF-α was not detected in response to poly(I:C), as mentioned earlier, although comprehensive ELISA data showed that it is usually released. IL-10, which is detected at low levels by ELISA was not detected at all on the array, probably as the signal was too weak. Another discrepancy between the array and ELISA data was that IL-8 appeared to decrease after LPS challenge on the array. This is likely to be due to the high levels of IL-8 generated being over the maximum detection limit of the array, leading to inaccurate pixel intensities. Caution also needs to be taken when comparing the different arrays, as different experiments/cell preparations were used for each one, therefore the quantitative pixel intensity levels of different arrays cannot be compared, only qualitiative expression. Therefore, it would be of benefit to investigate some of the wider cytokines released from macrophages that were identified on the arrays, by ELISA, such as MCP-1, MIP-1α, MIP-1β and RANTES.

In addition to being implicated in COPD, macrophages could have an important role in asthma (Sutherland and Martin, 2003). It has been demonstrated that macrophages

express high affinity and low affinity IgE receptors, known as FcεRI and FcεRII (CD23) respectively. It has been reported that the activation of the low affinity receptor results in release of pro- and anti-inflammatory cytokines (Gosset et al., 1999). Therefore, IgE-dependent responses were investigated to determine the possible involvement of macrophages in asthma. Only one cell preparation responded to anti-IgE with the extensive release of pro-inflammatory TNF-α, IL-6, IL-8 and anti-inflammatory IL-10. By contrast, all of the cell preparations responded to LPS with the release of these cytokines. As the asthmatic lung has generally been considered to be a Th2 environment, release of Th2 cytokines other than IL-10 was investigated (Barnes, 2008a). There was no release of IL-4 in response to anti-IgE or LPS. There was also no release of IL-17a, which has only over the last few years been reported to be a cytokine released from macrophages in allergic inflammation and not only from Th17 cells (Song et al., 2008). Passive sensitization of the cells with IgE specific for a particular antigen and subsequent challenge with the antigen did not elicit release of cytokines. This indicates that the isolated macrophages on the whole do not undergo IgE-dependent activation leading to the release of either the pro-inflammatory or anti-inflammatory cytokines measured in this study.

The lack of response from all but one cell preparation implies that donor variation could have been an issue. It has been reported that a greater percentage of macrophages from atopic asthmatics express IgE receptors than control subjects (Rankin, 1989, Williams et al., 1992). It may be that cells from patients with asthma respond better to IgE challenge to release pro-inflammatory cytokines (Gosset et al., 1999). However, the cell preparation that responded to anti-IgE with the release of pro-inflammatory cytokines in this study was not believed to be from an asthmatic patient. It is also possible that investigation of a larger number of cell preparations may have revealed more

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'responders'. In a human lung mast cell study that used the same allergic stimulus and a similar experimental design, most of the cell preparations responded to IgE activation. However, the study also demonstrated that 1 in 7 cell preparations do not respond to anti-IgE (Havard et al., 2011). Due to the lack of response of lung macrophages to IgE-dependent activation in the majority of experiments, further investigations into this process were not pursued in this study.

Macrophages are believed to exhibit plasticity (Giorgio, 2013, Mosser and Edwards, 2008). The phenotype is believed to be transient and able to switch upon changes in the local environment. Some preliminary experiments were performed to determine whether this may be the case in this system. The high release of pro-inflammatory cytokines observed upon stimulation with LPS indicated that the phenotype of the isolated macrophages could be closer to M1 than to M2. It has been reported that IL-4 or IL-13 stimulation can result in an M1 phenotype changing to M2. The TNF-α response to LPS was dampened with IL-4. It was thought that this was possibly due to an alteration in phenotype. Investigation into whether anti-inflammatory IL-10 was simultaneously increased with IL-4 pre-incubation was negative and furthermore, IL-10 was actually decreased. Therefore, this very preliminary investigation into plasticity was inconclusive but there is potential for further investigation.

Comparison of expression of cell surface markers by flow cytometry showed that both lung macrophage and MDM preparations expressed CD206 on most cells. MDMs expressed both CD206 and CD14, on an almost equal percentage of cells, producing a double-stained profile of expression. By contrast, the CD14 expression on lung macrophages was very low. CD206 is generally regarded as being an M2 phenotype marker. However, more recently it has been suggested that CD206 is not exclusively

present on M2 cells. In a study investigating macrophage polarization, MDMs incubated

with the M2 stimulus IL-4 resulted in up-regulation of CD206 but greater up-regulation

was seen upon stimulation with GM-CSF, which is believed to induce an M1 phenotype

(Ambarus et al., 2012). Therefore, in the present study, taking into account the high

macrophage purity of the cell preparations as demonstrated by cytospins, the data

suggest that all macrophages express CD206 to an extent. Therefore all of the cells

expressing CD206 in the flow cytometry experiments in this study were regarded as

being macrophages potentially of either M1 or M2 phenotype. To distinguish between

M1 and M2 macrophages, it would be necessary to compare the levels of M1 and M2

markers present simultaneously. The CD206 expression of M2 cells could be expected

to be high whilst the expression of an M1 marker on the same cells could be expected to

be low. For M1 cells, the opposite would be the case.

The expression of CD14, which is predominantly a monocyte marker, is expected to be

down-regulated during differentiation to a mature phenotype (Daigneault et al., 2010).

MDMs at 12 days of differentiation have possibly not reached the maturity status of

lung macrophages, as they still retain considerable CD14 expression. The preliminary

data indicate that the CD14/CD206 expression profile of lung macrophages and MDMs

is different. This suggests that it is difficult to make direct comparisons between MDM

and macrophage experiment results based on surface marker expression. Markers other

than CD14 would be used for determination of an M1 phenotype, although CD14 would

still be used as part of flow cytometry gating strategies. Suggestions based on other

flow cytometry studies are CD40 (Chana et al., 2014, Vogel et al., 2014) and CD80

(Ambarus et al., 2012). A suggested M2 surface marker in addition to CD206 is CD163

(Ambarus et al., 2012, Chana et al., 2014, Vogel et al., 2014). However, caution needs

to be exercised as some of these studies were performed using MDMs, not lung

macrophages. Also, macrophages in disease conditions can have altered cell surface marker expression. It has been reported that lung macrophages from COPD patients show reduced expression of both M1 and M2 surface markers (Chana et al., 2014, Pons et al., 2005).

Despite the CD14 expression on lung macrophages being low, this did not affect the consistent marked response to LPS seen in functional studies. It has been reported that LPS binding is not always related to the level of CD14, which could explain this (Haugen et al., 1998). In this current study, TLR2 surface marker expression was not investigated but no response to Pam3CSK4 was seen from lung macrophages. A recent study reported that MDMs had down-regulated surface expression of CD14 and TLR2 compared to monocytes. The cytokine response of the MDMs to Pam3CSK4 stimulation was more marked than to LPS stimulation (Daigneault et al., 2010). In another study, in which TLR2 was also down-regulated on MDMs and on alveolar macrophages compared to monocytes, the production of cytokines in response to the TLR2 agonist was lower compared to monocytes (Juarez et al., 2010). These studies indicate that surface expression of CD14 and TLR2 is down-regulated as cells undergo differentiation. However, there can be differences in functional responses to TLR agonists between different macrophage models, particularly as MDMs are often differentiated from monocytes using different methods.

As it is important to utilize more than one approach to investigate macrophage phenotypes, a preliminary qPCR experiment was carried out using a panel of primers for markers that can be regarded as being indicative of the M1 and M2 phenotypes. The combination of LPS+IFN-γ is reported to induce an M1 phenotype (Mosser, 2003). Originally, it was believed that IFN-γ was required to prime the cells and the LPS was

believed to cause induction of TNF-α, the second signal required. LPS alone was not considered to be M1-inducing. However it has since been suggested that LPS stimulation alone can induce TNF-α and also IFN-β, which can replace IFN-γ (Mosser and Edwards, 2008). In this experiment, expression of IL-1β was investigated. IL-1β was not detected by ELISAs in this study, as a two-step stimulation with a TLR agonist and adenosine triphosphate (ATP) is required for the release of IL-1β from macrophages (Netea et al., 2009). However, this two-step stimulation is not required for induction of IL-1β mRNA. In this experiment, IL-1β gene expression increased greatly in response to LPS+IFN-γ suggesting that the M1 phenotype was induced by the action of the stimuli. However, LPS alone up-regulated IL-1β expression to a greater level than LPS+IFN-γ. It is possible that LPS alone was stimulating macrophages that were M1 at baseline and already primed, resulting in the greater IL-1β expression seen compared to that with LPS+IFN-γ, or that LPS alone was inducing TNF-α and IFN-β.

Up-regulation of NOS2 was not seen in response to any of the stimuli in this experiment. There was a very slight increase above the level of the control in response to LPS+IFN-γ stimulation. The mRNA expression of NOS2 has been demonstrated in murine macrophages in response to M1 stimuli (Edwards et al., 2006). However, there is controversy in the literature regarding the expression of iNOS in human macrophages (Schneemann and Schoeden, 2007). Studies carried out in MDMs have shown no iNOS enzyme expression and activity (Schneemann et al., 1997) or none comparable to that seen in murine macrophages (Murray and Wynn, 2011b). A study using MDMs showed that the NOS2 gene was expressed in response to infection with mycobacterium (Jung et al., 2013). Another study using MDMs showed that iNOS protein expression was up-regulated in response to pneumococcal infection (Marriott et al., 2004). It appears that

expression of the NOS2 gene and protein is seen in human macrophages in response to certain conditions.

The expression of the M2 markers investigated was found to be up-regulated by both M2 and M1 stimuli. It is likely that the markers examined were not specific for the M2 phenotype. Alternatively, again, it is possible that the cells were M1 at baseline and not completely converting to an M2 phenotype. However, the different levels of up-regulation of markers seen in response to M1 and M2 stimuli could still assist in determining phenotype. An example in the present qPCR experiment is that greater up-regulation was seen of M2 markers than M1 markers by M2 stimuli, for example, MRC-1 expression in response to IL-13. Other gene expression markers that may be considered to be more reliable for determining the M2 phenotype are CD163 in response to IL-10 and CD206 in response to IL-4 (Ambarus et al., 2012, Vogel et al., 2014). Recent investigations have led to suggestions for other markers that may be suitable for determining M2 phenotypes, for example, CD200R upregulation in response to IL-4, although this was determined using MDMs and not lung macrophages (Ambarus et al., 2012, Hussell and Bell, 2014). Another suggestion is CD16 expression in response to IL-10 (Ambarus et al., 2012).

Arginase I and IL-10 were not up-regulated in the present experiment. Arginase I converts L-arginine to ornithine rather than nitric oxide (Chang et al., 1998). Arginase I is generally considered to be a murine M2 macrophage marker (Murray and Wynn, 2011b, Edwards et al., 2006, Martinez et al., 2009) but there are conflicting data demonstrating that expression can be induced by both M1 and M2 stimuli (Menzies et al., 2010). Although it has been reported that arginase can be expressed in human alveolar macrophages, for example in response to IL-4 in combination with cAMP-

increasing agents (Erdely et al., 2006), the majority of reports state that arginase is not expressed in human macrophages (Murray and Wynn, 2011a) but is expressed constitutively in human neutrophils (Munder et al., 2005). The lack of IL-10 expression observed in the experiment supports the results of the ELISAs performed throughout this study, in which very little IL-10 release was seen in response to both pro and anti-inflammatory stimuli.

Overall, the results of the qPCR experiment indicate that the isolated lung macrophages are likely to have an M1 or M1-like phenotype at baseline, which can be enhanced upon stimulation with M1 stimuli and altered, to an extent, towards an M2 phenotype upon stimulation with M2 stimuli. There are limitations to drawing definitive conclusions regarding the phenotype of the cells from the results obtained here. Only one experiment was performed, using one macrophage preparation. Only one concentration of each of the stimuli was used and the gene expression was only measured after 24 h stimulation of the cells. It would be interesting to investigate the gene expression at other time points. The results of the experiment indicate that further studies using this technique would be useful, in combination with other approaches used in this study.

The preliminary characterization of isolated lung macrophages demonstrates that the cells are active and respond to various stimuli to release a variety of cytokines and chemokines. The high release of pro-inflammatory cytokines compared to anti-inflammatory cytokines indicates that the cells are likely to have predominantly an M1 (classically activated) or M1-like phenotype upon stimulation with TLR agonists. Although the phenotype pre-stimulation is difficult to determine, particularly as phenotype is believed to be transient, the evidence suggests that it is closer to M1 than M2. For example, stimulation with IL-4 before LPS results in a decrease in pro-

inflammatory TNF-α but not an increase in anti-inflammatory IL-10, which could imply that the initial phenotype was perhaps M1 and not completely switching to M2. Lung macrophages are fully differentiated compared to MDMs and although major differences were not seen between the functional responses compared between the two models in this study, wider investigations could reveal different results in experiments. These preliminary studies provide the foundation for further study of the function and phenotype of lung macrophages in the context of inflammation.

Chapter 4: Characterization of the EP receptor that mediates the inhibitory effects of prostaglandin E_2 on human lung macrophages

4.1. Introduction

PGE_2 is known to have wide-ranging effects on a variety of tissues. These effects of PGE_2 are most likely to be mediated through specific EP receptors, of which four have been identified (Woodward et al., 2011, Coleman et al., 1994, Breyer et al., 2001). EP receptors are G protein-coupled receptors. EP_1 receptors appear to be coupled to phospholipase C, EP_2 and EP_4 receptors are linked to activation of adenylyl cyclase leading to increases in cellular cyclic-AMP and EP_3 receptors have variously been linked to adenylyl cyclase and phospholipase C (Breyer et al., 2001, Coleman et al., 1994, Woodward et al., 2011, Irie et al., 1993, An et al., 1994). Agonists and antagonists at these receptors have been described although developing selective and potent ligands has proved difficult (Abramovitz et al., 2000, Wilson et al., 2004, Jones et al., 2009).

The suggestion has been made that targeting EP receptors may be of benefit in the treatment of respiratory diseases. This stems from the finding that PGE_2 causes bronchodilation (Kawakami et al., 1973, Melillo et al., 1994, Gauvreau et al., 1999). An undesirable effect of PGE_2 is that it also induces cough (Maher et al., 2011). It appears, however, that cough and bronchodilation are mediated by different receptors suggesting that selective targeting of the beneficial receptor might be possible. The EP_3 receptor has been linked to cough (Maher et al., 2011) whereas the EP_2 receptor has been linked to bronchodilation (Norel et al., 1999). More recent studies suggest that EP_4 receptors

are more likely to be responsible for bronchodilation (Buckley et al., 2011, Benyahia et al., 2012).

In addition to effects on airway smooth muscle, PGE_2 may also be anti-inflammatory in the lung (Vancheri et al., 2004). The activity of a number of respiratory cells may be affected by PGE_2. In this context, PGE_2 has been shown to inhibit pro-inflammatory cytokine release from lung macrophages (Rowe et al., 1997, Buenestado et al., 2012, Ratcliffe et al., 2007). This effect of PGE_2 on human lung macrophages has been reported to be mediated by EP_2 and EP_4 receptors (Ratcliffe et al., 2007). However, this conclusion was drawn at a time when the availability of selective pharmacological ligands at especially the EP_2 receptor was limited.

In the present study, a range of pharmacological ligands was utilized to investigate the effects of PGE_2 on cytokine generation from LPS-stimulated human lung macrophages. A range of EP_2-selective and EP_4-selective antagonists that have emerged only recently was studied, including PF-04418948, the first potent and selective EP_2 receptor antagonist reported (af Forselles et al., 2011). These compounds enabled re-evaluation of the EP receptor mediating the anti-inflammatory effects of PGE_2 on human lung macrophages.

4.2. Results

4.2.1. Effects of PGE_2 on cytokine generation

In agreement with previous studies, PGE_2 was found to inhibit LPS-induced TNF-α generation from human lung macrophages in a concentration-dependent manner. Macrophages were incubated with PGE_2 (10^{-5}-10^{-11} M) for 30 min before being challenged with LPS (1 ng/ml) for 22 h. This experiment was carried out in the absence

(figure 4.1A) and presence (figure 4.1B) of the cyclooxygenase (COX) inhibitor indomethacin (1 μM). PGE$_2$ was a more potent (EC$_{50}$; 3.2 ± 0.6 cf 10.8 ± 2.0 nM) and efficacious (E$_{max}$; 77 ± 1.8 cf 53.5 ± 2.0 % inhibition) inhibitor of LPS-induced TNF-α generation in the presence of indomethacin (figure 4.1C). Moreover, in the presence of indomethacin (1 μM), TNF-α generation induced by LPS was significantly ($p<0.01$) higher than in its absence (2657 ± 496 cf 1648 ± 213 pg/ml; n=13). These experiments suggested that macrophages, in response to LPS, produce PGE$_2$ which acts in a paracrine fashion to limit TNF-α generation. Further experiments confirmed that macrophages generate a small amount of PGE$_2$ spontaneously and larger quantities following challenge with LPS (data shown in Chapter 5). In order to eliminate the potentially confounding influence of endogenous PGE$_2$ generation in the context of receptor characterizations, in all subsequent functional studies, indomethacin was also included.

In further studies, the effects of PGE$_2$ on LPS-induced IL-6 and IL-8 as well as TNF-α generation were determined (figure 4.2). PGE$_2$ inhibited TNF-α and IL-6 generation with similar potency (EC$_{50}$; ~1.6 nM) but was less efficacious as an inhibitor of IL-6 generation than of TNF-α. By contrast, PGE$_2$ was ineffective as an inhibitor of IL-8 generation.
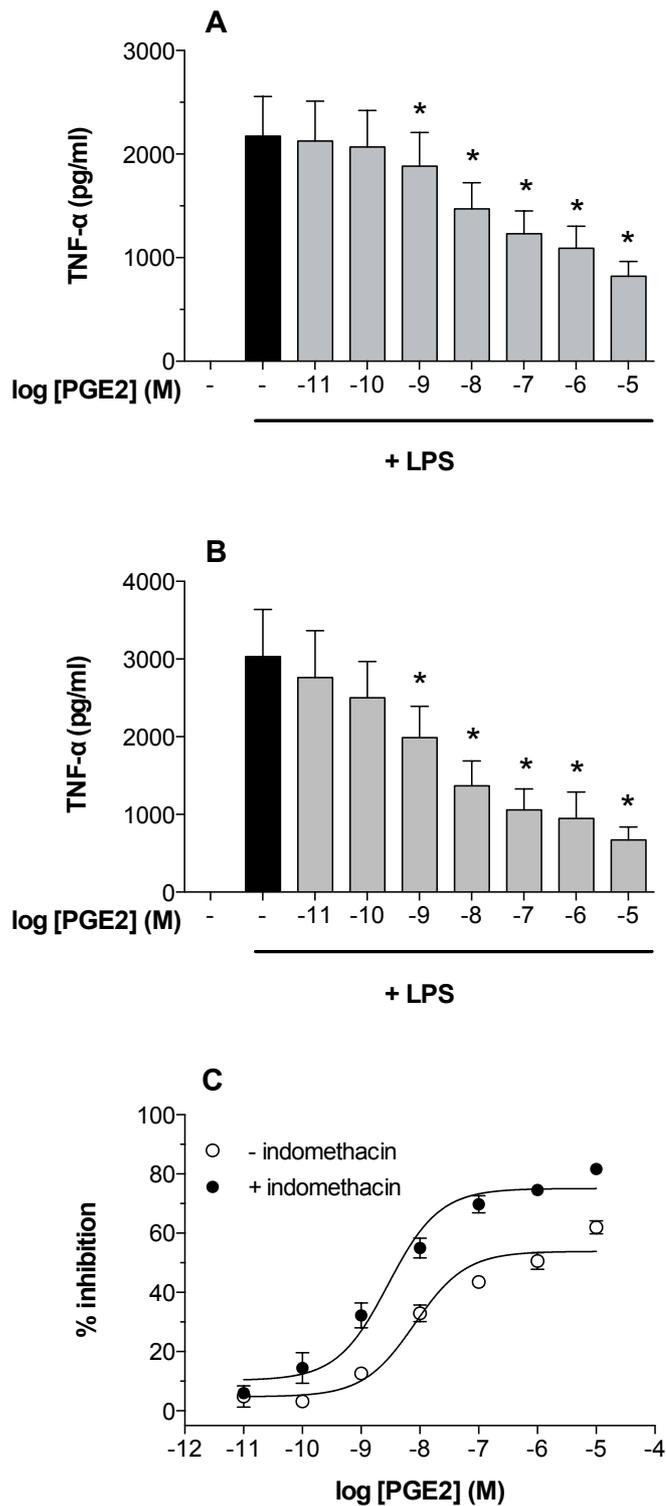
Figure 4.1. Effects of PGE$_2$ on TNF-α generation from macrophages.
Macrophages were pre-incubated without (A) or with (B) indomethacin (1 μM) for 30 min and then with or without PGE$_2$ for 30 min before challenge with LPS (1 ng/ml) for 22 h, after which supernatants were harvested and assayed for TNF-α generation. The data in (A) and (B) were reworked as % inhibition of the control unblocked release of TNF-α and this is shown in (C). Values are mean ± SEM, for 9 individual experiments. (One-way ANOVA with Dunnett's post test, *p<0.05, compared with unblocked control levels).
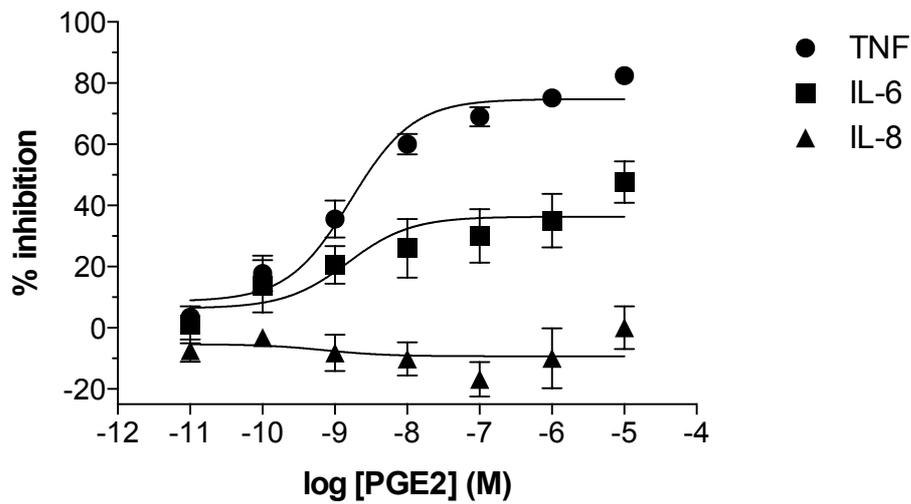
Figure 4.2. Effects of PGE$_2$ on cytokine generation from macrophages.
Macrophages were pre-incubated (30 min) with indomethacin (1 μM) and then with or without PGE$_2$ for 30 min before challenge with LPS (1 ng/ml) for 22 h, after which TNF-α, IL-6 and IL-8 were measured in the supernatants. Values are expressed as the % inhibition of control cytokine releases which were 2422 ± 510 pg/ml of TNF-α, 4992 ± 1980 pg/ml of IL-6 and 28877 ± 5554 pg/ml of IL-8. Values are mean ± SEM for 6 individual experiments.

4.2.2. Effects of prostanoids on LPS induced TNF-α generation

A comparison was made between the effects of PGE_2 and the effects of other prostanoids on LPS-induced TNF-α generation. The other prostanoids tested were prostaglandin D_2 (PGD_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), the prostaglandin H_2 analogue U46619 and the prostaglandin I_2 analogue iloprost. Macrophages were incubated for 30 min with indomethacin (1 μM) and for a further 30 min with the prostanoids (all 10^{-6}-10^{-8} M) before challenge with LPS (1 ng/ml) for 22 h. No effect on LPS-induced TNF-α generation was seen with PGD_2 and U46619 (figure 4.3A and 4.3C). Slight concentration-dependent inhibitions of TNF-α were observed with $PGF_{2\alpha}$ and iloprost (figure 4.3B and 4.3D) but these were not significant ($p > 0.05$). Only PGE_2 significantly ($p < 0.05$) inhibited LPS-induced TNF-α generation, in a concentration-dependent manner (figure 4.3E).

4.2.3. EP receptor expression in lung macrophages

RT-PCR was used to determine the expression of EP receptors by human lung macrophages. The data indicate that human lung macrophages express message for EP_2 and EP_4 receptors but do not express message for EP_1 or EP_3 receptors (figure 4.4). Densitometric analysis showed that the expression of EP_2 receptors remained consistent between cell preparations, relative to β-actin but there was some variability between cell preparations in the expression of EP_4 receptors. The expression of EP_2 and EP_4 receptors was further investigated after stimulation of the macrophages for 22 h with LPS (10 ng/ml). Up-regulation of both receptors was seen in response to LPS. A two-fold up-regulation of the expression of EP_2 receptors was seen in both cell preparations tested, whereas a 1.5-fold up-regulation of EP_4 receptors was seen (figure 4.5).
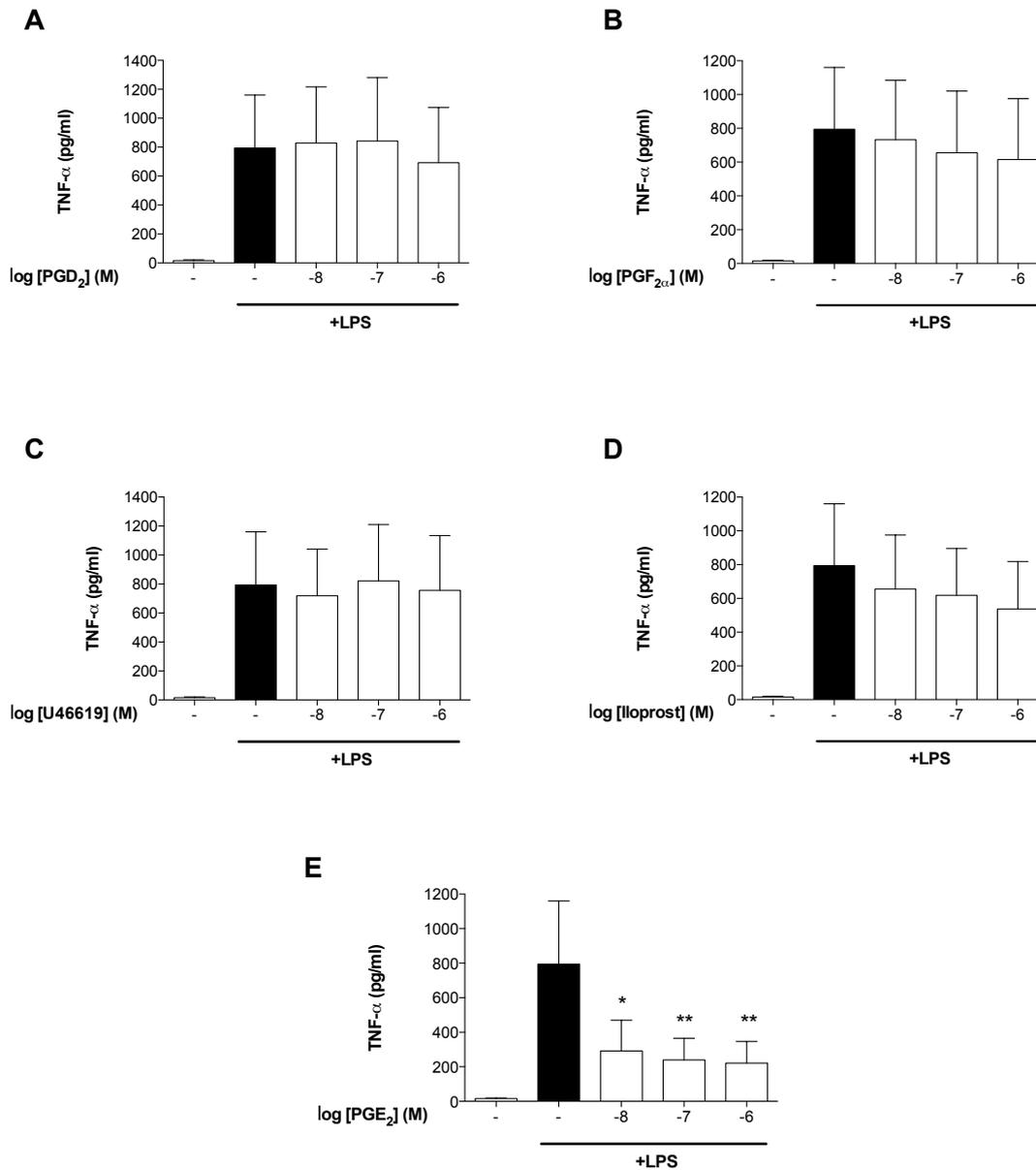
Figure 4.3. Effects of prostanoids on TNF-α generation from macrophages.
Macrophages were pre-incubated (30 min) with indomethacin (1 μM) and then with or without (A) PGD_2, (B) $PGF_{2\alpha}$, (C) U46619, (D) Iloprost or (E) PGE_2 for 30 min before challenge with LPS (1 ng/ml). After 22 h, TNF-α was measured in the supernatants. Values are mean ± SEM for 3 individual experiments. (One-way ANOVA with Dunnett's post test, *p<0.05, compared with LPS alone).
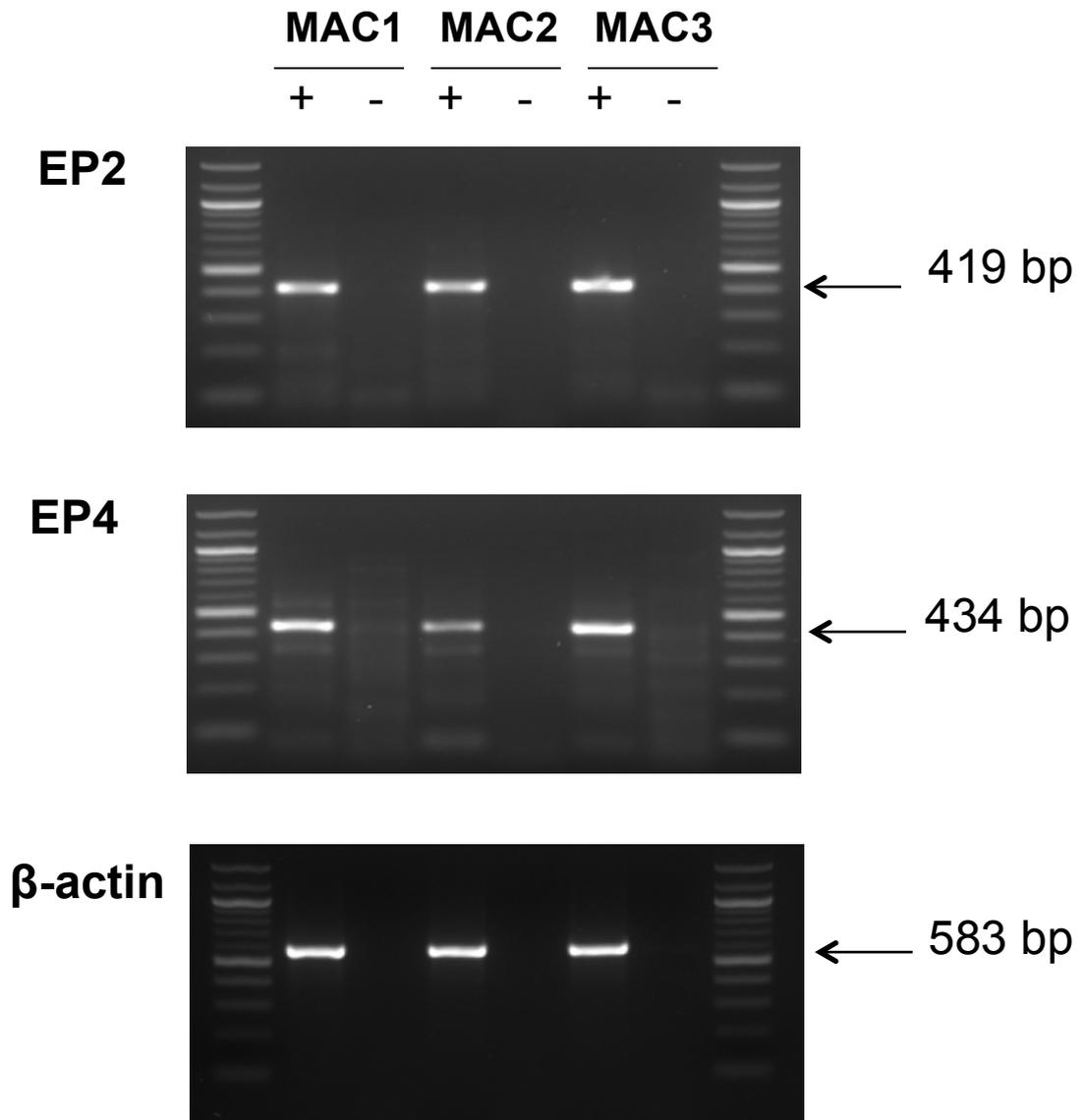
Figure 4.4. EP receptor expression in macrophages.
Isolated RNA was converted to cDNA in the presence (+) or absence (-) of reverse transcriptase. Amplification of cDNA was performed using primers specific for each of the EP receptor subtypes and β-actin. Expression profiles for three macrophage preparations (MAC1, MAC2 and MAC3) are shown. These findings are representative of a total of 4 different macrophage preparations in excess of 95% purity. Lanes at either end of each gel represent a 100 bp ladder.
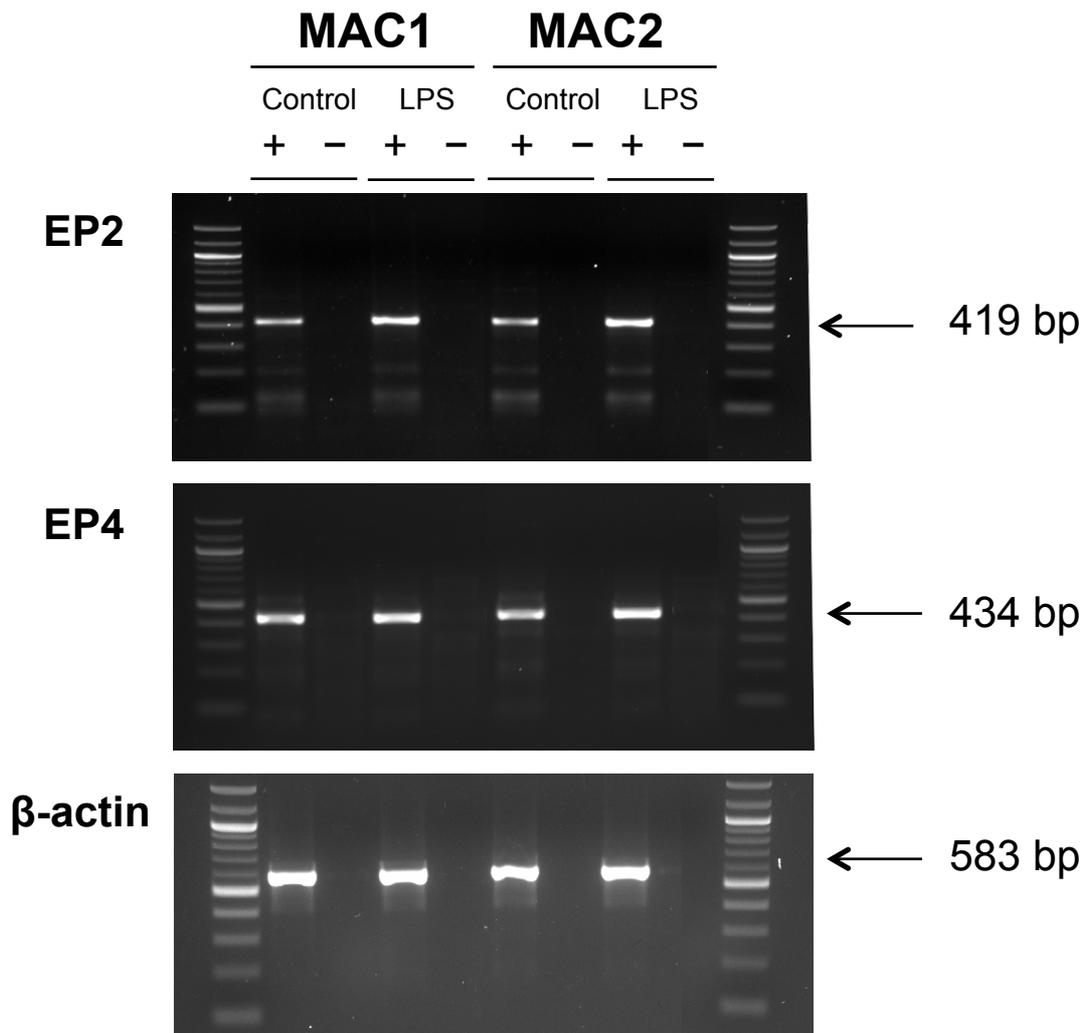
Figure 4.5. Effect of LPS on EP receptor expression in macrophages.
Macrophages were incubated without or with LPS (10 ng/ml) for 22 h. Isolated RNA
was converted to cDNA in the presence (+) or absence (-) of reverse transcriptase.
Amplification of cDNA was performed using primers specific for each of the EP
receptor subtypes and β-actin. Expression profiles for two macrophage preparations
(MAC1 and MAC2) are shown. These findings are representative of a total of 2
different macrophage preparations in excess of 95% purity. Lanes at either end of each
gel represent a 100 bp ladder.

4.2.4. Effects of PGE_2 on cyclic-AMP

Since EP_2 and EP_4 receptors are G-protein receptors coupled to adenylyl cyclase, it was investigated whether exposure (30 min) of macrophages to PGE_2 (1 µM) induced increases in total cell cyclic-AMP. The data demonstrated that PGE_2 induced statistically significant ($p < 0.05$) increases in total cell cyclic-AMP levels over basal (figure 4.6). Further studies demonstrated that PGE_2 maintained these increased cyclic-AMP levels in macrophages for up to 5 hours (data not shown).

4.2.5. Effects of EP agonists on cytokine generation

The effects of alternative EP agonists on macrophage function were explored. The effects of misoprostol (non-selective), butaprost (EP_2-selective) and L-902,688 (EP_4-selective) on LPS-induced TNF-α generation from macrophages were investigated. Macrophages were incubated with the agonists (all 10^{-5}-10^{-11} M) for 30 min before being challenged with LPS (1 ng/ml) for 22 h. The data show that misoprostol (figure 4.7A) was about 25-fold less potent than PGE_2 as an inhibitor of TNF-α generation (table 4.1). The EP_4 agonist, L-902,688 (figure 4.7B), was six-fold more potent than PGE_2 as an inhibitor of TNF-α generation whereas, by contrast, the EP_2-selective agonist, butaprost (figure 4.7C), was about 300-fold less potent than PGE_2 in this system (table 4.1). In further studies, the effects of an alternative EP_2-selective agonist, ONO-AE1-259, were determined (figure 4.7D) and ONO-AE1-259 was about 60-fold less potent than PGE_2 (table 4.1).
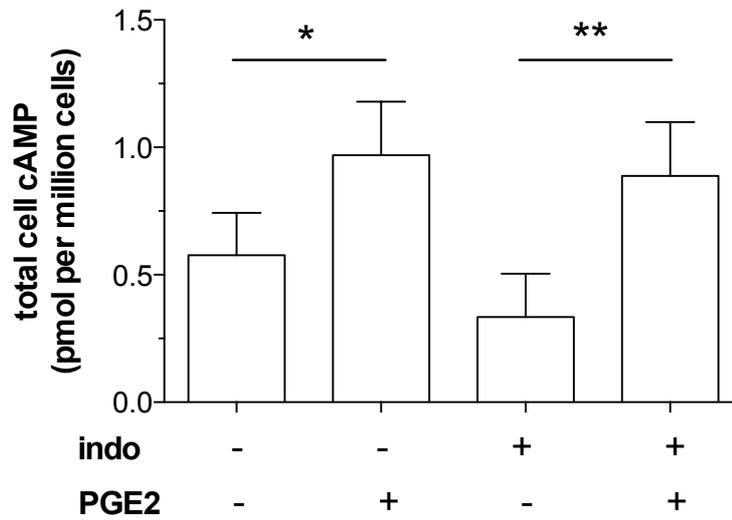
Figure 4.6. Effects of PGE$_2$ on cyclic-AMP.
Macrophages were pre-incubated (30 min) with or without indomethacin (indo; 1 µM) and then with or without PGE$_2$ (1 µM) for a further 30 min. After this treatment, the cells were solubilised and total cell cyclic-AMP levels measured. Values are mean ± SEM for 4 individual experiments. (Paired t tests, *p<0.05, **p<0.01).
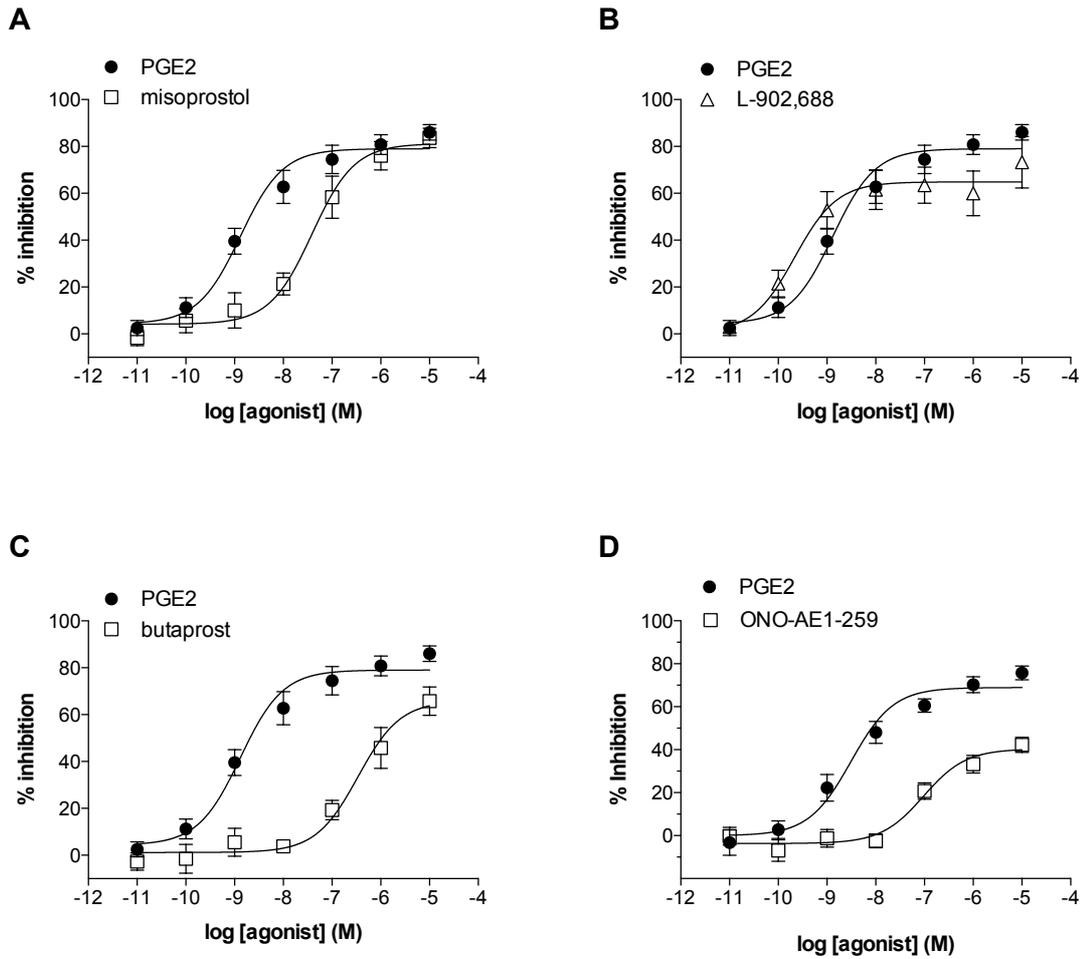
Figure 4.7. Effects of EP agonists on macrophages.
Macrophages were pre-incubated (30 min) with indomethacin (1 μM) and then with or without either (A) misoprostol, (B) L-902,688, (C) butaprost (D) ONO-AE1-259 or PGE$_2$ before challenge with LPS (1 ng/ml) for 22 h, after which TNF-α was measured in the supernatants. Values are expressed as the % inhibition of control cytokine releases which were (A, B, C) 1390 $\pm$ 505 pg/ml and (D) 2198 $\pm$ 1326 pg/ml, of TNF-α. Values are mean $\pm$ SEM for 4 (A, C, D) or 5 (B) individual experiments.

Table 4.1. EC$_{50}$ and E$_{max}$ values for the inhibition of TNF-α generation by EP agonists.

Agonist	EC$_{50}$ (nM)	E$_{max}$ (%)
PGE$_2$	1.8 ± 0.8	79 ± 3
misoprostol	46 ± 11	82 ± 5
L-902,688	0.3 ± 0.1	63 ± 7
butaprost	501 ± 262	67 ± 7
ONO-AE1-259	102 ± 18	41 ± 4

Table 4.1 shows the EC$_{50}$ and E$_{max}$ values for the inhibition of TNFα generation by EP agonists, calculated from the data shown in figure 4.7. Values are means ± SEM from 4 to 7 experiments.

4.2.6. Effects of EP antagonists on PGE$_2$ inhibition

The effects of the antagonists PF-04418948 (EP$_2$-selective) and CJ-042794 (EP$_4$-selective) were investigated (af Forselles et al., 2011, Murase et al., 2008) on the PGE$_2$ inhibition of LPS-induced TNF-α generation. Macrophages were incubated for 1 h with either PF-04418948 (300 nM) or CJ-042794 (300 nM) before incubation with PGE$_2$ for 30 min and then challenged with LPS (1 ng/ml) for 22 h. CJ-042794 effectively antagonized the PGE$_2$ inhibition of TNF-α generation (figure 4.8A). No antagonism of the PGE$_2$ inhibition was seen with PF-04418948 (figure 4.8B).

An alternative EP$_4$-selective antagonist, L-161,982 (Machwate et al., 2001), was also studied and in keeping with data obtained with CJ-042794, L-161,982 (300 nM) was found to be effective as an antagonist (figure 4.8C). An alternative EP$_2$-selective antagonist, PF-04852946, structurally distinct from PF-04418948, was also studied. PF-04852946 is about ten-fold more potent than PF-04418948 at EP$_2$ receptors (Kay et al., 2013). PF-04852946 (30 nM) was found to be an ineffective antagonist of the PGE$_2$ inhibition of TNF-α generation (figure 4.9).

The pK$_B$ (antagonist affinity) estimates for the antagonism of PGE$_2$ by CJ-042794 and L-161,982 were 8.77 ± 0.13 (K$_B$, 1.7 nM) and 8.46 ± 0.12 (K$_B$, 3.5 nM), respectively. These affinities are consistent with effects of these compounds at EP$_4$ receptors (Jones et al., 2009).
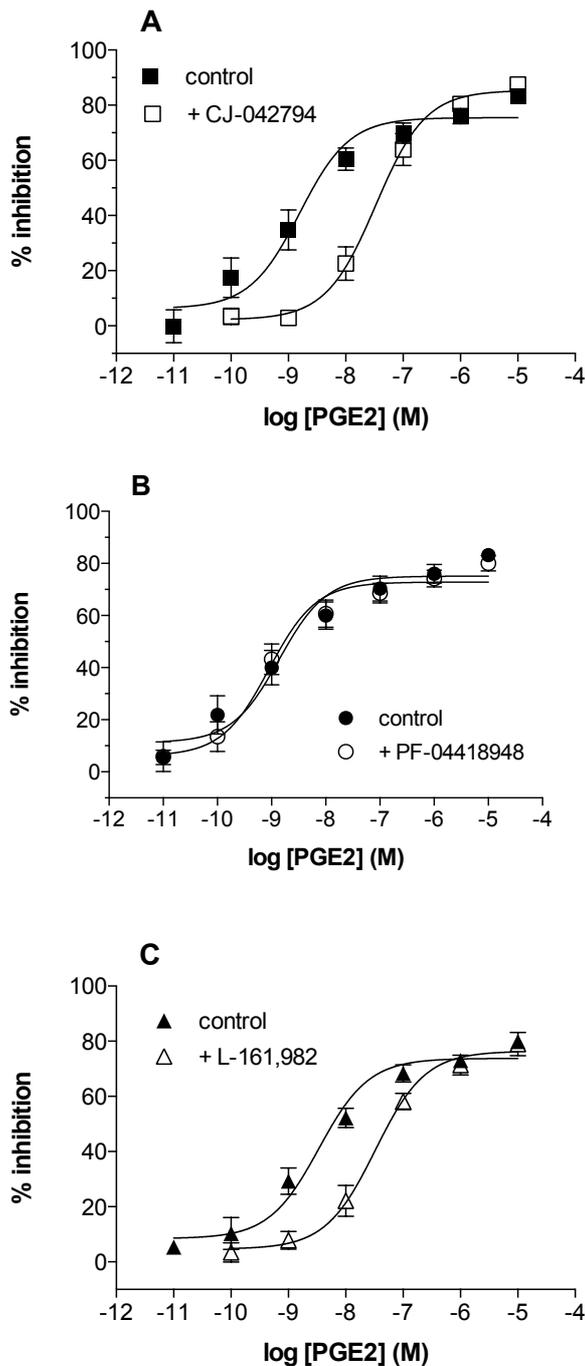
Figure 4.8. Effect of EP receptor antagonists on PGE$_2$ inhibition of TNF-α.
Macrophages were pre-incubated with indomethacin (1 μM) for 30 min and then
without or with EP-selective antagonists (300 nM) for 1 h and then without or with
PGE$_2$ for 30 min before challenge with LPS (1 ng/ml) for 22 h, after which TNF-α was
measured in the supernatants. The effects on PGE$_2$ of (A) the EP$_4$-selective antagonist
CJ-042794, (B) the EP$_2$-selective antagonist PF-04418948 and (C) the EP$_4$-selective
antagonist L-161,982 were evaluated. Values are expressed as the % inhibition of
control TNF-α releases which were, in the absence and presence of antagonist
respectively, (A) 2646 ± 562 and 2582 ± 496 pg/ml, (B) 3274 ± 502 and 3155 ± 551
pg/ml and (C) 2756 ± 882 and 2833 ± 862 pg/ml. Values are mean ± SEM for 5, 4 and
6 experiments for (A), (B) and (C), respectively.

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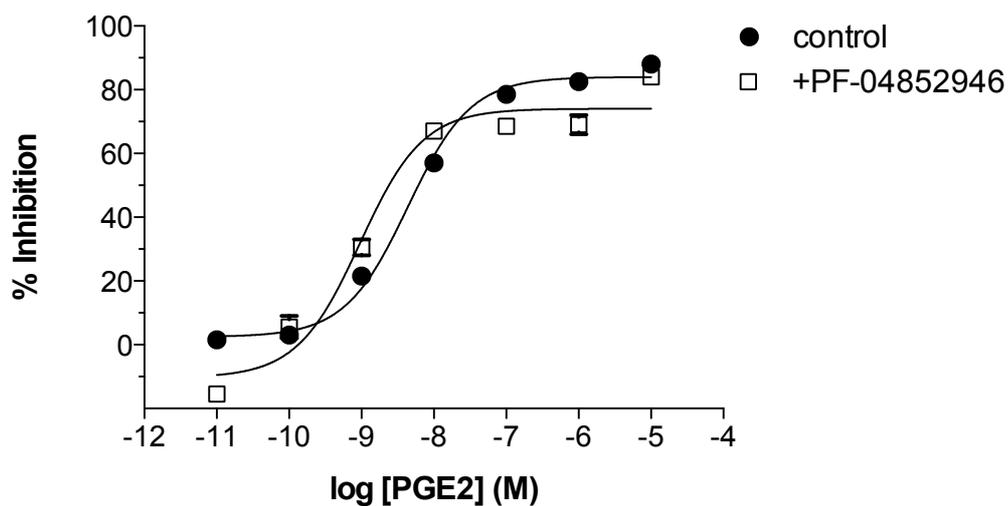
Figure 4.9. The effect of an alternative EP$_2$ antagonist on PGE$_2$ inhibition of TNF-α.
Macrophages were pre-incubated with indomethacin (1 μM) for 30 min and then without or with an EP-selective antagonist (30 nM) for 1 h and then without or with PGE$_2$ for 30 min before challenge with LPS (1 ng/ml) for 22 h, after which TNF-α was measured in the supernatants. The effects on PGE$_2$ of the EP$_2$-selective antagonist PF-04852946, was evaluated. Values are expressed as the % inhibition of control TNF-α releases which were, in the absence and presence of antagonist respectively, 243 ± 26 and 242 ± 21 pg/ml. Values are mean $\pm$ SEM for 2 individual experiments.

4.2.7. Effects of EP$_4$-selective antagonists on misoprostol inhibition

As antagonism of the PGE$_2$ inhibition of TNF-α generation was observed with the EP$_4$-selective antagonists CJ-042794 and L-161,982, the effects of the antagonists on inhibition by an alternative agonist, misoprostol, were explored. Macrophages were incubated with either CJ-042794 or L-161,982 (both 300 nM) for 1 h before incubation with misoprostol for 30 min and then challenged with LPS (1 ng/ml) for 22 h. Antagonism of the misoprostol inhibition was seen with both antagonists (figure 4.10). The pK$_B$ estimates for the antagonism of misoprostol by CJ-042794 and L-161,982 were approximately 8.07 for both antagonists (K$_B$, ~8.5 nM).

A

B
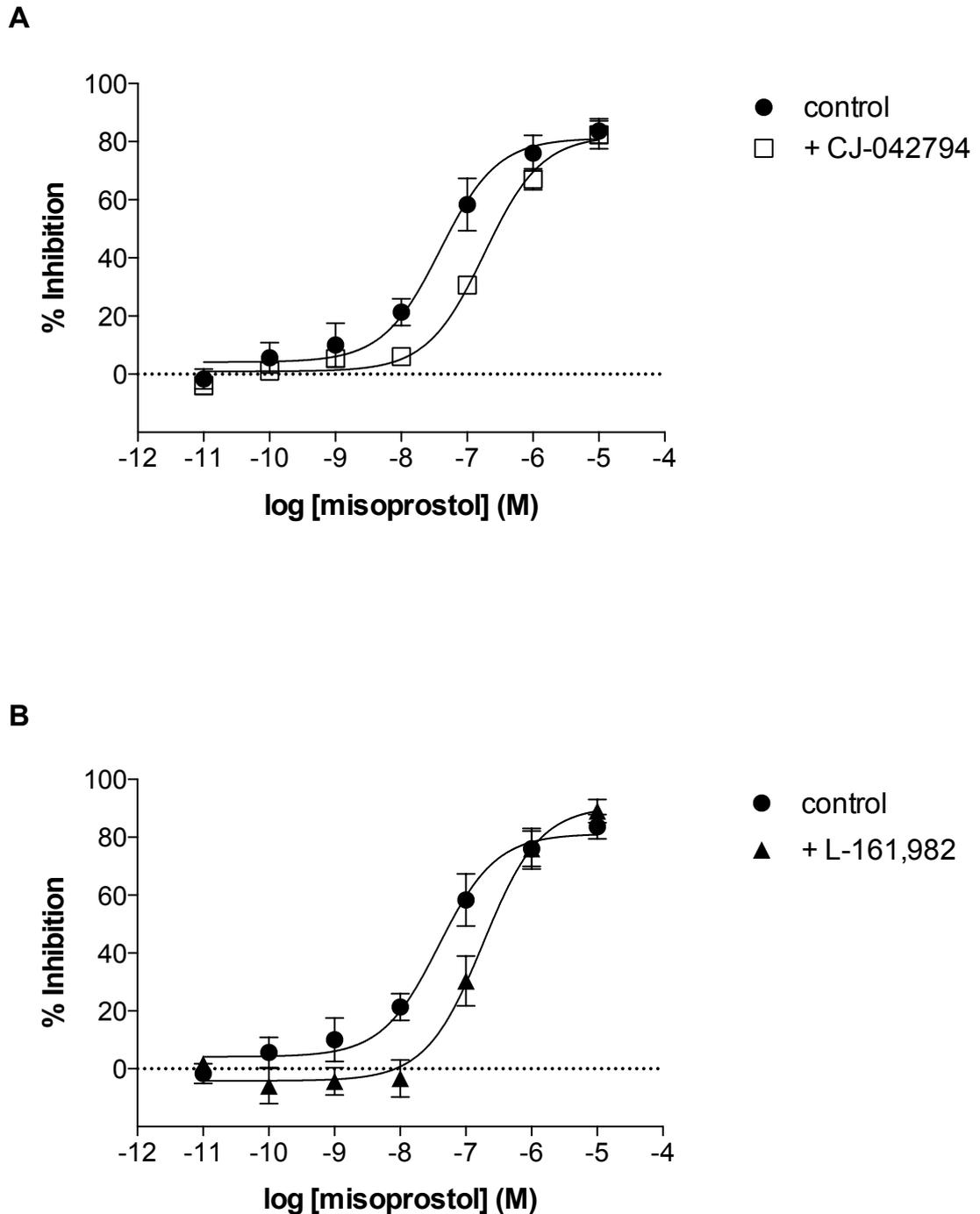
Figure 4.10. Effect of EP receptor antagonists on misoprostol inhibition of TNF-α.
Macrophages were pre-incubated with indomethacin (1 μM) for 30 min and then
without or with EP-selective antagonists (300 nM) for 1 h and then without or with
PGE_2 for 30 min before challenge with LPS (1 ng/ml) for 22 h, after which TNF-α was
measured in the supernatants. The effects on misoprostol of the EP_4-selective
antagonists CJ-042794 and L-161,982 were evaluated. Values are expressed as the %
inhibition of control TNF-α releases which were, in the absence and presence of
antagonist respectively, (A) 1061 ± 166 and 1216 ± 183 pg/ml and (B) 1061 ± 166 and
1199 ± 229 pg/ml. Values are mean ± SEM for 3 individual experiments.

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4.3. Discussion

Previous studies have shown that PGE_2 is an inhibitor of macrophage function (Rowe et al., 1997, Buenestado et al., 2012, Ratcliffe et al., 2007). However, the receptor involved in mediating this inhibitory effect has not been fully elucidated. This is largely because many of the pharmacological probes that have been available to study EP receptors have lacked selectivity. The recent emergence of selective agonists and especially antagonists, has enabled definitive characterization of the receptor involved in preventing cytokine generation from human lung macrophages, in this study.

In accord with previous studies, it has been shown that PGE_2 is an effective inhibitor of LPS-induced TNF-α generation from lung macrophages. This study also demonstrated that LPS stimulation of macrophages induced PGE_2 generation and it was evident that blocking this PGE_2 generation with the COX inhibitor indomethacin led to an increase in TNF-α generation by macrophages. This suggests that under normal circumstances, activated macrophages generate PGE_2 which acts in a paracrine fashion to limit the extent of TNF-α generation. These observations are followed up in more detail in Chapter 5. In further studies it was sought to establish the EP receptor through which PGE_2 acts in human lung macrophages.

In order to identify which EP receptors were expressed by human lung macrophages, RT-PCR studies were performed. These studies demonstrated that lung macrophages express mRNA for both EP_2 and EP_4 receptors but not EP_1 or EP_3 receptors. This suggests that EP_2 and/or EP_4 receptors are involved in mediating the effects of PGE_2. That PGE_2 induced increases in total cell cyclic-AMP in macrophages provides further support for the expression of EP_2 and/or EP_4 receptors in macrophages. This is because both EP_2 and EP_4 receptors are known to be coupled to adenylyl cyclase, so increases in

cyclic-AMP are consistent with the expression of EP_2 and/or EP_4 receptors in macrophages (Wilson et al., 2004).

The inhibition of LPS-induced TNF-α generation from lung macrophages by PGE_2 was compared to potential inhibition by other prostanoids. PGD_2 acts on DP receptors and U46619 acts on TP receptors (Ricciotti and FitzGerald, 2011). However, inhibition of TNF-α was not seen with either PGD_2 or U46619. A small amount of inhibition (although not significant) was seen with iloprost, which acts on IP receptors and with $PGF_{2\alpha}$ which acts on FP receptors (Ricciotti and FitzGerald, 2011). PGE_2 was the only one of the prostanoids tested, to effectively inhibit TNF-α generation. The expression of the receptors in the cells was not investigated. It may be the case that the receptors (other than EP receptors) that are linked to cAMP increases were expressed but not at a high enough level to lead to an inhibitory effect upon activation by their respective prostanoids. As the agonists utilized are not highly selective, it is also possible that iloprost and $PGF_{2\alpha}$ are acting on EP_2 or EP_4 receptors to cause some inhibition. Iloprost and $PGF_{2\alpha}$ are more potent agonists at the EP_2 and EP_4 receptors than PGD_2 and U46619 (Alexander et al., 2013b), which could explain the small inhibitory effects of iloprost and $PGF_{2\alpha}$ in this system. These data provide evidence that the PGE_2/EP axis is the only prostanoid system that effectively attenuates cytokine generation from lung macrophages. This further supports the rationale for investigating the inhibitory effects of PGE_2 and the EP receptor(s) mediating these effects, in lung macrophages.

In attempts to characterize EP receptors further, a range of EP agonists were studied for effects on cytokine generation. The non-selective agonist, misoprostol, was about 25-fold less potent than PGE_2 as an inhibitor of TNF-α generation. This potency ratio is consistent with an effect of misoprostol at EP_4 receptors since at EP_2 receptors

misoprostol is only about 7-fold less potent than PGE_2 (Abramovitz et al., 2000). Alternative agonists were also studied and it was of interest that the EP_4 agonist L-902,688, was about 6-fold more potent than PGE_2. By contrast, the EP_2-selective agonists, butaprost and ONO-AE1-259, were about 350 to 1500-fold less potent than L-902,688. Overall, these data provide preliminary evidence that the EP_4 receptor is responsible for mediating the effects of PGE_2 and argue against an EP_2 effect, since the concentrations of both butaprost and ONO-AE1-259 required for inhibition were higher than those usually associated with effects at EP_2 receptors (Wilson et al., 2004, Benyahia et al., 2012, Clarke et al., 2004).

In further studies the effects of EP_2 and EP_4 antagonists on the PGE_2 response in macrophages were investigated. It is noteworthy that the EP_2 antagonists, PF-04418948 and PF-04852946 that were used in this study are highly selective ligands and considerably superior to AH6809 which until now was the only EP_2 antagonist available (af Forselles et al., 2011, Kay et al., 2013). AH6809 shows poor selectivity and potency such that data generated with this antagonist are unlikely to be reliable (Abramovitz et al., 2000, Jones et al., 2009). Neither of the two EP_2 antagonists used in this study had any effect on the PGE_2 inhibition of TNF-α generation. By contrast, two EP_4 antagonists, CJ-042794 (K_B; 1.7 nM) and L-161,982 (K_B; 3.5 nM) effectively reversed the PGE_2 inhibition of TNF-α generation with affinities consistent with antagonism at EP_4 receptors (Jones et al., 2009). These data provide compelling evidence that the receptor that mediates the inhibitory effects of PGE_2 in human lung macrophages is the EP_4 receptor. Both EP_4 antagonists CJ-042794 and L-161,982 also antagonized misoprostol inhibition. Although both were less potent antagonists of misoprostol inhibition than of PGE_2 inhibition, these data further support the suggestion that PGE_2 is acting at the EP_4 receptor to attenuate TNF-α generation from activated macrophages.

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The suggestion has been made that the EP_4 receptor could be a target for respiratory diseases. This contention has been based largely on recent studies that have shown that PGE_2 mediates bronchodilation via the EP_4 receptor (Buckley et al., 2011, Benyahia et al., 2012). In addition to bronchodilation, the present study has demonstrated that targeting the EP_4 receptor may also provide additional desirable anti-inflammatory effects by stabilizing lung macrophage activity.

The prospect that the EP_4 receptor could be targeted in order to limit pro-inflammatory cytokine generation from macrophages is tempered by the finding that there are marked differences in the effects of PGE_2 on cytokine generation from macrophages. Whereas PGE_2 effectively blocks TNF-α and IL-6 generation from macrophages, it is completely ineffective against IL-8. Similar findings have been reported elsewhere (Standiford et al., 1992). The reason for this difference is not immediately obvious since it is thought that the LPS-mediated signalling pathways leading to the generation of these cytokines are similar (Thorley et al., 2007).

It has been reported in another study using human alveolar macrophages, that both EP_2 and EP_4 receptors are activated by PGE_2. The study reported that the activation of each receptor may be agonist concentration-dependent, where EP_4 receptors are activated at low concentrations of PGE_2 and EP_2 receptors at higher concentrations (Ratcliffe et al., 2007). However, it should be noted that this study was carried out using only the EP_4 antagonist ONO-AE2-227 and no EP_2 antagonist. Although butaprost was used as diagnostic for EP_2 receptors, butaprost can also have effects at EP_4 receptors at high concentrations (Wilson et al., 2004). In the present study, utilizing a range of agonists

and antagonists provides compelling evidence that PGE_2 acts at EP_4 receptors and not EP_2 receptors, to inhibit cytokine generation from lung macrophages.

Despite evidence from functional studies to the contrary, the mRNA studies carried out demonstrate expression of both EP_2 and EP_4 receptors in human lung macrophages, suggesting a possible role for EP_2 receptors in macrophages. The data also suggest that it is possible that the EP_2 receptor is induced in response to LPS. This indicates that the EP_2 receptor could also be a target for stabilization of macrophages following LPS activation. However, the protein expression of the receptors could not be demonstrated due to the lack of availability of reliable antibodies. Therefore, it is unknown whether translation to EP_2 receptor protein expression occurs. It is possible that the EP_2 receptor, if present, is involved in modulating other macrophage functions such as phagocytosis. Another study reported that PGE_2 acts at the EP_2 receptor to inhibit phagocytosis in rat alveolar macrophages (Aronoff et al., 2004). This was not investigated in the present study but would be of interest to explore in future investigations.

In summary, this study demonstrates that although human lung macrophages may express both EP_2 and EP_4 receptors, PGE_2 acts through the EP_4 receptor to inhibit pro-inflammatory cytokine generation.

Chapter 5: Characterization of the cyclooxygenase (COX) isoform responsible for PGE$_2$ generation in human lung macrophages

5.1. Introduction

Prostanoids are synthesized by the metabolism of arachidonic acid. The first step in the biosynthesis is the conversion of arachidonic acid to an endoperoxide intermediate, prostaglandin H$_2$. This reaction is catalyzed by cyclooxygenase enzymes, of which there are two isoforms, COX-1 and COX-2. COX-1 is believed to be constitutively expressed, whereas COX-2 is believed to be the inducible isoform, induced for example, by inflammatory stimuli (Tilley et al., 2001). Prostaglandin H$_2$ is converted by specific synthases, to specific prostanoids. These prostanoids include four prostaglandins and thromboxane A$_2$ (Ricciotti and FitzGerald, 2011), which are differentially produced by different cells (Tilley et al., 2001). One of the prostaglandins known to be produced by macrophages is prostaglandin E$_2$ (PGE$_2$) and this was confirmed by the studies in Chapter 4 of this thesis. PGE$_2$ has been demonstrated to have an inhibitory effect on cytokine release from human lung macrophages.

The timing of synthesis of PGE$_2$ resulting from COX-1 or COX-2 activation can be different, as COX-2 activation, unlike COX-1, requires de novo PGE$_2$ mRNA and protein synthesis to occur (Williams and Shacter, 1997). It is feasible that the roles of PGE$_2$ generated by the cell could be different, depending on whether it is driven by COX-1 or COX-2 activation.

In this study, a number of approaches were utilized to investigate the COX isoform(s) responsible for PGE_2 production in human lung macrophages. The constitutive expression of COX-1 and COX-2, in addition to the expression following challenge with inflammatory stimuli, was investigated. The inflammatory stimuli used were the TLR agonists LPS and poly(I:C). In addition to these, the bacteria *Staphylococcus aureus* and *Neisseria meningitidis* were also tested. Expression of the COX isoforms was investigated using RT-PCR and Western blotting. COX-1 and COX-2-selective inhibitors were also utilized to further determine the involvement of each of the isoforms in PGE_2 production from macrophages. The effect of the COX inhibitors on cytokine release was also investigated.

5.2. Results

5.2.1. Time dependency of LPS-induced PGE_2 generation from lung macrophages

Macrophages were challenged with LPS (10 ng/ml) for 2, 5, 16 and 22 h. The levels of spontaneously generated and LPS-induced PGE_2 were measured after each time point. Although there were differences between three experiments (figure 5.1A, B and C) in the overall levels of PGE_2 generated over the time course, generally the pattern was the same. Although PGE_2 was generated spontaneously in all of the experiments, in response to LPS challenge, there was a time-dependent increase in the levels of PGE_2. Maximal generation of PGE_2 was observed 16 h after activation with LPS.

A

B

C
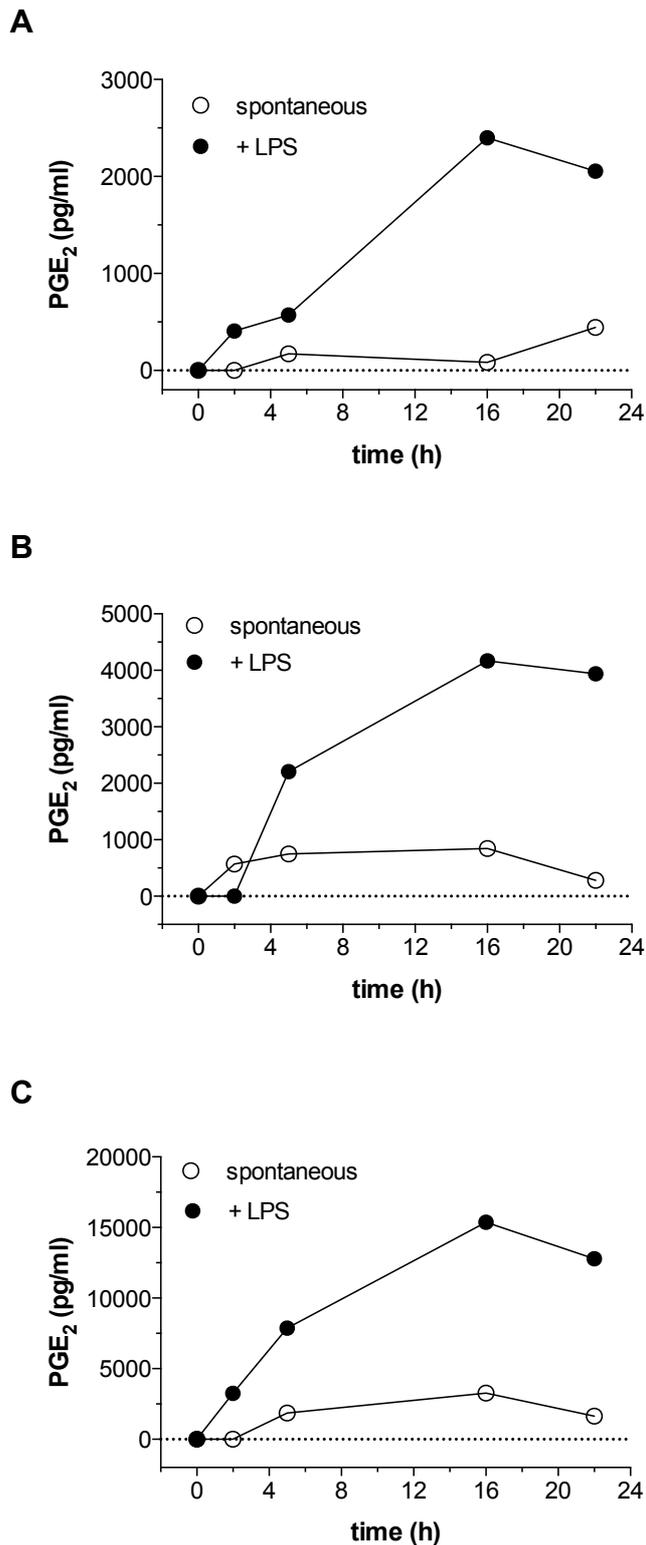
Figure 5.1. Time-dependent LPS-induced PGE$_2$ generation. Macrophages were incubated without or with LPS (10 ng/ml). At 2, 5, 16 and 22 h, cell culture supernatants were harvested and assayed for PGE$_2$. Graphs are of 3 individual experiments. (A) experiment 1, (B) experiment 2 and (C) experiment 3.

5.2.2. Constitutive and LPS-induced gene expression of COX-1 and COX-2

The expression of the two isoforms of COX was investigated in human lung macrophages at the mRNA level. Macrophages were solubilized with TRI Reagent® to enable RNA extraction. Reverse transcriptase PCR (RT-PCR) using primers specific for each of the COX isoforms demonstrated constitutive expression of both COX-1 and COX-2 in three separate cell preparations (figure 5.2). Densitometric analysis showed that although there was some variability between cell preparations, the expression of COX-1 and COX-2 was generally similar relative to β-actin.

To determine whether the expression of either isoform was altered in response to LPS, macrophages were challenged with LPS (10 ng/ml) for 22 h. The RT-PCR results and the corresponding densitometric analysis demonstrated that expression of COX-1 was up-regulated in response to LPS compared to the control in just one of the two cell preparations tested, by a 23% increase. Expression of COX-2 however, was up-regulated in response to LPS in both cell preparations tested and the up-regulation was greater than that of COX-1, at a mean increase of 55% (figure 5.3).
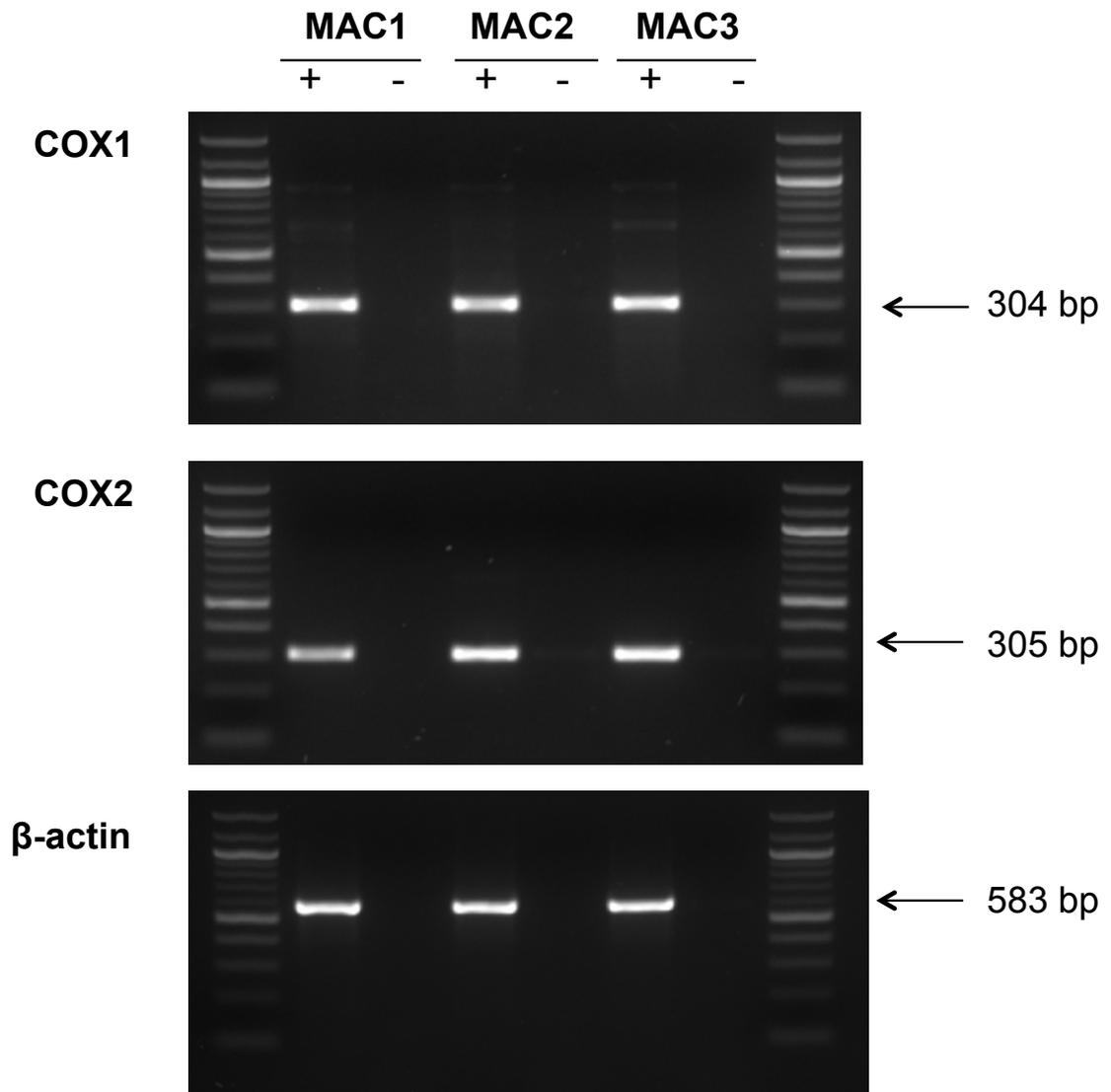
Figure 5.2. Expression of COX-1 and COX-2 by RT-PCR.
RNA from unstimulated macrophages was converted to cDNA in the presence (+) or absence (−) of reverse transcriptase. Amplification of cDNA was carried out using COX-1 and COX-2 specific primers. Data shown are of 3 different macrophage (MAC) preparations in excess of 95% purity. Lanes at either end of each gel represent a 100 bp ladder.
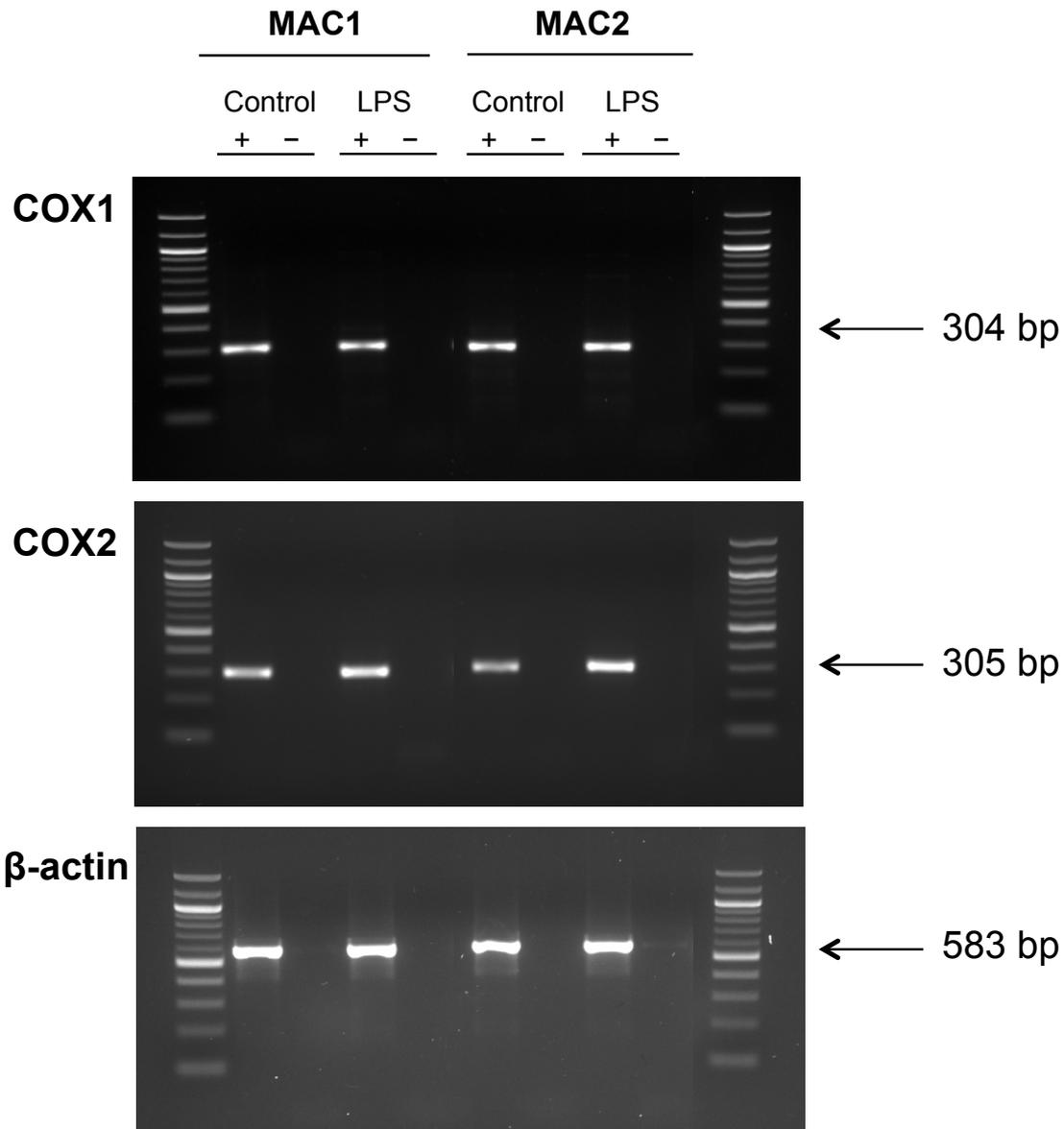
Figure 5.3. LPS-induced expression of COX-1 and COX-2.
Macrophages were incubated without or with LPS (10 ng/ml) for 22 h. Isolated RNA
from was converted to cDNA in the presence (+) or absence (−) of reverse transcriptase.
Amplification of cDNA was carried out using COX-1 and COX-2 specific primers.
Data shown are of 2 different macrophage (MAC) preparations in excess of 95% purity.
Lanes at either end of each gel represent a 100 bp ladder.

5.2.3. Effect of TLR agonists on COX induction

Macrophages were challenged without or with increasing concentrations of the TLR4 agonist LPS (0.1-10 ng/ml) for 22 h, after which protein lysis was performed. The protein lysates were analysed by SDS-PAGE followed by Western blotting using antibodies to COX-1 and COX-2. The constitutive expression of both isoforms was found to be low. COX-1 expression was not altered from constitutive levels, following LPS challenge, whereas there was an LPS concentration-dependent increase in COX-2 protein expression. Densitometric analysis showed that there was a two-fold increase in COX-2 expression from the control level with 1 ng/ml LPS and a five-fold increase over the control with 10 ng/ml LPS (figure 5.4).

Comparison of the effects of LPS and the TLR3 agonist poly(I:C) on protein expression in two separate cell preparations was then carried out. Macrophages were challenged with LPS (10 ng/ml) or poly(I:C) (10 μg/ml) for 22 h. LPS again induced up-regulation of COX-2 but not COX-1 in both cell preparations (figure 5.5A and 5.5B). Densitometric analysis showed that the up-regulation of COX-2 from the control was 19-fold in one cell preparation and four-fold in the other in response to LPS. Poly(I:C) did not result in up-regulation of COX-1 or COX-2 in either cell preparation (figure 5.5A and 5.5B).
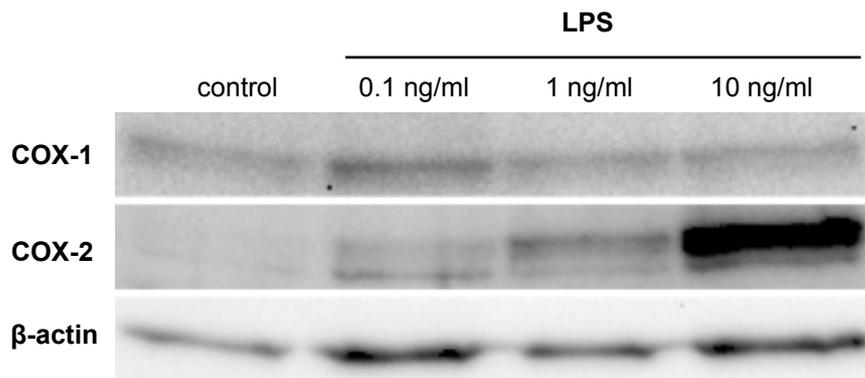
Figure 5.4. LPS-induced protein expression of COX-1 and COX-2.
Macrophages were incubated without or with LPS for 22 h, after which the cells were solubilized and cell lysates (9×10^5 cell equivalents) were subjected to SDS-PAGE electrophoresis. Separated proteins were transferred on to a nitrocellulose membrane and this was probed for COX-1, the membrane stripped and then probed for actin and following a further strip, probed for COX-2. Bands detected for COX-1 (~72 kDa), COX-2 (~72 kDa) and β-actin (42 kDa) are shown. Data are of 1 experiment.

A

B
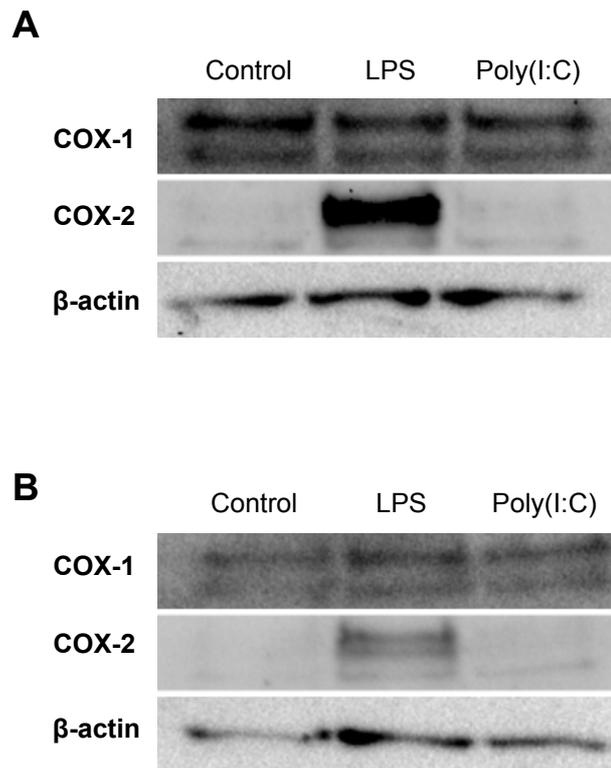
Figure 5.5. Poly(I:C)-induced protein expression of COX-1 and COX-2.
Macrophages were incubated without or with LPS (10 ng/ml) or poly(I:C) (1 μg/ml) for 22 h, after which the cells were solubilized and cell lysates (9 x 10^5 cell equivalents) were subjected to SDS-PAGE electrophoresis. Separated proteins were transferred on to a nitrocellulose membrane and this was probed for COX-1, the membrane stripped and then probed for actin and following a further strip, probed for COX-2. Bands detected for COX-1 (~72 kDa), COX-2 (~72 kDa) and β-actin (42 kDa) are shown. Data shown are of 2 individual experiments, (A) experiment 1 and (B) experiment 2.

5.2.4. Expression of COX-1 and COX-2 in response to pathogens

Macrophages were infected with *Staphylococcus aureus* (Newman strain) at a multiplicity of infection (MOI) of 0.05, 0.5 and 5 or *Neisseria meningitidis* (MC58 type B) at MOI 10 for 16 h. The protein lysates were analysed by Western blotting. Neither of the pathogens resulted in induction of COX-1 expression. Slight up-regulation of COX-2 was observed in response to *S.aureus* at the highest MOI investigated (figure 5.6). (The bands visible underneath the bands for COX-2 are likely to be a result of non-specific binding of the antibodies to *S.aureus* Protein A, approximately 55 kDa). Exposure of macrophages to *N. meningitidis* resulted in a dramatic up-regulation of COX-2 (figure 5.6). Densitometric analysis showed that this was a 29-fold increase in COX-2 expression over the control level.

5.2.5. The effect of COX inhibitors on LPS-induced PGE$_2$ generation

The effect of inhibiting COX on the generation of PGE$_2$ was investigated. Macrophages were pre-treated for 30 min with the non-selective COX inhibitor indomethacin, the COX-1 selective inhibitor FR122047 or the COX-2 selective inhibitor celecoxib (all 10^{-6} M), followed by incubation without or with LPS (10 ng/ml) for 22 h. In the absence of the inhibitors, PGE$_2$ was spontaneously generated and there was a significant ($p<0.01$) increase upon LPS stimulation. LPS-induced PGE$_2$ was significantly ($p<0.01$) inhibited by all three of the COX inhibitors, to levels below those of the spontaneous PGE$_2$. Indomethacin inhibited by ~100%, FR122047 inhibited by ~88% and celecoxib resulted in ~97% inhibition (figure 5.7).
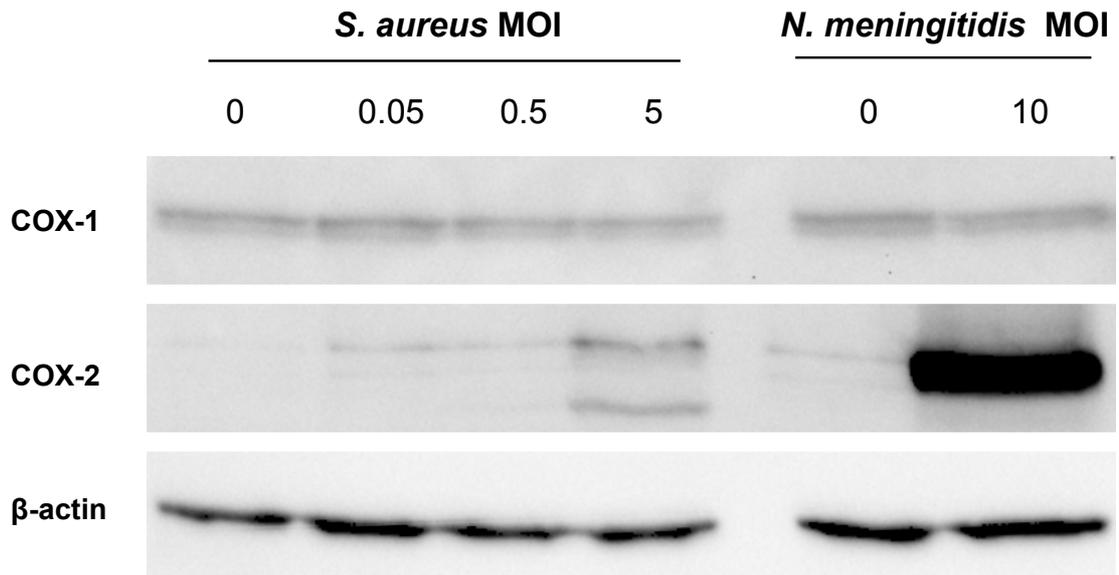
Figure 5.6. *S.aureus* **and** *N. meningitidis***-induced protein expression of COX-1 and COX-2.**

Macrophages were infected with *S.aureus* or *N. meningitidis* at the multiplicity of infection (MOI) indicated for 16 h. After this time, the cells were solubilized and cell lysates (9 x 10^5 cell equivalents) were subjected to SDS-PAGE electrophoresis. Separated proteins were transferred on to a nitrocellulose membrane and this was probed for COX-1, the membrane stripped and then probed for actin and following a further strip, probed for COX-2. Bands detected for COX-1 (~72 kDa), COX-2 (~72 kDa) and β-actin (42 kDa) are shown. Data shown are representative of a total of 3 individual experiments.
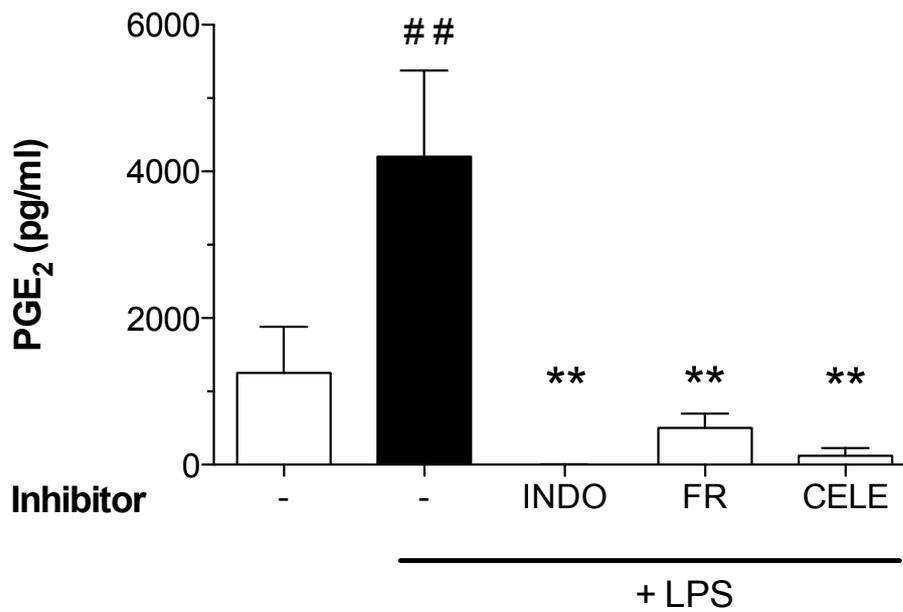
Figure 5.7. Effect of COX inhibitors on LPS-induced PGE$_2$ generation.
Macrophages were pre-treated for 30 min with indomethacin (INDO), FR122047 (FR) or celecoxib (CELE) (all 10^{-6} M) before challenge with LPS (10 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for PGE$_2$. Drugs alone had no effect on spontaneous PGE$_2$ release. Values are mean ± SEM for 4 individual experiments. (One-way ANOVA with Dunnett's post test, ##$p < 0.01$ compared with spontaneous release, **$p < 0.01$ compared with LPS alone).

To attempt to determine whether inhibition of PGE_2 generation by FR122047 and celecoxib was concentration-dependent, macrophages were incubated with a range of concentrations of the inhibitors (10^{-12}-10^{-7} M) for 30 min followed by challenge with LPS (1 ng/ml). Inhibition of LPS-induced PGE_2 was not seen in response to FR122047 (figure 5.8A). Celecoxib at the highest concentration studied (10^{-7} M) inhibited PGE_2 generation (figure 5.8B).

5.2.6. The effect of COX inhibitors on LPS-induced TNF-α generation

It has been demonstrated that macrophages produce PGE_2 endogenously upon LPS challenge and in Chapter 4 of this thesis it was demonstrated that this generated PGE_2 can act in a paracrine fashion to inhibit cytokine release. Therefore, the effect of the COX inhibitors on LPS-induced TNF-α release was investigated. Macrophages were treated with increasing concentrations of indomethacin, FR122047 or celecoxib for 30 min followed by challenge with LPS (1 ng/ml) for 22 h. Cell culture supernatants were assayed for TNF-α release. Indomethacin (figure 5.9A) and celecoxib (figure 5.9C) caused slight concentration-dependent increases in TNF-α release, whereas FR122047 only caused a slight increase at the highest concentration tested. However, none of the increases were significant compared to LPS-induced TNF-α in the absence of COX inhibitors.

To attempt to further determine whether there was a concentration-dependent effect of FR122047 and celecoxib on TNF-α release, macrophages were incubated with a range of concentrations (10^{-12}-10^{-7} M) of FR122047 (figure 5.10A) or celecoxib (figure 5.10B) for 30 min followed by challenge with LPS (1 ng/ml) for 22 h. Cell culture supernatants were assayed for TNF-α release. None of the COX inhibitors affected TNF-α generation.

A

B
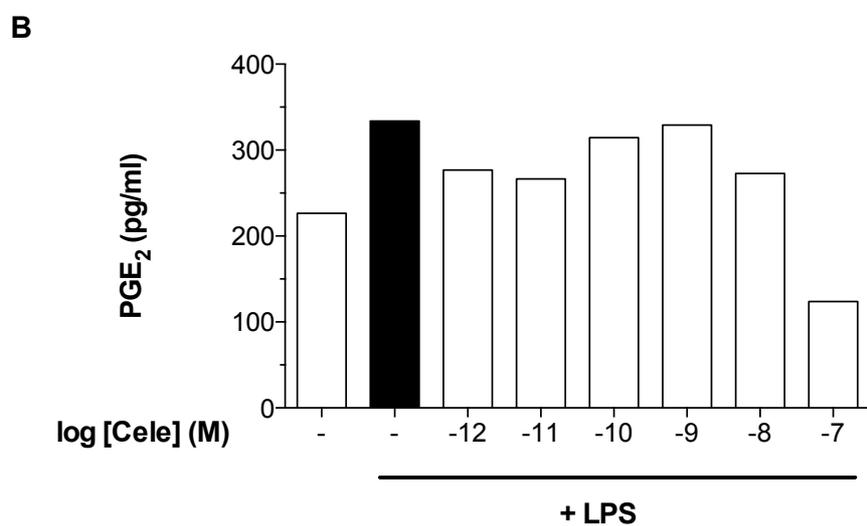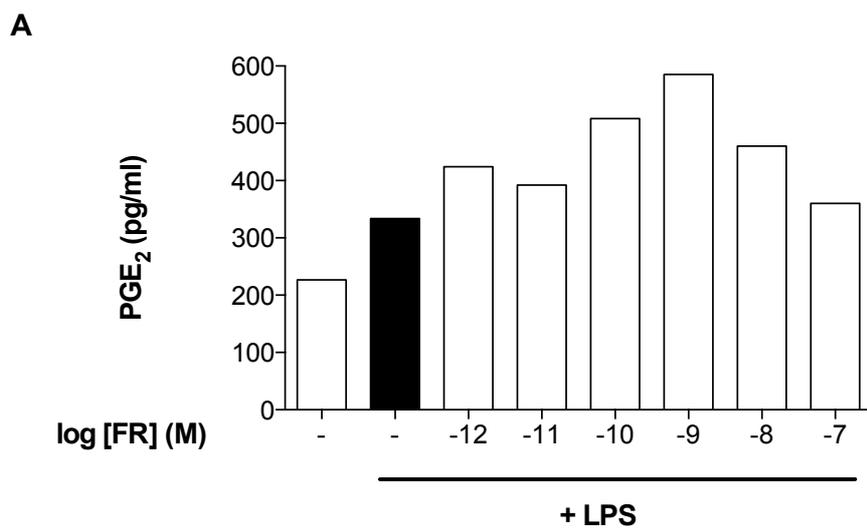
Figure 5.8. Effect of COX-1 and COX-2 selective inhibitors on PGE$_2$ generation.
Macrophages were pre-treated for 30 min with (A) FR122047 (FR) or (B) celecoxib
(Cele) before challenge with LPS (1 ng/ml). After 22 h, cell culture supernatants were
harvested and assayed for PGE$_2$. Drugs alone had no effect on spontaneous PGE$_2$
release. Values are for 1 experiment.

A

B

C
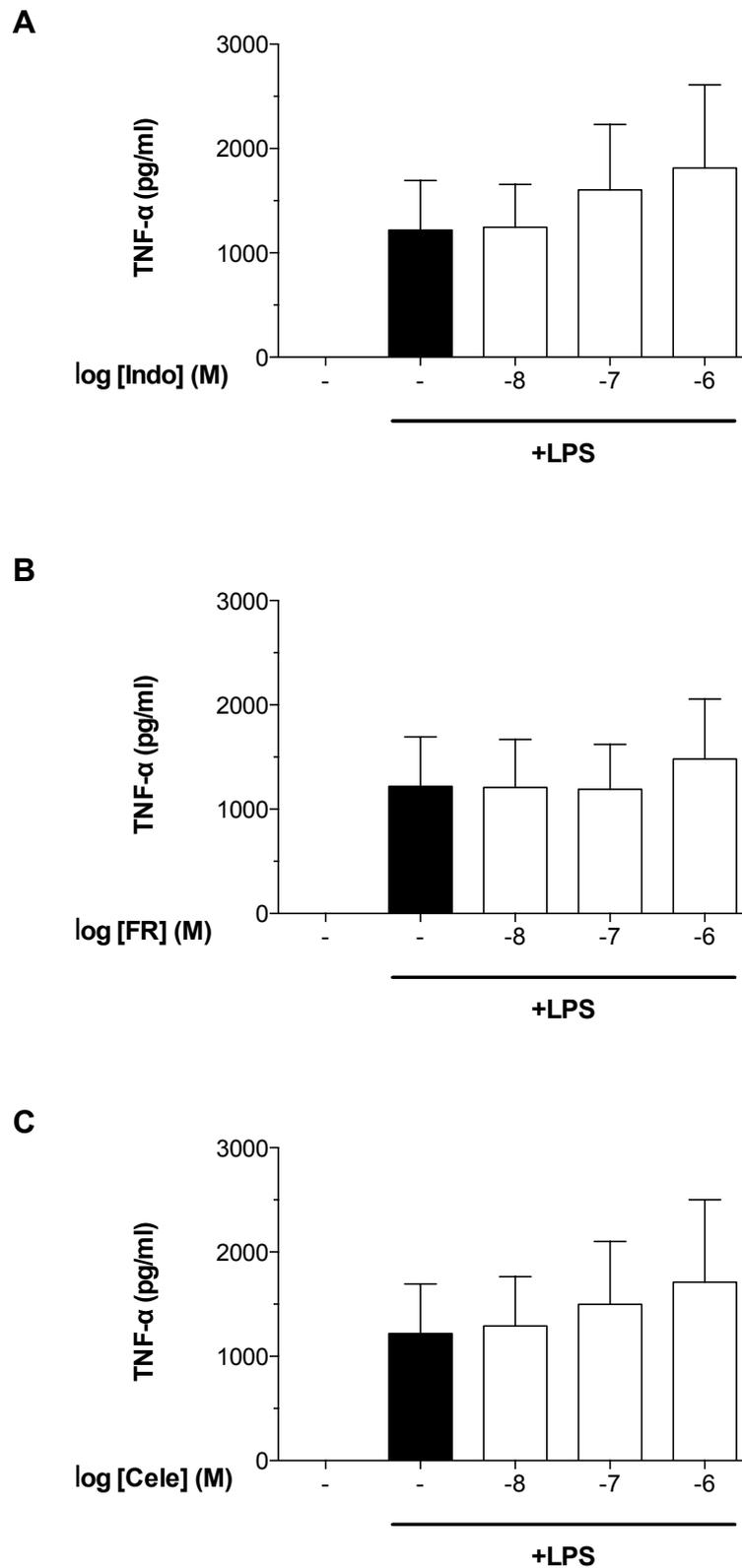
Figure 5.9. Effect of COX inhibitors on TNF-α generation.
Macrophages were pre-treated for 30 min with (A) indomethacin (Indo), (B) FR122047 (FR) or (C) celecoxib before challenge with LPS (1 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for TNF-α. Drugs alone had no effect on spontaneous cytokine release. Values are mean ± SEM for 4 individual experiments. (One-way ANOVA with Dunnett's post test, all $p>0.05$ compared with LPS alone)

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A
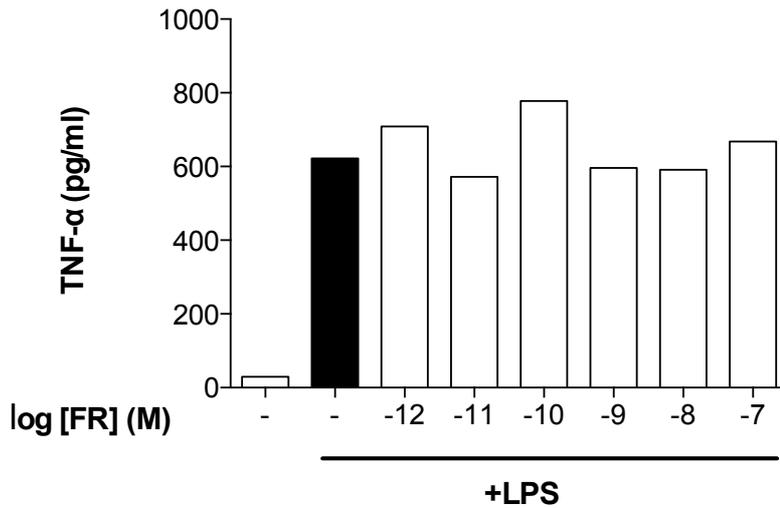
B
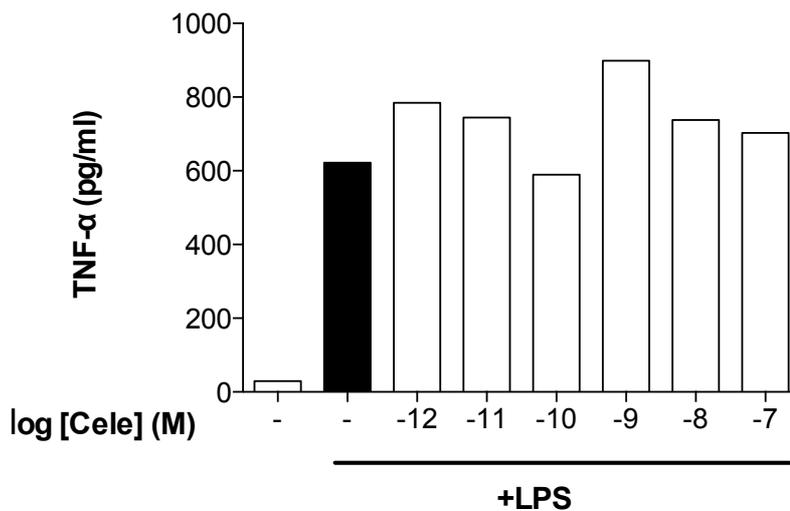
Figure 5.10. Effect of COX inhibitor concentration on TNF-α generation from macrophages.
Macrophages were pre-treated for 30 min with (A) FR122047 (FR) or (B) celecoxib (Cele) before challenge with LPS (1 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for TNF-α. Drugs alone had no effect on spontaneous cytokine release. Values are for 1 experiment.

5.2.7. The effect of COX inhibitors on LPS-induced IL-6 generation

The effect of COX inhibitors on LPS induced IL-6 release was investigated.

Macrophages were incubated with indomethacin (figure 5.11A), FR122047 (figure

5.11B) or celecoxib (figure 5.11C) (all 10^{-8}-10^{-6} M) for 30 min before challenge with

LPS (1 ng/ml) for 22 h. Cell culture supernatants were assayed for IL-6. All three COX

inhibitors had virtually no effect on IL-6 release.

5.2.8. Effects of EP antagonists on LPS-induced TNF-α generation

The effects of the antagonists PF-04418948 (EP_2-selective) and CJ-042794 (EP_4-

selective) were investigated on concentration-dependent LPS-induced cytokine release

(figure 5.12). Macrophages were incubated with the antagonists (300 nM) for 1 h before

being challenged with LPS (0.1-10 ng/ml) for 22 h. CJ-042794 caused a significant

increase ($p<0.01$) in TNF-α generation at all three concentrations of LPS. No significant

($p>0.05$) increase was observed in the presence of PF-04418948.

5.2.9. Comparison of the effect of COX inhibitors on cytokine generation from MDMs and lung macrophages

MDMs and lung macrophages were treated with indomethacin, FR122047 or celecoxib

(10^{-6} M) for 30 min followed by challenge with LPS (1 ng/ml) for 22 h. Cell culture

supernatants were assayed for TNF-α release. Indomethacin appeared to enhance LPS-

induced TNF-α release from MDMs, as did celecoxib to some extent. By contrast,

FR122047 did not have much of an effect on TNF-α release (figure 5.13A). A similar

trend in results was seen with these COX inhibitors on LPS-induced TNF-α release

from lung macrophages, although the effects were less pronounced (figure 5.13B).

A

B

C
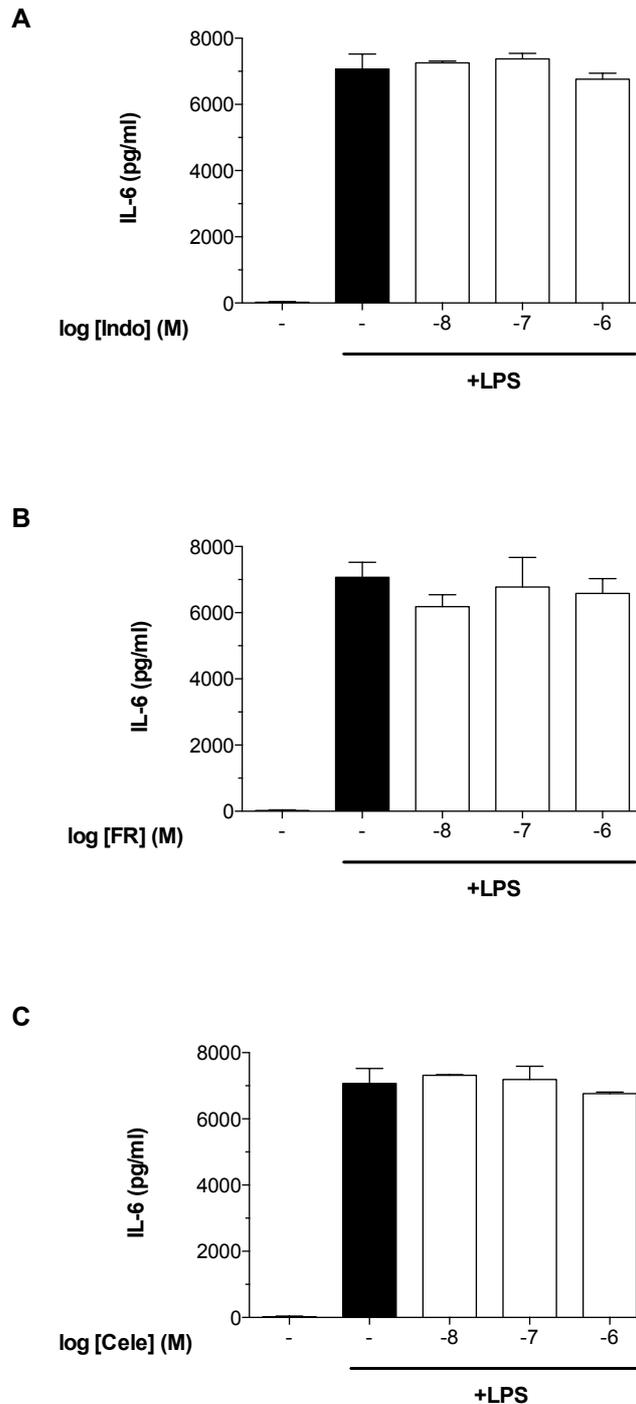
Figure 5.11. Effect of COX inhibitors on IL-6 generation.
Macrophages were pre-treated for 30 min with (A) indomethacin (Indo), (B) FR122047 (FR) or (C) celecoxib before challenge with LPS (1 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for IL-6. Drugs alone had no effect on spontaneous cytokine release. Values are mean ± SEM for 2 individual experiments. (One-way ANOVA with Dunnett's post test, all p>0.05 compared with LPS alone).
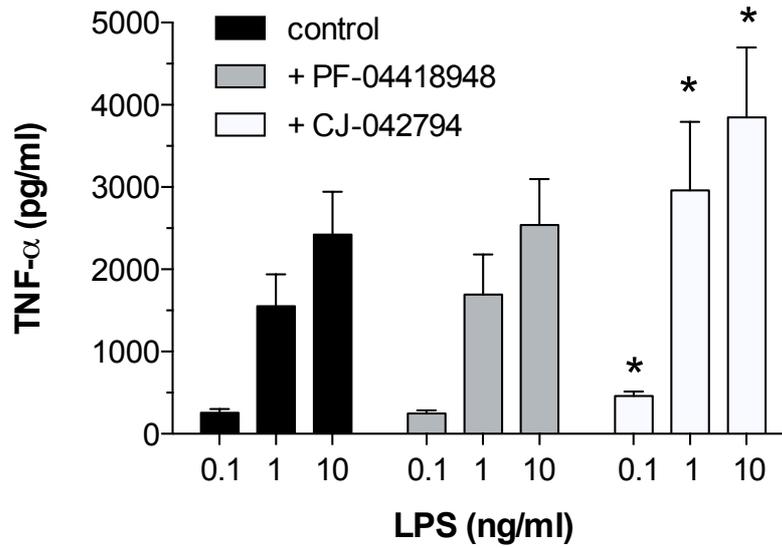
Figure 5.12. Effect of EP receptor antagonists on LPS-induced cytokine release.
Macrophages were incubated without or with the EP_2-selective antagonist PF-04418948
or the EP_4-selective antagonist CJ-042794 (both 300 nM) for 1 h before challenge with
LPS (0.1-10 ng/ml) for 22 h, after which TNF-α was measured in the supernatants.
Antagonists alone had no effect on spontaneous cytokine release. Values are mean ±
SEM for 4 individual experiments. (For each LPS concentration, one-way ANOVA
with Dunnett's post test, **$p<0.01$, compared with respective LPS alone controls).

A

B
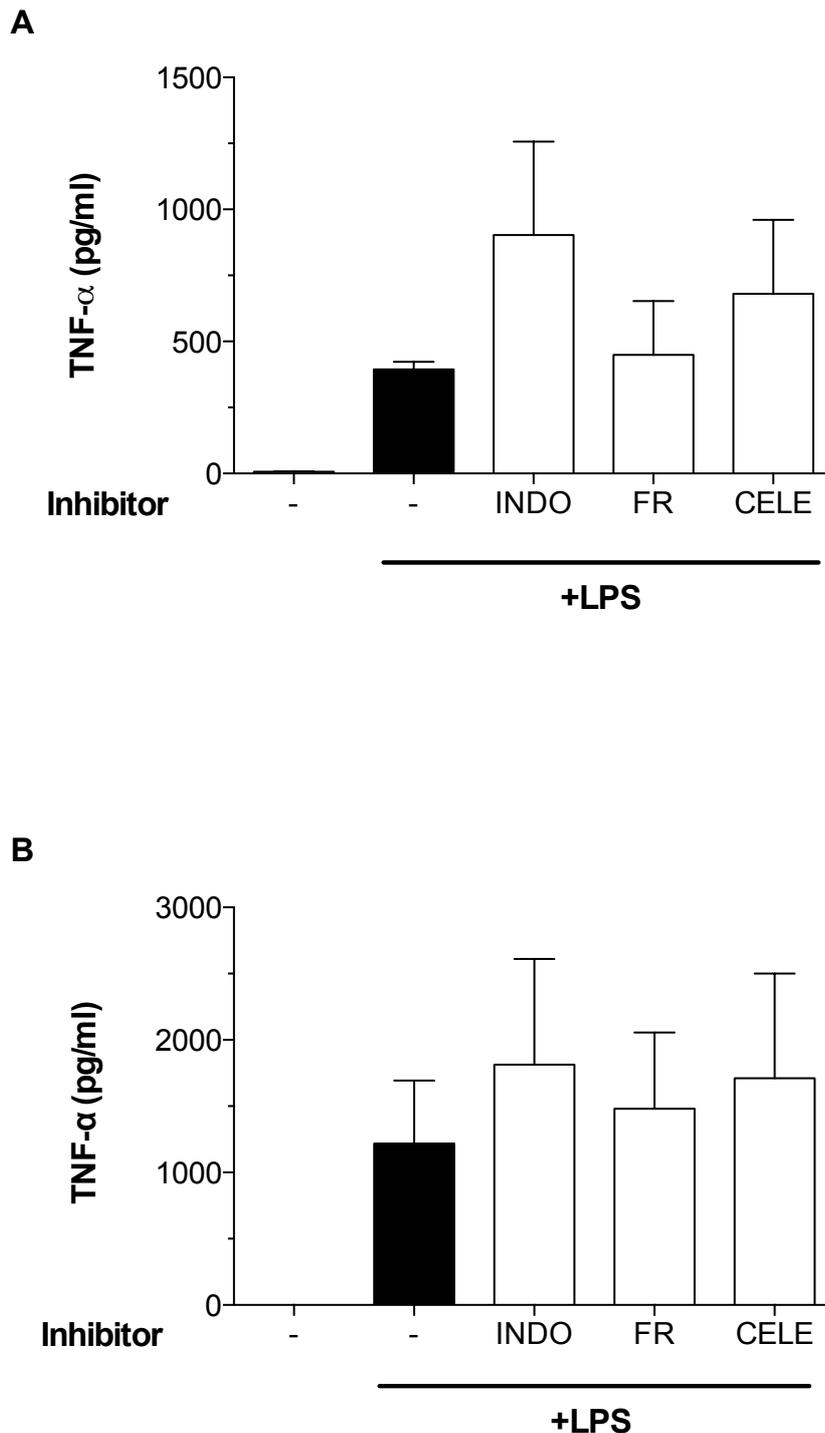
Figure 5.13. Comparison of the effect of COX inhibitors on TNF-α generation from MDMs and lung macrophages.

(A) MDMs and (B) lung macrophages were pre-treated for 30 min with indomethacin (INDO), FR122047 (FR) or celecoxib (CELE) (all 10^{-6} M) before challenge with LPS (1 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for TNF-α. Drugs alone had no effect on spontaneous cytokine release. Values are mean ± SEM for 2 individual experiments (MDMs) and mean ± SEM for 4 individual experiments (macrophages). (One-way ANOVA with Dunnett's post test, all p>0.05 compared with LPS alone for both cell types).

There may be some differences in COX inhibition between the two cell systems, which requires further investigation.

5.3. Discussion

Human lung macrophages produce PGE_2 (Monick et al., 2002, Hempel et al., 1994). This was confirmed in the present study. Macrophages were found to produce PGE_2 spontaneously and the levels of PGE_2 increased about 3-fold in response to LPS stimulation. Further work was performed to investigate which isoform of COX was responsible for LPS-induced PGE_2 generation in human lung macrophages.

Constitutive gene expression of COX-1 and COX-2 was investigated using RT-PCR. Generally, expression of COX-2 is tightly regulated and restricted in the absence of induction (Tilley et al., 2001). In this study, constitutive expression of both COX-1 and COX-2 was seen at the mRNA level in unstimulated macrophages. Stimulation with LPS resulted in increased expression of COX-2, whereas the increase in expression of COX-1 in the presence of LPS stimulation was less obvious and was much lower than the increase in COX-2 expression. Although the constitutive mRNA expression of both COX-1 and COX-2 was strong, the constitutive protein expression was weak. The protein expression of COX-1 was not up-regulated in response to LPS. By contrast, the COX-2 protein expression was up-regulated in response to increasing concentrations of LPS. This suggests that LPS stimulation may result in induction of the translation of COX-2 mRNA. Studies by other researchers have also demonstrated that LPS stimulation results in induction of COX-2 mRNA and protein in human alveolar macrophages from bronchoalveolar lavage (BAL) fluid (Monick et al., 2002).

In addition to LPS, the effects of another TLR agonist, the viral mimic poly(I:C), on COX-1 and COX-2 protein expression was investigated. The expression of COX-1 or COX-2 was not up-regulated at the protein level in response to poly(I:C), which is in contrast to the effect of LPS. It may be that TLR4 activation is required for COX-2 induction and therefore no COX-2 induction is observed in response to TLR3 activation by poly(I:C). Although COX-2 gene expression in response to poly(I:C) was not explored in the present study, in another study, LPS significantly induced COX-2 gene expression in murine lung tissue whereas the induction with poly(I:C) was negligible (Kirkby et al., 2013). However, it has been reported that COX-2 can be induced by poly(I:C) in murine macrophage-like RAW 264.7 cells (Pindado et al., 2007), which indicates that there are likely to be differences in responses to poly(I:C) between cell systems.

As modulation of expression of COX-2 protein was observed in response to a bacterial product but not a viral mimic, it was of interest to determine the effect of infection of macrophages with bacteria on COX expression. The expression of COX-1 protein in response to infection with *S. aureus* and with *N. meningitidis* was not up-regulated as the multiplicity of infection (MOI) was increased. The expression of COX-2, however, differed. In response to *S. aureus* at the highest MOI, the expression of COX-2 marginally increased. However, in response to *N. meningitidis*, COX-2 expression was strikingly up-regulated. This difference in response to the two pathogens could be attributed to *N. meningitidis* being gram-negative, whereas *S. aureus* is gram-positive. The LPS in the outer cell membrane of *N. meningitidis* could be activating the macrophages through TLR4 leading to the up-regulation of COX-2, whereas *S. aureus* is believed to be recognized by TLR2 (Pietrocola et al., 2011).

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To further investigate which isoform of COX may be responsible for LPS-induced PGE$_2$ generation from human lung macrophages, the cells were treated with selective COX inhibitors. Indomethacin is a non-selective COX inhibitor but shows slight selectivity for COX-1 over COX-2 (Mitchell et al., 1993). The selective COX-2 inhibitor celecoxib has been reported to be over 3000-fold more selective for COX-2 over COX-1 (Gierse et al., 1999). However, this value is disputed by studies utilizing alternative assays for analysis (Mardini and FitzGerald, 2001). The selective COX-1 inhibitor FR122047 is over 2000-fold more selective for COX-1 over COX-2 (Ochi et al., 2000). Overall, the reports in the literature support the assertion that the inhibitors are selective. The reported IC$_{50}$ values for both celecoxib and FR122047 are in the nanomolar range (Alexander et al., 2013a).

In the present study, when used at high concentrations (10^{-6} M), all of the COX inhibitors essentially abolished LPS-induced PGE$_2$ release. As the concentrations of celecoxib and FR122047 used were at least 1000-fold higher than their reported IC$_{50}$ values, it is possible that the inhibitors could have had non-selective effects. It follows that the COX isoform responsible for PGE$_2$ generation is not clear from the results of these experiments. Repeating these experiments using a wider concentration range of inhibitors may enable a clearer determination of their effects. Testing alternative selective COX inhibitors in this system may also be beneficial for confirmation of the effects observed.

As LPS-induced PGE$_2$ can act in a paracrine manner to inhibit pro-inflammatory cytokine release from macrophages, it was investigated whether inhibition of COX might modulate LPS-induced TNF-α release. Indomethacin and celecoxib caused concentration-dependent increases in LPS-induced TNF-α release, whereas FR122047

only had an effect at the highest concentration (10^{-6} M) used. Although the COX inhibitor-induced increases in TNF-α release were not statistically significant, the results provide an indication that inhibition of COX prevents production of PGE_2, which leads to a modest enhancement of TNF-α release.

In order to determine whether inhibition of COX might also affect the release of other cytokines, the effects of the COX inhibitors on LPS-induced IL-6 release were also investigated. It was found that none of the COX inhibitors had an effect on IL-6 release. This is in keeping with the demonstration in Chapter 4 that PGE_2 is not as effective at inhibiting LPS-induced IL-6 than LPS-induced TNF-α. It has been reported that COX-2 induced PGE_2 results in synthesis of IL-6 in murine peritoneal macrophages (Williams and Shacter, 1997). The study reported that this was related to timing and that the delayed production of PGE_2 by COX-2 in response to stimuli, as opposed to early production by COX-1, was required for IL-6 synthesis. The data in the present study compared to reports in the literature suggest that there are differences between model systems in the mechanism of PGE_2 synthesis and its subsequent effects.

It should also be noted that a brief investigation of whether there were any differences between MDMs and lung macrophages in the effects of COX inhibitors on TNF-α release suggested that the inhibitors may have a more pronounced effect on MDMs. This requires further investigation but it could be possible that results of studies using MDMs may not necessarily extrapolate to lung macrophages.

In Chapter 4 it was demonstrated that exogenous PGE_2 acts on the EP_4 receptor to inhibit LPS-induced TNF-α release. Following on from this observation, in work described in the present chapter, EP receptor antagonists were utilized (in the absence of

COX inhibitors) to block the effects of PGE_2 generated endogenously upon LPS stimulation. LPS-induced TNF-α generation increased in the presence of EP_4 antagonist CJ-042794 but not in the presence of EP_2 antagonist PF-04418948. This suggests that any PGE_2 generated by the macrophages endogenously in response to LPS may be acting on the EP_4 receptor to inhibit TNF-α release. These experiments also provide further confirmation that the prostanoid generated through COX activation by LPS to attenuate TNF-α generation was PGE_2.

The COX inhibitors used in this study are non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are commonly used therapeutically, as painkillers and antipyretics. NSAIDs such as aspirin and ibuprofen are non-selective, targeting both COX-1 and COX-2. A common side-effect of these compounds is gastrointestinal problems due to inhibition of PGE_2 driven by COX-1. PGE_2 is known to inhibit acid secretion from gastric parietal cells. Therefore, inhibition of PGE_2 due to COX-1 inhibition increases acid secretion leading to potential problems in the gut. Issues such as this with non-selective inhibitors led to the development of selective COX-2 inhibitors such as celecoxib (Celebrex™), which have a gut-sparing effect (Warner and Mitchell, 2004).

Other undesirable effects have also been reported with the use of NSAIDs. For example, 10-20 % of asthmatics are sensitive to aspirin and other non-selective NSAIDs (Bennett, 2000, Sanak, 2011). This condition is known as aspirin-exacerbated respiratory disease (AERD). Several mechanisms have been reported as being involved in AERD. The most widely cited of these indicates that inhibition of COX-1 leads to a build-up in arachidonic acid. This arachidonic acid is then converted via the 5-lipoxygenase pathway, to produce increased amounts of cysteinyl leukotrienes, rather than metabolized via the COX pathway (figure 5.14) (Picado, 2002, Varghese and Lockey,

2008). Cysteinyl leukotrienes are pro-inflammatory and cause bronchoconstriction. These deleterious effects may be further exacerbated by the decrease in PGE_2 resulting from COX inhibition, as PGE_2 is anti-inflammatory in the lung (Picado, 2002, Szczeklik, 1995). In keeping with this potential mechanism, the present study shows that inhibiting COX in lung macrophages leads to increased TNF-α generation. Therefore, caution needs to be exercised with the general use of any NSAIDs, including those that are COX-2 selective, for patients with underlying or accompanying respiratory conditions, as the drugs could potentially contribute to inflammation in the lungs.

In summary, the results of this study indicate that in response to different stimuli, there are differences in the expression of COX-1 and COX-2 in human lung macrophages. In the absence of stimuli, the expression of both COX-1 and COX-2 is weak. It is therefore not clear in this study, which COX isoform is responsible for spontaneous PGE_2 generation. In keeping with results of others, LPS but not poly(I:C) stimulation appears to induce COX-2 expression. Up-regulation of COX-2 is also seen in response to gram-negative but not gram-positive bacteria. Overall, the results indicate that LPS-induced PGE_2 production from human lung macrophages occurs principally through the activity of COX-2. However, it is not possible to exclude the possible involvement of COX-1. Further investigations will enable a more complete evaluation of the contribution of COX isoforms to the generation of PGE_2 from human lung macrophages.
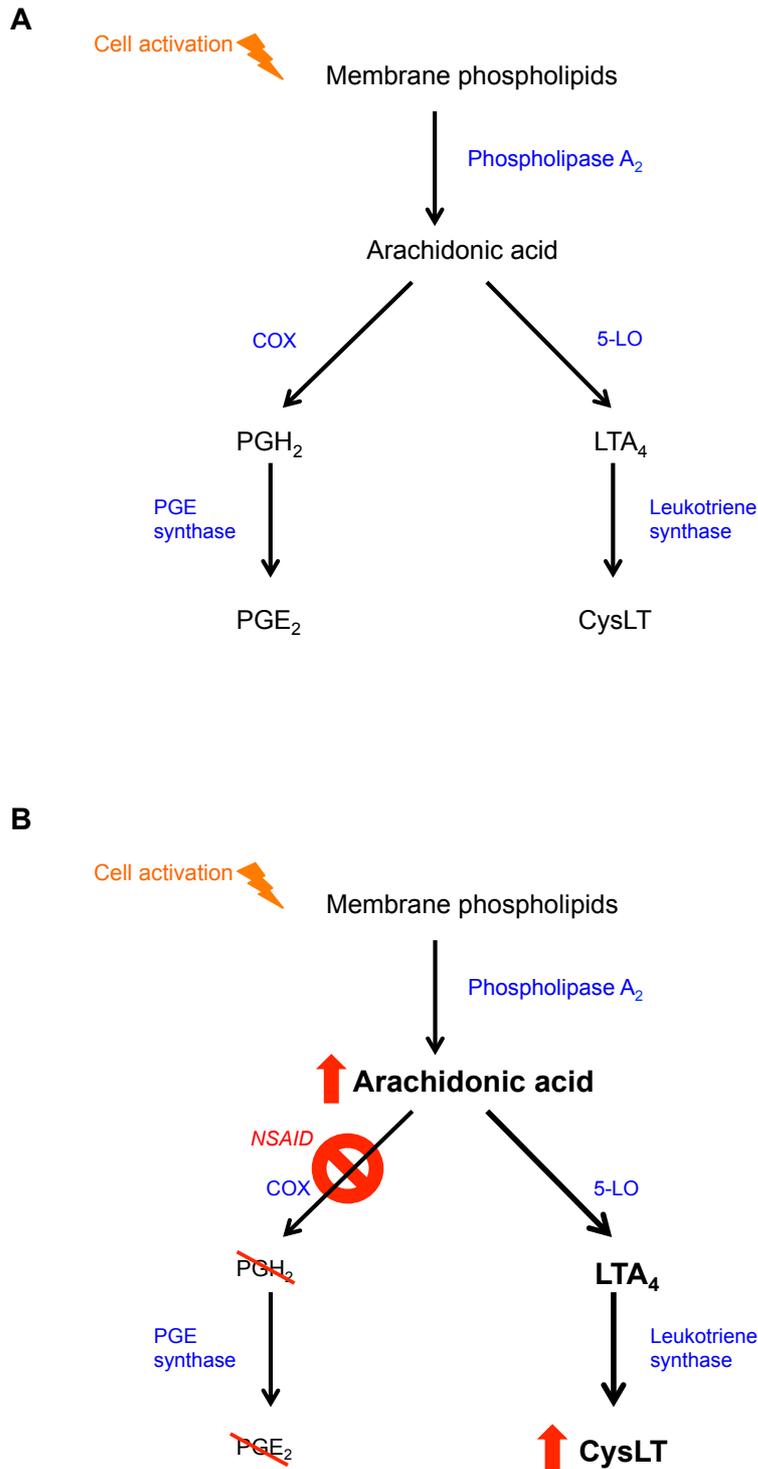
Figure 5.14. Inhibition of COX results in increased production of CysLT in AERD.
(A) A simplified figure of the eicosanoid and leukotriene production pathways. (B) A possible mechanism of AERD pathogenesis. The inhibition of COX by NSAIDS inhibits the production of PGE_2. This results in a build-up of arachidonic acid which is then metabolized via the 5-LO pathway as opposed to the COX pathway, resulting in increased production of cysteinyl leukotrienes (CysLT) (Medeiros et al., 2012, Peters-Golden et al., 2006).

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Chapter 6: Evaluation of the anti-inflammatory effects of β-adrenoceptor agonists on macrophages

6.1. Introduction

The current leading treatments available for chronic inflammatory lung diseases include β-adrenoceptor agonists (β_2-agonists) and corticosteroids (Barnes, 2006b, Theron et al., 2013). β_2-agonists are used principally to alleviate symptoms, as they act on airway smooth muscle to cause relaxation and therefore exert bronchodilatory effects. Corticosteroids are used as anti-inflammatories to target the underlying disease pathology. These pharmacological agents are also used in combination. In recent years, combination inhalers have become a commonly prescribed treatment for asthma and COPD (Domingo, 2013).

It has been reported that in addition to their bronchodilatory effects, β_2-agonists can have anti-inflammatory effects (Linden, 1992, Donnelly et al., 2010, Ezeamuzie and Shihab, 2010). The ability of β_2-agonists to act as effective anti-inflammatory agents would be useful as it could reduce the need for corticosteroids, which are not effective for all patients (Barnes, 2010), particularly those with severe cases of disease. High systemic concentrations of inhaled corticosteroids (ICS) can also result in side effects, particularly in children, such as endocrine disorders (Ahmet et al., 2011) and there is an increased risk of pneumonia in COPD patients (Calverley et al., 2011, Singh and Loke, 2010). Despite these issues with the use of ICS, they are currently the most effective anti-inflammatory treatments available for chronic lung diseases (Barnes, 2006b).

As macrophages are implicated in COPD and may also be important in asthma (Barnes, 2008b, Yang et al., 2012, Peters-Golden, 2004), it would be of benefit if β₂-agonists stabilized the macrophage inflammatory response. The reports in the literature regarding the anti-inflammatory effects of β₂-agonists on macrophages do not enable a clear conclusion to be drawn. Also, some of these studies were carried out using monocytes and macrophage models, not human lung macrophages (Donnelly et al., 2010, Zetterlund et al., 1998, Ezeamuzie and Shihab, 2010). Determining the anti-inflammatory effects of β₂-agonists would allow evaluation of their potential to target macrophages in the lung.

In this study, a range of β₂-agonists was evaluated for inhibitory effects on pro-inflammatory cytokine generation from human lung macrophages. The short-acting β-agonists (SABA), which have bronchodilatory effects for 4-6 hours (Tashkin and Fabbri, 2010) were salbutamol and terbutaline. The non-selective SABA isoprenaline was also studied, for comparative purposes, as it is known to act as a full agonist at β-adrenoceptors (Cazzola et al., 2012). The long-acting β-agonists (LABA) studied were formoterol, salmeterol and indacaterol. LABAs have bronchodilatory effects for 12 hours. Indacaterol is classed as ultra long-acting as it has a 24 hour effect (Domingo, 2013). Comparisons were made between the effects of these β₂-agonists and the effects of the corticosteroid dexamethasone.

6.2. Results

6.2.1. Comparison of the effects of salbutamol and dexamethasone on cytokine release

Macrophages were treated for 30 min with the β₂-agonist salbutamol (10^{-5} M) or the corticosteroid dexamethasone (10^{-7} M) before being challenged with LPS (10 ng/ml).

After 22 h, the release of the pro-inflammatory cytokines TNF-α (figure 6.1A), IL-6 (figure 6.1B) and IL-8 (figure 6.1C) into the cell culture media was measured by ELISA. Salbutamol did not cause significant inhibition of release of any of the cytokines ($p > 0.05$). However, dexamethasone significantly inhibited LPS-induced TNF-α by ~99% ($p < 0.05$), IL-6 by ~87% ($p < 0.001$) and IL-8 by ~78% ($p < 0.0001$).

6.2.2. Effects of long and short-acting β₂-agonists on cytokine release

Macrophages were treated for 30 min with a range of β₂-agonists. The short-acting β₂-agonists tested were isoprenaline, terbutaline or salbutamol (all 10^{-5} M). The long-acting β₂-agonists were formoterol, salmeterol or indacaterol (all 10^{-5} M). Dexamethasone (10^{-7} M) was used as a positive control for inhibition of LPS-induced cytokine release. The cells were then challenged with LPS (10 ng/ml). After 22 h, the cell culture supernatants were harvested and assayed for TNF-α (figure 6.2A), IL-6 (figure 6.2B) and IL-8 (figure 6.2C). Only salmeterol and indacaterol significantly inhibited TNF-α and IL-6 release. Salmeterol inhibited TNF-α by ~49% ($p < 0.01$) and indacaterol inhibited TNF-α by ~36% ($p < 0.05$). Salmeterol inhibited IL-6 by ~56% ($p < 0.01$) and indacaterol inhibited IL-6 by ~45% ($p < 0.05$). None of the β₂-agonists tested, including salmeterol and indacaterol, had any effect on IL-8 release. Dexamethasone, however, was an effective inhibitor of IL-8 release ($p < 0.001$) (figure 6.2C).

A

B

C
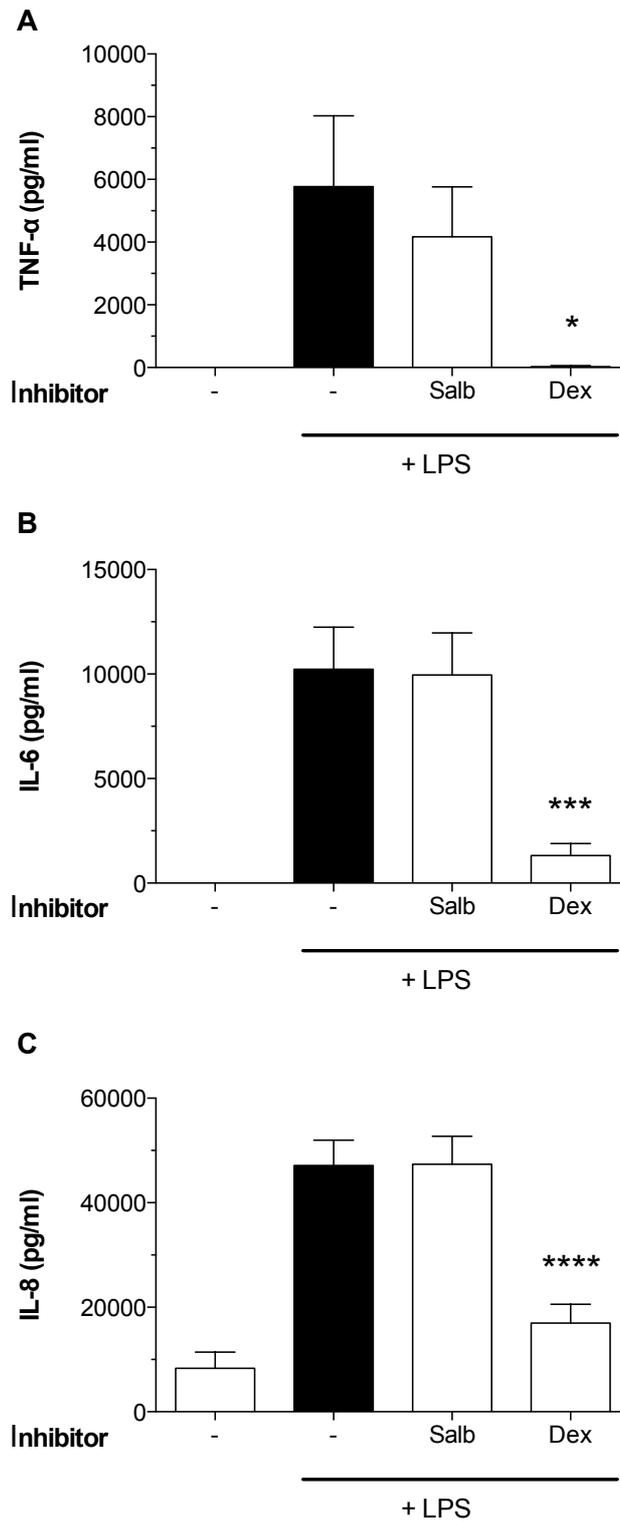
Figure 6.1. Effects of salbutamol and dexamethasone on LPS-mediated cytokine release. Macrophages were pre-treated for 30 min with salbutamol (Salb) (10^{-5} M) or dexamethasone (Dex) (10^{-7} M) before challenge with LPS (10 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for (A) TNF-α, (B) IL-6 and (C) IL-8. Drugs alone had no effect on spontaneous cytokine release. Values are mean ± SEM for 5 individual experiments. (One-way ANOVA with Dunnett's post test, *p<0.05, ***p<0.001, ****p<0.0001 compared with LPS alone).
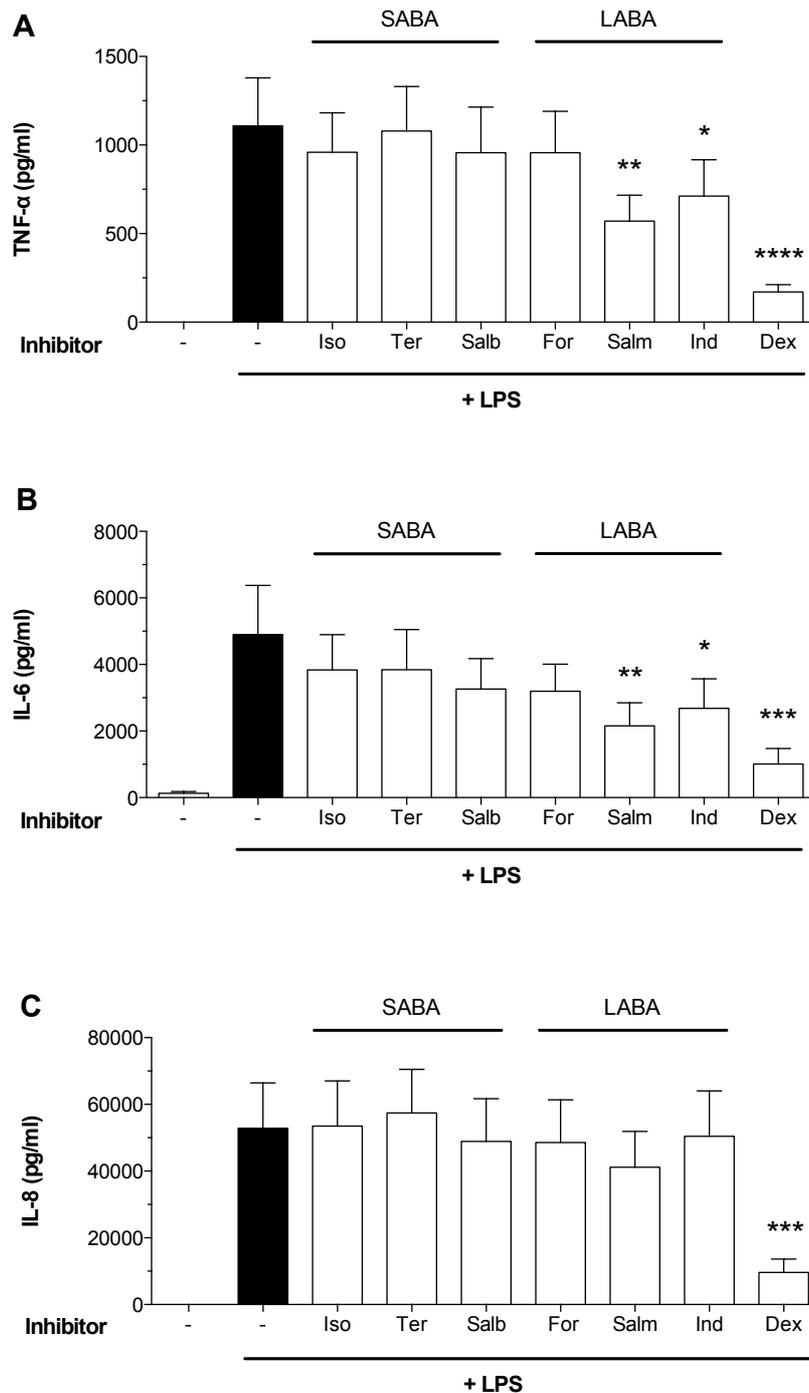
Figure 6.2. Effects of β₂-agonists on LPS-mediated cytokine release. Macrophages were pre-treated for 30 min with β₂-agonists (10^{-5} M) or dexamethasone (10^{-7} M) before challenge with LPS (10 ng/ml). Short-acting β-agonists (SABA): isoprenaline (Iso), terbutaline (Ter), salbutamol (Salb). Long-acting β-agonists (LABA): formoterol (For), salmeterol (Salm), indacaterol (Ind). After 22 h, cell culture supernatants were harvested and assayed for (A) TNF-α, (B) IL-6 and (C) IL-8. Drugs alone had no effect on spontaneous cytokine release. Values are mean ± SEM for 6 (TNF-α) or 5 (IL-6, IL-8) individual experiments. (One-way ANOVA with Dunnett's post test, *p< 0.05, **p<0.01, ****p<0.0001 compared with LPS alone).

6.2.3. Effects of long and short-acting β₂-agonists on cytokine release from MDMs

MDMs (monocyte-derived macrophages) were treated for 30 min with a range of β_2-agonists. The short-acting β_2-agonists tested were isoprenaline, terbutaline or salbutamol (all 10^{-5} M). The long-acting β_2-agonists were formoterol, salmeterol or indacaterol (all 10^{-5} M). Dexamethasone (10^{-7} M) was used as a positive control for inhibition of LPS-induced cytokine release. The cells were then challenged with LPS (10 ng/ml). After 22 h, the cell culture supernatants were harvested and assayed for TNF-α (figure 6.3). All of the β_2-agonists tested inhibited TNF-α release. The range of inhibition was between 35% and 75%, although it was not possible to determine if this inhibition was statistically significant, as only two experiments were performed. Incubation with salmeterol or indacaterol resulted in the highest level of inhibition. Salmeterol inhibited TNF-α by ~75% and indacaterol inhibited TNF-α by ~47%.

6.2.4. Influence of LPS concentration on inhibition of cytokine release by β₂-agonists

In order to determine whether the strength of the stimulus might influence the extent of inhibition, macrophages were activated with sub-optimal concentrations of LPS and the inhibitory effects of β_2-agonists were evaluated. Macrophages were incubated for 30 min with salbutamol, salmeterol or indacaterol (all 10^{-6} M) before challenge with 0.1, 1 or 10 ng/ml LPS. After 22 h, TNF-α release was measured. All three β_2-agonists significantly ($p < 0.01$) inhibited LPS-induced TNF-α release when the LPS concentration was 0.1 ng/ml (23-28% inhibition) or 1 ng/ml (14-22% inhibition) (figure 6.4). However, salmeterol and indacaterol did not inhibit 10 ng/ml LPS-induced TNF-α, in contrast to the results of the experiment in the previous section (figure 6.2), in which a significant inhibition was observed. However, it should be noted that in the present experiment, the concentration of β_2-agonists used was lower (10^{-6} M) than the concentration in the experiment in the previous section (10^{-5} M).
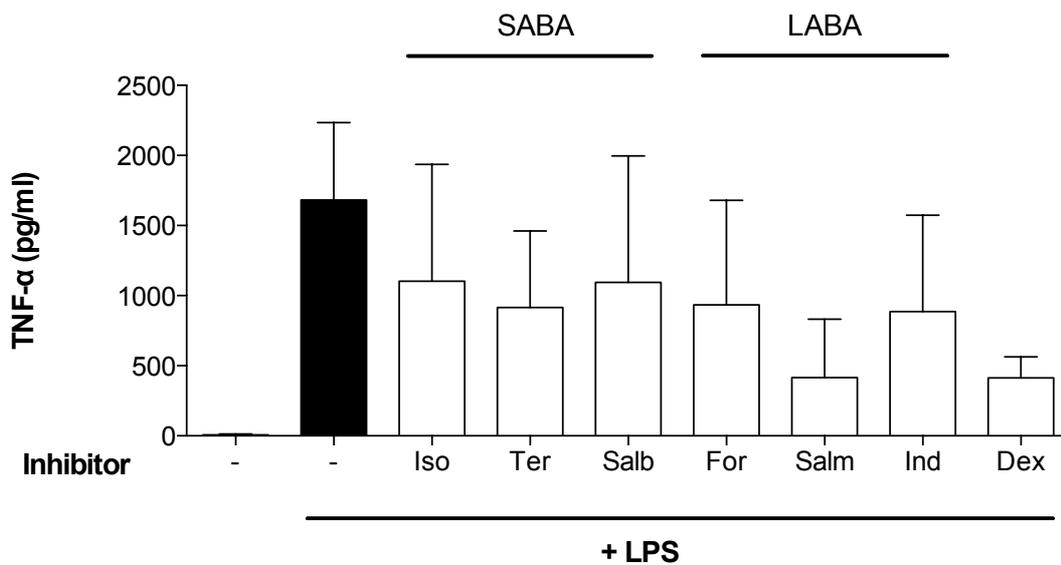
Figure 6.3. Effects of β₂-agonists on LPS-mediated cytokine release from MDMs.
MDMs were pre-treated for 30 min with β_2-agonists (10^{-5} M) or dexamethasone (10^{-7} M) before challenge with LPS (10 ng/ml). Short-acting β-agonists (SABA): isoprenaline (Iso), terbutaline (Ter), salbutamol (Salb). Long-acting β-agonists (LABA): formoterol (For), salmeterol (Salm), indacaterol (Ind). After 22 h, cell culture supernatants were harvested and assayed for (A) TNF-α. Drugs alone had no effect on spontaneous cytokine release. Values are mean ± SEM for 2 individual experiments.
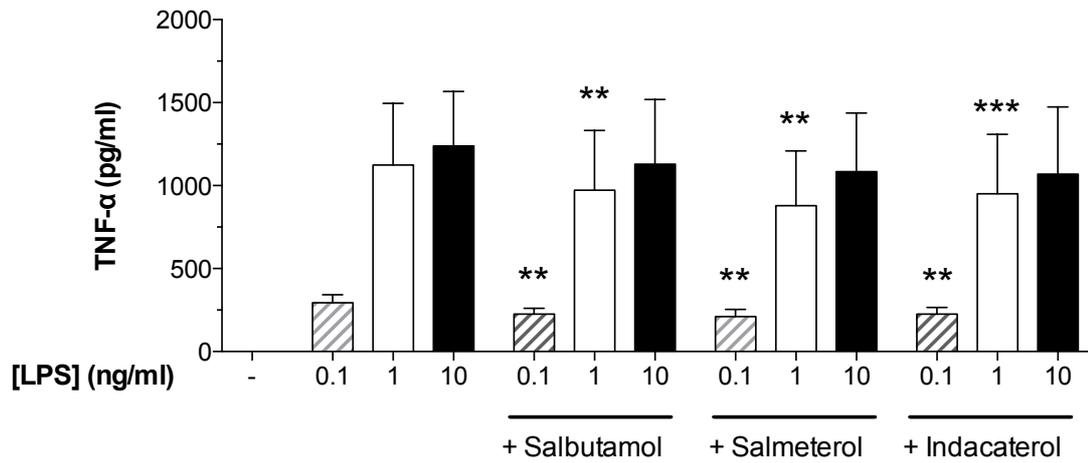
Figure 6.4. Effect of LPS concentration on β₂-agonist inhibition.
Macrophages were pre-treated for 30 min with salbutamol, salmeterol or indacaterol
(10^{-6} M) before challenge with LPS (0.1, 1 or 10 ng/ml). After 22 h, cell culture
supernatants were harvested and assayed for TNF-α. Drugs alone had no effect on
spontaneous cytokine release. Values are mean ± SEM for 3 individual experiments.
(For each LPS concentration, one-way ANOVA with Dunnett's post test, **$p < 0.01$,
***$p < 0.001$ compared with respective LPS alone controls).

6.2.5. Effects of indacaterol addition pre and post-LPS challenge

Routinely, β_2-agonists were added to cells 30 min before LPS challenge. It was

investigated whether addition of a β_2-agonist after LPS challenge instead could result in

a difference in the inhibition of TNF-α release. Macrophages were incubated with

indacaterol (10^{-6} M) for 30 min before challenge with LPS (1 ng/ml). This was

compared to indacaterol addition at 2, 4 or 6 h after LPS challenge. After a total of 22 h

of incubation with LPS, cell culture supernatants were assayed for TNF-α. There was no

significant ($p>0.05$) improvement in the inhibitory effects of indacaterol when added

after LPS challenge (figure 6.5).
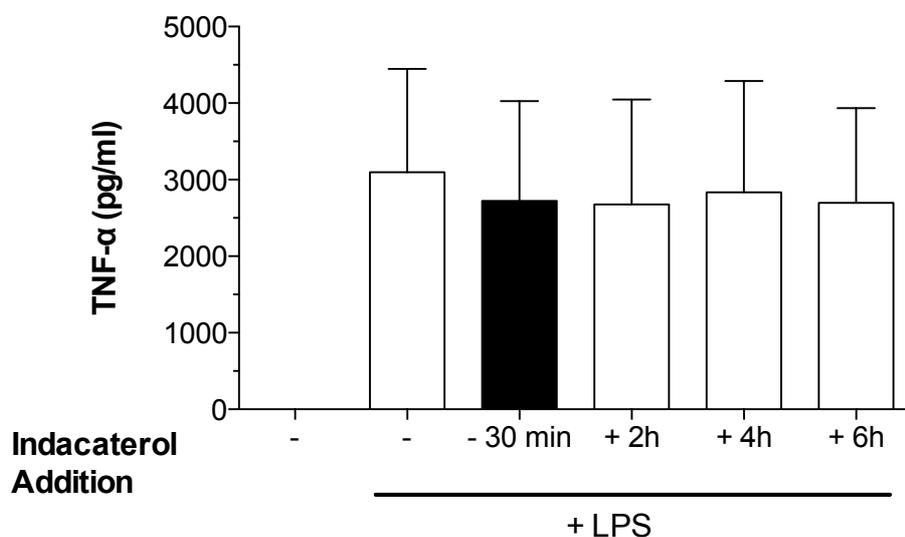
Figure 6.5. The effect of indacaterol addition at different timepoints pre or post-LPS challenge.
Macrophages were treated with indacaterol (10^{-6} M) at different timepoints before or after challenge with LPS (1 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for TNF-α. Indacaterol alone had no effect on spontaneous cytokine release. Values are mean ± SEM for 3 individual experiments. (One-way ANOVA with Dunnett's post test, all $p>0.05$ compared with LPS alone or LPS with 30 min indacaterol pre-treatment).

6.2.6. Effects of β₂-agonists on poly(I:C)-mediated cytokine release

Macrophages were treated for 30 min with salbutamol, salmeterol, indacaterol (all 10^{-5} M) or dexamethasone (10^{-7} M) before being challenged with LPS (10 ng/ml) or poly(I:C) (10 μg/ml). After 22 h cell culture supernatants were assayed for TNF-α release. In this experiment, only salmeterol significantly ($p<0.01$) inhibited LPS-induced TNF-α release (figure 6.6). None of the β₂-agonists significantly inhibited poly(I:C)-induced TNF-α release ($p>0.05$). Dexamethasone, however, significantly inhibited both LPS-induced ($p<0.0001$) and poly(I:C)-induced ($p<0.01$) TNF-α release.

6.2.7. Concentration-dependent effects of β₂-agonists on cytokine release

Macrophages were incubated with a range of concentrations of salbutamol (figure 6.7A), salmeterol (figure 6.7B) and indacaterol (figure 6.7C) for 30 min before challenge with LPS (1 ng/ml). After 22 h, cell culture supernatants were assayed for TNF-α release. It was observed that the concentration response 'curves' for each of the β₂-agonists were relatively flat and the highest concentration of drug used (10^{-5} M) resulted in slightly greater inhibition of LPS-induced TNF-α release than the lower concentrations.
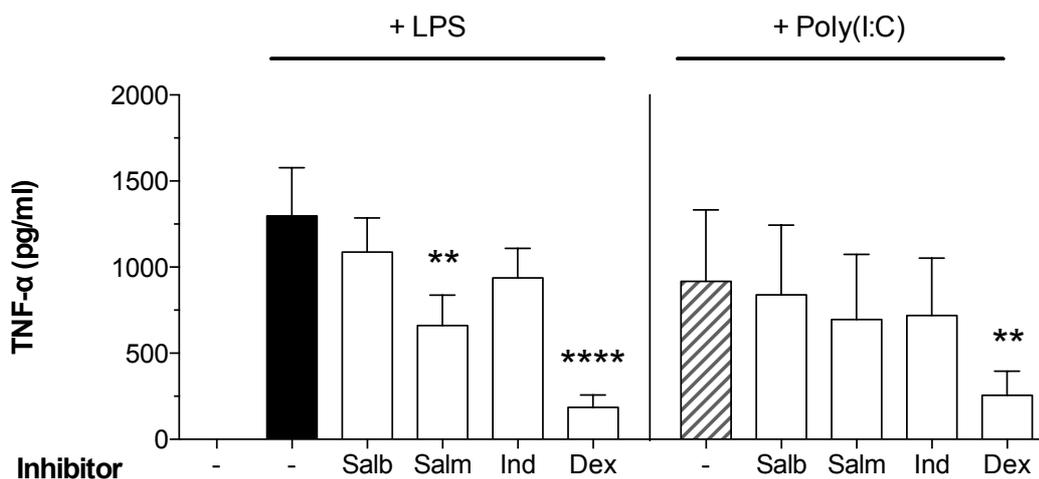
Figure 6.6. Effect of β₂-agonists on LPS or poly(I:C)-mediated cytokine release.
Macrophages were pre-treated for 30 min with either β₂-agonists salbutamol (Salb),
salmeterol (Salm) or indacaterol (Ind) (all 10^{-5} M) or with dexamethasone (10^{-7} M)
before challenge with LPS (10 ng/ml) or poly(I:C) (10 μg/ml). After 22 h, cell culture
supernatants were harvested and assayed for TNF-α. Drugs alone had no effect on
spontaneous cytokine release. Values are mean ± SEM for 4 individual experiments.
(One-way ANOVA with Dunnett's post test, **$p<0.01$, ****$p<0.0001$ compared with
LPS or poly(I:C) alone).

A

B

C
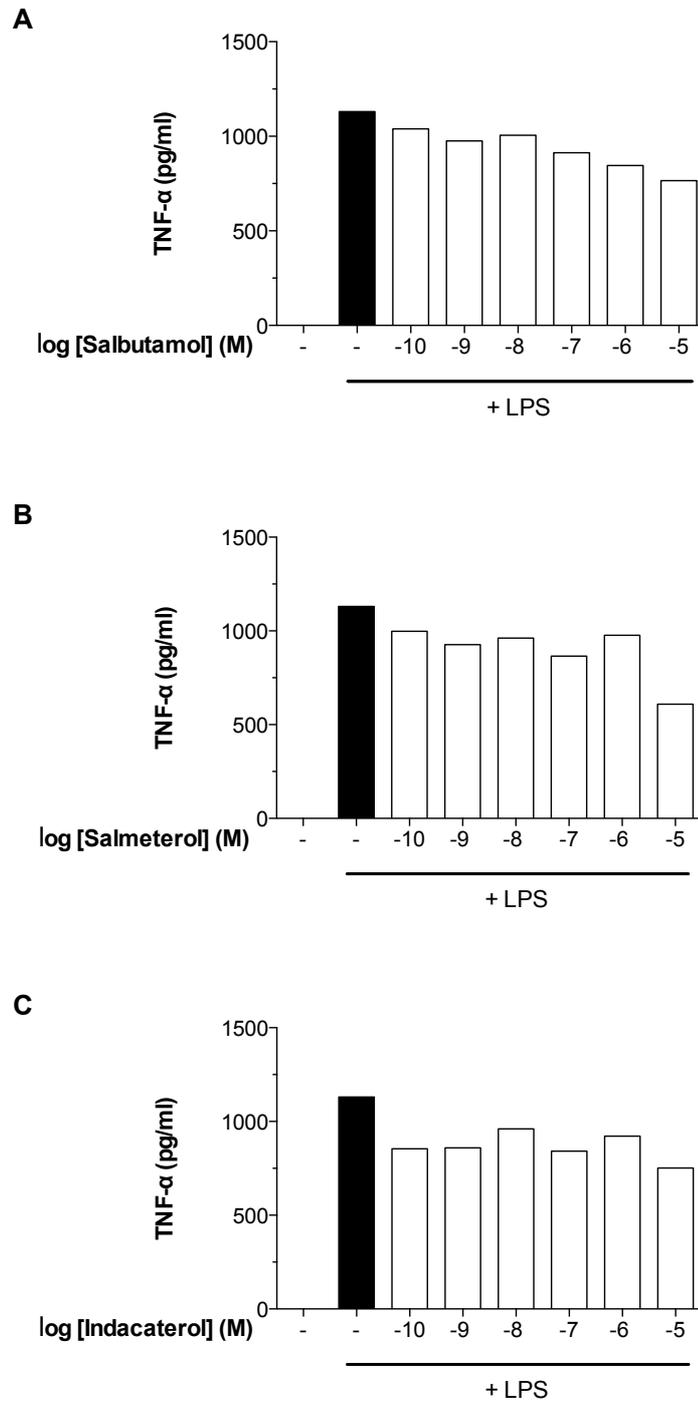
Figure 6.7. Effect of β₂-agonist concentration on LPS-mediated cytokine release.
Macrophages were pre-treated for 30 min with β₂-agonists (A) salbutamol, (B) salmeterol or (C) indacaterol before challenge with LPS (1 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for TNF-α. Drugs alone had no effect on spontaneous cytokine release. Values are for one experiment.

6.2.8. Effect of β₂-agonists on cAMP levels

The effect of β_2-agonists on total cyclic-AMP (cAMP) levels in macrophages was investigated. The short-acting β_2-agonists tested were isoprenaline, terbutaline or salbutamol (all 10^{-5} M). The long-acting β_2-agonists were formoterol, salmeterol or indacaterol (all 10^{-5} M). Macrophages were incubated with the drugs for 30 min before being solubilized and assayed for total cell cAMP levels. The values indicated that the cAMP levels increased over the spontaneous levels, in response to β_2-agonists (figure 6.8). However, no striking differences were observed between the responses to the different drugs.

6.2.9. Inhibition of cytokine release by indacaterol in combination with dexamethasone

As some current treatments combine long-acting β_2-agonists with corticosteroids, the effect of indacaterol in combination with dexamethasone on LPS-induced cytokine release was investigated. Macrophages were incubated with a range of indacaterol concentrations (10^{-5}-10^{-7} M) for 30 min before challenge with LPS (1 ng/ml) for 22 h. Indacaterol alone did not result in inhibition of LPS-induced TNF-α release (p>0.05) (figure 6.9A). In the same experiment, macrophages were incubated for 30 min with indacaterol and dexamethasone (10^{-9} M) before challenge with LPS (1 ng/ml) for 22 h. Cell culture supernatants were assayed for TNF-α release. Dexamethasone, at the sub-optimal concentration used (10^{-9} M), did not significantly (p>0.05) inhibit LPS-induced TNF-α release (figure 6.9B). However a significant (p<0.05) decrease in LPS-induced TNF-α release was seen with dexamethasone in combination with all three concentrations of β_2-agonists.
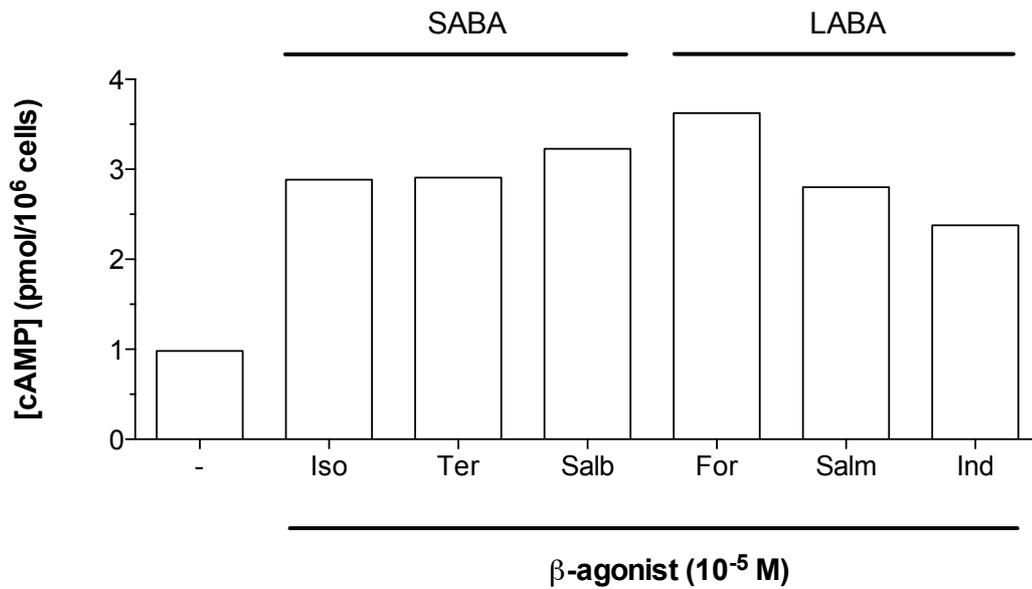
Figure 6.8. Release of cAMP in response to β₂-agonists. Macrophages were incubated without or with β_2-agonists (10^{-5} M) for 30 min. Short-acting β-agonists (SABA): isoprenaline (Iso), terbutaline (Ter), salbutamol (Salb). Long-acting β-agonists (LABA): formoterol (For), salmeterol (Salm), indacaterol (Ind). After this treatment, the cells were solubilized and assayed for total cell cyclic-AMP levels. Values are for one experiment.

A

B
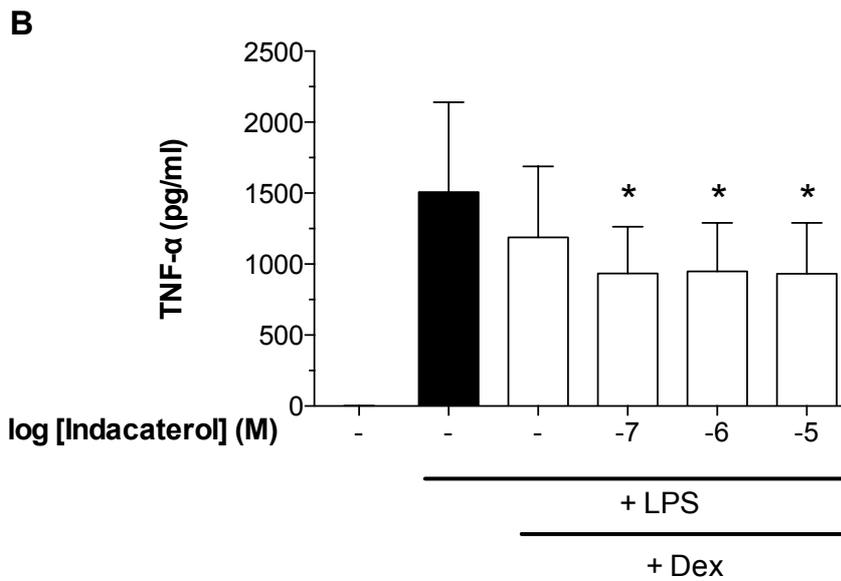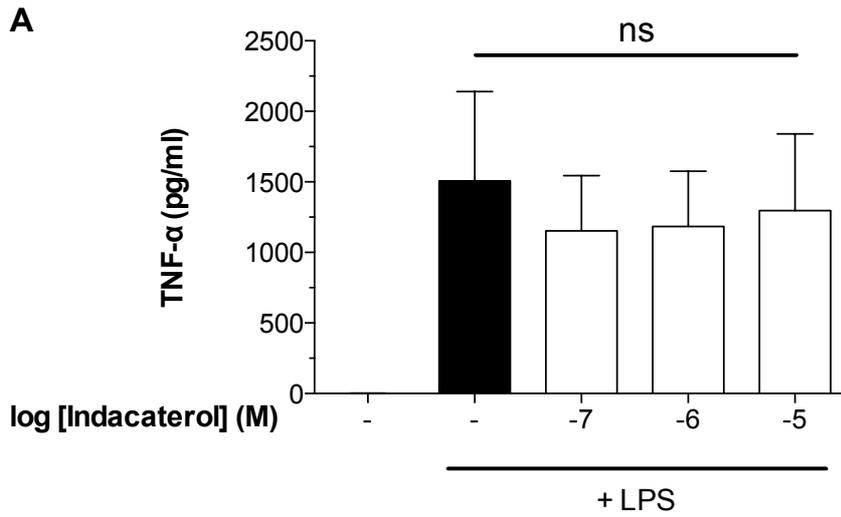
Figure 6.9. Effect of combination of indacaterol with dexamethasone on LPS-induced cytokine release. Macrophages were pre-treated for 30 min with indacaterol (A) and with dexamethasone (10^{-9} M) for 30 min (B) before challenge with LPS (1 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for TNF-α. Drugs alone had no effect on spontaneous cytokine release. Values are mean ± SEM for 4 individual experiments. (One-way ANOVA with Dunnett's post test, *p< 0.05, **p<0.01, compared with LPS alone).

6.2.10. Inhibition of cytokine release by indacaterol in combination with an EP_4 antagonist

In Chapter 4 of this thesis, it was demonstrated that PGE_2 acts on the EP_4 receptor to inhibit cytokine release from macrophages. It was possible that endogenously generated PGE_2 could have influenced the inhibition of cytokine release seen with β_2-agonists. Therefore, it was investigated whether blocking the effects of PGE_2 on the EP_4 receptor would alter the inhibition seen with β_2-agonists. In this experiment, indacaterol alone at a range of concentrations (10^{-5}-10^{-7} M) did not result in inhibition of LPS-induced TNF-α release ($p>0.05$) (figure 6.10A). In the same experiment, macrophages were incubated with EP4 antagonist (CJ-042794) for 1 h and for a further 30 min with indacaterol before challenge with LPS (1 ng/ml). After 22 h, cell culture supernatants were assayed for TNF-α release. The EP4 antagonist resulted in a significant ($p<0.01$) increase in LPS-induced TNF-α release. This TNF-α release was not significantly ($p>0.05$) inhibited by indacaterol (figure 6.10B). This meant that there was no improvement in the inhibitory effects of indacaterol on LPS-induced TNF-α when the EP_4 antagonist was present.

6.2.11. Effect of indomethacin on inhibition of cytokine release by salbutamol

Further investigation into the effects of the presence of endogenously generated PGE_2 on the inhibitory effects of β_2-agonists was carried out. In this experiment, the cyclooxygenase (COX) inhibitor indomethacin was used to block endogenous PGE_2 production. Macrophages were incubated without or with indomethacin (1 µM) for 30 min and then with salbutamol for 30 min. The cells were then challenged with LPS (1 ng/ml) for 22 h, after which cell culture supernatants were assayed for TNF-α release. The inhibition curves showed that the presence of indomethacin did not improve the inhibitory effects of salbutamol (figure 6.11).
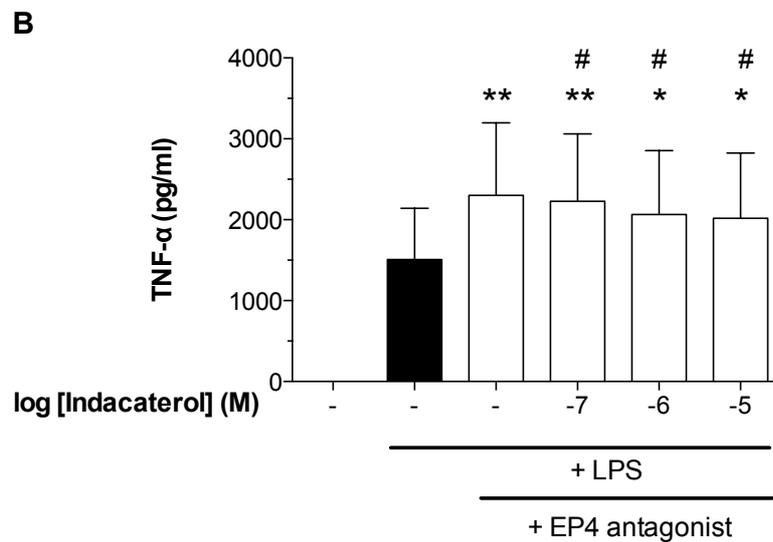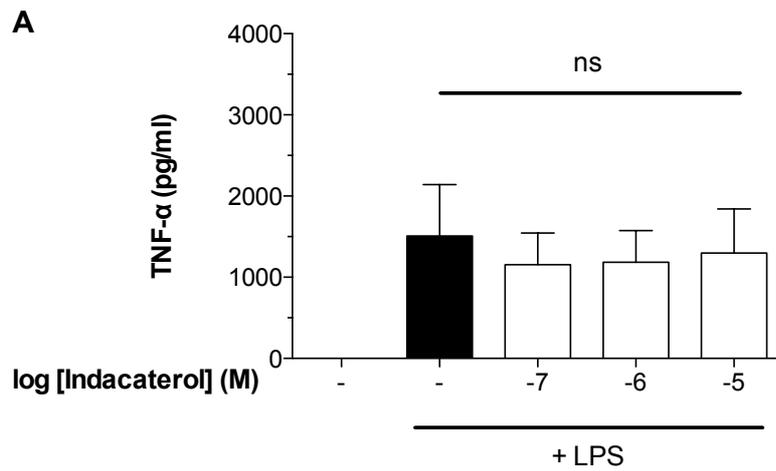
Figure 6.10. Effect of combination of indacaterol with EP$_4$ antagonist on LPS-induced cytokine release. Macrophages were pre-treated for 30 min with indacaterol (A) and with EP4 antagonist CJ-042794 (300 nM) for 1 h (B) before challenge with LPS (1 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for TNF-α. Drugs alone had no effect on spontaneous cytokine release. Values are mean ± SEM for 4 individual experiments. (One-way ANOVA with Dunnett's post test, *p< 0.05, **p<0.01, compared with LPS alone and #p>0.05, compared with LPS+EP4 antagonist alone).
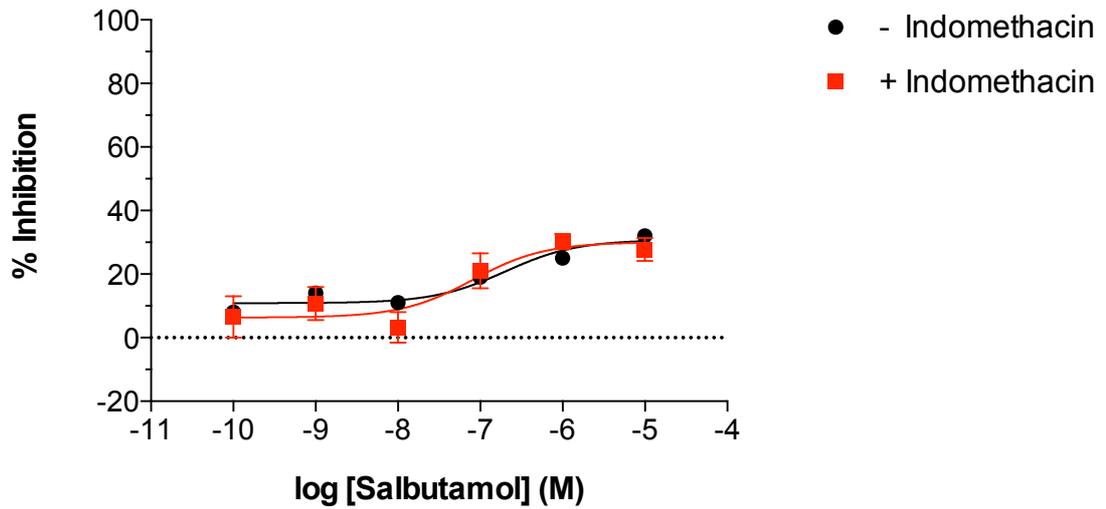
Figure 6.11. Effect of indomethacin on salbutamol inhibition. Macrophages were pre-incubated (30 min) without or with indomethacin (1 µM) and then with salbutamol for 30 min before challenge with LPS (1 ng/ml). After 22 h, cell culture supernatants were harvested and assayed for TNF-α. Drugs alone had no effect on spontaneous cytokine release. Values are expressed as the % inhibition of the unblocked control TNF-α release. Values are mean ± SEM for 4 individual experiments (+ indomethacin) and values for 1 experiment (- indomethacin).

6.2.12. Functional desensitization of β_2-agonist responses

Macrophages were incubated in media without or with either indacaterol (10^{-5} M) or salmeterol (10^{-5} M) for 22 h before being washed twice. The cells were then incubated for 30 min without or with either indacaterol (10^{-5} M) or salmeterol (10^{-5} M) before being challenged with LPS (1 ng/ml) for 22 h. Cell culture supernatants were then assayed for TNF-α release. The results demonstrated that indacaterol significantly ($p < 0.05$) inhibited LPS-induced TNF-α release (figure 6.12A). Salmeterol also significantly ($p < 0.05$) inhibited LPS-induced TNF-α release (figure 6.12B). When the cells were incubated for 22 h with indacaterol, the subsequent ability of indacaterol to inhibit TNF-α release was significantly ($p < 0.05$) abrogated (figure 6.12A). By contrast, after 22 h of pre-incubation with salmeterol, the subsequent inhibition by salmeterol was unaffected (figure 6.12B). This indicated that desensitization of the indacaterol response occurred whereas desensitization of the salmeterol response did not occur. Investigation of potential cross-desensitization indicated that pre-treatment with indacaterol had no effect on subsequent salmeterol inhibition (figure 6.13A) and that pre-treatment with salmeterol had no effect on subsequent indacaterol inhibition (figure 6.13B).

A

B
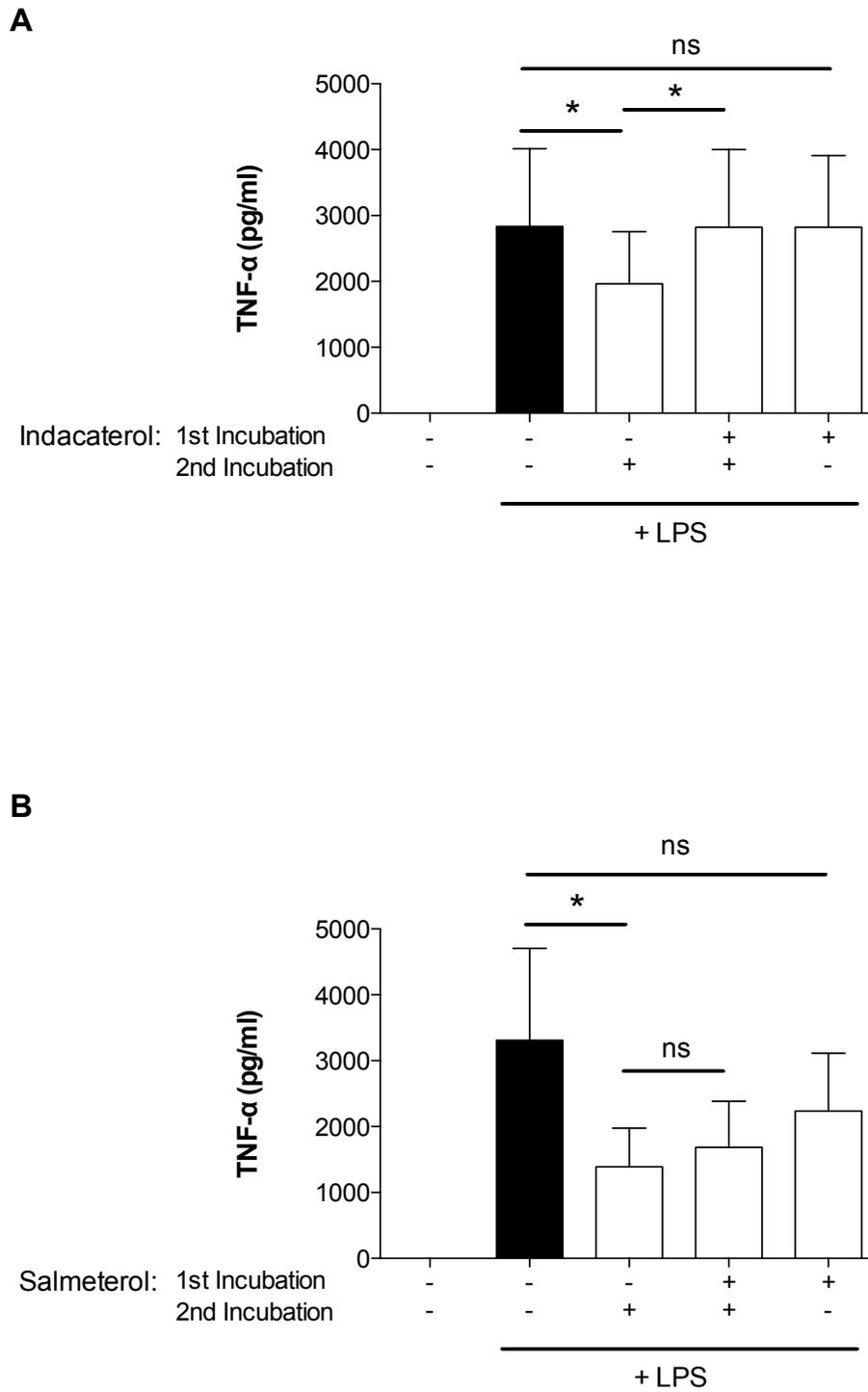
Figure 6.12. Effect of indacaterol and salmeterol on desensitization of the β₂-adrenoceptor mediated response. Macrophages were incubated without or with indacaterol or salmeterol (10^{-5} M) for 22 h, after which the cells were washed. The cells were then incubated without or with indacaterol or salmeterol (10^{-5} M) for 30 min before challenge with LPS (10 ng/ml) for 22 h. Cell culture supernatants were harvested and assayed for TNF-α. Drugs alone had no effect on spontaneous cytokine release. Values are mean ± SEM for (A) 5 and (B) 4 individual experiments. (One-way ANOVA with Dunnett's post test, *p< 0.05).

A

Indacaterol: 1st Incubation	-	-	-	+	+
2nd Incubation	-	-	+	+	-
Salmeterol: 1st Incubation	-	-	-	-	-
2nd Incubation	-	-	-	-	+

+ LPS

B

Salmeterol: 1st Incubation	-	-	-	+	+
2nd Incubation	-	-	+	+	-
Indacaterol: 1st Incubation	-	-	-	-	-
2nd Incubation	-	-	-	-	+

+ LPS
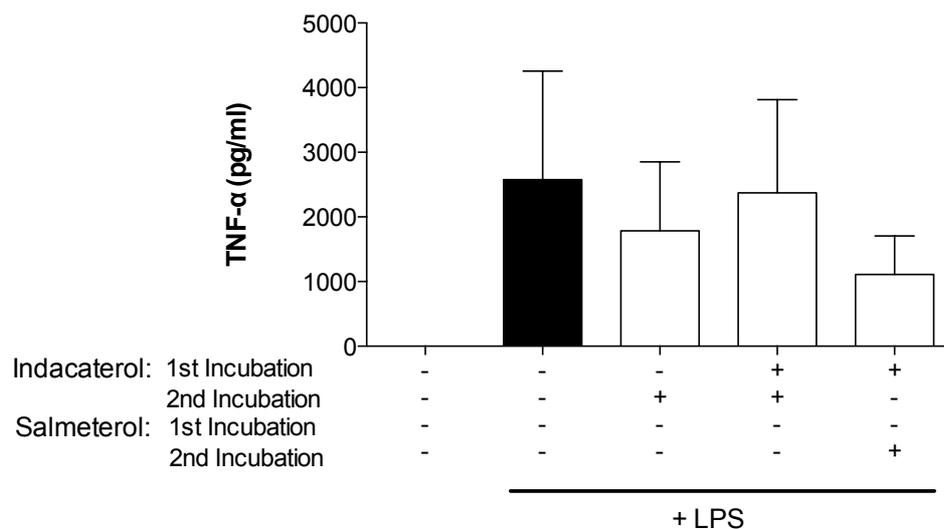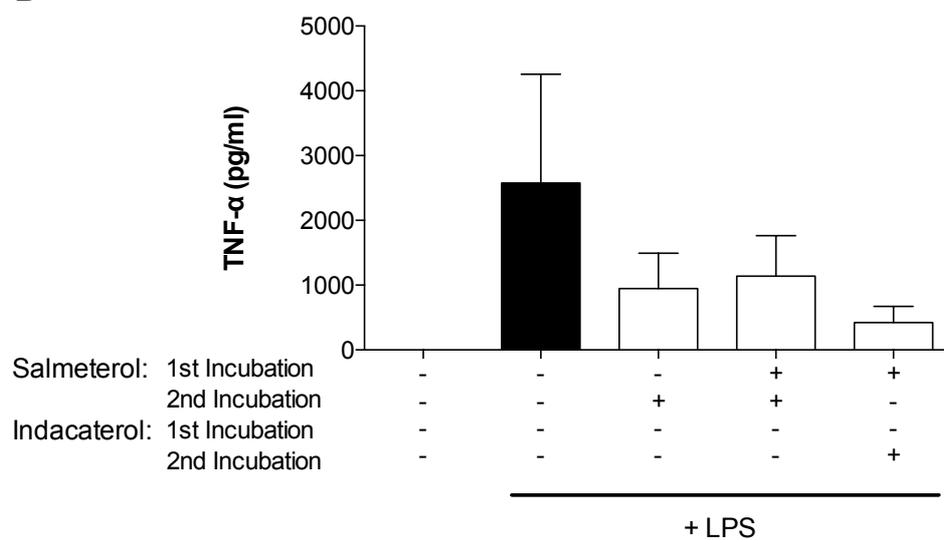
Figure 6.13. Effect of indacaterol and salmeterol on cross-desensitization of the β_2-adrenoceptor mediated response. Macrophages were incubated without or with indacaterol or salmeterol (10^{-5} M) for 22 h, after which the cells were washed. The cells were then incubated without or with indacaterol or salmeterol (10^{-5} M) for 30 min before challenge with LPS (10 ng/ml) for 22 h. Cell culture supernatants were harvested and assayed for TNF-α. Drugs alone had no effect on spontaneous cytokine release. Values are mean ± SEM for 2-3 individual experiments.

6.3. Discussion

Macrophage numbers are increased in the lung in inflammatory lung diseases and this could contribute to disease progression and severity (Barnes, 2008b). Although β_2-agonists used in current treatments are not designed to target macrophages specifically, their effects on the cells could be important to the overall benefits of the drugs. The reports of the effects of β_2-agonists on macrophages are conflicting. Some reports show that certain β_2-agonists have anti-inflammatory effects, whereas in other reports the same drugs do not. However, the same model systems were not used in all of the studies. In the present study, the effect of short and long-acting β_2-agonists on pro-inflammatory cytokine release from human lung macrophages was investigated.

Investigations were carried out into the effects of the SABA salbutamol and terbutaline, on LPS-induced TNF-α, IL-6 and IL-8 release. Neither drug significantly inhibited any of the cytokines in these initial studies. As salbutamol and terbutaline are partial agonists at the β_2-adrenoceptor (Cazzola et al., 2012), the effects of a full agonist, the SABA isoprenaline were also studied, for comparison. It was found that isoprenaline also did not cause significant inhibition of cytokine release. However, isoprenaline has a very short duration of action (1-2 h) as it is can be rapidly metabolized to 3-*O*-methyl-isoprenaline (Cazzola et al., 2012), and it is possible that this may have limited its effects in these studies.

Comparisons were made with the effects of LABAs, to determine whether drugs with a longer duration of action may be more effective at inhibiting pro-inflammatory cytokine release. Only two of the LABAs studied, salmeterol and indacaterol, consistently inhibited TNF-α and IL-6 release. Formoterol, surprisingly, did not cause any significant inhibition, despite being a full agonist at the β_2-adrenoceptor (Cazzola et al.,

2012). Formoterol, along with salmeterol, is reported to have inhibited TNF-α release in MDMs (Donnelly et al., 2010). Therefore the effects of the β_2-agonists investigated here were also very briefly explored using MDMs. Although further repetitions of the experiment are required, the preliminary data suggest that all of the SABAs and LABAs tested inhibit TNF-α release from MDMs to some extent. This indicates that potential differences exist between lung macrophages and MDMs in functional responses. It has been suggested that β_2-adrenoceptors are lost or down-regulated on monocytes as they differentiate into macrophages (Ezeamuzie et al., 2011). MDMs in vitro may not be as fully differentiated or as mature as lung macrophages, which could explain the better response to SABAs and LABAs that was observed.

High levels of the neutrophil chemoattractant IL-8 are found in sputum, lavage and blood of COPD patients (Nocker et al., 1996, Garcia-Rio et al., 2010). IL-8 release from lung macrophages was not inhibited by any of the β_2-agonists. As described in previous chapters in this thesis, the release of IL-8 from human lung macrophages was considerably higher than the other cytokines. In Chapter 3, investigation of the time course of IL-8 release in response to LPS revealed that IL-8 release continued to increase over 22 h whereas the release of the other cytokines was maximal at 16 h and then plateaued. Lack of inhibition of IL-8 by β_2-agonists has been reported elsewhere. Donnelly et al. showed that LPS-induced IL-8 release from MDMs was not attenuated by cAMP increases resulting from the action of β_2-agonists. This is the process that is generally believed to lead to inhibition of LPS-induced cytokines (Donnelly et al., 2010, Ezeamuzie and Shihab, 2010). The selective inhibition of certain cytokines but not IL-8 by β_2-agonists could be considered to be undesirable, as it could lead to an increase in neutrophilia in the lung.

Generally, it is believed that the mode of action of β_2-agonists to inhibit LPS-induced cytokine release is to act on the β_2-adrenoceptor to cause increases in intracellular cAMP (Donnelly et al., 2010, Ezeamuzie and Shihab, 2010). The transcription factor NF-κB can be inhibited by cAMP, resulting in inhibition of transcription of cytokine genes. Measurement of cAMP in response to a range of β_2-agonists including the ones found to be most effective at inhibiting cytokine release in this study (salmeterol and indacaterol) showed that there were no striking differences in the extent of cAMP levels induced. This could indicate that the inhibitory effects of LABAs seen in this study may not be linked to cAMP increases. It has been suggested elsewhere that not all β_2-agonists work through a cAMP-dependent pathway to exert anti-inflammatory effects (Donnelly et al., 2010). However, in the present study, cAMP was measured after 30 min exposure to agonists and whether the increased cAMP levels are sustained over longer time periods was not evaluated. It is possible that increased cAMP levels are sustained by LABAs but not by SABAs. These considerations make it difficult to determine the relationship between cAMP increases and cytokine inhibition.

Overall, the results of these initial investigations implied that β_2-agonists had limited effects as inhibitors of cytokine release. Therefore, it was investigated whether the inhibition could potentially be improved by modification of certain experimental conditions. The inhibition of cytokine release was found to be dependent on a combination of both the strength of the LPS stimulus (and therefore the level of cytokine generation) and on the concentration of drug used. In the presence of a sub-maximal concentration of stimulus, the SABA salbutamol was able to cause inhibition of TNF-α release, which it was unable to do when a maximally-effective concentration of stimulus was used. In the initial investigations discussed at the start of this section, the LABAs salmeterol and indacaterol inhibited TNF-α release when a maximally-

effective concentration of stimulus was used. However, when a lower concentration of salmeterol and indacaterol was used in the presence of this maximally-effective concentration of stimulus, they failed to inhibit TNF-α release. These results highlight that β2-agonists show some limited inhibitory effects on macrophages in this system. However, it is possible that *in vivo*, macrophages may produce lower cytokine levels in response to stimuli. Therefore, it is feasible that under such conditions, β2-agonists could be more effective *in vivo*.

In order to determine whether the moderate inhibitory activity observed with β2-agonists might be influenced by timing, this issue was investigated. Addition of indacaterol at time points up to six hours after LPS stimulation did not alter the inhibition of TNF-α release compared to pre-incubation with indacaterol. The results indicate that inhibition by indacaterol is not improved if added at a time point that is likely to be after the initiation of cytokine gene transcription.

In addition to inhibition of LPS-induced cytokine release, the inhibition of cytokines induced by another stimulus associated with macrophage activation was investigated. The viral mimic poly(I:C), a TLR3 agonist, was used. Salbutamol, salmeterol and indacaterol did not inhibit poly(I:C) induced TNF-α release. It could be that there is a difference in β2-agonists inhibiting cytokines generated by a viral infection than a bacterial infection, perhaps due to differences in the transcription factors activated that lead to cytokine generation. This could be important as it could be the case that β2-agonists have a slightly beneficial anti-inflammatory effect in bacterial infections but not in virus-induced exacerbations. This requires further investigation.

In Chapter 4 of this thesis, it was confirmed that macrophages produce PGE_2 endogenously and that PGE_2 inhibited cytokine release by acting at the EP_4 receptor. It was possible that the effects of endogenous PGE_2 produced upon LPS stimulation could be masking inhibition seen with β_2-agonists. It was of interest to determine whether inhibition by the β_2-agonists was altered after attenuation of the actions of PGE_2. Using an EP_4 antagonist to block endogenous PGE_2 acting on the EP_4 receptor resulted in an increase in TNF-α. However, combining the EP_4 antagonist with indacaterol did not improve the inhibitory effects of indacaterol. This indicates that the presence of endogenous PGE_2 did not affect inhibition by β_2-agonists. This was investigated further by using indomethacin to block endogenous PGE_2 generation and the effect of salbutamol on cytokine release was then examined. Salbutamol alone was found to inhibit TNF-α release by ~30% and the presence of indomethacin did not improve this inhibition.

The corticosteroid dexamethasone was used in this study as a positive control for inhibition of LPS-induced cytokine release. Dexamethasone inhibited all of the cytokines investigated, including IL-8, confirming that corticosteroids have potent anti-inflammatory activity in this system. It has been reported that macrophages in some cases can be corticosteroid insensitive, for example in COPD (Rossios et al., 2012). It is believed that the oxidative stress in COPD can affect the mechanism of action of corticosteroids at a molecular level. However, other researchers have reported that there are no issues of corticosteroid insensitivity in COPD macrophages compared to controls (Plumb et al., 2013, Higham et al., 2014). Corticosteroid insensitivity was not found to be an issue in any of the cell preparations (n=23) incubated with dexamethasone in the present study.

As β2-agonist and corticosteroid combination therapy is used for patients, the combination of indacaterol and dexamethasone on TNF-α release from macrophages was investigated. In this study, dexamethasone at a nanomolar concentration did not inhibit LPS-induced TNF-α release. However, in the presence of indacaterol, dexamethasone was able to cause inhibition. This could be considered to be a steroid-sparing effect (Taylor and Hancox, 2000). Considering the issues associated with corticosteroids in therapy, the ability to use a lower concentration could be beneficial. It has been proposed that β2-agonists and corticosteroids can work synergistically (Taylor and Hancox, 2000). There are several mechanisms that have been proposed for synergistic effects. For example, β2-agonists may activate transcription factors which cross-talk with glucocorticoid receptor transcription factors, leading to enhancement of glucocorticoid receptor function (Korn et al., 1998). Glucocorticoids may enhance β2-agonist responses by counteracting β2-adrenoceptor desensitization and downregulation (Johnson, 2004). The data in the present study suggest that there may be a modest additive inhibitory effect of dexamethasone and indacaterol on macrophage function.

To attempt to delineate the mode of action of salmeterol and indacaterol, experiments were carried out investigating functional desensitization of the β2-agonist responses. Long-term incubation of macrophages with indacaterol led to abrogation of the inhibitory effects of indacaterol. By contrast, long-term incubation of macrophages with salmeterol had no effect on the inhibitory effects of salmeterol on TNF-α release. These data suggest that the effects of indacaterol are receptor-mediated whereas those of salmeterol are not. Potential cross-desensitization of the functional response was also investigated. Incubation of macrophages with indacaterol followed by incubation with salmeterol did not affect the ability of salmeterol to inhibit cytokine release. This suggests that salmeterol and indacaterol exert their inhibitory effects through different

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mechanisms. These findings further support the idea that inhibition by salmeterol may not be β_2-adrenoceptor-mediated (Chong et al., 1998), whereas indacaterol is likely to be acting at the macrophage β_2-adrenoceptor to exert its inhibitory effects. It has been reported that salmeterol inhibits via a cAMP-independent mechanism in MDMs (Donnelly et al., 2010) but further work is required to determine whether this is specifically the case in this study. For any definitive conclusions to be drawn, it would be necessary to carry out further work to determine the mechanism by which these drugs work.

In this study, it was found that the LABAs salmeterol and indacaterol were more effective than the SABA in inhibiting LPS-induced cytokine release from human lung macrophages. The physicochemical properties of these two LABAs contribute to their long-acting effects. Both drugs are highly lipophilic and therefore likely to be sequestered in the plasma membrane of the cell. This means that they are well situated potentially to repeatedly activate the β_2-adrenoceptor (Domingo, 2013). Lipid rafts in the cell membrane have a high density of β_2-adrenoceptors (Domingo, 2013). Indacaterol has a high affinity for lipid raft microdomains and this affinity is higher than that of salmeterol (Cazzola et al., 2012). β_2-agonists exert their effects by binding to the active site of the β_2-adrenoceptor. In addition to this, reports suggest that salmeterol can also bind to the 'exosite' of the β_2-adrenoceptor by its lipid tail, which makes the association with the receptor more secure. Formoterol is less lipophilic than salmeterol (Anderson et al., 1994) but its lipophilicity is comparable to that of indacaterol (Cazzola et al., 2013). Like indacaterol, formoterol is also unable to access the 'exosite' of the β_2-adrenoceptors (Cazzola et al., 2012). Despite having similar physicochemical properties to indacaterol, formoterol lacked inhibitory activity on macrophages in this study. The reason for this is difficult to conclude. It could be the case that formoterol has a lower

affinity for lipid raft microdomains than indacaterol, which perhaps leads to the difference in effect on lung macrophages.

In summary, β_2-agonists inhibited the release of TNF-α and IL-6 to a limited extent but had no effect on IL-8 generation from macrophages. LABAs were found to be slightly better as inhibitors than SABAs. However, it is difficult to establish from the results of these studies whether these drugs would be effective in the clinical context. It is possible that the β_2-agonists could be more effective *in vivo* if, for example, the cytokine generation is lower than in these studies. Nevertheless, this is in contrast to the effective and blanket inhibition of cytokine generation seen with the corticosteroid dexamethasone. The limited effects of the β_2-agonists observed in this study suggest that any major anti-inflammatory effects that may be seen in patients following treatment with these drugs are unlikely to be accounted for by the action of β_2-agonists on macrophages.

Chapter 7: Final Discussion

Macrophages are important cells in the lungs in the context of homeostasis and in the physiological response to infections. However, inappropriate activation of macrophages could cause aberrant inflammation, which may implicate the cells in inflammatory diseases. A greater understanding of the characteristics of macrophages may enable determination of their possible roles in these diseases. At present, one of the major interests in macrophage biology research is the phenotypic heterogeneity of macrophages. This is a complex area and many questions remain to be answered. Although a body of knowledge is available based on studies using murine macrophages, surprisingly little is known of the different markers that define human macrophage phenotypes. Characterization of human macrophage phenotype may be an important contribution to determination of the potential roles macrophages play in different diseases. In addition to this, the plasticity of macrophages, which enables change of phenotype in different conditions, is also an exciting area, particularly in the context of how a particular phenotype might be linked to a certain disease state.

In the present study, macrophages were isolated from human lung tissue to be used as a model for investigations. It was found that the cells were highly responsive to stimulation by TLR agonists, releasing considerable amounts of pro-inflammatory cytokines and chemokines. This spectrum of cytokine generation indicated that the cells expressed an M1-like phenotype.

As well as generating cytokines in response to stimulation, macrophages also generated some cytokines spontaneously. In particular, considerable levels of spontaneous IL-8 release were measured. This was surprising, since activation of macrophages is believed

to be tightly regulated in the steady state *in vivo*. Comparison of the use of ultra-low endotoxin FCS with the FCS routinely used in the cell culture media allayed the initial concern that the cells may have been activated inappropriately by endotoxin contamination *in vitro*. It was also found that the cytokine response to LPS was not altered even when the cells were cultured over the course of five days, indicating that the cells were unlikely to be inappropriately activated whilst being cultured. However, the possibility of the responsiveness of macrophages changing after being removed from lung tissue into an artificial cell culture environment cannot be excluded as a reason for the cells being highly responsive to stimuli and for the elevated spontaneous generation of some cytokines.

Flow cytometry was used to investigate cell surface markers in an attempt to gain some insight of macrophage phenotype. It was found that CD206 was expressed on virtually all non-stimulated macrophages but the expression of CD14 was low. This suggested that the cells were mature macrophages, as CD14 is down-regulated during differentiation from monocyte to macrophage (Daigneault et al., 2010). This was in contrast to MDMs, which expressed similar levels of both CD206 and CD14. However, further studies are required to determine if this apparent difference in maturity translates to a difference in functional responses between the two cell types. As CD206 is generally regarded as being an M2 marker, the data implied that the phenotype of lung macrophages may be M2 but the functional data from this study suggested otherwise.

One thing that emerged when investigating macrophage markers was that simply investigating the expression of a certain set of markers in a static context is not sufficient for determining phenotypes. A comparison of expression levels before and after stimulation is required. For example, observation of whether the CD206

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expression changes upon stimulation of macrophages would possibly assist in revealing the phenotype and this approach would be taken in future studies. The flow cytometry studies also reinforced the notion that investigation of the expression of more than two markers is required to enable a clearer determination of phenotype. However, exploration of cell surface markers by flow cytometry was hampered by the high autofluorescence exhibited by many of the cell preparations. This is an issue for human macrophage researchers worldwide and a limitation of using cells from patients who are overwhelmingly likely to be current or ex-smokers. In any case, a multi-pronged approach consisting of determining markers in conjunction with functional studies is required for phenotype determination.

Another technique that can be used for investigating phenotypic markers is qPCR. Preliminary qPCR studies were found to be promising. The expression of certain genes was changed upon stimulation of the cells with certain M1 or M2 stimuli. The term 'spectrum of macrophage activation', that is used in the literature (Mosser and Edwards, 2008), was found to be accurate, as the data indicated skewing towards a particular phenotype and not complete switching following activation. The qPCR data indicate that the macrophages can exhibit plasticity when stimulated. The question is whether the cells have the ability to change phenotype repeatedly, or whether there are a finite number of changes that can take place.

Overall, it seemed that the isolated cells predominantly exhibited an M1 phenotype. This was based on the high levels of pro-inflammatory cytokines released in response to TLR agonists and low levels or virtually no release of anti-inflammatory cytokines. The qPCR data also indicated that the cells expressed an M1 phenotype at baseline before stimulation. Although the flow cytometry studies were inconclusive with regards to

determining phenotype, data from further studies investigating more markers may be useful when considered alongside the results of mediator release and gene expression studies.

Although macrophages are implicated in COPD, they may also be involved in asthma. It has been reported that IgE-dependent activation of macrophages can result in cytokine release, potentially contributing to asthma. However, this effect was not generally reproduced in this study, with the exception of one cell preparation, which responded with the release of pro-inflammatory cytokines. Passive sensitization of the cells with IgE and challenge with antigen also did not activate the cells. As direct IgE-dependent activation overwhelmingly did not occur, this line of research was not pursued further in this project. A greater number of cell preparations need to be tested to enable any firm conclusions to be drawn about the IgE-dependent responsiveness of the cells. It may be the case that cells from asthmatics express more IgE receptors and may therefore respond with the release of cytokines. Although only weak evidence for IgE-dependent activation of macrophages was found in this study, the possibility of macrophages contributing to exacerbations in asthma, following infections, cannot be excluded.

Although LPS stimulation was effective at inducing cytokine release, it also resulted in production of PGE_2. Previous studies have shown macrophages produce PGE_2 in response to LPS but the high levels produced by lung macrophages in the present study were surprising. This PGE_2 acts in paracrine fashion, presumably to inhibit cytokine release from macrophages as a mechanism to attenuate inflammatory responses. Induction of COX-2 is an important mechanism in the inflammatory response. Indeed, it was found that LPS and *N. meningitidis*, a gram-negative bacterium, up-regulated COX-

2. However, excessive PGE_2 production as a result of increased COX-2 induction may attenuate inflammatory responses excessively, decreasing the ability of the macrophage to manage an infection. Further studies would be beneficial, investigating the effects of other relevant pathogens such as *Haemophilus influenzae and Streptococcus pneumoniae* to determine whether these pathogens interfere with the COX/PGE_2 axis and how this might impact on macrophage function.

The utilization of novel, selective ligands for the EP_2 and EP_4 receptors enabled the determination that PGE_2 acts at the EP_4 receptor and not the EP_2 receptor to inhibit LPS-induced cytokine release. This is contrary to reports of both the EP_2 and EP_4 receptors being responsible for this effect (Ratcliffe et al., 2007). It has been suggested that it may be possible for an EP_4 agonist to be used to target the EP_4 receptor as a bronchodilator (Buckley et al., 2011). The present study suggests an EP_4 agonist could also be used as an anti-inflammatory.

In the present study, LPS caused up-regulation of both EP_2 and EP_4 receptors at the mRNA level. Although the EP_4 receptor is likely to be the principal functional receptor initially, it is possible that LPS activation induces EP_2 receptor expression in a time-dependent fashion. This could mean that after LPS activation the EP_2 receptor could also mediate the effects of PGE_2 but this was not investigated. Further studies investigating this and any changes to the functional responses are required. It may also be the case that EP_2 receptors regulate macrophage functions other than cytokine generation, such as phagocytosis, which warrants investigation.

It is of interest that EP agonists were found to be much more potent than β_2-agonists as anti-inflammatory agents. This is despite the fact that both EP receptors and β_2-

adrenoceptors activate adenylyl cyclase to cause cAMP increases. As Gs protein, adenylyl cyclase and downstream amplification events are expected to be the same for both receptors in the same cell preparation, the data suggest that the density of EP_4 receptors available for coupling with Gs is greater than the density of β_2-adrenoceptors. Alternatively, there are reports of cAMP signalling being compartmentalized, resulting in the cAMP generated upon activation of the different receptors leading to differential effects on the cell. The extent of cAMP signalling may be regulated by cAMP-dependent phosphodiesterases (PDEs) (Zaccolo, 2006). The cAMP signals generated by PGE_2 may be regulated by a different PDE to the PDE regulating cAMP generated by β_2-agonists. This may have some bearing on the efficacy of these agonists and may warrant further study.

The results of the studies in this thesis provide a contribution to the information available on human lung macrophages in the context of inflammation. The issues of performing these studies in lung macrophages have been highlighted. Preliminary characterization of phenotype has indicated that the cells have a predominantly M1, pro-inflammatory phenotype. Knowledge of the functional EP receptors expressed by macrophages that mediate the effects of PGE_2 was extended. These studies indicated that targeting EP_4 receptors may be a promising approach for stabilizing macrophages in inflammation. Although further work is clearly required, the studies in this thesis provide a sound framework for future investigations.

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Appendix I.

Publications arising from this thesis

Sharonjit K Gill, Yiwen Yao, Linda J Kay, Helen M Marriott and Peter T Peachell (2015), 'Characterization of the EP receptor that mediates the inhibitory effects of prostaglandin E_2 on lipopolysaccharide induced cytokine generation from human lung macrophages'. (In submission)

Oral presentations

Sharonjit Gill, Helen Marriott and Peter Peachell (2013), 'Endogenous PGE_2 activates EP_4 receptors to inhibit pro-inflammatory cytokine release from human lung macrophages', Yorkshire Immunology Group Symposium (British Society for Immunology), Sheffield

Poster presentations
External conferences

S.K.Gill, BSc, H.M.Marriott, PhD, P.T.Peachell, PhD (2014), 'Prostaglandin E_2 activates EP_4 receptors to inhibit pro-inflammatory cytokine release from human lung macrophages', American Thoracic Society International Conference, San Diego (Am J Respir Crit Care Med 189, 2014:A2762 at www.atsjournals.org)

Sharonjit Gill, Helen Marriott and Peter Peachell (2013), 'Endogenous PGE_2 activates EP_4 receptors to inhibit pro-inflammatory cytokine release from human lung macrophages', British Association for Lung Research Summer Meeting, Nottingham

SK Gill, HM Marriott, PT Peachell (2012), 'Differential effects of β_2-adrenoceptor agonists on cytokine generation from human lung macrophages', British Pharmacological Society Winter Meeting, London (Proceedings of the British Pharmacological Society at
http://www.pA2online.org/abstracts/Vol10Issue4abst074P.pdf)

Sharonjit Gill, Helen Marriott and Peter Peachell (2012), 'Heterogeneity in cytokine generation from human lung macrophages activated by bacterial or viral stimuli', British Association for Lung Research Summer Meeting, Southampton

Internal research days

Sharonjit Gill, Helen Marriott and Peter Peachell (2014), 'Prostaglandin E_2 activates EP_4 receptors to inhibit pro-inflammatory cytokine release from human lung macrophages', The Medical School Annual Research Meeting, University of Sheffield and Department of Infection & Immunity Annual Research Day, University of Sheffield

Sharonjit Gill, Helen Marriott and Peter Peachell (2013), 'Endogenous PGE_2 Activates EP4 Receptors to Inhibit Pro-inflammatory Cytokine Release from Human Lung Macrophages', The Medical School Annual Research Meeting, University of Sheffield

Sharonjit Gill, Helen Marriott and Peter Peachell (2013), 'Differential effects of β_2-adrenoceptor agonists on cytokine generation from human lung macrophages', Department of Infection & Immunity Annual Research Day, University of Sheffield

Sharonjit Gill, Helen Marriott and Peter Peachell (2012), 'Heterogeneity in Cytokine Generation from Human Lung Macrophages Activated by Bacterial or Viral Stimuli', The Medical School Annual Research Meeting, University of Sheffield

Sharonjit Gill, Helen Marriott and Peter Peachell (2012), 'The high level of pro-inflammatory cytokine release from human lung macrophages is effectively inhibited by steroids but not β_2-agonists', Department of Infection & Immunity Annual Research Day, University of Sheffield

Appendix II.

Consent forms

Information sheets and consent forms for the following studies:

i) 'Studies on lung cells involved in respiratory diseases'

ii) 'Investigation of how macrophage responses to micro-organisms programme the innate immune response to human disease'

Sheffield Teaching Hospitals _NHS_
NHS Foundation Trust

FORM 1: Information Sheet

STUDIES ON LUNG CELLS INVOLVED IN RESPIRATORY DISEASES

Introduction

We invite you to participate in studies aimed at understanding how lung cells are activated and how drugs affect the activation. Please read this leaflet carefully.

Background

We are interested in different types of lung cell and the triggers that activate these cells. Lung cell activation could contribute to respiratory diseases such as asthma. We are also interested in establishing mechanisms by which established drugs used for respiratory disorders, such as inhalers, work on cells found in the lung. We are also interested in testing alternative drugs on these cells either to assess how the cells work or because some of these drugs may be of potential therapeutic benefit. Some of these drugs will, therefore, have been developed by the pharmaceutical industry. Overall, these studies will identify (a) how lung cells are activated and (b) novel approaches to treating lung diseases.

What is involved?

During your operation, it is possible that lung tissue will be removed by the surgeons. Part of this tissue will be examined under the microscope for a diagnosis to be made and then the remainder disposed of according to proper hospital guidelines. We would like to take a part of the lung tissue, which would normally be disposed of, to use in our studies. You will not have extra lung tissue removed for research purposes. The tissue would be processed and analysed in our laboratory. A small portion of the tissue would be deposited in a research tissue bank designed to aid any future research into respiratory diseases. If you are happy with these

Version 1.5, August 2010

arrangements we would be grateful if you could please complete the accompanying short questionnaire and consent form.

What are the possible disadvantages and risks of taking part?

There are no disadvantages or risks associated with participation in this study other than the small inconvenience of filling in the accompanying short questionnaire and consent form.

What are the possible benefits of taking part?

There are no direct benefits in taking part but the information we get from the study may help to improve the treatment of people with respiratory diseases in the future.

What if I do not wish to take part?

This is entirely up to you. If you choose not to participate, you need not give a reason and this will not affect your current or future medical care in any way.

What about confidentiality?

The information provided is treated with the strictest confidence and is seen only by the people running the study. Indeed, the study will be conducted anonymously. Your name will not appear in any publications.

What if I wish to complain about the way the study has been conducted?

If you have any cause to complain about any aspect of the way in which you have been approached to take part in the study, the normal NHS complaints mechanisms are available to you.

If you have any complaints and concerns, please contact the project co-ordinator:

Dr P Peachell   Telephone   0114-271-2063

Otherwise, you can contact the Patient Advice and Liaison Service for further information.   Telephone   0114-271-5759

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Will I be able to find out the results?

If you wish, we can let you know the results at the end of the study.

What if I change my mind about participating?

You can withdraw from participation at any time that you so wish without giving a reason. Any information and materials that you may have contributed to the study will be deleted. If you decide to withdraw from the study then please contact Dr Peter Peachell by telephone (0114-271-2063) or in writing (see address on letterhead). Using standard operating procedures, any stored materials in the research tissue bank will be traced and disposed of. Also, any scientific information generated from the donated tissue, which has not been published, will be deleted. However, any data generated from the donated sample that has already been published will not be withdrawn.

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FORM 2: Short Questionnaire

STUDIES ON LUNG CELLS INVOLVED IN RESPIRATORY DISEASES

NAME   _____

CONTACT ADDRESS   _____

                                              _____

DATE OF BIRTH   _____   SEX _____

DO YOU HAVE ANY OF THE FOLLOWING MEDICAL CONDITIONS?

ASTHMA   YES/NO

EMPHYSEMA/COPD   YES/NO

ALLERGIES   YES/NO
(if yes, please specify what
triggers asthma and/or allergy)

_____

SMOKER   YES/NO/EX

PLEASE LIST YOUR CURRENT MEDICATION

_____   _____   _____

_____   _____   _____

_____   _____   _____

THANK YOU FOR YOUR HELP

                                                                                    P.T.O.

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RESEARCH CONSENT FORM

PROJECT TITLE:

STUDIES ON LUNG CELLS INVOLVED IN RESPIRATORY DISEASES

Please complete the following sections	Please complete	Please initial boxes
I confirm that I have read and understand the information sheet version 1.5, August 2010	YES/NO	
I have had an opportunity to ask questions and discuss the study	YES/NO	
I have been given enough information about the study	YES/NO	

I understand that my participation is voluntary and I am free to withdraw from participation:
    at any time
    without having to give a reason
    without affecting my future medical care
    without affecting my legal rights
	YES/NO	

I agree to donate a sample of my lung tissue for use in this study and for storage for potential use in future studies	YES/NO	
I agree to take part in the study	YES/NO	

Name of
Participant_____Date_____Signature_____

Name of
Witness_____Date_____Signature_____

(Please place this completed form in the provided envelope and bring with you
when you are admitted to hospital)

Version 1.5, August 2010

THE UNIVERSITY OF SHEFFIELD

Professor D H Dockrell
The University of Sheffield Medical School
Department of Infection and Immunity
Room K127, K Floor
Beech Hill Road
Sheffield
S10 2RX
United Kingdom
Tel: +44 (0)114 271 2160
Fax: +44 (0)114 271 3892
Email: d.h.dockrell@sheffield.ac.uk
Secretary - Mrs Sue Clary
Tel: +44 (0)114 226 8961
Email: s.clary@sheffield.ac.uk

PARTICIPANT INFORMATION SHEET

Study: Investigation of how macrophage responses to micro-organisms programme the innate immune response to human disease.

You are being asked to allow us to take a blood sample for use in a research study. Before you decide whether to give consent for this to be done it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and ask us about it if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?
We wish to determine how white blood cells called macrophages contribute to the bodies host defence against micro-organisms including bacteria and viruses. These cells represent the first line of resistance to infection and are able to respond in a variety of ways to these micro-organisms. These pre-programmed responses are general and non-specific and are often referred to as part of the body's innate immune system. We are discovering however that there is great diversity in the innate immune responses to different micro-organisms and this is particularly true of how the macrophages react when challenged by infections. Since the macrophages are able to both initiate the early response to micro-organisms but also to co-ordinate the overall immune response involving other components of the immune response we believe they are a central cell to investigate in order to build up a better understanding of how the innate immune system works. Improving our understanding of the diversity of macrophage responses will enable a more complete understanding of how these cells work.

To better characterise the macrophages response to infection we will isolate cells from your blood called peripheral blood mononuclear cells. A subset of these cells, when grown in the laboratory, will give rise to macrophages. Since it is only a small subset of your overall white blood cells that will become macrophages we need to collect 300-500 ml of blood. Once matured in the laboratory your macrophages will be exposed to certain bacteria or viruses and we will determine how they kill the micro-organisms, the proteins they express in response to challenge, how they interact with other components of the immune system (including cells and proteins) and how regulation of their lifespan influences their functions. In certain experiments we may isolate other cells or soluble factors from your blood and examine their response to micro-organisms, whether in the presence of macrophages or to contrast their responses to that of macrophages. By contrasting these responses to a variety of micro-organisms we will build up a better idea of the essential elements of this response and the ways in which micro-organisms subvert it.

What will be involved if I agree to take part in the study?
We would like to take a blood sample from you. The blood will be drawn using a phlebotomy bag similar to those used by the blood transfusion service and will be drawn by a qualified doctor. We will ask you to lie down while the blood is drawn. If necessary we can put some anaesthetic cream on your arm so that you will barely feel any discomfort at all. You may want a plaster to be put on your arm after the test has been taken. Occasionally people feel dizzy or sweaty during blood donation and if you do you can ask the doctor to stop drawing the blood. Some individuals experience a small bruise. There should be no other effects of the blood donation. If you wish to donate blood in the future (at least 4-6 months after the first donation) we will ask you some questions to determine if you have symptoms of anaemia and offer to perform a full blood count. If you agree to this test and it is abnormal we will ask that you contact your GP and we will also contact your GP to inform him/her of the result.

Do I have to take part?

No. You are free to refuse to participate. If you say no there will be no other consequences.

Are there any reasons why I should not take part?

In screening potential volunteers for the study we will ask if you have any ongoing medical conditions or take any regular medicines. If so we will ask you to not take part unless they are conditions such as minor asthma requiring use of an inhaler. We will also ask if you have had any illness in the last month or received a vaccine. We also want to make sure you have not donated blood for any

reason in the last 6 months, been told you are anaemic or have any symptoms to suggest possible anaemia such as shortness of breath, chest pain on exertion, fatigue or excessive pallor

How long will you keep my cells and DNA in the laboratory?

We will do experiments on your cells approximately 2 weeks after the blood sample is taken. Most of the experiments will be done on your cells or the proteins we isolate from your cells. Protein, RNA or DNA maybe isolated from your cells but will only be used by the investigators in this study or their collaborators and will only be used for our studies on the interactions of macrophages and related cells with micro-organisms. Any materials isolated from your cells will be stored for a maximun period of 5 years after which time it will be destroyed. The sample will be anonymised so that the results obtained will not be recognised as coming from your sample.

Will any genetic tests be done?

Genetic tests may be performed to see what 'Human Leucocyte Antigen (HLA) type' you are. HLAs are proteins on your cells that help the immune system recognise foreign matter like bacteria and viruses. Knowing what HLA type you are will allow in depth evaluation of your immune system's response to certain pathogens.

Will the information obtained in the study be confidential?

Anything you say will be treated in confidence, no names will be mentioned in any reports of the study, and care will be taken so that individuals cannot be identified from details in reports of the results of the study. In screening you for participation in the study we will certain questions such as do you have any medical conditions or take any medicines. The answers to these questions will be kept confidential and if they are deemed to result in your exclusion from participating the results will not be kept.

Will the results generated lead to any other possible outcomes?

It is possible that we may identify new ways of regulating the body's response to common infections. If that were to happen we may need to apply for a patent which would protect our right to try and exploit this finding in the diagnosis or treatment of relevant infections. We would do this through the University of Sheffield and would use the patent to support the development of research and treatment of infectious diseases.

Will I benefit from the study?

There will be no direct benefits to participation.

When can I next donate blood?

Since we take up to 500ml of your blood we recommend you do not participate in a further study involving blood donation and do not donate blood for 6 months after this study.

What if I wish to complain about the way in which the study has been conducted?

If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you. The research lead is Professor David Dockrell. If you wish to discuss the study further please contact Professor Dockrell, by telephoning him directly on 01142712160. Otherwise you can complain directly to the Medical Director who manages Professor Dockrell: Dr.David Throssell, Medical Directorate, 8 Beech Hill Road, Sheffield S10 2SB, Tel: 0114 271 2178

THE UNIVERSITY OF SHEFFIELD

Professor D H Dockrell
The University of Sheffield Medical School
Department of Infection and Immunity
Room K127, K Floor
Beech Hill Road
Sheffield
S10 2RX
United Kingdom
Tel: +44 (0)114 271 2160
Fax: +44 (0)114 271 3892
Email: d.h.dockrell@sheffield.ac.uk
Secretary - Mrs Sue Clary
Tel: +44 (0)114 226 8961
Email: s.clary@sheffield.ac.uk

STH14625   16-04-2014
Version 4.2

Consent form for healthy volunteers. *Version 4.2* 02/06/2014.
CONSENT FORM
Title of Project: Investigation of how macrophage responses to micro-organisms programme the innate immune response to human disease.
Name of Researchers: Professor. David Dockrell.

1.   I have read and understand the information sheet dated 16-04-2014
         (*Version5.1*) for the above study, answered the screening questions
         and have had the opportunity to ask questions.

2.   I understand that my participation is voluntary and that I am free
         to withdraw at any time, without giving any reason, without any
         medical care or legal rights being affected.

3.   I understand that my details as a donor will be recorded by Professor.
         Dockrell but that my blood sample will be anonymised.

4.   I understand that since I will donate up to 500ml of blood I should
         not donate blood or participate in other studies involving blood donation
         for 6 months.

5.   I give consent for my DNA to be used for this study

6.   I agree to take part in the above study.

_____   _____   _____
Name of volunteer   Date   Signature

_____   _____   _____
Name of person taking consent   Date   Signature
(if different from researcher)

_____   _____   _____
Researcher   Date   Signature

THE UNIVERSITY OF SHEFFIELD

Professor D H Dockrell
The University of Sheffield Medical School
Department of Infection and Immunity
Room K127, K Floor
Beech Hill Road
Sheffield
S10 2RX
United Kingdom

Tel: +44 (0)114 271 2160
Fax: +44 (0)114 271 3892
Email: d.h.dockrell@sheffield.ac.uk
Secretary - Mrs Sue Clary
Tel: +44 (0)114 226 8961
Email: s.clary@sheffield.ac.uk

Version 3 15 10 12
SCREENING FORM
Title of Project: Investigation of how macrophage responses to micro-organisms programme the innate immune response to human disease.
Name of Researchers: Professor. David Dockrell.

Name:

DOB:

Place √ in box to confirm

1.   The volunteer has received the patient information sheet, has had the principles of the study explained and has had the opportunity to ask questions.

The volunteer
2.   has not received a vaccine within the last week.

3.   has not had a febrile illness within the last week.

4.   has no history of chronic medical conditions.

5.   does not take regular medicines.

6.   is not known to be anaemic

7.   has not donated blood for any reason I the last 6 months

8.   has no symptoms of shortness of breath, chest pain, fatigue or excessive pallor

_____   _____   _____
Name of volunteer   Date   Signature

_____   _____   _____
Name of person screening   Date   Signature

_____   _____   _____
Researcher   Date   Signature

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Appendix III.

Lung preparations information.

Information table of the lung preparations carried out in this project. The table contains details of 230 preparations. The lung numbers relate to the coding system used in the laboratory. Any missing cell viability information is due to the viability assessment protocol initially not being well established or due to the type of experiments being performed. For example, some experiments required a high number of cells, which may have resulted in a lack of available cell culture wells to use for assessing viability. Very few of the macrophage purity values are missing. The reason for missing values is the inability to perform cytospins on those occasions.

Lung number	Gender	Age	Pre-chop weight (g)	Yield (million cells)	Macrophage purity (%)	Viability (%)
A417	M	68	14.91	48		
A418	M	65	6.1	6		
A419	F	72	9.19	8.9	98	
A420	M	64	23.48	146		
P1	M	79	11	9	97	
P2	M	79	11.84	35	96	
P3	F		10.4	13	92	
P5	F	36	6	8	91	
P9	M	42	15.05	22	92	
P14	M	55	9.2	59	96	65
P17	M	75	18.3	17	82	48
P19	M	82	16	18.6	98	
P22	M	60	7.5	31	76	
P23	F	83	14	21	88	
P24	M	53	4.04	9.5	96	
P25	F	67	3.5	0		
P26	M	74	7	1.5		
P27	F	72	12.4	23.5	81	
P29	F	64	8	21	82	
P33	M	69	8.9	54.5	96	
P35	M	76	7.85	44	94	96
P37	M	52	5.98	32	84	91
P39	M	64	7.3	33	76	
P42	F	66	12.8	5.2	68	
P47	M	24	11	3	64	
P51	F	72	13.25	22	87	92
P54	M	70	7.5	24	93	
P55	M	76	13	4.3	86	
P58	F	66	5.9	5.1	90	
P59	M	67	5	5.4	90	
P60	M	78	22	51	93	
P63	M	72	8.7	4.7	80	
P64	F	77	9.35	50	98	91
P66	M	56	23	21	81	91
P67	M	44	20.76	39	95	95
P69	M	68	8.18	18.6	93	90
P71	M	77	16.25	13	93	90
P72	F	59	23	26	90	94
P75	M	32	6.3	2.7	76	
P76	M	81	9	3.8	74	
P79	M	43	14.23	22.5	90	90
P80	M	67	6.45	11.8	93	95
P81	F	71	5.37	14	96	93
P85	M	71	9.2	38.8	97	82
P86	M	61	7.5	56.6	92	85
P88	M	60	20	51.8	88	92
P90	M	75	18	42	97	97
P93	F	79	7.28	7	84	93

P95	M	72	15.8	6	89	93
P97	F	65	11.8	14.9	81	82
P98	F	72	7.35	15.9	96	87
P102	M	80	26	18.7	71	92
P103	F	60	19	36	93	86
P104	F	72	9.5	48.7	90	96
P106	M	79	15	16.3	83	93
P111	F	52	5.5	35.5	95	85
P114	M	63	8	4.7		
P116	M	70	27.21	59.6	83	
P117	F	66	4.5	11.6	93	
P119	M	83	36	29.8	86	
P124	F	62	13.8	1.5	79	94
P125	F	79	6.5	6.3	90	95
P126	F	53	21	29	85	80
P128	M	67	19.75	66	90	88
P131	M	53	16.8	4.65		
P132	M	78	14	12	97	95
P133	F	75	5.77	5.5		
P134	F	78	11.8	3.8	86	93
P136	F	72	10.1	16		90
P139	F	58	14.22	0		
P140	F	73	13.75	23	92	96
P141	M	71	4.66	28	97	96
P143	M	75	10	5		
P156	F	54	17	14	84	98
P159	F	47	7.01	5.18	70	
P163	F	70	3	7.5	81	95
P165	F	62	18.7		83	91
P170	M	60	17	74	97	86
P171	F	60	5	4	77	
P173	F	59	26	11.6		
P176	M	77	14	32	80	91
P177	F	60	6	4	86	
P179	M	73	16.38	27	81	74
P180	F	68	4.2	8	90	
P185	M	88	15	18.5	88	92
P189	M	61	8.5	24	90	95
P190	M	64	17	30	92	87
P200	F	70	9.32	2.9	78	
P210	M	82	25.68	11	62	96
P214	F	52	13.68	13	92	90
P220	M	56	26	17.25	74	97
P221	M	61	9.5	28.5	99	96
P227	M	72	13.32	19.6	82	96
P228	F	76	15.89	5.3	87	92
P230	M	76	13.23	37.4	94	97
P231	F	68	10.19	2.3	82	98
P235	M	69	10	17.8	89	95
P241	M	79	20	25	87	94

P244	F	22	9.65	36	59	96
P248	F	86	10.53	7.6	91	95
P249	F	57	8.7	8	91	
P250	M	71	29	11.9	75	93
P258	M	27	15	32	86	94
P259	F	76	14	23		92
P262	M	70	13.58	42	91	96
P263	M	82	5.68	6.6	88	94
P265	M	61	20	12	88	90
P268	F	79	14.99	16	90	96
P270	M	78	14.8	18	95	92
P272	M	66	19.48	6	87	91
P275	M	69	14.76	14	86	97
P278	M	67	13	36	87	91
P282	F	80	3.75	7	68	92
P292	M	63	8.92	5.5	88	66
P293	F	59	23.16	44	96	96
P296	M	43	9.12	4.35	83	90
P297	M	77	10	42	86	93
P298	M	47	5	20	94	99
P302	M	77	14	15	73	94
P303	M	62	23.64	10.43	77	97
P306	M	69	13.42	20	93	84
P309	F	60	13.78	14.6	77	93
P313	M	68	12.6	20	89	
P317	M	77	11.07	49	84	90
P320	F	76	10.74	19	95	95
P324	M	41	11	2.25		94
P325	M	61	14	12		93
P329	M	74	20	11.5	72	97
P330	F	76	5.87	43		98
P331	M	72	17	41	76	95
P340	M	74	10	33	90	93
P345	F	65	8	5.6		59
P348	M	82	10.61	15		91
P355	F	62	9	21.5	95	88
P356	F	51	16	1.5	89	
P360	F	66	5	4.6	88	92
P361	F	59	5.76	29		87
P364	M	49	15	66	95	90
P369	M	74	4.5	10.4	82	90
P370	F	52	6.4	3.3	63	88
P371	M	76	20	68	89	98
P373	M	73	3.35	15	92	93
P378	F	73	10	7	90	95
P380	F	65	28.28	8.6	77	99
P381	F	52	8	23	94	92
P382	F	82	16.93	51	88	93
P383	F	74	6	9	87	
P384	M	60	10.4	8.3	68	93

P387	F	59	17	9	60	94
P389	M	70	7.47	19	86	95
P391	M	70	28	19.8	87	95
P393	M	68	13	24	94	
P394	M	48	8.3	7	87	
P397	M	69	3.5	7.6	90	
P398	F	59	11.7	9.8	90	
P401	M	53	16	14	91	
P403	F	68	3.6		57	
P405	F	73	18	7	82	
P406	F	77	5.4	7.5	93	85
P407	F	71	28	3	86	
P409	F	69	4	13	97	
P411	F	45	20	40	90	
P412	F	58	18	54	92	93
P413	F	74	10	21	91	93
P416	M	74	12.81	47	92	90
P421	M	73	11	6	88	80
P422	M	70	6.8	21	96	89
P423	M	84	19	14	69	98
P424	F	71	10.73	13	85	89
P438	F	61	19.06	7.8	59	85
P440	F	66	13	10.7	80	95
P441	F	61	10.42		92	
P444	M	63	23.56	14.8	91	81
P445	F	72	25	23.7	87	90
P447	M	79	16.2	11.8	89	
P448	F	51	25.13	24	86	
P450	F	65	9.9	7.4	80	
P455	M	56	15	10.4	92	92
P458	M	70				
P460	M	72	5.93	3.6	89	
P462	F	67	9	4.9	70	
P463	M	78	7.35	18	90	92
P464	F	74	10.36	3.3	63	
P465	F	54	9	7	90	90
P468	F	53	9.7	23	88	90
P469	M	77	10	23	87	82
P473	M	70	14.28	23.9	81	86
P476	M	66	16	24.6	91	92
P477	M	68	14.6	6	76	
P479	F	76	18.93	21.1	94	82
P484	M	59	6.11	19	81	92
P485	F	76			58	
P486	F	82	7.5	7	83	
P488	M	50	9.84	45	97	92
P489	M	68	13.91	6.8	68	
P490	F	70	12	6	89	92
P492	F	71	14.61	10	83	
P493	F	69	21	14	96	

P497	F	75	10.37	18.5	88	95
P498	F	80	10.6	44	92	93
P500	F	67	6.75	23	89	96
P505	F	69	10.5	16.9	78	95
P510	F	70	17.7	7.5	76	
P513	F	67	9.02	29.5	90	95
P522	F	62	16.43	11.4	71	
P524	F	77	13.86	75	81	93
P525	M	78	8.35	58	88	92
P527	M	84	9.51	5.9	89	
P530	F	54	15.33	7.3	82	93
P532	F	78	20	32.6		80
P534	M	59	15	47	94	94
P535	F	71	2.4	4.9	74	85
P536	F	66	9.1	2.2	76	78
P538	F	71	6.25	3.6	90	
P539	F	69	10.32	6.9	60	
P544	M	73	28	7.4	65	
P545	F	83	6.32	4.5	78	
P546	F	68	10.19	23.3	84	
P547	M	54	13	27.4	95	
P551	M	77	11.2	10.2	93	
P552	F	72	13.72	8.4	95	97
P554	F	56	8.14	45.9	90	
P556	F	73	19.32	45.9	82	
P559	F	72		7.6		91
P570	F	60	25.46	5.7	57	
P571	F	64	6.5	4.5	71	
P574	F	84	9.49	14.8	80	93
P576	F	65	7.85	24.9	83	91
P581	F	68	5.8	9.2	81	78
P582	F	58	6.7	60.6	97	93