# Expression and regulation of macrophage cAMP phosphodiesterase-4B2 in inflammatory airway diseases

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# Expression and regulation of macrophage cAMP phosphodiesterase-4B2 in inflammatory airway diseases

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## Abstract

The approval of roflumilast, a selective inhibitor of cAMP phosphodiesterase-4 (PDE4), provides clinical validation for selectively inhibiting cAMP degradation in COPD. Roflumilast, targets all known PDE4 isoforms coded by the PDE4A/B/C/D genes including PDE4D isoforms that are linked to dose-limiting side effects.

Animal studies demonstrate that PDE4B isoforms are critical for mediating the lipopolysaccharide (LPS)-induced inflammatory response. Since PDE4B2 is the only PDE4B isoform in macrophages – cells that help orchestrate the inflammatory response, an inhibitor designed to selectively target PDE4B2 may provide a better index of therapeutic efficacy over side effect profile than pan-PDE4 inhibitors like roflumilast.

Here I characterise PDE4 isoform expression in monocytes and monocyte-derived macrophage subsets in donors with COPD and without. Using RT-qPCR and DNA probes, I quantify the change in PDE4 isoform expression in response to TLR4 signalling with LPS and seek to evaluate if this is altered in COPD. Using different cell culture conditions and known macrophage markers, I define a model of ‘M1’ and ‘M2’ macrophages and show PDE4B2 expression is higher in M1, compared to M2 macrophages, and that, after LPS challenge, PDE4B2 levels become higher still. Using donated bronchial tissue I obtain data in support of reduced PDE4B expression in patients with atopic asthma.

I propose that PDE4B2 is degraded through ubiquitination. Using data from the USCD genome suite and Encode datasets I theorise that the transcriptional

repressor BCL-6 may regulate PDE4B2 expression in macrophages and provide data to support this.

I evaluate IL-4-mediated constraint of LPS-induced PDE4B2, showing that STAT6 regulates inducible PDE4B2 expression and that this signaling cascade is maintained in patients with COPD.

This work provides novel evidence and understanding concerning the importance of PDE4B2 and the regulatory pathways that allow it to orchestrate the macrophage inflammatory response. This may point to novel therapeutic approaches.

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Finally, I dedicate this thesis to my parents for the unlimited love and support they have given me and whom continue to be an inspiration for making me want to do better.

## Abbreviations

| Abbreviation | Meaning |
|---|---|
| AC | Adenylate cyclase |
| AKAP | A-kinase anchor proteins |
| AM | Alveolar macrophage |
| ANOVA | Analysis of variance |
| BSA | Bovine serum albumin |
| cAMP | cyclic adenosine 3’ 5’ monophosphate |
| DMSO | Dimethyl sulfoxide |
| EDTA | Ethylenediaminetetraacetic acid |
| EPAC | Exchange protein directly activated by cAMP |
| ERK | Extracellular signal-regulated kinase |
| FBS | Foetal bovine serum |
| G-CSF | Granulocyte macrophage colony stimulating factor |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GFP | Green fluorescent protein |
| GPCR | G protein-coupled receptor |
| IP | Immunoprecipitation |
| Jak | Janus kinase |
| M-CSF | Macrophage stimulating factor |
| MAPK | Mitogen-activated protein kinases |
| MMP | Matrix metalloprotein |
| NTR | N-terminal region |
| PBS | Phosphate buffered saline |
| PDE | Phosphodiesterase |
| PI3K | Phosphatidylinositol 3-kinase |
| PKA | Protein kinase A |
| RPMI | Roswell Park Memorial Institute |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SEM | Standard error of the mean |
| SOCS | Suppressor of cytokine signalling |
| STAT | Signal transducer and activator of transcription |
| UCR | Upstream conserved region |

# Chapter 1 **INTRODUCTION**

Macrophages have roles in almost every aspect of an organism's biology, from development, homeostasis and repair through to the immune response to pathogens (van Furth and Cohn, 1968, as cited in T. A. Wynn, Chawla, & Pollard, 2013). Macrophages are found in numerous tissue types where they exhibit significant anatomical and functional diversity. Within the lung, alveolar macrophages have a unique role as sentinel cells at the host – environment interface where they form the dominant immune cell in the steady state (Allard, Panariti, & Martin, 2018). However, it is now appreciated that the homeostatic and reparative functions of macrophages can be subverted by continuous insult, resulting in a causal association between macrophage responses and diseases including fibrosis, carcinoma, atherosclerosis and chronic inflammation (T. A. Wynn et al., 2013).

In recent years there has been a paradigm shift in our understanding of macrophage ontogeny, from one of monocyte derived macrophages seeding various tissues to one characterised by macrophage longevity and self-renewal. These insights, twinned with an understanding of how macrophages can help drive human disease has helped fuel interest in understanding macrophage activation states and the mechanisms that underlie them. Discovery here, could lead to novel therapeutic approaches and the promise of selectively targeting dysregulated macrophage responses to improve the treatment of chronic inflammatory diseases.

## 1.1 Macrophage origins

The discovery of the macrophage is credited to Elie Metchnikoff who in the late 19th century described the roles of macrophages and their ability to phagocytose infectious organisms and damaged cells. In the 1960s, van Furth and Cohn (van Furth and Cohn, 1968, as cited in Epelman, Lavine, & Randolph, 2014) proposed that all tissue macrophages originate from circulating adult monocytes, a view widely held for the next 40 years. In recent years, a series of seminal studies have drastically revised our understanding of macrophage ontogeny by demonstrating that in the steady state, many resident tissue macrophages are in fact established during embryonic development and persist in to adulthood independent of peripherally circulating blood monocytes, through a combination of longevity and limited self-renewal (Ginhoux et al., 2010; Schulz et al., 2012; Yona et al., 2013).

Macrophages first appear during early gestation (embryonic day (ED) 6.5 – ED 8.5) and expand in the extra-embryonic yolk sac during a phase of development termed primitive haematopoiesis. At this stage in development, haematopoietic progenitors in the yolk sac are still quite restricted and macrophages are the only type of leukocytes they give rise to. At E8.5 – E10.5, definitive haematopoietic stem cells (HSCs) emerge from the aorto-gonad-mesonephrons and give rise to all immune lineages (Epelman et al., 2014). Beginning at E10.5, HSCs migrate to the foetal liver which becomes the major haematopoietic organ during the remainder of embryonic development. It is not until the perinatal period do traditional bone marrow HSCs become the primary site of haematopoiesis.

Macrophages that develop during the embryonic period differ from those that develop from the yolk-sac in their dependence on transcription factors as well as surface marker expression. HSC-derived macrophages are dependent upon the transcription factor Myb whereas yolk-sac derived macrophages are not (Schulz et al., 2012). In the mouse, yolk-sac-derived macrophages – but not HSC-derived macrophages – display a characteristic CX3CR1$^{hi}$F4/80$^{hi}$CD11b$^{lo}$ expression pattern (Schulz et al., 2012; Yona et al., 2013). Discerning the functional relevance of these distinctly derived macrophage subset populations has proved challenging, as macrophages expressing CX3CR1 including those in the lung, lose this expression soon after birth (Epelman et al., 2014).

Fate mapping studies have been used to help establish that in most tissues, macrophage populations are seeded during early foetal development. Ablation of Myb-dependent bone marrow haematopoiesis followed by transplantation of genetically dissimilar bone marrow has showed that the major tissue resident macrophages (defined as F4/80 bright) in skin, spleen, pancreas, liver, brain and lung arise from yolk sac progenitors (Schulz et al., 2012). In some tissues including the lung and kidney, macrophages may have a chimeric origin being derived from both yolk sac (F4/80$^{high}$) and bone marrow (F4/80$^{low}$) (T. A. Wynn et al., 2013). The gut, however, appears to be an exception for its independence of yolk-sac derived macrophages as, in the steady state, intestinal macrophages derive directly from bone marrow derived monocytes that continuously seed the lamina propria and differentiate in to macrophages (Bogunovic et al., 2009; Tamoutounour et al., 2012).

### 1.1.1 Interstitial and tissue macrophages

Within the interstitial spaces of tissues, not all macrophages appear to completely fall into the category of embryonically derived macrophages. Lineage – tracing studies in mice have identified that postnatally, Ly6C+ monocytes, give rise to interstitial macrophages in the skin, heart and lung (Jakubzick, Randolph, & Henson, 2017). Hence, in some organs including the skin and lung, macrophages are derived both embryonically and postnatally.

The interplay between tissue resident and interstitial macrophages is not well understood. However, some insights have been gained, notably by collaborators of the Immunological Genome Project (ImmGen), who have studied transcriptional expression profiles in various mouse immune cells including macrophages (Gautier et al., 2012). In mice, there are major differences in the transcriptional profiles between tissue-specific macrophages and interstitial macrophages. Tissue-specific macrophages share a core macrophage signature, but in addition, have a unique transcriptional signature that is adapted to their local environment (Gautier et al., 2012). Tissue-specific macrophages also differ functionally, in parallel, with the variation in the transcriptomes. For example, Langerhans cells which are the tissue resident macrophages in skin help maintain the epidermal barrier, whereas alveolar macrophages in lung, are involved in clearance of pulmonary surfactant and cellular debris from the alveolar space (Okabe & Medzhitov, 2016). In contrast, interstitial macrophages from the skin, heart and lung, while also exhibiting standard macrophage features, seem to have more closely overlapping transcriptional profiles regardless of their local environment (Jakubzick et al., 2017).

### 1.1.2 Lung macrophages

In the steady state, alveolar macrophages (AM) replenish independently of circulating blood monocytes. However, depletion of AM using a model of CD163-DTR (a conditional system based upon susceptibility to Diphtheria toxin to selectively ablate macrophages expressing CD163 (Duffield et al., 2005)) results in repopulation by in situ proliferation, independently of blood monocytes (Hashimoto et al., 2013). However, if AM are depleted using lethal irradiation, then it is recruited monocytes that repopulate the AM population and not the existing tissue resident macrophages. Intriguingly, despite genotoxic injury, irradiated resident AM can re-expand if recruited monocytes cannot receive granulocyte-macrophage colony-stimulating factor (GM-CSF) signals (Epelman et al., 2014). Hence, it has been proposed that lung tissue macrophages maintain themselves indefinitely independently of blood monocyte input but if an acute and severe depletion occurs, circulating monocytes can readily re-populate macrophage populations.

Alveolar macrophage development is dependent upon environmental cues including the growth factor GM-CSF and macrophage stimulating factor (M-CSF) (Guilliams et al., 2013). Around the perinatal period, GM-CSF mRNA is highly expressed in epithelial cells and GM-CSF protein highly expressed in bronchoalveolar fluid, but in both cases, wanes soon after birth (Guilliams et al., 2013). Lungs from $Csf2^{-/-}$ mice, which lack the receptor to GM-CSF, are completely devoid of alveolar macrophages, but can be reconstituted with macrophages by perinatally-given recombinant GM-CSF. Thus, GM-CSF is proposed to be a driving cytokine for alveolar macrophage development.

Within the lung, there are two distinct macrophage populations, those that reside in the alveolus and those in the interstitium. As well as being anatomically distinct, AM and interstitial macrophages (IM) differ in surface marker expression and are proposed to have distinct functions.

Human AM are characterised by high expression of the integrin CD11c and low expression of CD11b (Hussell & Bell, 2014). AM appear to be derived from HSCs seeded during embryogenesis and develop into long-lived cells in response to GM-CSF (Guilliams et al., 2013). AM uniquely sit at the host-environment interface and are adapted to respond to commensal bacteria, inhaled particulates as well as host-epithelial derived factors, such as surfactants. In mice, it is reported that AM exhibit a greater microbicidal activity than IM, as evidenced by a higher release of reactive oxygen species, nitric oxides and TNF$\alpha$, following an appropriate stimulation (Franke-Ullmann et al., 1996).

IM, on the other hand, reside in the lung parenchyma and have contrasting expression of CD11, to that of AM - namely they express CD11b$^{high}$CD11c$^{low}$. IM functions are less well understood though using experimental models of disease, some important insights have been made. In a model of allergic asthma, IMs prevent the induction of a Th2 response in mice challenged with both lipopolysaccharide (LPS) – a prototypical ligand of Toll-like receptor 4 (TLR4) and a major component of the outer membrane of gram-negative bacteria – and an airborne allergen. Moreover, targeted elimination of IMs, led to an overt ‘asthmatic-like’ reaction to the same challenge (Bedoret et al., 2009). IM and not AM, produce high levels of anti-inflammatory IL-10 and inhibit LPS-induced maturation and migration of myeloid cells in an IL-10 dependent manner. Hence,

to summarise, it has been proposed that, while AMs are more intimately involved in direct killing and host defence, IM may exhibit a more regulatory function within lung tissue (Byrne, Mathie, Gregory, & Lloyd, 2015).

### 1.1.3 Macrophage phenotypes

When macrophages are exposed to micro-environmental stimuli, they acquire new functional properties in a dynamic and reversible fashion. Such plasticity affords them an ample repertoire of responses that contribute towards both innate and acquired immune functions (Byrne et al., 2015; Lavin et al., 2014; Lawrence & Natoli, 2011). Unfortunately for investigators of macrophage activations states, this plasticity also means there is a lack of consensus over the expression markers that can be used to help define them, the culture conditions in which they grow and even the appellations used to describe them. Despite this, there is a degree of consensus within the literature that macrophage activation states or 'phenotypes' can be broadly considered to be either 'classical' or 'non-classical'. Classical macrophages are defined by engagement with interferon (IFN)-$\gamma$ and toll-like receptors (TLRs) and are also known as M1 macrophages. Non-classical macrophages are activated by IL-4 and IL-13 and also known as M2 macrophages (T. A. Wynn et al., 2013).

The M1 phenotype is characterised by the expression of high levels of pro-inflammatory cytokines, high production of reactive nitrogen and oxygen species and strong microbicidal and tumoricidal activity (Sica & Mantovani, 2012). In contrast, M2 macrophages are reported to be involved in parasite containment, promotion of tissue remodelling and tumour progression as well as having immunoregulatory functions. M2 macrophages are characterized by efficient

phagocytic activity, high expression of scavenging molecules and the expression of mannose and galactose receptors (Gordon & Martinez, 2010; Mantovani, Sozzani, Locati, Allavena, & Sica, 2002).

Although a dichotomous classification between M1 and M2 macrophages provides a useful functional description encompassing the breath of macrophage polarising conditions, it is likely and widely accepted that within the tissue micro-environment, macrophages exist along a spectrum of activation states (Murray et al., 2014; Piccolo et al., 2017). This understanding has been underpinned by large transcriptomic data sets in human macrophages stimulated using diverse activation signals (Xue et al., 2014).

A further macrophage phenotype, M2c, distinct from M1 and M2 macrophages is said to be the product of engagement with IL-10 and functionally may exhibit a more regulatory role (Makita, Hizukuri, Yamashiro, Murakawa, & Hayashi, 2015).

## 1.2 Monocytes

Whilst in the majority of tissues, monocytes are non-essential towards populating resident tissue macrophages, monocytes have been shown to contribute to interstitial macrophage populations in the post-natal period (Jakubzick et al., 2017). Monocytes also serve an important function in supporting tissue macrophage responses during the response to injury. During acute lung injury, monocytes traffic to areas of inflammation to significantly increase the macrophage pool (Jakubzick et al., 2017). However, using experimental models, it has been shown that these ‘recruited’ monocyte-derived macrophages decline

in number and are not destined to remain a significant part of the tissue resident macrophage population (Janssen et al., 2011).

Using an influenza A and LPS instillation model of acute lung injury, followed by dye labelling techniques and bone marrow transplantation, Janssen and colleagues (Janssen et al., 2011) resolved that during the acute phase of lung injury, a high proportion of original resident macrophages persisted. In contrast, monocytes recruited to tissues that differentiated to macrophages progressively declined in number, mediated by apoptosis and local phagocytosis. These recruited macrophages expressed high levels of the death receptor Fas and were rapidly depleted by Fas-activating antibodies. Interestingly, clearance of recruited macrophages appears to be critical to the host reparative response as disruption to apoptosis, delayed the resolution of lung injury (Janssen et al., 2011). Hence, it may be proposed that although the developments in macrophage ontogeny have served to demote the role of monocytes in directly contributing to macrophage origins, monocytes continue to exert an important role in the acute response to inflammation and there is an important cross-talk with tissue resident macrophages.

### 1.2.1 Monocyte subsets

After birth, monocytes derive from precursors in bone marrow through a differentiation program involving progressively committed progenitors (Epelman et al., 2014). Monocyte trafficking studies in mice have provided significant insights into monocyte subsets and functions. In mice, at least two subsets of monocytes have been described; so-called ‘classical’ $Ly6c^{hi}CD43^{-}$ monocytes which are the direct descendants of a $Ly6c^{+}$ monocyte-specific progenitor and

‘non-classical’ Ly6c$^{lo}$CD43$^{+}$ monocytes that derive from an Nr4a1-dependent transcriptional program (R. N. Hanna et al., 2011; Hettinger et al., 2013).

Classical Ly6c$^{hi}$ monocytes display pro-inflammatory activity. They have a half-life in the circulation of approximately one day (Jakubzick et al., 2017). They extravasate into tissues where they patrol the extravascular spaces and are involved with antigen presentation without becoming committed to macrophages or dendritic cells (DCs). They may also give rise to blood non-classical Ly6c$^{lo}$ monocytes.

Non-classical monocytes appear to remain and perform their primary function within the vasculature itself. They ‘patrol’ alongside the endothelium of blood vessels and help to maintain the integrity of the blood vessels by clearing damaged endothelial cells (Carlin et al., 2013).

In response to an inflammatory stimulus, monocytes can undergo phenotypic switching under the control of the nuclear hormone receptor (NR) superfamily of early response genes. In response to LPS, oxidised lipids and cytokines, the orphan nuclear receptor Nur77 (Nr4a1) regulates the switch between inflammatory Ly6c$^{hi}$ and Ly6c$^{low}$ monocytes through regulation of differentiation from a myeloid dendritic precursor (Maxwell & Muscat, 2006). Elevated cAMP affects myeloid cell immunity in part by inducing Nur77 expression thus favouring a reparatory monocyte phenotype (Raker, Becker, & Steinbrink, 2016).

### 1.2.2 Monocyte functions

Ly6c is not expressed in humans. Instead, the relative expression of surface markers CD14 and CD16 have been used to help delineate and characterise three human monocyte subsets. In humans, the $CD14^{++}CD16^{-}$ population or 'classical' monocytes are the major monocyte subset representing 80–90% of circulating monocytes. Classical monocytes express high levels of the chemokine receptor CCR2 which together with its ligand monocyte chemoattractant protein-1 (MCP-1/CCL2) are important for monocyte recruitment to the site of inflammation (Cornwell, Vega, & Rogers, 2013). These 'classical' monocytes are counterparts to the $Ly6c^{hi}$ monocytes in mice and exhibit weak TNF$\alpha$ and IL-1β response but a strong IL-6, IL-8 and IL-10 response to LPS.

$CD14^{DIM}CD16^{+}$ non-classical monocytes which approximate to the $Ly6c^{low}$ monocytes in mice, contribute between 5–8% of total circulating monocytes and express low levels of CCR2 and CCR5, that limit their ability to extravasate in to tissues in response to inflammatory stimuli (Cornwell et al., 2013). Interestingly non-classical cells express the chemokine C-X3-C motif chemokine receptor 1 (CX3CR1) (fractalkine) which it is proposed enables them to interact with the CXC3CL1 receptor expressed on the luminal surface of vascular endothelial cells (Cros et al., 2010). This interaction enables these monocytes to 'crawl' along blood vessel surfaces and they maintain a complement of chemoattractant receptors to enable them to extravasate into sites of tissue inflammation.

The third monocyte subset, $CD14^{+}CD16^{+}$ population represents an 'intermediate' monocyte population that express low levels of CCR2 but high levels of CX3CR1

and CCR5 and produce an intermediate to high level of pro-inflammatory cytokines (Cros et al., 2010).

### 1.2.3 Monocyte to macrophage differentiation

When tissues are damaged following infection or injury, classical $CD14^+$ 'inflammatory' monocytes are recruited form the circulation by several pro-inflammatory cytokines, chemokines, and components and products of tissue matrix degradation (Geissmann et al., 2010). Once in the inflamed tissue, they begin to take on functional properties associated with macrophages including their ability to synthesise and secrete the inflammatory mediators, TNF$\alpha$, IL-1 and nitric oxide (T. A. Wynn et al., 2013). These cytokines participate in the activation of anti-microbial defences including oxidative processes that contributes to the killing of invading organisms (Murray & Wynn, 2011).

Recruited monocytes also exhibit an increase in size and complexity of their cellular machinery (Doerschuk et al., 1990). They produce IL-12 and IL-23, which direct the differentiation and expansion of anti-microbial Th1 and Th17 cells that help to propagate the inflammatory processes (T. A. Wynn et al., 2013). Although these inflammatory macrophage responses are initially beneficial in helping to clear invading organisms, they also trigger substantial collateral damage that if unchecked, have the potential to become pathogenic and contribute towards disease progression - as has been proposed occurs in the propagation of chronic inflammatory (Sindrilaru et al., 2011) and autoimmune diseases (Krausgruber et al., 2011). To counteract the potential tissue damage following an exuberant initial host response to stress or infection, macrophages either undergo apoptosis, or switch to an anti-inflammatory phenotype (T. A. Wynn et al., 2013). Such

‘regulatory’ macrophages produce ligands such as Wnt, that are associated with development and are essential for tissue repair.

It is becoming increasingly clear, that macrophages and the mechanisms that regulate switching between macrophage subsets are implicated within important disease processes. In the paragraphs below, macrophage responses are explored in further detail in selected major human diseases.

## 1.3 Macrophages in disease

### 1.3.1 Cancer

Tumours exist within a complex cellular environment in which innate immune cells, including macrophages are highly represented (Qian & Pollard, 2010). Macrophages promote both the initiation and malignant progression of cancer (Bingle, Brown, & Lewis, 2002). There is growing clinical and experimental evidence that Inflammation in particular, can be a key driver of many cancers (Mantovani & Sica, 2010). COPD is associated with an increased risk of cancer that is independent of the exposure to smoking (Durham & Adcock, 2015). Besides exposure to cigarette smoke, airway inflammation in COPD can also be caused by bacterial colonisation, such as with Haemophilus influenzae. This has been evaluated experimentally in the mouse, where bronchial exposure with lysate containing Haemophilus influenzae results in inflammation and increased tumourigenesis (Moghaddam et al., 2009).

Macrophages, in response to persistent infection, synthesise the inflammatory cytokines, IFN-$\gamma$, TNF$\alpha$ and IL-6 which engage other cells to sustain chronic inflammation (Balkwill & Mantovani, 2012). Since the immune system operates

on the basis of checks and balances, a failure of the homeostatic mechanisms that serve to counter inflammation is another important factor in tumorigenesis. In myeloid cells, ablation of STAT3, which suppresses inflammatory responses and is the downstream signalling effector of anti-inflammatory IL-10, leads to inflammation of the colon, chronic colitis and invasive colonic adenocarcinomas (Deng et al., 2010).

Once tumours develop, they appear to re-program macrophages from an immunologically active state to one that contributes to tumour tolerance and that may even be trophic for tumour progression and malignancy (Qian & Pollard, 2010). These, so called tumour associated macrophages (TAMs) are associated with an M2-like macrophage subset shaped by IL-10, IL-4 and IL-13 that together support tumour development (T. A. Wynn et al., 2013). Conversely, IFN-γ can switch TAMs purified from ascitic fluid of patients with ovarian cancer to a more M1 phenotype characterised by low levels of expression of IL-10, high levels of IL-12 and enhancement of CD4+ T cell responses (Duluc et al., 2009).

Tumours require angiogenesis for invasive growth and TAMs express the angiopoietin receptor TIE2 which is necessary for the development of a dense blood vessel network that connects the tumour and host circulation, termed the ‘angiogenic switch’ (E. Y. Lin & Pollard, 2007). Indeed, ablation of cells that express TIE2 regresses tumour growth and reduces metastasis (Mazzieri et al., 2011). TAMs also promote angiogenesis through the secretion of angiogenic molecules including members of the VEGF family (T. A. Wynn et al., 2013). Macrophages are also required for tumour cell migration and invasion (Condeelis & Pollard, 2006). In a model of mice mammary carcinoma, tumours produce

colony stimulating factor (CSF)-1, which stimulates macrophages to produce epidermal growth factor (EGF) that in turn activates migration of tumour cells. Inhibition of either EGF or CSF-1 signaling pathways, results in inhibition of migration and chemotaxis ((Wyckoff et al., 2004).

### 1.3.2 Pulmonary fibrosis

In addition to their innate phagocytic activity and role in anti-microbial immunity, macrophages are intimately involved in wound repair. M2 macrophages produce various mediators such as transforming growth factor-β1 (TGFβ1), platelet derived growth factor (PDGF) and insulin-like growth factor, that directly activate fibroblasts and are therefore intimately involved in wound healing (Barron & Wynn, 2011). These proteins regulate the proliferation, survival and activation status of myofibroblasts, which controls the deposition of extracellular matrix (ECM) (Nagaoka, Trapnell, & Crystal, 1990). Macrophages produce IL-1, a potent pro-fibrotic mediator in the lung (Kolb, Margetts, Anthony, Pitossi, & Gauldie, 2001). IL-1β in turn, also stimulates Th17 cells to produces IL-17, which has been identified as an important inducer of bleomycin-induced pulmonary fibrosis (M. S. Wilson et al., 2010).

Whilst there is significant experimental evidence to suggest macrophages are involved in the initiation and maintenance of fibrosis, other studies have also suggested that macrophages may be programmed to suppress and help resolve fibrosis (Duffield et al., 2005). Macrophages phagocytose dead cells and cellular debris and in doing so reduce the ‘danger signals’ that contribute to the production of pro-inflammatory mediators. Macrophages engulf ECM components and stimulate the production of collagen-degrading matrix

metalloproteinases (MMPs) in other cells, including myofibroblasts and neutrophils. Moreover, they produce IL-10, RELMα and ARG1 which have been shown to suppress fibrosis (Pesce et al., 2009; M. S. Wilson et al., 2007) .

### 1.3.3 Airway diseases

The airways are continuously challenged by a variety of foreign substances – including allergens, microbial pathogens, chemicals and particulates (Draijer & Peters-Golden, 2017). In the face of such assault, maintenance of homeostasis requires a carefully calibrated inflammatory response and where necessary, the ability to restrain them. Macrophages, help provide this, aided by their high degree of functional plasticity and ability to orchestrate the inflammatory response.

#### 1.3.3.1 COPD

Chronic obstructive pulmonary disease (COPD) is characterised by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases (GOLD, 2021). There is compelling evidence that the innate immune responses are dysfunctional in COPD (Belchamber et al., 2019; Marwick, Ito, Adcock, & Kirkham, 2007; Singh et al., 2021; Takanashi et al., 1999; Tetley, 2002). AM are exquisitely positioned to respond to inhaled noxious irritants including cigarette smoke. AM responses account for most of the pathophysiological features of COPD (P. J. Barnes, 2004b; Shapiro, 1999; Tetley, 2002). In the lungs of patients with COPD, there is a 5-10 fold increase in the numbers of macrophages in airways, lung parenchyma, bronchoalveolar lavage fluid and sputum (P. J. Barnes, 2004a). Macrophages are localised to sites

of destruction in patients with emphysema and there is a correlation between macrophage numbers in the airways and severity of COPD (Di Stefano et al., 1998; Meshi et al., 2002).

Macrophages activated by cigarette smoke recruit neutrophils through the release of IL-8 and leukotriene B4. They release matrix metalloproteinases (MMP) (Grumelli et al., 2004) and cathepsins that lead to elastolysis (P. J. Barnes, 2004a). Activated alveolar macrophages secrete transforming growth factor (TGF)-$\alpha$ (Madtes et al., 1988) which leads to epithelial growth factor receptor (EGFR) tyrosine phosphorylation and activation of EGFR downstream cascades and increased mucin production through the upregulation of MUC5AC (Takeyama, Fahy, & Nadel, 2001).

Macrophages exposed to cigarette smoke and oxidative stress release pro-inflammatory transcription factors, including NF-$\kappa$B and activator protein (AP)-1 (P. J. Barnes, Adcock, & Ito, 2005). These transcription factors interact with coactivator molecules, such as CREB-binding protein (CBP), p300 and p300/CBP-associated factor, that together help provide the molecular switches that control gene transcription through their intrinsic histone acetyltransferase (HAT) activity (P. J. Barnes et al., 2005). Countering HAT activity are histone deacetylases (HDACs) which help suppress gene transcription. HDAC is a key transcriptional repressor of inflammatory cytokines in AM and reduced expression of HDAC has been linked to the pathogenesis of COPD (Ito et al., 2005). Total HDAC activity is reduced in peripheral lung tissue, alveolar macrophage and bronchial biopsy specimens in patients with COPD compared to healthy non-smoker controls (Ito et al., 2005). Moreover in COPD, not only is

the expression of HDAC reduced, but also its activity, as has been shown following oxidative stress with HDAC2, (Marwick et al., 2007).

HDAC has been reported to mediate the action of steroids to help 'switch off' inflammatory genes and so, it is proposed, its reduced activity and expression in COPD may help account for the reduced anti-inflammatory efficacy of corticosteroids in COPD (P. J. Barnes, 2013; Milara et al., 2018). Interestingly, roflumilast (Daxas®), a selective inhibitor of cAMP-degrading phosphodiesterase-4 (PDE4) enzymes, which is approved for use in COPD, synergises with dexamethasone to reverse corticosteroid resistance in neutrophils from patients with COPD (Milara et al., 2014). The mechanism of this is not known, but it is proposed that roflumilast N-oxide the active metabolite of roflumilast, inhibits the release of cigarette-smoke induced reactive oxygen species and PI3K$\delta$ expression and activity and with it a break on HDAC2 activity, highlighting a further possible mechanism through which roflumilast may have therapeutic potential (Milara et al., 2014)

#### 1.3.3.2 Asthma

Asthma is a syndrome characterised by variable expiratory airflow limitation, bronchial hyper-responsiveness, airway inflammation and recurrent symptoms. In recent years there has been intense interest in the eosinophil-predominant endotype - Type 2 high inflammation (Johansson et al., 2004). This has been fuelled by the advent of a number of promising biological therapies. Consequently, it has been proposed that other mechanisms and cell types, including macrophages, have been overlooked in their contribution to the pathogenesis of asthma (Peters-Golden, 2004).

Macrophage dysfunction may contribute to the pathogenesis of asthma (Figure 1.1). Asthma is associated with distinct microbial signatures (Hilty et al., 2010; Marri, Stern, Wright, Billheimer, & Martinez, 2013) and macrophages play a key role in shaping microbial diversity in the airways of asthmatic patients. Several studies have shown that macrophage phagocytosis is dysfunctional in asthma (Fricker & Gibson, 2017; Liang et al., 2014), which may contribute to an altered microbiological environment and an increased frequency of exacerbations.

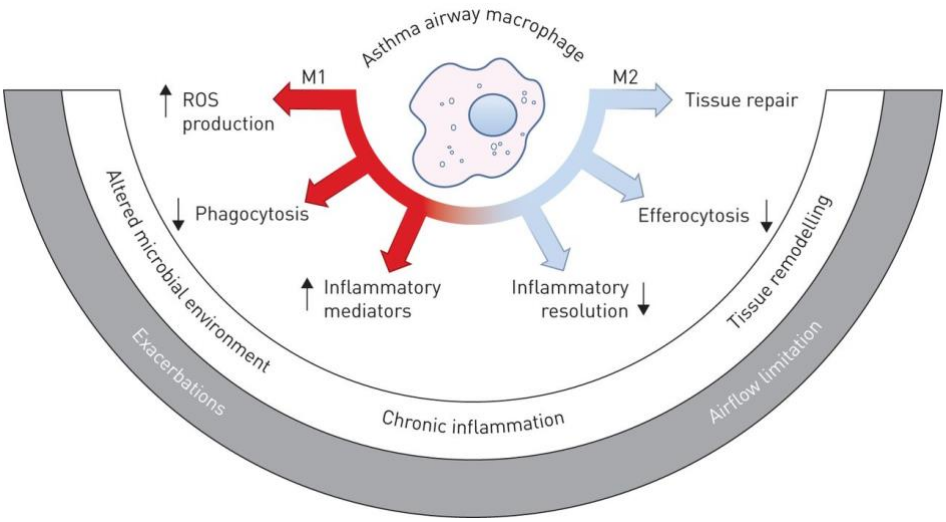



Figure 1.1 Macrophage dysfunction in asthma

(Taken from Fricker, 2017 #1593)

Macrophages are heterogenous cells that adopt a phenotype according to local micro-environmental signals. Broadly, macrophage phenotypes can be considered as either pro- or anti-inflammatory and classed in to M1 and M2 classes respectively. Macrophage effector and homeostatic functions are altered in patients with asthma and could contribute to many of the

pathophysiological hallmarks of asthma. ROS: reactive oxygen species.

Efferocytosis, which is the process by which dead, dying or stressed host cells are digested by neighbouring cells, has also be linked to the onset of asthma. Dead cells and damage-associated molecular patterns (DAMPs) are elevated in the airways of patients with asthma (Watanabe et al., 2011), which intriguingly may be linked to neutrophil persistence and contribute to another endotype of asthma – namely Type 2 *low* or neutrophil-asthma endotype.

In asthma, macrophage cytokine responses may be dysregulated toward a more pro-inflammatory phenotype. AM from asthmatic patients release more TNF$\alpha$, IL-1$\beta$, IL-6 and IL-9 than healthy controls (Ackerman et al., 1994; Hoshi et al., 1995). Meanwhile, in AM from asthmatic patients, anti-inflammatory IL-4 fails to constrain LPS-induced pro-inflammatory cytokine release, when compared to alveolar macrophages obtained from healthy individuals (Chanez et al., 1994).

Interestingly, and of particular relevance to the work presented here, there is evidence that macrophage polarisation may be dysregulated in asthma. In mice (Melgert et al., 2010) and in humans (Melgert et al., 2011), inflammation in the lungs of asthmatic patients is accompanied by increased numbers of alternatively activated macrophages in airway wall tissue. However, whether this merely reflects high levels of IL-4 and IL-13 and conditions favouring alternative macrophage activation or if higher levels of alternative activated macrophages are necessary to help drive the induction and progression of airway inflammation, remains unclear.

### 1.3.4 Extra-pulmonary inflammation

Macrophages have been implicated in the pathogenesis of autoimmune diseases including rheumatoid arthritis, multiple sclerosis and inflammatory bowel diseases (IBDs) (T. A. Wynn et al., 2013). Macrophages are an important source of many of the key cytokines that help drive these diseases including IL-12, IL-18, IL-23 and most notably TNFα (Murray & Wynn, 2011). Patients with Crohn's disease – a chronic gastrointestinal disease characterised by mucosal ulceration and inflammation – have reportedly highly levels of CD14+ intestinal macrophages that produce higher amounts of IL-23 and TNFα when compared to controls (Kamada et al., 2008). TNFα blocking approaches now form a critical part of the therapeutic armamentarium in a range of diverse diseases including rheumatoid arthritis, IBD, psoriatic arthritis and ankylosing spondylitis (Menegatti, Bianchi, & Rogge, 2019).

Microglial cells, the resident macrophages of the central nervous system are implicated in the pathogenesis of chronic demyelinating diseases. Inflammatory macrophages contribute to axon demyelination in mouse models of multiple sclerosis and can be inhibited using mesopram, a selective PDE4 inhibitor (Dinter et al., 2000).

Attenuating macrophage responses also holds therapeutic promise in other mechanisms of injury to the nervous system. In for example models of traumatic spinal cord injury (SCI), targeting the inflammatory response prevents expansion of the area involved and prevents progressive damage. Following on from work showing that agents that raise cAMP can have neuroprotective effects, Bao and

colleagues found the selective PDE4 inhibitor IC486051, preserved white matter and improved neurological function in a model of SCI (Bao et al., 2011). They observed that the use of IC486051 was associated with reductions of myeloperoxidase enzymatic activity and ED-1 – markers respectively of activated neutrophils and macrophages, providing data to support their hypothesis that the improvements in neurological recovery were associated with an anti-inflammatory mechanism of action.

Thus, macrophage responses are critically important in the pathogenesis of several disease states and helps to underscore the potential for therapeutic gain in selectively targeting harmful responses. Using the cAMP signaling cascade could provide a means through which macrophage responses can be selectively targeted.

In the paragraphs below, I describe the components of the cAMP signaling cascade and how targeting specific PDE4 isoforms may provide specificity of action on an otherwise ubiquitous signaling system.

## 1.4 Cyclic AMP signaling

Cyclic adenosine monophosphate (cAMP) is a critical secondary messenger that in immune cells can help regulate the response to injury and infection. In turn, cAMP is regulated by a complex set of cellular machinery, including a large family of cAMP-degrading phosphodiesterases (PDEs). Laboratory based studies have long since highlighted the potential of attenuating immune cell responses by modulating cAMP levels. However, it was only until the approval of the selective PDE4 inhibitors, roflumilast (Daxas®) in COPD and apremilast (Otezla®) in

psoriatic arthritis, that the clinical validity of raising cAMP within compartmentalised pools for therapeutic gain, was finally realised. The regulatory approval of these two novel medicines has fuelled interest in the expression and regulation of PDEs in chronic inflammatory disease as well as concentrating efforts to help identify which patients might benefit most from them.

This study explores the intersection between PDE4 expression and macrophage activation states toward identifying how PDE4 isoforms might be targeted in attenuating dysregulated immune responses.

Cells are charged with constantly having to integrate a myriad of extracellular and intracellular signals toward the maintenance of homeostasis. Dysregulation of this integration leads to maladaptive cellular functions and has been linked to human disease (Maurice et al., 2014). A host of signaling systems are involved in this integration including surface receptors and secondary messenger systems. Secondary messengers not only transmit extracellular signals toward the cell's nucleus and other cellular machinery, but also provides an efficient means of communicating within the cell enabling rapid and sometimes amplified cellular responses. The cyclic nucleotides, cAMP and cyclic guanosine monophosphate (cGMP) are amongst the most well studied and important secondary messenger systems (Houslay, 2010; Zaccolo, 2006; Zaccolo, Zerio, & Lobo, 2021). Indeed, it was investigations of cAMP signalling through glucagon in the liver that gave rise to the 'second messenger' concept itself (Berthet, Rall, & Sutherland, 1957).

Cyclic nucleotides are involved in a wide range of cellular physiological processes including cell proliferation and differentiation, gene expression, inflammation, apoptosis and metabolic pathways including those for steroidogenesis, insulin secretion and lipolysis (Ahmad et al., 2015; Francis, Blount, & Corbin, 2011). In recent years, cAMP has provided the basis for a further conceptual breakthrough, namely that signaling systems are organised in compartments within cells that are spatially and temporally distinct and have functionally important roles (Klussmann, 2016; Zaccolo et al., 2021).

### 1.4.1 Cyclic AMP compartmentation

The notion that intracellular signaling is compartmentalised originated in the early 1980s from work that explored the functional consequences of adenylyl cyclase activation in cardiomyocytes via two different G-protein coupled receptors (GPCRs), namely β-adrenergic and prostaglandin receptors (J. S. Hayes, Brunton, & Mayer, 1980). Although occupancy of these receptors led to similar increases in ‘global’ cAMP levels, the functional outputs were very different, with only β-adrenergic stimulation leading to increased force (inotropic) and rate (chronotropic) of contractions. Critically, occupancy of these different receptors leads to the activation of different protein kinase A (PKA) isoforms. Hayes and colleagues (J. S. Hayes et al., 1980), proposed that these observed differences could be explained if signal transduction was compartmentalised within these cells. Since then, a significant amount of work has led to our current understanding of the cellular machinery involved in achieving compartmentalisation, culminating in the direct visualisation of cAMP gradients in living cells using genetically engineered Fluorescent Resonance Energy Transfer (FRET) (Zaccolo & Pozzan, 2002; J. Zhang, Ma, Taylor, & Tsien, 2001).

cAMP compartmentation is made possible by a complex set of interactions involving GPCRs, adenylyl cyclases, isoforms of PKA, scaffolding proteins, PKA anchor proteins, two cAMP-stimulated GTP exchange proteins (Epacs) and a large family of PDEs (Figure 1.2). In the paragraphs below, I discuss the main components of the cAMP cascade in turn.

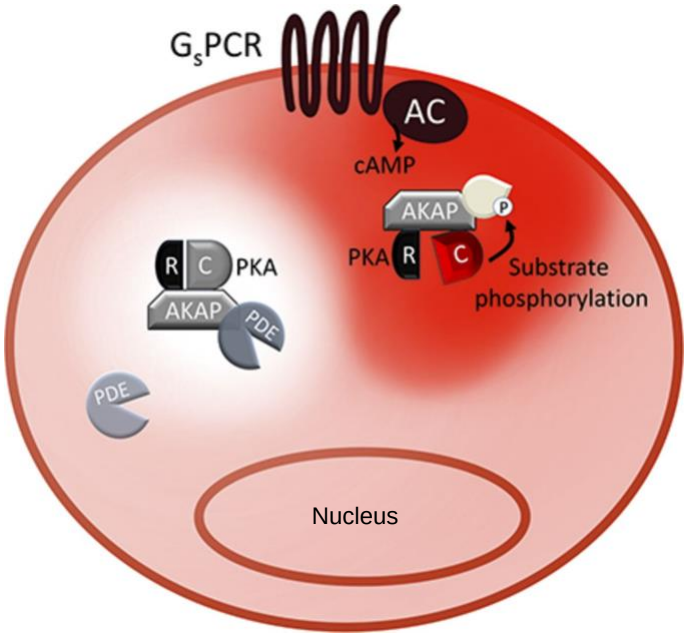



Figure 1.2 Subcellular organisation of the cAMP signaling pathway (Taken from (Zaccolo et al., 2021)

A schematic of a cell showing the subcellular machinery involved in the cAMP signaling pathway. Activated adenylyl cyclase (AC) generates intracellular cAMP (red), that leads to phosphorylation of its effector protein kinase A (PKA), that is localised to a specific subcellular domain by its anchoring protein A-kinase anchor protein (AKAP). Sequestered phosphodiesterase (PDE) (white) decreases local cAMP levels and helps gate the activation of PKA. GPCR: G-

protein coupled receptor, R: regulatory subunit, C: catalytic subunit, P: phosphorylation.

### 1.4.2 G – protein coupled receptors

The activation of cAMP signaling is initiated by the binding of agonists to GPCRs. GPCRs are the most widespread and diverse family of cell surface receptors (Lagerstrom & Schioth, 2008). Perhaps unsurprisingly, this ubiquity has also meant that they constitute the largest class of drug target in the human genome (E. Ghosh, Kumari, Jaiman, & Shukla, 2015). They consist of seven transmembrane α-helices linked by alternate intracellular and extracellular loops. The binding of an agonist ligand to a GPCR stabilises an active formation of the receptor, which then couples to heterotrimeric G proteins, composed of Gα Gβ and Gγ subunits. Subsequently, the heterotrimeric G proteins dissociate from the activated receptor and G protein signaling mediates the generation of cAMP through the activation of adenylate cyclase.

Unopposed G protein activation and generation of secondary messengers like cAMP could be harmful to the cell, so cells need a mechanism to terminate this signaling. This is provided by GPCR kinases that phosphorylate activated GPCRs, triggering the binding of a cytosolic scaffolding protein called β-arrestin. β-arrestins are multi-functional proteins that sterically hinder further G protein coupling and desensitise G protein signaling (Lefkowitz, 2007). As well as attenuating cAMP formation, β-arrestins also have the capacity to accelerate the degradation of cAMP. This has been demonstrated using PDE4D5, which is

sequestered by β-arrestin thereby delivering an active cAMP-degrading system to the site of cAMP synthesis (Lynch et al., 2005).

### 1.4.3 cAMP generation

Following agonist mediated GPCR activation, membrane bound adenylyl cyclases (ACs) catalyse the formation of cAMP from ATP. Membrane bound ACs exhibit a basal activity that is enhanced upon binding of the GTP-bound form of the *stimulatory* G protein α-subunit ($G_s\alpha$) and reduced upon binding of the GTP-bound form of the *inhibitory* G protein α-subunit ($G_i\alpha$). Agents that exploit this system for therapeutic benefit are well established and include the beta-agonist salmeterol, which is widely used in asthma (Tamm, Richards, Beghe, & Fabbri, 2012). In mammals, nine membrane-bound isoforms of AC have been identified, all of which have distinct regulatory properties (Hanoune & Defer, 2001). Different isoforms exhibit specific expression patterns that in some instances have been linked to specific functions. For example, in the central nervous system, AC 1 and 2 have been linked to learning and memory through their expression in the hippocampus and cerebellum (Tasken & Aandahl, 2004).

At subdomains of the surface plasma membrane, point sources of cAMP generation can be defined, which when coupled with tethered PDEs, help generate spatially distinct intracellular gradients of cAMP (Cooper & Tabbasum, 2014; Willoughby & Cooper, 2007). These gradients can be visualised within living cells using FRET sensors (Schleicher & Zaccolo, 2018; Zaccolo et al., 2021), based upon the cAMP effector proteins, Epac and PKA (J. Zhang et al., 2001). Genetic engineering allows these sensors to be manipulated and to be

targeted to specific intracellular sites and to specific intracellular signaling complexes.

### 1.4.4 cAMP effector proteins

Once cAMP is generated, it interacts with specific effector systems, namely PKA (Pidoux & Tasken, 2010), the GTP-exchange proteins Epac1 and Epac2 (Bos, 2006) cyclic nucleotide-gated (CNG) ion channels and POPDC proteins (Brand & Schindler, 2017). There are distinct binding sites for cAMP on all these effector proteins where occupancy by cAMP triggers a conformational change that formulates a distinct functional response (Houslay, 2010; Nakamura & Gold, 1987; Tasken & Aandahl, 2004). The different cellular distribution coupled with distinct intracellular patterns of these various effector proteins provides one key factor that underpins cAMP compartmentation.

PKA is a heterotetramer consisting of two cAMP-binding regulatory (R) subunits and two catalytic (C) units that are differentially expressed and able to form different isoforms of PKA (Tasken & Aandahl, 2004). cAMP binds co-operatively to two sites termed A and B on each R subunit. In the inactive state, only the B site is exposed and available for cAMP binding. When occupied, this enhances the binding to the A site by an intramolecular steric change (Tasken & Aandahl, 2004). Binding of four cAMP molecules, two to each R subunit, leads to a conformational change and dissociation into an R subunit dimer with four cAMP molecules bound and two C monomers (Kopperud et al., 2002). The C subunits then become catalytically active and phosphorylate specific serine and threonine residues on specific substrate proteins (Smith, Radzio-Andzelm, Madhusudan, Akamine, & Taylor, 1999). In addition, cAMP-activated PKA binds and

phosphorylates cAMP-responsive transcription factors that include cAMP-response element binding protein (CREB), cAMP-responsive element modulator/inducible cAMP early repressor (CREM/ICER) protein family, activating transcription factor (ATF-1), NF- $\kappa$B and other nuclear transcription factors

Figure 1.3 (Raker et al., 2016).

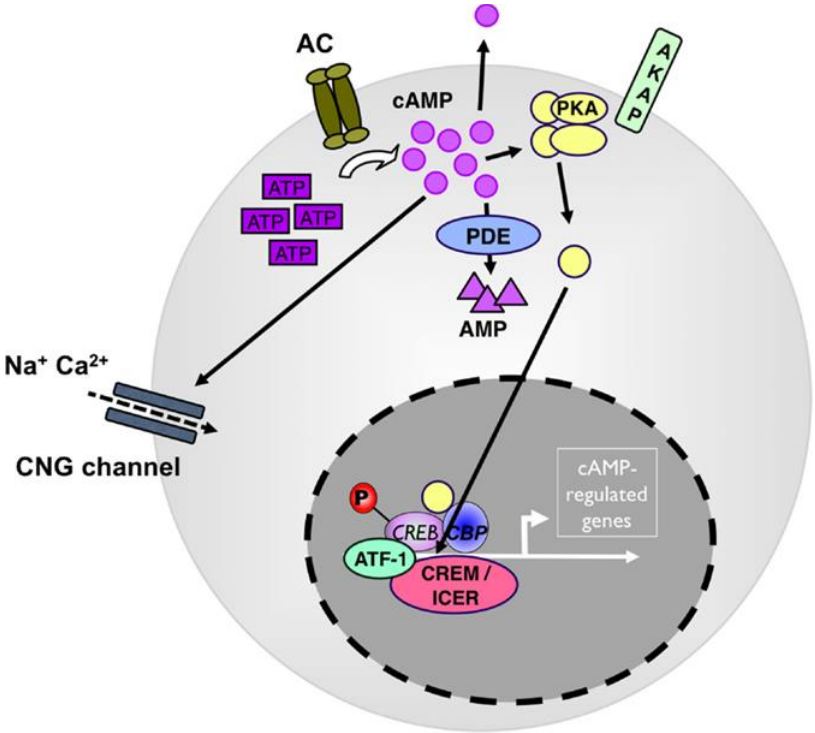



Figure 1.3 cAMP as a regular of immunity

(Taken from (Raker et al., 2016)

cAMP produced from adenosine-tri-phosphate (ATP) by adenylate cyclases (AC) leads to activation of protein kinase A (PKA). PKA stimulation induces the phosphorylation of transcription factors, such as cAMP response element-binding protein (CREB), inducible cAMP early repressor (ICER)/cAMP responsive element modulator (CREM), activating transcription factor-1 (ATF-1), and cAMP-

binding protein (CBP) to drive cAMP-driven genes. Phosphodiesterase (PDE)-4 decreases intracellular cAMP levels and counterbalances the intracellular cAMP effect. CNG: cyclic nucleotide-gated ion channel, P: phosphorylation.

Epac1 and Epac2 are multi-domain proteins that include a discrete cAMP binding domain as well as a guanine exchange factor domain (Szaszák, Christian, Rosenthal, & Klussmann, 2008). The binding of cAMP to Epac, triggers a conformational change that catalyses the exchange of GDP to GTP thus activating the small G-proteins RAP1 and RAP2 (Houslay, 2010). Various effectors downstream of RAP have been identified and it has been shown that Epac has roles in cardiac function, insulin secretion, cerebral function and the immune response. Indeed, in an *in vivo* model of four-day exposure to cigarette smoke, $Epac1^{-/-}$ mice exhibited a pro-fibrotic phenotype (as evidenced by increased expression of TGF-β1, collagen 1 and fibronectin) whilst Epac2 was linked to pro-inflammatory effects (Oldenburger et al., 2014).

cAMP also directly transduces signals by cyclic nucleotide-gated (CNG) ion channels. These channels open in direct response to binding of intracellular cyclic nucleotides and contribute to cellular control of the membrane potential and intracellular $Ca^{2+}$ levels. CNG channels were originally localised to retinal rod photoreceptors (Fesenko, Kolesnikov, & Lyubarsky, 1985) and olfactory sensory neurons (Nakamura & Gold, 1987), but have since been described in other neuronal and non-neuronal tissues (Kaupp & Seifert, 2002). All CNG channels respond to both cAMP and cGMP to some extent but amongst the rods and cone photoreceptors, there is a distinct ligand selectivity in favour of cGMP (Craven &

Zagotta, 2006). CNG channels form the targets of cGMP-signaling pathways that respond to light either by depolarising or hyperpolarising (Kaupp & Seifert, 2002)

In recent years, a novel class of cAMP effector proteins, the Popeye domain containing (POPDC) genes, have been recognised (Andree et al., 2000; Reese, Zavaljevski, Streiff, & Bader, 1999). The POPDC family consists of Popdc1, Popdc2 and Popdc3 genes and are known to be abundantly expressed in the heart and skeletal muscle but are also known to be expressed in smooth muscle tissue (including the lung, gastrointestinal tract and bladder) and epithelial cells (skin, cornea) (Andree et al., 2000). It is yet to be established what role POPDC proteins might play in cAMP signaling though a number of working models have been proposed (Brand, 2018). However, loss of function experiments in zebrafish (Schindler et al., 2016) and the mouse (Alcalay et al., 2013) have suggested an important role in cardiac conduction and heart rate adaption after stress. Indeed in support of this, in humans, loss of function mutations are associated with limb-girdle muscular dystrophy and atrio-ventricular heart block (Schindler et al., 2016).

### 1.4.5 cAMP scaffolding proteins

The cAMP effector proteins PKA and Epac are intrinsically soluble proteins which if left unchecked, might otherwise equilibrate within the cell. For compartmentalisation to ensue, sub-populations of PKA and Epac need to be sequestered to specific intracellular complexes, whether at the membrane or in the cytosol, together with their own downstream targets (Calejo & Tasken, 2015). A family of anchor proteins called A-kinase anchoring proteins (AKAPs) provides

this role and their discovery has been in critical to our understanding of compartmentalisation (Omar & Scott, 2020).

AKAPs help form spatially and temporally restricted multi-molecular complexes or ‘signalosomes’. There are over fifty known AKAPs (including alternative spliced forms) that target PKA to different sites within the cell (Carnegie, Means, & Scott, 2009). Despite a lack of sequence homology between AKAP isoforms, they have been identified through the presence of three common features: an anchoring PKA domain, their ability to bind other signaling enzymes (such as PDEs) and their ability to target these enzymes and kinases to specific sites within the cell (Wong & Scott, 2004).

AKAPs contribute to the specificity and versatility of the cAMP-PKA axis and formulate spatially discrete signaling complexes that respond to sculpted cAMP gradients (Tröger, Moutty, Skroblin, & Klussmann, 2012). By incorporating select ACs, AKAPs direct the specific phosphorylation of PKA substrates in response to a particular stimulus (Baldwin & Dessauer, 2018). An example of this was demonstrated in dorsal root ganglion where the activation of the transient receptor potential vanilloid (TRPV1) channel by forskolin is facilitated by a AKAP79-AC5-PKA-TRPV1 complex (Bauman et al., 2006). When these complexes also contain PDEs, they can help dictate the duration, amplitude and spatial extent of cAMP signaling, at a particular locale (Zaccolo, 2006).

Whilst many of the scaffolding proteins have not been identified, some have been the subject of significant interest including the disrupted in schizophrenia (DISC) 1 gene. Polymorphisms in DISC are a genetic susceptibility factor for

schizophrenia and related severe psychiatric conditions (further discussed in Chapter 1.6.3.1 PDE4B and schizophrenia).

### 1.4.6 Phosphodiesterases

The PDEs are a super-family of 11 structurally related but functionally distinct gene families (PDE 1-11) that differ in their cellular functions, primary structures, affinities for their substrates cAMP and cGMP, catalytic properties, response to activators and inhibitors, as well as in their mechanisms of regulation (Table 1.1). (Francis et al., 2011; Maurice et al., 2014). PDEs are found in many locations throughout the cell such as the cytosol, the plasma membrane, the cytoskeleton and the nucleus (Conti & Beavo, 2007; Houslay & Adams, 2003)

| PDE | Genes | Substrate | | Regulatory domain |
|---|---|---|---|---|
| | | cAMP | cGMP | |
| 1 | A, B, C | Y | Y | Ca2+/CaM-stimulated |
| 2 | A | Y | Y | GAF domains, cGMP activated |
| 3 | A, B | Y | Y | cGMP inhibited |
| 4 | A, B, C,D | Y | N | UCR1 and UCR2 targeting domain |
| 5 | A | N | Y | GAF domains, |
| 6 | A, B, C | N | Y | GAF domains, activated by rhodopsin and transducin |
| 7 | A, B, | Y | N | |
| 8 | A, B | Y | N | PAS |
| 9 | A | N | Y | REC |
| 10 | A | Y | Y | GAF-A, GAF-B |
| 11 | A | Y | Y | GAF-A, GAF-B, cAMP stimulated |

Table 1.1 Characteristics of the phosphodiesterase super-family

(adapted from (Ahmad et al., 2015; Maurice et al., 2014)

The phosphodiesterase (PDE) superfamily is comprised of 11 families of enzymes, derived from 21 genes, many of which encode multiple mRNAs. PDEs differ in their selectivity for cyclic adenosine monophosphate (AMP) and guanosine monophosphate (GMP). Individual PDEs share a conserved catalytic domain but have a more variable N-terminal regulatory domain. The N-terminal region contains PDE family-specific subdomains including the cGMP-binding PDEs (GAF) domains in PDEs 2, 5, 6, 10 and 11, calcium /calmodulin binding site for PDE1, $NH_2$-terminal hydrophobic

regions (NHR1 and NHR2) in PDE3, upstream conserved regions (UCRs) in PDE4, Per-Arnt-Sim (PAS) in PDE8 and receiver (REC) in PDE9.

PDEs are modular proteins that have a common structural organization ( Figure 1.4). The catalytic domain located at the C-terminal region of each PDE enzyme is highly conserved (Conti & Beavo, 2007). The N-terminus is quite diverse and confers unique regulatory properties and targeting sequences that result in tethering of PDEs to specific subcellular locations (Zaccolo et al., 2021). The N-terminal region can also carry post-translational modifications sites – for example phosphorylation of PDE3, PDE4 and PDE10 modulates their enzymatic activity and the ability of these enzymes to form complexes with other proteins (Zaccolo et al., 2021).

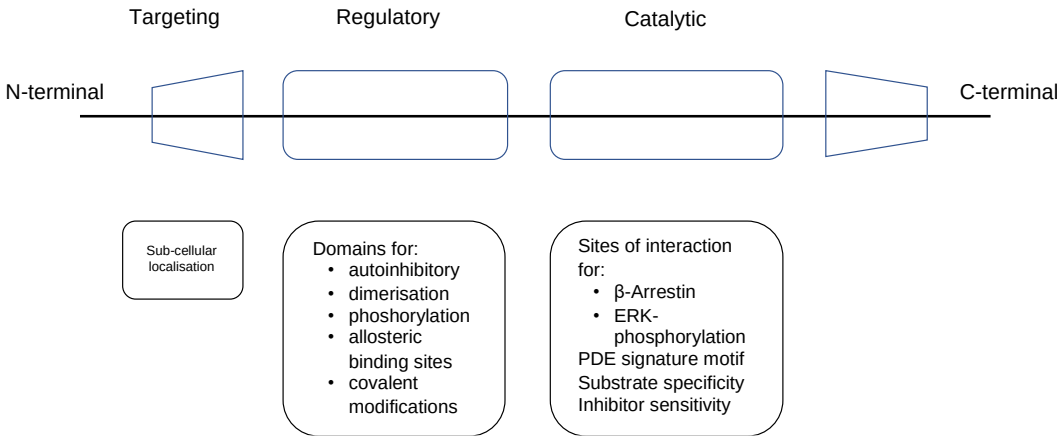



Figure 1.4 Schematic of structural pattern common to PDEs

(Adapted from (Ahmad et al., 2015)

Domain organisation common to PDE gene families. The N-terminal part contains targeting domains responsible for localisation of PDE isoforms to specific subcellular sites. The regulatory domain contains PDE family-specific sub-domains involved in the regulation of PDEs. The conserved catalytic domain provides sites for interaction including to β-Arrestin and extracellular-regulated kinase (ERK)

It is established that PDEs play an important role in compartmentalisation of cAMP signaling (Zaccolo et al., 2021), though it remains far from clear as to how PDEs achieve this. One challenge has been reconciling the apparent contradiction between the high diffusion rate of cAMP in aqueous solution and the catalytic activity of the PDEs (Koschinski & Zaccolo, 2017). For example, the diffusion coefficient of cAMP across the cell has been experimentally calculated to be around 40 $\mu m^2/s$, only around one order of magnitude lower than in water (Nikolaev, Bunemann, Hein, Hannawacker, & Lohse, 2004). Yet, considering the reported $K_M$ and $V_{max}$ values for PDEs, it is hard to see how PDEs are able to maintain the concentration of cAMP below the activation threshold of cAMP effectors such as PKA, even at basal levels, let alone following the higher concentrations generated after hormonal stimulation (Zaccolo et al., 2021). A number of factors may though contribute to reducing cAMP diffusion, including cAMP buffering (Lefkimmiatis, Moyer, Curci, & Hofer, 2009), cytosol viscosity (Feinstein, Zhu, Leavesley, Sayner, & Rich, 2012) and physical barriers (Feinstein et al., 2012). These factors may be particularly relevant in helping to create so called nanodomains (Bers, Xiang, & Zaccolo, 2019), in which PDEs may be able to effectively reduce the concentration of free cAMP, even when

compared with areas only nanometers farther away (Chao, Surdo, Pantano, & Zaccolo, 2019).

Most PDE families contain multiple PDE genes, which together generate nearly 100 PDE isoenzymes by alternative mRNA splicing or transcriptional processing. For many years, the basis for this apparent redundancy was not well understood. However, in recent years, it has become appreciated that a wide number of PDE isoforms are an inherent requirement towards individual cyclic nucleotide signaling within specific signalosomes (Maurice et al., 2014).

Appreciation of the 3-D structure of the catalytic units and cAMP binding pocket of the various PDE families has aided the development of family-specific selective inhibitors that have been used both experimentally to help decipher the role of particular PDEs and therapeutically as novel medicines. Successful examples include the PDE5-selective inhibitor sildenafil, which is used to treat erectile dysfunction (Yuan et al., 2013) and pulmonary hypertension (Oudiz et al., 2012), the PDE3 inhibitor milrinone (Primacor®), which is used to treat left ventricular dysfunction (Movsesian, Stehlik, Vandeput, & Bristow, 2009), the PDE3 inhibitor cilostazol (Pletal®), which is used to treat intermittent claudication (Dawson, Cutler, Meissner, & Strandness, 1998) and, of course, the PDE4-selective inhibitors roflumilast in COPD (Calverley et al., 2009; Calverley et al., 2007), apremilast in psoriatic arthritis (Kavanaugh et al., 2015) and most recently of all, crisaborole (Eucrisa®) in atopic dermatitis (Paller et al., 2016).

## 1.5 cAMP and inflammation

Inflammation is a protective pathophysiological response to infection or injury. Each step of the inflammatory process must be finely tuned to ensure an effective defence against harmful stimuli and later induction of resolution, with minimal collateral damage (Tavares et al., 2020). Indeed, uncontrolled inflammation is a common feature of the most common human diseases including those involving carcinoma, vascular and other chronic inflammatory disorders (Balkwill & Mantovani, 2012; Christenson et al., 2015; Hansson, Robertson, & Soderberg-Naucler, 2006). Within the immune system, cAMP is an established potent regulator of both innate and adaptive immune cell functions. It is then unsurprising that strategies to enhance cAMP concentrations or cAMP actions have attracted significant interest toward developing potential therapeutic drug targets to treat inflammatory and autoimmune disorders. Amongst anti-inflammatory medicines, these efforts have centred upon the development of inhibitors of cAMP-degrading PDEs rather than, for example, activators of ACs, although the recognition of different AC isoforms in recent years has helped single out these enzymes as potential drug targets in non-inflammatory disorders (Pierre, Eschenhagen, Geisslinger, & Scholich, 2009).

The cells of the immune system contain isoenzymes belonging to the families of PDE3, PDE4 and PDE7, though it is the isoenzymes belonging to PDE4 that predominate (Torphy, 1998). Amongst the four PDE4 sub-families, isoforms belonging to PDE4A, PDE4B and PDE4D have all been found in T and B cells, neutrophils, eosinophils, DCs, monocytes and macrophages. Isoforms of PDE4C on the other hand, are minimally active or absent (Press & Banner, 2009). Through increasing intracellular cAMP, PDE4 inhibitors show anti-inflammatory

effects in almost all inflammatory and immune cells. They have been linked to the suppression of a multitude of inflammatory responses including proliferation, chemotaxis, phagocytosis and release of pro-inflammatory mediators such as cytokines, chemokines, reactive oxygen species and lipid mediators (Press & Banner, 2009; Raker et al., 2016).

Targeting PDE4 has been advanced as an effective therapeutic strategy for inflammatory conditions in a variety of conditions including COPD, asthma, psoriasis, atopic dermatitis, inflammatory bowel disease, rheumatoid arthritis and neuro-inflammation (as shown in (Figure 1.5)

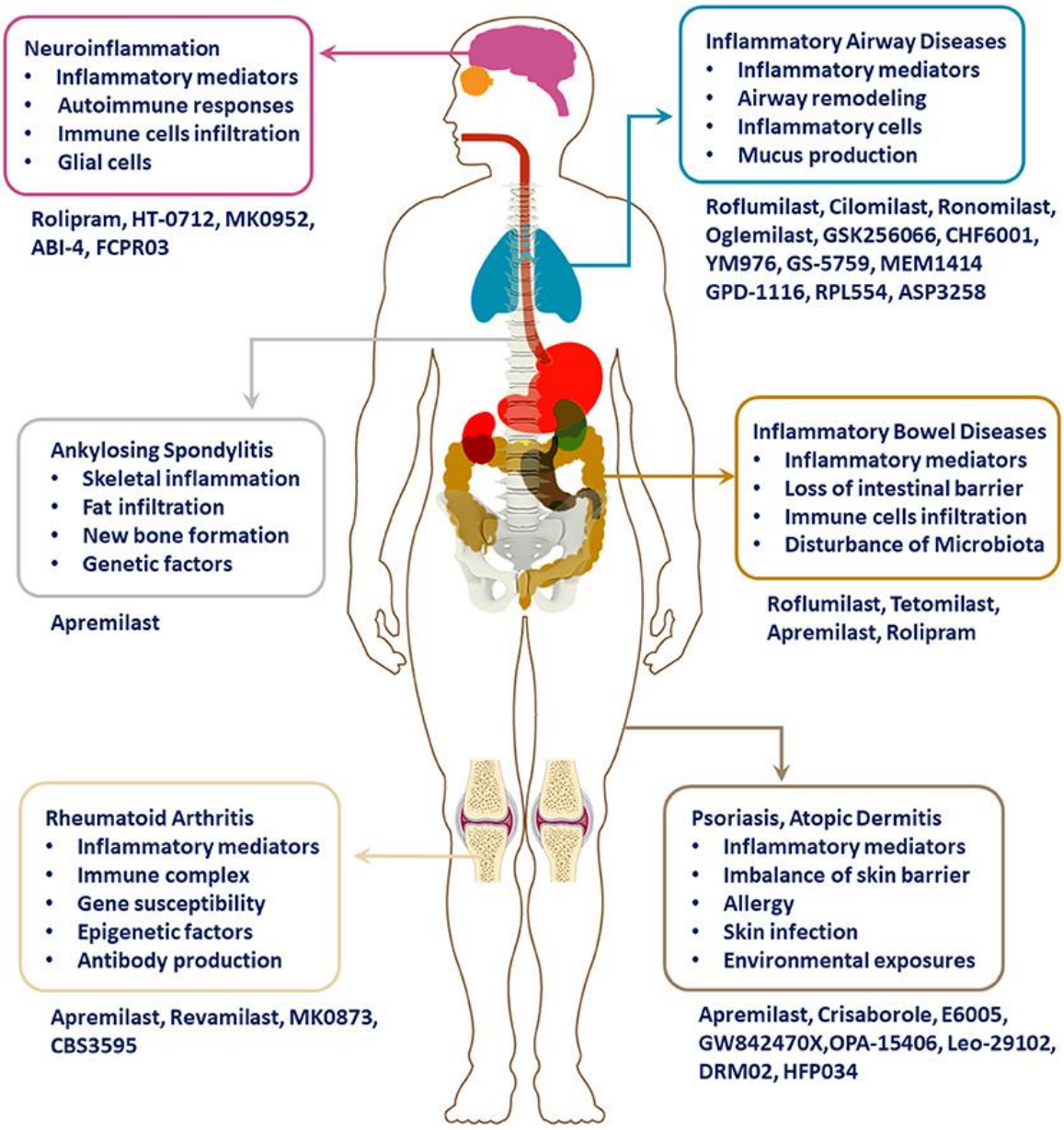



Figure 1.5 Characteristics of disorders targeted by PDE4 inhibitors

(Taken from (H. Li, Zuo, & Tang, 2018)

A schematic showing the range of inflammatory conditions for which PDE4 inhibitors have been proposed for therapeutic benefit, as well as the names of some of the compounds that have been evaluated in them.

cAMP-elevating agents have been shown to reduce levels of TNFα (D. M. Aronoff, Carstens, Chen, Toews, & Peters-Golden, 2006), IL-12 (van der Pouw Kraan, Boeije, Smeenk, Wijdenes, & Aarden, 1995), leukotriene $B_4$ (Luo et al., 2005) and chemokines such as CCL3 (Kawashita et al., 2011), CXCL1 (Tavares et al., 2016) and CCL11 (Silva et al., 2001). Therefore, it is not surprising that in pre-clinical and human studies, increased cAMP levels have also been shown to decrease T cell activation (Vang et al., 2001), neutrophil oxidative responses (P. Lin, Welch, Gao, Malik, & Ye, 2005), migration of eosinophils (Alves et al., 1997) as well as counter the expression of adhesion molecules (Kong et al., 2019). The reduced expression of chemokines and adhesion molecules leads to diminished levels of inflammatory leukocytes in tissues. One such example is the use of PDE4 inhibitors that have been deployed in a model of acute lung injury to reduce granulocyte recruitment (Miotla, Teixeira, & Hellewell, 1998) and shown to be protective in models of COPD and asthma (Huang & Mancini, 2006) as well as pneumonia (Tavares et al., 2016).

### 1.5.1 cAMP induces macrophage polarisation

As discussed earlier, macrophages adopt phenotypes according to the microenvironment in which they reside. Macrophage polarisation is a feature of the resolution of inflammation (Sugimoto, Sousa, Pinho, Perretti, & Teixeira, 2016). It has been proposed that this process is cAMP-dependent.

In a mouse model of autoimmune encephalomyelitis, activation of the cAMP pathway following treatment with the adenylate cyclase activator forskolin, increases expression of M2 macrophage markers (miR-124, Arg-1, MRC1, Fizz-1 and Ym-1) whilst simultaneously decreasing expression of M1 markers (NOS2

and CD86), a process dependent upon ERK signaling (Veremeyko et al., 2018). It has been proposed that cAMP induces M2 polarisation through phosphorylation of STAT3 (Negreiros-Lima et al., 2020) and STAT6 (Sheldon et al., 2013) signaling and also re-programs M1 polarised macrophages toward a M2 phenotype by decreasing STAT1 phosphorylation (Negreiros-Lima et al., 2020). The group working with Negreiros-Lima, have also shown that the cAMP analogue db-cAMP, decreases the proportion of M1 macrophages in LPS induced pleurisy while inhibition of the cAMP pathway using a PKA inhibitor prevents resolution of inflammation (Negreiros-Lima et al., 2020). These results provide evidence of an important role of cAMP in not only helping to determine macrophage polarisation, but also for the resolution of inflammation (Tavares et al., 2020).

## 1.6 Phosphodiesterase – 4

PDE4 inhibitors are under development to provide potential, novel therapeutics in a range of major disease areas (Figure 1.5). They are found in multiple cell types and tissues including airway and vascular smooth muscle, vascular endothelium, keratinocytes, the brain and as already discussed, cells of the immune system (Houslay, Schafer, & Zhang, 2005). PDE4 enzymes play major regulatory roles as can be deduced using a number of experimental techniques including highly selective inhibitors (Castro, Jerez, Gil, & Martinez, 2005), targeted gene knockout (Jin & Conti, 2002; H. T. Zhang et al., 2002), small interfering RNA (siRNA) ablation (Lynch et al., 2005) and dominant negative disruption of enzyme signaling (McCahill et al., 2005).

The large number of PDE4 isoforms presents a significant challenge to understanding the range of actions of this family (Houslay et al., 2005). However, this diversity and the selective expression and distinct regulation of individual isoforms, presents an opportunity for tailoring cAMP signaling on a cell-type specific basis and arguably, within the context of macrophage phenotypes, a context-specific basis too. In the sections below, I discuss some important aspects of PDE4 biology.

### 1.6.1 Introduction

The four PDE4 genes (PDE4A, B, C & D) encode for more than 25 different PDE4 isoforms as a result of variable mRNA splicing and the use of alternative promoters and transcriptional start sites (Cedervall, Aulabaugh, Geoghegan, McLellan, & Pandit, 2015). Each PDE4 sub-family has a highly conserved catalytic unit consisting of 17 α-helices organised in to three sub-domains, at the junction of which is a deep binding site for cAMP. This substrate binding site also contains binding sites for $Zn^{2+}$ and $Mg^{2+}$. (Shakur, Pryde, & Houslay, 1993). Whilst isoforms belonging to a PDE4 gene subfamily share a common C–terminal region, they have their own unique N–terminal region which confers isoform specific targeting to distinct intracellular sites and signalling complexes (Houslay & Adams, 2003), thereby underpinning compartmentalisation of cAMP signalling.

Unique amongst isoforms from the PDE4 sub-family, they contain regulatory domains named up-stream conserved region (UCR) 1 and UCR2 that together with less conserved regions of sequence, called linker region (LR1) 1 and LR2, help link the N–terminal region to the catalytic unit. The presence or part thereof of these regulatory domains confer a further organisational structure to classify

the numerous PDE4 isoforms that also informs their particular function within the cell. Long PDE4 isoforms contain both UCR1 and UCR2; short forms lack UCR1, super-short forms have only a truncated UCR2 and dead-short forms lack both UCR1 and UCR2 and are catalytically inactive (Figure 1.6). An important functional role of the UCR modules is in determining PDE4 regulation through PKA and ERK phosphorylation (Conti et al., 2003).

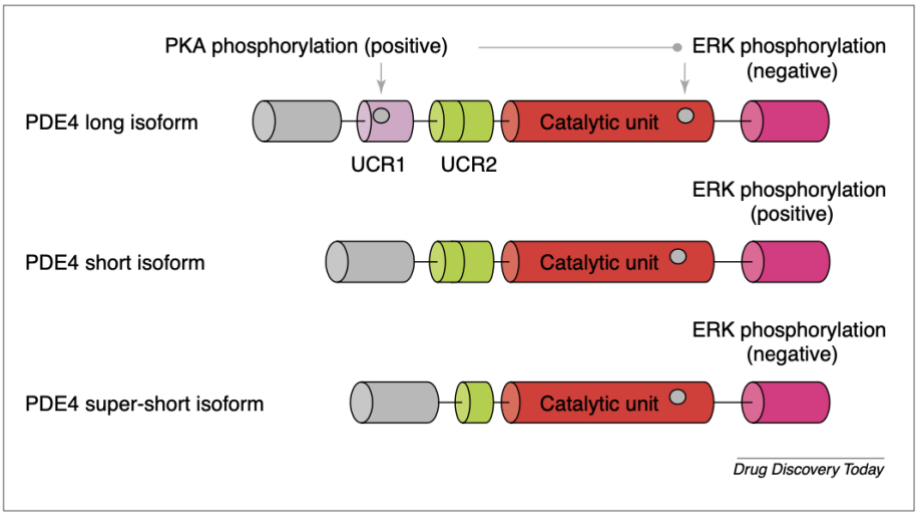



Figure 1.6 Schematic of the three main groups of PDE4 isoforms

(Taken from (Houslay et al., 2005)

All PDE4 isoforms are divided in to three groups according to the presence or part thereof of two regulatory domains, termed up-stream conserved region (UCR) 1 and UCR2. Long forms contain both UCR1 and UCR2, short forms lack UCR1 and super-short forms lack UCR1 and have a truncated UCR2 (a further category, so called dead-short forms – not shown here – lack both URC1 and UCR2 and are catalytically inactive). The UCR modules help determine PDE4 regulation through protein kinase A (PKA) and extracellular signal-regulated kinase (ERK) phosphorylation. Only long forms contain UCR1 and therefore are subject to activation by PKA whereas all

isoforms except those from the PDE4A sub-family, can be phosphorylated by ERK. The isoform- specific N-terminal region is shown in grey and the subfamily-specific C-terminal region is shown in pink.

UCR1 provides a binding site for PKA which then triggers a conformational change in the UCR1 and UCR2 module leading to increased catalytic activity (Houslay & Adams, 2003). It thereby also provides an important part of the cellular desensitisation machinery for cAMP signalling by increasing the cellular capacity for cAMP degradation (Houslay et al., 2005). This has been demonstrated in the long isoform PDE4D3 in which PKA mediated phosphorylation of $Ser^{54}$ in UCR1 was shown to enhance hydrolysis of cAMP (Sette & Conti, 1996).

The effects of ERK – a kinase that is activated by pro–inflammatory stimuli (Lucas, Luo, & Stow, 2022), are perhaps even more intriguing than those described for PKA. The catalytic unit of the PDE4B, PDE4C and PDE4D, but not the PDE4A families, contain a consensus site for phosphorylation by ERK, which in PDE4D3 is located at Ser579 (Baillie, MacKenzie, McPhee, & Houslay, 2000; Hoffmann, Baillie, MacKenzie, Yarwood, & Houslay, 1999; S. J. MacKenzie, Baillie, McPhee, Bolger, & Houslay, 2000). This catalytic site also contains both a KIM docking site for ERK (Val-Xaa-Xaa-Lys-Lys-$Xaa_6$-Leu-Leu-Leu-$Xaa_{122}$-phosphoSer) located on an exposed $\beta$-hairpin loop N-terminal to the target serine site, and a specificity site for ERK, conferred by the FQF motif, (Phe-Gln-Phe), located on an exposed $\alpha$-helix, C-terminal to the serine target (Houslay & Adams,

2003). These three key sites for ERK action are conserved across PDE4 enzymes and located on a single sub-domain of the PDE4 catalytic unit (S. J. MacKenzie et al., 2000) and are therefore well placed for interaction with ERK.

The presence or absence of UCR1/UCR2 helps determine the functional outcome of ERK phosphorylation of PDE4, with long forms being inhibited, short isoforms being activated and super-short forms being weakly inhibited (Houslay & Baillie, 2003). In such ways, the profile of PDE4 isoform expression, and the potential for cross-talk between ERK/cAMP signalling can help fashion the cell's response to a given environmental signal.

An example of the possible functional relevance of changes to the PDE4 isoform expression profile is elegantly demonstrated in monocyte to macrophage differentiation. In monocytes it has been proposed that the long forms PDE4D3 and PDE4D5 predominate whereas in macrophages, short PDE4B2 provides the dominant PDE4 activity (M. C. Shepherd, Baillie, Stirling, & Houslay, 2004). ERK, a kinase that is activated by pro-inflammatory stimuli may be expected to inhibit PDE4 activity across the long forms found in monocytes but stimulate short form activity, including for example the important PDE4B2 form, in macrophages.

Interestingly, ERK activation by PDE4 long isoforms can be negated by PKA phosphorylation (Hoffmann et al., 1999). ERK-induced inhibition of PDE4, raises cAMP, causing PKA to become activated and then phosphorylate (and activate) the long PDE4 isoform, thereby countering the inhibitory effect of ERK phosphorylation

The UCR modules have additional roles beyond their regulatory functions. They provide an interface for interaction with scaffolding proteins (G. B. Bolger et al., 2003; Verde et al., 2001), are capable of interacting with each other (Beard et al., 2000) and participate in PDE4 dimerisation (Richter & Conti, 2002).

The wide distribution of PDE4 across different cell types and tissues, together with the multitude of PDE4 isoforms coupled with the ubiquitous and critical nature of cAMP signalling in cellular function has spawned multiple areas of PDE4-related research. A complete review is beyond the scope of this study but in the sections below, I highlight three areas where there has been prolific and important PDE4-related research, namely inflammation, the cardiovascular system and cognition and learning.

### 1.6.2 PDE4A

The PDE4A sub-family can be sub-divided in to six splice variants: PDE4A 1/ 4/ 7/ 8/ 10 and 11. These variants are of different lengths (PDE4A1 belongs to the short form, PDE4A7 the catalytically inert dead-short form whilst PDE4A 4/ 8/ 10 and 11 all belong to the long forms (Hansen, Conti, & Zhang, 2014). Unlike other PDE4 enzymes, the catalytic unit of PDE4A family isoforms cannot be phosphorylated by ERK (Baillie et al., 2000) limiting the potential for an otherwise important means of cross-talk between cAMP and ERK signaling.

Whilst much of the focus around PDE4 isoforms has been around their catalytic activity for hydrolysing cAMP, it is also now appreciated that catalytically inert isoforms, such as dead-short PDE4A7 may also have important non-redundant functions. Transcripts for PDE4A7 have been identified in the human brain as well as a range of human cell types including macrophages, monocytes, T-cells and neutrophils (Johnston et al., 2004). PDE4A7 arises from both 5' and 3' splicing that gives PDE4A7 both a unique 32-residue N-terminal region and a unique 14-residue C-terminal region (Horton, Sullivan, & Houslay, 1995; Sullivan et al., 1998). It has been proposed that this configuration helps confer an exclusive targeting of PDE4A7 which in COS-7 cells, has been localised to the P1 particulate fraction (including cell membranes, granules and nuclei) (Johnston et al., 2004), and with it a role for PDE4A7 in interacting with other PDE4 isoforms and affecting intra-cellular targeting and functioning.

Since the 1970's, cAMP has been implicated in learning and memory formation (Ricciarelli & Fedele, 2015). The discovery of rolipram as a selective pan–PDE4

inhibitor aided the observation that increasing cAMP by blocking its PDE–mediated breakdown, could boost long term potentiation (LTP) and improve memory formation and consolidation in rodents (Barad, Bourtchouladze, Winder, Golan, & Kandel, 1998; Navakkode, Sajikumar, & Frey, 2005). Since then a variety of animal models utilising behavioural tasks, or models of pharmacologically–induced cognitive deficits or those mimicking human disease including Alzheimer's have all confirmed the importance of cAMP signaling in memory (Ricciarelli & Fedele, 2015). PDE4A, PDE4B and PDE4D have been of considerable interest in cognition-related research as well as in neuropsychiatric and neurodegenerative conditions. In contrast, the role of PDE4C in the brain has not been well explored, because research tools such as knock-out animals or sub-type specific inhibitors have not been available to study (Richter, Menniti, Zhang, & Conti, 2013)

PDE4A is expressed in multiple regions of the brain with high levels found in the cerebral cortex, hippocampus and cerebellum (Cherry & Davis, 1999; McPhee, Cochran, & Houslay, 2001). Moreover, PDE4A isoforms display clearly distinct localisation patterns (Kirsty F. Mackenzie et al., 2008; McPhee et al., 2001)PDE4A expression levels are altered in patients with bipolar disorder (Fatemi, Reutiman, Folsom, & Lee, 2008) and autism (Braun, Reutiman, Lee, Folsom, & Fatemi, 2007), linking PDE4A function and CNS disorders.

In experiments in mice, PDE4A has been linked to memory and anxiety. Mice deprived of sleep produce deficits of synaptic plasticity and hippocampus-dependent memory which is linked to increased expression of PDE4A and impaired cAMP/PKA signaling in the hippocampus (Vecsey et al., 2009).

Meanwhile, rolipram reverses these deficits. PDE4A$^{-/-}$ mice develop an anxiogenic profile in experimental behavioural tests as well as raised urinary corticosterone levels when compared with WT mice 24hr after the stress of food deprivation (Hansen et al., 2014). However, it may be noted that mice that are deficient in PDE4B also develop anxiogenic behaviours (H. T. Zhang et al., 2008) suggesting these observations are not specific to disrupted signaling associated with a particular PDE4 sub-family.

### 1.6.3 PDE4B

The gene for PDE4B lies on chromosome 1p31 (Szpirer et al., 1995) and gives rise to five PDE4B isoforms: PDE4B1-5. PDE4B has garnered significant interest as an important regulator of signaling within the brain (Tibbo & Baillie, 2020). Hippocampal long-term potentiation (LTP) – a prominent cellular model for learning and memory – was found to be linked to changes in expression and a peri-nuclear subcellular localisation of PDE4B (Ahmed & Frey, 2003). This is significant as in some cells, for example mouse fibroblasts, PDE4B isoforms have been shown to have a distinct distribution within the cell and that this is associated with their functional output (Blackman et al., 2011). However, in mice deficient in PDE4B, LTP is not affected though they do show features of long-term depression (Rutten et al., 2011). Further studies have also found that PDE4B$^{-/-}$ mice behave similarly to WT controls in behavioural tests assessing memory (Siuciak, McCarthy, Chapin, & Martin, 2008; H. T. Zhang et al., 2008). PDE4B has also been implicated in schizophrenia, which is further described in Chapter 1.6.3.1. However, beyond these possible links in learning and neuropsychiatric conditions, PDE4B has well-established anti-inflammatory properties.

There is compelling evidence that macrophage PDE4B2 is an important regulator of the inflammatory response. In a seminal study, Ma and colleagues (Ma, Wu, Egan, Billah, & Wang, 1999) were the first to demonstrate that in human monocytes, a factor other than one that increased cAMP activity could increase PDE4 gene expression, by showing that LPS selectively increased PDE4B sub-family expression and not the sub-family expression of any of PDE4A, PDE4C or PDE4D. In a follow-on study, the same group demonstrated that LPS induction of PDE4B and more specifically PDE4B2 was cell-type specific. Although PDE4B2 was the predominant species in both monocytes and neutrophils, only in monocytes and not in neutrophils, did LPS induce PDE4B2 expression (Wang, Wu, Ohleth, Egan, & Billah, 1999). However, it was the use of genetic ablation techniques that provided data for the functional significance in changes to PDE4B expression.

Jin and colleagues (Jin, Lan, Zoudilova, & Conti, 2005) used genetic ablation techniques to demonstrate that LPS-induced TNF$\alpha$ expression is dependent upon PDE4B and not either of PDE4A$^{-/-}$ or PDE4D$^{-/-}$ and furthermore that it was PKA-dependent. Moreover, whilst the PDE4 selective inhibitors rolipram and roflumilast suppressed the TNF$\alpha$ response in other PDE4 null cells, they had no additional inhibitory effect in macrophages deficient in PDE4B, suggesting PDE4B was critical for their inhibitory effects. Critically, the attenuation of LPS induced TNF$\alpha$ in PDE4B$^{-/-}$ macrophages, was not simply related to some generalised loss of function or cell viability as these cells retained their capacity to produce IL-6 at levels comparable to WT controls. Finally, PDE4B ablation of TLR responses was also shown to be retained *in vivo*, as mice deficient in PDE4B were protected in a model of LPS-induced septic shock (Jin et al., 2005). Taken

together, the data provides a firm basis for our hypothesis that PDE4B2 could be an important target for selective PDE4 inhibitors designed to attenuate the inflammatory response.

PDE4 is recognised to have an important role in the cardiovascular system where it has been extensively studied and found to help orchestrate complex, localised signaling that underpins many crucial functions of the heart (Fertig & Baillie, 2018). This is particularly during conditions of β-adrenergic stimulation. One example of this has been demonstrated using subfamily specific knockout mice that showed PDE4B has a dominant role in the regulation of the L-type calcium channel (LTCC) cardiac excitation-contraction coupling protein (Leroy et al., 2011). PDE4 inhibition had no effect on basal calcium current through the LTCC but led to a markedly increased calcium current under β-adrenergic stimulation. This was evident in both $PDE4B^{-/-}$ and $PDE4D^{-/-}$ mice leading to increased calcium current and contractility. However, it was only $PDE4B^{-/-}$ mice that *in vivo* cardiac pacing led to lethal ventricular tachycardia. The role of PDE4B in protection against arrhythmia is further evidenced by the observation of decreased PDE4B activity in cardiac hypertrophy (Abi-Gerges et al., 2009).

#### 1.6.3.1 PDE4B and schizophrenia

Genetic susceptibility factors have long been sought in schizophrenia. Twin studies have yielded heritability estimates of over 80% (Feng et al., 2016). The DISC (disrupted in schizophrenia) 1 gene was first identified as a candidate susceptibility gene in a large Scottish family presenting with schizophrenia and affective disorders (Millar et al., 2000). It has since been shown that DISC1, the scaffold protein encoded by the DISC1 gene, binds to UCR2 and as such is able

to interact with both PDE4B long forms and the short form PDE4B2 (Millar 2005). Using neuronal cell lines and primary rat hippocampal cells, Millar and colleagues, reported that DISC1 co-localised and co-precipitated with the long form PDE4B1. Agents that elevated cAMP levels including forskolin and the nonspecific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) dramatically reduced the amount of DISC1 co-precipitating with PDE4B. Using the PKA specific inhibitor H89, they also showed this effect was PKA dependent. Thus, a model emerges involving auto-regulation of DISC1 and PDE4B. In the resting state, DISC1 sequesters a predominantly dephosphorylated, low-activity form of PDE4B. Upon activation by cAMP, PKA phosphorylates DISC1 leading to its uncoupling with PDE4B and the removal of a functional break on PDE4B's cAMP degrading activity (Millar et al., 2005). Subsequent work has since shown that DISC1 can bind members of all four PDE4 sub-families and that point mutations in DISC1 may help confer phenotypes related to schizophrenia (Murdoch et al., 2007).

In addition to the evidence described above linking PDE4B and schizophrenia, several studies have identified single nucleotide polymorphisms (SNPs) in the PDE4B gene that are associated with an increased incidence of schizophrenia (Fatemi, King, et al., 2008; Feng et al., 2016; Kahler et al., 2010) and further that PDE4B expression levels were lower in brain tissue obtained post-mortem from patients diagnosed with schizophrenia (Fatemi, King, et al., 2008). However, these studies have been able to elucidate the exact functional significance of these genetic variants, for which it is likely even larger studies across multi-ethnic populations will be needed.

### 1.6.4 PDE4C

PDE4C isoforms are expressed at very low endogenous levels within cells. This has constrained the study of the functional properties of members of this sub-family. However three long isoforms of this enzyme have been identified, namely PDE4C1-3 (G. Bolger et al., 1993; Swinnen, Joseph, & Conti, 1989). Within the human brain, PDE4C has been detected in the cortex, thalamic nuclei and cerebellum, whereas, in monkeys, distribution is evident in the olfactory bulb (Perez-Torres et al., 2000).

PDE4C has also been implicated as part of a dysregulated cAMP signaling complex in polycystic kidney disease involving a ciliary calcium channel, polycystin-2 (PC2), and the anchor protein AKAP150 (Choi et al., 2011).

### 1.6.5 PDE4D

Within the brain, PDE4D is predominantly expressed in the hippocampus (Perez-Torres et al., 2000; H. T. Zhang et al., 2002). Contrary to findings that pan-PDE4 inhibitors exert memory and cognition enhancing effects (Barad et al., 1998; Huang et al., 2007), an early study of PDE4D$^{-/-}$, found mice exhibited memory impairment, not enhancement – though this might have been related to developmental changes. Since then, using either a pharmacological approach with the PDE4D selective inhibitor GEBR-7b or genetic silencing techniques, studies have shown that targeting PDE4D can improve recognition, spatial memory and even be neuro-protective in murine models of Alzheimer's disease (C. Zhang et al., 2014). However, despite the promise of this and other preliminary work, the promise of pan–PDE4 inhibitors as either cognitive enhancers in Alzheimer's Disease or as treatments for depression has yet to be

translated to clinical practice. Undoubtedly this is, at least in part, due to a narrow therapeutic window caused by the dose-limiting side effects of emesis, nausea and diarrhoea (Baillie, Tejeda, & Kelly, 2019; McDonough et al., 2020).

Pan-PDE4 inhibitors, whether the prototypical PDE4 inhibitor rolipram or more recently developed compounds such as roflumilast, induce strong emetic effects that have constrained their clinical utility. Multiple lines of evidence strongly implicate PDE4D as the basis of this emetic effect. Firstly, PDE4D is localised to regions of the brain associated with emesis including the area postrema and nucleus of the solitary tract (Lamontagne et al., 2001; Mori et al., 2010). Secondly, the deletion of PDE4D in transgenic mice reduced the anaesthetic effect of xylazine/ketamine, a behavioural correlate of emesis in non-vomiting species (Robichaud et al., 2002). Finally, and more translationally, cilomilast which was amongst the first of the second-generation PDE4 inhibitors developed for COPD, failed to gain regulatory approval due to its narrow index of therapeutic efficacy with dose-limiting side effects of emesis and nausea (Giembycz, 2006). Whereas roflumilast and its active metabolite, roflumilast N-oxide have a similar selectivity for PDE4 isoforms (Hatzelmann & Schudt, 2001; Manning et al., 1999), cilomilast has a reportedly 10-fold greater selectivity for PDE4D over other isoforms (Giembycz, 2006; Lipworth, 2005) and it seems very likely could not be given at a dose that would meet a therapeutic threshold without causing intolerable side effects.

### 1.6.6 PDE4 inhibitors in respiratory disease

Asthma and COPD are major causes of respiratory morbidity accounting for significant health utilisation, societal costs and reductions in quality of life. In the

UK alone, over eight million people have been diagnosed with asthma (British Lung Foundation, 2018) and it accounts for 2-3% of primary care consultations (M. Mukherjee et al., 2016). Yet, although the efficacy of inhaled corticosteroids is well established and there has been an almost prolific advent of novel biological treatments for certain asthma phenotypes (McCracken, Tripple, & Calhoun, 2016), there is an unmet need for medicines that exhibit non-steroidal anti-inflammatory activity and that are also well tolerated by patients.

In COPD, the clinical need is even more pressing. COPD is now the third largest cause of death worldwide and alongside ischaemic heart disease and stroke, an important rising cause of death in low- and middle-income countries (WHO, 2020). In less advanced economies, which are home to the majority of patients with COPD, the prevailing trends of urbanisation, pollution and an aging population are likely to only add to the burden of disease. Yet despite the higher and more immediate need, the available pharmaceutical armamentarium is far narrower in COPD than in asthma. It could be argued that in the nearly thirty years since inhaled anti-cholinergic medications were first described for use in COPD (Anthonisen et al., 1994), there have been only rather modest and largely iterative gains. It was therefore within this context of urgent clinical need that the anticipation of PDE4 inhibitors held so much promise, fuelled also by an array of promising pre-clinical data and potentially widespread desirable effects on cells and functions of the respiratory system.

#### 1.6.6.1 PDE4 inhibitors in asthma

The goal of treatment in asthma is to achieve good symptom control, to minimise risk of asthma related mortality and persistent airflow limitation (Bateman,

O'Byrne, Buhl, & Rabe, 2015). Amongst the majority of patients, existing treatments offer the opportunity to achieve good control of their disease. However, a proportion of patients do not achieve the current gold standards of care (Bateman et al., 2004), or can only do so with undesirable medication related adverse effects, leaving an unmet clinical need.

Cilomilast was amongst the first selective PDE4 inhibitor to be evaluated in asthma and COPD. Although it showed promise in early *challenge* and dosing studies (Lipworth, 2005), long-term large scale placebo controlled clinical studies failed to demonstrate consistent improvements in lung function or other important clinical outcomes (C. E. Compton CH, Nieman RB, Amit O, Langley SJ, Sapene M, 1999; D. M. Compton CH, Cedar E, et al, 2000) and in 2003, its manufacturer (GSK) discontinued its development for asthma.

Roflumilast has a higher *in vitro* potency than cilomilast (Hatzelmann & Schudt, 2001) and has fared better in clinical trials for asthma. One measure used to assess efficacy in asthma involves the assessment of both early and late phase asthmatic responses using allergen challenge and measurements of the forced expiration in 1 second ($FEV_1$). The early phase is characterised by immediate bronchoconstriction caused by the release of mediators such as histamine and cysteinyl leukotrienes whereas late phase bronchoconstriction is due to an influx of inflammatory cells (Lipworth, 2005).

In a study of 23 patients with mild asthma, roflumilast reduced late-stage bronchoconstriction by 43% in a dose dependent manner (in addition to reducing early stage bronchoconstriction to a lesser extent) (van Schalkwyk et al., 2005).

In a further study, targeting protection against exercise induced asthma, roflumilast attenuated the $FEV_1$ fall after exercise as well as reducing LPS induced TNF$\alpha$ in whole blood – used as a marker of inhibition of inflammatory cell activation (Timmer et al., 2002). Indeed, in a pooled mechanistic analysis of eight studies involving 197 patients, roflumilast was associated with reductions in allergen-induced bronchoconstriction and indices of airway inflammation including sputum neutrophil, eosinophil and TNF$\alpha$ concentrations (Bardin, Kanniess, Gauvreau, Bredenbroker, & Rabe, 2015).

In a dose-ranging study involving 690 patients, roflumilast led to significant increases in $FEV_1$ at 12 weeks across all three treatment groups (Bateman et al., 2006). In a follow up study lasting 12 months, the increases in $FEV_1$ were reportedly maintained in those patients taking the highest dose – though this observation was only published in abstract form (Izquierdo JL, 2003). Interestingly, this study also reported that roflumilast even at the highest dose range was well tolerated and that the most frequently reported side effects including headache (13%), diarrhoea (8%), nausea (8%) and abdominal pain (4%) all abated with time, with corresponding rates falling to respectively 6%, 3%, 1% and 1%. A further large non-inferiority study with 499 patients with mild to moderate asthma that compared roflumilast with inhaled beclomethasone, showed both medicines led to similar improvements in lung function, reductions in asthma symptom scores and also need for rescue treatments (Bousquet et al., 2006).

Although it has shown the greatest promise of all selective PDE4 inhibitors in asthma, the use of roflumilast in asthma is still not established and its true value in asthma continues to be debated (Bateman et al., 2015).

#### 1.6.6.2 PDE4 inhibitors in COPD

COPD is associated with exacerbations that are defined by a change in the patient's regular symptoms, beyond day to day variation that necessitates a change in their regular treatment. Exacerbations of COPD worsen health status and accelerate lung function decline (Vogelmeier et al., 2020) and therefore preventing exacerbations has been an important goal in the pharmacological management of COPD. The mainstay of treatment is inhaled bronchodilators and corticosteroids that provide modest improvements in symptoms and reduce the frequency of exacerbations. Other adjuncts include long term macrolide antibiotic therapy to reduce the exacerbation frequency (Albert et al., 2011), albeit with risks of adverse side effects and antibiotic resistance. It was thus with great promise, that roflumilast arrived as part of the therapeutic arsenal for treating COPD.

The selective PDE4 inhibitors, first cilomilast and then plainly, roflumilast have found greater therapeutic success in the treatment of COPD than asthma. In COPD, cilomilast improved lung function in both phase II dose-ranging studies (Compton et al., 2001) and short-term phase III studies (Edelson JD, 2001), though both these studies were reported in abstract form only. In a study to investigate the anti-inflammatory profile of cilomilast, investigators evaluated induced sputum and cell compositions in bronchial biopsies in patients randomised to cilomilast or placebo for 12 weeks (Gamble et al., 2003). When compared to placebo, cilomilast had no effect on baseline $FEV_1$ or sputum

differential cell counts but was associated with reductions in CD8 positive T cells, CD68 positive macrophages and neutrophils in bronchial biopsy tissue.

Roflumilast has been evaluated in several phase III/IV clinical trials involving over 10,000 patients (Wedzicha, Calverley, & Rabe, 2016). Two such early studies (M2-111 and M2-112) (Rennard, Calverley, Goehring, Bredenbroker, & Martinez, 2011) demonstrated that roflumilast was associated with modest benefits in lung function but no significant reduction in exacerbations. However, when the studies were pooled, a post-hoc analysis helped identify a responsive subset of patients with COPD who did have meaningful benefits. In patients who had symptoms of cough, sputum production and concurrent use of inhaled corticosteroids or short-acting anti-cholinergics, there was between 18-30% reduction in the incidence rate of moderate to severe COPD exacerbations (Rennard et al., 2011). The identified patient groups most responsive to roflumilast helped inform the design of subsequent studies including the REACT study (Fernando J. Martinez et al., 2015), a phase III/IV study that prospectively confirmed the reductions in exacerbations, observed in certain sub-groups in the aforementioned post-hoc analyses.

## 1.7 JAK-STAT and IL-4 signaling

In Chapter 1.5, I described how the cAMP signaling system has gained interest as a therapeutic target for the development of novel medicines to attenuate the effects of harmful inflammation, as evaluated in a range of chronic diseases (Figure 1.5). Another such critical system is the Janus kinase-signal transduction and activator of transcription (JAK-STAT) signalling pathway. Many cytokines involved in the pathogenesis of auto-immune and inflammatory diseases use

JAKs and STATs to transduce intracellular signals (O'Shea, Laurence, & McInnes, 2013). Mutations in JAK and STAT genes cause immunodeficiency syndromes (Casanova, Holland, & Notarangelo, 2012), myeloproliferative disorders (E. Chen, Staudt, & Green, 2012) and are also implicated in a range of inflammatory diseases including those of the bowel (C. Harris & Cummings, 2021) as well as lung (Tamura, Suzuki, Arakawa, Tokuyama, & Morikawa, 2003).

There are four intracellular tyrosine kinases in the JAK family (JAK1, JAK2, JAK3 and TYK2) and seven intracellular transcription factors of the STAT family (STAT1/ 2/ 3/ 4/ 5A /5B and 6). Upon ligand engagement, JAKs become activated and phosphorylate each other and the intracellular tail of their receptors, thereby creating docking sites for STATs (O'Shea et al., 2015). JAK mediated phosphorylation activates STATs which dimerize and then enter the nucleus where the directly bind to DNA and regulate gene expression (O'Shea, Holland, & Staudt, 2013). Genetic studies have also shown that STATs can regulate microRNAs, long non-coding RNAs and that they have important impacts on chromatin structure and distinctive enhancer landscapes (Vahedi et al., 2012).

Within the lung, STAT6 regulates many of the pathological features of lung inflammatory responses in animal models including airway eosinophilia, epithelial mucous production, smooth muscle changes and Th2 cell differentiation (Walford & Doherty, 2013). Binding of IL-4 and IL-13 through their receptors result in a common STAT6-mediated signaling pathway, critical to the development of Th2 inflammation (Hershey, 2003). IL-4 binds to a receptor complex consisting of the IL-4 receptor $\alpha$ chain (IL-4$\alpha$) and the common gamma chain $\gamma$C, to form the Type

I receptor, whilst both IL-4 and IL-13 bind to the shared type II receptor complex made up of IL-4Rα and IL-13Rα1(Villarino, Kanno, & O'Shea, 2017).

In recent years, extensive study of the Jak-STAT pathway has culminated in the deployment of two novel Jak inhibitors for therapeutic gain. Ruxolitinib and tofacitinib were the first in class of a novel group of compounds designed to target the Jak-STAT pathway. Ruxolitinib is licensed for use in myelofibrosis and is under evaluation in other myeloproliferative disorders (Villarino et al., 2017). Another FDA approved Jak inhibitor tofacitinib, is approved for use in rheumatoid arthritis (Villarino et al., 2017). Since then, Jak inhibitors have been evaluated in a range of inflammatory and immune related disorders (Fragoulis, McInnes, & Siebert, 2019) and have also been proposed for use in asthma and COPD (Peter J. Barnes, 2016).

## 1.8 Aims

The aim of this project was to profile the PDE4 isoform expression in monocytes and macrophages derived from them and obtain data as to whether the PDE4 isoform profile was dysregulated in COPD. I also set out to explore the expression and regulation of macrophage PDE4B2, which has been proposed to be an important regulator of the inflammatory response and a prime target through which PDE4 inhibitors exert their anti-inflammatory effect.

Using LPS challenge, I next evaluated the effect of TLR4 signaling on the PDE4 isoform expression in monocytes and macrophage subsets derived from them and obtained data as to whether the response to TLR4, was different in COPD. Using a limited supply of donated human bronchial tissue, I also compared the PDE4 sub-family expression in atopic asthma with non-asthma controls.

Turning to the regulation of PDE4B2 protein within the cell, I set out to map the degradation of PDE4B2 over a time-course experiment. Using data from the USCD genome suite and Encode datasets, a role for the transcriptional repressor BCL-6 in the regulation of PDE4B2 was hypothesised and data were subsequently provided to evaluate this hypothesis. Using the data herein obtained, it was further propose that macrophage PDE4B2 is maintained in a poised state under tonic regulation by BCL-6 but that this transcriptional break is relieved upon receptor ligation at the TLR4 receptor.

Finally, I explored the potential for cross-talk between prototypical cytokines involved in macrophage polarisation and LPS-induced PDE4B2. Based upon this work, I believe that I have provided novel insight into regulatory systems that help

underpin PDE4B2 as an important regulator of the inflammatory response in macrophages. Knowledge gained here, may offer new therapeutic targets for interdiction of macrophage inflammation and also systems to evaluate for any dysregulation in disease states in the future.

## 1.9 Main scientific objectives:

1. Quantify PDE4 isoform expression and the response to TLR4 signaling in monocytes and macrophage subsets from COPD.

2. Explore the degradation and regulation of macrophage PDE4B2 protein.

3. Determine the potential for cross-talk between cytokines involved in macrophage polarisation and LPS induced PDE4B2 and identify if these are maintained in COPD.

# Chapter 2 **METHODS AND MATERIALS**

## 2.1 Cell culture

All cell culture techniques were performed in a class II hood using aseptic techniques and reagents that had been filtered or autoclaved to ensure sterilisation.

### 2.1.1 U937 cell line

The U937 cell line is widely used in myeloid based cell research and has also been extensively deployed by members of the Houslay group in investigating the expression and regulation of PDE4 isoforms. U937 cells grow in suspension and resemble primary monocytes in morphology. A number of investigators (Fukunaga & Tsuruda, 2001; Kuroda, Sugiyama, Taki, Mino, & Kobayashi, 1997; Matheson, Labow, & Santerre, 2002; M. C. Shepherd et al., 2004; Twomey, McCallum, Isenberg, & Latchman, 1993), have demonstrated that chronic administration of phorbol 12-myristate 13-acetate (PMA) over several days, induces a differentiation of U937 toward a phenotype that resembles monocyte-derived macrophages.

U937 cells were supplied by Public Health England (PHE, UK). The cells were propagated in Roswell Park Memorial Institute (RPMI) 1640 cell culture medium with L-glutamine (GibCo Life Technologies, UK), enriched with 10% v/v foetal bovine serum (Gibco) and supplemented with 1% v/v penicillin/streptomycin 10,000U/ml (Gibco). This is described as complete RPMI in the present study. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$ and passaged at a density of approximately 2 x $10^6$ cells $ml^{-1,}$ .

U937 macrophages cells were obtained as described by Shepherd and colleagues (M. C. Shepherd et al., 2004). Early passage U937 cells were made to a concentration of 0.8 $x10^6$ cells $ml^{-1}$ using complete RMPI medium, supplemented with 4nM PMA for a total of 4 days. The medium was changed after two days and non-adherent cells were removed. After four days, the medium was changed and the cells were maintained in PMA-free complete RPMI for at least 24hours before use in cell experiments. Cells were considered to be differentiated if they were adherent, as determined using light microscopy (Hass et al., 1989).

### 2.1.2 Recruitment of blood donors

The study was approved by the Health Research Authority local Research Ethics Committee [REC 14/LO/1699]] and performed in accordance with all relevant institutional, national and international guidelines. Venous whole blood was collected from healthy subjects as well as patients with COPD. Donors without pre-existing health conditions or taking regular medications were identified from a pool of staff and students working at King's Healthcare Partners (Guy's Hospital, London, UK) who had agreed to participate in research studies in immunological research at KHP. Their age and sex are shown in Table 2.1 .

| **Donor** | H034 | H037 | H033 | H022 | H027 | H013 |
|---|---|---|---|---|---|---|
| **Sex** | F | M | M | F | F | F |
| **Age** | 49 | 52 | 57 | 43 | 22 | 28 |

| **Donor** | H003 | H005 | H023 | H019 | H032 | H040 |
|---|---|---|---|---|---|---|
| **Sex** | M | F | M | M | M | F |
| **Age** | 41 | 26 | 54 | 50 | 46 | 55 |

Table 2.1 Characteristics of blood donors who were healthy

Potential donors with COPD were identified using lists of patients attending a COPD clinic at Guy's Hospital. All patients had a physician made diagnosis of COPD in keeping with the Global initiative for Chronic Obstructive Lung Disease (GOLD) guidelines for the definition of COPD, were former smokers and had evidence of airflow limitation with a post-bronchodilator FEV1/FVC ratio of less than 0.7. Potential donors were first screened using their medical records for the presence of any condition or use of any medication that might reasonably interfere with the validity of immunological based research (for example but not limited to inflammatory conditions such as rheumatoid arthritis, diabetes mellitus inflammatory bowel disease, cancer as well as medications such as immune-suppressants including corticosteroids).

All participants were required to be over 18 years of age, willing to provide basic demographic and medical information and able to give written informed consent. Donors were excluded if they were pregnant or had any history of blood-borne viral disease (HBV, HCV and HIV). Healthy donors also had to be non-smokers

and free of any history of respiratory disease or significant other co-existent health condition. Donors were also asked not to donate if they were suffering from either a respiratory tract infection or in the case of donors with COPD - within four weeks of an exacerbation of COPD or of taking medication for one.

The characteristics of blood donors with COPD are shown in
Table 2.2. The severity of airflow limitation is defined by the $FEV_1$ where disease that is mild is considered to have a $FEV_1$ > 80% of that predicted for the individual, moderate between 50 – 80% and severe disease < 50% of the predicted value. The data also includes the donor's COPD assessment test (CAT) score which is a validated health related quality of life tool, often used in COPD clinical studies where the higher the score (max of 40) the greater the impact of COPD and risk of exacerbations (S.-D. Lee et al., 2014).

| **Donor** | **Sex** | **Age** | **$FEV_1$ (%)** | **$FEV_1$/FVC (%)** | **CAT** | **Smoking (pack yrs)** | **Inhaler class** |
|---|---|---|---|---|---|---|---|
| C02 | F | 74 | 71 | 67 | 19 | 60 | LABA/ICS/LAMA |
| C05 | M | 79 | 62 | 64 | 16 | 60 | LABA/LAMA |
| C08 | M | 77 | 43 | 43 | 27 | 60 | LABA/ICS/LAMA |
| C09 | F | 78 | 53 | 66 | 15 | 56 | LABA/ICS/LAMA |
| C10 | F | 76 | 94 | 69 | 13 | 23 | LABA/LAMA |

Table 2.2 Characteristics of blood donors with COPD

Donors with COPD were recruited from COPD clinics at Guy's Hospital. All donors had a physician made diagnosis of COPD, had accumulated a clinically significant burden of tobacco exposure (pack years) and had evidence of airflow obstruction using spirometry, as evidenced by a forced expiratory volume in 1 second (FEV1) to forced vital capacity (FVC) ratio of less than 0.7. The donor's COPD assessment (CAT) score was recorded as was the inhaler class they were prescribed; LABA; long acting beta agonist, ICS; inhaled corticosteroid and LAMA; long acting muscarinic agonist.

### 2.1.3 Bronchial tissue

Surplus RNA extracted from endobronchial biopsies were kindly donated from Dr Line Ohm-Laursen, (Randall Division of Cell and Molecular Biophysics, King's College London). The RNA was originally obtained for a study titled 'Role of IgE in the pathogenesis of non-atopic asthma'. The study was granted the appropriate institutional and ethical permissions (LREC 10/H0804/86). RNA from a total of six donors was obtained, three each from asthmatic and non – asthmatic groups. All study participants underwent clinical review, skin-prick testing and lung function tests. Current smokers were excluded. Donors with asthma all had a clinician-approved diagnosis of asthma. Atopic status was established through clinical review and the aid of skin prick testing. Donors with asthma were medicated with a combination of inhaled corticosteroid and long acting beta-agonist inhalers (Step 3, British Thoracic Society asthma guidelines, (BTS, 2019)). Donors without asthma were also non-atopic and not taking any medications used in asthma. The characteristics of the donors are shown in Table 2.3.

| Donor | M/F | Age | Asthma | Atopy | $FEV_1$ % | FEV1/FVC (%) | Smoking | Medication |
|---|---|---|---|---|---|---|---|---|
| HNA4 | M | 22 | N | N | 122 | 95.8 | Ex | None |
| HNA14 | M | 28 | N | N | 94 | 74 | N | None |
| HNA16 | F | 27 | N | N | 99 | 91 | N | None |
| AA3 | F | 65 | Y | Y | 64 | 61 | N | ICS/ LABA |
| AA4 | F | 32 | Y | Y | 115 | 77 | N | ICS/ LABA |
| AA6 | F | 31 | Y | Y | 130 | 83 | N | ICS/ LABA |

Table 2.3 Characteristics of donors of human bronchial tissue

Donors who were either healthy non-atopic (HNA) or had atopic asthma (AA), were recruited and underwent fibreoptic bronchoscopy and endobronchial biopsies. The biopsies were processed and RNA was extracted. The table shows the donor characteristics including lung function (forced expiratory volume in 1 second (FEV1) and FEV1/forced vital capacity (FVC) ratio as well as their use of medications; inhaled corticosteroid (ICS) and long acting beta agonists (LABA).

### 2.1.4 Isolation of PBMCs

Up to 120ml donor blood was collected using syringes pre-filled with acid citrate dextrose (ACD). ACD acts an anti-coagulant by the action of the citrate ion chelating free ionised calcium and making calcium unavailable to the coagulation system. Donor blood was processed individually and without delay. Whole blood was diluted 1:1 with Hank's Balanced Salt Solution (HBSS) (GibCo). 30mls of diluted blood was gently overlaid 15mls of Lymphoprep (Axis-shield) and centrifuged with the break off, at 800*g* for 20mins. Centrifugation leads to a

PBMC-rich interface which was carefully collected and then washed twice in HBSS supplemented with 2% v/v of FBS. Cells were pelleted by centrifugation at 300*g* for 10 mins. Cell viability was determined by trypan blue exclusion and was greater than 95%.

### 2.1.5 Positive selection of CD14+ cells

CD14+ monocytes were isolated by positive selection using anti-CD14 MicroBeads and a MACS® separator (both Miltenyi Biotec, Germany) according to the Manufacturer's instructions.

The cells were kept cold and solutions were pre-cooled before use. The cell pellet was re-suspended in 80ul of buffer per $10^7$ total cells. 20ul of CD14 MicroBeads (Miltenyi Biotec, Germany) per $10^7$ total cells were mixed well and kept on ice for 15minutes. Cells were washed using 1.5mls of MACS buffer (2% v/v HBSS + EDTA) per $10^7$ cells and centrifuged at 300*g* for 10 minutes. The supernatant was removed and up to $10^8$ cells were re-suspended in 500ul buffer (for higher cell numbers, the buffer was scaled up accordingly). Magnetic separation was performed using a MACS separator and MACS LS Column (both Miltenyi Biotec). The column was prepared using 3mls of MACS buffer. The cell suspension was applied on to the column and washed three times using buffer (3 x 3mls). The unlabelled cells and wash through effluent was kept aside and marked as PBMC-depleted CD14, for subsequent analysis using flow cytometry. The column was removed from the separator and placed on a collection tube labelled CD14+. 5mls of buffer was added to the column before being immediately flushed using the supplied plunger. The CD14+ cells were counted, an aliquot kept for analysis

using FACS and the remainder made up to a concentration of 1.5 x10^6 cells/ml in RPMI complete solution.

### 2.1.6 Flow cytometry

Flow cytometry (FACS) was used to help determine the purity of isolated CD14+ cells as well as the effectiveness of magnetic CD14+ bead isolation in isolating CD14+ PBMCs. Fluorescein isothiocyanate (FITC) (BioLegend) and Allophycocyanin (APC) (BioLegend) conjugated to anti-CD14+ and anti-CD3+-antibodies respectively were used according to the manufacturer's recommendation.

Approximately $1x10^5$ cells from both the CD14 depleted PBMC wash-through and the positively selected CD14+ cells were kept aside at 4°C. The cells were labelled with both FITC-CD14 and APC-CD3 alongside appropriate controls, mixed and incubated for 15mins at 4C. Non-viable cells were excluded by use of 7-amino actinomycin (7-AAD) (R&D Systems).

### 2.1.7 Differentiation of MDM and macrophage polarisation

A protocol for monocyte-derived macrophages and subsequent macrophage polarisation was adapted iteratively from those published previously by various investigators (Arnold et al., 2014; Krausgruber et al., 2011; Y. Liu et al., 2008; Tarique et al., 2015; H. M. Wilson, 2014), This is further discussed in Chapter 3.3.

CD14+ cells were seeded at density of 0.8 x10^6 cells/ml in complete RPMI supplemented with either 20ng/ml GM-CSF + 10ng/ml IFN-γ for M1 conditions or

10ng/ml M-CSF + IL-4 10ng/ml for M2 (R&D Systems) for four days. Cell media was changed after two days. The cells were cultured for a further 24hours in complete RPMI without CSF or cytokine supplementation before use in experiments.

## 2.2 Antibodies

| **Name** | **Application** | **Supplier** | **Catalogue No.** |
|---|---|---|---|
| Akt (pan)(C67E7)/p-Akt(Ser473) | WB | Cell signaling | 4691/4058 |
| APC – CD3+ (UCHT1) | FACS | BioLegend | 300411 |
| BCL-6 (D65C10) | WB | Cell signaling | 5650 |
| FITC Anti human CD14 (HCD14) | FACS | BioLegend | 325603 |
| Fk2 (UBCJ2) (mono & polyubiquitinylated) | WB, IP | Endo Life Sciences | BML-PW8810 |
| GAPDH (14C10) | WB | Cell signaling | 5174 |
| Jak1/p-Jak1(Tyr1022/1023) | WB | Cell signaling | 3332/3331 |
| Jak2 (D2E12)/p-Jak2 (Tyr1007/1008) | WB | Cell signaling | 3230/3776 |
| Jak3/p-Jak3 (Tyr980/981) | WB | Cell signaling | 3775/5031 |

| | | | |
|---|---|---|---|
| p44/42 MAPK (ERK 1/2) (137F5) / p-p44/42 (Thr202/Thr204) | WB | Cell signaling | 4695/ 9101 |
| PDE4B/ B2 | WB, IP | Houslay Laboratory (Glasgow) | None |
| PI3K p85 (19H8)/p-PI3K p85(Tyr458)/p55(Tyr199) | WB | Cell signaling | 4257/4228 |
| Stat1/p-Stat1(Tyr701) (58D6) | WB | Cell signaling | 9172/9167 |
| Stat3/-p-Stat3 (Tyr705) | WB | Cell signaling | 4904/9131 |
| Stat6/-p-Stat6 (Tyr641) | WB | Cell signaling | 9362/9361 |
| Tyk2/p-Tyk2 (Tyr1054/1055) | WB | Cell signaling | 9312/9321 |

Table 2.4 Suppliers of antibodies

The table shows a list of antibodies used as part of this study and their use in either western blot (WB), immunoprecipitation (IP) or both as well as their supplier and catalogue number. PDE4B/B2 antibodies were sourced from the Houslay Laboratory (Glasgow) and their use is referenced in Chapter 2.3.

## 2.3 Immunoblotting

Cells were washed x2 in PBS before being lysed in 150ul/12-well of lysate buffer: 0.76% w/v Tris-base, 0.013% w/v sodium pyrophosphate tetrabasic, 0.047% w/v EDTA, 1.25% w/v sodium dodecyl sulphate (SDS), 12.5% v/v glycerol, 0.05%

w/v bromophenol blue. β-mercaptoethanol (β-ME) 2% v/v was used as the reducing agent. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). A 1.5mm stacking gel was used for sample loading. SDS-PAGE was with 10% gel in running buffer (25mM 3.03% w/v Tris-base, 14.4% w/v Glycine, 1% w/v SDS). Samples were electrophoresed at 120V 40mA for approximately 100 minutes. Separated gels were transferred to nitrocellulose membranes (Bio-Rad) in transfer buffer (25mM 3.03% w/v Tris Base, 14.4% w/v glycine and 20% v/v methanol) at 100V 390mAs for 60minutes. Membranes were blocked for 1 hour at room temperature using a shaker, in either 5% w/v non-fat dry milk or 5% w/v bovine serum albumin made up using TBS-T (2.4% w/v Tris HCL, 0.56% w/v Tris base, 8.8% w/v NaCl, 0.1% v/v Tween 20). Next, the membrane was incubated with the desired primary antibody (Table 2.4), overnight at 4°C on a rocking platform. Membranes were washed three times for a minimum of 5 minutes in TBS-T on a shaker before incubation with secondary HRP conjugated antibodies diluted in blocking buffer for 1 hour at room temperature. Finally the membrane was washed three times for 5 minutes in TBS-T on a shaker, before developed using ECL Plus for western blot detection (GE Healthcare, Buckinghamshire UK).

Anti-sera specific for the extreme C-terminal portion of each PDE4 subfamily were supplied by the Houslay group and their use has been described before (Bolger 1997, MacKenzie 1998, McPhee 1999, MacKenzie and Houslay 2000).

## 2.4 Co–immunoprecipitation

Co-immunoprecipitation (Co-IP) is a widely used tool to help identify protein–protein interactions. I used Protein A/G magnetic beads and magnetic stand

(Pierce IP/Co-IP Kit, Thermo Scientific, UK) according to a slight modification of the manufacturer's protocol as outlined below. The antibody–bead complex was prepared according to the manufacturer's protocol using an anti-FK2 antibody (1:40 for IP) (Enzo Life Sciences) for both the pull-down and immunoblotting of mono and polyubiquitinated species. The FK2 antibody demonstrates a high affinity for protein A/G beads and has been widely deployed to detect ubiquitinylated proteins conjugated to target proteins, either as a monoubiquitin or as a polyubiquitin chain (Kharat et al., 2016; S. Kobayashi et al., 2015; Wright et al., 2015). Anti-sera against PDE4B (1:50 for IP) (Houslay group) was used for the pull-down and anti-sera raised against PDE4B2 was used for immunoblotting. The antibody–bead complex was cross–linked using disuccinimidyl suberate (DSS) to help prevent antibody elution and detection on immunoblot, as recommended by the manufacturer.

An NP40 lysis buffer was made using 2.42 w/v Tris HCL, 8% w/v NaCl, 1% v/v NP40 and 0.58% w/v EDTA in ddH20 supplemented with protease inhibitor cocktail (Roche). U937 macrophages were grown to confluence using a 10cm culture dish for each experimental condition. The culture medium was aspirated and cells washed using ice cold PBS. 500ul NP40 lysis buffer was added and the lysate transferred to a microcentrifuge tube. In a slight modification to the supplied protocol, the lysate was gently agitated using an orbital rotator for 30 minutes at 4°C and then centrifuge at 12,000*g* for 20 minutes at 4°C. Around 300ul of supernatant was removed (a larger amount risked disruption to the pelleted cell debris) The supernatant was made to 500μl using the NP40 lysis buffer described above, and added to the crosslinked antibody–bead complex and incubated overnight at 4°C under gentle agitation using an orbital rotator. The beads were

washed x3 times with 500μl NP40 lysis buffer and then with ultrapure water before addition of an elution and neutralisation buffer to collect the target antigen. The use of elution and neutralisation buffer was used as recommended in the manufacturer's protocol and may confer some resistance to antibody fragmentation that can be associated with the use of SDS buffer. Sample buffer was then added along with β-ME and the sample boiled at 95°C for 5 minutes. For the western blot, anti–PDE4B2 was used at 1:5000 in 5% milk protein and anti-FK2 was used at 1:1000 in 5% bovine serum albumin.

## 2.5 Cytokines

Human recombinant cytokines were purchased from R&D Systems and where recommended by the manufacturer, reconstituted in sterile PBS containing 0.1% bovine serum albumin, aliquoted and stored at -20°C.

## 2.6 RNA extraction and cDNA

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The yield and purity of RNA was assessed using Nanodrop ND-1000 spectrophotometer. The ratio of absorbance at 260nm and 280nm was used to assess the purity of RNA with a ratio of around 2.0 taken as highly pure for RNA. The ratio of absorbance at 260/230 nm was used as a secondary measure of purity with a ratio of above 1.8 considered relatively free of non-protein contaminants. cDNA was synthesised using a commercial synthesis kit (First Strand cDNA, ThermoFisher, UK) according to the manufacturer's instructions and made to a concentration of 2ng/μl and stored at -20°C.

## 2.7 RT - qPCR

The expression of PDE4 isoform mRNA was detected using real time quantitative PCR (RT-PCR). DNA probes to PDE4 sub-families and isoforms were supplied by the Houslay Laboratory (Henderson et al., 2014; Kirsty F. Mackenzie et al., 2008; M. C. Shepherd et al., 2004). In addition, a DNA probe against PDE4D was obtained from Applied Biosciences. Taq Man Universal PCR Mastermix (Applied Biosciences) and human 18S was used as an endogenous control. Reactions were run in triplicate and averaged. Gene expression was presented relative to the expression of 18S, as the internal control and using the $2^{-deltaCT}$ method. The $2^{-deltaCT}$ method is commonly used for the presentation of individual data points (Schmittgen & Livak, 2008). This method differs from the commonly used $2^{-delta\ delta\ CT}$ as the latter expresses the change in gene expression, relative to an untreated target sample, for example the fold change in gene expression of a given gene, between an untreated sample and a treated sample.

## 2.8 Nucleofection

Electroporation, using the Amaxa Nucleofector II (Lonza, UK) was used to transfect U937 macrophage like cells. The Amaxa system includes pre-optimised electrical programs that the manufacturer lists for individual cell lines including transfection of U937 monocytic cells. However, a pre-optimised program was not available for U937 macrophages. Technical advice was sought and the manufacturer recommended trialling three alternative electrical programs (W-001, T-001 and V-001) which was subsequently evaluated using a fluorescent positive control vector (pmaxGFP™) for both transfection efficiency and cell viability.

U937 cells that were up to passage 20, were cultured at a density of 5 x $10^5$ cells/ml, in line with the manufacturer's protocol. In a modification of the manufacturer's protocol, U937 cells were differentiated toward a macrophage like phenotype. Cells were cultured in T125$cm^2$ flasks in RPMI complete supplemented PMA 4ng/ml for four days before maintained in PMA-free medium for 24 hours. The medium was removed and the cells were washed using 15mls of pre-warmed Dulbecco's phosphate buffer saline calcium and magnesium free (DPBS). The DPBS was removed and 10ml of warmed trypsin/EDTA solution was added and cells incubated at 37◦C for 10minutes. The cells were gently agitated and then analysed under the microscope to ensure cell detachment. Growth media was added to inactivate the trypsin/EDTA solution. The cells were collected, counted and then aliquoted at 1x$10^6$ cells/100μl Nucleofector solution.

Commercially available siRNA for Jak1, Jak3, STAT6 was obtained from Ambion (Life Technologies, UK). Three different siRNAs designed to target mRNA from each gene target was obtained and pooled. A non-targeting control (NTC) (Silencer Select®) was also obtained. All siRNA was prepared according to the manufacture's recommendation and aliquoted prior to storage at -20C.

According to the manufacturer's recommendations, each nucleofection sample was prepared with 1 x$10^6$ cells, pooled target siRNA or a non-targeting control. The mixed sample was transferred to the supplied cuvette and then in to the Nucleofector which was programmed to the desired electrical program. The cuvette was removed and 500μl warmed growth media was immediately added. In a modification to the manufacturer's protocol, the contents of the cuvette were then centrifuged using 90*g* 10minutes to remove the Nucleofector solution which

the manufacturer lists as potentially harmful to cells. The cell pellet was resuspended and added to pre-warmed culture media in pre-prepared 12 well plate containing 1ml complete RPMI. The cells were cultured for up to 48 hours before lysed for immunoblot or 24 hours for RT-qPCR.

As an alternative to nucleofection, Oligofectamine (Invitrogen) was piloted in U937 macrophages as it has been successfully used in PMA-treated THP-1 cells, including by members of the Sethi group (MacKinnon et al., 2008). A matrix of different Oligofectamine and siRNA concentrations was used as part of an initial pilot to help demonstrate its effectiveness.

## 2.9 Statistical analysis

Statistical differences were determined by Student's t-test for parametric data with ANOVA comparison for more than two groups. For non-parametric data, Tukey's multiple comparisons test was performed. Probability of $P < 0.05$ was considered significant. GraphPad Prism and Microsoft Excel was used to prepare graphs and statistics. Experiments were completed in replicate as shown in each figure. Averages of multiple experiments are shown with the standard deviation.

# Chapter 3 **PDE4 EXPRESSION IN MYELOID CELLS**

As I describe through Chapter 1.1 through to 1.3, monocytes and macrophages are critical components of the host immune response and are implicated in the pathogenesis of chronic airway diseases, including COPD and asthma. cAMP meanwhile, regulates many of the functional outputs of monocytes and macrophages and is itself underpinned by a complex set of cellular machinery including multiple PDE4 isoforms (Chapter 1.4).

PDE4 is the dominant PDE family in monocytes and macrophages (G. Dent, Magnussen, & Rabe, 1994; Torphy, 1998) and is composed of over 25 PDE4 isoforms, each with non-redundant functions within cells (Houslay, 2001). Since PDEs provide the only route through which cAMP is degraded, characterising the expression profile of PDE4 isoforms in monocytes and in macrophages is critical to help understand the role of cAMP signaling in monocytes and macrophages and whether the expression of PDE4 is dysregulated in disease.

PDE4 expression has been reported to be altered in COPD. Firstly, in a study completed over 15 years ago using peripheral mononuclear cells and alveolar macrophages (AM) (Barber et al., 2004) and more recently, in AM taken from patients with COPD (Simon Lea, Metryka, Facchinetti, & Singh, 2011; S. Lea et al., 2019). However, in the case of the report by Barber and colleagues (Barber et al., 2004), this study was completed before the widespread availability of isoform-specific DNA probes and the discovery of a number of new PDE4 isoforms. As such, further investigation, using current techniques, is warranted. In the case of the study completed by Lea and colleagues (S. Lea et al., 2019),

their evaluation of PDE4 was limited to quantifying the expression of each of the four PDE4 sub-families, using ‘pan’ probes to detect all isoforms within a particular PDE4 sub-family and did not extend to analysing the expression of individual PDE4 isoforms within each sub-family.

After extensive study and at times an interminable wait, eventually two PDE4 inhibitors, roflumilast (Daxas®) in COPD and apremilast (Otezla®) in psoriatic arthritis, found clinical-trial success and gained regulatory approval. Regrettably, the therapeutic utility of these medicines have been limited due in part to off target effects (Kavanaugh et al., 2015; F. J. Martinez et al., 2015). To address this, two approaches have been taken. One approach focuses on providing a localised drug delivery system to avoid off-target systemic effects, exemplified by the topical use crisaborole (Eucrisa®) in a cream for the treatment of atopic dermatitis (H. Yang et al., 2019). Another focus of drug development has been to identify the PDE4 isoform(s) associated with the therapeutic response and try to selectively target said isoform(s) through the design of isoform-selective inhibitors. Progress here, combined with discovery of dysregulated PDE4 isoform expression in disease, could accelerate development toward a PDE4 isoform-specific inhibitor that could help improve the therapeutic index. Moreover, such discovery might also contribute to a biometric profile of disease toward identifying which patients might best respond to selective PDE4-inhibition and with it the advent of a more personalised therapeutic approach in treating patients with COPD.

The work described in this chapter describes an approach aimed at providing a comprehensive profile of PDE4 isoform expression in monocytes and monocyte-

derived macrophages (MDM) polarised to different activation states, with the use of RT-qPCR and isoform-specific probes. Here I evaluated the change in PDE4 isoform expression profile as monocytes differentiate to macrophages and sought to obtain data that might provide an indication as to whether the PDE4 isoform profile was altered in COPD. In addition, I evaluated the response to a pro-inflammatory stimulus using LPS as a prototypical ligand of TLR4 signaling (Bode, Ehlting, & Häussinger, 2012).

Beyond monocytes and MDM, I also profiled the PDE4 isoform expression in bronchial biopsies obtained from donors with asthma as well as donors without, as a further model of disease since PDE4 inhibitors have been proposed in asthma (Bardin et al., 2015; Bateman et al., 2015; Page, 2014).

## 3.1 CD14+ monocyte isolation

Monocytes comprise 5 – 10% of the circulating leukocytes in humans. Up to 120ml of whole blood was obtained from each donor to gain sufficient cells for experimental studies. Each 1ml of whole blood yielded approximately 1x10^6 peripheral blood mononuclear cells (PBMCs). CD14+ monocytes were isolated using positive magnetic bead isolation as described in the Methods and Materials Chapter. There was a wide range in the total yield of monocytes from individual donors, approximately between 10 – 20 x10^6 CD14+ cells per donation of 120mls of blood (data not shown). The cells from an individual donor were treated separately.

Donor blood from twelve healthy donors and five donors with COPD was used to obtain the data presented in this study. The donor group of healthy individuals

were younger than those with COPD (43.58 ± 12.03 yrs. vs 76.80 ± 1.92 yrs., respectively, $p < 0.0001$ $n = 12$ and 5). There was though a similar distribution of the sexes across both groups as 6/12 donors were female in the group of healthy individuals and 3/5 donors were female in the COPD group.

Flow cytometry and fluorophore – conjugated antibodies, were used to determine the composition of populations of cells following magnetic bead isolation. The purity and yield of magnetically isolated CD14+ labelled cells was determined using an anti–CD14+ antibody conjugated to fluorescein isothiocyanate (FITC) along with an anti–CD3 antibody conjugated to allophycocyanin (APC). CD14 is expressed by the majority of human monocytes (Schmidl et al., 2014) and was the basis upon which monocytes were isolated from the PBMC population. CD3 was used as the most common T cell marker and is expressed by both CD4+ and CD8+ T cells.

Figure 3.1 shows a representative flow cytometric analysis (FACS) of two cell fractions following CD14+ magnetic bead isolation of PBMCs. The figure on the left (A) shows the 'flow-through' of PBMCs that have been depleted of CD14+ cells. In this example, only 0.47% of cells expressed CD14+ indicating that magnetic bead isolation was able to select out the majority of CD14+ cells from PBMCs. In the figure on the right (B), 99.8% of cells that were positively selected, expressed CD14+ cells and not the T cell marker, CD3 consistent with a highly pure population of CD14+ monocytes. CD14+ magnetic bead isolation was undertaken in line with the manufacturer's instructions. Flow cytometry was repeated using isolated cells from at least three donors on different occasions and the results shown are representative.

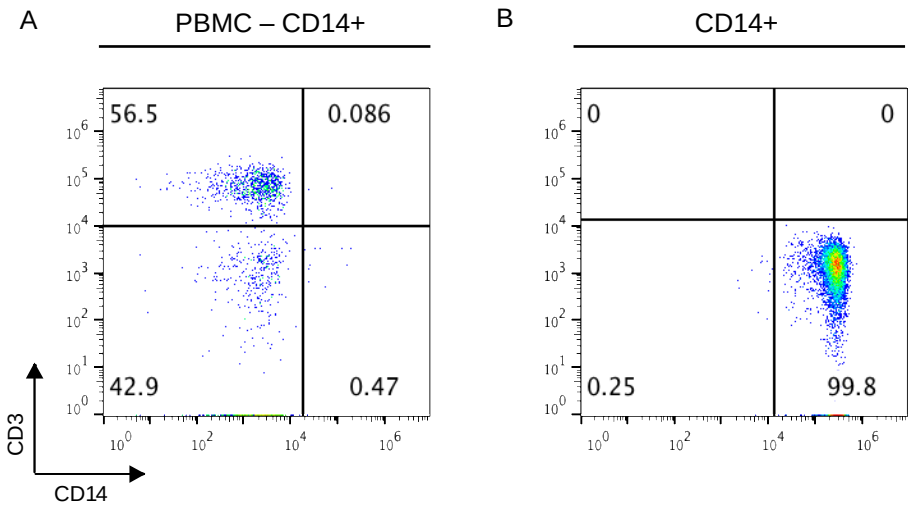



Figure 3.1 CD14+ monocyte isolation from PBMCs

CD14+ monocytes were isolated using magnetic bead separation of PBMCs derived from whole blood. PBMCs were labelled with anti–CD14 beads and magnetically separated. Flow cytometric analysis using CD3–APC and CD14–FITC antibodies, of (A) PBMC–CD14 and (B) CD14+ cell populations. Flow cytometry was repeated using isolated cells from at least three donors on different occasions and the results shown are representative.

## 3.2 PDE4 expression in monocytes

As the focus of investigation was on MDM, it was necessary to prioritise the use of CD14+ monocytes (hereafter, termed simply monocytes) toward macrophage differentiation and experiments in MDM subsets. However, sufficient donor blood was obtained to divert some monocytes toward analysis of their PDE4 expression as well as the effect, on expression, of treating such cells with LPS. It was also therefore possible to profile the PDE4 expression profile in monocyte to macrophage differentiation, which has been reported to be associated with a

reduction of long PDE4D isoforms and up-regulation of the short PDE4B2 (M. C. Shepherd et al., 2004).

Monocytes from a total of five healthy and five COPD donors were cultured with or without LPS 50 ng/ml in RPMI growth media for three hours. The RNA was extracted and the PDE4 sub-family and isoform expression was determined using RT-qPCR and PDE4 sub-family probes.

PDE4A and PDE4B sub-family expression was readily detected across all donors across both 'healthy' and 'COPD' groups (respectively 3.53 ± 0.73 and 1.77 ± 1.16 arbitrary units, mean ± SD) (Figure 3.2A). In contrast, PDE4C was only observed at vanishingly low levels (0.002 ± 0.0009). Vanishingly low expression of PDE4C has been previously reported in monocytes (M. C. Shepherd et al., 2004). PDE4D sub-family expression was also detected though less readily than might have been expected given previous reports that long PDED isoforms provide the dominant PDE4 activity in monocytes (M. C. Shepherd et al., 2004).

The observed lack of PDE4D sub-family expression was not expected but did prove to be a repetitive finding from RT-qPCR in both monocytes and MDMs and is further explored in Chapter 3.2.3. A positive control here, for example using genomic DNA and plasmid constructs, would have helped validate the efficiency of the DNA probes used in this PCR system and therefore help substantiate the observed lack of PDE4D sub-family expression. However, a positive control for the DNA probes was not included in the current study and would be important to include in a further study of PDE4 isoform expression in myeloid cells.

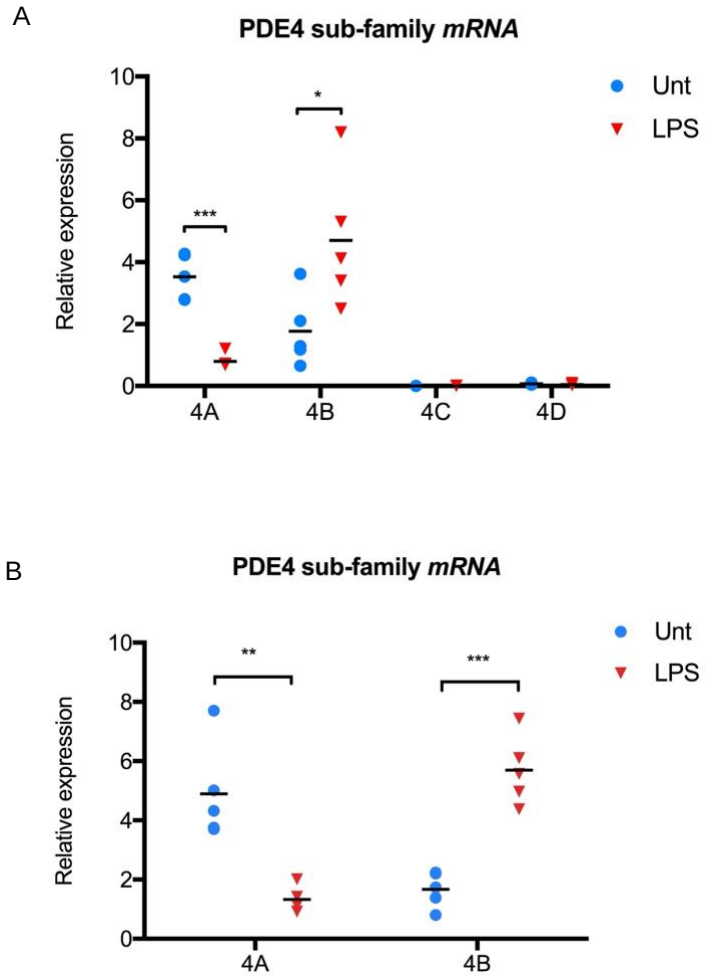



Figure 3.2 PDE4 sub-family expression in monocytes

CD14+ monocytes were isolated from PBMC's from donors who were healthy (A) and had COPD (B), before being cultured in RPMI complete ± lipopolysaccharide (LPS) 50 ng/ml for 3 hours. The mRNA of each PDE4 sub-family was detected using RT-qPCR. Mean shown, $n$ = 5 from each group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (response to LPS compared using 2-tailed Student's $t$-test).

I next explored the effect of LPS on PDE4 sub-family expression in recently harvested monocytes. Whilst LPS had no effect on the expression levels of either PDE4C or PDE4D, there was a marked and differential response in the expression of PDE4A and PDE4B sub-families. LPS increases PDE4B

expression by x2.7 ($P$ < 0.05, $n$ = 5, 2-tailed Student's $t$-test) but also simultaneously reduces PDE4A expression by 77% ($P$ < 0.001 $n$ = 5) (see above, Figure 3.2A). Thus, it could be proposed that, in monocytes, LPS leads to a switch away from PDE4A expression to one favouring PDE4B expression. However, to substantiate such a proposal, further study of transcript levels would need to be completed using absolute PCR quantification and samples with known copy number.

The effect that LPS had on simultaneously reducing PDE4A expression whilst increasing PDE4B sub-family expression in monocytes derived from healthy individuals, was mirrored in monocytes derived from patients with COPD. LPS led to a 72% reduction in PDE4A ($P$ < 0.01, $n$ = 5) and a x3.4 increase in PDE4B expression ($P$ < 0.001, $n$ = 5) (see above, Figure 3.2B). There was no significant difference between the magnitude of effect of LPS, on either PDE4A or PDE4B sub-family expression, between either healthy or COPD donor groups (0.77 ± 0.05 vs 0.72 ± 0.08, $P$ = 0.25 2-tailed Student's $t$-test; PDE4A reduction ratio in health vs COPD, 3.37 ± 2.19 vs 3.74 ± 1.28, $P$ = 0.75, 2-tailed Student's $t$-test, PDE4B induction ratio in health v's COPD).

Given that PDE4C and PDE4D sub-family expression was only modestly detected in monocytes and in an effort to prioritise the use of limited material, PDE4C and PDE4D sub-family expression was not determined in monocytes derived from individuals with COPD.

Changes in expression at the level of PDE4A/ B/ C/ D sub-families, using pan-PDE4 probes, reflects the aggregate change across all those isoforms within a

particular sub-family using probes defined by regions of their common core and C-terminal regions (Simon J. MacKenzie et al., 2002). As such, pan-PDE4 probes cannot, for example, distinguish between a scenario when a 'challenge' has no effect on the expression of all isoforms within a PDE4 sub-family to one where, for example, the induction of expression of one individual isoform is exactly mirrored by a reduction in expression in another within a particular PDE4 sub-family. Moreover, as the transcription of individual PDE4 isoforms is regulated by isoform-specific promoters it is critically important to characterise the effect of agents such as LPS, using probes that are specific for individual PDE4 isoforms.

Over 25 PDE4 isoforms have been described and, given the constraints in working with primary and in particular donor tissue from patients, it was necessary to prioritise which isoforms were selected for evaluation. As shown in Figure 3.2, using the pan PDE4 probes, LPS was associated with a significant change in PDE4A and PDE4B sub-family expression. In order to determine which isoform(s) might account for this change, I next proceeded to assay all isoforms for which there were available DNA probes in both PDE4A and PDE4B sub-families. I also sought to obtain data to help explore why the expression of PDE4D might be lower than has been reported previously (M. C. Shepherd et al., 2004).

### 3.2.1 PDE4A isoform expression

Isoform specific probes were available for all the widely reported PDE4A isoforms, namely PDE4A 1/ 4/ 7/ 8/ 10 and 11. Figure 3.3 shows the PDE4A isoform expression in monocytes derived from healthy donors and the effect of LPS. LPS reduced the expression of long PDE4A10 (PDE4A10 unt vs LPS, 67%

reduction, $P < 0.01$ $N = 5$) and not any of the long PDE4A4, long PDE4A8, short PDE4A1 or the catalytically inactive 'dead-short' PDE4A7 isoform.

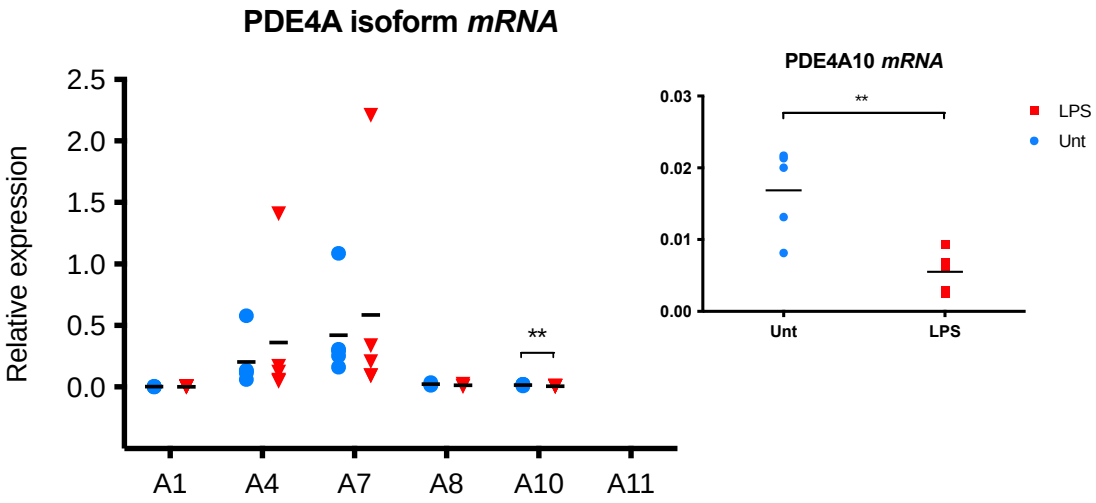



Figure 3.3 PDE4A isoform expression in monocytes from donors who were healthy

CD14+ monocytes were isolated from PBMCs and cultured in RPMI complete for 3 h ± lipopolysaccharide (LPS) 50 ng/ml. RT-qPCR was used to detect the expression of PDE4 1/ 4/ 7/ 8/ 10/ and 11, both without LPS (shown here in blue) and with (red). Mean shown. LPS was associated with a reduction of PDE4A10 expression as shown in the inset panel. Mean shown $n = 4$ (2-tailed Student's *t*-test, ** $P < 0.001$) .

Long PDE4A10 appears to be only very modestly expressed at the transcript level (Figure 3.3). As such it may be proposed that PDE4A10 is unlikely to account for the magnitude of change observed in PDE4A sub-family expression, following treatment with LPS (Figure 3.2). However, comparisons of relative template abundance using different DNA probes are problematic as the observed expression levels can depend upon the efficiency of amplification which itself can

differ between experiments using different DNA probes. This could be potentially reconciled with the help of a standard curve comparing the expression using DNA probes with known amounts of template, but this was not pursued in the current study. As such it was not possible to determine the contribution that changes in PDE4A10 expression might have made to the change in overall PDE4A sub-family expression. Additionally, comparative transcript levels do not necessarily reflect comparative protein levels as proteins can be subject to different rates of degradation that reflect differences in stability as well as any potential to targeted degradation through the proteasome system.

The reduction of PDE4A expression following treatment with LPS, may be explained by reductions in PDE4A isoforms that were not evaluated, namely PDE4A6 and PDE4A9, for which probes were not available. Intriguingly, another possibility remains that an, as yet unidentified, novel PDE4A isoform is expressed in human monocytes whose expression is attenuated by LPS action, though this would need further study.

The catalytically inactive dead-short PDE4A7 and long PDE4A4 isoforms were both detected across all five donors (Figure 3.3). The expression of short PDE4A1 as well as long PDE4A8 were both near negligible and, in two donors H01 and H05, long PDE4A8 was not detected at all. PDE4A11 was not detected in monocytes derived from any donor.

In monocytes from COPD donors (Figure 3.4), but not monocytes from healthy donors (Figure 3.3), I made the novel discovery that LPS led to reduced expression of the

dead-short PDE4A7 isoform (64% reduction, $P < 0.05$, $n = 5$). A change in PDE4A7 expression was also observed in monocyte to macrophage differentiation (

Figure 3.18). These observations around PDE4A7 expression are intriguing as PDE4A7 has no enzymatic activity, but may have an important function around protein-protein interactions, which I explore further in Chapter 3.4.2 on PDE4A expression in MDMs.

In another disease specific difference, in contrast to observations in monocytes from healthy donors, LPS did not reduce the expression of PDE4A10, though it may be observed that there was a trend toward this.

Interestingly, the expression of long PDE4A4, which has been reported to be upregulated in peripheral monocytes in smokers and is also upregulated in lung macrophages in individuals with COPD (Barber et al., 2004), was not found to be differentially expressed in monocytes derived from healthy and COPD donor groups (0.20 ± 0.21 v's 0.07 ± 0.06, $P = 0.09$ $n = 5$).

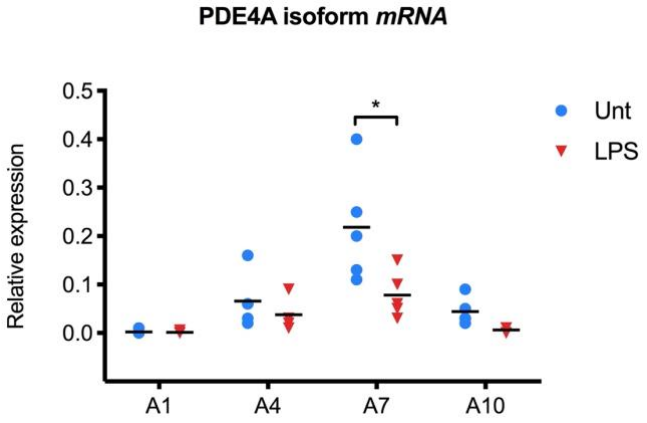



Figure 3.4 PDE4A isoform expression in monocytes from donors with COPD

CD14+ monocytes were isolated from PBMC's from donors with COPD and cultured in RPMI complete for 3h ± lipopolysaccharide (LPS) 50ng/ml. RT-qPCR was used to detect the expression of PDE4 1/ 4/ 7 and 10, both without LPS (shown here in blue) and with (red). Mean shown. LPS was associated with a reduction of PDE4A7 expression (2-tailed Student's *t*-test, * $P < 0.05$, $n = 5$ donors).

In order to prioritise the use of template, the isoforms PDE4A8 and PDE4A11, which were not detected in monocytes derived from five separate healthy donors, were not evaluated using template from patients with COPD.

Although isoform specific probes were available for all six widely reported PDE4A isoforms, GenBank® from the National institutes for Health lists a total of eight known PDE4A isoforms including PDE4A6 and PDE4A9. PDE4A6 expression has been reported in rat pulmonary microvascular endothelial cells (RPMVECs) and is subject to phosphorylation by PKA (B. Zhu, Kelly, Vemavarapu, Thompson, & Strada, 2004). However, the expression of PDE4A6 and PDE4A9

in humans is not well described and there is some doubt as to whether both species represent true isoforms (personal communication, Professor Miles Houslay).

### 3.2.2 PDE4B isoform expression

LPS-induced PDE4B sub-family expression, as shown in Figure 3.2, supports the scientific rationale for focusing on PDE4B as its positive induction is expected to drive the inflammatory response through lowering pools of cAMP in which it is localised. I focused first on monocytes obtained from healthy donors and examined the well-described short PDE4B2 isoform, which is the only PDE4 isoform reported to date that is induced by LPS. Doing this I observed that challenge of monocytes, with LPS, led to a x2.6 induction in PDE4B2 expression (transcript levels) (4.26 ± 2.78 vs 11.05 ± 4.73, $P < 0.05$, $n = 5$) (Figure 3.5).

Across five healthy donors, there was a wide distribution in the relative expression of short PDE4B2 with a mean of 4.26 and SD of 2.78 (arbitrary units). This may relate to the small sample size used or may reflect a wide variation in of PDE4B2 expression in humans, as others have also uncovered in 'freshly-isolated' human monocytes (Verghese, McConnell, Lenhard, Hamacher, & Jin, 1995).

The PDE4B long forms, PDE4B1 and PDE4B3 were detected at modest levels in monocytes from healthy donors. The super-short form PDE4B5, although expressed at low levels was, nevertheless, clearly detected in monocytes from all five donors, which represents a novel finding for this isoform whose expression in macrophages has not previously been reported. The short PDE4B5 isoform has, uniquely, been characterised as having a N-terminal region that is identical

to that of the short PDE4D6 isoform whose expression has been suggested to be ‘brain-specific’ (Cheung et al., 2007). PDE4B5 has gained interest at it is able to bind to the scaffolding protein DISC1, whose gene is linked to schizophrenia (Cheung et al., 2007).

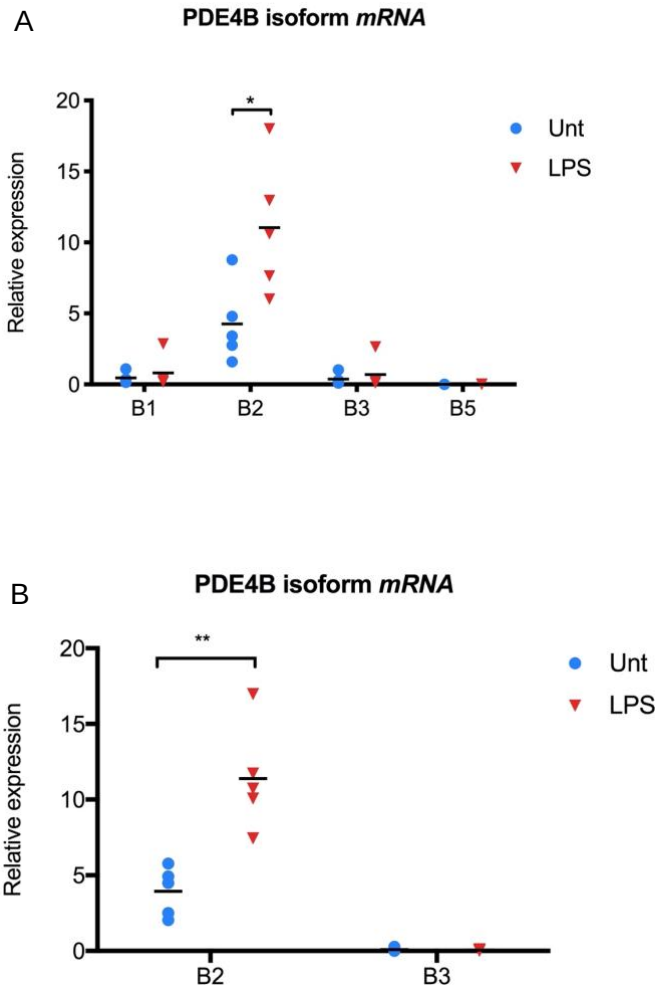



Figure 3.5 PDE4B isoform expression in monocytes

CD14+ monocytes were isolated from PBMCs from donors who were either healthy (A) or had COPD (B), before being cultured in RPMI complete for 3h ± lipopolysaccharide (LPS) 50 ng/ml. RT-qPCR and isoform-selective probes were used to detect the expression of PDE4B isoforms as shown, both without LPS (shown here in blue) and with (red).

Mean shown. Across both donor groups, LPS was associated with an increase in PDE4B2 expression (2-tailed Student's *t*-test, $n = 5$ donors, * $P < 0.05$, ** $P < 0.01$)

Turning to monocytes derived from patients with COPD, PDE4B2 was expressed across all five donors. LPS led to a x2.9 induction of PDE4B2 expression – similar to that seen in monocytes derived from healthy individuals (untreated vs LPS treated, 3.95 ± 1.60 vs 11.39 ± 1.57, mean ± SD, $P < 0.01$ $n = 5$). Long PDE4B3 was only modestly expressed and in one donor appeared not to be expressed at all (C09). LPS had no effect on PDE4B3 expression (0.086 ± 0.11 vs 0.05 ± 0.035 $P = 0.13$ $n = 5$). Long PDE4B1 and super-short PDE4B5 expression levels were not evaluated in monocytes from COPD donors due to their observed modest expression in monocytes from healthy donors and the need to prioritise template derived from COPD patients.

Since it has been hypothesised that PDE4B2 is associated with the inflammatory response (Borysiewicz, Fil, Dlaboga, O'Donnell, & Konat, 2009; Gobejishvili et al., 2011; Jin & Conti, 2002; Jin et al., 2005; Reyes-Irisarri, Sanchez, Garcia-Merino, & Mengod, 2007) - a hypothesis supported by the data shown in Figure 3.5 - I next sought to determine if the LPS-PDE4B2 expression of this key isoform was dysregulated in monocytes derived from donors who had COPD. Untreated monocytes show no difference in PDE4B2 expression between healthy and COPD donors (4.26 ± 2.78 vs 3.95 ± 1.60, $P = 0.83$, $n = 5$). There was also no difference in the degree to which LPS mediates PDE4B2 induction in monocytes derived from either donor group (matched donor pairs, 3.25 ± 2.01 vs 3.16 ± 1.05, $P = 0.93$, mean paired difference ± SD; health vs COPD)

The decision to focus on the expression of the PDE4B2 and PDE4B3 isoforms is underpinned by the observation that both isoforms are well conserved across mammalian species. For example, the sequences of the homologue isoforms from rats and humans are 90% identical (G. B. Bolger, Rodgers, & Riggs, 1994; Huston et al., 1997).

Long PDE4B4 has not been detected in humans and thus was not evaluated. Long PDE4B4 was first isolated and characterised by members of the Houslay group (M. Shepherd et al., 2003). It is found in multiple rat tissues including liver, skeletal muscle as well as parts of the brain. When expressed in COS-7 cells, it has minimal, or no, association with cellular particulate fractions and is part of only a small group of isoforms that are essentially cytosolic when expressed. The function of PDE4B4 is not well understood but its absence in humans implies that this function is not required in humans or that any function it may have, is performed by another (other) PDE4 isoform(s) expressed there.

### 3.2.3 PDE4D isoform expression

The expression of PDE4D in monocytes, as determined using the pan-PDE4D probe, appeared to be more modestly observed that might have been expected from the results of Shepherd and colleagues (M. C. Shepherd et al., 2004). This might be related to the relative efficiency of the pan-PDE4D probe within this assay.

Shepherd and co-workers (M. C. Shepherd et al., 2004), used a model monocytic system, namely the monocyte-like U937 cell line and employed anti-sera against

the specific C-terminal of each PDE4 sub-family to identify all active isoforms within a specific PDE4 sub-family. In their study, immunoblotting with PDE4D-specific anti-sera identified two species, one at 95kDa and another at 105kDa, which they concluded on the basis of accepted knowledge at that time was consistent with expression of the PDE4D3 and PDE4D5 long isoforms. They found that, within monocyte U937 cells, PDE4D3 expression predominated over PDE4D5 whereas in human monocytes this was reversed. Moreover, Shepherd (M. C. Shepherd et al., 2004) showed that the PDE4D sub-family - and so PDE4D3 and PDE4D5 long forms - provided the major fraction of total PDE4 cAMP hydrolysing activity in monocytic U937 cells, underscoring their importance in monocyte cAMP signaling. However, since the publication of the study by Shepherd (M. C. Shepherd et al., 2004) at least four new PDE4D variants (PDE4D6-9) have been identified (Gretarsdottir et al., 2003; D. Wang et al., 2003), including PDE4D8 and PDE4D9 which have been shown to have similar molecular masses and to co-migrate with PDE4D3 on SDS-PAGE (Levallet, Levallet, Bouraima-Lelong, & Bonnamy, 2007; Richter, Jin, & Conti, 2005). Thus, new studies require to be undertaken on U937 cells in order to define unequivocally the identity of the PDE4D species that migrates as a 95kDa species on SDS-PAGE.

To further evaluate the expression of PDE4D sub-family using the pan-PDE4D probe (Figure 3.2), I turned to evaluate individual PDE4D isoform expression using isoform-specific probes to long PDE4D3, long PDE4D5 as well as long PDE4D7 isoform, which is known to be dysregulated in prostate cancer (Bottcher et al., 2015; Henderson et al., 2014) and stroke (Gretarsdottir et al., 2003).

PDE4D3 was not detected in monocytes derived from both healthy and COPD donors – a combined total of 10 individuals (Figure 3.6). This observation seemingly contrasts with the conclusions made by Shepherd and co-workers (M. C. Shepherd et al., 2004) who concluded that PDE4D3 and PDE4D5 provided the dominant PDE4 activity in U937 monocytic cells. Absent or near absent PDE4D3 expression proved to be a consistent finding in the present study and this is explored further in Chapter 3.6.3. This finding underscores the likelihood that the 95kDa PDE4D species that Shepherd identified as PDE4D3, is in fact one of the PDE4 isoforms cloned and characterised after the Shepherd (M. C. Shepherd et al., 2004) study was published. Prior to such new PDE4D long isoforms being discovered it was accepted within the field that such a 95kDa species was PDE4D3 so this need will need reassessing throughout the literature.

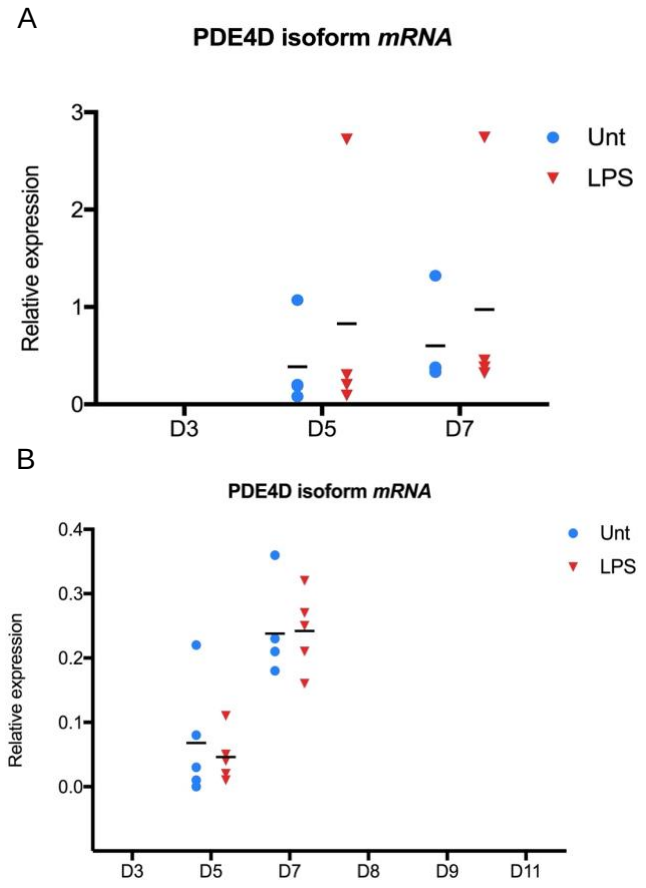


Figure 3.6 PDE4D isoform expression in monocytes

CD14+ monocytes were isolated from PBMCs from donors who were either healthy (A) or had COPD (B), before being cultured in RPMI complete for 3 h ± LPS 50 ng/ml. RT-qPCR and isoform selective probes were used to detect the expression of PDE4D isoforms as shown, both without LPS (shown here in blue) and with (red). PDE4D 8/ 9 and D11 isoform expression was not detected in monocytes derived from donors with COPD and not assessed in monocytes from healthy individuals, in order to prioritise the available template. Mean shown. *N* = 4 (healthy) and 5 (COPD). Response to LPS compared by 2-tailed Student's *t*-test.

The PDE4D5 and PDE4D7 long isoforms were detected in monocytes from all donors from both healthy and COPD groups. However, challenge with LPS had no effect on the expression of either isoform in either the healthy or COPD donor groups. Additionally, I found that there was no difference in expression between either the healthy or COPD groups for PDE4D5 (healthy vs COPD, 0.39 ± 0.46 v's 0.07 ± 0.10, $P = 0.17$ $n = 4 – 5$) or indeed between healthy and COPD donors' cells in PDE4D7 expression (0.60 ± 0.48 vs 0.25 ±0.08, $P = 0.13$ $n = 4 – 5$).

In order to further evaluate the lower than expected expression of PDE4D sub-family in monocytes and to gain further insight into how the PDE4D isoform specific probes were working, I prioritised the available template derived from COPD patients to also evaluate the expression of long forms PDE4D8, PDE4D9 and PDE4D11 (Figure 3.6). Across five donors and using the supplied DNA probes, I found no evidence of PDE4D8, PDE4D9 or PDE4D11 expression in monocytes that were either untreated or treated with LPS. However, it may be

proposed that the observed absence of these transcripts, was related to the efficiency of the isoform-specific probes and so these findings will need to be supported by the use of positive controls.

## 3.3 Defining a model of MDM subsets

Macrophages adopt different activation states or phenotypes according to environmental cues (Lavin et al., 2014; T. A. Wynn et al., 2013). Dysregulated macrophage phenotypes are implicated in the pathogenesis of chronic inflammation as well as diseases including COPD, fibrosis and cancer (Barron & Wynn, 2011; Bingle et al., 2002; T. A. Wynn et al., 2013). For their part, PDE4 inhibitors having been shown to have potent anti-inflammatory actions (Germain, Corbel, Belleguic, Boichot, & Lagente, 2001; Huang, Ducharme, Macdonald, & Robichaud, 2001; Jimenez, Punzon, Navarro, Munoz-Fernandez, & Fresno, 2001) and in addition, therapeutic potential in pulmonary fibrosis (Milara, Morcillo, Monleon, Tenor, & Cortijo, 2015; Sachs et al., 2007; Sisson et al., 2018) and cancer (Cassetta & Pollard, 2018; Domvri et al., 2017; Kelly et al., 2017; D. U. Kim, Kwak, & Kim, 2019). Yet, surprisingly, little is known about the PDE4 expression profile across macrophage phenotypes. Using a model of monocyte derived macrophages (MDM) polarised to different subsets, I set out here to explore the relationship between macrophage phenotype, PDE4 expression profile and the response to LPS.

The field of macrophage activation states, the descriptors that are used, as well as the protocols exploited to help derive them is contentious and confusing. In recent years this has given rise to concerted efforts amongst leaders in the field

to help provide a framework for the nomenclature as well as the macrophage markers used to help define macrophage activation states (Murray et al., 2014).

Often, researchers use either various cytokines or colony stimulating factors (CSFs) toward generating macrophages representative of different activation states (Murray et al., 2014; Ohradanova-Repic, Machacek, Fischer, & Stockinger, 2016; Seow et al., 2013). However, a wide number of published protocols combine macrophage growth factors and cytokines in order to better effect either a 'pro'- or 'anti'-inflammatory effect (Beyer et al., 2012; Lescoat et al., 2018; Martinez, Gordon, Locati, & Mantovani, 2006).

Classically activated or 'M1' macrophages, broadly promote inflammation and develop in response to engagement with the Th1 cytokine IFN-$\gamma$ as well as toll like receptor (TLR) ligands. Non-classical, also known as alternative or 'M2' macrophages, are considered to have a more regulatory role and are said to attenuate the inflammatory response following engagement with the prototypical Th2 cytokines IL-4 or IL-13 (Murray et al., 2014; Piccolo et al., 2017). Within the tissue environment, it is likely that this binary distinction is an over-simplification of macrophage biology and, instead, M1 and M2 macrophages sit at either end of a continuous spectrum of macrophages in varying activation states (Murray et al., 2014). Adding to this complexity, macrophages exhibit significant plasticity, enabling them to move between activation states, according to changing local environmental cues (Lavin et al., 2014).

Monocytes require a source of CSF to be sustained and to differentiate into macrophages. Both GM-CSF and M-CSF are used in protocols to develop MDM

and have been reported as driving, respectively, either M1 or M2 phenotypes (Krausgruber et al., 2011; Lescoat et al., 2018; Tarique et al., 2015). In order to test my hypothesis that the PDE4 profile may be context specific and altered in macrophage phenotypes, I set out to generate macrophages that were at the polar sides of the spectrum of macrophage activation states. A protocol adapted from those published previously by various investigators (Arnold et al., 2014; Krausgruber et al., 2011; Y. Liu et al., 2008; Tarique et al., 2015; H. M. Wilson, 2014) was developed, iteratively, in order to explore the impact of macrophage activation state upon PDE4 expression profile.

### 3.3.1 MDM activation states

Monocytes prefer to grow as a monolayer in semi-confluent populations but become (loosely) adherent within hours of cell culture. Over time, they become more firmly adherent, larger and more granular. After two days, monocytes cultured in conditions that were designed to be either more favourable to an ‘M1’ or ‘M2’ activation state, start to display morphological differences. After six days, monocytes cultured with GM-CSF and IFN-$\gamma$, hereafter termed M1 macrophages, have a typical ‘fried-egg’ or ‘lymphoblast-like’ appearance, whereas monocytes cultured in M-CSF and IL-4, hereafter termed M2 macrophages, appear more fibro-elastoid (

Figure 3.7).

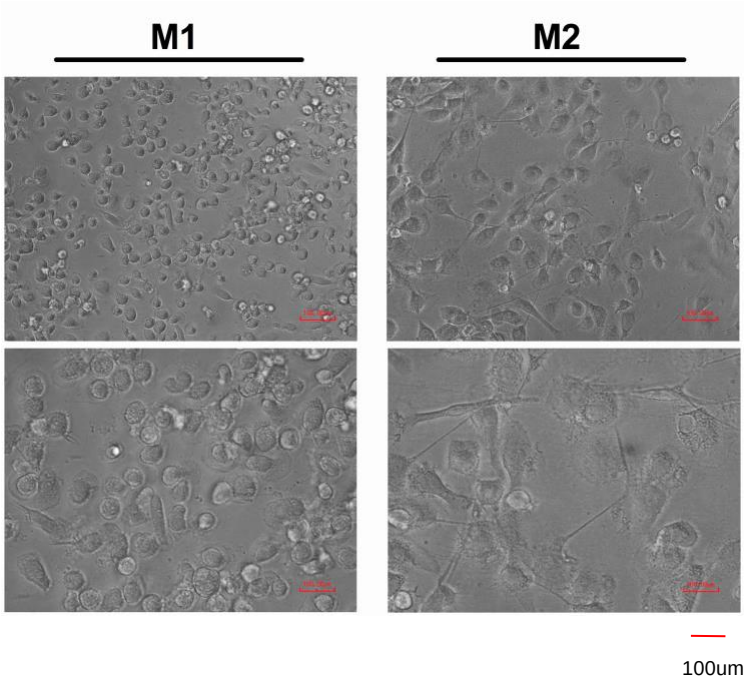



Figure 3.7 Photomicrographs of day 6 MDM

CD14+ monocytes were isolated from PBMCs and cultured in RPMI complete supplemented with either (M1) GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or (M2) M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete one day. Upper and lower panels shown respectively at x20 and x40 magnification. Scale bars 100μm.

A culture period of six days was found to be optimum between allowing sufficient time for monocytes to differentiate to toward a macrophage phenotype, whilst avoiding cell loss associated with prolonged culture.

The cellular morphological distinction between derived M1 and M2 MDMs has been reported by others and appears most closely related to the type of CSF used. Tarique and colleagues (Tarique et al., 2015), as well as Porcheray and colleagues (Porcheray et al., 2005), cultured CD14+ monocytes in, alternatively,

GM-CSF and M-CSF for six days, describing similar cell morphologies to those I observed in the current study (Figure 3.8).

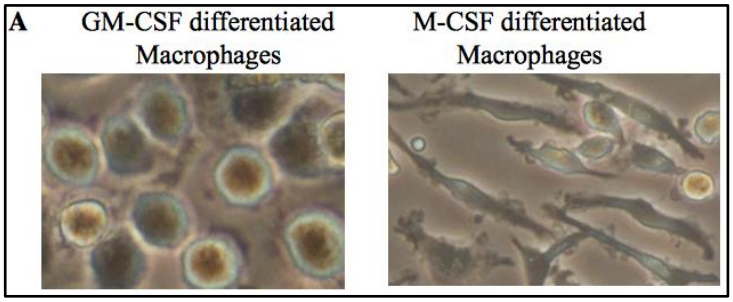



Figure 3.8 Photomicrograph of day 6 CD14+ monocytes cultured in CSF(Taken from (Tarique et al., 2015))

Photomicrograph showing the cell morphology of CD14+ monocytes alternatively cultured in GM-CSF or M-CSF as described by Tarique and colleagues (Tarique et al., 2015). Cells were obtained from the PBMCs of healthy donors using positive magnetic selection to CD14+, and cultured in RPMI-1640 supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin and either recombinant human (rh)GM-CSF (50ng/ml) or rhM-CSF (50ng/ml) for six days. Monocyte derived macrophages (MDM) obtained using GM-CSF retained a lymphoblast-like appearance, whereas MDMs differentiated in the presence of M-CSF were elongated and contained vacuoles. The morphological distinction between these cell groups appears to be a product of the type of CSF used in MDM culture and was also observed in the current study.

Beyond their morphological differences and the conditions in which they were derived, M1 and M2 populations of MDMs were also phenotypically distinct as determined using a suite of up to nine macrophage activation markers that were recommended for evaluation by Professor Heather Wilson (Aberdeen University,

UK) and reported previously (Arnold, Gordon, Barker, & Wilson, 2015; Whyte et al., 2011; H. M. Wilson, 2014). Due to the adapted protocol design, an initial preliminary determination of macrophage marker expression was performed using MDM subsets derived from three healthy donors, before those markers with the highest divergent expression, were prioritised for further evaluation in a minimum of five or more donors.

Markers of M1 activation state are expressed as multiples of the expression observed in M2 cells whilst markers of M2 activation state are expressed as multiples of the expression in M1 cells (see also
Table 3.1). In the paragraphs below, I describe the observed expression of each of nine macrophage markers and briefly describe their proposed role or significance.

### 3.3.2 Markers of M1 phenotype

TNFα, SOCS3, HLA-DR and IL-6 were evaluated as markers of M1 activation state. The expression of TNFα, SOCS3 and HLA-DR were respectively x28, x8 and x2 higher in M1 macrophages when compared to M2 macrophages (Figure 3.9).

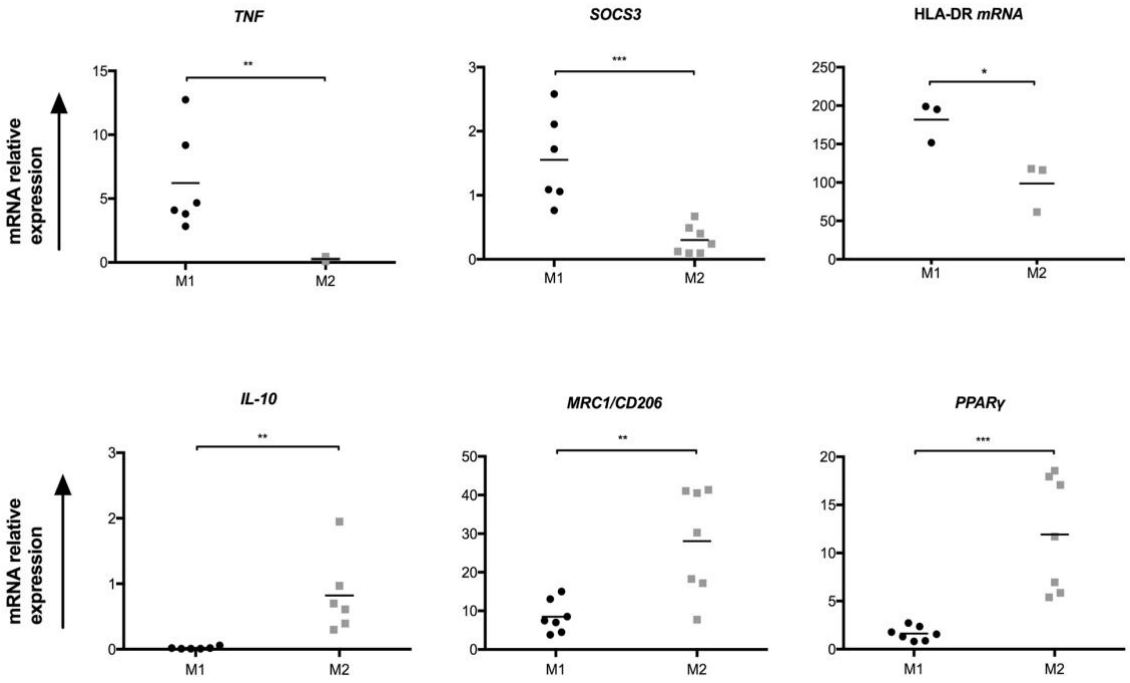



Figure 3.9 Macrophage marker expression in MDM subsets

Isolated CD14+ monocytes from healthy individuals were cultured in either GM-CSF 20 ng/ml + IFN-γ 10 ng/ml (M1 monocyte derived macrophages (MDM)) or M-CSF 10 ng/ml + IL-4 10 ng/ml (M2 MDM) for five days, followed by RPMI complete one day. The proposed markers of M1 macrophages, namely tumour necrosis factor (TNF), suppressor of cytokine signalling (SOCS) 3 and human leukocyte antigen (HLA)-DR were all more highly expressed in M1 MDM than M2, whereas the opposite pattern was observed in markers of M2 activation, namely interleukin (IL)-10, mannose receptor C-type1 (MRC1) and peroxisome

proliferator-activated receptor - γ (PPAR-γ). The mRNA was detected using RT-qPCR ($n$ = 6 – 7, $n$ = 3 in HLA-DR). Mean shown. Statistical significance was determined using a unpaired student $t$-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

TNFα is a powerful mediator of the inflammatory response and is firmly implicated in chronic inflammatory diseases including rheumatoid arthritis and Crohn's disease (I. A. Clark, 2007). TNFα and IFN-γ exhibit signaling cross-talk at the level of TNFR1, leading to enhanced TNFα-induced NF-κB activation (Wesemann & Benveniste, 2003).

Suppressor of cytokine signalling (SOCS) 3 belongs to a family of intracellular cytokine-inducible proteins consisting of eight members (CIS and SOCS1–SOCS7), that are known to negatively regulate the Jak-STAT signaling pathway (H. M. Wilson, 2014). SOCS3 is expressed in tissue macrophages activated in pro-inflammatory conditioning environments and in human MDMs. SOCS direct the production of IL-1β, IL-6 and IL-12 (Arnold et al., 2014; Y. Liu et al., 2008).

Human leukocyte antigen (HLA)-DR and another proposed M1 marker – interleukin (IL)-6 – are also expressed in monocytes cultured in 'Th1' conditions (Arnold et al., 2015). However, in this model of MDM, I observed that HLA-DR (see above, Figure 3.9) but not IL-6 (Figure 3.10) was significantly differentially expressed in M1 and not M2 cells.

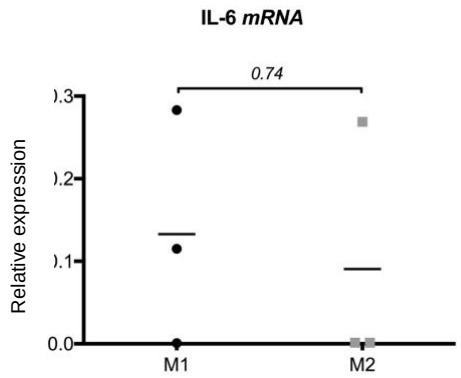



Figure 3.10 IL-6 expression in MDM subsets

Isolated CD14+ monocytes from healthy individuals were cultured in either GM-CSF 20 ng/ml + IFN-γ 10 ng/ml (M1 monocyte derived macrophages (MDM)) or M-CSF 10 ng/ml + IL-4 10 ng/ml (M2 MDM) for five days, followed by RPMI complete one day. The expression of a proposed M1 macrophage marker interleukin (IL)-6, was determined using RT-qPCR ($n$ = 3). Statistical significance was determined using a unpaired student $t$-test.

### 3.3.3 Markers of M2 phenotype

CD206, IL-10, PPAR-γ, CD163 and SOCS1 were evaluated as markers of M2 activation state. The mannose receptor, CD206, has long been described as a marker of alternative macrophage polarisation (Stein, Keshav, Harris, & Gordon, 1992) and is potently stimulated by IL-4. CD206 was expressed x3 higher in M2 than in M1 cells (Figure 3.9,

Table 3.1).

IL-10 is a potent anti-inflammatory cytokine that mediates anti-inflammatory effects directly by its actions on STAT3 as well as indirectly by antagonism of pro-inflammatory cytokines produced by macrophages. IL-10 has been shown to

augment IL-4 activity in M-CSF induced bone marrow derived macrophages (Makita et al., 2015). IL-10 was associated with x47 higher expression in M2 cells.

PPAR-γ was strongly associated with M2 activation with a x7 higher expression. The nuclear receptor peroxisome proliferator-activated receptor - γ (PPAR-γ) is a regulator of lipid metabolism and found in macrophages in atherosclerotic lesions, indicative of a role in cardiovascular disease (Ricote, Huang, et al., 1998; Ricote, Li, Willson, Kelly, & Glass, 1998). Within the lung, PPAR-γ is critical for perinatal differentiation of alveolar macrophages by a GM-CSF dependent process but appears to be less important for the development of macrophages within the liver, brain, heart as well as other organs (Schneider et al., 2014). PPAR-γ activity is augmented through IL-4 mediated STAT6 (Szanto et al., 2010).

The expression of both CD163 and SOCS1, was not significantly different between M1 and M2 MDM subsets (see below, Figure 3.11). CD163 is a scavenger receptor for the haemoglobin-haptoglobin complex and is a marker of alternative activation (Hussell & Bell, 2014). It has been shown to be up–regulated by M-CSF during differentiation of monocytes (Buechler et al., 2000) and its expression is enhanced in alveolar macrophages from patients with idiopathic pulmonary fibrosis (Gibbons et al., 2011).

SOSC1 expression is upregulated in M2 polarising environment (H. M. Wilson, 2014) where it is involved as a regulator of the M2 murine marker index between arginase $I^{high}$ and $iNOS^{low}$ (Whyte et al., 2011). SOCS1 is also involved in inhibiting IFN-γ-induced Jak2/STAT1 and TLR/NF-κB signaling associated with

M1 macrophage activation (Yoshimura, Naka, & Kubo, 2007) (H. M. Wilson, 2014).

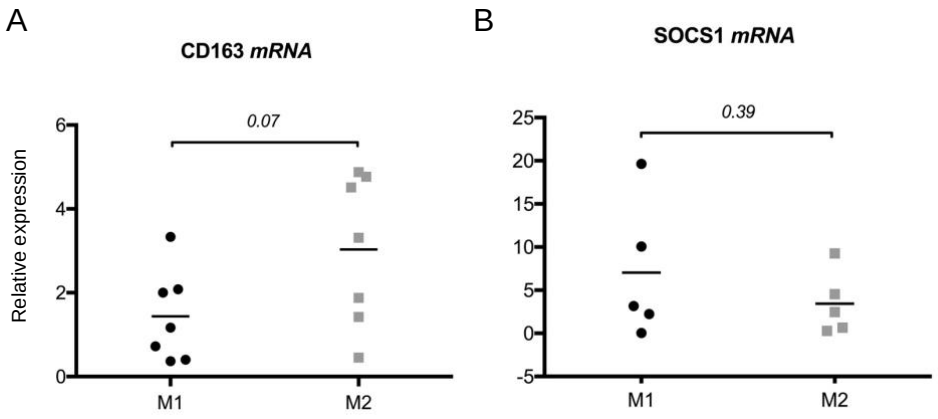



Figure 3.11 CD163 and SOCS1 expression in MDM subsets

The expression of CD163, a scavenger receptor and suppressor of cytokine signalling (SOCS)1 were determined using RT-qPCR, as markers of M2 (alternative) macrophage activation. CD14+ monocytes were isolated from PBMCs and cultured in RPMI complete supplemented with either GM-CSF 20 ng/ml + IFN-γ 10 ng/ml (M1) or M-CSF 10 ng/ml + IL-4 10 ng/ml (M2) for five days, followed by RPMI complete for one day. Relative expression was determined used RT-qPCR (*n* = 7 in CD163, *n* = 5 in SOCS1). Mean shown. Statistical significance was determined using a unpaired student *t*-test.

In summary, using an adapted protocol of different CSFs and Th1/Th2 cytokines I have been able to generate two morphologically and phenotypically distinct subsets of MDM that are proposed to be comparable to ‘M1’ and ‘M2’ macrophages. Out of a total of nine proposed markers of macrophage activation state, six showed a significant differential expression in this model of MDM.

Moreover, the greatest observed differences were seen with TNFα and IL-10 - respectively, the archetypal 'pro' and anti-inflammatory cytokines of the inflammatory response and those most closely associated with M1 and M2 functional responses.

| | **Relative difference** | | | |
|---|---|---|---|---|
| | **M1** | **M2** | ***n*** | ***P* value** |
| **M1 Gene Markers** | | | | |
| TNFα | 28.11 ± 16.12 | 1 | 6 | ** |
| SOCS3 | 7.73 ± 2.81 | 1 | 6 | *** |
| HLA-DR | 2.06 ± 1.03 | 1 | 3 | * |
| IL-6 | 92.49 ± 78.80 | 1 | 3 | 0.74 |
| **M2 Gene Markers** | | | | |
| MRC1/CD206 | 1 | 3.37 ± 1.13 | 7 | ** |
| IL-10 | 1 | 47.25 ± 25.16 | 6 | ** |
| PPAR-γ | 1 | 7.38 ± 2.38 | 7 | *** |
| CD163 | 1 | 3.54 ± 4.41 | 7 | 0.07 |
| SOCS1 | 1 | 1.86 ± 2.92 | 5 | 0.39 |

Table 3.1 Relative gene expression of M1 and M2 macrophage markers in MDM

A table showing the relative expression of nine proposed markers of macrophage activation state in monocyte derive macrophages (MDM), derived from healthy individuals. The expression of markers of M1 activation state, namely tumour necrosis factor (TNFα), suppressor of cytokine signalling (SOCS) 3, human leukocyte antigen (HLA)-DR and interleukin (IL)-6 are shown in M1 MDMs, in relation to their respective

expression in M2 cells. Mirroring this, the expression of markers of M2 activation state, namely mannose receptor C-type 1 (MRC1), IL-10, peroxisome proliferator-activated receptor - γ (PPAR-γ), CD163 and SOCS1 are shown in M2 MDMs, in relation to their expression in M1 cells. A total of six, out of nine markers of activation state show a differential expression across MDM subsets. Values shown are mean ± SD, *n* = as indicated, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Statistical significance was determined using a 2-tailed Student's *t*-test.

As an additional approach towards characterising macrophages subsets, pilot studies, using multicolour flow cytometry, were undertaken. These employed surface markers associated with macrophage activation states CD64, CD163, CD206, CD80, CD86. However, early attempts were hindered by the challenge of detaching strongly adherent cells to use in FACS, whilst maintaining cell viability. Both mechanically based approaches (initially through the use of vigorous tapping but progressing to use of cell scrapers) as well as chemical based ones (initially ice cold PBS + EDTA, through to Accutase™ and Trypsin) were all ultimately unsuccessful without an unacceptable loss of cell viability. This was compounded by the observation that M2 macrophages seemed more adherent than M1 macrophages frequently affecting the proportion of viable cell populations from each subset.

Further attempts at using FACS were not pursued, in part because of the technical challenges but also as the experimental outputs are constrained by the lack of commercially available fluorochromes conjugated to antibodies against PDE4 isoforms.

## 3.4 PDE4 expression in monocyte to macrophage differentiation

It has been reported that the PDE4 isoform profile can be remodelled upon monocyte to macrophage differentiation (M. C. Shepherd et al., 2004). However, the PDE4 expression from monocyte to macrophage subset has not been described.

### 3.4.1 PDE4B sub-family and isoforms

Starting with PDE4B sub-family expression, monocyte to macrophage differentiation elicited a marked reduction in PDE4B expression, as determined using the pan-PDE4B probe. In monocytes derived from healthy individuals, macrophage differentiation was associated with a 90 and 92% reduction in PDE4B expression in respectively M1 and M2 cells (1.77 ± 1.16 vs 0.19 ± 0.14, $P < 0.0001$, 1.77 ± 1.16 vs 0.14 ± 0.23, $P < 0.0001$, one-way ANOVA using CD14+ as control and a Dunnett test for multiple comparisons, mean ± SD, arbitrary units, CD14+ vs M1/M2, respectively) (

Figure 3.12). This reduction was matched in cells derived from COPD donors, with a respective 89 and 99% reduction in PDE4B expression in M1 and M2 macrophages (1.67 ± 0.18 vs 0.18 ± 0.06 $P < 0.0001$, 1.67 ± 0.18 vs 0.02 ± 0.02 $P < 0.0001$).

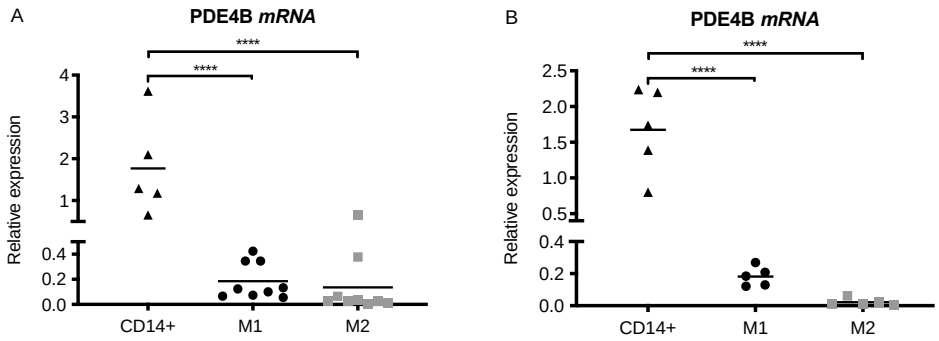



Figure 3.12 PDE4B sub-family expression in monocyte and MDM subsets from (A) healthy and (B) COPD donors

CD14+ monocytes were isolated from PBMCs from donors who were either healthy (A) or had COPD (B). A proportion of cells were used for RT-qPCR experiments after 3hrs culture in RPMI complete and represent the CD14+ cells shown. The balance of cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete for one day. Relative expression of PDE4B using the pan-PDE4B probe was determined used RT-qPCR ($n$ = 5-9). Mean shown. Statistical significance was determined using a one – way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons. **** $P <$ 0.0001.

Using isoform specific probes, it was possible to determine that short PDE4B2 expression and not either long PDE4B1 or long PDE4B3 accounted for the change in PDE4B sub-family expression upon monocyte to macrophage differentiation. Thus, in MDM derived from healthy donors there was a 79 and 97% reduction in PDE4B2 expression in respectively M1 and M2 MDM (CD14+

vs M1; 4.26 ± 2.78 vs 0.89 ± 0.82 *P* = 0.0002, *n* = 5 - 10 and CD14+ vs M2; 4.26 ± 2.78 vs 0.12 ± 0.09 < *P* = 0.0001 *n* = 5 - 10). The effect in MDM derived from COPD donors was similar at 91 and 99% reduction in M1 and M2 MDM respectively (3.95 ± 1.60 vs 0.37 ± 0.14 *P* = 0.0001, *n* = 5 CD14+ vs M1 and 3.95 ± 1.60 vs 0.05 ± 0.05 *P* = 0.0001 *n* = 5) (Figure 3.13).

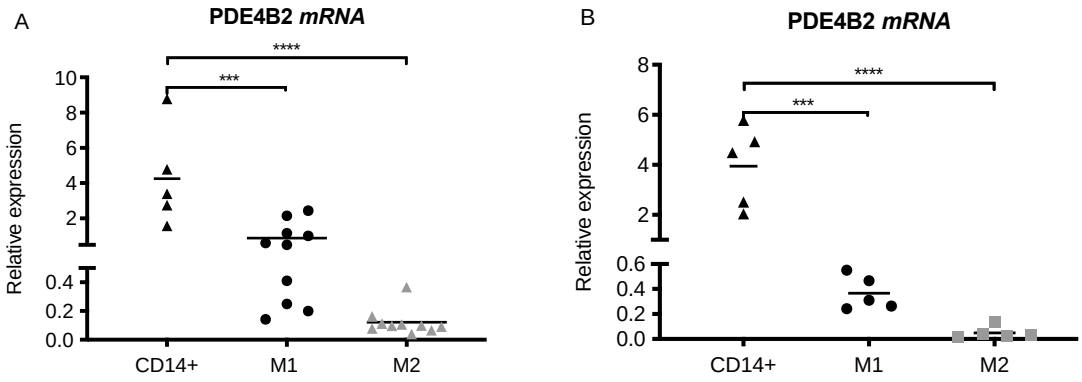



Figure 3.13 PDE4B2 expression in monocytes and MDM subsets from (A) healthy and (B) COPD donors

CD14+ monocytes were isolated from PBMCs from donors who were either healthy (A) or had COPD (B). A proportion of cells were used for RT-qPCR experiments after 3hrs culture in RPMI complete and represent the CD14+ cells shown. The balance of cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete for one day. Relative expression of PDE4B2 using the isoform-specific PDE4B2 probe was determined used RT-qPCR (*n* = 5-9). Mean shown. Statistical significance was determined using a one –

way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons. *** $P < 0.001$, **** $P < 0.0001$.

The expression of long PDE4B1 in monocytes derived from healthy individuals was not different in monocyte differentiation (

Figure 3.14) and was not evaluated in monocytes derived from COPD donors.

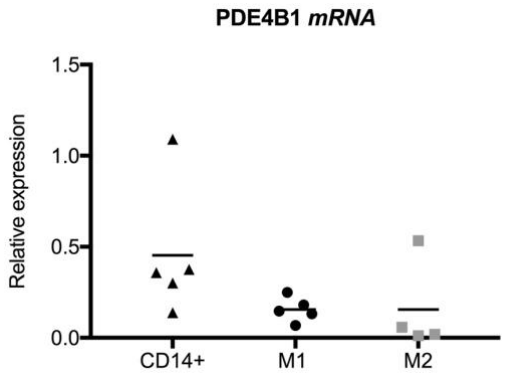



Figure 3.14 PDE4B1 expression in monocytes and MDM subsets from healthy donors

CD14+ monocytes were isolated from PBMCs from donors who were either healthy. A proportion of cells were used for RT-qPCR experiments after 3hrs culture in RPMI complete and represent the CD14+ cells shown. The balance of cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete for one day. Relative expression of PDE4B1 using the isoform-specific PDE4B1 probe was determined used RT-qPCR ($n$ = 5). Mean shown. Statistical significance was determined using a one – way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons.

Addressing the long PDE4B3, there was a 96% lower expression in this isoform in M1 MDM cells compared to levels seen in monocytes from healthy donors (CD14+ vs M1; 0.38 ± 0.37 vs 0.02 ± 0.01, $n$ = 5, $P$ < 0.05) (**Error! Reference source not found.**

While there appeared to be a comparable reduction in the expression of PDE4B3 in M2 macrophages, this did not reach significance (CD14+ vs M2; 0.38 ± 0.37 vs 0.03 ± 0.04, $P$ = 0.08, $N$ = 5). In cells derived from COPD donors, monocyte to macrophage differentiation was not associated with a change in PDE4B3 expression (0.09 ± 0.11 vs 0.03 ± 0.03, $P$ = 0.47, $n$ = 5 CD14+ vs M1 and 0.09 ± 0.11 vs 0.03 ± 0.05, $P$ = 0.60, $n$ = 5 CD14+ vs M2) (Figure 3.15B).

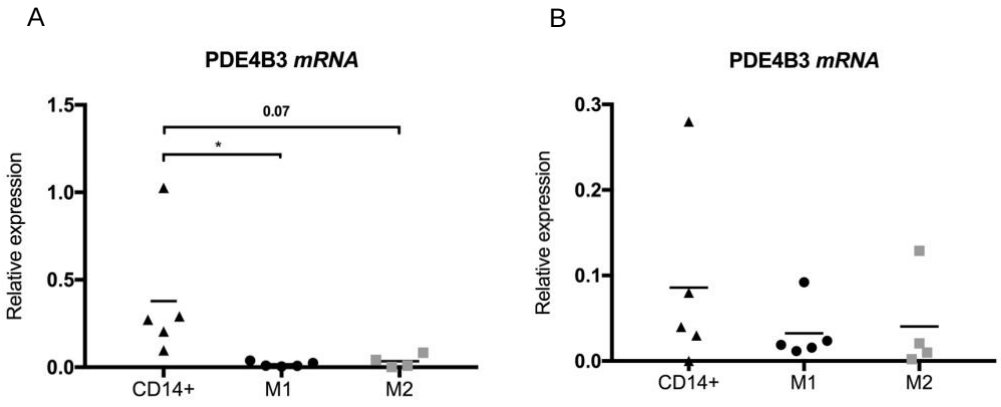



Figure 3.15 PDE4B3 expression in monocytes and MDM subsets from (A) healthy and (B) COPD donors

CD14+ monocytes were isolated from PBMCs from donors who were either healthy (A) or had COPD (B). A proportion of cells were used for RT-qPCR experiments after 3hrs culture in RPMI complete and represent the CD14+ cells shown. The balance of cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by

RPMI complete for one day. Relative expression of PDE4B3 using the isoform-specific PDE4B3 probe was determined used RT-qPCR ($n$ = 5). Mean shown. Statistical significance was determined using a one – way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons. *** $P < 0.001$, **** $P < 0.0001$.

In summary, monocyte to macrophage differentiation is associated with a reduction in PDE4B sub-family expression, which is attributable to a change in expression of the PDE4B2 isoform. This change is maintained in monocytes derived from COPD donors and occurs across both M1 and M2 subsets. Interestingly, the results contrast with those from Shepherd (M. C. Shepherd et al., 2004) who reported an increase in PDE4B2 expression in U937 cells differentiated to a macrophage phenotype – results that were reported to be similar to those obtained using peripheral monocytes.

### 3.4.2 PDE4A sub-family and isoforms

Using the pan-PDE4A probes, monocyte to macrophage differentiation was not associated with any change in PDE4A sub-family expression in cells derived from healthy donors (

Figure 3.16). In monocytes derived from COPD patients, PDE4A sub-family expression was reduced in M2 macrophages by 52% (CD14+ vs M2 macrophages; 4.90 ± 1.65 vs 2.36 ± 1.08, $P < 0.05$, $n$ = 5,) and trended towards a reduction in M1 macrophages (36%, 4.90 ± 1.65 vs 3.15 ± 0.96, monocytes vs M1, $P = 0.09$, $n$ = 5).

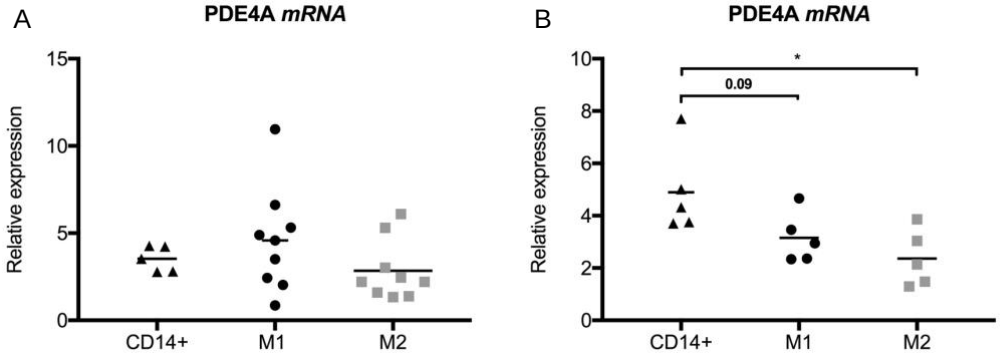



Figure 3.16 PDE4A sub-family expression in monocytes and MDM subsets from (A) healthy and (B) COPD donors

CD14+ monocytes were isolated from PBMCs from donors who were either healthy (A) or had COPD (B). A proportion of cells were used for RT-qPCR experiments after 3hrs culture in RPMI complete and represent the CD14+ cells shown. The balance of cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete for one day. Relative expression of PDE4A using the pan-PDE4A probe was determined used RT-qPCR ($n$ = 5-9). Mean shown. Statistical significance was determined using a one – way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons. * $P < 0.05$.

I next set out to determine which PDE4A isoforms might account for the change in PDE4A sub-family expression. Turning first to long PDE4A4, there was no change in the expression of this isoform in monocyte to M1 macrophage differentiation and similarly with monocyte to M2 macrophage differentiation in cells derived from either healthy or COPD donors (healthy; 0.20 ± 0.21 vs 0.06 ± 0.04, $P = 0.16$, $n = 5$ CD14+ vs M1 macrophages and 0.20 ± 0.21 vs 0.06 ± 0.03, $P = 0.17$, $n = 5$ CD14+ vs M2 macrophages; in COPD 0.20 ± 0.21 vs 0.04 ± 0.01,

*P* = 0.12, *n* = 5 CD14+ vs M1 macrophages and 0.20 ± 0.21 vs 0.06 ± 0.05, P = 0.17, *n* = 5, one-way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons) (Figure 3.17)

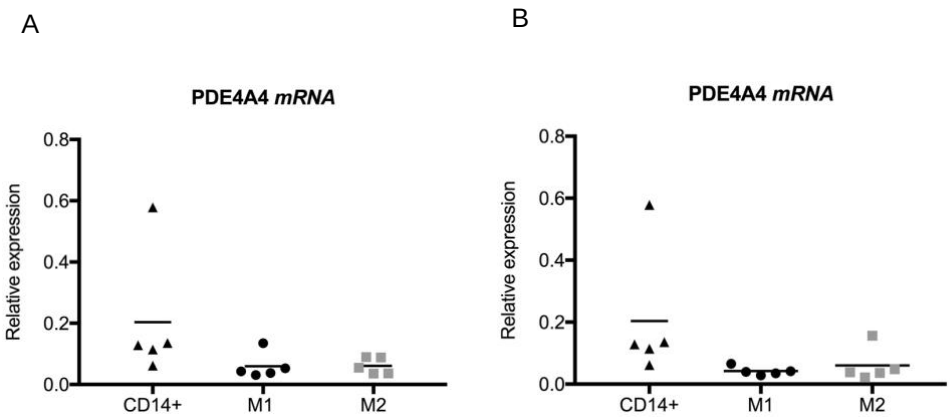



Figure 3.17 PDE4A4 expression in monocytes and MDM subsets from (A) healthy and (B) COPD donors

CD14+ monocytes were isolated from PBMCs from donors who were either healthy (A) or had COPD (B). A proportion of cells were used for RT-qPCR experiments after 3hrs culture in RPMI complete and represent the CD14+ cells shown. The balance of cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete for one day. Relative expression of PDE4A4 using the isoform specific PDE4A4 probe was determined used RT-qPCR (*n* = 5). Mean shown. Statistical significance was determined using a one – way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons.

In contrast to the expression of PDE4A4, there was a clear change in the expression of the dead short PDE4A7 isoform in monocyte to macrophage

differentiation. Indeed, in monocytes derived from healthy donors during differentiation to either M1 macrophages or to M2 macrophages, this was associated with, respectively, an 87% and an 84% reduction in PDE4A7 expression (0.42 ± 0.38 vs 0.06 ± 0.04, $P < 0.01$ $n = 5$ CD14+ vs M1 and 0.42 ± 0.38 vs 0.07 ± 0.04 $P < 0.01$ $n = 5$ CD14+ vs M2) (see below,

Figure 3.18). Similarly, in cells derived from COPD donors, PDE4A7 expression was reduced in monocyte to M1 macrophage differentiation and in monocyte to M2 macrophage differentiation by 72% and 79%, respectively (monocyte to M1; 0.22 ± 0.12 vs 0.06 ± 0.03 $P < 0.01$, $n = 5$ and monocyte to M2; 0.22 ± 0.12 vs 0.05 ± 0.04 $P < 0.01$, $n = 5$).

While the profiling of PDE4A7 expression in monocyte to macrophage differentiation highlights a consistent and profound change across multiple donors, the functional relevance of this change is uncertain as this ‘dead short’ isoform lacks both UCR regulatory regions and has a truncated catalytic site that renders it catalytically inactive. However, it is now appreciated that PDE4 isoforms can have important protein-protein interactions distinct to their ability to hydrolyse cAMP (H. W. Kim et al., 2010; Yarwood, Steele, Scotland, Houslay, & Bolger, 1999) including an ability to form signalling scaffold proteins (Houslay, 2010). Unusually amongst PDE4 isoforms, PDE4A7 arises from both 5’ and 3’ splicing that confers unique domains to PDE4A7 that may support a signalling/ scaffold function in macrophage / monocyte systems.

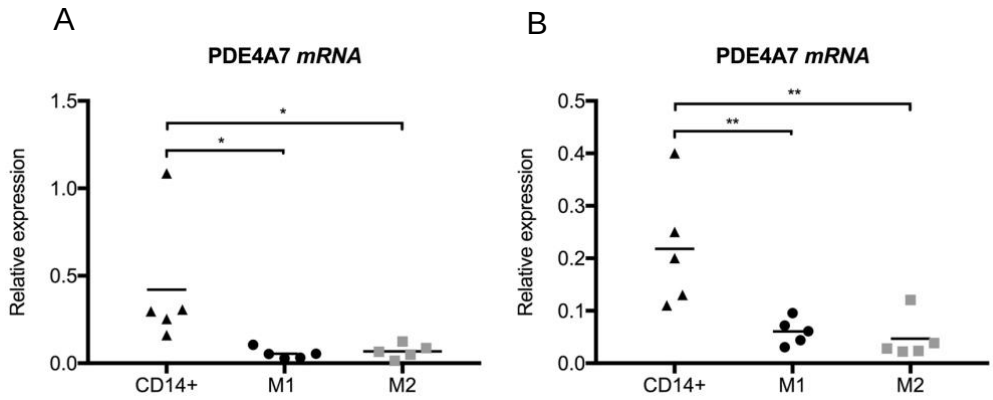



Figure 3.18 PDE4A7 expression in monocytes

and MDM subsets from (A) healthy and (B) COPD donors

CD14+ monocytes were isolated from PBMCs from donors who were either healthy (A) or had COPD (B). A proportion of cells were used for RT-qPCR experiments after 3hrs culture in RPMI complete and represent the CD14+ cells shown. The balance of cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete for one day. Relative expression of PDE4A7 using the isoform specific PDE4A7 probe was determined used RT-qPCR ($n$ = 5). Mean shown. Statistical significance was determined using a one – way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons. * $P < 0.05$, ** $P < 0.01$.

As described in Chapter 3.2.1, the expression of the short PDE4A1 isoform was near negligible and was not further defined in MDM.

Similarly to PDE4A1, long PDE4A8 expression was either very low in monocytes or absent altogether and in order to prioritise template, PDE4A8 expression was not assessed in cells from COPD donors. It was though possible to assess PDE4A8 using template from healthy donors and no change in its expression was observed in monocyte to macrophage differentiation (Figure 3.19).

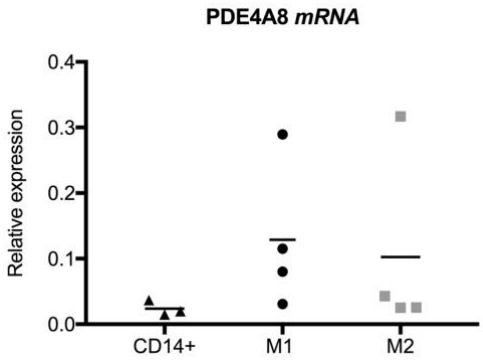



Figure 3.19 PDE4A8 expression in monocytes and macrophage subsets from healthy donors

CD14+ monocytes were isolated from PBMCs from donors who were either healthy. A proportion of cells were used for RT-qPCR experiments after 3hrs culture in RPMI complete and represent the CD14+ cells shown. The balance of cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete for one day. Relative expression of PDE4A8 using the isoform specific PDE4A8 probe was determined used RT-qPCR (*n* = 4). Mean shown. Statistical significance was determined using a one – way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons.

The expression of PDE4A10 was determined in MDM derived from donors with COPD but not those from healthy donors due to a limited amount of template. Both monocyte to M1 and monocyte to M2 macrophage differentiation was not associated with any change in PDE4A10 expression (CD14+ vs M1; 0.02 ± 0.006 vs 0.03 ± 0.02 $P$ = 0.30, $n$ = 5, and CD14+ vs M2 0.02 ± 0.006 vs 0.03 ± 0.01 $P$ = 0.19, $n$ = 5,) (see below, Figure 3.20). This is interesting as it contrasts with the findings of Shepherd and colleagues (M. C. Shepherd et al., 2004) who reported the presence of a species at 121kDa using immunoblotting techniques which they then further characterised as PDE4A10 using isoform-specific antisera and RT-PCR.

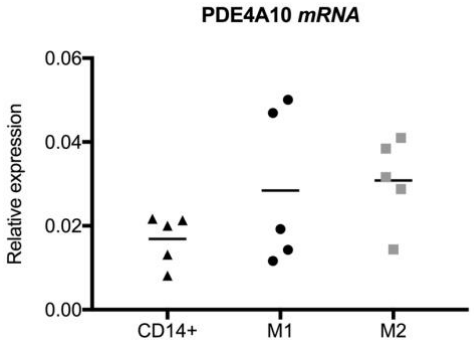



Figure 3.20 PDE4A10 expression in monocytes and MDM subsets from COPD donors

CD14+ monocytes were isolated from PBMCs from donors who had COPD. A proportion of cells were used for RT-qPCR experiments after 3hrs culture in RPMI complete and represent the CD14+ cells shown. The balance of cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete for one day. Relative expression of PDE4A10 using the isoform specific PDE4A10 probe was determined used RT-qPCR ($n$ = 5). Mean shown. Statistical

significance was determined using a one – way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons.

To summarise, in monocytes derived from COPD patients, but not those derived from healthy donors, there is a reduction in PDE4A sub-family expression in monocyte to M2 differentiation. This uncovers a disease specific related phenotype related to PDE4A. Profiling of PDE4A isoforms identifies a clear and consistent reduction of dead-short PDE4A7 in monocyte to macrophage differentiation, raising interest as to what its functional role could be.

### 3.4.3 PDE4D sub-family and isoforms

As described in Chapter 3.2, PDE4D sub-family expression was not determined in monocytes from COPD donors and so it was not possible to map the PDE4 expression upon monocyte to MDM differentiation in cells from COPD donors. In cells from healthy donors monocyte to M1 macrophage differentiation was associated with a 73% reduction in PDE4D sub-family expression (0.07 ± 0.03 vs 0.02 ± 0.01, $P < 0.01$, $n = 5–9$). In monocyte to M2 macrophage differentiation, there was a non-significant 54% reduction in PDE4D expression (0.07 ± 0.03 vs 0.03 ± 0.04, $P = 0.07$, $n = 5–9$) (Figure 3.21).

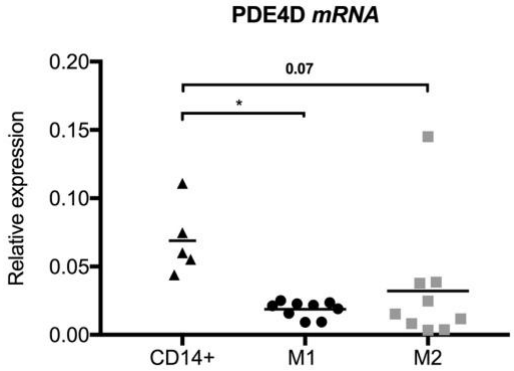



Figure 3.21 PDE4D sub-family expression in monocytes and MDM subsets from healthy donors

CD14+ monocytes were isolated from PBMCs from donors who were healthy. A proportion of cells were used for RT-qPCR experiments after 3hrs culture in RPMI complete and represent the CD14+ cells shown. The balance of cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete for one day. Relative expression of PDE4D using the pan-PDE4D probe was determined used RT-qPCR ($n$ = 5-9). Mean shown. Statistical significance was determined using a one – way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons, * $P < 0.05$.

Turning to the long PDE4D isoforms, I sought to prioritise the use of available template by evaluating the expression of long PDE4D5 and PDE4D7. Shepherd and colleagues (M. C. Shepherd et al., 2004) reported that PDE4D3 and PDE4D5 accounted for the major PDE4 activity in U937 monocytes and that both long forms were downregulated in the differentiation to macrophages. However, as shown in Figure 3.6, PDE4D3 was not detected in monocytes from 10 separate donors. Also and as described in Chapter 3.2.3, since the publication of the study

by Shepherd (M. C. Shepherd et al., 2004), a number of new isoforms have since been discovered, including PDE4D8 and PDE4D9 which have been shown to have similar molecular masses and to co-migrate with PDE4D3 on SDS-PAGE at 95kDa (Levallet et al., 2007; Richter et al., 2005). Therefore a new appraisal of the expression of PDE4D3 in myeloid cells is needed as is an assessment of the identity of the species that migrates at 95kDa on SDS-PAGE. The expression of PDE4D5 is described below, as is the expression of PDE4D7, which has been strongly implicated in disease (Henderson et al., 2014) and was therefore considered important to assess.

In both cells from healthy and COPD donors, there was a trend toward reduced expression of long PDE4D5 in monocyte to macrophage differentiation that was similar across MDM subsets and across donor groups. In cells from healthy donors, there was a 93% and 90% trend toward reduction in respectively monocyte to M1 macrophage and monocyte to M2 macrophages differentiation (0.31 ± 0.43 vs 0.02 ± 0.02 $P$ = 0.11, $n$ = 5 – 6 and 0.31 ± 0.43 vs 0.03 ± 0.03 $P$ = 0.12, $n$ = 5). In cells derived from COPD the comparable values were 97% and 96% - similarly not significant (0.07 ± 0.09 vs 0.01 ± 0.02 $P$ = 0.05 $n$ = 5 and 0.07 ± 0.09 vs 0.01 ± 0.02 $P$ = 0.05 $n$ =5).

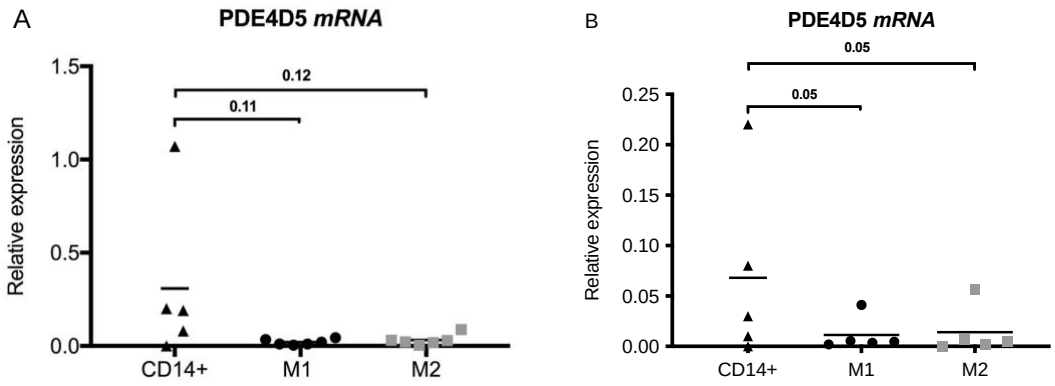



Figure 3.22 PDE4D5 expression in monocytes and MDM from (A) healthy and (B) COPD donors

CD14+ monocytes were isolated from PBMCs from donors who were healthy (A) and those that had COPD (B). A proportion of cells were used for RT-qPCR experiments after 3hrs culture in RPMI complete and represent the CD14+ cells shown. The balance of cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete for one day. Relative expression of PDE4D5 using the isoform-specific PDE4D5 probe was determined used RT-qPCR ($n$ = 5-9). Mean shown. Statistical significance was determined using a one – way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons

Turning to PDE4D7, in cells derived from COPD donors, monocyte to macrophage subset differentiation is associated with lower expression of PDE4D7. There was a 92% and 89% reduction in PDE4D7 expression in respectively monocyte to M1 and monocyte to M2 macrophages (0.24 ± 0.07 vs 0.05 ± 0.03, $P$ < 0.001, $n$ = 4–5 and 0.24 ± 0.07 vs 0.06 ± 0.05, $n$ = 4-5, $P$ <0.001)

(Figure 3.23). In cells from healthy donors, there was a non-significant trend toward a similar magnitude reduction at 83% and 78% in respectively monocyte to M1 and monocyte to M2 macrophages; (0.48 ± 0.50 vs 0.08 ± 0.03, *P* = 0.05, *n* = 5 and 0.48 ± 0.50 vs 0.11 ± 0.05, *P* = 0.07, *n* = 5, using a one–way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons).

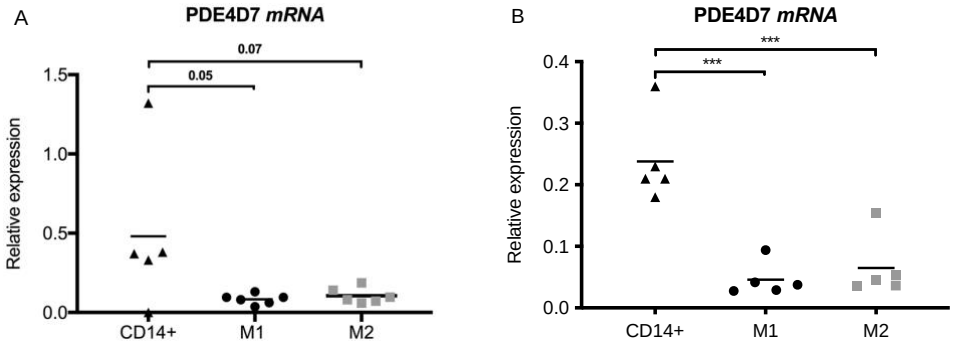



Figure 3.23 PDE4D7 expression in monocytes

and MDM subsets from (A) healthy and (B) COPD donors

CD14+ monocytes were isolated from PBMCs from donors who were healthy (A) and those that had COPD (B). A proportion of cells were used for RT-qPCR experiments after 3hrs culture in RPMI complete and represent the CD14+ cells shown. The balance of cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete for one day. Relative expression of PDE4D7 using the isoform-specific PDE4D7 probe was determined used RT-qPCR (*n* = 5-9). Mean shown. Statistical significance was determined using a one – way ANOVA with CD14+ as control and a Dunnett test for multiple comparisons. *** *P* < 0.001.

PDE4D7 was first cloned and characterised in human lung and to a lesser extent also kidney and brain tissue (Daguang Wang et al., 2003). Long PDE4D7 expression has been associated with vascular smooth muscle contractility (Houslay, Baillie, & Maurice, 2007) as well as ischaemic stroke (Gretarsdottir et al., 2003), but to our knowledge, its expression has not been reported before in monocytes and macrophages. Recently, PDE4D7 has been proposed as a novel marker of androgen insensitive prostate cancer (Henderson et al., 2014). This insight - combined with pre-clinical studies that suggest tumour associated macrophages promote prostate cancer cell proliferation and migration (Maolake et al., 2017; Nonomura et al., 2011) - may help fuel approaches as to whether PDE4D7-mediated control of cAMP signaling can be used for therapeutic gain.

## 3.5 PDE4 expression across MDM subsets

Macrophages fashion a response to environmental cues according to their activation states. For example, macrophages exposed to the cytokine IFN-γ have an enhanced response to the bacterial antigen LPS (J. Y. Lee & Sullivan, 2001; Mosser & Edwards, 2008).

It has been proposed that macrophage PDE4B2 is an important regulator of the inflammatory response (Jin & Conti, 2002; Jin et al., 2005; Ma et al., 1999; Wang et al., 1999) and that macrophages that highly express PDE4B2 may help drive the inflammatory response (discussed further in Chapter 1.6.3). I next set out to obtain experimental data to help address this hypothesis.

Starting with PDE4 sub-family expression using the pan-sub-family probes, I compared the expression of PDE4A, PDE4B and PDE4D across M1 and M2 macrophages in cells derived from donors who were healthy and those with COPD. A two–way ANOVA was used for statistical analysis using Tukey test for multiple comparisons. As shown in Figure 3.24, I observed no significant differences in either PDE4A, PDE4B or PDE4D sub-family expression between either macrophage subsets or between healthy and COPD donor groups

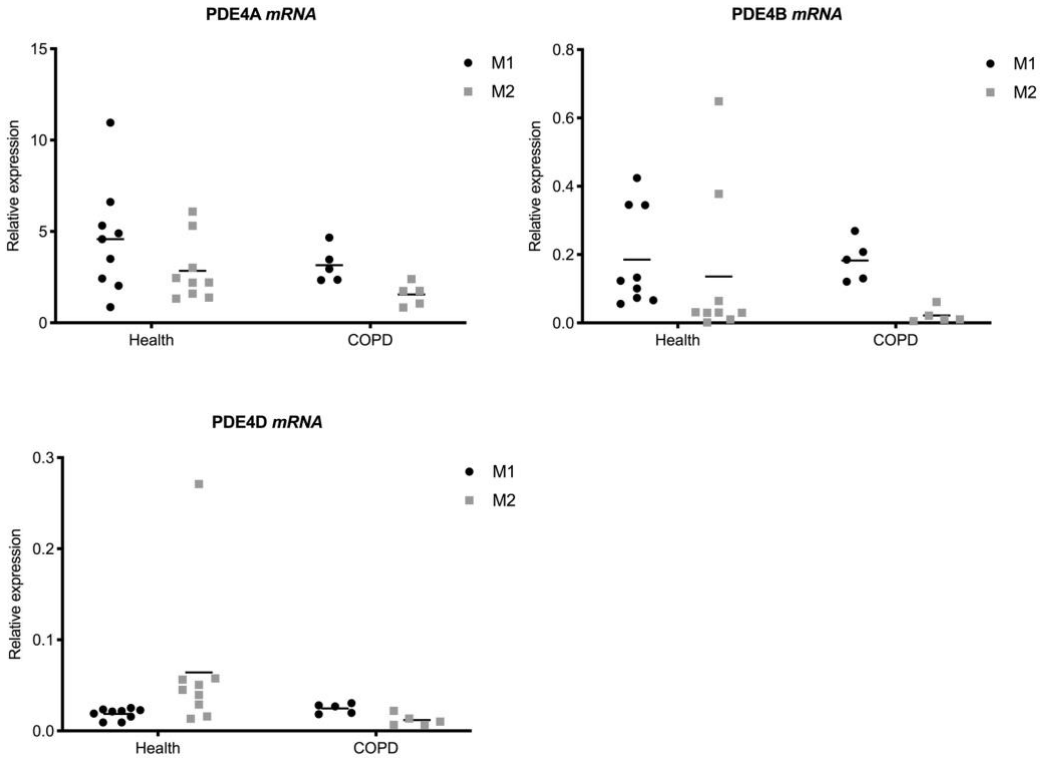



Figure 3.24 PDE4 sub-family expression in monocytes and MDM subsets

CD14+ monocytes were isolated from PBMCs from donors who were healthy and those that had COPD and differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml (circles) or M2 macrophages with M-CSF 10 ng/ml + IL-4 10 ng/ml (squares) for five

days, followed by RPMI complete for one day. Relative expression of PDE4A /B and D using the pan-PDE4 probes was determined used RT-qPCR (*n* = 5-9). Mean shown. Statistical significance was determined using a two – way ANOVA and Tukey test for multiple comparisons.

I then turned to evaluate the expression of eight selected PDE4 isoforms from PDE4A, PDE4B and PDE4D sub-families. Only long PDE4A8 and short PDE4B2 and not any of PDE4A4, PDE4A7, PDE4B1, PDE4B3, PDE4D5 or PDE4D7 had an expression pattern that was different across either macrophage subsets or across donor groups (Figure 3.25). In turn, macrophage PDE4A8 and PDE4B2 expression profiles are discussed below.

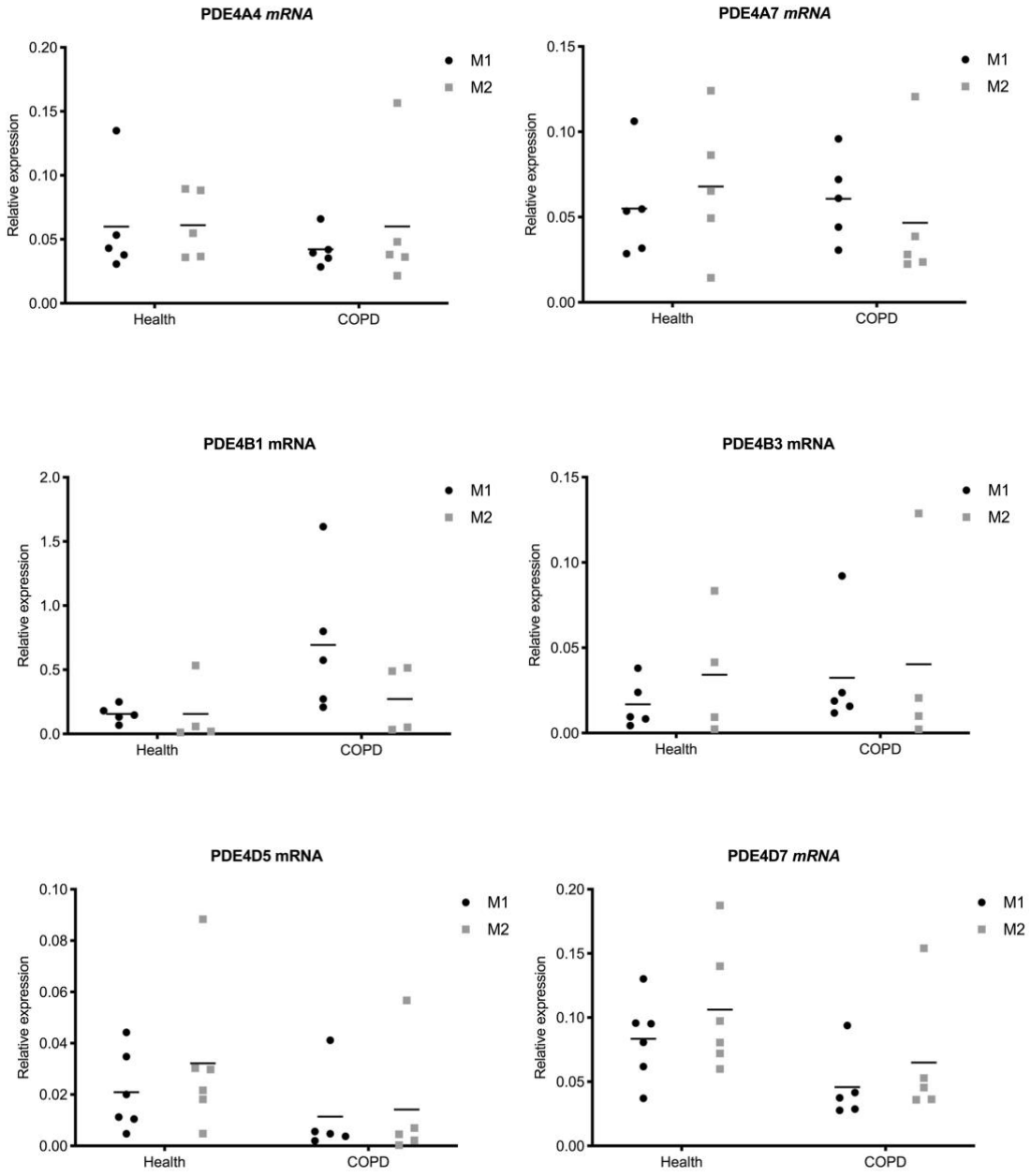



Figure 3.25 Selected PDE4 isoform expression in MDM subsets from healthy and COPD donors

CD14+ monocytes were isolated from PBMCs from donors who were healthy and those that had COPD and differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml (circles) or M2 macrophages with M-CSF 10 ng/ml + IL-4 10 ng/ml (squares) for five days, followed by RPMI complete for one day. Relative expression of selected PDE4 isoforms (as shown) using isoform-specific probes was

determined used RT-qPCR (*n* = 5-9). Mean shown. Statistical significance was determined using a two – way ANOVA and Tukey test for multiple comparisons.

Expression of PDE4A8 is highest in M1 macrophages derived from COPD donors (Figure 3.26). It is x4.5 higher in M1 macrophages from COPD donors when compared to M1 macrophages from healthy donors (0.57 ± 0.23 vs 0.13 ± 0.11 $P < 0.01$ $n$ = 4-5) and x2.2 higher when compared to M2 macrophages from COPD donors (0.57 ± 0.23 vs 0.26 ± 0.10, $P < 0.05$ $n$ = 5). This points toward a disease-and macrophage-subset specific difference in PDE4A8 isoform expression. The result is intriguing as one inference from these data might be that the PDE4A8 promoter is responsive to conditions that might favour inflammation – namely the pro-inflammatory cytokine milieu that might be expected to exist in the blood of COPD patients, as well as growth conditions that favoured M1 subset differentiation. However, this simplistic account is, seemingly, at odds with the experimental observation that monocyte challenge with LPS did not lead to induction of PDE4A8 expression (Figure 3.3).

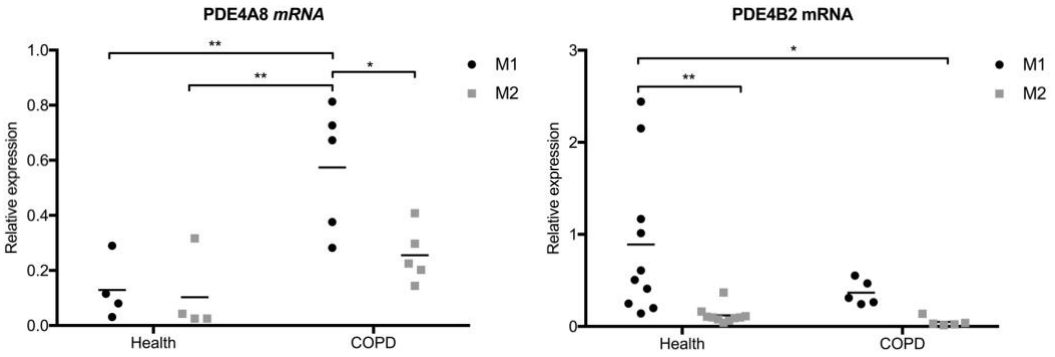



Figure 3.26 PDE4B2 and PDE4A8 isoform expression in MDM subsets from healthy and COPD donors

CD14+ monocytes were isolated from PBMCs from donors who were healthy and those that had COPD and differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml (circles) or M2 macrophages with M-CSF 10 ng/ml + IL-4 10 ng/ml (squares) for five days, followed by RPMI complete for one day. Relative expression of PDE4A8 and PDE4B2 using isoform-specific probes was determined used RT-qPCR ($n$ = 5-10). Mean shown. Statistical significance was determined using a two – way ANOVA and Tukey test for multiple comparisons, * $P < 0.05$, ** $P < 0.01$.

As expected, PDE4B2 expression was markedly higher in M1 macrophages compared to M2 macrophages (Figure 3.26). In cells derived from healthy donors, PDE4B2 expression was x7 higher in M1 macrophages compared to M2 macrophages (0.89 ± 0.82 vs 0.12 ± 0.09, $P < 0.01$ $n = 10$) and x18 higher than in M2 macrophages derived from COPD donors (0.89 ± 0.82 vs 0.05 ± 0.05, $P < 0.05$ $n = 5$ -10).

Perhaps less expectedly, PDE4B2 expression in M1 macrophages derived from COPD donors was not higher than in cells from healthy donors (M1 healthy vs COPD donors; 0.89 ± 0.82 vs 0.37 ± 0.14, $P = 0.23$ $n = 10$).

In summary, out of eight PDE4 isoform expression profiles that were evaluated across macrophage subsets and both donor groups, only two namely PDE4A8 and PDE4B2, showed any differences in isoform expression. Furthermore, I observed no difference in the expression of PDE4A4 in MDM derived from

healthy and COPD donors (Figure 3.25). This latter observation is contrary to the findings of Barber and colleagues who found that PDE4A4 expression was upregulated in lung macrophages in individuals with COPD when compared to those without (Barber et al., 2004). Two important methodology differences may go some way to account for why I did not observe a difference in PDE4A4 expression between healthy and COPD individuals when Barber and colleagues did. Firstly, Barber and colleagues reported their observation on PDE4A4 expression in smokers and did not employ a non-smoker control group in those who donated lung macrophages. In the present study, donors with COPD were all former smokers and those within the healthy group were never smokers. As such, it is not known if being a current smoker and having COPD, combines to dysregulate PDE4A4 expression in a way that wouldn't with a past smoking history. Secondly, whereas Barber and colleagues used lung macrophages obtained through bronchoalveolar lavage, I used a monocyte-derived macrophage model which it could be proposed is less representative of the lung tissue PDE4 expression profiles.

## 3.6 PDE4 expression in MDM

As critical components of the innate immune response, macrophages are highly adapted to mount a robust response to the bacterial endotoxin, lipopolysaccharide (LPS). In line with this response, it has been extensively shown that LPS leads to a marked induction of PDE4B expression in human monocytes (Ma et al., 1999; Wang et al., 1999) and mouse macrophages (Jin & Conti, 2002; Jin et al., 2005), but it is less well understood if this induction occurs uniformly in macrophages polarised to different subsets or if this induction is

dysregulated in COPD. I next set out to obtain data to help address this knowledge gap, which I describe below.

### 3.6.1 PDE4A isoform expression

M1 and M2 MDM derived from both healthy and COPD donors, were cultured for 3 hours with LPS (50 ng/ml) or without. In cells derived from healthy donors, LPS led to a 58% and 51% reduction in PDE4A sub-family expression in respectively M1 (4.58 ± 2.99 vs 1.94 ± 1.87 mean ± SD, $P < 0.05$ $n = 9$) and M2 MDM (2.84 ± 1.72 vs 1.40 ± 0.63, $P < 0.05$ $n = 9$,) (Figure 3.27). In cells derived from COPD donors and polarised to a M1 subset, there was a similar reduction of PDE4A sub-family expression (51% reduction; 3.15 ± 0.96 vs 1.55 ± 0.62 $P < 0.05$ $n = 5$) but only a trend reduction in cells polarised to a M2 subset (35% reduction, 2.36 ± 1.08 vs 1.54 ± 0.84, $P = 0.65$ $n = 5$).

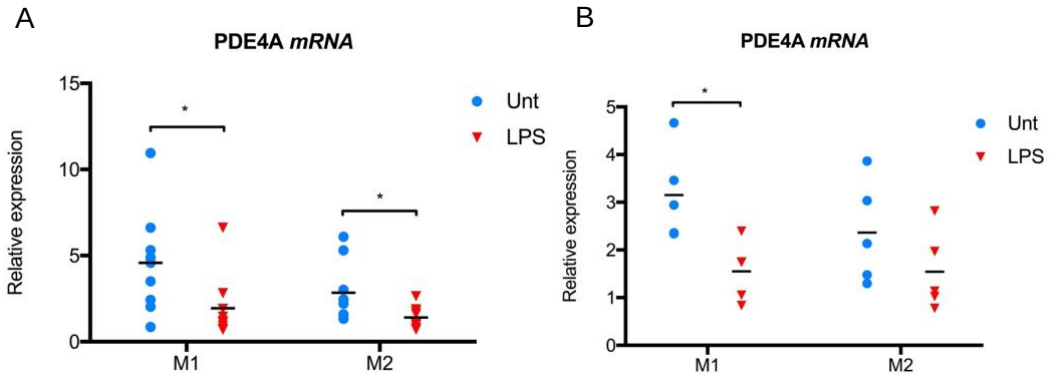



Figure 3.27 PDE4A sub-family expression in MDM subsets from (A) healthy and (B) COPD donors

CD14+ monocytes were obtained from donors who were either healthy (A) or had COPD (B) and differentiated to monocyte derived macrophages (MDM) in RPMI complete media supplemented with either GM-CSF 20

ng/ml + IFN-γ 10 ng/ml (M1) or M-CSF 10 ng/ml + IL-4 10 ng/ml (M2). The cells were cultured for five days in media supplemented with CSF, before culture for one day in media without. RT-qPCR and a pan-PDE4A probe was used to detect the expression of PDE4A sub-family expression, both without LPS 50ng/ml 3hrs (shown here in blue) and with (red). Mean shown. Statistical analysis was performed using a 2-tailed Student's t-test, * $P < 0.05$ ($n = 5 - 9$).

Next, I set out to evaluate which PDE4A isoforms might account for the LPS induced downregulation of macrophage PDE4A, focusing on those isoforms that had been previously detected in monocytes (Figure 3.3).

LPS had no effect on the expression of either PDE4A4 or PDE4A7 in either M1 or M2 subsets, in cells from either healthy or COPD donors (Figure 3.28). However, LPS did lead to a 58% reduction in long PDE4A8 expression in both M1 (0.57 ± 0.23 vs 0.24 ± 0.12, $P < 0.05$, $n = 5$) and M2 MDM (0.26 ± 0.10 vs 0.11 ± 0.07, $P < 0.05$, $n = 5$) derived from COPD donors. In cells from healthy donors, there was no LPS-effect on PDE4A8 expression. Interestingly, LPS also had no effect on monocyte PDE4A8 expression, which appears to point toward a disease specific response in the promoter region for macrophage PDE4A8.

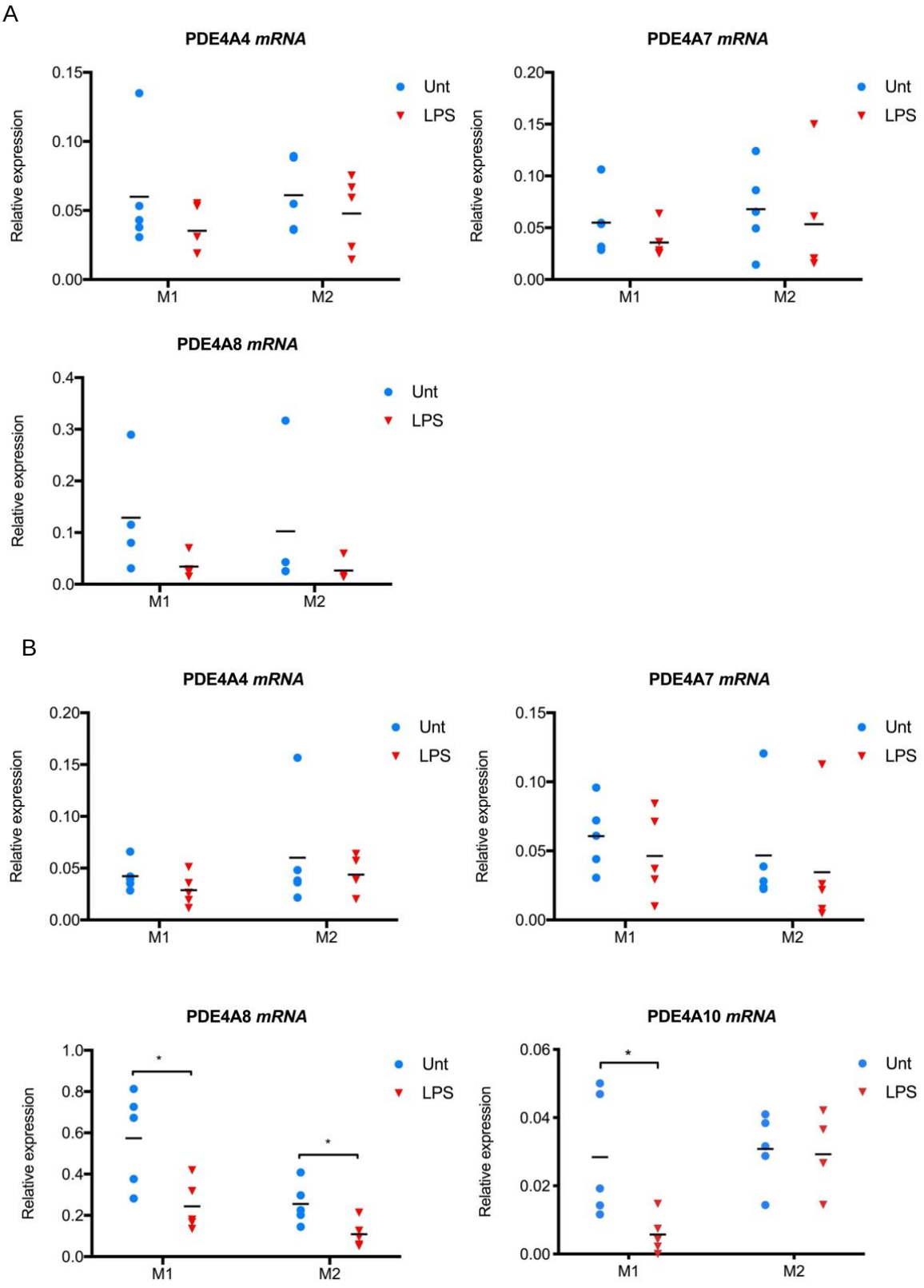



Figure 3.28 Selective PDE4A isoform expression in MDM subsets from (A) healthy and (B) COPD donors

CD14+ monocytes were obtained from donors who were either healthy (A) or had COPD (B) and differentiated to monocyte derived macrophages (MDM) in RPMI complete media supplemented with either GM-CSF 20 ng/ml + IFN-γ 10 ng/ml (M1) or M-CSF 10 ng/ml + IL-4 10 ng/ml (M2). The cells were cultured for five days in media supplemented with CSF, before culture for one day in media without. RT-qPCR and isoform selective probes were used to detect the expression of selected PDE4A isoforms as shown, both without LPS 50ng/ml 3hrs (shown here in blue) and with (red). Mean shown. Statistical analysis was performed using a 2-tailed Student's t-test, * $P$ <0.05 ($n$ = 5 - 9).

Interestingly, as was observed in monocytes derived from healthy but not COPD donors, LPS treatment led to reduced PDE4A10 expression in M1 macrophages derived from donors with COPD (80% reduction unt vs LPS 0.028 ± 0.019 vs 0.0057 ± 0.0057, $P$ <0.05, $n$ = 5). PDE4A10 expression was not determined in cells derived from healthy donors, due to a limited availability of template.

Hence, in both monocytes and MDM subsets, LPS leads to a reduction in PDE4A sub-family expression. Using isoform specific-probes it was not possible to clearly identify which, if any, of the isoforms evaluated accounted for this change but data were obtained suggesting a possible role for either PDE4A8 and/or PDE4A10.

### 3.6.2 PDE4B isoform expression

LPS is a potent inducer of PDE4B sub-family expression but interestingly this was only demonstrated in MDM polarised to a M1 subset and not MDM polarised to M2 subset. LPS led a x31 increase in PDE4B expression in M1 macrophages

derived from healthy donors (0.19 ± 0.14 vs 5.76 ± 7.62, $P < 0.05$, $n = 9$) and a x35 increased expression in M1 macrophages from COPD donors (0.18 ± 0.06 vs 6.43 ± 3.42, $P < 0.01$, $n = 5$) (Figure 3.29). The magnitude of this induction was not significantly different between healthy and COPD MDM groups.

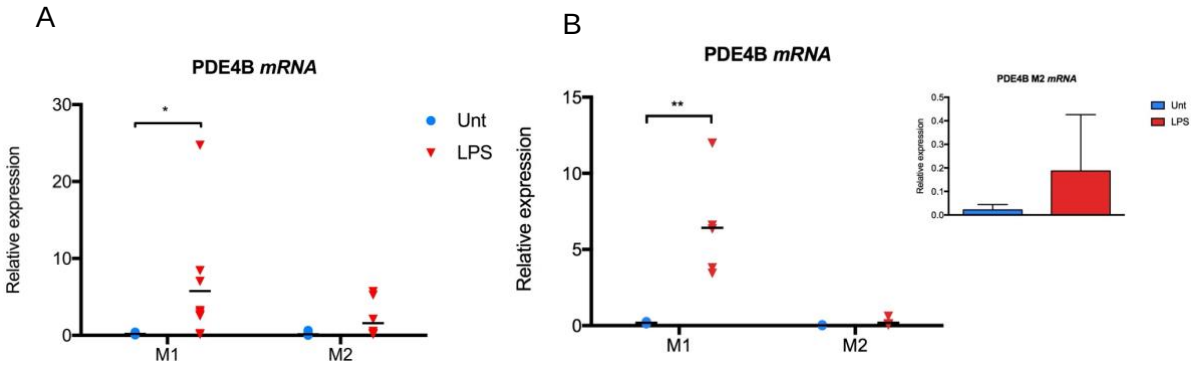



Figure 3.29 PDE4B sub-family expression in MDM subsets from (A) healthy and (B) COPD donors

CD14+ monocytes were obtained from donors who were either healthy (A) or had COPD (B) and differentiated to monocyte derived macrophages (MDM) in RPMI complete media supplemented with either GM-CSF 20 ng/ml + IFN-γ 10 ng/ml (M1) or M-CSF 10 ng/ml + IL-4 10 ng/ml (M2). The cells were cultured for five days in media supplemented with CSF, before culture for one day in media without. RT-qPCR and the pan-PDE4B probe was used to detect the expression of PDE4B, both without LPS 50ng/ml 3hrs (shown here in blue) and with (red). Mean shown. Statistical analysis was performed using a 2-tailed Student's t-test, * $P$ <0.05, ** $P$ <0.01 ($n$ = 5 - 9).

In MDM, polarised towards a M2 subset, there was a non-significant trend toward LPS-induction of PDE4B sub-family expression across both donor groups

(healthy donor group (x12 increase); 0.14 ± 0.23 vs 1.59 ± 2.28 $P = 0.07$ $n = 9$ and COPD donor group (x9 increase); 0.02 ± 0.02 vs 0.19 ± 0.24, $P = 0.16$ $n = 5$), (Figure 3.29).

Using PDE4B isoform-specific probes, it was possible to deduce that LPS induction of PDE4B sub-family expression is accounted for by the change in short PDE4B2 (Figure 3.30) and not by changes in the expression of either of the long isoforms PDE4B1 and PDE4B3 (and PDE4B5 which was evaluated in macrophages from COPD donors only, due to a need to prioritise the use of template from healthy donors) (see below Figure 3.31).

In MDM from healthy donors, LPS led to a x17 increase in PDE4B2 expression in M1 cells (0.89 ± 0.82 vs 15.02 ± 7.37, $P < 0.0001$, $n = 10$) and a x15 increase in M2 cells (0.12 ± 0.09 vs 1.82 ± 2.50, $P < 0.05$, $n = 10$) (Figure 3.30). Interestingly, whilst the magnitude of induction was similar across M1 and M2 subsets in cells derived from healthy donors, this was not the case in cells derived from COPD donors. In M1 MDM from COPD donors, LPS led to a x32 increase in PDE4B2 expression (0.37 ± 0.14 vs 11.90 ± 4.08, $P < 0.001$, $n = 5$) whereas in M2 macrophages there was a non-significant trend toward a x9 induction (0.05 ± 0.05 vs 0.45 ± 0.42, $P = 0.07$, $n = 5$).

It may be observed that whilst PDE4B2 expression is reduced in monocyte to macrophage differentiation (Figure 3.13), the PDE4B2 promoter region still retains the ability to significantly up-regulate PDE4B2 expression in response to engagement with LPS-activated TLR signaling.

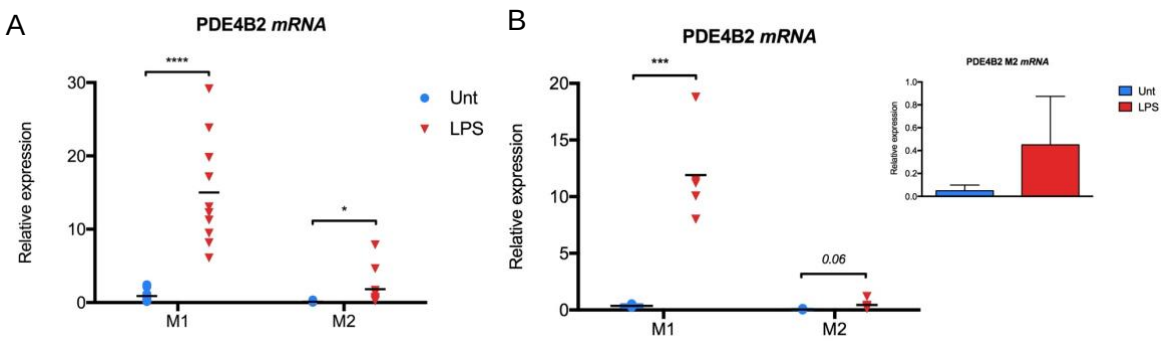



Figure 3.30 PDE4B2 expression in MDM derived from (A) healthy and (B) COPD donors

CD14+ monocytes were obtained from donors who were either healthy (A) or had COPD (B) and differentiated to monocyte derived macrophages (MDM) in RPMI complete media supplemented with either GM-CSF 20 ng/ml + IFN-γ 10 ng/ml (M1) or M-CSF 10 ng/ml + IL-4 10 ng/ml (M2). The cells were cultured for five days in media supplemented with CSF, before culture for one day in media without. RT-qPCR and an isoform-selective probe was used to detect the expression of PDE4B2 both without LPS 50ng/ml for 3h (shown here in blue) and with (red). Mean shown. LPS led to a marked induction of PDE4B2 expression across both A and B groups and both MDM subsets except in M2 MDMs derived from COPD donors, which trended toward induction but did not reach significance (inset panel, mean and SD shown). Statistics using 2-tailed Student's t-test, * $P$ <0.05, *** $P$ < 0.001, **** $P$ < 0.0001 * ($n$ = 5 - 10).

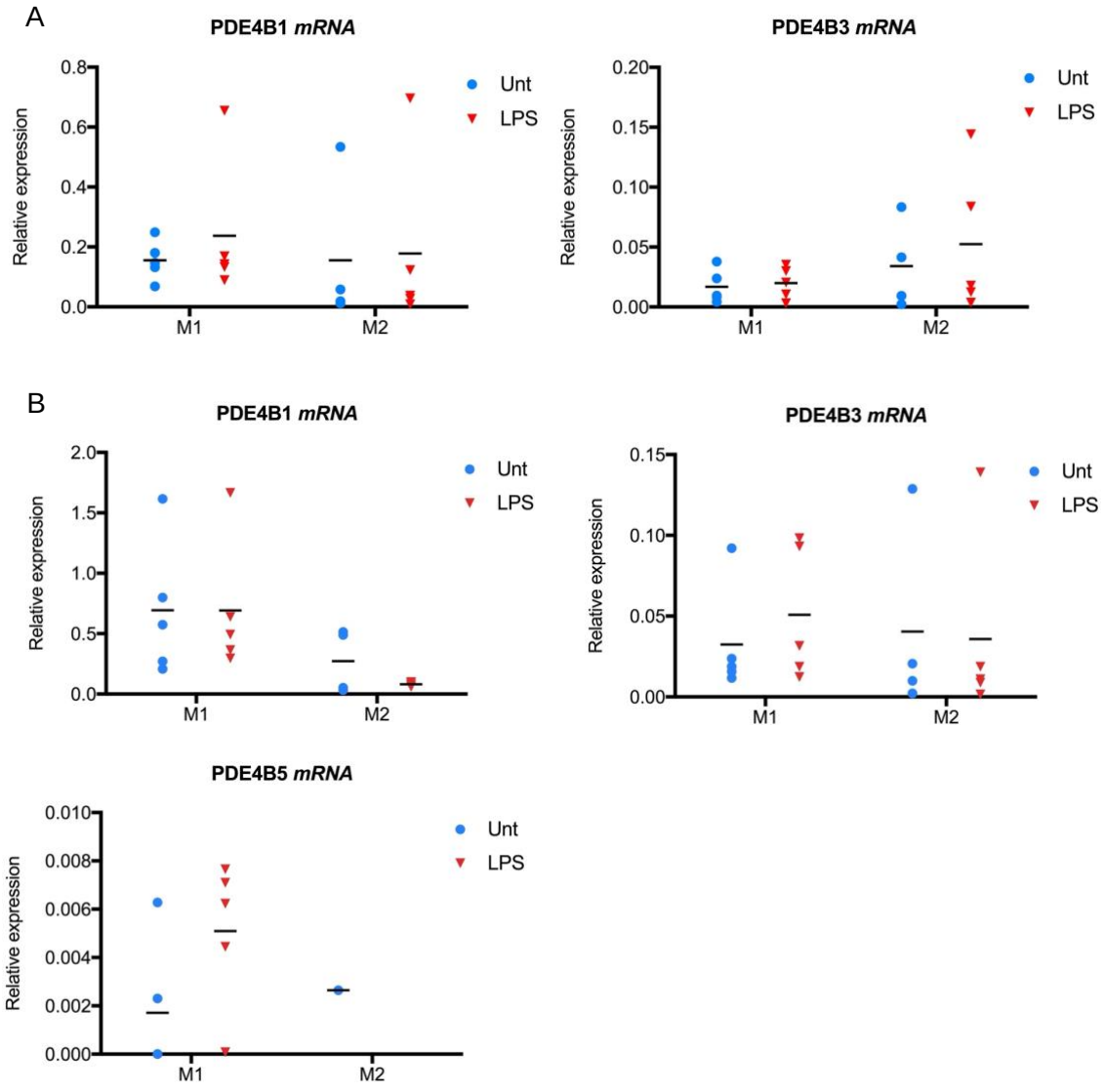



Figure 3.31 Selective PDE4B isoform expression in MDMs derived from (A) healthy and (B) COPD donors

CD14+ monocytes were obtained from donors who were either healthy (A) or had COPD (B) and differentiated to monocyte derived macrophages (MDM) in RPMI complete media supplemented with either GM-CSF 20 ng/ml + IFN-γ 10 ng/ml (M1) or M-CSF 10 ng/ml + IL-4 10 ng/ml (M2). The cells were cultured for five days in media supplemented with CSF, before culture for one day in media without. RT-qPCR and isoform selective probes were used to detect the expression of selected PDE4B isoforms as shown, both without LPS 50ng/ml for 3h (shown here

in blue) and with (red). Due to limited available template, it was not possible to assess PDE4B5 expression in MDMs from healthy donors. Mean shown. LPS had no effect on selected PDE4B isoform expression, using 2-tailed Student's t-test (*n* = 5).

### 3.6.3 PDE4D isoform expression

LPS led to a modest increase of PDE4D sub-family expression in M1 macrophages derived from COPD donors (x2; unt vs LPS; 0.02 ± 0.01 vs 0.05 ± 0.01, *P* <0.05 *n* = 4,) (Figure 3.32) but had no effect on M2 macrophages derived from the same donor group (x1.1; 0.012 ± 0.0064 vs 0.013 ± 0.0091 vs *P* = 0.80, *n* = 5). In MDM from healthy donors, LPS had no effect in either cells polarised to a M1 (x3.4; 0.02 ± 0.006 vs 0.06 ± 0.08, *P* = 0.10, *n* = 9) or M2 subset (0.03 ± 0.04 vs 0.025 ± 0.021, *P* = 0.67, *n* = 9)

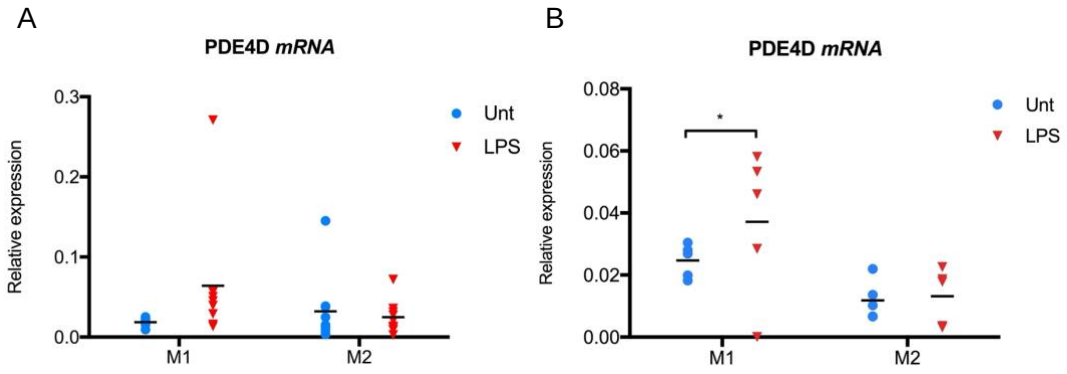
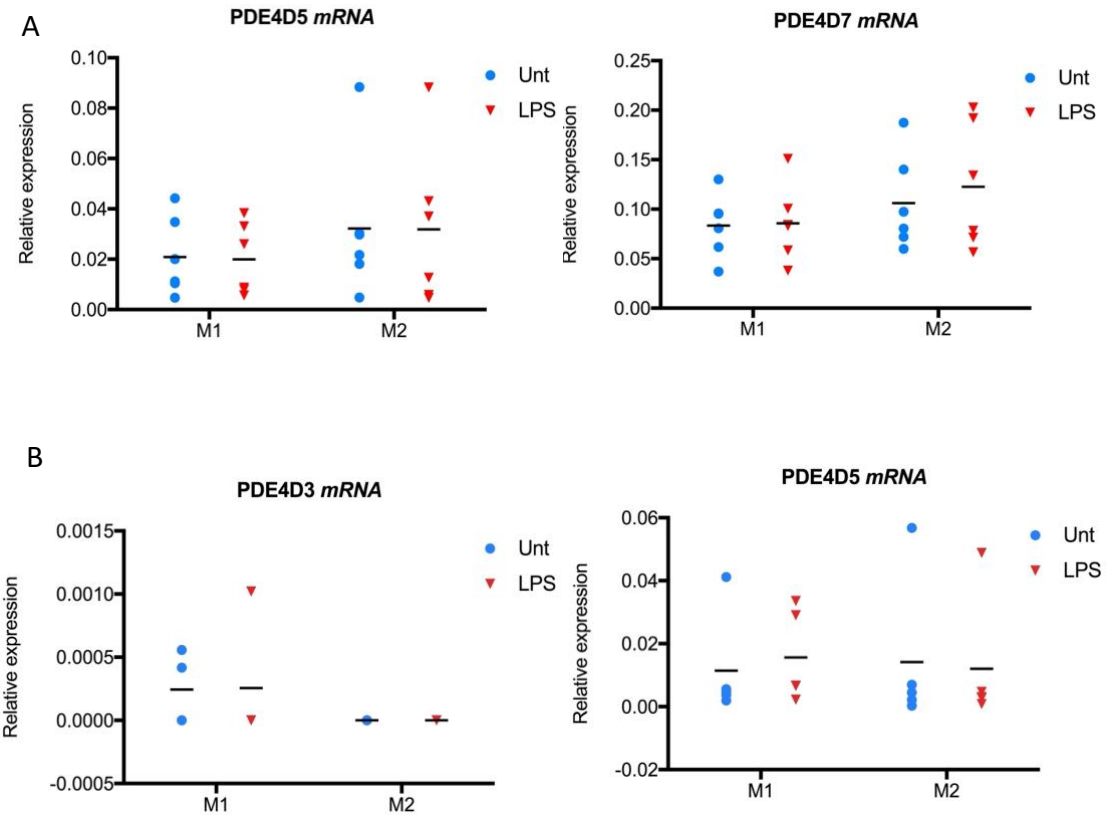



Figure 3.32 PDE4D sub-family expression in MDM subsets from (A) healthy and (B) COPD donors

CD14+ monocytes were obtained from donors who were either healthy (A) or had COPD (B) and differentiated to monocyte derived macrophages (MDM) in RPMI complete media supplemented with either

GM-CSF 20 ng/ml + IFN-γ 10 ng/ml (M1) or M-CSF 10 ng/ml + IL-4 10 ng/ml (M2). The cells were cultured for five days in media supplemented with CSF, before culture for one day in media without. RT-qPCR and the pan-PDE4D sub-family probe was used to detect the expression of PDE4D, both without LPS 50ng/ml for 3h (shown here in blue) and with (red). Mean shown. Statistical analysis using 2-tailed Student's t-test (*n* = 5 - 9).

Turning to the expression of long PDE4D isoforms, long PDE4D5 which I have detected in both monocytes and MDM, demonstrated no significant changes with LPS challenge in either M1 or M2 macrophages from healthy donors (M1; *P* = 0.91 *n* = 6 and M2 *P* = 0.98 *n* = 6, data abbreviated) or M1 or M2 macrophages from COPD donors (M1; *P* = 0.68, *n* = 5 and M2; *P* = 0.89 *n* = 5) (Figure 3.33).



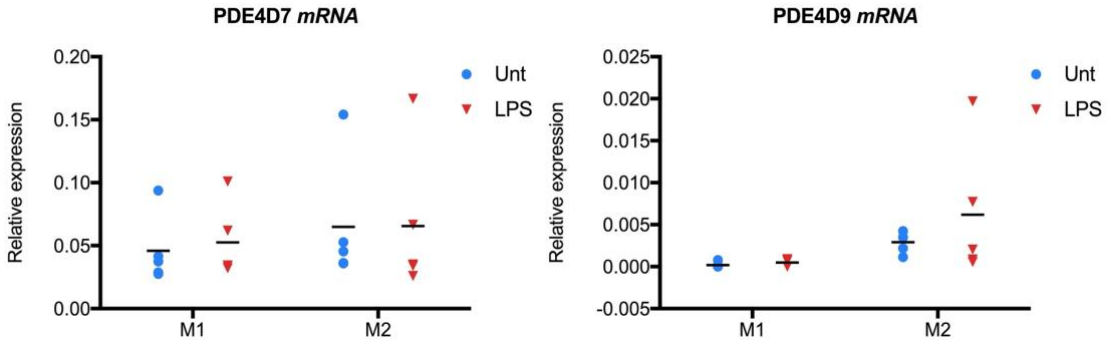



Figure 3.33 Selective PDE4D isoform expression in MDM subsets from (A) healthy and (B) COPD donors

CD14+ monocytes were obtained from donors who were either healthy (A) or had COPD (B) and differentiated to monocyte derived macrophages (MDM) in RPMI complete media supplemented with either GM-CSF 20 ng/ml + IFN-γ 10 ng/ml (M1) or M-CSF 10 ng/ml + IL-4 10 ng/ml (M2). The cells were cultured for five days in media supplemented with CSF, before culture for one day in media without. RT-qPCR and isoform-selective probes were used to detect the expression of selected PDE4D isoforms as shown, both without LPS 50ng/ml for 3h (shown here in blue) and with (red). Due to limited available template, it was not possible to assess PDE4D3 and PDE4D9 expression in MDMs from healthy donors. Mean shown. Statistical analysis using 2-tailed Student's t-test ($n$ = 5 - 9).

Long PDE4D7, which has been implicated in prostate cancer (Henderson et al., 2014), showed no change in expression upon challenge with LPS in either M1 or M2 subsets in both cells derived from healthy donors or those derived from COPD donors (Figure 3.33). As described in Chapter 3.2, the use of a plasmid control

including genomic DNA would have helped validate the use of the PDE4D-isoform specific probes including PDE4D7, but this was not included within the study. A control for LPS was not used since in other RT-qPCR analyses, LPS markedly and selectively induced PDE4B2, as has been widely reported (Ma et al., 1999; M. C. Shepherd et al., 2004), indicating LPS was working as expected in this system.

As part of a further evaluation of how the PDE4D sub-family and isoform specific probes were functioning, I also extended the assessment of PDE4D isoform expression to long PDE4D9 and long PDE4D3 in MDMs derived from COPD donors (in which there was sufficient template)

PDE4D9 expression was absent in four out of five donor cells polarised to M1 subset and in cells polarised to a M2 subset, did not alter following treatment with LPS (Figure 3.33).

3.6.3.1 PDE4D3 expression

PDE4D3 isoform expression was absent in two out of five donor cells polarised to M1 MDM and only detectable at near negligible levels in the remaining three donors. Meanwhile PDE4D3 was completely absent in cells polarised to a M2 subset (Figure 3.33). As described earlier, PDE4D3 expression was also completely absent in monocytes derived five healthy and also five COPD donors. How then is this reconciled with the observations from Shepherd and colleagues that PDE4D3 (and PDE4D5) provided the dominant PDE4 activity in U937 monocytic cells (M. C. Shepherd et al., 2004).

Although Shepherd and colleagues sought to assay PDE4 isoform activity rather than isoform expression the near complete absence of PDE4D3 in myeloid cells is notable. The consistency of the observations across different experiments, using different templates would argue against a simple experimental error.

One possibility that might account for the absence of PDE4D3 in myeloid cells as described the current study and its reported dominance of PDE4 activity in the study by Shepherd (M. C. Shepherd et al., 2004), is related to the model systems used and specifically how Shepherd and colleagues isolated primary monocytes. As was common practice at the time, Shepherd and colleagues used a density centrifugation method to isolate PBMCs and did not complete an enrichment step to help select out a monocyte cell fraction within the population of PBMCs. They also did not complete a determination of the isolated cell population and as such the derived cell populations would likely also have contained other PBMCs including T and B lymphocytes. Differences in PDE4 expression profiles according to cell types have been described in neutrophils (Wang et al., 1999) as well as T-lymphocytes (Lerner & Epstein, 2006) and their inclusion may have affected the resultant final expression profiles.

However, it may also be noted that Shepherd and colleagues also used the U937 monocytic cell line and a non-pure population of peripheral mononuclear cells would not account for the results obtained in cells derived from the U937 cell line.

Shepherd and colleagues, as was current practice at that time, deduced PDE4D3 expression simply from identifying a 95kDA migrating species, which was the only PDE4D long form species of that size known at that time. However, since the

publication of the study by Shepherd (M. C. Shepherd et al., 2004) at least four new PDE4D variants (PDE4D6-9) have been identified (Gretarsdottir et al., 2003; D. Wang et al., 2003), including PDE4D8 and PDE4D9 which have been shown to have similar molecular masses and to co-migrate with PDE4D3 on SDS-PAGE (Levallet et al., 2007; Richter et al., 2005). It has been reported that PDE4D3 is in fact very rarely expressed (Levallet 2007, PDE4 and M Houslay, personal communication). These observations no doubt account for the discrepancy between the results of Shepherd and colleagues and the current study, from which I conclude that the long form species identified by Shepherd and colleagues (M. C. Shepherd et al., 2004), was not in fact PDE4D3 but another PDE4D long isoform.

## 3.7 PDE4 expression in asthma

Since the late 1990s, pan-PDE inhibitors including theophylline, and latterly selective PDE4 inhibitors, have been extensively evaluated for use in asthma (Jin et al., 2010; M. Kobayashi et al., 2012; Landells, Spina, Souness, O'Connor, & Page, 2000). Yet, despite this and advocacy for its therapeutic potential in asthma (Bardin et al., 2015; Bateman et al., 2015; Page, 2014) selective PDE4 inhibitors have not met the threshold for regulatory approval in asthma, that they have met in COPD.

The PDE4 expression profile in bronchial tissue in asthma is not well described and discovery here, may provide insights as to why selective PDE4 inhibitors have not had the same clinical success in asthma, as they have had in COPD. The Division of Asthma and Lung Biology and allied laboratories at King’s College London maintains an extensive research interest in asthma biology. To further

explore the PDE4 expression in patients with chronic inflammatory airways disease, excess bronchial tissue from asthmatic patients and healthy controls was sought. RNA extracted from bronchial biopsies of three patients with atopic asthma along with three non-atopic healthy controls was generously donated by Dr Line Ohm-Laursen (Randall Division, King's College London). The groups were unmatched. The asthmatic donors tended to be older (43 vs 26 years, $P$ = 0.24 $n$ =3) and were all female compared with just one female in the healthy group (Table 2.3). The asthmatic donors were all prescribed inhaled corticosteroids in line with at least British Thoracic Society Step 3 management of asthma (BTS, 2019).

Bronchial biopsies were obtained using fibre-optic bronchoscopy under local anaesthetic and conscious sedation. Endobronchial biopsies obtained at bronchoscopy are small ranging in size 1-3mm and the adequacy of sampling is reduced if the procedure is not well tolerated by the donor individual. The RNA yield from bronchial biopsies is therefore often modest and varies according to the size and quality of the biopsy that was possible in that individual participant (personal communication, Dr Line Ohm-Laursen). RNA extracted from most donor samples were at concentrations less than 10 ng/μl which provides sufficient template for only a limited number of assays to be completed. It was therefore necessary to prioritise which PDE4 DNA probes were used. Notwithstanding the limitations in deducing changes in PDE4 isoform expression from changes in PDE4 sub-family expression, it was important to evaluate the PDE4 sub-family expression using the pan-PDE4 probes as the basis of determining PDE4 expression in bronchial tissue in health and disease. I also sought to evaluate the expression of PDE4B2 using the isoform-specific probes to obtain data that might

support the hypothesis that PDE4B2 was dysregulated in inflammatory airway diseases.

Using the pan-PDE4 sub-family probes, PDE4A, PDE4B and PDE4D sub-family expression was detected in all donor bronchial tissue from both asthmatics and non-asthmatics (Figure 3.34). PDE4C sub-family expression was not evaluated in order to prioritise template. Intriguingly, despite the small sample size, it was possible to identify a difference in PDE4 sub-family expression between asthma and healthy donors. PDE4B sub-family and not PDE4A or PDE4D expression is lower in bronchial tissue from those with asthma compared to healthy controls (51% lower, 1.09 ± 0.22 vs 0.54 ± 0.20, $P < 0.05$, $n = 3$, mean ± SD, arbitrary units).

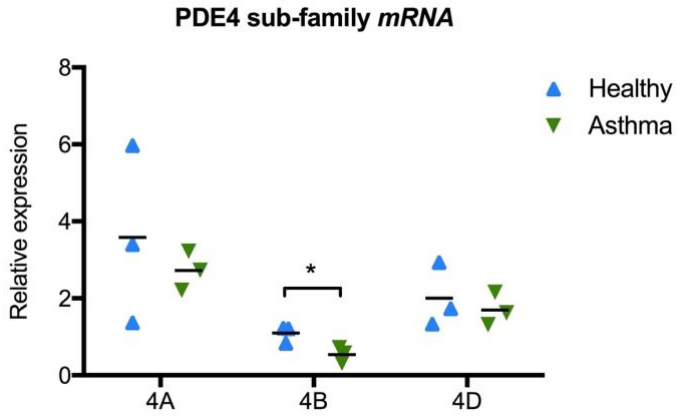



Figure 3.34 PDE4 sub-family expression in bronchial tissue

Endobronchial biopsy tissue was obtained using bronchoscopy in donors with asthma (green triangles) and without (blue). RT-qPCR and pan-PDE4 sub-family probes, were used to detect the relative expression of PDE4A/B and D sub-family expression across both groups. Mean shown. $n = 3$ in each donor group. 2-tailed Student's t-test * $P < 0.05$.

Using isoform specific DNA probes, I next sought to determine which PDE4B isoform might account for the reduced PDE4B sub-family expression in asthma. As the PDE4 isoform expression profile has not been well studied in bronchial tissue in asthma, I sought to assess the expression of all PDE4B isoforms. I also sought to obtain data about the expression of two separate isoforms for which earlier experiments had shown might be dysregulated in disease. First, PDE4A10 which in CD14+ monocytes is downregulated by LPS (Figure 3.3) and secondly PDE4A8 which I observed was upregulated in M1 macrophages derived from COPD donors (Figure 3.28).

Using the isoform specific probes, there were no differences in the expression of either PDE4B1/ 2/ 3/ or of PDE4A8 or of PDE4A10, between donors with asthma and donors without. There was though an observed difference in the expression of super-short PDE4B5, which was higher in bronchial tissue from asthmatics. PDE4B5 can interact with DISC1 and though it is reported to be brain specific in humans (Cheung et al., 2007), was detected in both monocytes and bronchial tissue in the current study. Reviewing PDE4B2 expression across disease states, there was an intriguing trend toward reduced expression of tissue from asthmatics which was 47% lower, when compared to healthy controls (2.92 ± 0.92 vs 1.56 ± 0.68, $P = 0.11$ $n = 3$,) (Figure 3.35), which though not significant, was similar in magnitude to the reduction observed in PDE4B sub-family expression

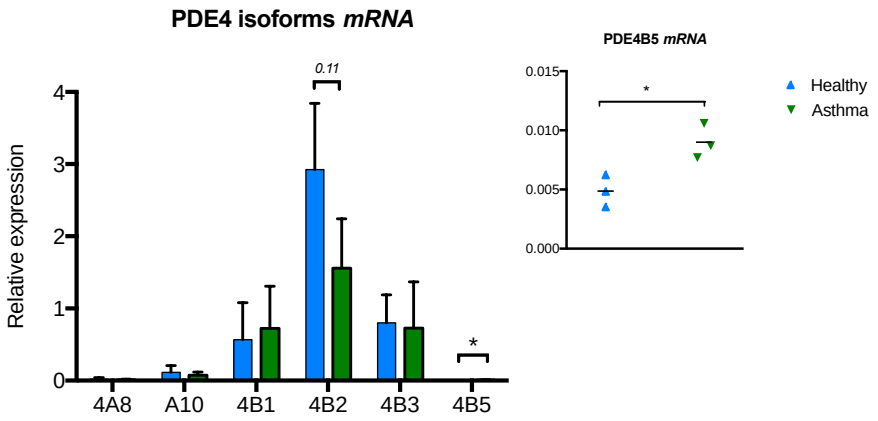



Figure 3.35 Selected PDE4 isoform expression in bronchial biopsies

Endobronchial biopsy tissue was obtained using bronchoscopy in donors with asthma (shown here in green) and without (blue). RT-qPCR and isoform-selective PDE4 probes, were used to detect the relative expression of PDE4 isoforms, as shown. Mean ± SD shown, (inset panel, mean shown) $n$ = 3 in each donor group. 2-tailed Student's t-test * $P < 0.05$.

## 3.8 Discussion

In this chapter, I have profiled the expression of up to 17 PDE4 isoforms in a highly pure population of human peripheral CD14+ monocytes and also determined the effect upon expression of challenge with the prototypical TLR4 agonist, LPS. I have developed and characterised a model of polarised monocyte-derived macrophage subsets and evaluated the change in PDE4 expression upon monocyte to macrophage subset differentiation.

Using, isoform specific DNA probes, I have obtained data to evaluate our hypothesis that the PDE4 expression might be context specific and macrophage

subset specific and further obtained to data as to whether the PDE4 expression profile is dysregulated in COPD. I have explored the effect of LPS upon PDE4 isoform expression in macrophage subsets.

Finally, I have assessed the PDE4 expression in another model of inflammatory airways disease using donated bronchial tissue from asthmatic donors and compared them to non-asthmatic controls.

In the paragraphs below, I first discuss important aspects with regard to the cell models including monocyte isolation and the development of MDMs, before discussing key thematic observations I have drawn from the data herein provided.

### 3.8.1 Monocyte selection

The use of magnetic beads and positive selection of monocytes based upon their expression of CD14+ provided a highly pure and consistent population of CD14+ monocytes. Other approaches at monocyte enrichment in the study of PDE4 expression profiles have been based upon elutriation (Ma et al., 1999; Verghese et al., 1995; Wang et al., 1999) or cell adherence (M. C. Shepherd et al., 2004). These methods of cell enrichment – though the prevailing methods of the time – are expected to yield cell populations of lower purity than one based upon selective antibody binding. The exclusion of other PBMCs that have been shown to have cell specific PDE4 expression profiles including neutrophils (Wang et al., 1999) as well as T-lymphocytes (Lerner & Epstein, 2006) helps provide a robustness to the monocyte model used in the current present study.

In recent years, it has been proposed that the peripheral circulating monocyte population is comprised of three monocyte subsets (Cornwell et al., 2013; Cros et al., 2010; Schmidl et al., 2014). Whilst the functional relevance of these individual monocyte cell populations is not fully understood, these cell populations can be distinguished by their relative expression – or lack thereof – of two cell surface makers – CD14 and CD16. CD14+CD16- and CD14+CD16+ are considered the two most numerous monocytes subsets (Cornwell et al., 2013; Ingersoll et al., 2010) accounting for the majority of circulating monocytes. It is likely that both of these subset populations are likely to have been included within a positive cell selection on the basis of their expression of CD14. However, it is as likely that $CD14^{DIM}$CD16+ monocytes may have been omitted by their absent/low expression of CD14. Although $CD14^{DIM}$CD16+ account for only between 5-8% of the total monocyte population (Cornwell et al., 2013), it is conceivable that their omission might have influenced the overall observed monocyte PDE4 expression profile in the present study.

$CD14^{DIM}$CD16+, also termed ‘non-classical monocytes’ (Cornwell et al., 2013) modestly express CD14, a co-receptor with TLR4 for bacterial LPS and consequently have been associated with a much weaker cytokine and chemokine response to LPS (Cros et al., 2010). Non-classical monocytes are very responsive to stimulation by viral pattern recognition receptors TLR7 and TLR8. Non-classical monocytes lack the chemokine receptors, CCR2 and CCR5, that enable monocytes to traffic into extravascular tissue in response to inflammatory stimuli (Mitchell, Roediger, & Weninger, 2014; Ziegler-Heitbrock, 2014). They do though express the chemokine receptor CX3CR1 which enables them to ‘crawl’ along luminal surfaces of vascular endothelial cells (Cornwell et al., 2013; Cros

et al., 2010). The implication of these differences in their chemokine repertoire, is that non-classical monocytes appear to be relatively less well equipped to respond to bacterial invasion and may instead serve to patrol sites for viral infection.

Despite these insights about monocyte subset surface receptor expression, the exact role and the contribution of individual monocyte subpopulations towards host defence remains contentious, in part due to the means by which subpopulations are isolated as well as due to a lack of a consistent gating strategy for immunophenotyping by flow cytometry (R. Mukherjee et al., 2015). Interestingly, in humans a small group of individuals from the same family who lack the CD16 receptor and therefore CD16+-expressing monocyte subpopulations, do not appear to have an impaired host defence or clinical phenotype (Wagner & Hansch, 2004).

Although CD14+ positive cell selection techniques do not isolate $CD14^{DIM}$ sub-populations, so called negative cell selection techniques can be used to help isolate pan-monocyte populations. Antibody cocktails can be used to help remove all non-monocyte cell populations including T, NK, B and dendritic cells. Pilot studies were completed using a commercially available pan-monocyte selection kit but this was associated with lower levels of purity using FACS as well as high levels of contamination with circulating platelets (data not shown). The presence of platelets within cell populations in *in vitro* studies has been reported to cause alterations in gene expression (Beliakova-Bethell et al., 2014) and further may display cross-talk with innate immune cells (Ribeiro, Migliari Branco, & Franklin,

2019). Pan-monocyte kits proved also prohibitively expensive and would have constrained the experimental approach and were therefore not further used.

In summary, despite its limitations, it is proposed that the model of CD14+ monocytes is likely to be representative of the monocyte population and provides a robust means to determine monocyte PDE4 expression.

### 3.8.2 Modelling macrophages

Monocyte derived macrophages (MDM) have been extensively used to model macrophage functions and responses (Haniffa, Bigley, & Collin, 2015) (Tarique et al., 2015) (Beyer et al., 2012; Martinez et al., 2006; Xue et al., 2014). Using *in vitro* differentiated monocytes to profile PDE4 isoform expression, not only overcomes the inability to obtain tissue-resident macrophages in sufficient quantities to achieve the experimental aims, it also enables the exploration of macrophage activation states or phenotypes, using reproducible and clearly defined maturation protocols.

The validity of the model used was supported by the generation of macrophage subsets that were morphologically distinct and in keeping with those reported elsewhere which used similar protocols (Figure 3.8) (Tarique et al., 2015). This distinction is likely to have been driven by the use of GM-CSF and M-CSF to generate respectively M1 and M2 macrophages. M-CSF appears critical for almost all macrophages irrespective of their embryological origin. Mice deficient in M-CSF or colony stimulating factor receptor (CSF1) lack tissue macrophages and display high rates of perinatal mortality (Epelman et al., 2014). Within the

lung and some other tissues, GM-CSF also has a critical role as mice lacking GM-CSF do not develop alveolar macrophages (Guilliams et al., 2013).

The robustness of the model of distinct macrophages subsets herein described, is further supported by the differential expression of up to six macrophage markers including TNF$\alpha$ and IL-10 – prototypical makers of respectively classical and alternatively activated macrophages (Arnold et al., 2015; Stein et al., 1992; Wesemann & Benveniste, 2003; H. M. Wilson, 2014; Yoshimura et al., 2007). As a model based on primary human cells, it is also more likely to provide a more representative basis of exploring PDE4 isoform profiles in humans than the use of humanised cell lines either U937 (M. C. Shepherd et al., 2004) or Mono Mac 6 (Verghese et al., 1995).

Although the use of a primary MDM has advantages over the use of cell lines, there are nevertheless important limitations in the use of this model. It may be considered that despite differences in maturation protocols as well as differences in the morphological appearances and expression profiles of each MDM subset, this may not adequately distinguish two cell populations that might behave differently. This could be further explored using functional studies including for example cytokine readouts or phagocytic assays (B. Ghosh et al., 2019).

Another possible limitation tends toward a more philosophical one, around the notion of macrophage subsets *ex vivo*. It has been argued that macrophage subsets, including those herein described represent a false dichotomy of true macrophage activation states as they exist in animal models. It is argued that macrophage activation states are as diverse as the variety of stimuli they are

exposed to (Murray et al., 2014) and that macrophage subsets do not represent terminal irreversible differentiation programs. Instead, it is argued that macrophages may exhibit significant plasticity and inter-convert between macrophage state (Lavin et al., 2014). However, in this study, to test the hypothesis that PDE4 isoform profile might be phenotype specific, it was necessary to generate macrophage subsets at the extremes of any spectrum of activation states, towards maximising the opportunity to identify a signal change in PDE4 expression. An animal model utilising a conditional ablation of macrophages, as for example as has been deployed using macrophage Fas-induced apoptosis (Hua, Shi, Shultz, & Ren, 2018), could potentially help address some of these issues about macrophage activation states within a tissue microenvironment.

A further contention around the use of a MDM model in assessing macrophage responses relates to a paradigm shift in our understanding of macrophage ontogeny. Recent studies have questioned the validity of the mononuclear phagocyte system and argued that tissue-resident macrophages are a separate lineage seeded during development and maintained by self-renewal. Fate mapping combined with parabiotic and adoptive-transplant studies demonstrate that during the steady state, monocytes do not make a significant contribution to tissue macrophage populations (Geissmann et al., 2010; Schulz et al., 2012). Depletion of lung macrophages leads to a repopulation by proliferation in situ, rather than replacement from bone marrow derived monocytes ((Hashimoto et al., 2013).

However, it may be observed that in times of tissue damage following either infection or injury, so called inflammatory monocytes (Ly6c+ in mice) are recruited from the circulation and differentiate into macrophages as they migrate in to affected tissues (R. L. Wynn, 2013). These recruited macrophages dominate the inflammatory response both directly – through the release of inflammatory mediators and oxidative processes – and indirectly through the release of IL-12 and IL-23 which leads to the differentiation of Th1 and Th17 helper T cells. As the threat subsides and to counter any collateral damage, these more 'inflammatory' macrophages undergo apoptosis or switch to an anti-inflammatory phenotype. Hence, in inflammatory states, as might for example occur in COPD, it may be proposed that monocytes do play an important part of the host response to injury and form part of a complex immune biology towards supporting homeostasis.

### 3.8.3 TLR signaling of macrophage PDE4B2

I have obtained data that clearly demonstrates LPS is an inducer of PDE4B2 expression in monocytes and MDMs. Moreover, LPS induction of PDE4 is specific to PDE4B2 as LPS did not induce the expression of up to 15 distinct PDE4 isoforms in monocytes (neither PDE4A1/ 4/ 7/ 8/ 10/ 11, PDE4B1/ 3/ 5 or PDE4D3/ 5/ 7/ 8/ 9/ 11) and also did not induce the expression of up to 11 distinct PDE4 isoforms in macrophages (PDE4A4/ 7/ 8/ 10, PDE4B1/ 3/ 5 or PDE4D3/ 5/ 7/ 9).

In monocytes, LPS led to a near x3 induction of PDE4B2 expression. The effect of LPS was no different in monocytes derived from COPD donors to those of monocytes derived from healthy donors. In MDMs, LPS proved to be a

particularly potent inducer of PDE4B2. In macrophages derived from healthy donors, LPS-PDE4B2 induction was broadly similar across M1 and M2 subsets with a respective x17 and x15 induction. However, in MDM derived from COPD donors, the rates of LPS-PDE4B2 induction seemed less equal between M1 and M2 subsets at x32 and x9 respectively.

Firstly, starting with LPS induction of PDE4B2 in monocytes, the results are in line those by Ma and colleagues (Ma et al., 1999) who also demonstrated that LPS selectively induced PDE4B2 in human monocytes. In the current study, I used LPS at a concentration of 50ng/ml - one half of that used by Ma and colleagues – who found LPS induced PDE4B2 with an $ED_{50}$ of 0.04 ng/ml. LPS induction of macrophage PDE4B2 has also been reported in a model of murine peritoneal macrophages (Jin et al., 2005). This latter study provides important mechanistic insights, in to the functional relevance of PDE4B2 induction.

Using a combination of genetic and pharmacological approaches Jin and colleagues (Jin et al., 2005) demonstrated that only macrophages deficient for PDE4B, and not those from $PDE4A^{-/-}$ or $PDE4D^{-/-}$ mice, attenuated the TNF$\alpha$ response to LPS. Moreover, they reasoned that this disruption, was not as a consequence of a generalised loss of macrophage function, as the ability for LPS to induce IL-6 in cells deficient for PDE4B was no different to that observed in wild-type cells. Hence, genetic ablation of PDE4B is associated with a specific interruption of the LPS- TNF$\alpha$ pathway.

The induction of PDE4B2 can be expected to lead to a reduction in compartmentalised pools of cAMP and subsequently reduced activation of

downstream effectors protein kinase A (PKA) and exchange proteins directly activated by cAMP (Epac) (David M. Aronoff, Canetti, Serezani, Luo, & Peters-Golden, 2005; Serezani et al., 2007). Activated PKA negatively regulates TNFα production and so loss of PKA activity uncouples the break on TNFα and with it, leads to conditions favouring inflammation. Hence, it is proposed that PDE4B2 through its degradation of compartmentalised cAMP, functions to regulate PKA activity and with it the TNFα production by TLR signaling. This is also evidenced by the observation that pharmacological inhibition of PKA, reverses the disruption of the LPS– TNFα pathway following genetic ablation of PDE4B (Jin et al., 2005).

TLR4 which is part of the Toll Like Receptor (TLR) family helps recognise distinct pathogen-associated molecular patterns (PAMPs) and are involved in the immune response to gram negative bacteria. However, the induction of PDE4B is not restricted to TLR4 mediated signaling. In astrocytes, which are the major innate cells of the CNS, PDE4B2 gene expression was profoundly up-regulated by ligands of TLR2, TLR3 as well as TLR4 (Borysiewicz et al., 2009). Although the promoter for PDE4B2 has not been fully established, it has been shown to contain several NF-κB binding sites as well as CRE loci (D'Sa, Tolbert, Conti, & Duman, 2002). Since TLR ligation activates both NF-κB and CREB (Mankan, Lawless, Gray, Kelleher, & McManus, 2009; Oeckinghaus & Ghosh, 2009)it is likely these transcription factors help drive the upregulation of PDE4B2 transcription and account for the effects of LPS in our study.

In the steady state, genetic transcription is regulated on multiple levels and involves cross-talk between transcription factors/co-factors, RNA editing, expression of long non-coding RNAs and epigenetic modifications. (Kuznetsova,

Prange, Glass, & de Winther, 2020). One such epigenetic modification involves chromatin accessibility by modifications to histone proteins which have been studied using the PDE4B2 promoter.

Watson and colleagues (Watson, Zhao, & Chawla, 1999), used the chromatin-modifying enzyme S-adenosylmethionine (SAM), to attenuate LPS-induced TNF$\alpha$ expression in monocytes and macrophages. Levels of chromatin-modifying metabolites including SAM, are controlled by multiple mechanisms including environmental inputs and provide a link between chromatin and intracellular metabolic status (Dai, Ramesh, & Locasale, 2020). In both RAW 264.7 (murine macrophage-like) cells and primary human CD14+ monocytes, pre-treatment with SAM leads to a significant decrease in LPS-induced up-regulation of PDE4B2 expression which has been associated with an increase in histone H3 lysine 9` trimethylation of the PDE4B2 intronic promoter region (Gobejishvili et al., 2011). Furthermore, SAM has been identified as a possible therapeutic in the treatment of inflammatory disorders (Bottiglieri, 2002).

Further insights into the possible negative modulation of PDE4B2 relate to the Rho GTPases. Since an exuberant and persistent inflammatory response can be damaging to tissues, cells need to maintain feedback mechanisms to control inflammatory responses including those mediated by TLRs. The Rho family are ubiquitously expressed cytoplasmic proteins involved in the regulation of multiple downstream effectors (Bros, Haas, Moll, & Grabbe, 2019). Inactivation of Rho proteins by Toxin B (a toxin derived from Clostridium difficile, used in cell biology to target Rho proteins (Popoff, 2014)) causes irreversible glycosylation, enhances TLR-induced transcription of PDE4B2 in astrocytes but does not

necessarily lead to enhanced levels of protein (Borysiewicz et al., 2009). For example, Borysiewicz and colleagues observed that using LPS alone led to a 5-fold increase in messenger RNA and 2.5-fold increase in PDE4B2 protein, but that when LPS was preceded by Toxin B, there was a 45-fold increase in messenger but only the same 2.5-fold increase in protein. This was in contrast to the expression and translation of IL-6 and NOS2 which were more closely matched and suggests the level of PDE4B2 protein is tight regulated in astrocytes. One mechanistic explanation for this may be related to how PDE4B2 is compartmentalised in cells through the formation of macromolecular complexes involving A-kinase anchoring proteins to provide spatiotemporal modulation of cAMP signaling. It could be envisaged that un-complexed PDE4B2 is targeted for rapid degradation and therefore an absence of complexing proteins to help sequester PDE4B2 may constrain its peak concentration. The degradation of PDE4B2 through the ubiquitin pathway is explored further in the following Chapter.

### 3.8.4 LPS down regulates PDE4A

Perhaps as striking as the effect of LPS induction on PDE4B2 expression in monocytes and MDM subsets, was the opposing effect of LPS downregulation of PDE4A sub-family expression. LPS leads to a two-third reduction of PDE4A expression in monocytes and up to one half reduction in macrophages subsets, as determined using the pan-PDE4A probes. Furthermore, LPS constraint of monocyte PDE4A expression is not different in monocytes whether they are derived from healthy donors or those with COPD and in monocytes derived from healthy donors, no different between M1 and M2 MDM subsets (Chapter 3.6.1 and Figure 3.27).

Consistent with our findings, a study of healthy human volunteers injected with LPS, found at two and four hours after injection with LPS, PDE4A sub-family expression was reduced (and PDE4B expression increased) in whole blood leukocytes using qRT-PCR (Lelubre et al., 2017). Negative modulation of PDE4A expression has also been reported in cultured astrocytes from new-born rats following challenge with polyinosinic-polycytidylic acid (PIC) that, is a ligand of TLR3 (Borysiewicz et al., 2009).

In contrast to the observed changes in PDE4A expression following treatment with LPS in the current study, Jin and colleagues found PDE4A activity in mouse peritoneal macrophages was increased after LPS. However, PDE4-hydrolysing activity does not necessarily correlate with PDE4 expression and these observations by Jin and colleagues did not meet the statistical threshold (Jin et al., 2005). Hence, to our knowledge, our study is the first report of LPS-mediated reduction of PDE4A expression in isolated human monocytes and macrophages derived from them.

PDE4A sub-family expression was readily observed in monocytes and macrophages and in all 14 donors (nine healthy and five with COPD). Other studies have also reported the presence of PDE4A in untreated monocytes or monocyte-like cells (Ma et al., 1999; Manning et al., 1996; Verghese et al., 1995) and also the presence of PDE4A in macrophages and macrophage-like cells (Barber et al., 2004; M. C. Shepherd et al., 2004). Although it was not possible to infer the abundance of PDE4A transcript levels relative to other PDE4 sub-families (to do so would require the use of a standard curve and samples with

known transcript quantities), PDE4A has been reported to be the dominantly expressed PDE4 sub-family (Ma et al., 1999).

Other studies that have looked at the potential significance of PDE4A in macrophages have focused on cAMP PDE4 activity. Shepherd (M. C. Shepherd et al., 2004) using U937 macrophages, found that in untreated cells long PDE4A isoforms accounted for less than 10% of the total cellular PDE4 activity In contrast, in a model of genetic ablation, only in untreated macrophages from mice that are PDE4A$^{-/-}$ and not PDE4B$^{-/-}$ or PDE4D$^{-/-}$, was the basal PDE4 activity reduced, implying that PDE4A sub-family accounts for the majority of PDE4 activity in macrophages during unstimulated conditions (Jin & Conti, 2002). However, the relative functional significance of each PDE4 sub-family changes in the stimulated state. Following treatment with LPS, only mice that were PDE4B$^{-/-}$, was there any absence of increased PDE4 activity (Jin & Conti, 2002).

Taking forward the aforementioned study by Jin and colleagues and the data obtained in the current study, it is proposed that in untreated macrophages, the PDE4A sub-family accounts for the majority of PDE4 activity in macrophages, but, following TLR signaling there is a switch to PDE4B dominant activity that is mediated both by the downregulation of PDE4A expression as well as the potent induction of PDE4B (PDE4B2 short form).

The switch from PDE4A to PDE4B2 following TLR signaling also represents a potentially important switch in the capacity for PDE4 post-translational regulation. Long isoforms contain the regulatory domain UCR1 which contains a PKA binding site, allowing long forms to be activated by PKA and so providing a direct

feedback mechanism through which cAMP homeostasis can be restored (Sette & Conti, 1996). The switch away from PDE4A isoforms also promotes a greater role for ERK. This is because the catalytic unit of PDE4B, PDE4C and PDE4D isoforms, but not PDE4A, all contain a single consensus binding site that allows phosphorylation by ERK to help regulate PDE4 activity (Houslay & Baillie, 2003). ERK is activated by pro-inflammatory stimuli and depending upon the configuration of UCR1/2 regions can lead to either inhibition or activation of PDE4 species.

The PDE4A sub-family and its isoforms have been less well studied than its related gene families; PDE4B and PDE4D. In contrast to PDE4D, selective knockout of PDE4A does not impair neonatal growth or like PDE4B attenuate the inflammatory response (Jin et al., 2005). PDE4A isoforms have been linked with learning and memory and in the hippocampus and their expression is increased following chronic exposure to anti-depressants (Y. Ye, Jackson, & O'Donnell, 2000).

Using isoform-specific probes, the data I obtained did not clearly demonstrate which PDE4A isoform might account for the reduction of LPS-mediated PDE4A sub-family expression. In monocytes derived from either healthy or COPD donors, there was no reduction in either PDE4A1, PDE4A4 or PDE4A11. In MDM derived from both donor groups there was no reduction in PDE4A4 or PDE4A7.

The reduction of PDE4A expression following treatment with LPS, may be explained by reductions in PDE4A isoforms that were not evaluated, namely PDE4A6 and PDE4A9, which are purported to exist in human tissue but for which

probes were not available. Intriguingly, the reduction of PDE4A expression may also represent the reduction in a PDE4A isoform, in an as yet undiscovered PDE4A isoform.

As part of the evaluation of PDE4A isoform expression in response to LPS, I did make the novel discovery that in monocytes from COPD donors, LPS leads to reduced expression of dead-short PDE4A7. I also observed that across both M1 and M2 subsets, monocyte to macrophage differentiation was also associated with reduced expression of PDE4A7 and that this affect was maintained in cells derived from COPD donors. This intriguing finding raises important questions as to what function PDE4A7 may be having in monocytes including whether it is involved in regulating other protein-protein interactions including through scaffolding complexes (Houslay, 2010).

### 3.8.5 PDE4 expression in COPD

In a landmark study, Barber and colleagues reported that PDE4A4 expression was upregulated in the lung macrophages of smokers with COPD compared to smokers without (Barber et al., 2004). In peripheral monocytes, they also identified that both PDE4A4 and PDE4B2 expression was upregulated in the cells from healthy smokers when compared to those from non-smoker controls. The study by Barber and colleagues was amongst the first report to identify that the PDE4 isoform expression might be dysregulated in disease.

In contrast to the findings from Barber and colleagues, I observed no difference in the expression of either PDE4A4 or the expression of PDE4B2 in monocytes

or in MDMs, when compared between healthy and COPD donors. A number of possible explanations may account for these differences which I discuss below.

Most noticeably, the study by Barber and colleagues used a different model system to that deployed in the current study. Barber obtained lung alveolar macrophages using bronchoalveolar lavage whereas I used a model of monocyte derived macrophages cultured over a six-day *ex vivo* maturation protocol. The use of monocytes and human monocytic cell lines is well described in PDE4 related research (S. J. MacKenzie & Houslay, 2000; M. C. Shepherd et al., 2004; Torphy et al., 1995) and in one study has been favourably compared to the PDE profile obtained from alveolar macrophages (Tenor et al., 1995). Despite this, the absence of a validation step using for example macrophage expression markers to compare monocyte derived macrophages to lung derived macrophages, is a limitation of the current study.

Barber and colleagues also reported differences in PDE4A4 and PDE4B2 expression in peripherally circulating monocytes - which were used in the current study. However, the differences in expression were between cells derived from smokers and non-smokers. In the current study, only former smokers were represented and all were within the COPD group. Thus, it is apposite to ask, what effect might smoking have on PDE4 expression?

Perhaps surprisingly - given the extensive evaluation of PDE4 inhibitors in COPD – the effect of chronic cigarette smoke (CS) on PDE4 expression in humans is not well defined. One study used microarrays and genome wide gene expression profiling of nasal and bronchial epithelium as well as whole lung tissue, to identify

differences in PDE expression profiles between healthy smokers and never smokers (Zuo et al., 2020). After correcting for age and sex, Zuo and colleagues reported the gene expression of multiple different PDEs in nasal and lung tissue is different in current smokers when compared with never smokers, suggesting that chronic CS leads to alterations in PDE expression. Indeed, this difference in PDE expression was observed across different types of airway tissue, adding perhaps more veracity to the findings. However, for the majority of PDEs, the changes were not matched across tissue groups – indeed PDE4 was the only PDE sub-family for which gene expression changes were observed in all three groups (nasal epithelium, bronchial epithelium and lung tissue). Despite this, even amongst PDE4 the changes were not consistent, as PDE4D expression was at the one hand increased in lung tissue from smokers but simultaneously also decreased in bronchial epithelial tissue in smokers. A further interesting observation in the study by Zuo and colleagues was that whilst they observed changes in PDE4A, PDE4C and PDED subfamily expression, they found no such changes in the expression of PDE4B - which it is proposed is more intimately linked to inflammatory related signaling and one would hypothesise, more likely to affected by CS.

Another study has provided further insights into the possible effect of CS on PDE4 expression. Using donated lung tissue in patients undergoing surgery for confirmed or suspected lung cancer, the expression of PDE4 sub-families was compared between three groups; non-smokers, smokers and patients with COPD (group sizes of eight, eight and eleven, respectively) (S. Lea et al., 2019). Between, smokers and non-smokers, there were no differences in the expression of either PDE4A, PDE4B or of PDE4D sub-family expression, in either lung

macrophages or whole lung tissue. Although it was a small sample size and not possible to match groups, the study found no evidence of an effect of CS on PDE4 sub-family expression. Interestingly, they did though report that PDE4A, PDE4B and PDE4D expression was higher in lung macrophages from COPD donors, when compared to non-smoker controls, implying a disease specific effect.

The study by Barber and colleagues was published over 15 years ago. Since that study was completed, there have been significant advancements in our understanding of PDE4 isoforms and the DNA probes designed to target them as well as RT-qPCR techniques. It is notable since their study was reported, that there have not been further reports identifying PDE4A4 or PDE4B2 expression to be dysregulated in COPD.

#### 3.8.5.1 PDE4A8 and PDE4B2 expression in COPD

The data in the current study, although different to the key findings of Barber and colleagues, did highlight important differences in the expression of PDE4A8 as well as PDE4B2 which warrant further consideration.

Starting first with PDE4A8 expression. In MDM derived from COPD donors, PDE4A8 expression is higher in cells polarised to an M1 subset when compared to cells polarised to a M2 subset (Figure 3.26). Moreover, PDE4A8 expression in M1 cells derived from COPD donors was also higher than PDE4A8 expression in M1 cells derived from healthy donors. One interpretation of this data might be that PDE4A8 expression was associated with pro-inflammatory conditions – both those that were experimentally generated through conditions favouring M1

polarisation and also intrinsic to the monocytes obtained from donors with COPD – a condition associated with systemic inflammation. Yet, this explanation is at odds with the experimental observation that treatment with LPS leads to a reduction in PDE4A8 expression.

Long PDE4A8 expression has been described in skeletal muscle and brain and is predominantly cytosolic (Kirsty F. Mackenzie et al., 2008). It has an N-terminal region quite different to other long PDE4A isoforms and within the brain has been linked to regions involved in sensation, co-ordination and higher cognitive functions. There are no reported associations between PDE4A8 and COPD.

Since increased PDE4 expression is expected to have broadly pro-inflammatory effects through selectively reducing cAMP within compartmentalised pools, it seems unlikely that that PDE4A8 could be both a driver of inflammation in COPD and yet be negatively modulated by LPS.

Turning next to short PDE4B2, PDE4B2 expression is seven times higher in M1 MDM from healthy donors when compared to MDM polarised to a M2 subset. In MDM derived from COPD donors, there was a non-significant trend toward higher PDE4B2 expression in M1 over M2 subsets. M1 macrophages, also termed classically activated macrophages exhibit the prototypical pro-inflammatory profile of high TNF$\alpha$ and low IL-10 expression. Together with long PDE4A8, I believe this is the first report that PDE4 isoform expression might be differentially expressed between macrophages subsets.

If confirmed by further study, differential expression of PDE4 isoforms across macrophage subsets might have important mechanistic insights in to the basis of macrophage phenotypes including those that heighten the inflammatory response and those that help constrain it. PDE4 isoforms help regulate compartmentalised cAMP and through this key secondary messenger are well positioned to impact a range of different important cell functions including the cellular response to ‘danger’ /PAMP signals.

Differential PDE4B2 expression across macrophage subsets also holds an intriguing possibility of providing a model through which to develop medicines that select macrophages based upon their phenotype – and so the functional role they are tasked with – rather than one that is purely cell based. Conditions characterised by chronic inflammation are underpinned by dysregulated macrophage responses including an imbalance between M1 ‘pro-inflammatory’ responses over M2 ‘regulatory’ responses. It is proposed that a medicine designed to target cAMP PDE4B2 may be expected to preferentially constrain M1 associated responses that might be harmful in chronic inflammation, over unwanted off-target effects on M2 macrophages that might otherwise help restore tissue homeostasis. Developing this idea, one could propose one such application of this could be to deploy selective PDE4B2 inhibitors in tissue in which there was evidence of chronic inflammation – the lung of a patient with COPD perhaps or an inflamed joint in a patient with psoriatic arthritis. Moreover, the identification of increased macrophage PDE4B2 expression could be exploited to help provide a biomarker of which patients might benefit from selective PDE4B2 inhibition towards a more personalised approach to treatment.

Beyond differences in the relative expression of PDE4B2 across macrophage subsets, the data I obtained also highlighted differences between macrophage subsets in the response to an inflammatory stimulus, using LPS. In monocytes from either healthy or COPD donor groups, the induction of PDE4B2 by LPS is not significantly different. However, in monocyte derived macrophages, there appears to be differences in the LPS induction of macrophage PDE4B2 that highlight a potential disease specific effect.

In M1 and M2 macrophages derived from healthy donors, LPS led to a similar induction of PDE4B2, 17 times and 15 times respectively over untreated macrophages. This might be described as a ratio of MDM responsiveness that could be expressed as 1.1: 1 (M1 vs M2) (Figure 3.30). However, in macrophage subsets derived from COPD donors, LPS led to a respective 32 times and nine times increase of PDE4B2 expression in M1 and M2 macrophages - a ratio of 3.6: 1. Qualifying this, it must be repeated that the induction of PDE4B2 in M2 cells derived from COPD donors did not meet the statistical threshold for significance. Although further study is required, one interpretation of the data, is that the induction of PDE4B2 by LPS is dysregulated in MDM subsets derived from donors with COPD. Indeed, MDM derived from patients with COPD show impaired phagocytosis (Belchamber et al., 2019) and have been further shown to have pro-inflammatory responses (Singh et al., 2021). Discovery here, might help underpin our hypothesis that PDE4B2 is an important mediator of the inflammatory response and its dysregulation in disease may present a treatable trait in COPD.

A heightened response to express PDE4B2 in M1 macrophages may be expected to drive the inflammatory response by selectively reducing pools of compartmentalised cAMP. This is important as whilst cAMP has long been recognised as an inducer of anti-inflammatory responses, in recent years it has also been marked out as a coordinator of the key mediators of resolution of inflammation. One such mediator is Annexin A1 (AnxA1). Lower levels of cAMP leads to less activation of PKA and with it, phosphorylated CREB which acts as a transcription factor of anti-inflammatory cytokines and production of pro-resolving mediators such as AnxA1 and the 5-lipoxygenase(5-LOX) (Figure 3.36). In addition, PKA inhibits glycogen synthase kinase 3 (GSK3), P13K/Akt and NF-κB signaling pathways and a reduction of activated PKA reduces the inhibitory capacity of PKA, on these pro-inflammatory pathways.

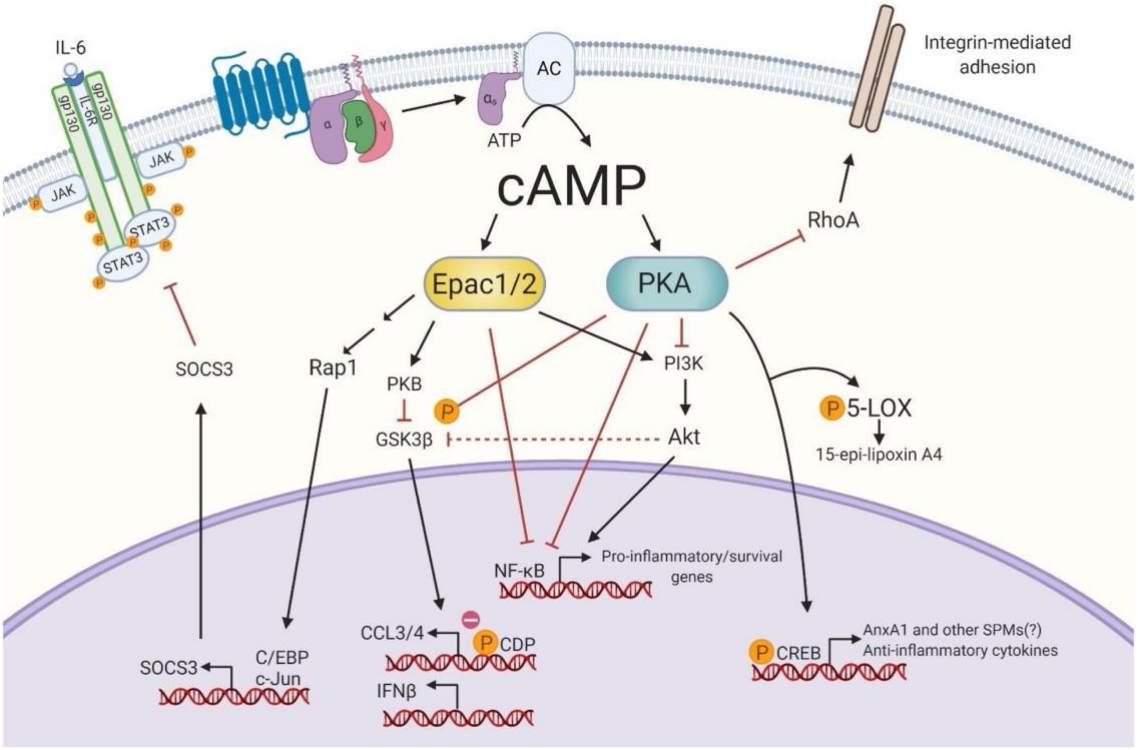



Figure 3.36 Schematic of cAMP dependent regulation

of pro-inflammatory cytokines and pro-resolving mediators

(Taken from (Tavares et al., 2020))

cAMP and downstream signalling pathways, help to orchestrate the anti-inflammatory response and also co-ordinates key mediators that resolve inflammation. Elevated cAMP activates protein kinase A (PKA) and exchange protein directly activated by cAMP (EPAC) – dependent pathways. PKA phosphorylates the transcription of cAMP-response element binding protein (CREB), leading to the transcription of anti-inflammatory cytokines such as Annexin A1 (AnxA1). PKA inhibits glycogen synthase kinase (GSK)-3, the PI3K/Akt pathway and NF-κB decreasing secretion of pro-inflammatory cytokines and pro-survival signals. PKA also inhibits the Ras Homolog Family Member A (RhoA)-dependent expression of integrins in granulocytes.

cAMP may also contribute to a ‘pro-resolving’ environment following the initiation of inflammation through a more indirect way, through effects on macrophage polarisation. Indeed, this has been explored experimentally using a compound termed A33 that is reported to be selective for PDE4B (Naganuma et al., 2009). In a murine model of Total Brain Injury (TBI), A33 treatment increased the percentage of microglial and infiltrating myeloid-lineage cells that expressed the murine alternative (M2) activation marker, Arg1 at three hours (N. M. Wilson, Gurney, Dietrich, & Atkins, 2017). Although the switch to Arg1 expression did not persist, the effects appeared to, as the investigators found that A33 treatment significantly reduced neutrophil accumulation and postulated that treatment with A33 led to a more anti-inflammatory pro-reparative environment.

The use of compounds to direct macrophages to a more regulatory macrophage phenotype has been exploited for use in the clinic to help develop novel anti-cancer treatments. The second-generation tyrosine kinase inhibitors bosutinib and dasatinib are approved for use in chronic phase chronic myeloid leukaemia (Hochhaus et al., 2020). These drugs help regulate macrophage polarisation by direct inhibition of salt-induced kinases (SIKs) and the dephosphorylation of the direct substrates of SIKs including CREB-regulated transcription coactivator 3 (CRTC3) (Ozanne, Prescott, & Clark, 2015). Non-phosphorylated CRTC3 then translocates to the nucleus where it interacts with CREB to induce transcription of M2 related genes and polarisation of macrophages to a more regulatory (M2) phenotype, characterised by high levels of IL-10, low levels of TNF$\alpha$, IL-6 and IL-12 as well as the expression of markers linked with regulatory macrophages including SPHK1, LIGHT and Arg1 (K. Clark et al., 2012). Observations such as those described above as well as those made by others (K. F. MacKenzie et al.,

2013; Wein, Foretz, Fisher, Xavier, & Kronenberg, 2018) help identify the importance of the cAMP→PKA→SIK→CRTC3→CREB→IL-10 axis in the polarisation of macrophages to an anti-inflammatory phenotype (Tavares et al., 2020). We hypothesise that by regulating cAMP, PDE4B2 thus may function as a master regulator of macrophage polarisation and that this could be further exploited for therapeutic gain.

I have already discussed some limitations in the current study, including the model system used – the absence of a validation step comparing derived MDM to tissue macrophages as well as the absence of a functional correlate of macrophage subset differentiation. Another limitation of the current study relates to differences between donor groups that could not be controlled. In line with many scientific studies, donor pools of healthy individuals often consist of young adults. Recognising that COPD patients tend to be within their $6^{th}$, $7^{th}$ or $8^{th}$ decade of life I attempted to recruit older donors from within the locally available healthy pool of volunteers. Despite this, the donor group of individuals with COPD were older that those without.

Also, as part of the very nature of studies involving donors with chronic health conditions, it was not possible to control for the effects of medications that donors with COPD were taking and that control donors were not. All donors with COPD were medicated with combination of long acting β-adrenergic agonists and long-acting muscarinic antagonists with/without inhaled corticosteroids (
Table 2.2). β-adrenergic agonists bind to β-adrenergic receptors leading to conformational changes and generation of cAMP through stimulation of

adenylate cyclase (Johnson, 2006) and thus could affect PDE4 expression profiles.

It may also be argued that that differences of PDE4B2 expression in M1 and M2 subsets reflect prior treatment with IFN-$\gamma$. Against this, it has been reported that IFN-$\gamma$ does not increase PDE4B activity in U937 like cells (S. J. MacKenzie & Houslay, 2000). In addition, as part of the cell culture protocol, MDMs were maintained in supplement-free media for 24hours prior to the use in experiments. However, the use of IFN-$\gamma$ is inextricably linked to M1 polarising conditions and as I discuss in Chapter 5.4.3, the effects of IFN-$\gamma$ can persist beyond 24 hours and as such it was difficult to control against this.

### 3.8.6 PDE4 expression in asthma

Despite extensive study, selective PDE4 inhibitors have not gained favour for use in asthma. Roflumilast, which has had most promise in asthma, is now approved for use in COPD (Bateman et al., 2015). However, as with COPD, the potential utility of roflumilast and other selective PDE4 inhibitors is constrained by the lack of understanding of PDE4 expression in tissues derived from individuals with disease.

Using RNA donated from healthy donors and patients with atopic asthma, I have been able to profile the PDE4 sub-family and selected PDE4 isoform expression in bronchial epithelial tissue. We believe this is the first report that PDE4B sub-family expression is reduced in human bronchial epithelial tissue derived from asthmatic patients. The reduction in PDE4B sub-family appears to be accounted for by short PDE4B2 which is the dominant PDE4B isoform in bronchial tissue

from non-asthmatic donors. Moreover, the reduction of PDE4B sub-family expression observed across asthmatic individuals does not appear to be offset by an opposing increase in the expression of PDE4A or PDE4D sub-family expression, or of long PDE4A isoforms, PDE4A8 or PDE4A10. As such, I have obtained data to support a hypothesis that PDE4 expression may be reduced in individuals with asthma.

A reduction in PDE4B transcripts, if also matched by a reduction in PDE4B2 protein, can be expected to lead to higher pools of cAMP in which PDE4B2 is localised and with it, lead to an attenuated cellular response to inflammation. Since asthma is characterised by chronic inflammation, reduced PDE4B2 could be part of an adaptive host response to counter excess inflammation through the cAMP pathway. The potential for this has been demonstrated using the adenyl cyclase activator forskolin as well as cAMP analogues. In a model of human bronchial epithelial tissue, increased intracellular cAMP attenuates the ability of respiratory syncytial virus (RSV) to disrupt the airway epithelial barrier by stabilising complexes of tight junctions and adherens junctions (Rezaee et al., 2017). These protective effects are mediated by PKA activation and do not involve Epac. Moreover, cAMP also displays direct anti-viral effects by inhibiting the expression of RSV F protein (Rezaee et al., 2017). Since RSV is the most common cause of respiratory tract infection and an important cause of exacerbations of asthma, modulation of the cAMP/PKA pathway could be an important host response in asthma.

Another consideration that might account for the reduction of PDE4B in bronchial tissue is that the two donor groups were not matched in age or sex and this may

have accounted for the difference in observed PDE4B expression. The donors with asthma were also all prescribed inhaled corticosteroids as part of their regular asthma treatments. Glucocorticoids including dexamethasone have the capacity to negatively modulate PDE4B expression in unstimulated cells (Ahlstrom, Pekkinen, Huttunen, & Lamberg-Allardt, 2005) as well as cells subject to different stimuli. For example, in harvested human pulmonary artery cells, dexamethasone suppresses the induction of PDE4A and PDE4B by cigarette smoke extract and has an additive effect when combined with rolipram (Ortiz et al., 2013). In human bronchial BEAS-2B cells as well as in mouse lung tissue, dexamethasone inhibits the synergistic induction of PDE4B expression induced by non-typable haemophilus influenzae (NTHi) and roflumilast (B. C. Lee, S. Susuki-Miyata, C. Yan, & J. D. Li, 2018). Moreover this combination inhibited the expression of a range of chemokines including CXCL1, CXCL2, CCL5 and CCL7 which are implicated in the recruitment of leukocytes in pulmonary diseases (Donnelly & Barnes, 2006). Although how dexamethasone down-regulates PDE4B expression is not known, it's affects are abolished by the glucocorticoid antagonist RU486 (mifepristone) (B. C. Lee et al., 2018) suggesting a glucocorticoid-receptor dependent pathway.

It is interesting to also consider that a reduction of PDE4B2 in bronchial epithelial tissue in asthma might be expected to reduce the available target for selective PDE4 inhibitors and as such may contribute to the limited therapeutic efficacy of PDE4 inhibitors – as for example observed with cilomilast.

The evidence from the literature for altered PDE4 expression in asthma has been conflicting. In one study using airway smooth muscle cells derived from asthmatic

and non-asthmatic individuals, tissue from asthmatic patients showed increased PDE activity which correlated with increased PDE4D protein expression (Trian et al., 2011) However, another study that evaluated PDE4 sub-family expression in peripheral leukocytes from healthy and asthmatic subjects found no difference in PDE4 expression in either CD4+ or CD8+ lymphocytes (Jones et al., 2007). It has been proposed that such conflicting findings may be related to patient selection, or the cell types studied (Jones et al., 2007).

In a rodent model of allergic asthma using sensitisation and challenge with ovalbumin, cAMP-PDE activity is increased which is accounted for by the expression of PDE4 and not PDE3 and not PDE5 (H.-F. Tang, Song, Chen, Chen, & Wang, 2005). Interestingly, in this same study, Tang and colleagues observed an increase in messenger PDE4A, PDE4C and PDE4D but not PDE4B. In a further model of allergic asthma and genetic ablation of PDE4A, PDE4B and PDE4D genes, Jin and colleagues (Jin et al., 2010) demonstrated that PDE4B is essential for the development of airway hyper-responsiveness (AHR) and induction of Th2-cell functions. Moreover, whilst PDE4D$^{-/-}$ mice also showed absent AHR, this latter effect was related to a disruption of muscarinic mediated bronchoconstriction – a feature preserved in PDE4A$^{-/-}$ and PDE4B$^{-/-}$ mice. Thus, despite the limited published evidence that PDE4 expression is altered in asthma, PDE4 and PDE4B responses appear significant in understanding the immune response and with it may still hold promise as a therapeutic target in asthma.

# Chapter 4 **REGULATION OF MACROPHAGE PDE4B2**

In this Chapter as well as the next, I turn to the regulation of macrophage PDE4B2. Here I explore the expression of PDE4B2 protein, its degradation within the cell over selected time points and provide data in support of its degradation through the proteasome. I also postulate a role for BCL-6 acting as a novel transcriptional repressor regulating PDE4B2 expression and provide data in support of this.

## 4.1 U937 macrophage model

U937 is a human monocytic-like cell line that is widely used in myeloid cell-based research. It use was first described over 40 years ago (Sundstrom & Nilsson, 1976) and like monocytes, grows as a cell suspension but can be made adherent. Chronic treatment with phorbol 12-myristate 13-acetate (PMA), cause U937s cells to differentiate to a macrophage-like phenotype (Hass et al., 1989; Kuroda et al., 1997; M. C. Shepherd et al., 2004; Twomey et al., 1993). It is not clearly known how PMA leads to monocyte to macrophage differentiation, but the use of RNA sequencing techniques, has highlighted a role for the PI3K/AKT and NF-κB pathways (Zeng et al., 2015). Indeed inhibition of PI3K activity using LY294002, inhibits PMA-induced monocyte cell differentiation (Zeng et al., 2015).

PMA-differentiated U937 macrophage-like cells, hereafter termed simply U937 macrophages, model the LPS induced TNFα response found in primary macrophages (S. J. MacKenzie & Houslay, 2000; M. C. Shepherd et al., 2004, Huston, 1997 #670). Following the protocol of these investigators, U937 cells were treated for four days with PMA (4nM) and then maintained in PMA free

complete medium for a further 24 hours before use in the experiments outlined below.

Using western blot and anti-sera targeted at PDE4B, I first sought to compare LPS induction of PDE4B2 in MDM subsets and U937 macrophages. U937 macrophages were obtained as described above and MDM subsets were cultured as described in Chapter 2. An equal number of cells was used to derive M1, M2 and U937 macrophages. GAPDH was used as a loading control.

Using SDS-PAGE, macrophage PDE4B2 was observed to migrate at 68kDA. In untreated cells, PDE4B2 expression was detected at only very modest levels across U937 as well as M1 and also M2 macrophages. At six hours after treatment with LPS (50ng/ml), there was a marked increase in PDE4B2 expression that was observed in U937 and primary macrophage subsets alike.

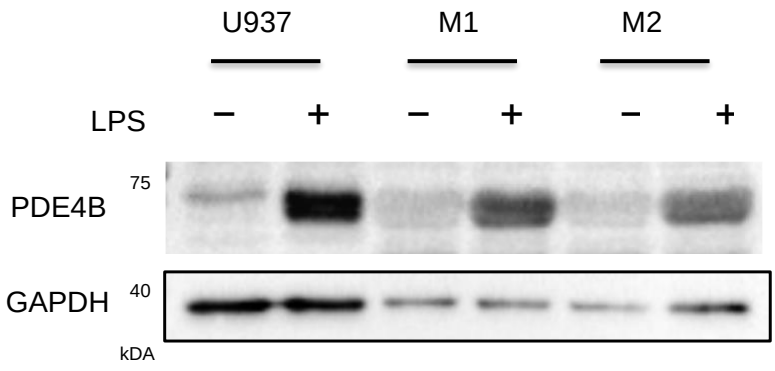



Figure 4.1 LPS induced PDE4B2 expression

in U937 and MDM subsets

U937 macrophages and primary monocyte-derived macrophages (MDM) subsets M1 (GM-CSF 20 ng/ml + IFN-γ 10 ng/ml) and M2 (M-CSF 10 ng/ml + IL-4 10 ng/ml) were obtained, before treatment with

lipopolysaccharide (LPS) 50ng/ml for six hours before total extract was produced. Lysates were probed with anti-sera to PDE4B. GAPDH was used as a loading control. The data shown are representative of three separate experiments. The molecular weight of the PDE4B immunoreactive species is 68kDa, consistent with it being PDE4B2.

## 4.2 PDE4B2 degradation

I next sought to obtain data that would help profile the expression of LPS-induced macrophage PDE4B2 over several time points. This was also used to help optimise the time at which LPS led to a maximal PDE4B2 readout using western blotting. Figure 4.2 shows a western blot of PDE4B2 expression in U937 macrophages at selected time points after the addition of LPS. LPS induces PDE4B2 expression at between one and three hours after treatment and from this time course, as well as other experiments, appears to reach peak induction at around six hours. All subsequent readouts for LPS-induced PDE4B2 protein expression using western blotting, were completed at six hours after treatment with LPS, unless otherwise stated. The time course experiment also demonstrates that LPS-induced PDE4B2 expression diminishes some 24 hours after LPS challenge.

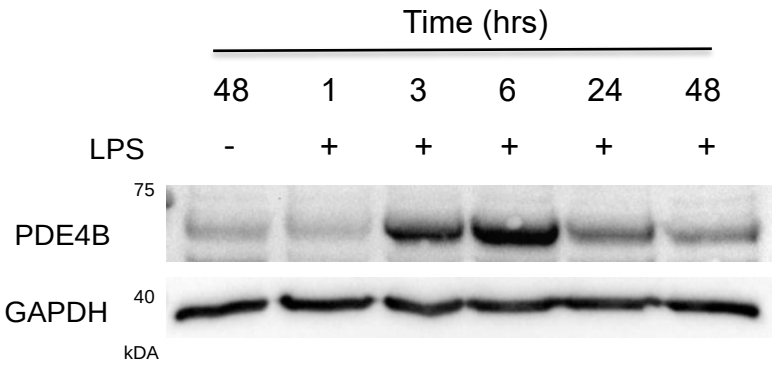



Figure 4.2 LPS-induced PDE4B2 expression

in U937 macrophages expression over time

U937 macrophages were treated with lipopolysaccharide (LPS) 50ng/ml, before total protein extract was obtained at the times indicated. Lysates were probed using anti sera to PDE4B and GADPH was used as the loading control. The data shown are representative of three separate experiments.

The determinants of protein expression, at any given moment, includes the rate at which messenger RNA is either translated to protein or degraded as well as the rate at which protein is degraded or otherwise, subject to any post-translation modification that might alter its ability to be degraded such as ubiquitination and delivery to the proteasome system, for example.

Since messenger RNA can be translated to protein continuously after a given treatment such as LPS, the use of a protein synthesis inhibitor such as cycloheximide can provide a truer estimate of protein turnover by preventing further addition to the protein pool (Eldeeb et al., 2019). I thus repeated a time course experiment of LPS induced PDE4B2 expression, but this time added cycloheximide three hours after the addition of LPS.

As shown in Figure 4.3, treatment with cycloheximide alone, does not induce PDE4B2 expression. However, when cycloheximide is added to LPS-treated U937 macrophages, PDE4B2 expression peaks at closer to four hours rather than at six hours, when LPS is used on its own. Moreover, the use of cycloheximide uncovers a rapid reduction in the expression of PDE4B2 protein, such that at six hours, the expression of PDE4B2 has almost returned to the levels at baseline (lane 5). Thus, within only around six hours, macrophage

PDE4B2 protein is both rapidly expressed and rapidly degraded, implying a highly dynamic process and one that may also be highly regulated.

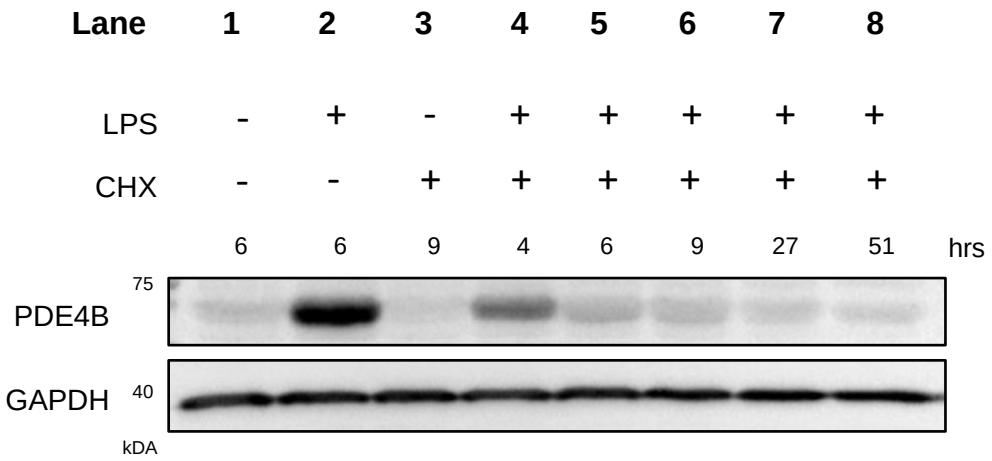



Figure 4.3 LPS induced PDE4B2 expression with cycloheximide.

U937 macrophages were treated with lipopolysaccharide (LPS) 50ng/ml for the times indicated. Cycloheximide (CHX) 50 ug/ml was added three hours after LPS, as indicated. Total extract was produced, and the lysates probed using anti-sera to PDE4B. GADPH was used as the loading control. The data shown are representative of two separate experiments.

Within eukaryotic cells, the ubiquitin-proteasome system (UPS) is the major pathway for the regulated degradation of most cytosolic, nuclear and membrane proteins (Livneh, Cohen-Kaplan, Cohen-Rosenzweig, Avni, & Ciechanover, 2016). Proteins destined for degradation are first conjugated to ubiquitin before processing by the 26S proteasome - a 2000-kDa ATP-dependent proteolytic complex (D. H. Lee & Goldberg, 1998).

A number of inhibitors have been used to investigate the UPS, but one of the most widely used is the selective proteasome inhibitor MG132 (Leu-Leu-Leu) (D. H. Lee & Goldberg, 1998). MG132 has also been deployed in PDE4-related research (Niimi et al., 2012) and was used to help demonstrate that the E3-ubiquitin ligase Mdm2, mediates PDE4D5 ubiquitination (X. Li, Baillie, & Houslay, 2009).

Using MG132, I first sought to determine if disruption of the proteasome pathway could 'rescue' LPS-induced PDE4B2 protein. As expected, LPS led to a marked induction of PDE4B2 expression at six hours. By 24 hours, PDE4B2 expression had returned to pre-treated levels (lane 4 (Figure 4.4).) I then observed the effect, on PDE4B2 expression, of increasing concentrations of MG132, added three hours after the addition of LPS. As shown below, MG132 at a concentration of 10uM, and also at 20uM, but not 1uM, was associated with an increased expression of PDE4B2 expression at 24hours. I propose that this change in expression is not due to changes in the induction of PDE4B2 transcript – as supported by data in a later experiment which shows that MG132 when given alone does not lead to induction of either PDE4B2 transcript or protein (Figure 4.6) – but through a direct inhibition on the proteasome pathway by MG132, that rescues PDE4B2 from degradation.

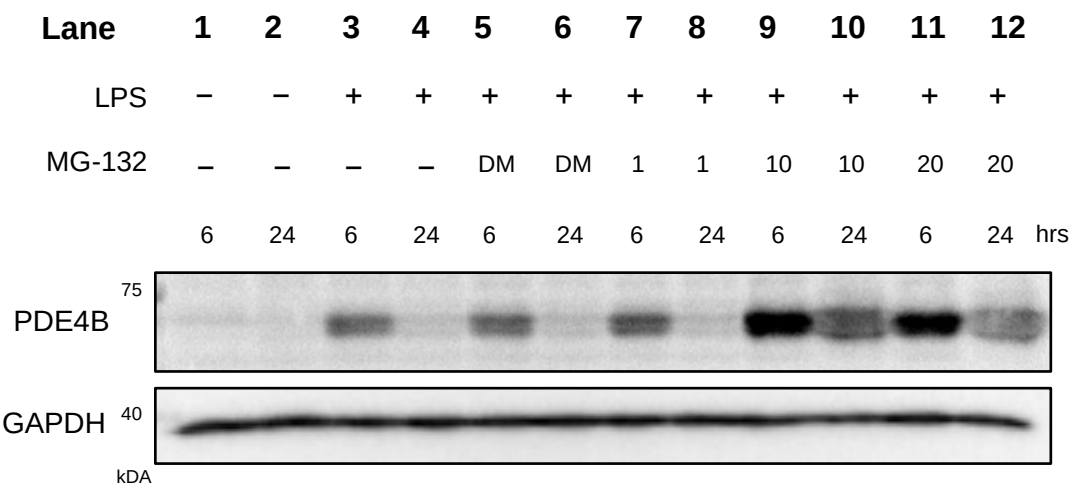



Figure 4.4 Proteasome inhibition of PDE4B2

U937 macrophages were treated with lipopolysaccharide (LPS) 50ng/ml as shown for either six or 24 hours. MG-132 (a proteasome inhibitor) or DMSO 0.2% v/v as a diluent control, was added three hours after the addition of LPS, as shown. Lysates were probed with antisera to PDE4B. GAPDH was used as a loading control. The data shown are representative of three separate experiments.

MG132 challenge (at both 10uM and 20uM), subsequent to LPS treatment, also enhanced PDE4B2 expression when compared with LPS treatment alone. An MG132 control was not added to the demonstrated immunoblot in order to prioritise the use of the available lanes. However, as described above and shown in Figure 4.6, I obtained data that suggests MG132 does not induce PDE4B2, either at the transcript level or at the protein level and therefore propose that the increased expression of PDE4B2 at both six and 24 hours is due to impaired degradation of PDE4B2, through the UPS.

The diluent control DMSO, was used at the highest equivalent concentration used for MG132, namely 0.2% v/v for a concentration of 20uM MG132 and had no effect on PDE4B2 expression.

Using co-immunoprecipitation (co-IP), I next set out to obtain data that would support an interaction between PDE4B2 and the ubiquitin system. Co-IP is a commonly used method using target protein-specific antibody in conjunction with Protein A/G affinity beads. The immunoprecipitates are fractionated by SDS-PAGE before co-immunoprecipiated proteins are then identified by western blot and an antibody directed against proteins of interest. I sought to pull down ubiquitinated species and to also pull down PDE4B2 using antibodies to respectively FK2 and PDE4B before blotting against PDE4B2 and FK2 to identify a protein-protein interaction.

LPS was used to increase PDE4B2 expression. MG132 was added 3 hours after LPS challenge in order to augment the amount of PDE4B2 conjugated to ubiquitin and therefore available for co-immunoprecipitation (Figure 4.5). In each blot, whole cell lysates (WCL) are shown alongside the target protein for immunoprecipitation. Starting first with Figure 4.5A and the blot on the left hand-side, the addition of MG132 given after LPS, enhances PDE4B2 expression – as was described also in Figure 4.4. Immunoprecipitation using anti-sera to PDE4B and then blotting using the antibody to PDE4B2, identifies a band migrating at 68kDa consistent with the selective immunoprecipitation of PDE4B2 from WCL. Turning to the blot on the right, also Figure 4.5A, using the same inputs of WCL and IP: 4B, I then blotted using an antibody against FK2, which identifies both mono- and poly-ubiquitin conjugates. As expected, this identified a smear across

the WCL where MG132 was added consistent with the disruption to proteasome degradation and an abundance of ubiquitinated species across a range of molecular sizes. Blotting using anti-FK2 did not though highlight any ubiquitinated species in the pull-down of PDE4B (lanes 3 and 4).

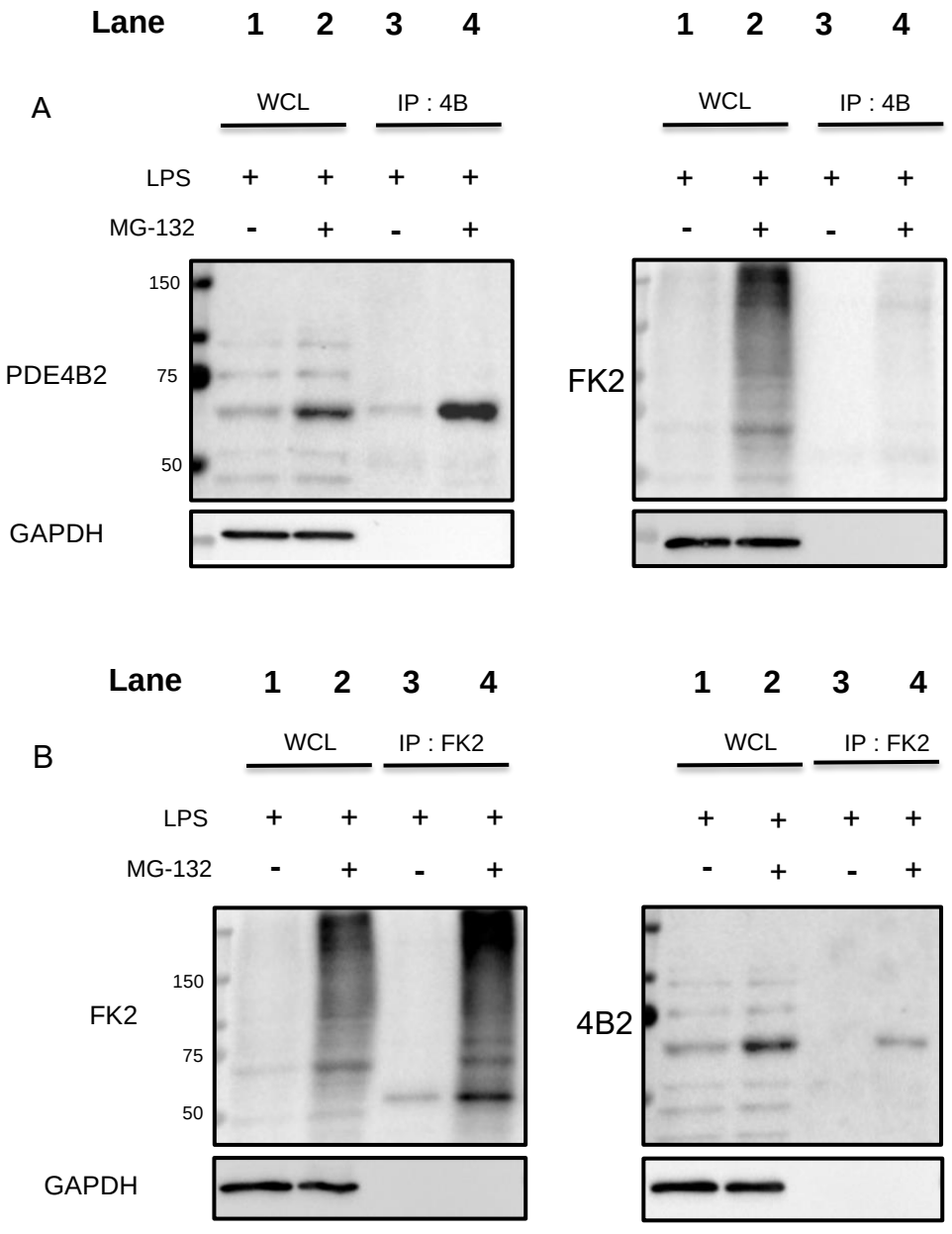



Figure 4.5 Co-IP of FK2 ubiquitin and PDE4B2

A co-immunoprecipitation (IP) was completed using alternately anti-PDE4B (A) or anti-FK2 (B), before blotting against both PDE4B2 and FK2 to identify a protein-protein interaction. U937 macrophages were treated with lipopolysaccharide (LPS) 50ng/ml for three hours, before the addition of the proteasome inhibitor MG132 20uM where shown and cultured for a further three hours, before cell lysates were obtained. The anti-ubiquitin FK2 antibody can be used to detect both mono and polyubiquitin species. The whole cell lysates (WCL) and immunoprecipitates were then separated using western blot and probed using alternately antisera to PDE4B2 and also with antisera to FK2. GAPDH was used as the loading control. Unfortunately an isotype IgG control was not included, which is discussed within the main text. The data shown are representative of three separate experiments.

Turning next to Figure 4.5B and, firstly, the blot on the left hand-side, immunoprecipitation using FK2 and then blotting against FK2, reveals a smear consistent with ubiquitinated proteins across a range of molecular weights, indicating a successful pull down. Turning to the blot on the right, when blotting against PDE4B2, using the same inputs, a single faint band is evident migrating at or around 68kDa, consistent with PDE4B2. This co-IP is consistent with the potential for interaction between PDE4B2 and ubiquitin and it is therefore proposed that, in macrophages, PDE4B2 is degraded by the ubiquitin-proteasome system.

However, there are a number of limitations of the data in Figure 4.5 that affect the conclusions that can be reliably drawn from data presented. This includes the absence of a non-binding antibody-bead control to help demonstrate the possible

effects of non-specific binding and/or eluted antibody fragments. Furthermore, and as is explored in the discussion Chapter 4.4.4, it may also be observed there was no clearly identified ubiquitinated species using anti-FK2, following the pull-down using anti-PDE4B (Figure 4.5A) and also that the band identified at around 68kDa, after blotting against PDE4B2 (Figure 4.5B) had not shifted higher as might have been expected with a PDE4B2 – ubiquitin interaction. This is explored further in Chapter 4.4.2.

## 4.3 BCL – 6 as a repressor to PDE4B2

The promoter to PDE4B2 has not been fully characterized. However, it is has been reported that the promoter region contains binding sites for both the transcription factors NF-κB and CREB (D'Sa et al., 2002). As part of an evaluation of the regulatory factors that might affect the transcription of macrophage PDE4B2, I undertook a collaboration with Dr David Henderson (Mironid Ltd, Glasgow, UK). Taking a bio-informatics approach, Dr Henderson interrogated the UCSD genome suite using Encode data to theorise which activator and repressor factors might interact with the PDE4B2 promoter. Such an approach predicted that the transcriptional repressor B-cell lymphoma 6 (BCL-6) might provide a novel means of regulating PDE4B2 transcription.

Although best known for its role in B-cell development and non-Hodgkin's lymphomas (A. L. Dent, Shaffer, Yu, Allman, & Staudt, 1997; B. H. Ye et al., 1997), BCL-6 has been shown to bind nuclear receptors and their corepressors that are linked to macrophage modulation of inflammation (Ghisletti et al., 2010; C. H. Lee et al., 2003; Ogawa et al., 2004). I thus set out to test a novel

hypothesis, namely that BCL-6 might provide a transcriptional brake on macrophage PDE4B2 whose uncoupling, by LPS, leads to enhanced PDE4B2 transcription.

An experiment was devised to stabilise any transcriptional repressors acting on the PDE4B2 promoter using the proteasome inhibitor MG-132. U937 macrophages were thus pre-treated with MG-132 and then, LPS was added to try to effect an induction of PDE4B2.

In untreated U937 macrophages, PDE4B2 transcripts and PDE4B2 protein are only modestly expressed, as detected by RT-qPCR and western blotting respectively. MG-132 does not lead to induction of PDE4B2, either at the transcript level or at the PDE4B2 protein level. However, I show here that MG-132 challenge is associated with the enhanced expression of BCL-6 (Figure 4.6B). This did not appear to be related to enhanced transcription of BCL-6 (data not shown). Thus I theorise that MG-132 acts to stabilise BCL-6 protein by inhibiting its degradation through the proteasome system.

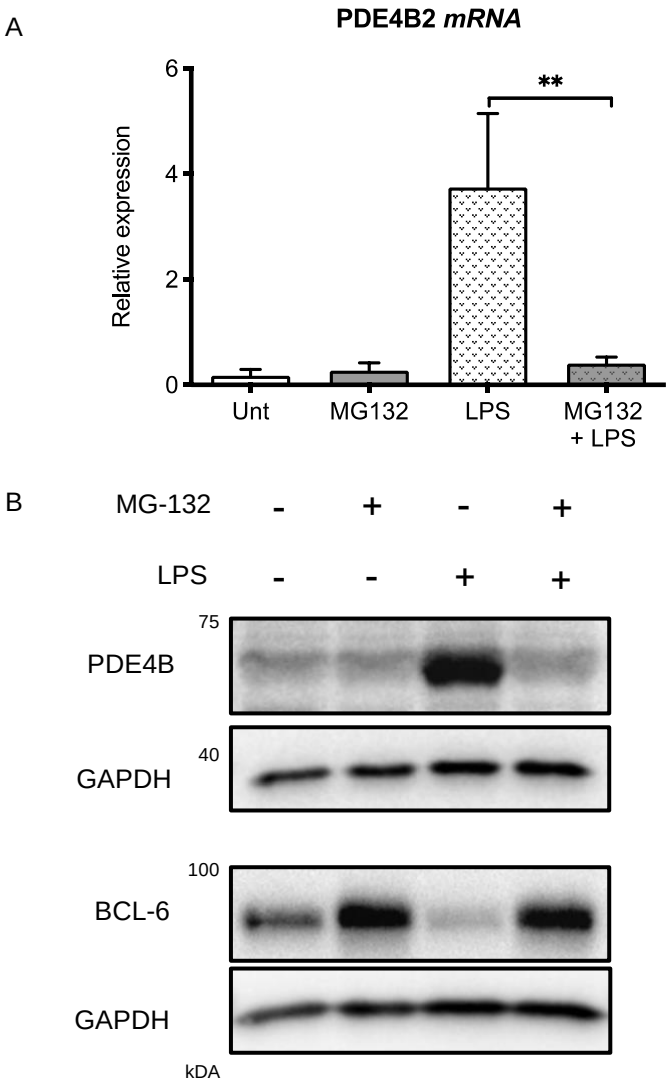



Figure 4.6 Inhibition of the proteasome attenuates LPS induction of PDE4B2

U937 macrophages were pre-treated with MG-132 (10uM) for one hour as shown, before the addition of lipopolysaccharide (LPS) 50ng/ml for three hours. The experiment was run in parallel and terminated before use for either RT-qPCR and the detection of PDE4B2 *m*RNA (A) or the lysates blotted and probed using anti-sera to PDE4B (B). Mean ± SD shown (A), $n = 4$ (RT-qPCR data). ** $P < 0.01$, using 2-tailed Student's *t*-test was

used. GAPDH was used as a loading control. The data shown are representative of three separate experiments.

As expected, LPS challenge led to a potent (x34) induction of messenger PDE4B2 (unt vs LPS 0.11 ± 0.02 v's 3.74 ± 1.40 $P$ <0.01 $n$ = 4). However, pre-treatment with MG-132, completely attenuates LPS induction of PDE4B2, with a 92% reduction of PDE4B2 transcription (3.74 ± 1.40 v's 0.28 ± 0.14 $P$ <0.01 $n$ = 4) and loss of inducible PDE4B2 protein. Intriguingly, this attenuation and the loss of PDE4B2 is mirrored by the expression of BCL-6. LPS, when given alone, leads to the loss of BCL-6 expression, but not when given after pre-treatment with MG132.

The rapid loss of BCL-6 following the addition of LPS is in line with findings reported by others. For example, in monocyte-derived dendritic cells, Pantano and colleagues (Pantano, Jarrossay, Saccani, Bosisio, & Natoli, 2006) used a protein synthesis inhibitor in a 'pulse-chase' assay to demonstrate that in unstimulated cells the half-life of BCL-6 was between 25–30minutes but that this reduced to a half-life of only 13 minutes following treatment with LPS.

Thus, it may be that, in untreated U937 macrophages, BCL-6 is constitutively expressed and is able to act as a brake on PDE4B2 expression. The addition of LPS, releases BCL-6 from the promoter to PDE4B2 (shunted it would appear to the proteasome) that leads inexorably to unchecked transcription of PDE4B2, mediated by downstream effectors of TLR4 engagement.

## 4.4 Discussion

I profiled the expression of inducible PDE4B2 protein over time and obtained data to support the proposition that induced PDE4B2 is rapidly degraded through ubiquitination. I also obtained evidence in support of our hypothesis that the transcriptional repressor BCL-6 may provide tonic negative control of PDE4B2 expression that is relieved by engagement with TLR4 agonists.

### 4.4.1 Evaluating the U937 macrophage model

An experimental objective of this Chapter was to gain mechanistic insight in to the regulation of PDE4B2 expression in macrophages. It was therefore necessary to secure an unlimited supply of cellular material to use to toward mapping signaling cascades and determinants of PDE4B2 protein expression. The human promonocytic U937 cell line is commonly used in myeloid based cell research and has further been established in PDE4 signaling by members of the Houslay group (S. J. MacKenzie & Houslay, 2000; M. C. Shepherd et al., 2004).

Chronically PMA treated U937 adopt a macrophage phenotype that models the TNF$\alpha$ response to LPS, observed in primary monocyte derived macrophages. U937 cells treated with PMA become adherent and start expressing ß2 integrin CD11b – a myeloid cell integrin involved in macrophage polarisation (Schmid et al., 2018). PMA treatment also induces expression of COX2 which is found in alveolar macrophages (Endo, Ogushi, Kawano, & Sone, 1998) and has been proposed as a marker of macrophage phenotypes (Koehler et al., 1990).

In studies of PDE4 biology, U937 macrophages were also found to model the expression of PDE4 isoforms found in primary cells. Indeed, the remodelling of

the PDE4 isoform profile in monocyte to macrophage differentiation in U937 macrophages phenocopies the change in PDE4 isoform profile found in human peripheral monocytes and macrophages derived from them (M. C. Shepherd et al., 2004). In totality, these observations helped underscore the robustness of using PMA differentiated U937 cells as a model to explore the regulatory mechanisms around macrophage PDE4B2 expression. Moreover, the use of a cell line also enabled a more tractable model system than for example the use of primary monocyte derived macrophages, to be used for studies in transfection and RNA interference.

One criticism of the use of a U937 cell model, is the absence of a standardised differentiation protocol for the use of PMA. The potential relevance of this has been neatly described in THP-1 cells – another immortalised monocyte-like cell derived from the blood of a childhood case of acute myeloid leukaemia (Tsuchiya et al., 1980). Using PMA at different concentrations and for varying length of exposure, leads to THP-1 macrophages that have different phenotypes and functions (Park et al., 2007).

Separate to the study by Park and colleagues, another study reported that in THP-1 macrophages, PMA at high concentrations (50-200ng/ml) and not low concentrations (8ng/ml) was associated with elevated expression of cytokines associated with the inflammatory response including TNF$\alpha$ and IL-8 (M. E. Lund, To, O'Brien, & Donnelly, 2016). However, these changes to gene expression were not followed by changes to protein readouts using ELISA, implying further regulatory breaks on protein translation. Moreover, Lund and colleagues also reported that a PMA-free period (more than or equal to 24hours) was associated

with an optimum yield of macrophage-like cells but also responsiveness to a stimulus with LPS. In the present study the use of low concentrations of PMA (4ng/ml) together with a PMA-free period of 24hours prior to their use in experiments is, it is proposed, likely to have enhanced the robustness of using a U937 based cell model.

In the present study, the effect of LPS on PDE4B2 protein expression was compared between U937 macrophages and MDMs polarised to M1 and M2 subsets, using western blot (Figure 4.1). In both U937 macrophages as well as MDM subsets, PDE4B2 protein is modestly expressed during basal conditions. However, LPS leads to a marked induction of PDE4B2 in both U937 and MDM macrophages that was evident at six hours.

There were notable differences in the loading control between U937 macrophages and primary macrophage subsets (Figure 4.1). These differences were not related to differences in the number of cells loaded for each experimental condition which was kept constant. The differences in protein loading were more likely related to the lower viability of primary cells in culture together with differences between U937 and MDM cell/protein ratios. As such, the absence of a standardisation step for protein loading may be considered a weakness of the protocol used.

The data shown in Figure 4.1 and described above, are in line with the findings of Jin and colleagues (Jin & Conti, 2002) who observed that, in THP-1 cells, PDE4B2 mRNA was barely detectable under basal conditions using Northern Blot. Indeed, data obtained in the present study, using RT-qPCR, also indicates

that PDE4B2 is modestly expressed in macrophages and when compared with monocytes (Figure 3.13). Yet, despite these observations that PDE4B2 is constitutively, only modestly expressed, Shepherd and colleagues reported that in resting U937 macrophages, PDE4B2 activity provided the dominant PDE4 activity (M. C. Shepherd et al., 2004). There are though important qualifications that must be noted when attempting to compare the results by Shepherd and colleagues who used functional assays of PDE4 activity rather than transcript (mRNA) readouts as done here and by Jin and colleagues. In this regard it should be noted that (i) there is not always a simple relationship between transcript numbers and protein expression and (ii) the relative specific activities of PDE4 isoforms is unknown and may not only differ between subfamilies and isoforms but also due to post-translational modification.

### 4.4.2 Inducible PDE4B2 is rapidly degraded

I next sought to gain insight into changes in the LPS-induced expression of PDE4B2 over time, using a time-course experiment of PDE4B2 expression between one and 48 hours. Using western blot, I observed LPS induced protein expression of PDE4B2 as early as three hours after treatment with LPS (Figure 4.2). This is line with the findings of Ma and colleagues who using Northern blot recorded that LPS stimulated PDE4B mRNA could be detected between 30 and 45mins (Ma et al., 1999).

Intriguingly, the rate at which PDE4B2 appears, is matched, and perhaps even surpassed, by the rate of its subsequent disappearance. Using a cycloheximide ‘chase’ assay, I observed that, within only six hours from the point at which U937 macrophages are exposed to LPS, PDE4B2 protein is both translated and then

degraded back to levels seen constituently (Figure 4.3). Thus, LPS challenge of macrophages leads to a transient and specific induction of the short PDE4B2 with the rapid rate of induction reaching a maximum after 4h of LPS challenge, and the consequent degradation of short PDE4B2 bringing its level back to basal levels after 6h. The transience of this effect is consistent with proteasomal activity – as it is stabilised by MG132. An alternative explanation, that for example the effects of LPS to induce PDE4B2 might wane, seems less likely, as it has been shown that LPS in culture with peritoneal macrophages is not readily degraded and displays slow kinetics (Forestier, Moreno, Pizarro-Cerda, & Gorvel, 1999).

It may be considered that the transience of PDE4B2 is not unexpected given the particular properties that govern the regulation of short PDE4 forms. Long PDE4 isoforms are characterised by the presence of both UCR1 and UCR2 regulatory domains and are regulated by post-translational modifications (Hoffmann et al., 1999; Oki, Takahashi, Hidaka, & Conti, 2000). The regulatory domain UCR1, which is unique to PDE4 long forms, provides a site for specific, endogenous regulation of activity through phosphorylation of this domain by various kinases such as PKA (Oki et al., 2000) and MAP kinase-activated protein kinase 2 (MK2) (K. F. MacKenzie et al., 2011) for example, and also by interaction with phosphatidic acid, which phenocopies activation elicited by PKA phosphorylation of UCR1 (Némoz, Sette, & Conti, 1997). In contrast, short PDE4 forms lack UCR1, and thus changes in the activity of PDE4 short forms are primarily determined by regulation of protein levels. This can be expected to be governed by manipulation of the rates of transcription, translation and degradation in response to extracellular signals.

In this study I set out to determine the processes underpinning the induction of macrophage PDE4B2 in response to LPS challenge. This is a crucially important process as activation of TLR4 by LPS promotes an inflammatory response (Jin & Conti, 2002) that from knockout studies of PDE4B (Jin et al., 2005) together with the inhibitory action of PDE4 specific inhibitors (Germain et al., 2001; J. X. Yang et al., 2017), it is clear the endogenous activation of PDE4B2 can be expected to facilitate macrophage activation and inflammatory activity subsequent to LPS challenge.

Inflammation is a co-ordinated process induced by infection or tissue injury, that once initiated must also be suppressed and brought to a halt to prevent additional tissue damage. It is proposed that the transient nature of LPS mediated induction of PDE4B2, affords the cell, the ability to help 'reset' and restore homeostatic balance, in order to prevent further harm. Thus, the rapid rate at which PDE4B2 is degraded from the cell appears to form an intrinsic part of its regulation within the cell. Such tight regulation of an important mediator of the inflammatory response confers an advantage to the cell's ability to respond to changing environmental cues as may occur in acute inflammation.

Having obtained data supporting the rapid turnover of PDE4B2 protein, I next considered what mechanisms might be involved in PDE4B2 degradation subsequent to LPS-induced induction. Since many signalling proteins can be regulated by being shunted through the proteasome pathway, I set out to evaluate the effect of a proteasome inhibitor MG-132 on this process. MG-132 added after LPS, rescues PDE4B2 from degradation, as observed at 24 hours. Interestingly, it could also be observed that at six hours after the addition of LPS, MG-132

combines with LPS to enhance PDE4B2 expression compared to the addition of LPS alone (Figure 4.4). This observation was due to the blockade of PDE4B2 degradation through the proteasome pathway – rather than MG-132 acting synergistically with LPS to induce PDE4B2 and supported by the findings of the cycloheximide chase assay that showed that even within 6 hours after treatment with LPS, the processes that drive PDE4B2 degradation are already firmly under way.

Using a co-IP, I then set out to obtain data that supported the interaction between PDE4B2 and ubiquitin. Using a system of Protein A/G magnetic beads and antibodies to FK2 and PDE4B, it was possible to alternately pull down ubiquitinated species and to also pull down PDE4B2. The FK2 (and FK1) antibodies are well characterised antibodies that do not recognise free ubiquitin but recognise specific forms of ubiquitin that have undergone confirmational change and are conjugated to a target protein either as a monoubiquitin or as a polyubiquitin chain (Danielson & Hope, 2013). Immunoprecipitation using anti-FK2 and blotting against PDE4B2 identifies a solitary band consistent with a PDE4B2 species. The data herein presented is consistent with an interaction between PDE4B2 and conjugated ubiquitin, but there are important limitations to the data, that warrant further discussion.

Whilst it was possible to pull down ubiquitinated species using anti-FK2 and blot against PDE4B2 to identify a solitary band around 68kDa – consistent with the predicted molecular weight of PDE4B2 – it was not possible to identify a clear ubiquitinated species using anti-FK2, following the pull down using anti-PDE4B (Figure 4.5A). The blot did though show the presence of faint bands with a

molecular weight towards 150kDa that may present polyubiquitinated species. However, this would need to be further studied with the inclusion of non-binding antibody-bead controls, which were unfortunately not included in this study and represents a limitation of the robustness of the data herein presented.

There are a number of factors that can contribute toward the outcome of a co-IP (J. S. Lin & Lai, 2017). One possibility that might account for the inconsistent co-IP results rests around the strength of interaction between PDE4B2 and ubiquitin proteins which might not have been strong enough to be detected in the experimental conditions used. This can be related to use of cell lysis buffers and further optimisation here may be helpful in a future study. Another possibility is epitope masking, where the binding site of the target protein is obscured under native conditions or as might occur following protein-protein interactions. However, it could also be noted that other causes that can affect the outcome of a co-IP including a lack of protein expression within the whole cell lysate, or the failure of the antibody to successfully pull-down the target protein are less likely to be relevant here, as the positive lysate control demonstrates a successful pull-down of PDE4B using anti-PDE4B.

Another interesting observation of the data provided in figure 4.5B concerns the absence of a shift in the molecular weight of the protein band identified using the anti-FK2 IP and then blotting using anti-PDE4B2. Ubiquitin is a small protein but it is estimated that mono-ubiquitination increases the apparent molecular weight in Western blot by approximately 8kDa and an even greater amount after polyubiquitination events (Seyfried et al., 2008). The presence therefore of a band

at 68 kDA is less consistent with a PDE4B2-ubiquitin interaction which it is expected would shift the observed molecular weight to be higher than 68kDa.

The degradation of PDE4 isoforms has been reported elsewhere. For example, Li and colleagues (X. Li et al., 2009) demonstrated the potential for PDE4 isoforms to be shunted to the UPS. They observed the β-agonist isoprenaline, triggers a rapid and transient ubiquitination of long PDE4D5 by the E3 ligase Mdm2 in primary cardiomyocytes and other cells. However Li as well as others (H. Zhu et al., 2010) have reported ubiquitination to occur only in PDE4D isoforms which contain a ubiquitin-interacting motif (UIM). If confirmed, the findings intriguingly raise the question as to whether ubiquitin binds to the unique N-terminal region of PDE4B2 or another binding motif.

As mentioned the co-IP lacked inclusion of a non-binding antibody-bead control but could be completed with further study. The use of a standard would also have brought clarity to the presence of multiple bands observed in the whole cell lysate input, when blotting against PDE4B2.

As the major pathway for the regulated degradation of the majority of cellular proteins, it may not be unexpected that the degradation of PDE4B2 would occur through the ubiquitin-proteasome system (UPS). Ubiquitination is induced by covalent binding of ubiquitin to lysine residues on target proteins and includes E1 activating, E2 conjugating and E3 ligase enzymes (Woo & Kwon, 2019). Ubiquitination is reversed by activation of deubiquitinases (DUBs) that depolymerise ubiquitin in polyubiquitin chains and cleaves isopeptide bonds between ubiquitin and target proteins (Leznicki & Kulathu, 2017).

The UPS performs a key role in determining individual protein abundance within cells. Critically, by controlling levels of key regulatory proteins, the UPS contributes to nearly every aspect of cellular function (J. Hanna, Guerra-Moreno, Ang, & Micoogullari, 2019). In disease, dysfunction of the UPS has been implicated in a diverse range of diseases including malignancy (J. Hanna et al., 2019) and the neurodegenerative diseases Alzheimer's and Huntington's (Lindquist & Kelly, 2011). In recent years, interest in the UPS has intensified following the approval of Bortezomib (Cavo, 2006) - a proteasome inhibitor for use in refractory multiple myeloma – and with it the validation that modulation of the proteasome system could be used for therapeutic gain.

Ubiquitination affects a range of cellular functions by regulating degradation of proteins by proteasomal and lysosomal processing. Central to this system are the E3 ubiquitin ligases, which interact with their substrates by direct binding (Cai et al., 2018). Smad ubiquitin regulatory factor 2 (Smurf2) is an E3 ubiquitin ligase that targets a range of protein substrates for degradation. In a model of liver fibrosis, over-expression of Smurf2 in livers in transgenic mice inhibits fibrosis through miR-132. Smurf2 activates the cAMP-PKA-CREB axis by facilitating the ubiquitination of PDE4B to increase miR-132 production which is regulated by CREB (Cai et al., 2018). Thus, in this model, manipulation of PDE4B could present an appealing target in attenuating liver fibrosis.

### 4.4.3 BCL – 6 and LPS induced PDE4B2

Given data that is published, together with data I have accrued then I believe that macrophage PDE4B2 is likely to serve as an important mediator of the

inflammatory response. An understanding of the factors that regulate the expression of PDE4B2 is critically important to help understand how PDE4B2 might contribute to the inflammatory environment, to determine if PDE4B2 is dysregulated in disease and also the therapeutic potential of targeting it with selective inhibitors. Using a combination of data from the USCD genome suite and Encode datasets on transcription factor binding sites, an analysis of conserved regions of the promoter to PDE4B2 was generously undertaken by Dr David Henderson (Mironid Ltd). A list of putative transcriptional activators and repressors was generated and, in a further analysis including experimental data from ChIP and whole genome analysis in the OCI-Ly1 cell line (a B cell lymphoma cell line) (Bertolo et al., 2013), a role for the transcriptional repressor BCL-6 at the promoter to PDE4B2 was theorised.

The proteasome inhibitor MG-132 led to increased BCL-6 expression, whereas treatment with LPS led to its complete absence (Figure 4.6). I further observed, that although pre-treatment here, with MG-132 blocks LPS induced PDE4B2 expression, in the Co-IP experiment (Figure 4.5) the combination of MG-132 and LPS, actually enhanced PDE4B2 expression. It is apposite to then ask, how can these seemingly contradictory results be reconciled? The likely explanation lies in the timing that MG-132 is given, relative to treatment with TLR agonists. This has also been described in THP-1 cells (A. C. Tang et al., 2018). In a study of novel anti-cancer strategies combining proteasome inhibitors and TLR adjuvants, Tang and colleagues demonstrated that MG-132 could have opposing effects on the expression of IL-1$\beta$1 – a pro-inflammatory mediator – contingent on whether LPS was given before or after the addition of LPS. Moreover, priming with LPS followed by MG-132 was associated with enhanced NF-$\kappa$B activity.

At least two possible scenarios emerge to help account for how proteasomal inhibition can be associated with an attenuated PDE4B2 expression response, to treatment with LPS. The first is that MG-132 blocks ubiquitin mediated proteolysis of the cytoplasmic inhibitor of NF-κB (IκB) (Nakajima, Kato, Takahashi, Johno, & Kitamura, 2011)– this has been further characterised as involving the E3 ubiquitin ligase and an E3 substrate component β-TrCP (Kanarek, London, Schueler-Furman, & Ben-Neriah, 2010). Since the promoter to PDE4B2, contains several NF-κB binding sites, it may be supposed that disruption to NF-κB signaling also leads to the disruption of PDE4B2 transcription at the promoter.

The second scenario as to how proteasomal inhibition attenuates LPS induced PDE4B2 expression, focuses on BCL-6 as a putative transcriptional repressor to PDE4B2. In this scenario, taking in to account the rapidity by which TLR agonists induce PDE4B2 expression, I'd hypothesize that the promoter to PDE4B2 may exist in a poised state, under transcriptional control by BCL-6, that is then evicted to the proteasome following activation by LPS (Figure 4.6).

#### 4.4.3.1 Linking BCL-6 and inflammation

There are multiple lines of evidence that highlight BCL-6 as an important regulator of the inflammatory response. BCL-6 deficient mice develop lethal neonatal pulmonary vasculitis as well as myocarditis (A. L. Dent et al., 1997; B. H. Ye et al., 1997). There is significant cistronic interplay between BCL-6 and NF-κB, which appears to provide a homeostatic break to limit the extent of NF-κB directed inflammatory responses in macrophages (Barish et al., 2010). In a model of BCL-$6^{-/-}$ bone-marrow derived macrophages, a third of the LPS-elicited

transcriptome was also controlled by BCL-6 (Barish et al., 2010). Moreover, in more than 60% of these co-regulated genes, the loss of BCL-6 mimicked LPS stimulation. BCL-6 has also been shown to be transcriptional repressor of chemokine gene expression in macrophages (Toney BCL-6 2000) and linked to another activator of the inflammatory response; lipopolysaccharide-induced TNF alpha (LITAF).

LITAF was initially identified as the P53-inducible gene 7 (therefore termed PIG7) in the DLD-1 colon cancer cell line (Polyak, Xia, Zweier, Kinzler, & Vogelstein, 1997). Subsequent studies have helped characterise some of the functions of LITAF including as a promoter of the secretion of inflammatory cytokines. In macrophages, it has been shown that LITAF binds to a sequence motif within the TNF$\alpha$ promoter - CTCCC (-515 to -511) – to activate the transcription of TNF$\alpha$ following stimulation with LPS (Myokai, Takashiba, Lebo, & Amar, 1999; X. Tang, Fenton, & Amar, 2003). Interestingly, in other cell types, LITAF can exert non-inflammatory functions. Mutations in LITAF cause abnormalities in protein degradation in the demyelinating neuropathy called Charcot-Marie-Tooth disease type 1C (Lacerda, Hartjes, & Brunetti, 2014; Somandin, Gerber, Pereira, Horn, & Suter, 2012; Street et al., 2003). In addition, LITAF has been implicated as a possible tumour suppressor in different malignancies (Bertolo et al., 2013) including in prostate cancer {Zhou, 2011 #401) and acute myeloid leukaemia (J. Liu et al., 2012).

In mature B cell lymphoma, LITAF is inactivated by epigenetic mutations {Mestre-Escorihuela, 2007}. LITAF expression is decreased in germinal centre (GC) B-cell-like diffuse large B–cell lymphoma which is characterised by constitutively

high expression of BCL-6. BCL-6 is normally expressed in the GCs of secondary follicles, structures where antibodies with high affinity for the antigen are generated during T-cell mediated humoral immune responses where it is described as the master regulator of the GC reaction (Basso & Dalla-Favera, 2010; Klein & Dalla-Favera, 2008). As well as being a target of BCL-6, LITAF may also regulate BCL-6 expression as it's silencing led to increased BCL-6 activity and expression of its target genes PRDM1 and c-Myc (Shi et al., 2016).

Expression microarray data of biopsy specimens from mature B-cell lymphoma patients as well as those derived from B cell lymphoma cells lines demonstrate that LITAF and BCL-6 share an oppositional expression (Bertolo et al., 2013; Shi et al., 2016). LITAF is a transcriptional target of BCL-6 through direct binding at its promoter (Bertolo et al., 2013).

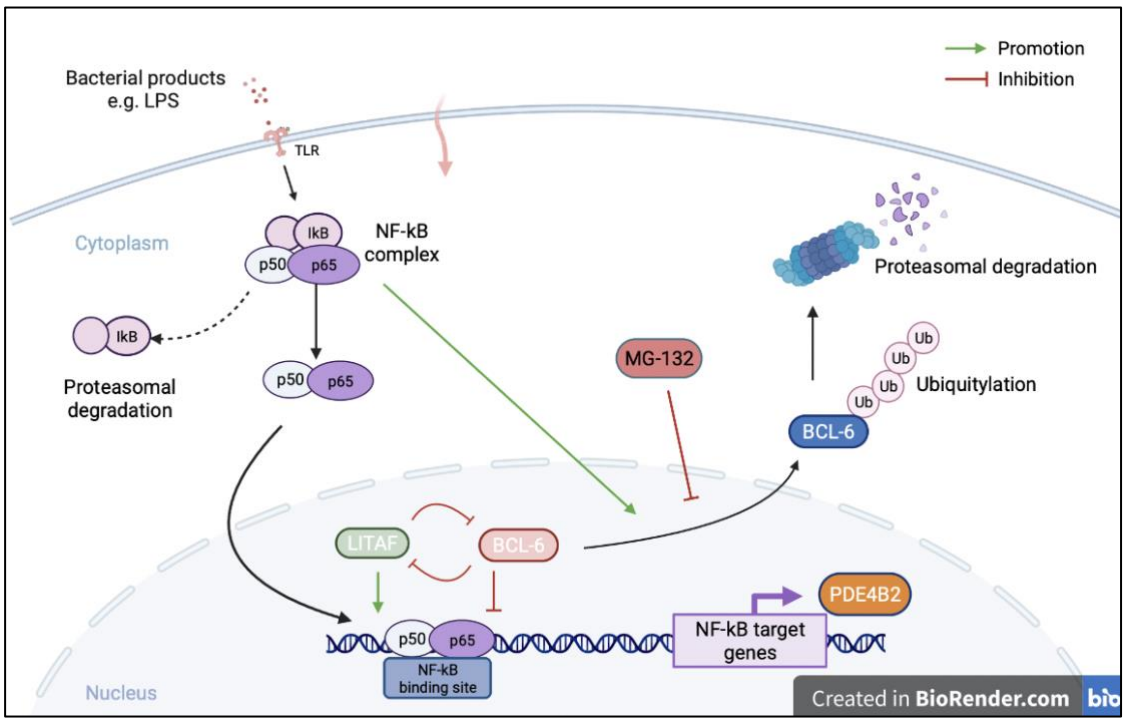



Figure 4.6. Schematic showing proposed transcriptional cross-talk between BCL-6, LITAF and LPS-induced transcription of PDE4B2

Bacterial products including lipopolysaccharide (LPS) engage through Toll-like receptors (TLR) to activate Nuclear factor-kappaB (NF-κB) signaling. In the resting state the NF-κB signaling complex (p65 (Rel A), RelB, c-Rel, p105/p50 (NF-κB1) and p100/52(NF-κB2), is under regulation by inhibitory IκB proteins (Oeckinghaus & Ghosh, 2009). TLR engagement leads to IκB ubiquitination and proteasomal degradation. This results in release of NF-κB dimers, which can then translocate to the nucleus and induce transcription of target genes including cAMP degrading phosphodiesterase-4B2 (PDE4B2). In the resting state PDE4B2 appears to be under tight transcriptional control, which is relieved upon engagement of TLR. We propose that B-cell lymphoma-6 (BCL-6) is a candidate transcriptional repressor of PDE4B2 and that pre-treatment with the proteasome inhibitor MG-132, stabilises BCL-6 and thereby constrains LPS (NF-κB)-mediated induction of PDE4B2. In the absence of MG-132, LPS leads to the loss of BCL-6 expression, which we propose is shunted to the proteasome by ubiquitination. A further regulatory control is provided by lipopolysaccharide tumour necrosis factor alpha (LITAF). In macrophages, LITAF can promote inflammation including the transcription of TNF (Myokai et al., 1999) and indirectly by inhibiting BCL-6. Indeed, in B-cell lymphoma, LITAF and BCL-6 share an oppositional expression (Bertolo et al., 2013).

Extending on from these reports, we propose that proteasomal inhibition and stabilisation of BCL-6, might also be associated with reduced expression of LITAF. Indeed, it may be expected that using either genetic interference with

siRNA or commercially available peptide inhibitors of BCL-6 (Polo et al., 2004), could uncouple the BCL-6-mediated repression of PDE4B2 and phenocopy the effect of treatment with LPS. Beyond these approaches, the use of ChIP and ChIP-seq would provide even more powerful data on the mechanistic link between BCL-6 and PDE4B2.

The possible role of BCL-6 in the regulation of PDE4B2, raises further interesting questions. For example, it has been shown that the transcriptomes of NF-κB and BCL-6 overlap in stimulated macrophages and a link between BCL-6 and PDE4B2 would help underscore the latter's role as part of the inflammation landscape. A direct link between BCL-6 and the expression of PDE4B2 would help identify that PDE4B2, perhaps uniquely amongst PDE4 isoforms is co-regulated by BCL-6 and TLR4 mediated signaling systems. Moreover, one might infer that such a cross-talk might be specific to macrophages since LPS does not regulate PDE4B2 in neutrophils (Wang et al., 1999).

Although genetic associations between BCL-6 and COPD have not been made, a study amongst Japanese patients with COPD has highlighted an association with BCL-2. In this study of 261 patients, Sata and colleagues (Sata et al., 2007) found an association of four SNPs of BCL-2 with lung function. Like BCL-6, BCL-2 is expressed in lymphoid cells and epithelial tissues including those that line the respiratory airways. The BCL-2 protein family is a regulator of apoptosis (Czabotar, Lessene, Strasser, & Adams, 2014) and intriguingly has been linked with the severity of emphysema (Sata et al., 2007). The identification of BCL-6 and PDE4B2, would though provide a truly game-changing paradigm and remains a priority for further study.

# Chapter 5 **CYTOKINE CROSS-TALK AND PDE4B2**

In an earlier Chapter, I used a well-characterised model of macrophage subsets to demonstrate that the TLR4 agonist LPS was a potent inducer of macrophage PDE4B2 in both M1 and in M2 monocyte-derived macrophages (MDMs).

Macrophage responses, including the induction of short PDE4B2, occur within a complex milieu of environmental signaling factors including cytokines, chemokines and cell-cell interactions that help shape the cellular response. In this Chapter, I investigate the potential for cross-talk between Th1 and Th2 cytokines involved in macrophage polarisation and LPS-mediated induction of PDE4B2. I explore IL-4-mediated constraint of LPS-induced PDE4B2 expression and use pharmacological inhibitors to help map the signaling pathways involved. I also develop a model of U937 nucleofection using silencing RNA that could form the basis of further study including targeting PDE4B2.

## 5.1 Th1 and Th2 Cytokines

In a previous Chapter, the Th1 and Th2 cytokines, IFN-$\gamma$ and IL-4, were used alongside colony stimulating factors (CSFs) to polarise MDMs towards, respectively, M1 and M2 macrophage subsets. Within the context of macrophage polarisation, I sought to explore if there could be cross-talk between these cytokines and LPS induction of macrophage PDE4B2. Moreover although a single study reports that both IL-4 and IL-10 constrains LPS-induced PDE4B2 expression in monocytes (Ma et al., 1999), this has not been reported in macrophages nor have the putative mechanisms by which it might occur been described.

In the next experiment, U937 macrophages were pre-treated with prototypical Th1 and Th2 cytokines, IFN-γ and IL-4 respectively, as well as other cytokines of interest, prior to treatment with LPS. Briefly, in the paragraphs below, I describe the relevance of assessing if IL-5, IL-13, IL-10 and also IL-6 had an effect on LPS-induced PDE4B2 expression.

IL-5 has a critical role in the maturation of eosinophils from bone marrow precursors as well as their trafficking in the lung to sites of inflammation (Kouro & Takatsu, 2009). Alongside IL-4 and IL-13, which are linked to allergy and atopic disease, IL-5 has gained significant interest as a therapeutic target in severe allergic asthma (McCracken et al., 2016).

IL-4 and IL-13 share similarities in their structure and their receptor usage and have long been considered to have overlapping roles with redundancy (see also Chapter 5.2). However, this has been called in to question following more recent discoveries that highlight differences in receptor distribution and receptor affinity to IL-4 and IL-13 (Gour & Wills-Karp, 2015).

IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of many inflammatory proteins, including several cytokines (TNFα, IL-1β, GM-CSF) as well as MMPs such as MMP-9 that are over expressed in COPD (P. J. Barnes, 2009). IL-10 concentrations are reduced in the sputum of patients with COPD (Takanashi et al., 1999) and the release of IL-10 is reduced in the peripheral lung of patients with COPD after LPS stimulation compared with lungs of smokers with normal function (Hackett, Holloway, Holgate, & Warner, 2008).

IL-6 is a pleiotropic cytokine and has been linked to both pro-inflammatory and anti-inflammatory responses. IL-6 appears to have an important protective role against infection, as evidenced for example in children who have auto-antibodies to IL-6 and have susceptibility to recurrent staphylococcal abscesses (Puel et al., 2008). However, its presence can also have a injurious effect, particularly in chronic inflammation where the monoclonal and anti-IL6R antibody Tocilizumab, has gained clinical utility in the treatment of inflammatory arthritis. Tocilizumab is now UK NICE approved for some types of vasculitis (Tocilizumab for treating giant cell arteritis, NICE Technology Appraisal 2018, UK). IL-6 is also of interest within PDE4 research as it has been associated with the development of mesenteric vasculitis in rats, that has complicated the development of novel PDE4 inhibitors (Daguès et al., 2007).

U937 macrophages were cultured separately with IFN-γ, IL-6, IL-5, IL-4, IL-13 and IL-10, first without LPS, and then with LPS (Figure 5.1A). PDE4B2 protein expression was determined using western blot at six hours.

Only IFN-γ and not any of IL-6, IL-5, IL-4, IL-13 or IL-10, used alone and separately, led to an induction of PDE4B2 expression (Figure 5.1A). Pre-treatment for 15min with IL-4, IL-13 and IL-10, but not with either IL-6 or IL-5, attenuates LPS-mediated induction of PDE4B2. On the other hand, pre-treatment with IFN-γ, led to enhance d expression of LPS induced PDE4B2.

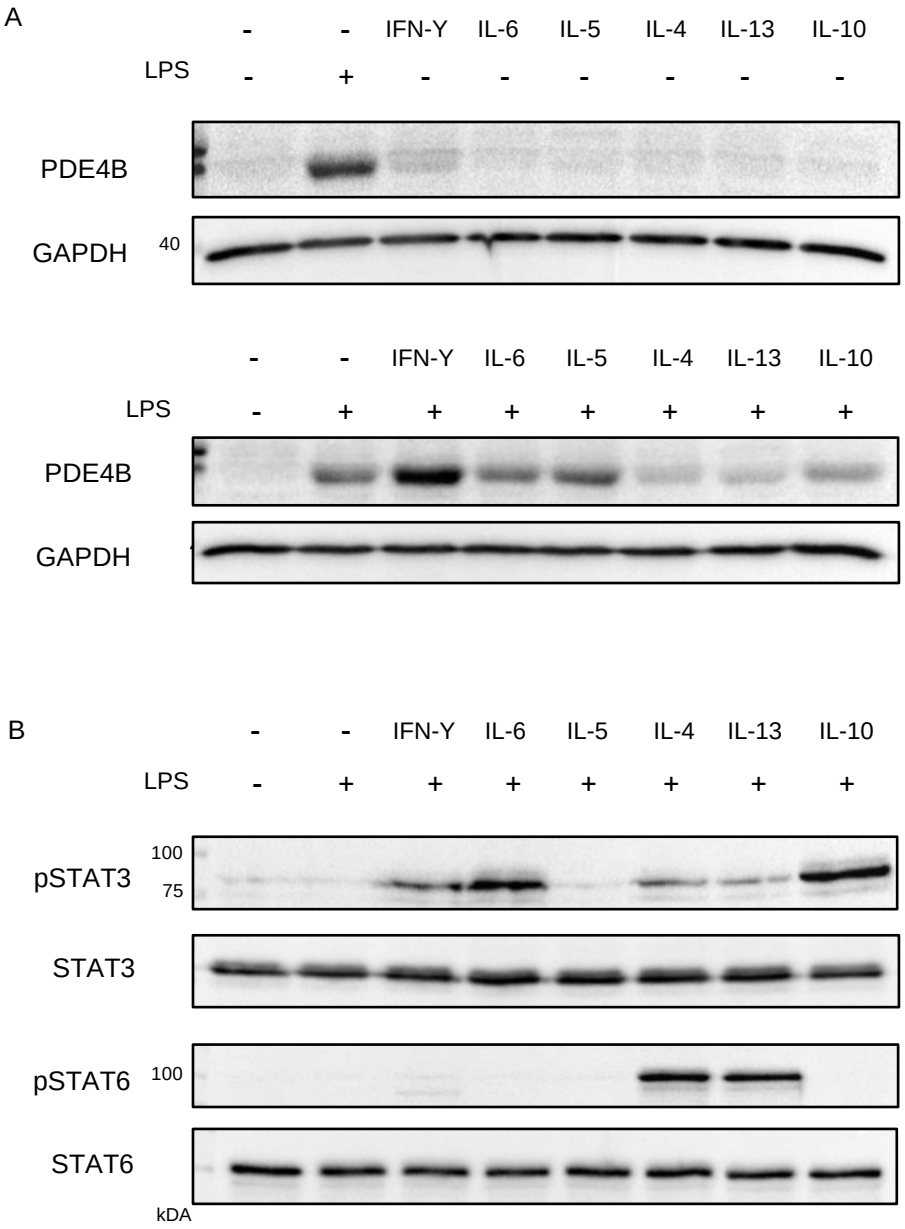



Figure 5.1 Effect of selected cytokines on LPS induced PDE4B2 and downstream effector signalling pathways

The effect of pre-incubation with various cytokines on LPS induced PDE4B2 was determined using U937 macrophages. (A) Cells were pre-treated to either IFN-Y (50ng/ml)/ IL-6 (50ng/ml)/ IL-5 (25ng/ml)/ IL-4 (40ng/ml)/ IL-13 (50ng/ml) or IL-10 (50ng/ml) for 15 mins, first without lipopolysaccharide (LPS) 50ng/ml (A; top blot) and then with LPS (A; lower blot), for six hours. The lysates were obtained and probed against PDE4B. GAPDH was used as a loading control. A parallel experiment was

completed (B) but terminated at 30 mins after the addition of LPS and 45 mins after pre-incubation with cytokines before lysates were obtained and probed for phosphorylated (p)STAT3(Tyr705) and pSTAT6(Tyr641), as well as appropriate controls. The data shown are representative of two separate experiments.

I next sought to explore the downstream signalling mechanisms that might be involved in mediating the effects of IL-4, IL-13 and IL-10 in constraining LPS-mediated induction of PDE4B2. Central to this, are the kinases of the janus kinase (Jak) family and the transcription factors of the signal transducer and activator of transcription (STAT) family. The Jak-STAT pathway underpins the signalling of over 50 cytokines which has helped make it the central communication node for the immune system (Villarino et al., 2017).

First turning to the structurally and functionally related IL-4 and IL-13. IL-4 and IL-13 are encoded by adjacent genes (chromosome 5q) and share about 25% sequence homology including several *cis*-activating transactivating regulatory regions. IL-4 and IL-13 also share a single functional receptor complex, namely IL-4$\alpha$/IL-13R$\alpha$ which affords overlapping but also some unique biological responses (Gour & Wills-Karp, 2015). Engagement by either IL-4 or IL-13 initiates activation of the STAT6 pathway (Kuperman & Schleimer, 2008).

IL-10 effects on the other hand, are mediated by STAT3. IL-10 is released by cells both of the myeloid and lymphoid lineage. It is a major suppressor of the immune response protecting the host from an over-exuberant response to pathogens. It is also implicated in wound healing, auto-immunity and disease where mice deficient in IL-10 develop colitis (Engelhardt & Grimbacher, 2014).

In Figure 5.1, it may be observed that IL-10 constrained LPS induced PDE4B2 induction. Since IL-10 activates STAT3, it may be reasoned that STAT3 is a repressor of PDE4B2 gene expression. However, it is has been proposed that STAT3 does not directly activate the anti-inflammatory response, but instead activates a number of effector genes (called anti-inflammatory response factors in one published Review (Murray, 2006), that themselves repress the expression of pro-inflammatory genes (Hutchins, Diez, & Miranda-Saavedra, 2013). In macrophages this may represent 20% of the pro-inflammatory genes activated by LPS (Lang, Patel, Morris, Rutschman, & Murray, 2002). In the present study, any contribution that STAT3 had on transcriptionally repressing PDE4B2 could be further assessed first using bioinformatics and a determination of whether the promoter to PDE4B2 had a binding site for STAT3 and also experimentally by selectively targeting STAT3 (Zou et al., 2020) to see if IL-4 mediated constraint was dependent upon it.

Antibodies specific to phosphorylated (p) STAT3 and pSTAT6, as well as to their non-phosphorylated controls, were used to identify the activation of both of these respective pathways following the addition of IFN-γ, IL-6, IL-5, IL-4, IL-13 and IL-10 (Figure 5.1B). In a repeat of the experiment described in Figure 5.1A, U937 macrophages were pre-treated with selected cytokines prior to the addition of LPS but this time, the lysate was obtained at 30 minutes after the addition of LPS – in line with the antibody manufacturer's recommendations and to better characterise phosphorylation of STAT3 and STAT6.

As shown in Figure 5.1B, STAT3 and STAT6 are not phosphorylated during basal conditions, as determined using western blot. STAT6 is phosphorylated by IL-4

and the related IL-13, but not any of the other cytokines included, namely IFN-γ, IL-6, IL-5 or IL-10. STAT3 meanwhile, appears to demonstrate more redundancy for cytokine engagement than for example STAT6 and was phosphorylated by both IL-6 and IL-10, as well as less robustly by IFN-γ, IL-4 and IL-13.

Interestingly, although treatment with both IL-6 and IL-10 leads to the phosphorylation of STAT3, only treatment with IL-10, leads to attenuation of LPS induced PDE4B2 expression. This has been described and may partly relate to the action of SOCS3 (Murray, 2007).

Challenge with IFN-γ was only weakly associated with phosphorylation of STAT3, since it predominantly activates phosphorylation of STAT1 (Murray, 2007). Meanwhile challenge with IL-5 is associated with Jak2-STAT5 but not STAT3 or STAT6 (Murray, 2007).

I next sought to obtain data to better characterise which downstream signalling pathways might be involved in cytokine mediated constraint of LPS induced PDE4B2 expression. Considering the candidate cytokines IL-4, IL-13 and IL-10 which all constrain induction of PDE4B2, IL-4 was chosen for further study.

## 5.2 IL-4 constraint of PDE4B2 expression

IL-4 and IL-13 are the signature cytokines of the Th2 response (Maier, Duschl, & Horejs-Hoeck, 2012). In allergy and asthma, IL-4 has a key role in some of the important pathogenic mechanisms that help drive these two diseases, including the differentiation of naïve CD4 T cells to Th2 cells, and the immunoglobulin (Ig)

class switch to IgG1 and IgE in B cells. In macrophages, IL-4 (and IL-13) induce alternative macrophage activation (Ilkka S. Junttila, 2018).

Focusing on IL-4, I first sought to identify the effect of increasing concentrations of IL-4, on the constraint of LPS induced PDE4B2 expression. IL-4 dose-dependently attenuates LPS-induced PDE4B2 expression (Figure 5.2). In the experiment shown in Figure 5.1, IL-4 was deployed at a concentration of 40ng/ml - a concentration sufficient to activate phosphorylation of STAT6 and lead to constraint of induced PDE4B2. However, as shown in Figure 5.2, doubling the concentration of IL-4, had either no more or only a modestly more, effect on constraining LPS induced PDE4B2 expression. Hence, in all other subsequent experiments, IL-4 was used at a concentration of 40ng/ml unless otherwise stated.

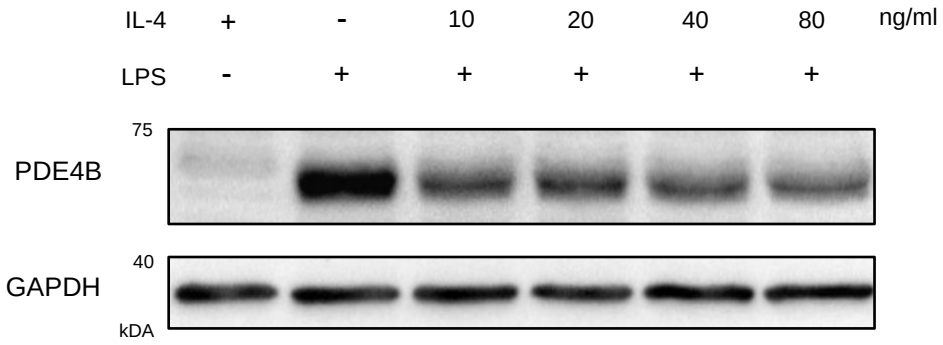



Figure 5.2 IL-4 constraint of LPS induced PDE4B2 expression

U937 macrophages were pre-treated with IL-4 for 15 mins at the concentrations shown, prior to the addition of lipopolysaccharide (LPS) 50ng/ml. Total extract was produced at six hours after the addition of LPS before blotting using anti-sera to PDE4B.. GAPDH was used as a loading control. The data shown are representative of two separate experiments.

The fate of IL-4 engagement at the cell surface is dependent upon which of two possible receptor complexes – type 1 or type II – are activated. The type I receptor is composed of IL-4Rα and common gamma chain (γC), while the type II receptor is composed of IL-4α and IL-13Rα1 (McCormick & Heller, 2015).

IL-4 binds to IL-4Rα and leads to the recruitment of either the common γC or IL-13Rα1 subunits. While the IL-4α and IL-13Rα1 components are widely expressed, the γC chain is primarily expressed on haematopoietic immune cells including macrophages. Therefore, it is the availability of each chain (either γC or IL-13Rα1) on the cell surface, that determines the signalling pathway, that is activated within the cell (I. S. Junttila et al., 2012).

IL-4 binding of the type I receptor complex leads to the phosphorylation of Jak 1/3, which in turn phosphorylates tyrosines within the IL-4Rα domain. These phospho-tyrosine residues create docking sites for STAT6 and insulin receptor substrate-2 (IRS-2) (McCormick & Heller, 2015). STAT6 Tyrosine phosphorylation of promotes its homo-dimerisation, nuclear translocation and gene transcription, while tyrosine phosphorylation of IRS-2 leads to activation of the PI3-K/ Akt/ mTOR pathway.

Although the Jak-STAT and PI3K pathways, appear to mediate most of the functional outcomes following IL-4R engagement, other pathways including the p38 MAPK pathway also serve an important role. For example, IL-4 induces phosphorylation of p38 MAPK to help induce alternative activation of peritoneal macrophages (Jiménez-Garcia et al., 2015).

Given, the possibility that IL-4 constraint of LPS-induced PDE4B2 expression could be mediated by more than one signalling cascade, I set out to deploy pharmacological inhibitors in turn to each of the Jak-STAT, PI3K and MAPK pathways to see which might reverse the effect IL-4 had on PDE4B2 expression. A schematic shown in Figure 5.3 illustrates the approach I used and the inhibitors that were deployed.

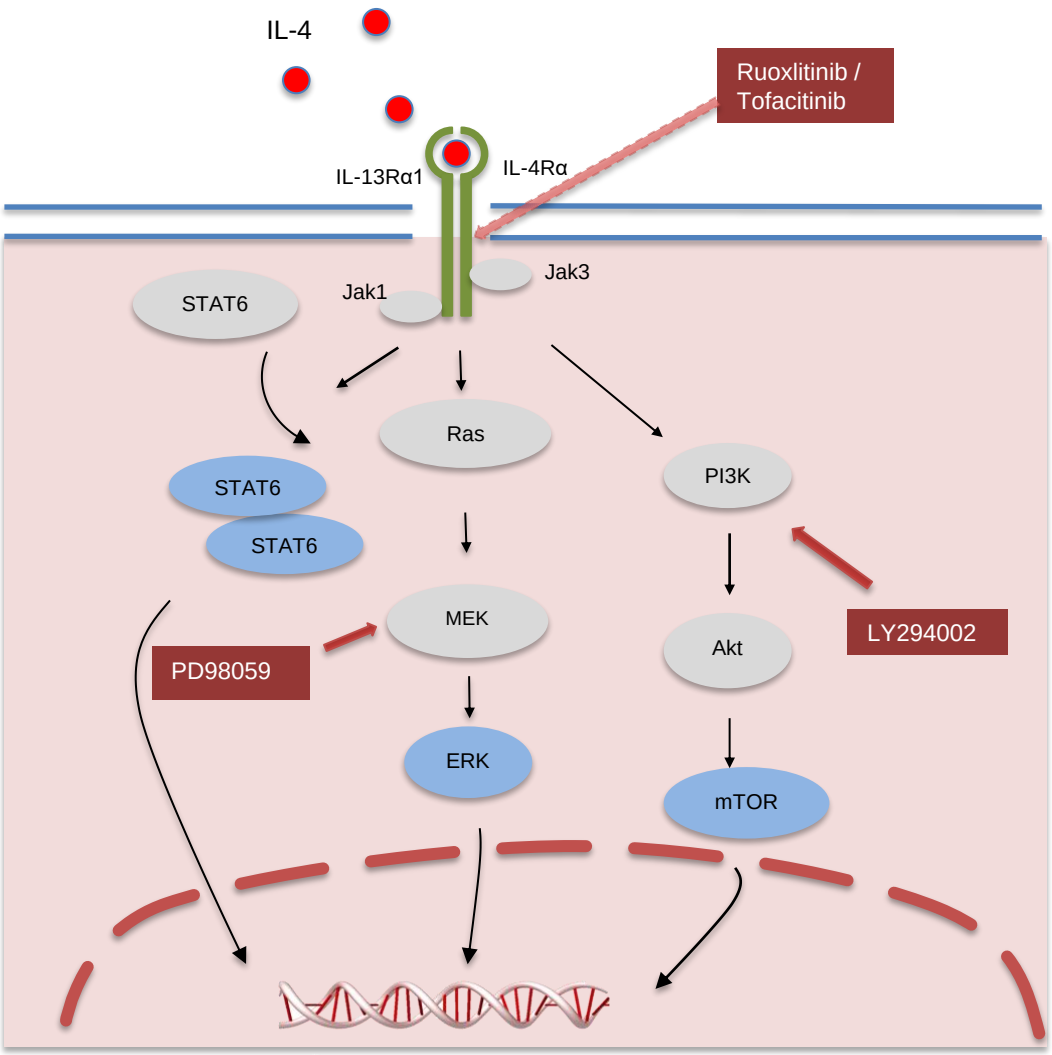



Figure 5.3 Schematic of IL-4 signalling pathways

and pharmacological blocking approaches

adapted from Jiménez-Garcia, Herránz, Luque, & Hortelano, 2015;

McCormick & Heller, 2015

Cellular responses to IL-4 are mediated by two different types of IL-4 receptor (R) complexes, type I and type II. The type I receptor is composed of IL-4Rα and the common gamma (γc) (not shown here) while the type II receptor is composed of IL-4α and IL-13Rα1. Binding of IL-4 to the type I receptor (the predominant type found in macrophages) activates janus kinase (Jak)1 and Jak3 which can then stimulate signal transducer and activator of transcription (STAT)6, phosphatidylinositol-3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinases (ERK), pathways. The use of pharmacological inhibitors including LY294002, an inhibitor of PI3K, PD98059 a inhibitor of MEK or the Jak inhibitors ruxolitinib and tofacitinib were deployed in turn, to selectively disrupt IL-4 signaling pathways, as part of a study of IL-4-constraint of LPS induced-PDE4B2 expression.

### 5.2.1 PI3K/Akt/mTOR pathway

Phosphatidylinositol 3-kinases (PI3Ks) regulate cellular signalling networks that are linked to survival, growth, proliferation, metabolism and specialist differentiated functions of cells (Gharbi et al., 2007). Owing to the critical role of the PI3K/Akt axis in cellular physiology, its perturbation has been linked to various diseases including cancer, diabetes and inflammatory diseases (Drees, Mills, Rommel, & Prestwich, 2004). LY294002 is a selective inhibitor of PI3K kinase-dependent phosphorylation of Akt and has been described as the ‘drug of choice’ in PI3K studies (Gharbi et al., 2007).

I started first with a time course to help optimise the time at which phosphorylated Akt (p-Akt) could be detected in U937 macrophages, using western blot. Using anti-sera to both p-Akt and total Akt, p-Akt was detectable from 15min through to around three hours after treatment with IL-4, peaking at around 30minutes (Figure 5.4). In line with the recommendations from the manufacturer of the antibody, U937 macrophages were serum starved and therefore, p-Akt was not evident under basal conditions.

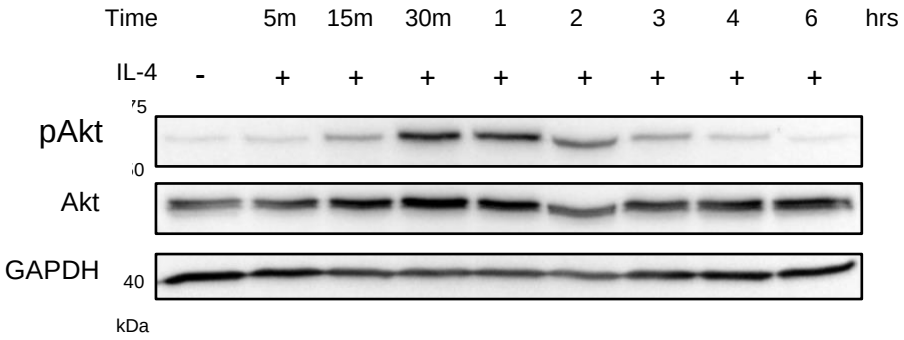



Figure 5.4 Time course of IL-4 phosphorylation of Akt

Serum starved U937 macrophages were treated with IL-4 (40ng/ml) for the times indicated. Total extract was produced and phosphorylation of Akt was detected using anti-pAkt(Ser473) antibody. Akt and GAPDH were used as loading controls.

Next, I used LY294002 to target the PI3K pathway in order to obtain data as to whether disruption of the PI3K-pAkt signalling pathway could uncouple IL-4 mediated constraint of LPS induced PDE4B2 expression. An escalating dose range of LY294002 was used at 1, 5 and 10uM. Dimethyl sulfoxide (DMSO) was the diluent control and used in this and in other experiments at a concentration equivalent to the highest dose of inhibitor.

U937 macrophages were prepared as previously described, before incubation with LY294002 or DMSO control. After 20 minutes, IL-4 was added, as shown and after a further 15 minutes, LPS was added, also as shown (Figure 5.5). The experiment was completed in parallel and lysates were obtained both at 30 minutes after addition of LPS to assess the phosphorylation of Akt and also at six hours after the addition of LPS, to assess the effect upon PDE4B2 expression.

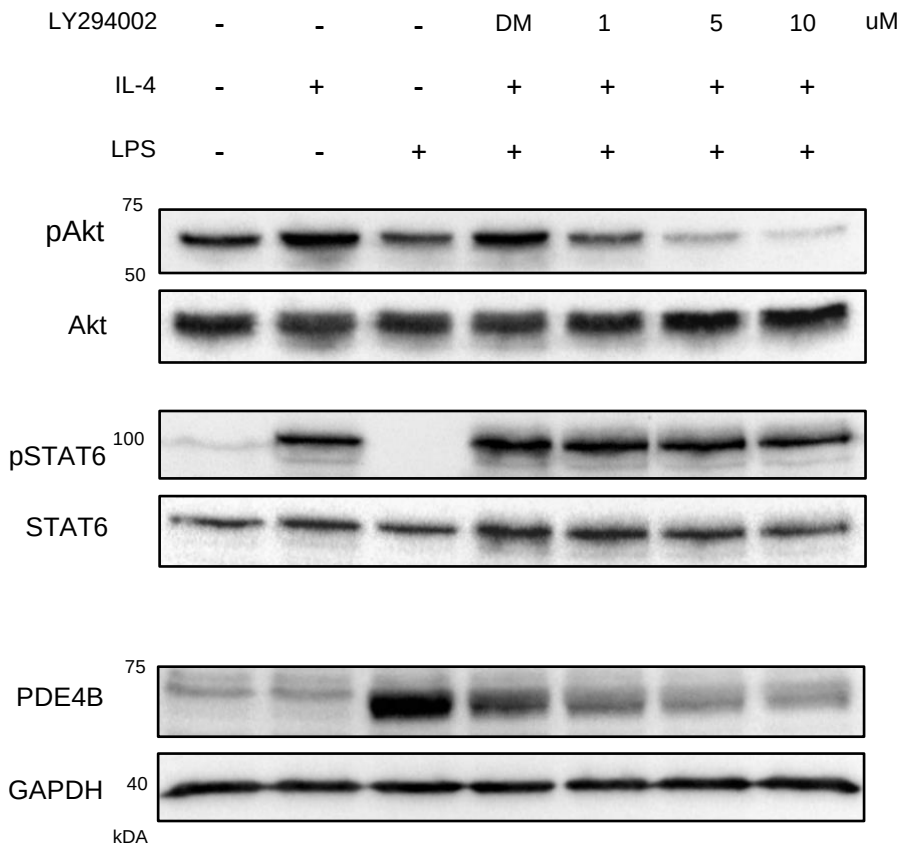



Figure 5.5 IL-4 inhibition of LPS induced PDE4B2 is not dependent upon PI3K signaling

U937 macrophages were pre-treated with LY294002, an inhibitor of the phosphatidylinositol-3-kinase (PI3K) pathway, at the doses indicated, 20 min prior to the addition of IL-4 (40ng/ml). Lipopolysaccharide (LPS) 50ng/ml was added 15 min after IL-4. DMSO (DM) 0.1% v/v was the

diluent control. Total extract was produced following incubation with LPS at 30 min pAkt(Ser473)/pSTAT6(Tyr641) and also at six hours for PDE4B2. Loading controls as shown. The data shown are representative of three separate experiments.

Unlike the experiment shown in Figure 5.4, the U937 cells were not maintained in serum starved media due to the extended culture period. As such, in this experiment p-Akt was detected during basal conditions. IL-4 but not LPS leads to increased p-Akt expression. LY294002 reduces p-Akt in a dose-dependent manner. At 5uM, LY294002 reduces p-Akt to less that that at basal conditions and at a concentration of 10uM, completely blocks p-Akt. Despite increasing blockade of p-Akt, LY294002 does not reverse the IL-4 constraint of PDE4B2 expression. Interestingly, LY294002 had no effect on p-STAT6 expression. This data leads me to propose that the IL-4 mediated constraint of LPS-induced PDE4B2 is not PI3K mediated.

### 5.2.2 MAPK

Next I moved on to explore the role that the mitogen activated protein kinase (MAPK) pathway might have in IL-4 mediated constraint of induced PDE4B2 expression. The MAPK system helps regulate cell growth, stress responses, apoptosis and immune defence (Grewal, Molina, & Bardwell, 2006). There are at least three well-studied distinct MAPK pathways: ERK 1/2, JNK and p38 MAPK. Although less well established than Jak-STAT and the PI3K pathways, IL-4 signalling through the MAPK pathway has been reported in macrophages and may account for IL-4 mediated activation of SOCS3 (Canfield, Lee, Schröder, & Rothman, 2005), a marker of M1 macrophages as discussed in Chapter 3.3.2.

The compound PD98059 is a selective inhibitor of the serine/threonine kinase, Mek. PD98059 inhibits Mek1 and Mek2 that, upon their phosphorylation by Raf – an effector belonging to the Ras superfamily, in turn phosphorylate ERK1/2, which then translocates to the nucleus Figure 5.3 (Grewal et al., 2006; Molina & Adjei, 2006).

A time course of IL-4 activation of phosphorylated p44/42 (ERK1/ERK2) was completed. IL-4 phosphorylation of p44/42 was evident from 15 minutes after the addition of IL-4, before peaking at around 30 minutes (Figure 5.6). In line with the experimental time points used for other phosphorylation events, and to allow time for LPS to also equilibrate during experimental conditions, whole cell lysates obtained to probe for p-p44/42, were obtained 45 minutes after the addition of IL-4.

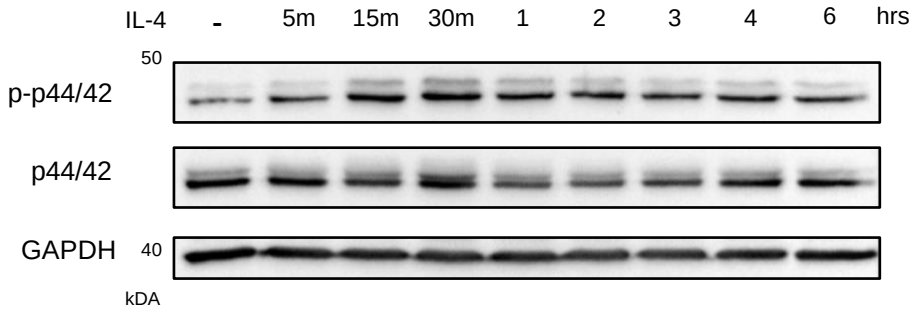



Figure 5.6 Time course of IL-4 phosphorylation of p44/42 (ERK 1/2)

Serum starved U937 macrophages were treated with IL-4 (40ng/ml) for the times indicated. Total extract was produced and phosphorylation of p44/42 was detected using anti-p-p44/42(Thr202/Thr204) antibody. p44/42 and GAPDH were used as loading controls. The data shown are representative of two separate experiments.

Mirroring the approach outlined in Figure 5.5 and the use of LY294002 to explore the PI3K/Akt pathway, I next completed a similarly designed experiment using the Mek inhibitor PD98059. As shown in Figure 5.7, IL-4, but not LPS, leads to increased phosphorylation of p44/42. Treatment with PD98059 reduces p-p44/42 dose-dependently. At 5uM, PD98059 reduces p-p44/42 to a level less than that detected during basal conditions and at 10uM, PD98059 blocks phosphorylation of p44/42 completely. Yet, as with LY294002 and pAkt, despite the effective blockade of p-p44/42, the disruption of ERK signalling does not constrain phosphorylation of STAT6 and it does not reverse IL-4 mediated constraint of LPS induced PDE4B2 expression. The data herein presented, supports the proposition that IL-4 constraint of induced PDE4B2 expression, is not ERK dependent.

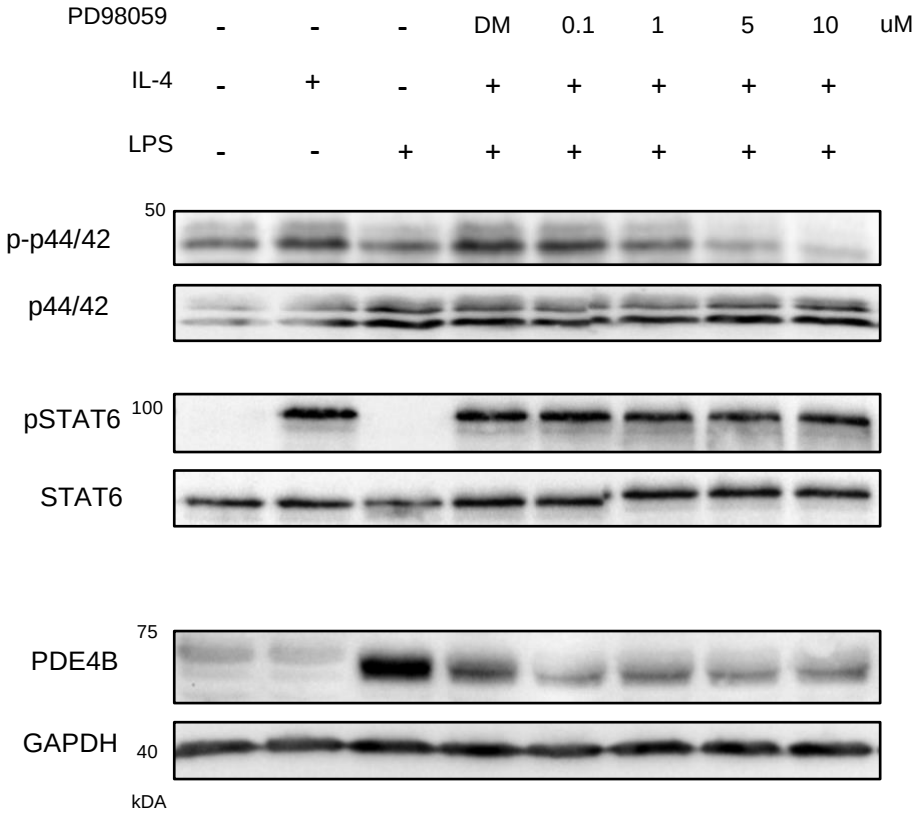



Figure 5.7 The ERK inhibitor PD98059 does not reverse IL-4 mediated constraint of LPS-induced PDE4B2 expression

U937 macrophages were pre-treated with PD98059, an inhibitor of mitogen-activated protein kinase (MEK)1, at the doses indicated, 60 min prior to the addition of IL-4 (40ng/ml). Lipopolysaccharide (LPS) 50ng/ml was added 15 min after IL-4. DMSO (DM) 0.1% v/v was the diluent control. Total extract was produced following incubation with LPS 30 min for p-p44/42(Thr202/Thr204) /pSTAT6(Tyr641) and also at six hours for PDE4B2. Loading controls as shown. The data shown are representative of three separate experiments.

### 5.2.3 Jak-STAT

Finally, I next turned to the well described Jak-STAT signalling system. Using selective inhibitors to block in turn Akt and then separately the MAPK pathways, I have provided data that shows IL-4 mediated constraint of LPS induced PDE4B2 expression is not dependent on either PI3K or ERK signalling. Moreover, using the selective inhibitors LY294002 and PD98059, I have also demonstrated that the phosphorylation of STAT6 is not dependent upon either p-Akt or p-p44/42 pathways.

I next exploited two clinically relevant Jak inhibitors – ruxolitinib and tofacitinib - to target the Jak-STAT signalling to further probe how IL-4 was mediating its effects on PDE4B2 expression. Both ruxolitinib and tofacitinib are not selective for an individual Jak protein but have a profile of selectivity with ruxolitinib known to target Jak1/Jak2 and Tofacitinib considered to target Jak1/Jak3 (Fragoulis et al., 2019; Villarino et al., 2017).

I first completed a time course of IL-4 induced phosphorylation of Jak3 and also phosphorylated STAT6. IL-4 mediated phosphorylation of Jak3 occurs from around 15 minutes and appears to peak at around 30 minutes, before reducing again from two hours. IL-4 mediated phosphorylation of STAT6, occurs even earlier than p-Jak3 and was evident at only five minutes from exposure to IL-4, persisting for several hours afterwards. A specific time course was not completed for Jak1 but the time points selected were found to be suitable to determine p-Jak1 signalling.

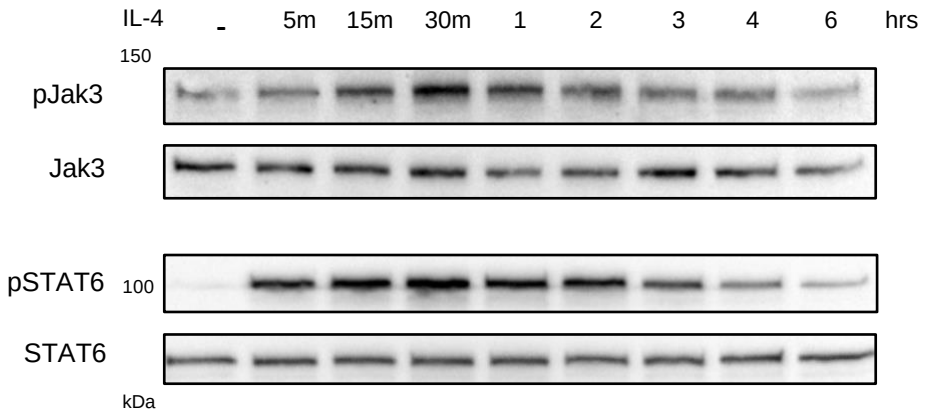



Figure 5.8 Time course of IL-4 mediated phosphorylation of Jak3 and STAT6

kDA

U937 macrophages were treated with IL-4 (40ng/ml) for the times indicated. Total extract was produced and phosphorylation of pJak3(Tyr980/981) and pSTAT6(Tyr641) was detected. Loading controls were as shown. The data shown are representative of two separate experiments.

In a similarly designed experiment to those described in Figure 5.5 and Figure 5.7,I next used the Jak1/Jak2 inhibitor ruxolitinib to attempt to reverse IL-4

mediated constraint of PDE4B2 expression. U937 macrophages were pre-treated with increasing concentrations of ruxolitinib for 60 minutes, before the addition of IL-4. LPS was added 15 minutes after the addition of IL-4 before lysates were obtained both at 30 minutes and also at six hours to characterise both phosphorylation of Jak-STAT proteins and determine the effect on PDE4B2 expression.

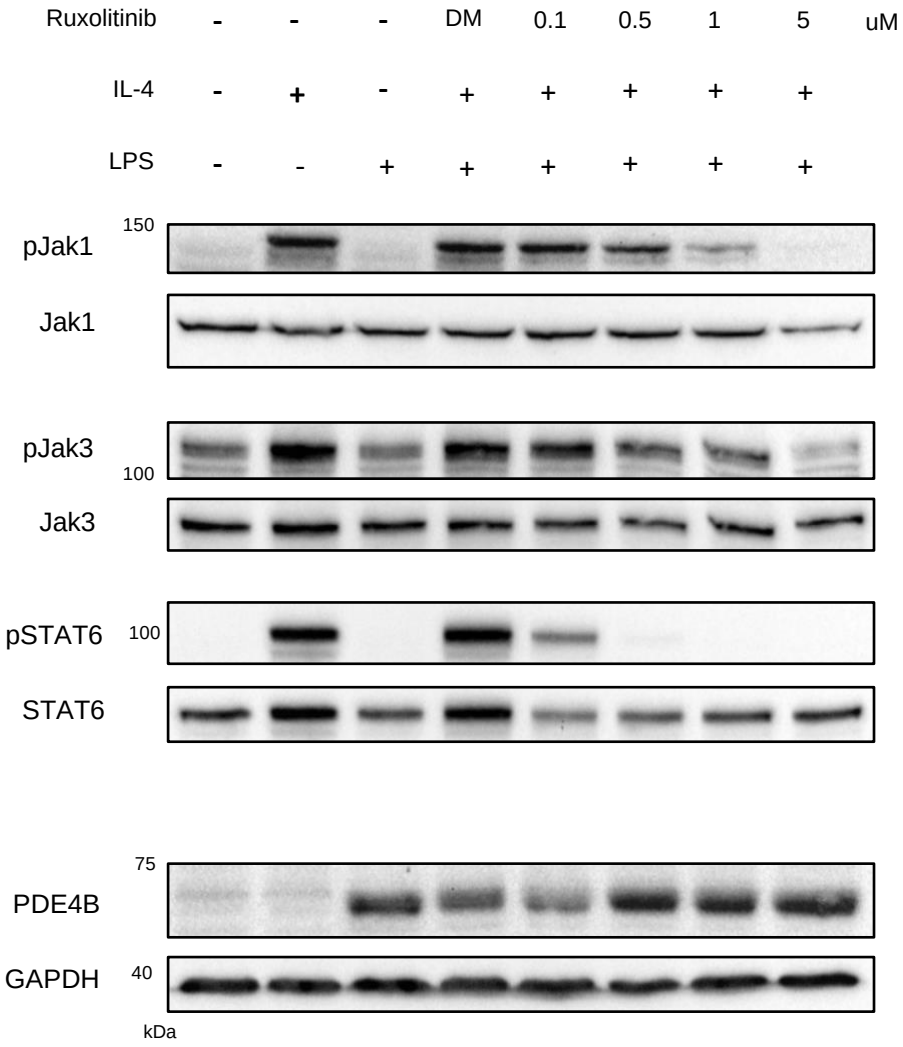



Figure 5.9 Ruxolitinib reverses IL-4 constraint of LPS induced PDE4B2

U937 macrophages cells were pre-treated with the Jak1/2 inhibitor, ruxolitinib, at the concentrations shown for 60 min, prior to the addition of IL-4 (40ng/ml). Lipopolysaccharide (LPS) 50ng/ml was added 15 mins after IL-4. DMSO (DM) 0.05% v/v was the diluent control. Total extract was produced following incubation with LPS at 30 min for pJak1(Tyr1022/1023), pJak3(Tyr980/981) and pSTAT6(Tyr641) and also at six hours for PDE4B2. Loading controls as shown. The data shown are representative of two separate experiments.

IL-4 and not LPS leads to the phosphorylation of Jak1, Jak3 and STAT6 (**Error! Reference source not found.**). During basal conditions Jak3 but not Jak1 was detected in its phosphorylated state. Ruxolitinib proved to be a potent inhibitor of Jak mediated phosphorylation of STAT6 and inhibited phosphorylation of Jak1 and also Jak3 at 1uM. Intriguingly, ruxolitinib constrained phosphorylation of STAT6 at an even lower level – in fact almost an order of magnitude lower concentration at 0.1uM. This, lack of correlation between phosphorylation of both Jak1 and Jak3 to that observed for STAT6, with increasing concentrations of ruxolitinib was also observed with another jak inhibitor, tofacitinib and is discussed further in Chapter 5.4.2. Following on from the data shown in Figure 5.9, it is proposed that Ruxolitinib 0.5uM stops phosphorylation of STAT6 and with it uncouples IL-4 constraint of LPS induced PDE4B2 expression.

A further observation may be made about the reconstitution of PDE4B2, following treatment with ruxolitinib in this model. Although ruxolitinib 0.5uM is associated with a return of PDE4B2 expression similar to that observed with LPS alone,

ruxolitinib given at both 1 and 5uM appear to be associated with an even greater expression that that observed following LPS treatment alone. This could be interpreted in various ways. For example, it could be that these higher concentrations of ruxolitinib are associated with off-target effects directly related to ruxolitinib, that lead to the induction of PDE4B2. It could also be related to the effect of further inhibition of pJak1 and pJak3, that might itself confer upon a further signalling system. Intriguingly, a further interpretation is that the expression of PDE4B2 is under tonal inhibition by the Jak-STAT cascade and that this is relieved upon engagement with a Jak inhibitor.

I next sought to deploy another Jak inhibitor, tofacitinib, to provide further data that the reversal of IL-4 constraint of LPS-induced PDE4B2 expression was mediated by inhibition of Jak-STAT6 and not any off-target effects specific to ruxolitinib. Tofacitinib is an inhibitor of Jak1 and Jak3, which are the upstream activators of STAT6

Tofacitinib dose-dependently inhibits phosphorylation of both Jak1 and Jak3 (Figure 5.10). At 0.5uM, tofacitinib completely inhibits pSTAT6 and with it, also uncouples of IL-4 constraint of induced PDE4B2 expression. Moreover, as was observed in the experiment with ruxolitinib (**Error! Reference source not found.**), tofacitinib given at higher concentrations appeared to be associated with PDE4B2 expression higher than that observed using LPS alone.

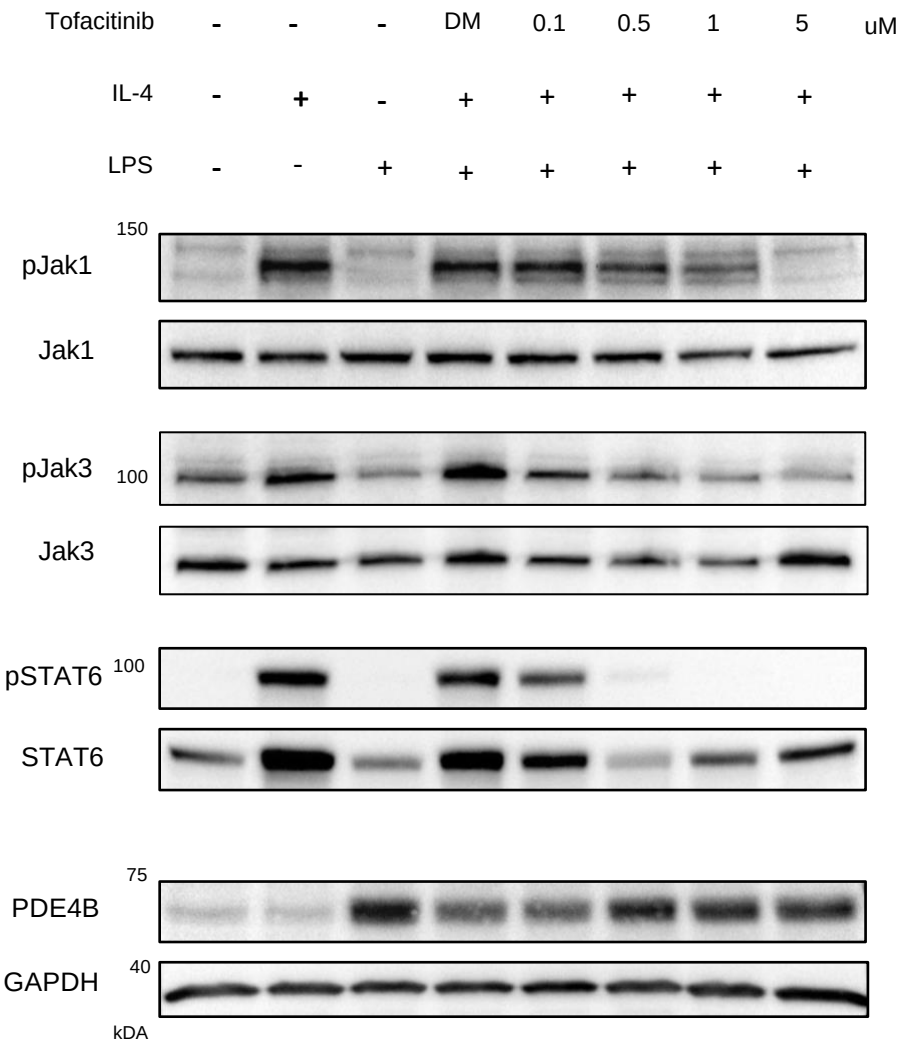



Figure 5.10 Tofacitinib reverses IL-4 constraint

of LPS induced PDE4B2

U937 macrophages cells were pre – treated with the Jak1/2 inhibitor, tofacitinib, at the concentrations shown for 60 min, prior to the addition of IL-4 (40ng/ml). Lipopolysaccharide (LPS) 50ng/ml was added 15 mins after IL-4. DMSO (DM) 0.05% v/v was the diluent control. Total extract was produced following incubation with LPS at 30 min for pJak1(Tyr1022/1023), pJak3(Tyr980/981) and pSTAT6(Tyr641) and also at six hours for PDE4B2. Loading controls as shown. The data shown are representative of two separate experiments.

So in summary, using a series of selective pharmacological inhibitors, I herein provide data in support of a role for STAT6 as a regulator of inducible PDE4B2 expression in macrophages.

### 5.2.4 STAT6 regulation of PDE4B2

In the previous section, using selective pharmacological inhibitors and western blot, the IL-4 constraint of LPS-induced PDE4B2 was mapped to Jak1, Jak3 and STAT6. Phosphorylation of STAT6 leads to its dimerization followed by translocation to the nucleus where it regulates gene expression (Walford & Doherty, 2013).

Beyond its role as an activator of transcription, STAT6 can also function as a transcriptional repressor by steric hinderance of other transcription factors (Bennett, Cruz, Lacson, & Manning, 1997; Maier et al., 2012). STAT6 activity is counterbalanced by SOCS1, which in Chapter 3.3.3, was explored as a marker of M2 macrophage polarisation. SOCS1 is itself induced by IL-4 and is a natural inhibitor of STAT6 activity. SOCS1 has gained interest as it is associated with the development and progression of cancers including haematopoietic malignancies (Melzner et al., 2005). Hence the balance between the respective activities of STAT6 and SOCS1, can help regulate the IL-4 mediated response.

I next sought to explore how Jak inhibitors relieved IL-4 constraint of LPS induced PDE4B2 expression and specifically if this could be observed at the level of gene transcription. Building upon the experiment with Jak inhibitors and western blot, I next used RT-qPCR and DNA probe to PDE4B2 to evaluate the effect of both

tofacitinib and ruxolitinib on IL-4 mediated constraint of LPS-induced PDE4B2 mRNA.

As described in earlier experiments, U937 macrophages were pre-treated separately with tofacitinib and ruxolitinib for 60 minutes, over a dose range between 0.5–5uM. IL-4 was added, as shown and 15 minutes after the addition of IL-4, LPS was added for 30 minutes before the experiment was stopped and RNA extracted. As shown in Figure 5.11, there was a trend reduction by both tofacitinib and ruxolitinib to reverse IL-4 mediated constraint of LPS induced PDE4B2, that appeared to be dose responsive (DMSO/IL-4/LPS v's tofacitinib 5uM 25.87 ± 12.77 v's 50.23 ± 22.01 $P = 0.17$ and DMSO/IL-4/LPS v's ruxolitinib 5uM 25.87 ± 12.77 v's 52.91 ± 20.15 $P = 0.12$, $n = 3$, Student's $t$ test).

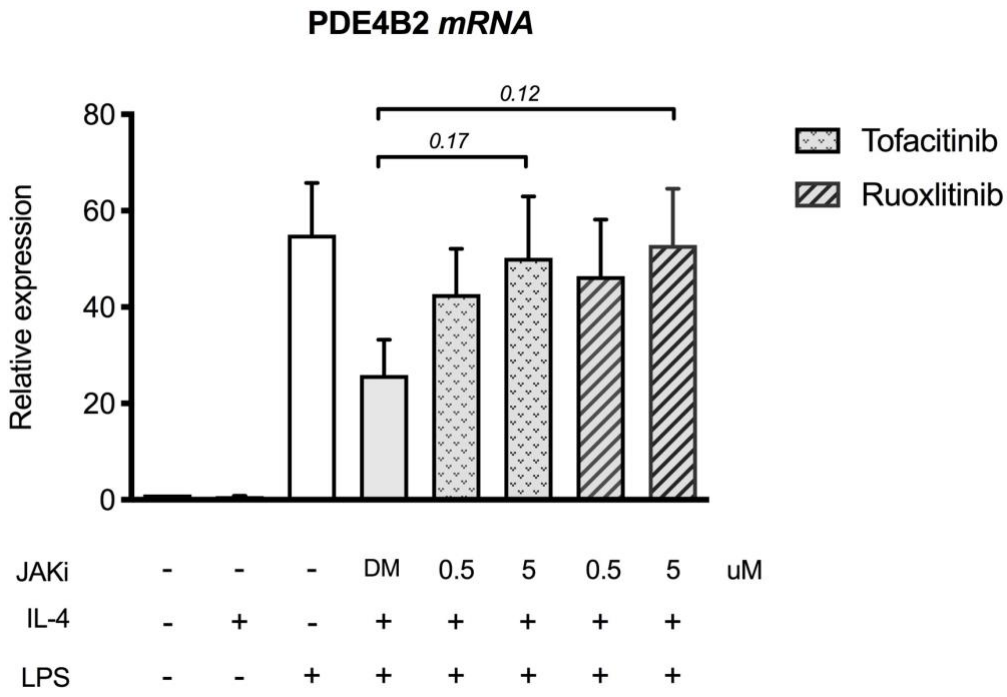



Figure 5.11 Jak inhibitors and IL-4 constraint of LPS induced PDE4B2 expression

U937 macrophages were pre-treated with the Jak inhibitors tofacitinib and ruxolitinib (both 0.5 and 5uM) for 60mins before addition of IL-4 (40ng/ml). Lipopolysaccharide (LPS) 50ng/ml was added 15 mins after IL-4. DMSO (DM) 0.05% v/v was the diluent control. Total extract was produced following incubation with LPS for 30 minutes and the RNA was extracted. mRNA was detected using RT – qPCR and the isoform selective probe to PDE4B2. Mean + SEM shown (*n* = 3). Statistical significance was determined using Student's *t*-test.

### 5.2.5 IL-4 and PDE4B2 in health and COPD

Using a model of U937 macrophages and western blot, I obtained data that maps the IL-4 constraint of LPS induced PDE4B2 to the Jak-STAT6 pathway. Using RT-qPCR, I next sought to observe if this effect was also phenocopied in primary MDMs and if the effect of IL-4 constraint on inducible PDE4B2 was different across macrophage subsets. Beyond this, I also sought to obtain data as to whether the regulation of PDE4B2 by IL-4 might be dysregulated in COPD, using MDM derived from COPD donors.

CD14+ peripheral monocytes were obtained from seven healthy donors and five donors with COPD and polarised to either an M1 or a M2 macrophage subset, as previously described. As expected, LPS proved a potent inducer of PDE4B2 expression - an effect that was more pronounced in M1 over M2 macrophages and an effect that persisted whether the MDM were derived from healthy donors (mean difference in induction following LPS in M1 vs in M2 healthy donors; 27.39 $\pm$ 15.93 v's 9.72 $\pm$ 7.0, $P < 0.05$, $n = 7$) or those with COPD (33.55 $\pm$ 14.20 v's

8.36 ± 3.10, $P < 0.01$, $n = 5$). However, the induction of PDE4B2 by LPS was no different between donor groups.

There was a trend toward IL-4 mediated constraint of LPS induced PDE4B2 expression, across both macrophage subsets and in both donor groups but only in M1 macrophages derived from healthy donors was this clearly demonstrated with a reduction of 41% (LPS v's IL-4 + LPS, 27.39 ± 17.84 v's 16.31 ± 5.87, $P < 0.05$, $n = 7$).

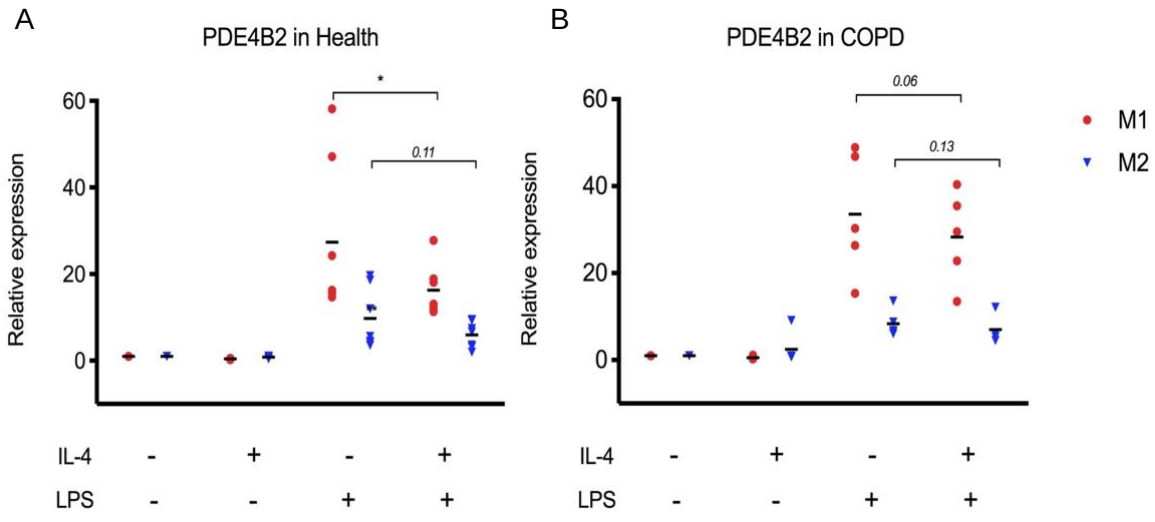



Figure 5.12 IL-4-mediated constraint of LPS-induced PDE4B2 appears maintained in M1 and M2 subsets, in both in health and COPD

CD14+ monocytes were isolated from PBMCs from donors who were either healthy (A) or had COPD (B). Cells were differentiated to either M1 macrophages with GM-CSF 20 ng/ml + IFN-γ 10 ng/ml or M2 macrophages; M-CSF 10 ng/ml + IL-4 10 ng/ml for five days, followed by RPMI complete for one day. Cells were pre-treated with IL-4 (40ng/ml) for 15 mins as shown before addition of lipopolysaccharide (50ng/ml) for

three hours. Relative expression of PDE4B2 was detected using RT – qPCR and an isoform-specific probe . Mean shown, ($n$ = 7, group A and $n$ = 5, group B). * $P < 0.05$. Statistical significance was determined using a paired Student's $t$-test (Wilcoxon test).

## 5.3 Using Nucleofection in U937 macrophages

Pharmacological inhibitors are a valuable experimental tool that can be used to help interrogate the signalling pathways involved in physiological cell responses. However, the use of small molecular inhibitors can also have unexpected off-target effects that can lead to incorrect conclusions about the involvement of their assumed target. In the current study, I used an deductive process to show that STAT6 mediated constraint of PDE4B2 expression was not dependent upon either the PI3K/Akt or MAPK signalling pathways and sought to add further validity to the observation that Jak-STAT6 signalling was involved by using two structurally distinct Jak inhibitors.

I next sought to use a genetic approach to provide further data in support of STAT6 as a regulator of PDE4B2 expression. In cell biology, macrophages are considered to be hard to transfect cells in part because they do not undergo rapid cell division and in part as they are adept at recognising foreign nucleic acids and mounting an immune response (Keller, Maeß, Schnoor, Scheiding, & Lorkowski, 2018; Warwick & Usachev, 2017). Nucleofection provides an alternative method to chemical transfection techniques and in THP-1 macrophages is reported to confer a high rate of transfection efficiency and cell viability (Keller et al., 2018; Maeß, Wittig, & Lorkowski, 2014; Martinet, Schrijvers, & Kockx, 2003). After a trial of different transfection techniques and a promising pilot, I used nucleofection

and small interfering RNA (siRNA) to disrupt STAT6 signalling toward further demonstrating its role in IL-4 mediated constraint of PDE4B2 expression.

An Amaxa Nucleofector II (Lonza, UK) was used according to the manufacturer's recommendations. The Nucleofector technology uses a specific combination of optimised electrical parameters and cell type specific solutions. However, at the time of study, protocols were only available for U937 suspension cells and not PMA differentiated U937 cells. Technical advice was sought and obtained (Lonza, UK) and on the basis of this advice, the protocol for U937 suspension cells was adapted for use in PMA-differentiated U937 cells. Pilot experiments were completed using the electrical programs W-001, T-001 and V-001 to help establish which electrical program might be associated with the best index of transfection efficiency to cell viability. A fluorescent plasmid – pmaxGFP, was used as a positive control and transfection efficiency assessed using fluorescence microscopy.

PMA differentiated U937 macrophages were prepared as described and then detached from their flasks using EDTA/Trypsin and gentle tapping. The cells were nucleofected with siRNA/control plasmid as described in Chapter 2.8, and then cultured for 24hours in complete media.

Using fluorescence microscopy, the electrical program W-001 and not T-001 or V-001 appeared to be associated with the highest transfection efficiency (Figure 5.13). Using light microscopy, the use of the W-001 program was also associated with high degree of cell adherence at 24hours post nucleofection, suggesting that cell viability was reasonably preserved. A quantitative assessment of cell viability

using 4',6-diamidino-2-phenylindole (DAPI) and flow cytometry was attempted but the cells did not survive further detachment techniques.

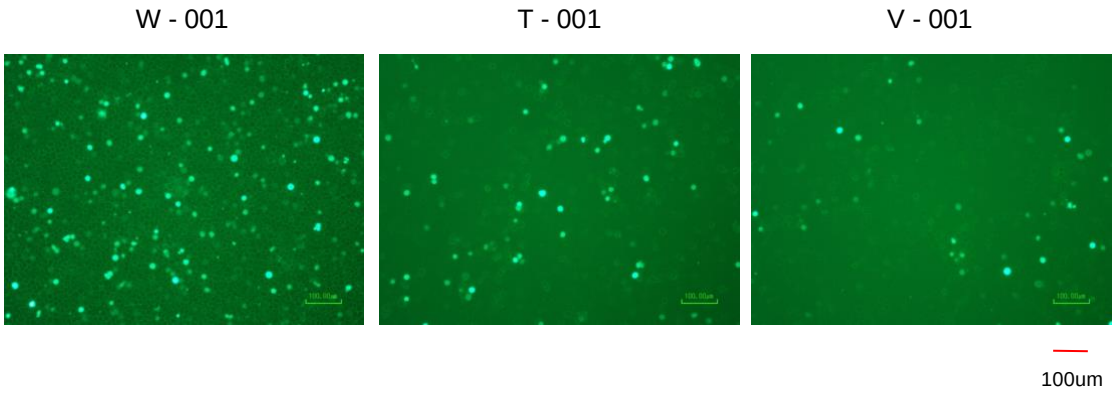



Figure 5.13 Photomicrographs using fluorescence microscopy showing the effect of different electrical programs using Nucleofection on the transfection of pmaxGFP, in U937 macrophages

U937 macrophages were transfected using the Amaxa Nucleofector II and 1ug of a plasmid encoding the enhanced protein eGFP. Three different programs were compared for transfection efficiency at 24 hours using fluorescence microscopy. Scale bar 100μum. The photomicrographs are representative of at least 2 separate experiments.

Having optimised the electrical program to be used in nucleofection, I next turned to deploy siRNA STAT6 to see if STAT6 knockdown would disrupt IL-4 mediated constraint of PDE4B2 expression. Pooled siRNA to STAT6 was first used in dose–response experiment to help optimise the amount siRNA to use to knockdown STAT6 protein. Readouts for mRNA and STAT6 protein were completed using respectively RT-qPCR and western blot.

Pooled siSTAT6 250nM/ml and higher appeared to reduce STAT6 mRNA at 24hours and completely knocked down STAT6 protein at 48hours.

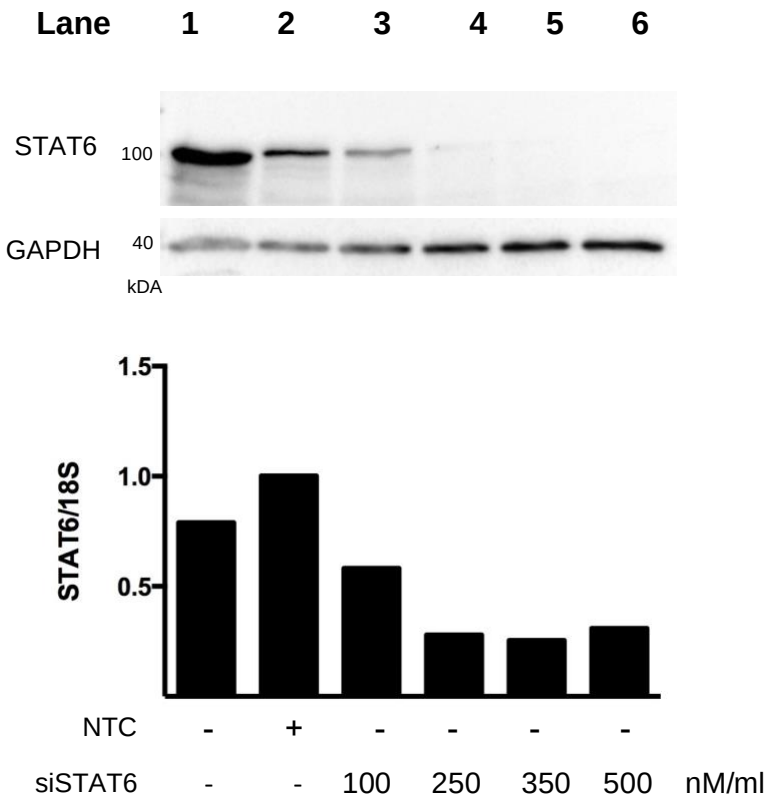



Figure 5.14 Dose response of siRNA STAT6

in U937 macrophages

U937 macrophages were nucleofected (lanes 2-6) using the Amaxa II Nucleofector with either non-targeting control (NTC) 500ng/ml or pooled siRNA to STAT6 at the concentrations shown. Lane 1 was a non-nucleofection control. The mRNA was detected using RT-qPCR at 24hrs (lower panel) and lysate produced for immunoblot at 48hrs (upper panel). The data shown are representative of two separate experiments.

Despite some initially successful optimisation experiments, further experiments proved technically challenging to produce consistent knockdowns of any of either Jak1 Jak3 or STAT6, whilst also maintaining adequate cell viability.

In parallel, pilot experiments were also attempted using custom designed pooled siRNA to PDE4B2. An experiment was designed using both NTC and siPDE4B2 in cells treated with LPS. However, in siPDE4B2 U937 macrophages, treatment with LPS was not associated with reduced PDE4B2 protein, suggesting the knockdown was not successful. A positive knockdown control was not included and so in these pilot experiments, it was not possible to know whether nucleofection had not been successful or if the pooled siPDE4B2 was not working as it was designed to.

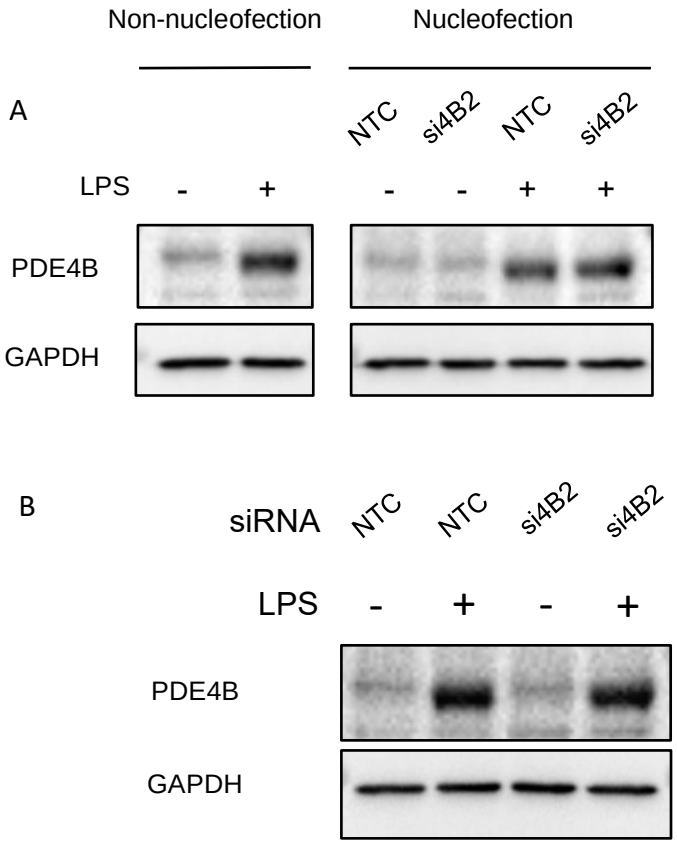



Figure 5.15 Pilot experiment targeting PDE4B2 using siRNA and nucleofection

Two pilot experiments, A and B, were completed in U937 macrophages using Amaxa II Nucleofector and either non-targeting controls (NTC) or

siPDE4B2. Blot A also includes non-nucleofected controls. Neither experiment A or B demonstrated an effective knockdown of PDE4B2 protein.

## 5.4 Discussion

### 5.4.1 Introduction

The aim of this chapter was to explore the potential for cross-talk between cytokines linked to macrophage polarisation and PDE4B2 expression. Focusing on IL-4, I obtained novel data in support of a role for Jak-STAT6 signalling in mediating IL-4 constraint of LPS induced PDE4B2 and explored the role of STAT6, as an inducible repressor of PDE4B2 expression. Using primary MDM including those derived from patients with COPD, I obtained evidence to suggest that the IL-4 – LPS – PDE4B2 regulatory cascade appears to be maintained in pulmonary disease (COPD).

I also completed pilot studies toward development of a model of nucleofection in U937 macrophages that with further optimisation could provide a basis for genetically targeting BCL-6 and STAT6 to further evaluate the findings in this Chapter. Critically, such a model could also help determine the consequences of genetic knockdown of PDE4B2 towards further delineating its role in inflammatory signalling in macrophages.

### 5.4.2 Mapping IL-4 constraint of PDE4B2

Cytokines help orchestrate a variety of different processes including the regulation of inflammation, cellular proliferation, metabolism, chemotaxis and tissue repair. The Jak-STAT pathway is amongst the most important of the signal

transduction systems and near universal to cytokine receptor signalling. STAT proteins are also closely linked with macrophage polarisation, for example STAT1 activation toward M1 subsets and STAT6 activation toward the M2 subset (Lawrence & Natoli, 2011).

In a study over twenty years ago, IL-10 and IL-4 were reported to constrain LPS-induced PDE4B expression (Ma et al., 1999). Since that study, there have been few reports about the effect of cytokines on inducible PDE4B expression and even less on the signalling cascades that might underlie them.

In support of the findings of Ma and colleagues, I observed that IL-4, IL-13 and IL-10 all attenuate LPS-induced PDE4B2 expression and that IL-4-mediated constraint was dose-dependent. IL-10 is the prototypical anti-inflammatory cytokine (Iyer, Ghaffari, & Cheng, 2010) and its effect on inducible PDE4B2 expression further underlines, PDE4B2's association with the inflammatory response. Meanwhile, IL-4 and IL-13 are of interest as the classical Type 2 cytokines linked to allergic disorders (Gour & Wills-Karp, 2015), their associations with alternatively activated macrophages and in recent years as druggable targets in asthma (McCracken et al., 2016).

Using a pharmacological based approach I then mapped the effect of IL-4 on PDE4B2 expression, to Jak and not either of the PI3K or MAPK signalling pathways. Moreover I obtained evidence that this regulatory pathway is maintained in primary cells, as observed in MDM from healthy donors, polarised to a M1 subset. In a further study, it will be important to evaluate if this negative

regulatory break on inducible PDE4B2 expression, is maintained across subsets and also in disease, as the data herein shown, fell short in demonstrating this.

An important observation may be made concerning the lack of correlation between phosphorylation of both Jak1 and Jak3 to that observed for STAT6, with increasing concentrations of ruxolitinib. As described above, ruxolitinib given at 0.1uM markedly reduces phosphorylation of STAT6 and at 0.5uM, completely abrogates it. However, ruxolitinib only appears to attenuate phosphorylation of Jak1 and Jak3 at a concentration of 0.5uM and possibly, only at a concentration of 1uM. How, it may be asked, can phosphorylation of STAT6 be inhibited at a concentration of Jak inhibitor, that doesn't appear to inhibit Jak phosphorylation?

It may be possible that a small reduction of phosphorylated Jak1 and Jak3 can have an amplified response on downstream signaling cascades and therefore account for a greater reduction in the observed phosphorylation of STAT6. Another possibility may be that ruxolitinib and tofacitinib target Jak2 (Villarino et al., 2017) and that blotting for p-Jak2 may have shown a closer correlation in the dose-response between p-Jaks and p-STAT6. Furthermore, cytokines display heterogenous signaling so that although IL-4 is the prototypical STAT6 stimulus, it also activates STAT5 (Lischke et al., 1998). It is also widely appreciated that Jak-STAT signaling is not limited to a simple, linear pathway but also involves non-canonical tangents including heterochromatin stability (Bousoik & Montazeri Aliabadi, 2018; W. X. Li, 2008; O'Shea et al., 2015). For example STAT6 can be activated by viruses without relying on Jaks (H. Chen et al., 2011) and unphosphorylated STAT3 can induce multiple STAT3 target gene expressions

without phosphorylation of its binding site at Serine 727 (X. Hu, Li, Fu, Zhao, & Wang, 2021) .

The inconsistency of the observed effect of IL-4 mediated constraint of inducible PDE4B2 in primary cells when compared to that observed using U937 macrophages, may reflect the greater heterogeneity of responses that is associated with working with primary cells when compared to cell lines. A future study may benefit from further optimising the experimental conditions including extending the exposure to pre-treatment with IL-4, that may provide a greater attenuated effect on LPS induced PDE4B2.

Turning back to the role of STAT6, it has been described that STAT signalling can be either cell type specific or core stereotypic (Murray, 2007). For example, whereas IFN-γ induced STAT1, induces a similar cohort of genes regardless of the cell type involved (van Boxel-Dezaire, Rani, & Stark, 2006), STAT6 activation by IL-4 has cell-type specific effects – in other words, IL-4 regulated genes in say T cells have a distinctive signature to IL-4 activated genes in for example macrophages (Z. Chen et al., 2003; R. Lund et al., 2005).

As well as being a transcriptional activator promoting Th2 development, STAT6 is also known to be an important transcriptional repressor in macrophages. STAT6 can directly inhibit transcription by steric hinderance to the binding of other transcription factors, as for example has been shown with the inhibition of IFN-γ CD40 expression in macrophages though binding to gamma-activated sites (Nguyen & Benveniste, 2000). Less directly, STAT6 can also repress the enhancer sequences in alternatively activated macrophages. This in turn reduces

the responsiveness to inflammatory signals including Nod-like and TLR signalling pathways and genes that compromise the LPS-induced inflammasome (Czimmerer et al., 2018).

In the Chapter 4.3, it was observed that using data from the USCD genome suite and Encode datasets, BCL-6 could be theorised to be a transcriptional repressor to the promoter to PDE4B2. As part of the same analysis of putative transcription factor binding sites to the promoter to PDE4B2, it was also observed that the promoter sequence contained a binding site to STAT6. Although this was not explored in the current study, this could for example be resolved using techniques such as Ch-IP.

Taking in to account the role of STAT6 in IL-4 mediated alternative macrophage activation, it is possible to hypothesise further about the role of STAT6 in the regulation of PDE4B2 expression. Alternatively activated macrophages are primed toward suppressing the inflammatory response and toward the restoration of homeostasis and wound healing. As part of this, alternative macrophages display reduced responsiveness to pro-inflammatory signalling. In the present study, I obtained data in support of reduced LPS-PDE4B2 expression in M2 macrophages when compared to that LPS-PDE4B2 expression in M1 macrophages. I hypothesise that STAT6, as well as driving alternative macrophage function also represses the promoter to PDE4B2 and with it, the reduced inflammatory response to TLR signalling.

### 5.4.3 IFN-γ primes LPS responses

As part of an evaluation of the effects of pre-treatment with various cytokines on LPS induced PDE4B2, I observed IFN-γ enhanced PDE4B2 expression in U937 macrophages (Figure 5.1). Moreover, when combined with LPS, IFN-γ has an additive effect and possibly may even potentiate the effect of LPS. The ability of IFN-γ to 'prime' cells, such that the effect of LPS is enhanced beyond that expected if given alone, is well described in macrophages (M. P. Hayes, Freeman, & Donnelly, 1995; J. Y. Lee & Sullivan, 2001; Ucla, Roux-Lombard, Fey, Dayer, & Mach, 1990). Indeed it can be conceived how such a priming effect may be advantageous as part of the host response to infection or other injurious insult.

Natural Killer (NK) are an important part of the innate immune response and in response to stress or infection are an early source of IFN-γ. Locally produced IFN-γ primes macrophages to secrete pro-inflammatory cytokines, superoxide anions and oxygen and nitrogen radicals and with this, enhances their ability to kill harmful pathogens (Mosser & Edwards, 2008). The importance of this enhancement can be demonstrated in animal models, as mice deficient for the gene to IFN-γ are more susceptible to bacterial, viral or protozoal infection as indeed are humans with genetic mutations in IFN-γ signalling (Filipe-Santos et al., 2006).

The difference I observed between different macrophage phenotypes in macrophage responsiveness to LPS-PDE4B2 appears related to the priming effects of respectively IFN-γ in M1 macrophages and IL-4/STAT6 in M2 macrophages. I have obtained data in support of reduced LPS responsiveness

to PDE4B2 expression in M2 macrophages in comparison to M1 macrophages (Figure 3.29 and Figure 5.12). IFN-γ signalling includes both MyD88-independent and dependent activation of NF-κB which can augment the pro-inflammatory response to TLR ligands (DiDonato, Hayakawa, Rothwarf, Zandi, & Karin, 1997). In contrast, in M2 macrophages, IL-4 mediated STAT6 is said to either provide steric hindrance to other transcription factors at the PDE4B2 promoter or induce epigenetic changes on inflammatory enhancers that have been shown to persist, even after the release of bound STAT6 from DNA (Czimmerer et al., 2018). These epigenetic changes may explain why STAT6 suppressive effects persisted in alternatively activated M2 macrophages, even when they were maintained in IL-4 free culture media for 24hours prior to their use in experiments.

Intriguingly, pSTAT6 and BCL-6 have been shown to have a mutually exclusive expression pattern in primary B-cell lymphoma cells (Ritz et al., 2013). Mice deficient for BCL-6 have an increased production of Th2 cytokines and develop Th2 inflammatory disease involving the heart and lungs characterised by eosinophilic rich cell infiltrates (A. L. Dent et al., 1997). The BCL-6 consensus binding site resembles the binding site recognised by the STAT family, raising the possibility that BCL-6 may bind competitively to some STAT-binding sites to repress STAT-dependent genes (A. L. Dent et al., 1997; M. B. Harris et al., 1999). In primary mediastinal B-cell lymphoma cells, it has been shown that pSTAT6 represses BCL-6 expression by binding to several GAS and DNA binding sites in the regulatory region of BCL-6 (Ritz et al., 2013). Indeed, within the context of allergic diseases, STAT6 and BCL-6 may provide opposing but complementary control, likened to the concept of “yin and yang” between those drivers of the Th2 response and those that would suppress it (Arima, Fukuda, & Tokuhisa, 2008).

### 5.4.4 Nucleofection in U937 macrophages

Genetic interference provides a powerful tool for investigating the functional consequences of knocking down endogenously expressed proteins. In the current study, an RNA interference approach was combined with nucleofection to help evaluate if a genetic approach targeting Jak-STAT6, could phenocopy the effect of selective inhibitors in uncoupling IL-4 mediated constraint of LPS - PDE4B2. I also set out to target PDE4B2 to obtain data as to determining its role in the macrophage response to inflammation.

Macrophages are considered to be notoriously difficult to transfect cells since in part because they do not undergo rapid cell division and in part as they are adept at recognising foreign nucleic acids and mounting an immune response (Keller et al., 2018; Warwick & Usachev, 2017). Nucleofection was selected as the most promising technique to ward deploying RNA interference in U937 macrophages

Using a positive control plasmid encoding a green fluorescent protein (GFP) and fluorescence microscopy I was able to establish a proof of concept of nucleofection in PMA-differentiated U937 macrophages (Figure 5.13). Using a visual comparison, the electrical program W-001 was associated with the highest transfection efficiency and this did not appear to be at the expense of an increased loss of cell viability.

In U937 suspension monocytes, the manufacturer of the nucleofection system, lists an expected transfection efficiency of between 45 - 55% at time points between 5 to 48 hours (using flow cytometry and a positive fluorescent plasmid control (Amaxa, U-937 [ATCC] DCV-1011 Vs. 06-2006). Although a quantitative

analysis of transfection efficiency or cell viability was not performed in the current study, the visualised results completed in U937 PMA differentiated macrophages appeared to be broadly in line with the results expected in U937 suspension cells. Cell viability was not directly measured but at 24hrs, confluent cultures of adherent cells were seen using light microscopy. This estimate of cell viability also compared favourably with reported rates of cell viability of 50% using flow cytometry (Optimised protocol U937 [ATCC] DCV=1011 Vs 06-2006).

Using a pooled siRNA STAT6, nucleofection was successfully used to knockdown STAT6 protein in U937 macrophages. However, the use of nucleofection did not prove to be a very tractable model system for RNA interference in U937 macrophages. The use of nucleofection in PMA-treated U937 macrophages has not been widely reported beyond two studies – the first citing its use in transfecting siRNA (Bertram et al., 2008) and another its use to transfect a luciferase reporter system (Vogel et al., 2012).

Although nucleofection in U937 may hold further promise, the challenges associated with it as well as the increasing adoption of alternative approaches including use of CRISPR may relegate its use in any further study.

# Chapter 6 **CONCLUSION**

The initial aims of my project were (i) to profile the PDE4 isoform expression in myeloid cells and (ii) to explore whether macrophage subsets and or macrophages derived from donors with COPD, had a signature PDE4 expression profile. Moreover, I sought to explore whether the expression of the short PDE4B2 isoform, which is believed to be a key anti-inflammatory target, differed across the various macrophage subsets and to determine whether this profile was dysregulated in COPD.

## 6.1 PDE4 expression profile

To date, our understanding of the PDE4 expression profile in primary monocytes and macrophages draws heavily upon the seminal findings of early investigators including work completed nearly two decades ago (Barber et al., 2004; M. C. Shepherd et al., 2004; Wang et al., 1999). As such, this project updates and refines our understanding of the PDE4 expression profile in monocytes and extends it, for the first time, to key macrophages subsets whose regulation is associated with inflammatory / pathological disease responses. As such, this study provides one of the most comprehensive accounts of the PDE4 isoform expression profile in myeloid cells that has been completed to date.

It has been reported that, in peripheral monocytes, the expression of the long PDE4A4 and the short PDE4B2 isoforms is higher in smokers than non-smokers (Barber et al., 2004). Although individuals that were ‘current smokers’ were not recruited into my studies, I found no evidence that either PDE4A4 or PDE4B2 expression was higher in both peripheral monocytes and MDMs from donors with COPD, when compared to donors without. However, in line with the study

reported by Barber and colleagues, I did find evidence that PDE4 isoform expression could be altered in end-organs that are the site of COPD. Additionally, I obtained novel evidence that that PDE4B expression is reduced in bronchial tissue from patients with asthma.

The data obtained from asthma donors is intriguing. Two hypotheses can be proposed to suggest why PDE4B expression might be reduced in bronchial airway tissue in asthma. The first is that it may be medication related in that all donor asthmatics, but not control individuals, were medicated with inhaled corticosteroids. Indeed, corticosteroids have been shown to constrain inducible PDE4B expression, possibly by negative cross-talk between the glucocorticoid receptor and NF-$\kappa$B (B.-C. Lee, S. Susuki-Miyata, C. Yan, & J.-D. Li, 2018). A second possibility is disease specific, namely that, in the respiratory airways of patients with asthma, PDE4B expression is reduced compared to those without (Chapter 3.7). This intriguing possibility may explain why selective (pan-) PDE4 inhibitors have been shown to have little or no therapeutic efficacy in asthma, in contrast to COPD (Bateman et al., 2015; Page, 2014).

However, the data that I obtained from donors with asthma should be treated with a degree of caution as it was derived from a small number of individuals and it was not possible to match donors by either age or sex. This constrains the ability to draw firm conclusions concerning the relationship between disease severity and PDE4B expression from these data. However, such findings could be explored in a follow-up study that encompassed a larger number of individuals with matched donors and where bronchial tissue was sampled from multiple sites as recommended by investigators assessing in airways diseases, see e.g.

(Labonte et al., 2008; Yick et al., 2013) and also with the inclusion of alveolar macrophages obtained using bronchoalveolar lavage. A study of such a format is likely to be required in order to gain robust insight into whether macrophage PDE4B2 is indeed dysregulated in asthma.

A further insight from profiling the PDE4 isoform expression in this study, concerns the expression of the dead-short, catalytically inactive PDE4A7 isoform. In monocytes from COPD but not healthy donors, I made the novel discovery that LPS led to reduced expression of PDE4A7. I also observed that in both M1 and M2 subsets, monocyte to macrophage differentiation was associated with reduced expression of PDE4A7 and furthermore that this affect was maintained in cells derived from COPD donors. The functional relevance of this change is uncertain as this 'dead short' isoform lacks both UCR regulatory regions and has a truncated catalytic site that renders it catalytically inactive. However, given that PDE4 isoforms have been shown to serve as signalling scaffold proteins (Houslay, 2010) and also one isoform can regulate mTor signalling through protein-protein interaction independent of catalytic function (H. W. Kim et al., 2010) it is tempting to suggest that PDE4A7 may provide a signalling / scaffold function in macrophage / monocyte systems whose functional role may be disrupted or re-programmed in COPD.

## 6.2 Macrophage phenotypes

Macrophage function is inextricably associated with macrophage phenotype and therefore it was apposite, indeed critical, to explore the relationship between PDE4 isoform expression profile and a model of macrophage polarisation. The MDM model provides such an opportunity.

I was able to develop a robust model of polarised macrophage subsets using contrasting cell maturation protocols that yielded cell populations that were not only morphologically distinct but that also displayed contrasting expression profiles in up to six macrophage markers. With further characterisation, perhaps including the use of functional assays and validation against tissue derived macrophages, this model could provide a highly tractable model to further explore PDE4 signalling in macrophage subsets.

Macrophage phenotypes afford macrophages with a large repertoire of responses that help equip them to play a number of diverse roles in the maintenance of homeostasis. However, these reparative functions can be subverted and in recent years dysregulated macrophage responses have been linked with the pathogenic mechanisms that underlie a range of chronic diseases including cancer, fibrosis and chronic inflammation (Cassetta & Pollard, 2018; Murray & Wynn, 2011; T. A. Wynn et al., 2013).

Broadly speaking, the spectrum of macrophage phenotypes encompass those that are classically activated M1 macrophages that follow the engagement with IFN-$\gamma$ and TLRs through to alternatively activated M2 macrophages, that follow engagement with IL-4, IL-13 (Piccolo et al., 2017; T. A. Wynn et al., 2013) and perhaps also IL-10 ((Makita et al., 2015). Within the airway, alveolar macrophages are exquisitely positioned to act as sentinel cells to respond to inhaled noxious irritants including cigarette smoke that are linked to COPD or viruses that help drive exacerbations of airway diseases including asthma. In COPD, macrophages help orchestrate the inflammatory response and are

localised to sites of disease whilst in asthma, macrophage responses to inhaled pathogens are reported to be dysregulated (Fricker & Gibson, 2017).

cAMP is a pivotal regulator of a variety of cell responses. However, such actions are critically dependent upon compartmentalisation within cells. In the case of macrophages / monocytes there is a distinct pool of cAMP that is regulated by PDE4 activity, namely that of PDE4B2, which is associated with regulation of the inflammatory response. Thus inhibitors of other PDE4 sub-families have little or no effect on inflammatory responses. We hypothesise that macrophage PDE4B2 is well positioned to be a key orchestrator of macrophage responses by regulating cAMP within cellular compartments that are critical for disease regulation by dampening down inflammation including TLR mediated responses including TNF$\alpha$ generation.

This study provides the first report that PDE4B2 expression is increased in classically activated, M1 macrophages, when compared to alternatively activated, M2 macrophages and is induced to yet higher levels still, following TLR4 activation. Moreover, I obtain preliminary evidence that in MDM derived from COPD donors, the TLR4-PDE4B2 response might be more divergent across macrophage subsets than that observed in cells from healthy donors. I would like to suggest that higher levels of PDE4B2 in M1 macrophages may be the key to the heightened response of LPS-TLR4 in M1 macrophages and therefore critical to promoting inflammation by decreasing cAMP in a key intracellular compartment where cAMP acts as a negative regulator of macrophage activation/ TNF$\alpha$ generation.

It is said that the priming of macrophages by IFN-γ (supplemented in M1 culture conditions) occurs through the canonical Jak-STAT1 pathway leading to the direct activation of IFN-γ stimulated genes. However, the activation through the Jak-STAT pathway is typically transient – reaching a peak signal between 15-60 minutes and resolution back to baseline within a few hours (Xiaoyu Hu & Ivashkiv, 2009). In the current study, it may be noted that macrophages were maintained in supplement free media (including IFN-γ) for 24 hours, prior to the addition of LPS. How then, one may ask is the increased expression of PDE4B2 in M1 macrophages and the hyper-responsiveness to TLR4 signalling reconciled with the fact that the direct effects of IFN-γ through the Jak-STAT pathway are likely to have elapsed? The answer, as I describe below appears to relate to epigenomic changes induced by IFN-γ that persist well beyond the point of IFN-γ engagement.

In macrophages, epigenomic changes are in part related to chromatin accessibility at the site of promoters and enhances (Glass & Natoli, 2016). For IFN-γ, this may be related to the priming of regulatory elements following binding by STAT1 at gamma interferon activation (GAS) sites (Ivashkiv, 2018) as well as the *de novo* formation of various latent enhancers (Ostuni et al., 2013). Furthermore, there may also be a contribution from the suppression of transcriptional repressor pathways that normally serve to counter IFN-γ mediated effects. Here I would suggest that it is likely that the exposure to IFN-γ, as part of a M1 macrophage polarising condition, instituted an epigenomic change including enhanced TLR4 responsiveness to PDE4B2 expression.

Beyond the linkage of dysfunctional macrophage responses to various chronic diseases (T. A. Wynn et al., 2013), it has also been proposed that a relative imbalance between macrophage phenotypes underlies the mechanisms that drive both COPD (Shaykhiev et al., 2009) as well as asthma (Madore et al., 2010). These observations have helped stimulate interest in the notion that targeting macrophage polarisation could be used for therapeutic gain (Cheng & Rong, 2018; Ponzoni, Pastorino, Di Paolo, Perri, & Brignole, 2018). For example, if macrophages polarised toward more pro-inflammatory responses (M1) were targeted and driven toward a more anti-inflammatory phenotype and one associated with the resolution of inflammation (M2), it could follow that this might help restore tissue homeostasis and be of therapeutic benefit.

The data in this project, adds to the considerable body of evidence that TLR4 signalling is a potent inducer of PDE4B2 expression in monocytes and macrophage subsets derived from them. Moreover, this signalling cascade is unique to PDE4B2 and not any of the 15 other PDE4 isoforms that I assessed in monocytes or the 11 other PDE4 isoforms that I assessed in macrophages. Since TLR4 mediated induction of macrophage PDE4 isoforms is specific to PDE4B2, the data presented in this study underlines the specificity of the macrophage TLR4-PDE4B2 response and provides novel insight into the capacity for macrophage PDE4B2 to play a key role as a mediator of the inflammatory response.

## 6.3 Regulation of PDE4B2 expression

Another aim of this study, was to explore the processes underpinning the induction of macrophage PDE4B2 in response to LPS challenge. This is a

critically important process as activation of TLR4 by LPS promotes an inflammatory response (Jin & Conti, 2002) that can help facilitate macrophage activation and inflammatory activity subsequent to LPS challenge (Germain et al., 2001). I have discussed previously the priming of M1 macrophages by IFN-γ and the likelihood that this leads to epigenomic changes that enhanced TLR4-PDE4B2 expression. Here within, I provide further data in support of other important transcriptional factors that I propose might help regulate the expression of PDE4B2. I have also obtained evidence that extends our understanding of cytokine-mediated constraint of inducible PDE4B2 expression to include IL-13, as well as IL-4 and IL-10, whose affects has been previously described (Ma et al., 1999).

Using a deductive process of blocking different signalling pathways in turn, I have mapped the effect of IL-4 to the Jak-STAT6 signalling pathway that is reversed using various clinically relevant Jak inhibitors. When considered alongside the interrogation of the PDE4B2 promoter that identified a STAT6 binding site, the data provided here in my study supports my hypothesis that STAT6 acts as an important transcriptional repressor to PDE4B2.

Analysis of the USCD and Encode datasets allowed for the proposal that BLC-6 may act as a transcriptional repressor to PDE4B2, which was then interrogated experimentally. This allowed me to obtain data showing the opposing expression of BCL-6 and PDE4B2 at both the steady state and in response to LPS challenge.

Using a proteasome inhibitor, I was able to identify the potential for cross-talk between BCL-6 and PDE4B2 that potentially occurred through the stabilisation of

BCL-6 at the PDE4B2 promoter. When considered alongside the rapidity by which PDE4B2 protein expression is induced, a model was conceived where, under resting cellular conditions, the promoter to PDE4B2 lies in a poised state that can be activated upon the release of the BCL-6 transcription repressor and its subsequent downregulation by targeted degradation through action of the proteasome. My hypothesis could be interrogated using Ch-IP methodology in order to define whether BCL-6 does indeed bind to the PDE4B2 promotor. It may also be possible to obtain further insight into this proposal by directly targeting BCL-6 using, for example, a peptide inhibitor of BCL-6 – PDE4B2 promoter interaction or by RNA interference to knock down BCL-6, both procedures which might be expected to phenocopy the effect of TLR4 activation on PDE4B2 expression if my hypothesis is correct. Such studies would also be expected to gain insight into the potential cross-talk with LITAF, which is said to offer transcriptional repression to BCL-6 (Bertolo et al., 2013).

It is thus possible that targeting PDE4B2 might provide a better index of therapeutic efficacy over off-target effects including emesis, than targeting all PDE4 isoforms using the pan-PDE4 inhibitors used currently in clinical practice. A parallel, albeit less direct, approach might be to augment the role of transcriptional repressors to PDE4B2, such as BCL-6 using proteasomal blockade, as demonstrated experimentally in the current study. In other therapeutic areas, proteasomal inhibitors have gained clinical validity. Thus, for example, the proteasomal inhibitor, Bortezomib is approved for use in multiple myeloma (Richardson, Hideshima, & Anderson, 2003) where it inhibits the 20S proteasome core leading to the accumulation of misfolded proteins in the endoplasmic reticulum (ER) and cytosol and eventually ER stress-related

apoptosis (Chauhan, Hideshima, Mitsiades, Richardson, & Anderson, 2005). It might thus be of interest to evaluate whether the proteasomal inhibitor, Bortezomib could be deployed in order help stabilise BCL-6 at the PDE4B2 promoter and determine if it served to attenuate the TLR4- PDE4B2 response *in vivo*.

My work has also provided pilot data in support of a model of transfection of U937 macrophages using nucleofection. The published use of this technology is limited, which may speak to its technical challenge. However, I believe that, with further optimisation, this may provide a useful model to exploit RNA interference technology to determine the functional significance of BCL-6 and PDE4B2 knockdown.

## 6.4 Down regulation of PDE4A

I also show, for the first time, that in both primary monocytes and also in macrophages derived from them, LPS challenge is associated with a marked reduction of PDE4A sub-family expression. My results provide insight into changes in the expression of various PDE4A isoforms in macrophages challenged with LPS, giving credence to previous reports that implied reduction in PDE4A sub-family expression subsequent to LPS challenge (Borysiewicz et al., 2009; Lelubre et al., 2017). Thus, it may be that, in macrophages, TLR signalling leads to a switch away from the expression of long PDE4A isoforms to the short form PDE4B2, much in manner that has been described in monocyte to macrophage differentiation (M. C. Shepherd et al., 2004).

The TLR4 mediated switch from long PDE4A forms to short PDE4B2 may be functionally significant, not only for the change in PDE4 catalytic activity that is associated with individual forms, but it also likely has important consequences for both the post-translational landscape mediated by both ERK and PKA signalling and, of course, in re-programming the landscape of compartmentalised cAMP signalling within such cells. With regard to cross-talk with ERK signalling, long forms, but not short forms, can be phosphorylated by PKA and thus the switch away from long forms represents the loss of a PKA-medicated mechanism for cellular desensitisation to cAMP and with it a homeostatic break to uncontrolled cAMP signalling. The switch to short PDE4B2, also heralds an increased capacity for regulation by ERK since PDE4A forms lack a binding site for ERK and, as such they are unable to be phosphorylated at their ERK phosphorylation consensus site. In contrast to this, the PDE4B2 short form can be phosphorylated by ERK, whereupon it is activated. It may thus be that the increased capacity for regulation by ERK of short PDE4B2 leads to a decrease in localised cAMP and with it one possible mechanism through which TLR activity may help drive macrophage inflammatory responses.

In summary, the well described induction of PDE4B2 by TLR4 activation is accompanied by the less well described, but I would suggest, functionally still important reduction of PDE4A.

## 6.5 Concluding remarks

Inflammatory conditions including asthma and COPD are major contributors to the burden of human disease. Selective PDE4 inhibitors including roflumilast provide a proof of principle that selectively raising cAMP within cellular compartments can be exploited for therapeutic gain. However, off-target effects have limited their widespread deployment leaving an unmet clinical need.

This project provides novel evidence that macrophage PDE4B2 is critically placed to act as an important regulator of the inflammatory response and that inhibitory targeting may successfully provide improved therapeutics in diseases such as COPD.

# Chapter 7 **BIBLIOGRAPHY**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