Regulation of leukocyte cytokine production by inhibitors of intracellular signalling pathways

Thesis

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Regulation of leukocyte cytokine production by inhibitors of intracellular signalling pathways

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I don't want to achieve immortality through my work...
I want to achieve it through not dying

Woody Allen

Abstract

The activation of leukocytes during inflammation results in the engagement of many related intracellular signalling pathways. The role of two key proteins families, phosphodiesterase (PDE) and Src kinase, has been assessed in these signalling pathways using specific inhibitors.

Inhibition of phosphodiesterase type 4 (PDE4) in lipopolysaccharide (LPS) stimulated human peripheral blood mononuclear cells (PBMC) resulted in the inhibition of TNF-α by an IL-10-independent mechanism. This is in contrast to other agents that elevate cAMP which in turn inhibit TNF-α synthesis by an IL-10-dependent mechanism. When PBMC were stimulated with LPS plus IFN-γ, inhibition of PDE4 resulted in an altered effect on cytokine production, elevating IL-10 while still inhibiting TNF-α.

Inhibition of PDE4 in activated T cells only weakly inhibited cellular proliferation. In contrast, PDE4 inhibitors were potent but non-selective inhibitors of T-cell cytokine production. In contrast to studies in murine cells, inhibition of T-cell activation correlates with inhibition of binding to a high-affinity Rolipram binding site on the PDE4 protein.

Kinases from the Src family (Lck, Fyn and Lyn) have been implicated in signalling via the T-cell receptor (TCR) on lymphocytes and the high affinity IgE receptor (FcεRI) on mast cells. Potent inhibitors of Src kinases blocked T-cell proliferation and cytokine generation in response to specific ligation of the TCR. However, activation of T-cells by multiple co-stimulatory pathways was much more resistant to Src kinase inhibition. Inhibition of Src kinase in human cord blood-derived mast cells inhibited IgE-dependent degranulation of these cells. This indicates that Src kinase inhibitors may be useful in down-regulating allergic responses. Conversely, inhibitors of PDE4 and PDE type 7 (PDE7) did not prevent mast cell degranulation.

The observation that PDE4 inhibitors can regulate monocyte cytokine generation further supports the view that these agents could have therapeutic benefit in a number of inflammatory diseases such as asthma, arthritis and inflammatory bowel disease. The failure of Src kinase inhibitors to modify T cell signalling activated by multiple pathways indicates redundancy in these signalling pathways and therefore Src kinases may not represent good targets for immunomodulation. However, a selective Src kinase inhibitor could represent a good target for an anti-allergic drug.

Acknowledgements

I would of course like to thank my supervisor and friend Dr. Rodger Allen, his help, understanding and ability to see the funnier side of life have made him of great help. I would also like to thank my external supervisor Prof. Brian (Binky) Henderson, yes for his advice and help but mostly for the fact that he was responsible for the one and only time I have been thrown out of a pub and to this day I still can't quite figure out why? I couldn't, though some people wish they could, leave out Dr. Gerry Higgs from proceedings. We on occasions didn't see eye to eye but his advice, strangely and usually days later, made sense though I never could quite see it at the time.

As my work was, at one time in the dim and distant past, part of a large team effort there are many people I need to thank and acknowledge. In particular, the whole of the high-throughput screening department who performed the kinases assays I used in my work. I must also not forget the help that Breda Twomey gave me in ironing out the glitches, I'm sure she had much better things to be doing. In addition, a mention goes out to L235, not a MI6 code word but the lab in which I work. I know that Nobby, Lisa, Emma *et al* had to put up with a lot of mindless gibberish from me but they still kept coming back for more.

Lastly, I would like to thank my wife Bernie. She was my tower of strength throughout this thesis.

Abbreviations

aa	amino acid
Ab	antibody
AMP	adenosine 3', 5' monophosphate
AP-1	activator protein-1
APC	antigen presenting cell
ATF-2	activating transcription factor 2
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
cAMP	cyclic adenosine 3', 5' monophosphate
cbMC	cord blood mast cells
cGMP	cyclic guanine 3', 5' monophosphate
Con A	concanavalin A
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
CsA	cyclosporin A
Csk	c-Src tyrosine kinase
CT-SKI	Celltech-Src kinase inhibitor
DMSO	dimethyl sulphoxide
EGF	epidermal growth factor
EGFR	epidermal growth factor-receptor
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
Erk	extracellular signal-regulated kinase
FCS	foetal calf serum
FcεRI	high affinity IgE receptor I
FcγRIII	low-affinity IgG receptor
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage-colony stimulating factor
GMP	guanine 3', 5' monophosphate
GST	glutathione S-transferase
HARBS	high-affinity Rolipram binding site
HLA	human leukocyte antigen

hr	human recombinant
HRP	horse radish peroxidase
IBMX	3-isobutyl-1-methylxanthine
IcbMC	immature cord blood derived mast cells
IFN-γ	interferon-γ
IkB	inhibitor of κB
IKK	IκB kinase
IL-	interleukin
IL-1Ra	IL-1 receptor antagonist
IRAK	IL-1 receptor activated kinase
ITAM	immunoreceptor tyrosine-based motif
Itk	IL-2 inducible T-cell kinase
Jak	janus kinase
Jnk	c-Jun N-terminal protein kinase
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
LTC_4	leukotriene C_4
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatability complex
MIP-1α	macrophage inhibitory protein-1 α
MLR	mixed lymphocyte reaction
mRNA	messenger RNA
MyD88	myeloid differentiation factor 88
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-κB
PAF	platelet activated factor
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PDE	phosphodiesterase
PDGF	platelet derived growth factor
PE	phycoerythrin
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2

PHA	phytohaemagglutinin
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLCγ1	phospholipase Cγ1
PMA	phorbol 12- myristate-13-acetate
RA	rheumatoid arthritis
RANTES	regulated upon activation normal T cell expressed and secreted
RIA	radioimmunoassay
RIP	receptor interacting protein
SCF	stem cell factor
SD	standard deviation
SEM	standard error of mean
SH2	Src homology 2 domain
Slp 76	SH2 domain leukocyte protein 76
SPA	scintillation proximity assay
SRF	serum response factor
STAT	signal transducer and activator of transcription
s-TNFR	soluble-TNF receptor
Syk	spleen tyrosine kinase
TCR	T cell receptor
Tec	tyrosine kinase expressed in hepatocellular carcinoma
TGF-β	transforming growth factor-β
Th1	T helper 1 cells
Th1	T helper 2 cells
TIR	toll-IL-1 receptor domain
TLR	toll-like receptor
TMB	tetramethylbenzidine
TNFR	tumour necrosis factor receptor
TNF-α	tumour necrosis factor-alpha
TRADD	TNFR1-associated death domain protein
TRAF	TNF receptor associated factor
VEGF	vascular endothelium growth factor
ZAP-70	zeta associated protein – 70 kDa

Contents

Chapter 1: Introduction

Chapter 2: Materials and Methods

Chapter 7: General discussion

Chapter 8: References

List of Figures

List of Tables

Chapter 1

Introduction

Chapter 1

Introduction

1.1 General introduction

The discovery of cytokines approximately 50 years ago has opened the door to the understanding of the inflammatory process. The family of cytokines (which are described in more detail in section 1.3) has dramatically increased as the impact of genomics, proteomics and informatics has uncovered an ever expanding family tree. Cytokines have many activities; they can induce activation, differentiation, proliferation, migration and apoptosis of cells. The wide spectrum of responses controlled and induced by cytokines, makes them attractive targets for therapeutic intervention. Cytokines, such as tumour necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), are regarded as excellent candidates and their blockade, using biological inhibitors, has already proved their importance in human clinical disease [1-4]. This thesis has examined how manipulation of enzymes that are involved in key intracellular signalling cascades can control cellular activation, particularly with respect to the control of cytokine production that is important in inflammation.

1.1.1 Acute inflammation

Since cytokines play an important role in inflammation it is necessary to define what is meant by 'inflammation'. Inflammation is classically defined as the response of a living tissue to damage. This damage can be due to: infection, physical injury, chemical substances or hypersensitivity reactions among other causes. Acute inflammation requires the rapid recruitment of cells capable of dealing with foreign invaders. This infiltration of cells to the site of inflammation is a complex process. Neutrophils normally are the first cell type recruited to the site of inflammation. The neutrophil is responsible for mounting a phagocytic response against any foreign material, leading to its destruction and removal. The danger signals emitted by the injured tissue during inflammation also serve to summon other cell types. At later stages of the inflammatory response, cells such as: eosinophils, basophils,

monocytes, T and B lymphocytes all appear at the site of injury. Resident tissue cells, such as mast cells, macrophages and dendritic cells also contribute to the innate and adaptive immune response. These cells are capable of mounting a sustained immune response to foreign bodies, resulting in the eventual control of the pathogen. The communication and co-ordination of the cells involved in the inflammatory response is, for the large part, mediated by the action of cytokines. When inflammation and hence cytokine production persist over time a state of chronic inflammation may ensue.

1.1.2 Chronic inflammation

Chronic inflammation is defined as an inflammatory response of prolonged duration - weeks, months or indefinitely. The persistence of inflammatory disease is due to the inflammatory agent remaining, either at the site of the inflammatory reaction, or systemically. This continuous inflammatory insult inevitably causes tissue damage and consequently the body attempts to repair that damage. Chronic inflammation may develop in a number of ways. This may be as a progression from a state of acute inflammation, or develop following repeat episodes of acute inflammation or develop *de novo*. The aetiological agents that cause chronic inflammation are summarised in **table 1.1**.

1.2 Inflammatory disease

The broad definition of inflammation, as defined above, encompasses many biological processes and involves many different cell types and tissues. Inflammatory disease is too wide a topic to cover extensively in this introduction. Instead, two diseases each with their own distinct aetiology and pathology – rheumatoid arthritis and asthma will be covered in some detail. These diseases will be used to introduce the concepts of inflammation, the cell types and cytokines involved in inflammation and as a general background to ideas introduced and studied throughout the thesis.

Table 1.1: Aetiological agents responsible for eliciting chronic inflammatory responses.

I	Infectious organisms that can avoid host defences and thus persist for prolonged periods. E.g. *Mycobacterium tuberculosis*, fungi and parasitic infections. Such organisms survive by either being able to withstand phagocytosis, by existing within phagocytic cells or by inducing only a mild inflammatory response.
II	Infectious organisms that are not resistant to host defences but exist in areas protected from host defences. For example, bacteria that grow in the pus within an undrained abscess cavity.
III	Irritant non-living and foreign material that cannot be removed by enzymatic breakdown or phagocytosis. For example, wood splinters, grit, metal and plastics. These foreign materials can be either: **Inhaled** e.g. silica dust and other particles/fibres. **Introduced** e.g. surgical prostheses etc.
IV	Stimulation from usually normal tissue components. As in the case of auto-antigens.
V	Where there is no other obvious cause, such as idiopathic diseases: Crohn's disease, rheumatoid arthritis etc.

1.2.1 Rheumatoid Arthritis

Like most other chronic inflammatory diseases, rheumatoid arthritis (RA) is poorly understood. Although it is known that RA has an autoimmune component, its exact aetiology is unknown. What is known, is that the disease can be associated with a number of risk factors, some of which are genetic. Identical twin studies, which are normally powerful indicators of the genetic linkage of diseases, show a relatively low rate of concordance, in the range of 15-35% for RA [5]. This places a limit upon the possible contribution of a genetic element to the disease. Though, undoubtedly factors such as HLA subtype, age and sex are important predictors of the likelihood of disease; they are not the full explanation.

RA is manifestly a disease of the joints, particularly the hands, wrists and feet. Despite this obvious outward phenotype, there is also clear evidence that RA has a systemic element. RA patients with active disease have elevated erythrocyte sedimentation rate (ESR), C reactive protein (CRP) and other acute phase proteins [6]. In more severe and prolonged cases there is an extra-articular disease, manifest as skin nodules which occur in regions prone to mild injury. Other features of RA include fibrosis, most commonly of the lung and in ~1% of cases 'Feltys syndrome' [7] (hypersplenism and increased white blood cell and platelet turnover).

The joints of patients with RA exhibit two main features; synovitis with hyperplasia and inflammation of the synovium inducing an inflammatory exudate into the joint causing erosion of bone and cartilage. Synovitis manifests itself symptomatically as joint pain, morning stiffness with swelling and tenderness of the joints. In RA there is a thickening of the synovium , the lining layer of the joint being 6-10 cells, as compared to 1-2 cells thick in a normal lining layer.

One consequence of synovitis is an increased vascularisation of the joint [8]. This ultimately leads to a large cellular infiltrate into and between the many newly formed small vessels. The most abundant infiltrating cells are T cells and macrophages with plasma cells, dendritic cells, activated fibroblasts and endothelial cells making up the rest of the cell mass [9]. As has been indicated, the majority of these cell types have trafficked to the joint and are blood-borne in nature. The erosion of cartilage and bone takes place mostly at the site where the fibrous capsule of the joint, lined by the synovium, abuts with the cartilage and bone. This region of synovium, known as the 'pannus', overlies and erodes the cartilage and invades the bone. Macrophages are the major cell type at this juncture with endothelial cells and fibroblasts (also known as synoviocytes) also present.

1.2.2 Asthma

The second disease used to illustrate the role that cytokines play in inflammation is asthma. Asthma is an inflammatory disease of the airways of the lung. In asthma, narrowing of the

airways occurs because of inflammation and hypersecretion of mucus. This is exacerbated as the smooth muscle of the bronchi become hyperresponsive to non-specific stimuli [10]. This intermittent airway constriction leads to wheeze, cough, tightness and shortness of breath. In the long term, this may lead to fibrosis and scarring of the bronchioles, and obstruction of the airways may become permanent.

Asthma is a disease whose prevalence has markedly increased over the last twenty years [11]. This increase has been postulated to be caused by such things as passive smoking [12], vaccination programmes (e.g. BCG) [13], viral infections [14], diet [15] and the length of time an individual spends indoors [16]. Either together, or on their own, none of these factors can truly explain the inexorable rise in the number of asthma cases.

Asthma has, like many diseases, been shown to have a genetic component with genetic loci being mapped as disease susceptibility factors. These have been associated with human chromosomes 4, 5, 6, 7, 11, 13 and 16 [10;17-19]. Genome screening techniques have been used to try to map individual genes or sectors of chromosomes that have either positive or negative effects upon asthma. Two such candidates are CD14 and the high-affinity IgE receptor.

A linkage with the CD14 gene was mapped to the short arm of chromosome 5 [20]. Interestingly this is the same location as the IL-4 gene and a number of other Th2 cytokines. CD14 acts as a co-receptor for lipopolysaccharide (LPS) so it's linkage, albeit as a negative regulator of disease, was a surprise. Baldini *et al* [20] discovered that a C→T base change 159 bases upstream of the transcription start site for CD14 was associated with high levels of soluble CD14 (sCD14) and low levels of IgE. This adds weight to the theories of those who think that exposure to allergens and the immune milieu encountered during infancy, programs immunity to asthma. Thus, exposure to bacterial antigens and stimulation of monocytes and macrophages (in particular the production of IL-12) may prime for cytokine responses that counteract IgE production.

The other gene product strongly linked to asthma is the high affinity IgE receptor (FcεRI) β-chain [21]. The β-chain of FcεRI, although not essential for IgE signalling, acts to amplify signals through the receptor. Regulating its expression may therefore be a way of modifying cell function and disease. Unlike CD14 there have been few changes identified in the FcεRIβ chain and those that have been discovered are conservative and do not seem to alter the gene function [22].

Although, like rheumatoid arthritis, asthma does have a genetic component, the disease can be best understood by examining the cell types and cell-cell interactions that occur both in normal and diseased states. At the centre of the asthmatic process lies the CD4 positive T helper memory cell [23;24]. These cells are critical because they produce an array of cytokines that programme the behaviour of other leukocytes, and ultimately control acute and chronic allergic inflammation in the airway (the role of cytokines in the pathogenesis of asthma will be covered in more detail in section 1.3.2). The various cell types and their interactions in asthma can be seen in **fig 1.1**.

Asthma exhibits a two stage progression from an immediate response, which starts in minutes, to a delayed response, which starts hours after the initial insult. The immediate response is mediated by acute phase components involved in classical immediate-type hypersensitivity responses. This is induced by allergen cross-linking specific IgE bound to mast cells via the high-affinity IgE receptor [25]. Mast cells release a range of granule-associated preformed mediators which are responsible for the immediate symptoms of the acute allergic response and which contribute to the late phase response [26]. Mast cells also produce a variety of chemokines and cytokines which contribute to the recruitment and activation of a second wave of response, in particular the recruitment and survival of eosinophils [27]. The late phase response is characterised by airway perivascular oedema, mucus plugging and activated Th2 cells which sustain the recruitment and activation of eosinophils.

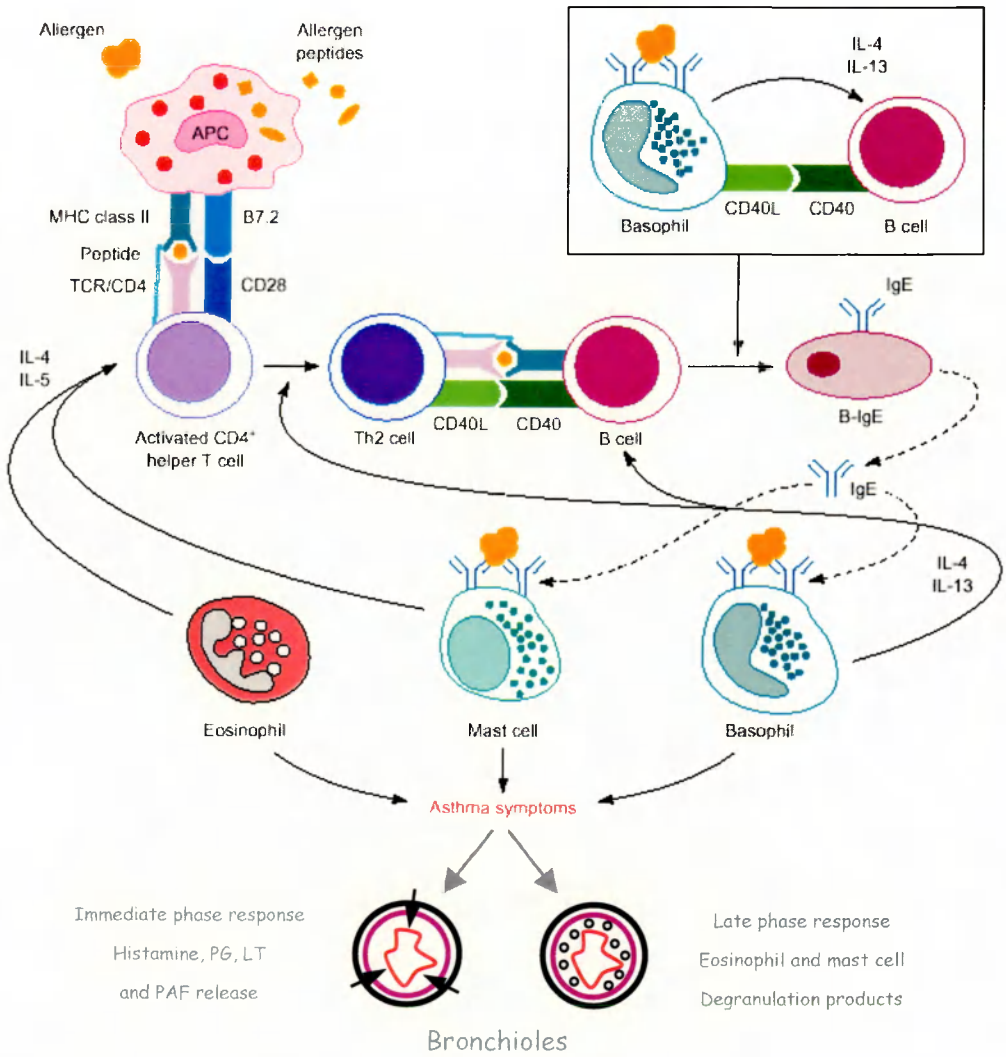

Figure 1.1: The cells co-ordinating the asthmatic response. Allergen is processed as peptides and presented to CD4 T helper cells in the context of MHC class II. IL-4 released by mast cells, basophils and eosinophils facilitates the differentiation of Th2 cells, which upon stimulation produce IL-4, IL-5, IL-10 and IL-13. Activated Th2 cells then interact with B cells via TCR, BCR and CD40/CD40L, and in the presence of IL-4 and IL-13 switch Ig synthesis in favour of IgE. IgE binds to IgE receptors (including FcεRI on mast cells and basophils) and is cross-linked by allergen to activate IgE receptor positive cells. Amplification of B cell Ig class switching may occur from interactions of basophils with CD40 positive B cells (see box). Activation of mast cells, basophils and eosinophils leads to the release of pro-inflammatory, vasoactive and fibrogenic factors (histamine, tryptase, chymase, LT, PG, PAF) that are responsible for the symptoms of bronchial asthma. Abbreviations: APC, antigen-presenting cell, BCR, B cell receptor, CD40L, CD40 ligand, Ig, immunoglobulin, LT, leukotrienes, MHC, major histocompatability complex, PG, prostaglandins, PAF, platelet activating factor, TCR, T cell receptor. Adapted from a diagram by Marone [23].

1.3 Cytokines and inflammation

Cytokines are often viewed as the hormones of the immune system. They bind to target receptors which are expressed at the cell surface but which can also be expressed in a secretable form. Cytokines are potent molecules that typically act at concentrations in the pico- to nano-molar range and can act in three separate ways. They can act upon the cell producing the cytokine in an autocrine manner, act locally on neighbouring cells in a paracrine manner or act over longer distances in an endocrine manner. Unlike classical endocrine hormones, cytokines are often pleiotropic in action. This can be exemplified by the actions of TNF-α, **fig 1.2**, an important pro-inflammatory cytokine involved in the pathogenesis of many diseases [2;28].

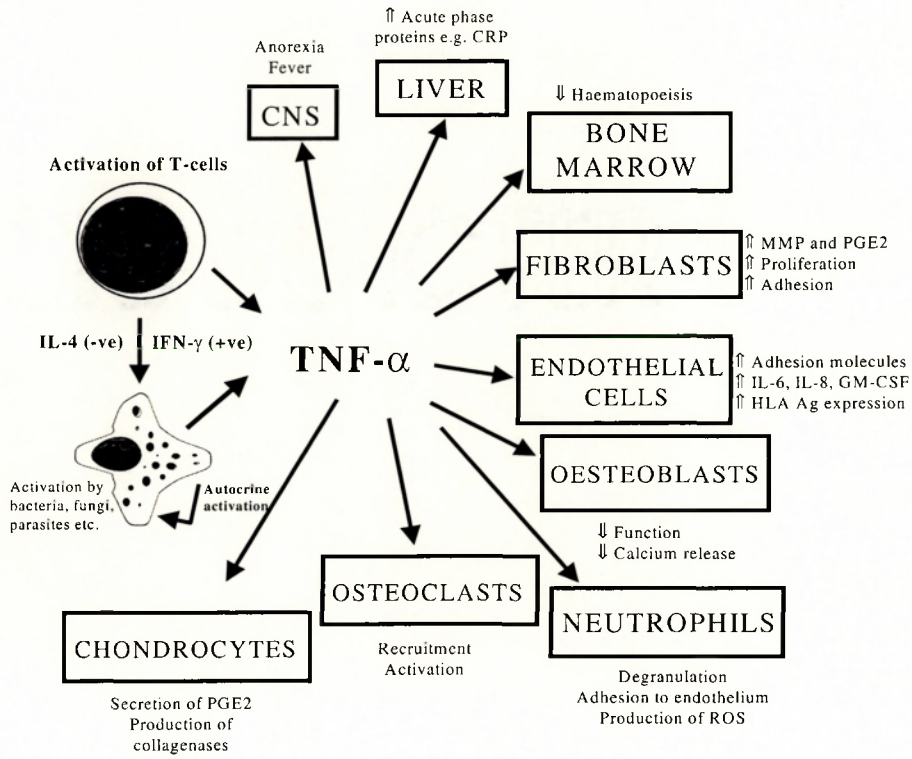

Figure 1.2: Overview of the effects of tumour necrosis factor-alpha. Abbreviations, Ag, antigen, HLA, human leukocyte antigen, MMP, matrix-metalloproteinases, PGE_2, prostaglandin E_2, ROS, reactive oxygen species.

Cytokines rarely act in isolation but form complex and interdependent regulatory pathways, termed 'cytokine networks' [29]. Individually cytokines have been shown to have distinct and

potent effects upon many cellular activities. These *in vitro* actions have not always been manifest when tested *in vivo*. To understand how cytokines act *in vivo* it is necessary to know how they interact with other cytokines in their local network. To understand how these networks are altered in diseases the examples of RA and asthma will again be used. RA will introduce the concept of a cytokine imbalance between pro- and anti-inflammatory cytokines that perturbs the cytokine network in the rheumatoid synovium and asthma will introduce the Th1/Th2 paradigm and how inappropriate cytokine production can lead to disease.

1.3.1 Cytokines and rheumatoid arthritis

In a chronic autoimmune diseases, such as RA, it can be predicted that cytokines will be expressed at high levels at the site of inflammation. From early studies measuring both mRNA and protein, in culture and *in situ*, it became apparent that the rheumatoid synovium was an abundant site of cytokine expression [30], these cytokines are shown in **table 1.2.** As can be seen from this list of cytokines, both pro- and anti-inflammatory cytokines can be found in the inflamed joint, and although informative it gives no indication as to which cytokines may be critically involved in the pathogenesis of the disease.

Using the knowledge that cytokines act within connected networks, researchers analysed the pattern of cytokine production in the hope of finding an immune bias to the cytokine network. It was discovered that macrophage derived proteins TNF-α, IL-1, IL-6 and IL-8 were found at high levels in the synovium [30-37]. These proteins, in particular TNF-α, IL-1 and IL-6, have many effects on different cells and organs of the body and were thought likely to play some role in the disease. T cell derived cytokines, such as IL-2 and IFN-γ were found to a lesser extent at the protein level, though were found to be more abundantly expressed at the mRNA level [38;39]. This imbalance in cytokine production formed the basis of a theory to explain the joint destruction seen in RA, **Fig 1.3.**

Table 1.2: The expression of cytokines in the rheumatoid synovium. *

Cytokines	Monocytes	Fibroblasts	T cells
IL-1	+	+	
TNF-α	+	+	
IL-6	+	+	
LIF	+	+	
GM-CSF	+	+	
PDGF	+	+	
bFGF	+		
IL-1Ra	+		
TGF-β	+	+	
IL-10	+		+
IL-13			+
MCP-1	+	+	
MIP-1α	+	+	
RANTES		+	
IL-8	+	+	
ENA-78	+	+	
VEGF	+		
IFN-γ			±
IL-2			±
IL-4			?

* Abbreviations, ENA-78, epithelial cell-derived protein 78, bFGF, basic fibroblast growth factor, GM-CSF, granulocyte/macrophage colony stimulating factor, LIF, leukaemia inhibitory factor, MCP-1, monocyte chemoattractant protein 1, MIP-1α, macrophage inflammatory protein 1 alpha, PDGF, platelet derived growth factor, RANTES, regulated and normal T cell expressed, TGF-β, transforming growth factor beta, VEGF, vascular endothelial growth factor.

The pattern of cytokine expression described in **figure 1.3** however, does not completely describe the network of cytokines involved in rheumatoid arthritis. Other cytokines, such as IL-11, IL-12, IL-15, IL-17 and IL-18 [40-44], which are also found to be expressed in the rheumatoid synovium are likely to play some role in the pathogenesis of the disease. Despite this obvious complexity, the key finding that inhibition of individual cytokines could alter the global cytokine profile has aided both the understanding and the therapy of RA. Work in animal models of arthritis [45] and ultimately in clinical trials [46], has shown that blockade of TNF-α can reduce disease severity.

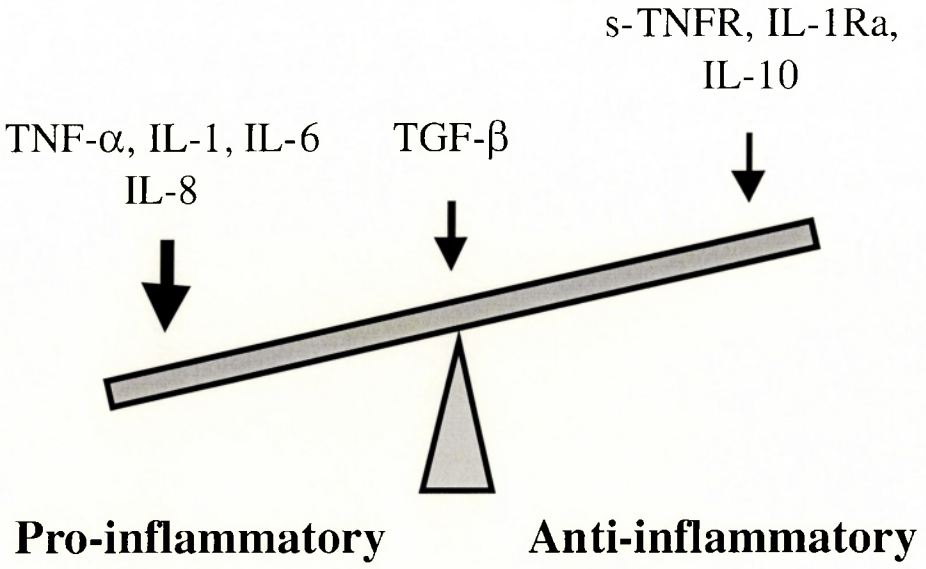

Figure 1.3: The balance of pro- and anti-inflammatory cytokines in the rheumatoid synovium. The overall outcome of cytokine production in the rheumatoid synovium can be seen as a balance of the actions of the cytokines produced in that environment. Many cytokines are pro-inflammatory and contribute to the pathogenesis of the disease, e.g. TNF-α and IL-1. Other cytokines are anti-inflammatory, and can antagonise the action of pro-inflammatory cytokines, e.g. soluble TNF receptors and IL-1 receptor antagonist. A third class of cytokines, that has both pro- and anti-inflammatory action can influence both arms of the inflammatory response, e.g. TGF-β. Adapted from a diagram by Feldmann and Brennan [47].

1.3.2 Cytokines and asthma

The adaptive immune response involves both humoral and cellular immunity. These twin arms of adaptive immunity are largely regulated by T cells and in particular the cytokines they produce. Pioneering work by Mosmann and Cofmann [48] led to the proposal that T helper (Th) cells could be separated into different subsets according to the cytokines they released. This distinction was also subsequently made for cytotoxic T cells [49]. The discovery of Th subsets has provided great insight into the probable disease mechanisms underpinning atopic inflammation and hence asthma. Th subsets are thought to be derived from a common precursor and, in response to environmental stimuli, differentiate into different populations with distinct cytokine secretion profiles, **Fig 1.4**. The so-called Th1 subset of cells secrete IL-2, IFN-γ, TNF-α and LT, i.e. cytokines involved in cellular defence mechanisms and lytic antibody responses against intracellular pathogens [50]. The other major subset, called Th2 cells, in contrast to Th1 cells produce an entirely different spectrum of cytokines including, IL-4, IL-5, IL-6, IL-9 and IL-13 [50]. These cytokines are involved in the immune response to

combat extracellular pathogens, e.g. parasitic worms. Th2 cytokines critically prime the immune response to make IgE antibodies. The presence of IgE and Th2 cytokines are strongly associated with asthma [10].

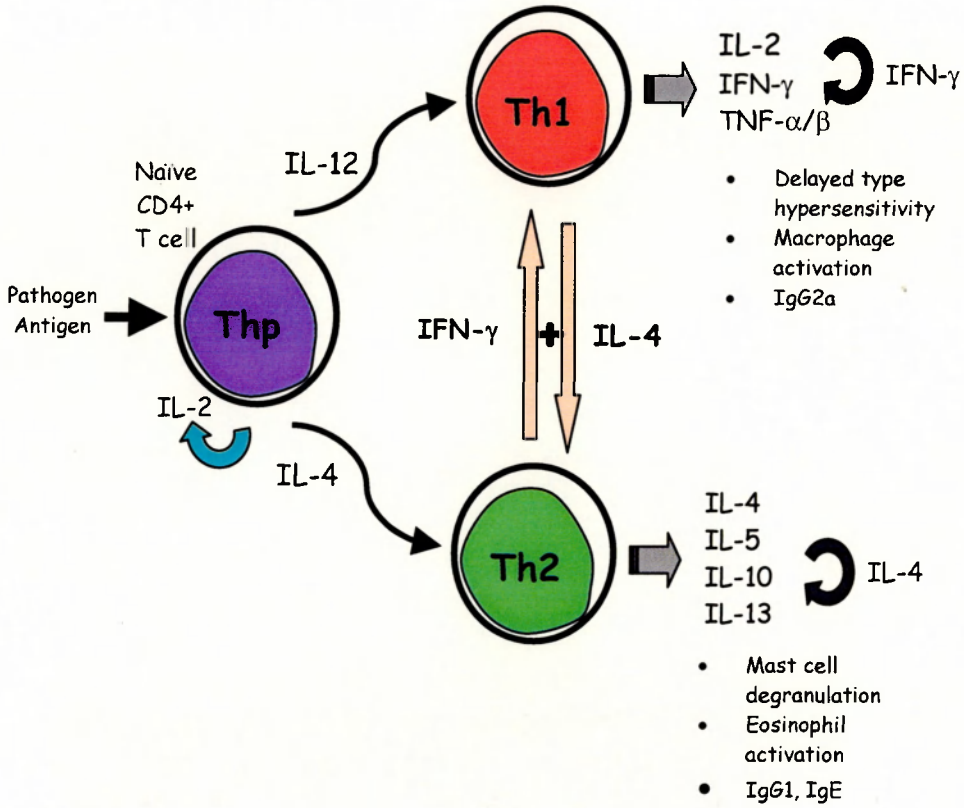

Figure 1.4: The balance of T helper subsets and cytokine production. Naïve CD4 positive T cells when experiencing antigen for the first time are not pre-committed to either a Th1 or Th2 lineage. Thp (T helper precursor cells) cells expand when stimulated with antigen under the influence of IL-2. The dominant differentiating cytokines, IL-12 (Th1) and IL-4 (Th2), skew T helper cells to becoming either Th1 or Th2 cells. These cells upon restimulation, produce distinct patterns of cytokines. Th1 cells produce, IL-2, IFN-γ, LT and TNF-α and Th2 cells produce, IL-4, IL-5, IL-10 and IL-13. IFN-γ is responsible for amplifying Th1 responses and IL-4 is responsible for amplifying Th2 responses. Th1 cells mediate delayed type hypersensitivity reactions and Th2 cells mediate allergic inflammatory reactions.

A major breakthrough in our understanding of asthma came about by the discovery that, in response to allergens, T lymphocytes produce a restricted array of cytokines [51]. The study of T cell lines and clones produced in response to such allergens lead to the discovery of a distinct cytokine production profile, dominated by cytokines that are pro-inflammatory for allergic inflammation [52]. It is the over-production of Th2 cytokines such as, IL-4, IL-5 and IL-13 (**Fig 1.1**), which contributes to events such as the production of IgE and recruitment of eosinophils to the asthmatic lung. A number of factors are thought to select for the Th2 response in asthma. These include low binding affinity of allergenic peptides in the groove

of MHC class II, selective engagement of CD28 with CD86 but not CD80 [53;54] and low IL-12 or IL-18 production in the microenvironment, which normally biases T cell/dendritic cell interactions to prime for Th1 responses [55].

1.4 Signal transduction pathways involved in inflammation

The activation of cells to produce factors, such as cytokines, requires the transduction of extracellular signals, usually through the binding of a ligand to a receptor, which leads to the transcription of genes and production of proteins. These activation pathways, in different cell types, while sharing common elements are distinct. Key signal transduction pathways activated in cells involved in RA and asthma will be emphasised. These signalling pathways can be activated *in vitro* and share common elements with those pathways likely to be activated during inflammation.

1.4.1 Activation of monocytes and macrophages with bacterial endotoxin (LPS)

Monocytes and macrophages are responsible for producing large amounts of inflammatory cytokines, e.g. TNF-α, IL-1 and IL-6. These cytokines, which are produced in large quantities in the rheumatoid synovium, play a role in joint destruction. To date the best established *in vitro* model of monocyte/macrophage activation utilises bacterial products such as LPS. Although there is no definitive evidence for the role of infection in rheumatoid arthritis, the signalling pathways activated by LPS are likely to be shared by cells in the synovial environment. The understanding of how LPS transduces downstream signals has recently undergone a paradigm shift [56;57].

In 1968 Sultzer *et al*, demonstrated that the C3H/HeJ inbred mouse strain was naturally tolerant to LPS [58]. These mice were able to withstand an LPS challenge 20-40 times the median lethal dose for most other laboratory strains [59]. This trait mapped to the major urinary protein (*mup1*) locus on chromosome 4 and was designated as the *lps* locus [60]. The *lps*

mutation not only manifests itself at a system level but also at the individual cell level and renders many diverse cell lineage's hypo-responsive to the effects of LPS.

Virulent Gram-negative bacteria induce inflammation by the shedding of their outer membrane [61]. Within the aqueous environment of the host's vascular compartment, amphipathic LPS molecules spontaneously aggregate as micelles [62]. These micelles, via a plasma LPS-binding protein, are catalytically transferred as monomers to the surface receptor CD14 [63]. CD14 is recognised as both a myeloid differentiation factor and a receptor for LPS and can either be glycosylphosphatidylinositol-linked into the plasma membrane or a soluble protein. Both the cell surface and soluble CD14 moieties facilitate LPS signalling despite the absence of a transmembrane domain [64]. This has led to speculation that a co-receptor is recruited to the LPS/CD14 complex and mediates signal transduction [65].

Recently the gene responsible for the *lps* phenotype in C3H/HeJ mice was discovered. A mis-sense mutation in the Toll-like receptor 4 (TLR4) was discovered and was found to result in a Pro 712→His amino acid substitution [66;67]. The Toll-like receptors are signalling receptors that, although initially thought to be involved in embryogenesis in *Drosophila*, were subsequently found to be implicated in innate immune defence responses against pathogens [57]. The mammalian homologues of Toll have a characteristic leucine rich repeat extracellular domain and a cytoplasmic domain responsible for signal transduction. Nine human Toll receptors have been cloned thus far but only one, TLR4, has been implicated in LPS signal transduction. The transfection of constitutively active TLR4 into a recipient cell line induces expression of cytokines and co-stimulatory molecules in a similar manner to endotoxin [68]. Wild-type TLR4 transfection alone is insufficient to confer LPS sensitivity, but in the presence of another protein, MD-2 it forms a complex at the cell surface which confers LPS sensitivity upon TLR4 negative cell lines [69]. Confusion regarding which toll receptor is responsible for mediating LPS signalling has recently been clarified as LPS preparations from distinct bacterial species (*Leptospira*) were found to activate cells through TLR2 and not TLR4 [70].

The cytoplasmic domain of the Toll-family proteins is homologous to the cytoplasmic domain of the IL-1 receptor and both receptors share many of the same signal transduction pathways [71-73]. The cytoplasmic domain of the Toll/IL-1 receptor (TIR) is referred to as a TIR domain. The TIR domain also resides in a second protein, known as myeloid differentiation factor 88 (MyD88). MyD88 is postulated, via its TIR domain, to interact with Toll-like receptor complexes [74]. In IL-1 signal transduction MyD88 immunoprecipitates with the functional IL-1R complex (IL-1R1, IL-1R accessory protein and IL-1R associated kinase (IRAK)) [75]. In MyD88 deficient mice, responses to IL-1, IL-18 and endotoxin are all deficient indicating its central role in all three pathways [76].

The death domain of MyD88 recruits the down stream serine/threonine kinase IRAK, in its unphosphorylated state [75;77]. IRAK is also a key molecule in the signalling cascade, as dominant-negative mutants of IRAK inhibit nuclear factor-κB (NF-κB) activation by upstream components of the cascade [78]. IRAK becomes autophosphorylated when the IL-1R is stimulated, but as yet no substrate for IRAK has been identified. IRAK interacts with a downstream protein, a member of the TNFR (tumour necrosis factor family receptor)-associated factors (TRAFs), known as TRAF6 [79]. The TRAF family of adapter proteins promotes protein oligomerisation and facilitates protein-protein interactions. TRAF6 is unique amongst the TRAF factors in that, apart from its association with CD40 [80], it is the only TRAF not to engage a receptor complex. LPS activates the production of both TNF and IL-1, the common elements between the two receptor signalling pathways are shown in **fig 1.5**. TRAF6 immunoprecipitates with the mitogen activated protein kinase kinase kinase (MAPKKK), NIK (NF-κB inducing kinase), this activates the IκB kinase complex and these enzymes are responsible for degrading the NF-κB inhibitor IκB [81]. Another MAPK, MEKK-1 (mitogen activated protein kinase Erk kinase kinase 1), is thought to activate IKK's but as yet this has no proven role in Toll receptor signalling. Finally the phosphorylation of IκB releases NF-κB and it translocates to the nucleus where it induces the expression of specific genes, many of which are cytokines involved in the inflammatory response [82].

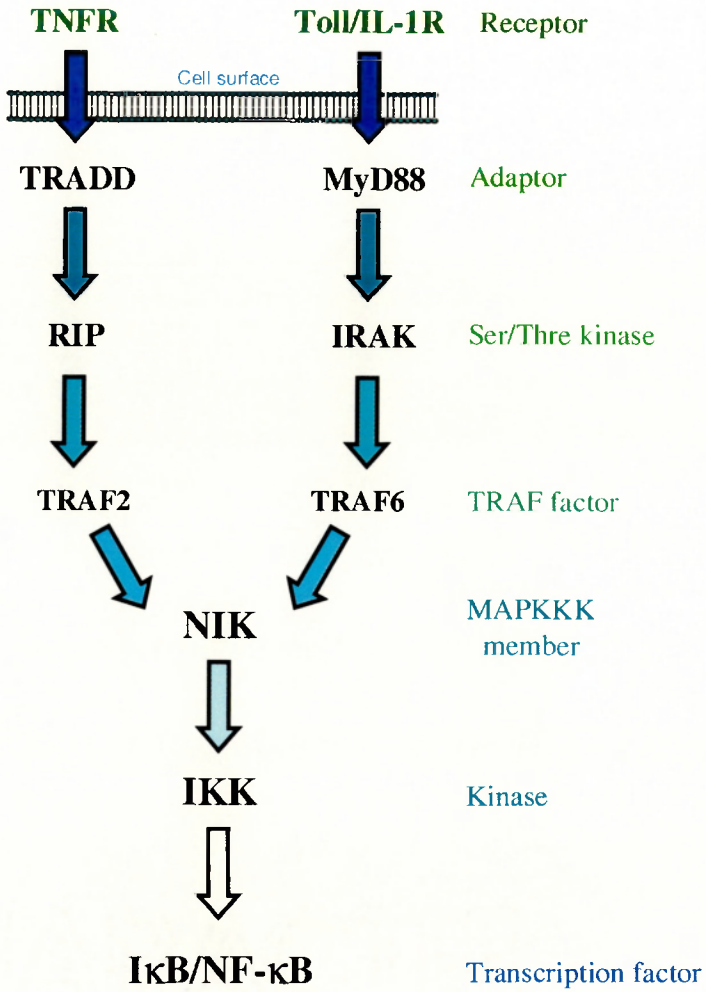

Figure 1.5: The similarities in signalling pathways of Toll/IL-1R and the TNFR family. Abbreviations, TNFR, TNF-α receptor, TRADD, TNFR1-associated death domain protein, RIP, receptor interacting protein, IRAK, IL-1 receptor-activated kinase, TRAF, TNF receptor-associated factors, NIK, NF-kβ inducing kinase, IKK, Ikβ kinase.

1.4.2 Activation of T cells through the T cell receptor

Alongside monocytes, activated T cells are critically involved in inflammatory cytokine production. The primary activation signal of T lymphocytes is one that is transduced via the T cell receptor (TCR), which recognises antigenic peptides in the binding groove of the major histocompatibility complex (MHC). Although there are many other pathways that can be activated in T cells, only a limited number of such pathways will be discussed in this introduction. The TCR is a complex multi-subunit structure composed of a ligand binding heterodimer (αβ or γδ) that can recognise peptides bound to MHC molecules. This complex also contains the CD3ε, γ, δ and TCRζ (zeta) chains [83]. The role of these chains is to target

the fully functional TCR complex to the cell surface. The CD3 and zeta chains contain immunoreceptor tyrosine-based motifs (ITAMs), three in the zeta chain and one in each of the CD3 chains [84]. These ITAMs may either be involved in amplification of signalling through the TCR, or determine signal specificity as they are thought to differentially bind intracellular signalling proteins, Zap-70 (Zeta associated protein – 70 kDa), phospholipase Cγ1 (PLCγ1), phosphatidylinositol 3-kinase (PI3 kinase) and shc (Sh2 containing sequence) [85-88].

Members of at least four families of tyrosine kinases are involved in the transduction of the signal generated by engaging the TCR. Src kinase members, $p56^{Lck}$ (Lck) and $p59^{Fyn}$ (Fyn) are two proteins that play an important role in TCR signalling. The role of Lck in TCR signalling was elucidated using a mutant of the Jurkat cell line, termed JCAM1 [89]. Upon TCR stimulation, these cells failed to give a calcium flux. Analysis of these cells revealed a defect in tyrosine phosphorylation, which was due to a defect in functionally active Lck [89]. Reconstitution of JCAM-1 cells with wildtype Lck restored functionality to the cell line. TCR signalling was also antagonised by the micro-injection of Lck antibodies into T cells [90]. Finally, Lck deficient mice were shown to have sub-optimal T cell proliferative responses, when T cells were stimulated through the TCR [91]. Fyn is also associated with the TCR directly, being co-expressed with the cytoplasmic domains of CD3ε and γ and TCR ζ and η chains. The role of Fyn in TCR signalling is not as clear as that of Lck. This is exemplified by the fact that overexpression of Fyn in T cells augments TCR stimulation, as measured by tyrosine phosphorylation and IL-2 production [92;93].

A member of the Syk (spleen tyrosine kinase) class of protein tyrosine kinases, Zap-70 is the dominant family member in T cells. Zap-70 is known to be a critical molecule in TCR signalling, as a rare Zap-70 immunodeficiency exists in humans, and such individuals exhibit a severely immunocompromised phenotype [94;95]. In transfection studies, Zap-70 associates with the zeta chain and its activation is dependent upon either Lck or Fyn [96]. In the JCAM-1 cell line Zap-70 is neither phosphorylated nor recruited to the T cell receptor complex [97]. Syk

augments TCR-triggered tyrosine phosphorylation of zeta and unlike Zap-70 was able to stimulate tyrosine phosphorylation of zeta when transfected into Cos-1 cells [98]. Another cell line P116 (which has defects of protein tyrosine phosphorylation, calcium flux and IL-2 transcription), that is also derived from Jurkat cells, is defective for both Syk and Zap-70. When either catalytically active protein was reintroduced into these cells their defects were reversed [99].

Csk (c-Src tyrosine kinase), another tyrosine kinase family protein, has a negative regulatory role upon Src kinase activation and as such upon T cell activation. Csk phosphorylates the carboxy-terminal of Lck and Fyn maintaining them in an inactive state [100]. The phosphatase CD45, (also associated with the TCR complex), dephosphorylates Lck and Fyn allowing their activation upon TCR engagement [84]. Another tyrosine kinase family member Tec (tyrosine kinase expressed in hepatocellular carcinoma), expressed as Itk (IL-2 inducible T cell kinase) and Tec, are also involved in TCR signalling. Itk signals downstream of Lck and is recruited to different signalling complexes when CD28 or the TCR are engaged [101;102]. In Itk-deficient mice T cell signal impairment is modest and hence its contribution to TCR signalling may not be as great as the Src family kinases [102]. Tec when activated by TCR/CD3 or CD28 ligation interacts with the CD28 receptor. Tec can phosphorylate $p62^{Dok}$ (a substrate of CD28), whereas Itk cannot. These differences may indicate different roles for these tyrosine kinases in T cell activation [102].

Further down stream events, in the TCR signalling cascade, occur via tyrosine phosphorylation of Slp-76 (SH2 domain leukocyte protein), a Zap-70 substrate, which mediates association with Vav. TCR stimulation induces tyrosine-phosphorylated proteins including Slp-76, $p120^{Cbl}$ and Shc which associate with the Grb-2/Sos complex which is known to be involved in the regulation of Ras [103-106]. The Ras/MAPK pathway is an important pathway activated upon TCR stimulation. The expression of T cell cytokines, such as IL-2, is largely inducible and is initiated by the cross-linking of the T cell receptor plus the activation of other co-stimulatory molecules. The signal transduction pathways detailed

above, ultimately leads to the activation of many transcription factors. The complex interplay between these factors, controls the cytokine production in T cells. In T cells these signal transduction pathways include a calcium-dependent/cyclosporin-sensitive pathway that regulates the nuclear factor of activated T cells (NFAT), a protein kinase C-dependent pathway that regulates activator protein-1 (AP-1), (NF-κB) and a p21/Ras activated MAPK cascade that affects AP-1 amongst other transcription factors [107].

1.4.3 Activation of mast cells through the high-affinity IgE receptor

As described earlier, mast cells are important in the pathogenesis of asthma and allergic disease. IgE, and more specifically the IgE that is cross-linked on the cell surface of a mast cell by antigen, is an important trigger of many of the symptoms of asthma. The receptors that bind IgE on mast cells are termed high-affinity IgE receptors or FcεRI.

FcεRI belongs to a family of multi-subunit immune receptors that do not have intrinsic kinase activity but associate reversibly with protein tyrosine kinases. FcεRI is expressed on mast cells as a heterodimeric complex comprising of α, β and two γ chains. IgE binds to the alpha chain, which is the major extracellular component of FcεRI [108]. The β and γ sub-units are responsible for down-stream propagation of signals through phosphorylation of their ITAM's (as seen in the TCR, section 1.4.2) [109]. The β chain has been ascribed an amplifying role within the signalling pathway and the γ units are essential for targeting the receptor to the cell surface and for signal transduction [110-112].

Upon receptor aggregation signals are transduced via an unknown mechanism to the β and γ-chain signalling subunits. The Src family tyrosine kinase, Lyn, is activated, or deactivated, via tyrosine phosphorylation or dephosphorylation at its carboxy terminus by Csk kinase and the phosphatase CD45 respectively [113;114]. Activated Lyn then phosphorylates the β and γ-chain ITAM's, the γ-chains are then able to selectively recruit Syk which in turn is activated by Lyn [108;115]. Targets of these tyrosine kinases include the activation of PI(3) kinase to

produce phosphotidylinositol (3,4,5)P_3 or PIP_3, phosphorylation of Bruton's tyrosine kinase (Btk) and phosphorylation of the membrane localised adapter protein (LAT) [116-119].

These pathways critically activate mobilisation of intra and extracellular calcium. Btk, when localised to the cell membrane by its binding to PIP_3, contributes to the activation of PLCγ1, which in turn is brought to the membrane by phosphorylated LAT. PLCγ1 acts upon membrane inositol phospholipids to generate Ins(1,4,5)P_3 or IP_3 and diacylglycerol (DAG) [120;121]. DAG targets various protein kinase C (PKC) isoforms, whereas IP_3 binds to IP_3 receptors on the surface of the endoplasmic reticulum containing calcium stores [122]. This leads to a depletion of intracellular calcium stores and an elevation of cytoplasmic calcium levels. The depletion of calcium stores leads to an opening of the plasma membrane channels required for calcium influx. The calcium release activated current is mediated by the store-operated calcium channels and is responsible for sustained elevations in cytosolic calcium and replenishment of ER (endoplasmic reticulum) calcium stores [120;123-125].

Activation of FcεRI also leads to the involvement of adapter proteins required to activate down-stream targets of the small GTP-binding protein and kinase cascades. Syk targets the membrane bound adapter LAT, which anchors further adapters Grb-2 and Slp-76 [126;127]. These adapters respond by recruiting and localising guanine nucleotide exchange factors for the Ras family of GTPases [128].

The Erk (extracellular signal-regulated kinase), Jnk (c-Jun N-terminal protein kinases) and p38 MAP kinases are downstream components of the signal cascade initiated by engagement of the high affinity IgE receptor [129;130]. The consequence of activation of these pathways is the nuclear import of a range of transcription factors including, NF-κB, ATF-2 (activating transcription factor 2), Elk-1, Jun and SRF (serum response factor) plus the activation of nuclear proteins, e.g. c-jun and c-fos. These transcription factors act in concert so the final effect on protein transcription depends upon their overall pattern of expression.

Finally, the activation of the signalling cascades described results in cytokine production and degranulation, with the subsequent release of pre-formed and newly synthesised inflammatory mediators. Purified lung mast cells constitutively express mRNA for IL-5, IL-6, IL-8 and TNF-α and upon stimulation with anti-IgE induce expression of IL-4 and GM-CSF, whilst increasing the expression of IL-5 and TNF-α [131-133]. In conjunction with pathways already described, a transient rise in free calcium and cyclic AMP enables the microtubular apparatus of the cell to mobilise and allow fusion of the secretory granules with the plasma membrane.

1.5 Inhibitors of cytokine production and signal transduction

The hypothesis that blockade of specific cytokines could be of therapeutic benefit has now been supported by clinical findings. This is especially true for TNF-α, where biological inhibitors may revolutionise the treatment of both RA and Crohn's disease [1;134;135]. The effectiveness of such an approach, has been to some extent limited by the expense of such treatments, which may curtail their eventual use. Orally available, small molecule inhibitors of signal transduction pathways, offer a cheaper alternative to biological inhibitors and as such are being pursued as alternative therapies.

1.5.1 Phosphodiesterase inhibitors

The phosphodiesterase (PDE) family of enzymes was discovered over thirty years ago by Butcher and Sutherland [136]. These enzymes were found to degrade the second messengers 3', 5'- cyclic adenosine monophosphate (cAMP) and 3', 5'- cyclic guanine monophosphate (cGMP), to 5'-AMP and 5'-GMP respectively. Both cAMP and cGMP act as second messengers propagating signals delivered by hormones, neurotransmitters and cytokines into the cell [137]. The family of PDE enzymes contains at least eleven isozymes [138] with different selectivity for cAMP and cGMP. The type 4 of the PDE family, termed PDE4, is known to specifically hydrolyse cAMP in preference to cGMP and is an abundant isoform in inflammatory cells, smooth muscle and vascular endothelium [139]. The PDE4 enzyme is

further split into four subtypes (A-D). These subtypes themselves are further divided, as alternative splicing of the PDE4 A-D genes produces additional splice variants [140].

The archetypal PDE4 inhibitor, Rolipram, was first described twenty five years ago [141]. Rolipram is a relatively weak competitive inhibitor of PDE4 (Kd ~0.5-1μM) and binds to an alternative conformation of the PDE4 enzyme in a steroselective manner and with a high affinity (Kd~1nM). This high-affinity confomer is often referred to as the high-affinity Rolipram binding site or HARBS [142;143]. As typified by Rolipram, the first generation of PDE4 inhibitors had dose limiting side effects in both animals and humans [144], the greatest manifestation of these side effects was the induction of a strong emetic response [145;146]. Second generation PDE4 inhibitors have been developed over the last ten years, which have reduced side effects and it is these inhibitors, plus Rolipram, that have been used to validate the therapeutic potential of PDE4 inhibitors

PDE4 inhibitors have been proposed as being useful in treating chronic inflammatory conditions such as RA. PDE4 is an abundant enzyme in monocytes and PDE4 inhibitors have proved potent modulators of some monocyte functions, such as the inhibition of LPS stimulated TNF-α production in humans and mice [147-151]. This reduction in TNF-α protein levels is mirrored by a decrease in TNF-α mRNA levels [149]. In rat models of arthritis, where anti-TNF-α antibodies are effective at stopping disease, PDE4 inhibitors had strong suppressive effects [152;153].

At the present time the main therapeutic target of PDE4 inhibitors has been asthma. PDE4 inhibitors effect diverse components of the allergic response from recruitment of eosinophils to the activation of T cell cytokines and show promise in many animal models of the disease, these are summarised in **table 1.3**. In man CDP840 (a specific PDE4 inhibitor) was well tolerated by volunteers and, although showed no effect on the early phase response in asthma, gave a 30% reduction in the late phase response [154].

Table 1.3: A list of the pharmacologically active PDE4 inhibitors tested in animal models of airway eosinophilia. *

Compound	Species	Route	Model	Refs.
CDP840	Rat	p.o.	IL-5 induced pleural eosinophilia	158
	Guinea pig	i.p.	OVA- sensitised and challenge (aerosol)	158
	Monkey	s.c.	Atopic challenge with *ascaris suum*	159
CP-80633	Guinea pig	p.o.	OVA sensitised and challenge	160
	Monkey	s.c.	Atopic challenge with *ascaris suum*	160
D-22888	Guinea pig	p.o.	OVA sensitised and challenge, late phase pulmonary eosinophilia	161
T-440	Guinea pig	p.o.	Allergen induced challenge early and late phase measurements	162
KF19514	Guinea pig	p.o.	PAF-induced lung eosinophilia	78;163;164
SB207499	Guinea pig	p.o.	LTD_4 and OVA induced eosinophilia	165

* Abbreviations; OVA, ovalbumin, PAF, platelet activating factor, LTD_4, leukotriene D_4, p.o., per oral, i.p., intra-peritonealy, s.c., sub-cutaneously.

1.5.2 Tyrosine kinase inhibitors

Signalling through the T cell receptor and high-affinity IgE receptor are both known to recruit tyrosine kinase enzymes to the receptor upon its engagement. The immunosuppression of T cell mediated immunity has been one of the driving forces behind the successful treatment of autoimmune disease and organ transplantation, in the modern era. The 'gold-standard' T cell immunosuppressive drug, cyclosporin A (CsA) has revolutionised the treatment of transplantation, being more efficient, effective and having fewer side-effects than treatments that preceded it [155;156]. CsA despite being a very effective treatment does however show dose-limiting side effects, particularly nephrotoxicity and a higher incidence

of cancer, that precludes the broader use of the drug. Other macrolide drugs such as FK-506 and Rapamycin, though effective, also suffer from dose-limiting side effects [157].

Newer T cell immunosuppressive drugs have emerged that target the IL-2R (monoclonal antibodies daclizumab and basiliximab) [166] and though useful in treating certain conditions [167], efficacy and cost prohibit their widespread use. Thus the search has been to replace cyclosporin with a synthetic inhibitor of T cell receptor signal transduction. Candidate targets, excluding the cyclophilins which are targeted by drugs such as CsA, include proteins such as Lck, Fyn, ZAP-70/Syk and MAP kinase, as well as non-TCR transduced signalling proteins such as those of the JAK/STAT cytokine receptor pathway. The Src-kinase family, which in T cells is represented by, Lck, Fyn and $p62^{Yes}$ (Yes), are attractive targets as they have a relatively restricted expression and in the case of Lck and Fyn are important in TCR signalling [168-170]. The Src-like family of enzymes is also thought to be involved in the activation of mast cells through the high-affinity IgE receptor.

A number of inhibitors of Src kinases exist with varying potencies and specificities, these are summarised in **table 1.4**. The most potent and selective inhibitors described to date are a series of pyrazolopyrimidines, which have specificity for Lck, Fyn, Src and $p59^{Hck}$ (Hck) over epidermal growth factor (EGF) receptor kinase, with selectivity against ZAP-70 and protein kinase A (PKA). These inhibitors, named PP1 and PP2, inhibited tyrosine phosphorylation of a number of proteins in human T cells stimulated through the TCR [171]; PP1 also inhibited the proliferation of peripheral blood lymphocytes (PBL) in response to anti-CD3 antibodies but not PMA/IL-2 dependent proliferation[171].

Table 1.4: The action of Src kinase inhibitors in *in vitro* assays. *

Compound	IC_{50} Lck	Specificity	Effects upon cell function
WIN61651	18-24μM	Selective compared to PKC and PKA, erb2, EGF kinase and Insulin receptor kinase	↓ tyrosine phosphorylation of cellular proteins ↓ IL-2 production in CD3/CD4/CD28 or CD3/PMA stimulated T cells ↓ proliferation to antigen and in MLR
A-125800	1-7μM	50-100 fold selectivity over MAPK but equipotent inhibition of Zap-70	↓ tyrosine phosphorylation and calcium flux ↓ proliferation to alloantigen and CD3/CD28 induced IL-2 production in T cell cultures Irreversible binding to Lck
PP1 & PP2	4-6nM	Selective over EGFR kinase Inactive against Jak-2, Zap-70 and PKA	↓ proliferation of T cells stimulated with CD3, influenza (antigen) and MLR Weakly active against PMA/IL-2 dependent proliferation Support Th2 differentiation in mice

* References, [171-174]

1.6 Aim of project

PDE4 and Src kinase enzymes are known to play a role in intracellular signalling processes. This project has capitalised on the fact that potent and specific inhibitors of these enzymes exist. Using such inhibitors it has been possible to assess the role played by PDE4 and Src in the activation of inflammatory cells.

Inhibiting PDE4 is known to block TNF-α production from LPS activated monocytes. To understand how this regulation occurs the effect of PDE4 blockade on the cytokine network induced by LPS activation of human PBMC was investigated.

The aims were:

- To investigate the effect of PDE4 inhibition on the overall pattern of cytokine production from LPS activated human PBMC.
- To investigate if the effect of inhibiting PDE4 blocks pro-inflammatory cytokine production via the elevation of anti-inflammatory cytokines, e.g. IL-10.

- To determine whether altering the stimulus delivered to PBMC, alters the effect PDE inhibitors have on cytokine production.

Inhibition of PDE4 is also thought to disrupt T cell function. To test this, the effect of PDE4 blockade on activated T cells was investigated.

The aims were:

- To investigate the effect of PDE4 inhibition on T cell proliferation.
- To investigate the effect of PDE4 inhibition on T cell cytokine production.
- To assess if the effect of PDE4 inhibitors on T cell function correlates with inhibition of the PDE4 enzyme in either its low-or high-affinity conformation.

Src kinase enzymes are implicated in transducing signals through the T cell receptor.

The aims were:

- To investigate the effect that Src kinase inhibition has on T cell proliferation induced by various stimuli.
- To investigate the effect of Src inhibition on T cell cytokine production.
- To investigate the point at which Src kinase inhibitors block TCR signal transduction.

Finally, to determine the role that Src kinase and phosphodiesterase type7 (PDE7) may play in mast cell activation.

The aims were:

- To develop a model of cord blood-derived mast cell activation.
- To investigate the effect of Src kinase and PDE7 inhibition on the IgE-dependent degranulation of mast cells.

Chapter 2

Materials and Methods

Chapter 2

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2.1 General reagents and equipment

Unless stated otherwise all chemical and biological reagents were obtained from Sigma Chemical company, Poole, UK. All tissue culture plastic ware was obtained from Falcon, Becton Dickinson labware, NJ, with the exception of 'U' bottomed 96 well tissue culture plates which were obtained from Costar, NY. Centrifugation was performed using bench top centrifuges 8R and GP8R, IEC, MA. All biological buffers, including tissue culture media were obtained from Gibco, Paisley, UK, unless stated otherwise. Foetal calf serum (FCS) was sourced from Helena Biosciences, #NS3005, lot 7329-2-NS31 (USA herd).

2.1.1 Recombinant proteins

Protein	Expression system	Source
IL-1α	*E.coli*	R&D systems, Oxford, UK
IL-β	*E.coli*	Biosource, UK
IL-2	*E.coli*	R&D systems, Oxford, UK
IL-4	*E.coli*	R&D systems, Oxford, UK
IL-6	*E.coli*	R&D systems, Oxford, UK
IL-8	*E.coli*	R&D systems, Oxford, UK
IL-10	SF21	R&D systems, Oxford, UK
IL-12p40	SF21	R&D systems, Oxford, UK
TNF-α	*E.coli*	Celltech, UK
IFN-γ	*E.coli*	R&D systems, Oxford, UK

2.1.2 Antibodies

Antibody	Isotype	Conjugate	Clone	Source
CCR1	Mouse IgG2b	Biotinylated	53504.11	R&D systems, Oxford, UK
CCR2	Mouse IgG2b	Biotinylated	48607.121	R&D systems, Oxford, UK

Cont.

CCR3	Rat IgG2a	PE	61828.111	R&D systems, Oxford, UK
CCR5	Mouse IgG2b	Biotinylated	45549.111	R&D systems, Oxford, UK
CCR6	Mouse IgG2b	PE	53103.111	R&D systems, Oxford, UK
CD117	Mouse IgG1	FITC	YB5.B8	Pharmingen, San Diego, CA
CD14	Mouse IgG2a	PE	M5E2	Pharmingen, San Diego, CA
CD16	Mouse IgG1	FITC	3G8	Pharmingen, San Diego, CA
CD25	Mouse IgG1	PE	2A3	BD, San Jose, CA
CD3	Mouse IgG1	FITC/PE	SK7	BD, San Jose, CA
CXCR1	Mouse IgG2b	PE	5A12	Pharmingen, San Diego, CA
CXCR2	Mouse IgG1	PE	6C6	Pharmingen, San Diego, CA
CXCR3	Mouse IgG1	FITC	49801.111	R&D systems, Oxford, UK
CXCR4	Mouse IgG2a	PE	12G5	Pharmingen, San Diego, CA
CXCR5	Mouse IgG2b	Biotinylated	51505.111	R&D systems, Oxford, UK
IFN-γ (c)	Mouse IgG1	Unconjugated	NIB42	Pharmingen, San Diego, CA
IFN-γ (d)	Mouse IgG1	Biotinylated	4S.B3	Pharmingen, San Diego, CA
IL-10	Mouse IgG2b	Unconjugated	23738.111	R&D systems, Oxford, UK
IL-10 (c)	Rat IgG2a	Unconjugated	JES3-19F1	Pharmingen, San Diego, CA
IL-10 (d)	Rat IgG2a	Biotinylated	JES3-12G8	Pharmingen, San Diego, CA
IL-10R	Mouse IgG1	Unconjugated	37607.11	R&D systems, Oxford, UK
IL-12p40 (c)	Mouse IgG1	Unconjugated	A08E6E5	Biosource, UK
IL-12p40 (d)	Mouse IgG1	Biotinylated	A25C4B6	Biosource, UK
IL-1α (c)	Mouse IgG1	Unconjugated	36-3B3-14	Pharmingen, San Diego, CA
IL-1α (d)	Mouse IgG1	Biotinylated	28.9	Pharmingen, San Diego, CA
IL-1β (c)	Mouse IgG1	Unconjugated	508A7G8	Biosource, UK
IL-1β (d)	Mouse IgG1	Biotinylated	508A3H12	Biosource, UK
IL-2 (c)	Mouse IgG1	Unconjugated	419A-7A3	Biosource, UK

Cont.

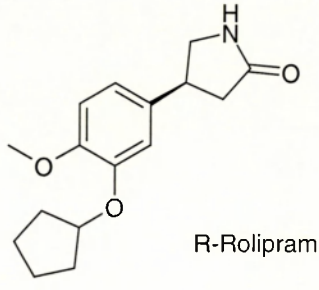
IL-2 (d)	Mouse IgG2b	Biotinylated	297C16G2	Biosource, UK
IL-4 (c)	Mouse IgG1	Unconjugated	8D4-8	Pharmingen, San Diego, CA
IL-4 (d)	Rat IgG1	Biotinylated	MP4-25D2	Pharmingen, San Diego, CA
IL-6 (c)	Rat IgG1	Unconjugated	MQ2-13A5	Pharmingen, San Diego, CA
IL-6 (d)	Rat IgG1	Biotinylated	MQ2-39C3	Pharmingen, San Diego, CA
IL-8 (c)	Mouse IgG2b	Unconjugated	G265-5	Pharmingen, San Diego, CA
IL-8 (d)	Mouse IgG2b	Biotinylated	G265-8	Pharmingen, San Diego, CA
mIgG1 control	Mouse IgG1	FITC/PE	A112-2	Pharmingen, San Diego, CA
mIgG2a control	Mouse IgG1	PE	G115-178	Pharmingen, San Diego, CA
mIgG2b control	Mouse IgG1	Biotin/PE	MPC-11	Pharmingen, San Diego, CA
rIgG2a control	Rat IgG2a	PE	35-95	Pharmingen, San Diego, CA
TNF-α (c)	Mouse IgG1	Unconjugated	MAB1	Pharmingen, San Diego, CA
TNF-α (d)	Mouse IgG1	Biotinylated	MAB11	Pharmingen, San Diego, CA

Notes : (c) = ELISA coating antibody, (d) = ELISA detection antibody, FITC = Fluorescein isothiocyanate, PE = Phycoerythrin.

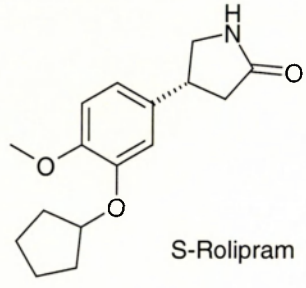
2.2 Chemical structures

All chemical inhibitors used throughout this thesis were synthesised by the medicinal chemistry department Celltech (Slough, UK).

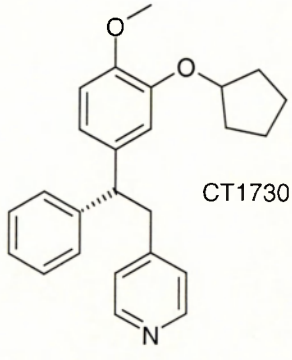
2.2.1 PDE4 inhibitors



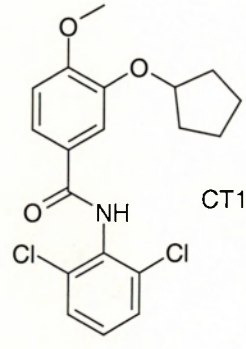
R-Rolipram



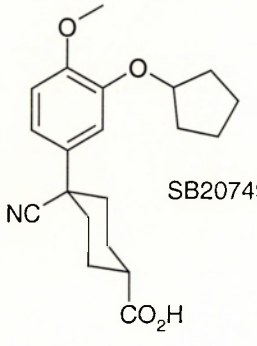
S-Rolipram



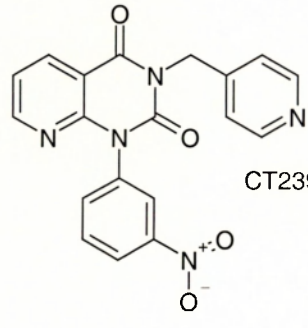
CT1730 (CDP840)



CT1881(RP73401)



SB207499 (Ariflo)

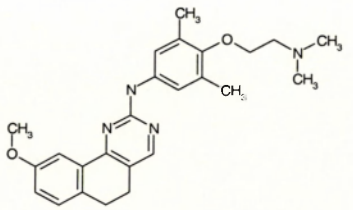


CT2396 (RS25344)

All PDE4 inhibitors were synthesised by the medicinal chemistry department of Celltech (Slough, UK) according to the details given in the references [141;158;175-179]. Compounds were kept as frozen stock solutions at a concentration of 20mM in DMSO, the repeated freeze thawing of compounds was avoided.

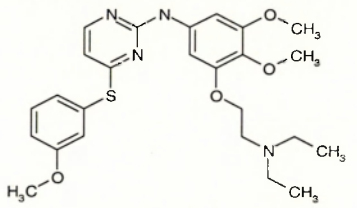
2.2.2 Kinase and PDE7 inhibitors

CT5102-00



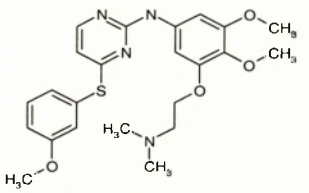
N-[3,5-Dimethyl-4-(2-dimethylaminoethyloxy)phenyl]-9-methoxybenzo[h]-5,6-dihydroquinazoline-2-amine

CT5215-10



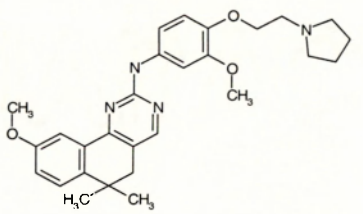
N-[3-(Diethylaminoethoxy)-4,5-dimethoxyphenyl]-4-(3-methoxyphenylsulfanyl) dihydrochloride

CT5227-10



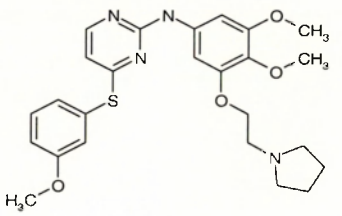
N-[3,4-Dimethoxy-5-(dimethylamino)ethoxyphenyl]-4-(3-methoxyphenylsulfanyl)pyrimidine 2-amine dihydrochloride

CT5263-00



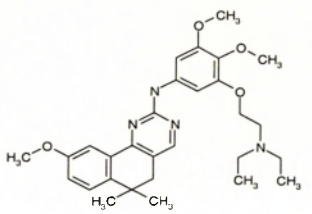
6,6-Dimethyl-9-methoxy-N-2-[3-methoxy-4-(2-pyrroldinyl)ethoxyphenyl]-benzo[h]-5,6-dihydroquinazoline-2-amine

CT5264-10



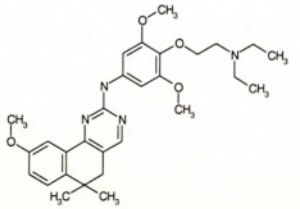
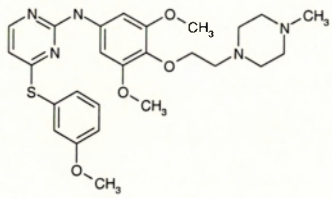
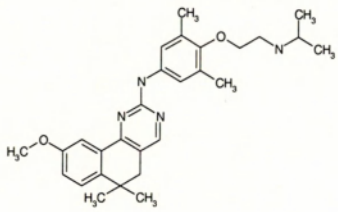
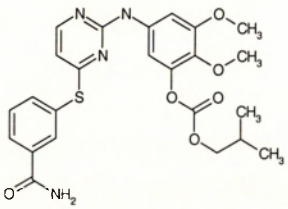
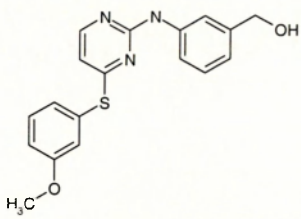
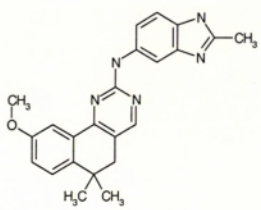
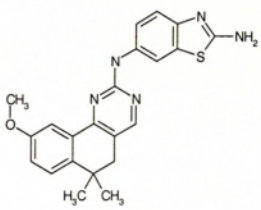
N-[3,4-Dimethoxy-5-(2-pyrrolidin-1-yl)ethoxyphenyl]-4-(3-methoxyphenylsulfanyl)pyrimidine 2-amine hydrochloride

CT5269-10

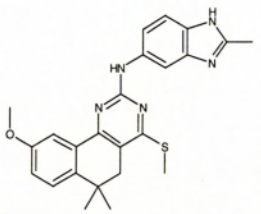


N-[3-(2-Diethylaminoethoxy)-4,5-dimethoxyphenyl]-9-methoxy-6,6-dimethylbenzo[h]5,6-dihydroquinazoline-2-amine dihydrochloride

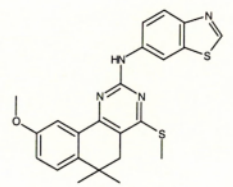
Cont.

CT5276-00		N-[3,5-Dimethoxy-4-(2-diethylaminoethoxy)phenyl]-9-methoxy-6,6-dimethyl-benzo[h]-5,6-dihydroquinazoline-2-amine
CT5378-10		N-{3,5-dimethoxy, 4-[2-(4-methoxypiperazin-1-yl)ethoxy]phenyl}-4 -(3-methoxyphenylthio)pyrimidine-2-amine dihydrochloride salt
CT5474-00		N-[3,5-Dimethyl-4-(2-isopropylaminoethoxy)phenyl]-6,6-diemthyl-9-methoxy-benzo[h]-5,6-dihydroquinazoline-2-amine
CT5475-00		4-(3-carboxamidophenylthio)-N-[3,4-dimethoxy-5-(isopropoxycarbonyloxy)phenyl]pyrimidine-2-amine
CT5605-10		N-(3-hydroxymethylphenyl) 4-(3-methoxyphenylthio)pyrimidine-2-amine hydrochloride
CT5651-00		9-methoxy-6,6-dimethyl-N-(2-methylbenzimadazol-5-yl)benzo[h]-5,6-dihydroquinazoline-2-amine
CT5733-00		9-methoxy-6,6-dimethyl-N-(2-aminobenzothiazol-6-yl)benzo[h]-5,6-dihydroquinazoline-2-amine

Cont.

CT5982-00 

6,6-Dimethyl-9-methoxy-N-(2-methylbenzimidazol-5-yl)-4-methylthio-benzo[h]-5,6-dihydroquinazoline-2-amine

CT6236-00 

N-(6-Benzothiazolyl)-6,6-dimethyl-9-methoxy-4-methylthio-benzo[h]-5,6-dihydroquinazoline-2-amine

All kinase/PDE7 inhibitors were synthesised by the medicinal chemistry department of Celltech (Slough, UK) according to the details given in patents WO 9841512, 9719065, 9828281, 9858926 [180-183]. Compounds were kept as frozen stock solutions at a concentration of 20mM in DMSO, the repeated freeze thawing of compounds was avoided.

2.3 Cell based assays

2.3.1 Purification of human PBMC

Peripheral blood mononuclear cells were isolated from normal healthy volunteers. Whole blood was taken by venous puncture using heparinised vacutainers (Becton Dickinson), diluted 1 in 4 in RPMI 1640 (Gibco, UK) and centrifuged at 400g for 35 min over a Ficoll-paque gradient (Amersham-Pharmacia Biotech, UK). Cells at the interface were removed and washed once followed by a low speed spin to remove platelets. Unless stated otherwise, cells were then resuspended in RPMI 1640 containing 10% FCS and penicillin 100 units ml^{-1}, streptomycin 50μg ml^{-1} and glutamine 2mM (Gibco, UK).

2.3.2 LPS stimulation of PBMC

PBMC were resuspended at a density of $2x10^5$ cells/well in flat bottomed 96 well tissue culture treated plates. Cells were stimulated with an optimal dose of LPS (*E.coli* strain

B5:055, Sigma, at 1μg ml^{-1}) and incubated at 37°C in 5%CO_2/95% air. Cytokine production was measured from cell free supernatants by sandwich ELISA (Chapter 2.4.).

2.3.3 Antigen stimulation of human PBMC

PBMC were resuspended at a density of $2x10^5$ cells/well in round bottomed 96 well tissue culture treated plates. Assays were performed in RPMI 1640 with 10% pooled human AB serum as a replacement for FCS. Healthy volunteers were chosen for their potential reactivity to tetanus toxoid and house dust mite antigen prior to use in assays. Only reliable and strong responders to each antigen were chosen. Cells were stimulated with an optimal dose of tetanus toxoid (Wellcome Laboratories, Beckenham, UK, at 1μg ml^{-1}) and incubated for five days at 37° in 5%CO_2/95% air. Cellular proliferation was measured by the incorporation of ^{3}H-thymidine. Cells were harvested onto glass fibre filter mats using a Skatron 96 well harvester (Molecular Devices, Sunnyvale, CA) and ^{3}H-thymidine incorporation measured using a β-plate counter (Wallac-Perkin Elmer, UK). Cells were also stimulated with an optimal dose of house dust mite antigen (part purified preparation of *Dermataphagoides pterrenyssinus*, ALK, Denmark, at 5000 units ml^{-1}) and incubated for five days at 37°C in 5%CO_2/95% air. Cellular proliferation was measured by the incorporation of ^{3}H-thymidine.

2.3.4 Superantigen stimulation of human PBMC

PBMC were resuspended at a density of $2x10^5$ cells/well in round bottomed 96 well tissue culture treated plates. Cells were stimulated with an optimal dose of superantigen (an equal mixture of staphylococcal enterotoxins A, B, D and E, Toxin Technologies, Sarasota, FL, at 0.1ng ml^{-1}) and incubated at 37°C in 5%CO_2/95% air. Cellular proliferation was measured by the incorporation of ^{3}H-thymidine.

2.3.5 Anti-CD3 stimulation of human PBMC

PBMC were resuspended at a density of $2x10^5$ cells/well in round bottomed 96 well tissue culture treated plates. Cells were stimulated with an optimal dose of the anti-CD3 antibody, OKT3, (Celltech, UK, at 0.05μg ml^{-1}) and incubated at 37°C in 5%CO_2/95% air. Cellular proliferation was measured by the incorporation of ^{3}H-thymidine. Where indicated, cells were resuspended in DMEM (Gibco, UK) containing 10% FCS and penicillin, streptomycin and glutamine (Low biotin preparation). The cytokine production from such cultures was measured using a multiplex cytokine assay (Section 2.4.4).

2.3.6 Concanavalin A (Con A) stimulation of human PBMC

PBMC were resuspended at a density of $2x10^5$ cells/well in round bottomed 96 well tissue culture treated plates. Cells were stimulated with an optimal dose of Con A (at 1μg ml^{-1}) and incubated at 37°C in 5%CO_2/95% air. Cellular proliferation was measured by the incorporation of ^{3}H-thymidine.

2.3.7 Phytohaemagglutinin (PHA) stimulation of human PBMC

PBMC were resuspended at a density of $2x10^5$ cells/well in round bottomed 96 well tissue culture treated plates. Cells were stimulated with an optimal dose of PHA (derived from *Phaseolus vulgaris*, at 1μg ml^{-1}) and incubated at 37°C in 5%CO_2/95% air. Cellular proliferation was measured by the incorporation of ^{3}H-thymidine.

2.3.8 Phorbol 12- myristate-13-acetate (PMA) and ionomycin stimulation of human PBMC

PBMC were resuspended at a density of $2x10^5$ cells/well in round bottomed 96 well tissue culture treated plates. Cells were stimulated with an optimal dose of PMA (at 1μg ml^{-1}) and ionomycin (Ca^{++} ionophore derived from *Streptomyces conglobatus*, at 10 ng ml^{-1}) and incubated at 37°C in 5%CO_2/95% air. Cellular proliferation was measured by the

incorporation of ^{3}H-thymidine. IL-2 production was measured in cell free supernatants by sandwich ELISA (Chapter 2.4).

2.3.9 Mixed lymphocyte reaction (MLR)

PBMC from two HLA mismatched donors were selected for use in a MLR. The first donor's cells, referred to as the 'responder', were resuspended at a density of $1x10^5$ cells/well in round bottomed 96 well tissue culture treated plates. These cells were stimulated with the second donors cells, referred to as the 'stimulator', which had been irradiated for a period of 45 min with a total dose of 2500 rads. These cells had no proliferative capacity but were determined to be alive by staining with trypan blue and propidium iodide. The stimulator cells were added to the responder cells at a density of $1x10^5$ in an equal volume. This gave a ratio of 1:1 responders to stimulators. Cells were was incubated for five days at 37°C in 5%CO_2/95% air. Cellular proliferation was measured by the incorporation of ^{3}H-thymidine.

2.3.10 Isolation of human mast cells derived from cord blood precursors

The purification of human mast cells was adapted from a technique developed by Saito *et al* [184]. Either heparin- or citrate-treated cord blood was taken from the umbilical cord vein of placental tissue (performed with parental consent). Cord blood was always used within 12 hours of collection. Cord blood mononuclear cells were separated over Ficoll-paque gradients as detailed in section 2.3.1. Briefly, cord blood was diluted 1 in 4 with RPMI 1640 and centrifuged at 400g for 35 min. Cells at the interface were removed and washed once followed by a low speed spin to remove platelets. Red cells were removed by suspension in red cell lysis buffer (155mM NH_4Cl; 10mM $KHCO_3$ and 0.1mM EDTA, in H_20 pH = 7.4) for 5 minutes. Cells were then resuspended at a concentration of $1x10^6$ cells ml^{-1} in a $75cm^3$ tissue culture flask in mast cell media (RPMI1640, 10% FCS, penicillin, streptomycin, glutamine, transferrin 5µg ml^{-1} , insulin 5µg ml^{-1}, sodium selinite 5ng ml^{-1} and HEPES 25mM). Cells were treated with a cocktail of human recombinant stem cell factor (SCF) 4.3nM (R&D Systems, Oxford, UK), interleukin-6 (IL-6) 2.5nM (R&D systems, Oxford, UK) and 300nM PGE_2 (prostaglandin E_2) and incubated at 37°C in 5%CO_2/95% air. Growth

factors were added at weekly intervals to cultures to which fresh mast cell media had also been added. Cellular viability was assessed weekly using trypan blue and cellular morphology was assessed using cytospin preparations stained with Giemsa/May-Grünwald.

2.3.11 Anti-IgE stimulated human mast cell degranulation

Cord blood derived mast cells (cbMC) were grown in a cocktail of SCF, IL-6 and PGE_2 for 70 days prior to harvest. cbMC were resuspended at a density of $2x10^4$ cells/well in flat bottomed 96 well tissue culture treated plates. Cells were preincubated with 10μg ml^{-1} of human IgE protein (Serotec, Oxford, UK) for 2 hours in mast cell media. Cells were then stimulated with an optimal dose of mouse anti-human IgE antibody (Pharmingen, at 5μg ml^{-1}), either in the presence or absence of SCF (10ng ml^{-1}), for 30 mins at 37°C in 5% CO_2/95% air. Plates were then spun at 200g for 3 minutes and supernatants taken and frozen at -70°C. The concentration of histamine and peptido-leukotriene was determined in supernatants by EIA (Section 2.4.5 and 2.4.6).

2.3.12 PMA and ionomycin stimulated human mast cell degranulation

cbMC were resuspended at a density of $2x10^4$ cells/well in flat bottomed 96 well tissue culture treated plates. Cells were stimulated with an optimal dose of PMA (1μg ml^{-1}) and ionomycin (10ng ml^{-1}) and incubated for 30 min at 37°C under conditions of 5% CO_2/95% air. Plates were then spun at 200g for 3 minutes and supernatants taken and frozen at -70°C. The concentration of histamine and peptido-leukotriene was determined from supernatants by EIA (Section 2.4.5 and 2.4.6).

2.3.13 Preparation of rat pleural mast cells

Mature Sprague-Dawley rats were killed according to an approved Home Office schedule. The underside of the rat was sprayed with 70% ethanol to reduce contamination by hair. Skin was removed from belly to ribs. The diaphragm was cut near the sternum and injected with 2mls of DPBS+ (Dulbecco's PBS with 0.1%BSA and 0.1%glucose); the pleural cavity

massaged and the washout placed into a 50ml centrifuge tube. Rat pleural lavages were centrifuged at 150g for 8 minutes. Pelleted cells were resuspended in 40ml cDMEM (DMEM + 10%FCS + glutamine + pen/strep) and centrifuged at 150g for 8 minutes. Pelleted cells were resuspended in 7.5 mls of 72.5% isotonic Percoll (Amersham Pharmacia Biotech) and placed into a 15ml centrifuge tube. 1ml of cDMEM was layered on top of the cell suspension and the tube centrifuged at 300g for 10 minutes. Cells at the interface and in the supernatant were discarded and the pellet resuspended in 10mls of cDMEM. Cells were washed once more in 10mls of cDMEM prior to use in assays.

2.3.14 Anti-IgE stimulated rat pleural mast cell degranulation

Rat pleural mast cells were resuspended in 1ml of cDMEM containing 2.5μg ml^{-1} of rat IgE (Zymed, San Francisco, CA) incubated overnight at 37°C in 5% CO_2/95% air. Cells were then washed and resuspended in cDMEM at a density of $2.5x10^4$ cells/well in round bottomed 96 well tissue culture treated plates. After 15 minutes incubation at 37°C, cells were stimulated with 1μg ml^{-1} anti-rat IgE and incubated for a further 30 minutes at 37°C. Plates were then centrifuged at 200g for 5 minutes and supernatants harvested. The concentration of histamine was determined from supernatants by EIA (Section 2.4.5).

2.4 Immunoassays

2.4.1 Assay buffers (ELISA)

Coating buffer : 4.3g $NaHCO_3$, 5.3g Na_2CO_3 made up to 1 litre with distilled H_2O, pH 9.4.

Blocking buffer: 8.0g NaCl, 1.42g $Na_2HPO_4.2H_2O$, 0.2g KH_2PO_4, 0.2g KCl, 5.0g bovine serum albumin (fraction V) made up to 1litre with distilled H_2O, pH 7.4.

Assay buffer: 8.0g NaCl, 1.42g $Na_2HPO_4.2H_2O$, 0.2g KH_2PO_4, 0.2g KCl, 5.0g bovine serum albumin (fraction V), 1ml Tween20 made up to 1 litre with distilled H_2O, pH 7.4.

Wash Buffer: 9.0g NaCl, 1ml Tween20 made up to 1 litre with distilled H_2O, pH 7.4.

Stop solution : 1.8M H_2SO_4

2.4.2 Cytokine sandwich ELISA protocol 1

Nunc Maxisorb plates (Nalge Nunc, Rochester, NY) were coated with 2.5 μg ml^{-1} of capture antibody (anti-IL-1α, -IL-4, -IL-6, -IL-8, -IL-10, -TNF-α and-IFN-γ) overnight at 4°C in coating buffer. Wells were then aspirated and blocking buffer added whilst plates were rotated (250rpm on an orbital shaker (Stuart Scientific, Bibby Sterilin, Staffordshire, UK)) at room temperature (RT) for 1.5h. Plates were then washed four times with wash buffer, using a Denley Wellwash 4 plate washer (Denley,Thermoquest). Standards were diluted in assay buffer and added along with samples to plates and incubated at RT for 2 h. The plates were washed a further four times and biotinylated detection antibody added at a concentration of 2.5 μg ml^{-1} in assay buffer. Plates were incubated at RT for a further 1.5 h. The plates were washed a further four times and streptavidin conjugated to horseradish peroxidase (Amdex, Amersham, UK) added at a concentration of 1 in 500 in assay buffer. The plates were incubated at RT for a further 30 min. Plates were washed four times and tetramethylbenzidine (TMB, Intergen, CA) substrate added. Plates were allowed to develop for between 10 to 30 min and the reaction terminated using stop solution. Plates were read at 450nm with a reference reading taken at 630nm using a Labsystems Multiskan Ex plate reader (Labsystems, Thermo Labsystems, UK). Standard curves were constructed and data analysed using Genesis II software (Labsystems, Thermo Labsystems, UK). Minimum detection limits of each assay were determined to be at least two standard deviations above background readings.

2.4.3 Cytokine sandwich ELISA protocol 2

As for cytokine sandwich ELISA protocol 1 except, capture antibodies (anti-IL-1β, -IL-2 and-IL-12p40) were used at a concentration of 5μg ml^{-1}, plates blocked for 2 h and the concentration of detection antibody used was 0.4 μg ml^{-1}.

2.4.4 Multiplex cytokine assay (Upstate biotech #48-001)

Cytokine production was measured from OKT3 stimulated PBMC as indicated in chapter 2.3.5. All assays were performed in 96 well 'U' bottomed polypropylene plates (Costar, NY). Recombinant standards, which contained 5000 pg ml^{-1} of IL-2, IL-4, TNF-α, IFN-γ and GM-CSF, were reconstituted with DMEM and diluted over a range from 5000 to 31.25 pg ml^{-1}. 50μl of standards and samples were added to 25μl of Luminex beads. Separate beads were conjugated to monoclonal antibodies that specifically recognised IL-2, IL-4, TNF-α, IFN-γ and GM-CSF. Standards and samples were vortexed gently and incubated at room temperature (RT) for 2 hours in the dark. 25μl of biotin-conjugated antibodies that specifically recognised IL-2, IL-4, TNF-α, IFN-γ and GM-CSF, were then added to samples, vortexed gently and incubated at RT for 1.5 hours in the dark. 25μl of a 20μg ml^{-1} solution of streptavidin-phycoerythrin (PE) (Pharmingen, SanDiego, CA) was added and samples incubated at RT for 30 minutes in the dark to reveal the presence of bound antibody on bead. The reaction was terminated by adding 25μl of a 0.2% (v/v) solution of formaldehyde in PBS. The fluorescence of each bead set was assayed at an emission wavelength of 532nm using a Luminex 100 analyser (Luminex, Austin, TX). 50 beads were counted per assay point and sample concentrations were determined by linear regression analysis of standard curves, using Graphpad-prism 3 software (Graphpad software, San Diego, CA).

2.4.5 Histamine EIA (IBL kit #RE 59221)

Standards and samples were added to soda glass tubes with an equal volume of indicator buffer. Samples were acetylated for 30 min by treatment with acetylating agent (acetic anhydride). Samples were then diluted 1 in 20 in assay buffer and added to 96 well plates. Tracer (histamine-HRP) was then added to the wells and anti-histamine anti-serum. Plates were then incubated for 3 h at RT and then washed four times in wash buffer. TMB substrate was added and the reaction terminated with stop solution 20 minutes after the addition of

substrate. Plates were read at an optical density of 450nm with a reference reading taken at 630nm. Standard curves were constructed and data analysed using GenesisII software.

2.4.6 Peptido-Leukotriene and leukotriene C4 (kit # 520501, 520211) and prostaglandin D2 (Kit # 512011) EIAs (Cayman Chemical Co.)

Assays for peptido-leukotrienes were determined to cross react with the following; Leukotriene C_4 (100%), D_4 (100%), E_4 (67%), D_5 (61%), C_5 (54%), E_5 (41%), A_3 (<0.01%), A_4 (<0.01%), B_3 (<0.01%), B_4 (<0.01%). Leukotrienes C_4, D_4 and E_4 are collectively termed peptido-leukotrienes. Plates pre-coated with mouse anti-rabbit IgG were treated with standards and samples plus peptido-leukotriene tracer (peptido-leukotriene linked to acetylcholinesterase) and peptido-leukotriene polyclonal antiserum. Plates were incubated for 18 hours at RT in the dark. Plates were then washed four times and Ellman's reagent added (acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid)) and colour allowed to develop in the dark for between 60 to 90 min. Plates were read at a wavelength 405nm and total and non-specific binding calculated to allow for the estimation of peptido-leukotriene levels. Due to the rapid degradation of PGD_2 methoxylamine (MOX) hydrochloride was added to cultures which formed stable PGD_2 – MOX derivatives, these were measured using specific antiserum raised against PGD_2 – MOX. Standard curves were constructed and data analysed using GenesisII software (Thermo Labsystems, UK).

2.4.7 cAMP EIA (Amersham kit #RPN225)

$5x10^5$ human PBMC were suspended in DPBS (0.1% glucose and 0.1% bovine serum albumin in Dulbecco's PBS). Cells were added to a 1.5ml Eppendorf tubes and mixed with inhibitor and incubated in a 37°C water bath for 10 minutes. 1μg ml^{-1} Con A was added and samples incubated at 37°C prior to harvesting cAMP. cAMP was retrieved from samples by firstly cooling cells on ice. Tubes were then micro-centrifuged and the assay supernatant discarded. Cell pellets were resuspended in assay buffer (0.05M sodium acetate buffer, pH

5.8 containing 0.02% BSA) and boiled for 10 minutes using a boiling water bath. Samples were snap frozen prior to assay using cAMP ELISA.

2.5 Flow cytometry

2.5.1 General procedure

$1x10^5$ cells were spun down in 5ml polypropylene Falcon tubes (Becton Dickinson, San Jose, CA). Cells were then washed once in PBS and then washed once more in 3ml of staining buffer (PBS, 0.5% heat-inactivated FCS and 0.1% sodium azide, pH 7.4). Cells were resuspended in 50µl of staining buffer with between 0.1 and 0.5µg μl^{-1} of either fluorochrome or biotinylated conjugated antibody added in a reaction volume of 10µl. Tubes were incubated at 4°C for 30 min in the dark. Cells treated with biotinylated antibodies were washed twice in 3ml of cold staining buffer and incubated with streptavidin-PE (0.1µg μl^{-1}) in a reaction volume of 50µl. These tubes were incubated for a further 30 min at 4°C . Before analysis, all cells were washed twice in cold PBS and resuspended in 500µl of PBS without azide. Cell staining data was acquired using CellQuest software (Becton Dickinson) on either a FACScan or FACSCalibur flow cytometer (Becton Dickinson). Specific antibody staining was compared to staining with isotype antibody controls. PMT (photo-multiplier tube) voltage, threshold and compensation settings were adjusted using calibrite beads™ (Becton Dickinson). Positivity was assessed using histogram markers for single stains and quadrant markers, on dot plots, for double stains.

2.5.2 IL-2 receptor alpha chain expression on OKT3 stimulated human PBMC

$1x10^5$ Human PBMC in round bottomed 96 well plates were stimulated with OKT3 (0.05µg ml^{-1}). Cells were assessed for the expression of CD25 (IL-2 receptor alpha chain) after 1, 2, 3 or 4 days post-stimulus. OKT3-stimulated cells were stained with CD3-FITC (fluorescein isothiocyanate) and CD25-PE according to the general staining protocol (Chapter 2.5.1). Lymphocytes were gated according to their characteristic forward and side light scattering properties. Lymphocytes that were deemed to be positive for CD3, using quadrant markers,

were also assessed for CD25 expression. CD25 positivity was expressed in mean (geometrical) fluorescent units.

2.5.3 CD14 and CD16 expression on LPS and LPS plus IFN-γ stimulated human PBMC

$1x10^6$ human PBMC in 24 well plates (Costar, UK) were stimulated with LPS (1μg ml^{-1}) or LPS (1μg ml^{-1}) plus IFN-γ (1ng ml^{-1}) or left untreated. PBMC were harvested by scraping all cells from the bottom of each well. Cells were assessed for the expression of CD14 and CD16, using CD14-PE and CD16-FITC directly conjugated antibodies, according to the general staining protocol (section 2.5.1). Monocyte/macrophages were gated according to their characteristic forward and side light scattering properties. Monocyte/macrophages were assessed for both CD14 and CD16 expression using quadrant gates, and CD14/CD16 positivity was assessed in terms of the percentage cells that stained double positive for both markers.

2.5.4 The expression of chemokine receptors on human cord blood derived mast cells

2.5.4.1 Light Scatter properties

Cord blood mast cells were analysed using flow cytometry to determine their physical phenotype. Cord blood mononuclear cells stimulated with SCF, IL-6 and PGE_2 (section 2.3.10) were assessed using flow cytometry at weekly intervals. Cellular light scattering properties, forward light scatter (a measure of cell size) and side or 90° light scatter (a measure of cell granularity) were measured. Forward and side scatter were plotted using dot plots and distinct populations were assessed using polygon gates applied to dot plots.

2.5.4.2 Chemokine receptor staining on cord blood mast cells

Distinct cbMC populations as determined in section 2.5.4.1. were stained with biotinylated antibodies against human CCR1, 2, 5 and CXCR5 and directly conjugated antibodies against human CCR3, 6 and CXCR1, 2, 3 and 4 according to the general staining protocol (section

2.5.1). Chemokine receptor expression was assessed on distinct cbMC populations at weekly intervals. Expression was assessed using histogram plots and positivity was expressed in mean (geometrical) fluorescent units.

2.6 Enzyme assays

2.6.1 Reagents

Staurosporin, ATP (Tris salt), DTT, HEPES, pEY (polyglutamic acid tyrosine ratio of 4:1), manganese chloride were obtained from Sigma. protein kinase C assay kit, streptavidin-SPA beads and ^{33}P–γATP were obtained from Amersham. Brij-35 was obtained from Pierce and magnesium chloride was obtained from BDH. Microtitre plates for SPA were purchased from Wallac. 6-amino hexanoyl AEEIYGVLAKKK Lck substrate was synthesised by IBMS Southampton.

2.6.2 Enzymes

GST (glutathione S-transferase) -Lck (GST fusion proteins refer to enzymes expressed with a GST affinity tag to aid enzyme purification), was cloned from a Jurkat cDNA library and expressed as a GST fusion protein in mammalian NS0 cells. GST-Lyn was produced in-house as a GST catalytic domain fusion by a Baculovirus-SF9 expression system. GST-Fyn was produced in-house as a GST catalytic domain fusion by a Baculovirus-SF9 expression system. PKC was purchased from Boehringer Mannheim. EGFR was produced in-house as a GST catalytic domain fusion by a Baculovirus-SF9 expression system. Zap-70 was produced in-house as a GST fusion by a Baculovirus-SF9 expression system. cdc-2 was purchased from Amersham. Full length PDE4A was harvested from baculovirus infected SF9 insect cells, partially purified from crude cell lysates using a Resource Q column with a 0 - 0.5M salt gradient. Truncated PDE4A enzyme, ΔQ44-L329, containing only the catalytic domain (without the high affinity Rolipram binding site (HARBS)) was purified from transfected COS cells [185].

2.6.3 GST-Lck enzyme assay

Reactions were carried out in a total volume of 200µl at room temperature in 96 well microtitre plates. The reaction mixture contained 20mM HEPES pH7.4, 10mM magnesium chloride, 10mM manganese chloride, 0.05% Brij 35, 0.5µM 6-amino hexanoyl AEEIYGVLAKKK peptide substrate, 0.6µM ATP (Tris salt) and 5µCi/ml 33P γ-ATP. The compounds were added in DMSO so that the final DMSO concentration was 1%. The assay was run for 15 minutes before being stopped with 50µl stop solution; 3mM ATP in 125mM EDTA. 200µl of the final mixture is then transferred to a Millipore MAPH filtration plate containing 100µl 75mM phosphoric acid. The plate was then left for at least 60 minutes at room temperature. The plate was then washed 100µl x6 with 75mM Phosphoric acid and then 100µl scintillant (Packard Ultima Gold) was added prior to counting in a Wallac Microbeta plate counter.

2.6.4 GST-Lyn enzyme assay

Reactions were carried out in a total volume of 200µl at room temperature in 96 well microtitre plates. The reaction mixture contained 20mM HEPES pH7.4, 2mM magnesium chloride, 2mM manganese chloride, 0.05% Brij 35, 5mM DTT, 1µM 6-amino hexanoyl AEEIYGVLAKKK peptide substrate, 0.6µM ATP (Tris salt) and 5µCi/ml 33P γ-ATP. The compounds were added in DMSO so that the final DMSO concentration was 1%. The assay was run for 15 minutes before being stopped with 50µl stop solution; 3mM ATP in 125mM EDTA. 200µl of the final mixture was then transferred to a Millipore MAPH filtration plate containing 100µl 75mM phosphoric acid. The plate was then left for at least 60 minutes at room temperature. The plate is then washed 100µl x6 with 75mM Phosphoric acid and then 100µl scintillant (Packard Ultima Gold) was added prior to counting in a Wallac Microbeta plate counter.

2.6.5 GST-FynT enzyme assay

Reactions were carried out in a total volume of 200µl at room temperature in 96 well microtitre plates. The reaction mixture contained 20mM HEPES pH7.4, 2mM manganese

chloride, 0.05% Brij 35, 10μM 6-amino hexanoyl AEEIYGVLAKKK peptide substrate, 0.6μM ATP (Tris salt) and 5μCi/ml 33P γ-ATP. The compounds were added in DMSO so that the final DMSO concentration was 1%. The assay was run for 15 minutes before being stopped with 50μl stop solution; 3mM ATP in 125mM EDTA. 200μl of the final mixture was then transferred to a Millipore MAPH filtration plate containing 100μl 75mM phosphoric acid. The plate was then left for at least 60 minutes at room temperature. The plate was then washed 100μl x6 with 75mM Phosphoric acid and then 100μl scintillant (Packard Ultima Gold) is added prior to counting in a Wallac Microbeta plate counter.

2.6.6 Zap-70 enzyme assay

Reactions were carried out in a total volume of 200μl at room temperature in 96 well microtitre plates. The reaction mixture contained 20mM HEPES pH7.4, 10mM magnesium chloride, 10mM manganese chloride, 5mM DTT, 0.05% Brij 35, 5μg/ml pEY, 0.6μM ATP (Tris salt) and 5μCi/ml 33P γ-ATP. The compounds were added in DMSO so that the final DMSO concentration was 1%. The assay was run for 10 minutes before being stopped with 50μl stop solution; 3mM ATP in 125mM EDTA. 200μl of the final mixture was then transferred to a Millipore MAFC filtration plate containing 100μl 30% cold TCA which was left at 4°C overnight. The plate was then washed 100μl x6 with 10% cold TCA, 100μl x3 with 100% ethanol and then 100μl scintillant (Packard Ultima Gold) was added prior to counting in a Wallac Microbeta plate counter.

2.6.7 PKC (Amersham Kit RPN77) enzyme assay

Reactions were carried out in a 30μl reaction volume 50mM Tris pH 7.5 buffer containing 1mM Ca2+, 15mM Mg2+, 0.6mole L-α phosphatidyl-L-serine and 2μg/ml^{-1} PMA, 2.25μM peptide, 2.5mM DTT, 1.2μM ATP, 0.2μCi 33P γ-ATP. For reactions that contained staurosporin, this was added in a DMSO solution and the final DMSO concentration did not exceed 1%. This was shown not to interfere with enzyme activity. (Data not shown.) The reaction was initiated with enzyme, either mix or individual isozymes and run for 10 minutes

at room temperature before being stopped with 20µl 0.5M phosphoric acid containing 1µM staurosporin. 30µl of the final mixture was then transferred to a Millipore MAPH filtration plate containing 100µl 75mM phosphoric acid. The plate was then left for at least 60 minutes at room temperature. The plate was then washed 100µl x6 with 75mM phosphoric acid and then 100µl scintillant (Packard Ultima Gold) was added prior to counting in a Wallac Microbeta plate counter.

2.6.8 EGFR enzyme assay

Reactions were carried out in a total volume of 200µl at room temperature in 96 well microtitre plate. The reaction mixture contained 20mM HEPES pH7.4, 25mM magnesium chloride, 1mM manganese chloride, 5mM DTT, 0.05% Brij 35, 5µg/ml pEY, 1µM ATP (Tris salt) and 5µCi/ml 33P γ-ATP. The compounds were added in DMSO so that the final DMSO concentration was 1%. The assay was run for 30 minutes before being stopped with 50µl stop solution; 3mM ATP in 125mM EDTA. 200µl of the final mixture was then transferred to a Millipore MAFC filtration plate containing 100µl 30% cold TCA and then this is left at 4°C overnight. The plate was then washed 100µl x6 with 10% cold TCA, 100µl x3 with 100% ethanol and then 100µl scintillant (Packard Ultima Gold) was added prior to counting in a Wallac Microbeta plate counter.

2.6.9 cdc-2 (Amersham SPA kit RPNQ 0170) enzyme assay

Reactions were carried out in a 40µl reaction volume containing, 50mM Tris-HCl pH 8.0 buffer, 10mM Mg2+, 100mM Na_2VO_3, 1mM DTT, 0.75µM (biotin–PKTPKKAKKL) peptide, 0.5µM ATP and 0.2µCi 33PγATP. For reactions that contained inhibitors, this was added in a DMSO solution and the final DMSO concentration did not exceed 1%. This was shown not to interfere with enzyme activity. The reaction was initiated with enzyme, and run for 30 minutes at room temperature before being stopped with 200µl streptavidin - PVT beads 5mg/ml in 50µM ATP in 5mM EDTA. The plate was left to stand for 30 minutes before being spun at 2000rpm for 10 minutes in a centrifuge and then read in the Wallac Microbeta plate counter.

2.6.10 PDE enzyme assay

PDE (3, 4 or 7) enzymatic activity was assayed in a homogeneous scintillation proximity assay (SPA) using yttrium silicate SPA beads (Amersham, UK). The buffer used for dilution of substrate and enzyme contained 50nM TES pH 7.6 with 10mM $MgCl_2$. Substrate was [^{3}H] cAMP/cGMP (Amersham) at 0.1 μm final concentration and the enzyme was titrated to give approximately 20% substrate hydrolysis at the 30 minute time-point. The inhibitors were added to the enzyme substrate in a DMSO solution prior to the addition of the enzyme. The reaction mixture was incubated at room temperature for 30 minutes then the reaction was terminated by the addition of PDE SPA beads at 20mg ml^{-1} in HPLC grade water. Samples were quantified in a Wallac Microbeta scintillation counter and IC_{50} values calculated using XL Fit (Microsoft) with a log dose inhibition curve. Background was given by the addition of an excess quantity of a potent reference PDE inhibitor.

2.6.11 [^{3}H]-Rolipram binding to guinea-pig membranes

R-Rolipram was iodinated and dispatched to Amersham International , where it was titrated by catalytic reduction with palladium charcoal to a specific radioactivity of 851 $Gbq/mmol^{-1}$. The ability of compounds to inhibit the binding of [^{3}H] R-Rolipram to guinea-pig membranes was investigated using the method devised by Schneider et al [186]. For saturation binding experiments , the concentration of [^{3}H] R-Rolipram was varied from 0.01 to 30nM. Sufficient enzyme was used to bind 10% of the total label at 5nM. Non-specific binding was assayed in the presence of 2μM unlabelled Rolipram. Specific binding was calculated by subtracting non-specific from total binding.

2.7 Statistical analysis of results

Where appropriate statistical analysis of data was performed using Graphpad-Prism version 3 (Graphpad software, San Diego, CA). To determine if parametric or non-parametric analysis of data was required Bartletts test of homogeneity of variance was performed. If the data was Gaussian or approximately Gaussian (using larger sample numbers) then parametric

analysis of variance (ANOVA) was performed. For sample comparisons data was analysed using a two-tailed student's T-test to which a P-value of =<0.05 was assigned as significant. To investigate the statistical relationship between two sets of data a Pearson correlation, assuming Gaussian distribution, was performed. The p-value for such correlation's was set at =/<0.05. The coefficient of determination (r^2) was derived from such calculations and used to explain the shared variance between two sets of data. If the data set was small, then no assumption of Gaussian distribution was made and a nonparametric (Spearman's) correlation was made. Spearman's (r) was calculated instead of an r^2 value. A two-tailed p-value of =/<0.05 was assigned as significant.

Chapter 3

Evaluation of the effect of inhibition of phosphodiesterase type 4 on human monocyte function

Chapter 3

Evaluation of the effect of inhibition of phosphodiesterase type 4 on human monocyte function

3.1 Introduction

Human monocytes are a rich source of both pro-inflammatory cytokines; TNF-α, IL-1α, IL-1β, IL-6, IL-8 and IL-12 and anti-inflammatory cytokines; IL-10, IL-1Ra (IL-1 receptor antagonist) and sTNF-R (Soluble TNF receptor). Regulation of the balance of these two types of mediator determines whether a response to infection or invasion by foreign material is either normal or inappropriate.

The elevation of cAMP is a key regulatory step in controlling inflammatory cell activation, and is an important mechanism for controlling the balance between pro- and anti-inflammatory responses. This need for control has resulted in many co-operative systems which act in unison to safeguard the production of cAMP. For instance many G protein coupled receptors, such as the histamine receptors, mediate intracellular signalling by controlling cAMP levels [187]. These pathways control the synthesis of cAMP by acting upon the enzyme, adenylyl cyclase, which is responsible for the synthesis of cAMP from AMP [188;189]. cAMP can also be controlled by another family of enzymes, the phosphodiesterases, which are responsible for the degradation of cAMP [190]. It is the eventual balance of the action of the phosphodiesterase and the adenylyl cyclase enzymes which determines the level of intracellular cAMP.

The cAMP dependent type 4 phosphodiesterase (PDE4) is abundantly present in inflammatory cells [191-195]. Thus the regulation of its activity has been proposed as a therapeutic target to control the level of cAMP in these cells [196-198]. PDE4 inhibitors potently inhibit TNF-α production by activated human and murine monocytes both *in vitro* and *in*

vivo [147;150;176 148;149 199;200]. The inhibition of TNF-α by PDE4 inhibitors has implications for their use in inflammatory diseases, such as rheumatoid arthritis, where blockade of TNF-α has been validated as a therapy for this disease [3;135;201].

The aim of the work presented in this chapter was to clarify how inhibition of PDE4 controls the production of cytokines from activated human peripheral blood mononuclear cells. Varying effects on the production of pro-inflammatory cytokines by elevating cAMP levels have been reported in the literature [202-205]. These variations, in both human and murine experiments, have suggested that apart from TNF-α, regulation of IL-1, IL-6 and IL-10 by cAMP is complex. The work in this chapter has attempted to quantify the effect PDE4 inhibition has on inflammatory cytokine synthesis. The concept that different activation signals delivered to the monocyte may alter the effect of PDE4 inhibition on cytokine release has also been explored.

3.2 Results

3.2.1 The effect of PDE4 inhibition on TNF-α release from human PBMC

In order to deduce the exact kinetics of cytokine production from activated human monocytes human PBMC were stimulated with purified LPS derived from *E.coli* (strain 055:B5) and the production of TNF-α, IL-1α, IL-1β, IL-6, IL-8 and IL-10 proteins was measured over a period of up to four days post stimulus, **fig 3.1**.

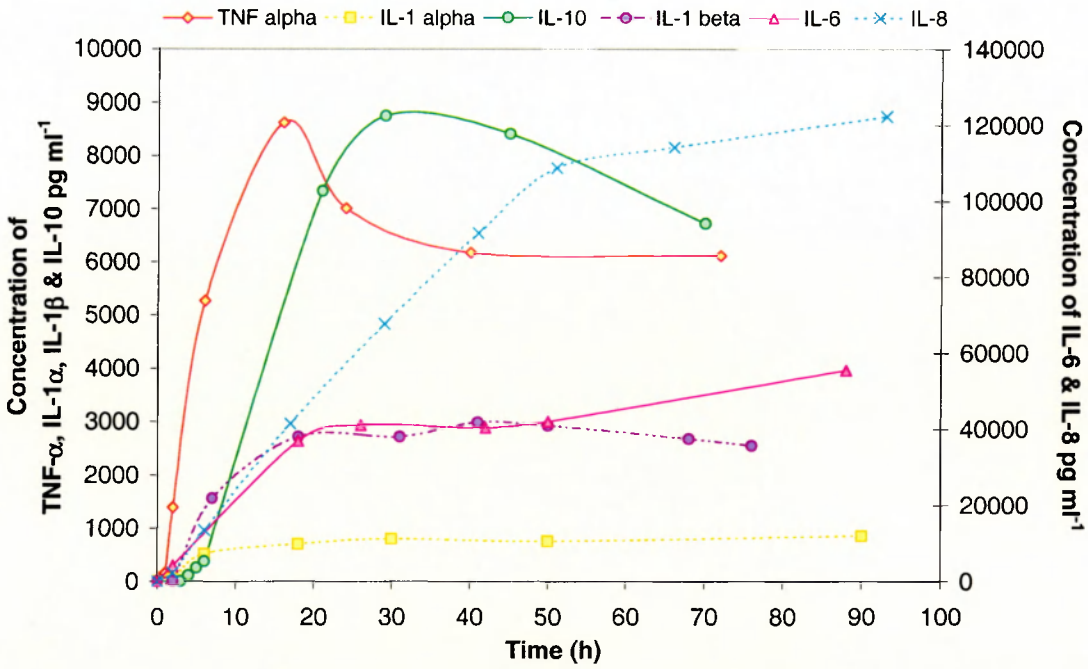


Figure 3.1: The production of cytokines from LPS stimulated human PBMC. $1x10^5$ human PBMC, were stimulated with 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain). TNF-α, IL-1α, IL-1β, IL-6, IL-8 and IL-10 were measured at the times indicated in cell free supernatants by sandwich ELISA. The amount of cytokine produced was quantified relative to standard curves for all cytokines tested. The data shown is representative of at least seven other experiments. The values shown are the mean of three separate points, standard deviations were omitted to allow the nature of the curves to be fully represented.

The maximum production of cytokines varied markedly. IL-8 was produced in the greatest quantities with a maximum concentration found in the culture fluid of 122.3 ng ml^{-1}. In contrast only 853 pg ml^{-1} of IL-1α was found to be produced.

When human PBMC were pre-treated with the potent PDE4 inhibitor RP73401 [175] (Chapter 2.2.1) prior to stimulation with LPS, RP73401 blocked the production of TNF-α. **Figure 3.2** indicates that RP73401 potently, and dose dependently, inhibited TNF-α protein production from LPS stimulated human PBMC. RP73401 inhibited the production of TNF-α for the duration of the experiment.

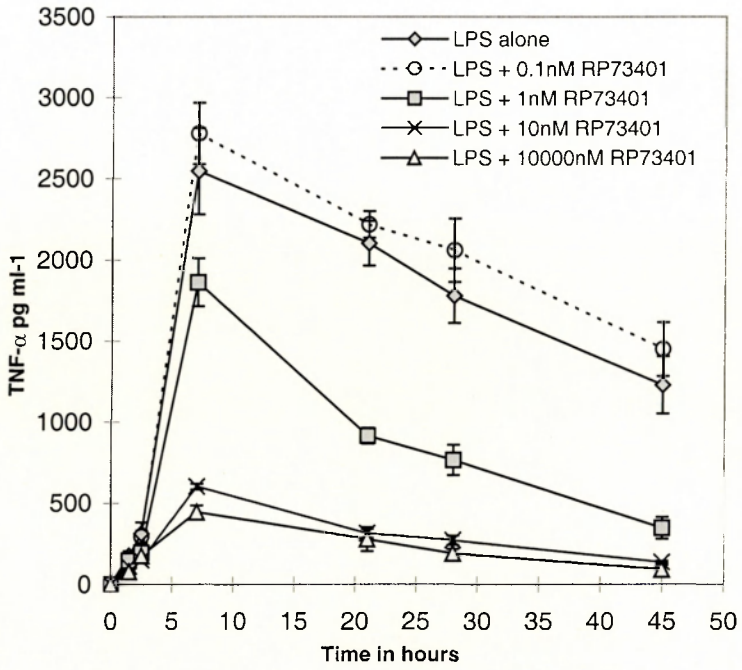


Figure 3.2: The effect of the PDE4 inhibitor RP73401 on the LPS stimulated release of TNF-α from human PBMC. $1x10^5$ human PBMC were incubated with the PDE4 inhibitor RP73401 at the concentrations indicated for 30 min prior to stimulation with 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain). TNF-α was measured at the times indicated in cell free supernatants by sandwich ELISA. The experiment shown is representative of six others. Mean values are indicated ± SD.

3.2.2 The effect of PDE4 inhibition on the production of IL-1α, IL-β, IL-6 and IL-8 by human PBMC

The effect of RP73401 on LPS induced IL-1α production by human PBMC was evaluated, **fig 3.3**. Pre-treatment of cells with RP73401 strongly inhibited the production of IL-1α. The effect of RP73401 was dose-dependent. The highest concentration of RP73401 (10μM) inhibited IL-1α production by 84 ± 5.1% when measured 9 hours after cytokine induction.

The effect of RP73401 on the other IL-1 family member, IL-1β, was also tested, **fig 3.4**. Unlike its effect on IL-1α, RP73401 inhibited IL-1β production to a lesser extent. However, the effect of RP73401 was dose-dependent. The highest concentration of RP73401 (10μM) inhibited IL-1β production by 39.1 ± 2.5%, 7 hours after LPS challenge.

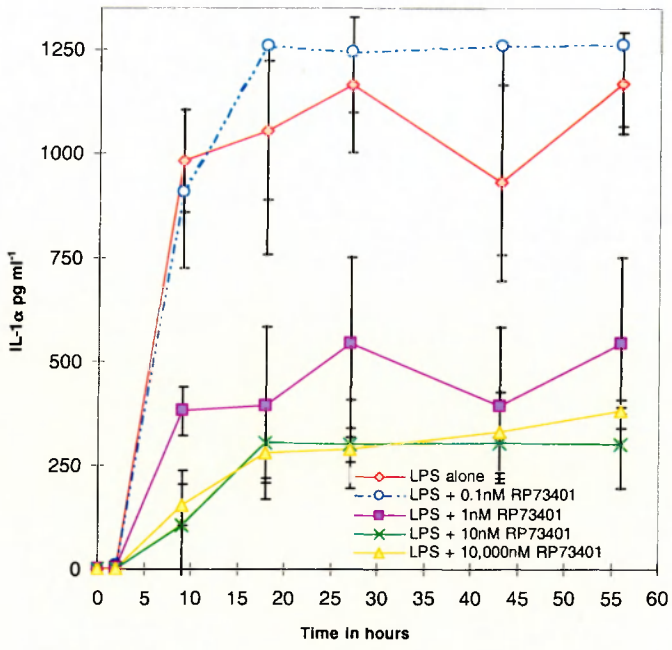


Figure 3.3: The effect of the PDE4 inhibitor RP73401 on the LPS stimulated release of IL-1α from human PBMC. $1x10^5$ human PBMC were incubated with the PDE4 inhibitor RP73401 at the concentrations indicated for 30 min prior to stimulation with 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain). IL-1α was measured at the times indicated in cell free supernatants by sandwich ELISA. The experiment shown is representative of four others. Mean values are indicated ± SD.

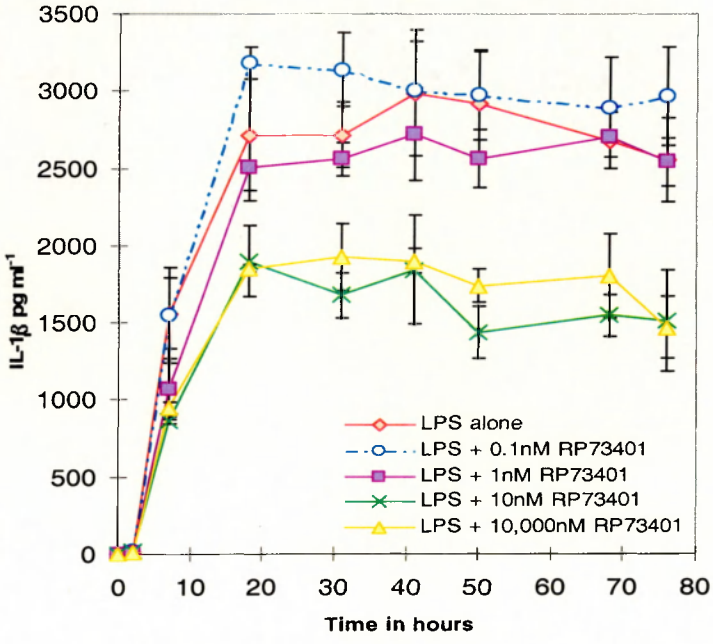


Figure 3.4: The effect of the PDE4 inhibitor RP73401 on the LPS stimulated release of IL-1β from human PBMC. $1x10^5$ human PBMC were incubated with the PDE4 inhibitor RP73401 at the concentrations indicated for 30 min prior to stimulation with 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain). IL-1β was measured at the times indicated in cell free supernatants by sandwich ELISA. The experiment shown is representative of three others. Mean values are indicated ± SD.

In contrast to its effects on TNF-α and IL-1, RP73401 did not inhibit IL-6 production by LPS stimulated human PBMC, **fig 3.5**. Even at the highest concentration tested (10μM), RP73401 showed no statistically significant inhibition of IL-6, p=0.799.

Like IL-6, RP73401 was unable to inhibit the production of IL-8 by LPS stimulated human PBMC cultures, **fig 3.6**. Even at the highest concentration tested (10μM), RP73401 showed no statistically significant inhibition of IL-8 production, p=0.720.

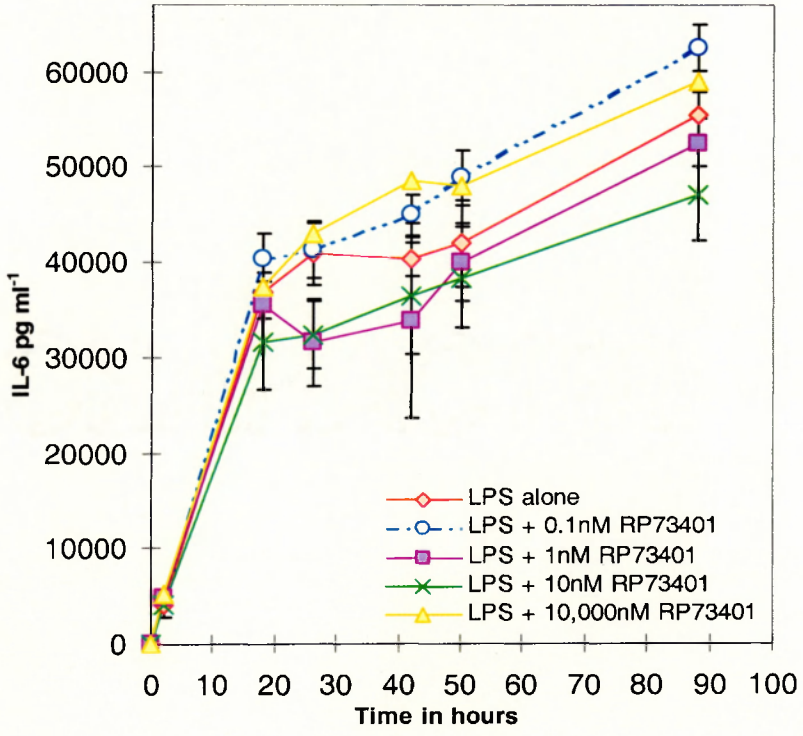


Figure 3.5: The effect of the PDE4 inhibitor RP73401 on the LPS stimulated release of IL-6 from human PBMC. $1x10^5$ human PBMC were incubated with the PDE4 inhibitor RP73401 at the concentrations indicated for 30 min prior to stimulation with 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain). IL-6 was measured at the times indicated in cell free supernatants by sandwich ELISA. The experiment shown is representative of four others. Mean values are indicated ± SD.

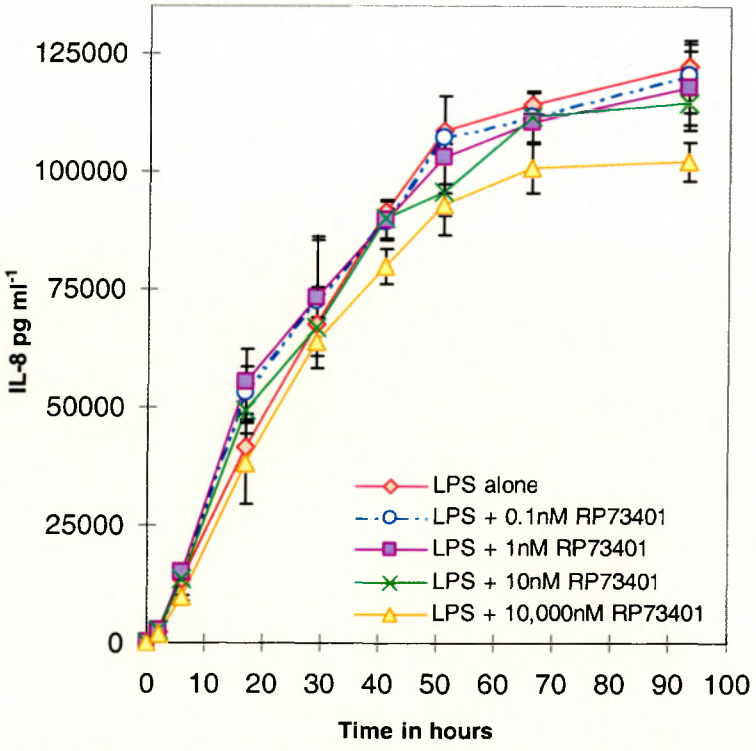


Figure 3.6: The effect of the PDE4 inhibitor RP73401 on the LPS stimulated release of IL-8 from human PBMC. $1x10^5$ human PBMC were incubated with the PDE4 inhibitor RP73401 at the concentrations indicated for 30 min prior to stimulation with 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain). IL-8 was measured at the times indicated in cell free supernatants by sandwich ELISA. The experiment shown is representative of three others. Mean values are indicated ± SD.

3.2.3 The role of IL-10 in PDE4 inhibitor-mediated blockade of TNF-α production

To investigate if elevated levels of IL-10 were responsible for the blockade of TNF-α caused by inhibition of PDE4, exogenous human recombinant (hr) IL-10 (hrIL-10) was added to human PBMC at the time of LPS challenge. The effect of the addition of hrIL-10 on TNF-α, IL-1β and IL-6 was measured and compared to the effect of RP73401 on the same cytokines (**figs 3.2, 3.4** and **3.5**). **Figure 3.7a** shows that hr IL-10 suppressed the production of TNF-α by LPS activated human PBMC. 1ng ml^{-1} of IL-10 inhibited TNF-α production for the duration of the experiment, blocking 73.4 ± 8.3% of TNF-α released 20 hours after stimulation. **Figure 3.7b** indicates that hrIL-10 also suppressed the production of IL-1β by LPS activated human PBMC. 1ng ml^{-1} of hrIL-10 inhibited IL-1β production for the duration of the experiment, blocking 55.8 ± 2.7% of IL-1β released 20 hours after stimulation. **Figure 3.7c** indicates that hr IL-10 weakly suppressed the production of IL-6 by

LPS activated PBMC. 1ng ml^{-1} of hr IL-10 inhibited IL-6 production for the duration of the experiment, blocking 21.8 ± 0.4% of IL-6 released 20 hours after stimulation, however this inhibition was not statistically significant, p=0.749. The effect of hrIL-10 on LPS induced cytokine production was similar to the effect RP73401 had on TNF-α production. To test if IL-10 mediated the effect of RP73401 on TNF, the IL-10 produced in LPS stimulated PBMC cultures was neutralised using an anti-IL-10 receptor antibody capable of neutralising bioactive IL-10.

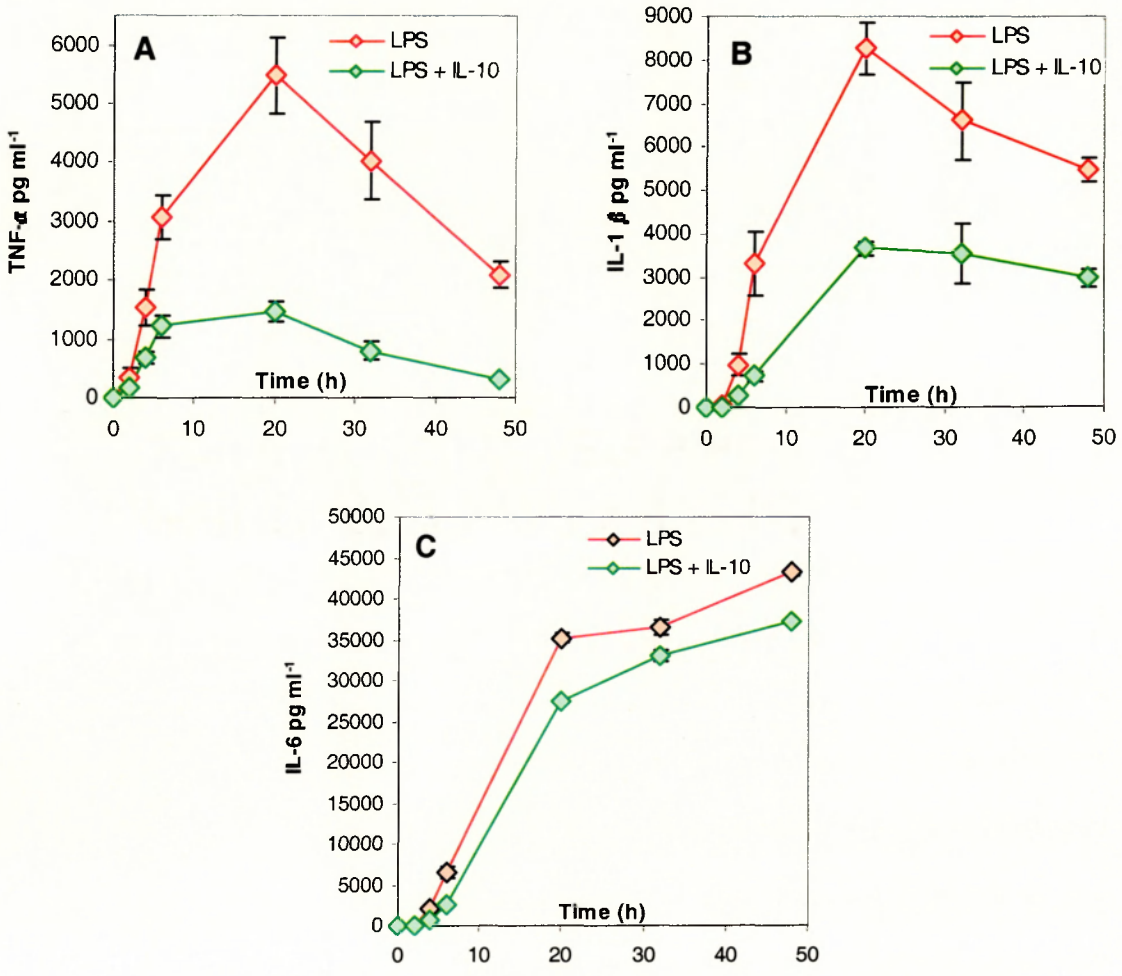


Figure 3.7: The effect of human recombinant IL-10 on the LPS stimulated release of TNF-α, IL-1β and TNF-α from human PBMC. 1x10^5 human PBMC were incubated with human recombinant IL-10 (1ng ml^{-1}) for 30 min prior to stimulation with 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain). TNF-α (A), IL-1β (B) and IL-6 (C) were measured at the times indicated in cell free supernatants by sandwich ELISA. The experiment shown is representative of three others. Mean values are indicated ± SD.

The antibody specifically recognised the IL-10 receptor, and at a dose of 5μg ml^{-1} blocked total IL-10 biological activity (according to the manufacturers data). **Table 3.1** indicates that at a dose of 5μg ml^{-1} an anti-IL-10 receptor antibody was unable to effect the ability of

RP73401 to inhibit TNF-α production. This effect was not dependent on the antibody used, as both neutralising monoclonal and polyclonal antibodies against IL-10 protein (as well as the anti-IL-10 receptor antibody used in this study) had no effect on the ability of RP73401 to block LPS stimulated TNF-α release (data not shown).

Table 3.1: The effect of an anti-IL-10 receptor antibody on the PDE4 inhibitor-mediated inhibition of TNF-α release from LPS stimulated human PBMC. *

Concentration of RP73401 (nM)	Anti-IL-10 receptor antibody (5μg ml^{-1})	SEM ±	MOPC21 control antibody (5μg ml^{-1})	SEM ±
1000	88.9%	5.5%	89.2%	4.6%
100	87.3%	5.0%	82.9%	4.4%

* $1x10^5$ human PBMC were incubated with 1000 and 100nM of the PDE4 inhibitor RP73401 for 30 min prior to the addition of either 5μg ml^{-1} of a mouse anti human IL-10 receptor antibody or 5μg ml^{-1} of an isotype control antibody (MOPC21) and 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain). TNF-α was measured after 24 hours from cell free supernatants by sandwich ELISA. The values shown are expressed as percentage inhibition of total specific TNF-α produced in the absence of any drug. The experiment shown is the mean of four other experiments ± SEM.

RP73401 was also tested to see if it effected IL-10 levels in LPS stimulated cultures of human PBMC. **Figure 3.8** indicates that RP73401 weakly inhibited IL-10 production. The effect of RP73401 on IL-10 levels was greatest between 20 and 40 hours after LPS stimulus. In the experiment shown the highest concentration of RP73401 (10μM) inhibited IL-10 production by between 48 ± 3.6% 20 hours after LPS challenge. In other experiments the effect of RP73401 was weaker, by 48 hours post LPS challenge RP73401 did not inhibit IL-10 production (**fig 3.12**).

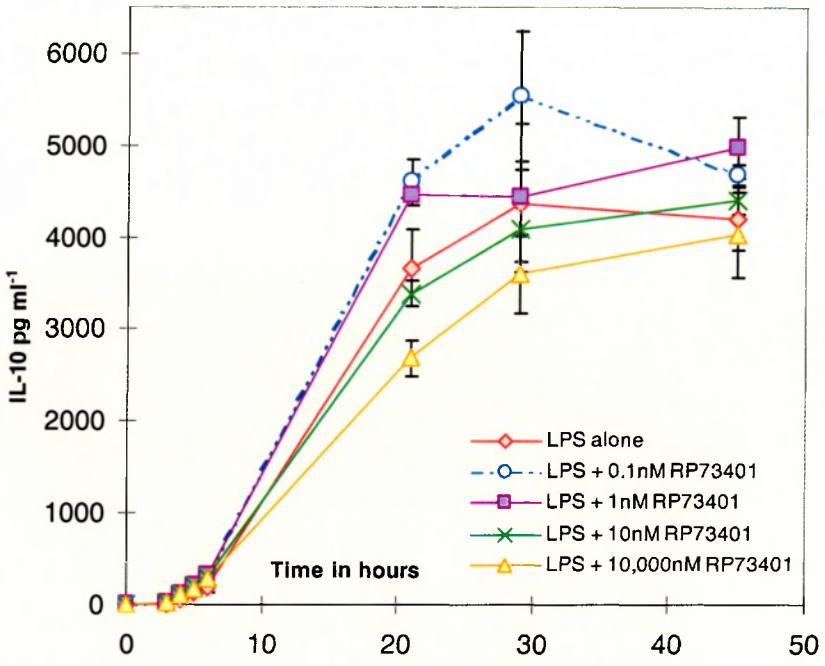


Figure 3.8: The effect of the PDE4 inhibitor RP73401 on the LPS stimulated release of IL-10 from human PBMC. $1x10^5$ human PBMC were incubated with the PDE4 inhibitor RP73401 at the concentrations indicated for 30 min prior to stimulation with 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain). IL-10 was measured at the times indicated in cell free supernatants by sandwich ELISA. Experiments shown is representative of five others. Mean values are indicated ± SD.

3.2.4 The effect of LPS plus IFN-γ on the phenotype of human monocytes and the effect of PDE4 inhibition on pro-inflammatory cytokine production by LPS plus IFN-γ stimulated human PBMC

In order to test if different activation conditions altered the phenotype of monocytes, the expression of two surface proteins, CD14 (co-receptor for the recognition of LPS) and CD16 (low-affinity receptor for IgG), was measured. **Figure 3.9** shows the effect of LPS, or LPS plus IFN-γ, or tissue culture media (containing 10% FCS) on the surface expression of CD16 in CD14 positive cells, measured over a six day period. After 2 days in culture 89.7 ± 1.9% of untreated CD14 positive cells expressed CD16, 43.7 ± 0.5% of LPS treated cells and 22.8 ± 4.6% of LPS plus IFN-γ treated cells expressed CD16. By day 4 these percentages had altered so, 77.4 ± 4.8% of untreated cells were positive, 62.2 ± 7.8% of LPS treated cells were positive and 18.1 ± 6.3% of LPS plus IFN-γ treated cells were positive. On the sixth day 53.9 ± 1.5% of untreated cells were positive, 22.4 ± 2.1% of LPS treated cells were positive and 10.9 ± 4.6% of LPS plus IFN-γ treated cells were positive.

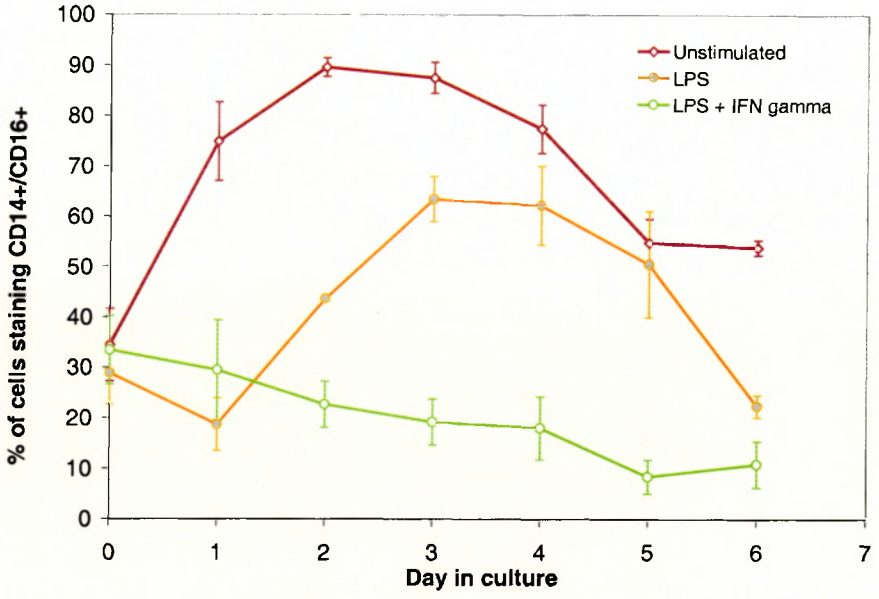


Figure 3.9: The effect of LPS plus IFN-γ on the maturation of CD14/CD16 double positive monocytes. $1x10^5$ human PBMC were incubated with 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain), 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain) plus 1ng ml^{-1} of human recombinant IFN-γ or left untreated. Prior to stimulation monocytes (identified using flow cytometry) were analysed for the dual expression of the surface markers CD14 and CD16. CD14/CD16 levels were enumerated using a mouse anti-human CD14 FITC antibody and a mouse anti-human CD16 PE antibody. Positivity for these markers was assessed by comparison with isotype control antibodies and the percentage of CD14 positive cells staining for CD16 was calculated. CD14 and CD16 expression were monitored daily. The values shown are the mean of six separate experiments ± SEM.

Using the same experimental conditions the effect of RP73401 (20μM) on CD16 expression in CD14 positive monocytes was investigated. **Figure 3.10a** shows the effect of 20μM RP73401, on CD16 expression in CD14 positive cells treated with tissue culture medium. After 2 days in culture 89.7 ± 1.9% of untreated CD14 positive cells expressed CD16 compared to 42.3 ± 11.7% of cells treated with RP73401. After 4 days in culture 77.4 ± 4.8% of untreated CD14 positive cells expressed CD16 compared to 54.3 ± 16.3% of cells treated with RP73401. On the sixth day in culture 53.9 ± 1.5% of untreated CD14 positive cells expressed CD16 compared to 38.4 ± 10.8% of cells treated with RP73401. **Figure 3.10b** shows the effect of 20μM RP73401, on CD16 expression in CD14 positive cells treated with LPS. After 2 days in culture 43.7 ± 0.5% of LPS treated CD14 positive cells expressed CD16 compared to 11.5 ± 4.0% of cells treated with RP73401. After 4 days in culture 62.2 ± 7.8% of LPS treated CD14 positive cells expressed CD16 compared to 12.1 ± 1.8% of cells treated with RP73401. On the sixth day in culture 22.4 ± 2.2% of LPS treated

CD14 positive cells expressed CD16 compared to 3.9 ± 1.2% of cells treated with RP73401. **Figure 3.10c** shows the effect of 20μM RP73401, on CD16 expression in CD14 positive cells treated with LPS plus IFN-γ. After 2 days in culture 22.7 ± 4.6% of LPS plus IFN-γ treated CD14 positive cells expressed CD16 compared to 8.3 ± 1.7% of cells treated with RP73401. After 4 days in culture 18.1 ± 6.3% of LPS plus IFN-γ treated CD14 positive cells expressed CD16 compared to 3.2 ± 1.3% of cells treated with RP73401. On the sixth day in culture 10.9 ± 4.6% of LPS plus IFN-γ treated CD14 positive cells expressed CD16 compared to 4.8 ± 0% of cells treated with RP73401.

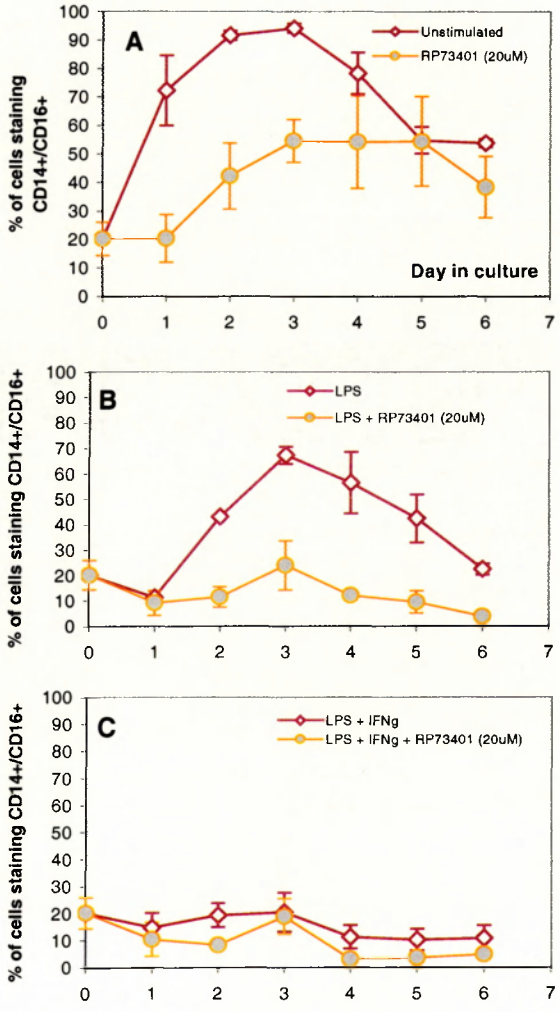


Figure 3.10: The effect of RP73401 on the maturation of CD14/CD16 double positive monocytes. 1x10^5 human PBMC were incubated with 1μg ml^{-1} of LPS, 1μg ml^{-1} of LPS plus 1ng ml^{-1} of IFN-γ or left untreated. Cells were either incubated with or without 20μM of RP73401. Prior to stimulation monocytes (identified using flow cytometry) were analysed for the dual expression of the surface markers CD14 and CD16. CD14/CD16 levels were enumerated using a mouse anti-human CD14 FITC antibody and a mouse anti-human CD16 PE antibody. Positivity for these markers was assessed by comparison with isotype control antibodies and the percentage of CD14 positive cells staining for CD16 was calculated. CD14 and CD16 expression were monitored daily. The values shown are the mean of three separate experiments ± SEM.

The PDE4 inhibitor RP73401 was compared in terms of its ability to alter the production of TNF-α and IL-10 from LPS and LPS plus IFN-γ stimulated human PBMC. **Figure 3.11** shows the percentage inhibition of TNF-α production by human PBMC stimulated with either LPS or LPS plus IFN-γ (1ng ml^{-1}). 48 hours after stimulation RP73401 inhibited LPS induced TNF-α production more potently and to a greater extent than LPS plus IFN-γ induced release. Using 10nM of RP73401 to compare effects, 90.9 ± 2.6% of LPS induced TNF-α was inhibited compared to 70.2 ± 2.6% of LPS plus IFN-γ induced TNF-α. The IC_{50} for RP73401 also shifted, being 0.4nM in LPS stimulated cultures and 3.5nM in LPS plus IFN-γ stimulated cultures. These differences were statistically significant, $p<0.05$.

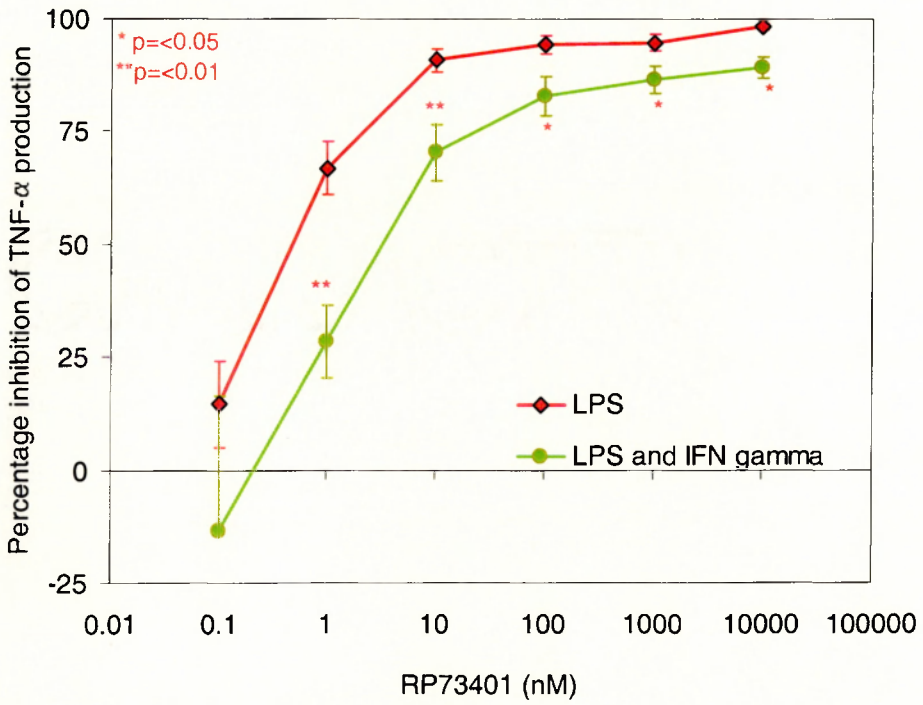


Figure 3.11: The effect of the PDE4 inhibitor RP73401 on LPS and LPS plus IFN-γ stimulated release of TNF-α from human PBMC. $1x10^5$ human PBMC were incubated with the PDE4 inhibitor RP73401 at the concentrations indicated for 30 min prior to stimulation with 1μg ml^{-1} of LPS derived from *E.coli* (055:b5 strain) or 1μg ml^{-1} of LPS derived from *E.coli* (055:b5 strain) plus 1ng ml^{-1} of human recombinant IFN-γ. TNF-α was measured after 48 hours from cell free supernatants by sandwich ELISA. The values shown are expressed in terms of percentage inhibition of total specific TNF-α produced in the absence of any drug. The data shown is the mean of seven experiments ± SEM. A single asterisk denotes a statistically significant difference in the ability of the same dose of RP73401 to inhibit TNF-α production from LPS versus LPS plus IFN-γ stimulated human PBMC, $p<0.05$. A double asterisk denotes a statistically significant difference in the ability of the same dose of RP73401 to inhibit TNF-α production from LPS versus LPS plus IFN-γ stimulated human PBMC, $p<0.01$.

The effect of RP73401 on LPS and LPS plus IFN-γ stimulated IL-10 release was also compared in the same way. **Figure 3.12** shows that 48 hrs after LPS stimulation RP73401 had little overall effect on IL-10 production by human PBMC, however RP73401 inhibited IL-10 production when measured at earlier time points (as indicated in **fig 3.8**). In contrast, RP73401 dose-dependently augmented IL-10 production by LPS plus IFN-γ stimulated human PBMC cultures. At a concentration of 10nM, RP73401 inhibited 2.4 ± 9.4% of LPS induced IL-10 compared to a stimulation of IL-10 levels by 83.9 ± 25.6 % in LPS plus IFN-γ stimulated cells. This effect was also seen at earlier time points (data not shown).

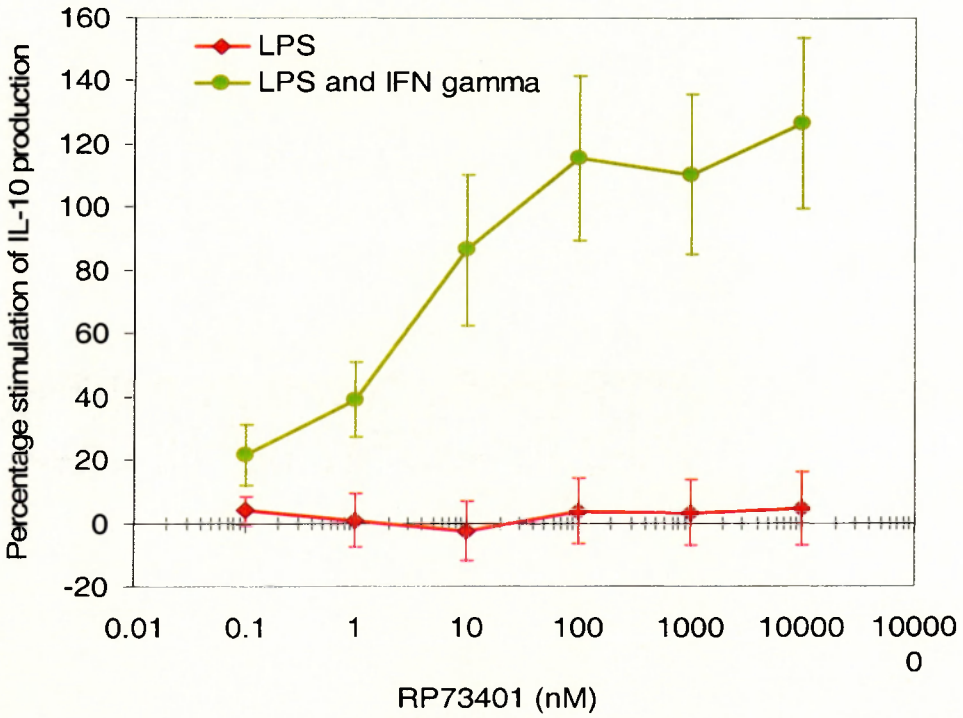


Figure 3.12: The effect of the PDE4 inhibitor RP73401 on the LPS and LPS plus IFN-γ stimulated release of IL-10 from human PBMC. $1x10^5$ human PBMC were incubated with the PDE4 inhibitor RP73401 at the concentrations indicated for 30 min prior to stimulation with 1μg ml^{-1} of LPS derived from *E.coli* (055:b5 strain) or 1μg ml^{-1} of LPS derived from *E.coli* (055:b5 strain) plus 1ng ml^{-1} of human recombinant IFN-γ. IL-10 was measured after 48 hours from cell free supernatants by sandwich ELISA. The values shown are expressed in terms of percentage stimulation of total specific IL-10 produced in the absence of any drug. The data shown is the mean of seven experiments ± SEM.

To evaluate if this increased IL-10 production caused by inhibiting PDE4 in LPS plus IFN-γ stimulated cells played a role in modulating TNF-α, bio-active IL-10 was neutralised using a monoclonal antibody to the IL-10 receptor. **Figure 3.13**, indicates that 5μg ml^{-1} of an anti-IL-10 receptor antibody did not alter the ability of RP73401 to inhibit TNF-α production 24 hours after human PBMC were stimulated with LPS plus IFN-γ. At a concentration of 10nM,

RP73401 inhibited 93.7 ± 0.6% of TNF-α in the presence of 5μg ml^{-1} of an anti IL-10 receptor antibody and inhibited 95.4 ± 0.7% of TNF-α in the presence of 5μg ml^{-1} of MOPC21 (isotype matched control antibody).

In contrast to PBMC stimulated with LPS alone, cells stimulated with LPS plus IFN-γ synthesised appreciable amounts of the pro-inflammatory cytokine IL-12. **Figure 3.14** indicates that RP73401, potently and dose dependently inhibited IL-12 p40 protein production by LPS plus IFN-γ stimulated human PBMC. RP73401 inhibited the production of IL-12 p40 for the duration of the experiment, 10μM of RP73401 inhibiting 81.8 ± 7.1% of IL-12 p40 released 7 hours after LPS plus IFN-γ challenge.

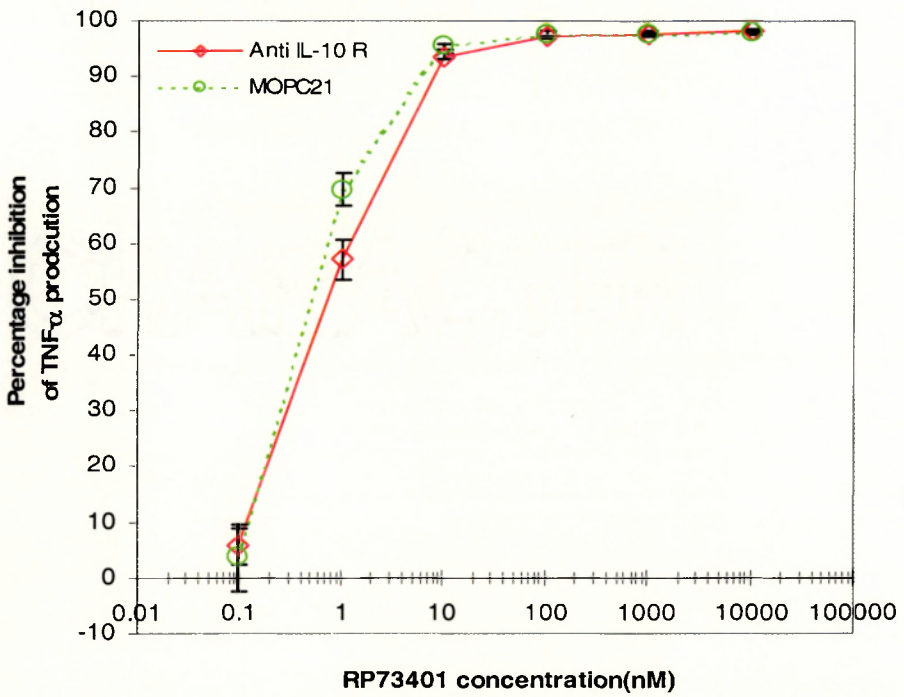


Figure 3.13: The effect of an anti IL-10 receptor antibody on the ability of RP73401 to block production of TNF-α from LPS plus IFN-γ stimulated human PBMC. $1x10^5$ human PBMC were incubated with the PDE4 inhibitor RP73401 at the concentrations indicated for 30 min prior to the addition of either 5μg ml^{-1} of a mouse anti human IL-10 receptor antibody or 5μg ml^{-1} of an isotype control antibody (MOPC21) and 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain) plus 1ng ml^{-1} of human recombinant IFN-γ. TNF-α was measured after 24 hours in cell free supernatants by sandwich ELISA. The values shown are expressed in terms of percentage inhibition of total specific TNF-α produced in the absence of any drug. The experiment shown is the mean of three other experiments ± SEM.

Elevated levels of IL-10, as well as being known to control TNF-α, have also been implicated in the control of IL-12 production from human monocytes [206-208]. The effect of

RP73401 on IL-12 production was tested in the presence of an anti-IL-10 receptor antibody that had previously been used to neutralise bioactive IL-10. **Figure 3.15** indicates that 5μg ml^{-1} of an anti-IL-10 receptor antibody did not alter the ability of RP73401 to inhibit IL-12 p40 production 24 hours after human PBMC were stimulated with LPS plus IFN-γ. At a concentration of 10nM, RP73401 inhibited 77.9 ± 5.5% of IL-12p 40 in the presence of 5μg ml^{-1} of an anti IL-10 receptor antibody and inhibited 87.0 ± 2.2% of IL-12 p40 in the presence of 5μg ml^{-1} of MOPC21 (isotype matched control antibody). These differences were not statistically significant, p=0.595.

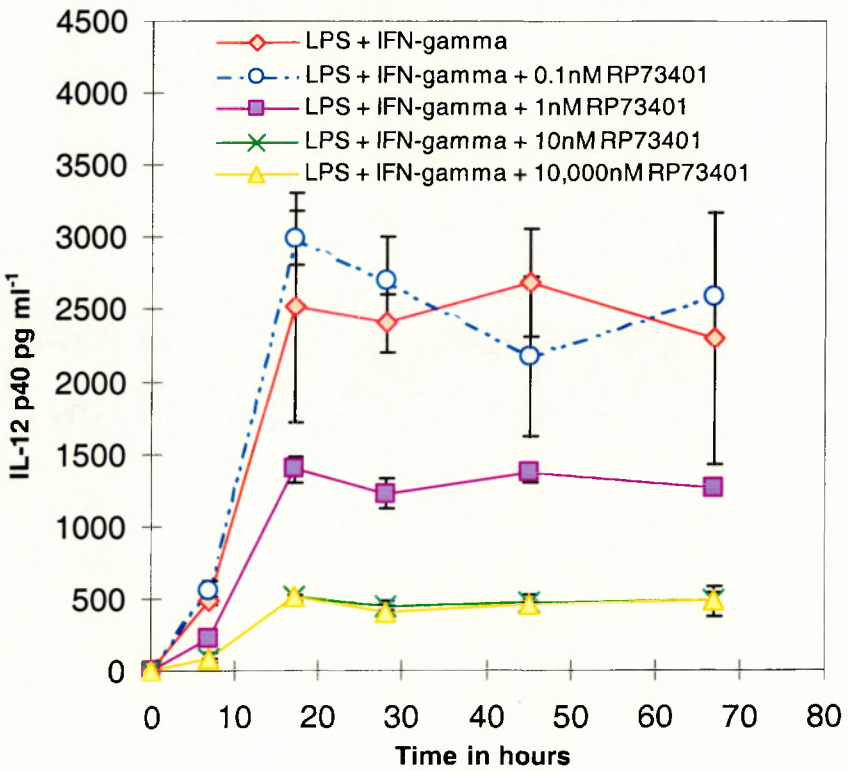


Figure 3.14: The effect of the PDE4 inhibitor RP73401 on the LPS plus IFN-γ stimulated release of IL-12 p40 from human PBMC. $1x10^5$ human PBMC were incubated with the PDE4 inhibitor RP73401 at the concentrations indicated for 30 min prior to stimulation with 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain) plus 1ng ml^{-1} of human recombinant IFN-γ. IL-12(p40) was measured at the times indicated in cell free supernatants by sandwich ELISA. The experiment shown is representative of three others. Mean values are indicated ± SD

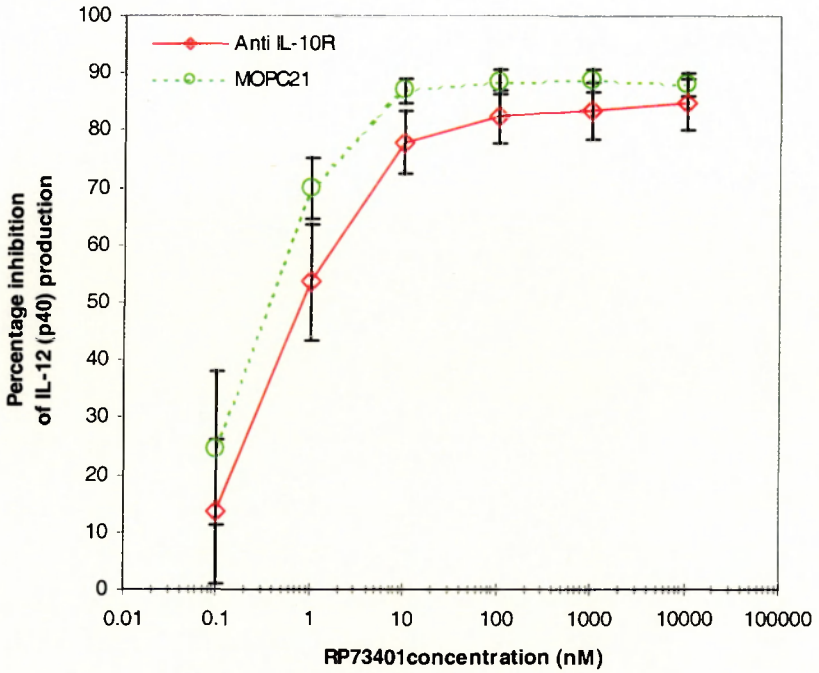


Figure 3.15: The effect of an anti-IL-10 receptor antibody on the ability of RP73401 to block production of IL-12p40 by LPS plus IFN-γ stimulated human PBMC. $1x10^5$ human PBMC were incubated with the PDE4 inhibitor RP73401 at the concentrations indicated for 30 min prior to the addition of either 5μg ml^{-1} of a mouse anti human IL-10 receptor antibody or 5μg ml^{-1} of an isotype control antibody (MOPC21) and 1μg ml^{-1} of LPS derived from *E.coli* (055:B5 strain) plus 1ng ml^{-1} of human recombinant IFN-γ. IL-12p40 was measured after 24 hours in cell free supernatants by sandwich ELISA. The values shown are expressed in terms of percentage inhibition of total specific TNF-α produced in the absence of any drug. The experiment shown is the mean of three other experiments ± SEM.

3.3 Discussion

Inhibitors of phosphodiesterase type 4 are currently being evaluated in a number of diseases and are showing clinical efficacy [154;196;198;209-214]. PDE4 inhibitors, which are non-discriminatory for the four PDE4 subtypes, have a wide range of action on cellular function. Particular interest has surrounded the capacity of PDE4 inhibitors to block TNF-α production from activated monocytes [147-150;176;199], as biological inhibitors of TNF-α are efficacious in the treatment of rheumatoid arthritis [1;215-218] and Crohn's disease [134;219;220]. Both IL-10-dependent and-independent mechanisms have been implicated to explain how PDE4 inhibitors might regulate TNF-α production. In light of these contradictions the effect of PDE4 inhibitors on key cytokines involved in inflammation was studied.

Using *in vitro* activation of human PBMC with LPS [61], studies were undertaken to detail the exact effect of a potent and specific PDE4 inhibitor upon pro- and anti-inflammatory cytokine production from activated cells. Human peripheral blood mononuclear cells activated with LPS release cytokines in an ordered and reproducible fashion. TNF-α is produced before either IL-1α and IL-1β which precede the release of IL-6 and IL-8 which in turn is followed by the production of IL-10. In accordance with literature reports [150] this study showed that, the potent selective PDE4 inhibitor RP73401, strongly and dose-dependently, blocked the production of TNF-α from LPS stimulated PBMC and this effect was sustained over the whole time course of the assay. This effect was not specific to this particular chemical class of PDE4 inhibitor (data not shown).

The inhibition of another key inflammatory regulator, IL-1, by either elevating cAMP levels directly or via inhibiting PDE4 has yielded conflicting data in the literature. In human monocytes or PBMC studies have shown either a suppression of IL-1β production [149] or a neutral effect on IL-1β production [221]. To confirm and extend these findings, experiments were performed to distinguish the inhibition of both IL-1α and IL-1β, as evidence points to distinct biological roles for these two proteins [222]. IL-1α production was, strongly and dose-dependently, blocked by RP73401 in a similar manner to TNF-α. In contrast IL-1β production, though blocked by RP73401, was inhibited to a lesser extent than IL-1α. IL-1β is thought to be a more physiologically important cytokine than IL-1α [222-224] and its overexpression may be responsible for some of the destructive effects seen in diseases such as rheumatoid arthritis [225-227]. The differential effect of RP73401 on IL-1α and β may relate to their different secretion pathways, both molecules are synthesised as pre-cursors with calpain cleaving pro-IL-1α [222] and IL-1β converting enzyme (ICE, also known as caspase-1) cleaving pro IL-1β [228].

The pro-inflammatory cytokine IL-6 can be induced by IL-1 [229-231], which is thought to play a regulating role in controlling IL-6 synthesis. Conflicting reports exist regarding the effect

of elevated (intracellular) cAMP on the production of IL-6 from LPS activated monocytes. These indicate that elevated cAMP (by PDE-dependent and-independent mechanisms) can either inhibit or have no effect on IL-6 production [168;202;221;232]. The work in this thesis has indicated that the PDE4 inhibitor RP73401 had no statistically significant effect on IL-6 production from LPS stimulated PBMC. This could be due to the weak effect on IL-1β caused by PDE4 inhibition and, since IL-1 is known to induce IL-6 [230], may mean that production of IL-1β rather than IL-1α controls the production of IL-6 in LPS stimulated human PBMC. In accordance with literature reports the PDE4 inhibitor RP73401 had no effect on IL-8 production from LPS stimulated human PBMC [233].

LPS stimulated PBMC produce IL-10 in response to increasing levels of TNF-α and as such TNF-α and IL-10 are reciprocally responsible for activating and deactivating gene transcription and protein production of each other [234-239]. Since IL-10 is a potent anti-inflammatory agent that is capable of controlling TNF-α the elevation of IL-10 could be an effective mechanism for inhibiting TNF-α production. This elevation in IL-10 has been shown to directly control TNF-α production [203;232]. One mechanism to increase IL-10 production in inflammatory cells is via the elevation of intracellular cAMP. The same hypothesis has been extended to explain why PDE4 inhibitors are such potent blockers of TNF-α production. In these studies IL-10 at low physiological doses was a potent suppresser of TNF-α production when added to PBMC at the time of LPS challenge, confirming literature findings [240]. In addition it also suppressed IL-1β production, inhibiting its production to the same extent as the PDE4 inhibitor RP73401. IL-6 production was only weakly inhibited by exogenous addition of IL-10.

The similarity between the effect of hrIL-10 and RP73401 on the inhibition of cytokine production gives credence to the hypothesis that an elevation of IL-10 may mediate the effect of PDE4 inhibition upon cytokine production. However, PDE4 inhibitors have been shown to inhibit TNF-α by both IL-10-dependent and-independent mechanisms [151;205;241]. In

this study the potent PDE4 inhibitor RP73401, did not induce increased IL-10 production. RP73401 at concentrations as low as 10nM inhibited IL-10 production from LPS stimulated PBMC, in contrast to the published observations of the effects of PDE4 inhibitors on IL-10 production [151;205]. This implies that elevated IL-10 did not mediate the inhibitory effect of PDE4 inhibition of TNF-α production. The IL-10-independent mechanism of RP73401 was confirmed, as it inhibited TNF-α production in the presence of an excess quantity of an anti-IL-10 receptor neutralising antibody. Thus, PDE4 inhibitors potently inhibit TNF-α production by an IL-10-independent mechanism.

Monocytes, like many other leukocytes, cannot be clearly defined as a single uniform population of cells. Indeed human blood derived monocytes can be defined as different types depending upon their relative expression of certain surface receptors. One population, typical of the majority of blood monocytes, expresses high levels of the LPS binding protein, CD14, but little or no low-affinity IgG receptor (CD16 or FcγRIIIa) [242;243]. The other population, which represents the minor sub-set in whole blood, expresses lower amounts of CD14 but much higher levels of CD16.

The occurrence of these two monocyte/macrophage phenotypes can be altered in disease and according to anatomical location, e.g. alveolar macrophages are $CD14^{+}/CD16^{+}$ [242], whereas peritoneal macrophages are $CD14^{++}$ [242] and pleural macrophages are intermediate between the two phenotypes [244]. In diseases, such as sepsis [245;246] and renal disease [247;248], the circulating levels of $CD14^{+}/CD16^{+}$ and $CD14^{++}$ monocytes are altered and may contribute to the specific pathogenesis of those diseases. As well as differing phenotypically these monocyte sub-sets also differ functionally. Although both sub-sets transcribe TNF-α mRNA and produce TNF-α protein in response to LPS [249], they differ in their regulation of IL-10. $CD14^{++}$ monocytes regulate IL-10 normally at both message and protein level, but cells that express CD16 produce little or no IL-10 protein when stimulated with LPS [249].

In light of the contradictory findings surrounding the action of PDE4 inhibitors on cytokine production, the contribution of monocyte phenotype on the effect of PDE4 inhibitors was investigated. Although certain cytokines, such as TGF-β and GM-CSF [249;250], can regulate CD16 expression, the effect of different activating agents on the maturation of $CD14^{+}/CD16^{+}$ monocytes is untested. In the present studies when monocytes were cultured in medium containing serum, they up-regulated CD16 expression in accordance with literature accounts [242;243]. However, when cells were stimulated with serum and LPS (stimulating through TLR4), cells initially suppressed CD16 expression but later reverted back to the phenotype of serum treated cells. In contrast, when LPS plus IFN-γ were added to human PBMC cultures, CD16 expression was strongly suppressed. This effect was not observed when IFN-γ alone was administered to monocytes [251].

$CD14^{+}/CD16^{+}$ and $CD14^{++}$ monocyte/macrophages as well as differing in their production of cytokines in response to LPS, also regulate their expression of phosphodiesterases differently. In blood, as monocytes mature (and increase CD16 expression) they also up-regulate the transcription of PDE1 and PDE3 whilst down-regulating the transcription of PDE4 [194]. This is in contrast to $CD14^{++}$ monocytes which maintain PDE4 activity [194] and are susceptible to the action of PDE4 inhibitors. Since PDE induction may be linked to monocyte phenotype, the effect of PDE4 inhibition on CD16 expression was investigated. Inhibition of PDE4 suppressed the expression of CD16 on serum, LPS and LPS plus IFN-γ treated monocytes. Paradoxically this would suggest that active PDE4 may be required for CD16 expression, though CD16 expressing cells are known to down-modulate PDE4 activity [194]. However, in cells treated with serum, for five days, RP73401 was unable to suppress CD16 expression. This fits with the known PDE expression profile of such cells, which lack PDE4 [194]. In both LPS and LPS plus IFN-γ treated cells the regulation of phosphodiesterases is unknown. Further studies will be required to see if such cells do regulate their PDE's differently, and if this accounts for the effect that PDE4 inhibitors have on CD16 expression.

Since LPS and LPS plus IFN-γ stimulated monocytes differ with respect to surface phenotype and may differ in PDE expression profile, the effect of PDE4 inhibition on cytokine release from such cells was studied. In contrast to LPS stimulated PBMC, cells stimulated with LPS plus IFN-γ were shown to produce large amounts of IL-12. RP73401 potently suppressed IL-12 production from PBMC stimulated with LPS plus IFN-γ, an effect seen in endotoxemic animals treated with PDE4 inhibitor [208]. This effect may be critical to the ability of PDE4 inhibitors to block inflammation since inhibiting IL-12 production can suppress Th1 mediated disease [252].

PDE4 inhibitors also inhibited TNF-α production by PBMC stimulated with LPS plus IFN-γ. However, this inhibition of TNF-α production was weaker than from PBMC stimulated with LPS alone. Expressed in terms of IC_{50} RP73401 was almost ten-fold weaker at inhibiting LPS plus IFN-γ than LPS induced TNF-α release. PDE4 inhibition also had markedly different effects on the regulation of IL-10 production from LPS versus LPS plus IFN-γ stimulated cells. Inhibition of PDE4 had no significant effect on IL-10 production from LPS stimulated PBMC measured 48 hours after LPS stimulation, though it did inhibit the production of IL-10 at earlier time points. However, it caused a dose-dependent elevation in IL-10 synthesis 48 hours after stimulation of PBMC with LPS plus IFN-γ.

It could be postulated that the increase in IL-10, caused by PDE4 inhibition, in LPS plus IFN-γ stimulated PBMC was responsible for the suppression of pro-inflammatory cytokines, such as TNF-α. However, despite the up-regulation of IL-10 caused by PDE4 inhibition this study found that the blockade of TNF-α production was independent of IL-10 release, as the inhibitor RP73401 worked equally well in the presence of excess amounts of an anti-IL-10 receptor antibody. Likewise, it was discovered that, as was the case for TNF-α, the inhibition of IL-12 production by PDE4 inhibitors was independent of any effect on IL-10 production.

To summarise, the results in this chapter confirm that PDE4 inhibitors have potent anti-inflammatory effects *in vitro*. They suppress the production of TNF-α by both LPS and LPS plus IFN-γ-stimulated PBMC. As well as TNF-α, they also suppress the production of IL-1α and to a lesser extent IL-1β. In addition, the suppression of IL-12 by PDE4 inhibitors represents an important point of blockade of Th1 (cellular) mediated immune responses. In contrast to other agents that elevate cAMP, PDE4 inhibitors do not inhibit TNF-α production by enhancing the synthesis of IL-10. It has been shown that PDE4 inhibitors, as well as suppressing cytokine production, can also suppress the expression of surface proteins, such as those of the B7 family [197]. To this list should be added the low-affinity IgG receptor, which is suppressed in CD14 positive cells. This may have important implications as CD16 expressing monocytes have been associated with specific diseases, e.g. sepsis [245]. The stimulation of monocytes with LPS plus IFN-γ, which results in a well documented effect on cytokine production, and now also an increase in $CD14^{++}$ cells, alters the pattern of cytokine inhibition achieved by PDE4 inhibitors. In such cells PDE4 inhibitors can up-regulate IL-10, though IL-10 is not responsible for the TNF-α inhibitory action of PDE4 inhibitors. The next results chapter will discuss the impact of inhibiting PDE4 upon T cell activation and cytokine release. This will focus on the therapeutic potential of PDE4 inhibitors to act as immunosuppressive or immunomodulatory agents.

Chapter 4

Evaluation of the effect of inhibition of phosphodiesterase type 4 on human T cell function

Chapter 4

Evaluation of the effect of inhibition of phosphodiesterase type 4 on human T cell function

4.1 Introduction

The effect of elevation of cAMP in T cells has been well documented. Elevating cAMP in T cells has a suppressive function, inhibiting both the production of IL-2 and cellular proliferation [253-261]. However, these suppressive effects are complex, and under certain circumstances cAMP can augment T cell responses, as in the case for the production of Th2 cytokines by Th2 cells or clones [262-265].

The elevation of cAMP through activation of adenylyl cyclase has emphasised the important role that cAMP plays in modulating T cell responses. Research aimed at defining the effect of PDE4 inhibition, as a means to elevate cAMP levels, has attracted far less study and has yielded some conflicting and contradictory data [199;266-269]. The archetypal PDE4 inhibitor, Rolipram, was the first PDE4-specific tool used to elucidate the effect of PDE4 in T cells [270] but further studies using PDE4 inhibitors to block T cell function have been compounded by a number of factors. The first of these relates to the pharmacological profile of PDE4 inhibitors. Rolipram binds, with a high affinity, to a site on the PDE4 enzyme. This binding was first ascribed to a phosphodiesterase species in brain membranes [271] and is often termed HARBS (high affinity Rolipram binding site). Rolipram binds to the catalytic site of the PDE4 enzyme, in what is thought to be an alternative conformation, with approximately 100-fold lower affinity. The pharmacological profile of inhibitors differs with respect to their inhibition at the catalytic (low-affinity) and HARB sites [272] and inhibition at these sites correlates with distinct physiological sequelae. This distinction occurs for inhibition of TNF-α production in LPS stimulated monocytes [147;150] and the inhibition of eosinophil superoxide production[273], which correlate with inhibition at the catalytic site. However, many of the so-called side effects of PDE4 inhibitors, such as the induction of emesis [146] and gastric acid

hyper-secretion [274], correlate with displacement of ^{3}H-Rolipam binding to the PDE4 enzyme (in its high-affinity state).

The pharmacological effect of PDE4 inhibitors is further complicated by the existence of sub-types of the PDE4 enzyme. The PDE4 enzyme can be transcribed by four distinct genes coding for four separate subtypes termed A, B, C and D. The expression patterns of these isoforms are tissue specific [275-277]. Human CD4 and CD8 positive T cells, in various studies, have been shown to express subtypes A, B and D but not C; this expression profile can be altered upon T cell activation [140;199;276]. In studies on resting human T cells messenger RNA for PDE4A was dominantly expressed. Upon activation both PDE4B and D were induced but not PDE4C [278]. These isoform expression patterns are complicated by the presence of many splice-variants [140], the expression patterns of which remain largely unknown.

These two factors, the unknown mode of binding to the PDE4 enzyme and the existence of PDE4 enzyme isoforms, have combined to confuse the studies evaluating the effect of PDE4 inhibitors on T cell function. Many studies conducted with PDE4 inhibitors have used either pan-specific PDE4 inhibitors or inhibitors of unknown specificity. Conclusions from such experiments have been interpreted without the consideration of which PDE4 isoforms or splice variants were involved. Work using inhibitors that preferentially inhibit PDE4A and B showed that inhibition of these subtypes correlated with inhibition of T cell activation [199], though a contribution of the PDE4D enzyme could not be ruled out. However PDE4D does not seem to be involved in controlling T cell proliferation as mice lacking PDE4D (PDE4D $^{-/-}$ knockout mice), showed no impairment of their T cell responses to antigen [279].

The differences in modulation of T cell responses by PDE4 inhibitors are most obvious when comparing *in vitro* and *in vivo* data between mammalian species. Elevation of cAMP has been shown to skew the Th cell cytokine profile toward a Th2 phenotype, yet in animal models PDE4 inhibitors have been shown to be efficacious in both Th1 and Th2 disease models [152;158;280;281]. In human *in vitro* experiments Rolipram inhibited Th2 cell proliferation

more potently than Th1 [267]. However, the effect of inhibition of PDE4 on Th1/Th2 cytokine production suggested Th1 cytokines may be inhibited to a greater extent than Th2 cytokines[266;267;282]. In contrast, inhibiting the action of PDE4 in murine T cell clones resulted in a skewed cytokine response in favour of Th2 cytokine production [264].

PDE4 inhibitors are currently undergoing clinical trials to test their efficacy in a number of diseases, such as asthma and chronic obstructive pulmonary disease (COPD) [154;209]. These diseases, particularly asthma, are either mediated or influenced by T cell activity and in particular the T helper cytokines produced by T cells. In light of the limited and contradictory studies on the effects of PDE4 inhibitors on human T cell function, this thesis has attempted to understand the therapeutic potential of inhibiting T cell PDE4. Using *in vitro* models of human T cell activation the contribution of PDE4, to both T cell proliferation and cytokine production, was studied in detail. This work has addressed how the pharmacological activity of PDE4 inhibitors may account for their inhibition of different T cell functions. In particular it addresses whether inhibition of the PDE4 enzyme in its low- or high-affinity state correlates with inhibition of T cell activation.

4.2 Results

4.2.1 The effect of PDE4 inhibitors on T cell activation

In order to assess the effect of PDE4 inhibitors on T cell activation initial experiments were carried out to determine the effect on cAMP levels in activated cells of PDE4 inhibition. Human PBMC were stimulated with the mitogen Con A for a period of 24 hours. The potent PDE4 inhibitor RS25344 (refer to chapter 2.2.1), when added to Con A stimulated PBMC cultures elevated the level of cAMP. **Figure 4.1** indicates that 10μM of RS25344 elevated cAMP levels by 303 ± 8% at thirty minutes and by 217 ± 31% at 24 hours after Con A stimulation of PBMC. The effect of RS25344 was dose-dependent as 100nM of RS25344 elevated cAMP levels by only 180 ± 12% thirty minutes after stimulation and by 21 ± 31% 24 hours after stimulation.

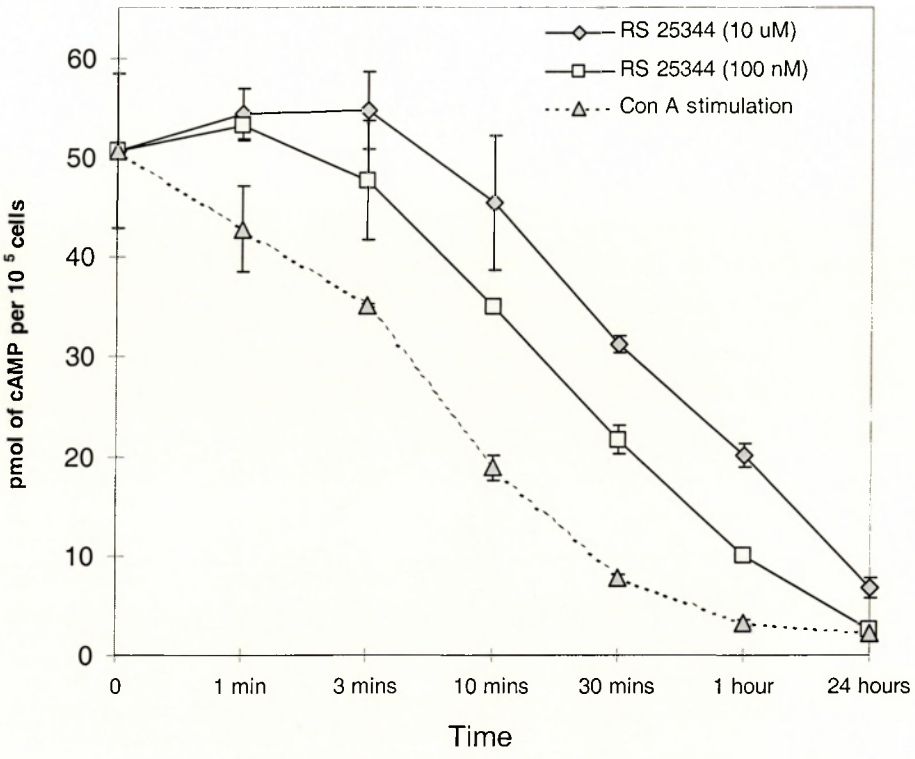


Figure 4.1: The effect of the PDE4 inhibitor RS25344 on intracellular cAMP accumulation in Con A stimulated human PBMC. $1x10^5$ human PBMC were incubated in the presence of 10μM and 100nM of the PDE4 inhibitor RS25344 prior to stimulation with 1μg ml^{-1} of Con A. The intracellular levels of cAMP were measured at the intervals indicated, using a RIA. The graph is representative of four other experiments, each point is the mean of four separate determinations ± SD.

In order to test if PDE4 inhibitors blocked T cell proliferation six phosphodiesterase inhibitors; CDP840, R-Rolipram, S-Rolipram, RS25344, RP73401 and SB 207499 (refer to Chapter 2.2.2) were used to inhibit the antigen specific proliferation of human PBMC. **Figure 4.2** shows that all six inhibitors evaluated inhibited tetanus toxoid stimulated human PBMC proliferation in a dose-dependent fashion. No compound achieved total inhibition of proliferation, maximal inhibition was in the range of 75 to 90%.

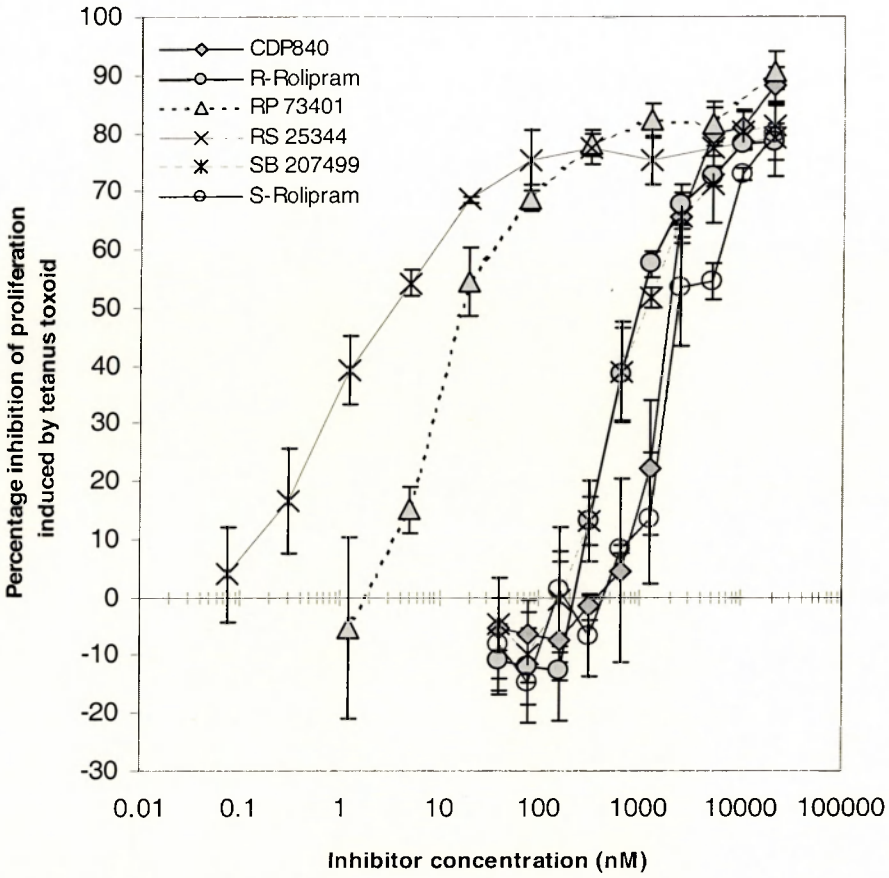


Figure 4.2: The effect of PDE4 inhibitors on tetanus toxoid induced proliferation of human PBMC. $1x10^5$ human PBMC were incubated in the presence of six PDE4 inhibitors; CDP840, R-Rolipram, S-Rolipram, RS25344, RP73401 and SB 207499 for 30min prior to stimulation with 1μg ml^{-1} of tetanus toxoid. PBMC were incubated for five days and ^{3}H-thymidine incorporation was measured for the last 12h of culture. PDE4 inhibitor inhibition of proliferation was expressed as a percentage of total proliferation induced by tetanus toxoid alone. The graph is a mean of three separate experiments ± SEM.

The same compounds also inhibited antigen proliferation of human PBMC in response to *Dermataphagoides pteronyssinus* (a Th2-like stimulus as opposed to tetanus toxoid which is a Th1-like stimulus) in a dose-dependent manner (data not shown). Inhibition of tetanus toxoid-and *D. pteronyssinus*-induced proliferation was compared in terms of the concentration of compound needed to inhibit 25% of the maximal proliferative response (IC_{25}) induced by each antigen. IC_{25} instead of IC_{50} values were used as not all compounds consistently achieved 50% inhibition of maximal proliferation. **Table 4.1** compared these IC_{25} values and indicates that all PDE4 inhibitors tested showed no significant differences in potency between tetanus toxoid-or *D. pteronyssinus*-induced PBMC proliferation.

Table 4.1: The effect of PDE4 inhibitors on Th1-and Th2-like antigen-induced proliferation of human PBMC. *

Compound	TT (IC_{25}) nM	SEM +/-	HDM (IC_{25}) nM	SEM +/-
S-Rolipram	3333	1333	3250	577
R-Rolipram	916	308	650	122
CDP840	2250	749	2625	509
RP73401	29	20.7	80	57.2
RS25344	8.53	4.21	10	0
SB207499	375	96	1000	288

* $1x10^5$ human PBMC were incubated in the presence of six PDE4 inhibitors; CDP840, R-Rolipram, S-Rolipram, RS25344, RP73401 and SB 207499 for 30min prior to stimulation with 1μg ml^{-1} of tetanus toxoid or $1x10^5$ units of *D.pteronyssinus* major antigen Der P1. PBMC were incubated for five days and ^{3}H-thymidine incorporation was measured for the last 12h of culture. PDE4 inhibitor inhibition of proliferation was expressed as a percentage of total proliferation induced by tetanus toxoid alone or *D.pteronyssinus*. The table is a mean of four separate experiments ± SEM. Abbreviations: TT, tetanus toxoid and HDM, house dust mite antigen.

The PDE4 inhibitor RP73401 was tested to see if it inhibited human PBMC proliferation induced by an equal mixture of the staphylococcal enterotoxins A, B, D and E (termed superantigen). RP73401 only weakly inhibited proliferation induced by superantigen, **fig 4.3.** At the highest concentration tested (10μM), RP73401 inhibited 77.5% of the response after 36 hours but only gave 17.8% inhibition 78 hours post-stimulus.

The inhibition of Con A stimulated PBMC proliferation by the PDE4 inhibitors CDP840, R-Rolipram, S-Rolipram, RS25344, RP73401 and SB 207499, like the effect on superantigen induced proliferation, was also transitory. IC_{25} values for each compound, when measured 24 and 48 hours after Con A stimulation, showed a decrease in potency in the range of 1.5 to 10 fold. The addition of 100nM of PGE_2 (which by itself elicited no effect upon PBMC proliferation) enhanced the anti-proliferative effects of the PDE4 inhibitors when measured 48 hours after stimulation **table 4.2.**

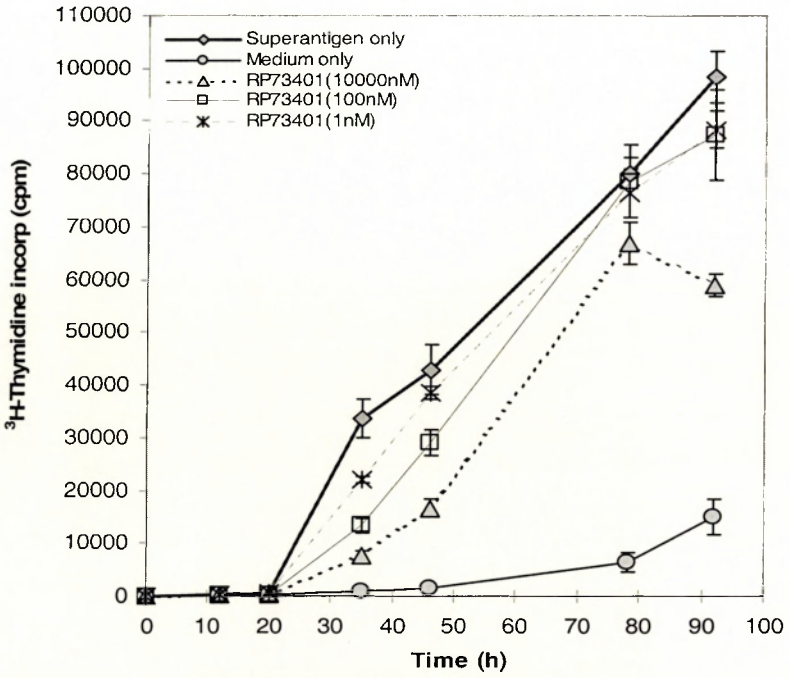


Figure 4.3: The effect of the PDE4 inhibitor RP73401 on superantigen induced proliferation of human PBMC. $1x10^5$ human PBMC were incubated in the presence of RP73401 for 30min prior to stimulation with 100pg ml^{-1} of an equal mixture of the staphylococcal enterotoxins A, B, D and E. ^{3}H-thymidine incorporation was measured at the intervals indicated for a period of 92h. The graph is a representative of three others; mean values are indicated ± SD.

Table 4.2: The effect of PGE_2 on the PDE4-mediated inhibition of Con A stimulated human PBMC proliferation. *

Compound	Con A 24hrs (IC_{25}) nM	SEM ±	Con A 48hrs (IC_{25}) nM	SEM ±	Con A 48hrs + PGE_2(IC_{25}) nM	SEM ±
S-Rolipram	500	218	2733	1246	550	122
R-Rolipram	253	130	1000	252	138	92
CDP840	33	19	46.7	27	15	7.5
RP73401	<2.2	/	5.3	1.8	<6.3	/
RS25344	<2	/	10.7	4.7	<3.8	/
SB207499	87	13	833	338	355	176

* $1x10^5$ human PBMC were incubated in the presence of six PDE4 inhibitors; CDP840, R-Rolipram, S-Rolipram, RS25344, RP73401 and SB 207499 for 30min prior to stimulation with 1µg ml^{-1} of Con A. ^{3}H-thymidine incorporation was measured after 24 and 48 h in culture. In separate experiments compounds were also incubated with 100nM of PGE_2 for 30min prior to stimulation with Con A and ^{3}H-thymidine incorporation was measured after 48h in culture. PDE4 inhibitor inhibition of proliferation was expressed as the concentration of compound needed to inhibit 25% of the total proliferation induced by Con A only. The table is a mean of four separate experiments ± SEM. Where ' < ' is shown this indicates values below lower detection limits of the assay.

4.2.2 The effect of PDE4 inhibition on T cell cytokine production and cytokine receptor expression.

Human PBMC were induced to produce cytokines using the mitogenic anti-CD3 antibody, OKT3. GM-CSF, IL-2, IFN-γ, IL-4 and TNF-α levels were then monitored at regular time intervals for a period of 72 hours. **Figure 4.4a** shows that RP73401 inhibited the OKT3 stimulated production of GM-CSF in a dose-dependent manner. At the highest concentration (1μM), 70.3 ± 7.4% of GM-CSF was inhibited 24 hours after OKT3 stimulation. In addition to GM-CSF, IL-2 levels were also inhibited by RP73401, **fig 4.4b**. However, IL-2 was only produced over the first 48 hours after OKT3 stimulation and its production peaked after 8 hours. The effect on IL-2 was again dose-dependent. The highest concentration of RP73401 blocked 69.5 ± 6.1% of IL-2 released 24 hours after OKT3 stimulation.

The cytokines IFN-γ and IL-4, are responsible for amplifying Th cytokine production and so are important immunomodulatory cytokines. **Figures 4.4c** and **d** indicate that RP73401 inhibited IFN-γ and IL-4 production in a dose-dependent manner, with the highest concentration of RP73401 inhibiting 72.1 ± 16.9% of IFN-γ and 53.2 ± 3.4% of IL-4 produced 24 hours after OKT3 stimulation. These differences were not statistically significant, p=0.211.

Finally, RP73401 potently blocked TNF-α production from OKT3 stimulated human PBMC cultures, **fig 4.4e**. Like its effect on other cytokines, RP73401 inhibited TNF-α production in a dose-dependent way. The highest concentration of RP73401 inhibited 94 ± 3.2% of TNF-α produced 24 hours after OKT3 stimulation. It should be noted that at later time points the inhibition of TNF-α production was total, though this was not the case for any of the other cytokines tested.

To test the effect of PDE4 inhibitors on cytokine receptor expression, human PBMC were induced to express the IL-2 receptor alpha chain (CD25) by stimulation with superantigen. **Figure 4.5** indicates that RP73401 weakly inhibited the expression of CD25 on CD3 positive

T cells. At the highest concentration tested (10μM), RP73401 inhibited 59 ± 20% of CD25 expression measured 18 hours after superantigen challenge. After 72 hours in culture RP73401 did not inhibit the expression of CD25 on CD3 positive cells.

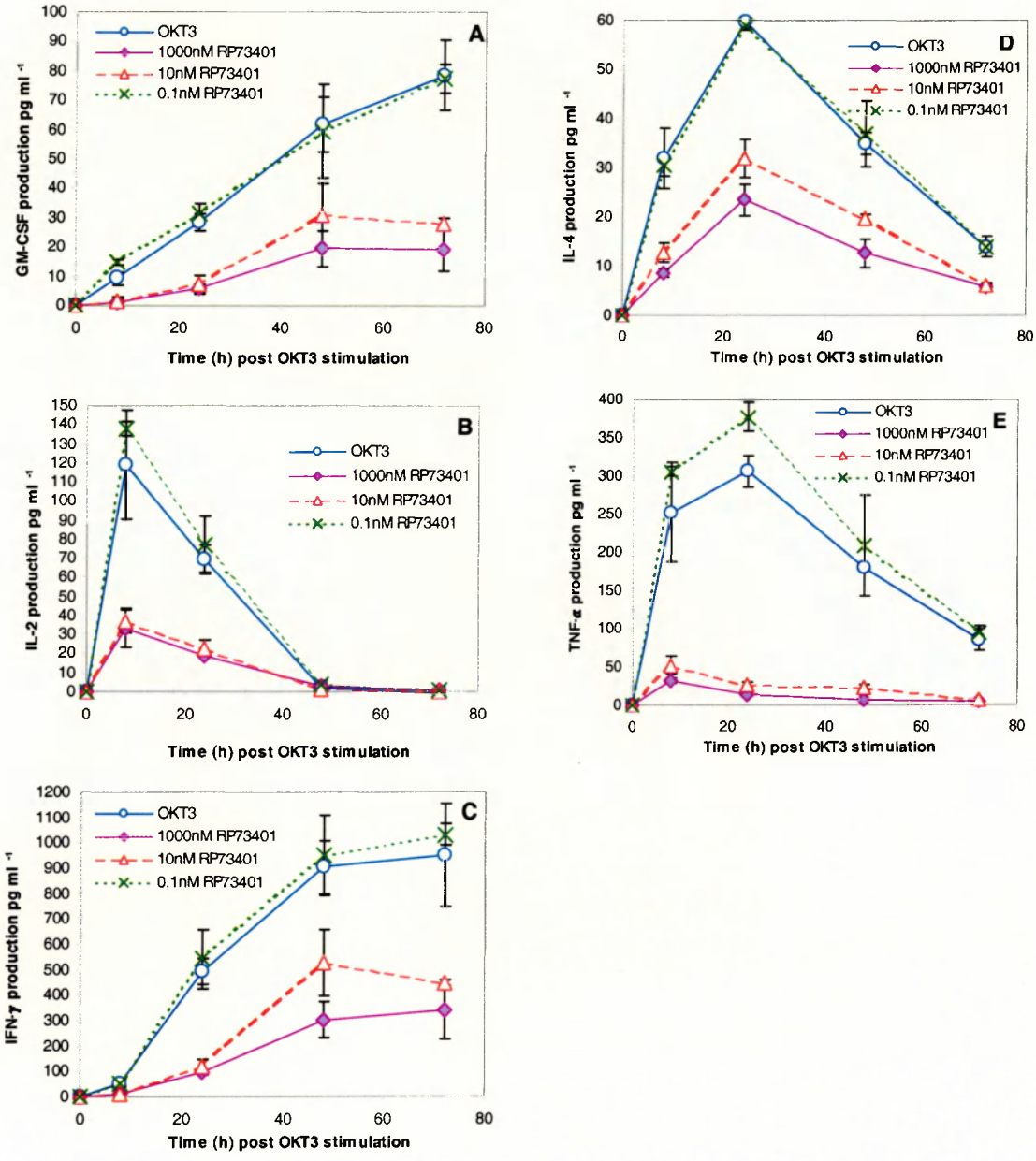


Figure 4.4: The effect of PDE4 inhibitor RP73401 on OKT3-induced cytokine (GM-CSF, IL-2, IFN-γ, IL-4 and TNF-α) production from human PBMC. $1x10^5$ human PBMC were incubated in the presence of RP73401 for 30min prior to stimulation with OKT3 (40ng ml^{-1}). GM-CSF (Figure 4.4a), IL-2 (Figure 4.4b), IFN-γ (Figure 4.4c), IL-4 (Figure 4.4d), and TNF-α (Figure 4.4e) production was monitored at regular intervals over a 72h period. Cytokine levels were measured in cell free supernatants by multiplex cytokine assay. The graphs shown are representative of five other experiments. The data shown represents mean values ± SD.

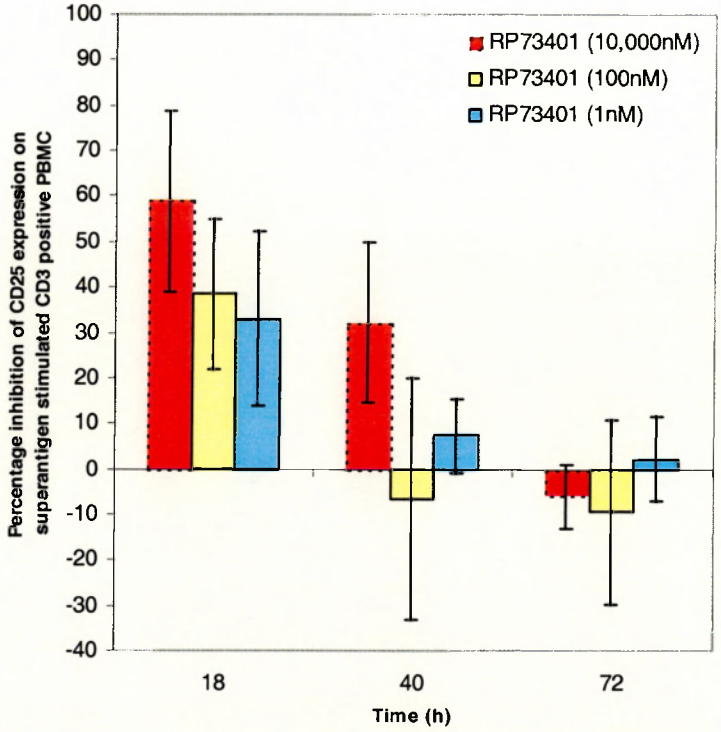


Figure 4.5: The effect of the PDE4 inhibitor RP73401 on superantigen induced expression of CD25 (IL-2 receptor alpha chain) on human PBMC. $1x10^5$ human PBMC were incubated in the presence of RP73401 for 30min prior to stimulation with 100pg ml^{-1} of an equal mixture of the staphylococcal enterotoxins A, B, D and E. CD25 (IL-2 receptor alpha chain) levels were monitored at regular intervals for a period of 72h. CD25 levels were determined on CD3 positive cells using a FACScan flow cytometer. The data shown is the mean of three separate experiments ± SEM.

4.2.3 PDE4 inhibition of superantigen stimulated PBMC proliferation: Data analysed for a correlation with inhibition of PDE4 in its high-affinity or low-affinity conformation.

Six PDE4 inhibitors; CDP840, R-Rolipram, S-Rolipram, RS25344, RP73401 and RS14203 were tested for their ability to block the activity of PDE4 enzyme preparations in an *in vitro* assay. **Table 4.3** indicates the IC_{50} values for each compound tested for their inhibition of the catalytic activity of PDE4A derived from SF9 cells, a truncated PDE4 enzyme PDE4A330 (A330 mutant lacking HARBS, [185]) which was purified from COS cells and inhibition of ^{3}H-Rolipram binding to guinea pig brain membranes (a PDE4 preparation demonstrated to exhibit the high-affinity Rolipram binding site). The IC_{50} values for all six compounds varied in potency from 0.26nM to 325nM against PDE4A, from 0.4nM to 753nM against PDE4A330 and from 1 to 91nM against antagonism of ^{3}H-Rolipram binding to guinea-pig membranes.

Table 4.3: The inhibition of PDE4 enzymatic activity and competition for ^{3}H-Rolipram binding by a selection of PDE4 inhibitors.*

Compound	PDE4A (SF9) IC_{50} (nM)	PDE4A330 (COS) IC_{50} (nM)	^{3}H-Rolipram binding to brain membranes IC_{50} (nM)
S-Rolipram	325	605	91
R-Rolipram	66	501	5
CDP840	2.7	3.9	60
RP73401	0.26	0.4	5
RS25344	37.4	753	1
RS14203	0.87	39	64

* PDE4 enzyme activity was assayed in a homogeneous scintillation proximity assay. Full length PDE4A was harvested from baculovirus infected SF9 cells and truncated PDE4A (ΔQ44-329) was harvested from transfected COS cells. Inhibitors were added to the enzyme substrate prior to enzyme addition. The reaction was incubated at room temperature for 30 min prior to termination of the reaction. Antagonism of ^{3}H-Rolipram binding to guinea pig brain membranes was measured using liquid scintillation counting The IC_{50} values shown represent the mean of five different experiments.

Figure 4.6a shows the relationship of the inhibition of PDE4A (derived from SF9 cells) by the six compounds CDP840, R-Rolipram, S-Rolipram, RS25344, RP73401 and RS14203 correlated against the inhibition of superantigen-induced human PBMC proliferation (measured after 36 hrs). The correlation between these two parameters was good (Spearman's r = 0.714) and though the result showed a trend towards significance it was not significant, p= 0.136. **Figure 4.6b** shows the relationship of the inhibition of ^{3}H-Rolipram binding to guinea-pig membranes for the same six compounds correlated against the inhibition of superantigen-induced human PBMC proliferation proliferation (measured after 36 hrs). The correlation between these two parameters was good (Spearman's r = 0.927) and the result was statistically significant, p= 0.007. **Figure 4.6c** shows the relationship of the inhibition of PDE4A330 (derived from COS cells) for the six compounds CDP840, R-Rolipram, S-Rolipram, RS25344, RP73401 and RS14203 correlated against the inhibition of superantigen-induced human PBMC proliferation proliferation (measured after 36 hrs). There was no correlation between these two parameters (Spearman's r = 0.143) and the result was not significant, p= 0.801.

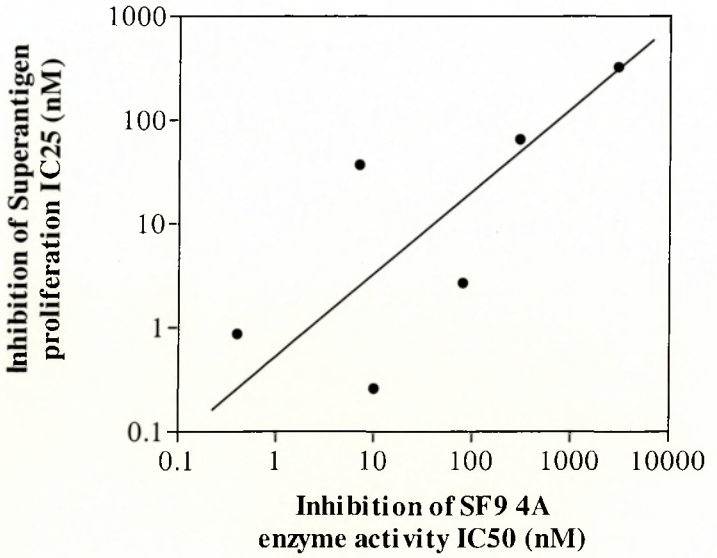


Figure 4.6a: Correlation of inhibition of superantigen induced proliferation and PDE4A enzyme activity by inhibitors of PDE4. PBMC were preincubated with PDE4 inhibitors 30 minutes prior to stimulation with 100pg ml^{-1} of staphylococcal enterotoxins A, B, D and E. Proliferation was measured after 36 h by incorporation of [^{3}H] thymidine. An IC_{25} was taken as not all responses consistently reached 50% inhibition. Independently the compounds were assayed for activity against the PDE4A enzyme derived from SF9 cells. These two data sets were plotted against each other using Prism-3. This graph represents the data from three separate experiments.

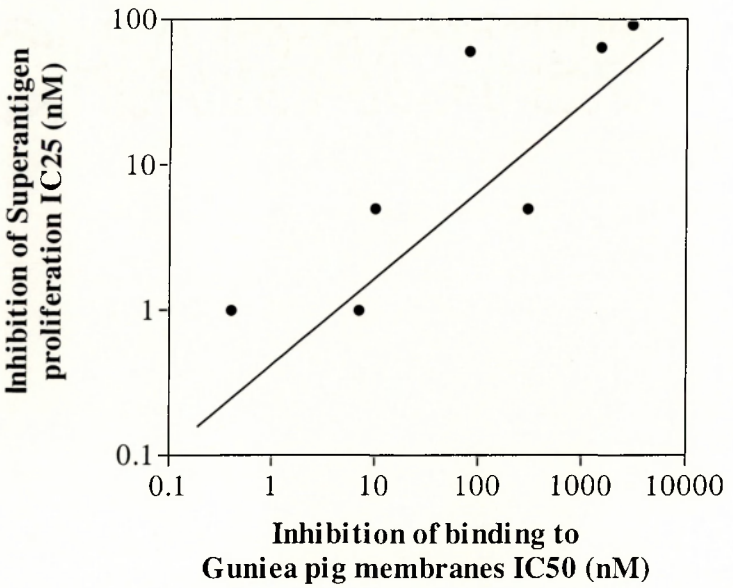


Figure 4.6b: Correlation of inhibition of superantigen induced proliferation and ^{3}H-Rolipram binding to brain membranes by inhibitors of PDE4. PBMC were preincubated with PDE4 inhibitors 30 minutes prior to stimulation with 100pg ml^{-1} of staphylococcal enterotoxins A, B, D and E. Proliferation was measured after 36 h by incorporation of [^{3}H] thymidine. An IC_{25} was taken as not all responses consistently reached 50% inhibition. Independently the compounds were assayed for ability to displace [^{3}H] R-Rolipram from guinea-pig brain membranes. These two data sets were plotted against each other using Prism-3. This graph represents the data from three separate experiments.

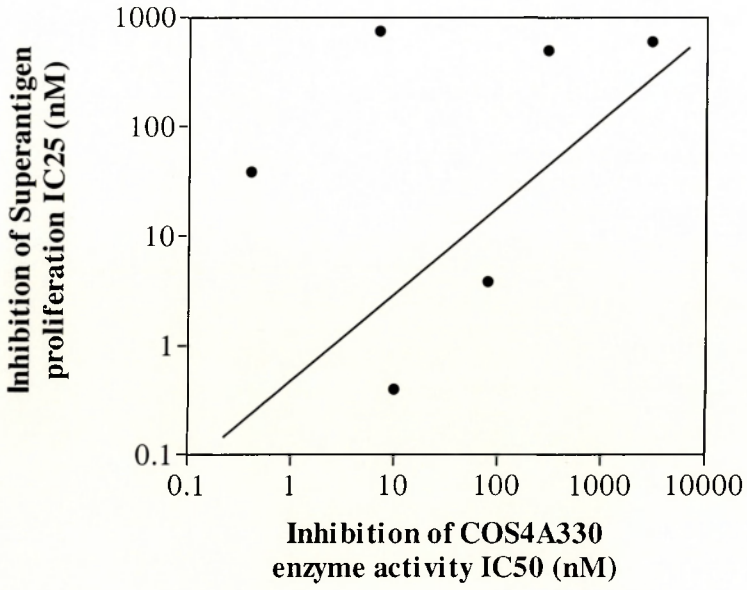


Figure 4.6c: Correlation of inhibition of superantigen induced proliferation and PDE4A330 enzyme activity by inhibitors of PDE4. PBMC were preincubated with PDE4 inhibitors 30 minutes prior to stimulation with 100pg ml^{-1} of staphylococcal enterotoxins A, B, D and E. Proliferation was measured after 36 h by incorporation of [^{3}H] thymidine. An IC_{25} was taken as not all responses consistently reached 50% inhibition. Independently the compounds were assayed for activity against the PDE4A330 truncated enzyme. These two data sets were plotted against each other using Prism-3. This graph represents the data from three separate experiments.

4.3 Discussion

As the results in this chapter indicate, PDE4 inhibitors were able to elevate cAMP levels in mitogen stimulated human PBMC. cAMP levels were still elevated in PDE4 inhibitor-treated cells 24 hours after Con A stimulation but overall cAMP levels were diminished by 86%. This may indicate that inhibition of PDE4 alone is insufficient to maintain cAMP levels as they are in resting cells and may require additional stimulatory signals, for example those delivered via adenylyl cyclase. This phenomenon (a decrease in cAMP following stimulation) is intriguing and may hint at the fact that PDE4 activity dominates over adenylyl cyclase stimulation in activated T cells.

The elevation of cAMP, as a result of inhibition of PDE4 in T cells, was manifest as an inhibition of PBMC proliferation in response to the recall antigen, tetanus toxoid. However, PDE4 inhibitors did not achieve total inhibition of tetanus toxoid induced proliferation. Since research has indicated that Th2 cells may be more susceptible to elevated levels of cyclic

AMP than Th1 cells, this possible effect was investigated [266;267;282]. However, the results for these experiments did not substantiate literature reports as all PDE4 inhibitors tested inhibited tetanus toxoid (Th1 priming) or *Dermataphagoides pteronyssinus* (Th2 priming) primed responses to the same extent.

In this study PDE4 inhibitors were unable to totally inhibit T cell proliferation induced by Con A or superantigen. Inhibition of mitogen-driven PBMC proliferation (or antigen, data not shown), became progressively weaker as time increased following the initial stimulation. This is in contrast to some the reported effects of PDE4 inhibitors [148;176;197;266], though none of these studies used human PBMC. It was postulated that the reduced potency of PDE4 inhibitors may be due to a reduced adenylyl cyclase stimulation. PGE_2 (which stimulates adenylyl cyclase), at a dose which did not interfere with PBMC proliferation, enhanced the potency of PDE4 inhibitors to block proliferation. PGE_2 production is known to be activated in human PBMC [283] but work in this chapter indicates that this may not be sufficient to maintain inhibition of T cell proliferation. Thus a lack of adenylyl cyclase stimulation could explain why PDE4 inhibitors are only transitory blockers of T cell proliferation.

As well as being anti-proliferative, PDE4 inhibitors have also been postulated to block cytokine production, either inhibiting Th1 responses or antagonising Th1 responses by augmenting Th2 responses [266-268;282]. Inhibition of PDE4 function, as exemplified by the inhibitor RP73401, was able to block the production of cytokines from PBMC activated via the T cell receptor. This inhibition of the PDE4 enzyme non-selectively blocked the production of GM-CSF, IL-2, IL-4 and IFN-γ. Unlike other studies [267], inhibition of PDE4 did not selectively enhance or diminish Th-polarising cytokines, in particular IFN-γ and IL-4. This evidence would indicate that, at least in human *in vitro* experimental models, PDE4 inhibitors do not bias Th cytokine expression but are general suppressants of cytokine production.

In contrast to the incomplete inhibition of IL-2, IL-4, IFN-γ and GM-CSF production, PDE4 inhibition completely blocked TNF-α production. This may indicate that TNF-α, produced by T cells stimulated through the TCR, lies on a pathway that is regulated by cAMP and hence PDE4. Further studies will need to establish that this effect on TNF-α production is solely directed at the T cell. These studies are needed as activated T cells, via the production of such factors as IL-17 [284], can induce monocytes to synthesise TNF-α. Regardless, this effect emphasises the potent anti-TNF-α activity of PDE4 inhibitors and reinforces their potential therapeutic benefit in TNF-α-mediated disease.

Inhibition of PDE4, as was the case for other cytokines with exception of TNF-α, did not completely block the production of IL-2. However, unlike the effect on proliferation, inhibition of cytokine production was maintained over the course of the experiment. To address if PDE4 inhibition altered cytokine receptor expression, CD25 levels on activated T cells were also measured. RP73401 only blocked CD25 expression for the first 24 hours following T cell activation. This may indicate that, as well as IL-2 receptor expression, signalling through the IL-2 receptor is unable to be blocked by inhibiting PDE4. Further studies are needed to distinguish the exact T cell signalling pathways that are PDE4 dependent. It is known that activation of certain pathways, e.g. using CD3, CD28 and PMA in combination, can overcome the inhibitory effect that cAMP has on T cell proliferation [285]. Thus it may be possible, using purified T cells, to distinguish PDE-sensitive and-insensitive signalling pathways.

The partial inhibition of T cell proliferation, by PDE4 inhibitors, is at odds with their potent suppressive effect in many other inflammatory cells [147;176;198;273;286]. Many of these effects correlate with inhibition of the enzyme in its low-affinity conformation [147;150]. However, a proportion of the biological effects of PDE4 inhibitors, including many unwanted side-effects, correlate with their ability of inhibitors to compete with Rolipram binding to the PDE4-HARB site [147], but not the enzyme in its low-affinity conformation.

It was discovered that, in contrast to studies that measured the production of IL-2 from activated murine T cells [269], PDE4-mediated inhibition of mitogen stimulated human PBMC proliferation correlated with displacement of ^{3}H-Rolipram binding to PDE4 and not inhibition of the low-affinity enzyme confomer. This was exemplified by a strong correlation with inhibition of superantigen-stimulated human PBMC proliferation and inhibition of ^{3}H-Rolipram binding to brain membranes. In an enzyme which is unable to adopt the high-affinity conformation (PDE4A330) [185], inhibition of proliferation did not correlate with inhibition of the enzyme. This effect is the opposite to that seen when correlating murine T cell cytokine production and may indicate that inhibition of cytokine production and T cell proliferation by PDE4 inhibitors correlates with inhibition of the PDE4 enzyme in its low- and high-affinity conformations respectively.

To summarise, though PDE4 inhibitors block T cell proliferation their effect is transitory. This may be due to both a lack of adenylyl cyclase stimulation and activation of signalling pathways that are insensitive to the inhibition of PDE4. Indeed, inhibition of T cell activity by PDE4 inhibitors does not correlate with inhibition of PDE4 enzyme, in its low-affinity state, but with inhibition of a high-affinity binding pocket on the enzyme, the function of which is unknown. In contrast to the effect on proliferation, inhibition of PDE4 activity does inhibit T cell cytokine release at all stages following T cell activation. This cytokine inhibition shows no Th1/Th2 bias and in particular indicates that PDE4 inhibitors completely inhibit TNF-α production.

Chapters 3 and 4 have discussed the effect of inhibiting PDE4 function in human monocytes (antigen-presenting cells) and T lymphocytes. The conclusions drawn from this work indicate that PDE4 inhibitors are potent blockers of cytokine production, particularly TNF-α. In the remaining chapters of the thesis another class of signal transduction inhibitor will be used in an attempt to block the function of leukocytes. These inhibitors are selective for the Src kinase family of enzymes, members of which play a role in signalling through multi-

subunit receptor complexes. Chapter 5 will concentrate on the consequences of inhibiting Src kinase in human T cells activated through the T cell receptor.

Chapter 5

Evaluation of the effect of inhibition of Src kinase enzymes on human T cell function

Chapter 5

Evaluation of the effect of inhibition of Src kinase enzymes on human T cell function

5.1 Introduction

Immunosuppressive drugs have radically altered the treatment of organ transplantation. The drug cyclosporin A (CsA) , which has proved very effective as an immunosuppressive agent, was the first drug in a class of relatively inexpensive small molecule inhibitors of T cell activation and function. Due to CsA's dose limiting side-effects [155], other immunosuppressive drugs have emerged that have sought to replace it. Many of these are biological inhibitors of T cell function, such as those that block activation of the IL-2 receptor alpha chain [166;287-289] and though effective in certain circumstances, their wide spread use has been limited by their cost. This has stimulated the search for new drugs that can block signal transduction pathways emanating from the T cell receptor.

The Src kinase Lck, as indicated in Chapter 1, plays a key role in mediating signals transduced through the T cell receptor. Lck has a restricted pattern of expression, being mainly expressed in cells of haematopoietic origin [290], and along with another Src family member, Fyn, it plays a critical role in mediating phosphorylation of proteins recruited to the TCR [93;291;292]. Although inhibitors of Lck currently exist (Chapter 1.5.2), they are limited in both their potency and selectivity for Lck over other tyrosine kinase enzymes. The work in this chapter has aimed, by utilising a panel of novel chemical inhibitors of Src family kinases, to assess whether inhibition of selected Src kinases inhibits T cell function in an *in vitro* assay system.

5.2 Results

5.2.1 The effect of Src kinase inhibitors on human PBMC proliferation induced by mitogenic stimuli

A novel series of chemical inhibitors (refer to Chapter 2.2.2) of the Src kinase family of enzymes (abbreviated to CT-SKI) was synthesised by the medicinal chemistry department, Celltech, UK. The selectivity of these compounds for the Src kinase family of enzymes was tested using a panel of *in vitro* enzyme assays. **Table 5.1** shows the IC_{50} values for six Src kinase inhibitors against Lck, Lyn and Fyn (Src kinase family members) as well as ZAP-70, protein kinase C (PKC), epidermal growth factor receptor (EGFR), Csk and cdc2. The known Src kinase inhibitor PP2 [171] was included to act as a reference standard. All six inhibitors tested were potent Lck inhibitors, each having IC_{50} values for the inhibition of Lck of less than 5nM. These inhibitors showed weak activity against ZAP-70 and PKC and an improved selectivity against EGFR and Csk as compared to PP2.

Table 5.1: IC_{50}s of different Src kinase inhibitors for a panel of different kinase enzymes.*

Compound	Lck	Zap-70	PKC	EGFR	Csk	cdc2	Fyn	Lyn
PP2	173	>10000	8900	700	281	ND	ND	51
CT5102	4.0	ND	ND	ND	1166	579	8	ND
CT5215	3.5	>10000	1531	606	330	9036	61	21
CT5263	4.0	ND	3590	631	330	1292	8	ND
CT5264	3.9	9209	1270	2938	126	>10000	24	15
CT5269	1.1	>10000	>10000	3155	86	8463	13	13
CT5276	3.0	ND	2245	795	135	6277	11	ND

* All values are expressed as nM IC_{50}'s. Values are the mean of five separate experiments. ND = not determined.

Figure 5.1 shows the effect of three Src kinase inhibitors, CT5102, CT5125 and CT5269 on human PBMC proliferation induced by OKT3. At a fixed concentration of 400nM, CT5269 inhibited PBMC proliferation by 65% when measured after 72 hours, CT5215 inhibited 30% of PBMC proliferation measured after 72 hours and CT5102 did not inhibit PBMC proliferation at the concentration tested. CT5269, which was the most potent of the three

inhibitors tested, inhibited OKT3 stimulated PBMC proliferation in a dose-dependent manner, **fig 5.2**. The IC_{50} of CT5269 was 130 ± 34nM. The other two inhibitors tested also inhibited OKT3 induced proliferation in dose-dependent manner (data not shown).

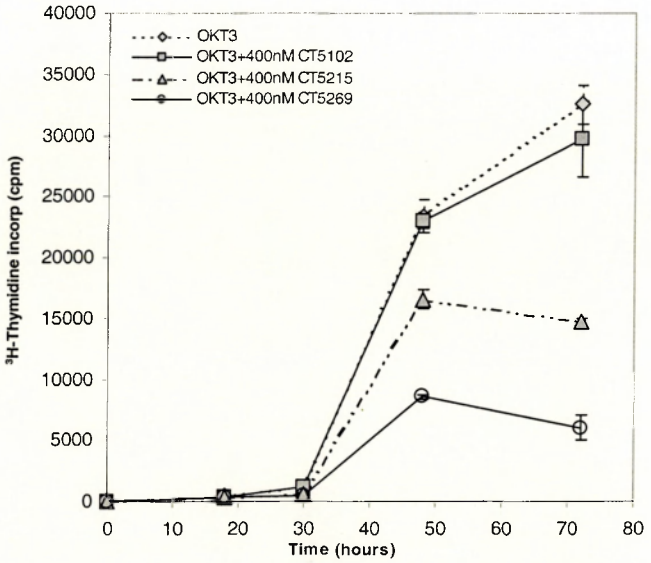


Figure 5.1: The effect of Src kinase inhibitors on the proliferation of human PBMC induced by OKT3. PBMC were pre-treated with 400nM of the Src kinase inhibitors for 30min, prior to stimulation with OKT3 (40ng ml^{-1}). Proliferation was measured at different times after stimulation of PBMC. Values are mean of triplicates ± SD and are representative of three separate experiments.

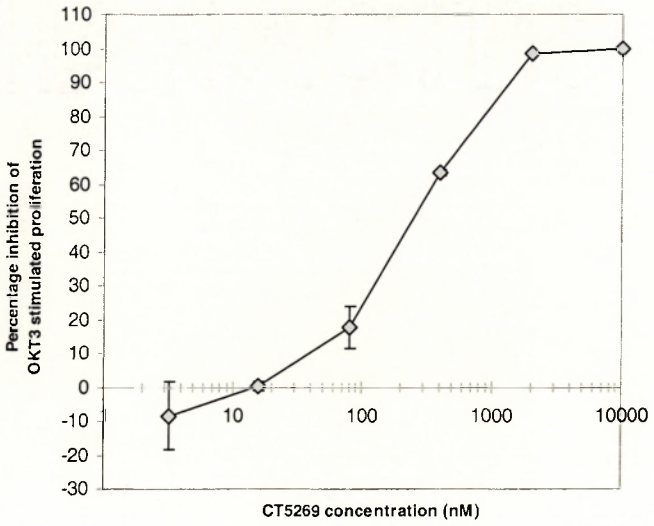


Figure 5.2: The effect of CT5269 on the proliferation of human PBMC activated by OKT3. PBMC were pre-treated with the concentrations of CT5269 indicated for 30min, prior to stimulation with OKT3 (40ng ml^{-1}). Proliferation was measured 48h post-stimulation with OKT3. Values are expressed as a percentage inhibition of OKT3 only stimulated cultures. Values are the mean of three separate experiments ± SEM.

A group of ten related Src kinase inhibitors were tested for their capacity to inhibit OKT3 and MLR induced proliferation of human PBMC. **Figure 5.3** indicates that the compounds blocked OKT3-more potently than MLR-induced proliferation. The mean IC_{50} value for the compounds, was 480nM for OKT3 and 2,000nM for MLR, these differences were statistically significant, $p<0.008$. **Figure 5.4** shows the correlation of the inhibition of Lck (expressed as an IC_{50}) with the inhibition of OKT3 induced PBMC proliferation (expressed as an IC_{50}) and the inhibition of an MLR (expressed as an IC_{50}) for fourteen Src kinase inhibitors. When OKT3 and Lck were correlated, the correlation was good ($r^2=0.853$) and the result was significant ($p<0.0001$). However, when the MLR and Lck were correlated the correlation was poor ($r^2=0.113$) and the result not significant ($p=0.241$).

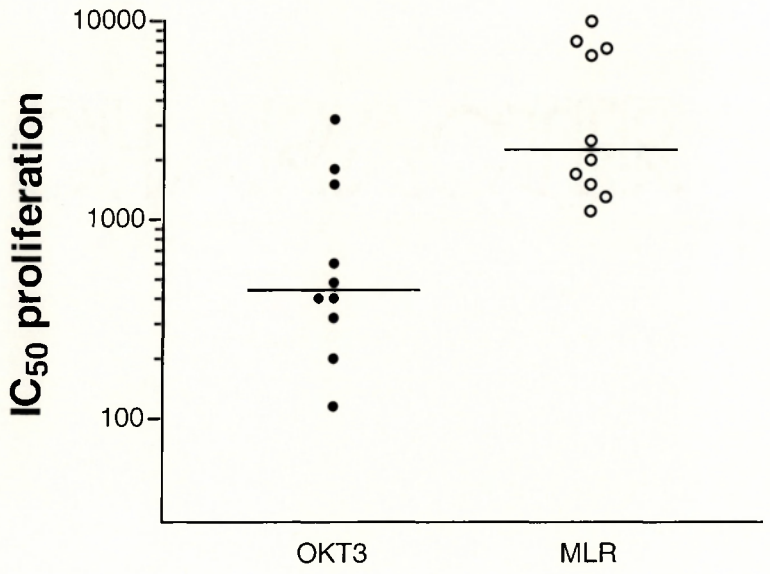


Figure 5.3: The effect of Src kinase inhibitors on the proliferation of human PBMC induced by OKT3 and in a MLR. PBMC were pre-treated with Src kinase inhibitors for 30 min prior to stimulation. Proliferation was measured 48h after stimulation with OKT3 and 5d after stimulation in the MLR. IC_{50} values for each compound were obtained from dose response curves. Values are mean of three separate experiments, errors are not shown. A straight line indicates the mean of all separate points.

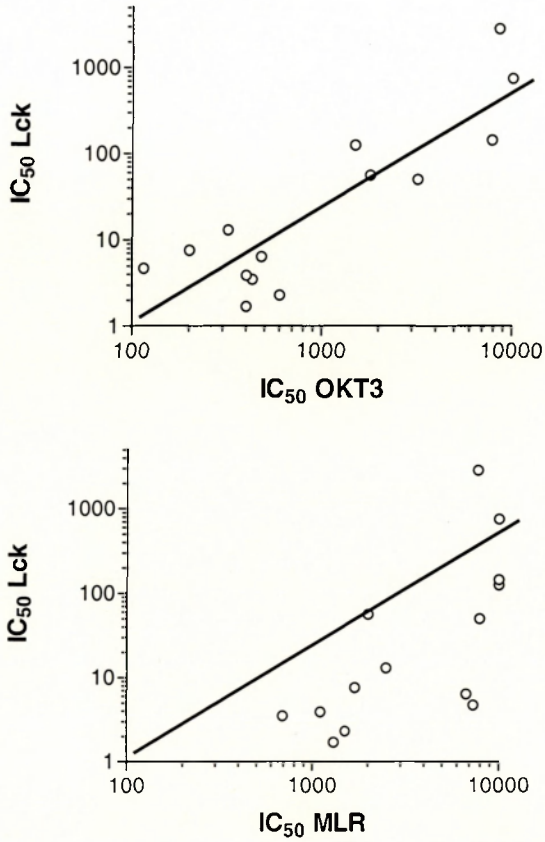


Figure 5.4.: Correlation of inhibition of Src kinase enzyme activity with inhibition of human PBMC proliferation induced by OKT3 and in a MLR. PBMC were pre-treated with Src kinase inhibitors for 30min, prior to stimulation with either OKT3 (40ng ml^{-1}) or an equal number of mismatched irradiated PBMC in the case of the MLR. IC_{50} s for each inhibitor were obtained 48h after stimulation of PBMC with OKT3 and 5d after stimulation in the MLR. Values are the mean of three separate IC_{50} determinations and are plotted without errors.

5.2.2 The effect of the Src kinase inhibitor CT5269 on T cell cytokine production

The Src kinase inhibitor CT5269 was evaluated for its effect on cytokine production from OKT3 stimulated human PBMC. **Figure 5.5a** shows that, at a fixed concentration of 500nM, CT5269 blocked 92.3% of IL-2 produced, as measured by area under the curve. The inhibition of IL-2 production was maintained over the course of the experiment.

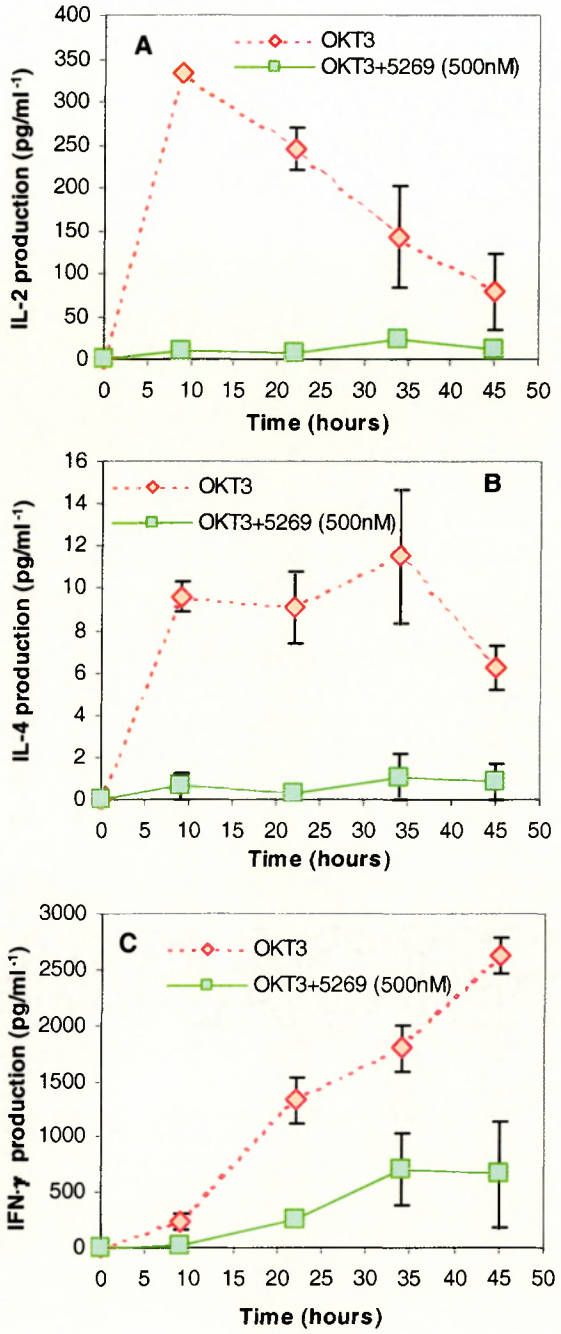


Figure 5.5: The effect of CT5269 on the synthesis of IL-2, IFN-γ and IL-4 by PBMC stimulated with OKT3. PBMC were pre-treated with 500nM of CT5269 for 30 min, prior to stimulation with OKT3 (40ng ml^{-1}). IL-2 (A), IL-4 (B) and IFN-γ (C) were measured in cell free culture supernatants at different times following stimulation with OKT3. Values are the mean of three separate experiments ± SD.

CT5269 was also evaluated for its effect on IL-4 production. **Figure 5.5b** shows that, like the effect on IL-2, CT5269 blocked 93% of IL-4 produced, as measured by area under the curve. Likewise the inhibition of IL-4 was maintained over the course of the experiment.

Finally, CT5269 was evaluated for its effect on IFN-γ production from OKT3 stimulated human PBMC. **Figure 5.5c** shows that CT5269 blocked IFN-γ production by 72%, as measured by area under the curve. This inhibition of IFN-γ production was maintained over the course of the experiment.

To test if inhibition of IL-2, IL-4 and IFN-γ production was dose-dependent, CT5269 was titrated from 20nM to 5μM and the percentage inhibition of cytokine production measured. **Figure 5.6** shows that CT5269 dose-dependently inhibited IL-2, IL-4 and IFN-γ production. The respective IC_{50}'s were 150nM for IL-2 and IL-4 and 300nM for IFN-γ. Although there was a trend suggesting that CT5269 was a weaker inhibitor of IFN-γ production this was not statistically significant, p= 0.879 when comparing IL-2 with IFN-γ.

5.2.3 The effect of Src kinase inhibitors on IL-2 production from human PBMC activated by either phorbol ester plus calcium ionophore or OKT3

A panel of thirty one related Src kinase inhibitors were chosen, which had a wide variation in their potency against isolated Src kinase enzymes. **Figure 5.7** shows the correlation of the inhibition of Lck, (expressed as an IC_{50}) with the inhibition of OKT3 induced IL-2 production (expressed as an IC_{50}), and the inhibition of PMA and ionomycin-induced IL-2 production (expressed as an IC_{50}), for all thirty one compounds. The inhibition of OKT3 induced IL-2 production and Lck showed good correlation (r^2=0.914) which was significant ($p<0.0001$). However, when inhibition of PMA/ionomycin induced IL-2 production and Lck were correlated the correlation was poor (r^2=0.110) and not significant (p=0.08).

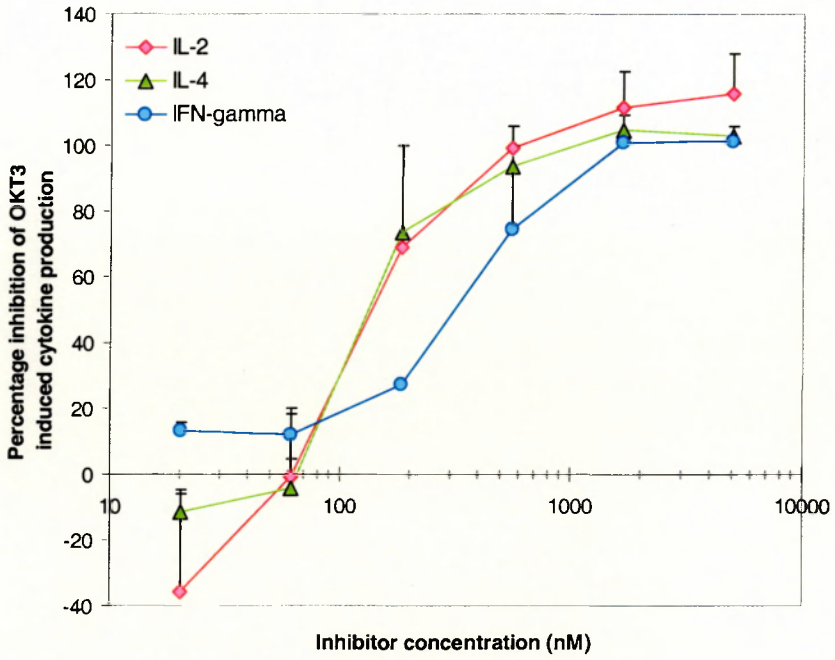


Figure 5.6: Titration of the effect of CT5269 on the synthesis of IL-2, IFN-γ and IL-4 by PBMC Stimulated with OKT3. PBMC were pre-treated with CT5269 at the concentrations indicated for 30 min prior to stimulation with OKT3 (40 ng ml^{-1}). Cytokine levels were measured 48 h after stimulation. Cytokine production was expressed as a percentage inhibition of total cytokine produced from OKT3 stimulated PBMC cultures only. Values are the mean of three separate experiments ± SD.

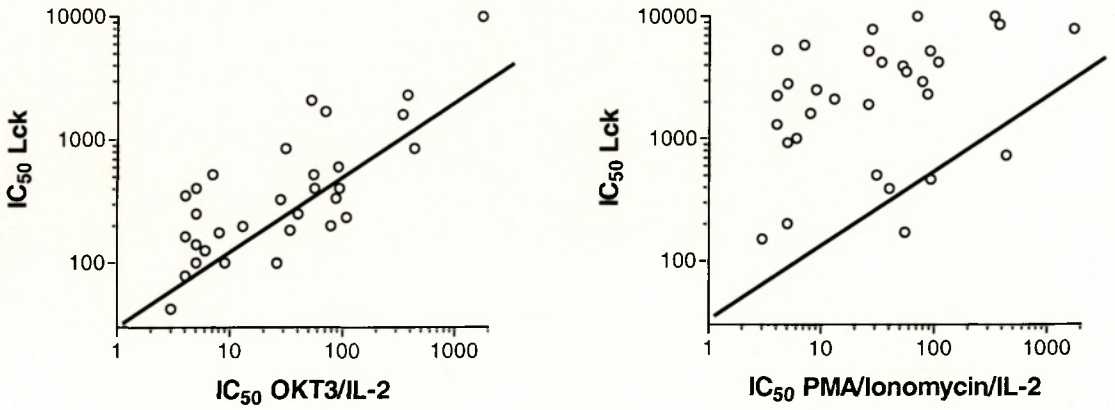


Figure 5.7: Correlation of inhibition of Src kinase enzyme with inhibition of IL-2 production from human PBMC stimulated with OKT3 and PMA/ionomycin. PBMC were treated with the Src kinase inhibitors for 30min, prior to stimulation with either OKT3 (40ng ml^{-1}) or PMA (1 μg ml^{-1}) and ionomycin (10 ng ml^{-1}). The IC_{50}s of each inhibitor were obtained 48h after stimulation of PBMC with either OKT3 or PMA and ionomycin. Values are the mean of three separate IC_{50} determinations and are plotted without errors.

5.2.4 The effect of the Src kinase inhibitor CT5269 on IL-2 receptor expression and IL-2 amplified proliferation in OKT3 stimulated human PBMC

Src kinase inhibitors were tested for their ability to block the expression of CD25 on OKT3 activated human CD3 positive T cells. **Figure 5.8** indicates that Src kinase inhibitors CT5102, CT5264 and CT5276 were effective inhibitors of CD25 expression. Their IC_{50} values being 900nM, 750nm and 400nm, respectively. The inhibition of CD25 expression was seen at all time intervals following OKT3 activation (data not shown).

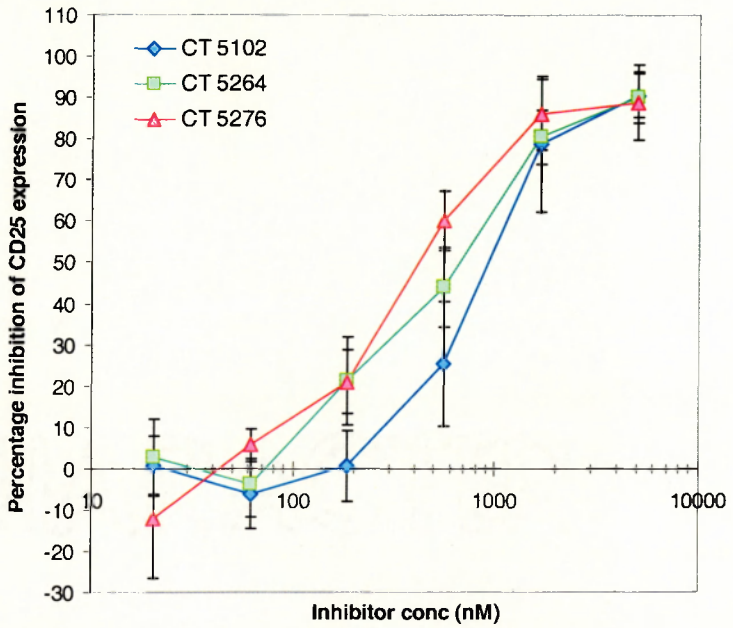


Figure 5.8: The effect of Src kinase inhibitors on the expression of CD25 (IL-2Rα chain) on human PBMC stimulated with OKT3. PBMC were pre-treated with Src kinase inhibitors for 30 min before stimulation with OKT3 (40 ng ml^{-1}). IL-2Rα chain (CD25) expression was measured 48 h after stimulation. CD25 expression was calculated as a percentage of CD25 mean expression levels on $CD3^{HI}$/CD25 double positive cells stimulated with OKT3 alone. Values are the mean of three separate experiments ± SD.

The ability of IL-2, added after TCR engagement, to reverse the inhibitory effect of CT5269 on proliferation was examined. **Figure 5.9** shows the effect of CT5269 (500nM) in the presence of increasing concentrations of hr IL-2. In the absence of IL-2 CT5269 inhibited 58.7 ± 15.3% of OKT3 induced proliferation. As the concentration of IL-2 was increased, the inhibitory effect of CT5269 was reversed. The highest concentration of IL-2 (10ng ml^{-1}) completely reversed the inhibition caused by 500nM of CT5269.

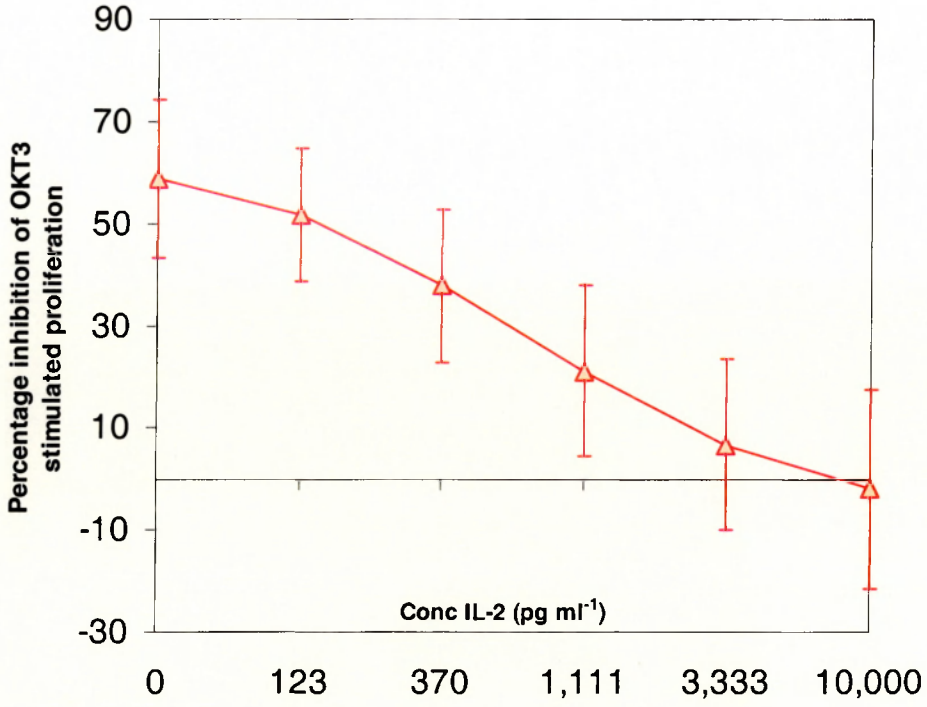


Figure 5.9: The effect of CT5269 on the proliferation of human PBMC induced by OKT3 and the addition of exogenous human recombinant IL-2. PBMC were preincubated with 500nM of CT5269 for 30min, prior to stimulation with OKT3 (40ng ml^{-1}). Four hours after stimulation PBMC were treated by the addition of human recombinant IL-2 at the concentrations indicated. Proliferation was measured 72h after OKT3 stimulation. Where a zero is indicated on the X-axis this refers to the percentage inhibition of CT5629 without the presence of exogenous IL-2. Data is represented as the percentage inhibition of OKT3 stimulated cultures. Values are the mean of three separate experiment ± SEM.

5.3 Discussion

The results in this chapter report the effect of a new class of Src kinase inhibitor on T cell function. These compounds were shown to be more potent than first generation inhibitors such as Genistein and WIN61651 [172;293]. They also improved upon second generation inhibitors, such as pyrazolopyrimidines (PP1 and PP2) [171], which until now were the most potent and specific inhibitors of the Src kinase family of enzymes.

This new class of Src inhibitor (abbreviated to CT-SKI, for Celltech Src kinase inhibitor) was shown to have little activity against other enzymes important to T cell receptor activation (e.g. PKC and ZAP-70) and showed selectivity of inhibition over the regulatory kinase Csk. This was in contrast to PP1/2 which showed equal activity against Src and Csk. This distinction between the two classes of compound may be important, as inhibition of Csk

may counteract any inhibitory effect towards Src kinases, Lck and Fyn, in T cell signalling [100]. It was confirmed that CT-SKI acted specifically to block phosphorylation of Lck and Fyn (auto-phosphorylation) in the T cell line E6.1 stimulated by cross-linking CD3 using an anti-CD3 antibody (data not included). The calcium flux induced in such cells was also blocked [294] these effects correlated with inhibition of isolated Src kinase enzyme (data also not included).

In order to study their effect at the cellular level, CT-SKI were tested in human *in vitro* T cell activation assays. Since T cell clonal expansion (i.e. proliferation) is an important step in the immune response mediated by T cells, CT-SKI were tested for their ability to interfere with this process. When T cells were activated through the T cell receptor, using cross-linking anti-CD3 antibodies, CT-SKI completely blocked cellular proliferation. It was noted, however, that when compared to inhibition of enzyme activity (e.g. Lck) CT-SKI potency was shifted by at least two orders of magnitude. When CT-SKI were tested in other models of T cell activation, such as the MLR, their ability to block proliferation was considerably weaker. This is in contrast to CsA which inhibits with an equal inhibitory potency OKT3-, PHA-and MLR-induced proliferation [295-297]. When the inhibition of proliferation induced by OKT3 and MLR were analysed, inhibition of OKT3-mediated proliferation but not MLR, correlated with inhibition of the activity of the Src enzyme p56Lck. These findings may indicate that separate signalling cascades may be activated by the different stimuli, OKT3 and MLR, whose requirement for Src kinases may differ.

Since proliferation is a multifactorial readout involving the activation and co-ordination of many genes and proteins, an earlier readout of cellular activation was chosen to test the effect of CT-SKI. T cell specific cytokines, such as IL-2, are inducible and highly regulated and also important in the proliferative response. CT-SKI were thus employed to block cytokine production from OKT3-stimulated T cells. CT-SKI were potent inhibitors of cytokine production and their effects were the same regardless of the time point when cytokine production was measured. The CT-SKI, CT5269, at a dose which inhibited

approximately 50% of OKT3 induced proliferation, completely blocked the production of both IL-2 and IL-4. The effect of CT5269 on IFN-γ synthesis was also potent, though it was weaker than on the other two cytokines tested. This may be due to the production of IFN-γ by non-T cells, e.g. NK cells, which may be refractory to the effects of Src kinase inhibitors. These effects were in contrast to those observed with the pyrazolopyrimidine compound, PP1, which acted differentially on Th cytokines, inhibiting IFN-γ production and augmenting IL-4 production [173].

CT-SKI have thus been shown to inhibit proliferation and IL-2 production under the same conditions. Since IL-2 production can act to amplify proliferation it is important to inhibit this pathway, since failing to do so may negate any inhibitory effects on the TCR pathway. To rule out a non-specific suppressive effect on IL-2 production, CT-SKI were tested using alternative stimuli to activate IL-2 production. When PBMC were stimulated with PMA and ionomycin, which are known to activate signalling at the level of PKC and calcium, CT-SKI were unable to block the production of IL-2. Inhibition of PMA/ionomycin induced IL-2 production by CT-SKI did not correlate with inhibition of Src, which is in contrast to inhibition of OTK3-induced IL-2 production which showed good correlation. This evidence would suggest that CT-SKI act to block IL-2 production at a point above PKC/calcium and would be expected if these inhibitors blocked Src kinase activity at the level of the TCR.

To localise the point of action of the inhibitors, assays were set up utilising stimuli that acted at different levels along the signalling cascade. Since CT-SKI were ineffective at inhibiting proliferation in an allogeneic MLR, but inhibited OKT3 stimulated proliferation and IL-2 production, the role of IL-2 receptor signalling was investigated in more detail. IL-2 receptor expression (IL-2 receptor α, β and common γ-chains) is required for full IL-2 signalling, so the effect of CT-SKI on IL-2 receptor α chain was measured. CT-SKI inhibited IL-2 receptor α expression in an equivalent manner to their effect on proliferation. This indicates that IL-2 receptor (α chain) expression may be dependent on active Src enzyme.

The final link in the signal transduction pathway from TCR through production of IL-2 and expression of IL-2 receptor is IL-2 binding and signalling through its receptor. If CT-SKI failed to block this pathway, this may limit their efficacy in blocking IL-2 driven proliferation. There is evidence in the literature that protein tyrosine kinases (PTK) are activated when IL-2 binds its receptor [298-300]. These PTK's include the Src kinase, Lck [301-303]. To test CT-SKI in IL-2 receptor-dependent T cell activation, an IL-2 driven model of proliferation was established. In OKT3 stimulated PBMC, exogenous IL-2 was added four hours after stimulation and its effect on proliferation measured. The addition of IL-2 under such circumstances reversed, in a dose-dependent manner, the inhibitory effect of CT-SKI. This is in accordance with the known role of Lck in IL-2 receptor signalling which is thought to act in parallel with Jak3 (Janus kinase 3)/STAT (signal transducer and activator of transcription), but is not involved in STAT activation [304].

To conclude, CT-SKI appear to be potent inhibitors of mitogen-stimulated T cell activation. However, when other stimuli are used, as in the case of the MLR, they are less potent. This shift in potency may represent the activation of distinct signalling pathways which may be Src-independent. In addition CT-SKI potency shows considerable drift going from the isolated enzyme to cell functional assays. This may be due to a number of factors, such as poor cell penetration, protein binding or more likely high (mM) intracellular ATP concentrations [305]. High ATP concentrations are likely to alter the potency of CT-SKI, as these compounds were discovered to be ATP-competitive (data not shown). In light of these studies, it could be suggested that CT-SKI would not make ideal immunosuppressive agents and Lck is not be a good therapeutic target for immunosuppression. Despite this, their capacity to block T cell cytokine production and proliferation may indicate that they can act in an immunomodulatory role, and further investigation, particularly of their effect *in vivo*, will be needed to verify this function. The final results chapter of this thesis will discuss the effect of inhibiting Src kinase enzymes in mast cells activated through the high-affinity IgE receptor. This chapter will also investigate the role of phosphodiesterase enzymes in IgE-dependent signalling.

Chapter 6

Evaluation of the effect of inhibition of Src kinase and PDE enzymes on mast cell function

Chapter 6

Evaluation of the effect of inhibition of Src kinase and PDE enzymes on mast cell function

6.1 Introduction

Mast cells have long been recognised as critical effector cells of the allergic inflammatory response [26;306]. When they act in an unregulated or dysfunctional manner, they can contribute to the pathogenesis of a number of diseases such as asthma, fibrosis, autoimmunity, neoplasia and inflammatory bowel disease amongst others [10;307 308-319]. Studies investigating the function of human mast cells have relied heavily upon the recovery of cells from cadaverous tissue. However, these cells are particularly heterogeneous in their phenotype and function according to anatomical location [320-322]. Current techniques of purifying mast cells from CD34 positive precursors have yielded large numbers of homogenous cells that have enabled an insight to be gained into both the function and development of these cells [323-325].

As the understanding of mast cell biology has increased, so too has the understanding of the molecular basis of mast cell activation and degranulation. The high-affinity IgE receptor (FcεRI) is the central molecule involved in mast cell activation in allergic inflammation [108;326]. The rapid degranulation of mast cells in response to allergens is mediated by the aggregation of surface bound IgE (bound to FcεRI) by multivalent antigen [108]. The consequence of these actions is ultimately the release and synthesis of histamine, prostaglandins, leukotrienes, proteases and cytokines [132;320;327-331].

Activation of FcεRI receptors on the surface of a mast cell initiates a complex signalling cascade. The Src family kinase Lyn is one protein involved in FcεRI-mediated signalling. Lyn, when activated by the phosphatase CD45, phosphorylates the ITAM's contained within the β and γ chains of the FcεRI receptor, [332] and is a critical controlling step in the activation

of FcεRI [326]. Evidence for the involvement of Lyn in FcεRI signalling comes from animals models in which Lyn activity has been abolished ($Lyn^{-/-}$ mice) [333;334]. Mast cells from these animals have impaired protein tyrosine phosphorylation and calcium mobilisation when activated via the FcεRI. In rat basophilic leukaemia-2H3 (RBL-2H3) cells a kinase inhibitor with specificity for the Src kinase family of enzymes, blocked activation by cross-linked IgE [335]. Other critical tyrosine kinases involved in mast cell signalling include Syk [336], a member of the Zap70 protein family and Bruton's tyrosine kinase which, may be a substrate of Lyn and has both redundant and opposing functions to Lyn in mast cells [333;337].

Another key molecule involved in regulating FcεRI signal transduction is the second messenger cAMP [338]. Elevated intracellular cAMP is a potent suppresser of mast cell degranulation [339]. Stimulation of Gs-coupled receptors [340] or direct stimulation of adenylyl cyclase [341;342] can suppress FcεRI signalling. Phosphodiesterases have been shown to control cAMP levels in mast cells and the pan-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) has been shown to block FcεRI activation [341;342]. However the specific phosphodiesterase thought to control cAMP levels in human mast cells is unknown, as both PDE3 and PDE4 specific inhibitors have no effect upon mast cell degranulation [148].

The aim of the work contained within this chapter was to discover which key intracellular targets, when blocked, could therapeutically modulate the activation of mast cells stimulated through the high-affinity IgE receptor. Using cord blood derived mast cells (as a model of IgE-dependent activation) a unique set of chemical inhibitors were used to probe FcεRI signalling. These inhibitors had selectivity for Src family kinase and phosphodiesterase type 7 (PDE7) and were used to elucidate the role of these proteins in mast cell activation.

6.2 Results

6.2.1 The generation of human mast cells from cord blood mononuclear cell precursors

Human mast cells were derived from cord blood mononuclear cells by growing them in the presence of SCF (Stem cell factor) at a concentration of 4.3nM, IL-6 at a concentration of 2.3nM and PGE_2 at a concentration of 300nM. The method is derived from Saito *et al* [184] and is detailed in chapter 2.3.10. The ability of these cord blood-derived mast cells to proliferate in culture was measured over a 45-day period. **Figure 6.1** indicates that, by day 15, cell number had increased by 177 ± 22%. From day 15 onwards cell numbers started to decline and by day 45 in culture 28 ± 9% of original cell numbers remained.

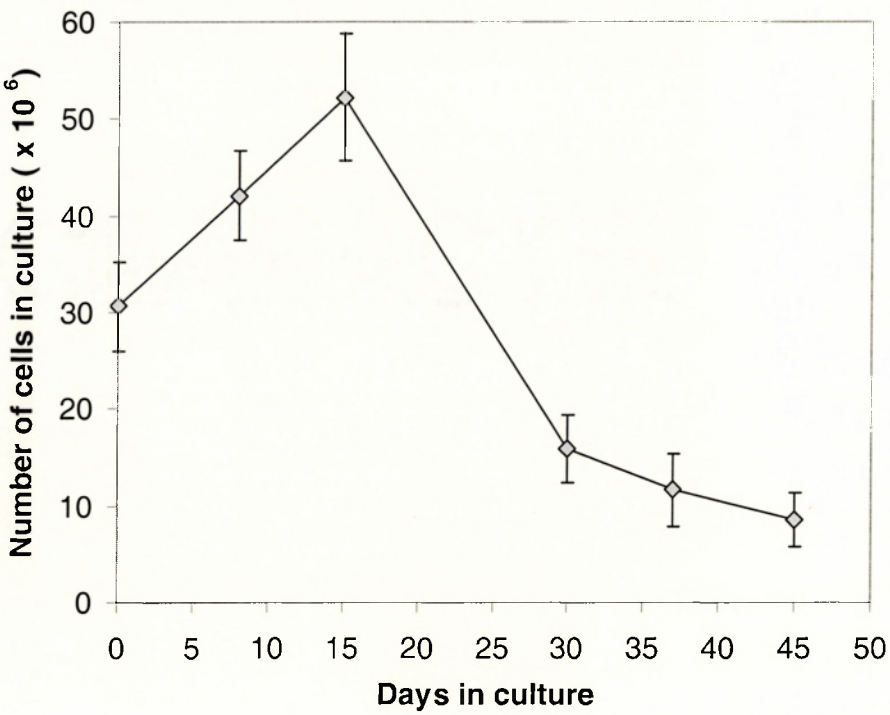


Figure 6.1: The effect of SCF, IL-6 and PGE_2 on cord blood mononuclear cell survival. 30×10^6 Cord blood PBMC were treated with SCF (4.3nM), IL-6 (2.5nM) and PGE_2 (300nM) in weekly doses. Viable cells were counted at the times indicated by their ability to exclude trypan blue. The values shown are the mean of five separate experiments ± SEM.

6.2.2 The phenotypical analysis of human mast cells derived from human cord blood mononuclear cell precursors

Cord blood mononuclear cells cultured with SCF, IL-6 and PGE_2 were monitored using a FACScalibur flow cytometer, **fig 6.2**. Forward light scatter (which was taken as an indicator of cell size) and side light scatter (which was taken as an indictor of cell granularity) were measured by flow cytometry for a period of 14 weeks. These measurements began after 6 days in order to eliminate contaminating cells, e.g. lymphocytes, from the plots. By day 20 the cells had formed into a single agranular population that was termed, immature cord blood-derived mast cells (IcbMC). This population is indicated on the day 27 plot of **fig 6.2** by an oval gate. IcbMC persisted until day 62 as a minor population. By day 34 in culture, the IcbMC population had started to split into another more granular population. These cells were termed cord blood-derived mast cells (cbMC), this population is indicated on the day 62 plot of **fig 6.2** by a circular gate.

These two populations, IcbMC and cbMC, were analysed using flow cytometry for the cell surface expression of a panel of chemokine receptors. **Figure 6.3** shows that IcbMC expressed the CC chemokine receptors CCR2 and CCR5 but did not express the chemokine receptors CCR1, CCR3 and CCR6. Expression of CCR2 and CCR5 progressively increased from day 13 to day 62. Weak expression of CCR6 was observed in some experiments, though this expression was variable.

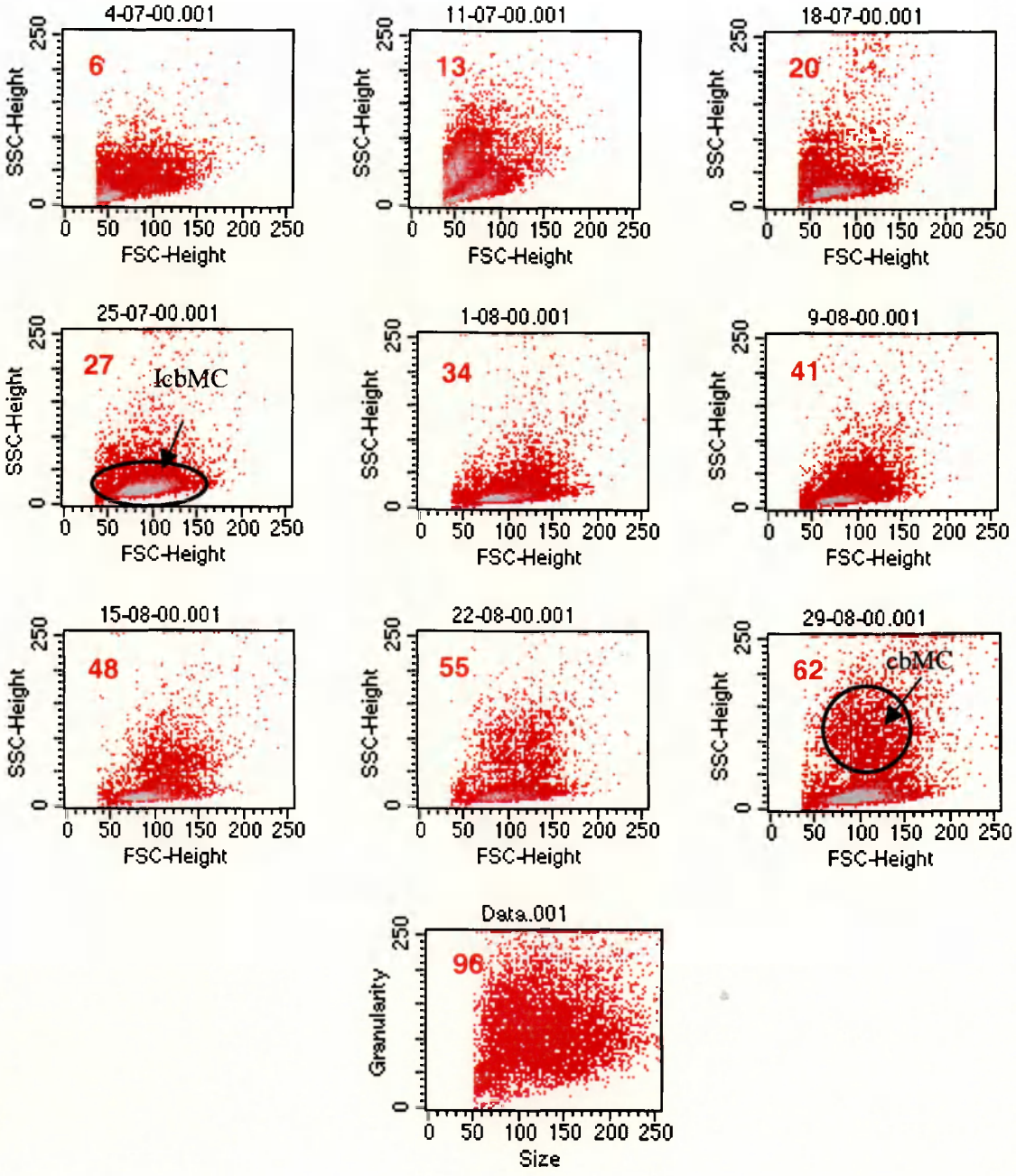


Figure 6.2: The light scatter properties of cord blood mononuclear cells treated with SCF, IL-6 and PGE_2. Cord blood PBMC were treated with SCF (4.3nM), IL-6 (2.5nM) and PGE_2 (300nM) in weekly doses. At the times indicated (day represented in the top left-hand corner of each plot) cells light scattering properties were analysed using flow cytometry. The data was expressed as plots of forward scatter (FSC or size) versus side scatter (SSC or granularity). The plots shown consist of at least 5000 events and the data shown is representative of five separate experiments.

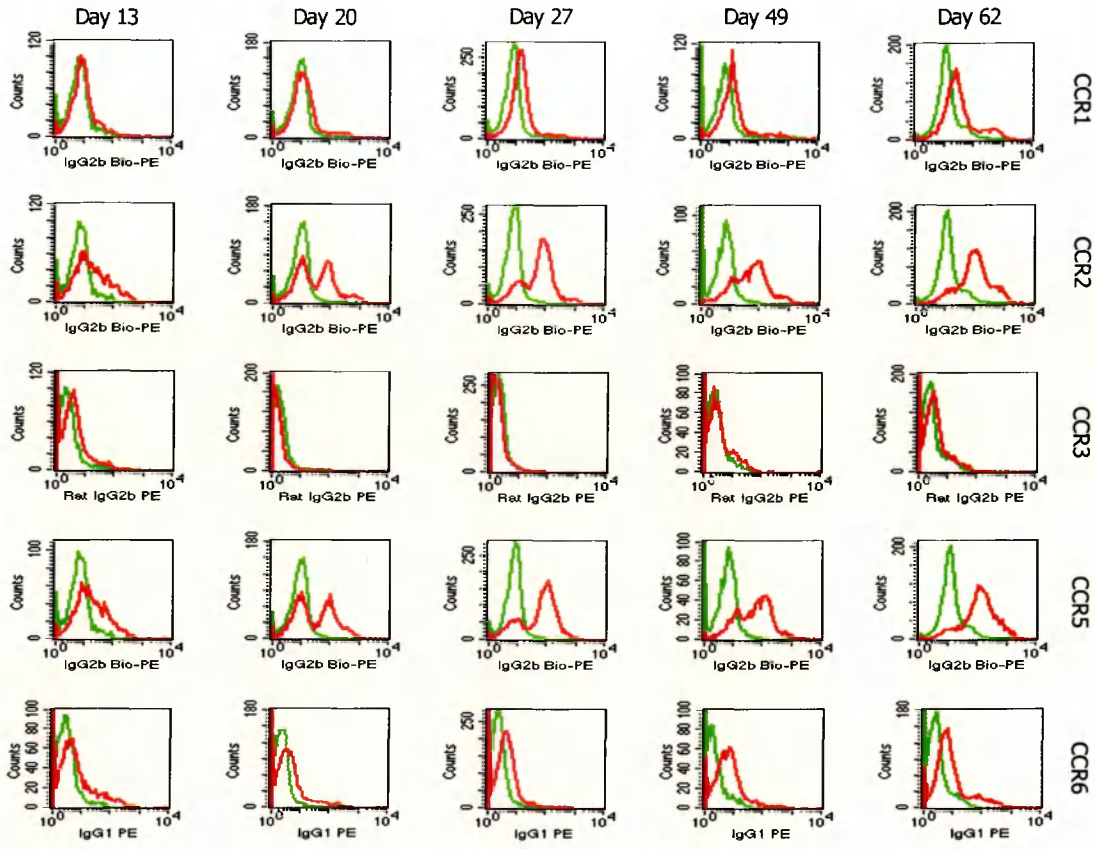


Figure 6.3: The expression of CCR1, CCR2, CCR3, CCR5 and CCR6 on immature cord blood derived human mast cells (IcbMC). Immature cord blood derived mast cells (IcbMC), were defined according to their unique light scattering properties, as analysed using flow cytometry techniques (see figure 6.2). IcbMC were stained with antibodies that recognised human CCR1, CCR2, CCR3, CCR5 and CCR6, represented by a red line. IcbMC were also stained with irrelevant isotype matched antibodies, represented by a green line. Data was plotted as overlaid histograms, each plot consists of at least 3000 events. The data shown is representative of five separate experiments.

IcbMC were also tested for the expression of CXC chemokine receptors. **Figure 6.4** shows that IcbMC expressed the CXC chemokine receptors CXCR2, CXCR3 and CXCR5 but did not express the CXC chemokine receptors CXCR1 and CXCR4. Expression of both CXCR3 and CXCR5, though consistently detectable, was weaker than the expression of the CC chemokine receptors CCR2 and CCR5 upon the same cells. Expression of CXCR3 and CXCR5 progressively increased from day 13 to day 62, in a similar fashion to the expression of CCR2 and CCR5. The expression of CXCR2 on IcbMC was transitory; CXCR2 levels peaked after 27 days in culture and had decreased by 62 days in culture.

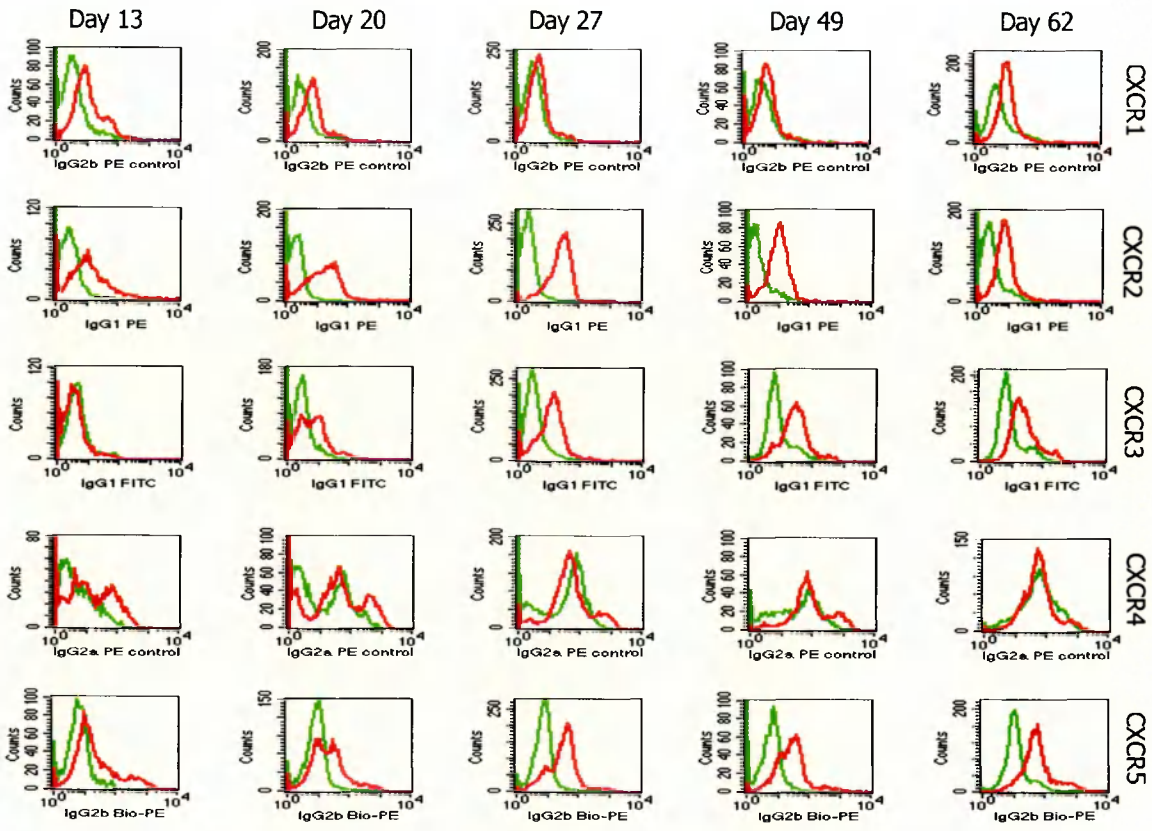


Figure 6.4: The expression of CXCR1, CXCR2, CXCR3, CXCR4 and CXCR5 on immature cord blood derived human mast cells (IcbMC). Immature cord blood derived mast cells (IcbMC), were defined according to their unique light scattering properties, as analysed using flow cytometry techniques (see figure 6.2). IcbMC were stained with antibodies that recognised human CXCR1, CXCR2, CXCR3, CXCR4 and CXCR5, represented by a red line. IcbMC were also stained with irrelevant isotype matched antibodies, represented by a green line. Data was plotted as overlaid histograms, each plot consists of at least 3000 events. The data shown is representative of five separate experiments.

Mature cbMC were also analysed for the expression of CC and CXC chemokine receptors. cbMC were assessed for the expression of chemokine receptors after 49 and 62 days in culture respectively. **Figure 6.5**, shows that cbMC did not express any of the CC chemokine receptors; CCR1, CCR2, CCR3, CCR5 and CCR6 nor did they express any of the CXC chemokine receptors; CXCR1, CXCR2, CXCR3, CXCR4 and CXCR5.

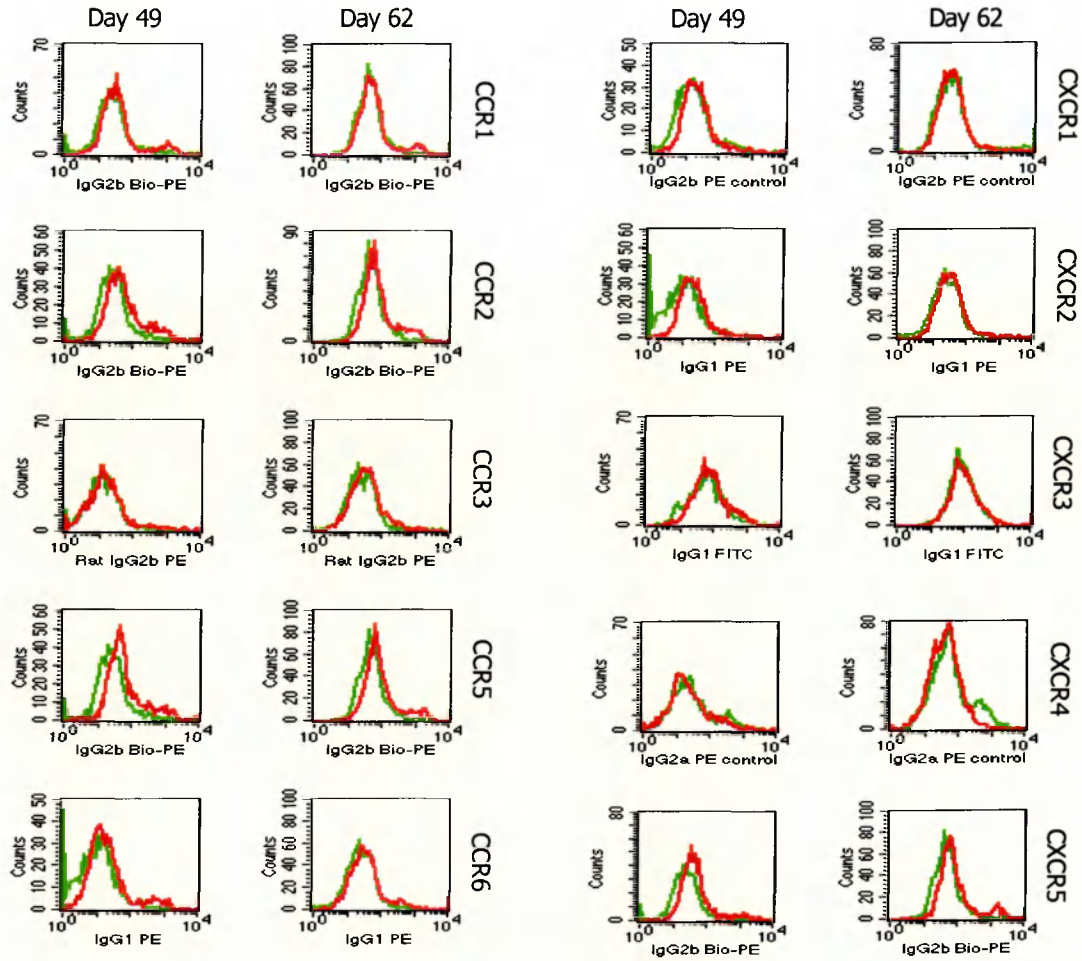


Figure 6.5: The expression of CCR1, CCR2, CCR3, CCR5, CCR6, CXCR1, CXCR2, CXCR3, CXCR4 and CXCR5 on mature cord blood derived mast cells (cbMC). Cord blood derived mast cells (cbMC), were defined according to their unique light scattering properties, as analysed using flow cytometry techniques (see figure 6.2). cbMC were stained with antibodies that recognised human CCR1, CCR2, CCR3, CCR5, CCR6, CXCR1, CXCR2, CXCR3, CXCR4 and CXCR5, represented by a red line. cbMC were also stained with irrelevant isotype matched antibodies, represented by a green line. Data was plotted as overlaid histograms, each plot consists of at least 3000 events. The data shown is representative of five separate experiments.

6.2.3 The functional responses of human mast cells derived from cord blood mononuclear cell precursors

Figure 6.6 indicates that cbMC (as defined in chapter 6.2.2) were found to contain histamine. Cells cultured for at least 70 days with SCF, IL-6 and PGE_2, contained 110 ± 37 ng ml^{-1} of histamine per 10^4 cells (11 ± 3.7 pg ml^{-1} of histamine per cell).

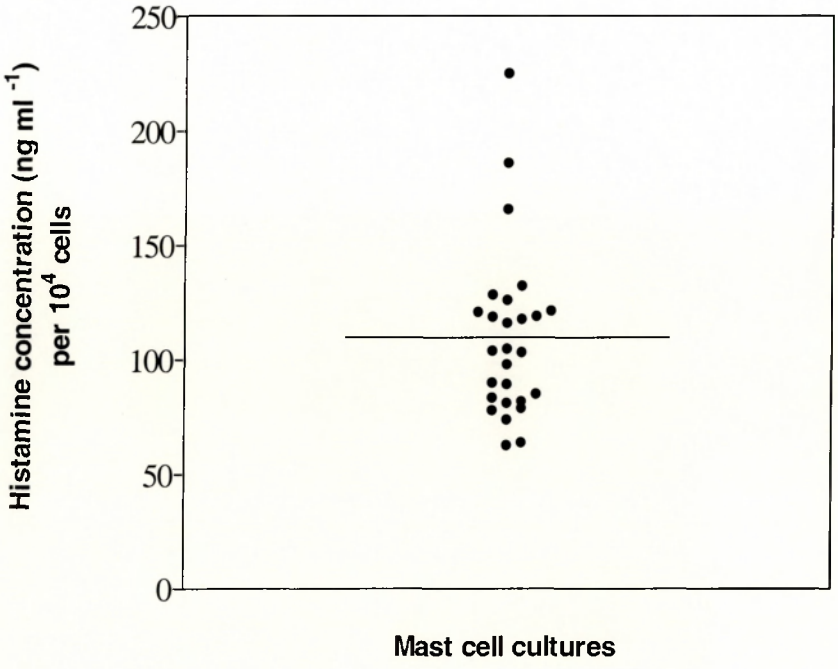


Figure 6.6: The histamine content of cord blood derived human mast cells. 1 x 10^4 cbMC were harvested after 70 days in culture (with SCF/IL-6/PGE_2) and lysed with 0.1% triton X-100. Histamine levels were determined in cell free culture supernatants by EIA. Each point is a mean of four separate values, each point represents a separate experiment. A straight line indicates the mean of all experiments.

cbMC (henceforward 'cbMC' will refer to cord blood mononuclear cells that have been cultured with SCF, IL-6 and PGE_2 for at least 70 days) when challenged with human IgE and an anti-human IgE antibody, released histamine, **fig 6.7a.** The release of histamine was specific and dose-dependent, 1 μg ml $^{-1}$ of IgE protein and 5 μg ml $^{-1}$ of anti-IgE antibody inducing a 6-fold increase in histamine release compared to cells treated with 5 μg ml $^{-1}$ of anti-IgE antibody alone. cbMC also synthesised peptido-leukotrienes when challenged with human IgE and an anti-IgE antibody, **fig 6.7b.** The synthesis of leukotrienes was specific and dose-dependent, 1 μg ml $^{-1}$ of IgE protein and 5 μg ml $^{-1}$ of anti-IgE antibody inducing a 58-fold increase in leukotriene synthesis compared to cells treated with 5 μg ml $^{-1}$ of anti IgE antibody alone.

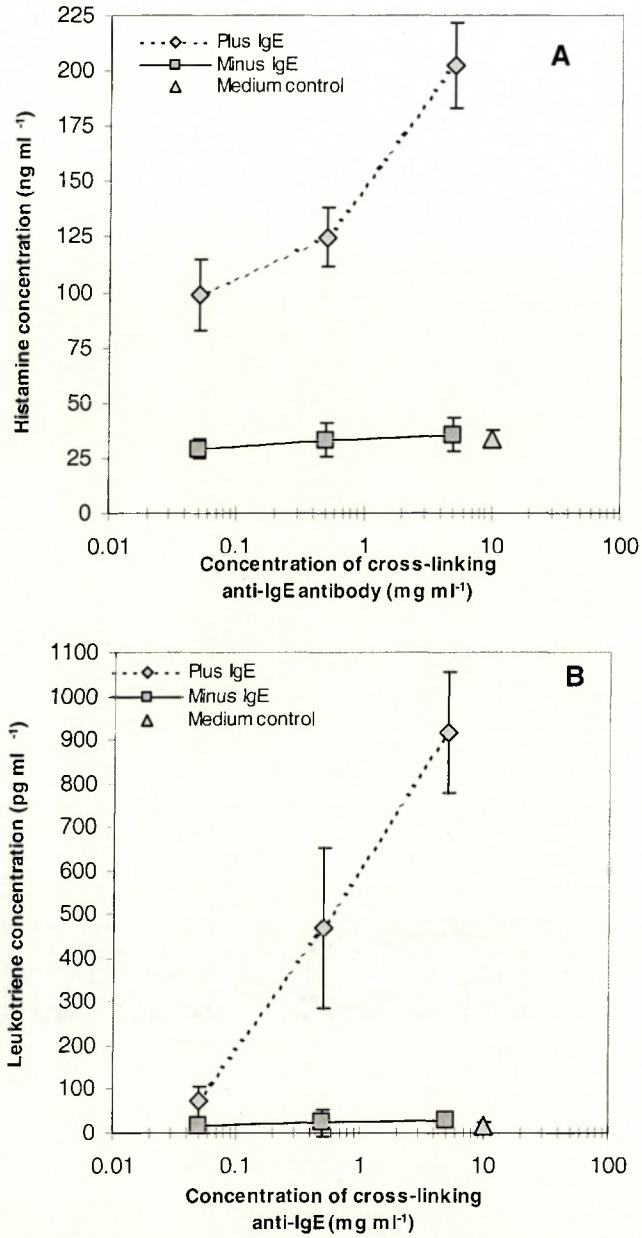


Figure 6.7: Dose-dependent release of histamine and synthesis of peptido-leukotrienes by cord blood derived human mast cells exposed to human IgE and anti-IgE antibodies. cbMC were pre-treated with or without human IgE (1 μg ml $^{-1}$) for 1h prior to stimulation with a mouse anti-human IgE antibody at the concentrations indicated. Histamine (A) and peptido-leukotrienes (B) were measured after 30 min in cell free culture supernatants by EIA. Values are in triplicate ± SD and the experiment is representative of three separate experiments.

Stem cell factor (10 ng ml $^{-1}$) when incubated with cells for one hour prior to culture, augmented the IgE-dependent release of histamine and synthesis of leukotrienes, **fig 6.8.** SCF augmented the release of histamine by 4-fold and leukotriene synthesis by 8 fold. cbMC when stimulated with SCF, IgE and an anti-IgE antibody preferentially synthesised leukotriene C_4 (LTC_4) in preference over prostaglandin D_2 (PGD_2). In three separate experiments the ratio of LTC_4 to PGD_2 varied from 9 to 19 fold, **fig 6.9.**

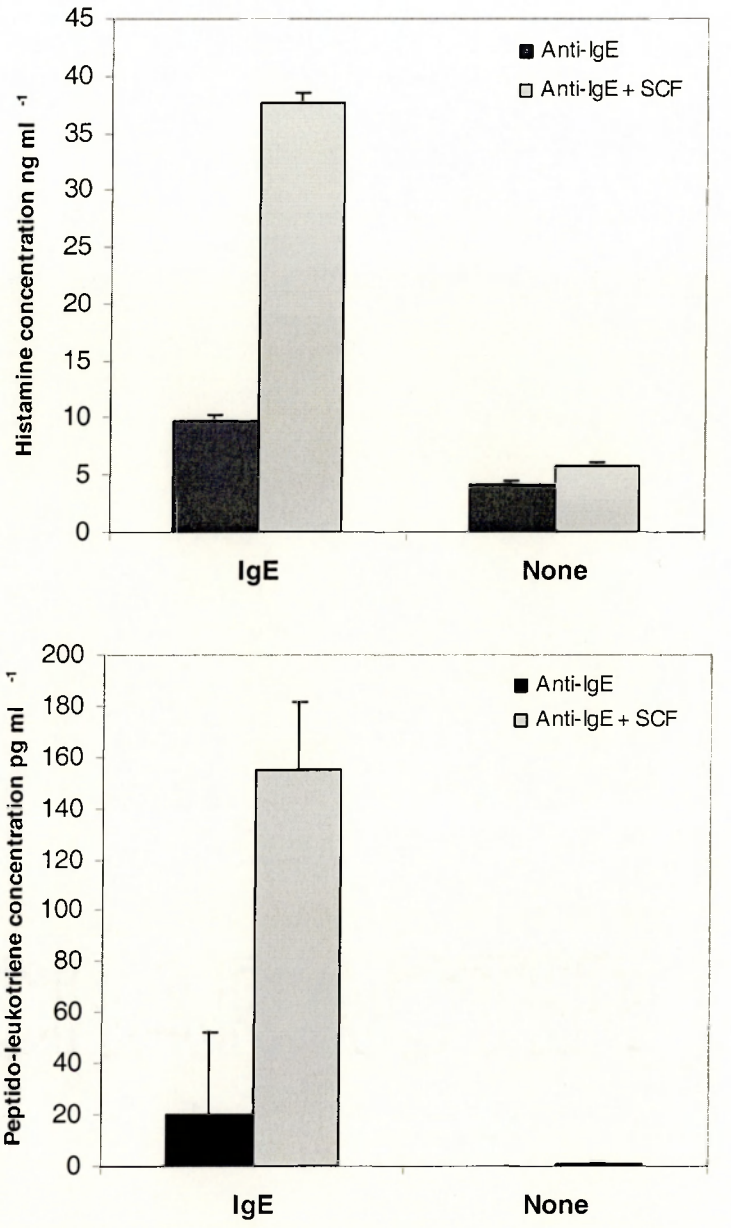


Figure 6.8: The release of histamine and synthesis of peptido-leukotrienes by cord blood derived human mast cells exposed to human IgE, anti-IgE and stem cell factor.
cbMC were pre-treated with or without human IgE (1 μg ml $^{-1}$) for 1h prior to stimulation with a mouse anti-human IgE antibody (5 μg ml $^{-1}$) in the presence or absence of SCF (10 ng ml $^{-1}$). Histamine and peptido-leukotrienes were measured after 30 min in cell free culture supernatants by EIA. Values are in triplicate ± SD and the experiment is representative of three separate experiments

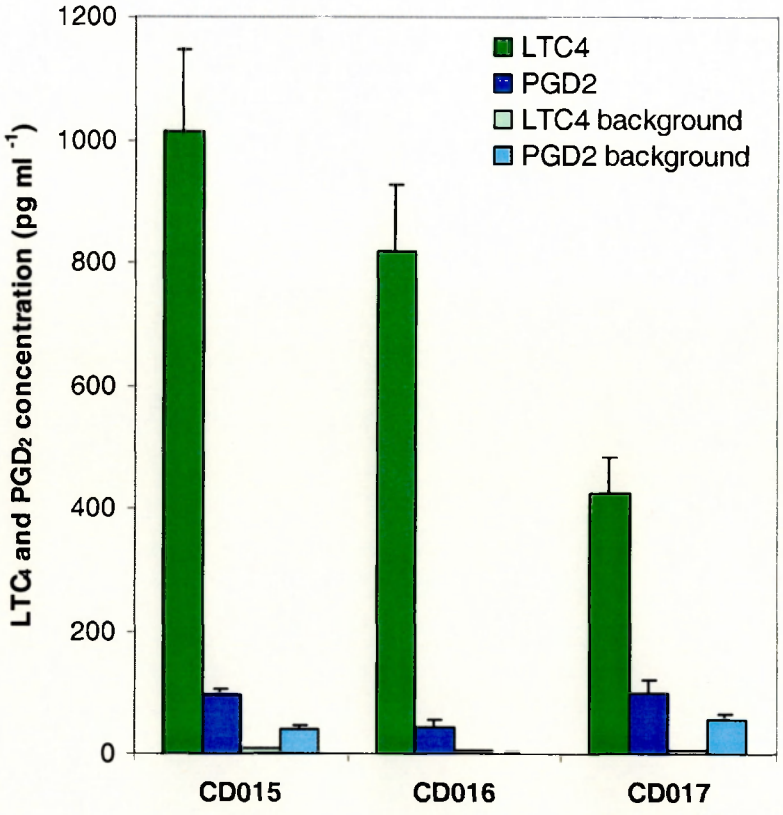


Figure 6.9: The production of LTC_4 and PGD_2 by cord blood derived mast cells stimulated with human IgE, anti-IgE antibodies and SCF. cbMC were pre-treated with human IgE (1 μg ml $^{-1}$) for 1h prior to stimulation with a mouse anti-human IgE antibody (5 μg ml $^{-1}$) and SCF (10 ng ml $^{-1}$). LTC_4 and PGD_2 were measured after 30min in cell free culture supernatants by EIA. Values are in triplicate ± SD. N.B. CD015-CD017 refers to separate cord blood cultures from different donors.

6.2.4 The effect of agents that elevate cAMP on the functional responses of human mast cells derived from cord blood mononuclear cell precursors

Figure 6.10 shows that forskolin (a direct adenylyl cyclase activator) dose-dependently inhibited the IgE-dependent release of histamine from cbMC. 25μM of forskolin inhibited 100% of the specific histamine released. **Figure 6.11** indicates that IBMX did not inhibit IgE-dependent histamine release from cbMC. Even at concentrations up to 500μM IBMX was unable to block histamine release. Two phosphodiesterase inhibitors, Trequinsin [343] (PDE3 selective inhibitor) and RP73401 [344;345](PDE4 specific inhibitor) both failed to inhibit the IgE-dependent release of histamine from cbMC, **fig 6.12.** When Trequinsin and RP73401 were both added to cbMC, they synergistically inhibited the IgE-dependent release of histamine. **Figure 6.13** indicates that 1μM of RP73401 and 10μM of Trequinsin inhibited 44% ± 1% of the specific histamine released from cbMC.

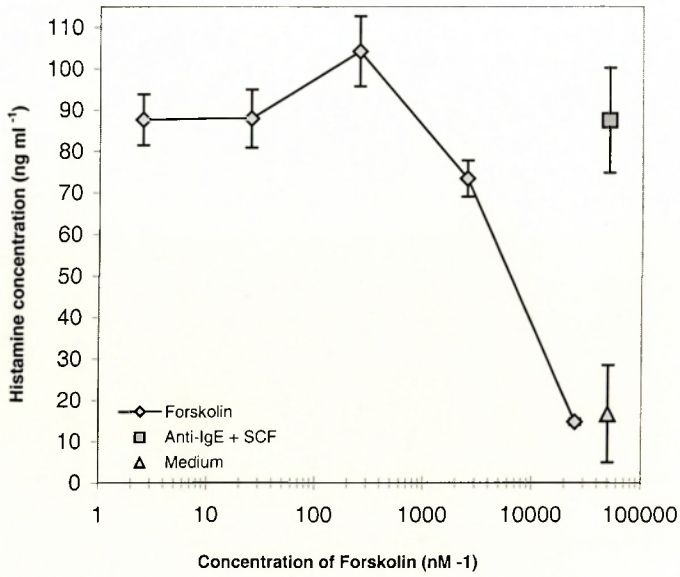


Figure 6.10: The effect of forskolin on the release of histamine from cord blood derived human mast cells exposed to human IgE, anti-IgE antibodies and SCF. cbMC were pre-treated with human IgE (1 μg ml $^{-1}$) and forskolin (at the concentrations indicated) for 1h prior to stimulation with a mouse anti-human IgE antibody (5 μg ml $^{-1}$) and SCF (10 ng ml $^{-1}$). Histamine was measured after 30 min in cell free culture supernatants by EIA. Values are in triplicate ± SD and the experiment is representative of three separate experiments.

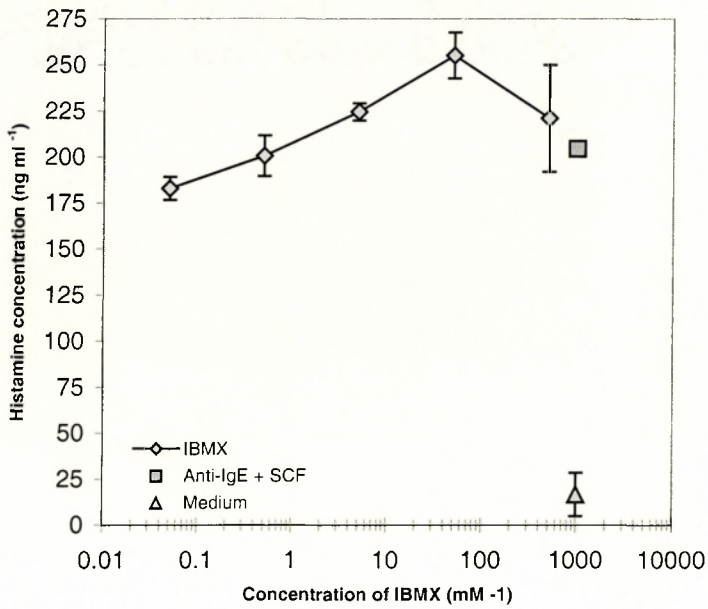


Figure 6.11: The effect of IBMX on the release of histamine from cord blood derived human mast cells exposed to human IgE, anti-IgE antibodies and SCF. cbMC were pre-treated with human IgE (1 μg ml $^{-1}$) and IBMX (at the concentrations indicated) for 1h prior to stimulation with a mouse anti-human IgE antibody (5 μg ml $^{-1}$) and SCF (10 ng ml $^{-1}$). Histamine was measured after 30 min in cell free culture supernatants by EIA. Values are in triplicate ± SD and the experiment is representative of three separate experiments

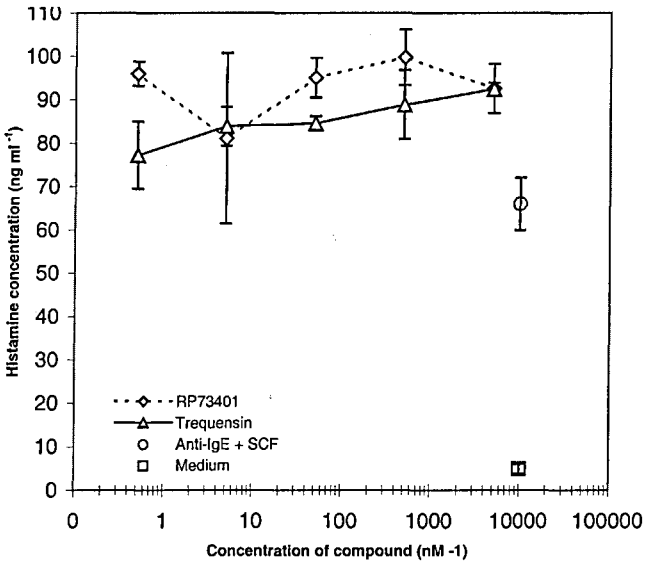


Figure 6.12: The effect of the PDE3 selective inhibitor Trequinsin and the PDE4 specific inhibitor RP73401 on the release of histamine from cord blood derived human mast cells exposed to human IgE, anti-IgE antibodies and SCF. cbMC were pre-treated with human IgE (1 μg ml $^{-1}$) and Trequinsin or RP73401 (at the concentrations indicated) for 1h prior to stimulation with a mouse anti-human IgE antibody (5 μg ml $^{-1}$) and SCF (10 ng ml $^{-1}$). Histamine was measured after 30 min in cell free culture supernatants by EIA. Values are in triplicate ± SD and the experiment is representative of three separate experiments

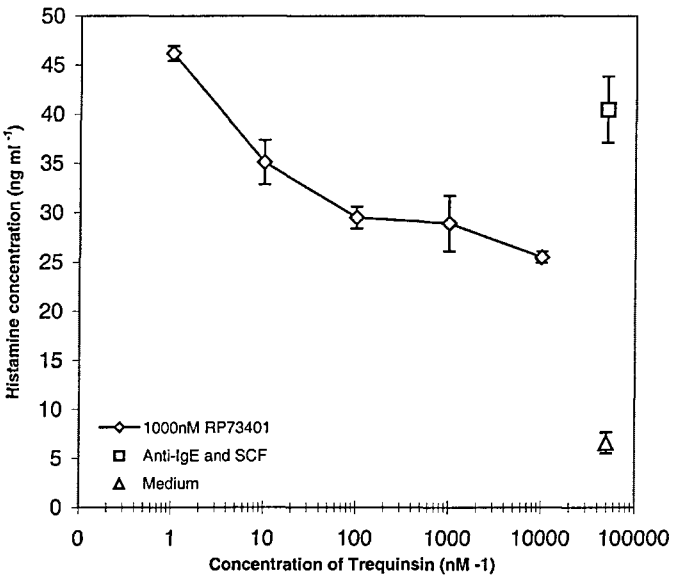


Figure 6.13: The effect of the PDE3 inhibitor Trequinsin in combination with the PDE4 inhibitor RP73401 on the release of histamine from cord blood derived human mast cells exposed to human IgE, anti-IgE antibodies and SCF. cbMC were pre-treated with human IgE (1 μg ml $^{-1}$), RP73401 (1μM) and Trequinsin (at the concentrations indicated) for 1h prior to stimulation with a mouse anti-human IgE antibody (5 μg ml $^{-1}$) and SCF (10 ng ml $^{-1}$). Histamine was measured after 30 min from cell free culture supernatants by EIA. Values are in triplicate ± SD and the experiment is representative of three separate experiments.

6.2.5 The effect of Src kinase and PDE 7 inhibitors on the functional responses of human mast cells derived from cord blood mononuclear cell precursors

A series of structurally similar inhibitors of the enzymes Lyn and/or PDE7, were used in these studies. These compounds; CT5474, CT5733, CT5651, CT5982, CT5227, CT5473, CT6236, CT5215, CT5378 and CT5605 are described in more detail in chapter 2.2.2. **Table 6.1** shows the selectivity for inhibition of the enzymes PDE7, Lck, Lyn, PDE3, PDE4 and PKC by these compounds. This series of compounds contained inhibitors of Lyn, which had no inhibitory activity against PDE7 e.g. CT5227. The series also contained inhibitors of PDE7, that had no inhibitory activity against Lyn e.g. CT5982 and inhibitors of both PDE7 and Lyn e.g. CT5474.

Table 6.1: The IC_{50}s of a series of structurally related compounds for the inhibition of the *in vitro* enzyme activity of PDE7, Lck, Lyn, PDE3, PDE4 and PKC.*

Inhibitors	PDE7	Lck	Lyn	PDE3	PDE4	PKC
CT5215	13,300	ND	21	ND	ND	ND
CT5227	13,300	6	11	13,300	ND	1872
CT5378	ND	ND	5.5	ND	ND	ND
CT5474	15	2.7	2.9	13,300	123	10,000
CT5475	2811	10,000	ND	ND	ND	ND
CT5605	13,300	ND	1273	ND	ND	ND
CT5651	4	165	191	13,300	164	10,000
CT5733	12	708	1287	6500	504	10,000
CT5982	13	10,000	10,000	1100	359	10,000
CT6236	117	13,000	13,000	13,000	13300	ND

* All values are expressed as nM IC_{50}s. Values are the mean of three separate experiments (standard deviations are not shown). ND = not determined.

Eight of the Src kinase and PDE7 inhibitors were tested for their ability to inhibit IgE-dependent histamine release and leukotriene synthesis from cbMC, **table 6.2.** The IC_{50} values for the compounds varied in potency from 38nM to 10μM for inhibition of histamine release and from 10nM to 10μM for the inhibition of leukotriene synthesis. The same eight compounds were also tested for their ability to block histamine release from rat pleural mast

cells that had been stimulated with cross-linking anti-IgE antibodies. **Table 6.2** shows that the IC_{50} values for these compounds ranged in potency from 110nM to 10μM for the inhibition of histamine release.

Table 6.2: IC_{50}s of Src kinase and PDE7 inhibitors for inhibition of histamine/leukotriene production by cbMC and histamine production by rat pleural mast cells.*

Compound	Mean IC_{50} (nM) histamine release (cbMC)	SEM ±	Mean IC_{50} (nM) leukotriene synthesis (cbMC)	SEM ±	Mean IC_{50} (nM) histamine release (Rat)	SEM ±
CT5227	198.7	114.7	190	130.6	350	47.3
CT5378	37.7	21.8	10	/	453.4	153.8
CT5474	82	47.3	125	61.2	110	55.4
CT5475	98. 7	56.9	80	16.3	1883	573.2
CT5651	266.7	154	250	40.8	2353	613.9
CT5733	1267	731.3	725	224.5	5367	1374
CT5982	6000	3464	6500	2857.7	5160	1642
CT6236	10000	5774	10000	0	10000	0

* cbMC were pre-treated with human IgE (1 μg ml $^{-1}$) and the compounds preincubated for 1h prior to stimulation with a mouse anti-human IgE antibody (5 μg ml $^{-1}$) and SCF (10 ng ml $^{-1}$). Histamine and peptido-leukotrienes were measured after 30 min from cell free culture supernatants by EIA. Rat pleural mast cells were pre-treated with rat IgE (2 μg ml $^{-1}$) and the compounds indicated for 1h prior to stimulation with a sheep anti-rat IgE antibody (1 μg ml $^{-1}$). Histamine was measured after 30 min from cell free culture supernatants by EIA. Values are the mean of three separate experiments ± SEM.

The inhibition of histamine release from cbMC by CT5227, CT5474, CT5651, CT5733, CT5982, CT5378 and CT6236 was correlated with inhibition of the Src kinase enzyme Lyn, **fig 6.14**. There was a good correlation between these two inhibitory activities (Spearman's r = 0.964) and the result was significant (p=0.003). **Figure 6.14** also shows the correlation of the inhibition of leukotriene synthesis with inhibition of Lyn for the same panel of compounds. The correlation between these two inhibitory activities was good (Spearman's r = 0.964) and the result was significant (p=0.003).

The inhibition of histamine release from cbMC by the compounds CT5227, CT5474, CT5475, CT5651, CT5733, CT5982, and CT6236 was also correlated with inhibition of the phosphodiesterase enzyme, PDE7, **figure 6.15**. There was no correlation between these two inhibitory activities (Spearman's r = -0.286) and the result was not significant (p=0.556).

Figure 6.15 also shows the correlation of the inhibition of leukotriene synthesis with inhibition of PDE7 for the same panel of compounds. There was no correlation between these two inhibitory activities (Spearman's r = -0.351) and the result was not significant (p=0.444). To investigate if inhibition of PDE4 correlated with inhibition of mast cell degranulation, five compounds that were tested for PDE4 inhibition were correlated with inhibition of mast cell histamine release. There was no correlation between these two activities (Spearman's r =0.700) and the result was not significant p=0.233, (data not shown).

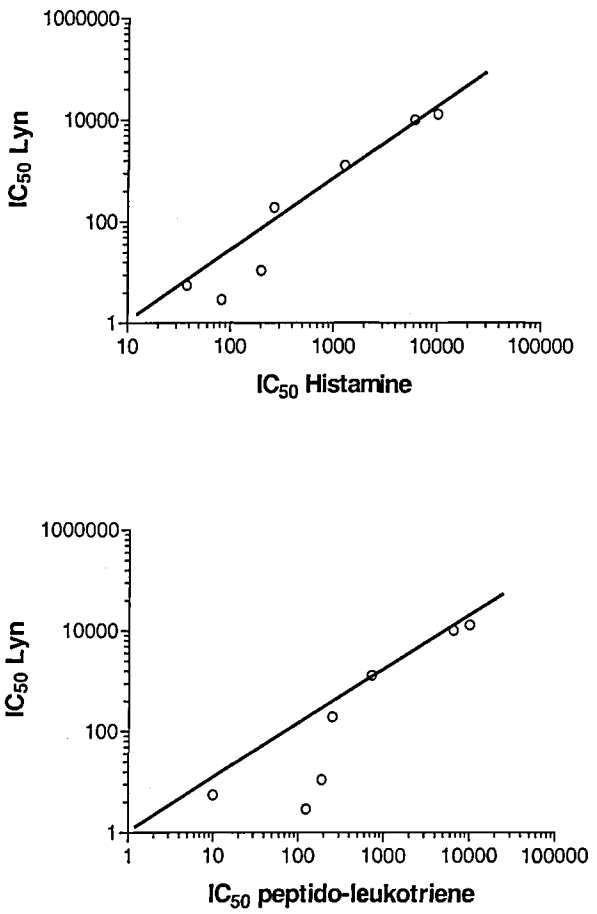


Figure 6.14: Correlation of inhibition of histamine release and leukotriene synthesis from cord blood derived mast cells stimulated with IgE, anti-IgE antibodies and SCF, with inhibition of $p56^{Lyn}$ for a series of structurally related inhibitors. cbMC were pre-treated with human IgE (1 μg ml $^{-1}$) and the compounds; CT5227, CT5474, CT5474, CT5651, CT5733, CT5982, CT5378 and CT6236 for 1h prior to stimulation with a mouse anti-human IgE antibody (5 μg ml $^{-1}$) in the presence of SCF (10 ng ml $^{-1}$). Histamine and peptido-leukotrienes were measured after 30 min from cell free culture supernatants by EIA. IC_{50} values obtained from this data were plotted against IC_{50} values obtained for the inhibition of $p56^{Lyn}$ in *in vitro* enzyme assays. This data is the mean of three separate experiments (errors not shown).

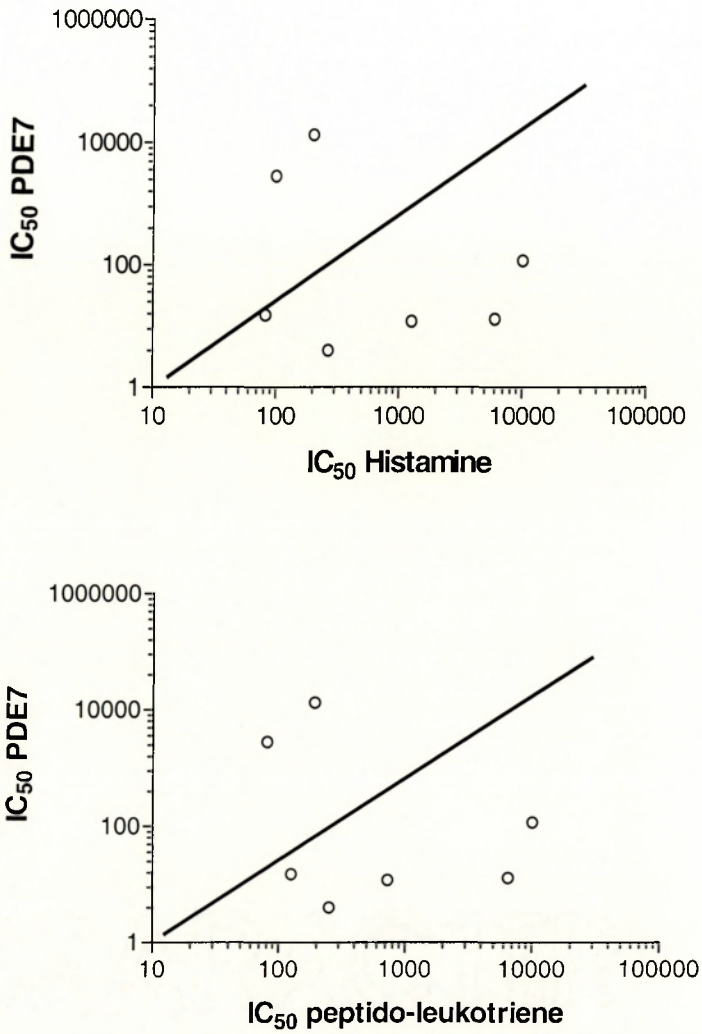


Figure 6.15: Correlation of inhibition of histamine release and leukotriene synthesis from cord blood derived mast cells stimulated with IgE, anti-IgE antibodies and SCF, with inhibition of PDE7 for a series of structurally related inhibitors. cbMC were pre-treated with human IgE (1 μg ml $^{-1}$) and the compounds; CT5227, CT5474, CT5474, CT5651, CT5733, CT5982, CT5378 and CT6236 for 1h prior to stimulation with a mouse anti-human IgE antibody (5 μg ml $^{-1}$) in the presence of SCF (10 ng ml $^{-1}$). Histamine and peptido-leukotrienes were measured after 30 min from cell free culture supernatants by EIA. IC_{50} values obtained from this data were plotted against IC_{50} values obtained for the inhibition of PDE7 in *in vitro* enzyme assays. This data is the mean of three separate experiments (errors not shown).

The inhibition of histamine release from rat pleural mast cells by the compounds CT5227, CT5474, CT5651, CT5733, CT5982, CT5378 and CT6236 was correlated with inhibition of the Src kinase enzyme Lyn, **fig 6.16**. The correlation between these two inhibitory activities was good (Spearman's r = 0.929) and highly significant (p=0.007). The inhibition of histamine release from rat pleural mast cells by the same panel of compounds was also correlated with their inhibition of the phosphodiesterase enzyme PDE7, **fig 6.16**. In contrast

the correlation between these two inhibitory activities was poor (Spearman's r = -0.357) and not significant (p=0.444).

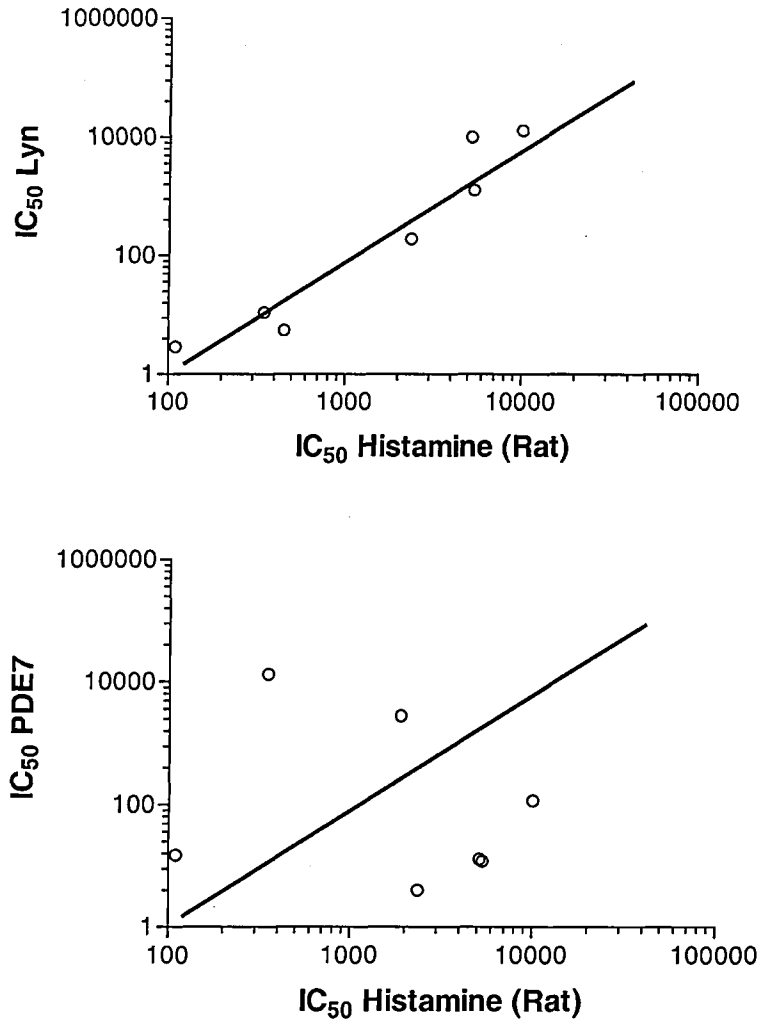


Figure 6.16: Correlation of inhibition of histamine release and leukotriene synthesis from rat pleural mast cells stimulated with rat IgE and anti-rat IgE antibodies, with inhibition of $p56^{Lyn}$ and PDE7 for a series of structurally related inhibitors. Rat pleural mast cells were pre-treated with IgE (2 μg ml $^{-1}$) and the compounds; CT5227, CT5474, CT5651, CT5733, CT5982, CT5378 and CT6236 for 1h prior to stimulation with a sheep anti-rat IgE antibody (1 μg ml $^{-1}$). Histamine levels were measured after 30 min from cell free culture supernatants by EIA. IC_{50} values obtained from this data were plotted against IC_{50} values obtained for the inhibition of $p56^{Lyn}$ and PDE7 in, *in vitro* enzyme assays. This data is the mean of three separate experiments (errors not shown).

The compounds CT5227, CT5733 and CT5982 (which represented potent, intermediate and weak inhibitors of IgE-dependent mast cell degraunlation), were examined for their ability to inhibit both histamine release and peptido-leukotriene synthesis from cbMC stimulated with PMA and ionomycin. None of these compounds inhibited the release of histamine and

synthesis of leukotrienes, with the exception of the compound CT5733, which at a concentration of 10μM inhibited 45% ± 9.5% of leukotriene synthesis, **fig 6.17**.

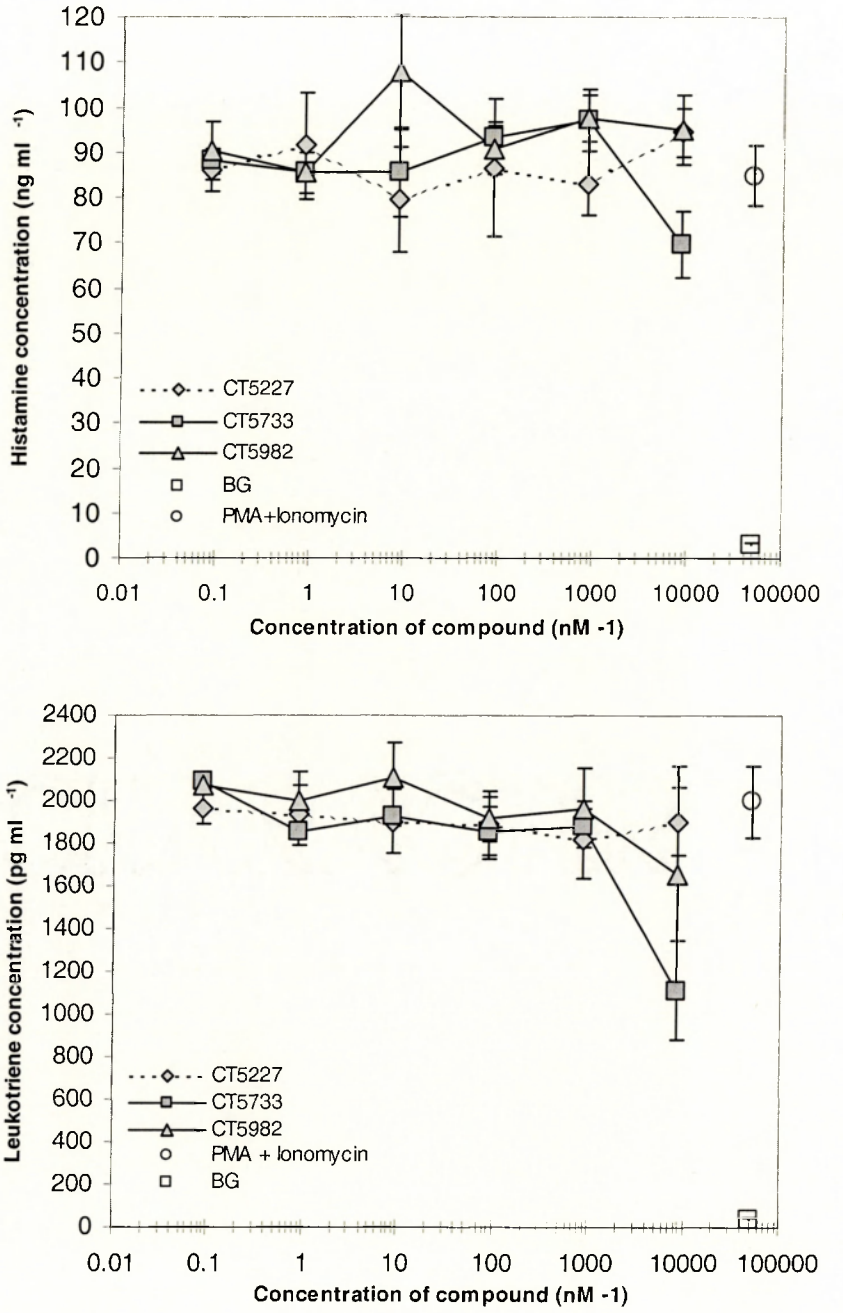


Figure 6.17: The effect of Src/PDE7 inhibitors on histamine release and peptido-leukotriene synthesis from cord blood derived mast cells exposed to PMA and ionomycin. cbMC were incubated with CT5227, CT5733 and CT5982 at the concentrations indicated for 30 minutes prior to stimulation with PMA (1 μg ml $^{-1}$) and ionomycin (10 ng ml $^{-1}$). Histamine and peptido-leukotrienes were measured after 30min in cell free supernatants by EIA. Values are shown in triplicate ± SD and the experiment is representative of three separate experiments.

6.3 Discussion

In order to study the effect of signal transduction inhibitors on mast cell function it was essential to obtain large numbers of purified cells. This need was met by deriving mast cells from CD34 positive precursors harvested from human cord blood. Cord blood-derived mast cells (cbMC) were characterised to ensure they were phenotypically and functionally representative of human mast cells. cbMC proliferated in response to high doses of the cytokines SCF and IL-6 in the presence of PGE_2. cbMC, after this initial period of proliferation formed a distinct agranular population as defined by their light scattering properties using flow cytometetry. This population termed IcbMC divided into another separate and more granular population at around 4 weeks. These granular cells increased in number relative to IcbMC and by week 8 were the dominant population. These cells were deemed mature mast cells and were termed cbMC.

cbMCs were extensively characterised to ensure that they phenotypically and functionally resembled human mast cells. Thirty separate cord blood cultures were used to characterise mast cell responses prior to use in this thesis. cbMC after 10 weeks in culture, contained levels of histamine similar to those found in human lung mast cells and cord blood-derived mast cells described in the literature [184;324;346]. These cells released histamine and synthesised peptido-leukotrienes when challenged with cross-linked human IgE but not IgE alone. In accordance with other studies SCF augmented this response [347;348]. In addition, cbMC also synthesised LTC_4 in preference to PGD_2, which may indicate the up-regulation of the 5-lipoxygenase-synthase pathway [349]. Although cbMC were found to express mast cell tryptase, technical difficulties precluded the measurement of mast cell chymase. However, cbMC did not, using light microscopy, resemble basophils nor did they express any basophil specific markers (data not shown). cbMC, thus behaved like human mast cells with respect to their functional activation using cross-linked IgE as an activating agent.

At the beginning of this project little was known of how mast cell precursors or mature mast cells migrate to the sites of allergic inflammation. To investigate this the expression of

chemokine receptors on differentiating and mature cord blood mast cells was studied. Evidence that mast cells express chemokine receptors has until recently been limited. Human mast cells were found to migrate, but not degranulate, in response to monocyte chemoattractant protein-1 (MCP-1) and RANTES (regulated upon activation normal T cell expressed and secreted) indicating that they express chemokine receptors [350;351]. The mast cell-like cell line HMC-1 expresses functional CXCR1 and 2 [352;353], though there is no evidence for the expression of such receptors on primary cells.

During the course of this thesis a paper by Ochi *et al* [354] supported some of the findings contained within this chapter. Ochi *et al* reported that cord blood derived mast cells express the chemokine receptor CCR3, a chemokine implicated in the pathogenesis of asthma. Subsequently, fully functional CCR3 was found to be expressed on human mast cells that stained positive for tryptase/chymase [355]. The findings of Ochi *et al*, also indicated that immature cord derived mast cells express functionally active C-C chemokine receptors CCR3 and 5 and CXC chemokine receptors CXCR2 and CXCR4.

Mast cells used in this study were also found to express a unique repertoire of chemokine receptors. IcbMC, which were less granular than fully mature mast cells and did not act as functional mast cells (data not shown), expressed the CC chemokine receptors CCR2 and 5 and the CXC chemokine receptors CXCR2, 3 and 5. IcbMC did not express the CC chemokine receptors CCR1, 3, or 6 nor the CXC chemokine receptors, CXCR1 and CXCR4. This pattern of expression differed from that reported by Ochi *et al* [354] who found expression of CCR3, CCR5, CXCR2 and CXCR4 on immature cells. Mature granular mast cells (detected from 5 weeks onwards) did not express the CC chemokine receptors CCR1, 2, 3, 5 and 6 nor did they express the CXC chemokine receptors CXCR1, 2, 3, 4 or 5. These findings are broadly in line with those described by Ochi *et al* [354], though they did find that mature mast cells expressed CCR3, a finding that could not be replicated in the present study.

These findings implicate a wide variety of chemokine receptors in the trafficking of mast cell-committed precursors to sites within the body. The expression of CCR2, CCR5, CXCR2, CXCR3 and CXCR5 receptors on mast cells precursors may mean they can migrate to many different sites within the body. This pattern of receptor expression has not been documented on haematopoetic cells. The lack of chemokine receptors on mature mast cells is contrary to that reported by Ochi *et al*, who found expression of CCR3. These differences in receptor expression may relate to the use of IL-10 in preference to PGE_2 as a differentiating factor. This may have induced the preferential expression of CCR3 on mast cells. For instance, it has been reported that different cytokines such as IL-4, IL-5 and IFN-γ can influence the differentiation of mast cells from their committed precursors [356 323 357]. More studies are needed to determine how these cytokines can influence the chemokine receptor expression on committed mast cell precursors and whether these processes occur *in vivo*.

Elevated levels of cAMP in cultured human mast cells correlate with the inhibition of mediator release and cytokine production [338]. The granular cbMC used in these studies were shown to be sensitive to the elevation of cAMP. The adenylyl cyclase activator, forskolin, inhibited IgE-mediated mast cell activation in accordance with the literature [340-342]. The question of which PDE controls cAMP in mast cells is unclear. Although PDE3 and 4 have been shown to be expressed in mast cells [339;342] the addition of specific PDE3 and PDE4 inhibitors was unable to block IgE-induced histamine release. In addition, IBMX did not inhibit histamine release from cbMC, in contrast to other experimental findings [341;342], but inhibitors of PDE3 and PDE4, when added together, did have a small synergistic inhibitory effect on histamine release at high concentrations. This effect has been observed with human mast cells using the mixed PDE3/4 inhibitor Benzafentrine [148]. This indicates that inhibition of PDE4, currently a therapeutic target for intervention in asthma [198;212;214], does not control FcεRI induced degranulation of mast cells and is, in contrast to FcεRI induced degranulation in other cells such as basophils, PDE4-insensitive [176;342]. This may imply that basophils and cbMC differ in their expression or utilisation of PDEs. These findings, along with reports in the literature, have led to the proposal that a PDE other than PDE3 or 4 must control cAMP

levels in mast cells. Inhibition of this PDE would control mast cell degranulation in response to IgE. One such candidate is PDE7, a cAMP selective PDE, that is thought to play a role in T cell activation [276;358]. Until now no potent and specific PDE7 inhibitors [359] have been available to test whether PDE7 plays a role in mast cells.

Using a series of Celltech inhibitors, the role of both Src kinase and PDE7 could be determined in mast cell activation. This series of inhibitors were potent and specific for Src kinase, Lyn without showing significant activity for PDE3, 4 and PKC (activity against PDE3,4 or PKC may inhibit mast cell degraulation). The inhibition of Lyn (as a representative Src kinase) but not PDE7 was able to control the activation of cbMC stimulated via the FcεRI receptor. Lyn kinase inhibitors controlled the IgE-mediated release of histamine and the *de novo* synthesis of leukotrienes. Under the same conditions low nano-molar inhibitors of PDE7 failed to inhibit the release of histamine and synthesis of leukotrienes. The inhibitor effects were not restricted to cord blood-derived mast cells as inhibition of Lyn, but not PDE7 also correlated with inhibition of histamine release from rat pleural mast cells. The effect was probably not due to a synergistic blockade of PDE3 and 4 as the most potent dual PDE3 and 4 inhibitor, CT5982, was a weak suppresser of mast cell activation. The effects of these inhibitors were confined to signalling pathways upstream of those activated by PMA and ionomycin.

This study has confirmed that Src kinases play an important role in FcεRI signal transduction. Studies using another Src kinase inhibitor, PP1, showed inhibition of markers of cellular activation in RBL (rat basophillic leukaemic cell line) cells activated through the FcεRI receptor [335]. During this thesis it emerged that the dominant role thought to be played by Lyn in FcεRI signalling may be incorrect. Work using Lyn negative mice indicates that Lyn does not play such a central role in FcεRI mediated degranulation [334]. To explain why CT-SKI inhibited FcεRI-dependent mast cell degranulation it may be that as well as inhibiting Lyn, other tyrosine kinases critical to FcεRI signalling are also blocked by these

inhibitors. Two such candidates Syk and Btk may account for the effects seen. Syk activity when absent, as in the case of the Syk deficient cell line (RBL-2H3), completely abolishes FcεRI induced degranulation and cytokine synthesis. When Btk's activity is abolished, together with Lyn, profound inhibition of mast cell degranulation is seen [333]. When Btk alone is absent from mast cells the synthesis of cytokines is impaired [360], but when Lyn alone is absent from mast cells cytokine synthesis can be increased [333]. Studies of the effect of inhibitors on mast cell cytokine production may delineate Lyn- and Btk-selective action of inhibitors.

Future work using these chemical inhibitors will be required to profile them against a panel of critical kinases involved in FcεRI activation. These inhibitors are likely to be pan-specific inhibitors of the Src family of kinases (as indicated by their inhibition of the related Src kinase Lck), whose family members Lyn, c-Src and c-Yes are all activated upon cross-linking the FcεRI [337]. Indeed, these inhibitors may mediate their effects by the concomitant inhibition of Lyn, Src and Yes in the same cells. A full understanding of these inhibitors profile against other key kinases, along with an understanding of their effects on other cellular phenomenon such as calcium mobilisation and cytokine production will be required to fully interpret their mechanism of action and possible therapeutic potential as anti-allergic drugs.

Chapter 7

General discussion

Chapter 7

General discussion

7.1 Introduction

Inflammation under normal circumstances is a protective phenomenon, but can be responsible for an enormous amount of morbidity and mortality worldwide. Infectious diseases, cardiovascular diseases, neuro-degenerative disorders, cancer and the many idiopathic chronic inflammatory conditions (asthma, rheumatoid arthritis etc.) all have inflammatory components which, if they could be blocked, would have therapeutic benefit. Inflammatory diseases have been the subject of study for well over a century and since the end of the second world war there has been an enormous effort made to understand these diseases at levels from the whole animal to the individual protein. More than 50 years of intensive research have made us realise the immense complexity of the inflammatory process and how deeply it is woven into the fabric of the individual. This has been brought into focus by the recent discoveries of the Toll interleukin-1 receptor domain which may be traced back through our genetic history for over 2 billion years [361].

How can one envisage controlling, for therapeutic benefit, this ancient and multifaceted protective mechanism? One hypothesis, that has been the subject of ongoing examination for the past two decades, is that cytokines represent a major control point in inflammation. When this thesis began the hypothesis that inhibition of particular cytokines could be of therapeutic use in controlling inflammation had not been formally tested. However, in the past few years sufficient clinical data has accrued to support the hypothesis that blockade of TNF-α has therapeutic benefit. The methods used to block this cytokine include the use of neutralising chimaeric anti-TNF antibodies [218] and soluble TNF receptor-Fc fusion proteins [134]. These macromolecular medicines are proving efficacious but may prove a mere stopgap before the introduction of small molecule drugs, which will provide the same end result but interfere, with different aspects of the control of the inflammatory process.

It is likely, given that the cytokines appear to make multi-point attachments with their receptors, that small molecule inhibitors of individual cytokines will prove difficult to produce. This leaves the intracellular signalling processes driven by cytokines and the interactions which induce the transcription of pro-inflammatory cytokine genes, as therapeutic targets. In this thesis the hypothesis under test is that blockade of specific intracellular signalling systems can inhibit cytokine synthesis or cytokine-driven patho-mechanisms.

This project has focused on two intracellular signalling enzyme classes which, when inhibited, may regulate the production of cytokines by leukocytes. Potent and selective inhibitors of phosphodiesterases and Src tyrosine kinases have been used to block the activity of these enzymes in leukocytes and in turn used to discover the role these proteins play in regulating cytokine production. Since a detailed discussion has accompanied each chapter, this final section will provide an abbreviated review of the findings of the thesis, comment on their implications and on further work that is indicated by the studies described.

7.2 Inhibition of the PDE4 enzyme

The PDE4 enzyme has a well defined role in regulating cytokine production [149;176;200;269]. Inhibiting PDE4 in monocytic cells greatly suppresses their ability to produce the pro-inflammatory cytokine TNF-α [150]. The ability of PDE4 inhibitors to block TNF-α production is thought to involve the elevation of intracellular cAMP. This method of suppressing TNF-α has been shown to be dependent upon elevating the level of the anti-inflammatory cytokine IL-10 [203], as IL-10 is a natural regulator of TNF-α production [234]. However, studies using PDE4 inhibitors have indicated that blockade of TNF-α can be both-dependent or-independent of IL-10 production [151;205;241] and has led to confusion over the exact way in which PDE4 inhibitors regulate monocyte synthesis of TNF.

Chapter 3 of this thesis supports the hypothesis that the suppression of monocyte TNF-α production by PDE4 inhibitors is not due to the elevation of IL-10 synthesis. To explain why conflicting reports suggested both IL-10-dependent and-independent mechanisms of TNF-α inhibition, it was proposed that studies comparing the effects of PDE4 inhibition on murine and human monocytic cells may not have taken into account the heterogeneous nature of monocytes when interpreting data.

In order to understand why different results using PDE4 inhibitors have been obtained it was necessary to study the effect of inhibiting PDE4 on cytokines other than TNF-α. It was discovered that inhibition of PDE4, in LPS activated human PBMC, resulted in a distinct pattern of cytokine inhibition as shown in **table 7.1.**

Table 7.1: PDE4-mediated inhibition of LPS induced human PBMC cytokine production. *

Cytokine	PDE4 inhibitor	hr IL-10
TNF-α	++++	++++
IL-1α	+++	ND
IL-1β	++	+++
IL-6	-	-
IL-8	-	ND

* ND, indicates measurements not determined. + indicates inhibition and – indicates no effect. hrIL-10 indicates the effect of 1ng ml^{-1} of hrIL-10 on LPS induced cytokine production.

As can be seen from the table PDE4 inhibitors mediated strong suppressive effects on both TNF and IL-1, an effect similar to the addition of exogenous IL-10 to LPS stimulated cultures. An interesting finding was the discovery that IL-1α was inhibited to a greater extent than IL-β. Both IL-1α and IL-1β are synthesised as precursors and secretion of mature protein is dependent upon calpain and caspase-1, respectively [222;228]. Apart from the requirement of these enzymes, the secretion pathways that lead to the release of IL-1α and IL-1β remains largely unknown. It may be that these pathways interact with the PDE4 enzyme differently. Future experiments will need to dissect apart these two pathways and

measure, for instance, the effect of inhibiting PDE4 activity on calpain and caspase-1 activity. Also, inhibition of PDE4 did not result in the inhibition of IL-6 or IL-8 *in vitro*. This suggests that neither IL-6 nor IL-8 lie on a pathway that is regulated by PDE4.

Using neutralising anti-IL-10 receptor antibodies, it was determined that PDE4 inhibitors did not mediate the inhibition of TNF via the production of IL-10. Indeed PDE4 inhibitors were found to suppress IL-10 production when measured early on after LPS stimulation. This could conceivably be due to the feedback mechanisms between TNF and IL-10 as it is known that when TNF production is suppressed, it can counteract the signals that induce IL-10 expression [239].

Monocytes are heterogeneous cells and the relative expression of two surface proteins, CD14 and CD16, has proved useful in determining different monocyte subsets [249]. The expression of CD16 on CD14 positive monocytes and macrophages correlates with downregulation of the expression of both IL-10 and PDE4 [194;249]. It was discovered that both activating stimuli and PDE4 inhibitors can alter monocyte phenotype, **fig 7.1**.

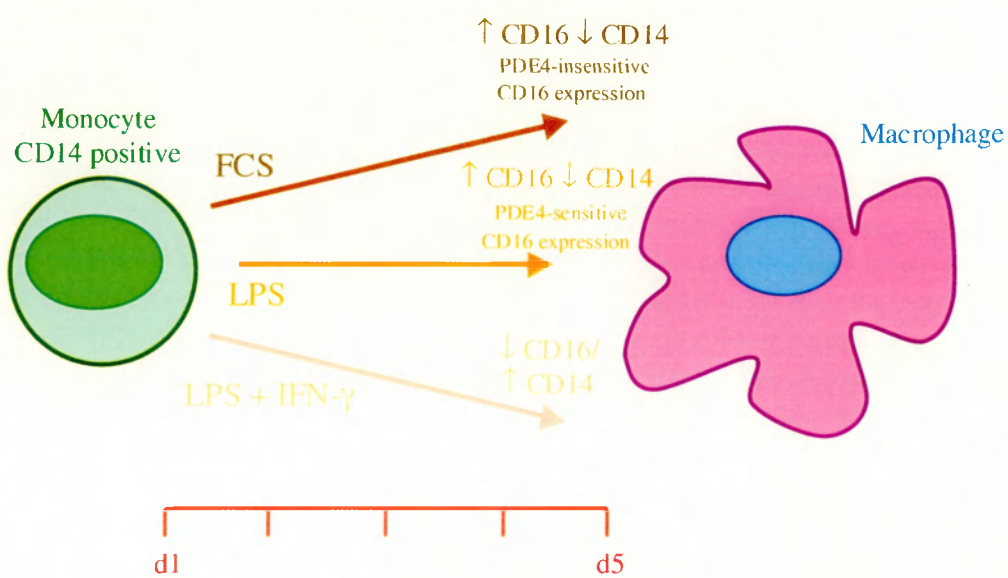


Figure 7.1: The effect of different stimuli on monocyte/macrophage maturation. FCS and to a lesser extent LPS upregulate the expression of CD16 on macrophages as they mature from human peripheral blood precursors. The expression of CD16 on LPS but not FCS stimulated macrophages was blocked by inhibiting the enzyme PDE4. In contrast, LPS plus IFN-γ suppressed the expression of CD16 and increased the expression of CD14 on macrophages.

Monocytes stimulated with LPS or LPS plus IFN-γ, when compared, showed altered regulation of IL-10 by PDE4 inhibitors, **table 7.2.**

Table 7.2: Comparison of PDE4-mediated inhibition of LPS and LPS plus IFN-γ induced human PBMC cytokine production. *

Cytokine	LPS	LPS plus IFN-γ
TNF-α	++++	+++
IL-1α	+++	ND
IL-1β	++	ND
IL-6	-	ND
IL-8	-	ND
IL-10	not detected	↑↑
IL-12	ND	++++

* ND, indicates measurements not determined. + indicates inhibition, – indicates no effect and ↑ indicates stimulation.

This table indicates that PDE4 inhibitors show altered regulation of the key inflammatory cytokine IL-10 when stimulated with LPS with and without IFN-γ. It was noted that PDE4 inhibitors increased IL-10 production only in PBMC stimulated with LPS plus IFN-γ. However despite elevating IL-10, PDE4-mediated inhibition of both TNF-α and IL-12 was found to be independent of IL-10 production. This may mean that PDE4-mediated inhibition of TNF-α is due to a direct effect on TNF-α production, which may for instance be due to regulation of TNF-α at a transcriptional level [149].

At the beginning of this thesis it was assumed by many that PDE4 inhibitors clearly elevated cAMP in monocytes and so, like other agents that elevate cAMP, inhibited TNF-α production via an increase in IL-10. In consideration of this, early attempts during this thesis were made to show that PDE4 inhibitors could augment LPS induced IL-10 production. This hypothesis proved unworkable. To reconcile these findings with studies that clearly showed that PDE4 inhibitors elevated IL-10 [151;205] it was proposed that the phenotype of the monocytes used in these studies could account for the different effects of PDE4 inhibitors on IL-10 synthesis. Since it was known that CD16 positive macrophages differ compared to

CD16 negative macrophages in their regulation of cytokines and phosphodiesterases, factors that alter CD16 expression could alter the effect of PDE4 inhibitors upon cytokine production. Indeed it was discovered that in CD16 negative monocytes (stimulated with LPS plus IFN-γ) PDE4 inhibitors could upregulate IL-10 production. Further studies are required to examine if CD16 negative cells stimulated with LPS alone also regulate IL-10 in the same way as cells stimulated with LPS plus IFN-γ. It would also be of interest to determine if cells from different species and anatomical sites, that are both CD16 positive and CD16 negative, regulate IL-10 the same way in response to PDE4 inhibitors. To know why PDE4 inhibitors act differently in different monocyte subsets, apart from the possible dysregulation of PDE4 protein expression, it is important to know if PDE4 inhibitors block interactions other than the hydrolysis of cAMP. For instance, it is known that PDE4 interacts with important signalling enzymes such as MAP kinase [362;363] and Src kinase [364] but it is not known if these interactions are functionally relevant with respect to the signalling cascade nor if PDE4 inhibitors can disrupt such interactions.

7.3 Inhibition of PDE4 in T cells

The work reported in chapter 4 strongly implicates PDE4 inhibitors as being antagonistic to Th1 mediated inflammation, exemplified by their inhibition of pro-inflammatory cytokine production by activated monocytes. In addition, it was shown that PDE4 inhibitors were equally effective at counteracting Th2 driven immune responses [158]. These effects may, in part, be due to the action on cells such as eosinophils [273;365] which may be critical to the pathogenesis of particular disease models. However, it is unclear whether PDE4 inhibitors differentially effect either Th1 or Th2 cytokine production [263;282]. In chapter 4 of this thesis it was hypothesised that PDE4 inhibitors have the potential to be both immunosuppressive and/or immunododulatory to T cells. To test if either or both of these hypotheses were correct the effect of inhibiting PDE4 on T cell proliferation and cytokine production was measured.

PDE4 is an enzyme that is expressed in many cells, including T cells. Since elevating cAMP can control T cell responses, an agent that can induce such an elevation would be a useful immunosuppressive drug. Work reported in this thesis has shown that PDE4 inhibitors can elevate cAMP levels in activated T cells. Despite this, PDE4 inhibitors were weak suppressants of T cell proliferation. These weak effects do not correlate with inhibition of the PDE4 enzyme in its low-affinity conformation but correlate with inhibition of the PDE4 enzyme in its, alternate, high-affinity conformation. The significance of these conformations is unclear but many of the side-effects of PDE4 inhibitors, e.g. the induction of emesis [145], have correlated with inhibition of the enzyme in its high-affinity state. The effect on proliferation is in contrast to the effect on T cell cytokine production which was found to correlate with inhibition of the PDE4 enzyme in its low-affinity state [269]. The weak suppressive effect of PDE4 inhibitors on proliferation showed no bias to antigens that primed for Th1 or Th2 responses, nor to the stimulus used to induce proliferation. Interestingly however, it was shown that the potency of PDE4 inhibitors at suppressing T cell proliferation could be enhanced by addition of exogenous PGE_2. Since PDE4 protein expression may regulate PGE_2 levels it has been suggested that the anti-proliferative effect of PDE4 inhibitors is due to PGE_2 [283]. This may indeed be the case, however in these studies PGE_2 was used at a dose that activated adenylyl cyclase but did not inhibit proliferation. To use an analogy, if inhibiting PDE4 is the equivalent of putting a plug in a bath, adenylyl cyclase is the equivalent of turning on the tap. Thus, if activated T cells have a decreased rate of cAMP synthesis then PDE4 inhibitors will not be able to maintain high cAMP levels. Indeed it was shown during this thesis that cAMP levels were greatly diminished 24 hours after activation, even in cells treated with high doses of PDE4 inhibitor.

In contrast to the inhibition of T cell proliferation PDE4 inhibitors were more effective at blocking cytokine production. The effects of inhibiting PDE4 on a number of T cell functions are compared in **table 7.3**.

Table 7.3: PDE4-mediated inhibition of T cell activation. *

Response	Stimulus	24hrs	48hrs
Proliferation	Con A	+++	+
Proliferation	Con A + PGE_2	+++	+++
IL-2	Anti-CD3	+++	ND
IL-4	Anti-CD3	+++	+++
IFN-γ	Anti-CD3	+++	+++
TNF-α	Anti-CD3	++++	++++
CD25 expression	Anti-CD3	++	-

* ND, indicates measurements not determined. + indicates inhibition and – indicates no effect.

PDE4 inhibitors blocked the expression of both Th1 (IFN-γ) and Th2 (IL-4) cytokines to the same extent indicating that in *in vitro* human cultures PDE4 inhibitors do not favour inhibition of either Th1 or Th2 cytokines. Under the same experimental conditions TNF-α production was completely blocked by PDE4 inhibitors. Although these studies were performed on mixed cell populations the TNF-α produced under such conditions could be completely blocked by the action of cyclosporin A [294], indicating that T cells were the sole source of cytokine production. If these findings can be extended to purified T cell cultures then this may indicate that unlike all other T cell responses, TNF-α production in T cells is largely regulated by PDE4. The inhibition of T cell PDE4 activity results in a spectrum of responses, ranging from the strong inhibition of cytokines such as TNF-α to the weak inhibition of T cell proliferation and CD25 expression. As some of these processes correlate with inhibition of the PDE4 enzyme in either one or other of its conformational states it may be of importance to investigate what factors can regulate PDE4 enzyme conformation. Such as the divalent cation magnesium [366] and the interaction of protein kinase A (PKA) with the PDE4 enzyme [367]. Understanding how such factors can regulate the PDE4 enzyme at the cellular level may explain the range of effects mediated by inhibiting PDE4 and how the compartmentalisation of responses can exist within the same cell.

7.4 Inhibition of Src kinase enzymes

Inhibition of kinase enzymes has until recently been of limited use as a therapeutic tool to treat disease. The complexity and supposed redundant nature of kinase enzymes has held back the development of kinase inhibitors. This has all changed with the introduction of Gleevec [368], an inhibitor of Bcr-Abl (Abelson tyrosine kinase) for the treatment of patients with CML (chronic myeloid leukaemia). This inhibitor has not only introduced the possibility of kinase inhibitors as effective treatments in cancer but has invoked a reassessment of kinase enzymes as targets in other non-cancerous diseases. In the second part of this thesis the activity of another class of signalling enzyme, Src tyrosine kinase, was inhibited in human T and mast cells. Since Src kinases are recruited to multi-chain immune recognition receptors, e.g. the T cell and high-affinity IgE receptors, they represent attractive targets to modulate immune responses. Such an undertaking required potent and specific chemical inhibitors. Through its medicinal chemistry department, Celltech (Slough, UK) developed a series of small molecule inhibitors of Src kinase. Although selectivity for individual Src kinase members was not achieved, a series of inhibitors were produced that specifically blocked Src kinases over other critical intracellular signalling enzymes. These small molecules improved upon existing pyrazolopyrimidine inhibitors of Src kinase [171]. As well as showing increased potency against Src enzymes, Celltech kinase inhibitors do not inhibit Csk, a kinase that can also regulate Src activity. This chemical series also contained molecules designed to inhibit a novel PDE, PDE7. These compounds were used to probe the function of both Src kinase and PDE7 in activated T and mast cells.

It was proposed that because Src kinase enzymes (Lck and Fyn) are implicated in TCR signalling, inhibiting their enzymatic function may block signalling through the T cell receptor. The data presented in chapter 5 indicates that Src kinase inhibitors were potent at suppressing T cell activation directed through the T cell receptor using anti-CD3 as a stimulus. This inhibition was exemplified by showing that inhibition of isolated Src kinase enzyme correlated with inhibition of anti-CD3-mediated T cell proliferation. This suppression was manifested as an inhibition of cellular proliferation, inhibition of the

synthesis of both Th1 and Th2 cytokines and inhibition of IL-2 receptor expression. However, when more complex antigen driven systems of T cell activation, such as the MLR, were used to activate T cells, Src kinase inhibitors were less effective. This implies that during antigen-driven proliferation other signalling pathways are activated that are Src kinase-independent. It was hypothesised that since responses such as the MLR are dependent upon IL-2 to amplify proliferative signals [369], signalling through the IL-2 receptor may be a Src-independent event. It was found that inhibition of anti-CD3 induced proliferation caused by blocking Src kinase activity could be short circuited by adding exogenous IL-2. This indicates that signalling through the IL-2 receptor does not critically require Src tyrosine kinases. To further define the point of action of the inhibitors, IL-2 production was activated below the level of the TCR using PMA and ionomycin (which act at the level of PKC and calcium signalling). Src inhibitors did not block IL-2 induced proliferation under such conditions suggesting that their activity can be localised to the part of the signal pathway downstream of the TCR but upstream of PMA and ionomycin.

More work is needed to define how antigen and mitogen driven systems differ in their requirement for Src kinases. It is not understood which (apart from signals that are forced through the IL-2 receptor) pathways activated by antigen are Src-independent. The complex response initiated by T cell recognition of antigen, i.e. antigen processing and presentation, TCR recognition of peptide and clonal expansion, means there are many co-stimulatory signals feeding into the T cell. Understanding the effect that inhibiting Src kinase has on events such as the production of cytokines or expression of activation markers may reveal possible Src-dependent-and-independent pathways. This however may be an academic exercise, as it is emerging that Src kinases do not represent suitable immunosuppressive targets as their inhibition is unable to block antigen-driven T cell proliferation. Instead, it may be of benefit to evaluate the ability of these compounds to modulate immune responses. Studies *in vivo* using models of inflammation and immune activation may be modulated by Src kinase inhibitors, especially if they are dependent upon overexpression or disregulation of T cell cytokines.

7.5 Inhibition of Src kinase and PDE enzymes in mast cells

Mast cells are recognised to be involved in allergic inflammation via activation of their high-affinity IgE receptors. Activation of these receptors results in the recruitment of many protein tyrosine kinases to the receptor complex and distinguishing which PTKs are crucial to activation is important in establishing therapeutic targets that can modulate mast cell function. The final results chapter introduced the hypothesis that inhibiting Src kinase enzymes may modulate acute mast cell responses, in particular the ability of mast cells to produce histamine and leukotrienes.

To perform these studies it was necessary to develop a model system of human mast cell activation. Human mast cells were generated from CD34 positive progenitors by stimulation with a cocktail of IL-6, SCF and PGE_2. Such cells were phenotypically and functionally similar to human tissue mast cells. As a consequence of the phenotypic analysis of these cells it emerged that, as cells mature from their progenitors, they express a distinct repertoire of chemokine receptors. Parallel to these studies, Ochi *et al* [354] also reported the expression of chemokine receptors on such mast cells. The similarities and differences between the two studies are summarised in **fig 7.2**.

The importance of these findings is yet to be fully understood, but differences between this and Ochi *et al's* work infer that the cytokines used to induce mast cell maturation may affect chemokine receptor expression. Indeed it is already known from work by other groups that cytokines such as IL-4 and-5 can influence the phenotype of mature mast cells differentiated from cord blood precursors [356;357].

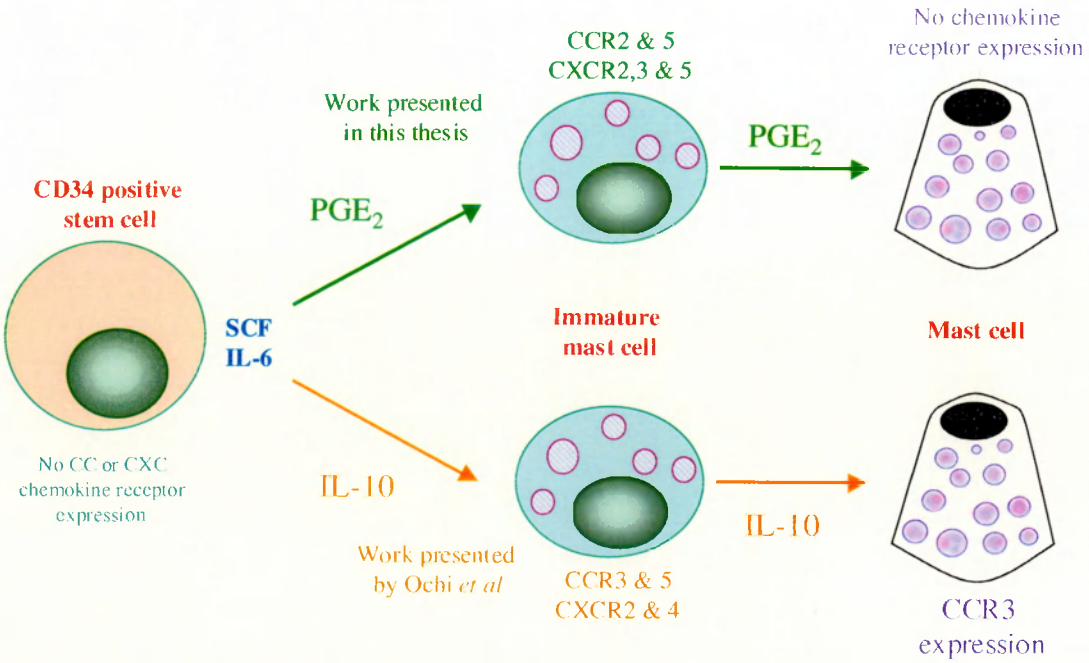


Figure 7.2: Chemokine receptor expression on cord blood derived mast cells. It is proposed that as mast cells mature from CD34 positive precursors they express distinct chemokine receptor repertoire. Agents such as PGE_2 and IL-10 can promote the alternative expression of chemokine receptors in the presence of SCF and IL-6. PGE_2 promotes the expression of CCR2, CXCR3 and CXCR5 where as IL-10 promotes the expression of CCR3 and CXCR4. PGE_2 is unable to induce mature mast cells to express chemokine receptors, however IL-10 (plus SCF and IL-6) can induce the expression of CCR3 [354].

In cord blood-derived mast cells elevation of intracellular cAMP was able to inhibit mast cell degranulation. As the PDE responsible for controlling mast cell cAMP was unknown, a panel of specific inhibitors to various members of the PDE family were used to identify the PDE responsible. No role could be ascribed to PDE3, 4 or 7 in FcεRI-dependent activation of mast cells but the ability of PDE3 and 4 inhibitors to partially inhibit mast cell degranulation suggests a complex regulation of cAMP by PDEs in the mast cell. This may be why pan-specific inhibitors of PDEs were unable to control cord-blood derived mast cell degranulation. However pan-specific PDE inhibitors did block degranulation of mast cells recovered from human tissue [341;342]. More studies are needed to ascertain which PDEs are expressed in cord-blood mast cells and to uncover what factors regulate PDE activity in tissue mast cells.

In fully functional mast cells, Src kinase inhibitors blocked the release of histamine and synthesis of leukotrienes, in response to IgE but not other downstream activators (e.g. PMA

and ionomycin). These effects correlated with inhibition of isolated Src kinase enzymes. In addition, these inhibitors also blocked rat pleural mast cell degranulation activated by cross-linked IgE. From studies using mice in which Lyn has been functionally deleted [333;334] it is apparent that the regulation of FcεRI by Src kinases is complex. In such mice deletion of Lyn alone does not inhibit mast cell degranulation [334]. To reconcile the effect of Celltech Src kinase inhibitors with the Lyn knockout mouse data, it must first be shown that these inhibitors do not inhibit Btk or Syk, as inhibition of either or both of these kinases could also inhibit FcεRI-induced degranulation [333;336]. This would then narrow the effect down to the Src kinases; Lyn, Src or Yes (all shown to be present in mast cells [370]). Mutant FcεRI positive cell lines (such as PT-18, a Lyn/Src deficient cell line [370]), which are positive for only one kinase would allow the role of each kinase to be assessed individually. It would be logical to test the effect of Celltech Src kinase inhibitors in such cells.

More work is also needed to define the effect of these compounds on other mast cell responses such as calcium influx or synthesis of cytokines in response to activation. Albeit, these Src kinase inhibitors represent an attractive starting point for a drug discovery programme aimed at developing Src kinase family specific inhibitors that can inhibit IgE-dependent mast cell degranulation. If these aims were met, then these current molecules, in their capacity to act as validating tools, would prove very useful.

7.6 Conclusions

In summary, these studies reinforce the premise that PDE4 inhibitors may be therapeutically beneficial for treating inflammatory diseases and can almost be called steroid-like in terms of their general broad suppressive qualities, particularly their suppression of cytokine production. Src kinase inhibitors, on the other hand, though less useful in suppressing T cell proliferation may be therapeutically useful in suppressing T cell cytokine production or mast cell activation. Though no role was found for PDE7 in mast cells activated through FcεRI, potent PDE7 inhibitors may be useful in determining the role of this PDE in other cells. In

particular, PDE7 is thought to be involved in signalling through the TCR complex [358] but as yet little evidence has supported this role.

This thesis has stimulated research on many fronts and has in particular emphasised the complexity of even simple *in vitro* assays, particularly with respect to the regulation of cytokines. Toward the end of this thesis a new method for analysing cytokine production became available. This technique using fluorescent polystyrene beads, has enabled the quantification of multiple analytes (in this case cytokines), measured simultaneously from the same sample [371]. Such a method will allow the measurement of many (up to 100) different cytokines from a sample. The application of such technology to understand the interplay of cytokines in simple *in vitro* systems, such as the LPS activation of monocytes, may be helpful in understanding how inhibition of single cytokines (e.g. TNF) affect other cytokines within a network. These methods are currently being applied to profiling the effect of drugs, such as PDE4 inhibitors, with the hope that they will reveal a unique fingerprint of cytokine inhibition. The usefulness of this technique has yet to be fully realised but preliminary work during this thesis has suggested that such experiments are possible and may allow a greater insight into the mechanism of action of cytokine inhibitors.

Chapter 8

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